**From:** Kuhn, Jens (NIH/NIAID) [C] <kuhnjens@niaid.nih.gov>
**Sent:** Friday, June 8, 2018 2:27 PM
**Cc:** Murilo Zerbini;Peter Simmonds
**Subject:** Virus Taxonomy 2018
**Attachments:** Piet Maes Bunyavirales DEmARC 1.pdf; Piet Maes Bunyavirales Comments.docx; TaxoProps SQ.DOCX

Dear All,

**The 2018 deadline** for submitting taxonomic proposals (TaxoProps) to the responsible ICTV Subcommittee Chairs (SCs) **has passed a couple of days ago on June 6**. Because I am the SC chair for animal dsRNA and (-)ssRNA viruses, **there is still some time to edit/establish TPs. We have until June 20**, which is when all SCs have to forward TPs to the ICTV Proposals Secretary. Likewise, the plant and fungal/protist SC chairs (Murilo and Peter, copied) may still accept new proposals. I received the following TPs (either in final form or currently being edited);

## *Bunyavirales*

*Peribunyaviridae*:

- Inclusion of 38 new species in genus *Orthobunyavirus*

*Phasmaviridae*:

- Inclusion of 5 new species in genus *Orthophasmavirus*

*Phenuiviridae*:

- Establishment of new genus *Coguvirus* including 1 new species

## *Mononegavirales*

*Mymonaviridae*:

- Inclusion of 1 new species in genus *Sclerotimonavirus*

*Paramyxoviridae*:

- Complete reorganization of the family: division of family into three subfamilies; establishment of 4 new genera; addition of several new species, abolishment of some species; renaming of some species

*Rhabdoviridae*:

- Establishment of novel genus *Caligrhavirus* including 3 new species
- Inclusion of 3 new species in genus *Dichorhavirus*
- Inclusion of 1 new species in genus *Ledantevirus*

## dsRNA

*Birnaviridae:*

- Complete reorganization of the family: division of family into three subfamilies; eight new genera, numerous new species



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I am further aware of efforts to establish or finish TPs. All involved: please finish these up now immediately as we have only 12 days left!

- to establish a new genus in the bunyaviral family *Arenaviridae*
- to establish a new genus in the mononegaviral family *Bornaviridae*
- to establish two new genera in the mononegaviral family *Filoviridae*
- to reorganize the bunyaviral family *Hantaviridae*
- to add several species to the bunyaviral family *Nairoviridae*

If I missed any TP in the list above, please let me know immediately. **Murilo, Peter:** please forward me any dsRNA or (-)ssRNA proposals that were submitted to you so we can all have a complete overview on what is happening in the "realm" of these viruses. **Non-animal Study Groups:** if you have any TPs in preparation, please copy me when you submit them to Murilo and/or Peter.

Piet Maes performed DEmARC on the order *Bunyavirales*. You can see the preliminary result in the attached PDF "Piet Maes Bunyavirales DEmARC 1" and his comments on potentially missing viruses in the attached Word file "Piet Maes Bunyavirales Comments". We will use this analysis to create an overarching TP to propose numerous new species and genera in the order. **All bunyavirus experts:** please review the list of green names in the Word file and let me know immediately if any of those proposed classifications contradicts your knowledge or understanding in any way.

I am still dreaming that Piet will miraculously pull a similar analysis out of his hat for mononegaviruses and/or orthomyxoviruses – but because he has been pulled into the Ebola virus outbreak response in Africa a few weeks ago, this is exceedingly unlikely. Thus, to all of **those who were waiting for DEmARC results: please assume they may not be forthcoming and therefore take a look which kind of proposals you could still pull together in absence of DEmARC in the 11th hour.**

Best,
Jens

Jens H. Kuhn, MD, PhD, PhD, MS
Virology Lead
Battelle Memorial Institute – Contractor supporting:

National Institute of Allergy and Infectious Diseases (NIH/NIAID)
Division of Clinical Research (DCR)
Integrated Research Facility at Fort Detrick (IRF-Frederick)
B-8200 Research Plaza
Fort Detrick, Frederick, MD 21702, USA
Office: +1.301.631.7245
Fax: +1.301.631.7389
Email: kuhnjens@mail.nih.gov



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**Xiangrong Geng**

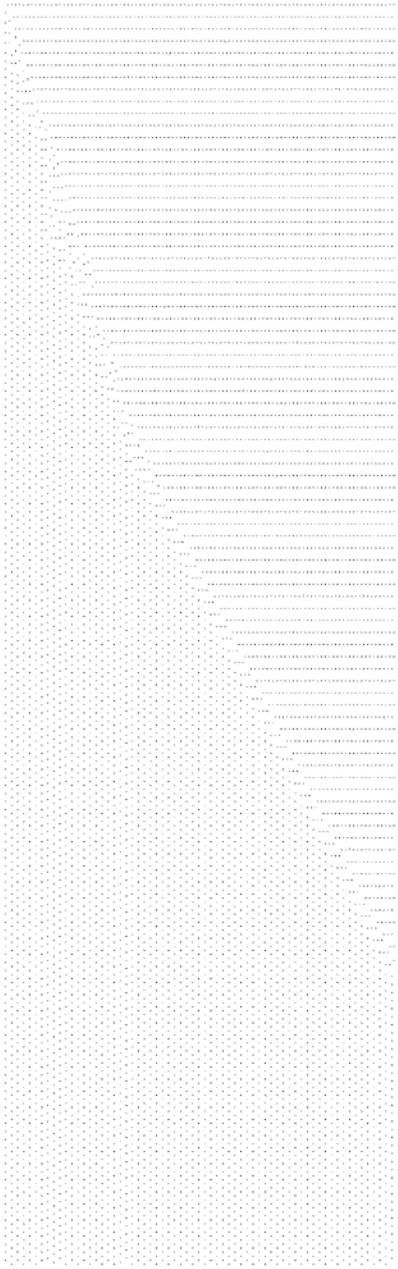
Ph.D. candidate (Expected December 2018)

[redacted]

Email: xgeng@oakland.edu Phone: [redacted]

(b)(6)

(b)(6)



| | |
|---|---|
| **MOU:** | **MOU – for the purpose of developing a research and training program** |
| **UTMB:** | **J. LeDuc** |
| **RECIPIENT:** | **Wuhan Institute of Virology** |
| | **Chinese Academy of I Sciences** |
| **EXPORT CONTROL:** | **N/A** |

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# MEMORANDUM OF UNDERSTANDING OF COOPERATION BETWEEN WUHAN INSTITUTE OF VIROLOGY, CHINESE ACADEMY OF SCIENCES AND THE UNIVERSITY OF TEXAS MEDICAL BRANCH AT GALVESTON

**Wuhan Institute of Virology, Chinese Academy of Sciences,** with its legal address and premises at No. 44, Xiao Hong Shan, Wuhan, Hubei Province, China 430071 (hereinafter referred to as "**WIV**")

**AND**

**The University of Texas Medical Branch,** with its legal address and premises at 301 University Blvd, Galveston, Texas 77550 (hereinafter referred to as "**UTMB**")

WIV and UTMB hereinafter also individually referred to as a "**Party**" or collectively as the "**Parties**"

The parties reached and confirmed with the Memorandum of Understanding (hereinafter referred to as "**Memorandum**") their consensus to establish exchanges and collaboration in areas of mutual interest and benefit.

## I. GENERAL PROVISION

i) **Relationship between the parties**

Nothing contained herein shall be construed as establishing a relationship of agent and principal or master and servant between the parties. Each party shall have full control of its operations and undertakings and shall have full responsibility for activities and duties carried by it and on its behalf.

ii) **Good faith and fairness**

a) The parties undertake to act in good faith with respect to each other's rights under the obligations and objectives of this MoU.

b) The parties, their representatives, their researchers and personnel shall not, either during or after the term of this MoU, disclose proprietary information relating to the



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undertaking by both parties and each other's operations without the consent of the other party.

c) The parties recognise the impracticality of providing for every contingency, which may arise during or after the life of the MoU and hereby agree to operate fairly and without detriment to the interests of either of them.

iii) **Notices**

Any notice, request or consent required or permission to be given or made pursuant to this MoU shall be in writing. Any such notice, request or consent shall be deemed to have been given or made when delivered either in person to the authorized representative at the Head Office of the party to whom communication is addressed or when sent by registered mail or by fax to such party at the following address:

For: Wuhan Institute of Virology

The Director General: Dr. Chen Xinwen

PO Box 430071

Xiao Hong Shan No.44

Wuhan, Hubei Province

The People's Republic of China

Tel: (0086)027-87199106

E-mail: chenxw@wh.iov.cn

For: The University of Texas Medical Branch

Dr. James LeDuc

301 University Blvd, 77550

Galveston, Texas

The United States

Tel:

E-mail:

Provided that a party may change its physical address and email for notice hereunder by giving the other party notice of such change pursuant to this clause.

Proof of posting or dispatch shall be deemed to be proof of receipt:

a) In the case of a letter, on the 10th day after posting



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b) In the case of email, on the day immediately following the date of dispatch, but the sender has to confirm receipt of the same by the other party by telephone.

Without prejudice to the foregoing provisions, any notice shall be sent by the quickest means reasonable available.

## II. OBJECTIVES OF THE COOPERATION

The objectives of the cooperation between WIV and UTMB are as follows:

- to promote the research cooperation between China and the United States for controlling infectious diseases, protecting laboratory safety and global health security;
- to deepen the training and education of all parties to this MoU; and
- to strengthen the academic and talent exchanges between the parties.

## II. CONTENTS OF THE COOPERATION

To achieve these objectives, WIV and UTMB will, insofar as the means of each allow:

- promote talent and education exchanges by initiating visiting scientists and post-doctoral education programs;
- receive researchers, technicians and students of both parties for periods of training, study and/or research;
- organize symposia, conferences, short courses, workshops and meetings on research issues;
- carry out joint research programs and apply for international grants with joint efforts;
- exchange information pertaining to developments in teaching, faculty, staff and student development and research at each institution and
- exchange the virus resources strictly for the scientific research purposes.

## III. MANAGEMENT SYSTEM OF THE MOU

i) WIV and UTMB will provide necessary supports to the MoU. If necessary the parties can communicate for asking other scientific research units in globe to participate in relevant scientific research activities in this MoU.

ii) Each party shall designate a coordinator to oversee and facilitate the implementation of this MoU. The coordinators, working with other appropriate administrators at the respective institutions, shall have the following responsibilities:

- to promote academic collaboration at both researchers, technicians and student levels for research, training and study;
- to act as principal contracts for individual and group activities and to plan and coordinate



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all activities under this MoU within their institutions as well as with the other party;

- to distribute to each party information about the faculty, facilities, research, publications, library materials and educational resources of the other party; and
- to meet periodically to review and evaluate past activities and to work out new ideas for future cooperative agreements.

## IV. INTELLECTUAL PROPERTY

Both parties do agree that:

i) Title rights, copy rights, patent rights or any other intellectual rights in Intellectual Property belonging to either party prior to this MoU shall remain with said Party. Title rights, copy rights, patent rights or any other intellectual rights in any Intellectual Property generated from information and data collected and analyzed under the MoU shall be the property of both WIV and UTMB.

ii) That prior to any disclosure of proprietary information by one party to the other concerning specific aspect of this collaboration, the one party may require the other to execute a confidentiality agreement.

iii) Once WIV and UTMB are satisfied that a given discovery ("Invention") arising from a joint project under the MoU is worth protecting by a patent, the Parties will negotiate in good faith to establish the terms of an "Inter-Institutional Agreement" or IIA, or such other agreements as may be appropriate, setting forth, amongst other things, the parties' agreement on the control of patent prosecution, administration and maintenance and on the responsibility for further research, development and commercialization of the Invention and associated rights. The parties intend that the terms of any Inter-Institutional Agreement shall outline each Party's assumption of equal responsibilities in administration of any patent obtained under this MoU. Responsibility for expenses relating to registration, administration and further development and exploitation of the invention (including funds to the inventors to carry out further work to bring the invention to a stage where it can be commercially exploited, researching for commercial outlets, advertising expenses, and fees for patent attorneys) will be agreed upon by both parties. Any decision relating to the commercial exploitation or to the manner of disposal of the patent shall be made jointly taking into consideration the patent regulations of each party in particular the role of the inventor with commercialization revenues received for the Invention or the rights therein to be shared equally among the parties according to their shares in the Invention.

iv) Material for publication or presentation arising from the joint research projects under the MoU shall be submitted for clearance to the two parties to ensure that no patentable discoveries are published prior to protection by patents.



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v) Any scientific publications resulting from the collaborative research under the MoU, including scientific papers, books and proceedings of conferences, seminars and workshops will be authored jointly to reflect where relevant contributions have been made and quoting the names of authors and the two parties as well as the donor agencies if applicable.

## V. FUNDING

It is understood and agreed that WIV and UTMB will each be responsible for their own expenses incurred in the performance of the collaborative research programs under this MoU. Neither party is under obligation to fund the portion of the collaborative research program conducted by the other party.

## VI. ADMINISTRATION OF THE MOU

Both parties agree (that):

a. This MoU shall be identified only as the current wish and intent of the Parties regarding the potential for developing a program agreement executed between the parties.

b. Further agreements concerning any program shall be independent of this MOU and shall each provide details concerning the specific commitments made by each party and shall not become effective until they have been reduced to writing and executed by the duly authorized representatives of the parties.

c. Any collaborative research agreement, or similar arrangement related to scientific development and/or discovery, shall be separately negotiated and a written agreement executed by and between the parties for each such arrangement.

d. To work out mechanisms of reviewing and evaluating the impact of the MoU every year and at the end of the MoU duration and to monitor implementation of any on-going activities.

e. To advice either party on available areas of collaboration and advice the Chief Executives of the institutions through a written report on the progress of the collaboration or on any need to amend or review the same.

## VII. DURATION OF THE MOU

The MoU will remain in force for five (5) years at the end of which both parties will review and may renew the collaboration.

## VIII. EFFECTIVE DATE

i) This memorandum shall become effective immediately from the date of the last signature by the appropriately authorized officers of each of the two institutions.



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ii) This memorandum may be extended by both parties' written consent after the expiration of the MoU.

## IX. MODIFICATION/AMENDMENTS

i) Modification of the terms and conditions of this MoU may only be made by written agreement between the parties, duly signed by authorized officers of both parties and the same will be incorporated in the annexure of this Memorandum of Understanding.

ii) Either party wishing to modify/ or amend the MoU shall give the other party two (2) months written notice of such intentions and shall send the proposed modifications/amendments within the two (2) months notice period.

iii) The modification/amendment proposal will be discussed between the parties who will make recommendations to the Chief Executives of the institutions who will execute the modified/amended MoU.

## X. SUSPENSION

i) In the event of one party not fulfilling its part of the obligations as spelt out in this MoU and the aggrieved party is being adversely affected, the aggrieved party is at liberty to suspend the MoU with immediate effect and call for renegotiating within 7 to 21 days.

ii) In the event of the other party not availing itself for renegotiating with the aggrieved, the aggrieved party may give the other party a notice of termination but in the meantime handle the cause of grievance/dispute in the best way possible to avoid further deterioration/harm occurring.

## XI. TERMINATION

i) Either party may terminate the MoU if the other party is in breach of terms and conditions of this MoU. The aggrieved party shall give the other party three (3) months notice of the termination of the MoU; provided that the other party has been given a one (1) month notice and the breach has not been rectified.

ii) Either party may terminate this MoU without cause and without penalty by giving three (3) months written notice to the other Party

iii) In the event of such termination of the MOU any on-going activity under the collaboration shall be dealt with as shall be mutually agreed by the two institutions.

iv) Where such activity involves students, the parties shall ensure that the students shall be able to complete their studies.



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v) Any gains or losses in the pursuance of the objectives of this MoU that shall have been incurred at the time of termination shall be shared in mutually agreed ratios.

## XII. SETTLEMENT OF DISPUTES

i) The parties shall use their efforts to settle amicably all disputes arising out of or in connection with this MoU and/or its interpretation hereof.

ii) Any dispute, difference or question which may arise at any time between the parties, which cannot be settled amicably within thirty (30) days after receipt by one party of the other party's request for such amicable settlement shall be referred to the decision of a single arbitrator to be agreed upon between the two parties or in default of agreement within 14 days of each party raising such dispute.

iii) Any party not satisfied with the arbitration, shall have the right to seek justice in the Courts of law.

## XIII. INDEMNITY

The parties do agree that if a legal suit arises with regard to the management and/or administrative in-capabilities and/or lapses that may arise as a result of action or inaction on the part of the party, which may have led to either party being sued individually, jointly or severally, each party shall be responsible for its own legal fees and expenses.

## XIV. EXPORT CONTROL

The parties acknowledge that this Agreement and the performance thereof are subject to compliance with any and all applicable Chinese and United States laws, regulations, or orders, including those that may relate to the export of technical data, and both Parties agree to comply with all such laws, regulations and orders, The Parties agree that if the U.S. export laws and/or those of the People's Republic of China are or become applicable, neither Party will export any Materials or information deemed Export Controlled, received under this Agreement to any countries for which the United States or Chinese government requires an export license or other supporting documentation at the time of export or transfer, unless said Party has obtained prior written authorization from the appropriate authority responsible for such matters.

## XV. FORCE MAJEURE

Both parties shall be released from their respective obligation in the event of national emergency, war, prohibitive governments' regulations or of any other cause beyond the reasonable control of the parties or either of those that render the performance of this MoU impossible whereupon moneys due under this agreement shall become due and payable.



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The MoU will consist of four numbered copies of equal value. Each Party will retain two copies and can make further copies as required.

## XVI. CONFIDENTIALITY

i) All cooperation and exchanges documents, data, details and materials shall be treated as confidential information by the parties.

ii) The party may not make use of their mutual dealings for advertising purposes without the other's prior written consent.

iii) The confidentiality obligation shall be applicable throughout the duration of this MoU and after it has been terminated. The party is entitled to ask the other to destroy and/or return the secret files, materials and equipment without any backups.

In witness whereof the parties have affixed their common seals on this .....day of .....2017.

**WUHAN INSTITUTE OF VIROLOGY, CHINESE ACADEMY OF SCIENCES**

(b)(6)

B

Name: Xinwen Chen
Title: Director General
Date: Dec. 12. 2017
Place: Wuhan, China

Read and understood:

(b)(6)

Signature of WIV Coordinator
Prof. Zhiming Yuan

**THE UNIVERSITY OF TEXAS MEDICAL BRANCH**

(b)(6)

B

Name: Carolee A. King, JD (b)(6)
Title: Senior VP and General Counsel
Date: 12/20/17
Place: UTMB Galveston, TX

Read and understood:

(b)(6)

Signature of UTMB Investigator (Coordinator)
Dr. James W. LeDuc

Content Reviewed
(b)(6)



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**From:** Shan, Chao
**Sent:** Saturday, July 21, 2018 10:00 AM
**To:** Shi, Pei yong;马海霞-中科院武汉病毒所
**Cc:** 袁志明;LeDuc, James W.;Xie, Xuping
**Subject:** RE: RE: Flight schedule and hotel at Galveston

Dear Dr. Yuan,

We are looking forward to meeting you at Galveston.

Have a great weekend!
Best,
Chao

**From:** Shi, Pei yong
**Sent:** Saturday, July 21, 2018 6:35 AM
**To:** 马海霞-中科院武汉病毒所 <haixiam@wh.iov.cn>
**Cc:** 袁志明 <yzm@wh.iov.cn>; LeDuc, James W. <jwleduc@UTMB.EDU>; Shan, Chao <chshan@UTMB.EDU>; Xie, Xuping <xuxie@UTMB.EDU>
**Subject:** RE: RE: Flight schedule and hotel at Galveston

Hi Haixia,
Thanks for the information. We will plan accordingly.

Hi Zhiming,
Xuping and Chao will meet you at the airport at on August 4th.

Have a great weekend!

Pei-Yong

**From:** 马海霞-中科院武汉病毒所 [mailto:haixiam@wh.iov.cn]
**Sent:** Friday, July 20, 2018 9:07 PM
**To:** Shi, Pei yong <peshi@UTMB.EDU>
**Cc:** 袁志明 <yzm@wh.iov.cn>; LeDuc, James W. <jwleduc@UTMB.EDU>
**Subject:** Re: RE: Flight schedule and hotel at Galveston

Hello, Professor Shi Peiyong,

Thanks for your preparation of Professor Yuan's visit.Wuhan Institute Of Virology，CAS will pay for Mr.Yuan's hotel expenses.



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The  flight information:
Flight number:CA995 ,Beijing to Houston ,
Arrival time: 15:40 in August 4th

The hotel information :
Homewood Suites By Hilton Galveston  Business trip
110 Seawall Boulevard , Galveston, 77550, United States of America
Phone: +1 409-497-2831

Best wishes

Haixia

-----原始邮件-----
**发件人:**"Shi, Pei yong" <peshi@UTMB.EDU>
**发送时间:**2018-07-20 22:35:31 (星期五)
**收件人:** "袁志明" <yzm@wh.iov.cn>
**抄送:** "马海霞" <haixiam@wh.iov.cn>, "LeDuc, James W." <jwleduc@UTMB.EDU>
**主题:** RE: Flight schedule and hotel at Galveston

Dear Zhiming,

Thanks! We all look forward to seeing you soon.

Once received you flight schedule, we will plan airport pickup.

Best,

Pei-Yong

**From:** 袁志明 [mailto:yzm@wh.iov.cn]
**Sent:** Friday, July 20, 2018 9:28 AM
**To:** Shi, Pei yong <peshi@UTMB.EDU>
**Cc:** 马海霞 <haixiam@wh.iov.cn>
**Subject:** Re: Flight schedule and hotel at Galveston

Dear Peiyong,

I have booked the flights and the hotel in Galveston, I will asked Miss Ma Haixia to send you my schedule and the hotel information.

thanks for your preparation of my visit there.



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Regards

Zhiming

-----原始邮件-----
发件人:"Shi, Pei yong" <peshi@UTMB.EDU>
发送时间:2018-07-19 21:47:20 (星期四)
收件人: "Yuan Zhiming" <yzm@wh.iov.cn>
抄送: "LeDuc, James W." <jwleduc@UTMB.EDU>
主题: Flight schedule and hotel at Galveston

Hi Zhiming,

We are looking forward to your visit. To prepare for the logistics, would you please send us the following information at your earliest convenience?

1. Flight schedule
2. Will CAS pay for your hotel expense?

Thanks!

Pei-Yong

--

祝：工作顺利！

马海霞 13477032090

---
中国科学院武汉病毒研究所
地址：湖北省武汉市武昌区小洪山中区中科院武汉分院行政楼514室
电话/传真：027-87198649



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**From:** Rusek, Benjamin <BRusek@nas.edu>
**Sent:** Monday, July 30, 2018 7:26 PM
**To:** Holubar, Connie J.
**Cc:** LeDuc, James W.
**Subject:** RE: NAS-CAS Draft Galveston bio dialogue summary 2018 v3-jwl
**Attachments:** NAS-CAS Galveston bio dialogue summary 2018 final.docx

Hi Connie,

I hope you are having a wonderful summer! I should have followed up sooner but did the summary from the January bilateral meeting (attached) ever get posted on the new UTMB website? If so could you point me to it?

PS Glad to hear that Zhiming is going to visit GNL in August.

Kind regards,

Ben

Benjamin Rusek
The U.S. National Academy of Sciences
1-202-334-3975

**From:** Holubar, Connie J. <cjholuba@UTMB.EDU>
**Sent:** Wednesday, February 28, 2018 5:33 PM
**To:** LeDuc, James W. <jwleduc@UTMB.EDU>; Rusek, Benjamin <BRusek@nas.edu>; Dave Franz (davidrfranz@gmail.com) <davidrfranz@gmail.com>; Shi, Pei yong <peshi@UTMB.EDU>
**Subject:** RE: NAS-CAS Draft Galveston bio dialogue summary 2018 v3-jwl

Yes, we can post that on our new website, which will launch in April.

Connie

**From:** LeDuc, James W.
**Sent:** Wednesday, February 28, 2018 4:26 PM
**To:** Benjamin Rusek (BRusek@nas.edu) <BRusek@nas.edu>; Dave Franz (davidrfranz@gmail.com) <davidrfranz@gmail.com>; Shi, Pei yong <peshi@UTMB.EDU>
**Cc:** Holubar, Connie J. <cjholuba@UTMB.EDU>
**Subject:** NAS-CAS Draft Galveston bio dialogue summary 2018 v3-jwl

Attached has a couple of edits on the agenda (dropped Scott's name as speaker) and dropped Geisbert's presentation that was not given. Otherwise, looks fine, thank you.

Connie, can we post the final on our GNL website?

Thanks, Jim

James W. Le Duc, Ph.D.



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Director
Galveston National Laboratory
University of Texas Medical Branch
Galveston, TX 77555-0610
(t) 409-266-6500
(f) 409-266-6810
(m) [redacted]



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**Third China-U.S. Workshop on**
**the Challenges of Emerging Infections, Laboratory Safety and Global Health Security**
Galveston National Laboratory
January 16-18 2018
Galveston, Texas, USA

## Summary

To address the interconnected life science challenges associated with emerging infectious diseases and enhance cooperation between China and the United States in the areas of global health, biosafety, and biosecurity, the U.S. National Academy of Sciences (NAS), the Galveston National Laboratory (GNL) at the University Texas Medical Branch (UTMB), the Chinese Academy of Sciences (CAS), Chinese Center for Disease Control and Prevention (CCDC), and other Chinese institutions cooperated to hold a third[1] *China-U.S. Workshop on the Challenges of Emerging Infections, Laboratory Safety and Global Health Security* in January 2018 in Galveston, Texas, USA.

The workshop brought more than 30 senior leaders from China and the U.S. to the GNL to discuss a range of infectious disease, public health and facility engineering topics of concern to scientists and leaders in China and the United States. Participants included Dr. James Le Duc, the director of the GNL and the workshop host, Dr. Xinwen Chen, the director of the Wuhan Institute of Virology, CAS and Dr. Zhigao Bu, the director of the Harbin Veterinary Research Institute, Chinese Academy of Agricultural Science (CAAS). Like the GNL, the Chinese laboratories represented are biosafety level four (BSL-4) laboratories. Other senior scientists represented the CCDC, Tsinghua University, Stanford University, Emory University, the University of North Carolina, the Biosecurity Research Institute at Kansas State University, and UTMB. Representatives from the U.S. Food and Drug Administration, the U.S. Department of Agriculture, and the NAS also participated.

The workshop aimed to build bridges between Chinese and American scientists and use the eminence and national leadership of the convening CAS and NAS organizations to improve scientific and technical cooperation in areas of mutual concern to China and the United States. The organizations recognize the benefits of sharing both scientific knowledge and new advancements in research addressing international public health threats and operational lessons learned in order to ensure safe and secure operations of biological laboratories.

At the workshop, experts gave presentations on the science behind combating emerging infections; high containment laboratories, including commissioning, construction, operation and sustainment; and the importance of conducting innovative life-science research while managing the risks to researchers and the public. The experts shared recent advances in the development of vaccines for Zika and Chikungunya viruses, as well as details of important drug discoveries and testing. Lectures on emerging and reemerging international threats from diseases like yellow

[1] This is the third workshop in a series. In September 2015 and May 2017 a similarly composed group of experts and representatives held workshops on emerging infections, laboratory safety, and global health security. The first took place at the CAS Institute of Microbiology in Beijing, China, and the second at the Wuhan Institute of Virology (CAS), in Wuhan, China.



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fever and Crimean-Congo hemorrhagic fever were also highlighted. Healthcare experts from the U.S. discussed their personal experience in care and management of patients infected with Ebola, and biosafety and engineering professionals shared important issues regarding the environmental and technical management of high-containment laboratories. Approaches to applying stringent biosafety and biosecurity measures in laboratories, life-science ethics, laboratory leadership and organizational cultures in China and the United States were addressed to advance a better understanding from the perspective of experts in each country and to seek agreement on best practices in these areas. Students and post docs from UTMB attended and took part in the robust discussions that followed each presentation. After the formal sessions the delegation from China toured the GNL and spent time discussing critical high-containment operations and maintenance issues, as well as training programs for scientific staff, students, and building operations personnel who ensure the ongoing maintenance and operations of critical safety systems.

A unique feature of the workshop was that it brought together the leadership of several BSL-4 laboratories. The number of BSL-4 facilities is growing; the World Health Organization (WHO) recently counted more than 50 BSL-4 labs operating, under construction, or being planned around the world. There are currently three BSL-4 labs in China that are either in the final stages of being commissioned or have recently begun research operations: the Wuhan Institute of Virology (CAS), the Harbin Veterinary Research Institute (CAAS), and the Kunming Institute of Medical Biology (Chinese Academy of Medical Sciences). This and previous meetings organized by NAS and CAS have laid the foundations for future exchanges and projects, both scientific and practical, between the leaders and researchers at these laboratories. Sharing advice and experiences and creating ties between institutions and personnel that operate these facilities is crucial to ensuring the safe operations of the laboratories and to prevent the misuse or accidental release of dangerous pathogens. GNL has negotiated bilateral memoranda of understanding (MOUs) between the GNL and the three Chinese BSL-4 labs so they can more easily work together on short-term and long-term joint projects.

One of the most important aspects of the meeting was to identify potential opportunities for collaboration between scientists in China and the U.S., two countries that account for some of the world's most important medical discoveries. Participants outlined some broad areas for further collaboration between the countries: (1) Issues related to emerging infections, understanding disease reservoirs, disease emergence, disease surveillance, natural versus human origin of disease, data sharing, and building capacity for research during public health emergencies. (2) Anticipating risk in the life sciences and medicine related to biotechnology innovation, such as identifying certain experiments that are too risky to conduct. (3) Governance of science to reduce risk—technical risk for the laboratory and societal risk for the community—such as doing research in centralized laboratories versus distributed labs, managing research costs, learning from past technical and bureaucratic mistakes, and ways to diminish risk without hindering science. (4) Training opportunities to ensure the safe and secure conduct of research in biocontainment and also the efficient and effective maintenance of high-containment laboratories to ensure the safety of the workforce as well as the surrounding community and environment.



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The experts identified several areas for promising future discussion in the NAS and CAS dialogue including creating joint BSL-4 research projects that are beneficial for both the United States and China, better ways to share samples, strains, and strain collections between laboratories, and improving and sharing best practices for training laboratory staff, particularly staff that work at the BSL-4 level. The group will work to build on this workshop and continue these discussions, subsequent meetings are planned to take place in China in 2018 and early 2019.

**Third China-U.S. Workshop on**
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**Sessions and Presentations**

***Opening Remarks and Keynote Addresses***

- Host jump of emerging viruses: Flu, MERS-CoV and Ebola
- "Going digital:" Opportunities and challenges for safety and security when life and advances in life sciences are expressed as O's and 1's

***Clinical Care and Product Development in Biocontainment***

- The care and management of Ebola patients
- Challenges of medical product development in biocontainment
- Ensuring data quality and integrity on studies supporting the advancement of medical countermeasures

***Vector-borne Diseases***

- Crimean-Congo hemorrhagic fever virus
- Vaccines for Alphaviruses
- Story of Arboviral Lifecycle: Acquisition Mechanism from Hosts to Mosquitoes
- Imported Yellow Fever and other important viral pathogens in China
- Zika vaccine development
- Flaviviral RNAs and host proteins: a love-hate relationship

***Agricultural Research in Biocontainment***

- Research and operations at the Biosecurity Research Institute: an agriculture biocontainment laboratory
- Development of candidate vaccines against MERS-CoV for camel and human
- Rift Valley fever virus
- Nipah virus
- Ecology and Epidemiology of Recent Highly Pathogenic Avian Influenza Outbreaks

***Recent Advances in Research on High Hazard Pathogens***

- Coronaviruses



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- Coronavirus from bats
- Interplay between coronaviruses and nonsense-mediated mRNA decay pathway
- Mammalian RNAi antiviral and host-virus interaction

***Biosafety, Biosecurity and Biocontainment Facilities Operation***

- Training in biosafety and biosecurity
- Training program for facilities operations
- National biosafety laboratory in China
- Next Steps: Building on the workshop for future cooperation and collaboration

Points of contact:


**James W. Le Duc, Ph.D.**
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**Zhiming Yuan, Ph.D.**
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**From:** Holubar, Connie J.
**Sent:** Tuesday, July 31, 2018 11:47 AM
**To:** Rusek, Benjamin
**Subject:** RE: NAS-CAS Draft Galveston bio dialogue summary 2018 v3-jwl
**Attachments:** NAS-CAS Galveston bio dialogue summary 2018 final (002).docx

Is this what you wanted us to post?

**From:** Rusek, Benjamin [mailto:BRusek@nas.edu]
**Sent:** Tuesday, July 31, 2018 10:54 AM
**To:** Holubar, Connie J. <cjholuba@UTMB.EDU>
**Subject:** RE: NAS-CAS Draft Galveston bio dialogue summary 2018 v3-jwl

Awesome! Thank you!

Kind regards,

Ben

Benjamin Rusek
The U.S. National Academy of Sciences
1-202-334-3975

**From:** Holubar, Connie J. <cjholuba@UTMB.EDU>
**Sent:** Tuesday, July 31, 2018 11:49 AM
**To:** Rusek, Benjamin <BRusek@nas.edu>
**Subject:** RE: NAS-CAS Draft Galveston bio dialogue summary 2018 v3-jwl

Here are some photos.

**From:** Rusek, Benjamin [mailto:BRusek@nas.edu]
**Sent:** Monday, July 30, 2018 8:15 PM
**To:** Holubar, Connie J. <cjholuba@UTMB.EDU>
**Subject:** RE: NAS-CAS Draft Galveston bio dialogue summary 2018 v3-jwl

Also do you have a copy of the group photo we took that you could send me? I don't think I ever got one

Kind regards,

Ben

Benjamin Rusek
The U.S. National Academy of Sciences
1-202-334-3975

**From:** Rusek, Benjamin
**Sent:** Monday, July 30, 2018 8:26 PM



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**To:** 'Holubar, Connie J.' <cjholuba@UTMB.EDU>
**Cc:** LeDuc, James W. <jwleduc@UTMB.EDU>
**Subject:** RE: NAS-CAS Draft Galveston bio dialogue summary 2018 v3-jwl

Hi Connie,

I hope you are having a wonderful summer! I should have followed up sooner but did the summary from the January bilateral meeting (attached) ever get posted on the new UTMB website? If so could you point me to it?

PS Glad to hear that Zhiming is going to visit GNL in August.

Kind regards,

Ben

Benjamin Rusek
The U.S. National Academy of Sciences
1-202-334-3975

**From:** Holubar, Connie J. <cjholuba@UTMB.EDU>
**Sent:** Wednesday, February 28, 2018 5:33 PM
**To:** LeDuc, James W. <jwleduc@UTMB.EDU>; Rusek, Benjamin <BRusek@nas.edu>; Dave Franz (davidrfranz@gmail.com) <davidrfranz@gmail.com>; Shi, Pei yong <peshi@UTMB.EDU>
**Subject:** RE: NAS-CAS Draft Galveston bio dialogue summary 2018 v3-jwl

Yes, we can post that on our new website, which will launch in April.

Connie

**From:** LeDuc, James W.
**Sent:** Wednesday, February 28, 2018 4:26 PM
**To:** Benjamin Rusek (BRusek@nas.edu) <BRusek@nas.edu>; Dave Franz (davidrfranz@gmail.com) <davidrfranz@gmail.com>; Shi, Pei yong <peshi@UTMB.EDU>
**Cc:** Holubar, Connie J. <cjholuba@UTMB.EDU>
**Subject:** NAS-CAS Draft Galveston bio dialogue summary 2018 v3-jwl

Attached has a couple of edits on the agenda (dropped Scott's name as speaker) and dropped Geisbert's presentation that was not given. Otherwise, looks fine, thank you.

Connie, can we post the final on our GNL website?

Thanks, Jim

James W. Le Duc, Ph.D.
Director
Galveston National Laboratory
University of Texas Medical Branch
Galveston, TX 77555-0610
(t) 409-266-6500
(f) 409-266-6810
(m) (b)(6)



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**Third China-U.S. Workshop on**
**the Challenges of Emerging Infections, Laboratory Safety and Global Health Security**
Galveston National Laboratory
January 16-18 2018
Galveston, Texas, USA

## Summary

To address the interconnected life science challenges associated with emerging infectious diseases and enhance cooperation between China and the United States in the areas of global health, biosafety, and biosecurity, the U.S. National Academy of Sciences (NAS), the Galveston National Laboratory (GNL) at the University Texas Medical Branch (UTMB), the Chinese Academy of Sciences (CAS), Chinese Center for Disease Control and Prevention (CCDC), and other Chinese institutions cooperated to hold a third[1] *China-U.S. Workshop on the Challenges of Emerging Infections, Laboratory Safety and Global Health Security* in January 2018 in Galveston, Texas, USA.

The workshop brought more than 30 senior leaders from China and the U.S. to the GNL to discuss a range of infectious disease, public health and facility engineering topics of concern to scientists and leaders in China and the United States. Participants included Dr. James Le Duc, the director of the GNL and the workshop host, Dr. Xinwen Chen, the director of the Wuhan Institute of Virology, CAS and Dr. Zhigao Bu, the director of the Harbin Veterinary Research Institute, Chinese Academy of Agricultural Science (CAAS). Like the GNL, the Chinese laboratories represented are biosafety level four (BSL-4) laboratories. Other senior scientists represented the CCDC, Tsinghua University, Stanford University, Emory University, the University of North Carolina, the Biosecurity Research Institute at Kansas State University, and UTMB. Representatives from the U.S. Food and Drug Administration, the U.S. Department of Agriculture, and the NAS also participated.

The workshop aimed to build bridges between Chinese and American scientists and use the eminence and national leadership of the convening CAS and NAS organizations to improve scientific and technical cooperation in areas of mutual concern to China and the United States. The organizations recognize the benefits of sharing both scientific knowledge and new advancements in research addressing international public health threats and operational lessons learned in order to ensure safe and secure operations of biological laboratories.

At the workshop, experts gave presentations on the science behind combating emerging infections; high containment laboratories, including commissioning, construction, operation and sustainment; and the importance of conducting innovative life-science research while managing the risks to researchers and the public. The experts shared recent advances in the development of vaccines for Zika and Chikungunya viruses, as well as details of important drug discoveries and testing. Lectures on emerging and reemerging international threats from diseases like yellow

[1] This is the third workshop in a series. In September 2015 and May 2017 a similarly composed group of experts and representatives held workshops on emerging infections, laboratory safety, and global health security. The first took place at the CAS Institute of Microbiology in Beijing, China, and the second at the Wuhan Institute of Virology (CAS), in Wuhan, China.



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fever and Crimean-Congo hemorrhagic fever were also highlighted. Healthcare experts from the U.S. discussed their personal experience in care and management of patients infected with Ebola, and biosafety and engineering professionals shared important issues regarding the environmental and technical management of high-containment laboratories. Approaches to applying stringent biosafety and biosecurity measures in laboratories, life-science ethics, laboratory leadership and organizational cultures in China and the United States were addressed to advance a better understanding from the perspective of experts in each country and to seek agreement on best practices in these areas. Students and post docs from UTMB attended and took part in the robust discussions that followed each presentation. After the formal sessions the delegation from China toured the GNL and spent time discussing critical high-containment operations and maintenance issues, as well as training programs for scientific staff, students, and building operations personnel who ensure the ongoing maintenance and operations of critical safety systems.

A unique feature of the workshop was that it brought together the leadership of several BSL-4 laboratories. The number of BSL-4 facilities is growing; the World Health Organization (WHO) recently counted more than 50 BSL-4 labs operating, under construction, or being planned around the world. There are currently three BSL-4 labs in China that are either in the final stages of being commissioned or have recently begun research operations: the Wuhan Institute of Virology (CAS), the Harbin Veterinary Research Institute (CAAS), and the Kunming Institute of Medical Biology (Chinese Academy of Medical Sciences). This and previous meetings organized by NAS and CAS have laid the foundations for future exchanges and projects, both scientific and practical, between the leaders and researchers at these laboratories. Sharing advice and experiences and creating ties between institutions and personnel that operate these facilities is crucial to ensuring the safe operations of the laboratories and to prevent the misuse or accidental release of dangerous pathogens. GNL has negotiated bilateral memoranda of understanding (MOUs) between the GNL and the three Chinese BSL-4 labs so they can more easily work together on short-term and long-term joint projects.

One of the most important aspects of the meeting was to identify potential opportunities for collaboration between scientists in China and the U.S., two countries that account for some of the world's most important medical discoveries. Participants outlined some broad areas for further collaboration between the countries: (1) Issues related to emerging infections, understanding disease reservoirs, disease emergence, disease surveillance, natural versus human origin of disease, data sharing, and building capacity for research during public health emergencies. (2) Anticipating risk in the life sciences and medicine related to biotechnology innovation, such as identifying certain experiments that are too risky to conduct. (3) Governance of science to reduce risk—technical risk for the laboratory and societal risk for the community—such as doing research in centralized laboratories versus distributed labs, managing research costs, learning from past technical and bureaucratic mistakes, and ways to diminish risk without hindering science. (4) Training opportunities to ensure the safe and secure conduct of research in biocontainment and also the efficient and effective maintenance of high-containment laboratories to ensure the safety of the workforce as well as the surrounding community and environment.



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The experts identified several areas for promising future discussion in the NAS and CAS dialogue including creating joint BSL-4 research projects that are beneficial for both the United States and China, better ways to share samples, strains, and strain collections between laboratories, and improving and sharing best practices for training laboratory staff, particularly staff that work at the BSL-4 level. The group will work to build on this workshop and continue these discussions, subsequent meetings are planned to take place in China in 2018 and early 2019.

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- Nipah virus
- Ecology and Epidemiology of Recent Highly Pathogenic Avian Influenza Outbreaks

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- Coronaviruses



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- Interplay between coronaviruses and nonsense-mediated mRNA decay pathway
- Mammalian RNAi antiviral and host-virus interaction

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- Training program for facilities operations
- National biosafety laboratory in China
- Next Steps: Building on the workshop for future cooperation and collaboration

Points of contact:


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Director, Wuhan Branch, CAS
yzm@wh.iov.cn

**David R. Franz, DVM, Ph.D**.
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**Benjamin J. Rusek**, Senior Program Officer
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utmb Health
Galveston National Laboratory

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**P** 409.266.6500 **F** 409.266.6810

*Itinerary*
***Zhiming Yuan, Ph.D.***
***Director***
***BSL-4 Laboratory at Wuhan Institute for Virology***
***Chinese Academy of Sciences***

**August 5-8, 2018**

## Sunday, August 5

| | | |
|---|---|---|
| | Leisure with Pei-Yong Shi, PhD. and Colleagues | |

## Monday, August 6

| | | |
|---|---|---|
| 8:30-12 noon | **Meet with:**<br>James Le Duc, PhD.<br>**Professor**<br>**Department of Microbiology & Immunology**<br>**Director, Galveston National Laboratory**<br><br>**Meet with:**<br>Pei-Yong Shi, PhD.<br>**I.H. Kempner Professor of Human Genetics**<br>**Vice Chair for Innovation and Commercialization**<br>**Department of Biochemistry & Molecular Biology** | 6.200C |
| 12:00-1:30 | **Informal seminar, "An Introduction to the Wuhan National Biosafety P4 Laboratory of the Chinese Academy of Sciences"**<br><br>**Lunch provided** | 1.100D, GNL |
| 1:30-5:00 | Meet with Pei-Yong Shi, PhD. lab members (Pei-Yong Shi, PhD. – Laboratory) | MRB, 5.136 |
| 6:00 pm | **Dinner by invitation (Dr. Le Duc's home)** | Galveston, Texas |

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## Tuesday, August 7

| | | |
|---|---|---|
| 8:30-9:30 | Discussions with Dr. Dennis Bente re:CCHF collaborations | 5.200E, GNL |
| 9:30-10:30 | Tour of GNL tick facility and Keiller insectary; discussion of research on ticks and hosts in BSL4 | |
| 10:30-11:30 | Discussions with Alex Freiberg on Nipah and other bat-borne viruses | |
| 12:00-1:00 | **Lunch – Olympia Grill (Le Duc, Shi, Bente, Freiberg and Zhiming)** | 100 21 st Street Galveston, Texas |
| 1:00-5:00 | Visit clinical biocontainment facilities<br>**Chad Connally, RN, BSN, CEN**<br>**Biocontainment and Emergency Management Program Manager**<br><br>Training of clinical care and transport personnel<br>**Corrie Ntiforo, MSPH, RBP**<br>**Biological Safety**<br>**Environmental Health and Safety**<br><br>Visit to biocontainment training facility<br>**Jet Newton MS, RBP**<br>**Senior Safety Consultant**<br>**Biological & Chemical Safety- EHS** | |
| 6 pm | **Dinner – Fisherman's Wharf** | Galveston, Texas |

## Wednesday, August 8

| | | |
|---|---|---|
| 8:30-12:00 | One-on-one meetings with UTMB Investigators | GNL |
| 8:30-9:00 | **Meet with:**<br>Miguel A. Grimaldo L., M. Eng.<br>**Assistant Professor, Department of Pathology**<br>**Director of Institutional Biocontainment Resources** | 6.200U |
| 9:00-9:30 | **Meet with:**<br>David W. C. Beasley, PhD.<br>**Sealy Institute for Vaccine Development, ORNcs** | 6.200C |
| 9:30-10:00 | **Meet with:**<br>**Slobodan Paessler, PhD.**<br>**Professor**<br>**Department of Pathology**<br><br>**Meet with:**<br>**Cheng Huang, PhD**<br>**Assistant Professor**<br>**Department of Pathology** | 6.200C |

| | | |
|---|---|---|
| 10:00-10:30 | **Meet with:**<br>Kent Tseng, PhD.<br>**Professor**<br>**Department of Microbiology & Immunology** | 6.200U |
| 10:30-11:00 | **Meet with:**<br>Tian (Tina) Wang, PhD.<br>**Professor**<br>**Department of Microbiology & Immunology**<br>**Department of Pathology** | 6.200U |
| 11:00-11:30 | **Meet with:**<br>Thomas Ksiazek, PhD.<br>**Professor**<br>**Department of Pathology** | 6.200U |
| 11:30-12:00 | **Meet with:**<br>Yingzi Cong, PhD.<br>**Professor of Microbiology, Immunology and Pathology**<br>**Department of Microbiology and Immunology** | 6.200U |
| 12:00-1:00 | **Lunch** | 6.200U |
| 1:00-3:00 | Next steps discussion with Dr. Le Duc and Dr. Pei-yong Shi | |
| 3:00 | Depart UTMB for Houston | |

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PLOS | NEGLECTED TROPICAL DISEASES

RESEARCH ARTICLE

# Phylogenomic analysis unravels evolution of yellow fever virus within hosts

**Chen Chen[1,2☯]*, Dong Jiang[1,2☯], Ming Ni[3☯], Jing Li[4☯], Zhihai Chen[1,2,5], Jingyuan Liu[6], Hanhui Ye[7], Gary Wong[4], Wei Li[4], Yuanyuan Zhang[1,2], Beibei Wang[1,2], Yuhai Bi[4], Danying Chen[1,2], Ping Zhang[7], Xuesen Zhao[1,2], Yaxian Kong[1,2], Weifeng Shi[8], Pengcheng Du[1,2], Gengfu Xiao[9], Juncai Ma[4], George F. Gao[4,10,11], Jie Cui[9], Fujie Zhang[1,2,5], Wenjun Liu[4,10], Xiaochen Bo[3], Ang Li[1,2]*, Hui Zeng[1,2]*, Di Liu[4,9,10]***

**1** Institute of Infectious Diseases, Beijing Ditan Hospital, Capital Medical University, Beijing, China, **2** Beijing Key Laboratory of Emerging Infectious Diseases, Beijing, China, **3** Beijing Institute of Radiation Medicine, Beijing, China, **4** Institute of Microbiology, Chinese Academy of Sciences, Beijing, China, **5** Clinical Center for Infectious Diseases, Beijing Ditan Hospital, Capital Medical University, Beijing, China, **6** Department of Intensive Care Unit, Beijing Ditan Hospital, Capital Medical University, Beijing, China, **7** Fuzhou Infectious Disease Hospital of Fujian Medical University, Fuzhou, China, **8** Institute of Pathogen Biology, Taishan Medical College, Taian, China, **9** Wuhan Institute of Virology, Chinese Academy of Sciences, Wuhan, China, **10** Medical School, University of Chinese Academy of Sciences, Beijing, China, **11** Chinese Center for Disease Control and Prevention (China CDC), Beijing, China

☯ These authors contributed equally to this work.
* liud@wh.iov.cn (DL); zenghui@ccmu.edu.cn (HZ); chenchen1@ccmu.edu.cn (CC); liang@ccmu.edu.cn) (AL)

Check for updates

OPEN ACCESS

**Citation:** Chen C, Jiang D, Ni M, Li J, Chen Z, Liu J, et al. (2018) Phylogenomic analysis unravels evolution of yellow fever virus within hosts. PLoS Negl Trop Dis 12(9): e0006738. https://doi.org/10.1371/journal.pntd.0006738

**Editor:** Eric Mossel, Center for Disease Control and Prevention, UNITED STATES

**Received:** May 22, 2018

**Accepted:** August 7, 2018

**Published:** September 6, 2018

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**Data Availability Statement:** All reads from next generation sequencing were deposited in the NCBI SRA database under the accession no. SRP096859. The genome sequences of YF-BJ1, YF-BJ2, YF-BJ3, YF-BJ4, YF-BJ5, YF-FZ2, YF-FZ4, YF-FZ6, and YF-FZ7 were deposited in the NCBI GenBank database under the accession no. MH633684-MH633692.

**Funding:** This work was supported by Grants from China Ministry of Science and Technology Key Research and Development Program [2016YFC1200804 and 2016YFC1200805], Beijing

## Abstract

The yellow fever virus (YFV) recently reemerged in the large outbreaks in Africa and Brazil, and the first imported patients into Asia have recalled the concerns of YFV evolution. Here we show phylogenomics of YFV with serial clinical samples of the 2016 YFV infections. Phylogenetics exhibited that the 2016 strains were close to Angola 1971 strains and only three amino acid changes presented new to other lineages. Deep sequencing of viral genomes discovered 101 intrahost single nucleotide variations (iSNVs) and 234 single nucleotide polymorphisms (SNPs). Analysis of iSNV distribution and mutated allele frequency revealed that the coding regions were under purifying selection. Comparison of the evolutionary rates estimated by iSNV and SNP showed that the intrahost rate was ~2.25 times higher than the epidemic rate, and both rates were higher than the long-term YFV substitution rate, as expected. In addition, the result also hinted that short viremia duration of YFV might further hinder the evolution of YFV.

## Author summary

The first importation of infections into China in 2016 and the following outbreaks in Africa and Brazil of yellow fever virus (YFV) have raised again the concerns of the potential viral spread into new territories. In this study, we aimed to know the evolution dynamics of YFV by using intrahost phylogenomics and to assess the risk of virus epidemics. Through deep sequencing of consecutive samples from 12 patients, we identified

UNCORRECTED PROOF



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Municipal Science & Technology Commission [Z161100000116049], National Natural Science Foundation of China [91631110 and U1435222], Beijing Municipal Administration of Hospitals Clinical Medicine Development of Special Funding Support [ZYLX201602NHFPC], Beijing Nova program [Z161100004916101, Z181100006218114], Beijing 215 Project [2015-3-107], China National Grand S&T Special Project [2016ZX10004222-010], and '863' Program [2015AA020108 & 2014AA021501]. WS is supported by the 'Taishan Scholar' project of Shandong Province. GW is supported by the President's International Fellowship Initiative from CAS. JC is supported by CAS Pioneer Hundred Talents Program. GFG is a leading principal investigator of the NSFC Innovative Research Group [81321063]. DL is supported by National Program for Support of Top-notch Young Professionals. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**Competing interests:** The authors have declared that no competing interests exist.

hundreds of genomic variations (iSNVs and SNPs), and noticed the nearly linear accumulation of variations within individuals. The estimated evolutionary rate within host is much higher than the epidemic evolutionary rate. In comparison with Dengue virus (DENV) and Zika virus (ZIKV), which share similar host vectors (Aedes spp.), life cycles, mutation rates and replication strategies to YFV, the lower epidemic evolutionary rate of YFV might have been hindered by the shorter viremia duration, which decreased the accumulated variations to get into the transmission cycle.

## Introduction

Yellow fever is a notorious mosquito-borne viral disease emerged during the 15th-19th centuries in the Americas, Africa and Europe, causing severe hemorrhagic fever and liver injury with high mortality rates. Although control of mosquitoes and the use of the live-attenuated yellow fever virus (YFV)-17D vaccine strain have effectively prevented and controlled the epidemics, YFV is estimated to cause approximately 30,000 deaths out of 200,000 infections annually worldwide, mostly in Africa (https://www.cdc.gov, last accessed 10th April, 2018). The etiological agent of the disease, YFV, is a single-stranded, positive-sense RNA virus with a ~11 kb genome in length. The virus is frequently transmitted between nonhuman primates and mosquitoes in African and American jungles, known as the sylvatic cycle [1]. Occasionally, YFV can escape from the sylvatic cycle to infect humans, with subsequent transmission between mosquitoes and humans, forming the urban cycle. Historically, YFV was believed to have originated from Central or East Africa, and transmitted to America during the slave trade [2, 3]. Currently, YFV in Africa and America are classified into seven genotypes, with two in South America, two in West Africa, and three in East and Central Africa [2, 4, 5].

In January 2016, the Ministry of Health of Angola notified the World Health Organization (WHO) of a yellow fever disease outbreak: as of October 28th, 4,347 suspected cases, including 377 deaths, were reported from all 18 provinces of Angola. Outbreaks were also reported simultaneously from the Democratic Republic of Congo and Uganda [6]. In February 2018, 464 confirmed human cases of yellow fever have also been reported in Brazil, with 154 deaths (http://www.who.int, last accessed 10th April, 2018). Meanwhile, epizootics have expanded to areas previously not considered at risk for yellow fever (http://www.who.int/csr/don/27-february-2018-yellow-fever-brazil/en/, last accessed on 10th April, 2018). All these recalled the concerns of the evolution of YFV. During the Angolan outbreak, YFV infections were detected in Chinese workers returning to China from Angola [7–9], marking the first time YFV infections were documented in Asia. By using the consecutive samples from those imported YFV patients, we unraveled the intrahost and epidemic evolutionary dynamics of YFV.

## Results

### Deep sequencing of YFV genomes from clinical samples

We collected samples from twelve out of thirteen YFV patients in China, sequenced the viral genomes, and performed analyses on phylogenetics, sequence comparison, and intra-host dynamics. Of the 12 patients, two had severe disease, in which one survived (YF-BJ3) and one died (YF-BJ1), and the remaining patients displayed mild symptoms (*Table 1*). We tested blood samples of these 12 patients from the first day of admittance to the hospital by real-time reverse transcription PCR (RT-PCR), and four patients (YF-BJ1, YF-BJ2, YF-BJ3, and YF-BJ5)



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**Table 1. Patient information, viral loads and genome sequencing in urine samples.**

| Patient ID | Group | Outcome | Days after onset | Blood virus (Ct)* | Urine virus (Ct)* | Sequencing Method† | # Total Reads | # Mapped Reads | Sequencing Depth (x1000) | Genome Coverage (bp) |
|---|---|---|---|---|---|---|---|---|---|---|
| YF-BJ1 | Severe | Death | 7 | 31 | 35.00 | AS | 1,695,368 | 1,509,993 | 30.98 | 10,732 |
| | | | 8 | 32 | 33.50 | AS | 1,661,085 | 953,063 | 19.67 | 10,650 |
| | | | 9 | 30 | 34.42 | AS | 3,303,973 | 1,952,626 | 35.68 | 10,725 |
| YF-BJ3 | Severe | Discharged | 8 | 27.6 | 25.60 | AS | 1,451,105 | 1,354,090 | 27.59 | 10,650 |
| | | | 10 | 27.9 | 25.32 | AS | 1,941,400 | 2,369,835 | 18.97 | 10,393 |
| | | | 14 | - | 32.85 | AS | 4,034,937 | 2,896,728 | 55.05 | 10,405 |
| | | | 17 | - | 31.66 | AS | 1,998,163 | 1,861,631 | 36.67 | 10,397 |
| | | | 20 | - | 34.00 | AS | 1,920,032 | 1,416,518 | 28.89 | 10,393 |
| | | | 32 | - | 39.83 | AS | 1,424,735 | 1,014,331 | 21.80 | 10,650 |
| YF-BJ5 | Mild | Discharged | 6 | 23.84 | 23.77 | AS | 2,001,845 | 1,866,672 | 37.82 | 9,918 |
| | | | 10 | - | 35.42 | AS | 1,684,026 | 1,014,398 | 18.54 | 10,674 |
| | | | 14 | - | 34.23 | AS | 2,090,128 | 840,862 | 16.56 | 10,394 |
| YF-BJ2 | Mild | Discharged | 16 | - | 30.95 | AS | 1,924,145 | 1,467,048 | 30.21 | 10,398 |
| YF-BJ4 | Mild | Discharged | 19 | - | 35.86 | TS | 56,728,408 | 873 | 0.02 | 10,393 |
| YF-FZ1§ | Mild | Discharged | 6 | - | - | TS | 77,238,978 | 129 | 0.00 | 1,012 |
| YF-FZ2¶ | Mild | Discharged | 16 | - | 39.37 | TS | 58,570,126 | 195 | 0.00 | 10,243 |
| YF-FZ3§ | Mild | Discharged | 14 | - | 39.40 | TS | 50,462,246 | 68 | 0.00 | 839 |
| YF-FZ4¶ | Mild | Discharged | 7 | - | - | TS | - | - | - | 10,222 |
| YF-FZ5§ | Mild | Discharged | 6 | - | - | TS | 56,605,654 | 68 | 0.00 | 2,795 |
| YF-FZ6¶ | Mild | Discharged | 20 | - | 32.44 | TS | 51,627,058 | 4,722 | 0.11 | 10,838 |
| YF-FZ7¶ | Mild | Discharged | 15 | - | 33.96 | TS | 64,345,650 | 3,945 | 0.09 | 10,311 |

* "-", Negative results (Ct value >45).

† AS, amplicon sequencing; TS, total RNA sequencing.

¶ Genome sequence assembled with Sanger sequencing data.

§ Genome sequence failed to assemble.

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were positive. YFV RNA fragments could be detected in serum until 9, 12, 10 and 6 days, respectively, after the onset of symptoms (*Table 1, and Fig 1*). Urine samples from all patients were also tested by real-time RT-PCR and all were YFV-positive. Higher viral RNA loads were observed in the urine compared to the blood samples, except for those from the non-survivor (*Table 1*). The virus persisted in urine samples for at least 15 days after the onset of symptoms in survivors. Specifically, the urine sample from patient YF-BJ3 was still PCR-positive when tested 32 days after the onset of symptoms (*Table 1 and Fig 1*). We then sequenced all available YFV-positive samples by using total RNA sequencing, amplicon sequencing and/or Sanger sequencing (*Table 1 and S1 Table*). In total, nearly complete virus genomes (>10,222 bp) from 9 patients, and deep-sequenced genome datasets (average sequence depth 34,978x) from 3 patients covering 12 time points were obtained (*Table 1*).

## Phylogenetics and sequence comparison of YFV genome

We performed phylogenetic analysis of the coding region of YFVs (*S2 Table*), and found that YFV sequences from the returning workers in 2016 outbreak closely clustered with the 1971 Angola strains (*Fig 2A*). Of note, the vaccine strain YF-17D and its derivatives are located far from the Angola strains on the phylogenetic tree. A closer inspection of the Angola strains shows that the viruses in the 2016 outbreak are likely from a single origin and genome sequences from both severely ill patients are closely clustered (*Fig 2B*). There are 188

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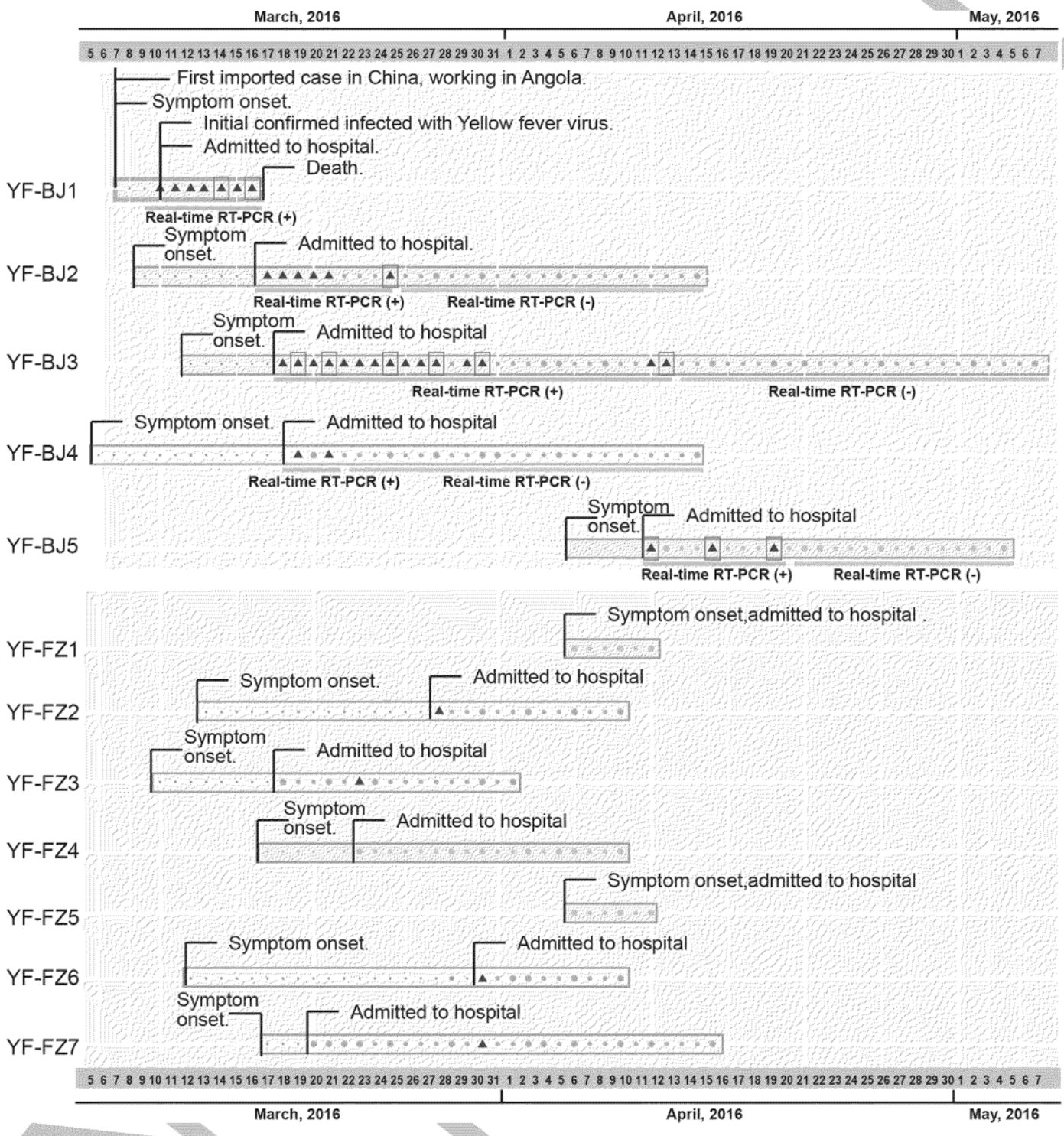



**Fig 1. Cases of twelve documented returning YFV patients in China by date in the outbreak of Angola from March to May 2016.** Timeline of events for each patient from symptom onset to the leaving from hospital are shown with different molecular detection methods and results. Dots denote that we did not get any positive molecular detection result in the time point, grey (before admitted to hospital) and blue (after admitted to hospital). The larger size dots means that we performed Real-time PCR detections in that point. The red triangles denote the sample have been detected by PCR and Real-time PCR with positive results. Blue boxes denote the samples that were subjected to NGS analysis at the time points.

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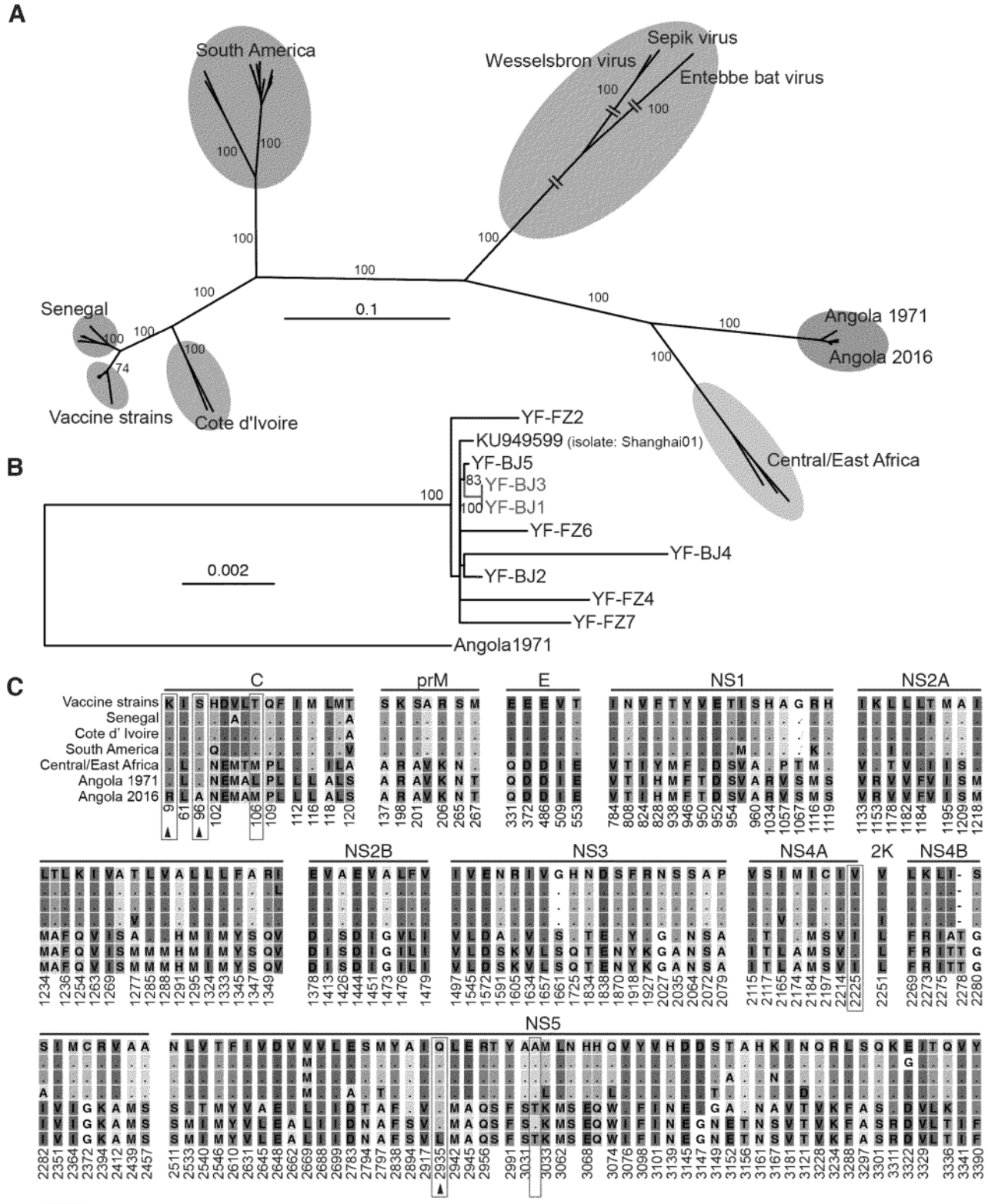



**Fig 2. Phylogenetics and amino acids comparison of YFV viruses.** (A) Maximum-likelihood phylogenetic tree of YFV genome sequences (the first 130 bps in 5'-UTR and the last 370 bps in 3'-UTR sequences are excluded). Bootstrap support values are shown along the branches. Wesselsbron virus, Sepik virus and Entebbe bat virus were used as outgroup. (B) Maximum-likelihood phylogenic tree of YFV genomes of Angola 2016 strains. Viruses from patients with severe



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disease are highlighted in magenta. Angola 1971(GenBank accession AY968064) was used as outgroup. Bootstrap support values (≥70) are shown. (C) Comparison of amino acids of YFV consensus sequence of each lineage in (A). Identical amino acid to vaccine strain (17D) lineage is denoted as a dot. The amino acid sites that were different in Angola 2016 and 1971, are highlight with red rectangles. Novel amino acid substitutions of Angola 2016 are denoted by arrowheads.

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nucleotide substitutions between the 1971 and 2016 consensus sequences and 6 of them are nonsynonymous substitutions. Comparison of the YFV polypeptides shows that only three amino acid changes that appear to be specific to the Angola 2016 strains (*Fig 2C*). Two are in the capsid protein and one in NS5, the RNA-dependent RNA polymerase. The above data are consistent with previous studies using partial or whole genomes showing that YFV exhibits a slow evolutionary rate [2, 4, 10].

## Intrahost genomic variations

For the deep-sequenced samples, we identified the iSNVs by using methods in a previous study for Ebola virus [11]. The mean sequencing depth of genomic regions is between 16,558x and 37,822x (*Fig 3A*), and a total of 101 iSNV sites were discovered, including 69 in the coding region and 32 in non-coding regions. In each iSNV sites, we only found two types of nucleotides. Taken that sequencing errors are generated randomly and may result in multi-nucleotide heterogeneity in a single site, it is unlikely those iSNVs were the results of sequencing bias. Fewer iSNVs appeared in the 1$^{st}$ and 2$^{nd}$ codon positions than the 3$^{rd}$ codon position (*Fig 3B*) and fewer non-synonymous than synonymous iSNVs (*Fig 3C*), implying that the coding region is generally under purifying selection. We plotted the distributions of all mutated allele frequencies of the iSNVs (*Fig 3D and 3E*). The mean mutated allele frequency of non-coding iSNVs was at 0.18, while that of synonymous and non-synonymous iSNVs was 0.12 and 0.09, respectively (*Fig 3D*). Among them, non-synonymous iSNV is significantly lower than that in non-coding regions (P = 0.02). The distribution of non-coding iSNVs is close to the expected neutrality, whereas curves of synonymous and nonsynonymous iSNVs have higher portions of iSNVs in the area of low mutated allele frequencies (*Fig 3E*). This further supports the notion that the coding region was under purifying selection. Additionally, we discovered two variant types at the 3' untranslated region (UTR), one with 5 concurrent iSNVs (G10360A, U10365G, C10367U, G10373A, and U10398C) and the other having an additional iSNV (A10425G) (*Fig 3F*). Phasing analysis reveals that these iSNVs tend to be concurrent in the same reads (*S3 Table and S1 Fig*). Predicted RNA structure shows that these substitutions are likely to affect the structure of the 3' UTR (*Fig 3F and S2 Fig*), and probably influence viral replication in hosts [12–14].

## Intrahost evolutionary dynamics

Subsequently, we placed all of the polymorphic sites (SNPs and iSNVs) along viral genome (*Fig 4A*). SNPs and iSNVs described the viral difference in two levels of between and within the host, respectively. Within 65 days (from the onset of the first patient to the sample of the last patient in this study) we observed a total of 234 SNPs in 148 sites in 18 samples, including 102 synonymous and 117 nonsynonymous SNPs. A total of 138 SNPs that appear only once are scattered along the viral genome. In particular, the SNPs of 5 sites (T900A, A2352C, C3918A, G6463A, and A7320G) could be used to characterize the 2016 YFVs. All five SNPs were detected in seven patients, four SNPs (without A7320G) were detected in one patient (YF-BJ5), and only one patient (YF-BJ3) does not possess any of the above SNPs (*Fig 4A*). Of note, YF-BJ1 is likely a co-infection with more than one YFV variant, as the virus genome on day 7 does not contain any of the five SNPs, whereas all five SNPs became dominant on day 8

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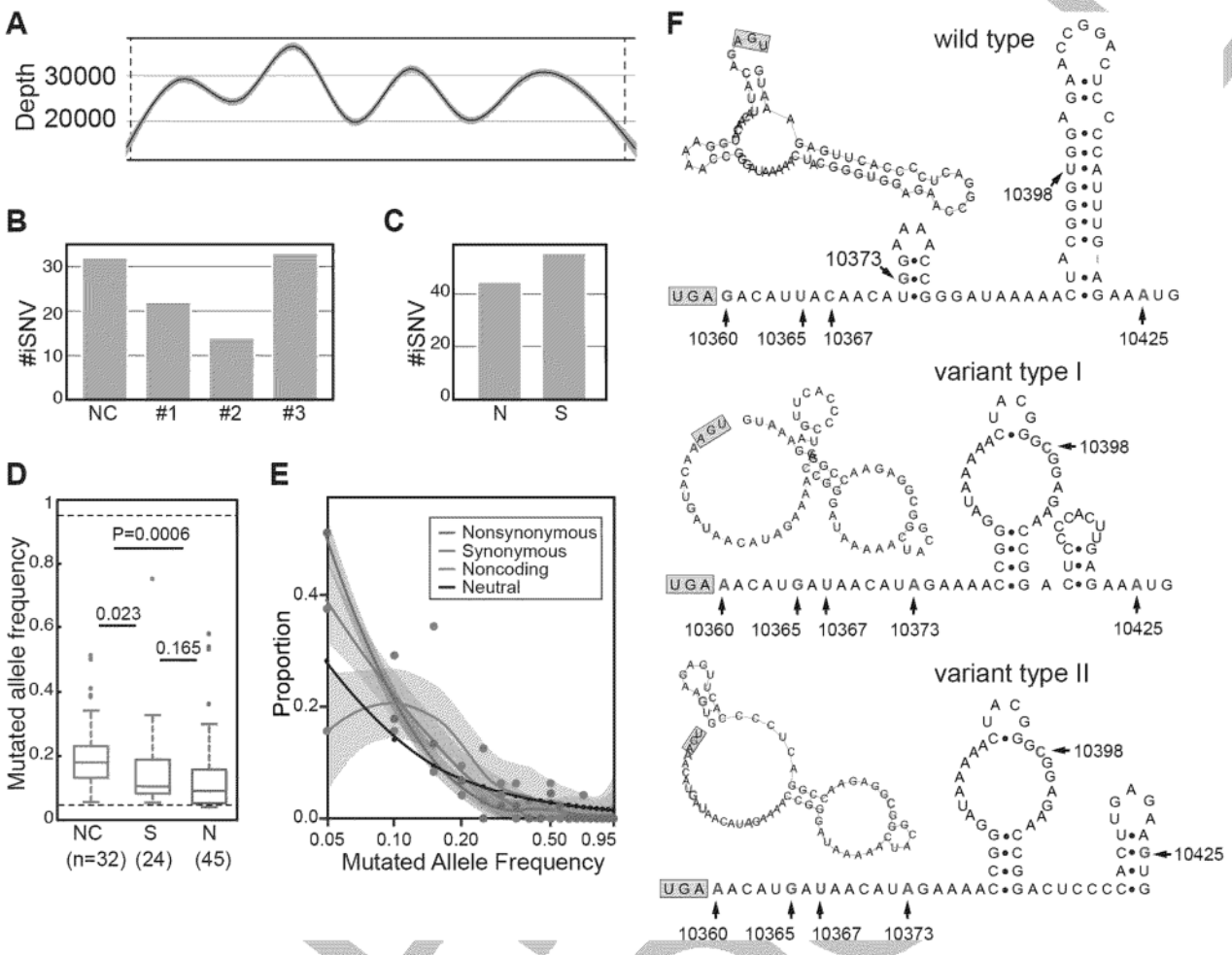



**Fig 3. The iSNVs of Angola 2016 YFVs from clinical samples.** (A) Sequencing depth across the sequenced genomes. The x-axis represents the YFV genome, with the ORF boundaries indicated by vertical dashed lines. The sequencing depth smoothed by locally weighted smooth regression (LOESS) is shown by the black curve, with 95% confidence interval as shown by shadow. (B) Numbers of iSNVs at non-coding (NC) regions and codon positions of the ORF. (C) Numbers of nonsynonymous (N) and synonymous (S) iSNVs of the ORF. (D) Box plots of the mutated allele frequencies (MuAFs) for non-coding, synonymous and nonsynonymous iSNVs. The MuAF values of three kinds of iSNVs are compared to each other with the Wilcoxon rank-sum test. Dashed lines denote the boundaries of MuAFs in iSNV identification. Boxes denote the interquartile range (IQR) between the first and third quartiles. Lines inside the boxes indicate the median, and the lines outside boxes represent values within 1.5 times the IRQ. Outliers are denoted as dots. (E) Comparison of MuAF spectra of non-coding, synonymous and nonsynonymous iSNVs. MuAFs (dots) are shown with LOESS lines (95% confidence interval in shadow). Expectation under neutral selection is shown by a black line. (F) Prediction of RNA secondary structures of 3' UTR (with 68 bp downstream of the stop codon). The earliest type of Angola 2016 (wildtype) and two nucleotides variant types are illustrated. Six variation sites are highlighted in magenta. The stop codon is in the gray box.

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(*Fig 4A*). Of the five SNPs, only G6463A is nonsynonymous, resulting in an amino acid substitution from Val to Ile in NS4A.

We then compared the accumulation of polymorphic sites within patients over time (*Fig 4B*). Each sample was set to days after onset of the symptoms of the patients it belongs to. Linear regressions revealed that iSNVs and SNPs accumulated from the day of onset of symptoms at a rate of 0.72 and 0.32 variations/day, respectively. The accumulation rate of iSNV may reflect the intrahost evolution. Intriguingly, the YFV evolutionary rate within humans is roughly 2.48 $x10^{-2}$ variations/site/year (0.72 variations/day = 0.72 x 365 / 10591 variations/site/year = 2.48 $x10^{-2}$ variations/site/year) in our study. Of those synonymous variations, the intrahost evolutionary rate estimated by iSNV was 0.7 $x10^{-2}$ variations/site/year; whereas the rate of



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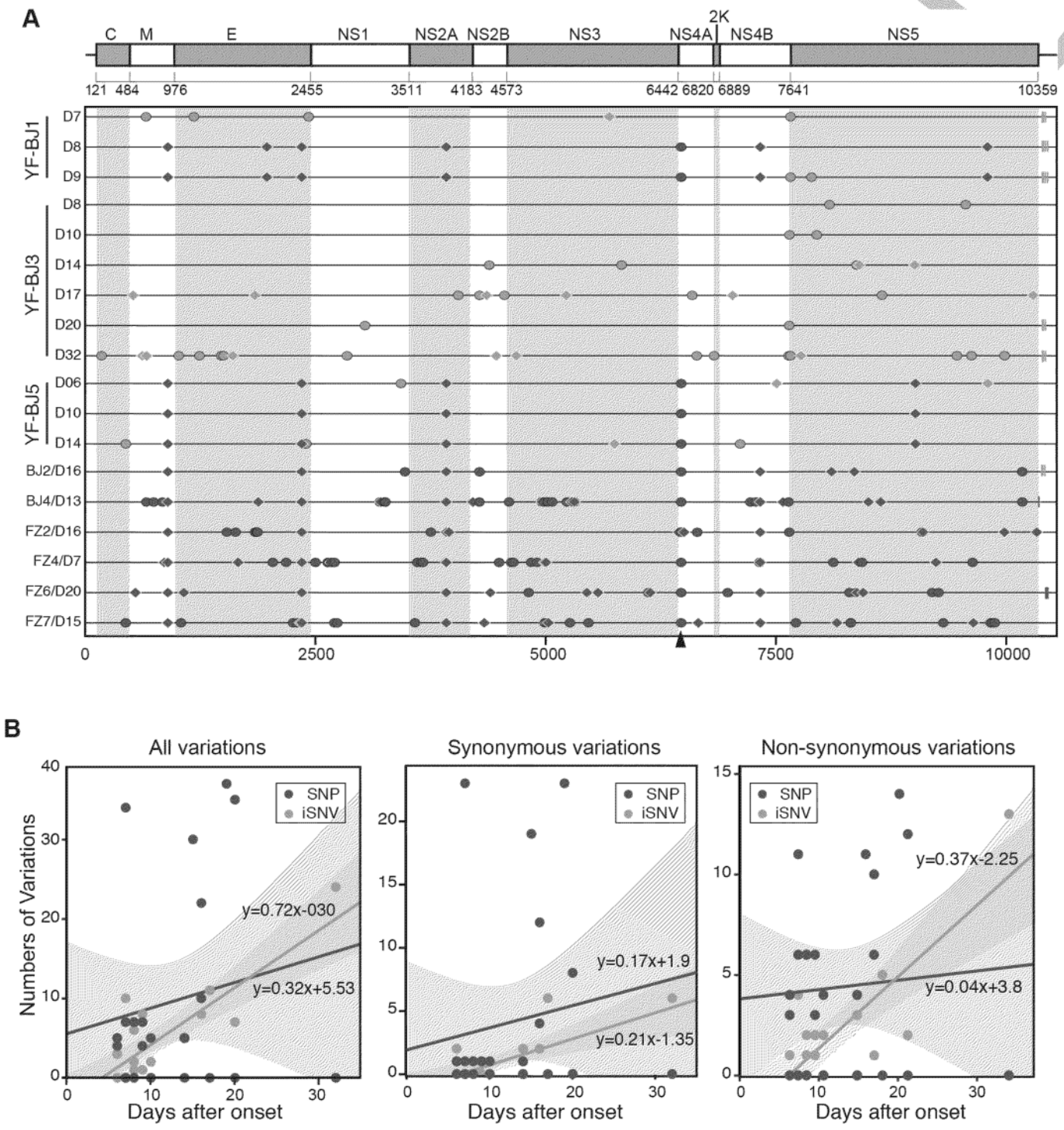



**Fig 4. Genomic variations of Angola 2016 YFV and evolutionary model.** (A) SNPs (in magenta) and iSNVs (in cyan) detected in Angola 2016 strains along the YFV genome. Non-synonymous variations are denoted by ovals, synonymous variations by diamonds, and variations in UTR by rectangles. Non-synonymous site at 6463 is denoted by arrowhead. YFV genome of patient YF-BJ1 at 7 days after the onset (YF-BJ1/7D) was used as the reference sequence (B) Linear regression of number of variations at all variations, synonymous and non-synonymous sites accumulated by days after onset. The smooth shadows denote the LOESS fit with 95% confidence interval.

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nonsynonymous variations was 1.2 x$10^{-2}$ variations/site/year. Compared to the rates estimated by SNP within these samples, the intrahost evolutionary rate of non-synonymous sites (by iSNV) is higher, while the rate of synonymous sites is slightly higher than the SNP rate.

## Discussion

The evolution of YFV is a basic question to unfold to predict the risk of the pathogens. Phylogenetic analysis suggests that the 2016 outbreak is likely the spillover of YFV viruses in Angola. The high identity between the 2016 strain and the vaccine strain YF-17D would explain the effectiveness of the current vaccines [15]. Although there is a diversity of yellow fever virus genotypes, all genotypes are indistinguishable in serological assays. There is not any evidence that the Angola outbreak was due to immune escape and it is accepted among public health professionals that low vaccine coverage in the area prior to the outbreak was the driving factor and the resulting aggressive mass vaccination campaign was vital for containing and eliminating the outbreak. However, it should be noted that the Angola lineage is clearly diversified from the 17D-lineage (*Fig 2A*). Moreover, we did notice 6 patients who had been vaccinated before [16], but the limited clinical and epidemiological data cannot tell the immune escape. Future immune escape from current YFV vaccines cannot be fully ruled out, although YFVs evolve very slowly [17]. A novel vaccine based on a Central/East African YFV isolate would be necessary as a complement vaccine to prepare against future outbreaks. Owing to the slow evolutionary rate and the past experience of using YF-17D, the novel vaccine is likely to work for another decades.

Virus evolution experienced mutation, selection within and between hosts, genetic drift, and transmission, and finally formed the genetic variants (lineages) over long-term evolutionary timescales [18]. Deep sequencing of viral genomes can provide novel insights into viral evolutionary dynamics [19, 20]. In this intra-host phylogenomic analysis, we depicted the evolutionary process of YFV from intra-host to epidemic substitutions, which were reflected by iSNV to SNP, respectively. Other than using the metric for long-term evolutionary rate, we applied the substitution/site/day to measure the intrahost evolutionary rate, which appeared more appropriate in outbreak or epidemic scenarios. Short-term rates may provide better insight into the intrahost YFV evolutionary trends (e.g., iSNV dynamics over time) relative to clinical symptomology, severity, and/or co-morbidities. However, further in vitro or in vivo studies would be needed. The estimated evolutionary rates clearly showed that the intrahost evolutionary rate is ~2.25 times higher than that of the epidemic evolutionary rate, and both are much higher (59 times and 26 times) than the substitution rate of YFV along longer evolutionary timescale [10]. The higher rate estimated by iSNV reflected purifying selection occurred, when intrahost variations were spreading out. The rates estimated by synonymous iSNV and SNP, respectively, were similar, implying that the synonymous variations were under similar evolutionary constraints during transmission. Meanwhile, only a few of the non-synonymous variations were spread out in different individuals, and only G6463A (Val to Ile in NS4A) were detected in multiple samples.

Compard to Dengue virus (DENV) and Zika virus (ZIKV), YFV shares similar host vectors (*Aedes spp*.) and life cycles [21, 22], and has similar mutation rates and replication strategies [10, 23, 24]. However, the long-term substitution rates of DENV (0.77–0.99 x$10^{-3}$ substitutions/site/year) and ZIKA (0.98–1.06 x$10^{-3}$ substitutions/site/year) are higher than that of YFV [23, 25, 26]. Previous studies have posed that additional constrained selection forces are probably existed [10, 27]. Based on our results, we noticed the nearly linear accumulation of variations within individuals. Since the transmission of YFV variants needs mosquitoes as vector, the window (viremia duration) that mosquitoes could use is essential, as only the variations

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within this period could be brought into the transmission cycle. A wider window size would possibly allow more non-synonymous variations into the transmission cycle (Cristina Domingo, EID, 2018). Given that YFV, DENV and ZIKV have ~3, 3–7, and 4–7 days of viremia, respectively, after symptom onset (*S4 Table*), it hinted that the shorter viremia duration in YFV infections might result in the lower evolutionary rate than that of DENV or ZIKV. Most Flaviviruses are vector-borne viruses and the evolutionary rates in mammals and arthropods are different. The intrahost analysis of virus could show the short-term evolution dynamics, especially from serial samples, and this would be a helpful complement to the long-term evolution of Flaviviruses.

In addition, deep sequencing and viral genomics have provided new insights into viral evolutionary dynamics, especially in a continuous mode. Our findings strongly suggest that the urine samples should be considered for the clinical diagnosis, genomic surveillance, and large-scale screening of YFV infections. Further investigations are also encouraged to characterize the factors that maintain viremia in vector-borne diseases.

## Methods

### Ethics statement

The study was conducted in accordance with the Declaration of Helsinki and approved by the ethics committee of Beijing Ditan Hospital (DSRB 2008/00293 and DSRB 2013/00209). Clinical samples and information were obtained after written informed consents from all participants. All human subjects were adult, and if not, whether a parent or guardian of any child participant provided informed consent on their behalf.

### Patients and data collection

Twelve YFV patients returning from Angola were recruited (*Table 1*). We sampled urine and blood sample of these patients from routine examination and collected their clinical data.

### YFV diagnosis and virus detection

YFV infection was diagnosed according to the WHO guidelines (http://www.who.int/csr/disease/yellowfev/case-definition/en/, last accessed 10$^{th}$ April, 2018), including clinical symptoms (fever, headache, jaundice, etc.) and laboratory detection of virus by using real-time RT PCR. The serum and urine samples were collected and stored at -80 $^{o}$C for further analyses, including real-time RT-PCR, PCR sequencing and RNA sequencing.

### Next generation sequencing

RNA from both serum and urine samples were deployed to two approaches of deep sequencing. Approach I: Total RNA sequencing. RNA was purified by Trizol (Thermo Scientific) for each sample, and then total RNA libraries were constructed with 500bps insertion size using the NEBNext UltraTM RNA Library Prep Kit (NEB, MA, USA), and then sequenced by Illumina Hi-seq platform, and generated 2 × 125 bp paired-end reads. Approach II: RNA amplicon sequencing. Two sets of YFV-specific primer pairs were designed (Set A: 5 pairs, amplicon length = 2,248 ± 168 nt; Set B: 12 pairs, amplicon length = 1,018 ± 48 nt; *S5 Table*). PCR products were sequenced by Illumina platform with 2 × 250 bp paired-end reads. Quality control and error correction were implemented according to previous report [28, 29]. The probably low quality regions in sequencing, including 1) Q<30, and 2) the first 10 bps after primers were removed from the high quality sequences, according to a previous study [28]. To minimize sequencing error that affects the accuracy of iSNV calling, we only kept the read-pair that

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has >100 high quality bases in both ends as clean reads. All reads were deposited in the NCBI SRA database under the accession no. SRP096859.

## Sequence alignment and phylogenetic analysis

Consensus sequences were aligned by MUSCLE [30]. Phylogenetic analyses were performed by using RAxML v8.1.6, with the GTR model of nucleotide substitution and γ-distributed rates among sites. Phylogenetic tree was constructed by maximum-likelihood using YFV genome sequences [31]. A NJ tree based on YFV genome sequences was also constructed to show the results were robust (S3 *Fig*). A total of 1,000 bootstrap replicates were performed. All SNPs were listed in S6 *Table*. The genome sequences of YF-BJ1, YF-BJ2, YF-BJ3, YF-BJ4, YF-BJ5, YF-FZ2, YF-FZ4, YF-FZ6, and YF-FZ7 were deposited in the NCBI GenBank database under the accession no. MH633684-MH633692.

## Calling of iSNVs

To minimize the potential sequencing errors generated by system, we performed the following two steps before we called iSNV, including 1) mapping the clean reads to the reference genome, 2) qualification of the samples. For mapping the clean reads to genome, we use the traditional protocol used widely. First, we mapped the clean reads with pair-ended aligned to the assembled genome from YF-BJ1/7D by using Bowtie2 v2.2.5 [32] with default parameters. SAMtools v1.2 [33] was used to generate 'mpileup' files with no limit for the maximum site depth. To find the potential mutation in each site, we do not use the following pipeline in SAMtools. Instead, we developed homemade PERL scripts (available at http://github.com/generality/iSNV-calling/), which were used for iSNV calling to identify all potential mutations by using the mpileup files as input. The depth of bases in each sites were used to measure the mutation rate of iSNV. Then, we selected the samples with more than 3,000 sites with a sequencing depth ≥300× as candidate samples for iSNV calling. Using this protocol, we called iSNV in 15 samples in this study (S1 *Table*), and obtained the frequencies of each allele in each genome site. Interestingly, we only found the heterogeneous nucleotides with two types in each sites. Thus, we defined the nucleotide with higher frequencies as the major allele, and fewer one as the minor allele in the following iSNV calling.

The iSNV calling was according to the site depth and strand bias with following: 1) remove the ambiguous iSNV and keep the following iSNVs: (1.1) minor allele frequency of ≥5%, a conservative cutoff based on an error rate estimation described before [11, 28]; (1.2) depth of the minor allele of ≥15; and (1.3) strand bias of the minor allele less than tenfold. 2) In our analysis, these two types of nucleotides in each site have contained the nucleotide in the reference genome. Mutated allele frequencies were calculated by the rate of the reads numbers for mutated alleles to the references and total reads. Minor allele and Major allele frequencies were calculated by the majority nucleotides and minor nucleotides in each site respectively. 3) The effect of iSNV to the gene was calculated by the mutation whether caused the amino acid changes.

## Phasing analysis of iSNVs

The iSNVs that co-occurred in same viral haplotype(s) within quasispecies was defined as phased iSNVs. 1)We screened the iSNV sites along genome with a window of 250 bp, and then identified all the windows which probably containing a phasing iSNVs. 2) All the sequencing reads aligned to YFV genome region harboring these iSNVs were extracted. 3) The stretches of nearest iSNV were determined by the mutations in the reads aligned to the genome region

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with the same phasing (supporting reads > = 2). 4) The fractions of reads supporting phased and non-phased neighbor iSNVs were respectively calculated.

### Prediction of RNA secondary structures

RNA secondary structures were predicted by using RNAfold program of ViennaRNA Package 2 with default settings [34]. Nucleotide sequences started at the beginning of 3' UTRs of Angola 2016 strain. The length of window for secondary structure prediction was equal to 70 nucleotides. We also extended the additional 200 nucleotides at the end of the sequence, with a step length equal to 50.

### Accession numbers

All reads from next generation sequencing were deposited in the NCBI SRA database under the accession no. SRP096859. The genome sequences of YF-BJ1, YF-BJ2, YF-BJ3, YF-BJ4, YF-BJ5, YF-FZ2, YF-FZ4, YF-FZ6, and YF-FZ7 were deposited in the NCBI GenBank database under the accession no. MH633684-MH633692.

## Supporting information

**S1 Fig. Phasing analysis of iSNVs at the 3' untranslated region (UTR).** For a given sample, the sequencing reads aligned to 3' UTR of the YFV genome were extracted to obtain the 5-mutations (at positions 10360, 10365, 10367, 10373 and 10398) or 6-mutations (10360, 10365, 10367, 10373, 10398 and 10425). The pie charts denote fractions of reads supporting wildtype 5/6-mers and phased mutant 5/6-mers (A10360G, G10365T, T10367C, A10373G, C10398T and G10425A), with read counts (or depth, x) shown in brackets. Sample IDs are indicated at the top of pie charts.
(PDF)

**S2 Fig. RNA secondary structure prediction of the 3' UTR.** RNA secondary structures were predicted based on sequences of YFV 3' UTR (started from the stop codon) with gradually increased lengths. The sequences were extracted from genomes of wild type Angola 2016 strain and two variant types. Blue arrows indicate variations corresponding to the phased iSNV sites at 3' UTR, A10360G, G10365T, T10367C, A10373G, C10398T and G10425A. The local structural alterations due to the variations are highlighted in blue shadow.
(PDF)

**S3 Fig. Phylogenetic tree constructed by neighbor-joining method using YFV genome sequences.** Viruses from patients with severe disease are highlighted in magenta. Angola 1971 (GenBank accession AY968064) was used as outgroup. Bootstrap support values over 70 are shown.
(PDF)

**S1 Table. Sequencing data from continuous clinical samples of YFV.**
(PDF)

**S2 Table. Public sequences used for phylogenic analysis.**
(PDF)

**S3 Table. Phasing analysis for continuous neighbor iSNV.**
(PDF)

**S4 Table. Duration of viremia and evolutionary rates of YFV, ZIKV and DENV.**
(PDF)

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**S5 Table. Two primer sets used for amplicon-sequencing.**
(PDF)

**S6 Table. SNPs observed in YFV.**
(PDF)

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PLOS | NEGLECTED TROPICAL DISEASES

RESEARCH ARTICLE

# Phylogenomic analysis unravels evolution of yellow fever virus within hosts

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**Data Availability Statement:** All reads from next generation sequencing were deposited in the NCBI SRA database under the accession no. SRP096859. The genome sequences of YF-BJ1, YF-BJ2, YF-BJ3, YF-BJ4, YF-BJ5, YF-FZ2, YF-FZ4, YF-FZ6, and YF-FZ7 were deposited in the NCBI GenBank database under the accession no. MH633684-MH633692.

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## Abstract

The yellow fever virus (YFV) recently reemerged in the large outbreaks in Africa and Brazil, and the first imported patients into Asia have recalled the concerns of YFV evolution. Here we show phylogenomics of YFV with serial clinical samples of the 2016 YFV infections. Phylogenetics exhibited that the 2016 strains were close to Angola 1971 strains and only three amino acid changes presented new to other lineages. Deep sequencing of viral genomes discovered 101 intrahost single nucleotide variations (iSNVs) and 234 single nucleotide polymorphisms (SNPs). Analysis of iSNV distribution and mutated allele frequency revealed that the coding regions were under purifying selection. Comparison of the evolutionary rates estimated by iSNV and SNP showed that the intrahost rate was ~2.25 times higher than the epidemic rate, and both rates were higher than the long-term YFV substitution rate, as expected. In addition, the result also hinted that short viremia duration of YFV might further hinder the evolution of YFV.

## Author summary

The first importation of infections into China in 2016 and the following outbreaks in Africa and Brazil of yellow fever virus (YFV) have raised again the concerns of the potential viral spread into new territories. In this study, we aimed to know the evolution dynamics of YFV by using intrahost phylogenomics and to assess the risk of virus epidemics. Through deep sequencing of consecutive samples from 12 patients, we identified

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hundreds of genomic variations (iSNVs and SNPs), and noticed the nearly linear accumulation of variations within individuals. The estimated evolutionary rate within host is much higher than the epidemic evolutionary rate. In comparison with Dengue virus (DENV) and Zika virus (ZIKV), which share similar host vectors (Aedes spp.), life cycles, mutation rates and replication strategies to YFV, the lower epidemic evolutionary rate of YFV might have been hindered by the shorter viremia duration, which decreased the accumulated variations to get into the transmission cycle.

## Introduction

Yellow fever is a notorious mosquito-borne viral disease emerged during the 15th-19th centuries in the Americas, Africa and Europe, causing severe hemorrhagic fever and liver injury with high mortality rates. Although control of mosquitoes and the use of the live-attenuated yellow fever virus (YFV)-17D vaccine strain have effectively prevented and controlled the epidemics, YFV is estimated to cause approximately 30,000 deaths out of 200,000 infections annually worldwide, mostly in Africa (https://www.cdc.gov, last accessed 10th April, 2018). The etiological agent of the disease, YFV, is a single-stranded, positive-sense RNA virus with a ~11 kb genome in length. The virus is frequently transmitted between nonhuman primates and mosquitoes in African and American jungles, known as the sylvatic cycle [1]. Occasionally, YFV can escape from the sylvatic cycle to infect humans, with subsequent transmission between mosquitoes and humans, forming the urban cycle. Historically, YFV was believed to have originated from Central or East Africa, and transmitted to America during the slave trade [2, 3]. Currently, YFV in Africa and America are classified into seven genotypes, with two in South America, two in West Africa, and three in East and Central Africa [2, 4, 5].

In January 2016, the Ministry of Health of Angola notified the World Health Organization (WHO) of a yellow fever disease outbreak: as of October 28th, 4,347 suspected cases, including 377 deaths, were reported from all 18 provinces of Angola. Outbreaks were also reported simultaneously from the Democratic Republic of Congo and Uganda [6]. In February 2018, 464 confirmed human cases of yellow fever have also been reported in Brazil, with 154 deaths (http://www.who.int, last accessed 10th April, 2018). Meanwhile, epizootics have expanded to areas previously not considered at risk for yellow fever (http://www.who.int/csr/don/27-february-2018-yellow-fever-brazil/en/, last accessed on 10th April, 2018). All these recalled the concerns of the evolution of YFV. During the Angolan outbreak, YFV infections were detected in Chinese workers returning to China from Angola [7–9], marking the first time YFV infections were documented in Asia. By using the consecutive samples from those imported YFV patients, we unraveled the intrahost and epidemic evolutionary dynamics of YFV.

## Results

### Deep sequencing of YFV genomes from clinical samples

We collected samples from twelve out of thirteen YFV patients in China, sequenced the viral genomes, and performed analyses on phylogenetics, sequence comparison, and intra-host dynamics. Of the 12 patients, two had severe disease, in which one survived (YF-BJ3) and one died (YF-BJ1), and the remaining patients displayed mild symptoms (*Table 1*). We tested blood samples of these 12 patients from the first day of admittance to the hospital by real-time reverse transcription PCR (RT-PCR), and four patients (YF-BJ1, YF-BJ2, YF-BJ3, and YF-BJ5)



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**Table 1. Patient information, viral loads and genome sequencing in urine samples.**

| Patient ID | Group | Outcome | Days after onset | Blood virus (Ct)* | Urine virus (Ct)* | Sequencing Method† | # Total Reads | # Mapped Reads | Sequencing Depth (x1000) | Genome Coverage (bp) |
|---|---|---|---|---|---|---|---|---|---|---|
| YF-BJ1 | Severe | Death | 7 | 31 | 35.00 | AS | 1,695,368 | 1,509,993 | 30.98 | 10,732 |
| | | | 8 | 32 | 33.50 | AS | 1,661,085 | 953,063 | 19.67 | 10,650 |
| | | | 9 | 30 | 34.42 | AS | 3,303,973 | 1,952,626 | 35.68 | 10,725 |
| YF-BJ3 | Severe | Discharged | 8 | 27.6 | 25.60 | AS | 1,451,105 | 1,354,090 | 27.59 | 10,650 |
| | | | 10 | 27.9 | 25.32 | AS | 1,941,400 | 2,369,835 | 18.97 | 10,393 |
| | | | 14 | - | 32.85 | AS | 4,034,937 | 2,896,728 | 55.05 | 10,405 |
| | | | 17 | - | 31.66 | AS | 1,998,163 | 1,861,631 | 36.67 | 10,397 |
| | | | 20 | - | 34.00 | AS | 1,920,032 | 1,416,518 | 28.89 | 10,393 |
| | | | 32 | - | 39.83 | AS | 1,424,735 | 1,014,331 | 21.80 | 10,650 |
| YF-BJ5 | Mild | Discharged | 6 | 23.84 | 23.77 | AS | 2,001,845 | 1,866,672 | 37.82 | 9,918 |
| | | | 10 | - | 35.42 | AS | 1,684,026 | 1,014,398 | 18.54 | 10,674 |
| | | | 14 | - | 34.23 | AS | 2,090,128 | 840,862 | 16.56 | 10,394 |
| YF-BJ2 | Mild | Discharged | 16 | - | 30.95 | AS | 1,924,145 | 1,467,048 | 30.21 | 10,398 |
| YF-BJ4 | Mild | Discharged | 19 | - | 35.86 | TS | 56,728,408 | 873 | 0.02 | 10,393 |
| YF-FZ1§ | Mild | Discharged | 6 | - | - | TS | 77,238,978 | 129 | 0.00 | 1,012 |
| YF-FZ2¶ | Mild | Discharged | 16 | - | 39.37 | TS | 58,570,126 | 195 | 0.00 | 10,243 |
| YF-FZ3§ | Mild | Discharged | 14 | - | 39.40 | TS | 50,462,246 | 68 | 0.00 | 839 |
| YF-FZ4¶ | Mild | Discharged | 7 | - | - | TS | - | - | - | 10,222 |
| YF-FZ5§ | Mild | Discharged | 6 | - | - | TS | 56,605,654 | 68 | 0.00 | 2,795 |
| YF-FZ6¶ | Mild | Discharged | 20 | - | 32.44 | TS | 51,627,058 | 4,722 | 0.11 | 10,838 |
| YF-FZ7¶ | Mild | Discharged | 15 | - | 33.96 | TS | 64,345,650 | 3,945 | 0.09 | 10,311 |

* "-", Negative results (Ct value >45).

† AS, amplicon sequencing; TS, total RNA sequencing.

¶ Genome sequence assembled with Sanger sequencing data.

§ Genome sequence failed to assemble.

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were positive. YFV RNA fragments could be detected in serum until 9, 12, 10 and 6 days, respectively, after the onset of symptoms (*Table 1, and Fig 1*). Urine samples from all patients were also tested by real-time RT-PCR and all were YFV-positive. Higher viral RNA loads were observed in the urine compared to the blood samples, except for those from the non-survivor (*Table 1*). The virus persisted in urine samples for at least 15 days after the onset of symptoms in survivors. Specifically, the urine sample from patient YF-BJ3 was still PCR-positive when tested 32 days after the onset of symptoms (*Table 1 and Fig 1*). We then sequenced all available YFV-positive samples by using total RNA sequencing, amplicon sequencing and/or Sanger sequencing (*Table 1 and S1 Table*). In total, nearly complete virus genomes (>10,222 bp) from 9 patients, and deep-sequenced genome datasets (average sequence depth 34,978x) from 3 patients covering 12 time points were obtained (*Table 1*).

## Phylogenetics and sequence comparison of YFV genome

We performed phylogenetic analysis of the coding region of YFVs (*S2 Table*), and found that YFV sequences from the returning workers in 2016 outbreak closely clustered with the 1971 Angola strains (*Fig 2A*). Of note, the vaccine strain YF-17D and its derivatives are located far from the Angola strains on the phylogenetic tree. A closer inspection of the Angola strains shows that the viruses in the 2016 outbreak are likely from a single origin and genome sequences from both severely ill patients are closely clustered (*Fig 2B*). There are 188

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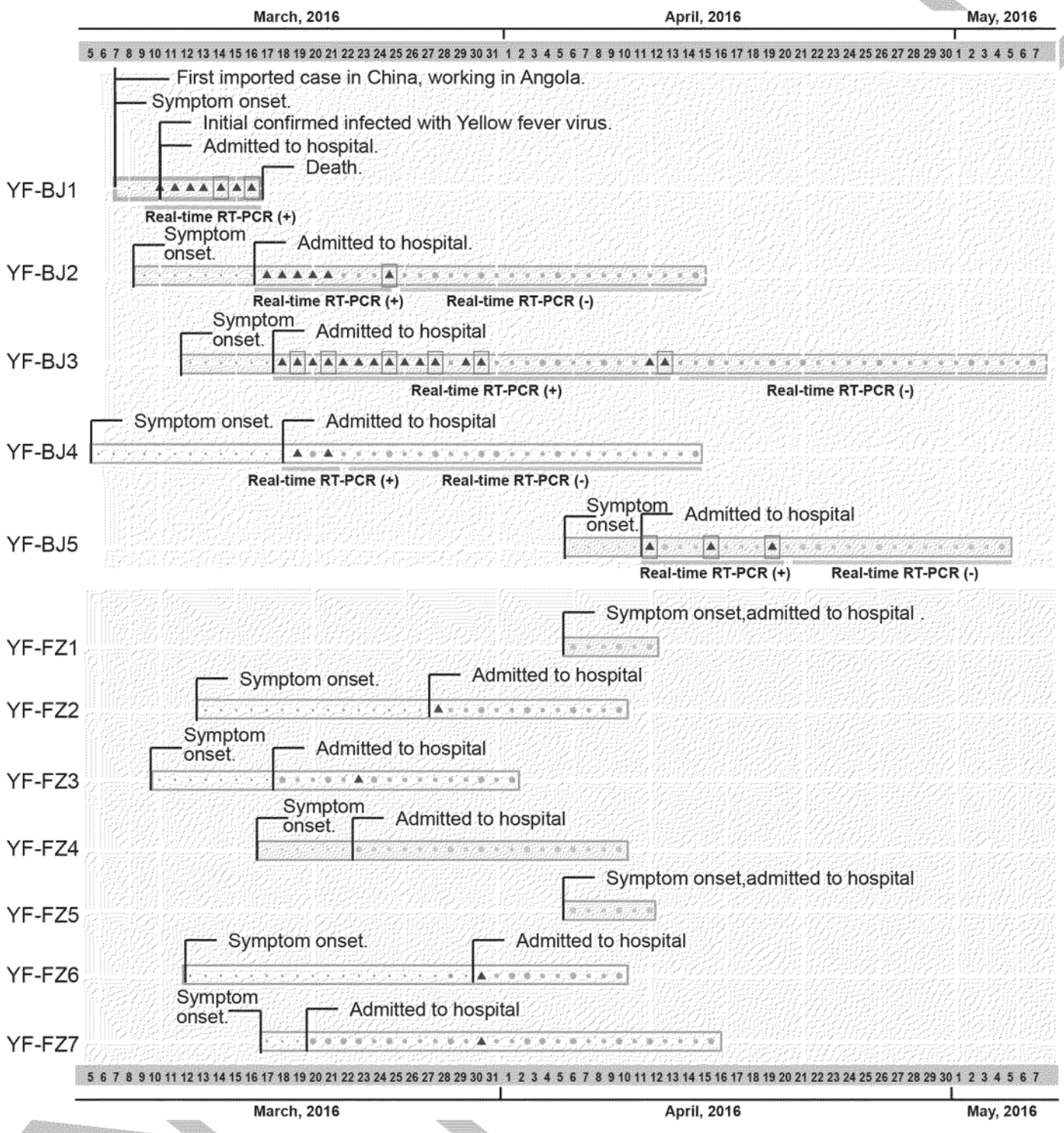



**Fig 1. Cases of twelve documented returning YFV patients in China by date in the outbreak of Angola from March to May 2016.** Timeline of events for each patient from symptom onset to the leaving from hospital are shown with different molecular detection methods and results. Dots denote that we did not get any positive molecular detection result in the time point, grey (before admitted to hospital) and blue (after admitted to hospital). The larger size dots means that we performed Real-time PCR detections in that point. The red triangles denote the sample have been detected by PCR and Real-time PCR with positive results. Blue boxes denote the samples that were subjected to NGS analysis at the time points.

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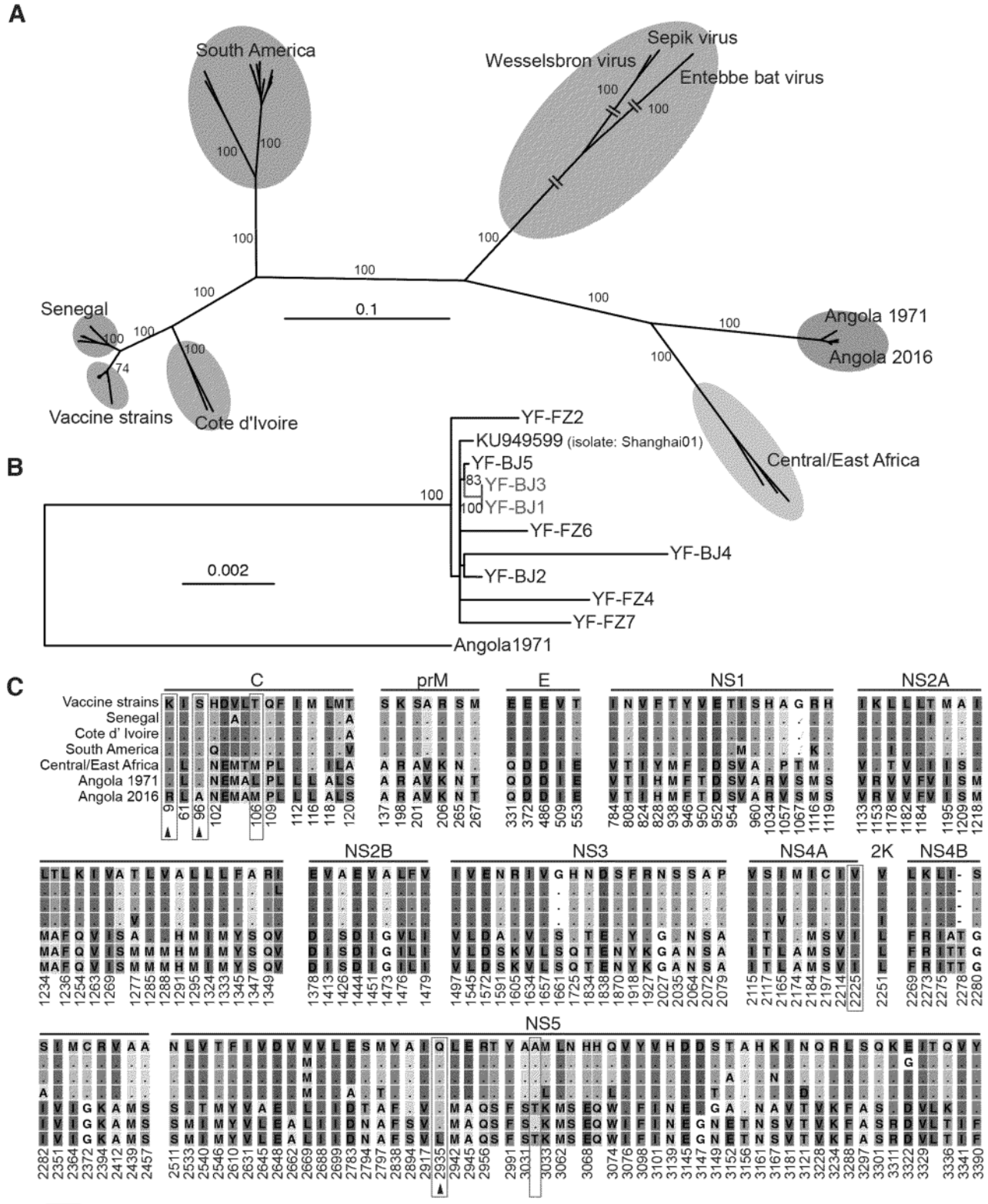



**Fig 2. Phylogenetics and amino acids comparison of YFV viruses.** (A) Maximum-likelihood phylogenetic tree of YFV genome sequences (the first 130 bps in 5'-UTR and the last 370 bps in 3'-UTR sequences are excluded). Bootstrap support values are shown along the branches. Wesselsbron virus, Sepik virus and Entebbe bat virus were used as outgroup. (B) Maximum-likelihood phylogenic tree of YFV genomes of Angola 2016 strains. Viruses from patients with severe



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disease are highlighted in magenta. Angola 1971(GenBank accession AY968064) was used as outgroup. Bootstrap support values (≥70) are shown. (C) Comparison of amino acids of YFV consensus sequence of each lineage in (A). Identical amino acid to vaccine strain (17D) lineage is denoted as a dot. The amino acid sites that were different in Angola 2016 and 1971, are highlight with red rectangles. Novel amino acid substitutions of Angola 2016 are denoted by arrowheads.

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nucleotide substitutions between the 1971 and 2016 consensus sequences and 6 of them are nonsynonymous substitutions. Comparison of the YFV polypeptides shows that only three amino acid changes that appear to be specific to the Angola 2016 strains (*Fig 2C*). Two are in the capsid protein and one in NS5, the RNA-dependent RNA polymerase. The above data are consistent with previous studies using partial or whole genomes showing that YFV exhibits a slow evolutionary rate [2, 4, 10].

## Intrahost genomic variations

For the deep-sequenced samples, we identified the iSNVs by using methods in a previous study for Ebola virus [11]. The mean sequencing depth of genomic regions is between 16,558x and 37,822x (*Fig 3A*), and a total of 101 iSNV sites were discovered, including 69 in the coding region and 32 in non-coding regions. In each iSNV sites, we only found two types of nucleotides. Taken that sequencing errors are generated randomly and may result in multi-nucleotide heterogeneity in a single site, it is unlikely those iSNVs were the results of sequencing bias. Fewer iSNVs appeared in the 1$^{st}$ and 2$^{nd}$ codon positions than the 3$^{rd}$ codon position (*Fig 3B*) and fewer non-synonymous than synonymous iSNVs (*Fig 3C*), implying that the coding region is generally under purifying selection. We plotted the distributions of all mutated allele frequencies of the iSNVs (*Fig 3D and 3E*). The mean mutated allele frequency of non-coding iSNVs was at 0.18, while that of synonymous and non-synonymous iSNVs was 0.12 and 0.09, respectively (*Fig 3D*). Among them, non-synonymous iSNV is significantly lower than that in non-coding regions (P = 0.02). The distribution of non-coding iSNVs is close to the expected neutrality, whereas curves of synonymous and nonsynonymous iSNVs have higher portions of iSNVs in the area of low mutated allele frequencies (*Fig 3E*). This further supports the notion that the coding region was under purifying selection. Additionally, we discovered two variant types at the 3' untranslated region (UTR), one with 5 concurrent iSNVs (G10360A, U10365G, C10367U, G10373A, and U10398C) and the other having an additional iSNV (A10425G) (*Fig 3F*). Phasing analysis reveals that these iSNVs tend to be concurrent in the same reads (*S3 Table and S1 Fig*). Predicted RNA structure shows that these substitutions are likely to affect the structure of the 3' UTR (*Fig 3F and S2 Fig*), and probably influence viral replication in hosts [12–14].

## Intrahost evolutionary dynamics

Subsequently, we placed all of the polymorphic sites (SNPs and iSNVs) along viral genome (*Fig 4A*). SNPs and iSNVs described the viral difference in two levels of between and within the host, respectively. Within 65 days (from the onset of the first patient to the sample of the last patient in this study) we observed a total of 234 SNPs in 148 sites in 18 samples, including 102 synonymous and 117 nonsynonymous SNPs. A total of 138 SNPs that appear only once are scattered along the viral genome. In particular, the SNPs of 5 sites (T900A, A2352C, C3918A, G6463A, and A7320G) could be used to characterize the 2016 YFVs. All five SNPs were detected in seven patients, four SNPs (without A7320G) were detected in one patient (YF-BJ5), and only one patient (YF-BJ3) does not possess any of the above SNPs (*Fig 4A*). Of note, YF-BJ1 is likely a co-infection with more than one YFV variant, as the virus genome on day 7 does not contain any of the five SNPs, whereas all five SNPs became dominant on day 8

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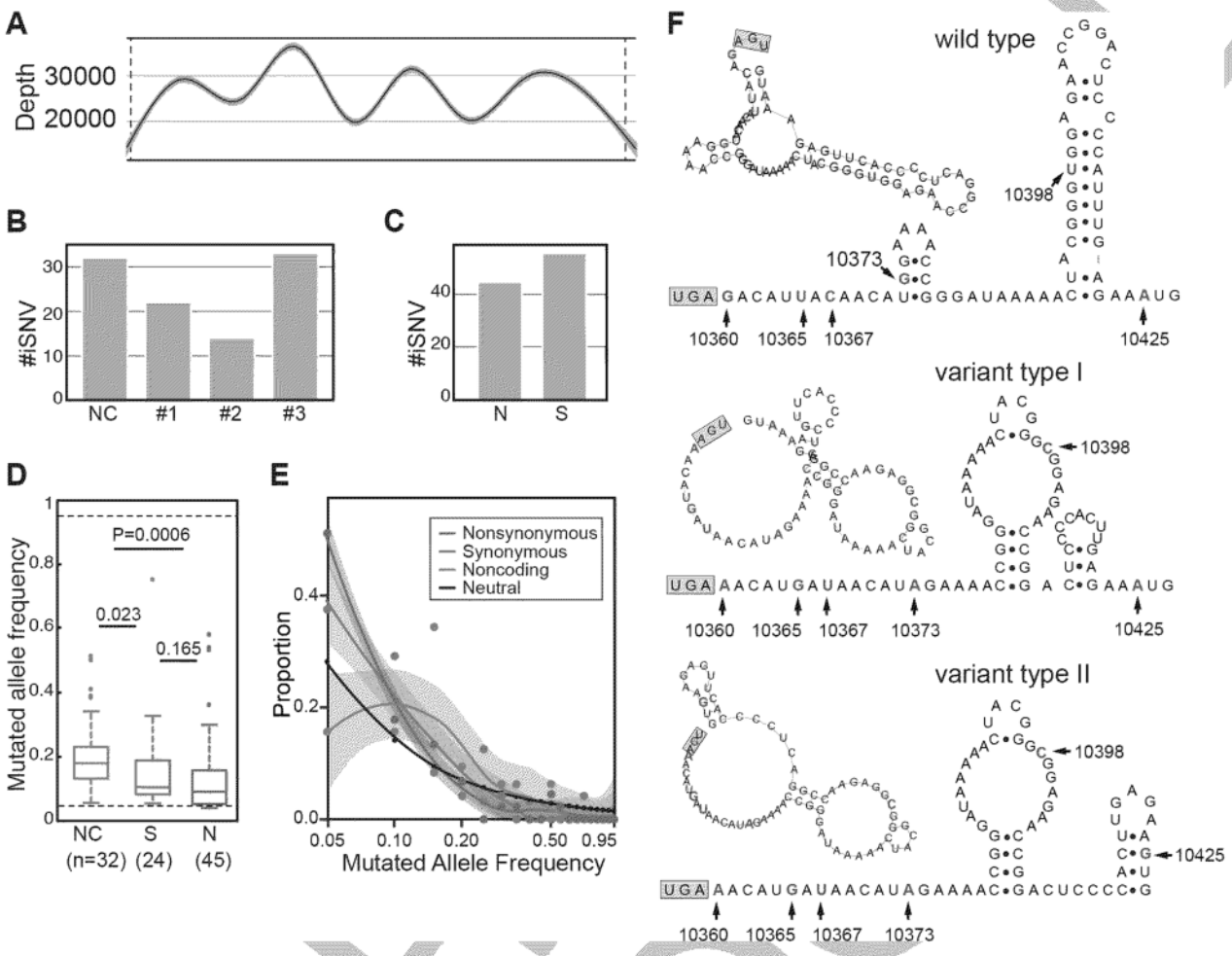



**Fig 3. The iSNVs of Angola 2016 YFVs from clinical samples.** (A) Sequencing depth across the sequenced genomes. The x-axis represents the YFV genome, with the ORF boundaries indicated by vertical dashed lines. The sequencing depth smoothed by locally weighted smooth regression (LOESS) is shown by the black curve, with 95% confidence interval as shown by shadow. (B) Numbers of iSNVs at non-coding (NC) regions and codon positions of the ORF. (C) Numbers of nonsynonymous (N) and synonymous (S) iSNVs of the ORF. (D) Box plots of the mutated allele frequencies (MuAFs) for non-coding, synonymous and nonsynonymous iSNVs. The MuAF values of three kinds of iSNVs are compared to each other with the Wilcoxon rank-sum test. Dashed lines denote the boundaries of MuAFs in iSNV identification. Boxes denote the interquartile range (IQR) between the first and third quartiles. Lines inside the boxes indicate the median, and the lines outside boxes represent values within 1.5 times the IRQ. Outliers are denoted as dots. (E) Comparison of MuAF spectra of non-coding, synonymous and nonsynonymous iSNVs. MuAFs (dots) are shown with LOESS lines (95% confidence interval in shadow). Expectation under neutral selection is shown by a black line. (F) Prediction of RNA secondary structures of 3' UTR (with 68 bp downstream of the stop codon). The earliest type of Angola 2016 (wildtype) and two nucleotides variant types are illustrated. Six variation sites are highlighted in magenta. The stop codon is in the gray box.

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(*Fig 4A*). Of the five SNPs, only G6463A is nonsynonymous, resulting in an amino acid substitution from Val to Ile in NS4A.

We then compared the accumulation of polymorphic sites within patients over time (*Fig 4B*). Each sample was set to days after onset of the symptoms of the patients it belongs to. Linear regressions revealed that iSNVs and SNPs accumulated from the day of onset of symptoms at a rate of 0.72 and 0.32 variations/day, respectively. The accumulation rate of iSNV may reflect the intrahost evolution. Intriguingly, the YFV evolutionary rate within humans is roughly 2.48 $x10^{-2}$ variations/site/year (0.72 variations/day = 0.72 x 365 / 10591 variations/site/year = 2.48 $x10^{-2}$ variations/site/year) in our study. Of those synonymous variations, the intrahost evolutionary rate estimated by iSNV was 0.7 $x10^{-2}$ variations/site/year; whereas the rate of



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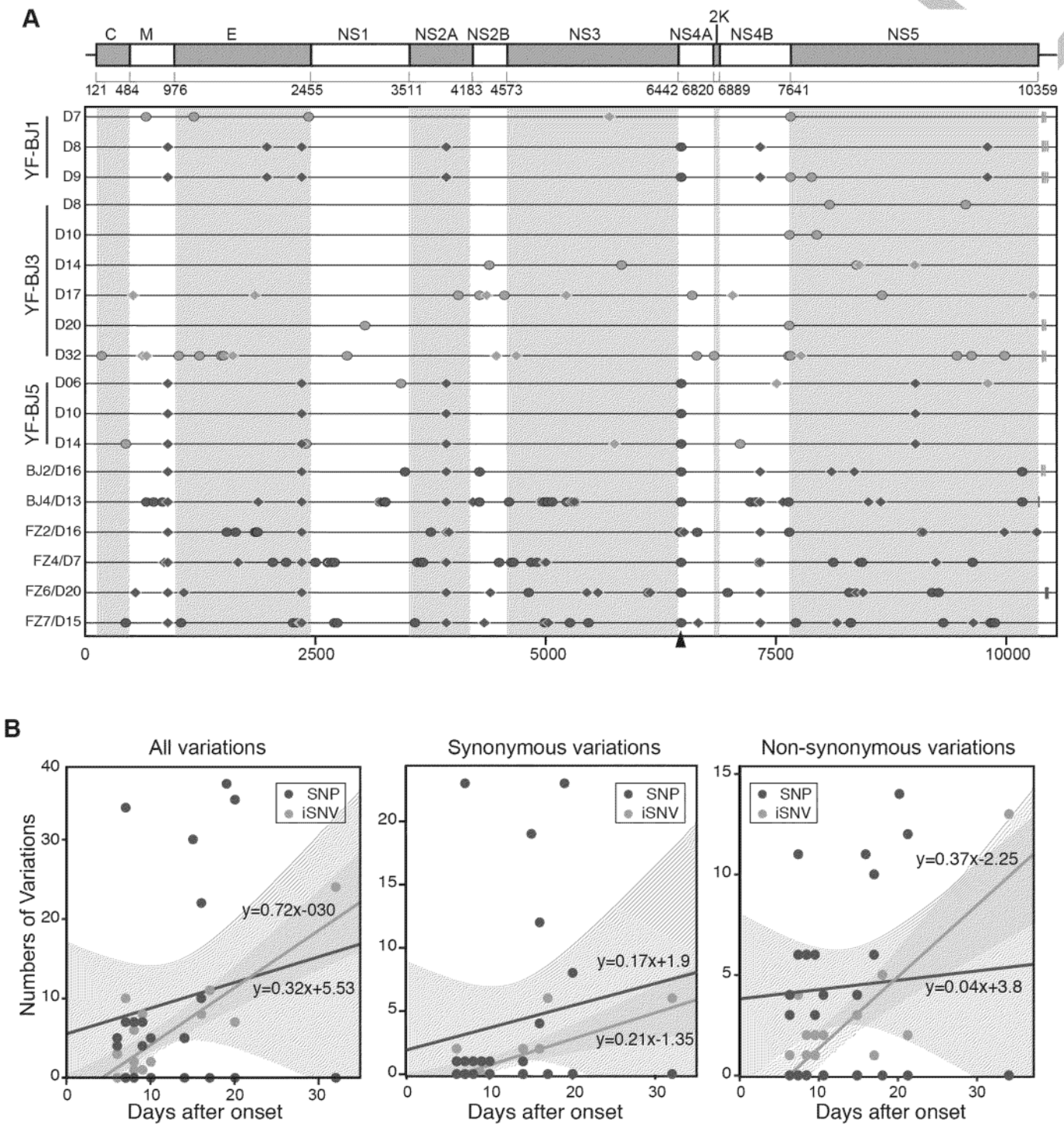



**Fig 4. Genomic variations of Angola 2016 YFV and evolutionary model.** (A) SNPs (in magenta) and iSNVs (in cyan) detected in Angola 2016 strains along the YFV genome. Non-synonymous variations are denoted by ovals, synonymous variations by diamonds, and variations in UTR by rectangles. Non-synonymous site at 6463 is denoted by arrowhead. YFV genome of patient YF-BJ1 at 7 days after the onset (YF-BJ1/7D) was used as the reference sequence (B) Linear regression of number of variations at all variations, synonymous and non-synonymous sites accumulated by days after onset. The smooth shadows denote the LOESS fit with 95% confidence interval.

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nonsynonymous variations was 1.2 x$10^{-2}$ variations/site/year. Compared to the rates estimated by SNP within these samples, the intrahost evolutionary rate of non-synonymous sites (by iSNV) is higher, while the rate of synonymous sites is slightly higher than the SNP rate.

## Discussion

The evolution of YFV is a basic question to unfold to predict the risk of the pathogens. Phylogenetic analysis suggests that the 2016 outbreak is likely the spillover of YFV viruses in Angola. The high identity between the 2016 strain and the vaccine strain YF-17D would explain the effectiveness of the current vaccines [15]. Although there is a diversity of yellow fever virus genotypes, all genotypes are indistinguishable in serological assays. There is not any evidence that the Angola outbreak was due to immune escape and it is accepted among public health professionals that low vaccine coverage in the area prior to the outbreak was the driving factor and the resulting aggressive mass vaccination campaign was vital for containing and eliminating the outbreak. However, it should be noted that the Angola lineage is clearly diversified from the 17D-lineage (*Fig 2A*). Moreover, we did notice 6 patients who had been vaccinated before [16], but the limited clinical and epidemiological data cannot tell the immune escape. Future immune escape from current YFV vaccines cannot be fully ruled out, although YFVs evolve very slowly [17]. A novel vaccine based on a Central/East African YFV isolate would be necessary as a complement vaccine to prepare against future outbreaks. Owing to the slow evolutionary rate and the past experience of using YF-17D, the novel vaccine is likely to work for another decades.

Virus evolution experienced mutation, selection within and between hosts, genetic drift, and transmission, and finally formed the genetic variants (lineages) over long-term evolutionary timescales [18]. Deep sequencing of viral genomes can provide novel insights into viral evolutionary dynamics [19, 20]. In this intra-host phylogenomic analysis, we depicted the evolutionary process of YFV from intra-host to epidemic substitutions, which were reflected by iSNV to SNP, respectively. Other than using the metric for long-term evolutionary rate, we applied the substitution/site/day to measure the intrahost evolutionary rate, which appeared more appropriate in outbreak or epidemic scenarios. Short-term rates may provide better insight into the intrahost YFV evolutionary trends (e.g., iSNV dynamics over time) relative to clinical symptomology, severity, and/or co-morbidities. However, further in vitro or in vivo studies would be needed. The estimated evolutionary rates clearly showed that the intrahost evolutionary rate is ~2.25 times higher than that of the epidemic evolutionary rate, and both are much higher (59 times and 26 times) than the substitution rate of YFV along longer evolutionary timescale [10]. The higher rate estimated by iSNV reflected purifying selection occurred, when intrahost variations were spreading out. The rates estimated by synonymous iSNV and SNP, respectively, were similar, implying that the synonymous variations were under similar evolutionary constraints during transmission. Meanwhile, only a few of the non-synonymous variations were spread out in different individuals, and only G6463A (Val to Ile in NS4A) were detected in multiple samples.

Compard to Dengue virus (DENV) and Zika virus (ZIKV), YFV shares similar host vectors (*Aedes spp*.) and life cycles [21, 22], and has similar mutation rates and replication strategies [10, 23, 24]. However, the long-term substitution rates of DENV (0.77–0.99 x$10^{-3}$ substitutions/site/year) and ZIKA (0.98–1.06 x$10^{-3}$ substitutions/site/year) are higher than that of YFV [23, 25, 26]. Previous studies have posed that additional constrained selection forces are probably existed [10, 27]. Based on our results, we noticed the nearly linear accumulation of variations within individuals. Since the transmission of YFV variants needs mosquitoes as vector, the window (viremia duration) that mosquitoes could use is essential, as only the variations

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within this period could be brought into the transmission cycle. A wider window size would possibly allow more non-synonymous variations into the transmission cycle (Cristina Domingo, EID, 2018). Given that YFV, DENV and ZIKV have ~3, 3–7, and 4–7 days of viremia, respectively, after symptom onset (*S4 Table*), it hinted that the shorter viremia duration in YFV infections might result in the lower evolutionary rate than that of DENV or ZIKV. Most Flaviviruses are vector-borne viruses and the evolutionary rates in mammals and arthropods are different. The intrahost analysis of virus could show the short-term evolution dynamics, especially from serial samples, and this would be a helpful complement to the long-term evolution of Flaviviruses.

In addition, deep sequencing and viral genomics have provided new insights into viral evolutionary dynamics, especially in a continuous mode. Our findings strongly suggest that the urine samples should be considered for the clinical diagnosis, genomic surveillance, and large-scale screening of YFV infections. Further investigations are also encouraged to characterize the factors that maintain viremia in vector-borne diseases.

## Methods

### Ethics statement

The study was conducted in accordance with the Declaration of Helsinki and approved by the ethics committee of Beijing Ditan Hospital (DSRB 2008/00293 and DSRB 2013/00209). Clinical samples and information were obtained after written informed consents from all participants. All human subjects were adult, and if not, whether a parent or guardian of any child participant provided informed consent on their behalf.

### Patients and data collection

Twelve YFV patients returning from Angola were recruited (*Table 1*). We sampled urine and blood sample of these patients from routine examination and collected their clinical data.

### YFV diagnosis and virus detection

YFV infection was diagnosed according to the WHO guidelines (http://www.who.int/csr/disease/yellowfev/case-definition/en/, last accessed 10th April, 2018), including clinical symptoms (fever, headache, jaundice, etc.) and laboratory detection of virus by using real-time RT PCR. The serum and urine samples were collected and stored at -80 °C for further analyses, including real-time RT-PCR, PCR sequencing and RNA sequencing.

### Next generation sequencing

RNA from both serum and urine samples were deployed to two approaches of deep sequencing. Approach I: Total RNA sequencing. RNA was purified by Trizol (Thermo Scientific) for each sample, and then total RNA libraries were constructed with 500bps insertion size using the NEBNext UltraTM RNA Library Prep Kit (NEB, MA, USA), and then sequenced by Illumina Hi-seq platform, and generated 2 × 125 bp paired-end reads. Approach II: RNA amplicon sequencing. Two sets of YFV-specific primer pairs were designed (Set A: 5 pairs, amplicon length = 2,248 ± 168 nt; Set B: 12 pairs, amplicon length = 1,018 ± 48 nt; *S5 Table*). PCR products were sequenced by Illumina platform with 2 × 250 bp paired-end reads. Quality control and error correction were implemented according to previous report [28, 29]. The probably low quality regions in sequencing, including 1) Q<30, and 2) the first 10 bps after primers were removed from the high quality sequences, according to a previous study [28]. To minimize sequencing error that affects the accuracy of iSNV calling, we only kept the read-pair that

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has >100 high quality bases in both ends as clean reads. All reads were deposited in the NCBI SRA database under the accession no. SRP096859.

## Sequence alignment and phylogenetic analysis

Consensus sequences were aligned by MUSCLE [30]. Phylogenetic analyses were performed by using RAxML v8.1.6, with the GTR model of nucleotide substitution and γ-distributed rates among sites. Phylogenetic tree was constructed by maximum-likelihood using YFV genome sequences [31]. A NJ tree based on YFV genome sequences was also constructed to show the results were robust (S3 *Fig*). A total of 1,000 bootstrap replicates were performed. All SNPs were listed in S6 *Table*. The genome sequences of YF-BJ1, YF-BJ2, YF-BJ3, YF-BJ4, YF-BJ5, YF-FZ2, YF-FZ4, YF-FZ6, and YF-FZ7 were deposited in the NCBI GenBank database under the accession no. MH633684-MH633692.

## Calling of iSNVs

To minimize the potential sequencing errors generated by system, we performed the following two steps before we called iSNV, including 1) mapping the clean reads to the reference genome, 2) qualification of the samples. For mapping the clean reads to genome, we use the traditional protocol used widely. First, we mapped the clean reads with pair-ended aligned to the assembled genome from YF-BJ1/7D by using Bowtie2 v2.2.5 [32] with default parameters. SAMtools v1.2 [33] was used to generate 'mpileup' files with no limit for the maximum site depth. To find the potential mutation in each site, we do not use the following pipeline in SAMtools. Instead, we developed homemade PERL scripts (available at http://github.com/generality/iSNV-calling/), which were used for iSNV calling to identify all potential mutations by using the mpileup files as input. The depth of bases in each sites were used to measure the mutation rate of iSNV. Then, we selected the samples with more than 3,000 sites with a sequencing depth ≥300× as candidate samples for iSNV calling. Using this protocol, we called iSNV in 15 samples in this study (S1 *Table*), and obtained the frequencies of each allele in each genome site. Interestingly, we only found the heterogeneous nucleotides with two types in each sites. Thus, we defined the nucleotide with higher frequencies as the major allele, and fewer one as the minor allele in the following iSNV calling.

The iSNV calling was according to the site depth and strand bias with following: 1) remove the ambiguous iSNV and keep the following iSNVs: (1.1) minor allele frequency of ≥5%, a conservative cutoff based on an error rate estimation described before [11, 28]; (1.2) depth of the minor allele of ≥15; and (1.3) strand bias of the minor allele less than tenfold. 2) In our analysis, these two types of nucleotides in each site have contained the nucleotide in the reference genome. Mutated allele frequencies were calculated by the rate of the reads numbers for mutated alleles to the references and total reads. Minor allele and Major allele frequencies were calculated by the majority nucleotides and minor nucleotides in each site respectively. 3) The effect of iSNV to the gene was calculated by the mutation whether caused the amino acid changes.

## Phasing analysis of iSNVs

The iSNVs that co-occurred in same viral haplotype(s) within quasispecies was defined as phased iSNVs. 1)We screened the iSNV sites along genome with a window of 250 bp, and then identified all the windows which probably containing a phasing iSNVs. 2) All the sequencing reads aligned to YFV genome region harboring these iSNVs were extracted. 3) The stretches of nearest iSNV were determined by the mutations in the reads aligned to the genome region

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with the same phasing (supporting reads > = 2). 4) The fractions of reads supporting phased and non-phased neighbor iSNVs were respectively calculated.

### Prediction of RNA secondary structures

RNA secondary structures were predicted by using RNAfold program of ViennaRNA Package 2 with default settings [34]. Nucleotide sequences started at the beginning of 3' UTRs of Angola 2016 strain. The length of window for secondary structure prediction was equal to 70 nucleotides. We also extended the additional 200 nucleotides at the end of the sequence, with a step length equal to 50.

### Accession numbers

All reads from next generation sequencing were deposited in the NCBI SRA database under the accession no. SRP096859. The genome sequences of YF-BJ1, YF-BJ2, YF-BJ3, YF-BJ4, YF-BJ5, YF-FZ2, YF-FZ4, YF-FZ6, and YF-FZ7 were deposited in the NCBI GenBank database under the accession no. MH633684-MH633692.

## Supporting information

**S1 Fig. Phasing analysis of iSNVs at the 3' untranslated region (UTR).** For a given sample, the sequencing reads aligned to 3' UTR of the YFV genome were extracted to obtain the 5-mutations (at positions 10360, 10365, 10367, 10373 and 10398) or 6-mutations (10360, 10365, 10367, 10373, 10398 and 10425). The pie charts denote fractions of reads supporting wildtype 5/6-mers and phased mutant 5/6-mers (A10360G, G10365T, T10367C, A10373G, C10398T and G10425A), with read counts (or depth, x) shown in brackets. Sample IDs are indicated at the top of pie charts.
(PDF)

**S2 Fig. RNA secondary structure prediction of the 3' UTR.** RNA secondary structures were predicted based on sequences of YFV 3' UTR (started from the stop codon) with gradually increased lengths. The sequences were extracted from genomes of wild type Angola 2016 strain and two variant types. Blue arrows indicate variations corresponding to the phased iSNV sites at 3' UTR, A10360G, G10365T, T10367C, A10373G, C10398T and G10425A. The local structural alterations due to the variations are highlighted in blue shadow.
(PDF)

**S3 Fig. Phylogenetic tree constructed by neighbor-joining method using YFV genome sequences.** Viruses from patients with severe disease are highlighted in magenta. Angola 1971 (GenBank accession AY968064) was used as outgroup. Bootstrap support values over 70 are shown.
(PDF)

**S1 Table. Sequencing data from continuous clinical samples of YFV.**
(PDF)

**S2 Table. Public sequences used for phylogenic analysis.**
(PDF)

**S3 Table. Phasing analysis for continuous neighbor iSNV.**
(PDF)

**S4 Table. Duration of viremia and evolutionary rates of YFV, ZIKV and DENV.**
(PDF)

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**S5 Table. Two primer sets used for amplicon-sequencing.**
(PDF)

**S6 Table. SNPs observed in YFV.**
(PDF)

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**Writing – review & editing:** Gary Wong, Fujie Zhang, Wenjun Liu, Ang Li, Di Liu.

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The Pirbright Institute, UK. chris.netherton@pirbright.ac.uk
*(need to cover the flight tickets and accommodation）*

Dr. Pip Beard, Group of Large DNA Virus, The Pirbright Institute, UK.
pip.beard@pirbright.ac.uk
*(need to cover the flight tickets and accommodation）*

袁志明（中国科学院武汉病毒研究所，研究员）
Prof. Zhiming Yuan (Wuhan Institute of Virology, CAS)

唐　红（上海巴斯德研究所，研究员）
Prof. Hong Tang (Institute Pasture of Shanghai, CAS)

石正丽（中国科学院武汉病毒研究所，研究员）
Prof. Zhengli Shi (Wuhan Institute of Virology, CAS)

龚睿（中国科学院武汉病毒研究所，研究员）
Prof. Ruigong (Wuhan Institute of Virology, CAS)

关武强（中科院武汉病毒研究所，研究员）
Prof. Wuqiang Guan (Wuhan Institute of Virology, CAS)

刘文军（中科院微生物研究所，研究员）
Prof. Wenjun Liu (Institute of Microbiology, CAS)

左建平（中国科学院上海药物研究所，研究员）
Prof. Jianping Zuo（Shanghai Institute of Materia Medica, CAS）

郑永唐（中国科学院昆明动物研究所，研究员）
Prof. Yongtang Zheng (Kunming Institute of Zoology, CAS)

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梁米芳（中国疾病预防控制中心病毒病预防控制所，研究员）
Prof. Mifang Liang (National Institute for Viral Disease Control and Prevention, China CDC)

胡云章（中国医学科学院医学生物学研究所，研究员）
Prof. Yunzhang Hu (Institute of Medical Biology, Chinese Academy of Medical Sciences)

高玉伟（军事医学研究院， 研究员）
Prof. Yuwei Gao（Academy of Military Medical Sciences）

王恒梁（军事医学科学院生物工程研究所，研究员）
Prof. Hengliang Wang（Academy of Military Medical Sciences）

杨瑞馥（军事医学研究院， 研究员）
Prof. Ruifu Yang（Academy of Military Medical Sciences）

程功（清华大学，研究员）
Prof. Gong Cheng (Tsinghua University)

郭德银（中山大学，教授）
Prof. Deyin Guo (Sun Yat-sen University)

张伟文（天津大学，研究员）
Prof. Weiwen Zhang (Tianjin University)

陆家海（中山大学医学院，教授）
Prof. Jiahai Lu (Sun Yat-sen University)

翟培军（中国合格评定国家认可委员会，认可四处处长）
Dr. Peijun Zhai (CNAS)

王君伟（中国动物卫生与流行病学中心，实验室管理处长）
Dr. Junwei Wang (China Animal Health and Epidemiology Center)

段赵军（中国疾病预防控制中心，实验室管理处副处长）
Dr. Zhaojun Duan (China CDC)

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### Keynote Lecture

**Prof. Fu Gao** (Director-General, Chinese Center for Disease Control and Prevention (China CDC)

### African Swine Fever

**Prof. Xiaodong Wu** (China Animal Health and Epidemiology Center)
Current Situation and Control Strategy of African Swine Fever in China

**Prof. Zhigao Bu**（Harbin Veterinary Research Institute, CAAS）
Characterization of African Swine Fever Virus isolated from China

### Influenza

**Prof. Hualan Chen**（Harbin Veterinary Research Institute, CAAS）
TBD

**Prof. Yuwei Gao**（Academy of Military Medical Sciences）
The Diversity of Avian Influenza Virus in Wild Bird in China

**Prof. Dayan Wang** (National Institute for Viral Disease Control and Prevention, China CDC)
Human Infection with Zoonotic Influenza

**Prof. Wenjun Liu** (Institute of Microbiology, CAS)
The Anti-Influenza Drug Development by Targeting Nucleoprotein Nuclear Export

**Prof. Chengjun Li**（Harbin Veterinary Research Institute, CAAS）
Interactions between Influenza Viral RNP Complex Proteins and Host Cellular Factors

### Mosquito Vector

**Prof. Tongyan Zhao**（Institute of Microbiology and Epidemiology, Academy of Military Medical Sciences）
Vector Competence of Emerging Mosquito Borne Virus in China

**Prof. Gong Cheng** (Tsinghua University，P.R.China)
A Gut Commensal Bacterium Promotes Arbovirus Infection in Mosquitoes

### Emerging Infectious Diseases

**Prof. Jianguo Xu** (Director General, National Insitute for Communicable Disease Control and Prevention Chinese Center for Diseases control and Prevention)
TBD

**Prof. Zhengli Shi** (Wuhan Institute of Virology, CAS)
From SARS, MERS to SADS: Interspecies Transmission of Bat Coronaviruses.

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**Prof. Chengfeng Qin**（Institute of Microbiology and Epidemiology, Academy of Military Medical Sciences）
Zika Virus Neurotropism the Bad and the Good

**Prof. Changjiang Weng** (Harbin Veterinary Research Institute, CAAS)
DDX19，A novel Viral RNA Sensor, Regulates IFN Signaling upon Viral Infection

### Vaccines & Therapeutics

**Prof. Haixue Zheng**（Lanzhou Veterinary Research Institute, CAAS）
Rational Design of Master Seed Virus to Improve the Safety and Effectiveness of Foot and Mouth Disease Vaccine

**Prof. Longding Liu** (Institute of Medical Biology, Chinese Academy of Medical Sciences)
Development of inactivated EV71 Vaccine (Human diploid cell) and Pathogenic Study of EV71 Infection in Rhesus Macaque

**Prof. Ruigong** (Wuhan Institute of Virology, CAS)
Development of Neutralizing Antibodies Domains as Therapeutic Candidates Against Infectious Diseases

**Prof. Yunzhang Hu** (Institute of Medical Biology, Chinese Academy of Medical Sciences)
Kunming National Primate Research Center of High-Level Biosafety

### Ethics

**Prof. Weiwen Zhang** (Tianjin University)
Cutting Edging New Biotechnology: Ethic and Biosafety Concerns and Possible solution

### High Containment Safety and Operations

**Prof. Zhiming Yuan** (Institute of Medical Biology, Chinese Academy of Medical Sciences)
Biosafey and Bioethics in Medical Research

**Dr. Peijun Zhai** (CNAS)
Overview of Laboratory Biosafety Management System in China

**Prof. Jiancheng Qi**(National Biological Protection Engineering Center, China )
Development and Application of Laboratory Biosafety Equipment in China

**Prof. Ruifu Yang**（Institute of Microbiology and Epidemiology, Academy of Military Medical Sciences）
Microbial Forensics: Building Capability for Source Tracing of Bioterrorism Pathogen.

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One Health

**Prof. Jiahai Lu** (Sun Yat-sen University)

One Health in China

**From**: Mike Bray (b)(6)
**Sent**: 12/19/2018 3:31:36 PM
**To**: Shi, Pei yong [peshi@UTMB.EDU]
**Subject**: Your help with a title for Yan-Yi's lecture

WARNING: This email originated from outside of UTMB's email system. Do not click links or open attachments unless you recognize the sender and know the content is safe.

Hello Pei-Yong,

We're closing in on a final program for ICAR, and I need to ask your help in communicating with Yan-Yi, to make sure that we avoid misunderstandings. I've attached the current draft program (please don't distribute), and you'll see that we have two 45-minute keynote lectures on the first afternoon (Diane Griffin and Richard Mackman), and all the rest of the invited lectures will be 30 minutes.

Because Yan-Yi is an institute director (and she would certainly win the award for longest travel to attend ICAR), I want to highlight her presentation, so I'm scheduling it as a "special talk," after the Holy Award lecture on Tuesday morning, and before the first session on "What's new in antiviral research." I would be quite happy to simply hear about the Wuhan institute, but some others on the organizing committee have insisted that she present some of her own research, so I'm thinking that a good title for her talk would be "Antiviral research at the Wuhan Institute of Virology." That would allow her to both present some of her own work and give an overview of the lab and its capabilities.

You'll also notice on the schedule that the talk by Amadou Sall, scientific director of the Institut Pasteur in Dakar, is also listed as a "special talk", preceding a session. I don't think we'll use those labels in the final program, but for the moment they indicate a lecture that is less than a keynote, but more than one piece of a multi-speaker session.

Please let me know your thoughts about Yan-Yi's presentation, and I'll then write to her.

Best,

Mike

Mike Bray, MD MPH
Editor-in-chief
Antiviral Research

DRAFT 12-17-18

# U.S. China Dialogue and Workshop on the Challenges of Emerging Infections, Laboratory Safety, Global Health Security and Responsible Conduct in the Use of Gene Editing in Viral Infectious Disease Research

Harbin Veterinary Research Institute
Chinese Academy of Agricultural Sciences
Harbin, China

8-10 January 2019

**[Please review the entire agenda and make/ suggest changes. Please confirm that listed Chinese participates are attending, especially the speakers that are listed in red text, and that each speaker is speaking in the right session.]**

## Monday, 7 January

Some participants arrive at the Aoluguya Hotel (No. 800, the 3rd Road of Chuangxin, Harbin)

Welcome reception for those who are at the hotel (proposed)

Evening activity for those that have arrived: group tour of the Harbin Ice Festival (proposed)

## Tuesday, 8 January

**8:30** Shuttle bus from Aoluguya Hotel to Harbin Veterinary Research Institute (HVRI)
(Weihai N Rd, Xiangfang Qu, Haerbin Shi, Heilongjiang Sheng, China 150060)

**WELCOME AND OPENING REMARKS**

**9:00** **Welcome, Introductions, and Opening Remarks**

1. Welcome on behalf of Chinese Academy of Agricultural Sciences (CAAS): **Zhigao Bu**, Harbin Veterinary Research Institute
2. Remarks on Behalf of U.S. National Academies of Sciences, Engineering, and Medicine: **Benjamin Rusek**, Policy and Global Affairs, U.S. National Academy of Sciences (NAS) and **Diane Griffin**, Johns Hopkins Bloomberg School of Public Health and Vice-President of NAS

**9:15** **Session 1. Keynote Addresses** *[20 min each]*
**Chair:** TBD

1. **Prof. Fu Gao**, Director-General, Chinese Center for Disease Control and Prevention
   *[Talk title TBD]*
2. **Prof. Linda Saif**, Ohio State University
   *Proposed title: Applied Agricultural Virology and Emerging Veterinary Diseases*



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**Q&A/Discussion** *[15-20 min]*

**10:15** Coffee and Tea Break

**EMERGING INFECTIOUS DISEASES**

Goal for Sessions 2 and 3: Consider progress in understanding two diseases of concern to China and the US, including the health and economic implications and what advances in understanding may be enabled by recent scientific and technical developments such as gene editing.

**10:30** **Session 2. African Swine Fever** *[15 min each]*

**Chair:** TBD

1. **Prof. Xiaodong Wu**, China Animal Health and Epidemiology Center)
   *Current Situation and Control Strategy of African Swine Fever in China*
2. **Prof. Zhigao Bu,** Harbin Veterinary Research Institute, CAAS *Characterization of African Swine Fever Virus Isolated from China*
3. **Dr. Douglas Gladue**, U.S. Department of Agriculture
   *Proposed title: African Swine Fever research update*

**Q&A/Discussion** *[10-15 min]*

***Proposed discussion questions***

- What is known about the epidemiology, health, and economic impacts of Africa Swine Fever?
- What is the latest progress on developing effective countermeasures and limiting the spread of this disease?
- What critical areas remain unknown or what are key challenges to preventing and responding to Africa Swine Fever outbreaks? How can new scientific and technical advances contribute to research and development?

**11:30** **Session 3. Influenza**

**Chairs: Hualan Chen** and **David Swayne**

1. **Overview: Perspectives on international collaborations for influenza research** *[10 min each]*
   **Hualan Chen** Harbin Veterinary Research Institute, CAAS
   *Influenza vaccine research in China and Globally*
   **David Swayne,** U.S. Department of Agriculture
   *Collaborating on Influenza Research from the U.S. perspective*
2. **Panel Discussion**
   Speakers provide 5 minutes opening remarks on the topics identified below, followed by discussion facilitated by the session Chairs
   **Prof. Yuwei Gao,** Academy of Military Medical Sciences
   *The Diversity of Avian Influenza Virus in Wild Bird in China*
   **Prof. Dayan Wang**, National Institute for Viral Disease Control and Prevention, China CDC
   *Human Infection with Zoonotic Influenza*
   **Prof. Wenjun Liu**, Institute of Microbiology, CAS



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*The Anti-Influenza Drug Development by Targeting Nucleoprotein Nuclear Export*
**Prof. Chengjun Li,** Harbin Veterinary Research Institute, CAAS
*Interactions between Influenza Viral RNP Complex Proteins and Host Cellular Factors*

***Proposed discussion questions***

- What is known about the epidemiology, health, and economic impacts of Influenza?
- What is the latest progress on developing effective countermeasures?
- What critical areas remain unknown or what are key challenges to preventing and responding to influenza outbreaks? How can new scientific and technical advances contribute to research and development?

**12:45** **Group Photo**

**13:00** **Lunch**

**VIRAL PATHOGENS: SCIENCE AND RESPONSIBILITY**

Goal for Sessions 4, 5, and 6: Discuss relevant pathogen properties that researchers may seek to alter, and why, as part of studying viral pathogens with pandemic potential. Illustrate challenges and opportunities posed by new strategies such as use of gene editing, rational design approaches, other strategies enable by advancing biotechnology. Discuss the associated issues in the ethical planning and conduct of such research so that it can be conducted safety and securely.

**14:30** **Session 4. Understanding and Engineering Viral Pathogens with Pandemic Potential** *[20 min each]*
Chairs: **Diane Griffin** and **Fu Gao**

1. **Ralph Baric**, University of North Carolina
   *Advances in Understanding and Altering Pathogen Properties*
2. **Prof. Weiwen Zhang**, Tianjin University
   *Cutting Edging New Biotechnology: Strategies and Tools for Engineering*

**15:15** Coffee and Tea break

**15:45** **Session 5. Bioethics and Responsible Planning for Pathogen Research** *[15 min each]*
**Chairs: Diane Griffin** and **Fu Gao**

1. **Prof. Zhiming Yuan**, Institute of Medical Biology, Chinese Academy of Medical Sciences
   *Biosafey and Bioethics in Medical Research*
2. **David Relman**, Stanford University
   *Proposed title: Bioethical Considerations for the Conception and Design of pathogen Research and Approaches for Assessing Potential Risks and Benefits of Research*
3. **Prof. Jianwie Wang**, Institute of Pathogen Biology, Chinese Academy of Medical Sciences & Peking Union Medical College
   *Biosafety Management of Synthetic Biology: Challenges and Progress*
4. **Associate Prof. Yang Xue**, Tianjin University



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*Biosafety and Bioethis Considerations in Dual-Use Risks Management – China's Efforts and Future Works*

**16:45** **Session 6. Panel on Science and Ethics in Research with Pathogens with Pandemic Potential**
**Chairs**: **Diane Griffin** and **Fu Gao**
Chairs facilitate a discussion between **Ralph Baric, Weiwen Zhang, Zhiming Yuan, and David Relman, Jianwie Wang, Yang Xue** and the audience. *[45 min]*

***Proposed discussion questions***

- *What do recent advances in biotechnology, such as genome editing, enable researchers to achieve in improved characterization and alteration of the properties of pathogenic viruses?*
- *What do you see coming in the near term? What still remains very challenging and why?*
- *Because research on viral pathogens can raise security concerns, how do you think about making decisions on whether or not to conduct such research or about modifying research proposals? What are the responsibilities and best practices of the PI to ensure that pathogen research is conducted safely and securely over the course of the project?*
- *What ethical and policy challenges remain and what actions do you think are needed to help overcome these challenges?*

**17:30** Transportation from HVRI to hotel

**19:00** Dinner at Hotel (proposed)

Optional: Harbin city tour/snowmobile/dogsledding? (proposed)

**Wednesday, 9 January**

**08:30** Transportation to HVRI

**VECTOR AND HOST ENGINEERING**
Goal for Session 7: Research to understand and prepare for viral pathogens may also involve work conducted on the hosts or vectors of such pathogens, for example to better understand host-pathogen responses and vector transmission. Discuss how advances in areas such as gene editing are affecting such research and best practices for safely and responsibly conducting such studies

**9:00** **Session 7. Vector and Host Engineering: Safety and Security** *[15-20 min each]*
**Chair: Jim Le Duc**, University of Texas Medical Branch and **Zhiming Yuan**

1. **Prof. Qian Han**, Hainan University
   *Biochemical Pathway of a Mosquito Innate Immunity, Melanization*
2. **Stephen Higgs**, Kansas State University
   *Proposed title: Best practices in safety and security for working with arthropods that serve as pathogen vectors, including responsible research with gene drive modified vectors*

**Q&A/ Discussion** *[20 min]*

**10:00** Coffee and tea break



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**10:30 Session 7 resumes**

3. **Highlights of Recent Research:**
   **Prof. Tongyan Zhao**, Institute of Microbiology and Epidemiology, Academy of Military Medical Sciences
   *Vector Competence of Emerging Mosquito Borne Virus in China*
4. **Prof. Gong Cheng**, Tsinghua University
   *A Gut Commensal Bacterium Promotes Arbovirus Infection in Mosquitoes*

**Q&A/ Discussion** *[30 min]*

***Proposed discussion questions***

- *What do recent advances in biotechnology, such as genome editing, enable researchers to achieve in studying and manipulating hosts and vectors of viral pathogens*
- *What do you see coming in the near term? What still remains very challenging and why?*
- *What are the responsibilities and best practices of the PI to ensure that pathogen research is conducted safely and securely over the course of the project?*
- *What ethical and policy challenges remain and what actions do you think are needed to help overcome these challenges?*

**12:00 Lunch**

**EFFECTIVE OVERSIGHT SYSTEMS**

Goal for Session 8: Discuss the systems of institutional review and oversight in China and in the US for safe and secure conduct of laboratory research on viral pathogens with pandeminc potential. Focus on the institutional systems including the important role of leadership in promoting institutional cultures that support responsible research practices.

**13:30 Session 8. Best Practices in Laboratory Safety and Security for Pathogen Research**

**Chair:** TBD

1. **Prof. Jianguo Xu**, Director General, National Institute for Communicable Disease Control and Prevention Chinese Center for Diseases control and Prevention. *[15 min]*
   *Proposed topic: Institutional review and oversight for research on viral pathogens with pandemic potential—the perspective from China*
2. **Joseph Kanabrocki**, ***University of Chicago*** *[15 min]*
   *Institutional review and oversight for research on viral pathogens with pandemic potential – a U.S. perspective*
3. **David Franz** and **Zhiming Yuan** *[20 min together]*
   *The Role of Leaders in Laboratory Safety: Culture and governance to steer toward behavior changes*

**Facilitated Discussion** *[xx min]*

Chair facilitates a discussion with the speakers and audience [20 min]

***Proposed discussion questions***



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*If assessment of anticipated risks and benefits results in the decision to undertake an experiment that involves characterizing or manipulating a pathogen with pandemic potential:*

1. *How should the research-conducting institution oversee this research to ensure that it is conducted safely and securely over the course of the project? Does this oversight happen at one point or at multiple points over the course of the project?*
2. *What are the responsibilities of institutional leadership in promoting the culture of responsible conduct of science?*
3. *Are there particular scientific or experimental practices that should be incorporated into research design and conduct that can maximize safety and security for studies carried out to understand or alter the properties of pathogens with pandemic potential?*

**14:40** **Special Topic: One Health in China** *[20 min]*
Understand how a One Health approach is shaping research to understand and address viral pathogens in China
**Prof. Jiahai Lu**, Sun Yat-sen University
**Q&A/Discussion** *[10 min]*

**15:10** Coffee and Tea Break

**RECENT RESEARCH AND PERSPECTIVES**
Goal for Sessions 9 and 10: Gain further shared insights into how research communities are addressing issues in biosafety, biosecurity, and bioethics, particularly as applied to research on emerging infectious diseases caused by viral pathogens

**15:30** **Session 9. Chinese Biosafety, Biosecurity, and Bioethics** *[15 min each]*
**Chair:** Mifang Liang, China CDC and **Joe Kanabrocki** (give some opening comments)

1. **Dr. Peijun Zhai**, CNAS
   *Overview of Laboratory Biosafety Management System in China*
2. **Prof. Jiancheng Qi**, National Biological Protection Engineering Center, China
   *Development and Application of Laboratory Biosafety Equipment in China*
3. **Prof. Yunzhang Hu**, Institute of Medical Biology, Chinese Academy of Medical Sciences
   *Kunming National Primate Research Center of High-Level Biosafety*
4. **Prof. Ruifu Yang**, Institute of Microbiology and Epidemiology, Academy of Military Medical Sciences
   *Microbial Forensics: Building Capability for Source Tracing of Bioterrorism Pathogen.*

**Q&A/Discussion** *[30 min]*

**17:00** Transportation from HVRI to hotel

**19:00** Optional Dinner and or group outing

**Thursday, 10 January**

**8:30** Hotel shuttle bus transport to HVRI



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**9:00** **Special Topic: Update on Acute Flaccid Myelitis**
(Review and discuss what is currently known about the cause of this condition)
**Diane Griffin,** Johns Hopkins Bloomberg School of Public Health [20 min]
*Talk title TBD*
**Q&A/Discussion** *[10 min]*

**9:30** **Session 10: Chinese Perspectives on Emerging Infectious Diseases** *[15 min each]*
**Chair: Pei-yong Shi**

1. **Prof. Changjiang Weng,** Harbin Veterinary Research Institute, CAAS
   *DDX19, A novel Viral RNA Sensor, Regulates IFN Signaling upon Viral Infection*
2. **Prof. Zhengli Shi,** Wuhan Institute of Virology, CAS
   *From SARS, MERS to SADS: Interspecies Transmission of Bat Coronaviruses*
3. **Prof. Haixue Zheng**, Lanzhou Veterinary Research Institute, CAAS,
   *Rational Design of Master Seed Virus to Improve the Safety and Effectiveness of Foot and Mouth Disease Vaccine*

**10:15** Coffee and Tea Break

**10:45** **Session 10, cont'd**

4. **Prof. Ruigong**, Wuhan Institute of Virology, CAS
   *Development of Neutralizing Antibodies Domains as Therapeutic Candidates Against Infectious Diseases*
5. **Prof. Longding Liu**, Institute of Medical Biology, Chinese Academy of Medical Sciences
   *Development of inactivated EV71 Vaccine (Human diploid cell) and Pathogenic Study of EV71 Infection in Rhesus Macaque*
6. **Prof. Chengfeng Qin**, Institute of Microbiology and Epidemiology, Academy of Military Medical Sciences
   *Zika Virus Neurotropism the Bad and the Good*

**Q&A/Discussion** *[30 min]*

**12:00** Special Session 11 Application of Gene Editing technology

1. **Prof. Hongsheng Ouyang, Jili** University
   Genome-edited Pigs are Protected from Classical Swine Fever Virus
2. **Prof. Chunbo Lou,** Institute of Microbiology, CAS
   *Large Gene Cluster and Regulatory Manipulation for Activating the Cryptic Natural Product Clusters in Streptomyces*
3. **Prof. Xiaoli Xue,** Key Laboratory of Synthetic Biology, Shanghai Institute of Plant Physiology, CAS
   *Creating a Functional Single-chromosome Yeast*

**12:30** Lunch

**LOOKING FORWARD**

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Goal for Sessions 12: Identify shared priorities and potential opportunities for future discussions to advance safety and security

**13:30** **Session 12: China-U.S. Collaboration: Opportunities to Prevent Future Pandemics**
**Chairs: James Le Duc, Zhiming Yuan, Diane Griffin, and Zhigao Bu**
Cooperating to combat emerging infectious diseases that affect China and the U.S. - Cooperation on laboratory safety: biocontainment research and collaboration across borders (including discussion on building a network of BSL-4 laboratories concept http://science.sciencemag.org/content/362/6412/267 and other collaborations between Chinese and American scientists)

**15:00** Tour of HVRI (proposed)

**17:00** Transport from HVRI to hotel

**17:00** Some participants depart for airport

**19:00** Optional Dinner and or group outing

**Friday, 11 January**

AM Optional: Group tour of Unit 731 Museum (proposed)

AM and PM: transportation provided to airport

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# U.S. China Dialogue and Workshop on the Challenges of Emerging Infections, Laboratory Safety, Global Health Security and Responsible Conduct in the Use of Gene Editing in Viral Infectious Disease Research

Harbin Veterinary Research Institute
Chinese Academy of Agricultural Sciences
Harbin, China

8-10 January 2019

**[Please review the entire agenda and make/ suggest changes. Please confirm that listed Chinese participates are attending, especially the speakers that are listed in red text, and that each speaker is speaking in the right session.]** **Hengsheng Ouyang, Chunbo Lou, and Xiaoli Xue are now on the agenda.**

## Monday, 7 January

Some participants arrive at the Aoluguya Hotel (No. 800, the 3rd Road of Chuangxin, Harbin)

Welcome reception for those who are at the hotel (proposed)

Evening activity for those that have arrived: group tour of the Harbin Ice Festival (proposed)

## Tuesday, 8 January

**8:30** Shuttle bus from Aoluguya Hotel to Harbin Veterinary Research Institute (HVRI)
(Weihai N Rd, Xiangfang Qu, Haerbin Shi, Heilongjiang Sheng, China 150060)

**WELCOME AND OPENING REMARKS**

**9:00 Welcome, Introductions, and Opening Remarks**

1. Welcome on behalf of Chinese Academy of Agricultural Sciences (CAAS): **Zhigao Bu**, Harbin Veterinary Research Institute
2. Remarks on Behalf of U.S. National Academies of Sciences, Engineering, and Medicine: **Benjamin Rusek**, Policy and Global Affairs, U.S. National Academy of Sciences (NAS) and **Diane Griffin**, Johns Hopkins Bloomberg School of Public Health and Vice-President of NAS

**9:15 Session 1. Keynote Addresses** *[20 min each]*
**Chair:** TBD

1. **Prof. Fu Gao**, Director-General, Chinese Center for Disease Control and Prevention
   *[Talk title TBD]*
2. **Prof. Linda Saif**, Ohio State University
   *Emerging swine coronaviruses and their interspecies transmission*



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**Q&A/Discussion** *[15-20 min]*

**10:15** Coffee and Tea Break

**EMERGING INFECTIOUS DISEASES**

Goal for Sessions 2 and 3: Consider progress in understanding two diseases of concern to China and the US, including the health and economic implications and what advances in understanding may be enabled by recent scientific and technical developments such as gene editing.

**10:30 Session 2. Swine Fever** *[15 min each]*

**Chair:** TBD

1. **Prof. Xiaodong Wu**, China Animal Health and Epidemiology Center)
   *Current Situation and Control Strategy of African Swine Fever in China*
2. **Prof. Zhigao Bu,** Harbin Veterinary Research Institute, CAAS *Characterization of African Swine Fever Virus Isolated from China*
3. **Dr. Douglas Gladue**, U.S. Department of Agriculture
   *ASFV vaccine status: Rapid production of recombinant ASFV for live attenuated vaccines*
4. *Prof. Hengsheng Ouyang, Jilin University*
   *Genome-edited pigs protected from classical swine fever virus*

**Q&A/Discussion** *[10-15 min]*

***Proposed discussion questions***

- What is known about the epidemiology, health, and economic impacts of Africa Swine Fever?
- What is the latest progress on developing effective countermeasures and limiting the spread of this disease?
- What critical areas remain unknown or what are key challenges to preventing and responding to Africa Swine Fever outbreaks? How can new scientific and technical advances contribute to research and development?

**11:30 Session 3. Influenza**

**Chairs: Hualan Chen** and **David Swayne**

1. **Overview: Perspectives on international collaborations for influenza research** *[10 min each]*
   **Hualan Chen** Harbin Veterinary Research Institute, CAAS
   *Influenza vaccine research in China and Globally*
   **David Swayne,** U.S. Department of Agriculture
   *Collaborating on Influenza Research from the U.S. perspective*
2. **Panel Discussion**
   Speakers provide 5 minutes opening remarks on the topics identified below, followed by discussion facilitated by the session Chairs
   **Prof. Yuwei Gao,** Academy of Military Medical Sciences
   *The Diversity of Avian Influenza Virus in Wild Bird in China*



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**Prof. Dayan Wang**, National Institute for Viral Disease Control and Prevention, China CDC
*Human Infection with Zoonotic Influenza*
**Prof. Wenjun Liu**, Institute of Microbiology, CAS
*The Anti-Influenza Drug Development by Targeting Nucleoprotein Nuclear Export*
**Prof. Chengjun Li,** Harbin Veterinary Research Institute, CAAS
*Interactions between Influenza Viral RNP Complex Proteins and Host Cellular Factors*

***Proposed discussion questions***

- What is known about the epidemiology, health, and economic impacts of Influenza?
- What is the latest progress on developing effective countermeasures?
- What critical areas remain unknown or what are key challenges to preventing and responding to influenza outbreaks? How can new scientific and technical advances contribute to research and development?

**12:45** **Group Photo**

**13:00** **Lunch**

**VIRAL PATHOGENS: SCIENCE AND RESPONSIBILITY**

Goal for Sessions 4, 5, and 6: Discuss relevant pathogen properties that researchers may seek to alter, and why, as part of studying viral pathogens with pandemic potential. Illustrate challenges and opportunities posed by new strategies such as use of gene editing, rational design approaches, other strategies enable by advancing biotechnology. Discuss the associated issues in the ethical planning and conduct of such research so that it can be conducted safety and securely.

**14:30** **Session 4. Understanding and Engineering Viral Pathogens with Pandemic Potential**
*[20 min each]*
Chairs**: Diane Griffin** and **Fu Gao**

1. **Ralph Baric**, University of North Carolina
   *Advances in Understanding and Altering Pathogen Properties*
2. **Prof. Weiwen Zhang**, Tianjin University
   *Cutting Edging New Biotechnology: Strategies and Tools for Engineering*

**15:15** Coffee and Tea break

**15:45** **Session 5. Bioethics and Responsible Planning for Pathogen Research** *[15 min each]*
**Chairs: Diane Griffin** and **Fu Gao**

1. **Prof. Zhiming Yuan**, Institute of Medical Biology, Chinese Academy of Medical Sciences
   *Biosafey and Bioethics in Medical Research*
2. **David Relman**, Stanford University
   *Proposed title: Bioethical Considerations for the Conception and Design of pathogen Research and Approaches for Assessing Potential Risks and Benefits of Research*
3. **Prof. Jianwie Wang,** Institute of Pathogen Biology, Chinese Academy of Medical Sciences & Peking Union Medical College



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*Biosafety Management of Synthetic Biology: Challenges and Progress*

4. **Associate Prof. Yang Xue**, Tianjin University
   *Biosafety and Bioethis Considerations in Dual-Use Risks Management – China's Efforts and Future Works*

**16:45** **Session 6. Panel on Science and Ethics in Research with Pathogens with Pandemic Potential**
**Chairs**: **Diane Griffin** and **Fu Gao**
Chairs facilitate a discussion between **Ralph Baric, Weiwen Zhang, Zhiming Yuan, and David Relman, Jianwie Wang, Yang Xue** and the audience. *[45 min]*

***Proposed discussion questions***

- *What do recent advances in biotechnology, such as genome editing, enable researchers to achieve in improved characterization and alteration of the properties of pathogenic viruses?*
- *What do you see coming in the near term? What still remains very challenging and why?*
- *Because research on viral pathogens can raise security concerns, how do you think about making decisions on whether or not to conduct such research or about modifying research proposals? What are the responsibilities and best practices of the PI to ensure that pathogen research is conducted safely and securely over the course of the project?*
- *What ethical and policy challenges remain and what actions do you think are needed to help overcome these challenges?*

**17:30** Transportation from HVRI to hotel

**19:00** Dinner at Hotel (proposed)

Optional: Harbin city tour/snowmobile/dogsledding? (proposed)

**Wednesday, 9 January**

**08:30** Transportation to HVRI

**VECTOR AND HOST ENGINEERING**
Goal for Session 7: Research to understand and prepare for viral pathogens may also involve work conducted on the hosts or vectors of such pathogens, for example to better understand host-pathogen responses and vector transmission. Discuss how advances in areas such as gene editing are affecting such research and best practices for safely and responsibly conducting such studies

**9:00** **Session 7. Vector and Host Engineering: Safety and Security** *[15-20 min each]*
**Chair: Jim Le Duc**, University of Texas Medical Branch and **Zhiming Yuan**

1. **Prof. Qian Han**, Hainan University
   *Biochemical Pathway of a Mosquito Innate Immunity, Melanization*
2. **Stephen Higgs**, Kansas State University
   *Proposed title: Best practices in safety and security for working with arthropods that serve as pathogen vectors, including responsible research with gene drive modified vectors*

**Q&A/ Discussion** *[20 min]*



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**10:00** Coffee and tea break

**10:30 Session 7 resumes**

3. **Highlights of Recent Research:**
   **Prof. Tongyan Zhao**, Institute of Microbiology and Epidemiology, Academy of Military Medical Sciences
   *Vector Competence of Emerging Mosquito Borne Virus in China*
4. **Prof. Gong Cheng**, Tsinghua University
   *A Gut Commensal Bacterium Promotes Arbovirus Infection in Mosquitoes*

**Q&A/ Discussion** *[30 min]*

***Proposed discussion questions***

- *What do recent advances in biotechnology, such as genome editing, enable researchers to achieve in studying and manipulating hosts and vectors of viral pathogens*
- *What do you see coming in the near term? What still remains very challenging and why?*
- *What are the responsibilities and best practices of the PI to ensure that pathogen research is conducted safely and securely over the course of the project?*
- *What ethical and policy challenges remain and what actions do you think are needed to help overcome these challenges?*

**12:00 Lunch**

**EFFECTIVE OVERSIGHT SYSTEMS**

Goal for Session 8: Discuss the systems of institutional review and oversight in China and in the US for safe and secure conduct of laboratory research on viral pathogens with pandeminc potential. Focus on the institutional systems including the important role of leadership in promoting institutional cultures that support responsible research practices.

**13:30 Session 8. Best Practices in Laboratory Safety and Security for Pathogen Research**
**Chair:** TBD

1. **Prof. Jianguo Xu**, Director General, National Institute for Communicable Disease Control and Prevention Chinese Center for Diseases control and Prevention. *[15 min]*
   *Proposed topic: Institutional review and oversight for research on viral pathogens with pandemic potential—the perspective from China*
2. **Joseph Kanabrocki**, ***University of Chicago*** *[15 min]*
   *Institutional review and oversight for research on viral pathogens with pandemic potential – a U.S. perspective*
3. **David Franz** and **Zhiming Yuan** *[20 min together]*
   *The Role of Leaders in Laboratory Safety: Culture and governance to steer toward behavior changes*

**Facilitated Discussion** *[xx min]*
Chair facilitates a discussion with the speakers and audience [20 min]



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***Proposed discussion questions***

*If assessment of anticipated risks and benefits results in the decision to undertake an experiment that involves characterizing or manipulating a pathogen with pandemic potential:*

1. *How should the research-conducting institution oversee this research to ensure that it is conducted safely and securely over the course of the project? Does this oversight happen at one point or at multiple points over the course of the project?*
2. *What are the responsibilities of institutional leadership in promoting the culture of responsible conduct of science?*
3. *Are there particular scientific or experimental practices that should be incorporated into research design and conduct that can maximize safety and security for studies carried out to understand or alter the properties of pathogens with pandemic potential?*

**14:40** **Special Topic: One Health in China** *[20 min]*
Understand how a One Health approach is shaping research to understand and address viral pathogens in China
**Prof. Jiahai Lu**, Sun Yat-sen University
**Q&A/Discussion** *[10 min]*

**15:10** Coffee and Tea Break

**RECENT RESEARCH AND PERSPECTIVES**

Goal for Sessions 9 and 10: Gain further shared insights into how research communities are addressing issues in biosafety, biosecurity, and bioethics, particularly as applied to research on emerging infectious diseases caused by viral pathogens

**15:30** **Session 9. Chinese Biosafety, Biosecurity, and Bioethics** *[15 min each]*
**Chair:** **Mifang Liang**, China CDC and **Joe Kanabrocki** (give some opening comments)

1. **Dr. Peijun Zhai**, CNAS
   *Overview of Laboratory Biosafety Management System in China*
2. **Prof. Jiancheng Qi**, National Biological Protection Engineering Center, China
   *Development and Application of Laboratory Biosafety Equipment in China*
3. **Prof. Yunzhang Hu**, Institute of Medical Biology, Chinese Academy of Medical Sciences
   *Kunming National Primate Research Center of High-Level Biosafety*
4. **Prof. Ruifu Yang**, Institute of Microbiology and Epidemiology, Academy of Military Medical Sciences
   *Microbial Forensics: Building Capability for Source Tracing of Bioterrorism Pathogen.*

**Q&A/Discussion** *[30 min]*

**17:00** Transportation from HVRI to hotel

**19:00** Optional Dinner and or group outing

**Thursday, 10 January**



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**8:30** Hotel shuttle bus transport to HVRI

**9:00** **Special Topic: Update on Acute Flaccid Myelitis**
(Review and discuss what is currently known about the cause of this condition)
**Diane Griffin,** Johns Hopkins Bloomberg School of Public Health [20 min]
*Talk title TBD*
**Q&A/Discussion** [*10 min]*

**9:30** **Session 10: Chinese Perspectives on Emerging Infectious Diseases** *[15 min each]*
**Chair: Pei-yong Shi**

1. **Prof. Changjiang Weng,** Harbin Veterinary Research Institute, CAAS
   *DDX19, A novel Viral RNA Sensor, Regulates IFN Signaling upon Viral Infection*
2. **Prof. Zhengli Shi,** Wuhan Institute of Virology, CAS
   *From SARS, MERS to SADS: Interspecies Transmission of Bat Coronaviruses*
3. **Prof. Haixue Zheng**, Lanzhou Veterinary Research Institute, CAAS,
   *Rational Design of Master Seed Virus to Improve the Safety and Effectiveness of Foot and Mouth Disease Vaccine*

**10:15** Coffee and Tea Break

**10:45** **Session 10, cont'd**

4. **Prof. Ruigong**, Wuhan Institute of Virology, CAS
   *Development of Neutralizing Antibodies Domains as Therapeutic Candidates Against Infectious Diseases*
5. **Prof. Longding Liu**, Institute of Medical Biology, Chinese Academy of Medical Sciences
   *Development of inactivated EV71 Vaccine (Human diploid cell) and Pathogenic Study of EV71 Infection in Rhesus Macaque*
6. **Prof. Chengfeng Qin**, Institute of Microbiology and Epidemiology, Academy of Military Medical Sciences
   *Zika Virus Neurotropism the Bad and the Good*

**Q&A/Discussion** *[30 min]*

**12:00** **Special Session 11**

1. **Prof. Chunbo Lou,** Institute of Microbiology, CAS
   *Large Gene Cluster and Regulatory Manipulation for Activating the Cryptic Natural Product Clusters in Streptomyces*
2. **Prof. Xiaoli Xue,** Key Laboratory of Synthetic Biology, Shanghai Institute of Plant Physiology, CAS
   *Creating a Functional Single-chromosome Yeast*

**12:30** Lunch

**LOOKING FORWARD (CLOSED SESSION, BY INVITATION ONLY)**



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Goal for Sessions 12: Identify shared priorities and potential opportunities for future discussions to advance safety and security

**13:30** **Session 12: China-U.S. Collaboration: Opportunities to Prevent Future Pandemics**
**Chairs: James Le Duc, Zhiming Yuan, Diane Griffin, and Zhigao Bu**
Cooperating to combat emerging infectious diseases that affect China and the U.S. - Cooperation on laboratory safety: biocontainment research and collaboration across borders (including discussion on building a network of BSL-4 laboratories concept http://science.sciencemag.org/content/362/6412/267 and other collaborations between Chinese and American scientists)

**15:00** Tour of HVRI (proposed)

**17:00** Transport from HVRI to hotel

**17:00** Some participants depart for airport

**19:00** Optional Dinner and or group outing

**Friday, 11 January**

AM Optional: Group tour of Unit 731 Museum (proposed)

AM and PM: transportation provided to airport

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2018-12-25

# U.S. China Dialogue and Workshop on the Challenges of Emerging Infections, Laboratory Safety, Global Health Security and Responsible Conduct in the Use of Gene Editing in Viral Infectious Disease Research

Harbin Veterinary Research Institute
Chinese Academy of Agricultural Sciences
Harbin, China

8-10 January 2019

## Monday, 7 January

Some participants arrive at the Aoluguya Hotel (No. 800, the 3rd Road of Chuangxin, Harbin)

Welcome reception for those who are at the hotel (proposed)

Evening activity for those that have arrived: group tour of the Harbin Ice Festival (proposed)

## Tuesday, 8 January

**8:30** Shuttle bus from Aoluguya Hotel to Harbin Veterinary Research Institute (HVRI)
(Weihai N Rd, Xiangfang Qu, Haerbin Shi, Heilongjiang Sheng, China 150060)

**WELCOME AND OPENING REMARKS**

**9:00** **Welcome, Introductions, and Opening Remarks**

1. Welcome on behalf of Chinese Academy of Agricultural Sciences (CAAS): **Zhigao Bu**, Harbin Veterinary Research Institute
2. Remarks on Behalf of U.S. National Academies of Sciences, Engineering, and Medicine: **Benjamin Rusek**, Policy and Global Affairs, U.S. National Academy of Sciences (NAS) and **Diane Griffin**, Johns Hopkins Bloomberg School of Public Health and Vice-President of NAS

**9:15** **Session 1. Keynote Addresses** *[20 min each]*
**Chair:** TBD

1. **Prof. Fu Gao**, Director-General, Chinese Center for Disease Control and Prevention
   *[Talk title TBD]*
2. **Prof. Linda Saif**, Ohio State University
   *Emerging swine coronaviruses and their interspecies transmission*

**Q&A/Discussion** *[15-20 min]*

**10:15** Coffee and Tea Break



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**EMERGING INFECTIOUS DISEASES**

Goal for Sessions 2 and 3: Consider progress in understanding two diseases of concern to China and the US, including the health and economic implications and what advances in understanding may be enabled by recent scientific and technical developments such as gene editing.

**10:30 Session 2. Swine Fever** *[15 min each]*

**Chair:** TBD

1. **Prof. Xiaodong Wu**, China Animal Health and Epidemiology Center)
   *Current Situation and Control Strategy of African Swine Fever in China*
2. **Prof. Zhigao Bu,** Harbin Veterinary Research Institute, CAAS *Characterization of African Swine Fever Virus Isolated from China*
3. **Dr. Douglas Gladue**, U.S. Department of Agriculture
   *ASFV vaccine status: Rapid production of recombinant ASFV for live attenuated vaccines*
4. *Prof. Hengsheng Ouyang, Jilin University*
   *Genome-edited pigs protected from classical swine fever virus*
   **Q&A/Discussion** *[10-15 min]*

***Proposed discussion questions***

- What is known about the epidemiology, health, and economic impacts of Africa Swine Fever?
- What is the latest progress on developing effective countermeasures and limiting the spread of this disease?
- What critical areas remain unknown or what are key challenges to preventing and responding to Africa Swine Fever outbreaks? How can new scientific and technical advances contribute to research and development?

**11:30 Session 3. Influenza**

**Chairs**: **Hualan Chen** and **David Swayne**

1. **Overview: Perspectives on international collaborations for influenza research** *[10 min each]*
   **Hualan Chen** Harbin Veterinary Research Institute, CAAS
   *Influenza vaccine research in China and Globally*
   **David Swayne,** U.S. Department of Agriculture
   *Collaborating on Influenza Research from the U.S. perspective*
2. **Panel Discussion**
   Speakers provide 5 minutes opening remarks on the topics identified below, followed by discussion facilitated by the session Chairs
   **Prof. Yuwei Gao,** Academy of Military Medical Sciences
   *The Diversity of Avian Influenza Virus in Wild Bird in China*
   **Prof. Dayan Wang**, National Institute for Viral Disease Control and Prevention, China CDC
   *Human Infection with Zoonotic Influenza*
   **Prof. Wenjun Liu**, Institute of Microbiology, CAS
   *The Anti-Influenza Drug Development by Targeting Nucleoprotein Nuclear Export*
   **Prof. Chengjun Li,** Harbin Veterinary Research Institute, CAAS



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*Interactions between Influenza Viral RNP Complex Proteins and Host Cellular Factors*

***Proposed discussion questions***

- What is known about the epidemiology, health, and economic impacts of Influenza?
- What is the latest progress on developing effective countermeasures?
- What critical areas remain unknown or what are key challenges to preventing and responding to influenza outbreaks? How can new scientific and technical advances contribute to research and development?

**12:45** **Group Photo**

**13:00** **Lunch**

**VIRAL PATHOGENS: SCIENCE AND RESPONSIBILITY**

Goal for Sessions 4, 5, and 6: Discuss relevant pathogen properties that researchers may seek to alter, and why, as part of studying viral pathogens with pandemic potential. Illustrate challenges and opportunities posed by new strategies such as use of gene editing, rational design approaches, other strategies enable by advancing biotechnology. Discuss the associated issues in the ethical planning and conduct of such research so that it can be conducted safety and securely.

**14:30** **Session 4. Understanding and Engineering Viral Pathogens with Pandemic Potential** *[20 min each]*

Chairs: **Diane Griffin** and **Fu Gao**

1. **Ralph Baric**, University of North Carolina
   *Advances in Understanding and Altering Pathogen Properties*
2. **Prof. Weiwen Zhang**, Tianjin University
   *Cutting Edging New Biotechnology: Strategies and Tools for Engineering*

**15:15** Coffee and Tea break

**15:45** **Session 5. Bioethics and Responsible Planning for Pathogen Research** *[15 min each]*

**Chairs**: **Diane Griffin** and **Fu Gao**

1. **Prof. Zhiming Yuan**, Institute of Medical Biology, Chinese Academy of Medical Sciences
   *Biosafey and Bioethics in Medical Research*
2. **David Relman**, Stanford University
   *Proposed title: Bioethical Considerations for the Conception and Design of pathogen Research and Approaches for Assessing Potential Risks and Benefits of Research*
3. **Prof. Jianwie Wang,** Institute of Pathogen Biology, Chinese Academy of Medical Sciences & Peking Union Medical College
   *Biosafety Management of Synthetic Biology: Challenges and Progress*
4. **Associate Prof. Yang Xue**, Tianjin University
   *Biosafety and Bioethis Considerations in Dual-Use Risks Management – China's Efforts and Future Works*



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**16:45** **Session 6. Panel on Science and Ethics in Research with Pathogens with Pandemic Potential**
**Chairs**: **Diane Griffin** and **Fu Gao**
Chairs facilitate a discussion between **Ralph Baric, Weiwen Zhang, Zhiming Yuan, and David Relman, Jianwie Wang, Yang Xue** and the audience. *[45 min]*

***Proposed discussion questions***

- *What do recent advances in biotechnology, such as genome editing, enable researchers to achieve in improved characterization and alteration of the properties of pathogenic viruses?*
- *What do you see coming in the near term? What still remains very challenging and why?*
- *Because research on viral pathogens can raise security concerns, how do you think about making decisions on whether or not to conduct such research or about modifying research proposals? What are the responsibilities and best practices of the PI to ensure that pathogen research is conducted safely and securely over the course of the project?*
- *What ethical and policy challenges remain and what actions do you think are needed to help overcome these challenges?*

**17:30** Transportation from HVRI to hotel

**19:00** Dinner at Hotel (proposed)

Optional: Harbin city tour/snowmobile/dogsledding? (proposed)

**Wednesday, 9 January**

**08:30** Transportation to HVRI

**VECTOR AND HOST ENGINEERING**
Goal for Session 7: Research to understand and prepare for viral pathogens may also involve work conducted on the hosts or vectors of such pathogens, for example to better understand host-pathogen responses and vector transmission. Discuss how advances in areas such as gene editing are affecting such research and best practices for safely and responsibly conducting such studies

**9:00** **Session 7. Vector and Host Engineering: Safety and Security** *[15-20 min each]*
**Chair: Jim Le Duc**, University of Texas Medical Branch and **Zhiming Yuan**

1. **Prof. Qian Han**, Hainan University
   *Biochemical Pathway of a Mosquito Innate Immunity, Melanization*
2. **Stephen Higgs**, Kansas State University
   *Proposed title: Best practices in safety and security for working with arthropods that serve as pathogen vectors, including responsible research with gene drive modified vectors*

**Q&A/ Discussion** *[20 min]*

**10:00** Coffee and tea break

**10:30** **Session 7 resumes**

3. **Highlights of Recent Research:**



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**Prof. Tongyan Zhao**, Institute of Microbiology and Epidemiology, Academy of Military Medical Sciences
*Vector Competence of Emerging Mosquito Borne Virus in China*

4. **Prof. Gong Cheng**, Tsinghua University
*A Gut Commensal Bacterium Promotes Arbovirus Infection in Mosquitoes*

**Q&A/ Discussion** *[30 min]*

***Proposed discussion questions***

- *What do recent advances in biotechnology, such as genome editing, enable researchers to achieve in studying and manipulating hosts and vectors of viral pathogens*
- *What do you see coming in the near term? What still remains very challenging and why?*
- *What are the responsibilities and best practices of the PI to ensure that pathogen research is conducted safely and securely over the course of the project?*
- *What ethical and policy challenges remain and what actions do you think are needed to help overcome these challenges?*

**12:00 Lunch**

**EFFECTIVE OVERSIGHT SYSTEMS**

Goal for Session 8: Discuss the systems of institutional review and oversight in China and in the US for safe and secure conduct of laboratory research on viral pathogens with pandeminc potential. Focus on the institutional systems including the important role of leadership in promoting institutional cultures that support responsible research practices.

**13:30 Session 8. Best Practices in Laboratory Safety and Security for Pathogen Research**
**Chair:** TBD

1. **Joseph Kanabrocki**, ***University of Chicago*** *[15 min]*
*Institutional review and oversight for research on viral pathogens with pandemic potential – a U.S. perspective*
2. **David Franz** and **Zhiming Yuan** *[20 min together]*
*The Role of Leaders in Laboratory Safety: Culture and governance to steer toward behavior changes*

**Facilitated Discussion** *[xx min]*
Chair facilitates a discussion with the speakers and audience [20 min]

***Proposed discussion questions***

*If assessment of anticipated risks and benefits results in the decision to undertake an experiment that involves characterizing or manipulating a pathogen with pandemic potential:*

1. *How should the research-conducting institution oversee this research to ensure that it is conducted safely and securely over the course of the project? Does this oversight happen at one point or at multiple points over the course of the project?*
2. *What are the responsibilities of institutional leadership in promoting the culture of responsible conduct of science?*

**Deleted:** <#>Prof. Jianguo Xu, Director General, National Institute for Communicable Disease Control and Prevention Chinese Center for Diseases control and Prevention. *[15 min]*¶
*Proposed topic: Institutional review and oversight for research on viral pathogens with pandemic potential—the perspective from China* ¶



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3. *Are there particular scientific or experimental practices that should be incorporated into research design and conduct that can maximize safety and security for studies carried out to understand or alter the properties of pathogens with pandemic potential?*

**14:40** **Special Topic: One Health in China** *[20 min]*
Understand how a One Health approach is shaping research to understand and address viral pathogens in China
**Prof. Jiahai Lu**, Sun Yat-sen University
**Q&A/Discussion** *[10 min]*

**15:10** Coffee and Tea Break

**RECENT RESEARCH AND PERSPECTIVES**
Goal for Sessions 9 and 10: Gain further shared insights into how research communities are addressing issues in biosafety, biosecurity, and bioethics, particularly as applied to research on emerging infectious diseases caused by viral pathogens

**15:30** **Session 9. Chinese Biosafety, Biosecurity, and Bioethics** *[15 min each]*
**Chair: Mifang Liang**, China CDC and **Joe Kanabrocki** (give some opening comments)
1. **Dr. Peijun Zhai**, CNAS
   *Overview of Laboratory Biosafety Management System in China*
2. **Prof. Jiancheng Qi**, National Biological Protection Engineering Center, China
   *Development and Application of Laboratory Biosafety Equipment in China*
3. **Prof. Yunzhang Hu**, Institute of Medical Biology, Chinese Academy of Medical Sciences
   *Kunming National Primate Research Center of High-Level Biosafety*
4. **Prof. Ruifu Yang**, Institute of Microbiology and Epidemiology, Academy of Military Medical Sciences
   *Microbial Forensics: Building Capability for Source Tracing of Bioterrorism Pathogen.*

**Q&A/Discussion** *[30 min]*

**17:00** Transportation from HVRI to hotel

**19:00** Optional Dinner and or group outing

**Thursday, 10 January**

**8:30** Hotel shuttle bus transport to HVRI

**9:00** **Special Topic: Update on Acute Flaccid Myelitis**
(Review and discuss what is currently known about the cause of this condition)
**Diane Griffin,** Johns Hopkins Bloomberg School of Public Health [20 min]
*Talk title TBD*
**Q&A/Discussion** [*10 min]*



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**9:30** **Session 10: Chinese Perspectives on Emerging Infectious Diseases** *[15 min each]*
**Chair: Pei-yong Shi**

1. **Prof. Changjiang Weng,** Harbin Veterinary Research Institute, CAAS
   *DDX19, A novel Viral RNA Sensor, Regulates IFN Signaling upon Viral Infection*
2. **Prof. Zhengli Shi,** Wuhan Institute of Virology, CAS
   *From SARS, MERS to SADS: Interspecies Transmission of Bat Coronaviruses*
3. **Prof. Haixue Zheng**, Lanzhou Veterinary Research Institute, CAAS,
   *Rational Design of Master Seed Virus to Improve the Safety and Effectiveness of Foot and Mouth Disease Vaccine*

**10:15** Coffee and Tea Break

**10:45** **Session 10, cont'd**

4. **Prof. Ruigong**, Wuhan Institute of Virology, CAS
   *Development of Neutralizing Antibodies Domains as Therapeutic Candidates Against Infectious Diseases*
5. **Prof. Longding Liu**, Institute of Medical Biology, Chinese Academy of Medical Sciences
   *Development of inactivated EV71 Vaccine (Human diploid cell) and Pathogenic Study of EV71 Infection in Rhesus Macaque*
6. **Prof. Chengfeng Qin**, Institute of Microbiology and Epidemiology, Academy of Military Medical Sciences
   *Zika Virus Neurotropism the Bad and the Good*

**Q&A/Discussion** *[30 min]*

**12:00** **Special Session 11**

1. **Prof. Chunbo Lou,** Institute of Microbiology, CAS
   *Large Gene Cluster and Regulatory Manipulation for Activating the Cryptic Natural Product Clusters in Streptomyces*
2. **Prof. Xiaoli Xue,** Key Laboratory of Synthetic Biology, Shanghai Institute of Plant Physiology, CAS
   *Creating a Functional Single-chromosome Yeast*

**12:30** Lunch

**LOOKING FORWARD (CLOSED SESSION, BY INVITATION ONLY)**
Goal for Sessions 12: Identify shared priorities and potential opportunities for future discussions to advance safety and security

**13:30** **Session 12: China-U.S. Collaboration: Opportunities to Prevent Future Pandemics**
**Chairs: James Le Duc, Zhiming Yuan, Diane Griffin, and Zhigao Bu**
Cooperating to combat emerging infectious diseases that affect China and the U.S. -
Cooperation on laboratory safety: biocontainment research and collaboration across borders (including discussion on building a network of BSL-4 laboratories concept



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http://science.sciencemag.org/content/362/6412/267 and other collaborations between Chinese and American scientists)

**15:00** Tour of HVRI (proposed)

**17:00** Transport from HVRI to hotel

**17:00** Some participants depart for airport

**19:00** Optional Dinner and or group outing

**Friday, 11 January**

AM Optional: Group tour of Unit 731 Museum (proposed)

AM and PM: transportation provided to airport

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2018-12-27

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# U.S. China Dialogue and Workshop on the Challenges of Emerging Infections, Laboratory Safety, Global Health Security and Responsible Conduct in the Use of Gene Editing in Viral Infectious Disease Research

Harbin Veterinary Research Institute
Chinese Academy of Agricultural Sciences
Harbin, China

8-10 January 2019

**Monday, 7 January**

Some participants arrive at the Aoluguya Hotel (No. 800, the 3rd Road of Chuangxin, Harbin)

Evening activity for those that have arrived: group tour of the Harbin Ice Festival (proposed)

Deleted: Welcome reception for those who are at the hotel (proposed) ¶
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**Tuesday, 8 January**

**8:30** Shuttle bus from Aoluguya Hotel to Harbin Veterinary Research Institute (HVRI)
(Weihai N Rd, Xiangfang Qu, Haerbin Shi, Heilongjiang Sheng, China 150060)

**WELCOME AND OPENING REMARKS**

**9:00** **Welcome, Introductions, and Opening Remarks**

1. Welcome on behalf of Chinese Academy of Agricultural Sciences (CAAS): **Zhigao Bu**, Harbin Veterinary Research Institute
2. Remarks on Behalf of U.S. National Academies of Sciences, Engineering, and Medicine: **Benjamin Rusek**, Policy and Global Affairs, U.S. National Academy of Sciences (NAS) and **Diane Griffin**, Johns Hopkins Bloomberg School of Public Health and Vice-President of NAS

**9:15** **Session 1. Keynote Addresses** *[20 min each]*
**Chair:** TBD

1. **Prof. Fu Gao**, Director-General, Chinese Center for Disease Control and Prevention
   *Talk title TBD*
2. **Prof. Linda Saif**, Ohio State University
   *Emerging swine coronaviruses and their interspecies transmission*

**Q&A/Discussion** *[15-20 min]*

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**10:15** Coffee and Tea Break

**EMERGING INFECTIOUS DISEASES**



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Goal for Sessions 2 and 3: Consider progress in understanding two diseases of concern to China and the US, including the health and economic implications and what advances in understanding may be enabled by recent scientific and technical developments such as gene editing.

**10:30 Session 2. Swine Fever** *[15 min each]*
**Chair:** TBD

1. **Prof. Xiaodong Wu**, China Animal Health and Epidemiology Center)
   *Current Situation and Control Strategy of African Swine Fever in China*
2. **Prof. Zhigao Bu,** Harbin Veterinary Research Institute, CAAS *Characterization of African Swine Fever Virus Isolated from China*
3. **Dr. Douglas Gladue**, U.S. Department of Agriculture
   *ASFV vaccine status: Rapid production of recombinant ASFV for live attenuated vaccines*
4. *Prof. Hengsheng Ouyang, Jilin University*
   *Genome-edited pigs protected from classical swine fever virus*
   **Q&A/Discussion** *[10-15 min]*

***Proposed discussion questions***

- What is known about the epidemiology, health, and economic impacts of Africa Swine Fever?
- What is the latest progress on developing effective countermeasures and limiting the spread of this disease?
- What critical areas remain unknown or what are key challenges to preventing and responding to Africa Swine Fever outbreaks? How can new scientific and technical advances contribute to research and development?

**11:30 Session 3. Influenza**
**Chairs: Hualan Chen** and **David Swayne**

1. **Overview: Perspectives on international collaborations for influenza research** *[10 min each]*
   **Hualan Chen** Harbin Veterinary Research Institute, CAAS
   *Influenza vaccine research in China and Globally*
   **David Swayne,** U.S. Department of Agriculture
   *Collaborating on Influenza Research from the U.S. perspective*
2. **Panel Discussion**
   Speakers provide 5 minutes opening remarks on the topics identified below, followed by discussion facilitated by the session Chairs
   ~~**Prof. Yuwei Gao,** Academy of Military Medical Sciences~~
   ~~*The Diversity of Avian Influenza Virus in Wild Bird in China*~~
   **Prof. Dayan Wang**, National Institute for Viral Disease Control and Prevention, China CDC
   *Human Infection with Zoonotic Influenza*
   **Prof. Wenjun Liu**, Institute of Microbiology, CAS
   *The Anti-Influenza Drug Development by Targeting Nucleoprotein Nuclear Export*
   **Prof. Chengjun Li,** Harbin Veterinary Research Institute, CAAS
   *Interactions between Influenza Viral RNP Complex Proteins and Host Cellular Factors*

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***Proposed discussion questions***

- What is known about the epidemiology, health, and economic impacts of Influenza?
- What is the latest progress on developing effective countermeasures?
- What critical areas remain unknown or what are key challenges to preventing and responding to influenza outbreaks? How can new scientific and technical advances contribute to research and development?

**12:45** **Group Photo**

**13:00** **Lunch**

**VIRAL PATHOGENS: SCIENCE AND RESPONSIBILITY**

Goal for Sessions 4, 5, and 6: Discuss relevant pathogen properties that researchers may seek to alter, and why, as part of studying viral pathogens with pandemic potential. Illustrate challenges and opportunities posed by new strategies such as use of gene editing, rational design approaches, other strategies enable by advancing biotechnology. Discuss the associated issues in the ethical planning and conduct of such research so that it can be conducted safety and securely.

**14:30** **Session 4. Understanding and Engineering Viral Pathogens with Pandemic Potential** *[20 min each]*

Chairs**: Diane Griffin** and **Fu Gao**

1. **Ralph Baric**, University of North Carolina
   *Advances in Understanding and Altering Pathogen Properties*
2. **Prof. Weiwen Zhang**, Tianjin University
   *Cutting Edging New Biotechnology: Strategies and Tools for Engineering*

**15:15** Coffee and Tea break

**15:45** **Session 5. Bioethics and Responsible Planning for Pathogen Research** *[15 min each]*

**Chairs: Diane Griffin** and **Fu Gao**

1. **Prof. Zhiming Yuan**, Institute of Medical Biology, Chinese Academy of Medical Sciences
   *Biosafey and Bioethics in Medical Research*
2. **David Relman**, Stanford University
   *Proposed title: Bioethical Considerations for the Conception and Design of pathogen Research and Approaches for Assessing Potential Risks and Benefits of Research*
3. **Prof. Jianwie Wang,** Institute of Pathogen Biology, Chinese Academy of Medical Sciences & Peking Union Medical College
   *Biosafety Management of Synthetic Biology: Challenges and Progress*
4. **Associate Prof. Yang Xue**, Tianjin University
   *Biosafety and Bioethis Considerations in Dual-Use Risks Management – China's Efforts and Future Works*

**16:45** **Session 6. Panel on Science and Ethics in Research with Pathogens with Pandemic Potential**



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**Chairs**: **Diane Griffin** and **Fu Gao**
Chairs facilitate a discussion between **Ralph Baric, Weiwen Zhang, Zhiming Yuan, and David Relman, Jianwie Wang, Yang Xue** and the audience. *[45 min]*

***Proposed discussion questions***

- *What do recent advances in biotechnology, such as genome editing, enable researchers to achieve in improved characterization and alteration of the properties of pathogenic viruses?*
- *What do you see coming in the near term? What still remains very challenging and why?*
- *Because research on viral pathogens can raise security concerns, how do you think about making decisions on whether or not to conduct such research or about modifying research proposals? What are the responsibilities and best practices of the PI to ensure that pathogen research is conducted safely and securely over the course of the project?*
- *What ethical and policy challenges remain and what actions do you think are needed to help overcome these challenges?*

**17:30** Transportation from HVRI to hotel

**19:00** Dinner at Hotel (proposed)

Optional: Harbin city tour/snowmobile/dogsledding? (proposed)

**Wednesday, 9 January**

**08:30** Transportation to HVRI

**VECTOR AND HOST ENGINEERING**
Goal for Session 7: Research to understand and prepare for viral pathogens may also involve work conducted on the hosts or vectors of such pathogens, for example to better understand host-pathogen responses and vector transmission. Discuss how advances in areas such as gene editing are affecting such research and best practices for safely and responsibly conducting such studies

**9:00** **Session 7. Vector and Host Engineering: Safety and Security** *[15-20 min each]*
**Chair: Jim Le Duc**, University of Texas Medical Branch and **Zhiming Yuan**

1. **Prof. Qian Han**, Hainan University
   *Biochemical Pathway of a Mosquito Innate Immunity, Melanization*
2. **Stephen Higgs**, Kansas State University
   *Best practices in safety and security for working with arthropods that serve as pathogen vectors, including responsible research with gene drive modified vectors*

Deleted: *Proposed title:*

**Q&A/ Discussion** *[20 min]*

**10:00** Coffee and tea break

**10:30** **Session 7 resumes**

3. **Highlights of Recent Research:**



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**Prof. Tongyan Zhao**, Institute of Microbiology and Epidemiology, Academy of Military Medical Sciences
*Vector Competence of Emerging Mosquito Borne Virus in China*

4. **Prof. Gong Cheng**, Tsinghua University
   *A Gut Commensal Bacterium Promotes Arbovirus Infection in Mosquitoes*

**Q&A/ Discussion** *[30 min]*

***Proposed discussion questions***

- *What do recent advances in biotechnology, such as genome editing, enable researchers to achieve in studying and manipulating hosts and vectors of viral pathogens*
- *What do you see coming in the near term? What still remains very challenging and why?*
- *What are the responsibilities and best practices of the PI to ensure that pathogen research is conducted safely and securely over the course of the project?*
- *What ethical and policy challenges remain and what actions do you think are needed to help overcome these challenges?*

**12:00 Lunch**

**EFFECTIVE OVERSIGHT SYSTEMS**

Goal for Session 8: Discuss the systems of institutional review and oversight in China and in the US for safe and secure conduct of laboratory research on viral pathogens with pandeminc potential. Focus on the institutional systems including the important role of leadership in promoting institutional cultures that support responsible research practices.

**13:30 Session 8. Best Practices in Laboratory Safety and Security for Pathogen Research**
**Chair:** TBD

1. **Joseph Kanabrocki**, ***University of Chicago*** *[15 min]*
   *Institutional review and oversight for research on viral pathogens with pandemic potential – a U.S. perspective*
2. **David Franz** and **Zhiming Yuan** *[20 min together]*
   *The Role of Leaders in Laboratory Safety: Culture and governance to steer toward behavior changes*

**Facilitated Discussion** *[xx min]*
Chair facilitates a discussion with the speakers and audience [20 min]

Deleted: <#> Prof. Jianguo Xu, Director General, National Institute for Communicable Disease Control and Prevention Chinese Center for Diseases control and Prevention. *[15 min]* ¶
<#> *Proposed topic: Institutional review and oversight for research on viral pathogens with pandemic potential— the perspective from China* ¶

***Proposed discussion questions***

*If assessment of anticipated risks and benefits results in the decision to undertake an experiment that involves characterizing or manipulating a pathogen with pandemic potential:*

1. *How should the research-conducting institution oversee this research to ensure that it is conducted safely and securely over the course of the project? Does this oversight happen at one point or at multiple points over the course of the project?*
2. *What are the responsibilities of institutional leadership in promoting the culture of responsible conduct of science?*



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3. *Are there particular scientific or experimental practices that should be incorporated into research design and conduct that can maximize safety and security for studies carried out to understand or alter the properties of pathogens with pandemic potential?*

**14:40** **Special Topic: One Health in China** *[20 min]*
Understand how a One Health approach is shaping research to understand and address viral pathogens in China
**Prof. Jiahai Lu**, Sun Yat-sen University
**Q&A/Discussion** *[10 min]*

**15:10** Coffee and Tea Break

**RECENT RESEARCH AND PERSPECTIVES**

Goal for Sessions 9 and 10: Gain further shared insights into how research communities are addressing issues in biosafety, biosecurity, and bioethics, particularly as applied to research on emerging infectious diseases caused by viral pathogens

**15:30** **Session 9. Chinese Biosafety, Biosecurity, and Bioethics** *[15 min each]*
**Chair: Mifang Liang**, China CDC and **Joe Kanabrocki** (give some opening comments)

1. **Dr. Peijun Zhai**, CNAS
   *Overview of Laboratory Biosafety Management System in China*
2. **Prof. Jiancheng Qi**, National Biological Protection Engineering Center, China
   *Development and Application of Laboratory Biosafety Equipment in China*
3. **Prof. Yunzhang Hu**, Institute of Medical Biology, Chinese Academy of Medical Sciences
   *Kunming National Primate Research Center of High-Level Biosafety*
4. **Prof. Ruifu Yang**, Institute of Microbiology and Epidemiology, Academy of Military Medical Sciences
   *Microbial Forensics: Building Capability for Source Tracing of Bioterrorism Pathogen.*

**Q&A/Discussion** *[30 min]*

**17:00** Transportation from HVRI to hotel

**19:00** Optional Dinner and or group outing

**Thursday, 10 January**

**8:30** Hotel shuttle bus transport to HVRI

**9:00** **Special Topic: Update on Acute Flaccid Myelitis**
**Diane Griffin,** Johns Hopkins Bloomberg School of Public Health [20 min]
*Review of what is currently known about the cause of Acute Flaccid Myelitis*
**Q&A/Discussion** [*10 min]*

**9:30** **Session 10: Chinese Perspectives on Emerging Infectious Diseases** *[15 min each]*

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Deleted: this condition)
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**Chair: Pei-yong Shi**

1. **Prof. Changjiang Weng,** Harbin Veterinary Research Institute, CAAS
   *DDX19, A novel Viral RNA Sensor, Regulates IFN Signaling upon Viral Infection*
2. **Prof. Zhengli Shi,** Wuhan Institute of Virology, CAS
   *From SARS, MERS to SADS: Interspecies Transmission of Bat Coronaviruses*
3. **Prof. Haixue Zheng**, Lanzhou Veterinary Research Institute, CAAS,
   *Rational Design of Master Seed Virus to Improve the Safety and Effectiveness of Foot and Mouth Disease Vaccine*

**10:15** Coffee and Tea Break

**10:45 Session 10, cont'd**

4. **Prof. Ruigong**, Wuhan Institute of Virology, CAS
   *Development of Neutralizing Antibodies Domains as Therapeutic Candidates Against Infectious Diseases*
5. **Prof. Longding Liu**, Institute of Medical Biology, Chinese Academy of Medical Sciences
   *Development of inactivated EV71 Vaccine (Human diploid cell) and Pathogenic Study of EV71 Infection in Rhesus Macaque*
6. **Prof. Chengfeng Qin**, Institute of Microbiology and Epidemiology, Academy of Military Medical Sciences
   *Zika Virus Neurotropism the Bad and the Good*

**Q&A/Discussion** *[30 min]*

**12:00 Special Session 11**

1. **Prof. Chunbo Lou,** Institute of Microbiology, CAS
   *Large Gene Cluster and Regulatory Manipulation for Activating the Cryptic Natural Product Clusters in Streptomyces*
2. **Prof. Xiaoli Xue,** Key Laboratory of Synthetic Biology, Shanghai Institute of Plant Physiology, CAS
   *Creating a Functional Single-chromosome Yeast*

**12:30** Lunch

**LOOKING FORWARD (CLOSED SESSION, BY INVITATION ONLY)**

Goal for Sessions 12: Identify shared priorities and potential opportunities for future discussions to advance safety and security

**13:30 Session 12: China-U.S. Collaboration: Opportunities to Prevent Future Pandemics**
**Chairs: James Le Duc, Zhiming Yuan, Diane Griffin, and Zhigao Bu**
Cooperating to combat emerging infectious diseases that affect China and the U.S. - Cooperation on laboratory safety: biocontainment research and collaboration across borders (including discussion on building a network of BSL-4 laboratories concept http://science.sciencemag.org/content/362/6412/267 and other collaborations between Chinese and American scientists)



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**15:00** Tour of HVRI (proposed)

**17:00** Transport from HVRI to hotel

**17:00** Some participants depart for airport

**19:00** Optional Dinner and or group outing

**Friday, 11 January**

AM: Optional group tour of Unit 731 Museum (proposed)

Deleted: Optional

Deleted: G

AM and PM: transportation provided to airport



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**From:** Gallo, Robert <rgallo@ihv.umaryland.edu>
**Sent:** Friday, January 4, 2019 1:07 PM
**To:** LeDuc, James W. (b)(6) Gallo, Robert
**Subject:** RE: Biosecurity

**WARNING:** This email originated from outside of UTMB's email system. Do not click links or open attachments unless you recognize the sender and know the content is safe.

Dear James,

Yes, I am available Saturday for this call. Please let me know the best time.

Thank you,

**Robert C. Gallo, MD**
The Homer & Martha Gudelsky Distinguished Professor in Medicine
Co-Founder & Director, Institute of Human Virology *at the*
University of Maryland School of Medicine
Co-Founder & Scientific Director, Global Virus Network
Institute of Human Virology at the
University of Maryland School of Medicine
725 W Lombard Street, S307
Baltimore, MD 21201
(410) 706-8614

**From:** LeDuc, James W. [mailto:jwleduc@UTMB.EDU]
**Sent:** Friday, January 04, 2019 10:56 AM
**To:** (b)(6) Gallo, Robert <rgallo@ihv.umaryland.edu>
**Subject:** Biosecurity

Dear John and Bob,

I hope you both had a pleasant holiday break and that the new year is off to a great start.

I wanted to follow up on our earlier discussions on the biosecurity activities and the GVN. Following our meeting in Annecy, I had a chance to talk with several leaders at WHO including Mike Ryan, the assistant director general in charge of infectious diseases. I briefed them on the work we have been doing bilaterally between the US National Academy of Science and the Chinese Academy of Science and shared with him the attached editorial published in the attached October 2018 in *Science*. As a result of those meetings, I was introduced (virtually) to Mark Perkins, and American working at WHO in lab issues. My email exchange with Mark is below and I spoke with him at length yesterday about the need for a global network of BSL4 labs. I think we are in general agreement and it is clear to me that we need to engage with WHO going forward, but we can't leave it to them to make it happen.

With that background, I leave early Sunday morning for another joint meeting with the US and China National Academies of Science, this time to be held in Harbin at their BSL4 agriculture research center. The agenda of our meeting is attached just FYI. During that meeting, I have scheduled time to meet with a good friend, George Gao, the



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director of the China CDC to discuss next steps in developing the global network of BSL4 labs. We will continue the bilateral collaborations between the US and China National Academies of Science, but I'm concerned about the move to global engagement in building this network. **I wonder if we could have a brief call to discuss before I leave for Harbin?** I'm free all day today and tomorrow (Saturday) through about mid-day. My direct office line is 409-266-6516 and cell is (b)(6)

Thanks, Jim

James W. Le Duc, Ph.D.
Director
Galveston National Laboratory
University of Texas Medical Branch
Galveston, TX 77555-0610
(t) 409-266-6500
(f) 409-266-6810
(m) (b)(6)

**From:** LeDuc, James W.
**Sent:** Tuesday, December 18, 2018 10:28 AM
**To:** 'PERKINS, Mark' <perkinsm@who.int>
**Cc:** BRIAND, Sylvie <briands@who.int>; KOJIMA, Kazunobu <kojimak@who.int>; Mike Ryan (ryanm@who.int) <ryanm@who.int>; Yuan Zhiming <yzm@wh.iov.cn>
**Subject:** RE: GLAD-HP

Hi Mark,

Thanks for the follow up. As I mentioned to Sylvie, we have been working closely with colleagues in China where new BSL4 laboratories have been constructed and are now becoming operational. These collaborations have been facilitated by the US National Academy of Sciences and the Chinese Academy of Sciences. At a meeting hosted by WHO in Lyon last December and organized by Kaz Kojima, I was struck by the global proliferation of BSL4 biocontainment laboratories (not to mention the BSL3s), and the attached editorial followed. The editorial has gained some traction and hopefully over the coming year there will be an opportunity to establish a more active global network of directors of these facilities. I discussed this in general terms with Mike Ryan following last week's GOARN meeting and agreed to keep him abreast of activities and to coordinate with WHO as closely as possible.

My thoughts going forward with a network would be to:

- Establish a functional network so that points of contact are known for each facility and general information about capabilities and proposed scope of activities. Ideally this would involve a face-to-face meeting.
- Facilitate information exchange among network members on both operational issues and scientific opportunities.
- Offer a credible source of information from experienced leaders for those considering the construction a new BSL4 facility
- Provide hands on training and capacity building in:
    - Biosafety—working in biocontainment
    - Biosecurity—ensuring the security of pathogens under their control
    - Building operations and maintenance—this is especially challenging and expensive
- Identify areas of possible scientific collaborations.
- Facilitate personnel exchanges.
- Create a forum where international rules, regulations, norms and standards can be discussed and allow credible feed-back to policy development.



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I look forward to learning how these thoughts might align with your plans. I'm happy to discuss further, although it may be easier by email given the Holidays.

Best wishes,

Jim

James W. Le Duc, Ph.D.
Director
Galveston National Laboratory
University of Texas Medical Branch
Galveston, TX 77555-0610
(t) 409-266-6500
(f) 409-266-6810
(m) (b)(6)

**From:** PERKINS, Mark <perkinsm@who.int>
**Sent:** Monday, December 17, 2018 10:48 PM
**To:** LeDuc, James W. <jwleduc@UTMB.EDU>
**Cc:** BRIAND, Sylvie <briands@who.int>
**Subject:** GLAD-HP

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Jim,

Sylvie Briand, under who's direction I work at WHO, suggested that I contact you. I recently joined WHO to lead lab networks in the WHO Health Emergencies cluster in the IHM department, and specifically to establish an umbrella network for high-threat pathogens that would try to answer some of the unresolved sample and data sharing issues, and more broadly to prepare WHO to offer leadership and a forum for engagement of the scientific community in a rapidly evolving technical and social landscape. If you have a half hour in the coming two weeks, it would be great to connect by phone to get your thoughts and share ideas.

Mark Perkins

Dr Mark D. Perkins
Leader, Laboratory Networks
Infectious Hazards Management, WHO Health Emergencies Programme
Email: perkinsm@who.int
Desk phone: +4122-7911649 Cell: (b)(6)



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**From:** LeDuc, James W.
**Sent:** Friday, January 4, 2019 2:46 PM
**To:** Gallo, Robert (b)(6)
**Subject:** RE: Biosecurity

Thanks very much Bob. I have not heard back from John but it would be good for us to chat before I head to Harbin. May I call you at say 10:00 am CT/11:00 am ET? If so, should I use the (410) 706-8614 number? If that's not a convenient time, let me know when would be. I need to drive to the airport in mid-afternoon Saturday to start the trek. My mobile number is highlighted below if you prefer to call me.

Thanks, Jim

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(t) 409-266-6500
(f) 409-266-6810
([redacted])(b)(6)

**From:** Gallo, Robert <rgallo@ihv.umaryland.edu>
**Sent:** Friday, January 04, 2019 1:07 PM
**To:** LeDuc, James W. <jwleduc@UTMB.EDU>; (b)(6) Gallo, Robert <rgallo@ihv.umaryland.edu>
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725 W Lombard Street, S307



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Baltimore, MD 21201
(410) 706-8614

**From:** LeDuc, James W. [mailto:jwleduc@UTMB.EDU]
**Sent:** Friday, January 04, 2019 10:56 AM
**To:** (b)(6) Gallo, Robert <rgallo@ihv.umaryland.edu>
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**Subject:** GLAD-HP

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Mark Perkins

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Email: perkinsm@who.int
Desk phone: +4122-7911649 Cell: (b)(6)



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**From:** Gallo, Robert <rgallo@ihv.umaryland.edu>
**Sent:** Friday, January 4, 2019 2:52 PM
**To:** LeDuc, James W. (b)(6)
**Subject:** RE: Biosecurity

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Yes—I am OK with 11 a.m. I hope you (we) do hear from John soon. I will be **home at** (b)(6)

Bob

**From:** LeDuc, James W. [mailto:jwleduc@UTMB.EDU]
**Sent:** Friday, January 04, 2019 3:46 PM
**To:** Gallo, Robert <rgallo@ihv.umaryland.edu>; (b)(6)
**Subject:** RE: Biosecurity

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**From:** Gallo, Robert <rgallo@ihv.umaryland.edu>
**Sent:** Friday, January 04, 2019 1:07 PM
**To:** LeDuc, James W. <jwleduc@UTMB.EDU>; (b)(6) Gallo, Robert <rgallo@ihv.umaryland.edu>
**Subject:** RE: Biosecurity

**WARNING:** This email originated from outside of UTMB's email system. Do not click links or open attachments unless you recognize the sender and know the content is safe.

Dear James,



FOIA Confidential Treatment Requested by Univ of Texas System UTSystem_004000

Yes, I am available Saturday for this call. Please let me know the best time.

Thank you,

**Robert C. Gallo, MD**
The Homer & Martha Gudelsky Distinguished Professor in Medicine
Co-Founder & Director, Institute of Human Virology *at the*
University of Maryland School of Medicine
Co-Founder & Scientific Director, Global Virus Network
Institute of Human Virology at the
University of Maryland School of Medicine
725 W Lombard Street, S307
Baltimore, MD 21201
(410) 706-8614

**From:** LeDuc, James W. [mailto:jwleduc@UTMB.EDU]
**Sent:** Friday, January 04, 2019 10:56 AM
**To:** (b)(6) Gallo, Robert <rgallo@ihv.umaryland.edu>
**Subject:** Biosecurity

Dear John and Bob,

I hope you both had a pleasant holiday break and that the new year is off to a great start.

I wanted to follow up on our earlier discussions on the biosecurity activities and the GVN. Following our meeting in Annecy, I had a chance to talk with several leaders at WHO including Mike Ryan, the assistant director general in charge of infectious diseases. I briefed them on the work we have been doing bilaterally between the US National Academy of Science and the Chinese Academy of Science and shared with him the attached editorial published in the attached October 2018 in *Science*. As a result of those meetings, I was introduced (virtually) to Mark Perkins, and American working at WHO in lab issues. My email exchange with Mark is below and I spoke with him at length yesterday about the need for a global network of BSL4 labs. I think we are in general agreement and it is clear to me that we need to engage with WHO going forward, but we can't leave it to them to make it happen.

With that background, I leave early Sunday morning for another joint meeting with the US and China National Academies of Science, this time to be held in Harbin at their BSL4 agriculture research center. The agenda of our meeting is attached just FYI. During that meeting, I have scheduled time to meet with a good friend, George Gao, the director of the China CDC to discuss next steps in developing the global network of BSL4 labs. We will continue the bilateral collaborations between the US and China National Academies of Science, but I'm concerned about the move to global engagement in building this network. **I wonder if we could have a brief call to discuss before I leave for Harbin?** I'm free all day today and tomorrow (Saturday) through about mid-day. My direct office line is 409-266-6516 and cell is (b)(6)

Thanks, Jim

James W. Le Duc, Ph.D.
Director
Galveston National Laboratory
University of Texas Medical Branch
Galveston, TX 77555-0610
(t) 409-266-6500



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(f) 409-266-6810
(m) (b)(6)

**From:** LeDuc, James W.
**Sent:** Tuesday, December 18, 2018 10:28 AM
**To:** 'PERKINS, Mark' <perkinsm@who.int>
**Cc:** BRIAND, Sylvie <briands@who.int>; KOJIMA, Kazunobu <kojimak@who.int>; Mike Ryan (ryanm@who.int) <ryanm@who.int>; Yuan Zhiming <yzm@wh.iov.cn>
**Subject:** RE: GLAD-HP

Hi Mark,

Thanks for the follow up. As I mentioned to Sylvie, we have been working closely with colleagues in China where new BSL4 laboratories have been constructed and are now becoming operational. These collaborations have been facilitated by the US National Academy of Sciences and the Chinese Academy of Sciences. At a meeting hosted by WHO in Lyon last December and organized by Kaz Kojima, I was struck by the global proliferation of BSL4 biocontainment laboratories (not to mention the BSL3s), and the attached editorial followed. The editorial has gained some traction and hopefully over the coming year there will be an opportunity to establish a more active global network of directors of these facilities. I discussed this in general terms with Mike Ryan following last week's GOARN meeting and agreed to keep him abreast of activities and to coordinate with WHO as closely as possible.

My thoughts going forward with a network would be to:

- Establish a functional network so that points of contact are known for each facility and general information about capabilities and proposed scope of activities. Ideally this would involve a face-to-face meeting.
- Facilitate information exchange among network members on both operational issues and scientific opportunities.
- Offer a credible source of information from experienced leaders for those considering the construction a new BSL4 facility
- Provide hands on training and capacity building in:
    - Biosafety—working in biocontainment
    - Biosecurity—ensuring the security of pathogens under their control
    - Building operations and maintenance—this is especially challenging and expensive
- Identify areas of possible scientific collaborations.
- Facilitate personnel exchanges.
- Create a forum where international rules, regulations, norms and standards can be discussed and allow credible feed-back to policy development.

I look forward to learning how these thoughts might align with your plans. I'm happy to discuss further, although it may be easier by email given the Holidays.

Best wishes,

Jim

James W. Le Duc, Ph.D.
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University of Texas Medical Branch
Galveston, TX 77555-0610
(t) 409-266-6500



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(f) 409-266-6810
(m) (b)(6)

**From:** PERKINS, Mark <perkinsm@who.int>
**Sent:** Monday, December 17, 2018 10:48 PM
**To:** LeDuc, James W. <jwleduc@UTMB.EDU>
**Cc:** BRIAND, Sylvie <briands@who.int>
**Subject:** GLAD-HP

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Jim,

Sylvie Briand, under who's direction I work at WHO, suggested that I contact you. I recently joined WHO to lead lab networks in the WHO Health Emergencies cluster in the IHM department, and specifically to establish an umbrella network for high-threat pathogens that would try to answer some of the unresolved sample and data sharing issues, and more broadly to prepare WHO to offer leadership and a forum for engagement of the scientific community in a rapidly evolving technical and social landscape. If you have a half hour in the coming two weeks, it would be great to connect by phone to get your thoughts and share ideas.

Mark Perkins

Dr Mark D. Perkins
Leader, Laboratory Networks
Infectious Hazards Management, WHO Health Emergencies Programme
Email: perkinsm@who.int
Desk phone: +4122-7911649 Cell: (b)(6)



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**From:** LeDuc, James W.
**Sent:** Friday, January 4, 2019 3:24 PM
**To:** Gallo, Robert
**Subject:** RE: Biosecurity

Perfect, thanks

**From:** Gallo, Robert <rgallo@ihv.umaryland.edu>
**Sent:** Friday, January 04, 2019 2:52 PM
**To:** LeDuc, James W. <jwleduc@UTMB.EDU>; (b)(6)
**Subject:** RE: Biosecurity

**WARNING:** This email originated from outside of UTMB's email system. Do not click links or open attachments unless you recognize the sender and know the content is safe.

Yes—I am OK with 11 a.m. I hope you (we) do hear from John soon. I will be **home at** (b)(6)

Bob

**From:** LeDuc, James W. [mailto:jwleduc@UTMB.EDU]
**Sent:** Friday, January 04, 2019 3:46 PM
**To:** Gallo, Robert <rgallo@ihv.umaryland.edu>; (b)(6)
**Subject:** RE: Biosecurity

Thanks very much Bob. I have not heard back from John but it would be good for us to chat before I head to Harbin. May I call you at say 10:00 am CT/11:00 am ET? If so, should I use the (410) 706-8614 number? If that's not a convenient time, let me know when would be. I need to drive to the airport in mid-afternoon Saturday to start the trek. My mobile number is highlighted below if you prefer to call me.

Thanks, Jim

James W. Le Duc, Ph.D.
Director
Galveston National Laboratory
University of Texas Medical Branch
Galveston, TX 77555-0610
(t) 409-266-6500
(f) 409-266-6810
(b)(6)

**From:** Gallo, Robert <rgallo@ihv.umaryland.edu>
**Sent:** Friday, January 04, 2019 1:07 PM



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**To:** LeDuc, James W. <jwleduc@UTMB.EDU>; (b)(6) Gallo, Robert <rgallo@ihv.umaryland.edu>
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The Homer & Martha Gudelsky Distinguished Professor in Medicine
Co-Founder & Director, Institute of Human Virology *at the*
University of Maryland School of Medicine
Co-Founder & Scientific Director, Global Virus Network
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725 W Lombard Street, S307
Baltimore, MD 21201
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Thanks, Jim



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James W. Le Duc, Ph.D.
Director
Galveston National Laboratory
University of Texas Medical Branch
Galveston, TX 77555-0610
(t) 409-266-6500
(f) 409-266-6810
(m) (b)(6)

**From:** LeDuc, James W.
**Sent:** Tuesday, December 18, 2018 10:28 AM
**To:** 'PERKINS, Mark' <perkinsm@who.int>
**Cc:** BRIAND, Sylvie <briands@who.int>; KOJIMA, Kazunobu <kojimak@who.int>; Mike Ryan (ryanm@who.int) <ryanm@who.int>; Yuan Zhiming <yzm@wh.iov.cn>
**Subject:** RE: GLAD-HP

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- Facilitate personnel exchanges.
- Create a forum where international rules, regulations, norms and standards can be discussed and allow credible feed-back to policy development.

I look forward to learning how these thoughts might align with your plans. I'm happy to discuss further, although it may be easier by email given the Holidays.

Best wishes,

Jim



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James W. Le Duc, Ph.D.
Director
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Galveston, TX 77555-0610
(t) 409-266-6500
(f) 409-266-6810
(m) (b)(6)

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**To:** LeDuc, James W. <jwleduc@UTMB.EDU>
**Cc:** BRIAND, Sylvie <briands@who.int>
**Subject:** GLAD-HP

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Jim,

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Mark Perkins

Dr Mark D. Perkins
Leader, Laboratory Networks
Infectious Hazards Management, WHO Health Emergencies Programme
Email: perkinsm@who.int
Desk phone: +4122-7911649 Cell: (b)(6)



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## CONCENTS

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# CONFERENCE NOTICE

**1. Accommodation and Registration**

1）For all participants, accommodation are provided by Aoluguya Hotel (No. 800, the 3rd Road of Chuangxin, Harbin), accommodation fee will be paid by guests and invoice will be provided by the hotel.

2）The registration time is 7th January and the registration area is located in level 42 of Aoluguya Hotel.

3）The conference is held in Room 1058 of the Comprehensive Laboratory Building, Harbin Veterinary Research Institute (HVRI).

4）The Shuttle bus leaves from Aoluguya Hotel to HVRI at 7:50am every day during 8th-10th January.

**2. Chief organizers' contact information in the conference committee**

1) Reception: Jinxiong Liu 13796020235

2) Conference-related affairs: Jing Zhang 18645117376

**3. City tour**

On the 10th January, a visit to the Unit 731 Museum at 1:00pm and a visit to Harbin International Ice and Snow Festival at 4:00pm can be arranged at own expenses. For those guests who are interested in this trip, please register during you registration.



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**U.S.-China Dialogue and Workshop on the Challenges of Emerging Infections, Laboratory Safety, Global Health Security and Responsible Conduct in the Use of Gene Editing in Viral Infectious Disease Research**

Room 1058, Comprehensive Laboratory Building
Harbin Veterinary Research Institute, CAAS
Harbin, China

8-10 January 2019

| Time | Topic | Chairs |
|---|---|---|
| **Tuesday, 8 January** | | |
| **Welcome and Opening Remarks** | | **Zhigao Bu** |
| 9:00-9:15 | **Kongming Wu**, Vice President of the Chinese Academy of Agricultural Sciences<br>**Benjamin Rusek**, Policy and Global Affairs, U.S. National Academy of Sciences (NAS)<br>**Diane Griffin**, Johns Hopkins Bloomberg School of Public Health and Vice-President of NAS | |
| **Session 1: Keynote Addresses** | | **Benjamin Rusek** |
| 9:15-9:35 | **Prof. George F. Gao**, Director-General, Chinese Center for Disease Control and Prevention<br>*Oversight for Research on Viral Pathogens with Pandemic Potential – the Perspective from China* | |
| 9:35-9:55 | **Prof. Linda Saif**, Ohio State University<br>*Emerging swine coronaviruses and their interspecies transmission* | |
| 9:55-10:15 | **Prof. Hualan Chen** Harbin Veterinary Research Institute, CAAS<br>*Vaccination in poultry eliminated human infections with H7N9 virus in China* | |
| 10:15-10:30 | Q&A/Discussion | |
| 10:30-10:40 | **Coffee and Tea Break** | |
| **Session 2: Influenza** | | **Linda Saif**<br>**Hualan Chen** |
| 10:40-10:55 | **Prof. David Swayne,** U.S. Department of Agriculture<br>*Collaborating on Influenza Research from the U.S. perspective* | |
| 10:55-11:10 | **Prof. Dayan Wang**, National Institute for Viral Disease Control and Prevention, China CDC<br>*Human Infection with Zoonotic Influenza* | |
| 11:10-11:25 | **Prof. Chengjun Li,** Harbin Veterinary Research Institute, CAAS<br>*Interactions between Influenza Viral RNP Complex Proteins and Host Cellular Factors* | |
| 11:25-11:35 | Q&A/Discussion | |



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| | | |
|---|---|---|
| **Session 3: African Swine Fever** | | **Linda Saif<br>Hualan Chen** |
| 11:35-11:50 | **Prof. Xiaodong Wu**, China Animal Health and Epidemiology Center<br>*Current Situation and Control Strategy of African Swine Fever in China* | |
| 11:50-12:15 | **Prof. Zhigao Bu,** Harbin Veterinary Research Institute, CAAS<br>*Characterization of African Swine Fever Virus Isolated from China* | |
| 12:15-12:30 | **Dr. Douglas Gladue**, U.S. Department of Agriculture<br>*ASFV vaccine status: Rapid production of recombinant ASFV for live attenuated vaccines* | |
| 12:30-12:45 | **Prof. Hongsheng Ouyang,** Jilin University<br>*Genome-edited pigs protected from classical swine fever virus* | |
| 12:45-13:00 | Q&A/Discussion | |
| 13:00-13:10 | **Group Photo** | |
| 13:10-14:30 | **Lunch** | |
| **Session 4: Understanding and Engineering Viral Pathogens with Pandemic Potential** | | **Diane Griffin<br>George F. Gao** |
| 14:30-14:50 | **Prof. Ralph Baric**, University of North Carolina<br>*Advances in Understanding and Altering Pathogen Properties* | |
| 14:50-15:10 | **Prof. Weiwen Zhang**, Tianjin University<br>*Cutting Edging New Biotechnology: Strategies and Tools for Engineering* | |
| 15:10-15:45 | **Coffee and Tea Break** | |
| **Session 5: Bioethics and Responsible Planning for Pathogen Research** | | **Diane Griffin<br>George F. Gao** |
| 15:45-16:00 | **Prof. Zhiming Yuan**, Wuhan Institute of Virology, CAS<br>*Biosafety and Bioethics in Medical Research* | |
| 16:00-16:15 | **Prof. David Relman**, Stanford University<br>*Proposed title: Bioethical Considerations for the Conception and Design of pathogen Research and Approaches for Assessing Potential Risks and Benefits of Research* | |
| 16:15-16:30 | **Prof. Jianwei Wang,** Institute of Pathogen Biology, Chinese Academy of Medical Sciences & Peking Union Medical College<br>*Biosafety Management of Synthetic Biology: Challenges and Progress* | |
| 16:30-16:45 | **Associate Prof. Yang Xue**, Tianjin University<br>*Biosafety and Bioethics Considerations in Dual-Use Risks Management – China's Efforts and Future Works* | |
| 16:45-17:30 | **Session 6: Panel on Science and Ethics in Research with Pathogens with Pandemic Potential** | **Diane Griffin<br>George F. Gao** |



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| Wednesday, 9 January | | |
|---|---|---|
| **Session 7: Vector and Host Engineering : Safety and Security** | | **Jim LeDuc<br>Zhiming Yuan** |
| 9:00-9:20 | **Prof. Qian Han,** Hainan University<br>*Biochemical Pathway of a Mosquito Innate Immunity, Melanization* | |
| 9:20-9:40 | **Prof. Stephen Higgs**, Kansas State University<br>*Best practices in safety and security for working with arthropods that serve as pathogen vectors, including responsible research with gene drive modified vectors* | |
| 9:40-10:00 | Q&A/Discussion | |
| 10:00-10:30 | **Coffee and Tea Break** | |
| 10:30-10:50 | **Prof. Tongyan Zhao**, Institute of Microbiology and Epidemiology, Academy of Military Medical Sciences<br>*Vector Competence of Emerging Mosquito Borne Virus in China* | |
| 10:50-11:10 | **Prof. Gong Cheng**, Tsinghua University<br>*A Gut Commensal Bacterium Promotes Arbovirus Infection in Mosquitoes* | |
| 11:10-12:00 | Q&A/Discussion | |
| 12:00-13:30 | **Lunch** | |
| **Session 8: Best Practices in Laboratory Safety and Security for Pathogen Research** | | **Mifang Liang<br>Joe Kanabrocki** |
| 13:30-13:45 | **Prof. Joseph Kanabrocki**, ***University of Chicago***<br>*Institutional review and oversight for research on viral pathogens with pandemic potential – a U.S. perspective* | |
| 13:45-14:15 | **Prof. David Franz** and **Prof. Zhiming Yuan**<br>*The Role of Leaders in Laboratory Safety: Culture and governance to steer toward behavior changes* | |
| 14:15-14:40 | Facilitated Discussion | |
| **Special Topic: One Health in China** | | **Mifang Liang<br>Joe Kanabrocki** |
| 14:40-15:00 | **Prof. Jiahai Lu**, Sun Yat-sen University<br>*Understand how a One Health approach is shaping research to understand and address viral pathogens in China* | |
| 15:00-15:10 | Q&A/Discussion | |
| 15:10-15:30 | **Coffee and Tea Break** | |



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| Session 9: Chinese Biosafety, Biosecurity, and Bioethics | Mifang Liang<br>Joe Kanabrocki |
|---|---|
| 15:30-15:45 | **Prof. Peijun Zhai**, CNAS<br>*Overview of Laboratory Biosafety Management System in China* |
| 15:45-16:00 | **Prof. Jiancheng Qi**, National Biological Protection Engineering Center, China<br>*Development and Application of Laboratory Biosafety Equipment in China* |
| 16:00-16:15 | **Prof. Yunzhang Hu**, Institute of Medical Biology, Chinese Academy of Medical Sciences<br>*Kunming National Primate Research Center of High-Level Biosafety* |
| 16:15-16:30 | **Prof. Ruifu Yang**, Institute of Microbiology and Epidemiology, Academy of Military Medical Sciences<br>*Microbial Forensics: Building Capability for Source Tracing of Bioterrorism Pathogen* |
| 16:30-17:00 | Q&A/Discussion |
| **Thursday, 10 January** | |
| **Special Topic: Update on Acute Flaccid Myelitis** | **Pei-yong Shi<br>Zhengli Shi** |
| 9:00-9:20 | **Prof. Diane Griffin,** Johns Hopkins Bloomberg School of Public Health<br>*Review of what is currently known about the cause of Acute Flaccid Myelitis* |
| 9:20-9:30 | Q&A/Discussion |
| **Session 10: Chinese Perspectives on Emerging Infectious Diseases** | **Pei-yong Shi<br>Zhengli Shi** |
| 9:30-9:45 | **Prof. Changjiang Weng,** Harbin Veterinary Research Institute, CAAS<br>*DDX19, A novel Viral RNA Sensor, Regulates IFN Signaling upon Viral Infection* |
| 9:45-10:00 | **Prof. Zhengli Shi,** Wuhan Institute of Virology, CAS<br>*Risks of MERS-cluster coronaviruses in China* |
| 10:00-10:15 | **Prof. Haixue Zheng**, Lanzhou Veterinary Research Institute, CAAS,<br>*Rational Design of Master Seed Virus to Improve the Safety and Effectiveness of Foot and Mouth Disease Vaccine* |
| 10:15-10:45 | **Coffee and Tea Break** |
| 10:45-11:00 | **Prof. Rui Gong**, Wuhan Institute of Virology, CAS<br>*Development of Neutralizing Antibodies Domains as Therapeutic Candidates Against Infectious Diseases* |
| 11:00-11:15 | **Prof. Longding Liu**, Institute of Medical Biology, Chinese Academy of Medical Sciences<br>*Development of inactivated EV71 Vaccine (Human diploid cell) and Pathogenic Study of EV71 Infection in Rhesus Macaque* |
| 11:15-11:30 | **Prof. Chengfeng Qin**, Institute of Microbiology and Epidemiology, Academy of Military Medical Sciences<br>*Zika Virus Neurotropism the Bad and the Good* |



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| | | |
|---|---|---|
| 11:30-12:00 | Q&A/Discussion | |
| **Special Session 11** | | **Pei-yong Shi**<br>**Zhengli Shi** |
| 12:00-12:15 | **Prof. Chunbo Lou,** Institute of Microbiology, CAS<br>*Large Gene Cluster and Regulatory Manipulation for Activating the Cryptic Natural Product Clusters in Streptomyces* | |
| 12:15-12:30 | **Prof. Xiaoli Xue,** Key Laboratory of Synthetic Biology, Shanghai Institute of Plant Physiology, CAS<br>*Creating a Functional Single-chromosome Yeast* | |
| 12:30-13:30 | **Lunch** | |
| **Session 12: China-U.S. Collaboration: Opportunities to Prevent Future Pandemics**<br>**(Room 1046, Comprehensive laboratory Building, HVRI)** | | **James LeDuc**<br>**Zhiming Yuan**<br>**Diane Griffin**<br>**Zhigao Bu** |



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# U.S.-China Dialogue and Workshop on the Challenges of Emerging Infections, Laboratory Safety, Global Health Security and Responsible Conduct in the Use of Gene Editing in Viral Infectious Disease Research

Room 1058, Comprehensive Laboratory Building
Harbin Veterinary Research Institute, CAAS
Harbin, China
8-10 January 2019

**Monday, 7 January**

Some participants arrive at the Aoluguya Hotel (No. 800, the 3rd Road of Chuangxin, Harbin)

**Tuesday, 8 January**

**7:50** Shuttle bus from Aoluguya Hotel to Harbin Veterinary Research Institute (HVRI)
(No. 678 Haping Road, Xiangfang District, Harbin, Heilongjiang Province, China 150069)

**WELCOME AND OPENING REMARKS**

**9:00** **Welcome, Introductions, and Opening Remarks**
**Chairs: Zhigao Bu**

1. Welcome on behalf of Chinese Academy of Agricultural Sciences (CAAS): **Kongming Wu**, Vice-President of CAAS
2. Remarks on Behalf of U.S. National Academies of Sciences, Engineering, and Medicine: **Benjamin Rusek**, Policy and Global Affairs, U.S. National Academy of Sciences (NAS) and **Diane Griffin**, Johns Hopkins Bloomberg School of Public Health and Vice-President of NAS

**9:15** **Session 1. Keynote Addresses** *[20 min each]*
**Chairs: Benjamin Rusek**

1. **Prof. George F. Gao**, Director-General, Chinese Center for Disease Control and Prevention
   *Oversight for Research on Viral Pathogens with Pandemic Potential – the Perspective from China*
2. **Prof. Linda Saif**, Ohio State University
   *Emerging swine coronaviruses and their interspecies transmission*
3. **Prof. Hualan Chen** Harbin Veterinary Research Institute, CAAS
   *Vaccination in poultry eliminated human infections with H7N9 virus in China*

**Q&A/Discussion** *[15-20 min]*

**10:30** Coffee and Tea Break



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**EMERGING INFECTIOUS DISEASES**

Goal for Sessions 2 and 3: Consider progress in understanding two diseases of concern to China and the US, including the health and economic implications and what advances in understanding may be enabled by recent scientific and technical developments such as gene editing.

**10:40 Session 2. Influenza**

**Chairs: Linda Saif** and **Hualan Chen**

1. **Overview: Perspectives on international collaborations for influenza research** *[15 min each]*
   **Dr. David Swayne,** U.S. Department of Agriculture
   *Collaborating on Influenza Research from the U.S. perspective*
2. **Panel Discussion**
   Speakers provide 15 minutes opening remarks on the topics identified below, followed by discussion facilitated by the session Chairs
   **Prof. Dayan Wang**, National Institute for Viral Disease Control and Prevention, China CDC *Human Infection with Zoonotic Influenza*
   **Prof. Chengjun Li,** Harbin Veterinary Research Institute, CAAS
   *Interactions between Influenza Viral RNP Complex Proteins and Host Cellular Factors*

***Proposed discussion questions***

- What is known about the epidemiology, health, and economic impacts of Influenza?
- What is the latest progress on developing effective countermeasures?
- What critical areas remain unknown or what are key challenges to preventing and responding to influenza outbreaks? How can new scientific and technical advances contribute to research and development?

**11:35 Session 3. African Swine Fever** *[15 min each]*

**Chairs: Linda Saif** and **Hualan Chen**

1. **Prof. Xiaodong Wu**, China Animal Health and Epidemiology Center
   *Current Situation and Control Strategy of African Swine Fever in China*
2. **Prof. Zhigao Bu,** Harbin Veterinary Research Institute, CAAS *Characterization of African Swine Fever Virus Isolated from China*
3. **Dr. Douglas Gladue**, U.S. Department of Agriculture
   *ASFV vaccine status: Rapid production of recombinant ASFV for live attenuated vaccines*
4. **Prof. Hongsheng Ouyang,** Jilin University
   *Genome-edited pigs protected from classical swine fever virus*
   **Q&A/Discussion** *[10-15 min]*

***Proposed discussion questions***



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- What is known about the epidemiology, health, and economic impacts of Africa Swine Fever?
- What is the latest progress on developing effective countermeasures and limiting the spread of this disease?
- What critical areas remain unknown or what are key challenges to preventing and responding to Africa Swine Fever outbreaks? How can new scientific and technical advances contribute to research and development?

**13:00** **Group Photo**

**13:10** **Lunch**

**VIRAL PATHOGENS: SCIENCE AND RESPONSIBILITY**

Goal for Sessions 4, 5, and 6: Discuss relevant pathogen properties that researchers may seek to alter, and why, as part of studying viral pathogens with pandemic potential. Illustrate challenges and opportunities posed by new strategies such as use of gene editing, rational design approaches, other strategies enable by advancing biotechnology. Discuss the associated issues in the ethical planning and conduct of such research so that it can be conducted safety and securely.

**14:30** **Session 4. Understanding and Engineering Viral Pathogens with Pandemic Potential** *[20 min each]*

**Chairs: Diane Griffin** and **George F. Gao**

1. **Prof. Ralph Baric**, University of North Carolina
   *Advances in Understanding and Altering Pathogen Properties*
2. **Prof. Weiwen Zhang**, Tianjin University
   *Cutting Edging New Biotechnology: Strategies and Tools for Engineering*

**15:10** Coffee and Tea break

**15:45** **Session 5. Bioethics and Responsible Planning for Pathogen Research** *[15 min each]*

**Chairs: Diane Griffin** and **George F. Gao**

1. **Prof. Zhiming Yuan**, Wuhan Institute of Virology, CAS *Biosafey and Bioethics in Medical Research*
2. **Prof.David Relman**, Stanford University
   *Proposed title: Bioethical Considerations for the Conception and Design of pathogen Research and Approaches for Assessing Potential Risks and Benefits of Research*
3. **Prof. Jianwei Wang,** Institute of Pathogen Biology, Chinese Academy of Medical Sciences & Peking Union Medical College
   *Biosafety Management of Synthetic Biology: Challenges and Progress*
4. **Associate Prof. Yang Xue**, Tianjin University
   *Biosafety and Bioethis Considerations in Dual-Use Risks Management – China's Efforts and Future Works*



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**16:45** **Session 6. Panel on Science and Ethics in Research with Pathogens with Pandemic Potential**

**Chairs**: **Diane Griffin** and **George F. Gao**

Chairs facilitate a discussion between **Ralph Baric, Weiwen Zhang, Zhiming Yuan, and David Relman, Jianwei Wang, Yang Xue** and the audience. *[45 min]*

***Proposed discussion questions***

- *What do recent advances in biotechnology, such as genome editing, enable researchers to achieve in improved characterization and alteration of the properties of pathogenic viruses?*
- *What do you see coming in the near term? What still remains very challenging and why?*
- *Because research on viral pathogens can raise security concerns, how do you think about making decisions on whether or not to conduct such research or about modifying research proposals? What are the responsibilities and best practices of the PI to ensure that pathogen research is conducted safely and securely over the course of the project?*
- *What ethical and policy challenges remain and what actions do you think are needed to help overcome these challenges?*

**17:30** Transportation from HVRI to Restaurant

**18:30** Dinner at Restaurant

**Wednesday, 9 January**

**07:50** Shuttle bus from Aoluguya Hotel to Harbin Veterinary Research Institute (HVRI)

**VECTOR AND HOST ENGINEERING**

Goal for Session 7: Research to understand and prepare for viral pathogens may also involve work conducted on the hosts or vectors of such pathogens, for example to better understand host-pathogen responses and vector transmission. Discuss how advances in areas such as gene editing are affecting such research and best practices for safely and responsibly conducting such studies

**9:00** **Session 7. Vector and Host Engineering: Safety and Security** *[15-20 min each]*

**Chairs: Jim LeDuc** and **Zhiming Yuan**

1. **Prof. Qian Han**, Hainan University
   *Biochemical Pathway of a Mosquito Innate Immunity, Melanization*
2. **Prof. Stephen Higgs**, Kansas State University



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*Best practices in safety and security for working with arthropods that serve as pathogen vectors, including responsible research with gene drive modified vectors*

**Q&A/ Discussion** *[20 min]*

**10:00** Coffee and tea break

**10:30** **Session 7 resumes**

3. **Highlights of Recent Research:**
   **Prof. Tongyan Zhao**, Institute of Microbiology and Epidemiology, Academy of Military Medical Sciences
   *Vector Competence of Emerging Mosquito Borne Virus in China*
4. **Prof. Gong Cheng**, Tsinghua University

   *A Gut Commensal Bacterium Promotes Arbovirus Infection in Mosquitoes*

**Q&A/ Discussion** *[30 min]*

***Proposed discussion questions***

- *What do recent advances in biotechnology, such as genome editing, enable researchers to achieve in studying and manipulating hosts and vectors of viral pathogens*
- *What do you see coming in the near term? What still remains very challenging and why?*
- *What are the responsibilities and best practices of the PI to ensure that pathogen research is conducted safely and securely over the course of the project?*
- *What ethical and policy challenges remain and what actions do you think are needed to help overcome these challenges?*

**12:00** **Lunch**

**EFFECTIVE OVERSIGHT SYSTEMS**

Goal for Session 8: Discuss the systems of institutional review and oversight in China and in the US for safe and secure conduct of laboratory research on viral pathogens with pandemic potential. Focus on the institutional systems including the important role of leadership in promoting institutional cultures that support responsible research practices.

**13:30** **Session 8. Best Practices in Laboratory Safety and Security for Pathogen Research**
**Chairs: Mifang Liang** and **Joe Kanabrocki**

1. **Prof. Joseph Kanabrocki**, ***University of Chicago*** *[15 min]*
   *Institutional review and oversight for research on viral pathogens with pandemic potential – a U.S. perspective*
2. **Prof. David Franz** and **Prof**. **Zhiming Yuan** *[30 min together]*



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*The Role of Leaders in Laboratory Safety: Culture and governance to steer toward behavior changes*

**Facilitated Discussion**

Chair facilitates a discussion with the speakers and audience [25 min]

***Proposed discussion questions***

*If assessment of anticipated risks and benefits results in the decision to undertake an experiment that involves characterizing or manipulating a pathogen with pandemic potential:*

1. *How should the research-conducting institution oversee this research to ensure that it is conducted safely and securely over the course of the project? Does this oversight happen at one point or at multiple points over the course of the project?*
2. *What are the responsibilities of institutional leadership in promoting the culture of responsible conduct of science?*
3. *Are there particular scientific or experimental practices that should be incorporated into research design and conduct that can maximize safety and security for studies carried out to understand or alter the properties of pathogens with pandemic potential?*

**14:40** **Special Topic: One Health in China**

**Chairs: Mifang Liang** and **Joe Kanabrocki**

1. **Prof. Jiahai Lu**, Sun Yat-sen University *[20 min]*
   Understand how a One Health approach is shaping research to understand and address viral pathogens in China

**Q&A/Discussion** *[10 min]*

**15:10** Coffee and Tea Break

**RECENT RESEARCH AND PERSPECTIVES**

Goal for Sessions 9 and 10: Gain further shared insights into how research communities are addressing issues in biosafety, biosecurity, and bioethics, particularly as applied to research on emerging infectious diseases caused by viral pathogens

**15:30** **Session 9. Chinese Biosafety, Biosecurity, and Bioethics** *[15 min each]*

**Chairs: Mifang Liang** and **Joe Kanabrocki** (give some opening comments)

1. **Dr. Peijun Zhai**, CNAS
   *Overview of Laboratory Biosafety Management System in China*
2. **Prof. Jiancheng Qi**, National Biological Protection Engineering Center, China
   *Development and Application of Laboratory Biosafety Equipment in China*
3. **Prof. Yunzhang Hu**, Institute of Medical Biology, Chinese Academy of Medical Sciences
   *Kunming National Primate Research Center of High-Level Biosafety*



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4. **Prof. Ruifu Yang**, Institute of Microbiology and Epidemiology, Academy of Military Medical Sciences
   *Microbial Forensics: Building Capability for Source Tracing of Bioterrorism Pathogen.*

**Q&A/Discussion** *[30 min]*

**17:00** Transportation from HVRI to Restaurant

**18:30** Dinner

**Thursday, 10 January**

**7:50** Shuttle bus from Aoluguya Hotel to Harbin Veterinary Research Institute (HVRI)

**9:00** **Special Topic: Update on Acute Flaccid Myelitis**
**Diane Griffin,** Johns Hopkins Bloomberg School of Public Health [20 min]
*Review of what is currently known about the cause of Acute Flaccid Myelitis*
**Q&A/Discussion** [*10 min]*

**9:30** **Session 10: Chinese Perspectives on Emerging Infectious Diseases** *[15 min each]*
**Chair: Pei-yong Shi** and **Zhengli Shi**

1. **Prof. Changjiang Weng,** Harbin Veterinary Research Institute, CAAS
   *DDX19, A novel Viral RNA Sensor, Regulates IFN Signaling upon Viral Infection*
2. **Prof. Zhengli Shi,** Wuhan Institute of Virology, CAS
   *Risks of MERS-cluster coronaviruses in China*
3. **Prof. Haixue Zheng**, Lanzhou Veterinary Research Institute, CAAS,
   *Rational Design of Master Seed Virus to Improve the Safety and Effectiveness of Foot and Mouth Disease Vaccine*

**10:15** Coffee and Tea Break

**10:45** **Session 10, cont'd**

4. **Prof. Rui Gong**, Wuhan Institute of Virology, CAS
   *Development of Neutralizing Antibodies Domains as Therapeutic Candidates Against Infectious Diseases*
5. **Prof. Longding Liu**, Institute of Medical Biology, Chinese Academy of Medical Sciences
   *Development of inactivated EV71 Vaccine (Human diploid cell) and Pathogenic Study of EV71 Infection in Rhesus Macaque*
6. **Prof. Chengfeng Qin**, Institute of Microbiology and Epidemiology, Academy of Military Medical Sciences
   *Zika Virus Neurotropism the Bad and the Good*

**Q&A/Discussion** *[30 min]*



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**12:00 Special Session 11**

**Chairs: Pei-yong Shi** and **Zhengli Shi**

1. **Prof. Chunbo Lou,** Institute of Microbiology, CAS
   *Large Gene Cluster and Regulatory Manipulation for Activating the Cryptic Natural Product Clusters in Streptomyces*
2. **Prof. Xiaoli Xue,** Key Laboratory of Synthetic Biology, Shanghai Institute of Plant Physiology, CAS
   *Creating a Functional Single-chromosome Yeast*

**12:30 Lunch**

13:00 Visit the Unit 731 Museum

**LOOKING FORWARD (CLOSED SESSION, BY INVITATION ONLY)**

Goal for Sessions 12: Identify shared priorities and potential opportunities for future discussions to advance safety and security (Room 1046 of the Comprehensive laboratory Building)

**14:30 Session 12: China-U.S. Collaboration: Opportunities to Prevent Future Pandemics**

**Chairs: James LeDuc, Zhiming Yuan, Diane Griffin,** and **Zhigao Bu**

Cooperating to combat emerging infectious diseases that affect China and the U.S. - Cooperation on laboratory safety: biocontainment research and collaboration across borders (including discussion on building a network of BSL-4 laboratories concept http://science.sciencemag.org/content/362/6412/267 and other collaborations between Chinese and American scientists)

**16:00 Visit Harbin International Ice and Snow Festival**

**18:30** Transport to Restaurant

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**Friday, 11 January**

AM and PM: transportation provided to airport



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VIROLOGY DIVISION NEWS

# Taxonomy of the order *Bunyavirales*: second update 2018

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## Abstract

In October 2018, the order *Bunyavirales* was amended by inclusion of the family *Arenaviridae*, abolishment of three families, creation of three new families, 19 new genera, and 14 new species, and renaming of three genera and 22 species. This article presents the updated taxonomy of the order *Bunyavirales* as now accepted by the International Committee on Taxonomy of Viruses (ICTV).

Handling Editor: Sead Sabanadzovic.

Michael J. Buchmeier, Rémi N. Charrel, J. Christopher S. Clegg, Juan Carlos de la Torre, Jean-Paul J. Gonzalez, Stephan Günther, Igor S. Lukashevich, Sheli R. Radoshitzky, Víctor Romanowski, Maria S. Salvato, Manuela Sironi, Mark D. Stenglein, Jens H. Kuhn: The members of the 2017–2020 International Committee on Taxonomy of Viruses (ICTV) *Arenaviridae* Study Group; Piet Maes, Scott Adkins, Rémi N. Charrel, Juan Carlos de la Torre, Sandra Junglen, Amy J. Lambert, Gustavo Palacios, Takahide Sasaya, Yǒng-Zhèn Zhāng, Jens H. Kuhn: the members of the 2017–2020 ICTV *Bunyavirales* Study Group; Michele Digiaro, Toufic Elbeaino, Giovanni P. Martelli, Nicole Mielke-Ehret, Hans-Peter Mühlbach: the members of the 2017–2020 ICTV *Fimoviridae* Study Group; Piet Maes, Charles H. Calisher, Charles F. Fulhorst, Boris Klempa, Jonas Klingström, Jin-Won Song, Yǒng-Zhèn Zhāng: the members of the 2017–2020 ICTV *Hantaviridae* Study Group; Sergey V. Alkhovsky, Tatjana Avšič-Županc, Dennis A. Bente, Éric Bergeron, Felicity J. Burt, Mike Drebot, Koray Ergünay, Aura R. Garrison, Roger Hewson, Holly R. Hughes, Ali Mirazimi, Gustavo Palacios, Anna Papa, Janusz T. Pawęska, Amadou Alpha Sall, Jessica R. Spengler, Jens H. Kuhn: the members of the 2017–2020 ICTV *Nairoviridae* Study Group; Scott Adkins, Sergey V. Alkhovsky, Martin Beer, Carol D. Blair, Charles H. Calisher, Amy J. Lambert, Marco Marklewitz, Márcio Roberto Teixeira Nunes, Xiǎohóng Shí: the members of the 2017–2020 ICTV *Peribunyaviridae* Study Group; Matthew J. Ballinger, Roy A. Hall, Sandra Junglen, Stanley A. Langevin: the members of the 2017–2020 ICTV *Phasmaviridae* Study Group; Thomas Briese, Rémi N. Charrel, Xavier de Lamballerie, Hideki Ebihara, George Fú Gāo, Martin H. Groschup, Márcio Roberto Teixeira Nunes, Gustavo Palacios, Takahide Sasaya, Jin-Won Song: the members of the 2017–2020 ICTV *Phenuiviridae* Study Group; Il Ryong Choi, Anne-Lise Haenni, Miranda Gilda Jonson, Takahide Sasaya, Yukio Shirako, Tàiyún Wèi, Xueping Zhou: the members of the 2017–2020 ICTV *Tenuivirus* Study Group; Scott Adkins, Amy J. Lambert, Rayapati Naidu, Renato O. Resende, Massimo Turina, Anna E. Whitfield: the 2017–2020 ICTV *Tospovirus* Study Group; Peter Simmonds, the 2017–2020 ICTV Chair of the Fungal and Protist Viruses Subcommittee; F. Murilo Zerbini: the 2017–2020 ICTV Chair of the Plant Viruses Subcommittee; the 2017–2020 ICTV Chair of the Animal dsRNA and ssRNA- Viruses Subcommittee.

Extended author information available on the last page of the article



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## Introduction

The virus order *Bunyavirales* was established in 2017 to accommodate related viruses with segmented, linear, single-stranded, negative-sense or ambisense RNA genomes classified into nine families [2]. Here, we present the changes that were proposed via an official ICTV taxonomic proposal (TaxoProp 2017.012M.A.v1.Bunyavirales_rev) at http://www.ictvonline.org/ in 2017 and were accepted by the ICTV Executive Committee (EC) in October 2018. These changes are now part of the official ICTV taxonomy as of October 2018.

## Taxonomic changes at the order rank

In October 2018, the order *Bunyavirales* was amended by inclusion of the previously unassigned family *Arenaviridae*. The families *Feraviridae*, *Jonviridae*, and *Tospoviridae* were dissolved, and their genera (*Orthoferavirus*, *Orthojonvirus*, and *Orthotospovirus*) were renamed (*Feravirus*, *Jonvirus*, and *Tospovirus*, respectively) and moved into established families (*Feravirus*, *Jonvirus→Phasmaviridae* and *Tospovirus→Peribunyaviridae*). Three new families were created for novel invertebrate viruses: *Cruliviridae* for Wēnlǐng crustacean virus 9 (WlCV-9), *Mypoviridae* for Húběi myriapoda virus 5 (HbMV-5), and *Wupedeviridae* for Wǔhàn millipede virus 2 (WhMV-2) [4].

## Taxonomic changes at the family rank

### Fimoviridae

In 2018, no changes were made at the family rank.

### Hantaviridae

In October 2018, the family was expanded by the addition of three new genera (*Loanvirus*, *Mobatvirus*, and *Thottimvirus*) for the species *Longquan orthohantavirus*, *Laibin orthohantavirus*, *Nova orthohantavirus*, *Quezon orthohantavirus*, *Imjin orthohantavirus*, and *Thottapalayam orthohantavirus* (now renamed *Longquan loanvirus*, *Laibin mobatvirus*, *Nova mobatvirus*, *Quezon mobatvirus*, *Imjin thottimvirus*, and *Thottapalayam thottimvirus*, respectively).

### Nairoviridae

In October 2018, the family was expanded by the addition of two new genera, *Shaspivirus* and *Striwavirus*, for Shāyáng spider virus 1 (SySV-1) and Sānxiá water strider virus 1 (SxWSV-1) discovered in invertebrates, respectively [1, 4].

### Peribunyaviridae

In October 2018, the family was expanded by the addition of the new genus *Shangavirus* for the species *Shuangao insect herbevirus* (now renamed *Insect shangavirus*).

### Phasmaviridae

In October 2018, the family was expanded by the addition of the new genus *Inshuvirus* for the species *Shuangao insect orthophasmavirus 2* (now renamed *Insect inshuvirus*) and the new genus *Wuhivirus* for Wǔhàn insect virus 2 (WhIV-2) discovered in invertebrates [1].

### Phenuiviridae

In October 2018, the family was expanded by the addition of the new genus *Banyangvirus* for the species *SFTS phlebovirus* (now renamed *Huaiyangshan banyangvirus*) and the new genera *Beidivirus*, *Horwuvirus*, *Hudivirus*, *Hudovirus*, *Mobuvirus*, *Pidchovirus*, and *Wubeivirus* for Húběi diptera virus 3 (HbDV-3), Wǔhàn horsefly virus (WhHV), Húběi diptera virus 4 (HbDV-4), Húběi lepidoptera virus 1 (HbLV-1), Mothra virus (MTHV), Pidgey virus (PGYV), and Húběi diptera virus 5 (HbDV-5)/Wǔhàn fly virus 1 (WhFV-1), respectively, discovered in invertebrates [1, 3, 4].

## Summary

A summary of the current, ICTV-accepted taxonomy of the order *Bunyavirales* is presented in Table 1.

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**Table 1** ICTV-accepted taxonomy of the order *Bunyavirales* as of October 2018. Listed are all bunyaviruses that are classified into species

| Genus | Species[¶] | Virus (abbreviation)[¶] |
|---|---|---|
| **Family *Arenaviridae*** | | |
| *Hartmanivirus* | *Haartman hartmanivirus** | Haartman Institute snake virus 1 (HISV-1) |
| *Mammarenavirus* | *Allpahuayo mammarenavirus* | Allpahuayo virus (ALLV) |
| | *Argentinian mammarenavirus* | Junín virus (JUNV) |
| | *Bear Canyon mammarenavirus* | Bear Canyon virus (BCNV) |
| | *Brazilian mammarenavirus* | Sabiá virus (SBAV) |
| | *Chapare mammarenavirus* | Chapare virus (CHAPV) |
| | *Cupixi mammarenavirus* | Cupixi virus (CUPXV) |
| | *Flexal mammarenavirus* | Flexal virus (FLEV) |
| | *Gairo mammarenavirus* | Gairo virus (GAIV) |
| | *Guanarito mammarenavirus* | Guanarito virus (GTOV) |
| | *Ippy mammarenavirus* | Ippy virus (IPPYV) |
| | *Lassa mammarenavirus* | Lassa virus (LASV) |
| | *Latino mammarenavirus* | Latino virus (LATV) |
| | *Loei River mammarenavirus* | Loei River virus (LORV) |
| | *Lujo mammarenavirus* | Lujo virus (LUJV) |
| | *Luna mammarenavirus* | Luna virus (LUAV) |
| | | Luli virus (LULV) |
| | *Lunk mammarenavirus* | Lunk virus (LNKV) |
| | *Lymphocytic choriomeningitis mammarenavirus** | Dandenong virus (DANV) |
| | | lymphocytic choriomeningitis virus (LCMV) |
| | *Machupo mammarenavirus* | Machupo virus (MACV) |
| | *Mariental mammarenavirus* | Mariental virus (MRLV) |
| | *Merino Walk mammarenavirus* | Merino Walk virus (MRWV) |
| | *Mobala mammarenavirus* | mobala virus (MOBV) |
| | *Mopeia mammarenavirus* | Mopeia virus (MPOV) |
| | | Morogoro virus (MORV) |
| | *Okahandja mammarenavirus* | Okahandja virus (OKAV) |
| | *Oliveros mammarenavirus* | Oliveros virus (OLVV) |
| | *Paraguayan mammarenavirus* | Paraná virus (PRAV) |
| | *Cali mammarenavirus* | Pichindé virus (PICHV) |
| | *Pirital mammarenavirus* | Pirital virus (PIRV) |
| | *Ryukyu mammarenavirus* | Ryukyu virus (RYKV) |
| | *Serra do Navio mammarenavirus* | Amaparí virus (AMAV) |
| | *Solwezi mammarenavirus* | Solwezi virus (SOLV) |
| | *Souris mammarenavirus* | souris virus (SOUV) |
| | *Tacaribe mammarenavirus* | Tacaribe virus (TCRV) |
| | *Tamiami mammarenavirus* | Tamiami virus (TMMV) |
| | *Wenzhou mammarenavirus* | Wēnzhōu virus (WENV)[1] |
| | *Whitewater Arroyo mammarenavirus* | Big Brushy Tank virus (BBRTV) |
| | | Catarina virus (CTNV) |
| | | Skinner Tank virus (SKTV) |
| | | Tonto Creek virus (TTCV) |
| | | Whitewater Arroyo virus (WWAV) |
| *Reptarenavirus* | *California reptarenavirus* | CAS virus (CASV) |
| | *Giessen reptarenavirus* | University of Giessen virus 1 (UGV-1) |
| | | University of Giessen virus 2 (UGV-2) |
| | | University of Giessen virus 3 (UGV-3) |
| | *Golden reptarenavirus** | Golden Gate virus (GOGV) |

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**Table 1** (continued)

| Genus | Species[¶] | Virus (abbreviation)[¶] |
|---|---|---|
| | *Ordinary reptarenavirus* | tavallinen suomalainen mies virus 2 (TSMV-2) |
| | *Rotterdam reptarenavirus* | ROUT virus (ROUTV) |
| | | University of Helsinki virus 1 (UHV-1) |
| **Family *Cruliviridae*** | | |
| *Lincruvirus* | *Crustacean lincruvirus** | Wēnlǐng crustacean virus 9 (WlCV-9) |
| **Family *Fimoviridae*** | | |
| *Emaravirus* | *Actinidia chlorotic ringspot-associated emaravirus* | Actinidia chlorotic ringspot-associated virus (AcCRaV) |
| | *European mountain ash ringspot-associated emaravirus** | European mountain ash ringspot-associated virus (EMARaV) |
| | *Fig mosaic emaravirus* | fig mosaic virus (FMV) |
| | *High Plains wheat mosaic emaravirus* | High Plains wheat mosaic virus (HPWMoV) |
| | *Pigeonpea sterility mosaic emaravirus 1* | pigeonpea sterility mosaic virus (PPSMV) |
| | *Pigeonpea sterility mosaic emaravirus 2* | pigeonpea sterility mosaic virus 2 (PPSMV-2) |
| | *Raspberry leaf blotch emaravirus* | raspberry leaf blotch virus (RLBV) |
| | *Redbud yellow ringspot-associated emaravirus* | redbud yellow ringspot-associated virus (RYRaV) |
| | *Rose rosette emaravirus* | rose rosette virus (RRV) |
| **Family *Hantaviridae*** | | |
| *Orthohantavirus* | *Amga orthohantavirus* | Amga virus (MGAV)[2] |
| | *Andes orthohantavirus* | Andes virus (ANDV) |
| | | Castelo dos Sonhos virus (CASV) |
| | | Lechiguanas virus (LECV = LECHV) |
| | | Orán virus (ORNV) |
| | *Asama orthohantavirus* | Asama virus (ASAV) |
| | *Asikkala orthohantavirus* | Asikkala virus (ASIV) |
| | *Bayou orthohantavirus* | bayou virus (BAYV) |
| | | Catacamas virus (CATV) |
| | *Black Creek Canal orthohantavirus* | Black Creek Canal virus (BCCV) |
| | *Bowe orthohantavirus* | Bowé virus (BOWV) |
| | *Bruges orthohantavirus* | Bruges virus (BRGV) |
| | *Cano Delgadito orthohantavirus* | Caño Delgadito virus (CADV) |
| | *Cao Bang orthohantavirus* | Cao Bằng virus (CBNV) |
| | | Liánghé virus (LHEV) |
| | *Choclo orthohantavirus* | Choclo virus (CHOV) |
| | *Dabieshan orthohantavirus* | Dàbiéshān virus (DBSV) |
| | *Dobrava-Belgrade orthohantavirus* | Dobrava virus (DOBV) |
| | | Kurkino virus (KURV) |
| | | Saaremaa virus (SAAV) |
| | | Sochi virus (SOCV) |
| | *El Moro Canyon orthohantavirus* | Carrizal virus (CARV) |
| | | El Moro Canyon virus (ELMCV) |
| | | Huitzilac virus (HUIV) |
| | *Fugong orthohantavirus* | Fúgòng virus (FUGV) |
| | *Fusong orthohantavirus* | Fŭsōng virus (FUSV) |
| | *Hantaan orthohantavirus** | Amur virus (AMRV) |
| | | Hantaan virus (HTNV) |
| | | Soochong virus (SOOV) |
| | *Jeju orthohantavirus* | Jeju virus (JJUV) |
| | *Kenkeme orthohantavirus* | Kenkeme virus (KKMV) |
| | *Khabarovsk orthohantavirus* | Khabarovsk virus (KHAV) |
| | | Topografov virus (TOPV) |

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**Table 1** (continued)

| Genus | Species[¶] | Virus (abbreviation)[¶] |
|---|---|---|
| | *Laguna Negra orthohantavirus* | Laguna Negra virus (LANV) |
| | | Maripa virus (MARV) |
| | | Río Mamoré virus (RIOMV) |
| | *Luxi orthohantavirus* | Lúxī virus (LUXV) |
| | *Maporal orthohantavirus* | Maporal virus (MAPV) |
| | *Montano orthohantavirus* | Montaño virus (MTNV) |
| | *Necocli orthohantavirus* | Necoclí virus (NECV) |
| | *Oxbow orthohantavirus* | Oxbow virus (OXBV) |
| | *Prospect Hill orthohantavirus* | Prospect Hill virus (PHV) |
| | *Puumala orthohantavirus* | Hokkaido virus (HOKV) |
| | | Muju virus (MUJV) |
| | | Puumala virus (PUUV) |
| | *Rockport orthohantavirus* | Rockport virus (RKPV) |
| | *Sangassou orthohantavirus* | Sangassou virus (SANGV) |
| | *Seoul orthohantavirus* | gōu virus (GOUV) |
| | | Seoul virus (SEOV) |
| | *Sin Nombre orthohantavirus* | New York virus (NYV)[3] |
| | | Sin Nombre virus (SNV) |
| | *Thailand orthohantavirus* | Anjozorobe virus |
| | | Serang virus (SERV)[4] |
| | | Thailand virus (THAIV) |
| | *Tula orthohantavirus* | Adler virus (ADLV) |
| | | Tula virus (TULV) |
| | *Yakeshi orthohantavirus* | Yákèshí virus (YKSV) |
| *Loanvirus* | *Longquan loanvirus** | Lóngquán virus (LQUV) |
| *Mobatvirus* | *Laibin mobatvirus* | Láibīn virus (LAIV) |
| | *Nova mobatvirus** | Nova virus (NVAV) |
| | *Quezon mobatvirus* | Quezon virus (QZNV) |
| *Thottimvirus* | *Imjin thottimvirus* | Imjin virus (MJNV) |
| | *Thottapalayam thottimvirus** | Thottapalayam virus (TPMV) |
| **Family *Mypoviridae*** | | |
| *Hubavirus* | *Myriapod hubavirus** | Húběi myriapoda virus 5 (HbMV-5) |
| **Family *Nairoviridae*** | | |
| *Orthonairovirus* | *Artashat orthonairovirus* | Artashat virus (ARTSV) |
| | *Chim orthonairovirus* | Chim virus (CHIMV) |
| | *Crimean-Congo hemorrhagic fever orthonairovirus* | Crimean-Congo hemorrhagic fever virus (CCHFV) |
| | *Dera Ghazi Khan orthonairovirus* | Abu Hammad virus (AHV)[5] |
| | | Abu Mina virus (AMV) |
| | | Dera Ghazi Khan virus (DGKV) |
| | | Sapphire II virus (SAPV) |
| | *Dugbe orthonairovirus** | Dugbe virus (DUGV) |
| | | kupe virus (KUPEV) |
| | *Hazara orthonairovirus* | Hazara virus (HAZV) |
| | | Tofla virus (TFLV) |
| | *Hughes orthonairovirus* | Caspiy virus (CASV) |
| | | Farallon virus (FARV) |
| | | Great Saltee virus (GRSV) |
| | | Hughes virus (HUGV) |
| | | Punta Salinas virus (PSV) |
| | | Raza virus (RAZAV) |

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**Table 1** (continued)

| Genus | Species[¶] | Virus (abbreviation)[¶] |
|---|---|---|
| | | Soldado virus (SOLV) |
| | | Zirqa virus (ZIRV) |
| | *Kasokero orthonairovirus* | Kasokero virus (KASV = KASOV) |
| | | Leopards Hill virus (LPHV) |
| | | Yogue virus (YOGV) |
| | *Keterah orthonairovirus* | Gossas virus (GOSV) |
| | | Issyk-kul virus (ISKV) |
| | | Keterah virus (KTRV)[6] |
| | | Uzun-Agach virus (UZAV) |
| | *Nairobi sheep disease orthonairovirus* | Nairobi sheep disease virus (NSDV)[7] |
| | *Qalyub orthonairovirus* | Bandia virus (BDAV) |
| | | Geran virus (GERV) |
| | | Qalyub virus (QYBV) |
| | *Sakhalin orthonairovirus* | Avalon virus (AVAV) |
| | | Clo Mor virus (CMV = CLMV) |
| | | Sakhalin virus (SAKV) |
| | | Taggert virus (TAGV) |
| | | Tillamook virus (TILLV) |
| | *Tamdy orthonairovirus* | Burana virus (BURV) |
| | | Huángpí tick virus 1 (HpTV-1) |
| | | Tamdy virus (TAMV) |
| | | Tǎchéng tick virus 1 (TcTV-1) |
| | | Wēnzhōu tick virus (WzTV) |
| | *Thiafora orthonairovirus* | Erve virus (ERVEV) |
| | | Thiafora virus (TFAV) |
| *Shaspivirus* | *Spider shaspivirus** | Shāyáng spider virus 1 (SySV-1) |
| *Striwavirus* | *Strider striwavirus** | Sānxiá water strider virus 1 (SxWSV-1) |
| **Family *Peribunyaviridae*** | | |
| *Herbevirus* | *Herbert herbevirus** | Herbert virus (HEBV) |
| | *Kibale herbevirus* | Kibale virus (KIBV) |
| | *Tai herbevirus* | Taï virus (TAIV) |
| *Orthobunyavirus* | *Acara orthobunyavirus* | Acará virus (ACAV) |
| | | Moriche virus (MORV) |
| | *Akabane orthobunyavirus* | Akabane virus (AKAV) |
| | | Sabo virus (SABOV) |
| | | Tinaroo virus (TINV) |
| | | Yaba-7 virus (Y7V) |
| | *Alajuela orthobunyavirus* | Alajuela virus (ALJV) |
| | | Brus Laguna virus (BLAV) |
| | | San Juan virus (SJV) |
| | *Anopheles A orthobunyavirus* | Anopheles A virus (ANAV) |
| | | Arumateua virus (ARTV = ARMTV) |
| | | Caraipé virus (CPEV = CRPV) |
| | | Las Maloyas virus (LMV) |
| | | Lukuni virus (LUKV) |
| | | Trombetas virus (TRMV) |
| | | Tucuruí virus (TUCV = TUCRV) |
| | *Anopheles B orthobunyavirus* | Anopheles B virus (ANBV) |
| | | Boracéia virus (BORV) |
| | *Bakau orthobunyavirus* | Bakau virus (BAKV) |

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**Table 1** (continued)

| Genus | Species[¶] | Virus (abbreviation)[¶] |
|---|---|---|
| | | Ketapang virus (KETV) |
| | | Nola virus (NOLAV) |
| | | Tanjong Rabok virus (TRV) |
| | | Telok Forest virus (TFV) |
| | *Batama orthobunyavirus* | Batama virus (BMAV) |
| | *Benevides orthobunyavirus* | Benevides virus (BVSV = BENV) |
| | *Bertioga orthobunyavirus* | Bertioga virus (BERV) |
| | | Cananéia virus (CNAV) |
| | | Guaratuba virus (GTBV) |
| | | Itimirim virus (ITIV) |
| | | Mirim virus (MIRV) |
| | *Bimiti orthobunyavirus* | bimiti virus (BIMV) |
| | *Botambi orthobunyavirus* | Botambi virus (BOTV) |
| | *Bunyamwera orthobunyavirus** | Anadyr virus (ANADV) |
| | | Batai virus (BATV)[8] |
| | | Birao virus (BIRV) |
| | | Bozo virus (BOZOV) |
| | | Bunyamwera virus (BUNV) |
| | | Cache Valley virus (CVV) |
| | | Fort Sherman virus (FSV) |
| | | Germiston virus (GERV) |
| | | Ilesha virus (ILEV) |
| | | Lokern virus (LOKV) |
| | | Maguari virus (MAGV) |
| | | Mboké virus (MBOV) |
| | | Ngari virus (NRIV)[9] |
| | | Northway virus (NORV) |
| | | Playas virus (PLAV) |
| | | Potosi virus (POTV) |
| | | Santa Rosa virus (SARV) |
| | | Shokwe virus (SHOV) |
| | | Stanfield virus (STAV) |
| | | Tensaw virus (TENV) |
| | | Tlacotalpan virus (TLAV) |
| | | Xingu virus (XINV) |
| | *Bushbush orthobunyavirus* | Benfica virus (BENV = BNFV) |
| | | Bushbush virus (BSBV) |
| | | Juan Díaz virus (JDV) |
| | *Bwamba orthobunyavirus* | Bwamba virus (BWAV) |
| | | Pongola virus (PGAV) |
| | *California encephalitis orthobunyavirus* | Achiote virus (ACHOV) |
| | | California encephalitis virus (CEV) |
| | | infirmatus virus (INFV) |
| | | Inkoo virus (INKV) |
| | | Jamestown Canyon virus (JCV) |
| | | Jerry Slough virus (JSV) |
| | | Keystone virus (KEYV) |
| | | Khatanga virus (KHATV)[10] |
| | | La Crosse virus (LACV) |

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**Table 1** (continued)

| Genus | Species[¶] | Virus (abbreviation)[¶] |
|---|---|---|
| | | Lumbo virus (LUMV) |
| | | Melao virus (MELV) |
| | | Morro Bay virus (MBV) |
| | | San Angelo virus (SAV) |
| | | Serra do Navio virus (SDNV) |
| | | snowshoe hare virus (SSHV) |
| | | South River virus (SORV) |
| | | Ťahyňa virus (TAHV) |
| | | trivittatus virus (TVTV) |
| | *Capim orthobunyavirus* | Capim virus (CAPV) |
| | *Caraparu orthobunyavirus* | Apeú virus (APEUV) |
| | | Bruconha virus (BRUV) |
| | | Caraparú virus (CARV) |
| | | El Huayo virus (EHUV) |
| | | Itaya virus (ITYV) |
| | | Ossa virus (OSSAV) |
| | | Vinces virus (VINV) |
| | *Catu orthobunyavirus* | Catú virus (CATUV) |
| | *Estero Real orthobunyavirus* | Estero Real virus (ERV) |
| | *Gamboa orthobunyavirus* | Calchaquí virus (CQIV) |
| | | Gamboa virus (GAMV) |
| | | Pueblo Viejo virus (PVV) |
| | | Soberanía virus (SOBV) |
| | *Guajara orthobunyavirus* | Guajará virus (GJAV) |
| | *Guama orthobunyavirus* | Ananindeua virus (ANUV) |
| | | Guamá virus (GMAV) |
| | | Mahogany Hammock virus (MHV) |
| | | Moju virus (MOJUV) |
| | *Guaroa orthobunyavirus* | Guaroa virus (GROV) |
| | *Kaeng Khoi orthobunyavirus* | Kaeng Khoi virus (KKV) |
| | *Kairi orthobunyavirus* | Kairi virus (KRIV) |
| | *Koongol orthobunyavirus* | koongol virus (KOOV) |
| | | wongal virus (WONV) |
| | *Madrid orthobunyavirus* | Madrid virus (MADV) |
| | *Main Drain orthobunyavirus* | Main Drain virus (MDV) |
| | *Manzanilla orthobunyavirus* | Buttonwillow virus (BUTV) |
| | | Cát Quế virus (CQV) |
| | | Ingwavuma virus (INGV) |
| | | Inini virus (INIV) |
| | | Manzanilla virus (MANV) |
| | | Mermet virus (MERV) |
| | *Marituba orthobunyavirus* | Gumbo Limbo virus (GLV) |
| | | Marituba virus (MTBV) |
| | | Murutucú virus (MURV) |
| | | Nepuyo virus (NEPV) |
| | | Restan virus (RESV) |
| | | Zungarococha virus (ZUNV) |
| | *Minatitlan orthobunyavirus* | Minatitlán virus (MNTV) |
| | | Palestina virus (PLSV) |
| | *MPoko orthobunyavirus* | M'Poko virus (MPOV) |

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**Table 1** (continued)

| Genus | Species[¶] | Virus (abbreviation)[¶] |
|---|---|---|
| | | Yaba-1 virus (Y1V) |
| | *Nyando orthobunyavirus* | Nyando virus (NDV) |
| | | Eretmapodites virus (ERETV) |
| | *Olifantsvlei orthobunyavirus* | Bobia virus (BIAV) |
| | | Dabakala virus (DABV) |
| | | Olifantsvlei virus (OLIV) |
| | | Oubi virus (OUBIV) |
| | *Oriboca orthobunyavirus* | Itaquí virus (ITQV) |
| | | Oriboca virus (ORIV) |
| | *Oropouche orthobunyavirus* | Facey's Paddock virus (FPV) |
| | | Iquitos virus (IQTV)[d] |
| | | Madre de Dios virus (MDDV) |
| | | Oropouche virus (OROV) |
| | | Perdões virus (PDEV) |
| | | Pintupo virus (PINTV) |
| | | Utinga virus (UTIV) |
| | | Utivé virus (UVV = UTVEV) |
| | *Patois orthobunyavirus* | Abras virus (ABRV) |
| | | Babahoya virus (BABV) |
| | | Pahayokee virus (PAHV) |
| | | Patois virus (PATV) |
| | | Shark River virus (SRV) |
| | *Sathuperi orthobunyavirus* | Douglas virus (DOUV) |
| | | Sathuperi virus (SATV) |
| | *Shamonda orthobunyavirus* | Peaton virus (PEAV) |
| | | Sango virus (SANV) |
| | | Shamonda virus (SHAV) |
| | *Shuni orthobunyavirus* | Aino virus (AINOV) |
| | | Kaikalur virus (KAIV) |
| | | Shuni virus (SHUV) |
| | *Simbu orthobunyavirus* | Simbu virus (SIMV) |
| | | Oya virus (OYAV) |
| | *Tacaiuma orthobunyavirus* | Tacaiuma virus (TCMV) |
| | | CoAr 1071 virus (CA1071V) |
| | | CoAr 3627 virus (CA3626V) |
| | | Virgin River virus (VRV) |
| | *Tete orthobunyavirus* | Bahig virus (BAHV) |
| | | Matruh virus (MTRV) |
| | | Tete virus (TETEV) |
| | | Tsuruse virus (TSUV) |
| | | Weldona virus (WELV) |
| | *Thimiri orthobunyavirus* | Thimiri virus (THIV) |
| | *Timboteua orthobunyavirus* | Timboteua virus (TBTV) |
| | *Turlock orthobunyavirus* | Lednice virus (LEDV) |
| | | Turlock virus (TURV) |
| | | Umbre virus (UMBV) |
| | *Wolkberg orthobunyavirus* | Wolkberg virus (WBV) |
| | *Wyeomyia orthobunyavirus* | Anhembi virus (AMBV) |
| | | BeAr 328208 virus (BAV) |
| | | Cachoeira Porteira virus (CPOV) |

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**Table 1** (continued)

| Genus | Species[¶] | Virus (abbreviation)[¶] |
|---|---|---|
| | | Iaco virus (IACOV) |
| | | Macauã virus (MCAV) |
| | | Rio Pracupi virus |
| | | Sororoca virus (SORV) |
| | | Taiassui virus (TAIAV) |
| | | Tucunduba virus (TUCV) |
| | | Wyeomyia virus (WYOV) |
| | *Zegla orthobunyavirus* | Zegla virus (ZEGV) |
| *Shangavirus* | *Insect shangavirus* | Shuāngào insect virus 1 (SgIV-1) |
| *Tospovirus* | *Groundnut bud necrosis tospovirus* | groundnut bud necrosis virus (GBNV)[11] |
| | *Groundnut ringspot tospovirus* | groundnut ringspot virus (GRSV) |
| | *Groundnut yellow spot tospovirus* | groundnut yellow spot virus (GYSV)[12] |
| | *Impatiens necrotic spot tospovirus* | impatiens necrotic spot virus (INSV) |
| | *Iris yellow spot tospovirus* | iris yellow spot virus (IYSV) |
| | *Polygonum ringspot tospovirus* | Polygonum ringspot virus (PolRSV) |
| | *Tomato chlorotic spot tospovirus* | tomato chlorotic spot virus (TCSV) |
| | *Tomato spotted wilt tospovirus** | tomato spotted wilt virus (TSWV) |
| | *Watermelon bud necrosis tospovirus* | watermelon bud necrosis virus (WBNV) |
| | *Watermelon silver mottle tospovirus* | watermelon silver mottle virus (WSMoV) |
| | *Zucchini lethal chlorosis tospovirus* | zucchini lethal chlorosis virus (ZLCV) |
| **Family *Phasmaviridae*** | | |
| *Feravirus* | *Ferak feravirus** | Ferak virus (FERV) |
| *Inshuvirus* | *Insect inshuvirus** | Shuāngào insect virus 2 (SgIV-2) |
| *Jonvirus* | *Jonchet jonvirus** | jonchet virus (JONV) |
| *Orthophasmavirus* | *Kigluaik phantom orthophasmavirus** | Kigluaik phantom virus (KIGV) |
| | *Nome phantom orthophasmavirus* | Nome phantom virus (NOMV) |
| | *Wuchang cockroach orthophasmavirus 1* | Wǔchāng cockroach virus 1 (WcCV-1) |
| | *Wuhan mosquito orthophasmavirus 1* | Wǔhàn mosquito virus 1 (WhMV-1) |
| | *Wuhan mosquito orthophasmavirus 2* | Wǔhàn mosquito virus 2 (WhMV-2) |
| *Wuhivirus* | *Insect feravirus** | Wǔhàn insect virus 2 (WhIV-2) |
| **Family *Phenuiviridae*** | | |
| *Banyangvirus* | *Huaiyangshan banyangvirus** | severe fever with thrombocytopenia syndrome virus (SFTSV) |
| *Beidivirus* | *Dipteran beidivirus** | Húběi diptera virus 3 (HbDV-3) |
| *Goukovirus* | *Cumuto goukovirus* | Cumuto virus (CUMV) |
| | *Gouleako goukovirus** | Gouléako virus (GOLV) |
| | *Yichang insect goukovirus* | Yíchāng insect virus (YcIV) |
| *Horwuvirus* | *Horsefly horwuvirus** | Wǔhàn horsefly virus (WhHV) |
| *Hudivirus* | *Dipteran hudivirus** | Húběi diptera virus 4 (HbDV-4) |
| *Hudovirus* | *Lepidopteran hudovirus** | Húběi lepidoptera virus 1 (HbLV-1) |
| *Mobuvirus* | *Mothra mobuvirus** | Mothra virus (MTHV) |
| *Phasivirus* | *Badu phasivirus** | Badu virus (BADUV) |
| | *Phasi Charoen-like phasivirus* | Phasi Chaeron-like virus (PCLV) |
| | *Wuhan fly phasivirus*[#] | |
| | *Wutai mosquito phasivirus* | Wǔtái mosquito virus (WtMV) |
| *Phlebovirus* | *Bujaru phlebovirus* | Bujaru virus (BUJV) |
| | | Munguba virus (MUNV) |
| | *Candiru phlebovirus* | Alenquer virus (ALEV) |
| | | Ariquemes virus (ARQV) |
| | | Candirú virus (CDUV) |

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**Table 1** (continued)

| Genus | Species¶ | Virus (abbreviation)¶ |
|---|---|---|
| | | Itaituba virus (ITAV) |
| | | Jacundá virus (JCNV) |
| | | Maldonado virus (MLOV) |
| | | Morumbi virus (MR(M)BV) |
| | | Mucura virus (MCRV/MRAV) |
| | | Nique virus (NIQV) |
| | | Oriximiná virus (ORXV) |
| | | Serra Norte virus (SRNV) |
| | | Turuna virus (TUAV) |
| | *Chilibre phlebovirus* | Cacao virus (CACV) |
| | | Chilibre virus (CHIV) |
| | *Frijoles phlebovirus* | frijoles virus (FRIV) |
| | | Joá virus (JOAV) |
| | *Punta Toro phlebovirus* | Buenaventura virus (BUEV) |
| | | Campana virus (CMAV) |
| | | Capira virus (CAPIV) |
| | | Coclé virus (CCLV) |
| | | Leticia virus (LTCV) |
| | | Punta Toro virus (PTV) |
| | *Rift Valley fever phlebovirus** | Rift Valley fever virus (RVFV) |
| | *Salehabad phlebovirus* | Adana virus (ADAV) |
| | | Adria virus (ADRV) |
| | | Alcube virus |
| | | Arbia virus (ARBV) |
| | | Arumowot virus (AMTV) |
| | | Medjerda Valley virus (MVV) |
| | | Odrénisrou virus (ODRV) |
| | | Olbia virus (OLBV) |
| | | Salehabad virus (SALV) |
| | | Bregalaka virus (BREV) |
| | | Zaba virus (ZABAV) |
| | *Sandfly fever Naples phlebovirus* | Arrábida virus (ARRV) |
| | | Balkan virus (BALKV) |
| | | Fermo virus (FERV) |
| | | Gordil virus (GORV) |
| | | Granada virus (GRV = GRAV) |
| | | Massilia virus (MASV) |
| | | Punique virus (PUNV) |
| | | Saddaguia virus (SADV) |
| | | Saint-Floris virus (SAFV) |
| | | sandfly fever Naples virus (SFNV) |
| | | Tehran virus (THEV) |
| | | Toscana virus (TOSV) |
| | | Zerdali virus (ZERV) |
| | *Uukuniemi phlebovirus* | Chizé virus (CHZV) |
| | | EgAN 1825-61 virus (EGAV) |
| | | Fin V 707 virus (FINV) |
| | | Oceanside virus (OCV = OCEV) |
| | | Pontevès virus (PTVV) |

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**Table 1** (continued)

| Genus | Species¶ | Virus (abbreviation)¶ |
|---|---|---|
| | | St. Abbs Head virus (SAHV) |
| | | Uukuniemi virus (UUKV) |
| | | Zaliv Terpenyia virus (ZTV) |
| *Pidchovirus* | *Pidgey pidchovirus** | Pidgey virus (PGYV) |
| *Tenuivirus* | *Echinochloa hoja blanca tenuivirus* | Echinochloa hoja blanca virus (EHBV) |
| | *Iranian wheat stripe tenuivirus* | Iranian wheat stripe virus (IWSV) |
| | *Maize stripe tenuivirus* | maize stripe virus (MStV = MSpV) |
| | *Rice grassy stunt tenuivirus* | rice grassy stunt virus (RGSV) |
| | *Rice hoja blanca tenuivirus* | rice hoja blanca virus (RHBV) |
| | *Rice stripe tenuivirus** | rice stripe virus (RSV = RStV) |
| | *Urochloa hoja blanca tenuivirus* | Urochloa hoja blanca virus (UHBV) |
| *Wubeivirus* | *Dipteran wubeivirus** | Húběi diptera virus 5 (HbDV-5) |
| | *Fly wubeivirus* | Wǔhàn fly virus 1 (WhFV-1) |
| **Family *Wupedeviridae*** | | |
| *Wumivirus* | *Millipede wumivirus** | Wǔhàn millipede virus 2 (WhMV-2) |

*Asterisks denote type species

[1]Junior synonyms: Cardamones virus, Hǎinán Medical University virus, Xīngyì virus

[2]Synonym: Artybash virus (ARTV)

[3]synonym: New York 1 virus (NY-1V)

[4]synonym: Jurong virus

[5]includes the strain previously referred to as Tunis virus (TUNV)

[6]includes the strain previously referred to as soft tick bunyavirus (STBV)

[7]includes the strain previously referred to as Ganjam virus (GANV)

[8]synonyms: Čalovo virus (CVOV), Chittoor virus (CHITV), Olkya virus, Olyka virus, UgMP-6830 virus

[9]includes the strain previously referred to as Garissa virus

[10]also mistakenly referred to in the literature as Chantanga virus (CHATV) and Chatanga virus (CHATV)

[11]synonym: peanut bud necrosis virus (PDNV)

[12]synonym: peanut yellow spot virus (PYSV)

[#]The virus of this species was moved into a new genus (*Wubeivirus*) and species (*Fly wubeivirus*), but due to a formal error, the species *Wuhan fly phasivirus* was not deleted. A taxonomic proposal to rectify this oversight has been submitted

[¶]Please note that viruses are real objects that are assigned to concepts that are called taxa. Species, genera, families, and orders are taxa. Taxon names are always italicized and always begin with a capital letter. Virus names, on the other hand, are not italicized and are not capitalized, except if the name or a name component is a proper noun. This column lists the virus names with their correct (lack of) capitalization. Lists of viruses within a given species are provisional at this point and will likely be amended in the near future

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## Compliance with ethical standards

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**Ethical approval** This article does not contain any studies with human participants or animals performed by any of the authors.

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VIROLOGY DIVISION NEWS

# Taxonomy of the order *Mononegavirales*: second update 2018

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## Abstract

In October 2018, the order *Mononegavirales* was amended by the establishment of three new families and three new genera, abolishment of two genera, and creation of 28 novel species. This article presents the updated taxonomy of the order *Mononegavirales* as now accepted by the International Committee on Taxonomy of Viruses (ICTV).

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## Introduction

The virus order *Mononegavirales* was established in 1991 to accommodate related viruses with nonsegmented, linear, single-stranded, negative-sense RNA genomes classified into three families [12, 13]. According to the previous ratification vote held in March 2018, the order included eight families [7]. Amended/emended order descriptions were published in 1995 [4], 1997 [14], 2000 [15], 2005 [16], 2011 [5], 2016 [1], 2017 [2], and March 2018 [3]. Here, we present the changes that were proposed via an official ICTV taxonomic proposal (TaxoProp 2017.016M.A.v1. Mononegavirales_rev) at http://www.ictvonline.org/ in 2017 and were accepted by the ICTV Executive Committee (EC) in October 2018. These changes are now part of the official ICTV taxonomy as of October 2018.

## Taxonomic changes at the order rank

In October 2018, the order *Mononegavirales* was expanded by three families. The family *Artoviridae* was created for the established genus *Peropuvirus* (moved out of *Nyamiviridae*) and six new species for Běihǎi barnacle virus 8 (BhBV-8), Běihǎi rhabdo-like virus 1 (BhRLV-1), Běihǎi rhabdo-like virus 2 (BhRLV-2), Húběi rhabdo-like virus 5 (HbRLV-5), Húběi rhabdo-like virus 6 (HbRLV-6), and Húběi rhabdo-like virus 8 (HbRLV-8), all discovered in invertebrates [8, 17]. The family *Lispiviridae* was established to include the floating genus *Arlivirus*; inclusion of the novel species *Hubei arlivirus*, *Odonate arlivirus*, and *Wuchang arlivirus* for Húběi rhabdo-like virus 3 (HbRLV-3), Húběi odonate virus 10 (HbOV-10), and Wǔchāng romanomermis nematode virus 2 (WcRNV-2) discovered in invertebrates; and inclusion of the (renamed) species of the now abandoned free-floating genera *Chengtivirus* and *Wastrivirus* [8, 17]. The family *Xinmoviridae* was established for the floating genus *Anphevirus*, and six novel species were added to the genus for Bolahun virus (BLHV), Drosophila unispina virus 1 (DuniV-1), Húběi diptera virus 11 (HbDV-11), Húběi orthoptera virus 5 (HbOV-5), Húběi rhabdo-like virus 7 (HbRLV-7), and Shuāngào fly virus 2 (SgFV-2) discovered in invertebrates [6, 8, 9, 17].

## Taxonomic changes at the family rank

### Bornaviridae

In October 2018, no changes were made at the family rank.

### Filoviridae

In October 2018, no changes were made at the family rank.

### Mymonaviridae

In October 2018, six new species were added to the genus *Sclerotimonavirus* for Húběi rhabdo-like virus 4 (HbRLV-4), Sclerotinia sclerotiorum negative-stranded RNA virus 4 (SsNSRV-4), soybean leaf-associated negative-stranded RNA virus 1 (SLaNSRV-1), soybean leaf-associated negative-stranded RNA virus 2 (SLaNSRV-2), soybean leaf-associated negative-stranded RNA virus 3 (SLaNSRV-3), and soybean leaf-associated negative-stranded RNA virus 4 (SLaNSRV-4) associated with soybean leaves, invertebrates, and fungi [10, 11, 17].

### Nyamiviridae

In October 2018, the free-floating genus *Crustavirus* was included in the family and two novel species were added to that genus for Běihǎi rhabdo-like virus 6 (BhRLV-6) and Wēnlíng crustacean virus 12 (WlCV-12) discovered in invertebrates. One genus, *Peropuvirus*, was moved from the family into the new family *Artoviridae*. Finally, the family was expanded by three novel genera that include a total of five new species for Běihǎi rhabdo-like virus 3 (BhRLV-3), Běihǎi rhabdo-like virus 4 (BhRLV-4), Běihǎi rhabdo-like virus 5 (BhRLV-5), Orinoco virus (ONCV), and Wēnzhōu tapeworm virus 1 (WzTWV-1) discovered in invertebrates [8, 17].

### Paramyxoviridae

In October 2018, no changes were made at the family rank.

### Pneumoviridae

In October 2018, no changes were made at the family rank.

### Rhabdoviridae

In October 2018, no changes were made at the family rank.

### Sunviridae

In October 2018, no changes were made at the family rank.

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**Table 1** ICTV-accepted taxonomy of the order *Mononegavirales* as of October 2018. Listed are all mononegaviruses that are classified into species

| Genus | Species[¶] | Virus (Abbreviation)[¶] |
|---|---|---|
| **Family *Artoviridae*** | | |
| *Peropuvirus* | *Barnacle peropuvirus* | Běihǎi barnacle virus 8 (BhBV-8) |
| | *Beihai peropuvirus* | Běihǎi rhabdo-like virus 1 (BhRLV-1) |
| | *Hubei peropuvirus* | Húběi rhabdo-like virus 6 (HbRLV-6) |
| | *Odonate peropuvirus* | Húběi rhabdo-like virus 8 (HbRLV-8) |
| | *Pillworm peropuvirus* | Húběi rhabdo-like virus 5 (HbRLV-5) |
| | *Pteromalus puparum peropuvirus** | Pteromalus puparum negative-strand RNA virus 1 (PpNSRV-1) |
| | *Woodlouse peropuvirus* | Běihǎi rhabdo-like virus 2 (BhRLV-2) |
| **Family *Bornaviridae*** | | |
| *Carbovirus* | *Queensland carbovirus** | jungle carpet python virus (JCPV) |
| | *Southwest carbovirus* | southwest carpet python virus (SWCPV) |
| *Orthobornavirus* | *Elapid 1 orthobornavirus* | Loveridge's garter snake virus 1 (LGSV-1) |
| | *Mammalian 1 orthobornavirus** | Borna disease virus 1 (BoDV-1) |
| | | Borna disease virus 2 (BoDV-2) |
| | *Mammalian 2 orthobornavirus* | variegated squirrel bornavirus 1 (VSBV-1) |
| | *Passeriform 1 orthobornavirus* | canary bornavirus 1 (CnBV-1) |
| | | canary bornavirus 2 (CnBV-2) |
| | | canary bornavirus 3 (CnBV-3) |
| | *Passeriform 2 orthobornavirus* | estrildid finch bornavirus 1 (EsBV-1) |
| | *Psittaciform 1 orthobornavirus* | parrot bornavirus 1 (PaBV-1) |
| | | parrot bornavirus 2 (PaBV-2) |
| | | parrot bornavirus 3 (PaBV-3) |
| | | parrot bornavirus 4 (PaBV-4) |
| | | parrot bornavirus 7 (PaBV-7) |
| | *Psittaciform 2 orthobornavirus* | parrot bornavirus 5 (PaBV-5) |
| | *Waterbird 1 orthobornavirus* | aquatic bird bornavirus 1 (ABBV-1) |
| | | aquatic bird bornavirus 2 (ABBV-2) |
| **Family *Filoviridae*** | | |
| *Cuevavirus* | *Lloviu cuevavirus** | Lloviu virus (LLOV) |
| *Ebolavirus* | *Bundibugyo ebolavirus* | Bundibugyo virus (BDBV) |
| | *Reston ebolavirus* | Reston virus (RESTV) |
| | *Sudan ebolavirus* | Sudan virus (SUDV) |
| | *Tai Forest ebolavirus* | Taï Forest virus (TAFV) |
| | *Zaire ebolavirus** | Ebola virus (EBOV) |
| *Marburgvirus* | *Marburg marburgvirus** | Marburg virus (MARV) |
| | | Ravn virus (RAVV) |
| **Family *Lispiviridae*** | | |
| *Arlivirus* | *Hubei arlivirus* | Húběi rhabdo-like virus 3 (HbRLV-3) |
| | *Lishi arlivirus** | Líshí spider virus 2 (LsSV-2) |
| | *Odonate arlivirus* | Húběi odonate virus 10 (HbOV-10) |
| | *Gerrid arlivirus* | Sānxiá water strider virus 4 (SxWSV-4) |
| | *Tacheng arlivirus* | Tǎchéng tick virus 6 (TcTV-6) |
| | *Wuchang arlivirus* | Wǔchāng romanomermis nematode virus 2 (WcRNV-2) |
| **Family *Mymonaviridae*** | | |
| *Sclerotimonavirus* | *Dadou sclerotimonavirus* | soybean leaf-associated negative-stranded RNA virus 3 (SLaNSRV-3) |
| | *Drop sclerotimonavirus* | Sclerotinia sclerotiorum negative-stranded RNA virus 2 (SsNSRV-2) |
| | | Sclerotinia sclerotiorum negative-stranded RNA virus 4 (SsNSRV-4) |
| | *Glycine sclerotimonavirus* | Fusarium graminearum negative-stranded RNA virus 1 (FgNSRV-1) |
| | | soybean leaf-associated negative-stranded RNA virus 1 (SLaNSRV-1) |

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**Table 1** (continued)

| Genus | Species[¶] | Virus (Abbreviation)[¶] |
|---|---|---|
| | *Hubei sclerotimonavirus* | Húběi rhabdo-like virus 4 (HbRLV-4) |
| | *Illinois sclerotimonavirus* | soybean leaf-associated negative-stranded RNA virus 2 (SLaNSRV-2) |
| | *Phyllosphere sclerotimonavirus* | soybean leaf-associated negative-stranded RNA virus 4 (SLaNSRV-4) |
| | *Sclerotinia sclerotimonavirus** | Sclerotinia sclerotiorum negative-stranded RNA virus 1 (SsNSRV-1) |
| | | Sclerotinia sclerotiorum negative-stranded RNA virus 3 (SsNSRV-3) |
| **Family *Nyamiviridae*** | | |
| *Berhavirus* | *Beihai berhavirus* | Běihǎi rhabdo-like virus 4 (BhRLV-4) |
| | *Echinoderm berhavirus* | Běihǎi rhabdo-like virus 5 (BhRLV-5) |
| | *Sipunculid berhavirus** | Běihǎi rhabdo-like virus 3 (BhRLV-3) |
| *Crustavirus* | *Beihai crustavirus* | Běihǎi rhabdo-like virus 6 (BhRLV-6) |
| | *Wenling crustavirus* | Wēnlǐng crustacean virus 12 (WlCV-12) |
| | *Wenzhou crustavirus** | Wēnzhōu crab virus 1 (WzCV-1) |
| *Nyavirus* | *Midway nyavirus* | Midway virus (MIDWV) |
| | *Nyamanini nyavirus** | Nyamanini virus (NYMV) |
| | *Sierra Nevada nyavirus* | Sierra Nevada virus (SNVV) |
| *Orinovirus* | *Orinoco orinovirus** | Orinoco virus (ONCV) |
| *Socyvirus* | *Soybean cyst nematode socyvirus** | soybean cyst nematode virus 1 (SbCNV-1) |
| *Tapwovirus* | *Tapeworm tapwovirus** | Wēnzhōu tapeworm virus 1 (WzTWV-1) |
| **Family *Paramyxoviridae*** | | |
| *Aquaparamyxovirus* | *Salmon aquaparamyxovirus** | Atlantic salmon paramyxovirus (AsaPV) |
| *Avulavirus* | *Avian avulavirus 1** | avian paramyxovirus 1 (APMV-1)[1] |
| | *Avian avulavirus 2* | avian paramyxovirus 2 (APMV-2) |
| | *Avian avulavirus 3* | avian paramyxovirus 3 (APMV-3) |
| | *Avian avulavirus 4* | avian paramyxovirus 4 (APMV-4) |
| | *Avian avulavirus 5* | avian paramyxovirus 5 (APMV-5) |
| | *Avian avulavirus 6* | avian paramyxovirus 6 (APMV-6) |
| | *Avian avulavirus 7* | avian paramyxovirus 7 (APMV-7) |
| | *Avian avulavirus 8* | avian paramyxovirus 8 (APMV-8) |
| | *Avian avulavirus 9* | avian paramyxovirus 9 (APMV-9) |
| | *Avian avulavirus 10* | avian paramyxovirus 10 (APMV-10) |
| | *Avian avulavirus 11* | avian paramyxovirus 11 (APMV-11) |
| | *Avian avulavirus 12* | avian paramyxovirus 12 (APMV-12) |
| | *Avian avulavirus 13* | avian paramyxovirus 13 (APMV-13) |
| | *Avian avulavirus 14* | avian paramyxovirus 14 (APMV-14) |
| | *Avian avulavirus 15* | avian paramyxovirus 15 (APMV-15) |
| | *Avian avulavirus 16* | avian paramyxovirus 16 (APMV-16) |
| | *Avian avulavirus 17* | Antarctic penguin virus A (APV-A) |
| | *Avian avulavirus 18* | Antarctic penguin virus B (APV-B) |
| | *Avian avulavirus 19* | Antarctic penguin virus C (APV-C) |
| *Ferlavirus* | *Reptilian ferlavirus** | Fer-de-Lance virus (FDLV) |
| *Henipavirus* | *Cedar henipavirus* | Cedar virus (CedV) |
| | *Ghanaian bat henipavirus* | Ghana virus (GhV)[2] |
| | *Hendra henipavirus** | Hendra virus (HeV) |
| | *Mojiang henipavirus* | Mòjiāng virus (MojV) |
| | *Nipah henipavirus* | Nipah virus (NiV) |
| *Morbillivirus* | *Canine morbillivirus* | canine distemper virus (CDV) |
| | *Cetacean morbillivirus* | cetacean morbillivirus (CeMV) |
| | *Feline morbillivirus* | feline morbillivirus (FeMV) |
| | *Measles morbillivirus** | measles virus (MeV) |
| | *Small ruminant morbillivirus* | peste-des-petits-ruminants virus (PPRV) |

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**Table 1** (continued)

| Genus | Species[¶] | Virus (Abbreviation)[¶] |
|---|---|---|
| | *Phocine morbillivirus* | phocine distemper virus (PDV) |
| | *Rinderpest morbillivirus* | rinderpest virus (RPV) |
| *Respirovirus* | *Bovine respirovirus 3* | bovine parainfluenza virus 3 (BPIV-3) |
| | *Human respirovirus 1* | human parainfluenza virus 1 (HPIV-1) |
| | *Human respirovirus 3* | human parainfluenza virus 3 (HPIV-3) |
| | *Porcine respirovirus 1* | porcine parainfluenza virus 1 (PPIV-1) |
| | *Murine respirovirus** | Sendai virus (SeV)[3] |
| *Rubulavirus* | *Achimota rubulavirus 1* | Achimota virus 1 (AchPV-1) |
| | *Achimota rubulavirus 2* | Achimota virus 2 (AchPV-2) |
| | *Bat mumps rubulavirus* | bat mumps virus (BMV)[4] |
| | *Human rubulavirus 2* | human parainfluenza virus 2 (HPIV-2) |
| | *Human rubulavirus 4* | human parainfluenza virus 4a (HPIV-4a) |
| | | human parainfluenza virus 4b (HPIV-4b) |
| | *Mammalian rubulavirus 5* | parainfluenza virus 5 (PIV-5)[5] |
| | *Mapuera rubulavirus* | Mapuera virus (MapV) |
| | *Menangle rubulavirus* | Menangle virus (MenPV) |
| | *Mumps rubulavirus** | mumps virus (MuV) |
| | *Porcine rubulavirus* | La Piedad Michoacán Mexico virus (LPMV)[6] |
| | *Simian rubulavirus* | simian virus 41 (SV-41) |
| | *Sosuga rubulavirus* | Sosuga virus (SOSV) |
| | *Teviot rubulavirus* | Teviot virus (TevPV) |
| | *Tioman rubulavirus* | Tioman virus (TioPV) |
| | *Tuhoko rubulavirus 1* | Tuhoko virus 1 (ThkPV-1) |
| | *Tuhoko rubulavirus 2* | Tuhoko virus 2 (ThkPV-2) |
| | *Tuhoko rubulavirus 3* | Tuhoko virus 3 (ThkPV-3) |
| **Family *Pneumoviridae*** | | |
| *Metapneumovirus* | *Avian metapneumovirus** | avian metapneumovirus (AMPV)[7] |
| | *Human metapneumovirus* | human metapneumovirus (HMPV) |
| *Orthopneumovirus* | *Bovine orthopneumovirus* | bovine respiratory syncytial virus (BRSV) |
| | *Human orthopneumovirus** | human respiratory syncytial virus (HRSV) |
| | *Murine orthopneumovirus* | murine pneumonia virus (MPV) |
| **Family *Rhabdoviridae*** | | |
| *Almendravirus* | *Arboretum almendravirus* | Arboretum virus (ABTV) |
| | *Balsa almendravirus* | Balsa virus (BALV) |
| | *Coot Bay almendravirus* | Coot Bay virus (CBV) |
| | *Puerto Almendras almendravirus** | Puerto Almendras virus (PTAMV) |
| | *Rio Chico almendravirus* | Rio Chico virus (RCHV) |
| *Curiovirus* | *Curionopolis curiovirus** | Curionopolis virus (CURV) |
| | *Iriri curiovirus* | Iriri virus (IRIRV) |
| | *Itacaiunas curiovirus* | Itacaiunas virus (ITAV) |
| | *Rochambeau curiovirus* | Rochambeau virus (RBUV) |
| *Cytorhabdovirus* | *Alfalfa dwarf cytorhabdovirus* | alfalfa dwarf virus (ADV) |
| | *Barley yellow striate mosaic cytorhabdovirus* | barley yellow striate mosaic virus (BYSMV) |
| | *Broccoli necrotic yellows cytorhabdovirus* | broccoli necrotic yellows virus (BNYV) |
| | *Colocasia bobone disease-associated cytorhabdovirus* | Colocasia bobone disease-associated virus (CBDaV) |
| | *Festuca leaf streak cytorhabdovirus* | Festuca leaf streak virus (FLSV) |
| | *Lettuce necrotic yellows cytorhabdovirus** | lettuce necrotic yellows virus (LNYV) |
| | *Lettuce yellow mottle cytorhabdovirus* | lettuce yellow mottle virus (LYMoV) |

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**Table 1** (continued)

| Genus | Species[¶] | Virus (Abbreviation)[¶] |
|---|---|---|
| | *Northern cereal mosaic cytorhabdovirus* | northern cereal mosaic virus (NCMV) |
| | *Sonchus cytorhabdovirus 1* | Sonchus virus (SonV) |
| | *Strawberry crinkle cytorhabdovirus* | strawberry crinkle virus (SCV) |
| | *Wheat American striate mosaic cytorhabdovirus* | wheat American striate mosaic virus (WASMV) |
| *Dichorhavirus* | *Coffee ringspot dichorhavirus* | coffee ringspot virus (CoRSV) |
| | *Orchid fleck dichorhavirus** | orchid fleck virus (OFV) |
| *Ephemerovirus* | *Adelaide River ephemerovirus* | Adelaide River virus (ARV) |
| | *Berrimah ephemerovirus* | Berrimah virus (BRMV) |
| | *Bovine fever ephemerovirus** | bovine ephemeral fever virus (BEFV) |
| | *Kimberley ephemerovirus* | Kimberley virus (KIMV) |
| | | Malakal virus (MALV) |
| | *Koolpinyah ephemerovirus* | Koolpinyah virus (KOOLV) |
| | *Kotonkan ephemerovirus* | kotonkan virus (KOTV) |
| | *Obodhiang ephemerovirus* | Obodhiang virus (OBOV) |
| | *Yata ephemerovirus* | Yata virus (YATV) |
| *Hapavirus* | *Flanders hapavirus* | Flanders virus (FLAV) |
| | *Hart Park hapavirus* | Hart Park virus (HPV) |
| | *Gray Lodge hapavirus* | Gray Lodge virus (GLOV) |
| | *Joinjakaka hapavirus* | Joinjakaka virus (JOIV) |
| | *La Joya hapavirus* | La Joya virus (LJV) |
| | *Kamese hapavirus* | Kamese virus (KAMV) |
| | *Landjia hapavirus* | Landjia virus (LANV = LJAV) |
| | *Manitoba hapavirus* | Manitoba virus (MANV = MNTBV) |
| | *Marco hapavirus* | Marco virus (MCOV) |
| | *Mosqueiro hapavirus* | Mosqueiro virus (MQOV) |
| | *Mossuril hapavirus* | Mossuril virus (MOSV) |
| | *Ngaingan hapavirus* | Ngaingan virus (NGAV) |
| | *Ord River hapavirus* | Ord River virus (ORV) |
| | *Parry Creek hapavirus* | Parry Creek virus (PCV) |
| | *Wongabel hapavirus** | Wongabel virus (WONV) |
| *Ledantevirus* | *Barur ledantevirus* | Barur virus (BARV) |
| | *Fikirini ledantevirus* | Fikirini virus (FKRV) |
| | *Fukuoka ledantevirus* | Fukuoka virus (FUKV) |
| | *Kanyawara ledantevirus* | Kanyawara virus (KYAV) |
| | *Kern Canyon ledantevirus* | Kern Canyon virus (KCV) |
| | *Keuraliba ledantevirus* | Keuraliba virus (KEUV) |
| | *Kolente ledantevirus* | Kolente virus (KOLEV) |
| | *Kumasi ledantevirus* | Kumasi rhabdovirus (KRV) |
| | *Le Dantec ledantevirus** | Le Dantec virus (LDV) |
| | *Mount Elgon bat ledantevirus* | Mount Elgon bat virus (MEBV) |
| | *Nkolbisson ledantevirus* | Nkolbisson virus (NKOV) |
| | *Nishimuro ledantevirus* | Nishimuro virus (NISV)[8] |
| | *Oita ledantevirus* | Oita virus (OITAV) |
| | *Wuhan ledantevirus* | Wuhan louse fly virus 5 (WLFV-5) |
| | *Yongjia ledantevirus* | Yongjia tick virus 2 (YTV-2) |
| *Lyssavirus* | *Aravan lyssavirus* | Aravan virus (ARAV) |
| | *Australian bat lyssavirus* | Australian bat lyssavirus (ABLV) |
| | *Bokeloh bat lyssavirus* | Bokeloh bat lyssavirus (BBLV) |
| | *Duvenhage lyssavirus* | Duvenhage virus (DUVV) |

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**Table 1** (continued)

| Genus | Species[¶] | Virus (Abbreviation)[¶] |
|---|---|---|
| | *European bat 1 lyssavirus* | European bat lyssavirus 1 (EBLV-1) |
| | *European bat 2 lyssavirus* | European bat lyssavirus 2 (EBLV-2) |
| | *Gannoruwa bat lyssavirus* | Gannoruwa bat lyssavirus (GBLV) |
| | *Ikoma lyssavirus* | Ikoma lyssavirus (IKOV) |
| | *Irkut lyssavirus* | Irkut virus (IRKV) |
| | *Khujand lyssavirus* | Khujand virus (KHUV) |
| | *Lagos bat lyssavirus* | Lagos bat virus (LBV) |
| | *Lleida bat lyssavirus* | Lleida bat virus (LLEBV) |
| | *Mokola lyssavirus* | Mokola virus (MOKV) |
| | *Rabies lyssavirus** | rabies virus (RABV) |
| | *Shimoni bat lyssavirus* | Shimoni bat virus (SHIBV) |
| | *West Caucasian bat lyssavirus* | West Caucasian bat virus (WCBV) |
| *Novirhabdovirus* | *Hirame novirhabdovirus* | hirame rhabdovirus (HIRV) |
| | *Piscine novirhabdovirus* | viral hemorrhagic septicemia virus (VHSV)[9] |
| | *Salmonid novirhabdovirus** | infectious hematopoietic necrosis virus (IHNV) |
| | *Snakehead novirhabdovirus* | snakehead rhabdovirus (SHRV) |
| *Nucleorhabdovirus* | *Datura yellow vein nucleorhabdovirus* | datura yellow vein virus (DYVV) |
| | *Eggplant mottled dwarf nucleorhabdovirus* | eggplant mottled dwarf virus (EMDV) |
| | *Maize fine streak nucleorhabdovirus* | maize fine streak virus (MSFV) |
| | *Maize Iranian mosaic nucleorhabdovirus* | maize Iranian mosaic virus (MIMV) |
| | *Maize mosaic nucleorhabdovirus* | maize mosaic virus (MMV) |
| | *Potato yellow dwarf nucleorhabdovirus** | potato yellow dwarf virus (PYDV) |
| | *Rice yellow stunt nucleorhabdovirus* | rice yellow stunt virus (RYSV) |
| | | rice transitory yellowing virus (RTYV) |
| | *Sonchus yellow net nucleorhabdovirus* | Sonchus yellow net virus (SYNV) |
| | *Sowthistle yellow vein nucleorhabdovirus* | sowthistle yellow vein virus (SYVV) |
| | *Taro vein chlorosis nucleorhabdovirus* | taro vein chlorosis virus (TaVCV) |
| *Perhabdovirus* | *Anguillid perhabdovirus* | eel virus European X (EVEX) |
| | *Perch perhabdovirus** | perch rhabdovirus (PRV) |
| | *Sea trout perhabdovirus* | lake trout rhabdovirus (LTRV) |
| *Sigmavirus* | *Drosophila affinis sigmavirus* | Drosophila affinis sigmavirus (DAffSV) |
| | *Drosophila ananassae sigmavirus* | Drosophila ananassae sigmavirus (DAnaSV) |
| | *Drosophila immigrans sigmavirus* | Drosophila immigrans sigmavirus (DImmSV) |
| | *Drosophila melanogaster sigmavirus** | Drosophila melanogaster sigmavirus (DMelSV) |
| | *Drosophila obscura sigmavirus* | Drosophila obscura sigmavirus (DObsSV) |
| | *Drosophila tristis sigmavirus* | Drosophila tristis sigmavirus (DTriSV) |
| | *Muscina stabulans sigmavirus* | Muscina stabulans sigmavirus (MStaSV) |
| *Sprivivirus* | *Carp sprivivirus** | spring viremia of carp virus (SVCV) |
| | *Pike fry sprivivirus* | grass carp rhabdovirus (GrCRV) |
| | | pike fry rhabdovirus (PFRV) |
| | | tench rhabdovirus (TenRV) |
| *Sripuvirus* | *Almpiwar sripuvirus* | Almpiwar virus (ALMV) |
| | *Chaco sripuvirus* | Chaco virus (CHOV) |
| | *Niakha sripuvirus** | Niakha virus (NIAV) |
| | *Sena Madureira sripuvirus* | Sena Madureira virus (SMV) |
| | *Sripur sripuvirus* | Sripur virus (SRIV) |
| *Tibrovirus* | *Bas Congo tibrovirus* | Bas-Congo virus (BASV) |
| | *Beatrice Hill tibrovirus* | Beatrice Hill virus (BHV) |
| | *Coastal Plains tibrovirus* | Coastal Plains virus (CPV) |
| | *Ekpoma 1 tibrovirus* | Ekpoma virus 1 (EKV-1) |

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**Table 1** (continued)

| Genus | Species[¶] | Virus (Abbreviation)[¶] |
|---|---|---|
| | *Ekpoma 2 tibrovirus* | Ekpoma virus 2 (EKV-2) |
| | *Sweetwater Branch tibrovirus* | Sweetwater Branch virus (SWBV) |
| | *Tibrogargan tibrovirus** | Bivens Arm virus (BAV) |
| | | Tibrogargan virus (TIBV) |
| *Tupavirus* | *Durham tupavirus** | Durham virus (DURV) |
| | *Klamath tupavirus* | Klamath virus (KLAV) |
| | *Tupaia tupavirus* | tupaia virus (TUPV) |
| *Varicosavirus* | *Lettuce big-vein associated varicosavirus** | lettuce big-vein associated virus (LBVaV)[10] |
| *Vesiculovirus* | *Alagoas vesiculovirus* | vesicular stomatitis Alagoas virus (VSAV) |
| | *American bat vesiculovirus* | American bat vesiculovirus (ABVV) |
| | *Carajas vesiculovirus* | Carajas virus (CJSV) |
| | *Chandipura vesiculovirus* | Chandipura virus (CHPV) |
| | *Cocal vesiculovirus* | Cocal virus (COCV) |
| | *Indiana vesiculovirus** | vesicular stomatitis Indiana virus (VSIV) |
| | *Isfahan vesiculovirus* | Isfahan virus (ISFV) |
| | *Jurona vesiculovirus* | Jurona virus (JURV) |
| | *Malpais Spring vesiculovirus* | Malpais Spring virus (MSPV) |
| | *Maraba vesiculovirus* | Maraba virus (MARAV) |
| | *Morreton vesiculovirus* | Morreton virus (MORV) |
| | *New Jersey vesiculovirus* | vesicular stomatitis New Jersey virus (VSNJV) |
| | *Perinet vesiculovirus* | Perinet virus (PERV) |
| | *Piry vesiculovirus* | Piry virus (PIRYV) |
| | *Radi vesiculovirus* | Radi virus (RADV) |
| | *Yug Bogdanovac vesiculovirus* | Yug Bogdanovac virus (YBV) |
| Unassigned | *Moussa virus* | Moussa virus (MOUV) |
| **Family *Sunviridae*** | | |
| *Sunshinevirus* | *Reptile sunshinevirus 1** | Sunshine Coast virus (SunCV) |
| **Family *Xinmoviridae*** | | |
| *Anphevirus* | *Bolahun anphevirus* | Bolahun virus (BLHV) |
| | | Gambie virus (GAMV) |
| | *Dipteran anphevirus* | Húběi diptera virus 11 (HbDV-11) |
| | *Drosophilid anphevirus* | Drosophila unispina virus 1 (DuniV-1) |
| | *Odonate anphevirus* | Húběi rhabdo-like virus 7 (HbRLV-7) |
| | *Orthopteran anphevirus* | Húběi orthoptera virus 5 (HbOV-5) |
| | *Shuangao anphevirus* | Shuāngào fly virus 2 (SgFV-2) |
| | *Xincheng anphevirus** | Xīnchéng mosquito virus (XcMV) |

*Asterisks denote type species

[1]Includes Newcastle disease virus (NDV) and pigeon paramyxovirus 1 (PPMV-1)

[2]synonym: GH-M74a virus, Kumasi virus (KV)

[3]synonym: murine parainfluenza virus 1

[4]synonym: bat paramyxovirus

[5]synonym: simian virus 5

[6]synonym: porcine rubulavirus

[7]synonyms: avian pneumovirus, turkey rhinotracheitis virus

[8]synonym: wild boar rhabdovirus 1 (WBRV1)

[9]synonyms: Egtved virus, Paralichthys olivaceus rhabdovirus

[10]synonym: tobacco stunt virus

[¶]Please note that viruses are real objects that are assigned to concepts that are called taxa. Species, genera, families, and orders are taxa. Taxon names are always italicized and always begin with a capital letter. Virus names, on the other hand, are not italicized and are not capitalized, except if the name or a name component is a proper noun. This column lists the virus names with their correct (lack of) capitalization. Lists of viruses within a given species are provisional at this point and will likely be amended in the near future



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## Summary

A summary of the current, ICTV-accepted taxonomy of the order *Mononegavirales* is presented in Table 1.

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## Compliance with ethical standards

The views and conclusions contained in this document are those of the authors and should not be interpreted as necessarily representing the official policies, either expressed or implied, of the US Department of the Army, the US Department of Defense, the US Department of Health and Human Services, the Department of Homeland Security (DHS) Science and Technology Directorate (S&T), or of the institutions and companies affiliated with the authors. In no event shall any of these entities have any responsibility or liability for any use, misuse, inability to use, or reliance upon the information contained herein. The US departments do not endorse any products or commercial services mentioned in this publication.

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| AQ2 | Please check and confirm that the authors and their respective affiliations have been correctly identified and amend if necessary. | |
| AQ3 | Journal instruction requires a city for affiliations; however, this is missing in affiliations 37, 55, 56, 66. Please verify if the provided city and country are correct and amend if necessary. | |
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| AQ5 | Affiliations 61, 65 has been split into two different affiliations. Please check if action taken is appropriate and amend if necessary. | |
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**MATERIAL TRANSFER AND LIMITED USE AGREEMENT**

The World Reference Center for Emerging Viruses and Arboviruses ("WRCEVA"), through the University of Texas Medical Branch at Galveston, d/b/a UTMB Health ("UTMB"), a health institution of The University of Texas System ("System"), an agency of the state of Texas, located at 301 University, Galveston, TX 77555-0926, is willing to transfer the requested materials to you on the following conditions.

The parties to this Agreement are Institut Pasteur of Shanghai, Chinese Academy of Sciences, hereinafter "RECIPIENT", and UTMB. The research (as hereinafter defined) will be conducted by RECIPIENT under the supervision of Prof. Dr. Dimitri LAVILLETTE, hereinafter "SCIENTIST".

1. The Material that is covered by this Agreement includes molecular clone of Eilat virus EILV and EILV eRFP that will be provided by UTMB or received by RECIPIENT from UTMB, hereinafter "Material." For the purpose of this Agreement, "Material" means any material or portion thereof shipped to the RECIPIENT by WRCEVA. For the purpose of this Agreement, "Replicates" means any biological or chemical material that represents a substantially unmodified copy of the Material. Replicates include but are not limited to material produced by growth of cells or microorganisms or amplification of Material. For the purpose of this Agreement, "Derivative" means material created from the Material that is substantially modified to have new properties. Derivative includes, but is not limited to, recombinant DNA clones made using a vector. UTMB shall be free, in its sole discretion, to distribute the Material to others and to use it for its own purpose. Recipient shall not distribute, transfer, recreate for commercial use, or release the Material to any person or entity other than laboratory personnel under Recipient Scientist's direct supervision, unless written permission is obtained from UTMB. In addition, Recipient shall refer all requests for materials transferred hereunder to UTMB.

2. This Agreement applies to the use, handling, sale, distribution and any disposition of the Material, Replicates, and Derivatives.

3. Subject to the terms and conditions of this Agreement and any statutory, regulatory or other restriction imposed by law or any third party interest, the RECIPIENT may use the Material, Replicates, or Derivatives in any lawful manner for the purpose of scientific research.

4. RECIPIENT agrees that all of its SCIENTISTS who are involved in the Research, will have read the terms and conditions of this Agreement, and are obligated to abide by the terms and conditions of this Agreement as if each were a signatory hereto.

5. If a scientific disclosure or publication comes about, RECIPIENT agrees to acknowledge World Reference Center for Emerging Viruses and Arboviruses (WRCEVA), as academically and scientifically appropriate, based on provision of the Material or other direct contribution to the Research.



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6. RECIPIENT represents that within their laboratory (i) access to the Material, Replicates or Derivatives will be restricted to personnel capable and qualified to safely handle the Material, Replicates, or Derivatives and (ii) RECIPIENT shall exercise the utmost care, taking into account the unique characteristics of the Material, to maintain and use the Material, Replicates, or Derivatives and appropriate precautions to minimize any risk of harm to persons and property and to safeguard them from theft or misuse. RECIPIENT agrees that Material or Replicates designated Class II, III, or IV constitute known pathogens and that other Material, not so designated or Derivatives may be pathogenic under certain conditions.

7. RECIPIENT represents, that all information provided to WRCEVA in connection with this request is true, correct and complete, including, without limitation, any information provided for use in obtaining any license, permit or other authorization with respect to orders hereunder or otherwise complying with applicable law and regulations. RECIPIENT agrees to comply with all restrictions on export from the United States and re-export from other countries set forth in the export license and any other permit or authorization required by law for the Material, Replicates, and Derivatives and/or any information provided therewith or derived there from, and are responsible for obtaining any re-export authorization before transferring the Material, Replicates, or Derivatives provided hereunder or derived therefrom to another country or national thereof. With respect to transfers of Material, Replicates, or Derivatives to destinations outside the United States, (i) RECIPIENT assumes all risk and responsibility in connection with complying with applicable US and foreign laws and regulations concerning the import, handling, transportation, storage, use, and misuse or other wrongdoing with respect to Material, Replicates or Derivatives and (ii) RECIPIENT has advised WRCEVA, when placing its order, of any foreign legal or regulatory requirements pertaining to the requested shipment to be implemented within the United States in connection with such shipment.

8. **THE RECIPIENT RECOGNIZES THE POTENTIAL HAZARD OF UTILIZING THE MATERIAL AND UNDERSTANDS THAT THE APPROPRIATE PRECAUTIONS TO MINIMIZE ANY HEALTH RISK BECOMES FULLY THE RECIPIENT'S RESPONSIBILITY. THE RECIPIENT ASSUMES ALL RISK AND RESPONSIBILITY IN CONNECTION WITH THE RECEIPT, HANDLING, STORAGE, DISPOSAL, USE AND ANY MISUSE OR OTHER WRONGDOING WITH RESPECT TO MATERIAL TRANSFERRED HEREUNDER, THE REPLICATES, AND ANY DERIVATIVES.** In no event shall UTMB, including the UT System, UT Regents, officers, agents, employees and contributors of materials, nor the US government be liable for any use by SCIENTIST or RECIPIENT of the Material or any loss, claim, damage or liability, of whatsoever kind of nature, which may arise from or in connection with RECIPIENT's breach of this Agreement or RECIPIENT's use, handling or storage, including the receipt and/or improper, inappropriate, negligent or other wrongful handling or use of the Material, and/or from any misidentification, misrepresentation, lack of purity or typicality of the Material, unless such loss, claim, damage or liability results from the negligence of The University of Texas System, UTMB, their Regents, officers, agents and employees. Unless a US State or Federal agency, RECIPIENT agrees to



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indemnify and hold harmless The US Government, The University of Texas System, UTMB, their Regents, officers, agents and employees and the contributors of the Materials, from any liability, loss or damage they may suffer as a result of claims, demands, costs or judgments against them arising out of the RECIPIENT's activities to be carried out pursuant to this Agreement and the use by RECIPIENT of the results obtained from Research.

9. **RECIPIENT ACKNOWLEDGES, AGREES AND REPRESENTS THAT THE MATERIAL IS NOT INTENDED FOR USE IN HUMANS, AND AGREES NOT TO CONDUCT RESEARCH IN HUMANS USING THE MATERIAL.**

10. The Material is experimental in nature and it is provided WITHOUT WARRANTY OF MERCHANTABILITY OR FITNESS FOR A PARTICULAR PURPOSE OR SAFETY OR ANY OTHER WARRANTY, EXPRESS OR IMPLIED. UTMB MAKES NO REPRESENTATION OR WARRANTY THAT THE USE OF THE MATERIAL WILL NOT INFRINGE ANY PATENT OR OTHER PROPRIETARY RIGHT.

11. This Agreement is not assignable, whether by operation of law or otherwise, without the prior written consent of UTMB.

12. RECIPIENT may not use the name of UTMB, System or Board, or the US government without express written consent.

13. If any provision of this Agreement shall be found by a court to be void, invalid or unenforceable, the same shall be reformed to comply with applicable law or stricken if not so conformable, so as not to affect the validity or enforceability of this Agreement.

14. The following attachments are incorporated herein and made a part of this Agreement for all purposes:

    - Attachment A - List of all requested Materials and proposed scope of Research
    - Attachment B – list of all required permits to receive Materials and carry out Research.

By signing below the RECIPIENT hereby certifies that an appropriate plan is in place for managing all health and safety risks involved with receiving and handling the transferred Materials. The Authorized Official who cosigns must be someone who can legally bind your institution, such as a president, vice-president, dean, provost, or corporate officer. A department chairman cannot serve as a cosigner for this form. This agreement is effective as of the date of the last signature below:

**RECIPIENT Organization:** Institut Pasteur of Shanghai, Chinese Academy of Sciences

RECIPIENT Address:

Life Science Builing Rm B0809

MTA from WRCEVA



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No.320 Yueyang Road

Materials will be shipped to the following address:

Life Science Builing Rm.B0809

No.320 Yueyang Road

Shanghai, PR China, 200031

**Shipping Contact: _Ruoyu ZHOU**

Email: ryzhou@ips.ac.cn>

Telephone: _ 18565215940

Name of Authorized Official: _Pr Lubin JIANG

Title of Authorized Official: ___Deputy Director, Institut Pasteur of Shanghai

Signature of Authorized Official:

Date:

ACKNOWLEDGED BY:

RECIPIENT Scientist Name: ___ Prof. Dr. Dimitri Lavillette _____

RECIPIENT Scientist Signature: _____

Name of the RECIPIENT'S Biosafety/Compliance Officer: __Pr Jian LUBIN

Signature of RECIPIENT'S Biosafety/Compliance Officer::

**The University of Texas Medical Branch**

301 University Blvd., Galveston, TX 77555-0609

Name of Authorized Official: Carolee A. King, JD

Title of Authorized Official: Sr. VP and General Counsel

Signature of Authorized Official: _____

Date: _____

MTA from WRCEVA



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ACKNOWLEDGED BY:

UTMB Scientist: _____

UTMB Scientist Signature: _____

Name of the Biosafety/Compliance Officer: Anne-Sophie Brocard, Ph.D.

Signature of Biosafety/Compliance Officer: _____



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## Attachment A

**Materials Requested**

Molecular clones of Eilat virus EILV and EILV eRFP_



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**Scope of Work**

*Please write in 500 words, or less, the Scope of Work*

The project aims at studying EILAT virus - host interaction and Flavivirus-alphavirus interaction. We will derive mutated viruses and replicon to analyze different phenotypes.



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## Attachment B

*Please list all required permits to receive Materials and carry out the Research.*

The Eilat virus derived from the molecular clone need to be manipulated in BSL2. It will be rescued in Biosafety level 2+ laboratory in Institut Pasteur of Shanghai, China.



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**U.S. China Dialogue and Workshop on the Challenges of Emerging Infections, Laboratory Safety, Global Health Security and Responsible Conduct in the Use of Gene Editing in Viral Infectious Disease Research**

Harbin Veterinary Research Institute | Chinese Academy of Agricultural Sciences
January 8-10, 2019
Harbin, China

**Trip Report**

To address the interconnected life science challenges associated with combating infectious diseases and further enhance cooperation between China and the United States in the areas of global health, biosafety, and biosecurity, the U.S. National Academy of Sciences (NAS), and the Chinese Academy of Agricultural Sciences (CAAS) in partnership with representatives from other Chinese institutions held the fourth *U.S. China Dialogue on the Challenges of Emerging Infections, Laboratory Safety and Global Health Security* and a *Workshop on Responsible Conduct in the Use of Gene Editing in Pathogen Research* in tandem on January 8-10, 2019 at the Harbin Veterinary Research Institute (HVRI) in Harbin, China. The event was held in the conference facility of the HVRI of the Chinese Academy of Agricultural Sciences, one of three institutes in China that currently operate Biological Safety Level-4 (BSL-4) laboratories.

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The meeting, ultimately titled *U.S. China Dialogue and Workshop on the Challenges of Emerging Infections, Laboratory Safety, Global Health Security and Responsible Conduct in the Use of Gene Editing in Viral Infectious Disease Research* was the latest in a series designed to share information on research relevant to combating emerging diseases of concern to the U.S. and China, promote common understanding of high biological containment laboratory safety, security and responsible research risk management, and build relationships among members of the U.S. and Chinese research communities who conduct infectious disease research.

The meeting was also designed to have a special focus on research that uses gene-editing technology to allow participants to discuss ways to create norms and reduce risks when using these tools for research. (The co-chairs of the meeting will produce a short summary of this portion of the workshop discussion.)

Several themes emerged during the workshop. The first was a focus on the importance of pursuing an integrated, One Health-based approach to understanding the causes and addressing the effects of animal and human diseases. Some of the issues raised included the short-term intended and unintended effects of recent disease control strategies, as well as a need for collaboration amongst experts and across regions on medical, ecological and ecosystems science to ensure human and animal health.

The second was a growing appreciation for the widespread use of gene editing tools, particularly those based on the CRISPR-Cas9 system, that are being integrated into many new research



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efforts. New gene editing techniques to cut, edit, activate and repress genes with greater accuracy and at much lower costs well beyond longstanding methods of selective breeding of hosts and pathogens allow researchers to quickly explore changes not readily achieved in the past. Although the benefits to society are potentially enormous, there is the potential for careless or even malevolent application thus for the technology to contribute to dangerous "bioerror"; that is, for researchers to create a modified organism that has an unintended disastrous impact. Many of the technical talks including those on vaccine development, vector engineering, host engineering, and synthetic biology included details on the researchers' use of gene editing tools and techniques. During the presentations the importance of gene editing to facilitate genetic modifications was highlighted but participants also discussed how they assessed risks and potential untended consequence of using the tools and best practices to ensure the appropriate and safe use of this technology in the future.

A third theme was the importance of establishing and maintaining strong institutional cultures and norms to support the safe and responsible conduct of science. Although the meeting agenda was not designed to focus on human gene editing, the research project led by He Jiankui formally an associate professor in the Department of Biology of the Southern University of Science and Technology in Shenzhen, China that lead to the birth of two children with edited DNA was widely condemned by the meeting participants. The sessions included several discussions on possible collaborations between Chinese and American scientists and institutions on the responsible conduct of science and other ways to ensure that gene editing techniques are used safely and ethically across the life science enterprise in the future.

Throughout the workshop and during tours of the HVRI security and training centers, experts also shared information and practices regarding the safe and secure operation of high containment biological laboratories. Topics discussed included combating emerging infectious diseases in livestock, safely doing research on insect vectors in high biological containment, inactivation/decontamination procedures for African swine fever virus, improving the public health response to disease outbreaks, training and vetting high containment laboratory workers, and the safe and sustainable operation of high containment laboratories generally.

NAS has helped to shape the international dialogue on the risks and the multitude of challenges associated with planning for, building, sustaining, and safely and securely running high containment laboratories. Before the meeting the directors of the Galveston National Laboratory (of the University of Texas Medical Branch) and the Wuhan Institute of Virology (of the Chinese Academy of Sciences) jointly published an editorial in Science that calls for the creation of a global network to bring together the leaders of institutes that operate laboratories at the highest biological safety level, BSL-4, to ensure the safe, secure and sustainable operation of these valuable facilities.[1] The final session of the meeting included a discussion on the concept led by

[1] "One solution is to establish an [BSL-4] network that enables the sharing of best practices, collaboration, transparency, and exchange of specimens and technology." The authors noted that their partnership "benefited from meetings jointly sponsored by the U.S. National Academy of Sciences and the Chinese National Academy of Sciences…" Network for Safe and Secure Labs. James W. Le Duc and Zhiming Yuan, Science. 19 Oct 2018. Vol. 362, Issue 6412, pp. 267 http://science.sciencemag.org/content/362/6412/267.



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the authors of the editorial. During the discussion the director of China's Centers for Disease Control and the leadership of the Chinese institutes that operate China's three BSL-4 labs indicated that they support the idea of building an even broader network and will work with NAS to develop and create a network; they have started planning an international meeting of laboratory leaders to take place later in 2019. The next bilateral meeting will likely take place in Kunming, China at the Kunming National Primate Research Center, another Chinese institute that operates a BSL-4 laboratory, later in 2019.

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**Sessions and Presentations**

**Keynote addresses**

- Oversight for Research on Viral Pathogens with Pandemic Potential – the Perspective from China
- Emerging Swine Coronaviruses and their Interspecies Transmission
- Vaccination in Poultry Eliminated Human Infections with H7N9 Virus in China

**Influenza**

- Collaborating on Influenza Research from the U.S. perspective
- Human Infection with Zoonotic Influenza
- Interactions between Influenza Viral RNP Complex Proteins and Host Cellular Factors

**African swine fever**

- Current Situation and Control Strategy of African Swine Fever in China
- Characterization of African Swine Fever Virus Isolated from China
- ASFV Vaccine Status: Rapid Production of Recombinant ASFV for Live Attenuated Vaccines
- Genome-edited Pigs Protected From Classical Swine Fever Virus

**Understanding and engineering viral pathogens with pandemic potential**

- Advances in Understanding and Altering Pathogen Properties
- Cutting Edging New Biotechnology: Strategies and Tools for Engineering

**Bioethics and responsible planning for pathogen research**

- Biosafety and Bioethics in Medical Research
- Bioethical Considerations for the Conception and Design of pathogen Research and Approaches for Assessing Potential Risks and Benefits of Research
- Biosafety and Bioethics Considerations in Dual-Use Risks Management - China's Efforts and Future Works

**Vector and host engineering: safety and security**

- Biochemical Pathway of a Mosquito Innate Immunity, Melanization
- Best Practices in Safety and Security for Working with Arthropods that Serve as Pathogen Vectors, Including Responsible Research with Gene Drive Modified Vectors
- Vector Competence of Emerging Mosquito Borne Virus in China
- A Gut Commensal Bacterium Promotes Arbovirus Infection in Mosquitoes



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**Acute Flaccid Myelitis**

- Review of what is currently known about the cause of Acute Flaccid Myelitis

**Best practices in laboratory safety and security for pathogen research**

- Institutional Review and Oversight for Research on Viral Pathogens with Pandemic Potential - a U.S. Perspective
- The Role of Leaders in Laboratory Safety: Culture and governance to steer toward behaviorial changes
- Overview of Laboratory Biosafety Management System in China

**One Health in China**

- Understanding how a One Health Approach is Shaping Research to Understand and Address Viral Pathogens in China

**Chinese biosafety, biosecurity, and bioethics**

- Development and Application of Laboratory Biosafety Equipment in China
- Kunming National Primate Research Center of High-Level Biosafety

**Chinese perspectives on emerging infectious diseases**

- DDX19, A novel Viral RNA Sensor, Regulates IFN Signaling upon Viral Infection
- Risks of MERS-cluster Coronaviruses in China
- Rational Design of Master Seed Virus to Improve the Safety and Effectiveness of Foot and Mouth Disease Vaccine
- Development of Inactivated EV71 Vaccine (Human Diploid Cell) and Pathogenic Study of EV71 Infection in Rhesus Macaques
- Zika Virus Neurotropism: the Bad and the Good

**Special session (gene editing and synthetic biology)**

- Large Gene Cluster and Regulatory Manipulation for Activating the Cryptic Natural Product Clusters in Streotomvces
- Creating a Functional Single-chromosome Yeast

**China-U.S. Collaboration: Opportunities to Prevent Future Pandemics**



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**American Participants**

**Ralph Baric**, Professor
Department of Epidemiology, University of North Carolina, Chapel Hill

**David Franz**, Professor
USAMRIID (retired)

**Douglas Gladue**, Scientist
U.S. Department of Agriculture

**Diane Griffin**, Professor
Department of Molecular Microbiology and Immunology of Johns Hopkins Bloomberg School of Public Health

**Stephen Higgs,** Professor
Department of Diagnostic Medicine/Pathobiology, College of Veterinary Medicine, Kansas State University

**Joseph Kanabrocki**, Professor
Microbiology in the Biological Sciences Division of the University of Chicago

**James Le Duc**, Professor
Galveston National Laboratory, Department of Microbiology and Immunology, University of Texas Medical Branch, Galveston

**David Relman**, Professor
Department of Microbiology and Immunology, Stanford University School of Medicine

**Linda Saif**, Professor
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**Pei-Yong Shi**, Professor
Department of Biochemistry and Molecular Biology, University of Texas Medical Branch

**David Swayne**, Center Director
Agricultural Research Service, United States Department of Agriculture

**Katherine Bowman**, Senior Program Officer
National Academy of Sciences

**Benjamin Rusek**, Senior Program Officer
National Academy of Sciences

**Chinese Participants**

**George F. Gao**, Academician,
Director-General Chinese Center for Disease Control and Prevention

**Zhigao Bu**, Professor
Harbin Veterinary Research Institute, CAAS

**Wen Dang**, Doctor
Lanzhou Veterinary Research Institute, CAAS

**Wuxiang Guan**, Professor Wuhan Institute of Virology, CAS

**Yunzhang Hu**, Professor
Institute of Medical Biology, Chinese Academy of Medical Sciences

**Mifang Liang**, Professor
National Institute for Viral Disease Control and Prevention, China CDC

**Chunbo Lou**, Professor Institute of Microbiology, CAS

**Hongsheng Ouyang**, Professor
Jilin University



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**Hualan Chen**, Academician
Harbin Veterinary Research Institute, CAAS

**Gong Cheng**, Professor
Tsinghua University

**Rui Gong**, Professor
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**Qian Han**, Professor
Hainan University

**Chengjun Li**, Professor
Harbin Veterinary Research Institute, CAAS

**Longding Liu**, Professor
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**Jiahai Lu**, Professor
Sun Yat-sen University

**Jiancheng Qi**, Professor
National Biological Protection Engineering Center

**Zhengli Shi**, Professor
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**Kongming Wu**, Vice President
Chinese Academy of Agricultural Sciences

**Xiaodong Wu**, Professor
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**Yang Xue**, Associate Professor
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**Ruifu Yang**, Professor
Institute of Microbiology and Epidemiology, Academy of Military Medical Sciences

**Zhiming Yuan**, Professor
Wuhan Institute of Virology, CAS

**Peijun Zhai**, Professor
China National Accreditation Service for Conformity Assessment (CNAS)

**Pingping Zhang**, Doctor
Institute of Microbiology and Epidemiology
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**Tietao Zhang**, Professor
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**Weiwen Zhang**, Professor
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National Institute for Viral Disease Control and Prevention, China CDC

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**Haixue Zheng**, Professor
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# Genetic and biochemical characterizations of Zika virus NS2A protein

Xianwen Zhang[1,2,3], Xuping Xie[3,*], Jing Zou[3], Hongjie Xia[3], Chao Shan[3], Xinwen Chen[1,2,4,*], Pei-Yong Shi[3,5,6,7,8*]

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Lead contact: peshi@utmb.edu



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## Abstract

Zika virus (ZIKV) can cause devastating congenital Zika syndromes in pregnant women and Guillain-Barre syndrome in adults. Understanding the molecular mechanism of ZIKV replication is essential for antiviral and vaccine development. Here we report, for the first time, the structural and functional characterization of ZIKV NS2A protein. Biochemical structural probing suggests that ZIKV NS2A has a single segment that traverses the ER membrane and six segments that peripherally associate with the ER membrane. Functional analysis has defined distinct NS2A residues essential for viral RNA synthesis or virion assembly. Only the virion assembly-defective mutants, but not the RNA synthesis-defective mutants, could be rescued through *trans* complementation with a wide-type NS2A protein. These results suggest that the NS2A molecules in virion assembly complex could be recruited in *trans*, whereas the NS2A molecules in viral replication complex must be recruited in *cis*. Together with previous results, we propose a flavivirus assembly model where NS2A plays a central role in modulating viral structural and nonstructural proteins as well as genomic RNA during virion assembly.



**Keywords:** Zika virus; flavivirus replication; virion assembly; flavivirus NS2A; membrane topology





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## Introduction

Zika virus (ZIKV) is a recently emerged mosquito-borne flavivirus that is primarily transmitted by *Aedes spp.* mosquitoes. It can also be transmitted through sexual, maternal-to-fetal, and blood transfusion routes [1, 2, 3, 4, 5]. ZIKV was first identified in 1947 from a sentinel rhesus monkey in Uganda [6]. Since 2007, it emerged in the Pacific region, South America, Central America, and the Caribbean, posing a global public health threat [7, 8, 9, 10, 11]. ZIKV infection causes mild illnesses, including fever, pruritis, arthritis, conjunctivitis, and asthenia [12]. Importantly, it can cause devastating congenital Zika syndromes (CZS; including microcephaly, congenital malformation, and fetal demise) in pregnant women and Guillain-Barre syndrome (GBS) in adults [13, 14, 15, 16]. The explosive epidemics and its association with CZS had led the World Health Organization (WTO) to declare ZIKV as a Public Health Emergency of International Concern from February to November in 2016 [17]. Since then, a number of vaccine candidates have been developed, some of which have already advanced to clinical trials [18]. In addition, small molecule inhibitors and therapeutic antibodies have also been identified for antiviral development [19, 20, 21].

ZIKV belongs to the *Flavivirus* genus within the *Flaviviridae* family. Besides ZIKV, many other flaviviruses are significant human pathogens, including dengue (DENV), yellow fever (YFV), West Nile (WNV), Japanese encephalitis (JEV), Saint Louis encephalitis (SLEV), and tick-borne encephalitis virus (TBEV) [22]. Flavivirus genome



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58 is a positive-sense, single-stranded RNA of ~11,000 nucleotides. It contains a 5'
59 untranslated region (UTR), a long open-reading-frame (ORF), and a 3' UTR. The single
60 ORF encodes a polyprotein that is processed into three structure proteins (capsid [C],
61 pre-membrane [prM] and envelope [E]) and seven nonstructural proteins (NS1, NS2A,
62 NS2B, NS3, NS4A, NS4B, and NS5). The structural proteins, together with genomic
63 RNA, are components of virions. The nonstructural proteins participate in viral RNA
64 replication, virion assembly, and evasion of host immune response [23, 24, 25].

65 Flavivirus NS2A is an ER-resident, transmembrane protein with a calculated
66 molecular weight of ~22 kDa. The N-terminus and C-terminus of NS2A are formed by
67 cleavages with an unknown host protease and viral protease NS3 (in complex with
68 NS2B), respectively [26, 27]. Flavivirus NS2A has multiple functions: (i) Viral
69 replication. Kunjin virus (KUNV) NS2A co-localizes with viral double-stranded RNA
70 and interacts with the 3'UTR of genomic RNA, NS3, and NS5 protein [28]. Mutagenesis
71 studies confirmed the role of NS2A in viral RNA synthesis [29, 30]. (ii) Virion
72 assembly/secretion. Mutagenesis analyses of YFV, KUNV, and DENV have identified
73 distinct NS2A amino acids that are essential for virion assembly/secretion [29, 30, 31, 32,
74 33]. The virion assembly defects can be restored by second site mutations in NS2B or
75 NS3 in YFV and DENV [31, 34, 35]. (iii) Evasion of innate immunity. ZIKV NS2A
76 inhibits type-I IFN induction at the step of TBK1 [25]. DENV NS2A antagonizes type-I
77 IFN signaling [24]. JEV NS2A blocks dsRNA-activated protein kinase PKR [36]. In



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78 WNV and KUNV, the NS2A-mediated inhibition of type-I IFN induction can be
79 abrogated by specific NS2A mutations [37, 38]. (iv) Disease pathogenesis. WNV NS2A
80 modulates virus-induced cytopathic effect (CPE) and pathogenesis in mice [39, 40].
81 ZIKV NS2A impairs mammalian cortical neurogenesis through depleting adherens
82 junction proteins [41].

83 The structure and function of ZIKV NS2A have not been characterized. Although
84 NS2A proteins from different flaviviruses perform similar functions (described above),
85 each flavivirus may exert its NS2A functions in a virus-specific manner [34]. The goals
86 of this study are (i) to define the membrane topology of ZIKV NS2A and (ii) to
87 characterize its roles in viral RNA synthesis and virion assembly.

88

89 **Materials and methods**

90 ***Cell lines***

91 Vero cells, baby hamster kidney (BHK-21) cells and human embryo kidney 293T cells
92 were cultured at 37°C with 5% CO2 in high-glucose Dulbecco's modified Eagle medium
93 (DMEM; Life Technologies) supplemented with 2 mM L-glutamine, 100U/ml penicillin,
94 100g/ml streptomycin and 10% fetal bovine serum (FBS; HyClone Laboratories).

95 ***Bioinformatics***



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NS2A protein sequences from ZIKV, DENV1, DENV2, DENV3, DENV4, WNV, JEV, YFV, SLEV were aligned using CLC Main Workbench software (CLC Bio). Prediction of ZIKV NS2A was carried out using various web servers, which including TOPCONS, Philius, Polyphobius, SCAMPI, and TMHMM2. The amphipathic helices of ZIKV NS2A were predicted by HeliQuest.

***Antibody***

The following antibodies were used in this study: a mouse monoclonal antibody 4G2 cross-reactive with flavivirus E protein (American Type Culture Collection); Rabbit anti-HA monoclonal antibody (Cell Signaling Technology); Alexa Fluor®488 conjugated mouse anti-HA monoclonal antibody (Cell Signaling Technology); Rabbit anti-Calnexin polyclonal antibody(Abcam); Mouse anti-GFP monoclonal antibody (Sigma-Aldrich); Horseradish peroxidase(HRP) conjugated goat anti-mouse and anti-rabbit IgG polyclonal antibodies (Sigma-Aldrich); Alexa Fluor®488 conjugated goat anti-mouse IgG polyclonal antibody (Thermo Fisher Scientific); Alexa Fluor®568 conjugated goat anti-rabbit IgG polyclonal antibody (Thermo Fisher Scientific).

***Plasmid construction***

The Infectious cDNA clone (pFLZIKV) bearing the full-length genome of ZIKV (strain FSS13025) or Renilla luciferase (Rluc) reporter ZIKV replicon (pZIKV Rep WT) were used as the backbone for introducing NS2A mutations [42, 43]. XhoI and KpnI restriction



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115 sites were initially generated into pFLZIKV and pZIKV Rep WT replicon at positions
116 3018 and 5010 in ZIKV genome without changing amino acid sequences. The NS2A
117 alanine-scanning mutagenesis was performed by using a QuikChange II XL Site-Directed
118 Mutagenesis kit (Agilent Technologies) as described previously [29].

119 A mammalian expression vector pXJ with a cytomegalovirus (CMV) promoter was
120 used for constructing plasmids expressing NS2A and its derivatives. To construct the
121 pXJ-$E_{24}$-NS1-NS2A-HA plasmid, a gene cassette encoding the last 24 amino acids of E
122 protein ($E_{24}$), NS1 and NS2A with a C-terminal hemagglutinin (HA) tag was firstly
123 amplified from the pFLZIKV and cloned into the pXJ vector. Next, 35 NS2A mutations
124 were individually introduced into the pXJ-$E_{24}$-NS1-NS2A-HA by overlap PCR, resulting
125 in plasmids expressing NS2A mutants.

126 To construct the pXJ-SPG-$C_{16}$-NS2A-HA that was used for selecting cell line
127 constitutively expressing NS2A protein, a DNA fragment containing the Gaussia
128 luciferase signal peptide (SPG), the last 16 amino acids of NS1 protein ($C_{16}$) and NS2A
129 tagged with a C-terminal HA tag sequence were constructed by overlap PCR and cloned
130 into the pXJ vector via NotI/XhoI restriction sites.

131 Plasmid pXJ-eGFP was constructed by inserting the enhanced green fluorescence
132 protein (eGFP) sequence into the pXJ vector via BamHI/XhoI restriction sites. DNA
133 fragments that encode different NS2A segments were individually inserted into above
134 pXJ-eGFP vector, resulting various pXJ-smNS2A-eGFP plasmids (smNS2A represents



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NS2A segment). A DNA fragment that express NS2A or truncated NS2A and an eGFP with a C-terminal N-glycosylation acceptor site (Glyc) were amplified and cloned into the pXJ-SPG-$C_{16}$-NS2A-HA vector, resulting in the plasmid pXJ-SPG-$C_{16}$-NS2A-eGFP-Glyc and various pXJ-SPG-$C_{16}$-ctNS2A-eGFP-Glyc plasmids (ctNS2A represents C-terminal truncated NS2A).

***RNA in vitro transcription and electroporation***

All full-length cDNA clone plasmids and subgenomic replicons of ZIKV were linearized with restriction enzyme ClaI, followed by phenol-chloroform extraction and ethanol precipitation. RNA transcripts were prepared by using the T7 mMessage mMachine kit (Ambion). For RNA transfection, 10 μg *in vitro* transcribed RNA was electroporated into $8\times10^6$ cells (BHK-21 or Vero) by using Gene Pulser Xcell™ Electroporation Systems (Bio-rad) as described previously [29, 42].

***Immunofluorescence assay***

For indirect immunofluorescence assay (IFA), cells were seeded in an 8-well Nunc Lab-Tek II Chamber Slide (Thermo Fisher Scientific). At various time points, cells were washed three times with phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde in PBS at room temperature for 30 min and permeabilized with 0.1% Triton X-100 in PBS at room temperature for 10 min. The permeabilized cells were then washed three times with PBS and incubated in a blocking buffer containing 1% FBS in



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PBS at room temperature for 1h. E protein was stained with a 1:1000 dilution of mouse monoclonal antibody 4G2 in blocking buffer. After incubation for 1h, the cells were washed three times with a washing buffer (1% FBS and 0.5% Tween 20 in PBS). After 1 hour of incubation with a secondary antibody (Alexa Fluor®488 conjugated goat anti-mouse IgG), the cells were washed three times with washing buffer and counterstained with DAPI (4',6-diamidino-2-phenylindole). Images were acquired using an Olympus fluorescence microscope. Images were processed in ImageJ software (National Institutes of Health, MD).

***Plaque assay***

$2\times10^5$ Vero cells per well were grown in 24-well plates one day prior to viral infection. 10-fold serial dilutions of virus samples were prepared in DMEM containing 2% FBS and 1% penicillin/streptomycin and inoculated (100 µl) into each well of 24-well plates. After 1 h of incubation at 37°C with 5% $CO_2$, the inoculum was replaced with 500 µl overlay medium (DMEM medium containing 0.8% methyl-cellulose, 2% FBS and 1% penicillin/streptomycin). Plaques formed after incubation at 37°C with 5% $CO_2$ for 4-5 days. Cells were fixed with 4% paraformaldehyde for 30 min, followed by staining with 1% crystal violet. The numbers of plaque per milliliter (PFU/ml) of ZIKV were calculated.

***Replicon transient-transfection assay***

Briefly, $8\times10^6$ Vero cells were electroporated with 10 µg wild type or mutant Rluc-ZIKV



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replicon RNAs. After electroporation, $3\times10^5$ cells per well were seeded in 12-well plates. At given time points, the cells were washed twice with PBS and lysed in 200 μl of luciferase lysis buffer (Promega). The luciferase activity assay was performed according to the manufacturer's protocol.

***RNA extraction and cDNA sequencing***

The culture fluids from infected cells were centrifuged at 1000 g for 10 min and then filtered through a 0.22 μm pore size polyethersulfone membrane (Millipore) to remove cellular debris. Viral RNAs in the supernatants were extracted using a QIAamp viral-RNA Minikit (Qiagen) by following the manufacturer's protocol. Reverse transcription PCR (RT-PCR) was performed by using the SuperScript® III One-Step RT-PCR System (Thermo Fisher Scientific). The amplified cDNA fragments were purified and sent for Sanger sequencing at the GENEWIZ facilities.

***Selection of NS2A-HA BHK-21 cell line***

$4\times10^5$ BHK-21 cells per well of were seeded into a 6-well plate. The plasmid pXJ-SPG-$C_{16}$-NS2A-HA that contains a neomycin resistance gene was transfected into BHK-21 cells (4 μg per well) using X-tremeGENE 9 DNA transfection reagent (Roche). At 24h post-transfection, geneticin (G418) was added to the culture medium with a concentration of 1 mg/ml. The medium was changed every three days. After two weeks, several neomycin-resistant individual colonies were selected for propagation. The



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192 expression of NS2A-HA protein in stable cell lines was analyzed by immunofluorescence
193 assay and Western blot.

194 ***In vitro proteinase K protection assay***

195 Proteinase K protection assay was performed as previously described with minor
196 modifications [44, 45]. BHK-21 cells were transfected in 6-well plates with various
197 pXJ-SPG-$C_{16}$-ctNS2A-eGFP-Glyc plasmids. After incubated at 30°C for 24h, cells were
198 trypsinized, washed twice with PBS and centrifuged at 450×g for 5 min. The cell pellets
199 were resuspended in a hypotonic lysis buffer (10 mM Tris-HCl, pH 7.5, and 2 mM $MgCl_2$)
200 and kept on ice for 10 min. Subsequently, the samples were homogenized with 40 strokes
201 and centrifuged at 1,000×g for 5 min. The postnuclear supernatants (PNS) were divided
202 into three aliquots and centrifuged at 15,000×rpm for 40 min. The pellets from three
203 aliquots were then resuspended in PBS, 60 μg/ml proteinase K in PBS, or 60 μg/ml
204 proteinase K in PBS in the presence of 0.5% Triton X-100, respectively. The samples
205 were incubated on ice for 90 min. 20 mM phenylmethylsulfonyl fluoride (PMSF) was
206 finally added to terminate the reaction. After further incubation on ice for 15 min, the
207 samples were mixed with 4× Laemmli sample buffer, heated at 95°C for 15 min and
208 analyzed by Western blotting.

209 ***In vitro deglycosylation assay***

210 The samples were prepared by following a previously described protocol [45, 46]. Briefly,



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211 the transfected BHK-21 cells in 6-well plates were incubated at 30°C for 24 h. Cells were
212 trypsinized and suspended in DMEM medium containing 10%FBS. The cells were
213 pelleted by centrifuging at 450×g for 5 min. After two PBS washes, the cell pellets were
214 resuspended in a solubilization buffer (20 mM Tris-HCl, pH 7.5, 50 mM NaCl, 10 mM
215 $Mg(CH_3COO)_2$, 1% Triton X-100, and EDTA-free protease inhibitors [Roche]) and
216 incubated at 4°C for 2 h with gentle agitation. After centrifugation at 15,000 rpm 4°C for
217 20 min to remove cell debris, the supernatants were split into two aliquots. 50,000
218 units/ml N-glycosidase F (PNGase F) or PBS (no-treatment control) were added to those
219 two aliquots. After incubation at 37°C for 2 h, the samples were mixed with 4× Laemmli
220 sample buffer, heated at 70°C for 15 min and analyzed by Western blot.

221 ***SDS-PAGE and western blot***

222 Protein samples were separated by 12% SDS-polyacrylamide gel electrophoresis and
223 transferred onto a polyvinylidene difluoride (PVDF) membrane using Trans-Blot Turbo
224 transfer system (Bio-Rad), after which the membrane was blocked in TBST (10 mM
225 Tris-HCl, pH 8.0, 150 mM NaCl, and 0.1% Tween 20) supplemented with 5% nonfat
226 milk for 1h at room temperature. The blots were probed with primary antibodies (1:1,000
227 dilution) overnight at 4°C, washed three times with TBST and incubated with horseradish
228 peroxidase (HRP) conjugated goat anti-mouse and anti-rabbit IgG polyclonal antibodies
229 (1:10,000 dilution) for 1h. After three thorough TBST buffer washes, the blots were
230 incubated with SuperSignal™ West Femto Maximum Sensitivity Substrate (Thermo



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231 Fisher Scientific). Chemiluminescence signals were detected in ChemiDoc System
232 (Bio-Rad).

233 ***Quantification and statistical analysis***

234 IFA images processing and cell counting were performed in software ImageJ (NIH).
235 Densitometry analysis was performed using Image lab version 6.0 (Bio-Rad Laboratories,
236 Hercules, CA). All numerical data are presented as the mean ± SEM (standard error of
237 mean). The size of each study or number of replicates, along with the statistical tests
238 performed can be found in corresponding Figure Legends.

239

240 **Results**

241 ***The rationale for ZIKV NS2A mutagenesis***

242 To identify critical residues in ZIKV NS2A, we performed a systematic alanine-scanning
243 mutagenesis using an infectious cDNA clone of ZIKV strain FSS13025 [42]. Sequence
244 alignment of NS2A proteins from ZIKV, DENV, WNV, JEV, SLEV, and YFV revealed a
245 high diversity: only 31 out of 218-227 amino acids with a consensus of >80% among
246 different flaviviruses (Fig. 1A). A total of 35 residues were selected for mutagenesis: (i)
247 All 8 identical non-alanine residues, including G12, G47, D53, G71, F83, P87, E103, and
248 K193. The amino acid numbering is based on the ZIKV sequence. (ii) All charged



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249 residues, including D4, D7, E22, K31, K56, E67, D73, H76, K84, R86, R96, R102, E122,
250 D124, R140, R146, D148, R163, R171, K186, K188, K192, R207, D210, and R222.
251 Charged residues were selected because they likely participate in inter- or intra-molecular
252 interactions. (iii) A K25/K26/R27 motif that was previously reported to be critical for
253 YFV and DENV virion assembly (32, 42). (iv) Residue N130 that was previously shown
254 to be required for NS1-NS2A cleavage and viral replication in DENV [26, 29]. The
255 selected residues were mutated to alanine in the context of ZIKV genomic RNA. Equal
256 amounts of wild-type (WT) and mutant RNAs were electroporated into Vero cells. After
257 electroporation, RNA replication and virus spread were monitored by
258 immunofluorescence assay (IFA); the production of infectious viruses was quantified by
259 plaque assay and RT-PCR. The 35 mutants exhibited distinct phenotypes that could be
260 categorized into five groups (Fig. 1B), as detailed in the following sections.

261 ***Class I mutations have no or weak effects on viral replication***

262 Fourteen of the 35 NS2A mutant RNAs, including D4A, E22A, K31A, G47A, D53A,
263 E67A, H76A, E122A, R146A, D148A, R163A, K186A, K188A, and D210A, replicated
264 at levels comparable to the WT RNA. These mutants were categorized as class I (Fig. 1B).
265 The IFA images and plaque morphologies of three representatives (D53A, D148A, and
266 R163A), as well as the WT, are shown in Fig. 2A and 2B, respectively. Most class I
267 mutants (*e.g.*, D148A and R163A) yielded comparable E-expressing cells as the WT from
268 24 to 48 h post-transfection (p.t.). At 72 h p.t., severe CPE occurred among D148A,



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269 R163A, and WT RNA-transfected cells. Some class I mutants (*e.g.*, D53A) produced
270 fewer E-positive cells than the WT at 48 h p.t., and spread to infect more cells at 72 h p.t
271 (Fig. 2A). All class I mutants produced viruses with plaques similar to the WT virus (Fig.
272 2B). At 72 h p.t., the class I mutants produced infectious titers <4-fold lower than the WT
273 did (Fig. 2C). Sequencing of the mutant viruses (collected at 72 h p.t.) confirmed the
274 retaining of the engineered mutations without other changes (data not shown).

275 ***Class II mutations significantly attenuate viral replication***

276 Nine NS2A mutants, including K56A, F83A, K84A, R86A, P87A, R96A, R102A,
277 D124A, and R207A, significantly attenuated viral replication. These mutants were
278 categorized into class II (Fig. 1B). Class II RNA-transfected cells produced fewer
279 E-positive cells than the WT did (Fig. 2D). At 72 h p.t., only 30-50% of E-positive cells
280 were detected in the class II RNA-transfected cells, whereas the WT RNA-transfected
281 cells exhibited CPE. Class II mutant viruses formed smaller, opaque plaques (Fig. 2E),
282 and the transfected cells produced >10-fold lower infectious titers than the WT
283 RNA-transfected cells at 72 h p.t. (Fig. 2F). Complete genome sequencing of the class II
284 mutant viruses (collected at 96 h p.t.) did not reveal reversions or any other mutations
285 (data not shown). Because of the weak phenotypes of class I and II mutants, we did not to
286 further characterize them.

287 ***Class III mutations are lethal for viral RNA synthesis***



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288 Class III includes two mutants, G12A and N130A, which were completely defective in
289 viral RNA synthesis (Fig. 1B). The G12A and N130A genomic RNA-transfected cells did
290 not produce any E-positive cells at 24 and 48 h p.t. (Fig. 2G). On day 5 to 8 p.t., no
291 infectious viruses were detected from culture medium by plaque assay (Fig. 2H) and
292 RT-PCR (Fig. 2I). To confirm the above results, we introduced G12A or N130A mutation
293 into a ZIKV luciferase replicon (Fig. 2J). A replication-defective replicon, containing an
294 NS5 polymerase active site ΔGDD mutation, was included as a negative control [43].
295 Vero cells were electroporated with equal amounts of WT, G12A, N130A, or NS5 ΔGDD
296 replicon RNAs. At 2-6 h p.t., all replicons generated similar levels of luciferase signals,
297 suggesting comparable levels of viral RNA translation and transfection efficiencies (Fig.
298 2K). However, at 18-30 h p.t., G12A and N130A replicons generated low luciferase
299 signals as the non-replicative NS5 ΔGDD replicon did, whereas the WT replicon
300 produced increasing luciferase signals (Fig. 2K). These results demonstrate that class III
301 mutations completely abolish viral RNA synthesis.

302 ***Class IV mutations impair both viral RNA synthesis and virion production***

303 Class IV includes three mutants, including D7A, R140A, and R222A (Fig. 1B). These
304 mutant genomic RNAs produced fewer E-positive cells in the transfected cells than the
305 WT RNA (Fig. 3A). For D7A or R140A mutants, the RNA-transfected cells did not
306 produce infectious viruses, as suggested by plaque assay (Fig. 3B) and RT-PCR (Fig. 3C).
307 Incubation of naïve Vero cells with culture supernatants from the D7A or R140A



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308 transfected cells did not generate any viral E-positive cells (Fig. 3D). Replicon analysis
309 showed that D7A or R140A significantly compromised viral RNA synthesis (Fig. 3E).
310 Since neither mutants could produce extracellular viruses (Fig. 3B&C), and could
311 replicate at attenuated levels inside cells (Fig. 3E), D7A and R140A mutations may
312 impair both viral RNA synthesis and virion production.

313 For R222A, the number of E-positive cells slightly increased in the genomic
314 RNA-transfected cells from 24 to 72 h p.t. (Fig. 3A). A low level of infectious virus was
315 detected from the culture medium by plaque assay (Fig. 3B) and RT-PCR (Fig. 3C).
316 These data were supported by the positive-IFA results after incubating naive Vero cells
317 with the supernatant from the R222A RNA-transfected cells (Fig. 3D). Sequencing the
318 virus (collected at 72 h p.t.) revealed that, besides the engineered R222A mutation, the
319 virus had acquired a second site mutation of L220F in NS2A (Fig. 3C). Replicon analysis
320 showed that R222A significantly lowered viral RNA synthesis (Fig. 3E); no secondary
321 mutation (*i.e.*, L220F) was detected in the replicon RNA at 30 h p.t. (data not shown).
322 These results suggest that (i) R222A mutant is defective in viral RNA synthesis and/or
323 virion assembly, and (ii) a second site mutation L220F in NS2A could rescue the defects.

324 ***Class III and IV mutations do not affect NS2A maturation***

325 Since cleavage between NS1 and NS2A is essential for flavivirus RNA synthesis [29], we
326 examined whether the replication defects of class III and IV mutants were caused by
327 defective maturation of NS2A. To test this hypothesis, we introduced individual



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mutations into an expression plasmid pXJ-$E_{24}$-NS1-NS2A-HA that encodes a polyprotein containing the C-terminal 24 amino acids of the E protein (serving as a signal peptide to translocate NS1 to ER lumen), NS1, and NS2A with a C-terminal HA tag. After transfecting the plasmids into 293T cells, the expression of un-cleaved NS1-NS2A-HA intermediate and cleaved NS2A-HA product were analyzed using Western blot (Fig. 3F). None of the mutations significantly affected the cleavage efficiencies of NS1-NS2A-HA to NS2A-HA, indicating that the replication defects of class III and IV mutants are not caused by impaired NS2A cleavage.

***Class V mutations block virion assembly***

Class V includes G71A, D73A, E103A, R171A, K192A, K193A, and a triple K25A/K26A/R27A. Cells transfected with class V mutant genomic RNAs yielded E-positive cells at 24 h p.t., but the number of E-positive cells did not increase afterward (Fig. 4A), suggesting that the mutants are defective in virus spread. No infectious mutant viruses, except E103A, were detected in the supernatants of transfected cells by plaque assay (Fig. 4B) or RT-PCR (Fig. 4C). No IFA-positive cells were detected after naïve cells were incubated with the supernatants from transfected cells (Fig. 4D). Sequencing of the recovered E103A mutant virus revealed an A103D change in the NS2A gene (Fig. 4C), suggesting that an E103A-to-A103D substitution is responsible for the rescue of viral replication.

To determine the stage of viral replication that was disrupted by the class V



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mutations, we analyzed the mutational effect on viral replication in ZIKV replicon (Fig. 4E). Compared with the WT replicon, all class V mutants exhibited equivalent levels of viral translation and RNA synthesis (<4-fold difference), suggesting that the mutations only marginally affect viral RNA synthesis. Next, we tested if the class V genomic RNAs could generate intracellular infectious viruses. Incubating naïve Vero cells with WT RNA-transfected cell extracts yielded IFA-positive cells, whereas no IFA-positive cells were detected after incubating naive cells with the mutant RNA-transfected cell extracts (Fig. 4F). Altogether, the results suggest that class V mutants are competent in viral RNA replication, but defective in assembly of infectious viruses.

***WT NS2A rescues virion assembly for class V mutants***

To test *trans* complementation of NS2A mutants, we constructed a BHK-21 cell line that constitutively expressed WT ZIKV NS2A protein with a C-terminal HA-tagged (NS2A-HA). The cell line was constructed by transfecting plasmid pXJ-SPG-$C_{16}$-NS2A-HA encoding a Gaussia luciferase signal peptide (SPG), the C-terminal 16 amino acids of NS1 protein ($C_{16}$), and NS2A-HA, as well as a neomycin resistance gene (*Neo*). The SPG and $C_{16}$ sequences were engineered to enable the correct formation of NS2A membrane topology when expressed in cells. Individual BHK-NS2A-HA cell lines were generated through colony purification under G418 (1 mg/ml) selection. For each BHK-NS2A-HA cell line, all cells were IFA-positive for HA-NS2A expression (Fig. 5A). Western blot revealed an expected band of ~22-kDa



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band (Fig. 5B). The expression level of NS2A-HA did not decrease in the BHK-NS2A-HA cells after 20 passages in culture (4 days per passage; Fig. 5B), indicating the stability of the cell line.

We tested *trans* complementation by transfecting class V genomic RNAs to BHK-NS2A-HA cells. The transfected cells developed an increasing number of E-positive cells from 48 to 96 h p.t. (Fig. 5C). Culture supernatants were collected at 96 h p.t. to infect naïve Vero cells. The infected cells developed E-positive cells for all class V mutants (Fig. 5D), demonstrating the rescue of infectious viruses through *trans* complementation. Quantification of the E-positive cells allowed us to determine the titers of rescued infectious viruses (Fig. 5E). Among the class V mutants, E103A, K192A, and K193A exhibited the highest *trans* complementation efficiencies, producing $1.9\times10^5$ to $3.04\times10^5$ IFU/ml viruses at 96 h p.i. (Fig. 5E). Sequencing of the rescued virion genomes confirmed the retention of the engineered class V mutations without any other changes in NS2A (Fig. 5E).

Next, supernatants from the first round of infection collected at 72 h p.i. (from Fig. 5D) were used to infect naïve Vero cells for a second round. In contrast to the first round of infection (Fig. 5D), no E-positive cells were detected in the second round of infection for any of the class V mutants (Fig. 5F). As a positive control, WT RNA produced high numbers of E-positive cells in both the first and second rounds of infections (Fig. 5D&F). Collectively, the results indicate that (i) the defect in virion assembly of class V mutants



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388 could be rescued through *trans* supply of WT NS2A protein and (ii) the rescued virions
389 could only infect cells for a single round.

390 ***DENV-2 NS2A could not trans complement ZIKV class V mutants***

391 We tested whether DENV NS2A could rescue class V mutants of ZIKV. Each class V
392 mutant of ZIKV was transfected into a previously established BHK-21 cell line that
393 constitutively expresses WT DENV-2 NS2A protein [29]. For all class V mutants, no
394 increase in E-positive cells or infections viruses was detected from the transfected cells
395 from 24 to 96 h p.t. (data not shown). The results suggest that flavivirus NS2A modulates
396 virion assembly in a virus-type specific manner.

397 ***WT NS2A could not rescue viral RNA synthesis of class III mutants***

398 We tested whether the defect in viral RNA synthesis of class III mutants could be *trans*
399 complemented. To test this possibility, we transfected class III genomic RNAs (G12A and
400 N130A) into ZIKV BHK-NS2A-HA cells. Neither mutants generated any E-positive cells
401 in the transfected BHK-NS2A-HA cells (Fig. 5C). Infection of naïve Vero cells with
402 supernatants of the transfected cells did not produce any IFA-positive cells (Fig. 5D-F).
403 The results suggest that the RNA synthesis defect of class III mutants could not be
404 rescued through *trans* complementation.

405 ***Probing the topology of ZIKV NS2A on the ER membrane***

406 The distinct functions of amino acids in viral RNA synthesis and virion assembly



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prompted us to probe the topology of ZIKV NS2A on ER membrane. Bioinformatics analysis suggests that ZIKV NS2A may form seven transmembrane segments (pTMS1-7; Fig. 6A). To examine whether each pTMS associates with the ER membrane, we constructed a panel of plasmids encoding full-length or various NS2A fragments with a C-terminally fused eGFP reporter (Fig. 6B). The length of each segment was slightly modified from the bioinformatic prediction to allow some flexibility between pTMS and eGFP. Upon transfection into Vero cells, eGFP alone (without any NS2A fragment) exhibited an even distribution of fluorescence throughout the cell, whereas the full-length NS2A-eGFP (construct 1–226) displayed fluorescence co-localized with calnexin (an ER membrane protein) in the perinuclear region (Fig. 6B), indicating that ZIKV NS2A is an integral membrane protein. Expression of seven fragments spanning each pTMS, represented by constructs 1-25 (pTMS1), 30-58 (pTMS2) , 74-97 (pTMS3), 101-123 (pTMS4), 125-145 (pTMS5), 150-185 (pTMS6), and 198-221 (pTMS7), exhibited fluorescence patterns that co-localized with calnexin in the perinuclear region (Fig. 6B). The results indicate that individual of the seven pTMSes is associated with the ER membrane. In contrast, expression of fragments between different pTMS, represented by constructs 55-73, 186-197, and 221-226, showed fluorescence throughout the cells (Fig. 6B), indicating that these fragments are not membrane-associated.

An *in vitro* deglycosylation analysis was performed to determine the orientation of each NS2A pTMS on the ER membrane. A panel of plasmids were prepared to express



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427 truncated NS2A proteins that were C-terminally fused with an eGFP followed by an
428 N-glycosylation acceptor peptide (Glyc; Fig. 7A). An SPG-$C_{16}$ peptide (Gaussia
429 luciferase signal peptide SPG and the C-terminal 16 amino acids of NS1) was fused to
430 the N terminus of each NS2A-eGFP-Glyc construct to ensure a proper translocation of
431 the expressed protein on the ER membrane. Western blot showed that, upon transfection
432 into BHK-21 cells, all constructs expressed fusion proteins with expected molecular
433 masses (Fig. 7B). Because N-glycosylation enzymes reside in the ER lumen, the
434 glycosylation status could be used to indicate the location of the C-terminus of each
435 NS2A-eGFP-Glyc protein (Fig. 7C). As a negative control, treatment of EGFP-Glyc
436 (without any viral sequence) with PNGase F (an enzyme that removes high mannose and
437 complex carbohydrates) did not change the mobility of the protein (Fig. 7D), indicating a
438 cytosol localization of EGFP-Glyc. As a positive control, PNGaseF treatment increased
439 the mobility of SPG-$C_{16}$-EGFP-Glyc (construct 0), confirming that the C-terminus of this
440 protein resides in the ER lumen. Among the NS2A constructs, PNGase F treatment
441 increased the mobility of fusion proteins 1-26 and 1-56, but not 1-103, 1-123, 1-147,
442 1-192, and 1-226 (Fig. 7D). The results suggest that the N-terminal 56 amino acids of
443 NS2A reside in the ER lumen, and the C-terminal region from residues 103 to 226 is
444 located in the cytosol.

445 Next, we performed an *in vitro* proteinase K protection assay [45] to validate the
446 above *in vitro* deglycosylation results. BHK-21 cells were transfected with the same



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447 constructs depicted in Fig. 7A. At 24 h p.t., membrane fractions from the transfected cells
448 were treated with proteinase K in the presence or absence of 0.5% Triton X-100 and
449 analyzed by Western blot using an eGFP antibody. In the absence of Triton X-100, the
450 eGFP residing in the cytosol would be susceptible to proteinase K digestion, whereas the
451 eGFP residing in the ER lumen would be protected from proteinase K digestion (Fig. 7E).
452 As shown in Fig. 7F, the eGFPs from constructs 0, 1-26, and 1-56 were protected from
453 protease K digestion, again indicating that the first 56 residues are located in the ER
454 lumen. In contrast, the eGFPs from constructs 1-103, 1-123, 1-147, 1-192, 1-226, and
455 control eGFP-Glyc were completely degraded by the protease K treatment (Fig. 7F),
456 supporting that the C-terminal region from residues 103 to 226 is located in the cytosol.
457 As expected, in the presence of Triton X-100, the eGFPs from all constructs were
458 degraded by proteinase K (Fig. 7F). The results of proteinase K assay are in full
459 agreement with those of deglycosylation assay.

460 ***A topology model for ZIKV NS2A***

461 The above biochemical results support a topology model for ZIKV NS2A on the ER
462 membrane (Fig. 8A): (i) The N-terminal region (including pTMS1 and pTMS2) resides in
463 the ER lumen; (ii) the region spanning residues 74 to 97 (pTMS3) traverses the ER
464 membrane in a lumen-to-cytosol direction; and (iii) the C-terminal region from residues
465 103 to 226 (including pTMS4 to pTMS7) is located in the cytosol side. Since pTMS1,
466 pTMS2, pTMS4, PTMS5, PTM6, and pTMS7 associate with ER membraned when



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467 expressed individually (Fig. 6B), they may interact with the ER membrane in a peripheral
468 manner. In support of this notion, informatics analyses using Jpred 4 and HeliQuest
469 programs suggest that amino acids 9-26 (pTMS1), 41-58 (pTMS2), 104-121 (pTMS4),
470 125-142 (pTMS5), 172-189 (pTMS6), and 203-220 (pTM7) may form amphipathic
471 helices with hydrophobic and hydrophilic surfaces (Fig. 8B), where the hydrophobic
472 surface may interact with the ER membrane.

473

474 **Discussion**

475 Flavivirus NS2A functions in viral RNA replication, virion assembly, evasion of innate
476 immune response, and disease pathogenesis in a virus-specific manner [34]. This is
477 evidenced by its high sequence diversity (Fig. 1A), distinct membrane topology [34, 45],
478 and association with different disease pathways [40, 41]. Thus, it is critical to perform a
479 systematic genetic and biochemical characterization of ZIKV NS2A to delineate its
480 structure and function. Our biochemical results showed that, unlike other flaviviruses,
481 ZIKV NS2A has only a single transmembrane segment that traverses the ER membrane,
482 the other six pTMSes are peripherally associated with the ER membrane (Fig. 8A). Our
483 functional analysis revealed five classes of mutants with distinct replication phenotypes,
484 among which class III, IV, and V mutants could not produce infectious viruses through
485 different mechanisms (Fig. 1B). The critical mutations from class III to V are not
486 clustered in specific regions of NS2A (Fig. 8A), suggesting that multiple domains of



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NS2A are essential for viral replication.

The membrane topology of ZIKV NS2A is in stark contrast with the topologies of other flavivirus NS2A proteins: DENV-2 NS2A has five membrane-traversing segments [45] and YFV NS2A has three membrane-traversing segments [34]. Interestingly, these topologically diverse flavivirus NS2A proteins are able to converge their functions in viral RNA replication, virion assembly, and evasion of immune response. High resolution, three-dimensional structures of flavivirus NS2A are needed to address this question. It should be noted that the membrane topology of NS2A presented here is in the absence of other viral proteins. In the context of viral replication, the NS2A protein is associated with various viral and cellular proteins that would modulate the structure of NS2A and perform biological functions.

Our mutagenesis study has identified distinct NS2A mutations that selectively abolish ZIKV RNA synthesis (class III) or virion assembly (class V), among which only the virion assembly-defective mutants could be rescued through *trans* complementation (Fig. 5). The results suggest that there are two species of NS2A molecules in ZIKV-infected cells. One NS2A species is located at the viral replication complex that could only be formed in *cis*; another NS2A species is located at virion assembly complex that could be formed in *trans* or *cis*. These results are in agreement with the previous studies from DENV and YFV NS2A [29, 34, 35]. Compared with the viral replication complex, the virion assembly complex is much less well defined for flaviviruses.



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507 Previously studies showed that second-site mutations in prM, E, NS2B, and NS3 could
508 rescue the defect of virion assembly caused by DENV-2 and YFV NS2A mutations,
509 suggesting that these viral proteins participate in virion assembly. Besides the genetic
510 evidence, the role of NS3 in virion assembly is also supported by (i) mutations in the NS3
511 helicase domain alone affect YFV assembly [47], (ii) the helicase domain of DENV or
512 YFV NS3 directly binds to NS2A [34, 35], (iii) the cleavage between C and prM is
513 tightly modulated by NS2B/NS3 protease and cellular signalase during YFV and Murray
514 Valey encephalitis virus assembly [48, 49, 50]. These data, together with the result that
515 recombinant NS2A of Kunjin binds to viral 3'UTR RNA [28], suggest a flavivirus
516 assembly model. During flavivirus assembly, the NS2A protein recruits NS2B/NS3
517 through binding to the NS3 helicase domain. Once the NS2A-NS2B/NS3 complex
518 reaches the virion assembly site, the NS3B/NS3 protease cleaves the C protein from the
519 C/prM/E polyprotein. The C protein assembles onto genomic RNA (presented by NS2A)
520 to form nucleocapsid, which is subsequently enveloped by prM/E complex to form
521 virions. Since flavivirus assembly has to be coupled to viral RNA replication [51], the
522 newly synthesized genomic RNA has to be transported from the replication site to the
523 virion assembly site [52], possibly through the NS2A protein.

524 The *trans* complemented class V mutants could potentially be used as vaccine
525 candidates, as recently reported for DENV-2 [35]. Since the *trans* complemented virions
526 are able to infect cells for only a single cycle, such vaccine platform is safer than the



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multi-round infectious live-attenuated vaccines [53, 54]. This is particularly important for ZIKV vaccine when immunizing immune-compromised individuals and pregnant women [55]. However, for development of class V mutants as a vaccine platform, the *trans* complementation efficiency needs to be improved to higher titers before they could be tested for *in vivo* efficacy.

In summary, we report the first genetic and biochemical characterization of ZIKV NS2A protein. The results indicate that different flavivirus NS2A proteins have distinct membrane topologies, but converge to function in viral RNA synthesis and virion assembly. Together with previous results, we propose a model for flavivirus assembly in which NS2A plays a central role in mediating viral structural and nonstructural proteins as well as genomic RNA during virion formation.

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## Declaration of interests

The authors declare no competing interests.



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762 **Figure legend**

763 ***Fig. 1. ZIKV NS2A mutagenesis.***

764 (A) Sequence alignment of NS2A proteins among ZIKV (strain FSS13025), DENV-1-4
765 serotypes, WNV, JEV, SLEV, and YFV using CLC Main Workbench software (CLC Bio).
766 Thirty-five amino acids were selected for mutagenesis: (i) All 8 identical non-alanine
767 residues (indicated by triangles), (ii) 25 charged residues (arrows), (iii) a K25/K26/R27
768 motif (line), (iv) residue N130 (circle). The consensus and conservation percentage are
769 indicated below each amino acids. (B) A summary of ZIKV NS2A mutagenesis results.
770 An infectious cDNA clone and a luciferase replicon of ZIKV strain FSS13025 were used
771 to analyze the function of each amino acid. Based on the replication phenotypes, the 35
772 mutants could be categorized into five classes as indicated.

773 ***Fig. 2. Characterization of class I, II, and III NS2A mutants.***

774 The results of representative mutants from class I (A-C), II (D-F), and III (G-K) are
775 shown. (A) Vero cells were electroporated with equal amounts of ZIKV wild-type (WT)
776 or class I mutant genomic RNAs. At indicated time points, the cells were fixed and
777 subjected to IFA. Viral E protein and nuclei were stained with antibody 4G2 (green) and
778 DAPI (blue), respectively. Cytopathic effect (CPE) is indicated at 72 h post-transfection.
779 (B) Plaque morphologies. Recombinant viruses were subjected to plaque assay. Plaques
780 were developed at day 4.5 post-infection. (C) Viral titers of class I mutants at 72 h



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781 post-transfection. The mean values from two independent experiments are shown. Error
782 bars indicate the standard deviations. The limit of detection (LOD) of plaque assay is 10
783 PFU/ml. (D) IFA of class II mutant RNA-transfected Vero cells. (E) Plaque morphology
784 of class II mutant viruses. (F) Viral titers of class II mutant viruses at 72 h
785 post-transfection. (G) IFA of class III mutant RNA-transfected Vero cells. (H) Plaque
786 morphologies of class III mutant viruses. Plaque assay was performed using culture
787 supernatants collected at day 3 (for WT) and day 5 (for G12A and N130A)
788 post-transfection. (I) RT-PCR analysis. At 5 day p.t., the mutant RNA-transfected cells
789 were washed twice with PBS to remove residual input RNAs. At day 8 p.t., the
790 supernatants of G12A and N130A RNA-transfected cells were subjected to RT-PCR. The
791 WT virus (collected at 72 h p.t.) was used as a positive control. (J) A ZIKV luciferase
792 replicon. $C_{38}$ and $E_{30}$ represent the first 38 amino acids of C protein and the last 30 amino
793 acids of E protein, respectively. *Renilla* luciferase: Rluc. Foot-and-mouse-disease virus
794 2A sequence: 2A. (K) Transient replicon assay. WT, G12A, N130A, and NS5ΔGDD
795 replicon RNAs were electroporated into Vero cells. Luciferase activities were measured
796 at indicated time points. An average of 3 experiments are presented. Bars represent
797 standard deviations.

798 ***Fig. 3. Characterization of class IV NS2A mutants.***

799 (A) IFA of transfected Vero cells. (B) Plaque morphologies of WT, D7A, R140A, and
800 R222A viruses. (C) Extracellular viral titers were measured at 24, 48, and 72 h p.t. using



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plaque assay. At 72 h p.t., extracellular viral RNAs were subjected RT-PCR analysis. The RT-PCR products were sequenced to confirm the engineered mutations and to detect potential second site mutation(s) in the NS2A gene. N.A., not applicable. (D) The supernatants of transfected cells were collected on day 8 p.t. to infect naïve Vero cells. At 24 h p.t., the cells were analyzed for viral E expression by IFA. (E) Transient luciferase replicon analysis. Vero cells were electroporated with ZIKV replicon RNAs and measured for luciferase activities at indicated time points. An average of 3 experiments are presented. Bars represent standard deviations. (F) Analysis of NS2A proteolytic processes. NS2A D7A, G12A, N130A, R140A, and R222A mutations were individually engineered into expression plasmid pXJ-$E_{24}$-NS1-NS2A-HA. After transfecting the plasmids into 293T cells, total cellular protein samples were harvested at 24 h p.t. and analyzed for NS2A process using Western blot. An HA-tag antibody was used to detect both unprocessed NS1-NS2A-HA and processed NS2A-HA proteins.

***Fig. 4. Characterization of class V NS2A mutants.***

(A) IFA of Vero cells that were transfected with WT or class V genomic RNAs. Viral E-positive cells are indicated in green. (B) Plaque morphologies of class V mutant viruses. Only the WT and E103A mutant generated plaques. (C) Supernatants from the RNA-transfected cells were quantified for viral titers by plaque assay. RT-PCR was also performed on the supernatants at day 3 p.t. for the WT virus and at day 8 p.t. for the mutants. Positive RT-PCR products were sequenced for potential second site mutation(s).



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821 N.A., not applicable. (D) Naïve Vero cells were infected with supernatants from the
822 RNA-transfected cells and subjected to IFA for viral E protein expression. (E) Luciferase
823 replicon analysis. Equal amounts of WT and mutant replicon RNAs were electroporated
824 into Vero cells. Luciferase activities were measured at the indicated time points. (F)
825 Analysis of intracellular infectious viruses. Intracellular viruses from ZIKV
826 RNA-transfected cells were harvested at 24 h p.t. as previously described [45]. The
827 harvested samples were used to infect naïve Vero cells. IFA was performed to examine
828 viral E protein expression at 24 h post-infection.

829 ***Fig. 5. Trans complementation of class III and V mutants.***

830 (A) IFA of BHK-NS2A-HA cells. The BHK-NS2A-HA cells constitutively express WT
831 ZIKV NS2A fused with a C-terminal HA-tag. The cells were fixed with
832 paraformaldehyde and stained with an HA-tag antibody. (B) Western blot of
833 BHK-NS2A-HA cells. The expression of WT NS2A-HA from BHK-NS2A-HA cells was
834 detected using an HA-tag antibody. Both passage one (P1) and 20 (P20) BHK-NS2A-HA
835 cells were analyzed. Naïve BHK-21 cells were included as a negative control. (C) WT
836 and class III and V genomic RNAs were electroporated into BHK-NS2A-HA cells. IFA
837 was performed on the transfected cells to detect viral E protein expression. (D) Viruses
838 rescued from the BHK-NS2A-HA cells (harvested at 96 h p.t.) were used to infect naïve
839 Vero cells. The infected Vero cells were subjected to IFA for E protein expression. (E)
840 Extracellular viruses were harvested from the supernatants of RNA-transfected



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841 BHK-NS2A-HA cells. The harvested viruses were serially diluted to infect naïve Vero
842 cells in 96-well plates. At 24 h p.i., IFA was performed to quantify the viral titers by
843 counting the number of E-positive cells. The results show averages and standard
844 deviations from triplicate samples. Viral RNAs in the supernatants of transfected
845 BHK-NS2A-HA cells were isolated at 96 h p.t. and subjected to RT-PCR. Positive
846 RT-PCR products were sequenced to confirm the engineered mutations and to identify
847 potential second site change(s). (F) The supernatants of infected Vero cells from (D) were
848 collected at 72 h p.i. to infect naïve Vero cells for a second round. IFA was performed on
849 these cells for viral E-positive cells.

850 ***Fig. 6. Membrane association of ZIKV NS2A transmembrane segments.***

851 (A) Bioinformatics analysis of ZIKV NS2A for transmembrane segments. ZIKV NS2A
852 sequence was analyzed by bioinformatics program TOPCONS, Philius, PolyPhobius,
853 SCAMPI, and TMHMM. The compiled transmembrane segments are shown at the
854 bottom. Seven predicted transmembrane segments (pTMS) are presented as thick bars.
855 The amino acid positions of ZIKV NS2A are indicated. (B) Membrane association of
856 individual pTMS. Expression plasmid pXJ-eGFP (without NS2A sequence) and
857 pXJ-smNS2A-eGFP (smNS2A represents different NS2A segments) were transfected into
858 Vero cell by using X-tremeGENE 9 DNA transfection reagent (Roche). For each plasmid
859 construct, the NS2A segment is indicated by amino acid positions. At 24 h p.t., IFA was
860 performed to monitor eGFP expression. ER and nuclei were stained with anti-Calnexin



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antibody and DAPI, respectively. The merged images are shown on the right panels.

***Fig. 7. Probing the membrane topology of ZIKV NS2A by in vitro deglycosylation assay and in vitro proteinase K protection assay.***

(A) Schematic representation of C-terminally truncated ZIKV NS2A proteins. The amino acid positions of each truncated NS2A are indicated. A "SPG-C16" sequence was fused to the N terminus of NS2A to ensure correct translocation of NS2A on the ER membrane. An "eGFP-Glyc" sequence was fused to the C terminus of NS2A to probe its location at cytosol or ER lumen. See text for details. (B) BHK-21 cells were transfected with the expression plasmids described in (A). At 24 h p.t., the expression levels of various NS2A truncates were examined by Western blot using an eGFP antibody. The amino acid positions are indicated for each NS2A truncates. The molecular masses of marker proteins are shown to the left of Western blot image. (C) Diagram of glycosylation of eGFP-Glyc when located at ER lumen (*left*). No glycosylation occurs when eGFP-Glyc is located at cytosol (*right*). The glycosylation residue Asn is indicated. (D) *In vitro* deglycosylation assay. Plasmid-transfected cells were treated with PNGase F or PBS according to the protocol of *in vitro* deglycosylation assay (see Materials and Methods). The samples were examined for glycosylation status by Western blot using an eGFP antibody. (E) Diagram of *in vitro* proteinase K protection assay. When eGFP is located at ER lumen, it is protected from proteinase K cleavage (*left*); when located in the cytosol, eGFP is degraded by proteinase K (*right*). (F) *In vitro* proteinase K protection assay. Cell



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881 lysates from plasmid-transfected BHK-21 cells were treated with proteinase K or PBS
882 according to the protocol of *in vitro* proteinase K protection assay (see Materials and
883 Methods). The samples were detected for eGFP by Western blot using an eGFP antibody.
884 Triton X-100 treatment is indicated.

885 ***Fig. 8. A topological model of ZIKV NS2A.***

886 (A) A model of ZIKV NS2A membrane topology. See text for details. Class III, IV, and V
887 mutations are depicted on the topology model. (B) Helical wheel representations of
888 NS2A fragment 9-26, 41-58, 104-121, 125-142, 172-189, and 203-220. The amphiphilic
889 helix regions were analyzed using Heliquest server (heliquest.ipmc.cnrs.fr/). The best
890 18-residue windows are presented. The amino acids forming the hydrophobic surface of
891 each helix wheel are depicted. Yellow, gray, blue, red, purple, pink, and green circles
892 indicate nonpolar, glycine/alanine, positively charged, negatively charged, polar, amide,
893 and proline amino acids, respectively. The first and last residues in each helical wheel are
894 indicated by a blue and red arrow, respectively.



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# Genetic and biochemical characterizations of Zika virus NS2A protein

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**Abstract**

Zika virus (ZIKV) can cause devastating congenital Zika syndromes in pregnant women and Guillain-Barre syndrome in adults. Understanding the molecular mechanism of ZIKV replication is essential for antiviral and vaccine development. Here we report the structural and functional characterization of ZIKV NS2A protein. Biochemical structural probing suggests that ZIKV NS2A has a single segment that traverses the ER membrane and six segments that peripherally associate with the ER membrane. Functional analysis has defined distinct NS2A residues essential for viral RNA synthesis or virion assembly. Only the virion assembly-defective mutants, but not the RNA synthesis-defective mutants, could be rescued through *trans* complementation with a wide-type NS2A protein. These results suggest that the NS2A molecules in virion assembly complex could be recruited in *trans*, whereas the NS2A molecules in viral replication complex must be recruited in *cis*. Together with previous results, we propose a flavivirus assembly model where NS2A plays a central role in modulating viral structural and nonstructural proteins as well as genomic RNA during virion assembly.

**Keywords:** Zika virus; flavivirus replication; virion assembly; flavivirus NS2A; membrane topology



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## Introduction

Zika virus (ZIKV) is a recently emerged mosquito-borne flavivirus that is primarily transmitted by *Aedes spp.* mosquitoes. It can also be transmitted through sexual, maternal-to-fetal, and blood transfusion routes [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. ZIKV was first identified in 1947 from a sentinel rhesus monkey in Uganda [ ADDIN EN.CITE <EndNote><Cite><Author>Dick</Author><Year>1952</Year><RecNum>10</RecNum><DisplayText>[6]</DisplayText><record><rec-number>10</rec-number><foreign-keys><key app="EN" db-id="sva5pexpet29snerv2jpeedvrdwz0fzfs2ee">10</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Dick, G. W. A.</author><author>Kitchen, S. F.</author><author>Haddow, A. J.</author></authors></contributors><titles><title>Zika Virus .1. Isolations and Serological Specificity</title><secondary-title>Transactions of the Royal Society of Tropical Medicine and Hygiene</secondary-title><alt-title>T Roy Soc Trop Med H</alt-title></titles><periodical><full-title>Transactions of the Royal Society of Tropical Medicine and Hygiene</full-title><abbr-1>T Roy Soc Trop Med H</abbr-1></periodical><alt-periodical><full-title>Transactions of the Royal Society of Tropical Medicine and Hygiene</full-title><abbr-1>T Roy Soc Trop Med H</abbr-1></alt-periodical><pages>509-520</pages><volume>46</volume><number>



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5</number><dates><year>1952</year></dates><isbn>0035-9203</isbn><accession-num>WOS:A1952XT98500007</accession-num><urls><related-urls><url><Go to ISI>://WOS:A1952XT98500007</url></related-urls></urls><electronic-resource-num>Doi 10.1016/0035-9203(52)90042-4</electronic-resource-num><language>English</language></record></Cite></EndNote>]. Since 2007, it emerged in the Pacific region, South America, Central America, and the Caribbean, posing a global public health threat [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. ZIKV infection causes mild illnesses, including fever, pruritis, arthritis, conjunctivitis, and asthenia [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. Importantly, it can cause devastating congenital Zika syndromes (CZS; including microcephaly, congenital malformation, and fetal demise) in pregnant women and Guillain-Barre syndrome (GBS) in adults [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. The explosive epidemics and its association with CZS had led the World Health Organization (WTO) to declare ZIKV as a Public Health Emergency of International Concern from February to November in 2016 [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. Since then, a number of vaccine candidates have been developed, some of which have already advanced to clinical trials [ ADDIN EN.CITE

<EndNote><Cite><Author>Shan</Author><Year>2018</Year><RecNum>454</RecNum><DisplayText>[18]</DisplayText><record><rec-number>454</rec-number><foreign-keys><key app="EN"



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103 112613</custom2><electronic-resource-num>10.1016/j.chom.2018.05.021</electronic-r
104 esource-num></record></Cite></EndNote>]. In addition, small molecule inhibitors and
105 therapeutic antibodies have also been identified for antiviral development [ ADDIN
106 EN.CITE ADDIN EN.CITE.DATA ].

Comment [SPy1]: Add reference
Jing Zou, Pei-Yong Shi. 2019. **Strategies for Zika Drug Discovery.** Current Opinion in Virology (in press)

107 ZIKV belongs to the *Flavivirus* genus within the *Flaviviridae* family. Besides ZIKV,
108 many other flaviviruses are significant human pathogens, including dengue (DENV),
109 yellow fever (YFV), West Nile (WNV), Japanese encephalitis (JEV), Saint Louis
110 encephalitis (SLEV), and tick-borne encephalitis virus (TBEV) [ ADDIN EN.CITE
111 <EndNote><Cite><Author>Mlakar</Author><Year>2016</Year><RecNum>265</Rec
112 Num><DisplayText>[22]</DisplayText><record><rec-number>265</rec-number><forei
113 gn-keys><key app="EN"
114 db-id="sva5pexpet29snerv2jpeedvrdwz0fzfs2ee">265</key></foreign-keys><ref-type
115 name="Journal Article">17</ref-type><contributors><authors><author>Mlakar,
116 J.</author><author>Korva, M.</author><author>Tul, N.</author><author>Popovic,
117 M.</author><author>Poljsak-Prijatelj, M.</author><author>Mraz,
118 J.</author><author>Kolenc, M.</author><author>Rus, K. R.</author><author>Vipotnik,
119 T. V.</author><author>Vodusek, V. F.</author><author>Vizjak,



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A.</author><author>Pizem, J.</author><author>Petrovec, M.</author><author>Zupanc, T. A.</author></authors></contributors><auth-address>Univ Ljubljana, Inst Pathol, Fac Med, Ljubljana, SloveniaUniv Ljubljana, Inst Microbiol & Immunol, Fac Med, Zaloska 4, Ljubljana 1000, SloveniaUniv Med Ctr Ljubljana, Dept Perinatol, Div Gynecol & Obstet, Ljubljana, SloveniaUniv Med Ctr Ljubljana, Inst Radiol, Ljubljana, Slovenia</auth-address><titles><title>Zika Virus Associated with Microcephaly</title><secondary-title>New England Journal of Medicine</secondary-title><alt-title>New Engl J Med</alt-title></titles><periodical><full-title>New England Journal of Medicine</full-title><abbr-1>New Engl J Med</abbr-1></periodical><alt-periodical><full-title>New England Journal of Medicine</full-title><abbr-1>New Engl J Med</abbr-1></alt-periodical><pages>951-958</pages><volume>374</volume><number>10</number><keywords><keyword>micronesia</keyword><keyword>brain</keyword><keyword>pcr</keyword></keywords><dates><year>2016</year><pub-dates><date>Mar 10</date></pub-dates></dates><isbn>0028-4793</isbn><accession-num>WOS:000371660000008</accession-num><urls><related-urls><url><Go to ISI>://WOS:000371660000008</url></related-urls></urls><electronic-resource-num>10.1056/Nejmoa1600651</electronic-resource-num><language>English</language></record></Cite></EndNote>]. Flavivirus genome is a positive-sense, single-stranded RNA



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of ~11,000 nucleotides. It contains a 5' untranslated region (UTR), a long open-reading-frame (ORF), and a 3' UTR. The single ORF encodes a polyprotein that is processed into three structure proteins (capsid [C], pre-membrane [prM] and envelope [E]) and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5). The structural proteins, together with genomic RNA, are components of virions. The nonstructural proteins participate in viral RNA replication, virion assembly, and evasion of host immune response [ ADDIN EN.CITE ADDIN EN.CITE.DATA ].

Flavivirus NS2A is an ER-resident, transmembrane protein with a calculated molecular weight of ~22 kDa. The N-terminus and C-terminus of NS2A are formed by cleavages with an unknown host protease and viral protease NS3 (in complex with NS2B), respectively [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. Flavivirus NS2A has multiple functions: (i) Viral replication. Kunjin virus (KUNV) NS2A co-localizes with viral double-stranded RNA and interacts with the 3'UTR of genomic RNA, NS3, and NS5 protein [ ADDIN EN.CITE <EndNote><Cite><Author>Mackenzie</Author><Year>1998</Year><RecNum>405</RecNum><DisplayText>[28]</DisplayText><record><rec-number>405</rec-number><foreign-keys><key app="EN" db-id="sva5pexpet29snerv2jpeedvrdwz0fzfs2ee">405</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Mackenzie, J. M.</author><author>Khromykh, A. A.</author><author>Jones, M.



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Sakzewski Virus Res Ctr, Brisbane, Qld 4029, AustraliaUniv Queensland, Ctr
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Australia</auth-address><titles><title>Subcellular localization and some biochemical
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182 10.1006/viro.1998.9156</electronic-resource-num><language>English</language></rec
183 ord></Cite></EndNote>]. Mutagenesis studies confirmed the role of NS2A in viral RNA
184 synthesis [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. (ii) Virion
185 assembly/secretion. Mutagenesis analyses of YFV, KUNV, and DENV have identified
186 distinct NS2A amino acids that are essential for virion assembly/secretion [ ADDIN
187 EN.CITE ADDIN EN.CITE.DATA ]. The virion assembly defects can be restored by
188 second site mutations in NS2B or NS3 in YFV and DENV [ ADDIN EN.CITE ADDIN
189 EN.CITE.DATA ]. (iii) Evasion of innate immunity. ZIKV NS2A inhibits type-I IFN
190 induction at the step of TBK1 [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. DENV
191 NS2A antagonizes type-I IFN signaling [ ADDIN EN.CITE
192 <EndNote><Cite><Author>Munoz-Jordan</Author><Year>2003</Year><RecNum>389
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197 J. L.</author><author>Sanchez-Burgos, G. G.</author><author>Laurent-Rolle,
198 M.</author><author>Garcia-Sastre,
199 A.</author></authors></contributors><auth-address>Mt Sinai Sch Med, Dept Microbiol,
200 New York, NY 10029 USA</auth-address><titles><title>Inhibition of interferon
201 signaling by dengue virus</title><secondary-title>Proceedings of the National Academy
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Sci, Taipei, TaiwanNatl Def Med Ctr, Dept Microbiol & Immunol, Taipei,
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245 virus</keyword><keyword>5a

246 protein</keyword><keyword>viral-rna</keyword><keyword>infection</keyword></ke

247 ywords><dates><year>2012</year><pub-dates><date>Oct</date></pub-dates></dates>

248 <isbn>0022-538X</isbn><accession-num>WOS:000308740700010</accession-num><u

249 rls><related-urls><url><Go to

250 ISI>://WOS:000308740700010</url></related-urls></urls><electronic-resource-num

251 >10.1128/Jvi.00525-12</electronic-resource-num><language>English</language></reco

252 rd></Cite></EndNote>]. In WNV and KUNV, the NS2A-mediated inhibition of type-I

253 IFN induction can be abrogated by specific NS2A mutations [ ADDIN EN.CITE

254 ADDIN EN.CITE.DATA ]. (iv) Disease pathogenesis. WNV NS2A modulates

255 virus-induced cytopathic effect (CPE) and pathogenesis in mice [ ADDIN EN.CITE

256 ADDIN EN.CITE.DATA ]. ZIKV NS2A impairs mammalian cortical neurogenesis

257 through depleting adherens junction proteins [ ADDIN EN.CITE ADDIN

258 EN.CITE.DATA ].

259 The structure and function of ZIKV NS2A have not been characterized. Although

260 NS2A proteins from different flaviviruses perform similar functions (described above),

261 each flavivirus may exert its NS2A functions in a virus-specific manner [ ADDIN

262 EN.CITE

263 <EndNote><Cite><Author>Vossmann</Author><Year>2015</Year><RecNum>423</R

264 ecNum><DisplayText>[34]</DisplayText><record><rec-number>423</rec-number><fo



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S.</author><author>Wieseler, J.</author><author>Kerber,
R.</author><author>Kummerer, B.
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Dept Virol, D-20359 Hamburg, GermanyUniv Bonn, Inst Virol, Med Ctr, Bonn,
Germany</auth-address><titles><title>A Basic Cluster in the N Terminus of Yellow
Fever Virus NS2A Contributes to Infectious Particle
Production</title><secondary-title>Journal of Virology</secondary-title><alt-title>J
Virol</alt-title></titles><periodical><full-title>Journal of Virology</full-title><abbr-1>J
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n-num><urls><related-urls><url><Go to



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ISI>://WOS:000352219600023</url></related-urls></urls><electronic-resource-num >10.1128/Jvi.03351-14</electronic-resource-num><language>English</language></reco rd></Cite></EndNote>]. The goals of this study are (i) to define the membrane topology of ZIKV NS2A and (ii) to characterize its roles in viral RNA synthesis and virion assembly.

## Materials and methods

### *Cell lines*

Vero cells, baby hamster kidney (BHK-21) cells and human embryo kidney 293T cells were cultured at 37°C with 5% CO2 in high-glucose Dulbecco's modified Eagle medium (DMEM; Life Technologies) supplemented with 2 mM L-glutamine, 100U/ml penicillin, 100g/ml streptomycin and 10% fetal bovine serum (FBS; HyClone Laboratories).

### *Bioinformatics*

NS2A protein sequences from ZIKV, DENV1, DENV2, DENV3, DENV4, WNV, JEV, YFV, SLEV were aligned using CLC Main Workbench software (CLC Bio). Prediction of ZIKV NS2A was carried out using various web servers, which including TOPCONS, Philius, Polyphobius, SCAMPI, and TMHMM2. The amphipathic helices of ZIKV NS2A were predicted by HeliQuest.



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304 ***Antibody***

305 The following antibodies were used in this study: a mouse monoclonal antibody 4G2
306 cross-reactive with flavivirus E protein (American Type Culture Collection); Rabbit
307 anti-HA monoclonal antibody (Cell Signaling Technology); Alexa Fluor®488 conjugated
308 mouse anti-HA monoclonal antibody (Cell Signaling Technology); Rabbit anti-Calnexin
309 polyclonal antibody(Abcam); Mouse anti-GFP monoclonal antibody (Sigma-Aldrich);
310 Horseradish peroxidase(HRP) conjugated goat anti-mouse and anti-rabbit IgG polyclonal
311 antibodies (Sigma-Aldrich); Alexa Fluor®488 conjugated goat anti-mouse IgG
312 polyclonal antibody (Thermo Fisher Scientific); Alexa Fluor®568 conjugated goat
313 anti-rabbit IgG polyclonal antibody (Thermo Fisher Scientific).

314 ***Plasmid construction***

315 The Infectious cDNA clone (pFLZIKV) bearing the full-length genome of ZIKV (strain
316 FSS13025) or Renilla luciferase (Rluc) reporter ZIKV replicon (pZIKV Rep WT) were
317 used as the backbone for introducing NS2A mutations [ ADDIN EN.CITE ADDIN
318 EN.CITE.DATA ]. XhoI and KpnI restriction sites were initially generated into
319 pFLZIKV and pZIKV Rep WT replicon at positions 3018 and 5010 in ZIKV genome
320 without changing amino acid sequences. The NS2A alanine-scanning mutagenesis was
321 performed by using a QuikChange II XL Site-Directed Mutagenesis kit (Agilent
322 Technologies) as described previously [ ADDIN EN.CITE
323 <EndNote><Cite><Author>Xie</Author><Year>2015</Year><RecNum>279</RecNum



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327 Article">17</ref-type><contributors><authors><author>Xie, X.
328 P.</author><author>Zou, J.</author><author>Puttikhunt, C.</author><author>Yuan, Z.
329 M.</author><author>Shi, P.
330 Y.</author></authors></contributors><auth-address>Novartis Inst Trop Dis, Singapore,
331 SingaporeChinese Acad Sci, State Key Lab Virol, Wuhan Inst Virol, Wuhan,
332 Peoples R ChinaNatl Sci & Technol Dev Agcy, Med Biotechnol Unit, Natl
333 Ctr Genet Engn & Biotechnol, Bangkok,
334 Thailand</auth-address><titles><title>Two Distinct Sets of NS2A Molecules Are
335 Responsible for Dengue Virus RNA Synthesis and Virion
336 Assembly</title><secondary-title>Journal of Virology</secondary-title><alt-title>J
337 Virol</alt-title></titles><alt-periodical><full-title>J
338 Virol</full-title></alt-periodical><pages>1298-1313</pages><volume>89</volume><nu
339 mber>2</number><keywords><keyword>nonstructural protein
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342 architecture</keyword><keyword>subcellular-localization</keyword><keyword>endopl
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ord>inhibition</keyword></keywords><dates><year>2015</year><pub-dates><date>Ja n</date></pub-dates></dates><isbn>0022-538X</isbn><accession-num>WOS:000347178900035</accession-num><urls><related-urls><url><Go to ISI>://WOS:000347178900035</url></related-urls></urls><electronic-resource-num>10.1128/Jvi.02882-14</electronic-resource-num><language>English</language></record></Cite></EndNote>].

A mammalian expression vector pXJ with a cytomegalovirus (CMV) promoter was used for constructing plasmids expressing NS2A and its derivatives. To construct the pXJ-$E_{24}$-NS1-NS2A-HA plasmid, a gene cassette encoding the last 24 amino acids of E protein ($E_{24}$), NS1 and NS2A with a C-terminal hemagglutinin (HA) tag was firstly amplified from the pFLZIKV and cloned into the pXJ vector. Next, 35 NS2A mutations were individually introduced into the pXJ-$E_{24}$-NS1-NS2A-HA by overlap PCR, resulting in plasmids expressing NS2A mutants.

To construct the pXJ-SPG-$C_{16}$-NS2A-HA that was used for selecting cell line constitutively expressing NS2A protein, a DNA fragment containing the Gaussia luciferase signal peptide (SPG), the last 16 amino acids of NS1 protein ($C_{16}$) and NS2A tagged with a C-terminal HA tag sequence were constructed by overlap PCR and cloned into the pXJ vector via NotI/XhoI restriction sites.

Plasmid pXJ-eGFP was constructed by inserting the enhanced green fluorescence protein (eGFP) sequence into the pXJ vector via BamHI/XhoI restriction sites. DNA



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fragments that encode different NS2A segments were individually inserted into above pXJ-eGFP vector, resulting various pXJ-smNS2A-eGFP plasmids (smNS2A represents NS2A segment). A DNA fragment that express NS2A or truncated NS2A and an eGFP with a C-terminal N-glycosylation acceptor site (Glyc) were amplified and cloned into the pXJ-SPG-$C_{16}$-NS2A-HA vector, resulting in the plasmid pXJ-SPG-$C_{16}$-NS2A-eGFP-Glyc and various pXJ-SPG-$C_{16}$-ctNS2A-eGFP-Glyc plasmids (ctNS2A represents C-terminal truncated NS2A).

***RNA in vitro transcription and electroporation***

All full-length cDNA clone plasmids and subgenomic replicons of ZIKV were linearized with restriction enzyme ClaI, followed by phenol-chloroform extraction and ethanol precipitation. RNA transcripts were prepared by using the T7 mMessage mMachine kit (Ambion). For RNA transfection, 10 μg *in vitro* transcribed RNA was electroporated into $8\times10^6$ cells (BHK-21 or Vero) by using Gene Pulser Xcell™ Electroporation Systems (Bio-rad) as described previously [ ADDIN EN.CITE ADDIN EN.CITE.DATA ].

***Immunofluorescence assay***

For indirect immunofluorescence assay (IFA), cells were seeded in an 8-well Nunc Lab-Tek II Chamber Slide (Thermo Fisher Scientific). At various time points, cells were washed three times with phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde in PBS at room temperature for 30 min and permeabilized with 0.1%



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Triton X-100 in PBS at room temperature for 10 min. The permeabilized cells were then washed three times with PBS and incubated in a blocking buffer containing 1% FBS in PBS at room temperature for 1h. E protein was stained with a 1:1000 dilution of mouse monoclonal antibody 4G2 in blocking buffer. After incubation for 1h, the cells were washed three times with a washing buffer (1% FBS and 0.5% Tween 20 in PBS). After 1 hour of incubation with a secondary antibody (Alexa Fluor®488 conjugated goat anti-mouse IgG), the cells were washed three times with washing buffer and counterstained with DAPI (4',6-diamidino-2-phenylindole). Images were acquired using an Olympus fluorescence microscope. Images were processed in ImageJ software (National Institutes of Health, MD).

***Plaque assay***

$2\times10^5$ Vero cells per well were grown in 24-well plates one day prior to viral infection. 10-fold serial dilutions of virus samples were prepared in DMEM containing 2% FBS and 1% penicillin/streptomycin and inoculated (100 µl) into each well of 24-well plates. After 1 h of incubation at 37°C with 5% $CO_2$, the inoculum was replaced with 500 µl overlay medium (DMEM medium containing 0.8% methyl-cellulose, 2% FBS and 1% penicillin/streptomycin). Plaques formed after incubation at 37°C with 5% $CO_2$ for 4-5 days. Cells were fixed with 4% paraformaldehyde for 30 min, followed by staining with 1% crystal violet. The numbers of plaque per milliliter (PFU/ml) of ZIKV were calculated.



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***Replicon transient-transfection assay***

Briefly, $8\times10^6$ Vero cells were electroporated with 10 μg wild type or mutant Rluc-ZIKV replicon RNAs. After electroporation, $3\times10^5$ cells per well were seeded in 12-well plates. At given time points, the cells were washed twice with PBS and lysed in 200 μl of luciferase lysis buffer (Promega). The luciferase activity assay was performed according to the manufacturer's protocol.

***RNA extraction and cDNA sequencing***

The culture fluids from infected cells were centrifuged at 1000 g for 10 min and then filtered through a 0.22 μm pore size polyethersulfone membrane (Millipore) to remove cellular debris. Viral RNAs in the supernatants were extracted using a QIAamp viral-RNA Minikit (Qiagen) by following the manufacturer's protocol. Reverse transcription PCR (RT-PCR) was performed by using the SuperScript® III One-Step RT-PCR System (Thermo Fisher Scientific). The amplified cDNA fragments were purified and sent for Sanger sequencing at the GENEWIZ facilities.

***Selection of NS2A-HA BHK-21 cell line***

$4\times10^5$ BHK-21 cells per well of were seeded into a 6-well plate. The plasmid pXJ-SPG-$C_{16}$-NS2A-HA that contains a neomycin resistance gene was transfected into BHK-21 cells (4 μg per well) using X-tremeGENE 9 DNA transfection reagent (Roche). At 24h post-transfection, geneticin (G418) was added to the culture medium with a



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concentration of 1 mg/ml. The medium was changed every three days. After two weeks, several neomycin-resistant individual colonies were selected for propagation. The expression of NS2A-HA protein in stable cell lines was analyzed by immunofluorescence assay and Western blot.

***In vitro proteinase K protection assay***

Proteinase K protection assay was performed as previously described with minor modifications [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. BHK-21 cells were transfected in 6-well plates with various pXJ-SPG-$C_{16}$-ctNS2A-eGFP-Glyc plasmids. After incubated at 30°C for 24h, cells were trypsinized, washed twice with PBS and centrifuged at 450×g for 5 min. The cell pellets were resuspended in a hypotonic lysis buffer (10 mM Tris-HCl, pH 7.5, and 2 mM $MgCl_2$) and kept on ice for 10 min. Subsequently, the samples were homogenized with 40 strokes and centrifuged at 1,000×g for 5 min. The postnuclear supernatants (PNS) were divided into three aliquots and centrifuged at 15,000×rpm for 40 min. The pellets from three aliquots were then resuspended in PBS, 60 μg/ml proteinase K in PBS, or 60 μg/ml proteinase K in PBS in the presence of 0.5% Triton X-100, respectively. The samples were incubated on ice for 90 min. 20 mM phenylmethylsulfonyl fluoride (PMSF) was finally added to terminate the reaction. After further incubation on ice for 15 min, the samples were mixed with 4× Laemmli sample buffer, heated at 95°C for 15 min and analyzed by Western blotting.



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441 ***In vitro deglycosylation assay***

442 The samples were prepared by following a previously described protocol [ ADDIN
443 EN.CITE ADDIN EN.CITE.DATA ]. Briefly, the transfected BHK-21 cells in 6-well
444 plates were incubated at 30°C for 24 h. Cells were trypsinized and suspended in DMEM
445 medium containing 10%FBS. The cells were pelleted by centrifuging at 450×g for 5 min.
446 After two PBS washes, the cell pellets were resuspended in a solubilization buffer (20
447 mM Tris-HCl, pH 7.5, 50 mM NaCl, 10 mM $Mg(CH_3COO)_2$, 1% Triton X-100, and
448 EDTA-free protease inhibitors [Roche]) and incubated at 4°C for 2 h with gentle agitation.
449 After centrifugation at 15,000 rpm 4°C for 20 min to remove cell debris, the supernatants
450 were split into two aliquots. 50,000 units/ml N-glycosidase F (PNGase F) or PBS
451 (no-treatment control) were added to those two aliquots. After incubation at 37°C for 2 h,
452 the samples were mixed with 4× Laemmli sample buffer, heated at 70°C for 15 min and
453 analyzed by Western blot.

454 ***SDS-PAGE and western blot***

455 Protein samples were separated by 12% SDS-polyacrylamide gel electrophoresis and
456 transferred onto a polyvinylidene difluoride (PVDF) membrane using Trans-Blot Turbo
457 transfer system (Bio-Rad), after which the membrane was blocked in TBST (10 mM
458 Tris-HCl, pH 8.0, 150 mM NaCl, and 0.1% Tween 20) supplemented with 5% nonfat
459 milk for 1h at room temperature. The blots were probed with primary antibodies (1:1,000
460 dilution) overnight at 4°C, washed three times with TBST and incubated with horseradish



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peroxidase (HRP) conjugated goat anti-mouse and anti-rabbit IgG polyclonal antibodies (1:10,000 dilution) for 1h. After three thorough TBST buffer washes, the blots were incubated with SuperSignal™ West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific). Chemiluminescence signals were detected in ChemiDoc System (Bio-Rad).

### *Quantification and statistical analysis*

IFA images processing and cell counting were performed in software ImageJ (NIH). Densitometry analysis was performed using Image lab version 6.0 (Bio-Rad Laboratories, Hercules, CA). All numerical data are presented as the mean ± SEM (standard error of mean). The size of each study or number of replicates, along with the statistical tests performed can be found in corresponding Figure Legends.

## Results

### *The rationale for ZIKV NS2A mutagenesis*

To identify critical residues in ZIKV NS2A, we performed a systematic alanine-scanning mutagenesis using an infectious cDNA clone of ZIKV strain FSS13025 [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. Sequence alignment of NS2A proteins from ZIKV, DENV, WNV, JEV, SLEV, and YFV revealed a high diversity: only 31 out of



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218-227 amino acids with a consensus of >80% among different flaviviruses (Fig. 1A). A total of 35 residues were selected for mutagenesis: (i) All 8 identical non-alanine residues, including G12, G47, D53, G71, F83, P87, E103, and K193. The amino acid numbering is based on the ZIKV sequence. (ii) All charged residues, including D4, D7, E22, K31, K56, E67, D73, H76, K84, R86, R96, R102, E122, D124, R140, R146, D148, R163, R171, K186, K188, K192, R207, D210, and R222. Charged residues were selected because they likely participate in inter- or intra-molecular interactions; alternatively, they may alter the locations where NS2A would traverse the ER membrane since charged residues are hydrophilic. In addition, NS2A D73H mutation was previously shown to attenuate WNV replication (ref). (iii) A K25/K26/R27 motif that was previously reported to be critical for YFV and DENV virion assembly (32, 42). (iv) Residue N130 that was previously shown to be required for NS1-NS2A cleavage and viral replication in DENV [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. The selected residues were mutated to alanine in the context of ZIKV genomic RNA. Equal amounts of wild-type (WT) and mutant RNAs were electroporated into Vero cells. After electroporation, RNA replication and virus spread were monitored by immunofluorescence assay (IFA); the production of infectious viruses was quantified by plaque assay and RT-PCR. The 35 mutants exhibited distinct phenotypes that could be categorized into five groups (Fig. 1B), as detailed in the following sections.

***Class I mutations have no or weak effects on viral replication***



**Comment [SPy2]:** [ HYPERLINK "https://www.ncbi.nlm.nih.gov/pubmed/17382364" \o "Virology." ] 2007 Jul 20;364(1):184-95. Epub 2007 Mar 23. **Mutations in West Nile virus nonstructural proteins that facilitate replicon persistence in vitro attenuate virus replication in vitro and in vivo.** [ HYPERLINK "https://www.ncbi.nlm.nih.gov/pubmed/?term=Rossi%20SL%5BAuthor%5D&cauthor=true&cauthor_uid=17382364" ][1], [ HYPERLINK "https://www.ncbi.nlm.nih.gov/pubmed/?term=Fayzulin%20R%5BAuthor%5D&cauthor=true&cauthor_uid=17382364" ], [ HYPERLINK "https://www.ncbi.nlm.nih.gov/pubmed/?term=Dewsbury%20N%5BAuthor%5D&cauthor=true&cauthor_uid=17382364" ], [ HYPERLINK "https://www.ncbi.nlm.nih.gov/pubmed/?term=Bourne%20N%5BAuthor%5D&cauthor=true&cauthor_uid=17382364" ], [ HYPERLINK "https://www.ncbi.nlm.nih.gov/pubmed/?term=Mason%20PW%5BAuthor%5D&cauthor=true&cauthor_uid=17382364" ].

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Fourteen of the 35 NS2A mutant RNAs, including D4A, E22A, K31A, G47A, D53A, E67A, H76A, E122A, R146A, D148A, R163A, K186A, K188A, and D210A, replicated at levels comparable to the WT RNA. These mutants were categorized as class I (Fig. 1B). The IFA images and plaque morphologies of three representatives (D53A, D148A, and R163A), as well as the WT, are shown in Fig. 2A and 2B, respectively. Most class I mutants (*e.g.*, D148A and R163A) yielded comparable E-expressing cells as the WT from 24 to 48 h post-transfection (p.t.). At 72 h p.t., severe CPE occurred among D148A, R163A, and WT RNA-transfected cells. Some class I mutants (*e.g.*, D53A) produced fewer E-positive cells than the WT at 48 h p.t., and spread to infect more cells at 72 h p.t (Fig. 2A). All class I mutants produced viruses with plaques similar to the WT virus (Fig. 2B). At 72 h p.t., the class I mutants produced infectious titers <4-fold lower than the WT did (Fig. 2C). Sequencing of the mutant viruses (collected at 72 h p.t.) confirmed the retaining of the engineered mutations without other changes (data not shown).

***Class II mutations significantly attenuate viral replication***

Nine NS2A mutants, including K56A, F83A, K84A, R86A, P87A, R96A, R102A, D124A, and R207A, significantly attenuated viral replication. These mutants were categorized into class II (Fig. 1B). Class II RNA-transfected cells produced fewer E-positive cells than the WT did (Fig. 2D). At 72 h p.t., only 30-50% of E-positive cells were detected in the class II RNA-transfected cells, whereas the WT RNA-transfected cells exhibited CPE. Class II mutant viruses formed smaller, opaque plaques (Fig. 2E),



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519 and the transfected cells produced >10-fold lower infectious titers than the WT
520 RNA-transfected cells at 72 h p.t. (Fig. 2F). Complete genome sequencing of the class II
521 mutant viruses (collected at 96 h p.t.) did not reveal reversions or any other mutations
522 (data not shown). Because of the weak phenotypes of class I and II mutants, we did not
523 further characterize them.

524 ***Class III mutations are lethal for viral RNA synthesis***

525 Class III includes two mutants, G12A and N130A, which were completely defective in
526 viral RNA synthesis (Fig. 1B). The G12A and N130A genomic RNA-transfected cells did
527 not produce any E-positive cells at 24 and 48 h p.t. (Fig. 2G). On day 5 to 8 p.t., no
528 infectious viruses were detected from culture medium by plaque assay (Fig. 2H) and
529 RT-PCR (Fig. 2I). To confirm the above results, we introduced G12A or N130A mutation
530 into a ZIKV luciferase replicon (Fig. 2J). A replication-defective replicon, containing an
531 NS5 polymerase active site ΔGDD mutation, was included as a negative control [ ADDIN
532 EN.CITE ADDIN EN.CITE.DATA ]. Vero cells were electroporated with equal
533 amounts of WT, G12A, N130A, or NS5 ΔGDD replicon RNAs. At 2-6 h p.t., all replicons
534 generated similar levels of luciferase signals, suggesting comparable levels of viral RNA
535 translation and transfection efficiencies (Fig. 2K). However, at 18-30 h p.t., G12A and
536 N130A replicons generated low luciferase signals as the non-replicative NS5 ΔGDD
537 replicon did, whereas the WT replicon produced increasing luciferase signals (Fig. 2K).
538 These results demonstrate that class III mutations completely abolish viral RNA



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synthesis.

***Class IV mutations impair both viral RNA synthesis and virion production***

Class IV includes three mutants, including D7A, R140A, and R222A (Fig. 1B). These mutant genomic RNAs produced fewer E-positive cells in the transfected cells than the WT RNA (Fig. 3A). For D7A or R140A mutants, the RNA-transfected cells did not produce infectious viruses, as suggested by plaque assay (Fig. 3B) and RT-PCR (Fig. 3C). Incubation of naïve Vero cells with culture supernatants from the D7A or R140A transfected cells did not generate any viral E-positive cells (Fig. 3D). Replicon analysis showed that D7A or R140A significantly compromised viral RNA synthesis (Fig. 3E). Since neither mutants could produce extracellular viruses (Fig. 3B&C), and could replicate at attenuated levels inside cells (Fig. 3E), D7A and R140A mutations may impair both viral RNA synthesis and virion production.

For R222A, the number of E-positive cells slightly increased in the genomic RNA-transfected cells from 24 to 72 h p.t. (Fig. 3A). A low level of infectious virus was detected from the culture medium by plaque assay (Fig. 3B) and RT-PCR (Fig. 3C). These data were supported by the positive-IFA results after incubating naive Vero cells with the supernatant from the R222A RNA-transfected cells (Fig. 3D). Sequencing the virus (collected at 72 h p.t.) revealed that, besides the engineered R222A mutation, the virus had acquired a second site mutation of L220F in NS2A (Fig. 3C). Replicon analysis showed that R222A significantly lowered viral RNA synthesis (Fig. 3E); no secondary



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mutation (*i.e.*, L220F) was detected in the replicon RNA at 30 h p.t. (data not shown). These results suggest that (i) R222A mutant is defective in viral RNA synthesis and/or virion assembly, and (ii) a second site mutation L220F in NS2A could rescue the defects.

***Class III and IV mutations do not affect NS2A maturation***

Since cleavage between NS1 and NS2A is essential for flavivirus RNA synthesis [ ADDIN EN.CITE <EndNote><Cite><Author>Xie</Author><Year>2015</Year><RecNum>406</RecNum><DisplayText>[29]</DisplayText><record><rec-number>406</rec-number><foreign-keys><key app="EN" db-id="sva5pexpet29snerv2jpeedvrdwz0fzfs2ee">406</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Xie, X. P.</author><author>Zou, J.</author><author>Puttikhunt, C.</author><author>Yuan, Z. M.</author><author>Shi, P. Y.</author></authors></contributors><auth-address>Novartis Inst Trop Dis, Singapore, SingaporeChinese Acad Sci, State Key Lab Virol, Wuhan Inst Virol, Wuhan, Peoples R ChinaNatl Sci & Technol Dev Agcy, Med Biotechnol Unit, Natl Ctr Genet Engn & Biotechnol, Bangkok, Thailand</auth-address><titles><title>Two Distinct Sets of NS2A Molecules Are Responsible for Dengue Virus RNA Synthesis and Virion Assembly</title><secondary-title>Journal of Virology</secondary-title><alt-title>J



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583 mber>2</number><keywords><keyword>nonstructural protein
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585 protein</keyword><keyword>3-dimensional
586 architecture</keyword><keyword>subcellular-localization</keyword><keyword>endopl
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589 ord>inhibition</keyword></keywords><dates><year>2015</year><pub-dates><date>Ja
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592 ISI>://WOS:000347178900035</url></related-urls></urls><electronic-resource-num
593 >10.1128/Jvi.02882-14</electronic-resource-num><language>English</language></reco
594 rd></Cite></EndNote>], we examined whether the replication defects of class III and IV
595 mutants were caused by defective maturation of NS2A. To test this hypothesis, we
596 introduced individual mutations into an expression plasmid pXJ-$E_{24}$-NS1-NS2A-HA that
597 encodes a polyprotein containing the C-terminal 24 amino acids of the E protein (serving
598 as a signal peptide to translocate NS1 to ER lumen), NS1, and NS2A with a C-terminal
599 HA tag. After transfecting the plasmids into 293T cells, the expression of un-cleaved



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NS1-NS2A-HA intermediate and cleaved NS2A-HA product were analyzed using Western blot (Fig. 3F). None of the mutations significantly affected the cleavage efficiencies of NS1-NS2A-HA to NS2A-HA, indicating that the replication defects of class III and IV mutants are not caused by impaired NS2A cleavage.

***Class V mutations block virion assembly***

Class V includes G71A, D73A, E103A, R171A, K192A, K193A, and a triple K25A/K26A/R27A. Cells transfected with class V mutant genomic RNAs yielded E-positive cells at 24 h p.t., but the number of E-positive cells did not increase afterward (Fig. 4A), suggesting that the mutants are defective in virus spread. No infectious mutant viruses, except E103A, were detected in the supernatants of transfected cells by plaque assay (Fig. 4B) or RT-PCR (Fig. 4C). No IFA-positive cells were detected after naïve cells were incubated with the supernatants from transfected cells (Fig. 4D). Sequencing of the recovered E103A mutant virus revealed an A103D change in the NS2A gene (Fig. 4C), suggesting that an E103A-to-A103D substitution is responsible for the rescue of viral replication.

To determine the stage of viral replication that was disrupted by the class V mutations, we analyzed the mutational effect on viral replication in ZIKV replicon (Fig. 4E). Compared with the WT replicon, all class V mutants exhibited equivalent levels of viral translation and RNA synthesis (<4-fold difference), suggesting that the mutations only marginally affect viral RNA synthesis. Next, we tested if the class V genomic RNAs



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could generate intracellular infectious viruses. Incubating naïve Vero cells with WT RNA-transfected cell extracts yielded IFA-positive cells, whereas no IFA-positive cells were detected after incubating naive cells with the mutant RNA-transfected cell extracts (Fig. 4F). Altogether, the results suggest that class V mutants are competent in viral RNA replication, but defective in assembly of infectious viruses.

***WT NS2A rescues virion assembly for class V mutants***

To test *trans* complementation of NS2A mutants, we constructed a BHK-21 cell line that constitutively expressed WT ZIKV NS2A protein with a C-terminal HA-tagged (NS2A-HA). The cell line was constructed by transfecting plasmid pXJ-SPG-$C_{16}$-NS2A-HA encoding a Gaussia luciferase signal peptide (SPG), the C-terminal 16 amino acids of NS1 protein ($C_{16}$), and NS2A-HA, as well as a neomycin resistance gene (*Neo*). The SPG and $C_{16}$ sequences were engineered to enable the correct formation of NS2A membrane topology when expressed in cells. Individual BHK-NS2A-HA cell lines were generated through colony purification under G418 (1 mg/ml) selection. For each BHK-NS2A-HA cell line, all cells were IFA-positive for HA-NS2A expression (Fig. 5A). Western blot revealed an expected band of ~22-kDa band (Fig. 5B). The expression level of NS2A-HA did not decrease in the BHK-NS2A-HA cells after 20 passages in culture (4 days per passage; Fig. 5B), indicating the stability of the cell line.

We tested *trans* complementation by transfecting class V genomic RNAs to



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BHK-NS2A-HA cells. The transfected cells developed an increasing number of E-positive cells from 48 to 96 h p.t. (Fig. 5C). Culture supernatants were collected at 96 h p.t. to infect naïve Vero cells. The infected cells developed E-positive cells for all class V mutants (Fig. 5D), demonstrating the rescue of infectious viruses through *trans* complementation. Quantification of the E-positive cells allowed us to determine the titers of rescued infectious viruses (Fig. 5E). Among the class V mutants, E103A, K192A, and K193A exhibited the highest *trans* complementation efficiencies, producing $1.9\times10^5$ to $3.04\times10^5$ IFU/ml viruses at 96 h p.i. (Fig. 5E). Sequencing of the rescued virion genomes confirmed the retention of the engineered class V mutations without any other changes in NS2A (Fig. 5E).

Next, supernatants from the first round of infection collected at 72 h p.i. (from Fig. 5D) were used to infect naïve Vero cells for a second round. In contrast to the first round of infection (Fig. 5D), no E-positive cells were detected in the second round of infection for any of the class V mutants (Fig. 5F). As a positive control, WT RNA produced high numbers of E-positive cells in both the first and second rounds of infections (Fig. 5D&F). Collectively, the results indicate that (i) the defect in virion assembly of class V mutants could be rescued through *trans* supply of WT NS2A protein and (ii) the rescued virions could only infect cells for a single round.

***DENV-2 NS2A could not trans complement ZIKV class V mutants***

We tested whether DENV NS2A could rescue class V mutants of ZIKV. Each class V



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mutant of ZIKV was transfected into a previously established BHK-21 cell line that constitutively expresses WT DENV-2 NS2A protein [ ADDIN EN.CITE <EndNote><Cite><Author>Xie</Author><Year>2015</Year><RecNum>279</RecNum><DisplayText>[29]</DisplayText><record><rec-number>279</rec-number><foreign-keys><key app="EN" db-id="fawazxzw2e20pteerwsvxwel59p2ffetexz2" timestamp="1532700599">279</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Xie, X. P.</author><author>Zou, J.</author><author>Puttikhunt, C.</author><author>Yuan, Z. M.</author><author>Shi, P. Y.</author></authors></contributors><auth-address>Novartis Inst Trop Dis, Singapore, SingaporeChinese Acad Sci, State Key Lab Virol, Wuhan Inst Virol, Wuhan, Peoples R ChinaNatl Sci & Technol Dev Agcy, Med Biotechnol Unit, Natl Ctr Genet Engn & Biotechnol, Bangkok, Thailand</auth-address><titles><title>Two Distinct Sets of NS2A Molecules Are Responsible for Dengue Virus RNA Synthesis and Virion Assembly</title><secondary-title>Journal of Virology</secondary-title><alt-title>J Virol</alt-title></titles><alt-periodical><full-title>J Virol</full-title></alt-periodical><pages>1298-1313</pages><volume>89</volume><number>2</number><keywords><keyword>nonstructural protein ns2a</keyword><keyword>double-stranded-rna</keyword><keyword>ns4b protein</keyword><keyword>3-dimensional



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681 architecture</keyword><keyword>subcellular-localization</keyword><keyword>endopl
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688 >10.1128/Jvi.02882-14</electronic-resource-num><language>English</language></reco
689 rd></Cite></EndNote>]. For all class V mutants, no increase in E-positive cells or
690 infections viruses was detected from the transfected cells from 24 to 96 h p.t. (data not
691 shown). The results suggest that flavivirus NS2A modulates virion assembly in a
692 virus-type specific manner.

693 ***WT NS2A could not rescue viral RNA synthesis of class III mutants***

694 We tested whether the defect in viral RNA synthesis of class III mutants could be *trans*
695 complemented. To test this possibility, we transfected class III genomic RNAs (G12A and
696 N130A) into ZIKV BHK-NS2A-HA cells. Neither mutants generated any E-positive cells
697 in the transfected BHK-NS2A-HA cells (Fig. 5C). Infection of naïve Vero cells with
698 supernatants of the transfected cells did not produce any IFA-positive cells (Fig. 5D-F).
699 The results suggest that the RNA synthesis defect of class III mutants could not be
700 rescued through *trans* complementation. Due to the dual defects of class IV mutants in



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both viral RNA synthesis and virion assembly, we did not pursue *trans* complementation experiments for this class of mutants; such complementation experiments may not reveal clear mechanistic insights as compared with the class III and V mutants.

***Probing the topology of ZIKV NS2A on the ER membrane***

The distinct functions of amino acids in viral RNA synthesis and virion assembly prompted us to probe the topology of ZIKV NS2A on ER membrane. Bioinformatics analysis suggests that ZIKV NS2A may form seven transmembrane segments (pTMS1-7; Fig. 6A). To examine whether each pTMS associates with the ER membrane, we constructed a panel of plasmids encoding full-length or various NS2A fragments with a C-terminally fused eGFP reporter (Fig. 6B). The length of each segment was slightly modified from the bioinformatic prediction to allow some flexibility between pTMS and eGFP. Upon transfection into Vero cells, eGFP alone (without any NS2A fragment) exhibited an even distribution of fluorescence throughout the cell, whereas the full-length NS2A-eGFP (construct 1–226) displayed fluorescence co-localized with calnexin (an ER membrane protein) in the perinuclear region (Fig. 6B), indicating that ZIKV NS2A is an integral membrane protein. Expression of seven fragments spanning each pTMS, represented by constructs 1-25 (pTMS1), 30-58 (pTMS2) , 74-97 (pTMS3), 101-123 (pTMS4), 125-145 (pTMS5), 150-185 (pTMS6), and 198-221 (pTMS7), exhibited fluorescence patterns that co-localized with calnexin in the perinuclear region (Fig. 6B). The results indicate that individual of the seven pTMSes is associated with the ER



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721 membrane. In contrast, expression of fragments between different pTMS, represented by
722 constructs 55-73, 186-197, and 221-226, showed fluorescence throughout the cells (Fig.
723 6B), indicating that these fragments are not membrane-associated.

724 An *in vitro* deglycosylation analysis was performed to determine the orientation of
725 each NS2A pTMS on the ER membrane. A panel of plasmids were prepared to express
726 truncated NS2A proteins that were C-terminally fused with an eGFP followed by an
727 N-glycosylation acceptor peptide (Glyc; Fig. 7A). An SPG-$C_{16}$ peptide (Gaussia
728 luciferase signal peptide SPG and the C-terminal 16 amino acids of NS1) was fused to
729 the N terminus of each NS2A-eGFP-Glyc construct to ensure a proper translocation of
730 the expressed protein on the ER membrane. Western blot showed that, upon transfection
731 into BHK-21 cells, all constructs expressed fusion proteins with expected molecular
732 masses (Fig. 7B). Because N-glycosylation enzymes reside in the ER lumen, the
733 glycosylation status could be used to indicate the location of the C-terminus of each
734 NS2A-eGFP-Glyc protein (Fig. 7C). As a negative control, treatment of EGFP-Glyc
735 (without any viral sequence) with PNGase F (an enzyme that removes high mannose and
736 complex carbohydrates) did not change the mobility of the protein (Fig. 7D), indicating a
737 cytosol localization of EGFP-Glyc. As a positive control, PNGaseF treatment increased
738 the mobility of SPG-$C_{16}$-EGFP-Glyc (construct 0), confirming that the C-terminus of this
739 protein resides in the ER lumen. Among the NS2A constructs, PNGase F treatment
740 increased the mobility of fusion proteins 1-26 and 1-56, but not 1-103, 1-123, 1-147,



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741 1-192, and 1-226 (Fig. 7D). The results suggest that the N-terminal 56 amino acids of
742 NS2A reside in the ER lumen, and the C-terminal region from residues 103 to 226 is
743 located in the cytosol.

744 Next, we performed an *in vitro* proteinase K protection assay [ ADDIN EN.CITE
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751 M.</author><author>Shi, P.
752 Y.</author></authors></contributors><auth-address>Chinese Acad Sci, Wuhan Inst Virol,
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756 Dengue Virus NS2A Protein</title><secondary-title>Journal of
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769 >10.1128/Jvi.02424-12</electronic-resource-num><language>English</language></reco
770 rd></Cite></EndNote>] to validate the above *in vitro* deglycosylation results. BHK-21
771 cells were transfected with the same constructs depicted in Fig. 7A. At 24 h p.t.,
772 membrane fractions from the transfected cells were treated with proteinase K in the
773 presence or absence of 0.5% Triton X-100 and analyzed by Western blot using an eGFP
774 antibody. In the absence of Triton X-100, the eGFP residing in the cytosol would be
775 susceptible to proteinase K digestion, whereas the eGFP residing in the ER lumen would
776 be protected from proteinase K digestion (Fig. 7E). As shown in Fig. 7F, the eGFPs from
777 constructs 0, 1-26, and 1-56 were protected from protease K digestion, again indicating
778 that the first 56 residues are located in the ER lumen. In contrast, the eGFPs from
779 constructs 1-103, 1-123, 1-147, 1-192, 1-226, and control eGFP-Glyc were completely
780 degraded by the protease K treatment (Fig. 7F), supporting that the C-terminal region
781 from residues 103 to 226 is located in the cytosol. As expected, in the presence of Triton



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X-100, the eGFPs from all constructs were degraded by proteinase K (Fig. 7F). The results of proteinase K assay are in full agreement with those of deglycosylation assay.

***A topology model for ZIKV NS2A***

The above biochemical results support a topology model for ZIKV NS2A on the ER membrane (Fig. 8A): (i) The N-terminal region (including pTMS1 and pTMS2) resides in the ER lumen; (ii) the region spanning residues 74 to 97 (pTMS3) traverses the ER membrane in a lumen-to-cytosol direction; and (iii) the C-terminal region from residues 103 to 226 (including pTMS4 to pTMS7) is located in the cytosol side. Since pTMS1, pTMS2, pTMS4, PTMS5, PTM6, and pTMS7 associate with ER membraned when expressed individually (Fig. 6B), they may interact with the ER membrane in a peripheral manner. In support of this notion, informatics analyses using Jpred 4 and HeliQuest programs suggest that amino acids 9-26 (pTMS1), 41-58 (pTMS2), 104-121 (pTMS4), 125-142 (pTMS5), 172-189 (pTMS6), and 203-220 (pTM7) may form amphipathic helices with hydrophobic and hydrophilic surfaces (Fig. 8B), where the hydrophobic surface may interact with the ER membrane.

## Discussion

Flavivirus NS2A functions in viral RNA replication, virion assembly, evasion of innate immune response, and disease pathogenesis in a virus-specific manner [ ADDIN



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807 S.</author><author>Wieseler, J.</author><author>Kerber,
808 R.</author><author>Kummerer, B.
809 M.</author></authors></contributors><auth-address>Bernhard Nocht Inst Trop Med,
810 Dept Virol, D-20359 Hamburg, GermanyUniv Bonn, Inst Virol, Med Ctr, Bonn,
811 Germany</auth-address><titles><title>A Basic Cluster in the N Terminus of Yellow
812 Fever Virus NS2A Contributes to Infectious Particle
813 Production</title><secondary-title>Journal of Virology</secondary-title><alt-title>J
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821 cleavage</keyword><keyword>identification</keyword><keyword>vaccination</keyw


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826 >10.1128/Jvi.03351-14</electronic-resource-num><language>English</language></reco
827 rd></Cite></EndNote>]. This is evidenced by its high sequence diversity (Fig. 1A),
828 distinct membrane topology [ ADDIN EN.CITE ADDIN EN.CITE.DATA ], and
829 association with different disease pathways [ ADDIN EN.CITE ADDIN
830 EN.CITE.DATA ]. Thus, it is critical to perform a systematic genetic and biochemical
831 characterization of ZIKV NS2A to delineate its structure and function. Our biochemical
832 results showed that, unlike other flaviviruses, ZIKV NS2A has only a single
833 transmembrane segment that traverses the ER membrane, the other six pTMSes are
834 peripherally associated with the ER membrane (Fig. 8A). Our functional analysis
835 revealed five classes of mutants with distinct replication phenotypes, among which class
836 III, IV, and V mutants could not produce infectious viruses through different mechanisms
837 (Fig. 1B). The critical mutations from class III to V are not clustered in specific regions
838 of NS2A (Fig. 8A), suggesting that multiple domains of NS2A are essential for viral
839 replication.

840 The membrane topology of ZIKV NS2A is in stark contrast with the topologies of
841 other flavivirus NS2A proteins: DENV-2 NS2A has five membrane-traversing segments



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848 P.</author><author>Gayen, S.</author><author>Kang, C. B.</author><author>Yuan, Z.

849 M.</author><author>Shi, P.

850 Y.</author></authors></contributors><auth-address>Chinese Acad Sci, Wuhan Inst Virol,

851 State Key Lab Virol, Beijing 100864, Peoples R ChinaNovartis Inst Trop Dis,

852 Singapore, SingaporeAgcy Sci Technol & Res, Ctr Expt Therapeut,

853 Singapore, Singapore</auth-address><titles><title>Membrane Topology and Function of

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855 Virology</secondary-title><alt-title>J

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861 >viral replication</keyword><keyword>ns4b

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bition</keyword><keyword>cleavage</keyword><keyword>identification</keyword><keyword>flaviviruses</keyword><keyword>complement</keyword></keywords><dates><year>2013</year><pub-dates><date>Apr</date></pub-dates></dates><isbn>0022-538X</isbn><accession-num>WOS:000316671000043</accession-num><urls><related-urls><url><Go to ISI>://WOS:000316671000043</url></related-urls></urls><electronic-resource-num>10.1128/Jvi.02424-12</electronic-resource-num><language>English</language></record></Cite></EndNote>] and YFV NS2A has three membrane-traversing segments [ ADDIN EN.CITE <EndNote><Cite><Author>Vossmann</Author><Year>2015</Year><RecNum>423</RecNum><DisplayText>[34]</DisplayText><record><rec-number>423</rec-number><foreign-keys><key app="EN" db-id="sva5pexpet29snerv2jpeedvrdwz0fzfs2ee">423</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Vossmann, S.</author><author>Wieseler, J.</author><author>Kerber, R.</author><author>Kummerer, B. M.</author></authors></contributors><auth-address>Bernhard Nocht Inst Trop Med, Dept Virol, D-20359 Hamburg, GermanyUniv Bonn, Inst Virol, Med Ctr, Bonn, Germany</auth-address><titles><title>A Basic Cluster in the N Terminus of Yellow Fever Virus NS2A Contributes to Infectious Particle Production</title><secondary-title>Journal of Virology</secondary-title><alt-title>J



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892 ord></keywords><dates><year>2015</year><pub-dates><date>May</date></pub-dates
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894 n-num><urls><related-urls><url><Go to
895 ISI>://WOS:000352219600023</url></related-urls></urls><electronic-resource-num
896 >10.1128/Jvi.03351-14</electronic-resource-num><language>English</language></reco
897 rd></Cite></EndNote>]. Interestingly, these topologically diverse flavivirus NS2A
898 proteins are able to converge their functions in viral RNA replication, virion assembly,
899 and evasion of immune response. High resolution, three-dimensional structures of
900 flavivirus NS2A are needed to address this question. It should be noted that the
901 membrane topology of NS2A presented here is in the absence of other viral proteins. In
902 the context of viral replication, the NS2A protein is associated with various viral and
903 cellular proteins that would modulate the structure of NS2A and perform biological
904 functions.



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Our mutagenesis study has identified distinct NS2A mutations that selectively abolish ZIKV RNA synthesis (class III) or virion assembly (class V), among which only the virion assembly-defective mutants could be rescued through *trans* complementation (Fig. 5). The results suggest that there are two species of NS2A molecules in ZIKV-infected cells. One NS2A species is located at the viral replication complex that could only be formed in *cis*; another NS2A species is located at virion assembly complex that could be formed in *trans* or *cis*. These results are in agreement with the previous studies from DENV and YFV NS2A [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. Compared with the viral replication complex, the virion assembly complex is much less well defined for flaviviruses. Previously studies showed that second-site mutations in prM, E, NS2B, and NS3 could rescue the defect of virion assembly caused by DENV-2 and YFV NS2A mutations, suggesting that these viral proteins participate in virion assembly. Besides the genetic evidence, the role of NS3 in virion assembly is also supported by (i) mutations in the NS3 helicase domain alone affect YFV assembly [ ADDIN EN.CITE <EndNote><Cite><Author>Patkar</Author><Year>2008</Year><RecNum>467</RecNum><DisplayText>[47]</DisplayText><record><rec-number>467</rec-number><foreign-keys><key app="EN" db-id="sva5pexpet29snerv2jpeedvrdwz0fzfs2ee">467</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Patkar, C. G.</author><author>Kuhn, R.



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926 J.</author></authors></contributors><auth-address>Purdue Univ, Dept Biol Sci, W
927 Lafayette, IN 47907 USA</auth-address><titles><title>Yellow fever virus NS3 plays an
928 essential role in virus assembly independent of its known enzymatic
929 functions</title><secondary-title>Journal of Virology</secondary-title><alt-title>J
930 Virol</alt-title></titles><periodical><full-title>Journal of Virology</full-title><abbr-1>J
931 Virol</abbr-1></periodical><alt-periodical><full-title>Journal of
932 Virology</full-title><abbr-1>J
933 Virol</abbr-1></alt-periodical><pages>3342-3352</pages><volume>82</volume><nu
934 mber>7</number><keywords><keyword>tick-borne
935 encephalitis</keyword><keyword>flavivirus kunjin
936 ns3</keyword><keyword>west-nile-virus</keyword><keyword>dengue
937 virus</keyword><keyword>nonstructural protein</keyword><keyword>rna
938 replication</keyword><keyword>cdna-clones</keyword><keyword>particles</keyword
939 ><keyword>ns2a</keyword><keyword>organization</keyword></keywords><dates><y
940 ear>2008</year><pub-dates><date>Apr</date></pub-dates></dates><isbn>0022-538X
941 </isbn><accession-num>WOS:000254139800015</accession-num><urls><related-urls>
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943 ISI>://WOS:000254139800015</url></related-urls></urls><electronic-resource-num
944 >10.1128/Jvi.02447-07</electronic-resource-num><language>English</language></reco
945 rd></Cite></EndNote>], (ii) the helicase domain of DENV or YFV NS3 directly binds to
946 NS2A [ ADDIN EN.CITE ADDIN EN.CITE.DATA ], (iii) the cleavage between C



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947 and prM is tightly modulated by NS2B/NS3 protease and cellular signalase during YFV
948 and Murray Valey encephalitis virus assembly [ ADDIN EN.CITE ADDIN
949 EN.CITE.DATA ]. These data, together with the result that recombinant NS2A of
950 Kunjin binds to viral 3'UTR RNA [ ADDIN EN.CITE
951 <EndNote><Cite><Author>Mackenzie</Author><Year>1998</Year><RecNum>405</R
952 ecNum><DisplayText>[28]</DisplayText><record><rec-number>405</rec-number><fo
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954 db-id="sva5pexpet29snerv2jpeedvrdwz0fzfs2ee">405</key></foreign-keys><ref-type
955 name="Journal Article">17</ref-type><contributors><authors><author>Mackenzie, J.
956 M.</author><author>Khromykh, A. A.</author><author>Jones, M.
957 K.</author><author>Westaway, E.
958 G.</author></authors></contributors><auth-address>Royal Childrens Hosp, Sir Albert
959 Sakzewski Virus Res Ctr, Brisbane, Qld 4029, AustraliaUniv Queensland, Ctr
960 Microscopy & Microanal, St Lucia, Qld 4072,
961 Australia</auth-address><titles><title>Subcellular localization and some biochemical
962 properties of the flavivirus Kunjin nonstructural proteins NS2A and
963 NS4A</title><secondary-title>Virology</secondary-title><alt-title>Virology</alt-title><
964 /titles><periodical><full-title>Virology</full-title><abbr-1>Virology</abbr-1></periodic
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967 ds><keyword>japanese encephalitis-virus</keyword><keyword>yellow-fever



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968 virus</keyword><keyword>dependent rna-polymerase</keyword><keyword>west nile
969 flavivirus</keyword><keyword>escherichia-coli</keyword><keyword>positive
970 identification</keyword><keyword>replication
971 strategy</keyword><keyword>infected-cells</keyword><keyword>core
972 protein</keyword><keyword>ns5
973 protein</keyword></keywords><dates><year>1998</year><pub-dates><date>Jun
974 5</date></pub-dates></dates><isbn>0042-6822</isbn><accession-num>WOS:0000741
975 86800004</accession-num><urls><related-urls><url><Go to
976 ISI>://WOS:000074186800004</url></related-urls></urls><electronic-resource-num
977 >DOI
978 10.1006/viro.1998.9156</electronic-resource-num><language>English</language></rec
979 ord></Cite></EndNote>], suggest a flavivirus assembly model. During flavivirus
980 assembly, the NS2A protein recruits NS2B/NS3 through binding to the NS3 helicase
981 domain. Once the NS2A-NS2B/NS3 complex reaches the virion assembly site, the
982 NS3B/NS3 protease cleaves the C protein from the C/prM/E polyprotein. The C protein
983 assembles onto genomic RNA (presented by NS2A) to form nucleocapsid, which is
984 subsequently enveloped by prM/E complex to form virions. Since flavivirus assembly has
985 to be coupled to viral RNA replication [ ADDIN EN.CITE
986 <EndNote><Cite><Author>Khromykh</Author><Year>2001</Year><RecNum>471</R
987 ecNum><DisplayText>[51]</DisplayText><record><rec-number>471</rec-number><fo
988 reign-keys><key app="EN"



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db-id="sva5pexpet29snerv2jpeedvrdwz0fzfs2ee">471</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Khromykh, A. A.</author><author>Varnavski, A. N.</author><author>Sedlak, P. L.</author><author>Westaway, E. G.</author></authors></contributors><auth-address>Royal Childrens Hosp, Sir Albert Sakzewski Virus Res Ctr, Brisbane, Qld 4029, AustraliaUniv Queensland, Clin Med Virol Ctr, Brisbane, Qld, Australia</auth-address><titles><title>Coupling between replication and packaging of flavivirus RNA: Evidence derived from the use of DNA-based full-length cDNA clones of kunjin virus</title><secondary-title>Journal of Virology</secondary-title><alt-title>J Virol</alt-title></titles><periodical><full-title>Journal of Virology</full-title><abbr-1>J Virol</abbr-1></periodical><alt-periodical><full-title>Journal of Virology</full-title><abbr-1>J Virol</abbr-1></alt-periodical><pages>4633-4640</pages><volume>75</volume><number>10</number><keywords><keyword>double-stranded-rna</keyword><keyword>replicon rna</keyword><keyword>heterologous genes</keyword><keyword>infected cells</keyword><keyword>transcomplementation</keyword><keyword>proteins</keyword><keyword>ns1</keyword><keyword>trans</keyword><keyword>components</keyword><keyword>expression</keyword></keywords><dates><year>2001</year><pub-dates><date>May</date></pub-dates></dates><isbn>0022-538X</isbn><accession-num>WOS:000168309300017</accession-num><urls><related-urls><url><Go to



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ISI>://WOS:000168309300017</url></related-urls></urls><electronic-resource-num >Doi 10.1128/Jvi.75.10.4633-4640.2001</electronic-resource-num><language>English</language></record></Cite></EndNote>], the newly synthesized genomic RNA has to be transported from the replication site to the virion assembly site [ ADDIN EN.CITE ADDIN EN.CITE.DATA ], possibly through the NS2A protein.

The *trans* complemented class V mutants could potentially be used as vaccine candidates, as recently reported for DENV-2 [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. Since the *trans* complemented virions are able to infect cells for only a single cycle, such vaccine platform is safer than the multi-round infectious live-attenuated vaccines [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. This is particularly important for ZIKV vaccine when immunizing immune-compromised individuals and pregnant women [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. However, for development of class V mutants as a vaccine platform, the *trans* complementation efficiency needs to be improved to higher titers before they could be tested for *in vivo* efficacy.

In summary, we report the genetic and biochemical characterization of ZIKV NS2A protein. The results indicate that different flavivirus NS2A proteins have distinct membrane topologies, but converge to function in viral RNA synthesis and virion assembly. Together with previous results, we propose a model for flavivirus assembly in



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which NS2A plays a central role in mediating viral structural and nonstructural proteins as well as genomic RNA during virion formation.


## Acknowledgments

We thank Mariano Garcia-Blanco, Shelton Bradrick, and other colleagues at University of Texas Medical Branch for helpful discussions during the course of this study. P.-Y.S. lab was supported by a Kleberg Foundation Award, UTMB CTSA UL1TR-001439, NIH grants AI127744 and AI136126, and Cooperative Agreement Number U01CK000512 from the Centers for Disease Control and Prevention.


## Declaration of interests

The authors declare no competing interests.



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## References

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**Figure legend**

***Fig. 1. ZIKV NS2A mutagenesis.***

(A) Sequence alignment of NS2A proteins among ZIKV (strain FSS13025), DENV-1-4 serotypes, WNV, JEV, SLEV, and YFV using CLC Main Workbench software (CLC Bio). Thirty-five amino acids were selected for mutagenesis: (i) All 8 identical non-alanine residues (indicated by triangles), (ii) 25 charged residues (arrows), (iii) a K25/K26/R27 motif (line), (iv) residue N130 (circle). The consensus and conservation percentage are indicated below each amino acids. (B) A summary of ZIKV NS2A mutagenesis results. An infectious cDNA clone and a luciferase replicon of ZIKV strain FSS13025 were used to analyze the function of each amino acid. Based on the replication phenotypes, the 35 mutants could be categorized into five classes as indicated.

***Fig. 2. Characterization of class I, II, and III NS2A mutants.***

The results of representative mutants from class I (A-C), II (D-F), and III (G-K) are shown. (A) Vero cells were electroporated with equal amounts of ZIKV wild-type (WT) or class I mutant genomic RNAs. At indicated time points, the cells were fixed and subjected to IFA. Viral E protein and nuclei were stained with antibody 4G2 (green) and DAPI (blue), respectively. Cytopathic effect (CPE) is indicated at 72 h post-transfection. (B) Plaque morphologies. Recombinant viruses were subjected to plaque assay. Plaques were developed at day 4.5 post-infection. (C) Viral titers of class I mutants at 72 h



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post-transfection. The mean values from two independent experiments are shown. Error bars indicate the standard deviations. The limit of detection (LOD) of plaque assay is 10 PFU/ml. (D) IFA of class II mutant RNA-transfected Vero cells. (E) Plaque morphology of class II mutant viruses. (F) Viral titers of class II mutant viruses at 72 h post-transfection. (G) IFA of class III mutant RNA-transfected Vero cells. (H) Plaque morphologies of class III mutant viruses. Plaque assay was performed using culture supernatants collected at day 3 (for WT) and day 5 (for G12A and N130A) post-transfection. (I) RT-PCR analysis. At 5 day p.t., the mutant RNA-transfected cells were washed twice with PBS to remove residual input RNAs. At day 8 p.t., the supernatants of G12A and N130A RNA-transfected cells were subjected to RT-PCR. The WT virus (collected at 72 h p.t.) was used as a positive control. (J) A ZIKV luciferase replicon. $C_{38}$ and $E_{30}$ represent the first 38 amino acids of C protein and the last 30 amino acids of E protein, respectively. *Renilla* luciferase: Rluc. Foot-and-mouse-disease virus 2A sequence: 2A. (K) Transient replicon assay. WT, G12A, N130A, and NS5ΔGDD replicon RNAs were electroporated into Vero cells. Luciferase activities were measured at indicated time points. An average of 3 experiments are presented. Bars represent standard deviations.

***Fig. 3. Characterization of class IV NS2A mutants.***

(A) IFA of transfected Vero cells. (B) Plaque morphologies of WT, D7A, R140A, and R222A viruses. (C) Extracellular viral titers were measured at 24, 48, and 72 h p.t. using



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plaque assay. At 72 h p.t., extracellular viral RNAs were subjected RT-PCR analysis. The RT-PCR products were sequenced to confirm the engineered mutations and to detect potential second site mutation(s) in the NS2A gene. N.A., not applicable. (D) The supernatants of transfected cells were collected on day 8 p.t. to infect naïve Vero cells. At 24 h p.t., the cells were analyzed for viral E expression by IFA. (E) Transient luciferase replicon analysis. Vero cells were electroporated with ZIKV replicon RNAs and measured for luciferase activities at indicated time points. An average of 3 experiments are presented. Bars represent standard deviations. (F) Analysis of NS2A proteolytic processes. NS2A D7A, G12A, N130A, R140A, and R222A mutations were individually engineered into expression plasmid pXJ-$E_{24}$-NS1-NS2A-HA. After transfecting the plasmids into 293T cells, total cellular protein samples were harvested at 24 h p.t. and analyzed for NS2A process using Western blot. An HA-tag antibody was used to detect both unprocessed NS1-NS2A-HA and processed NS2A-HA proteins.

***Fig. 4. Characterization of class V NS2A mutants.***

(A) IFA of Vero cells that were transfected with WT or class V genomic RNAs. Viral E-positive cells are indicated in green. (B) Plaque morphologies of class V mutant viruses. Only the WT and E103A mutant generated plaques. (C) Supernatants from the RNA-transfected cells were quantified for viral titers by plaque assay. RT-PCR was also performed on the supernatants at day 3 p.t. for the WT virus and at day 8 p.t. for the mutants. Positive RT-PCR products were sequenced for potential second site mutation(s).



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1104 N.A., not applicable. (D) Naïve Vero cells were infected with supernatants from the
1105 RNA-transfected cells and subjected to IFA for viral E protein expression. (E) Luciferase
1106 replicon analysis. Equal amounts of WT and mutant replicon RNAs were electroporated
1107 into Vero cells. Luciferase activities were measured at the indicated time points. (F)
1108 Analysis of intracellular infectious viruses. Intracellular viruses from ZIKV
1109 RNA-transfected cells were harvested at 24 h p.t. as previously described [ ADDIN
1110 EN.CITE ADDIN EN.CITE.DATA ]. The harvested samples were used to infect
1111 naïve Vero cells. IFA was performed to examine viral E protein expression at 24 h
1112 post-infection.

1113 ***Fig. 5. Trans complementation of class III and V mutants.***

1114 (A) IFA of BHK-NS2A-HA cells. The BHK-NS2A-HA cells constitutively express WT
1115 ZIKV NS2A fused with a C-terminal HA-tag. The cells were fixed with
1116 paraformaldehyde and stained with an HA-tag antibody. (B) Western blot of
1117 BHK-NS2A-HA cells. The expression of WT NS2A-HA from BHK-NS2A-HA cells was
1118 detected using an HA-tag antibody. Both passage one (P1) and 20 (P20) BHK-NS2A-HA
1119 cells were analyzed. Naïve BHK-21 cells were included as a negative control. (C) WT
1120 and class III and V genomic RNAs were electroporated into BHK-NS2A-HA cells. IFA
1121 was performed on the transfected cells to detect viral E protein expression. (D) Viruses
1122 rescued from the BHK-NS2A-HA cells (harvested at 96 h p.t.) were used to infect naïve
1123 Vero cells. The infected Vero cells were subjected to IFA for E protein expression. (E)



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1124 Extracellular viruses were harvested from the supernatants of RNA-transfected
1125 BHK-NS2A-HA cells. The harvested viruses were serially diluted to infect naïve Vero
1126 cells in 96-well plates. At 24 h p.i., IFA was performed to quantify the viral titers by
1127 counting the number of E-positive cells. The results show averages and standard
1128 deviations from triplicate samples. Viral RNAs in the supernatants of transfected
1129 BHK-NS2A-HA cells were isolated at 96 h p.t. and subjected to RT-PCR. Positive
1130 RT-PCR products were sequenced to confirm the engineered mutations and to identify
1131 potential second site change(s). (F) The supernatants of infected Vero cells from (D) were
1132 collected at 72 h p.i. to infect naïve Vero cells for a second round. IFA was performed on
1133 these cells for viral E-positive cells.

1134 ***Fig. 6. Membrane association of ZIKV NS2A transmembrane segments.***

1135 (A) Bioinformatics analysis of ZIKV NS2A for transmembrane segments. ZIKV NS2A
1136 sequence was analyzed by bioinformatics program TOPCONS, Philius, PolyPhobius,
1137 SCAMPI, and TMHMM. The compiled transmembrane segments are shown at the
1138 bottom. Seven predicted transmembrane segments (pTMS) are presented as thick bars.
1139 The amino acid positions of ZIKV NS2A are indicated. (B) Membrane association of
1140 individual pTMS. Expression plasmid pXJ-eGFP (without NS2A sequence) and
1141 pXJ-smNS2A-eGFP (smNS2A represents different NS2A segments) were transfected into
1142 Vero cell by using X-tremeGENE 9 DNA transfection reagent (Roche). For each plasmid
1143 construct, the NS2A segment is indicated by amino acid positions. At 24 h p.t., IFA was



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performed to monitor eGFP expression. ER and nuclei were stained with anti-Calnexin antibody and DAPI, respectively. The merged images are shown on the right panels.

***Fig. 7. Probing the membrane topology of ZIKV NS2A by in vitro deglycosylation assay and in vitro proteinase K protection assay.***

(A) Schematic representation of C-terminally truncated ZIKV NS2A proteins. The amino acid positions of each truncated NS2A are indicated. A "SPG-C16" sequence was fused to the N terminus of NS2A to ensure correct translocation of NS2A on the ER membrane. An "eGFP-Glyc" sequence was fused to the C terminus of NS2A to probe its location at cytosol or ER lumen. See text for details. (B) BHK-21 cells were transfected with the expression plasmids described in (A). At 24 h p.t., the expression levels of various NS2A truncates were examined by Western blot using an eGFP antibody. The amino acid positions are indicated for each NS2A truncates. The molecular masses of marker proteins are shown to the left of Western blot image. (C) Diagram of glycosylation of eGFP-Glyc when located at ER lumen (*left*). No glycosylation occurs when eGFP-Glyc is located at cytosol (*right*). The glycosylation residue Asn is indicated. (D) *In vitro* deglycosylation assay. Plasmid-transfected cells were treated with PNGase F or PBS according to the protocol of *in vitro* deglycosylation assay (see Materials and Methods). The samples were examined for glycosylation status by Western blot using an eGFP antibody. (E) Diagram of *in vitro* proteinase K protection assay. When eGFP is located at ER lumen, it is protected from proteinase K cleavage (*left*); when located in the cytosol,



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1164 eGFP is degraded by proteinase K (*right*). (F) *In vitro* proteinase K protection assay. Cell
1165 lysates from plasmid-transfected BHK-21 cells were treated with proteinase K or PBS
1166 according to the protocol of *in vitro* proteinase K protection assay (see Materials and
1167 Methods). The samples were detected for eGFP by Western blot using an eGFP antibody.
1168 Triton X-100 treatment is indicated.

1169 ***Fig. 8. A topological model of ZIKV NS2A.***

1170 (A) A model of ZIKV NS2A membrane topology. See text for details. Class III, IV, and V
1171 mutations are depicted on the topology model. (B) Helical wheel representations of
1172 NS2A fragment 9-26, 41-58, 104-121, 125-142, 172-189, and 203-220. The amphiphilic
1173 helix regions were analyzed using Heliquest server (heliquest.ipmc.cnrs.fr/). The best
1174 18-residue windows are presented. The amino acids forming the hydrophobic surface of
1175 each helix wheel are depicted. Yellow, gray, blue, red, purple, pink, and green circles
1176 indicate nonpolar, glycine/alanine, positively charged, negatively charged, polar, amide,
1177 and proline amino acids, respectively. The first and last residues in each helical wheel are
1178 indicated by a blue and red arrow, respectively.



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# Genetic and biochemical characterizations of Zika virus NS2A protein

Xianwen Zhang[1,2,3], Xuping Xie[3,*], Jing Zou[3], Hongjie Xia[3], Chao Shan[3], Xinwen Chen[1,2,4,*], Pei-Yong Shi[3,5,6,7,8,*]

*[1]State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Sciences, Wuhan, China. [2]University of Chinese Academy of Sciences, Beijing, China. [3]Department of Biochemistry & Molecular Biology, University of Texas Medical Branch, Galveston, TX 77555, USA. [4]Guangzhou Institute of Biomedicine and Health, Chinese Academy of Sciences, Guangzhou, China. [5]Institute for Human Infections & Immunity, University of Texas Medical Branch, Galveston, Texas, USA. [6]Sealy Institute for Vaccine Sciences, University of Texas Medical Branch, Galveston, Texas, USA. [7]Sealy Center for Structural Biology & Molecular Biophysics, University of Texas Medical Branch, Galveston, Texas, USA. [8]Department of Pharmacology & Toxicology, University of Texas Medical Branch, Galveston, Texas, USA*

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**Abstract**

Zika virus (ZIKV) can cause devastating congenital Zika syndromes in pregnant women and Guillain-Barre syndrome in adults. Understanding the molecular mechanism of ZIKV replication is essential for antiviral and vaccine development. Here we report the structural and functional characterization of ZIKV NS2A protein. Biochemical structural probing suggests that ZIKV NS2A has a single segment that traverses the ER membrane and six segments that peripherally associate with the ER membrane. Functional analysis has defined distinct NS2A residues essential for viral RNA synthesis or virion assembly. Only the virion assembly-defective mutants, but not the RNA synthesis-defective mutants, could be rescued through *trans* complementation with a wide-type NS2A protein. These results suggest that the NS2A molecules in virion assembly complex could be recruited in *trans*, whereas the NS2A molecules in viral replication complex must be recruited in *cis*. Together with previous results, we propose a flavivirus assembly model where NS2A plays a central role in modulating viral structural and nonstructural proteins as well as genomic RNA during virion assembly.

**Keywords:** Zika virus; flavivirus replication; virion assembly; flavivirus NS2A; membrane topology



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## Introduction

Zika virus (ZIKV) is a recently emerged mosquito-borne flavivirus that is primarily transmitted by *Aedes spp.* mosquitoes. It can also be transmitted through sexual, maternal-to-fetal, and blood transfusion routes [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. ZIKV was first identified in 1947 from a sentinel rhesus monkey in Uganda [ ADDIN EN.CITE <EndNote><Cite><Author>Dick</Author><Year>1952</Year><RecNum>10</RecNum><DisplayText>[6]</DisplayText><record><rec-number>10</rec-number><foreign-keys><key app="EN" db-id="sva5pexpet29snerv2jpeedvrdwz0fzfs2ee">10</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Dick, G. W. A.</author><author>Kitchen, S. F.</author><author>Haddow, A. J.</author></authors></contributors><titles><title>Zika Virus .1. Isolations and Serological Specificity</title><secondary-title>Transactions of the Royal Society of Tropical Medicine and Hygiene</secondary-title><alt-title>T Roy Soc Trop Med H</alt-title></titles><periodical><full-title>Transactions of the Royal Society of Tropical Medicine and Hygiene</full-title><abbr-1>T Roy Soc Trop Med H</abbr-1></periodical><alt-periodical><full-title>Transactions of the Royal Society of Tropical Medicine and Hygiene</full-title><abbr-1>T Roy Soc Trop Med H</abbr-1></alt-periodical><pages>509-520</pages><volume>46</volume><number>



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5</number><dates><year>1952</year></dates><isbn>0035-9203</isbn><accession-num>WOS:A1952XT98500007</accession-num><urls><related-urls><url><Go to ISI>://WOS:A1952XT98500007</url></related-urls></urls><electronic-resource-num>Doi 10.1016/0035-9203(52)90042-4</electronic-resource-num><language>English</language></record></Cite></EndNote>]. Since 2007, it emerged in the Pacific region, South America, Central America, and the Caribbean, posing a global public health threat [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. ZIKV infection causes mild illnesses, including fever, pruritis, arthritis, conjunctivitis, and asthenia [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. Importantly, it can cause devastating congenital Zika syndromes (CZS; including microcephaly, congenital malformation, and fetal demise) in pregnant women and Guillain-Barre syndrome (GBS) in adults [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. The explosive epidemics and its association with CZS had led the World Health Organization (WTO) to declare ZIKV as a Public Health Emergency of International Concern from February to November in 2016 [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. Since then, a number of vaccine candidates have been developed, some of which have already advanced to clinical trials [ ADDIN EN.CITE

<EndNote><Cite><Author>Shan</Author><Year>2018</Year><RecNum>454</RecNum><DisplayText>[18]</DisplayText><record><rec-number>454</rec-number><foreign-keys><key app="EN"



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101 (Linking)</isbn><accession-num>30008291</accession-num><urls><related-urls><url>
102 http://www.ncbi.nlm.nih.gov/pubmed/30008291</url></related-urls></urls><custom2>6
103 112613</custom2><electronic-resource-num>10.1016/j.chom.2018.05.021</electronic-r
104 esource-num></record></Cite></EndNote>]. In addition, small molecule inhibitors and
105 therapeutic antibodies have also been identified for antiviral development [ ADDIN
106 EN.CITE ADDIN EN.CITE.DATA ].

Comment [SPy1]: Add reference
Jing Zou, Pei-Yong Shi. 2019. **Strategies for Zika Drug Discovery.** Current Opinion in Virology (in press)

107 ZIKV belongs to the *Flavivirus* genus within the *Flaviviridae* family. Besides ZIKV,
108 many other flaviviruses are significant human pathogens, including dengue (DENV),
109 yellow fever (YFV), West Nile (WNV), Japanese encephalitis (JEV), Saint Louis
110 encephalitis (SLEV), and tick-borne encephalitis virus (TBEV) [ ADDIN EN.CITE
111 <EndNote><Cite><Author>Mlakar</Author><Year>2016</Year><RecNum>265</Rec
112 Num><DisplayText>[22]</DisplayText><record><rec-number>265</rec-number><forei
113 gn-keys><key app="EN"
114 db-id="sva5pexpet29snerv2jpeedvrdwz0fzfs2ee">265</key></foreign-keys><ref-type
115 name="Journal Article">17</ref-type><contributors><authors><author>Mlakar,
116 J.</author><author>Korva, M.</author><author>Tul, N.</author><author>Popovic,
117 M.</author><author>Poljsak-Prijatelj, M.</author><author>Mraz,
118 J.</author><author>Kolenc, M.</author><author>Rus, K. R.</author><author>Vipotnik,
119 T. V.</author><author>Vodusek, V. F.</author><author>Vizjak,



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A.</author><author>Pizem, J.</author><author>Petrovec, M.</author><author>Zupanc, T. A.</author></authors></contributors><auth-address>Univ Ljubljana, Inst Pathol, Fac Med, Ljubljana, SloveniaUniv Ljubljana, Inst Microbiol & Immunol, Fac Med, Zaloska 4, Ljubljana 1000, SloveniaUniv Med Ctr Ljubljana, Dept Perinatol, Div Gynecol & Obstet, Ljubljana, SloveniaUniv Med Ctr Ljubljana, Inst Radiol, Ljubljana, Slovenia</auth-address><titles><title>Zika Virus Associated with Microcephaly</title><secondary-title>New England Journal of Medicine</secondary-title><alt-title>New Engl J Med</alt-title></titles><periodical><full-title>New England Journal of Medicine</full-title><abbr-1>New Engl J Med</abbr-1></periodical><alt-periodical><full-title>New England Journal of Medicine</full-title><abbr-1>New Engl J Med</abbr-1></alt-periodical><pages>951-958</pages><volume>374</volume><number>10</number><keywords><keyword>micronesia</keyword><keyword>brain</keyword><keyword>pcr</keyword></keywords><dates><year>2016</year><pub-dates><date>Mar 10</date></pub-dates></dates><isbn>0028-4793</isbn><accession-num>WOS:000371660000008</accession-num><urls><related-urls><url><Go to ISI>://WOS:000371660000008</url></related-urls></urls><electronic-resource-num>10.1056/Nejmoa1600651</electronic-resource-num><language>English</language></record></Cite></EndNote>]. Flavivirus genome is a positive-sense, single-stranded RNA



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of ~11,000 nucleotides. It contains a 5' untranslated region (UTR), a long open-reading-frame (ORF), and a 3' UTR. The single ORF encodes a polyprotein that is processed into three structure proteins (capsid [C], pre-membrane [prM] and envelope [E]) and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5). The structural proteins, together with genomic RNA, are components of virions. The nonstructural proteins participate in viral RNA replication, virion assembly, and evasion of host immune response [ ADDIN EN.CITE ADDIN EN.CITE.DATA ].

Flavivirus NS2A is an ER-resident, transmembrane protein with a calculated molecular weight of ~22 kDa. The N-terminus and C-terminus of NS2A are formed by cleavages with an unknown host protease and viral protease NS3 (in complex with NS2B), respectively [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. Flavivirus NS2A has multiple functions: (i) Viral replication. Kunjin virus (KUNV) NS2A co-localizes with viral double-stranded RNA and interacts with the 3'UTR of genomic RNA, NS3, and NS5 protein [ ADDIN EN.CITE <EndNote><Cite><Author>Mackenzie</Author><Year>1998</Year><RecNum>405</RecNum><DisplayText>[28]</DisplayText><record><rec-number>405</rec-number><foreign-keys><key app="EN" db-id="sva5pexpet29snerv2jpeedvrdwz0fzfs2ee">405</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Mackenzie, J. M.</author><author>Khromykh, A. A.</author><author>Jones, M.



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K.</author><author>Westaway, E.
G.</author></authors></contributors><auth-address>Royal Childrens Hosp, Sir Albert
Sakzewski Virus Res Ctr, Brisbane, Qld 4029, AustraliaUniv Queensland, Ctr
Microscopy & Microanal, St Lucia, Qld 4072,
Australia</auth-address><titles><title>Subcellular localization and some biochemical
properties of the flavivirus Kunjin nonstructural proteins NS2A and
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182 10.1006/viro.1998.9156</electronic-resource-num><language>English</language></rec
183 ord></Cite></EndNote>]. Mutagenesis studies confirmed the role of NS2A in viral RNA
184 synthesis [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. (ii) Virion
185 assembly/secretion. Mutagenesis analyses of YFV, KUNV, and DENV have identified
186 distinct NS2A amino acids that are essential for virion assembly/secretion [ ADDIN
187 EN.CITE ADDIN EN.CITE.DATA ]. The virion assembly defects can be restored by
188 second site mutations in NS2B or NS3 in YFV and DENV [ ADDIN EN.CITE ADDIN
189 EN.CITE.DATA ]. (iii) Evasion of innate immunity. ZIKV NS2A inhibits type-I IFN
190 induction at the step of TBK1 [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. DENV
191 NS2A antagonizes type-I IFN signaling [ ADDIN EN.CITE
192 <EndNote><Cite><Author>Munoz-Jordan</Author><Year>2003</Year><RecNum>389
193 </RecNum><DisplayText>[24]</DisplayText><record><rec-number>389</rec-number>
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196 name="Journal Article">17</ref-type><contributors><authors><author>Munoz-Jordan,
197 J. L.</author><author>Sanchez-Burgos, G. G.</author><author>Laurent-Rolle,
198 M.</author><author>Garcia-Sastre,
199 A.</author></authors></contributors><auth-address>Mt Sinai Sch Med, Dept Microbiol,
200 New York, NY 10029 USA</auth-address><titles><title>Inhibition of interferon
201 signaling by dengue virus</title><secondary-title>Proceedings of the National Academy
202 of Sciences of the United States of America</secondary-title><alt-title>P Natl Acad Sci



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C.</author><author>Yu, C. Y.</author><author>Liang, J. J.</author><author>Lin, E.
L.</author><author>Liao, C. L.</author><author>Lin, Y.
L.</author></authors></contributors><auth-address>Natl Def Med Ctr, Grad Inst Life
Sci, Taipei, TaiwanNatl Def Med Ctr, Dept Microbiol & Immunol, Taipei,
TaiwanAcad Sinica, Inst Biomed Sci, Taipei, TaiwanAcad Sinica, Genom
Res Ctr, Taipei 115, TaiwanCent Taiwan Univ Sci & Technol, Dept Food Sci
& Technol, Taichung, Taiwan</auth-address><titles><title>Blocking
Double-Stranded RNA-Activated Protein Kinase PKR by Japanese Encephalitis Virus
Nonstructural Protein 2A</title><secondary-title>Journal of
Virology</secondary-title><alt-title>J
Virol</alt-title></titles><periodical><full-title>Journal of Virology</full-title><abbr-1>J
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245 virus</keyword><keyword>5a

246 protein</keyword><keyword>viral-rna</keyword><keyword>infection</keyword></ke

247 ywords><dates><year>2012</year><pub-dates><date>Oct</date></pub-dates></dates>

248 <isbn>0022-538X</isbn><accession-num>WOS:000308740700010</accession-num><u

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250 ISI>://WOS:000308740700010</url></related-urls></urls><electronic-resource-num

251 >10.1128/Jvi.00525-12</electronic-resource-num><language>English</language></reco

252 rd></Cite></EndNote>]. In WNV and KUNV, the NS2A-mediated inhibition of type-I

253 IFN induction can be abrogated by specific NS2A mutations [ ADDIN EN.CITE

254 ADDIN EN.CITE.DATA ]. (iv) Disease pathogenesis. WNV NS2A modulates

255 virus-induced cytopathic effect (CPE) and pathogenesis in mice [ ADDIN EN.CITE

256 ADDIN EN.CITE.DATA ]. ZIKV NS2A impairs mammalian cortical neurogenesis

257 through depleting adherens junction proteins [ ADDIN EN.CITE ADDIN

258 EN.CITE.DATA ].

259 The structure and function of ZIKV NS2A have not been characterized. Although

260 NS2A proteins from different flaviviruses perform similar functions (described above),

261 each flavivirus may exert its NS2A functions in a virus-specific manner [ ADDIN

262 EN.CITE

263 <EndNote><Cite><Author>Vossmann</Author><Year>2015</Year><RecNum>423</R

264 ecNum><DisplayText>[34]</DisplayText><record><rec-number>423</rec-number><fo



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S.</author><author>Wieseler, J.</author><author>Kerber,
R.</author><author>Kummerer, B.
M.</author></authors></contributors><auth-address>Bernhard Nocht Inst Trop Med,
Dept Virol, D-20359 Hamburg, GermanyUniv Bonn, Inst Virol, Med Ctr, Bonn,
Germany</auth-address><titles><title>A Basic Cluster in the N Terminus of Yellow
Fever Virus NS2A Contributes to Infectious Particle
Production</title><secondary-title>Journal of Virology</secondary-title><alt-title>J
Virol</alt-title></titles><periodical><full-title>Journal of Virology</full-title><abbr-1>J
Virol</abbr-1></periodical><alt-periodical><full-title>Journal of
Virology</full-title><abbr-1>J
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ns2a</keyword><keyword>dengue virus</keyword><keyword>west
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## Materials and methods

### *Cell lines*

Vero cells, baby hamster kidney (BHK-21) cells and human embryo kidney 293T cells were cultured at 37°C with 5% CO2 in high-glucose Dulbecco's modified Eagle medium (DMEM; Life Technologies) supplemented with 2 mM L-glutamine, 100U/ml penicillin, 100g/ml streptomycin and 10% fetal bovine serum (FBS; HyClone Laboratories).

### *Bioinformatics*

NS2A protein sequences from ZIKV, DENV1, DENV2, DENV3, DENV4, WNV, JEV, YFV, SLEV were aligned using CLC Main Workbench software (CLC Bio). Prediction of ZIKV NS2A was carried out using various web servers, which including TOPCONS, Philius, Polyphobius, SCAMPI, and TMHMM2. The amphipathic helices of ZIKV NS2A were predicted by HeliQuest.



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304 ***Antibody***

305 The following antibodies were used in this study: a mouse monoclonal antibody 4G2
306 cross-reactive with flavivirus E protein (American Type Culture Collection); Rabbit
307 anti-HA monoclonal antibody (Cell Signaling Technology); Alexa Fluor®488 conjugated
308 mouse anti-HA monoclonal antibody (Cell Signaling Technology); Rabbit anti-Calnexin
309 polyclonal antibody(Abcam); Mouse anti-GFP monoclonal antibody (Sigma-Aldrich);
310 Horseradish peroxidase(HRP) conjugated goat anti-mouse and anti-rabbit IgG polyclonal
311 antibodies (Sigma-Aldrich); Alexa Fluor®488 conjugated goat anti-mouse IgG
312 polyclonal antibody (Thermo Fisher Scientific); Alexa Fluor®568 conjugated goat
313 anti-rabbit IgG polyclonal antibody (Thermo Fisher Scientific).

314 ***Plasmid construction***

315 The Infectious cDNA clone (pFLZIKV) bearing the full-length genome of ZIKV (strain
316 FSS13025) or Renilla luciferase (Rluc) reporter ZIKV replicon (pZIKV Rep WT) were
317 used as the backbone for introducing NS2A mutations [ ADDIN EN.CITE ADDIN
318 EN.CITE.DATA ]. XhoI and KpnI restriction sites were initially generated into
319 pFLZIKV and pZIKV Rep WT replicon at positions 3018 and 5010 in ZIKV genome
320 without changing amino acid sequences. The NS2A alanine-scanning mutagenesis was
321 performed by using a QuikChange II XL Site-Directed Mutagenesis kit (Agilent
322 Technologies) as described previously [ ADDIN EN.CITE
323 <EndNote><Cite><Author>Xie</Author><Year>2015</Year><RecNum>279</RecNum



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326 timestamp="1532700599">279</key></foreign-keys><ref-type name="Journal
327 Article">17</ref-type><contributors><authors><author>Xie, X.
328 P.</author><author>Zou, J.</author><author>Puttikhunt, C.</author><author>Yuan, Z.
329 M.</author><author>Shi, P.
330 Y.</author></authors></contributors><auth-address>Novartis Inst Trop Dis, Singapore,
331 SingaporeChinese Acad Sci, State Key Lab Virol, Wuhan Inst Virol, Wuhan,
332 Peoples R ChinaNatl Sci & Technol Dev Agcy, Med Biotechnol Unit, Natl
333 Ctr Genet Engn & Biotechnol, Bangkok,
334 Thailand</auth-address><titles><title>Two Distinct Sets of NS2A Molecules Are
335 Responsible for Dengue Virus RNA Synthesis and Virion
336 Assembly</title><secondary-title>Journal of Virology</secondary-title><alt-title>J
337 Virol</alt-title></titles><alt-periodical><full-title>J
338 Virol</full-title></alt-periodical><pages>1298-1313</pages><volume>89</volume><nu
339 mber>2</number><keywords><keyword>nonstructural protein
340 ns2a</keyword><keyword>double-stranded-rna</keyword><keyword>ns4b
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342 architecture</keyword><keyword>subcellular-localization</keyword><keyword>endopl
343 asmic-reticulum</keyword><keyword>membrane
344 topology</keyword><keyword>replication</keyword><keyword>ns3</keyword><keyw



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345 ord>inhibition</keyword></keywords><dates><year>2015</year><pub-dates><date>Ja
346 n</date></pub-dates></dates><isbn>0022-538X</isbn><accession-num>WOS:0003471
347 78900035</accession-num><urls><related-urls><url><Go to
348 ISI>://WOS:000347178900035</url></related-urls></urls><electronic-resource-num
349 >10.1128/Jvi.02882-14</electronic-resource-num><language>English</language></reco
350 rd></Cite></EndNote>].

351 A mammalian expression vector pXJ with a cytomegalovirus (CMV) promoter was
352 used for constructing plasmids expressing NS2A and its derivatives. To construct the
353 pXJ-$E_{24}$-NS1-NS2A-HA plasmid, a gene cassette encoding the last 24 amino acids of E
354 protein ($E_{24}$), NS1 and NS2A with a C-terminal hemagglutinin (HA) tag was firstly
355 amplified from the pFLZIKV and cloned into the pXJ vector. Next, 35 NS2A mutations
356 were individually introduced into the pXJ-$E_{24}$-NS1-NS2A-HA by overlap PCR, resulting
357 in plasmids expressing NS2A mutants.

358 To construct the pXJ-SPG-$C_{16}$-NS2A-HA that was used for selecting cell line
359 constitutively expressing NS2A protein, a DNA fragment containing the Gaussia
360 luciferase signal peptide (SPG), the last 16 amino acids of NS1 protein ($C_{16}$) and NS2A
361 tagged with a C-terminal HA tag sequence were constructed by overlap PCR and cloned
362 into the pXJ vector via NotI/XhoI restriction sites.

363 Plasmid pXJ-eGFP was constructed by inserting the enhanced green fluorescence
364 protein (eGFP) sequence into the pXJ vector via BamHI/XhoI restriction sites. DNA



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fragments that encode different NS2A segments were individually inserted into above pXJ-eGFP vector, resulting various pXJ-smNS2A-eGFP plasmids (smNS2A represents NS2A segment). A DNA fragment that express NS2A or truncated NS2A and an eGFP with a C-terminal N-glycosylation acceptor site (Glyc) were amplified and cloned into the pXJ-SPG-$C_{16}$-NS2A-HA vector, resulting in the plasmid pXJ-SPG-$C_{16}$-NS2A-eGFP-Glyc and various pXJ-SPG-$C_{16}$-ctNS2A-eGFP-Glyc plasmids (ctNS2A represents C-terminal truncated NS2A).

***RNA in vitro transcription and electroporation***

All full-length cDNA clone plasmids and subgenomic replicons of ZIKV were linearized with restriction enzyme ClaI, followed by phenol-chloroform extraction and ethanol precipitation. RNA transcripts were prepared by using the T7 mMessage mMachine kit (Ambion). For RNA transfection, 10 μg *in vitro* transcribed RNA was electroporated into $8\times10^6$ cells (BHK-21 or Vero) by using Gene Pulser Xcell™ Electroporation Systems (Bio-rad) as described previously [ ADDIN EN.CITE ADDIN EN.CITE.DATA ].

***Immunofluorescence assay***

For indirect immunofluorescence assay (IFA), cells were seeded in an 8-well Nunc Lab-Tek II Chamber Slide (Thermo Fisher Scientific). At various time points, cells were washed three times with phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde in PBS at room temperature for 30 min and permeabilized with 0.1%



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Triton X-100 in PBS at room temperature for 10 min. The permeabilized cells were then washed three times with PBS and incubated in a blocking buffer containing 1% FBS in PBS at room temperature for 1h. E protein was stained with a 1:1000 dilution of mouse monoclonal antibody 4G2 in blocking buffer. After incubation for 1h, the cells were washed three times with a washing buffer (1% FBS and 0.5% Tween 20 in PBS). After 1 hour of incubation with a secondary antibody (Alexa Fluor®488 conjugated goat anti-mouse IgG), the cells were washed three times with washing buffer and counterstained with DAPI (4',6-diamidino-2-phenylindole). Images were acquired using an Olympus fluorescence microscope. Images were processed in ImageJ software (National Institutes of Health, MD).

***Plaque assay***

$2\times10^5$ Vero cells per well were grown in 24-well plates one day prior to viral infection. 10-fold serial dilutions of virus samples were prepared in DMEM containing 2% FBS and 1% penicillin/streptomycin and inoculated (100 µl) into each well of 24-well plates. After 1 h of incubation at 37°C with 5% $CO_2$, the inoculum was replaced with 500 µl overlay medium (DMEM medium containing 0.8% methyl-cellulose, 2% FBS and 1% penicillin/streptomycin). Plaques formed after incubation at 37°C with 5% $CO_2$ for 4-5 days. Cells were fixed with 4% paraformaldehyde for 30 min, followed by staining with 1% crystal violet. The numbers of plaque per milliliter (PFU/ml) of ZIKV were calculated.



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403 ***Replicon transient-transfection assay***

404 Briefly, $8\times10^6$ Vero cells were electroporated with 10 μg wild type or mutant Rluc-ZIKV
405 replicon RNAs. After electroporation, $3\times10^5$ cells per well were seeded in 12-well plates.
406 At given time points, the cells were washed twice with PBS and lysed in 200 μl of
407 luciferase lysis buffer (Promega). The luciferase activity assay was performed according
408 to the manufacturer's protocol.

409 ***RNA extraction and cDNA sequencing***

410 The culture fluids from infected cells were centrifuged at 1000 g for 10 min and then
411 filtered through a 0.22 μm pore size polyethersulfone membrane (Millipore) to remove
412 cellular debris. Viral RNAs in the supernatants were extracted using a QIAamp
413 viral-RNA Minikit (Qiagen) by following the manufacturer's protocol. Reverse
414 transcription PCR (RT-PCR) was performed by using the SuperScript® III One-Step
415 RT-PCR System (Thermo Fisher Scientific). The amplified cDNA fragments were
416 purified and sent for Sanger sequencing at the GENEWIZ facilities.

417 ***Selection of NS2A-HA BHK-21 cell line***

418 $4\times10^5$ BHK-21 cells per well of were seeded into a 6-well plate. The plasmid
419 pXJ-SPG-$C_{16}$-NS2A-HA that contains a neomycin resistance gene was transfected into
420 BHK-21 cells (4 μg per well) using X-tremeGENE 9 DNA transfection reagent (Roche).
421 At 24h post-transfection, geneticin (G418) was added to the culture medium with a



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concentration of 1 mg/ml. The medium was changed every three days. After two weeks, several neomycin-resistant individual colonies were selected for propagation. The expression of NS2A-HA protein in stable cell lines was analyzed by immunofluorescence assay and Western blot.

***In vitro proteinase K protection assay***

Proteinase K protection assay was performed as previously described with minor modifications [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. BHK-21 cells were transfected in 6-well plates with various pXJ-SPG-$C_{16}$-ctNS2A-eGFP-Glyc plasmids. After incubated at 30°C for 24h, cells were trypsinized, washed twice with PBS and centrifuged at 450×g for 5 min. The cell pellets were resuspended in a hypotonic lysis buffer (10 mM Tris-HCl, pH 7.5, and 2 mM $MgCl_2$) and kept on ice for 10 min. Subsequently, the samples were homogenized with 40 strokes and centrifuged at 1,000×g for 5 min. The postnuclear supernatants (PNS) were divided into three aliquots and centrifuged at 15,000×rpm for 40 min. The pellets from three aliquots were then resuspended in PBS, 60 μg/ml proteinase K in PBS, or 60 μg/ml proteinase K in PBS in the presence of 0.5% Triton X-100, respectively. The samples were incubated on ice for 90 min. 20 mM phenylmethylsulfonyl fluoride (PMSF) was finally added to terminate the reaction. After further incubation on ice for 15 min, the samples were mixed with 4× Laemmli sample buffer, heated at 95°C for 15 min and analyzed by Western blotting.



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441 ***In vitro deglycosylation assay***

442 The samples were prepared by following a previously described protocol [ ADDIN
443 EN.CITE ADDIN EN.CITE.DATA ]. Briefly, the transfected BHK-21 cells in 6-well
444 plates were incubated at 30°C for 24 h. Cells were trypsinized and suspended in DMEM
445 medium containing 10%FBS. The cells were pelleted by centrifuging at 450×g for 5 min.
446 After two PBS washes, the cell pellets were resuspended in a solubilization buffer (20
447 mM Tris-HCl, pH 7.5, 50 mM NaCl, 10 mM $Mg(CH_3COO)_2$, 1% Triton X-100, and
448 EDTA-free protease inhibitors [Roche]) and incubated at 4°C for 2 h with gentle agitation.
449 After centrifugation at 15,000 rpm 4°C for 20 min to remove cell debris, the supernatants
450 were split into two aliquots. 50,000 units/ml N-glycosidase F (PNGase F) or PBS
451 (no-treatment control) were added to those two aliquots. After incubation at 37°C for 2 h,
452 the samples were mixed with 4× Laemmli sample buffer, heated at 70°C for 15 min and
453 analyzed by Western blot.

454 ***SDS-PAGE and western blot***

455 Protein samples were separated by 12% SDS-polyacrylamide gel electrophoresis and
456 transferred onto a polyvinylidene difluoride (PVDF) membrane using Trans-Blot Turbo
457 transfer system (Bio-Rad), after which the membrane was blocked in TBST (10 mM
458 Tris-HCl, pH 8.0, 150 mM NaCl, and 0.1% Tween 20) supplemented with 5% nonfat
459 milk for 1h at room temperature. The blots were probed with primary antibodies (1:1,000
460 dilution) overnight at 4°C, washed three times with TBST and incubated with horseradish



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peroxidase (HRP) conjugated goat anti-mouse and anti-rabbit IgG polyclonal antibodies (1:10,000 dilution) for 1h. After three thorough TBST buffer washes, the blots were incubated with SuperSignal™ West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific). Chemiluminescence signals were detected in ChemiDoc System (Bio-Rad).

### *Quantification and statistical analysis*

IFA images processing and cell counting were performed in software ImageJ (NIH). Densitometry analysis was performed using Image lab version 6.0 (Bio-Rad Laboratories, Hercules, CA). All numerical data are presented as the mean ± SEM (standard error of mean). The size of each study or number of replicates, along with the statistical tests performed can be found in corresponding Figure Legends.

## Results

### *The rationale for ZIKV NS2A mutagenesis*

To identify critical residues in ZIKV NS2A, we performed a systematic alanine-scanning mutagenesis using an infectious cDNA clone of ZIKV strain FSS13025 [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. Sequence alignment of NS2A proteins from ZIKV, DENV, WNV, JEV, SLEV, and YFV revealed a high diversity: only 31 out of



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218-227 amino acids with a consensus of >80% among different flaviviruses (Fig. 1A). A total of 35 residues were selected for mutagenesis: (i) All 8 identical non-alanine residues, including G12, G47, D53, G71, F83, P87, E103, and K193. The amino acid numbering is based on the ZIKV sequence. (ii) All charged residues, including D4, D7, E22, K31, K56, E67, D73, H76, K84, R86, R96, R102, E122, D124, R140, R146, D148, R163, R171, K186, K188, K192, R207, D210, and R222. Charged residues were selected because they likely participate in inter- or intra-molecular interactions; alternatively, they may alter the locations where NS2A would traverse the ER membrane since charged residues are hydrophilic. In addition, NS2A D73H mutation was previously shown to attenuate WNV replication (ref). (iii) A K25/K26/R27 motif that was previously reported to be critical for YFV and DENV virion assembly (32, 42). (iv) Residue N130 that was previously shown to be required for NS1-NS2A cleavage and viral replication in DENV [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. The selected residues were mutated to alanine in the context of ZIKV genomic RNA. Equal amounts of wild-type (WT) and mutant RNAs were electroporated into Vero cells. After electroporation, RNA replication and virus spread were monitored by immunofluorescence assay (IFA); the production of infectious viruses was quantified by plaque assay and RT-PCR. The 35 mutants exhibited distinct phenotypes that could be categorized into five groups (Fig. 1B), as detailed in the following sections.

***Class I mutations have no or weak effects on viral replication***



**Comment [SPy2]:** [ HYPERLINK "https://www.ncbi.nlm.nih.gov/pubmed/17382364" \o "Virology." ] 2007 Jul 20;364(1):184-95. Epub 2007 Mar 23. **Mutations in West Nile virus nonstructural proteins that facilitate replicon persistence in vitro attenuate virus replication in vitro and in vivo.** [ HYPERLINK "https://www.ncbi.nlm.nih.gov/pubmed/?term=Rossi%20SL%5BAuthor%5D&cauthor=true&cauthor_uid=17382364" ][1], [ HYPERLINK "https://www.ncbi.nlm.nih.gov/pubmed/?term=Fayzulin%20R%5BAuthor%5D&cauthor=true&cauthor_uid=17382364" ], [ HYPERLINK "https://www.ncbi.nlm.nih.gov/pubmed/?term=Dewsbury%20N%5BAuthor%5D&cauthor=true&cauthor_uid=17382364" ], [ HYPERLINK "https://www.ncbi.nlm.nih.gov/pubmed/?term=Bourne%20N%5BAuthor%5D&cauthor=true&cauthor_uid=17382364" ], [ HYPERLINK "https://www.ncbi.nlm.nih.gov/pubmed/?term=Mason%20PW%5BAuthor%5D&cauthor=true&cauthor_uid=17382364" ].

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Fourteen of the 35 NS2A mutant RNAs, including D4A, E22A, K31A, G47A, D53A, E67A, H76A, E122A, R146A, D148A, R163A, K186A, K188A, and D210A, replicated at levels comparable to the WT RNA. These mutants were categorized as class I (Fig. 1B). The IFA images and plaque morphologies of three representatives (D53A, D148A, and R163A), as well as the WT, are shown in Fig. 2A and 2B, respectively. Most class I mutants (*e.g.*, D148A and R163A) yielded comparable E-expressing cells as the WT from 24 to 48 h post-transfection (p.t.). At 72 h p.t., severe CPE occurred among D148A, R163A, and WT RNA-transfected cells. Some class I mutants (*e.g.*, D53A) produced fewer E-positive cells than the WT at 48 h p.t., and spread to infect more cells at 72 h p.t (Fig. 2A). All class I mutants produced viruses with plaques similar to the WT virus (Fig. 2B). At 72 h p.t., the class I mutants produced infectious titers <4-fold lower than the WT did (Fig. 2C). Sequencing of the mutant viruses (collected at 72 h p.t.) confirmed the retaining of the engineered mutations without other changes (data not shown).

***Class II mutations significantly attenuate viral replication***

Nine NS2A mutants, including K56A, F83A, K84A, R86A, P87A, R96A, R102A, D124A, and R207A, significantly attenuated viral replication. These mutants were categorized into class II (Fig. 1B). Class II RNA-transfected cells produced fewer E-positive cells than the WT did (Fig. 2D). At 72 h p.t., only 30-50% of E-positive cells were detected in the class II RNA-transfected cells, whereas the WT RNA-transfected cells exhibited CPE. Class II mutant viruses formed smaller, opaque plaques (Fig. 2E),



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519 and the transfected cells produced >10-fold lower infectious titers than the WT
520 RNA-transfected cells at 72 h p.t. (Fig. 2F). Complete genome sequencing of the class II
521 mutant viruses (collected at 96 h p.t.) did not reveal reversions or any other mutations
522 (data not shown). Because of the weak phenotypes of class I and II mutants, we did not
523 further characterize them.

524 ***Class III mutations are lethal for viral RNA synthesis***

525 Class III includes two mutants, G12A and N130A, which were completely defective in
526 viral RNA synthesis (Fig. 1B). The G12A and N130A genomic RNA-transfected cells did
527 not produce any E-positive cells at 24 and 48 h p.t. (Fig. 2G). On day 5 to 8 p.t., no
528 infectious viruses were detected from culture medium by plaque assay (Fig. 2H) and
529 RT-PCR (Fig. 2I). To confirm the above results, we introduced G12A or N130A mutation
530 into a ZIKV luciferase replicon (Fig. 2J). A replication-defective replicon, containing an
531 NS5 polymerase active site ΔGDD mutation, was included as a negative control [ ADDIN
532 EN.CITE ADDIN EN.CITE.DATA ]. Vero cells were electroporated with equal
533 amounts of WT, G12A, N130A, or NS5 ΔGDD replicon RNAs. At 2-6 h p.t., all replicons
534 generated similar levels of luciferase signals, suggesting comparable levels of viral RNA
535 translation and transfection efficiencies (Fig. 2K). However, at 18-30 h p.t., G12A and
536 N130A replicons generated low luciferase signals as the non-replicative NS5 ΔGDD
537 replicon did, whereas the WT replicon produced increasing luciferase signals (Fig. 2K).
538 These results demonstrate that class III mutations completely abolish viral RNA



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synthesis.

***Class IV mutations impair both viral RNA synthesis and virion production***

Class IV includes three mutants, including D7A, R140A, and R222A (Fig. 1B). These mutant genomic RNAs produced fewer E-positive cells in the transfected cells than the WT RNA (Fig. 3A). For D7A or R140A mutants, the RNA-transfected cells did not produce infectious viruses, as suggested by plaque assay (Fig. 3B) and RT-PCR (Fig. 3C). Incubation of naïve Vero cells with culture supernatants from the D7A or R140A transfected cells did not generate any viral E-positive cells (Fig. 3D). Replicon analysis showed that D7A or R140A significantly compromised viral RNA synthesis (Fig. 3E). Since neither mutants could produce extracellular viruses (Fig. 3B&C), and could replicate at attenuated levels inside cells (Fig. 3E), D7A and R140A mutations may impair both viral RNA synthesis and virion production.

For R222A, the number of E-positive cells slightly increased in the genomic RNA-transfected cells from 24 to 72 h p.t. (Fig. 3A). A low level of infectious virus was detected from the culture medium by plaque assay (Fig. 3B) and RT-PCR (Fig. 3C). These data were supported by the positive-IFA results after incubating naive Vero cells with the supernatant from the R222A RNA-transfected cells (Fig. 3D). Sequencing the virus (collected at 72 h p.t.) revealed that, besides the engineered R222A mutation, the virus had acquired a second site mutation of L220F in NS2A (Fig. 3C). Replicon analysis showed that R222A significantly lowered viral RNA synthesis (Fig. 3E); no secondary



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559 mutation (*i.e.*, L220F) was detected in the replicon RNA at 30 h p.t. (data not shown).
560 These results suggest that (i) R222A mutant is defective in viral RNA synthesis and/or
561 virion assembly, and (ii) a second site mutation L220F in NS2A could rescue the defects.

562 ***Class III and IV mutations do not affect NS2A maturation***

563 Since cleavage between NS1 and NS2A is essential for flavivirus RNA synthesis
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573 SingaporeChinese Acad Sci, State Key Lab Virol, Wuhan Inst Virol, Wuhan,
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577 Responsible for Dengue Virus RNA Synthesis and Virion
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593 >10.1128/Jvi.02882-14</electronic-resource-num><language>English</language></reco
594 rd></Cite></EndNote>], we examined whether the replication defects of class III and IV
595 mutants were caused by defective maturation of NS2A. To test this hypothesis, we
596 introduced individual mutations into an expression plasmid pXJ-$E_{24}$-NS1-NS2A-HA that
597 encodes a polyprotein containing the C-terminal 24 amino acids of the E protein (serving
598 as a signal peptide to translocate NS1 to ER lumen), NS1, and NS2A with a C-terminal
599 HA tag. After transfecting the plasmids into 293T cells, the expression of un-cleaved



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NS1-NS2A-HA intermediate and cleaved NS2A-HA product were analyzed using Western blot (Fig. 3F). None of the mutations significantly affected the cleavage efficiencies of NS1-NS2A-HA to NS2A-HA, indicating that the replication defects of class III and IV mutants are not caused by impaired NS2A cleavage.

***Class V mutations block virion assembly***

Class V includes G71A, D73A, E103A, R171A, K192A, K193A, and a triple K25A/K26A/R27A. Cells transfected with class V mutant genomic RNAs yielded E-positive cells at 24 h p.t., but the number of E-positive cells did not increase afterward (Fig. 4A), suggesting that the mutants are defective in virus spread. No infectious mutant viruses, except E103A, were detected in the supernatants of transfected cells by plaque assay (Fig. 4B) or RT-PCR (Fig. 4C). No IFA-positive cells were detected after naïve cells were incubated with the supernatants from transfected cells (Fig. 4D). Sequencing of the recovered E103A mutant virus revealed an A103D change in the NS2A gene (Fig. 4C), suggesting that an E103A-to-A103D substitution is responsible for the rescue of viral replication.

To determine the stage of viral replication that was disrupted by the class V mutations, we analyzed the mutational effect on viral replication in ZIKV replicon (Fig. 4E). Compared with the WT replicon, all class V mutants exhibited equivalent levels of viral translation and RNA synthesis (<4-fold difference), suggesting that the mutations only marginally affect viral RNA synthesis. Next, we tested if the class V genomic RNAs



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could generate intracellular infectious viruses. Incubating naïve Vero cells with WT RNA-transfected cell extracts yielded IFA-positive cells, whereas no IFA-positive cells were detected after incubating naive cells with the mutant RNA-transfected cell extracts (Fig. 4F). Altogether, the results suggest that class V mutants are competent in viral RNA replication, but defective in assembly of infectious viruses.

***WT NS2A rescues virion assembly for class V mutants***

To test *trans* complementation of NS2A mutants, we constructed a BHK-21 cell line that constitutively expressed WT ZIKV NS2A protein with a C-terminal HA-tagged (NS2A-HA). The cell line was constructed by transfecting plasmid pXJ-SPG-$C_{16}$-NS2A-HA encoding a Gaussia luciferase signal peptide (SPG), the C-terminal 16 amino acids of NS1 protein ($C_{16}$), and NS2A-HA, as well as a neomycin resistance gene (*Neo*). The SPG and $C_{16}$ sequences were engineered to enable the correct formation of NS2A membrane topology when expressed in cells. Individual BHK-NS2A-HA cell lines were generated through colony purification under G418 (1 mg/ml) selection. For each BHK-NS2A-HA cell line, all cells were IFA-positive for HA-NS2A expression (Fig. 5A). Western blot revealed an expected band of ~22-kDa band (Fig. 5B). The expression level of NS2A-HA did not decrease in the BHK-NS2A-HA cells after 20 passages in culture (4 days per passage; Fig. 5B), indicating the stability of the cell line.

We tested *trans* complementation by transfecting class V genomic RNAs to



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BHK-NS2A-HA cells. The transfected cells developed an increasing number of E-positive cells from 48 to 96 h p.t. (Fig. 5C). Culture supernatants were collected at 96 h p.t. to infect naïve Vero cells. The infected cells developed E-positive cells for all class V mutants (Fig. 5D), demonstrating the rescue of infectious viruses through *trans* complementation. Quantification of the E-positive cells allowed us to determine the titers of rescued infectious viruses (Fig. 5E). Among the class V mutants, E103A, K192A, and K193A exhibited the highest *trans* complementation efficiencies, producing $1.9\times10^5$ to $3.04\times10^5$ IFU/ml viruses at 96 h p.i. (Fig. 5E). Sequencing of the rescued virion genomes confirmed the retention of the engineered class V mutations without any other changes in NS2A (Fig. 5E).

Next, supernatants from the first round of infection collected at 72 h p.i. (from Fig. 5D) were used to infect naïve Vero cells for a second round. In contrast to the first round of infection (Fig. 5D), no E-positive cells were detected in the second round of infection for any of the class V mutants (Fig. 5F). As a positive control, WT RNA produced high numbers of E-positive cells in both the first and second rounds of infections (Fig. 5D&F). Collectively, the results indicate that (i) the defect in virion assembly of class V mutants could be rescued through *trans* supply of WT NS2A protein and (ii) the rescued virions could only infect cells for a single round.

***DENV-2 NS2A could not trans complement ZIKV class V mutants***

We tested whether DENV NS2A could rescue class V mutants of ZIKV. Each class V



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660 mutant of ZIKV was transfected into a previously established BHK-21 cell line that
661 constitutively expresses WT DENV-2 NS2A protein [ ADDIN EN.CITE
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>10.1128/Jvi.02882-14</electronic-resource-num><language>English</language></reco
rd></Cite></EndNote>]. For all class V mutants, no increase in E-positive cells or infections viruses was detected from the transfected cells from 24 to 96 h p.t. (data not shown). The results suggest that flavivirus NS2A modulates virion assembly in a virus-type specific manner.

***WT NS2A could not rescue viral RNA synthesis of class III mutants***

We tested whether the defect in viral RNA synthesis of class III mutants could be *trans* complemented. To test this possibility, we transfected class III genomic RNAs (G12A and N130A) into ZIKV BHK-NS2A-HA cells. Neither mutants generated any E-positive cells in the transfected BHK-NS2A-HA cells (Fig. 5C). Infection of naïve Vero cells with supernatants of the transfected cells did not produce any IFA-positive cells (Fig. 5D-F). The results suggest that the RNA synthesis defect of class III mutants could not be rescued through *trans* complementation. Due to the dual defects of class IV mutants in



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both viral RNA synthesis and virion assembly, we did not pursue *trans* complementation experiments for this class of mutants; such complementation experiments may not reveal clear mechanistic insights as compared with the class III and V mutants.

***Probing the topology of ZIKV NS2A on the ER membrane***

The distinct functions of amino acids in viral RNA synthesis and virion assembly prompted us to probe the topology of ZIKV NS2A on ER membrane. Bioinformatics analysis suggests that ZIKV NS2A may form seven transmembrane segments (pTMS1-7; Fig. 6A). To examine whether each pTMS associates with the ER membrane, we constructed a panel of plasmids encoding full-length or various NS2A fragments with a C-terminally fused eGFP reporter (Fig. 6B). The length of each segment was slightly modified from the bioinformatic prediction to allow some flexibility between pTMS and eGFP. Upon transfection into Vero cells, eGFP alone (without any NS2A fragment) exhibited an even distribution of fluorescence throughout the cell, whereas the full-length NS2A-eGFP (construct 1–226) displayed fluorescence co-localized with calnexin (an ER membrane protein) in the perinuclear region (Fig. 6B), indicating that ZIKV NS2A is an integral membrane protein. Expression of seven fragments spanning each pTMS, represented by constructs 1-25 (pTMS1), 30-58 (pTMS2) , 74-97 (pTMS3), 101-123 (pTMS4), 125-145 (pTMS5), 150-185 (pTMS6), and 198-221 (pTMS7), exhibited fluorescence patterns that co-localized with calnexin in the perinuclear region (Fig. 6B). The results indicate that individual of the seven pTMSes is associated with the ER



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721 membrane. In contrast, expression of fragments between different pTMS, represented by
722 constructs 55-73, 186-197, and 221-226, showed fluorescence throughout the cells (Fig.
723 6B), indicating that these fragments are not membrane-associated.

724 An *in vitro* deglycosylation analysis was performed to determine the orientation of
725 each NS2A pTMS on the ER membrane. A panel of plasmids were prepared to express
726 truncated NS2A proteins that were C-terminally fused with an eGFP followed by an
727 N-glycosylation acceptor peptide (Glyc; Fig. 7A). An SPG-$C_{16}$ peptide (Gaussia
728 luciferase signal peptide SPG and the C-terminal 16 amino acids of NS1) was fused to
729 the N terminus of each NS2A-eGFP-Glyc construct to ensure a proper translocation of
730 the expressed protein on the ER membrane. Western blot showed that, upon transfection
731 into BHK-21 cells, all constructs expressed fusion proteins with expected molecular
732 masses (Fig. 7B). Because N-glycosylation enzymes reside in the ER lumen, the
733 glycosylation status could be used to indicate the location of the C-terminus of each
734 NS2A-eGFP-Glyc protein (Fig. 7C). As a negative control, treatment of EGFP-Glyc
735 (without any viral sequence) with PNGase F (an enzyme that removes high mannose and
736 complex carbohydrates) did not change the mobility of the protein (Fig. 7D), indicating a
737 cytosol localization of EGFP-Glyc. As a positive control, PNGaseF treatment increased
738 the mobility of SPG-$C_{16}$-EGFP-Glyc (construct 0), confirming that the C-terminus of this
739 protein resides in the ER lumen. Among the NS2A constructs, PNGase F treatment
740 increased the mobility of fusion proteins 1-26 and 1-56, but not 1-103, 1-123, 1-147,



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741 1-192, and 1-226 (Fig. 7D). The results suggest that the N-terminal 56 amino acids of
742 NS2A reside in the ER lumen, and the C-terminal region from residues 103 to 226 is
743 located in the cytosol.

744 Next, we performed an *in vitro* proteinase K protection assay [ ADDIN EN.CITE
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766 38X</isbn><accession-num>WOS:000316671000043</accession-num><urls><related-u
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769 >10.1128/Jvi.02424-12</electronic-resource-num><language>English</language></reco
770 rd></Cite></EndNote>] to validate the above *in vitro* deglycosylation results. BHK-21
771 cells were transfected with the same constructs depicted in Fig. 7A. At 24 h p.t.,
772 membrane fractions from the transfected cells were treated with proteinase K in the
773 presence or absence of 0.5% Triton X-100 and analyzed by Western blot using an eGFP
774 antibody. In the absence of Triton X-100, the eGFP residing in the cytosol would be
775 susceptible to proteinase K digestion, whereas the eGFP residing in the ER lumen would
776 be protected from proteinase K digestion (Fig. 7E). As shown in Fig. 7F, the eGFPs from
777 constructs 0, 1-26, and 1-56 were protected from protease K digestion, again indicating
778 that the first 56 residues are located in the ER lumen. In contrast, the eGFPs from
779 constructs 1-103, 1-123, 1-147, 1-192, 1-226, and control eGFP-Glyc were completely
780 degraded by the protease K treatment (Fig. 7F), supporting that the C-terminal region
781 from residues 103 to 226 is located in the cytosol. As expected, in the presence of Triton



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X-100, the eGFPs from all constructs were degraded by proteinase K (Fig. 7F). The results of proteinase K assay are in full agreement with those of deglycosylation assay.

***A topology model for ZIKV NS2A***

The above biochemical results support a topology model for ZIKV NS2A on the ER membrane (Fig. 8A): (i) The N-terminal region (including pTMS1 and pTMS2) resides in the ER lumen; (ii) the region spanning residues 74 to 97 (pTMS3) traverses the ER membrane in a lumen-to-cytosol direction; and (iii) the C-terminal region from residues 103 to 226 (including pTMS4 to pTMS7) is located in the cytosol side. Since pTMS1, pTMS2, pTMS4, PTMS5, PTM6, and pTMS7 associate with ER membraned when expressed individually (Fig. 6B), they may interact with the ER membrane in a peripheral manner. In support of this notion, informatics analyses using Jpred 4 and HeliQuest programs suggest that amino acids 9-26 (pTMS1), 41-58 (pTMS2), 104-121 (pTMS4), 125-142 (pTMS5), 172-189 (pTMS6), and 203-220 (pTM7) may form amphipathic helices with hydrophobic and hydrophilic surfaces (Fig. 8B), where the hydrophobic surface may interact with the ER membrane.

## Discussion

Flavivirus NS2A functions in viral RNA replication, virion assembly, evasion of innate immune response, and disease pathogenesis in a virus-specific manner [ ADDIN



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806 name="Journal Article">17</ref-type><contributors><authors><author>Vossmann,
807 S.</author><author>Wieseler, J.</author><author>Kerber,
808 R.</author><author>Kummerer, B.
809 M.</author></authors></contributors><auth-address>Bernhard Nocht Inst Trop Med,
810 Dept Virol, D-20359 Hamburg, GermanyUniv Bonn, Inst Virol, Med Ctr, Bonn,
811 Germany</auth-address><titles><title>A Basic Cluster in the N Terminus of Yellow
812 Fever Virus NS2A Contributes to Infectious Particle
813 Production</title><secondary-title>Journal of Virology</secondary-title><alt-title>J
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817 Virol</abbr-1></alt-periodical><pages>4951-4965</pages><volume>89</volume><nu
818 mber>9</number><keywords><keyword>nonstructural protein
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821 cleavage</keyword><keyword>identification</keyword><keyword>vaccination</keyw


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823 ></dates><isbn>0022-538X</isbn><accession-num>WOS:000352219600023</accessio
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826 >10.1128/Jvi.03351-14</electronic-resource-num><language>English</language></reco
827 rd></Cite></EndNote>]. This is evidenced by its high sequence diversity (Fig. 1A),
828 distinct membrane topology [ ADDIN EN.CITE ADDIN EN.CITE.DATA ], and
829 association with different disease pathways [ ADDIN EN.CITE ADDIN
830 EN.CITE.DATA ]. Thus, it is critical to perform a systematic genetic and biochemical
831 characterization of ZIKV NS2A to delineate its structure and function. Our biochemical
832 results showed that, unlike other flaviviruses, ZIKV NS2A has only a single
833 transmembrane segment that traverses the ER membrane, the other six pTMSes are
834 peripherally associated with the ER membrane (Fig. 8A). Our functional analysis
835 revealed five classes of mutants with distinct replication phenotypes, among which class
836 III, IV, and V mutants could not produce infectious viruses through different mechanisms
837 (Fig. 1B). The critical mutations from class III to V are not clustered in specific regions
838 of NS2A (Fig. 8A), suggesting that multiple domains of NS2A are essential for viral
839 replication.

840 The membrane topology of ZIKV NS2A is in stark contrast with the topologies of
841 other flavivirus NS2A proteins: DENV-2 NS2A has five membrane-traversing segments



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846 db-id="sva5pexpet29snerv2jpeedvrdwz0fzfs2ee">399</key></foreign-keys><ref-type

847 name="Journal Article">17</ref-type><contributors><authors><author>Xie, X.

848 P.</author><author>Gayen, S.</author><author>Kang, C. B.</author><author>Yuan, Z.

849 M.</author><author>Shi, P.

850 Y.</author></authors></contributors><auth-address>Chinese Acad Sci, Wuhan Inst Virol,

851 State Key Lab Virol, Beijing 100864, Peoples R ChinaNovartis Inst Trop Dis,

852 Singapore, SingaporeAgcy Sci Technol & Res, Ctr Expt Therapeut,

853 Singapore, Singapore</auth-address><titles><title>Membrane Topology and Function of

854 Dengue Virus NS2A Protein</title><secondary-title>Journal of

855 Virology</secondary-title><alt-title>J

856 Virol</alt-title></titles><periodical><full-title>Journal of Virology</full-title><abbr-1>J

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859 Virol</abbr-1></alt-periodical><pages>4609-4622</pages><volume>87</volume><nu

860 mber>8</number><keywords><keyword>subcellular-localization</keyword><keyword

861 >viral replication</keyword><keyword>ns4b

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bition</keyword><keyword>cleavage</keyword><keyword>identification</keyword><keyword>flaviviruses</keyword><keyword>complement</keyword></keywords><dates><year>2013</year><pub-dates><date>Apr</date></pub-dates></dates><isbn>0022-538X</isbn><accession-num>WOS:000316671000043</accession-num><urls><related-urls><url><Go to ISI>://WOS:000316671000043</url></related-urls></urls><electronic-resource-num>10.1128/Jvi.02424-12</electronic-resource-num><language>English</language></record></Cite></EndNote>] and YFV NS2A has three membrane-traversing segments [ ADDIN EN.CITE <EndNote><Cite><Author>Vossmann</Author><Year>2015</Year><RecNum>423</RecNum><DisplayText>[34]</DisplayText><record><rec-number>423</rec-number><foreign-keys><key app="EN" db-id="sva5pexpet29snerv2jpeedvrdwz0fzfs2ee">423</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Vossmann, S.</author><author>Wieseler, J.</author><author>Kerber, R.</author><author>Kummerer, B. M.</author></authors></contributors><auth-address>Bernhard Nocht Inst Trop Med, Dept Virol, D-20359 Hamburg, GermanyUniv Bonn, Inst Virol, Med Ctr, Bonn, Germany</auth-address><titles><title>A Basic Cluster in the N Terminus of Yellow Fever Virus NS2A Contributes to Infectious Particle Production</title><secondary-title>Journal of Virology</secondary-title><alt-title>J



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885 Virol</abbr-1></periodical><alt-periodical><full-title>Journal of
886 Virology</full-title><abbr-1>J
887 Virol</abbr-1></alt-periodical><pages>4951-4965</pages><volume>89</volume><nu
888 mber>9</number><keywords><keyword>nonstructural protein
889 ns2a</keyword><keyword>dengue virus</keyword><keyword>west
890 nile</keyword><keyword>rna</keyword><keyword>replication</keyword><keyword>
891 cleavage</keyword><keyword>identification</keyword><keyword>vaccination</keyw
892 ord></keywords><dates><year>2015</year><pub-dates><date>May</date></pub-dates
893 ></dates><isbn>0022-538X</isbn><accession-num>WOS:000352219600023</accessio
894 n-num><urls><related-urls><url><Go to
895 ISI>://WOS:000352219600023</url></related-urls></urls><electronic-resource-num
896 >10.1128/Jvi.03351-14</electronic-resource-num><language>English</language></reco
897 rd></Cite></EndNote>]. Interestingly, these topologically diverse flavivirus NS2A
898 proteins are able to converge their functions in viral RNA replication, virion assembly,
899 and evasion of immune response. High resolution, three-dimensional structures of
900 flavivirus NS2A are needed to address this question. It should be noted that the
901 membrane topology of NS2A presented here is in the absence of other viral proteins. In
902 the context of viral replication, the NS2A protein is associated with various viral and
903 cellular proteins that would modulate the structure of NS2A and perform biological
904 functions.



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Our mutagenesis study has identified distinct NS2A mutations that selectively abolish ZIKV RNA synthesis (class III) or virion assembly (class V), among which only the virion assembly-defective mutants could be rescued through *trans* complementation (Fig. 5). The results suggest that there are two species of NS2A molecules in ZIKV-infected cells. One NS2A species is located at the viral replication complex that could only be formed in *cis*; another NS2A species is located at virion assembly complex that could be formed in *trans* or *cis*. These results are in agreement with the previous studies from DENV and YFV NS2A [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. Compared with the viral replication complex, the virion assembly complex is much less well defined for flaviviruses. Previously studies showed that second-site mutations in prM, E, NS2B, and NS3 could rescue the defect of virion assembly caused by DENV-2 and YFV NS2A mutations, suggesting that these viral proteins participate in virion assembly. Besides the genetic evidence, the role of NS3 in virion assembly is also supported by (i) mutations in the NS3 helicase domain alone affect YFV assembly [ ADDIN EN.CITE <EndNote><Cite><Author>Patkar</Author><Year>2008</Year><RecNum>467</RecNum><DisplayText>[47]</DisplayText><record><rec-number>467</rec-number><foreign-keys><key app="EN" db-id="sva5pexpet29snerv2jpeedvrdwz0fzfs2ee">467</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Patkar, C. G.</author><author>Kuhn, R.



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926 J.</author></authors></contributors><auth-address>Purdue Univ, Dept Biol Sci, W
927 Lafayette, IN 47907 USA</auth-address><titles><title>Yellow fever virus NS3 plays an
928 essential role in virus assembly independent of its known enzymatic
929 functions</title><secondary-title>Journal of Virology</secondary-title><alt-title>J
930 Virol</alt-title></titles><periodical><full-title>Journal of Virology</full-title><abbr-1>J
931 Virol</abbr-1></periodical><alt-periodical><full-title>Journal of
932 Virology</full-title><abbr-1>J
933 Virol</abbr-1></alt-periodical><pages>3342-3352</pages><volume>82</volume><nu
934 mber>7</number><keywords><keyword>tick-borne
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936 ns3</keyword><keyword>west-nile-virus</keyword><keyword>dengue
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939 ><keyword>ns2a</keyword><keyword>organization</keyword></keywords><dates><y
940 ear>2008</year><pub-dates><date>Apr</date></pub-dates></dates><isbn>0022-538X
941 </isbn><accession-num>WOS:000254139800015</accession-num><urls><related-urls>
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943 ISI>://WOS:000254139800015</url></related-urls></urls><electronic-resource-num
944 >10.1128/Jvi.02447-07</electronic-resource-num><language>English</language></reco
945 rd></Cite></EndNote>], (ii) the helicase domain of DENV or YFV NS3 directly binds to
946 NS2A [ ADDIN EN.CITE ADDIN EN.CITE.DATA ], (iii) the cleavage between C



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947 and prM is tightly modulated by NS2B/NS3 protease and cellular signalase during YFV
948 and Murray Valey encephalitis virus assembly [ ADDIN EN.CITE ADDIN
949 EN.CITE.DATA ]. These data, together with the result that recombinant NS2A of
950 Kunjin binds to viral 3'UTR RNA [ ADDIN EN.CITE
951 <EndNote><Cite><Author>Mackenzie</Author><Year>1998</Year><RecNum>405</R
952 ecNum><DisplayText>[28]</DisplayText><record><rec-number>405</rec-number><fo
953 reign-keys><key app="EN"
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955 name="Journal Article">17</ref-type><contributors><authors><author>Mackenzie, J.
956 M.</author><author>Khromykh, A. A.</author><author>Jones, M.
957 K.</author><author>Westaway, E.
958 G.</author></authors></contributors><auth-address>Royal Childrens Hosp, Sir Albert
959 Sakzewski Virus Res Ctr, Brisbane, Qld 4029, AustraliaUniv Queensland, Ctr
960 Microscopy & Microanal, St Lucia, Qld 4072,
961 Australia</auth-address><titles><title>Subcellular localization and some biochemical
962 properties of the flavivirus Kunjin nonstructural proteins NS2A and
963 NS4A</title><secondary-title>Virology</secondary-title><alt-title>Virology</alt-title><
964 /titles><periodical><full-title>Virology</full-title><abbr-1>Virology</abbr-1></periodic
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967 ds><keyword>japanese encephalitis-virus</keyword><keyword>yellow-fever



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virus</keyword><keyword>dependent rna-polymerase</keyword><keyword>west nile flavivirus</keyword><keyword>escherichia-coli</keyword><keyword>positive identification</keyword><keyword>replication strategy</keyword><keyword>infected-cells</keyword><keyword>core protein</keyword><keyword>ns5 protein</keyword></keywords><dates><year>1998</year><pub-dates><date>Jun 5</date></pub-dates></dates><isbn>0042-6822</isbn><accession-num>WOS:000074186800004</accession-num><urls><related-urls><url><Go to ISI>://WOS:000074186800004</url></related-urls></urls><electronic-resource-num>DOI 10.1006/viro.1998.9156</electronic-resource-num><language>English</language></record></Cite></EndNote>], suggest a flavivirus assembly model. During flavivirus assembly, the NS2A protein recruits NS2B/NS3 through binding to the NS3 helicase domain. Once the NS2A-NS2B/NS3 complex reaches the virion assembly site, the NS3B/NS3 protease cleaves the C protein from the C/prM/E polyprotein. The C protein assembles onto genomic RNA (presented by NS2A) to form nucleocapsid, which is subsequently enveloped by prM/E complex to form virions. Since flavivirus assembly has to be coupled to viral RNA replication [ ADDIN EN.CITE <EndNote><Cite><Author>Khromykh</Author><Year>2001</Year><RecNum>471</RecNum><DisplayText>[51]</DisplayText><record><rec-number>471</rec-number><foreign-keys><key app="EN"



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db-id="sva5pexpet29snerv2jpeedvrdwz0fzfs2ee">471</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Khromykh, A. A.</author><author>Varnavski, A. N.</author><author>Sedlak, P. L.</author><author>Westaway, E. G.</author></authors></contributors><auth-address>Royal Childrens Hosp, Sir Albert Sakzewski Virus Res Ctr, Brisbane, Qld 4029, AustraliaUniv Queensland, Clin Med Virol Ctr, Brisbane, Qld, Australia</auth-address><titles><title>Coupling between replication and packaging of flavivirus RNA: Evidence derived from the use of DNA-based full-length cDNA clones of kunjin virus</title><secondary-title>Journal of Virology</secondary-title><alt-title>J Virol</alt-title></titles><periodical><full-title>Journal of Virology</full-title><abbr-1>J Virol</abbr-1></periodical><alt-periodical><full-title>Journal of Virology</full-title><abbr-1>J Virol</abbr-1></alt-periodical><pages>4633-4640</pages><volume>75</volume><number>10</number><keywords><keyword>double-stranded-rna</keyword><keyword>replicon rna</keyword><keyword>heterologous genes</keyword><keyword>infected cells</keyword><keyword>transcomplementation</keyword><keyword>proteins</keyword><keyword>ns1</keyword><keyword>trans</keyword><keyword>components</keyword><keyword>expression</keyword></keywords><dates><year>2001</year><pub-dates><date>May</date></pub-dates></dates><isbn>0022-538X</isbn><accession-num>WOS:000168309300017</accession-num><urls><related-urls><url><Go to



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ISI>://WOS:000168309300017</url></related-urls></urls><electronic-resource-num >Doi 10.1128/Jvi.75.10.4633-4640.2001</electronic-resource-num><language>English</language></record></Cite></EndNote>], the newly synthesized genomic RNA has to be transported from the replication site to the virion assembly site [ ADDIN EN.CITE ADDIN EN.CITE.DATA ], possibly through the NS2A protein.

The *trans* complemented class V mutants could potentially be used as vaccine candidates, as recently reported for DENV-2 [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. Since the *trans* complemented virions are able to infect cells for only a single cycle, such vaccine platform is safer than the multi-round infectious live-attenuated vaccines [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. This is particularly important for ZIKV vaccine when immunizing immune-compromised individuals and pregnant women [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. However, for development of class V mutants as a vaccine platform, the *trans* complementation efficiency needs to be improved to higher titers before they could be tested for *in vivo* efficacy.

In summary, we report the genetic and biochemical characterization of ZIKV NS2A protein. The results indicate that different flavivirus NS2A proteins have distinct membrane topologies, but converge to function in viral RNA synthesis and virion assembly. Together with previous results, we propose a model for flavivirus assembly in



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which NS2A plays a central role in mediating viral structural and nonstructural proteins as well as genomic RNA during virion formation.


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We thank Mariano Garcia-Blanco, Shelton Bradrick, and other colleagues at University of Texas Medical Branch for helpful discussions during the course of this study. P.-Y.S. lab was supported by a Kleberg Foundation Award, UTMB CTSA UL1TR-001439, NIH grants AI127744 and AI136126, and Cooperative Agreement Number U01CK000512 from the Centers for Disease Control and Prevention.


## Declaration of interests

The authors declare no competing interests.



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## References

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**Figure legend**

***Fig. 1. ZIKV NS2A mutagenesis.***

(A) Sequence alignment of NS2A proteins among ZIKV (strain FSS13025), DENV-1-4 serotypes, WNV, JEV, SLEV, and YFV using CLC Main Workbench software (CLC Bio). Thirty-five amino acids were selected for mutagenesis: (i) All 8 identical non-alanine residues (indicated by triangles), (ii) 25 charged residues (arrows), (iii) a K25/K26/R27 motif (line), (iv) residue N130 (circle). The consensus and conservation percentage are indicated below each amino acids. (B) A summary of ZIKV NS2A mutagenesis results. An infectious cDNA clone and a luciferase replicon of ZIKV strain FSS13025 were used to analyze the function of each amino acid. Based on the replication phenotypes, the 35 mutants could be categorized into five classes as indicated.

***Fig. 2. Characterization of class I, II, and III NS2A mutants.***

The results of representative mutants from class I (A-C), II (D-F), and III (G-K) are shown. (A) Vero cells were electroporated with equal amounts of ZIKV wild-type (WT) or class I mutant genomic RNAs. At indicated time points, the cells were fixed and subjected to IFA. Viral E protein and nuclei were stained with antibody 4G2 (green) and DAPI (blue), respectively. Cytopathic effect (CPE) is indicated at 72 h post-transfection. (B) Plaque morphologies. Recombinant viruses were subjected to plaque assay. Plaques were developed at day 4.5 post-infection. (C) Viral titers of class I mutants at 72 h



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post-transfection. The mean values from two independent experiments are shown. Error bars indicate the standard deviations. The limit of detection (LOD) of plaque assay is 10 PFU/ml. (D) IFA of class II mutant RNA-transfected Vero cells. (E) Plaque morphology of class II mutant viruses. (F) Viral titers of class II mutant viruses at 72 h post-transfection. (G) IFA of class III mutant RNA-transfected Vero cells. (H) Plaque morphologies of class III mutant viruses. Plaque assay was performed using culture supernatants collected at day 3 (for WT) and day 5 (for G12A and N130A) post-transfection. (I) RT-PCR analysis. At 5 day p.t., the mutant RNA-transfected cells were washed twice with PBS to remove residual input RNAs. At day 8 p.t., the supernatants of G12A and N130A RNA-transfected cells were subjected to RT-PCR. The WT virus (collected at 72 h p.t.) was used as a positive control. (J) A ZIKV luciferase replicon. $C_{38}$ and $E_{30}$ represent the first 38 amino acids of C protein and the last 30 amino acids of E protein, respectively. *Renilla* luciferase: Rluc. Foot-and-mouse-disease virus 2A sequence: 2A. (K) Transient replicon assay. WT, G12A, N130A, and NS5ΔGDD replicon RNAs were electroporated into Vero cells. Luciferase activities were measured at indicated time points. An average of 3 experiments are presented. Bars represent standard deviations.

***Fig. 3. Characterization of class IV NS2A mutants.***

(A) IFA of transfected Vero cells. (B) Plaque morphologies of WT, D7A, R140A, and R222A viruses. (C) Extracellular viral titers were measured at 24, 48, and 72 h p.t. using



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plaque assay. At 72 h p.t., extracellular viral RNAs were subjected RT-PCR analysis. The RT-PCR products were sequenced to confirm the engineered mutations and to detect potential second site mutation(s) in the NS2A gene. N.A., not applicable. (D) The supernatants of transfected cells were collected on day 8 p.t. to infect naïve Vero cells. At 24 h p.t., the cells were analyzed for viral E expression by IFA. (E) Transient luciferase replicon analysis. Vero cells were electroporated with ZIKV replicon RNAs and measured for luciferase activities at indicated time points. An average of 3 experiments are presented. Bars represent standard deviations. (F) Analysis of NS2A proteolytic processes. NS2A D7A, G12A, N130A, R140A, and R222A mutations were individually engineered into expression plasmid pXJ-$E_{24}$-NS1-NS2A-HA. After transfecting the plasmids into 293T cells, total cellular protein samples were harvested at 24 h p.t. and analyzed for NS2A process using Western blot. An HA-tag antibody was used to detect both unprocessed NS1-NS2A-HA and processed NS2A-HA proteins.

***Fig. 4. Characterization of class V NS2A mutants.***

(A) IFA of Vero cells that were transfected with WT or class V genomic RNAs. Viral E-positive cells are indicated in green. (B) Plaque morphologies of class V mutant viruses. Only the WT and E103A mutant generated plaques. (C) Supernatants from the RNA-transfected cells were quantified for viral titers by plaque assay. RT-PCR was also performed on the supernatants at day 3 p.t. for the WT virus and at day 8 p.t. for the mutants. Positive RT-PCR products were sequenced for potential second site mutation(s).



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N.A., not applicable. (D) Naïve Vero cells were infected with supernatants from the RNA-transfected cells and subjected to IFA for viral E protein expression. (E) Luciferase replicon analysis. Equal amounts of WT and mutant replicon RNAs were electroporated into Vero cells. Luciferase activities were measured at the indicated time points. (F) Analysis of intracellular infectious viruses. Intracellular viruses from ZIKV RNA-transfected cells were harvested at 24 h p.t. as previously described [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. The harvested samples were used to infect naïve Vero cells. IFA was performed to examine viral E protein expression at 24 h post-infection.

***Fig. 5. Trans complementation of class III and V mutants.***

(A) IFA of BHK-NS2A-HA cells. The BHK-NS2A-HA cells constitutively express WT ZIKV NS2A fused with a C-terminal HA-tag. The cells were fixed with paraformaldehyde and stained with an HA-tag antibody. (B) Western blot of BHK-NS2A-HA cells. The expression of WT NS2A-HA from BHK-NS2A-HA cells was detected using an HA-tag antibody. Both passage one (P1) and 20 (P20) BHK-NS2A-HA cells were analyzed. Naïve BHK-21 cells were included as a negative control. (C) WT and class III and V genomic RNAs were electroporated into BHK-NS2A-HA cells. IFA was performed on the transfected cells to detect viral E protein expression. (D) Viruses rescued from the BHK-NS2A-HA cells (harvested at 96 h p.t.) were used to infect naïve Vero cells. The infected Vero cells were subjected to IFA for E protein expression. (E)



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Extracellular viruses were harvested from the supernatants of RNA-transfected BHK-NS2A-HA cells. The harvested viruses were serially diluted to infect naïve Vero cells in 96-well plates. At 24 h p.i., IFA was performed to quantify the viral titers by counting the number of E-positive cells. The results show averages and standard deviations from triplicate samples. Viral RNAs in the supernatants of transfected BHK-NS2A-HA cells were isolated at 96 h p.t. and subjected to RT-PCR. Positive RT-PCR products were sequenced to confirm the engineered mutations and to identify potential second site change(s). (F) The supernatants of infected Vero cells from (D) were collected at 72 h p.i. to infect naïve Vero cells for a second round. IFA was performed on these cells for viral E-positive cells.

***Fig. 6. Membrane association of ZIKV NS2A transmembrane segments.***

(A) Bioinformatics analysis of ZIKV NS2A for transmembrane segments. ZIKV NS2A sequence was analyzed by bioinformatics program TOPCONS, Philius, PolyPhobius, SCAMPI, and TMHMM. The compiled transmembrane segments are shown at the bottom. Seven predicted transmembrane segments (pTMS) are presented as thick bars. The amino acid positions of ZIKV NS2A are indicated. (B) Membrane association of individual pTMS. Expression plasmid pXJ-eGFP (without NS2A sequence) and pXJ-smNS2A-eGFP (smNS2A represents different NS2A segments) were transfected into Vero cell by using X-tremeGENE 9 DNA transfection reagent (Roche). For each plasmid construct, the NS2A segment is indicated by amino acid positions. At 24 h p.t., IFA was



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performed to monitor eGFP expression. ER and nuclei were stained with anti-Calnexin antibody and DAPI, respectively. The merged images are shown on the right panels.

***Fig. 7. Probing the membrane topology of ZIKV NS2A by in vitro deglycosylation assay and in vitro proteinase K protection assay.***

(A) Schematic representation of C-terminally truncated ZIKV NS2A proteins. The amino acid positions of each truncated NS2A are indicated. A "SPG-C16" sequence was fused to the N terminus of NS2A to ensure correct translocation of NS2A on the ER membrane. An "eGFP-Glyc" sequence was fused to the C terminus of NS2A to probe its location at cytosol or ER lumen. See text for details. (B) BHK-21 cells were transfected with the expression plasmids described in (A). At 24 h p.t., the expression levels of various NS2A truncates were examined by Western blot using an eGFP antibody. The amino acid positions are indicated for each NS2A truncates. The molecular masses of marker proteins are shown to the left of Western blot image. (C) Diagram of glycosylation of eGFP-Glyc when located at ER lumen (*left*). No glycosylation occurs when eGFP-Glyc is located at cytosol (*right*). The glycosylation residue Asn is indicated. (D) *In vitro* deglycosylation assay. Plasmid-transfected cells were treated with PNGase F or PBS according to the protocol of *in vitro* deglycosylation assay (see Materials and Methods). The samples were examined for glycosylation status by Western blot using an eGFP antibody. (E) Diagram of *in vitro* proteinase K protection assay. When eGFP is located at ER lumen, it is protected from proteinase K cleavage (*left*); when located in the cytosol,



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1164 eGFP is degraded by proteinase K (*right*). (F) *In vitro* proteinase K protection assay. Cell
1165 lysates from plasmid-transfected BHK-21 cells were treated with proteinase K or PBS
1166 according to the protocol of *in vitro* proteinase K protection assay (see Materials and
1167 Methods). The samples were detected for eGFP by Western blot using an eGFP antibody.
1168 Triton X-100 treatment is indicated.

1169 ***Fig. 8. A topological model of ZIKV NS2A.***

1170 (A) A model of ZIKV NS2A membrane topology. See text for details. Class III, IV, and V
1171 mutations are depicted on the topology model. (B) Helical wheel representations of
1172 NS2A fragment 9-26, 41-58, 104-121, 125-142, 172-189, and 203-220. The amphiphilic
1173 helix regions were analyzed using Heliquest server (heliquest.ipmc.cnrs.fr/). The best
1174 18-residue windows are presented. The amino acids forming the hydrophobic surface of
1175 each helix wheel are depicted. Yellow, gray, blue, red, purple, pink, and green circles
1176 indicate nonpolar, glycine/alanine, positively charged, negatively charged, polar, amide,
1177 and proline amino acids, respectively. The first and last residues in each helical wheel are
1178 indicated by a blue and red arrow, respectively.



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**Subject:** VDN Bunyavirales update article 2019



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**Attachments:** Bunyavirales 2019 VDN 02152019.docx

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Dear All,

Please find attached a short *Archives of Virology* Virology Division News (VDN) update article draft on the current composition of the order *Bunyavirales*. The primary purpose of this article (as in the years before) is to a) give credit to all of those who are actively involved in virus taxonomy by writing or judging taxonomic proposals and b) to provide an easy, up-to-date, and citable go-to-point for virologists who are not only interested in taxa but also would like to see which viruses are assigned to each taxon and how these viruses are abbreviated.

The attached article is in exactly the same format as the previous ones. This one is based on all taxonomic proposals that were submitted and accepted by the ICTV Executive Committee in 2018 and are currently under ratification (ratification vote deadline is today – the article is written under the assumption that ratification went smoothly as planned).

**Please review the attached document and**

- **All: please review whether your name is spelled correctly and completely (missing middle initials, diacritics/Pīnyīn, original names?)**
- **All: please double-check your affiliation(s)**
- **All: please double-check your ORCIDs (or let me know which ORCID to add)**
- **Study Group Chairs: please double-check whether all your Study Group members are listed**
- **Study Group Members: please double-check whether you are assigned to the correct Study Group(s)**
- **TaxoProp authors: please double-check whether all your co-authors are listed. Dr. Deng: please forward this email to Shu Shen, Xiaomei Duan, Bo Wang, Liying Zhu, Yanfang Zhang, Jingyuan Zhang, Jun Wang, Tao Luo, Chun Kou, Dan Liu, Chuanwei Lv, Lei Zhang, Chenchen Chang, Zhengyuan Su, Shuang Tang, Jie Qiao, Abulimiti Moming, Cheng Wang, Abulikemu Abudurexiti, Hualin Wang, Zhihong Hu, Yujiang Zhang, and Surong Sun as I do not have their contact addresses**
- **All: please double-check the funding and disclaimer section and let me know what to add**
- **All: please then review the very short text and, in particular, the references**

It would be great if we could now get rid of outdated virus name synonyms (bottom/legend of the table). Study Groups: please look over these and let me know which one should *not* be deleted. Also, for any virus name having more than one abbreviation, it would be nice if the Study Groups could make a recommendation which one should be used (ideally one that is not in use for other viruses, though).

**Please email me your corrections/and edits by Wednesday, Feb. 20, midnight US EST. Please also write me an explicit "OK" if you are ok with publication of the article.**

**Please Do NOT "reply to all" to prevent Inbox clogging. I will consolidate all corrections and send out a new version.**

Thanks, as always, for all your work on virus taxonomy!

Best,
Jens

Jens H. Kuhn, MD, PhD, PhD, MS
Virology Lead
Battelle Memorial Institute – Contractor supporting:

National Institute of Allergy and Infectious Diseases (NIH/NIAID)
Division of Clinical Research (DCR)



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Lambert: orcid.org/0000-0002-7571-3529

Marklewitz: orcid.org/0000-0003-1828-8770

Nunes: orcid.org/0000-0001-9739-5499

Palacios: orcid.org/0000-0001-5062-1938

Pawęska: orcid.org/0000-0001-8776-7519

Radoshitzky: orcid.org/0000-0001-8976-8056

Salvato: orchid.org/0000-0002-3357-5391

Sasaya: orcid.org/0000-0002-5020-9377

Shí: orcid.org/0000-0002-5289-7222

Simmonds: 0000-0002-7964-4700

Sironi: orcid.org/0000-0002-2267-5266

Spengler: orcid.org/0000-0002-5383-0513

Stenglein: orcid.org/0000-0002-0993-813X

Turina: orcid.org/0000-0002-9659-9470

Whitfield: orcid.org/0000-0002-3538-015X

Zerbini: orcid.org/0000-0001-8617-0200

Kuhn: orcid.org/0000-0002-7800-6045

**Keywords:** *Arenaviridae*; arenavirid; arenavirus; bunyavirad; *Bunyavirales*; bunyavirid; *Bunyaviridae*; bunyavirus; emaravirus; *Feraviridae*; feravirid, feravirus; fimovirid; *Fimoviridae*; fimovirus; goukovirus; hantavirid; *Hantaviridae*; hantavirus; hartmanivirus; herbevirus; ICTV; International Committee on Taxonomy of Viruses; jonvirid; *Jonviridae*; jonvirus; leishbuvirid; *Leishbuviridae*; leishbuvirus; mammarenavirus; nairovirid; *Nairoviridae*; nairovirus;



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184 orthobunyavirus; orthoferavirus; orthohantavirus; orthojonvirus; orthonairovirus;
185 orthophasmavirus; orthotospovirus; peribunyavirid; *Peribunyaviridae*; peribunyavirus;
186 phasmavirid; phasivirus; *Phasmaviridae*; phasmavirus; phenuivirid; *Phenuiviridae*; phenuivirus;
187 phlebovirus; reptarenavirus; tenuivirus; tospovirid; *Tospoviridae*; tospovirus; virus classification;
188 virus nomenclature; virus taxonomy



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## ABSTRACT

In February 2019, the order *Bunyavirales* was amended by creation of two new families, four new subfamilies, eleven new genera, and 77 new species, merging of two species, and deletion of one species. This article presents the updated taxonomy of the order *Bunyavirales* now accepted by the International Committee on Taxonomy of Viruses (ICTV).


## INTRODUCTION

The virus order *Bunyavirales* was established in 2017 to accommodate related viruses with segmented, linear, single-stranded, negative-sense or ambisense RNA genomes classified into 9 families [18]. An amended/emended order description was published in early 2019 [19]. Here we present the changes that were proposed via official ICTV taxonomic proposals that were accepted by the ICTV Executive Committee (EC) in February 2019. These changes are now part of the official ICTV taxonomy as of February 2019.

## TAXONOMIC CHANGES AT THE ORDER RANK

In February 2019, the order was expanded by addition of two new families. Family *Leishbuviridae* was created to accommodate one new genus, *Shilevirus*, including one new species, *Leptomonas shilevirus*, for Leptomonas moramango leishbunyavirus (LEPMV) discovered in a trypanosomatid protist (*Leptomonas moramango*) [2]. Family *Tospoviridae* was recreated for the already established genus *Orthotospovirus*. (TaxoProp 2018.017M.A.v1.Bunyavirales_2fam5gen) and expanded by 7 new species (TaxoProp 2018.025P.A.v1.Orthotospovirus_7sp):

- *Bean necrotic mosaic orthotospovirus* for bean necrotic mosaic virus (BeNMV) discovered



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in common beans (*Phaseolus vulgaris*) [8];

- *Calla lily chlorotic spot orthotospovirus* for calla lily chlorotic spot virus (CCSV) found in calla lilies (*Zantedeschia* sp.) [5, 17];
- *Capsicum chlorosis orthotospovirus* for Capsicum chlorosis virus (CaCV) found in capsicums, chillies, and tomatoes [15, 22];
- *Chrysanthemum stem necrosis orthotospovirus* for Chrysanthemum stem necrosis virus (CSNV) infecting chrysanthemums [3, 10];
- *Melon severe mosaic orthotospovirus* for melon severe mosaic virus (MSMV) in cucurbit crops [6, 7];
- *Melon yellow spot orthotospovirus* for melon yellow spot virus (MYSV) found in netted melon (*Cucumis melo*) [14]; and
- *Soybean vein necrosis orthotospovirus* for soybean vein necrosis virus (SVNV) discovered in soybeans (Glycine max) [35].

A genus unassigned to any family, *Coguvirus*, was established to include species *Citrus coguvirus* for Citrus concave gum-associated virus (CCGaV) found in citrus trees [25] (TaxoProp 2018.020P.A.v1.Coguvirus).

## TAXONOMIC CHANGES AT THE FAMILY RANK

### *Arenaviridae*

In February 2019, the family *Arenaviridae* was expanded by one genus, *Antennavirus*, to include two species, *Hairy antennavirus* and *Striated antennavirus*, for Wēnlǐngfrogfish arenavirus 2 (WlFV-2) and Wēnlǐng frogfish arenavirus 1 (WlFV-1) found in striated frogfish (*Antennarius*



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231 *striatus*), respectively [32] (TaxoProp 2018.005M.A.v1.Antennavirus).

232 ***Cruliviridae***

233 In February 2019, no changes were made at the family rank.

234 ***Fimoviridae***

235 In February 2019, no changes were made at the family rank.

236 ***Hantaviridae***

237 In February 2019, the family (TaxoProp 2018.010M.A.v2.Hantaviridae_4subfam) was
238 subdivided into four subfamilies:

239 • subfamily *Actantavirinae* was created for new genus *Actinavirus* to accommodate 3 novel
240 species: *Batfish actinovirus* for Wēnlǐng minipizza batfish virus (WEMV) discovered in
241 minipizza batfish (*Halieutaea stellata*); *Goosefish actinovirus* for Wēnlǐng yellow goosefish
242 virus (WEYGV) found in yellow goosefish (*Lophius litulon*); and *Spikefish actinovirus* for
243 Wēnlǐng red spikefish virus (WERSV) of red spikefish (*Triacanthodes anomalus*) [32];
244 • subfamily *Agantavirinae* was created for new genus *Agnathovirus* to accommodate one new
245 species, *Hagfish agnathovirus*, for Wēnlǐng hagfish virus (WEHV) of inshore hagfish
246 (*Eptatretus burgeri*) [32];
247 • subfamily *Mammantavirinae* was created to accommodate the established genera *Loanvirus*,
248 *Mobatvirus*, and *Orthohantavirus*. Two new orthohantavirus species, *Seewis orhtohantavirus*
249 and *Tigray orthohantavirus*, were created for Seewis virus (SWSV) of Eurasian common
250 shrews (*Sorex araneus*) [33] and Tigray virus (TIGV) of Ethiopian white-footed mice



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(*Stenocephalemys albipes*) [24], respectively; and

- subfamily *Repantavirinae* was created for new genus *Reptillovirus* to accommodate one new species, *Gecko reptillovirus*, for Hǎinán oriental lead-toed gecko virus (HOLGV) discovered in oriental leaf-toed geckos (*Hemidactylus bowringii*) [32].

***Mypoviridae***

In February 2019, no changes were made at the family rank.

***Nairoviridae***

In February 2019, species *Estero Real orthonairovirus* was created for Estero Real virus (ERV) (moved from genus *Orthobunyavirus*, family *Peribunyaviridae*) [1] (TaxoProp 2018.012M.A.v1.Bunyavirales_spmov).

***Peribunyaviridae***

In February 2019, the family was expanded by one new genus, *Pacuvirus*, to accommodate three new species: *Pacui pacuvirus* for Pacui virus (PACV) discovered in a rice rat (*Oryzomys* sp.); *Rio Preto da Eva pacuvirus* for Rio Preto da Eva virus discovered in a sewer gnat (Psychodidae sp.); and *Tapirape pacuvirus* for Tapirapé virus found in a hocicudo (*Oxymycterus* sp.) [26] (TaxoProp 2018.017M.A.v1.Bunyavirales_2fam5gen). Genus *Orthotospovirus* was removed from the family and placed into the new family *Tospoviridae* (TaxoProp 2018.017M.A.v1.Bunyavirales_2fam5gen). The genus *Orthobunyavirus* was reorganized by moving previously classified viruses into a total of 38 new species (one resulting from a merger of two previously established species) (TaxoProp 2018.008M.A.v1.Orthobunyavirus_38sp). Five additional novel species were added: *Bellavista orthobunyavirus* for Bellavista virus isolated



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from mosquitoes (*Culex portesi*) [13]; *Enseada orthobunyavirus* for Enseada virus isolated from Culex mosquitoes [4, 9]; *Maguari orthobunyavirus* for Maguari virus (MAGV) isolated from mosquitoes [12]; *Tataguine orthobunyavirus* for Tataguine virus (TATV) found in a human sample, and *Witwatersrand orthobunyavirus* for Witwatersrand virus (WITV) from mosquitoes [28] (TaxoProp 2018.017M.A.v1.Bunyavirales_2fam5gen). Species *Estero Real orthobunyavirus* dissolved, and its member, Estero Real virus (ERV), was moved into family *Nairoviridae* [1] (TaxoProp 2018.012M.A.v1.Bunyavirales_spmov).

***Phasmaviridae***

In February 2019, the family was expanded by addition of one new genus, *Sawastrivirus*, to include one new species, *Sanxia sawastrivirus*, for Sānxiá water strider virus 2 (SxWSV-2) (TaxoProp 2018.017M.A.v1.Bunyavirales_2fam5gen) detected in gerrid water striders [30]. The genus *Orthophasmavirus* was expanded by the addition of five new species: *Culex orthophasmavirus* for Culex phasma-like virus (CPLV) detected in *Culex* mosquitoes [31]; *Ganda orthophasmavirus* for Ganda bee virus (GBEEV) of European orchard bees (*Osmia cornuta*) [27]; *Odonate orthophasmavirus* for Húběi odonate virus 8 (HbOV-8) [30]; *Qingling orthophasmavirus* for Húběi odonate virus 9 (HbOV-9) of odonates [30]; and *Seattle orthophasmavirus* for Seattle Prectang virus (SEPV) found in a moth (*Pasiphila rectangulata*) [20] (TaxoProp 2018.009M.A.v1.Phasmavirus_5sp).

***Phenuiviridae***

In February 2019, the family *Phenuiviridae* was expanded three new genera. Genus *Kabutovirus* was created to include two new species, *Huangpi kabutovirus* and *Kabuto mountain kabutovirus*,



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293 for Huángpí tick virus 1 (HpTV-1) of ticks (*Haemaphysalis doenitzi*) [16] and Kabuto mountain
294 virus (KAMV) of ticks (*Haemaphysalis flava*) [11], respectively. Genus *Laulavirus* was created
295 to include one species, *Laurel Lake laulavirus*, for Laurel Lake virus (LLV) of ticks (*Ixodes*
296 *scapularis*) [34]. Genus *Wenrivirus* was created to include one species, *Shrimp wenrivirus*, for
297 Wēnzhōu shrimp virus 1 (WzSV-1) [16] found in giant tiger prawns (Penaeus monodon)
298 [16](TaxoProp 2018.017M.A.v1.Bunyavirales_2fam5gen). The established genus *Banyangvirus*
299 was expanded by two species, *Guertu banyangvirus* and *Heartland banyangvirus*, for Guertu
300 virus (GTV) found in *Dermacentor nuttalli* ticks [29] and Heartland virus (HRTV), a tick-borne
301 virus originally discovered in human samples [23], respectively (TaxoProp
302 2018.013M.A.v1.Banyangvirus_sp; TaxoProp 2018.017M.A.v1.Bunyavirales_2fam5gen).
303 Genus *Phlebovirus* was expanded by one species, *Mukawa phlebovirus*, for Mukawa virus
304 (MKWV) of ticks (*Ixodes persulcatus*) [21] (TaxoProp 2018.014M.A.v1.Phlebovirus_sp). In
305 genus *Phasivirus*, species *Wuhan fly phasivirus* was deleted (TaxoProp
306 2018.019M.A.v1.Phenuiviridae_Remsp).

307 ***Wupedeviridae***

308 In February 2019, no changes were made at the family rank.

309 **SUMMARY**

310 A summary of the current, ICTV-accepted taxonomy of the order *Bunyavirales* is presented in
311 Table 1.

312 **ACKNOWLEDGMENTS**



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**Table 1. ICTV-accepted taxonomy of the order *Bunyavirales* as of February 2019.** Listed are all bunyaviruses that are classified into species.

| Genus | Species¶ | Virus (Abbreviation)¶ |
|---|---|---|
| **Family *Arenaviridae*** | | |
| *Antennavirus* | *Hairy antennavirus* | Wēnlǐng frogfish arenavirus 2 (WlFAV-2) |
| | *Striated antennavirus** | Wēnlǐng frogfish arenavirus 1 (WlFAV-1) |
| *Hartmanivirus* | *Haartman hartmanivirus** | Haartman Institute snake virus 1 (HISV-1) |
| *Mammarenavirus* | *Allpahuayo mammarenavirus* | Allpahuayo virus (ALLV) |
| | *Argentinian mammarenavirus* | Junín virus (JUNV) |
| | *Bear Canyon mammarenavirus* | Bear Canyon virus (BCNV) |
| | *Brazilian mammarenavirus* | Sabiá virus (SBAV) |
| | *Cali mammarenavirus* | Pichindé virus (PICHV) |
| | *Chapare mammarenavirus* | Chapare virus (CHAPV) |
| | *Cupixi mammarenavirus* | Cupixi virus (CUPXV) |
| | *Flexal mammarenavirus* | Flexal virus (FLEV) |
| | *Gairo mammarenavirus* | Gairo virus (GAIV) |

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| | | |
|---|---|---|
| | *Guanarito mammarenavirus* | Guanarito virus (GTOV) |
| | *Ippy mammarenavirus* | Ippy virus (IPPYV) |
| | *Lassa mammarenavirus* | Lassa virus (LASV) |
| | *Latino mammarenavirus* | Latino virus (LATV) |
| | *Loei River mammarenavirus* | Loei River virus (LORV) |
| | *Lujo mammarenavirus* | Lujo virus (LUJV) |
| | *Luna mammarenavirus* | Luli virus (LULV) |
| | | Luna virus (LUAV) |
| | *Lunk mammarenavirus* | Lunk virus (LNKV) |
| | *Lymphocytic choriomeningitis mammarenavirus** | Dandenong virus (DANV) |
| | | lymphocytic choriomeningitis virus (LCMV) |
| | *Machupo mammarenavirus* | Machupo virus (MACV) |
| | *Mariental mammarenavirus* | Mariental virus (MRLV) |



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| | | |
|---|---|---|
| | *Merino Walk mammarenavirus* | Merino Walk virus (MRWV) |
| | *Mobala mammarenavirus* | mobala virus (MOBV) |
| | *Mopeia mammarenavirus* | Mopeia virus (MPOV) |
| | | Morogoro virus (MORV) |
| | *Okahandja mammarenavirus* | Okahandja virus (OKAV) |
| | *Oliveros mammarenavirus* | Oliveros virus (OLVV) |
| | *Paraguayan mammarenavirus* | Paraná virus (PRAV) |
| | *Pirital mammarenavirus* | Pirital virus (PIRV) |
| | *Ryukyu mammarenavirus* | Ryukyu virus (RYKV) |
| | *Serra do Navio mammarenavirus* | Amaparí virus (AMAV) |
| | *Solwezi mammarenavirus* | Solwezi virus (SOLV) |
| | *Souris mammarenavirus* | souris virus (SOUV) |
| | *Tacaribe mammarenavirus* | Tacaribe virus (TCRV) |
| | *Tamiami mammarenavirus* | Tamiami virus (TMMV) |
| | *Wenzhou mammarenavirus* | Wēnzhōu virus (WENV)[1] |



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| | | |
|---|---|---|
| | *Whitewater Arroyo mammarenavirus* | Big Brushy Tank virus (BBRTV) |
| | | Catarina virus (CTNV) |
| | | Skinner Tank virus (SKTV) |
| | | Tonto Creek virus (TTCV) |
| | | Whitewater Arroyo virus (WWAV) |
| *Reptarenavirus* | *California reptarenavirus* | CAS virus (CASV) |
| | *Giessen reptarenavirus* | University of Giessen virus 1 (UGV-1) |
| | | University of Giessen virus 2 (UGV-2) |
| | | University of Giessen virus 3 (UGV-3) |
| | *Golden reptarenavirus** | Golden Gate virus (GOGV) |
| | *Ordinary reptarenavirus* | tavallinen suomalainen mies virus 2 (TSMV-2) |
| | *Rotterdam reptarenavirus* | ROUT virus (ROUTV) |
| | | University of Helsinki virus 1 (UHV-1) |
| **Family *Cruliviridae*** | | |



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| | | |
|---|---|---|
| *Lincruvirus* | *Crustacean lincruvirus** | Wēnlǐng crustacean virus 9 (WlCV-9) |
| **Family *Fimoviridae*** | | |
| *Emaravirus* | *Actinidia chlorotic ringspot-associated emaravirus* | Actinidia chlorotic ringspot-associated virus (AcCRaV) |
| | *European mountain ash ringspot-associated emaravirus** | European mountain ash ringspot-associated virus (EMARaV) |
| | *Fig mosaic emaravirus* | fig mosaic virus (FMV) |
| | *High Plains wheat mosaic emaravirus* | High Plains wheat mosaic virus (HPWMoV) |
| | *Pigeonpea sterility mosaic emaravirus 1* | pigeonpea sterility mosaic virus (PPSMV) |
| | *Pigeonpea sterility mosaic emaravirus 2* | pigeonpea sterility mosaic virus 2 (PPSMV-2) |
| | *Raspberry leaf blotch emaravirus* | raspberry leaf blotch virus (RLBV) |
| | *Redbud yellow ringspot-associated emaravirus* | redbud yellow ringspot-associated virus (RYRaV) |
| | *Rose rosette emaravirus* | rose rosette virus (RRV) |



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| **Family *Hantaviridae*** | | |
|---|---|---|
| Subfamily *Actantavirinae* | | |
| *Actinovirus* | *Batfish actinovirus* | Wēnlǐng minipizza batfish virus (WEMBV) |
| | *Goosefish actinovirus* | Wēnlǐng yellow goosefish virus (WEYGV) |
| | *Spikefish actinovirus* | Wēnlǐng red spikefish virus (WERSV) |
| Subfamily *Agantavirinae* | | |
| *Agnathovirus* | *Hagfish agnathovirus* | Wēnlǐng hagfish virus (WEHV) |
| Subfamily *Mammantavirinae* | | |
| *Loanvirus* | *Longquan loanvirus** | Lóngquán virus (LQUV) |
| *Mobatvirus* | *Laibin mobatvirus* | Láibīn virus (LAIV) |
| | *Nova mobatvirus** | Nova virus (NVAV) |
| | *Quezon mobatvirus* | Quezon virus (QZNV) |
| *Orthohantavirus* | *Andes orthohantavirus* | Andes virus (ANDV) |
| | | Castelo dos Sonhos virus (CASV) |



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| | | |
|---|---|---|
| | | Lechiguanas virus (LECV = LECHV) |
| | | Orán virus (ORNV) |
| | *Asama orthohantavirus* | Asama virus (ASAV) |
| | *Asikkala orthohantavirus* | Asikkala virus (ASIV) |
| | *Bayou orthohantavirus* | bayou virus (BAYV) |
| | | Catacamas virus (CATV) |
| | *Black Creek Canal orthohantavirus* | Black Creek Canal virus (BCCV) |
| | *Bowe orthohantavirus* | Bowé virus (BOWV) |
| | *Bruges orthohantavirus* | Bruges virus (BRGV) |
| | *Cano Delgadito orthohantavirus* | Caño Delgadito virus (CADV) |
| | *Cao Bang orthohantavirus* | Cao Bằng virus (CBNV) |
| | | Liánghé virus (LHEV) |
| | *Choclo orthohantavirus* | Choclo virus (CHOV) |
| | *Dabieshan orthohantavirus* | Dàbiéshān virus (DBSV) |
| | *Dobrava-Belgrade orthohantavirus* | Dobrava virus (DOBV) |



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| | | |
|---|---|---|
| | | Kurkino virus (KURV) |
| | | Saaremaa virus (SAAV) |
| | | Sochi virus (SOCV) |
| | *El Moro Canyon orthohantavirus* | Carrizal virus (CARV) |
| | | El Moro Canyon virus (ELMCV) |
| | | Huitzilac virus (HUIV) |
| | *Fugong orthohantavirus* | Fúgòng virus (FUGV) |
| | *Fusong orthohantavirus* | Fŭsōng virus (FUSV) |
| | *Hantaan orthohantavirus** | Amur virus (AMRV) |
| | | Hantaan virus (HTNV) |
| | | Soochong virus (SOOV) |
| | *Jeju orthohantavirus* | Jeju virus (JJUV) |
| | *Kenkeme orthohantavirus* | Kenkeme virus (KKMV) |
| | *Khabarovsk orthohantavirus* | Khabarovsk virus (KHAV) |
| | | Topografov virus (TOPV) |



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| | | |
|---|---|---|
| | *Laguna Negra orthohantavirus* | Laguna Negra virus (LANV) |
| | | Maripa virus (MARV) |
| | | Río Mamoré virus (RIOMV) |
| | *Luxi orthohantavirus* | Lúxī virus (LUXV) |
| | *Maporal orthohantavirus* | Maporal virus (MAPV) |
| | *Montano orthohantavirus* | Montaño virus (MTNV) |
| | *Necocli orthohantavirus* | Necoclí virus (NECV) |
| | *Oxbow orthohantavirus* | Oxbow virus (OXBV) |
| | *Prospect Hill orthohantavirus* | Prospect Hill virus (PHV) |
| | *Puumala orthohantavirus* | Hokkaido virus (HOKV) |
| | | Muju virus (MUJV) |
| | | Puumala virus (PUUV) |
| | *Rockport orthohantavirus* | Rockport virus (RKPV) |
| | *Sangassou orthohantavirus* | Sangassou virus (SANGV) |
| | *Seewis orthohantavirus* | Seewis virus (SWSV)[2] |



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| | | |
|---|---|---|
| | *Seoul orthohantavirus* | gōu virus (GOUV) |
| | | Seoul virus (SEOV) |
| | *Sin Nombre orthohantavirus* | New York virus (NYV)[3] |
| | | Sin Nombre virus (SNV) |
| | *Thailand orthohantavirus* | Anjozorobe virus (ANJZV) |
| | | Serang virus (SERV)[4] |
| | | Thailand virus (THAIV) |
| | *Tigray orthohantavirus* | Tigray virus (TIGV) |
| | *Tula orthohantavirus* | Adler virus (ADLV) |
| | | Tula virus (TULV) |
| | *Yakeshi orthohantavirus* | Yákèshí virus (YKSV) |
| *Thottimvirus* | *Imjin thottimvirus* | Imjin virus (MJNV) |
| | *Thottapalayam thottimvirus** | Thottapalayam virus (TPMV) |
| Subfamily *Repantavirinae* | | |
| *Reptillovirus* | *Gecko reptillovirus* | Hǎinán oriental leaf-toed gecko virus |



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| | | |
|---|---|---|
| | | (HOLGV) |
| **Family *Leishbuviridae*** | | |
| *Shilevirus* | *Leptomonas shilevirus** | Leptomonas moramango virus (LEPMV) |
| **Family *Mypoviridae*** | | |
| *Hubavirus* | *Myriapod hubavirus** | Húběi myriapoda virus 5 (HbMV-5) |
| **Family *Nairoviridae*** | | |
| *Orthonairovirus* | *Artashat orthonairovirus* | Artashat virus (ARTSV) |
| | *Chim orthonairovirus* | Chim virus (CHIMV) |
| | *Crimean-Congo hemorrhagic fever orthonairovirus* | Crimean-Congo hemorrhagic fever virus (CCHFV) |
| | *Dera Ghazi Khan orthonairovirus* | Abu Hammad virus (AHV)[5] |
| | | Abu Mina virus (AMV) |
| | | Dera Ghazi Khan virus (DGKV) |
| | | Sapphire II virus (SAPV) |



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| | | |
|---|---|---|
| | *Dugbe orthonairovirus** | Dugbe virus (DUGV) |
| | | kupe virus (KUPEV) |
| | *Estero Real orthonairovirus* | Estero Real virus (ERV) |
| | *Hazara orthonairovirus* | Hazara virus (HAZV) |
| | | Tofla virus (TFLV) |
| | *Hughes orthonairovirus* | Caspiy virus (CASV) |
| | | Farallon virus (FARV) |
| | | Great Saltee virus (GRSV) |
| | | Hughes virus (HUGV) |
| | | Punta Salinas virus (PSV) |
| | | Raza virus (RAZAV) |
| | | Soldado virus (SOLV) |
| | | Zirqa virus (ZIRV) |
| | *Kasokero orthonairovirus* | Kasokero virus (KASV = KASOV) |
| | | Leopards Hill virus (LPHV) |



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| | | |
|---|---|---|
| | | Yogue virus (YOGV) |
| | *Keterah orthonairovirus* | Gossas virus (GOSV) |
| | | Issyk-kul virus (ISKV) |
| | | Keterah virus (KTRV)[6] |
| | | Uzun-Agach virus (UZAV) |
| | *Nairobi sheep disease orthonairovirus* | Nairobi sheep disease virus (NSDV)[7] |
| | *Qalyub orthonairovirus* | Bandia virus (BDAV) |
| | | Geran virus (GERV) |
| | | Qalyub virus (QYBV) |
| | *Sakhalin orthonairovirus* | Avalon virus (AVAV) |
| | | Clo Mor virus (CMV = CLMV) |
| | | Sakhalin virus (SAKV) |
| | | Taggert virus (TAGV) |
| | | Tillamook virus (TILLV) |
| | *Tamdy orthonairovirus* | Burana virus (BURV) |



FOIA Confidential Treatment Requested by Univ of Texas System UTSystem_054497

| | | |
|---|---|---|
| | | Huángpí tick virus 1 (HpTV-1) |
| | | Tăchéng tick virus 1 (TcTV-1) |
| | | Tamdy virus (TAMV) |
| | | Wēnzhōu tick virus (WzTV) |
| | *Thiafora orthonairovirus* | Erve virus (ERVEV) |
| | | Thiafora virus (TFAV) |
| *Shaspivirus* | *Spider shaspivirus** | Shāyáng spider virus 1 (SySV-1) |
| *Striwavirus* | *Strider striwavirus** | Sānxiá water strider virus 1 (SxWSV-1) |
| **Family *Peribunyaviridae*** | | |
| *Herbevirus* | *Herbert herbevirus** | Herbert virus (HEBV) |
| | *Kibale herbevirus* | Kibale virus (KIBV) |
| | *Tai herbevirus* | Taï virus (TAIV) |
| *Orthobunyavirus* | *Acara orthobunyavirus* | Acará virus (ACAV) |
| | | Moriche virus (MORV) |
| | *Akabane orthobunyavirus* | Akabane virus (AKAV) |



FOIA Confidential Treatment Requested by Univ of Texas System UTSystem_054498

| | | |
|---|---|---|
| | | Tinaroo virus (TINV) |
| | | Yaba-7 virus (Y7V) |
| | *Aino orthobunyavirus* | Aino virus (AINOV) |
| | *Alajuela orthobunyavirus* | Alajuela virus (ALJV) |
| | | San Juan virus (SJV) |
| | *Anadyr orthobunyavirus* | Anadyr virus (ANADV) |
| | *Anhembi orthobunyavirus* | Anhembi virus (AMBV) |
| | *Anopheles A orthobunyavirus* | Anopheles A virus (ANAV) |
| | | Arumateua virus (ARTV = ARMTV) |
| | | Caraipé virus (CPEV = CRPV) |
| | | Las Maloyas virus (LMV) |
| | | Lukuni virus (LUKV) |
| | | Trombetas virus (TRMV) |
| | | Tucuruí virus (TUCV = TUCRV) |
| | *Anopheles B orthobunyavirus* | Anopheles B virus (ANBV) |



FOIA Confidential Treatment Requested by Univ of Texas System UTSystem_054499

| | | |
|---|---|---|
| | | Boracéia virus (BORV) |
| | *Bakau orthobunyavirus* | Bakau virus (BAKV) |
| | | Ketapang virus (KETV) |
| | | Nola virus (NOLAV) |
| | | Tanjong Rabok virus (TRV) |
| | | Telok Forest virus (TFV) |
| | *Batai orthobunyavirus* | Batai virus (BATV)[8] |
| | *Batama orthobunyavirus* | Batama virus (BMAV) |
| | *Bellavista orthobunyavirus* | Bellavista virus (BELLV) |
| | *Benevides orthobunyavirus* | Benevides virus (BVSV = BENV) |
| | *Bertioga orthobunyavirus* | Bertioga virus (BERV) |
| | | Cananéia virus (CNAV) |
| | | Guaratuba virus (GTBV) |
| | | Itimirim virus (ITIV) |
| | | Mirim virus (MIRV) |



FOIA Confidential Treatment Requested by Univ of Texas System UTSystem_054500

| | | |
|---|---|---|
| | *Bimiti orthobunyavirus* | bimiti virus (BIMV) |
| | *Birao orthobunyavirus* | Birao virus (BIRV) |
| | *Botambi orthobunyavirus* | Botambi virus (BOTV) |
| | *Bozo orthobunyavirus* | Bozo virus (BOZOV) |
| | *Bunyamwera orthobunyavirus** | Bunyamwera virus (BUNV) |
| | | Germiston virus (GERV) |
| | | Lokern virus (LOKV) |
| | | Mboké virus (MBOV) |
| | | Ngari virus (NRIV)[9] |
| | | Northway virus (NORV) |
| | | Santa Rosa virus (SARV) |
| | | Shokwe virus (SHOV) |
| | | Stanfield virus (STAV) |
| | | Xingu virus (XINV) |
| | *Bushbush orthobunyavirus* | Benfica virus (BENV = BNFV) |



FOIA Confidential Treatment Requested by Univ of Texas System UTSystem_054501

| | | |
|---|---|---|
| | | Bushbush virus (BSBV) |
| | | Juan Díaz virus (JDV) |
| | *Buttonwillow orthobunyavirus* | Buttonwillow virus (BUTV) |
| | *Bwamba orthobunyavirus* | Bwamba virus (BWAV) |
| | | Pongola virus (PGAV) |
| | *Cache Valley orthobunyavirus* | Cache Valley virus (CVV) |
| | | Cholul virus (CHLV) |
| | | Tlacotalpan virus (TALV) |
| | *Cachoeira Porteira orthobunyavirus* | Cachoeira Porteira virus (CPOV) |
| | *California encephalitis orthobunyavirus* | California encephalitis virus (CEV) |
| | | Morro Bay virus (MBV) |
| | *Capim orthobunyavirus* | Capim virus (CAPV) |
| | *Caraparu orthobunyavirus* | Apeú virus (APEUV) |
| | | Bruconha virus (BRUV) |
| | | Caraparú virus (CARV) |



FOIA Confidential Treatment Requested by Univ of Texas System UTSystem_054502

| | | |
|---|---|---|
| | | El Huayo virus (EHUV) |
| | | Itaya virus (ITYV) |
| | | Ossa virus (OSSAV) |
| | | Vinces virus (VINV) |
| | *Cat Que orthobunyavirus* | Cát Quế virus (CQV) |
| | | Oya virus (OYAV) |
| | *Catu orthobunyavirus* | Catú virus (CATUV) |
| | *Enseada orthobunyavirus* | Enseada virus (ENSV) |
| | *Faceys paddock orthobunyavirus* | Facey's paddock virus (FPV) |
| | *Fort Sherman orthobunyavirus* | Fort Sherman virus (FSV) |
| | *Gamboa orthobunyavirus* | Brus Laguna virus (BLAV) |
| | | Calchaquí virus (CQIV) |
| | | Gamboa virus (GAMV) |
| | | Pueblo Viejo virus (PVV) |
| | | Soberanía virus (SOBV) |



FOIA Confidential Treatment Requested by Univ of Texas System UTSystem_054503

| | | |
|---|---|---|
| | *Guajara orthobunyavirus* | Guajará virus (GJAV) |
| | *Guama orthobunyavirus* | Ananindeua virus (ANUV) |
| | | Guamá virus (GMAV) |
| | | Mahogany Hammock virus (MHV) |
| | | Moju virus (MOJUV) |
| | *Guaroa orthobunyavirus* | Guaroa virus (GROV) |
| | *Iaco orthobunyavirus* | Iaco virus (IACOV) |
| | *Ilesha orthobunyavirus* | Ilesha virus (ILEV) |
| | *Ingwavuma orthobunyavirus* | Ingwavuma virus (INGV) |
| | *Jamestown Canyon orthobunyavirus* | Inkoo virus (INKV) |
| | | Jamestown Canyon virus (JCV) |
| | | Jerry Slough virus (JSV) |
| | | South River virus (SORV) |
| | *Jatobal orthobunyavirus* | Jatobal virus (JASTV) |
| | *Kaeng Khoi orthobunyavirus* | Kaeng Khoi virus (KKV) |



FOIA Confidential Treatment Requested by Univ of Texas System UTSystem_054504

| | | |
|---|---|---|
| | *Kairi orthobunyavirus* | Kairi virus (KRIV) |
| | *Keystone orthobunyavirus* | Keystone virus (KEYV) |
| | *Koongol orthobunyavirus* | koongol virus (KOOV) |
| | | wongal virus (WONV) |
| | *La Crosse orthobunyavirus* | La Crosse virus (LACV) |
| | *Leanyer orthobunyavirus* | Leanyer virus (LEAV) |
| | *Lumbo orthbunyavirus* | Lumbo virus (LUMV) |
| | *Macaua orthobunyavirus* | Macauã virus (MCAV) |
| | *Madrid orthobunyavirus* | Madrid virus (MADV) |
| | *Maguari orthobunyavirus* | Maguari virus (MAGV) |
| | | Playas virus (PLAV) |
| | *Main Drain orthobunyavirus* | Main Drain virus (MDV) |
| | *Manzanilla orthobunyavirus* | Manzanilla virus (MANV) |
| | | Inini virus (INIV) |
| | *Marituba orthobunyavirus* | Gumbo Limbo virus (GLV) |



FOIA Confidential Treatment Requested by Univ of Texas System UTSystem_054505

| | | |
|---|---|---|
| | | Marituba virus (MTBV) |
| | | Murutucú virus (MURV) |
| | | Nepuyo virus (NEPV) |
| | | Restan virus (RESV) |
| | | Zungarococha virus (ZUNV) |
| | *Melao orthobunyavirus* | Melao virus (MELV) |
| | *Mermet orthobunyavirus* | Mermet virus (MERV) |
| | *Minatitlan orthobunyavirus* | Minatitlán virus (MNTV) |
| | | Palestina virus (PLSV) |
| | *MPoko orthobunyavirus* | M'Poko virus (MPOV) |
| | | Yaba-1 virus (Y1V) |
| | *Nyando orthobunyavirus* | Eretmapodites virus (ERETV) |
| | | |
| | | Mojuí dos Campos virus (MDCV) |
| | | Nyando virus (NDV) |



FOIA Confidential Treatment Requested by Univ of Texas System UTSystem_054506

| | | |
|---|---|---|
| | *Olifantsvlei orthobunyavirus* | Bobia virus (BIAV) |
| | | Dabakala virus (DABV) |
| | | Olifantsvlei virus (OLIV) |
| | | Oubi virus (OUBIV) |
| | *Oriboca orthobunyavirus* | Itaquí virus (ITQV) |
| | | Oriboca virus (ORIV) |
| | *Oropouche orthobunyavirus* | Iquitos virus (IQTV)[d] |
| | | Madre de Dios virus (MDDV) |
| | | Oropouche virus (OROV) |
| | | Perdões virus (PDEV) |
| | | Pintupo virus (PINTV) |
| | *Patois orthobunyavirus* | Abras virus (ABRV) |
| | | Babahoya virus (BABV) |
| | | Pahayokee virus (PAHV) |
| | | Patois virus (PATV) |



FOIA Confidential Treatment Requested by Univ of Texas System UTSystem_054507

| | | |
|---|---|---|
| | | Shark River virus (SRV) |
| | *Peaton orthobunyavirus* | Peaton virus (PEAV) |
| | *Potosi orthobunyavirus* | Potosi virus (POTV) |
| | *Sabo orthobunyavirus* | Sabo virus (SABOV) |
| | *San Angelo orthobunyavirus* | San Angelo virus (SAV) |
| | *Sango orthobunyavirus* | Sango virus (SANV) |
| | *Schmallenberg orthobunyavirus* | Douglas virus (DOUV) |
| | | Sathuperi virus (SATV) |
| | | Schmallenberg virus (SBV) |
| | | Shamonda virus (SHAV) |
| | *Serra do Navio orthobunyavirus* | Serra do Navio virus (SDNV) |
| | *Shuni orthobunyavirus* | Kaikalur virus (KAIV) |
| | | Shuni virus (SHUV) |
| | *Simbu orthobunyavirus* | Para virus (PARAV) |
| | | Simbu virus (SIMV) |



FOIA Confidential Treatment Requested by Univ of Texas System UTSystem_054508

| | | |
|---|---|---|
| | *Snowshoe hare orthobunyavirus* | Khatanga virus (KHATV)[10] |
| | | snowshoe hare virus (SSHV) |
| | *Sororoca orthobunyavirus* | Sororoca virus (SORV) |
| | *Tacaiuma orthobunyavirus* | CoAr 1071 virus (CA1071V) |
| | | CoAr 3627 virus (CA3626V) |
| | | Tacaiuma virus (TCMV) |
| | | Virgin River virus (VRV) |
| | *Tahyna orthobunyavirus* | Ťahyňa virus (TAHV) |
| | *Tataguine orthobunyavirus* | Tataguine virus (TATV) |
| | *Tensaw orthobunyavirus* | Tensaw virus (TENV) |
| | *Tete orthobunyavirus* | Bahig virus (BAHV) |
| | | Matruh virus (MTRV) |
| | | Tete virus (TETEV) |
| | | Tsuruse virus (TSUV) |
| | | Weldona virus (WELV) |



FOIA Confidential Treatment Requested by Univ of Texas System UTSystem_054509

| | | |
|---|---|---|
| | *Thimiri orthobunyavirus* | Thimiri virus (THIV) |
| | *Timboteua orthobunyavirus* | Timboteua virus (TBTV) |
| | *Trivittatus orthobunyavirus* | Achiote virus (ACHOV) |
| | | Trivittatus virus (TVTV) |
| | *Turlock orthobunyavirus* | Lednice virus (LEDV) |
| | | Turlock virus (TURV) |
| | | Umbre virus (UMBV) |
| | *Utinga orthobunyavirus* | Utinga virus (UTIV) |
| | *Wolkberg orthobunyavirus* | Wolkberg virus (WBV) |
| | *Wyeomyia orthobunyavirus* | BeAr 328208 virus (BAV) |
| | | Rio Pracupi virus |
| | | Taiassui virus (TAIAV) |
| | | Tucunduba virus (TUCV) |
| | | Wyeomyia virus (WYOV) |
| | *Zegla orthobunyavirus* | Zegla virus (ZEGV) |



FOIA Confidential Treatment Requested by Univ of Texas System UTSystem_054510

| | | |
|---|---|---|
| *Pacuvirus* | *Pacui pacuvirus** | Pacui virus (PACV) |
| | *Rio Preto da Eva pacuvirus* | Rio Preto da Eva virus (RPEV) |
| | *Tapirape pacuvirus* | Tapirapé virus (TAPV) |
| *Shangavirus* | *Insect shangavirus* | Shuāngào insect virus 1 (SgIV-1) |
| **Family *Phasmaviridae*** | | |
| *Feravirus* | *Ferak feravirus** | Ferak virus (FRKV) |
| *Inshuvirus* | *Insect inshuvirus** | Shuāngào insect virus 2 (SgIV-2) |
| *Jonvirus* | *Jonchet jonvirus** | jonchet virus (JONV) |
| *Orthophasmavirus* | *Culex orthophasmavirus* | Culex orthophasmavirus (CPLV) |
| | *Ganda orthophasmavirus* | Ganda orthophasmavirus (GBEEV) |
| | *Kigluaik phantom orthophasmavirus** | Kigluaik phantom virus (KIGV) |
| | *Nome phantom orthophasmavirus* | Nome phantom virus (NOMV) |
| | *Odonate orthophasmavirus* | Odonate orthophasmavirus (HbOV-8) |
| | *Qingling orthophasmavirus* | Qingling orthophasmavirus (HbOV-9) |
| | *Seattle orthophasmavirus* | Seattle orthophasmavirus (SEPV) |



FOIA Confidential Treatment Requested by Univ of Texas System UTSystem_054511

| | | |
|---|---|---|
| | *Wuchang cockroach orthophasmavirus 1* | Wǔchāng cockroach virus 1 (WcCV-1) |
| | *Wuhan mosquito orthophasmavirus 1* | Wǔhàn mosquito virus 1 (WhMV-1) |
| | *Wuhan mosquito orthophasmavirus 2* | Wǔhàn mosquito virus 2 (WhMV-2) |
| *Sawastrivirus* | *Sanxia sawastrivirus** | Sānxiá water strider virus 2 (SxWSV-2) |
| *Wuhivirus* | *Insect wuhivirus** | Wǔhàn insect virus 2 (WhIV-2) |
| **Family *Phenuiviridae*** | | |
| *Banyangvirus* | *Guertu banyangvirus* | Guertu virus (GTV) |
| | *Heartland banyangvirus* | Heartland virus (HRTV) |
| | *Huaiyangshan banyangvirus** | severe fever with thrombocytopenia syndrome virus (SFTSV) |
| *Beidivirus* | *Dipteran beidivirus** | Húběi diptera virus 3 (HbDV-3) |
| *Goukovirus* | *Cumuto goukovirus* | Cumuto virus (CUMV) |
| | *Gouleako goukovirus** | Gouléako virus (GOLV) |
| | *Yichang insect goukovirus* | Yíchāng insect virus (YcIV) |
| *Horwuvirus* | *Horsefly horwuvirus** | Wǔhàn horsefly virus (WhHV) |



FOIA Confidential Treatment Requested by Univ of Texas System UTSystem_054512

| | | |
|---|---|---|
| *Hudivirus* | *Dipteran hudivirus** | Húběi diptera virus 4 (HbDV-4) |
| *Hudovirus* | *Lepidopteran hudovirus** | Húběi lepidoptera virus 1 (HbLV-1) |
| *Kabutovirus* | *Huangpi kabutovirus** | Huángpí tick virus 2 (HpTV-2) |
| | *Kabuto mountain kabutovirus* | Kabuto mountain virus (KAMV) |
| *Laulavirus* | *Laurel Lake laulavirus** | Laurel Lake virus (LLV) |
| *Mobuvirus* | *Mothra mobuvirus** | Mothra virus (MTHV) |
| *Phasivirus* | *Badu phasivirus** | Badu virus (BADUV) |
| | *Phasi Charoen-like phasivirus* | Phasi Chaeron-like virus (PCLV) |
| | *Wutai mosquito phasivirus* | Wǔtái mosquito virus (WtMV) |
| *Phlebovirus* | *Bujaru phlebovirus* | Bujaru virus (BUJV) |
| | | Munguba virus (MUNV) |
| | *Candiru phlebovirus* | Alenquer virus (ALEV) |
| | | Ariquemes virus (ARQV) |
| | | Candirú virus (CDUV) |
| | | Itaituba virus (ITAV) |



FOIA Confidential Treatment Requested by Univ of Texas System UTSystem_054513

| | | |
|---|---|---|
| | | Jacundá virus (JCNV) |
| | | Maldonado virus (MLOV) |
| | | Morumbi virus (MR(M)BV) |
| | | Mucura virus (MCRV/MRAV) |
| | | Nique virus (NIQV) |
| | | Oriximiná virus (ORXV) |
| | | Serra Norte virus (SRNV) |
| | | Turuna virus (TUAV) |
| | *Chilibre phlebovirus* | Cacao virus (CACV) |
| | | Chilibre virus (CHIV) |
| | *Frijoles phlebovirus* | Frijoles virus (FRIV) |
| | | Joá virus (JOAV) |
| | *Mukawa phlebovirus* | Mukawa virus (MKWV) |
| | *Punta Toro phlebovirus* | Buenaventura virus (BUEV) |
| | | Campana virus (CMAV) |



FOIA Confidential Treatment Requested by Univ of Texas System UTSystem_054514

| | | |
|---|---|---|
| | | Capira virus (CAPIV) |
| | | Coclé virus (CCLV) |
| | | Leticia virus (LTCV) |
| | | Punta Toro virus (PTV) |
| | *Rift Valley fever phlebovirus** | Rift Valley fever virus (RVFV) |
| | *Salehabad phlebovirus* | Adana virus (ADAV) |
| | | Adria virus (ADRV) |
| | | Alcube virus |
| | | Arbia virus (ARBV) |
| | | Arumowot virus (AMTV) |
| | | Bregalaka virus (BREV) |
| | | Medjerda Valley virus (MVV) |
| | | Odrénisrou virus (ODRV) |
| | | Olbia virus (OLBV) |
| | | Salehabad virus (SALV) |



FOIA Confidential Treatment Requested by Univ of Texas System UTSystem_054515

| | | |
|---|---|---|
| | | Zaba virus (ZABAV) |
| | *Sandfly fever Naples phlebovirus* | Arrábida virus (ARRV) |
| | | Balkan virus (BALKV) |
| | | Fermo virus (FERV) |
| | | Gordil virus (GORV) |
| | | Granada virus (GRV = GRAV) |
| | | Massilia virus (MASV) |
| | | Punique virus (PUNV) |
| | | Saddaguia virus (SADV) |
| | | Saint-Floris virus (SAFV) |
| | | sandfly fever Naples virus (SFNV) |
| | | Tehran virus (THEV) |
| | | Toscana virus (TOSV) |
| | | Zerdali virus (ZERV) |
| | *Uukuniemi phlebovirus* | Chizé virus (CHZV) |



FOIA Confidential Treatment Requested by Univ of Texas System UTSystem_054516

| | | |
|---|---|---|
| | | EgAN 1825-61 virus (EGAV) |
| | | Fin V 707 virus (FINV) |
| | | Oceanside virus (OCV = OCEV) |
| | | Pontevès virus (PTVV) |
| | | St. Abbs Head virus (SAHV) |
| | | Uukuniemi virus (UUKV) |
| | | Zaliv Terpenyia virus (ZTV) |
| *Pidchovirus* | *Pidgey pidchovirus** | Pidgey virus (PGYV) |
| *Tenuivirus* | *Echinochloa hoja blanca tenuivirus* | Echinochloa hoja blanca virus (EHBV) |
| | *Iranian wheat stripe tenuivirus* | Iranian wheat stripe virus (IWSV) |
| | *Maize stripe tenuivirus* | maize stripe virus (MStV = MSpV) |
| | *Rice grassy stunt tenuivirus* | rice grassy stunt virus (RGSV) |
| | *Rice hoja blanca tenuivirus* | rice hoja blanca virus (RHBV) |
| | *Rice stripe tenuivirus** | rice stripe virus (RSV = RStV) |
| | *Urochloa hoja blanca tenuivirus* | Urochloa hoja blanca virus (UHBV) |



FOIA Confidential Treatment Requested by Univ of Texas System UTSystem_054517

| | | |
|---|---|---|
| *Wenrivirus* | *Shrimp wenrivirus** | Wēnzhōu shrimp virus 1 WzSV-1 |
| *Wubeivirus* | *Dipteran wubeivirus** | Húběi diptera virus 5 (HbDV-5) |
| | *Fly wubeivirus* | Wǔhàn fly virus 1 (WhFV-1) |
| **Family *Tospoviridae*** | | |
| *Orthotospovirus* | *Bean necrotic mosaic orthotospovirus* | bean necrotic mosaic virus (BeNMV) |
| | *Calla lily chlorotic spot orthotospovirus* | calla lily chlorotic spot virus (CCSV) |
| | *Capsicum chlorosis orthotospovirus* | Capsicum chlorosis virus (CaCV) |
| | *Chrysanthemum stem necrosis orthotospovirus* | Chrysanthemum stem necrosis virus (CSNV) |
| | *Groundnut bud necrosis orthotospovirus* | groundnut bud necrosis virus (GBNV)[11] |
| | *Groundnut ringspot orthotospovirus* | groundnut ringspot virus (GRSV) |
| | *Groundnut yellow spot orthotospovirus* | groundnut yellow spot virus (GYSV)[12] |
| | *Impatiens necrotic spot orthotospovirus* | impatiens necrotic spot virus (INSV) |
| | *Iris yellow spot orthotospovirus* | iris yellow spot virus (IYSV) |
| | *Melon severe mosaic orthotospovirus* | melon severe mosaic virus (MSMV) |



FOIA Confidential Treatment Requested by Univ of Texas System UTSystem_054518

| | | |
|---|---|---|
| | *Melon yellow spot orthotospovirus* | melon yellow spot virus (MYSV) |
| | *Polygonum ringspot orthotospovirus* | Polygonum ringspot virus (PolRSV) |
| | *Soybean vein necrosis orthotospovirus* | soybean vein necrosis virus (SVNV) |
| | *Tomato chlorotic spot orthotospovirus* | tomato chlorotic spot virus (TCSV) |
| | *Tomato spotted wilt orthotospovirus** | tomato spotted wilt virus (TSWV) |
| | *Watermelon bud necrosis orthotospovirus* | watermelon bud necrosis virus (WBNV) |
| | *Watermelon silver mottle orthotospovirus* | watermelon silver mottle virus (WSMoV) |
| | *Zucchini lethal chlorosis orthotospovirus* | zucchini lethal chlorosis virus (ZLCV) |
| **Family *Wupedeviridae*** | | |
| *Wumivirus* | *Millipede wumivirus** | Wǔhàn millipede virus 2 (WhMV-2) |
| **Unassigned** | | |
| *Coguvirus* | *Citrus coguvirus** | citrus concave gum-associated virus (CCGaV) |

*type species



FOIA Confidential Treatment Requested by Univ of Texas System UTSystem_054519

[1]Synonyms: Cardamones virus, Hǎinán Medical University virus, Xīngyì virus; [2]Synonyms: Amga virus (MGAB), Artybash virus (ARTV); [3]synonym: New York 1 virus (NY-1V); [4]synonym: Jurong virus; [5]includes the strain previously referred to as Tunis virus (TUNV); [6]includes the strain previously referred to as soft tick bunyavirus (STBV); [7]includes the strain previously referred to as Ganjam virus (GANV); [8]synonyms: Čalovo virus (CVOV), Chittoor virus (CHITV), Olkya virus, Olyka virus, UgMP-6830 virus; [9]includes the strain previously referred to as Garissa virus; [10]also mistakenly referred to in the literature as Chantanga virus (CHATV) and Chatanga virus (CHATV); [11]synonym: peanut bud necrosis virus (PDNV); [12]synonym: peanut yellow spot virus (PYSV).

Caption: ¶Please note that viruses are real objects that are assigned to concepts that are called taxa. Species, genera, families, and orders are taxa. Taxon names are always italicized and always begin with a capital letter. Virus names, on the other hand, are not italicized and are not capitalized, except if the name or a name component is a proper noun. This column lists the virus names with their correct (lack of) capitalization. Lists of viruses within a given species are provisional at this point and will likely be amended in the near future.



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## COMPLIANCE WITH ETHICAL STANDARDS

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## Conflict of Interest

The authors declare no conflicts of interest.

## Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

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Buchmeier: orcid/org/0000-0002-2387-9287

Burt: orcid.org/0000-0002-7238-7799

Charrel: orcid.org/0000-0002-7675-8251

de la Torre: orcid.org/0000-0002-8171-8115

Deng: orcid.org/ 0000-0002-5385-083X

Di Serio: orcid.org/0000-0003-2822-704X



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159 Ebihara: orcid.org/0000-0002-2576-9735

160 Ergünay: orcid.org/0000-0001-5422-1982

161 Garrison: orcid.org/0000-0003-3034-6400

162 Gonzalez: orcid.org/0000-0003-3063-1770

163 Groschup: orcid.org/0000-0003-0215-185X

164 Günther: orcid.org/0000-0002-6562-0230

165 Hepojoki: orcid.org/0000-0001-5699-214X

166 Hewson: orcid.org/0000-0003-2273-3152

167 Hu: orcid.org/0000-0002-1560-0928

168 Hughes: orcid.org/0000-0003-1380-8263

169 Klempa: orcid.org/0000-0002-6931-1224

170 Klingström: orcid.org/0000-0001-9076-1441

171 Laenen: orcid.org/0000-0001-9118-8608

172 Lambert: orcid.org/0000-0002-7571-3529

173 Lukashevich: orcid.org/0000-0001-6523-5116

174 Maes: orcid.org/0000-0002-4571-5232

175 Marciel de Souza: orcid.org/0000-0002-0025-8293

176 Marklewitz: orcid.org/0000-0003-1828-8770

177 Matsuno: orcid.org/0000-0002-4205-6526

178 Minutolo: orcid.org/0000-0001-7816-2657

179 Navarro: orcid.org/0000-0001-8981-1018

180 Nunes: orcid.org/0000-0001-9739-5499

181 Palacios: orcid.org/0000-0001-5062-1938



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Pawęska: orcid.org/0000-0001-8776-7519

Radoshitzky: orcid.org/0000-0001-8976-8056

Resende: orcid.org/0000-0003-3852-1576

Salvato: orchid.org/0000-0002-3357-5391

Sasaya: orcid.org/0000-0002-5020-9377

Shen: orcid.org/ 0000-0002-0013-5365

Shí: orcid.org/0000-0002-5289-7222

Simmonds: 0000-0002-7964-4700

Sironi: orcid.org/0000-0002-2267-5266

Spengler: orcid.org/0000-0002-5383-0513

Stenglein: orcid.org/0000-0002-0993-813X

Sun: orcid.org/0000-0003-3633-1904

Tang: orcid. org/0000-0002-3416-8739

Turina: orcid.org/0000-0002-9659-9470

Wang: orcid.org/0000-0001-8916-4578

Whitfield: orcid.org/0000-0002-3538-015X

Zerbini: orcid.org/0000-0001-8617-0200

Kuhn: orcid.org/0000-0002-7800-6045

**Keywords:** *Arenaviridae*; arenavirid; arenavirus; bunyavirad; *Bunyavirales*; bunyavirid; *Bunyaviridae*; bunyavirus; emaravirus; *Feraviridae*; feravirid, feravirus; fimovirid; *Fimoviridae*; fimovirus; goukovirus; hantavirid; *Hantaviridae*; hantavirus; hartmanivirus; herbevirus; ICTV; International Committee on Taxonomy of Viruses; jonvirid; *Jonviridae*; jonvirus; leishbuvirid;



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*Leishbuviridae*; leishbuvirus; mammarenavirus; nairovirid; *Nairoviridae*; nairovirus; orthobunyavirus; orthoferavirus; orthohantavirus; orthojonvirus; orthonairovirus; orthophasmavirus; orthotospovirus; peribunyavirid; *Peribunyaviridae*; peribunyavirus; phasmavirid; phasivirus; *Phasmaviridae*; phasmavirus; phenuivirid; *Phenuiviridae*; phenuivirus; phlebovirus; reptarenavirus; tenuivirus; tospovirid; *Tospoviridae*; tospovirus; virus classification; virus nomenclature; virus taxonomy



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## ABSTRACT

In February 2019, the order *Bunyavirales* was amended by creation of two new families, four new subfamilies, eleven new genera, and 77 new species, merging of two species, and deletion of one species. This article presents the updated taxonomy of the order *Bunyavirales* now accepted by the International Committee on Taxonomy of Viruses (ICTV).


## INTRODUCTION

The virus order *Bunyavirales* was established in 2017 to accommodate related viruses with segmented, linear, single-stranded, negative-sense or ambisense RNA genomes classified into 9 families [19]. An amended/emended order description was published in early 2019 [20]. Here we present the changes that were proposed via official ICTV taxonomic proposals that were accepted by the ICTV Executive Committee (EC) in February 2019. These changes are now part of the official ICTV taxonomy as of February 2019.

## TAXONOMIC CHANGES AT THE ORDER RANK

The order was expanded by addition of two new families. Family *Leishbuviridae* was created to accommodate one new genus, *Shilevirus*, including one new species, *Leptomonas shilevirus*, for Leptomonas moramango leishbunyavirus (LEPMV) discovered in a trypanosomatid protist (*Leptomonas moramango*) [2]. Family *Tospoviridae* was recreated for the already established genus *Orthotospovirus*. (TaxoProp 2018.017M.A.v1.Bunyavirales_2fam5gen) and expanded by 7 new species (TaxoProp 2018.025P.A.v1.Orthotospovirus_7sp):

- *Bean necrotic mosaic orthotospovirus* for bean necrotic mosaic virus (BeNMV) discovered in common beans (*Phaseolus vulgaris*) [8];



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- *Calla lily chlorotic spot orthotospovirus* for calla lily chlorotic spot virus (CCSV) found in calla lilies (*Zantedeschia* sp.) [5, 18];
- *Capsicum chlorosis orthotospovirus* for Capsicum chlorosis virus (CaCV) found in capsicums, chillies, and tomatoes [16, 23];
- *Chrysanthemum stem necrosis orthotospovirus* for Chrysanthemum stem necrosis virus (CSNV) infecting chrysanthemums [3, 10];
- *Melon severe mosaic orthotospovirus* for melon severe mosaic virus (MSMV) in cucurbit crops [6, 7];
- *Melon yellow spot orthotospovirus* for melon yellow spot virus (MYSV) found in netted melon (*Cucumis melo*) [15]; and
- *Soybean vein necrosis orthotospovirus* for soybean vein necrosis virus (SVNV) discovered in soybeans (Glycine max) [36].

A genus unassigned to any family, *Coguvirus*, was established to include species *Citrus coguvirus* for Citrus concave gum-associated virus (CCGaV) found in citrus trees [26] (TaxoProp 2018.020P.A.v1.Coguvirus).

## TAXONOMIC CHANGES AT THE FAMILY RANK

### *Arenaviridae*

The family *Arenaviridae* was expanded by one genus, *Antennavirus*, to include two species, *Hairy antennavirus* and *Striated antennavirus*, for Wēnlǐngfrogfish arenavirus 2 (WlFV-2) and Wēnlǐng frogfish arenavirus 1 (WlFV-1) both found in striated frogfish (*Antennarius striatus*) [33] (TaxoProp 2018.005M.A.v1.Antennavirus).



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253 ***Cruliviridae***

254 No changes were made at the family rank.

255 ***Fimoviridae***

256 No changes were made at the family rank.

257 ***Hantaviridae***

258 The family (TaxoProp 2018.010M.A.v2.Hantaviridae_4subfam) was subdivided into four
259 subfamilies:

260 • subfamily *Actantavirinae* was created for new genus *Actinavirus* to accommodate 3 novel
261 species: *Batfish actinovirus* for Wēnlǐng minipizza batfish virus (WEMV) discovered in
262 minipizza batfish (*Halieutaea stellata*); *Goosefish actinovirus* for Wēnlǐng yellow goosefish
263 virus (WEYGV) found in yellow goosefish (*Lophius litulon*); and *Spikefish actinovirus* for
264 Wēnlǐng red spikefish virus (WERSV) of red spikefish (*Triacanthodes anomalus*) [33];
265 • subfamily *Agantavirinae* was created for new genus *Agnathovirus* to accommodate one new
266 species, *Hagfish agnathovirus*, for Wēnlǐng hagfish virus (WEHV) of inshore hagfish
267 (*Eptatretus burgeri*) [33];
268 • subfamily *Mammantavirinae* was created to accommodate the established genera *Loanvirus*,
269 *Mobatvirus*, and *Orthohantavirus*. Two new orthohantavirus species, *Seewis orhtohantavirus*
270 and *Tigray orthohantavirus*, were created for Seewis virus (SWSV) of Eurasian common
271 shrews (*Sorex araneus*) [34] and Tigray virus (TIGV) of Ethiopian white-footed mice
272 (*Stenocephalemys albipes*) [12, 25], respectively; and



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- subfamily *Repantavirinae* was created for new genus *Reptillovirus* to accommodate one new species, *Gecko reptillovirus*, for Hǎinán oriental lead-toed gecko virus (HOLGV) discovered in oriental leaf-toed geckos (*Hemidactylus bowringii*) [33].

***Mypoviridae***

No changes were made at the family rank.

***Nairoviridae***

The species *Estero Real orthonairovirus* was created for Estero Real virus (ERV) (moved from genus *Orthobunyavirus*, family *Peribunyaviridae*) [1] (TaxoProp 2018.012M.A.v1.Bunyavirales_spmov).

***Peribunyaviridae***

The family was expanded by one new genus, *Pacuvirus*, to accommodate three new species: *Pacui pacuvirus* for Pacui virus (PACV) discovered in a rice rat (*Oryzomys* sp.); *Rio Preto da Eva pacuvirus* for Rio Preto da Eva virus discovered in a sewer gnat (Psychodidae sp.); and *Tapirape pacuvirus* for Tapirapé virus found in a hocicudo (*Oxymycterus* sp.) [27] (TaxoProp 2018.017M.A.v1.Bunyavirales_2fam5gen). Genus *Orthotospovirus* was removed from the family and placed into the new family *Tospoviridae* (TaxoProp 2018.017M.A.v1.Bunyavirales_2fam5gen). The genus *Orthobunyavirus* was reorganized by moving previously classified viruses into a total of 38 new species (one resulting from a merger of two previously established species) (TaxoProp 2018.008M.A.v1.Orthobunyavirus_38sp). Five additional novel species were added: *Bellavista orthobunyavirus* for Bellavista virus isolated from mosquitoes (*Culex portesi*) [14]; *Enseada orthobunyavirus* for Enseada virus isolated from



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294 *Culex* mosquitoes [4, 9]; *Maguari orthobunyavirus* for Maguari virus (MAGV) isolated from
295 mosquitoes [13]; *Tataguine orthobunyavirus* for Tataguine virus (TATV) found in a human
296 sample, and *Witwatersrand orthobunyavirus* for Witwatersrand virus (WITV) from mosquitoes
297 [29] (TaxoProp 2018.017M.A.v1.Bunyavirales_2fam5gen). Species *Estero Real orthobunyavirus*
298 dissolved, and its member, Estero Real virus (ERV), was moved into family *Nairoviridae* [1]
299 (TaxoProp 2018.012M.A.v1.Bunyavirales_spmov).

300 ***Phasmaviridae***

301 The family was expanded by addition of one new genus, *Sawastrivirus*, to include one new
302 species, *Sanxia sawastrivirus*, for Sānxiá water strider virus 2 (SxWSV-2) (TaxoProp
303 2018.017M.A.v1.Bunyavirales_2fam5gen) detected in gerrid water striders [31]. The genus
304 *Orthophasmavirus* was expanded by the addition of five new species: *Culex orthophasmavirus*
305 for Culex phasma-like virus (CPLV) detected in *Culex* mosquitoes [32]; *Ganda*
306 *orthophasmavirus* for Ganda bee virus (GBEEV) of European orchard bees (*Osmia cornuta*)
307 [28]; *Odonate orthophasmavirus* for Húběi odonate virus 8 (HbOV-8) [31]; *Qingling*
308 *orthophasmavirus* for Húběi odonate virus 9 (HbOV-9) of odonates [31]; and *Seattle*
309 *orthophasmavirus* for Seattle Prectang virus (SEPV) found in a moth (*Pasiphila rectangulata*)
310 [21] (TaxoProp 2018.009M.A.v1.Phasmavirus_5sp).

311 ***Phenuiviridae***

312 The family *Phenuiviridae* was expanded three new genera. Genus *Kabutovirus* was created to
313 include two new species, *Huangpi kabutovirus* and *Kabuto mountain kabutovirus*, for Huángpí
314 tick virus 1 (HpTV-1) of ticks (*Haemaphysalis doenitzi*) [17] and Kabuto mountain virus



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(KAMV) of ticks (*Haemaphysalis flava*) [11], respectively. Genus *Laulavirus* was created to include one species, *Laurel Lake laulavirus*, for Laurel Lake virus (LLV) of ticks (*Ixodes scapularis*) [35]. Genus *Wenrivirus* was created to include one species, *Shrimp wenrivirus*, for Wēnzhōu shrimp virus 1 (WzSV-1) [17] found in giant tiger prawns (Penaeus monodon) [17](TaxoProp 2018.017M.A.v1.Bunyavirales_2fam5gen). The established genus *Banyangvirus* was expanded by two species, *Guertu banyangvirus* and *Heartland banyangvirus*, for Guertu virus (GTV) found in *Dermacentor nuttalli* ticks [30] and Heartland virus (HRTV), a tick-borne virus originally discovered in human samples [24], respectively (TaxoProp 2018.013M.A.v1.Banyangvirus_sp; TaxoProp 2018.017M.A.v1.Bunyavirales_2fam5gen). Genus *Phlebovirus* was expanded by one species, *Mukawa phlebovirus*, for Mukawa virus (MKWV) of ticks (*Ixodes persulcatus*) [22] (TaxoProp 2018.014M.A.v1.Phlebovirus_sp). In genus *Phasivirus*, species *Wuhan fly phasivirus* was deleted (TaxoProp 2018.019M.A.v1.Phenuiviridae_Remsp).

***Wupedeviridae***

No changes were made at the family rank.

## SUMMARY

A summary of the current, ICTV-accepted taxonomy of the order *Bunyavirales* is presented in Table 1.

## ACKNOWLEDGMENTS

We thank Laura Bollinger (NIH/NIAID Integrated Research Facility at Fort Detrick, Frederick, MD, USA) for critically editing the manuscript.



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**Table 1. ICTV-accepted taxonomy of the order *Bunyavirales* as of February 2019.** Listed are all bunyaviruses that are classified into species.

| Genus | Species¶ | Virus (Abbreviation)¶ |
|---|---|---|
| **Family *Arenaviridae*** | | |
| *Antennavirus* | *Hairy antennavirus* | Wēnlǐng frogfish arenavirus 2 (WlFAV-2) |
| | *Striated antennavirus** | Wēnlǐng frogfish arenavirus 1 (WlFAV-1) |
| *Hartmanivirus* | *Haartman hartmanivirus** | Haartman Institute snake virus 1 (HISV-1) |
| *Mammarenavirus* | *Allpahuayo mammarenavirus* | Allpahuayo virus (ALLV) |
| | *Argentinian mammarenavirus* | Junín virus (JUNV) |
| | *Bear Canyon mammarenavirus* | Bear Canyon virus (BCNV) |
| | *Brazilian mammarenavirus* | Sabiá virus (SBAV) |
| | *Cali mammarenavirus* | Pichindé virus (PICHV) |
| | *Chapare mammarenavirus* | Chapare virus (CHAPV) |
| | *Cupixi mammarenavirus* | Cupixi virus (CUPXV) |
| | *Flexal mammarenavirus* | Flexal virus (FLEV) |
| | *Gairo mammarenavirus* | Gairo virus (GAIV) |

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| | | |
|---|---|---|
| | *Guanarito mammarenavirus* | Guanarito virus (GTOV) |
| | *Ippy mammarenavirus* | Ippy virus (IPPYV) |
| | *Lassa mammarenavirus* | Lassa virus (LASV) |
| | *Latino mammarenavirus* | Latino virus (LATV) |
| | *Loei River mammarenavirus* | Loei River virus (LORV) |
| | *Lujo mammarenavirus* | Lujo virus (LUJV) |
| | *Luna mammarenavirus* | Luli virus (LULV) |
| | | Luna virus (LUAV) |
| | *Lunk mammarenavirus* | Lunk virus (LNKV) |
| | *Lymphocytic choriomeningitis mammarenavirus** | Dandenong virus (DANV) |
| | | lymphocytic choriomeningitis virus (LCMV) |
| | *Machupo mammarenavirus* | Machupo virus (MACV) |
| | *Mariental mammarenavirus* | Mariental virus (MRLV) |



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| | | |
|---|---|---|
| | *Merino Walk mammarenavirus* | Merino Walk virus (MRWV) |
| | *Mobala mammarenavirus* | mobala virus (MOBV) |
| | *Mopeia mammarenavirus* | Mopeia virus (MPOV) |
| | | Morogoro virus (MORV) |
| | *Okahandja mammarenavirus* | Okahandja virus (OKAV) |
| | *Oliveros mammarenavirus* | Oliveros virus (OLVV) |
| | *Paraguayan mammarenavirus* | Paraná virus (PRAV) |
| | *Pirital mammarenavirus* | Pirital virus (PIRV) |
| | *Ryukyu mammarenavirus* | Ryukyu virus (RYKV) |
| | *Serra do Navio mammarenavirus* | Amaparí virus (AMAV) |
| | *Solwezi mammarenavirus* | Solwezi virus (SOLV) |
| | *Souris mammarenavirus* | souris virus (SOUV) |
| | *Tacaribe mammarenavirus* | Tacaribe virus (TCRV) |
| | *Tamiami mammarenavirus* | Tamiami virus (TMMV) |
| | *Wenzhou mammarenavirus* | Wēnzhōu virus (WENV)[1] |



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| | | |
|---|---|---|
| | *Whitewater Arroyo mammarenavirus* | Big Brushy Tank virus (BBRTV) |
| | | Catarina virus (CTNV) |
| | | Skinner Tank virus (SKTV) |
| | | Tonto Creek virus (TTCV) |
| | | Whitewater Arroyo virus (WWAV) |
| *Reptarenavirus* | *California reptarenavirus* | CAS virus (CASV) |
| | *Giessen reptarenavirus* | University of Giessen virus 1 (UGV-1) |
| | | University of Giessen virus 2 (UGV-2) |
| | | University of Giessen virus 3 (UGV-3) |
| | *Golden reptarenavirus** | Golden Gate virus (GOGV) |
| | *Ordinary reptarenavirus* | tavallinen suomalainen mies virus 2 (TSMV-2) |
| | *Rotterdam reptarenavirus* | ROUT virus (ROUTV) |
| | | University of Helsinki virus 1 (UHV-1) |
| **Family *Cruliviridae*** | | |



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| | | |
|---|---|---|
| *Lincruvirus* | *Crustacean lincruvirus** | Wēnlǐng crustacean virus 9 (WlCV-9) |
| **Family *Fimoviridae*** | | |
| *Emaravirus* | *Actinidia chlorotic ringspot-associated emaravirus* | Actinidia chlorotic ringspot-associated virus (AcCRaV) |
| | *European mountain ash ringspot-associated emaravirus** | European mountain ash ringspot-associated virus (EMARaV) |
| | *Fig mosaic emaravirus* | fig mosaic virus (FMV) |
| | *High Plains wheat mosaic emaravirus* | High Plains wheat mosaic virus (HPWMoV) |
| | *Pigeonpea sterility mosaic emaravirus 1* | pigeonpea sterility mosaic virus (PPSMV) |
| | *Pigeonpea sterility mosaic emaravirus 2* | pigeonpea sterility mosaic virus 2 (PPSMV-2) |
| | *Raspberry leaf blotch emaravirus* | raspberry leaf blotch virus (RLBV) |
| | *Redbud yellow ringspot-associated emaravirus* | redbud yellow ringspot-associated virus (RYRaV) |
| | *Rose rosette emaravirus* | rose rosette virus (RRV) |



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| **Family *Hantaviridae*** | | |
|---|---|---|
| Subfamily *Actantavirinae* | | |
| *Actinovirus* | *Batfish actinovirus* | Wēnlǐng minipizza batfish virus (WEMBV) |
| | *Goosefish actinovirus* | Wēnlǐng yellow goosefish virus (WEYGV) |
| | *Spikefish actinovirus* | Wēnlǐng red spikefish virus (WERSV) |
| Subfamily *Agantavirinae* | | |
| *Agnathovirus* | *Hagfish agnathovirus* | Wēnlǐng hagfish virus (WEHV) |
| Subfamily *Mammantavirinae* | | |
| *Loanvirus* | *Longquan loanvirus** | Lóngquán virus (LQUV) |
| *Mobatvirus* | *Laibin mobatvirus* | Láibīn virus (LAIV) |
| | *Nova mobatvirus** | Nova virus (NVAV) |
| | *Quezon mobatvirus* | Quezon virus (QZNV) |
| *Orthohantavirus* | *Andes orthohantavirus* | Andes virus (ANDV) |
| | | Castelo dos Sonhos virus (CASV) |



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| | | |
|---|---|---|
| | | Lechiguanas virus (LECV = LECHV) |
| | | Orán virus (ORNV) |
| | *Asama orthohantavirus* | Asama virus (ASAV) |
| | *Asikkala orthohantavirus* | Asikkala virus (ASIV) |
| | *Bayou orthohantavirus* | bayou virus (BAYV) |
| | | Catacamas virus (CATV) |
| | *Black Creek Canal orthohantavirus* | Black Creek Canal virus (BCCV) |
| | *Bowe orthohantavirus* | Bowé virus (BOWV) |
| | *Bruges orthohantavirus* | Bruges virus (BRGV) |
| | *Cano Delgadito orthohantavirus* | Caño Delgadito virus (CADV) |
| | *Cao Bang orthohantavirus* | Cao Bằng virus (CBNV) |
| | | Liánghé virus (LHEV) |
| | *Choclo orthohantavirus* | Choclo virus (CHOV) |
| | *Dabieshan orthohantavirus* | Dàbiéshān virus (DBSV) |
| | *Dobrava-Belgrade orthohantavirus* | Dobrava virus (DOBV) |



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| | | |
|---|---|---|
| | | Kurkino virus (KURV) |
| | | Saaremaa virus (SAAV) |
| | | Sochi virus (SOCV) |
| | *El Moro Canyon orthohantavirus* | Carrizal virus (CARV) |
| | | El Moro Canyon virus (ELMCV) |
| | | Huitzilac virus (HUIV) |
| | *Fugong orthohantavirus* | Fúgòng virus (FUGV) |
| | *Fusong orthohantavirus* | Fŭsōng virus (FUSV) |
| | *Hantaan orthohantavirus** | Amur virus (AMRV) |
| | | Hantaan virus (HTNV) |
| | | Soochong virus (SOOV) |
| | *Jeju orthohantavirus* | Jeju virus (JJUV) |
| | *Kenkeme orthohantavirus* | Kenkeme virus (KKMV) |
| | *Khabarovsk orthohantavirus* | Khabarovsk virus (KHAV) |
| | | Topografov virus (TOPV) |



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| | | |
|---|---|---|
| | *Laguna Negra orthohantavirus* | Laguna Negra virus (LANV) |
| | | Maripa virus (MARV) |
| | | Río Mamoré virus (RIOMV) |
| | *Luxi orthohantavirus* | Lúxī virus (LUXV) |
| | *Maporal orthohantavirus* | Maporal virus (MAPV) |
| | *Montano orthohantavirus* | Montaño virus (MTNV) |
| | *Necocli orthohantavirus* | Necoclí virus (NECV) |
| | *Oxbow orthohantavirus* | Oxbow virus (OXBV) |
| | *Prospect Hill orthohantavirus* | Prospect Hill virus (PHV) |
| | *Puumala orthohantavirus* | Hokkaido virus (HOKV) |
| | | Muju virus (MUJV) |
| | | Puumala virus (PUUV) |
| | *Rockport orthohantavirus* | Rockport virus (RKPV) |
| | *Sangassou orthohantavirus* | Sangassou virus (SANGV) |
| | *Seewis orthohantavirus* | Seewis virus (SWSV)[2] |



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| | | |
|---|---|---|
| | *Seoul orthohantavirus* | gōu virus (GOUV) |
| | | Seoul virus (SEOV) |
| | *Sin Nombre orthohantavirus* | New York virus (NYV) |
| | | Sin Nombre virus (SNV) |
| | *Thailand orthohantavirus* | Anjozorobe virus (ANJZV) |
| | | Serang virus (SERV)[3] |
| | | Thailand virus (THAIV) |
| | *Tigray orthohantavirus* | Tigray virus (TIGV) |
| | *Tula orthohantavirus* | Adler virus (ADLV) |
| | | Tula virus (TULV) |
| | *Yakeshi orthohantavirus* | Yákèshí virus (YKSV) |
| *Thottimvirus* | *Imjin thottimvirus* | Imjin virus (MJNV) |
| | *Thottapalayam thottimvirus** | Thottapalayam virus (TPMV) |
| Subfamily *Repantavirinae* | | |
| *Reptillovirus* | *Gecko reptillovirus* | Hǎinán oriental leaf-toed gecko virus |



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| | | |
|---|---|---|
| | | (HOLGV) |
| **Family *Leishbuviridae*** | | |
| *Shilevirus* | *Leptomonas shilevirus** | Leptomonas moramango virus (LEPMV) |
| **Family *Mypoviridae*** | | |
| *Hubavirus* | *Myriapod hubavirus** | Húběi myriapoda virus 5 (HbMV-5) |
| **Family *Nairoviridae*** | | |
| *Orthonairovirus* | *Artashat orthonairovirus* | Artashat virus (ARTSV) |
| | *Chim orthonairovirus* | Chim virus (CHIMV) |
| | *Crimean-Congo hemorrhagic fever orthonairovirus* | Crimean-Congo hemorrhagic fever virus (CCHFV) |
| | *Dera Ghazi Khan orthonairovirus* | Abu Hammad virus (AHV)[4] |
| | | Abu Mina virus (AMV) |
| | | Dera Ghazi Khan virus (DGKV) |
| | | Sapphire II virus (SAPV) |



FOIA Confidential Treatment Requested by Univ of Texas System UTSystem_054432

| | | |
|---|---|---|
| | *Dugbe orthonairovirus** | Dugbe virus (DUGV) |
| | | kupe virus (KUPEV) |
| | *Estero Real orthonairovirus* | Estero Real virus (ERV) |
| | *Hazara orthonairovirus* | Hazara virus (HAZV) |
| | | Tofla virus (TFLV) |
| | *Hughes orthonairovirus* | Caspiy virus (CASV) |
| | | Farallon virus (FARV) |
| | | Great Saltee virus (GRSV) |
| | | Hughes virus (HUGV) |
| | | Punta Salinas virus (PSV) |
| | | Raza virus (RAZAV) |
| | | Soldado virus (SOLV) |
| | | Zirqa virus (ZIRV) |
| | *Kasokero orthonairovirus* | Kasokero virus (KASV = KASOV) |
| | | Leopards Hill virus (LPHV) |



FOIA Confidential Treatment Requested by Univ of Texas System UTSystem_054433

| | | |
|---|---|---|
| | | Yogue virus (YOGV) |
| | *Keterah orthonairovirus* | Gossas virus (GOSV) |
| | | Issyk-kul virus (ISKV) |
| | | Keterah virus (KTRV)[5] |
| | | Uzun-Agach virus (UZAV) |
| | *Nairobi sheep disease orthonairovirus* | Nairobi sheep disease virus (NSDV)[6] |
| | *Qalyub orthonairovirus* | Bandia virus (BDAV) |
| | | Geran virus (GERV) |
| | | Qalyub virus (QYBV) |
| | *Sakhalin orthonairovirus* | Avalon virus (AVAV) |
| | | Clo Mor virus (CMV = CLMV) |
| | | Sakhalin virus (SAKV) |
| | | Taggert virus (TAGV) |
| | | Tillamook virus (TILLV) |
| | *Tamdy orthonairovirus* | Burana virus (BURV) |



FOIA Confidential Treatment Requested by Univ of Texas System UTSystem_054434

| | | |
|---|---|---|
| | | Huángpí tick virus 1 (HpTV-1) |
| | | Tăchéng tick virus 1 (TcTV-1) |
| | | Tamdy virus (TAMV) |
| | | Wēnzhōu tick virus (WzTV) |
| | *Thiafora orthonairovirus* | Erve virus (ERVEV) |
| | | Thiafora virus (TFAV) |
| *Shaspivirus* | *Spider shaspivirus** | Shāyáng spider virus 1 (SySV-1) |
| *Striwavirus* | *Strider striwavirus** | Sānxiá water strider virus 1 (SxWSV-1) |
| **Family *Peribunyaviridae*** | | |
| *Herbevirus* | *Herbert herbevirus** | Herbert virus (HEBV) |
| | *Kibale herbevirus* | Kibale virus (KIBV) |
| | *Tai herbevirus* | Taï virus (TAIV) |
| *Orthobunyavirus* | *Acara orthobunyavirus* | Acará virus (ACAV) |
| | | Moriche virus (MORV) |
| | *Akabane orthobunyavirus* | Akabane virus (AKAV) |



FOIA Confidential Treatment Requested by Univ of Texas System UTSystem_054435

| | | |
|---|---|---|
| | | Tinaroo virus (TINV) |
| | | Yaba-7 virus (Y7V) |
| | *Aino orthobunyavirus* | Aino virus (AINOV) |
| | *Alajuela orthobunyavirus* | Alajuela virus (ALJV) |
| | | San Juan virus (SJV) |
| | *Anadyr orthobunyavirus* | Anadyr virus (ANADV) |
| | *Anhembi orthobunyavirus* | Anhembi virus (AMBV) |
| | *Anopheles A orthobunyavirus* | Anopheles A virus (ANAV) |
| | | Arumateua virus (ARTV = ARMTV) |
| | | Caraipé virus (CPEV = CRPV) |
| | | Las Maloyas virus (LMV) |
| | | Lukuni virus (LUKV) |
| | | Trombetas virus (TRMV) |
| | | Tucuruí virus (TUCV = TUCRV) |
| | *Anopheles B orthobunyavirus* | Anopheles B virus (ANBV) |



FOIA Confidential Treatment Requested by Univ of Texas System UTSystem_054436

| | | |
|---|---|---|
| | | Boracéia virus (BORV) |
| | *Bakau orthobunyavirus* | Bakau virus (BAKV) |
| | | Ketapang virus (KETV) |
| | | Nola virus (NOLAV) |
| | | Tanjong Rabok virus (TRV) |
| | | Telok Forest virus (TFV) |
| | *Batai orthobunyavirus* | Batai virus (BATV)[7] |
| | *Batama orthobunyavirus* | Batama virus (BMAV) |
| | *Bellavista orthobunyavirus* | Bellavista virus (BELLV) |
| | *Benevides orthobunyavirus* | Benevides virus (BVSV = BENV) |
| | *Bertioga orthobunyavirus* | Bertioga virus (BERV) |
| | | Cananéia virus (CNAV) |
| | | Guaratuba virus (GTBV) |
| | | Itimirim virus (ITIV) |
| | | Mirim virus (MIRV) |



FOIA Confidential Treatment Requested by Univ of Texas System UTSystem_054437

| | | |
|---|---|---|
| | *Bimiti orthobunyavirus* | bimiti virus (BIMV) |
| | *Birao orthobunyavirus* | Birao virus (BIRV) |
| | *Botambi orthobunyavirus* | Botambi virus (BOTV) |
| | *Bozo orthobunyavirus* | Bozo virus (BOZOV) |
| | *Bunyamwera orthobunyavirus** | Bunyamwera virus (BUNV) |
| | | Germiston virus (GERV) |
| | | Lokern virus (LOKV) |
| | | Mboké virus (MBOV) |
| | | Ngari virus (NRIV)[8] |
| | | Northway virus (NORV) |
| | | Santa Rosa virus (SARV) |
| | | Shokwe virus (SHOV) |
| | | Stanfield virus (STAV) |
| | | Xingu virus (XINV) |
| | *Bushbush orthobunyavirus* | Benfica virus (BENV = BNFV) |



FOIA Confidential Treatment Requested by Univ of Texas System UTSystem_054438

| | | |
|---|---|---|
| | | Bushbush virus (BSBV) |
| | | Juan Díaz virus (JDV) |
| | *Buttonwillow orthobunyavirus* | Buttonwillow virus (BUTV) |
| | *Bwamba orthobunyavirus* | Bwamba virus (BWAV) |
| | | Pongola virus (PGAV) |
| | *Cache Valley orthobunyavirus* | Cache Valley virus (CVV) |
| | | Cholul virus (CHLV) |
| | | Tlacotalpan virus (TLAV) |
| | *Cachoeira Porteira orthobunyavirus* | Cachoeira Porteira virus (CPOV) |
| | *California encephalitis orthobunyavirus* | California encephalitis virus (CEV) |
| | | Morro Bay virus (MBV) |
| | *Capim orthobunyavirus* | Capim virus (CAPV) |
| | *Caraparu orthobunyavirus* | Apeú virus (APEUV) |
| | | Bruconha virus (BRUV) |
| | | Caraparú virus (CARV) |



FOIA Confidential Treatment Requested by Univ of Texas System UTSystem_054439

| | | |
|---|---|---|
| | | El Huayo virus (EHUV) |
| | | Itaya virus (ITYV) |
| | | Ossa virus (OSSAV) |
| | | Vinces virus (VINV) |
| | *Cat Que orthobunyavirus* | Cát Quế virus (CQV) |
| | | Oya virus (OYAV) |
| | *Catu orthobunyavirus* | Catú virus (CATUV) |
| | *Enseada orthobunyavirus* | Enseada virus (ENSV) |
| | *Faceys paddock orthobunyavirus* | Facey's paddock virus (FPV) |
| | *Fort Sherman orthobunyavirus* | Fort Sherman virus (FSV) |
| | *Gamboa orthobunyavirus* | Brus Laguna virus (BLAV) |
| | | Calchaquí virus (CQIV) |
| | | Gamboa virus (GAMV) |
| | | Pueblo Viejo virus (PVV) |
| | | Soberanía virus (SOBV) |



FOIA Confidential Treatment Requested by Univ of Texas System UTSystem_054440

| | | |
|---|---|---|
| | *Guajara orthobunyavirus* | Guajará virus (GJAV) |
| | *Guama orthobunyavirus* | Ananindeua virus (ANUV) |
| | | Guamá virus (GMAV) |
| | | Mahogany Hammock virus (MHV) |
| | | Moju virus (MOJUV) |
| | *Guaroa orthobunyavirus* | Guaroa virus (GROV) |
| | *Iaco orthobunyavirus* | Iaco virus (IACOV) |
| | *Ilesha orthobunyavirus* | Ilesha virus (ILEV) |
| | *Ingwavuma orthobunyavirus* | Ingwavuma virus (INGV) |
| | *Jamestown Canyon orthobunyavirus* | Inkoo virus (INKV) |
| | | Jamestown Canyon virus (JCV) |
| | | Jerry Slough virus (JSV) |
| | | South River virus (SORV) |
| | *Jatobal orthobunyavirus* | Jatobal virus (JATV) |
| | *Kaeng Khoi orthobunyavirus* | Kaeng Khoi virus (KKV) |



FOIA Confidential Treatment Requested by Univ of Texas System UTSystem_054441

| | | |
|---|---|---|
| | *Kairi orthobunyavirus* | Kairi virus (KRIV) |
| | *Keystone orthobunyavirus* | Keystone virus (KEYV) |
| | *Koongol orthobunyavirus* | koongol virus (KOOV) |
| | | wongal virus (WONV) |
| | *La Crosse orthobunyavirus* | La Crosse virus (LACV) |
| | *Leanyer orthobunyavirus* | Leanyer virus (LEAV) |
| | *Lumbo orthbunyavirus* | Lumbo virus (LUMV) |
| | *Macaua orthobunyavirus* | Macauã virus (MCAV) |
| | *Madrid orthobunyavirus* | Madrid virus (MADV) |
| | *Maguari orthobunyavirus* | Maguari virus (MAGV) |
| | | Playas virus (PLAV) |
| | *Main Drain orthobunyavirus* | Main Drain virus (MDV) |
| | *Manzanilla orthobunyavirus* | Manzanilla virus (MANV) |
| | | Inini virus (INIV) |
| | *Marituba orthobunyavirus* | Gumbo Limbo virus (GLV) |



FOIA Confidential Treatment Requested by Univ of Texas System UTSystem_054442

| | | |
|---|---|---|
| | | Marituba virus (MTBV) |
| | | Murutucú virus (MURV) |
| | | Nepuyo virus (NEPV) |
| | | Restan virus (RESV) |
| | | Zungarococha virus (ZUNV) |
| | *Melao orthobunyavirus* | Melao virus (MELV) |
| | *Mermet orthobunyavirus* | Mermet virus (MERV) |
| | *Minatitlan orthobunyavirus* | Minatitlán virus (MNTV) |
| | | Palestina virus (PLSV) |
| | *MPoko orthobunyavirus* | M'Poko virus (MPOV) |
| | | Yaba-1 virus (Y1V) |
| | *Nyando orthobunyavirus* | Eretmapodites virus (ERETV) |
| | | Mojuí dos Campos virus (MDCV) |
| | | Nyando virus (NDV) |
| | *Olifantsvlei orthobunyavirus* | Bobia virus (BIAV) |



FOIA Confidential Treatment Requested by Univ of Texas System UTSystem_054443

| | | |
|---|---|---|
| | | Dabakala virus (DABV) |
| | | Olifantsvlei virus (OLIV) |
| | | Oubi virus (OUBIV) |
| | *Oriboca orthobunyavirus* | Itaquí virus (ITQV) |
| | | Oriboca virus (ORIV) |
| | *Oropouche orthobunyavirus* | Iquitos virus (IQTV)[d] |
| | | Madre de Dios virus (MDDV) |
| | | Oropouche virus (OROV) |
| | | Perdões virus (PDEV) |
| | | Pintupo virus (PINTV) |
| | *Patois orthobunyavirus* | Abras virus (ABRV) |
| | | Babahoya virus (BABV) |
| | | Pahayokee virus (PAHV) |
| | | Patois virus (PATV) |
| | | Shark River virus (SRV) |



FOIA Confidential Treatment Requested by Univ of Texas System UTSystem_054444

| | | |
|---|---|---|
| | *Peaton orthobunyavirus* | Peaton virus (PEAV) |
| | *Potosi orthobunyavirus* | Potosi virus (POTV) |
| | *Sabo orthobunyavirus* | Sabo virus (SABOV) |
| | *San Angelo orthobunyavirus* | San Angelo virus (SAV) |
| | *Sango orthobunyavirus* | Sango virus (SANV) |
| | *Schmallenberg orthobunyavirus* | Douglas virus (DOUV) |
| | | Sathuperi virus (SATV) |
| | | Schmallenberg virus (SBV) |
| | | Shamonda virus (SHAV) |
| | *Serra do Navio orthobunyavirus* | Serra do Navio virus (SDNV) |
| | *Shuni orthobunyavirus* | Kaikalur virus (KAIV) |
| | | Shuni virus (SHUV) |
| | *Simbu orthobunyavirus* | Para virus (PARAV) |
| | | Simbu virus (SIMV) |
| | *Snowshoe hare orthobunyavirus* | Khatanga virus (KHATV)[9] |



FOIA Confidential Treatment Requested by Univ of Texas System UTSystem_054445

| | | |
|---|---|---|
| | | snowshoe hare virus (SSHV) |
| | *Sororoca orthobunyavirus* | Sororoca virus (SORV) |
| | *Tacaiuma orthobunyavirus* | CoAr 1071 virus (CA1071V) |
| | | CoAr 3627 virus (CA3626V) |
| | | Tacaiuma virus (TCMV) |
| | | Virgin River virus (VRV) |
| | *Tahyna orthobunyavirus* | Ťahyňa virus (TAHV) |
| | *Tataguine orthobunyavirus* | Tataguine virus (TATV) |
| | *Tensaw orthobunyavirus* | Tensaw virus (TENV) |
| | *Tete orthobunyavirus* | Bahig virus (BAHV) |
| | | Matruh virus (MTRV) |
| | | Tete virus (TETEV) |
| | | Tsuruse virus (TSUV) |
| | | Weldona virus (WELV) |
| | *Thimiri orthobunyavirus* | Thimiri virus (THIV) |



FOIA Confidential Treatment Requested by Univ of Texas System UTSystem_054446

| | | |
|---|---|---|
| | *Timboteua orthobunyavirus* | Timboteua virus (TBTV) |
| | *Trivittatus orthobunyavirus* | Achiote virus (ACHOV) |
| | | Trivittatus virus (TVTV) |
| | *Turlock orthobunyavirus* | Lednice virus (LEDV) |
| | | Turlock virus (TURV) |
| | | Umbre virus (UMBV) |
| | *Utinga orthobunyavirus* | Utinga virus (UTIV) |
| | *Wolkberg orthobunyavirus* | Wolkberg virus (WBV) |
| | *Wyeomyia orthobunyavirus* | Rio Pracupi virus |
| | | Taiassui virus (TAIAV) |
| | | Tucunduba virus (TUCV) |
| | | Wyeomyia virus (WYOV) |
| | *Zegla orthobunyavirus* | Zegla virus (ZEGV) |
| *Pacuvirus* | *Pacui pacuvirus** | Pacui virus (PACV) |
| | *Rio Preto da Eva pacuvirus* | Rio Preto da Eva virus (RPEV) |



FOIA Confidential Treatment Requested by Univ of Texas System UTSystem_054447

| | | |
|---|---|---|
| | *Tapirape pacuvirus* | Tapirapé virus (TAPV) |
| *Shangavirus* | *Insect shangavirus* | Shuāngào insect virus 1 (SgIV-1) |
| **Family *Phasmaviridae*** | | |
| *Feravirus* | *Ferak feravirus** | Ferak virus (FRKV) |
| *Inshuvirus* | *Insect inshuvirus** | Shuāngào insect virus 2 (SgIV-2) |
| *Jonvirus* | *Jonchet jonvirus** | jonchet virus (JONV) |
| *Orthophasmavirus* | *Culex orthophasmavirus* | Culex orthophasmavirus (CPLV) |
| | *Ganda orthophasmavirus* | Ganda orthophasmavirus (GBEEV) |
| | *Kigluaik phantom orthophasmavirus** | Kigluaik phantom virus (KIGV) |
| | *Nome phantom orthophasmavirus* | Nome phantom virus (NOMV) |
| | *Odonate orthophasmavirus* | Odonate orthophasmavirus (HbOV-8) |
| | *Qingling orthophasmavirus* | Qingling orthophasmavirus (HbOV-9) |
| | *Seattle orthophasmavirus* | Seattle orthophasmavirus (SEPV) |
| | *Wuchang cockroach orthophasmavirus 1* | Wǔchāng cockroach virus 1 (WcCV-1) |
| | *Wuhan mosquito orthophasmavirus 1* | Wǔhàn mosquito virus 1 (WhMV-1) |



FOIA Confidential Treatment Requested by Univ of Texas System UTSystem_054448

| | | |
|---|---|---|
| | *Wuhan mosquito orthophasmavirus 2* | Wǔhàn mosquito virus 2 (WhMV-2) |
| *Sawastrivirus* | *Sanxia sawastrivirus** | Sānxiá water strider virus 2 (SxWSV-2) |
| *Wuhivirus* | *Insect wuhivirus** | Wǔhàn insect virus 2 (WhIV-2) |
| **Family *Phenuiviridae*** | | |
| *Banyangvirus* | *Guertu banyangvirus* | Guertu virus (GTV) |
| | *Heartland banyangvirus* | Heartland virus (HRTV) |
| | *Huaiyangshan banyangvirus** | severe fever with thrombocytopenia syndrome virus (SFTSV) |
| *Beidivirus* | *Dipteran beidivirus** | Húběi diptera virus 3 (HbDV-3) |
| *Goukovirus* | *Cumuto goukovirus* | Cumuto virus (CUMV) |
| | *Gouleako goukovirus** | Gouléako virus (GOLV) |
| | *Yichang insect goukovirus* | Yíchāng insect virus (YcIV) |
| *Horwuvirus* | *Horsefly horwuvirus** | Wǔhàn horsefly virus (WhHV) |
| *Hudivirus* | *Dipteran hudivirus** | Húběi diptera virus 4 (HbDV-4) |
| *Hudovirus* | *Lepidopteran hudovirus** | Húběi lepidoptera virus 1 (HbLV-1) |



FOIA Confidential Treatment Requested by Univ of Texas System UTSystem_054449

| | | |
|---|---|---|
| *Kabutovirus* | *Huangpi kabutovirus** | Huángpí tick virus 2 (HpTV-2) |
| | *Kabuto mountain kabutovirus* | Kabuto mountain virus (KAMV) |
| *Laulavirus* | *Laurel Lake laulavirus** | Laurel Lake virus (LLV) |
| *Mobuvirus* | *Mothra mobuvirus** | Mothra virus (MTHV) |
| *Phasivirus* | *Badu phasivirus** | Badu virus (BADUV) |
| | *Phasi Charoen-like phasivirus* | Phasi Chaeron-like virus (PCLV) |
| | *Wutai mosquito phasivirus* | Wǔtái mosquito virus (WtMV) |
| *Phlebovirus* | *Bujaru phlebovirus* | Bujaru virus (BUJV) |
| | | Munguba virus (MUNV) |
| | *Candiru phlebovirus* | Alenquer virus (ALEV) |
| | | Ariquemes virus (ARQV) |
| | | Candirú virus (CDUV) |
| | | Itaituba virus (ITAV) |
| | | Jacundá virus (JCNV) |
| | | Maldonado virus (MLOV) |



FOIA Confidential Treatment Requested by Univ of Texas System UTSystem_054450

| | | |
|---|---|---|
| | | Morumbi virus (MR(M)BV) |
| | | Mucura virus (MCRV/MRAV) |
| | | Nique virus (NIQV) |
| | | Oriximiná virus (ORXV) |
| | | Serra Norte virus (SRNV) |
| | | Turuna virus (TUAV) |
| | *Chilibre phlebovirus* | Cacao virus (CACV) |
| | | Chilibre virus (CHIV) |
| | *Frijoles phlebovirus* | Frijoles virus (FRIV) |
| | | Joá virus (JOAV) |
| | *Mukawa phlebovirus* | Mukawa virus (MKWV) |
| | *Punta Toro phlebovirus* | Buenaventura virus (BUEV) |
| | | Campana virus (CMAV) |
| | | Capira virus (CAPIV) |
| | | Coclé virus (CCLV) |



FOIA Confidential Treatment Requested by Univ of Texas System UTSystem_054451

| | | |
|---|---|---|
| | | Leticia virus (LTCV) |
| | | Punta Toro virus (PTV) |
| | *Rift Valley fever phlebovirus** | Rift Valley fever virus (RVFV) |
| | *Salehabad phlebovirus* | Adana virus (ADAV) |
| | | Adria virus (ADRV) |
| | | Alcube virus |
| | | Arbia virus (ARBV) |
| | | Arumowot virus (AMTV) |
| | | Bregalaka virus (BREV) |
| | | Medjerda Valley virus (MVV) |
| | | Odrénisrou virus (ODRV) |
| | | Olbia virus (OLBV) |
| | | Salehabad virus (SALV) |
| | | Zaba virus (ZABAV) |
| | *Sandfly fever Naples phlebovirus* | Arrábida virus (ARRV) |



FOIA Confidential Treatment Requested by Univ of Texas System UTSystem_054452

| | | |
|---|---|---|
| | | Balkan virus (BALKV) |
| | | Fermo virus (FERV) |
| | | Gordil virus (GORV) |
| | | Granada virus (GRV = GRAV) |
| | | Massilia virus (MASV) |
| | | Punique virus (PUNV) |
| | | Saddaguia virus (SADV) |
| | | Saint-Floris virus (SAFV) |
| | | sandfly fever Naples virus (SFNV) |
| | | Tehran virus (THEV) |
| | | Toscana virus (TOSV) |
| | | Zerdali virus (ZERV) |
| | *Uukuniemi phlebovirus* | Chizé virus (CHZV) |
| | | EgAN 1825-61 virus (EGAV) |
| | | Fin V 707 virus (FINV) |



FOIA Confidential Treatment Requested by Univ of Texas System UTSystem_054453

| | | |
|---|---|---|
| | | Oceanside virus (OCV = OCEV) |
| | | Pontevès virus (PTVV) |
| | | St. Abbs Head virus (SAHV) |
| | | Uukuniemi virus (UUKV) |
| | | Zaliv Terpenyia virus (ZTV) |
| *Pidchovirus* | *Pidgey pidchovirus** | Pidgey virus (PGYV) |
| *Tenuivirus* | *Echinochloa hoja blanca tenuivirus* | Echinochloa hoja blanca virus (EHBV) |
| | *Iranian wheat stripe tenuivirus* | Iranian wheat stripe virus (IWSV) |
| | *Maize stripe tenuivirus* | maize stripe virus (MStV = MSpV) |
| | *Rice grassy stunt tenuivirus* | rice grassy stunt virus (RGSV) |
| | *Rice hoja blanca tenuivirus* | rice hoja blanca virus (RHBV) |
| | *Rice stripe tenuivirus** | rice stripe virus (RSV = RStV) |
| | *Urochloa hoja blanca tenuivirus* | Urochloa hoja blanca virus (UHBV) |
| *Wenrivirus* | *Shrimp wenrivirus** | Wēnzhōu shrimp virus 1 WzSV-1 |
| *Wubeivirus* | *Dipteran wubeivirus** | Húběi diptera virus 5 (HbDV-5) |



FOIA Confidential Treatment Requested by Univ of Texas System UTSystem_054454

| | | |
|---|---|---|
| | *Fly wubeivirus* | Wǔhàn fly virus 1 (WhFV-1) |
| **Family *Tospoviridae*** | | |
| *Orthotospovirus* | *Bean necrotic mosaic orthotospovirus* | bean necrotic mosaic virus (BeNMV) |
| | *Calla lily chlorotic spot orthotospovirus* | calla lily chlorotic spot virus (CCSV) |
| | *Capsicum chlorosis orthotospovirus* | Capsicum chlorosis virus (CaCV) |
| | *Chrysanthemum stem necrosis orthotospovirus* | Chrysanthemum stem necrosis virus (CSNV) |
| | *Groundnut bud necrosis orthotospovirus* | groundnut bud necrosis virus (GBNV)[10] |
| | *Groundnut ringspot orthotospovirus* | groundnut ringspot virus (GRSV) |
| | *Groundnut yellow spot orthotospovirus* | groundnut yellow spot virus (GYSV)[11] |
| | *Impatiens necrotic spot orthotospovirus* | impatiens necrotic spot virus (INSV) |
| | *Iris yellow spot orthotospovirus* | iris yellow spot virus (IYSV) |
| | *Melon severe mosaic orthotospovirus* | melon severe mosaic virus (MSMV) |
| | *Melon yellow spot orthotospovirus* | melon yellow spot virus (MYSV) |
| | *Polygonum ringspot orthotospovirus* | Polygonum ringspot virus (PolRSV) |



FOIA Confidential Treatment Requested by Univ of Texas System UTSystem_054455

| | | |
|---|---|---|
| | *Soybean vein necrosis orthotospovirus* | soybean vein necrosis virus (SVNV) |
| | *Tomato chlorotic spot orthotospovirus* | tomato chlorotic spot virus (TCSV) |
| | *Tomato spotted wilt orthotospovirus** | tomato spotted wilt virus (TSWV) |
| | *Watermelon bud necrosis orthotospovirus* | watermelon bud necrosis virus (WBNV) |
| | *Watermelon silver mottle orthotospovirus* | watermelon silver mottle virus (WSMoV) |
| | *Zucchini lethal chlorosis orthotospovirus* | zucchini lethal chlorosis virus (ZLCV) |
| **Family *Wupedeviridae*** | | |
| *Wumivirus* | *Millipede wumivirus** | Wǔhàn millipede virus 2 (WhMV-2) |
| **Unassigned** | | |
| *Coguvirus* | *Citrus coguvirus** | citrus concave gum-associated virus (CCGaV) |

*type species

[1]Synonyms: Cardamones virus, Hǎinán Medical University virus, Xīngyì virus; [2]Synonyms: Amga virus (MGAB), Artybash virus (ARTV); [3]synonym: Jurong virus; [4]includes the strain previously referred to as Tunis virus (TUNV); [5]includes the strain previously referred to as soft tick bunyavirus (STBV); [6]includes the strain previously referred to as Ganjam virus (GANV); [7]synonyms: Čalovo virus (CVOV), Chittoor virus (CHITV), Olkya virus, Olyka virus, UgMP-6830 virus; [8]includes the strain previously referred to as



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Garissa virus; [9]also mistakenly referred to in the literature as Chantanga virus (CHATV) and Chatanga virus (CHATV); [10]synonym: peanut bud necrosis virus (PDNV); [11]synonym: peanut yellow spot virus (PYSV).

¶Please note that viruses are real objects that are assigned to concepts that are called taxa. Species, genera, families, and orders are taxa. Taxon names are always italicized and always begin with a capital letter. Virus names, on the other hand, are not italicized and are not capitalized, except if the name or a name component is a proper noun. This column lists the virus names with their correct (lack of) capitalization. Lists of viruses within a given species are provisional at this point and will likely be amended in the near future.



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## COMPLIANCE WITH ETHICAL STANDARDS

The views and conclusions contained in this document are those of the authors and should not be interpreted as necessarily representing the official policies, either expressed or implied, of the US Department of the Army, the US Department of Defense, the US Department of Health and Human Services, or of the institutions and companies affiliated with the authors. In no event shall any of these entities have any responsibility or liability for any use, misuse, inability to use, or reliance upon the information contained herein. The US departments do not endorse any products or commercial services mentioned in this publication.


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## Conflict of Interest

The authors declare no conflicts of interest.

## Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

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# A universal vaccine platform and glioblastoma therapy

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## Research portfolio







- **Viral replication and pathogenesis**
  - Basic research
- **Antiviral drug discovery**
  - Partner with leading pharmaceutical companies
- **Vaccine development**
  - Partner with Brazilian Ministry of Health
- **Diagnostics**
  - Partner with diagnostics companies and CDC
- **Zika virus-based oncolytic therapy for glioblastoma**
  - Partner with Washington University, MD Anderson, and institutions in China

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## Flaviviruses: Unmet Medical Needs

- Dengue virus: The most important mosquito-borne viral pathogen
  - >2.5 billion people at risk
  - 390 million infections each year
  - 96 million infections with clinical diseases
- West Nile virus
- Yellow fever virus
- Japanese encephalitis virus
- Tick-borne encephalitis virus
- Zika virus

Zika virus



Sirohi et al., 2016

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## Zika virus transmission and epidemics/outbreaks

- First identified in the Zika forest in Uganda in 1947
- Transmission
  - *Aedes* mosquitoes (also transmit dengue virus, yellow fever virus, and chikungunya virus)
  - Sexual transmission
  - Blood and transplantation

***Aedes aegypti***

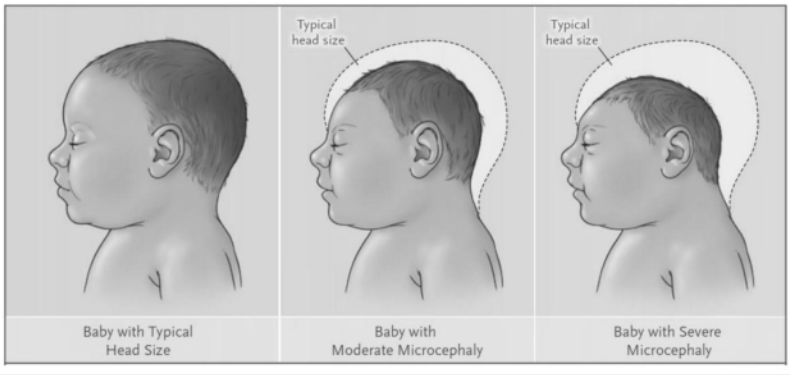


- Epidemics and outbreaks
  - 13 naturally acquired ZIKV infection from 1947-2006
  - Frequent outbreaks/epidemics since 2007, with >220,000 confirmed and 580,000 suspected cases by 2017 (WHO/PAHO)

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# Zika Disease

- Up to 80% of ZIKV infections may be asymptomatic. Persistent infection in male reproductive system
- Disease symptoms: Fever, lethargy, eye pain, conjunctivitis, rash, muscle aches, and arthralgia
- Severe diseases: Congenital Zika syndrome (including microcephaly) in fetuses and Guillain-Barré syndrome in adults

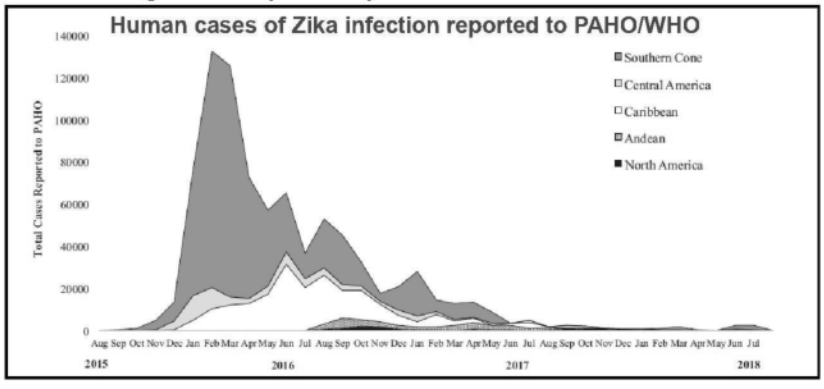



Petersen et al., 2016

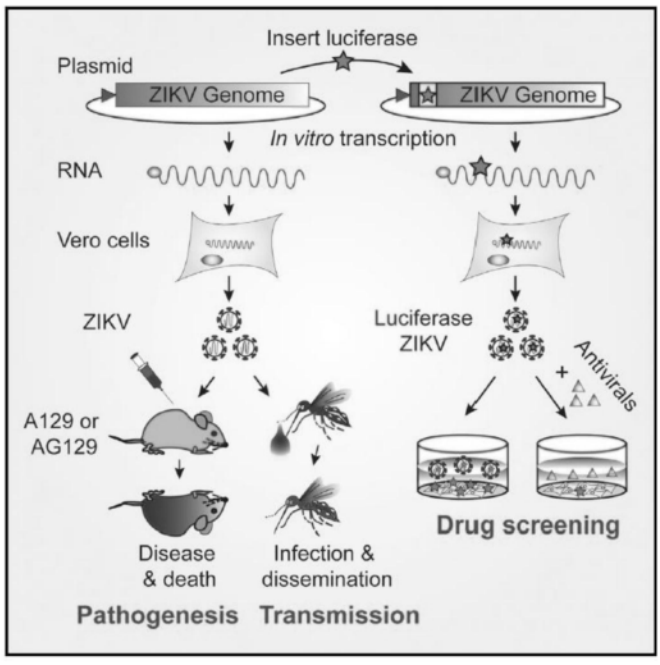
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## What triggered ZIKV epidemics and severe diseases?

- Introduction to a naïve population
- Viral evolution
- Previous infection and immunity of dengue virus
- Co-infection with Chikungunya virus
- Human genetic predisposition to disease




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- **An infectious cDNA clone of ZIKV**
  Shan et al. 2016. Cell Host & Microbe
- **A single amino acid change in ZIKV NS1 gene (A188V) enhances mosquito transmission**
  Lui et al., 2017. Nature
- **An evolutionary NS1 mutation (A188V) enhances Zika virus evasion of host interference induction**
  Xia et al., 2018. Nature Communications
- **A single mutation in ZIKV prM gene (S17N) enhances microcephaly**
  Yuan et al., 2017. Science

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# A Universal Vaccine Platform



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## Current Zika vaccine landscape

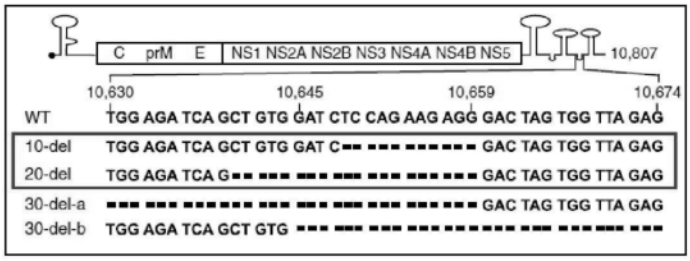
- **Vaccine platforms (*Currently in clinical trial)**
  - **Inactivated virus***
  - **Subunit vaccine: empty ZIKV particles expressed from DNA plasmid*, mRNA*, or viral vectors (such as measles virus vector*)**
  - **Chimeric virus: dengue vaccine (rZIKV/D4Δ30-713*), yellow fever vaccine, Japanese encephalitis vaccine, or other viral vaccines to express ZIKV structural proteins**
  - **Live-attenuated virus**
- **Inactivated vaccine and subunit vaccine: Multiple shots and periodic booster to maintain immunity; better safety compared with live-attenuated vaccine**
- **Live-attenuated vaccine: Single shot vaccine with potential life time protection, rapid immunity; higher safety barrier**

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# A single-dose live-attenuated Zika vaccine



- Excellent safety and efficacy
  - Prevents infection in non-human primates
  - Elicit rapid immune protection within 14 days post-vaccination in non-human primates
  - Prevents *in utero* transmission during pregnancy
  - Protects testis damage and sperm counts in mice
- Currently being transferred to Brazil for clinical development

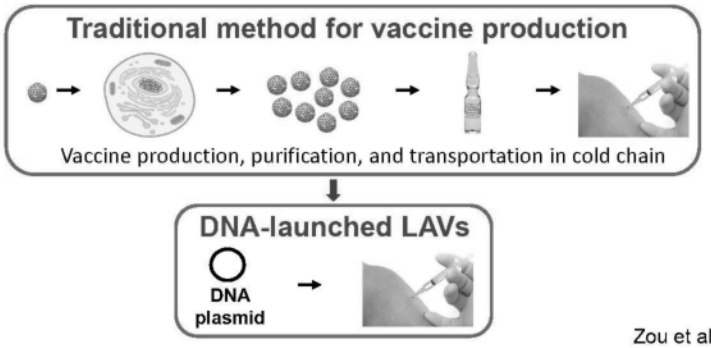



Shan et al., 2017, *Nature Medicine*
Richner et al., 2017, *Cell*
Shan et al., 2017, *Nature Communications*

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## DNA-launched ZIKV live-attenuated vaccine

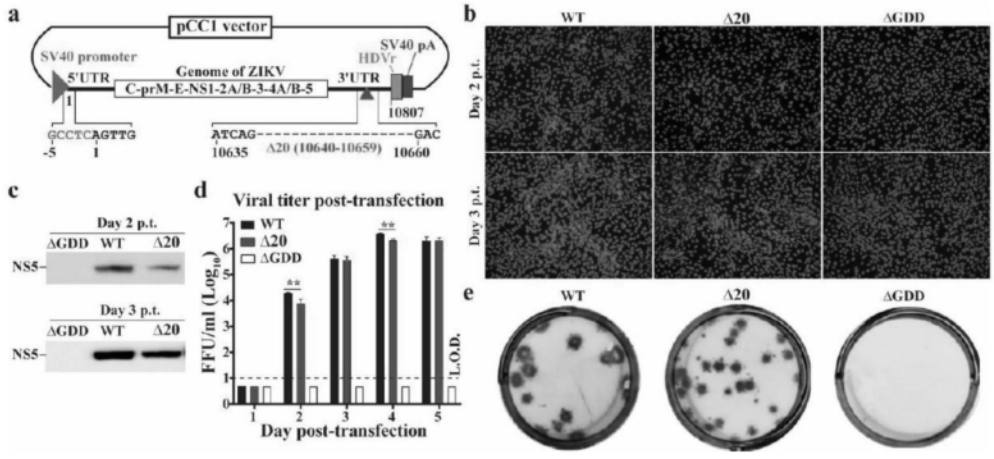
- **Advantages**
  - No cell culture or eggs required for vaccine production
  - No cold chain
  - The platform is applicable to other plus-sense RNA viruses
  - Viral vector for a plug-and-play universal vaccine platform




Zou et al., 2018, EBioMedicine

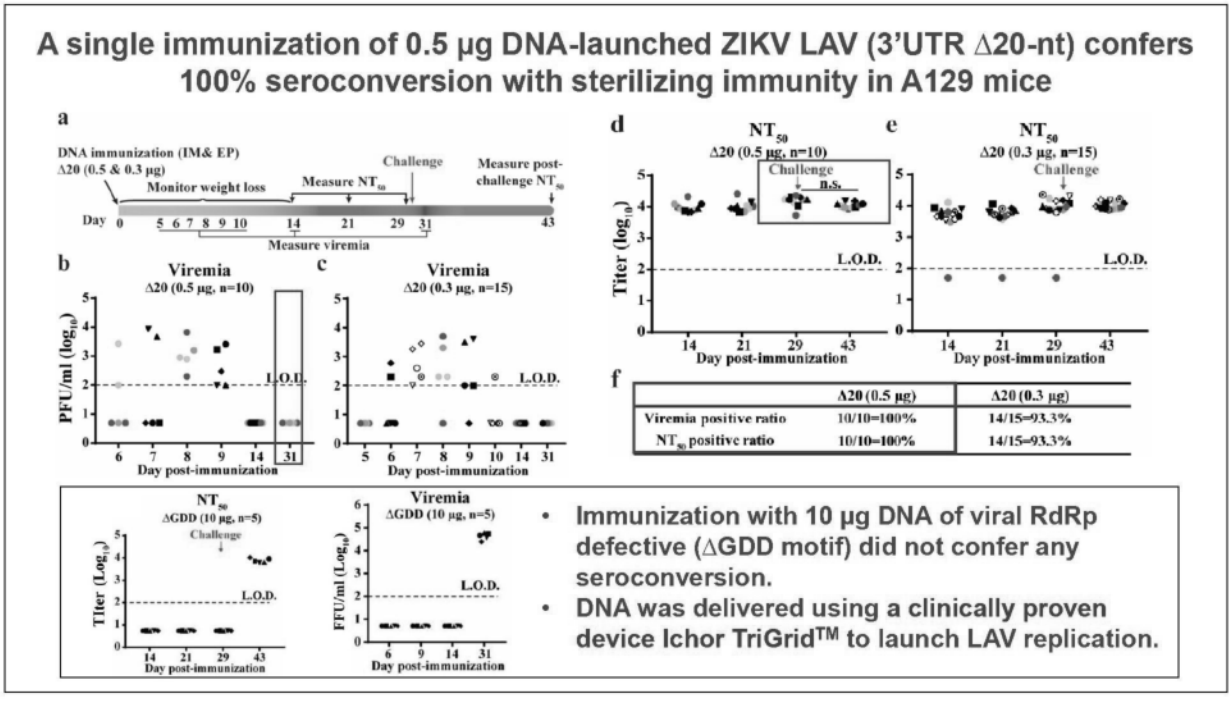
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## A DNA-launched ZIKV LAV (3'UTR Δ20-nt) in cell culture




- **A single-dose ZIKV LAV 3'UTR Δ20-nt ($10^3$ PFU) confers sterilizing immunity in HNPs with an excellent safety profile** (Nat. Med. 2017. Chao et al.; Nat. Commun. 2017. Chao et al.)

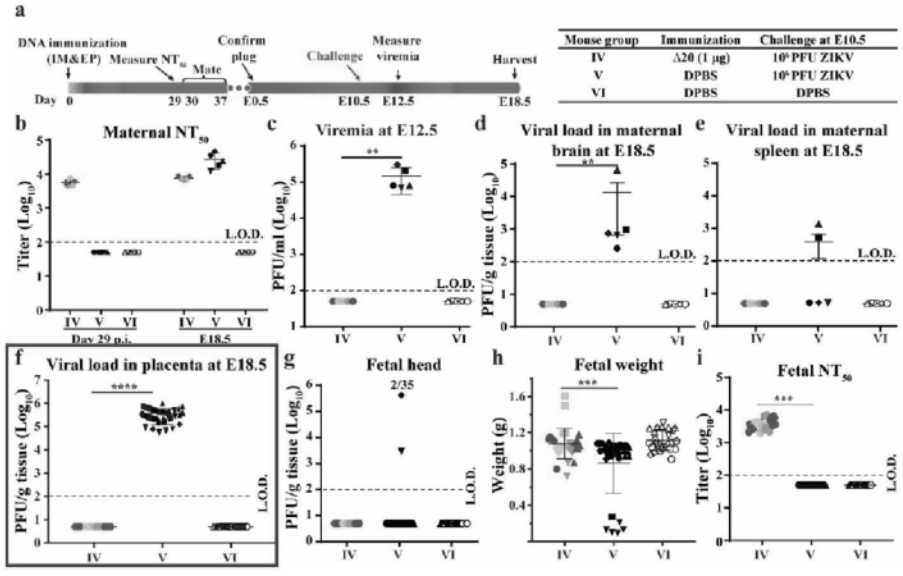
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DNA prME plasmid vaccine uses 50 ug and could not reach sterilizing immunity

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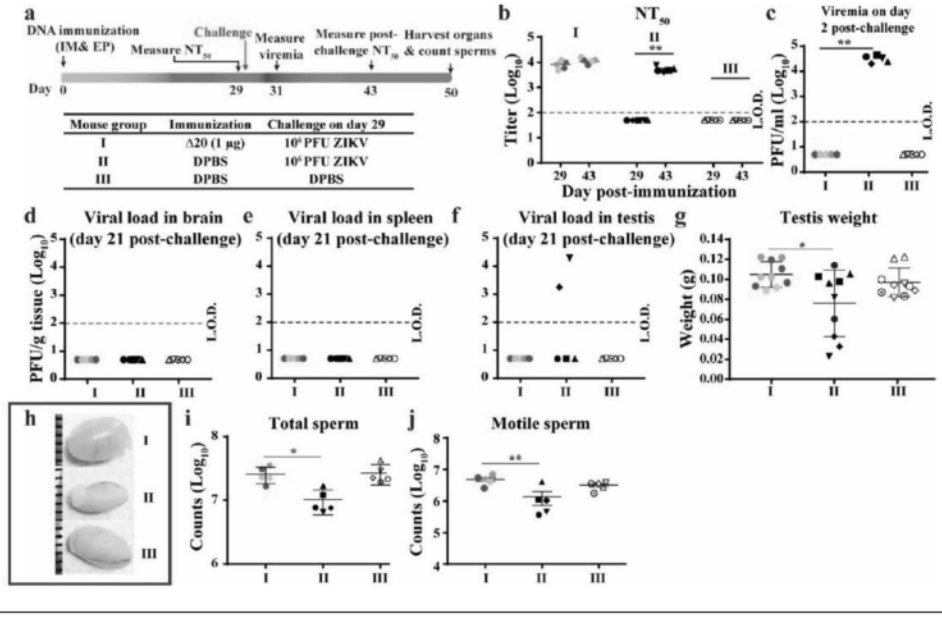
# Prevention of maternal-to-fetal transmission in pregnant mice




| Mouse group | Immunization | Challenge at E10.5 |
|---|---|---|
| IV | Δ20 (1 μg) | $10^5$ PFU ZIKV |
| V | DPBS | $10^5$ PFU ZIKV |
| VI | DPBS | DPBS |

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# Protection of male mice from ZIKV-induced damage to testis




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## Future directions

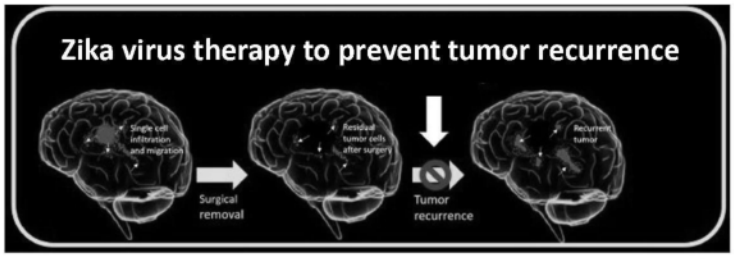
- Development path
  - Non-human primates: safety and efficacy
  - Apply to other flavivirus live-attenuated vaccines: YFV 17D and JEV 14-14-2
  - GMP production and non-human primate toxicity test
  - Clinical trial using YFV 17D or JEV 14-14-2
- **A universal vaccine platform: Use DNA-launched, FDA-approved RNA virus vaccines as vehicles to develop a "plug-and-play" technology**



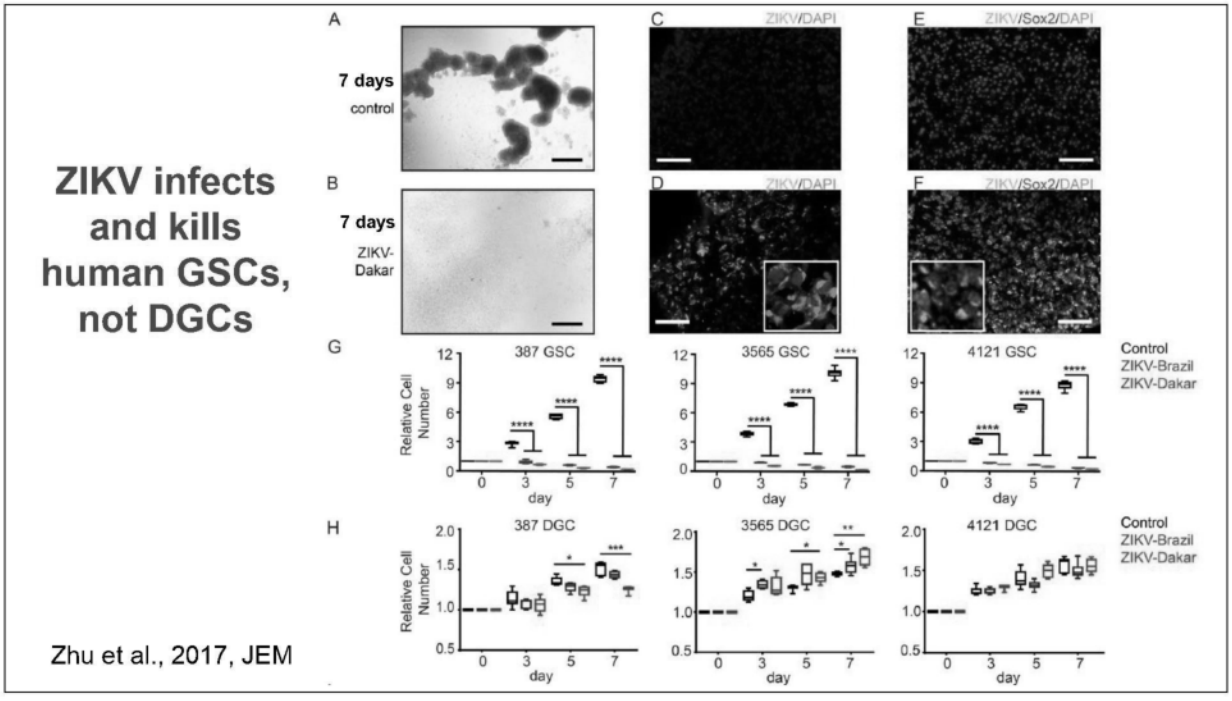

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## Engineering Zika virus to treat glioblastoma

- Publications
  - Zhu et al., 2017, Journal of Experimental Medicine
  - Chen et al., 2018, mBio
- Patent filed jointly by UTMB and Washington University
- Patent filed for a live-attenuated Zika virus vaccine

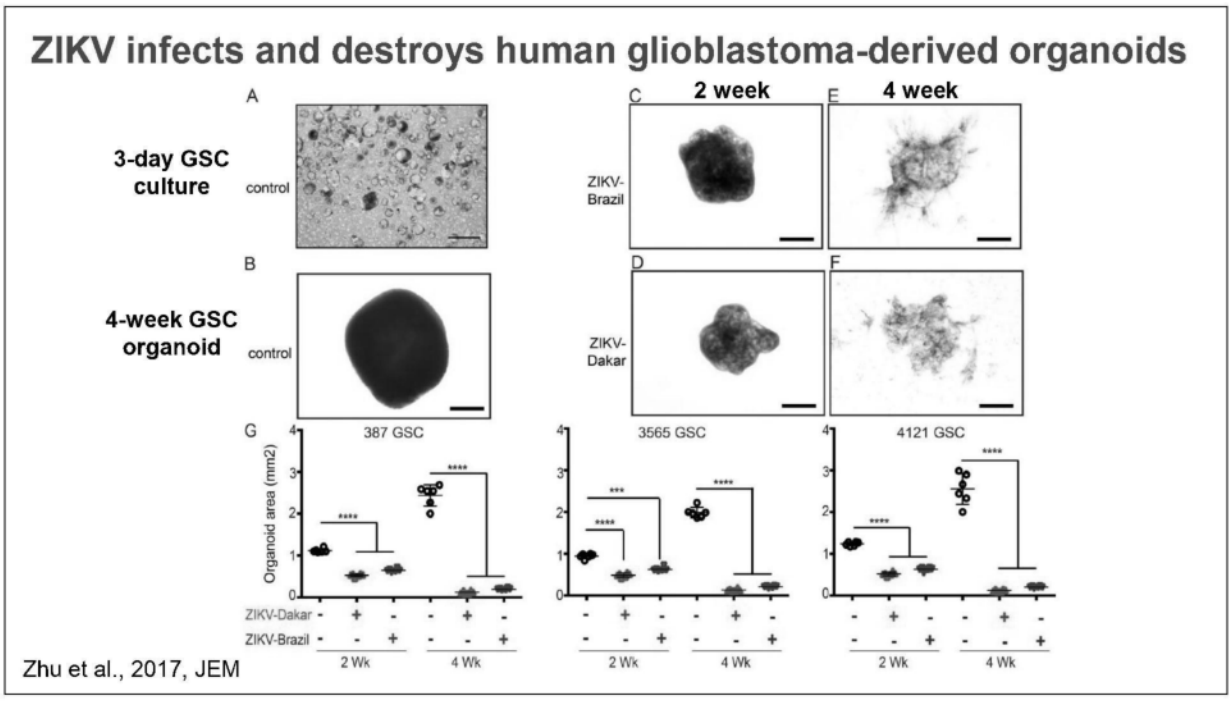



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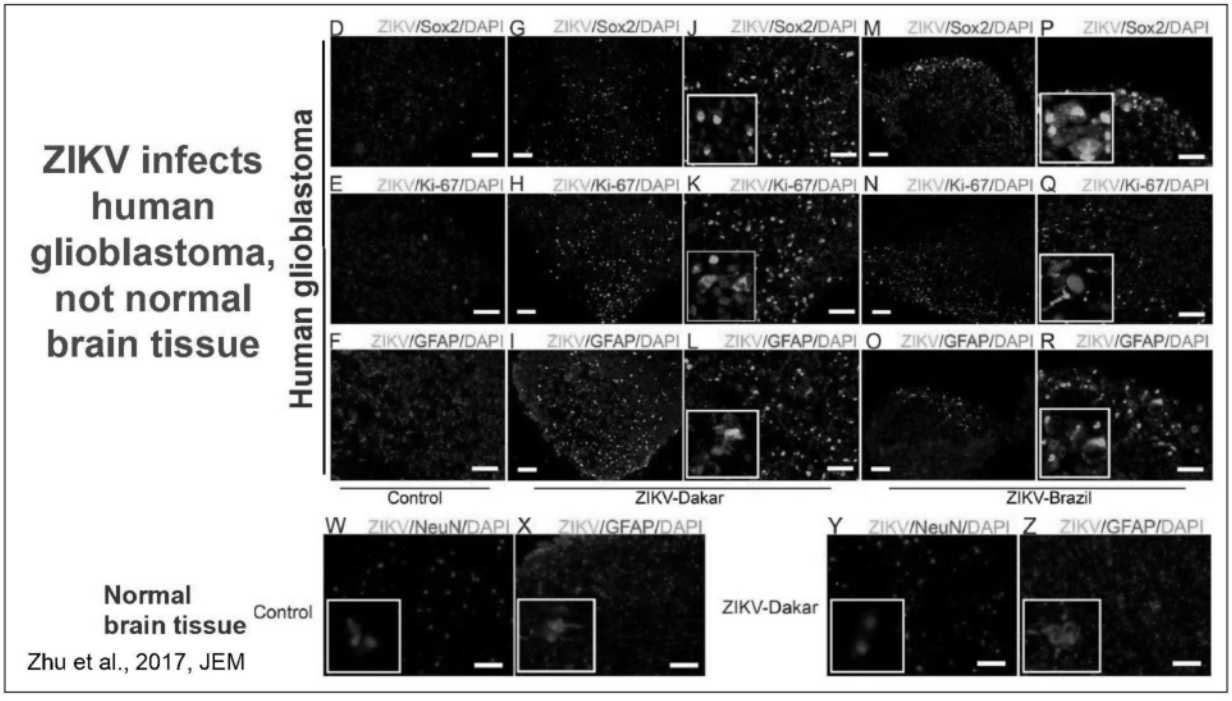

Bar: 725 um; DGC: differentiated glioma cells

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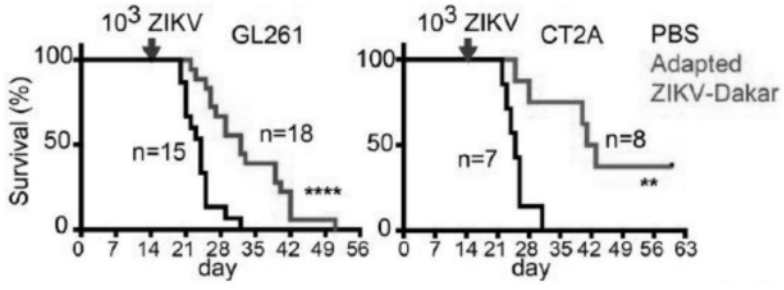
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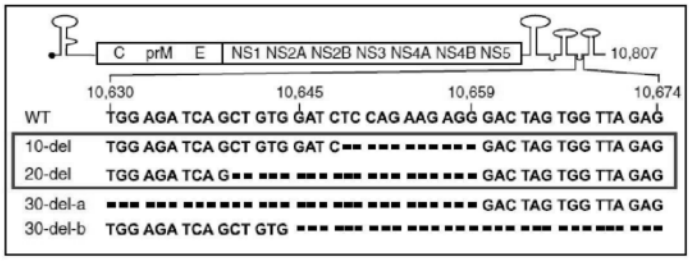
- Safety improvement
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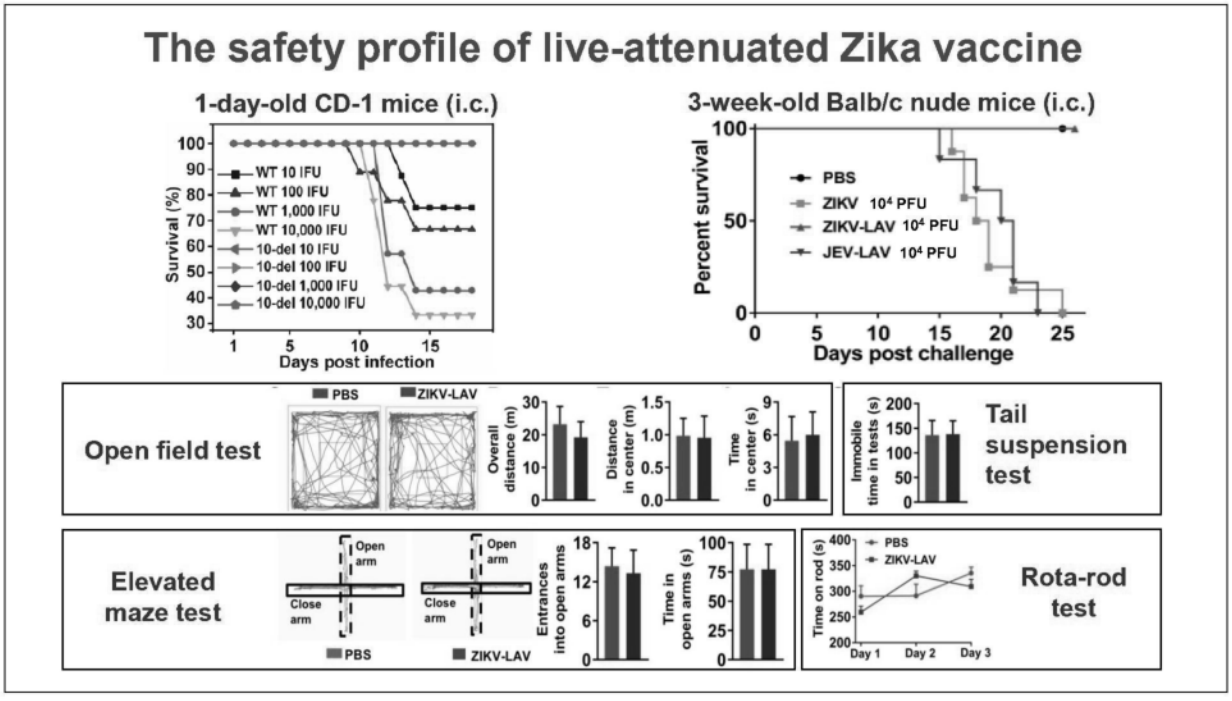


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Shan et al., 2017, Nature Medicine
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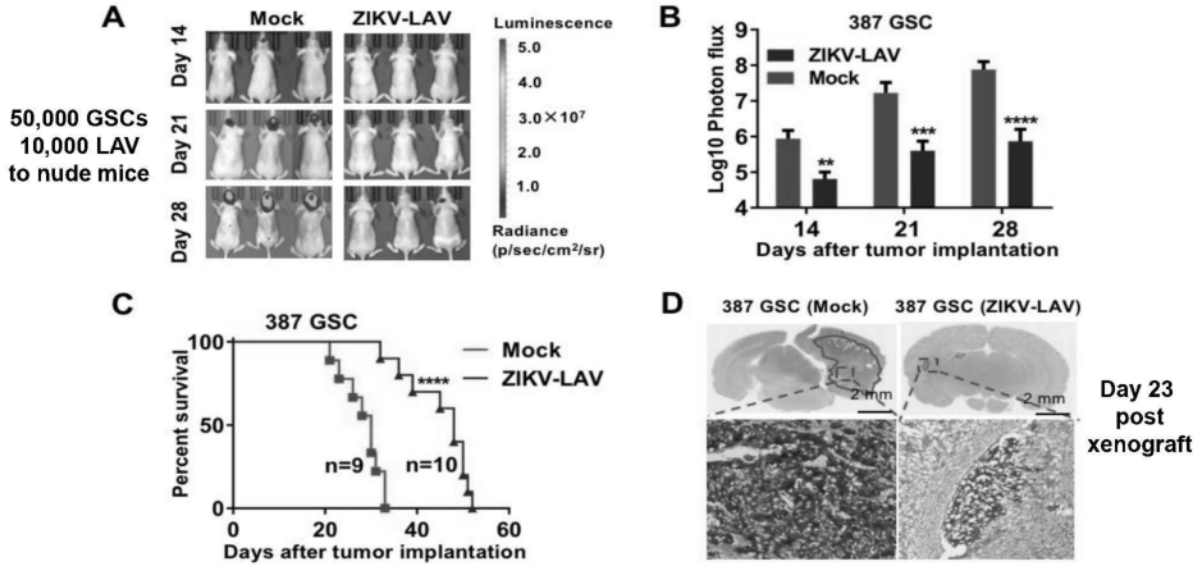
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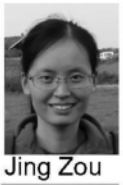

Chao Shan

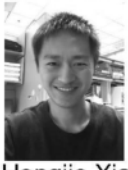

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Tony Muruato


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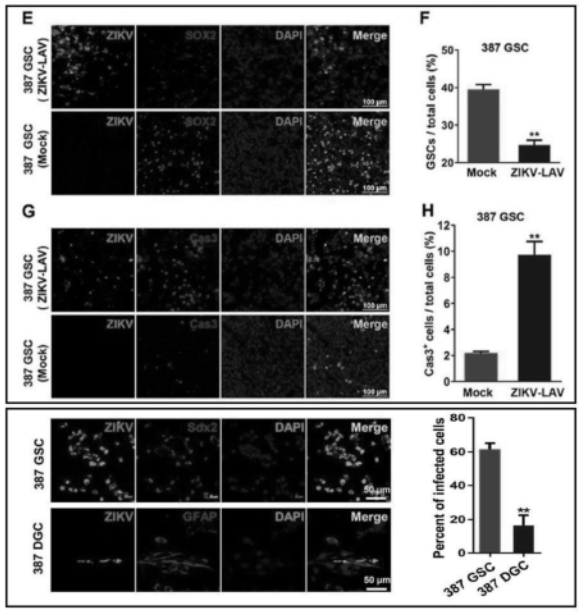

Jianying Liu


**UTMB**
Scott Weaver
Tina Wang
Alan Barrett
**Washington University**
Michael Diamond
**Beijing Institute of Microbiology & Epidemiology**
Cheng-Feng Qin
**Tsinghua University**
Gong Cheng
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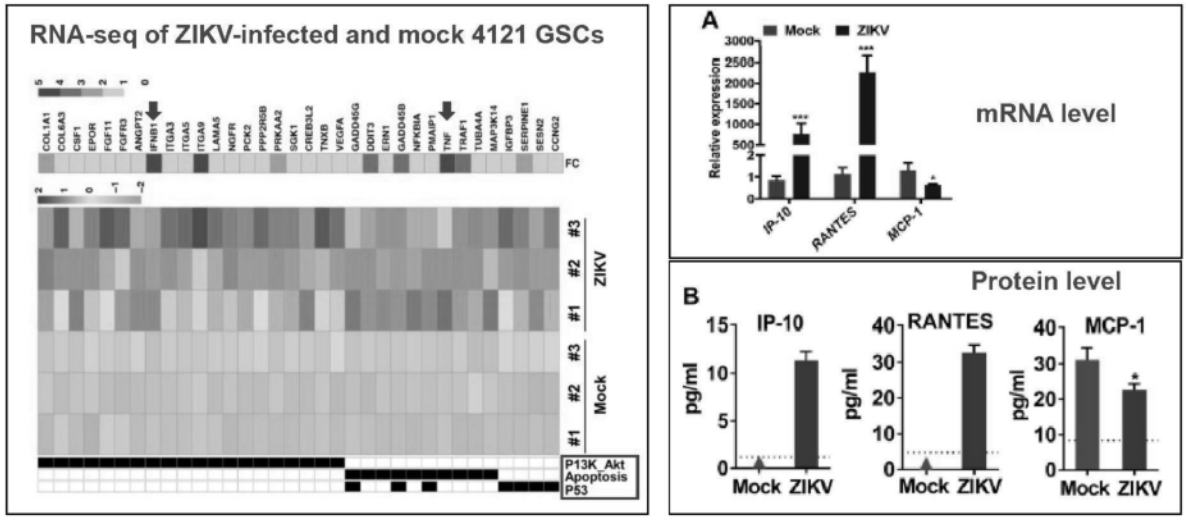
**Funding:** WHO/PAHO, NIH, Gilead Sciences, Kleberg Foundation, John S. Dunn Foundation, Summerfield Roberts Foundation


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# ZIKV infection triggers antiviral immunity, inflammation response, cell cycle p53, apoptosis, and PI3K signaling pathways




Type 4121 GSCs were infected with ZIKV (MOI of 1) analyzed at 48 h post-infection

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# A universal vaccine platform and glioblastoma therapy

Pei-Yong Shi, Ph.D.
Department of Biochemistry & Molecular Biology
Department of Pharmacology & Toxicology
Sealy Center for Structural Biology & Molecular Biophysics

utmb Health

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## Research portfolio







- **Viral replication and pathogenesis**
  - Basic research
- **Antiviral drug discovery**
  - Partner with leading pharmaceutical companies
- **Vaccine development**
  - Partner with Brazilian Ministry of Health
- **Diagnostics**
  - Partner with diagnostics companies and CDC
- **Zika virus-based oncolytic therapy for glioblastoma**
  - Partner with Washington University, MD Anderson, and institutions in China

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## Flaviviruses: Unmet Medical Needs

- Dengue virus: The most important mosquito-borne viral pathogen
  - >2.5 billion people at risk
  - 390 million infections each year
  - 96 million infections with clinical diseases
- West Nile virus
- Yellow fever virus
- Japanese encephalitis virus
- Tick-borne encephalitis virus
- Zika virus

Zika virus



Sirohi et al., 2016

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## Zika virus transmission and epidemics/outbreaks

- First identified in the Zika forest in Uganda in 1947
- Transmission
  - *Aedes* mosquitoes (also transmit dengue virus, yellow fever virus, and chikungunya virus)
  - Sexual transmission
  - Blood and transplantation

***Aedes aegypti***

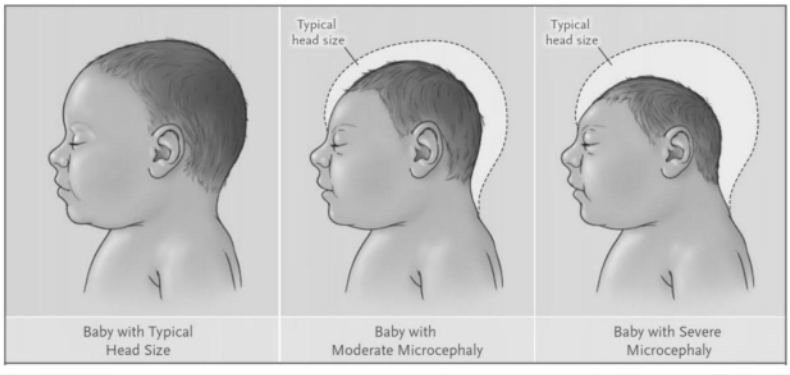


- Epidemics and outbreaks
  - 13 naturally acquired ZIKV infection from 1947-2006
  - Frequent outbreaks/epidemics since 2007, with >220,000 confirmed and 580,000 suspected cases by 2017 (WHO/PAHO)

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# Zika Disease

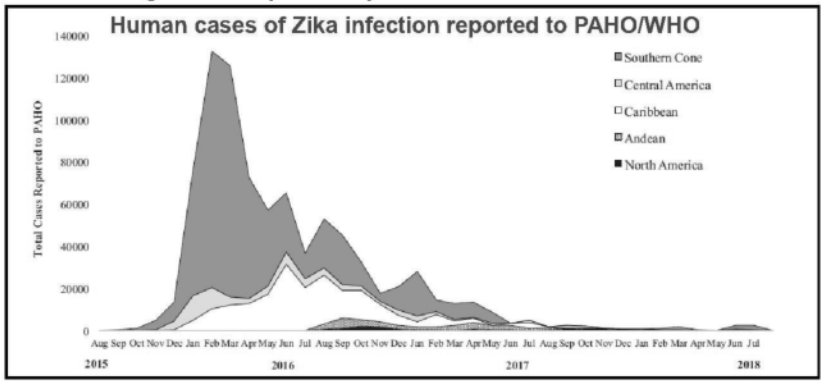
- Up to 80% of ZIKV infections may be asymptomatic. Persistent infection in male reproductive system
- Disease symptoms: Fever, lethargy, eye pain, conjunctivitis, rash, muscle aches, and arthralgia
- Severe diseases: Congenital Zika syndrome (including microcephaly) in fetuses and Guillain-Barré syndrome in adults



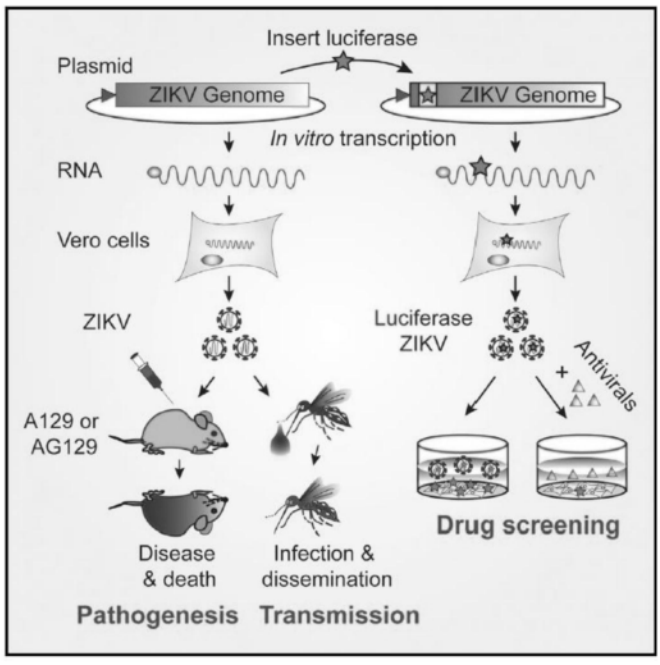

Petersen et al., 2016

## What triggered ZIKV epidemics and severe diseases?

- Introduction to a naïve population
- Viral evolution
- Previous infection and immunity of dengue virus
- Co-infection with Chikungunya virus
- Human genetic predisposition to disease




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- **An infectious cDNA clone of ZIKV**
  Shan et al. 2016. Cell Host & Microbe
- **A single amino acid change in ZIKV NS1 gene (A188V) enhances mosquito transmission**
  Lui et al., 2017. Nature
- **An evolutionary NS1 mutation (A188V) enhances Zika virus evasion of host interference induction**
  Xia et al., 2018. Nature Communications
- **A single mutation in ZIKV prM gene (S17N) enhances microcephaly**
  Yuan et al., 2017. Science

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# A Universal Vaccine Platform



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## Current Zika vaccine landscape

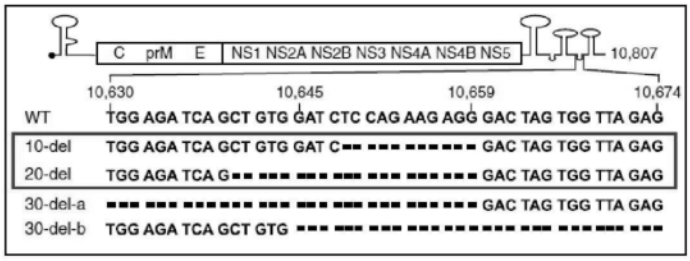
- **Vaccine platforms (*Currently in clinical trial)**
  - **Inactivated virus***
  - **Subunit vaccine: empty ZIKV particles expressed from DNA plasmid*, mRNA*, or viral vectors (such as measles virus vector*)**
  - **Chimeric virus: dengue vaccine (rZIKV/D4Δ30-713*), yellow fever vaccine, Japanese encephalitis vaccine, or other viral vaccines to express ZIKV structural proteins**
  - **Live-attenuated virus**
- **Inactivated vaccine and subunit vaccine: Multiple shots and periodic booster to maintain immunity; better safety compared with live-attenuated vaccine**
- **Live-attenuated vaccine: Single shot vaccine with potential life time protection, rapid immunity; higher safety barrier**

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# A single-dose live-attenuated Zika vaccine



- Excellent safety and efficacy
  - Prevents infection in non-human primates
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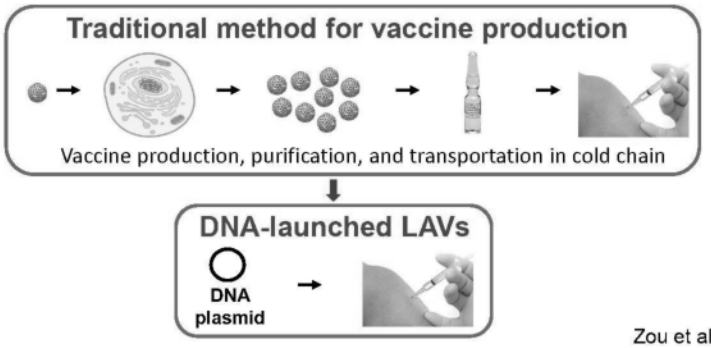



Shan et al., 2017, *Nature Medicine*
Richner et al., 2017, *Cell*
Shan et al., 2017, *Nature Communications*

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## DNA-launched ZIKV live-attenuated vaccine

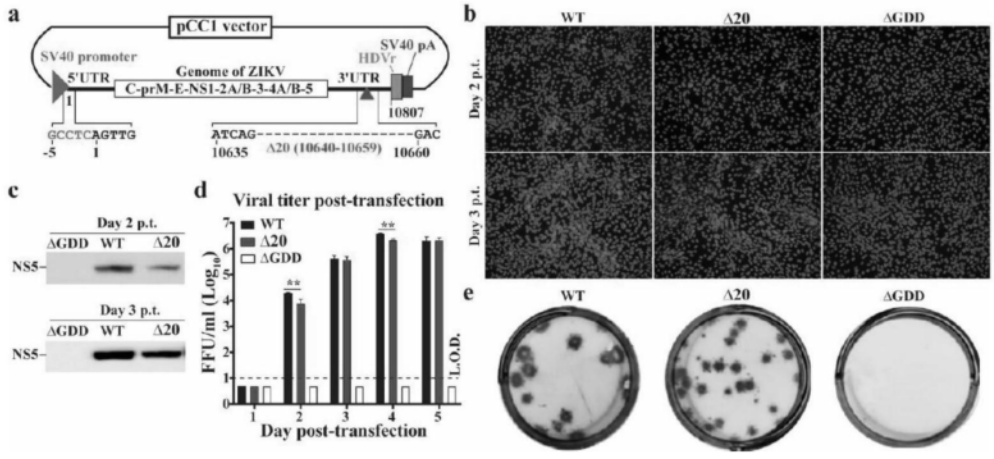
- **Advantages**
  - No cell culture or eggs required for vaccine production
  - No cold chain
  - The platform is applicable to other plus-sense RNA viruses
  - Viral vector for a plug-and-play universal vaccine platform




Zou et al., 2018, EBioMedicine

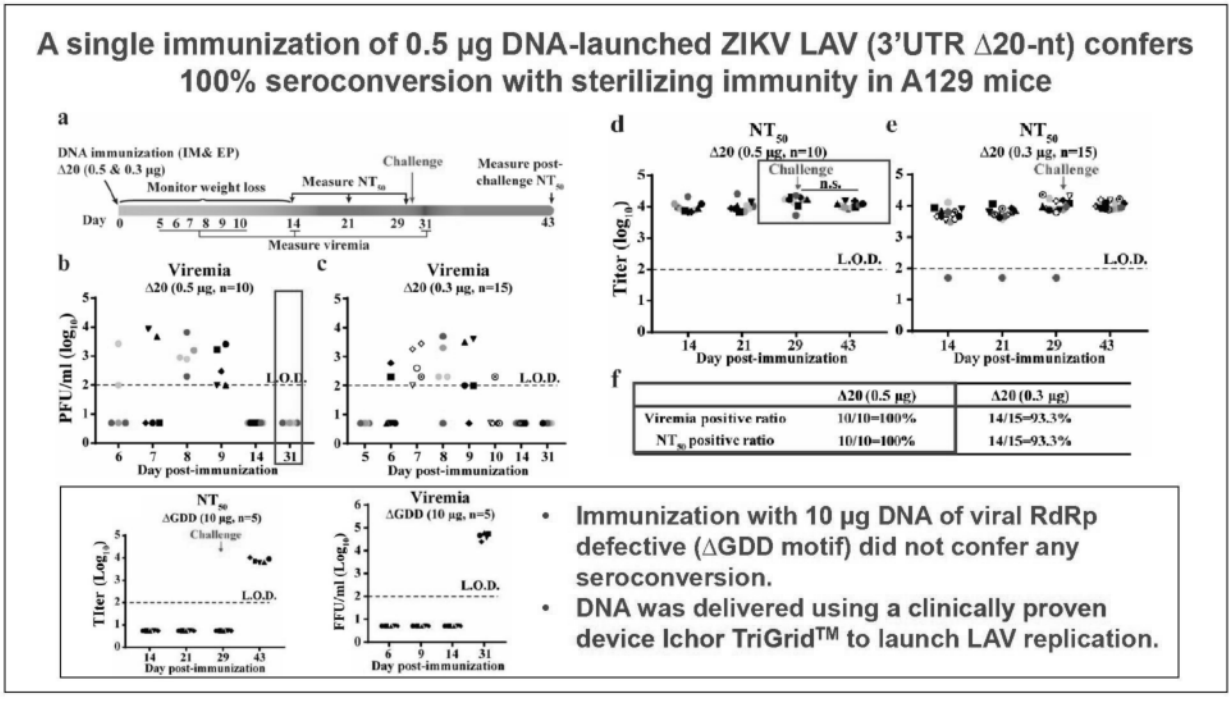
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## A DNA-launched ZIKV LAV (3'UTR Δ20-nt) in cell culture



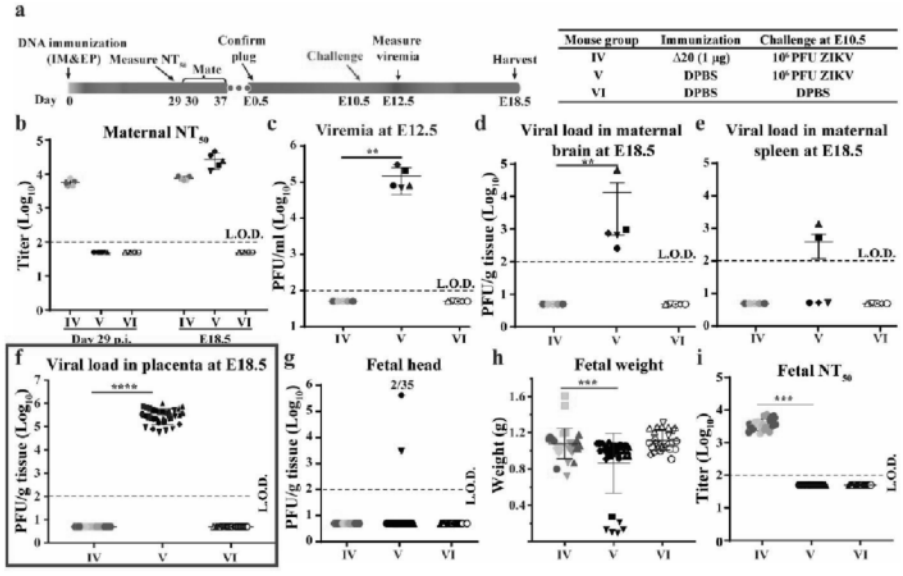

- **A single-dose ZIKV LAV 3'UTR Δ20-nt ($10^3$ PFU) confers sterilizing immunity in HNPs with an excellent safety profile** (Nat. Med. 2017. Chao et al.; Nat. Commun. 2017. Chao et al.)

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DNA prME plasmid vaccine uses 50 ug and could not reach sterilizing immunity

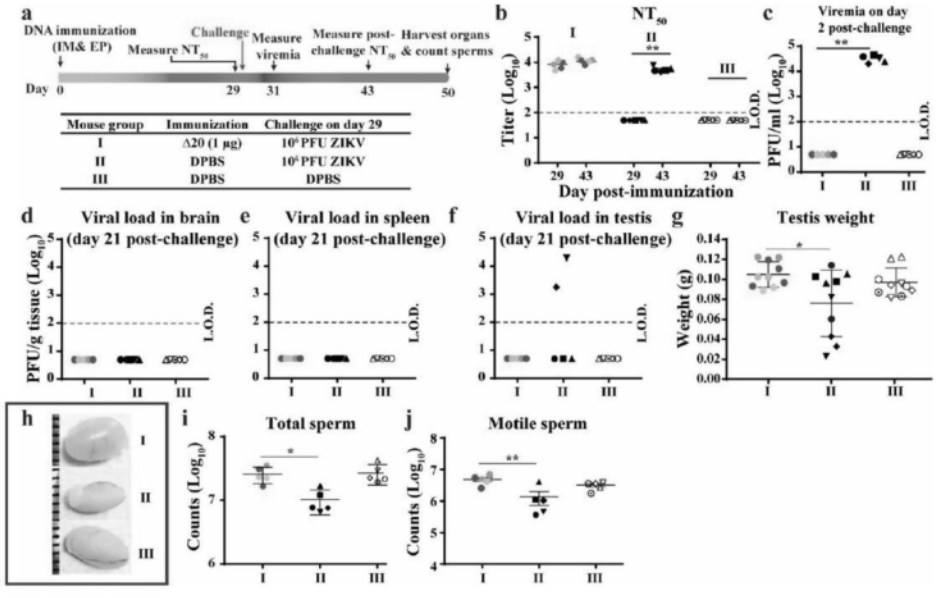
# Prevention of maternal-to-fetal transmission in pregnant mice




| Mouse group | Immunization | Challenge at E10.5 |
|---|---|---|
| IV | Δ20 (1 μg) | $10^5$ PFU ZIKV |
| V | DPBS | $10^5$ PFU ZIKV |
| VI | DPBS | DPBS |

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# Protection of male mice from ZIKV-induced damage to testis




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## Future directions

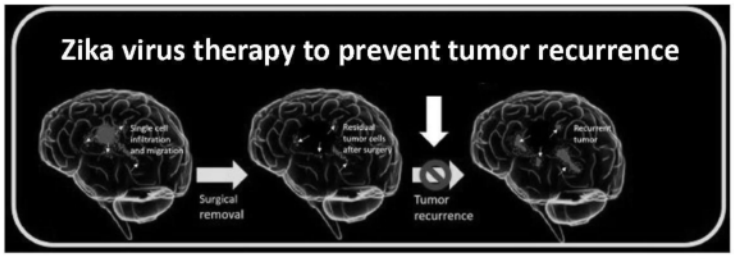
- Development path
  - Non-human primates: safety and efficacy
  - Apply to other flavivirus live-attenuated vaccines: YFV 17D and JEV 14-14-2
  - GMP production and non-human primate toxicity test
  - Clinical trial using YFV 17D or JEV 14-14-2
- **A universal vaccine platform: Use DNA-launched, FDA-approved RNA virus vaccines as vehicles to develop a "plug-and-play" technology**



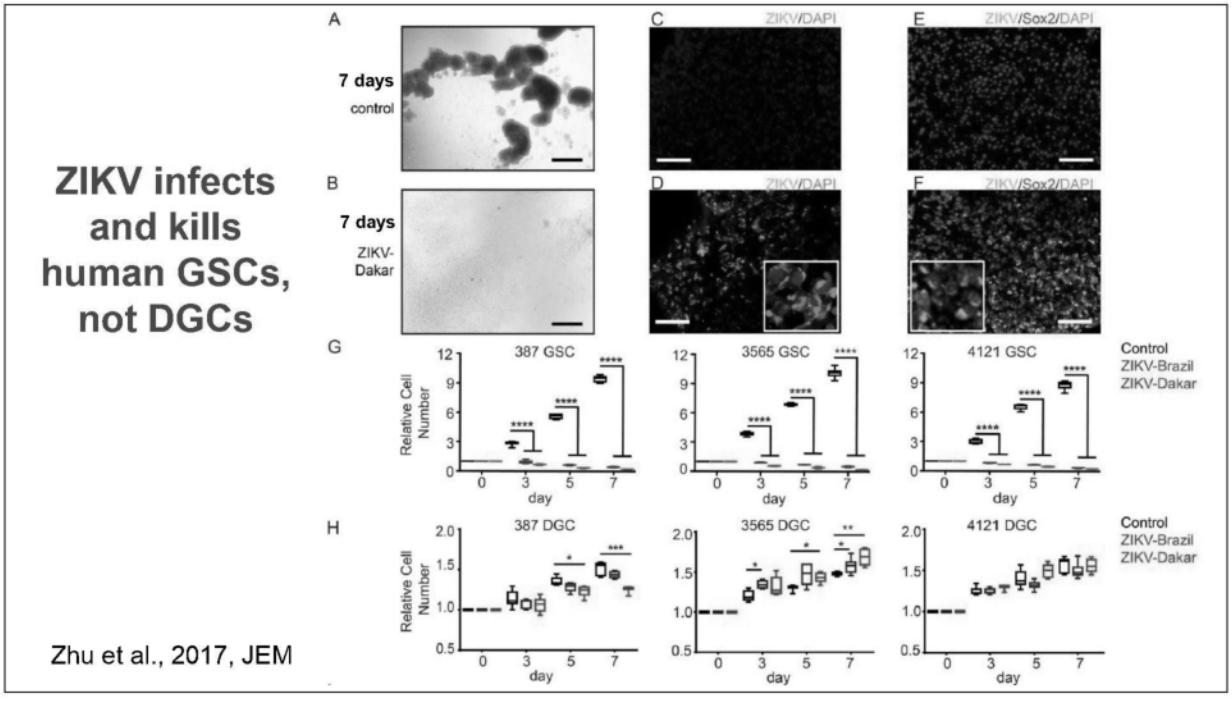

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## Engineering Zika virus to treat glioblastoma

- Publications
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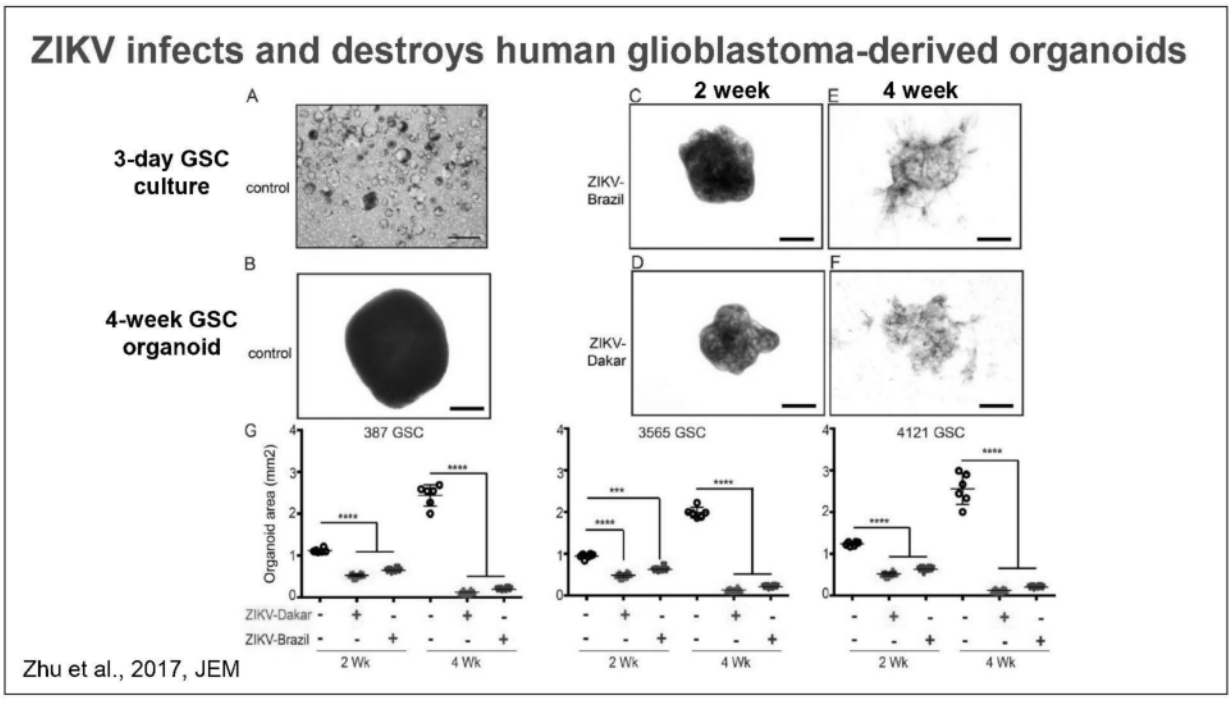



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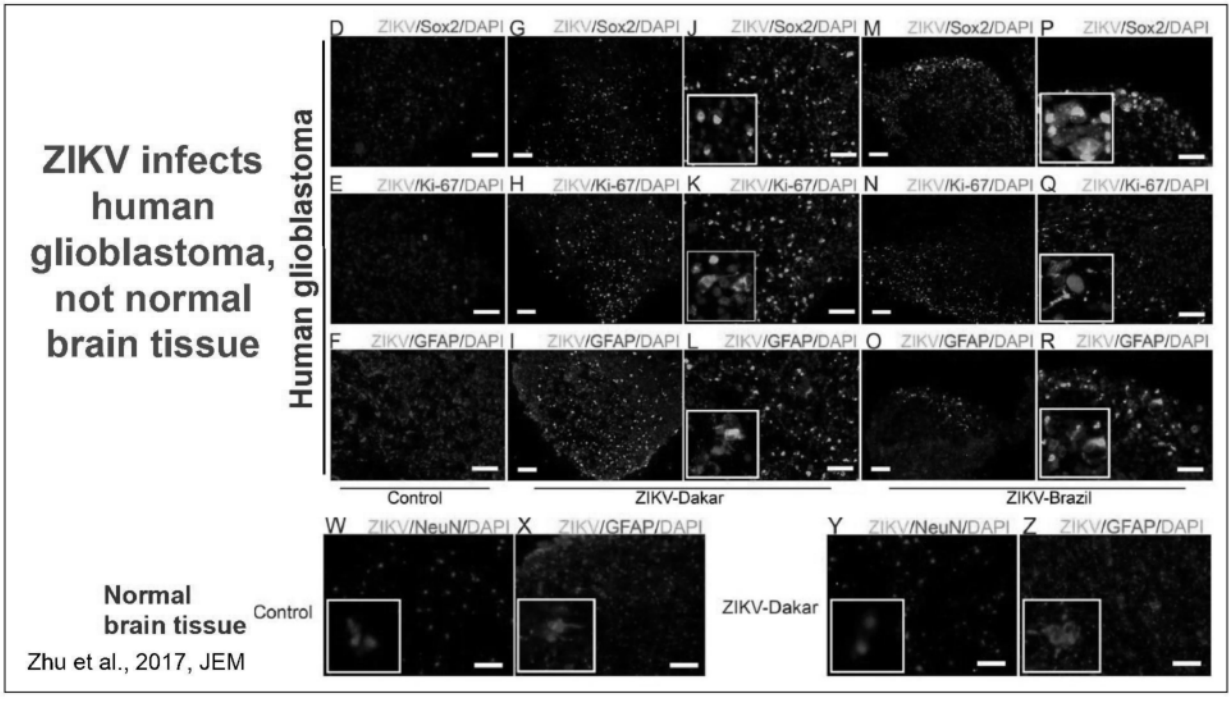

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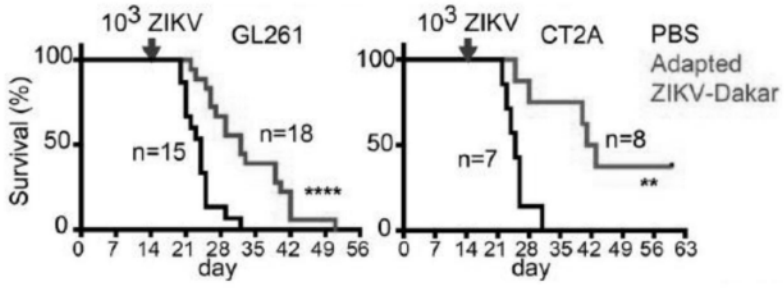
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## Using Zika vaccine to treat human glioblastoma

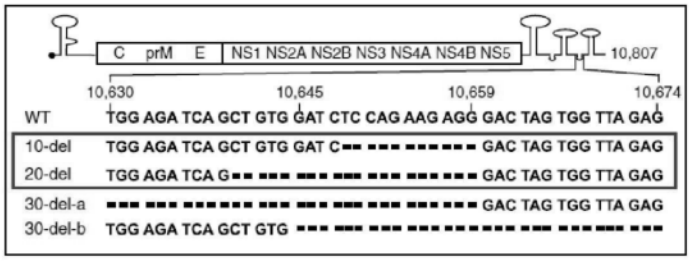
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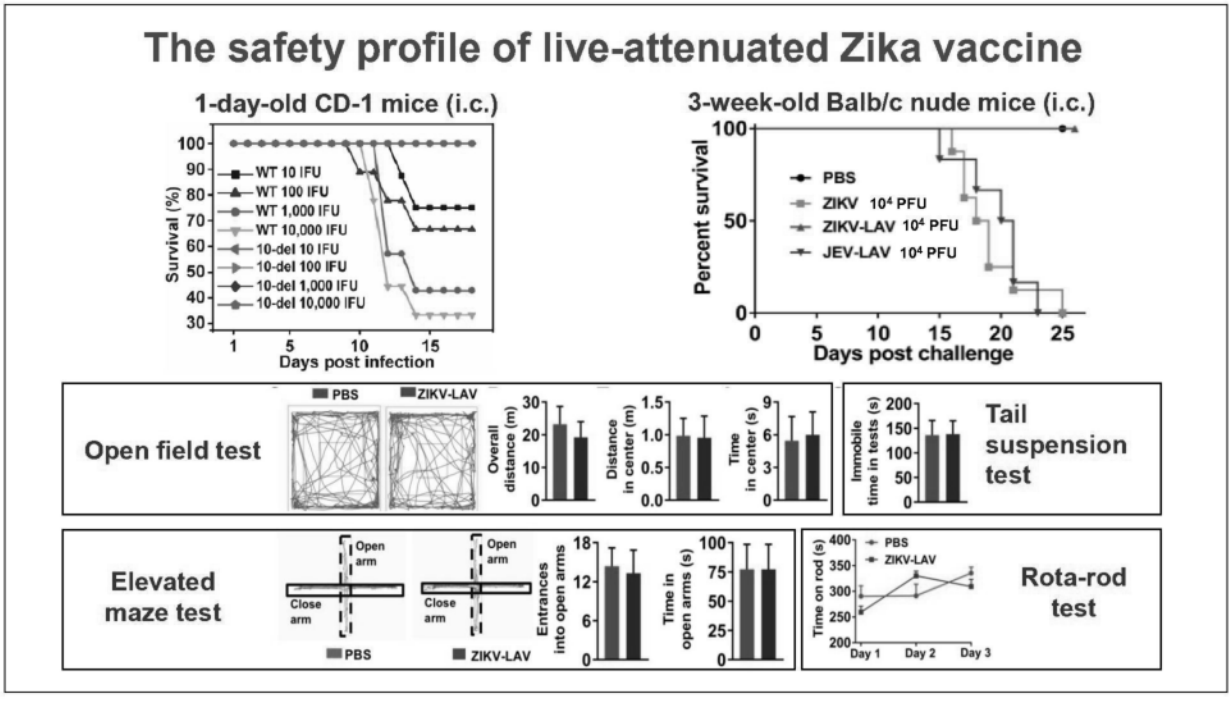


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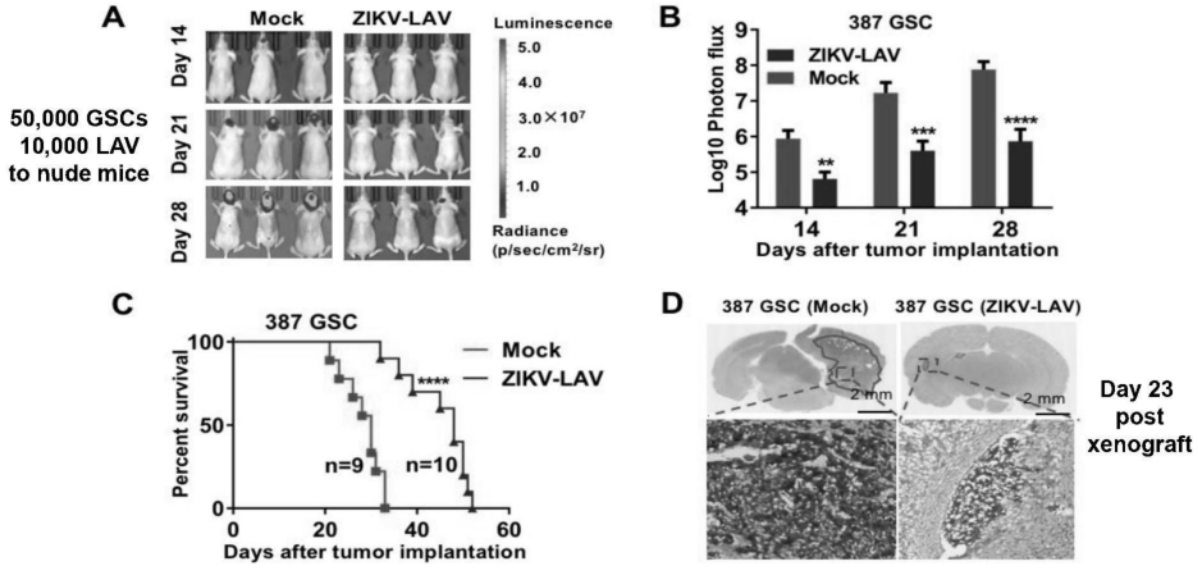
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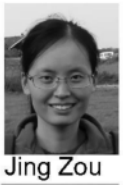

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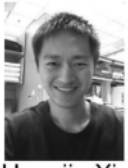

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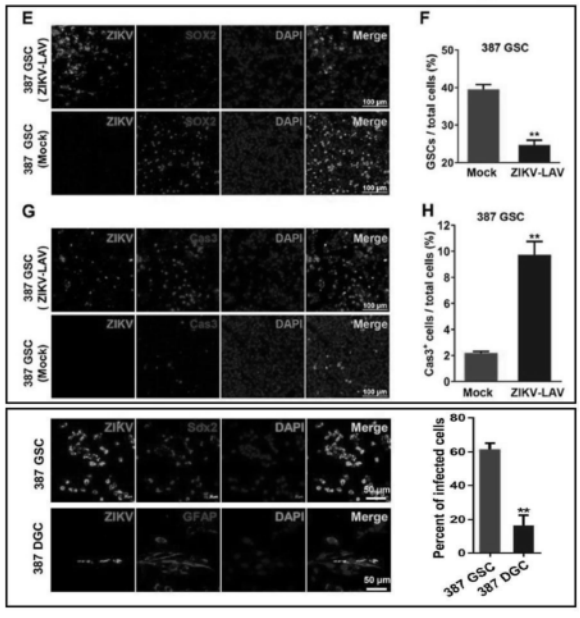

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Cheng-Feng Qin
**Tsinghua University**
Gong Cheng
**Wuhan Institute of Virology**
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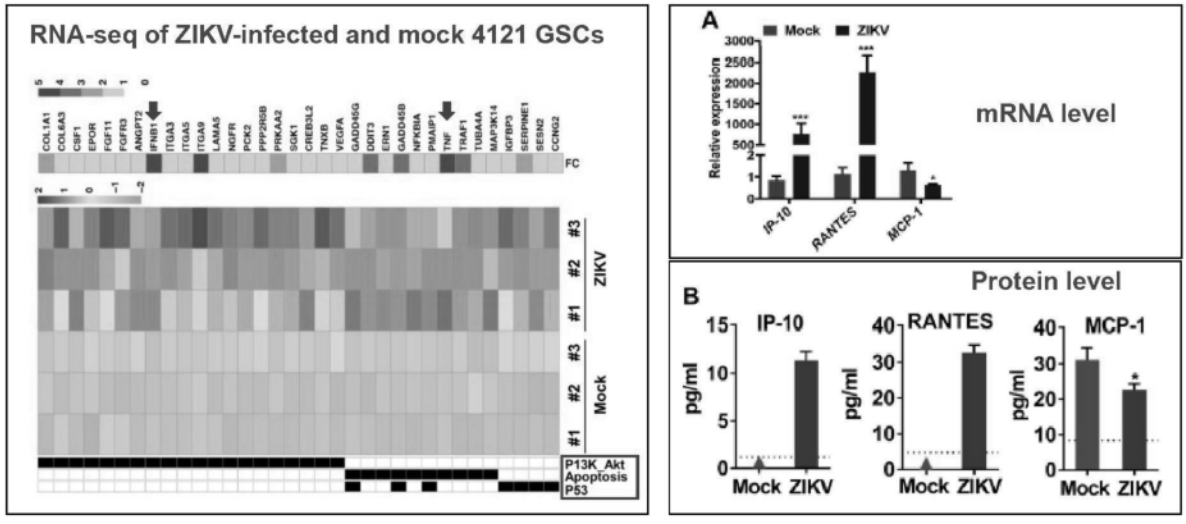
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# TAXONOMY OF THE ORDER *BUNYAVIRALES*: UPDATE 2019

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*Leishbuviridae*; leishbuvirus; mammarenavirus; nairovirid; *Nairoviridae*; nairovirus; orthobunyavirus; orthoferavirus; orthohantavirus; orthojonvirus; orthonairovirus; orthophasmavirus; orthotospovirus; peribunyavirid; *Peribunyaviridae*; peribunyavirus; phasmavirid; phasivirus; *Phasmaviridae*; phasmavirus; phenuivirid; *Phenuiviridae*; phenuivirus; phlebovirus; reptarenavirus; tenuivirus; tospovirid; *Tospoviridae*; tospovirus; virus classification; virus nomenclature; virus taxonomy



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## ABSTRACT

In February 2019, the order *Bunyavirales* was amended by creation of two new families, four new subfamilies, eleven new genera, and 77 new species, merging of two species, and deletion of one species. This article presents the updated taxonomy of the order *Bunyavirales* now accepted by the International Committee on Taxonomy of Viruses (ICTV).


## INTRODUCTION

The virus order *Bunyavirales* was established in 2017 to accommodate related viruses with segmented, linear, single-stranded, negative-sense or ambisense RNA genomes classified into 9 families [19]. An amended/emended order description was published in early 2019 [20]. Here we present the changes that were proposed via official ICTV taxonomic proposals that were accepted by the ICTV Executive Committee (EC) in February 2019. These changes are now part of the official ICTV taxonomy as of February 2019.

## TAXONOMIC CHANGES AT THE ORDER RANK

The order was expanded by addition of two new families. Family *Leishbuviridae* was created to accommodate one new genus, *Shilevirus*, including one new species, *Leptomonas shilevirus*, for Leptomonas moramango leishbunyavirus (LEPMV) discovered in a trypanosomatid protist (*Leptomonas moramango*) [2]. Family *Tospoviridae* was recreated for the already established genus *Tospovirus* (TaxoProp 2018.017M.A.v1.Bunyavirales_2fam5gen) and expanded by 7 new species (TaxoProp 2018.025P.A.v1.Orthotospovirus_7sp):

- *Bean necrotic mosaic orthotospovirus* for bean necrotic mosaic virus (BeNMV) discovered in common beans (*Phaseolus vulgaris*) [8];



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- *Calla lily chlorotic spot orthotospovirus* for calla lily chlorotic spot virus (CCSV) found in calla lilies (*Zantedeschia* sp.) [5, 18];
- *Capsicum chlorosis orthotospovirus* for Capsicum chlorosis virus (CaCV) found in capsicums, chillies, and tomatoes [16, 23];
- *Chrysanthemum stem necrosis orthotospovirus* for Chrysanthemum stem necrosis virus (CSNV) infecting chrysanthemums [3, 10];
- *Melon severe mosaic orthotospovirus* for melon severe mosaic virus (MSMV) in cucurbit crops [6, 7];
- *Melon yellow spot orthotospovirus* for melon yellow spot virus (MYSV) found in netted melon (*Cucumis melo*) [15]; and
- *Soybean vein necrosis orthotospovirus* for soybean vein necrosis virus (SVNV) discovered in soybeans (Glycine max) [36].

A genus unassigned to any family, *Coguvirus*, was established to include species *Citrus coguvirus* for Citrus concave gum-associated virus (CCGaV) found in citrus trees [26] (TaxoProp 2018.020P.A.v1.Coguvirus).

## TAXONOMIC CHANGES AT THE FAMILY RANK

### *Arenaviridae*

The family *Arenaviridae* was expanded by one genus, *Antennavirus*, to include two species, *Hairy antennavirus* and *Striated antennavirus*, for Wēnlǐngfrogfish arenavirus 2 (WlFV-2) and Wēnlǐng frogfish arenavirus 1 (WlFV-1) both found in striated frogfish (*Antennarius striatus*) [33] (TaxoProp 2018.005M.A.v1.Antennavirus).



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253 ***Cruliviridae***

254 No changes were made at the family rank.

255 ***Fimoviridae***

256 No changes were made at the family rank.

257 ***Hantaviridae***

258 The family (TaxoProp 2018.010M.A.v2.Hantaviridae_4subfam) was subdivided into four
259 subfamilies:

260 • subfamily *Actantavirinae* was created for new genus *Actinavirus* to accommodate 3 novel
261 species: *Batfish actinovirus* for Wēnlǐng minipizza batfish virus (WEMV) discovered in
262 minipizza batfish (*Halieutaea stellata*); *Goosefish actinovirus* for Wēnlǐng yellow goosefish
263 virus (WEYGV) found in yellow goosefish (*Lophius litulon*); and *Spikefish actinovirus* for
264 Wēnlǐng red spikefish virus (WERSV) of red spikefish (*Triacanthodes anomalus*) [33];
265 • subfamily *Agantavirinae* was created for new genus *Agnathovirus* to accommodate one new
266 species, *Hagfish agnathovirus*, for Wēnlǐng hagfish virus (WEHV) of inshore hagfish
267 (*Eptatretus burgeri*) [33];
268 • subfamily *Mammantavirinae* was created to accommodate the established genera *Loanvirus*,
269 *Mobatvirus*, and *Orthohantavirus*. Two new orthohantavirus species, *Seewis orhtohantavirus*
270 and *Tigray orthohantavirus*, were created for Seewis virus (SWSV) of Eurasian common
271 shrews (*Sorex araneus*) [34] and Tigray virus (TIGV) of Ethiopian white-footed mice
272 (*Stenocephalemys albipes*) [12, 25], respectively; and



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- subfamily *Repantavirinae* was created for new genus *Reptillovirus* to accommodate one new species, *Gecko reptillovirus*, for Hǎinán oriental lead-toed gecko virus (HOLGV) discovered in oriental leaf-toed geckos (*Hemidactylus bowringii*) [33].

***Mypoviridae***

No changes were made at the family rank.

***Nairoviridae***

The species *Estero Real orthonairovirus* was created for Estero Real virus (ERV) (moved from genus *Orthobunyavirus*, family *Peribunyaviridae*) [1] (TaxoProp 2018.012M.A.v1.Bunyavirales_spmov).

***Peribunyaviridae***

The family was expanded by one new genus, *Pacuvirus*, to accommodate three new species: *Pacui pacuvirus* for Pacui virus (PACV) discovered in a rice rat (*Oryzomys* sp.); *Rio Preto da Eva pacuvirus* for Rio Preto da Eva virus discovered in a sewer gnat (Psychodidae sp.); and *Tapirape pacuvirus* for Tapirapé virus found in a hocicudo (*Oxymycterus* sp.) [27] (TaxoProp 2018.017M.A.v1.Bunyavirales_2fam5gen). Genus *Tospovirus* was removed from the family and placed into the new family *Tospoviridae* (TaxoProp 2018.017M.A.v1.Bunyavirales_2fam5gen). The genus *Orthobunyavirus* was reorganized by moving previously classified viruses into a total of 38 new species (one resulting from a merger of two previously established species) (TaxoProp 2018.008M.A.v1.Orthobunyavirus_38sp). Five additional novel species were added: *Bellavista orthobunyavirus* for Bellavista virus isolated from mosquitoes (*Culex portesi*) [14]; *Enseada orthobunyavirus* for Enseada virus isolated from *Culex* mosquitoes [4, 9]; *Maguari*



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294 *orthobunyavirus* for Maguari virus (MAGV) isolated from mosquitoes [13]; *Tataguine*
295 *orthobunyavirus* for Tataguine virus (TATV) found in a human sample, and *Witwatersrand*
296 *orthobunyavirus* for Witwatersrand virus (WITV) from mosquitoes [29] (TaxoProp
297 2018.017M.A.v1.Bunyavirales_2fam5gen). Species *Estero Real orthobunyavirus* dissolved, and
298 its member, Estero Real virus (ERV), was moved into family *Nairoviridae* [1] (TaxoProp
299 2018.012M.A.v1.Bunyavirales_spmov).

300 ***Phasmaviridae***

301 The family was expanded by addition of one new genus, *Sawastrivirus*, to include one new
302 species, *Sanxia sawastrivirus*, for Sānxiá water strider virus 2 (SxWSV-2) (TaxoProp
303 2018.017M.A.v1.Bunyavirales_2fam5gen) detected in gerrid water striders [31]. The genus
304 *Orthophasmavirus* was expanded by the addition of five new species: *Culex orthophasmavirus*
305 for Culex phasma-like virus (CPLV) detected in *Culex* mosquitoes [32]; *Ganda*
306 *orthophasmavirus* for Ganda bee virus (GBEEV) of European orchard bees (*Osmia cornuta*)
307 [28]; *Odonate orthophasmavirus* for Húběi odonate virus 8 (HbOV-8) [31]; *Qingling*
308 *orthophasmavirus* for Húběi odonate virus 9 (HbOV-9) of odonates [31]; and *Seattle*
309 *orthophasmavirus* for Seattle Prectang virus (SEPV) found in a moth (*Pasiphila rectangulata*)
310 [21] (TaxoProp 2018.009M.A.v1.Phasmavirus_5sp).

311 ***Phenuiviridae***

312 The family *Phenuiviridae* was expanded three new genera. Genus *Kabutovirus* was created to
313 include two new species, *Huangpi kabutovirus* and *Kabuto mountain kabutovirus*, for Huángpí
314 tick virus 1 (HpTV-1) of ticks (*Haemaphysalis doenitzi*) [17] and Kabuto mountain virus



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(KAMV) of ticks (*Haemaphysalis flava*) [11], respectively. Genus *Laulavirus* was created to include one species, *Laurel Lake laulavirus*, for Laurel Lake virus (LLV) of ticks (*Ixodes scapularis*) [35]. Genus *Wenrivirus* was created to include one species, *Shrimp wenrivirus*, for Wēnzhōu shrimp virus 1 (WzSV-1) [17] found in giant tiger prawns (Penaeus monodon) [17](TaxoProp 2018.017M.A.v1.Bunyavirales_2fam5gen). The established genus *Banyangvirus* was expanded by two species, *Guertu banyangvirus* and *Heartland banyangvirus*, for Guertu virus (GTV) found in *Dermacentor nuttalli* ticks [30] and Heartland virus (HRTV), a tick-borne virus originally discovered in human samples [24], respectively (TaxoProp 2018.013M.A.v1.Banyangvirus_sp; TaxoProp 2018.017M.A.v1.Bunyavirales_2fam5gen). Genus *Phlebovirus* was expanded by one species, *Mukawa phlebovirus*, for Mukawa virus (MKWV) of ticks (*Ixodes persulcatus*) [22] (TaxoProp 2018.014M.A.v1.Phlebovirus_sp). In genus *Phasivirus*, species *Wuhan fly phasivirus* was deleted (TaxoProp 2018.019M.A.v1.Phenuiviridae_Remsp).

***Wupedeviridae***

No changes were made at the family rank.

## SUMMARY

A summary of the current, ICTV-accepted taxonomy of the order *Bunyavirales* is presented in Table 1.

## ACKNOWLEDGMENTS

We thank Laura Bollinger (NIH/NIAID Integrated Research Facility at Fort Detrick, Frederick, MD, USA) for critically editing the manuscript.



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**Table 1. ICTV-accepted taxonomy of the order *Bunyavirales* as of February 2019.** Listed are all bunyaviruses that are classified into species.

| Genus | Species¶ | Virus (Abbreviation)¶ |
|---|---|---|
| **Family *Arenaviridae*** | | |
| *Antennavirus* | *Hairy antennavirus* | Wēnlǐng frogfish arenavirus 2 (WlFAV-2) |
| | *Striated antennavirus** | Wēnlǐng frogfish arenavirus 1 (WlFAV-1) |
| *Hartmanivirus* | *Haartman hartmanivirus** | Haartman Institute snake virus 1 (HISV-1) |
| *Mammarenavirus* | *Allpahuayo mammarenavirus* | Allpahuayo virus (ALLV) |
| | *Argentinian mammarenavirus* | Junín virus (JUNV) |
| | *Bear Canyon mammarenavirus* | Bear Canyon virus (BCNV) |
| | *Brazilian mammarenavirus* | Sabiá virus (SBAV) |
| | *Cali mammarenavirus* | Pichindé virus (PICHV) |
| | *Chapare mammarenavirus* | Chapare virus (CHAPV) |
| | *Cupixi mammarenavirus* | Cupixi virus (CUPXV) |
| | *Flexal mammarenavirus* | Flexal virus (FLEV) |
| | *Gairo mammarenavirus* | Gairo virus (GAIV) |

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| | | |
|---|---|---|
| | *Guanarito mammarenavirus* | Guanarito virus (GTOV) |
| | *Ippy mammarenavirus* | Ippy virus (IPPYV) |
| | *Lassa mammarenavirus* | Lassa virus (LASV) |
| | *Latino mammarenavirus* | Latino virus (LATV) |
| | *Loei River mammarenavirus* | Loei River virus (LORV) |
| | *Lujo mammarenavirus* | Lujo virus (LUJV) |
| | *Luna mammarenavirus* | Luli virus (LULV) |
| | | Luna virus (LUAV) |
| | *Lunk mammarenavirus* | Lunk virus (LNKV) |
| | *Lymphocytic choriomeningitis mammarenavirus** | Dandenong virus (DANV) |
| | | lymphocytic choriomeningitis virus (LCMV) |
| | *Machupo mammarenavirus* | Machupo virus (MACV) |
| | *Mariental mammarenavirus* | Mariental virus (MRLV) |



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| | | |
|---|---|---|
| | *Merino Walk mammarenavirus* | Merino Walk virus (MRWV) |
| | *Mobala mammarenavirus* | mobala virus (MOBV) |
| | *Mopeia mammarenavirus* | Mopeia virus (MPOV) |
| | | Morogoro virus (MORV) |
| | *Okahandja mammarenavirus* | Okahandja virus (OKAV) |
| | *Oliveros mammarenavirus* | Oliveros virus (OLVV) |
| | *Paraguayan mammarenavirus* | Paraná virus (PRAV) |
| | *Pirital mammarenavirus* | Pirital virus (PIRV) |
| | *Ryukyu mammarenavirus* | Ryukyu virus (RYKV) |
| | *Serra do Navio mammarenavirus* | Amaparí virus (AMAV) |
| | *Solwezi mammarenavirus* | Solwezi virus (SOLV) |
| | *Souris mammarenavirus* | souris virus (SOUV) |
| | *Tacaribe mammarenavirus* | Tacaribe virus (TCRV) |
| | *Tamiami mammarenavirus* | Tamiami virus (TMMV) |
| | *Wenzhou mammarenavirus* | Wēnzhōu virus (WENV)[1] |



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| | | |
|---|---|---|
| | *Whitewater Arroyo mammarenavirus* | Big Brushy Tank virus (BBRTV) |
| | | Catarina virus (CTNV) |
| | | Skinner Tank virus (SKTV) |
| | | Tonto Creek virus (TTCV) |
| | | Whitewater Arroyo virus (WWAV) |
| *Reptarenavirus* | *California reptarenavirus* | CAS virus (CASV) |
| | *Giessen reptarenavirus* | University of Giessen virus 1 (UGV-1) |
| | | University of Giessen virus 2 (UGV-2) |
| | | University of Giessen virus 3 (UGV-3) |
| | *Golden reptarenavirus** | Golden Gate virus (GOGV) |
| | *Ordinary reptarenavirus* | tavallinen suomalainen mies virus 2 (TSMV-2) |
| | *Rotterdam reptarenavirus* | ROUT virus (ROUTV) |
| | | University of Helsinki virus 1 (UHV-1) |
| **Family *Cruliviridae*** | | |



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| | | |
|---|---|---|
| *Lincruvirus* | *Crustacean lincruvirus** | Wēnlǐng crustacean virus 9 (WlCV-9) |
| **Family *Fimoviridae*** | | |
| *Emaravirus* | *Actinidia chlorotic ringspot-associated emaravirus* | Actinidia chlorotic ringspot-associated virus (AcCRaV) |
| | *European mountain ash ringspot-associated emaravirus** | European mountain ash ringspot-associated virus (EMARaV) |
| | *Fig mosaic emaravirus* | fig mosaic virus (FMV) |
| | *High Plains wheat mosaic emaravirus* | High Plains wheat mosaic virus (HPWMoV) |
| | *Pigeonpea sterility mosaic emaravirus 1* | pigeonpea sterility mosaic virus (PPSMV) |
| | *Pigeonpea sterility mosaic emaravirus 2* | pigeonpea sterility mosaic virus 2 (PPSMV-2) |
| | *Raspberry leaf blotch emaravirus* | raspberry leaf blotch virus (RLBV) |
| | *Redbud yellow ringspot-associated emaravirus* | redbud yellow ringspot-associated virus (RYRaV) |
| | *Rose rosette emaravirus* | rose rosette virus (RRV) |



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| **Family *Hantaviridae*** | | |
|---|---|---|
| Subfamily *Actantavirinae* | | |
| *Actinovirus* | *Batfish actinovirus* | Wēnlǐng minipizza batfish virus (WEMBV) |
| | *Goosefish actinovirus* | Wēnlǐng yellow goosefish virus (WEYGV) |
| | *Spikefish actinovirus* | Wēnlǐng red spikefish virus (WERSV) |
| Subfamily *Agantavirinae* | | |
| *Agnathovirus* | *Hagfish agnathovirus* | Wēnlǐng hagfish virus (WEHV) |
| Subfamily *Mammantavirinae* | | |
| *Loanvirus* | *Longquan loanvirus** | Lóngquán virus (LQUV) |
| *Mobatvirus* | *Laibin mobatvirus* | Láibīn virus (LAIV) |
| | *Nova mobatvirus** | Nova virus (NVAV) |
| | *Quezon mobatvirus* | Quezon virus (QZNV) |
| *Orthohantavirus* | *Andes orthohantavirus* | Andes virus (ANDV) |
| | | Castelo dos Sonhos virus (CASV) |



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| | | |
|---|---|---|
| | | Lechiguanas virus (LECV = LECHV) |
| | | Orán virus (ORNV) |
| | *Asama orthohantavirus* | Asama virus (ASAV) |
| | *Asikkala orthohantavirus* | Asikkala virus (ASIV) |
| | *Bayou orthohantavirus* | bayou virus (BAYV) |
| | | Catacamas virus (CATV) |
| | *Black Creek Canal orthohantavirus* | Black Creek Canal virus (BCCV) |
| | *Bowe orthohantavirus* | Bowé virus (BOWV) |
| | *Bruges orthohantavirus* | Bruges virus (BRGV) |
| | *Cano Delgadito orthohantavirus* | Caño Delgadito virus (CADV) |
| | *Cao Bang orthohantavirus* | Cao Bằng virus (CBNV) |
| | | Liánghé virus (LHEV) |
| | *Choclo orthohantavirus* | Choclo virus (CHOV) |
| | *Dabieshan orthohantavirus* | Dàbiéshān virus (DBSV) |
| | *Dobrava-Belgrade orthohantavirus* | Dobrava virus (DOBV) |



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| | | |
|---|---|---|
| | | Kurkino virus (KURV) |
| | | Saaremaa virus (SAAV) |
| | | Sochi virus (SOCV) |
| | *El Moro Canyon orthohantavirus* | Carrizal virus (CARV) |
| | | El Moro Canyon virus (ELMCV) |
| | | Huitzilac virus (HUIV) |
| | *Fugong orthohantavirus* | Fúgòng virus (FUGV) |
| | *Fusong orthohantavirus* | Fŭsōng virus (FUSV) |
| | *Hantaan orthohantavirus** | Amur virus (AMRV) |
| | | Hantaan virus (HTNV) |
| | | Soochong virus (SOOV) |
| | *Jeju orthohantavirus* | Jeju virus (JJUV) |
| | *Kenkeme orthohantavirus* | Kenkeme virus (KKMV) |
| | *Khabarovsk orthohantavirus* | Khabarovsk virus (KHAV) |
| | | Topografov virus (TOPV) |



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| | | |
|---|---|---|
| | *Laguna Negra orthohantavirus* | Laguna Negra virus (LANV) |
| | | Maripa virus (MARV) |
| | | Río Mamoré virus (RIOMV) |
| | *Luxi orthohantavirus* | Lúxī virus (LUXV) |
| | *Maporal orthohantavirus* | Maporal virus (MAPV) |
| | *Montano orthohantavirus* | Montaño virus (MTNV) |
| | *Necocli orthohantavirus* | Necoclí virus (NECV) |
| | *Oxbow orthohantavirus* | Oxbow virus (OXBV) |
| | *Prospect Hill orthohantavirus* | Prospect Hill virus (PHV) |
| | *Puumala orthohantavirus* | Hokkaido virus (HOKV) |
| | | Muju virus (MUJV) |
| | | Puumala virus (PUUV) |
| | *Rockport orthohantavirus* | Rockport virus (RKPV) |
| | *Sangassou orthohantavirus* | Sangassou virus (SANGV) |
| | *Seewis orthohantavirus* | Seewis virus (SWSV)[2] |



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| | | |
|---|---|---|
| | *Seoul orthohantavirus* | gōu virus (GOUV) |
| | | Seoul virus (SEOV) |
| | *Sin Nombre orthohantavirus* | New York virus (NYV) |
| | | Sin Nombre virus (SNV) |
| | *Thailand orthohantavirus* | Anjozorobe virus (ANJZV) |
| | | Serang virus (SERV)[3] |
| | | Thailand virus (THAIV) |
| | *Tigray orthohantavirus* | Tigray virus (TIGV) |
| | *Tula orthohantavirus* | Adler virus (ADLV) |
| | | Tula virus (TULV) |
| | *Yakeshi orthohantavirus* | Yákèshí virus (YKSV) |
| *Thottimvirus* | *Imjin thottimvirus* | Imjin virus (MJNV) |
| | *Thottapalayam thottimvirus** | Thottapalayam virus (TPMV) |
| Subfamily *Repantavirinae* | | |
| *Reptillovirus* | *Gecko reptillovirus* | Hǎinán oriental leaf-toed gecko virus |



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| | | |
|---|---|---|
| | | (HOLGV) |
| **Family *Leishbuviridae*** | | |
| *Shilevirus* | *Leptomonas shilevirus** | Leptomonas moramango virus (LEPMV) |
| **Family *Mypoviridae*** | | |
| *Hubavirus* | *Myriapod hubavirus** | Húběi myriapoda virus 5 (HbMV-5) |
| **Family *Nairoviridae*** | | |
| *Orthonairovirus* | *Artashat orthonairovirus* | Artashat virus (ARTSV) |
| | *Chim orthonairovirus* | Chim virus (CHIMV) |
| | *Crimean-Congo hemorrhagic fever orthonairovirus* | Crimean-Congo hemorrhagic fever virus (CCHFV) |
| | *Dera Ghazi Khan orthonairovirus* | Abu Hammad virus (AHV)[4] |
| | | Abu Mina virus (AMV) |
| | | Dera Ghazi Khan virus (DGKV) |
| | | Sapphire II virus (SAPV) |



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| | | |
|---|---|---|
| | *Dugbe orthonairovirus** | Dugbe virus (DUGV) |
| | | kupe virus (KUPEV) |
| | *Estero Real orthonairovirus* | Estero Real virus (ERV) |
| | *Hazara orthonairovirus* | Hazara virus (HAZV) |
| | | Tofla virus (TFLV) |
| | *Hughes orthonairovirus* | Caspiy virus (CASV) |
| | | Farallon virus (FARV) |
| | | Great Saltee virus (GRSV) |
| | | Hughes virus (HUGV) |
| | | Punta Salinas virus (PSV) |
| | | Raza virus (RAZAV) |
| | | Soldado virus (SOLV) |
| | | Zirqa virus (ZIRV) |
| | *Kasokero orthonairovirus* | Kasokero virus (KASV = KASOV) |
| | | Leopards Hill virus (LPHV) |



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| | | |
|---|---|---|
| | | Yogue virus (YOGV) |
| | *Keterah orthonairovirus* | Gossas virus (GOSV) |
| | | Issyk-kul virus (ISKV) |
| | | Keterah virus (KTRV)[5] |
| | | Uzun-Agach virus (UZAV) |
| | *Nairobi sheep disease orthonairovirus* | Nairobi sheep disease virus (NSDV)[6] |
| | *Qalyub orthonairovirus* | Bandia virus (BDAV) |
| | | Geran virus (GERV) |
| | | Qalyub virus (QYBV) |
| | *Sakhalin orthonairovirus* | Avalon virus (AVAV) |
| | | Clo Mor virus (CMV = CLMV) |
| | | Sakhalin virus (SAKV) |
| | | Taggert virus (TAGV) |
| | | Tillamook virus (TILLV) |
| | *Tamdy orthonairovirus* | Burana virus (BURV) |



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| | | |
|---|---|---|
| | | Huángpí tick virus 1 (HpTV-1) |
| | | Tăchéng tick virus 1 (TcTV-1) |
| | | Tamdy virus (TAMV) |
| | | Wēnzhōu tick virus (WzTV) |
| | *Thiafora orthonairovirus* | Erve virus (ERVEV) |
| | | Thiafora virus (TFAV) |
| *Shaspivirus* | *Spider shaspivirus** | Shāyáng spider virus 1 (SySV-1) |
| *Striwavirus* | *Strider striwavirus** | Sānxiá water strider virus 1 (SxWSV-1) |
| **Family *Peribunyaviridae*** | | |
| *Herbevirus* | *Herbert herbevirus** | Herbert virus (HEBV) |
| | *Kibale herbevirus* | Kibale virus (KIBV) |
| | *Tai herbevirus* | Taï virus (TAIV) |
| *Orthobunyavirus* | *Acara orthobunyavirus* | Acará virus (ACAV) |
| | | Moriche virus (MORV) |
| | *Akabane orthobunyavirus* | Akabane virus (AKAV) |



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| | | |
|---|---|---|
| | | Tinaroo virus (TINV) |
| | | Yaba-7 virus (Y7V) |
| | *Aino orthobunyavirus* | Aino virus (AINOV) |
| | *Alajuela orthobunyavirus* | Alajuela virus (ALJV) |
| | | San Juan virus (SJV) |
| | *Anadyr orthobunyavirus* | Anadyr virus (ANADV) |
| | *Anhembi orthobunyavirus* | Anhembi virus (AMBV) |
| | *Anopheles A orthobunyavirus* | Anopheles A virus (ANAV) |
| | | Arumateua virus (ARTV = ARMTV) |
| | | Caraipé virus (CPEV = CRPV) |
| | | Las Maloyas virus (LMV) |
| | | Lukuni virus (LUKV) |
| | | Trombetas virus (TRMV) |
| | | Tucuruí virus (TUCV = TUCRV) |
| | *Anopheles B orthobunyavirus* | Anopheles B virus (ANBV) |



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| | | |
|---|---|---|
| | | Boracéia virus (BORV) |
| | *Bakau orthobunyavirus* | Bakau virus (BAKV) |
| | | Ketapang virus (KETV) |
| | | Nola virus (NOLAV) |
| | | Tanjong Rabok virus (TRV) |
| | | Telok Forest virus (TFV) |
| | *Batai orthobunyavirus* | Batai virus (BATV)[7] |
| | *Batama orthobunyavirus* | Batama virus (BMAV) |
| | *Bellavista orthobunyavirus* | Bellavista virus (BELLV) |
| | *Benevides orthobunyavirus* | Benevides virus (BVSV = BENV) |
| | *Bertioga orthobunyavirus* | Bertioga virus (BERV) |
| | | Cananéia virus (CNAV) |
| | | Guaratuba virus (GTBV) |
| | | Itimirim virus (ITIV) |
| | | Mirim virus (MIRV) |



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| | | |
|---|---|---|
| | *Bimiti orthobunyavirus* | bimiti virus (BIMV) |
| | *Birao orthobunyavirus* | Birao virus (BIRV) |
| | *Botambi orthobunyavirus* | Botambi virus (BOTV) |
| | *Bozo orthobunyavirus* | Bozo virus (BOZOV) |
| | *Bunyamwera orthobunyavirus** | Bunyamwera virus (BUNV) |
| | | Germiston virus (GERV) |
| | | Lokern virus (LOKV) |
| | | Mboké virus (MBOV) |
| | | Ngari virus (NRIV)[8] |
| | | Northway virus (NORV) |
| | | Santa Rosa virus (SARV) |
| | | Shokwe virus (SHOV) |
| | | Stanfield virus (STAV) |
| | | Xingu virus (XINV) |
| | *Bushbush orthobunyavirus* | Benfica virus (BENV = BNFV) |



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| | | |
|---|---|---|
| | | Bushbush virus (BSBV) |
| | | Juan Díaz virus (JDV) |
| | *Buttonwillow orthobunyavirus* | Buttonwillow virus (BUTV) |
| | *Bwamba orthobunyavirus* | Bwamba virus (BWAV) |
| | | Pongola virus (PGAV) |
| | *Cache Valley orthobunyavirus* | Cache Valley virus (CVV) |
| | | Cholul virus (CHLV) |
| | | Tlacotalpan virus (TLAV) |
| | *Cachoeira Porteira orthobunyavirus* | Cachoeira Porteira virus (CPOV) |
| | *California encephalitis orthobunyavirus* | California encephalitis virus (CEV) |
| | | Morro Bay virus (MBV) |
| | *Capim orthobunyavirus* | Capim virus (CAPV) |
| | *Caraparu orthobunyavirus* | Apeú virus (APEUV) |
| | | Bruconha virus (BRUV) |
| | | Caraparú virus (CARV) |



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| | | |
|---|---|---|
| | | El Huayo virus (EHUV) |
| | | Itaya virus (ITYV) |
| | | Ossa virus (OSSAV) |
| | | Vinces virus (VINV) |
| | *Cat Que orthobunyavirus* | Cát Quế virus (CQV) |
| | | Oya virus (OYAV) |
| | *Catu orthobunyavirus* | Catú virus (CATUV) |
| | *Enseada orthobunyavirus* | Enseada virus (ENSV) |
| | *Faceys paddock orthobunyavirus* | Facey's paddock virus (FPV) |
| | *Fort Sherman orthobunyavirus* | Fort Sherman virus (FSV) |
| | *Gamboa orthobunyavirus* | Brus Laguna virus (BLAV) |
| | | Calchaquí virus (CQIV) |
| | | Gamboa virus (GAMV) |
| | | Pueblo Viejo virus (PVV) |
| | | Soberanía virus (SOBV) |



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| | | |
|---|---|---|
| | *Guajara orthobunyavirus* | Guajará virus (GJAV) |
| | *Guama orthobunyavirus* | Ananindeua virus (ANUV) |
| | | Guamá virus (GMAV) |
| | | Mahogany Hammock virus (MHV) |
| | | Moju virus (MOJUV) |
| | *Guaroa orthobunyavirus* | Guaroa virus (GROV) |
| | *Iaco orthobunyavirus* | Iaco virus (IACOV) |
| | *Ilesha orthobunyavirus* | Ilesha virus (ILEV) |
| | *Ingwavuma orthobunyavirus* | Ingwavuma virus (INGV) |
| | *Jamestown Canyon orthobunyavirus* | Inkoo virus (INKV) |
| | | Jamestown Canyon virus (JCV) |
| | | Jerry Slough virus (JSV) |
| | | South River virus (SORV) |
| | *Jatobal orthobunyavirus* | Jatobal virus (JATV) |
| | *Kaeng Khoi orthobunyavirus* | Kaeng Khoi virus (KKV) |



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| | | |
|---|---|---|
| | *Kairi orthobunyavirus* | Kairi virus (KRIV) |
| | *Keystone orthobunyavirus* | Keystone virus (KEYV) |
| | *Koongol orthobunyavirus* | koongol virus (KOOV) |
| | | wongal virus (WONV) |
| | *La Crosse orthobunyavirus* | La Crosse virus (LACV) |
| | *Leanyer orthobunyavirus* | Leanyer virus (LEAV) |
| | *Lumbo orthbunyavirus* | Lumbo virus (LUMV) |
| | *Macaua orthobunyavirus* | Macauã virus (MCAV) |
| | *Madrid orthobunyavirus* | Madrid virus (MADV) |
| | *Maguari orthobunyavirus* | Maguari virus (MAGV) |
| | | Playas virus (PLAV) |
| | *Main Drain orthobunyavirus* | Main Drain virus (MDV) |
| | *Manzanilla orthobunyavirus* | Manzanilla virus (MANV) |
| | | Inini virus (INIV) |
| | *Marituba orthobunyavirus* | Gumbo Limbo virus (GLV) |



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| | | |
|---|---|---|
| | | Marituba virus (MTBV) |
| | | Murutucú virus (MURV) |
| | | Nepuyo virus (NEPV) |
| | | Restan virus (RESV) |
| | | Zungarococha virus (ZUNV) |
| | *Melao orthobunyavirus* | Melao virus (MELV) |
| | *Mermet orthobunyavirus* | Mermet virus (MERV) |
| | *Minatitlan orthobunyavirus* | Minatitlán virus (MNTV) |
| | | Palestina virus (PLSV) |
| | *MPoko orthobunyavirus* | M'Poko virus (MPOV) |
| | | Yaba-1 virus (Y1V) |
| | *Nyando orthobunyavirus* | Eretmapodites virus (ERETV) |
| | | Mojuí dos Campos virus (MDCV) |
| | | Nyando virus (NDV) |
| | *Olifantsvlei orthobunyavirus* | Bobia virus (BIAV) |



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| | | |
|---|---|---|
| | | Dabakala virus (DABV) |
| | | Olifantsvlei virus (OLIV) |
| | | Oubi virus (OUBIV) |
| | *Oriboca orthobunyavirus* | Itaquí virus (ITQV) |
| | | Oriboca virus (ORIV) |
| | *Oropouche orthobunyavirus* | Iquitos virus (IQTV)[d] |
| | | Madre de Dios virus (MDDV) |
| | | Oropouche virus (OROV) |
| | | Perdões virus (PDEV) |
| | | Pintupo virus (PINTV) |
| | *Patois orthobunyavirus* | Abras virus (ABRV) |
| | | Babahoya virus (BABV) |
| | | Pahayokee virus (PAHV) |
| | | Patois virus (PATV) |
| | | Shark River virus (SRV) |



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| | | |
|---|---|---|
| | *Peaton orthobunyavirus* | Peaton virus (PEAV) |
| | *Potosi orthobunyavirus* | Potosi virus (POTV) |
| | *Sabo orthobunyavirus* | Sabo virus (SABOV) |
| | *San Angelo orthobunyavirus* | San Angelo virus (SAV) |
| | *Sango orthobunyavirus* | Sango virus (SANV) |
| | *Schmallenberg orthobunyavirus* | Douglas virus (DOUV) |
| | | Sathuperi virus (SATV) |
| | | Schmallenberg virus (SBV) |
| | | Shamonda virus (SHAV) |
| | *Serra do Navio orthobunyavirus* | Serra do Navio virus (SDNV) |
| | *Shuni orthobunyavirus* | Kaikalur virus (KAIV) |
| | | Shuni virus (SHUV) |
| | *Simbu orthobunyavirus* | Para virus (PARAV) |
| | | Simbu virus (SIMV) |
| | *Snowshoe hare orthobunyavirus* | Khatanga virus (KHATV)[9] |



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| | | |
|---|---|---|
| | | snowshoe hare virus (SSHV) |
| | *Sororoca orthobunyavirus* | Sororoca virus (SORV) |
| | *Tacaiuma orthobunyavirus* | CoAr 1071 virus (CA1071V) |
| | | CoAr 3627 virus (CA3626V) |
| | | Tacaiuma virus (TCMV) |
| | | Virgin River virus (VRV) |
| | *Tahyna orthobunyavirus* | Ťahyňa virus (TAHV) |
| | *Tataguine orthobunyavirus* | Tataguine virus (TATV) |
| | *Tensaw orthobunyavirus* | Tensaw virus (TENV) |
| | *Tete orthobunyavirus* | Bahig virus (BAHV) |
| | | Matruh virus (MTRV) |
| | | Tete virus (TETEV) |
| | | Tsuruse virus (TSUV) |
| | | Weldona virus (WELV) |
| | *Thimiri orthobunyavirus* | Thimiri virus (THIV) |



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| | | |
|---|---|---|
| | *Timboteua orthobunyavirus* | Timboteua virus (TBTV) |
| | *Trivittatus orthobunyavirus* | Achiote virus (ACHOV) |
| | | Trivittatus virus (TVTV) |
| | *Turlock orthobunyavirus* | Lednice virus (LEDV) |
| | | Turlock virus (TURV) |
| | | Umbre virus (UMBV) |
| | *Utinga orthobunyavirus* | Utinga virus (UTIV) |
| | *Wolkberg orthobunyavirus* | Wolkberg virus (WBV) |
| | *Wyeomyia orthobunyavirus* | Rio Pracupi virus |
| | | Taiassui virus (TAIAV) |
| | | Tucunduba virus (TUCV) |
| | | Wyeomyia virus (WYOV) |
| | *Zegla orthobunyavirus* | Zegla virus (ZEGV) |
| *Pacuvirus* | *Pacui pacuvirus** | Pacui virus (PACV) |
| | *Rio Preto da Eva pacuvirus* | Rio Preto da Eva virus (RPEV) |



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| | | |
|---|---|---|
| | *Tapirape pacuvirus* | Tapirapé virus (TAPV) |
| *Shangavirus* | *Insect shangavirus* | Shuāngào insect virus 1 (SgIV-1) |
| **Family *Phasmaviridae*** | | |
| *Feravirus* | *Ferak feravirus** | Ferak virus (FRKV) |
| *Inshuvirus* | *Insect inshuvirus** | Shuāngào insect virus 2 (SgIV-2) |
| *Jonvirus* | *Jonchet jonvirus** | jonchet virus (JONV) |
| *Orthophasmavirus* | *Culex orthophasmavirus* | Culex orthophasmavirus (CPLV) |
| | *Ganda orthophasmavirus* | Ganda orthophasmavirus (GBEEV) |
| | *Kigluaik phantom orthophasmavirus** | Kigluaik phantom virus (KIGV) |
| | *Nome phantom orthophasmavirus* | Nome phantom virus (NOMV) |
| | *Odonate orthophasmavirus* | Odonate orthophasmavirus (HbOV-8) |
| | *Qingling orthophasmavirus* | Qingling orthophasmavirus (HbOV-9) |
| | *Seattle orthophasmavirus* | Seattle orthophasmavirus (SEPV) |
| | *Wuchang cockroach orthophasmavirus 1* | Wǔchāng cockroach virus 1 (WcCV-1) |
| | *Wuhan mosquito orthophasmavirus 1* | Wǔhàn mosquito virus 1 (WhMV-1) |



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| | | |
|---|---|---|
| | *Wuhan mosquito orthophasmavirus 2* | Wǔhàn mosquito virus 2 (WhMV-2) |
| *Sawastrivirus* | *Sanxia sawastrivirus** | Sānxiá water strider virus 2 (SxWSV-2) |
| *Wuhivirus* | *Insect wuhivirus** | Wǔhàn insect virus 2 (WhIV-2) |
| **Family *Phenuiviridae*** | | |
| *Banyangvirus* | *Guertu banyangvirus* | Guertu virus (GTV) |
| | *Heartland banyangvirus* | Heartland virus (HRTV) |
| | *Huaiyangshan banyangvirus** | severe fever with thrombocytopenia syndrome virus (SFTSV) |
| *Beidivirus* | *Dipteran beidivirus** | Húběi diptera virus 3 (HbDV-3) |
| *Goukovirus* | *Cumuto goukovirus* | Cumuto virus (CUMV) |
| | *Gouleako goukovirus** | Gouléako virus (GOLV) |
| | *Yichang insect goukovirus* | Yíchāng insect virus (YcIV) |
| *Horwuvirus* | *Horsefly horwuvirus** | Wǔhàn horsefly virus (WhHV) |
| *Hudivirus* | *Dipteran hudivirus** | Húběi diptera virus 4 (HbDV-4) |
| *Hudovirus* | *Lepidopteran hudovirus** | Húběi lepidoptera virus 1 (HbLV-1) |



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| | | |
|---|---|---|
| *Kabutovirus* | *Huangpi kabutovirus** | Huángpí tick virus 2 (HpTV-2) |
| | *Kabuto mountain kabutovirus* | Kabuto mountain virus (KAMV) |
| *Laulavirus* | *Laurel Lake laulavirus** | Laurel Lake virus (LLV) |
| *Mobuvirus* | *Mothra mobuvirus** | Mothra virus (MTHV) |
| *Phasivirus* | *Badu phasivirus** | Badu virus (BADUV) |
| | *Phasi Charoen-like phasivirus* | Phasi Chaeron-like virus (PCLV) |
| | *Wutai mosquito phasivirus* | Wǔtái mosquito virus (WtMV) |
| *Phlebovirus* | *Bujaru phlebovirus* | Bujaru virus (BUJV) |
| | | Munguba virus (MUNV) |
| | *Candiru phlebovirus* | Alenquer virus (ALEV) |
| | | Ariquemes virus (ARQV) |
| | | Candirú virus (CDUV) |
| | | Itaituba virus (ITAV) |
| | | Jacundá virus (JCNV) |
| | | Maldonado virus (MLOV) |



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| | | |
|---|---|---|
| | | Morumbi virus (MR(M)BV) |
| | | Mucura virus (MCRV/MRAV) |
| | | Nique virus (NIQV) |
| | | Oriximiná virus (ORXV) |
| | | Serra Norte virus (SRNV) |
| | | Turuna virus (TUAV) |
| | *Chilibre phlebovirus* | Cacao virus (CACV) |
| | | Chilibre virus (CHIV) |
| | *Frijoles phlebovirus* | Frijoles virus (FRIV) |
| | | Joá virus (JOAV) |
| | *Mukawa phlebovirus* | Mukawa virus (MKWV) |
| | *Punta Toro phlebovirus* | Buenaventura virus (BUEV) |
| | | Campana virus (CMAV) |
| | | Capira virus (CAPIV) |
| | | Coclé virus (CCLV) |



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| | | |
|---|---|---|
| | | Leticia virus (LTCV) |
| | | Punta Toro virus (PTV) |
| | *Rift Valley fever phlebovirus** | Rift Valley fever virus (RVFV) |
| | *Salehabad phlebovirus* | Adana virus (ADAV) |
| | | Adria virus (ADRV) |
| | | Alcube virus |
| | | Arbia virus (ARBV) |
| | | Arumowot virus (AMTV) |
| | | Bregalaka virus (BREV) |
| | | Medjerda Valley virus (MVV) |
| | | Odrénisrou virus (ODRV) |
| | | Olbia virus (OLBV) |
| | | Salehabad virus (SALV) |
| | | Zaba virus (ZABAV) |
| | *Sandfly fever Naples phlebovirus* | Arrábida virus (ARRV) |



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| | | |
|---|---|---|
| | | Balkan virus (BALKV) |
| | | Fermo virus (FERV) |
| | | Gordil virus (GORV) |
| | | Granada virus (GRV = GRAV) |
| | | Massilia virus (MASV) |
| | | Punique virus (PUNV) |
| | | Saddaguia virus (SADV) |
| | | Saint-Floris virus (SAFV) |
| | | sandfly fever Naples virus (SFNV) |
| | | Tehran virus (THEV) |
| | | Toscana virus (TOSV) |
| | | Zerdali virus (ZERV) |
| | *Uukuniemi phlebovirus* | Chizé virus (CHZV) |
| | | EgAN 1825-61 virus (EGAV) |
| | | Fin V 707 virus (FINV) |



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| | | |
|---|---|---|
| | | Oceanside virus (OCV = OCEV) |
| | | Pontevès virus (PTVV) |
| | | St. Abbs Head virus (SAHV) |
| | | Uukuniemi virus (UUKV) |
| | | Zaliv Terpenyia virus (ZTV) |
| *Pidchovirus* | *Pidgey pidchovirus** | Pidgey virus (PGYV) |
| *Tenuivirus* | *Echinochloa hoja blanca tenuivirus* | Echinochloa hoja blanca virus (EHBV) |
| | *Iranian wheat stripe tenuivirus* | Iranian wheat stripe virus (IWSV) |
| | *Maize stripe tenuivirus* | maize stripe virus (MStV = MSpV) |
| | *Rice grassy stunt tenuivirus* | rice grassy stunt virus (RGSV) |
| | *Rice hoja blanca tenuivirus* | rice hoja blanca virus (RHBV) |
| | *Rice stripe tenuivirus** | rice stripe virus (RSV = RStV) |
| | *Urochloa hoja blanca tenuivirus* | Urochloa hoja blanca virus (UHBV) |
| *Wenrivirus* | *Shrimp wenrivirus** | Wēnzhōu shrimp virus 1 WzSV-1 |
| *Wubeivirus* | *Dipteran wubeivirus** | Húběi diptera virus 5 (HbDV-5) |



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| | | |
|---|---|---|
| | *Fly wubeivirus* | Wǔhàn fly virus 1 (WhFV-1) |
| **Family *Tospoviridae*** | | |
| *Tospovirus* | *Bean necrotic mosaic orthotospovirus* | bean necrotic mosaic virus (BeNMV) |
| | *Calla lily chlorotic spot orthotospovirus* | calla lily chlorotic spot virus (CCSV) |
| | *Capsicum chlorosis orthotospovirus* | Capsicum chlorosis virus (CaCV) |
| | *Chrysanthemum stem necrosis orthotospovirus* | Chrysanthemum stem necrosis virus (CSNV) |
| | *Groundnut bud necrosis tospovirus* | groundnut bud necrosis virus (GBNV)[10] |
| | *Groundnut ringspot tospovirus* | groundnut ringspot virus (GRSV) |
| | *Groundnut yellow spot tospovirus* | groundnut yellow spot virus (GYSV)[11] |
| | *Impatiens necrotic spot tospovirus* | impatiens necrotic spot virus (INSV) |
| | *Iris yellow spot tospovirus* | iris yellow spot virus (IYSV) |
| | *Melon severe mosaic orthotospovirus* | melon severe mosaic virus (MSMV) |
| | *Melon yellow spot orthotospovirus* | melon yellow spot virus (MYSV) |
| | *Polygonum ringspot tospovirus* | Polygonum ringspot virus (PolRSV) |



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| | | |
|---|---|---|
| | *Soybean vein necrosis orthotospovirus* | soybean vein necrosis virus (SVNV) |
| | *Tomato chlorotic spot tospovirus* | tomato chlorotic spot virus (TCSV) |
| | *Tomato spotted wilt tospovirus** | tomato spotted wilt virus (TSWV) |
| | *Watermelon bud necrosis tospovirus* | watermelon bud necrosis virus (WBNV) |
| | *Watermelon silver mottle tospovirus* | watermelon silver mottle virus (WSMoV) |
| | *Zucchini lethal chlorosis tospovirus* | zucchini lethal chlorosis virus (ZLCV) |
| **Family *Wupedeviridae*** | | |
| *Wumivirus* | *Millipede wumivirus** | Wǔhàn millipede virus 2 (WhMV-2) |
| **Unassigned** | | |
| *Coguvirus* | *Citrus coguvirus** | citrus concave gum-associated virus (CCGaV) |

*type species

[1]Synonyms: Cardamones virus, Hǎinán Medical University virus, Xīngyì virus; [2]Synonyms: Amga virus (MGAB), Artybash virus (ARTV); [3]synonym: Jurong virus; [4]includes the strain previously referred to as Tunis virus (TUNV); [5]includes the strain previously referred to as soft tick bunyavirus (STBV); [6]includes the strain previously referred to as Ganjam virus (GANV); [7]synonyms: Čalovo virus (CVOV), Chittoor virus (CHITV), Olkya virus, Olyka virus, UgMP-6830 virus; [8]includes the strain previously referred to as



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Garissa virus; [9]also mistakenly referred to in the literature as Chantanga virus (CHATV) and Chatanga virus (CHATV); [10]synonym: peanut bud necrosis virus (PDNV); [11]synonym: peanut yellow spot virus (PYSV).

¶Please note that viruses are real objects that are assigned to concepts that are called taxa. Species, genera, families, and orders are taxa. Taxon names are always italicized and always begin with a capital letter. Virus names, on the other hand, are not italicized and are not capitalized, except if the name or a name component is a proper noun. This column lists the virus names with their correct (lack of) capitalization. Lists of viruses within a given species are provisional at this point and will likely be amended in the near future.



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## COMPLIANCE WITH ETHICAL STANDARDS

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## Conflict of Interest

The authors declare no conflicts of interest.

## Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

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# Genetic and biochemical characterizations of Zika virus NS2A protein

Xianwen Zhang, Xuping Xie, Jing Zou, Hongjie Xia, Chao Shan, Xinwen Chen, and Pei-Yong Shi

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# Genetic and biochemical characterizations of Zika virus NS2A protein

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**ABSTRACT**

Zika virus (ZIKV) can cause devastating congenital Zika syndromes in pregnant women and Guillain-Barre syndrome in adults. Understanding the molecular mechanism of ZIKV replication is essential for antiviral and vaccine development. Here we report the structural and functional characterization of ZIKV NS2A protein. Biochemical structural probing suggests that ZIKV NS2A has a single segment that traverses the ER membrane and six segments that peripherally associate with the ER membrane. Functional analysis has defined distinct NS2A residues essential for viral RNA synthesis or virion assembly. Only the virion assembly-defective mutants, but not the RNA synthesis-defective mutants, could be rescued through *trans* complementation with a wide-type NS2A protein. These results suggest that the NS2A molecules in virion assembly complex could be recruited in *trans*, whereas the NS2A molecules in viral replication complex must be recruited in *cis*. Together with previous results, we propose a flavivirus assembly model where NS2A plays a central role in modulating viral structural and nonstructural proteins as well as genomic RNA during virion assembly.

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**KEYWORDS** Zika virus; flavivirus replication; virion assembly; flavivirus NS2A; membrane topology

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## Introduction

Zika virus (ZIKV) is a recently emerged mosquito-borne flavivirus that is primarily transmitted by *Aedes spp.* mosquitoes. It can also be transmitted through sexual, maternal-to-fetal, and blood transfusion routes [1–5]. ZIKV was first identified in 1947 from a sentinel rhesus monkey in Uganda [6]. Since 2007, it emerged in the Pacific region, South America, Central America, and the Caribbean, posing a global public health threat [7–11]. ZIKV infection causes mild illnesses, including fever, pruritis, arthritis, conjunctivitis, and asthenia [12]. Importantly, it can cause devastating congenital Zika syndromes (CZS; including microcephaly, congenital malformation, and fetal demise) in pregnant women and Guillain-Barre syndrome (GBS) in adults [13–16]. The explosive epidemics and its association with CZS had led the World Health Organization (WTO) to declare ZIKV as a Public Health Emergency of International Concern from February to November in 2016 [17]. Since then, a number of vaccine candidates have been developed, some of which have already advanced to clinical trials [18]. In addition, small molecule inhibitors and therapeutic antibodies have also been identified for antiviral development [19–22].

ZIKV belongs to the *Flavivirus* genus within the *Flaviviridae* family. Besides ZIKV, many other flaviviruses are significant human pathogens, including dengue (DENV), yellow fever (YFV), West Nile (WNV), Japanese encephalitis (JEV), Saint Louis encephalitis (SLEV), and tick-borne encephalitis virus (TBEV) [23]. Flavivirus genome is a positive-sense, single-stranded RNA of ~11,000 nucleotides. It contains a 5' untranslated region (UTR), a long open-reading-frame (ORF), and a 3' UTR. The single ORF encodes

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a polyprotein that is processed into three structure proteins (capsid [C], pre-membrane [prM] and envelope [E]) and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5). The structural proteins, together with genomic RNA, are components of virions. The nonstructural proteins participate in viral RNA replication, virion assembly, and evasion of host immune response [24–26].

Flavivirus NS2A is an ER-resident, transmembrane protein with a calculated molecular weight of ~22 kDa. The N-terminus and C-terminus of NS2A are formed by cleavages with an unknown host protease and viral protease NS3 (in complex with NS2B), respectively [27,28]. Flavivirus NS2A has multiple functions: (i) Viral replication. Kunjin virus (KUNV) NS2A co-localizes with viral double-stranded RNA and interacts with the 3'UTR of genomic RNA, NS3, and NS5 protein [29]. Mutagenesis studies confirmed the role of NS2A in viral RNA synthesis [30,31]. (ii) Virion assembly/secretion. Mutagenesis analyses of YFV, KUNV, and DENV have identified distinct NS2A amino acids that are essential for virion assembly/secretion [30–34]. The virion assembly defects can be restored by second site mutations in NS2B or NS3 in YFV and DENV [32,35,36]. (iii) Evasion of innate immunity. ZIKV NS2A inhibits type-I IFN induction at the step of TBK1 [26]. DENV NS2A antagonizes type-I IFN signalling [25]. JEV NS2A blocks dsRNA-activated protein kinase PKR [37]. In WNV and KUNV, the NS2A-mediated inhibition of type-I IFN induction can be abrogated by specific NS2A mutations [38,39]. (iv) Disease pathogenesis. WNV NS2A modulates virus-induced cytopathic effect (CPE) and pathogenesis in mice [40,41]. ZIKV NS2A impairs mammalian cortical neurogenesis through depleting adherens junction proteins [42].

The structure and function of ZIKV NS2A have not been characterized. Although NS2A proteins from different flaviviruses perform similar functions (described above), each flavivirus may exert its NS2A functions in a virus-specific manner [35]. The goals of this study are (i) to define the membrane topology of ZIKV NS2A and (ii) to characterize its roles in viral RNA synthesis and virion assembly.

## Materials and methods

### Cell lines

Vero cells, baby hamster kidney (BHK-21) cells and human embryo kidney 293 T cells were cultured at 37°C with 5% CO2 in high-glucose Dulbecco's modified Eagle medium (DMEM; Life Technologies) supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 g/ml streptomycin and 10% fetal bovine serum (FBS; HyClone Laboratories).

### Bioinformatics

NS2A protein sequences from ZIKV, DENV1, DENV2, DENV3, DENV4, WNV, JEV, YFV, SLEV were aligned using CLC Main Workbench software (CLC Bio). Prediction of ZIKV NS2A was carried out using various web servers, which including TOPCONS, Philius, Polyphobius, SCAMPI, and TMHMM2. The amphipathic helices of ZIKV NS2A were predicted by HeliQuest.

### Antibody

The following antibodies were used in this study: a mouse monoclonal antibody 4G2 cross-reactive with flavivirus E protein (American Type Culture Collection); Rabbit anti-HA monoclonal antibody (Cell Signaling Technology); Alexa Fluor®488 conjugated mouse anti-HA monoclonal antibody (Cell Signaling Technology); Rabbit anti-Calnexin polyclonal antibody(Abcam); Mouse anti-GFP monoclonal antibody (Sigma-Aldrich); Horseradish peroxidase(HRP) conjugated goat anti-mouse and anti-rabbit IgG polyclonal antibodies (Sigma-Aldrich); Alexa Fluor®488 conjugated goat anti-mouse IgG polyclonal antibody (Thermo Fisher Scientific); Alexa Fluor®568 conjugated goat anti-rabbit IgG polyclonal antibody (Thermo Fisher Scientific).

### Plasmid construction

The [redacted]ctious cDNA clone (pFLZIKV) bearing the full-length genome of ZIKV (strain FSS13025) or Renilla luciferase (Rluc) reporter ZIKV replicon (pZIKV Rep WT) were used as the backbone for introducing NS2A mutations [43,44]. XhoI and KpnI restriction sites were initially generated into pFLZIKV and pZIKV Rep WT replicon at positions 3018 and 5010 in ZIKV genome without changing amino acid sequences. The NS2A alanine-scanning mutagenesis was performed by using a QuikChange II XL Site-Directed Mutagenesis kit (Agilent Technologies) as described previously [30].

A mammalian expression vector pXJ with a cytomegalovirus (CMV) promoter was used for constructing plasmids expressing NS2A and its derivatives. To construct the pXJ-$E_{24}$-NS1-NS2A-HA plasmid, a gene cassette encoding the last 24 amino acids of E protein ($E_{24}$), NS1 and NS2A with a C-terminal hemagglutinin (HA) tag was firstly amplified from the pFLZIKV and cloned into the pXJ vector. Next, 35[redacted]2A mutations were individually introduced into the pXJ-$E_{24}$-NS1-NS2A-HA by overlap PCR, resulting in plasmids expressing NS2A mutants.

To construct the pXJ-SPG-$C_{16}$-NS2A-HA that was used for selecting cell line constitutively expressing NS2A protein, a DNA fragment containing the Gaussia luciferase signal peptide (SPG), the last 16 amino acids

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of NS1 protein ($C_{16}$) and NS2A tagged with a C-terminal HA tag sequence were constructed by overlap PCR and cloned into the pXJ vector via NotI/XhoI restriction sites.

Plasmid pXJ-eGFP was constructed by inserting the enhanced green fluorescence protein (eGFP) sequence into the pXJ vector via BamHI/XhoI restriction sites. DNA fragments that encode different NS2A segments were individually inserted into above pXJ-eGFP vector, resulting various pXJ-smNS2A-eGFP plasmids (smNS2A represents NS2A segment). A DNA fragment that express NS2A or truncated NS2A and an eGFP with a C-terminal N-glycosylation acceptor site (Glyc) were amplified and cloned into the pXJ-SPG-$C_{16}$-NS2A-HA vector, resulting in the plasmid pXJ-SPG-$C_{16}$-NS2A-eGFP-Glyc and various pXJ-SPG-$C_{16}$-ctNS2A-eGFP-Glyc plasmids (ctNS2A represents C-terminal truncated NS2A).

### RNA in vitro transcription and electroporation

All full-length cDNA clone plasmids and subgenomic replicons of ZIKV were linearized with restriction enzyme ClaI, followed by phenol-chloroform extraction and ethanol precipitation. RNA transcripts were prepared by using the T7 mMessage mMachine kit (Ambion). For RNA transfection, 10 μg *in vitro* transcribed RNA was electroporated into $8 \times 10^6$ cells (BHK-21 or Vero) by using Gene Pulser Xcell™ Electroporation Systems (Bio-rad) as described previously [30,43].

### Immunofluorescence assay

For indirect immunofluorescence assay (IFA), cells were seeded in an 8-well Nunc Lab-Tek II Chamber Slide (Thermo Fisher Scientific). At various time points, cells were washed three times with phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde in PBS at room temperature for 30 min and permeabilized with 0.1% Triton X-100 in PBS at room temperature for 10 min. The permeabilized cells were then washed three times with PBS and incubated in a blocking buffer containing 1% FBS in PBS at room temperature for 1 h. E protein was stained with a 1:1000 dilution of mouse monoclonal antibody 4G2 in blocking buffer. After incubation for 1 h, the cells were washed three times with a washing buffer (1% FBS and 0.5% Tween 20 in PBS). After 1 h of incubation with a secondary antibody (Alexa Fluor®488 conjugated goat anti-mouse IgG), the cells were washed three times with washing buffer and counterstained with DAPI (4',6-diamidino-2-phenylindole). Images were acquired using an Olympus fluorescence microscope. Images were processed in ImageJ software (National Institutes of Health, MD).

### Plaque assay

$2 \times 10^5$ Vero cells per well were grown in 24-well plates one day prior to viral infection. 10-fold serial dilutions of virus samples were prepared in DMEM containing 2% FBS and 1% penicillin/streptomycin and inoculated (100 μl) into each well of 24-well plates. After 1 h of incubation at 37°C with 5% $CO_2$, the inoculum was replaced with 500 μl overlay medium (DMEM medium containing 0.8% methyl-cellulose, 2% FBS and 1% penicillin/streptomycin). Plaques formed after incubation at 37°C with 5% $CO_2$ for 4–5 days. Cells were fixed with 4% paraformaldehyde for 30 min, followed by staining with 1% crystal violet. The numbers of plaque per millilitre (PFU/ml) of ZIKV were calculated.

### Replicon transient-transfection assay

Briefly, $8 \times 10^6$ Vero cells were electroporated with 10 μg wild type or mutant Rluc-ZIKV replicon RNAs. After electroporation, $3 \times 10^5$ cells per well were seeded in 12-well plates. At given time points, the cells were washed twice with PBS and lysed in 200 μl of luciferase lysis buffer (Promega). The luciferase activity assay was performed according to the manufacturer's protocol.

### RNA extraction and cDNA sequencing

The culture fluids from infected cells were centrifuged at 1000 g for 10 min and then filtered through a 0.22 μm pore size polyethersulfone membrane (Millipore) to remove cellular debris. Viral RNAs in the supernatants were extracted using a QIAamp viral-RNA Minikit (Qiagen) by following the manufacturer's protocol. Reverse transcription PCR (RT-PCR) was performed by using the SuperScript® III One-Step RT-PCR System (Thermo Fisher Scientific). The amplified cDNA fragments were purified and sent for Sanger sequencing at the GENEWIZ facilities.

### Selection of NS2A-HA BHK-21 cell line

$4 \times 10^5$ BHK-21 cells per well of [illegible]re seeded into a 6-well plate. The plasmid pXJ-SPG-$C_{16}$-NS2A-HA that contains a neomycin resistance gene was transfected into BHK-21 cells (4 μg per well) using X-tremeGENE 9 DNA transfection reagent (Roche). At 24 h post-transfection, geneticin (G418) was added to the culture medium with a concentration of 1 mg/ml. The medium was changed every three days. After two weeks, several neomycin-resistant individual colonies were selected for propagation. The expression of NS2A-HA protein in stable cell lines was analyzed by immunofluorescence assay and Western blot.

### In vitro proteinase K protection assay

Proteinase K protection assay was performed as previously described with minor modifications [45,46].

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BHK-21 cells were transfected in 6-well plates with various pXJ-SPG-$C_{16}$-ctNS2A-eGFP-Glyc plasmids. After incubated at 30°C for 24 h, cells were trypsinized, washed twice with PBS and centrifuged at 450 × g for 5 min. The cell pellets were resuspended in a hypotonic lysis buffer (10 mM Tris-HCl, pH 7.5, and 2 mM $MgCl_2$) and kept on ice for 10 min. Subsequently, the samples were homogenized with 40 strokes and centrifuged at 1000 × g for 5 min. The postnuclear supernatants (PNS) were divided into three aliquots and centrifuged at 15,000 × rpm for 40 min. The pellets from three aliquots were then resuspended in PBS, 60 μg/ml proteinase K in PBS, or 60 μg/ml proteinase K in PBS in the presence of 0.5% Triton X-100, respectively. The samples were incubated on ice for 90 min. 20 mM phenylmethylsulfonyl fluoride (PMSF) was finally added to terminate the reaction. After further incubation on ice for 15 min, the samples were mixed with 4× Laemmli sample buffer, heated at 95°C for 15 min and analyzed by Western blotting.

### *In vitro deglycosylation assay*

The samples were prepared by following a previously described protocol [46,47]. Briefly, the transfected BHK-21 cells in 6-well plates were incubated at 30°C for 24 h. Cells were trypsinized and suspended in DMEM medium containing 10%FBS. The cells were pelleted by centrifuging at 450 × g for 5 min. After two PBS washes, the cell pellets were resuspended in a solubilization buffer (20 mM Tris-HCl, pH 7.5, 50 mM NaCl, 10 mM $Mg(CH_3COO)_2$, 1% Triton X-100, and EDTA-free protease inhibitors [Roche]) and incubated at 4°C for 2 h with gentle agitation. After centrifugation at 15,000 rpm 4°C for 20 min to remove cell debris, the supernatants were split into two aliquots. 50,000 units/ml N-glycosidase F (PNGase F) or PBS (no-treatment control) were added to those two aliquots. After incubation at 37°C for 2 h, the samples were mixed with 4× Laemmli sample buffer, heated at 70°C for 15 min and analyzed by Western blot.

### *SDS-PAGE and western blot*

Protein samples were separated by 12% SDS-polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride (PVDF) membrane using Trans-Blot Turbo transfer system (Bio-Rad), after which the membrane was blocked in TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 0.1% Tween 20) supplemented with 5% nonfat milk for 1 h at room temperature. The blots were probed with primary antibodies (1:1,000 dilution) overnight at 4°C, washed three times with TBST and incubated with horseradish peroxidase (HRP) conjugated goat anti-mouse and anti-rabbit IgG polyclonal antibodies (1:10,000 dilution) for 1 h. After three thorough TBST buffer washes, the blots were incubated with SuperSignal™ West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific). Chemiluminescence signals were detected in ChemiDoc System (Bio-Rad).

### *Quantification and statistical analysis*

IFA images processing and cell counting were performed in software ImageJ (NIH). Densitometry analysis was performed using Image lab version 6.0 (Bio-Rad Laboratories, Hercules, CA). All numerical data are presented as the mean ± SEM (standard error of mean). The size of each study or number of replicates, along with the statistical tests performed can be found in corresponding Figure Legends.

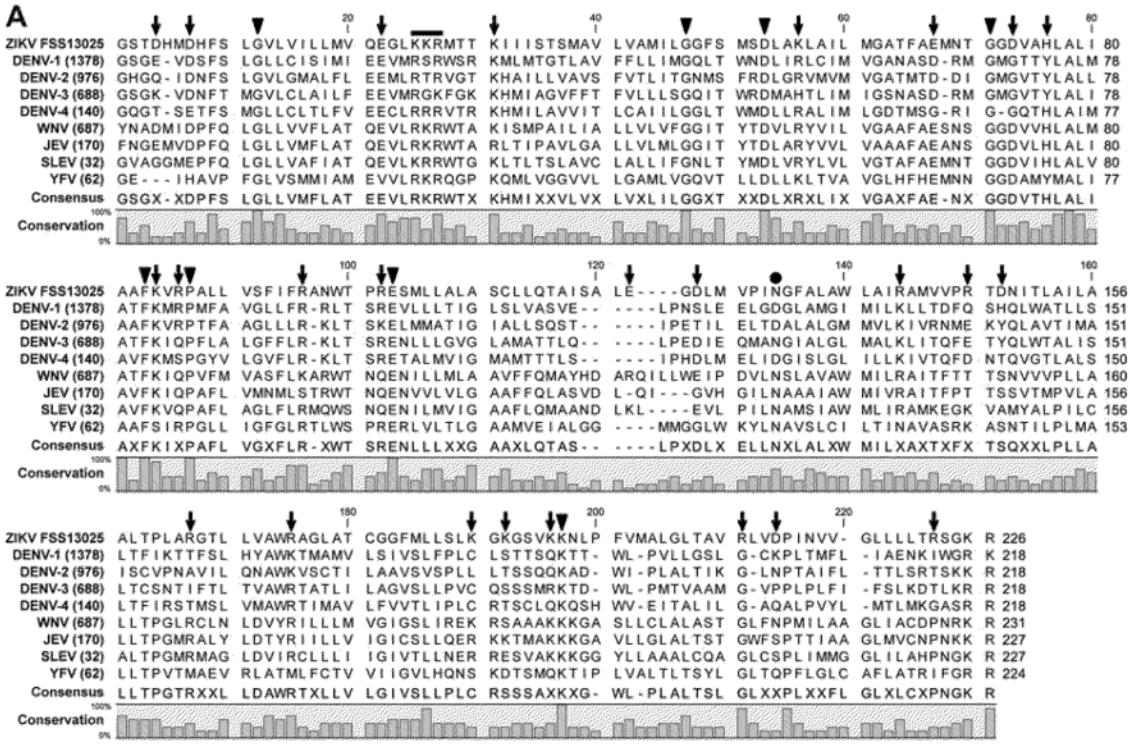
## Results

### *The rationale for ZIKV NS2A mutagenesis*

To identify critical residues in ZIKV NS2A, we performed a systematic alanine-scanning mutagenesis using an infectious cDNA clone of ZIKV strain FSS13025 [43]. Sequence alignment of NS2A proteins from ZIKV, DENV, WNV, JEV, SLEV, and YFV revealed a high diversity: only 31 out of 218–227 amino acids with a consensus of >80% among different flaviviruses (Figure 1A). A total of 35 residues were selected for mutagenesis: (i) All 8 identical non-alanine residues, including G12, G47, D53, G71, F83, P87, E103, and K193. The amino acid numbering is based on the ZIKV sequence. (ii) All charged residues, including D4, D7, E22, K31, K56, E67, D73, H76, K84, R86, R96, R102, E122, D124, R140, R146, D148, R163, R171, K186, K188, K192, R207, D210, and R222. Charged residues were selected because they likely participate in inter- or intra-molecular interactions; alternatively, they may alter the locations where NS2A would traverse the ER membrane since charged residues are hydrophilic. In addition, NS2A D73H mutation was previously shown to attenuate WNV replication [48]. (iii) A K25/K26/R27 motif that was previously reported to be critical for YFV and DENV virion assembly [35,40]. (iv) Residue N130 that was previously shown to be required for NS1-NS2A cleavage and viral replication in DENV [27,30]. The selected residues were mutated to alanine in the context of ZIKV genomic RNA. Equal amounts of wild-type (WT) and mutant RNAs were electroporated into Vero cells. After electroporation, RNA replication and virus spread were monitored by immunofluorescence assay (IFA); the production of infectious viruses was quantified by plaque assay and RT-PCR. The 35 mutants exhibited distinct phenotypes that could be categorized into five groups (Figure 1B), as detailed in the following sections.

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**B**

| Class | NS2A Mutations | Phenotypes |
|---|---|---|
| I | D4A, E22A, K31A, G47A, D53A, E67A, H76A, E122A, R146A, D148A, R163A, K186A, K188A, D210A | Mutants do not or weakly affect viral production |
| II | K56A , F83A, K84A, R86A, P87A, R96A, R102A, D124A, R207A | Mutants dramatically reduce viral production |
| III | G12A, N130A | Mutants abolish viral RNA synthesis |
| IV | D7A, R140A, R222A | Mutants abolish virus production by affecting RNA synthesis and viron assembly/release |
| V | K25A/K26A/R27A, G71A, D73A, E103A, R171A, K192A, K193A | Mutants abolish virion assembly |

**Figure 1.** ZIKV NS2A mutagenesis. (A) Sequence alignment of NS2A proteins among ZIKV (strain FSS13025), DENV-1-4 serotypes, WNV, JEV, SLEV, and YFV using CLC Main Workbench software (CLC Bio). Thirty-five amino acids were selected for mutagenesis: (i) All 8 identical non-alanine residues (indicated by triangles), (ii) 25 charged residues (arrows), (iii) a K25/K26/R27 motif (line), (iv) residue N130 (circle). The consensus and conservation percentage are indicated below each amino acids. (B) A summary of ZIKV NS2A mutagenesis results. An infectious cDNA clone and a luciferase replicon of ZIKV strain FSS13025 were used to analyze the function of each amino acid. Based on the replication phenotypes, the 35 mutants could be categorized into five classes as indicated.

### *Class I mutations have no or weak effects on viral replication*

Fourteen of the 35 NS2A mutant RNAs, including D4A, E22A, K31A, G47A, D53A, E67A, H76A, E122A, R146A, D148A, R163A, K186A, K188A, and D210A, replicated at levels comparable to the WT RNA. These mutants were categorized as class I (Figure 1B). The IFA images and plaque morphologies of three representatives (D53A, D148A, and R163A), as well as the WT, are shown in Figure 2(A and B), respectively. Most class I mutants (e.g. D148A and R163A) yielded comparable E-expressing cells as the WT from 24 to 48 h post-transfection (p.t.). At 72 h p.t., severe CPE occurred among D148A, R163A, and WT RNA-transfected cells. Some class I mutants (e.g. D53A) produced fewer E-positive cells than the WT at 48 h p.t., and spread to infect more cells at 72 h p.t (Figure 2A). All class I mutants produced viruses with plaques similar to the WT virus (Figure 2B). At 72 h p.t., the class I mutants produced infectious titers <4-fold lower than the WT did (Figure 2C). Sequencing of the mutant viruses (collected at 72 h p.t.) confirmed the retaining of the engineered mutations without other changes (data not shown).

### *Class II mutations significantly attenuate viral replication*

Nine NS2A mutants, including K56A, F83A, K84A, R86A, P87A, R96A, R102A, D124A, and R207A,

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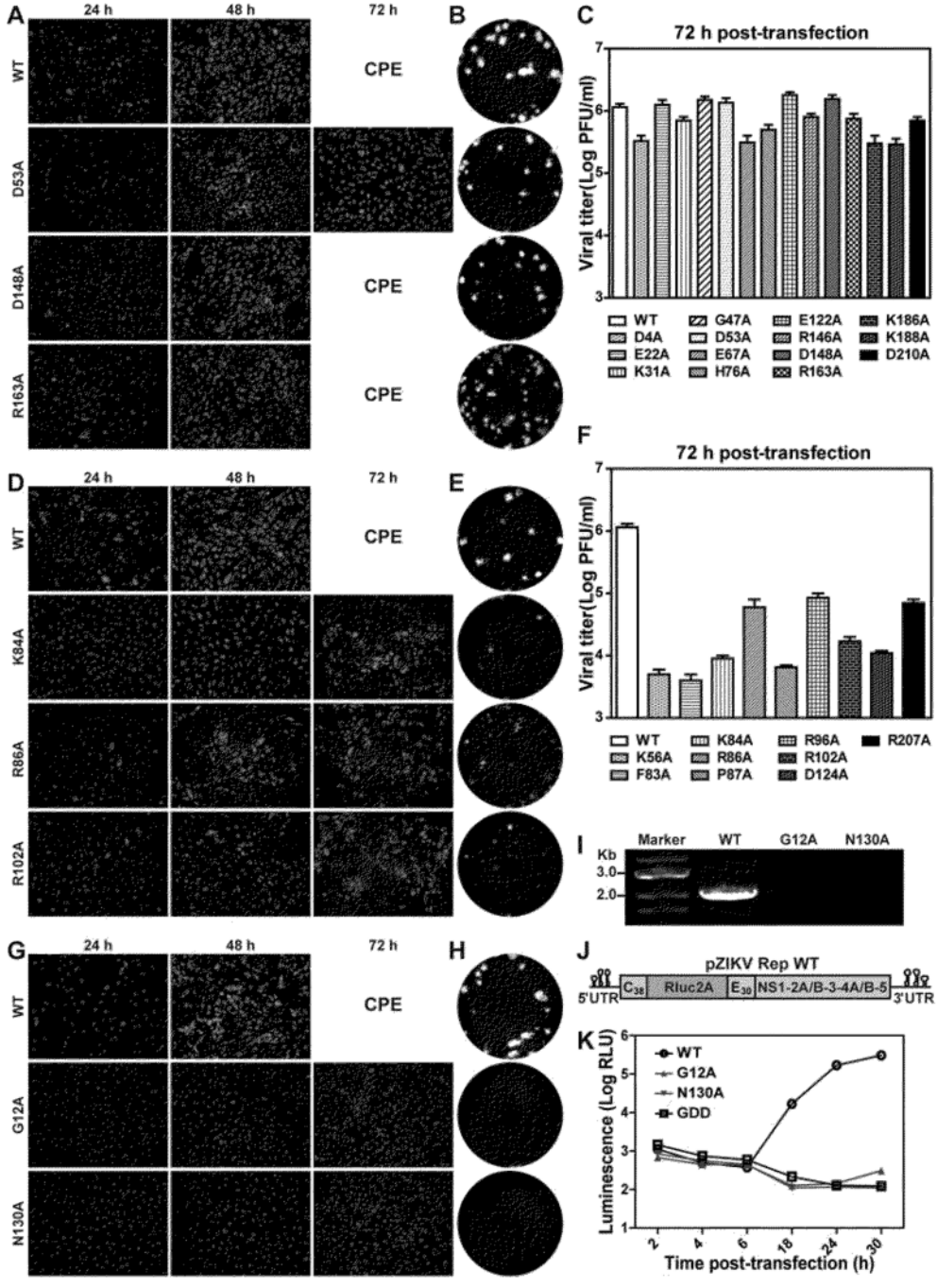



**Figure 2.** Characterization of class I, II, and III NS2A mutants. The results of representative mutants from class I (A–C), II (D–F), and III (G–K) are shown. (A) Vero cells were electroporated with equal amounts of ZIKV wild-type (WT) or class I mutant genomic RNAs. At indicated time points, the cells were fixed and subjected to IFA. Viral E protein and nuclei were stained with antibody 4G2 (green) and DAPI (blue), respectively. Cytopathic effect (CPE) is indicated at 72 h post-transfection. (B) Plaque morphologies. Recombinant viruses were subjected to plaque assay. Plaques were developed at day 4.5 post-infection. (C) Viral titers of class I mutants at 72 h post-transfection. The mean values from two independent experiments are shown. Error bars indicate the standard deviations. The limit of detection (LOD) of plaque assay is 10 PFU/ml. (D) IFA of class II mutant RNA-transfected Vero cells. (E) Plaque morphology of class II mutant viruses. (F) Viral titers of class II mutant viruses at 72 h post-transfection. (G) IFA of class III mutant RNA-transfected Vero cells. (H) Plaque morphologies of class III mutant viruses. Plaque assay was performed using culture supernatants collected at day 3 (for WT) and day 5 (for G12A and N130A) post-transfection. (I) RT-PCR analysis. At 5 day p.t., the mutant RNA-transfected cells were washed twice with PBS to remove residual input RNAs. At day 8 p.t., the supernatants of G12A and N130A RNA-transfected cells were subjected to RT-PCR. The WT virus (collected at 72 h p.t.) was used as a positive control. (J) A ZIKV luciferase replicon. $C_{38}$ and $E_{30}$ represent the first 38 amino acids of C protein and the last 30 amino acids of E protein, respectively. *Renilla* luciferase: Rluc. Foot-and-mouse-disease virus 2A sequence: 2A. (K) Transient replicon assay. WT, G12A, N130A, and NS5ΔGDD replicon RNAs were electroporated into Vero cells. Luciferase activities were measured at indicated time points. An average of 3 experiments are presented. Bars represent standard deviations.

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significantly attenuated viral replication. These mutants were categorized into class II (Figure 1B). Class II RNA-transfected cells produced fewer E-positive cells than the WT did (Figure 2D). At 72 h p.t., only 30–50% of E-positive cells were detected in the class II RNA-transfected cells, whereas the WT RNA-transfected cells exhibited CPE. Class II mutant viruses formed smaller, opaque plaques (Figure 2E), and the transfected cells produced >10-fold lower infectious titers than the WT RNA-transfected cells at 72 h p.t. (Figure 2F). Complete genome sequencing of the class II mutant viruses (collected at 96 h p.t.) did not reveal reversions or any other mutations (data not shown). Because of the weak phenotypes of class I and II mutants, we did not further characterize them.

### *Class III mutations are lethal for viral RNA synthesis*

Class III includes two mutants, G12A and N130A, which were completely defective in viral RNA synthesis (Figure 1B). The G12A and N130A genomic RNA-transfected cells did not produce any E-positive cells at 24 and 48 h p.t. (Figure 2G). On day 5–8 p.t., no infectious viruses were detected from culture medium by plaque assay (Figure 2H) and RT-PCR (Figure 2I). To confirm the above results, we introduced G12A or N130A mutation into a ZIKV luciferase replicon (Figure 2J). A replication-defective replicon, containing an NS5 polymerase active site ΔGDD mutation, was included as a negative control [44]. Vero cells were electroporated with equal amounts of WT, G12A, N130A, or NS5 ΔGDD replicon RNAs. At 2–6 h p.t., all replicons generated similar levels of luciferase signals, suggesting comparable levels of viral RNA translation and transfection efficiencies (Figure 2K). However, at 18–30 h p.t., G12A and N130A replicons generated low luciferase signals as the non-replicative NS5 ΔGDD replicon did, whereas the WT replicon produced increasing luciferase signals (Figure 2K). These results demonstrate that class III mutations completely abolish viral RNA synthesis.

### *Class IV mutations impair both viral RNA synthesis and virion production*

Class IV includes three mutants, including D7A, R140A, and R222A (Figure 1B). These mutant genomic RNAs produced fewer E-positive cells in the transfected cells than the WT RNA (Figure 3A). For D7A or R140A mutants, the RNA-transfected cells did not produce infectious viruses, as suggested by plaque assay (Figure 3B) and RT-PCR (Figure 3C). Incubation of naïve Vero cells with culture supernatants from the D7A or R140A transfected cells did not generate any viral E-positive cells (Figure 3D). Replicon analysis showed that D7A or R140A significantly compromised viral RNA synthesis (Figure 3E). Since neither mutants could produce extracellular viruses (Figure 3B and C), and could replicate at attenuated levels inside cells (Figure 3E), D7A and R140A mutations may impair both viral RNA synthesis and virion production.

For R222A, the number of E-positive cells slightly increased in the genomic RNA-transfected cells from 24 to 72 h p.t. (Figure 3A). A low level of infectious virus was detected from the culture medium by plaque assay (Figure 3B) and RT-PCR (Figure 3C). These data were supported by the positive-IFA results after incubating naive Vero cells with the supernatant from the R222A RNA-transfected cells (Figure 3D). Sequencing the virus (collected at 72 h p.t.) revealed that, besides the engineered R222A mutation, the virus had acquired a second site mutation of L220F in NS2A (Figure 3C). Replicon analysis showed that R222A significantly lowered viral RNA synthesis (Figure 3E); no secondary mutation (i.e. L220F) was detected in the replicon RNA at 30 h p.t. (data not shown). These results suggest that (i) R222A mutant is defective in viral RNA synthesis and/or virion assembly, and (ii) a second site mutation L220F in NS2A could rescue the defects.

### *Class III and IV mutations do not affect NS2A maturation*

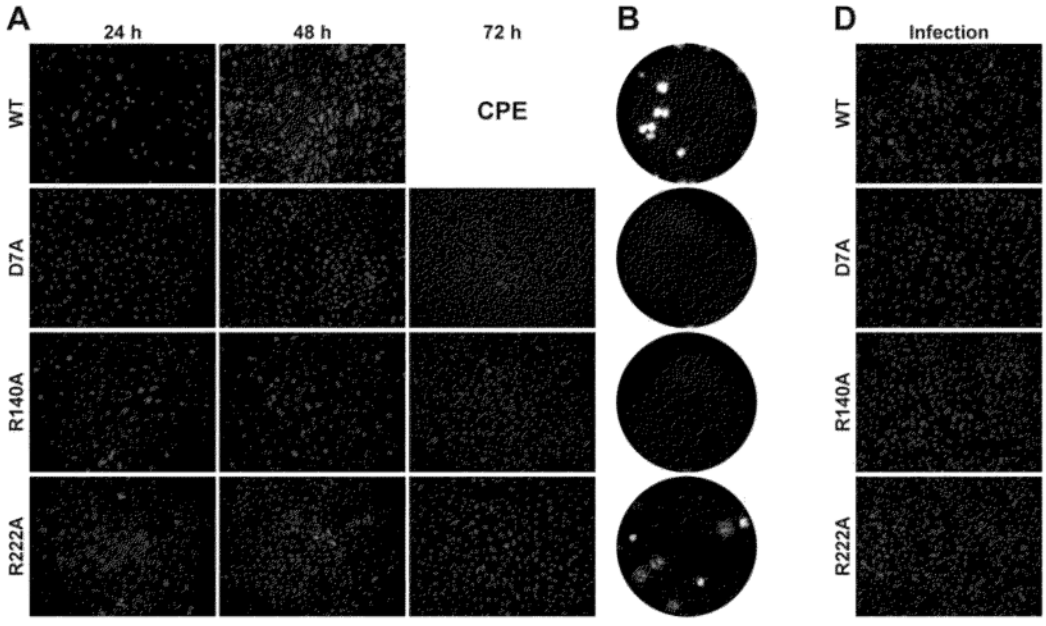
Since cleavage between NS1 and NS2A is essential for flavivirus RNA synthesis [30], we examined whether the replication defects of class III and IV mutants were caused by defective maturation of NS2A. To test this hypothesis, we introduced individual mutations into an expression plasmid pXJ-$E_{24}$-NS1-NS2A-HA that encodes a polyprotein containing the C-terminal 24 amino acids of the E protein (serving as a signal peptide to translocate NS1 to ER lumen), NS1, and NS2A with a C-terminal HA tag. After transfecting the plasmids into 293 T cells, the expression of un-cleaved NS1-NS2A-HA intermediate and cleaved NS2A-HA product were analyzed using Western blot (Figure 3F). None of the mutations significantly affected the cleavage efficiencies of NS1-NS2A-HA to NS2A-HA, indicating that the replication defects of class III and IV mutants are not caused by impaired NS2A cleavage.

### *Class V mutations block virion assembly*

Class V includes G71A, D73A, E103A, R171A, K192A, K193A, and a triple K25A/K26A/R27A. Cells transfected with class V mutant genomic RNAs yielded E-positive cells at 24 h p.t., but the number of E-positive cells did not increase afterward (Figure 4A), suggesting that the mutants are defective in virus spread. No infectious mutant viruses, except E103A, were detected in the supernatants of transfected cells by plaque assay (Figure 4B) or RT-PCR (Figure 4C). No IFA-positive

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C

| | Viral titer (IFU/ml) | | | Extracellular viral RNA RT-PCR | Second site mutation |
|---|---|---|---|---|---|
| | 24 h | 48 h | 72 h | | |
| WT | $(1.70\pm0.30)\times10^5$ | $(4.30\pm0.70)\times10^5$ | $(1.15\pm0.15)\times10^6$ | Positive | None |
| D7A | <10 | <10 | <10 | Negative | N.A. |
| R140A | <10 | <10 | <10 | Negative | N.A. |
| R222A | <10 | 35±15 | 400±100 | Positive | L220F(NS2A) |

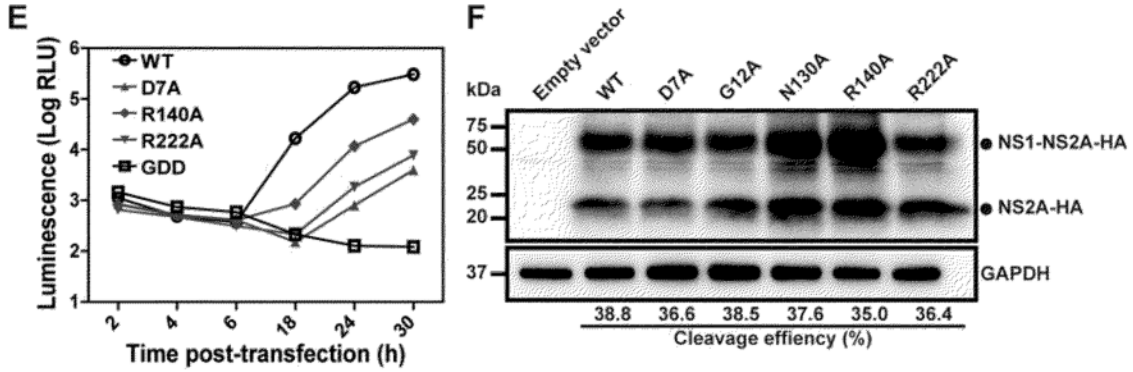
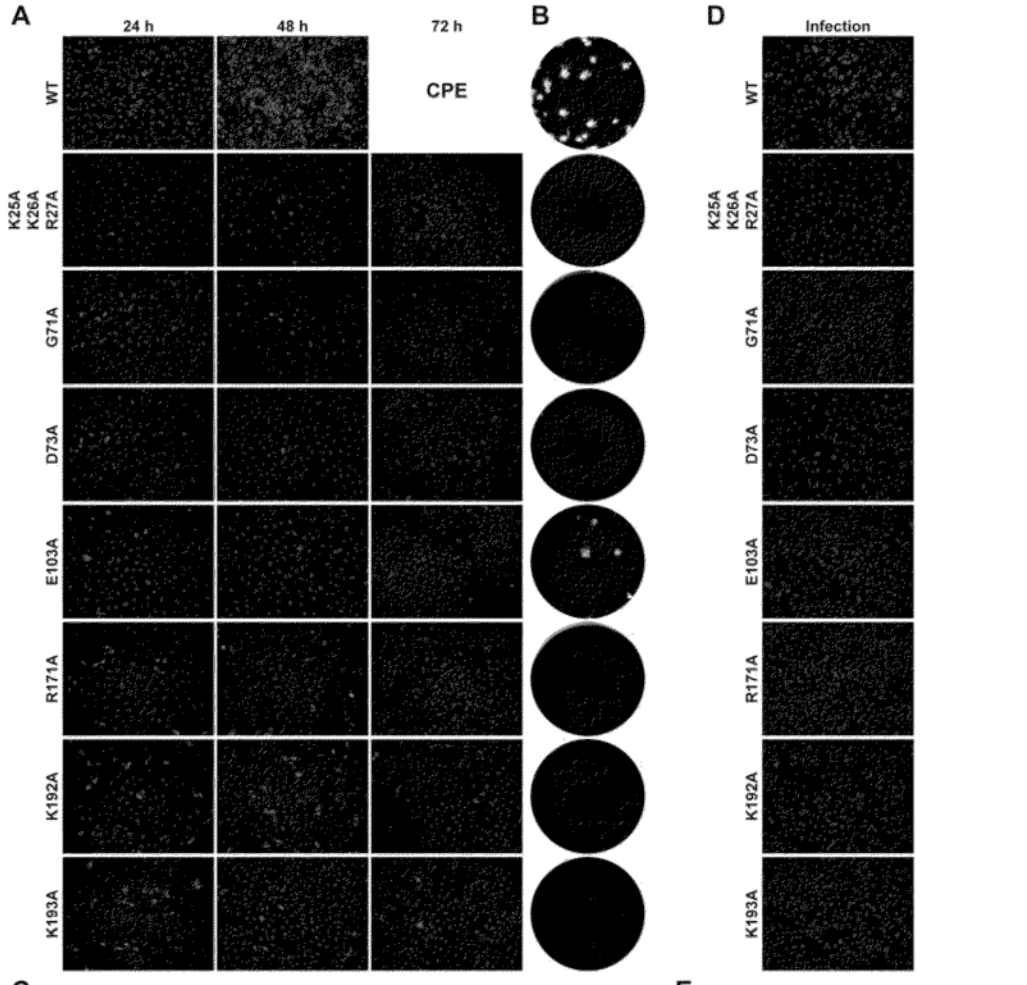



**Figure 3.** Characterization of class IV NS2A mutants. (A) IFA of transfected Vero cells. (B) Plaque morphologies of WT, D7A, R140A, and R222A viruses. (C) Extracellular viral titers were measured at 24, 48, and 72 h p.t. using plaque assay. At 72 h p.t., extracellular viral RNAs were subjected RT-PCR analysis. The RT-PCR products were sequenced to confirm the engineered mutations and to detect potential second site mutation(s) in the NS2A gene. N.A., not applicable. (D) The supernatants of transfected cells were collected on day 8 p.t. to infect naïve Vero cells. At 24 h p.t., the cells were analyzed for viral E expression by IFA. (E) Transient luciferase replicon analysis. Vero cells were electroporated with ZIKV replicon RNAs and measured for luciferase activities at indicated time points. An average of 3 experiments are presented. Bars represent standard deviations. (F) Analysis of NS2A proteolytic processes. NS2A D7A, G12A, N130A, R140A, and R222A mutations were individually engineered into expression plasmid pXJ-$E_{24}$-NS1-NS2A-HA. After transfecting the plasmids into 293 T cells, total cellular protein samples were harvested at 24 h p.t. and analyzed for NS2A process using Western blot. An HA-tag antibody was used to detect both unprocessed NS1-NS2A-HA and processed NS2A-HA proteins.

cells were detected after naïve cells were incubated with the supernatants from transfected cells (Figure 4D). Sequencing of the recovered E103A mutant virus revealed an A103D change in the NS2A gene (Figure 4C), suggesting that an E103A-to-A103D substitution is responsible for the rescue of viral replication.

To determine the stage of viral replication that was disrupted by the class V mutations, we analyzed the mutational effect on viral replication in ZIKV replicon (Figure 4E). Compared with the WT replicon, all class V mutants exhibited equivalent levels of viral translation and RNA synthesis (<4-fold difference),

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| | Viral titer (PFU/ml) | | | Extracellular viral RNA RT-PCR | Second site mutation |
|---|---|---|---|---|---|
| | 24h | 48h | 72h | | |
| Wild Type | $(1.7\pm0.3)\times10^5$ | $(4.3\pm0.7)\times10^5$ | $(1.15\pm0.15)\times10^6$ | Positive | None |
| K25AK26AR27A | <10 | <10 | <10 | Negative | N.A. |
| G71A | <10 | <10 | <10 | Negative | N.A. |
| D73A | <10 | <10 | <10 | Negative | N.A. |
| E103A | <10 | <10 | 175±25 | Positive | A103D(NS2A) |
| R171A | <10 | <10 | <10 | Negative | N.A. |
| K192A | <10 | <10 | <10 | Negative | N.A. |
| K193A | <10 | <10 | <10 | Negative | N.A. |

**Figure 4.** Characterization of class V NS2A mutants. (A) IFA of Vero cells that were transfected with WT or class V genomic RNAs. Viral E-positive cells are indicated in green. (B) Plaque morphologies of class V mutant viruses. Only the WT and E103A mutant generated plaques. (C) Supernatants from the RNA-transfected cells were quantified for viral titers by plaque assay. RT-PCR was also performed on the supernatants at day 3 p.t. for the WT virus and at day 8 p.t. for the mutants. Positive RT-PCR products were sequenced for potential second site mutation(s). N.A., not applicable. (D) Naïve Vero cells were infected with supernatants from the RNA-transfected cells and subjected to IFA for viral E protein expression. (E) Luciferase replicon analysis. Equal amounts of WT and mutant replicon RNAs were electroporated into Vero cells. Luciferase activities were measured at the indicated time points. (F) Analysis of intracellular infectious viruses. Intracellular viruses from ZIKV RNA-transfected cells were harvested at 24 h p.t. as previously described [46]. The harvested samples were used to infect naïve Vero cells. IFA was performed to examine viral E protein expression at 24 h post-infection.

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suggesting that the mutations only marginally affect viral RNA synthesis. Next, we tested if the class V genomic RNAs could generate intracellular infectious viruses. Incubating naïve Vero cells with WT RNA-transfected cell extracts yielded IFA-positive cells, whereas no IFA-positive cells were detected after incubating naive cells with the mutant RNA-transfected cell extracts (Figure 4F). Altogether, the results suggest that class V mutants are competent in viral RNA replication, but defective in assembly of infectious viruses.

### WT NS2A rescues virion assembly for class V mutants

To test *trans* complementation of NS2A mutants, we constructed a BHK-21 cell line that constitutively expressed WT ZIKV NS2A protein with a C-terminal HA-tagged (NS2A-HA). The cell line was constructed by transfecting plasmid pXJ-SPG-$C_{16}$-NS2A-HA encoding a Gaussia luciferase signal peptide (SPG), the C-terminal 16 amino acids of NS1 protein ($C_{16}$), and NS2A-HA, as well as a neomycin resistance gene (*Neo*). The SPG and $C_{16}$ sequences were engineered to enable the correct formation of NS2A membrane topology when expressed in cells. Individual BHK-NS2A-HA cell lines were generated through colony purification under G418 (1 mg/ml) selection. For each BHK-NS2A-HA cell line, all cells were IFA-positive for HA-NS2A expression (Figure 5A). Western blot revealed an expected band of ~22-kDa band (Figure 5B). The expression level of NS2A-HA did not decrease in the BHK-NS2A-HA cells after 20 passages in culture (4 days per passage; Figure 5B), indicating the stability of the cell line.

We tested *trans* complementation by transfecting class V genomic RNAs to BHK-NS2A-HA cells. The transfected cells developed an increasing number of E-positive cells from 48 to 96 h p.t. (Figure 5C). Culture supernatants were collected at 96 h p.t. to infect naïve Vero cells. The infected cells developed E-positive cells for all class V mutants (Figure 5D), demonstrating the rescue of infectious viruses through *trans* complementation. Quantification of the E-positive cells allowed us to determine the titers of rescued infectious viruses (Figure 5E). Among the class V mutants, E103A, K192A, and K193A exhibited the highest *trans* complementation efficiencies, producing $1.9 \times 10^5$–$3.04 \times 10^5$ IFU/ml viruses at 96 h p.i. (Figure 5E). Sequencing of the rescued virion genomes confirmed the retention of the engineered class V mutations without any other changes in NS2A (Figure 5E).

Next, supernatants from the first round of infection collected at 72 h p.i. (from Figure 5D) were used to infect naïve Vero cells for a second round. In contrast to the first round of infection (Figure 5D), no E-positive cells were detected in the second round of infection for any of the class V mutants (Figure 5F). As a positive control, WT RNA produced high numbers of E-positive cells in both the first and second rounds of infections (Figure 5D and F). Collectively, the results indicate that (i) the defect in virion assembly of class V mutants could be rescued through *trans* supply of WT NS2A protein and (ii) the rescued virions could only infect cells for a single round.

### DENV-2 NS2A could not trans complement ZIKV class V mutants

We tested whether DENV NS2A could rescue class V mutants of ZIKV. Each class V mutant of ZIKV was transfected into a previously established BHK-21 cell line that constitutively expresses WT DENV-2 NS2A protein [30]. For all class V mutants, no increase in E-positive cells or infections viruses was detected from the transfected cells from 24 to 96 h p.t. (data not shown). The results suggest that flavivirus NS2A modulates virion assembly in a virus-type specific manner.

### WT NS2A could not rescue viral RNA synthesis of class III mutants

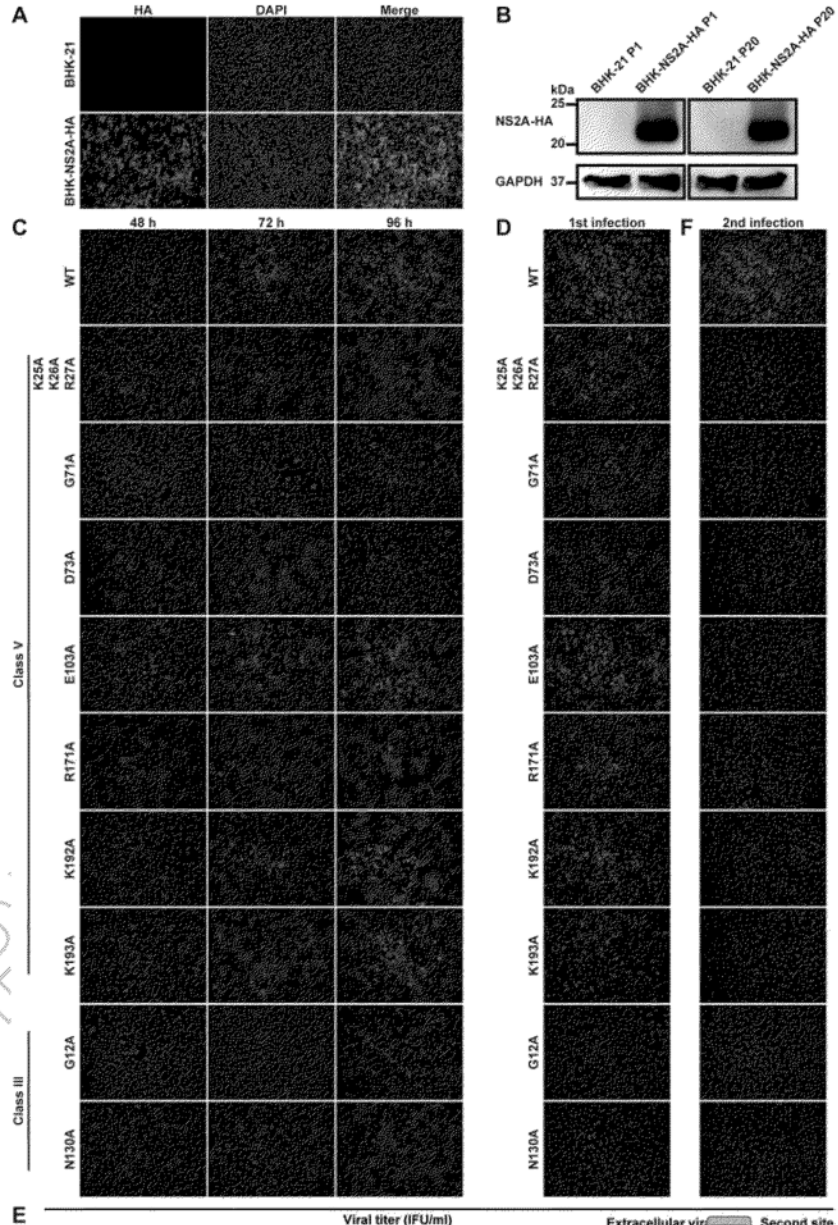
We tested whether the defect in viral RNA synthesis of class III mutants could be *trans* complemented. To test this possibility, we transfected class III genomic RNAs (G12A and N130A) into ZIKV BHK-NS2A-HA cells. Neither mutants generated any E-positive cells in the transfected BHK-NS2A-HA cells (Figure 5C). Infection of naïve Vero cells with supernatants of the transfected cells did not produce any IFA-positive cells (Figure 5D–F). The results suggest that the RNA synthesis defect of class III mutants could not be rescued through *trans* complementation. Due to the dual defects of class IV mutants in both viral RNA synthesis and virion assembly, we did not pursue *trans* complementation experiments for this class of mutants; such complementation experiments may not reveal clear mechanistic insights as compared with the class III and V mutants.

### Probing the topology of ZIKV NS2A on the ER membrane

The distinct functions of amino acids in viral RNA synthesis and virion assembly prompted us to probe the topology of ZIKV NS2A on ER membrane. Bioinformatics analysis suggests that ZIKV NS2A may form seven transmembrane segments (pTMS1–7; Figure 6A). To examine whether each pTMS associates with the ER membrane, we constructed a panel of plasmids encoding full-length or various NS2A fragments with a C-terminally fused eGFP reporter (Figure 6B). The length of each segment was slightly modified from the bioinformatic prediction to allow some

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E

| | | Viral titer (IFU/ml) 48 h | 72 h | 96 h | Extracellular virus RT-PCR | Second site mutation |
|---|---|---|---|---|---|---|
| Class V | K25AK26AR27A | (7.16±1.01)×10$^3$ | (9.69±2.65)×10$^3$ | (5.97±1.18)×10$^4$ | Positive | None |
| | G71A | (1.48±0.78)×10$^3$ | (1.97±0.66)×10$^3$ | (5.42±0.66)×10$^3$ | Positive | None |
| | D73A | (2.10±0.57)×10$^2$ | (1.97±0.12)×10$^3$ | (8.60±1.10)×10$^3$ | Positive | None |
| | E103A | (1.13±0.14)×10$^4$ | (2.30±0.20)×10$^4$ | (3.04±0.25)×10$^5$ | Positive | None |
| | R171A | (6.41±1.58)×10$^3$ | (8.90±0.32)×10$^3$ | (6.06±1.06)×10$^4$ | Positive | None |
| | K192A | (7.85±2.04)×10$^3$ | (1.90±0.02)×10$^4$ | (2.17±0.17)×10$^5$ | Positive | None |
| | K193A | (2.69±0.32)×10$^3$ | (1.85±0.05)×10$^4$ | (1.90±0.08)×10$^5$ | Positive | None |
| Class III | G12A | <10 | <10 | <10 | Negative | N.A. |
| | N130A | <10 | <10 | <10 | Negative | N.A. |

**Figure 5.** Trans complementation of class III and V mutants. (A) IFA of BHK-NS2A-HA cells. The BHK-NS2A-HA cells constitutively express WT ZIKV NS2A fused with a C-terminal HA-tag. The cells were fixed with paraformaldehyde and stained with an HA-tag antibody. (B) Western blot of BHK-NS2A-HA cells. The expression of WT NS2A-HA from BHK-NS2A-HA cells was detected using an HA-tag antibody. Both passage one (P1) and 20 (P20) BHK-NS2A-HA cells were analyzed. Naïve BHK-21 cells were included as a negative control. (C) WT and class III and V genomic RNAs were electroporated into BHK-NS2A-HA cells. IFA was performed on the transfected cells to detect viral E protein expression. (D) Viruses rescued from the BHK-NS2A-HA cells (harvested at 96 h p.t.) were used to infect naïve Vero cells. The infected Vero cells were subjected to IFA for E protein expression. (E) Extracellular viruses were harvested from the supernatants of RNA-transfected BHK-NS2A-HA cells. The harvested viruses were serially diluted to infect naïve Vero cells in 96-well plates. At 24 h p.i., IFA was performed to quantify the viral titers by counting the number of E-positive cells. The results show averages and standard deviations from triplicate samples. Viral RNAs in the supernatants of transfected BHK-NS2A-HA cells were isolated at 96 h p.t. and subjected to RT-PCR. Positive RT-PCR products were sequenced to confirm the engineered mutations and to identify potential second site change(s). (F) The supernatants of infected Vero cells from (D) were collected at 72 h p.i. to infect naïve Vero cells for a second round. IFA was performed on these cells for viral E-positive cells.

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flexibility between pTMS and eGFP. Upon transfection into Vero cells, eGFP alone (without any NS2A fragment) exhibited an even distribution of fluorescence throughout the cell, whereas the full-length NS2A-eGFP (construct 1–226) displayed fluorescence co-localized with calnexin (an ER membrane protein) in the perinuclear region (Figure 6B), indicating that ZIKV NS2A is an integral membrane protein. Expression of seven fragments spanning each pTMS, represented by constructs 1–25 (pTMS1), 30–58 (pTMS2), 74–97 (pTMS3), 101–123 (pTMS4), 125–145 (pTMS5), 150–185 (pTMS6), and 198–221 (pTMS7), exhibited fluorescence patterns that co-localized with calnexin in the perinuclear region (Figure 6B). The results indicate that individual of the seven pTMSes is associated with the ER membrane. In contrast, expression of fragments between different pTMS, represented by constructs 55–73, 186–197, and 221–226, showed fluorescence throughout the cells (Figure 6B), indicating that these fragments are not membrane-associated.

An *in vitro* deglycosylation analysis was performed to determine the orientation of each NS2A pTMS on the ER membrane. A panel of plasmids were prepared to express truncated NS2A proteins that were C-terminally fused with an eGFP followed by an N-glycosylation acceptor peptide (Glyc; Figure 7A). An SPG-$C_{16}$ peptide (Gaussia luciferase signal peptide SPG and the C-terminal 16 amino acids of NS1) was fused to the N terminus of each NS2A-eGFP-Glyc construct to ensure a proper translocation of the expressed protein on the ER membrane. Western blot showed that, upon transfection into BHK-21 cells, all constructs expressed fusion proteins with expected molecular masses (Figure 7B). Because N-glycosylation enzymes reside in the ER lumen, the glycosylation status could be used to indicate the location of the C-terminus of each NS2A-eGFP-Glyc protein (Figure 7C). As a negative control, treatment of EGFP-Glyc (without any viral sequence) with PNGase F (an enzyme that removes high mannose and complex carbohydrates) did not change the mobility of the protein (Figure 7D), indicating a cytosol localization of EGFP-Glyc. As a positive control, PNGaseF treatment increased the mobility of SPG-$C_{16}$-EGFP-Glyc (construct 0), confirming that the C-terminus of this protein resides in the ER lumen. Among the NS2A constructs, PNGase F treatment increased the mobility of fusion proteins 1–26 and 1–56, but not 1–103, 1–123, 1–147, 1–192, and 1–226 (Figure 7D). The results suggest that the N-terminal 56 amino acids of NS2A reside in the ER lumen, and the C-terminal region from residues 103–226 is located in the cytosol.

Next, we performed an *in vitro* proteinase K protection assay [46] to validate the above *in vitro* deglycosylation results. BHK-21 cells were transfected with the same constructs depicted in Figure 7A. At 24 h p.t., membrane fractions from the transfected cells were treated with proteinase K in the presence or absence of 0.5% Triton X-100 and analyzed by Western blot using an eGFP antibody. In the absence of Triton X-100, the eGFP residing in the cytosol would be susceptible to proteinase K digestion, whereas the eGFP residing in the ER lumen would be protected from proteinase K digestion (Figure 7E). As shown in Figure 7F, the eGFPs from constructs 0, 1–26, and 1–56 were protected from protease K digestion, again indicating that the first 56 residues are located in the ER lumen. In contrast, the eGFPs from constructs 1–103, 1–123, 1–147, 1–192, 1–226, and control eGFP-Glyc were completely degraded by the protease K treatment (Figure 7F), supporting that the C-terminal region from residues 103–226 is located in the cytosol. As expected, in the presence of Triton X-100, the eGFPs from all constructs were degraded by proteinase K (Figure 7F). The results of proteinase K assay are in full agreement with those of deglycosylation assay.

### *A topology model for ZIKV NS2A*

The above biochemical results support a topology model for ZIKV NS2A on the ER membrane (Figure 8A): (i) The N-terminal region (including pTMS1 and pTMS2) resides in the ER lumen; (ii) the region spanning residues 74–97 (pTMS3) traverses the ER membrane in a lumen-to-cytosol direction; and (iii) the C-terminal region from residues 103–226 (including pTMS4 to pTMS7) is located in the cytosol side. Since pTMS1, pTMS2, pTMS4, PTMS5, PTM6, and pTMS7 associate with ER membraned when expressed individually (Figure 6B), they may interact with the ER membrane in a peripheral manner. In support of this notion, informatics analyses using Jpred 4 and HeliQuest programmes suggest that amino acids 9–26 (pTMS1), 41–58 (pTMS2), 104–121 (pTMS4), 125–142 (pTMS5), 172–189 (pTMS6), and 203–220 (pTM7) may form amphipathic helices with hydrophobic and hydrophilic surfaces (Figure 8B), where the hydrophobic surface may interact with the ER membrane.

## Discussion

Flavivirus NS2A functions in viral RNA replication, virion assembly, evasion of innate immune response, and disease pathogenesis in a virus-specific manner [35]. This is evidenced by its high sequence diversity (Figure 1A), distinct membrane topology [35,46], and association with different disease pathways [41,42]. Thus, it is critical to perform a systematic genetic and biochemical characterization of ZIKV NS2A to delineate its structure and function. Our biochemical results showed that, unlike other flaviviruses, ZIKV NS2A has only a single transmembrane segment that traverses the

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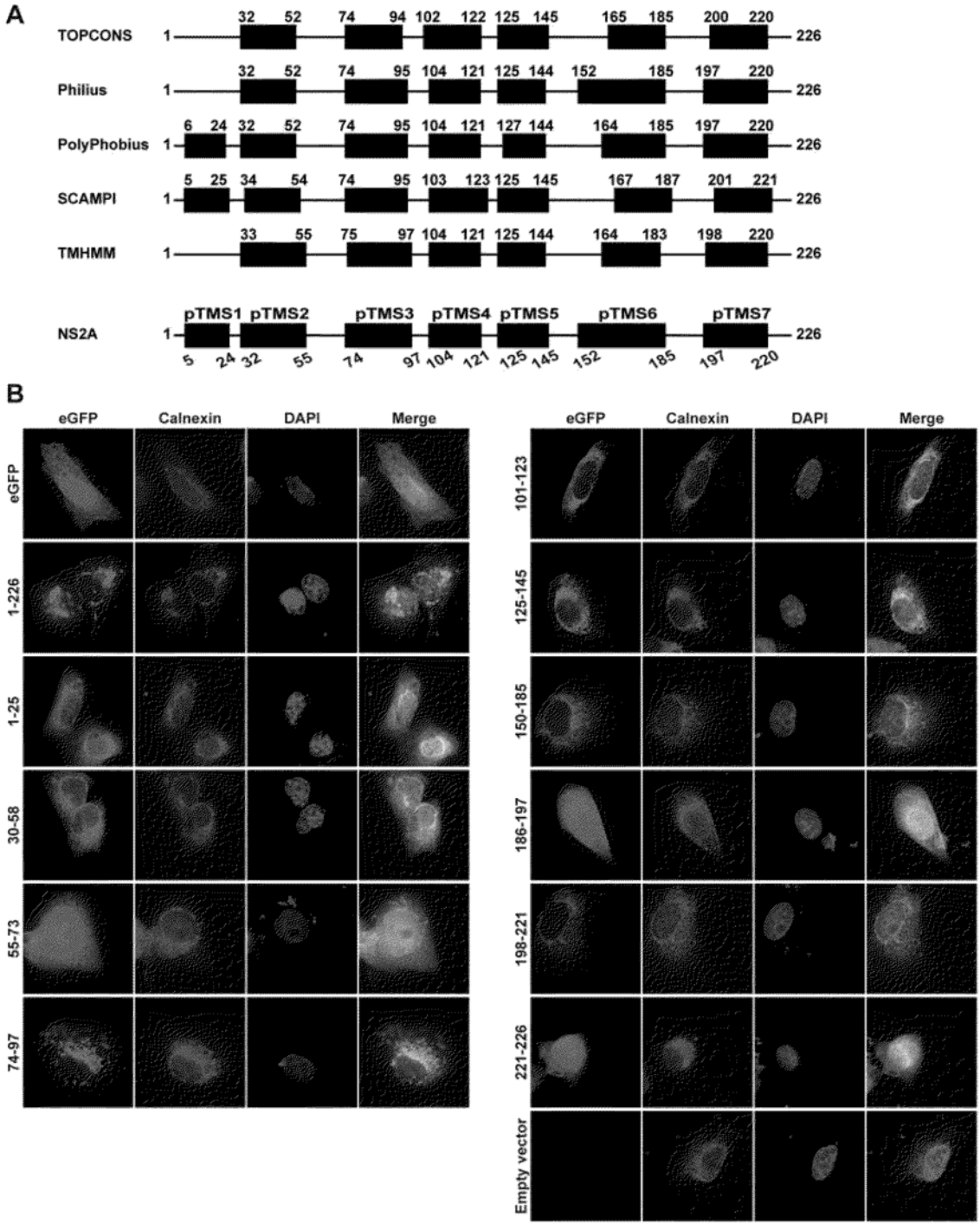



**Figure 6.** Membrane association of ZIKV NS2A transmembrane segments. (A) Bioinformatics analysis of ZIKV NS2A for transmembrane segments. ZIKV NS2A sequence was analyzed by bioinformatics programme TOPCONS, Philius, PolyPhobius, SCAMPI, and TMHMM. The compiled transmembrane segments are shown at the bottom. Seven predicted transmembrane segments (pTMS) are presented as thick bars. The amino acid positions of ZIKV NS2A are indicated. (B) Membrane association of individual pTMS. Expression plasmid pXJ-eGFP (without NS2A sequence) and pXJ-smNS2A-eGFP (smNS2A represents different NS2A segments) were transfected into Vero cell by using X-tremeGENE 9 DNA transfection reagent (Roche). For each plasmid construct, the NS2A segment is indicated by amino acid positions. At 24 h p.t., IFA was performed to monitor eGFP expression. ER and nuclei were stained with anti-Calnexin antibody and DAPI, respectively. The merged images are shown on the right panels.

ER membrane, the other six pTMSes are peripherally associated with the ER membrane (Figure 8A). Our functional analysis revealed five classes of mutants with distinct replication phenotypes, among which class III, IV, and V mutants could not produce infectious viruses through different mechanisms (Figure 1B). The critical mutations from class III to V are not clustered in specific regions of NS2A (Figure 8A),

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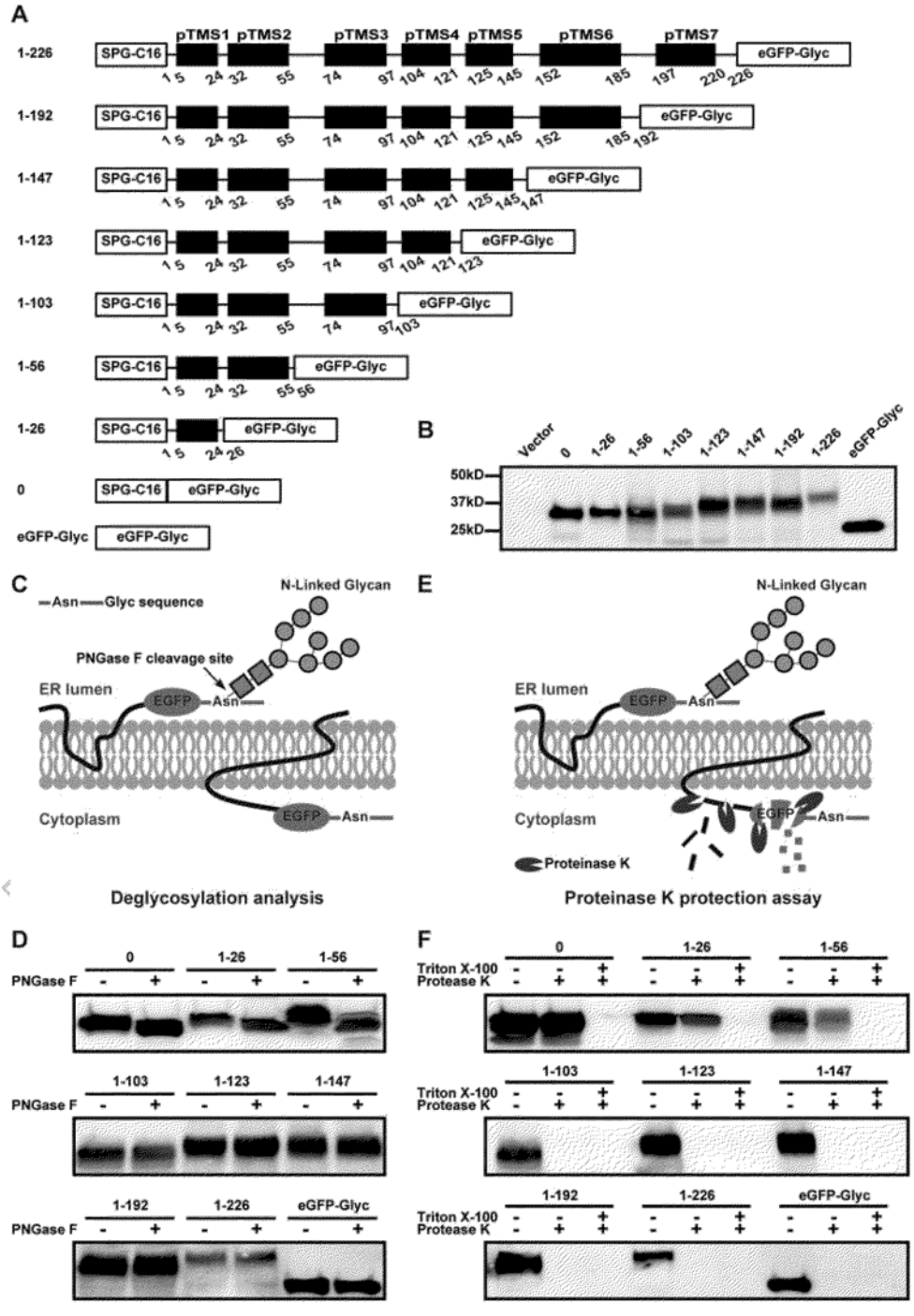



**Figure 7.** Probing the membrane topology of ZIKV NS2A by in vitro deglycosylation assay and in vitro proteinase K protection assay. (A) Schematic representation of C-terminally truncated ZIKV NS2A proteins. The amino acid positions of each truncated NS2A are indicated. A 'SPG-C16' sequence was fused to the N terminus of NS2A to ensure correct translocation of NS2A on the ER membrane. An 'eGFP-Glyc' sequence was fused to the C terminus of NS2A to probe its location at cytosol or ER lumen. See text for details. (B) BHK-21 cells were transfected with the expression plasmids described in (A). At 24 h p.t., the expression levels of various NS2A truncates were examined by Western blot using an eGFP antibody. The amino acid positions are indicated for each NS2A truncates. The molecular masses of marker proteins are shown to the left of Western blot image. (C) Diagram of glycosylation of eGFP-Glyc when located at ER lumen (*left*). No glycosylation occurs when eGFP-Glyc is located at cytosol (*right*). The glycosylation residue Asn is indicated. (D) *In vitro* deglycosylation assay. Plasmid-transfected cells were treated with PNGase F or PBS according to the protocol of *in vitro* deglycosylation assay (see Materials and Methods). The samples were examined for glycosylation status by Western blot using an eGFP antibody. (E) Diagram of *in vitro* proteinase K protection assay. When eGFP is located at ER lumen, it is protected from proteinase K cleavage (*left*); when located in the cytosol, eGFP is degraded by proteinase K (*right*). (F) *In vitro* proteinase K protection assay. Cell lysates from plasmid-transfected BHK-21 cells were treated with proteinase K or PBS according to the protocol of *in vitro* proteinase K protection assay (see Materials and Methods). The samples were detected for eGFP by Western blot using an eGFP antibody. Triton X-100 treatment is indicated.

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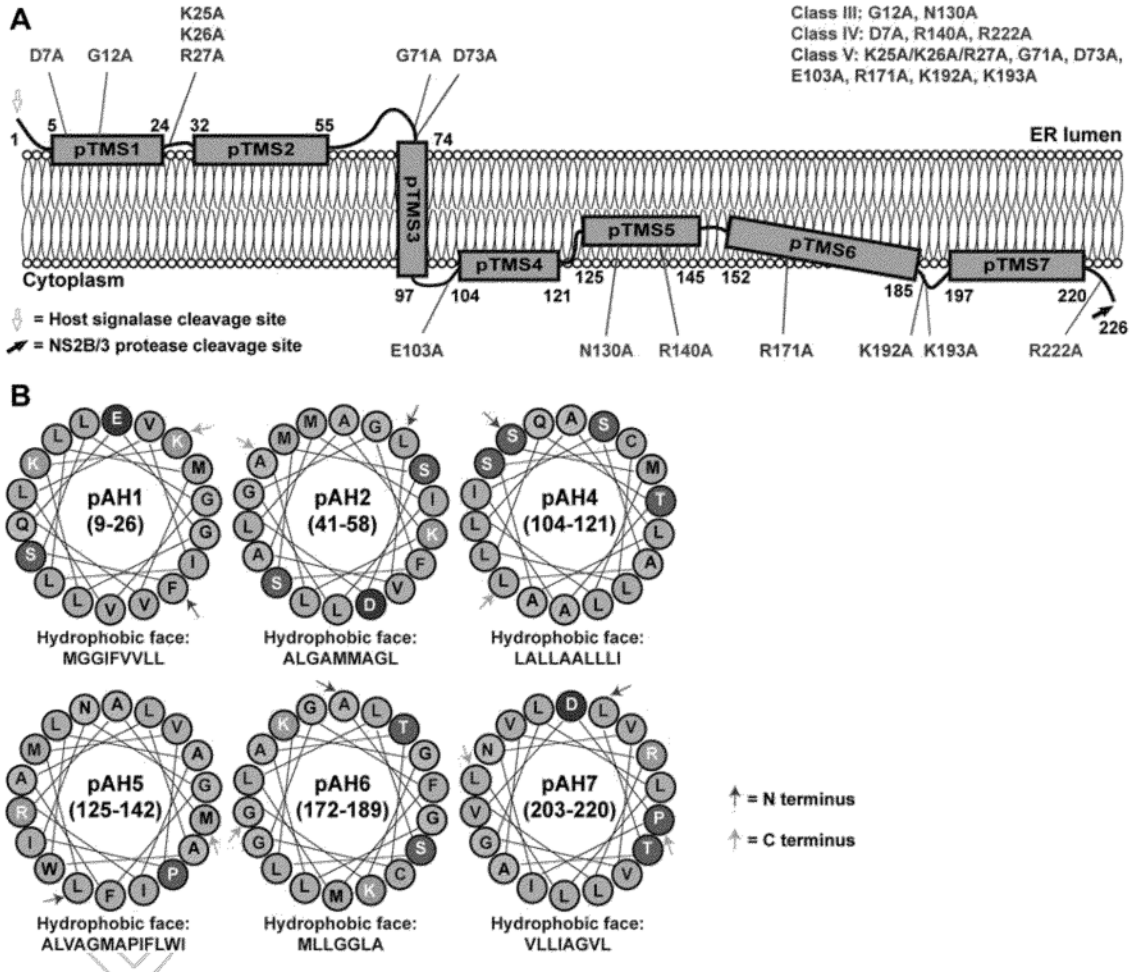



**Figure 8.** A topological model of ZIKV NS2A. (A) A model of ZIKV NS2A membrane topology. See text for details. Class III, IV, and V mutations are depicted on the topology model. (B) Helical wheel representations of NS2A fragment 9–26, 41–58, 104–121, 125–142, 172–189, and 203–220. The amphiphilic helix regions were analyzed using Heliquest server (heliquest.ipmc.cnrs.fr/). The best 18-residue windows are presented. The amino acids forming the hydrophobic surface of each helix wheel are depicted. Yellow, gray, blue, red, purple, pink, and green circles indicate nonpolar, glycine/alanine, positively charged, negatively charged, polar, amide, and proline amino acids, respectively. The first and last residues in each helical wheel are indicated by a blu[illegible] red arrow, respectively.

suggesting that multiple domains of NS2A are essential for viral replication.

The membrane topology of ZIKV NS2A is in stark contrast with the topologies of other flavivirus NS2A proteins: DENV-2 NS2A has five membrane-traversing segments [46] and YFV NS2A has three membrane-traversing segments [35]. Interestingly, these topologically diverse flavivirus NS2A proteins are able to converge their functions in viral RNA replication, virion assembly, and evasion of immune response. High resolution, three-dimensional structures of flavivirus NS2A are needed to address this question. It should be noted that the membrane topology of NS2A presented here is in the absence of other viral proteins. In the context of viral replication, the NS2A protein is associated with various viral and cellular proteins that would modulate the structure of NS2A and perform biological functions.

Our mutagenesis study has identified distinct NS2A mutations that selectively abolish ZIKV RNA synthesis (class III) or virion assembly (class V), among which only the virion assembly-defective mutants could be rescued through *trans* complementation (Figure 5). The results suggest that there are two species of NS2A molecules in ZIKV-infected cells. One NS2A species is located at the viral replication complex that could only be formed in *cis*; another NS2A species is located at virion assembly complex that could be formed in *trans* or *cis*. These results are in agreement with the previous studies from DENV and YFV NS2A [30,35,36]. Compared with the viral replication complex, the virion assembly complex is much less well defined for flaviviruses. Previously studies showed that second-site mutations in prM, E, NS2B, and NS3 could rescue the defect of virion assembly caused by DENV-2 and YFV NS2A mutations, suggesting that these viral proteins

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participate in virion assembly. Besides the genetic evidence, the role of NS3 in virion assembly is also supported by (i) mutations in the NS3 helicase domain alone affect YFV assembly [49], (ii) the helicase domain of DENV or YFV NS3 directly binds to NS2A [35,36], (iii) the cleavage between C and prM is tightly modulated by NS2B/NS3 protease and cellular signalase during YFV and Murray Valey encephalitis virus assembly [50–52]. These data, together with the result that recombinant NS2A of Kunjin binds to viral 3'UTR RNA [29], suggest a flavivirus assembly model. During flavivirus assembly, the NS2A protein recruits NS2B/NS3 through binding to the NS3 helicase domain. Once the NS2A-NS2B/NS3 complex reaches the virion assembly site, the NS3B/NS3 protease cleaves the C protein from the C/prM/E polyprotein. The C protein assembles onto genomic RNA (presented by NS2A) to form nucleocapsid, which is subsequently enveloped by prM/E complex to form virions. Since flavivirus assembly has to be coupled to viral RNA replication [53], the newly synthesized genomic RNA has to be transported from the replication site to the virion assembly site [54], possibly through the NS2A protein.

The *trans* complemented class V mutants could potentially be used as vaccine candidates, as recently reported for DENV-2 [36]. Since the *trans* complemented virions are able to infect cells for only a single cycle, such vaccine platform is safer than the multi-round infectious live-attenuated vaccines [55,56]. This is particularly important for ZIKV vaccine when immunizing immune-compromised individuals and pregnant women [57]. However, for development of class V mutants as a vaccine platform, the *trans* complementation efficiency needs to be improved to higher titers before they could be tested for *in vivo* efficacy.

In summary, we report the genetic and biochemical characterization of ZIKV NS2A protein. The results indicate that different flavivirus NS2A proteins have distinct membrane topologies, but converge to function in viral RNA synthesis and virion assembly. Together with previous results, we propose a model for flavivirus assembly in which NS2A plays a central role in mediating viral structural and nonstructural proteins as well as genomic RNA during virion formation.

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## Disclosure statement

No potential conflict of interest was reported by the authors.

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# Genetic and biochemical characterizations of Zika virus NS2A protein

Xianwen Zhang, Xuping Xie, Jing Zou, Hongjie Xia, Chao Shan, Xinwen Chen, and Pei-Yong Shi

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# Genetic and biochemical characterizations of Zika virus NS2A protein

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**ABSTRACT**

Zika virus (ZIKV) can cause devastating congenital Zika syndromes in pregnant women and Guillain-Barre syndrome in adults. Understanding the molecular mechanism of ZIKV replication is essential for antiviral and vaccine development. Here we report the structural and functional characterization of ZIKV NS2A protein. Biochemical structural probing suggests that ZIKV NS2A has a single segment that traverses the ER membrane and six segments that peripherally associate with the ER membrane. Functional analysis has defined distinct NS2A residues essential for viral RNA synthesis or virion assembly. Only the virion assembly-defective mutants, but not the RNA synthesis-defective mutants, could be rescued through *trans* complementation with a wide-type NS2A protein. These results suggest that the NS2A molecules in virion assembly complex could be recruited in *trans*, whereas the NS2A molecules in viral replication complex must be recruited in *cis*. Together with previous results, we propose a flavivirus assembly model where NS2A plays a central role in modulating viral structural and nonstructural proteins as well as genomic RNA during virion assembly.

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**KEYWORDS** Zika virus; flavivirus replication; virion assembly; flavivirus NS2A; membrane topology

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## Introduction

Zika virus (ZIKV) is a recently emerged mosquito-borne flavivirus that is primarily transmitted by *Aedes spp.* mosquitoes. It can also be transmitted through sexual, maternal-to-fetal, and blood transfusion routes [1–5]. ZIKV was first identified in 1947 from a sentinel rhesus monkey in Uganda [6]. Since 2007, it emerged in the Pacific region, South America, Central America, and the Caribbean, posing a global public health threat [7–11]. ZIKV infection causes mild illnesses, including fever, pruritis, arthritis, conjunctivitis, and asthenia [12]. Importantly, it can cause devastating congenital Zika syndromes (CZS; including microcephaly, congenital malformation, and fetal demise) in pregnant women and Guillain-Barre syndrome (GBS) in adults [13–16]. The explosive epidemics and its association with CZS had led the World Health Organization (WTO) to declare ZIKV as a Public Health Emergency of International Concern from February to November in 2016 [17]. Since then, a number of vaccine candidates have been developed, some of which have already advanced to clinical trials [18]. In addition, small molecule inhibitors and therapeutic antibodies have also been identified for antiviral development [19–22].

ZIKV belongs to the *Flavivirus* genus within the *Flaviviridae* family. Besides ZIKV, many other flaviviruses are significant human pathogens, including dengue (DENV), yellow fever (YFV), West Nile (WNV), Japanese encephalitis (JEV), Saint Louis encephalitis (SLEV), and tick-borne encephalitis virus (TBEV) [23]. Flavivirus genome is a positive-sense, single-stranded RNA of ~11,000 nucleotides. It contains a 5' untranslated region (UTR), a long open-reading-frame (ORF), and a 3' UTR. The single ORF encodes

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a polyprotein that is processed into three structure proteins (capsid [C], pre-membrane [prM] and envelope [E]) and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5). The structural proteins, together with genomic RNA, are components of virions. The nonstructural proteins participate in viral RNA replication, virion assembly, and evasion of host immune response [24–26].

Flavivirus NS2A is an ER-resident, transmembrane protein with a calculated molecular weight of ~22 kDa. The N-terminus and C-terminus of NS2A are formed by cleavages with an unknown host protease and viral protease NS3 (in complex with NS2B), respectively [27,28]. Flavivirus NS2A has multiple functions: (i) Viral replication. Kunjin virus (KUNV) NS2A co-localizes with viral double-stranded RNA and interacts with the 3'UTR of genomic RNA, NS3, and NS5 protein [29]. Mutagenesis studies confirmed the role of NS2A in viral RNA synthesis [30,31]. (ii) Virion assembly/secretion. Mutagenesis analyses of YFV, KUNV, and DENV have identified distinct NS2A amino acids that are essential for virion assembly/secretion [30–34]. The virion assembly defects can be restored by second site mutations in NS2B or NS3 in YFV and DENV [32,35,36]. (iii) Evasion of innate immunity. ZIKV NS2A inhibits type-I IFN induction at the step of TBK1 [26]. DENV NS2A antagonizes type-I IFN signalling [25]. JEV NS2A blocks dsRNA-activated protein kinase PKR [37]. In WNV and KUNV, the NS2A-mediated inhibition of type-I IFN induction can be abrogated by specific NS2A mutations [38,39]. (iv) Disease pathogenesis. WNV NS2A modulates virus-induced cytopathic effect (CPE) and pathogenesis in mice [40,41]. ZIKV NS2A impairs mammalian cortical neurogenesis through depleting adherens junction proteins [42].

The structure and function of ZIKV NS2A have not been characterized. Although NS2A proteins from different flaviviruses perform similar functions (described above), each flavivirus may exert its NS2A functions in a virus-specific manner [35]. The goals of this study are (i) to define the membrane topology of ZIKV NS2A and (ii) to characterize its roles in viral RNA synthesis and virion assembly.

## Materials and methods

### Cell lines

Vero cells, baby hamster kidney (BHK-21) cells and human embryo kidney 293-T cells were cultured at 37°C with 5% ~~CO2~~ in high-glucose Dulbecco's modified Eagle medium (DMEM; Life Technologies) supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 g/ml streptomycin and 10% fetal bovine serum (FBS; HyClone Laboratories).

### Bioinformatics

NS2A protein sequences from ZIKV, DENV1, DENV2, DENV3, DENV4, WNV, JEV, YFV, SLEV were aligned using CLC Main Workbench software (CLC Bio). Prediction of ZIKV NS2A was carried out using various web servers, which including TOPCONS, Philius, Polyphobius, SCAMPI, and TMHMM2. The amphipathic helices of ZIKV NS2A were predicted by HeliQuest.

### Antibody

The following antibodies were used in this study: a mouse monoclonal antibody 4G2 cross-reactive with flavivirus E protein (American Type Culture Collection); Rabbit anti-HA monoclonal antibody (Cell Signaling Technology); Alexa Fluor®488 conjugated mouse anti-HA monoclonal antibody (Cell Signaling Technology); Rabbit anti-Calnexin polyclonal antibody(Abcam); Mouse anti-GFP monoclonal antibody (Sigma-Aldrich); Horseradish peroxidase(HRP) conjugated goat anti-mouse and anti-rabbit IgG polyclonal antibodies (Sigma-Aldrich); Alexa Fluor®488 conjugated goat anti-mouse IgG polyclonal antibody (Thermo Fisher Scientific); Alexa Fluor®568 conjugated goat anti-rabbit IgG polyclonal antibody (Thermo Fisher Scientific).

### Plasmid construction

The ~~Infectious~~ cDNA clone (pFLZIKV) bearing the full-length genome of ZIKV (strain FSS13025) or Renilla luciferase (Rluc) reporter ZIKV replicon (pZIKV Rep WT) were used as the backbone for introducing NS2A mutations [43,44]. XhoI and KpnI restriction sites were initially generated into pFLZIKV and pZIKV Rep WT replicon at positions 3018 and 5010 in ZIKV genome without changing amino acid sequences. The NS2A alanine-scanning mutagenesis was performed by using a QuikChange II XL Site-Directed Mutagenesis kit (Agilent Technologies) as described previously [30].

A mammalian expression vector pXJ with a cytomegalovirus (CMV) promoter was used for constructing plasmids expressing NS2A and its derivatives. To construct the pXJ-$E_{24}$-NS1-NS2A-HA plasmid, a gene cassette encoding the last 24 amino acids of E protein ($E_{24}$), NS1 and NS2A with a C-terminal hemagglutinin (HA) tag was firstly amplified from the pFLZIKV and cloned into the pXJ vector. Next, ~~35~~ NS2A mutations were individually introduced into the pXJ-$E_{24}$-NS1-NS2A-HA by overlap PCR, resulting in plasmids expressing NS2A mutants.

To construct the pXJ-SPG-$C_{16}$-NS2A-HA that was used for selecting cell line constitutively expressing NS2A protein, a DNA fragment containing the Gaussia luciferase signal peptide (SPG), the last 16 amino acids

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of NS1 protein ($C_{16}$) and NS2A tagged with a C-terminal HA tag sequence were constructed by overlap PCR and cloned into the pXJ vector via NotI/XhoI restriction sites.

Plasmid pXJ-eGFP was constructed by inserting the enhanced green fluorescence protein (eGFP) sequence into the pXJ vector via BamHI/XhoI restriction sites. DNA fragments that encode different NS2A segments were individually inserted into above pXJ-eGFP vector, resulting various pXJ-smNS2A-eGFP plasmids (smNS2A represents NS2A segment). A DNA fragment that express NS2A or truncated NS2A and an eGFP with a C-terminal N-glycosylation acceptor site (Glyc) were amplified and cloned into the pXJ-SPG-$C_{16}$-NS2A-HA vector, resulting in the plasmid pXJ-SPG-$C_{16}$-NS2A-eGFP-Glyc and various pXJ-SPG-$C_{16}$-ctNS2A-eGFP-Glyc plasmids (ctNS2A represents C-terminal truncated NS2A).

### *RNA in vitro transcription and electroporation*

All full-length cDNA clone plasmids and subgenomic replicons of ZIKV were linearized with restriction enzyme ClaI, followed by phenol-chloroform extraction and ethanol precipitation. RNA transcripts were prepared by using the T7 mMessage mMachine kit (Ambion). For RNA transfection, 10 μg *in vitro* transcribed RNA was electroporated into $8 \times 10^6$ cells (BHK-21 or Vero) by using Gene Pulser Xcell™ Electroporation Systems (Bio-rad) as described previously [30,43].

### *Immunofluorescence assay*

For indirect immunofluorescence assay (IFA), cells were seeded in an 8-well Nunc Lab-Tek II Chamber Slide (Thermo Fisher Scientific). At various time points, cells were washed three times with phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde in PBS at room temperature for 30 min and permeabilized with 0.1% Triton X-100 in PBS at room temperature for 10 min. The permeabilized cells were then washed three times with PBS and incubated in a blocking buffer containing 1% FBS in PBS at room temperature for 1 h. E protein was stained with a 1:1000 dilution of mouse monoclonal antibody 4G2 in blocking buffer. After incubation for 1 h, the cells were washed three times with a washing buffer (1% FBS and 0.5% Tween 20 in PBS). After 1 h of incubation with a secondary antibody (Alexa Fluor®488 conjugated goat anti-mouse IgG), the cells were washed three times with washing buffer and counterstained with DAPI (4',6-diamidino-2-phenylindole). Images were acquired using an Olympus fluorescence microscope. Images were processed in ImageJ software (National Institutes of Health, MD).

### *Plaque assay*

$2 \times 10^5$ Vero cells per well were grown in 24-well plates one day prior to viral infection. 10-fold serial dilutions of virus samples were prepared in DMEM containing 2% FBS and 1% penicillin/streptomycin and inoculated (100 μl) into each well of 24-well plates. After 1 h of incubation at 37°C with 5% $CO_2$, the inoculum was replaced with 500 μl overlay medium (DMEM medium containing 0.8% methyl-cellulose, 2% FBS and 1% penicillin/streptomycin). Plaques formed after incubation at 37°C with 5% $CO_2$ for 4–5 days. Cells were fixed with 4% paraformaldehyde for 30 min, followed by staining with 1% crystal violet. The numbers of plaque per millilitre (PFU/ml) of ZIKV were calculated.

### *Replicon transient-transfection assay*

Briefly, $8 \times 10^6$ Vero cells were electroporated with 10 μg wild type or mutant Rluc-ZIKV replicon RNAs. After electroporation, $3 \times 10^5$ cells per well were seeded in 12-well plates. At given time points, the cells were washed twice with PBS and lysed in 200 μl of luciferase lysis buffer (Promega). The luciferase activity assay was performed according to the manufacturer's protocol.

### *RNA extraction and cDNA sequencing*

The culture fluids from infected cells were centrifuged at 1000 g for 10 min and then filtered through a 0.22 μm pore size polyethersulfone membrane (Millipore) to remove cellular debris. Viral RNAs in the supernatants were extracted using a QIAamp viral-RNA Minikit (Qiagen) by following the manufacturer's protocol. Reverse transcription PCR (RT-PCR) was performed by using the SuperScript® III One-Step RT-PCR System (Thermo Fisher Scientific). The amplified cDNA fragments were purified and sent for Sanger sequencing at the GENEWIZ facilities.

### *Selection of NS2A-HA BHK-21 cell line*

$4 \times 10^5$ BHK-21 cells per well ~~of~~ were seeded into a 6-well plate. The plasmid pXJ-SPG-$C_{16}$-NS2A-HA that contains a neomycin resistance gene was transfected into BHK-21 cells (4 μg per well) using X-tremeGENE 9 DNA transfection reagent (Roche). At 24 h post-transfection, geneticin (G418) was added to the culture medium with a concentration of 1 mg/ml. The medium was changed every three days. After two weeks, several neomycin-resistant individual colonies were selected for propagation. The expression of NS2A-HA protein in stable cell lines was analyzed by immunofluorescence assay and Western blot.

### *In vitro proteinase K protection assay*

Proteinase K protection assay was performed as previously described with minor modifications [45,46].

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BHK-21 cells were transfected in 6-well plates with various pXJ-SPG-$C_{16}$-ctNS2A-eGFP-Glyc plasmids. After incubated at 30°C for 24 h, cells were trypsinized, washed twice with PBS and centrifuged at 450 × g for 5 min. The cell pellets were resuspended in a hypotonic lysis buffer (10 mM Tris-HCl, pH 7.5, and 2 mM $MgCl_2$) and kept on ice for 10 min. Subsequently, the samples were homogenized with 40 strokes and centrifuged at 1000 × g for 5 min. The postnuclear supernatants (PNS) were divided into three aliquots and centrifuged at 15,000 × rpm for 40 min. The pellets from three aliquots were then resuspended in PBS, 60 μg/ml proteinase K in PBS, or 60 μg/ml proteinase K in PBS in the presence of 0.5% Triton X-100, respectively. The samples were incubated on ice for 90 min. 20 mM phenylmethylsulfonyl fluoride (PMSF) was finally added to terminate the reaction. After further incubation on ice for 15 min, the samples were mixed with 4× Laemmli sample buffer, heated at 95°C for 15 min and analyzed by Western blotting.

### *In vitro deglycosylation assay*

The samples were prepared by following a previously described protocol [46,47]. Briefly, the transfected BHK-21 cells in 6-well plates were incubated at 30°C for 24 h. Cells were trypsinized and suspended in DMEM medium containing 10%FBS. The cells were pelleted by centrifuging at 450 × g for 5 min. After two PBS washes, the cell pellets were resuspended in a solubilization buffer (20 mM Tris-HCl, pH 7.5, 50 mM NaCl, 10 mM $Mg(CH_3COO)_2$, 1% Triton X-100, and EDTA-free protease inhibitors [Roche]) and incubated at 4°C for 2 h with gentle agitation. After centrifugation at 15,000 rpm 4°C for 20 min to remove cell debris, the supernatants were split into two aliquots. 50,000 units/ml N-glycosidase F (PNGase F) or PBS (no-treatment control) were added to those two aliquots. After incubation at 37°C for 2 h, the samples were mixed with 4× Laemmli sample buffer, heated at 70°C for 15 min and analyzed by Western blot.

### *SDS-PAGE and western blot*

Protein samples were separated by 12% SDS-polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride (PVDF) membrane using Trans-Blot Turbo transfer system (Bio-Rad), after which the membrane was blocked in TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 0.1% Tween 20) supplemented with 5% nonfat milk for 1 h at room temperature. The blots were probed with primary antibodies (1:1,000 dilution) overnight at 4°C, washed three times with TBST and incubated with horseradish peroxidase (HRP) conjugated goat anti-mouse and anti-rabbit IgG polyclonal antibodies (1:10,000 dilution) for 1 h. After three thorough TBST buffer washes, the blots were incubated with SuperSignal™ West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific). Chemiluminescence signals were detected in ChemiDoc System (Bio-Rad).

### *Quantification and statistical analysis*

IFA images processing and cell counting were performed in software ImageJ (NIH). Densitometry analysis was performed using Image lab version 6.0 (Bio-Rad Laboratories, Hercules, CA). All numerical data are presented as the mean ± SEM (standard error of mean). The size of each study or number of replicates, along with the statistical tests performed can be found in corresponding Figure Legends.

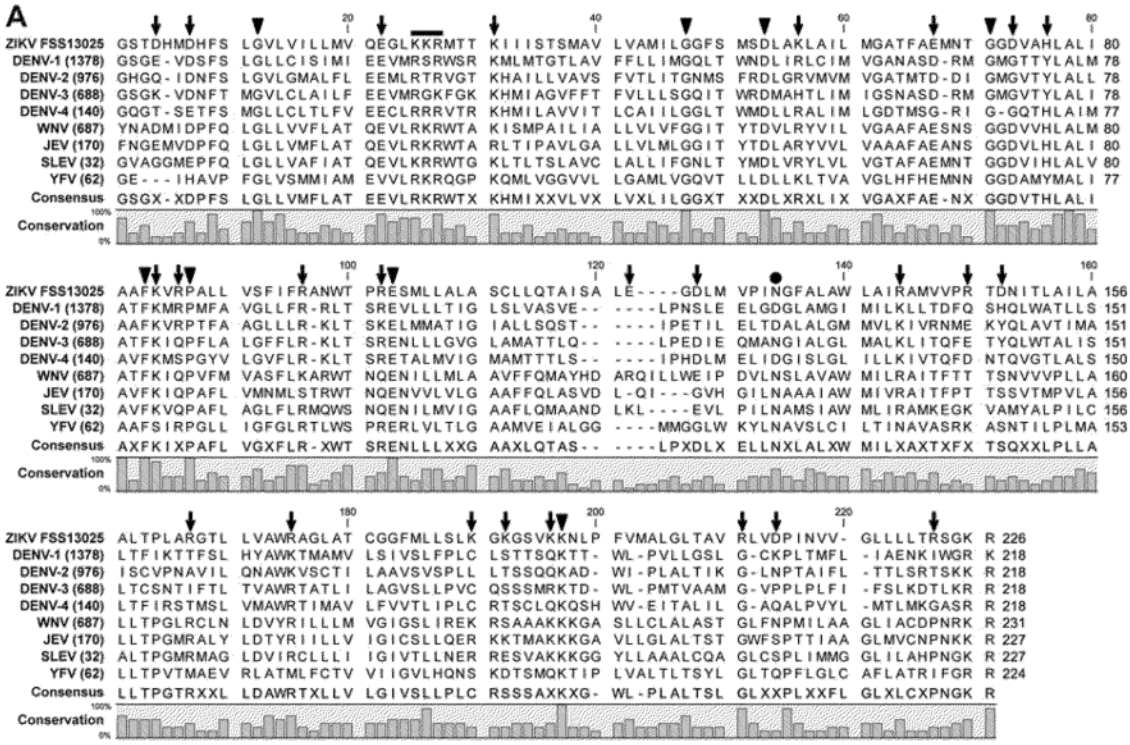
## Results

### *The rationale for ZIKV NS2A mutagenesis*

To identify critical residues in ZIKV NS2A, we performed a systematic alanine-scanning mutagenesis using an infectious cDNA clone of ZIKV strain FSS13025 [43]. Sequence alignment of NS2A proteins from ZIKV, DENV, WNV, JEV, SLEV, and YFV revealed a high diversity: only 31 out of 218–227 amino acids with a consensus of >80% among different flaviviruses (Figure 1A). A total of 35 residues were selected for mutagenesis: (i) All 8 identical non-alanine residues, including G12, G47, D53, G71, F83, P87, E103, and K193. The amino acid numbering is based on the ZIKV sequence. (ii) All charged residues, including D4, D7, E22, K31, K56, E67, D73, H76, K84, R86, R96, R102, E122, D124, R140, R146, D148, R163, R171, K186, K188, K192, R207, D210, and R222. Charged residues were selected because they likely participate in inter- or intra-molecular interactions; alternatively, they may alter the locations where NS2A would traverse the ER membrane since charged residues are hydrophilic. In addition, NS2A D73H mutation was previously shown to attenuate WNV replication [48]. (iii) A K25/K26/R27 motif that was previously reported to be critical for YFV and DENV virion assembly [35,40]. (iv) Residue N130 that was previously shown to be required for NS1-NS2A cleavage and viral replication in DENV [27,30]. The selected residues were mutated to alanine in the context of ZIKV genomic RNA. Equal amounts of wild-type (WT) and mutant RNAs were electroporated into Vero cells. After electroporation, RNA replication and virus spread were monitored by immunofluorescence assay (IFA); the production of infectious viruses was quantified by plaque assay and RT-PCR. The 35 mutants exhibited distinct phenotypes that could be categorized into five groups (Figure 1B), as detailed in the following sections.

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**B**

| Class | NS2A Mutations | Phenotypes |
|---|---|---|
| I | D4A, E22A, K31A, G47A, D53A, E67A, H76A, E122A, R146A, D148A, R163A, K186A, K188A, D210A | Mutants do not or weakly affect viral production |
| II | K56A , F83A, K84A, R86A, P87A, R96A, R102A, D124A, R207A | Mutants dramatically reduce viral production |
| III | G12A, N130A | Mutants abolish viral RNA synthesis |
| IV | D7A, R140A, R222A | Mutants abolish virus production by affecting RNA synthesis and viron assembly/release |
| V | K25A/K26A/R27A, G71A, D73A, E103A, R171A, K192A, K193A | Mutants abolish virion assembly |

**Figure 1.** ZIKV NS2A mutagenesis. (A) Sequence alignment of NS2A proteins among ZIKV (strain FSS13025), DENV-1-4 serotypes, WNV, JEV, SLEV, and YFV using CLC Main Workbench software (CLC Bio). Thirty-five amino acids were selected for mutagenesis: (i) All 8 identical non-alanine residues (indicated by triangles), (ii) 25 charged residues (arrows), (iii) a K25/K26/R27 motif (line), (iv) residue N130 (circle). The consensus and conservation percentage are indicated below each amino acids. (B) A summary of ZIKV NS2A mutagenesis results. An infectious cDNA clone and a luciferase replicon of ZIKV strain FSS13025 were used to analyze the function of each amino acid. Based on the replication phenotypes, the 35 mutants could be categorized into five classes as indicated.

### *Class I mutations have no or weak effects on viral replication*

Fourteen of the 35 NS2A mutant RNAs, including D4A, E22A, K31A, G47A, D53A, E67A, H76A, E122A, R146A, D148A, R163A, K186A, K188A, and D210A, replicated at levels comparable to the WT RNA. These mutants were categorized as class I (Figure 1B). The IFA images and plaque morphologies of three representatives (D53A, D148A, and R163A), as well as the WT, are shown in Figure 2(A and B), respectively. Most class I mutants (e.g. D148A and R163A) yielded comparable E-expressing cells as the WT from 24 to 48 h post-transfection (p.t.). At 72 h p.t., severe CPE occurred among D148A, R163A, and WT RNA-transfected cells. Some class I mutants (e.g. D53A) produced fewer E-positive cells than the WT at 48 h p.t., and spread to infect more cells at 72 h p.t (Figure 2A). All class I mutants produced viruses with plaques similar to the WT virus (Figure 2B). At 72 h p.t., the class I mutants produced infectious titers <4-fold lower than the WT did (Figure 2C). Sequencing of the mutant viruses (collected at 72 h p.t.) confirmed the retaining of the engineered mutations without other changes (data not shown).

### *Class II mutations significantly attenuate viral replication*

Nine NS2A mutants, including K56A, F83A, K84A, R86A, P87A, R96A, R102A, D124A, and R207A,

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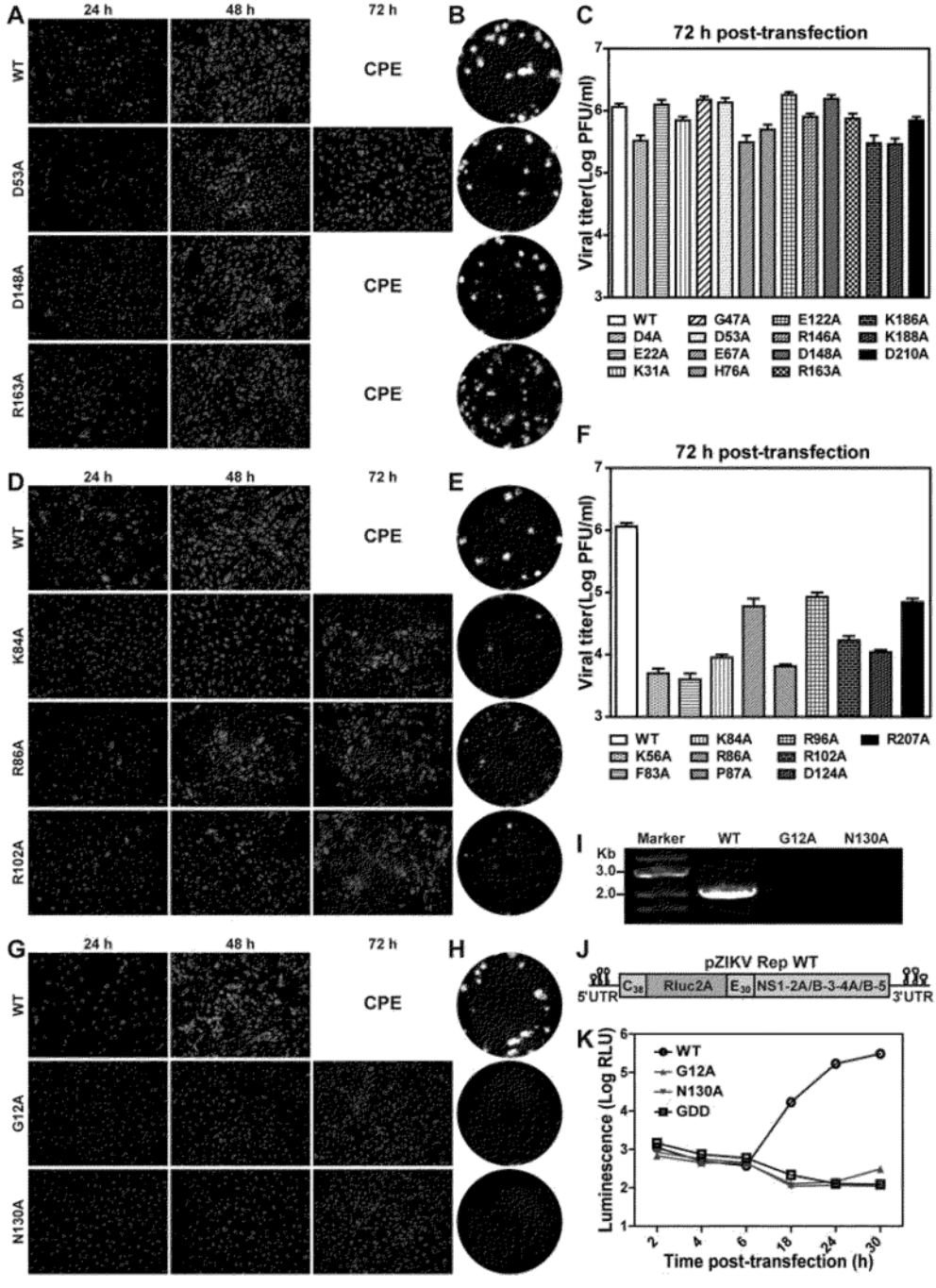



**Figure 2.** Characterization of class I, II, and III NS2A mutants. The results of representative mutants from class I (A–C), II (D–F), and III (G–K) are shown. (A) Vero cells were electroporated with equal amounts of ZIKV wild-type (WT) or class I mutant genomic RNAs. At indicated time points, the cells were fixed and subjected to IFA. Viral E protein and nuclei were stained with antibody 4G2 (green) and DAPI (blue), respectively. Cytopathic effect (CPE) is indicated at 72 h post-transfection. (B) Plaque morphologies. Recombinant viruses were subjected to plaque assay. Plaques were developed at day 4.5 post-infection. (C) Viral titers of class I mutants at 72 h post-transfection. The mean values from two independent experiments are shown. Error bars indicate the standard deviations. The limit of detection (LOD) of plaque assay is 10 PFU/ml. (D) IFA of class II mutant RNA-transfected Vero cells. (E) Plaque morphology of class II mutant viruses. (F) Viral titers of class II mutant viruses at 72 h post-transfection. (G) IFA of class III mutant RNA-transfected Vero cells. (H) Plaque morphologies of class III mutant viruses. Plaque assay was performed using culture supernatants collected at day 3 (for WT) and day 5 (for G12A and N130A) post-transfection. (I) RT-PCR analysis. At 5 day p.t., the mutant RNA-transfected cells were washed twice with PBS to remove residual input RNAs. At day 8 p.t., the supernatants of G12A and N130A RNA-transfected cells were subjected to RT-PCR. The WT virus (collected at 72 h p.t.) was used as a positive control. (J) A ZIKV luciferase replicon. $C_{38}$ and $E_{30}$ represent the first 38 amino acids of C protein and the last 30 amino acids of E protein, respectively. *Renilla* luciferase: Rluc. Foot-and-mouse-disease virus 2A sequence: 2A. (K) Transient replicon assay. WT, G12A, N130A, and NS5ΔGDD replicon RNAs were electroporated into Vero cells. Luciferase activities were measured at indicated time points. An average of 3 experiments are presented. Bars represent standard deviations.

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significantly attenuated viral replication. These mutants were categorized into class II (Figure 1B). Class II RNA-transfected cells produced fewer E-positive cells than the WT did (Figure 2D). At 72 h p.t., only 30–50% of E-positive cells were detected in the class II RNA-transfected cells, whereas the WT RNA-transfected cells exhibited CPE. Class II mutant viruses formed smaller, opaque plaques (Figure 2E), and the transfected cells produced >10-fold lower infectious titers than the WT RNA-transfected cells at 72 h p.t. (Figure 2F). Complete genome sequencing of the class II mutant viruses (collected at 96 h p.t.) did not reveal reversions or any other mutations (data not shown). Because of the weak phenotypes of class I and II mutants, we did not further characterize them.

### *Class III mutations are lethal for viral RNA synthesis*

Class III includes two mutants, G12A and N130A, which were completely defective in viral RNA synthesis (Figure 1B). The G12A and N130A genomic RNA-transfected cells did not produce any E-positive cells at 24 and 48 h p.t. (Figure 2G). On day 5–8 p.t., no infectious viruses were detected from culture medium by plaque assay (Figure 2H) and RT-PCR (Figure 2I). To confirm the above results, we introduced G12A or N130A mutation into a ZIKV luciferase replicon (Figure 2J). A replication-defective replicon, containing an NS5 polymerase active site ΔGDD mutation, was included as a negative control [44]. Vero cells were electroporated with equal amounts of WT, G12A, N130A, or NS5 ΔGDD replicon RNAs. At 2–6 h p.t., all replicons generated similar levels of luciferase signals, suggesting comparable levels of viral RNA translation and transfection efficiencies (Figure 2K). However, at 18–30 h p.t., G12A and N130A replicons generated low luciferase signals as the non-replicative NS5 ΔGDD replicon did, whereas the WT replicon produced increasing luciferase signals (Figure 2K). These results demonstrate that class III mutations completely abolish viral RNA synthesis.

### *Class IV mutations impair both viral RNA synthesis and virion production*

Class IV includes three mutants, including D7A, R140A, and R222A (Figure 1B). These mutant genomic RNAs produced fewer E-positive cells in the transfected cells than the WT RNA (Figure 3A). For D7A or R140A mutants, the RNA-transfected cells did not produce infectious viruses, as suggested by plaque assay (Figure 3B) and RT-PCR (Figure 3C). Incubation of naïve Vero cells with culture supernatants from the D7A or R140A transfected cells did not generate any viral E-positive cells (Figure 3D). Replicon analysis showed that D7A or R140A significantly compromised viral RNA synthesis (Figure 3E). Since neither mutants could produce extracellular viruses (Figure 3B and C), and could replicate at attenuated levels inside cells (Figure 3E), D7A and R140A mutations may impair both viral RNA synthesis and virion production.

For R222A, the number of E-positive cells slightly increased in the genomic RNA-transfected cells from 24 to 72 h p.t. (Figure 3A). A low level of infectious virus was detected from the culture medium by plaque assay (Figure 3B) and RT-PCR (Figure 3C). These data were supported by the positive-IFA results after incubating naive Vero cells with the supernatant from the R222A RNA-transfected cells (Figure 3D). Sequencing the virus (collected at 72 h p.t.) revealed that, besides the engineered R222A mutation, the virus had acquired a second site mutation of L220F in NS2A (Figure 3C). Replicon analysis showed that R222A significantly lowered viral RNA synthesis (Figure 3E); no secondary mutation (i.e. L220F) was detected in the replicon RNA at 30 h p.t. (data not shown). These results suggest that (i) R222A mutant is defective in viral RNA synthesis and/or virion assembly, and (ii) a second site mutation L220F in NS2A could rescue the defects.

### *Class III and IV mutations do not affect NS2A maturation*

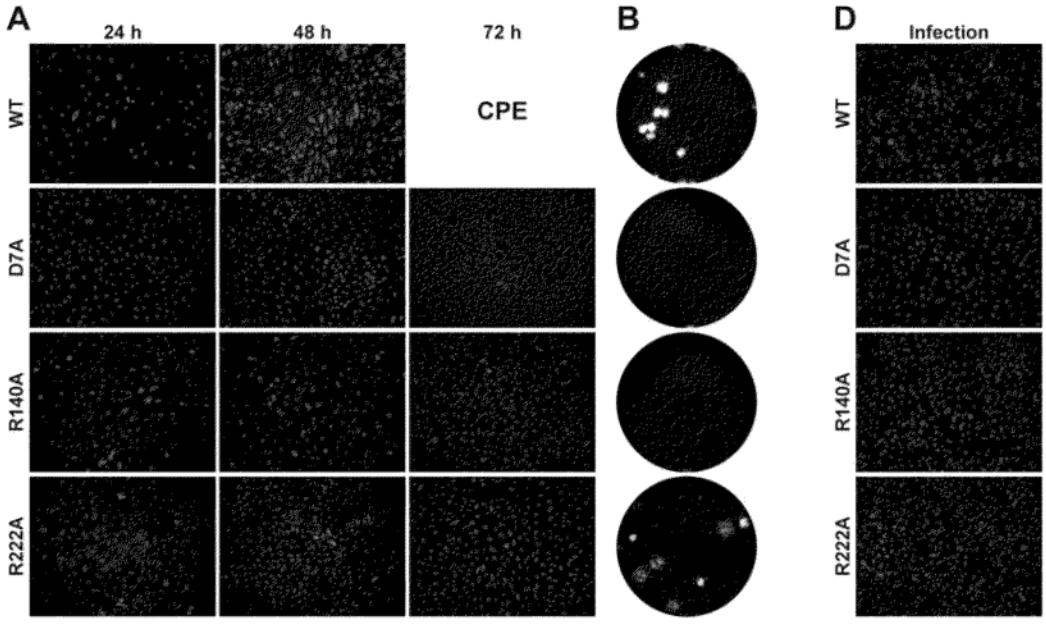
Since cleavage between NS1 and NS2A is essential for flavivirus RNA synthesis [30], we examined whether the replication defects of class III and IV mutants were caused by defective maturation of NS2A. To test this hypothesis, we introduced individual mutations into an expression plasmid pXJ-$E_{24}$-NS1-NS2A-HA that encodes a polyprotein containing the C-terminal 24 amino acids of the E protein (serving as a signal peptide to translocate NS1 to ER lumen), NS1, and NS2A with a C-terminal HA tag. After transfecting the plasmids into 293 T cells, the expression of un-cleaved NS1-NS2A-HA intermediate and cleaved NS2A-HA product were analyzed using Western blot (Figure 3F). None of the mutations significantly affected the cleavage efficiencies of NS1-NS2A-HA to NS2A-HA, indicating that the replication defects of class III and IV mutants are not caused by impaired NS2A cleavage.

### *Class V mutations block virion assembly*

Class V includes G71A, D73A, E103A, R171A, K192A, K193A, and a triple K25A/K26A/R27A. Cells transfected with class V mutant genomic RNAs yielded E-positive cells at 24 h p.t., but the number of E-positive cells did not increase afterward (Figure 4A), suggesting that the mutants are defective in virus spread. No infectious mutant viruses, except E103A, were detected in the supernatants of transfected cells by plaque assay (Figure 4B) or RT-PCR (Figure 4C). No IFA-positive

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C

| | Viral titer (IFU/ml) | | | Extracellular viral RNA RT-PCR | Second site mutation |
|---|---|---|---|---|---|
| | 24 h | 48 h | 72 h | | |
| WT | $(1.70\pm0.30)\times10^5$ | $(4.30\pm0.70)\times10^5$ | $(1.15\pm0.15)\times10^6$ | Positive | None |
| D7A | <10 | <10 | <10 | Negative | N.A. |
| R140A | <10 | <10 | <10 | Negative | N.A. |
| R222A | <10 | 35±15 | 400±100 | Positive | L220F(NS2A) |

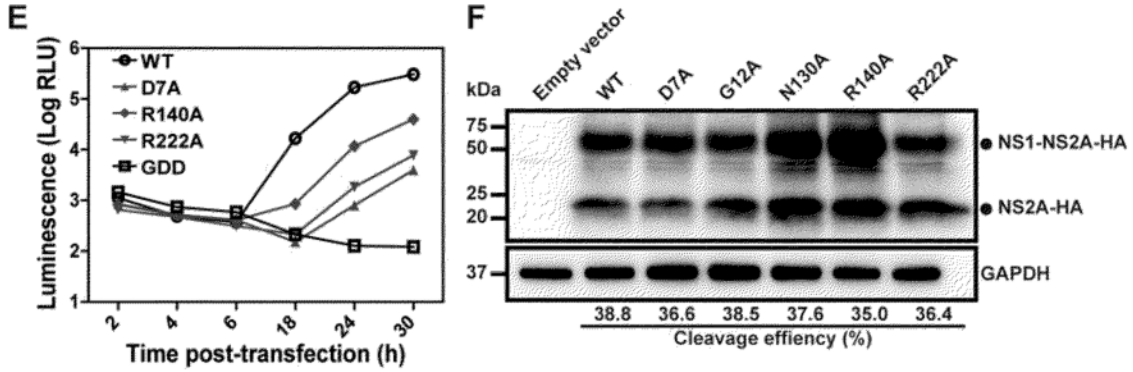
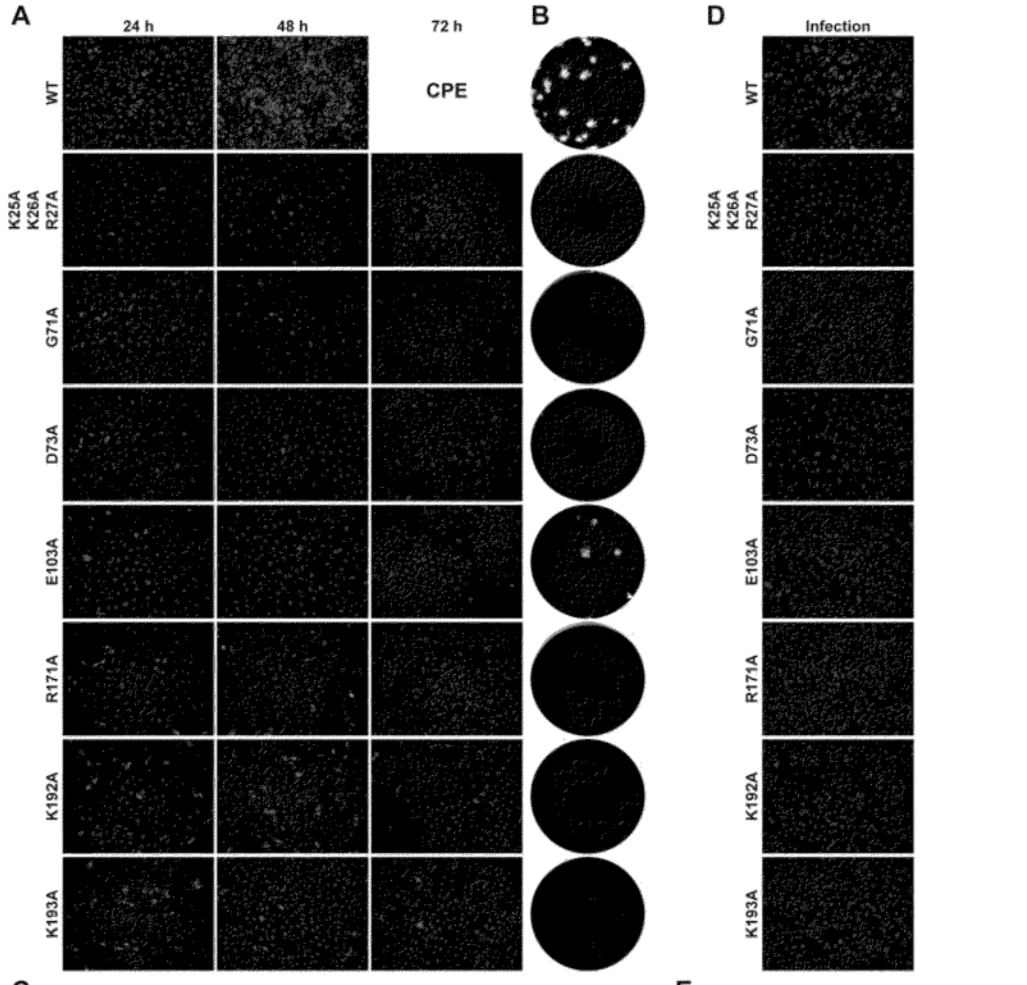



**Figure 3.** Characterization of class IV NS2A mutants. (A) IFA of transfected Vero cells. (B) Plaque morphologies of WT, D7A, R140A, and R222A viruses. (C) Extracellular viral titers were measured at 24, 48, and 72 h p.t. using plaque assay. At 72 h p.t., extracellular viral RNAs were subjected RT-PCR analysis. The RT-PCR products were sequenced to confirm the engineered mutations and to detect potential second site mutation(s) in the NS2A gene. N.A., not applicable. (D) The supernatants of transfected cells were collected on day 8 p.t. to infect naïve Vero cells. At 24 h p.t., the cells were analyzed for viral E expression by IFA. (E) Transient luciferase replicon analysis. Vero cells were electroporated with ZIKV replicon RNAs and measured for luciferase activities at indicated time points. An average of 3 experiments are presented. Bars represent standard deviations. (F) Analysis of NS2A proteolytic processes. NS2A D7A, G12A, N130A, R140A, and R222A mutations were individually engineered into expression plasmid pXJ-$E_{24}$-NS1-NS2A-HA. After transfecting the plasmids into 293 T cells, total cellular protein samples were harvested at 24 h p.t. and analyzed for NS2A process using Western blot. An HA-tag antibody was used to detect both unprocessed NS1-NS2A-HA and processed NS2A-HA proteins.

cells were detected after naïve cells were incubated with the supernatants from transfected cells (Figure 4D). Sequencing of the recovered E103A mutant virus revealed an A103D change in the NS2A gene (Figure 4C), suggesting that an E103A-to-A103D substitution is responsible for the rescue of viral replication.

To determine the stage of viral replication that was disrupted by the class V mutations, we analyzed the mutational effect on viral replication in ZIKV replicon (Figure 4E). Compared with the WT replicon, all class V mutants exhibited equivalent levels of viral translation and RNA synthesis (<4-fold difference),

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| | Viral titer (PFU/ml) | | | Extracellular viral RNA RT-PCR | Second site mutation |
|---|---|---|---|---|---|
| | 24h | 48h | 72h | | |
| Wild Type | $(1.7\pm0.3)\times10^5$ | $(4.3\pm0.7)\times10^5$ | $(1.15\pm0.15)\times10^6$ | Positive | None |
| K25AK26AR27A | <10 | <10 | <10 | Negative | N.A. |
| G71A | <10 | <10 | <10 | Negative | N.A. |
| D73A | <10 | <10 | <10 | Negative | N.A. |
| E103A | <10 | <10 | 175±25 | Positive | A103D(NS2A) |
| R171A | <10 | <10 | <10 | Negative | N.A. |
| K192A | <10 | <10 | <10 | Negative | N.A. |
| K193A | <10 | <10 | <10 | Negative | N.A. |

**Figure 4.** Characterization of class V NS2A mutants. (A) IFA of Vero cells that were transfected with WT or class V genomic RNAs. Viral E-positive cells are indicated in green. (B) Plaque morphologies of class V mutant viruses. Only the WT and E103A mutant generated plaques. (C) Supernatants from the RNA-transfected cells were quantified for viral titers by plaque assay. RT-PCR was also performed on the supernatants at day 3 p.t. for the WT virus and at day 8 p.t. for the mutants. Positive RT-PCR products were sequenced for potential second site mutation(s). N.A., not applicable. (D) Naïve Vero cells were infected with supernatants from the RNA-transfected cells and subjected to IFA for viral E protein expression. (E) Luciferase replicon analysis. Equal amounts of WT and mutant replicon RNAs were electroporated into Vero cells. Luciferase activities were measured at the indicated time points. (F) Analysis of intracellular infectious viruses. Intracellular viruses from ZIKV RNA-transfected cells were harvested at 24 h p.t. as previously described [46]. The harvested samples were used to infect naïve Vero cells. IFA was performed to examine viral E protein expression at 24 h post-infection.

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suggesting that the mutations only marginally affect viral RNA synthesis. Next, we tested if the class V genomic RNAs could generate intracellular infectious viruses. Incubating naïve Vero cells with WT RNA-transfected cell extracts yielded IFA-positive cells, whereas no IFA-positive cells were detected after incubating naive cells with the mutant RNA-transfected cell extracts (Figure 4F). Altogether, the results suggest that class V mutants are competent in viral RNA replication, but defective in assembly of infectious viruses.

### WT NS2A rescues virion assembly for class V mutants

To test *trans* complementation of NS2A mutants, we constructed a BHK-21 cell line that constitutively expressed WT ZIKV NS2A protein with a C-terminal HA-tagged (NS2A-HA). The cell line was constructed by transfecting plasmid pXJ-SPG-$C_{16}$-NS2A-HA encoding a Gaussia luciferase signal peptide (SPG), the C-terminal 16 amino acids of NS1 protein ($C_{16}$), and NS2A-HA, as well as a neomycin resistance gene (*Neo*). The SPG and $C_{16}$ sequences were engineered to enable the correct formation of NS2A membrane topology when expressed in cells. Individual BHK-NS2A-HA cell lines were generated through colony purification under G418 (1 mg/ml) selection. For each BHK-NS2A-HA cell line, all cells were IFA-positive for HA-NS2A expression (Figure 5A). Western blot revealed an expected band of ~22-kDa band (Figure 5B). The expression level of NS2A-HA did not decrease in the BHK-NS2A-HA cells after 20 passages in culture (4 days per passage; Figure 5B), indicating the stability of the cell line.

We tested *trans* complementation by transfecting class V genomic RNAs to BHK-NS2A-HA cells. The transfected cells developed an increasing number of E-positive cells from 48 to 96 h p.t. (Figure 5C). Culture supernatants were collected at 96 h p.t. to infect naïve Vero cells. The infected cells developed E-positive cells for all class V mutants (Figure 5D), demonstrating the rescue of infectious viruses through *trans* complementation. Quantification of the E-positive cells allowed us to determine the titers of rescued infectious viruses (Figure 5E). Among the class V mutants, E103A, K192A, and K193A exhibited the highest *trans* complementation efficiencies, producing $1.9 \times 10^5$–$3.04 \times 10^5$ IFU/ml viruses at 96 h p.i. (Figure 5E). Sequencing of the rescued virion genomes confirmed the retention of the engineered class V mutations without any other changes in NS2A (Figure 5E).

Next, supernatants from the first round of infection collected at 72 h p.i. (from Figure 5D) were used to infect naïve Vero cells for a second round. In contrast to the first round of infection (Figure 5D), no E-positive cells were detected in the second round of infection for any of the class V mutants (Figure 5F). As a positive control, WT RNA produced high numbers of E-positive cells in both the first and second rounds of infections (Figure 5D and F). Collectively, the results indicate that (i) the defect in virion assembly of class V mutants could be rescued through *trans* supply of WT NS2A protein and (ii) the rescued virions could only infect cells for a single round.

### DENV-2 NS2A could not trans complement ZIKV class V mutants

We tested whether DENV NS2A could rescue class V mutants of ZIKV. Each class V mutant of ZIKV was transfected into a previously established BHK-21 cell line that constitutively expresses WT DENV-2 NS2A protein [30]. For all class V mutants, no increase in E-positive cells or infections viruses was detected from the transfected cells from 24 to 96 h p.t. (data not shown). The results suggest that flavivirus NS2A modulates virion assembly in a virus-type specific manner.

### WT NS2A could not rescue viral RNA synthesis of class III mutants

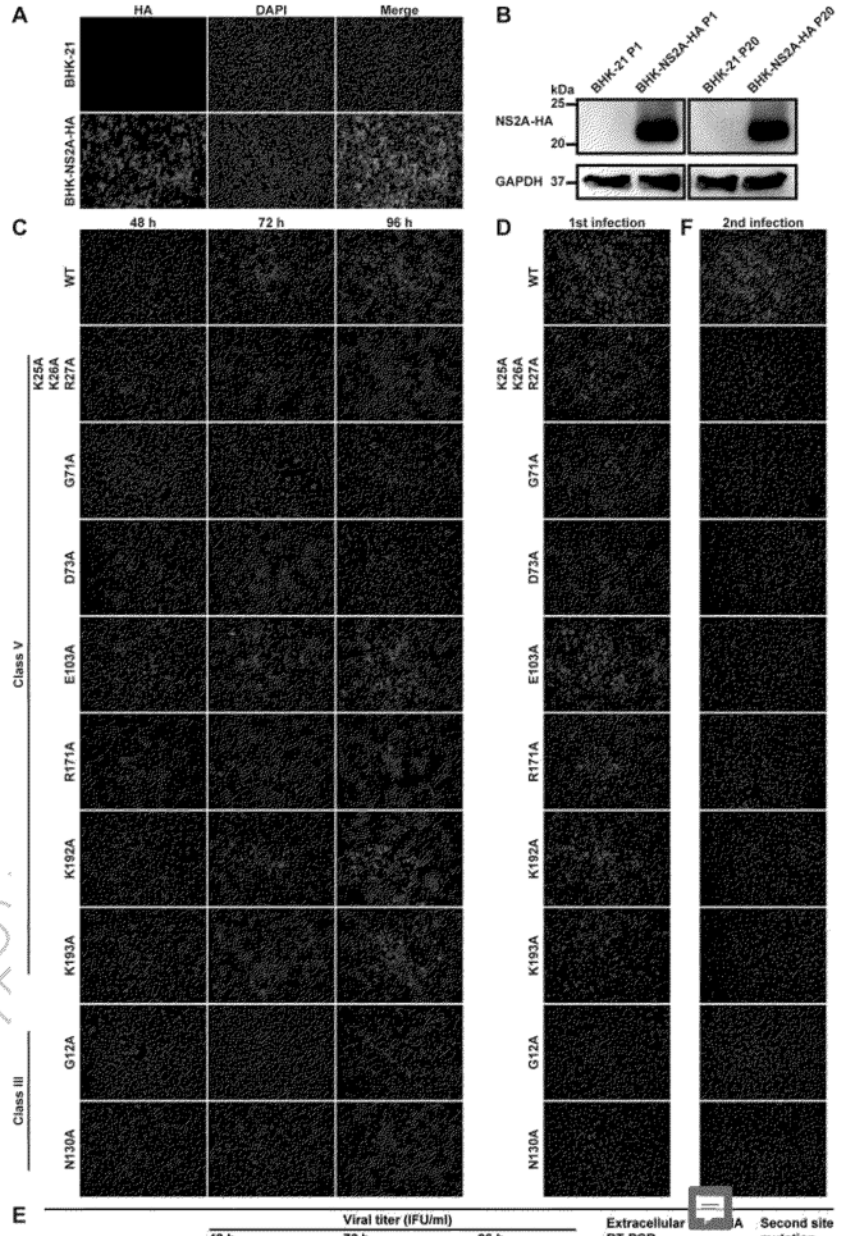
We tested whether the defect in viral RNA synthesis of class III mutants could be *trans* complemented. To test this possibility, we transfected class III genomic RNAs (G12A and N130A) into ZIKV BHK-NS2A-HA cells. Neither mutants generated any E-positive cells in the transfected BHK-NS2A-HA cells (Figure 5C). Infection of naïve Vero cells with supernatants of the transfected cells did not produce any IFA-positive cells (Figure 5D–F). The results suggest that the RNA synthesis defect of class III mutants could not be rescued through *trans* complementation. Due to the dual defects of class IV mutants in both viral RNA synthesis and virion assembly, we did not pursue *trans* complementation experiments for this class of mutants; such complementation experiments may not reveal clear mechanistic insights as compared with the class III and V mutants.

### Probing the topology of ZIKV NS2A on the ER membrane

The distinct functions of amino acids in viral RNA synthesis and virion assembly prompted us to probe the topology of ZIKV NS2A on ER membrane. Bioinformatics analysis suggests that ZIKV NS2A may form seven transmembrane segments (pTMS1–7; Figure 6A). To examine whether each pTMS associates with the ER membrane, we constructed a panel of plasmids encoding full-length or various NS2A fragments with a C-terminally fused eGFP reporter (Figure 6B). The length of each segment was slightly modified from the bioinformatic prediction to allow some

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E

| | | Viral titer (IFU/ml) | | | Extracellular viral RNA | Second site mutation |
|---|---|---|---|---|---|---|
| | | 48 h | 72 h | 96 h | RT-PCR | |
| Class V | K25AK26AR27A | $(7.16\pm1.01)\times10^3$ | $(9.69\pm2.65)\times10^3$ | $(5.97\pm1.18)\times10^4$ | Positive | None |
| | G71A | $(1.48\pm0.78)\times10^3$ | $(1.97\pm0.66)\times10^3$ | $(5.42\pm0.66)\times10^3$ | Positive | None |
| | D73A | $(2.10\pm0.57)\times10^2$ | $(1.97\pm0.12)\times10^3$ | $(8.60\pm1.10)\times10^3$ | Positive | None |
| | E103A | $(1.13\pm0.14)\times10^4$ | $(2.30\pm0.20)\times10^4$ | $(3.04\pm0.25)\times10^5$ | Positive | None |
| | R171A | $(6.41\pm1.58)\times10^3$ | $(8.90\pm0.32)\times10^3$ | $(6.06\pm1.06)\times10^4$ | Positive | None |
| | K192A | $(7.85\pm2.04)\times10^3$ | $(1.90\pm0.02)\times10^4$ | $(2.17\pm0.17)\times10^5$ | Positive | None |
| | K193A | $(2.69\pm0.32)\times10^3$ | $(1.85\pm0.05)\times10^4$ | $(1.90\pm0.08)\times10^5$ | Positive | None |
| Class III | G12A | <10 | <10 | <10 | Negative | N.A. |
| | N130A | <10 | <10 | <10 | Negative | N.A. |

**Figure 5.** Trans complementation of class III and V mutants. (A) IFA of BHK-NS2A-HA cells. The BHK-NS2A-HA cells constitutively express WT ZIKV NS2A fused with a C-terminal HA-tag. The cells were fixed with paraformaldehyde and stained with an HA-tag antibody. (B) Western blot of BHK-NS2A-HA cells. The expression of WT NS2A-HA from BHK-NS2A-HA cells was detected using an HA-tag antibody. Both passage one (P1) and 20 (P20) BHK-NS2A-HA cells were analyzed. Naïve BHK-21 cells were included as a negative control. (C) WT and class III and V genomic RNAs were electroporated into BHK-NS2A-HA cells. IFA was performed on the transfected cells to detect viral E protein expression. (D) Viruses rescued from the BHK-NS2A-HA cells (harvested at 96 h p.t.) were used to infect naïve Vero cells. The infected Vero cells were subjected to IFA for E protein expression. (E) Extracellular viruses were harvested from the supernatants of RNA-transfected BHK-NS2A-HA cells. The harvested viruses were serially diluted to infect naïve Vero cells in 96-well plates. At 24 h p.i., IFA was performed to quantify the viral titers by counting the number of E-positive cells. The results show averages and standard deviations from triplicate samples. Viral RNAs in the supernatants of transfected BHK-NS2A-HA cells were isolated at 96 h p.t. and subjected to RT-PCR. Positive RT-PCR products were sequenced to confirm the engineered mutations and to identify potential second site change(s). (F) The supernatants of infected Vero cells from (D) were collected at 72 h p.i. to infect naïve Vero cells for a second round. IFA was performed on these cells for viral E-positive cells.

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flexibility between pTMS and eGFP. Upon transfection into Vero cells, eGFP alone (without any NS2A fragment) exhibited an even distribution of fluorescence throughout the cell, whereas the full-length NS2A-eGFP (construct 1–226) displayed fluorescence co-localized with calnexin (an ER membrane protein) in the perinuclear region (Figure 6B), indicating that ZIKV NS2A is an integral membrane protein. Expression of seven fragments spanning each pTMS, represented by constructs 1–25 (pTMS1), 30–58 (pTMS2), 74–97 (pTMS3), 101–123 (pTMS4), 125–145 (pTMS5), 150–185 (pTMS6), and 198–221 (pTMS7), exhibited fluorescence patterns that co-localized with calnexin in the perinuclear region (Figure 6B). The results indicate that individual of the seven pTMSes is associated with the ER membrane. In contrast, expression of fragments between different pTMS, represented by constructs 55–73, 186–197, and 221–226, showed fluorescence throughout the cells (Figure 6B), indicating that these fragments are not membrane-associated.

An *in vitro* deglycosylation analysis was performed to determine the orientation of each NS2A pTMS on the ER membrane. A panel of plasmids were prepared to express truncated NS2A proteins that were C-terminally fused with an eGFP followed by an N-glycosylation acceptor peptide (Glyc; Figure 7A). An SPG-$C_{16}$ peptide (Gaussia luciferase signal peptide SPG and the C-terminal 16 amino acids of NS1) was fused to the N terminus of each NS2A-eGFP-Glyc construct to ensure a proper translocation of the expressed protein on the ER membrane. Western blot showed that, upon transfection into BHK-21 cells, all constructs expressed fusion proteins with expected molecular masses (Figure 7B). Because N-glycosylation enzymes reside in the ER lumen, the glycosylation status could be used to indicate the location of the C-terminus of each NS2A-eGFP-Glyc protein (Figure 7C). As a negative control, treatment of EGFP-Glyc (without any viral sequence) with PNGase F (an enzyme that removes high mannose and complex carbohydrates) did not change the mobility of the protein (Figure 7D), indicating a cytosol localization of EGFP-Glyc. As a positive control, PNGaseF treatment increased the mobility of SPG-$C_{16}$-EGFP-Glyc (construct 0), confirming that the C-terminus of this protein resides in the ER lumen. Among the NS2A constructs, PNGase F treatment increased the mobility of fusion proteins 1–26 and 1–56, but not 1–103, 1–123, 1–147, 1–192, and 1–226 (Figure 7D). The results suggest that the N-terminal 56 amino acids of NS2A reside in the ER lumen, and the C-terminal region from residues 103–226 is located in the cytosol.

Next, we performed an *in vitro* proteinase K protection assay [46] to validate the above *in vitro* deglycosylation results. BHK-21 cells were transfected with the same constructs depicted in Figure 7A. At 24 h p.t., membrane fractions from the transfected cells were treated with proteinase K in the presence or absence of 0.5% Triton X-100 and analyzed by Western blot using an eGFP antibody. In the absence of Triton X-100, the eGFP residing in the cytosol would be susceptible to proteinase K digestion, whereas the eGFP residing in the ER lumen would be protected from proteinase K digestion (Figure 7E). As shown in Figure 7F, the eGFPs from constructs 0, 1–26, and 1–56 were protected from protease K digestion, again indicating that the first 56 residues are located in the ER lumen. In contrast, the eGFPs from constructs 1–103, 1–123, 1–147, 1–192, 1–226, and control eGFP-Glyc were completely degraded by the protease K treatment (Figure 7F), supporting that the C-terminal region from residues 103–226 is located in the cytosol. As expected, in the presence of Triton X-100, the eGFPs from all constructs were degraded by proteinase K (Figure 7F). The results of proteinase K assay are in full agreement with those of deglycosylation assay.

### *A topology model for ZIKV NS2A*

The above biochemical results support a topology model for ZIKV NS2A on the ER membrane (Figure 8A): (i) The N-terminal region (including pTMS1 and pTMS2) resides in the ER lumen; (ii) the region spanning residues 74–97 (pTMS3) traverses the ER membrane in a lumen-to-cytosol direction; and (iii) the C-terminal region from residues 103–226 (including pTMS4 to pTMS7) is located in the cytosol side. Since pTMS1, pTMS2, pTMS4, PTMS5, PTM6, and pTMS7 associate with ER membraned when expressed individually (Figure 6B), they may interact with the ER membrane in a peripheral manner. In support of this notion, informatics analyses using Jpred 4 and HeliQuest programmes suggest that amino acids 9–26 (pTMS1), 41–58 (pTMS2), 104–121 (pTMS4), 125–142 (pTMS5), 172–189 (pTMS6), and 203–220 (pTM7) may form amphipathic helices with hydrophobic and hydrophilic surfaces (Figure 8B), where the hydrophobic surface may interact with the ER membrane.

## Discussion

Flavivirus NS2A functions in viral RNA replication, virion assembly, evasion of innate immune response, and disease pathogenesis in a virus-specific manner [35]. This is evidenced by its high sequence diversity (Figure 1A), distinct membrane topology [35,46], and association with different disease pathways [41,42]. Thus, it is critical to perform a systematic genetic and biochemical characterization of ZIKV NS2A to delineate its structure and function. Our biochemical results showed that, unlike other flaviviruses, ZIKV NS2A has only a single transmembrane segment that traverses the

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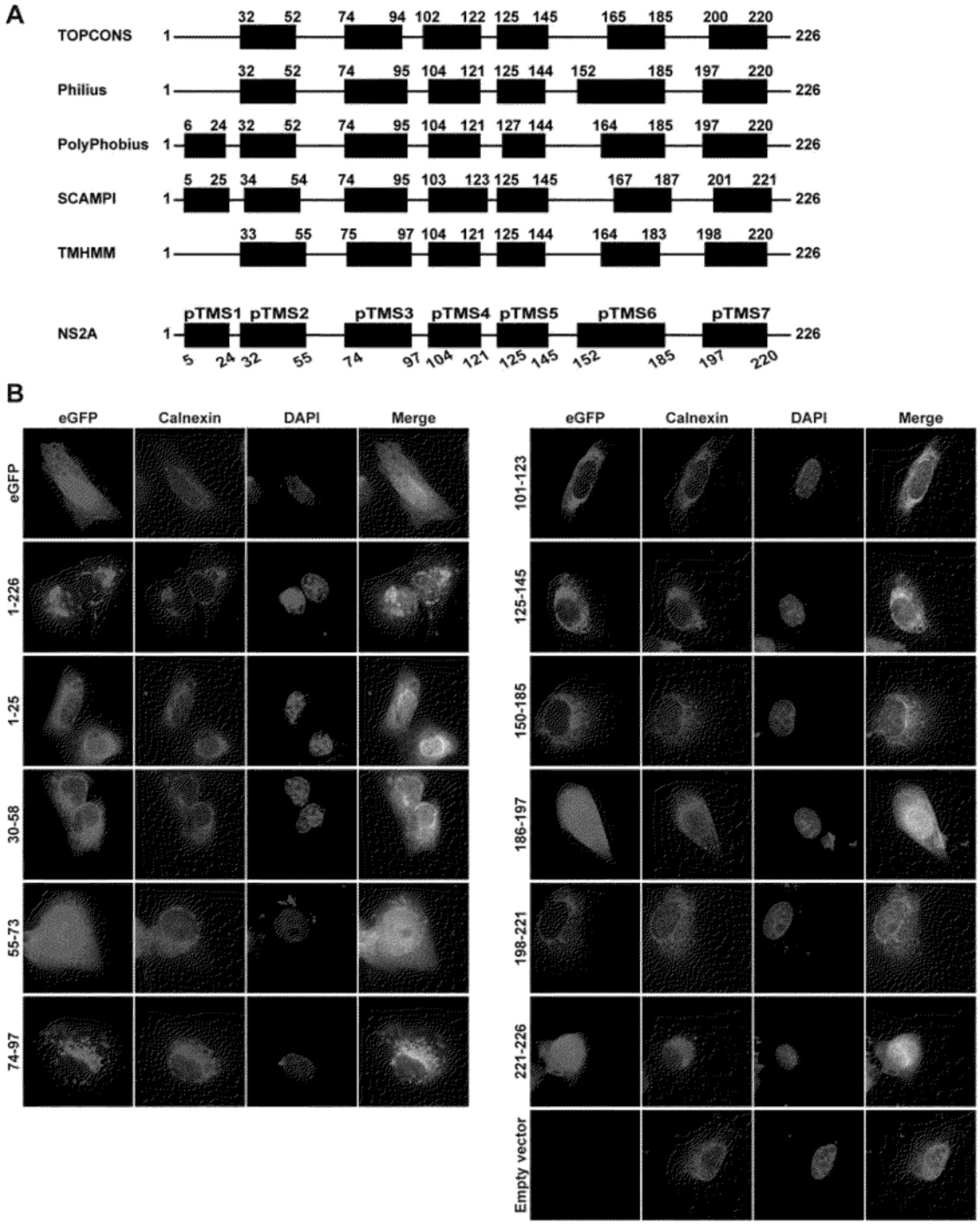



**Figure 6.** Membrane association of ZIKV NS2A transmembrane segments. (A) Bioinformatics analysis of ZIKV NS2A for transmembrane segments. ZIKV NS2A sequence was analyzed by bioinformatics programme TOPCONS, Philius, PolyPhobius, SCAMPI, and TMHMM. The compiled transmembrane segments are shown at the bottom. Seven predicted transmembrane segments (pTMS) are presented as thick bars. The amino acid positions of ZIKV NS2A are indicated. (B) Membrane association of individual pTMS. Expression plasmid pXJ-eGFP (without NS2A sequence) and pXJ-smNS2A-eGFP (smNS2A represents different NS2A segments) were transfected into Vero cell by using X-tremeGENE 9 DNA transfection reagent (Roche). For each plasmid construct, the NS2A segment is indicated by amino acid positions. At 24 h p.t., IFA was performed to monitor eGFP expression. ER and nuclei were stained with anti-Calnexin antibody and DAPI, respectively. The merged images are shown on the right panels.

ER membrane, the other six pTMSes are peripherally associated with the ER membrane (Figure 8A). Our functional analysis revealed five classes of mutants with distinct replication phenotypes, among which class III, IV, and V mutants could not produce infectious viruses through different mechanisms (Figure 1B). The critical mutations from class III to V are not clustered in specific regions of NS2A (Figure 8A),

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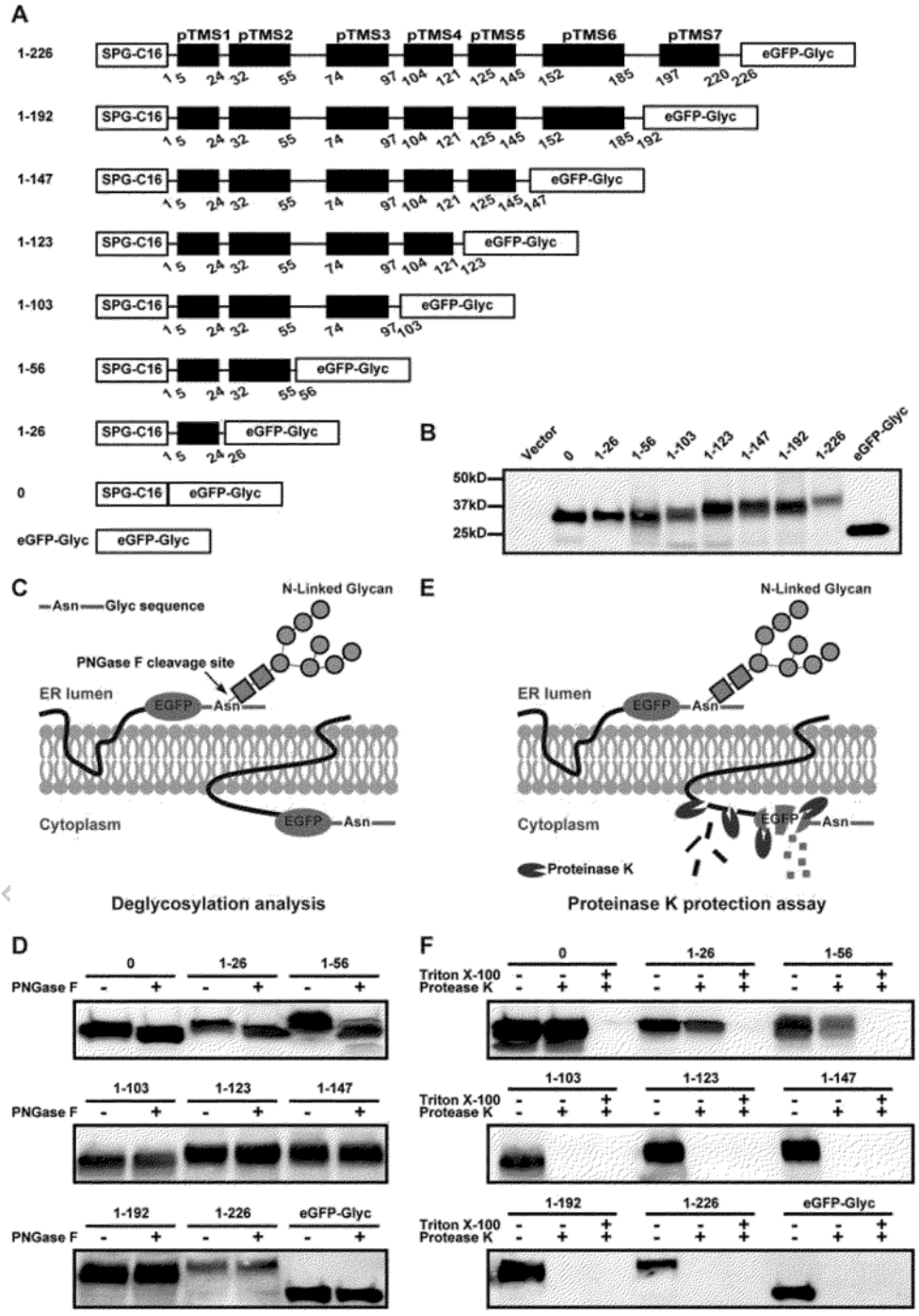



**Figure 7.** Probing the membrane topology of ZIKV NS2A by in vitro deglycosylation assay and in vitro proteinase K protection assay. (A) Schematic representation of C-terminally truncated ZIKV NS2A proteins. The amino acid positions of each truncated NS2A are indicated. A 'SPG-C16' sequence was fused to the N terminus of NS2A to ensure correct translocation of NS2A on the ER membrane. An 'eGFP-Glyc' sequence was fused to the C terminus of NS2A to probe its location at cytosol or ER lumen. See text for details. (B) BHK-21 cells were transfected with the expression plasmids described in (A). At 24 h p.t., the expression levels of various NS2A truncates were examined by Western blot using an eGFP antibody. The amino acid positions are indicated for each NS2A truncates. The molecular masses of marker proteins are shown to the left of Western blot image. (C) Diagram of glycosylation of eGFP-Glyc when located at ER lumen (*left*). No glycosylation occurs when eGFP-Glyc is located at cytosol (*right*). The glycosylation residue Asn is indicated. (D) *In vitro* deglycosylation assay. Plasmid-transfected cells were treated with PNGase F or PBS according to the protocol of *in vitro* deglycosylation assay (see Materials and Methods). The samples were examined for glycosylation status by Western blot using an eGFP antibody. (E) Diagram of *in vitro* proteinase K protection assay. When eGFP is located at ER lumen, it is protected from proteinase K cleavage (*left*); when located in the cytosol, eGFP is degraded by proteinase K (*right*). (F) *In vitro* proteinase K protection assay. Cell lysates from plasmid-transfected BHK-21 cells were treated with proteinase K or PBS according to the protocol of *in vitro* proteinase K protection assay (see Materials and Methods). The samples were detected for eGFP by Western blot using an eGFP antibody. Triton X-100 treatment is indicated.

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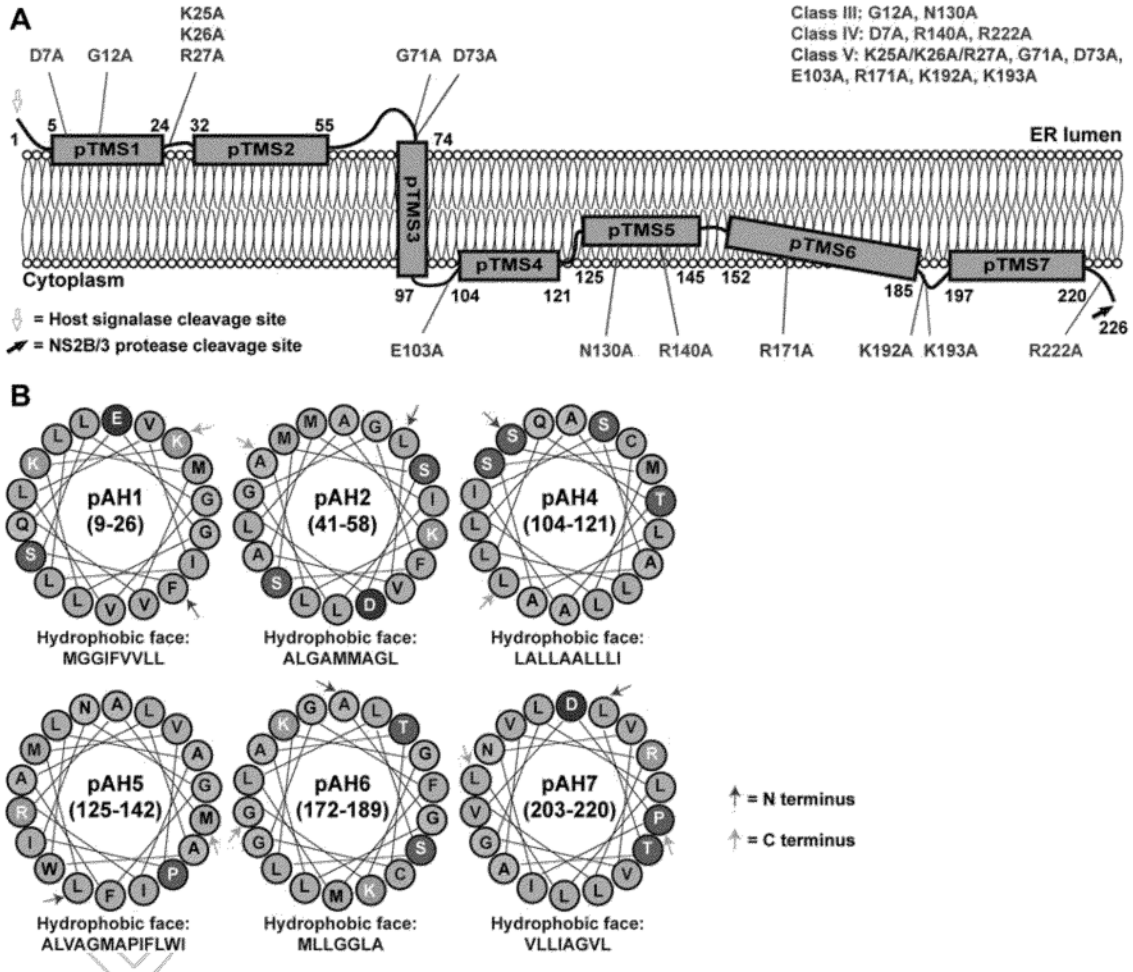



**Figure 8.** A topological model of ZIKV NS2A. (A) A model of ZIKV NS2A membrane topology. See text for details. Class III, IV, and V mutations are depicted on the topology model. (B) Helical wheel representations of NS2A fragment 9–26, 41–58, 104–121, 125–142, 172–189, and 203–220. The amphiphilic helix regions were analyzed using Heliquest server (heliquest.ipmc.cnrs.fr/). The best 18-residue windows are presented. The amino acids forming the hydrophobic surface of each helical wheel are depicted. Yellow, gray, blue, red, purple, pink, and green circles indicate nonpolar, glycine/alanine, positively charged, negatively charged, polar, amide, and proline amino acids, respectively. The first and last residues in each helical wheel are indicated by a blue and red arrow, respectively.

suggesting that multiple domains of NS2A are essential for viral replication.

The membrane topology of ZIKV NS2A is in stark contrast with the topologies of other flavivirus NS2A proteins: DENV-2 NS2A has five membrane-traversing segments [46] and YFV NS2A has three membrane-traversing segments [35]. Interestingly, these topologically diverse flavivirus NS2A proteins are able to converge their functions in viral RNA replication, virion assembly, and evasion of immune response. High resolution, three-dimensional structures of flavivirus NS2A are needed to address this question. It should be noted that the membrane topology of NS2A presented here is in the absence of other viral proteins. In the context of viral replication, the NS2A protein is associated with various viral and cellular proteins that would modulate the structure of NS2A and perform biological functions.

Our mutagenesis study has identified distinct NS2A mutations that selectively abolish ZIKV RNA synthesis (class III) or virion assembly (class V), among which only the virion assembly-defective mutants could be rescued through *trans* complementation (Figure 5). The results suggest that there are two species of NS2A molecules in ZIKV-infected cells. One NS2A species is located at the viral replication complex that could only be formed in *cis*; another NS2A species is located at virion assembly complex that could be formed in *trans* or *cis*. These results are in agreement with the previous studies from DENV and YFV NS2A [30,35,36]. Compared with the viral replication complex, the virion assembly complex is much less well defined for flaviviruses. Previously studies showed that second-site mutations in prM, E, NS2B, and NS3 could rescue the defect of virion assembly caused by DENV-2 and YFV NS2A mutations, suggesting that these viral proteins

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participate in virion assembly. Besides the genetic evidence, the role of NS3 in virion assembly is also supported by (i) mutations in the NS3 helicase domain alone affect YFV assembly [49], (ii) the helicase domain of DENV or YFV NS3 directly binds to NS2A [35,36], (iii) the cleavage between C and prM is tightly modulated by NS2B/NS3 protease and cellular signalase during YFV and Murray Valey encephalitis virus assembly [50–52]. These data, together with the result that recombinant NS2A of Kunjin binds to viral 3'UTR RNA [29], suggest a flavivirus assembly model. During flavivirus assembly, the NS2A protein recruits NS2B/NS3 through binding to the NS3 helicase domain. Once the NS2A-NS2B/NS3 complex reaches the virion assembly site, the NS3B/NS3 protease cleaves the C protein from the C/prM/E polyprotein. The C protein assembles onto genomic RNA (presented by NS2A) to form nucleocapsid, which is subsequently enveloped by prM/E complex to form virions. Since flavivirus assembly has to be coupled to viral RNA replication [53], the newly synthesized genomic RNA has to be transported from the replication site to the virion assembly site [54], possibly through the NS2A protein.

The *trans* complemented class V mutants could potentially be used as vaccine candidates, as recently reported for DENV-2 [36]. Since the *trans* complemented virions are able to infect cells for only a single cycle, such vaccine platform is safer than the multi-round infectious live-attenuated vaccines [55,56]. This is particularly important for ZIKV vaccine when immunizing immune-compromised individuals and pregnant women [57]. However, for development of class V mutants as a vaccine platform, the *trans* complementation efficiency needs to be improved to higher titers before they could be tested for *in vivo* efficacy.

In summary, we report the genetic and biochemical characterization of ZIKV NS2A protein. The results indicate that different flavivirus NS2A proteins have distinct membrane topologies, but converge to function in viral RNA synthesis and virion assembly. Together with previous results, we propose a model for flavivirus assembly in which NS2A plays a central role in mediating viral structural and nonstructural proteins as well as genomic RNA during virion formation.

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## Disclosure statement

No potential conflict of interest was reported by the authors.

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| | ORCID | http://orcid.org/0000-0002-2387-9287 |
| Author | Family Name | **Burt** |
| | Particle | |
| | Given Name | **Felicity J.** |
| | Suffix | |
| | Division | Division of Virology, National Health Laboratory Service and Division of Virology |
| | Organization | University of the Free State |
| | Address | Bloemfontein, Republic of South Africa |
| | Phone | |

FOIA Confidential Treatment Requested by Univ of Texas System UTSystem_054250

| | | |
|---|---|---|
| | Fax | |
| | Email | |
| | URL | |
| | ORCID | http://orcid.org/0000-0002-7238-7799 |
| Author | Family Name | **Calisher** |
| | Particle | |
| | Given Name | **Charles H.** |
| | Suffix | |
| | Division | |
| | Organization | Colorado State University |
| | Address | Fort Collins, CO, USA |
| | Phone | |
| | Fax | |
| | Email | |
| | URL | |
| | ORCID | |
| Author | Family Name | **Cháng** |
| | Particle | |
| | Given Name | **Chénchén** |
| | Suffix | |
| | Division | Xinjiang Key Laboratory of Biological Resources and Genetic Engineering, College of Life Science and Technology |
| | Organization | Xinjiang University |
| | Address | Ürümqi, China |
| | Phone | |
| | Fax | |
| | Email | |
| | URL | |
| | ORCID | |
| Author | Family Name | **Charrel** |
| | Particle | |
| | Given Name | **Rémi N.** |
| | Suffix | |
| | Division | |
| | Organization | Unité des Virus Emergents (Aix-Marseille Univ-IRD 190-Inserm 1207-IHU Méditerranée Infection) |
| | Address | Marseille, France |
| | Phone | |
| | Fax | |
| | Email | |
| | URL | |
| | ORCID | http://orcid.org/0000-0002-7675-8251 |
| Author | Family Name | **Choi** |
| | Particle | |
| | Given Name | **Il Ryong** |
| | Suffix | |

FOIA Confidential Treatment Requested by Univ of Texas System UTSystem_054251

| | | |
|---|---|---|
| | Division | |
| | Organization | Plant Breeding Genetics and Biotechnology Division and International Rice Research Institute |
| | Address | Los Baños, Philippines |
| | Phone | |
| | Fax | |
| | Email | |
| | URL | |
| | ORCID | |
| Author | Family Name | **Clegg** |
| | Particle | |
| | Given Name | **J. Christopher S.** |
| | Suffix | |
| | Division | |
| | Organization | Les Mandinaux |
| | Address | Le Grand Madieu, France |
| | Phone | |
| | Fax | |
| | Email | |
| | URL | |
| | ORCID | |
| Author | Family Name | **Torre** |
| | Particle | **de la** |
| | Given Name | **Juan Carlos** |
| | Suffix | |
| | Division | Department of Immunology and Microbiology IMM-6 |
| | Organization | The Scripps Research Institute |
| | Address | La Jolla, CA, USA |
| | Phone | |
| | Fax | |
| | Email | |
| | URL | |
| | ORCID | http://orcid.org/0000-0002-8171-8115 |
| Author | Family Name | **Lamballerie** |
| | Particle | **de** |
| | Given Name | **Xavier** |
| | Suffix | |
| | Division | |
| | Organization | Unité des Virus Emergents (Aix-Marseille Univ-IRD 190-Inserm 1207-IHU Méditerranée Infection) |
| | Address | Marseille, France |
| | Phone | |
| | Fax | |
| | Email | |
| | URL | |
| | ORCID | |

FOIA Confidential Treatment Requested by Univ of Texas System UTSystem_054252

| | | |
|---|---|---|
| Author | Family Name | **Dèng** |
| | Particle | |
| | Given Name | **Fēi** |
| | Suffix | |
| | Division | State Key Laboratory of Virology |
| | Organization | Wuhan Institute of Virology, Chinese Academy of Sciences |
| | Address | Wuhan, China |
| | Phone | |
| | Fax | |
| | Email | |
| | URL | |
| | ORCID | http://orcid.org/0000-0002-5385-083X |
| Author | Family Name | **Serio** |
| | Particle | **Di** |
| | Given Name | **Francesco** |
| | Suffix | |
| | Division | |
| | Organization | Istituto per la Protezione Sostenibile delle Piante, Consiglio Nazionale delle Ricerche |
| | Address | Bari, Italy |
| | Phone | |
| | Fax | |
| | Email | |
| | URL | |
| | ORCID | http://orcid.org/0000-0003-2822-704X |
| Author | Family Name | **Digiaro** |
| | Particle | |
| | Given Name | **Michele** |
| | Suffix | |
| | Division | |
| | Organization | Istituto Agronomico Mediterraneo di Bari |
| | Address | Valenzano, Italy |
| | Phone | |
| | Fax | |
| | Email | |
| | URL | |
| | ORCID | |
| Author | Family Name | **Drebot** |
| | Particle | |
| | Given Name | **Michael A.** |
| | Suffix | |
| | Division | Zoonotic Diseases and Special Pathogens |
| | Organization | National Microbiology Laboratory, Public Health Agency of Canada |
| | Address | Winnipeg, MB, Canada |
| | Phone | |

FOIA Confidential Treatment Requested by Univ of Texas System UTSystem_054253

| | | |
|---|---|---|
| | Fax | |
| | Email | |
| | URL | |
| | ORCID | |
| Author | Family Name | **Duàn** |
| | Particle | |
| | Given Name | **Xiǎoméi** |
| | Suffix | |
| | Division | Xinjiang Key Laboratory of Biological Resources and Genetic Engineering, College of Life Science and Technology |
| | Organization | Xinjiang University |
| | Address | Ürümqi, China |
| | Phone | |
| | Fax | |
| | Email | |
| | URL | |
| | ORCID | |
| Author | Family Name | **Ebihara** |
| | Particle | |
| | Given Name | **Hideki** |
| | Suffix | |
| | Division | Department of Molecular Medicine |
| | Organization | Mayo Clinic |
| | Address | Rochester, MN, USA |
| | Phone | |
| | Fax | |
| | Email | |
| | URL | |
| | ORCID | http://orcid.org/0000-0002-2576-9735 |
| Author | Family Name | **Elbeaino** |
| | Particle | |
| | Given Name | **Toufic** |
| | Suffix | |
| | Division | |
| | Organization | Istituto Agronomico Mediterraneo di Bari |
| | Address | Valenzano, Italy |
| | Phone | |
| | Fax | |
| | Email | |
| | URL | |
| | ORCID | |
| Author | Family Name | **Ergünay** |
| | Particle | |
| | Given Name | **Koray** |
| | Suffix | |

FOIA Confidential Treatment Requested by Univ of Texas System UTSystem_054254

| | | |
|---|---|---|
| | Division | Virology Unit, Department of Medical Microbiology |
| | Organization | Hacettepe University, Faculty of Medicine |
| | Address | Ankara, Turkey |
| | Phone | |
| | Fax | |
| | Email | |
| | URL | |
| | ORCID | http://orcid.org/0000-0001-5422-1982 |
| Author | Family Name | **Fulhorst** |
| | Particle | |
| | Given Name | **Charles F.** |
| | Suffix | |
| | Division | |
| | Organization | University of Texas Medical Branch |
| | Address | Galveston, TX, USA |
| | Phone | |
| | Fax | |
| | Email | |
| | URL | |
| | ORCID | |
| Author | Family Name | **Garrison** |
| | Particle | |
| | Given Name | **Aura R.** |
| | Suffix | |
| | Division | |
| | Organization | United States Army Medical Research Institute of Infectious Diseases, Fort Detrick |
| | Address | Frederick, MD, USA |
| | Phone | |
| | Fax | |
| | Email | |
| | URL | |
| | ORCID | http://orcid.org/0000-0003-3034-6400 |
| Author | Family Name | **Gão** |
| | Particle | |
| | Given Name | **George Fú** |
| | Suffix | |
| | Division | National Institute for Viral Disease Control and Prevention |
| | Organization | Chinese Center for Disease Control and Prevention |
| | Address | Beijing, China |
| | Phone | |
| | Fax | |
| | Email | |
| | URL | |
| | ORCID | |

FOIA Confidential Treatment Requested by Univ of Texas System UTSystem_054255

| | | |
|---|---|---|
| Author | Family Name | **Gonzalez** |
| | Particle | |
| | Given Name | **Jean-Paul J.** |
| | Suffix | |
| | Division | Center of Excellence for Emerging and Zoonotic Animal Disease |
| | Organization | Kansas State University |
| | Address | Manhattan, KS, USA |
| | Phone | |
| | Fax | |
| | Email | |
| | URL | |
| | ORCID | http://orcid.org/0000-0003-3063-1770 |
| Author | Family Name | **Groschup** |
| | Particle | |
| | Given Name | **Martin H.** |
| | Suffix | |
| | Division | Institute of Novel and Emerging Infectious Diseases |
| | Organization | Friedrich-Loeffler-Institut |
| | Address | Greifswald-Insel Riems, Germany |
| | Phone | |
| | Fax | |
| | Email | |
| | URL | |
| | ORCID | http://orcid.org/0000-0003-0215-185X |
| Author | Family Name | **Günther** |
| | Particle | |
| | Given Name | **Stephan** |
| | Suffix | |
| | Division | Department of Virology |
| | Organization | Bernhard-Nocht Institute for Tropical Medicine, WHO Collaborating Centre for Arboviruses and Hemorrhagic Fever Reference and Research |
| | Address | Hamburg, Germany |
| | Phone | |
| | Fax | |
| | Email | |
| | URL | |
| | ORCID | http://orcid.org/0000-0002-6562-0230 |
| Author | Family Name | **Haenni** |
| | Particle | |
| | Given Name | **Anne-Lise** |
| | Suffix | |
| | Division | |
| | Organization | Institut Jacques Monod, CNRS-Université Paris-Diderot |
| | Address | Paris, France |
| | Phone | |

FOIA Confidential Treatment Requested by Univ of Texas System UTSystem_054256

| | | |
|---|---|---|
| | Fax | |
| | Email | |
| | URL | |
| | ORCID | |
| Author | Family Name | **Hall** |
| | Particle | |
| | Given Name | **Roy A.** |
| | Suffix | |
| | Division | |
| | Organization | Australian Infectious Diseases Research Centre, School of Chemistry and Molecular Biosciences, The University of Queensland |
| | Address | Brisbane, Australia |
| | Phone | |
| | Fax | |
| | Email | |
| | URL | |
| | ORCID | |
| Author | Family Name | **Hepojoki** |
| | Particle | |
| | Given Name | **Jussi** |
| | Suffix | |
| | Division | Department of Virology |
| | Organization | University of Helsinki, Faculty of Medicine, Medicum |
| | Address | Helsinki, Finland |
| | Division | Institute of Veterinary Pathology |
| | Organization | Vetsuisse Faculty, University of Zurich |
| | Address | Zurich, Switzerland |
| | Phone | |
| | Fax | |
| | Email | |
| | URL | |
| | ORCID | http://orcid.org/0000-0001-5699-214X |
| Author | Family Name | **Hewson** |
| | Particle | |
| | Given Name | **Roger** |
| | Suffix | |
| | Division | |
| | Organization | Public Health England |
| | Address | Porton Down, Wiltshire, Salisbury, UK |
| | Phone | |
| | Fax | |
| | Email | |
| | URL | |
| | ORCID | http://orcid.org/0000-0003-2273-3152 |
| Author | Family Name | **Hú** |

FOIA Confidential Treatment Requested by Univ of Texas System UTSystem_054257

| | | |
|---|---|---|
| | Particle | |
| | Given Name | **Zhihóng** |
| | Suffix | |
| | Division | State Key Laboratory of Virology |
| | Organization | Wuhan Institute of Virology, Chinese Academy of Sciences |
| | Address | Wuhan, China |
| | Phone | |
| | Fax | |
| | Email | |
| | URL | |
| | ORCID | http://orcid.org/0000-0002-1560-0928 |
| Author | Family Name | **Hughes** |
| | Particle | |
| | Given Name | **Holly R.** |
| | Suffix | |
| | Division | |
| | Organization | Centers for Disease Control and Prevention |
| | Address | Fort Collins, CO, USA |
| | Phone | |
| | Fax | |
| | Email | |
| | URL | |
| | ORCID | http://orcid.org/0000-0003-1380-8263 |
| Author | Family Name | **Jonson** |
| | Particle | |
| | Given Name | **Miranda Gilda** |
| | Suffix | |
| | Division | Department of Agricultural Biotechnology, Center for Fungal Pathogenesis, College of Agriculture and Life Sciences |
| | Organization | Seoul National University |
| | Address | Seoul, Korea |
| | Phone | |
| | Fax | |
| | Email | |
| | URL | |
| | ORCID | |
| Author | Family Name | **Junglen** |
| | Particle | |
| | Given Name | **Sandra** |
| | Suffix | |
| | Division | |
| | Organization | Charité-Universitätsmedizin Berlin, Corporate Member of Free University Berlin, Humboldt-University Berlin, and Berlin Institute of Health |
| | Address | Berlin, Germany |
| | Division | |
| | Organization | German Centre for Infection Research |

FOIA Confidential Treatment Requested by Univ of Texas System UTSystem_054258

| | | |
|---|---|---|
| | Address | Berlin, Germany |
| | Phone | |
| | Fax | |
| | Email | |
| | URL | |
| | ORCID | |
| Author | Family Name | **Klempa** |
| | Particle | |
| | Given Name | **Boris** |
| | Suffix | |
| | Division | Biomedical Research Center |
| | Organization | Slovak Academy of Sciences |
| | Address | Bratislava, Slovakia |
| | Phone | |
| | Fax | |
| | Email | |
| | URL | |
| | ORCID | http://orcid.org/0000-0002-6931-1224 |
| Author | Family Name | **Klingström** |
| | Particle | |
| | Given Name | **Jonas** |
| | Suffix | |
| | Division | Center for Infectious Medicine, Department of Medicine Huddinge |
| | Organization | Karolinska Institutet, Karolinska University Hospital |
| | Address | Stockholm, Sweden |
| | Phone | |
| | Fax | |
| | Email | |
| | URL | |
| | ORCID | http://orcid.org/0000-0001-9076-1441 |
| Author | Family Name | **Kòu** |
| | Particle | |
| | Given Name | **Chūn** |
| | Suffix | |
| | Division | Xinjiang Key Laboratory of Biological Resources and Genetic Engineering, College of Life Science and Technology |
| | Organization | Xinjiang University |
| | Address | Ürümqi, China |
| | Phone | |
| | Fax | |
| | Email | |
| | URL | |
| | ORCID | |
| Author | Family Name | **Laenen** |
| | Particle | |

FOIA Confidential Treatment Requested by Univ of Texas System

| | | |
|---|---|---|
| | Given Name | **Lies** |
| | Suffix | |
| | Division | |
| | Organization | KU Leuven, Rega Institute, Zoonotic Infectious Diseases Unit |
| | Address | Leuven, Belgium |
| | Division | Department of Laboratory Medicine |
| | Organization | University Hospitals Leuven |
| | Address | Leuven, Belgium |
| | Phone | |
| | Fax | |
| | Email | |
| | URL | |
| | ORCID | http://orcid.org/0000-0001-9118-8608 |
| Author | Family Name | **Lambert** |
| | Particle | |
| | Given Name | **Amy J.** |
| | Suffix | |
| | Division | |
| | Organization | Centers for Disease Control and Prevention |
| | Address | Fort Collins, CO, USA |
| | Phone | |
| | Fax | |
| | Email | |
| | URL | |
| | ORCID | http://orcid.org/0000-0002-7571-3529 |
| Author | Family Name | **Langevin** |
| | Particle | |
| | Given Name | **Stanley A.** |
| | Suffix | |
| | Division | Department of Microbiology |
| | Organization | University of Washington |
| | Address | Washington, USA |
| | Phone | |
| | Fax | |
| | Email | |
| | URL | |
| | ORCID | |
| Author | Family Name | **Liu** |
| | Particle | |
| | Given Name | **Dan** |
| | Suffix | |
| | Division | School of Medicine |
| | Organization | Wuhan University of Science and Technology |
| | Address | Wuhan, China |
| | Phone | |

FOIA Confidential Treatment Requested by Univ of Texas System UTSystem_054260

| | | |
|---|---|---|
| | Fax | |
| | Email | |
| | URL | |
| | ORCID | |
| Author | Family Name | **Lukashevich** |
| | Particle | |
| | Given Name | **Igor S.** |
| | Suffix | |
| | Division | Department of Pharmacology and Toxicology, School of Medicine, and the Center for Predictive Medicine for Biodefense and Emerging Infectious Diseases |
| | Organization | University of Louisville |
| | Address | Louisville, KY, USA |
| | Phone | |
| | Fax | |
| | Email | |
| | URL | |
| | ORCID | http://orcid.org/0000-0001-6523-5116 |
| Author | Family Name | **Luò** |
| | Particle | |
| | Given Name | **Tāo** |
| | Suffix | |
| | Division | |
| | Organization | Center for Disease Control and Prevention of Xinjiang Uygur Autonomous Region |
| | Address | Ürümqi, China |
| | Phone | |
| | Fax | |
| | Email | |
| | URL | |
| | ORCID | |
| Author | Family Name | **Lǚ** |
| | Particle | |
| | Given Name | **Chuánwèi** |
| | Suffix | |
| | Division | State Key Laboratory of Virology |
| | Organization | Wuhan Institute of Virology, Chinese Academy of Sciences |
| | Address | Wuhan, China |
| | Phone | |
| | Fax | |
| | Email | |
| | URL | |
| | ORCID | |
| Author | Family Name | **Maes** |
| | Particle | |
| | Given Name | **Piet** |

FOIA Confidential Treatment Requested by Univ of Texas System

| | | |
|---|---|---|
| | Suffix | |
| | Division | |
| | Organization | KU Leuven, Rega Institute, Zoonotic Infectious Diseases Unit |
| | Address | Leuven, Belgium |
| | Phone | |
| | Fax | |
| | Email | |
| | URL | |
| | ORCID | http://orcid.org/0000-0002-4571-5232 |
| Author | Family Name | **Souza** |
| | Particle | **de** |
| | Given Name | **William Marciel** |
| | Suffix | |
| | Division | Virology Research Center |
| | Organization | School of Medicine of Ribeirão Preto, University of São Paulo |
| | Address | Ribeirão Preto, São Paulo, Brazil |
| | Phone | |
| | Fax | |
| | Email | |
| | URL | |
| | ORCID | http://orcid.org/0000-0002-0025-8293 |
| Author | Family Name | **Marklewitz** |
| | Particle | |
| | Given Name | **Marco** |
| | Suffix | |
| | Division | |
| | Organization | Charité-Universitätsmedizin Berlin, Corporate Member of Free University Berlin, Humboldt-University Berlin, and Berlin Institute of Health |
| | Address | Berlin, Germany |
| | Division | |
| | Organization | German Centre for Infection Research |
| | Address | Berlin, Germany |
| | Phone | |
| | Fax | |
| | Email | |
| | URL | |
| | ORCID | http://orcid.org/0000-0003-1828-8770 |
| Author | Family Name | **Martelli** |
| | Particle | |
| | Given Name | **Giovanni P.** |
| | Suffix | |
| | Division | Department of Plant, Soil and Food Sciences |
| | Organization | University “Aldo Moro” |
| | Address | Bari, Italy |
| | Phone | |

FOIA Confidential Treatment Requested by Univ of Texas System UTSystem_054262

| | | |
|---|---|---|
| | Fax | |
| | Email | |
| | URL | |
| | ORCID | |
| Author | Family Name | **Matsuno** |
| | Particle | |
| | Given Name | **Keita** |
| | Suffix | |
| | Division | Laboratory of Microbiology, Faculty of Veterinary Medicine |
| | Organization | Hokkaido University |
| | Address | Sapporo, Japan |
| | Division | Global Institution for Collaborative Research and Education (GI-CoRE) |
| | Organization | Hokkaido University |
| | Address | Sapporo, Japan |
| | Phone | |
| | Fax | |
| | Email | |
| | URL | |
| | ORCID | http://orcid.org/0000-0002-4205-6526 |
| Author | Family Name | **Mielke-Ehret** |
| | Particle | |
| | Given Name | **Nicole** |
| | Suffix | |
| | Division | |
| | Organization | Biocentre Klein Flottbek, University of Hamburg |
| | Address | Hamburg, Germany |
| | Phone | |
| | Fax | |
| | Email | |
| | URL | |
| | ORCID | |
| Author | Family Name | **Minutolo** |
| | Particle | |
| | Given Name | **Maria** |
| | Suffix | |
| | Division | Dipartimento di Agraria |
| | Organization | Università degli Studi di Napoli Federico II |
| | Address | Portici, Italy |
| | Phone | |
| | Fax | |
| | Email | |
| | URL | |
| | ORCID | http://orcid.org/0000-0001-7816-2657 |
| Author | Family Name | **Mirazimi** |
| | Particle | |

FOIA Confidential Treatment Requested by Univ of Texas System

| | | |
|---|---|---|
| | Given Name | **Ali** |
| | Suffix | |
| | Division | |
| | Organization | Folkhalsomyndigheten |
| | Address | Stockholm, Sweden |
| | Phone | |
| | Fax | |
| | Email | |
| | URL | |
| | ORCID | |
| Author | Family Name | **Moming** |
| | Particle | |
| | Given Name | **Abulimiti** |
| | Suffix | |
| | Division | Xinjiang Key Laboratory of Biological Resources and Genetic Engineering, College of Life Science and Technology |
| | Organization | Xinjiang University |
| | Address | Ürümqi, China |
| | Phone | |
| | Fax | |
| | Email | |
| | URL | |
| | ORCID | |
| Author | Family Name | **Mühlbach** |
| | Particle | |
| | Given Name | **Hans-Peter** |
| | Suffix | |
| | Division | |
| | Organization | Biocentre Klein Flottbek, University of Hamburg |
| | Address | Hamburg, Germany |
| | Phone | |
| | Fax | |
| | Email | |
| | URL | |
| | ORCID | |
| Author | Family Name | **Naidu** |
| | Particle | |
| | Given Name | **Rayapati** |
| | Suffix | |
| | Division | Department of Plant Pathology, Irrigated Agricultural Research and Extension Center |
| | Organization | Washington State University |
| | Address | Prosser, WA, USA |
| | Phone | |
| | Fax | |
| | Email | |

FOIA Confidential Treatment Requested by Univ of Texas System UTSystem_054264

| | | |
|---|---|---|
| | URL | |
| | ORCID | |
| Author | Family Name | **Navarro** |
| | Particle | |
| | Given Name | **Beatriz** |
| | Suffix | |
| | Division | |
| | Organization | Istituto per la Protezione Sostenibile delle Piante, Consiglio Nazionale delle Ricerche |
| | Address | Bari, Italy |
| | Phone | |
| | Fax | |
| | Email | |
| | URL | |
| | ORCID | http://orcid.org/0000-0001-8981-1018 |
| Author | Family Name | **Nunes** |
| | Particle | |
| | Given Name | **Márcio Roberto Teixeira** |
| | Suffix | |
| | Division | |
| | Organization | Evandro Chagas Institute, Ministry of Health |
| | Address | Pará, Brazil |
| | Phone | |
| | Fax | |
| | Email | |
| | URL | |
| | ORCID | http://orcid.org/0000-0001-9739-5499 |
| Author | Family Name | **Palacios** |
| | Particle | |
| | Given Name | **Gustavo** |
| | Suffix | |
| | Division | |
| | Organization | United States Army Medical Research Institute of Infectious Diseases, Fort Detrick |
| | Address | Frederick, MD, USA |
| | Phone | |
| | Fax | |
| | Email | |
| | URL | |
| | ORCID | http://orcid.org/0000-0001-5062-1938 |
| Author | Family Name | **Papa** |
| | Particle | |
| | Given Name | **Anna** |
| | Suffix | |
| | Division | National Reference Centre for Arboviruses and Haemorrhagic Fever Viruses, Department of Microbiology, Medical School |

FOIA Confidential Treatment Requested by Univ of Texas System UTSystem_054265

| | | |
|---|---|---|
| | Organization | Aristotle University of Thessaloniki |
| | Address | Thessaloníki, Greece |
| | Phone | |
| | Fax | |
| | Email | |
| | URL | |
| | ORCID | |
| Author | Family Name | **Pauvolid-Corrêa** |
| | Particle | |
| | Given Name | **Alex** |
| | Suffix | |
| | Division | Flavivirus Laboratory |
| | Organization | Oswaldo Cruz Foundation, Ministry of Health |
| | Address | Rio de Janeiro, Brazil |
| | Phone | |
| | Fax | |
| | Email | |
| | URL | |
| | ORCID | |
| Author | Family Name | **Pawęska** |
| | Particle | |
| | Given Name | **Janusz T.** |
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| Abstract | In February 2019, following the annual taxon ratification vote, the order *Bunyavirales* was amended by creation of two new families, four new subfamilies, 11 new genera and 77 new species, merging of two species, and deletion of one species. This article presents the updated taxonomy of the order *Bunyavirales* now accepted by the International Committee on Taxonomy of Viruses (ICTV). |


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VIROLOGY DIVISION NEWS

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**Abstract**
In February 2019, following the annual taxon ratification vote, the order *Bunyavirales* was amended by creation of two new families, four new subfamilies, 11 new genera and 77 new species, merging of two species, and deletion of one species. This article presents the updated taxonomy of the order *Bunyavirales* now accepted by the International Committee on Taxonomy of Viruses (ICTV).

## Introduction

The virus order *Bunyavirales* was established in 2017 to accommodate related viruses with segmented, linear, single-stranded, negative-sense or ambisense RNA genomes classified into nine families [19]. An amended/emended order description was published in early 2019 [20]. Here, we present the changes that were proposed via official ICTV taxonomic proposals that were accepted by the ICTV Executive Committee (EC) in February 2019. Therefore, these changes are now part of the official ICTV taxonomy.

## Taxonomic changes at the order rank

The order was expanded by addition of two new families. Family *Leishbuviridae* was created to accommodate one new genus, *Shilevirus*, including one new species, *Leptomonas shilevirus*, for Leptomonas moramango leishbunyavirus (LEPMV) discovered in a trypanosomatid protist (*Leptomonas moramango*) [2]. Family *Tospoviridae* was recreated for the already established genus *Tospovirus* (now renamed *Orthotospovirus*; TaxoProp 2018.017M.A.v1. Bunyavirales_2fam5gen) and expanded by seven new species (TaxoProp 2018.025P.A.v1.Orthotospovirus_7sp):

- *Bean necrotic mosaic orthotospovirus* for bean necrotic mosaic virus (BeNMV) discovered in common beans (*Phaseolus vulgaris*) [8];
- *Calla lily chlorotic spot orthotospovirus* for calla lily chlorotic spot virus (CCSV) found in calla lilies (*Zantedeschia* sp.) [5, 18];
- *Capsicum chlorosis orthotospovirus* for capsicum chlorosis virus (CaCV) found in capsicums, chillies, and tomatoes [16, 23];
- *Chrysanthemum stem necrosis orthotospovirus* for chrysanthemum stem necrosis virus (CSNV) infecting chrysanthemums [3, 10];
- *Melon severe mosaic orthotospovirus* for melon severe mosaic virus (MSMV) infecting cucurbit crops [6, 7];
- *Melon yellow spot orthotospovirus* for melon yellow spot virus (MYSV) found in netted melon (*Cucumis melo*) [15]; and
- *Soybean vein necrosis orthotospovirus* for soybean vein necrosis virus (SVNV) discovered in soybeans (*Glycine max*) [36].

A genus unassigned to any family, *Coguvirus*, was established to include species *Citrus coguvirus* for citrus concave gum-associated virus (CCGaV) found in citrus trees [26] (TaxoProp 2018.020P.A.v1.Coguvirus).

## Taxonomic changes at the family rank

### Arenaviridae

The family *Arenaviridae* was expanded by one genus, *Antennavirus*, to include two new species, *Hairy antennavirus* and *Striated antennavirus*, for Wēnlǐng frogfish arenavirus 2 (WlFV-2) and Wēnlǐng frogfish arenavirus 1 (WlFV-1), both found in striated frogfish (*Antennarius striatus*) [33] (TaxoProp 2018.005M.A.v1.Antennavirus).

### Cruliviridae

No changes were made at the family rank.

### Fimoviridae

No changes were made at the family rank.

### Hantaviridae

The family (TaxoProp 2018.010M.A.v2. Hantaviridae_4subfam) was reorganized into four subfamilies:

- subfamily *Actantavirinae* was created for the new genus *Actinavirus* to accommodate three novel species: *Batfish actinovirus* for Wēnlǐng minipizza batfish virus (WEMV) discovered in minipizza batfish (*Halieutaea stellata*); *Goosefish actinovirus* for Wēnlǐng yellow goosefish virus (WEYGV) found in yellow goosefish (*Lophius litulon*); and *Spikefish actinovirus* for Wēnlǐng red spikefish virus (WERSV) of red spikefish (*Triacanthodes anomalus*) [33];
- subfamily *Agantavirinae* was created for the new genus *Agnathovirus* to accommodate one new species, *Hag-*

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*fish agnathovirus*, for Wēnlǐng hagfish virus (WEHV) of inshore hagfish (*Eptatretus burgeri*) [33];

- subfamily *Mammantavirinae* was created to accommodate the established genera *Loanvirus*, *Mobatvirus*, and *Orthohantavirus*. Two new orthohantavirus species, *Seewis orhtohantavirus* and *Tigray orthohantavirus*, were created for Seewis virus (SWSV) of Eurasian common shrews (*Sorex araneus*) [34] and Tigray virus (TIGV) of Ethiopian white-footed mice (*Stenocephalemys albipes*) [12, 25], respectively; and
- subfamily *Repantavirinae* was created for the new genus *Reptillovirus* to accommodate one new species, *Gecko reptillovirus*, for Hǎinán oriental leaf-toed gecko virus (HOLGV) discovered in oriental leaf-toed geckos (*Hemidactylus bowringii*) [33].

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## Mypoviridae

No changes were made at the family rank.

## Nairoviridae

The species *Estero Real orthonairovirus* was created for Estero Real virus (ERV) (moved from genus *Orthobunyavirus*, family *Peribunyaviridae*) [1] (TaxoProp 2018.012M.A.v1.Bunyavirales_spmov).

## Peribunyaviridae

The family was expanded by one new genus, *Pacuvirus*, to accommodate three new species: *Pacui pacuvirus* for Pacui virus (PACV) discovered in a rice rat (*Oryzomys* sp.); *Rio Preto da Eva pacuvirus* for Rio Preto da Eva virus discovered in a sewer gnat (Psychodidae sp.); and *Tapirape pacuvirus* for Tapirapé virus found in a hocicudo (*Oxymycterus* sp.) [27] (TaxoProp 2018.017M.A.v1.Bunyavirales_2fam5gen). Genus *Tospovirus* was removed from the family and placed into the new family *Tospoviridae* as genus *Orthotospovirus* (TaxoProp 2018.017M.A.v1.Bunyavirales_2fam5gen). The genus *Orthobunyavirus* was reorganized by moving previously classified viruses into a total of 38 new species (one resulting from a merger of two previously established species) (TaxoProp 2018.008M.A.v1.Orthobunyavirus_38sp). Five additional novel species were added: *Bellavista orthobunyavirus* for Bellavista virus isolated from mosquitoes (*Culex portesi*) [14]; *Enseada orthobunyavirus* for Enseada virus isolated from *Culex* mosquitoes [4, 9]; *Maguari orthobunyavirus* for Maguari virus (MAGV) isolated from mosquitoes [13]; *Tataguine orthobunyavirus* for Tataguine virus (TATV) found in a human sample, and *Witwatersrand orthobunyavirus* for Witwatersrand virus (WITV) from mosquitoes [29] (TaxoProp 2018.017M.A.v1.Bunyavirales_2fam5gen). Species *Estero Real orthobunyavirus* was abolished, and its member, Estero Real virus (ERV), was moved into family *Nairoviridae* [1] (TaxoProp 2018.012M.A.v1.Bunyavirales_spmov).

## Phasmaviridae

The family was expanded by addition of one new genus, *Sawastrivirus*, to include one new species, *Sanxia sawastrivirus*, for Sānxiá water strider virus 2 (SxWSV-2) (TaxoProp 2018.017M.A.v1.Bunyavirales_2fam5gen) detected in gerrid water striders [31]. The genus *Orthophasmavirus* was expanded by the addition of five new species: *Culex orthophasmavirus* for Culex phasma-like virus (CPLV) detected in *Culex* mosquitoes [32]; *Ganda orthophasmavirus* for Ganda bee virus (GBEEV) of European orchard bees (*Osmia cornuta*) [28]; *Odonate orthophasmavirus* for Húběi odonate virus 8 (HbOV-8) [31]; *Qingling orthophasmavirus* for Húběi odonate virus 9 (HbOV-9) of odonates [31]; and *Seattle orthophasmavirus* for Seattle Prectang virus (SEPV) found in a moth (*Pasiphila rectangulata*) [21] (TaxoProp 2018.009M.A.v1.Phasmavirus_5sp).

## Phenuiviridae

The family *Phenuiviridae* was expanded by addition of three new genera. Genus *Kabutovirus* was created to include two new species, *Huangpi kabutovirus* and *Kabuto mountain kabutovirus*, for Huángpí tick virus 1 (HpTV-1) of ticks (*Haemaphysalis doenitzi*) [17] and Kabuto mountain virus (KAMV) of ticks (*Haemaphysalis flava*) [11], respectively. Genus *Laulavirus* was created to include one species, *Laurel Lake laulavirus*, for Laurel Lake virus (LLV) of ticks (*Ixodes scapularis*) [35]. Genus *Wenrivirus* was created to include one species, *Shrimp wenrivirus*, for Wēnzhōu shrimp virus 1 (WzSV-1) [17] found in giant tiger prawns (*Penaeus monodon*) [17] (TaxoProp 2018.017M.A.v1.Bunyavirales_2fam5gen). The established genus *Banyangvirus* was expanded by two species, *Guertu banyangvirus* and *Heartland banyangvirus*, for Guertu virus (GTV) found in *Dermacentor nuttalli* ticks [30] and Heartland virus (HRTV), a tick-borne virus originally discovered in human samples [24], respectively (TaxoProp 2018.013M.A.v1.Banyangvirus_sp; TaxoProp 2018.017M.A.v1.Bunyavirales_2fam5gen). Genus *Phlebovirus* was expanded by one species, *Mukawa phlebovirus*, for Mukawa virus (MKWV) of ticks (*Ixodes persulcatus*) [22] (TaxoProp 2018.014M.A.v1.Phlebovirus_sp). In genus *Phasivirus*, species *Wuhan fly phasivirus* was abolished (TaxoProp 2018.019M.A.v1.Phenuiviridae_Remsp).

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**Table 1** ICTV-accepted taxonomy of the order *Bunyavirales* as of February 2019. Listed are all bunyaviruses that are classified into species

| Genus | Species[¶] | Virus (abbreviation)[¶] |
|---|---|---|
| Family *Arenaviridae* | | |
| *Antennavirus* | *Hairy antennavirus* | Wēnlǐng frogfish arenavirus 2 (WlFAV-2) |
| | *Striated antennavirus** | Wēnlǐng frogfish arenavirus 1 (WlFAV-1) |
| *Hartmanivirus* | *Haartman hartmanivirus** | Haartman Institute snake virus 1 (HISV-1) |
| *Mammarenavirus* | *Allpahuayo mammarenavirus* | Allpahuayo virus (ALLV) |
| | *Argentinian mammarenavirus* | Junín virus (JUNV) |
| | *Bear Canyon mammarenavirus* | Bear Canyon virus (BCNV) |
| | *Brazilian mammarenavirus* | Sabiá virus (SBAV) |
| | *Cali mammarenavirus* | Pichindé virus (PICHV) |
| | *Chapare mammarenavirus* | Chapare virus (CHAPV) |
| | *Cupixi mammarenavirus* | Cupixi virus (CUPXV) |
| | *Flexal mammarenavirus* | Flexal virus (FLEV) |
| | *Gairo mammarenavirus* | Gairo virus (GAIV) |
| | *Guanarito mammarenavirus* | Guanarito virus (GTOV) |
| | *Ippy mammarenavirus* | Ippy virus (IPPYV) |
| | *Lassa mammarenavirus* | Lassa virus (LASV) |
| | *Latino mammarenavirus* | Latino virus (LATV) |
| | *Loei River mammarenavirus* | Loei River virus (LORV) |
| | *Lujo mammarenavirus* | Lujo virus (LUJV) |
| | *Luna mammarenavirus* | Luli virus (LULV) |
| | | Luna virus (LUAV) |
| | *Lunk mammarenavirus* | Lunk virus (LNKV) |
| | *Lymphocytic choriomeningitis mammarenavirus** | Dandenong virus (DANV) |
| | | lymphocytic choriomeningitis virus (LCMV) |
| | *Machupo mammarenavirus* | Machupo virus (MACV) |
| | *Mariental mammarenavirus* | Mariental virus (MRLV) |
| | *Merino Walk mammarenavirus* | Merino Walk virus (MRWV) |
| | *Mobala mammarenavirus* | mobala virus (MOBV) |
| | *Mopeia mammarenavirus* | Mopeia virus (MPOV) |
| | | Morogoro virus (MORV) |
| | *Okahandja mammarenavirus* | Okahandja virus (OKAV) |
| | *Oliveros mammarenavirus* | Oliveros virus (OLVV) |
| | *Paraguayan mammarenavirus* | Paraná virus (PRAV) |
| | *Pirital mammarenavirus* | Pirital virus (PIRV) |
| | *Ryukyu mammarenavirus* | Ryukyu virus (RYKV) |
| | *Serra do Navio mammarenavirus* | Amaparí virus (AMAV) |
| | *Solwezi mammarenavirus* | Solwezi virus (SOLV) |
| | *Souris mammarenavirus* | souris virus (SOUV) |
| | *Tacaribe mammarenavirus* | Tacaribe virus (TCRV) |
| | *Tamiami mammarenavirus* | Tamiami virus (TMMV) |
| | *Wenzhou mammarenavirus* | Wēnzhōu virus (WENV) |
| | *Whitewater Arroyo mammarenavirus* | Big Brushy Tank virus (BBRTV) |
| | | Catarina virus (CTNV) |
| | | Skinner Tank virus (SKTV) |
| | | Tonto Creek virus (TTCV) |
| | | Whitewater Arroyo virus (WWAV) |
| *Reptarenavirus* | *California reptarenavirus* | CAS virus (CASV) |
| | *Giessen reptarenavirus* | University of Giessen virus 1 (UGV-1) |
| | | University of Giessen virus 2 (UGV-2) |
| | | University of Giessen virus 3 (UGV-3) |
| | *Golden reptarenavirus** | Golden Gate virus (GOGV) |
| | *Ordinary reptarenavirus* | tavallinen suomalainen mies virus 2 (TSMV-2) |
| | *Rotterdam reptarenavirus* | ROUT virus (ROUTV) |
| | | University of Helsinki virus 1 (UHV-1) |

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**Table 1** (continued)

| Genus | Species[¶] | Virus (abbreviation)[¶] |
|---|---|---|
| Family *Cruliviridae* | | |
| *Lincruvirus* | *Crustacean lincruvirus** | Wēnlǐng crustacean virus 9 (WlCV-9) |
| Family *Fimoviridae* | | |
| *Emaravirus* | *Actinidia chlorotic ringspot-associated emaravirus* | Actinidia chlorotic ringspot-associated virus (AcCRaV) |
| | *European mountain ash ringspot-associated emaravirus** | European mountain ash ringspot-associated virus (EMARaV) |
| | *Fig mosaic emaravirus* | fig mosaic virus (FMV) |
| | *High Plains wheat mosaic emaravirus* | High Plains wheat mosaic virus (HPWMoV) |
| | *Pigeonpea sterility mosaic emaravirus 1* | pigeonpea sterility mosaic virus (PPSMV) |
| | *Pigeonpea sterility mosaic emaravirus 2* | pigeonpea sterility mosaic virus 2 (PPSMV-2) |
| | *Raspberry leaf blotch emaravirus* | raspberry leaf blotch virus (RLBV) |
| | *Redbud yellow ringspot-associated emaravirus* | redbud yellow ringspot-associated virus (RYRaV) |
| | *Rose rosette emaravirus* | rose rosette virus (RRV) |
| Family *Hantaviridae* | | |
| Subfamily *Actantavirinae* | | |
| *Actinovirus* | *Batfish actinovirus** | Wēnlǐng minipizza batfish virus (WEMBV) |
| | *Goosefish actinovirus* | Wēnlǐng yellow goosefish virus (WEYGV) |
| | *Spikefish actinovirus* | Wēnlǐng red spikefish virus (WERSV) |
| Subfamily *Agantavirinae* | | |
| *Agnathovirus* | *Hagfish agnathovirus** | Wēnlǐng hagfish virus (WEHV) |
| Subfamily *Mammantavirinae* | | |
| *Loanvirus* | *Longquan loanvirus** | Lóngquán virus (LQUV) |
| *Mobatvirus* | *Laibin mobatvirus* | Láibīn virus (LAIV) |
| | *Nova mobatvirus** | Nova virus (NVAV) |
| | *Quezon mobatvirus* | Quezon virus (QZNV) |
| *Orthohantavirus* | *Andes orthohantavirus* | Andes virus (ANDV) |
| | | Castelo dos Sonhos virus (CASV) |
| | | Lechiguanas virus (LECV = LECHV) |
| | | Orán virus (ORNV) |
| | *Asama orthohantavirus* | Asama virus (ASAV) |
| | *Asikkala orthohantavirus* | Asikkala virus (ASIV) |
| | *Bayou orthohantavirus* | bayou virus (BAYV) |
| | | Catacamas virus (CATV) |
| | *Black Creek Canal orthohantavirus* | Black Creek Canal virus (BCCV) |
| | *Bowe orthohantavirus* | Bowé virus (BOWV) |
| | *Bruges orthohantavirus* | Bruges virus (BRGV) |
| | *Caño Delgadito orthohantavirus* | Caño Delgadito virus (CADV) |
| | *Cao Bang orthohantavirus* | Cao Bằng virus (CBNV) |
| | | Liánghé virus (LHEV) |
| | *Choclo orthohantavirus* | Choclo virus (CHOV) |
| | *Dabieshan orthohantavirus* | Dàbiéshān virus (DBSV) |
| | *Dobrava-Belgrade orthohantavirus* | Dobrava virus (DOBV) |
| | | Kurkino virus (KURV) |
| | | Saaremaa virus (SAAV) |
| | | Sochi virus (SOCV) |
| | *El Moro Canyon orthohantavirus* | Carrizal virus (CARV) |
| | | El Moro Canyon virus (ELMCV) |
| | | Huitzilac virus (HUIV) |
| | *Fugong orthohantavirus* | Fúgòng virus (FUGV) |
| | *Fusong orthohantavirus* | Fūsōng virus (FUSV) |
| | *Hantaan orthohantavirus** | Amur virus (AMRV) |
| | | Hantaan virus (HTNV) |
| | | Soochong virus (SOOV) |
| | *Jeju orthohantavirus* | Jeju virus (JJUV) |
| | *Kenkeme orthohantavirus* | Kenkeme virus (KKMV) |

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**Table 1** (continued)

| Genus | Species[¶] | Virus (abbreviation)[¶] |
|---|---|---|
| | *Khabarovsk orthohantavirus* | Khabarovsk virus (KHAV) |
| | | Topografov virus (TOPV) |
| | *Laguna Negra orthohantavirus* | Laguna Negra virus (LANV) |
| | | Maripa virus (MARV) |
| | | Río Mamoré virus (RIOMV) |
| | *Luxi orthohantavirus* | Lúxī virus (LUXV) |
| | *Maporal orthohantavirus* | Maporal virus (MAPV) |
| | *Montano orthohantavirus* | Montaño virus (MTNV) |
| | *Necocli orthohantavirus* | Necoclí virus (NECV) |
| | *Oxbow orthohantavirus* | Oxbow virus (OXBV) |
| | *Prospect Hill orthohantavirus* | Prospect Hill virus (PHV) |
| | *Puumala orthohantavirus* | Hokkaido virus (HOKV) |
| | | Muju virus (MUJV) |
| | | Puumala virus (PUUV) |
| | *Rockport orthohantavirus* | Rockport virus (RKPV) |
| | *Sangassou orthohantavirus* | Sangassou virus (SANGV) |
| | *Seewis orthohantavirus* | Seewis virus (SWSV) |
| | *Seoul orthohantavirus* | gōu virus (GOUV) |
| | | Seoul virus (SEOV) |
| | *Sin Nombre orthohantavirus* | New York virus (NYV) |
| | | Sin Nombre virus (SNV) |
| | *Thailand orthohantavirus* | Anjozorobe virus (ANJZV) |
| | | Serang virus (SERV) |
| | | Thailand virus (THAIV) |
| | *Tigray orthohantavirus* | Tigray virus (TIGV) |
| | *Tula orthohantavirus* | Adler virus (ADLV) |
| | | Tula virus (TULV) |
| | *Yakeshi orthohantavirus* | Yákèshí virus (YKSV) |
| *Thottimvirus* | *Imjin thottimvirus* | Imjin virus (MJNV) |
| | *Thottapalayam thottimvirus** | Thottapalayam virus (TPMV) |
| Subfamily *Repantavirinae* | | |
| *Reptillovirus* | *Gecko reptillovirus** | Hǎinán oriental leaf-toed gecko virus (HOLGV) |
| Family *Leishbuviridae* | | |
| *Shilevirus* | *Leptomonas shilevirus** | Leptomonas moramango virus (LEPMV) |
| Family *Mypoviridae* | | |
| *Hubavirus* | *Myriapod hubavirus** | Húběi myriapoda virus 5 (HbMV-5) |
| Family *Nairoviridae* | | |
| *Orthonairovirus* | *Artashat orthonairovirus* | Artashat virus (ARTSV) |
| | *Chim orthonairovirus* | Chim virus (CHIMV) |
| | *Crimean-Congo hemorrhagic fever orthonairovirus* | Crimean-Congo hemorrhagic fever virus (CCHFV) |
| | *Dera Ghazi Khan orthonairovirus* | Abu Hammad virus (AHV) |
| | | Abu Mina virus (AMV) |
| | | Dera Ghazi Khan virus (DGKV) |
| | | Sapphire II virus (SAPV) |
| | *Dugbe orthonairovirus** | Dugbe virus (DUGV) |
| | | kupe virus (KUPEV) |
| | *Estero Real orthonairovirus* | Estero Real virus (ERV) |
| | *Hazara orthonairovirus* | Hazara virus (HAZV) |
| | | Tofla virus (TFLV) |
| | *Hughes orthonairovirus* | Caspiy virus (CASV) |
| | | Farallon virus (FARV) |
| | | Great Saltee virus (GRSV) |
| | | Hughes virus (HUGV) |
| | | Punta Salinas virus (PSV) |

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**Table 1** (continued)

| Genus | Species[¶] | Virus (abbreviation)[¶] |
|---|---|---|
| | | Raza virus (RAZAV) |
| | | Soldado virus (SOLV) |
| | | Zirqa virus (ZIRV) |
| | *Kasokero orthonairovirus* | Kasokero virus (KASV = KASOV) |
| | | Leopards Hill virus (LPHV) |
| | | Yogue virus (YOGV) |
| | *Keterah orthonairovirus* | Gossas virus (GOSV) |
| | | Issyk-kul virus (ISKV) |
| | | Keterah virus (KTRV) |
| | | Uzun-Agach virus (UZAV) |
| | *Nairobi sheep disease orthonairovirus* | Nairobi sheep disease virus (NSDV) |
| | *Qalyub orthonairovirus* | Bandia virus (BDAV) |
| | | Geran virus (GERV) |
| | | Qalyub virus (QYBV) |
| | *Sakhalin orthonairovirus* | Avalon virus (AVAV) |
| | | Clo Mor virus (CMV = CLMV) |
| | | Sakhalin virus (SAKV) |
| | | Taggert virus (TAGV) |
| | | Tillamook virus (TILLV) |
| | *Tamdy orthonairovirus* | Burana virus (BURV) |
| | | Huángpí tick virus 1 (HpTV-1) |
| | | Tǎchéng tick virus 1 (TcTV-1) |
| | | Tamdy virus (TAMV) |
| | | Wēnzhōu tick virus (WzTV) |
| | *Thiafora orthonairovirus* | Erve virus (ERVEV) |
| | | Thiafora virus (TFAV) |
| *Shaspivirus* | *Spider shaspivirus** | Shāyáng spider virus 1 (SySV-1) |
| *Striwavirus* | *Strider striwavirus** | Sānxiá water strider virus 1 (SxWSV-1) |
| Family *Peribunyaviridae* | | |
| *Herbevirus* | *Herbert herbevirus** | Herbert virus (HEBV) |
| | *Kibale herbevirus* | Kibale virus (KIBV) |
| | *Tai herbevirus* | Taï virus (TAIV) |
| *Orthobunyavirus* | *Acara orthobunyavirus* | Acará virus (ACAV) |
| | | Moriche virus (MORV) |
| | *Aino orthobunyavirus* | Aino virus (AINOV) |
| | *Akabane orthobunyavirus* | Akabane virus (AKAV) |
| | | Tinaroo virus (TINV) |
| | | Yaba-7 virus (Y7V) |
| | *Alajuela orthobunyavirus* | Alajuela virus (ALJV) |
| | | San Juan virus (SJV) |
| | *Anadyr orthobunyavirus* | Anadyr virus (ANADV) |
| | *Anhembi orthobunyavirus* | Anhembi virus (AMBV) |
| | *Anopheles A orthobunyavirus* | Anopheles A virus (ANAV) |
| | | Arumateua virus (ARTV = ARMTV) |
| | | Caraipé virus (CPEV = CRPV) |
| | | Las Maloyas virus (LMV) |
| | | Lukuni virus (LUKV) |
| | | Trombetas virus (TRMV) |
| | | Tucuruí virus (TUCV = TUCRV) |
| | *Anopheles B orthobunyavirus* | Anopheles B virus (ANBV) |
| | | Boracéia virus (BORV) |
| | *Bakau orthobunyavirus* | Bakau virus (BAKV) |
| | | Ketapang virus (KETV) |
| | | Nola virus (NOLAV) |
| | | Tanjong Rabok virus (TRV) |

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**Table 1** (continued)

| Genus | Species[¶] | Virus (abbreviation)[¶] |
|---|---|---|
| | | Telok Forest virus (TFV) |
| | *Batai orthobunyavirus* | Batai virus (BATV) |
| | *Batama orthobunyavirus* | Batama virus (BMAV) |
| | *Bellavista orthobunyavirus* | Bellavista virus (BELLV) |
| | *Benevides orthobunyavirus* | Benevides virus (BVSV = BENV) |
| | *Bertioga orthobunyavirus* | Bertioga virus (BERV) |
| | | Cananéia virus (CNAV) |
| | | Guaratuba virus (GTBV) |
| | | Itimirim virus (ITIV) |
| | | Mirim virus (MIRV) |
| | *Bimiti orthobunyavirus* | bimiti virus (BIMV) |
| | *Birao orthobunyavirus* | Birao virus (BIRV) |
| | *Botambi orthobunyavirus* | Botambi virus (BOTV) |
| | *Bozo orthobunyavirus* | Bozo virus (BOZOV) |
| | *Bunyamwera orthobunyavirus** | Bunyamwera virus (BUNV) |
| | | Germiston virus (GERV) |
| | | Lokern virus (LOKV) |
| | | Mboké virus (MBOV) |
| | | Ngari virus (NRIV) |
| | | Northway virus (NORV) |
| | | Santa Rosa virus (SARV) |
| | | Shokwe virus (SHOV) |
| | | Stanfield virus (STAV) |
| | | Xingu virus (XINV) |
| | *Bushbush orthobunyavirus* | Benfica virus (BENV = BNFV) |
| | | Bushbush virus (BSBV) |
| | | Juan Díaz virus (JDV) |
| | *Buttonwillow orthobunyavirus* | Buttonwillow virus (BUTV) |
| | *Bwamba orthobunyavirus* | Bwamba virus (BWAV) |
| | | Pongola virus (PGAV) |
| | *Cache Valley orthobunyavirus* | Cache Valley virus (CVV) |
| | | Cholul virus (CHLV) |
| | | Tlacotalpan virus (TLAV) |
| | *Cachoeira Porteira orthobunyavirus* | Cachoeira Porteira virus (CPOV) |
| | *California encephalitis orthobunyavirus* | California encephalitis virus (CEV) |
| | | Morro Bay virus (MBV) |
| | *Capim orthobunyavirus* | Capim virus (CAPV) |
| | *Caraparu orthobunyavirus* | Apeú virus (APEUV) |
| | | Bruconha virus (BRUV) |
| | | Caraparú virus (CARV) |
| | | El Huayo virus (EHUV) |
| | | Itaya virus (ITYV) |
| | | Ossa virus (OSSAV) |
| | | Vinces virus (VINV) |
| | *Cat Que orthobunyavirus* | Cát Quế virus (CQV) |
| | | Oya virus (OYAV) |
| | *Catu orthobunyavirus* | Catú virus (CATUV) |
| | *Enseada orthobunyavirus* | Enseada virus (ENSV) |
| | *Faceys paddock orthobunyavirus* | Facey's paddock virus (FPV) |
| | *Fort Sherman orthobunyavirus* | Fort Sherman virus (FSV) |
| | *Gamboa orthobunyavirus* | Brus Laguna virus (BLAV) |
| | | Calchaquí virus (CQIV) |
| | | Gamboa virus (GAMV) |
| | | Pueblo Viejo virus (PVV) |
| | | Soberanía virus (SOBV) |

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**Table 1** (continued)

| Genus | Species[¶] | Virus (abbreviation)[¶] |
|---|---|---|
| | *Guajara orthobunyavirus* | Guajará virus (GJAV) |
| | *Guama orthobunyavirus* | Ananindeua virus (ANUV) |
| | | Guamá virus (GMAV) |
| | | Mahogany Hammock virus (MHV) |
| | | Moju virus (MOJUV) |
| | *Guaroa orthobunyavirus* | Guaroa virus (GROV) |
| | *Iaco orthobunyavirus* | Iaco virus (IACOV) |
| | *Ilesha orthobunyavirus* | Ilesha virus (ILEV) |
| | *Ingwavuma orthobunyavirus* | Ingwavuma virus (INGV) |
| | *Jamestown Canyon orthobunyavirus* | Inkoo virus (INKV) |
| | | Jamestown Canyon virus (JCV) |
| | | Jerry Slough virus (JSV) |
| | | South River virus (SORV) |
| | *Jatobal orthobunyavirus* | Jatobal virus (JATV) |
| | *Kaeng Khoi orthobunyavirus* | Kaeng Khoi virus (KKV) |
| | *Kairi orthobunyavirus* | Kairi virus (KRIV) |
| | *Keystone orthobunyavirus* | Keystone virus (KEYV) |
| | *Koongol orthobunyavirus* | koongol virus (KOOV) |
| | | wongal virus (WONV) |
| | *La Crosse orthobunyavirus* | La Crosse virus (LACV) |
| | *Leanyer orthobunyavirus* | Leanyer virus (LEAV) |
| | *Lumbo orthbunyavirus* | Lumbo virus (LUMV) |
| | *Macaua orthobunyavirus* | Macauã virus (MCAV) |
| | *Madrid orthobunyavirus* | Madrid virus (MADV) |
| | *Maguari orthobunyavirus* | Maguari virus (MAGV) |
| | | Playas virus (PLAV) |
| | *Main Drain orthobunyavirus* | Main Drain virus (MDV) |
| | *Manzanilla orthobunyavirus* | Manzanilla virus (MANV) |
| | | Inini virus (ININ) |
| | *Marituba orthobunyavirus* | Gumbo Limbo virus (GLV) |
| | | Marituba virus (MTBV) |
| | | Murutucú virus (MURV) |
| | | Nepuyo virus (NEPV) |
| | | Restan virus (RESV) |
| | | Zungarococha virus (ZUNV) |
| | *Melao orthobunyavirus* | Melao virus (MELV) |
| | *Mermet orthobunyavirus* | Mermet virus (MERV) |
| | *Minatitlan orthobunyavirus* | Minatitlán virus (MNTV) |
| | | Palestina virus (PLSV) |
| | *MPoko orthobunyavirus* | M'Poko virus (MPOV) |
| | | Yaba-1 virus (Y1V) |
| | *Nyando orthobunyavirus* | Eretmapodites virus (ERETV) |
| | | Mojuí dos Campos virus (MDCV) |
| | | Nyando virus (NDV) |
| | *Olifantsvlei orthobunyavirus* | Bobia virus (BIAV) |
| | | Dabakala virus (DABV) |
| | | Olifantsvlei virus (OLIV) |
| | | Oubi virus (OUBIV) |
| | *Oriboca orthobunyavirus* | Itaquí virus (ITQV) |
| | | Oriboca virus (ORIV) |
| | *Oropouche orthobunyavirus* | Iquitos virus (IQTV)[d] |
| | | Madre de Dios virus (MDDV) |
| | | Oropouche virus (OROV) |
| | | Perdões virus (PDEV) |
| | | Pintupo virus (PINTV) |

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**Table 1** (continued)

| Genus | Species[¶] | Virus (abbreviation)[¶] |
|---|---|---|
| | *Patois orthobunyavirus* | Abras virus (ABRV) |
| | | Babahoya virus (BABV) |
| | | Pahayokee virus (PAHV) |
| | | Patois virus (PATV) |
| | | Shark River virus (SRV) |
| | *Peaton orthobunyavirus* | Peaton virus (PEAV) |
| | *Potosi orthobunyavirus* | Potosi virus (POTV) |
| | *Sabo orthobunyavirus* | Sabo virus (SABOV) |
| | *San Angelo orthobunyavirus* | San Angelo virus (SAV) |
| | *Sango orthobunyavirus* | Sango virus (SANV) |
| | *Schmallenberg orthobunyavirus* | Douglas virus (DOUV) |
| | | Sathuperi virus (SATV) |
| | | Schmallenberg virus (SBV) |
| | | Shamonda virus (SHAV) |
| | *Serra do Navio orthobunyavirus* | Serra do Navio virus (SDNV) |
| | *Shuni orthobunyavirus* | Kaikalur virus (KAIV) |
| | | Shuni virus (SHUV) |
| | *Simbu orthobunyavirus* | Para virus (PARAV) |
| | | Simbu virus (SIMV) |
| | *Snowshoe hare orthobunyavirus* | Khatanga virus (KHATV)[9] |
| | | snowshoe hare virus (SSHV) |
| | *Sororoca orthobunyavirus* | Sororoca virus (SORV) |
| | *Tacaiuma orthobunyavirus* | CoAr 1071 virus (CA1071V) |
| | | CoAr 3627 virus (CA3626V) |
| | | Tacaiuma virus (TCMV) |
| | | Virgin River virus (VRV) |
| | *Tahyna orthobunyavirus* | Ťahyňa virus (TAHV) |
| | *Tataguine orthobunyavirus* | Tataguine virus (TATV) |
| | *Tensaw orthobunyavirus* | Tensaw virus (TENV) |
| | *Tete orthobunyavirus* | Bahig virus (BAHV) |
| | | Matruh virus (MTRV) |
| | | Tete virus (TETEV) |
| | | Tsuruse virus (TSUV) |
| | | Weldona virus (WELV) |
| | *Thimiri orthobunyavirus* | Thimiri virus (THIV) |
| | *Timboteua orthobunyavirus* | Timboteua virus (TBTV) |
| | *Trivittatus orthobunyavirus* | Achiote virus (ACHOV) |
| | | Trivittatus virus (TVTV) |
| | *Turlock orthobunyavirus* | Lednice virus (LEDV) |
| | | Turlock virus (TURV) |
| | | Umbre virus (UMBV) |
| | *Utinga orthobunyavirus* | Utinga virus (UTIV) |
| | *Witwatersrand orthobunyavirus* | Witwatersrand virus (WITV) |
| | *Wolkberg orthobunyavirus* | Wolkberg virus (WBV) |
| | *Wyeomyia orthobunyavirus* | Rio Pracupi virus |
| | | Taiassui virus (TAIAV) |
| | | Tucunduba virus (TUCV) |
| | | Wyeomyia virus (WYOV) |
| | *Zegla orthobunyavirus* | Zegla virus (ZEGV) |
| *Pacuvirus* | *Pacui pacuvirus** | Pacui virus (PACV) |
| | *Rio Preto da Eva pacuvirus* | Rio Preto da Eva virus (RPEV) |
| | *Tapirape pacuvirus* | Tapirapé virus (TAPV) |
| *Shangavirus* | *Insect shangavirus** | Shuāngào insect virus 1 (SgIV-1) |
| Family *Phasmaviridae* | | |
| *Feravirus* | *Ferak feravirus** | Ferak virus (FRKV) |

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**Table 1** (continued)

| Genus | Species[¶] | Virus (abbreviation)[¶] |
|---|---|---|
| *Insluvirus* | *Insect insluvirus** | Shuāngào insect virus 2 (SgIV-2) |
| *Jonvirus* | *Jonchet jonvirus** | jonchet virus (JONV) |
| *Orthophasmavirus* | *Culex orthophasmavirus* | Culex orthophasmavirus (CPLV) |
| | *Ganda orthophasmavirus* | Ganda orthophasmavirus (GAEV) |
| | *Kigluaik phantom orthophasmavirus** | Kigluaik phantom virus (KIGV) |
| | *Nome phantom orthophasmavirus* | Nome phantom virus (NOMV) |
| | *Odonate orthophasmavirus* | Odonate orthophasmavirus (HbOV-8) |
| | *Qingling orthophasmavirus* | Qingling orthophasmavirus (HbOV-9) |
| | *Seattle orthophasmavirus* | Seattle orthophasmavirus (SEPV) |
| | *Wuchang cockroach orthophasmavirus 1* | Wǔchāng cockroach virus 1 (WcCV-1) |
| | *Wuhan mosquito orthophasmavirus 1* | Wǔhàn mosquito virus 1 (WhMV-1) |
| | *Wuhan mosquito orthophasmavirus 2* | Wǔhàn mosquito virus 2 (WhMV-2) |
| *Sawastrivirus* | *Sanxia sawastrivirus** | Sānxiá water strider virus 2 (SxWSV-2) |
| *Wuhivirus* | *Insect wuhivirus** | Wǔhàn insect virus 2 (WhIV-2) |
| Family *Phenuiviridae* | | |
| *Banyangvirus* | *Guertu banyangvirus* | Guertu virus (GTV) |
| | *Heartland banyangvirus* | Heartland virus (HRTV) |
| | *Huaiyangshan banyangvirus** | severe fever with thrombocytopenia syndrome virus (SFTSV) |
| *Beidivirus* | *Dipteran beidivirus** | Húběi diptera virus 3 (HbDV-3) |
| *Goukovirus* | *Cumuto goukovirus* | Cumuto virus (CUMV) |
| | *Gouleako goukovirus** | Gouléako virus (GOLV) |
| | *Yichang insect goukovirus* | Yíchāng insect virus (YcIV) |
| *Horwuvirus* | *Horsefly horwuvirus** | Wǔhàn horsefly virus (WhHV) |
| *Hudivirus* | *Dipteran hudivirus** | Húběi diptera virus 4 (HbDV-4) |
| *Hudovirus* | *Lepidopteran hudovirus** | Húběi lepidoptera virus 1 (HbLV-1) |
| *Kabutovirus* | *Huangpi kabutovirus** | Huángpí tick virus 2 (HpTV-2) |
| | *Kabuto mountain kabutovirus* | Kabuto mountain virus (KAMV) |
| *Laulavirus* | *Laurel Lake laulavirus** | Laurel Lake virus (LLV) |
| *Mobuvirus* | *Mothra mobuvirus** | Mothra virus (MTHV) |
| *Phasivirus* | *Badu phasivirus** | Badu virus (BADUV) |
| | *Phasi Charoen-like phasivirus* | Phasi Chaeron-like virus (PCLV) |
| | *Wutai mosquito phasivirus* | Wǔtái mosquito virus (WtMV) |
| *Phlebovirus* | *Bujaru phlebovirus* | Bujaru virus (BUJV) |
| | | Munguba virus (MUNV) |
| | *Candiru phlebovirus* | Alenquer virus (ALEV) |
| | | Ariquemes virus (ARQV) |
| | | Candirú virus (CDUV) |
| | | Itaituba virus (ITAV) |
| | | Jacundá virus (JCNV) |
| | | Maldonado virus (MLOV) |
| | | Morumbi virus (MR(M)BV) |
| | | Mucura virus (MCRV/MRAV) |
| | | Nique virus (NIQV) |
| | | Oriximiná virus (ORXV) |
| | | Serra Norte virus (SRNV) |
| | | Turuna virus (TUAV) |
| | *Chilibre phlebovirus* | Cacao virus (CACV) |
| | | Chilibre virus (CHIV) |
| | *Frijoles phlebovirus* | Frijoles virus (FRIV) |
| | | Joá virus (JOAV) |
| | *Mukawa phlebovirus* | Mukawa virus (MKWV) |
| | *Punta Toro phlebovirus* | Buenaventura virus (BUEV) |
| | | Campana virus (CMAV) |
| | | Capira virus (CAPIV) |
| | | Coclé virus (CCLV) |

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**Table 1** (continued)

| Genus | Species[¶] | Virus (abbreviation)[¶] |
|---|---|---|
| | | Leticia virus (LTCV) |
| | | Punta Toro virus (PTV) |
| | *Rift Valley fever phlebovirus** | Rift Valley fever virus (RVFV) |
| | *Salehabad phlebovirus* | Adana virus (ADAV) |
| | | Adria virus (ADRV) |
| | | Alcube virus |
| | | Arbia virus (ARBV) |
| | | Arumowot virus (AMTV) |
| | | Bregalaka virus (BREV) |
| | | Medjerda Valley virus (MVV) |
| | | Odrénisrou virus (ODRV) |
| | | Olbia virus (OLBV) |
| | | Salehabad virus (SALV) |
| | | Zaba virus (ZABAV) |
| | *Sandfly fever Naples phlebovirus* | Arrábida virus (ARRV) |
| | | Balkan virus (BALKV) |
| | | Fermo virus (FERV) |
| | | Gordil virus (GORV) |
| | | Granada virus (GRV = GRAV) |
| | | Massilia virus (MASV) |
| | | Punique virus (PUNV) |
| | | Saddaguia virus (SADV) |
| | | Saint-Floris virus (SAFV) |
| | | sandfly fever Naples virus (SFNV) |
| | | Tehran virus (THEV) |
| | | Toscana virus (TOSV) |
| | | Zerdali virus (ZERV) |
| | *Uukuniemi phlebovirus* | Chizé virus (CHZV) |
| | | EgAN 1825-61 virus (EGAV) |
| | | Fin V 707 virus (FINV) |
| | | Oceanside virus (OCV = OCEV) |
| | | Pontevès virus (PTVV) |
| | | St. Abbs Head virus (SAHV) |
| | | Uukuniemi virus (UUKV) |
| | | Zaliv Terpenyia virus (ZTV) |
| *Pidchovirus* | *Pidgey pidchovirus** | Pidgey virus (PGYV) |
| *Tenuivirus* | *Echinochloa hoja blanca tenuivirus* | Echinochloa hoja blanca virus (EHBV) |
| | *Iranian wheat stripe tenuivirus* | Iranian wheat stripe virus (IWSV) |
| | *Maize stripe tenuivirus* | maize stripe virus (MStV = MSpV) |
| | *Rice grassy stunt tenuivirus* | rice grassy stunt virus (RGSV) |
| | *Rice hoja blanca tenuivirus* | rice hoja blanca virus (RHBV) |
| | *Rice stripe tenuivirus** | rice stripe virus (RSV = RStV) |
| | *Urochloa hoja blanca tenuivirus* | Urochloa hoja blanca virus (UHBV) |
| *Wenrivirus* | *Shrimp wenrivirus** | Wēnzhōu shrimp virus 1 WzSV-1 |
| *Wubeivirus* | *Dipteran wubeivirus** | Húběi diptera virus 5 (HbDV-5) |
| | *Fly wubeivirus* | Wǔhàn fly virus 1 (WhFV-1) |
| Family *Tospoviridae* | | |
| *Orthotospovirus* | *Bean necrotic mosaic orthotospovirus* | bean necrotic mosaic virus (BeNMV) |
| | *Calla lily chlorotic spot orthotospovirus* | calla lily chlorotic spot virus (CCSV) |
| | *Capsicum chlorosis orthotospovirus* | capsicum chlorosis virus (CaCV) |
| | *Chrysanthemum stem necrosis orthotospovirus* | chrysanthemum stem necrosis virus (CSNV) |
| | *Groundnut bud necrosis tospovirus*[1] | groundnut bud necrosis virus (GBNV) |
| | *Groundnut ringspot tospovirus*[1] | groundnut ringspot virus (GRSV) |
| | *Groundnut yellow spot tospovirus*[1] | groundnut yellow spot virus (GYSV) |
| | *Impatiens necrotic spot tospovirus*[1] | impatiens necrotic spot virus (INSV) |

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**Table 1** (continued)

| Genus | Species[¶] | Virus (abbreviation)[¶] |
|---|---|---|
| | *Iris yellow spot tospovirus*[1] | iris yellow spot virus (IYSV) |
| | *Melon severe mosaic orthotospovirus* | melon severe mosaic virus (MSMV) |
| | *Melon yellow spot orthotospovirus* | melon yellow spot virus (MYSV) |
| | *Polygonum ringspot tospovirus*[1] | polygonum ringspot virus (PolRSV) |
| | *Soybean vein necrosis orthotospovirus* | soybean vein necrosis virus (SVNV) |
| | *Tomato chlorotic spot tospovirus*[1] | tomato chlorotic spot virus (TCSV) |
| | *Tomato spotted wilt tospovirus**[1] | tomato spotted wilt virus (TSWV) |
| | *Watermelon bud necrosis tospovirus*[1] | watermelon bud necrosis virus (WBNV) |
| | *Watermelon silver mottle tospovirus*[1] | watermelon silver mottle virus (WSMoV) |
| | *Zucchini lethal chlorosis tospovirus*[1] | zucchini lethal chlorosis virus (ZLCV) |
| Family *Wupedeviridae* | | |
| *Wumivirus* | *Millipede wumivirus** | Wǔhàn millipede virus 2 (WhMV-2) |
| Unassigned | | |
| *Coguvirus* | *Citrus coguvirus** | citrus concave gum-associated virus (CCGaV) |

*type species

[1]Due to a formal classification mistake, this species was not correctly renamed to include the genus epithet "*orthotospovirus*". A proposal to ensure that all species included in the genus *Orthotospovirus* are named uniformly ending in "*orthotospovirus*" will be submitted prior to the next taxonomic proposal submission deadline

[¶]Please note that viruses are real objects that are assigned to concepts that are called taxa. Species, genera, families, and orders are taxa. Taxon names are always italicized and always begin with a capital letter. Virus names, on the other hand, are not italicized and are not capitalized, except if the name or a name component is a proper noun. This column lists the virus names with their correct (lack of) capitalization. Lists of viruses within a given species are provisional at this point and will likely be amended in the near future

## Wupedeviridae

No changes were made at the family rank.

## Summary

A summary of the current, ICTV-accepted taxonomy of the order *Bunyavirales* is presented in Table 1.

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## Compliance with ethical standards

The views and conclusions contained in this document are those of the authors and should not be interpreted as necessarily representing the official policies, either expressed or implied, of the US Department of the Army, the US Department of Defense, the US Department of Health and Human Services, or of the institutions and companies affiliated with the authors. In no event shall any of these entities have any responsibility or liability for any use, misuse, inability to use, or reliance upon the information contained herein. The US departments do not endorse any products or commercial services mentioned in this publication.

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**Conflict of interest** The authors declare no conflicts of interest.

**Ethical approval** This article does not contain any studies with human participants or animals performed by any of the authors.

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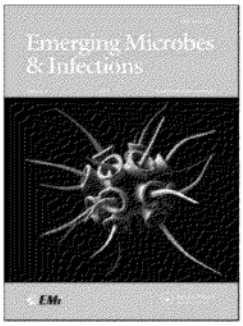
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# Genetic and biochemical characterizations of Zika virus NS2A protein

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# Genetic and biochemical characterizations of Zika virus NS2A protein

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**ABSTRACT**

Zika virus (ZIKV) can cause devastating congenital Zika syndromes in pregnant women and Guillain-Barre syndrome in adults. Understanding the molecular mechanism of ZIKV replication is essential for antiviral and vaccine development. Here we report the structural and functional characterization of ZIKV NS2A protein. Biochemical structural probing suggests that ZIKV NS2A has a single segment that traverses the ER membrane and six segments that peripherally associate with the ER membrane. Functional analysis has defined distinct NS2A residues essential for viral RNA synthesis or virion assembly. Only the virion assembly-defective mutants, but not the RNA synthesis-defective mutants, could be rescued through *trans* complementation with a wide-type NS2A protein. These results suggest that the NS2A molecules in virion assembly complex could be recruited in *trans*, whereas the NS2A molecules in viral replication complex must be recruited in *cis*. Together with previous results, we propose a flavivirus assembly model where NS2A plays a central role in modulating viral structural and nonstructural proteins as well as genomic RNA during virion assembly.

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**KEYWORDS** Zika virus; flavivirus replication; virion assembly; flavivirus NS2A; membrane topology

## Introduction

Zika virus (ZIKV) is a recently emerged mosquito-borne flavivirus that is primarily transmitted by *Aedes spp.* mosquitoes. It can also be transmitted through sexual, maternal-to-fetal, and blood transfusion routes [1–5]. ZIKV was first identified in 1947 from a sentinel rhesus monkey in Uganda [6]. Since 2007, it emerged in the Pacific region, South America, Central America, and the Caribbean, posing a global public health threat [7–11]. ZIKV infection causes mild illnesses, including fever, pruritis, arthritis, conjunctivitis, and asthenia [12]. Importantly, it can cause devastating congenital Zika syndromes (CZS; including microcephaly, congenital malformation, and fetal demise) in pregnant women and Guillain-Barre syndrome (GBS) in adults [13–16]. The explosive epidemics and its association with CZS had led the World Health Organization (WTO) to declare ZIKV as a Public Health Emergency of International Concern from February to November in 2016 [17]. Since then, a number of vaccine candidates have been developed, some of which have already advanced to clinical trials [18]. In addition, small molecule inhibitors and therapeutic antibodies have also been identified for antiviral development [19–22].

ZIKV belongs to the *Flavivirus* genus within the *Flaviviridae* family. Besides ZIKV, many other flaviviruses are significant human pathogens, including dengue (DENV), yellow fever (YFV), West Nile (WNV), Japanese encephalitis (JEV), Saint Louis encephalitis (SLEV), and tick-borne encephalitis virus (TBEV) [23]. Flavivirus genome is a positive-sense, single-stranded RNA of ~11,000 nucleotides. It contains a 5' untranslated region (UTR), a long open-reading-frame (ORF), and a 3' UTR. The single ORF encodes a polyprotein that is processed into three structure proteins (capsid [C], pre-membrane

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[prM] and envelope [E]) and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5). The structural proteins, together with genomic RNA, are components of virions. The nonstructural proteins participate in viral RNA replication, virion assembly, and evasion of host immune response [24–26].

Flavivirus NS2A is an ER-resident, transmembrane protein with a calculated molecular weight of ~22 kDa. The N-terminus and C-terminus of NS2A are formed by cleavages with an unknown host protease and viral protease NS3 (in complex with NS2B), respectively [27,28]. Flavivirus NS2A has multiple functions: (i) Viral replication. Kunjin virus (KUNV) NS2A co-localizes with viral double-stranded RNA and interacts with the 3'UTR of genomic RNA, NS3, and NS5 protein [29]. Mutagenesis studies confirmed the role of NS2A in viral RNA synthesis [30,31]. (ii) Virion assembly/secretion. Mutagenesis analyses of YFV, KUNV, and DENV have identified distinct NS2A amino acids that are essential for virion assembly/secretion [30–34]. The virion assembly defects can be restored by second site mutations in NS2B or NS3 in YFV and DENV [32,35,36]. (iii) Evasion of innate immunity. ZIKV NS2A inhibits type-I IFN induction at the step of TBK1 [26]. DENV NS2A antagonizes type-I IFN signalling [25]. JEV NS2A blocks dsRNA-activated protein kinase PKR [37]. In WNV and KUNV, the NS2A-mediated inhibition of type-I IFN induction can be abrogated by specific NS2A mutations [38,39]. (iv) Disease pathogenesis. WNV NS2A modulates virus-induced cytopathic effect (CPE) and pathogenesis in mice [40,41]. ZIKV NS2A impairs mammalian cortical neurogenesis through depleting adherens junction proteins [42].

The structure and function of ZIKV NS2A have not been characterized. Although NS2A proteins from different flaviviruses perform similar functions (described above), each flavivirus may exert its NS2A functions in a virus-specific manner [35]. The goals of this study are (i) to define the membrane topology of ZIKV NS2A and (ii) to characterize its roles in viral RNA synthesis and virion assembly.

## Materials and methods

### Cell lines

Vero cells, baby hamster kidney (BHK-21) cells and human embryo kidney 293T cells were cultured at 37°C with 5% $CO_2$ in high-glucose Dulbecco's modified Eagle medium (DMEM; Life Technologies) supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 g/ml streptomycin and 10% fetal bovine serum (FBS; HyClone Laboratories).

### Bioinformatics

NS2A protein sequences from ZIKV, DENV1, DENV2, DENV3, DENV4, WNV, JEV, YFV, SLEV were aligned using CLC Main Workbench software (CLC Bio). Prediction of ZIKV NS2A was carried out using various web servers, which including TOPCONS, Philius, Polyphobius, SCAMPI, and TMHMM2. The amphipathic helices of ZIKV NS2A were predicted by HeliQuest.

### Antibody

The following antibodies were used in this study: a mouse monoclonal antibody 4G2 cross-reactive with flavivirus E protein (American Type Culture Collection); Rabbit anti-HA monoclonal antibody (Cell Signaling Technology); Alexa Fluor®488 conjugated mouse anti-HA monoclonal antibody (Cell Signaling Technology); Rabbit anti-Calnexin polyclonal antibody(Abcam); Mouse anti-GFP monoclonal antibody (Sigma-Aldrich); Horseradish peroxidase(HRP) conjugated goat anti-mouse and anti-rabbit IgG polyclonal antibodies (Sigma-Aldrich); Alexa Fluor®488 conjugated goat anti-mouse IgG polyclonal antibody (Thermo Fisher Scientific); Alexa Fluor®568 conjugated goat anti-rabbit IgG polyclonal antibody (Thermo Fisher Scientific).

### Plasmid construction

The infectious cDNA clone (pFLZIKV) bearing the full-length genome of ZIKV (strain FSS13025) or Renilla luciferase (Rluc) reporter ZIKV replicon (pZIKV Rep WT) were used as the backbone for introducing NS2A mutations [43,44]. XhoI and KpnI restriction sites were initially generated into pFLZIKV and pZIKV Rep WT replicon at positions 3018 and 5010 in ZIKV genome without changing amino acid sequences. The NS2A alanine-scanning mutagenesis was performed by using a QuikChange II XL Site-Directed Mutagenesis kit (Agilent Technologies) as described previously [30].

A mammalian expression vector pXJ with a cytomegalovirus (CMV) promoter was used for constructing plasmids expressing NS2A and its derivatives. To construct the pXJ-$E_{24}$-NS1-NS2A-HA plasmid, a gene cassette encoding the last 24 amino acids of E protein ($E_{24}$), NS1 and NS2A with a C-terminal hemagglutinin (HA) tag was firstly amplified from the pFLZIKV and cloned into the pXJ vector. Next, NS2A mutations were individually introduced into the pXJ-$E_{24}$-NS1-NS2A-HA by overlap PCR, resulting in plasmids expressing NS2A mutants.

To construct the pXJ-SPG-$C_{16}$-NS2A-HA that was used for selecting cell line constitutively expressing NS2A protein, a DNA fragment containing the Gaussia luciferase signal peptide (SPG), the last 16 amino acids

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of NS1 protein ($C_{16}$) and NS2A tagged with a C-terminal HA tag sequence were constructed by overlap PCR and cloned into the pXJ vector via NotI/XhoI restriction sites.

Plasmid pXJ-eGFP was constructed by inserting the enhanced green fluorescence protein (eGFP) sequence into the pXJ vector via BamHI/XhoI restriction sites. DNA fragments that encode different NS2A segments were individually inserted into above pXJ-eGFP vector, resulting various pXJ-smNS2A-eGFP plasmids (smNS2A represents NS2A segment). A DNA fragment that express NS2A or truncated NS2A and an eGFP with a C-terminal N-glycosylation acceptor site (Glyc) were amplified and cloned into the pXJ-SPG-$C_{16}$-NS2A-HA vector, resulting in the plasmid pXJ-SPG-$C_{16}$-NS2A-eGFP-Glyc and various pXJ-SPG-$C_{16}$-ctNS2A-eGFP-Glyc plasmids (ctNS2A represents C-terminal truncated NS2A).

### *RNA in vitro transcription and electroporation*

All full-length cDNA clone plasmids and subgenomic replicons of ZIKV were linearized with restriction enzyme ClaI, followed by phenol-chloroform extraction and ethanol precipitation. RNA transcripts were prepared by using the T7 mMessage mMachine kit (Ambion). For RNA transfection, 10 μg *in vitro* transcribed RNA was electroporated into $8 \times 10^6$ cells (BHK-21 or Vero) by using Gene Pulser Xcell™ Electroporation Systems (Bio-rad) as described previously [30,43].

### *Immunofluorescence assay*

For indirect immunofluorescence assay (IFA), cells were seeded in an 8-well Nunc Lab-Tek II Chamber Slide (Thermo Fisher Scientific). At various time points, cells were washed three times with phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde in PBS at room temperature for 30 min and permeabilized with 0.1% Triton X-100 in PBS at room temperature for 10 min. The permeabilized cells were then washed three times with PBS and incubated in a blocking buffer containing 1% FBS in PBS at room temperature for 1 h. E protein was stained with a 1:1000 dilution of mouse monoclonal antibody 4G2 in blocking buffer. After incubation for 1 h, the cells were washed three times with a washing buffer (1% FBS and 0.5% Tween 20 in PBS). After 1 h of incubation with a secondary antibody (Alexa Fluor®488 conjugated goat anti-mouse IgG), the cells were washed three times with washing buffer and counterstained with DAPI (4',6-diamidino-2-phenylindole). Images were acquired using an Olympus fluorescence microscope. Images were processed in ImageJ software (National Institutes of Health, MD).

### *Plaque assay*

$2 \times 10^5$ Vero cells per well were grown in 24-well plates one day prior to viral infection. 10-fold serial dilutions of virus samples were prepared in DMEM containing 2% FBS and 1% penicillin/streptomycin and inoculated (100 μl) into each well of 24-well plates. After 1 h of incubation at 37°C with 5% $CO_2$, the inoculum was replaced with 500 μl overlay medium (DMEM medium containing 0.8% methyl-cellulose, 2% FBS and 1% penicillin/streptomycin). Plaques formed after incubation at 37°C with 5% $CO_2$ for 4–5 days. Cells were fixed with 4% paraformaldehyde for 30 min, followed by staining with 1% crystal violet. The numbers of plaque per millilitre (PFU/ml) of ZIKV were calculated.

### *Replicon transient-transfection assay*

Briefly, $8 \times 10^6$ Vero cells were electroporated with 10 μg wild type or mutant Rluc-ZIKV replicon RNAs. After electroporation, $3 \times 10^5$ cells per well were seeded in 12-well plates. At given time points, the cells were washed twice with PBS and lysed in 200 μl of luciferase lysis buffer (Promega). The luciferase activity assay was performed according to the manufacturer's protocol.

### *RNA extraction and cDNA sequencing*

The culture fluids from infected cells were centrifuged at 1000 g for 10 min and then filtered through a 0.22 μm pore size polyethersulfone membrane (Millipore) to remove cellular debris. Viral RNAs in the supernatants were extracted using a QIAamp viral-RNA Minikit (Qiagen) by following the manufacturer's protocol. Reverse transcription PCR (RT-PCR) was performed by using the SuperScript® III One-Step RT-PCR System (Thermo Fisher Scientific). The amplified cDNA fragments were purified and sent for Sanger sequencing at the GENEWIZ facilities.

### *Selection of NS2A-HA BHK-21 cell line*

$4 \times 10^5$ BHK-21 cells per well were seeded into a 6-well plate. The plasmid pXJ-SPG-$C_{16}$-NS2A-HA that contains a neomycin resistance gene was transfected into BHK-21 cells (4 μg per well) using X-tremeGENE 9 DNA transfection reagent (Roche). At 24 h post-transfection, geneticin (G418) was added to the culture medium with a concentration of 1 mg/ml. The medium was changed every three days. After two weeks, several neomycin-resistant individual colonies were selected for propagation. The expression of NS2A-HA protein in stable cell lines was analyzed by immunofluorescence assay and Western blot.

### *In vitro proteinase K protection assay*

Proteinase K protection assay was performed as previously described with minor modifications [45,46].

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BHK-21 cells were transfected in 6-well plates with various pXJ-SPG-$C_{16}$-ctNS2A-eGFP-Glyc plasmids. After incubated at 30°C for 24 h, cells were trypsinized, washed twice with PBS and centrifuged at 450 × g for 5 min. The cell pellets were resuspended in a hypotonic lysis buffer (10 mM Tris-HCl, pH 7.5, and 2 mM $MgCl_2$) and kept on ice for 10 min. Subsequently, the samples were homogenized with 40 strokes and centrifuged at 1000 × g for 5 min. The postnuclear supernatants (PNS) were divided into three aliquots and centrifuged at 15,000 × rpm for 40 min. The pellets from three aliquots were then resuspended in PBS, 60 μg/ml proteinase K in PBS, or 60 μg/ml proteinase K in PBS in the presence of 0.5% Triton X-100, respectively. The samples were incubated on ice for 90 min. 20 mM phenylmethylsulfonyl fluoride (PMSF) was finally added to terminate the reaction. After further incubation on ice for 15 min, the samples were mixed with 4× Laemmli sample buffer, heated at 95°C for 15 min and analyzed by Western blotting.

### *In vitro deglycosylation assay*

The samples were prepared by following a previously described protocol [46,47]. Briefly, the transfected BHK-21 cells in 6-well plates were incubated at 30°C for 24 h. Cells were trypsinized and suspended in DMEM medium containing 10%FBS. The cells were pelleted by centrifuging at 450 × g for 5 min. After two PBS washes, the cell pellets were resuspended in a solubilization buffer (20 mM Tris-HCl, pH 7.5, 50 mM NaCl, 10 mM $Mg(CH_3COO)_2$, 1% Triton X-100, and EDTA-free protease inhibitors [Roche]) and incubated at 4°C for 2 h with gentle agitation. After centrifugation at 15,000 rpm 4°C for 20 min to remove cell debris, the supernatants were split into two aliquots. 50,000 units/ml N-glycosidase F (PNGase F) or PBS (no-treatment control) were added to those two aliquots. After incubation at 37°C for 2 h, the samples were mixed with 4× Laemmli sample buffer, heated at 70°C for 15 min and analyzed by Western blot.

### *SDS-PAGE and Western blot*

Protein samples were separated by 12% SDS-polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride (PVDF) membrane using Trans-Blot Turbo transfer system (Bio-Rad), after which the membrane was blocked in TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 0.1% Tween 20) supplemented with 5% nonfat milk for 1 h at room temperature. The blots were probed with primary antibodies (1:1,000 dilution) overnight at 4°C, washed three times with TBST and incubated with horseradish peroxidase (HRP) conjugated goat anti-mouse and anti-rabbit IgG polyclonal antibodies (1:10,000 dilution) for 1 h. After three thorough TBST buffer washes, the blots were incubated with SuperSignal™ West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific). Chemiluminescence signals were detected in ChemiDoc System (Bio-Rad).

### *Quantification and statistical analysis*

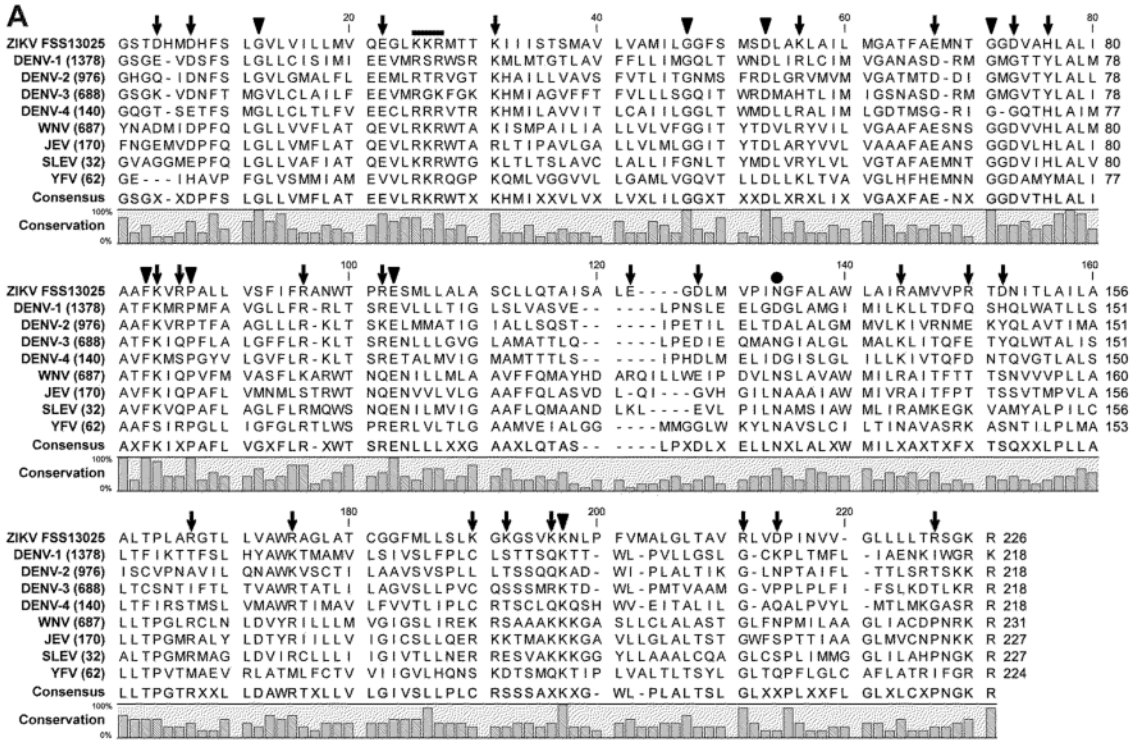
IFA images processing and cell counting were performed in software ImageJ (NIH). Densitometry analysis was performed using Image lab version 6.0 (Bio-Rad Laboratories, Hercules, CA). All numerical data are presented as the mean ± SEM (standard error of mean). The size of each study or number of replicates, along with the statistical tests performed can be found in corresponding Figure Legends.

## Results

### *The rationale for ZIKV NS2A mutagenesis*

To identify critical residues in ZIKV NS2A, we performed a systematic alanine-scanning mutagenesis using an infectious cDNA clone of ZIKV strain FSS13025 [43]. Sequence alignment of NS2A proteins from ZIKV, DENV, WNV, JEV, SLEV, and YFV revealed a high diversity: only 31 out of 218–227 amino acids with a consensus of >80% among different flaviviruses (Figure 1A). A total of 35 residues were selected for mutagenesis: (i) All 8 identical non-alanine residues, including G12, G47, D53, G71, F83, P87, E103, and K193. The amino acid numbering is based on the ZIKV sequence. (ii) All charged residues, including D4, D7, E22, K31, K56, E67, D73, H76, K84, R86, R96, R102, E122, D124, R140, R146, D148, R163, R171, K186, K188, K192, R207, D210, and R222. Charged residues were selected because they likely participate in inter- or intra-molecular interactions; alternatively, they may alter the locations where NS2A would traverse the ER membrane since charged residues are hydrophilic. In addition, NS2A D73H mutation was previously shown to attenuate WNV replication [48]. (iii) A K25/K26/R27 motif that was previously reported to be critical for YFV and DENV virion assembly [35,40]. (iv) Residue N130 that was previously shown to be required for NS1-NS2A cleavage and viral replication in DENV [27,30]. The selected residues were mutated to alanine in the context of ZIKV genomic RNA. Equal amounts of wild-type (WT) and mutant RNAs were electroporated into Vero cells. After electroporation, RNA replication and virus spread were monitored by immunofluorescence assay (IFA); the production of infectious viruses was quantified by plaque assay and RT-PCR. The 35 mutants exhibited distinct phenotypes that could be categorized into five groups (Figure 1B), as detailed in the following sections.

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**B**

| Class | NS2A Mutations | Phenotypes |
|---|---|---|
| I | D4A, E22A, K31A, G47A, D53A, E67A, H76A, E122A, R146A, D148A, R163A, K186A, K188A, D210A | Mutants do not or weakly affect viral production |
| II | K56A , F83A, K84A, R86A, P87A, R96A, R102A, D124A, R207A | Mutants dramatically reduce viral production |
| III | G12A, N130A | Mutants abolish viral RNA synthesis |
| IV | D7A, R140A, R222A | Mutants abolish virus production by affecting RNA synthesis and viron assembly/release |
| V | K25A/K26A/R27A, G71A, D73A, E103A, R171A, K192A, K193A | Mutants abolish virion assembly |

**Figure 1.** ZIKV NS2A mutagenesis. (A) Sequence alignment of NS2A proteins among ZIKV (strain FSS13025), DENV-1-4 serotypes, WNV, JEV, SLEV, and YFV using CLC Main Workbench software (CLC Bio). Thirty-five amino acids were selected for mutagenesis: (i) All 8 identical non-alanine residues (indicated by triangles), (ii) 25 charged residues (arrows), (iii) a K25/K26/R27 motif (line), (iv) residue N130 (circle). The consensus and conservation percentage are indicated below each amino acids. (B) A summary of ZIKV NS2A mutagenesis results. An infectious cDNA clone and a luciferase replicon of ZIKV strain FSS13025 were used to analyze the function of each amino acid. Based on the replication phenotypes, the 35 mutants could be categorized into five classes as indicated.

### *Class I mutations have no or weak effects on viral replication*

Fourteen of the 35 NS2A mutant RNAs, including D4A, E22A, K31A, G47A, D53A, E67A, H76A, E122A, R146A, D148A, R163A, K186A, K188A, and D210A, replicated at levels comparable to the WT RNA. These mutants were categorized as class I (Figure 1B). The IFA images and plaque morphologies of three representatives (D53A, D148A, and R163A), as well as the WT, are shown in Figure 2(A and B), respectively. Most class I mutants (e.g. D148A and R163A) yielded comparable E-expressing cells as the WT from 24 to 48 h post-transfection (p.t.). At 72 h p.t., severe CPE occurred among D148A, R163A, and WT RNA-transfected cells. Some class I mutants (e.g. D53A) produced fewer E-positive cells than the WT at 48 h p.t., and spread to infect more cells at 72 h p.t (Figure 2A). All class I mutants produced viruses with plaques similar to the WT virus (Figure 2B). At 72 h p.t., the class I mutants produced infectious titers <4-fold lower than the WT did (Figure 2C). Sequencing of the mutant viruses (collected at 72 h p.t.) confirmed the retaining of the engineered mutations without other changes (data not shown).

### *Class II mutations significantly attenuate viral replication*

Nine NS2A mutants, including K56A, F83A, K84A, R86A, P87A, R96A, R102A, D124A, and R207A,

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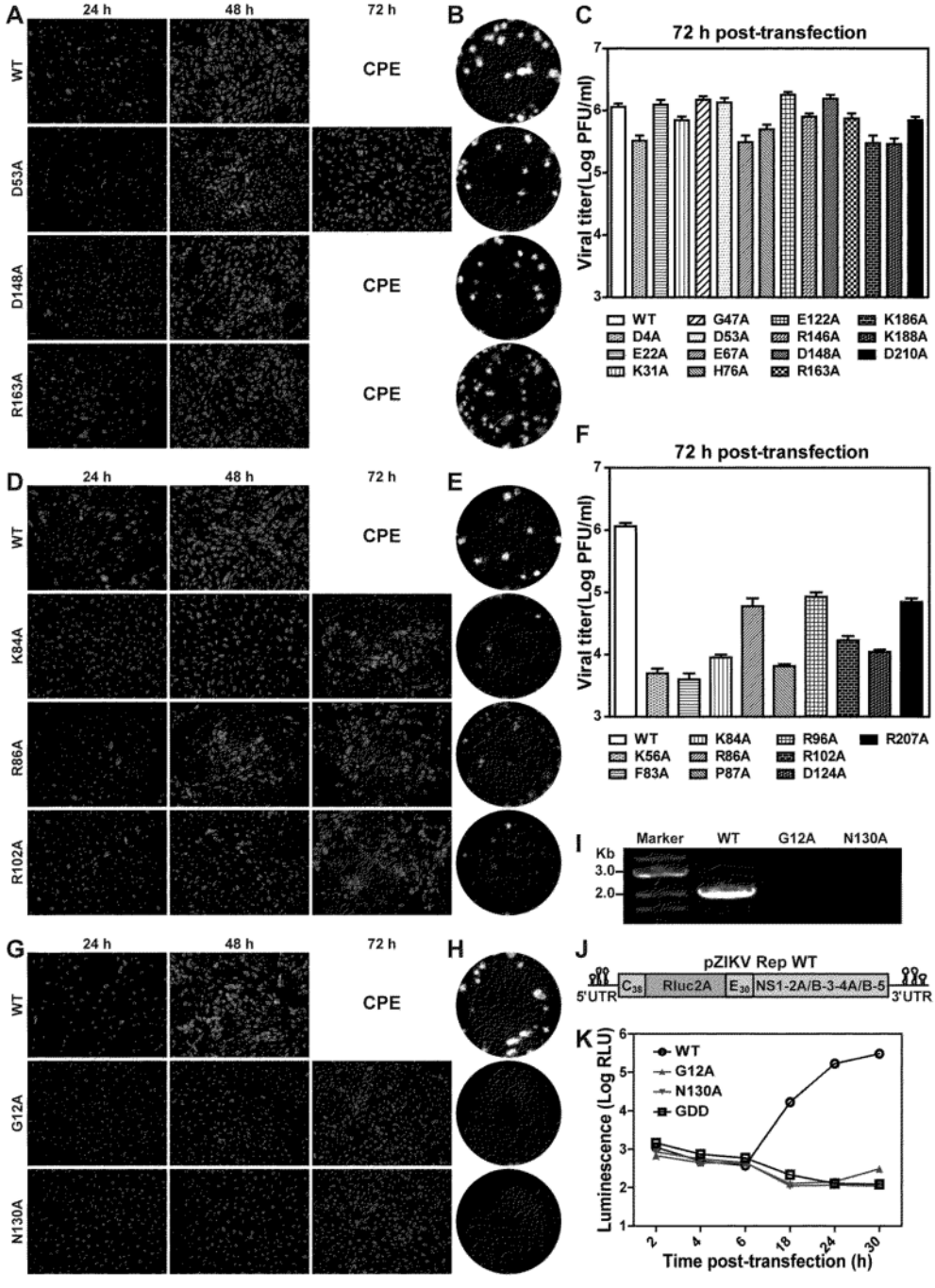



**Figure 2.** Characterization of class I, II, and III NS2A mutants. The results of representative mutants from class I (A–C), II (D–F), and III (G–K) are shown. (A) Vero cells were electroporated with equal amounts of ZIKV wild-type (WT) or class I mutant genomic RNAs. At indicated time points, the cells were fixed and subjected to IFA. Viral E protein and nuclei were stained with antibody 4G2 (green) and DAPI (blue), respectively. Cytopathic effect (CPE) is indicated at 72 h post-transfection. (B) Plaque morphologies. Recombinant viruses were subjected to plaque assay. Plaques were developed at day 4.5 post-infection. (C) Viral titers of class I mutants at 72 h post-transfection. The mean values from two independent experiments are shown. Error bars indicate the standard deviations. The limit of detection (LOD) of plaque assay is 10 PFU/ml. (D) IFA of class II mutant RNA-transfected Vero cells. (E) Plaque morphology of class II mutant viruses. (F) Viral titers of class II mutant viruses at 72 h post-transfection. (G) IFA of class III mutant RNA-transfected Vero cells. (H) Plaque morphologies of class III mutant viruses. Plaque assay was performed using culture supernatants collected at day 3 (for WT) and day 5 (for G12A and N130A) post-transfection. (I) RT-PCR analysis. At 5 day p.t., the mutant RNA-transfected cells were washed twice with PBS to remove residual input RNAs. At day 8 p.t., the supernatants of G12A and N130A RNA-transfected cells were subjected to RT-PCR. The WT virus (collected at 72 h p.t.) was used as a positive control. (J) A ZIKV luciferase replicon. $C_{38}$ and $E_{30}$ represent the first 38 amino acids of C protein and the last 30 amino acids of E protein, respectively. *Renilla* luciferase: Rluc. Foot-and-mouse-disease virus 2A sequence: 2A. (K) Transient replicon assay. WT, G12A, N130A, and NS5ΔGDD replicon RNAs were electroporated into Vero cells. Luciferase activities were measured at indicated time points. An average of 3 experiments are presented. Bars represent standard deviations.

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significantly attenuated viral replication. These mutants were categorized into class II (Figure 1B). Class II RNA-transfected cells produced fewer E-positive cells than the WT did (Figure 2D). At 72 h p.t., only 30–50% of E-positive cells were detected in the class II RNA-transfected cells, whereas the WT RNA-transfected cells exhibited CPE. Class II mutant viruses formed smaller, opaque plaques (Figure 2E), and the transfected cells produced >10-fold lower infectious titers than the WT RNA-transfected cells at 72 h p.t. (Figure 2F). Complete genome sequencing of the class II mutant viruses (collected at 96 h p.t.) did not reveal reversions or any other mutations (data not shown). Because of the weak phenotypes of class I and II mutants, we did not further characterize them.

### *Class III mutations are lethal for viral RNA synthesis*

Class III includes two mutants, G12A and N130A, which were completely defective in viral RNA synthesis (Figure 1B). The G12A and N130A genomic RNA-transfected cells did not produce any E-positive cells at 24 and 48 h p.t. (Figure 2G). On day 5–8 p.t., no infectious viruses were detected from culture medium by plaque assay (Figure 2H) and RT-PCR (Figure 2I). To confirm the above results, we introduced G12A or N130A mutation into a ZIKV luciferase replicon (Figure 2J). A replication-defective replicon, containing an NS5 polymerase active site ΔGDD mutation, was included as a negative control [44]. Vero cells were electroporated with equal amounts of WT, G12A, N130A, or NS5 ΔGDD replicon RNAs. At 2–6 h p.t., all replicons generated similar levels of luciferase signals, suggesting comparable levels of viral RNA translation and transfection efficiencies (Figure 2K). However, at 18–30 h p.t., G12A and N130A replicons generated low luciferase signals as the non-replicative NS5 ΔGDD replicon did, whereas the WT replicon produced increasing luciferase signals (Figure 2K). These results demonstrate that class III mutations completely abolish viral RNA synthesis.

### *Class IV mutations impair both viral RNA synthesis and virion production*

Class IV includes three mutants, including D7A, R140A, and R222A (Figure 1B). These mutant genomic RNAs produced fewer E-positive cells in the transfected cells than the WT RNA (Figure 3A). For D7A or R140A mutants, the RNA-transfected cells did not produce infectious viruses, as suggested by plaque assay (Figure 3B) and RT-PCR (Figure 3C). Incubation of naïve Vero cells with culture supernatants from the D7A or R140A transfected cells did not generate any viral E-positive cells (Figure 3D). Replicon analysis showed that D7A or R140A significantly compromised viral RNA synthesis (Figure 3E). Since neither mutants could produce extracellular viruses (Figure 3B and C), and could replicate at attenuated levels inside cells (Figure 3E), D7A and R140A mutations may impair both viral RNA synthesis and virion production.

For R222A, the number of E-positive cells slightly increased in the genomic RNA-transfected cells from 24 to 72 h p.t. (Figure 3A). A low level of infectious virus was detected from the culture medium by plaque assay (Figure 3B) and RT-PCR (Figure 3C). These data were supported by the positive-IFA results after incubating naive Vero cells with the supernatant from the R222A RNA-transfected cells (Figure 3D). Sequencing the virus (collected at 72 h p.t.) revealed that, besides the engineered R222A mutation, the virus had acquired a second site mutation of L220F in NS2A (Figure 3C). Replicon analysis showed that R222A significantly lowered viral RNA synthesis (Figure 3E); no secondary mutation (i.e. L220F) was detected in the replicon RNA at 30 h p.t. (data not shown). These results suggest that (i) R222A mutant is defective in viral RNA synthesis and/or virion assembly, and (ii) a second site mutation L220F in NS2A could rescue the defects.

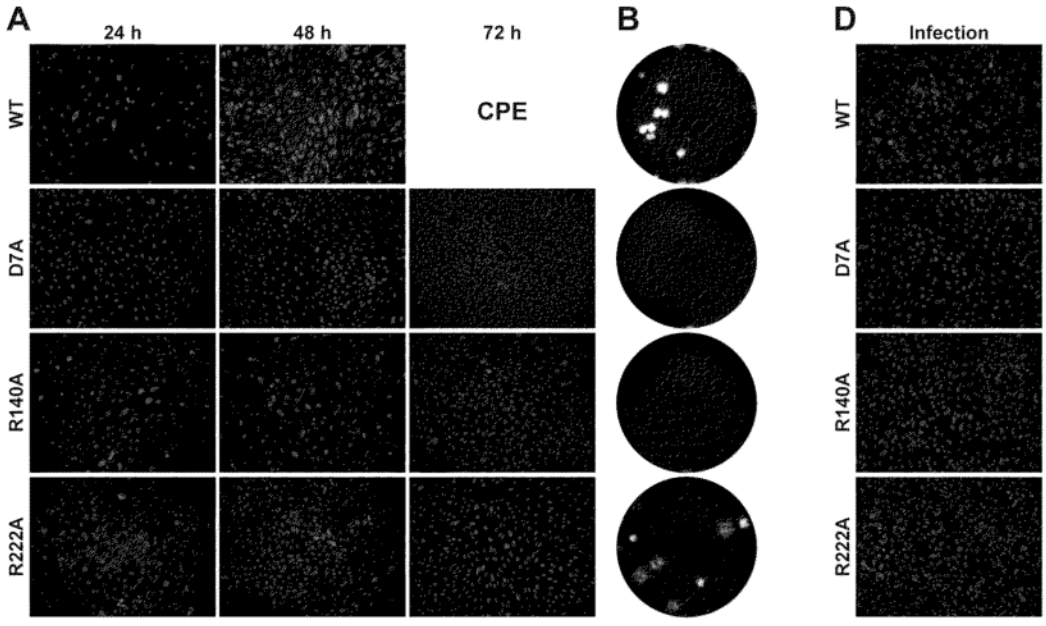
### *Class III and IV mutations do not affect NS2A maturation*

Since cleavage between NS1 and NS2A is essential for flavivirus RNA synthesis [30], we examined whether the replication defects of class III and IV mutants were caused by defective maturation of NS2A. To test this hypothesis, we introduced individual mutations into an expression plasmid pXJ-$E_{24}$-NS1-NS2A-HA that encodes a polyprotein containing the C-terminal 24 amino acids of the E protein (serving as a signal peptide to translocate NS1 to ER lumen), NS1, and NS2A with a C-terminal HA tag. After transfecting the plasmids into 293 T cells, the expression of un-cleaved NS1-NS2A-HA intermediate and cleaved NS2A-HA product were analyzed using Western blot (Figure 3F). None of the mutations significantly affected the cleavage efficiencies of NS1-NS2A-HA to NS2A-HA, indicating that the replication defects of class III and IV mutants are not caused by impaired NS2A cleavage.

### *Class V mutations block virion assembly*

Class V includes G71A, D73A, E103A, R171A, K192A, K193A, and a triple K25A/K26A/R27A. Cells transfected with class V mutant genomic RNAs yielded E-positive cells at 24 h p.t., but the number of E-positive cells did not increase afterward (Figure 4A), suggesting that the mutants are defective in virus spread. No infectious mutant viruses, except E103A, were detected in the supernatants of transfected cells by plaque assay (Figure 4B) or RT-PCR (Figure 4C). No IFA-positive

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C

| | Viral titer (IFU/ml) | | | Extracellular viral RNA RT-PCR | Second site mutation |
|---|---|---|---|---|---|
| | 24 h | 48 h | 72 h | | |
| WT | $(1.70\pm0.30)\times10^5$ | $(4.30\pm0.70)\times10^5$ | $(1.15\pm0.15)\times10^6$ | Positive | None |
| D7A | <10 | <10 | <10 | Negative | N.A. |
| R140A | <10 | <10 | <10 | Negative | N.A. |
| R222A | <10 | 35±15 | 400±100 | Positive | L220F(NS2A) |

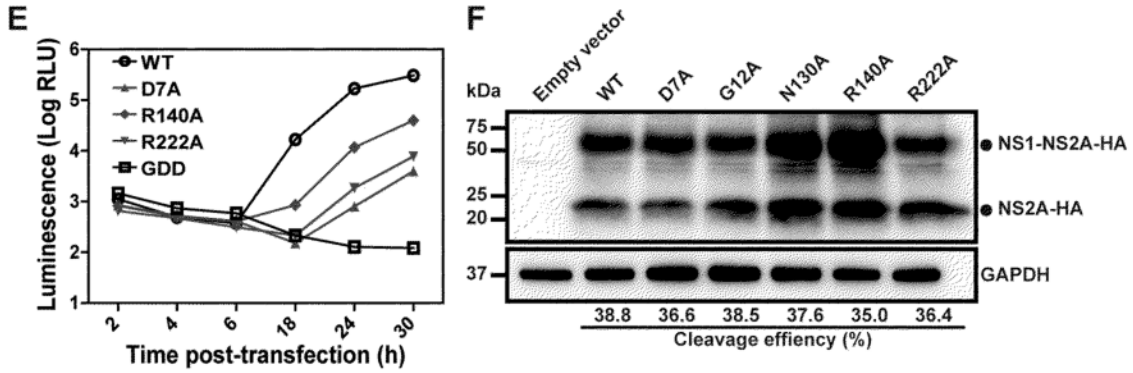
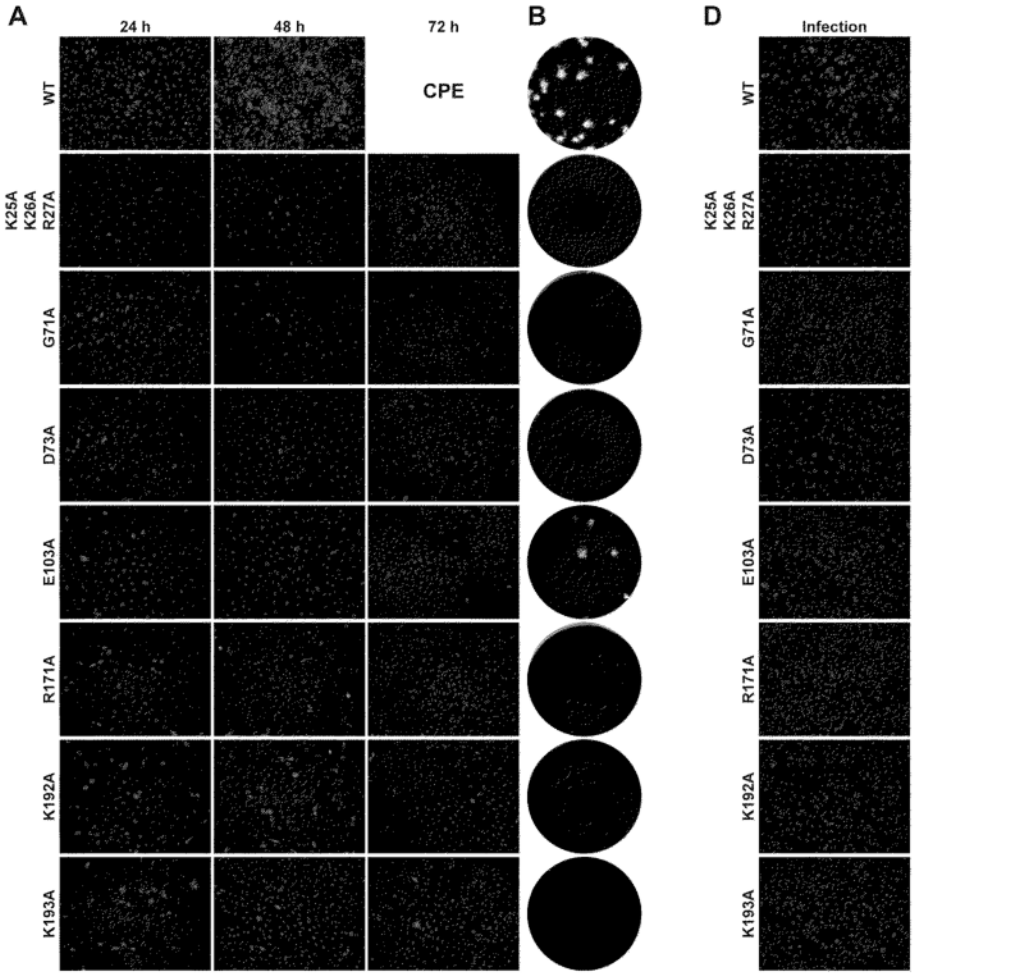



**Figure 3.** Characterization of class IV NS2A mutants. (A) IFA of transfected Vero cells. (B) Plaque morphologies of WT, D7A, R140A, and R222A viruses. (C) Extracellular viral titers were measured at 24, 48, and 72 h p.t. using plaque assay. At 72 h p.t., extracellular viral RNAs were subjected RT-PCR analysis. The RT-PCR products were sequenced to confirm the engineered mutations and to detect potential second site mutation(s) in the NS2A gene. N.A., not applicable. (D) The supernatants of transfected cells were collected on day 8 p.t. to infect naïve Vero cells. At 24 h p.t., the cells were analyzed for viral E expression by IFA. (E) Transient luciferase replicon analysis. Vero cells were electroporated with ZIKV replicon RNAs and measured for luciferase activities at indicated time points. An average of 3 experiments are presented. Bars represent standard deviations. (F) Analysis of NS2A proteolytic processes. NS2A D7A, G12A, N130A, R140A, and R222A mutations were individually engineered into expression plasmid pXJ-$E_{24}$-NS1-NS2A-HA. After transfecting the plasmids into 293 T cells, total cellular protein samples were harvested at 24 h p.t. and analyzed for NS2A process using Western blot. An HA-tag antibody was used to detect both unprocessed NS1-NS2A-HA and processed NS2A-HA proteins.

cells were detected after naïve cells were incubated with the supernatants from transfected cells (Figure 4D). Sequencing of the recovered E103A mutant virus revealed an A103D change in the NS2A gene (Figure 4C), suggesting that an E103A-to-A103D substitution is responsible for the rescue of viral replication.

To determine the stage of viral replication that was disrupted by the class V mutations, we analyzed the mutational effect on viral replication in ZIKV replicon (Figure 4E). Compared with the WT replicon, all class V mutants exhibited equivalent levels of viral translation and RNA synthesis (<4-fold difference),

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| | Viral titer (PFU/ml) | | | Extracellular viral RNA RT-PCR | Second site mutation |
|---|---|---|---|---|---|
| | 24h | 48h | 72h | | |
| Wild Type | $(1.7\pm0.3)\times10^5$ | $(4.3\pm0.7)\times10^5$ | $(1.15\pm0.15)\times10^6$ | Positive | None |
| K25AK26AR27A | <10 | <10 | <10 | Negative | N.A. |
| G71A | <10 | <10 | <10 | Negative | N.A. |
| D73A | <10 | <10 | <10 | Negative | N.A. |
| E103A | <10 | <10 | 175±25 | Positive | A103D(NS2A) |
| R171A | <10 | <10 | <10 | Negative | N.A. |
| K192A | <10 | <10 | <10 | Negative | N.A. |
| K193A | <10 | <10 | <10 | Negative | N.A. |

**Figure 4.** Characterization of class V NS2A mutants. (A) IFA of Vero cells that were transfected with WT or class V genomic RNAs. Viral E-positive cells are indicated in green. (B) Plaque morphologies of class V mutant viruses. Only the WT and E103A mutant generated plaques. (C) Supernatants from the RNA-transfected cells were quantified for viral titers by plaque assay. RT-PCR was also performed on the supernatants at day 3 p.t. for the WT virus and at day 8 p.t. for the mutants. Positive RT-PCR products were sequenced for potential second site mutation(s). N.A., not applicable. (D) Naïve Vero cells were infected with supernatants from the RNA-transfected cells and subjected to IFA for viral E protein expression. (E) Luciferase replicon analysis. Equal amounts of WT and mutant replicon RNAs were electroporated into Vero cells. Luciferase activities were measured at the indicated time points. (F) Analysis of intracellular infectious viruses. Intracellular viruses from ZIKV RNA-transfected cells were harvested at 24 h p.t. as previously described [46]. The harvested samples were used to infect naïve Vero cells. IFA was performed to examine viral E protein expression at 24 h post-infection.

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suggesting that the mutations only marginally affect viral RNA synthesis. Next, we tested if the class V genomic RNAs could generate intracellular infectious viruses. Incubating naïve Vero cells with WT RNA-transfected cell extracts yielded IFA-positive cells, whereas no IFA-positive cells were detected after incubating naive cells with the mutant RNA-transfected cell extracts (Figure 4F). Altogether, the results suggest that class V mutants are competent in viral RNA replication, but defective in assembly of infectious viruses.

### *WT NS2A rescues virion assembly for class V mutants*

To test *trans* complementation of NS2A mutants, we constructed a BHK-21 cell line that constitutively expressed WT ZIKV NS2A protein with a C-terminal HA-tagged (NS2A-HA). The cell line was constructed by transfecting plasmid pXJ-SPG-$C_{16}$-NS2A-HA encoding a Gaussia luciferase signal peptide (SPG), the C-terminal 16 amino acids of NS1 protein ($C_{16}$), and NS2A-HA, as well as a neomycin resistance gene (*Neo*). The SPG and $C_{16}$ sequences were engineered to enable the correct formation of NS2A membrane topology when expressed in cells. Individual BHK-NS2A-HA cell lines were generated through colony purification under G418 (1 mg/ml) selection. For each BHK-NS2A-HA cell line, all cells were IFA-positive for HA-NS2A expression (Figure 5A). Western blot revealed an expected band of ~22-kDa band (Figure 5B). The expression level of NS2A-HA did not decrease in the BHK-NS2A-HA cells after 20 passages in culture (4 days per passage; Figure 5B), indicating the stability of the cell line.

We tested *trans* complementation by transfecting class V genomic RNAs to BHK-NS2A-HA cells. The transfected cells developed an increasing number of E-positive cells from 48 to 96 h p.t. (Figure 5C). Culture supernatants were collected at 96 h p.t. to infect naïve Vero cells. The infected cells developed E-positive cells for all class V mutants (Figure 5D), demonstrating the rescue of infectious viruses through *trans* complementation. Quantification of the E-positive cells allowed us to determine the titers of rescued infectious viruses (Figure 5E). Among the class V mutants, E103A, K192A, and K193A exhibited the highest *trans* complementation efficiencies, producing $1.9 \times 10^5$–$3.04 \times 10^5$ IFU/ml viruses at 96 h p.i. (Figure 5E). Sequencing of the rescued virion genomes confirmed the retention of the engineered class V mutations without any other changes in NS2A (Figure 5E).

Next, supernatants from the first round of infection collected at 72 h p.i. (from Figure 5D) were used to infect naïve Vero cells for a second round. In contrast to the first round of infection (Figure 5D), no E-positive cells were detected in the second round of infection for any of the class V mutants (Figure 5F). As a positive control, WT RNA produced high numbers of E-positive cells in both the first and second rounds of infections (Figure 5D and F). Collectively, the results indicate that (i) the defect in virion assembly of class V mutants could be rescued through *trans* supply of WT NS2A protein and (ii) the rescued virions could only infect cells for a single round.

### *DENV-2 NS2A could not trans complement ZIKV class V mutants*

We tested whether DENV NS2A could rescue class V mutants of ZIKV. Each class V mutant of ZIKV was transfected into a previously established BHK-21 cell line that constitutively expresses WT DENV-2 NS2A protein [30]. For all class V mutants, no increase in E-positive cells or infections viruses was detected from the transfected cells from 24 to 96 h p.t. (data not shown). The results suggest that flavivirus NS2A modulates virion assembly in a virus-type specific manner.

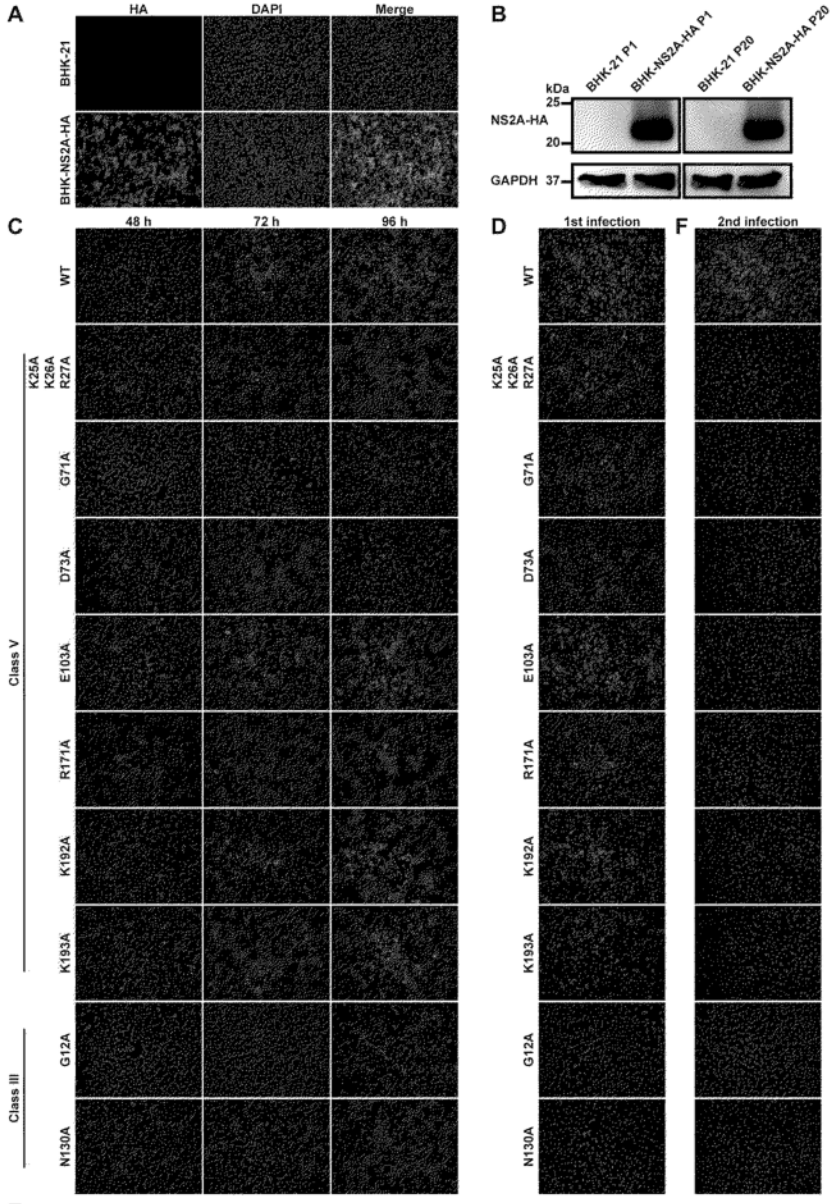
### *WT NS2A could not rescue viral RNA synthesis of class III mutants*

We tested whether the defect in viral RNA synthesis of class III mutants could be *trans* complemented. To test this possibility, we transfected class III genomic RNAs (G12A and N130A) into ZIKV BHK-NS2A-HA cells. Neither mutants generated any E-positive cells in the transfected BHK-NS2A-HA cells (Figure 5C). Infection of naïve Vero cells with supernatants of the transfected cells did not produce any IFA-positive cells (Figure 5D–F). The results suggest that the RNA synthesis defect of class III mutants could not be rescued through *trans* complementation. Due to the dual defects of class IV mutants in both viral RNA synthesis and virion assembly, we did not pursue *trans* complementation experiments for this class of mutants; such complementation experiments may not reveal clear mechanistic insights as compared with the class III and V mutants.

### *Probing the topology of ZIKV NS2A on the ER membrane*

The distinct functions of amino acids in viral RNA synthesis and virion assembly prompted us to probe the topology of ZIKV NS2A on ER membrane. Bioinformatics analysis suggests that ZIKV NS2A may form seven transmembrane segments (pTMS1–7; Figure 6A). To examine whether each pTMS associates with the ER membrane, we constructed a panel of plasmids encoding full-length or various NS2A fragments with a C-terminally fused eGFP reporter (Figure 6B). The length of each segment was slightly modified from the bioinformatic prediction to allow some

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E

| | | Viral titer (IFU/ml) | | | Extracellular viral RNA RT-PCR | Second site mutation |
|---|---|---|---|---|---|---|
| | | 48 h | 72 h | 96 h | | |
| Class V | K25AK26AR27A | $(7.16\pm1.01)\times10^3$ | $(9.69\pm2.65)\times10^3$ | $(5.97\pm1.18)\times10^4$ | Positive | None |
| | G71A | $(1.48\pm0.78)\times10^3$ | $(1.97\pm0.66)\times10^3$ | $(5.42\pm0.66)\times10^3$ | Positive | None |
| | D73A | $(2.10\pm0.57)\times10^2$ | $(1.97\pm0.12)\times10^3$ | $(8.60\pm1.10)\times10^3$ | Positive | None |
| | E103A | $(1.13\pm0.14)\times10^4$ | $(2.30\pm0.20)\times10^4$ | $(3.04\pm0.25)\times10^5$ | Positive | None |
| | R171A | $(6.41\pm1.58)\times10^3$ | $(8.90\pm0.32)\times10^3$ | $(6.06\pm1.06)\times10^4$ | Positive | None |
| | K192A | $(7.85\pm2.04)\times10^3$ | $(1.90\pm0.02)\times10^4$ | $(2.17\pm0.17)\times10^5$ | Positive | None |
| | K193A | $(2.69\pm0.32)\times10^3$ | $(1.85\pm0.05)\times10^4$ | $(1.90\pm0.08)\times10^5$ | Positive | None |
| Class III | G12A | <10 | <10 | <10 | Negative | N.A. |
| | N130A | <10 | <10 | <10 | Negative | N.A. |

**Figure 5.** Trans complementation of class III and V mutants. (A) IFA of BHK-NS2A-HA cells. The BHK-NS2A-HA cells constitutively express WT ZIKV NS2A fused with a C-terminal HA-tag. The cells were fixed with paraformaldehyde and stained with an HA-tag antibody. (B) Western blot of BHK-NS2A-HA cells. The expression of WT NS2A-HA from BHK-NS2A-HA cells was detected using an HA-tag antibody. Both passage one (P1) and 20 (P20) BHK-NS2A-HA cells were analyzed. Naïve BHK-21 cells were included as a negative control. (C) WT and class III and V genomic RNAs were electroporated into BHK-NS2A-HA cells. IFA was performed on the transfected cells to detect viral E protein expression. (D) Viruses rescued from the BHK-NS2A-HA cells (harvested at 96 h p.t.) were used to infect naïve Vero cells. The infected Vero cells were subjected to IFA for E protein expression. (E) Extracellular viruses were harvested from the supernatants of RNA-transfected BHK-NS2A-HA cells. The harvested viruses were serially diluted to infect naïve Vero cells in 96-well plates. At 24 h p.i., IFA was performed to quantify the viral titers by counting the number of E-positive cells. The results show averages and standard deviations from triplicate samples. Viral RNAs in the supernatants of transfected BHK-NS2A-HA cells were isolated at 96 h p.t. and subjected to RT-PCR. Positive RT-PCR products were sequenced to confirm the engineered mutations and to identify potential second site change(s). (F) The supernatants of infected Vero cells from (D) were collected at 72 h p.i. to infect naïve Vero cells for a second round. IFA was performed on these cells for viral E-positive cells.

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flexibility between pTMS and eGFP. Upon transfection into Vero cells, eGFP alone (without any NS2A fragment) exhibited an even distribution of fluorescence throughout the cell, whereas the full-length NS2A-eGFP (construct 1–226) displayed fluorescence co-localized with calnexin (an ER membrane protein) in the perinuclear region (Figure 6B), indicating that ZIKV NS2A is an integral membrane protein. Expression of seven fragments spanning each pTMS, represented by constructs 1–25 (pTMS1), 30–58 (pTMS2), 74–97 (pTMS3), 101–123 (pTMS4), 125–145 (pTMS5), 150–185 (pTMS6), and 198–221 (pTMS7), exhibited fluorescence patterns that co-localized with calnexin in the perinuclear region (Figure 6B). The results indicate that individual of the seven pTMSes is associated with the ER membrane. In contrast, expression of fragments between different pTMS, represented by constructs 55–73, 186–197, and 221–226, showed fluorescence throughout the cells (Figure 6B), indicating that these fragments are not membrane-associated.

An *in vitro* deglycosylation analysis was performed to determine the orientation of each NS2A pTMS on the ER membrane. A panel of plasmids were prepared to express truncated NS2A proteins that were C-terminally fused with an eGFP followed by an N-glycosylation acceptor peptide (Glyc; Figure 7A). An SPG-$C_{16}$ peptide (Gaussia luciferase signal peptide SPG and the C-terminal 16 amino acids of NS1) was fused to the N terminus of each NS2A-eGFP-Glyc construct to ensure a proper translocation of the expressed protein on the ER membrane. Western blot showed that, upon transfection into BHK-21 cells, all constructs expressed fusion proteins with expected molecular masses (Figure 7B). Because N-glycosylation enzymes reside in the ER lumen, the glycosylation status could be used to indicate the location of the C-terminus of each NS2A-eGFP-Glyc protein (Figure 7C). As a negative control, treatment of EGFP-Glyc (without any viral sequence) with PNGase F (an enzyme that removes high mannose and complex carbohydrates) did not change the mobility of the protein (Figure 7D), indicating a cytosol localization of EGFP-Glyc. As a positive control, PNGaseF treatment increased the mobility of SPG-$C_{16}$-EGFP-Glyc (construct 0), confirming that the C-terminus of this protein resides in the ER lumen. Among the NS2A constructs, PNGase F treatment increased the mobility of fusion proteins 1–26 and 1–56, but not 1–103, 1–123, 1–147, 1–192, and 1–226 (Figure 7D). The results suggest that the N-terminal 56 amino acids of NS2A reside in the ER lumen, and the C-terminal region from residues 103–226 is located in the cytosol.

Next, we performed an *in vitro* proteinase K protection assay [46] to validate the above *in vitro* deglycosylation results. BHK-21 cells were transfected with the same constructs depicted in Figure 7A. At 24 h p.t., membrane fractions from the transfected cells were treated with proteinase K in the presence or absence of 0.5% Triton X-100 and analyzed by Western blot using an eGFP antibody. In the absence of Triton X-100, the eGFP residing in the cytosol would be susceptible to proteinase K digestion, whereas the eGFP residing in the ER lumen would be protected from proteinase K digestion (Figure 7E). As shown in Figure 7F, the eGFPs from constructs 0, 1–26, and 1–56 were protected from protease K digestion, again indicating that the first 56 residues are located in the ER lumen. In contrast, the eGFPs from constructs 1–103, 1–123, 1–147, 1–192, 1–226, and control eGFP-Glyc were completely degraded by the protease K treatment (Figure 7F), supporting that the C-terminal region from residues 103–226 is located in the cytosol. As expected, in the presence of Triton X-100, the eGFPs from all constructs were degraded by proteinase K (Figure 7F). The results of proteinase K assay are in full agreement with those of deglycosylation assay.

### *A topology model for ZIKV NS2A*

The above biochemical results support a topology model for ZIKV NS2A on the ER membrane (Figure 8A): (i) The N-terminal region (including pTMS1 and pTMS2) resides in the ER lumen; (ii) the region spanning residues 74–97 (pTMS3) traverses the ER membrane in a lumen-to-cytosol direction; and (iii) the C-terminal region from residues 103–226 (including pTMS4 to pTMS7) is located in the cytosol side. Since pTMS1, pTMS2, pTMS4, PTMS5, PTM6, and pTMS7 associate with ER membraned when expressed individually (Figure 6B), they may interact with the ER membrane in a peripheral manner. In support of this notion, informatics analyses using Jpred 4 and HeliQuest programmes suggest that amino acids 9–26 (pTMS1), 41–58 (pTMS2), 104–121 (pTMS4), 125–142 (pTMS5), 172–189 (pTMS6), and 203–220 (pTM7) may form amphipathic helices with hydrophobic and hydrophilic surfaces (Figure 8B), where the hydrophobic surface may interact with the ER membrane.

## Discussion

Flavivirus NS2A functions in viral RNA replication, virion assembly, evasion of innate immune response, and disease pathogenesis in a virus-specific manner [35]. This is evidenced by its high sequence diversity (Figure 1A), distinct membrane topology [35,46], and association with different disease pathways [41,42]. Thus, it is critical to perform a systematic genetic and biochemical characterization of ZIKV NS2A to delineate its structure and function. Our biochemical results showed that, unlike other flaviviruses, ZIKV NS2A has only a single transmembrane segment that traverses the

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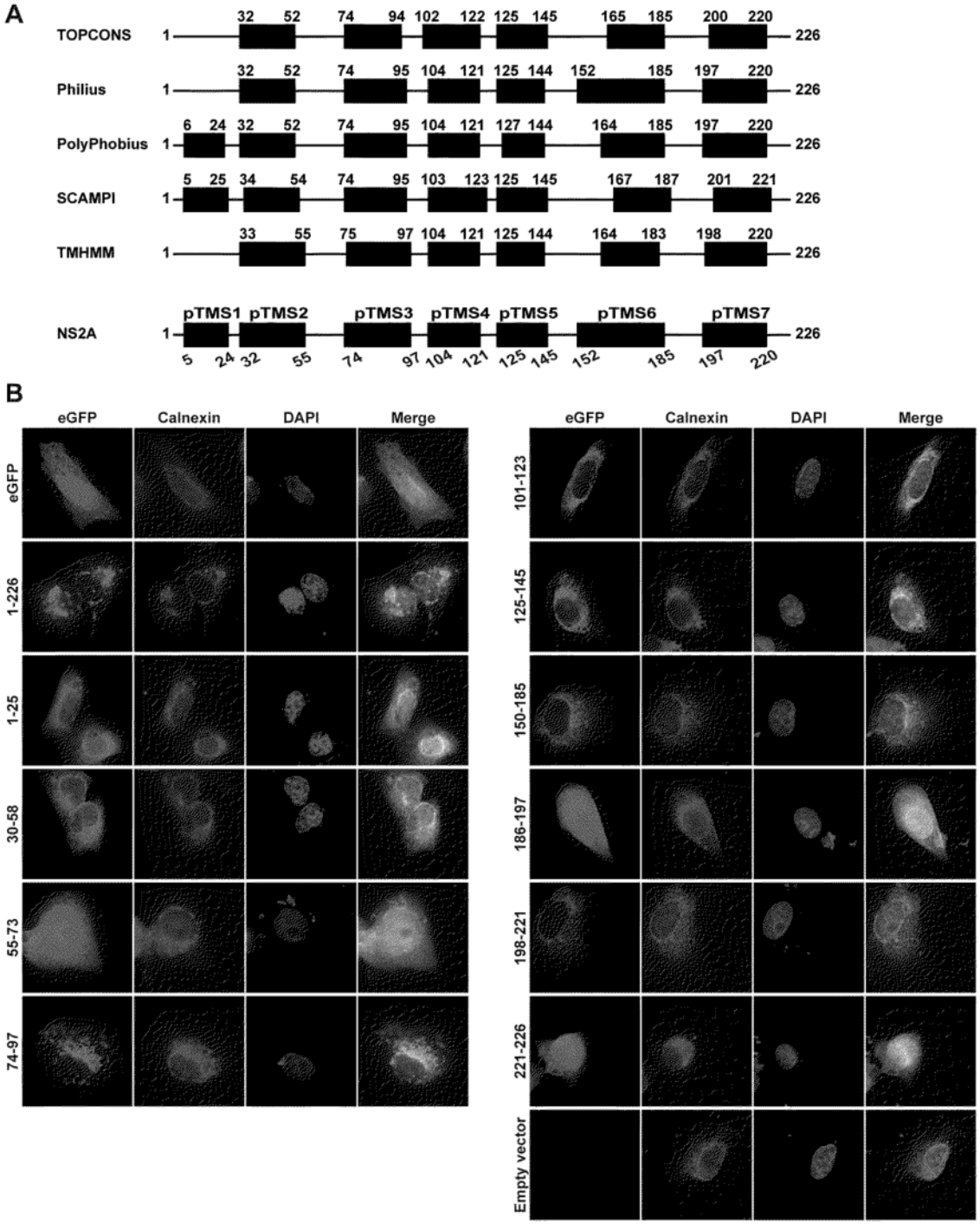



**Figure 6.** Membrane association of ZIKV NS2A transmembrane segments. (A) Bioinformatics analysis of ZIKV NS2A for transmembrane segments. ZIKV NS2A sequence was analyzed by bioinformatics programme TOPCONS, Philius, PolyPhobius, SCAMPI, and TMHMM. The compiled transmembrane segments are shown at the bottom. Seven predicted transmembrane segments (pTMS) are presented as thick bars. The amino acid positions of ZIKV NS2A are indicated. (B) Membrane association of individual pTMS. Expression plasmid pXJ-eGFP (without NS2A sequence) and pXJ-smNS2A-eGFP (smNS2A represents different NS2A segments) were transfected into Vero cell by using X-tremeGENE 9 DNA transfection reagent (Roche). For each plasmid construct, the NS2A segment is indicated by amino acid positions. At 24 h p.t., IFA was performed to monitor eGFP expression. ER and nuclei were stained with anti-Calnexin antibody and DAPI, respectively. The merged images are shown on the right panels.

ER membrane, the other six pTMSes are peripherally associated with the ER membrane (Figure 8A). Our functional analysis revealed five classes of mutants with distinct replication phenotypes, among which class III, IV, and V mutants could not produce infectious viruses through different mechanisms (Figure 1B). The critical mutations from class III to V are not clustered in specific regions of NS2A (Figure 8A),

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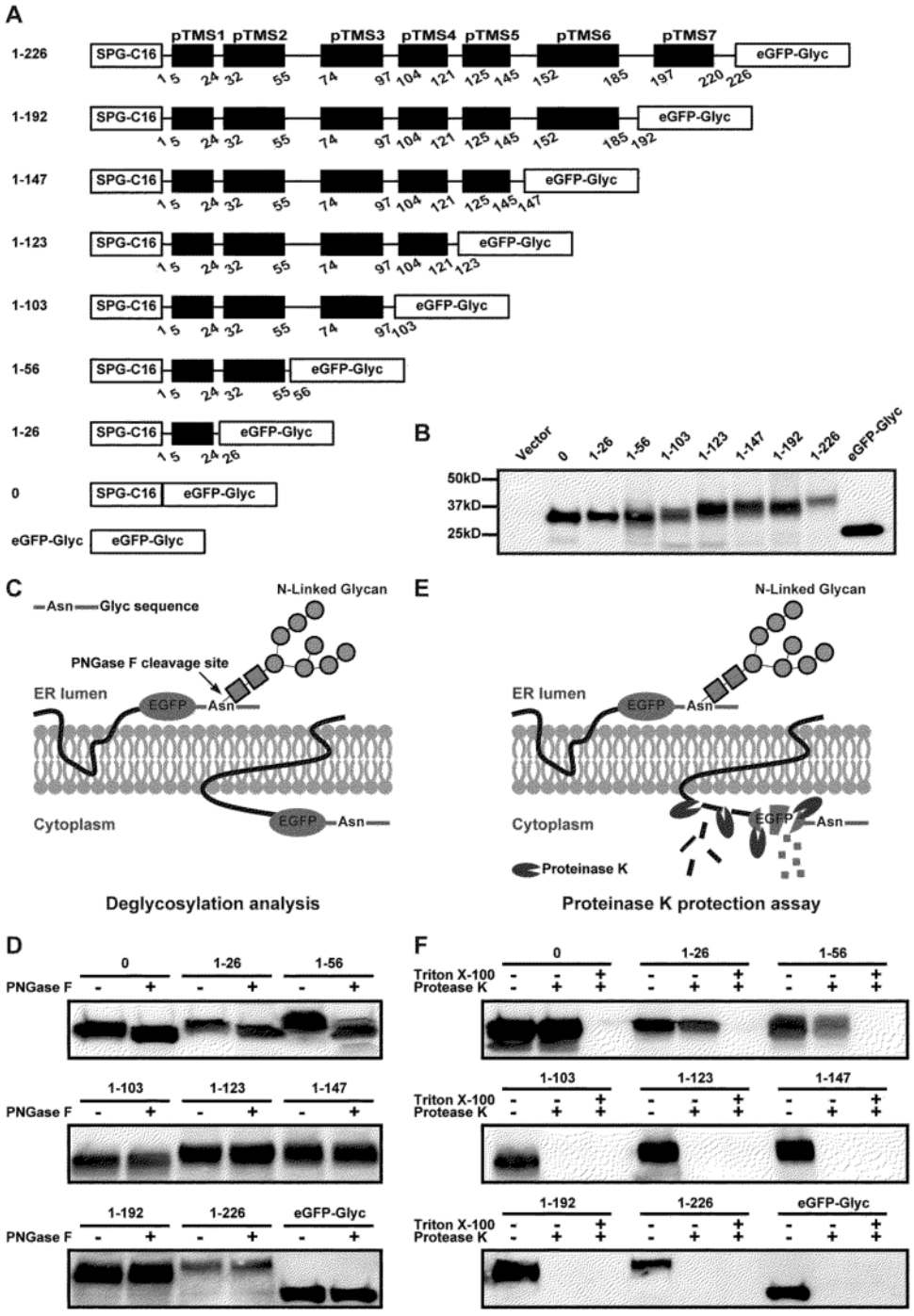



**Figure 7.** Probing the membrane topology of ZIKV NS2A by in vitro deglycosylation assay and in vitro proteinase K protection assay. (A) Schematic representation of C-terminally truncated ZIKV NS2A proteins. The amino acid positions of each truncated NS2A are indicated. A 'SPG-C16' sequence was fused to the N terminus of NS2A to ensure correct translocation of NS2A on the ER membrane. An 'eGFP-Glyc' sequence was fused to the C terminus of NS2A to probe its location at cytosol or ER lumen. See text for details. (B) BHK-21 cells were transfected with the expression plasmids described in (A). At 24 h p.t., the expression levels of various NS2A truncates were examined by Western blot using an eGFP antibody. The amino acid positions are indicated for each NS2A truncates. The molecular masses of marker proteins are shown to the left of Western blot image. (C) Diagram of glycosylation of eGFP-Glyc when located at ER lumen (*left*). No glycosylation occurs when eGFP-Glyc is located at cytosol (*right*). The glycosylation residue Asn is indicated. (D) *In vitro* deglycosylation assay. Plasmid-transfected cells were treated with PNGase F or PBS according to the protocol of *in vitro* deglycosylation assay (see Materials and Methods). The samples were examined for glycosylation status by Western blot using an eGFP antibody. (E) Diagram of *in vitro* proteinase K protection assay. When eGFP is located at ER lumen, it is protected from proteinase K cleavage (*left*); when located in the cytosol, eGFP is degraded by proteinase K (*right*). (F) *In vitro* proteinase K protection assay. Cell lysates from plasmid-transfected BHK-21 cells were treated with proteinase K or PBS according to the protocol of *in vitro* proteinase K protection assay (see Materials and Methods). The samples were detected for eGFP by Western blot using an eGFP antibody. Triton X-100 treatment is indicated.

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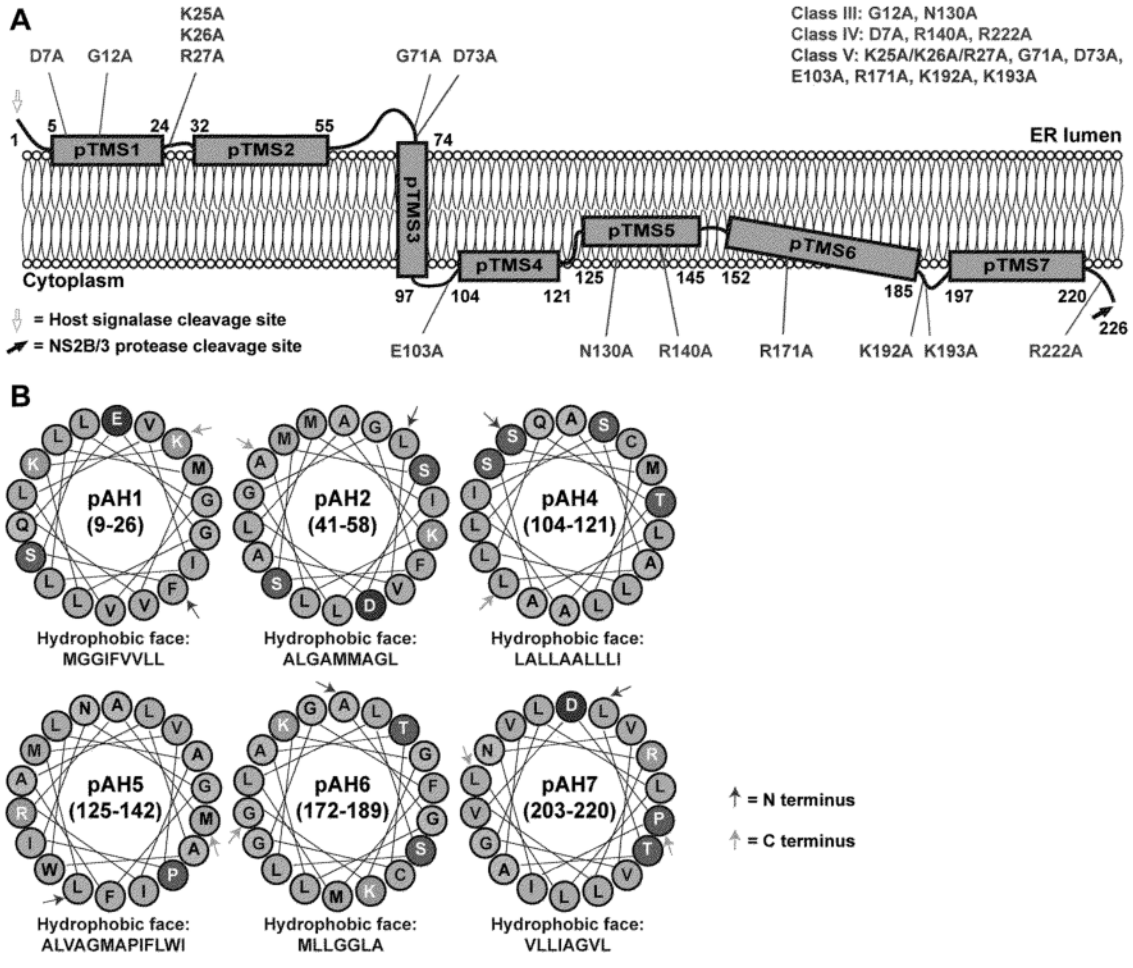



**Figure 8.** A topological model of ZIKV NS2A. (A) A model of ZIKV NS2A membrane topology. See text for details. Class III, IV, and V mutations are depicted on the topology model. (B) Helical wheel representations of NS2A fragment 9–26, 41–58, 104–121, 125–142, 172–189, and 203–220. The amphiphilic helix regions were analyzed using Heliquest server (heliquest.ipmc.cnrs.fr/). The best 18-residue windows are presented. The amino acids forming the hydrophobic surface of each helix wheel are depicted. Yellow, gray, blue, red, purple, pink, and green circles indicate nonpolar, glycine/alanine, positively charged, negatively charged, polar, amide, and proline amino acids, respectively. The first and last residues in each helical wheel are indicated by a red and blue arrow, respectively.

suggesting that multiple domains of NS2A are essential for viral replication.

The membrane topology of ZIKV NS2A is in stark contrast with the topologies of other flavivirus NS2A proteins: DENV-2 NS2A has five membrane-traversing segments [46] and YFV NS2A has three membrane-traversing segments [35]. Interestingly, these topologically diverse flavivirus NS2A proteins are able to converge their functions in viral RNA replication, virion assembly, and evasion of immune response. High resolution, three-dimensional structures of flavivirus NS2A are needed to address this question. It should be noted that the membrane topology of NS2A presented here is in the absence of other viral proteins. In the context of viral replication, the NS2A protein is associated with various viral and cellular proteins that would modulate the structure of NS2A and perform biological functions.

Our mutagenesis study has identified distinct NS2A mutations that selectively abolish ZIKV RNA synthesis (class III) or virion assembly (class V), among which only the virion assembly-defective mutants could be rescued through *trans* complementation (Figure 5). The results suggest that there are two species of NS2A molecules in ZIKV-infected cells. One NS2A species is located at the viral replication complex that could only be formed in *cis*; another NS2A species is located at virion assembly complex that could be formed in *trans* or *cis*. These results are in agreement with the previous studies from DENV and YFV NS2A [30,35,36]. Compared with the viral replication complex, the virion assembly complex is much less well defined for flaviviruses. Previously studies showed that second-site mutations in prM, E, NS2B, and NS3 could rescue the defect of virion assembly caused by DENV-2 and YFV NS2A mutations, suggesting that these viral proteins

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participate in virion assembly. Besides the genetic evidence, the role of NS3 in virion assembly is also supported by (i) mutations in the NS3 helicase domain alone affect YFV assembly [49], (ii) the helicase domain of DENV or YFV NS3 directly binds to NS2A [35,36], (iii) the cleavage between C and prM is tightly modulated by NS2B/NS3 protease and cellular signalase during YFV and Murray Valey encephalitis virus assembly [50–52]. These data, together with the result that recombinant NS2A of Kunjin binds to viral 3'UTR RNA [29], suggest a flavivirus assembly model. During flavivirus assembly, the NS2A protein recruits NS2B/NS3 through binding to the NS3 helicase domain. Once the NS2A-NS2B/NS3 complex reaches the virion assembly site, the NS3B/NS3 protease cleaves the C protein from the C/prM/E polyprotein. The C protein assembles onto genomic RNA (presented by NS2A) to form nucleocapsid, which is subsequently enveloped by prM/E complex to form virions. Since flavivirus assembly has to be coupled to viral RNA replication [53], the newly synthesized genomic RNA has to be transported from the replication site to the virion assembly site [54], possibly through the NS2A protein.

The *trans* complemented class V mutants could potentially be used as vaccine candidates, as recently reported for DENV-2 [36]. Since the *trans* complemented virions are able to infect cells for only a single cycle, such vaccine platform is safer than the multi-round infectious live-attenuated vaccines [55,56]. This is particularly important for ZIKV vaccine when immunizing immune-compromised individuals and pregnant women [57]. However, for development of class V mutants as a vaccine platform, the *trans* complementation efficiency needs to be improved to higher titers before they could be tested for *in vivo* efficacy.

In summary, we report the genetic and biochemical characterization of ZIKV NS2A protein. The results indicate that different flavivirus NS2A proteins have distinct membrane topologies, but converge to function in viral RNA synthesis and virion assembly. Together with previous results, we propose a model for flavivirus assembly in which NS2A plays a central role in mediating viral structural and nonstructural proteins as well as genomic RNA during virion formation.

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## Disclosure statement

No potential conflict of interest was reported by the authors.

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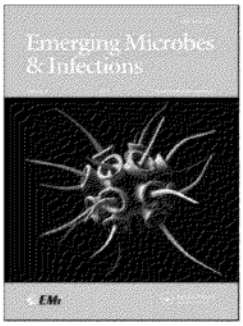
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# Genetic and biochemical characterizations of Zika virus NS2A protein

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**ABSTRACT**

Zika virus (ZIKV) can cause devastating congenital Zika syndromes in pregnant women and Guillain-Barre syndrome in adults. Understanding the molecular mechanism of ZIKV replication is essential for antiviral and vaccine development. Here we report the structural and functional characterization of ZIKV NS2A protein. Biochemical structural probing suggests that ZIKV NS2A has a single segment that traverses the ER membrane and six segments that peripherally associate with the ER membrane. Functional analysis has defined distinct NS2A residues essential for viral RNA synthesis or virion assembly. Only the virion assembly-defective mutants, but not the RNA synthesis-defective mutants, could be rescued through *trans* complementation with a wide-type NS2A protein. These results suggest that the NS2A molecules in virion assembly complex could be recruited in *trans*, whereas the NS2A molecules in viral replication complex must be recruited in *cis*. Together with previous results, we propose a flavivirus assembly model where NS2A plays a central role in modulating viral structural and nonstructural proteins as well as genomic RNA during virion assembly.

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**KEYWORDS** Zika virus; flavivirus replication; virion assembly; flavivirus NS2A; membrane topology

## Introduction

Zika virus (ZIKV) is a recently emerged mosquito-borne flavivirus that is primarily transmitted by *Aedes spp.* mosquitoes. It can also be transmitted through sexual, maternal-to-fetal, and blood transfusion routes [1–5]. ZIKV was first identified in 1947 from a sentinel rhesus monkey in Uganda [6]. Since 2007, it emerged in the Pacific region, South America, Central America, and the Caribbean, posing a global public health threat [7–11]. ZIKV infection causes mild illnesses, including fever, pruritis, arthritis, conjunctivitis, and asthenia [12]. Importantly, it can cause devastating congenital Zika syndromes (CZS; including microcephaly, congenital malformation, and fetal demise) in pregnant women and Guillain-Barre syndrome (GBS) in adults [13–16]. The explosive epidemics and its association with CZS had led the World Health Organization (WTO) to declare ZIKV as a Public Health Emergency of International Concern from February to November in 2016 [17]. Since then, a number of vaccine candidates have been developed, some of which have already advanced to clinical trials [18]. In addition, small molecule inhibitors and therapeutic antibodies have also been identified for antiviral development [19–22].

ZIKV belongs to the *Flavivirus* genus within the *Flaviviridae* family. Besides ZIKV, many other flaviviruses are significant human pathogens, including dengue (DENV), yellow fever (YFV), West Nile (WNV), Japanese encephalitis (JEV), Saint Louis encephalitis (SLEV), and tick-borne encephalitis virus (TBEV) [23]. Flavivirus genome is a positive-sense, single-stranded RNA of ~11,000 nucleotides. It contains a 5' untranslated region (UTR), a long open-reading-frame (ORF), and a 3' UTR. The single ORF encodes a polyprotein that is processed into three structure proteins (capsid [C], pre-membrane

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[prM] and envelope [E]) and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5). The structural proteins, together with genomic RNA, are components of virions. The nonstructural proteins participate in viral RNA replication, virion assembly, and evasion of host immune response [24–26].

Flavivirus NS2A is an ER-resident, transmembrane protein with a calculated molecular weight of ~22 kDa. The N-terminus and C-terminus of NS2A are formed by cleavages with an unknown host protease and viral protease NS3 (in complex with NS2B), respectively [27,28]. Flavivirus NS2A has multiple functions: (i) Viral replication. Kunjin virus (KUNV) NS2A co-localizes with viral double-stranded RNA and interacts with the 3'UTR of genomic RNA, NS3, and NS5 protein [29]. Mutagenesis studies confirmed the role of NS2A in viral RNA synthesis [30,31]. (ii) Virion assembly/secretion. Mutagenesis analyses of YFV, KUNV, and DENV have identified distinct NS2A amino acids that are essential for virion assembly/secretion [30–34]. The virion assembly defects can be restored by second site mutations in NS2B or NS3 in YFV and DENV [32,35,36]. (iii) Evasion of innate immunity. ZIKV NS2A inhibits type-I IFN induction at the step of TBK1 [26]. DENV NS2A antagonizes type-I IFN signalling [25]. JEV NS2A blocks dsRNA-activated protein kinase PKR [37]. In WNV and KUNV, the NS2A-mediated inhibition of type-I IFN induction can be abrogated by specific NS2A mutations [38,39]. (iv) Disease pathogenesis. WNV NS2A modulates virus-induced cytopathic effect (CPE) and pathogenesis in mice [40,41]. ZIKV NS2A impairs mammalian cortical neurogenesis through depleting adherens junction proteins [42].

The structure and function of ZIKV NS2A have not been characterized. Although NS2A proteins from different flaviviruses perform similar functions (described above), each flavivirus may exert its NS2A functions in a virus-specific manner [35]. The goals of this study are (i) to define the membrane topology of ZIKV NS2A and (ii) to characterize its roles in viral RNA synthesis and virion assembly.

## Materials and methods

### Cell lines

Vero cells, baby hamster kidney (BHK-21) cells and human embryo kidney 293T cells were cultured at 37°C with 5% $CO_2$ in high-glucose Dulbecco's modified Eagle medium (DMEM; Life Technologies) supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 g/ml streptomycin and 10% fetal bovine serum (FBS; HyClone Laboratories).

### Bioinformatics

NS2A protein sequences from ZIKV, DENV1, DENV2, DENV3, DENV4, WNV, JEV, YFV, SLEV were aligned using CLC Main Workbench software (CLC Bio). Prediction of ZIKV NS2A was carried out using various web servers, which including TOPCONS, Philius, Polyphobius, SCAMPI, and TMHMM2. The amphipathic helices of ZIKV NS2A were predicted by HeliQuest.

### Antibody

The following antibodies were used in this study: a mouse monoclonal antibody 4G2 cross-reactive with flavivirus E protein (American Type Culture Collection); Rabbit anti-HA monoclonal antibody (Cell Signaling Technology); Alexa Fluor®488 conjugated mouse anti-HA monoclonal antibody (Cell Signaling Technology); Rabbit anti-Calnexin polyclonal antibody(Abcam); Mouse anti-GFP monoclonal antibody (Sigma-Aldrich); Horseradish peroxidase(HRP) conjugated goat anti-mouse and anti-rabbit IgG polyclonal antibodies (Sigma-Aldrich); Alexa Fluor®488 conjugated goat anti-mouse IgG polyclonal antibody (Thermo Fisher Scientific); Alexa Fluor®568 conjugated goat anti-rabbit IgG polyclonal antibody (Thermo Fisher Scientific).

### Plasmid construction

The infectious cDNA clone (pFLZIKV) bearing the full-length genome of ZIKV (strain FSS13025) or Renilla luciferase (Rluc) reporter ZIKV replicon (pZIKV Rep WT) were used as the backbone for introducing NS2A mutations [43,44]. XhoI and KpnI restriction sites were initially generated into pFLZIKV and pZIKV Rep WT replicon at positions 3018 and 5010 in ZIKV genome without changing amino acid sequences. The NS2A alanine-scanning mutagenesis was performed by using a QuikChange II XL Site-Directed Mutagenesis kit (Agilent Technologies) as described previously [30].

A mammalian expression vector pXJ with a cytomegalovirus (CMV) promoter was used for constructing plasmids expressing NS2A and its derivatives. To construct the pXJ-$E_{24}$-NS1-NS2A-HA plasmid, a gene cassette encoding the last 24 amino acids of E protein ($E_{24}$), NS1 and NS2A with a C-terminal hemagglutinin (HA) tag was firstly amplified from the pFLZIKV and cloned into the pXJ vector. Next, NS2A mutations were individually introduced into the pXJ-$E_{24}$-NS1-NS2A-HA by overlap PCR, resulting in plasmids expressing NS2A mutants.

To construct the pXJ-SPG-$C_{16}$-NS2A-HA that was used for selecting cell line constitutively expressing NS2A protein, a DNA fragment containing the Gaussia luciferase signal peptide (SPG), the last 16 amino acids

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of NS1 protein ($C_{16}$) and NS2A tagged with a C-terminal HA tag sequence were constructed by overlap PCR and cloned into the pXJ vector via NotI/XhoI restriction sites.

Plasmid pXJ-eGFP was constructed by inserting the enhanced green fluorescence protein (eGFP) sequence into the pXJ vector via BamHI/XhoI restriction sites. DNA fragments that encode different NS2A segments were individually inserted into above pXJ-eGFP vector, resulting various pXJ-smNS2A-eGFP plasmids (smNS2A represents NS2A segment). A DNA fragment that express NS2A or truncated NS2A and an eGFP with a C-terminal N-glycosylation acceptor site (Glyc) were amplified and cloned into the pXJ-SPG-$C_{16}$-NS2A-HA vector, resulting in the plasmid pXJ-SPG-$C_{16}$-NS2A-eGFP-Glyc and various pXJ-SPG-$C_{16}$-ctNS2A-eGFP-Glyc plasmids (ctNS2A represents C-terminal truncated NS2A).

### *RNA in vitro transcription and electroporation*

All full-length cDNA clone plasmids and subgenomic replicons of ZIKV were linearized with restriction enzyme ClaI, followed by phenol-chloroform extraction and ethanol precipitation. RNA transcripts were prepared by using the T7 mMessage mMachine kit (Ambion). For RNA transfection, 10 μg *in vitro* transcribed RNA was electroporated into $8 \times 10^6$ cells (BHK-21 or Vero) by using Gene Pulser Xcell™ Electroporation Systems (Bio-rad) as described previously [30,43].

### *Immunofluorescence assay*

For indirect immunofluorescence assay (IFA), cells were seeded in an 8-well Nunc Lab-Tek II Chamber Slide (Thermo Fisher Scientific). At various time points, cells were washed three times with phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde in PBS at room temperature for 30 min and permeabilized with 0.1% Triton X-100 in PBS at room temperature for 10 min. The permeabilized cells were then washed three times with PBS and incubated in a blocking buffer containing 1% FBS in PBS at room temperature for 1 h. E protein was stained with a 1:1000 dilution of mouse monoclonal antibody 4G2 in blocking buffer. After incubation for 1 h, the cells were washed three times with a washing buffer (1% FBS and 0.5% Tween 20 in PBS). After 1 h of incubation with a secondary antibody (Alexa Fluor®488 conjugated goat anti-mouse IgG), the cells were washed three times with washing buffer and counterstained with DAPI (4',6-diamidino-2-phenylindole). Images were acquired using an Olympus fluorescence microscope. Images were processed in ImageJ software (National Institutes of Health, MD).

### *Plaque assay*

$2 \times 10^5$ Vero cells per well were grown in 24-well plates one day prior to viral infection. 10-fold serial dilutions of virus samples were prepared in DMEM containing 2% FBS and 1% penicillin/streptomycin and inoculated (100 μl) into each well of 24-well plates. After 1 h of incubation at 37°C with 5% $CO_2$, the inoculum was replaced with 500 μl overlay medium (DMEM medium containing 0.8% methyl-cellulose, 2% FBS and 1% penicillin/streptomycin). Plaques formed after incubation at 37°C with 5% $CO_2$ for 4–5 days. Cells were fixed with 4% paraformaldehyde for 30 min, followed by staining with 1% crystal violet. The numbers of plaque per millilitre (PFU/ml) of ZIKV were calculated.

### *Replicon transient-transfection assay*

Briefly, $8 \times 10^6$ Vero cells were electroporated with 10 μg wild type or mutant Rluc-ZIKV replicon RNAs. After electroporation, $3 \times 10^5$ cells per well were seeded in 12-well plates. At given time points, the cells were washed twice with PBS and lysed in 200 μl of luciferase lysis buffer (Promega). The luciferase activity assay was performed according to the manufacturer's protocol.

### *RNA extraction and cDNA sequencing*

The culture fluids from infected cells were centrifuged at 1000 g for 10 min and then filtered through a 0.22 μm pore size polyethersulfone membrane (Millipore) to remove cellular debris. Viral RNAs in the supernatants were extracted using a QIAamp viral-RNA Minikit (Qiagen) by following the manufacturer's protocol. Reverse transcription PCR (RT-PCR) was performed by using the SuperScript® III One-Step RT-PCR System (Thermo Fisher Scientific). The amplified cDNA fragments were purified and sent for Sanger sequencing at the GENEWIZ facilities.

### *Selection of NS2A-HA BHK-21 cell line*

$4 \times 10^5$ BHK-21 cells per well were seeded into a 6-well plate. The plasmid pXJ-SPG-$C_{16}$-NS2A-HA that contains a neomycin resistance gene was transfected into BHK-21 cells (4 μg per well) using X-tremeGENE 9 DNA transfection reagent (Roche). At 24 h post-transfection, geneticin (G418) was added to the culture medium with a concentration of 1 mg/ml. The medium was changed every three days. After two weeks, several neomycin-resistant individual colonies were selected for propagation. The expression of NS2A-HA protein in stable cell lines was analyzed by immunofluorescence assay and Western blot.

### *In vitro proteinase K protection assay*

Proteinase K protection assay was performed as previously described with minor modifications [45,46].

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BHK-21 cells were transfected in 6-well plates with various pXJ-SPG-$C_{16}$-ctNS2A-eGFP-Glyc plasmids. After incubated at 30°C for 24 h, cells were trypsinized, washed twice with PBS and centrifuged at 450 × g for 5 min. The cell pellets were resuspended in a hypotonic lysis buffer (10 mM Tris-HCl, pH 7.5, and 2 mM $MgCl_2$) and kept on ice for 10 min. Subsequently, the samples were homogenized with 40 strokes and centrifuged at 1000 × g for 5 min. The postnuclear supernatants (PNS) were divided into three aliquots and centrifuged at 15,000 × rpm for 40 min. The pellets from three aliquots were then resuspended in PBS, 60 μg/ml proteinase K in PBS, or 60 μg/ml proteinase K in PBS in the presence of 0.5% Triton X-100, respectively. The samples were incubated on ice for 90 min. 20 mM phenylmethylsulfonyl fluoride (PMSF) was finally added to terminate the reaction. After further incubation on ice for 15 min, the samples were mixed with 4× Laemmli sample buffer, heated at 95°C for 15 min and analyzed by Western blotting.

### *In vitro deglycosylation assay*

The samples were prepared by following a previously described protocol [46,47]. Briefly, the transfected BHK-21 cells in 6-well plates were incubated at 30°C for 24 h. Cells were trypsinized and suspended in DMEM medium containing 10%FBS. The cells were pelleted by centrifuging at 450 × g for 5 min. After two PBS washes, the cell pellets were resuspended in a solubilization buffer (20 mM Tris-HCl, pH 7.5, 50 mM NaCl, 10 mM $Mg(CH_3COO)_2$, 1% Triton X-100, and EDTA-free protease inhibitors [Roche]) and incubated at 4°C for 2 h with gentle agitation. After centrifugation at 15,000 rpm 4°C for 20 min to remove cell debris, the supernatants were split into two aliquots. 50,000 units/ml N-glycosidase F (PNGase F) or PBS (no-treatment control) were added to those two aliquots. After incubation at 37°C for 2 h, the samples were mixed with 4× Laemmli sample buffer, heated at 70°C for 15 min and analyzed by Western blot.

### *SDS-PAGE and Western blot*

Protein samples were separated by 12% SDS-polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride (PVDF) membrane using Trans-Blot Turbo transfer system (Bio-Rad), after which the membrane was blocked in TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 0.1% Tween 20) supplemented with 5% nonfat milk for 1 h at room temperature. The blots were probed with primary antibodies (1:1,000 dilution) overnight at 4°C, washed three times with TBST and incubated with horseradish peroxidase (HRP) conjugated goat anti-mouse and anti-rabbit IgG polyclonal antibodies (1:10,000 dilution) for 1 h. After three thorough TBST buffer washes, the blots were incubated with SuperSignal™ West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific). Chemiluminescence signals were detected in ChemiDoc System (Bio-Rad).

### *Quantification and statistical analysis*

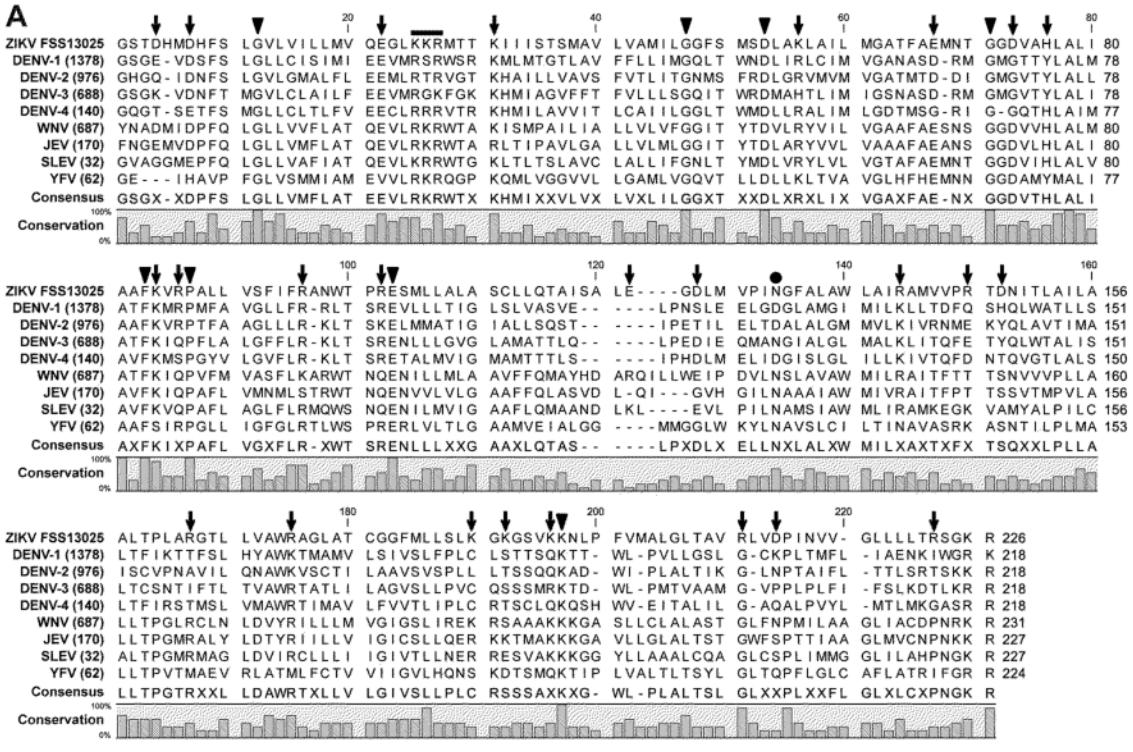
IFA images processing and cell counting were performed in software ImageJ (NIH). Densitometry analysis was performed using Image lab version 6.0 (Bio-Rad Laboratories, Hercules, CA). All numerical data are presented as the mean ± SEM (standard error of mean). The size of each study or number of replicates, along with the statistical tests performed can be found in corresponding Figure Legends.

## Results

### *The rationale for ZIKV NS2A mutagenesis*

To identify critical residues in ZIKV NS2A, we performed a systematic alanine-scanning mutagenesis using an infectious cDNA clone of ZIKV strain FSS13025 [43]. Sequence alignment of NS2A proteins from ZIKV, DENV, WNV, JEV, SLEV, and YFV revealed a high diversity: only 31 out of 218–227 amino acids with a consensus of >80% among different flaviviruses (Figure 1A). A total of 35 residues were selected for mutagenesis: (i) All 8 identical non-alanine residues, including G12, G47, D53, G71, F83, P87, E103, and K193. The amino acid numbering is based on the ZIKV sequence. (ii) All charged residues, including D4, D7, E22, K31, K56, E67, D73, H76, K84, R86, R96, R102, E122, D124, R140, R146, D148, R163, R171, K186, K188, K192, R207, D210, and R222. Charged residues were selected because they likely participate in inter- or intra-molecular interactions; alternatively, they may alter the locations where NS2A would traverse the ER membrane since charged residues are hydrophilic. In addition, NS2A D73H mutation was previously shown to attenuate WNV replication [48]. (iii) A K25/K26/R27 motif that was previously reported to be critical for YFV and DENV virion assembly [35,40]. (iv) Residue N130 that was previously shown to be required for NS1-NS2A cleavage and viral replication in DENV [27,30]. The selected residues were mutated to alanine in the context of ZIKV genomic RNA. Equal amounts of wild-type (WT) and mutant RNAs were electroporated into Vero cells. After electroporation, RNA replication and virus spread were monitored by immunofluorescence assay (IFA); the production of infectious viruses was quantified by plaque assay and RT-PCR. The 35 mutants exhibited distinct phenotypes that could be categorized into five groups (Figure 1B), as detailed in the following sections.

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**B**

| Class | NS2A Mutations | Phenotypes |
|---|---|---|
| I | D4A, E22A, K31A, G47A, D53A, E67A, H76A, E122A, R146A, D148A, R163A, K186A, K188A, D210A | Mutants do not or weakly affect viral production |
| II | K56A , F83A, K84A, R86A, P87A, R96A, R102A, D124A, R207A | Mutants dramatically reduce viral production |
| III | G12A, N130A | Mutants abolish viral RNA synthesis |
| IV | D7A, R140A, R222A | Mutants abolish virus production by affecting RNA synthesis and viron assembly/release |
| V | K25A/K26A/R27A, G71A, D73A, E103A, R171A, K192A, K193A | Mutants abolish virion assembly |

**Figure 1.** ZIKV NS2A mutagenesis. (A) Sequence alignment of NS2A proteins among ZIKV (strain FSS13025), DENV-1-4 serotypes, WNV, JEV, SLEV, and YFV using CLC Main Workbench software (CLC Bio). Thirty-five amino acids were selected for mutagenesis: (i) All 8 identical non-alanine residues (indicated by triangles), (ii) 25 charged residues (arrows), (iii) a K25/K26/R27 motif (line), (iv) residue N130 (circle). The consensus and conservation percentage are indicated below each amino acids. (B) A summary of ZIKV NS2A mutagenesis results. An infectious cDNA clone and a luciferase replicon of ZIKV strain FSS13025 were used to analyze the function of each amino acid. Based on the replication phenotypes, the 35 mutants could be categorized into five classes as indicated.

### *Class I mutations have no or weak effects on viral replication*

Fourteen of the 35 NS2A mutant RNAs, including D4A, E22A, K31A, G47A, D53A, E67A, H76A, E122A, R146A, D148A, R163A, K186A, K188A, and D210A, replicated at levels comparable to the WT RNA. These mutants were categorized as class I (Figure 1B). The IFA images and plaque morphologies of three representatives (D53A, D148A, and R163A), as well as the WT, are shown in Figure 2(A and B), respectively. Most class I mutants (e.g. D148A and R163A) yielded comparable E-expressing cells as the WT from 24 to 48 h post-transfection (p.t.). At 72 h p.t., severe CPE occurred among D148A, R163A, and WT RNA-transfected cells. Some class I mutants (e.g. D53A) produced fewer E-positive cells than the WT at 48 h p.t., and spread to infect more cells at 72 h p.t (Figure 2A). All class I mutants produced viruses with plaques similar to the WT virus (Figure 2B). At 72 h p.t., the class I mutants produced infectious titers <4-fold lower than the WT did (Figure 2C). Sequencing of the mutant viruses (collected at 72 h p.t.) confirmed the retaining of the engineered mutations without other changes (data not shown).

### *Class II mutations significantly attenuate viral replication*

Nine NS2A mutants, including K56A, F83A, K84A, R86A, P87A, R96A, R102A, D124A, and R207A,

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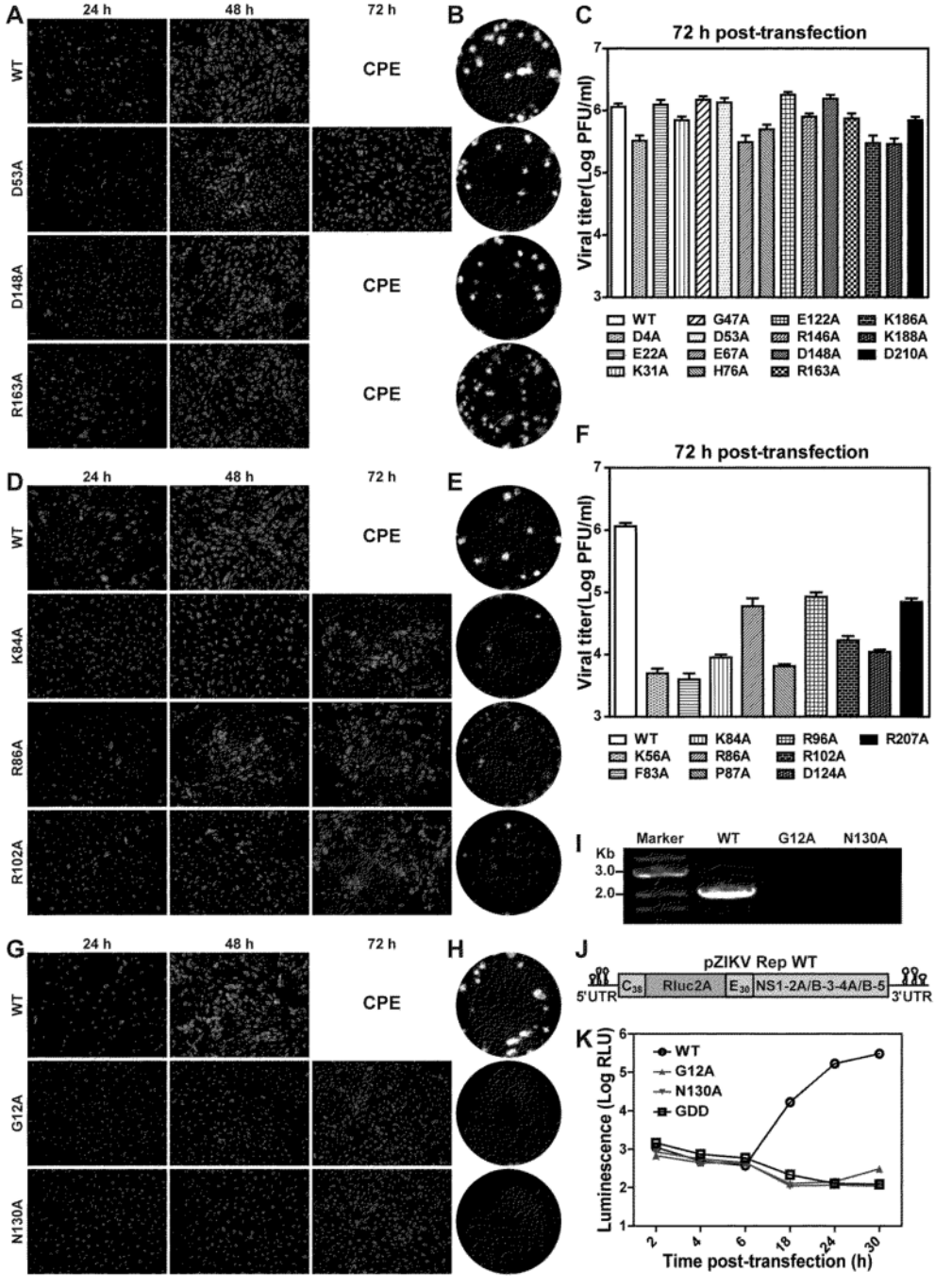



**Figure 2.** Characterization of class I, II, and III NS2A mutants. The results of representative mutants from class I (A–C), II (D–F), and III (G–K) are shown. (A) Vero cells were electroporated with equal amounts of ZIKV wild-type (WT) or class I mutant genomic RNAs. At indicated time points, the cells were fixed and subjected to IFA. Viral E protein and nuclei were stained with antibody 4G2 (green) and DAPI (blue), respectively. Cytopathic effect (CPE) is indicated at 72 h post-transfection. (B) Plaque morphologies. Recombinant viruses were subjected to plaque assay. Plaques were developed at day 4.5 post-infection. (C) Viral titers of class I mutants at 72 h post-transfection. The mean values from two independent experiments are shown. Error bars indicate the standard deviations. The limit of detection (LOD) of plaque assay is 10 PFU/ml. (D) IFA of class II mutant RNA-transfected Vero cells. (E) Plaque morphology of class II mutant viruses. (F) Viral titers of class II mutant viruses at 72 h post-transfection. (G) IFA of class III mutant RNA-transfected Vero cells. (H) Plaque morphologies of class III mutant viruses. Plaque assay was performed using culture supernatants collected at day 3 (for WT) and day 5 (for G12A and N130A) post-transfection. (I) RT-PCR analysis. At 5 day p.t., the mutant RNA-transfected cells were washed twice with PBS to remove residual input RNAs. At day 8 p.t., the supernatants of G12A and N130A RNA-transfected cells were subjected to RT-PCR. The WT virus (collected at 72 h p.t.) was used as a positive control. (J) A ZIKV luciferase replicon. $C_{38}$ and $E_{30}$ represent the first 38 amino acids of C protein and the last 30 amino acids of E protein, respectively. *Renilla* luciferase: Rluc. Foot-and-mouse-disease virus 2A sequence: 2A. (K) Transient replicon assay. WT, G12A, N130A, and NS5ΔGDD replicon RNAs were electroporated into Vero cells. Luciferase activities were measured at indicated time points. An average of 3 experiments are presented. Bars represent standard deviations.

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significantly attenuated viral replication. These mutants were categorized into class II (Figure 1B). Class II RNA-transfected cells produced fewer E-positive cells than the WT did (Figure 2D). At 72 h p.t., only 30–50% of E-positive cells were detected in the class II RNA-transfected cells, whereas the WT RNA-transfected cells exhibited CPE. Class II mutant viruses formed smaller, opaque plaques (Figure 2E), and the transfected cells produced >10-fold lower infectious titers than the WT RNA-transfected cells at 72 h p.t. (Figure 2F). Complete genome sequencing of the class II mutant viruses (collected at 96 h p.t.) did not reveal reversions or any other mutations (data not shown). Because of the weak phenotypes of class I and II mutants, we did not further characterize them.

### *Class III mutations are lethal for viral RNA synthesis*

Class III includes two mutants, G12A and N130A, which were completely defective in viral RNA synthesis (Figure 1B). The G12A and N130A genomic RNA-transfected cells did not produce any E-positive cells at 24 and 48 h p.t. (Figure 2G). On day 5–8 p.t., no infectious viruses were detected from culture medium by plaque assay (Figure 2H) and RT-PCR (Figure 2I). To confirm the above results, we introduced G12A or N130A mutation into a ZIKV luciferase replicon (Figure 2J). A replication-defective replicon, containing an NS5 polymerase active site ΔGDD mutation, was included as a negative control [44]. Vero cells were electroporated with equal amounts of WT, G12A, N130A, or NS5 ΔGDD replicon RNAs. At 2–6 h p.t., all replicons generated similar levels of luciferase signals, suggesting comparable levels of viral RNA translation and transfection efficiencies (Figure 2K). However, at 18–30 h p.t., G12A and N130A replicons generated low luciferase signals as the non-replicative NS5 ΔGDD replicon did, whereas the WT replicon produced increasing luciferase signals (Figure 2K). These results demonstrate that class III mutations completely abolish viral RNA synthesis.

### *Class IV mutations impair both viral RNA synthesis and virion production*

Class IV includes three mutants, including D7A, R140A, and R222A (Figure 1B). These mutant genomic RNAs produced fewer E-positive cells in the transfected cells than the WT RNA (Figure 3A). For D7A or R140A mutants, the RNA-transfected cells did not produce infectious viruses, as suggested by plaque assay (Figure 3B) and RT-PCR (Figure 3C). Incubation of naïve Vero cells with culture supernatants from the D7A or R140A transfected cells did not generate any viral E-positive cells (Figure 3D). Replicon analysis showed that D7A or R140A significantly compromised viral RNA synthesis (Figure 3E). Since neither mutants could produce extracellular viruses (Figure 3B and C), and could replicate at attenuated levels inside cells (Figure 3E), D7A and R140A mutations may impair both viral RNA synthesis and virion production.

For R222A, the number of E-positive cells slightly increased in the genomic RNA-transfected cells from 24 to 72 h p.t. (Figure 3A). A low level of infectious virus was detected from the culture medium by plaque assay (Figure 3B) and RT-PCR (Figure 3C). These data were supported by the positive-IFA results after incubating naive Vero cells with the supernatant from the R222A RNA-transfected cells (Figure 3D). Sequencing the virus (collected at 72 h p.t.) revealed that, besides the engineered R222A mutation, the virus had acquired a second site mutation of L220F in NS2A (Figure 3C). Replicon analysis showed that R222A significantly lowered viral RNA synthesis (Figure 3E); no secondary mutation (i.e. L220F) was detected in the replicon RNA at 30 h p.t. (data not shown). These results suggest that (i) R222A mutant is defective in viral RNA synthesis and/or virion assembly, and (ii) a second site mutation L220F in NS2A could rescue the defects.

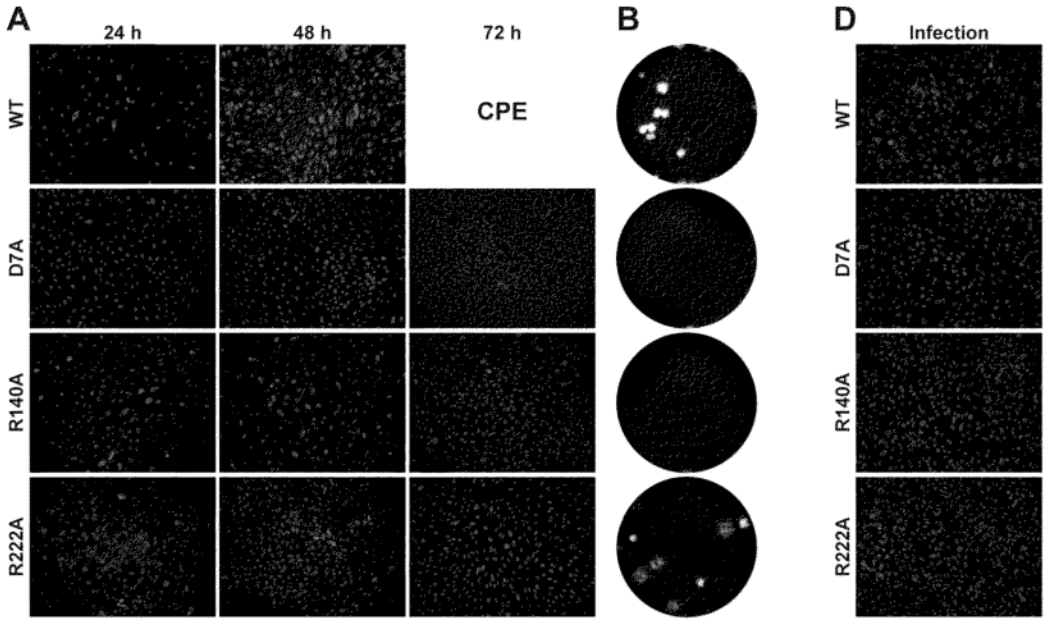
### *Class III and IV mutations do not affect NS2A maturation*

Since cleavage between NS1 and NS2A is essential for flavivirus RNA synthesis [30], we examined whether the replication defects of class III and IV mutants were caused by defective maturation of NS2A. To test this hypothesis, we introduced individual mutations into an expression plasmid pXJ-$E_{24}$-NS1-NS2A-HA that encodes a polyprotein containing the C-terminal 24 amino acids of the E protein (serving as a signal peptide to translocate NS1 to ER lumen), NS1, and NS2A with a C-terminal HA tag. After transfecting the plasmids into 293 T cells, the expression of un-cleaved NS1-NS2A-HA intermediate and cleaved NS2A-HA product were analyzed using Western blot (Figure 3F). None of the mutations significantly affected the cleavage efficiencies of NS1-NS2A-HA to NS2A-HA, indicating that the replication defects of class III and IV mutants are not caused by impaired NS2A cleavage.

### *Class V mutations block virion assembly*

Class V includes G71A, D73A, E103A, R171A, K192A, K193A, and a triple K25A/K26A/R27A. Cells transfected with class V mutant genomic RNAs yielded E-positive cells at 24 h p.t., but the number of E-positive cells did not increase afterward (Figure 4A), suggesting that the mutants are defective in virus spread. No infectious mutant viruses, except E103A, were detected in the supernatants of transfected cells by plaque assay (Figure 4B) or RT-PCR (Figure 4C). No IFA-positive

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C

| | Viral titer (IFU/ml) | | | Extracellular viral RNA RT-PCR | Second site mutation |
|---|---|---|---|---|---|
| | 24 h | 48 h | 72 h | | |
| WT | $(1.70\pm0.30)\times10^5$ | $(4.30\pm0.70)\times10^5$ | $(1.15\pm0.15)\times10^6$ | Positive | None |
| D7A | <10 | <10 | <10 | Negative | N.A. |
| R140A | <10 | <10 | <10 | Negative | N.A. |
| R222A | <10 | 35±15 | 400±100 | Positive | L220F(NS2A) |

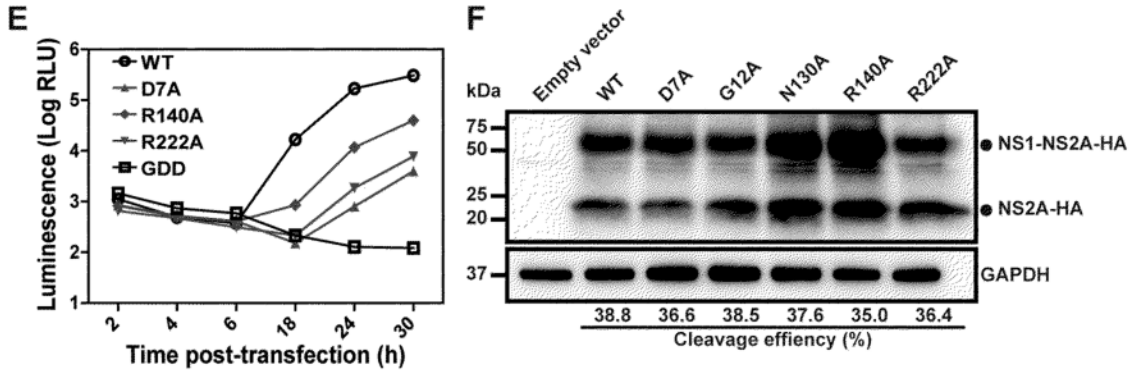
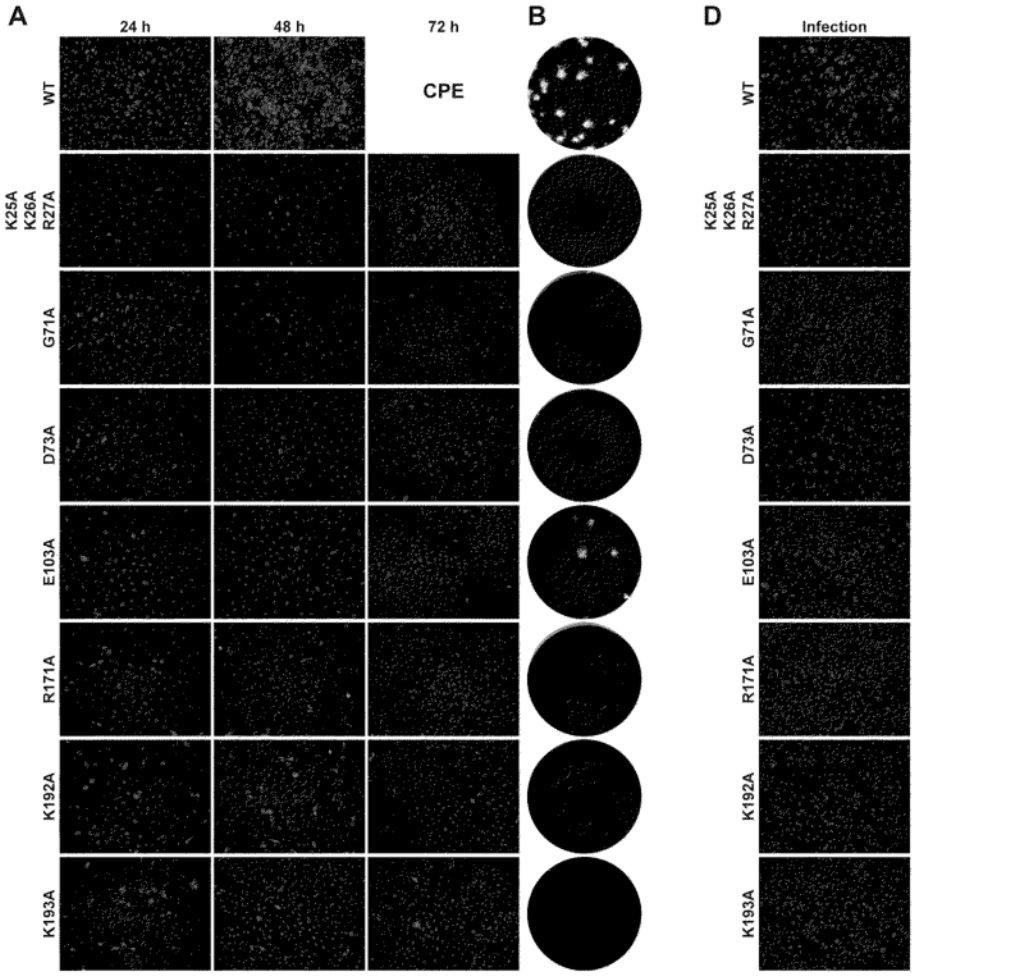



**Figure 3.** Characterization of class IV NS2A mutants. (A) IFA of transfected Vero cells. (B) Plaque morphologies of WT, D7A, R140A, and R222A viruses. (C) Extracellular viral titers were measured at 24, 48, and 72 h p.t. using plaque assay. At 72 h p.t., extracellular viral RNAs were subjected RT-PCR analysis. The RT-PCR products were sequenced to confirm the engineered mutations and to detect potential second site mutation(s) in the NS2A gene. N.A., not applicable. (D) The supernatants of transfected cells were collected on day 8 p.t. to infect naïve Vero cells. At 24 h p.t., the cells were analyzed for viral E expression by IFA. (E) Transient luciferase replicon analysis. Vero cells were electroporated with ZIKV replicon RNAs and measured for luciferase activities at indicated time points. An average of 3 experiments are presented. Bars represent standard deviations. (F) Analysis of NS2A proteolytic processes. NS2A D7A, G12A, N130A, R140A, and R222A mutations were individually engineered into expression plasmid pXJ-$E_{24}$-NS1-NS2A-HA. After transfecting the plasmids into 293 T cells, total cellular protein samples were harvested at 24 h p.t. and analyzed for NS2A process using Western blot. An HA-tag antibody was used to detect both unprocessed NS1-NS2A-HA and processed NS2A-HA proteins.

cells were detected after naïve cells were incubated with the supernatants from transfected cells (Figure 4D). Sequencing of the recovered E103A mutant virus revealed an A103D change in the NS2A gene (Figure 4C), suggesting that an E103A-to-A103D substitution is responsible for the rescue of viral replication.

To determine the stage of viral replication that was disrupted by the class V mutations, we analyzed the mutational effect on viral replication in ZIKV replicon (Figure 4E). Compared with the WT replicon, all class V mutants exhibited equivalent levels of viral translation and RNA synthesis (<4-fold difference),

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| | Viral titer (PFU/ml) | | | Extracellular viral RNA RT-PCR | Second site mutation |
|---|---|---|---|---|---|
| | 24h | 48h | 72h | | |
| Wild Type | $(1.7\pm0.3)\times10^5$ | $(4.3\pm0.7)\times10^5$ | $(1.15\pm0.15)\times10^6$ | Positive | None |
| K25AK26AR27A | <10 | <10 | <10 | Negative | N.A. |
| G71A | <10 | <10 | <10 | Negative | N.A. |
| D73A | <10 | <10 | <10 | Negative | N.A. |
| E103A | <10 | <10 | 175±25 | Positive | A103D(NS2A) |
| R171A | <10 | <10 | <10 | Negative | N.A. |
| K192A | <10 | <10 | <10 | Negative | N.A. |
| K193A | <10 | <10 | <10 | Negative | N.A. |

**Figure 4.** Characterization of class V NS2A mutants. (A) IFA of Vero cells that were transfected with WT or class V genomic RNAs. Viral E-positive cells are indicated in green. (B) Plaque morphologies of class V mutant viruses. Only the WT and E103A mutant generated plaques. (C) Supernatants from the RNA-transfected cells were quantified for viral titers by plaque assay. RT-PCR was also performed on the supernatants at day 3 p.t. for the WT virus and at day 8 p.t. for the mutants. Positive RT-PCR products were sequenced for potential second site mutation(s). N.A., not applicable. (D) Naïve Vero cells were infected with supernatants from the RNA-transfected cells and subjected to IFA for viral E protein expression. (E) Luciferase replicon analysis. Equal amounts of WT and mutant replicon RNAs were electroporated into Vero cells. Luciferase activities were measured at the indicated time points. (F) Analysis of intracellular infectious viruses. Intracellular viruses from ZIKV RNA-transfected cells were harvested at 24 h p.t. as previously described [46]. The harvested samples were used to infect naïve Vero cells. IFA was performed to examine viral E protein expression at 24 h post-infection.

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suggesting that the mutations only marginally affect viral RNA synthesis. Next, we tested if the class V genomic RNAs could generate intracellular infectious viruses. Incubating naïve Vero cells with WT RNA-transfected cell extracts yielded IFA-positive cells, whereas no IFA-positive cells were detected after incubating naive cells with the mutant RNA-transfected cell extracts (Figure 4F). Altogether, the results suggest that class V mutants are competent in viral RNA replication, but defective in assembly of infectious viruses.

### *WT NS2A rescues virion assembly for class V mutants*

To test *trans* complementation of NS2A mutants, we constructed a BHK-21 cell line that constitutively expressed WT ZIKV NS2A protein with a C-terminal HA-tagged (NS2A-HA). The cell line was constructed by transfecting plasmid pXJ-SPG-$C_{16}$-NS2A-HA encoding a Gaussia luciferase signal peptide (SPG), the C-terminal 16 amino acids of NS1 protein ($C_{16}$), and NS2A-HA, as well as a neomycin resistance gene (*Neo*). The SPG and $C_{16}$ sequences were engineered to enable the correct formation of NS2A membrane topology when expressed in cells. Individual BHK-NS2A-HA cell lines were generated through colony purification under G418 (1 mg/ml) selection. For each BHK-NS2A-HA cell line, all cells were IFA-positive for HA-NS2A expression (Figure 5A). Western blot revealed an expected band of ~22-kDa band (Figure 5B). The expression level of NS2A-HA did not decrease in the BHK-NS2A-HA cells after 20 passages in culture (4 days per passage; Figure 5B), indicating the stability of the cell line.

We tested *trans* complementation by transfecting class V genomic RNAs to BHK-NS2A-HA cells. The transfected cells developed an increasing number of E-positive cells from 48 to 96 h p.t. (Figure 5C). Culture supernatants were collected at 96 h p.t. to infect naïve Vero cells. The infected cells developed E-positive cells for all class V mutants (Figure 5D), demonstrating the rescue of infectious viruses through *trans* complementation. Quantification of the E-positive cells allowed us to determine the titers of rescued infectious viruses (Figure 5E). Among the class V mutants, E103A, K192A, and K193A exhibited the highest *trans* complementation efficiencies, producing $1.9 \times 10^5$–$3.04 \times 10^5$ IFU/ml viruses at 96 h p.i. (Figure 5E). Sequencing of the rescued virion genomes confirmed the retention of the engineered class V mutations without any other changes in NS2A (Figure 5E).

Next, supernatants from the first round of infection collected at 72 h p.i. (from Figure 5D) were used to infect naïve Vero cells for a second round. In contrast to the first round of infection (Figure 5D), no E-positive cells were detected in the second round of infection for any of the class V mutants (Figure 5F). As a positive control, WT RNA produced high numbers of E-positive cells in both the first and second rounds of infections (Figure 5D and F). Collectively, the results indicate that (i) the defect in virion assembly of class V mutants could be rescued through *trans* supply of WT NS2A protein and (ii) the rescued virions could only infect cells for a single round.

### *DENV-2 NS2A could not trans complement ZIKV class V mutants*

We tested whether DENV NS2A could rescue class V mutants of ZIKV. Each class V mutant of ZIKV was transfected into a previously established BHK-21 cell line that constitutively expresses WT DENV-2 NS2A protein [30]. For all class V mutants, no increase in E-positive cells or infections viruses was detected from the transfected cells from 24 to 96 h p.t. (data not shown). The results suggest that flavivirus NS2A modulates virion assembly in a virus-type specific manner.

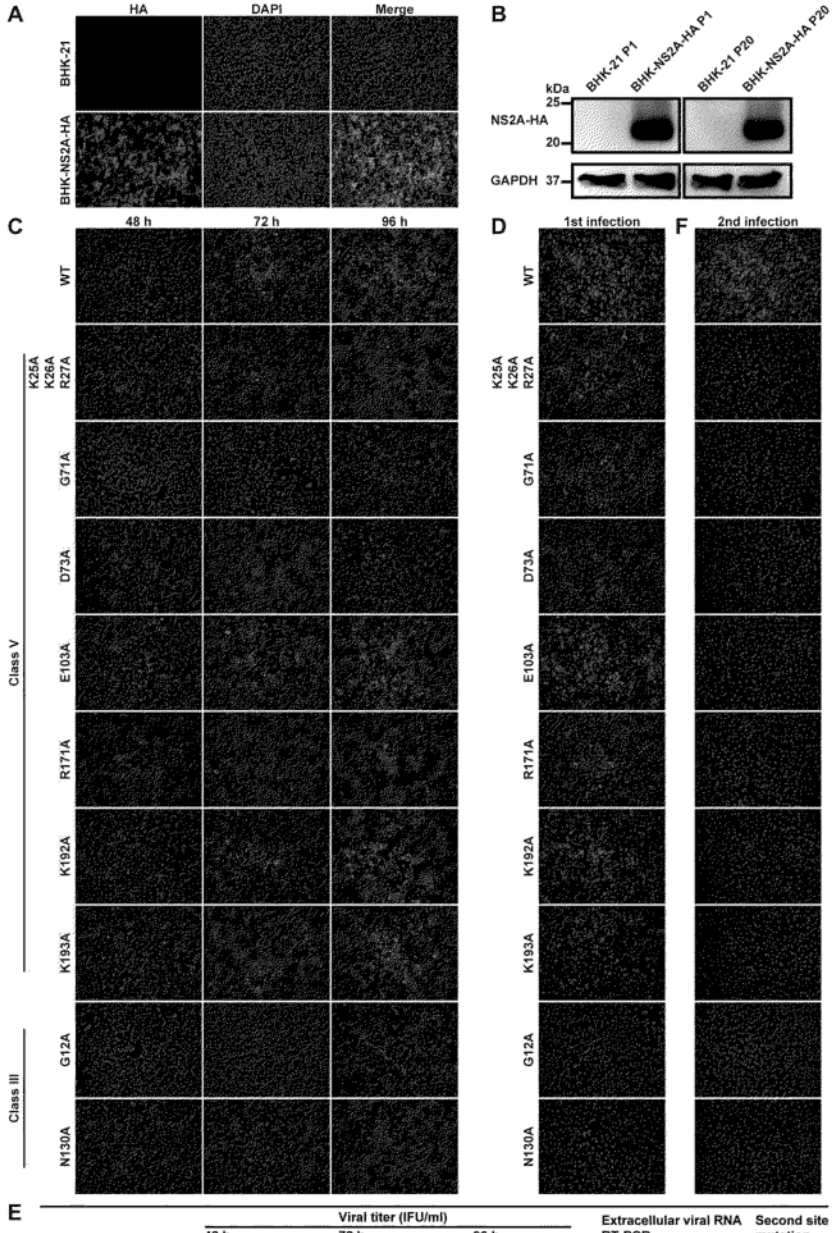
### *WT NS2A could not rescue viral RNA synthesis of class III mutants*

We tested whether the defect in viral RNA synthesis of class III mutants could be *trans* complemented. To test this possibility, we transfected class III genomic RNAs (G12A and N130A) into ZIKV BHK-NS2A-HA cells. Neither mutants generated any E-positive cells in the transfected BHK-NS2A-HA cells (Figure 5C). Infection of naïve Vero cells with supernatants of the transfected cells did not produce any IFA-positive cells (Figure 5D–F). The results suggest that the RNA synthesis defect of class III mutants could not be rescued through *trans* complementation. Due to the dual defects of class IV mutants in both viral RNA synthesis and virion assembly, we did not pursue *trans* complementation experiments for this class of mutants; such complementation experiments may not reveal clear mechanistic insights as compared with the class III and V mutants.

### *Probing the topology of ZIKV NS2A on the ER membrane*

The distinct functions of amino acids in viral RNA synthesis and virion assembly prompted us to probe the topology of ZIKV NS2A on ER membrane. Bioinformatics analysis suggests that ZIKV NS2A may form seven transmembrane segments (pTMS1–7; Figure 6A). To examine whether each pTMS associates with the ER membrane, we constructed a panel of plasmids encoding full-length or various NS2A fragments with a C-terminally fused eGFP reporter (Figure 6B). The length of each segment was slightly modified from the bioinformatic prediction to allow some

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E

| | | Viral titer (IFU/ml) 48 h | 72 h | 96 h | Extracellular viral RNA RT-PCR | Second site mutation |
|---|---|---|---|---|---|---|
| Class V | K25AK26AR27A | (7.16±1.01)×10$^3$ | (9.69±2.65)×10$^3$ | (5.97±1.18)×10$^4$ | Positive | None |
| | G71A | (1.48±0.78)×10$^3$ | (1.97±0.66)×10$^3$ | (5.42±0.66)×10$^3$ | Positive | None |
| | D73A | (2.10±0.57)×10$^2$ | (1.97±0.12)×10$^3$ | (8.60±1.10)×10$^3$ | Positive | None |
| | E103A | (1.13±0.14)×10$^4$ | (2.30±0.20)×10$^4$ | (3.04±0.25)×10$^5$ | Positive | None |
| | R171A | (6.41±1.58)×10$^3$ | (8.90±0.32)×10$^3$ | (6.06±1.06)×10$^4$ | Positive | None |
| | K192A | (7.85±2.04)×10$^3$ | (1.90±0.02)×10$^4$ | (2.17±0.17)×10$^5$ | Positive | None |
| | K193A | (2.69±0.32)×10$^3$ | (1.85±0.05)×10$^4$ | (1.90±0.08)×10$^5$ | Positive | None |
| Class III | G12A | <10 | <10 | <10 | Negative | N.A. |
| | N130A | <10 | <10 | <10 | Negative | N.A. |

**Figure 5.** Trans complementation of class III and V mutants. (A) IFA of BHK-NS2A-HA cells. The BHK-NS2A-HA cells constitutively express WT ZIKV NS2A fused with a C-terminal HA-tag. The cells were fixed with paraformaldehyde and stained with an HA-tag antibody. (B) Western blot of BHK-NS2A-HA cells. The expression of WT NS2A-HA from BHK-NS2A-HA cells was detected using an HA-tag antibody. Both passage one (P1) and 20 (P20) BHK-NS2A-HA cells were analyzed. Naïve BHK-21 cells were included as a negative control. (C) WT and class III and V genomic RNAs were electroporated into BHK-NS2A-HA cells. IFA was performed on the transfected cells to detect viral E protein expression. (D) Viruses rescued from the BHK-NS2A-HA cells (harvested at 96 h p.t.) were used to infect naïve Vero cells. The infected Vero cells were subjected to IFA for E protein expression. (E) Extracellular viruses were harvested from the supernatants of RNA-transfected BHK-NS2A-HA cells. The harvested viruses were serially diluted to infect naïve Vero cells in 96-well plates. At 24 h p.i., IFA was performed to quantify the viral titers by counting the number of E-positive cells. The results show averages and standard deviations from triplicate samples. Viral RNAs in the supernatants of transfected BHK-NS2A-HA cells were isolated at 96 h p.t. and subjected to RT-PCR. Positive RT-PCR products were sequenced to confirm the engineered mutations and to identify potential second site change(s). (F) The supernatants of infected Vero cells from (D) were collected at 72 h p.i. to infect naïve Vero cells for a second round. IFA was performed on these cells for viral E-positive cells.

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flexibility between pTMS and eGFP. Upon transfection into Vero cells, eGFP alone (without any NS2A fragment) exhibited an even distribution of fluorescence throughout the cell, whereas the full-length NS2A-eGFP (construct 1–226) displayed fluorescence co-localized with calnexin (an ER membrane protein) in the perinuclear region (Figure 6B), indicating that ZIKV NS2A is an integral membrane protein. Expression of seven fragments spanning each pTMS, represented by constructs 1–25 (pTMS1), 30–58 (pTMS2), 74–97 (pTMS3), 101–123 (pTMS4), 125–145 (pTMS5), 150–185 (pTMS6), and 198–221 (pTMS7), exhibited fluorescence patterns that co-localized with calnexin in the perinuclear region (Figure 6B). The results indicate that individual of the seven pTMSes is associated with the ER membrane. In contrast, expression of fragments between different pTMS, represented by constructs 55–73, 186–197, and 221–226, showed fluorescence throughout the cells (Figure 6B), indicating that these fragments are not membrane-associated.

An *in vitro* deglycosylation analysis was performed to determine the orientation of each NS2A pTMS on the ER membrane. A panel of plasmids were prepared to express truncated NS2A proteins that were C-terminally fused with an eGFP followed by an N-glycosylation acceptor peptide (Glyc; Figure 7A). An SPG-$C_{16}$ peptide (Gaussia luciferase signal peptide SPG and the C-terminal 16 amino acids of NS1) was fused to the N terminus of each NS2A-eGFP-Glyc construct to ensure a proper translocation of the expressed protein on the ER membrane. Western blot showed that, upon transfection into BHK-21 cells, all constructs expressed fusion proteins with expected molecular masses (Figure 7B). Because N-glycosylation enzymes reside in the ER lumen, the glycosylation status could be used to indicate the location of the C-terminus of each NS2A-eGFP-Glyc protein (Figure 7C). As a negative control, treatment of EGFP-Glyc (without any viral sequence) with PNGase F (an enzyme that removes high mannose and complex carbohydrates) did not change the mobility of the protein (Figure 7D), indicating a cytosol localization of EGFP-Glyc. As a positive control, PNGaseF treatment increased the mobility of SPG-$C_{16}$-EGFP-Glyc (construct 0), confirming that the C-terminus of this protein resides in the ER lumen. Among the NS2A constructs, PNGase F treatment increased the mobility of fusion proteins 1–26 and 1–56, but not 1–103, 1–123, 1–147, 1–192, and 1–226 (Figure 7D). The results suggest that the N-terminal 56 amino acids of NS2A reside in the ER lumen, and the C-terminal region from residues 103–226 is located in the cytosol.

Next, we performed an *in vitro* proteinase K protection assay [46] to validate the above *in vitro* deglycosylation results. BHK-21 cells were transfected with the same constructs depicted in Figure 7A. At 24 h p.t., membrane fractions from the transfected cells were treated with proteinase K in the presence or absence of 0.5% Triton X-100 and analyzed by Western blot using an eGFP antibody. In the absence of Triton X-100, the eGFP residing in the cytosol would be susceptible to proteinase K digestion, whereas the eGFP residing in the ER lumen would be protected from proteinase K digestion (Figure 7E). As shown in Figure 7F, the eGFPs from constructs 0, 1–26, and 1–56 were protected from protease K digestion, again indicating that the first 56 residues are located in the ER lumen. In contrast, the eGFPs from constructs 1–103, 1–123, 1–147, 1–192, 1–226, and control eGFP-Glyc were completely degraded by the protease K treatment (Figure 7F), supporting that the C-terminal region from residues 103–226 is located in the cytosol. As expected, in the presence of Triton X-100, the eGFPs from all constructs were degraded by proteinase K (Figure 7F). The results of proteinase K assay are in full agreement with those of deglycosylation assay.

### *A topology model for ZIKV NS2A*

The above biochemical results support a topology model for ZIKV NS2A on the ER membrane (Figure 8A): (i) The N-terminal region (including pTMS1 and pTMS2) resides in the ER lumen; (ii) the region spanning residues 74–97 (pTMS3) traverses the ER membrane in a lumen-to-cytosol direction; and (iii) the C-terminal region from residues 103–226 (including pTMS4 to pTMS7) is located in the cytosol side. Since pTMS1, pTMS2, pTMS4, PTMS5, PTM6, and pTMS7 associate with ER membraned when expressed individually (Figure 6B), they may interact with the ER membrane in a peripheral manner. In support of this notion, informatics analyses using Jpred 4 and HeliQuest programmes suggest that amino acids 9–26 (pTMS1), 41–58 (pTMS2), 104–121 (pTMS4), 125–142 (pTMS5), 172–189 (pTMS6), and 203–220 (pTM7) may form amphipathic helices with hydrophobic and hydrophilic surfaces (Figure 8B), where the hydrophobic surface may interact with the ER membrane.

## Discussion

Flavivirus NS2A functions in viral RNA replication, virion assembly, evasion of innate immune response, and disease pathogenesis in a virus-specific manner [35]. This is evidenced by its high sequence diversity (Figure 1A), distinct membrane topology [35,46], and association with different disease pathways [41,42]. Thus, it is critical to perform a systematic genetic and biochemical characterization of ZIKV NS2A to delineate its structure and function. Our biochemical results showed that, unlike other flaviviruses, ZIKV NS2A has only a single transmembrane segment that traverses the

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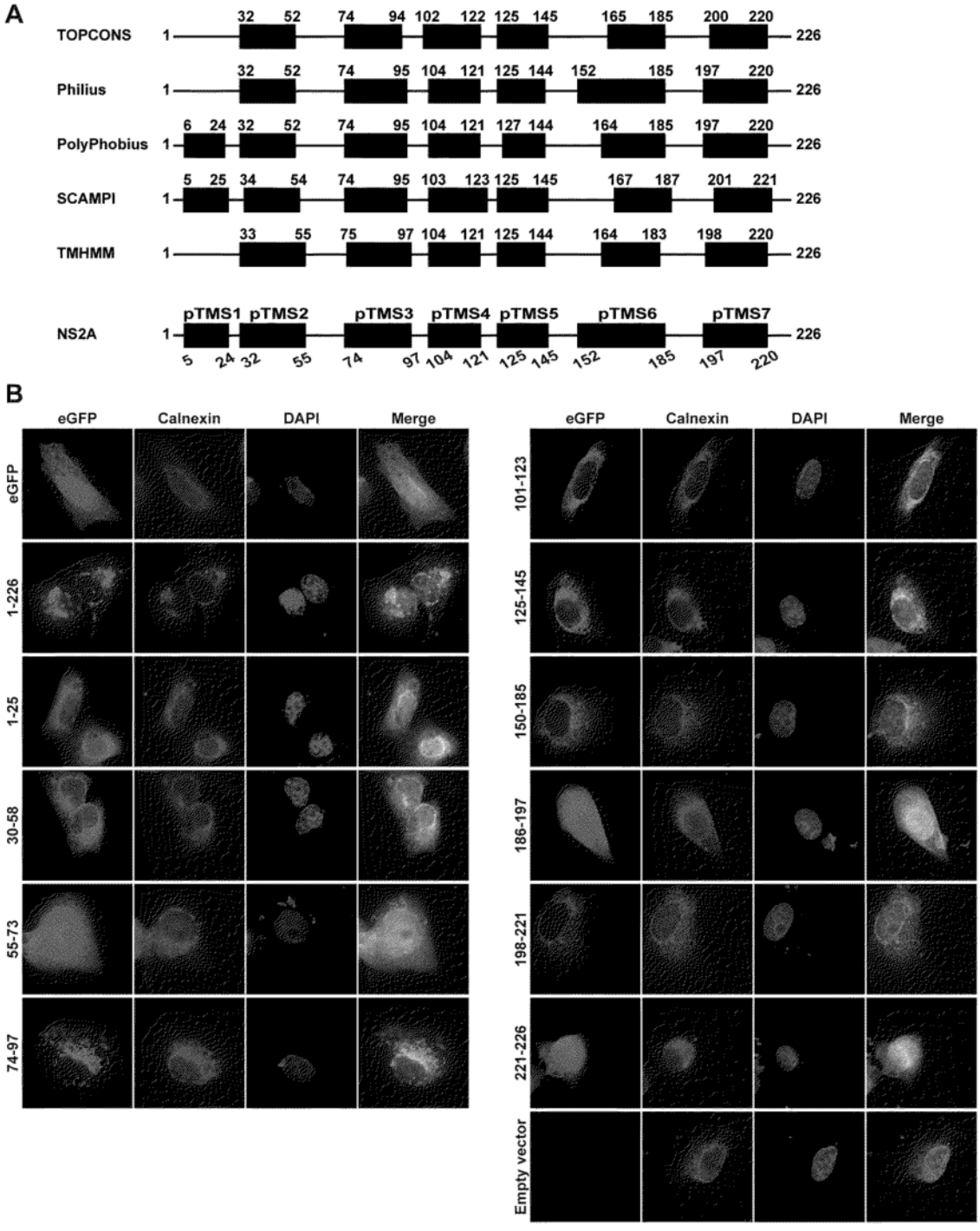



**Figure 6.** Membrane association of ZIKV NS2A transmembrane segments. (A) Bioinformatics analysis of ZIKV NS2A for transmembrane segments. ZIKV NS2A sequence was analyzed by bioinformatics programme TOPCONS, Philius, PolyPhobius, SCAMPI, and TMHMM. The compiled transmembrane segments are shown at the bottom. Seven predicted transmembrane segments (pTMS) are presented as thick bars. The amino acid positions of ZIKV NS2A are indicated. (B) Membrane association of individual pTMS. Expression plasmid pXJ-eGFP (without NS2A sequence) and pXJ-smNS2A-eGFP (smNS2A represents different NS2A segments) were transfected into Vero cell by using X-tremeGENE 9 DNA transfection reagent (Roche). For each plasmid construct, the NS2A segment is indicated by amino acid positions. At 24 h p.t., IFA was performed to monitor eGFP expression. ER and nuclei were stained with anti-Calnexin antibody and DAPI, respectively. The merged images are shown on the right panels.

ER membrane, the other six pTMSes are peripherally associated with the ER membrane (Figure 8A). Our functional analysis revealed five classes of mutants with distinct replication phenotypes, among which class III, IV, and V mutants could not produce infectious viruses through different mechanisms (Figure 1B). The critical mutations from class III to V are not clustered in specific regions of NS2A (Figure 8A),

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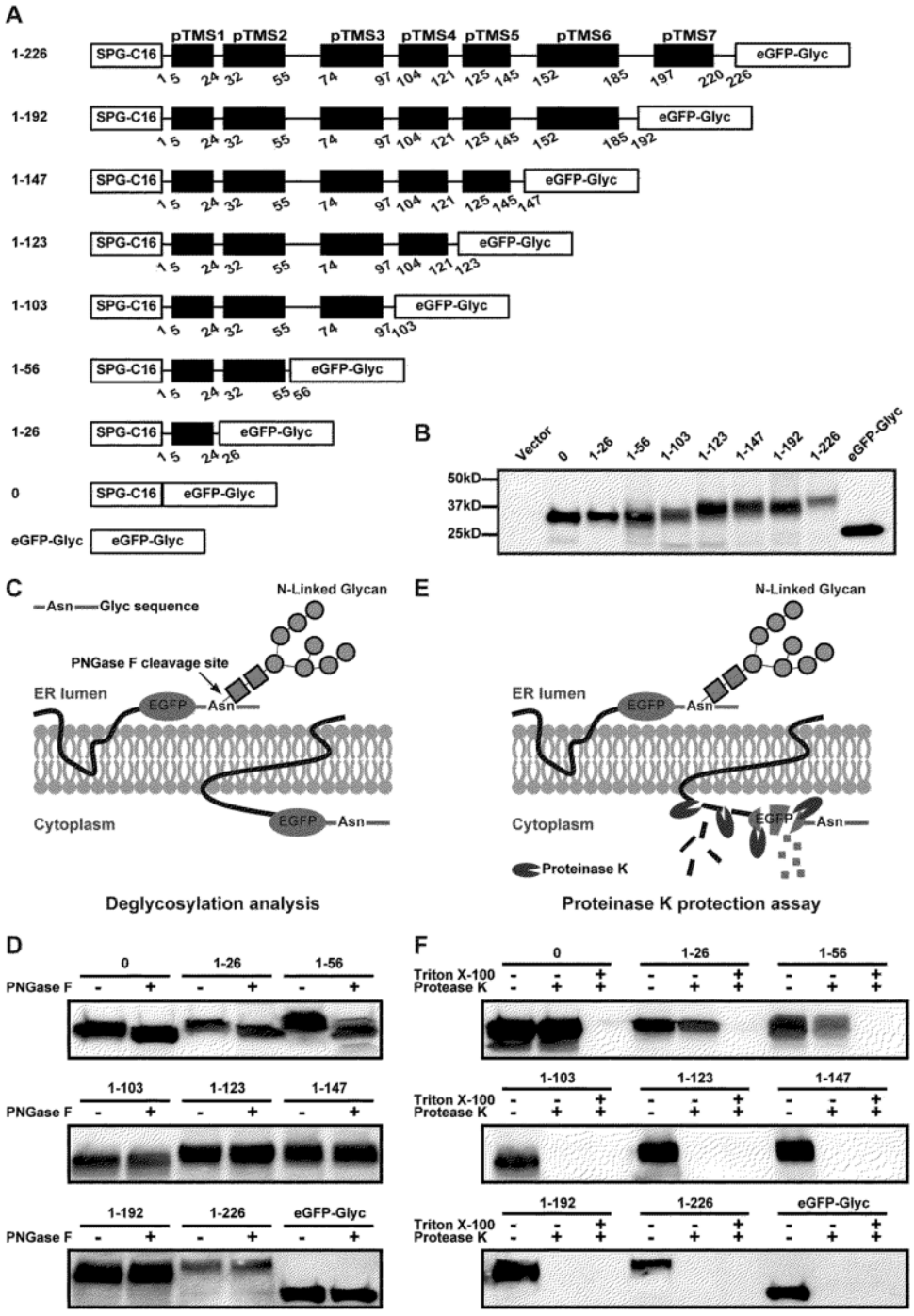



**Figure 7.** Probing the membrane topology of ZIKV NS2A by in vitro deglycosylation assay and in vitro proteinase K protection assay. (A) Schematic representation of C-terminally truncated ZIKV NS2A proteins. The amino acid positions of each truncated NS2A are indicated. A 'SPG-C16' sequence was fused to the N terminus of NS2A to ensure correct translocation of NS2A on the ER membrane. An 'eGFP-Glyc' sequence was fused to the C terminus of NS2A to probe its location at cytosol or ER lumen. See text for details. (B) BHK-21 cells were transfected with the expression plasmids described in (A). At 24 h p.t., the expression levels of various NS2A truncates were examined by Western blot using an eGFP antibody. The amino acid positions are indicated for each NS2A truncates. The molecular masses of marker proteins are shown to the left of Western blot image. (C) Diagram of glycosylation of eGFP-Glyc when located at ER lumen (*left*). No glycosylation occurs when eGFP-Glyc is located at cytosol (*right*). The glycosylation residue Asn is indicated. (D) *In vitro* deglycosylation assay. Plasmid-transfected cells were treated with PNGase F or PBS according to the protocol of *in vitro* deglycosylation assay (see Materials and Methods). The samples were examined for glycosylation status by Western blot using an eGFP antibody. (E) Diagram of *in vitro* proteinase K protection assay. When eGFP is located at ER lumen, it is protected from proteinase K cleavage (*left*); when located in the cytosol, eGFP is degraded by proteinase K (*right*). (F) *In vitro* proteinase K protection assay. Cell lysates from plasmid-transfected BHK-21 cells were treated with proteinase K or PBS according to the protocol of *in vitro* proteinase K protection assay (see Materials and Methods). The samples were detected for eGFP by Western blot using an eGFP antibody. Triton X-100 treatment is indicated.

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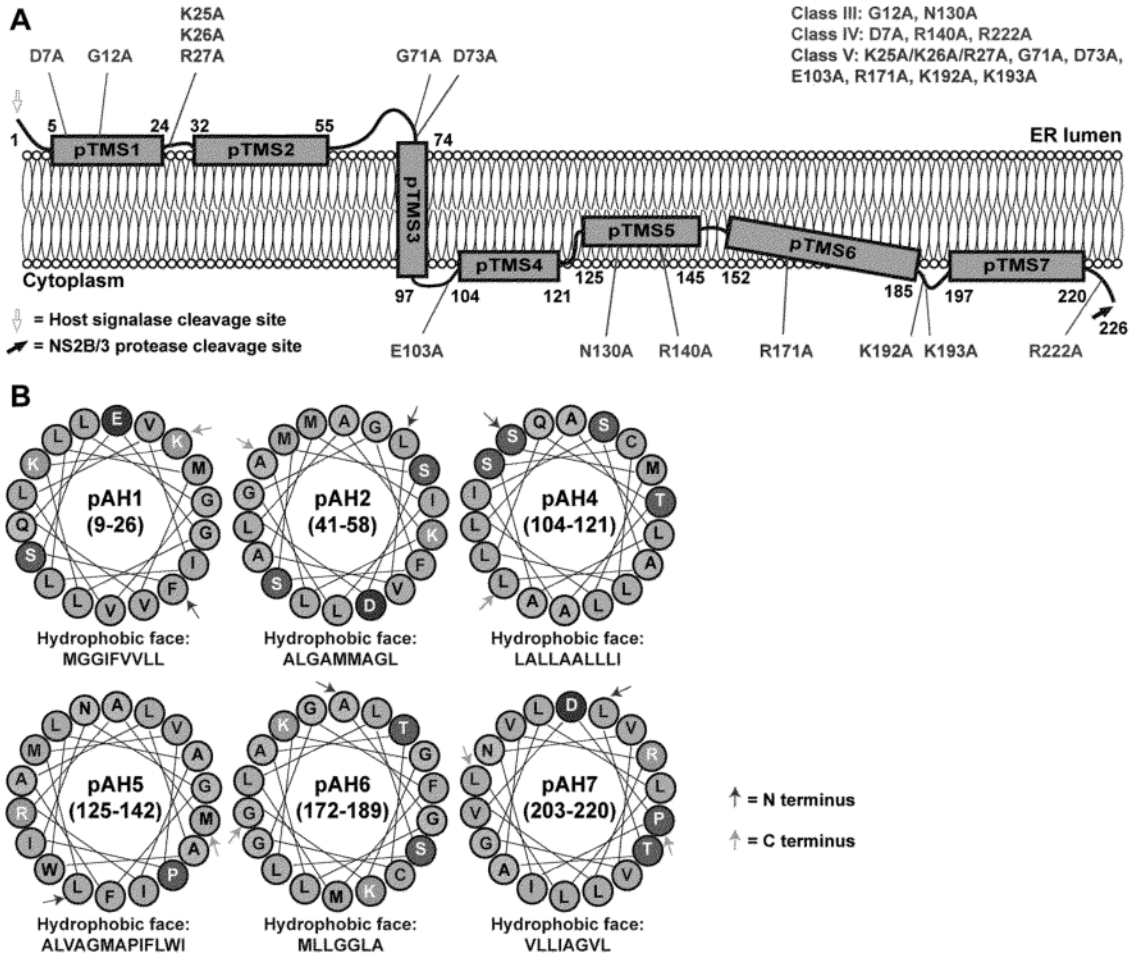



**Figure 8.** A topological model of ZIKV NS2A. (A) A model of ZIKV NS2A membrane topology. See text for details. Class III, IV, and V mutations are depicted on the topology model. (B) Helical wheel representations of NS2A fragment 9–26, 41–58, 104–121, 125–142, 172–189, and 203–220. The amphiphilic helix regions were analyzed using Heliquest server (heliquest.ipmc.cnrs.fr/). The best 18-residue windows are presented. The amino acids forming the hydrophobic surface of each helix wheel are depicted. Yellow, gray, blue, red, purple, pink, and green circles indicate nonpolar, glycine/alanine, positively charged, negatively charged, polar, amide, and proline amino acids, respectively. The first and last residues in each helical wheel are indicated by a red and blue arrow, respectively.

suggesting that multiple domains of NS2A are essential for viral replication.

The membrane topology of ZIKV NS2A is in stark contrast with the topologies of other flavivirus NS2A proteins: DENV-2 NS2A has five membrane-traversing segments [46] and YFV NS2A has three membrane-traversing segments [35]. Interestingly, these topologically diverse flavivirus NS2A proteins are able to converge their functions in viral RNA replication, virion assembly, and evasion of immune response. High resolution, three-dimensional structures of flavivirus NS2A are needed to address this question. It should be noted that the membrane topology of NS2A presented here is in the absence of other viral proteins. In the context of viral replication, the NS2A protein is associated with various viral and cellular proteins that would modulate the structure of NS2A and perform biological functions.

Our mutagenesis study has identified distinct NS2A mutations that selectively abolish ZIKV RNA synthesis (class III) or virion assembly (class V), among which only the virion assembly-defective mutants could be rescued through *trans* complementation (Figure 5). The results suggest that there are two species of NS2A molecules in ZIKV-infected cells. One NS2A species is located at the viral replication complex that could only be formed in *cis*; another NS2A species is located at virion assembly complex that could be formed in *trans* or *cis*. These results are in agreement with the previous studies from DENV and YFV NS2A [30,35,36]. Compared with the viral replication complex, the virion assembly complex is much less well defined for flaviviruses. Previously studies showed that second-site mutations in prM, E, NS2B, and NS3 could rescue the defect of virion assembly caused by DENV-2 and YFV NS2A mutations, suggesting that these viral proteins

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participate in virion assembly. Besides the genetic evidence, the role of NS3 in virion assembly is also supported by (i) mutations in the NS3 helicase domain alone affect YFV assembly [49], (ii) the helicase domain of DENV or YFV NS3 directly binds to NS2A [35,36], (iii) the cleavage between C and prM is tightly modulated by NS2B/NS3 protease and cellular signalase during YFV and Murray Valey encephalitis virus assembly [50–52]. These data, together with the result that recombinant NS2A of Kunjin binds to viral 3'UTR RNA [29], suggest a flavivirus assembly model. During flavivirus assembly, the NS2A protein recruits NS2B/NS3 through binding to the NS3 helicase domain. Once the NS2A-NS2B/NS3 complex reaches the virion assembly site, the NS3B/NS3 protease cleaves the C protein from the C/prM/E polyprotein. The C protein assembles onto genomic RNA (presented by NS2A) to form nucleocapsid, which is subsequently enveloped by prM/E complex to form virions. Since flavivirus assembly has to be coupled to viral RNA replication [53], the newly synthesized genomic RNA has to be transported from the replication site to the virion assembly site [54], possibly through the NS2A protein.

The *trans* complemented class V mutants could potentially be used as vaccine candidates, as recently reported for DENV-2 [36]. Since the *trans* complemented virions are able to infect cells for only a single cycle, such vaccine platform is safer than the multi-round infectious live-attenuated vaccines [55,56]. This is particularly important for ZIKV vaccine when immunizing immune-compromised individuals and pregnant women [57]. However, for development of class V mutants as a vaccine platform, the *trans* complementation efficiency needs to be improved to higher titers before they could be tested for *in vivo* efficacy.

In summary, we report the genetic and biochemical characterization of ZIKV NS2A protein. The results indicate that different flavivirus NS2A proteins have distinct membrane topologies, but converge to function in viral RNA synthesis and virion assembly. Together with previous results, we propose a model for flavivirus assembly in which NS2A plays a central role in mediating viral structural and nonstructural proteins as well as genomic RNA during virion formation.

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## Disclosure statement

No potential conflict of interest was reported by the authors.

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PLOS ONE

RESEARCH ARTICLE

# MITA/STING and Its Alternative Splicing Isoform MRP Restrict Hepatitis B Virus Replication

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## Abstract

An efficient clearance of hepatitis B virus (HBV) requires the coordinated work of both the innate and adaptive immune responses. MITA/STING, an adapter protein of the innate immune signaling pathways, plays a key role in regulating innate and adaptive immune responses to DNA virus infection. Previously, we identified an alternatively spliced isoform of MITA/STING, called MITA-related protein (MRP), and found that MRP could specifically block MITA-mediated interferon (IFN) induction while retaining the ability to activate NF-κB. Here, we asked whether MITA/STING and MRP were able to control the HBV replication. Both MITA/STING and MRP significantly inhibited HBV replication *in vitro*. MITA overexpression stimulated IRF3-IFN pathway; while MRP overexpression activated NF-κB pathway, suggesting these two isoforms may inhibit HBV replication through different ways. Using a hydrodynamic injection (HI) mouse model, we found that HBV replication was reduced following MITA/STING and MRP expression vectors in mice and was enhanced by the knockout of MITA/STING (MITA/STING$^{-/-}$). The HBV specific humoral and CD8$^{+}$ T cell responses were impaired in MITA/STING deficient mice, suggesting the participation of MITA/STING in the initiation of host adaptive immune responses. In summary, our data suggest that MITA/STING and MRP contribute to HBV control via modulation of the innate and adaptive responses.

## Introduction

Hepatitis B virus (HBV) is a noncytopathic, hepatotropic double-stranded DNA virus that causes acute and chronic hepatitis and increases the risk of hepatic cirrhosis and hepatocellular carcinoma (HCC). It is generally accepted that the vigor and quality of the cellular immune response determined whether HBV infection is cleared or persists. The adaptive immune responses to viral antigens, especially the destruction of infected cells and the non-lytic mechanisms eliminating



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HBV cccDNA mediated by CD8 cytotoxic T lymphocytes (CTLs), are responsible for HBV clearance in HBV acute infection [1–3].

In addition to the adaptive immune response, the innate immune response induced by the recognition of pathogen-associated molecular patterns (PAMPs) via pattern recognition receptors (PRRs) plays an indispensable role in controlling viral infection [4]. Many PRRs have been identified to be nucleic acid sensors, such as toll-like receptors (TLRs) [5], nucleotide-binding oligomerization domain leucine-rich repeat proteins (NOD-like receptors, NLRs) [6], retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) [7], the member of the PYHIN protein family Interferon Gamma Inducible Protein 16 (IFI16) and proteins absent in melanoma 2 (AIM2) [8–11], DNA-dependent activator of IFN regulatory factors (DAI) [12], the member of the DEXDc family of helicases DDX41 and cyclic GMP-AMP (cGAMP) synthase (cGAS) [13–15]. Once the PRR senses PAMPs, a series of signaling cascades are triggered, resulting in the activation of transcription factors IRF3 and NF-κB, then the following production of interferon (IFN) and inflammatory cytokines, which restrict microbial invasion and further evocate the adaptive immune responses [16].

With regard to HBV, it was thought to be a stealthy virus because the innate responses are predominantly weak or absent during the natural course of HBV infection [17, 18]. However, it is possible that HBV is detected by the innate immune system, while the virus can actively suppress or evade the early antiviral response. Indeed, recent studies have shown that the pgRNA (pregenomic RNA) of HBV and the cytoplasmic exposed HBV rcDNA (relaxed circular DNA), a result of nucleocapsid destabilization, could be detected by RIG-I and MITA/STING, respectively [19, 20]. The activation of different innate immune signaling pathways may induce type III IFN response and inhibit HBV replication [20]. On the other hand, accumulating evidences have demonstrated that distinct HBV proteins and HBV virion particles could impair the innate immune signaling such as RIG-I, TLR3 and STING/MITA-stimulated signaling through various mechanisms. HBV-induced miR146a could target RIG-I thus attenuate IFN production [21]. The HBV X protein interacts with MAVS and the P protein competes for DDX3 binding with TBK1, to inhibit RIG-I-mediated type I IFN pathway signaling [22, 23]. HBV P protein blocks STING/MITA mediated innate immunity response by disrupting K63-linked ubiquitination of STING/MITA [24].

MITA/STING is an adapter protein in the cellular signaling cascade downstream of the DNA sensor cGAS and RNA sensor RIG-I [16]. As reported previously, the cGAS-STING signaling pathway could recognize several invading viruses such as herpes simplex virus type 1 (HSV-1), vaccinia virus (VACV), and human immunodeficiency virus (HIV) [14, 25–27]. In addition, MITA/STING also plays an important role in IFNs production during Dengue virus (DENV) infection [28]. An alternatively spliced variance of MITA/STING, designated as MITA-related protein (MRP), was found to act as a dominant negative mutant of MITA/STING, blocking MITA/STING-mediated IFN induction by disrupting the interaction between MITA/STING and TBK1; interestingly, MRP is still able to activate NF-κB [29]. Because dsDNA and ssDNA are produced during HBV replication, we propose that MITA and MRP are involved in the recognition of HBV replicative intermediates and the regulation of HBV clearance. A recent study demonstrated that activation of cGAS-STING pathway could contribute to the control of HBV replication [30]. Here, we compared the effect of MITA/STING and MRP on cellular signaling as well as their effect on HBV replication in Huh7, HepG2.2.15 cells and in mice. We found that MRP, despite its inability to trigger IRF3 activation, could restrict HBV replication, suggesting the importance of NF-κB-mediated antiviral functions. Interestingly, MITA/STING deficiency resulted in enhanced HBV replication and lower specific humoral and CD8+ T cell immune responses to HBV in the hydrodynamic injection mouse model, suggesting that MITA/STING plays an important role in triggering HBV specific adaptive immune responses.



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## Materials and Methods

### Ethics statement

This study was performed in strict accordance with recommendations in the Guide for the Care and Use of Laboratory Animals according to the regulation in the People's Republic of China. MITA/STING$^{-/-}$ mice, kindly provided by Prof. H. B. Shu (Wuhan University, Wuhan) [31], and littermate control wild-type (WT) mice were bred and maintained under specific pathogen-free conditions at the Central Animal Laboratory of Wuhan Institute of Virology, Chinese Academy of Sciences (WIV, CAS. License number: SYXK2014-0034). All animal experiments were approved by the Institutional Animal Ethical Committee of WIV, CAS (Serial number: WIVA02201404). All procedures were carried out under isoflurane anesthesia and all mice were monitored, weighted daily. None of the mice became severely ill or died prior to the experimental endpoint. The mice were anesthetized with isoflurane and then euthanized by cervical dislocation at the experimental endpoint. All efforts were made to minimize any suffering and the number of animals used in the study.

The knockout mice were screened by genotyping. The mice genomic DNA was extracted from digested tail tissue and genotyping was performed by PCR with the following primers. Knockout and wild type mice shared common forward primer: `5′-CTCCTAGACAGGTGCTGTAGGATG-3′`. Reverse primer for knockout mice, `5′-AAGGGTTATTGAATATGATCGGA-3′`; reverse primer for wild type, `5′-TGGAGACCACAGAGGGTTACCTG-3′` (data not shown). The bone marrow cells were collected from mice long bones and the progenitor cells were differentiated into bone marrow derived macrophage (BMDM) with L929 cell condition medium as previously described [32]. The knockout of MITA/STING was further confirmed by enhanced susceptibility of BMDM to vesicular stomatitis virus (VSV) (data not shown).

### Plasmids, reagent and cell culture

The HBV replication competent plasmid pSM2 was kindly provided by Prof. Dr. Hans Will (Heinrich-Pette-Institute, Hamburg, Germany) [33]. The plasmid was constructed by cloning the *Eco*RI head-to-tail dimer of HBV genome (genotype D), subtype *ayw* (GenBank accession number, V01460) into plasmid pMa5-8 [33, 34], using the authentic HBV promoter for HBV transcription. The human MITA/STING overexpression plasmid pFlag-MITA (pMITA), human MRP overexpression plasmid pHA-MRP (pMRP) and control vector plasmid pcDNA 3.1(+) were described previously [29]. The reporter plasmids pIFN-β-luc, pIRF3-luc, pNF-κB-luc, pISRE-luc were purchased from Clontech. pRL-TK was purchased from Promega. The human β-actin expression plasmid pHA-β-actin was generated by cloning the β-actin sequence into pXJ-40 with *Xho*I and *Kpn*I and used as a control for assessing the transfection efficiency. The MITA/STING ligand c-di-GMP was purchased from InvivoGen. The NF-κB inhibitor ammonium pyrrolidinedithiocarbamic (PDTC) was purchased from Beyotime (Shanghai, China). The human hepatoma cell lines Huh7 and HepG2 were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) supplemented with 10% fetal bovine serum (FBS) (Invitrogen), 100 U/ml penicillin (Invitrogen), 100 mg/ml streptomycin sulfate (Invitrogen), 2 mM L-glutamine (Invitrogen) and nonessential amino acids (Invitrogen). HepG2.2.15 cells with integrated dimers of the HBV genome (GenBank accession number, U95551) was cultured with 500 μg/ml of G418 (Sigma-Aldrich).

### Transfection and luciferase assays

Transient transfections of Huh7, HepG2 and HepG2.2.15 cells were performed with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Cells were transfected



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with the indicated amount of pFlag-MITA or pHA-MRP combined with pSM2. For the luciferase assays, cells were transfected with the indicated amount of pFlag-MITA or pHA-MRP and 100 ng of the indicated reporter plasmids. For the inner control, 10 ng of pRL-TK was co-transfected. The reporter activity was measured at 18–24 hours post-transfection (hpt) using the Dual-Luciferase reporter assay system (Promega).

## Enzyme-linked immunosorbent assay (ELISA) and Western blot analysis

To measure the levels of HBsAg and HBeAg, culture supernatants of transfected cells were diluted 5-fold and analyzed by ELISA as described previously [35]. Levels of HBsAg, HBeAg and antibody to HBsAg in mouse sera were measured by ELISA after a 1:10 dilution [35]. Western blot analysis was performed as previously described [35]. The following antibodies were used: rabbit polyclonal anti-HBc and mouse monoclonal antibodies (mAbs) against β-actin from Santa Cruz Biotechnology, anti-GAPDH (Proteintech), anti-FLAG-tag and rabbit monoclonal Abs against HA-tag from Sigma-Aldrich, anti-phospho-IRF3 (Ser396), anti-IRF3, anti-phospho-IκB (Ser32), anti-IκB, anti-phospho-NF-κB p65 (Ser536) and anti-NF-κB p65 from Cell Signaling Technology. Proteins were visualized with appropriate HRP-conjugated secondary antibodies (Jackson Immuno Research) and SuperSignal-Femto chemiluminescent substrate (Pierce).

## Detection of HBV DNA replicative intermediates and HBV core-associated DNA by Southern blot or real-time PCR

For extraction of HBV replicative intermediates, Huh7 or HepG2.2.15 cells were lysed in 800 μl of DNA extraction buffer (50 mM Tris-HCl, 50 mM NaCl, 1 mM EDTA, 1% NP-40, pH 7.4) with 10 mM $MgCl_2$ and 100 μg/ml DNase I (Sigma-Aldrich, St. Louis, MO) and maintained at 37˚C for 0.5 h. The DNase I digestion was stopped using 25 mM EDTA (pH 8.5). Then, the samples were further digested with 5 mg/ml proteinase K (Qiagen) and 1% SDS at 55˚C for 2 h. The HBV replicative intermediates were extracted with a phenol-chloroform mixture (1:1 ratio) and subjected to Southern blot analysis or quantitative real-time PCR as described previously [36].

Serum HBV core-associated DNA was extracted using a QIAamp DNA Blood Mini Kit (Qiagen). HBV DNA was quantitatively detected by real-time PCR using the SYBR green real-time PCR master mix (Roche). The primers used for real-time PCR were as follows: Forward, 5′–ACCAATCGCCAGTCAGGAAG–3′; Reverse, 5′–ACCAGCAGGGAAATACAGGC–3′.

## RNA interference

The following siRNAs were used: siControl (Qiagen #1027281); siRNA to human MITA/STING (Qiagen #SI04287626) and siRNA synthesized specific to MRP (GenePharm$^{TM}$, 5′–CGGGCAGCGGAACCUGCA–3′). The siMRP was designed and had been proved to be specific to MRP [29]. The siRNAs were transfected using Lipofectamine RNAiMax (Invitrogen) at a final concentration of 80 nM referring to the manufacturer's instructions. To maintain the gene silencing effect from the beginning of virus replication until the last time point analyzed, the cells were split 24 hours after the initial transfection and then transfected with the same siRNA together with pSM2.

## HBV capsid detection

The procedure used to prepare intracellular capsid was adapted from the procedure by Roychouhury *et al*. [37]. Generally, the cultured cells were lysed in extraction buffer (10 mM Tris-HCl, 50 mM NaCl, 1 mM EDTA, 0.25% NP-40, 8% sucrose, pH 7.4) for 10 min on the



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ice. After centrifugation, the supernatant was collected and digested with 6 μM $MgCl_2$, 0.2 mg/ml DNase I (Sigma-Aldrich, St. Louis, MO) and 1.5 mg/ml RNase (Omega) for 20 min at 37˚C. After centrifugation, the supernatant was collected and subjected to electrophoresis on a 1.6% native agarose gel. Then the capsid was transferred to nitrocellulose filtermembrane and subjected to immunoblot analysis with rabbit polyclonal anti-HBV core antibody (Dako, GB058629).

## Immunoprecipitation of mature virions in supernatant

The supernatant of cells were collected and pre-incubated with 2 μg of monoclonal anti-HBs antibodies (A11, S11, and S1, kindly provided by Yan Bin, WIV, CAS, China) for overnight at 4˚C [38]. Then the agarose beads protein A or protein G (Millipore) were added into mixture and rotated for 4 h at 4˚C. After four times wash with phosphate buffered saline, the mixture was resuspended in 200 μl phosphate buffered saline and subjected to HBV core-associated DNA extraction and detection by real-time PCR [39].

## RNA extraction, quantitative real-time RT-PCR, and Northern blot analysis

Total RNA from cultured cells was extracted with TRIzol reagent (Invitrogen) and digested with RNase-free DNase (Promega). Specific mRNAs were quantified by one-step qRT-PCR using the QuantiTect SYBR Green RT-PCR kit (Qiagen). Primers used are listed in Table 1. The mRNA levels were normalized against the copy number of human beta-actin mRNA. Intracellular HBV mRNAs were detected using Northern blot analysis as described previously [40].

## Mouse model of hydrodynamic injection

Hydrodynamic injection (HI) was performed as previously described [38]. In brief, 10 μg of HBV plasmid DNA was injected (5 to 8 s injection) alone or in combination with 10 μg of

**Table 1. Primers used for qRT-PCR.**

| primer | sequence |
|---|---|
| *Ifnb* Forward | 5′-CACGACAGCTCTTTCCATGA-3′ |
| *Ifnb* Reverse | 5′-AGCCAGTGCTCGATGAATCT-3′ |
| *Tnfa* Forward | 5′-CACAGTGAAGTGCTGGCAAC-3′ |
| *Tnfa* Reverse | 5′-AGGAAGGCCTAAGGTCCACT-3′ |
| *Il-6* Forward | 5′-ACCCCCAATAAATATAGGACTGGA-3′ |
| *Il-6* Reverse | 5′-TTCTCTTTCGTTCCCGGTGG-3′ |
| *Cxcl2* Forward | 5′-CAAGAACATCCAAAGTGTGA-3′ |
| *Cxcl2* Reverse | 5′-CCATTCTTGAGTGTGGCTAT-3′ |
| *Isg56* Forward | 5′-GAAAGCCTCAGTCTTGCAGC-3′ |
| *Isg56* Reverse | 5′-CCTGTTGTAAGAGGCCAGCA-3′ |
| *MxA* Forward | 5′- CTCCGACACGAGTTCCACAA-3′ |
| *MxA* Reverse | 5′-GGCTCTTCCAGTGCCTTGAT-3′ |
| *Cxcl10* Forward | 5′-CCTGCAAGCCAATTTTGTCCA-3′ |
| *Cxcl10* Reverse | 5′-TGCATCGATTTTGCTCCCCT-3′ |
| *Oas1* Forward | 5′-ATTCTGCTGGCTGAAAGCAAC-3′ |
| *Oas1* Reverse | 5′-GGAGTGTGCTGGGTCTATGA-3′ |
| *Beta-actin* Forward | 5' TGGAATCCTGTGGCATCCATGAAAC 3' |
| *Beta-actin* Reverse | 5' TAAAACGCAGCTCAGTAACAGTCCG 3' |

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vector plasmid, pFlag-MITA or pHA-MRP into the tail veins of mice aged 6–8 weeks in a volume of phosphate buffered saline (PBS) equivalent to 8% of the body weight.

## Liver tissue preparation and immunohistochemistry (IHC) staining

Liver tissues were collected from mice sacrificed at 4 days post-hydrodynamic injection (days post-HI). To detect intrahepatic HBV replicative intermediates, 30 mg of mouse liver was lysed in 1 ml DNA extraction buffer, extracted as described above and subjected to Southern blot analysis.

To detect intrahepatic HBV RNAs, 50 mg of mouse liver was splintered and subjected to RNA extraction as described above. Of the purified RNA, 10 μg was used for Northern blot and another 10 μg was subjected to agarose gel electrophoresis as an RNA loading control (18S/28S). Relative band intensities of viral mRNAs were quantified using Image J software (NIH) [41].

For IHC staining, the collected liver samples were fixed in formalin and embedded in paraffin. Expression of pMRP, pMITA and intrahepatic HBcAg was detected by immunohistochemical staining with anti-HA, anti-Flag (Sigma-Aldrich) and anti-HBc antibodies (Dako, Carpinteria, CA), respectively, as described previously [42]. The samples were then probed with an appropriate horseradish peroxidase (HRP)-conjugated secondary antibody and visualized with the Envision system.

## Intrahepatic leukocytes and splenocytes isolation, intracellular cytokine staining and flow cytometry

To analyze T cell responses, mouse livers were perfused with phosphate buffered saline, homogenized, passed through a nylon mesh (BD, Biosciences, San Jose, CA), and then digested with 0.2 mg/ml collagenase-IV and 0.002% DNase I (approximately 50–100 U/ml) (Sigma-Aldrich, St. Louis, MO) for 30 min at 37˚C. Hepatocytes were removed by centrifugation for 5 min at 60×g and the supernatant containing intrahepatic leukocytes (IHLs) was retained. The precipitant was washed with RPMI 1640 twice at 50×g for 5 min and collect the supernatant. The supernatant was then pelleted by centrifugation at 300×g for 10 min at 4˚C and resuspended in RPMI 1640. The cell resuspension was gently layered on Mouse Lymphocyte Separation Medium (Dakewe, Beijing, China) and leukocytes were isolated according to the manufacturer's protocols.

To isolate splenocytes, mouse spleens were homogenized and passed through a nylon mesh. The splenocytes were purified directly using Mouse Lymphocyte Separation Medium (Dakewe, Beijing, China) according to the manufacturer's protocols.

Intracellular cytokine staining and flow cytometry analysis of intrahepatic lymphocytes and splenocytes were performed as previously described [35]. Peptides corresponding to the HBsAg $CD8^+$ T cell epitope ($K^b/S_{190-197}$, VWLSVIWM) and the HBcAg $CD8^+$ T cell epitope ($K^b/C_{93-100}$, MGLKFRQL) were used for lymphocyte stimulation. Allophycocyanin (APC)-conjugated anti-mouse IFN-γ, phycoerythrin (PE)-conjugated anti-mouse IL-2, fluorescein isothiocyanate (FITC)-conjugated anti-mouse TNF-α and APC-Cy7-conjugated anti-mouse CD8 mAbs (BioLegend, San Diego, CA) were used for flow cytometry. Dead cells were excluded by staining with 7-aminoactinomycin D (7-AAD; BioLegend, San Diego, CA). Flow cytometry data were acquired on FACS Caliburor or LSRII flow cytometers (BD, Biosciences). Data analysis was performed using FlowJo (Tree Star) software.

## Statistical analysis

Statistical analysis was performed using GraphPad Prism version 5 (GraphPad Software Inc., San Diego, CA). Significant differences were analyzed using a two-tailed unpaired *t*-test. The two ways ANOVA followed by Bonferroni's test was used to determine the differences in



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multiple comparisons. P-values were calculated, and statistical significance is reported as highly significant using $^{*}(p<0.05)$, $^{**}$ $(p<0.01)$, or $^{***}$ $(p<0.001)$. Data are presented as the mean ± standard deviation.

## Results

### The activity of MITA/STING and MRP in Huh7 cells

Although MRP was identified as a dominant negative mutant of MITA/STING, it retains the ability to activate NF-κB in 293T cells [29]. Therefore, it is worthwhile to investigate and compare the function of MITA/STING and MRP during HBV replication. The activities of MITA/STING and MRP in the IFN-β signaling pathway in Huh7 cells, a hepatoma cell line supporting HBV replication, were first analyzed using reporter assays. The results showed that MITA/STING overexpression activated IRF3- but not NF-κB-dependent luciferase expression in Huh7 cells (Fig 1A). The IFN-β promoter and the downstream ISRE-dependent reporter expressions were stimulated by MITA/STING in a dose-dependent manner (Fig 1B and 1C), but MRP overexpression only stimulated the NF-κB-dependent reporter and had no effect on IFN-β, IRF3 or the ISRE-dependent reporter expression (Fig 1D and 1F). Consistent with reporter results, the phosphorylation of IRF3 but not IκB was induced by MITA/STING in a dose-dependent way, while the phosphorylation of IκB but not IRF3 was increased by MRP (Fig 1G and 1H). These data suggested that in Huh7 cells MITA activated the IFN-β signaling pathway through IRF3, while MRP activated the NF-κB pathway.

### MITA/STING and MRP inhibited HBV replication in Huh7 cells

To investigate the potential roles of MITA/STING and MRP in HBV replication, the HBV replication plasmid pSM2 was cotransfected with pFlag-MITA or pHA-MRP into Huh7 cells [43]. HBV replication was analyzed by monitoring HBV replicative intermediates, transcripts and the expression levels of HBeAg, HBsAg and HBcAg. As shown in Fig 2A, HBV DNA replicative intermediates were significantly inhibited by both MITA/STING and MRP as detected by Southern blot (Fig 2A, upper panel). MITA/STING and MRP also inhibited the transcription of HBV mRNAs (Fig 2A, lower panel), the expression of HBeAg and HBsAg in supernatant (Fig 2B and 2C) as well as the expression of the intracellular HBV core protein (Fig 3A). In all cases, MITA/STING expression achieved a much stronger inhibition of HBV replication and gene expression than MRP.

To further confirm the inhibitory function of MITA/STING and MRP on HBV replication, siRNAs specific to human MITA/STING and MRP were transfected into Huh7 cells. Since the expression levels of MITA/STING and MRP in Huh7 cells are very low, the siRNAs were transfected twice at concentration of 80 nM to achieve the knockdown effect (data not shown). Silencing of MITA/STING and MRP increased the cytoplasmic HBV core-associated DNA levels as detected by real-time PCR (Fig 2D) and the amounts of intracellular HBc protein (Fig 2E). The levels of HBsAg and HBeAg in the supernatant were significantly elevated after MITA/STING and MRP silencing as well (Fig 2F and 2G). Altogether, these results indicated that both MITA/STING and MRP inhibited HBV replication in hepatoma cells. Clearly, however, MITA overexpression resulted in a more significant inhibition of HBV replication than MRP overexpression did.

### MITA/STING and MRP suppressed HBV replication by inducing innate immune signaling in Huh7 cells

The activation of innate immune signaling in Huh7 cells by MITA/STING and MRP was analyzed at the present of the HBV replication. It is notable that HBV replication did not significantly



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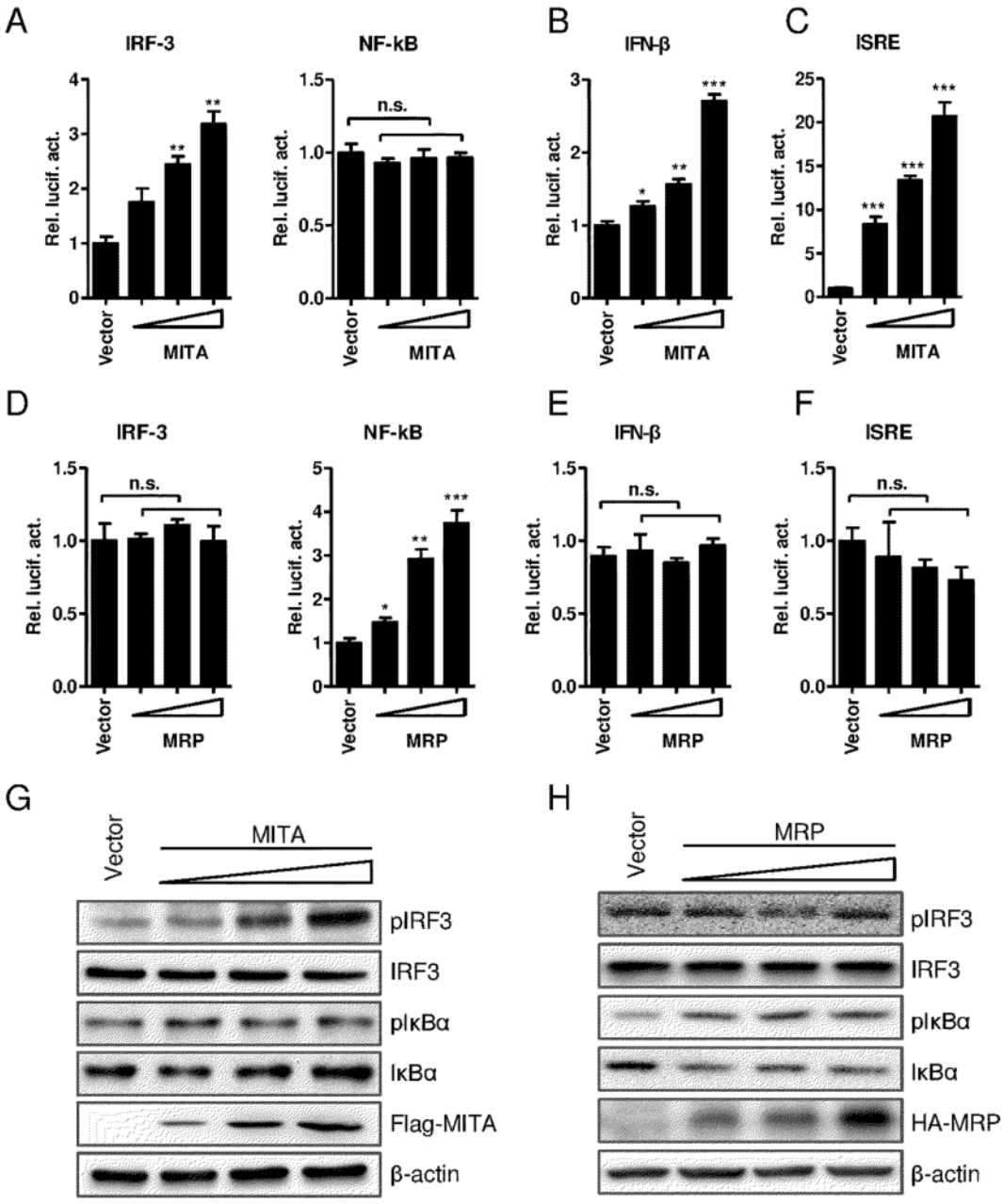



**Fig 1. The functions of MITA/STING and MRP in hepatoma cell line Huh7 which supports HBV replication.** (A) pIRF3-luc and pNF-κB-luc, (B) pIFN-β-luc and (C) pISRE-luc reporter plasmids were cotransfected with a serial concentration of pFlag-MITA/STING plasmids into Huh7 cells. (D) pIRF3-luc and pNF-κB-luc, (E) pIFN-β-luc and (F) pISRE-luc reporter plasmids were cotransfected with a serial density of pHA-MRP plasmids into Huh7 cells. The reporter activity was measured at 18–24 hpt with the Dual-Luciferase reporter assay system. (G-H) A serial increasing amount of pFlag-MITA or pHA-MRP plasmids were transfected into Huh7. The intracellular pIRF3, IRF3, pIκB and IκB protein levels were detected by Western blot. The two ways ANOVA followed by Bonferroni's test was used to determine the differences in multiple comparisons (*, P <0.05; **, P <0.01; ***, P <0.001).

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induce the IFN-β pathway as the phosphorylation level of IRF3 did not change after pSM2 transfection. However, co-transfection of pMITA and pSM2 induced greater phosphorylation of IRF3 compared with that induced by pMITA expression alone (Fig 3A). The phosphorylation level of



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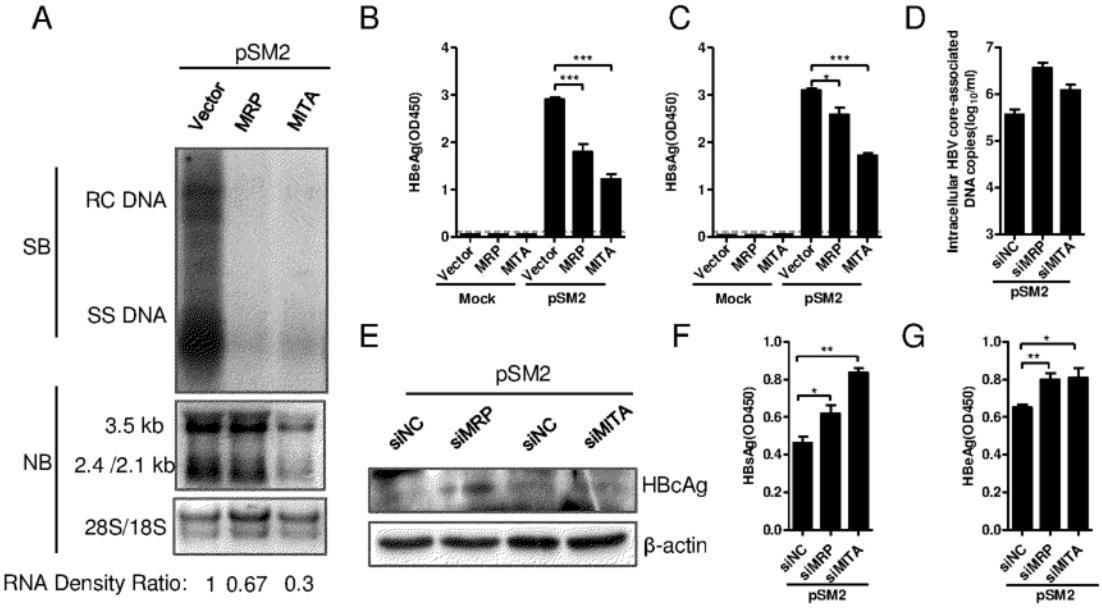



**Fig 2. MITA/STING and MRP inhibited HBV replication *in vitro*.** (A-C) The overexpression plasmid pFlag-MITA/STING or pHA-MRP was co-transfected with HBV plasmid pSM2 into Huh7 cells. (A) HBV DNA replicative intermediates (upper) and mRNAs (lower) were detected by Southern blot and Northern blot, respectively. HBV mRNA density signals on Northern blot were normalized to 18S/28S and showed as RNA density ratio. (B) Levels of HBeAg and (C) HBsAg secreted into supernatant were detected by ELISA after 5-fold dilution. Results were presented as the optical density at 450 nm (OD450). (D-G) siRNA specific to human MITA/STING or MRP was transfected into Huh7 for twice. pSM2 was cotransfected with siRNA at the second time. (D) Intracellular HBV core-associated DNA was extracted and quantified with real-time PCR. (E) Intracellular HBV core protein was detected by Western blot. (F) HBsAg and (G) HBeAg expressed in supernatant were diluted 5-fold and measured by ELISA. The dashed line represents the cutoff value (CoV), which was assumed to be 2.1-fold mean value of the negative samples. Significant differences were analyzed using a two-tailed unpaired *t*-test (*, P <0.05; **, P <0.01; ***, P <0.001).

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IκB was slightly increased by pMRP, pMITA and pSM2 transfection (Fig 3A). The mRNA levels of IFN-β, pro-inflammatory cytokines TNF-α and IL-6, and several ISGs, such as ISG56, OAS1, MxA and CXCL10, were significantly increased in MITA/STING and pSM2 co-transfected cells (Fig 3B–3D), suggesting that both NF-κB and IFN-β were activated when MITA/STING and pSM2 were co-expressed. In contrast, the IFN-β or ISGs mRNA levels were not changed in pMRP and pSM2 co-transfected Huh7 cells (Fig 3B and 3C), while the chemokine CXCL2 mRNA but not TNF-α or IL-6 was significantly up-regulated (Fig 3C), implying that MRP inhibited HBV replication via a mechanism that was different from MITA/STING and independent of IFN induction.

To further confirm the role of NF-κB activation in the anti-HBV effect of MRP, the inhibitor PDTC was used to block NF-κB activation. As shown in Fig 3E, the HBV DNA replicative intermediates and the HBV mRNA transcripts, which were inhibited by MRP, were partially rescued by PDTC treatment. PDTC also partially restored the HBsAg, HBeAg levels in supernatant (Fig 3F and 3G) and the intracellular HBcAg level (Fig 3H upper). Interestingly, the HBV capsid formation as well as the matured HBV particle level in supernatant (Fig 3H lower panel and 3I) was significantly rescued by PDTC. These results demonstrated that the activation of NF-κB by MRP was important for the anti-HBV effect of MRP.

## MITA/STING, MRP and c-di-GMP inhibited HBV replication in HepG2.2.15 cells

The effects of MITA/STING and MRP on HBV replication were further studied in HepG2.2.15 cells. The overexpression of MITA/STING enhanced the phosphorylation level of IRF3, while



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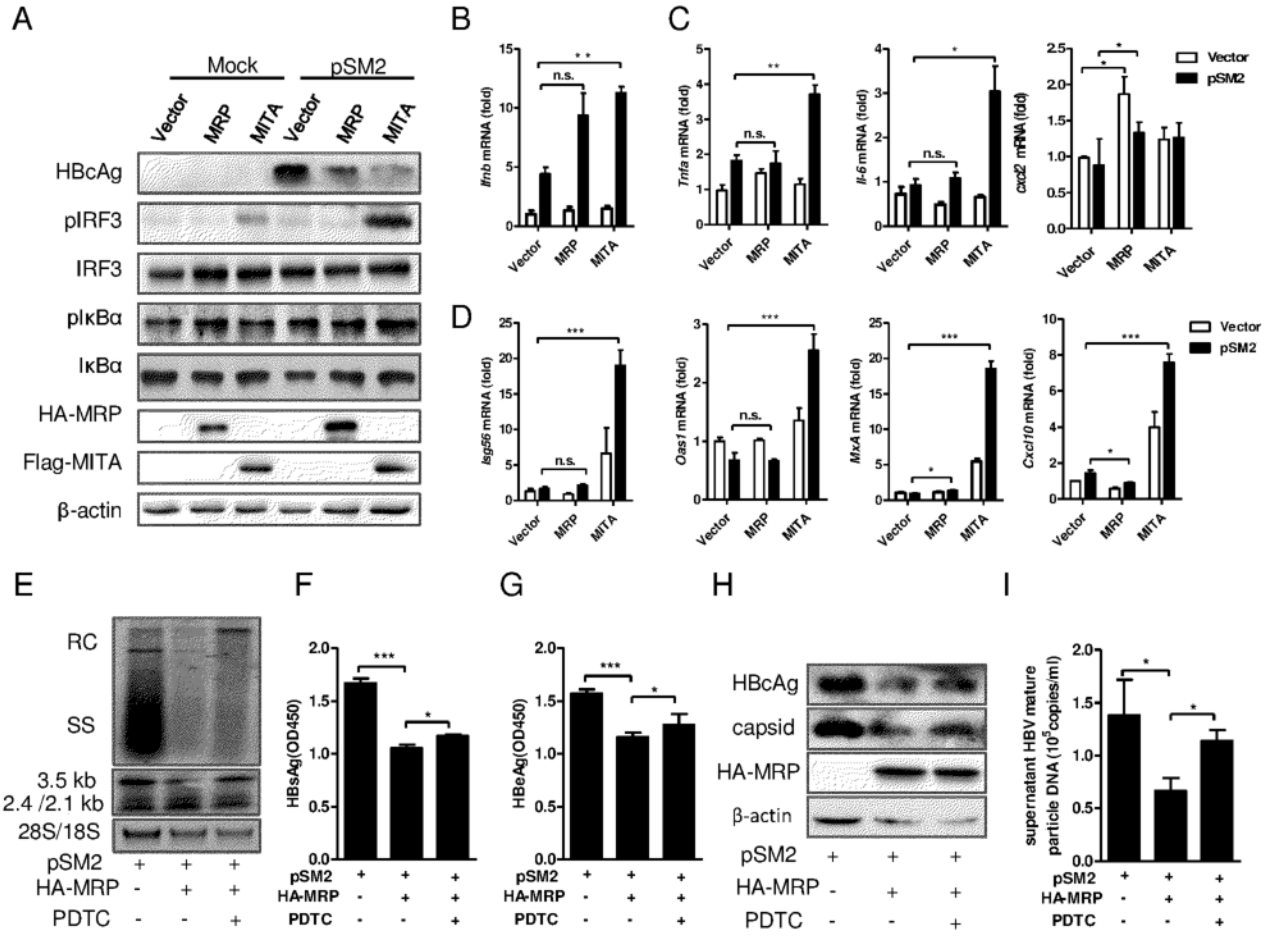



**Fig 3. MITA/STING and MRP suppressed HBV replication by inducing innate immune signaling.** (A-D) The overexpression plasmid pFlag-MITA/STING or pHA-MRP was co-transfected with HBV plasmid pSM2 into Huh7 cells. (A) Intracellular HBV core protein, pIRF3, IRF3, pIκB and IκB protein levels were detected by Western Blot with indicated antibody. The expression of MRP and MITA/STING were detected with anti-HA and anti-Flag antibody, respectively. (B) The levels of *Ifnb*, (C) pro-inflammatory cytokines *Tnfa, Il-6*, chemokine *Cxcl2* and (D) ISGs *Isg56*, *Oas1*, *MxA*, *Cxcl10* mRNAs in MRP or MITA overexpressed with or without pSM2 transfected Huh7 cells were detected by qRT-PCR. (E-I) HepG2 cells were transfected with pHA-MRP and pSM2 together and then treated with PDTC. (E) HBV DNA replicative intermediates and mRNA transcripts were detected by Southern blot and Northern blot, respectively. (F) The HBsAg and (G) HBeAg level in supernatant were measured by ELISA after 5-fold dilution of the supernatant. (H) HBcAg and capsid were detected by Western blot. (I) The supernatant mature virions were immunoprecipitated with anti-HBs antibodies and subjected to HBV core-associated DNA extraction, then quantified by real-time PCR. Significant differences were analyzed using a two-tailed unpaired *t*-test (*, P <0.05; **, P <0.01; ***, P <0.001).

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MRP enhanced the phosphorylation level of IκB and p65, suggesting that the activation of innate immune responses in HepG2.2.15 cells by MITA/STING and MRP were different (Fig 4A). The intracellular HBcAg, HBV capsid level (Fig 4A) and the transcription of HBV mRNAs (Fig 4B) were reduced by MITA/STING and MRP overexpression. Consistent with the reduced capsid level, the intracellular HBV core-associated DNA level was decreased in MITA/STING and MRP overexpressing cells (Fig 4C). Interestingly, the mature particles in supernatant were strongly inhibited by MITA/STING but not MRP overexpression (Fig 4D). It's clear that both MITA/STING and MRP have an inhibitory effect on HBV replication in HepG2.2.15 cells, while MITA/STING showed a stronger effect than MRP, consistent with the results in pSM2 transfected Huh7 cells.



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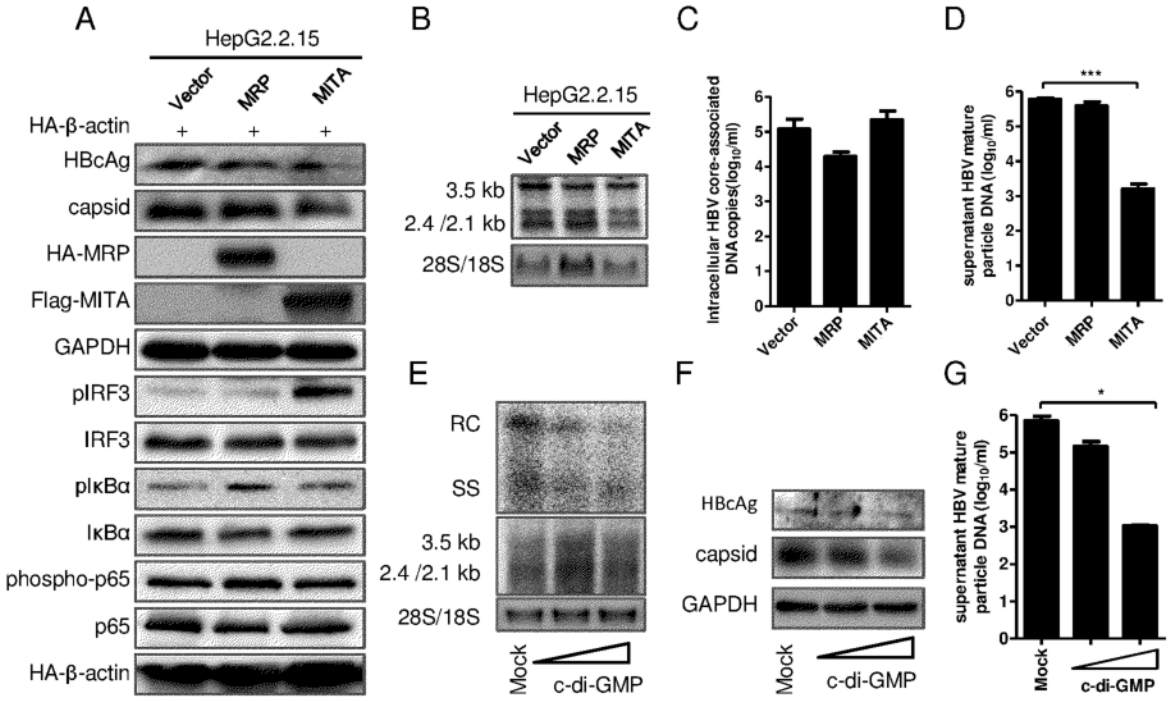



**Fig 4. MITA/STING, MRP and c-di-GMP inhibited HBV replication in HepG2.2.15 cells.** (A-E) The overexpression plasmid pHA-MRP, pFlag-MITA/STING or vector plasmid was transfected into HepG2.2.15 cells. The pHA-β-actin was cotransfected as an inner control. (A) The levels of HBV capsid, intracellular core antigen, pIRF3, IRF3, pIκBα, IκBα, phospho-p65, p65, MRP, MITA, GAPDH and β-actin were detected by immunoblotting with indicated antibodies. (B) The HBV mRNA replicative intermediates were detected by Northern blot. (C) Intracellular HBV core-associated DNA were extracted and quantified with real-time PCR. (D) The HBV mature particles in supernatant were immunoprecipitated with anti-HBs, and then HBV core-associated DNA were extracted and quantified with real-time PCR. (E-G) HepG2.2.15 cells were transfected with c-di-GMP of different concentrations and incubated for 24 h. (E) The HBV DNA replicative intermediates (upper) and mRNAs (lower) were investigated by Southern blot and Northern blot, respectively. (F) HBcAg (upper) and HBV capsid (lower) were immunoblotted with indicated antibody. (G) The supernatant HBV mature particles were immunoprecipitated and HBV core-associated DNA were extracted and quantified with real-time PCR. Significant differences were analyzed using a two-tailed unpaired *t*-test (*, P <0.05; **, P <0.01; ***, P <0.001).

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To further confirm the control of HBV replication by MITA/STING pathway, the HBV replication was analyzed in HepG2.2.15 cells treated with c-di-GMP, the ligand of MITA/STING. The HBcAg protein expression, capsid formation, HBV DNA replicative intermediates in cells and the mature particles in supernatant were decreased by c-di-GMP treatment (Fig 4E–4G), although the HBV mRNA transcript level in cells was not apparently changed by c-di-GMP treatment (Fig 4F lower). These results demonstrated that c-di-GMP treatment inhibited HBV replication, which was in line with that MITA/STING overexpression behaved.

## MITA/STING and MRP suppressed HBV replication in mice

We further investigated the influence of MRP and MITA/STING on HBV replication using a hydrodynamic injection mouse model. Empty vector pcDNA 3.1(+), pMRP or pMITA was mixed with pSM2 and were delivered into wild type C57BL/6 mice by HI. The expression of MRP and MITA/STING was then detected by immunohistochemical staining (IHC) with anti-HA and anti-Flag antibodies, respectively (Fig 5A). After HI of plasmid mixtures into C57BL/6 mice, serum HBsAg levels were monitored from day 1 through day 21 post-HI. A high serum HBsAg level was detected in mice receiving pSM2/Vector at 1 through 7



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days post-HI (Fig 5B). Although seroconversion occurred at 11 days post-HI in all treatment groups, serum HBsAg levels as well as HBeAg levels, were significantly lower in mice that received pSM2/pMITA and pSM2/pMRP than in mice that received pSM2/Vector (Fig 5B and 5C). The levels of serum HBV core-associated DNA reached a peak at day 7 post-HI and were undetectable at day 17 post-HI in all treatments. A reduction in serum HBV core-associated DNA was observed in pMITA- and pMRP-delivered mice at day 4 post-HI (Fig 5D). Compared with control group, the percentages of mice with detectable serum HBV DNA were reduced in the pSM2/MITA and pSM2/MRP groups at day 10 and day 14 post-HI (Fig 5E).

The intrahepatic HBV DNA replicative intermediates and mRNAs at day 4 post-HI were detected by Southern blot and Northern blot, respectively. The results showed an obvious reduction of HBV DNA and RNA levels in the pSM2/MITA and pSM2/MRP groups (Fig 5F). Furthermore, the percentages of HBcAg-positive hepatocytes in liver tissue were also

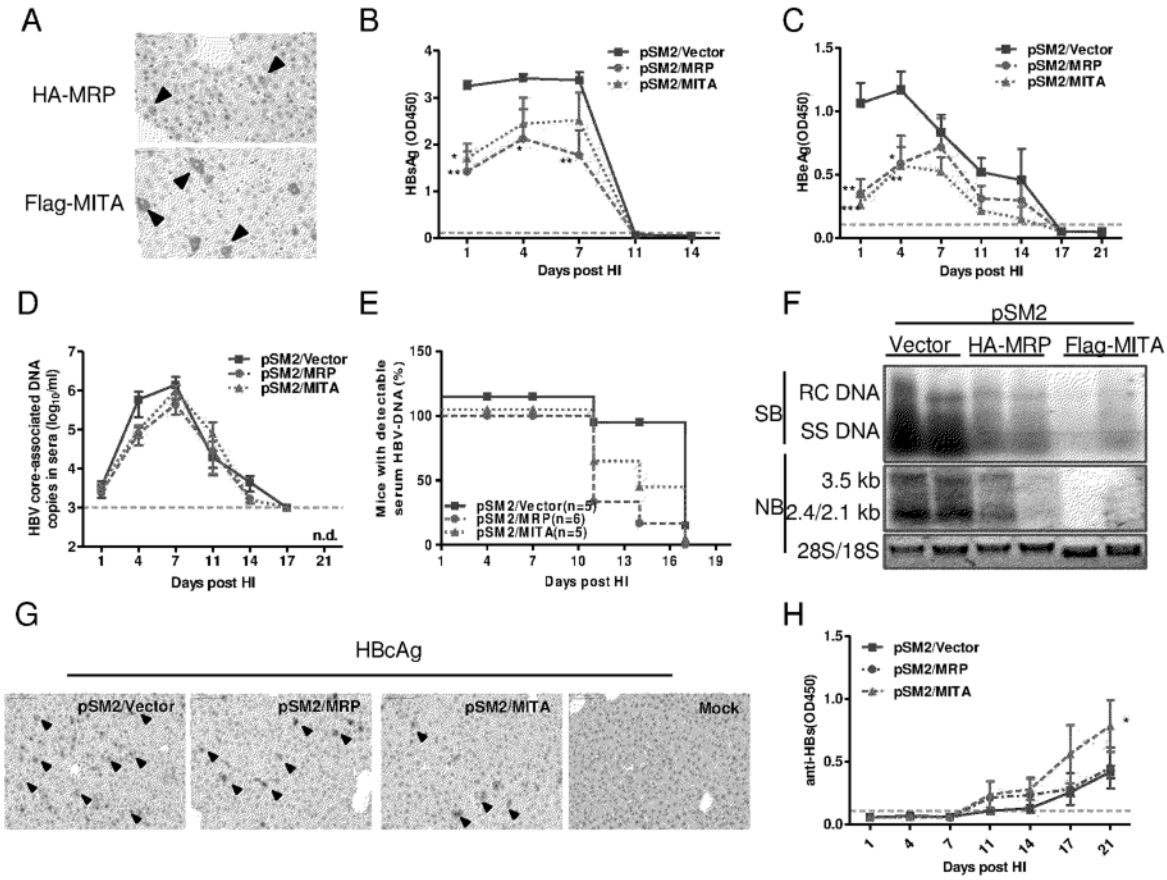



**Fig 5. Overexpressed MRP and MITA/STING significantly suppressed HBV replication *in vivo*.** 6~8 weeks old wild type C57BL/6 mice were hydrodynamically co-injected with 10 μg pSM2 plasmid and 10 μg pHA-MRP or pFlag-MITA/STING or control plasmid by tail-vein. (A) Expression of MRP or MITA/STING protein was detected in liver sample collected at 4 days post-HI by IHC staining with antibody against HA- or Flag-tag. (B) The levels of HBsAg or (C) HBeAg in 10-fold diluted sera were detected by ELISA. (D) The serum HBV DNA in mice was quantified by real-time PCR at the indicated time points. The detection limit for HBV DNA in our system was 1000 copies per milliliter. (E) The percentage of mice with detectable serum HBV DNA at indicated time point was calculated as the percentage of total mice in each group. (F) HBV DNA and RNA replicative intermediates in liver samples collected at 4 days post-HI were measured with Southern blot and Northern blot, respectively. Each lane represented a liver sample from a mouse. (G) Immunohistochemical staining showed HBcAg levels in liver at 4 days post-HI. (H) The dynamic levels of antibody specific to HBV S antigen in 10-fold diluted sera at indicated time points were detected by ELISA. The black arrow indicated the HA-MRP, Flag-MITA or HBcAg expression positive hepatocytes. The dashed line represents the cutoff value, which was assumed to be 2.1-fold mean value of the negative samples. Five or six mice per group were analyzed. The two ways ANOVA followed by Bonferroni's test was used to determine the differences in multiple comparisons (*, P <0.05; **, P <0.01; ***, P <0.001).

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decreased significantly in the pSM2/pMITA and pSM2/pMRP groups compared with the pSM2/Vector group as shown by IHC staining (Fig 5G). Interestingly, the level of antibodies specific for HBsAg (anti-HBs) in serum was increased in the pSM2/pMITA group, suggesting that MITA/STING enhanced HBV-specific antibody production *in vivo* (Fig 5H). These data demonstrated that both MITA/STING and MRP inhibited HBV replication, and that MITA/STING played a role in triggering HBV-specific adaptive immune responses.

## HBV replication was enhanced in MITA/STING knockout mice

The influences of MITA/STING and MRP on HBV replication were further analyzed in MITA/STING$^{-/-}$ and wild type littermate mice (MITA/STING$^{+/+}$). The HBV replication plasmids were delivered into mice by HI. The quantification of HBV core-associated DNA copies in mouse serum samples showed that HBV DNA levels were higher in MITA/STING$^{-/-}$ mice than in wild type mice at day 7 post-HI (Fig 6A). The ELISA results showed that the HBsAg level in serum of MITA/STING$^{-/-}$ mice was also higher at day 7 post-HI (Fig 6B). Compared with that in wild type mice, the serum HBeAg level in MITA/STING$^{-/-}$ mice was higher during all infection courses (Fig 6C), indicating an enhanced replication of HBV in MITA/STING$^{-/-}$ mice.

## HBV antibody response and specific T-cell immune response in MITA/STING knockout mice

MITA/STING as an adapter protein not only instigates transcription of cytoplasmic DNA-induced cellular defense genes but also facilitates adaptive responses required for viral control

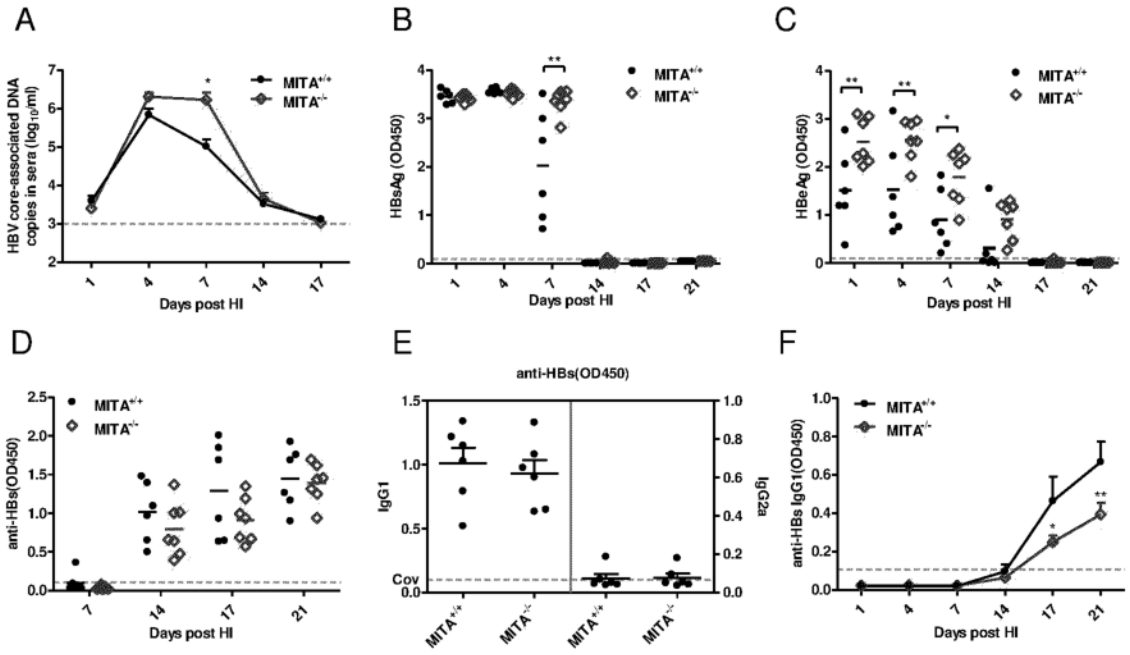



**Fig 6. MITA deficiency induced elevation of serum HBV viral loads and impaired HBV humoral immune response *in vivo*.** Wild type (MITA$^{+/+}$) and MITA knockout (MITA$^{-/-}$) mice were hydrodynamically (HI) injected with 10 μg pSM2 plasmid. (A) The serum HBV DNA in mice was quantified by real-time PCR at the indicated time points. (B) The levels of HBsAg and (C) HBeAg in 10-fold diluted sera were detected by ELISA. (D) The dynamic anti-HBs antibodies in 10-fold diluted sera were monitored and detected by ELISA. (E) The levels of HBsAg-specific IgG subtypes were determined by ELISA. (F) The dynamic of HBsAg-specific IgG1 subtype were also detected by ELISA. The dashed line represents the cutoff value, which was assumed to be 2.1-fold mean value of the negative samples. Six or seven mice per group were analyzed. The two ways ANOVA followed by Bonferroni's test was used to determine the differences in multiple comparisons (*, P <0.05; **, P <0.01).

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and elimination [44]. Therefore, we assessed the production of anti-HBs antibodies in MITA/STING$^{+/+}$ and MITA/STING$^{-/-}$ mice receiving pSM2. As shown in Fig 6D, following the decline of serum HBsAg, the anti-HBs levels rose after day 7 post-HI and increased thereafter in both groups. However, the antibody level in MITA/STING$^{-/-}$ mice was weaker compared to that in MITA/STING$^{+/+}$ mice, which was consistent with previous findings that overexpression of MITA/STING enhanced HBV-specific antibody levels in wild type mice (Fig 5H). IgG1/IgG2a antibody responses were measured as an indicator of the Th bias of specific immune responses [45]. The different subtypes of HBsAg-specific IgGs present post-HI indicated that pSM2 induced a significantly stronger Th2 response (Fig 6E). Then, the HBsAg-specific IgG1 subtype was monitored at different time points after HI, and results showed that the IgG1 response was weakened in MITA/STING$^{-/-}$ mice (Fig 6F).

We also analyzed HBV-specific CD8$^+$ T cell response. Intrahepatic lymphocytes and splenocytes were isolated at day 28 post-HI, and the cells were stimulated with H-2k$^b$-restricted CTL epitope peptides derived from HBsAg or HBcAg. The populations of HBsAg- or HBcAg-specific IFN-γ-, TNF-α-, and IL-2-producing CD8$^+$ T cells were measured by intracellular cytokine staining and analyzed via flow cytometry. Compared to those in MITA/STING$^{+/+}$ mice, the percentages of HBcAg- and HBsAg-specific mono-functional CD8$^+$ T cells producing IFN-γ or TNF-α among the liver-infiltrating CD8$^+$ T cells were reduced in MITA/STING$^{-/-}$ mice. In addition, the frequency of IFN-γ$^+$ CD8$^+$ T cells was also distinctly lower among spleen CD8$^+$ T cells of MITA/STING$^{-/-}$ mice than that of MITA/STING$^{+/+}$ mice. There was little difference in the levels of IL-2-producing CD8$^+$ T cells between the two groups in the liver as well as in the spleen (Fig 7A). The majority of HBV peptide-stimulated CD8$^+$ T cells in the MITA/STING$^{+/+}$ and MITA/STING$^{-/-}$ mice were monofunctional, producing one cytokine (IFN-γ, TNF-α or IL-2). However, the percentages of polyfunctional CD8$^+$ T cells, which simultaneously produced two or three cytokines (IFN-γ/ TNF-α/ IL-2), were significantly reduced among the liver-infiltrating CD8$^+$ T cells, as well as among the spleen CD8$^+$ T cells isolated from the MITA/STING$^{-/-}$ mice compared with that observed from the MITA/STING$^{+/+}$ mice (Fig 7B and 7C). These findings suggest that the cytokine producing function of CD8$^+$ T cells was impaired when MITA/STING was deficient. Taken together, a MITA/STING deficiency impaired HBV-specific antibody responses and reduced the intensity of the CD8$^+$ T cell response.

## Discussion

Previously, we found that despite a negative modulation of MITA/STING induced IFN-β activation, MRP played a different role in response to RNA and DNA virus infection since MRP blocked MITA-mediated IFN signaling pathway induced by SeV infection but enhanced HSV-1 induced IFN response [29]. In this present study, we found that MITA/STING and MRP overexpression inhibited HBV replication *in vitro* and *in vivo*. In addition to activating the innate immune response, MITA/STING also regulates the adaptive immune response to HBV.

Our study found that MITA/STING was a better inhibitor of HBV replication than MRP (Fig 2 and Fig 4), which could be attributed to the different signaling pathways and cytokine profiles activated by MITA/STING versus MRP. While MITA/STING was able to activate the IFN-β pathway through IRF3 phosphorylation and consequently increase the expression of ISGs, pro-inflammatory cytokines and chemokines, MRP was able to active the NF-κB reporter, up-regulate IκB phosphorylation and chemokine CXCL2 expression in Huh7 cells. Although MITA/STING overexpression did not activate the NF-κB reporter in Huh7 cells, it stimulated the IFN-β promoter whose activation requires the cooperative action of IRF3 and NF-κB. It may possibly attribute to that the baseline of NF-κB activation in Huh7 cells is higher than in other cell lines because p65 phosphorylation was readily detectable in Huh7



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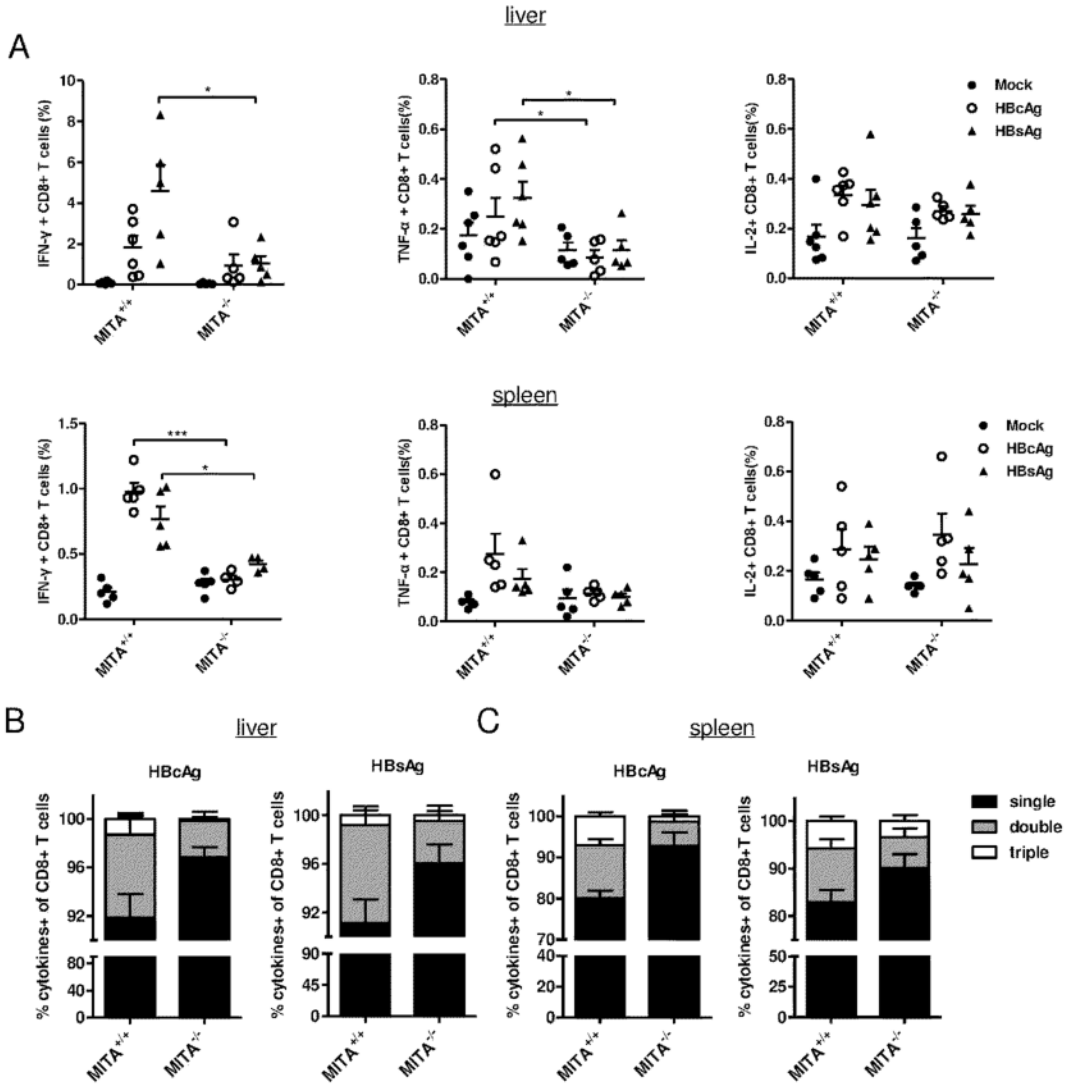



**Fig 7. Liver-infiltrating CD8⁺ T lymphocytes and splenocytes in MITA knockout mice displayed deficient cytokines producing phenotype and impaired specific CTL response.** (A) Wild type ($MITA^{+/+}$) and MITA knockout ($MITA^{-/-}$) mice were hydrodynamically injected with pSM2 plasmids. Twenty-eight days after injection, intrahepatic lymphocytes and splenocytes from HBsAg-positive mice were isolated and unstimulated (Mock) or stimulated with HBcAg- or HBsAg-derived peptides *ex vivo*. The frequencies of IFN-γ+ or TNF-α+ or IL-2+ CD8+ T cells were measured by intracellular staining and analyzed by FACS assay. (B) Quantitative analysis showed the percentages of one or two or three kinds of cytokines (IFN-γ/ TNF-α/ IL-2) simultaneously producing cells within the CD8+ T cell population from liver or (C) spleen. Five or six mice per group were analyzed. Significant differences were analyzed using a two-tailed unpaired *t*-test (*, P <0.05; **, P <0.01; ***, P <0.001).

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cells without stimulation. This phenomenon is consistent with previous reports that NF-κB is constitutively active in hepatoma tissues and Huh7 cells [46]. Activations of both IFN-β and NF-κB signaling have been shown to inhibit HBV replication through the induction of different effectors. A number of previous studies demonstrated that type I IFN could induce ISGs, such as MxA and IFIT1/2 (ISG56/ISG54), and suppress HBV replication *in vitro* and *in vivo* [47–49]. Generally, activation of the NF-κB pathway leads to the production of pro-inflammatory cytokines, such as TNF-α, which has a known direct anti-viral effect on HBV. TNF-α was shown to suppress HBV replication by damaging the formation or stability of cytoplasmic



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viral capsids [50]. In the present study, MRP reduced the levels of HBV DNA replicative intermediates strongly and the levels of HBV transcripts slightly. In contrast, MITA/STING significantly suppressed the formation of both HBV DNA replicative intermediates and transcripts. The precise mechanism of how MITA/STING and MRP function in this capacity needs further investigation.

The presence of HBV significantly enhanced the MITA/STING-mediated IRF3-IFN pathway, as shown by the increased level of phosphorylated IRF3, the upregulation of IFN-β mRNA and the elevated expression levels of the downstream ISGs, pro-inflammatory cytokines and chemokines (Fig 3). These findings suggest that the MITA/STING signaling pathway may sense HBV components and in turn be further activated. Indeed, Dansako *et al*. demonstrated that HBV-derived synthetic dsDNA and the HBV virus could induce ISG56 expression in a cGAS/STING-dependent manner [51]. Consistently, Cui *et al*. showed that HBV DNA could be detected by DNA sensors, specifically the cGAS/STING signaling pathway, in an immortalized mouse hepatocyte cell line [19]. On the other hand, HBV was shown to counteract the MITA/STING-mediated antiviral responses by disrupting K63-linked ubiquitination of MITA/STING [24].

The innate immunity signals are essential for the development of adaptive immune response [52]. Soon after discovery, MITA/STING was found to be required for efficient T-cell response to antigen induced by DNA-vaccine and CTL response triggered by baculovirus [26, 53]. A recent report also showed that activation of the cGAS-STING pathway boosts antigen-specific T cell activation and antibody production in mice [54]. Consistent with previous reports, our results showed that MITA/STING deficiency impaired HBV-specific humoral and T-cell mediated responses in HI mouse model, indicated by the weakened production of HBV-specific antibodies and the multiple cytokines producing ability of $CD8^+$ T cells specific to HBV in MITA/STING knockout mice. On the other hand, overexpression of MITA/STING enhanced the antibody response to HBV *in vivo*.

In the HI mouse model, overexpression of MITA/STING and MRP restricted HBV replication and the deficiency of MITA/STING enhanced HBV replication. Thomsen *et al*. also showed that overexpression of MITA/STING in mice hepatocytes could reduce HBV replication. However, they found that MITA/STING deficiency has no influence on HBV replication in a adenovirus-HBV model [55]. The discrepancy may attribute to the different mechanisms of host recognition and responses to systemic infected adenovirus vector and naked plasmid DNA delivered by HI. Adenovirus DNA was demonstrated to induce IFN-β production by TLR9/MyD88 cascade in plasmacytoid dendritic cells (pDCs) and MyD88-independent pathway in conventional DCs and macrophages [56]. Daniela *et al*. demonstrated that cGAS/STING pathway was essential in MyD88-independent pathway for type I IFN induction and antiviral response to recombinant adenovirus (rAdV). Although the rAdV could trigger robust type I IFN production through STING-IRF3 pathway as well as Ad-HBV proved by Thomsen [55], loss of cGAS/STING minimally impacted viral clearance and persistence of transgene expression delivered by rAdV [57], which indicated that cGAS/STING was dispensable to the induction of adaptive immune response to rAdV. The minimal impact of MTIA/STING-mediated type I IFN pathway on adaptive immune response to rAdV was also previously confirmed in STAT2 knockout Syrian hamster model and IFNRI and STING knock out mouse [58, 59]. Different from the receptor mediated uptake of rAdV DNA, the naked HBV plasmids were delivered directly into cytoplasm by hydrodynamic injection. In the cytoplasm, the HBV DNAs were mainly recognized by cGAS/STING firstly, especially in MITA/STING-abundant nonparenchymal cells (NPCs). Since hepatocytes express low level of MITA/STING, the enhanced HBV replication in MITA/$STING^{-/-}$ mice probably was due to the deficiency of MITA/STING in NPCs, as lack of MITA/STING would influence host innate immune



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response as well as DCs maturation, and defective antigen presentation of DCs consequently impaired the expansion of leukemia specific T cells and CTL response to control HBV replication.

In conclusion, although the baseline of MITA/STING expression was low in hepatoma cells, the complement of MITA/STING by overexpression restricted HBV replication significantly, suggesting an important role of MITA/STING in HBV persistence. The spliced variant of MITA/STING, MRP also inhibits HBV replication *in vitro* and *in vivo*, via the activation of NF-κB pathway. Deficiency of MITA/STING weakened HBV specific humoral and CTL responses, and consequently enhanced HBV replication in MITA/STING$^{-/-}$ mice, which indicated indispensable role of MITA/STING in adaptive response initiation. Due to the important role of MITA/STING in linking the innate response to adaptive response, the agonists to MITA/STING have been explored as vaccine adjuvants and for tumor treatment. Similarly, the MITA/STING agonists probably could be used as adjuvants of therapeutic vaccination for chronic HBV infection.

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**Investigation:** SL KZ XS LL.

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**Supervision:** RP XC.

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**U.S. China Dialogue and Workshop on the Challenges of Emerging Infections, Laboratory Safety, Global Health Security and Responsible Conduct in the Use of Gene Editing in Viral Infectious Disease Research**

Harbin Veterinary Research Institute | Chinese Academy of Agricultural Sciences

January 8-10, 2019
Harbin, China

To address the interconnected life science challenges associated with combating infectious diseases and further enhance cooperation between China and the United States in the areas of global health, biosafety, and biosecurity, the U.S. National Academy of Sciences (NAS), and the Chinese Academy of Agricultural Sciences (CAAS) in partnership with representatives from other Chinese institutions held the fourth *U.S. China Dialogue on the Challenges of Emerging Infections, Laboratory Safety and Global Health Security* in tandem with a *Workshop on Responsible Conduct in the Use of Gene Editing in Pathogen Research* on January 8-10, 2019 at the CAAS Harbin Veterinary Research Institute (HVRI) in Harbin, China. HVRI is one of three institutes in China that operate a Biological Safety Level-4 (BSL-4) laboratory.

The meeting, ultimately titled *U.S. China Dialogue and Workshop on the Challenges of Emerging Infections, Laboratory Safety, Global Health Security and Responsible Conduct in the Use of Gene Editing in Viral Infectious Disease Research* was the latest in a series designed to share information on research relevant to combating emerging diseases of concern to the U.S. and China, promote common understanding of high biological containment laboratory safety, security and responsible research risk management, and build relationships among members of the U.S. and Chinese research communities who conduct infectious disease research. The special focus on research that uses gene-editing technology gave the opportunity for participants to discuss ways to create norms and reduce risks when using these tools for research.

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Several themes emerged during the workshop. The first was a focus on the importance of pursuing an integrated, One Health-based approach to understanding the causes and addressing the effects of animal and human diseases. Some of the issues raised included the short-term intended and unintended effects of recent disease control strategies, as well as a need for collaboration amongst experts and across regions on medical, ecological and ecosystems science to ensure human and animal health.

The second was a growing appreciation for the widespread use of gene-editing tools, particularly those based on the CRISPR-Cas9 system, that are being integrated into many new research efforts. New gene editing techniques to cut, edit, activate and repress genes with greater accuracy and at much lower costs go well beyond longstanding methods of selective breeding of hosts and pathogens and allow researchers to quickly explore changes not readily achieved in the past. Although the benefits to society are potentially enormous, there is the potential for malevolent application or for the technology to contribute to potentially (?) dangerous



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"bioerror," that is, for researchers to inadvertently create a modified organism that has an unintended disastrous impact. Many of the technical talks including those on vaccine development, vector engineering, host engineering, and synthetic biology included details on the researchers' use of gene editing tools and techniques. During the presentations the importance of gene editing to facilitate genetic modifications were highlighted but participants also discussed how they assessed risks and untended consequence of using the tools as well as best practices to ensure the appropriate and safe use of this technology in the future. See box on Gene Editing below.

A third theme was the importance of establishing and maintaining strong institutional cultures and norms to support the safe and responsible conduct of science. The sessions included several discussions on possible collaborations between Chinese and American scientists and institutions to ensure the responsible conduct of scientific research, ways to reduce research risk, and ways to ensure that gene editing techniques are used safely and ethically across the life science enterprise in the future.[1]

Throughout the workshop and during tours of the HVRI high containment security and training centers, experts also shared information regarding the safe and secure operation of high containment biological laboratories. Topics discussed included combating emerging infectious diseases in livestock, safely doing research on insect vectors in high biological containment, inactivation/decontamination procedures for African swine fever virus, improving the public health response to disease outbreaks, training and vetting high containment laboratory workers, and the safe and sustainable operation of high containment laboratories more broadly.

NAS has helped to shape the international dialogue on the risks and the multitude of challenges associated with planning for, building, sustaining, and safely and securely operating high containment laboratories. Before the meeting the directors of the Galveston National Laboratory (of the University of Texas Medical Branch) and the Wuhan Institute of Virology (of the Chinese Academy of Sciences) jointly published an editorial in *Science* that calls for the creation of a global network to bring together the leaders of institutes that operate laboratories at the highest biological safety level, BSL-4, to ensure the safe, secure and sustainable operation of these facilities.[2] The final session of the meeting included a discussion of the idea led by the authors of th editorial. During the discussion the director of China's Centers for Disease Control and the leadership of the Chinese institutes that operate China's three BSL-4 labs indicated that they support the idea and will work with NAS to develop and create a network. After the workshop

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[1] Although the meeting agenda was not designed to focus on human gene editing, the research project led by He Jiankui, formally an associate professor in the Department of Biology of the Southern University of Science and Technology in Shenzhen, China that lead to the birth of two children with edited DNA was universally condemned by the meeting participants.

[2] "One solution is to establish an [BSL-4] network that enables the sharing of best practices, collaboration, transparency, and exchange of specimens and technology." The authors noted that their partnership "benefited from meetings jointly sponsored by the U.S. National Academy of Sciences and the Chinese National Academy of Sciences…" Network for Safe and Secure Labs. James W. Le Duc and Zhiming Yuan, Science. 19 Oct 2018. Vol. 362, Issue 6412, pp. 267 http://science.sciencemag.org/content/362/6412/267.



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the group started planning (and raising funds to support) an international meeting of BSL-4 laboratory leaders to take place later in 2019 or in 2020 at a location to be determined. The next bilateral dialogue between the groups will likely take place in Kunming, China at the Kunming National Primate Research Center, another Chinese institute that operates a BSL-4 laboratory, towards the end of 2019.

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**BOX 1**

**GENE EDITING**

Commented [MOU1]: Assume the Spin Box and extra discussion of Steve's work is to satisfy the 'other half' of the mission of the meeting.

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Gene editing involves altering the DNA of a cell or organism. Modifications can include deletions, insertions, or other changes to the genetic code, generally carried out to change characteristics of a gene's expression. A particular edit might aim to "knock out" the gene and render it non-functional. Alternatively, precise changes to the nucleic acid sequence may be made to affect properties of the protein that results from the gene's transcription and translation. Edits are carried out for a number of other purposes, as well.

Researchers have been making alterations to nucleic acid sequences for many years, but tools developed since approximately 2012 – particularly the CRISPR-Cas9 editing system – have significantly increased the flexibility, efficiency, accuracy, speed, and ease (including lower cost) of making these changes. CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) and Cas9 (CRISPR-associated protein 9) were discovered in bacteria, where they form part of an immune mechanism for responding to infection. The basic system has since been adapted and repurposed as a key research tool and the use of gene editing is now widespread in research laboratories (Doudna and Charpentier 2014). A designed guide RNA sequence aligns with and binds to a target DNA sequence in the genome. The Cas9 protein then makes a double strand DNA cut. This cut can be repaired in various ways, including end joining or filling in with a donor DNA template to lead to sequence alterations.

Approximately a third of the presentations during the workshop referred to CRISPR and Cas9-based gene editing tools in some fashion. The key areas discussed during the workshop included:

- **Vaccine development efforts**. Use of gene editing to generate an attenuated version of a double-stranded DNA virus (Gladue)
- **Vector engineering**. Use in generating a mosquito that lacks an immune system associated enzyme (Han)
- **Host engineering**. Introduction of a human receptor domain into the mouse to create a model for viral infection (Baric); Creation of a pig designed to be resistant to a viral disease of interest (Ouyang)
- **Synthetic biology**. Design of gene circuits in bacteria to produce or respond to signaling molecules (Lou); Changes to eukaryotic genome organization to create a single chromosome yeast (X. Xue).



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- **Ethics**. Considerations for responsible use and governance of gene manipulations in synthetic biology (W. Zhang) and in human genome editing applications (Y. Xue).

Professor Steven Higgs also briefly discussed a special use of CRISPR-Cas9 in the creation of a gene drive. Gene drives "are systems of biased inheritance in which the ability of a genetic element to pass from a parent to its offspring through sexual reproduction is enhanced" (NASEM 2016). The design of these systems includes a mechanism in which an edited gene can be copied to its homologous chromosome and will continue to spread as the organism reproduces (Esvelt et al. 2014). The advent of CRISPR-Cas9 genome editing enabled scientists to create a gene drive and the field has continued to progress rapidly, with gene drive modified mosquitos reported by late 2015 (Gantz et al., 2015). Scientists are exploring genetically modified and/or even gene drive modified insect vectors as one strategy for control of devastating insect-borne diseases. But if used in this way, modified insects ultimately would be released from the laboratory into an ecosystem. Special considerations arise from use of modified species in open field trials and potential deployment. Researchers will need to understand not only how a modification affects the target population, but also how it affects the broader ecological community. A number of reports have discussed scientific, ethical and social considerations, particularly around gene drives (for example, NASEM 2016), while funders and scientists are developing norms to guide responsible use (Emerson et al. 2017). Dr. Higgs highlighted these special considerations in his presentation on the use of insect vectors for infectious disease research.

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**Sessions and Presentations**

**Keynote addresses**

- Oversight for Research on Viral Pathogens with Pandemic Potential – the Perspective from China
- Emerging Swine Coronaviruses and their Interspecies Transmission
- Vaccination in Poultry Eliminated Human Infections with H7N9 Virus in China

**Influenza**

- Collaborating on Influenza Research from the U.S. perspective
- Human Infection with Zoonotic Influenza
- Interactions between Influenza Viral RNP Complex Proteins and Host Cellular Factors

**African swine fever**

- Current Situation and Control Strategy of African Swine Fever in China
- Characterization of African Swine Fever Virus Isolated from China
- ASFV Vaccine Status: Rapid Production of Recombinant ASFV for Live Attenuated Vaccines
- Genome-edited Pigs Protected From Classical Swine Fever Virus

**Understanding and engineering viral pathogens with pandemic potential**

- Advances in Understanding and Altering Pathogen Properties
- Cutting Edging New Biotechnology: Strategies and Tools for Engineering

**Bioethics and responsible planning for pathogen research**

- Biosafety and Bioethics in Medical Research
- Bioethical Considerations for the Conception and Design of pathogen Research and Approaches for Assessing Potential Risks and Benefits of Research



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- Biosafety and Bioethics Considerations in Dual-Use Risks Management - China's Efforts and Future Works

**Vector and host engineering: safety and security**

- Biochemical Pathway of a Mosquito Innate Immunity, Melanization
- Best Practices in Safety and Security for Working with Arthropods that Serve as Pathogen Vectors, Including Responsible Research with Gene Drive Modified Vectors
- Vector Competence of Emerging Mosquito Borne Virus in China
- A Gut Commensal Bacterium Promotes Arbovirus Infection in Mosquitoes

**Acute Flaccid Myelitis**

- Review of what is currently known about the cause of Acute Flaccid Myelitis

**Best practices in laboratory safety and security for pathogen research**

- Institutional Review and Oversight for Research on Viral Pathogens with Pandemic Potential - a U.S. Perspective
- The Role of Leaders in Laboratory Safety: Culture and governance to steer toward behavior changes
- Overview of Laboratory Biosafety Management System in China

**One Health in China**

- Understanding how a One Health Approach is Shaping Research to Understand and Address Viral Pathogens in China

**Chinese biosafety, biosecurity, and bioethics**

- Development and Application of Laboratory Biosafety Equipment in China
- Kunming National Primate Research Center of High-Level Biosafety

**Chinese perspectives on emerging infectious diseases**

- DDX19, A novel Viral RNA Sensor, Regulates IFN Signaling upon Viral Infection
- Risks of MERS-cluster Coronaviruses in China
- Rational Design of Master Seed Virus to Improve the Safety and Effectiveness of Foot and Mouth Disease Vaccine
- Development of Inactivated EV71 Vaccine (Human Diploid Cell) and Pathogenic Study of EV71 Infection in Rhesus Macaques
- Zika Virus Neurotropism: the Bad and the Good

**Special session (gene editing and synthetic biology)**

- Large Gene Cluster and Regulatory Manipulation for Activating the Cryptic Natural Product Clusters in Streotomvces
- Creating a Functional Single-chromosome Yeast

**China-U.S. Collaboration: Opportunities to Prevent Future Pandemics**



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**American Participants**

**Ralph Baric**, Professor
Department of Epidemiology, University of North Carolina, Chapel Hill

**David Franz**, Professor
USAMRIID (retired)

**Douglas Gladue**, Scientist
U.S. Department of Agriculture

**Diane Griffin**, Professor
Department of Molecular Microbiology and Immunology of Johns Hopkins Bloomberg School of Public Health

**Stephen Higgs,** Professor
Department of Diagnostic Medicine/Pathobiology, College of Veterinary Medicine, Kansas State University

**Joseph Kanabrocki**, Professor
Microbiology in the Biological Sciences Division of the University of Chicago

**James Le Duc**, Professor
Galveston National Laboratory, Department of Microbiology and Immunology, University of Texas Medical Branch, Galveston

**David Relman**, Professor
Department of Microbiology and Immunology, Stanford University School of Medicine

**Linda Saif**, Professor
Department of Veterinary Preventive Medicine, Food Animal Health Research Program, Ohio Agricultural Research and Development Center, The Ohio State University

**Pei-Yong Shi**, Professor
Department of Biochemistry and Molecular Biology, University of Texas Medical Branch

**David Swayne**, Center Director
Agricultural Research Service, United States Department of Agriculture

**Katherine Bowman**, Senior Program Officer
National Academy of Sciences

**Benjamin Rusek**, Senior Program Officer
National Academy of Sciences

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**Chinese Participants**

**George F. Gao**, Academician,
Director-General Chinese Center for Disease Control and Prevention

**Zhigao Bu**, Professor
Harbin Veterinary Research Institute, CAAS

**Wen Dang**, Doctor
Lanzhou Veterinary Research Institute, CAAS

**Wuxiang Guan**, Professor Wuhan Institute of Virology, CAS

**Yunzhang Hu**, Professor
Institute of Medical Biology, Chinese Academy of Medical Sciences

**Mifang Liang**, Professor
National Institute for Viral Disease Control and Prevention, China CDC

**Chunbo Lou**, Professor Institute of Microbiology, CAS

**Hongsheng Ouyang**, Professor
Jilin University



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**Chengfeng Qin**, Professor
Institute of Microbiology and Epidemiology, Academy of Military Medical Sciences

**Hualan Chen**, Academician
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**Gong Cheng**, Professor
Tsinghua University

**Rui Gong**, Professor
Wuhan Institute of Virology, CAS

**Qian Han**, Professor
Hainan University

**Chengjun Li**, Professor
Harbin Veterinary Research Institute, CAAS

**Longding Liu**, Professor
Institute of Medical Biology, Chinese Academy of Medical Sciences

**Jiahai Lu**, Professor
Sun Yat-sen University

**Jiancheng Qi**, Professor
National Biological Protection Engineering Center

**Zhengli Shi**, Professor
Wuhan Institute of Virology, CAS

**Dayan Wang**, Professor
Institute of Pathogen Biology, Chinese Academy of Medical Sciences & Peking Union Medical College

**Jianwei Wang**, Professor
National Institute for Viral Disease Control and Prevention, China CDC

**Changjiang Weng**, Professor
Harbin Veterinary Research Institute, CAAS

**Kongming Wu**, Vice President
Chinese Academy of Agricultural Sciences

**Xiaodong Wu**, Professor
China Animal Health and Epidemiology Center

**Xaoli Xue**, Professor
Laboratory of Synthetic Biology, Shanghai Institute of Plant Physiology, CAS

**Yang Xue**, Associate Professor
Tianjin University

**Ruifu Yang**, Professor
Institute of Microbiology and Epidemiology, Academy of Military Medical Sciences

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**Zhiming Yuan**, Professor
Wuhan Institute of Virology, CAS

**Peijun Zhai**, Professor
China National Accreditation Service for Conformity Assessment (CNAS)

**Pingping Zhang**, Doctor
Institute of Microbiology and Epidemiology
Academy of Military Medical Sciences

**Tietao Zhang**, Professor
International bureau, CAAS

**Weiwen Zhang**, Professor
Tianjin University

**Chihong Zhao**, Professor
National Institute for Viral Disease Control and Prevention, China CDC

**Tongyan Zhao**, Professor
Institute of Microbiology and Epidemiology, Academy of Military Medical Sciences

**Yong Zhao**, Doctor
Institute of Microbiology and Epidemiology, Academy of Military Medical Sciences

**Haixue Zheng**, Professor
Lanzhou Veterinary Research Institute, CAAS



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**Emerging technologies to investigate coronavirus emergence – an interview with Vineet Menachery**

Recent decades have seen the emergence of several zoonotic coronaviruses (CoV), from the first outbreak of SARS to the ongoing cases of MERS in the Middle East. However, with major advances in fields such as genomics and structural biology, can more be uncovered about coronaviruses and their emergence?

In this interview we speak to Vineet Menachery about his recent talk at the Microbiology Society's Annual Conference (8–11 April, Belfast, UK) suggesting that CoV emergence is more complicated than receptor binding alone.

**First, could you introduce yourself and give a brief summary your career to date?**

I started as a PhD student at Washington University in Saint Louis (MO, USA). I was in the immunology program, but always interested in virology. I did my PhD with David Leib (Geisel School of Medicine at Dartmouth, NH, USA), who was a herpes virologist, and then from David's lab, I went to Ralph Baric's lab at the University of North Carolina (NC, USA) in 2010 to study coronaviruses.

So, I had been interested in type one interferon and interferon-stimulated genes and that is what I worked on initially when I was at Ralph's lab. Then the opportunity came up to work with bat CoVs, which was obviously a great opportunity. My lab also works on the host side looking at aging in the context of SARS and MERS infections and we have funding from the NIH to work on that, in addition to our research on bat viruses.

**Could you outline the research you're presenting here?**

So, in my talk I talked about a longitudinal study that had been done by Zhengli Shi's group at the Wuhan Institute [1]on Chinese horseshoe bats, which led to a big shift in our thinking about these bat CoVs because it revealed they are much closer to the epidemic SARS strains than previously thought. Normally, we don't evaluate anything beyond being reactive to the next emergent strain, but building on this study using our infectious clone system we had the opportunity to ask more questions – and that's how this project started.

The study that I showed was using a chimeric approach – taking the spike protein of bat CoVs and putting them into the backbone of a virus we knew was functional. Coronaviruses are really large, so with generating the viruses there are any number of other things that could go wrong, such as incompatibilities, that might not allow that virus replicate even though the spike might be viable – by putting just the spike in a known backbone we could gain some insight into that. We chose a mouse-adapted strain for the backbone because then we could take the chimeric virus into animals, and

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what surprised us was that the SARS-like CoV spikes, WIV1 and SHC014, which were isolated from bats, caused robust disease.

Our results indicated that the SHC014 spike in a wild-type backbone could use the human SARS receptor ACE2 – this is a known interaction and we thought there was no way this could bind. What we discovered is that it appears to bind but in a slightly different groove, and this groove actually mimics the binding angle of a spike protein from a mouse-adapted strain, which could explain why the SHC014 chimeric virus causes disease in mice.

Then we wanted to take it further, so we generated the full-length bat virus and showed that it could replicate. In addition, we evaluated SARS vaccines and therapeutics and assessed whether they were be effective – For example, we investigated combinations of monoclonal antibodies against SARS and whether these would neutralize WIV1 and SHC014. This highlighted holes in our therapeutic options, possibly a result of the shifted receptor binding domain. However, we only tested a selection, and there may be antibodies that bind the entire group so it would be ideal to have a neutralization profile that would allow us to treat the full spectrum of CoVs [2,3].

**If CoV emergence is more complicated than receptor binding alone, what other factors do you think are involved?**

I think in reality things are never simple. The bat strains that I have talked about that are SARS-like and they cause some disease, such as SHC014 spike causing disease in mice, but when you compare the mouse-adapted spike and the SHC spike, the viruses aren't that different. And we think that if we make these chimeric viruses or at least chimeric spikes, we can actually figure out what is driving the difference in pathogenesis and that might have implications for infection, pathogenesis, transmission – emergence could be driven by whatever we find. I don't know if that is true yet or not, my expectation is that these viruses are not that different, but hopefully it is not just receptor binding mediating emergence, and we think we probably have evidence for that.

**How has emerging technology in metagenomics and structural biology impacted your research?**

Metagenomics has been huge – there is more and more sequence and this is only going to get bigger with new tools like MinION. If we had that in 2002–2003, I imagine you would have found SARS in a heartbeat! Sequence capacities have increased quite a bit and the nice thing about having all these sequences is that we have done a lot of work previously on guessing what mutations might be important, but now we can actually populate this with viruses that exist in nature.

The other thing that has really helped has been that about 3–4 years ago the structural biologists really began working on the coronavirus spike. Andrew Ward (Scripps Research Institute, CA, USA), David Veesler (University of Washington, WA, USA), George Gao (Chinese Center for Disease Control and Prevention (China CDC), Beijing, China) and Jason McLellan (University of Texas, TX, USA) groups all have made structures based on the trimer forms of the spike, whether it be HKU1 or SARS, MERS.

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These are really important because now we also have this breadth of sequences, we can now overlay them on the structure of the proteins and identify conserved regions and differences. In addition, these proteins don't self-trimerize, which was challenging for research with psuedotype viruses, so a trimer form of the spike is an important step forward.

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Finally, new technology pioneered by Ralph Baric's lab has allowed the chimeric approach we use in our infectious clone system and this allows us to test viruses in addition to assessing various therapeutics and vaccines.

**How can your work be translated into a framework for surveillance of zoonotic strains?**

People have asked me; do you think you can identify the new strain? I think that we might get lucky, but I think it is unlikely we will predict that this is the next strain. Although viruses like SHC014 are definitely threats, I don't think there is any evidence that they will emerge. What we should focus on is what we know is different between bat CoVs and epidemics strain and whether we can use that to inform what actually has to change for zoonotic CoVs to become human pathogens.

**What do you consider to be the greatest challenges hindering preparedness for zoonotic CoVs?**

The regulatory burden and the difficulty in working with CoVs in the United States. For example, MERS and SARS are BSL 3 agents, so you have to have the facilities and I think that's entirely reasonable, however, if you look at SARS research after the CDC (GA, USA) put SARS in the select agent category, many labs are forced to make a choice. You either do what my lab does and carry out almost all BSL 3 work because that is the only way to justify the cost of the facilities needed – the 'in for a penny in for a pound' approach! But on the other hand, these hurdles can force people to pick and choose coronaviruses that are much easier to work with, like the mouse CoV MHV, which is a great model system. For example, if I was really interested in nitty-gritty things about coronavirus, I would probably do an MHV study, but if you are interested in pathogenesis or emergence SARS is the best model and this brings regulatory burden and special facilities.

**Any other comments you'd like to add?**

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Another barrier in the field is; how comfortable are you making a virus that could be highly virulent? There is the potential for gain-of-function experiments that you may not have considered if you are studying. Scientists have a public health obligation to make sure that they are not making things that could be risky and if it is potentially risky, to mitigate those risks. For all of these viruses that we have talked about, we have had several discussion with our institutional biosafety committee and clear plans on anything we see as potential gain-of-function or relative gain. You need to consider all potential implications in your work, not just the immediate, and I think that's an important aspect. Many CoVs aren't yet human pathogens, so let's make sure it stays that way.

References


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**Deleted:** for example, the mouse MHV virus, but if you discover something that allows it to bind human cells – now you may have created a human pathogen. I really rely on my institutional biosafety committee to be an objective, reasonable party that's well-versed in these matters but my concern would be that not every board has the same expertise. This could be good or bad – they might let things that they shouldn't go through or they may be cracking down on the things that should be done.¶

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References


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3. Menachery VD, Yount Jr. BL, Debbink K *et al*. [ HYPERLINK "https://www.nature.com/articles/nm.3985" ]. *Nat Med.* 21, 1508-1513 (2015).


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**Sent:** Wednesday, May 8, 2019 10:18 PM
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**Cc:** chenxw@wh.iov.cn;Shi, Pei yong;Xie, Xuping
**Subject:** abot the publication fee (CUSTOMER NO.3798492)
**Attachments:** SD00058933350003.PDF

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MR Xinwen Chen
Wuhan Institute of Virology,
Chinese Academy of Sciences,
44 Xiao Hong Shan, Wuhan,
430071, P.R.China

With My sincere greetings~Thanks!

Yours: Yuan

--
周缘
中国科学院武汉病毒研究所
肝炎病毒分子生物学学科组，武汉市武昌区小洪山中区44号



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Email: zhouyuan@wh.iov.cn
Phone: 86-27-87197575

-----原始邮件-----
发件人:"Shi, Pei yong" <peshi@UTMB.EDU>
发送时间:2019-05-09 09:52:26 (星期四)
**收件人:** "Veasey, Erica" <Erica.C.Veasey@informa.com>, "Xie, Xuping" <xuxie@UTMB.EDU>, "chenxw@wh.iov.cn" <chenxw@wh.iov.cn>
**抄送:** "zhouyuan@wh.iov.cn" <zhouyuan@wh.iov.cn>
**主题:** RE: 3798492 University of Texas Medical Branch at Galveston tel; +1 409-772-1011

Dear Erica,

Dr. Xinwen Chen from Wuhan Institute of Virology will pay for the publication fee. Dr. Chen's assistant Yuan (cc'd) will contact you for the payment arrangement shortly.

Best regards,

Pei-Yong Shi



FOIA Confidential Treatment Requested by Univ of Texas System UTSystem_010326

**From:** Veasey, Erica [mailto:Erica.C.Veasey@informa.com]
**Sent:** Wednesday, May 08, 2019 4:22 AM
**To:** Xie, Xuping <xuxie@UTMB.EDU>; chenxw@wh.iov.cn; Shi, Pei yong <peshi@UTMB.EDU>
**Subject:** 3798492 University of Texas Medical Branch at Galveston tel; +1 409-772-1011
**Importance:** High

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Dear Professors,

Please find attached your APC invoice.

This invoice was sent to you on **03/25/2019** and payment is now required. We would appreciate an update as to when the money could be expected in.

If you are having problems paying this invoice, or if you are currently working with co-authors, funders or your institution to arrange payment please do update us so that we are able to provide assistance or update your invoice where necessary.

Alternatively, if you have paid this invoice, please could you provide us with proof of payment so we can check our records?

If you wish to pay by credit card, please use our online payment link: https://secure.taylorandfrancis.com/payment#/?product=tandf.

We look forward to hearing from you with an update.

Kind Regards

**Erica Veasey**



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erica.c.veasey@informa.com

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**If you are unable to pay by the dates specified or you wish to pay by debit or credit card please kindly contact me on the number above.**





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Information Classification: General



FOIA Confidential Treatment Requested by Univ of Texas System UTSystem_010329

**From:** Marcus Gallo <mgallo@gvn.org>
**Sent:** Wednesday, May 15, 2019 2:43 PM
**To:** LeDuc, James W.
**Cc:** Gallo, Robert;Christian Brechot
**Subject:** Re: Biosecurity

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James,
Thank you very much for sharing the information.

You have begun with a good model, US/China ASs formed good contacts,
but are correct in noticing the need for an outline, vision and
even secondary plans. This could really lead somewhere.

Marcus

Marcus Gallo, MS
Research Analyst & Center Outreach Coordinator
Global Virus Network
725 W. Lombard St. Rm S416
Baltimore, MD 21201
(410) 706-5609
mgallo@gvn.org

---

**From:** LeDuc, James W. <jwleduc@UTMB.EDU>
**Sent:** Wednesday, May 15, 2019 3:33:52 PM
**To:** Marcus Gallo
**Cc:** Gallo, Robert; Christian Brechot
**Subject:** FW: Biosecurity

Marcus, below is additional information on the biosecurity initiative we discussed. Some potential objectives highlighted in my note below to Mark Perkins.

Jim

**From:** LeDuc, James W.
**Sent:** Friday, January 04, 2019 9:56 AM
**To:** (b)(6) Gallo, Robert <rgallo@ihv.umaryland.edu>
**Subject:** Biosecurity

Dear John and Bob,

I hope you both had a pleasant holiday break and that the new year is off to a great start.



FOIA Confidential Treatment Requested by Univ of Texas System UTSystem_007979

I wanted to follow up on our earlier discussions on the biosecurity activities and the GVN. Following our meeting in Annecy, I had a chance to talk with several leaders at WHO including Mike Ryan, the assistant director general in charge of infectious diseases. I briefed them on the work we have been doing bilaterally between the US National Academy of Science and the Chinese Academy of Science and shared with him the attached editorial published in the attached October 2018 in *Science*. As a result of those meetings, I was introduced (virtually) to Mark Perkins, and American working at WHO in lab issues. My email exchange with Mark is below and I spoke with him at length yesterday about the need for a global network of BSL4 labs. I think we are in general agreement and it is clear to me that we need to engage with WHO going forward, but we can't leave it to them to make it happen.

With that background, I leave early Sunday morning for another joint meeting with the US and China National Academies of Science, this time to be held in Harbin at their BSL4 agriculture research center. The agenda of our meeting is attached just FYI. During that meeting, I have scheduled time to meet with a good friend, George Gao, the director of the China CDC to discuss next steps in developing the global network of BSL4 labs. We will continue the bilateral collaborations between the US and China National Academies of Science, but I'm concerned about the move to global engagement in building this network. **I wonder if we could have a brief call to discuss before I leave for Harbin?** I'm free all day today and tomorrow (Saturday) through about mid-day. My direct office line is 409-266-6516 and cell is (b)(6)

Thanks, Jim

James W. Le Duc, Ph.D.
Director
Galveston National Laboratory
University of Texas Medical Branch
Galveston, TX 77555-0610
(t) 409-266-6500
(f) 409-266-6810
(m) (b)(6)

**From:** LeDuc, James W.
**Sent:** Tuesday, December 18, 2018 10:28 AM
**To:** 'PERKINS, Mark' <perkinsm@who.int>
**Cc:** BRIAND, Sylvie <briands@who.int>; KOJIMA, Kazunobu <kojimak@who.int>; Mike Ryan (ryanm@who.int) <ryanm@who.int>; Yuan Zhiming <yzm@wh.iov.cn>
**Subject:** RE: GLAD-HP

Hi Mark,

Thanks for the follow up. As I mentioned to Sylvie, we have been working closely with colleagues in China where new BSL4 laboratories have been constructed and are now becoming operational. These collaborations have been facilitated by the US National Academy of Sciences and the Chinese Academy of Sciences. At a meeting hosted by WHO in Lyon last December and organized by Kaz Kojima, I was struck by the global proliferation of BSL4 biocontainment laboratories (not to mention the BSL3s), and the attached editorial followed. The editorial has gained some traction and hopefully over the coming year there will be an opportunity to establish a more active global network of directors of these facilities. I discussed this in general terms with Mike Ryan following last week's GOARN meeting and agreed to keep him abreast of activities and to coordinate with WHO as closely as possible.

My thoughts going forward with a network would be to:



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- Establish a functional network so that points of contact are known for each facility and general information about capabilities and proposed scope of activities. Ideally this would involve a face-to-face meeting.
- Facilitate information exchange among network members on both operational issues and scientific opportunities.
- Offer a credible source of information from experienced leaders for those considering the construction a new BSL4 facility
- Provide hands on training and capacity building in:
    - Biosafety—working in biocontainment
    - Biosecurity—ensuring the security of pathogens under their control
    - Building operations and maintenance—this is especially challenging and expensive
- Identify areas of possible scientific collaborations.
- Facilitate personnel exchanges.
- Create a forum where international rules, regulations, norms and standards can be discussed and allow credible feed-back to policy development.

I look forward to learning how these thoughts might align with your plans. I'm happy to discuss further, although it may be easier by email given the Holidays.

Best wishes,

Jim

James W. Le Duc, Ph.D.
Director
Galveston National Laboratory
University of Texas Medical Branch
Galveston, TX 77555-0610
(t) 409-266-6500
(f) 409-266-6810
(m) (b)(6)

**From:** PERKINS, Mark <perkinsm@who.int>
**Sent:** Monday, December 17, 2018 10:48 PM
**To:** LeDuc, James W. <jwleduc@UTMB.EDU>
**Cc:** BRIAND, Sylvie <briands@who.int>
**Subject:** GLAD-HP

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Jim,

Sylvie Briand, under who's direction I work at WHO, suggested that I contact you. I recently joined WHO to lead lab networks in the WHO Health Emergencies cluster in the IHM department, and specifically to establish an umbrella network for high-threat pathogens that would try to answer some of the unresolved sample and data sharing issues, and more broadly to prepare WHO to offer leadership and a forum for engagement of the scientific community in a rapidly evolving technical and social landscape. If you have a half hour in the coming two weeks, it would be great to connect by phone to get your thoughts and share ideas.



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Mark Perkins

Dr Mark D. Perkins
Leader, Laboratory Networks
Infectious Hazards Management, WHO Health Emergencies Programme
Email: perkinsm@who.int
Desk phone: +4122-7911649 Cell: [redacted]



FOIA Confidential Treatment Requested by Univ of Texas System UTSystem_007982

**From:** Marcus Gallo <mgallo@gvn.org>
**Sent:** Tuesday, May 21, 2019 9:53 AM
**To:** LeDuc, James W.
**Cc:** Gallo, Robert;Christian Brechot
**Subject:** Re: Biosecurity

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Dear James,

Is it acceptable for me to share with Dr. Christopher Kratochvil your email and attachments related to biosecurity?

Can we still plan a future Call regarding the same material: global engagement of building bi-lateral collaborations of BSL-4 facility with USA/China-NASs, and actors Mark Perkins and George Gao, upon your return?

Thank you,
Marcus

Marcus Gallo, MS
Research Analyst & Center Outreach Coordinator
Global Virus Network
725 W. Lombard St. Rm S416
Baltimore, MD 21201
(410) 706-5609
mgallo@gvn.org

---

**From:** LeDuc, James W. <jwleduc@UTMB.EDU>
**Sent:** Wednesday, May 15, 2019 3:33 PM
**To:** Marcus Gallo
**Cc:** Gallo, Robert; Christian Brechot
**Subject:** FW: Biosecurity

Marcus, below is additional information on the biosecurity initiative we discussed. Some potential objectives highlighted in my note below to Mark Perkins.

Jim

**From:** LeDuc, James W.
**Sent:** Friday, January 04, 2019 9:56 AM



FOIA Confidential Treatment Requested by Univ of Texas System 

**To:** (b)(6) Gallo, Robert <rgallo@ihv.umaryland.edu>
**Subject:** Biosecurity

Dear John and Bob,

I hope you both had a pleasant holiday break and that the new year is off to a great start.

I wanted to follow up on our earlier discussions on the biosecurity activities and the GVN. Following our meeting in Annecy, I had a chance to talk with several leaders at WHO including Mike Ryan, the assistant director general in charge of infectious diseases. I briefed them on the work we have been doing bilaterally between the US National Academy of Science and the Chinese Academy of Science and shared with him the attached editorial published in the attached October 2018 in *Science*. As a result of those meetings, I was introduced (virtually) to Mark Perkins, and American working at WHO in lab issues. My email exchange with Mark is below and I spoke with him at length yesterday about the need for a global network of BSL4 labs. I think we are in general agreement and it is clear to me that we need to engage with WHO going forward, but we can't leave it to them to make it happen.

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Thanks, Jim

James W. Le Duc, Ph.D.
Director
Galveston National Laboratory
University of Texas Medical Branch
Galveston, TX 77555-0610
(t) 409-266-6500
(f) 409-266-6810
(m) (b)(6)

**From:** LeDuc, James W.
**Sent:** Tuesday, December 18, 2018 10:28 AM
**To:** 'PERKINS, Mark' <perkinsm@who.int>
**Cc:** BRIAND, Sylvie <briands@who.int>; KOJIMA, Kazunobu <kojimak@who.int>; Mike Ryan (ryanm@who.int) <ryanm@who.int>; Yuan Zhiming <yzm@wh.iov.cn>
**Subject:** RE: GLAD-HP

Hi Mark,



FOIA Confidential Treatment Requested by Univ of Texas System 

Thanks for the follow up. As I mentioned to Sylvie, we have been working closely with colleagues in China where new BSL4 laboratories have been constructed and are now becoming operational. These collaborations have been facilitated by the US National Academy of Sciences and the Chinese Academy of Sciences. At a meeting hosted by WHO in Lyon last December and organized by Kaz Kojima, I was struck by the global proliferation of BSL4 biocontainment laboratories (not to mention the BSL3s), and the attached editorial followed. The editorial has gained some traction and hopefully over the coming year there will be an opportunity to establish a more active global network of directors of these facilities. I discussed this in general terms with Mike Ryan following last week's GOARN meeting and agreed to keep him abreast of activities and to coordinate with WHO as closely as possible.

My thoughts going forward with a network would be to:

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- Facilitate information exchange among network members on both operational issues and scientific opportunities.
- Offer a credible source of information from experienced leaders for those considering the construction a new BSL4 facility
- Provide hands on training and capacity building in:
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    - Biosecurity—ensuring the security of pathogens under their control
    - Building operations and maintenance—this is especially challenging and expensive
- Identify areas of possible scientific collaborations.
- Facilitate personnel exchanges.
- Create a forum where international rules, regulations, norms and standards can be discussed and allow credible feed-back to policy development.

I look forward to learning how these thoughts might align with your plans. I'm happy to discuss further, although it may be easier by email given the Holidays.

Best wishes,

Jim

James W. Le Duc, Ph.D.
Director
Galveston National Laboratory
University of Texas Medical Branch
Galveston, TX 77555-0610
(t) 409-266-6500
(f) 409-266-6810
(m) (b)(6)

**From:** PERKINS, Mark <perkinsm@who.int>
**Sent:** Monday, December 17, 2018 10:48 PM
**To:** LeDuc, James W. <jwleduc@UTMB.EDU>



FOIA Confidential Treatment Requested by Univ of Texas System UTSystem_007452

**Cc:** BRIAND, Sylvie <briands@who.int>
**Subject:** GLAD-HP

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Jim,

Sylvie Briand, under who's direction I work at WHO, suggested that I contact you. I recently joined WHO to lead lab networks in the WHO Health Emergencies cluster in the IHM department, and specifically to establish an umbrella network for high-threat pathogens that would try to answer some of the unresolved sample and data sharing issues, and more broadly to prepare WHO to offer leadership and a forum for engagement of the scientific community in a rapidly evolving technical and social landscape. If you have a half hour in the coming two weeks, it would be great to connect by phone to get your thoughts and share ideas.

Mark Perkins

Dr Mark D. Perkins
Leader, Laboratory Networks
Infectious Hazards Management, WHO Health Emergencies Programme
Email: perkinsm@who.int
Desk phone: +4122-7911649 Cell: (b)(6)



FOIA Confidential Treatment Requested by Univ of Texas System UTSystem_007453

**Full Name:** Hong Tang (tangh@wh.iov.cn)
**Last Name:** Tang
**First Name:** Hong
**Job Title:** Deputy Dir General
**Company:** Wuhan Institute of Virology

**Business:** +86 (27) 87197727

**E-mail:** tangh@wh.iov.cn
**E-mail Display As:** Hong Tang (tangh@wh.iov.cn)



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**Full Name:** chenxw
**Last Name:** Chen
**First Name:** Xinwen
**Company:** Wuhan Institute of Virology

**E-mail:** chenxw@wh.iov.cn
**E-mail Display As:** chenxw



FOIA Confidential Treatment Requested by Univ of Texas System UTSystem_059322

**From:** David Franz <davidrfranz@gmail.com>
**Sent:** Thursday, June 20, 2019 6:32 PM
**To:** Shi, Pei yong
**Cc:** Rusek, Benjamin;LeDuc, James W.;Lowenthal, Micah
**Subject:** Re: Kungmin update

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Sounds good Pei-Yong. Thanks! Dave

Sent from my iPhone

On Jun 20, 2019, at 6:41 PM, Shi, Pei yong <peshi@utmb.edu> wrote:

> Hi Ben, Dave, and Jim,
>
> I talked with Longding and Zhiming. Here is the summary.
>
> Longding was reluctant to go back to Kunming's leadership team for another trial. This is because their new Party Secretary (who makes the decision) is newly appointed from Beijing. So, I was not able to get the name from Longding whom the NAS letter should address to. We will discuss this further at our TC tomorrow.
>
> Zhiming was very supportive to host the next meeting under CAS. The location could be Wuhan or any other cities in China. If we decide to work with Zhiming for the next meeting, NAS needs to send a letter to President of CAS (Dr. Chunli Bai), as we did for the Wuhan meeting in 2016.
>
> I have not talked with George Gao yet and am planning to do so after our TC tomorrow.
>
> Best, Pei-Yong
>
> **From:** Rusek, Benjamin [mailto:BRusek@nas.edu]
> **Sent:** Thursday, June 20, 2019 4:38 PM
> **To:** LeDuc, James W. <jwleduc@UTMB.EDU>; 'David Franz' <davidrfranz@gmail.com>; Shi, Pei yong <peshi@UTMB.EDU>
> **Cc:** Lowenthal, Micah <mlowenth@nas.edu>
> **Subject:** RE: Kungmin update
> **Importance:** High
>
> **WARNING:** This email originated from outside of UTMB's email system. Do not click links or open attachments unless you recognize the sender and know the content is safe.
>
> Greetings,



FOIA Confidential Treatment Requested by Univ of Texas System UTSystem_071627

I have attached a draft letter for Diane and Peggy for your review, Pei-yong, we will need to know who to send it to…

Re the call tomorrow, please call 1-888-537-7715 and enter participant code 12223052# at 1:00 PM ET.

Kind regards,

Ben

Benjamin Rusek
The U.S. National Academy of Sciences
1-202-334-3975

**From:** LeDuc, James W. <jwleduc@UTMB.EDU>
**Sent:** Tuesday, June 18, 2019 2:45 PM
**To:** Rusek, Benjamin <BRusek@nas.edu>; 'David Franz' <davidrfranz@gmail.com>; Shi, Pei yong <peshi@UTMB.EDU>
**Subject:** RE: Kungmin update

OK—added to my calendar. Please share call in information when available.

Thanks, Jim

**From:** Rusek, Benjamin <BRusek@nas.edu>
**Sent:** Tuesday, June 18, 2019 12:52 PM
**To:** 'David Franz' <davidrfranz@gmail.com>; Shi, Pei yong <peshi@UTMB.EDU>
**Cc:** LeDuc, James W. <jwleduc@UTMB.EDU>
**Subject:** RE: Kungmin update

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Pei-yong and I spoke on the phone, he is on vacation until Thursday. Lets plan to talk on Friday. How about 1 PM ET?

In the interim, I will write a draft letter and contact Peggy and Diane.

And yes I will leave the funding support offer out of the letter.

Ben

Benjamin Rusek
The U.S. National Academy of Sciences
1-202-334-3975

**From:** David Franz <davidrfranz@gmail.com>
**Sent:** Tuesday, June 18, 2019 1:49 PM
**To:** Shi, Pei yong <peshi@utmb.edu>
**Cc:** LeDuc, James W. <jwleduc@utmb.edu>; Rusek, Benjamin <BRusek@nas.edu>
**Subject:** Re: Kungmin update



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If I don't make it on the call, the only thing I'm not comfortable with is offering them funding support. Whatever you guys decide is fine. I like Pei-Yong talking to Zhiming or George. Home tomorrow afternoon, dave

Sent from my iPhone

On Jun 18, 2019, at 4:18 PM, Shi, Pei yong <peshi@utmb.edu> wrote:

> Hi all,
>
> Longding's institution is very conservative. This has limited their growth. I'll be back to office on Thursday. We could have a TC on Thursday or Friday.
>
> One suggestion is to talk to Zhiming and George for options. We could decide after our TC.
>
> Best, Pei-Yong
>
> ---
>
> **From:** LeDuc, James W.
> **Sent:** Tuesday, June 18, 2019 9:11 AM
> **To:** Shi, Pei yong; Rusek, Benjamin
> **Cc:** Dave Franz
> **Subject:** RE: Kungmin update
>
> Thanks for the update Pei-Yong. This is very sad and frustrating, but certainly understandable from their perspective. We should probably have a brief teleconference to discuss options. I'm available all day.
>
> Thanks, Jim
>
> **From:** Shi, Pei yong <peshi@UTMB.EDU>
> **Sent:** Tuesday, June 18, 2019 7:04 AM
> **To:** Rusek, Benjamin <BRusek@nas.edu>
> **Cc:** Dave Franz <davidrfranz@gmail.com>; LeDuc, James W. <jwleduc@UTMB.EDU>
> **Subject:** Kungmin update
>
> Hi Ben, Dave, and Jim,
>
> I just received a negative response from Longding that, due to the current China-US relationship, their institute has decided not to host our next meeting. I personally understand why they are changing their position. Let's talk about it through a TC or email.
>
> Sorry about the negative development. Since Longding wrote to me in Chinese through WeChat. I am not forwarding it to you. However, I have asked him to email us about it in English.
>
> Best regards,
> Pei-Yong
>
> On Jun 17, 2019 5:27 PM, "Rusek, Benjamin" <BRusek@nas.edu> wrote:



FOIA Confidential Treatment Requested by Univ of Texas System UTSystem_071629

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Hi Jim,

Here are my two cents. You might want to send the next draft to Katie Bowman (Bowman, Katherine KBowman@nas.edu)

Kind regards,

Ben

Benjamin Rusek

The U.S. National Academy of Sciences

1-202-334-3975

**From:** LeDuc, James W. <jwleduc@UTMB.EDU>
**Sent:** Monday, June 17, 2019 6:10 PM
**To:** Dave Franz <davidrfranz@gmail.com>
**Cc:** Rusek, Benjamin <BRusek@nas.edu>; Shi, Pei yong <peshi@UTMB.EDU>
**Subject:** Re: Draft editorial

Thanks very much Dave. Received just fine and I will make the adjustments.

Jim

Sent from my iPhone

On Jun 17, 2019, at 2:48 PM, Dave Franz <davidrfranz@gmail.com> wrote:

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FOIA Confidential Treatment Requested by Univ of Texas System UTSystem_071630

Trying t send from Chromebook, with which I'm still a little unsure. Let me know if a marked up version comes through. d

James W. Le Duc, Ph.D.

Director

Galveston National Laboratory

University of Texas Medical Branch

Galveston, TX 77555-0610

(t) 409-266-6500

(f) 409-266-6810

(m) (b)(6)

<Biosecurity in the age to synthetic biology June 16 2019.docx>



FOIA Confidential Treatment Requested by Univ of Texas System UTSystem_071631

**From:** David Franz <davidrfranz@gmail.com>
**Sent:** Thursday, June 20, 2019 6:32 PM
**To:** Shi, Pei yong
**Cc:** Rusek, Benjamin;LeDuc, James W.;Lowenthal, Micah
**Subject:** Re: Kungmin update

**WARNING:** This email originated from outside of UTMB's email system. Do not click links or open attachments unless you recognize the sender and know the content is safe.

Sounds good Pei-Yong. Thanks! Dave

Sent from my iPhone

On Jun 20, 2019, at 6:41 PM, Shi, Pei yong <peshi@utmb.edu> wrote:

> Hi Ben, Dave, and Jim,
>
> I talked with Longding and Zhiming. Here is the summary.
>
> Longding was reluctant to go back to Kunming's leadership team for another trial. This is because their new Party Secretary (who makes the decision) is newly appointed from Beijing. So, I was not able to get the name from Longding whom the NAS letter should address to. We will discuss this further at our TC tomorrow.
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> I have not talked with George Gao yet and am planning to do so after our TC tomorrow.
>
> Best, Pei-Yong
>
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> **Sent:** Thursday, June 20, 2019 4:38 PM
> **To:** LeDuc, James W. <jwleduc@UTMB.EDU>; 'David Franz' <davidrfranz@gmail.com>; Shi, Pei yong <peshi@UTMB.EDU>
> **Cc:** Lowenthal, Micah <mlowenth@nas.edu>
> **Subject:** RE: Kungmin update
> **Importance:** High
>
> **WARNING:** This email originated from outside of UTMB's email system. Do not click links or open attachments unless you recognize the sender and know the content is safe.
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> Greetings,



FOIA Confidential Treatment Requested by Univ of Texas System 

I have attached a draft letter for Diane and Peggy for your review, Pei-yong, we will need to know who to send it to…

Re the call tomorrow, please call 1-888-537-7715 and enter participant code 12223052# at 1:00 PM ET.

Kind regards,

Ben

Benjamin Rusek
The U.S. National Academy of Sciences
1-202-334-3975

**From:** LeDuc, James W. <jwleduc@UTMB.EDU>
**Sent:** Tuesday, June 18, 2019 2:45 PM
**To:** Rusek, Benjamin <BRusek@nas.edu>; 'David Franz' <davidrfranz@gmail.com>; Shi, Pei yong <peshi@UTMB.EDU>
**Subject:** RE: Kungmin update

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And yes I will leave the funding support offer out of the letter.

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Sent from my iPhone

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> On Jun 17, 2019 5:27 PM, "Rusek, Benjamin" <BRusek@nas.edu> wrote:



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Hi Jim,

Here are my two cents. You might want to send the next draft to Katie Bowman (Bowman, Katherine KBowman@nas.edu)

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FOIA Confidential Treatment Requested by Univ of Texas System UTSystem_007698

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James W. Le Duc, Ph.D.

Director

Galveston National Laboratory

University of Texas Medical Branch

Galveston, TX 77555-0610

(t) 409-266-6500

(f) 409-266-6810

(m) (b)(6)

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FOIA Confidential Treatment Requested by Univ of Texas System UTSystem_007699

**From:** LeDuc, James W.
**Sent:** Thursday, June 20, 2019 9:12 PM
**To:** David Franz
**Cc:** Shi, Pei yong;Rusek, Benjamin;Lowenthal, Micah
**Subject:** Re: Kungmin update

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Sent from my iPhone

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Sounds good Pei-Yong. Thanks! Dave

Sent from my iPhone

On Jun 20, 2019, at 6:41 PM, Shi, Pei yong <peshi@utmb.edu> wrote:

Hi Ben, Dave, and Jim,

I talked with Longding and Zhiming. Here is the summary.

Longding was reluctant to go back to Kunming's leadership team for another trial. This is because their new Party Secretary (who makes the decision) is newly appointed from Beijing. So, I was not able to get the name from Longding whom the NAS letter should address to. We will discuss this further at our TC tomorrow.

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I have not talked with George Gao yet and am planning to do so after our TC tomorrow.

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**To:** LeDuc, James W. <jwleduc@UTMB.EDU>; 'David Franz' <davidrfranz@gmail.com>; Shi, Pei yong <peshi@UTMB.EDU>
**Cc:** Lowenthal, Micah <mlowenth@nas.edu>
**Subject:** RE: Kungmin update
**Importance:** High



FOIA Confidential Treatment Requested by Univ of Texas System 

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FOIA Confidential Treatment Requested by Univ of Texas System UTSystem_008756

Sorry about the negative development. Since Longding wrote to me in Chinese through WeChat. I am not forwarding it to you. However, I have asked him to email us about it in English.

Best regards,
Pei-Yong

On Jun 17, 2019 5:27 PM, "Rusek, Benjamin" <BRusek@nas.edu> wrote:

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Hi Jim,

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1-202-334-3975

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**To:** Dave Franz <davidrfranz@gmail.com>
**Cc:** Rusek, Benjamin <BRusek@nas.edu>; Shi, Pei yong <peshi@UTMB.EDU>
**Subject:** Re: Draft editorial

Thanks very much Dave. Received just fine and I will make the adjustments.



FOIA Confidential Treatment Requested by Univ of Texas System UTSystem_008757

Jim

Sent from my iPhone

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Director

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(m) (b)(6)

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FOIA Confidential Treatment Requested by Univ of Texas System UTSystem_008758

**From:** Rusek, Benjamin <BRusek@nas.edu>
**Sent:** Friday, June 21, 2019 2:49 PM
**To:** LeDuc, James W.;David Franz
**Cc:** Shi, Pei yong;Lowenthal, Micah
**Subject:** RE: Kungmin update

**Follow Up Flag:** FollowUp
**Flag Status:** Flagged

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Good to talk to you all today.

FYI Xuetao Cao, M.D., Ph.D., president, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, China was elected a foreign member of NAM in 2017 https://nam.edu/national-academy-of-medicine-elects-80-new-members/

**Qi Han Li**, is listed as the Director, Kunming Institute of Medical Biology, Kunming, People's Republic of China (from 2013)

There was a Virology con in Kunming in 2017 http://www.imbcams.ac.cn/Item/17194.aspx. Lists De Yin Guo, George Fu Gao, and **Qi Han Li** as the meeting Chairmen.

Kind regards,

Ben

Benjamin Rusek
The U.S. National Academy of Sciences
1-202-334-3975

**From:** LeDuc, James W. <jwleduc@UTMB.EDU>
**Sent:** Thursday, June 20, 2019 10:12 PM
**To:** David Franz <davidrfranz@gmail.com>
**Cc:** Shi, Pei yong <peshi@UTMB.EDU>; Rusek, Benjamin <BRusek@nas.edu>; Lowenthal, Micah <mlowenth@nas.edu>
**Subject:** Re: Kungmin update

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Sounds good Pei-Yong. Thanks! Dave



FOIA Confidential Treatment Requested by Univ of Texas System UTSystem_071621

Sent from my iPhone

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> Ben
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> Benjamin Rusek
> The U.S. National Academy of Sciences
> 1-202-334-3975



FOIA Confidential Treatment Requested by Univ of Texas System UTSystem_071622

**From:** LeDuc, James W. <jwleduc@UTMB.EDU>
**Sent:** Tuesday, June 18, 2019 2:45 PM
**To:** Rusek, Benjamin <BRusek@nas.edu>; 'David Franz' <davidrfranz@gmail.com>; Shi, Pei yong <peshi@UTMB.EDU>
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FOIA Confidential Treatment Requested by Univ of Texas System UTSystem_071623

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Best, Pei-Yong

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FOIA Confidential Treatment Requested by Univ of Texas System UTSystem_071624

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Benjamin Rusek

The U.S. National Academy of Sciences

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FOIA Confidential Treatment Requested by Univ of Texas System UTSystem_071625

James W. Le Duc, Ph.D.

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Galveston National Laboratory

University of Texas Medical Branch

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(m) (b)(6)

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FOIA Confidential Treatment Requested by Univ of Texas System UTSystem_071626

# Dengue NS2A protein orchestrates virus assembly

Xuping Xie[1]*, Jing Zou[1], Xianwen Zhang[1,2,3], Yiyang Zhou[1], Andrew Routh[1], Vsevolod L. Popov[4], Xinwen Chen[2,3,5], Qingyin Wang[6], Hong-Ping Dong[6], Pei-Yong Shi[1,7,8,9,10]*

[1]Department of Biochemistry & Molecular Biology, University of Texas Medical Branch, Galveston, Texas, USA; [2]State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Sciences, Wuhan, China. [3]University of Chinese Academy of Sciences, Beijing, China. [4]Department of Pathology, University of Texas Medical Branch, Galveston, Texas, USA; [5]Guangzhou Institute of Biomedicine and Health, Chinese Academy of Sciences, Guangzhou, China. [6]Clearbridge Health, Singapore; [7]Institute for Translational Science, University of Texas Medical Branch, Galveston, Texas, USA; [8]Sealy Institute for Vaccine Sciences, University of Texas Medical Branch, Galveston, Texas, USA; [9]Sealy Center for Structural Biology & Molecular Biophysics, University of Texas Medical Branch, Galveston, Texas, USA; [10]Institute for Human Infections & Immunity, University of Texas Medical Branch, Galveston, Texas, USA;

*Correspondence: [ HYPERLINK "mailto:xuxie@UTMB.edu" ] (X.X.), [ HYPERLINK "mailto:peshi@UTMB.edu" ] (P.-Y.S.)

Running title: The role of NS2A protein in dengue virus assembly



FOIA Confidential Treatment Requested by Univ of Texas System UTSystem_140040

## SUMMARY

Dengue virus (DENV) is the most important mosquito-borne viral pathogen in humans. DENV assembly requires cleavages of viral C-prM-E polyprotein into three structural proteins (capsid, pre-membrane, and envelope), packaging of viral RNA with C protein into nucleocapsid, and budding of prM/E proteins into virion. No study has addressed how these assembly events are orchestrated. Here we show that DENV-2 NS2A protein recruits viral RNA, structural proteins, and protease to virion assembly site, and coordinates nucleocapsid and virus formation. The last 285 nucleotides of viral 3'UTR serve as a "packaging signal" that binds to a cytosolic loop of NS2A protein. This interaction allows NS2A to recruit nascent RNA from the replication complex to virion assembly site. Mutations in NS2A that weaken the 3'UTR/NS2A interaction abolish virus assembly. In addition, NS2A interacts with prM, E, and NS3 to recruit C-prM-E polyprotein and NS2B/NS3 protease to the virion assembly site, leading to coordinated C-prM-E cleavage. Mature C protein assembles onto genomic RNA to form nucleocapsid, followed by prM/E envelopment and virion formation. An NS2A mutation that alters the prM/NS2A interaction reduces C-prM cleavage and abolishes virion assembly; second-site mutations in C protein that restore the C-prM cleavage rescue virion assembly. The results support a DENV assembly model that NS2A recruits viral RNA to virion assembly site where coordinated cleavage of C-prM-E ensues infections virus morphogenesis.




FOIA Confidential Treatment Requested by Univ of Texas System UTSystem_140041

## INTRODUCTION

The genus *Flavivirus* of the family *Flaviviridae* comprises over 70 viruses, many of which are important human pathogens, including dengue (DENV), yellow fever (YFV), Zika (ZIKV), West Nile (WNV), Japanese encephalitis (JEV), St. Louis encephalitis (SLE), and tick-borne encephalitis (TBEV) viruses[ ADDIN EN.CITE <EndNote><Cite><Author>Pierson</Author><Year>2013</Year><RecNum>3</RecNum><DisplayText>(Pierson and Diamond, 2013)</DisplayText><record><rec-number>3</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1536704974">3</key><key app="ENWeb" db-id="">0</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Pierson, T.C.</author><author>Diamond, M. S.</author></authors></contributors><titles><secondary-title>Flaviviruses p. 747-794. In D.M. Knipe and P.M. Howley (ed), Fields virology, 6th., vol. 1.</secondary-title></titles><periodical><full-title>Flaviviruses p. 747-794. In D.M. Knipe and P.M. Howley (ed), Fields virology, 6th., vol. 1.</full-title></periodical><dates><year>2013</year></dates><urls></urls></record></Cite></EndNote>]. The four serotypes of DENV alone cause about 390 million human infections each year, leading to dengue fever, dengue hemorrhagic fever, and dengue shock syndrome [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. Because of the huge social economic burden, DENV was included in the top 10 global health threats by the World Health Organization (WHO). The development of vaccine and therapeutics for DENV has been proven challenging [ ADDIN EN.CITE <EndNote><Cite><Author>Lim</Author><Year>2013</Year><RecNum>58</RecNum><DisplayText>(Lim et al., 2013)</DisplayText><record><rec-number>58</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv"



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timestamp="1560793214">58</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Lim, S. P.</author><author>Wang, Q. Y.</author><author>Noble, C. G.</author><author>Chen, Y. L.</author><author>Dong, H.</author><author>Zou, B.</author><author>Yokokawa, F.</author><author>Nilar, S.</author><author>Smith, P.</author><author>Beer, D.</author><author>Lescar, J.</author><author>Shi, P. Y.</author></authors></contributors><auth-address>Novartis Institute for Tropical Diseases, 10 Biopolis Road, 05-01 Chromos, Singapore 138670, Singapore.</auth-address><titles><title>Ten years of dengue drug discovery: Progress and prospects</title><secondary-title>Antiviral Res</secondary-title><alt-title>Antiviral research</alt-title></titles><periodical><full-title>Antiviral Res</full-title></periodical><pages>500-19</pages><volume>100</volume><number>2</number><edition>2013/10/01</edition><dates><year>2013</year><pub-dates><date>Nov</date></pub-dates></dates><isbn>1872-9096 (Electronic)0166-3542 (Linking)</isbn><accession-num>24076358</accession-num><urls><related-urls><url>http://www.ncbi.nlm.nih.gov/pubmed/24076358</url></related-urls></urls><electronic-resource-num>10.1016/j.antiviral.2013.09.013</electronic-resource-num><language>eng</language></record></Cite></EndNote>]. The first FDA approved dengue vaccine has a low efficacy of 30-61% [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. Due to the risk of vaccine-induced severe disease in children who are seronegative at the time of vaccination, WHO recommended only use of the vaccine in areas where the disease is highly prevalent [ ADDIN EN.CITE <EndNote><Cite><Author>Whitehead</Author><Year>2016</Year><RecNum>63</RecNum><DisplayText>(Whitehead, 2016)</DisplayText><record><rec-number>63</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793215">63</key></foreign-keys><ref-type name="Journal



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Article">17</ref-type><contributors><authors><author>Whitehead, S. S.</author></authors></contributors><auth-address>a Laboratory of Infectious Diseases , NIAID, National Institutes of Health , Bethesda , MD , USA.</auth-address><titles><title>Development of TV003/TV005, a single dose, highly immunogenic live attenuated dengue vaccine; what makes this vaccine different from the Sanofi-Pasteur CYD vaccine?</title><secondary-title>Expert Rev Vaccines</secondary-title></titles><periodical><full-title>Expert Rev Vaccines</full-title></periodical><pages>509-17</pages><volume>15</volume><number>4</number><keywords><keyword>Dengue</keyword><keyword>live attenuated dengue vaccine</keyword><keyword>vaccine attenuation strategies</keyword><keyword>vaccine efficacy</keyword><keyword>vaccine immunogenicity</keyword><keyword>vaccine infectivity</keyword></keywords><dates><year>2016</year></dates><isbn>1744-8395 (Electronic)1476-0584 (Linking)</isbn><accession-num>26559731</accession-num><urls><related-urls><url>http://www.ncbi.nlm.nih.gov/pubmed/26559731</url></related-urls></urls><custom2>PMC4956407</custom2><electronic-resource-num>10.1586/14760584.2016.1115727</electronic-resource-num></record></Cite></EndNote>]. No clinically approved antivirals are available for flavivirus treatment, despite major effort in the past two decades[ ADDIN EN.CITE <EndNote><Cite><Author>Zou</Author><Year>2019</Year><RecNum>64</RecNum><DisplayText>(Zou and Shi, 2019)</DisplayText><record><rec-number>64</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793215">64</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Zou, J.</author><author>Shi, P. Y.</author></authors></contributors><auth-address>Department of Biochemistry and



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Molecular Biology, University of Texas Medical Branch, Galveston, TX, USA.Department of Biochemistry and Molecular Biology, University of Texas Medical Branch, Galveston, TX, USA; Department of Phamarcology and Toxicology, University of Texas Medical Branch, Galveston, TX, USA; Sealy Center for Structural Biology and Molecular Biophysics, University of Texas Medical Branch, Galveston, TX, USA; Sealy Institute for Vaccine Sciences, University of Texas Medical Branch, Galveston, TX, USA. Electronic address: peshi@utmb.edu.</auth-address><titles><title>Strategies for Zika drug discovery</title><secondary-title>Curr Opin Virol</secondary-title></titles><periodical><full-title>Curr Opin Virol</full-title></periodical><pages>19-26</pages><volume>35</volume><dates><year>2019</year><pub-dates><date>Mar 7</date></pub-dates></dates><isbn>1879-6265 (Electronic)1879-6257 (Linking)</isbn><accession-num>30852345</accession-num><urls><related-urls><url>http://www.ncbi.nlm.nih.gov/pubmed/30852345</url></related-urls></urls><electronic-resource-num>10.1016/j.coviro.2019.01.005</electronic-resource-num></record></Cite></EndNote>]. It is therefore critical to study the molecular mechanism of flavivirus replication, which may lead to new approaches for countermeasure development.

Flavivirus has a plus-sense, single-stranded RNA of about 11,000-nucleotide in length. The genomic RNA contains a 5' untranslated region (UTR), a single open-reading-frame (ORF), and a 3' UTR. The ORF translates a polyprotein that is processed by viral and host proteases into three structure proteins [capsid (C), pre-membrane (prM), and envelope (E)] and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5). The structural proteins, together with genomic RNA, assemble into virions. The nonstructural proteins participate in viral RNA replication, virion assembly, and evasion of host immune response. Upon viral translation, a minus-sense RNA is synthesized from the



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genomic RNA template, forming a double-stranded RNA. The minus-strand RNA then serves as a template to make many copies of plus-sense RNA that are packaged to form progeny virions [ ADDIN EN.CITE <EndNote><Cite><Author>Pierson</Author><Year>2013</Year><RecNum>3</RecNum><DisplayText>(Pierson and Diamond, 2013)</DisplayText><record><rec-number>3</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1536704974">3</key><key app="ENWeb" db-id="">0</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Pierson, T.C.</author><author>Diamond, M. S.</author></authors></contributors><titles><secondary-title>Flaviviruses p. 747-794. In D.M. Knipe and P.M. Howley (ed), Fields virology, 6th., vol. 1.</secondary-title></titles><periodical><full-title>Flaviviruses p. 747-794. In D.M. Knipe and P.M. Howley (ed), Fields virology, 6th., vol. 1.</full-title></periodical><dates><year>2013</year></dates><urls></urls></record></Cite></EndNote>]. Like other RNA viruses, the assembly of flavivirus is coupled with RNA replication [ ADDIN EN.CITE ADDIN EN.CITE.DATA ].

Flavivirus infection induces invaginations of the endoplasmic reticulum (ER) to form clusters of double-membrane vesicles (Ve) enclosed in the vesicle packets (VPs). Electron tomography has revealed the three-dimensional architectures of Ve and VPs formed during DENV, WNV, TBEV, and ZIKV infections [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. Ve houses viral replication complex (including the double-stranded viral RNA, viral nonstructural proteins, and cellular proteins) where RNA synthesis occurs [ ADDIN EN.CITE <EndNote><Cite><Author>Junjhon</Author><Year>2014</Year><RecNum>71</RecNum><DisplayText>(Junjhon et al., 2014)</DisplayText><record><rec-number>71</rec-



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number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793216">71</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Junjhon, J.</author><author>Pennington, J. G.</author><author>Edwards, T. J.</author><author>Perera, R.</author><author>Lanman, J.</author><author>Kuhn, R. J.</author></authors></contributors><auth-address>Markey Center for Structural Biology, Department of Biological Sciences, Purdue University, West Lafayette, Indiana, USA.</auth-address><titles><title>Ultrastructural characterization and three-dimensional architecture of replication sites in dengue virus-infected mosquito cells</title><secondary-title>J Virol</secondary-title></titles><periodical><full-title>J Virol</full-title></periodical><pages>4687-97</pages><volume>88</volume><number>9</number><keywords><keyword>Animals</keyword><keyword>Cell Line</keyword><keyword>Culicidae/*virology</keyword><keyword>Dengue Virus/*physiology/*ultrastructure</keyword><keyword>Electron Microscope Tomography</keyword><keyword>Imaging, Three-Dimensional</keyword><keyword>Intracellular Membranes/*ultrastructure/*virology</keyword><keyword>*Virus Replication</keyword></keywords><dates><year>2014</year><pub-dates><date>May</date></pub-dates></dates><isbn>1098-5514 (Electronic)0022-538X (Linking)</isbn><accession-num>24522909</accession-num><urls><related-urls><url>http://www.ncbi.nlm.nih.gov/pubmed/24522909</url></related-urls></urls><custom2>PMC3993787</custom2><electronic-resource-num>10.1128/JVI.00118-14</electronic-resource-num></record></Cite></EndNote>]. Newly synthesized plus-sense RNA is released from the Ve pore to the rough ER for new rounds of RNA translation and replication. Alternatively, the nascent RNA is assembled



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into virions through encapsidation (with C protein) and envelopment (with prM and E proteins) through budding into the ER lumen. The virion assembly occurs at the ER sites juxtaposed to the VPs. An initial immature virion contains 60 icosahedrally arranged prM/E heterotrimer. As the immature virion transits through the Golgi network, the acidic environment triggers the cleavage of prM by cellular furin protease, resulting in an infectious mature virion [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. Cryo-EM of flavivirus virions exhibit two structural features: (i) The nucleocapsid core lacks organized structures and (ii) there are no observable connections between nucleocapsid and the prM/E membrane shell, suggesting that the C protein may bind to genomic RNA in a random manner without direct interaction with prM/E proteins [ ADDIN EN.CITE ADDIN EN.CITE.DATA ].

The molecular mechanism of flavivirus assembly remains largely obscure. Besides structural proteins, several nonstructural proteins, such as NS2A, NS2B, and NS3, have been shown to regulate flavivirus assembly. Different amino acids of NS2A protein were reported to play distinct roles in flavivirus RNA replication and virion assembly [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. The cleavage between C and prM is tightly modulated by NS2B/NS3 protease and cellular signalase during YFV and Murray Valley encephalitis virus assembly [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. Mutations in the NS3 helicase domain alone affect YFV assembly [ ADDIN EN.CITE <EndNote><Cite><Author>Patkar</Author><Year>2008</Year><RecNum>90</RecNum><DisplayText>(Patkar and Kuhn, 2008a)</DisplayText><record><rec-number>90</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793218">90</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Patkar, C. G.</author><author>Kuhn, R. J.</author></authors></contributors><auth-



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address>Purdue Univ, Dept Biol Sci, W Lafayette, IN 47907 USA</auth-address><titles><title>Yellow fever virus NS3 plays an essential role in virus assembly independent of its known enzymatic functions</title><secondary-title>Journal of Virology</secondary-title><alt-title>J Virol</alt-title></titles><alt-periodical><full-title>J Virol</full-title></alt-periodical><pages>3342-3352</pages><volume>82</volume><number>7</number><keywords><keyword>tick-borne encephalitis</keyword><keyword>flavivirus kunjin ns3</keyword><keyword>west-nile-virus</keyword><keyword>dengue virus</keyword><keyword>nonstructural protein</keyword><keyword>rna replication</keyword><keyword>cdna-clones</keyword><keyword>particles</keyword><keyword>ns2a</keyword><keyword>organization</keyword></keywords><dates><year>2008</year><pub-dates><date>Apr</date></pub-dates></dates><isbn>0022-538X</isbn><accession-num>WOS:000254139800015</accession-num><urls><related-urls><url><Go to ISI>://WOS:000254139800015</url></related-urls></urls><electronic-resource-num>10.1128/Jvi.02447-07</electronic-resource-num><language>English</language></record></Cite></EndNote>]. Although expression of prM/E alone is sufficient to generate empty virus-like particles, it is not known how nucleocapsid is formed and subsequently enveloped with prM/E to form infectious virions. The goal of this study is to define the molecular mechanism that orchestrates three structural proteins and viral RNA to form nucleocapsid and infectious virus in a highly coordinated manner. Using genetic, biochemical, and imaging approaches, we demonstrate that DENV NS2A protein choreographs virion morphogenesis by (i) interacting with viral prM and E proteins, (ii) regulating the processing of C-prM polyprotein by viral protease, and (iii) recruiting genomic RNA through binding to the 3'UTR. These results support a model that DENV NS2A recruits nascent viral RNA from the replication complex (housed in Ve) to the virion assembly site (on the ER membrane juxtaposed to



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241 VPs), where an coordinated C-prM cleavage facilitates genomic RNA encapsidation with C
242 protein and budding with prM and E proteins into the ER lumen, resulting in progeny
243 virions.



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## RESULTS

**NS2A G11A mutation abolishes virion assembly**. Using DENV-2 as a model, we previously identified a panel of NS2A mutant viruses that were selectively defective in virion assembly without compromising other steps of an infection cycle [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. Here, we performed four experiments to demonstrate that NS2A mutation G11A of DENV-2 caused a defect in virion assembly. (i) A DENV-2 luciferase replicon was used to examine the effect of NS2A G11A on viral translation and RNA synthesis. Upon transfection into BHK-21 cells, the G11A replicon produced a luciferase profile comparable to the WT replicon (Fig. 1A), suggesting that the mutation does not significantly reduce viral translation and RNA replication. (ii) When full-length viral RNA of DENV-2 was transfected into BHK-21 cells, the NS2A G11A mutant failed to release extracellular viral RNA (Fig. 1B). No infectious virus was detected from the culture medium of G11A RNA-transfected cells (Fig. 1C). (iii) In a *trans* packaging system, WT luciferase replicon could be packaged into pseudo-infectious particles (PIPs) when structural C-prM-E proteins were expressed from Semliki Forest virus replicon (Fig. S1A). The WT reporter replicon PIPs infected Vero cells and generated robust luciferase signals (Fig. S1B). In contrast, no PIPs were produced with the NS2A G11A replicon. (iv) Transmission electron microscopy revealed substantial virus-induced single-membrane vesicles (within the lumen of the rough ER) or double-membrane vesicles (closely associated with cisternae of the rough ER) in both WT and G11A RNA-transfected cells at 48 h post-transfection (Fig. 1D). A lot of virus particles appeared in the WT RNA-transfected cells, but no virus particles were detected in the G11A RNA-transfected cells. The results demonstrate that NS2A G11A mutation specifically abolishes virion assembly.

**NS2A G11A mutation alters the expression and localization of viral structural proteins.** We compared the subcellular localization of viral proteins in BHK-21 cells that



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were transfected with WT or NS2A G11A genomic RNA. Surprisingly, the NS2A G11A mutation altered the intracellular distribution of three structural proteins. (i) C protein disseminated in both nucleus and cytoplasm in the WT RNA-transfected cells, as previously reported [ ADDIN EN.CITE <EndNote><Cite><Author>Wang</Author><Year>2004</Year><RecNum>91</RecNum><DisplayText>(Wang et al., 2004)</DisplayText><record><rec-number>91</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793218">91</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Wang, S. H.</author><author>Syu, W. J.</author><author>Hu, S. T.</author></authors></contributors><auth-address>Institute of Microbiology and Immunology, School of Life Science, National Yang-Ming University, Taipei, Taiwan, Republic of China.</auth-address><titles><title>Identification of the homotypic interaction domain of the core protein of dengue virus type 2</title><secondary-title>J Gen Virol</secondary-title><alt-title>The Journal of general virology</alt-title></titles><periodical><full-title>J Gen Virol</full-title><abbr-1>The Journal of general virology</abbr-1></periodical><alt-periodical><full-title>J Gen Virol</full-title><abbr-1>The Journal of general virology</abbr-1></alt-periodical><pages>2307-14</pages><volume>85</volume><number>Pt 8</number><keywords><keyword>Active Transport, Cell Nucleus</keyword><keyword>Dengue Virus/*chemistry/classification</keyword><keyword>Precipitin Tests</keyword><keyword>Viral Core Proteins/*chemistry</keyword></keywords><dates><year>2004</year><pub-dates><date>Aug</date></pub-dates></dates><isbn>0022-1317 (Print)0022-1317 (Linking)</isbn><accession-num>15269372</accession-num><urls><related-urls><url>http://www.ncbi.nlm.nih.gov/pubmed/15269372</url></related-



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urls></urls><electronic-resource-num>10.1099/vir.0.80067-0</electronic-resource-num></record></Cite></EndNote>]. However, in the G11A RNA-transfected cells, the C protein was mainly restricted to the cytoplasm with rare nucleus localization (Fig. 1E). (ii) prM protein was located at the ER region in the WT RNA-transfected cells, whereas the prM protein was undetectable in the G11A RNA-transfected cells (Fig. 1F). (iii) E protein spread broadly like an ER web in the WT RNA-transfected cells. In contrast, in the G11A RNA-transfected cells, the E protein formed larger speckles at restricted subcellular areas (Fig. 1G). Compared with the structural proteins, the effect of NS2A G11A on the subcellular expression of viral nonstructural proteins (including NS1, NS3, NS4A, NS4B, and NS5) and dsRNA was not apparent (Fig. 1E-G, *top panels* for NS3, and data not shown).

The distinct effect of NS2A G11A mutation on subcellular expression between the viral structural and nonstructural proteins were further supported by immunofluorescent co-staining experiments. NS2A G11A altered the co-staining profile of E and NS3 proteins (Fig. S2). In the NS2A G11A RNA-transfected cells, 100% of NS3-expressing cells were E-positive at 24 h post-transfection; however, at 48 and 72 h post-transfection, only 59% and 20% of the NS3-expressing cells were E-positive, respectively (Fig. S2). In the WT RNA-transfected cells, 100% of NS3-expressing cells were E-positive from 24 to 72 h post-transfection (Fig. S2). The results demonstrate that NS2A G11A mutation changes the expression and subcellular localization of viral structural proteins.

**NS2A G11A mutation impairs the cleavage of viral C-prM polyprotein.** To determine the molecular mechanism of the above observations, we examined the processing of viral proteins in the WT and NS2A G11A viral RNA-transfected cells at 48 h post-transfection by Western blot. As expected, the amounts of C, NS3, and NS5 proteins from the G11A RNA-transfected cells were 15-26% of those from the WT RNA-transfected



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cells (Fig. 1H-I), due to the lack of virion production or multiple rounds of infection for the G11A mutant. Notably, the amounts of prM and E proteins were further lower in the G11A RNA-transfected cells (only 1-4% of those in the WT RNA-transfected cells), suggesting an asymmetric degradation in cells. Moreover, the NS2A G11A mutation impaired the cleavage of C-prM to individual C and prM proteins: 90% of C-prM was cleaved to mature C protein in the WT RNA-transfected cells, whereas only 20% of C-prM was processed to C protein in the G11A RNA-transfected cells (Fig. 1H, *compare lanes 1 and 5 in the top panel*). The impaired C-prM cleavage could account for the altered subcellular distribution of viral structural proteins in the NS2A C11A RNA-transfected cells: Restricting the un-cleaved C-prM to the ER membrane, preventing C protein from translocating to the nucleus, and consequently blocking prM/E heterodimer formation and virion budding.

Next, we examined the role of ubiquitin-proteasome pathway in degrading viral proteins in the G11A RNA-transfected cells. Treatment with proteasome inhibitor MG132 increased the amounts of C, prM, E, and NS5 (but not NS3) by 1-3 folds in the NS2A G11A RNA-transfected cells (Fig. 1H, *compare lanes 5 and 6*). In contrast, in the WT RNA-transfected cells, MG132 slightly reduced the amounts of C, prM, and E to different extents (Fig. 1H, *compare lanes 1 and 2*) because MG132 was known to inhibit flavivirus replication [ ADDIN EN.CITE <EndNote><Cite><Author>Bhuvanakantham</Author><Year>2013</Year><RecNum>108</RecNum><DisplayText>(Bhuvanakantham and Ng, 2013)</DisplayText><record><rec-number>108</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793288">108</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Bhuvanakantham, R.</author><author>Ng, M. L.</author></authors></contributors><auth-



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address>Flavivirology Laboratory, Department of Microbiology, Yong Loo Lin School of Medicine, National University of Singapore, 5 Science Drive 2, Singapore, 117597.</auth-address><titles><title>West Nile virus and dengue virus capsid protein negates the antiviral activity of human Sec3 protein through the proteasome pathway</title><secondary-title>Cell Microbiol</secondary-title></titles><periodical><full-title>Cell Microbiol</full-title></periodical><pages>1688-706</pages><volume>15</volume><number>10</number><keywords><keyword>Antiviral Agents/*antagonists & inhibitors</keyword><keyword>Capsid Proteins/genetics/*metabolism</keyword><keyword>Cell Line</keyword><keyword>DNA Mutational Analysis</keyword><keyword>Dengue Virus/*physiology</keyword><keyword>*Host-Pathogen Interactions</keyword><keyword>Humans</keyword><keyword>Models, Biological</keyword><keyword>Proteasome Endopeptidase Complex/*metabolism</keyword><keyword>Protein Binding</keyword><keyword>Protein Interaction Mapping</keyword><keyword>Proteolysis</keyword><keyword>Vesicular Transport Proteins/*antagonists & inhibitors</keyword><keyword>West Nile virus/*physiology</keyword></keywords><dates><year>2013</year><pub-dates><date>Oct</date></pub-dates></dates><isbn>1462-5822 (Electronic)1462-5814 (Linking)</isbn><accession-num>23522008</accession-num><urls><related-urls><url>https://www.ncbi.nlm.nih.gov/pubmed/23522008</url></related-urls></urls><electronic-resource-num>10.1111/cmi.12143</electronic-resource-num></record></Cite></EndNote>]. Distinct responses to MG132 treatment among different viral proteins were probably due to their subcellular distributions and involvement in various biological microenvironments. Nevertheless, the results suggest that ubiquitin-proteasome pathway is responsible for viral protein degradation in the NS2A G11A RNA-transfected cells.



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**WT NS2A rescues virion assembly of NS2A G11A mutant through restoring C-prM cleavage.** We examined if *trans* supply of WT NS2A could restore virion assembly of NS2A G11A RNA and rescue C-prM protein processing (Fig.S3A). Indeed, after transfection into NS2A-EGFP BHK-21 cells that constitutively expressed WT NS2A protein, the NS2A G11A RNA produced infectious virions that could infect naïve BHK-21 cells, as indicated by E-positive cells (Fig. S3B); further passaging of culture supernatants to new BHK-21 cells did not yield any E-positive cells (Fig. S3C), suggesting that the *trans* packaged virions could only infect cells for a single-round. Remarkably, the *trans* supplied WT NS2A protein restored the C-prM cleavage in the G11A RNA-transfected cells to WT level, exhibiting >89% C protein processing (Fig. S3D, *top panel*). Two prM bands, representing glycosylated and un-glycosylated forms of the protein (see below), were detected in both NS2A G11A and WT RNA-transfected NS2A-EGFP cells (Fig. S3D, *second panel*). The relative abundances of C, prM, and NS1 proteins were similar in the NS2A G11A and WT RNA-transfected cells (Fig. S3D). Because of the restored C-prM cleavage, the nucleus localization of C protein and prM-positive cells were detected in the G11A RNA-transfected NS2A-EGFP cells (Fig. S3E). These results demonstrate that WT NS2A can rescue virion assembly of NS2A G11A mutant through restoring the C-prM cleavage.

Next, we examined the effect of NS2A on the glycosylation of prM by co-expressing NS2A and prM in HEK293T cells. As a control, when EGFP was co-expressed, only the glycosylated form of prM was detected (Fig. S3F); after PGNaseF treatment, the prM was completely deglycoslated and migrated as a glycosylation-knockout mutant N69Q prM (Fig. S3G). In contrast, when NS2A-EGFP were co-expressed, both glycosylated and un-glycosylated forms of prM were detected (Fig. S3F), indicating that NS2A suppresses the glycosylation of prM. Furthermore, the G11A mutation seemed to increase the



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suppression effect of NS2A on prM glycosylation (Fig. 3F). These results suggest that NS2A may interact with prM, and that the G11A mutation may modulate the prM/NS2A interaction.

**Capsid R99S mutation can rescue virion assembly of NS2A G11A RNA.** We selected revertants by continuously passaging the NS2A G11A RNA-transfected BHK-21 cells for five rounds (Fig. S4A). Culture medium from each passaging (P1-P5) were tested for emergence of infectious viruses using plaque assay. The P5 culture produced infectious viruses with titers of $>10^4$ PFU/ml and plaques smaller than the WT virus (Fig. S4B). In addition, almost all P5 cells were E protein-positive (Fig. S4C). Whole genome sequencing of the P5 viruses from three independent selections consistently showed a consensus C-to-A substitution at nucleotide position 391 of viral genome, resulting in an Arg-to-Ser change at amino acid position 99 of the C protein (C_R99S; Fig. 2A). The original NS2A G11A mutation was retained in all P5 viruses.

To validate the role of C_R99S mutation in rescuing virion assembly of NS2A G11A RNA, we engineered C_R99S into a DENV-2 infectious clone [ ADDIN EN.CITE <EndNote><Cite><Author>Xie</Author><Year>2013</Year><RecNum>85</RecNum><DisplayText>(Xie et al., 2013)</DisplayText><record><rec-number>85</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793217">85</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Xie, X.</author><author>Gayen, S.</author><author>Kang, C.</author><author>Yuan, Z.</author><author>Shi, P. Y.</author></authors></contributors><auth-address>State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Science, China.</auth-address><titles><title>Membrane topology and function of dengue virus NS2A protein</title><secondary-title>J Virol</secondary-title><alt-title>Journal of virology</alt-



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title></titles><periodical><full-title>J Virol</full-title></periodical><pages>4609-22</pages><volume>87</volume><number>8</number><edition>2013/02/15</edition><keywords><keyword>Animals</keyword><keyword>Cell Line</keyword><keyword>Cricetinae</keyword><keyword>Dengue Virus/*physiology</keyword><keyword>Endoplasmic Reticulum/*virology</keyword><keyword>Humans</keyword><keyword>Intracellular Membranes/*metabolism</keyword><keyword>Magnetic Resonance Spectroscopy</keyword><keyword>Models, Molecular</keyword><keyword>Protein Conformation</keyword><keyword>Viral Nonstructural Proteins/*chemistry/metabolism</keyword><keyword>*Virus Assembly</keyword></keywords><dates><year>2013</year><pub-dates><date>Apr</date></pub-dates></dates><isbn>1098-5514 (Electronic)0022-538X (Linking)</isbn><accession-num>23408612</accession-num><work-type>Research Support, Non-U.S. Gov't</work-type><urls><related-urls><url>http://www.ncbi.nlm.nih.gov/pubmed/23408612</url></related-urls></urls><custom2>3624351</custom2><electronic-resource-num>10.1128/JVI.02424-12</electronic-resource-num><language>eng</language></record></Cite></EndNote>] in the presence or absence of NS2A G11A. BHK-21 cells were electroporated with WT, NS2A G11A, C_R99S, or NS2A G11A+C_R99S viral RNA. The transfected cells were analyzed for virus production and C-prM cleavage. In the NS2A G11A+C_R99S RNA-transfected cells, the percentage of E-positive cells increased from 10% to 50% at 24 h and 96 h post-transfection, suggesting the spread of infectious virus (Fig. 2B); indeed, the transfected cells produced infectious viruses with plaques smaller than the WT virus (Fig. 2C); the viral titer increased over time, reaching $>10^5$ PFU/ml at 96-120 h post-transfection (Fig. 2D). The prM and NS1 proteins exhibited ER-like distributions in the NS2A G11A+C_R99S RNA-transfected cells, similar to that observed in the WT RNA-transfected



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cells (Fig. 2E). Viral C protein was detected in both cytoplasm and nucleus in the NS2A G11A+C_R99S RNA-transfected cells (Fig. 2F-G). Furthermore, 90.5% of the C-prM protein was cleaved to mature C and prM in the NS2A G11A+C_R99S RNA-transfected cells (Fig. 2G). As controls, NS2A G11A RNA showed 15.4% of C-prM cleavage and did not produce any infectious virus, whereas C_R99S RNA exhibited 99.9% of prM-C cleavage and produced a WT level of infectious virus (Fig. 2B-H). Taken together, the results demonstrate that C_R99S mutation efficiently restores C-prM cleavage and virion assembly of NS2A G11A RNA.

**Capsid R99S cannot rescue virion assembly of other NS2A mutants.** We tested if C_R99S could rescue other NS2A mutants that were previously shown to be defective in virion assembly, including E20A, E100A, and K188A [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. The C_R99S was engineered into those NS2A mutants, resulting in NS2A E20A+C_R99S, NS2A E100A+C_R99S, and NS2A K188A+C_R99S viral RNAs. After transfection into BHK-12 cells, none of the mutant RNAs produced increasing number of E-positive cells from 24 to 96 h (Fig. S5A). No infectious virus was detected in the culture medium from the mutant RNA-transfected cells (Fig. S5B). Viral C protein was detected in both nucleus and cytoplasm of all mutant RNA-transfected cells (Fig. S5C). In agreement with the C nucleus localization, the NS2A single mutant E20A and E100A cleaved 36% and 79% of C-prM, and the addition of C_R99S increased the C-prM cleavage to 72% and 96%, respectively. In contrast, NS2A K188A mutant cleaved 100% of C-prM in the presence or absence of C_R99S (Fig. 5D). The results demonstrate that C_R99S can only rescue the assembly defect of NS2A G11A possibly because different NS2A mutations abolish virion assembly through distinct mechanisms.

**Capsid R99S shifts the C-prM cleavage site by two amino acids.** The above results prompted us to investigate how C_R99S rescues the virion assembly of NS2A



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G11A. Fig. S6 depicts the topology of viral polyprotein C-prM-E-NS1-NS2A on the ER membrane. NS2A G11 resides at the first amphipathic helix on the ER lumen side [ ADDIN EN.CITE

<EndNote><Cite><Author>Xie</Author><Year>2013</Year><RecNum>85</RecNum><DisplayText>(Xie et al., 2013)</DisplayText><record><rec-number>85</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793217">85</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Xie, X.</author><author>Gayen, S.</author><author>Kang, C.</author><author>Yuan, Z.</author><author>Shi, P. Y.</author></authors></contributors><auth-address>State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Science, China.</auth-address><titles><title>Membrane topology and function of dengue virus NS2A protein</title><secondary-title>J Virol</secondary-title><alt-title>Journal of virology</alt-title></titles><periodical><full-title>J Virol</full-title></periodical><pages>4609-22</pages><volume>87</volume><number>8</number><edition>2013/02/15</edition><keywords><keyword>Animals</keyword><keyword>Cell Line</keyword><keyword>Cricetinae</keyword><keyword>Dengue Virus/*physiology</keyword><keyword>Endoplasmic Reticulum/*virology</keyword><keyword>Humans</keyword><keyword>Intracellular Membranes/*metabolism</keyword><keyword>Magnetic Resonance Spectroscopy</keyword><keyword>Models, Molecular</keyword><keyword>Protein Conformation</keyword><keyword>Viral Nonstructural Proteins/*chemistry/metabolism</keyword><keyword>*Virus Assembly</keyword></keywords><dates><year>2013</year><pub-dates><date>Apr</date></pub-dates></dates><isbn>1098-5514 (Electronic)0022-538X (Linking)</isbn><accession-num>23408612</accession-num><work-type>Research



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Support, Non-U.S. Gov't</work-type><urls><related-urls><url>http://www.ncbi.nlm.nih.gov/pubmed/23408612</url></related-urls></urls><custom2>3624351</custom2><electronic-resource-num>10.1128/JVI.02424-12</electronic-resource-num><language>eng</language></record></Cite></EndNote>], while C_R99 is the penultimate amino acid of the C protein on the cytosolic side (Fig. S6B). Since flavivirus NS2B/NS3 protease cleaves a substrate with two basic residues (Arg or Lys) at the P1 and P2 sites and a residue with short side chain (Gly, Ser, or Ala) at the P1' site [ ADDIN EN.CITE ADDIN EN.CITE.DATA ], sequence analysis suggests that the C_R99S substitution may shift the capsid C-terminal cleavage by two amino acids from position 100 to 98 (Fig. S6B). Specifically, viral NS2B/NS3 protease cleaves the WT capsid at NRRRR↓TA and the R99S capsid at NRR↓SRTA (↓ denoting cleavage site and residue R99S underlined). To test this hypothesis, we performed a biochemical protease assay using synthetic WT peptide (NILNRRRRTAGMII) and R99S peptide (NILNRRSRTAGMII). After incubating the peptides with recombinant DENV-2 NS2B/NS3 protease, the reaction products were analyzed by liquid chromatography-mass spectrometry (LC-MS). The WT peptide was cleaved into two smaller peptides with molecular weights of 1096.73 Da and 604.77 Da, corresponding to peptides NILNRRRR and TAGMII, respectively (Fig. S6C). In contrast, the R99S peptide was cleaved into two fragments with molecular weights of 784.47 Da and 847.46 Da, corresponding to peptide NILNRR and SRTAGMII, respectively (Fig. S6D). The LC-MS also detected some unknown peptides, probably due to nonspecific degradations of the protease reaction (Fig. S6C-D). Nevertheless, the data indicate that the C_R99S mutation shifts the capsid cleavage site upstream by two amino acids.

**NS2A G11A enhances prM/NS2A interaction.** Since NS2A G11A alters the processing of C-prM (Fig. 1H) and prM glycosylation (Fig. S3F), we hypothesized that



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NS2A directly interacts with prM and the G11A mutation affects the prM/NS2A interaction. To test this hypothesis, we co-transfected two plasmids into HEK293T cells. One plasmid expressed the authentic signal peptide anchorC ($C_{14}$) fused with the full-length prM. Another plasmid expressed a fusion protein containing the C-terminal 24 amino acids of the E protein, the N-terminal 50 amino acids of the NS1 protein, the C-terminal 50 amino acids of NS1, full length NS2A, and an EGFP [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]; this plasmid was previously shown to express NS2A-EGFP with a correct topology on the ER membrane [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. Co-immunoprecipitation showed that NS2A-EGFP, but not EGFP, pulled down prM (Fig. 3B, lanes 2 and 3). As controls, mouse IgG did not pull down any NS2A-EGFP or prM (Lane 1), and EGFP antibody did not pull down prM in the absence of NS2A-EGFP (Lane 6). To map the region of prM that binds to NS2A, we expressed the first 130 amino acids of prM [prM(1-130)] that lacked the C-terminal two transmembrane domains (Fig. 3A). Co-immunoprecipitation showed that prM(1-130) could not pull down NS2A-EGFP (Fig. 3B, lane 4), suggesting that (i) NS2A may bind to the transmembrane region of prM or (ii) the transmembrane region of prM may affect the conformation of its ectodomain and, consequently, abolish its interaction with NS2A.

Next, we examined if NS2A mutation G11A affects the prM/NS2A interaction. Co-immunoprecipitation experiments showed that G11A NS2A-EGFP pulled down about 2.5-fold more prM than the WT NS2A-EGFP did (Fig. 4C). Reciprocally, prM pulled down about 1.7-fold more G11A NS2A-EGFP than WT NS2A-EGFP (Fig. 4D). The results indicate that the G11A mutation enhances NS2A binding to prM.

To further support the prM/NS2A interaction, we used confocal microscope to examine the co-localization of prM/NS2A in cells. In this case, prM-E were co-expressed with NS2A-EGFP in HEK293T cells. As shown in Fig. 3E, NS2A-EGFP co-localized with



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prM. The G11A mutation did not change the co-localization of NS2A-EGFP with prM (Fig. 3E). Taken together, the results demonstrate that (i) NS2A interacts with prM and (ii) NS2A G11A significantly enhances the prM/NS2A interaction.

**The G11A mutation does not affect NS2A binding to E or NS3.** We also examined the interactions between NS2A and E or NS3 proteins that were co-expressed in HEK293T cells. For testing the E/NS2A interaction, we fused a GST tag to the C-terminus of NS2A for the co-immunoprecipitation experiment [because the EGFP tag used in NS2A-EGFP in Fig. 3B-D non-specifically interacts with E protein (data not shown)]. As shown in Fig. 3F, both WT and G11A NS2A-GST pulled down E protein at comparable efficiencies. As a control, GST alone did not pull down E protein. The result suggests that NS2A interacts with E and the G11A mutation does not affect this interaction.

Since flavivirus NS3 is known to participate in viron assembly [ ADDIN EN.CITE ADDIN EN.CITE.DATA ], we also tested if NS2A G11A mutation affects the NS2A/NS3 interaction. When co-expressed in HEK293T cells, GST alone pulled down a trace amount of HA-tagged NS3 (NS3-HA) protein (Fig. S7, lane 1), whereas NS2A-GST pulled down 10-fold more NS3-HA (Lane 2). Equivalent amounts of NS3-HA were pulled down by the WT and G11A NS2A-GST (Compare lanes 2 and 3), suggesting that NS2A G11A does not affect NS2A/NS3 binding.

**Extension of two or three residues at the N-terminus of anchorC efficiently rescues virion assembly of NS2A G11A**. So far, our results showed that NS2A G11A mutation increases its binding to prM on the ER lumen side, leading to impaired C-prM processing and defective virion assembly. These defects can be rescued by a capsid mutation R99S that shifts the C-terminal cleavage site by two amino acids. As a result of this shift, the N-terminus of transmembrane anchorC is extended by two amino acids on



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572 the cytosolic side of ER membrane. These data support a model of coordinated C-prM
573 processing during virion assembly (Fig. 4A) [ ADDIN EN.CITE
574 <EndNote><Cite><Author>Amberg</Author><Year>1999</Year><RecNum>295</RecNu
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579 Article">17</ref-type><contributors><authors><author>Amberg, S.
580 M.</author><author>Rice, C. M.</author></authors></contributors><auth-
581 address>Department of Molecular Microbiology, Washington University School of
582 Medicine, St. Louis, Missouri 63110-1093, USA.</auth-
583 address><titles><title>Mutagenesis of the NS2B-NS3-mediated cleavage site in the
584 flavivirus capsid protein demonstrates a requirement for coordinated
585 processing</title><secondary-title>J Virol</secondary-title><alt-title>Journal of
586 virology</alt-title></titles><periodical><full-title>J Virol</full-title><abbr-1>Journal of
587 virology</abbr-1></periodical><alt-periodical><full-title>J Virol</full-title><abbr-1>Journal
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589 94</pages><volume>73</volume><number>10</number><keywords><keyword>Capsid/*
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597 urls><url>http://www.ncbi.nlm.nih.gov/pubmed/10482557</url></related-



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598 urls></urls><custom2>112824</custom2></record></Cite></EndNote>]. During WT virus
599 assembly, polyprotein C-anchorC-prM was first cleaved at the C-terminus of capsid protein
600 (*i.e.*, R100) by NS2B/NS3 protease, producing mature C and anchorC-prM. The anchorC-
601 prM was then cleaved by a host signalase at the N-terminus of prM, generating anchorC
602 and prM. The cleaved prM coordinates with E protein for virion budding (Fig. 4A, *left*
603 *panel*). In the presence of NS2A G11A mutation, NS2A binds to prM with a higher affinity,
604 pulling anchorC (embedded in the ER membrane) towards the lumen side. The viral
605 protease cleavage site of the "pulled anchorC" is blocked, possibly by the ER membrane
606 (*middle panel*), leading to unprocessed C-prM. To overcome this defect, the C_R99S
607 mutation creates a new NS2A/NS3 cleavage site at R98 of capsid protein, restoring the C-
608 prM processing and virion assembly of NS2A G11A (*right panel*).

609 The key of the above model is the blockage of viral protease cleavage site at the
610 C-terminus of capsid protein, which is caused by the NS2A G11A mutation. If the model is
611 correct, an insertion of one or two amino acids to the N-terminus of anchorC should
612 restore the C-prM cleavage and virion assembly in the presence of NS2A G11A mutation
613 (Fig. 4B). To test this hypothesis, we inserted Thr, The-Ala, or Ser-Arg to the C-terminus of
614 capsid protein (after R100) in the context of WT or NS2A G11A viral RNA. The inserted
615 amino acids were selected based on the cleavage requirement for viral protease (Fig. 4B).
616 Transfection of each viral RNA into BHK-21 cells yielded increasing number of E-positive
617 cells from day 1 to 4 (Fig. 4C), produced infectious viruses (Fig. 4D-E), displayed cytosolic
618 and nucleus localizations of C protein (Fig. 4F), and exhibited efficient C-prM cleavage
619 (Fig. 4H). In the absence of NS2A G11A, the capsid insertion mutant RNAs behaved
620 similarly to the WT RNA. In the presence of NS2A G11A, the capsid single-amino acid
621 insertion (*i.e.*, NS2A G11A+C_T101) partially restored virion assembly ($1.2\times10^3$ PFU/ml;
622 Fig. 4D-E), prM-positive cells (Fig. 4G), and C-prM cleavage (60%; Fig. 4H); while the



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capsid double-amino acid insertion (*i.e.*, NS2A G11A+C_TA and NS2A G11A+C_SR) almost fully restored virion assembly to the WT level (2.3-2.5×$10^6$ PFU/ml; Fig. 4D-E) and C-prM cleavage (92-98%; Fig. 4H). These results demonstrate that addition of one or two amino acids to the N-terminus of anchorC can rescue virion assembly of NS2A G11A.

Since the single-amino acid C_T101 insertion only partially rescued virion assembly of NS2A G11A, we selected revertants for NS2A G11A+C_T101 virus. After continuous passaging of the virus on Vero cells for eight rounds, the P8 viruses were subjected to plaque assay (Fig. S8A-B) and whole-genome sequencing (Fig. S8C). The P8 viruses from all four independent selections produced viral titers (Fig. S8B) that were ≥100-fold higher than the original NS2A G11A+C_T101 virus (Fig. 4E). Among them, selection I P8 virus retained the original NS2A G11A+C_T101 mutations. In addition, an extra mutation R100S was recovered in the capsid protein. Sequence analysis suggests that the R100S could alter the protease cleavage site from the original RRRR↓T to RRR↓ST (R100S underlined) and extends the N-terminus of anchorC by one more amino acid. Selection II P8 virus also retained the original NS2A G11A+C_T101 mutations and accumulated another mutation R99S in the capsid protein. The R99S may change the protease cleavage site to RR↓SRT and extends the N-terminus of anchorC by two more amino acids. In addition, selection II P8 virus contained the R100S mutation in 27% of the viral population. Selection III P8 virus retained the NS2A G11A mutation and showed mixed sequences of R99S and/or R100S. Selection IV P8 virus exhibited WT plaque morphology; sequencing results showed that the NS2A G11A was reverted to WT Gly, but the C_T101 insertion was retained. Collectively, the results again support the conclusion that extension of the N-terminus of anchorC by two to three residues could efficiently rescue virion assembly of NS2A G11A.



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**Analysis of NS2A G11A in mosquito cells.** To test if the above findings from mammalian cells could be extended to mosquito host, we analyzed NS2A G11A+capsid mutant RNAs in C6/36 cells. Viral RNAs containing NS2A G11A alone, NS2A G11A+C_T101, NS2A G11A+C_R99S, or NS2A G11A+C_TA were transfected into C6/36 cells. The transfected cells were analyzed for capsid protein-positive cells (Fig. S9A), infectious virus production (Fig. S9B-C), extracellular viral RNA release (Fig. S9D), and C-prM cleavage (Fig. S9E). Similar to the results obtained from mammalian cells, NS2A G11A mutant was defective in C-prM processing and virion production in C6/36 cells. The defects could be rescued by the capsid mutations, with the rescuing efficiency of C_TA > C_R99S > C_T101 (Fig. S9). The results indicate that NS2A G11A mutation exerts the same mechanism in abolishing virion assembly in mammalian and mosquito hosts.

**NS2A protein binds to 3'UTR RNA**. To further explore the role of DENV-2 NS2A in virion assembly, we examined the ability of NS2A to bind viral genomic RNA. A series of pulldown experiments were performed using biotinylated viral RNAs and NS2A protein (Fig. 5A). A 616-nucleotide viral RNA (5'UTR-$C_{66}$-3'UTR) comprising the 5'UTR, the first 66 nucleotides from the C gene, and the 3'UTR regions of DENV-2 genome was *in vitro* synthesized (Fig. S10A). The 3' end of the RNA was ligated to a biotinylated cytidine using T4 RNA ligase, resulting in 5'UTR-$C_{66}$-3'UTR-biotin RNA (Fig. S10B). Two types of GST-tagged NS2A protein were prepared for the pulldown experiment: One type NS2A was expressed from *E. coli*, partially purified, and reconstituted in detergent DDM-containing buffer (Fig. 5B); another type NS2A was from the cell lysates of HEK293T cells transiently expressing the NS2A protein (Fig. 5C). As shown in Fig. 5B-C, the 5'UTR-$C_{66}$-3'UTR-biotin RNA efficiently pulled down GST-tagged NS2A (Lanes 4 and 6), but not GST (Lanes 2 and 5); in contrast, neither GST nor GST-tagged NS2A proteins was pulled down by a biotinylated non-viral RNA control (Lanes 1 and 3). To further demonstrate the specificity



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of NS2A/5'UTR-$C_{66}$-3'UTR RNA interaction, we performed an RNA competition experiment. Increasing amounts un-biotinylated 5'UTR-$C_{66}$-3'UTR RNA competed away the NS2A/5'UTR-$C_{66}$-3'UTR-biotin RNA interaction, whereas yeast tRNA did not (Fig. 5D). These results suggest that 5'UTR-$C_{66}$-3'UTR RNA specifically binds to NS2A protein.

We estimated the binding affinity of NS2A/viral RNA interaction. 5'UTR-$C_{66}$-3'UTR-biotin RNA was incubated with increasing amounts of recombinant GST-NS2A protein. The reactions were separated on a native polyacrylamide gel (PAGE), transferred to nylon membrane, and probed with streptavidin-HRP (Fig. 5E). Quantification of free RNA and RNA/protein complex estimated a *Kd* of 35 nM for DENV-2 NS2A biding to viral RNA.

**The last 285 nucleotides of 3'UTR RNA is responsible for binding to NS2A protein**. To map the region of viral RNA responsible for binding to NS2A protein, we performed a competition assay using a set of un-biotinylated RNA fragments from 5'UTR-$C_{66}$-3'UTR (Fig. S10C). The fragments were prepared based on the previously proposed RNA structure [ ADDIN EN.CITE <EndNote><Cite><Author>Villordo</Author><Year>2015</Year><RecNum>109</RecNum><DisplayText>(Villordo et al., 2015)</DisplayText><record><rec-number>109</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1561296709">109</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Villordo, S. M.</author><author>Filomatori, C. V.</author><author>Sanchez-Vargas, I.</author><author>Blair, C. D.</author><author>Gamarnik, A. V.</author></authors></contributors><auth-address>Fundacion Instituto Leloir-CONICET, Buenos Aires, Argentina.Arthropod-borne and Infectious Diseases Laboratory, Department of Microbiology, Immunology and Pathology, Colorado State University, Fort Collins, Colorado, United States of America.</auth-address><titles><title>Dengue virus



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RNA structure specialization facilitates host adaptation</title><secondary-title>PLoS Pathog</secondary-title></titles><periodical><full-title>PLoS Pathog</full-title></periodical><pages>e1004604</pages><volume>11</volume><number>1</number><keywords><keyword>3' Untranslated Regions</keyword><keyword>Adaptation, Biological/genetics</keyword><keyword>Animals</keyword><keyword>Cells, Cultured</keyword><keyword>Cricetinae</keyword><keyword>Culicidae</keyword><keyword>Dengue Virus/*genetics</keyword><keyword>Host Specificity/genetics</keyword><keyword>Host-Pathogen Interactions/*genetics</keyword><keyword>Humans</keyword><keyword>Mutation</keyword><keyword>*Nucleic Acid Conformation</keyword><keyword>RNA, Viral/*chemistry/genetics</keyword></keywords><dates><year>2015</year><pub-dates><date>Jan</date></pub-dates></dates><isbn>1553-7374 (Electronic)1553-7366 (Linking)</isbn><accession-num>25635835</accession-num><urls><related-urls><url>https://www.ncbi.nlm.nih.gov/pubmed/25635835</url></related-urls></urls><custom2>PMC4311971</custom2><electronic-resource-num>10.1371/journal.ppat.1004604</electronic-resource-num></record></Cite></EndNote>]. As shown in Fig. 5F, 3'UTR, but not 5'UTR-$C_{66}$, competed away the NS2A/5'UTR-$C_{66}$-3'UTR-biotin RNA interaction, indicating that 3'UTR RNA binds to NS2A protein. To further map the region of 3'UTR for NS2A binding, we deleted pseudoknot 1 (Δpk1), pseudoknots 1-2 [Δpk(1-2)], and pseudoknots 1-3 [Δpk(1-3)] from the 3'UTR (Fig. S10C). Only Δpk1 and Δpk(1-2) RNAs, not Δpk(1-3) RNA, could compete away the NS2A/5'UTR-$C_{66}$-3'UTR-biotin RNA interaction (Fig. 5F), suggesting that Δpk(1-2) RNA, representing the last 285 nucleotides of 3'UTR RNA, is required for binding to NS2A protein. This conclusion was further supported by the results that different fragments from Δpk(1-2) RNA, including pK(3-4), pk3, and 3'SL, did not compete away the NS2A/5'UTR-$C_{66}$-3'UTR-biotin RNA interaction (Fig. 5F).



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Next, we performed a photo-activatable ribonucleoside-enhanced crosslinking (PAR-CL) experiment to define the nucleotides that may interact with NS2A protein (Fig. 5G). The 3'UTR RNA containing [ HYPERLINK "https://en.wikipedia.org/wiki/Photosensitivity" \o "Photosensitivity" ] 4-thiouridine was prepared through *in vitro* transcription. After RNA/protein complex formation, the 4-thiouridine in the 3'UTR RNA was cross-linked to NS2A protein under UV light (at 365-nm wavelength). The RNA was then subjected to deep sequencing. Due to the crosslinking of 4-thiouridine to protein, a T-to-C mutation was recovered at the RNA sites that interacted with NS2A protein (Figs. 5G and S11). Fig. 5H summarizes the U-to-C mutations in the 3'UTR RNA when complexed with NS2A protein. About 67% mutations occurred within the last 285 nucleotides of 3'UTR. The result further supports the NS2A/3'UTR RNA interaction.

**NS2A requires amino acids R94, K95, and K99A for binding to 3'UTR RNA and virion assembly.** To map the region of NS2A required for viral 3'UTR RNA binding, we prepared a panel of purified NS2A truncates with an N-terminal GST tag from *E. coli* (Fig. 6A). The binding efficiencies of NS2A truncates to 5'UTR-$C_{66}$-3'UTR-biotin RNA were compared using a pulldown assay. As shown in Fig. 6B, different NS2A truncates were pulled down by 5'UTR-$C_{66}$-3'UTR-biotin at varied efficiencies. The RNA pulled down NS2A 1-145, 1-168, and 1-218 (full-length) at comparable efficiencies. Compared to the full-length NS2A, the pulldown efficiencies for NS2A 1-101 and 1-71 decreased to 40% and 0%, respectively. These data suggest that amino acids 72-144 are required for NS2A binding to viral RNA.

According to the membrane topology of DENV-2 NS2A [ ADDIN EN.CITE <EndNote><Cite><Author>Nemesio</Author><Year>2014</Year><RecNum>82</RecNum><DisplayText>(Nemesio and Villalain, 2014)</DisplayText><record><rec-number>82</rec-number><foreign-keys><key app="EN" db-



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id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793217">82</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Nemesio, H.</author><author>Villalain, J.</author></authors></contributors><auth-address>Molecular and Cellular Biology Institute, Universitas "Miguel Hernandez" , E-03202 Elche-Alicante, Spain.</auth-address><titles><title>Membrane interacting regions of Dengue virus NS2A protein</title><secondary-title>J Phys Chem B</secondary-title></titles><periodical><full-title>J Phys Chem B</full-title></periodical><pages>10142-55</pages><volume>118</volume><number>34</number><keywords><keyword>Calorimetry, Differential Scanning</keyword><keyword>Cell Membrane/*metabolism</keyword><keyword>Fluorescence Polarization</keyword><keyword>*Hydrophobic and Hydrophilic Interactions</keyword><keyword>Peptide Library</keyword><keyword>Spectrophotometry, Infrared</keyword><keyword>Viral Nonstructural Proteins/*chemistry/*metabolism</keyword></keywords><dates><year>2014</year><pub-dates><date>Aug 28</date></pub-dates></dates><isbn>1520-5207 (Electronic)1520-5207 (Linking)</isbn><accession-num>25119664</accession-num><urls><related-urls><url>http://www.ncbi.nlm.nih.gov/pubmed/25119664</url></related-urls></urls><custom2>PMC4148155</custom2><electronic-resource-num>10.1021/jp504911r</electronic-resource-num></record></Cite></EndNote>], amino acids 72-144 form two transmembrane domains (72-92 and 101-118), one short cytosolic loop (93-100), and a luminal segment (118-144; Fig. 6A). Since viral RNA resides in the cytoplasm, we examined the cytosolic loop of NS2A for viral RNA binding. The cytosolic



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loop has three positively charged residues (R94, K95, and K99) that are conserved among the four DENV serotypes (Fig. 6C). To examine the function of these residues, we prepared a panel of recombinant DENV-2 containing Ala substitutions at single (R94A, K95A, or K99A), double (R94A+K95A), or triple residues (R94A+K95A+K99A). Upon transfection to BHK-21 cells, all mutant RNAs generated viral E protein-positive cells at day 1 (Fig. 6D). Except for the triple mutant, other RNAs produced more E-positive cells on day 4 post-transfection. Mutants R94A and K95A generated WT level of infectious viruses and plaque morphology (Fig. 6E-F). Mutants K99A and K94A+K95A generated 22- to 3-fold less viruses than the WT, respectively (Fig. 6F), and exhibited smaller plaques (Fig. 6E). Remarkably, triple mutant R94A+K95A+K99A RNA yielded almost undetectable amount of virus that was 50,000-fold lower than the WT (Fig. 6F). To examine the effect of R94A+K95A+K99A on viral RNA synthesis, we engineered the triple mutations into a luciferase replicon of DENV-2 (Fig. 6G). The R94A+K95A+K99A replicon generated a luciferase profile similar to the WT replicon, indicating that the mutations do not affect viral translation and RNA synthesis. Taken together, the results demonstrate that R94A+K95A+K99A triple mutant is defective in virion assembly.

Next, we examined the effect of R94A+K95A+K99A mutations on NS2A binding to viral RNA. Since the result from Fig. 6B indicates that NS2A fragment 72-144 is required for viral RNA binding, we expressed a short NS2A peptide 64-128 (comprising the first two NS2A transmembrane domains, the cytosolic region 93-100, and the N- and C-terminal luminal tails for correct folding of the peptide on membrane) fused with an N-terminal GST-tag (Fig. 6H). As shown in Fig. 6I, peptide 64-128 could be pulled down by 5'UTR-$C_{66}$-3'UTR-biotin RNA (Lane 1). Un-biotinylated 5'UTR-$C_{66}$-3'UTR or 3'UTR RNA (Lanes 2 and 4), but not 5'UTR-$C_{66}$ RNA or yeast tRNA (Lanes 3 and 5), could compete away the NS2A peptide 64-128/5'UTR-$C_{66}$-3'UTR-biotin RNA interaction. The result demonstrates that



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NS2A peptide 64-128 specifically binds to viral 3'UTR RNA. Importantly, mutant R94A+K95A+K99A reduced the pulldown efficiency of NS2A peptide 64-128 by over 80% (Fig. 6J). Notably, some degraded GST-tag was found to associate with the NS2A truncates in the above pulldown experiments (Figs. 6B I-J), most likely due to the dimerization property of GST[ ADDIN EN.CITE <EndNote><Cite><Author>Maru</Author><Year>1996</Year><RecNum>110</RecNum><DisplayText>(Maru et al., 1996)</DisplayText><record><rec-number>110</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1561302524">110</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Maru, Y.</author><author>Afar, D. E.</author><author>Witte, O. N.</author><author>Shibuya, M.</author></authors></contributors><auth-address>Department of Genetics, Institute of Medical Science, University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108, Japan.</auth-address><titles><title>The dimerization property of glutathione S-transferase partially reactivates Bcr-Abl lacking the oligomerization domain</title><secondary-title>J Biol Chem</secondary-title></titles><periodical><full-title>J Biol Chem</full-title></periodical><pages>15353-7</pages><volume>271</volume><number>26</number><keywords><keyword>*Adaptor Proteins, Signal Transducing</keyword><keyword>Animals</keyword><keyword>Bone Marrow Cells</keyword><keyword>Cell Transformation, Neoplastic</keyword><keyword>Cells, Cultured</keyword><keyword>Fusion Proteins, bcr-abl/*metabolism</keyword><keyword>GRB2 Adaptor Protein</keyword><keyword>Glutathione Transferase/*metabolism</keyword><keyword>Macromolecular Substances</keyword><keyword>Mice</keyword><keyword>Phosphotyrosine/metabolism</keyword><keyword>Protein Binding</keyword><keyword>Protein-Tyrosine



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825 Kinases/metabolism</keyword><keyword>Proteins/metabolism</keyword><keyword>Rat
826 s</keyword><keyword>Recombinant Fusion Proteins</keyword><keyword>Structure-
827 Activity Relationship</keyword></keywords><dates><year>1996</year><pub-
828 dates><date>Jun 28</date></pub-dates></dates><isbn>0021-9258 (Print)0021-
829 9258 (Linking)</isbn><accession-num>8663064</accession-num><urls><related-
830 urls><url>https://www.ncbi.nlm.nih.gov/pubmed/8663064</url></related-
831 urls></urls><electronic-resource-num>10.1074/jbc.271.26.15353</electronic-resource-
832 num></record></Cite></EndNote>]. Overall, the results indicate that R94, K95, and K99
833 are important for NS2A binding to viral 3'UTR.

834 **DISCUSSION**

835 Flavivirus assembly consists of a tightly controlled cascade of events: export of
836 newly synthesized viral RNA from the replication complex to assembly sites, encapsidation
837 of viral RNA with C protein, and envelopment of nucleocapsid with prM and E proteins.
838 Many host and viral factors (proteins and lipids) participate in this temporal and spatial
839 process [ ADDIN EN.CITE <EndNote><Cite><Author>Apte-
840 Sengupta</Author><Year>2014</Year><RecNum>94</RecNum><DisplayText>(Apte-
841 Sengupta et al., 2014)</DisplayText><record><rec-number>94</rec-number><foreign-
842 keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv"
843 timestamp="1560793218">94</key></foreign-keys><ref-type name="Journal
844 Article">17</ref-type><contributors><authors><author>Apte-Sengupta,
845 S.</author><author>Sirohi, D.</author><author>Kuhn, R.
846 J.</author></authors></contributors><auth-address>Markey Center for Structural Biology,
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849 Biological Sciences and Bindley Bioscience Center, Purdue University, West Lafayette, IN,
850 USA. Electronic address: kuhnr@purdue.edu.</auth-address><titles><title>Coupling of
851 replication and assembly in flaviviruses</title><secondary-title>Curr Opin
852 Virol</secondary-title></titles><periodical><full-title>Curr Opin Virol</full-
853 title></periodical><pages>134-
854 42</pages><volume>9</volume><keywords><keyword>Flavivirus/*physiology</keyword>
855 <keyword>Host-Pathogen
856 Interactions</keyword><keyword>Humans</keyword><keyword>Lipid
857 Metabolism</keyword><keyword>Viral Proteins/metabolism</keyword><keyword>*Virus
858 Assembly</keyword><keyword>*Virus
859 Replication</keyword></keywords><dates><year>2014</year><pub-
860 dates><date>Dec</date></pub-dates></dates><isbn>1879-6265 (Electronic)1879-
861 6257 (Linking)</isbn><accession-num>25462445</accession-num><urls><related-
862 urls><url>http://www.ncbi.nlm.nih.gov/pubmed/25462445</url></related-
863 urls></urls><custom2>PMC4268268</custom2><electronic-resource-
864 num>10.1016/j.coviro.2014.09.020</electronic-resource-
865 num></record></Cite></EndNote>]. Among them, flaviviral NS2A has emerged as a key
866 player to orchestrate virion assembly, as evidenced by the following findings. (i) In infected
867 cells, there are two distinct NS2A species that are required for viral RNA synthesis and
868 virion assembly. The NS2A species required for RNA synthesis is located at the viral
869 replication complex that could only be formed in *cis*; the other NS2A species required for
870 virus morphogenesis is located at the virion assembly complex that could be formed in *cis*
871 or *trans* [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. (ii) The defect in virion assembly
872 caused by DENV-2 and YFV NS2A mutations could be rescued by adaptive mutations in
873 prM, E, NS2B, and NS3 proteins, suggesting that NS2A genetically modulates other viral
874 proteins during virion assembly [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. (iii)



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875 Recombinant NS2A of Kunjin virus binds to viral 3'UTR RNA [ ADDIN EN.CITE
876 <EndNote><Cite><Author>Mackenzie</Author><Year>1998</Year><RecNum>81</RecN
877 um><DisplayText>(Mackenzie et al., 1998)</DisplayText><record><rec-number>81</rec-
878 number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv"
879 timestamp="1560793217">81</key></foreign-keys><ref-type name="Journal
880 Article">17</ref-type><contributors><authors><author>Mackenzie, J.
881 M.</author><author>Khromykh, A. A.</author><author>Jones, M.
882 K.</author><author>Westaway, E.
883 G.</author></authors></contributors><titles><title>Subcellular localization and some
884 biochemical properties of the flavivirus Kunjin nonstructural proteins NS2A and
885 NS4A</title><secondary-title>Virology</secondary-title></titles><periodical><full-
886 title>Virology</full-title></periodical><pages>203-
887 15</pages><volume>245</volume><number>2</number><keywords><keyword>Animal<
888 /keyword><keyword>Cercopithecus aethiops</keyword><keyword>*Flavivirus/ph
889 [Physiology]</keyword><keyword>Immunohistochemistry</keyword><keyword>RNA,
890 Viral/me [Metabolism]</keyword><keyword>Support, Non-U.S.
891 Gov't</keyword><keyword>Vero Cells</keyword><keyword>*Viral Nonstructural
892 Proteins/me [Metabolism]</keyword><keyword>Virus
893 Replication</keyword></keywords><dates><year>1998</year></dates><urls></urls></re
894 cord></Cite></EndNote>]. These studies indicate that NS2A interacts with two of the three
895 structural proteins (prM and E, not C) and viral RNA. However, how viral RNA and C
896 protein are recruited to form nucleocapsid and how nucleocapsid is enveloped with prM-E
897 proteins to form virion remain unknown. The current study aims to address these
898 questions.



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First, we showed that DENV-2 NS2A interacted with prM to modulate virion assembly. WT NS2A directly bound to prM in the absence of other viral proteins (Fig. 3B). The biological relevance of this interaction was supported by the correlation between virion assembly and prM/NS2A interaction. Specifically, an NS2A mutation G11A, which abolished virion assembly (Fig. 1), enhanced its binding to prM by 1.7-2.5 folds (Fig. 3). Mechanistically, the enhanced interaction between the mutant NS2A and prM "over pulled" the membrane-associated anchorC towards the ER lumen, leading to a blockage of the viral protease cleavage site (most likely by the ER membrane) at the C-anchorC junction. The uncleaved C-prM resulted in a rapid degradation of all three structural proteins (Fig. 1). This is not surprising because the unprocessed C-prM is retained on the ER membrane, misfolded, and degraded via the ubiquitin-proteasome machinery (Fig. 1H). Since prM is a chaperone for E protein [ ADDIN EN.CITE <EndNote><Cite><Author>Lorenz</Author><Year>2002</Year><RecNum>96</RecNum><DisplayText>(Lorenz et al., 2002)</DisplayText><record><rec-number>96</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793218">96</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Lorenz, I. C.</author><author>Allison, S. L.</author><author>Heinz, F. X.</author><author>Helenius, A.</author></authors></contributors><auth-address>Institute of Biochemistry, Swiss Federal Institute of Technology, CH-8093 Zurich, Switzerland.</auth-address><titles><title>Folding and dimerization of tick-borne encephalitis virus envelope proteins prM and E in the endoplasmic reticulum</title><secondary-title>J Virol</secondary-title></titles><periodical><full-title>J Virol</full-title></periodical><pages>5480-91</pages><volume>76</volume><number>11</number><keywords><keyword>Animals</keyword><keyword>COS Cells</keyword><keyword>Cercopithecus



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925 aethiops</keyword><keyword>Dimerization</keyword><keyword>Dithiothreitol</keyword
926 ><keyword>Encephalitis Viruses, Tick-
927 Borne/genetics/*metabolism</keyword><keyword>Hexosaminidases/metabolism</keywor
928 d><keyword>Polyproteins/genetics/metabolism</keyword><keyword>*Protein
929 Folding</keyword><keyword>Protein
930 Precursors/genetics/metabolism</keyword><keyword>Recombinant Fusion
931 Proteins/genetics/metabolism</keyword><keyword>Viral Envelope
932 Proteins/genetics/*metabolism</keyword></keywords><dates><year>2002</year><pub-
933 dates><date>Jun</date></pub-dates></dates><isbn>0022-538X (Print)0022-538X
934 (Linking)</isbn><accession-num>11991976</accession-num><urls><related-
935 urls><url>http://www.ncbi.nlm.nih.gov/pubmed/11991976</url></related-
936 urls></urls><custom2>PMC137023</custom2><electronic-resource-
937 num>10.1128/jvi.76.11.5480-5491.2002</electronic-resource-
938 num></record></Cite></EndNote>], the degradation of C-prM leads to the degradation of
939 E protein. These results are in agreement with a recent report that ZIKV anchorC plays an
940 important role in the stability of E protein and virion morphogenesis [ ADDIN EN.CITE
941 <EndNote><Cite><Author>Rana</Author><Year>2018</Year><RecNum>97</RecNum><
942 DisplayText>(Rana et al., 2018)</DisplayText><record><rec-number>97</rec-
943 number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv"
944 timestamp="1560793219">97</key></foreign-keys><ref-type name="Journal
945 Article">17</ref-type><contributors><authors><author>Rana, J.</author><author>Slon
946 Campos, J. L.</author><author>Leccese, G.</author><author>Francolini,
947 M.</author><author>Bestagno, M.</author><author>Poggianella,
948 M.</author><author>Burrone, O. R.</author></authors></contributors><auth-
949 address>Molecular Immunology Group, International Centre for Genetic Engineering and
950 Biotechnology, Trieste, Italy rana@icgeb.org burrone@icgeb.org.Molecular



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951 Immunology Group, International Centre for Genetic Engineering and Biotechnology,
952 Trieste, Italy.Department of Medical Biotechnology and Translational Medicine,
953 Universita degli Studi di Milano, Milan, Italy.</auth-address><titles><title>Role of Capsid
954 Anchor in the Morphogenesis of Zika Virus</title><secondary-title>J Virol</secondary-
955 title></titles><periodical><full-title>J Virol</full-
956 title></periodical><volume>92</volume><number>22</number><keywords><keyword>Zi
957 ka virus</keyword><keyword>capsid
958 anchor</keyword><keyword>flavivirus</keyword><keyword>morphogenesis</keyword><
959 keyword>pseudovirus</keyword><keyword>viral
960 assembly</keyword></keywords><dates><year>2018</year><pub-dates><date>Nov
961 15</date></pub-dates></dates><isbn>1098-5514 (Electronic)0022-538X
962 (Linking)</isbn><accession-num>30158295</accession-num><urls><related-
963 urls><url>http://www.ncbi.nlm.nih.gov/pubmed/30158295</url></related-
964 urls></urls><custom2>PMC6206493</custom2><electronic-resource-
965 num>10.1128/JVI.01174-18</electronic-resource-num></record></Cite></EndNote>].

966 Second, we demonstrated that the defect in virion assembly of NS2A G11A could
967 be restored by two different means. Creation of a new viral protease cleavage site by
968 extending the N-terminus of anchorC by two amino acids restored the capsid-anchorC
969 processing and, consequently, restored virion assembly. This conclusion was supported
970 by revertant analysis of the NS2A G11A mutant (Figs. 2 and S4) and amino acid insertion
971 experiment (Fig. 4). The two-amino acid extension exposes the new protease cleavage
972 site to NS2B/NS3. Alternatively, the virion assembly defect of NS2A G11A could be
973 rescued by *trans* supply of WT NS2A (Fig. S3). Under this condition, the WT NS2A
974 replaced the G11A NS2A to interact with C-prM, restored the C-prM cleavage, and ensued
975 a productive virion morphogenesis. Collectively, our results support a previous model that



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the two cleavages at C-anchorC-prM junctions are coordinated in a sequential manner during flavivirus assembly. The C-anchorC junction was first cleaved by viral protease on the cytosolic side, followed by the anchorC-prM cleavage by the cellular signalase on the ER lumen side [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. Although expression of flavivirus prM-E alone could form empty virus-like particles, the coordinated C-anchorC-prM processing couples the encapsidation and envelopment events; such coupling is required for the production of infectious virus. This conclusion is also supported by the previous reports that separate expression of C and prM-E significantly compromised the assembly efficiency of infectious virions [ ADDIN EN.CITE ADDIN EN.CITE.DATA ].

Third, we characterized the specific interaction between DENV-2 NS2A and the 3'UTR of viral genome. Although Kunjin virus NS2A was reported to bind to the 3'UTR of viral RNA [ ADDIN EN.CITE <EndNote><Cite><Author>Mackenzie</Author><Year>1998</Year><RecNum>81</RecNum><DisplayText>(Mackenzie et al., 1998)</DisplayText><record><rec-number>81</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793217">81</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Mackenzie, J. M.</author><author>Khromykh, A. A.</author><author>Jones, M. K.</author><author>Westaway, E. G.</author></authors></contributors><titles><title>Subcellular localization and some biochemical properties of the flavivirus Kunjin nonstructural proteins NS2A and NS4A</title><secondary-title>Virology</secondary-title></titles><periodical><full-title>Virology</full-title></periodical><pages>203-15</pages><volume>245</volume><number>2</number><keywords><keyword>Animal</keyword><keyword>Cercopithecus aethiops</keyword><keyword>*Flavivirus/ph



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1001 [Physiology]</keyword><keyword>Immunohistochemistry</keyword><keyword>RNA,
1002 Viral/me [Metabolism]</keyword><keyword>Support, Non-U.S.
1003 Gov't</keyword><keyword>Vero Cells</keyword><keyword>*Viral Nonstructural
1004 Proteins/me [Metabolism]</keyword><keyword>Virus
1005 Replication</keyword></keywords><dates><year>1998</year></dates><urls></urls></re
1006 cord></Cite></EndNote>], the molecular details of the interaction has not been delineated.
1007 Using competition assay and PAR-CLIP technique, we mapped that DENV-2 NS2A bound
1008 to the last 285 nucleotides of the 3'UTR, including the pk3, pk4, and 3'SL. Interestingly, the
1009 pk3, pk3-4, or 3'SL RNA alone did not efficiently compete out the NS2A/3'UTR RNA
1010 interaction (Fig. 5F), suggesting that a complex RNA structure in the 3'UTR is required for
1011 efficient binding to NS2A protein. Since no RNA “packaging signal” has been identified for
1012 flavivirus assembly and the C protein was shown to non-specifically bind to any RNA
1013 [ ADDIN EN.CITE
1014 <EndNote><Cite><Author>Pong</Author><Year>2011</Year><RecNum>102</RecNum>
1015 <DisplayText>(Pong et al., 2011)</DisplayText><record><rec-number>102</rec-
1016 number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv"
1017 timestamp="1560793219">102</key></foreign-keys><ref-type name="Journal
1018 Article">17</ref-type><contributors><authors><author>Pong, W.
1019 L.</author><author>Huang, Z. S.</author><author>Teoh, P. G.</author><author>Wang,
1020 C. C.</author><author>Wu, H. N.</author></authors></contributors><auth-
1021 address>Institute of Molecular Biology, Academia Sinica, Taipei, Taiwan.</auth-
1022 address><titles><title>RNA binding property and RNA chaperone activity of dengue virus
1023 core protein and other viral RNA-interacting proteins</title><secondary-title>FEBS
1024 Lett</secondary-title><alt-title>FEBS letters</alt-title></titles><periodical><full-title>FEBS
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81</pages><volume>585</volume><number>16</number><keywords><keyword>Base Sequence</keyword><keyword>*Dengue Virus</keyword><keyword>Genome, Viral/genetics</keyword><keyword>Molecular Chaperones/*metabolism</keyword><keyword>Nucleic Acid Conformation</keyword><keyword>Protein Binding</keyword><keyword>Protein Engineering</keyword><keyword>RNA Helicases/metabolism</keyword><keyword>RNA, Catalytic/genetics</keyword><keyword>RNA, Viral/chemistry/genetics/*metabolism</keyword><keyword>Serine Endopeptidases/metabolism</keyword><keyword>Viral Core Proteins/*metabolism</keyword><keyword>Viral Nonstructural Proteins/metabolism</keyword></keywords><dates><year>2011</year><pub-dates><date>Aug 19</date></pub-dates></dates><isbn>1873-3468 (Electronic)0014-5793 (Linking)</isbn><accession-num>21771593</accession-num><urls><related-urls><url>http://www.ncbi.nlm.nih.gov/pubmed/21771593</url></related-urls></urls><electronic-resource-num>10.1016/j.febslet.2011.06.038</electronic-resource-num></record></Cite></EndNote>], the last 285 nucleotides of 3'UTR may serve as the "packaging signal" to recruit viral RNA to the virion assembly site through specific binding to NS2A. From the NS2A side, we have mapped the 3'UTR-binding site to a cytosolic loop (containing R94, K95, and K99) between the first and second transmembrane domains (Fig. 6). Triple mutant R94A+K95A+K99A was defective in virion assembly and lost over 80% of the NS2A binding activity to 3'UTR RNA (Fig. 6). Caution should be taken when applying the DENV-2 findings to other flaviviruses because of the variations of NS2A proteins. The three positively charged R94, K95, and K99 of NS2A are only conserved among the four serotypes of DENV. In addition, DENV-2, YFV, and ZIKV NS2A proteins



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exhibit different topologies on the ER membrane [ ADDIN EN.CITE ADDIN EN.CITE.DATA ].

Our findings support a general model for flavivirus virion assembly (Fig. 7). In infected cells, NS2A molecules reside in both replication complex (housed in Ve) and viron assembly complex (located on the juxtaposed ER membrane). When plus-sense RNAs are made and released from the replication complex (through the pore on Ve), they are initially destined to new rounds of viral translation and replication on the ER. Once the amounts of viral RNA and proteins have accumulated over certain “thresholds”, nascent RNAs are switched for virion assembly. At this time, the newly synthesized RNA exits the replication complex and binds to the NS2A located at the assembly site through the 3'UTR RNA. The NS2A molecules at the assembly site also recruits C-prM-E and NS2B/NS3 through binding to prM and NS3, respectively. One NS2A molecule does not have to bind to the 3’UTR RNA, C-prM-E, and NS2B/NS3 at the same time. These interactions could be sequential or through different NS2A molecules that oligomerize together on the ER membrane [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. Next, the C-prM-E polyprotein is sequentially cleaved by the NS2B/NS3 protease and host signalase to produce mature C, prM, and E [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. The C molecules, many of which have been previously made and stored at the nearby lipid droplets [ ADDIN EN.CITE ADDIN EN.CITE.DATA ], bind viral RNA to form nucleocapsid. The processed prM undergoes conformational change and dimerizes with E. The prM/E dimerization and oligomerization dissociate prM from NS2A. When 180 copies of prM/E oligomerize on the ER membrane, envelopment of the nucleocapsid occurs, generating spiky immature virions. During the above process, NS3 may also use its helicase activity to transport viral RNA from the replication complex to the assembly site and condense the



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RNA during encapsidation. Besides NS3, NS2A or other proteins may also contribute to the translocation of viral RNA from the replication complex to the assembly site.

The above model is centered on the multifunctional role of NS2A in flavivirus replication and virion assembly. Our results have functionally demonstrated the molecular mechanism for two important steps during DENV-2 assembly: (i) The binding of 3'UTR RNA to NS2A servers as a "packaging signal" and (ii) in the presence of viral RNA-NS2A complex, the coordinated cleavage of C-prM-E facilitates the envelopment of nucleocapsid. The proposed model could serve as a framework for future investigations and antiviral development.

**Author contribution**

X.X., J.Z., X.Z., Y.Z., Q.W., H.-P.D. performed experiments and data analysis. X.X., J.Z., X.Z., Y.Z., A.R., V.L.P., X.W., Q.W., H.-P.D, P.-Y.S. designed the experiments and interpreted the results. X.X., P.-Y.S. wrote the manuscript.

**Acknowledgements**

We are grateful to Dr. Mariano A. Garcia-Blanco, Shelton S. Brarick from University of Texas Medical Branch for helpful discussions during this work. P.-Y.S. was supported by NIH grants AI142759, AI[ HYPERLINK "https://public.era.nih.gov/grantfolder/piAppDetails/genericStatus.do?encryptedParam=vXeG1axw2cY.YZxjFgAisBblI6OqVj4LxDR99WdVyTI-X4FuJbCLt4g." \t "_blank" ], and AI136126, and awards from the Kleberg Foundation, John S. Dunn Foundation, Amon G. Carter Foundation, Gilson Longenbaugh Foundation, and Summerfield Robert Foundation. X.X. and J.Z. were awarded with postdoctoral fellowships from the Novartis Institutes for



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Biomedical Research (NIBR) and the McLaughlin Endowment at University of Texas Medical Branch, respectively.



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## Figure titles and legends

**Figure 1 Characterization of NS2A G11A mutant**. (A) Replicon transient transfection assay. Top panel depicts the DENV-2 luciferase replicon. Bottom panel shows the luciferase activities after transfecting BHK-21 cells with equal amounts (10 μg) of WT or NS2A G11A mutant replicon RNA. (B) Quantification of extracellular viral RNA. Top panel depicts the full-length genome of DENV-2. Equal amounts of WT or NS2A G11A mutant viral RNA (10 μg) were electroporated into BHK-21 cells. At different time post-transfection (p.t.), extracellular viral RNAs were quantified by RT-qPCR. The DENV-2 replicon RNA was used as a control for determining the basal level of viral RNA released from dead cells. Bottom panel shows the CT values of the RT-qPCR results. Data show the means and standard deviations from three independent experiments. (C) Plaque morphology. Culture supernatants from (B) were analyzed for virus production using plaque assay. Only WT, not NS2A G11A mutant (N.D.), produced plaques. Plaques were developed on BHK-21 cells after 5 days of infection. (D) Thin-section transmission electron microscope (TEM) images of viral RNA-transfected BHK-21 cells. At 48 h p.t., cells were fixed for TEM analysis. Ve, virus-induced vesicles; T, membrane tubes; Vi, virus particles; ER, endoplasmic reticulum. Mock transfected cells were included as a control. (E-G) Confocal microscopy analysis of subcellular localizations of C, prM, and E in viral RNA-transfected BHK-21 cells. At 48 h p.t., transfected cells were analyzed by IFA. NS3 staining (red) was used as a control. Nuclei (blue) were stained by DAPI. Scale bar, 10 μm. (H) Viral protein processing in viral RNA-transfected BHK-21 cells. At 42 h p.t., BHK-21 (Mock) or viral RNA-transfected cells were treated with 0.01% DMSO (-) or 10 μM MG132 (+). After 6 h treatment, the cells were analyzed by Western blot. (I) Densitometry analysis. The intensities of bands representing specific viral proteins from DMSO-treated cells in (H) was quantified. The amounts of viral proteins derived from WT RNA-transfected cells were set as 100%. Relative intensities are calculated by normalizing the protein intensities derived from NS2A G11A mutant RNA-transfected cells to those from WT RNA-transfected cells. Data shows the means and standard deviations from three independent experiments.

**Figure 2 Restoration of NS2A G11A virion assembly by capsid R99S mutation.** (A) Revertant mutation. BHK-21 cells were transfected with NS2A G11A viral RNA. The transfected cells were consecutively passaged for five rounds to select for revertant viruses. The P5 virus was sequenced for revertant mutations. Changes in nucleotide



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positions and amino acids are indicated. (B) IFA of viral RNA-transfected cells. WT and mutant viral RNAs were electroporated to BHK-21 cells. The transfected cells were analyzed for E protein expression at 24 and 96 h p.t. by IFA. Scale bar, 100 μm. (C) Plaque morphology. Infectious viruses, derived from the RNA-transfected cells, were analyzed by plaque assay. Plaques were developed on day 5 post-infection on BHK-21 cells. N.D., not detectable. (D) Virus production. The culture fluids from (B) were quantified for infectious viruses. Data represent the means and standard deviations from three independent measurements. Limit of detection (L.O.D.), 10 PFU/ml. (E-F) Subcellular distributions of prM, NS1, and C proteins. prM, NS1 and C proteins were detected by DM1, human IgG anti-NS1 and D2C1 antibodies, respectively. Representative IFA images of viral RNA-transfected cells are shown at 24 h post-transfection. White arrows point to nucleus with C protein speckles. (G) Quantitative analysis of C-positive cells. The C-positive cells with cytoplasm or cytoplasm & nucleus distribution from 4-5 random fields were counted and normalized to the total cell numbers. The averages and standard deviations are shown. (H) Viral protein processing in viral RNA-transfected BHK-21 cells at 48 h post-transfection. The percentages of mature C protein were calculated from the following the formula: [C intensity/(C intensity + C-prM intensity)]×100%.

**Figure 3 DENV-2 NS2A interacts with prM and E proteins**. (A) Membrane topologies of DENV-2 prM, E, and NS2A proteins. The NS2A G11 position is indicated. (B) Co-IP of WT NS2A with prM. HEK293T cells were transfected with one plasmid (encoding prM only) or two plasmids (one encoding leader-NS2A-EGFP and the other encoding full-length or the ectodomain of prM). At 40 h p.t., IP was performed using mouse anti-EGFP or control IgG antibodies. Immunoblot (IB) was performed using rabbit anti-EGFP antibody or anti-prM antibody. (C) Co-IP of WT or G11A NS2A-EGFP with prM. Mouse anti-EGFP antibody was used for IP. Rabbit anti-EGFP or anti-prM antibodies were used for IB. The intensity of prM, co-precipitated with WT NS2A-EGFP, was set as 100%. The relative efficiency of prM co-precipitated with G11A NS2A-EGFP was compared with the WT NS2A-EGFP. Statistical analysis was performed using the unpaired parametric t-test. $*p < 0.05$, significant; $**p < 0.01$, very significant. (D) Reciprocal co-IP of prM with WT or G11A NS2A-EGFP. Rabbit anti-prM antibodies were used for IP. The amounts of co-precipitated WT and G11A NS2A-EGFP were detected using mouse anti-EGFP antibody. prM in eluates were detected by rabbit anti-prM antibody followed by protein G conjugated with HRP. (E) Co-localization analysis of NS2A and prM proteins. BHK-21 cells were co-



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transfected with two plasmids: One encoding the $C_{14}$-prM-E and the other encoding SPG-$C_{16}$-NS2A-EGFP. At 24 h p.t., the cells were examined for prM and NS2A-EGFP co-localization under a confocal microscope. Scale bar, 10 μm. (F) Co-IP of NS2A with E protein. HEK293T cells were co-transfected with two plasmids: One encoding the last 17 amino acids of prM and complete E protein ($M_{17}$-E), and another encoding NS2A with a C-terminal GST tag (NS2A-GST). GST tag alone was used as a negative control. IPs were performed using rabbit anti-GST antibody. E and GST fusion proteins were detected in cell lysates and eluates by mouse anti-E monoclonal antibody (9F12) and anti-GST antibody, respectively. The relative pulldown efficiency was calculated by normalizing the intensity of E co-precipitated with G11A NS2A-GST to the intensity of E co-precipitated with the WT NS2A-GST. The means and standard deviations from three independent experiments are shown. N.S., no significance.

**Figure 4 Two-residue insertion at the capsid-anchorC junction rescues the defect of NS2A G11A**. (A) A model depicting how NS2A modulates C-anchorC-prM cleavage. On the cytosolic side, viral NS2B/NS3 protease cleaves the C-anchorC junction. On the ER lumen side, host signalase cleaves the anchorC-prM junction. Three cleavage scenarios are presented for WT, NS2A G11A, and NS2A G11A+C_R99S constructs. (B) Constructs of insertion mutants of capsid protein. One residue (C_101) or two residues (C_TA or C_SR) were inserted to the C-anchorC junction. The potential cleavage sites for viral NS2B/NS3 protease are indicated. (C) IFA of viral RNA-transfected cells. Viral RNAs containing the NS2A G11A mutation with or without capsid insertions were electroporated into BHK-21 cells. The transfected cells were analyzed for E protein expression on day 1 and 4 by IFA. Scale bar, 100 μm. (D) Plaque morphology. Viruses recovered from (C) were analyzed by plaque assay on BHK-21 cells. (E) Viral titers. Culture supernatants from (C) were measured for viral titers on day 5 post-transfection. (F) Subcellular localization of C protein in viral RNA-transfected cells. White arrows point to nucleus with C protein speckles. Scale bar, 25 μm. (G) Expression of prM and NS1 proteins in viral RNA-transfected cells. Scale bar, 50 μm. (H) C-prM processing in viral RNA-transfected cells. At 48 h p.t., WT or NS2A G11A+C_T101 RNA-transfected cells were analyzed for C, NS1, and β-actin expression by Western blot. The percentages of C-prM to mature C conversion are presented for each construct.

**Figure 5 DENV-2 NS2A protein binds to 3'UTR RNA**. (A) Diagram of pulldown assay using biotinylated RNAs. 3'-end biotinylated RNA binds to streptavidin-conjugated



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magnetic beads. After incubating the bead-bound biotinylated RNA with NS2A protein, the RNA-bound NS2A was enriched and eluted for SDS-PAGE and Western blot analysis. (B) Pulldown analysis using recombinant NS2A protein. N-terminally GST-tagged NS2A protein (GST-NS2A) was expressed in *E. coli* and affinity purified. The 5'UTR-$C_{66}$-3'UTR-biotin RNA was prepared by ligating biotinylated cytidine (bis)phosphate to the 3' end of the 5'UTR-$C_{66}$-3'UTR RNA (containing the 5'UTR, the first 66 nucleotides of C gene, and the 3'UTR). The amounts of GST-NS2A in the input and eluates were analyzed using rabbit anti-GST antibody by Western blot. (C) Pulldown analysis using HEK293T cell-expressed NS2A-GST protein. HEK293T cells were transfected with a plasmid expressing GST or C-terminally GST-tagged NS2A protein (NS2A-GST). At 42 h p.t., cells were harvested, lysed, and used for RNA pull down-assay. The pulldown assay was performed as described in (B). (D) RNA competition assay. Various amounts of unlabeled 5'UTR-$C_{66}$-3'UTR RNA were used to compete the binding of 5'UTR-$C_{66}$-3'UTR-biotin RNA to GST-NS2A protein. tRNA was used as a negative control. (E) Viral RNA/NS2A binding affinity. Electrophoretic mobility-shift assay (EMSA) was used to determine the binding affinity of DENV-2 5'UTR-$C_{66}$-3'UTR-biotin RNA with NS2A protein. 5'UTR-$C_{66}$-3'UTR-biotin RNA was incubated with increasing amounts of GST-NS2A protein. Free and bound RNAs were resolved by native agarose gel electrophoresis. The equilibrium dissociation constant (*Kd*) was determined by nonlinear regression with least-squares fitting method. The coefficient of determination ($R^2$) is shown. Data represent the means and standard deviations from three independent experiments. (F) Mapping the RNA region responsible for NS2A binding. A panel of unlabeled RNA truncates of the 5'UTR-$C_{66}$-3'UTR were used as competitors for the binding of 5'UTR-$C_{66}$-3'UTR-biotin RNA to GST-NS2A protein. The intensity of GST-NS2A (bound to the 5'UTR-$C_{66}$-3'UTR-biotin) without competitor was set as 100%. Relative GST-NS2A intensities were calculated as [(signal in the presence competitor)/(signal in the absence of competitor)]×100%. Statistical analysis was performed using the unpaired parametric t-test. (G) PAR-CLIP workflow [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. See text for detail. (H) PAR-CLIP result summary. Uridines in the 3'UTR that may participate in NS2A-binding are indicated. The dash line indicates the threshold (equal to 5.76, representing the average fold change of mutation rate of all uridines determined by Illumina sequencing). The uridine positions with fold changes of mutation rate over the threshold are indicated in the 3'UTR sequence (GenBank no. AF038403).



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**Figure 6 NS2A residues responsible for the NS2A/3'UTR RNA interaction**. (A) Diagram of NS2A truncates used in RNA pulldown assay. All proteins were fused with an N-terminal GST tag, expressed in *E. coli*, and affinity purified. (B) Interactions between NS2A truncates with 5'UTR-$C_{66}$-3'UTR-biotin RNA. The interactions were measured using pulldown experiments. The pulldown efficiency of full-length NS2A (1-218) was set as 100%. Relative efficiency was calculated by comparing the intensity of NS2A truncate with that of the full-length NS2A. (C) Sequence alignment of NS2A among flaviviruses. Amino acids of a cytosolic loop (amino acids 93-101) are presented. The number of viral strains used for the NS2A alignment are indicated in parentheses. Amino acid positions of NS2A are labeled according to DENV-2 strain New Guinea C. Residues R94, K95, and K99 are shaded. The conserved (:) and identical (*) residues are indicated. (D) IFA of BHK-21 cells transfected with viral RNA. WT and mutant viral RNAs were electroporated into BHK-21 cells. The cells were assayed for E protein expression. (E) Plaque morphology. Viruses derived from the transfected cells in (D) were analyzed by plaque assay. (F) Viral titers. Culture supernatants from (D) were measured for virus yields on day 5 post-transfection. (G) Replicon transient transfection assay. NS2A triple mutations (R94A+K95A+K99A) were engineered into a luciferase replicon of DENV-2. Luciferase activities were presented after the WT or mutant replicon RNAs were electroporated into BHK-21 cells. (H) Diagram of N-terminally GST-tagged NS2A peptide 64-128 [GST-NS2A (64-128)]. (I) Interaction of GST-NS2A (64-128) protein with 3'UTR RNA. GST-NS2A (64-128) was expressed in *E. coli* and affinity purified. The pulldown efficiency of GST-NS2A (64-128) by 5'UTR-$C_{66}$-3'UTR-biotin was set as 100%. The binding efficiencies of RNA variants were indicated through competition assay. In the presence of competitor RNAs, the remaining amounts of WT GST-NS2A (64-128)/5'UTR-$C_{66}$-3'UTR-biotin interaction are presented. (J) R94A+K95A+K99A mutations reduce GST-NS2A (64-128) binding to 5'UTR-$C_{66}$-3'UTR-biotin RNA. The R94A+K95A+K99A variant retained only 19.1% of the WT activity.

**Figure 7 A model depicting the role of NS2A in DENV-2 assembly.** Viral replication complex (top) and virion assembly site (bottom) are depicted. DENV-2 NS2A protein recruits viral RNA, three structural proteins, and NS2B/NS3 protease to virion assembly site, where coordinated cleavage of C-prM-E ensues infections virus formation. C molecules accumulated on lipid droplets (LD) could bind viral RNA to form nucleocapsid. See text for details.



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**Reference**

[ ADDIN EN.REFLIST ]



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## STAR METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---|---|---|
| Antibodies | | |
| Mouse IgG Anti-C (D2C1) | [ ADDIN EN.CITE ADDIN EN.CITE.DATA ] | N/A |
| Mouse IgG anti-prM (DM1) | Abcam | Cat# ab41473, RRID:AB_873758 |
| Rabbit IgG anti-prM | GeneTex | GTX128093 |
| Mouse IgG anti-E (4G2) | ATCC | Cat# HB-112, RRID:CVCL_J890 |
| Mouse IgG anti-E (9F12) | [ ADDIN EN.CITE ADDIN EN.CITE.DATA ] | N/A |
| Mouse IgG anti-NS1 | Abcam | Cat# ab41616, RRID:AB_869448 |
| Human IgG anti-NS1 | This study. Generated by Novartis Institute for Tropical Diseases in Singapore | N/A |
| Rabbit IgG anti-NS1 | GeneTex | GTX103346 |
| Rabbit IgG anti-NS3 | GeneTex | GTX124252 |
| Mouse IgG anti-NS5 | GeneTex | GTX629447 |
| Rabbit IgG anti-calnexin | SIGMA-AlDRICH | Cat# C4731, RRID:AB_476845 |
| Mouse IgG anti-EGFP | SIGMA-AlDRICH | Cat# 11814460001, RRID:AB_390913 |
| Moue IgG1 Isotype Control | SIGMA-AlDRICH | Cat# M5284, RRID:AB_1163685 |
| Rabbit IgG anti-EGFP | Abcam | Cat# ab13970, RRID:AB_300798 |
| protein G horseradish peroxidase (HRP) conjugate | Thermo Fisher Scientific | Cat# P21041 |
| Mouse IgG anti-GST | Abcam | Cat# ab19256, RRID:AB_444809 |
| Rabbit IgG anti-GST | SIGMA-AlDRICH | Cat# G7781, RRID:AB_259965 |
| Mouse IgG anti-β-actin | Abcam | Cat# ab8226, RRID:AB_306371 |
| Mouse IgG anti-HA | Abcam | Cat# ab18181, RRID:AB_444303 |
| Goat anti-mouse IgG conjugated with HRP | SIGMA-AlDRICH | Cat# A5278, RRID:AB_258232 |
| Goat anti-rabbit IgG conjugated with HRP | SIGMA-AlDRICH | Cat#AP307P, RRID:AB_92641 |
| Goat anti-mouse IgGs conjugated with Alexa 488 | Thermo Fisher Scientific | Cat#A-1100, RRID:AB_2534069 |
| Goat anti-mouse IgGs conjugated with Alexa 594 | Thermo Fisher Scientific | Cat# A-11005, RRID:AB_2534073 |
| Goat anti-rabbit IgGs conjugated with Alexa 488 | Thermo Fisher Scientific | Cat# A-11008, RRID:AB_143165 |



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| | | |
|---|---|---|
| Goat anti-rabbit IgGs conjugated with Alexa 594 | Thermo Fisher Scientific | Cat# A-11072, RRID:AB_2534116 |
| Goat anti-Human IgG (H+L) cross-adsorbed secondary antibody, Alexa Fluor 594 | Thermo Fisher Scientific | Cat# A-11014, RRID:AB_2534081 |
| Bacterial and Virus Strains | | |
| *E.* coli strain Top 10 | ThermoFisher Scientific | Cat#C404006 |
| *E.* coli strain Rosetta™ 2(DE3) pLysS | MilliporeSigma | Cat#71403-4 |



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| | | |
|---|---|---|
| DENV-2 strain New Guinea C | [ ADDIN EN.CITE <EndNote><Cite><Author>Xie</Author><Year>2013</Year><RecNum>85</RecNum><DisplayText>(Xie et al., 2013)</DisplayText><record><rec-number>85</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793217">85</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Xie, X.</author><author>Gayen, S.</author><author>Kang, C.</author><author>Yuan, Z.</author><author>Shi, P. Y.</author></authors></contributors><auth-address>State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Science, China.</auth-address><titles><title>Membrane topology and function of dengue virus NS2A protein</title><secondary-title>J Virol</secondary-title><alt-title>Journal of virology</alt-title></titles><periodical><full-title>J Virol</full-title></periodical><pages>4609-22</pages><volume>87</volume><number>8</number><edition>2013/02/15</edition><keywords><keyword>Animals</keyword><keyword>Cell Line</keyword><keyword>Cricetinae</keyword><keyword>Dengue | N.A. |



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| | | |
|---|---|---|
| Biological Samples | | |
| None | | |
| Chemicals, Peptides, and Recombinant Proteins | | |
| Peptide WT (NILNRRRRTAGMII) | GenScript | N/A |
| Peptide C_R99S (NILNRRSRTAGMII) | GenScript | N/A |
| Recombinant protein: GST | This study | N/A |
| Recombinant protein: GST-NS2A | This study | N/A |
| Recombinant protein: GST-NS2A(1-71) | This study | N/A |
| Recombinant protein: GST-NS2A(1-101) | This study | N/A |
| Recombinant protein: GST-NS2A(1-145) | This study | N/A |
| Recombinant protein: GST-NS2A(1-168) | This study | N/A |
| Recombinant protein: GST-NS2A(64-128) | This study | N/A |
| Critical Commercial Assays | | |
| iScript SYBR Green One-Step kit | Bio-Rad | Cat#170-8893 |
| QIAamp viral RNA mini kit | Qiagen | Cat#52906 |
| T7 mMessage mMachine kit | Thermo Fisher Scientific | Cat# AM1344 |
| Renilla Luciferase Assay System | Promega Corporation | Cat# E2810 |
| Pierce™ RNA 3' End Biotinylation Kit | Thermo Fisher Scientific | Cat# 20160 |
| Pierce™ Magnetic RNA-Protein Pull-Down kit | Thermo Fisher Scientific | Cat# 20164 |
| Chemiluminescent Detection Module | Thermo Fisher Scientific | Cat# 89880 |
| | | |
| Deposited Data | | |
| Raw image data for illumina sequencing | | |
| Experimental Models: Cell Lines | | |
| BHK-21 cell | ATCC | Cat#CCL-10, RRID:CVCL_1915 |
| Vero cell | ATCC | Cat#CCL-81, RRID:CVCL_0059 |
| HEK293T cell | ATCC | Cat# CRL-3216, RRID:CVCL_0063 |
| NS2A-EGFP BHK-21 cell | [ ADDIN EN.CITE ADDIN EN.CITE.DATA ] | N/A |
| | | |
| Experimental Models: Organisms/Strains | | |
| N.A. | | |
| Oligonucleotides | | |
| Primer P1(5'-ACACTCTTTCCCTACACGA CGCTCTTCCGATCTAAGGCAAAACTAACATGAA AC-3') | Integrated DNA Technologies (Skokie, Illinois) | N/A |
| Primer P2 (5'-GTGACTGGAGTTCAGACG TGTGCTCTTCCGATCTAGAACCTGTTGATTCAA CAGCAC-3') | Integrated DNA Technologies (Skokie, Illinois) | N/A |



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| | | |
|---|---|---|
| 5' universal adaptor (5'-AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGAC-3') | Illumine (San Diego, CA) | N/A |
| 3' indexing primer (5'-CAAGCAGAAGACGGCATACGAGATnnnnnnGTGACTGGAGTTCAGACGTGT-3') | Illumine (San Diego, CA) | N/A |
| Primer NGC 7764V (5'-CGTCGAGAGAAATATGGTCACACC-3') | Integrated DNA Technologies (Skokie, Illinois) | N/A |
| Primer NGC 7844C (5'CCACAATAGTATGACCAGCCT-3') | Integrated DNA Technologies (Skokie, Illinois) | N/A |
| Recombinant DNA | | |



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| | | |
|---|---|---|
| pACYC-NGC FL | [ ADDIN EN.CITE <EndNote><Cite><Author>Xie</Author><Year>2013</Year><RecNum>85</RecNum><DisplayText>(Xie et al., 2013)</DisplayText><record><rec-number>85</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793217">85</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Xie, X.</author><author>Gayen, S.</author><author>Kang, C.</author><author>Yuan, Z.</author><author>Shi, P. Y.</author></authors></contributors><auth-address>State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Science, China.</auth-address><titles><title>Membrane topology and function of dengue virus NS2A protein</title><secondary-title>J Virol</secondary-title><alt-title>Journal of virology</alt-title></titles><periodical><full-title>J Virol</full-title></periodical><pages>4609-22</pages><volume>87</volume><number>8</number><edition>2013/02/15</edition><keywords><keyword>Animals</keyword><keyword>Cell Line</keyword><keyword>Cricetinae</keyword><keyword>Dengu | N/A |



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| | | |
|---|---|---|
| pACYC-NGC replicon | [ ADDIN EN.CITE <EndNote><Cite><Author>Xie</Author><Year>2013</Year><RecNum>85</RecNum><DisplayText>(Xie et al., 2013)</DisplayText><record><rec-number>85</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793217">85</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Xie, X.</author><author>Gayen, S.</author><author>Kang, C.</author><author>Yuan, Z.</author><author>Shi, P. Y.</author></authors></contributors><auth-address>State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Science, China.</auth-address><titles><title>Membrane topology and function of dengue virus NS2A protein</title><secondary-title>J Virol</secondary-title><alt-title>Journal of virology</alt-title></titles><periodical><full-title>J Virol</full-title></periodical><pages>4609-22</pages><volume>87</volume><number>8</number><edition>2013/02/15</edition><keywords><keyword>Animals</keyword><keyword>Cell Line</keyword><keyword>Cricetinae</keyword><keyword>Dengue | N/A |



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| | | |
|---|---|---|
| pACYC-NGC replicon G11A | [ ADDIN EN.CITE ADDIN EN.CITE.DATA ] | N/A |
| pACYC-NGC replicon R94A+K95A+K99A | This study | N/A |
| pACYC-NGC FL C_R99S | This study | N/A |
| pACYC-NGC FL G11A | This study | N/A |
| pACYC-NGC FL G11A+C_R99S | This study | N/A |
| pACYC-NGC FL E20A | This study | N/A |
| pACYC-NGC FL E100A | This study | N/A |
| pACYC-NGC FL K188A | This study | N/A |
| pACYC-NGC FL E20A+C_R99S | This study | N/A |
| pACYC-NGC FL E100A+C_R99S | This study | N/A |
| pACYC-NGC FL K188A+C_R99S | This study | N/A |
| pACYC-NGC FL C_TA | This study | N/A |
| pACYC-NGC FL C_SR | This study | N/A |
| pACYC-NGC FL G11A+C_TA | This study | N/A |
| pACYC-NGC FL G11A+C_SR | This study | N/A |
| pACYC-NGC FL C_T101 | This study | N/A |
| pACYC-NGC FL G11A+C_T101 | This study | N/A |
| pACYC-NGC FL R94A | This study | N/A |
| pACYC-NGC FL K95A | This study | N/A |
| pACYC-NGC FL K99A | This study | N/A |
| pACYC-NGC FL R94A+K95A+K99A | This study | N/A |
| pACYC-NGC FL R94A+K95A | This study | N/A |



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| | | |
|---|---|---|
| SFV-DENV2 CprME | [ ADDIN EN.CITE <EndNote><Cite><Author>Qing</Author><Year>2010</Year><RecNum>105</RecNum><DisplayText>(Qing et al., 2010)</DisplayText><record><rec-number>105</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793220">105</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Qing, M.</author><author>Liu, W.</author><author>Yuan, Z.</author><author>Gu, F.</author><author>Shi, P. Y.</author></authors></contributors><auth-address>Novartis Institute for Tropical Diseases, 10, Biopolis Road, #05-01 Chromos, Singapore, Singapore.</auth-address><titles><title>A high-throughput assay using dengue-1 virus-like particles for drug discovery</title><secondary-title>Antiviral Res</secondary-title></titles><periodical><full-title>Antiviral Res</full-title></periodical><pages>163-71</pages><volume>86</volume><number>2</number><dates><year>2010</year><pub-dates><date>May</date></pub-dates></dates><accession-num>20153777</accession-num><urls><related- | N/A |



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| | | |
|---|---|---|
| pXJ-SPG-$C_{16}$-NS2A-EGFP | This study | N/A |
| pXJ-SPG-$C_{16}$-NS2A(G11A)-EGFP | This study | N/A |
| pXJ-EGFP | [ ADDIN EN.CITE ADDIN EN.CITE.DATA ] | N/A |
| pXJ-$C_{14}$-prM | This study | N/A |
| pXJ-$C_{14}$-prM(1-130) | This study | N/A |
| pXJ-$C_{14}$-prM (N69Q) | This study | N/A |
| pXJ-SPG-$C_{16}$-NS2A-GST | This study | N/A |
| pXJ-SPG-$C_{16}$-NS2A(G11A)-GST | This study | N/A |
| pXJ-GST | This study | N/A |
| pXJ-HA-NS3 | This study | N/A |
| pXJ-$M_{17}$-E | This study | N/A |
| pGEX-4T-1 | GE Healthcare Life Sciences | Cat# 28954549 |
| pGEX-4T-GST-NS2A | This study | N/A |
| pGEX-4T-GST-NS2A (1-71) | This study | N/A |
| pGEX-4T-GST-NS2A (1-101) | This study | N/A |
| pGEX-4T-GST-NS2A (1-145) | This study | N/A |
| pGEX-4T-GST-NS2A (1-168) | This study | N/A |
| pGEX-4T-GST-NS2A (64-128) | This study | N/A |
| pGEX-4T-GST-NS2A (64-128) R94A+K95A+K99A | This study | N/A |
| | | |
| Software and Algorithms | | |
| Illustrator CC | Adobe | [ HYPERLINK "https://www.adobe.com/products/illustrator.html" ] |
| ImageJ | NIH | [ HYPERLINK "https://fiji.sc/" ] |
| CLC main workbench | Qiagen | [ HYPERLINK "https://www.qiagenbioinformatics.com/products/clc-main-workbench/" ] |
| Prism 8.0 software | GraphPad | [ HYPERLINK "https://www.graphpad.com/scientific-software/prism/" ] |
| | | |

1271

1272 **Contact for Reagent and Resource Sharing**

1273 Further information and requests for resources and reagents should be directed to and will
1274 be fulfilled by Lead Contact, Dr. Pei-Yong Shi (peshi@utmb.edu)

1275 **Experimental Model and Subject Details**



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**Cell Lines**

The Baby hamster kidney fibroblast (BHK-21) cells, African green monkey kidney epithelial (Vero) cells were purchased from the American Type Culture Collection (ATCC, Bethesda, MD) and maintained in a high-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (HyClone Laboratories, South Logan, UT) and 1% penicillin/streptomycin (P/S). The NS2A-EGFP BHK-21 cells constitutively expressing the C-terminally EGFP-tagged NS2A protein was maintained in the high-glucose DMEM medium supplemented with 10%FBS, 1% P/S and 1 mg/ml Geneticin. HEK293T cells were grown in low-glucose DMEM (Invitrogen) containing 10% FBS and 1% penicillin-streptomycin. All mammalian cell lines were grown at 37°C with 5% $CO_2$. Mosquito C6/36 cells were grown in RPMI 1640 medium (Invitrogen) with 10% FBS and 1% penicillin-streptomycin at 28°C without $CO_2$. All culture medium and antibiotics were purchased from ThermoFisher Scientific (Waltham, MA). All cell lines were tested negative for mycoplasma.

**Pseudo-infectious Particle**

Pseudo-infectious particles were produced using the a previously described protocol [ ADDIN EN.CITE <EndNote><Cite><Author>Qing</Author><Year>2010</Year><RecNum>114</RecNum><DisplayText>(Qing et al., 2010)</DisplayText><record><rec-number>114</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1561745637">114</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Qing, M.</author><author>Liu, W.</author><author>Yuan, Z.</author><author>Gu, F.</author><author>Shi, P. Y.</author></authors></contributors><auth-address>Novartis Institute for Tropical Diseases, 10, Biopolis Road, #05-01 Chromos, Singapore, Singapore.</auth-address><titles><title>A high-throughput assay using dengue-1 virus-like particles for drug discovery</title><secondary-title>Antiviral Res</secondary-title></titles><periodical><full-title>Antiviral Res</full-title></periodical><pages>163-71</pages><volume>86</volume><number>2</number><keywords><keyword>Animals</keyword><keyword>Antiviral Agents/*isolation & purification/*pharmacology</keyword><keyword>Biological Assay/methods</keyword><keyword>Cell Line</keyword><keyword>Cercopithecus



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aethiops</keyword><keyword>Cricetinae</keyword><keyword>Dengue Virus/*drug effects/genetics</keyword><keyword>Drug Discovery/*methods</keyword><keyword>Drug Evaluation, Preclinical/*methods</keyword><keyword>Electroporation</keyword><keyword>Genes, Reporter</keyword><keyword>Luciferases, Renilla/genetics/metabolism</keyword><keyword>Macaca mulatta</keyword><keyword>Viral Proteins/genetics/metabolism</keyword><keyword>Virosomes/*drug effects/genetics</keyword></keywords><dates><year>2010</year><pub-dates><date>May</date></pub-dates></dates><isbn>1872-9096 (Electronic)0166-3542 (Linking)</isbn><accession-num>20153777</accession-num><urls><related-urls><url>https://www.ncbi.nlm.nih.gov/pubmed/20153777</url></related-urls></urls><electronic-resource-num>10.1016/j.antiviral.2010.02.313</electronic-resource-num></record></Cite></EndNote>] with several modifications. 10 µg of replicon RNA was electroporated into BHK-21 cells as described above. After transfection, the cells were maintained at 37°C with 5% $CO_2$ for 24 h. Afterwards, cells were harvested and electroporated with 10 µg of a helper replicon RNA SFV-DENV2 CprME (replicon derived from Semliki forest virus that express the DENV-2 encoding the CprME). After the second electroporation, cells were cultured at 30°C with 5% $CO_2$. At 20, 28 and 48 h after the second electroporation, culture medium was collected and stored at -80 °C. Afterwards, the collected medium was used ton infection naïve Vero cells in 96-well plates. At 48 h post-infection, cells were lysed, and luciferase signals were measured using the Renilla luciferase assay system (Promega) in the Clarity luminescence microplate reader (BioTek) according to the manufacturer's protocol.

## METHOD DETAILS

### Plasmid Construction

The full-length cDNA infectious clone of DENV-2 strain New Guinea C (NGC), pACYC-NGC FL, and its derived replicon (pACYC-NGC replicon) clone [ ADDIN EN.CITE <EndNote><Cite><Author>Xie</Author><Year>2013</Year><RecNum>85</RecNum><DisplayText>(Xie et al., 2013)</DisplayText><record><rec-number>85</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793217">85</key></foreign-keys><ref-type name="Journal



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Article">17</ref-type><contributors><authors><author>Xie, X.</author><author>Gayen, S.</author><author>Kang, C.</author><author>Yuan, Z.</author><author>Shi, P. Y.</author></authors></contributors><auth-address>State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Science, China.</auth-address><titles><title>Membrane topology and function of dengue virus NS2A protein</title><secondary-title>J Virol</secondary-title><alt-title>Journal of virology</alt-title></titles><periodical><full-title>J Virol</full-title></periodical><pages>4609-22</pages><volume>87</volume><number>8</number><edition>2013/02/15</edition><keywords><keyword>Animals</keyword><keyword>Cell Line</keyword><keyword>Cricetinae</keyword><keyword>Dengue Virus/*physiology</keyword><keyword>Endoplasmic Reticulum/*virology</keyword><keyword>Humans</keyword><keyword>Intracellular Membranes/*metabolism</keyword><keyword>Magnetic Resonance Spectroscopy</keyword><keyword>Models, Molecular</keyword><keyword>Protein Conformation</keyword><keyword>Viral Nonstructural Proteins/*chemistry/metabolism</keyword><keyword>*Virus Assembly</keyword></keywords><dates><year>2013</year><pub-dates><date>Apr</date></pub-dates></dates><isbn>1098-5514 (Electronic)0022-538X (Linking)</isbn><accession-num>23408612</accession-num><work-type>Research Support, Non-U.S. Gov't</work-type><urls><related-urls><url>http://www.ncbi.nlm.nih.gov/pubmed/23408612</url></related-urls></urls><custom2>3624351</custom2><electronic-resource-num>10.1128/JVI.02424-12</electronic-resource-num><language>eng</language></record></Cite></EndNote>] were used as the backbones for mutagenesis study. The replicon was constructed by replacing most of the structural genes (C, prM and E) with a gene cassette (Rluc2A) encoding the Renilla luciferase and a 2A peptide from Foot-and-mouth disease virus. The first 38 amino acids of C protein and the last 31 amino acids of E protein were retained in the replicon. Standard overlap PCR was performed to introduce the mutations in C and NS2A genes. The DNA fragments containing the mutations in C gene were cloned into pACYC-NGC FL clone through restriction enzyme sites NotI (upstream of the T7 promoter and 5'UTR of DENV-2) and SphI (at the position of 1384 in DENV-2 genome). The DNA fragments containing mutations in NS2A gene were cloned into pACYC-NGC FL or replicon through restriction enzyme sites and NheI (at the position of 2544 in DENV-2 genome) and SacI (at the position of 4342 in the DENV-2 genome).



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A mammalian expression vector, pXJ [ ADDIN EN.CITE <EndNote><Cite><Author>Xie</Author><Year>2013</Year><RecNum>85</RecNum><DisplayText>(Xie et al., 2013)</DisplayText><record><rec-number>85</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793217">85</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Xie, X.</author><author>Gayen, S.</author><author>Kang, C.</author><author>Yuan, Z.</author><author>Shi, P. Y.</author></authors></contributors><auth-address>State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Science, China.</auth-address><titles><title>Membrane topology and function of dengue virus NS2A protein</title><secondary-title>J Virol</secondary-title><alt-title>Journal of virology</alt-title></titles><periodical><full-title>J Virol</full-title></periodical><pages>4609-22</pages><volume>87</volume><number>8</number><edition>2013/02/15</edition><keywords><keyword>Animals</keyword><keyword>Cell Line</keyword><keyword>Cricetinae</keyword><keyword>Dengue Virus/*physiology</keyword><keyword>Endoplasmic Reticulum/*virology</keyword><keyword>Humans</keyword><keyword>Intracellular Membranes/*metabolism</keyword><keyword>Magnetic Resonance Spectroscopy</keyword><keyword>Models, Molecular</keyword><keyword>Protein Conformation</keyword><keyword>Viral Nonstructural Proteins/*chemistry/metabolism</keyword><keyword>*Virus Assembly</keyword></keywords><dates><year>2013</year><pub-dates><date>Apr</date></pub-dates></dates><isbn>1098-5514 (Electronic)0022-538X (Linking)</isbn><accession-num>23408612</accession-num><work-type>Research Support, Non-U.S. Gov't</work-type><urls><related-urls><url>http://www.ncbi.nlm.nih.gov/pubmed/23408612</url></related-urls></urls><custom2>3624351</custom2><electronic-resource-num>10.1128/JVI.02424-12</electronic-resource-num><language>eng</language></record></Cite></EndNote>], driven by a cytomegalovirus (CMV) promoter was used to express various viral proteins prM, E, NS2A and NS3 fusion proteins in HEK293T cells. For constructs pXJ-$C_{14}$-prM and pXJ-$C_{14}$-prM (1-130), the anchor C signal peptide ($C_{14}$) was retained at the N-terminus of prM to ensure the correct targeting on ER membrane. For construct pXJ-$M_{17}$-E, the last 17 amino acids from M protein was retained to serve as a signal peptide for translocating the E protein into the ER lumen. For constructs pXJ-SPG-$C_{16}$-NS2A-EGFP and pXJ-SPG-$C_{16}$-



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NS2A-GST, the signal peptide derived from Gaussia luciferase (SPG) [ ADDIN EN.CITE <EndNote><Cite><Author>Verhaegent</Author><Year>2002</Year><RecNum>112</RecNum><DisplayText>(Verhaegent and Christopoulos, 2002)</DisplayText><record><rec-number>112</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1561735944">112</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Verhaegent, M.</author><author>Christopoulos, T. K.</author></authors></contributors><auth-address>Department of Chemistry and Biochemistry, University of Windsor, Ontario, Canada.</auth-address><titles><title>Recombinant Gaussia luciferase. Overexpression, purification, and analytical application of a bioluminescent reporter for DNA hybridization</title><secondary-title>Anal Chem</secondary-title></titles><periodical><full-title>Anal Chem</full-title></periodical><pages>4378-85</pages><volume>74</volume><number>17</number><keywords><keyword>Animals</keyword><keyword>DNA/*analysis</keyword><keyword>DNA Probes</keyword><keyword>Genes, Reporter</keyword><keyword>*Luciferases/genetics/isolation & purification</keyword><keyword>Luminescent Proteins/genetics/isolation & purification</keyword><keyword>Plankton/*enzymology</keyword><keyword>Recombinant Proteins/genetics/isolation & purification</keyword></keywords><dates><year>2002</year><pub-dates><date>Sep 1</date></pub-dates></dates><isbn>0003-2700 (Print)0003-2700 (Linking)</isbn><accession-num>12236345</accession-num><urls><related-urls><url>https://www.ncbi.nlm.nih.gov/pubmed/12236345</url></related-urls></urls></record></Cite></EndNote>] and the last 16 amino acids of NS1 ($C_{16}$) was fused at the N-terminus of NS2A to ensure the correct processing and membrane topology of NS2A. The EGFP and GST were amplified from pEGFPluc (Clontech) and pGEX-4T-1 (Sigma-Aldrich) respectively. The Human influenza hemagglutinin (HA) tag was fused to the N-terminus of NS3 and cloned into pXJ vector, resulting the construct pXJ-HA-NS3.

pGEX-4T-1 (GE Healthcare Life Sciences) was used as the vector for expressing all N-terminally glutathione S-transferase (GST)-tagged NS2A fusion proteins. The DNA fragments containing various length of NS2A were cloned into pGEX-4T-1 through restriction enzyme sites BamHI and XhoI.



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All plasmids were propagated in *E. coli* strain Top10 (ThermoFisher Scientific). All restriction enzymes were purchased from New England BioLabs (Ipswitch, MA). The plasmids were validated by restriction enzyme digestion and Sanger DNA sequencing. All primers were synthesized from Integrated DNA Technologies (Skokie, Illinois) and are available upon request.

**RNA Transcription and Electroporation**

The NGC FL or replicon plasmids were linearized by restriction enzyme XbaI. RNA transcription and electroporation were performed as described previously [ ADDIN EN.CITE <EndNote><Cite><Author>Xie</Author><Year>2013</Year><RecNum>85</RecNum><DisplayText>(Xie et al., 2013)</DisplayText><record><rec-number>85</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793217">85</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Xie, X.</author><author>Gayen, S.</author><author>Kang, C.</author><author>Yuan, Z.</author><author>Shi, P. Y.</author></authors></contributors><auth-address>State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Science, China.</auth-address><titles><title>Membrane topology and function of dengue virus NS2A protein</title><secondary-title>J Virol</secondary-title><alt-title>Journal of virology</alt-title></titles><periodical><full-title>J Virol</full-title></periodical><pages>4609-22</pages><volume>87</volume><number>8</number><edition>2013/02/15</edition><keywords><keyword>Animals</keyword><keyword>Cell Line</keyword><keyword>Cricetinae</keyword><keyword>Dengue Virus/*physiology</keyword><keyword>Endoplasmic Reticulum/*virology</keyword><keyword>Humans</keyword><keyword>Intracellular Membranes/*metabolism</keyword><keyword>Magnetic Resonance Spectroscopy</keyword><keyword>Models, Molecular</keyword><keyword>Protein Conformation</keyword><keyword>Viral Nonstructural Proteins/*chemistry/metabolism</keyword><keyword>*Virus Assembly</keyword></keywords><dates><year>2013</year><pub-dates><date>Apr</date></pub-dates></dates><isbn>1098-5514 (Electronic)0022-538X (Linking)</isbn><accession-num>23408612</accession-num><work-type>Research Support, Non-U.S. Gov't</work-type><urls><related-



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urls><url>http://www.ncbi.nlm.nih.gov/pubmed/23408612</url></related-urls></urls><custom2>3624351</custom2><electronic-resource-num>10.1128/JVI.02424-12</electronic-resource-num><language>eng</language></record></Cite></EndNote>]. Briefly, 10 μg RNA was electroporated into 8 × $10^6$ cells in a 4-mm cuvette using the GenePulser apparatus (Bio-Rad) at settings of 0.45 kV (C6/36 cells) or 0.85 kV (BHK-21 cells) and 25 mF, pulsing three times, with 3 s intervals. After a 10-min recovery at room temperature, transfected cells were suspended in culture media and incubated at corresponding conditions: 28°C (C6/36 cells) or 37°C with 5% $CO_2$ (BHK-21).

**Virus Production and Quantification**

After electroporation, the transfected BHK-21 cells were seeded in one T-75 flask. After incubation at 37°C for 24 h, the cells were cultured in DMEM with 2% FBS at 30°C for another 4 days. At every 24 h p.t., culture fluids was collected and stored at -80°C. Viral titer was determined by crystal violet-based plaque assay using BHK-21 cells according to the protocol as described previously [ ADDIN EN.CITE <EndNote><Cite><Author>Xie</Author><Year>2013</Year><RecNum>85</RecNum><DisplayText>(Xie et al., 2013)</DisplayText><record><rec-number>85</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793217">85</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Xie, X.</author><author>Gayen, S.</author><author>Kang, C.</author><author>Yuan, Z.</author><author>Shi, P. Y.</author></authors></contributors><auth-address>State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Science, China.</auth-address><titles><title>Membrane topology and function of dengue virus NS2A protein</title><secondary-title>J Virol</secondary-title><alt-title>Journal of virology</alt-title></titles><periodical><full-title>J Virol</full-title></periodical><pages>4609-22</pages><volume>87</volume><number>8</number><edition>2013/02/15</edition><keywords><keyword>Animals</keyword><keyword>Cell Line</keyword><keyword>Cricetinae</keyword><keyword>Dengue Virus/*physiology</keyword><keyword>Endoplasmic Reticulum/*virology</keyword><keyword>Humans</keyword><keyword>Intracellular Membranes/*metabolism</keyword><keyword>Magnetic Resonance Spectroscopy</keyword><keyword>Models, Molecular</keyword><keyword>Protein Conformation</keyword><keyword>Viral Nonstructural



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Proteins/*chemistry/metabolism</keyword><keyword>*Virus Assembly</keyword></keywords><dates><year>2013</year><pub-dates><date>Apr</date></pub-dates></dates><isbn>1098-5514 (Electronic)0022-538X (Linking)</isbn><accession-num>23408612</accession-num><work-type>Research Support, Non-U.S. Gov't</work-type><urls><related-urls><url>http://www.ncbi.nlm.nih.gov/pubmed/23408612</url></related-urls></urls><custom2>3624351</custom2><electronic-resource-num>10.1128/JVI.02424-12</electronic-resource-num><language>eng</language></record></Cite></EndNote>]. Plaques were developed on day 5 post-infection.

**RNA extraction and RT-PCR**

Viral RNAs in culture fluids were extracted using a QIAamp viral RNA minikit (Qiagen). Extracted RNAs were eluted in 50 μl RNase-free water. Reverse transcription (RT)-qPCR was performed to measure the amounts of viral RNA copy in the culture medium. Viral RNAs were measured using an iScript one-Step RT-PCR kit with SYBR green (Bio-Rad) and the primer pair NGC 7764V (sequence: 5'-CGTCGAGAGAAATATGGTCACACC-3') and 7844C (sequence: 5'CCACAATAGTATGACCAGCCT-3') [ ADDIN EN.CITE <EndNote><Cite><Author>Xie</Author><Year>2013</Year><RecNum>85</RecNum><DisplayText>(Xie et al., 2013)</DisplayText><record><rec-number>85</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793217">85</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Xie, X.</author><author>Gayen, S.</author><author>Kang, C.</author><author>Yuan, Z.</author><author>Shi, P. Y.</author></authors></contributors><auth-address>State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Science, China.</auth-address><titles><title>Membrane topology and function of dengue virus NS2A protein</title><secondary-title>J Virol</secondary-title><alt-title>Journal of virology</alt-title></titles><periodical><full-title>J Virol</full-title></periodical><pages>4609-22</pages><volume>87</volume><number>8</number><edition>2013/02/15</edition><keywords><keyword>Animals</keyword><keyword>Cell Line</keyword><keyword>Cricetinae</keyword><keyword>Dengue Virus/*physiology</keyword><keyword>Endoplasmic Reticulum/*virology</keyword><keyword>Humans</keyword><keyword>Intracellular Membranes/*metabolism</keyword><keyword>Magnetic Resonance



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Spectroscopy</keyword><keyword>Models, Molecular</keyword><keyword>Protein Conformation</keyword><keyword>Viral Nonstructural Proteins/*chemistry/metabolism</keyword><keyword>*Virus Assembly</keyword></keywords><dates><year>2013</year><pub-dates><date>Apr</date></pub-dates></dates><isbn>1098-5514 (Electronic)0022-538X (Linking)</isbn><accession-num>23408612</accession-num><work-type>Research Support, Non-U.S. Gov't</work-type><urls><related-urls><url>http://www.ncbi.nlm.nih.gov/pubmed/23408612</url></related-urls></urls><custom2>3624351</custom2><electronic-resource-num>10.1128/JVI.02424-12</electronic-resource-num><language>eng</language></record></Cite></EndNote>]. Alternatively, the extracted viral RNA was reversely transcribed and amplified using SuperScript One-Step (RT)-PCR with Platinum Taq (Invitrogen). The RT-PCR products were then gel purified and subjected to Sanger DNA sequencing.

**Replicon Transient Transfection**

After electroporation of replicon RNA into BHK-21 cells, the cells were resuspended in culture medium and seeded in a 12-well plate ($2 \times 10^5$ cells/well). At various time points, the cells were washed once with phosphate-buffered saline (PBS) and lysed using lysis buffer (Promega). The plates containing the lysis buffer were sealed with Parafilm and stored at -80°C. Once samples for all time points had been collected, luciferase signals were measured in a Clarity luminescence microplate reader (BioTek) according to the manufacturer's protocol. The luciferase signals at early time points (2-6 h post-transfection) represent the translation of input viral RNA; and the luciferase signals at later points (24-46 h post-transfection) represent the stage of RNA replication.

**Immunofluorescence assay (IFA)**

Cells were seeded in an 8-well Lab-Tek II chamber slide (ThermoFisher Scientific). At given time points, cells were fixed in 4% paraformaldehyde in PBS for 20 min at room temperature. Afterwards, cells were permeabilized by 0.1% Triton X-100 (Sigma-Aldrich) for 5 min followed by 1-h incubation in blocking buffer containing 1% FBS and 0.05% Tween-20 (Sigma-Aldrich) in PBS. Cells were then incubated with primary antibodies (diluted in the blocking buffer) at room temperature for 1 h. After three PBS washes, cells were incubated with goat anti-mouse or rabbit IgG (1:1000 diluted in blocking buffer) conjugated with Alexa Fluor 488 or 594 (ThermoFisher Scientific) for 1 h in blocking buffer.



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Cells were finally washed by PBS for three times, counter-stained by Hoechst 33342 Solution (ThermoFisher Scientific) and mounted in ProLong® Diamond Antifade Mountant (ThermoFisher Scientific). Epifluorescence images were acquired by Leica DM4000 B system. Confocal fluorescence images were acquired by Zeiss LSM 5 META systems.

**Virus Revertant Selection**

For selecting revertants from G11A-transfected cells, we consecutively passaged the transfected cells for 5 rounds. In brief, after electroporation, the cells were seeded in 96-well plates and incubate at 30°C for 3 days. Afterwards, cells for each well were detached by trypsin and suspended in culture medium. About 20% of the suspended cells were transferred into the well of new 96-well plates and incubated at 37°C for one day. The cells were then incubated at 30°C for two additional days. Again, the confluent cells were detached for next passage. At every passage, the supernatants were collected. After 5 rounds of passaging, IFA was performed to determine the intracellular E protein expression by 4G2 antibody. Plaque assay was performed to examine the amounts of infectious virus particle in the culture fluids. Viral RNA from the P5 supernatants were extracted and used as template for RT-PCR. Sanger sequencing was performed to sequence the entire viral genome.

**Co-immunoprecipitation (Co-IP)**

The NS2A/prM, NS2A/E and NS2A/NS3 interactions were examined by Co-IP analysis according to the protocol as described previously[ ADDIN EN.CITE <EndNote><Cite><Author>Zou</Author><Year>2014</Year><RecNum>113</RecNum><DisplayText>(Zou et al., 2014)</DisplayText><record><rec-number>113</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1561742592">113</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Zou, J.</author><author>Xie, X.</author><author>Lee le, T.</author><author>Chandrasekaran, R.</author><author>Reynaud, A.</author><author>Yap, L.</author><author>Wang, Q. Y.</author><author>Dong, H.</author><author>Kang, C.</author><author>Yuan, Z.</author><author>Lescar, J.</author><author>Shi, P. Y.</author></authors></contributors><auth-address>State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Sciences, Wuhan, China.</auth-address><titles><title>Dimerization of flavivirus NS4B protein</title><secondary-title>J



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Virol</secondary-title></titles><periodical><full-title>J Virol</full-title></periodical><pages>3379-91</pages><volume>88</volume><number>6</number><keywords><keyword>Amino Acid Motifs</keyword><keyword>Dengue/*virology</keyword><keyword>Dengue Virus/chemistry/genetics/*metabolism</keyword><keyword>Dimerization</keyword><keyword>Humans</keyword><keyword>Viral Nonstructural Proteins/*chemistry/genetics/*metabolism</keyword><keyword>West Nile Fever/*virology</keyword><keyword>West Nile virus/chemistry/genetics/*metabolism</keyword></keywords><dates><year>2014</year><pub-dates><date>Mar</date></pub-dates></dates><isbn>1098-5514 (Electronic)0022-538X (Linking)</isbn><accession-num>24390334</accession-num><urls><related-urls><url>https://www.ncbi.nlm.nih.gov/pubmed/24390334</url></related-urls></urls><custom2>PMC3957939</custom2><electronic-resource-num>10.1128/JVI.02782-13</electronic-resource-num></record></Cite></EndNote>].
Various combinations of plasmids were transfected into HEK293T cells in 10-cm dishes using the X-tremeGENE 9 DNA transfection reagent (Roche). At 40-42 h post-transfection, cells were lysed in 1 ml immunoprecipitation (IP) buffer (20 mM Tris, pH 7.5, 100 mM NaCl, 0.5% DDM, and EDTA-free protease inhibitor cocktail [Roche]) with rotation at 4°C for 1 h. Lysates were clarified by centrifugation at 20,000 × g and 4°C for 30 min and subjected to co-IP using protein G-conjugated magnetic beads according to the manufacturer's instructions (Millipore). Briefly, immune complexes were formed at 4°C overnight by mixing 200 µl of cell lysate with 2 µg corresponding antibodies in a 500-µl reaction system containing 400 mM sodium chloride. Subsequently, the complexes were precipitated with protein G-conjugated magnetic beads at 4°C for 1 h with rotation, followed by washing extensively with phosphate-buffered saline (PBS) containing 0.1% Tween-20. Finally, proteins were eluted with 4× lithium dodecyl sulfate (LDS) sample buffer (ThermoFisher Scientific) supplemented with 100 mM 1,4-dithiothreitol (DTT), heated at 70°C for 10 min, and analyzed by Western blot.

**SDS-PAGE and Western Blot**

Protein samples were mixed with 4×LDS sample buffer (ThermoFisher Scientific). After denaturing at 70°C for 10 min, 10-30 µl samples were loaded onto to a 12% or 15% SDS-PAGE gel (Bio-Rad Laboratories). After electrophoresis, proteins were resolved and



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transferred onto a polyvinylidene difluoride (PVDF) membrane using the Bio-Rad TransBlot Turbo Blotting System. Blots were soaked for 1 hour in a blocking buffer containing TBST (10 mM Tris-HCl [pH 7.5], 150 mM NaCl, and 0.1% Tween-20) and 5% skim milk, followed by 1 h incubation with primary antibodies (diluted in blocking buffer). After three washes with TBST buffer, blots were incubated with goat anti-mouse or rabbit antibodies conjugated to HRP (1:10,000 diluted in blocking buffer). After three thorough washes with TBST buffer, blots were incubated with the ECL substrates (GE Healthcare). Chemiluminescence signals were detected in ChemiDoc System (Bio-Rad).

**Transmission Electron Microscopy**

Viral genomic RNAs were electroporated into BHK-21 cells as described above. At 48 h post-transfection, the cells were fixed for at least 1 h in a mixture of 2.5% formaldehyde (prepared from paraformaldehyde powder) and 0.1% glutaraldehyde in 0.05M cacodylate buffer (pH 7.3) to which 0.03% picric acid and 0.03% $CaCl_2$ were added. After washing in 0.1 M cacodylate buffer, the cells were scraped off and processed further as a pellet. The pellets were post-fixed in 1% OsO4 in 0.1M cacodylate buffer (pH 7.3) for 1 h, washed with distilled water and *en bloc* stained with 2% aqueous uranyl acetate for 20 min at 60°C. The pellets were dehydrated in ethanol, processed through propylene oxide and embedded in Poly/Bed 812 (Polysciences, Warrington, PA). Ultrathin sections were cut on Leica EM UC7 ultramicrotome (Leica Microsystems, Buffalo Grove, IL), stained with lead citrate and examined in a Philips 201 transmission electron microscope at 60 kV.

**Expression and purification of recombinant NS2A proteins**

The GST-tagged DENV-2 NGC NS2A and its truncates was expressed and purified by following a previously described protocol [ ADDIN EN.CITE <EndNote><Cite><Author>Zou</Author><Year>2014</Year><RecNum>113</RecNum><DisplayText>(Zou et al., 2014)</DisplayText><record><rec-number>113</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1561742592">113</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Zou, J.</author><author>Xie, X.</author><author>Lee le, T.</author><author>Chandrasekaran, R.</author><author>Reynaud, A.</author><author>Yap, L.</author><author>Wang, Q. Y.</author><author>Dong, H.</author><author>Kang, C.</author><author>Yuan, Z.</author><author>Lescar, J.</author><author>Shi, P.



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Y.</author></authors></contributors><auth-address>State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Sciences, Wuhan, China.</auth-address><titles><title>Dimerization of flavivirus NS4B protein</title><secondary-title>J Virol</secondary-title></titles><periodical><full-title>J Virol</full-title></periodical><pages>3379-91</pages><volume>88</volume><number>6</number><keywords><keyword>Amino Acid Motifs</keyword><keyword>Dengue/*virology</keyword><keyword>Dengue Virus/chemistry/genetics/*metabolism</keyword><keyword>Dimerization</keyword><keyword>Humans</keyword><keyword>Viral Nonstructural Proteins/*chemistry/genetics/*metabolism</keyword><keyword>West Nile Fever/*virology</keyword><keyword>West Nile virus/chemistry/genetics/*metabolism</keyword></keywords><dates><year>2014</year><pub-dates><date>Mar</date></pub-dates></dates><isbn>1098-5514 (Electronic)0022-538X (Linking)</isbn><accession-num>24390334</accession-num><urls><related-urls><url>https://www.ncbi.nlm.nih.gov/pubmed/24390334</url></related-urls></urls><custom2>PMC3957939</custom2><electronic-resource-num>10.1128/JVI.02782-13</electronic-resource-num></record></Cite></EndNote>] with modifications. The pGEX-4T GST-NS2A plasmids were transformed into *E. coli* strain Rosetta™ 2 (pLysS) (Novagen) cells. Bacteria culture was grown in Tefferic broth containing 50 µg/ml Kanamycin and 34 µg/ml Chloramphenicol. When the cells reached an optical density at 600 nm (OD600) of 0.8-1.0, isopropyl β-d-1-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM. After growth for 16 h at 18°C, the cells were harvested, resuspended in a lysis buffer (10 mM $Na_2HPO_4$, 1.8 mM $KH_2PO_4$, 2.7 mM KCl, 400 mM NaCl, 1 mM EDTA, 0.5× Roche protease inhibitor cocktail, pH7.5) and disrupted by sonication using a Digital Sonifier 450 (Branson). The cell debris was removed by centrifugation at 10,000 rpm for 10 min at 4°C. The supernatants were collected and pelleted down by centrifugation at 45,000 rpm for 1 h at 4°C using a Beckman type 70 Ti rotor. The pellets containing the membrane fractions were collected and solubilized in the lysis buffer supplemented with 1.5% n-Dodecyl β-D-maltoside (DDM; Anatrace) at 4°C for 2 hours. Following another centrifugation step at 45,000 rpm for 1 h, the supernatants were collected and loaded onto a GSTrap HP Fast Flow column (GE Healthcare) which was preequilibrated with buffer A (10 mM $Na_2HPO_4$, 1.8 mM $KH_2PO_4$, 2.7 mM KCl, 400 mM NaCl, 2 mM tris(2-carboxyethyl) phosphine [TCEP], 0.05% DDM, pH



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7.5). After extensively wash with buffer B (10 mM $Na_2HPO_4$, 1.8 mM $KH_2PO_4$, 2.7 mM KCl, 1M NaCl, 2 mM tris(2-carboxyethyl) phosphine [TCEP], 0.05% DDM, pH 7.5), the protein was eluted in buffer A supplied with 20 mM glutathione. The fractions containing GST-NS2A proteins were pooled, concentrated, and subjected to gel filtration using a HiLoad Superdex 200 10/300 column (GE Healthcare) in buffer C (10 mM $Na_2HPO_4$, 1.8 mM $KH_2PO_4$, 2.7 mM KCl, 150 mM NaCl, 2 mM tris(2-carboxyethyl) phosphine [TCEP], 0.05% DDM, pH 7.5) with 5% glycerol. The peak fractions containing GST-NS2A proteins were pooled and concentrated before storage at -80°C.

**RNA Pull-down Assay**

The 5'UTR-$C_{66}$-3'UTR-biotin RNA was prepared by ligating a single biotinylated nucleotide to the 3' terminus of the RNA 5'UTR-$C_{66}$-3'UTR using the Pierce™ RNA 3' End Biotinylation Kit (ThermoFisher Scientific) according to the manufacturer's instructions. The RNA 5'UTR-$C_{66}$-3'UTR was *in vitro* transcribed from a PCR template using a T7 MEGAscript kit (ThermoFisher Scientific). The PCR template contained a T7 promoter, followed by a cDNA fragment representing a DENV-2 subgenome with a deletion from nucleotides 163 to 10269 (GenBank accession number AF038403). After *in vitro* transcription, the products were passed twice through the Microspin™ G-25 Columns (GE Healthcare Life Sciences) to remove excessive UTPs. The final products were subjected to phenol-chloroform extraction followed by alcohol precipitation. The purified RNAs were resuspended in nuclease-free water prior to storage at -80°C. Other shorter RNAs 3'UTR, Δpk1, 5'UTR-$C_{66}$, Δpk(1-2), Δpk(1-3), 3'SL, pk(3-4) and pk3 were *in vitro* synthesized from corresponding PCR templates using the same method.

RNA pull-down experiments were performed using the Pierce™ Magnetic RNA-Protein Pull-Down Kit (ThermoFisher Scientific) according to the manufacturer's instructions. Briefly, 2 µg of biotin-labeled RNAs were pre-captured by 50 µl Streptavidin magnetic beads in 1 × RNA capture buffer (10mL, 20mM Tris (pH 7.5), 1M NaCl, 1mM EDTA) at room temperature for 20 min with agitation. A 28-mer biotinylated IRE RNA (5'-UCCUGCUUCAACAGUGCUUGGACGGAAC-3'-biotin) from the Pierce™ RNA 3' End Biotinylation Kit was used as a control. The RNA-bound beads were then mixed with 2 µg purified GST-tagged NS2A proteins in a 100-µl reaction system containing 20 mM Tris (pH 7.5), 200 mM NaCl, 2 mM $MgCl_2$, 0.1% Tween-20 and 15% glycerol and incubated for 45 minutes at 4°C with agitation. For RNA competition assay, the various amounts of



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competitors (tRNA and other shorter RNAs from DENV-2 genome) were immediately added to above the mixtures containing RNA-bound beads and GST-NS2A proteins. After 5 times of wash by 20 mM Tris (pH 7.5), 10 mM NaCl, 0.1% Tween-20 Detergent, the proteins were eluted in 30 µl biotin elution buffer. The eluates were mixed with 4×LDS sample buffer and heated at 70°C for 10 min. 10 µl protein samples were loaded onto a 12% SDS-PAGE gel and followed by detection by Western blot using rabbit anti-GST antibodies.

**Electrophoretic Mobility-Shift Assay**

Electrophoretic mobility-shift assay (EMSA) was performed to estimate the binding affinity of DENV-2 5'UTR-$C_{66}$-3'UTR RNA to GST-NS2A proteins. 100 pM 5'UTR-$C_{66}$-3'UTR-biotin RNA was incubated with a serial concentration of recombinant GST-NS2A protein (0-250 nM) in a 50-µl reaction system containing 50mM Tris-HCl, pH 7.5, 10 mM 2-mercaptoethanol, 2 mM $MgCl_2$, 50 mM KCl at 24°C for 15 min. Afterwards, 10 µl 6x Gel Loading Dye without SDS (New England Biolabs Inc.) were added to stop the reaction. Sample were then loaded onto a 0.4% agarose gel (prepared in 0.5× Tris/Borate/EDTA[TBE]) to resolve the RNA-protein complexes and free RNAs by electrophoresis in 1× TBE buffer (120 V for 30 min) at room temperature. After electrophoresis, RNAs were transferred from the agarose gel to a BrightStar-Plus positively charged nylon membrane (ThermoFisher Scientific) according to the procedure as described in the manual of NorthernMax Gly Kit (ThermoFisher Scientific). The nylon membrane was irradiated with 200 mJ/$cm^2$ of Ultraviolet (UV) light at 254 nm in a CX-2000 UV crosslinker (Analytic Jene, CA) with default settings. The biotinylated RNA was finally detected by the Streptavidin-horseradish peroxidase conjugate by using the Chemiluminescent Detection Module (ThermoFisher Scientific). Chemiluminescent signals were detected in the ChemiDoc™ Imaging Systems (Bio-rad).

**PAR-CLIP**

The Photoactivatable-Ribonucleoside-Enhanced Crosslinking and Immunoprecipitation (PAR-CLIP) was performed according to the protocol as described previously [ ADDIN EN.CITE ADDIN EN.CITE.DATA ] with modifications. In brief, 4-thiouridine labelled DENV-2 3'UTR RNA was prepared by adding a final concentration 0.75 mM of 4-Thio-UTP (TriLink BioTechnologies) to a 20-µl *in vitro* transcription reaction (4-Thio-UTR to UTP



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ration is 1:10) using the MEGAscript T7 transcription Kit (Thermo Fisher Scientific). After 2 hours of reaction at 37°C, the reaction was stopped and the 4-thiouridine labelled RNAs were purified and resuspended in nuclease-free water. 15 µg of 4-thiouridine labelled DENV-2 3'UTR RNA was incubated with 6 µg recombinant DENV-2 GST-NS2A protein in a 100-µl reaction system containing 50 mM Tris-HCl, pH 7.5, 2 mM $MgCl_2$, 50 mM KCl and 5% glycerol at 24°C for 15 min. The RNA-protein mixtures were then irradiated on ice with 150 mJ/cm$^2$ at UV light at 365 nm. An aliquot of the same reaction without UV irradiation was used as the uncross-linked control. Prior to immunoprecipitation, 30 µl of PureProteome™ Protein G Magnetic Beads (MilliporeSigma) were washed twice with 1 ml citrate-phosphate buffer (4.7 g/L citric acid, 9.2 g/L $Na_2HPO_4$, pH 5.0), resuspended in 100 µl citrate-phosphate buffer and followed by in addition of 1 µg of rabbit anti-GST polyclonal antibody (Abcam). The bead-antibody mixtures were incubated at 4°C for 45 min. After two washed with citrate-phosphate buffer, the antibody-bound beads were resuspended in 30 µl of citrate-phosphate buffer. Upon immunoprecipitation, the RNA-protein mixtures were mixed with antibody-bound beads and incubated at 4°C for 1 h. Afterwards, the beads were subjected to three washes in buffer I (50mM HEPES-KOH, pH 7.5, 300 mM KCl, 0.05% IGEPAL CA-630L[Sigma-Aldrich] and 0.5 mM DTT) followed by two washes in buffer II (50 mM HEPES-KOH, pH 7.5, 500 KCl, 0.05% IGEPAL CA-630 and 0.5 mM DTT). The beads were then resuspended in 100 µl Proteinase K digestion buffer (100 mM Tris, pH 7.5, 50 mM NaCl, 10 mM EDTA and 1%SDS) supplemented with proteinase K (New England BioLabs) at a final concentration of 2 mg/ml. The reaction was placed into the Eppendorf ThermoMixer® C and incubated at 37°C for 1 h with agitation at 1100 rpm. Thereafter, the RNA in the reaction was extracted by phenol-chloroform, alcohol-precipitated, and resuspended in 20 µl of nuclease-free water. The extracted RNA was subjected to reversely transcribed and followed by amplification (4 PCR-cycles) using the SuperScript® III One-Step RT-PCR System (ThermoFisher Scientific) with a pair of specific primers P1 (sequence: 5'-ACACTCTTTCCCTACACGA CGCTCTTCCGATCTAAGGCAAAACTAACATGAAAC-3'; underline indicates the sequence identical to the sequence at the 5' end of the DENV-2 3'UTR) and P2 (sequence: 5'-GTGACTGGAGTTCAGACG TGTGCTCTTCCGATCTAGAACCTGTTGATTCAACAGCAC-3'; underline indicates the sequence complimentary to the sequence at the 3' end of the DENV-2 3'UTR) targeting the DENV-2 3'UTR. Afterwards, the RT-PCR product was cleaned up using the QIAquick PCR Purification Kit (Qiagen) and subjected to another round of amplification (15 PCR-



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cycles) using the KAPA HiFi PCR Kit (Kapa Biosystems) with a pair of Illumina adapter/index primers (5' universal adaptor sequence: 5'-AATGATACGGCGAC CACCGAGATCTACACTCTTTCCCTACACGAC-3'; and 3' indexing primer sequence: 5'-CAAGCAGAAGACGGCATACGAGATnnnnnnGTGACTGGAGTTCAGACGTGT-3'. The "nnnnnn" here indicates the TruSeq index sequence). Thereafter, the PCR product was cleaned up using the QIAquick PCR Purification Kit (Qiagen) and followed by separation on 2% E-Gel™ EX Agarose Gels (ThermoFisher Scientific). The cDNAs fragment between 500 to 650 bp was excised, purified using the Zymo Research Gel DNA Recovery Kit (Zymo Research) and resuspended in nuclease-free water. The final cDNA libraries were quantified using a Qubit dsDNA Assay Kit (Invitrogen) and sequenced using a MiSeq Reagent Kit v3 (600-cycle) (illumina).

## QUANTIFICATION AND STATISTICAL ANALYSIS

### Sequence Alignment, Image Process and Densitometry Analysis

The amino acid sequences of flavivirus NS2A proteins were download from GenBank. Sequence alignment was performed with default settings using the CLC main workbench software (Qiagen). IFA images processing and cell counting was performed in software ImageJ (National Institutes of Health, NIH). At least four different IFA images were used to estimate the mean and standard deviations of percentage of viral protein staining positive-cells in Figure 2G and S2. Densitometry analysis was performed using Image lab version 6.0 (Bio-Rad Laboratories, Hercules, CA). All numerical data are presented as the mean ± SD (standard deviations). Group comparisons were performed using the unpaired parametric t-test in software Prism 8.0 (GraphPad). *$p<0.05$, significant; **$p<0.01$, very significant; ***$p<0.001$, highly significant; n.s., not significant. Images were assembled using the software Adobe illustrator CC.

### Calculating the equilibrium dissociation constant (Kd)

The intensities of bands representing the RNA/GST-NS2A complexes and free RNA in Figure 5E were quantitated by software ImageJ (NIH). The ratio of bound RNA (in the RNA/GST-NS2A complexes) to total RNA (free and bound RNA) in each lane was calculated. The ratio of bound RNA to total RNA (y-axis) versus the concentration of GST-NS2A protein (x-axis) was plotted using the software Prism 8 (Graphad). The value of Kd



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was obtained using a nonlinear regression with least-squares fitting method, where the Bmax was set as 1. All data points come from three independent experiments.

**Illumina sequencing data process and analysis**

The indexed sequencing reads of both forward and reverse strands were proceeded with the following bioinformatic pipelines: first, an initial quality filtering were conducted with Fastp [ ADDIN EN.CITE <EndNote><Cite><Author>Chen</Author><Year>2018</Year><RecNum>115</RecNum><DisplayText>(Chen et al., 2018)</DisplayText><record><rec-number>115</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1562011493">115</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Chen, S.</author><author>Zhou, Y.</author><author>Chen, Y.</author><author>Gu, J.</author></authors><translated-authors><author>Bioinformatics,</author></translated-authors></contributors><auth-address>Department of Bioinformatics, HaploX Biotechnology, Shenzhen, China.Shenzhen Institutes of Advanced Technology, Chinese Academy of Sciences, Shenzhen, China.</auth-address><titles><title>fastp: an ultra-fast all-in-one FASTQ preprocessor</title><secondary-title>Bioinformatics</secondary-title><alt-title>Bioinformatics</alt-title></titles><periodical><full-title>Bioinformatics</full-title><abbr-1>Bioinformatics</abbr-1></periodical><alt-periodical><full-title>Bioinformatics</full-title><abbr-1>Bioinformatics</abbr-1></alt-periodical><pages>i884-i890</pages><volume>34</volume><number>17</number><dates><year>2018</year><pub-dates><date>Sep 1</date></pub-dates></dates><isbn>1367-4811 (Electronic)1367-4803 (Linking)</isbn><accession-num>30423086</accession-num><urls><related-urls><url>http://www.ncbi.nlm.nih.gov/pubmed/30423086</url></related-urls></urls><custom2>6129281</custom2><electronic-resource-num>10.1093/bioinformatics/bty560</electronic-resource-num><remote-database-provider>2018 Sep 1</remote-database-provider><language>eng</language></record></Cite></EndNote>] to remove bad reads (too low in quality score, too short, too many “N”, etc.). Then, the illumina sequencing adapter sequence “AGATCGGAAGAGC” was trimmed with cutadapt [ ADDIN EN.CITE <EndNote><Cite><Author>Martin</Author><Year>2011</Year><RecNum>116</RecNum><DisplayText>(Martin, 2011)</DisplayText><record><rec-number>116</rec-



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number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1562011493">116</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Martin, Marcel</author></authors></contributors><titles><title>Cutadapt removes adapter sequences from high-throughput sequencing reads</title><secondary-title>EMBnet.journal; Vol 17, No 1: Next Generation Sequencing Data AnalysisDO - 10.14806/ej.17.1.200</secondary-title></titles><periodical><full-title>EMBnet.journal; Vol 17, No 1: Next Generation Sequencing Data AnalysisDO - 10.14806/ej.17.1.200</full-title></periodical><keywords><keyword>next generation sequencing, small RNA, microRNA, adapter removal</keyword></keywords><dates><year>2011</year><pub-dates><date>05/02/</date></pub-dates></dates><urls><related-urls><url>https://journal.embnet.org/index.php/embnetjournal/article/view/200/458</url></related-urls></urls></record></Cite></EndNote>] (command line parameters: -b AGATCGGAAGAGC -m 40). A secondary quality filter was applied with FASTX toolkit ([ HYPERLINK "http://hannonlab.cshl.edu/fastx_toolkit/index.html" ]) to remove any reads that contained more than 4% nucleotides with a PHRED score <20 (command line parameters: fastq_quality_filter -Q33 -q 20 -p 96). The remaining reads were aligned to 3'UTR of DENV genome (GenBank accession number AF038403), with Bowtie2 [ ADDIN EN.CITE <EndNote><Cite><Author>Langmead</Author><Year>2012</Year><RecNum>117</RecNum><DisplayText>(Langmead and Salzberg, 2012)</DisplayText><record><rec-number>117</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1562011493">117</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Langmead, Ben</author><author>Salzberg, Steven L.</author></authors></contributors><titles><title>Fast gapped-read alignment with Bowtie 2</title><secondary-title>Nature Methods</secondary-title></titles><periodical><full-title>Nature Methods</full-title></periodical><pages>357</pages><volume>9</volume><dates><year>2012</year><pub-dates><date>03/04/online</date></pub-dates></dates><publisher>Nature Publishing Group, a division of Macmillan Publishers Limited. All Rights Reserved.</publisher><urls><related-urls><url>https://doi.org/10.1038/nmeth.1923</url></related-urls></urls><electronic-resource-num>10.1038/nmeth.1923</electronic-resource-



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num></record></Cite></EndNote>] (command line parameters: --local). Using SAMtools [ ADDIN EN.CITE <EndNote><Cite><Author>Li</Author><RecNum>118</RecNum><DisplayText>(Li et al.)</DisplayText><record><rec-number>118</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1562011493">118</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Li, H.</author><author>Handsaker B Fau - Wysoker, Alec</author><author>Wysoker A Fau - Fennell, Tim</author><author>Fennell T Fau - Ruan, Jue</author><author>Ruan J Fau - Homer, Nils</author><author>Homer N Fau - Marth, Gabor</author><author>Marth G Fau - Abecasis, Goncalo</author><author>Abecasis G Fau - Durbin, Richard</author><author>Durbin, R.</author></authors><translated-authors><author>Bioinformatics,</author></translated-authors></contributors><auth-address>Wellcome Trust Sanger Institute, Wellcome Trust Genome Campus, Cambridge, CB10 1SA, UK, Broad Institute of MIT and Harvard, Cambridge, MA 02141, USA. FAU - Handsaker, Bob</auth-address><titles><title>The Sequence Alignment/Map format and SAMtools</title></titles><number>1367-4811 (Electronic)</number><dates></dates><call-num>1000 Genome Project Data Processing Subgroup</call-num><urls></urls><remote-database-provider>2009 Aug 15</remote-database-provider><language>eng</language></record></Cite></EndNote>], the aligned forward and reverse reads were binarily converted, merged, indexed, sorted, and mathematically noted (command line parameters: view -buS, merge, sort, index, mpileup). This is followed by extracting and calculating the T/U-to-C mutation rate at each genome position and the mutation frequencies of each of the four nucleotides.

## DATA AND SOFTWARE AVAILABILITY

Illumina sequencing data access. The raw data of illumine sequencing data can be accessed, queried and downloaded using the following link: .



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# Dengue NS2A protein orchestrates virus assembly

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Running title: The role of NS2A protein in dengue virus assembly



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## SUMMARY

Dengue virus (DENV) is the most important mosquito-borne viral pathogen in humans. DENV assembly requires cleavages of viral C-prM-E polyprotein into three structural proteins (capsid, pre-membrane, and envelope), packaging of viral RNA with C protein into nucleocapsid, and budding of prM/E proteins into virion. No study has addressed how these assembly events are orchestrated. Here we show that DENV-2 NS2A protein recruits viral RNA, structural proteins, and protease to virion assembly site, and coordinates nucleocapsid and virus formation. The last 285 nucleotides of viral 3'UTR serve as a "packaging signal" that binds to a cytosolic loop of NS2A protein. This interaction allows NS2A to recruit nascent RNA from the replication complex to virion assembly site. Mutations in NS2A that weaken the 3'UTR/NS2A interaction abolish virus assembly. In addition, NS2A interacts with prM, E, and NS3 to recruit C-prM-E polyprotein and NS2B/NS3 protease to the virion assembly site, leading to coordinated C-prM-E cleavage. Mature C protein assembles onto genomic RNA to form nucleocapsid, followed by prM/E envelopment and virion formation. An NS2A mutation that alters the prM/NS2A interaction reduces C-prM cleavage and abolishes virion assembly; second-site mutations in C protein that restore the C-prM cleavage rescue virion assembly. The results support a DENV assembly model that NS2A recruits viral RNA to virion assembly site where coordinated cleavage of C-prM-E ensues infections virus morphogenesis.



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## INTRODUCTION

The genus *Flavivirus* of the family *Flaviviridae* comprises over 70 viruses, many of which are important human pathogens, including dengue (DENV), yellow fever (YFV), Zika (ZIKV), West Nile (WNV), Japanese encephalitis (JEV), St. Louis encephalitis (SLE), and tick-borne encephalitis (TBEV) viruses[ ADDIN EN.CITE <EndNote><Cite><Author>Pierson</Author><Year>2013</Year><RecNum>3</RecNum><DisplayText>(Pierson and Diamond, 2013)</DisplayText><record><rec-number>3</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1536704974">3</key><key app="ENWeb" db-id="">0</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Pierson, T.C.</author><author>Diamond, M. S.</author></authors></contributors><titles><secondary-title>Flaviviruses p. 747-794. In D.M. Knipe and P.M. Howley (ed), Fields virology, 6th., vol. 1.</secondary-title></titles><periodical><full-title>Flaviviruses p. 747-794. In D.M. Knipe and P.M. Howley (ed), Fields virology, 6th., vol. 1.</full-title></periodical><dates><year>2013</year></dates><urls></urls></record></Cite></EndNote>]. The four serotypes of DENV alone cause about 390 million human infections each year, leading to dengue fever, dengue hemorrhagic fever, and dengue shock syndrome [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. Because of the huge social economic burden, DENV was included in the top 10 global health threats by the World Health Organization (WHO). The development of vaccine and therapeutics for DENV has been proven challenging [ ADDIN EN.CITE <EndNote><Cite><Author>Lim</Author><Year>2013</Year><RecNum>58</RecNum><DisplayText>(Lim et al., 2013)</DisplayText><record><rec-number>58</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv"



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timestamp="1560793214">58</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Lim, S. P.</author><author>Wang, Q. Y.</author><author>Noble, C. G.</author><author>Chen, Y. L.</author><author>Dong, H.</author><author>Zou, B.</author><author>Yokokawa, F.</author><author>Nilar, S.</author><author>Smith, P.</author><author>Beer, D.</author><author>Lescar, J.</author><author>Shi, P. Y.</author></authors></contributors><auth-address>Novartis Institute for Tropical Diseases, 10 Biopolis Road, 05-01 Chromos, Singapore 138670, Singapore.</auth-address><titles><title>Ten years of dengue drug discovery: Progress and prospects</title><secondary-title>Antiviral Res</secondary-title><alt-title>Antiviral research</alt-title></titles><periodical><full-title>Antiviral Res</full-title></periodical><pages>500-19</pages><volume>100</volume><number>2</number><edition>2013/10/01</edition><dates><year>2013</year><pub-dates><date>Nov</date></pub-dates></dates><isbn>1872-9096 (Electronic)0166-3542 (Linking)</isbn><accession-num>24076358</accession-num><urls><related-urls><url>http://www.ncbi.nlm.nih.gov/pubmed/24076358</url></related-urls></urls><electronic-resource-num>10.1016/j.antiviral.2013.09.013</electronic-resource-num><language>eng</language></record></Cite></EndNote>]. The first FDA approved dengue vaccine has a low efficacy of 30-61% [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. Due to the risk of vaccine-induced severe disease in children who are seronegative at the time of vaccination, WHO recommended only use of the vaccine in areas where the disease is highly prevalent [ ADDIN EN.CITE <EndNote><Cite><Author>Whitehead</Author><Year>2016</Year><RecNum>63</RecNum><DisplayText>(Whitehead, 2016)</DisplayText><record><rec-number>63</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793215">63</key></foreign-keys><ref-type name="Journal



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Article">17</ref-type><contributors><authors><author>Whitehead, S. S.</author></authors></contributors><auth-address>a Laboratory of Infectious Diseases , NIAID, National Institutes of Health , Bethesda , MD , USA.</auth-address><titles><title>Development of TV003/TV005, a single dose, highly immunogenic live attenuated dengue vaccine; what makes this vaccine different from the Sanofi-Pasteur CYD vaccine?</title><secondary-title>Expert Rev Vaccines</secondary-title></titles><periodical><full-title>Expert Rev Vaccines</full-title></periodical><pages>509-17</pages><volume>15</volume><number>4</number><keywords><keyword>Dengue</keyword><keyword>live attenuated dengue vaccine</keyword><keyword>vaccine attenuation strategies</keyword><keyword>vaccine efficacy</keyword><keyword>vaccine immunogenicity</keyword><keyword>vaccine infectivity</keyword></keywords><dates><year>2016</year></dates><isbn>1744-8395 (Electronic)1476-0584 (Linking)</isbn><accession-num>26559731</accession-num><urls><related-urls><url>http://www.ncbi.nlm.nih.gov/pubmed/26559731</url></related-urls></urls><custom2>PMC4956407</custom2><electronic-resource-num>10.1586/14760584.2016.1115727</electronic-resource-num></record></Cite></EndNote>]. No clinically approved antivirals are available for flavivirus treatment, despite major effort in the past two decades[ ADDIN EN.CITE <EndNote><Cite><Author>Zou</Author><Year>2019</Year><RecNum>64</RecNum><DisplayText>(Zou and Shi, 2019)</DisplayText><record><rec-number>64</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793215">64</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Zou, J.</author><author>Shi, P. Y.</author></authors></contributors><auth-address>Department of Biochemistry and



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Molecular Biology, University of Texas Medical Branch, Galveston, TX, USA.Department of Biochemistry and Molecular Biology, University of Texas Medical Branch, Galveston, TX, USA; Department of Phamarcology and Toxicology, University of Texas Medical Branch, Galveston, TX, USA; Sealy Center for Structural Biology and Molecular Biophysics, University of Texas Medical Branch, Galveston, TX, USA; Sealy Institute for Vaccine Sciences, University of Texas Medical Branch, Galveston, TX, USA. Electronic address: peshi@utmb.edu.</auth-address><titles><title>Strategies for Zika drug discovery</title><secondary-title>Curr Opin Virol</secondary-title></titles><periodical><full-title>Curr Opin Virol</full-title></periodical><pages>19-26</pages><volume>35</volume><dates><year>2019</year><pub-dates><date>Mar 7</date></pub-dates></dates><isbn>1879-6265 (Electronic)1879-6257 (Linking)</isbn><accession-num>30852345</accession-num><urls><related-urls><url>http://www.ncbi.nlm.nih.gov/pubmed/30852345</url></related-urls></urls><electronic-resource-num>10.1016/j.coviro.2019.01.005</electronic-resource-num></record></Cite></EndNote>]. It is therefore critical to study the molecular mechanism of flavivirus replication, which may lead to new approaches for countermeasure development.

Flavivirus has a plus-sense, single-stranded RNA of about 11,000-nucleotide in length. The genomic RNA contains a 5' untranslated region (UTR), a single open-reading-frame (ORF), and a 3' UTR. The ORF translates a polyprotein that is processed by viral and host proteases into three structure proteins [capsid (C), pre-membrane (prM), and envelope (E)] and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5). The structural proteins, together with genomic RNA, assemble into virions. The nonstructural proteins participate in viral RNA replication, virion assembly, and evasion of host immune response. Upon viral translation, a minus-sense RNA is synthesized from the



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genomic RNA template, forming a double-stranded RNA. The minus-strand RNA then serves as a template to make many copies of plus-sense RNA that are packaged to form progeny virions [ ADDIN EN.CITE <EndNote><Cite><Author>Pierson</Author><Year>2013</Year><RecNum>3</RecNum><DisplayText>(Pierson and Diamond, 2013)</DisplayText><record><rec-number>3</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1536704974">3</key><key app="ENWeb" db-id="">0</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Pierson, T.C.</author><author>Diamond, M. S.</author></authors></contributors><titles><secondary-title>Flaviviruses p. 747-794. In D.M. Knipe and P.M. Howley (ed), Fields virology, 6th., vol. 1.</secondary-title></titles><periodical><full-title>Flaviviruses p. 747-794. In D.M. Knipe and P.M. Howley (ed), Fields virology, 6th., vol. 1.</full-title></periodical><dates><year>2013</year></dates><urls></urls></record></Cite></EndNote>]. Like other RNA viruses, the assembly of flavivirus is coupled with RNA replication [ ADDIN EN.CITE ADDIN EN.CITE.DATA ].

Flavivirus infection induces invaginations of the endoplasmic reticulum (ER) to form clusters of double-membrane vesicles (Ve) enclosed in the vesicle packets (VPs). Electron tomography has revealed the three-dimensional architectures of Ve and VPs formed during DENV, WNV, TBEV, and ZIKV infections [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. Ve houses viral replication complex (including the double-stranded viral RNA, viral nonstructural proteins, and cellular proteins) where RNA synthesis occurs [ ADDIN EN.CITE <EndNote><Cite><Author>Junjhon</Author><Year>2014</Year><RecNum>71</RecNum><DisplayText>(Junjhon et al., 2014)</DisplayText><record><rec-number>71</rec-



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number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793216">71</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Junjhon, J.</author><author>Pennington, J. G.</author><author>Edwards, T. J.</author><author>Perera, R.</author><author>Lanman, J.</author><author>Kuhn, R. J.</author></authors></contributors><auth-address>Markey Center for Structural Biology, Department of Biological Sciences, Purdue University, West Lafayette, Indiana, USA.</auth-address><titles><title>Ultrastructural characterization and three-dimensional architecture of replication sites in dengue virus-infected mosquito cells</title><secondary-title>J Virol</secondary-title></titles><periodical><full-title>J Virol</full-title></periodical><pages>4687-97</pages><volume>88</volume><number>9</number><keywords><keyword>Animals</keyword><keyword>Cell Line</keyword><keyword>Culicidae/*virology</keyword><keyword>Dengue Virus/*physiology/*ultrastructure</keyword><keyword>Electron Microscope Tomography</keyword><keyword>Imaging, Three-Dimensional</keyword><keyword>Intracellular Membranes/*ultrastructure/*virology</keyword><keyword>*Virus Replication</keyword></keywords><dates><year>2014</year><pub-dates><date>May</date></pub-dates></dates><isbn>1098-5514 (Electronic)0022-538X (Linking)</isbn><accession-num>24522909</accession-num><urls><related-urls><url>http://www.ncbi.nlm.nih.gov/pubmed/24522909</url></related-urls></urls><custom2>PMC3993787</custom2><electronic-resource-num>10.1128/JVI.00118-14</electronic-resource-num></record></Cite></EndNote>]. Newly synthesized plus-sense RNA is released from the Ve pore to the rough ER for new rounds of RNA translation and replication. Alternatively, the nascent RNA is assembled



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into virions through encapsidation (with C protein) and envelopment (with prM and E proteins) through budding into the ER lumen. The virion assembly occurs at the ER sites juxtaposed to the VPs. An initial immature virion contains 60 icosahedrally arranged prM/E heterotrimer. As the immature virion transits through the Golgi network, the acidic environment triggers the cleavage of prM by cellular furin protease, resulting in an infectious mature virion [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. Cryo-EM of flavivirus virions exhibit two structural features: (i) The nucleocapsid core lacks organized structures and (ii) there are no observable connections between nucleocapsid and the prM/E membrane shell, suggesting that the C protein may bind to genomic RNA in a random manner without direct interaction with prM/E proteins [ ADDIN EN.CITE ADDIN EN.CITE.DATA ].

The molecular mechanism of flavivirus assembly remains largely obscure. Besides structural proteins, several nonstructural proteins, such as NS2A, NS2B, and NS3, have been shown to regulate flavivirus assembly. Different amino acids of NS2A protein were reported to play distinct roles in flavivirus RNA replication and virion assembly [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. The cleavage between C and prM is tightly modulated by NS2B/NS3 protease and cellular signalase during YFV and Murray Valley encephalitis virus assembly [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. Mutations in the NS3 helicase domain alone affect YFV assembly [ ADDIN EN.CITE <EndNote><Cite><Author>Patkar</Author><Year>2008</Year><RecNum>90</RecNum><DisplayText>(Patkar and Kuhn, 2008a)</DisplayText><record><rec-number>90</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793218">90</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Patkar, C. G.</author><author>Kuhn, R. J.</author></authors></contributors><auth-



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address>Purdue Univ, Dept Biol Sci, W Lafayette, IN 47907 USA</auth-address><titles><title>Yellow fever virus NS3 plays an essential role in virus assembly independent of its known enzymatic functions</title><secondary-title>Journal of Virology</secondary-title><alt-title>J Virol</alt-title></titles><alt-periodical><full-title>J Virol</full-title></alt-periodical><pages>3342-3352</pages><volume>82</volume><number>7</number><keywords><keyword>tick-borne encephalitis</keyword><keyword>flavivirus kunjin ns3</keyword><keyword>west-nile-virus</keyword><keyword>dengue virus</keyword><keyword>nonstructural protein</keyword><keyword>rna replication</keyword><keyword>cdna-clones</keyword><keyword>particles</keyword><keyword>ns2a</keyword><keyword>organization</keyword></keywords><dates><year>2008</year><pub-dates><date>Apr</date></pub-dates></dates><isbn>0022-538X</isbn><accession-num>WOS:000254139800015</accession-num><urls><related-urls><url><Go to ISI>://WOS:000254139800015</url></related-urls></urls><electronic-resource-num>10.1128/Jvi.02447-07</electronic-resource-num><language>English</language></record></Cite></EndNote>]. Although expression of prM/E alone is sufficient to generate empty virus-like particles, it is not known how nucleocapsid is formed and subsequently enveloped with prM/E to form infectious virions. The goal of this study is to define the molecular mechanism that orchestrates three structural proteins and viral RNA to form nucleocapsid and infectious virus in a highly coordinated manner. Using genetic, biochemical, and imaging approaches, we demonstrate that DENV NS2A protein choreographs virion morphogenesis by (i) interacting with viral prM and E proteins, (ii) regulating the processing of C-prM polyprotein by viral protease, and (iii) recruiting genomic RNA through binding to the 3'UTR. These results support a model that DENV NS2A recruits nascent viral RNA from the replication complex (housed in Ve) to the virion assembly site (on the ER membrane juxtaposed to



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VPs), where an coordinated C-prM cleavage facilitates genomic RNA encapsidation with C protein and budding with prM and E proteins into the ER lumen, resulting in progeny virions.



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## RESULTS

**NS2A G11A mutation abolishes virion assembly**. Using DENV-2 as a model, we previously identified a panel of NS2A mutant viruses that were selectively defective in virion assembly without compromising other steps of an infection cycle [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. Here, we performed four experiments to demonstrate that NS2A mutation G11A of DENV-2 caused a defect in virion assembly. (i) A DENV-2 luciferase replicon was used to examine the effect of NS2A G11A on viral translation and RNA synthesis. Upon transfection into BHK-21 cells, the G11A replicon produced a luciferase profile comparable to the WT replicon (Fig. 1A), suggesting that the mutation does not significantly reduce viral translation and RNA replication. (ii) When full-length viral RNA of DENV-2 was transfected into BHK-21 cells, the NS2A G11A mutant failed to release extracellular viral RNA (Fig. 1B). No infectious virus was detected from the culture medium of G11A RNA-transfected cells (Fig. 1C). (iii) In a *trans* packaging system, WT luciferase replicon could be packaged into pseudo-infectious particles (PIPs) when structural C-prM-E proteins were expressed from Semliki Forest virus replicon (Fig. S1A). The WT reporter replicon PIPs infected Vero cells and generated robust luciferase signals (Fig. S1B). In contrast, no PIPs were produced with the NS2A G11A replicon. (iv) Transmission electron microscopy revealed substantial virus-induced single-membrane vesicles (within the lumen of the rough ER) or double-membrane vesicles (closely associated with cisternae of the rough ER) in both WT and G11A RNA-transfected cells at 48 h post-transfection (Fig. 1D). A lot of virus particles appeared in the WT RNA-transfected cells, but no virus particles were detected in the G11A RNA-transfected cells. The results demonstrate that NS2A G11A mutation specifically abolishes virion assembly.

**NS2A G11A mutation alters the expression and localization of viral structural proteins.** We compared the subcellular localization of viral proteins in BHK-21 cells that



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were transfected with WT or NS2A G11A genomic RNA. Surprisingly, the NS2A G11A mutation altered the intracellular distribution of three structural proteins. (i) C protein disseminated in both nucleus and cytoplasm in the WT RNA-transfected cells, as previously reported [ ADDIN EN.CITE <EndNote><Cite><Author>Wang</Author><Year>2004</Year><RecNum>91</RecNum><DisplayText>(Wang et al., 2004)</DisplayText><record><rec-number>91</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793218">91</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Wang, S. H.</author><author>Syu, W. J.</author><author>Hu, S. T.</author></authors></contributors><auth-address>Institute of Microbiology and Immunology, School of Life Science, National Yang-Ming University, Taipei, Taiwan, Republic of China.</auth-address><titles><title>Identification of the homotypic interaction domain of the core protein of dengue virus type 2</title><secondary-title>J Gen Virol</secondary-title><alt-title>The Journal of general virology</alt-title></titles><periodical><full-title>J Gen Virol</full-title><abbr-1>The Journal of general virology</abbr-1></periodical><alt-periodical><full-title>J Gen Virol</full-title><abbr-1>The Journal of general virology</abbr-1></alt-periodical><pages>2307-14</pages><volume>85</volume><number>Pt 8</number><keywords><keyword>Active Transport, Cell Nucleus</keyword><keyword>Dengue Virus/*chemistry/classification</keyword><keyword>Precipitin Tests</keyword><keyword>Viral Core Proteins/*chemistry</keyword></keywords><dates><year>2004</year><pub-dates><date>Aug</date></pub-dates></dates><isbn>0022-1317 (Print)0022-1317 (Linking)</isbn><accession-num>15269372</accession-num><urls><related-urls><url>http://www.ncbi.nlm.nih.gov/pubmed/15269372</url></related-



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urls></urls><electronic-resource-num>10.1099/vir.0.80067-0</electronic-resource-num></record></Cite></EndNote>]. However, in the G11A RNA-transfected cells, the C protein was mainly restricted to the cytoplasm with rare nucleus localization (Fig. 1E). (ii) prM protein was located at the ER region in the WT RNA-transfected cells, whereas the prM protein was undetectable in the G11A RNA-transfected cells (Fig. 1F). (iii) E protein spread broadly like an ER web in the WT RNA-transfected cells. In contrast, in the G11A RNA-transfected cells, the E protein formed larger speckles at restricted subcellular areas (Fig. 1G). Compared with the structural proteins, the effect of NS2A G11A on the subcellular expression of viral nonstructural proteins (including NS1, NS3, NS4A, NS4B, and NS5) and dsRNA was not apparent (Fig. 1E-G, *top panels* for NS3, and data not shown).

The distinct effect of NS2A G11A mutation on subcellular expression between the viral structural and nonstructural proteins were further supported by immunofluorescent co-staining experiments. NS2A G11A altered the co-staining profile of E and NS3 proteins (Fig. S2). In the NS2A G11A RNA-transfected cells, 100% of NS3-expressing cells were E-positive at 24 h post-transfection; however, at 48 and 72 h post-transfection, only 59% and 20% of the NS3-expressing cells were E-positive, respectively (Fig. S2). In the WT RNA-transfected cells, 100% of NS3-expressing cells were E-positive from 24 to 72 h post-transfection (Fig. S2). The results demonstrate that NS2A G11A mutation changes the expression and subcellular localization of viral structural proteins.

**NS2A G11A mutation impairs the cleavage of viral C-prM polyprotein.** To determine the molecular mechanism of the above observations, we examined the processing of viral proteins in the WT and NS2A G11A viral RNA-transfected cells at 48 h post-transfection by Western blot. As expected, the amounts of C, NS3, and NS5 proteins from the G11A RNA-transfected cells were 15-26% of those from the WT RNA-transfected



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cells (Fig. 1H-I), due to the lack of virion production or multiple rounds of infection for the G11A mutant. Notably, the amounts of prM and E proteins were further lower in the G11A RNA-transfected cells (only 1-4% of those in the WT RNA-transfected cells), suggesting an asymmetric degradation in cells. Moreover, the NS2A G11A mutation impaired the cleavage of C-prM to individual C and prM proteins: 90% of C-prM was cleaved to mature C protein in the WT RNA-transfected cells, whereas only 20% of C-prM was processed to C protein in the G11A RNA-transfected cells (Fig. 1H, *compare lanes 1 and 5 in the top panel*). The impaired C-prM cleavage could account for the altered subcellular distribution of viral structural proteins in the NS2A C11A RNA-transfected cells: Restricting the un-cleaved C-prM to the ER membrane, preventing C protein from translocating to the nucleus, and consequently blocking prM/E heterodimer formation and virion budding.

Next, we examined the role of ubiquitin-proteasome pathway in degrading viral proteins in the G11A RNA-transfected cells. Treatment with proteasome inhibitor MG132 increased the amounts of C, prM, E, and NS5 (but not NS3) by 1-3 folds in the NS2A G11A RNA-transfected cells (Fig. 1H, *compare lanes 5 and 6*). In contrast, in the WT RNA-transfected cells, MG132 slightly reduced the amounts of C, prM, and E to different extents (Fig. 1H, *compare lanes 1 and 2*) because MG132 was known to inhibit flavivirus replication [ ADDIN EN.CITE <EndNote><Cite><Author>Bhuvanakantham</Author><Year>2013</Year><RecNum>108</RecNum><DisplayText>(Bhuvanakantham and Ng, 2013)</DisplayText><record><rec-number>108</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793288">108</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Bhuvanakantham, R.</author><author>Ng, M. L.</author></authors></contributors><auth-



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address>Flavivirology Laboratory, Department of Microbiology, Yong Loo Lin School of Medicine, National University of Singapore, 5 Science Drive 2, Singapore, 117597.</auth-address><titles><title>West Nile virus and dengue virus capsid protein negates the antiviral activity of human Sec3 protein through the proteasome pathway</title><secondary-title>Cell Microbiol</secondary-title></titles><periodical><full-title>Cell Microbiol</full-title></periodical><pages>1688-706</pages><volume>15</volume><number>10</number><keywords><keyword>Antiviral Agents/*antagonists & inhibitors</keyword><keyword>Capsid Proteins/genetics/*metabolism</keyword><keyword>Cell Line</keyword><keyword>DNA Mutational Analysis</keyword><keyword>Dengue Virus/*physiology</keyword><keyword>*Host-Pathogen Interactions</keyword><keyword>Humans</keyword><keyword>Models, Biological</keyword><keyword>Proteasome Endopeptidase Complex/*metabolism</keyword><keyword>Protein Binding</keyword><keyword>Protein Interaction Mapping</keyword><keyword>Proteolysis</keyword><keyword>Vesicular Transport Proteins/*antagonists & inhibitors</keyword><keyword>West Nile virus/*physiology</keyword></keywords><dates><year>2013</year><pub-dates><date>Oct</date></pub-dates></dates><isbn>1462-5822 (Electronic)1462-5814 (Linking)</isbn><accession-num>23522008</accession-num><urls><related-urls><url>https://www.ncbi.nlm.nih.gov/pubmed/23522008</url></related-urls></urls><electronic-resource-num>10.1111/cmi.12143</electronic-resource-num></record></Cite></EndNote>]. Distinct responses to MG132 treatment among different viral proteins were probably due to their subcellular distributions and involvement in various biological microenvironments. Nevertheless, the results suggest that ubiquitin-proteasome pathway is responsible for viral protein degradation in the NS2A G11A RNA-transfected cells.



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**WT NS2A rescues virion assembly of NS2A G11A mutant through restoring C-prM cleavage.** We examined if *trans* supply of WT NS2A could restore virion assembly of NS2A G11A RNA and rescue C-prM protein processing (Fig.S3A). Indeed, after transfection into NS2A-EGFP BHK-21 cells that constitutively expressed WT NS2A protein, the NS2A G11A RNA produced infectious virions that could infect naïve BHK-21 cells, as indicated by E-positive cells (Fig. S3B); further passaging of culture supernatants to new BHK-21 cells did not yield any E-positive cells (Fig. S3C), suggesting that the *trans* packaged virions could only infect cells for a single-round. Remarkably, the *trans* supplied WT NS2A protein restored the C-prM cleavage in the G11A RNA-transfected cells to WT level, exhibiting >89% C protein processing (Fig. S3D, *top panel*). Two prM bands, representing glycosylated and un-glycosylated forms of the protein (see below), were detected in both NS2A G11A and WT RNA-transfected NS2A-EGFP cells (Fig. S3D, *second panel*). The relative abundances of C, prM, and NS1 proteins were similar in the NS2A G11A and WT RNA-transfected cells (Fig. S3D). Because of the restored C-prM cleavage, the nucleus localization of C protein and prM-positive cells were detected in the G11A RNA-transfected NS2A-EGFP cells (Fig. S3E). These results demonstrate that WT NS2A can rescue virion assembly of NS2A G11A mutant through restoring the C-prM cleavage.

Next, we examined the effect of NS2A on the glycosylation of prM by co-expressing NS2A and prM in HEK293T cells. As a control, when EGFP was co-expressed, only the glycosylated form of prM was detected (Fig. S3F); after PGNaseF treatment, the prM was completely deglycoslated and migrated as a glycosylation-knockout mutant N69Q prM (Fig. S3G). In contrast, when NS2A-EGFP were co-expressed, both glycosylated and un-glycosylated forms of prM were detected (Fig. S3F), indicating that NS2A suppresses the glycosylation of prM. Furthermore, the G11A mutation seemed to increase the



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suppression effect of NS2A on prM glycosylation (Fig. 3F). These results suggest that NS2A may interact with prM, and that the G11A mutation may modulate the prM/NS2A interaction.

**Capsid R99S mutation can rescue virion assembly of NS2A G11A RNA.** We selected revertants by continuously passaging the NS2A G11A RNA-transfected BHK-21 cells for five rounds (Fig. S4A). Culture medium from each passaging (P1-P5) were tested for emergence of infectious viruses using plaque assay. The P5 culture produced infectious viruses with titers of $>10^4$ PFU/ml and plaques smaller than the WT virus (Fig. S4B). In addition, almost all P5 cells were E protein-positive (Fig. S4C). Whole genome sequencing of the P5 viruses from three independent selections consistently showed a consensus C-to-A substitution at nucleotide position 391 of viral genome, resulting in an Arg-to-Ser change at amino acid position 99 of the C protein (C_R99S; Fig. 2A). The original NS2A G11A mutation was retained in all P5 viruses.

To validate the role of C_R99S mutation in rescuing virion assembly of NS2A G11A RNA, we engineered C_R99S into a DENV-2 infectious clone [ ADDIN EN.CITE <EndNote><Cite><Author>Xie</Author><Year>2013</Year><RecNum>85</RecNum><DisplayText>(Xie et al., 2013)</DisplayText><record><rec-number>85</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793217">85</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Xie, X.</author><author>Gayen, S.</author><author>Kang, C.</author><author>Yuan, Z.</author><author>Shi, P. Y.</author></authors></contributors><auth-address>State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Science, China.</auth-address><titles><title>Membrane topology and function of dengue virus NS2A protein</title><secondary-title>J Virol</secondary-title><alt-title>Journal of virology</alt-



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title></titles><periodical><full-title>J Virol</full-title></periodical><pages>4609-22</pages><volume>87</volume><number>8</number><edition>2013/02/15</edition><keywords><keyword>Animals</keyword><keyword>Cell Line</keyword><keyword>Cricetinae</keyword><keyword>Dengue Virus/*physiology</keyword><keyword>Endoplasmic Reticulum/*virology</keyword><keyword>Humans</keyword><keyword>Intracellular Membranes/*metabolism</keyword><keyword>Magnetic Resonance Spectroscopy</keyword><keyword>Models, Molecular</keyword><keyword>Protein Conformation</keyword><keyword>Viral Nonstructural Proteins/*chemistry/metabolism</keyword><keyword>*Virus Assembly</keyword></keywords><dates><year>2013</year><pub-dates><date>Apr</date></pub-dates></dates><isbn>1098-5514 (Electronic)0022-538X (Linking)</isbn><accession-num>23408612</accession-num><work-type>Research Support, Non-U.S. Gov't</work-type><urls><related-urls><url>http://www.ncbi.nlm.nih.gov/pubmed/23408612</url></related-urls></urls><custom2>3624351</custom2><electronic-resource-num>10.1128/JVI.02424-12</electronic-resource-num><language>eng</language></record></Cite></EndNote>] in the presence or absence of NS2A G11A. BHK-21 cells were electroporated with WT, NS2A G11A, C_R99S, or NS2A G11A+C_R99S viral RNA. The transfected cells were analyzed for virus production and C-prM cleavage. In the NS2A G11A+C_R99S RNA-transfected cells, the percentage of E-positive cells increased from 10% to 50% at 24 h and 96 h post-transfection, suggesting the spread of infectious virus (Fig. 2B); indeed, the transfected cells produced infectious viruses with plaques smaller than the WT virus (Fig. 2C); the viral titer increased over time, reaching >$10^5$ PFU/ml at 96-120 h post-transfection (Fig. 2D). The prM and NS1 proteins exhibited ER-like distributions in the NS2A G11A+C_R99S RNA-transfected cells, similar to that observed in the WT RNA-transfected



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cells (Fig. 2E). Viral C protein was detected in both cytoplasm and nucleus in the NS2A G11A+C_R99S RNA-transfected cells (Fig. 2F-G). Furthermore, 90.5% of the C-prM protein was cleaved to mature C and prM in the NS2A G11A+C_R99S RNA-transfected cells (Fig. 2G). As controls, NS2A G11A RNA showed 15.4% of C-prM cleavage and did not produce any infectious virus, whereas C_R99S RNA exhibited 99.9% of prM-C cleavage and produced a WT level of infectious virus (Fig. 2B-H). Taken together, the results demonstrate that C_R99S mutation efficiently restores C-prM cleavage and virion assembly of NS2A G11A RNA.

**Capsid R99S cannot rescue virion assembly of other NS2A mutants.** We tested if C_R99S could rescue other NS2A mutants that were previously shown to be defective in virion assembly, including E20A, E100A, and K188A [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. The C_R99S was engineered into those NS2A mutants, resulting in NS2A E20A+C_R99S, NS2A E100A+C_R99S, and NS2A K188A+C_R99S viral RNAs. After transfection into BHK-12 cells, none of the mutant RNAs produced increasing number of E-positive cells from 24 to 96 h (Fig. S5A). No infectious virus was detected in the culture medium from the mutant RNA-transfected cells (Fig. S5B). Viral C protein was detected in both nucleus and cytoplasm of all mutant RNA-transfected cells (Fig. S5C). In agreement with the C nucleus localization, the NS2A single mutant E20A and E100A cleaved 36% and 79% of C-prM, and the addition of C_R99S increased the C-prM cleavage to 72% and 96%, respectively. In contrast, NS2A K188A mutant cleaved 100% of C-prM in the presence or absence of C_R99S (Fig. 5D). The results demonstrate that C_R99S can only rescue the assembly defect of NS2A G11A possibly because different NS2A mutations abolish virion assembly through distinct mechanisms.

**Capsid R99S shifts the C-prM cleavage site by two amino acids.** The above results prompted us to investigate how C_R99S rescues the virion assembly of NS2A



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G11A. Fig. S6 depicts the topology of viral polyprotein C-prM-E-NS1-NS2A on the ER membrane. NS2A G11 resides at the first amphipathic helix on the ER lumen side [ ADDIN EN.CITE

<EndNote><Cite><Author>Xie</Author><Year>2013</Year><RecNum>85</RecNum><DisplayText>(Xie et al., 2013)</DisplayText><record><rec-number>85</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793217">85</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Xie, X.</author><author>Gayen, S.</author><author>Kang, C.</author><author>Yuan, Z.</author><author>Shi, P. Y.</author></authors></contributors><auth-address>State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Science, China.</auth-address><titles><title>Membrane topology and function of dengue virus NS2A protein</title><secondary-title>J Virol</secondary-title><alt-title>Journal of virology</alt-title></titles><periodical><full-title>J Virol</full-title></periodical><pages>4609-22</pages><volume>87</volume><number>8</number><edition>2013/02/15</edition><keywords><keyword>Animals</keyword><keyword>Cell Line</keyword><keyword>Cricetinae</keyword><keyword>Dengue Virus/*physiology</keyword><keyword>Endoplasmic Reticulum/*virology</keyword><keyword>Humans</keyword><keyword>Intracellular Membranes/*metabolism</keyword><keyword>Magnetic Resonance Spectroscopy</keyword><keyword>Models, Molecular</keyword><keyword>Protein Conformation</keyword><keyword>Viral Nonstructural Proteins/*chemistry/metabolism</keyword><keyword>*Virus Assembly</keyword></keywords><dates><year>2013</year><pub-dates><date>Apr</date></pub-dates></dates><isbn>1098-5514 (Electronic)0022-538X (Linking)</isbn><accession-num>23408612</accession-num><work-type>Research



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Support, Non-U.S. Gov't</work-type><urls><related-urls><url>http://www.ncbi.nlm.nih.gov/pubmed/23408612</url></related-urls></urls><custom2>3624351</custom2><electronic-resource-num>10.1128/JVI.02424-12</electronic-resource-num><language>eng</language></record></Cite></EndNote>], while C_R99 is the penultimate amino acid of the C protein on the cytosolic side (Fig. S6B). Since flavivirus NS2B/NS3 protease cleaves a substrate with two basic residues (Arg or Lys) at the P1 and P2 sites and a residue with short side chain (Gly, Ser, or Ala) at the P1' site [ ADDIN EN.CITE ADDIN EN.CITE.DATA ], sequence analysis suggests that the C_R99S substitution may shift the capsid C-terminal cleavage by two amino acids from position 100 to 98 (Fig. S6B). Specifically, viral NS2B/NS3 protease cleaves the WT capsid at NRRRR↓TA and the R99S capsid at NRR↓SRTA (↓ denoting cleavage site and residue R99S underlined). To test this hypothesis, we performed a biochemical protease assay using synthetic WT peptide (NILNRRRRTAGMII) and R99S peptide (NILNRRSRTAGMII). After incubating the peptides with recombinant DENV-2 NS2B/NS3 protease, the reaction products were analyzed by liquid chromatography-mass spectrometry (LC-MS). The WT peptide was cleaved into two smaller peptides with molecular weights of 1096.73 Da and 604.77 Da, corresponding to peptides NILNRRRR and TAGMII, respectively (Fig. S6C). In contrast, the R99S peptide was cleaved into two fragments with molecular weights of 784.47 Da and 847.46 Da, corresponding to peptide NILNRR and SRTAGMII, respectively (Fig. S6D). The LC-MS also detected some unknown peptides, probably due to nonspecific degradations of the protease reaction (Fig. S6C-D). Nevertheless, the data indicate that the C_R99S mutation shifts the capsid cleavage site upstream by two amino acids.

**NS2A G11A enhances prM/NS2A interaction.** Since NS2A G11A alters the processing of C-prM (Fig. 1H) and prM glycosylation (Fig. S3F), we hypothesized that



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NS2A directly interacts with prM and the G11A mutation affects the prM/NS2A interaction. To test this hypothesis, we co-transfected two plasmids into HEK293T cells. One plasmid expressed the authentic signal peptide anchorC ($C_{14}$) fused with the full-length prM. Another plasmid expressed a fusion protein containing the C-terminal 24 amino acids of the E protein, the N-terminal 50 amino acids of the NS1 protein, the C-terminal 50 amino acids of NS1, full length NS2A, and an EGFP [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]; this plasmid was previously shown to express NS2A-EGFP with a correct topology on the ER membrane [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. Co-immunoprecipitation showed that NS2A-EGFP, but not EGFP, pulled down prM (Fig. 3B, lanes 2 and 3). As controls, mouse IgG did not pull down any NS2A-EGFP or prM (Lane 1), and EGFP antibody did not pull down prM in the absence of NS2A-EGFP (Lane 6). To map the region of prM that binds to NS2A, we expressed the first 130 amino acids of prM [prM(1-130)] that lacked the C-terminal two transmembrane domains (Fig. 3A). Co-immunoprecipitation showed that prM(1-130) could not pull down NS2A-EGFP (Fig. 3B, lane 4), suggesting that (i) NS2A may bind to the transmembrane region of prM or (ii) the transmembrane region of prM may affect the conformation of its ectodomain and, consequently, abolish its interaction with NS2A.

Next, we examined if NS2A mutation G11A affects the prM/NS2A interaction. Co-immunoprecipitation experiments showed that G11A NS2A-EGFP pulled down about 2.5-fold more prM than the WT NS2A-EGFP did (Fig. 4C). Reciprocally, prM pulled down about 1.7-fold more G11A NS2A-EGFP than WT NS2A-EGFP (Fig. 4D). The results indicate that the G11A mutation enhances NS2A binding to prM.

To further support the prM/NS2A interaction, we used confocal microscope to examine the co-localization of prM/NS2A in cells. In this case, prM-E were co-expressed with NS2A-EGFP in HEK293T cells. As shown in Fig. 3E, NS2A-EGFP co-localized with



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prM. The G11A mutation did not change the co-localization of NS2A-EGFP with prM (Fig. 3E). Taken together, the results demonstrate that (i) NS2A interacts with prM and (ii) NS2A G11A significantly enhances the prM/NS2A interaction.

**The G11A mutation does not affect NS2A binding to E or NS3.** We also examined the interactions between NS2A and E or NS3 proteins that were co-expressed in HEK293T cells. For testing the E/NS2A interaction, we fused a GST tag to the C-terminus of NS2A for the co-immunoprecipitation experiment [because the EGFP tag used in NS2A-EGFP in Fig. 3B-D non-specifically interacts with E protein (data not shown)]. As shown in Fig. 3F, both WT and G11A NS2A-GST pulled down E protein at comparable efficiencies. As a control, GST alone did not pull down E protein. The result suggests that NS2A interacts with E and the G11A mutation does not affect this interaction.

Since flavivirus NS3 is known to participate in viron assembly [ ADDIN EN.CITE ADDIN EN.CITE.DATA ], we also tested if NS2A G11A mutation affects the NS2A/NS3 interaction. When co-expressed in HEK293T cells, GST alone pulled down a trace amount of HA-tagged NS3 (NS3-HA) protein (Fig. S7, lane 1), whereas NS2A-GST pulled down 10-fold more NS3-HA (Lane 2). Equivalent amounts of NS3-HA were pulled down by the WT and G11A NS2A-GST (Compare lanes 2 and 3), suggesting that NS2A G11A does not affect NS2A/NS3 binding.

**Extension of two or three residues at the N-terminus of anchorC efficiently rescues virion assembly of NS2A G11A**. So far, our results showed that NS2A G11A mutation increases its binding to prM on the ER lumen side, leading to impaired C-prM processing and defective virion assembly. These defects can be rescued by a capsid mutation R99S that shifts the C-terminal cleavage site by two amino acids. As a result of this shift, the N-terminus of transmembrane anchorC is extended by two amino acids on



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572 the cytosolic side of ER membrane. These data support a model of coordinated C-prM
573 processing during virion assembly (Fig. 4A) [ ADDIN EN.CITE
574 <EndNote><Cite><Author>Amberg</Author><Year>1999</Year><RecNum>295</RecNu
575 m><DisplayText>(Amberg and Rice, 1999a)</DisplayText><record><rec-
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579 Article">17</ref-type><contributors><authors><author>Amberg, S.
580 M.</author><author>Rice, C. M.</author></authors></contributors><auth-
581 address>Department of Molecular Microbiology, Washington University School of
582 Medicine, St. Louis, Missouri 63110-1093, USA.</auth-
583 address><titles><title>Mutagenesis of the NS2B-NS3-mediated cleavage site in the
584 flavivirus capsid protein demonstrates a requirement for coordinated
585 processing</title><secondary-title>J Virol</secondary-title><alt-title>Journal of
586 virology</alt-title></titles><periodical><full-title>J Virol</full-title><abbr-1>Journal of
587 virology</abbr-1></periodical><alt-periodical><full-title>J Virol</full-title><abbr-1>Journal
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589 94</pages><volume>73</volume><number>10</number><keywords><keyword>Capsid/*
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597 urls><url>http://www.ncbi.nlm.nih.gov/pubmed/10482557</url></related-



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598 urls></urls><custom2>112824</custom2></record></Cite></EndNote>]. During WT virus
599 assembly, polyprotein C-anchorC-prM was first cleaved at the C-terminus of capsid protein
600 (*i.e.*, R100) by NS2B/NS3 protease, producing mature C and anchorC-prM. The anchorC-
601 prM was then cleaved by a host signalase at the N-terminus of prM, generating anchorC
602 and prM. The cleaved prM coordinates with E protein for virion budding (Fig. 4A, *left*
603 *panel*). In the presence of NS2A G11A mutation, NS2A binds to prM with a higher affinity,
604 pulling anchorC (embedded in the ER membrane) towards the lumen side. The viral
605 protease cleavage site of the "pulled anchorC" is blocked, possibly by the ER membrane
606 (*middle panel*), leading to unprocessed C-prM. To overcome this defect, the C_R99S
607 mutation creates a new NS2A/NS3 cleavage site at R98 of capsid protein, restoring the C-
608 prM processing and virion assembly of NS2A G11A (*right panel*).

609 The key of the above model is the blockage of viral protease cleavage site at the
610 C-terminus of capsid protein, which is caused by the NS2A G11A mutation. If the model is
611 correct, an insertion of one or two amino acids to the N-terminus of anchorC should
612 restore the C-prM cleavage and virion assembly in the presence of NS2A G11A mutation
613 (Fig. 4B). To test this hypothesis, we inserted Thr, The-Ala, or Ser-Arg to the C-terminus of
614 capsid protein (after R100) in the context of WT or NS2A G11A viral RNA. The inserted
615 amino acids were selected based on the cleavage requirement for viral protease (Fig. 4B).
616 Transfection of each viral RNA into BHK-21 cells yielded increasing number of E-positive
617 cells from day 1 to 4 (Fig. 4C), produced infectious viruses (Fig. 4D-E), displayed cytosolic
618 and nucleus localizations of C protein (Fig. 4F), and exhibited efficient C-prM cleavage
619 (Fig. 4H). In the absence of NS2A G11A, the capsid insertion mutant RNAs behaved
620 similarly to the WT RNA. In the presence of NS2A G11A, the capsid single-amino acid
621 insertion (*i.e.*, NS2A G11A+C_T101) partially restored virion assembly ($1.2\times10^3$ PFU/ml;
622 Fig. 4D-E), prM-positive cells (Fig. 4G), and C-prM cleavage (60%; Fig. 4H); while the



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capsid double-amino acid insertion (*i.e.*, NS2A G11A+C_TA and NS2A G11A+C_SR) almost fully restored virion assembly to the WT level (2.3-2.5×$10^6$ PFU/ml; Fig. 4D-E) and C-prM cleavage (92-98%; Fig. 4H). These results demonstrate that addition of one or two amino acids to the N-terminus of anchorC can rescue virion assembly of NS2A G11A.

Since the single-amino acid C_T101 insertion only partially rescued virion assembly of NS2A G11A, we selected revertants for NS2A G11A+C_T101 virus. After continuous passaging of the virus on Vero cells for eight rounds, the P8 viruses were subjected to plaque assay (Fig. S8A-B) and whole-genome sequencing (Fig. S8C). The P8 viruses from all four independent selections produced viral titers (Fig. S8B) that were ≥100-fold higher than the original NS2A G11A+C_T101 virus (Fig. 4E). Among them, selection I P8 virus retained the original NS2A G11A+C_T101 mutations. In addition, an extra mutation R100S was recovered in the capsid protein. Sequence analysis suggests that the R100S could alter the protease cleavage site from the original RRRR↓T to RRR↓ST (R100S underlined) and extends the N-terminus of anchorC by one more amino acid. Selection II P8 virus also retained the original NS2A G11A+C_T101 mutations and accumulated another mutation R99S in the capsid protein. The R99S may change the protease cleavage site to RR↓SRT and extends the N-terminus of anchorC by two more amino acids. In addition, selection II P8 virus contained the R100S mutation in 27% of the viral population. Selection III P8 virus retained the NS2A G11A mutation and showed mixed sequences of R99S and/or R100S. Selection IV P8 virus exhibited WT plaque morphology; sequencing results showed that the NS2A G11A was reverted to WT Gly, but the C_T101 insertion was retained. Collectively, the results again support the conclusion that extension of the N-terminus of anchorC by two to three residues could efficiently rescue virion assembly of NS2A G11A.



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**Analysis of NS2A G11A in mosquito cells.** To test if the above findings from mammalian cells could be extended to mosquito host, we analyzed NS2A G11A+capsid mutant RNAs in C6/36 cells. Viral RNAs containing NS2A G11A alone, NS2A G11A+C_T101, NS2A G11A+C_R99S, or NS2A G11A+C_TA were transfected into C6/36 cells. The transfected cells were analyzed for capsid protein-positive cells (Fig. S9A), infectious virus production (Fig. S9B-C), extracellular viral RNA release (Fig. S9D), and C-prM cleavage (Fig. S9E). Similar to the results obtained from mammalian cells, NS2A G11A mutant was defective in C-prM processing and virion production in C6/36 cells. The defects could be rescued by the capsid mutations, with the rescuing efficiency of C_TA > C_R99S > C_T101 (Fig. S9). The results indicate that NS2A G11A mutation exerts the same mechanism in abolishing virion assembly in mammalian and mosquito hosts.

**NS2A protein binds to 3'UTR RNA**. To further explore the role of DENV-2 NS2A in virion assembly, we examined the ability of NS2A to bind viral genomic RNA. A series of pulldown experiments were performed using biotinylated viral RNAs and NS2A protein (Fig. 5A). A 616-nucleotide viral RNA (5'UTR-$C_{66}$-3'UTR) comprising the 5'UTR, the first 66 nucleotides from the C gene, and the 3'UTR regions of DENV-2 genome was *in vitro* synthesized (Fig. S10A). The 3' end of the RNA was ligated to a biotinylated cytidine using T4 RNA ligase, resulting in 5'UTR-$C_{66}$-3'UTR-biotin RNA (Fig. S10B). Two types of GST-tagged NS2A protein were prepared for the pulldown experiment: One type NS2A was expressed from *E. coli*, partially purified, and reconstituted in detergent DDM-containing buffer (Fig. 5B); another type NS2A was from the cell lysates of HEK293T cells transiently expressing the NS2A protein (Fig. 5C). As shown in Fig. 5B-C, the 5'UTR-$C_{66}$-3'UTR-biotin RNA efficiently pulled down GST-tagged NS2A (Lanes 4 and 6), but not GST (Lanes 2 and 5); in contrast, neither GST nor GST-tagged NS2A proteins was pulled down by a biotinylated non-viral RNA control (Lanes 1 and 3). To further demonstrate the specificity



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of NS2A/5'UTR-$C_{66}$-3'UTR RNA interaction, we performed an RNA competition experiment. Increasing amounts un-biotinylated 5'UTR-$C_{66}$-3'UTR RNA competed away the NS2A/5'UTR-$C_{66}$-3'UTR-biotin RNA interaction, whereas yeast tRNA did not (Fig. 5D). These results suggest that 5'UTR-$C_{66}$-3'UTR RNA specifically binds to NS2A protein.

We estimated the binding affinity of NS2A/viral RNA interaction. 5'UTR-$C_{66}$-3'UTR-biotin RNA was incubated with increasing amounts of recombinant GST-NS2A protein. The reactions were separated on a native polyacrylamide gel (PAGE), transferred to nylon membrane, and probed with streptavidin-HRP (Fig. 5E). Quantification of free RNA and RNA/protein complex estimated a *Kd* of 35 nM for DENV-2 NS2A biding to viral RNA.

**The last 285 nucleotides of 3'UTR RNA is responsible for binding to NS2A protein**. To map the region of viral RNA responsible for binding to NS2A protein, we performed a competition assay using a set of un-biotinylated RNA fragments from 5'UTR-$C_{66}$-3'UTR (Fig. S10C). The fragments were prepared based on the previously proposed RNA structure [ ADDIN EN.CITE <EndNote><Cite><Author>Villordo</Author><Year>2015</Year><RecNum>109</RecNum><DisplayText>(Villordo et al., 2015)</DisplayText><record><rec-number>109</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1561296709">109</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Villordo, S. M.</author><author>Filomatori, C. V.</author><author>Sanchez-Vargas, I.</author><author>Blair, C. D.</author><author>Gamarnik, A. V.</author></authors></contributors><auth-address>Fundacion Instituto Leloir-CONICET, Buenos Aires, Argentina.Arthropod-borne and Infectious Diseases Laboratory, Department of Microbiology, Immunology and Pathology, Colorado State University, Fort Collins, Colorado, United States of America.</auth-address><titles><title>Dengue virus



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697 RNA structure specialization facilitates host adaptation</title><secondary-title>PLoS
698 Pathog</secondary-title></titles><periodical><full-title>PLoS Pathog</full-
699 title></periodical><pages>e1004604</pages><volume>11</volume><number>1</number
700 ><keywords><keyword>3' Untranslated Regions</keyword><keyword>Adaptation,
701 Biological/genetics</keyword><keyword>Animals</keyword><keyword>Cells,
702 Cultured</keyword><keyword>Cricetinae</keyword><keyword>Culicidae</keyword><key
703 word>Dengue Virus/*genetics</keyword><keyword>Host
704 Specificity/genetics</keyword><keyword>Host-Pathogen
705 Interactions/*genetics</keyword><keyword>Humans</keyword><keyword>Mutation</key
706 word><keyword>*Nucleic Acid Conformation</keyword><keyword>RNA,
707 Viral/*chemistry/genetics</keyword></keywords><dates><year>2015</year><pub-
708 dates><date>Jan</date></pub-dates></dates><isbn>1553-7374 (Electronic)1553-
709 7366 (Linking)</isbn><accession-num>25635835</accession-num><urls><related-
710 urls><url>https://www.ncbi.nlm.nih.gov/pubmed/25635835</url></related-
711 urls></urls><custom2>PMC4311971</custom2><electronic-resource-
712 num>10.1371/journal.ppat.1004604</electronic-resource-
713 num></record></Cite></EndNote>]. As shown in Fig. 5F, 3'UTR, but not 5'UTR-$C_{66}$,
714 competed away the NS2A/5'UTR-$C_{66}$-3'UTR-biotin RNA interaction, indicating that 3'UTR
715 RNA binds to NS2A protein. To further map the region of 3'UTR for NS2A binding, we
716 deleted pseudoknot 1 (Δpk1), pseudoknots 1-2 [Δpk(1-2)], and pseudoknots 1-3 [Δpk(1-3)]
717 from the 3'UTR (Fig. S10C). Only Δpk1 and Δpk(1-2) RNAs, not Δpk(1-3) RNA, could
718 compete away the NS2A/5'UTR-$C_{66}$-3'UTR-biotin RNA interaction (Fig. 5F), suggesting
719 that Δpk(1-2) RNA, representing the last 285 nucleotides of 3'UTR RNA, is required for
720 binding to NS2A protein. This conclusion was further supported by the results that different
721 fragments from Δpk(1-2) RNA, including pK(3-4), pk3, and 3'SL, did not compete away the
722 NS2A/5'UTR-$C_{66}$-3'UTR-biotin RNA interaction (Fig. 5F).



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Next, we performed a photo-activatable ribonucleoside-enhanced crosslinking (PAR-CL) experiment to define the nucleotides that may interact with NS2A protein (Fig. 5G). The 3'UTR RNA containing [ HYPERLINK "https://en.wikipedia.org/wiki/Photosensitivity" \o "Photosensitivity" ] 4-thiouridine was prepared through *in vitro* transcription. After RNA/protein complex formation, the 4-thiouridine in the 3'UTR RNA was cross-linked to NS2A protein under UV light (at 365-nm wavelength). The RNA was then subjected to deep sequencing. Due to the crosslinking of 4-thiouridine to protein, a T-to-C mutation was recovered at the RNA sites that interacted with NS2A protein (Figs. 5G and S11). Fig. 5H summarizes the U-to-C mutations in the 3'UTR RNA when complexed with NS2A protein. About 67% mutations occurred within the last 285 nucleotides of 3'UTR. The result further supports the NS2A/3'UTR RNA interaction.

**NS2A requires amino acids R94, K95, and K99A for binding to 3'UTR RNA and virion assembly.** To map the region of NS2A required for viral 3'UTR RNA binding, we prepared a panel of purified NS2A truncates with an N-terminal GST tag from *E. coli* (Fig. 6A). The binding efficiencies of NS2A truncates to 5'UTR-$C_{66}$-3'UTR-biotin RNA were compared using a pulldown assay. As shown in Fig. 6B, different NS2A truncates were pulled down by 5'UTR-$C_{66}$-3'UTR-biotin at varied efficiencies. The RNA pulled down NS2A 1-145, 1-168, and 1-218 (full-length) at comparable efficiencies. Compared to the full-length NS2A, the pulldown efficiencies for NS2A 1-101 and 1-71 decreased to 40% and 0%, respectively. These data suggest that amino acids 72-144 are required for NS2A binding to viral RNA.

According to the membrane topology of DENV-2 NS2A [ ADDIN EN.CITE <EndNote><Cite><Author>Nemesio</Author><Year>2014</Year><RecNum>82</RecNum><DisplayText>(Nemesio and Villalain, 2014)</DisplayText><record><rec-number>82</rec-number><foreign-keys><key app="EN" db-



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id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793217">82</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Nemesio, H.</author><author>Villalain, J.</author></authors></contributors><auth-address>Molecular and Cellular Biology Institute, Universitas "Miguel Hernandez" , E-03202 Elche-Alicante, Spain.</auth-address><titles><title>Membrane interacting regions of Dengue virus NS2A protein</title><secondary-title>J Phys Chem B</secondary-title></titles><periodical><full-title>J Phys Chem B</full-title></periodical><pages>10142-55</pages><volume>118</volume><number>34</number><keywords><keyword>Calorimetry, Differential Scanning</keyword><keyword>Cell Membrane/*metabolism</keyword><keyword>Fluorescence Polarization</keyword><keyword>*Hydrophobic and Hydrophilic Interactions</keyword><keyword>Peptide Library</keyword><keyword>Spectrophotometry, Infrared</keyword><keyword>Viral Nonstructural Proteins/*chemistry/*metabolism</keyword></keywords><dates><year>2014</year><pub-dates><date>Aug 28</date></pub-dates></dates><isbn>1520-5207 (Electronic)1520-5207 (Linking)</isbn><accession-num>25119664</accession-num><urls><related-urls><url>http://www.ncbi.nlm.nih.gov/pubmed/25119664</url></related-urls></urls><custom2>PMC4148155</custom2><electronic-resource-num>10.1021/jp504911r</electronic-resource-num></record></Cite></EndNote>], amino acids 72-144 form two transmembrane domains (72-92 and 101-118), one short cytosolic loop (93-100), and a luminal segment (118-144; Fig. 6A). Since viral RNA resides in the cytoplasm, we examined the cytosolic loop of NS2A for viral RNA binding. The cytosolic



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loop has three positively charged residues (R94, K95, and K99) that are conserved among the four DENV serotypes (Fig. 6C). To examine the function of these residues, we prepared a panel of recombinant DENV-2 containing Ala substitutions at single (R94A, K95A, or K99A), double (R94A+K95A), or triple residues (R94A+K95A+K99A). Upon transfection to BHK-21 cells, all mutant RNAs generated viral E protein-positive cells at day 1 (Fig. 6D). Except for the triple mutant, other RNAs produced more E-positive cells on day 4 post-transfection. Mutants R94A and K95A generated WT level of infectious viruses and plaque morphology (Fig. 6E-F). Mutants K99A and K94A+K95A generated 22- to 3-fold less viruses than the WT, respectively (Fig. 6F), and exhibited smaller plaques (Fig. 6E). Remarkably, triple mutant R94A+K95A+K99A RNA yielded almost undetectable amount of virus that was 50,000-fold lower than the WT (Fig. 6F). To examine the effect of R94A+K95A+K99A on viral RNA synthesis, we engineered the triple mutations into a luciferase replicon of DENV-2 (Fig. 6G). The R94A+K95A+K99A replicon generated a luciferase profile similar to the WT replicon, indicating that the mutations do not affect viral translation and RNA synthesis. Taken together, the results demonstrate that R94A+K95A+K99A triple mutant is defective in virion assembly.

Next, we examined the effect of R94A+K95A+K99A mutations on NS2A binding to viral RNA. Since the result from Fig. 6B indicates that NS2A fragment 72-144 is required for viral RNA binding, we expressed a short NS2A peptide 64-128 (comprising the first two NS2A transmembrane domains, the cytosolic region 93-100, and the N- and C-terminal luminal tails for correct folding of the peptide on membrane) fused with an N-terminal GST-tag (Fig. 6H). As shown in Fig. 6I, peptide 64-128 could be pulled down by 5'UTR-$C_{66}$-3'UTR-biotin RNA (Lane 1). Un-biotinylated 5'UTR-$C_{66}$-3'UTR or 3'UTR RNA (Lanes 2 and 4), but not 5'UTR-$C_{66}$ RNA or yeast tRNA (Lanes 3 and 5), could compete away the NS2A peptide 64-128/5'UTR-$C_{66}$-3'UTR-biotin RNA interaction. The result demonstrates that



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NS2A peptide 64-128 specifically binds to viral 3'UTR RNA. Importantly, mutant R94A+K95A+K99A reduced the pulldown efficiency of NS2A peptide 64-128 by over 80% (Fig. 6J). Notably, some degraded GST-tag was found to associate with the NS2A truncates in the above pulldown experiments (Figs. 6B I-J), most likely due to the dimerization property of GST[ ADDIN EN.CITE <EndNote><Cite><Author>Maru</Author><Year>1996</Year><RecNum>110</RecNum><DisplayText>(Maru et al., 1996)</DisplayText><record><rec-number>110</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1561302524">110</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Maru, Y.</author><author>Afar, D. E.</author><author>Witte, O. N.</author><author>Shibuya, M.</author></authors></contributors><auth-address>Department of Genetics, Institute of Medical Science, University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108, Japan.</auth-address><titles><title>The dimerization property of glutathione S-transferase partially reactivates Bcr-Abl lacking the oligomerization domain</title><secondary-title>J Biol Chem</secondary-title></titles><periodical><full-title>J Biol Chem</full-title></periodical><pages>15353-7</pages><volume>271</volume><number>26</number><keywords><keyword>*Adaptor Proteins, Signal Transducing</keyword><keyword>Animals</keyword><keyword>Bone Marrow Cells</keyword><keyword>Cell Transformation, Neoplastic</keyword><keyword>Cells, Cultured</keyword><keyword>Fusion Proteins, bcr-abl/*metabolism</keyword><keyword>GRB2 Adaptor Protein</keyword><keyword>Glutathione Transferase/*metabolism</keyword><keyword>Macromolecular Substances</keyword><keyword>Mice</keyword><keyword>Phosphotyrosine/metabolism</keyword><keyword>Protein Binding</keyword><keyword>Protein-Tyrosine



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825 Kinases/metabolism</keyword><keyword>Proteins/metabolism</keyword><keyword>Rat
826 s</keyword><keyword>Recombinant Fusion Proteins</keyword><keyword>Structure-
827 Activity Relationship</keyword></keywords><dates><year>1996</year><pub-
828 dates><date>Jun 28</date></pub-dates></dates><isbn>0021-9258 (Print)0021-
829 9258 (Linking)</isbn><accession-num>8663064</accession-num><urls><related-
830 urls><url>https://www.ncbi.nlm.nih.gov/pubmed/8663064</url></related-
831 urls></urls><electronic-resource-num>10.1074/jbc.271.26.15353</electronic-resource-
832 num></record></Cite></EndNote>]. Overall, the results indicate that R94, K95, and K99
833 are important for NS2A binding to viral 3'UTR.

834 **DISCUSSION**

835 Flavivirus assembly consists of a tightly controlled cascade of events: export of
836 newly synthesized viral RNA from the replication complex to assembly sites, encapsidation
837 of viral RNA with C protein, and envelopment of nucleocapsid with prM and E proteins.
838 Many host and viral factors (proteins and lipids) participate in this temporal and spatial
839 process [ ADDIN EN.CITE <EndNote><Cite><Author>Apte-
840 Sengupta</Author><Year>2014</Year><RecNum>94</RecNum><DisplayText>(Apte-
841 Sengupta et al., 2014)</DisplayText><record><rec-number>94</rec-number><foreign-
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843 timestamp="1560793218">94</key></foreign-keys><ref-type name="Journal
844 Article">17</ref-type><contributors><authors><author>Apte-Sengupta,
845 S.</author><author>Sirohi, D.</author><author>Kuhn, R.
846 J.</author></authors></contributors><auth-address>Markey Center for Structural Biology,
847 Department of Biological Sciences and Bindley Bioscience Center, Purdue University,
848 West Lafayette, IN, USA.Markey Center for Structural Biology, Department of



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849 Biological Sciences and Bindley Bioscience Center, Purdue University, West Lafayette, IN,
850 USA. Electronic address: kuhnr@purdue.edu.</auth-address><titles><title>Coupling of
851 replication and assembly in flaviviruses</title><secondary-title>Curr Opin
852 Virol</secondary-title></titles><periodical><full-title>Curr Opin Virol</full-
853 title></periodical><pages>134-
854 42</pages><volume>9</volume><keywords><keyword>Flavivirus/*physiology</keyword>
855 <keyword>Host-Pathogen
856 Interactions</keyword><keyword>Humans</keyword><keyword>Lipid
857 Metabolism</keyword><keyword>Viral Proteins/metabolism</keyword><keyword>*Virus
858 Assembly</keyword><keyword>*Virus
859 Replication</keyword></keywords><dates><year>2014</year><pub-
860 dates><date>Dec</date></pub-dates></dates><isbn>1879-6265 (Electronic)1879-
861 6257 (Linking)</isbn><accession-num>25462445</accession-num><urls><related-
862 urls><url>http://www.ncbi.nlm.nih.gov/pubmed/25462445</url></related-
863 urls></urls><custom2>PMC4268268</custom2><electronic-resource-
864 num>10.1016/j.coviro.2014.09.020</electronic-resource-
865 num></record></Cite></EndNote>]. Among them, flaviviral NS2A has emerged as a key
866 player to orchestrate virion assembly, as evidenced by the following findings. (i) In infected
867 cells, there are two distinct NS2A species that are required for viral RNA synthesis and
868 virion assembly. The NS2A species required for RNA synthesis is located at the viral
869 replication complex that could only be formed in *cis*; the other NS2A species required for
870 virus morphogenesis is located at the virion assembly complex that could be formed in *cis*
871 or *trans* [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. (ii) The defect in virion assembly
872 caused by DENV-2 and YFV NS2A mutations could be rescued by adaptive mutations in
873 prM, E, NS2B, and NS3 proteins, suggesting that NS2A genetically modulates other viral
874 proteins during virion assembly [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. (iii)



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Recombinant NS2A of Kunjin virus binds to viral 3'UTR RNA [ ADDIN EN.CITE <EndNote><Cite><Author>Mackenzie</Author><Year>1998</Year><RecNum>81</RecNum><DisplayText>(Mackenzie et al., 1998)</DisplayText><record><rec-number>81</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793217">81</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Mackenzie, J. M.</author><author>Khromykh, A. A.</author><author>Jones, M. K.</author><author>Westaway, E. G.</author></authors></contributors><titles><title>Subcellular localization and some biochemical properties of the flavivirus Kunjin nonstructural proteins NS2A and NS4A</title><secondary-title>Virology</secondary-title></titles><periodical><full-title>Virology</full-title></periodical><pages>203-15</pages><volume>245</volume><number>2</number><keywords><keyword>Animal</keyword><keyword>Cercopithecus aethiops</keyword><keyword>*Flavivirus/ph [Physiology]</keyword><keyword>Immunohistochemistry</keyword><keyword>RNA, Viral/me [Metabolism]</keyword><keyword>Support, Non-U.S. Gov't</keyword><keyword>Vero Cells</keyword><keyword>*Viral Nonstructural Proteins/me [Metabolism]</keyword><keyword>Virus Replication</keyword></keywords><dates><year>1998</year></dates><urls></urls></record></Cite></EndNote>]. These studies indicate that NS2A interacts with two of the three structural proteins (prM and E, not C) and viral RNA. However, how viral RNA and C protein are recruited to form nucleocapsid and how nucleocapsid is enveloped with prM-E proteins to form virion remain unknown. The current study aims to address these questions.



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First, we showed that DENV-2 NS2A interacted with prM to modulate virion assembly. WT NS2A directly bound to prM in the absence of other viral proteins (Fig. 3B). The biological relevance of this interaction was supported by the correlation between virion assembly and prM/NS2A interaction. Specifically, an NS2A mutation G11A, which abolished virion assembly (Fig. 1), enhanced its binding to prM by 1.7-2.5 folds (Fig. 3). Mechanistically, the enhanced interaction between the mutant NS2A and prM "over pulled" the membrane-associated anchorC towards the ER lumen, leading to a blockage of the viral protease cleavage site (most likely by the ER membrane) at the C-anchorC junction. The uncleaved C-prM resulted in a rapid degradation of all three structural proteins (Fig. 1). This is not surprising because the unprocessed C-prM is retained on the ER membrane, misfolded, and degraded via the ubiquitin-proteasome machinery (Fig. 1H). Since prM is a chaperone for E protein [ ADDIN EN.CITE <EndNote><Cite><Author>Lorenz</Author><Year>2002</Year><RecNum>96</RecNum><DisplayText>(Lorenz et al., 2002)</DisplayText><record><rec-number>96</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793218">96</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Lorenz, I. C.</author><author>Allison, S. L.</author><author>Heinz, F. X.</author><author>Helenius, A.</author></authors></contributors><auth-address>Institute of Biochemistry, Swiss Federal Institute of Technology, CH-8093 Zurich, Switzerland.</auth-address><titles><title>Folding and dimerization of tick-borne encephalitis virus envelope proteins prM and E in the endoplasmic reticulum</title><secondary-title>J Virol</secondary-title></titles><periodical><full-title>J Virol</full-title></periodical><pages>5480-91</pages><volume>76</volume><number>11</number><keywords><keyword>Animals</keyword><keyword>COS Cells</keyword><keyword>Cercopithecus



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925 aethiops</keyword><keyword>Dimerization</keyword><keyword>Dithiothreitol</keyword
926 ><keyword>Encephalitis Viruses, Tick-
927 Borne/genetics/*metabolism</keyword><keyword>Hexosaminidases/metabolism</keywor
928 d><keyword>Polyproteins/genetics/metabolism</keyword><keyword>*Protein
929 Folding</keyword><keyword>Protein
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934 (Linking)</isbn><accession-num>11991976</accession-num><urls><related-
935 urls><url>http://www.ncbi.nlm.nih.gov/pubmed/11991976</url></related-
936 urls></urls><custom2>PMC137023</custom2><electronic-resource-
937 num>10.1128/jvi.76.11.5480-5491.2002</electronic-resource-
938 num></record></Cite></EndNote>], the degradation of C-prM leads to the degradation of
939 E protein. These results are in agreement with a recent report that ZIKV anchorC plays an
940 important role in the stability of E protein and virion morphogenesis [ ADDIN EN.CITE
941 <EndNote><Cite><Author>Rana</Author><Year>2018</Year><RecNum>97</RecNum><
942 DisplayText>(Rana et al., 2018)</DisplayText><record><rec-number>97</rec-
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944 timestamp="1560793219">97</key></foreign-keys><ref-type name="Journal
945 Article">17</ref-type><contributors><authors><author>Rana, J.</author><author>Slon
946 Campos, J. L.</author><author>Leccese, G.</author><author>Francolini,
947 M.</author><author>Bestagno, M.</author><author>Poggianella,
948 M.</author><author>Burrone, O. R.</author></authors></contributors><auth-
949 address>Molecular Immunology Group, International Centre for Genetic Engineering and
950 Biotechnology, Trieste, Italy rana@icgeb.org burrone@icgeb.org.Molecular



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951 Immunology Group, International Centre for Genetic Engineering and Biotechnology,
952 Trieste, Italy.Department of Medical Biotechnology and Translational Medicine,
953 Universita degli Studi di Milano, Milan, Italy.</auth-address><titles><title>Role of Capsid
954 Anchor in the Morphogenesis of Zika Virus</title><secondary-title>J Virol</secondary-
955 title></titles><periodical><full-title>J Virol</full-
956 title></periodical><volume>92</volume><number>22</number><keywords><keyword>Zi
957 ka virus</keyword><keyword>capsid
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963 urls><url>http://www.ncbi.nlm.nih.gov/pubmed/30158295</url></related-
964 urls></urls><custom2>PMC6206493</custom2><electronic-resource-
965 num>10.1128/JVI.01174-18</electronic-resource-num></record></Cite></EndNote>].

966 Second, we demonstrated that the defect in virion assembly of NS2A G11A could
967 be restored by two different means. Creation of a new viral protease cleavage site by
968 extending the N-terminus of anchorC by two amino acids restored the capsid-anchorC
969 processing and, consequently, restored virion assembly. This conclusion was supported
970 by revertant analysis of the NS2A G11A mutant (Figs. 2 and S4) and amino acid insertion
971 experiment (Fig. 4). The two-amino acid extension exposes the new protease cleavage
972 site to NS2B/NS3. Alternatively, the virion assembly defect of NS2A G11A could be
973 rescued by *trans* supply of WT NS2A (Fig. S3). Under this condition, the WT NS2A
974 replaced the G11A NS2A to interact with C-prM, restored the C-prM cleavage, and ensued
975 a productive virion morphogenesis. Collectively, our results support a previous model that



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the two cleavages at C-anchorC-prM junctions are coordinated in a sequential manner during flavivirus assembly. The C-anchorC junction was first cleaved by viral protease on the cytosolic side, followed by the anchorC-prM cleavage by the cellular signalase on the ER lumen side [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. Although expression of flavivirus prM-E alone could form empty virus-like particles, the coordinated C-anchorC-prM processing couples the encapsidation and envelopment events; such coupling is required for the production of infectious virus. This conclusion is also supported by the previous reports that separate expression of C and prM-E significantly compromised the assembly efficiency of infectious virions [ ADDIN EN.CITE ADDIN EN.CITE.DATA ].

Third, we characterized the specific interaction between DENV-2 NS2A and the 3'UTR of viral genome. Although Kunjin virus NS2A was reported to bind to the 3'UTR of viral RNA [ ADDIN EN.CITE <EndNote><Cite><Author>Mackenzie</Author><Year>1998</Year><RecNum>81</RecNum><DisplayText>(Mackenzie et al., 1998)</DisplayText><record><rec-number>81</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793217">81</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Mackenzie, J. M.</author><author>Khromykh, A. A.</author><author>Jones, M. K.</author><author>Westaway, E. G.</author></authors></contributors><titles><title>Subcellular localization and some biochemical properties of the flavivirus Kunjin nonstructural proteins NS2A and NS4A</title><secondary-title>Virology</secondary-title></titles><periodical><full-title>Virology</full-title></periodical><pages>203-15</pages><volume>245</volume><number>2</number><keywords><keyword>Animal</keyword><keyword>Cercopithecus aethiops</keyword><keyword>*Flavivirus/ph



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1001 [Physiology]</keyword><keyword>Immunohistochemistry</keyword><keyword>RNA,
1002 Viral/me [Metabolism]</keyword><keyword>Support, Non-U.S.
1003 Gov't</keyword><keyword>Vero Cells</keyword><keyword>*Viral Nonstructural
1004 Proteins/me [Metabolism]</keyword><keyword>Virus
1005 Replication</keyword></keywords><dates><year>1998</year></dates><urls></urls></re
1006 cord></Cite></EndNote>], the molecular details of the interaction has not been delineated.
1007 Using competition assay and PAR-CLIP technique, we mapped that DENV-2 NS2A bound
1008 to the last 285 nucleotides of the 3'UTR, including the pk3, pk4, and 3'SL. Interestingly, the
1009 pk3, pk3-4, or 3'SL RNA alone did not efficiently compete out the NS2A/3'UTR RNA
1010 interaction (Fig. 5F), suggesting that a complex RNA structure in the 3'UTR is required for
1011 efficient binding to NS2A protein. Since no RNA “packaging signal” has been identified for
1012 flavivirus assembly and the C protein was shown to non-specifically bind to any RNA
1013 [ ADDIN EN.CITE
1014 <EndNote><Cite><Author>Pong</Author><Year>2011</Year><RecNum>102</RecNum>
1015 <DisplayText>(Pong et al., 2011)</DisplayText><record><rec-number>102</rec-
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1017 timestamp="1560793219">102</key></foreign-keys><ref-type name="Journal
1018 Article">17</ref-type><contributors><authors><author>Pong, W.
1019 L.</author><author>Huang, Z. S.</author><author>Teoh, P. G.</author><author>Wang,
1020 C. C.</author><author>Wu, H. N.</author></authors></contributors><auth-
1021 address>Institute of Molecular Biology, Academia Sinica, Taipei, Taiwan.</auth-
1022 address><titles><title>RNA binding property and RNA chaperone activity of dengue virus
1023 core protein and other viral RNA-interacting proteins</title><secondary-title>FEBS
1024 Lett</secondary-title><alt-title>FEBS letters</alt-title></titles><periodical><full-title>FEBS
1025 Lett</full-title><abbr-1>FEBS letters</abbr-1></periodical><alt-periodical><full-title>FEBS
1026 Lett</full-title><abbr-1>FEBS letters</abbr-1></alt-periodical><pages>2575-



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81</pages><volume>585</volume><number>16</number><keywords><keyword>Base Sequence</keyword><keyword>*Dengue Virus</keyword><keyword>Genome, Viral/genetics</keyword><keyword>Molecular Chaperones/*metabolism</keyword><keyword>Nucleic Acid Conformation</keyword><keyword>Protein Binding</keyword><keyword>Protein Engineering</keyword><keyword>RNA Helicases/metabolism</keyword><keyword>RNA, Catalytic/genetics</keyword><keyword>RNA, Viral/chemistry/genetics/*metabolism</keyword><keyword>Serine Endopeptidases/metabolism</keyword><keyword>Viral Core Proteins/*metabolism</keyword><keyword>Viral Nonstructural Proteins/metabolism</keyword></keywords><dates><year>2011</year><pub-dates><date>Aug 19</date></pub-dates></dates><isbn>1873-3468 (Electronic)0014-5793 (Linking)</isbn><accession-num>21771593</accession-num><urls><related-urls><url>http://www.ncbi.nlm.nih.gov/pubmed/21771593</url></related-urls></urls><electronic-resource-num>10.1016/j.febslet.2011.06.038</electronic-resource-num></record></Cite></EndNote>], the last 285 nucleotides of 3'UTR may serve as the "packaging signal" to recruit viral RNA to the virion assembly site through specific binding to NS2A. From the NS2A side, we have mapped the 3'UTR-binding site to a cytosolic loop (containing R94, K95, and K99) between the first and second transmembrane domains (Fig. 6). Triple mutant R94A+K95A+K99A was defective in virion assembly and lost over 80% of the NS2A binding activity to 3'UTR RNA (Fig. 6). Caution should be taken when applying the DENV-2 findings to other flaviviruses because of the variations of NS2A proteins. The three positively charged R94, K95, and K99 of NS2A are only conserved among the four serotypes of DENV. In addition, DENV-2, YFV, and ZIKV NS2A proteins



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exhibit different topologies on the ER membrane [ ADDIN EN.CITE ADDIN EN.CITE.DATA ].

Our findings support a general model for flavivirus virion assembly (Fig. 7). In infected cells, NS2A molecules reside in both replication complex (housed in Ve) and viron assembly complex (located on the juxtaposed ER membrane). When plus-sense RNAs are made and released from the replication complex (through the pore on Ve), they are initially destined to new rounds of viral translation and replication on the ER. Once the amounts of viral RNA and proteins have accumulated over certain “thresholds”, nascent RNAs are switched for virion assembly. At this time, the newly synthesized RNA exits the replication complex and binds to the NS2A located at the assembly site through the 3'UTR RNA. The NS2A molecules at the assembly site also recruits C-prM-E and NS2B/NS3 through binding to prM and NS3, respectively. One NS2A molecule does not have to bind to the 3’UTR RNA, C-prM-E, and NS2B/NS3 at the same time. These interactions could be sequential or through different NS2A molecules that oligomerize together on the ER membrane [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. Next, the C-prM-E polyprotein is sequentially cleaved by the NS2B/NS3 protease and host signalase to produce mature C, prM, and E [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. The C molecules, many of which have been previously made and stored at the nearby lipid droplets [ ADDIN EN.CITE ADDIN EN.CITE.DATA ], bind viral RNA to form nucleocapsid. The processed prM undergoes conformational change and dimerizes with E. The prM/E dimerization and oligomerization dissociate prM from NS2A. When 180 copies of prM/E oligomerize on the ER membrane, envelopment of the nucleocapsid occurs, generating spiky immature virions. During the above process, NS3 may also use its helicase activity to transport viral RNA from the replication complex to the assembly site and condense the



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RNA during encapsidation. Besides NS3, NS2A or other proteins may also contribute to the translocation of viral RNA from the replication complex to the assembly site.

The above model is centered on the multifunctional role of NS2A in flavivirus replication and virion assembly. Our results have functionally demonstrated the molecular mechanism for two important steps during DENV-2 assembly: (i) The binding of 3'UTR RNA to NS2A servers as a "packaging signal" and (ii) in the presence of viral RNA-NS2A complex, the coordinated cleavage of C-prM-E facilitates the envelopment of nucleocapsid. The proposed model could serve as a framework for future investigations and antiviral development.

**Author contribution**

X.X., J.Z., X.Z., Y.Z., Q.W., H.-P.D. performed experiments and data analysis. X.X., J.Z., X.Z., Y.Z., A.R., V.L.P., X.W., Q.W., H.-P.D, P.-Y.S. designed the experiments and interpreted the results. X.X., P.-Y.S. wrote the manuscript.

**Acknowledgements**

We are grateful to Dr. Mariano A. Garcia-Blanco, Shelton S. Brarick from University of Texas Medical Branch for helpful discussions during this work. P.-Y.S. was supported by NIH grants AI142759, AI[ HYPERLINK "https://public.era.nih.gov/grantfolder/piAppDetails/genericStatus.do?encryptedParam=vXeG1axw2cY.YZxjFgAisBblI6OqVj4LxDR99WdVyTI-X4FuJbCLt4g." \t "_blank" ], and AI136126, and awards from the Kleberg Foundation, John S. Dunn Foundation, Amon G. Carter Foundation, Gilson Longenbaugh Foundation, and Summerfield Robert Foundation. X.X. and J.Z. were awarded with postdoctoral fellowships from the Novartis Institutes for



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Biomedical Research (NIBR) and the McLaughlin Endowment at University of Texas Medical Branch, respectively.



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## Figure titles and legends

**Figure 1 Characterization of NS2A G11A mutant**. (A) Replicon transient transfection assay. Top panel depicts the DENV-2 luciferase replicon. Bottom panel shows the luciferase activities after transfecting BHK-21 cells with equal amounts (10 μg) of WT or NS2A G11A mutant replicon RNA. (B) Quantification of extracellular viral RNA. Top panel depicts the full-length genome of DENV-2. Equal amounts of WT or NS2A G11A mutant viral RNA (10 μg) were electroporated into BHK-21 cells. At different time post-transfection (p.t.), extracellular viral RNAs were quantified by RT-qPCR. The DENV-2 replicon RNA was used as a control for determining the basal level of viral RNA released from dead cells. Bottom panel shows the CT values of the RT-qPCR results. Data show the means and standard deviations from three independent experiments. (C) Plaque morphology. Culture supernatants from (B) were analyzed for virus production using plaque assay. Only WT, not NS2A G11A mutant (N.D.), produced plaques. Plaques were developed on BHK-21 cells after 5 days of infection. (D) Thin-section transmission electron microscope (TEM) images of viral RNA-transfected BHK-21 cells. At 48 h p.t., cells were fixed for TEM analysis. Ve, virus-induced vesicles; T, membrane tubes; Vi, virus particles; ER, endoplasmic reticulum. Mock transfected cells were included as a control. (E-G) Confocal microscopy analysis of subcellular localizations of C, prM, and E in viral RNA-transfected BHK-21 cells. At 48 h p.t., transfected cells were analyzed by IFA. NS3 staining (red) was used as a control. Nuclei (blue) were stained by DAPI. Scale bar, 10 μm. (H) Viral protein processing in viral RNA-transfected BHK-21 cells. At 42 h p.t., BHK-21 (Mock) or viral RNA-transfected cells were treated with 0.01% DMSO (-) or 10 μM MG132 (+). After 6 h treatment, the cells were analyzed by Western blot. (I) Densitometry analysis. The intensities of bands representing specific viral proteins from DMSO-treated cells in (H) was quantified. The amounts of viral proteins derived from WT RNA-transfected cells were set as 100%. Relative intensities are calculated by normalizing the protein intensities derived from NS2A G11A mutant RNA-transfected cells to those from WT RNA-transfected cells. Data shows the means and standard deviations from three independent experiments.

**Figure 2 Restoration of NS2A G11A virion assembly by capsid R99S mutation.** (A) Revertant mutation. BHK-21 cells were transfected with NS2A G11A viral RNA. The transfected cells were consecutively passaged for five rounds to select for revertant viruses. The P5 virus was sequenced for revertant mutations. Changes in nucleotide



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positions and amino acids are indicated. (B) IFA of viral RNA-transfected cells. WT and mutant viral RNAs were electroporated to BHK-21 cells. The transfected cells were analyzed for E protein expression at 24 and 96 h p.t. by IFA. Scale bar, 100 μm. (C) Plaque morphology. Infectious viruses, derived from the RNA-transfected cells, were analyzed by plaque assay. Plaques were developed on day 5 post-infection on BHK-21 cells. N.D., not detectable. (D) Virus production. The culture fluids from (B) were quantified for infectious viruses. Data represent the means and standard deviations from three independent measurements. Limit of detection (L.O.D.), 10 PFU/ml. (E-F) Subcellular distributions of prM, NS1, and C proteins. prM, NS1 and C proteins were detected by DM1, human IgG anti-NS1 and D2C1 antibodies, respectively. Representative IFA images of viral RNA-transfected cells are shown at 24 h post-transfection. White arrows point to nucleus with C protein speckles. (G) Quantitative analysis of C-positive cells. The C-positive cells with cytoplasm or cytoplasm & nucleus distribution from 4-5 random fields were counted and normalized to the total cell numbers. The averages and standard deviations are shown. (H) Viral protein processing in viral RNA-transfected BHK-21 cells at 48 h post-transfection. The percentages of mature C protein were calculated from the following the formula: [C intensity/(C intensity + C-prM intensity)]×100%.

**Figure 3 DENV-2 NS2A interacts with prM and E proteins**. (A) Membrane topologies of DENV-2 prM, E, and NS2A proteins. The NS2A G11 position is indicated. (B) Co-IP of WT NS2A with prM. HEK293T cells were transfected with one plasmid (encoding prM only) or two plasmids (one encoding leader-NS2A-EGFP and the other encoding full-length or the ectodomain of prM). At 40 h p.t., IP was performed using mouse anti-EGFP or control IgG antibodies. Immunoblot (IB) was performed using rabbit anti-EGFP antibody or anti-prM antibody. (C) Co-IP of WT or G11A NS2A-EGFP with prM. Mouse anti-EGFP antibody was used for IP. Rabbit anti-EGFP or anti-prM antibodies were used for IB. The intensity of prM, co-precipitated with WT NS2A-EGFP, was set as 100%. The relative efficiency of prM co-precipitated with G11A NS2A-EGFP was compared with the WT NS2A-EGFP. Statistical analysis was performed using the unpaired parametric t-test. $*p < 0.05$, significant; $**p < 0.01$, very significant. (D) Reciprocal co-IP of prM with WT or G11A NS2A-EGFP. Rabbit anti-prM antibodies were used for IP. The amounts of co-precipitated WT and G11A NS2A-EGFP were detected using mouse anti-EGFP antibody. prM in eluates were detected by rabbit anti-prM antibody followed by protein G conjugated with HRP. (E) Co-localization analysis of NS2A and prM proteins. BHK-21 cells were co-



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transfected with two plasmids: One encoding the $C_{14}$-prM-E and the other encoding SPG-$C_{16}$-NS2A-EGFP. At 24 h p.t., the cells were examined for prM and NS2A-EGFP co-localization under a confocal microscope. Scale bar, 10 μm. (F) Co-IP of NS2A with E protein. HEK293T cells were co-transfected with two plasmids: One encoding the last 17 amino acids of prM and complete E protein ($M_{17}$-E), and another encoding NS2A with a C-terminal GST tag (NS2A-GST). GST tag alone was used as a negative control. IPs were performed using rabbit anti-GST antibody. E and GST fusion proteins were detected in cell lysates and eluates by mouse anti-E monoclonal antibody (9F12) and anti-GST antibody, respectively. The relative pulldown efficiency was calculated by normalizing the intensity of E co-precipitated with G11A NS2A-GST to the intensity of E co-precipitated with the WT NS2A-GST. The means and standard deviations from three independent experiments are shown. N.S., no significance.

**Figure 4 Two-residue insertion at the capsid-anchorC junction rescues the defect of NS2A G11A**. (A) A model depicting how NS2A modulates C-anchorC-prM cleavage. On the cytosolic side, viral NS2B/NS3 protease cleaves the C-anchorC junction. On the ER lumen side, host signalase cleaves the anchorC-prM junction. Three cleavage scenarios are presented for WT, NS2A G11A, and NS2A G11A+C_R99S constructs. (B) Constructs of insertion mutants of capsid protein. One residue (C_101) or two residues (C_TA or C_SR) were inserted to the C-anchorC junction. The potential cleavage sites for viral NS2B/NS3 protease are indicated. (C) IFA of viral RNA-transfected cells. Viral RNAs containing the NS2A G11A mutation with or without capsid insertions were electroporated into BHK-21 cells. The transfected cells were analyzed for E protein expression on day 1 and 4 by IFA. Scale bar, 100 μm. (D) Plaque morphology. Viruses recovered from (C) were analyzed by plaque assay on BHK-21 cells. (E) Viral titers. Culture supernatants from (C) were measured for viral titers on day 5 post-transfection. (F) Subcellular localization of C protein in viral RNA-transfected cells. White arrows point to nucleus with C protein speckles. Scale bar, 25 μm. (G) Expression of prM and NS1 proteins in viral RNA-transfected cells. Scale bar, 50 μm. (H) C-prM processing in viral RNA-transfected cells. At 48 h p.t., WT or NS2A G11A+C_T101 RNA-transfected cells were analyzed for C, NS1, and β-actin expression by Western blot. The percentages of C-prM to mature C conversion are presented for each construct.

**Figure 5 DENV-2 NS2A protein binds to 3'UTR RNA**. (A) Diagram of pulldown assay using biotinylated RNAs. 3'-end biotinylated RNA binds to streptavidin-conjugated



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magnetic beads. After incubating the bead-bound biotinylated RNA with NS2A protein, the RNA-bound NS2A was enriched and eluted for SDS-PAGE and Western blot analysis. (B) Pulldown analysis using recombinant NS2A protein. N-terminally GST-tagged NS2A protein (GST-NS2A) was expressed in *E. coli* and affinity purified. The 5'UTR-$C_{66}$-3'UTR-biotin RNA was prepared by ligating biotinylated cytidine (bis)phosphate to the 3' end of the 5'UTR-$C_{66}$-3'UTR RNA (containing the 5'UTR, the first 66 nucleotides of C gene, and the 3'UTR). The amounts of GST-NS2A in the input and eluates were analyzed using rabbit anti-GST antibody by Western blot. (C) Pulldown analysis using HEK293T cell-expressed NS2A-GST protein. HEK293T cells were transfected with a plasmid expressing GST or C-terminally GST-tagged NS2A protein (NS2A-GST). At 42 h p.t., cells were harvested, lysed, and used for RNA pull down-assay. The pulldown assay was performed as described in (B). (D) RNA competition assay. Various amounts of unlabeled 5'UTR-$C_{66}$-3'UTR RNA were used to compete the binding of 5'UTR-$C_{66}$-3'UTR-biotin RNA to GST-NS2A protein. tRNA was used as a negative control. (E) Viral RNA/NS2A binding affinity. Electrophoretic mobility-shift assay (EMSA) was used to determine the binding affinity of DENV-2 5'UTR-$C_{66}$-3'UTR-biotin RNA with NS2A protein. 5'UTR-$C_{66}$-3'UTR-biotin RNA was incubated with increasing amounts of GST-NS2A protein. Free and bound RNAs were resolved by native agarose gel electrophoresis. The equilibrium dissociation constant (*Kd*) was determined by nonlinear regression with least-squares fitting method. The coefficient of determination ($R^2$) is shown. Data represent the means and standard deviations from three independent experiments. (F) Mapping the RNA region responsible for NS2A binding. A panel of unlabeled RNA truncates of the 5'UTR-$C_{66}$-3'UTR were used as competitors for the binding of 5'UTR-$C_{66}$-3'UTR-biotin RNA to GST-NS2A protein. The intensity of GST-NS2A (bound to the 5'UTR-$C_{66}$-3'UTR-biotin) without competitor was set as 100%. Relative GST-NS2A intensities were calculated as [(signal in the presence competitor)/(signal in the absence of competitor)]×100%. Statistical analysis was performed using the unpaired parametric t-test. (G) PAR-CLIP workflow [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. See text for detail. (H) PAR-CLIP result summary. Uridines in the 3'UTR that may participate in NS2A-binding are indicated. The dash line indicates the threshold (equal to 5.76, representing the average fold change of mutation rate of all uridines determined by Illumina sequencing). The uridine positions with fold changes of mutation rate over the threshold are indicated in the 3'UTR sequence (GenBank no. AF038403).



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**Figure 6 NS2A residues responsible for the NS2A/3'UTR RNA interaction**. (A) Diagram of NS2A truncates used in RNA pulldown assay. All proteins were fused with an N-terminal GST tag, expressed in *E. coli*, and affinity purified. (B) Interactions between NS2A truncates with 5'UTR-$C_{66}$-3'UTR-biotin RNA. The interactions were measured using pulldown experiments. The pulldown efficiency of full-length NS2A (1-218) was set as 100%. Relative efficiency was calculated by comparing the intensity of NS2A truncate with that of the full-length NS2A. (C) Sequence alignment of NS2A among flaviviruses. Amino acids of a cytosolic loop (amino acids 93-101) are presented. The number of viral strains used for the NS2A alignment are indicated in parentheses. Amino acid positions of NS2A are labeled according to DENV-2 strain New Guinea C. Residues R94, K95, and K99 are shaded. The conserved (:) and identical (*) residues are indicated. (D) IFA of BHK-21 cells transfected with viral RNA. WT and mutant viral RNAs were electroporated into BHK-21 cells. The cells were assayed for E protein expression. (E) Plaque morphology. Viruses derived from the transfected cells in (D) were analyzed by plaque assay. (F) Viral titers. Culture supernatants from (D) were measured for virus yields on day 5 post-transfection. (G) Replicon transient transfection assay. NS2A triple mutations (R94A+K95A+K99A) were engineered into a luciferase replicon of DENV-2. Luciferase activities were presented after the WT or mutant replicon RNAs were electroporated into BHK-21 cells. (H) Diagram of N-terminally GST-tagged NS2A peptide 64-128 [GST-NS2A (64-128)]. (I) Interaction of GST-NS2A (64-128) protein with 3'UTR RNA. GST-NS2A (64-128) was expressed in *E. coli* and affinity purified. The pulldown efficiency of GST-NS2A (64-128) by 5'UTR-$C_{66}$-3'UTR-biotin was set as 100%. The binding efficiencies of RNA variants were indicated through competition assay. In the presence of competitor RNAs, the remaining amounts of WT GST-NS2A (64-128)/5'UTR-$C_{66}$-3'UTR-biotin interaction are presented. (J) R94A+K95A+K99A mutations reduce GST-NS2A (64-128) binding to 5'UTR-$C_{66}$-3'UTR-biotin RNA. The R94A+K95A+K99A variant retained only 19.1% of the WT activity.

**Figure 7 A model depicting the role of NS2A in DENV-2 assembly.** Viral replication complex (top) and virion assembly site (bottom) are depicted. DENV-2 NS2A protein recruits viral RNA, three structural proteins, and NS2B/NS3 protease to virion assembly site, where coordinated cleavage of C-prM-E ensues infections virus formation. C molecules accumulated on lipid droplets (LD) could bind viral RNA to form nucleocapsid. See text for details.



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**Reference**

[ ADDIN EN.REFLIST ]



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## STAR METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---|---|---|
| Antibodies | | |
| Mouse IgG Anti-C (D2C1) | [ ADDIN EN.CITE ADDIN EN.CITE.DATA ] | N/A |
| Mouse IgG anti-prM (DM1) | Abcam | Cat# ab41473, RRID:AB_873758 |
| Rabbit IgG anti-prM | GeneTex | GTX128093 |
| Mouse IgG anti-E (4G2) | ATCC | Cat# HB-112, RRID:CVCL_J890 |
| Mouse IgG anti-E (9F12) | [ ADDIN EN.CITE ADDIN EN.CITE.DATA ] | N/A |
| Mouse IgG anti-NS1 | Abcam | Cat# ab41616, RRID:AB_869448 |
| Human IgG anti-NS1 | This study. Generated by Novartis Institute for Tropical Diseases in Singapore | N/A |
| Rabbit IgG anti-NS1 | GeneTex | GTX103346 |
| Rabbit IgG anti-NS3 | GeneTex | GTX124252 |
| Mouse IgG anti-NS5 | GeneTex | GTX629447 |
| Rabbit IgG anti-calnexin | SIGMA-AlDRICH | Cat# C4731, RRID:AB_476845 |
| Mouse IgG anti-EGFP | SIGMA-AlDRICH | Cat# 11814460001, RRID:AB_390913 |
| Moue IgG1 Isotype Control | SIGMA-AlDRICH | Cat# M5284, RRID:AB_1163685 |
| Rabbit IgG anti-EGFP | Abcam | Cat# ab13970, RRID:AB_300798 |
| protein G horseradish peroxidase (HRP) conjugate | Thermo Fisher Scientific | Cat# P21041 |
| Mouse IgG anti-GST | Abcam | Cat# ab19256, RRID:AB_444809 |
| Rabbit IgG anti-GST | SIGMA-AlDRICH | Cat# G7781, RRID:AB_259965 |
| Mouse IgG anti-β-actin | Abcam | Cat# ab8226, RRID:AB_306371 |
| Mouse IgG anti-HA | Abcam | Cat# ab18181, RRID:AB_444303 |
| Goat anti-mouse IgG conjugated with HRP | SIGMA-AlDRICH | Cat# A5278, RRID:AB_258232 |
| Goat anti-rabbit IgG conjugated with HRP | SIGMA-AlDRICH | Cat#AP307P, RRID:AB_92641 |
| Goat anti-mouse IgGs conjugated with Alexa 488 | Thermo Fisher Scientific | Cat#A-1100, RRID:AB_2534069 |
| Goat anti-mouse IgGs conjugated with Alexa 594 | Thermo Fisher Scientific | Cat# A-11005, RRID:AB_2534073 |
| Goat anti-rabbit IgGs conjugated with Alexa 488 | Thermo Fisher Scientific | Cat# A-11008, RRID:AB_143165 |



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| | | |
|---|---|---|
| Goat anti-rabbit IgGs conjugated with Alexa 594 | Thermo Fisher Scientific | Cat# A-11072, RRID:AB_2534116 |
| Goat anti-Human IgG (H+L) cross-adsorbed secondary antibody, Alexa Fluor 594 | Thermo Fisher Scientific | Cat# A-11014, RRID:AB_2534081 |
| Bacterial and Virus Strains | | |
| *E.* coli strain Top 10 | ThermoFisher Scientific | Cat#C404006 |
| *E.* coli strain Rosetta™ 2(DE3) pLysS | MilliporeSigma | Cat#71403-4 |



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|---|---|---|
| DENV-2 strain New Guinea C | [ ADDIN EN.CITE <EndNote><Cite><Author>Xie</Author><Year>2013</Year><RecNum>85</RecNum><DisplayText>(Xie et al., 2013)</DisplayText><record><rec-number>85</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793217">85</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Xie, X.</author><author>Gayen, S.</author><author>Kang, C.</author><author>Yuan, Z.</author><author>Shi, P. Y.</author></authors></contributors><auth-address>State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Science, China.</auth-address><titles><title>Membrane topology and function of dengue virus NS2A protein</title><secondary-title>J Virol</secondary-title><alt-title>Journal of virology</alt-title></titles><periodical><full-title>J Virol</full-title></periodical><pages>4609-22</pages><volume>87</volume><number>8</number><edition>2013/02/15</edition><keywords><keyword>Animals</keyword><keyword>Cell Line</keyword><keyword>Cricetinae</keyword><keyword>Dengue | N.A. |



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| | | |
|---|---|---|
| Biological Samples | | |
| None | | |
| Chemicals, Peptides, and Recombinant Proteins | | |
| Peptide WT (NILNRRRRTAGMII) | GenScript | N/A |
| Peptide C_R99S (NILNRRSRTAGMII) | GenScript | N/A |
| Recombinant protein: GST | This study | N/A |
| Recombinant protein: GST-NS2A | This study | N/A |
| Recombinant protein: GST-NS2A(1-71) | This study | N/A |
| Recombinant protein: GST-NS2A(1-101) | This study | N/A |
| Recombinant protein: GST-NS2A(1-145) | This study | N/A |
| Recombinant protein: GST-NS2A(1-168) | This study | N/A |
| Recombinant protein: GST-NS2A(64-128) | This study | N/A |
| Critical Commercial Assays | | |
| iScript SYBR Green One-Step kit | Bio-Rad | Cat#170-8893 |
| QIAamp viral RNA mini kit | Qiagen | Cat#52906 |
| T7 mMessage mMachine kit | Thermo Fisher Scientific | Cat# AM1344 |
| Renilla Luciferase Assay System | Promega Corporation | Cat# E2810 |
| Pierce™ RNA 3' End Biotinylation Kit | Thermo Fisher Scientific | Cat# 20160 |
| Pierce™ Magnetic RNA-Protein Pull-Down kit | Thermo Fisher Scientific | Cat# 20164 |
| Chemiluminescent Detection Module | Thermo Fisher Scientific | Cat# 89880 |
| | | |
| Deposited Data | | |
| Raw image data for illumina sequencing | | |
| Experimental Models: Cell Lines | | |
| BHK-21 cell | ATCC | Cat#CCL-10, RRID:CVCL_1915 |
| Vero cell | ATCC | Cat#CCL-81, RRID:CVCL_0059 |
| HEK293T cell | ATCC | Cat# CRL-3216, RRID:CVCL_0063 |
| NS2A-EGFP BHK-21 cell | [ ADDIN EN.CITE ADDIN EN.CITE.DATA ] | N/A |
| | | |
| Experimental Models: Organisms/Strains | | |
| N.A. | | |
| Oligonucleotides | | |
| Primer P1(5'-ACACTCTTTCCCTACACGA CGCTCTTCCGATCTAAGGCAAAACTAACATGAA AC-3') | Integrated DNA Technologies (Skokie, Illinois) | N/A |
| Primer P2 (5'-GTGACTGGAGTTCAGACG TGTGCTCTTCCGATCTAGAACCTGTTGATTCAA CAGCAC-3') | Integrated DNA Technologies (Skokie, Illinois) | N/A |



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|---|---|---|
| 5' universal adaptor (5'-AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGAC-3') | Illumine (San Diego, CA) | N/A |
| 3' indexing primer (5'-CAAGCAGAAGACGGCATACGAGATnnnnnnGTGACTGGAGTTCAGACGTGT-3') | Illumine (San Diego, CA) | N/A |
| Primer NGC 7764V (5'-CGTCGAGAGAAATATGGTCACACC-3') | Integrated DNA Technologies (Skokie, Illinois) | N/A |
| Primer NGC 7844C (5'CCACAATAGTATGACCAGCCT-3') | Integrated DNA Technologies (Skokie, Illinois) | N/A |
| Recombinant DNA | | |



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| | | |
|---|---|---|
| pACYC-NGC FL | [ ADDIN EN.CITE <EndNote><Cite><Author>Xie</Author><Year>2013</Year><RecNum>85</RecNum><DisplayText>(Xie et al., 2013)</DisplayText><record><rec-number>85</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793217">85</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Xie, X.</author><author>Gayen, S.</author><author>Kang, C.</author><author>Yuan, Z.</author><author>Shi, P. Y.</author></authors></contributors><auth-address>State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Science, China.</auth-address><titles><title>Membrane topology and function of dengue virus NS2A protein</title><secondary-title>J Virol</secondary-title><alt-title>Journal of virology</alt-title></titles><periodical><full-title>J Virol</full-title></periodical><pages>4609-22</pages><volume>87</volume><number>8</number><edition>2013/02/15</edition><keywords><keyword>Animals</keyword><keyword>Cell Line</keyword><keyword>Cricetinae</keyword><keyword>Dengu | N/A |



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| | | |
|---|---|---|
| pACYC-NGC replicon | [ ADDIN EN.CITE <EndNote><Cite><Author>Xie</Author><Year>2013</Year><RecNum>85</RecNum><DisplayText>(Xie et al., 2013)</DisplayText><record><rec-number>85</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793217">85</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Xie, X.</author><author>Gayen, S.</author><author>Kang, C.</author><author>Yuan, Z.</author><author>Shi, P. Y.</author></authors></contributors><auth-address>State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Science, China.</auth-address><titles><title>Membrane topology and function of dengue virus NS2A protein</title><secondary-title>J Virol</secondary-title><alt-title>Journal of virology</alt-title></titles><periodical><full-title>J Virol</full-title></periodical><pages>4609-22</pages><volume>87</volume><number>8</number><edition>2013/02/15</edition><keywords><keyword>Animals</keyword><keyword>Cell Line</keyword><keyword>Cricetinae</keyword><keyword>Dengue | N/A |



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| | | |
|---|---|---|
| pACYC-NGC replicon G11A | [ ADDIN EN.CITE ADDIN EN.CITE.DATA ] | N/A |
| pACYC-NGC replicon R94A+K95A+K99A | This study | N/A |
| pACYC-NGC FL C_R99S | This study | N/A |
| pACYC-NGC FL G11A | This study | N/A |
| pACYC-NGC FL G11A+C_R99S | This study | N/A |
| pACYC-NGC FL E20A | This study | N/A |
| pACYC-NGC FL E100A | This study | N/A |
| pACYC-NGC FL K188A | This study | N/A |
| pACYC-NGC FL E20A+C_R99S | This study | N/A |
| pACYC-NGC FL E100A+C_R99S | This study | N/A |
| pACYC-NGC FL K188A+C_R99S | This study | N/A |
| pACYC-NGC FL C_TA | This study | N/A |
| pACYC-NGC FL C_SR | This study | N/A |
| pACYC-NGC FL G11A+C_TA | This study | N/A |
| pACYC-NGC FL G11A+C_SR | This study | N/A |
| pACYC-NGC FL C_T101 | This study | N/A |
| pACYC-NGC FL G11A+C_T101 | This study | N/A |
| pACYC-NGC FL R94A | This study | N/A |
| pACYC-NGC FL K95A | This study | N/A |
| pACYC-NGC FL K99A | This study | N/A |
| pACYC-NGC FL R94A+K95A+K99A | This study | N/A |
| pACYC-NGC FL R94A+K95A | This study | N/A |



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| | | |
|---|---|---|
| SFV-DENV2 CprME | [ ADDIN EN.CITE <EndNote><Cite><Author>Qing</Author><Year>2010</Year><RecNum>105</RecNum><DisplayText>(Qing et al., 2010)</DisplayText><record><rec-number>105</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793220">105</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Qing, M.</author><author>Liu, W.</author><author>Yuan, Z.</author><author>Gu, F.</author><author>Shi, P. Y.</author></authors></contributors><auth-address>Novartis Institute for Tropical Diseases, 10, Biopolis Road, #05-01 Chromos, Singapore, Singapore.</auth-address><titles><title>A high-throughput assay using dengue-1 virus-like particles for drug discovery</title><secondary-title>Antiviral Res</secondary-title></titles><periodical><full-title>Antiviral Res</full-title></periodical><pages>163-71</pages><volume>86</volume><number>2</number><dates><year>2010</year><pub-dates><date>May</date></pub- [ PAGE * MERGEFORMAT ] dates></dates><accession-num>20153777</accession-num><urls><related- | N/A |

| | | |
|---|---|---|
| pXJ-SPG-$C_{16}$-NS2A-EGFP | This study | N/A |
| pXJ-SPG-$C_{16}$-NS2A(G11A)-EGFP | This study | N/A |
| pXJ-EGFP | [ ADDIN EN.CITE ADDIN EN.CITE.DATA ] | N/A |
| pXJ-$C_{14}$-prM | This study | N/A |
| pXJ-$C_{14}$-prM(1-130) | This study | N/A |
| pXJ-$C_{14}$-prM (N69Q) | This study | N/A |
| pXJ-SPG-$C_{16}$-NS2A-GST | This study | N/A |
| pXJ-SPG-$C_{16}$-NS2A(G11A)-GST | This study | N/A |
| pXJ-GST | This study | N/A |
| pXJ-HA-NS3 | This study | N/A |
| pXJ-$M_{17}$-E | This study | N/A |
| pGEX-4T-1 | GE Healthcare Life Sciences | Cat# 28954549 |
| pGEX-4T-GST-NS2A | This study | N/A |
| pGEX-4T-GST-NS2A (1-71) | This study | N/A |
| pGEX-4T-GST-NS2A (1-101) | This study | N/A |
| pGEX-4T-GST-NS2A (1-145) | This study | N/A |
| pGEX-4T-GST-NS2A (1-168) | This study | N/A |
| pGEX-4T-GST-NS2A (64-128) | This study | N/A |
| pGEX-4T-GST-NS2A (64-128) R94A+K95A+K99A | This study | N/A |
| | | |
| Software and Algorithms | | |
| Illustrator CC | Adobe | [ HYPERLINK "https://www.adobe.com/products/illustrator.html" ] |
| ImageJ | NIH | [ HYPERLINK "https://fiji.sc/" ] |
| CLC main workbench | Qiagen | [ HYPERLINK "https://www.qiagenbioinformatics.com/products/clc-main-workbench/" ] |
| Prism 8.0 software | GraphPad | [ HYPERLINK "https://www.graphpad.com/scientific-software/prism/" ] |
| | | |

1271

1272 **Contact for Reagent and Resource Sharing**

1273 Further information and requests for resources and reagents should be directed to and will
1274 be fulfilled by Lead Contact, Dr. Pei-Yong Shi (peshi@utmb.edu)

1275 **Experimental Model and Subject Details**



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## Cell Lines

The Baby hamster kidney fibroblast (BHK-21) cells, African green monkey kidney epithelial (Vero) cells were purchased from the American Type Culture Collection (ATCC, Bethesda, MD) and maintained in a high-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (HyClone Laboratories, South Logan, UT) and 1% penicillin/streptomycin (P/S). The NS2A-EGFP BHK-21 cells constitutively expressing the C-terminally EGFP-tagged NS2A protein was maintained in the high-glucose DMEM medium supplemented with 10%FBS, 1% P/S and 1 mg/ml Geneticin. HEK293T cells were grown in low-glucose DMEM (Invitrogen) containing 10% FBS and 1% penicillin-streptomycin. All mammalian cell lines were grown at 37°C with 5% $CO_2$. Mosquito C6/36 cells were grown in RPMI 1640 medium (Invitrogen) with 10% FBS and 1% penicillin-streptomycin at 28°C without $CO_2$. All culture medium and antibiotics were purchased from ThermoFisher Scientific (Waltham, MA). All cell lines were tested negative for mycoplasma.

## Pseudo-infectious Particle

Pseudo-infectious particles were produced using the a previously described protocol [ ADDIN EN.CITE <EndNote><Cite><Author>Qing</Author><Year>2010</Year><RecNum>114</RecNum><DisplayText>(Qing et al., 2010)</DisplayText><record><rec-number>114</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1561745637">114</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Qing, M.</author><author>Liu, W.</author><author>Yuan, Z.</author><author>Gu, F.</author><author>Shi, P. Y.</author></authors></contributors><auth-address>Novartis Institute for Tropical Diseases, 10, Biopolis Road, #05-01 Chromos, Singapore, Singapore.</auth-address><titles><title>A high-throughput assay using dengue-1 virus-like particles for drug discovery</title><secondary-title>Antiviral Res</secondary-title></titles><periodical><full-title>Antiviral Res</full-title></periodical><pages>163-71</pages><volume>86</volume><number>2</number><keywords><keyword>Animals</keyword><keyword>Antiviral Agents/*isolation & purification/*pharmacology</keyword><keyword>Biological Assay/methods</keyword><keyword>Cell Line</keyword><keyword>Cercopithecus



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aethiops</keyword><keyword>Cricetinae</keyword><keyword>Dengue Virus/*drug effects/genetics</keyword><keyword>Drug Discovery/*methods</keyword><keyword>Drug Evaluation, Preclinical/*methods</keyword><keyword>Electroporation</keyword><keyword>Genes, Reporter</keyword><keyword>Luciferases, Renilla/genetics/metabolism</keyword><keyword>Macaca mulatta</keyword><keyword>Viral Proteins/genetics/metabolism</keyword><keyword>Virosomes/*drug effects/genetics</keyword></keywords><dates><year>2010</year><pub-dates><date>May</date></pub-dates></dates><isbn>1872-9096 (Electronic)0166-3542 (Linking)</isbn><accession-num>20153777</accession-num><urls><related-urls><url>https://www.ncbi.nlm.nih.gov/pubmed/20153777</url></related-urls></urls><electronic-resource-num>10.1016/j.antiviral.2010.02.313</electronic-resource-num></record></Cite></EndNote>] with several modifications. 10 µg of replicon RNA was electroporated into BHK-21 cells as described above. After transfection, the cells were maintained at 37°C with 5% $CO_2$ for 24 h. Afterwards, cells were harvested and electroporated with 10 µg of a helper replicon RNA SFV-DENV2 CprME (replicon derived from Semliki forest virus that express the DENV-2 encoding the CprME). After the second electroporation, cells were cultured at 30°C with 5% $CO_2$. At 20, 28 and 48 h after the second electroporation, culture medium was collected and stored at -80 °C. Afterwards, the collected medium was used ton infection naïve Vero cells in 96-well plates. At 48 h post-infection, cells were lysed, and luciferase signals were measured using the Renilla luciferase assay system (Promega) in the Clarity luminescence microplate reader (BioTek) according to the manufacturer's protocol.

## METHOD DETAILS

### Plasmid Construction

The full-length cDNA infectious clone of DENV-2 strain New Guinea C (NGC), pACYC-NGC FL, and its derived replicon (pACYC-NGC replicon) clone [ ADDIN EN.CITE <EndNote><Cite><Author>Xie</Author><Year>2013</Year><RecNum>85</RecNum><DisplayText>(Xie et al., 2013)</DisplayText><record><rec-number>85</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793217">85</key></foreign-keys><ref-type name="Journal



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Article">17</ref-type><contributors><authors><author>Xie, X.</author><author>Gayen, S.</author><author>Kang, C.</author><author>Yuan, Z.</author><author>Shi, P. Y.</author></authors></contributors><auth-address>State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Science, China.</auth-address><titles><title>Membrane topology and function of dengue virus NS2A protein</title><secondary-title>J Virol</secondary-title><alt-title>Journal of virology</alt-title></titles><periodical><full-title>J Virol</full-title></periodical><pages>4609-22</pages><volume>87</volume><number>8</number><edition>2013/02/15</edition><keywords><keyword>Animals</keyword><keyword>Cell Line</keyword><keyword>Cricetinae</keyword><keyword>Dengue Virus/*physiology</keyword><keyword>Endoplasmic Reticulum/*virology</keyword><keyword>Humans</keyword><keyword>Intracellular Membranes/*metabolism</keyword><keyword>Magnetic Resonance Spectroscopy</keyword><keyword>Models, Molecular</keyword><keyword>Protein Conformation</keyword><keyword>Viral Nonstructural Proteins/*chemistry/metabolism</keyword><keyword>*Virus Assembly</keyword></keywords><dates><year>2013</year><pub-dates><date>Apr</date></pub-dates></dates><isbn>1098-5514 (Electronic)0022-538X (Linking)</isbn><accession-num>23408612</accession-num><work-type>Research Support, Non-U.S. Gov't</work-type><urls><related-urls><url>http://www.ncbi.nlm.nih.gov/pubmed/23408612</url></related-urls></urls><custom2>3624351</custom2><electronic-resource-num>10.1128/JVI.02424-12</electronic-resource-num><language>eng</language></record></Cite></EndNote>] were used as the backbones for mutagenesis study. The replicon was constructed by replacing most of the structural genes (C, prM and E) with a gene cassette (Rluc2A) encoding the Renilla luciferase and a 2A peptide from Foot-and-mouth disease virus. The first 38 amino acids of C protein and the last 31 amino acids of E protein were retained in the replicon. Standard overlap PCR was performed to introduce the mutations in C and NS2A genes. The DNA fragments containing the mutations in C gene were cloned into pACYC-NGC FL clone through restriction enzyme sites NotI (upstream of the T7 promoter and 5'UTR of DENV-2) and SphI (at the position of 1384 in DENV-2 genome). The DNA fragments containing mutations in NS2A gene were cloned into pACYC-NGC FL or replicon through restriction enzyme sites and NheI (at the position of 2544 in DENV-2 genome) and SacI (at the position of 4342 in the DENV-2 genome).



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A mammalian expression vector, pXJ [ ADDIN EN.CITE <EndNote><Cite><Author>Xie</Author><Year>2013</Year><RecNum>85</RecNum><DisplayText>(Xie et al., 2013)</DisplayText><record><rec-number>85</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793217">85</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Xie, X.</author><author>Gayen, S.</author><author>Kang, C.</author><author>Yuan, Z.</author><author>Shi, P. Y.</author></authors></contributors><auth-address>State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Science, China.</auth-address><titles><title>Membrane topology and function of dengue virus NS2A protein</title><secondary-title>J Virol</secondary-title><alt-title>Journal of virology</alt-title></titles><periodical><full-title>J Virol</full-title></periodical><pages>4609-22</pages><volume>87</volume><number>8</number><edition>2013/02/15</edition><keywords><keyword>Animals</keyword><keyword>Cell Line</keyword><keyword>Cricetinae</keyword><keyword>Dengue Virus/*physiology</keyword><keyword>Endoplasmic Reticulum/*virology</keyword><keyword>Humans</keyword><keyword>Intracellular Membranes/*metabolism</keyword><keyword>Magnetic Resonance Spectroscopy</keyword><keyword>Models, Molecular</keyword><keyword>Protein Conformation</keyword><keyword>Viral Nonstructural Proteins/*chemistry/metabolism</keyword><keyword>*Virus Assembly</keyword></keywords><dates><year>2013</year><pub-dates><date>Apr</date></pub-dates></dates><isbn>1098-5514 (Electronic)0022-538X (Linking)</isbn><accession-num>23408612</accession-num><work-type>Research Support, Non-U.S. Gov't</work-type><urls><related-urls><url>http://www.ncbi.nlm.nih.gov/pubmed/23408612</url></related-urls></urls><custom2>3624351</custom2><electronic-resource-num>10.1128/JVI.02424-12</electronic-resource-num><language>eng</language></record></Cite></EndNote>], driven by a cytomegalovirus (CMV) promoter was used to express various viral proteins prM, E, NS2A and NS3 fusion proteins in HEK293T cells. For constructs pXJ-$C_{14}$-prM and pXJ-$C_{14}$-prM (1-130), the anchor C signal peptide ($C_{14}$) was retained at the N-terminus of prM to ensure the correct targeting on ER membrane. For construct pXJ-$M_{17}$-E, the last 17 amino acids from M protein was retained to serve as a signal peptide for translocating the E protein into the ER lumen. For constructs pXJ-SPG-$C_{16}$-NS2A-EGFP and pXJ-SPG-$C_{16}$-



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NS2A-GST, the signal peptide derived from Gaussia luciferase (SPG) [ ADDIN EN.CITE <EndNote><Cite><Author>Verhaegent</Author><Year>2002</Year><RecNum>112</RecNum><DisplayText>(Verhaegent and Christopoulos, 2002)</DisplayText><record><rec-number>112</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1561735944">112</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Verhaegent, M.</author><author>Christopoulos, T. K.</author></authors></contributors><auth-address>Department of Chemistry and Biochemistry, University of Windsor, Ontario, Canada.</auth-address><titles><title>Recombinant Gaussia luciferase. Overexpression, purification, and analytical application of a bioluminescent reporter for DNA hybridization</title><secondary-title>Anal Chem</secondary-title></titles><periodical><full-title>Anal Chem</full-title></periodical><pages>4378-85</pages><volume>74</volume><number>17</number><keywords><keyword>Animals</keyword><keyword>DNA/*analysis</keyword><keyword>DNA Probes</keyword><keyword>Genes, Reporter</keyword><keyword>*Luciferases/genetics/isolation & purification</keyword><keyword>Luminescent Proteins/genetics/isolation & purification</keyword><keyword>Plankton/*enzymology</keyword><keyword>Recombinant Proteins/genetics/isolation & purification</keyword></keywords><dates><year>2002</year><pub-dates><date>Sep 1</date></pub-dates></dates><isbn>0003-2700 (Print)0003-2700 (Linking)</isbn><accession-num>12236345</accession-num><urls><related-urls><url>https://www.ncbi.nlm.nih.gov/pubmed/12236345</url></related-urls></urls></record></Cite></EndNote>] and the last 16 amino acids of NS1 ($C_{16}$) was fused at the N-terminus of NS2A to ensure the correct processing and membrane topology of NS2A. The EGFP and GST were amplified from pEGFPluc (Clontech) and pGEX-4T-1 (Sigma-Aldrich) respectively. The Human influenza hemagglutinin (HA) tag was fused to the N-terminus of NS3 and cloned into pXJ vector, resulting the construct pXJ-HA-NS3.

pGEX-4T-1 (GE Healthcare Life Sciences) was used as the vector for expressing all N-terminally glutathione S-transferase (GST)-tagged NS2A fusion proteins. The DNA fragments containing various length of NS2A were cloned into pGEX-4T-1 through restriction enzyme sites BamHI and XhoI.



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All plasmids were propagated in *E. coli* strain Top10 (ThermoFisher Scientific). All restriction enzymes were purchased from New England BioLabs (Ipswitch, MA). The plasmids were validated by restriction enzyme digestion and Sanger DNA sequencing. All primers were synthesized from Integrated DNA Technologies (Skokie, Illinois) and are available upon request.

**RNA Transcription and Electroporation**

The NGC FL or replicon plasmids were linearized by restriction enzyme XbaI. RNA transcription and electroporation were performed as described previously [ ADDIN EN.CITE <EndNote><Cite><Author>Xie</Author><Year>2013</Year><RecNum>85</RecNum><DisplayText>(Xie et al., 2013)</DisplayText><record><rec-number>85</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793217">85</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Xie, X.</author><author>Gayen, S.</author><author>Kang, C.</author><author>Yuan, Z.</author><author>Shi, P. Y.</author></authors></contributors><auth-address>State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Science, China.</auth-address><titles><title>Membrane topology and function of dengue virus NS2A protein</title><secondary-title>J Virol</secondary-title><alt-title>Journal of virology</alt-title></titles><periodical><full-title>J Virol</full-title></periodical><pages>4609-22</pages><volume>87</volume><number>8</number><edition>2013/02/15</edition><keywords><keyword>Animals</keyword><keyword>Cell Line</keyword><keyword>Cricetinae</keyword><keyword>Dengue Virus/*physiology</keyword><keyword>Endoplasmic Reticulum/*virology</keyword><keyword>Humans</keyword><keyword>Intracellular Membranes/*metabolism</keyword><keyword>Magnetic Resonance Spectroscopy</keyword><keyword>Models, Molecular</keyword><keyword>Protein Conformation</keyword><keyword>Viral Nonstructural Proteins/*chemistry/metabolism</keyword><keyword>*Virus Assembly</keyword></keywords><dates><year>2013</year><pub-dates><date>Apr</date></pub-dates></dates><isbn>1098-5514 (Electronic)0022-538X (Linking)</isbn><accession-num>23408612</accession-num><work-type>Research Support, Non-U.S. Gov't</work-type><urls><related-



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urls><url>http://www.ncbi.nlm.nih.gov/pubmed/23408612</url></related-urls></urls><custom2>3624351</custom2><electronic-resource-num>10.1128/JVI.02424-12</electronic-resource-num><language>eng</language></record></Cite></EndNote>]. Briefly, 10 μg RNA was electroporated into 8 × $10^6$ cells in a 4-mm cuvette using the GenePulser apparatus (Bio-Rad) at settings of 0.45 kV (C6/36 cells) or 0.85 kV (BHK-21 cells) and 25 mF, pulsing three times, with 3 s intervals. After a 10-min recovery at room temperature, transfected cells were suspended in culture media and incubated at corresponding conditions: 28°C (C6/36 cells) or 37°C with 5% $CO_2$ (BHK-21).

**Virus Production and Quantification**

After electroporation, the transfected BHK-21 cells were seeded in one T-75 flask. After incubation at 37°C for 24 h, the cells were cultured in DMEM with 2% FBS at 30°C for another 4 days. At every 24 h p.t., culture fluids was collected and stored at -80°C. Viral titer was determined by crystal violet-based plaque assay using BHK-21 cells according to the protocol as described previously [ ADDIN EN.CITE <EndNote><Cite><Author>Xie</Author><Year>2013</Year><RecNum>85</RecNum><DisplayText>(Xie et al., 2013)</DisplayText><record><rec-number>85</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793217">85</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Xie, X.</author><author>Gayen, S.</author><author>Kang, C.</author><author>Yuan, Z.</author><author>Shi, P. Y.</author></authors></contributors><auth-address>State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Science, China.</auth-address><titles><title>Membrane topology and function of dengue virus NS2A protein</title><secondary-title>J Virol</secondary-title><alt-title>Journal of virology</alt-title></titles><periodical><full-title>J Virol</full-title></periodical><pages>4609-22</pages><volume>87</volume><number>8</number><edition>2013/02/15</edition><keywords><keyword>Animals</keyword><keyword>Cell Line</keyword><keyword>Cricetinae</keyword><keyword>Dengue Virus/*physiology</keyword><keyword>Endoplasmic Reticulum/*virology</keyword><keyword>Humans</keyword><keyword>Intracellular Membranes/*metabolism</keyword><keyword>Magnetic Resonance Spectroscopy</keyword><keyword>Models, Molecular</keyword><keyword>Protein Conformation</keyword><keyword>Viral Nonstructural



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Proteins/*chemistry/metabolism</keyword><keyword>*Virus Assembly</keyword></keywords><dates><year>2013</year><pub-dates><date>Apr</date></pub-dates></dates><isbn>1098-5514 (Electronic)0022-538X (Linking)</isbn><accession-num>23408612</accession-num><work-type>Research Support, Non-U.S. Gov't</work-type><urls><related-urls><url>http://www.ncbi.nlm.nih.gov/pubmed/23408612</url></related-urls></urls><custom2>3624351</custom2><electronic-resource-num>10.1128/JVI.02424-12</electronic-resource-num><language>eng</language></record></Cite></EndNote>]. Plaques were developed on day 5 post-infection.

**RNA extraction and RT-PCR**

Viral RNAs in culture fluids were extracted using a QIAamp viral RNA minikit (Qiagen). Extracted RNAs were eluted in 50 μl RNase-free water. Reverse transcription (RT)-qPCR was performed to measure the amounts of viral RNA copy in the culture medium. Viral RNAs were measured using an iScript one-Step RT-PCR kit with SYBR green (Bio-Rad) and the primer pair NGC 7764V (sequence: 5'-CGTCGAGAGAAATATGGTCACACC-3') and 7844C (sequence: 5'CCACAATAGTATGACCAGCCT-3') [ ADDIN EN.CITE <EndNote><Cite><Author>Xie</Author><Year>2013</Year><RecNum>85</RecNum><DisplayText>(Xie et al., 2013)</DisplayText><record><rec-number>85</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793217">85</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Xie, X.</author><author>Gayen, S.</author><author>Kang, C.</author><author>Yuan, Z.</author><author>Shi, P. Y.</author></authors></contributors><auth-address>State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Science, China.</auth-address><titles><title>Membrane topology and function of dengue virus NS2A protein</title><secondary-title>J Virol</secondary-title><alt-title>Journal of virology</alt-title></titles><periodical><full-title>J Virol</full-title></periodical><pages>4609-22</pages><volume>87</volume><number>8</number><edition>2013/02/15</edition><keywords><keyword>Animals</keyword><keyword>Cell Line</keyword><keyword>Cricetinae</keyword><keyword>Dengue Virus/*physiology</keyword><keyword>Endoplasmic Reticulum/*virology</keyword><keyword>Humans</keyword><keyword>Intracellular Membranes/*metabolism</keyword><keyword>Magnetic Resonance



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Spectroscopy</keyword><keyword>Models, Molecular</keyword><keyword>Protein Conformation</keyword><keyword>Viral Nonstructural Proteins/*chemistry/metabolism</keyword><keyword>*Virus Assembly</keyword></keywords><dates><year>2013</year><pub-dates><date>Apr</date></pub-dates></dates><isbn>1098-5514 (Electronic)0022-538X (Linking)</isbn><accession-num>23408612</accession-num><work-type>Research Support, Non-U.S. Gov't</work-type><urls><related-urls><url>http://www.ncbi.nlm.nih.gov/pubmed/23408612</url></related-urls></urls><custom2>3624351</custom2><electronic-resource-num>10.1128/JVI.02424-12</electronic-resource-num><language>eng</language></record></Cite></EndNote>]. Alternatively, the extracted viral RNA was reversely transcribed and amplified using SuperScript One-Step (RT)-PCR with Platinum Taq (Invitrogen). The RT-PCR products were then gel purified and subjected to Sanger DNA sequencing.

**Replicon Transient Transfection**

After electroporation of replicon RNA into BHK-21 cells, the cells were resuspended in culture medium and seeded in a 12-well plate ($2 \times 10^5$ cells/well). At various time points, the cells were washed once with phosphate-buffered saline (PBS) and lysed using lysis buffer (Promega). The plates containing the lysis buffer were sealed with Parafilm and stored at -80°C. Once samples for all time points had been collected, luciferase signals were measured in a Clarity luminescence microplate reader (BioTek) according to the manufacturer's protocol. The luciferase signals at early time points (2-6 h post-transfection) represent the translation of input viral RNA; and the luciferase signals at later points (24-46 h post-transfection) represent the stage of RNA replication.

**Immunofluorescence assay (IFA)**

Cells were seeded in an 8-well Lab-Tek II chamber slide (ThermoFisher Scientific). At given time points, cells were fixed in 4% paraformaldehyde in PBS for 20 min at room temperature. Afterwards, cells were permeabilized by 0.1% Triton X-100 (Sigma-Aldrich) for 5 min followed by 1-h incubation in blocking buffer containing 1% FBS and 0.05% Tween-20 (Sigma-Aldrich) in PBS. Cells were then incubated with primary antibodies (diluted in the blocking buffer) at room temperature for 1 h. After three PBS washes, cells were incubated with goat anti-mouse or rabbit IgG (1:1000 diluted in blocking buffer) conjugated with Alexa Fluor 488 or 594 (ThermoFisher Scientific) for 1 h in blocking buffer.



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Cells were finally washed by PBS for three times, counter-stained by Hoechst 33342 Solution (ThermoFisher Scientific) and mounted in ProLong® Diamond Antifade Mountant (ThermoFisher Scientific). Epifluorescence images were acquired by Leica DM4000 B system. Confocal fluorescence images were acquired by Zeiss LSM 5 META systems.

**Virus Revertant Selection**

For selecting revertants from G11A-transfected cells, we consecutively passaged the transfected cells for 5 rounds. In brief, after electroporation, the cells were seeded in 96-well plates and incubate at 30°C for 3 days. Afterwards, cells for each well were detached by trypsin and suspended in culture medium. About 20% of the suspended cells were transferred into the well of new 96-well plates and incubated at 37°C for one day. The cells were then incubated at 30°C for two additional days. Again, the confluent cells were detached for next passage. At every passage, the supernatants were collected. After 5 rounds of passaging, IFA was performed to determine the intracellular E protein expression by 4G2 antibody. Plaque assay was performed to examine the amounts of infectious virus particle in the culture fluids. Viral RNA from the P5 supernatants were extracted and used as template for RT-PCR. Sanger sequencing was performed to sequence the entire viral genome.

**Co-immunoprecipitation (Co-IP)**

The NS2A/prM, NS2A/E and NS2A/NS3 interactions were examined by Co-IP analysis according to the protocol as described previously[ ADDIN EN.CITE <EndNote><Cite><Author>Zou</Author><Year>2014</Year><RecNum>113</RecNum><DisplayText>(Zou et al., 2014)</DisplayText><record><rec-number>113</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1561742592">113</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Zou, J.</author><author>Xie, X.</author><author>Lee le, T.</author><author>Chandrasekaran, R.</author><author>Reynaud, A.</author><author>Yap, L.</author><author>Wang, Q. Y.</author><author>Dong, H.</author><author>Kang, C.</author><author>Yuan, Z.</author><author>Lescar, J.</author><author>Shi, P. Y.</author></authors></contributors><auth-address>State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Sciences, Wuhan, China.</auth-address><titles><title>Dimerization of flavivirus NS4B protein</title><secondary-title>J



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Virol</secondary-title></titles><periodical><full-title>J Virol</full-title></periodical><pages>3379-91</pages><volume>88</volume><number>6</number><keywords><keyword>Amino Acid Motifs</keyword><keyword>Dengue/*virology</keyword><keyword>Dengue Virus/chemistry/genetics/*metabolism</keyword><keyword>Dimerization</keyword><keyword>Humans</keyword><keyword>Viral Nonstructural Proteins/*chemistry/genetics/*metabolism</keyword><keyword>West Nile Fever/*virology</keyword><keyword>West Nile virus/chemistry/genetics/*metabolism</keyword></keywords><dates><year>2014</year><pub-dates><date>Mar</date></pub-dates></dates><isbn>1098-5514 (Electronic)0022-538X (Linking)</isbn><accession-num>24390334</accession-num><urls><related-urls><url>https://www.ncbi.nlm.nih.gov/pubmed/24390334</url></related-urls></urls><custom2>PMC3957939</custom2><electronic-resource-num>10.1128/JVI.02782-13</electronic-resource-num></record></Cite></EndNote>].
Various combinations of plasmids were transfected into HEK293T cells in 10-cm dishes using the X-tremeGENE 9 DNA transfection reagent (Roche). At 40-42 h post-transfection, cells were lysed in 1 ml immunoprecipitation (IP) buffer (20 mM Tris, pH 7.5, 100 mM NaCl, 0.5% DDM, and EDTA-free protease inhibitor cocktail [Roche]) with rotation at 4°C for 1 h. Lysates were clarified by centrifugation at 20,000 × g and 4°C for 30 min and subjected to co-IP using protein G-conjugated magnetic beads according to the manufacturer's instructions (Millipore). Briefly, immune complexes were formed at 4°C overnight by mixing 200 µl of cell lysate with 2 µg corresponding antibodies in a 500-µl reaction system containing 400 mM sodium chloride. Subsequently, the complexes were precipitated with protein G-conjugated magnetic beads at 4°C for 1 h with rotation, followed by washing extensively with phosphate-buffered saline (PBS) containing 0.1% Tween-20. Finally, proteins were eluted with 4× lithium dodecyl sulfate (LDS) sample buffer (ThermoFisher Scientific) supplemented with 100 mM 1,4-dithiothreitol (DTT), heated at 70°C for 10 min, and analyzed by Western blot.

**SDS-PAGE and Western Blot**

Protein samples were mixed with 4×LDS sample buffer (ThermoFisher Scientific). After denaturing at 70°C for 10 min, 10-30 µl samples were loaded onto to a 12% or 15% SDS-PAGE gel (Bio-Rad Laboratories). After electrophoresis, proteins were resolved and



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transferred onto a polyvinylidene difluoride (PVDF) membrane using the Bio-Rad TransBlot Turbo Blotting System. Blots were soaked for 1 hour in a blocking buffer containing TBST (10 mM Tris-HCl [pH 7.5], 150 mM NaCl, and 0.1% Tween-20) and 5% skim milk, followed by 1 h incubation with primary antibodies (diluted in blocking buffer). After three washes with TBST buffer, blots were incubated with goat anti-mouse or rabbit antibodies conjugated to HRP (1:10,000 diluted in blocking buffer). After three thorough washes with TBST buffer, blots were incubated with the ECL substrates (GE Healthcare). Chemiluminescence signals were detected in ChemiDoc System (Bio-Rad).

**Transmission Electron Microscopy**

Viral genomic RNAs were electroporated into BHK-21 cells as described above. At 48 h post-transfection, the cells were fixed for at least 1 h in a mixture of 2.5% formaldehyde (prepared from paraformaldehyde powder) and 0.1% glutaraldehyde in 0.05M cacodylate buffer (pH 7.3) to which 0.03% picric acid and 0.03% $CaCl_2$ were added. After washing in 0.1 M cacodylate buffer, the cells were scraped off and processed further as a pellet. The pellets were post-fixed in 1% OsO4 in 0.1M cacodylate buffer (pH 7.3) for 1 h, washed with distilled water and *en bloc* stained with 2% aqueous uranyl acetate for 20 min at 60°C. The pellets were dehydrated in ethanol, processed through propylene oxide and embedded in Poly/Bed 812 (Polysciences, Warrington, PA). Ultrathin sections were cut on Leica EM UC7 ultramicrotome (Leica Microsystems, Buffalo Grove, IL), stained with lead citrate and examined in a Philips 201 transmission electron microscope at 60 kV.

**Expression and purification of recombinant NS2A proteins**

The GST-tagged DENV-2 NGC NS2A and its truncates was expressed and purified by following a previously described protocol [ ADDIN EN.CITE <EndNote><Cite><Author>Zou</Author><Year>2014</Year><RecNum>113</RecNum><DisplayText>(Zou et al., 2014)</DisplayText><record><rec-number>113</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1561742592">113</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Zou, J.</author><author>Xie, X.</author><author>Lee le, T.</author><author>Chandrasekaran, R.</author><author>Reynaud, A.</author><author>Yap, L.</author><author>Wang, Q. Y.</author><author>Dong, H.</author><author>Kang, C.</author><author>Yuan, Z.</author><author>Lescar, J.</author><author>Shi, P.



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Y.</author></authors></contributors><auth-address>State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Sciences, Wuhan, China.</auth-address><titles><title>Dimerization of flavivirus NS4B protein</title><secondary-title>J Virol</secondary-title></titles><periodical><full-title>J Virol</full-title></periodical><pages>3379-91</pages><volume>88</volume><number>6</number><keywords><keyword>Amino Acid Motifs</keyword><keyword>Dengue/*virology</keyword><keyword>Dengue Virus/chemistry/genetics/*metabolism</keyword><keyword>Dimerization</keyword><keyword>Humans</keyword><keyword>Viral Nonstructural Proteins/*chemistry/genetics/*metabolism</keyword><keyword>West Nile Fever/*virology</keyword><keyword>West Nile virus/chemistry/genetics/*metabolism</keyword></keywords><dates><year>2014</year><pub-dates><date>Mar</date></pub-dates></dates><isbn>1098-5514 (Electronic)0022-538X (Linking)</isbn><accession-num>24390334</accession-num><urls><related-urls><url>https://www.ncbi.nlm.nih.gov/pubmed/24390334</url></related-urls></urls><custom2>PMC3957939</custom2><electronic-resource-num>10.1128/JVI.02782-13</electronic-resource-num></record></Cite></EndNote>] with modifications. The pGEX-4T GST-NS2A plasmids were transformed into *E. coli* strain Rosetta™ 2 (pLysS) (Novagen) cells. Bacteria culture was grown in Tefferic broth containing 50 µg/ml Kanamycin and 34 µg/ml Chloramphenicol. When the cells reached an optical density at 600 nm (OD600) of 0.8-1.0, isopropyl β-d-1-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM. After growth for 16 h at 18°C, the cells were harvested, resuspended in a lysis buffer (10 mM $Na_2HPO_4$, 1.8 mM $KH_2PO_4$, 2.7 mM KCl, 400 mM NaCl, 1 mM EDTA, 0.5× Roche protease inhibitor cocktail, pH7.5) and disrupted by sonication using a Digital Sonifier 450 (Branson). The cell debris was removed by centrifugation at 10,000 rpm for 10 min at 4°C. The supernatants were collected and pelleted down by centrifugation at 45,000 rpm for 1 h at 4°C using a Beckman type 70 Ti rotor. The pellets containing the membrane fractions were collected and solubilized in the lysis buffer supplemented with 1.5% n-Dodecyl β-D-maltoside (DDM; Anatrace) at 4°C for 2 hours. Following another centrifugation step at 45,000 rpm for 1 h, the supernatants were collected and loaded onto a GSTrap HP Fast Flow column (GE Healthcare) which was preequilibrated with buffer A (10 mM $Na_2HPO_4$, 1.8 mM $KH_2PO_4$, 2.7 mM KCl, 400 mM NaCl, 2 mM tris(2-carboxyethyl) phosphine [TCEP], 0.05% DDM, pH



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7.5). After extensively wash with buffer B (10 mM $Na_2HPO_4$, 1.8 mM $KH_2PO_4$, 2.7 mM KCl, 1M NaCl, 2 mM tris(2-carboxyethyl) phosphine [TCEP], 0.05% DDM, pH 7.5), the protein was eluted in buffer A supplied with 20 mM glutathione. The fractions containing GST-NS2A proteins were pooled, concentrated, and subjected to gel filtration using a HiLoad Superdex 200 10/300 column (GE Healthcare) in buffer C (10 mM $Na_2HPO_4$, 1.8 mM $KH_2PO_4$, 2.7 mM KCl, 150 mM NaCl, 2 mM tris(2-carboxyethyl) phosphine [TCEP], 0.05% DDM, pH 7.5) with 5% glycerol. The peak fractions containing GST-NS2A proteins were pooled and concentrated before storage at -80°C.

**RNA Pull-down Assay**

The 5'UTR-$C_{66}$-3'UTR-biotin RNA was prepared by ligating a single biotinylated nucleotide to the 3' terminus of the RNA 5'UTR-$C_{66}$-3'UTR using the Pierce™ RNA 3' End Biotinylation Kit (ThermoFisher Scientific) according to the manufacturer's instructions. The RNA 5'UTR-$C_{66}$-3'UTR was *in vitro* transcribed from a PCR template using a T7 MEGAscript kit (ThermoFisher Scientific). The PCR template contained a T7 promoter, followed by a cDNA fragment representing a DENV-2 subgenome with a deletion from nucleotides 163 to 10269 (GenBank accession number AF038403). After *in vitro* transcription, the products were passed twice through the Microspin™ G-25 Columns (GE Healthcare Life Sciences) to remove excessive UTPs. The final products were subjected to phenol-chloroform extraction followed by alcohol precipitation. The purified RNAs were resuspended in nuclease-free water prior to storage at -80°C. Other shorter RNAs 3'UTR, Δpk1, 5'UTR-$C_{66}$, Δpk(1-2), Δpk(1-3), 3'SL, pk(3-4) and pk3 were *in vitro* synthesized from corresponding PCR templates using the same method.

RNA pull-down experiments were performed using the Pierce™ Magnetic RNA-Protein Pull-Down Kit (ThermoFisher Scientific) according to the manufacturer's instructions. Briefly, 2 µg of biotin-labeled RNAs were pre-captured by 50 µl Streptavidin magnetic beads in 1 × RNA capture buffer (10mL, 20mM Tris (pH 7.5), 1M NaCl, 1mM EDTA) at room temperature for 20 min with agitation. A 28-mer biotinylated IRE RNA (5'-UCCUGCUUCAACAGUGCUUGGACGGAAC-3'-biotin) from the Pierce™ RNA 3' End Biotinylation Kit was used as a control. The RNA-bound beads were then mixed with 2 µg purified GST-tagged NS2A proteins in a 100-µl reaction system containing 20 mM Tris (pH 7.5), 200 mM NaCl, 2 mM $MgCl_2$, 0.1% Tween-20 and 15% glycerol and incubated for 45 minutes at 4°C with agitation. For RNA competition assay, the various amounts of



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competitors (tRNA and other shorter RNAs from DENV-2 genome) were immediately added to above the mixtures containing RNA-bound beads and GST-NS2A proteins. After 5 times of wash by 20 mM Tris (pH 7.5), 10 mM NaCl, 0.1% Tween-20 Detergent, the proteins were eluted in 30 µl biotin elution buffer. The eluates were mixed with 4×LDS sample buffer and heated at 70°C for 10 min. 10 µl protein samples were loaded onto a 12% SDS-PAGE gel and followed by detection by Western blot using rabbit anti-GST antibodies.

**Electrophoretic Mobility-Shift Assay**

Electrophoretic mobility-shift assay (EMSA) was performed to estimate the binding affinity of DENV-2 5'UTR-$C_{66}$-3'UTR RNA to GST-NS2A proteins. 100 pM 5'UTR-$C_{66}$-3'UTR-biotin RNA was incubated with a serial concentration of recombinant GST-NS2A protein (0-250 nM) in a 50-µl reaction system containing 50mM Tris-HCl, pH 7.5, 10 mM 2-mercaptoethanol, 2 mM $MgCl_2$, 50 mM KCl at 24°C for 15 min. Afterwards, 10 µl 6x Gel Loading Dye without SDS (New England Biolabs Inc.) were added to stop the reaction. Sample were then loaded onto a 0.4% agarose gel (prepared in 0.5× Tris/Borate/EDTA[TBE]) to resolve the RNA-protein complexes and free RNAs by electrophoresis in 1× TBE buffer (120 V for 30 min) at room temperature. After electrophoresis, RNAs were transferred from the agarose gel to a BrightStar-Plus positively charged nylon membrane (ThermoFisher Scientific) according to the procedure as described in the manual of NorthernMax Gly Kit (ThermoFisher Scientific). The nylon membrane was irradiated with 200 mJ/$cm^2$ of Ultraviolet (UV) light at 254 nm in a CX-2000 UV crosslinker (Analytic Jene, CA) with default settings. The biotinylated RNA was finally detected by the Streptavidin-horseradish peroxidase conjugate by using the Chemiluminescent Detection Module (ThermoFisher Scientific). Chemiluminescent signals were detected in the ChemiDoc™ Imaging Systems (Bio-rad).

**PAR-CLIP**

The Photoactivatable-Ribonucleoside-Enhanced Crosslinking and Immunoprecipitation (PAR-CLIP) was performed according to the protocol as described previously [ ADDIN EN.CITE ADDIN EN.CITE.DATA ] with modifications. In brief, 4-thiouridine labelled DENV-2 3'UTR RNA was prepared by adding a final concentration 0.75 mM of 4-Thio-UTP (TriLink BioTechnologies) to a 20-µl *in vitro* transcription reaction (4-Thio-UTR to UTP



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ration is 1:10) using the MEGAscript T7 transcription Kit (Thermo Fisher Scientific). After 2 hours of reaction at 37°C, the reaction was stopped and the 4-thiouridine labelled RNAs were purified and resuspended in nuclease-free water. 15 µg of 4-thiouridine labelled DENV-2 3'UTR RNA was incubated with 6 µg recombinant DENV-2 GST-NS2A protein in a 100-µl reaction system containing 50 mM Tris-HCl, pH 7.5, 2 mM $MgCl_2$, 50 mM KCl and 5% glycerol at 24°C for 15 min. The RNA-protein mixtures were then irradiated on ice with 150 mJ/cm$^2$ at UV light at 365 nm. An aliquot of the same reaction without UV irradiation was used as the uncross-linked control. Prior to immunoprecipitation, 30 µl of PureProteome™ Protein G Magnetic Beads (MilliporeSigma) were washed twice with 1 ml citrate-phosphate buffer (4.7 g/L citric acid, 9.2 g/L $Na_2HPO_4$, pH 5.0), resuspended in 100 µl citrate-phosphate buffer and followed by in addition of 1 µg of rabbit anti-GST polyclonal antibody (Abcam). The bead-antibody mixtures were incubated at 4°C for 45 min. After two washed with citrate-phosphate buffer, the antibody-bound beads were resuspended in 30 µl of citrate-phosphate buffer. Upon immunoprecipitation, the RNA-protein mixtures were mixed with antibody-bound beads and incubated at 4°C for 1 h. Afterwards, the beads were subjected to three washes in buffer I (50mM HEPES-KOH, pH 7.5, 300 mM KCl, 0.05% IGEPAL CA-630L[Sigma-Aldrich] and 0.5 mM DTT) followed by two washes in buffer II (50 mM HEPES-KOH, pH 7.5, 500 KCl, 0.05% IGEPAL CA-630 and 0.5 mM DTT). The beads were then resuspended in 100 µl Proteinase K digestion buffer (100 mM Tris, pH 7.5, 50 mM NaCl, 10 mM EDTA and 1%SDS) supplemented with proteinase K (New England BioLabs) at a final concentration of 2 mg/ml. The reaction was placed into the Eppendorf ThermoMixer® C and incubated at 37°C for 1 h with agitation at 1100 rpm. Thereafter, the RNA in the reaction was extracted by phenol-chloroform, alcohol-precipitated, and resuspended in 20 µl of nuclease-free water. The extracted RNA was subjected to reversely transcribed and followed by amplification (4 PCR-cycles) using the SuperScript® III One-Step RT-PCR System (ThermoFisher Scientific) with a pair of specific primers P1 (sequence: 5'-ACACTCTTTCCCTACACGA CGCTCTTCCGATCTAAGGCAAAACTAACATGAAAC-3'; underline indicates the sequence identical to the sequence at the 5' end of the DENV-2 3'UTR) and P2 (sequence: 5'-GTGACTGGAGTTCAGACG TGTGCTCTTCCGATCTAGAACCTGTTGATTCAACAGCAC-3'; underline indicates the sequence complimentary to the sequence at the 3' end of the DENV-2 3'UTR) targeting the DENV-2 3'UTR. Afterwards, the RT-PCR product was cleaned up using the QIAquick PCR Purification Kit (Qiagen) and subjected to another round of amplification (15 PCR-



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cycles) using the KAPA HiFi PCR Kit (Kapa Biosystems) with a pair of Illumina adapter/index primers (5' universal adaptor sequence: 5'-AATGATACGGCGAC CACCGAGATCTACACTCTTTCCCTACACGAC-3'; and 3' indexing primer sequence: 5'-CAAGCAGAAGACGGCATACGAGATnnnnnnGTGACTGGAGTTCAGACGTGT-3'. The "nnnnnn" here indicates the TruSeq index sequence). Thereafter, the PCR product was cleaned up using the QIAquick PCR Purification Kit (Qiagen) and followed by separation on 2% E-Gel™ EX Agarose Gels (ThermoFisher Scientific). The cDNAs fragment between 500 to 650 bp was excised, purified using the Zymo Research Gel DNA Recovery Kit (Zymo Research) and resuspended in nuclease-free water. The final cDNA libraries were quantified using a Qubit dsDNA Assay Kit (Invitrogen) and sequenced using a MiSeq Reagent Kit v3 (600-cycle) (illumina).

## QUANTIFICATION AND STATISTICAL ANALYSIS

### Sequence Alignment, Image Process and Densitometry Analysis

The amino acid sequences of flavivirus NS2A proteins were download from GenBank. Sequence alignment was performed with default settings using the CLC main workbench software (Qiagen). IFA images processing and cell counting was performed in software ImageJ (National Institutes of Health, NIH). At least four different IFA images were used to estimate the mean and standard deviations of percentage of viral protein staining positive-cells in Figure 2G and S2. Densitometry analysis was performed using Image lab version 6.0 (Bio-Rad Laboratories, Hercules, CA). All numerical data are presented as the mean ± SD (standard deviations). Group comparisons were performed using the unpaired parametric t-test in software Prism 8.0 (GraphPad). *$p<0.05$, significant; **$p<0.01$, very significant; ***$p<0.001$, highly significant; n.s., not significant. Images were assembled using the software Adobe illustrator CC.

### Calculating the equilibrium dissociation constant (Kd)

The intensities of bands representing the RNA/GST-NS2A complexes and free RNA in Figure 5E were quantitated by software ImageJ (NIH). The ratio of bound RNA (in the RNA/GST-NS2A complexes) to total RNA (free and bound RNA) in each lane was calculated. The ratio of bound RNA to total RNA (y-axis) versus the concentration of GST-NS2A protein (x-axis) was plotted using the software Prism 8 (Graphad). The value of Kd



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was obtained using a nonlinear regression with least-squares fitting method, where the Bmax was set as 1. All data points come from three independent experiments.

**Illumina sequencing data process and analysis**

The indexed sequencing reads of both forward and reverse strands were proceeded with the following bioinformatic pipelines: first, an initial quality filtering were conducted with Fastp [ ADDIN EN.CITE <EndNote><Cite><Author>Chen</Author><Year>2018</Year><RecNum>115</RecNum><DisplayText>(Chen et al., 2018)</DisplayText><record><rec-number>115</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1562011493">115</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Chen, S.</author><author>Zhou, Y.</author><author>Chen, Y.</author><author>Gu, J.</author></authors><translated-authors><author>Bioinformatics,</author></translated-authors></contributors><auth-address>Department of Bioinformatics, HaploX Biotechnology, Shenzhen, China.Shenzhen Institutes of Advanced Technology, Chinese Academy of Sciences, Shenzhen, China.</auth-address><titles><title>fastp: an ultra-fast all-in-one FASTQ preprocessor</title><secondary-title>Bioinformatics</secondary-title><alt-title>Bioinformatics</alt-title></titles><periodical><full-title>Bioinformatics</full-title><abbr-1>Bioinformatics</abbr-1></periodical><alt-periodical><full-title>Bioinformatics</full-title><abbr-1>Bioinformatics</abbr-1></alt-periodical><pages>i884-i890</pages><volume>34</volume><number>17</number><dates><year>2018</year><pub-dates><date>Sep 1</date></pub-dates></dates><isbn>1367-4811 (Electronic)1367-4803 (Linking)</isbn><accession-num>30423086</accession-num><urls><related-urls><url>http://www.ncbi.nlm.nih.gov/pubmed/30423086</url></related-urls></urls><custom2>6129281</custom2><electronic-resource-num>10.1093/bioinformatics/bty560</electronic-resource-num><remote-database-provider>2018 Sep 1</remote-database-provider><language>eng</language></record></Cite></EndNote>] to remove bad reads (too low in quality score, too short, too many “N”, etc.). Then, the illumina sequencing adapter sequence “AGATCGGAAGAGC” was trimmed with cutadapt [ ADDIN EN.CITE <EndNote><Cite><Author>Martin</Author><Year>2011</Year><RecNum>116</RecNum><DisplayText>(Martin, 2011)</DisplayText><record><rec-number>116</rec-



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number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1562011493">116</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Martin, Marcel</author></authors></contributors><titles><title>Cutadapt removes adapter sequences from high-throughput sequencing reads</title><secondary-title>EMBnet.journal; Vol 17, No 1: Next Generation Sequencing Data AnalysisDO - 10.14806/ej.17.1.200</secondary-title></titles><periodical><full-title>EMBnet.journal; Vol 17, No 1: Next Generation Sequencing Data AnalysisDO - 10.14806/ej.17.1.200</full-title></periodical><keywords><keyword>next generation sequencing, small RNA, microRNA, adapter removal</keyword></keywords><dates><year>2011</year><pub-dates><date>05/02/</date></pub-dates></dates><urls><related-urls><url>https://journal.embnet.org/index.php/embnetjournal/article/view/200/458</url></related-urls></urls></record></Cite></EndNote>] (command line parameters: -b AGATCGGAAGAGC -m 40). A secondary quality filter was applied with FASTX toolkit ([ HYPERLINK "http://hannonlab.cshl.edu/fastx_toolkit/index.html" ]) to remove any reads that contained more than 4% nucleotides with a PHRED score <20 (command line parameters: fastq_quality_filter -Q33 -q 20 -p 96). The remaining reads were aligned to 3'UTR of DENV genome (GenBank accession number AF038403), with Bowtie2 [ ADDIN EN.CITE <EndNote><Cite><Author>Langmead</Author><Year>2012</Year><RecNum>117</RecNum><DisplayText>(Langmead and Salzberg, 2012)</DisplayText><record><rec-number>117</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1562011493">117</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Langmead, Ben</author><author>Salzberg, Steven L.</author></authors></contributors><titles><title>Fast gapped-read alignment with Bowtie 2</title><secondary-title>Nature Methods</secondary-title></titles><periodical><full-title>Nature Methods</full-title></periodical><pages>357</pages><volume>9</volume><dates><year>2012</year><pub-dates><date>03/04/online</date></pub-dates></dates><publisher>Nature Publishing Group, a division of Macmillan Publishers Limited. All Rights Reserved.</publisher><urls><related-urls><url>https://doi.org/10.1038/nmeth.1923</url></related-urls></urls><electronic-resource-num>10.1038/nmeth.1923</electronic-resource-



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num></record></Cite></EndNote>] (command line parameters: --local). Using SAMtools [ ADDIN EN.CITE <EndNote><Cite><Author>Li</Author><RecNum>118</RecNum><DisplayText>(Li et al.)</DisplayText><record><rec-number>118</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1562011493">118</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Li, H.</author><author>Handsaker B Fau - Wysoker, Alec</author><author>Wysoker A Fau - Fennell, Tim</author><author>Fennell T Fau - Ruan, Jue</author><author>Ruan J Fau - Homer, Nils</author><author>Homer N Fau - Marth, Gabor</author><author>Marth G Fau - Abecasis, Goncalo</author><author>Abecasis G Fau - Durbin, Richard</author><author>Durbin, R.</author></authors><translated-authors><author>Bioinformatics,</author></translated-authors></contributors><auth-address>Wellcome Trust Sanger Institute, Wellcome Trust Genome Campus, Cambridge, CB10 1SA, UK, Broad Institute of MIT and Harvard, Cambridge, MA 02141, USA. FAU - Handsaker, Bob</auth-address><titles><title>The Sequence Alignment/Map format and SAMtools</title></titles><number>1367-4811 (Electronic)</number><dates></dates><call-num>1000 Genome Project Data Processing Subgroup</call-num><urls></urls><remote-database-provider>2009 Aug 15</remote-database-provider><language>eng</language></record></Cite></EndNote>], the aligned forward and reverse reads were binarily converted, merged, indexed, sorted, and mathematically noted (command line parameters: view -buS, merge, sort, index, mpileup). This is followed by extracting and calculating the T/U-to-C mutation rate at each genome position and the mutation frequencies of each of the four nucleotides.

## DATA AND SOFTWARE AVAILABILITY

Illumina sequencing data access. The raw data of illumine sequencing data can be accessed, queried and downloaded using the following link: .



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# Dengue NS2A protein orchestrates virus assembly

Xuping Xie[1]*, Jing Zou[1], Xianwen Zhang[1,2,3], Yiyang Zhou[1], Andrew Routh[1], Vsevolod L. Popov[4], Xinwen Chen[2,3,5], Qingyin Wang[6], Hong-Ping Dong[6], Pei-Yong Shi[1,7,8,9,10]*

[1]Department of Biochemistry & Molecular Biology, University of Texas Medical Branch, Galveston, Texas, USA; [2]State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Sciences, Wuhan, China. [3]University of Chinese Academy of Sciences, Beijing, China. [4]Department of Pathology, University of Texas Medical Branch, Galveston, Texas, USA; [5]Guangzhou Institute of Biomedicine and Health, Chinese Academy of Sciences, Guangzhou, China. [6]Clearbridge Health, Singapore; [7]Institute for Translational Science, University of Texas Medical Branch, Galveston, Texas, USA; [8]Sealy Institute for Vaccine Sciences, University of Texas Medical Branch, Galveston, Texas, USA; [9]Sealy Center for Structural Biology & Molecular Biophysics, University of Texas Medical Branch, Galveston, Texas, USA; [10]Institute for Human Infections & Immunity, University of Texas Medical Branch, Galveston, Texas, USA;

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Running title: The role of NS2A protein in dengue virus assembly



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## SUMMARY

Dengue virus (DENV) is the most important mosquito-borne viral pathogen in humans. DENV assembly requires cleavages of viral C-prM-E polyprotein into three structural proteins (capsid, pre-membrane, and envelope), packaging of viral RNA with C protein into nucleocapsid, and budding of prM/E proteins into virion. No study has addressed how these assembly events are orchestrated. Here we show that DENV-2 NS2A protein recruits viral RNA, structural proteins, and protease to virion assembly site, and coordinates nucleocapsid and virus formation. The last 285 nucleotides of viral 3'UTR serve as a "packaging signal" that binds to a cytosolic loop of NS2A protein. This interaction allows NS2A to recruit nascent RNA from the replication complex to virion assembly site. Mutations in NS2A that weaken the 3'UTR/NS2A interaction abolish virus assembly. In addition, NS2A interacts with prM, E, and NS3 to recruit C-prM-E polyprotein and NS2B/NS3 protease to the virion assembly site, leading to coordinated C-prM-E cleavage. Mature C protein assembles onto genomic RNA to form nucleocapsid, followed by prM/E envelopment and virion formation. An NS2A mutation that alters the prM/NS2A interaction reduces C-prM cleavage and abolishes virion assembly; second-site mutations in C protein that restore the C-prM cleavage rescue virion assembly. The results support a DENV assembly model that NS2A recruits viral RNA to virion assembly site where coordinated cleavage of C-prM-E ensues infections virus morphogenesis.




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## INTRODUCTION

The genus *Flavivirus* of the family *Flaviviridae* comprises over 70 viruses, many of which are important human pathogens, including dengue (DENV), yellow fever (YFV), Zika (ZIKV), West Nile (WNV), Japanese encephalitis (JEV), St. Louis encephalitis (SLE), and tick-borne encephalitis (TBEV) viruses[ ADDIN EN.CITE <EndNote><Cite><Author>Pierson</Author><Year>2013</Year><RecNum>3</RecNum><DisplayText>(Pierson and Diamond, 2013)</DisplayText><record><rec-number>3</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1536704974">3</key><key app="ENWeb" db-id="">0</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Pierson, T.C.</author><author>Diamond, M. S.</author></authors></contributors><titles><secondary-title>Flaviviruses p. 747-794. In D.M. Knipe and P.M. Howley (ed), Fields virology, 6th., vol. 1.</secondary-title></titles><periodical><full-title>Flaviviruses p. 747-794. In D.M. Knipe and P.M. Howley (ed), Fields virology, 6th., vol. 1.</full-title></periodical><dates><year>2013</year></dates><urls></urls></record></Cite></EndNote>]. The four serotypes of DENV alone cause about 390 million human infections each year, leading to dengue fever, dengue hemorrhagic fever, and dengue shock syndrome [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. Because of the huge social economic burden, DENV was included in the top 10 global health threats by the World Health Organization (WHO). The development of vaccine and therapeutics for DENV has been proven challenging [ ADDIN EN.CITE <EndNote><Cite><Author>Lim</Author><Year>2013</Year><RecNum>58</RecNum><DisplayText>(Lim et al., 2013)</DisplayText><record><rec-number>58</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv"



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timestamp="1560793214">58</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Lim, S. P.</author><author>Wang, Q. Y.</author><author>Noble, C. G.</author><author>Chen, Y. L.</author><author>Dong, H.</author><author>Zou, B.</author><author>Yokokawa, F.</author><author>Nilar, S.</author><author>Smith, P.</author><author>Beer, D.</author><author>Lescar, J.</author><author>Shi, P. Y.</author></authors></contributors><auth-address>Novartis Institute for Tropical Diseases, 10 Biopolis Road, 05-01 Chromos, Singapore 138670, Singapore.</auth-address><titles><title>Ten years of dengue drug discovery: Progress and prospects</title><secondary-title>Antiviral Res</secondary-title><alt-title>Antiviral research</alt-title></titles><periodical><full-title>Antiviral Res</full-title></periodical><pages>500-19</pages><volume>100</volume><number>2</number><edition>2013/10/01</edition><dates><year>2013</year><pub-dates><date>Nov</date></pub-dates></dates><isbn>1872-9096 (Electronic)0166-3542 (Linking)</isbn><accession-num>24076358</accession-num><urls><related-urls><url>http://www.ncbi.nlm.nih.gov/pubmed/24076358</url></related-urls></urls><electronic-resource-num>10.1016/j.antiviral.2013.09.013</electronic-resource-num><language>eng</language></record></Cite></EndNote>]. The first FDA approved dengue vaccine has a low efficacy of 30-61% [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. Due to the risk of vaccine-induced severe disease in children who are seronegative at the time of vaccination, WHO recommended only use of the vaccine in areas where the disease is highly prevalent [ ADDIN EN.CITE <EndNote><Cite><Author>Whitehead</Author><Year>2016</Year><RecNum>63</RecNum><DisplayText>(Whitehead, 2016)</DisplayText><record><rec-number>63</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793215">63</key></foreign-keys><ref-type name="Journal



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Article">17</ref-type><contributors><authors><author>Whitehead, S. S.</author></authors></contributors><auth-address>a Laboratory of Infectious Diseases , NIAID, National Institutes of Health , Bethesda , MD , USA.</auth-address><titles><title>Development of TV003/TV005, a single dose, highly immunogenic live attenuated dengue vaccine; what makes this vaccine different from the Sanofi-Pasteur CYD vaccine?</title><secondary-title>Expert Rev Vaccines</secondary-title></titles><periodical><full-title>Expert Rev Vaccines</full-title></periodical><pages>509-17</pages><volume>15</volume><number>4</number><keywords><keyword>Dengue</keyword><keyword>live attenuated dengue vaccine</keyword><keyword>vaccine attenuation strategies</keyword><keyword>vaccine efficacy</keyword><keyword>vaccine immunogenicity</keyword><keyword>vaccine infectivity</keyword></keywords><dates><year>2016</year></dates><isbn>1744-8395 (Electronic)1476-0584 (Linking)</isbn><accession-num>26559731</accession-num><urls><related-urls><url>http://www.ncbi.nlm.nih.gov/pubmed/26559731</url></related-urls></urls><custom2>PMC4956407</custom2><electronic-resource-num>10.1586/14760584.2016.1115727</electronic-resource-num></record></Cite></EndNote>]. No clinically approved antivirals are available for flavivirus treatment, despite major effort in the past two decades[ ADDIN EN.CITE <EndNote><Cite><Author>Zou</Author><Year>2019</Year><RecNum>64</RecNum><DisplayText>(Zou and Shi, 2019)</DisplayText><record><rec-number>64</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793215">64</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Zou, J.</author><author>Shi, P. Y.</author></authors></contributors><auth-address>Department of Biochemistry and



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Molecular Biology, University of Texas Medical Branch, Galveston, TX, USA.Department of Biochemistry and Molecular Biology, University of Texas Medical Branch, Galveston, TX, USA; Department of Phamarcology and Toxicology, University of Texas Medical Branch, Galveston, TX, USA; Sealy Center for Structural Biology and Molecular Biophysics, University of Texas Medical Branch, Galveston, TX, USA; Sealy Institute for Vaccine Sciences, University of Texas Medical Branch, Galveston, TX, USA. Electronic address: peshi@utmb.edu.</auth-address><titles><title>Strategies for Zika drug discovery</title><secondary-title>Curr Opin Virol</secondary-title></titles><periodical><full-title>Curr Opin Virol</full-title></periodical><pages>19-26</pages><volume>35</volume><dates><year>2019</year><pub-dates><date>Mar 7</date></pub-dates></dates><isbn>1879-6265 (Electronic)1879-6257 (Linking)</isbn><accession-num>30852345</accession-num><urls><related-urls><url>http://www.ncbi.nlm.nih.gov/pubmed/30852345</url></related-urls></urls><electronic-resource-num>10.1016/j.coviro.2019.01.005</electronic-resource-num></record></Cite></EndNote>]. It is therefore critical to study the molecular mechanism of flavivirus replication, which may lead to new approaches for countermeasure development.

Flavivirus has a plus-sense, single-stranded RNA of about 11,000-nucleotide in length. The genomic RNA contains a 5' untranslated region (UTR), a single open-reading-frame (ORF), and a 3' UTR. The ORF translates a polyprotein that is processed by viral and host proteases into three structure proteins [capsid (C), pre-membrane (prM), and envelope (E)] and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5). The structural proteins, together with genomic RNA, assemble into virions. The nonstructural proteins participate in viral RNA replication, virion assembly, and evasion of host immune response. Upon viral translation, a minus-sense RNA is synthesized from the



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genomic RNA template, forming a double-stranded RNA. The minus-strand RNA then serves as a template to make many copies of plus-sense RNA that are packaged to form progeny virions [ ADDIN EN.CITE <EndNote><Cite><Author>Pierson</Author><Year>2013</Year><RecNum>3</RecNum><DisplayText>(Pierson and Diamond, 2013)</DisplayText><record><rec-number>3</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1536704974">3</key><key app="ENWeb" db-id="">0</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Pierson, T.C.</author><author>Diamond, M. S.</author></authors></contributors><titles><secondary-title>Flaviviruses p. 747-794. In D.M. Knipe and P.M. Howley (ed), Fields virology, 6th., vol. 1.</secondary-title></titles><periodical><full-title>Flaviviruses p. 747-794. In D.M. Knipe and P.M. Howley (ed), Fields virology, 6th., vol. 1.</full-title></periodical><dates><year>2013</year></dates><urls></urls></record></Cite></EndNote>]. Like other RNA viruses, the assembly of flavivirus is coupled with RNA replication [ ADDIN EN.CITE ADDIN EN.CITE.DATA ].

Flavivirus infection induces invaginations of the endoplasmic reticulum (ER) to form clusters of double-membrane vesicles (Ve) enclosed in the vesicle packets (VPs). Electron tomography has revealed the three-dimensional architectures of Ve and VPs formed during DENV, WNV, TBEV, and ZIKV infections [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. Ve houses viral replication complex (including the double-stranded viral RNA, viral nonstructural proteins, and cellular proteins) where RNA synthesis occurs [ ADDIN EN.CITE <EndNote><Cite><Author>Junjhon</Author><Year>2014</Year><RecNum>71</RecNum><DisplayText>(Junjhon et al., 2014)</DisplayText><record><rec-number>71</rec-



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number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793216">71</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Junjhon, J.</author><author>Pennington, J. G.</author><author>Edwards, T. J.</author><author>Perera, R.</author><author>Lanman, J.</author><author>Kuhn, R. J.</author></authors></contributors><auth-address>Markey Center for Structural Biology, Department of Biological Sciences, Purdue University, West Lafayette, Indiana, USA.</auth-address><titles><title>Ultrastructural characterization and three-dimensional architecture of replication sites in dengue virus-infected mosquito cells</title><secondary-title>J Virol</secondary-title></titles><periodical><full-title>J Virol</full-title></periodical><pages>4687-97</pages><volume>88</volume><number>9</number><keywords><keyword>Animals</keyword><keyword>Cell Line</keyword><keyword>Culicidae/*virology</keyword><keyword>Dengue Virus/*physiology/*ultrastructure</keyword><keyword>Electron Microscope Tomography</keyword><keyword>Imaging, Three-Dimensional</keyword><keyword>Intracellular Membranes/*ultrastructure/*virology</keyword><keyword>*Virus Replication</keyword></keywords><dates><year>2014</year><pub-dates><date>May</date></pub-dates></dates><isbn>1098-5514 (Electronic)0022-538X (Linking)</isbn><accession-num>24522909</accession-num><urls><related-urls><url>http://www.ncbi.nlm.nih.gov/pubmed/24522909</url></related-urls></urls><custom2>PMC3993787</custom2><electronic-resource-num>10.1128/JVI.00118-14</electronic-resource-num></record></Cite></EndNote>]. Newly synthesized plus-sense RNA is released from the Ve pore to the rough ER for new rounds of RNA translation and replication. Alternatively, the nascent RNA is assembled



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into virions through encapsidation (with C protein) and envelopment (with prM and E proteins) through budding into the ER lumen. The virion assembly occurs at the ER sites juxtaposed to the VPs. An initial immature virion contains 60 icosahedrally arranged prM/E heterotrimer. As the immature virion transits through the Golgi network, the acidic environment triggers the cleavage of prM by cellular furin protease, resulting in an infectious mature virion [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. Cryo-EM of flavivirus virions exhibit two structural features: (i) The nucleocapsid core lacks organized structures and (ii) there are no observable connections between nucleocapsid and the prM/E membrane shell, suggesting that the C protein may bind to genomic RNA in a random manner without direct interaction with prM/E proteins [ ADDIN EN.CITE ADDIN EN.CITE.DATA ].

The molecular mechanism of flavivirus assembly remains largely obscure. Besides structural proteins, several nonstructural proteins, such as NS2A, NS2B, and NS3, have been shown to regulate flavivirus assembly. Different amino acids of NS2A protein were reported to play distinct roles in flavivirus RNA replication and virion assembly [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. The cleavage between C and prM is tightly modulated by NS2B/NS3 protease and cellular signalase during YFV and Murray Valley encephalitis virus assembly [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. Mutations in the NS3 helicase domain alone affect YFV assembly [ ADDIN EN.CITE <EndNote><Cite><Author>Patkar</Author><Year>2008</Year><RecNum>90</RecNum><DisplayText>(Patkar and Kuhn, 2008a)</DisplayText><record><rec-number>90</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793218">90</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Patkar, C. G.</author><author>Kuhn, R. J.</author></authors></contributors><auth-



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address>Purdue Univ, Dept Biol Sci, W Lafayette, IN 47907 USA</auth-address><titles><title>Yellow fever virus NS3 plays an essential role in virus assembly independent of its known enzymatic functions</title><secondary-title>Journal of Virology</secondary-title><alt-title>J Virol</alt-title></titles><alt-periodical><full-title>J Virol</full-title></alt-periodical><pages>3342-3352</pages><volume>82</volume><number>7</number><keywords><keyword>tick-borne encephalitis</keyword><keyword>flavivirus kunjin ns3</keyword><keyword>west-nile-virus</keyword><keyword>dengue virus</keyword><keyword>nonstructural protein</keyword><keyword>rna replication</keyword><keyword>cdna-clones</keyword><keyword>particles</keyword><keyword>ns2a</keyword><keyword>organization</keyword></keywords><dates><year>2008</year><pub-dates><date>Apr</date></pub-dates></dates><isbn>0022-538X</isbn><accession-num>WOS:000254139800015</accession-num><urls><related-urls><url><Go to ISI>://WOS:000254139800015</url></related-urls></urls><electronic-resource-num>10.1128/Jvi.02447-07</electronic-resource-num><language>English</language></record></Cite></EndNote>]. Although expression of prM/E alone is sufficient to generate empty virus-like particles, it is not known how nucleocapsid is formed and subsequently enveloped with prM/E to form infectious virions. The goal of this study is to define the molecular mechanism that orchestrates three structural proteins and viral RNA to form nucleocapsid and infectious virus in a highly coordinated manner. Using genetic, biochemical, and imaging approaches, we demonstrate that DENV NS2A protein choreographs virion morphogenesis by (i) interacting with viral prM and E proteins, (ii) regulating the processing of C-prM polyprotein by viral protease, and (iii) recruiting genomic RNA through binding to the 3'UTR. These results support a model that DENV NS2A recruits nascent viral RNA from the replication complex (housed in Ve) to the virion assembly site (on the ER membrane juxtaposed to



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241 VPs), where an coordinated C-prM cleavage facilitates genomic RNA encapsidation with C
242 protein and budding with prM and E proteins into the ER lumen, resulting in progeny
243 virions.



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## RESULTS

**NS2A G11A mutation abolishes virion assembly**. Using DENV-2 as a model, we previously identified a panel of NS2A mutant viruses that were selectively defective in virion assembly without compromising other steps of an infection cycle [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. Here, we performed four experiments to demonstrate that NS2A mutation G11A of DENV-2 caused a defect in virion assembly. (i) A DENV-2 luciferase replicon was used to examine the effect of NS2A G11A on viral translation and RNA synthesis. Upon transfection into BHK-21 cells, the G11A replicon produced a luciferase profile comparable to the WT replicon (Fig. 1A), suggesting that the mutation does not significantly reduce viral translation and RNA replication. (ii) When full-length viral RNA of DENV-2 was transfected into BHK-21 cells, the NS2A G11A mutant failed to release extracellular viral RNA (Fig. 1B). No infectious virus was detected from the culture medium of G11A RNA-transfected cells (Fig. 1C). (iii) In a *trans* packaging system, WT luciferase replicon could be packaged into pseudo-infectious particles (PIPs) when structural C-prM-E proteins were expressed from Semliki Forest virus replicon (Fig. S1A). The WT reporter replicon PIPs infected Vero cells and generated robust luciferase signals (Fig. S1B). In contrast, no PIPs were produced with the NS2A G11A replicon. (iv) Transmission electron microscopy revealed substantial virus-induced single-membrane vesicles (within the lumen of the rough ER) or double-membrane vesicles (closely associated with cisternae of the rough ER) in both WT and G11A RNA-transfected cells at 48 h post-transfection (Fig. 1D). A lot of virus particles appeared in the WT RNA-transfected cells, but no virus particles were detected in the G11A RNA-transfected cells. The results demonstrate that NS2A G11A mutation specifically abolishes virion assembly.

**NS2A G11A mutation alters the expression and localization of viral structural proteins.** We compared the subcellular localization of viral proteins in BHK-21 cells that



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were transfected with WT or NS2A G11A genomic RNA. Surprisingly, the NS2A G11A mutation altered the intracellular distribution of three structural proteins. (i) C protein disseminated in both nucleus and cytoplasm in the WT RNA-transfected cells, as previously reported [ ADDIN EN.CITE <EndNote><Cite><Author>Wang</Author><Year>2004</Year><RecNum>91</RecNum><DisplayText>(Wang et al., 2004)</DisplayText><record><rec-number>91</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793218">91</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Wang, S. H.</author><author>Syu, W. J.</author><author>Hu, S. T.</author></authors></contributors><auth-address>Institute of Microbiology and Immunology, School of Life Science, National Yang-Ming University, Taipei, Taiwan, Republic of China.</auth-address><titles><title>Identification of the homotypic interaction domain of the core protein of dengue virus type 2</title><secondary-title>J Gen Virol</secondary-title><alt-title>The Journal of general virology</alt-title></titles><periodical><full-title>J Gen Virol</full-title><abbr-1>The Journal of general virology</abbr-1></periodical><alt-periodical><full-title>J Gen Virol</full-title><abbr-1>The Journal of general virology</abbr-1></alt-periodical><pages>2307-14</pages><volume>85</volume><number>Pt 8</number><keywords><keyword>Active Transport, Cell Nucleus</keyword><keyword>Dengue Virus/*chemistry/classification</keyword><keyword>Precipitin Tests</keyword><keyword>Viral Core Proteins/*chemistry</keyword></keywords><dates><year>2004</year><pub-dates><date>Aug</date></pub-dates></dates><isbn>0022-1317 (Print)0022-1317 (Linking)</isbn><accession-num>15269372</accession-num><urls><related-urls><url>http://www.ncbi.nlm.nih.gov/pubmed/15269372</url></related-



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urls></urls><electronic-resource-num>10.1099/vir.0.80067-0</electronic-resource-num></record></Cite></EndNote>]. However, in the G11A RNA-transfected cells, the C protein was mainly restricted to the cytoplasm with rare nucleus localization (Fig. 1E). (ii) prM protein was located at the ER region in the WT RNA-transfected cells, whereas the prM protein was undetectable in the G11A RNA-transfected cells (Fig. 1F). (iii) E protein spread broadly like an ER web in the WT RNA-transfected cells. In contrast, in the G11A RNA-transfected cells, the E protein formed larger speckles at restricted subcellular areas (Fig. 1G). Compared with the structural proteins, the effect of NS2A G11A on the subcellular expression of viral nonstructural proteins (including NS1, NS3, NS4A, NS4B, and NS5) and dsRNA was not apparent (Fig. 1E-G, *top panels* for NS3, and data not shown).

The distinct effect of NS2A G11A mutation on subcellular expression between the viral structural and nonstructural proteins were further supported by immunofluorescent co-staining experiments. NS2A G11A altered the co-staining profile of E and NS3 proteins (Fig. S2). In the NS2A G11A RNA-transfected cells, 100% of NS3-expressing cells were E-positive at 24 h post-transfection; however, at 48 and 72 h post-transfection, only 59% and 20% of the NS3-expressing cells were E-positive, respectively (Fig. S2). In the WT RNA-transfected cells, 100% of NS3-expressing cells were E-positive from 24 to 72 h post-transfection (Fig. S2). The results demonstrate that NS2A G11A mutation changes the expression and subcellular localization of viral structural proteins.

**NS2A G11A mutation impairs the cleavage of viral C-prM polyprotein.** To determine the molecular mechanism of the above observations, we examined the processing of viral proteins in the WT and NS2A G11A viral RNA-transfected cells at 48 h post-transfection by Western blot. As expected, the amounts of C, NS3, and NS5 proteins from the G11A RNA-transfected cells were 15-26% of those from the WT RNA-transfected



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cells (Fig. 1H-I), due to the lack of virion production or multiple rounds of infection for the G11A mutant. Notably, the amounts of prM and E proteins were further lower in the G11A RNA-transfected cells (only 1-4% of those in the WT RNA-transfected cells), suggesting an asymmetric degradation in cells. Moreover, the NS2A G11A mutation impaired the cleavage of C-prM to individual C and prM proteins: 90% of C-prM was cleaved to mature C protein in the WT RNA-transfected cells, whereas only 20% of C-prM was processed to C protein in the G11A RNA-transfected cells (Fig. 1H, *compare lanes 1 and 5 in the top panel*). The impaired C-prM cleavage could account for the altered subcellular distribution of viral structural proteins in the NS2A C11A RNA-transfected cells: Restricting the un-cleaved C-prM to the ER membrane, preventing C protein from translocating to the nucleus, and consequently blocking prM/E heterodimer formation and virion budding.

Next, we examined the role of ubiquitin-proteasome pathway in degrading viral proteins in the G11A RNA-transfected cells. Treatment with proteasome inhibitor MG132 increased the amounts of C, prM, E, and NS5 (but not NS3) by 1-3 folds in the NS2A G11A RNA-transfected cells (Fig. 1H, *compare lanes 5 and 6*). In contrast, in the WT RNA-transfected cells, MG132 slightly reduced the amounts of C, prM, and E to different extents (Fig. 1H, *compare lanes 1 and 2*) because MG132 was known to inhibit flavivirus replication [ ADDIN EN.CITE <EndNote><Cite><Author>Bhuvanakantham</Author><Year>2013</Year><RecNum>108</RecNum><DisplayText>(Bhuvanakantham and Ng, 2013)</DisplayText><record><rec-number>108</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793288">108</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Bhuvanakantham, R.</author><author>Ng, M. L.</author></authors></contributors><auth-



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address>Flavivirology Laboratory, Department of Microbiology, Yong Loo Lin School of Medicine, National University of Singapore, 5 Science Drive 2, Singapore, 117597.</auth-address><titles><title>West Nile virus and dengue virus capsid protein negates the antiviral activity of human Sec3 protein through the proteasome pathway</title><secondary-title>Cell Microbiol</secondary-title></titles><periodical><full-title>Cell Microbiol</full-title></periodical><pages>1688-706</pages><volume>15</volume><number>10</number><keywords><keyword>Antiviral Agents/*antagonists & inhibitors</keyword><keyword>Capsid Proteins/genetics/*metabolism</keyword><keyword>Cell Line</keyword><keyword>DNA Mutational Analysis</keyword><keyword>Dengue Virus/*physiology</keyword><keyword>*Host-Pathogen Interactions</keyword><keyword>Humans</keyword><keyword>Models, Biological</keyword><keyword>Proteasome Endopeptidase Complex/*metabolism</keyword><keyword>Protein Binding</keyword><keyword>Protein Interaction Mapping</keyword><keyword>Proteolysis</keyword><keyword>Vesicular Transport Proteins/*antagonists & inhibitors</keyword><keyword>West Nile virus/*physiology</keyword></keywords><dates><year>2013</year><pub-dates><date>Oct</date></pub-dates></dates><isbn>1462-5822 (Electronic)1462-5814 (Linking)</isbn><accession-num>23522008</accession-num><urls><related-urls><url>https://www.ncbi.nlm.nih.gov/pubmed/23522008</url></related-urls></urls><electronic-resource-num>10.1111/cmi.12143</electronic-resource-num></record></Cite></EndNote>]. Distinct responses to MG132 treatment among different viral proteins were probably due to their subcellular distributions and involvement in various biological microenvironments. Nevertheless, the results suggest that ubiquitin-proteasome pathway is responsible for viral protein degradation in the NS2A G11A RNA-transfected cells.



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**WT NS2A rescues virion assembly of NS2A G11A mutant through restoring C-prM cleavage.** We examined if *trans* supply of WT NS2A could restore virion assembly of NS2A G11A RNA and rescue C-prM protein processing (Fig.S3A). Indeed, after transfection into NS2A-EGFP BHK-21 cells that constitutively expressed WT NS2A protein, the NS2A G11A RNA produced infectious virions that could infect naïve BHK-21 cells, as indicated by E-positive cells (Fig. S3B); further passaging of culture supernatants to new BHK-21 cells did not yield any E-positive cells (Fig. S3C), suggesting that the *trans* packaged virions could only infect cells for a single-round. Remarkably, the *trans* supplied WT NS2A protein restored the C-prM cleavage in the G11A RNA-transfected cells to WT level, exhibiting >89% C protein processing (Fig. S3D, *top panel*). Two prM bands, representing glycosylated and un-glycosylated forms of the protein (see below), were detected in both NS2A G11A and WT RNA-transfected NS2A-EGFP cells (Fig. S3D, *second panel*). The relative abundances of C, prM, and NS1 proteins were similar in the NS2A G11A and WT RNA-transfected cells (Fig. S3D). Because of the restored C-prM cleavage, the nucleus localization of C protein and prM-positive cells were detected in the G11A RNA-transfected NS2A-EGFP cells (Fig. S3E). These results demonstrate that WT NS2A can rescue virion assembly of NS2A G11A mutant through restoring the C-prM cleavage.

Next, we examined the effect of NS2A on the glycosylation of prM by co-expressing NS2A and prM in HEK293T cells. As a control, when EGFP was co-expressed, only the glycosylated form of prM was detected (Fig. S3F); after PGNaseF treatment, the prM was completely deglycoslated and migrated as a glycosylation-knockout mutant N69Q prM (Fig. S3G). In contrast, when NS2A-EGFP were co-expressed, both glycosylated and un-glycosylated forms of prM were detected (Fig. S3F), indicating that NS2A suppresses the glycosylation of prM. Furthermore, the G11A mutation seemed to increase the



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suppression effect of NS2A on prM glycosylation (Fig. 3F). These results suggest that NS2A may interact with prM, and that the G11A mutation may modulate the prM/NS2A interaction.

**Capsid R99S mutation can rescue virion assembly of NS2A G11A RNA.** We selected revertants by continuously passaging the NS2A G11A RNA-transfected BHK-21 cells for five rounds (Fig. S4A). Culture medium from each passaging (P1-P5) were tested for emergence of infectious viruses using plaque assay. The P5 culture produced infectious viruses with titers of $>10^4$ PFU/ml and plaques smaller than the WT virus (Fig. S4B). In addition, almost all P5 cells were E protein-positive (Fig. S4C). Whole genome sequencing of the P5 viruses from three independent selections consistently showed a consensus C-to-A substitution at nucleotide position 391 of viral genome, resulting in an Arg-to-Ser change at amino acid position 99 of the C protein (C_R99S; Fig. 2A). The original NS2A G11A mutation was retained in all P5 viruses.

To validate the role of C_R99S mutation in rescuing virion assembly of NS2A G11A RNA, we engineered C_R99S into a DENV-2 infectious clone [ ADDIN EN.CITE <EndNote><Cite><Author>Xie</Author><Year>2013</Year><RecNum>85</RecNum><DisplayText>(Xie et al., 2013)</DisplayText><record><rec-number>85</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793217">85</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Xie, X.</author><author>Gayen, S.</author><author>Kang, C.</author><author>Yuan, Z.</author><author>Shi, P. Y.</author></authors></contributors><auth-address>State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Science, China.</auth-address><titles><title>Membrane topology and function of dengue virus NS2A protein</title><secondary-title>J Virol</secondary-title><alt-title>Journal of virology</alt-



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title></titles><periodical><full-title>J Virol</full-title></periodical><pages>4609-22</pages><volume>87</volume><number>8</number><edition>2013/02/15</edition><keywords><keyword>Animals</keyword><keyword>Cell Line</keyword><keyword>Cricetinae</keyword><keyword>Dengue Virus/*physiology</keyword><keyword>Endoplasmic Reticulum/*virology</keyword><keyword>Humans</keyword><keyword>Intracellular Membranes/*metabolism</keyword><keyword>Magnetic Resonance Spectroscopy</keyword><keyword>Models, Molecular</keyword><keyword>Protein Conformation</keyword><keyword>Viral Nonstructural Proteins/*chemistry/metabolism</keyword><keyword>*Virus Assembly</keyword></keywords><dates><year>2013</year><pub-dates><date>Apr</date></pub-dates></dates><isbn>1098-5514 (Electronic)0022-538X (Linking)</isbn><accession-num>23408612</accession-num><work-type>Research Support, Non-U.S. Gov't</work-type><urls><related-urls><url>http://www.ncbi.nlm.nih.gov/pubmed/23408612</url></related-urls></urls><custom2>3624351</custom2><electronic-resource-num>10.1128/JVI.02424-12</electronic-resource-num><language>eng</language></record></Cite></EndNote>] in the presence or absence of NS2A G11A. BHK-21 cells were electroporated with WT, NS2A G11A, C_R99S, or NS2A G11A+C_R99S viral RNA. The transfected cells were analyzed for virus production and C-prM cleavage. In the NS2A G11A+C_R99S RNA-transfected cells, the percentage of E-positive cells increased from 10% to 50% at 24 h and 96 h post-transfection, suggesting the spread of infectious virus (Fig. 2B); indeed, the transfected cells produced infectious viruses with plaques smaller than the WT virus (Fig. 2C); the viral titer increased over time, reaching >$10^5$ PFU/ml at 96-120 h post-transfection (Fig. 2D). The prM and NS1 proteins exhibited ER-like distributions in the NS2A G11A+C_R99S RNA-transfected cells, similar to that observed in the WT RNA-transfected



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447 cells (Fig. 2E). Viral C protein was detected in both cytoplasm and nucleus in the NS2A
448 G11A+C_R99S RNA-transfected cells (Fig. 2F-G). Furthermore, 90.5% of the C-prM
449 protein was cleaved to mature C and prM in the NS2A G11A+C_R99S RNA-transfected
450 cells (Fig. 2G). As controls, NS2A G11A RNA showed 15.4% of C-prM cleavage and did
451 not produce any infectious virus, whereas C_R99S RNA exhibited 99.9% of prM-C
452 cleavage and produced a WT level of infectious virus (Fig. 2B-H). Taken together, the
453 results demonstrate that C_R99S mutation efficiently restores C-prM cleavage and virion
454 assembly of NS2A G11A RNA.

455 **Capsid R99S cannot rescue virion assembly of other NS2A mutants.** We
456 tested if C_R99S could rescue other NS2A mutants that were previously shown to be
457 defective in virion assembly, including E20A, E100A, and K188A [ ADDIN EN.CITE
458 ADDIN EN.CITE.DATA ]. The C_R99S was engineered into those NS2A mutants,
459 resulting in NS2A E20A+C_R99S, NS2A E100A+C_R99S, and NS2A K188A+C_R99S
460 viral RNAs. After transfection into BHK-12 cells, none of the mutant RNAs produced
461 increasing number of E-positive cells from 24 to 96 h (Fig. S5A). No infectious virus was
462 detected in the culture medium from the mutant RNA-transfected cells (Fig. S5B). Viral C
463 protein was detected in both nucleus and cytoplasm of all mutant RNA-transfected cells
464 (Fig. S5C). In agreement with the C nucleus localization, the NS2A single mutant E20A
465 and E100A cleaved 36% and 79% of C-prM, and the addition of C_R99S increased the C-
466 prM cleavage to 72% and 96%, respectively. In contrast, NS2A K188A mutant cleaved
467 100% of C-prM in the presence or absence of C_R99S (Fig. 5D). The results demonstrate
468 that C_R99S can only rescue the assembly defect of NS2A G11A possibly because
469 different NS2A mutations abolish virion assembly through distinct mechanisms.

470 **Capsid R99S shifts the C-prM cleavage site by two amino acids.** The above
471 results prompted us to investigate how C_R99S rescues the virion assembly of NS2A



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G11A. Fig. S6 depicts the topology of viral polyprotein C-prM-E-NS1-NS2A on the ER membrane. NS2A G11 resides at the first amphipathic helix on the ER lumen side [ ADDIN EN.CITE

<EndNote><Cite><Author>Xie</Author><Year>2013</Year><RecNum>85</RecNum><DisplayText>(Xie et al., 2013)</DisplayText><record><rec-number>85</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793217">85</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Xie, X.</author><author>Gayen, S.</author><author>Kang, C.</author><author>Yuan, Z.</author><author>Shi, P. Y.</author></authors></contributors><auth-address>State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Science, China.</auth-address><titles><title>Membrane topology and function of dengue virus NS2A protein</title><secondary-title>J Virol</secondary-title><alt-title>Journal of virology</alt-title></titles><periodical><full-title>J Virol</full-title></periodical><pages>4609-22</pages><volume>87</volume><number>8</number><edition>2013/02/15</edition><keywords><keyword>Animals</keyword><keyword>Cell Line</keyword><keyword>Cricetinae</keyword><keyword>Dengue Virus/*physiology</keyword><keyword>Endoplasmic Reticulum/*virology</keyword><keyword>Humans</keyword><keyword>Intracellular Membranes/*metabolism</keyword><keyword>Magnetic Resonance Spectroscopy</keyword><keyword>Models, Molecular</keyword><keyword>Protein Conformation</keyword><keyword>Viral Nonstructural Proteins/*chemistry/metabolism</keyword><keyword>*Virus Assembly</keyword></keywords><dates><year>2013</year><pub-dates><date>Apr</date></pub-dates></dates><isbn>1098-5514 (Electronic)0022-538X (Linking)</isbn><accession-num>23408612</accession-num><work-type>Research



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Support, Non-U.S. Gov't</work-type><urls><related-urls><url>http://www.ncbi.nlm.nih.gov/pubmed/23408612</url></related-urls></urls><custom2>3624351</custom2><electronic-resource-num>10.1128/JVI.02424-12</electronic-resource-num><language>eng</language></record></Cite></EndNote>], while C_R99 is the penultimate amino acid of the C protein on the cytosolic side (Fig. S6B). Since flavivirus NS2B/NS3 protease cleaves a substrate with two basic residues (Arg or Lys) at the P1 and P2 sites and a residue with short side chain (Gly, Ser, or Ala) at the P1' site [ ADDIN EN.CITE ADDIN EN.CITE.DATA ], sequence analysis suggests that the C_R99S substitution may shift the capsid C-terminal cleavage by two amino acids from position 100 to 98 (Fig. S6B). Specifically, viral NS2B/NS3 protease cleaves the WT capsid at NRRRR↓TA and the R99S capsid at NRR↓SRTA (↓ denoting cleavage site and residue R99S underlined). To test this hypothesis, we performed a biochemical protease assay using synthetic WT peptide (NILNRRRRTAGMII) and R99S peptide (NILNRRSRTAGMII). After incubating the peptides with recombinant DENV-2 NS2B/NS3 protease, the reaction products were analyzed by liquid chromatography-mass spectrometry (LC-MS). The WT peptide was cleaved into two smaller peptides with molecular weights of 1096.73 Da and 604.77 Da, corresponding to peptides NILNRRRR and TAGMII, respectively (Fig. S6C). In contrast, the R99S peptide was cleaved into two fragments with molecular weights of 784.47 Da and 847.46 Da, corresponding to peptide NILNRR and SRTAGMII, respectively (Fig. S6D). The LC-MS also detected some unknown peptides, probably due to nonspecific degradations of the protease reaction (Fig. S6C-D). Nevertheless, the data indicate that the C_R99S mutation shifts the capsid cleavage site upstream by two amino acids.

**NS2A G11A enhances prM/NS2A interaction.** Since NS2A G11A alters the processing of C-prM (Fig. 1H) and prM glycosylation (Fig. S3F), we hypothesized that



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NS2A directly interacts with prM and the G11A mutation affects the prM/NS2A interaction. To test this hypothesis, we co-transfected two plasmids into HEK293T cells. One plasmid expressed the authentic signal peptide anchorC ($C_{14}$) fused with the full-length prM. Another plasmid expressed a fusion protein containing the C-terminal 24 amino acids of the E protein, the N-terminal 50 amino acids of the NS1 protein, the C-terminal 50 amino acids of NS1, full length NS2A, and an EGFP [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]; this plasmid was previously shown to express NS2A-EGFP with a correct topology on the ER membrane [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. Co-immunoprecipitation showed that NS2A-EGFP, but not EGFP, pulled down prM (Fig. 3B, lanes 2 and 3). As controls, mouse IgG did not pull down any NS2A-EGFP or prM (Lane 1), and EGFP antibody did not pull down prM in the absence of NS2A-EGFP (Lane 6). To map the region of prM that binds to NS2A, we expressed the first 130 amino acids of prM [prM(1-130)] that lacked the C-terminal two transmembrane domains (Fig. 3A). Co-immunoprecipitation showed that prM(1-130) could not pull down NS2A-EGFP (Fig. 3B, lane 4), suggesting that (i) NS2A may bind to the transmembrane region of prM or (ii) the transmembrane region of prM may affect the conformation of its ectodomain and, consequently, abolish its interaction with NS2A.

Next, we examined if NS2A mutation G11A affects the prM/NS2A interaction. Co-immunoprecipitation experiments showed that G11A NS2A-EGFP pulled down about 2.5-fold more prM than the WT NS2A-EGFP did (Fig. 4C). Reciprocally, prM pulled down about 1.7-fold more G11A NS2A-EGFP than WT NS2A-EGFP (Fig. 4D). The results indicate that the G11A mutation enhances NS2A binding to prM.

To further support the prM/NS2A interaction, we used confocal microscope to examine the co-localization of prM/NS2A in cells. In this case, prM-E were co-expressed with NS2A-EGFP in HEK293T cells. As shown in Fig. 3E, NS2A-EGFP co-localized with



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prM. The G11A mutation did not change the co-localization of NS2A-EGFP with prM (Fig. 3E). Taken together, the results demonstrate that (i) NS2A interacts with prM and (ii) NS2A G11A significantly enhances the prM/NS2A interaction.

**The G11A mutation does not affect NS2A binding to E or NS3.** We also examined the interactions between NS2A and E or NS3 proteins that were co-expressed in HEK293T cells. For testing the E/NS2A interaction, we fused a GST tag to the C-terminus of NS2A for the co-immunoprecipitation experiment [because the EGFP tag used in NS2A-EGFP in Fig. 3B-D non-specifically interacts with E protein (data not shown)]. As shown in Fig. 3F, both WT and G11A NS2A-GST pulled down E protein at comparable efficiencies. As a control, GST alone did not pull down E protein. The result suggests that NS2A interacts with E and the G11A mutation does not affect this interaction.

Since flavivirus NS3 is known to participate in viron assembly [ ADDIN EN.CITE ADDIN EN.CITE.DATA ], we also tested if NS2A G11A mutation affects the NS2A/NS3 interaction. When co-expressed in HEK293T cells, GST alone pulled down a trace amount of HA-tagged NS3 (NS3-HA) protein (Fig. S7, lane 1), whereas NS2A-GST pulled down 10-fold more NS3-HA (Lane 2). Equivalent amounts of NS3-HA were pulled down by the WT and G11A NS2A-GST (Compare lanes 2 and 3), suggesting that NS2A G11A does not affect NS2A/NS3 binding.

**Extension of two or three residues at the N-terminus of anchorC efficiently rescues virion assembly of NS2A G11A**. So far, our results showed that NS2A G11A mutation increases its binding to prM on the ER lumen side, leading to impaired C-prM processing and defective virion assembly. These defects can be rescued by a capsid mutation R99S that shifts the C-terminal cleavage site by two amino acids. As a result of this shift, the N-terminus of transmembrane anchorC is extended by two amino acids on



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572 the cytosolic side of ER membrane. These data support a model of coordinated C-prM
573 processing during virion assembly (Fig. 4A) [ ADDIN EN.CITE
574 <EndNote><Cite><Author>Amberg</Author><Year>1999</Year><RecNum>295</RecNu
575 m><DisplayText>(Amberg and Rice, 1999a)</DisplayText><record><rec-
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580 M.</author><author>Rice, C. M.</author></authors></contributors><auth-
581 address>Department of Molecular Microbiology, Washington University School of
582 Medicine, St. Louis, Missouri 63110-1093, USA.</auth-
583 address><titles><title>Mutagenesis of the NS2B-NS3-mediated cleavage site in the
584 flavivirus capsid protein demonstrates a requirement for coordinated
585 processing</title><secondary-title>J Virol</secondary-title><alt-title>Journal of
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598 urls></urls><custom2>112824</custom2></record></Cite></EndNote>]. During WT virus
599 assembly, polyprotein C-anchorC-prM was first cleaved at the C-terminus of capsid protein
600 (*i.e.*, R100) by NS2B/NS3 protease, producing mature C and anchorC-prM. The anchorC-
601 prM was then cleaved by a host signalase at the N-terminus of prM, generating anchorC
602 and prM. The cleaved prM coordinates with E protein for virion budding (Fig. 4A, *left*
603 *panel*). In the presence of NS2A G11A mutation, NS2A binds to prM with a higher affinity,
604 pulling anchorC (embedded in the ER membrane) towards the lumen side. The viral
605 protease cleavage site of the "pulled anchorC" is blocked, possibly by the ER membrane
606 (*middle panel*), leading to unprocessed C-prM. To overcome this defect, the C_R99S
607 mutation creates a new NS2A/NS3 cleavage site at R98 of capsid protein, restoring the C-
608 prM processing and virion assembly of NS2A G11A (*right panel*).

609 The key of the above model is the blockage of viral protease cleavage site at the
610 C-terminus of capsid protein, which is caused by the NS2A G11A mutation. If the model is
611 correct, an insertion of one or two amino acids to the N-terminus of anchorC should
612 restore the C-prM cleavage and virion assembly in the presence of NS2A G11A mutation
613 (Fig. 4B). To test this hypothesis, we inserted Thr, The-Ala, or Ser-Arg to the C-terminus of
614 capsid protein (after R100) in the context of WT or NS2A G11A viral RNA. The inserted
615 amino acids were selected based on the cleavage requirement for viral protease (Fig. 4B).
616 Transfection of each viral RNA into BHK-21 cells yielded increasing number of E-positive
617 cells from day 1 to 4 (Fig. 4C), produced infectious viruses (Fig. 4D-E), displayed cytosolic
618 and nucleus localizations of C protein (Fig. 4F), and exhibited efficient C-prM cleavage
619 (Fig. 4H). In the absence of NS2A G11A, the capsid insertion mutant RNAs behaved
620 similarly to the WT RNA. In the presence of NS2A G11A, the capsid single-amino acid
621 insertion (*i.e.*, NS2A G11A+C_T101) partially restored virion assembly ($1.2\times10^3$ PFU/ml;
622 Fig. 4D-E), prM-positive cells (Fig. 4G), and C-prM cleavage (60%; Fig. 4H); while the



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capsid double-amino acid insertion (*i.e.*, NS2A G11A+C_TA and NS2A G11A+C_SR) almost fully restored virion assembly to the WT level (2.3-2.5×$10^6$ PFU/ml; Fig. 4D-E) and C-prM cleavage (92-98%; Fig. 4H). These results demonstrate that addition of one or two amino acids to the N-terminus of anchorC can rescue virion assembly of NS2A G11A.

Since the single-amino acid C_T101 insertion only partially rescued virion assembly of NS2A G11A, we selected revertants for NS2A G11A+C_T101 virus. After continuous passaging of the virus on Vero cells for eight rounds, the P8 viruses were subjected to plaque assay (Fig. S8A-B) and whole-genome sequencing (Fig. S8C). The P8 viruses from all four independent selections produced viral titers (Fig. S8B) that were ≥100-fold higher than the original NS2A G11A+C_T101 virus (Fig. 4E). Among them, selection I P8 virus retained the original NS2A G11A+C_T101 mutations. In addition, an extra mutation R100S was recovered in the capsid protein. Sequence analysis suggests that the R100S could alter the protease cleavage site from the original RRRR↓T to RRR↓ST (R100S underlined) and extends the N-terminus of anchorC by one more amino acid. Selection II P8 virus also retained the original NS2A G11A+C_T101 mutations and accumulated another mutation R99S in the capsid protein. The R99S may change the protease cleavage site to RR↓SRT and extends the N-terminus of anchorC by two more amino acids. In addition, selection II P8 virus contained the R100S mutation in 27% of the viral population. Selection III P8 virus retained the NS2A G11A mutation and showed mixed sequences of R99S and/or R100S. Selection IV P8 virus exhibited WT plaque morphology; sequencing results showed that the NS2A G11A was reverted to WT Gly, but the C_T101 insertion was retained. Collectively, the results again support the conclusion that extension of the N-terminus of anchorC by two to three residues could efficiently rescue virion assembly of NS2A G11A.



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**Analysis of NS2A G11A in mosquito cells.** To test if the above findings from mammalian cells could be extended to mosquito host, we analyzed NS2A G11A+capsid mutant RNAs in C6/36 cells. Viral RNAs containing NS2A G11A alone, NS2A G11A+C_T101, NS2A G11A+C_R99S, or NS2A G11A+C_TA were transfected into C6/36 cells. The transfected cells were analyzed for capsid protein-positive cells (Fig. S9A), infectious virus production (Fig. S9B-C), extracellular viral RNA release (Fig. S9D), and C-prM cleavage (Fig. S9E). Similar to the results obtained from mammalian cells, NS2A G11A mutant was defective in C-prM processing and virion production in C6/36 cells. The defects could be rescued by the capsid mutations, with the rescuing efficiency of C_TA > C_R99S > C_T101 (Fig. S9). The results indicate that NS2A G11A mutation exerts the same mechanism in abolishing virion assembly in mammalian and mosquito hosts.

**NS2A protein binds to 3'UTR RNA**. To further explore the role of DENV-2 NS2A in virion assembly, we examined the ability of NS2A to bind viral genomic RNA. A series of pulldown experiments were performed using biotinylated viral RNAs and NS2A protein (Fig. 5A). A 616-nucleotide viral RNA (5'UTR-$C_{66}$-3'UTR) comprising the 5'UTR, the first 66 nucleotides from the C gene, and the 3'UTR regions of DENV-2 genome was *in vitro* synthesized (Fig. S10A). The 3' end of the RNA was ligated to a biotinylated cytidine using T4 RNA ligase, resulting in 5'UTR-$C_{66}$-3'UTR-biotin RNA (Fig. S10B). Two types of GST-tagged NS2A protein were prepared for the pulldown experiment: One type NS2A was expressed from *E. coli*, partially purified, and reconstituted in detergent DDM-containing buffer (Fig. 5B); another type NS2A was from the cell lysates of HEK293T cells transiently expressing the NS2A protein (Fig. 5C). As shown in Fig. 5B-C, the 5'UTR-$C_{66}$-3'UTR-biotin RNA efficiently pulled down GST-tagged NS2A (Lanes 4 and 6), but not GST (Lanes 2 and 5); in contrast, neither GST nor GST-tagged NS2A proteins was pulled down by a biotinylated non-viral RNA control (Lanes 1 and 3). To further demonstrate the specificity



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of NS2A/5'UTR-$C_{66}$-3'UTR RNA interaction, we performed an RNA competition experiment. Increasing amounts un-biotinylated 5'UTR-$C_{66}$-3'UTR RNA competed away the NS2A/5'UTR-$C_{66}$-3'UTR-biotin RNA interaction, whereas yeast tRNA did not (Fig. 5D). These results suggest that 5'UTR-$C_{66}$-3'UTR RNA specifically binds to NS2A protein.

We estimated the binding affinity of NS2A/viral RNA interaction. 5'UTR-$C_{66}$-3'UTR-biotin RNA was incubated with increasing amounts of recombinant GST-NS2A protein. The reactions were separated on a native polyacrylamide gel (PAGE), transferred to nylon membrane, and probed with streptavidin-HRP (Fig. 5E). Quantification of free RNA and RNA/protein complex estimated a *Kd* of 35 nM for DENV-2 NS2A biding to viral RNA.

**The last 285 nucleotides of 3'UTR RNA is responsible for binding to NS2A protein**. To map the region of viral RNA responsible for binding to NS2A protein, we performed a competition assay using a set of un-biotinylated RNA fragments from 5'UTR-$C_{66}$-3'UTR (Fig. S10C). The fragments were prepared based on the previously proposed RNA structure [ ADDIN EN.CITE <EndNote><Cite><Author>Villordo</Author><Year>2015</Year><RecNum>109</RecNum><DisplayText>(Villordo et al., 2015)</DisplayText><record><rec-number>109</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1561296709">109</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Villordo, S. M.</author><author>Filomatori, C. V.</author><author>Sanchez-Vargas, I.</author><author>Blair, C. D.</author><author>Gamarnik, A. V.</author></authors></contributors><auth-address>Fundacion Instituto Leloir-CONICET, Buenos Aires, Argentina.Arthropod-borne and Infectious Diseases Laboratory, Department of Microbiology, Immunology and Pathology, Colorado State University, Fort Collins, Colorado, United States of America.</auth-address><titles><title>Dengue virus



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RNA structure specialization facilitates host adaptation</title><secondary-title>PLoS Pathog</secondary-title></titles><periodical><full-title>PLoS Pathog</full-title></periodical><pages>e1004604</pages><volume>11</volume><number>1</number><keywords><keyword>3' Untranslated Regions</keyword><keyword>Adaptation, Biological/genetics</keyword><keyword>Animals</keyword><keyword>Cells, Cultured</keyword><keyword>Cricetinae</keyword><keyword>Culicidae</keyword><keyword>Dengue Virus/*genetics</keyword><keyword>Host Specificity/genetics</keyword><keyword>Host-Pathogen Interactions/*genetics</keyword><keyword>Humans</keyword><keyword>Mutation</keyword><keyword>*Nucleic Acid Conformation</keyword><keyword>RNA, Viral/*chemistry/genetics</keyword></keywords><dates><year>2015</year><pub-dates><date>Jan</date></pub-dates></dates><isbn>1553-7374 (Electronic)1553-7366 (Linking)</isbn><accession-num>25635835</accession-num><urls><related-urls><url>https://www.ncbi.nlm.nih.gov/pubmed/25635835</url></related-urls></urls><custom2>PMC4311971</custom2><electronic-resource-num>10.1371/journal.ppat.1004604</electronic-resource-num></record></Cite></EndNote>]. As shown in Fig. 5F, 3'UTR, but not 5'UTR-$C_{66}$, competed away the NS2A/5'UTR-$C_{66}$-3'UTR-biotin RNA interaction, indicating that 3'UTR RNA binds to NS2A protein. To further map the region of 3'UTR for NS2A binding, we deleted pseudoknot 1 (Δpk1), pseudoknots 1-2 [Δpk(1-2)], and pseudoknots 1-3 [Δpk(1-3)] from the 3'UTR (Fig. S10C). Only Δpk1 and Δpk(1-2) RNAs, not Δpk(1-3) RNA, could compete away the NS2A/5'UTR-$C_{66}$-3'UTR-biotin RNA interaction (Fig. 5F), suggesting that Δpk(1-2) RNA, representing the last 285 nucleotides of 3'UTR RNA, is required for binding to NS2A protein. This conclusion was further supported by the results that different fragments from Δpk(1-2) RNA, including pK(3-4), pk3, and 3'SL, did not compete away the NS2A/5'UTR-$C_{66}$-3'UTR-biotin RNA interaction (Fig. 5F).



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Next, we performed a photo-activatable ribonucleoside-enhanced crosslinking (PAR-CL) experiment to define the nucleotides that may interact with NS2A protein (Fig. 5G). The 3'UTR RNA containing [ HYPERLINK "https://en.wikipedia.org/wiki/Photosensitivity" \o "Photosensitivity" ] 4-thiouridine was prepared through *in vitro* transcription. After RNA/protein complex formation, the 4-thiouridine in the 3'UTR RNA was cross-linked to NS2A protein under UV light (at 365-nm wavelength). The RNA was then subjected to deep sequencing. Due to the crosslinking of 4-thiouridine to protein, a T-to-C mutation was recovered at the RNA sites that interacted with NS2A protein (Figs. 5G and S11). Fig. 5H summarizes the U-to-C mutations in the 3'UTR RNA when complexed with NS2A protein. About 67% mutations occurred within the last 285 nucleotides of 3'UTR. The result further supports the NS2A/3'UTR RNA interaction.

**NS2A requires amino acids R94, K95, and K99A for binding to 3'UTR RNA and virion assembly.** To map the region of NS2A required for viral 3'UTR RNA binding, we prepared a panel of purified NS2A truncates with an N-terminal GST tag from *E. coli* (Fig. 6A). The binding efficiencies of NS2A truncates to 5'UTR-$C_{66}$-3'UTR-biotin RNA were compared using a pulldown assay. As shown in Fig. 6B, different NS2A truncates were pulled down by 5'UTR-$C_{66}$-3'UTR-biotin at varied efficiencies. The RNA pulled down NS2A 1-145, 1-168, and 1-218 (full-length) at comparable efficiencies. Compared to the full-length NS2A, the pulldown efficiencies for NS2A 1-101 and 1-71 decreased to 40% and 0%, respectively. These data suggest that amino acids 72-144 are required for NS2A binding to viral RNA.

According to the membrane topology of DENV-2 NS2A [ ADDIN EN.CITE <EndNote><Cite><Author>Nemesio</Author><Year>2014</Year><RecNum>82</RecNum><DisplayText>(Nemesio and Villalain, 2014)</DisplayText><record><rec-number>82</rec-number><foreign-keys><key app="EN" db-



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id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793217">82</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Nemesio, H.</author><author>Villalain, J.</author></authors></contributors><auth-address>Molecular and Cellular Biology Institute, Universitas "Miguel Hernandez" , E-03202 Elche-Alicante, Spain.</auth-address><titles><title>Membrane interacting regions of Dengue virus NS2A protein</title><secondary-title>J Phys Chem B</secondary-title></titles><periodical><full-title>J Phys Chem B</full-title></periodical><pages>10142-55</pages><volume>118</volume><number>34</number><keywords><keyword>Calorimetry, Differential Scanning</keyword><keyword>Cell Membrane/*metabolism</keyword><keyword>Fluorescence Polarization</keyword><keyword>*Hydrophobic and Hydrophilic Interactions</keyword><keyword>Peptide Library</keyword><keyword>Spectrophotometry, Infrared</keyword><keyword>Viral Nonstructural Proteins/*chemistry/*metabolism</keyword></keywords><dates><year>2014</year><pub-dates><date>Aug 28</date></pub-dates></dates><isbn>1520-5207 (Electronic)1520-5207 (Linking)</isbn><accession-num>25119664</accession-num><urls><related-urls><url>http://www.ncbi.nlm.nih.gov/pubmed/25119664</url></related-urls></urls><custom2>PMC4148155</custom2><electronic-resource-num>10.1021/jp504911r</electronic-resource-num></record></Cite></EndNote>], amino acids 72-144 form two transmembrane domains (72-92 and 101-118), one short cytosolic loop (93-100), and a luminal segment (118-144; Fig. 6A). Since viral RNA resides in the cytoplasm, we examined the cytosolic loop of NS2A for viral RNA binding. The cytosolic



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loop has three positively charged residues (R94, K95, and K99) that are conserved among the four DENV serotypes (Fig. 6C). To examine the function of these residues, we prepared a panel of recombinant DENV-2 containing Ala substitutions at single (R94A, K95A, or K99A), double (R94A+K95A), or triple residues (R94A+K95A+K99A). Upon transfection to BHK-21 cells, all mutant RNAs generated viral E protein-positive cells at day 1 (Fig. 6D). Except for the triple mutant, other RNAs produced more E-positive cells on day 4 post-transfection. Mutants R94A and K95A generated WT level of infectious viruses and plaque morphology (Fig. 6E-F). Mutants K99A and K94A+K95A generated 22- to 3-fold less viruses than the WT, respectively (Fig. 6F), and exhibited smaller plaques (Fig. 6E). Remarkably, triple mutant R94A+K95A+K99A RNA yielded almost undetectable amount of virus that was 50,000-fold lower than the WT (Fig. 6F). To examine the effect of R94A+K95A+K99A on viral RNA synthesis, we engineered the triple mutations into a luciferase replicon of DENV-2 (Fig. 6G). The R94A+K95A+K99A replicon generated a luciferase profile similar to the WT replicon, indicating that the mutations do not affect viral translation and RNA synthesis. Taken together, the results demonstrate that R94A+K95A+K99A triple mutant is defective in virion assembly.

Next, we examined the effect of R94A+K95A+K99A mutations on NS2A binding to viral RNA. Since the result from Fig. 6B indicates that NS2A fragment 72-144 is required for viral RNA binding, we expressed a short NS2A peptide 64-128 (comprising the first two NS2A transmembrane domains, the cytosolic region 93-100, and the N- and C-terminal luminal tails for correct folding of the peptide on membrane) fused with an N-terminal GST-tag (Fig. 6H). As shown in Fig. 6I, peptide 64-128 could be pulled down by 5'UTR-$C_{66}$-3'UTR-biotin RNA (Lane 1). Un-biotinylated 5'UTR-$C_{66}$-3'UTR or 3'UTR RNA (Lanes 2 and 4), but not 5'UTR-$C_{66}$ RNA or yeast tRNA (Lanes 3 and 5), could compete away the NS2A peptide 64-128/5'UTR-$C_{66}$-3'UTR-biotin RNA interaction. The result demonstrates that



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NS2A peptide 64-128 specifically binds to viral 3'UTR RNA. Importantly, mutant R94A+K95A+K99A reduced the pulldown efficiency of NS2A peptide 64-128 by over 80% (Fig. 6J). Notably, some degraded GST-tag was found to associate with the NS2A truncates in the above pulldown experiments (Figs. 6B I-J), most likely due to the dimerization property of GST[ ADDIN EN.CITE <EndNote><Cite><Author>Maru</Author><Year>1996</Year><RecNum>110</RecNum><DisplayText>(Maru et al., 1996)</DisplayText><record><rec-number>110</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1561302524">110</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Maru, Y.</author><author>Afar, D. E.</author><author>Witte, O. N.</author><author>Shibuya, M.</author></authors></contributors><auth-address>Department of Genetics, Institute of Medical Science, University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108, Japan.</auth-address><titles><title>The dimerization property of glutathione S-transferase partially reactivates Bcr-Abl lacking the oligomerization domain</title><secondary-title>J Biol Chem</secondary-title></titles><periodical><full-title>J Biol Chem</full-title></periodical><pages>15353-7</pages><volume>271</volume><number>26</number><keywords><keyword>*Adaptor Proteins, Signal Transducing</keyword><keyword>Animals</keyword><keyword>Bone Marrow Cells</keyword><keyword>Cell Transformation, Neoplastic</keyword><keyword>Cells, Cultured</keyword><keyword>Fusion Proteins, bcr-abl/*metabolism</keyword><keyword>GRB2 Adaptor Protein</keyword><keyword>Glutathione Transferase/*metabolism</keyword><keyword>Macromolecular Substances</keyword><keyword>Mice</keyword><keyword>Phosphotyrosine/metabolism</keyword><keyword>Protein Binding</keyword><keyword>Protein-Tyrosine



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825 Kinases/metabolism</keyword><keyword>Proteins/metabolism</keyword><keyword>Rat
826 s</keyword><keyword>Recombinant Fusion Proteins</keyword><keyword>Structure-
827 Activity Relationship</keyword></keywords><dates><year>1996</year><pub-
828 dates><date>Jun 28</date></pub-dates></dates><isbn>0021-9258 (Print)0021-
829 9258 (Linking)</isbn><accession-num>8663064</accession-num><urls><related-
830 urls><url>https://www.ncbi.nlm.nih.gov/pubmed/8663064</url></related-
831 urls></urls><electronic-resource-num>10.1074/jbc.271.26.15353</electronic-resource-
832 num></record></Cite></EndNote>]. Overall, the results indicate that R94, K95, and K99
833 are important for NS2A binding to viral 3'UTR.

834 **DISCUSSION**

835 Flavivirus assembly consists of a tightly controlled cascade of events: export of
836 newly synthesized viral RNA from the replication complex to assembly sites, encapsidation
837 of viral RNA with C protein, and envelopment of nucleocapsid with prM and E proteins.
838 Many host and viral factors (proteins and lipids) participate in this temporal and spatial
839 process [ ADDIN EN.CITE <EndNote><Cite><Author>Apte-
840 Sengupta</Author><Year>2014</Year><RecNum>94</RecNum><DisplayText>(Apte-
841 Sengupta et al., 2014)</DisplayText><record><rec-number>94</rec-number><foreign-
842 keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv"
843 timestamp="1560793218">94</key></foreign-keys><ref-type name="Journal
844 Article">17</ref-type><contributors><authors><author>Apte-Sengupta,
845 S.</author><author>Sirohi, D.</author><author>Kuhn, R.
846 J.</author></authors></contributors><auth-address>Markey Center for Structural Biology,
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850 USA. Electronic address: kuhnr@purdue.edu.</auth-address><titles><title>Coupling of
851 replication and assembly in flaviviruses</title><secondary-title>Curr Opin
852 Virol</secondary-title></titles><periodical><full-title>Curr Opin Virol</full-
853 title></periodical><pages>134-
854 42</pages><volume>9</volume><keywords><keyword>Flavivirus/*physiology</keyword>
855 <keyword>Host-Pathogen
856 Interactions</keyword><keyword>Humans</keyword><keyword>Lipid
857 Metabolism</keyword><keyword>Viral Proteins/metabolism</keyword><keyword>*Virus
858 Assembly</keyword><keyword>*Virus
859 Replication</keyword></keywords><dates><year>2014</year><pub-
860 dates><date>Dec</date></pub-dates></dates><isbn>1879-6265 (Electronic)1879-
861 6257 (Linking)</isbn><accession-num>25462445</accession-num><urls><related-
862 urls><url>http://www.ncbi.nlm.nih.gov/pubmed/25462445</url></related-
863 urls></urls><custom2>PMC4268268</custom2><electronic-resource-
864 num>10.1016/j.coviro.2014.09.020</electronic-resource-
865 num></record></Cite></EndNote>]. Among them, flaviviral NS2A has emerged as a key
866 player to orchestrate virion assembly, as evidenced by the following findings. (i) In infected
867 cells, there are two distinct NS2A species that are required for viral RNA synthesis and
868 virion assembly. The NS2A species required for RNA synthesis is located at the viral
869 replication complex that could only be formed in *cis*; the other NS2A species required for
870 virus morphogenesis is located at the virion assembly complex that could be formed in *cis*
871 or *trans* [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. (ii) The defect in virion assembly
872 caused by DENV-2 and YFV NS2A mutations could be rescued by adaptive mutations in
873 prM, E, NS2B, and NS3 proteins, suggesting that NS2A genetically modulates other viral
874 proteins during virion assembly [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. (iii)



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875 Recombinant NS2A of Kunjin virus binds to viral 3'UTR RNA [ ADDIN EN.CITE
876 <EndNote><Cite><Author>Mackenzie</Author><Year>1998</Year><RecNum>81</RecN
877 um><DisplayText>(Mackenzie et al., 1998)</DisplayText><record><rec-number>81</rec-
878 number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv"
879 timestamp="1560793217">81</key></foreign-keys><ref-type name="Journal
880 Article">17</ref-type><contributors><authors><author>Mackenzie, J.
881 M.</author><author>Khromykh, A. A.</author><author>Jones, M.
882 K.</author><author>Westaway, E.
883 G.</author></authors></contributors><titles><title>Subcellular localization and some
884 biochemical properties of the flavivirus Kunjin nonstructural proteins NS2A and
885 NS4A</title><secondary-title>Virology</secondary-title></titles><periodical><full-
886 title>Virology</full-title></periodical><pages>203-
887 15</pages><volume>245</volume><number>2</number><keywords><keyword>Animal<
888 /keyword><keyword>Cercopithecus aethiops</keyword><keyword>*Flavivirus/ph
889 [Physiology]</keyword><keyword>Immunohistochemistry</keyword><keyword>RNA,
890 Viral/me [Metabolism]</keyword><keyword>Support, Non-U.S.
891 Gov't</keyword><keyword>Vero Cells</keyword><keyword>*Viral Nonstructural
892 Proteins/me [Metabolism]</keyword><keyword>Virus
893 Replication</keyword></keywords><dates><year>1998</year></dates><urls></urls></re
894 cord></Cite></EndNote>]. These studies indicate that NS2A interacts with two of the three
895 structural proteins (prM and E, not C) and viral RNA. However, how viral RNA and C
896 protein are recruited to form nucleocapsid and how nucleocapsid is enveloped with prM-E
897 proteins to form virion remain unknown. The current study aims to address these
898 questions.



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First, we showed that DENV-2 NS2A interacted with prM to modulate virion assembly. WT NS2A directly bound to prM in the absence of other viral proteins (Fig. 3B). The biological relevance of this interaction was supported by the correlation between virion assembly and prM/NS2A interaction. Specifically, an NS2A mutation G11A, which abolished virion assembly (Fig. 1), enhanced its binding to prM by 1.7-2.5 folds (Fig. 3). Mechanistically, the enhanced interaction between the mutant NS2A and prM "over pulled" the membrane-associated anchorC towards the ER lumen, leading to a blockage of the viral protease cleavage site (most likely by the ER membrane) at the C-anchorC junction. The uncleaved C-prM resulted in a rapid degradation of all three structural proteins (Fig. 1). This is not surprising because the unprocessed C-prM is retained on the ER membrane, misfolded, and degraded via the ubiquitin-proteasome machinery (Fig. 1H). Since prM is a chaperone for E protein [ ADDIN EN.CITE <EndNote><Cite><Author>Lorenz</Author><Year>2002</Year><RecNum>96</RecNum><DisplayText>(Lorenz et al., 2002)</DisplayText><record><rec-number>96</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793218">96</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Lorenz, I. C.</author><author>Allison, S. L.</author><author>Heinz, F. X.</author><author>Helenius, A.</author></authors></contributors><auth-address>Institute of Biochemistry, Swiss Federal Institute of Technology, CH-8093 Zurich, Switzerland.</auth-address><titles><title>Folding and dimerization of tick-borne encephalitis virus envelope proteins prM and E in the endoplasmic reticulum</title><secondary-title>J Virol</secondary-title></titles><periodical><full-title>J Virol</full-title></periodical><pages>5480-91</pages><volume>76</volume><number>11</number><keywords><keyword>Animals</keyword><keyword>COS Cells</keyword><keyword>Cercopithecus



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925 aethiops</keyword><keyword>Dimerization</keyword><keyword>Dithiothreitol</keyword
926 ><keyword>Encephalitis Viruses, Tick-
927 Borne/genetics/*metabolism</keyword><keyword>Hexosaminidases/metabolism</keywor
928 d><keyword>Polyproteins/genetics/metabolism</keyword><keyword>*Protein
929 Folding</keyword><keyword>Protein
930 Precursors/genetics/metabolism</keyword><keyword>Recombinant Fusion
931 Proteins/genetics/metabolism</keyword><keyword>Viral Envelope
932 Proteins/genetics/*metabolism</keyword></keywords><dates><year>2002</year><pub-
933 dates><date>Jun</date></pub-dates></dates><isbn>0022-538X (Print)0022-538X
934 (Linking)</isbn><accession-num>11991976</accession-num><urls><related-
935 urls><url>http://www.ncbi.nlm.nih.gov/pubmed/11991976</url></related-
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937 num>10.1128/jvi.76.11.5480-5491.2002</electronic-resource-
938 num></record></Cite></EndNote>], the degradation of C-prM leads to the degradation of
939 E protein. These results are in agreement with a recent report that ZIKV anchorC plays an
940 important role in the stability of E protein and virion morphogenesis [ ADDIN EN.CITE
941 <EndNote><Cite><Author>Rana</Author><Year>2018</Year><RecNum>97</RecNum><
942 DisplayText>(Rana et al., 2018)</DisplayText><record><rec-number>97</rec-
943 number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv"
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946 Campos, J. L.</author><author>Leccese, G.</author><author>Francolini,
947 M.</author><author>Bestagno, M.</author><author>Poggianella,
948 M.</author><author>Burrone, O. R.</author></authors></contributors><auth-
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950 Biotechnology, Trieste, Italy rana@icgeb.org burrone@icgeb.org.Molecular



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951 Immunology Group, International Centre for Genetic Engineering and Biotechnology,
952 Trieste, Italy.Department of Medical Biotechnology and Translational Medicine,
953 Universita degli Studi di Milano, Milan, Italy.</auth-address><titles><title>Role of Capsid
954 Anchor in the Morphogenesis of Zika Virus</title><secondary-title>J Virol</secondary-
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956 title></periodical><volume>92</volume><number>22</number><keywords><keyword>Zi
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965 num>10.1128/JVI.01174-18</electronic-resource-num></record></Cite></EndNote>].

966 Second, we demonstrated that the defect in virion assembly of NS2A G11A could
967 be restored by two different means. Creation of a new viral protease cleavage site by
968 extending the N-terminus of anchorC by two amino acids restored the capsid-anchorC
969 processing and, consequently, restored virion assembly. This conclusion was supported
970 by revertant analysis of the NS2A G11A mutant (Figs. 2 and S4) and amino acid insertion
971 experiment (Fig. 4). The two-amino acid extension exposes the new protease cleavage
972 site to NS2B/NS3. Alternatively, the virion assembly defect of NS2A G11A could be
973 rescued by *trans* supply of WT NS2A (Fig. S3). Under this condition, the WT NS2A
974 replaced the G11A NS2A to interact with C-prM, restored the C-prM cleavage, and ensued
975 a productive virion morphogenesis. Collectively, our results support a previous model that



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the two cleavages at C-anchorC-prM junctions are coordinated in a sequential manner during flavivirus assembly. The C-anchorC junction was first cleaved by viral protease on the cytosolic side, followed by the anchorC-prM cleavage by the cellular signalase on the ER lumen side [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. Although expression of flavivirus prM-E alone could form empty virus-like particles, the coordinated C-anchorC-prM processing couples the encapsidation and envelopment events; such coupling is required for the production of infectious virus. This conclusion is also supported by the previous reports that separate expression of C and prM-E significantly compromised the assembly efficiency of infectious virions [ ADDIN EN.CITE ADDIN EN.CITE.DATA ].

Third, we characterized the specific interaction between DENV-2 NS2A and the 3'UTR of viral genome. Although Kunjin virus NS2A was reported to bind to the 3'UTR of viral RNA [ ADDIN EN.CITE <EndNote><Cite><Author>Mackenzie</Author><Year>1998</Year><RecNum>81</RecNum><DisplayText>(Mackenzie et al., 1998)</DisplayText><record><rec-number>81</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793217">81</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Mackenzie, J. M.</author><author>Khromykh, A. A.</author><author>Jones, M. K.</author><author>Westaway, E. G.</author></authors></contributors><titles><title>Subcellular localization and some biochemical properties of the flavivirus Kunjin nonstructural proteins NS2A and NS4A</title><secondary-title>Virology</secondary-title></titles><periodical><full-title>Virology</full-title></periodical><pages>203-15</pages><volume>245</volume><number>2</number><keywords><keyword>Animal</keyword><keyword>Cercopithecus aethiops</keyword><keyword>*Flavivirus/ph



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1001 [Physiology]</keyword><keyword>Immunohistochemistry</keyword><keyword>RNA,
1002 Viral/me [Metabolism]</keyword><keyword>Support, Non-U.S.
1003 Gov't</keyword><keyword>Vero Cells</keyword><keyword>*Viral Nonstructural
1004 Proteins/me [Metabolism]</keyword><keyword>Virus
1005 Replication</keyword></keywords><dates><year>1998</year></dates><urls></urls></re
1006 cord></Cite></EndNote>], the molecular details of the interaction has not been delineated.
1007 Using competition assay and PAR-CLIP technique, we mapped that DENV-2 NS2A bound
1008 to the last 285 nucleotides of the 3'UTR, including the pk3, pk4, and 3'SL. Interestingly, the
1009 pk3, pk3-4, or 3'SL RNA alone did not efficiently compete out the NS2A/3'UTR RNA
1010 interaction (Fig. 5F), suggesting that a complex RNA structure in the 3'UTR is required for
1011 efficient binding to NS2A protein. Since no RNA “packaging signal” has been identified for
1012 flavivirus assembly and the C protein was shown to non-specifically bind to any RNA
1013 [ ADDIN EN.CITE
1014 <EndNote><Cite><Author>Pong</Author><Year>2011</Year><RecNum>102</RecNum>
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1019 L.</author><author>Huang, Z. S.</author><author>Teoh, P. G.</author><author>Wang,
1020 C. C.</author><author>Wu, H. N.</author></authors></contributors><auth-
1021 address>Institute of Molecular Biology, Academia Sinica, Taipei, Taiwan.</auth-
1022 address><titles><title>RNA binding property and RNA chaperone activity of dengue virus
1023 core protein and other viral RNA-interacting proteins</title><secondary-title>FEBS
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81</pages><volume>585</volume><number>16</number><keywords><keyword>Base Sequence</keyword><keyword>*Dengue Virus</keyword><keyword>Genome, Viral/genetics</keyword><keyword>Molecular Chaperones/*metabolism</keyword><keyword>Nucleic Acid Conformation</keyword><keyword>Protein Binding</keyword><keyword>Protein Engineering</keyword><keyword>RNA Helicases/metabolism</keyword><keyword>RNA, Catalytic/genetics</keyword><keyword>RNA, Viral/chemistry/genetics/*metabolism</keyword><keyword>Serine Endopeptidases/metabolism</keyword><keyword>Viral Core Proteins/*metabolism</keyword><keyword>Viral Nonstructural Proteins/metabolism</keyword></keywords><dates><year>2011</year><pub-dates><date>Aug 19</date></pub-dates></dates><isbn>1873-3468 (Electronic)0014-5793 (Linking)</isbn><accession-num>21771593</accession-num><urls><related-urls><url>http://www.ncbi.nlm.nih.gov/pubmed/21771593</url></related-urls></urls><electronic-resource-num>10.1016/j.febslet.2011.06.038</electronic-resource-num></record></Cite></EndNote>], the last 285 nucleotides of 3'UTR may serve as the "packaging signal" to recruit viral RNA to the virion assembly site through specific binding to NS2A. From the NS2A side, we have mapped the 3'UTR-binding site to a cytosolic loop (containing R94, K95, and K99) between the first and second transmembrane domains (Fig. 6). Triple mutant R94A+K95A+K99A was defective in virion assembly and lost over 80% of the NS2A binding activity to 3'UTR RNA (Fig. 6). Caution should be taken when applying the DENV-2 findings to other flaviviruses because of the variations of NS2A proteins. The three positively charged R94, K95, and K99 of NS2A are only conserved among the four serotypes of DENV. In addition, DENV-2, YFV, and ZIKV NS2A proteins



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exhibit different topologies on the ER membrane [ ADDIN EN.CITE ADDIN EN.CITE.DATA ].

Our findings support a general model for flavivirus virion assembly (Fig. 7). In infected cells, NS2A molecules reside in both replication complex (housed in Ve) and viron assembly complex (located on the juxtaposed ER membrane). When plus-sense RNAs are made and released from the replication complex (through the pore on Ve), they are initially destined to new rounds of viral translation and replication on the ER. Once the amounts of viral RNA and proteins have accumulated over certain “thresholds”, nascent RNAs are switched for virion assembly. At this time, the newly synthesized RNA exits the replication complex and binds to the NS2A located at the assembly site through the 3'UTR RNA. The NS2A molecules at the assembly site also recruits C-prM-E and NS2B/NS3 through binding to prM and NS3, respectively. One NS2A molecule does not have to bind to the 3’UTR RNA, C-prM-E, and NS2B/NS3 at the same time. These interactions could be sequential or through different NS2A molecules that oligomerize together on the ER membrane [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. Next, the C-prM-E polyprotein is sequentially cleaved by the NS2B/NS3 protease and host signalase to produce mature C, prM, and E [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. The C molecules, many of which have been previously made and stored at the nearby lipid droplets [ ADDIN EN.CITE ADDIN EN.CITE.DATA ], bind viral RNA to form nucleocapsid. The processed prM undergoes conformational change and dimerizes with E. The prM/E dimerization and oligomerization dissociate prM from NS2A. When 180 copies of prM/E oligomerize on the ER membrane, envelopment of the nucleocapsid occurs, generating spiky immature virions. During the above process, NS3 may also use its helicase activity to transport viral RNA from the replication complex to the assembly site and condense the



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RNA during encapsidation. Besides NS3, NS2A or other proteins may also contribute to the translocation of viral RNA from the replication complex to the assembly site.

The above model is centered on the multifunctional role of NS2A in flavivirus replication and virion assembly. Our results have functionally demonstrated the molecular mechanism for two important steps during DENV-2 assembly: (i) The binding of 3'UTR RNA to NS2A servers as a "packaging signal" and (ii) in the presence of viral RNA-NS2A complex, the coordinated cleavage of C-prM-E facilitates the envelopment of nucleocapsid. The proposed model could serve as a framework for future investigations and antiviral development.

**Author contribution**

X.X., J.Z., X.Z., Y.Z., Q.W., H.-P.D. performed experiments and data analysis. X.X., J.Z., X.Z., Y.Z., A.R., V.L.P., X.W., Q.W., H.-P.D, P.-Y.S. designed the experiments and interpreted the results. X.X., P.-Y.S. wrote the manuscript.

**Acknowledgements**

We are grateful to Dr. Mariano A. Garcia-Blanco, Shelton S. Brarick from University of Texas Medical Branch for helpful discussions during this work. P.-Y.S. was supported by NIH grants AI142759, AI[ HYPERLINK "https://public.era.nih.gov/grantfolder/piAppDetails/genericStatus.do?encryptedParam=vXeG1axw2cY.YZxjFgAisBblI6OqVj4LxDR99WdVyTI-X4FuJbCLt4g." \t "_blank" ], and AI136126, and awards from the Kleberg Foundation, John S. Dunn Foundation, Amon G. Carter Foundation, Gilson Longenbaugh Foundation, and Summerfield Robert Foundation. X.X. and J.Z. were awarded with postdoctoral fellowships from the Novartis Institutes for



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Biomedical Research (NIBR) and the McLaughlin Endowment at University of Texas Medical Branch, respectively.



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## Figure titles and legends

**Figure 1 Characterization of NS2A G11A mutant**. (A) Replicon transient transfection assay. Top panel depicts the DENV-2 luciferase replicon. Bottom panel shows the luciferase activities after transfecting BHK-21 cells with equal amounts (10 μg) of WT or NS2A G11A mutant replicon RNA. (B) Quantification of extracellular viral RNA. Top panel depicts the full-length genome of DENV-2. Equal amounts of WT or NS2A G11A mutant viral RNA (10 μg) were electroporated into BHK-21 cells. At different time post-transfection (p.t.), extracellular viral RNAs were quantified by RT-qPCR. The DENV-2 replicon RNA was used as a control for determining the basal level of viral RNA released from dead cells. Bottom panel shows the CT values of the RT-qPCR results. Data show the means and standard deviations from three independent experiments. (C) Plaque morphology. Culture supernatants from (B) were analyzed for virus production using plaque assay. Only WT, not NS2A G11A mutant (N.D.), produced plaques. Plaques were developed on BHK-21 cells after 5 days of infection. (D) Thin-section transmission electron microscope (TEM) images of viral RNA-transfected BHK-21 cells. At 48 h p.t., cells were fixed for TEM analysis. Ve, virus-induced vesicles; T, membrane tubes; Vi, virus particles; ER, endoplasmic reticulum. Mock transfected cells were included as a control. (E-G) Confocal microscopy analysis of subcellular localizations of C, prM, and E in viral RNA-transfected BHK-21 cells. At 48 h p.t., transfected cells were analyzed by IFA. NS3 staining (red) was used as a control. Nuclei (blue) were stained by DAPI. Scale bar, 10 μm. (H) Viral protein processing in viral RNA-transfected BHK-21 cells. At 42 h p.t., BHK-21 (Mock) or viral RNA-transfected cells were treated with 0.01% DMSO (-) or 10 μM MG132 (+). After 6 h treatment, the cells were analyzed by Western blot. (I) Densitometry analysis. The intensities of bands representing specific viral proteins from DMSO-treated cells in (H) was quantified. The amounts of viral proteins derived from WT RNA-transfected cells were set as 100%. Relative intensities are calculated by normalizing the protein intensities derived from NS2A G11A mutant RNA-transfected cells to those from WT RNA-transfected cells. Data shows the means and standard deviations from three independent experiments.

**Figure 2 Restoration of NS2A G11A virion assembly by capsid R99S mutation.** (A) Revertant mutation. BHK-21 cells were transfected with NS2A G11A viral RNA. The transfected cells were consecutively passaged for five rounds to select for revertant viruses. The P5 virus was sequenced for revertant mutations. Changes in nucleotide



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positions and amino acids are indicated. (B) IFA of viral RNA-transfected cells. WT and mutant viral RNAs were electroporated to BHK-21 cells. The transfected cells were analyzed for E protein expression at 24 and 96 h p.t. by IFA. Scale bar, 100 μm. (C) Plaque morphology. Infectious viruses, derived from the RNA-transfected cells, were analyzed by plaque assay. Plaques were developed on day 5 post-infection on BHK-21 cells. N.D., not detectable. (D) Virus production. The culture fluids from (B) were quantified for infectious viruses. Data represent the means and standard deviations from three independent measurements. Limit of detection (L.O.D.), 10 PFU/ml. (E-F) Subcellular distributions of prM, NS1, and C proteins. prM, NS1 and C proteins were detected by DM1, human IgG anti-NS1 and D2C1 antibodies, respectively. Representative IFA images of viral RNA-transfected cells are shown at 24 h post-transfection. White arrows point to nucleus with C protein speckles. (G) Quantitative analysis of C-positive cells. The C-positive cells with cytoplasm or cytoplasm & nucleus distribution from 4-5 random fields were counted and normalized to the total cell numbers. The averages and standard deviations are shown. (H) Viral protein processing in viral RNA-transfected BHK-21 cells at 48 h post-transfection. The percentages of mature C protein were calculated from the following the formula: [C intensity/(C intensity + C-prM intensity)]×100%.

**Figure 3 DENV-2 NS2A interacts with prM and E proteins**. (A) Membrane topologies of DENV-2 prM, E, and NS2A proteins. The NS2A G11 position is indicated. (B) Co-IP of WT NS2A with prM. HEK293T cells were transfected with one plasmid (encoding prM only) or two plasmids (one encoding leader-NS2A-EGFP and the other encoding full-length or the ectodomain of prM). At 40 h p.t., IP was performed using mouse anti-EGFP or control IgG antibodies. Immunoblot (IB) was performed using rabbit anti-EGFP antibody or anti-prM antibody. (C) Co-IP of WT or G11A NS2A-EGFP with prM. Mouse anti-EGFP antibody was used for IP. Rabbit anti-EGFP or anti-prM antibodies were used for IB. The intensity of prM, co-precipitated with WT NS2A-EGFP, was set as 100%. The relative efficiency of prM co-precipitated with G11A NS2A-EGFP was compared with the WT NS2A-EGFP. Statistical analysis was performed using the unpaired parametric t-test. $*p < 0.05$, significant; $**p < 0.01$, very significant. (D) Reciprocal co-IP of prM with WT or G11A NS2A-EGFP. Rabbit anti-prM antibodies were used for IP. The amounts of co-precipitated WT and G11A NS2A-EGFP were detected using mouse anti-EGFP antibody. prM in eluates were detected by rabbit anti-prM antibody followed by protein G conjugated with HRP. (E) Co-localization analysis of NS2A and prM proteins. BHK-21 cells were co-



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transfected with two plasmids: One encoding the $C_{14}$-prM-E and the other encoding SPG-$C_{16}$-NS2A-EGFP. At 24 h p.t., the cells were examined for prM and NS2A-EGFP co-localization under a confocal microscope. Scale bar, 10 μm. (F) Co-IP of NS2A with E protein. HEK293T cells were co-transfected with two plasmids: One encoding the last 17 amino acids of prM and complete E protein ($M_{17}$-E), and another encoding NS2A with a C-terminal GST tag (NS2A-GST). GST tag alone was used as a negative control. IPs were performed using rabbit anti-GST antibody. E and GST fusion proteins were detected in cell lysates and eluates by mouse anti-E monoclonal antibody (9F12) and anti-GST antibody, respectively. The relative pulldown efficiency was calculated by normalizing the intensity of E co-precipitated with G11A NS2A-GST to the intensity of E co-precipitated with the WT NS2A-GST. The means and standard deviations from three independent experiments are shown. N.S., no significance.

**Figure 4 Two-residue insertion at the capsid-anchorC junction rescues the defect of NS2A G11A**. (A) A model depicting how NS2A modulates C-anchorC-prM cleavage. On the cytosolic side, viral NS2B/NS3 protease cleaves the C-anchorC junction. On the ER lumen side, host signalase cleaves the anchorC-prM junction. Three cleavage scenarios are presented for WT, NS2A G11A, and NS2A G11A+C_R99S constructs. (B) Constructs of insertion mutants of capsid protein. One residue (C_101) or two residues (C_TA or C_SR) were inserted to the C-anchorC junction. The potential cleavage sites for viral NS2B/NS3 protease are indicated. (C) IFA of viral RNA-transfected cells. Viral RNAs containing the NS2A G11A mutation with or without capsid insertions were electroporated into BHK-21 cells. The transfected cells were analyzed for E protein expression on day 1 and 4 by IFA. Scale bar, 100 μm. (D) Plaque morphology. Viruses recovered from (C) were analyzed by plaque assay on BHK-21 cells. (E) Viral titers. Culture supernatants from (C) were measured for viral titers on day 5 post-transfection. (F) Subcellular localization of C protein in viral RNA-transfected cells. White arrows point to nucleus with C protein speckles. Scale bar, 25 μm. (G) Expression of prM and NS1 proteins in viral RNA-transfected cells. Scale bar, 50 μm. (H) C-prM processing in viral RNA-transfected cells. At 48 h p.t., WT or NS2A G11A+C_T101 RNA-transfected cells were analyzed for C, NS1, and β-actin expression by Western blot. The percentages of C-prM to mature C conversion are presented for each construct.

**Figure 5 DENV-2 NS2A protein binds to 3'UTR RNA**. (A) Diagram of pulldown assay using biotinylated RNAs. 3'-end biotinylated RNA binds to streptavidin-conjugated



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magnetic beads. After incubating the bead-bound biotinylated RNA with NS2A protein, the RNA-bound NS2A was enriched and eluted for SDS-PAGE and Western blot analysis. (B) Pulldown analysis using recombinant NS2A protein. N-terminally GST-tagged NS2A protein (GST-NS2A) was expressed in *E. coli* and affinity purified. The 5'UTR-$C_{66}$-3'UTR-biotin RNA was prepared by ligating biotinylated cytidine (bis)phosphate to the 3' end of the 5'UTR-$C_{66}$-3'UTR RNA (containing the 5'UTR, the first 66 nucleotides of C gene, and the 3'UTR). The amounts of GST-NS2A in the input and eluates were analyzed using rabbit anti-GST antibody by Western blot. (C) Pulldown analysis using HEK293T cell-expressed NS2A-GST protein. HEK293T cells were transfected with a plasmid expressing GST or C-terminally GST-tagged NS2A protein (NS2A-GST). At 42 h p.t., cells were harvested, lysed, and used for RNA pull down-assay. The pulldown assay was performed as described in (B). (D) RNA competition assay. Various amounts of unlabeled 5'UTR-$C_{66}$-3'UTR RNA were used to compete the binding of 5'UTR-$C_{66}$-3'UTR-biotin RNA to GST-NS2A protein. tRNA was used as a negative control. (E) Viral RNA/NS2A binding affinity. Electrophoretic mobility-shift assay (EMSA) was used to determine the binding affinity of DENV-2 5'UTR-$C_{66}$-3'UTR-biotin RNA with NS2A protein. 5'UTR-$C_{66}$-3'UTR-biotin RNA was incubated with increasing amounts of GST-NS2A protein. Free and bound RNAs were resolved by native agarose gel electrophoresis. The equilibrium dissociation constant (*Kd*) was determined by nonlinear regression with least-squares fitting method. The coefficient of determination ($R^2$) is shown. Data represent the means and standard deviations from three independent experiments. (F) Mapping the RNA region responsible for NS2A binding. A panel of unlabeled RNA truncates of the 5'UTR-$C_{66}$-3'UTR were used as competitors for the binding of 5'UTR-$C_{66}$-3'UTR-biotin RNA to GST-NS2A protein. The intensity of GST-NS2A (bound to the 5'UTR-$C_{66}$-3'UTR-biotin) without competitor was set as 100%. Relative GST-NS2A intensities were calculated as [(signal in the presence competitor)/(signal in the absence of competitor)]×100%. Statistical analysis was performed using the unpaired parametric t-test. (G) PAR-CLIP workflow [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. See text for detail. (H) PAR-CLIP result summary. Uridines in the 3'UTR that may participate in NS2A-binding are indicated. The dash line indicates the threshold (equal to 5.76, representing the average fold change of mutation rate of all uridines determined by Illumina sequencing). The uridine positions with fold changes of mutation rate over the threshold are indicated in the 3'UTR sequence (GenBank no. AF038403).



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**Figure 6 NS2A residues responsible for the NS2A/3'UTR RNA interaction**. (A) Diagram of NS2A truncates used in RNA pulldown assay. All proteins were fused with an N-terminal GST tag, expressed in *E. coli*, and affinity purified. (B) Interactions between NS2A truncates with 5'UTR-$C_{66}$-3'UTR-biotin RNA. The interactions were measured using pulldown experiments. The pulldown efficiency of full-length NS2A (1-218) was set as 100%. Relative efficiency was calculated by comparing the intensity of NS2A truncate with that of the full-length NS2A. (C) Sequence alignment of NS2A among flaviviruses. Amino acids of a cytosolic loop (amino acids 93-101) are presented. The number of viral strains used for the NS2A alignment are indicated in parentheses. Amino acid positions of NS2A are labeled according to DENV-2 strain New Guinea C. Residues R94, K95, and K99 are shaded. The conserved (:) and identical (*) residues are indicated. (D) IFA of BHK-21 cells transfected with viral RNA. WT and mutant viral RNAs were electroporated into BHK-21 cells. The cells were assayed for E protein expression. (E) Plaque morphology. Viruses derived from the transfected cells in (D) were analyzed by plaque assay. (F) Viral titers. Culture supernatants from (D) were measured for virus yields on day 5 post-transfection. (G) Replicon transient transfection assay. NS2A triple mutations (R94A+K95A+K99A) were engineered into a luciferase replicon of DENV-2. Luciferase activities were presented after the WT or mutant replicon RNAs were electroporated into BHK-21 cells. (H) Diagram of N-terminally GST-tagged NS2A peptide 64-128 [GST-NS2A (64-128)]. (I) Interaction of GST-NS2A (64-128) protein with 3'UTR RNA. GST-NS2A (64-128) was expressed in *E. coli* and affinity purified. The pulldown efficiency of GST-NS2A (64-128) by 5'UTR-$C_{66}$-3'UTR-biotin was set as 100%. The binding efficiencies of RNA variants were indicated through competition assay. In the presence of competitor RNAs, the remaining amounts of WT GST-NS2A (64-128)/5'UTR-$C_{66}$-3'UTR-biotin interaction are presented. (J) R94A+K95A+K99A mutations reduce GST-NS2A (64-128) binding to 5'UTR-$C_{66}$-3'UTR-biotin RNA. The R94A+K95A+K99A variant retained only 19.1% of the WT activity.

**Figure 7 A model depicting the role of NS2A in DENV-2 assembly.** Viral replication complex (top) and virion assembly site (bottom) are depicted. DENV-2 NS2A protein recruits viral RNA, three structural proteins, and NS2B/NS3 protease to virion assembly site, where coordinated cleavage of C-prM-E ensues infections virus formation. C molecules accumulated on lipid droplets (LD) could bind viral RNA to form nucleocapsid. See text for details.



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**Reference**

[ ADDIN EN.REFLIST ]



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## STAR METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---|---|---|
| Antibodies | | |
| Mouse IgG Anti-C (D2C1) | [ ADDIN EN.CITE ADDIN EN.CITE.DATA ] | N/A |
| Mouse IgG anti-prM (DM1) | Abcam | Cat# ab41473, RRID:AB_873758 |
| Rabbit IgG anti-prM | GeneTex | GTX128093 |
| Mouse IgG anti-E (4G2) | ATCC | Cat# HB-112, RRID:CVCL_J890 |
| Mouse IgG anti-E (9F12) | [ ADDIN EN.CITE ADDIN EN.CITE.DATA ] | N/A |
| Mouse IgG anti-NS1 | Abcam | Cat# ab41616, RRID:AB_869448 |
| Human IgG anti-NS1 | This study. Generated by Novartis Institute for Tropical Diseases in Singapore | N/A |
| Rabbit IgG anti-NS1 | GeneTex | GTX103346 |
| Rabbit IgG anti-NS3 | GeneTex | GTX124252 |
| Mouse IgG anti-NS5 | GeneTex | GTX629447 |
| Rabbit IgG anti-calnexin | SIGMA-AlDRICH | Cat# C4731, RRID:AB_476845 |
| Mouse IgG anti-EGFP | SIGMA-AlDRICH | Cat# 11814460001, RRID:AB_390913 |
| Moue IgG1 Isotype Control | SIGMA-AlDRICH | Cat# M5284, RRID:AB_1163685 |
| Rabbit IgG anti-EGFP | Abcam | Cat# ab13970, RRID:AB_300798 |
| protein G horseradish peroxidase (HRP) conjugate | Thermo Fisher Scientific | Cat# P21041 |
| Mouse IgG anti-GST | Abcam | Cat# ab19256, RRID:AB_444809 |
| Rabbit IgG anti-GST | SIGMA-AlDRICH | Cat# G7781, RRID:AB_259965 |
| Mouse IgG anti-β-actin | Abcam | Cat# ab8226, RRID:AB_306371 |
| Mouse IgG anti-HA | Abcam | Cat# ab18181, RRID:AB_444303 |
| Goat anti-mouse IgG conjugated with HRP | SIGMA-AlDRICH | Cat# A5278, RRID:AB_258232 |
| Goat anti-rabbit IgG conjugated with HRP | SIGMA-AlDRICH | Cat#AP307P, RRID:AB_92641 |
| Goat anti-mouse IgGs conjugated with Alexa 488 | Thermo Fisher Scientific | Cat#A-1100, RRID:AB_2534069 |
| Goat anti-mouse IgGs conjugated with Alexa 594 | Thermo Fisher Scientific | Cat# A-11005, RRID:AB_2534073 |
| Goat anti-rabbit IgGs conjugated with Alexa 488 | Thermo Fisher Scientific | Cat# A-11008, RRID:AB_143165 |



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| | | |
|---|---|---|
| Goat anti-rabbit IgGs conjugated with Alexa 594 | Thermo Fisher Scientific | Cat# A-11072, RRID:AB_2534116 |
| Goat anti-Human IgG (H+L) cross-adsorbed secondary antibody, Alexa Fluor 594 | Thermo Fisher Scientific | Cat# A-11014, RRID:AB_2534081 |
| Bacterial and Virus Strains | | |
| *E.* coli strain Top 10 | ThermoFisher Scientific | Cat#C404006 |
| *E.* coli strain Rosetta™ 2(DE3) pLysS | MilliporeSigma | Cat#71403-4 |



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| | | |
|---|---|---|
| DENV-2 strain New Guinea C | [ ADDIN EN.CITE <EndNote><Cite><Author>Xie</Author><Year>2013</Year><RecNum>85</RecNum><DisplayText>(Xie et al., 2013)</DisplayText><record><rec-number>85</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793217">85</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Xie, X.</author><author>Gayen, S.</author><author>Kang, C.</author><author>Yuan, Z.</author><author>Shi, P. Y.</author></authors></contributors><auth-address>State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Science, China.</auth-address><titles><title>Membrane topology and function of dengue virus NS2A protein</title><secondary-title>J Virol</secondary-title><alt-title>Journal of virology</alt-title></titles><periodical><full-title>J Virol</full-title></periodical><pages>4609-22</pages><volume>87</volume><number>8</number><edition>2013/02/15</edition><keywords><keyword>Animals</keyword><keyword>Cell Line</keyword><keyword>Cricetinae</keyword><keyword>Dengue | N.A. |



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| | | |
|---|---|---|
| Biological Samples | | |
| None | | |
| Chemicals, Peptides, and Recombinant Proteins | | |
| Peptide WT (NILNRRRRTAGMII) | GenScript | N/A |
| Peptide C_R99S (NILNRRSRTAGMII) | GenScript | N/A |
| Recombinant protein: GST | This study | N/A |
| Recombinant protein: GST-NS2A | This study | N/A |
| Recombinant protein: GST-NS2A(1-71) | This study | N/A |
| Recombinant protein: GST-NS2A(1-101) | This study | N/A |
| Recombinant protein: GST-NS2A(1-145) | This study | N/A |
| Recombinant protein: GST-NS2A(1-168) | This study | N/A |
| Recombinant protein: GST-NS2A(64-128) | This study | N/A |
| Critical Commercial Assays | | |
| iScript SYBR Green One-Step kit | Bio-Rad | Cat#170-8893 |
| QIAamp viral RNA mini kit | Qiagen | Cat#52906 |
| T7 mMessage mMachine kit | Thermo Fisher Scientific | Cat# AM1344 |
| Renilla Luciferase Assay System | Promega Corporation | Cat# E2810 |
| Pierce™ RNA 3' End Biotinylation Kit | Thermo Fisher Scientific | Cat# 20160 |
| Pierce™ Magnetic RNA-Protein Pull-Down kit | Thermo Fisher Scientific | Cat# 20164 |
| Chemiluminescent Detection Module | Thermo Fisher Scientific | Cat# 89880 |
| | | |
| Deposited Data | | |
| Raw image data for illumina sequencing | | |
| Experimental Models: Cell Lines | | |
| BHK-21 cell | ATCC | Cat#CCL-10, RRID:CVCL_1915 |
| Vero cell | ATCC | Cat#CCL-81, RRID:CVCL_0059 |
| HEK293T cell | ATCC | Cat# CRL-3216, RRID:CVCL_0063 |
| NS2A-EGFP BHK-21 cell | [ ADDIN EN.CITE ADDIN EN.CITE.DATA ] | N/A |
| | | |
| Experimental Models: Organisms/Strains | | |
| N.A. | | |
| Oligonucleotides | | |
| Primer P1(5'-ACACTCTTTCCCTACACGA CGCTCTTCCGATCTAAGGCAAAACTAACATGAA AC-3') | Integrated DNA Technologies (Skokie, Illinois) | N/A |
| Primer P2 (5'-GTGACTGGAGTTCAGACG TGTGCTCTTCCGATCTAGAACCTGTTGATTCAA CAGCAC-3') | Integrated DNA Technologies (Skokie, Illinois) | N/A |



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| | | |
|---|---|---|
| 5' universal adaptor (5'-AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGAC-3') | Illumine (San Diego, CA) | N/A |
| 3' indexing primer (5'-CAAGCAGAAGACGGCATACGAGATnnnnnnGTGACTGGAGTTCAGACGTGT-3') | Illumine (San Diego, CA) | N/A |
| Primer NGC 7764V (5'-CGTCGAGAGAAATATGGTCACACC-3') | Integrated DNA Technologies (Skokie, Illinois) | N/A |
| Primer NGC 7844C (5'CCACAATAGTATGACCAGCCT-3') | Integrated DNA Technologies (Skokie, Illinois) | N/A |
| Recombinant DNA | | |



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| | | |
|---|---|---|
| pACYC-NGC FL | [ ADDIN EN.CITE <EndNote><Cite><Author>Xie</Author><Year>2013</Year><RecNum>85</RecNum><DisplayText>(Xie et al., 2013)</DisplayText><record><rec-number>85</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793217">85</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Xie, X.</author><author>Gayen, S.</author><author>Kang, C.</author><author>Yuan, Z.</author><author>Shi, P. Y.</author></authors></contributors><auth-address>State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Science, China.</auth-address><titles><title>Membrane topology and function of dengue virus NS2A protein</title><secondary-title>J Virol</secondary-title><alt-title>Journal of virology</alt-title></titles><periodical><full-title>J Virol</full-title></periodical><pages>4609-22</pages><volume>87</volume><number>8</number><edition>2013/02/15</edition><keywords><keyword>Animals</keyword><keyword>Cell Line</keyword><keyword>Cricetinae</keyword><keyword>Dengu | N/A |



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| | | |
|---|---|---|
| pACYC-NGC replicon | [ ADDIN EN.CITE <EndNote><Cite><Author>Xie</Author><Year>2013</Year><RecNum>85</RecNum><DisplayText>(Xie et al., 2013)</DisplayText><record><rec-number>85</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793217">85</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Xie, X.</author><author>Gayen, S.</author><author>Kang, C.</author><author>Yuan, Z.</author><author>Shi, P. Y.</author></authors></contributors><auth-address>State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Science, China.</auth-address><titles><title>Membrane topology and function of dengue virus NS2A protein</title><secondary-title>J Virol</secondary-title><alt-title>Journal of virology</alt-title></titles><periodical><full-title>J Virol</full-title></periodical><pages>4609-22</pages><volume>87</volume><number>8</number><edition>2013/02/15</edition><keywords><keyword>Animals</keyword><keyword>Cell Line</keyword><keyword>Cricetinae</keyword><keyword>Dengue | N/A |



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| | | |
|---|---|---|
| pACYC-NGC replicon G11A | [ ADDIN EN.CITE ADDIN EN.CITE.DATA ] | N/A |
| pACYC-NGC replicon R94A+K95A+K99A | This study | N/A |
| pACYC-NGC FL C_R99S | This study | N/A |
| pACYC-NGC FL G11A | This study | N/A |
| pACYC-NGC FL G11A+C_R99S | This study | N/A |
| pACYC-NGC FL E20A | This study | N/A |
| pACYC-NGC FL E100A | This study | N/A |
| pACYC-NGC FL K188A | This study | N/A |
| pACYC-NGC FL E20A+C_R99S | This study | N/A |
| pACYC-NGC FL E100A+C_R99S | This study | N/A |
| pACYC-NGC FL K188A+C_R99S | This study | N/A |
| pACYC-NGC FL C_TA | This study | N/A |
| pACYC-NGC FL C_SR | This study | N/A |
| pACYC-NGC FL G11A+C_TA | This study | N/A |
| pACYC-NGC FL G11A+C_SR | This study | N/A |
| pACYC-NGC FL C_T101 | This study | N/A |
| pACYC-NGC FL G11A+C_T101 | This study | N/A |
| pACYC-NGC FL R94A | This study | N/A |
| pACYC-NGC FL K95A | This study | N/A |
| pACYC-NGC FL K99A | This study | N/A |
| pACYC-NGC FL R94A+K95A+K99A | This study | N/A |
| pACYC-NGC FL R94A+K95A | This study | N/A |



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| | | |
|---|---|---|
| SFV-DENV2 CprME | [ ADDIN EN.CITE <EndNote><Cite><Author>Qing</Author><Year>2010</Year><RecNum>105</RecNum><DisplayText>(Qing et al., 2010)</DisplayText><record><rec-number>105</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793220">105</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Qing, M.</author><author>Liu, W.</author><author>Yuan, Z.</author><author>Gu, F.</author><author>Shi, P. Y.</author></authors></contributors><auth-address>Novartis Institute for Tropical Diseases, 10, Biopolis Road, #05-01 Chromos, Singapore, Singapore.</auth-address><titles><title>A high-throughput assay using dengue-1 virus-like particles for drug discovery</title><secondary-title>Antiviral Res</secondary-title></titles><periodical><full-title>Antiviral Res</full-title></periodical><pages>163-71</pages><volume>86</volume><number>2</number><dates><year>2010</year><pub-dates><date>May</date></pub-dates></dates><accession-num>20153777</accession-num><urls><related- | N/A |



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| | | |
|---|---|---|
| pXJ-SPG-$C_{16}$-NS2A-EGFP | This study | N/A |
| pXJ-SPG-$C_{16}$-NS2A(G11A)-EGFP | This study | N/A |
| pXJ-EGFP | [ ADDIN EN.CITE ADDIN EN.CITE.DATA ] | N/A |
| pXJ-$C_{14}$-prM | This study | N/A |
| pXJ-$C_{14}$-prM(1-130) | This study | N/A |
| pXJ-$C_{14}$-prM (N69Q) | This study | N/A |
| pXJ-SPG-$C_{16}$-NS2A-GST | This study | N/A |
| pXJ-SPG-$C_{16}$-NS2A(G11A)-GST | This study | N/A |
| pXJ-GST | This study | N/A |
| pXJ-HA-NS3 | This study | N/A |
| pXJ-$M_{17}$-E | This study | N/A |
| pGEX-4T-1 | GE Healthcare Life Sciences | Cat# 28954549 |
| pGEX-4T-GST-NS2A | This study | N/A |
| pGEX-4T-GST-NS2A (1-71) | This study | N/A |
| pGEX-4T-GST-NS2A (1-101) | This study | N/A |
| pGEX-4T-GST-NS2A (1-145) | This study | N/A |
| pGEX-4T-GST-NS2A (1-168) | This study | N/A |
| pGEX-4T-GST-NS2A (64-128) | This study | N/A |
| pGEX-4T-GST-NS2A (64-128) R94A+K95A+K99A | This study | N/A |
| | | |
| Software and Algorithms | | |
| Illustrator CC | Adobe | [ HYPERLINK "https://www.adobe.com/products/illustrator.html" ] |
| ImageJ | NIH | [ HYPERLINK "https://fiji.sc/" ] |
| CLC main workbench | Qiagen | [ HYPERLINK "https://www.qiagenbioinformatics.com/products/clc-main-workbench/" ] |
| Prism 8.0 software | GraphPad | [ HYPERLINK "https://www.graphpad.com/scientific-software/prism/" ] |
| | | |

1271

1272 **Contact for Reagent and Resource Sharing**

1273 Further information and requests for resources and reagents should be directed to and will
1274 be fulfilled by Lead Contact, Dr. Pei-Yong Shi (peshi@utmb.edu)

1275 **Experimental Model and Subject Details**



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## Cell Lines

The Baby hamster kidney fibroblast (BHK-21) cells, African green monkey kidney epithelial (Vero) cells were purchased from the American Type Culture Collection (ATCC, Bethesda, MD) and maintained in a high-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (HyClone Laboratories, South Logan, UT) and 1% penicillin/streptomycin (P/S). The NS2A-EGFP BHK-21 cells constitutively expressing the C-terminally EGFP-tagged NS2A protein was maintained in the high-glucose DMEM medium supplemented with 10%FBS, 1% P/S and 1 mg/ml Geneticin. HEK293T cells were grown in low-glucose DMEM (Invitrogen) containing 10% FBS and 1% penicillin-streptomycin. All mammalian cell lines were grown at 37°C with 5% $CO_2$. Mosquito C6/36 cells were grown in RPMI 1640 medium (Invitrogen) with 10% FBS and 1% penicillin-streptomycin at 28°C without $CO_2$. All culture medium and antibiotics were purchased from ThermoFisher Scientific (Waltham, MA). All cell lines were tested negative for mycoplasma.

## Pseudo-infectious Particle

Pseudo-infectious particles were produced using the a previously described protocol [ ADDIN EN.CITE <EndNote><Cite><Author>Qing</Author><Year>2010</Year><RecNum>114</RecNum><DisplayText>(Qing et al., 2010)</DisplayText><record><rec-number>114</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1561745637">114</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Qing, M.</author><author>Liu, W.</author><author>Yuan, Z.</author><author>Gu, F.</author><author>Shi, P. Y.</author></authors></contributors><auth-address>Novartis Institute for Tropical Diseases, 10, Biopolis Road, #05-01 Chromos, Singapore, Singapore.</auth-address><titles><title>A high-throughput assay using dengue-1 virus-like particles for drug discovery</title><secondary-title>Antiviral Res</secondary-title></titles><periodical><full-title>Antiviral Res</full-title></periodical><pages>163-71</pages><volume>86</volume><number>2</number><keywords><keyword>Animals</keyword><keyword>Antiviral Agents/*isolation & purification/*pharmacology</keyword><keyword>Biological Assay/methods</keyword><keyword>Cell Line</keyword><keyword>Cercopithecus



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aethiops</keyword><keyword>Cricetinae</keyword><keyword>Dengue Virus/*drug effects/genetics</keyword><keyword>Drug Discovery/*methods</keyword><keyword>Drug Evaluation, Preclinical/*methods</keyword><keyword>Electroporation</keyword><keyword>Genes, Reporter</keyword><keyword>Luciferases, Renilla/genetics/metabolism</keyword><keyword>Macaca mulatta</keyword><keyword>Viral Proteins/genetics/metabolism</keyword><keyword>Virosomes/*drug effects/genetics</keyword></keywords><dates><year>2010</year><pub-dates><date>May</date></pub-dates></dates><isbn>1872-9096 (Electronic)0166-3542 (Linking)</isbn><accession-num>20153777</accession-num><urls><related-urls><url>https://www.ncbi.nlm.nih.gov/pubmed/20153777</url></related-urls></urls><electronic-resource-num>10.1016/j.antiviral.2010.02.313</electronic-resource-num></record></Cite></EndNote>] with several modifications. 10 µg of replicon RNA was electroporated into BHK-21 cells as described above. After transfection, the cells were maintained at 37°C with 5% $CO_2$ for 24 h. Afterwards, cells were harvested and electroporated with 10 µg of a helper replicon RNA SFV-DENV2 CprME (replicon derived from Semliki forest virus that express the DENV-2 encoding the CprME). After the second electroporation, cells were cultured at 30°C with 5% $CO_2$. At 20, 28 and 48 h after the second electroporation, culture medium was collected and stored at -80 °C. Afterwards, the collected medium was used ton infection naïve Vero cells in 96-well plates. At 48 h post-infection, cells were lysed, and luciferase signals were measured using the Renilla luciferase assay system (Promega) in the Clarity luminescence microplate reader (BioTek) according to the manufacturer's protocol.

## METHOD DETAILS

### Plasmid Construction

The full-length cDNA infectious clone of DENV-2 strain New Guinea C (NGC), pACYC-NGC FL, and its derived replicon (pACYC-NGC replicon) clone [ ADDIN EN.CITE <EndNote><Cite><Author>Xie</Author><Year>2013</Year><RecNum>85</RecNum><DisplayText>(Xie et al., 2013)</DisplayText><record><rec-number>85</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793217">85</key></foreign-keys><ref-type name="Journal



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Article">17</ref-type><contributors><authors><author>Xie, X.</author><author>Gayen, S.</author><author>Kang, C.</author><author>Yuan, Z.</author><author>Shi, P. Y.</author></authors></contributors><auth-address>State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Science, China.</auth-address><titles><title>Membrane topology and function of dengue virus NS2A protein</title><secondary-title>J Virol</secondary-title><alt-title>Journal of virology</alt-title></titles><periodical><full-title>J Virol</full-title></periodical><pages>4609-22</pages><volume>87</volume><number>8</number><edition>2013/02/15</edition><keywords><keyword>Animals</keyword><keyword>Cell Line</keyword><keyword>Cricetinae</keyword><keyword>Dengue Virus/*physiology</keyword><keyword>Endoplasmic Reticulum/*virology</keyword><keyword>Humans</keyword><keyword>Intracellular Membranes/*metabolism</keyword><keyword>Magnetic Resonance Spectroscopy</keyword><keyword>Models, Molecular</keyword><keyword>Protein Conformation</keyword><keyword>Viral Nonstructural Proteins/*chemistry/metabolism</keyword><keyword>*Virus Assembly</keyword></keywords><dates><year>2013</year><pub-dates><date>Apr</date></pub-dates></dates><isbn>1098-5514 (Electronic)0022-538X (Linking)</isbn><accession-num>23408612</accession-num><work-type>Research Support, Non-U.S. Gov't</work-type><urls><related-urls><url>http://www.ncbi.nlm.nih.gov/pubmed/23408612</url></related-urls></urls><custom2>3624351</custom2><electronic-resource-num>10.1128/JVI.02424-12</electronic-resource-num><language>eng</language></record></Cite></EndNote>] were used as the backbones for mutagenesis study. The replicon was constructed by replacing most of the structural genes (C, prM and E) with a gene cassette (Rluc2A) encoding the Renilla luciferase and a 2A peptide from Foot-and-mouth disease virus. The first 38 amino acids of C protein and the last 31 amino acids of E protein were retained in the replicon. Standard overlap PCR was performed to introduce the mutations in C and NS2A genes. The DNA fragments containing the mutations in C gene were cloned into pACYC-NGC FL clone through restriction enzyme sites NotI (upstream of the T7 promoter and 5'UTR of DENV-2) and SphI (at the position of 1384 in DENV-2 genome). The DNA fragments containing mutations in NS2A gene were cloned into pACYC-NGC FL or replicon through restriction enzyme sites and NheI (at the position of 2544 in DENV-2 genome) and SacI (at the position of 4342 in the DENV-2 genome).



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A mammalian expression vector, pXJ [ ADDIN EN.CITE <EndNote><Cite><Author>Xie</Author><Year>2013</Year><RecNum>85</RecNum><DisplayText>(Xie et al., 2013)</DisplayText><record><rec-number>85</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793217">85</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Xie, X.</author><author>Gayen, S.</author><author>Kang, C.</author><author>Yuan, Z.</author><author>Shi, P. Y.</author></authors></contributors><auth-address>State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Science, China.</auth-address><titles><title>Membrane topology and function of dengue virus NS2A protein</title><secondary-title>J Virol</secondary-title><alt-title>Journal of virology</alt-title></titles><periodical><full-title>J Virol</full-title></periodical><pages>4609-22</pages><volume>87</volume><number>8</number><edition>2013/02/15</edition><keywords><keyword>Animals</keyword><keyword>Cell Line</keyword><keyword>Cricetinae</keyword><keyword>Dengue Virus/*physiology</keyword><keyword>Endoplasmic Reticulum/*virology</keyword><keyword>Humans</keyword><keyword>Intracellular Membranes/*metabolism</keyword><keyword>Magnetic Resonance Spectroscopy</keyword><keyword>Models, Molecular</keyword><keyword>Protein Conformation</keyword><keyword>Viral Nonstructural Proteins/*chemistry/metabolism</keyword><keyword>*Virus Assembly</keyword></keywords><dates><year>2013</year><pub-dates><date>Apr</date></pub-dates></dates><isbn>1098-5514 (Electronic)0022-538X (Linking)</isbn><accession-num>23408612</accession-num><work-type>Research Support, Non-U.S. Gov't</work-type><urls><related-urls><url>http://www.ncbi.nlm.nih.gov/pubmed/23408612</url></related-urls></urls><custom2>3624351</custom2><electronic-resource-num>10.1128/JVI.02424-12</electronic-resource-num><language>eng</language></record></Cite></EndNote>], driven by a cytomegalovirus (CMV) promoter was used to express various viral proteins prM, E, NS2A and NS3 fusion proteins in HEK293T cells. For constructs pXJ-$C_{14}$-prM and pXJ-$C_{14}$-prM (1-130), the anchor C signal peptide ($C_{14}$) was retained at the N-terminus of prM to ensure the correct targeting on ER membrane. For construct pXJ-$M_{17}$-E, the last 17 amino acids from M protein was retained to serve as a signal peptide for translocating the E protein into the ER lumen. For constructs pXJ-SPG-$C_{16}$-NS2A-EGFP and pXJ-SPG-$C_{16}$-



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NS2A-GST, the signal peptide derived from Gaussia luciferase (SPG) [ ADDIN EN.CITE <EndNote><Cite><Author>Verhaegent</Author><Year>2002</Year><RecNum>112</RecNum><DisplayText>(Verhaegent and Christopoulos, 2002)</DisplayText><record><rec-number>112</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1561735944">112</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Verhaegent, M.</author><author>Christopoulos, T. K.</author></authors></contributors><auth-address>Department of Chemistry and Biochemistry, University of Windsor, Ontario, Canada.</auth-address><titles><title>Recombinant Gaussia luciferase. Overexpression, purification, and analytical application of a bioluminescent reporter for DNA hybridization</title><secondary-title>Anal Chem</secondary-title></titles><periodical><full-title>Anal Chem</full-title></periodical><pages>4378-85</pages><volume>74</volume><number>17</number><keywords><keyword>Animals</keyword><keyword>DNA/*analysis</keyword><keyword>DNA Probes</keyword><keyword>Genes, Reporter</keyword><keyword>*Luciferases/genetics/isolation & purification</keyword><keyword>Luminescent Proteins/genetics/isolation & purification</keyword><keyword>Plankton/*enzymology</keyword><keyword>Recombinant Proteins/genetics/isolation & purification</keyword></keywords><dates><year>2002</year><pub-dates><date>Sep 1</date></pub-dates></dates><isbn>0003-2700 (Print)0003-2700 (Linking)</isbn><accession-num>12236345</accession-num><urls><related-urls><url>https://www.ncbi.nlm.nih.gov/pubmed/12236345</url></related-urls></urls></record></Cite></EndNote>] and the last 16 amino acids of NS1 ($C_{16}$) was fused at the N-terminus of NS2A to ensure the correct processing and membrane topology of NS2A. The EGFP and GST were amplified from pEGFPluc (Clontech) and pGEX-4T-1 (Sigma-Aldrich) respectively. The Human influenza hemagglutinin (HA) tag was fused to the N-terminus of NS3 and cloned into pXJ vector, resulting the construct pXJ-HA-NS3.

pGEX-4T-1 (GE Healthcare Life Sciences) was used as the vector for expressing all N-terminally glutathione S-transferase (GST)-tagged NS2A fusion proteins. The DNA fragments containing various length of NS2A were cloned into pGEX-4T-1 through restriction enzyme sites BamHI and XhoI.



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All plasmids were propagated in *E. coli* strain Top10 (ThermoFisher Scientific). All restriction enzymes were purchased from New England BioLabs (Ipswitch, MA). The plasmids were validated by restriction enzyme digestion and Sanger DNA sequencing. All primers were synthesized from Integrated DNA Technologies (Skokie, Illinois) and are available upon request.

**RNA Transcription and Electroporation**

The NGC FL or replicon plasmids were linearized by restriction enzyme XbaI. RNA transcription and electroporation were performed as described previously [ ADDIN EN.CITE <EndNote><Cite><Author>Xie</Author><Year>2013</Year><RecNum>85</RecNum><DisplayText>(Xie et al., 2013)</DisplayText><record><rec-number>85</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793217">85</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Xie, X.</author><author>Gayen, S.</author><author>Kang, C.</author><author>Yuan, Z.</author><author>Shi, P. Y.</author></authors></contributors><auth-address>State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Science, China.</auth-address><titles><title>Membrane topology and function of dengue virus NS2A protein</title><secondary-title>J Virol</secondary-title><alt-title>Journal of virology</alt-title></titles><periodical><full-title>J Virol</full-title></periodical><pages>4609-22</pages><volume>87</volume><number>8</number><edition>2013/02/15</edition><keywords><keyword>Animals</keyword><keyword>Cell Line</keyword><keyword>Cricetinae</keyword><keyword>Dengue Virus/*physiology</keyword><keyword>Endoplasmic Reticulum/*virology</keyword><keyword>Humans</keyword><keyword>Intracellular Membranes/*metabolism</keyword><keyword>Magnetic Resonance Spectroscopy</keyword><keyword>Models, Molecular</keyword><keyword>Protein Conformation</keyword><keyword>Viral Nonstructural Proteins/*chemistry/metabolism</keyword><keyword>*Virus Assembly</keyword></keywords><dates><year>2013</year><pub-dates><date>Apr</date></pub-dates></dates><isbn>1098-5514 (Electronic)0022-538X (Linking)</isbn><accession-num>23408612</accession-num><work-type>Research Support, Non-U.S. Gov't</work-type><urls><related-



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urls><url>http://www.ncbi.nlm.nih.gov/pubmed/23408612</url></related-urls></urls><custom2>3624351</custom2><electronic-resource-num>10.1128/JVI.02424-12</electronic-resource-num><language>eng</language></record></Cite></EndNote>]. Briefly, 10 μg RNA was electroporated into 8 × $10^6$ cells in a 4-mm cuvette using the GenePulser apparatus (Bio-Rad) at settings of 0.45 kV (C6/36 cells) or 0.85 kV (BHK-21 cells) and 25 mF, pulsing three times, with 3 s intervals. After a 10-min recovery at room temperature, transfected cells were suspended in culture media and incubated at corresponding conditions: 28°C (C6/36 cells) or 37°C with 5% $CO_2$ (BHK-21).

**Virus Production and Quantification**

After electroporation, the transfected BHK-21 cells were seeded in one T-75 flask. After incubation at 37°C for 24 h, the cells were cultured in DMEM with 2% FBS at 30°C for another 4 days. At every 24 h p.t., culture fluids was collected and stored at -80°C. Viral titer was determined by crystal violet-based plaque assay using BHK-21 cells according to the protocol as described previously [ ADDIN EN.CITE <EndNote><Cite><Author>Xie</Author><Year>2013</Year><RecNum>85</RecNum><DisplayText>(Xie et al., 2013)</DisplayText><record><rec-number>85</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793217">85</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Xie, X.</author><author>Gayen, S.</author><author>Kang, C.</author><author>Yuan, Z.</author><author>Shi, P. Y.</author></authors></contributors><auth-address>State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Science, China.</auth-address><titles><title>Membrane topology and function of dengue virus NS2A protein</title><secondary-title>J Virol</secondary-title><alt-title>Journal of virology</alt-title></titles><periodical><full-title>J Virol</full-title></periodical><pages>4609-22</pages><volume>87</volume><number>8</number><edition>2013/02/15</edition><keywords><keyword>Animals</keyword><keyword>Cell Line</keyword><keyword>Cricetinae</keyword><keyword>Dengue Virus/*physiology</keyword><keyword>Endoplasmic Reticulum/*virology</keyword><keyword>Humans</keyword><keyword>Intracellular Membranes/*metabolism</keyword><keyword>Magnetic Resonance Spectroscopy</keyword><keyword>Models, Molecular</keyword><keyword>Protein Conformation</keyword><keyword>Viral Nonstructural



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1507 Proteins/*chemistry/metabolism</keyword><keyword>*Virus
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1512 urls><url>http://www.ncbi.nlm.nih.gov/pubmed/23408612</url></related-
1513 urls></urls><custom2>3624351</custom2><electronic-resource-num>10.1128/JVI.02424-
1514 12</electronic-resource-num><language>eng</language></record></Cite></EndNote>].
1515 Plaques were developed on day 5 post-infection.

1516 **RNA extraction and RT-PCR**

1517 Viral RNAs in culture fluids were extracted using a QIAamp viral RNA minikit (Qiagen).
1518 Extracted RNAs were eluted in 50 μl RNase-free water. Reverse transcription (RT)-qPCR
1519 was performed to measure the amounts of viral RNA copy in the culture medium. Viral
1520 RNAs were measured using an iScript one-Step RT-PCR kit with SYBR green (Bio-Rad)
1521 and the primer pair NGC 7764V (sequence: 5'-CGTCGAGAGAAATATGGTCACACC-3')
1522 and 7844C (sequence: 5'CCACAATAGTATGACCAGCCT-3') [ ADDIN EN.CITE
1523 <EndNote><Cite><Author>Xie</Author><Year>2013</Year><RecNum>85</RecNum><Di
1524 splayText>(Xie et al., 2013)</DisplayText><record><rec-number>85</rec-
1525 number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv"
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1527 Article">17</ref-type><contributors><authors><author>Xie, X.</author><author>Gayen,
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1531 address><titles><title>Membrane topology and function of dengue virus NS2A
1532 protein</title><secondary-title>J Virol</secondary-title><alt-title>Journal of virology</alt-
1533 title></titles><periodical><full-title>J Virol</full-title></periodical><pages>4609-
1534 22</pages><volume>87</volume><number>8</number><edition>2013/02/15</edition><k
1535 eywords><keyword>Animals</keyword><keyword>Cell
1536 Line</keyword><keyword>Cricetinae</keyword><keyword>Dengue
1537 Virus/*physiology</keyword><keyword>Endoplasmic
1538 Reticulum/*virology</keyword><keyword>Humans</keyword><keyword>Intracellular
1539 Membranes/*metabolism</keyword><keyword>Magnetic Resonance



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Spectroscopy</keyword><keyword>Models, Molecular</keyword><keyword>Protein Conformation</keyword><keyword>Viral Nonstructural Proteins/*chemistry/metabolism</keyword><keyword>*Virus Assembly</keyword></keywords><dates><year>2013</year><pub-dates><date>Apr</date></pub-dates></dates><isbn>1098-5514 (Electronic)0022-538X (Linking)</isbn><accession-num>23408612</accession-num><work-type>Research Support, Non-U.S. Gov't</work-type><urls><related-urls><url>http://www.ncbi.nlm.nih.gov/pubmed/23408612</url></related-urls></urls><custom2>3624351</custom2><electronic-resource-num>10.1128/JVI.02424-12</electronic-resource-num><language>eng</language></record></Cite></EndNote>]. Alternatively, the extracted viral RNA was reversely transcribed and amplified using SuperScript One-Step (RT)-PCR with Platinum Taq (Invitrogen). The RT-PCR products were then gel purified and subjected to Sanger DNA sequencing.

**Replicon Transient Transfection**

After electroporation of replicon RNA into BHK-21 cells, the cells were resuspended in culture medium and seeded in a 12-well plate ($2 \times 10^5$ cells/well). At various time points, the cells were washed once with phosphate-buffered saline (PBS) and lysed using lysis buffer (Promega). The plates containing the lysis buffer were sealed with Parafilm and stored at -80°C. Once samples for all time points had been collected, luciferase signals were measured in a Clarity luminescence microplate reader (BioTek) according to the manufacturer's protocol. The luciferase signals at early time points (2-6 h post-transfection) represent the translation of input viral RNA; and the luciferase signals at later points (24-46 h post-transfection) represent the stage of RNA replication.

**Immunofluorescence assay (IFA)**

Cells were seeded in an 8-well Lab-Tek II chamber slide (ThermoFisher Scientific). At given time points, cells were fixed in 4% paraformaldehyde in PBS for 20 min at room temperature. Afterwards, cells were permeabilized by 0.1% Triton X-100 (Sigma-Aldrich) for 5 min followed by 1-h incubation in blocking buffer containing 1% FBS and 0.05% Tween-20 (Sigma-Aldrich) in PBS. Cells were then incubated with primary antibodies (diluted in the blocking buffer) at room temperature for 1 h. After three PBS washes, cells were incubated with goat anti-mouse or rabbit IgG (1:1000 diluted in blocking buffer) conjugated with Alexa Fluor 488 or 594 (ThermoFisher Scientific) for 1 h in blocking buffer.



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Cells were finally washed by PBS for three times, counter-stained by Hoechst 33342 Solution (ThermoFisher Scientific) and mounted in ProLong® Diamond Antifade Mountant (ThermoFisher Scientific). Epifluorescence images were acquired by Leica DM4000 B system. Confocal fluorescence images were acquired by Zeiss LSM 5 META systems.

**Virus Revertant Selection**

For selecting revertants from G11A-transfected cells, we consecutively passaged the transfected cells for 5 rounds. In brief, after electroporation, the cells were seeded in 96-well plates and incubate at 30°C for 3 days. Afterwards, cells for each well were detached by trypsin and suspended in culture medium. About 20% of the suspended cells were transferred into the well of new 96-well plates and incubated at 37°C for one day. The cells were then incubated at 30°C for two additional days. Again, the confluent cells were detached for next passage. At every passage, the supernatants were collected. After 5 rounds of passaging, IFA was performed to determine the intracellular E protein expression by 4G2 antibody. Plaque assay was performed to examine the amounts of infectious virus particle in the culture fluids. Viral RNA from the P5 supernatants were extracted and used as template for RT-PCR. Sanger sequencing was performed to sequence the entire viral genome.

**Co-immunoprecipitation (Co-IP)**

The NS2A/prM, NS2A/E and NS2A/NS3 interactions were examined by Co-IP analysis according to the protocol as described previously[ ADDIN EN.CITE <EndNote><Cite><Author>Zou</Author><Year>2014</Year><RecNum>113</RecNum><DisplayText>(Zou et al., 2014)</DisplayText><record><rec-number>113</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1561742592">113</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Zou, J.</author><author>Xie, X.</author><author>Lee le, T.</author><author>Chandrasekaran, R.</author><author>Reynaud, A.</author><author>Yap, L.</author><author>Wang, Q. Y.</author><author>Dong, H.</author><author>Kang, C.</author><author>Yuan, Z.</author><author>Lescar, J.</author><author>Shi, P. Y.</author></authors></contributors><auth-address>State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Sciences, Wuhan, China.</auth-address><titles><title>Dimerization of flavivirus NS4B protein</title><secondary-title>J



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Virol</secondary-title></titles><periodical><full-title>J Virol</full-title></periodical><pages>3379-91</pages><volume>88</volume><number>6</number><keywords><keyword>Amino Acid Motifs</keyword><keyword>Dengue/*virology</keyword><keyword>Dengue Virus/chemistry/genetics/*metabolism</keyword><keyword>Dimerization</keyword><keyword>Humans</keyword><keyword>Viral Nonstructural Proteins/*chemistry/genetics/*metabolism</keyword><keyword>West Nile Fever/*virology</keyword><keyword>West Nile virus/chemistry/genetics/*metabolism</keyword></keywords><dates><year>2014</year><pub-dates><date>Mar</date></pub-dates></dates><isbn>1098-5514 (Electronic)0022-538X (Linking)</isbn><accession-num>24390334</accession-num><urls><related-urls><url>https://www.ncbi.nlm.nih.gov/pubmed/24390334</url></related-urls></urls><custom2>PMC3957939</custom2><electronic-resource-num>10.1128/JVI.02782-13</electronic-resource-num></record></Cite></EndNote>].
Various combinations of plasmids were transfected into HEK293T cells in 10-cm dishes using the X-tremeGENE 9 DNA transfection reagent (Roche). At 40-42 h post-transfection, cells were lysed in 1 ml immunoprecipitation (IP) buffer (20 mM Tris, pH 7.5, 100 mM NaCl, 0.5% DDM, and EDTA-free protease inhibitor cocktail [Roche]) with rotation at 4°C for 1 h. Lysates were clarified by centrifugation at 20,000 × g and 4°C for 30 min and subjected to co-IP using protein G-conjugated magnetic beads according to the manufacturer's instructions (Millipore). Briefly, immune complexes were formed at 4°C overnight by mixing 200 µl of cell lysate with 2 µg corresponding antibodies in a 500-µl reaction system containing 400 mM sodium chloride. Subsequently, the complexes were precipitated with protein G-conjugated magnetic beads at 4°C for 1 h with rotation, followed by washing extensively with phosphate-buffered saline (PBS) containing 0.1% Tween-20. Finally, proteins were eluted with 4× lithium dodecyl sulfate (LDS) sample buffer (ThermoFisher Scientific) supplemented with 100 mM 1,4-dithiothreitol (DTT), heated at 70°C for 10 min, and analyzed by Western blot.

**SDS-PAGE and Western Blot**

Protein samples were mixed with 4×LDS sample buffer (ThermoFisher Scientific). After denaturing at 70°C for 10 min, 10-30 µl samples were loaded onto to a 12% or 15% SDS-PAGE gel (Bio-Rad Laboratories). After electrophoresis, proteins were resolved and



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transferred onto a polyvinylidene difluoride (PVDF) membrane using the Bio-Rad TransBlot Turbo Blotting System. Blots were soaked for 1 hour in a blocking buffer containing TBST (10 mM Tris-HCl [pH 7.5], 150 mM NaCl, and 0.1% Tween-20) and 5% skim milk, followed by 1 h incubation with primary antibodies (diluted in blocking buffer). After three washes with TBST buffer, blots were incubated with goat anti-mouse or rabbit antibodies conjugated to HRP (1:10,000 diluted in blocking buffer). After three thorough washes with TBST buffer, blots were incubated with the ECL substrates (GE Healthcare). Chemiluminescence signals were detected in ChemiDoc System (Bio-Rad).

**Transmission Electron Microscopy**

Viral genomic RNAs were electroporated into BHK-21 cells as described above. At 48 h post-transfection, the cells were fixed for at least 1 h in a mixture of 2.5% formaldehyde (prepared from paraformaldehyde powder) and 0.1% glutaraldehyde in 0.05M cacodylate buffer (pH 7.3) to which 0.03% picric acid and 0.03% $CaCl_2$ were added. After washing in 0.1 M cacodylate buffer, the cells were scraped off and processed further as a pellet. The pellets were post-fixed in 1% OsO4 in 0.1M cacodylate buffer (pH 7.3) for 1 h, washed with distilled water and *en bloc* stained with 2% aqueous uranyl acetate for 20 min at 60°C. The pellets were dehydrated in ethanol, processed through propylene oxide and embedded in Poly/Bed 812 (Polysciences, Warrington, PA). Ultrathin sections were cut on Leica EM UC7 ultramicrotome (Leica Microsystems, Buffalo Grove, IL), stained with lead citrate and examined in a Philips 201 transmission electron microscope at 60 kV.

**Expression and purification of recombinant NS2A proteins**

The GST-tagged DENV-2 NGC NS2A and its truncates was expressed and purified by following a previously described protocol [ ADDIN EN.CITE <EndNote><Cite><Author>Zou</Author><Year>2014</Year><RecNum>113</RecNum><DisplayText>(Zou et al., 2014)</DisplayText><record><rec-number>113</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1561742592">113</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Zou, J.</author><author>Xie, X.</author><author>Lee le, T.</author><author>Chandrasekaran, R.</author><author>Reynaud, A.</author><author>Yap, L.</author><author>Wang, Q. Y.</author><author>Dong, H.</author><author>Kang, C.</author><author>Yuan, Z.</author><author>Lescar, J.</author><author>Shi, P.



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Y.</author></authors></contributors><auth-address>State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Sciences, Wuhan, China.</auth-address><titles><title>Dimerization of flavivirus NS4B protein</title><secondary-title>J Virol</secondary-title></titles><periodical><full-title>J Virol</full-title></periodical><pages>3379-91</pages><volume>88</volume><number>6</number><keywords><keyword>Amino Acid Motifs</keyword><keyword>Dengue/*virology</keyword><keyword>Dengue Virus/chemistry/genetics/*metabolism</keyword><keyword>Dimerization</keyword><keyword>Humans</keyword><keyword>Viral Nonstructural Proteins/*chemistry/genetics/*metabolism</keyword><keyword>West Nile Fever/*virology</keyword><keyword>West Nile virus/chemistry/genetics/*metabolism</keyword></keywords><dates><year>2014</year><pub-dates><date>Mar</date></pub-dates></dates><isbn>1098-5514 (Electronic)0022-538X (Linking)</isbn><accession-num>24390334</accession-num><urls><related-urls><url>https://www.ncbi.nlm.nih.gov/pubmed/24390334</url></related-urls></urls><custom2>PMC3957939</custom2><electronic-resource-num>10.1128/JVI.02782-13</electronic-resource-num></record></Cite></EndNote>] with modifications. The pGEX-4T GST-NS2A plasmids were transformed into *E. coli* strain Rosetta™ 2 (pLysS) (Novagen) cells. Bacteria culture was grown in Tefferic broth containing 50 µg/ml Kanamycin and 34 µg/ml Chloramphenicol. When the cells reached an optical density at 600 nm (OD600) of 0.8-1.0, isopropyl β-d-1-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM. After growth for 16 h at 18°C, the cells were harvested, resuspended in a lysis buffer (10 mM $Na_2HPO_4$, 1.8 mM $KH_2PO_4$, 2.7 mM KCl, 400 mM NaCl, 1 mM EDTA, 0.5× Roche protease inhibitor cocktail, pH7.5) and disrupted by sonication using a Digital Sonifier 450 (Branson). The cell debris was removed by centrifugation at 10,000 rpm for 10 min at 4°C. The supernatants were collected and pelleted down by centrifugation at 45,000 rpm for 1 h at 4°C using a Beckman type 70 Ti rotor. The pellets containing the membrane fractions were collected and solubilized in the lysis buffer supplemented with 1.5% n-Dodecyl β-D-maltoside (DDM; Anatrace) at 4°C for 2 hours. Following another centrifugation step at 45,000 rpm for 1 h, the supernatants were collected and loaded onto a GSTrap HP Fast Flow column (GE Healthcare) which was preequilibrated with buffer A (10 mM $Na_2HPO_4$, 1.8 mM $KH_2PO_4$, 2.7 mM KCl, 400 mM NaCl, 2 mM tris(2-carboxyethyl) phosphine [TCEP], 0.05% DDM, pH



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7.5). After extensively wash with buffer B (10 mM $Na_2HPO_4$, 1.8 mM $KH_2PO_4$, 2.7 mM KCl, 1M NaCl, 2 mM tris(2-carboxyethyl) phosphine [TCEP], 0.05% DDM, pH 7.5), the protein was eluted in buffer A supplied with 20 mM glutathione. The fractions containing GST-NS2A proteins were pooled, concentrated, and subjected to gel filtration using a HiLoad Superdex 200 10/300 column (GE Healthcare) in buffer C (10 mM $Na_2HPO_4$, 1.8 mM $KH_2PO_4$, 2.7 mM KCl, 150 mM NaCl, 2 mM tris(2-carboxyethyl) phosphine [TCEP], 0.05% DDM, pH 7.5) with 5% glycerol. The peak fractions containing GST-NS2A proteins were pooled and concentrated before storage at -80°C.

**RNA Pull-down Assay**

The 5'UTR-$C_{66}$-3'UTR-biotin RNA was prepared by ligating a single biotinylated nucleotide to the 3' terminus of the RNA 5'UTR-$C_{66}$-3'UTR using the Pierce™ RNA 3' End Biotinylation Kit (ThermoFisher Scientific) according to the manufacturer's instructions. The RNA 5'UTR-$C_{66}$-3'UTR was *in vitro* transcribed from a PCR template using a T7 MEGAscript kit (ThermoFisher Scientific). The PCR template contained a T7 promoter, followed by a cDNA fragment representing a DENV-2 subgenome with a deletion from nucleotides 163 to 10269 (GenBank accession number AF038403). After *in vitro* transcription, the products were passed twice through the Microspin™ G-25 Columns (GE Healthcare Life Sciences) to remove excessive UTPs. The final products were subjected to phenol-chloroform extraction followed by alcohol precipitation. The purified RNAs were resuspended in nuclease-free water prior to storage at -80°C. Other shorter RNAs 3'UTR, Δpk1, 5'UTR-$C_{66}$, Δpk(1-2), Δpk(1-3), 3'SL, pk(3-4) and pk3 were *in vitro* synthesized from corresponding PCR templates using the same method.

RNA pull-down experiments were performed using the Pierce™ Magnetic RNA-Protein Pull-Down Kit (ThermoFisher Scientific) according to the manufacturer's instructions. Briefly, 2 µg of biotin-labeled RNAs were pre-captured by 50 µl Streptavidin magnetic beads in 1 × RNA capture buffer (10mL, 20mM Tris (pH 7.5), 1M NaCl, 1mM EDTA) at room temperature for 20 min with agitation. A 28-mer biotinylated IRE RNA (5'-UCCUGCUUCAACAGUGCUUGGACGGAAC-3'-biotin) from the Pierce™ RNA 3' End Biotinylation Kit was used as a control. The RNA-bound beads were then mixed with 2 µg purified GST-tagged NS2A proteins in a 100-µl reaction system containing 20 mM Tris (pH 7.5), 200 mM NaCl, 2 mM $MgCl_2$, 0.1% Tween-20 and 15% glycerol and incubated for 45 minutes at 4°C with agitation. For RNA competition assay, the various amounts of



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competitors (tRNA and other shorter RNAs from DENV-2 genome) were immediately added to above the mixtures containing RNA-bound beads and GST-NS2A proteins. After 5 times of wash by 20 mM Tris (pH 7.5), 10 mM NaCl, 0.1% Tween-20 Detergent, the proteins were eluted in 30 µl biotin elution buffer. The eluates were mixed with 4×LDS sample buffer and heated at 70°C for 10 min. 10 µl protein samples were loaded onto a 12% SDS-PAGE gel and followed by detection by Western blot using rabbit anti-GST antibodies.

**Electrophoretic Mobility-Shift Assay**

Electrophoretic mobility-shift assay (EMSA) was performed to estimate the binding affinity of DENV-2 5'UTR-$C_{66}$-3'UTR RNA to GST-NS2A proteins. 100 pM 5'UTR-$C_{66}$-3'UTR-biotin RNA was incubated with a serial concentration of recombinant GST-NS2A protein (0-250 nM) in a 50-µl reaction system containing 50mM Tris-HCl, pH 7.5, 10 mM 2-mercaptoethanol, 2 mM $MgCl_2$, 50 mM KCl at 24°C for 15 min. Afterwards, 10 µl 6x Gel Loading Dye without SDS (New England Biolabs Inc.) were added to stop the reaction. Sample were then loaded onto a 0.4% agarose gel (prepared in 0.5× Tris/Borate/EDTA[TBE]) to resolve the RNA-protein complexes and free RNAs by electrophoresis in 1× TBE buffer (120 V for 30 min) at room temperature. After electrophoresis, RNAs were transferred from the agarose gel to a BrightStar-Plus positively charged nylon membrane (ThermoFisher Scientific) according to the procedure as described in the manual of NorthernMax Gly Kit (ThermoFisher Scientific). The nylon membrane was irradiated with 200 mJ/$cm^2$ of Ultraviolet (UV) light at 254 nm in a CX-2000 UV crosslinker (Analytic Jene, CA) with default settings. The biotinylated RNA was finally detected by the Streptavidin-horseradish peroxidase conjugate by using the Chemiluminescent Detection Module (ThermoFisher Scientific). Chemiluminescent signals were detected in the ChemiDoc™ Imaging Systems (Bio-rad).

**PAR-CLIP**

The Photoactivatable-Ribonucleoside-Enhanced Crosslinking and Immunoprecipitation (PAR-CLIP) was performed according to the protocol as described previously [ ADDIN EN.CITE ADDIN EN.CITE.DATA ] with modifications. In brief, 4-thiouridine labelled DENV-2 3'UTR RNA was prepared by adding a final concentration 0.75 mM of 4-Thio-UTP (TriLink BioTechnologies) to a 20-µl *in vitro* transcription reaction (4-Thio-UTR to UTP



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ration is 1:10) using the MEGAscript T7 transcription Kit (Thermo Fisher Scientific). After 2 hours of reaction at 37°C, the reaction was stopped and the 4-thiouridine labelled RNAs were purified and resuspended in nuclease-free water. 15 µg of 4-thiouridine labelled DENV-2 3'UTR RNA was incubated with 6 µg recombinant DENV-2 GST-NS2A protein in a 100-µl reaction system containing 50 mM Tris-HCl, pH 7.5, 2 mM $MgCl_2$, 50 mM KCl and 5% glycerol at 24°C for 15 min. The RNA-protein mixtures were then irradiated on ice with 150 mJ/cm$^2$ at UV light at 365 nm. An aliquot of the same reaction without UV irradiation was used as the uncross-linked control. Prior to immunoprecipitation, 30 µl of PureProteome™ Protein G Magnetic Beads (MilliporeSigma) were washed twice with 1 ml citrate-phosphate buffer (4.7 g/L citric acid, 9.2 g/L $Na_2HPO_4$, pH 5.0), resuspended in 100 µl citrate-phosphate buffer and followed by in addition of 1 µg of rabbit anti-GST polyclonal antibody (Abcam). The bead-antibody mixtures were incubated at 4°C for 45 min. After two washed with citrate-phosphate buffer, the antibody-bound beads were resuspended in 30 µl of citrate-phosphate buffer. Upon immunoprecipitation, the RNA-protein mixtures were mixed with antibody-bound beads and incubated at 4°C for 1 h. Afterwards, the beads were subjected to three washes in buffer I (50mM HEPES-KOH, pH 7.5, 300 mM KCl, 0.05% IGEPAL CA-630L[Sigma-Aldrich] and 0.5 mM DTT) followed by two washes in buffer II (50 mM HEPES-KOH, pH 7.5, 500 KCl, 0.05% IGEPAL CA-630 and 0.5 mM DTT). The beads were then resuspended in 100 µl Proteinase K digestion buffer (100 mM Tris, pH 7.5, 50 mM NaCl, 10 mM EDTA and 1%SDS) supplemented with proteinase K (New England BioLabs) at a final concentration of 2 mg/ml. The reaction was placed into the Eppendorf ThermoMixer® C and incubated at 37°C for 1 h with agitation at 1100 rpm. Thereafter, the RNA in the reaction was extracted by phenol-chloroform, alcohol-precipitated, and resuspended in 20 µl of nuclease-free water. The extracted RNA was subjected to reversely transcribed and followed by amplification (4 PCR-cycles) using the SuperScript® III One-Step RT-PCR System (ThermoFisher Scientific) with a pair of specific primers P1 (sequence: 5'-ACACTCTTTCCCTACACGA CGCTCTTCCGATCTAAGGCAAAACTAACATGAAAC-3'; underline indicates the sequence identical to the sequence at the 5' end of the DENV-2 3'UTR) and P2 (sequence: 5'-GTGACTGGAGTTCAGACG TGTGCTCTTCCGATCTAGAACCTGTTGATTCAACAGCAC-3'; underline indicates the sequence complimentary to the sequence at the 3' end of the DENV-2 3'UTR) targeting the DENV-2 3'UTR. Afterwards, the RT-PCR product was cleaned up using the QIAquick PCR Purification Kit (Qiagen) and subjected to another round of amplification (15 PCR-



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cycles) using the KAPA HiFi PCR Kit (Kapa Biosystems) with a pair of Illumina adapter/index primers (5' universal adaptor sequence: 5'-AATGATACGGCGAC CACCGAGATCTACACTCTTTCCCTACACGAC-3'; and 3' indexing primer sequence: 5'-CAAGCAGAAGACGGCATACGAGATnnnnnnGTGACTGGAGTTCAGACGTGT-3'. The "nnnnnn" here indicates the TruSeq index sequence). Thereafter, the PCR product was cleaned up using the QIAquick PCR Purification Kit (Qiagen) and followed by separation on 2% E-Gel™ EX Agarose Gels (ThermoFisher Scientific). The cDNAs fragment between 500 to 650 bp was excised, purified using the Zymo Research Gel DNA Recovery Kit (Zymo Research) and resuspended in nuclease-free water. The final cDNA libraries were quantified using a Qubit dsDNA Assay Kit (Invitrogen) and sequenced using a MiSeq Reagent Kit v3 (600-cycle) (illumina).

## QUANTIFICATION AND STATISTICAL ANALYSIS

### Sequence Alignment, Image Process and Densitometry Analysis

The amino acid sequences of flavivirus NS2A proteins were download from GenBank. Sequence alignment was performed with default settings using the CLC main workbench software (Qiagen). IFA images processing and cell counting was performed in software ImageJ (National Institutes of Health, NIH). At least four different IFA images were used to estimate the mean and standard deviations of percentage of viral protein staining positive-cells in Figure 2G and S2. Densitometry analysis was performed using Image lab version 6.0 (Bio-Rad Laboratories, Hercules, CA). All numerical data are presented as the mean ± SD (standard deviations). Group comparisons were performed using the unpaired parametric t-test in software Prism 8.0 (GraphPad). *$p<0.05$, significant; **$p<0.01$, very significant; ***$p<0.001$, highly significant; n.s., not significant. Images were assembled using the software Adobe illustrator CC.

### Calculating the equilibrium dissociation constant (Kd)

The intensities of bands representing the RNA/GST-NS2A complexes and free RNA in Figure 5E were quantitated by software ImageJ (NIH). The ratio of bound RNA (in the RNA/GST-NS2A complexes) to total RNA (free and bound RNA) in each lane was calculated. The ratio of bound RNA to total RNA (y-axis) versus the concentration of GST-NS2A protein (x-axis) was plotted using the software Prism 8 (Graphad). The value of Kd



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was obtained using a nonlinear regression with least-squares fitting method, where the Bmax was set as 1. All data points come from three independent experiments.

**Illumina sequencing data process and analysis**

The indexed sequencing reads of both forward and reverse strands were proceeded with the following bioinformatic pipelines: first, an initial quality filtering were conducted with Fastp [ ADDIN EN.CITE <EndNote><Cite><Author>Chen</Author><Year>2018</Year><RecNum>115</RecNum><DisplayText>(Chen et al., 2018)</DisplayText><record><rec-number>115</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1562011493">115</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Chen, S.</author><author>Zhou, Y.</author><author>Chen, Y.</author><author>Gu, J.</author></authors><translated-authors><author>Bioinformatics,</author></translated-authors></contributors><auth-address>Department of Bioinformatics, HaploX Biotechnology, Shenzhen, China.Shenzhen Institutes of Advanced Technology, Chinese Academy of Sciences, Shenzhen, China.</auth-address><titles><title>fastp: an ultra-fast all-in-one FASTQ preprocessor</title><secondary-title>Bioinformatics</secondary-title><alt-title>Bioinformatics</alt-title></titles><periodical><full-title>Bioinformatics</full-title><abbr-1>Bioinformatics</abbr-1></periodical><alt-periodical><full-title>Bioinformatics</full-title><abbr-1>Bioinformatics</abbr-1></alt-periodical><pages>i884-i890</pages><volume>34</volume><number>17</number><dates><year>2018</year><pub-dates><date>Sep 1</date></pub-dates></dates><isbn>1367-4811 (Electronic)1367-4803 (Linking)</isbn><accession-num>30423086</accession-num><urls><related-urls><url>http://www.ncbi.nlm.nih.gov/pubmed/30423086</url></related-urls></urls><custom2>6129281</custom2><electronic-resource-num>10.1093/bioinformatics/bty560</electronic-resource-num><remote-database-provider>2018 Sep 1</remote-database-provider><language>eng</language></record></Cite></EndNote>] to remove bad reads (too low in quality score, too short, too many “N”, etc.). Then, the illumina sequencing adapter sequence “AGATCGGAAGAGC” was trimmed with cutadapt [ ADDIN EN.CITE <EndNote><Cite><Author>Martin</Author><Year>2011</Year><RecNum>116</RecNum><DisplayText>(Martin, 2011)</DisplayText><record><rec-number>116</rec-



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number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1562011493">116</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Martin, Marcel</author></authors></contributors><titles><title>Cutadapt removes adapter sequences from high-throughput sequencing reads</title><secondary-title>EMBnet.journal; Vol 17, No 1: Next Generation Sequencing Data AnalysisDO - 10.14806/ej.17.1.200</secondary-title></titles><periodical><full-title>EMBnet.journal; Vol 17, No 1: Next Generation Sequencing Data AnalysisDO - 10.14806/ej.17.1.200</full-title></periodical><keywords><keyword>next generation sequencing, small RNA, microRNA, adapter removal</keyword></keywords><dates><year>2011</year><pub-dates><date>05/02/</date></pub-dates></dates><urls><related-urls><url>https://journal.embnet.org/index.php/embnetjournal/article/view/200/458</url></related-urls></urls></record></Cite></EndNote>] (command line parameters: -b AGATCGGAAGAGC -m 40). A secondary quality filter was applied with FASTX toolkit ([ HYPERLINK "http://hannonlab.cshl.edu/fastx_toolkit/index.html" ]) to remove any reads that contained more than 4% nucleotides with a PHRED score <20 (command line parameters: fastq_quality_filter -Q33 -q 20 -p 96). The remaining reads were aligned to 3'UTR of DENV genome (GenBank accession number AF038403), with Bowtie2 [ ADDIN EN.CITE <EndNote><Cite><Author>Langmead</Author><Year>2012</Year><RecNum>117</RecNum><DisplayText>(Langmead and Salzberg, 2012)</DisplayText><record><rec-number>117</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1562011493">117</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Langmead, Ben</author><author>Salzberg, Steven L.</author></authors></contributors><titles><title>Fast gapped-read alignment with Bowtie 2</title><secondary-title>Nature Methods</secondary-title></titles><periodical><full-title>Nature Methods</full-title></periodical><pages>357</pages><volume>9</volume><dates><year>2012</year><pub-dates><date>03/04/online</date></pub-dates></dates><publisher>Nature Publishing Group, a division of Macmillan Publishers Limited. All Rights Reserved.</publisher><urls><related-urls><url>https://doi.org/10.1038/nmeth.1923</url></related-urls></urls><electronic-resource-num>10.1038/nmeth.1923</electronic-resource-



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num></record></Cite></EndNote>] (command line parameters: --local). Using SAMtools [ ADDIN EN.CITE <EndNote><Cite><Author>Li</Author><RecNum>118</RecNum><DisplayText>(Li et al.)</DisplayText><record><rec-number>118</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1562011493">118</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Li, H.</author><author>Handsaker B Fau - Wysoker, Alec</author><author>Wysoker A Fau - Fennell, Tim</author><author>Fennell T Fau - Ruan, Jue</author><author>Ruan J Fau - Homer, Nils</author><author>Homer N Fau - Marth, Gabor</author><author>Marth G Fau - Abecasis, Goncalo</author><author>Abecasis G Fau - Durbin, Richard</author><author>Durbin, R.</author></authors><translated-authors><author>Bioinformatics,</author></translated-authors></contributors><auth-address>Wellcome Trust Sanger Institute, Wellcome Trust Genome Campus, Cambridge, CB10 1SA, UK, Broad Institute of MIT and Harvard, Cambridge, MA 02141, USA. FAU - Handsaker, Bob</auth-address><titles><title>The Sequence Alignment/Map format and SAMtools</title></titles><number>1367-4811 (Electronic)</number><dates></dates><call-num>1000 Genome Project Data Processing Subgroup</call-num><urls></urls><remote-database-provider>2009 Aug 15</remote-database-provider><language>eng</language></record></Cite></EndNote>], the aligned forward and reverse reads were binarily converted, merged, indexed, sorted, and mathematically noted (command line parameters: view -buS, merge, sort, index, mpileup). This is followed by extracting and calculating the T/U-to-C mutation rate at each genome position and the mutation frequencies of each of the four nucleotides.

## DATA AND SOFTWARE AVAILABILITY

Illumina sequencing data access. The raw data of illumine sequencing data can be accessed, queried and downloaded using the following link: .



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**From**: Popov, Vsevolod [/O=EXCHANGELABS/OU=EXCHANGE ADMINISTRATIVE GROUP (FYDIBOHF23SPDLT)/CN=RECIPIENTS/CN=DC1D76129A3C488A962774D19C013230-POPOV, VSEV]
**Sent**: 7/3/2019 10:01:03 AM
**To**: Xie, Xuping [xuxie@UTMB.EDU]
**Subject**: RE: Review MS

Hi, Xuping,
Your EMs look convincing to me. Interesting double membrane vesicles in WT though I do not see any ribosomes at their surface. Are they a different compartment from granular ER? With HepC virus they assign important role to DMVs in viral replication. Ve in center left is in ER with ribosomes at its surface. In G11A you mention "membrane tubes" (T). What is their function? Has it to be discussed? Can some vesicles be just cross sections of the tubes? In some flaviviruses grown in insect cells I observed very long tubes, up to 1-2 micrometers.
In M&M (page 44, line1100 – please add: "Images were acquired with Orius SC2001 digital camera (Gatan, Pleasanton, CA)".
In my affiliations (page 1) please add "10" for IHII.
Good luck,

Vsevolod L. Popov, Ph.D., D.Sc.
Professor
Director, Electron Microscopy
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Center for Biodefense and Emerging Infectious Diseases
World Reference Center for Emerging Viruses and Arboviruses
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The University of Texas Medical Branch
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Tel: (409) 747-2423
e-mail: vpopov@utmb.edu

**From:** Xie, Xuping <xuxie@UTMB.EDU>
**Sent:** Tuesday, July 2, 2019 12:29 PM
**To:** Zhou, Yiyang <yizhou@UTMB.EDU>; Routh, Andrew L. <alrouth@UTMB.EDU>; Popov, Vsevolod <vpopov@UTMB.EDU>; chenxw@wh.iov.cn; hongping@biolidics.com; qingyin.wang@clearbridgemedical.com
**Cc:** Shi, Pei yong <peshi@UTMB.EDU>; Zhang, Xianwen <zhxianwe@UTMB.EDU>; Zou, Jing <jizou@UTMB.EDU>
**Subject:** Review MS

Dear all,

Thank you for your contribution to this work. Can you help review the manuscript and send me your feedbacks by Friday (Jul 5, 2019)? We are going to submit this manuscript once I have your comments/suggestions.

Best regards,

Xuping Xie
Postdoctoral Fellow
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TX 77550, USA
Email: xuxie@utmb.edu
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**From**: Popov, Vsevolod [vpopov@UTMB.EDU]
**Sent**: 7/3/2019 10:01:03 AM
**To**: Xie, Xuping [xuxie@UTMB.EDU]
**Subject**: RE: Review MS

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Good luck,

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Director, Electron Microscopy
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**Sent:** Tuesday, July 2, 2019 12:29 PM
**To:** Zhou, Yiyang <yizhou@UTMB.EDU>; Routh, Andrew L. <alrouth@UTMB.EDU>; Popov, Vsevolod <vpopov@UTMB.EDU>; chenxw@wh.iov.cn; hongping@biolidics.com; qingyin.wang@clearbridgemedical.com
**Cc:** Shi, Pei yong <peshi@UTMB.EDU>; Zhang, Xianwen <zhxianwe@UTMB.EDU>; Zou, Jing <jizou@UTMB.EDU>
**Subject:** Review MS

Dear all,

Thank you for your contribution to this work. Can you help review the manuscript and send me your feedbacks by Friday (Jul 5, 2019)? We are going to submit this manuscript once I have your comments/suggestions.

Best regards,

Xuping Xie
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# Dengue NS2A protein orchestrates virus assembly

Xuping Xie[1]*, Jing Zou[1], Xianwen Zhang[1,2,3], Yiyang Zhou[1], Andrew Routh[1], Vsevolod L. Popov[4], Xinwen Chen[2,3,5], Qingyin Wang[6], Hong-Ping Dong[6], Pei-Yong Shi[1,7,8,9,10]*

[1]Department of Biochemistry & Molecular Biology, University of Texas Medical Branch, Galveston, Texas, USA; [2]State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Sciences, Wuhan, China. [3]University of Chinese Academy of Sciences, Beijing, China. [4]Department of Pathology, University of Texas Medical Branch, Galveston, Texas, USA; [5]Guangzhou Institute of Biomedicine and Health, Chinese Academy of Sciences, Guangzhou, China. [6]Clearbridge Health, Singapore; [7]Institute for Translational Science, University of Texas Medical Branch, Galveston, Texas, USA; [8]Sealy Institute for Vaccine Sciences, University of Texas Medical Branch, Galveston, Texas, USA; [9]Sealy Center for Structural Biology & Molecular Biophysics, University of Texas Medical Branch, Galveston, Texas, USA; [10]Institute for Human Infections & Immunity, University of Texas Medical Branch, Galveston, Texas, USA;

*Correspondence: [ HYPERLINK "mailto:xuxie@UTMB.edu" ] (X.X.), [ HYPERLINK "mailto:peshi@UTMB.edu" ] (P.-Y.S.)

Running title: The role of NS2A protein in dengue virus assembly



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## SUMMARY

Dengue virus (DENV) is the most important mosquito-borne viral pathogen in humans. DENV assembly requires cleavages of viral C-prM-E polyprotein into three structural proteins (capsid, pre-membrane, and envelope), packaging of viral RNA with C protein into nucleocapsid, and budding of prM/E proteins into virion. No study has addressed how these assembly events are orchestrated. Here we show that DENV-2 NS2A protein recruits viral RNA, structural proteins, and protease to virion assembly site, and coordinates nucleocapsid and virus formation. The last 285 nucleotides of viral 3'UTR serve as a "packaging signal" that binds to a cytosolic loop of NS2A protein. This interaction allows NS2A to recruit nascent RNA from the replication complex to virion assembly site. Mutations in NS2A that weaken the 3'UTR/NS2A interaction abolish virus assembly. In addition, NS2A interacts with prM, E, and NS3 to recruit C-prM-E polyprotein and NS2B/NS3 protease to the virion assembly site, leading to coordinated C-prM-E cleavage. Mature C protein assembles onto genomic RNA to form nucleocapsid, followed by prM/E envelopment and virion formation. An NS2A mutation that alters the prM/NS2A interaction reduces C-prM cleavage and abolishes virion assembly; second-site mutations in C protein that restore the C-prM cleavage rescue virion assembly. The results support a DENV assembly model that NS2A recruits viral RNA to virion assembly site where coordinated cleavage of C-prM-E ensues infections virus morphogenesis.




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## INTRODUCTION

The genus *Flavivirus* of the family *Flaviviridae* comprises over 70 viruses, many of which are important human pathogens, including dengue (DENV), yellow fever (YFV), Zika (ZIKV), West Nile (WNV), Japanese encephalitis (JEV), St. Louis encephalitis (SLE), and tick-borne encephalitis (TBEV) viruses[ ADDIN EN.CITE <EndNote><Cite><Author>Pierson</Author><Year>2013</Year><RecNum>3</RecNum><DisplayText>(Pierson and Diamond, 2013)</DisplayText><record><rec-number>3</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1536704974">3</key><key app="ENWeb" db-id="">0</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Pierson, T.C.</author><author>Diamond, M. S.</author></authors></contributors><titles><secondary-title>Flaviviruses p. 747-794. In D.M. Knipe and P.M. Howley (ed), Fields virology, 6th., vol. 1.</secondary-title></titles><periodical><full-title>Flaviviruses p. 747-794. In D.M. Knipe and P.M. Howley (ed), Fields virology, 6th., vol. 1.</full-title></periodical><dates><year>2013</year></dates><urls></urls></record></Cite></EndNote>]. The four serotypes of DENV alone cause about 390 million human infections each year, leading to dengue fever, dengue hemorrhagic fever, and dengue shock syndrome [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. Because of the huge social economic burden, DENV was included in the top 10 global health threats by the World Health Organization (WHO). The development of vaccine and therapeutics for DENV has been proven challenging [ ADDIN EN.CITE <EndNote><Cite><Author>Lim</Author><Year>2013</Year><RecNum>58</RecNum><DisplayText>(Lim et al., 2013)</DisplayText><record><rec-number>58</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv"



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timestamp="1560793214">58</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Lim, S. P.</author><author>Wang, Q. Y.</author><author>Noble, C. G.</author><author>Chen, Y. L.</author><author>Dong, H.</author><author>Zou, B.</author><author>Yokokawa, F.</author><author>Nilar, S.</author><author>Smith, P.</author><author>Beer, D.</author><author>Lescar, J.</author><author>Shi, P. Y.</author></authors></contributors><auth-address>Novartis Institute for Tropical Diseases, 10 Biopolis Road, 05-01 Chromos, Singapore 138670, Singapore.</auth-address><titles><title>Ten years of dengue drug discovery: Progress and prospects</title><secondary-title>Antiviral Res</secondary-title><alt-title>Antiviral research</alt-title></titles><periodical><full-title>Antiviral Res</full-title></periodical><pages>500-19</pages><volume>100</volume><number>2</number><edition>2013/10/01</edition><dates><year>2013</year><pub-dates><date>Nov</date></pub-dates></dates><isbn>1872-9096 (Electronic)0166-3542 (Linking)</isbn><accession-num>24076358</accession-num><urls><related-urls><url>http://www.ncbi.nlm.nih.gov/pubmed/24076358</url></related-urls></urls><electronic-resource-num>10.1016/j.antiviral.2013.09.013</electronic-resource-num><language>eng</language></record></Cite></EndNote>]. The first FDA approved dengue vaccine has a low efficacy of 30-61% [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. Due to the risk of vaccine-induced severe disease in children who are seronegative at the time of vaccination, WHO recommended only use of the vaccine in areas where the disease is highly prevalent [ ADDIN EN.CITE <EndNote><Cite><Author>Whitehead</Author><Year>2016</Year><RecNum>63</RecNum><DisplayText>(Whitehead, 2016)</DisplayText><record><rec-number>63</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793215">63</key></foreign-keys><ref-type name="Journal



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88 Article">17</ref-type><contributors><authors><author>Whitehead, S.
89 S.</author></authors></contributors><auth-address>a Laboratory of Infectious Diseases ,
90 NIAID, National Institutes of Health , Bethesda , MD , USA.</auth-
91 address><titles><title>Development of TV003/TV005, a single dose, highly immunogenic
92 live attenuated dengue vaccine; what makes this vaccine different from the Sanofi-Pasteur
93 CYD vaccine?</title><secondary-title>Expert Rev Vaccines</secondary-
94 title></titles><periodical><full-title>Expert Rev Vaccines</full-
95 title></periodical><pages>509-
96 17</pages><volume>15</volume><number>4</number><keywords><keyword>Dengue</
97 keyword><keyword>live attenuated dengue vaccine</keyword><keyword>vaccine
98 attenuation strategies</keyword><keyword>vaccine
99 efficacy</keyword><keyword>vaccine immunogenicity</keyword><keyword>vaccine
100 infectivity</keyword></keywords><dates><year>2016</year></dates><isbn>1744-8395
101 (Electronic)1476-0584 (Linking)</isbn><accession-num>26559731</accession-
102 num><urls><related-
103 urls><url>http://www.ncbi.nlm.nih.gov/pubmed/26559731</url></related-
104 urls></urls><custom2>PMC4956407</custom2><electronic-resource-
105 num>10.1586/14760584.2016.1115727</electronic-resource-
106 num></record></Cite></EndNote>]. No clinically approved antivirals are available for
107 flavivirus treatment, despite major effort in the past two decades[ ADDIN EN.CITE
108 <EndNote><Cite><Author>Zou</Author><Year>2019</Year><RecNum>64</RecNum><
109 DisplayText>(Zou and Shi, 2019)</DisplayText><record><rec-number>64</rec-
110 number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv"
111 timestamp="1560793215">64</key></foreign-keys><ref-type name="Journal
112 Article">17</ref-type><contributors><authors><author>Zou, J.</author><author>Shi, P.
113 Y.</author></authors></contributors><auth-address>Department of Biochemistry and



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Molecular Biology, University of Texas Medical Branch, Galveston, TX, USA.Department of Biochemistry and Molecular Biology, University of Texas Medical Branch, Galveston, TX, USA; Department of Phamarcology and Toxicology, University of Texas Medical Branch, Galveston, TX, USA; Sealy Center for Structural Biology and Molecular Biophysics, University of Texas Medical Branch, Galveston, TX, USA; Sealy Institute for Vaccine Sciences, University of Texas Medical Branch, Galveston, TX, USA. Electronic address: peshi@utmb.edu.</auth-address><titles><title>Strategies for Zika drug discovery</title><secondary-title>Curr Opin Virol</secondary-title></titles><periodical><full-title>Curr Opin Virol</full-title></periodical><pages>19-26</pages><volume>35</volume><dates><year>2019</year><pub-dates><date>Mar 7</date></pub-dates></dates><isbn>1879-6265 (Electronic)1879-6257 (Linking)</isbn><accession-num>30852345</accession-num><urls><related-urls><url>http://www.ncbi.nlm.nih.gov/pubmed/30852345</url></related-urls></urls><electronic-resource-num>10.1016/j.coviro.2019.01.005</electronic-resource-num></record></Cite></EndNote>]. It is therefore critical to study the molecular mechanism of flavivirus replication, which may lead to new approaches for countermeasure development.

Flavivirus has a plus-sense, single-stranded RNA of about 11,000-nucleotide in length. The genomic RNA contains a 5' untranslated region (UTR), a single open-reading-frame (ORF), and a 3' UTR. The ORF translates a polyprotein that is processed by viral and host proteases into three structure proteins [capsid (C), pre-membrane (prM), and envelope (E)] and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5). The structural proteins, together with genomic RNA, assemble into virions. The nonstructural proteins participate in viral RNA replication, virion assembly, and evasion of host immune response. Upon viral translation, a minus-sense RNA is synthesized from the



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genomic RNA template, forming a double-stranded RNA. The minus-strand RNA then serves as a template to make many copies of plus-sense RNA that are packaged to form progeny virions [ ADDIN EN.CITE <EndNote><Cite><Author>Pierson</Author><Year>2013</Year><RecNum>3</RecNum><DisplayText>(Pierson and Diamond, 2013)</DisplayText><record><rec-number>3</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1536704974">3</key><key app="ENWeb" db-id="">0</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Pierson, T.C.</author><author>Diamond, M. S.</author></authors></contributors><titles><secondary-title>Flaviviruses p. 747-794. In D.M. Knipe and P.M. Howley (ed), Fields virology, 6th., vol. 1.</secondary-title></titles><periodical><full-title>Flaviviruses p. 747-794. In D.M. Knipe and P.M. Howley (ed), Fields virology, 6th., vol. 1.</full-title></periodical><dates><year>2013</year></dates><urls></urls></record></Cite></EndNote>]. Like other RNA viruses, the assembly of flavivirus is coupled with RNA replication [ ADDIN EN.CITE ADDIN EN.CITE.DATA ].

Flavivirus infection induces invaginations of the endoplasmic reticulum (ER) to form clusters of double-membrane vesicles (Ve) enclosed in the vesicle packets (VPs). Electron tomography has revealed the three-dimensional architectures of Ve and VPs formed during DENV, WNV, TBEV, and ZIKV infections [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. Ve houses viral replication complex (including the double-stranded viral RNA, viral nonstructural proteins, and cellular proteins) where RNA synthesis occurs [ ADDIN EN.CITE <EndNote><Cite><Author>Junjhon</Author><Year>2014</Year><RecNum>71</RecNum><DisplayText>(Junjhon et al., 2014)</DisplayText><record><rec-number>71</rec-



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number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793216">71</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Junjhon, J.</author><author>Pennington, J. G.</author><author>Edwards, T. J.</author><author>Perera, R.</author><author>Lanman, J.</author><author>Kuhn, R. J.</author></authors></contributors><auth-address>Markey Center for Structural Biology, Department of Biological Sciences, Purdue University, West Lafayette, Indiana, USA.</auth-address><titles><title>Ultrastructural characterization and three-dimensional architecture of replication sites in dengue virus-infected mosquito cells</title><secondary-title>J Virol</secondary-title></titles><periodical><full-title>J Virol</full-title></periodical><pages>4687-97</pages><volume>88</volume><number>9</number><keywords><keyword>Animals</keyword><keyword>Cell Line</keyword><keyword>Culicidae/*virology</keyword><keyword>Dengue Virus/*physiology/*ultrastructure</keyword><keyword>Electron Microscope Tomography</keyword><keyword>Imaging, Three-Dimensional</keyword><keyword>Intracellular Membranes/*ultrastructure/*virology</keyword><keyword>*Virus Replication</keyword></keywords><dates><year>2014</year><pub-dates><date>May</date></pub-dates></dates><isbn>1098-5514 (Electronic)0022-538X (Linking)</isbn><accession-num>24522909</accession-num><urls><related-urls><url>http://www.ncbi.nlm.nih.gov/pubmed/24522909</url></related-urls></urls><custom2>PMC3993787</custom2><electronic-resource-num>10.1128/JVI.00118-14</electronic-resource-num></record></Cite></EndNote>]. Newly synthesized plus-sense RNA is released from the Ve pore to the rough ER for new rounds of RNA translation and replication. Alternatively, the nascent RNA is assembled



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into virions through encapsidation (with C protein) and envelopment (with prM and E proteins) through budding into the ER lumen. The virion assembly occurs at the ER sites juxtaposed to the VPs. An initial immature virion contains 60 icosahedrally arranged prM/E heterotrimer. As the immature virion transits through the Golgi network, the acidic environment triggers the cleavage of prM by cellular furin protease, resulting in an infectious mature virion [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. Cryo-EM of flavivirus virions exhibit two structural features: (i) The nucleocapsid core lacks organized structures and (ii) there are no observable connections between nucleocapsid and the prM/E membrane shell, suggesting that the C protein may bind to genomic RNA in a random manner without direct interaction with prM/E proteins [ ADDIN EN.CITE ADDIN EN.CITE.DATA ].

The molecular mechanism of flavivirus assembly remains largely obscure. Besides structural proteins, several nonstructural proteins, such as NS2A, NS2B, and NS3, have been shown to regulate flavivirus assembly. Different amino acids of NS2A protein were reported to play distinct roles in flavivirus RNA replication and virion assembly [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. The cleavage between C and prM is tightly modulated by NS2B/NS3 protease and cellular signalase during YFV and Murray Valley encephalitis virus assembly [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. Mutations in the NS3 helicase domain alone affect YFV assembly [ ADDIN EN.CITE <EndNote><Cite><Author>Patkar</Author><Year>2008</Year><RecNum>90</RecNum><DisplayText>(Patkar and Kuhn, 2008a)</DisplayText><record><rec-number>90</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793218">90</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Patkar, C. G.</author><author>Kuhn, R. J.</author></authors></contributors><auth-



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address>Purdue Univ, Dept Biol Sci, W Lafayette, IN 47907 USA</auth-address><titles><title>Yellow fever virus NS3 plays an essential role in virus assembly independent of its known enzymatic functions</title><secondary-title>Journal of Virology</secondary-title><alt-title>J Virol</alt-title></titles><alt-periodical><full-title>J Virol</full-title></alt-periodical><pages>3342-3352</pages><volume>82</volume><number>7</number><keywords><keyword>tick-borne encephalitis</keyword><keyword>flavivirus kunjin ns3</keyword><keyword>west-nile-virus</keyword><keyword>dengue virus</keyword><keyword>nonstructural protein</keyword><keyword>rna replication</keyword><keyword>cdna-clones</keyword><keyword>particles</keyword><keyword>ns2a</keyword><keyword>organization</keyword></keywords><dates><year>2008</year><pub-dates><date>Apr</date></pub-dates></dates><isbn>0022-538X</isbn><accession-num>WOS:000254139800015</accession-num><urls><related-urls><url><Go to ISI>://WOS:000254139800015</url></related-urls></urls><electronic-resource-num>10.1128/Jvi.02447-07</electronic-resource-num><language>English</language></record></Cite></EndNote>]. Although expression of prM/E alone is sufficient to generate empty virus-like particles, it is not known how nucleocapsid is formed and subsequently enveloped with prM/E to form infectious virions. The goal of this study is to define the molecular mechanism that orchestrates three structural proteins and viral RNA to form nucleocapsid and infectious virus in a highly coordinated manner. Using genetic, biochemical, and imaging approaches, we demonstrate that DENV NS2A protein choreographs virion morphogenesis by (i) interacting with viral prM and E proteins, (ii) regulating the processing of C-prM polyprotein by viral protease, and (iii) recruiting genomic RNA through binding to the 3'UTR. These results support a model that DENV NS2A recruits nascent viral RNA from the replication complex (housed in Ve) to the virion assembly site (on the ER membrane juxtaposed to



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VPs), where an coordinated C-prM cleavage facilitates genomic RNA encapsidation with C protein and budding with prM and E proteins into the ER lumen, resulting in progeny virions.



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## RESULTS

**NS2A G11A mutation abolishes virion assembly**. Using DENV-2 as a model, we previously identified a panel of NS2A mutant viruses that were selectively defective in virion assembly without compromising other steps of an infection cycle [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. Here, we performed four experiments to demonstrate that NS2A mutation G11A of DENV-2 caused a defect in virion assembly. (i) A DENV-2 luciferase replicon was used to examine the effect of NS2A G11A on viral translation and RNA synthesis. Upon transfection into BHK-21 cells, the G11A replicon produced a luciferase profile comparable to the WT replicon (Fig. 1A), suggesting that the mutation does not significantly reduce viral translation and RNA replication. (ii) When full-length viral RNA of DENV-2 was transfected into BHK-21 cells, the NS2A G11A mutant failed to release extracellular viral RNA (Fig. 1B). No infectious virus was detected from the culture medium of G11A RNA-transfected cells (Fig. 1C). (iii) In a *trans* packaging system, WT luciferase replicon could be packaged into pseudo-infectious particles (PIPs) when structural C-prM-E proteins were expressed from Semliki Forest virus replicon (Fig. S1A). The WT reporter replicon PIPs infected Vero cells and generated robust luciferase signals (Fig. S1B). In contrast, no PIPs were produced with the NS2A G11A replicon. (iv) Transmission electron microscopy revealed substantial virus-induced single-membrane vesicles (within the lumen of the rough ER) or double-membrane vesicles (closely associated with cisternae of the rough ER) in both WT and G11A RNA-transfected cells at 48 h post-transfection (Fig. 1D). A lot of virus particles appeared in the WT RNA-transfected cells, but no virus particles were detected in the G11A RNA-transfected cells. The results demonstrate that NS2A G11A mutation specifically abolishes virion assembly.

**NS2A G11A mutation alters the expression and localization of viral structural proteins.** We compared the subcellular localization of viral proteins in BHK-21 cells that



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were transfected with WT or NS2A G11A genomic RNA. Surprisingly, the NS2A G11A mutation altered the intracellular distribution of three structural proteins. (i) C protein disseminated in both nucleus and cytoplasm in the WT RNA-transfected cells, as previously reported [ ADDIN EN.CITE <EndNote><Cite><Author>Wang</Author><Year>2004</Year><RecNum>91</RecNum><DisplayText>(Wang et al., 2004)</DisplayText><record><rec-number>91</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793218">91</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Wang, S. H.</author><author>Syu, W. J.</author><author>Hu, S. T.</author></authors></contributors><auth-address>Institute of Microbiology and Immunology, School of Life Science, National Yang-Ming University, Taipei, Taiwan, Republic of China.</auth-address><titles><title>Identification of the homotypic interaction domain of the core protein of dengue virus type 2</title><secondary-title>J Gen Virol</secondary-title><alt-title>The Journal of general virology</alt-title></titles><periodical><full-title>J Gen Virol</full-title><abbr-1>The Journal of general virology</abbr-1></periodical><alt-periodical><full-title>J Gen Virol</full-title><abbr-1>The Journal of general virology</abbr-1></alt-periodical><pages>2307-14</pages><volume>85</volume><number>Pt 8</number><keywords><keyword>Active Transport, Cell Nucleus</keyword><keyword>Dengue Virus/*chemistry/classification</keyword><keyword>Precipitin Tests</keyword><keyword>Viral Core Proteins/*chemistry</keyword></keywords><dates><year>2004</year><pub-dates><date>Aug</date></pub-dates></dates><isbn>0022-1317 (Print)0022-1317 (Linking)</isbn><accession-num>15269372</accession-num><urls><related-urls><url>http://www.ncbi.nlm.nih.gov/pubmed/15269372</url></related-



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urls></urls><electronic-resource-num>10.1099/vir.0.80067-0</electronic-resource-num></record></Cite></EndNote>]. However, in the G11A RNA-transfected cells, the C protein was mainly restricted to the cytoplasm with rare nucleus localization (Fig. 1E). (ii) prM protein was located at the ER region in the WT RNA-transfected cells, whereas the prM protein was undetectable in the G11A RNA-transfected cells (Fig. 1F). (iii) E protein spread broadly like an ER web in the WT RNA-transfected cells. In contrast, in the G11A RNA-transfected cells, the E protein formed larger speckles at restricted subcellular areas (Fig. 1G). Compared with the structural proteins, the effect of NS2A G11A on the subcellular expression of viral nonstructural proteins (including NS1, NS3, NS4A, NS4B, and NS5) and dsRNA was not apparent (Fig. 1E-G, *top panels* for NS3, and data not shown).

The distinct effect of NS2A G11A mutation on subcellular expression between the viral structural and nonstructural proteins were further supported by immunofluorescent co-staining experiments. NS2A G11A altered the co-staining profile of E and NS3 proteins (Fig. S2). In the NS2A G11A RNA-transfected cells, 100% of NS3-expressing cells were E-positive at 24 h post-transfection; however, at 48 and 72 h post-transfection, only 59% and 20% of the NS3-expressing cells were E-positive, respectively (Fig. S2). In the WT RNA-transfected cells, 100% of NS3-expressing cells were E-positive from 24 to 72 h post-transfection (Fig. S2). The results demonstrate that NS2A G11A mutation changes the expression and subcellular localization of viral structural proteins.

**NS2A G11A mutation impairs the cleavage of viral C-prM polyprotein.** To determine the molecular mechanism of the above observations, we examined the processing of viral proteins in the WT and NS2A G11A viral RNA-transfected cells at 48 h post-transfection by Western blot. As expected, the amounts of C, NS3, and NS5 proteins from the G11A RNA-transfected cells were 15-26% of those from the WT RNA-transfected



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cells (Fig. 1H-I), due to the lack of virion production or multiple rounds of infection for the G11A mutant. Notably, the amounts of prM and E proteins were further lower in the G11A RNA-transfected cells (only 1-4% of those in the WT RNA-transfected cells), suggesting an asymmetric degradation in cells. Moreover, the NS2A G11A mutation impaired the cleavage of C-prM to individual C and prM proteins: 90% of C-prM was cleaved to mature C protein in the WT RNA-transfected cells, whereas only 20% of C-prM was processed to C protein in the G11A RNA-transfected cells (Fig. 1H, *compare lanes 1 and 5 in the top panel*). The impaired C-prM cleavage could account for the altered subcellular distribution of viral structural proteins in the NS2A C11A RNA-transfected cells: Restricting the un-cleaved C-prM to the ER membrane, preventing C protein from translocating to the nucleus, and consequently blocking prM/E heterodimer formation and virion budding.

Next, we examined the role of ubiquitin-proteasome pathway in degrading viral proteins in the G11A RNA-transfected cells. Treatment with proteasome inhibitor MG132 increased the amounts of C, prM, E, and NS5 (but not NS3) by 1-3 folds in the NS2A G11A RNA-transfected cells (Fig. 1H, *compare lanes 5 and 6*). In contrast, in the WT RNA-transfected cells, MG132 slightly reduced the amounts of C, prM, and E to different extents (Fig. 1H, *compare lanes 1 and 2*) because MG132 was known to inhibit flavivirus replication [ ADDIN EN.CITE <EndNote><Cite><Author>Bhuvanakantham</Author><Year>2013</Year><RecNum>108</RecNum><DisplayText>(Bhuvanakantham and Ng, 2013)</DisplayText><record><rec-number>108</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793288">108</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Bhuvanakantham, R.</author><author>Ng, M. L.</author></authors></contributors><auth-



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address>Flavivirology Laboratory, Department of Microbiology, Yong Loo Lin School of Medicine, National University of Singapore, 5 Science Drive 2, Singapore, 117597.</auth-address><titles><title>West Nile virus and dengue virus capsid protein negates the antiviral activity of human Sec3 protein through the proteasome pathway</title><secondary-title>Cell Microbiol</secondary-title></titles><periodical><full-title>Cell Microbiol</full-title></periodical><pages>1688-706</pages><volume>15</volume><number>10</number><keywords><keyword>Antiviral Agents/*antagonists & inhibitors</keyword><keyword>Capsid Proteins/genetics/*metabolism</keyword><keyword>Cell Line</keyword><keyword>DNA Mutational Analysis</keyword><keyword>Dengue Virus/*physiology</keyword><keyword>*Host-Pathogen Interactions</keyword><keyword>Humans</keyword><keyword>Models, Biological</keyword><keyword>Proteasome Endopeptidase Complex/*metabolism</keyword><keyword>Protein Binding</keyword><keyword>Protein Interaction Mapping</keyword><keyword>Proteolysis</keyword><keyword>Vesicular Transport Proteins/*antagonists & inhibitors</keyword><keyword>West Nile virus/*physiology</keyword></keywords><dates><year>2013</year><pub-dates><date>Oct</date></pub-dates></dates><isbn>1462-5822 (Electronic)1462-5814 (Linking)</isbn><accession-num>23522008</accession-num><urls><related-urls><url>https://www.ncbi.nlm.nih.gov/pubmed/23522008</url></related-urls></urls><electronic-resource-num>10.1111/cmi.12143</electronic-resource-num></record></Cite></EndNote>]. Distinct responses to MG132 treatment among different viral proteins were probably due to their subcellular distributions and involvement in various biological microenvironments. Nevertheless, the results suggest that ubiquitin-proteasome pathway is responsible for viral protein degradation in the NS2A G11A RNA-transfected cells.



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**WT NS2A rescues virion assembly of NS2A G11A mutant through restoring C-prM cleavage.** We examined if *trans* supply of WT NS2A could restore virion assembly of NS2A G11A RNA and rescue C-prM protein processing (Fig.S3A). Indeed, after transfection into NS2A-EGFP BHK-21 cells that constitutively expressed WT NS2A protein, the NS2A G11A RNA produced infectious virions that could infect naïve BHK-21 cells, as indicated by E-positive cells (Fig. S3B); further passaging of culture supernatants to new BHK-21 cells did not yield any E-positive cells (Fig. S3C), suggesting that the *trans* packaged virions could only infect cells for a single-round. Remarkably, the *trans* supplied WT NS2A protein restored the C-prM cleavage in the G11A RNA-transfected cells to WT level, exhibiting >89% C protein processing (Fig. S3D, *top panel*). Two prM bands, representing glycosylated and un-glycosylated forms of the protein (see below), were detected in both NS2A G11A and WT RNA-transfected NS2A-EGFP cells (Fig. S3D, *second panel*). The relative abundances of C, prM, and NS1 proteins were similar in the NS2A G11A and WT RNA-transfected cells (Fig. S3D). Because of the restored C-prM cleavage, the nucleus localization of C protein and prM-positive cells were detected in the G11A RNA-transfected NS2A-EGFP cells (Fig. S3E). These results demonstrate that WT NS2A can rescue virion assembly of NS2A G11A mutant through restoring the C-prM cleavage.

Next, we examined the effect of NS2A on the glycosylation of prM by co-expressing NS2A and prM in HEK293T cells. As a control, when EGFP was co-expressed, only the glycosylated form of prM was detected (Fig. S3F); after PGNaseF treatment, the prM was completely deglycoslated and migrated as a glycosylation-knockout mutant N69Q prM (Fig. S3G). In contrast, when NS2A-EGFP were co-expressed, both glycosylated and un-glycosylated forms of prM were detected (Fig. S3F), indicating that NS2A suppresses the glycosylation of prM. Furthermore, the G11A mutation seemed to increase the



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suppression effect of NS2A on prM glycosylation (Fig. 3F). These results suggest that NS2A may interact with prM, and that the G11A mutation may modulate the prM/NS2A interaction.

**Capsid R99S mutation can rescue virion assembly of NS2A G11A RNA.** We selected revertants by continuously passaging the NS2A G11A RNA-transfected BHK-21 cells for five rounds (Fig. S4A). Culture medium from each passaging (P1-P5) were tested for emergence of infectious viruses using plaque assay. The P5 culture produced infectious viruses with titers of $>10^4$ PFU/ml and plaques smaller than the WT virus (Fig. S4B). In addition, almost all P5 cells were E protein-positive (Fig. S4C). Whole genome sequencing of the P5 viruses from three independent selections consistently showed a consensus C-to-A substitution at nucleotide position 391 of viral genome, resulting in an Arg-to-Ser change at amino acid position 99 of the C protein (C_R99S; Fig. 2A). The original NS2A G11A mutation was retained in all P5 viruses.

To validate the role of C_R99S mutation in rescuing virion assembly of NS2A G11A RNA, we engineered C_R99S into a DENV-2 infectious clone [ ADDIN EN.CITE <EndNote><Cite><Author>Xie</Author><Year>2013</Year><RecNum>85</RecNum><DisplayText>(Xie et al., 2013)</DisplayText><record><rec-number>85</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793217">85</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Xie, X.</author><author>Gayen, S.</author><author>Kang, C.</author><author>Yuan, Z.</author><author>Shi, P. Y.</author></authors></contributors><auth-address>State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Science, China.</auth-address><titles><title>Membrane topology and function of dengue virus NS2A protein</title><secondary-title>J Virol</secondary-title><alt-title>Journal of virology</alt-



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421 title></titles><periodical><full-title>J Virol</full-title></periodical><pages>4609-
422 22</pages><volume>87</volume><number>8</number><edition>2013/02/15</edition><k
423 eywords><keyword>Animals</keyword><keyword>Cell
424 Line</keyword><keyword>Cricetinae</keyword><keyword>Dengue
425 Virus/*physiology</keyword><keyword>Endoplasmic
426 Reticulum/*virology</keyword><keyword>Humans</keyword><keyword>Intracellular
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429 Conformation</keyword><keyword>Viral Nonstructural
430 Proteins/*chemistry/metabolism</keyword><keyword>*Virus
431 Assembly</keyword></keywords><dates><year>2013</year><pub-
432 dates><date>Apr</date></pub-dates></dates><isbn>1098-5514 (Electronic)0022-
433 538X (Linking)</isbn><accession-num>23408612</accession-num><work-type>Research
434 Support, Non-U.S. Gov't</work-type><urls><related-
435 urls><url>http://www.ncbi.nlm.nih.gov/pubmed/23408612</url></related-
436 urls></urls><custom2>3624351</custom2><electronic-resource-num>10.1128/JVI.02424-
437 12</electronic-resource-num><language>eng</language></record></Cite></EndNote>] in
438 the presence or absence of NS2A G11A. BHK-21 cells were electroporated with WT,
439 NS2A G11A, C_R99S, or NS2A G11A+C_R99S viral RNA. The transfected cells were
440 analyzed for virus production and C-prM cleavage. In the NS2A G11A+C_R99S RNA-
441 transfected cells, the percentage of E-positive cells increased from 10% to 50% at 24 h
442 and 96 h post-transfection, suggesting the spread of infectious virus (Fig. 2B); indeed, the
443 transfected cells produced infectious viruses with plaques smaller than the WT virus (Fig.
444 2C); the viral titer increased over time, reaching >$10^5$ PFU/ml at 96-120 h post-transfection
445 (Fig. 2D). The prM and NS1 proteins exhibited ER-like distributions in the NS2A
446 G11A+C_R99S RNA-transfected cells, similar to that observed in the WT RNA-transfected



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cells (Fig. 2E). Viral C protein was detected in both cytoplasm and nucleus in the NS2A G11A+C_R99S RNA-transfected cells (Fig. 2F-G). Furthermore, 90.5% of the C-prM protein was cleaved to mature C and prM in the NS2A G11A+C_R99S RNA-transfected cells (Fig. 2G). As controls, NS2A G11A RNA showed 15.4% of C-prM cleavage and did not produce any infectious virus, whereas C_R99S RNA exhibited 99.9% of prM-C cleavage and produced a WT level of infectious virus (Fig. 2B-H). Taken together, the results demonstrate that C_R99S mutation efficiently restores C-prM cleavage and virion assembly of NS2A G11A RNA.

**Capsid R99S cannot rescue virion assembly of other NS2A mutants.** We tested if C_R99S could rescue other NS2A mutants that were previously shown to be defective in virion assembly, including E20A, E100A, and K188A [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. The C_R99S was engineered into those NS2A mutants, resulting in NS2A E20A+C_R99S, NS2A E100A+C_R99S, and NS2A K188A+C_R99S viral RNAs. After transfection into BHK-12 cells, none of the mutant RNAs produced increasing number of E-positive cells from 24 to 96 h (Fig. S5A). No infectious virus was detected in the culture medium from the mutant RNA-transfected cells (Fig. S5B). Viral C protein was detected in both nucleus and cytoplasm of all mutant RNA-transfected cells (Fig. S5C). In agreement with the C nucleus localization, the NS2A single mutant E20A and E100A cleaved 36% and 79% of C-prM, and the addition of C_R99S increased the C-prM cleavage to 72% and 96%, respectively. In contrast, NS2A K188A mutant cleaved 100% of C-prM in the presence or absence of C_R99S (Fig. 5D). The results demonstrate that C_R99S can only rescue the assembly defect of NS2A G11A possibly because different NS2A mutations abolish virion assembly through distinct mechanisms.

**Capsid R99S shifts the C-prM cleavage site by two amino acids.** The above results prompted us to investigate how C_R99S rescues the virion assembly of NS2A



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G11A. Fig. S6 depicts the topology of viral polyprotein C-prM-E-NS1-NS2A on the ER membrane. NS2A G11 resides at the first amphipathic helix on the ER lumen side [ ADDIN EN.CITE

<EndNote><Cite><Author>Xie</Author><Year>2013</Year><RecNum>85</RecNum><DisplayText>(Xie et al., 2013)</DisplayText><record><rec-number>85</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793217">85</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Xie, X.</author><author>Gayen, S.</author><author>Kang, C.</author><author>Yuan, Z.</author><author>Shi, P. Y.</author></authors></contributors><auth-address>State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Science, China.</auth-address><titles><title>Membrane topology and function of dengue virus NS2A protein</title><secondary-title>J Virol</secondary-title><alt-title>Journal of virology</alt-title></titles><periodical><full-title>J Virol</full-title></periodical><pages>4609-22</pages><volume>87</volume><number>8</number><edition>2013/02/15</edition><keywords><keyword>Animals</keyword><keyword>Cell Line</keyword><keyword>Cricetinae</keyword><keyword>Dengue Virus/*physiology</keyword><keyword>Endoplasmic Reticulum/*virology</keyword><keyword>Humans</keyword><keyword>Intracellular Membranes/*metabolism</keyword><keyword>Magnetic Resonance Spectroscopy</keyword><keyword>Models, Molecular</keyword><keyword>Protein Conformation</keyword><keyword>Viral Nonstructural Proteins/*chemistry/metabolism</keyword><keyword>*Virus Assembly</keyword></keywords><dates><year>2013</year><pub-dates><date>Apr</date></pub-dates></dates><isbn>1098-5514 (Electronic)0022-538X (Linking)</isbn><accession-num>23408612</accession-num><work-type>Research



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Support, Non-U.S. Gov't</work-type><urls><related-urls><url>http://www.ncbi.nlm.nih.gov/pubmed/23408612</url></related-urls></urls><custom2>3624351</custom2><electronic-resource-num>10.1128/JVI.02424-12</electronic-resource-num><language>eng</language></record></Cite></EndNote>], while C_R99 is the penultimate amino acid of the C protein on the cytosolic side (Fig. S6B). Since flavivirus NS2B/NS3 protease cleaves a substrate with two basic residues (Arg or Lys) at the P1 and P2 sites and a residue with short side chain (Gly, Ser, or Ala) at the P1' site [ ADDIN EN.CITE ADDIN EN.CITE.DATA ], sequence analysis suggests that the C_R99S substitution may shift the capsid C-terminal cleavage by two amino acids from position 100 to 98 (Fig. S6B). Specifically, viral NS2B/NS3 protease cleaves the WT capsid at NRRRR↓TA and the R99S capsid at NRR↓SRTA (↓ denoting cleavage site and residue R99S underlined). To test this hypothesis, we performed a biochemical protease assay using synthetic WT peptide (NILNRRRRTAGMII) and R99S peptide (NILNRRSRTAGMII). After incubating the peptides with recombinant DENV-2 NS2B/NS3 protease, the reaction products were analyzed by liquid chromatography-mass spectrometry (LC-MS). The WT peptide was cleaved into two smaller peptides with molecular weights of 1096.73 Da and 604.77 Da, corresponding to peptides NILNRRRR and TAGMII, respectively (Fig. S6C). In contrast, the R99S peptide was cleaved into two fragments with molecular weights of 784.47 Da and 847.46 Da, corresponding to peptide NILNRR and SRTAGMII, respectively (Fig. S6D). The LC-MS also detected some unknown peptides, probably due to nonspecific degradations of the protease reaction (Fig. S6C-D). Nevertheless, the data indicate that the C_R99S mutation shifts the capsid cleavage site upstream by two amino acids.

**NS2A G11A enhances prM/NS2A interaction.** Since NS2A G11A alters the processing of C-prM (Fig. 1H) and prM glycosylation (Fig. S3F), we hypothesized that



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NS2A directly interacts with prM and the G11A mutation affects the prM/NS2A interaction. To test this hypothesis, we co-transfected two plasmids into HEK293T cells. One plasmid expressed the authentic signal peptide anchorC ($C_{14}$) fused with the full-length prM. Another plasmid expressed a fusion protein containing the C-terminal 24 amino acids of the E protein, the N-terminal 50 amino acids of the NS1 protein, the C-terminal 50 amino acids of NS1, full length NS2A, and an EGFP [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]; this plasmid was previously shown to express NS2A-EGFP with a correct topology on the ER membrane [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. Co-immunoprecipitation showed that NS2A-EGFP, but not EGFP, pulled down prM (Fig. 3B, lanes 2 and 3). As controls, mouse IgG did not pull down any NS2A-EGFP or prM (Lane 1), and EGFP antibody did not pull down prM in the absence of NS2A-EGFP (Lane 6). To map the region of prM that binds to NS2A, we expressed the first 130 amino acids of prM [prM(1-130)] that lacked the C-terminal two transmembrane domains (Fig. 3A). Co-immunoprecipitation showed that prM(1-130) could not pull down NS2A-EGFP (Fig. 3B, lane 4), suggesting that (i) NS2A may bind to the transmembrane region of prM or (ii) the transmembrane region of prM may affect the conformation of its ectodomain and, consequently, abolish its interaction with NS2A.

Next, we examined if NS2A mutation G11A affects the prM/NS2A interaction. Co-immunoprecipitation experiments showed that G11A NS2A-EGFP pulled down about 2.5-fold more prM than the WT NS2A-EGFP did (Fig. 4C). Reciprocally, prM pulled down about 1.7-fold more G11A NS2A-EGFP than WT NS2A-EGFP (Fig. 4D). The results indicate that the G11A mutation enhances NS2A binding to prM.

To further support the prM/NS2A interaction, we used confocal microscope to examine the co-localization of prM/NS2A in cells. In this case, prM-E were co-expressed with NS2A-EGFP in HEK293T cells. As shown in Fig. 3E, NS2A-EGFP co-localized with



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prM. The G11A mutation did not change the co-localization of NS2A-EGFP with prM (Fig. 3E). Taken together, the results demonstrate that (i) NS2A interacts with prM and (ii) NS2A G11A significantly enhances the prM/NS2A interaction.

**The G11A mutation does not affect NS2A binding to E or NS3.** We also examined the interactions between NS2A and E or NS3 proteins that were co-expressed in HEK293T cells. For testing the E/NS2A interaction, we fused a GST tag to the C-terminus of NS2A for the co-immunoprecipitation experiment [because the EGFP tag used in NS2A-EGFP in Fig. 3B-D non-specifically interacts with E protein (data not shown)]. As shown in Fig. 3F, both WT and G11A NS2A-GST pulled down E protein at comparable efficiencies. As a control, GST alone did not pull down E protein. The result suggests that NS2A interacts with E and the G11A mutation does not affect this interaction.

Since flavivirus NS3 is known to participate in viron assembly [ ADDIN EN.CITE ADDIN EN.CITE.DATA ], we also tested if NS2A G11A mutation affects the NS2A/NS3 interaction. When co-expressed in HEK293T cells, GST alone pulled down a trace amount of HA-tagged NS3 (NS3-HA) protein (Fig. S7, lane 1), whereas NS2A-GST pulled down 10-fold more NS3-HA (Lane 2). Equivalent amounts of NS3-HA were pulled down by the WT and G11A NS2A-GST (Compare lanes 2 and 3), suggesting that NS2A G11A does not affect NS2A/NS3 binding.

**Extension of two or three residues at the N-terminus of anchorC efficiently rescues virion assembly of NS2A G11A**. So far, our results showed that NS2A G11A mutation increases its binding to prM on the ER lumen side, leading to impaired C-prM processing and defective virion assembly. These defects can be rescued by a capsid mutation R99S that shifts the C-terminal cleavage site by two amino acids. As a result of this shift, the N-terminus of transmembrane anchorC is extended by two amino acids on



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572 the cytosolic side of ER membrane. These data support a model of coordinated C-prM
573 processing during virion assembly (Fig. 4A) [ ADDIN EN.CITE
574 <EndNote><Cite><Author>Amberg</Author><Year>1999</Year><RecNum>295</RecNu
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579 Article">17</ref-type><contributors><authors><author>Amberg, S.
580 M.</author><author>Rice, C. M.</author></authors></contributors><auth-
581 address>Department of Molecular Microbiology, Washington University School of
582 Medicine, St. Louis, Missouri 63110-1093, USA.</auth-
583 address><titles><title>Mutagenesis of the NS2B-NS3-mediated cleavage site in the
584 flavivirus capsid protein demonstrates a requirement for coordinated
585 processing</title><secondary-title>J Virol</secondary-title><alt-title>Journal of
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598 urls></urls><custom2>112824</custom2></record></Cite></EndNote>]. During WT virus
599 assembly, polyprotein C-anchorC-prM was first cleaved at the C-terminus of capsid protein
600 (*i.e.*, R100) by NS2B/NS3 protease, producing mature C and anchorC-prM. The anchorC-
601 prM was then cleaved by a host signalase at the N-terminus of prM, generating anchorC
602 and prM. The cleaved prM coordinates with E protein for virion budding (Fig. 4A, *left*
603 *panel*). In the presence of NS2A G11A mutation, NS2A binds to prM with a higher affinity,
604 pulling anchorC (embedded in the ER membrane) towards the lumen side. The viral
605 protease cleavage site of the "pulled anchorC" is blocked, possibly by the ER membrane
606 (*middle panel*), leading to unprocessed C-prM. To overcome this defect, the C_R99S
607 mutation creates a new NS2A/NS3 cleavage site at R98 of capsid protein, restoring the C-
608 prM processing and virion assembly of NS2A G11A (*right panel*).

609 The key of the above model is the blockage of viral protease cleavage site at the
610 C-terminus of capsid protein, which is caused by the NS2A G11A mutation. If the model is
611 correct, an insertion of one or two amino acids to the N-terminus of anchorC should
612 restore the C-prM cleavage and virion assembly in the presence of NS2A G11A mutation
613 (Fig. 4B). To test this hypothesis, we inserted Thr, The-Ala, or Ser-Arg to the C-terminus of
614 capsid protein (after R100) in the context of WT or NS2A G11A viral RNA. The inserted
615 amino acids were selected based on the cleavage requirement for viral protease (Fig. 4B).
616 Transfection of each viral RNA into BHK-21 cells yielded increasing number of E-positive
617 cells from day 1 to 4 (Fig. 4C), produced infectious viruses (Fig. 4D-E), displayed cytosolic
618 and nucleus localizations of C protein (Fig. 4F), and exhibited efficient C-prM cleavage
619 (Fig. 4H). In the absence of NS2A G11A, the capsid insertion mutant RNAs behaved
620 similarly to the WT RNA. In the presence of NS2A G11A, the capsid single-amino acid
621 insertion (*i.e.*, NS2A G11A+C_T101) partially restored virion assembly ($1.2\times10^3$ PFU/ml;
622 Fig. 4D-E), prM-positive cells (Fig. 4G), and C-prM cleavage (60%; Fig. 4H); while the



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capsid double-amino acid insertion (*i.e.*, NS2A G11A+C_TA and NS2A G11A+C_SR) almost fully restored virion assembly to the WT level (2.3-2.5×$10^6$ PFU/ml; Fig. 4D-E) and C-prM cleavage (92-98%; Fig. 4H). These results demonstrate that addition of one or two amino acids to the N-terminus of anchorC can rescue virion assembly of NS2A G11A.

Since the single-amino acid C_T101 insertion only partially rescued virion assembly of NS2A G11A, we selected revertants for NS2A G11A+C_T101 virus. After continuous passaging of the virus on Vero cells for eight rounds, the P8 viruses were subjected to plaque assay (Fig. S8A-B) and whole-genome sequencing (Fig. S8C). The P8 viruses from all four independent selections produced viral titers (Fig. S8B) that were ≥100-fold higher than the original NS2A G11A+C_T101 virus (Fig. 4E). Among them, selection I P8 virus retained the original NS2A G11A+C_T101 mutations. In addition, an extra mutation R100S was recovered in the capsid protein. Sequence analysis suggests that the R100S could alter the protease cleavage site from the original RRRR↓T to RRR↓ST (R100S underlined) and extends the N-terminus of anchorC by one more amino acid. Selection II P8 virus also retained the original NS2A G11A+C_T101 mutations and accumulated another mutation R99S in the capsid protein. The R99S may change the protease cleavage site to RR↓SRT and extends the N-terminus of anchorC by two more amino acids. In addition, selection II P8 virus contained the R100S mutation in 27% of the viral population. Selection III P8 virus retained the NS2A G11A mutation and showed mixed sequences of R99S and/or R100S. Selection IV P8 virus exhibited WT plaque morphology; sequencing results showed that the NS2A G11A was reverted to WT Gly, but the C_T101 insertion was retained. Collectively, the results again support the conclusion that extension of the N-terminus of anchorC by two to three residues could efficiently rescue virion assembly of NS2A G11A.



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**Analysis of NS2A G11A in mosquito cells.** To test if the above findings from mammalian cells could be extended to mosquito host, we analyzed NS2A G11A+capsid mutant RNAs in C6/36 cells. Viral RNAs containing NS2A G11A alone, NS2A G11A+C_T101, NS2A G11A+C_R99S, or NS2A G11A+C_TA were transfected into C6/36 cells. The transfected cells were analyzed for capsid protein-positive cells (Fig. S9A), infectious virus production (Fig. S9B-C), extracellular viral RNA release (Fig. S9D), and C-prM cleavage (Fig. S9E). Similar to the results obtained from mammalian cells, NS2A G11A mutant was defective in C-prM processing and virion production in C6/36 cells. The defects could be rescued by the capsid mutations, with the rescuing efficiency of C_TA > C_R99S > C_T101 (Fig. S9). The results indicate that NS2A G11A mutation exerts the same mechanism in abolishing virion assembly in mammalian and mosquito hosts.

**NS2A protein binds to 3'UTR RNA**. To further explore the role of DENV-2 NS2A in virion assembly, we examined the ability of NS2A to bind viral genomic RNA. A series of pulldown experiments were performed using biotinylated viral RNAs and NS2A protein (Fig. 5A). A 616-nucleotide viral RNA (5'UTR-$C_{66}$-3'UTR) comprising the 5'UTR, the first 66 nucleotides from the C gene, and the 3'UTR regions of DENV-2 genome was *in vitro* synthesized (Fig. S10A). The 3' end of the RNA was ligated to a biotinylated cytidine using T4 RNA ligase, resulting in 5'UTR-$C_{66}$-3'UTR-biotin RNA (Fig. S10B). Two types of GST-tagged NS2A protein were prepared for the pulldown experiment: One type NS2A was expressed from *E. coli*, partially purified, and reconstituted in detergent DDM-containing buffer (Fig. 5B); another type NS2A was from the cell lysates of HEK293T cells transiently expressing the NS2A protein (Fig. 5C). As shown in Fig. 5B-C, the 5'UTR-$C_{66}$-3'UTR-biotin RNA efficiently pulled down GST-tagged NS2A (Lanes 4 and 6), but not GST (Lanes 2 and 5); in contrast, neither GST nor GST-tagged NS2A proteins was pulled down by a biotinylated non-viral RNA control (Lanes 1 and 3). To further demonstrate the specificity



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of NS2A/5'UTR-$C_{66}$-3'UTR RNA interaction, we performed an RNA competition experiment. Increasing amounts un-biotinylated 5'UTR-$C_{66}$-3'UTR RNA competed away the NS2A/5'UTR-$C_{66}$-3'UTR-biotin RNA interaction, whereas yeast tRNA did not (Fig. 5D). These results suggest that 5'UTR-$C_{66}$-3'UTR RNA specifically binds to NS2A protein.

We estimated the binding affinity of NS2A/viral RNA interaction. 5'UTR-$C_{66}$-3'UTR-biotin RNA was incubated with increasing amounts of recombinant GST-NS2A protein. The reactions were separated on a native polyacrylamide gel (PAGE), transferred to nylon membrane, and probed with streptavidin-HRP (Fig. 5E). Quantification of free RNA and RNA/protein complex estimated a *Kd* of 35 nM for DENV-2 NS2A biding to viral RNA.

**The last 285 nucleotides of 3'UTR RNA is responsible for binding to NS2A protein**. To map the region of viral RNA responsible for binding to NS2A protein, we performed a competition assay using a set of un-biotinylated RNA fragments from 5'UTR-$C_{66}$-3'UTR (Fig. S10C). The fragments were prepared based on the previously proposed RNA structure [ ADDIN EN.CITE <EndNote><Cite><Author>Villordo</Author><Year>2015</Year><RecNum>109</RecNum><DisplayText>(Villordo et al., 2015)</DisplayText><record><rec-number>109</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1561296709">109</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Villordo, S. M.</author><author>Filomatori, C. V.</author><author>Sanchez-Vargas, I.</author><author>Blair, C. D.</author><author>Gamarnik, A. V.</author></authors></contributors><auth-address>Fundacion Instituto Leloir-CONICET, Buenos Aires, Argentina.Arthropod-borne and Infectious Diseases Laboratory, Department of Microbiology, Immunology and Pathology, Colorado State University, Fort Collins, Colorado, United States of America.</auth-address><titles><title>Dengue virus



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711 urls></urls><custom2>PMC4311971</custom2><electronic-resource-
712 num>10.1371/journal.ppat.1004604</electronic-resource-
713 num></record></Cite></EndNote>]. As shown in Fig. 5F, 3'UTR, but not 5'UTR-$C_{66}$,
714 competed away the NS2A/5'UTR-$C_{66}$-3'UTR-biotin RNA interaction, indicating that 3'UTR
715 RNA binds to NS2A protein. To further map the region of 3'UTR for NS2A binding, we
716 deleted pseudoknot 1 (Δpk1), pseudoknots 1-2 [Δpk(1-2)], and pseudoknots 1-3 [Δpk(1-3)]
717 from the 3'UTR (Fig. S10C). Only Δpk1 and Δpk(1-2) RNAs, not Δpk(1-3) RNA, could
718 compete away the NS2A/5'UTR-$C_{66}$-3'UTR-biotin RNA interaction (Fig. 5F), suggesting
719 that Δpk(1-2) RNA, representing the last 285 nucleotides of 3'UTR RNA, is required for
720 binding to NS2A protein. This conclusion was further supported by the results that different
721 fragments from Δpk(1-2) RNA, including pK(3-4), pk3, and 3'SL, did not compete away the
722 NS2A/5'UTR-$C_{66}$-3'UTR-biotin RNA interaction (Fig. 5F).



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Next, we performed a photo-activatable ribonucleoside-enhanced crosslinking (PAR-CL) experiment to define the nucleotides that may interact with NS2A protein (Fig. 5G). The 3'UTR RNA containing [ HYPERLINK "https://en.wikipedia.org/wiki/Photosensitivity" \o "Photosensitivity" ] 4-thiouridine was prepared through *in vitro* transcription. After RNA/protein complex formation, the 4-thiouridine in the 3'UTR RNA was cross-linked to NS2A protein under UV light (at 365-nm wavelength). The RNA was then subjected to deep sequencing. Due to the crosslinking of 4-thiouridine to protein, a T-to-C mutation was recovered at the RNA sites that interacted with NS2A protein (Figs. 5G and S11). Fig. 5H summarizes the U-to-C mutations in the 3'UTR RNA when complexed with NS2A protein. About 67% mutations occurred within the last 285 nucleotides of 3'UTR. The result further supports the NS2A/3'UTR RNA interaction.

**NS2A requires amino acids R94, K95, and K99A for binding to 3'UTR RNA and virion assembly.** To map the region of NS2A required for viral 3'UTR RNA binding, we prepared a panel of purified NS2A truncates with an N-terminal GST tag from *E. coli* (Fig. 6A). The binding efficiencies of NS2A truncates to 5'UTR-$C_{66}$-3'UTR-biotin RNA were compared using a pulldown assay. As shown in Fig. 6B, different NS2A truncates were pulled down by 5'UTR-$C_{66}$-3'UTR-biotin at varied efficiencies. The RNA pulled down NS2A 1-145, 1-168, and 1-218 (full-length) at comparable efficiencies. Compared to the full-length NS2A, the pulldown efficiencies for NS2A 1-101 and 1-71 decreased to 40% and 0%, respectively. These data suggest that amino acids 72-144 are required for NS2A binding to viral RNA.

According to the membrane topology of DENV-2 NS2A [ ADDIN EN.CITE <EndNote><Cite><Author>Nemesio</Author><Year>2014</Year><RecNum>82</RecNum><DisplayText>(Nemesio and Villalain, 2014)</DisplayText><record><rec-number>82</rec-number><foreign-keys><key app="EN" db-



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id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793217">82</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Nemesio, H.</author><author>Villalain, J.</author></authors></contributors><auth-address>Molecular and Cellular Biology Institute, Universitas "Miguel Hernandez" , E-03202 Elche-Alicante, Spain.</auth-address><titles><title>Membrane interacting regions of Dengue virus NS2A protein</title><secondary-title>J Phys Chem B</secondary-title></titles><periodical><full-title>J Phys Chem B</full-title></periodical><pages>10142-55</pages><volume>118</volume><number>34</number><keywords><keyword>Calorimetry, Differential Scanning</keyword><keyword>Cell Membrane/*metabolism</keyword><keyword>Fluorescence Polarization</keyword><keyword>*Hydrophobic and Hydrophilic Interactions</keyword><keyword>Peptide Library</keyword><keyword>Spectrophotometry, Infrared</keyword><keyword>Viral Nonstructural Proteins/*chemistry/*metabolism</keyword></keywords><dates><year>2014</year><pub-dates><date>Aug 28</date></pub-dates></dates><isbn>1520-5207 (Electronic)1520-5207 (Linking)</isbn><accession-num>25119664</accession-num><urls><related-urls><url>http://www.ncbi.nlm.nih.gov/pubmed/25119664</url></related-urls></urls><custom2>PMC4148155</custom2><electronic-resource-num>10.1021/jp504911r</electronic-resource-num></record></Cite></EndNote>], amino acids 72-144 form two transmembrane domains (72-92 and 101-118), one short cytosolic loop (93-100), and a luminal segment (118-144; Fig. 6A). Since viral RNA resides in the cytoplasm, we examined the cytosolic loop of NS2A for viral RNA binding. The cytosolic



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loop has three positively charged residues (R94, K95, and K99) that are conserved among the four DENV serotypes (Fig. 6C). To examine the function of these residues, we prepared a panel of recombinant DENV-2 containing Ala substitutions at single (R94A, K95A, or K99A), double (R94A+K95A), or triple residues (R94A+K95A+K99A). Upon transfection to BHK-21 cells, all mutant RNAs generated viral E protein-positive cells at day 1 (Fig. 6D). Except for the triple mutant, other RNAs produced more E-positive cells on day 4 post-transfection. Mutants R94A and K95A generated WT level of infectious viruses and plaque morphology (Fig. 6E-F). Mutants K99A and K94A+K95A generated 22- to 3-fold less viruses than the WT, respectively (Fig. 6F), and exhibited smaller plaques (Fig. 6E). Remarkably, triple mutant R94A+K95A+K99A RNA yielded almost undetectable amount of virus that was 50,000-fold lower than the WT (Fig. 6F). To examine the effect of R94A+K95A+K99A on viral RNA synthesis, we engineered the triple mutations into a luciferase replicon of DENV-2 (Fig. 6G). The R94A+K95A+K99A replicon generated a luciferase profile similar to the WT replicon, indicating that the mutations do not affect viral translation and RNA synthesis. Taken together, the results demonstrate that R94A+K95A+K99A triple mutant is defective in virion assembly.

Next, we examined the effect of R94A+K95A+K99A mutations on NS2A binding to viral RNA. Since the result from Fig. 6B indicates that NS2A fragment 72-144 is required for viral RNA binding, we expressed a short NS2A peptide 64-128 (comprising the first two NS2A transmembrane domains, the cytosolic region 93-100, and the N- and C-terminal luminal tails for correct folding of the peptide on membrane) fused with an N-terminal GST-tag (Fig. 6H). As shown in Fig. 6I, peptide 64-128 could be pulled down by 5'UTR-$C_{66}$-3'UTR-biotin RNA (Lane 1). Un-biotinylated 5'UTR-$C_{66}$-3'UTR or 3'UTR RNA (Lanes 2 and 4), but not 5'UTR-$C_{66}$ RNA or yeast tRNA (Lanes 3 and 5), could compete away the NS2A peptide 64-128/5'UTR-$C_{66}$-3'UTR-biotin RNA interaction. The result demonstrates that



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NS2A peptide 64-128 specifically binds to viral 3'UTR RNA. Importantly, mutant R94A+K95A+K99A reduced the pulldown efficiency of NS2A peptide 64-128 by over 80% (Fig. 6J). Notably, some degraded GST-tag was found to associate with the NS2A truncates in the above pulldown experiments (Figs. 6B I-J), most likely due to the dimerization property of GST[ ADDIN EN.CITE <EndNote><Cite><Author>Maru</Author><Year>1996</Year><RecNum>110</RecNum><DisplayText>(Maru et al., 1996)</DisplayText><record><rec-number>110</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1561302524">110</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Maru, Y.</author><author>Afar, D. E.</author><author>Witte, O. N.</author><author>Shibuya, M.</author></authors></contributors><auth-address>Department of Genetics, Institute of Medical Science, University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108, Japan.</auth-address><titles><title>The dimerization property of glutathione S-transferase partially reactivates Bcr-Abl lacking the oligomerization domain</title><secondary-title>J Biol Chem</secondary-title></titles><periodical><full-title>J Biol Chem</full-title></periodical><pages>15353-7</pages><volume>271</volume><number>26</number><keywords><keyword>*Adaptor Proteins, Signal Transducing</keyword><keyword>Animals</keyword><keyword>Bone Marrow Cells</keyword><keyword>Cell Transformation, Neoplastic</keyword><keyword>Cells, Cultured</keyword><keyword>Fusion Proteins, bcr-abl/*metabolism</keyword><keyword>GRB2 Adaptor Protein</keyword><keyword>Glutathione Transferase/*metabolism</keyword><keyword>Macromolecular Substances</keyword><keyword>Mice</keyword><keyword>Phosphotyrosine/metabolism</keyword><keyword>Protein Binding</keyword><keyword>Protein-Tyrosine



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825 Kinases/metabolism</keyword><keyword>Proteins/metabolism</keyword><keyword>Rat
826 s</keyword><keyword>Recombinant Fusion Proteins</keyword><keyword>Structure-
827 Activity Relationship</keyword></keywords><dates><year>1996</year><pub-
828 dates><date>Jun 28</date></pub-dates></dates><isbn>0021-9258 (Print)0021-
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830 urls><url>https://www.ncbi.nlm.nih.gov/pubmed/8663064</url></related-
831 urls></urls><electronic-resource-num>10.1074/jbc.271.26.15353</electronic-resource-
832 num></record></Cite></EndNote>]. Overall, the results indicate that R94, K95, and K99
833 are important for NS2A binding to viral 3'UTR.

834 **DISCUSSION**

835 Flavivirus assembly consists of a tightly controlled cascade of events: export of
836 newly synthesized viral RNA from the replication complex to assembly sites, encapsidation
837 of viral RNA with C protein, and envelopment of nucleocapsid with prM and E proteins.
838 Many host and viral factors (proteins and lipids) participate in this temporal and spatial
839 process [ ADDIN EN.CITE <EndNote><Cite><Author>Apte-
840 Sengupta</Author><Year>2014</Year><RecNum>94</RecNum><DisplayText>(Apte-
841 Sengupta et al., 2014)</DisplayText><record><rec-number>94</rec-number><foreign-
842 keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv"
843 timestamp="1560793218">94</key></foreign-keys><ref-type name="Journal
844 Article">17</ref-type><contributors><authors><author>Apte-Sengupta,
845 S.</author><author>Sirohi, D.</author><author>Kuhn, R.
846 J.</author></authors></contributors><auth-address>Markey Center for Structural Biology,
847 Department of Biological Sciences and Bindley Bioscience Center, Purdue University,
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849 Biological Sciences and Bindley Bioscience Center, Purdue University, West Lafayette, IN,
850 USA. Electronic address: kuhnr@purdue.edu.</auth-address><titles><title>Coupling of
851 replication and assembly in flaviviruses</title><secondary-title>Curr Opin
852 Virol</secondary-title></titles><periodical><full-title>Curr Opin Virol</full-
853 title></periodical><pages>134-
854 42</pages><volume>9</volume><keywords><keyword>Flavivirus/*physiology</keyword>
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857 Metabolism</keyword><keyword>Viral Proteins/metabolism</keyword><keyword>*Virus
858 Assembly</keyword><keyword>*Virus
859 Replication</keyword></keywords><dates><year>2014</year><pub-
860 dates><date>Dec</date></pub-dates></dates><isbn>1879-6265 (Electronic)1879-
861 6257 (Linking)</isbn><accession-num>25462445</accession-num><urls><related-
862 urls><url>http://www.ncbi.nlm.nih.gov/pubmed/25462445</url></related-
863 urls></urls><custom2>PMC4268268</custom2><electronic-resource-
864 num>10.1016/j.coviro.2014.09.020</electronic-resource-
865 num></record></Cite></EndNote>]. Among them, flaviviral NS2A has emerged as a key
866 player to orchestrate virion assembly, as evidenced by the following findings. (i) In infected
867 cells, there are two distinct NS2A species that are required for viral RNA synthesis and
868 virion assembly. The NS2A species required for RNA synthesis is located at the viral
869 replication complex that could only be formed in *cis*; the other NS2A species required for
870 virus morphogenesis is located at the virion assembly complex that could be formed in *cis*
871 or *trans* [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. (ii) The defect in virion assembly
872 caused by DENV-2 and YFV NS2A mutations could be rescued by adaptive mutations in
873 prM, E, NS2B, and NS3 proteins, suggesting that NS2A genetically modulates other viral
874 proteins during virion assembly [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. (iii)



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875 Recombinant NS2A of Kunjin virus binds to viral 3'UTR RNA [ ADDIN EN.CITE
876 <EndNote><Cite><Author>Mackenzie</Author><Year>1998</Year><RecNum>81</RecN
877 um><DisplayText>(Mackenzie et al., 1998)</DisplayText><record><rec-number>81</rec-
878 number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv"
879 timestamp="1560793217">81</key></foreign-keys><ref-type name="Journal
880 Article">17</ref-type><contributors><authors><author>Mackenzie, J.
881 M.</author><author>Khromykh, A. A.</author><author>Jones, M.
882 K.</author><author>Westaway, E.
883 G.</author></authors></contributors><titles><title>Subcellular localization and some
884 biochemical properties of the flavivirus Kunjin nonstructural proteins NS2A and
885 NS4A</title><secondary-title>Virology</secondary-title></titles><periodical><full-
886 title>Virology</full-title></periodical><pages>203-
887 15</pages><volume>245</volume><number>2</number><keywords><keyword>Animal<
888 /keyword><keyword>Cercopithecus aethiops</keyword><keyword>*Flavivirus/ph
889 [Physiology]</keyword><keyword>Immunohistochemistry</keyword><keyword>RNA,
890 Viral/me [Metabolism]</keyword><keyword>Support, Non-U.S.
891 Gov't</keyword><keyword>Vero Cells</keyword><keyword>*Viral Nonstructural
892 Proteins/me [Metabolism]</keyword><keyword>Virus
893 Replication</keyword></keywords><dates><year>1998</year></dates><urls></urls></re
894 cord></Cite></EndNote>]. These studies indicate that NS2A interacts with two of the three
895 structural proteins (prM and E, not C) and viral RNA. However, how viral RNA and C
896 protein are recruited to form nucleocapsid and how nucleocapsid is enveloped with prM-E
897 proteins to form virion remain unknown. The current study aims to address these
898 questions.



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First, we showed that DENV-2 NS2A interacted with prM to modulate virion assembly. WT NS2A directly bound to prM in the absence of other viral proteins (Fig. 3B). The biological relevance of this interaction was supported by the correlation between virion assembly and prM/NS2A interaction. Specifically, an NS2A mutation G11A, which abolished virion assembly (Fig. 1), enhanced its binding to prM by 1.7-2.5 folds (Fig. 3). Mechanistically, the enhanced interaction between the mutant NS2A and prM "over pulled" the membrane-associated anchorC towards the ER lumen, leading to a blockage of the viral protease cleavage site (most likely by the ER membrane) at the C-anchorC junction. The uncleaved C-prM resulted in a rapid degradation of all three structural proteins (Fig. 1). This is not surprising because the unprocessed C-prM is retained on the ER membrane, misfolded, and degraded via the ubiquitin-proteasome machinery (Fig. 1H). Since prM is a chaperone for E protein [ ADDIN EN.CITE <EndNote><Cite><Author>Lorenz</Author><Year>2002</Year><RecNum>96</RecNum><DisplayText>(Lorenz et al., 2002)</DisplayText><record><rec-number>96</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793218">96</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Lorenz, I. C.</author><author>Allison, S. L.</author><author>Heinz, F. X.</author><author>Helenius, A.</author></authors></contributors><auth-address>Institute of Biochemistry, Swiss Federal Institute of Technology, CH-8093 Zurich, Switzerland.</auth-address><titles><title>Folding and dimerization of tick-borne encephalitis virus envelope proteins prM and E in the endoplasmic reticulum</title><secondary-title>J Virol</secondary-title></titles><periodical><full-title>J Virol</full-title></periodical><pages>5480-91</pages><volume>76</volume><number>11</number><keywords><keyword>Animals</keyword><keyword>COS Cells</keyword><keyword>Cercopithecus



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925 aethiops</keyword><keyword>Dimerization</keyword><keyword>Dithiothreitol</keyword
926 ><keyword>Encephalitis Viruses, Tick-
927 Borne/genetics/*metabolism</keyword><keyword>Hexosaminidases/metabolism</keywor
928 d><keyword>Polyproteins/genetics/metabolism</keyword><keyword>*Protein
929 Folding</keyword><keyword>Protein
930 Precursors/genetics/metabolism</keyword><keyword>Recombinant Fusion
931 Proteins/genetics/metabolism</keyword><keyword>Viral Envelope
932 Proteins/genetics/*metabolism</keyword></keywords><dates><year>2002</year><pub-
933 dates><date>Jun</date></pub-dates></dates><isbn>0022-538X (Print)0022-538X
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935 urls><url>http://www.ncbi.nlm.nih.gov/pubmed/11991976</url></related-
936 urls></urls><custom2>PMC137023</custom2><electronic-resource-
937 num>10.1128/jvi.76.11.5480-5491.2002</electronic-resource-
938 num></record></Cite></EndNote>], the degradation of C-prM leads to the degradation of
939 E protein. These results are in agreement with a recent report that ZIKV anchorC plays an
940 important role in the stability of E protein and virion morphogenesis [ ADDIN EN.CITE
941 <EndNote><Cite><Author>Rana</Author><Year>2018</Year><RecNum>97</RecNum><
942 DisplayText>(Rana et al., 2018)</DisplayText><record><rec-number>97</rec-
943 number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv"
944 timestamp="1560793219">97</key></foreign-keys><ref-type name="Journal
945 Article">17</ref-type><contributors><authors><author>Rana, J.</author><author>Slon
946 Campos, J. L.</author><author>Leccese, G.</author><author>Francolini,
947 M.</author><author>Bestagno, M.</author><author>Poggianella,
948 M.</author><author>Burrone, O. R.</author></authors></contributors><auth-
949 address>Molecular Immunology Group, International Centre for Genetic Engineering and
950 Biotechnology, Trieste, Italy rana@icgeb.org burrone@icgeb.org.Molecular



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951 Immunology Group, International Centre for Genetic Engineering and Biotechnology,
952 Trieste, Italy.Department of Medical Biotechnology and Translational Medicine,
953 Universita degli Studi di Milano, Milan, Italy.</auth-address><titles><title>Role of Capsid
954 Anchor in the Morphogenesis of Zika Virus</title><secondary-title>J Virol</secondary-
955 title></titles><periodical><full-title>J Virol</full-
956 title></periodical><volume>92</volume><number>22</number><keywords><keyword>Zi
957 ka virus</keyword><keyword>capsid
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959 keyword>pseudovirus</keyword><keyword>viral
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963 urls><url>http://www.ncbi.nlm.nih.gov/pubmed/30158295</url></related-
964 urls></urls><custom2>PMC6206493</custom2><electronic-resource-
965 num>10.1128/JVI.01174-18</electronic-resource-num></record></Cite></EndNote>].

966 Second, we demonstrated that the defect in virion assembly of NS2A G11A could
967 be restored by two different means. Creation of a new viral protease cleavage site by
968 extending the N-terminus of anchorC by two amino acids restored the capsid-anchorC
969 processing and, consequently, restored virion assembly. This conclusion was supported
970 by revertant analysis of the NS2A G11A mutant (Figs. 2 and S4) and amino acid insertion
971 experiment (Fig. 4). The two-amino acid extension exposes the new protease cleavage
972 site to NS2B/NS3. Alternatively, the virion assembly defect of NS2A G11A could be
973 rescued by *trans* supply of WT NS2A (Fig. S3). Under this condition, the WT NS2A
974 replaced the G11A NS2A to interact with C-prM, restored the C-prM cleavage, and ensued
975 a productive virion morphogenesis. Collectively, our results support a previous model that



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the two cleavages at C-anchorC-prM junctions are coordinated in a sequential manner during flavivirus assembly. The C-anchorC junction was first cleaved by viral protease on the cytosolic side, followed by the anchorC-prM cleavage by the cellular signalase on the ER lumen side [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. Although expression of flavivirus prM-E alone could form empty virus-like particles, the coordinated C-anchorC-prM processing couples the encapsidation and envelopment events; such coupling is required for the production of infectious virus. This conclusion is also supported by the previous reports that separate expression of C and prM-E significantly compromised the assembly efficiency of infectious virions [ ADDIN EN.CITE ADDIN EN.CITE.DATA ].

Third, we characterized the specific interaction between DENV-2 NS2A and the 3'UTR of viral genome. Although Kunjin virus NS2A was reported to bind to the 3'UTR of viral RNA [ ADDIN EN.CITE <EndNote><Cite><Author>Mackenzie</Author><Year>1998</Year><RecNum>81</RecNum><DisplayText>(Mackenzie et al., 1998)</DisplayText><record><rec-number>81</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793217">81</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Mackenzie, J. M.</author><author>Khromykh, A. A.</author><author>Jones, M. K.</author><author>Westaway, E. G.</author></authors></contributors><titles><title>Subcellular localization and some biochemical properties of the flavivirus Kunjin nonstructural proteins NS2A and NS4A</title><secondary-title>Virology</secondary-title></titles><periodical><full-title>Virology</full-title></periodical><pages>203-15</pages><volume>245</volume><number>2</number><keywords><keyword>Animal</keyword><keyword>Cercopithecus aethiops</keyword><keyword>*Flavivirus/ph



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1001 [Physiology]</keyword><keyword>Immunohistochemistry</keyword><keyword>RNA,
1002 Viral/me [Metabolism]</keyword><keyword>Support, Non-U.S.
1003 Gov't</keyword><keyword>Vero Cells</keyword><keyword>*Viral Nonstructural
1004 Proteins/me [Metabolism]</keyword><keyword>Virus
1005 Replication</keyword></keywords><dates><year>1998</year></dates><urls></urls></re
1006 cord></Cite></EndNote>], the molecular details of the interaction has not been delineated.
1007 Using competition assay and PAR-CLIP technique, we mapped that DENV-2 NS2A bound
1008 to the last 285 nucleotides of the 3'UTR, including the pk3, pk4, and 3'SL. Interestingly, the
1009 pk3, pk3-4, or 3'SL RNA alone did not efficiently compete out the NS2A/3'UTR RNA
1010 interaction (Fig. 5F), suggesting that a complex RNA structure in the 3'UTR is required for
1011 efficient binding to NS2A protein. Since no RNA “packaging signal” has been identified for
1012 flavivirus assembly and the C protein was shown to non-specifically bind to any RNA
1013 [ ADDIN EN.CITE
1014 <EndNote><Cite><Author>Pong</Author><Year>2011</Year><RecNum>102</RecNum>
1015 <DisplayText>(Pong et al., 2011)</DisplayText><record><rec-number>102</rec-
1016 number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv"
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1019 L.</author><author>Huang, Z. S.</author><author>Teoh, P. G.</author><author>Wang,
1020 C. C.</author><author>Wu, H. N.</author></authors></contributors><auth-
1021 address>Institute of Molecular Biology, Academia Sinica, Taipei, Taiwan.</auth-
1022 address><titles><title>RNA binding property and RNA chaperone activity of dengue virus
1023 core protein and other viral RNA-interacting proteins</title><secondary-title>FEBS
1024 Lett</secondary-title><alt-title>FEBS letters</alt-title></titles><periodical><full-title>FEBS
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1026 Lett</full-title><abbr-1>FEBS letters</abbr-1></alt-periodical><pages>2575-



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81</pages><volume>585</volume><number>16</number><keywords><keyword>Base Sequence</keyword><keyword>*Dengue Virus</keyword><keyword>Genome, Viral/genetics</keyword><keyword>Molecular Chaperones/*metabolism</keyword><keyword>Nucleic Acid Conformation</keyword><keyword>Protein Binding</keyword><keyword>Protein Engineering</keyword><keyword>RNA Helicases/metabolism</keyword><keyword>RNA, Catalytic/genetics</keyword><keyword>RNA, Viral/chemistry/genetics/*metabolism</keyword><keyword>Serine Endopeptidases/metabolism</keyword><keyword>Viral Core Proteins/*metabolism</keyword><keyword>Viral Nonstructural Proteins/metabolism</keyword></keywords><dates><year>2011</year><pub-dates><date>Aug 19</date></pub-dates></dates><isbn>1873-3468 (Electronic)0014-5793 (Linking)</isbn><accession-num>21771593</accession-num><urls><related-urls><url>http://www.ncbi.nlm.nih.gov/pubmed/21771593</url></related-urls></urls><electronic-resource-num>10.1016/j.febslet.2011.06.038</electronic-resource-num></record></Cite></EndNote>], the last 285 nucleotides of 3'UTR may serve as the "packaging signal" to recruit viral RNA to the virion assembly site through specific binding to NS2A. From the NS2A side, we have mapped the 3'UTR-binding site to a cytosolic loop (containing R94, K95, and K99) between the first and second transmembrane domains (Fig. 6). Triple mutant R94A+K95A+K99A was defective in virion assembly and lost over 80% of the NS2A binding activity to 3'UTR RNA (Fig. 6). Caution should be taken when applying the DENV-2 findings to other flaviviruses because of the variations of NS2A proteins. The three positively charged R94, K95, and K99 of NS2A are only conserved among the four serotypes of DENV. In addition, DENV-2, YFV, and ZIKV NS2A proteins



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exhibit different topologies on the ER membrane [ ADDIN EN.CITE ADDIN EN.CITE.DATA ].

Our findings support a general model for flavivirus virion assembly (Fig. 7). In infected cells, NS2A molecules reside in both replication complex (housed in Ve) and viron assembly complex (located on the juxtaposed ER membrane). When plus-sense RNAs are made and released from the replication complex (through the pore on Ve), they are initially destined to new rounds of viral translation and replication on the ER. Once the amounts of viral RNA and proteins have accumulated over certain “thresholds”, nascent RNAs are switched for virion assembly. At this time, the newly synthesized RNA exits the replication complex and binds to the NS2A located at the assembly site through the 3'UTR RNA. The NS2A molecules at the assembly site also recruits C-prM-E and NS2B/NS3 through binding to prM and NS3, respectively. One NS2A molecule does not have to bind to the 3’UTR RNA, C-prM-E, and NS2B/NS3 at the same time. These interactions could be sequential or through different NS2A molecules that oligomerize together on the ER membrane [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. Next, the C-prM-E polyprotein is sequentially cleaved by the NS2B/NS3 protease and host signalase to produce mature C, prM, and E [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. The C molecules, many of which have been previously made and stored at the nearby lipid droplets [ ADDIN EN.CITE ADDIN EN.CITE.DATA ], bind viral RNA to form nucleocapsid. The processed prM undergoes conformational change and dimerizes with E. The prM/E dimerization and oligomerization dissociate prM from NS2A. When 180 copies of prM/E oligomerize on the ER membrane, envelopment of the nucleocapsid occurs, generating spiky immature virions. During the above process, NS3 may also use its helicase activity to transport viral RNA from the replication complex to the assembly site and condense the



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RNA during encapsidation. Besides NS3, NS2A or other proteins may also contribute to the translocation of viral RNA from the replication complex to the assembly site.

The above model is centered on the multifunctional role of NS2A in flavivirus replication and virion assembly. Our results have functionally demonstrated the molecular mechanism for two important steps during DENV-2 assembly: (i) The binding of 3'UTR RNA to NS2A servers as a "packaging signal" and (ii) in the presence of viral RNA-NS2A complex, the coordinated cleavage of C-prM-E facilitates the envelopment of nucleocapsid. The proposed model could serve as a framework for future investigations and antiviral development.

**Author contribution**

X.X., J.Z., X.Z., Y.Z., Q.W., H.-P.D. performed experiments and data analysis. X.X., J.Z., X.Z., Y.Z., A.R., V.L.P., X.W., Q.W., H.-P.D, P.-Y.S. designed the experiments and interpreted the results. X.X., P.-Y.S. wrote the manuscript.

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We are grateful to Dr. Mariano A. Garcia-Blanco, Shelton S. Brarick from University of Texas Medical Branch for helpful discussions during this work. P.-Y.S. was supported by NIH grants AI142759, AI[ HYPERLINK "https://public.era.nih.gov/grantfolder/piAppDetails/genericStatus.do?encryptedParam=vXeG1axw2cY.YZxjFgAisBblI6OqVj4LxDR99WdVyTI-X4FuJbCLt4g." \t "_blank" ], and AI136126, and awards from the Kleberg Foundation, John S. Dunn Foundation, Amon G. Carter Foundation, Gilson Longenbaugh Foundation, and Summerfield Robert Foundation. X.X. and J.Z. were awarded with postdoctoral fellowships from the Novartis Institutes for



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Biomedical Research (NIBR) and the McLaughlin Endowment at University of Texas Medical Branch, respectively.



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## Figure titles and legends

**Figure 1 Characterization of NS2A G11A mutant**. (A) Replicon transient transfection assay. Top panel depicts the DENV-2 luciferase replicon. Bottom panel shows the luciferase activities after transfecting BHK-21 cells with equal amounts (10 μg) of WT or NS2A G11A mutant replicon RNA. (B) Quantification of extracellular viral RNA. Top panel depicts the full-length genome of DENV-2. Equal amounts of WT or NS2A G11A mutant viral RNA (10 μg) were electroporated into BHK-21 cells. At different time post-transfection (p.t.), extracellular viral RNAs were quantified by RT-qPCR. The DENV-2 replicon RNA was used as a control for determining the basal level of viral RNA released from dead cells. Bottom panel shows the CT values of the RT-qPCR results. Data show the means and standard deviations from three independent experiments. (C) Plaque morphology. Culture supernatants from (B) were analyzed for virus production using plaque assay. Only WT, not NS2A G11A mutant (N.D.), produced plaques. Plaques were developed on BHK-21 cells after 5 days of infection. (D) Thin-section transmission electron microscope (TEM) images of viral RNA-transfected BHK-21 cells. At 48 h p.t., cells were fixed for TEM analysis. Ve, virus-induced vesicles; T, membrane tubes; Vi, virus particles; ER, endoplasmic reticulum. Mock transfected cells were included as a control. (E-G) Confocal microscopy analysis of subcellular localizations of C, prM, and E in viral RNA-transfected BHK-21 cells. At 48 h p.t., transfected cells were analyzed by IFA. NS3 staining (red) was used as a control. Nuclei (blue) were stained by DAPI. Scale bar, 10 μm. (H) Viral protein processing in viral RNA-transfected BHK-21 cells. At 42 h p.t., BHK-21 (Mock) or viral RNA-transfected cells were treated with 0.01% DMSO (-) or 10 μM MG132 (+). After 6 h treatment, the cells were analyzed by Western blot. (I) Densitometry analysis. The intensities of bands representing specific viral proteins from DMSO-treated cells in (H) was quantified. The amounts of viral proteins derived from WT RNA-transfected cells were set as 100%. Relative intensities are calculated by normalizing the protein intensities derived from NS2A G11A mutant RNA-transfected cells to those from WT RNA-transfected cells. Data shows the means and standard deviations from three independent experiments.

**Figure 2 Restoration of NS2A G11A virion assembly by capsid R99S mutation.** (A) Revertant mutation. BHK-21 cells were transfected with NS2A G11A viral RNA. The transfected cells were consecutively passaged for five rounds to select for revertant viruses. The P5 virus was sequenced for revertant mutations. Changes in nucleotide



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positions and amino acids are indicated. (B) IFA of viral RNA-transfected cells. WT and mutant viral RNAs were electroporated to BHK-21 cells. The transfected cells were analyzed for E protein expression at 24 and 96 h p.t. by IFA. Scale bar, 100 μm. (C) Plaque morphology. Infectious viruses, derived from the RNA-transfected cells, were analyzed by plaque assay. Plaques were developed on day 5 post-infection on BHK-21 cells. N.D., not detectable. (D) Virus production. The culture fluids from (B) were quantified for infectious viruses. Data represent the means and standard deviations from three independent measurements. Limit of detection (L.O.D.), 10 PFU/ml. (E-F) Subcellular distributions of prM, NS1, and C proteins. prM, NS1 and C proteins were detected by DM1, human IgG anti-NS1 and D2C1 antibodies, respectively. Representative IFA images of viral RNA-transfected cells are shown at 24 h post-transfection. White arrows point to nucleus with C protein speckles. (G) Quantitative analysis of C-positive cells. The C-positive cells with cytoplasm or cytoplasm & nucleus distribution from 4-5 random fields were counted and normalized to the total cell numbers. The averages and standard deviations are shown. (H) Viral protein processing in viral RNA-transfected BHK-21 cells at 48 h post-transfection. The percentages of mature C protein were calculated from the following the formula: [C intensity/(C intensity + C-prM intensity)]×100%.

**Figure 3 DENV-2 NS2A interacts with prM and E proteins**. (A) Membrane topologies of DENV-2 prM, E, and NS2A proteins. The NS2A G11 position is indicated. (B) Co-IP of WT NS2A with prM. HEK293T cells were transfected with one plasmid (encoding prM only) or two plasmids (one encoding leader-NS2A-EGFP and the other encoding full-length or the ectodomain of prM). At 40 h p.t., IP was performed using mouse anti-EGFP or control IgG antibodies. Immunoblot (IB) was performed using rabbit anti-EGFP antibody or anti-prM antibody. (C) Co-IP of WT or G11A NS2A-EGFP with prM. Mouse anti-EGFP antibody was used for IP. Rabbit anti-EGFP or anti-prM antibodies were used for IB. The intensity of prM, co-precipitated with WT NS2A-EGFP, was set as 100%. The relative efficiency of prM co-precipitated with G11A NS2A-EGFP was compared with the WT NS2A-EGFP. Statistical analysis was performed using the unpaired parametric t-test. $*p < 0.05$, significant; $**p < 0.01$, very significant. (D) Reciprocal co-IP of prM with WT or G11A NS2A-EGFP. Rabbit anti-prM antibodies were used for IP. The amounts of co-precipitated WT and G11A NS2A-EGFP were detected using mouse anti-EGFP antibody. prM in eluates were detected by rabbit anti-prM antibody followed by protein G conjugated with HRP. (E) Co-localization analysis of NS2A and prM proteins. BHK-21 cells were co-



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transfected with two plasmids: One encoding the $C_{14}$-prM-E and the other encoding SPG-$C_{16}$-NS2A-EGFP. At 24 h p.t., the cells were examined for prM and NS2A-EGFP co-localization under a confocal microscope. Scale bar, 10 μm. (F) Co-IP of NS2A with E protein. HEK293T cells were co-transfected with two plasmids: One encoding the last 17 amino acids of prM and complete E protein ($M_{17}$-E), and another encoding NS2A with a C-terminal GST tag (NS2A-GST). GST tag alone was used as a negative control. IPs were performed using rabbit anti-GST antibody. E and GST fusion proteins were detected in cell lysates and eluates by mouse anti-E monoclonal antibody (9F12) and anti-GST antibody, respectively. The relative pulldown efficiency was calculated by normalizing the intensity of E co-precipitated with G11A NS2A-GST to the intensity of E co-precipitated with the WT NS2A-GST. The means and standard deviations from three independent experiments are shown. N.S., no significance.

**Figure 4 Two-residue insertion at the capsid-anchorC junction rescues the defect of NS2A G11A**. (A) A model depicting how NS2A modulates C-anchorC-prM cleavage. On the cytosolic side, viral NS2B/NS3 protease cleaves the C-anchorC junction. On the ER lumen side, host signalase cleaves the anchorC-prM junction. Three cleavage scenarios are presented for WT, NS2A G11A, and NS2A G11A+C_R99S constructs. (B) Constructs of insertion mutants of capsid protein. One residue (C_101) or two residues (C_TA or C_SR) were inserted to the C-anchorC junction. The potential cleavage sites for viral NS2B/NS3 protease are indicated. (C) IFA of viral RNA-transfected cells. Viral RNAs containing the NS2A G11A mutation with or without capsid insertions were electroporated into BHK-21 cells. The transfected cells were analyzed for E protein expression on day 1 and 4 by IFA. Scale bar, 100 μm. (D) Plaque morphology. Viruses recovered from (C) were analyzed by plaque assay on BHK-21 cells. (E) Viral titers. Culture supernatants from (C) were measured for viral titers on day 5 post-transfection. (F) Subcellular localization of C protein in viral RNA-transfected cells. White arrows point to nucleus with C protein speckles. Scale bar, 25 μm. (G) Expression of prM and NS1 proteins in viral RNA-transfected cells. Scale bar, 50 μm. (H) C-prM processing in viral RNA-transfected cells. At 48 h p.t., WT or NS2A G11A+C_T101 RNA-transfected cells were analyzed for C, NS1, and β-actin expression by Western blot. The percentages of C-prM to mature C conversion are presented for each construct.

**Figure 5 DENV-2 NS2A protein binds to 3'UTR RNA**. (A) Diagram of pulldown assay using biotinylated RNAs. 3'-end biotinylated RNA binds to streptavidin-conjugated



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magnetic beads. After incubating the bead-bound biotinylated RNA with NS2A protein, the RNA-bound NS2A was enriched and eluted for SDS-PAGE and Western blot analysis. (B) Pulldown analysis using recombinant NS2A protein. N-terminally GST-tagged NS2A protein (GST-NS2A) was expressed in *E. coli* and affinity purified. The 5'UTR-$C_{66}$-3'UTR-biotin RNA was prepared by ligating biotinylated cytidine (bis)phosphate to the 3' end of the 5'UTR-$C_{66}$-3'UTR RNA (containing the 5'UTR, the first 66 nucleotides of C gene, and the 3'UTR). The amounts of GST-NS2A in the input and eluates were analyzed using rabbit anti-GST antibody by Western blot. (C) Pulldown analysis using HEK293T cell-expressed NS2A-GST protein. HEK293T cells were transfected with a plasmid expressing GST or C-terminally GST-tagged NS2A protein (NS2A-GST). At 42 h p.t., cells were harvested, lysed, and used for RNA pull down-assay. The pulldown assay was performed as described in (B). (D) RNA competition assay. Various amounts of unlabeled 5'UTR-$C_{66}$-3'UTR RNA were used to compete the binding of 5'UTR-$C_{66}$-3'UTR-biotin RNA to GST-NS2A protein. tRNA was used as a negative control. (E) Viral RNA/NS2A binding affinity. Electrophoretic mobility-shift assay (EMSA) was used to determine the binding affinity of DENV-2 5'UTR-$C_{66}$-3'UTR-biotin RNA with NS2A protein. 5'UTR-$C_{66}$-3'UTR-biotin RNA was incubated with increasing amounts of GST-NS2A protein. Free and bound RNAs were resolved by native agarose gel electrophoresis. The equilibrium dissociation constant (*Kd*) was determined by nonlinear regression with least-squares fitting method. The coefficient of determination ($R^2$) is shown. Data represent the means and standard deviations from three independent experiments. (F) Mapping the RNA region responsible for NS2A binding. A panel of unlabeled RNA truncates of the 5'UTR-$C_{66}$-3'UTR were used as competitors for the binding of 5'UTR-$C_{66}$-3'UTR-biotin RNA to GST-NS2A protein. The intensity of GST-NS2A (bound to the 5'UTR-$C_{66}$-3'UTR-biotin) without competitor was set as 100%. Relative GST-NS2A intensities were calculated as [(signal in the presence competitor)/(signal in the absence of competitor)]×100%. Statistical analysis was performed using the unpaired parametric t-test. (G) PAR-CLIP workflow [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. See text for detail. (H) PAR-CLIP result summary. Uridines in the 3'UTR that may participate in NS2A-binding are indicated. The dash line indicates the threshold (equal to 5.76, representing the average fold change of mutation rate of all uridines determined by Illumina sequencing). The uridine positions with fold changes of mutation rate over the threshold are indicated in the 3'UTR sequence (GenBank no. AF038403).



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**Figure 6 NS2A residues responsible for the NS2A/3'UTR RNA interaction**. (A) Diagram of NS2A truncates used in RNA pulldown assay. All proteins were fused with an N-terminal GST tag, expressed in *E. coli*, and affinity purified. (B) Interactions between NS2A truncates with 5'UTR-$C_{66}$-3'UTR-biotin RNA. The interactions were measured using pulldown experiments. The pulldown efficiency of full-length NS2A (1-218) was set as 100%. Relative efficiency was calculated by comparing the intensity of NS2A truncate with that of the full-length NS2A. (C) Sequence alignment of NS2A among flaviviruses. Amino acids of a cytosolic loop (amino acids 93-101) are presented. The number of viral strains used for the NS2A alignment are indicated in parentheses. Amino acid positions of NS2A are labeled according to DENV-2 strain New Guinea C. Residues R94, K95, and K99 are shaded. The conserved (:) and identical (*) residues are indicated. (D) IFA of BHK-21 cells transfected with viral RNA. WT and mutant viral RNAs were electroporated into BHK-21 cells. The cells were assayed for E protein expression. (E) Plaque morphology. Viruses derived from the transfected cells in (D) were analyzed by plaque assay. (F) Viral titers. Culture supernatants from (D) were measured for virus yields on day 5 post-transfection. (G) Replicon transient transfection assay. NS2A triple mutations (R94A+K95A+K99A) were engineered into a luciferase replicon of DENV-2. Luciferase activities were presented after the WT or mutant replicon RNAs were electroporated into BHK-21 cells. (H) Diagram of N-terminally GST-tagged NS2A peptide 64-128 [GST-NS2A (64-128)]. (I) Interaction of GST-NS2A (64-128) protein with 3'UTR RNA. GST-NS2A (64-128) was expressed in *E. coli* and affinity purified. The pulldown efficiency of GST-NS2A (64-128) by 5'UTR-$C_{66}$-3'UTR-biotin was set as 100%. The binding efficiencies of RNA variants were indicated through competition assay. In the presence of competitor RNAs, the remaining amounts of WT GST-NS2A (64-128)/5'UTR-$C_{66}$-3'UTR-biotin interaction are presented. (J) R94A+K95A+K99A mutations reduce GST-NS2A (64-128) binding to 5'UTR-$C_{66}$-3'UTR-biotin RNA. The R94A+K95A+K99A variant retained only 19.1% of the WT activity.

**Figure 7 A model depicting the role of NS2A in DENV-2 assembly.** Viral replication complex (top) and virion assembly site (bottom) are depicted. DENV-2 NS2A protein recruits viral RNA, three structural proteins, and NS2B/NS3 protease to virion assembly site, where coordinated cleavage of C-prM-E ensues infections virus formation. C molecules accumulated on lipid droplets (LD) could bind viral RNA to form nucleocapsid. See text for details.



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**Reference**

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## STAR METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---|---|---|
| Antibodies | | |
| Mouse IgG Anti-C (D2C1) | [ ADDIN EN.CITE ADDIN EN.CITE.DATA ] | N/A |
| Mouse IgG anti-prM (DM1) | Abcam | Cat# ab41473, RRID:AB_873758 |
| Rabbit IgG anti-prM | GeneTex | GTX128093 |
| Mouse IgG anti-E (4G2) | ATCC | Cat# HB-112, RRID:CVCL_J890 |
| Mouse IgG anti-E (9F12) | [ ADDIN EN.CITE ADDIN EN.CITE.DATA ] | N/A |
| Mouse IgG anti-NS1 | Abcam | Cat# ab41616, RRID:AB_869448 |
| Human IgG anti-NS1 | This study. Generated by Novartis Institute for Tropical Diseases in Singapore | N/A |
| Rabbit IgG anti-NS1 | GeneTex | GTX103346 |
| Rabbit IgG anti-NS3 | GeneTex | GTX124252 |
| Mouse IgG anti-NS5 | GeneTex | GTX629447 |
| Rabbit IgG anti-calnexin | SIGMA-AlDRICH | Cat# C4731, RRID:AB_476845 |
| Mouse IgG anti-EGFP | SIGMA-AlDRICH | Cat# 11814460001, RRID:AB_390913 |
| Moue IgG1 Isotype Control | SIGMA-AlDRICH | Cat# M5284, RRID:AB_1163685 |
| Rabbit IgG anti-EGFP | Abcam | Cat# ab13970, RRID:AB_300798 |
| protein G horseradish peroxidase (HRP) conjugate | Thermo Fisher Scientific | Cat# P21041 |
| Mouse IgG anti-GST | Abcam | Cat# ab19256, RRID:AB_444809 |
| Rabbit IgG anti-GST | SIGMA-AlDRICH | Cat# G7781, RRID:AB_259965 |
| Mouse IgG anti-β-actin | Abcam | Cat# ab8226, RRID:AB_306371 |
| Mouse IgG anti-HA | Abcam | Cat# ab18181, RRID:AB_444303 |
| Goat anti-mouse IgG conjugated with HRP | SIGMA-AlDRICH | Cat# A5278, RRID:AB_258232 |
| Goat anti-rabbit IgG conjugated with HRP | SIGMA-AlDRICH | Cat#AP307P, RRID:AB_92641 |
| Goat anti-mouse IgGs conjugated with Alexa 488 | Thermo Fisher Scientific | Cat#A-1100, RRID:AB_2534069 |
| Goat anti-mouse IgGs conjugated with Alexa 594 | Thermo Fisher Scientific | Cat# A-11005, RRID:AB_2534073 |
| Goat anti-rabbit IgGs conjugated with Alexa 488 | Thermo Fisher Scientific | Cat# A-11008, RRID:AB_143165 |



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| | | |
|---|---|---|
| Goat anti-rabbit IgGs conjugated with Alexa 594 | Thermo Fisher Scientific | Cat# A-11072, RRID:AB_2534116 |
| Goat anti-Human IgG (H+L) cross-adsorbed secondary antibody, Alexa Fluor 594 | Thermo Fisher Scientific | Cat# A-11014, RRID:AB_2534081 |
| Bacterial and Virus Strains | | |
| *E.* coli strain Top 10 | ThermoFisher Scientific | Cat#C404006 |
| *E.* coli strain Rosetta™ 2(DE3) pLysS | MilliporeSigma | Cat#71403-4 |



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| | | |
|---|---|---|
| DENV-2 strain New Guinea C | [ ADDIN EN.CITE <EndNote><Cite><Author>Xie</Author><Year>2013</Year><RecNum>85</RecNum><DisplayText>(Xie et al., 2013)</DisplayText><record><rec-number>85</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793217">85</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Xie, X.</author><author>Gayen, S.</author><author>Kang, C.</author><author>Yuan, Z.</author><author>Shi, P. Y.</author></authors></contributors><auth-address>State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Science, China.</auth-address><titles><title>Membrane topology and function of dengue virus NS2A protein</title><secondary-title>J Virol</secondary-title><alt-title>Journal of virology</alt-title></titles><periodical><full-title>J Virol</full-title></periodical><pages>4609-22</pages><volume>87</volume><number>8</number><edition>2013/02/15</edition><keywords><keyword>Animals</keyword><keyword>Cell Line</keyword><keyword>Cricetinae</keyword><keyword>Dengue | N.A. |



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| | | |
|---|---|---|
| Biological Samples | | |
| None | | |
| Chemicals, Peptides, and Recombinant Proteins | | |
| Peptide WT (NILNRRRRTAGMII) | GenScript | N/A |
| Peptide C_R99S (NILNRRSRTAGMII) | GenScript | N/A |
| Recombinant protein: GST | This study | N/A |
| Recombinant protein: GST-NS2A | This study | N/A |
| Recombinant protein: GST-NS2A(1-71) | This study | N/A |
| Recombinant protein: GST-NS2A(1-101) | This study | N/A |
| Recombinant protein: GST-NS2A(1-145) | This study | N/A |
| Recombinant protein: GST-NS2A(1-168) | This study | N/A |
| Recombinant protein: GST-NS2A(64-128) | This study | N/A |
| Critical Commercial Assays | | |
| iScript SYBR Green One-Step kit | Bio-Rad | Cat#170-8893 |
| QIAamp viral RNA mini kit | Qiagen | Cat#52906 |
| T7 mMessage mMachine kit | Thermo Fisher Scientific | Cat# AM1344 |
| Renilla Luciferase Assay System | Promega Corporation | Cat# E2810 |
| Pierce™ RNA 3' End Biotinylation Kit | Thermo Fisher Scientific | Cat# 20160 |
| Pierce™ Magnetic RNA-Protein Pull-Down kit | Thermo Fisher Scientific | Cat# 20164 |
| Chemiluminescent Detection Module | Thermo Fisher Scientific | Cat# 89880 |
| | | |
| Deposited Data | | |
| Raw image data for illumina sequencing | | |
| Experimental Models: Cell Lines | | |
| BHK-21 cell | ATCC | Cat#CCL-10, RRID:CVCL_1915 |
| Vero cell | ATCC | Cat#CCL-81, RRID:CVCL_0059 |
| HEK293T cell | ATCC | Cat# CRL-3216, RRID:CVCL_0063 |
| NS2A-EGFP BHK-21 cell | [ ADDIN EN.CITE ADDIN EN.CITE.DATA ] | N/A |
| | | |
| Experimental Models: Organisms/Strains | | |
| N.A. | | |
| Oligonucleotides | | |
| Primer P1(5'-ACACTCTTTCCCTACACGA CGCTCTTCCGATCTAAGGCAAAACTAACATGAA AC-3') | Integrated DNA Technologies (Skokie, Illinois) | N/A |
| Primer P2 (5'-GTGACTGGAGTTCAGACG TGTGCTCTTCCGATCTAGAACCTGTTGATTCAA CAGCAC-3') | Integrated DNA Technologies (Skokie, Illinois) | N/A |



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|---|---|---|
| 5' universal adaptor (5'-AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGAC-3') | Illumine (San Diego, CA) | N/A |
| 3' indexing primer (5'-CAAGCAGAAGACGGCATACGAGATnnnnnnGTGACTGGAGTTCAGACGTGT-3') | Illumine (San Diego, CA) | N/A |
| Primer NGC 7764V (5'-CGTCGAGAGAAATATGGTCACACC-3') | Integrated DNA Technologies (Skokie, Illinois) | N/A |
| Primer NGC 7844C (5'CCACAATAGTATGACCAGCCT-3') | Integrated DNA Technologies (Skokie, Illinois) | N/A |
| Recombinant DNA | | |



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|---|---|---|
| pACYC-NGC FL | [ ADDIN EN.CITE <EndNote><Cite><Author>Xie</Author><Year>2013</Year><RecNum>85</RecNum><DisplayText>(Xie et al., 2013)</DisplayText><record><rec-number>85</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793217">85</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Xie, X.</author><author>Gayen, S.</author><author>Kang, C.</author><author>Yuan, Z.</author><author>Shi, P. Y.</author></authors></contributors><auth-address>State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Science, China.</auth-address><titles><title>Membrane topology and function of dengue virus NS2A protein</title><secondary-title>J Virol</secondary-title><alt-title>Journal of virology</alt-title></titles><periodical><full-title>J Virol</full-title></periodical><pages>4609-22</pages><volume>87</volume><number>8</number><edition>2013/02/15</edition><keywords><keyword>Animals</keyword><keyword>Cell Line</keyword><keyword>Cricetinae</keyword><keyword>Dengu | N/A |



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| | | |
|---|---|---|
| pACYC-NGC replicon | [ ADDIN EN.CITE <EndNote><Cite><Author>Xie</Author><Year>2013</Year><RecNum>85</RecNum><DisplayText>(Xie et al., 2013)</DisplayText><record><rec-number>85</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793217">85</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Xie, X.</author><author>Gayen, S.</author><author>Kang, C.</author><author>Yuan, Z.</author><author>Shi, P. Y.</author></authors></contributors><auth-address>State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Science, China.</auth-address><titles><title>Membrane topology and function of dengue virus NS2A protein</title><secondary-title>J Virol</secondary-title><alt-title>Journal of virology</alt-title></titles><periodical><full-title>J Virol</full-title></periodical><pages>4609-22</pages><volume>87</volume><number>8</number><edition>2013/02/15</edition><keywords><keyword>Animals</keyword><keyword>Cell Line</keyword><keyword>Cricetinae</keyword><keyword>Dengue | N/A |



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| | | |
|---|---|---|
| pACYC-NGC replicon G11A | [ ADDIN EN.CITE ADDIN EN.CITE.DATA ] | N/A |
| pACYC-NGC replicon R94A+K95A+K99A | This study | N/A |
| pACYC-NGC FL C_R99S | This study | N/A |
| pACYC-NGC FL G11A | This study | N/A |
| pACYC-NGC FL G11A+C_R99S | This study | N/A |
| pACYC-NGC FL E20A | This study | N/A |
| pACYC-NGC FL E100A | This study | N/A |
| pACYC-NGC FL K188A | This study | N/A |
| pACYC-NGC FL E20A+C_R99S | This study | N/A |
| pACYC-NGC FL E100A+C_R99S | This study | N/A |
| pACYC-NGC FL K188A+C_R99S | This study | N/A |
| pACYC-NGC FL C_TA | This study | N/A |
| pACYC-NGC FL C_SR | This study | N/A |
| pACYC-NGC FL G11A+C_TA | This study | N/A |
| pACYC-NGC FL G11A+C_SR | This study | N/A |
| pACYC-NGC FL C_T101 | This study | N/A |
| pACYC-NGC FL G11A+C_T101 | This study | N/A |
| pACYC-NGC FL R94A | This study | N/A |
| pACYC-NGC FL K95A | This study | N/A |
| pACYC-NGC FL K99A | This study | N/A |
| pACYC-NGC FL R94A+K95A+K99A | This study | N/A |
| pACYC-NGC FL R94A+K95A | This study | N/A |



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| | | |
|---|---|---|
| SFV-DENV2 CprME | [ ADDIN EN.CITE <EndNote><Cite><Author>Qing</Author><Year>2010</Year><RecNum>105</RecNum><DisplayText>(Qing et al., 2010)</DisplayText><record><rec-number>105</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793220">105</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Qing, M.</author><author>Liu, W.</author><author>Yuan, Z.</author><author>Gu, F.</author><author>Shi, P. Y.</author></authors></contributors><auth-address>Novartis Institute for Tropical Diseases, 10, Biopolis Road, #05-01 Chromos, Singapore, Singapore.</auth-address><titles><title>A high-throughput assay using dengue-1 virus-like particles for drug discovery</title><secondary-title>Antiviral Res</secondary-title></titles><periodical><full-title>Antiviral Res</full-title></periodical><pages>163-71</pages><volume>86</volume><number>2</number><dates><year>2010</year><pub-dates><date>May</date></pub- [ PAGE * MERGEFORMAT ] dates></dates><accession-num>20153777</accession-num><urls><related- | N/A |

| | | |
|---|---|---|
| pXJ-SPG-$C_{16}$-NS2A-EGFP | This study | N/A |
| pXJ-SPG-$C_{16}$-NS2A(G11A)-EGFP | This study | N/A |
| pXJ-EGFP | [ ADDIN EN.CITE ADDIN EN.CITE.DATA ] | N/A |
| pXJ-$C_{14}$-prM | This study | N/A |
| pXJ-$C_{14}$-prM(1-130) | This study | N/A |
| pXJ-$C_{14}$-prM (N69Q) | This study | N/A |
| pXJ-SPG-$C_{16}$-NS2A-GST | This study | N/A |
| pXJ-SPG-$C_{16}$-NS2A(G11A)-GST | This study | N/A |
| pXJ-GST | This study | N/A |
| pXJ-HA-NS3 | This study | N/A |
| pXJ-$M_{17}$-E | This study | N/A |
| pGEX-4T-1 | GE Healthcare Life Sciences | Cat# 28954549 |
| pGEX-4T-GST-NS2A | This study | N/A |
| pGEX-4T-GST-NS2A (1-71) | This study | N/A |
| pGEX-4T-GST-NS2A (1-101) | This study | N/A |
| pGEX-4T-GST-NS2A (1-145) | This study | N/A |
| pGEX-4T-GST-NS2A (1-168) | This study | N/A |
| pGEX-4T-GST-NS2A (64-128) | This study | N/A |
| pGEX-4T-GST-NS2A (64-128) R94A+K95A+K99A | This study | N/A |
| | | |
| Software and Algorithms | | |
| Illustrator CC | Adobe | [ HYPERLINK "https://www.adobe.com/products/illustrator.html" ] |
| ImageJ | NIH | [ HYPERLINK "https://fiji.sc/" ] |
| CLC main workbench | Qiagen | [ HYPERLINK "https://www.qiagenbioinformatics.com/products/clc-main-workbench/" ] |
| Prism 8.0 software | GraphPad | [ HYPERLINK "https://www.graphpad.com/scientific-software/prism/" ] |
| | | |

1271

1272 **Contact for Reagent and Resource Sharing**

1273 Further information and requests for resources and reagents should be directed to and will
1274 be fulfilled by Lead Contact, Dr. Pei-Yong Shi (peshi@utmb.edu)

1275 **Experimental Model and Subject Details**



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## Cell Lines

The Baby hamster kidney fibroblast (BHK-21) cells, African green monkey kidney epithelial (Vero) cells were purchased from the American Type Culture Collection (ATCC, Bethesda, MD) and maintained in a high-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (HyClone Laboratories, South Logan, UT) and 1% penicillin/streptomycin (P/S). The NS2A-EGFP BHK-21 cells constitutively expressing the C-terminally EGFP-tagged NS2A protein was maintained in the high-glucose DMEM medium supplemented with 10%FBS, 1% P/S and 1 mg/ml Geneticin. HEK293T cells were grown in low-glucose DMEM (Invitrogen) containing 10% FBS and 1% penicillin-streptomycin. All mammalian cell lines were grown at 37°C with 5% $CO_2$. Mosquito C6/36 cells were grown in RPMI 1640 medium (Invitrogen) with 10% FBS and 1% penicillin-streptomycin at 28°C without $CO_2$. All culture medium and antibiotics were purchased from ThermoFisher Scientific (Waltham, MA). All cell lines were tested negative for mycoplasma.

## Pseudo-infectious Particle

Pseudo-infectious particles were produced using the a previously described protocol [ ADDIN EN.CITE <EndNote><Cite><Author>Qing</Author><Year>2010</Year><RecNum>114</RecNum><DisplayText>(Qing et al., 2010)</DisplayText><record><rec-number>114</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1561745637">114</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Qing, M.</author><author>Liu, W.</author><author>Yuan, Z.</author><author>Gu, F.</author><author>Shi, P. Y.</author></authors></contributors><auth-address>Novartis Institute for Tropical Diseases, 10, Biopolis Road, #05-01 Chromos, Singapore, Singapore.</auth-address><titles><title>A high-throughput assay using dengue-1 virus-like particles for drug discovery</title><secondary-title>Antiviral Res</secondary-title></titles><periodical><full-title>Antiviral Res</full-title></periodical><pages>163-71</pages><volume>86</volume><number>2</number><keywords><keyword>Animals</keyword><keyword>Antiviral Agents/*isolation & purification/*pharmacology</keyword><keyword>Biological Assay/methods</keyword><keyword>Cell Line</keyword><keyword>Cercopithecus



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aethiops</keyword><keyword>Cricetinae</keyword><keyword>Dengue Virus/*drug effects/genetics</keyword><keyword>Drug Discovery/*methods</keyword><keyword>Drug Evaluation, Preclinical/*methods</keyword><keyword>Electroporation</keyword><keyword>Genes, Reporter</keyword><keyword>Luciferases, Renilla/genetics/metabolism</keyword><keyword>Macaca mulatta</keyword><keyword>Viral Proteins/genetics/metabolism</keyword><keyword>Virosomes/*drug effects/genetics</keyword></keywords><dates><year>2010</year><pub-dates><date>May</date></pub-dates></dates><isbn>1872-9096 (Electronic)0166-3542 (Linking)</isbn><accession-num>20153777</accession-num><urls><related-urls><url>https://www.ncbi.nlm.nih.gov/pubmed/20153777</url></related-urls></urls><electronic-resource-num>10.1016/j.antiviral.2010.02.313</electronic-resource-num></record></Cite></EndNote>] with several modifications. 10 µg of replicon RNA was electroporated into BHK-21 cells as described above. After transfection, the cells were maintained at 37°C with 5% $CO_2$ for 24 h. Afterwards, cells were harvested and electroporated with 10 µg of a helper replicon RNA SFV-DENV2 CprME (replicon derived from Semliki forest virus that express the DENV-2 encoding the CprME). After the second electroporation, cells were cultured at 30°C with 5% $CO_2$. At 20, 28 and 48 h after the second electroporation, culture medium was collected and stored at -80 °C. Afterwards, the collected medium was used ton infection naïve Vero cells in 96-well plates. At 48 h post-infection, cells were lysed, and luciferase signals were measured using the Renilla luciferase assay system (Promega) in the Clarity luminescence microplate reader (BioTek) according to the manufacturer's protocol.

## METHOD DETAILS

### Plasmid Construction

The full-length cDNA infectious clone of DENV-2 strain New Guinea C (NGC), pACYC-NGC FL, and its derived replicon (pACYC-NGC replicon) clone [ ADDIN EN.CITE <EndNote><Cite><Author>Xie</Author><Year>2013</Year><RecNum>85</RecNum><DisplayText>(Xie et al., 2013)</DisplayText><record><rec-number>85</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793217">85</key></foreign-keys><ref-type name="Journal



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Article">17</ref-type><contributors><authors><author>Xie, X.</author><author>Gayen, S.</author><author>Kang, C.</author><author>Yuan, Z.</author><author>Shi, P. Y.</author></authors></contributors><auth-address>State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Science, China.</auth-address><titles><title>Membrane topology and function of dengue virus NS2A protein</title><secondary-title>J Virol</secondary-title><alt-title>Journal of virology</alt-title></titles><periodical><full-title>J Virol</full-title></periodical><pages>4609-22</pages><volume>87</volume><number>8</number><edition>2013/02/15</edition><keywords><keyword>Animals</keyword><keyword>Cell Line</keyword><keyword>Cricetinae</keyword><keyword>Dengue Virus/*physiology</keyword><keyword>Endoplasmic Reticulum/*virology</keyword><keyword>Humans</keyword><keyword>Intracellular Membranes/*metabolism</keyword><keyword>Magnetic Resonance Spectroscopy</keyword><keyword>Models, Molecular</keyword><keyword>Protein Conformation</keyword><keyword>Viral Nonstructural Proteins/*chemistry/metabolism</keyword><keyword>*Virus Assembly</keyword></keywords><dates><year>2013</year><pub-dates><date>Apr</date></pub-dates></dates><isbn>1098-5514 (Electronic)0022-538X (Linking)</isbn><accession-num>23408612</accession-num><work-type>Research Support, Non-U.S. Gov't</work-type><urls><related-urls><url>http://www.ncbi.nlm.nih.gov/pubmed/23408612</url></related-urls></urls><custom2>3624351</custom2><electronic-resource-num>10.1128/JVI.02424-12</electronic-resource-num><language>eng</language></record></Cite></EndNote>] were used as the backbones for mutagenesis study. The replicon was constructed by replacing most of the structural genes (C, prM and E) with a gene cassette (Rluc2A) encoding the Renilla luciferase and a 2A peptide from Foot-and-mouth disease virus. The first 38 amino acids of C protein and the last 31 amino acids of E protein were retained in the replicon. Standard overlap PCR was performed to introduce the mutations in C and NS2A genes. The DNA fragments containing the mutations in C gene were cloned into pACYC-NGC FL clone through restriction enzyme sites NotI (upstream of the T7 promoter and 5'UTR of DENV-2) and SphI (at the position of 1384 in DENV-2 genome). The DNA fragments containing mutations in NS2A gene were cloned into pACYC-NGC FL or replicon through restriction enzyme sites and NheI (at the position of 2544 in DENV-2 genome) and SacI (at the position of 4342 in the DENV-2 genome).



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A mammalian expression vector, pXJ [ ADDIN EN.CITE <EndNote><Cite><Author>Xie</Author><Year>2013</Year><RecNum>85</RecNum><DisplayText>(Xie et al., 2013)</DisplayText><record><rec-number>85</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793217">85</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Xie, X.</author><author>Gayen, S.</author><author>Kang, C.</author><author>Yuan, Z.</author><author>Shi, P. Y.</author></authors></contributors><auth-address>State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Science, China.</auth-address><titles><title>Membrane topology and function of dengue virus NS2A protein</title><secondary-title>J Virol</secondary-title><alt-title>Journal of virology</alt-title></titles><periodical><full-title>J Virol</full-title></periodical><pages>4609-22</pages><volume>87</volume><number>8</number><edition>2013/02/15</edition><keywords><keyword>Animals</keyword><keyword>Cell Line</keyword><keyword>Cricetinae</keyword><keyword>Dengue Virus/*physiology</keyword><keyword>Endoplasmic Reticulum/*virology</keyword><keyword>Humans</keyword><keyword>Intracellular Membranes/*metabolism</keyword><keyword>Magnetic Resonance Spectroscopy</keyword><keyword>Models, Molecular</keyword><keyword>Protein Conformation</keyword><keyword>Viral Nonstructural Proteins/*chemistry/metabolism</keyword><keyword>*Virus Assembly</keyword></keywords><dates><year>2013</year><pub-dates><date>Apr</date></pub-dates></dates><isbn>1098-5514 (Electronic)0022-538X (Linking)</isbn><accession-num>23408612</accession-num><work-type>Research Support, Non-U.S. Gov't</work-type><urls><related-urls><url>http://www.ncbi.nlm.nih.gov/pubmed/23408612</url></related-urls></urls><custom2>3624351</custom2><electronic-resource-num>10.1128/JVI.02424-12</electronic-resource-num><language>eng</language></record></Cite></EndNote>], driven by a cytomegalovirus (CMV) promoter was used to express various viral proteins prM, E, NS2A and NS3 fusion proteins in HEK293T cells. For constructs pXJ-$C_{14}$-prM and pXJ-$C_{14}$-prM (1-130), the anchor C signal peptide ($C_{14}$) was retained at the N-terminus of prM to ensure the correct targeting on ER membrane. For construct pXJ-$M_{17}$-E, the last 17 amino acids from M protein was retained to serve as a signal peptide for translocating the E protein into the ER lumen. For constructs pXJ-SPG-$C_{16}$-NS2A-EGFP and pXJ-SPG-$C_{16}$-



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NS2A-GST, the signal peptide derived from Gaussia luciferase (SPG) [ ADDIN EN.CITE <EndNote><Cite><Author>Verhaegent</Author><Year>2002</Year><RecNum>112</RecNum><DisplayText>(Verhaegent and Christopoulos, 2002)</DisplayText><record><rec-number>112</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1561735944">112</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Verhaegent, M.</author><author>Christopoulos, T. K.</author></authors></contributors><auth-address>Department of Chemistry and Biochemistry, University of Windsor, Ontario, Canada.</auth-address><titles><title>Recombinant Gaussia luciferase. Overexpression, purification, and analytical application of a bioluminescent reporter for DNA hybridization</title><secondary-title>Anal Chem</secondary-title></titles><periodical><full-title>Anal Chem</full-title></periodical><pages>4378-85</pages><volume>74</volume><number>17</number><keywords><keyword>Animals</keyword><keyword>DNA/*analysis</keyword><keyword>DNA Probes</keyword><keyword>Genes, Reporter</keyword><keyword>*Luciferases/genetics/isolation & purification</keyword><keyword>Luminescent Proteins/genetics/isolation & purification</keyword><keyword>Plankton/*enzymology</keyword><keyword>Recombinant Proteins/genetics/isolation & purification</keyword></keywords><dates><year>2002</year><pub-dates><date>Sep 1</date></pub-dates></dates><isbn>0003-2700 (Print)0003-2700 (Linking)</isbn><accession-num>12236345</accession-num><urls><related-urls><url>https://www.ncbi.nlm.nih.gov/pubmed/12236345</url></related-urls></urls></record></Cite></EndNote>] and the last 16 amino acids of NS1 ($C_{16}$) was fused at the N-terminus of NS2A to ensure the correct processing and membrane topology of NS2A. The EGFP and GST were amplified from pEGFPluc (Clontech) and pGEX-4T-1 (Sigma-Aldrich) respectively. The Human influenza hemagglutinin (HA) tag was fused to the N-terminus of NS3 and cloned into pXJ vector, resulting the construct pXJ-HA-NS3.

pGEX-4T-1 (GE Healthcare Life Sciences) was used as the vector for expressing all N-terminally glutathione S-transferase (GST)-tagged NS2A fusion proteins. The DNA fragments containing various length of NS2A were cloned into pGEX-4T-1 through restriction enzyme sites BamHI and XhoI.



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All plasmids were propagated in *E. coli* strain Top10 (ThermoFisher Scientific). All restriction enzymes were purchased from New England BioLabs (Ipswitch, MA). The plasmids were validated by restriction enzyme digestion and Sanger DNA sequencing. All primers were synthesized from Integrated DNA Technologies (Skokie, Illinois) and are available upon request.

**RNA Transcription and Electroporation**

The NGC FL or replicon plasmids were linearized by restriction enzyme XbaI. RNA transcription and electroporation were performed as described previously [ ADDIN EN.CITE <EndNote><Cite><Author>Xie</Author><Year>2013</Year><RecNum>85</RecNum><DisplayText>(Xie et al., 2013)</DisplayText><record><rec-number>85</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793217">85</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Xie, X.</author><author>Gayen, S.</author><author>Kang, C.</author><author>Yuan, Z.</author><author>Shi, P. Y.</author></authors></contributors><auth-address>State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Science, China.</auth-address><titles><title>Membrane topology and function of dengue virus NS2A protein</title><secondary-title>J Virol</secondary-title><alt-title>Journal of virology</alt-title></titles><periodical><full-title>J Virol</full-title></periodical><pages>4609-22</pages><volume>87</volume><number>8</number><edition>2013/02/15</edition><keywords><keyword>Animals</keyword><keyword>Cell Line</keyword><keyword>Cricetinae</keyword><keyword>Dengue Virus/*physiology</keyword><keyword>Endoplasmic Reticulum/*virology</keyword><keyword>Humans</keyword><keyword>Intracellular Membranes/*metabolism</keyword><keyword>Magnetic Resonance Spectroscopy</keyword><keyword>Models, Molecular</keyword><keyword>Protein Conformation</keyword><keyword>Viral Nonstructural Proteins/*chemistry/metabolism</keyword><keyword>*Virus Assembly</keyword></keywords><dates><year>2013</year><pub-dates><date>Apr</date></pub-dates></dates><isbn>1098-5514 (Electronic)0022-538X (Linking)</isbn><accession-num>23408612</accession-num><work-type>Research Support, Non-U.S. Gov't</work-type><urls><related-



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urls><url>http://www.ncbi.nlm.nih.gov/pubmed/23408612</url></related-urls></urls><custom2>3624351</custom2><electronic-resource-num>10.1128/JVI.02424-12</electronic-resource-num><language>eng</language></record></Cite></EndNote>]. Briefly, 10 μg RNA was electroporated into 8 × $10^6$ cells in a 4-mm cuvette using the GenePulser apparatus (Bio-Rad) at settings of 0.45 kV (C6/36 cells) or 0.85 kV (BHK-21 cells) and 25 mF, pulsing three times, with 3 s intervals. After a 10-min recovery at room temperature, transfected cells were suspended in culture media and incubated at corresponding conditions: 28°C (C6/36 cells) or 37°C with 5% $CO_2$ (BHK-21).

**Virus Production and Quantification**

After electroporation, the transfected BHK-21 cells were seeded in one T-75 flask. After incubation at 37°C for 24 h, the cells were cultured in DMEM with 2% FBS at 30°C for another 4 days. At every 24 h p.t., culture fluids was collected and stored at -80°C. Viral titer was determined by crystal violet-based plaque assay using BHK-21 cells according to the protocol as described previously [ ADDIN EN.CITE <EndNote><Cite><Author>Xie</Author><Year>2013</Year><RecNum>85</RecNum><DisplayText>(Xie et al., 2013)</DisplayText><record><rec-number>85</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793217">85</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Xie, X.</author><author>Gayen, S.</author><author>Kang, C.</author><author>Yuan, Z.</author><author>Shi, P. Y.</author></authors></contributors><auth-address>State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Science, China.</auth-address><titles><title>Membrane topology and function of dengue virus NS2A protein</title><secondary-title>J Virol</secondary-title><alt-title>Journal of virology</alt-title></titles><periodical><full-title>J Virol</full-title></periodical><pages>4609-22</pages><volume>87</volume><number>8</number><edition>2013/02/15</edition><keywords><keyword>Animals</keyword><keyword>Cell Line</keyword><keyword>Cricetinae</keyword><keyword>Dengue Virus/*physiology</keyword><keyword>Endoplasmic Reticulum/*virology</keyword><keyword>Humans</keyword><keyword>Intracellular Membranes/*metabolism</keyword><keyword>Magnetic Resonance Spectroscopy</keyword><keyword>Models, Molecular</keyword><keyword>Protein Conformation</keyword><keyword>Viral Nonstructural



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Proteins/*chemistry/metabolism</keyword><keyword>*Virus Assembly</keyword></keywords><dates><year>2013</year><pub-dates><date>Apr</date></pub-dates></dates><isbn>1098-5514 (Electronic)0022-538X (Linking)</isbn><accession-num>23408612</accession-num><work-type>Research Support, Non-U.S. Gov't</work-type><urls><related-urls><url>http://www.ncbi.nlm.nih.gov/pubmed/23408612</url></related-urls></urls><custom2>3624351</custom2><electronic-resource-num>10.1128/JVI.02424-12</electronic-resource-num><language>eng</language></record></Cite></EndNote>]. Plaques were developed on day 5 post-infection.

**RNA extraction and RT-PCR**

Viral RNAs in culture fluids were extracted using a QIAamp viral RNA minikit (Qiagen). Extracted RNAs were eluted in 50 μl RNase-free water. Reverse transcription (RT)-qPCR was performed to measure the amounts of viral RNA copy in the culture medium. Viral RNAs were measured using an iScript one-Step RT-PCR kit with SYBR green (Bio-Rad) and the primer pair NGC 7764V (sequence: 5'-CGTCGAGAGAAATATGGTCACACC-3') and 7844C (sequence: 5'CCACAATAGTATGACCAGCCT-3') [ ADDIN EN.CITE <EndNote><Cite><Author>Xie</Author><Year>2013</Year><RecNum>85</RecNum><DisplayText>(Xie et al., 2013)</DisplayText><record><rec-number>85</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793217">85</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Xie, X.</author><author>Gayen, S.</author><author>Kang, C.</author><author>Yuan, Z.</author><author>Shi, P. Y.</author></authors></contributors><auth-address>State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Science, China.</auth-address><titles><title>Membrane topology and function of dengue virus NS2A protein</title><secondary-title>J Virol</secondary-title><alt-title>Journal of virology</alt-title></titles><periodical><full-title>J Virol</full-title></periodical><pages>4609-22</pages><volume>87</volume><number>8</number><edition>2013/02/15</edition><keywords><keyword>Animals</keyword><keyword>Cell Line</keyword><keyword>Cricetinae</keyword><keyword>Dengue Virus/*physiology</keyword><keyword>Endoplasmic Reticulum/*virology</keyword><keyword>Humans</keyword><keyword>Intracellular Membranes/*metabolism</keyword><keyword>Magnetic Resonance



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Spectroscopy</keyword><keyword>Models, Molecular</keyword><keyword>Protein Conformation</keyword><keyword>Viral Nonstructural Proteins/*chemistry/metabolism</keyword><keyword>*Virus Assembly</keyword></keywords><dates><year>2013</year><pub-dates><date>Apr</date></pub-dates></dates><isbn>1098-5514 (Electronic)0022-538X (Linking)</isbn><accession-num>23408612</accession-num><work-type>Research Support, Non-U.S. Gov't</work-type><urls><related-urls><url>http://www.ncbi.nlm.nih.gov/pubmed/23408612</url></related-urls></urls><custom2>3624351</custom2><electronic-resource-num>10.1128/JVI.02424-12</electronic-resource-num><language>eng</language></record></Cite></EndNote>]. Alternatively, the extracted viral RNA was reversely transcribed and amplified using SuperScript One-Step (RT)-PCR with Platinum Taq (Invitrogen). The RT-PCR products were then gel purified and subjected to Sanger DNA sequencing.

**Replicon Transient Transfection**

After electroporation of replicon RNA into BHK-21 cells, the cells were resuspended in culture medium and seeded in a 12-well plate ($2 \times 10^5$ cells/well). At various time points, the cells were washed once with phosphate-buffered saline (PBS) and lysed using lysis buffer (Promega). The plates containing the lysis buffer were sealed with Parafilm and stored at -80°C. Once samples for all time points had been collected, luciferase signals were measured in a Clarity luminescence microplate reader (BioTek) according to the manufacturer's protocol. The luciferase signals at early time points (2-6 h post-transfection) represent the translation of input viral RNA; and the luciferase signals at later points (24-46 h post-transfection) represent the stage of RNA replication.

**Immunofluorescence assay (IFA)**

Cells were seeded in an 8-well Lab-Tek II chamber slide (ThermoFisher Scientific). At given time points, cells were fixed in 4% paraformaldehyde in PBS for 20 min at room temperature. Afterwards, cells were permeabilized by 0.1% Triton X-100 (Sigma-Aldrich) for 5 min followed by 1-h incubation in blocking buffer containing 1% FBS and 0.05% Tween-20 (Sigma-Aldrich) in PBS. Cells were then incubated with primary antibodies (diluted in the blocking buffer) at room temperature for 1 h. After three PBS washes, cells were incubated with goat anti-mouse or rabbit IgG (1:1000 diluted in blocking buffer) conjugated with Alexa Fluor 488 or 594 (ThermoFisher Scientific) for 1 h in blocking buffer.



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Cells were finally washed by PBS for three times, counter-stained by Hoechst 33342 Solution (ThermoFisher Scientific) and mounted in ProLong® Diamond Antifade Mountant (ThermoFisher Scientific). Epifluorescence images were acquired by Leica DM4000 B system. Confocal fluorescence images were acquired by Zeiss LSM 5 META systems.

**Virus Revertant Selection**

For selecting revertants from G11A-transfected cells, we consecutively passaged the transfected cells for 5 rounds. In brief, after electroporation, the cells were seeded in 96-well plates and incubate at 30°C for 3 days. Afterwards, cells for each well were detached by trypsin and suspended in culture medium. About 20% of the suspended cells were transferred into the well of new 96-well plates and incubated at 37°C for one day. The cells were then incubated at 30°C for two additional days. Again, the confluent cells were detached for next passage. At every passage, the supernatants were collected. After 5 rounds of passaging, IFA was performed to determine the intracellular E protein expression by 4G2 antibody. Plaque assay was performed to examine the amounts of infectious virus particle in the culture fluids. Viral RNA from the P5 supernatants were extracted and used as template for RT-PCR. Sanger sequencing was performed to sequence the entire viral genome.

**Co-immunoprecipitation (Co-IP)**

The NS2A/prM, NS2A/E and NS2A/NS3 interactions were examined by Co-IP analysis according to the protocol as described previously[ ADDIN EN.CITE <EndNote><Cite><Author>Zou</Author><Year>2014</Year><RecNum>113</RecNum><DisplayText>(Zou et al., 2014)</DisplayText><record><rec-number>113</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1561742592">113</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Zou, J.</author><author>Xie, X.</author><author>Lee le, T.</author><author>Chandrasekaran, R.</author><author>Reynaud, A.</author><author>Yap, L.</author><author>Wang, Q. Y.</author><author>Dong, H.</author><author>Kang, C.</author><author>Yuan, Z.</author><author>Lescar, J.</author><author>Shi, P. Y.</author></authors></contributors><auth-address>State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Sciences, Wuhan, China.</auth-address><titles><title>Dimerization of flavivirus NS4B protein</title><secondary-title>J



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Virol</secondary-title></titles><periodical><full-title>J Virol</full-title></periodical><pages>3379-91</pages><volume>88</volume><number>6</number><keywords><keyword>Amino Acid Motifs</keyword><keyword>Dengue/*virology</keyword><keyword>Dengue Virus/chemistry/genetics/*metabolism</keyword><keyword>Dimerization</keyword><keyword>Humans</keyword><keyword>Viral Nonstructural Proteins/*chemistry/genetics/*metabolism</keyword><keyword>West Nile Fever/*virology</keyword><keyword>West Nile virus/chemistry/genetics/*metabolism</keyword></keywords><dates><year>2014</year><pub-dates><date>Mar</date></pub-dates></dates><isbn>1098-5514 (Electronic)0022-538X (Linking)</isbn><accession-num>24390334</accession-num><urls><related-urls><url>https://www.ncbi.nlm.nih.gov/pubmed/24390334</url></related-urls></urls><custom2>PMC3957939</custom2><electronic-resource-num>10.1128/JVI.02782-13</electronic-resource-num></record></Cite></EndNote>].
Various combinations of plasmids were transfected into HEK293T cells in 10-cm dishes using the X-tremeGENE 9 DNA transfection reagent (Roche). At 40-42 h post-transfection, cells were lysed in 1 ml immunoprecipitation (IP) buffer (20 mM Tris, pH 7.5, 100 mM NaCl, 0.5% DDM, and EDTA-free protease inhibitor cocktail [Roche]) with rotation at 4°C for 1 h. Lysates were clarified by centrifugation at 20,000 × g and 4°C for 30 min and subjected to co-IP using protein G-conjugated magnetic beads according to the manufacturer's instructions (Millipore). Briefly, immune complexes were formed at 4°C overnight by mixing 200 µl of cell lysate with 2 µg corresponding antibodies in a 500-µl reaction system containing 400 mM sodium chloride. Subsequently, the complexes were precipitated with protein G-conjugated magnetic beads at 4°C for 1 h with rotation, followed by washing extensively with phosphate-buffered saline (PBS) containing 0.1% Tween-20. Finally, proteins were eluted with 4× lithium dodecyl sulfate (LDS) sample buffer (ThermoFisher Scientific) supplemented with 100 mM 1,4-dithiothreitol (DTT), heated at 70°C for 10 min, and analyzed by Western blot.

**SDS-PAGE and Western Blot**

Protein samples were mixed with 4×LDS sample buffer (ThermoFisher Scientific). After denaturing at 70°C for 10 min, 10-30 µl samples were loaded onto to a 12% or 15% SDS-PAGE gel (Bio-Rad Laboratories). After electrophoresis, proteins were resolved and



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transferred onto a polyvinylidene difluoride (PVDF) membrane using the Bio-Rad TransBlot Turbo Blotting System. Blots were soaked for 1 hour in a blocking buffer containing TBST (10 mM Tris-HCl [pH 7.5], 150 mM NaCl, and 0.1% Tween-20) and 5% skim milk, followed by 1 h incubation with primary antibodies (diluted in blocking buffer). After three washes with TBST buffer, blots were incubated with goat anti-mouse or rabbit antibodies conjugated to HRP (1:10,000 diluted in blocking buffer). After three thorough washes with TBST buffer, blots were incubated with the ECL substrates (GE Healthcare). Chemiluminescence signals were detected in ChemiDoc System (Bio-Rad).

**Transmission Electron Microscopy**

Viral genomic RNAs were electroporated into BHK-21 cells as described above. At 48 h post-transfection, the cells were fixed for at least 1 h in a mixture of 2.5% formaldehyde (prepared from paraformaldehyde powder) and 0.1% glutaraldehyde in 0.05M cacodylate buffer (pH 7.3) to which 0.03% picric acid and 0.03% $CaCl_2$ were added. After washing in 0.1 M cacodylate buffer, the cells were scraped off and processed further as a pellet. The pellets were post-fixed in 1% OsO4 in 0.1M cacodylate buffer (pH 7.3) for 1 h, washed with distilled water and *en bloc* stained with 2% aqueous uranyl acetate for 20 min at 60°C. The pellets were dehydrated in ethanol, processed through propylene oxide and embedded in Poly/Bed 812 (Polysciences, Warrington, PA). Ultrathin sections were cut on Leica EM UC7 ultramicrotome (Leica Microsystems, Buffalo Grove, IL), stained with lead citrate and examined in a Philips 201 transmission electron microscope at 60 kV.

**Expression and purification of recombinant NS2A proteins**

The GST-tagged DENV-2 NGC NS2A and its truncates was expressed and purified by following a previously described protocol [ ADDIN EN.CITE <EndNote><Cite><Author>Zou</Author><Year>2014</Year><RecNum>113</RecNum><DisplayText>(Zou et al., 2014)</DisplayText><record><rec-number>113</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1561742592">113</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Zou, J.</author><author>Xie, X.</author><author>Lee le, T.</author><author>Chandrasekaran, R.</author><author>Reynaud, A.</author><author>Yap, L.</author><author>Wang, Q. Y.</author><author>Dong, H.</author><author>Kang, C.</author><author>Yuan, Z.</author><author>Lescar, J.</author><author>Shi, P.



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Y.</author></authors></contributors><auth-address>State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Sciences, Wuhan, China.</auth-address><titles><title>Dimerization of flavivirus NS4B protein</title><secondary-title>J Virol</secondary-title></titles><periodical><full-title>J Virol</full-title></periodical><pages>3379-91</pages><volume>88</volume><number>6</number><keywords><keyword>Amino Acid Motifs</keyword><keyword>Dengue/*virology</keyword><keyword>Dengue Virus/chemistry/genetics/*metabolism</keyword><keyword>Dimerization</keyword><keyword>Humans</keyword><keyword>Viral Nonstructural Proteins/*chemistry/genetics/*metabolism</keyword><keyword>West Nile Fever/*virology</keyword><keyword>West Nile virus/chemistry/genetics/*metabolism</keyword></keywords><dates><year>2014</year><pub-dates><date>Mar</date></pub-dates></dates><isbn>1098-5514 (Electronic)0022-538X (Linking)</isbn><accession-num>24390334</accession-num><urls><related-urls><url>https://www.ncbi.nlm.nih.gov/pubmed/24390334</url></related-urls></urls><custom2>PMC3957939</custom2><electronic-resource-num>10.1128/JVI.02782-13</electronic-resource-num></record></Cite></EndNote>] with modifications. The pGEX-4T GST-NS2A plasmids were transformed into *E. coli* strain Rosetta™ 2 (pLysS) (Novagen) cells. Bacteria culture was grown in Tefferic broth containing 50 µg/ml Kanamycin and 34 µg/ml Chloramphenicol. When the cells reached an optical density at 600 nm (OD600) of 0.8-1.0, isopropyl β-d-1-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM. After growth for 16 h at 18°C, the cells were harvested, resuspended in a lysis buffer (10 mM $Na_2HPO_4$, 1.8 mM $KH_2PO_4$, 2.7 mM KCl, 400 mM NaCl, 1 mM EDTA, 0.5× Roche protease inhibitor cocktail, pH7.5) and disrupted by sonication using a Digital Sonifier 450 (Branson). The cell debris was removed by centrifugation at 10,000 rpm for 10 min at 4°C. The supernatants were collected and pelleted down by centrifugation at 45,000 rpm for 1 h at 4°C using a Beckman type 70 Ti rotor. The pellets containing the membrane fractions were collected and solubilized in the lysis buffer supplemented with 1.5% n-Dodecyl β-D-maltoside (DDM; Anatrace) at 4°C for 2 hours. Following another centrifugation step at 45,000 rpm for 1 h, the supernatants were collected and loaded onto a GSTrap HP Fast Flow column (GE Healthcare) which was preequilibrated with buffer A (10 mM $Na_2HPO_4$, 1.8 mM $KH_2PO_4$, 2.7 mM KCl, 400 mM NaCl, 2 mM tris(2-carboxyethyl) phosphine [TCEP], 0.05% DDM, pH



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7.5). After extensively wash with buffer B (10 mM $Na_2HPO_4$, 1.8 mM $KH_2PO_4$, 2.7 mM KCl, 1M NaCl, 2 mM tris(2-carboxyethyl) phosphine [TCEP], 0.05% DDM, pH 7.5), the protein was eluted in buffer A supplied with 20 mM glutathione. The fractions containing GST-NS2A proteins were pooled, concentrated, and subjected to gel filtration using a HiLoad Superdex 200 10/300 column (GE Healthcare) in buffer C (10 mM $Na_2HPO_4$, 1.8 mM $KH_2PO_4$, 2.7 mM KCl, 150 mM NaCl, 2 mM tris(2-carboxyethyl) phosphine [TCEP], 0.05% DDM, pH 7.5) with 5% glycerol. The peak fractions containing GST-NS2A proteins were pooled and concentrated before storage at -80°C.

**RNA Pull-down Assay**

The 5'UTR-$C_{66}$-3'UTR-biotin RNA was prepared by ligating a single biotinylated nucleotide to the 3' terminus of the RNA 5'UTR-$C_{66}$-3'UTR using the Pierce™ RNA 3' End Biotinylation Kit (ThermoFisher Scientific) according to the manufacturer's instructions. The RNA 5'UTR-$C_{66}$-3'UTR was *in vitro* transcribed from a PCR template using a T7 MEGAscript kit (ThermoFisher Scientific). The PCR template contained a T7 promoter, followed by a cDNA fragment representing a DENV-2 subgenome with a deletion from nucleotides 163 to 10269 (GenBank accession number AF038403). After *in vitro* transcription, the products were passed twice through the Microspin™ G-25 Columns (GE Healthcare Life Sciences) to remove excessive UTPs. The final products were subjected to phenol-chloroform extraction followed by alcohol precipitation. The purified RNAs were resuspended in nuclease-free water prior to storage at -80°C. Other shorter RNAs 3'UTR, Δpk1, 5'UTR-$C_{66}$, Δpk(1-2), Δpk(1-3), 3'SL, pk(3-4) and pk3 were *in vitro* synthesized from corresponding PCR templates using the same method.

RNA pull-down experiments were performed using the Pierce™ Magnetic RNA-Protein Pull-Down Kit (ThermoFisher Scientific) according to the manufacturer's instructions. Briefly, 2 µg of biotin-labeled RNAs were pre-captured by 50 µl Streptavidin magnetic beads in 1 × RNA capture buffer (10mL, 20mM Tris (pH 7.5), 1M NaCl, 1mM EDTA) at room temperature for 20 min with agitation. A 28-mer biotinylated IRE RNA (5'-UCCUGCUUCAACAGUGCUUGGACGGAAC-3'-biotin) from the Pierce™ RNA 3' End Biotinylation Kit was used as a control. The RNA-bound beads were then mixed with 2 µg purified GST-tagged NS2A proteins in a 100-µl reaction system containing 20 mM Tris (pH 7.5), 200 mM NaCl, 2 mM $MgCl_2$, 0.1% Tween-20 and 15% glycerol and incubated for 45 minutes at 4°C with agitation. For RNA competition assay, the various amounts of



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competitors (tRNA and other shorter RNAs from DENV-2 genome) were immediately added to above the mixtures containing RNA-bound beads and GST-NS2A proteins. After 5 times of wash by 20 mM Tris (pH 7.5), 10 mM NaCl, 0.1% Tween-20 Detergent, the proteins were eluted in 30 µl biotin elution buffer. The eluates were mixed with 4×LDS sample buffer and heated at 70°C for 10 min. 10 µl protein samples were loaded onto a 12% SDS-PAGE gel and followed by detection by Western blot using rabbit anti-GST antibodies.

**Electrophoretic Mobility-Shift Assay**

Electrophoretic mobility-shift assay (EMSA) was performed to estimate the binding affinity of DENV-2 5'UTR-$C_{66}$-3'UTR RNA to GST-NS2A proteins. 100 pM 5'UTR-$C_{66}$-3'UTR-biotin RNA was incubated with a serial concentration of recombinant GST-NS2A protein (0-250 nM) in a 50-µl reaction system containing 50mM Tris-HCl, pH 7.5, 10 mM 2-mercaptoethanol, 2 mM $MgCl_2$, 50 mM KCl at 24°C for 15 min. Afterwards, 10 µl 6x Gel Loading Dye without SDS (New England Biolabs Inc.) were added to stop the reaction. Sample were then loaded onto a 0.4% agarose gel (prepared in 0.5× Tris/Borate/EDTA[TBE]) to resolve the RNA-protein complexes and free RNAs by electrophoresis in 1× TBE buffer (120 V for 30 min) at room temperature. After electrophoresis, RNAs were transferred from the agarose gel to a BrightStar-Plus positively charged nylon membrane (ThermoFisher Scientific) according to the procedure as described in the manual of NorthernMax Gly Kit (ThermoFisher Scientific). The nylon membrane was irradiated with 200 mJ/$cm^2$ of Ultraviolet (UV) light at 254 nm in a CX-2000 UV crosslinker (Analytic Jene, CA) with default settings. The biotinylated RNA was finally detected by the Streptavidin-horseradish peroxidase conjugate by using the Chemiluminescent Detection Module (ThermoFisher Scientific). Chemiluminescent signals were detected in the ChemiDoc™ Imaging Systems (Bio-rad).

**PAR-CLIP**

The Photoactivatable-Ribonucleoside-Enhanced Crosslinking and Immunoprecipitation (PAR-CLIP) was performed according to the protocol as described previously [ ADDIN EN.CITE ADDIN EN.CITE.DATA ] with modifications. In brief, 4-thiouridine labelled DENV-2 3'UTR RNA was prepared by adding a final concentration 0.75 mM of 4-Thio-UTP (TriLink BioTechnologies) to a 20-µl *in vitro* transcription reaction (4-Thio-UTR to UTP



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ration is 1:10) using the MEGAscript T7 transcription Kit (Thermo Fisher Scientific). After 2 hours of reaction at 37°C, the reaction was stopped and the 4-thiouridine labelled RNAs were purified and resuspended in nuclease-free water. 15 µg of 4-thiouridine labelled DENV-2 3'UTR RNA was incubated with 6 µg recombinant DENV-2 GST-NS2A protein in a 100-µl reaction system containing 50 mM Tris-HCl, pH 7.5, 2 mM $MgCl_2$, 50 mM KCl and 5% glycerol at 24°C for 15 min. The RNA-protein mixtures were then irradiated on ice with 150 mJ/cm$^2$ at UV light at 365 nm. An aliquot of the same reaction without UV irradiation was used as the uncross-linked control. Prior to immunoprecipitation, 30 µl of PureProteome™ Protein G Magnetic Beads (MilliporeSigma) were washed twice with 1 ml citrate-phosphate buffer (4.7 g/L citric acid, 9.2 g/L $Na_2HPO_4$, pH 5.0), resuspended in 100 µl citrate-phosphate buffer and followed by in addition of 1 µg of rabbit anti-GST polyclonal antibody (Abcam). The bead-antibody mixtures were incubated at 4°C for 45 min. After two washed with citrate-phosphate buffer, the antibody-bound beads were resuspended in 30 µl of citrate-phosphate buffer. Upon immunoprecipitation, the RNA-protein mixtures were mixed with antibody-bound beads and incubated at 4°C for 1 h. Afterwards, the beads were subjected to three washes in buffer I (50mM HEPES-KOH, pH 7.5, 300 mM KCl, 0.05% IGEPAL CA-630L[Sigma-Aldrich] and 0.5 mM DTT) followed by two washes in buffer II (50 mM HEPES-KOH, pH 7.5, 500 KCl, 0.05% IGEPAL CA-630 and 0.5 mM DTT). The beads were then resuspended in 100 µl Proteinase K digestion buffer (100 mM Tris, pH 7.5, 50 mM NaCl, 10 mM EDTA and 1%SDS) supplemented with proteinase K (New England BioLabs) at a final concentration of 2 mg/ml. The reaction was placed into the Eppendorf ThermoMixer® C and incubated at 37°C for 1 h with agitation at 1100 rpm. Thereafter, the RNA in the reaction was extracted by phenol-chloroform, alcohol-precipitated, and resuspended in 20 µl of nuclease-free water. The extracted RNA was subjected to reversely transcribed and followed by amplification (4 PCR-cycles) using the SuperScript® III One-Step RT-PCR System (ThermoFisher Scientific) with a pair of specific primers P1 (sequence: 5'-ACACTCTTTCCCTACACGA CGCTCTTCCGATCTAAGGCAAAACTAACATGAAAC-3'; underline indicates the sequence identical to the sequence at the 5' end of the DENV-2 3'UTR) and P2 (sequence: 5'-GTGACTGGAGTTCAGACG TGTGCTCTTCCGATCTAGAACCTGTTGATTCAACAGCAC-3'; underline indicates the sequence complimentary to the sequence at the 3' end of the DENV-2 3'UTR) targeting the DENV-2 3'UTR. Afterwards, the RT-PCR product was cleaned up using the QIAquick PCR Purification Kit (Qiagen) and subjected to another round of amplification (15 PCR-



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cycles) using the KAPA HiFi PCR Kit (Kapa Biosystems) with a pair of Illumina adapter/index primers (5' universal adaptor sequence: 5'-AATGATACGGCGAC CACCGAGATCTACACTCTTTCCCTACACGAC-3'; and 3' indexing primer sequence: 5'-CAAGCAGAAGACGGCATACGAGATnnnnnnGTGACTGGAGTTCAGACGTGT-3'. The "nnnnnn" here indicates the TruSeq index sequence). Thereafter, the PCR product was cleaned up using the QIAquick PCR Purification Kit (Qiagen) and followed by separation on 2% E-Gel™ EX Agarose Gels (ThermoFisher Scientific). The cDNAs fragment between 500 to 650 bp was excised, purified using the Zymo Research Gel DNA Recovery Kit (Zymo Research) and resuspended in nuclease-free water. The final cDNA libraries were quantified using a Qubit dsDNA Assay Kit (Invitrogen) and sequenced using a MiSeq Reagent Kit v3 (600-cycle) (illumina).

## QUANTIFICATION AND STATISTICAL ANALYSIS

### Sequence Alignment, Image Process and Densitometry Analysis

The amino acid sequences of flavivirus NS2A proteins were download from GenBank. Sequence alignment was performed with default settings using the CLC main workbench software (Qiagen). IFA images processing and cell counting was performed in software ImageJ (National Institutes of Health, NIH). At least four different IFA images were used to estimate the mean and standard deviations of percentage of viral protein staining positive-cells in Figure 2G and S2. Densitometry analysis was performed using Image lab version 6.0 (Bio-Rad Laboratories, Hercules, CA). All numerical data are presented as the mean ± SD (standard deviations). Group comparisons were performed using the unpaired parametric t-test in software Prism 8.0 (GraphPad). *$p<0.05$, significant; **$p<0.01$, very significant; ***$p<0.001$, highly significant; n.s., not significant. Images were assembled using the software Adobe illustrator CC.

### Calculating the equilibrium dissociation constant (Kd)

The intensities of bands representing the RNA/GST-NS2A complexes and free RNA in Figure 5E were quantitated by software ImageJ (NIH). The ratio of bound RNA (in the RNA/GST-NS2A complexes) to total RNA (free and bound RNA) in each lane was calculated. The ratio of bound RNA to total RNA (y-axis) versus the concentration of GST-NS2A protein (x-axis) was plotted using the software Prism 8 (Graphad). The value of Kd



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was obtained using a nonlinear regression with least-squares fitting method, where the Bmax was set as 1. All data points come from three independent experiments.

**Illumina sequencing data process and analysis**

The indexed sequencing reads of both forward and reverse strands were proceeded with the following bioinformatic pipelines: first, an initial quality filtering were conducted with Fastp [ ADDIN EN.CITE <EndNote><Cite><Author>Chen</Author><Year>2018</Year><RecNum>115</RecNum><DisplayText>(Chen et al., 2018)</DisplayText><record><rec-number>115</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1562011493">115</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Chen, S.</author><author>Zhou, Y.</author><author>Chen, Y.</author><author>Gu, J.</author></authors><translated-authors><author>Bioinformatics,</author></translated-authors></contributors><auth-address>Department of Bioinformatics, HaploX Biotechnology, Shenzhen, China.Shenzhen Institutes of Advanced Technology, Chinese Academy of Sciences, Shenzhen, China.</auth-address><titles><title>fastp: an ultra-fast all-in-one FASTQ preprocessor</title><secondary-title>Bioinformatics</secondary-title><alt-title>Bioinformatics</alt-title></titles><periodical><full-title>Bioinformatics</full-title><abbr-1>Bioinformatics</abbr-1></periodical><alt-periodical><full-title>Bioinformatics</full-title><abbr-1>Bioinformatics</abbr-1></alt-periodical><pages>i884-i890</pages><volume>34</volume><number>17</number><dates><year>2018</year><pub-dates><date>Sep 1</date></pub-dates></dates><isbn>1367-4811 (Electronic)1367-4803 (Linking)</isbn><accession-num>30423086</accession-num><urls><related-urls><url>http://www.ncbi.nlm.nih.gov/pubmed/30423086</url></related-urls></urls><custom2>6129281</custom2><electronic-resource-num>10.1093/bioinformatics/bty560</electronic-resource-num><remote-database-provider>2018 Sep 1</remote-database-provider><language>eng</language></record></Cite></EndNote>] to remove bad reads (too low in quality score, too short, too many “N”, etc.). Then, the illumina sequencing adapter sequence “AGATCGGAAGAGC” was trimmed with cutadapt [ ADDIN EN.CITE <EndNote><Cite><Author>Martin</Author><Year>2011</Year><RecNum>116</RecNum><DisplayText>(Martin, 2011)</DisplayText><record><rec-number>116</rec-



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number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1562011493">116</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Martin, Marcel</author></authors></contributors><titles><title>Cutadapt removes adapter sequences from high-throughput sequencing reads</title><secondary-title>EMBnet.journal; Vol 17, No 1: Next Generation Sequencing Data AnalysisDO - 10.14806/ej.17.1.200</secondary-title></titles><periodical><full-title>EMBnet.journal; Vol 17, No 1: Next Generation Sequencing Data AnalysisDO - 10.14806/ej.17.1.200</full-title></periodical><keywords><keyword>next generation sequencing, small RNA, microRNA, adapter removal</keyword></keywords><dates><year>2011</year><pub-dates><date>05/02/</date></pub-dates></dates><urls><related-urls><url>https://journal.embnet.org/index.php/embnetjournal/article/view/200/458</url></related-urls></urls></record></Cite></EndNote>] (command line parameters: -b AGATCGGAAGAGC -m 40). A secondary quality filter was applied with FASTX toolkit ([ HYPERLINK "http://hannonlab.cshl.edu/fastx_toolkit/index.html" ]) to remove any reads that contained more than 4% nucleotides with a PHRED score <20 (command line parameters: fastq_quality_filter -Q33 -q 20 -p 96). The remaining reads were aligned to 3'UTR of DENV genome (GenBank accession number AF038403), with Bowtie2 [ ADDIN EN.CITE <EndNote><Cite><Author>Langmead</Author><Year>2012</Year><RecNum>117</RecNum><DisplayText>(Langmead and Salzberg, 2012)</DisplayText><record><rec-number>117</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1562011493">117</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Langmead, Ben</author><author>Salzberg, Steven L.</author></authors></contributors><titles><title>Fast gapped-read alignment with Bowtie 2</title><secondary-title>Nature Methods</secondary-title></titles><periodical><full-title>Nature Methods</full-title></periodical><pages>357</pages><volume>9</volume><dates><year>2012</year><pub-dates><date>03/04/online</date></pub-dates></dates><publisher>Nature Publishing Group, a division of Macmillan Publishers Limited. All Rights Reserved.</publisher><urls><related-urls><url>https://doi.org/10.1038/nmeth.1923</url></related-urls></urls><electronic-resource-num>10.1038/nmeth.1923</electronic-resource-



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num></record></Cite></EndNote>] (command line parameters: --local). Using SAMtools [ ADDIN EN.CITE <EndNote><Cite><Author>Li</Author><RecNum>118</RecNum><DisplayText>(Li et al.)</DisplayText><record><rec-number>118</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1562011493">118</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Li, H.</author><author>Handsaker B Fau - Wysoker, Alec</author><author>Wysoker A Fau - Fennell, Tim</author><author>Fennell T Fau - Ruan, Jue</author><author>Ruan J Fau - Homer, Nils</author><author>Homer N Fau - Marth, Gabor</author><author>Marth G Fau - Abecasis, Goncalo</author><author>Abecasis G Fau - Durbin, Richard</author><author>Durbin, R.</author></authors><translated-authors><author>Bioinformatics,</author></translated-authors></contributors><auth-address>Wellcome Trust Sanger Institute, Wellcome Trust Genome Campus, Cambridge, CB10 1SA, UK, Broad Institute of MIT and Harvard, Cambridge, MA 02141, USA. FAU - Handsaker, Bob</auth-address><titles><title>The Sequence Alignment/Map format and SAMtools</title></titles><number>1367-4811 (Electronic)</number><dates></dates><call-num>1000 Genome Project Data Processing Subgroup</call-num><urls></urls><remote-database-provider>2009 Aug 15</remote-database-provider><language>eng</language></record></Cite></EndNote>], the aligned forward and reverse reads were binarily converted, merged, indexed, sorted, and mathematically noted (command line parameters: view -buS, merge, sort, index, mpileup). This is followed by extracting and calculating the T/U-to-C mutation rate at each genome position and the mutation frequencies of each of the four nucleotides.

## DATA AND SOFTWARE AVAILABILITY

Illumina sequencing data access. The raw data of illumine sequencing data can be accessed, queried and downloaded using the following link: .



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# Dengue NS2A protein orchestrates virus assembly

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*Correspondence: [ HYPERLINK "mailto:xuxie@UTMB.edu" ] (X.X.), [ HYPERLINK "mailto:peshi@UTMB.edu" ] (P.-Y.S.)

Running title: The role of NS2A protein in dengue virus assembly



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## SUMMARY

Dengue virus (DENV) is the most important mosquito-borne viral pathogen in humans. DENV assembly requires cleavages of viral C-prM-E polyprotein into three structural proteins (capsid, pre-membrane, and envelope), packaging of viral RNA with C protein into nucleocapsid, and budding of prM/E proteins into virion. No study has addressed how these assembly events are orchestrated. Here we show that DENV-2 NS2A protein recruits viral RNA, structural proteins, and protease to virion assembly site, and coordinates nucleocapsid and virus formation. The last 285 nucleotides of viral 3'UTR serve as a "packaging signal" that binds to a cytosolic loop of NS2A protein. This interaction allows NS2A to recruit nascent RNA from the replication complex to virion assembly site. Mutations in NS2A that weaken the 3'UTR/NS2A interaction abolish virus assembly. In addition, NS2A interacts with prM, E, and NS3 to recruit C-prM-E polyprotein and NS2B/NS3 protease to the virion assembly site, leading to coordinated C-prM-E cleavage. Mature C protein assembles onto genomic RNA to form nucleocapsid, followed by prM/E envelopment and virion formation. An NS2A mutation that alters the prM/NS2A interaction reduces C-prM cleavage and abolishes virion assembly; second-site mutations in C protein that restore the C-prM cleavage rescue virion assembly. The results support a DENV assembly model that NS2A recruits viral RNA to virion assembly site where coordinated cleavage of C-prM-E ensues infections virus morphogenesis.




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## INTRODUCTION

The genus *Flavivirus* of the family *Flaviviridae* comprises over 70 viruses, many of which are important human pathogens, including dengue (DENV), yellow fever (YFV), Zika (ZIKV), West Nile (WNV), Japanese encephalitis (JEV), St. Louis encephalitis (SLE), and tick-borne encephalitis (TBEV) viruses[ ADDIN EN.CITE <EndNote><Cite><Author>Pierson</Author><Year>2013</Year><RecNum>3</RecNum><DisplayText>(Pierson and Diamond, 2013)</DisplayText><record><rec-number>3</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1536704974">3</key><key app="ENWeb" db-id="">0</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Pierson, T.C.</author><author>Diamond, M. S.</author></authors></contributors><titles><secondary-title>Flaviviruses p. 747-794. In D.M. Knipe and P.M. Howley (ed), Fields virology, 6th., vol. 1.</secondary-title></titles><periodical><full-title>Flaviviruses p. 747-794. In D.M. Knipe and P.M. Howley (ed), Fields virology, 6th., vol. 1.</full-title></periodical><dates><year>2013</year></dates><urls></urls></record></Cite></EndNote>]. The four serotypes of DENV alone cause about 390 million human infections each year, leading to dengue fever, dengue hemorrhagic fever, and dengue shock syndrome [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. Because of the huge social economic burden, DENV was included in the top 10 global health threats by the World Health Organization (WHO). The development of vaccine and therapeutics for DENV has been proven challenging [ ADDIN EN.CITE <EndNote><Cite><Author>Lim</Author><Year>2013</Year><RecNum>58</RecNum><DisplayText>(Lim et al., 2013)</DisplayText><record><rec-number>58</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv"



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timestamp="1560793214">58</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Lim, S. P.</author><author>Wang, Q. Y.</author><author>Noble, C. G.</author><author>Chen, Y. L.</author><author>Dong, H.</author><author>Zou, B.</author><author>Yokokawa, F.</author><author>Nilar, S.</author><author>Smith, P.</author><author>Beer, D.</author><author>Lescar, J.</author><author>Shi, P. Y.</author></authors></contributors><auth-address>Novartis Institute for Tropical Diseases, 10 Biopolis Road, 05-01 Chromos, Singapore 138670, Singapore.</auth-address><titles><title>Ten years of dengue drug discovery: Progress and prospects</title><secondary-title>Antiviral Res</secondary-title><alt-title>Antiviral research</alt-title></titles><periodical><full-title>Antiviral Res</full-title></periodical><pages>500-19</pages><volume>100</volume><number>2</number><edition>2013/10/01</edition><dates><year>2013</year><pub-dates><date>Nov</date></pub-dates></dates><isbn>1872-9096 (Electronic)0166-3542 (Linking)</isbn><accession-num>24076358</accession-num><urls><related-urls><url>http://www.ncbi.nlm.nih.gov/pubmed/24076358</url></related-urls></urls><electronic-resource-num>10.1016/j.antiviral.2013.09.013</electronic-resource-num><language>eng</language></record></Cite></EndNote>]. The first FDA approved dengue vaccine has a low efficacy of 30-61% [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. Due to the risk of vaccine-induced severe disease in children who are seronegative at the time of vaccination, WHO recommended only use of the vaccine in areas where the disease is highly prevalent [ ADDIN EN.CITE <EndNote><Cite><Author>Whitehead</Author><Year>2016</Year><RecNum>63</RecNum><DisplayText>(Whitehead, 2016)</DisplayText><record><rec-number>63</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793215">63</key></foreign-keys><ref-type name="Journal



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88 Article">17</ref-type><contributors><authors><author>Whitehead, S.
89 S.</author></authors></contributors><auth-address>a Laboratory of Infectious Diseases ,
90 NIAID, National Institutes of Health , Bethesda , MD , USA.</auth-
91 address><titles><title>Development of TV003/TV005, a single dose, highly immunogenic
92 live attenuated dengue vaccine; what makes this vaccine different from the Sanofi-Pasteur
93 CYD vaccine?</title><secondary-title>Expert Rev Vaccines</secondary-
94 title></titles><periodical><full-title>Expert Rev Vaccines</full-
95 title></periodical><pages>509-
96 17</pages><volume>15</volume><number>4</number><keywords><keyword>Dengue</
97 keyword><keyword>live attenuated dengue vaccine</keyword><keyword>vaccine
98 attenuation strategies</keyword><keyword>vaccine
99 efficacy</keyword><keyword>vaccine immunogenicity</keyword><keyword>vaccine
100 infectivity</keyword></keywords><dates><year>2016</year></dates><isbn>1744-8395
101 (Electronic)1476-0584 (Linking)</isbn><accession-num>26559731</accession-
102 num><urls><related-
103 urls><url>http://www.ncbi.nlm.nih.gov/pubmed/26559731</url></related-
104 urls></urls><custom2>PMC4956407</custom2><electronic-resource-
105 num>10.1586/14760584.2016.1115727</electronic-resource-
106 num></record></Cite></EndNote>]. No clinically approved antivirals are available for
107 flavivirus treatment, despite major effort in the past two decades[ ADDIN EN.CITE
108 <EndNote><Cite><Author>Zou</Author><Year>2019</Year><RecNum>64</RecNum><
109 DisplayText>(Zou and Shi, 2019)</DisplayText><record><rec-number>64</rec-
110 number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv"
111 timestamp="1560793215">64</key></foreign-keys><ref-type name="Journal
112 Article">17</ref-type><contributors><authors><author>Zou, J.</author><author>Shi, P.
113 Y.</author></authors></contributors><auth-address>Department of Biochemistry and



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Molecular Biology, University of Texas Medical Branch, Galveston, TX, USA.Department of Biochemistry and Molecular Biology, University of Texas Medical Branch, Galveston, TX, USA; Department of Phamarcology and Toxicology, University of Texas Medical Branch, Galveston, TX, USA; Sealy Center for Structural Biology and Molecular Biophysics, University of Texas Medical Branch, Galveston, TX, USA; Sealy Institute for Vaccine Sciences, University of Texas Medical Branch, Galveston, TX, USA. Electronic address: peshi@utmb.edu.</auth-address><titles><title>Strategies for Zika drug discovery</title><secondary-title>Curr Opin Virol</secondary-title></titles><periodical><full-title>Curr Opin Virol</full-title></periodical><pages>19-26</pages><volume>35</volume><dates><year>2019</year><pub-dates><date>Mar 7</date></pub-dates></dates><isbn>1879-6265 (Electronic)1879-6257 (Linking)</isbn><accession-num>30852345</accession-num><urls><related-urls><url>http://www.ncbi.nlm.nih.gov/pubmed/30852345</url></related-urls></urls><electronic-resource-num>10.1016/j.coviro.2019.01.005</electronic-resource-num></record></Cite></EndNote>]. It is therefore critical to study the molecular mechanism of flavivirus replication, which may lead to new approaches for countermeasure development.

Flavivirus has a plus-sense, single-stranded RNA of about 11,000-nucleotide in length. The genomic RNA contains a 5' untranslated region (UTR), a single open-reading-frame (ORF), and a 3' UTR. The ORF translates a polyprotein that is processed by viral and host proteases into three structure proteins [capsid (C), pre-membrane (prM), and envelope (E)] and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5). The structural proteins, together with genomic RNA, assemble into virions. The nonstructural proteins participate in viral RNA replication, virion assembly, and evasion of host immune response. Upon viral translation, a minus-sense RNA is synthesized from the



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genomic RNA template, forming a double-stranded RNA. The minus-strand RNA then serves as a template to make many copies of plus-sense RNA that are packaged to form progeny virions [ ADDIN EN.CITE <EndNote><Cite><Author>Pierson</Author><Year>2013</Year><RecNum>3</RecNum><DisplayText>(Pierson and Diamond, 2013)</DisplayText><record><rec-number>3</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1536704974">3</key><key app="ENWeb" db-id="">0</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Pierson, T.C.</author><author>Diamond, M. S.</author></authors></contributors><titles><secondary-title>Flaviviruses p. 747-794. In D.M. Knipe and P.M. Howley (ed), Fields virology, 6th., vol. 1.</secondary-title></titles><periodical><full-title>Flaviviruses p. 747-794. In D.M. Knipe and P.M. Howley (ed), Fields virology, 6th., vol. 1.</full-title></periodical><dates><year>2013</year></dates><urls></urls></record></Cite></EndNote>]. Like other RNA viruses, the assembly of flavivirus is coupled with RNA replication [ ADDIN EN.CITE ADDIN EN.CITE.DATA ].

Flavivirus infection induces invaginations of the endoplasmic reticulum (ER) to form clusters of double-membrane vesicles (Ve) enclosed in the vesicle packets (VPs). Electron tomography has revealed the three-dimensional architectures of Ve and VPs formed during DENV, WNV, TBEV, and ZIKV infections [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. Ve houses viral replication complex (including the double-stranded viral RNA, viral nonstructural proteins, and cellular proteins) where RNA synthesis occurs [ ADDIN EN.CITE <EndNote><Cite><Author>Junjhon</Author><Year>2014</Year><RecNum>71</RecNum><DisplayText>(Junjhon et al., 2014)</DisplayText><record><rec-number>71</rec-



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number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793216">71</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Junjhon, J.</author><author>Pennington, J. G.</author><author>Edwards, T. J.</author><author>Perera, R.</author><author>Lanman, J.</author><author>Kuhn, R. J.</author></authors></contributors><auth-address>Markey Center for Structural Biology, Department of Biological Sciences, Purdue University, West Lafayette, Indiana, USA.</auth-address><titles><title>Ultrastructural characterization and three-dimensional architecture of replication sites in dengue virus-infected mosquito cells</title><secondary-title>J Virol</secondary-title></titles><periodical><full-title>J Virol</full-title></periodical><pages>4687-97</pages><volume>88</volume><number>9</number><keywords><keyword>Animals</keyword><keyword>Cell Line</keyword><keyword>Culicidae/*virology</keyword><keyword>Dengue Virus/*physiology/*ultrastructure</keyword><keyword>Electron Microscope Tomography</keyword><keyword>Imaging, Three-Dimensional</keyword><keyword>Intracellular Membranes/*ultrastructure/*virology</keyword><keyword>*Virus Replication</keyword></keywords><dates><year>2014</year><pub-dates><date>May</date></pub-dates></dates><isbn>1098-5514 (Electronic)0022-538X (Linking)</isbn><accession-num>24522909</accession-num><urls><related-urls><url>http://www.ncbi.nlm.nih.gov/pubmed/24522909</url></related-urls></urls><custom2>PMC3993787</custom2><electronic-resource-num>10.1128/JVI.00118-14</electronic-resource-num></record></Cite></EndNote>].
Newly synthesized plus-sense RNA is released from the Ve pore to the rough ER for new rounds of RNA translation and replication. Alternatively, the nascent RNA is assembled



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into virions through encapsidation (with C protein) and envelopment (with prM and E proteins) through budding into the ER lumen. The virion assembly occurs at the ER sites juxtaposed to the VPs. An initial immature virion contains 60 icosahedrally arranged prM/E heterotrimer. As the immature virion transits through the Golgi network, the acidic environment triggers the cleavage of prM by cellular furin protease, resulting in an infectious mature virion [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. Cryo-EM of flavivirus virions exhibit two structural features: (i) The nucleocapsid core lacks organized structures and (ii) there are no observable connections between nucleocapsid and the prM/E membrane shell, suggesting that the C protein may bind to genomic RNA in a random manner without direct interaction with prM/E proteins [ ADDIN EN.CITE ADDIN EN.CITE.DATA ].

The molecular mechanism of flavivirus assembly remains largely obscure. Besides structural proteins, several nonstructural proteins, such as NS2A, NS2B, and NS3, have been shown to regulate flavivirus assembly. Different amino acids of NS2A protein were reported to play distinct roles in flavivirus RNA replication and virion assembly [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. The cleavage between C and prM is tightly modulated by NS2B/NS3 protease and cellular signalase during YFV and Murray Valley encephalitis virus assembly [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. Mutations in the NS3 helicase domain alone affect YFV assembly [ ADDIN EN.CITE <EndNote><Cite><Author>Patkar</Author><Year>2008</Year><RecNum>90</RecNum><DisplayText>(Patkar and Kuhn, 2008a)</DisplayText><record><rec-number>90</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793218">90</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Patkar, C. G.</author><author>Kuhn, R. J.</author></authors></contributors><auth-



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address>Purdue Univ, Dept Biol Sci, W Lafayette, IN 47907 USA</auth-address><titles><title>Yellow fever virus NS3 plays an essential role in virus assembly independent of its known enzymatic functions</title><secondary-title>Journal of Virology</secondary-title><alt-title>J Virol</alt-title></titles><alt-periodical><full-title>J Virol</full-title></alt-periodical><pages>3342-3352</pages><volume>82</volume><number>7</number><keywords><keyword>tick-borne encephalitis</keyword><keyword>flavivirus kunjin ns3</keyword><keyword>west-nile-virus</keyword><keyword>dengue virus</keyword><keyword>nonstructural protein</keyword><keyword>rna replication</keyword><keyword>cdna-clones</keyword><keyword>particles</keyword><keyword>ns2a</keyword><keyword>organization</keyword></keywords><dates><year>2008</year><pub-dates><date>Apr</date></pub-dates></dates><isbn>0022-538X</isbn><accession-num>WOS:000254139800015</accession-num><urls><related-urls><url><Go to ISI>://WOS:000254139800015</url></related-urls></urls><electronic-resource-num>10.1128/Jvi.02447-07</electronic-resource-num><language>English</language></record></Cite></EndNote>]. Although expression of prM/E alone is sufficient to generate empty virus-like particles, it is not known how nucleocapsid is formed and subsequently enveloped with prM/E to form infectious virions. The goal of this study is to define the molecular mechanism that orchestrates three structural proteins and viral RNA to form nucleocapsid and infectious virus in a highly coordinated manner. Using genetic, biochemical, and imaging approaches, we demonstrate that DENV NS2A protein choreographs virion morphogenesis by (i) interacting with viral prM and E proteins, (ii) regulating the processing of C-prM polyprotein by viral protease, and (iii) recruiting genomic RNA through binding to the 3'UTR. These results support a model that DENV NS2A recruits nascent viral RNA from the replication complex (housed in Ve) to the virion assembly site (on the ER membrane juxtaposed to



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VPs), where an coordinated C-prM cleavage facilitates genomic RNA encapsidation with C protein and budding with prM and E proteins into the ER lumen, resulting in progeny virions.



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## RESULTS

**NS2A G11A mutation abolishes virion assembly**. Using DENV-2 as a model, we previously identified a panel of NS2A mutant viruses that were selectively defective in virion assembly without compromising other steps of an infection cycle [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. Here, we performed four experiments to demonstrate that NS2A mutation G11A of DENV-2 caused a defect in virion assembly. (i) A DENV-2 luciferase replicon was used to examine the effect of NS2A G11A on viral translation and RNA synthesis. Upon transfection into BHK-21 cells, the G11A replicon produced a luciferase profile comparable to the WT replicon (Fig. 1A), suggesting that the mutation does not significantly reduce viral translation and RNA replication. (ii) When full-length viral RNA of DENV-2 was transfected into BHK-21 cells, the NS2A G11A mutant failed to release extracellular viral RNA (Fig. 1B). No infectious virus was detected from the culture medium of G11A RNA-transfected cells (Fig. 1C). (iii) In a *trans* packaging system, WT luciferase replicon could be packaged into pseudo-infectious particles (PIPs) when structural C-prM-E proteins were expressed from Semliki Forest virus replicon (Fig. S1A). The WT reporter replicon PIPs infected Vero cells and generated robust luciferase signals (Fig. S1B). In contrast, no PIPs were produced with the NS2A G11A replicon. (iv) Transmission electron microscopy revealed substantial virus-induced single-membrane vesicles (within the lumen of the rough ER) or double-membrane vesicles (closely associated with cisternae of the rough ER) in both WT and G11A RNA-transfected cells at 48 h post-transfection (Fig. 1D). A lot of virus particles appeared in the WT RNA-transfected cells, but no virus particles were detected in the G11A RNA-transfected cells. The results demonstrate that NS2A G11A mutation specifically abolishes virion assembly.

**NS2A G11A mutation alters the expression and localization of viral structural proteins.** We compared the subcellular localization of viral proteins in BHK-21 cells that



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were transfected with WT or NS2A G11A genomic RNA. Surprisingly, the NS2A G11A mutation altered the intracellular distribution of three structural proteins. (i) C protein disseminated in both nucleus and cytoplasm in the WT RNA-transfected cells, as previously reported [ ADDIN EN.CITE <EndNote><Cite><Author>Wang</Author><Year>2004</Year><RecNum>91</RecNum><DisplayText>(Wang et al., 2004)</DisplayText><record><rec-number>91</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793218">91</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Wang, S. H.</author><author>Syu, W. J.</author><author>Hu, S. T.</author></authors></contributors><auth-address>Institute of Microbiology and Immunology, School of Life Science, National Yang-Ming University, Taipei, Taiwan, Republic of China.</auth-address><titles><title>Identification of the homotypic interaction domain of the core protein of dengue virus type 2</title><secondary-title>J Gen Virol</secondary-title><alt-title>The Journal of general virology</alt-title></titles><periodical><full-title>J Gen Virol</full-title><abbr-1>The Journal of general virology</abbr-1></periodical><alt-periodical><full-title>J Gen Virol</full-title><abbr-1>The Journal of general virology</abbr-1></alt-periodical><pages>2307-14</pages><volume>85</volume><number>Pt 8</number><keywords><keyword>Active Transport, Cell Nucleus</keyword><keyword>Dengue Virus/*chemistry/classification</keyword><keyword>Precipitin Tests</keyword><keyword>Viral Core Proteins/*chemistry</keyword></keywords><dates><year>2004</year><pub-dates><date>Aug</date></pub-dates></dates><isbn>0022-1317 (Print)0022-1317 (Linking)</isbn><accession-num>15269372</accession-num><urls><related-urls><url>http://www.ncbi.nlm.nih.gov/pubmed/15269372</url></related-



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urls></urls><electronic-resource-num>10.1099/vir.0.80067-0</electronic-resource-num></record></Cite></EndNote>]. However, in the G11A RNA-transfected cells, the C protein was mainly restricted to the cytoplasm with rare nucleus localization (Fig. 1E). (ii) prM protein was located at the ER region in the WT RNA-transfected cells, whereas the prM protein was undetectable in the G11A RNA-transfected cells (Fig. 1F). (iii) E protein spread broadly like an ER web in the WT RNA-transfected cells. In contrast, in the G11A RNA-transfected cells, the E protein formed larger speckles at restricted subcellular areas (Fig. 1G). Compared with the structural proteins, the effect of NS2A G11A on the subcellular expression of viral nonstructural proteins (including NS1, NS3, NS4A, NS4B, and NS5) and dsRNA was not apparent (Fig. 1E-G, *top panels* for NS3, and data not shown).

The distinct effect of NS2A G11A mutation on subcellular expression between the viral structural and nonstructural proteins were further supported by immunofluorescent co-staining experiments. NS2A G11A altered the co-staining profile of E and NS3 proteins (Fig. S2). In the NS2A G11A RNA-transfected cells, 100% of NS3-expressing cells were E-positive at 24 h post-transfection; however, at 48 and 72 h post-transfection, only 59% and 20% of the NS3-expressing cells were E-positive, respectively (Fig. S2). In the WT RNA-transfected cells, 100% of NS3-expressing cells were E-positive from 24 to 72 h post-transfection (Fig. S2). The results demonstrate that NS2A G11A mutation changes the expression and subcellular localization of viral structural proteins.

**NS2A G11A mutation impairs the cleavage of viral C-prM polyprotein.** To determine the molecular mechanism of the above observations, we examined the processing of viral proteins in the WT and NS2A G11A viral RNA-transfected cells at 48 h post-transfection by Western blot. As expected, the amounts of C, NS3, and NS5 proteins from the G11A RNA-transfected cells were 15-26% of those from the WT RNA-transfected



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cells (Fig. 1H-I), due to the lack of virion production or multiple rounds of infection for the G11A mutant. Notably, the amounts of prM and E proteins were further lower in the G11A RNA-transfected cells (only 1-4% of those in the WT RNA-transfected cells), suggesting an asymmetric degradation in cells. Moreover, the NS2A G11A mutation impaired the cleavage of C-prM to individual C and prM proteins: 90% of C-prM was cleaved to mature C protein in the WT RNA-transfected cells, whereas only 20% of C-prM was processed to C protein in the G11A RNA-transfected cells (Fig. 1H, *compare lanes 1 and 5 in the top panel*). The impaired C-prM cleavage could account for the altered subcellular distribution of viral structural proteins in the NS2A C11A RNA-transfected cells: Restricting the un-cleaved C-prM to the ER membrane, preventing C protein from translocating to the nucleus, and consequently blocking prM/E heterodimer formation and virion budding.

Next, we examined the role of ubiquitin-proteasome pathway in degrading viral proteins in the G11A RNA-transfected cells. Treatment with proteasome inhibitor MG132 increased the amounts of C, prM, E, and NS5 (but not NS3) by 1-3 folds in the NS2A G11A RNA-transfected cells (Fig. 1H, *compare lanes 5 and 6*). In contrast, in the WT RNA-transfected cells, MG132 slightly reduced the amounts of C, prM, and E to different extents (Fig. 1H, *compare lanes 1 and 2*) because MG132 was known to inhibit flavivirus replication [ ADDIN EN.CITE <EndNote><Cite><Author>Bhuvanakantham</Author><Year>2013</Year><RecNum>108</RecNum><DisplayText>(Bhuvanakantham and Ng, 2013)</DisplayText><record><rec-number>108</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793288">108</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Bhuvanakantham, R.</author><author>Ng, M. L.</author></authors></contributors><auth-



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address>Flavivirology Laboratory, Department of Microbiology, Yong Loo Lin School of Medicine, National University of Singapore, 5 Science Drive 2, Singapore, 117597.</auth-address><titles><title>West Nile virus and dengue virus capsid protein negates the antiviral activity of human Sec3 protein through the proteasome pathway</title><secondary-title>Cell Microbiol</secondary-title></titles><periodical><full-title>Cell Microbiol</full-title></periodical><pages>1688-706</pages><volume>15</volume><number>10</number><keywords><keyword>Antiviral Agents/*antagonists & inhibitors</keyword><keyword>Capsid Proteins/genetics/*metabolism</keyword><keyword>Cell Line</keyword><keyword>DNA Mutational Analysis</keyword><keyword>Dengue Virus/*physiology</keyword><keyword>*Host-Pathogen Interactions</keyword><keyword>Humans</keyword><keyword>Models, Biological</keyword><keyword>Proteasome Endopeptidase Complex/*metabolism</keyword><keyword>Protein Binding</keyword><keyword>Protein Interaction Mapping</keyword><keyword>Proteolysis</keyword><keyword>Vesicular Transport Proteins/*antagonists & inhibitors</keyword><keyword>West Nile virus/*physiology</keyword></keywords><dates><year>2013</year><pub-dates><date>Oct</date></pub-dates></dates><isbn>1462-5822 (Electronic)1462-5814 (Linking)</isbn><accession-num>23522008</accession-num><urls><related-urls><url>https://www.ncbi.nlm.nih.gov/pubmed/23522008</url></related-urls></urls><electronic-resource-num>10.1111/cmi.12143</electronic-resource-num></record></Cite></EndNote>]. Distinct responses to MG132 treatment among different viral proteins were probably due to their subcellular distributions and involvement in various biological microenvironments. Nevertheless, the results suggest that ubiquitin-proteasome pathway is responsible for viral protein degradation in the NS2A G11A RNA-transfected cells.



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**WT NS2A rescues virion assembly of NS2A G11A mutant through restoring C-prM cleavage.** We examined if *trans* supply of WT NS2A could restore virion assembly of NS2A G11A RNA and rescue C-prM protein processing (Fig.S3A). Indeed, after transfection into NS2A-EGFP BHK-21 cells that constitutively expressed WT NS2A protein, the NS2A G11A RNA produced infectious virions that could infect naïve BHK-21 cells, as indicated by E-positive cells (Fig. S3B); further passaging of culture supernatants to new BHK-21 cells did not yield any E-positive cells (Fig. S3C), suggesting that the *trans* packaged virions could only infect cells for a single-round. Remarkably, the *trans* supplied WT NS2A protein restored the C-prM cleavage in the G11A RNA-transfected cells to WT level, exhibiting >89% C protein processing (Fig. S3D, *top panel*). Two prM bands, representing glycosylated and un-glycosylated forms of the protein (see below), were detected in both NS2A G11A and WT RNA-transfected NS2A-EGFP cells (Fig. S3D, *second panel*). The relative abundances of C, prM, and NS1 proteins were similar in the NS2A G11A and WT RNA-transfected cells (Fig. S3D). Because of the restored C-prM cleavage, the nucleus localization of C protein and prM-positive cells were detected in the G11A RNA-transfected NS2A-EGFP cells (Fig. S3E). These results demonstrate that WT NS2A can rescue virion assembly of NS2A G11A mutant through restoring the C-prM cleavage.

Next, we examined the effect of NS2A on the glycosylation of prM by co-expressing NS2A and prM in HEK293T cells. As a control, when EGFP was co-expressed, only the glycosylated form of prM was detected (Fig. S3F); after PGNaseF treatment, the prM was completely deglycoslated and migrated as a glycosylation-knockout mutant N69Q prM (Fig. S3G). In contrast, when NS2A-EGFP were co-expressed, both glycosylated and un-glycosylated forms of prM were detected (Fig. S3F), indicating that NS2A suppresses the glycosylation of prM. Furthermore, the G11A mutation seemed to increase the



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suppression effect of NS2A on prM glycosylation (Fig. 3F). These results suggest that NS2A may interact with prM, and that the G11A mutation may modulate the prM/NS2A interaction.

**Capsid R99S mutation can rescue virion assembly of NS2A G11A RNA.** We selected revertants by continuously passaging the NS2A G11A RNA-transfected BHK-21 cells for five rounds (Fig. S4A). Culture medium from each passaging (P1-P5) were tested for emergence of infectious viruses using plaque assay. The P5 culture produced infectious viruses with titers of $>10^4$ PFU/ml and plaques smaller than the WT virus (Fig. S4B). In addition, almost all P5 cells were E protein-positive (Fig. S4C). Whole genome sequencing of the P5 viruses from three independent selections consistently showed a consensus C-to-A substitution at nucleotide position 391 of viral genome, resulting in an Arg-to-Ser change at amino acid position 99 of the C protein (C_R99S; Fig. 2A). The original NS2A G11A mutation was retained in all P5 viruses.

To validate the role of C_R99S mutation in rescuing virion assembly of NS2A G11A RNA, we engineered C_R99S into a DENV-2 infectious clone [ ADDIN EN.CITE <EndNote><Cite><Author>Xie</Author><Year>2013</Year><RecNum>85</RecNum><DisplayText>(Xie et al., 2013)</DisplayText><record><rec-number>85</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793217">85</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Xie, X.</author><author>Gayen, S.</author><author>Kang, C.</author><author>Yuan, Z.</author><author>Shi, P. Y.</author></authors></contributors><auth-address>State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Science, China.</auth-address><titles><title>Membrane topology and function of dengue virus NS2A protein</title><secondary-title>J Virol</secondary-title><alt-title>Journal of virology</alt-



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cells (Fig. 2E). Viral C protein was detected in both cytoplasm and nucleus in the NS2A G11A+C_R99S RNA-transfected cells (Fig. 2F-G). Furthermore, 90.5% of the C-prM protein was cleaved to mature C and prM in the NS2A G11A+C_R99S RNA-transfected cells (Fig. 2G). As controls, NS2A G11A RNA showed 15.4% of C-prM cleavage and did not produce any infectious virus, whereas C_R99S RNA exhibited 99.9% of prM-C cleavage and produced a WT level of infectious virus (Fig. 2B-H). Taken together, the results demonstrate that C_R99S mutation efficiently restores C-prM cleavage and virion assembly of NS2A G11A RNA.

**Capsid R99S cannot rescue virion assembly of other NS2A mutants.** We tested if C_R99S could rescue other NS2A mutants that were previously shown to be defective in virion assembly, including E20A, E100A, and K188A [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. The C_R99S was engineered into those NS2A mutants, resulting in NS2A E20A+C_R99S, NS2A E100A+C_R99S, and NS2A K188A+C_R99S viral RNAs. After transfection into BHK-12 cells, none of the mutant RNAs produced increasing number of E-positive cells from 24 to 96 h (Fig. S5A). No infectious virus was detected in the culture medium from the mutant RNA-transfected cells (Fig. S5B). Viral C protein was detected in both nucleus and cytoplasm of all mutant RNA-transfected cells (Fig. S5C). In agreement with the C nucleus localization, the NS2A single mutant E20A and E100A cleaved 36% and 79% of C-prM, and the addition of C_R99S increased the C-prM cleavage to 72% and 96%, respectively. In contrast, NS2A K188A mutant cleaved 100% of C-prM in the presence or absence of C_R99S (Fig. 5D). The results demonstrate that C_R99S can only rescue the assembly defect of NS2A G11A possibly because different NS2A mutations abolish virion assembly through distinct mechanisms.

**Capsid R99S shifts the C-prM cleavage site by two amino acids.** The above results prompted us to investigate how C_R99S rescues the virion assembly of NS2A



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G11A. Fig. S6 depicts the topology of viral polyprotein C-prM-E-NS1-NS2A on the ER membrane. NS2A G11 resides at the first amphipathic helix on the ER lumen side [ ADDIN EN.CITE

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501 12</electronic-resource-num><language>eng</language></record></Cite></EndNote>],
502 while C_R99 is the penultimate amino acid of the C protein on the cytosolic side (Fig. S6B).
503 Since flavivirus NS2B/NS3 protease cleaves a substrate with two basic residues (Arg or
504 Lys) at the P1 and P2 sites and a residue with short side chain (Gly, Ser, or Ala) at the P1'
505 site [ ADDIN EN.CITE ADDIN EN.CITE.DATA ], sequence analysis suggests that the
506 C_R99S substitution may shift the capsid C-terminal cleavage by two amino acids from
507 position 100 to 98 (Fig. S6B). Specifically, viral NS2B/NS3 protease cleaves the WT
508 capsid at NRRRR↓TA and the R99S capsid at NRR↓SRTA (↓ denoting cleavage site and
509 residue R99S underlined). To test this hypothesis, we performed a biochemical protease
510 assay using synthetic WT peptide (NILNRRRRTAGMII) and R99S peptide
511 (NILNRRSRTAGMII). After incubating the peptides with recombinant DENV-2 NS2B/NS3
512 protease, the reaction products were analyzed by liquid chromatography-mass
513 spectrometry (LC-MS). The WT peptide was cleaved into two smaller peptides with
514 molecular weights of 1096.73 Da and 604.77 Da, corresponding to peptides NILNRRRR
515 and TAGMII, respectively (Fig. S6C). In contrast, the R99S peptide was cleaved into two
516 fragments with molecular weights of 784.47 Da and 847.46 Da, corresponding to peptide
517 NILNRR and SRTAGMII, respectively (Fig. S6D). The LC-MS also detected some
518 unknown peptides, probably due to nonspecific degradations of the protease reaction (Fig.
519 S6C-D). Nevertheless, the data indicate that the C_R99S mutation shifts the capsid
520 cleavage site upstream by two amino acids.

521 **NS2A G11A enhances prM/NS2A interaction.** Since NS2A G11A alters the
522 processing of C-prM (Fig. 1H) and prM glycosylation (Fig. S3F), we hypothesized that



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NS2A directly interacts with prM and the G11A mutation affects the prM/NS2A interaction. To test this hypothesis, we co-transfected two plasmids into HEK293T cells. One plasmid expressed the authentic signal peptide anchorC ($C_{14}$) fused with the full-length prM. Another plasmid expressed a fusion protein containing the C-terminal 24 amino acids of the E protein, the N-terminal 50 amino acids of the NS1 protein, the C-terminal 50 amino acids of NS1, full length NS2A, and an EGFP [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]; this plasmid was previously shown to express NS2A-EGFP with a correct topology on the ER membrane [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. Co-immunoprecipitation showed that NS2A-EGFP, but not EGFP, pulled down prM (Fig. 3B, lanes 2 and 3). As controls, mouse IgG did not pull down any NS2A-EGFP or prM (Lane 1), and EGFP antibody did not pull down prM in the absence of NS2A-EGFP (Lane 6). To map the region of prM that binds to NS2A, we expressed the first 130 amino acids of prM [prM(1-130)] that lacked the C-terminal two transmembrane domains (Fig. 3A). Co-immunoprecipitation showed that prM(1-130) could not pull down NS2A-EGFP (Fig. 3B, lane 4), suggesting that (i) NS2A may bind to the transmembrane region of prM or (ii) the transmembrane region of prM may affect the conformation of its ectodomain and, consequently, abolish its interaction with NS2A.

Next, we examined if NS2A mutation G11A affects the prM/NS2A interaction. Co-immunoprecipitation experiments showed that G11A NS2A-EGFP pulled down about 2.5-fold more prM than the WT NS2A-EGFP did (Fig. 4C). Reciprocally, prM pulled down about 1.7-fold more G11A NS2A-EGFP than WT NS2A-EGFP (Fig. 4D). The results indicate that the G11A mutation enhances NS2A binding to prM.

To further support the prM/NS2A interaction, we used confocal microscope to examine the co-localization of prM/NS2A in cells. In this case, prM-E were co-expressed with NS2A-EGFP in HEK293T cells. As shown in Fig. 3E, NS2A-EGFP co-localized with



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prM. The G11A mutation did not change the co-localization of NS2A-EGFP with prM (Fig. 3E). Taken together, the results demonstrate that (i) NS2A interacts with prM and (ii) NS2A G11A significantly enhances the prM/NS2A interaction.

**The G11A mutation does not affect NS2A binding to E or NS3.** We also examined the interactions between NS2A and E or NS3 proteins that were co-expressed in HEK293T cells. For testing the E/NS2A interaction, we fused a GST tag to the C-terminus of NS2A for the co-immunoprecipitation experiment [because the EGFP tag used in NS2A-EGFP in Fig. 3B-D non-specifically interacts with E protein (data not shown)]. As shown in Fig. 3F, both WT and G11A NS2A-GST pulled down E protein at comparable efficiencies. As a control, GST alone did not pull down E protein. The result suggests that NS2A interacts with E and the G11A mutation does not affect this interaction.

Since flavivirus NS3 is known to participate in viron assembly [ ADDIN EN.CITE ADDIN EN.CITE.DATA ], we also tested if NS2A G11A mutation affects the NS2A/NS3 interaction. When co-expressed in HEK293T cells, GST alone pulled down a trace amount of HA-tagged NS3 (NS3-HA) protein (Fig. S7, lane 1), whereas NS2A-GST pulled down 10-fold more NS3-HA (Lane 2). Equivalent amounts of NS3-HA were pulled down by the WT and G11A NS2A-GST (Compare lanes 2 and 3), suggesting that NS2A G11A does not affect NS2A/NS3 binding.

**Extension of two or three residues at the N-terminus of anchorC efficiently rescues virion assembly of NS2A G11A**. So far, our results showed that NS2A G11A mutation increases its binding to prM on the ER lumen side, leading to impaired C-prM processing and defective virion assembly. These defects can be rescued by a capsid mutation R99S that shifts the C-terminal cleavage site by two amino acids. As a result of this shift, the N-terminus of transmembrane anchorC is extended by two amino acids on



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572 the cytosolic side of ER membrane. These data support a model of coordinated C-prM
573 processing during virion assembly (Fig. 4A) [ ADDIN EN.CITE
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585 processing</title><secondary-title>J Virol</secondary-title><alt-title>Journal of
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598 urls></urls><custom2>112824</custom2></record></Cite></EndNote>]. During WT virus
599 assembly, polyprotein C-anchorC-prM was first cleaved at the C-terminus of capsid protein
600 (*i.e.*, R100) by NS2B/NS3 protease, producing mature C and anchorC-prM. The anchorC-
601 prM was then cleaved by a host signalase at the N-terminus of prM, generating anchorC
602 and prM. The cleaved prM coordinates with E protein for virion budding (Fig. 4A, *left*
603 *panel*). In the presence of NS2A G11A mutation, NS2A binds to prM with a higher affinity,
604 pulling anchorC (embedded in the ER membrane) towards the lumen side. The viral
605 protease cleavage site of the "pulled anchorC" is blocked, possibly by the ER membrane
606 (*middle panel*), leading to unprocessed C-prM. To overcome this defect, the C_R99S
607 mutation creates a new NS2A/NS3 cleavage site at R98 of capsid protein, restoring the C-
608 prM processing and virion assembly of NS2A G11A (*right panel*).

609 The key of the above model is the blockage of viral protease cleavage site at the
610 C-terminus of capsid protein, which is caused by the NS2A G11A mutation. If the model is
611 correct, an insertion of one or two amino acids to the N-terminus of anchorC should
612 restore the C-prM cleavage and virion assembly in the presence of NS2A G11A mutation
613 (Fig. 4B). To test this hypothesis, we inserted Thr, The-Ala, or Ser-Arg to the C-terminus of
614 capsid protein (after R100) in the context of WT or NS2A G11A viral RNA. The inserted
615 amino acids were selected based on the cleavage requirement for viral protease (Fig. 4B).
616 Transfection of each viral RNA into BHK-21 cells yielded increasing number of E-positive
617 cells from day 1 to 4 (Fig. 4C), produced infectious viruses (Fig. 4D-E), displayed cytosolic
618 and nucleus localizations of C protein (Fig. 4F), and exhibited efficient C-prM cleavage
619 (Fig. 4H). In the absence of NS2A G11A, the capsid insertion mutant RNAs behaved
620 similarly to the WT RNA. In the presence of NS2A G11A, the capsid single-amino acid
621 insertion (*i.e.*, NS2A G11A+C_T101) partially restored virion assembly ($1.2\times10^3$ PFU/ml;
622 Fig. 4D-E), prM-positive cells (Fig. 4G), and C-prM cleavage (60%; Fig. 4H); while the



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capsid double-amino acid insertion (*i.e.*, NS2A G11A+C_TA and NS2A G11A+C_SR) almost fully restored virion assembly to the WT level (2.3-2.5×$10^6$ PFU/ml; Fig. 4D-E) and C-prM cleavage (92-98%; Fig. 4H). These results demonstrate that addition of one or two amino acids to the N-terminus of anchorC can rescue virion assembly of NS2A G11A.

Since the single-amino acid C_T101 insertion only partially rescued virion assembly of NS2A G11A, we selected revertants for NS2A G11A+C_T101 virus. After continuous passaging of the virus on Vero cells for eight rounds, the P8 viruses were subjected to plaque assay (Fig. S8A-B) and whole-genome sequencing (Fig. S8C). The P8 viruses from all four independent selections produced viral titers (Fig. S8B) that were ≥100-fold higher than the original NS2A G11A+C_T101 virus (Fig. 4E). Among them, selection I P8 virus retained the original NS2A G11A+C_T101 mutations. In addition, an extra mutation R100S was recovered in the capsid protein. Sequence analysis suggests that the R100S could alter the protease cleavage site from the original RRRR↓T to RRR↓ST (R100S underlined) and extends the N-terminus of anchorC by one more amino acid. Selection II P8 virus also retained the original NS2A G11A+C_T101 mutations and accumulated another mutation R99S in the capsid protein. The R99S may change the protease cleavage site to RR↓SRT and extends the N-terminus of anchorC by two more amino acids. In addition, selection II P8 virus contained the R100S mutation in 27% of the viral population. Selection III P8 virus retained the NS2A G11A mutation and showed mixed sequences of R99S and/or R100S. Selection IV P8 virus exhibited WT plaque morphology; sequencing results showed that the NS2A G11A was reverted to WT Gly, but the C_T101 insertion was retained. Collectively, the results again support the conclusion that extension of the N-terminus of anchorC by two to three residues could efficiently rescue virion assembly of NS2A G11A.



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**Analysis of NS2A G11A in mosquito cells.** To test if the above findings from mammalian cells could be extended to mosquito host, we analyzed NS2A G11A+capsid mutant RNAs in C6/36 cells. Viral RNAs containing NS2A G11A alone, NS2A G11A+C_T101, NS2A G11A+C_R99S, or NS2A G11A+C_TA were transfected into C6/36 cells. The transfected cells were analyzed for capsid protein-positive cells (Fig. S9A), infectious virus production (Fig. S9B-C), extracellular viral RNA release (Fig. S9D), and C-prM cleavage (Fig. S9E). Similar to the results obtained from mammalian cells, NS2A G11A mutant was defective in C-prM processing and virion production in C6/36 cells. The defects could be rescued by the capsid mutations, with the rescuing efficiency of C_TA > C_R99S > C_T101 (Fig. S9). The results indicate that NS2A G11A mutation exerts the same mechanism in abolishing virion assembly in mammalian and mosquito hosts.

**NS2A protein binds to 3'UTR RNA**. To further explore the role of DENV-2 NS2A in virion assembly, we examined the ability of NS2A to bind viral genomic RNA. A series of pulldown experiments were performed using biotinylated viral RNAs and NS2A protein (Fig. 5A). A 616-nucleotide viral RNA (5'UTR-$C_{66}$-3'UTR) comprising the 5'UTR, the first 66 nucleotides from the C gene, and the 3'UTR regions of DENV-2 genome was *in vitro* synthesized (Fig. S10A). The 3' end of the RNA was ligated to a biotinylated cytidine using T4 RNA ligase, resulting in 5'UTR-$C_{66}$-3'UTR-biotin RNA (Fig. S10B). Two types of GST-tagged NS2A protein were prepared for the pulldown experiment: One type NS2A was expressed from *E. coli*, partially purified, and reconstituted in detergent DDM-containing buffer (Fig. 5B); another type NS2A was from the cell lysates of HEK293T cells transiently expressing the NS2A protein (Fig. 5C). As shown in Fig. 5B-C, the 5'UTR-$C_{66}$-3'UTR-biotin RNA efficiently pulled down GST-tagged NS2A (Lanes 4 and 6), but not GST (Lanes 2 and 5); in contrast, neither GST nor GST-tagged NS2A proteins was pulled down by a biotinylated non-viral RNA control (Lanes 1 and 3). To further demonstrate the specificity



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of NS2A/5'UTR-$C_{66}$-3'UTR RNA interaction, we performed an RNA competition experiment. Increasing amounts un-biotinylated 5'UTR-$C_{66}$-3'UTR RNA competed away the NS2A/5'UTR-$C_{66}$-3'UTR-biotin RNA interaction, whereas yeast tRNA did not (Fig. 5D). These results suggest that 5'UTR-$C_{66}$-3'UTR RNA specifically binds to NS2A protein.

We estimated the binding affinity of NS2A/viral RNA interaction. 5'UTR-$C_{66}$-3'UTR-biotin RNA was incubated with increasing amounts of recombinant GST-NS2A protein. The reactions were separated on a native polyacrylamide gel (PAGE), transferred to nylon membrane, and probed with streptavidin-HRP (Fig. 5E). Quantification of free RNA and RNA/protein complex estimated a *Kd* of 35 nM for DENV-2 NS2A biding to viral RNA.

**The last 285 nucleotides of 3'UTR RNA is responsible for binding to NS2A protein**. To map the region of viral RNA responsible for binding to NS2A protein, we performed a competition assay using a set of un-biotinylated RNA fragments from 5'UTR-$C_{66}$-3'UTR (Fig. S10C). The fragments were prepared based on the previously proposed RNA structure [ ADDIN EN.CITE <EndNote><Cite><Author>Villordo</Author><Year>2015</Year><RecNum>109</RecNum><DisplayText>(Villordo et al., 2015)</DisplayText><record><rec-number>109</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1561296709">109</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Villordo, S. M.</author><author>Filomatori, C. V.</author><author>Sanchez-Vargas, I.</author><author>Blair, C. D.</author><author>Gamarnik, A. V.</author></authors></contributors><auth-address>Fundacion Instituto Leloir-CONICET, Buenos Aires, Argentina.Arthropod-borne and Infectious Diseases Laboratory, Department of Microbiology, Immunology and Pathology, Colorado State University, Fort Collins, Colorado, United States of America.</auth-address><titles><title>Dengue virus



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711 urls></urls><custom2>PMC4311971</custom2><electronic-resource-
712 num>10.1371/journal.ppat.1004604</electronic-resource-
713 num></record></Cite></EndNote>]. As shown in Fig. 5F, 3'UTR, but not 5'UTR-$C_{66}$,
714 competed away the NS2A/5'UTR-$C_{66}$-3'UTR-biotin RNA interaction, indicating that 3'UTR
715 RNA binds to NS2A protein. To further map the region of 3'UTR for NS2A binding, we
716 deleted pseudoknot 1 (Δpk1), pseudoknots 1-2 [Δpk(1-2)], and pseudoknots 1-3 [Δpk(1-3)]
717 from the 3'UTR (Fig. S10C). Only Δpk1 and Δpk(1-2) RNAs, not Δpk(1-3) RNA, could
718 compete away the NS2A/5'UTR-$C_{66}$-3'UTR-biotin RNA interaction (Fig. 5F), suggesting
719 that Δpk(1-2) RNA, representing the last 285 nucleotides of 3'UTR RNA, is required for
720 binding to NS2A protein. This conclusion was further supported by the results that different
721 fragments from Δpk(1-2) RNA, including pK(3-4), pk3, and 3'SL, did not compete away the
722 NS2A/5'UTR-$C_{66}$-3'UTR-biotin RNA interaction (Fig. 5F).



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723 Next, we performed a photo-activatable ribonucleoside-enhanced crosslinking
724 (PAR-CL) experiment to define the nucleotides that may interact with NS2A protein (Fig.
725 5G). The 3'UTR RNA containing [ HYPERLINK "https://en.wikipedia.org/wiki/Photosensitivity"
726 \o "Photosensitivity" ] 4-thiouridine was prepared through *in vitro* transcription. After
727 RNA/protein complex formation, the 4-thiouridine in the 3'UTR RNA was cross-linked to
728 NS2A protein under UV light (at 365-nm wavelength). The RNA was then subjected to
729 deep sequencing. Due to the crosslinking of 4-thiouridine to protein, a T-to-C mutation was
730 recovered at the RNA sites that interacted with NS2A protein (Figs. 5G and S11). Fig. 5H
731 summarizes the U-to-C mutations in the 3'UTR RNA when complexed with NS2A protein.
732 About 67% mutations occurred within the last 285 nucleotides of 3'UTR. The result further
733 supports the NS2A/3'UTR RNA interaction.

734 **NS2A requires amino acids R94, K95, and K99A for binding to 3'UTR RNA**
735 **and virion assembly.** To map the region of NS2A required for viral 3'UTR RNA binding,
736 we prepared a panel of purified NS2A truncates with an N-terminal GST tag from *E. coli*
737 (Fig. 6A). The binding efficiencies of NS2A truncates to 5'UTR-$C_{66}$-3'UTR-biotin RNA were
738 compared using a pulldown assay. As shown in Fig. 6B, different NS2A truncates were
739 pulled down by 5'UTR-$C_{66}$-3'UTR-biotin at varied efficiencies. The RNA pulled down NS2A
740 1-145, 1-168, and 1-218 (full-length) at comparable efficiencies. Compared to the full-
741 length NS2A, the pulldown efficiencies for NS2A 1-101 and 1-71 decreased to 40% and
742 0%, respectively. These data suggest that amino acids 72-144 are required for NS2A
743 binding to viral RNA.

744 According to the membrane topology of DENV-2 NS2A [ ADDIN EN.CITE
745 <EndNote><Cite><Author>Nemesio</Author><Year>2014</Year><RecNum>82</RecNu
746 m><DisplayText>(Nemesio and Villalain, 2014)</DisplayText><record><rec-
747 number>82</rec-number><foreign-keys><key app="EN" db-



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id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793217">82</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Nemesio, H.</author><author>Villalain, J.</author></authors></contributors><auth-address>Molecular and Cellular Biology Institute, Universitas "Miguel Hernandez" , E-03202 Elche-Alicante, Spain.</auth-address><titles><title>Membrane interacting regions of Dengue virus NS2A protein</title><secondary-title>J Phys Chem B</secondary-title></titles><periodical><full-title>J Phys Chem B</full-title></periodical><pages>10142-55</pages><volume>118</volume><number>34</number><keywords><keyword>Calorimetry, Differential Scanning</keyword><keyword>Cell Membrane/*metabolism</keyword><keyword>Fluorescence Polarization</keyword><keyword>*Hydrophobic and Hydrophilic Interactions</keyword><keyword>Peptide Library</keyword><keyword>Spectrophotometry, Infrared</keyword><keyword>Viral Nonstructural Proteins/*chemistry/*metabolism</keyword></keywords><dates><year>2014</year><pub-dates><date>Aug 28</date></pub-dates></dates><isbn>1520-5207 (Electronic)1520-5207 (Linking)</isbn><accession-num>25119664</accession-num><urls><related-urls><url>http://www.ncbi.nlm.nih.gov/pubmed/25119664</url></related-urls></urls><custom2>PMC4148155</custom2><electronic-resource-num>10.1021/jp504911r</electronic-resource-num></record></Cite></EndNote>], amino acids 72-144 form two transmembrane domains (72-92 and 101-118), one short cytosolic loop (93-100), and a luminal segment (118-144; Fig. 6A). Since viral RNA resides in the cytoplasm, we examined the cytosolic loop of NS2A for viral RNA binding. The cytosolic



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loop has three positively charged residues (R94, K95, and K99) that are conserved among the four DENV serotypes (Fig. 6C). To examine the function of these residues, we prepared a panel of recombinant DENV-2 containing Ala substitutions at single (R94A, K95A, or K99A), double (R94A+K95A), or triple residues (R94A+K95A+K99A). Upon transfection to BHK-21 cells, all mutant RNAs generated viral E protein-positive cells at day 1 (Fig. 6D). Except for the triple mutant, other RNAs produced more E-positive cells on day 4 post-transfection. Mutants R94A and K95A generated WT level of infectious viruses and plaque morphology (Fig. 6E-F). Mutants K99A and K94A+K95A generated 22- to 3-fold less viruses than the WT, respectively (Fig. 6F), and exhibited smaller plaques (Fig. 6E). Remarkably, triple mutant R94A+K95A+K99A RNA yielded almost undetectable amount of virus that was 50,000-fold lower than the WT (Fig. 6F). To examine the effect of R94A+K95A+K99A on viral RNA synthesis, we engineered the triple mutations into a luciferase replicon of DENV-2 (Fig. 6G). The R94A+K95A+K99A replicon generated a luciferase profile similar to the WT replicon, indicating that the mutations do not affect viral translation and RNA synthesis. Taken together, the results demonstrate that R94A+K95A+K99A triple mutant is defective in virion assembly.

Next, we examined the effect of R94A+K95A+K99A mutations on NS2A binding to viral RNA. Since the result from Fig. 6B indicates that NS2A fragment 72-144 is required for viral RNA binding, we expressed a short NS2A peptide 64-128 (comprising the first two NS2A transmembrane domains, the cytosolic region 93-100, and the N- and C-terminal luminal tails for correct folding of the peptide on membrane) fused with an N-terminal GST-tag (Fig. 6H). As shown in Fig. 6I, peptide 64-128 could be pulled down by 5'UTR-$C_{66}$-3'UTR-biotin RNA (Lane 1). Un-biotinylated 5'UTR-$C_{66}$-3'UTR or 3'UTR RNA (Lanes 2 and 4), but not 5'UTR-$C_{66}$ RNA or yeast tRNA (Lanes 3 and 5), could compete away the NS2A peptide 64-128/5'UTR-$C_{66}$-3'UTR-biotin RNA interaction. The result demonstrates that



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NS2A peptide 64-128 specifically binds to viral 3'UTR RNA. Importantly, mutant R94A+K95A+K99A reduced the pulldown efficiency of NS2A peptide 64-128 by over 80% (Fig. 6J). Notably, some degraded GST-tag was found to associate with the NS2A truncates in the above pulldown experiments (Figs. 6B I-J), most likely due to the dimerization property of GST[ ADDIN EN.CITE <EndNote><Cite><Author>Maru</Author><Year>1996</Year><RecNum>110</RecNum><DisplayText>(Maru et al., 1996)</DisplayText><record><rec-number>110</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1561302524">110</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Maru, Y.</author><author>Afar, D. E.</author><author>Witte, O. N.</author><author>Shibuya, M.</author></authors></contributors><auth-address>Department of Genetics, Institute of Medical Science, University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108, Japan.</auth-address><titles><title>The dimerization property of glutathione S-transferase partially reactivates Bcr-Abl lacking the oligomerization domain</title><secondary-title>J Biol Chem</secondary-title></titles><periodical><full-title>J Biol Chem</full-title></periodical><pages>15353-7</pages><volume>271</volume><number>26</number><keywords><keyword>*Adaptor Proteins, Signal Transducing</keyword><keyword>Animals</keyword><keyword>Bone Marrow Cells</keyword><keyword>Cell Transformation, Neoplastic</keyword><keyword>Cells, Cultured</keyword><keyword>Fusion Proteins, bcr-abl/*metabolism</keyword><keyword>GRB2 Adaptor Protein</keyword><keyword>Glutathione Transferase/*metabolism</keyword><keyword>Macromolecular Substances</keyword><keyword>Mice</keyword><keyword>Phosphotyrosine/metabolism</keyword><keyword>Protein Binding</keyword><keyword>Protein-Tyrosine



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825 Kinases/metabolism</keyword><keyword>Proteins/metabolism</keyword><keyword>Rat
826 s</keyword><keyword>Recombinant Fusion Proteins</keyword><keyword>Structure-
827 Activity Relationship</keyword></keywords><dates><year>1996</year><pub-
828 dates><date>Jun 28</date></pub-dates></dates><isbn>0021-9258 (Print)0021-
829 9258 (Linking)</isbn><accession-num>8663064</accession-num><urls><related-
830 urls><url>https://www.ncbi.nlm.nih.gov/pubmed/8663064</url></related-
831 urls></urls><electronic-resource-num>10.1074/jbc.271.26.15353</electronic-resource-
832 num></record></Cite></EndNote>]. Overall, the results indicate that R94, K95, and K99
833 are important for NS2A binding to viral 3'UTR.

834 **DISCUSSION**

835 Flavivirus assembly consists of a tightly controlled cascade of events: export of
836 newly synthesized viral RNA from the replication complex to assembly sites, encapsidation
837 of viral RNA with C protein, and envelopment of nucleocapsid with prM and E proteins.
838 Many host and viral factors (proteins and lipids) participate in this temporal and spatial
839 process [ ADDIN EN.CITE <EndNote><Cite><Author>Apte-
840 Sengupta</Author><Year>2014</Year><RecNum>94</RecNum><DisplayText>(Apte-
841 Sengupta et al., 2014)</DisplayText><record><rec-number>94</rec-number><foreign-
842 keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv"
843 timestamp="1560793218">94</key></foreign-keys><ref-type name="Journal
844 Article">17</ref-type><contributors><authors><author>Apte-Sengupta,
845 S.</author><author>Sirohi, D.</author><author>Kuhn, R.
846 J.</author></authors></contributors><auth-address>Markey Center for Structural Biology,
847 Department of Biological Sciences and Bindley Bioscience Center, Purdue University,
848 West Lafayette, IN, USA.Markey Center for Structural Biology, Department of



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849 Biological Sciences and Bindley Bioscience Center, Purdue University, West Lafayette, IN,
850 USA. Electronic address: kuhnr@purdue.edu.</auth-address><titles><title>Coupling of
851 replication and assembly in flaviviruses</title><secondary-title>Curr Opin
852 Virol</secondary-title></titles><periodical><full-title>Curr Opin Virol</full-
853 title></periodical><pages>134-
854 42</pages><volume>9</volume><keywords><keyword>Flavivirus/*physiology</keyword>
855 <keyword>Host-Pathogen
856 Interactions</keyword><keyword>Humans</keyword><keyword>Lipid
857 Metabolism</keyword><keyword>Viral Proteins/metabolism</keyword><keyword>*Virus
858 Assembly</keyword><keyword>*Virus
859 Replication</keyword></keywords><dates><year>2014</year><pub-
860 dates><date>Dec</date></pub-dates></dates><isbn>1879-6265 (Electronic)1879-
861 6257 (Linking)</isbn><accession-num>25462445</accession-num><urls><related-
862 urls><url>http://www.ncbi.nlm.nih.gov/pubmed/25462445</url></related-
863 urls></urls><custom2>PMC4268268</custom2><electronic-resource-
864 num>10.1016/j.coviro.2014.09.020</electronic-resource-
865 num></record></Cite></EndNote>]. Among them, flaviviral NS2A has emerged as a key
866 player to orchestrate virion assembly, as evidenced by the following findings. (i) In infected
867 cells, there are two distinct NS2A species that are required for viral RNA synthesis and
868 virion assembly. The NS2A species required for RNA synthesis is located at the viral
869 replication complex that could only be formed in *cis*; the other NS2A species required for
870 virus morphogenesis is located at the virion assembly complex that could be formed in *cis*
871 or *trans* [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. (ii) The defect in virion assembly
872 caused by DENV-2 and YFV NS2A mutations could be rescued by adaptive mutations in
873 prM, E, NS2B, and NS3 proteins, suggesting that NS2A genetically modulates other viral
874 proteins during virion assembly [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. (iii)



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Recombinant NS2A of Kunjin virus binds to viral 3'UTR RNA [ ADDIN EN.CITE <EndNote><Cite><Author>Mackenzie</Author><Year>1998</Year><RecNum>81</RecNum><DisplayText>(Mackenzie et al., 1998)</DisplayText><record><rec-number>81</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793217">81</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Mackenzie, J. M.</author><author>Khromykh, A. A.</author><author>Jones, M. K.</author><author>Westaway, E. G.</author></authors></contributors><titles><title>Subcellular localization and some biochemical properties of the flavivirus Kunjin nonstructural proteins NS2A and NS4A</title><secondary-title>Virology</secondary-title></titles><periodical><full-title>Virology</full-title></periodical><pages>203-15</pages><volume>245</volume><number>2</number><keywords><keyword>Animal</keyword><keyword>Cercopithecus aethiops</keyword><keyword>*Flavivirus/ph [Physiology]</keyword><keyword>Immunohistochemistry</keyword><keyword>RNA, Viral/me [Metabolism]</keyword><keyword>Support, Non-U.S. Gov't</keyword><keyword>Vero Cells</keyword><keyword>*Viral Nonstructural Proteins/me [Metabolism]</keyword><keyword>Virus Replication</keyword></keywords><dates><year>1998</year></dates><urls></urls></record></Cite></EndNote>]. These studies indicate that NS2A interacts with two of the three structural proteins (prM and E, not C) and viral RNA. However, how viral RNA and C protein are recruited to form nucleocapsid and how nucleocapsid is enveloped with prM-E proteins to form virion remain unknown. The current study aims to address these questions.



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First, we showed that DENV-2 NS2A interacted with prM to modulate virion assembly. WT NS2A directly bound to prM in the absence of other viral proteins (Fig. 3B). The biological relevance of this interaction was supported by the correlation between virion assembly and prM/NS2A interaction. Specifically, an NS2A mutation G11A, which abolished virion assembly (Fig. 1), enhanced its binding to prM by 1.7-2.5 folds (Fig. 3). Mechanistically, the enhanced interaction between the mutant NS2A and prM "over pulled" the membrane-associated anchorC towards the ER lumen, leading to a blockage of the viral protease cleavage site (most likely by the ER membrane) at the C-anchorC junction. The uncleaved C-prM resulted in a rapid degradation of all three structural proteins (Fig. 1). This is not surprising because the unprocessed C-prM is retained on the ER membrane, misfolded, and degraded via the ubiquitin-proteasome machinery (Fig. 1H). Since prM is a chaperone for E protein [ ADDIN EN.CITE <EndNote><Cite><Author>Lorenz</Author><Year>2002</Year><RecNum>96</RecNum><DisplayText>(Lorenz et al., 2002)</DisplayText><record><rec-number>96</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793218">96</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Lorenz, I. C.</author><author>Allison, S. L.</author><author>Heinz, F. X.</author><author>Helenius, A.</author></authors></contributors><auth-address>Institute of Biochemistry, Swiss Federal Institute of Technology, CH-8093 Zurich, Switzerland.</auth-address><titles><title>Folding and dimerization of tick-borne encephalitis virus envelope proteins prM and E in the endoplasmic reticulum</title><secondary-title>J Virol</secondary-title></titles><periodical><full-title>J Virol</full-title></periodical><pages>5480-91</pages><volume>76</volume><number>11</number><keywords><keyword>Animals</keyword><keyword>COS Cells</keyword><keyword>Cercopithecus



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925 aethiops</keyword><keyword>Dimerization</keyword><keyword>Dithiothreitol</keyword
926 ><keyword>Encephalitis Viruses, Tick-
927 Borne/genetics/*metabolism</keyword><keyword>Hexosaminidases/metabolism</keywor
928 d><keyword>Polyproteins/genetics/metabolism</keyword><keyword>*Protein
929 Folding</keyword><keyword>Protein
930 Precursors/genetics/metabolism</keyword><keyword>Recombinant Fusion
931 Proteins/genetics/metabolism</keyword><keyword>Viral Envelope
932 Proteins/genetics/*metabolism</keyword></keywords><dates><year>2002</year><pub-
933 dates><date>Jun</date></pub-dates></dates><isbn>0022-538X (Print)0022-538X
934 (Linking)</isbn><accession-num>11991976</accession-num><urls><related-
935 urls><url>http://www.ncbi.nlm.nih.gov/pubmed/11991976</url></related-
936 urls></urls><custom2>PMC137023</custom2><electronic-resource-
937 num>10.1128/jvi.76.11.5480-5491.2002</electronic-resource-
938 num></record></Cite></EndNote>], the degradation of C-prM leads to the degradation of
939 E protein. These results are in agreement with a recent report that ZIKV anchorC plays an
940 important role in the stability of E protein and virion morphogenesis [ ADDIN EN.CITE
941 <EndNote><Cite><Author>Rana</Author><Year>2018</Year><RecNum>97</RecNum><
942 DisplayText>(Rana et al., 2018)</DisplayText><record><rec-number>97</rec-
943 number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv"
944 timestamp="1560793219">97</key></foreign-keys><ref-type name="Journal
945 Article">17</ref-type><contributors><authors><author>Rana, J.</author><author>Slon
946 Campos, J. L.</author><author>Leccese, G.</author><author>Francolini,
947 M.</author><author>Bestagno, M.</author><author>Poggianella,
948 M.</author><author>Burrone, O. R.</author></authors></contributors><auth-
949 address>Molecular Immunology Group, International Centre for Genetic Engineering and
950 Biotechnology, Trieste, Italy rana@icgeb.org burrone@icgeb.org.Molecular



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951 Immunology Group, International Centre for Genetic Engineering and Biotechnology,
952 Trieste, Italy.Department of Medical Biotechnology and Translational Medicine,
953 Universita degli Studi di Milano, Milan, Italy.</auth-address><titles><title>Role of Capsid
954 Anchor in the Morphogenesis of Zika Virus</title><secondary-title>J Virol</secondary-
955 title></titles><periodical><full-title>J Virol</full-
956 title></periodical><volume>92</volume><number>22</number><keywords><keyword>Zi
957 ka virus</keyword><keyword>capsid
958 anchor</keyword><keyword>flavivirus</keyword><keyword>morphogenesis</keyword><
959 keyword>pseudovirus</keyword><keyword>viral
960 assembly</keyword></keywords><dates><year>2018</year><pub-dates><date>Nov
961 15</date></pub-dates></dates><isbn>1098-5514 (Electronic)0022-538X
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963 urls><url>http://www.ncbi.nlm.nih.gov/pubmed/30158295</url></related-
964 urls></urls><custom2>PMC6206493</custom2><electronic-resource-
965 num>10.1128/JVI.01174-18</electronic-resource-num></record></Cite></EndNote>].

966 Second, we demonstrated that the defect in virion assembly of NS2A G11A could
967 be restored by two different means. Creation of a new viral protease cleavage site by
968 extending the N-terminus of anchorC by two amino acids restored the capsid-anchorC
969 processing and, consequently, restored virion assembly. This conclusion was supported
970 by revertant analysis of the NS2A G11A mutant (Figs. 2 and S4) and amino acid insertion
971 experiment (Fig. 4). The two-amino acid extension exposes the new protease cleavage
972 site to NS2B/NS3. Alternatively, the virion assembly defect of NS2A G11A could be
973 rescued by *trans* supply of WT NS2A (Fig. S3). Under this condition, the WT NS2A
974 replaced the G11A NS2A to interact with C-prM, restored the C-prM cleavage, and ensued
975 a productive virion morphogenesis. Collectively, our results support a previous model that



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the two cleavages at C-anchorC-prM junctions are coordinated in a sequential manner during flavivirus assembly. The C-anchorC junction was first cleaved by viral protease on the cytosolic side, followed by the anchorC-prM cleavage by the cellular signalase on the ER lumen side [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. Although expression of flavivirus prM-E alone could form empty virus-like particles, the coordinated C-anchorC-prM processing couples the encapsidation and envelopment events; such coupling is required for the production of infectious virus. This conclusion is also supported by the previous reports that separate expression of C and prM-E significantly compromised the assembly efficiency of infectious virions [ ADDIN EN.CITE ADDIN EN.CITE.DATA ].

Third, we characterized the specific interaction between DENV-2 NS2A and the 3'UTR of viral genome. Although Kunjin virus NS2A was reported to bind to the 3'UTR of viral RNA [ ADDIN EN.CITE <EndNote><Cite><Author>Mackenzie</Author><Year>1998</Year><RecNum>81</RecNum><DisplayText>(Mackenzie et al., 1998)</DisplayText><record><rec-number>81</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793217">81</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Mackenzie, J. M.</author><author>Khromykh, A. A.</author><author>Jones, M. K.</author><author>Westaway, E. G.</author></authors></contributors><titles><title>Subcellular localization and some biochemical properties of the flavivirus Kunjin nonstructural proteins NS2A and NS4A</title><secondary-title>Virology</secondary-title></titles><periodical><full-title>Virology</full-title></periodical><pages>203-15</pages><volume>245</volume><number>2</number><keywords><keyword>Animal</keyword><keyword>Cercopithecus aethiops</keyword><keyword>*Flavivirus/ph



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1001 [Physiology]</keyword><keyword>Immunohistochemistry</keyword><keyword>RNA,
1002 Viral/me [Metabolism]</keyword><keyword>Support, Non-U.S.
1003 Gov't</keyword><keyword>Vero Cells</keyword><keyword>*Viral Nonstructural
1004 Proteins/me [Metabolism]</keyword><keyword>Virus
1005 Replication</keyword></keywords><dates><year>1998</year></dates><urls></urls></re
1006 cord></Cite></EndNote>], the molecular details of the interaction has not been delineated.
1007 Using competition assay and PAR-CLIP technique, we mapped that DENV-2 NS2A bound
1008 to the last 285 nucleotides of the 3'UTR, including the pk3, pk4, and 3'SL. Interestingly, the
1009 pk3, pk3-4, or 3'SL RNA alone did not efficiently compete out the NS2A/3'UTR RNA
1010 interaction (Fig. 5F), suggesting that a complex RNA structure in the 3'UTR is required for
1011 efficient binding to NS2A protein. Since no RNA “packaging signal” has been identified for
1012 flavivirus assembly and the C protein was shown to non-specifically bind to any RNA
1013 [ ADDIN EN.CITE
1014 <EndNote><Cite><Author>Pong</Author><Year>2011</Year><RecNum>102</RecNum>
1015 <DisplayText>(Pong et al., 2011)</DisplayText><record><rec-number>102</rec-
1016 number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv"
1017 timestamp="1560793219">102</key></foreign-keys><ref-type name="Journal
1018 Article">17</ref-type><contributors><authors><author>Pong, W.
1019 L.</author><author>Huang, Z. S.</author><author>Teoh, P. G.</author><author>Wang,
1020 C. C.</author><author>Wu, H. N.</author></authors></contributors><auth-
1021 address>Institute of Molecular Biology, Academia Sinica, Taipei, Taiwan.</auth-
1022 address><titles><title>RNA binding property and RNA chaperone activity of dengue virus
1023 core protein and other viral RNA-interacting proteins</title><secondary-title>FEBS
1024 Lett</secondary-title><alt-title>FEBS letters</alt-title></titles><periodical><full-title>FEBS
1025 Lett</full-title><abbr-1>FEBS letters</abbr-1></periodical><alt-periodical><full-title>FEBS
1026 Lett</full-title><abbr-1>FEBS letters</abbr-1></alt-periodical><pages>2575-



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81</pages><volume>585</volume><number>16</number><keywords><keyword>Base Sequence</keyword><keyword>*Dengue Virus</keyword><keyword>Genome, Viral/genetics</keyword><keyword>Molecular Chaperones/*metabolism</keyword><keyword>Nucleic Acid Conformation</keyword><keyword>Protein Binding</keyword><keyword>Protein Engineering</keyword><keyword>RNA Helicases/metabolism</keyword><keyword>RNA, Catalytic/genetics</keyword><keyword>RNA, Viral/chemistry/genetics/*metabolism</keyword><keyword>Serine Endopeptidases/metabolism</keyword><keyword>Viral Core Proteins/*metabolism</keyword><keyword>Viral Nonstructural Proteins/metabolism</keyword></keywords><dates><year>2011</year><pub-dates><date>Aug 19</date></pub-dates></dates><isbn>1873-3468 (Electronic)0014-5793 (Linking)</isbn><accession-num>21771593</accession-num><urls><related-urls><url>http://www.ncbi.nlm.nih.gov/pubmed/21771593</url></related-urls></urls><electronic-resource-num>10.1016/j.febslet.2011.06.038</electronic-resource-num></record></Cite></EndNote>], the last 285 nucleotides of 3'UTR may serve as the "packaging signal" to recruit viral RNA to the virion assembly site through specific binding to NS2A. From the NS2A side, we have mapped the 3'UTR-binding site to a cytosolic loop (containing R94, K95, and K99) between the first and second transmembrane domains (Fig. 6). Triple mutant R94A+K95A+K99A was defective in virion assembly and lost over 80% of the NS2A binding activity to 3'UTR RNA (Fig. 6). Caution should be taken when applying the DENV-2 findings to other flaviviruses because of the variations of NS2A proteins. The three positively charged R94, K95, and K99 of NS2A are only conserved among the four serotypes of DENV. In addition, DENV-2, YFV, and ZIKV NS2A proteins



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exhibit different topologies on the ER membrane [ ADDIN EN.CITE ADDIN EN.CITE.DATA ].

Our findings support a general model for flavivirus virion assembly (Fig. 7). In infected cells, NS2A molecules reside in both replication complex (housed in Ve) and viron assembly complex (located on the juxtaposed ER membrane). When plus-sense RNAs are made and released from the replication complex (through the pore on Ve), they are initially destined to new rounds of viral translation and replication on the ER. Once the amounts of viral RNA and proteins have accumulated over certain “thresholds”, nascent RNAs are switched for virion assembly. At this time, the newly synthesized RNA exits the replication complex and binds to the NS2A located at the assembly site through the 3'UTR RNA. The NS2A molecules at the assembly site also recruits C-prM-E and NS2B/NS3 through binding to prM and NS3, respectively. One NS2A molecule does not have to bind to the 3’UTR RNA, C-prM-E, and NS2B/NS3 at the same time. These interactions could be sequential or through different NS2A molecules that oligomerize together on the ER membrane [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. Next, the C-prM-E polyprotein is sequentially cleaved by the NS2B/NS3 protease and host signalase to produce mature C, prM, and E [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. The C molecules, many of which have been previously made and stored at the nearby lipid droplets [ ADDIN EN.CITE ADDIN EN.CITE.DATA ], bind viral RNA to form nucleocapsid. The processed prM undergoes conformational change and dimerizes with E. The prM/E dimerization and oligomerization dissociate prM from NS2A. When 180 copies of prM/E oligomerize on the ER membrane, envelopment of the nucleocapsid occurs, generating spiky immature virions. During the above process, NS3 may also use its helicase activity to transport viral RNA from the replication complex to the assembly site and condense the



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RNA during encapsidation. Besides NS3, NS2A or other proteins may also contribute to the translocation of viral RNA from the replication complex to the assembly site.

The above model is centered on the multifunctional role of NS2A in flavivirus replication and virion assembly. Our results have functionally demonstrated the molecular mechanism for two important steps during DENV-2 assembly: (i) The binding of 3'UTR RNA to NS2A servers as a "packaging signal" and (ii) in the presence of viral RNA-NS2A complex, the coordinated cleavage of C-prM-E facilitates the envelopment of nucleocapsid. The proposed model could serve as a framework for future investigations and antiviral development.

**Author contribution**

X.X., J.Z., X.Z., Y.Z., Q.W., H.-P.D. performed experiments and data analysis. X.X., J.Z., X.Z., Y.Z., A.R., V.L.P., X.W., Q.W., H.-P.D, P.-Y.S. designed the experiments and interpreted the results. X.X., P.-Y.S. wrote the manuscript.

**Acknowledgements**

We are grateful to Dr. Mariano A. Garcia-Blanco, Shelton S. Brarick from University of Texas Medical Branch for helpful discussions during this work. P.-Y.S. was supported by NIH grants AI142759, AI[ HYPERLINK "https://public.era.nih.gov/grantfolder/piAppDetails/genericStatus.do?encryptedParam=vXeG1axw2cY.YZxjFgAisBblI6OqVj4LxDR99WdVyTI-X4FuJbCLt4g." \t "_blank" ], and AI136126, and awards from the Kleberg Foundation, John S. Dunn Foundation, Amon G. Carter Foundation, Gilson Longenbaugh Foundation, and Summerfield Robert Foundation. X.X. and J.Z. were awarded with postdoctoral fellowships from the Novartis Institutes for



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Biomedical Research (NIBR) and the McLaughlin Endowment at University of Texas Medical Branch, respectively.



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## Figure titles and legends

**Figure 1 Characterization of NS2A G11A mutant**. (A) Replicon transient transfection assay. Top panel depicts the DENV-2 luciferase replicon. Bottom panel shows the luciferase activities after transfecting BHK-21 cells with equal amounts (10 μg) of WT or NS2A G11A mutant replicon RNA. (B) Quantification of extracellular viral RNA. Top panel depicts the full-length genome of DENV-2. Equal amounts of WT or NS2A G11A mutant viral RNA (10 μg) were electroporated into BHK-21 cells. At different time post-transfection (p.t.), extracellular viral RNAs were quantified by RT-qPCR. The DENV-2 replicon RNA was used as a control for determining the basal level of viral RNA released from dead cells. Bottom panel shows the CT values of the RT-qPCR results. Data show the means and standard deviations from three independent experiments. (C) Plaque morphology. Culture supernatants from (B) were analyzed for virus production using plaque assay. Only WT, not NS2A G11A mutant (N.D.), produced plaques. Plaques were developed on BHK-21 cells after 5 days of infection. (D) Thin-section transmission electron microscope (TEM) images of viral RNA-transfected BHK-21 cells. At 48 h p.t., cells were fixed for TEM analysis. Ve, virus-induced vesicles; T, membrane tubes; Vi, virus particles; ER, endoplasmic reticulum. Mock transfected cells were included as a control. (E-G) Confocal microscopy analysis of subcellular localizations of C, prM, and E in viral RNA-transfected BHK-21 cells. At 48 h p.t., transfected cells were analyzed by IFA. NS3 staining (red) was used as a control. Nuclei (blue) were stained by DAPI. Scale bar, 10 μm. (H) Viral protein processing in viral RNA-transfected BHK-21 cells. At 42 h p.t., BHK-21 (Mock) or viral RNA-transfected cells were treated with 0.01% DMSO (-) or 10 μM MG132 (+). After 6 h treatment, the cells were analyzed by Western blot. (I) Densitometry analysis. The intensities of bands representing specific viral proteins from DMSO-treated cells in (H) was quantified. The amounts of viral proteins derived from WT RNA-transfected cells were set as 100%. Relative intensities are calculated by normalizing the protein intensities derived from NS2A G11A mutant RNA-transfected cells to those from WT RNA-transfected cells. Data shows the means and standard deviations from three independent experiments.

**Figure 2 Restoration of NS2A G11A virion assembly by capsid R99S mutation.** (A) Revertant mutation. BHK-21 cells were transfected with NS2A G11A viral RNA. The transfected cells were consecutively passaged for five rounds to select for revertant viruses. The P5 virus was sequenced for revertant mutations. Changes in nucleotide



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positions and amino acids are indicated. (B) IFA of viral RNA-transfected cells. WT and mutant viral RNAs were electroporated to BHK-21 cells. The transfected cells were analyzed for E protein expression at 24 and 96 h p.t. by IFA. Scale bar, 100 μm. (C) Plaque morphology. Infectious viruses, derived from the RNA-transfected cells, were analyzed by plaque assay. Plaques were developed on day 5 post-infection on BHK-21 cells. N.D., not detectable. (D) Virus production. The culture fluids from (B) were quantified for infectious viruses. Data represent the means and standard deviations from three independent measurements. Limit of detection (L.O.D.), 10 PFU/ml. (E-F) Subcellular distributions of prM, NS1, and C proteins. prM, NS1 and C proteins were detected by DM1, human IgG anti-NS1 and D2C1 antibodies, respectively. Representative IFA images of viral RNA-transfected cells are shown at 24 h post-transfection. White arrows point to nucleus with C protein speckles. (G) Quantitative analysis of C-positive cells. The C-positive cells with cytoplasm or cytoplasm & nucleus distribution from 4-5 random fields were counted and normalized to the total cell numbers. The averages and standard deviations are shown. (H) Viral protein processing in viral RNA-transfected BHK-21 cells at 48 h post-transfection. The percentages of mature C protein were calculated from the following the formula: [C intensity/(C intensity + C-prM intensity)]×100%.

**Figure 3 DENV-2 NS2A interacts with prM and E proteins**. (A) Membrane topologies of DENV-2 prM, E, and NS2A proteins. The NS2A G11 position is indicated. (B) Co-IP of WT NS2A with prM. HEK293T cells were transfected with one plasmid (encoding prM only) or two plasmids (one encoding leader-NS2A-EGFP and the other encoding full-length or the ectodomain of prM). At 40 h p.t., IP was performed using mouse anti-EGFP or control IgG antibodies. Immunoblot (IB) was performed using rabbit anti-EGFP antibody or anti-prM antibody. (C) Co-IP of WT or G11A NS2A-EGFP with prM. Mouse anti-EGFP antibody was used for IP. Rabbit anti-EGFP or anti-prM antibodies were used for IB. The intensity of prM, co-precipitated with WT NS2A-EGFP, was set as 100%. The relative efficiency of prM co-precipitated with G11A NS2A-EGFP was compared with the WT NS2A-EGFP. Statistical analysis was performed using the unpaired parametric t-test. $*p < 0.05$, significant; $**p < 0.01$, very significant. (D) Reciprocal co-IP of prM with WT or G11A NS2A-EGFP. Rabbit anti-prM antibodies were used for IP. The amounts of co-precipitated WT and G11A NS2A-EGFP were detected using mouse anti-EGFP antibody. prM in eluates were detected by rabbit anti-prM antibody followed by protein G conjugated with HRP. (E) Co-localization analysis of NS2A and prM proteins. BHK-21 cells were co-



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transfected with two plasmids: One encoding the $C_{14}$-prM-E and the other encoding SPG-$C_{16}$-NS2A-EGFP. At 24 h p.t., the cells were examined for prM and NS2A-EGFP co-localization under a confocal microscope. Scale bar, 10 μm. (F) Co-IP of NS2A with E protein. HEK293T cells were co-transfected with two plasmids: One encoding the last 17 amino acids of prM and complete E protein ($M_{17}$-E), and another encoding NS2A with a C-terminal GST tag (NS2A-GST). GST tag alone was used as a negative control. IPs were performed using rabbit anti-GST antibody. E and GST fusion proteins were detected in cell lysates and eluates by mouse anti-E monoclonal antibody (9F12) and anti-GST antibody, respectively. The relative pulldown efficiency was calculated by normalizing the intensity of E co-precipitated with G11A NS2A-GST to the intensity of E co-precipitated with the WT NS2A-GST. The means and standard deviations from three independent experiments are shown. N.S., no significance.

**Figure 4 Two-residue insertion at the capsid-anchorC junction rescues the defect of NS2A G11A**. (A) A model depicting how NS2A modulates C-anchorC-prM cleavage. On the cytosolic side, viral NS2B/NS3 protease cleaves the C-anchorC junction. On the ER lumen side, host signalase cleaves the anchorC-prM junction. Three cleavage scenarios are presented for WT, NS2A G11A, and NS2A G11A+C_R99S constructs. (B) Constructs of insertion mutants of capsid protein. One residue (C_101) or two residues (C_TA or C_SR) were inserted to the C-anchorC junction. The potential cleavage sites for viral NS2B/NS3 protease are indicated. (C) IFA of viral RNA-transfected cells. Viral RNAs containing the NS2A G11A mutation with or without capsid insertions were electroporated into BHK-21 cells. The transfected cells were analyzed for E protein expression on day 1 and 4 by IFA. Scale bar, 100 μm. (D) Plaque morphology. Viruses recovered from (C) were analyzed by plaque assay on BHK-21 cells. (E) Viral titers. Culture supernatants from (C) were measured for viral titers on day 5 post-transfection. (F) Subcellular localization of C protein in viral RNA-transfected cells. White arrows point to nucleus with C protein speckles. Scale bar, 25 μm. (G) Expression of prM and NS1 proteins in viral RNA-transfected cells. Scale bar, 50 μm. (H) C-prM processing in viral RNA-transfected cells. At 48 h p.t., WT or NS2A G11A+C_T101 RNA-transfected cells were analyzed for C, NS1, and β-actin expression by Western blot. The percentages of C-prM to mature C conversion are presented for each construct.

**Figure 5 DENV-2 NS2A protein binds to 3'UTR RNA**. (A) Diagram of pulldown assay using biotinylated RNAs. 3'-end biotinylated RNA binds to streptavidin-conjugated



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magnetic beads. After incubating the bead-bound biotinylated RNA with NS2A protein, the RNA-bound NS2A was enriched and eluted for SDS-PAGE and Western blot analysis. (B) Pulldown analysis using recombinant NS2A protein. N-terminally GST-tagged NS2A protein (GST-NS2A) was expressed in *E. coli* and affinity purified. The 5'UTR-$C_{66}$-3'UTR-biotin RNA was prepared by ligating biotinylated cytidine (bis)phosphate to the 3' end of the 5'UTR-$C_{66}$-3'UTR RNA (containing the 5'UTR, the first 66 nucleotides of C gene, and the 3'UTR). The amounts of GST-NS2A in the input and eluates were analyzed using rabbit anti-GST antibody by Western blot. (C) Pulldown analysis using HEK293T cell-expressed NS2A-GST protein. HEK293T cells were transfected with a plasmid expressing GST or C-terminally GST-tagged NS2A protein (NS2A-GST). At 42 h p.t., cells were harvested, lysed, and used for RNA pull down-assay. The pulldown assay was performed as described in (B). (D) RNA competition assay. Various amounts of unlabeled 5'UTR-$C_{66}$-3'UTR RNA were used to compete the binding of 5'UTR-$C_{66}$-3'UTR-biotin RNA to GST-NS2A protein. tRNA was used as a negative control. (E) Viral RNA/NS2A binding affinity. Electrophoretic mobility-shift assay (EMSA) was used to determine the binding affinity of DENV-2 5'UTR-$C_{66}$-3'UTR-biotin RNA with NS2A protein. 5'UTR-$C_{66}$-3'UTR-biotin RNA was incubated with increasing amounts of GST-NS2A protein. Free and bound RNAs were resolved by native agarose gel electrophoresis. The equilibrium dissociation constant (*Kd*) was determined by nonlinear regression with least-squares fitting method. The coefficient of determination ($R^2$) is shown. Data represent the means and standard deviations from three independent experiments. (F) Mapping the RNA region responsible for NS2A binding. A panel of unlabeled RNA truncates of the 5'UTR-$C_{66}$-3'UTR were used as competitors for the binding of 5'UTR-$C_{66}$-3'UTR-biotin RNA to GST-NS2A protein. The intensity of GST-NS2A (bound to the 5'UTR-$C_{66}$-3'UTR-biotin) without competitor was set as 100%. Relative GST-NS2A intensities were calculated as [(signal in the presence competitor)/(signal in the absence of competitor)]×100%. Statistical analysis was performed using the unpaired parametric t-test. (G) PAR-CLIP workflow [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. See text for detail. (H) PAR-CLIP result summary. Uridines in the 3'UTR that may participate in NS2A-binding are indicated. The dash line indicates the threshold (equal to 5.76, representing the average fold change of mutation rate of all uridines determined by Illumina sequencing). The uridine positions with fold changes of mutation rate over the threshold are indicated in the 3'UTR sequence (GenBank no. AF038403).



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**Figure 6 NS2A residues responsible for the NS2A/3'UTR RNA interaction**. (A) Diagram of NS2A truncates used in RNA pulldown assay. All proteins were fused with an N-terminal GST tag, expressed in *E. coli*, and affinity purified. (B) Interactions between NS2A truncates with 5'UTR-$C_{66}$-3'UTR-biotin RNA. The interactions were measured using pulldown experiments. The pulldown efficiency of full-length NS2A (1-218) was set as 100%. Relative efficiency was calculated by comparing the intensity of NS2A truncate with that of the full-length NS2A. (C) Sequence alignment of NS2A among flaviviruses. Amino acids of a cytosolic loop (amino acids 93-101) are presented. The number of viral strains used for the NS2A alignment are indicated in parentheses. Amino acid positions of NS2A are labeled according to DENV-2 strain New Guinea C. Residues R94, K95, and K99 are shaded. The conserved (:) and identical (*) residues are indicated. (D) IFA of BHK-21 cells transfected with viral RNA. WT and mutant viral RNAs were electroporated into BHK-21 cells. The cells were assayed for E protein expression. (E) Plaque morphology. Viruses derived from the transfected cells in (D) were analyzed by plaque assay. (F) Viral titers. Culture supernatants from (D) were measured for virus yields on day 5 post-transfection. (G) Replicon transient transfection assay. NS2A triple mutations (R94A+K95A+K99A) were engineered into a luciferase replicon of DENV-2. Luciferase activities were presented after the WT or mutant replicon RNAs were electroporated into BHK-21 cells. (H) Diagram of N-terminally GST-tagged NS2A peptide 64-128 [GST-NS2A (64-128)]. (I) Interaction of GST-NS2A (64-128) protein with 3'UTR RNA. GST-NS2A (64-128) was expressed in *E. coli* and affinity purified. The pulldown efficiency of GST-NS2A (64-128) by 5'UTR-$C_{66}$-3'UTR-biotin was set as 100%. The binding efficiencies of RNA variants were indicated through competition assay. In the presence of competitor RNAs, the remaining amounts of WT GST-NS2A (64-128)/5'UTR-$C_{66}$-3'UTR-biotin interaction are presented. (J) R94A+K95A+K99A mutations reduce GST-NS2A (64-128) binding to 5'UTR-$C_{66}$-3'UTR-biotin RNA. The R94A+K95A+K99A variant retained only 19.1% of the WT activity.

**Figure 7 A model depicting the role of NS2A in DENV-2 assembly.** Viral replication complex (top) and virion assembly site (bottom) are depicted. DENV-2 NS2A protein recruits viral RNA, three structural proteins, and NS2B/NS3 protease to virion assembly site, where coordinated cleavage of C-prM-E ensues infections virus formation. C molecules accumulated on lipid droplets (LD) could bind viral RNA to form nucleocapsid. See text for details.



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**Reference**

[ ADDIN EN.REFLIST ]



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## STAR METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---|---|---|
| Antibodies | | |
| Mouse IgG Anti-C (D2C1) | [ ADDIN EN.CITE ADDIN EN.CITE.DATA ] | N/A |
| Mouse IgG anti-prM (DM1) | Abcam | Cat# ab41473, RRID:AB_873758 |
| Rabbit IgG anti-prM | GeneTex | GTX128093 |
| Mouse IgG anti-E (4G2) | ATCC | Cat# HB-112, RRID:CVCL_J890 |
| Mouse IgG anti-E (9F12) | [ ADDIN EN.CITE ADDIN EN.CITE.DATA ] | N/A |
| Mouse IgG anti-NS1 | Abcam | Cat# ab41616, RRID:AB_869448 |
| Human IgG anti-NS1 | This study. Generated by Novartis Institute for Tropical Diseases in Singapore | N/A |
| Rabbit IgG anti-NS1 | GeneTex | GTX103346 |
| Rabbit IgG anti-NS3 | GeneTex | GTX124252 |
| Mouse IgG anti-NS5 | GeneTex | GTX629447 |
| Rabbit IgG anti-calnexin | SIGMA-AlDRICH | Cat# C4731, RRID:AB_476845 |
| Mouse IgG anti-EGFP | SIGMA-AlDRICH | Cat# 11814460001, RRID:AB_390913 |
| Moue IgG1 Isotype Control | SIGMA-AlDRICH | Cat# M5284, RRID:AB_1163685 |
| Rabbit IgG anti-EGFP | Abcam | Cat# ab13970, RRID:AB_300798 |
| protein G horseradish peroxidase (HRP) conjugate | Thermo Fisher Scientific | Cat# P21041 |
| Mouse IgG anti-GST | Abcam | Cat# ab19256, RRID:AB_444809 |
| Rabbit IgG anti-GST | SIGMA-AlDRICH | Cat# G7781, RRID:AB_259965 |
| Mouse IgG anti-β-actin | Abcam | Cat# ab8226, RRID:AB_306371 |
| Mouse IgG anti-HA | Abcam | Cat# ab18181, RRID:AB_444303 |
| Goat anti-mouse IgG conjugated with HRP | SIGMA-AlDRICH | Cat# A5278, RRID:AB_258232 |
| Goat anti-rabbit IgG conjugated with HRP | SIGMA-AlDRICH | Cat#AP307P, RRID:AB_92641 |
| Goat anti-mouse IgGs conjugated with Alexa 488 | Thermo Fisher Scientific | Cat#A-1100, RRID:AB_2534069 |
| Goat anti-mouse IgGs conjugated with Alexa 594 | Thermo Fisher Scientific | Cat# A-11005, RRID:AB_2534073 |
| Goat anti-rabbit IgGs conjugated with Alexa 488 | Thermo Fisher Scientific | Cat# A-11008, RRID:AB_143165 |



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| | | |
|---|---|---|
| Goat anti-rabbit IgGs conjugated with Alexa 594 | Thermo Fisher Scientific | Cat# A-11072, RRID:AB_2534116 |
| Goat anti-Human IgG (H+L) cross-adsorbed secondary antibody, Alexa Fluor 594 | Thermo Fisher Scientific | Cat# A-11014, RRID:AB_2534081 |
| Bacterial and Virus Strains | | |
| *E.* coli strain Top 10 | ThermoFisher Scientific | Cat#C404006 |
| *E.* coli strain Rosetta™ 2(DE3) pLysS | MilliporeSigma | Cat#71403-4 |



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| | | |
|---|---|---|
| DENV-2 strain New Guinea C | [ ADDIN EN.CITE <EndNote><Cite><Author>Xie</Author><Year>2013</Year><RecNum>85</RecNum><DisplayText>(Xie et al., 2013)</DisplayText><record><rec-number>85</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793217">85</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Xie, X.</author><author>Gayen, S.</author><author>Kang, C.</author><author>Yuan, Z.</author><author>Shi, P. Y.</author></authors></contributors><auth-address>State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Science, China.</auth-address><titles><title>Membrane topology and function of dengue virus NS2A protein</title><secondary-title>J Virol</secondary-title><alt-title>Journal of virology</alt-title></titles><periodical><full-title>J Virol</full-title></periodical><pages>4609-22</pages><volume>87</volume><number>8</number><edition>2013/02/15</edition><keywords><keyword>Animals</keyword><keyword>Cell Line</keyword><keyword>Cricetinae</keyword><keyword>Dengue | N.A. |



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| | | |
|---|---|---|
| Biological Samples | | |
| None | | |
| Chemicals, Peptides, and Recombinant Proteins | | |
| Peptide WT (NILNRRRRTAGMII) | GenScript | N/A |
| Peptide C_R99S (NILNRRSRTAGMII) | GenScript | N/A |
| Recombinant protein: GST | This study | N/A |
| Recombinant protein: GST-NS2A | This study | N/A |
| Recombinant protein: GST-NS2A(1-71) | This study | N/A |
| Recombinant protein: GST-NS2A(1-101) | This study | N/A |
| Recombinant protein: GST-NS2A(1-145) | This study | N/A |
| Recombinant protein: GST-NS2A(1-168) | This study | N/A |
| Recombinant protein: GST-NS2A(64-128) | This study | N/A |
| Critical Commercial Assays | | |
| iScript SYBR Green One-Step kit | Bio-Rad | Cat#170-8893 |
| QIAamp viral RNA mini kit | Qiagen | Cat#52906 |
| T7 mMessage mMachine kit | Thermo Fisher Scientific | Cat# AM1344 |
| Renilla Luciferase Assay System | Promega Corporation | Cat# E2810 |
| Pierce™ RNA 3' End Biotinylation Kit | Thermo Fisher Scientific | Cat# 20160 |
| Pierce™ Magnetic RNA-Protein Pull-Down kit | Thermo Fisher Scientific | Cat# 20164 |
| Chemiluminescent Detection Module | Thermo Fisher Scientific | Cat# 89880 |
| | | |
| Deposited Data | | |
| Raw image data for illumina sequencing | | |
| Experimental Models: Cell Lines | | |
| BHK-21 cell | ATCC | Cat#CCL-10, RRID:CVCL_1915 |
| Vero cell | ATCC | Cat#CCL-81, RRID:CVCL_0059 |
| HEK293T cell | ATCC | Cat# CRL-3216, RRID:CVCL_0063 |
| NS2A-EGFP BHK-21 cell | [ ADDIN EN.CITE ADDIN EN.CITE.DATA ] | N/A |
| | | |
| Experimental Models: Organisms/Strains | | |
| N.A. | | |
| Oligonucleotides | | |
| Primer P1(5'-ACACTCTTTCCCTACACGA CGCTCTTCCGATCTAAGGCAAAACTAACATGAA AC-3') | Integrated DNA Technologies (Skokie, Illinois) | N/A |
| Primer P2 (5'-GTGACTGGAGTTCAGACG TGTGCTCTTCCGATCTAGAACCTGTTGATTCAA CAGCAC-3') | Integrated DNA Technologies (Skokie, Illinois) | N/A |



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| | | |
|---|---|---|
| 5' universal adaptor (5'-AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGAC-3') | Illumine (San Diego, CA) | N/A |
| 3' indexing primer (5'-CAAGCAGAAGACGGCATACGAGATnnnnnnGTGACTGGAGTTCAGACGTGT-3') | Illumine (San Diego, CA) | N/A |
| Primer NGC 7764V (5'-CGTCGAGAGAAATATGGTCACACC-3') | Integrated DNA Technologies (Skokie, Illinois) | N/A |
| Primer NGC 7844C (5'CCACAATAGTATGACCAGCCT-3') | Integrated DNA Technologies (Skokie, Illinois) | N/A |
| Recombinant DNA | | |



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| | | |
|---|---|---|
| pACYC-NGC FL | [ ADDIN EN.CITE <EndNote><Cite><Author>Xie</Author><Year>2013</Year><RecNum>85</RecNum><DisplayText>(Xie et al., 2013)</DisplayText><record><rec-number>85</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793217">85</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Xie, X.</author><author>Gayen, S.</author><author>Kang, C.</author><author>Yuan, Z.</author><author>Shi, P. Y.</author></authors></contributors><auth-address>State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Science, China.</auth-address><titles><title>Membrane topology and function of dengue virus NS2A protein</title><secondary-title>J Virol</secondary-title><alt-title>Journal of virology</alt-title></titles><periodical><full-title>J Virol</full-title></periodical><pages>4609-22</pages><volume>87</volume><number>8</number><edition>2013/02/15</edition><keywords><keyword>Animals</keyword><keyword>Cell Line</keyword><keyword>Cricetinae</keyword><keyword>Dengu | N/A |



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| | | |
|---|---|---|
| pACYC-NGC replicon | [ ADDIN EN.CITE <EndNote><Cite><Author>Xie</Author><Year>2013</Year><RecNum>85</RecNum><DisplayText>(Xie et al., 2013)</DisplayText><record><rec-number>85</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793217">85</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Xie, X.</author><author>Gayen, S.</author><author>Kang, C.</author><author>Yuan, Z.</author><author>Shi, P. Y.</author></authors></contributors><auth-address>State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Science, China.</auth-address><titles><title>Membrane topology and function of dengue virus NS2A protein</title><secondary-title>J Virol</secondary-title><alt-title>Journal of virology</alt-title></titles><periodical><full-title>J Virol</full-title></periodical><pages>4609-22</pages><volume>87</volume><number>8</number><edition>2013/02/15</edition><keywords><keyword>Animals</keyword><keyword>Cell Line</keyword><keyword>Cricetinae</keyword><keyword>Dengue | N/A |



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| | | |
|---|---|---|
| pACYC-NGC replicon G11A | [ ADDIN EN.CITE ADDIN EN.CITE.DATA ] | N/A |
| pACYC-NGC replicon R94A+K95A+K99A | This study | N/A |
| pACYC-NGC FL C_R99S | This study | N/A |
| pACYC-NGC FL G11A | This study | N/A |
| pACYC-NGC FL G11A+C_R99S | This study | N/A |
| pACYC-NGC FL E20A | This study | N/A |
| pACYC-NGC FL E100A | This study | N/A |
| pACYC-NGC FL K188A | This study | N/A |
| pACYC-NGC FL E20A+C_R99S | This study | N/A |
| pACYC-NGC FL E100A+C_R99S | This study | N/A |
| pACYC-NGC FL K188A+C_R99S | This study | N/A |
| pACYC-NGC FL C_TA | This study | N/A |
| pACYC-NGC FL C_SR | This study | N/A |
| pACYC-NGC FL G11A+C_TA | This study | N/A |
| pACYC-NGC FL G11A+C_SR | This study | N/A |
| pACYC-NGC FL C_T101 | This study | N/A |
| pACYC-NGC FL G11A+C_T101 | This study | N/A |
| pACYC-NGC FL R94A | This study | N/A |
| pACYC-NGC FL K95A | This study | N/A |
| pACYC-NGC FL K99A | This study | N/A |
| pACYC-NGC FL R94A+K95A+K99A | This study | N/A |
| pACYC-NGC FL R94A+K95A | This study | N/A |



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| | | |
|---|---|---|
| SFV-DENV2 CprME | [ ADDIN EN.CITE <EndNote><Cite><Author>Qing</Author><Year>2010</Year><RecNum>105</RecNum><DisplayText>(Qing et al., 2010)</DisplayText><record><rec-number>105</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793220">105</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Qing, M.</author><author>Liu, W.</author><author>Yuan, Z.</author><author>Gu, F.</author><author>Shi, P. Y.</author></authors></contributors><auth-address>Novartis Institute for Tropical Diseases, 10, Biopolis Road, #05-01 Chromos, Singapore, Singapore.</auth-address><titles><title>A high-throughput assay using dengue-1 virus-like particles for drug discovery</title><secondary-title>Antiviral Res</secondary-title></titles><periodical><full-title>Antiviral Res</full-title></periodical><pages>163-71</pages><volume>86</volume><number>2</number><dates><year>2010</year><pub-dates><date>May</date></pub-dates></dates><accession-num>20153777</accession-num><urls><related- | N/A |



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| | | |
|---|---|---|
| pXJ-SPG-$C_{16}$-NS2A-EGFP | This study | N/A |
| pXJ-SPG-$C_{16}$-NS2A(G11A)-EGFP | This study | N/A |
| pXJ-EGFP | [ ADDIN EN.CITE ADDIN EN.CITE.DATA ] | N/A |
| pXJ-$C_{14}$-prM | This study | N/A |
| pXJ-$C_{14}$-prM(1-130) | This study | N/A |
| pXJ-$C_{14}$-prM (N69Q) | This study | N/A |
| pXJ-SPG-$C_{16}$-NS2A-GST | This study | N/A |
| pXJ-SPG-$C_{16}$-NS2A(G11A)-GST | This study | N/A |
| pXJ-GST | This study | N/A |
| pXJ-HA-NS3 | This study | N/A |
| pXJ-$M_{17}$-E | This study | N/A |
| pGEX-4T-1 | GE Healthcare Life Sciences | Cat# 28954549 |
| pGEX-4T-GST-NS2A | This study | N/A |
| pGEX-4T-GST-NS2A (1-71) | This study | N/A |
| pGEX-4T-GST-NS2A (1-101) | This study | N/A |
| pGEX-4T-GST-NS2A (1-145) | This study | N/A |
| pGEX-4T-GST-NS2A (1-168) | This study | N/A |
| pGEX-4T-GST-NS2A (64-128) | This study | N/A |
| pGEX-4T-GST-NS2A (64-128) R94A+K95A+K99A | This study | N/A |
| | | |
| Software and Algorithms | | |
| Illustrator CC | Adobe | [ HYPERLINK "https://www.adobe.com/products/illustrator.html" ] |
| ImageJ | NIH | [ HYPERLINK "https://fiji.sc/" ] |
| CLC main workbench | Qiagen | [ HYPERLINK "https://www.qiagenbioinformatics.com/products/clc-main-workbench/" ] |
| Prism 8.0 software | GraphPad | [ HYPERLINK "https://www.graphpad.com/scientific-software/prism/" ] |
| | | |

1271

1272 **Contact for Reagent and Resource Sharing**

1273 Further information and requests for resources and reagents should be directed to and will
1274 be fulfilled by Lead Contact, Dr. Pei-Yong Shi (peshi@utmb.edu)

1275 **Experimental Model and Subject Details**



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## Cell Lines

The Baby hamster kidney fibroblast (BHK-21) cells, African green monkey kidney epithelial (Vero) cells were purchased from the American Type Culture Collection (ATCC, Bethesda, MD) and maintained in a high-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (HyClone Laboratories, South Logan, UT) and 1% penicillin/streptomycin (P/S). The NS2A-EGFP BHK-21 cells constitutively expressing the C-terminally EGFP-tagged NS2A protein was maintained in the high-glucose DMEM medium supplemented with 10%FBS, 1% P/S and 1 mg/ml Geneticin. HEK293T cells were grown in low-glucose DMEM (Invitrogen) containing 10% FBS and 1% penicillin-streptomycin. All mammalian cell lines were grown at 37°C with 5% $CO_2$. Mosquito C6/36 cells were grown in RPMI 1640 medium (Invitrogen) with 10% FBS and 1% penicillin-streptomycin at 28°C without $CO_2$. All culture medium and antibiotics were purchased from ThermoFisher Scientific (Waltham, MA). All cell lines were tested negative for mycoplasma.

## Pseudo-infectious Particle

Pseudo-infectious particles were produced using the a previously described protocol [ ADDIN EN.CITE <EndNote><Cite><Author>Qing</Author><Year>2010</Year><RecNum>114</RecNum><DisplayText>(Qing et al., 2010)</DisplayText><record><rec-number>114</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1561745637">114</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Qing, M.</author><author>Liu, W.</author><author>Yuan, Z.</author><author>Gu, F.</author><author>Shi, P. Y.</author></authors></contributors><auth-address>Novartis Institute for Tropical Diseases, 10, Biopolis Road, #05-01 Chromos, Singapore, Singapore.</auth-address><titles><title>A high-throughput assay using dengue-1 virus-like particles for drug discovery</title><secondary-title>Antiviral Res</secondary-title></titles><periodical><full-title>Antiviral Res</full-title></periodical><pages>163-71</pages><volume>86</volume><number>2</number><keywords><keyword>Animals</keyword><keyword>Antiviral Agents/*isolation & purification/*pharmacology</keyword><keyword>Biological Assay/methods</keyword><keyword>Cell Line</keyword><keyword>Cercopithecus



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aethiops</keyword><keyword>Cricetinae</keyword><keyword>Dengue Virus/*drug effects/genetics</keyword><keyword>Drug Discovery/*methods</keyword><keyword>Drug Evaluation, Preclinical/*methods</keyword><keyword>Electroporation</keyword><keyword>Genes, Reporter</keyword><keyword>Luciferases, Renilla/genetics/metabolism</keyword><keyword>Macaca mulatta</keyword><keyword>Viral Proteins/genetics/metabolism</keyword><keyword>Virosomes/*drug effects/genetics</keyword></keywords><dates><year>2010</year><pub-dates><date>May</date></pub-dates></dates><isbn>1872-9096 (Electronic)0166-3542 (Linking)</isbn><accession-num>20153777</accession-num><urls><related-urls><url>https://www.ncbi.nlm.nih.gov/pubmed/20153777</url></related-urls></urls><electronic-resource-num>10.1016/j.antiviral.2010.02.313</electronic-resource-num></record></Cite></EndNote>] with several modifications. 10 µg of replicon RNA was electroporated into BHK-21 cells as described above. After transfection, the cells were maintained at 37°C with 5% $CO_2$ for 24 h. Afterwards, cells were harvested and electroporated with 10 µg of a helper replicon RNA SFV-DENV2 CprME (replicon derived from Semliki forest virus that express the DENV-2 encoding the CprME). After the second electroporation, cells were cultured at 30°C with 5% $CO_2$. At 20, 28 and 48 h after the second electroporation, culture medium was collected and stored at -80 °C. Afterwards, the collected medium was used ton infection naïve Vero cells in 96-well plates. At 48 h post-infection, cells were lysed, and luciferase signals were measured using the Renilla luciferase assay system (Promega) in the Clarity luminescence microplate reader (BioTek) according to the manufacturer's protocol.

## METHOD DETAILS

### Plasmid Construction

The full-length cDNA infectious clone of DENV-2 strain New Guinea C (NGC), pACYC-NGC FL, and its derived replicon (pACYC-NGC replicon) clone [ ADDIN EN.CITE <EndNote><Cite><Author>Xie</Author><Year>2013</Year><RecNum>85</RecNum><DisplayText>(Xie et al., 2013)</DisplayText><record><rec-number>85</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793217">85</key></foreign-keys><ref-type name="Journal



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Article">17</ref-type><contributors><authors><author>Xie, X.</author><author>Gayen, S.</author><author>Kang, C.</author><author>Yuan, Z.</author><author>Shi, P. Y.</author></authors></contributors><auth-address>State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Science, China.</auth-address><titles><title>Membrane topology and function of dengue virus NS2A protein</title><secondary-title>J Virol</secondary-title><alt-title>Journal of virology</alt-title></titles><periodical><full-title>J Virol</full-title></periodical><pages>4609-22</pages><volume>87</volume><number>8</number><edition>2013/02/15</edition><keywords><keyword>Animals</keyword><keyword>Cell Line</keyword><keyword>Cricetinae</keyword><keyword>Dengue Virus/*physiology</keyword><keyword>Endoplasmic Reticulum/*virology</keyword><keyword>Humans</keyword><keyword>Intracellular Membranes/*metabolism</keyword><keyword>Magnetic Resonance Spectroscopy</keyword><keyword>Models, Molecular</keyword><keyword>Protein Conformation</keyword><keyword>Viral Nonstructural Proteins/*chemistry/metabolism</keyword><keyword>*Virus Assembly</keyword></keywords><dates><year>2013</year><pub-dates><date>Apr</date></pub-dates></dates><isbn>1098-5514 (Electronic)0022-538X (Linking)</isbn><accession-num>23408612</accession-num><work-type>Research Support, Non-U.S. Gov't</work-type><urls><related-urls><url>http://www.ncbi.nlm.nih.gov/pubmed/23408612</url></related-urls></urls><custom2>3624351</custom2><electronic-resource-num>10.1128/JVI.02424-12</electronic-resource-num><language>eng</language></record></Cite></EndNote>] were used as the backbones for mutagenesis study. The replicon was constructed by replacing most of the structural genes (C, prM and E) with a gene cassette (Rluc2A) encoding the Renilla luciferase and a 2A peptide from Foot-and-mouth disease virus. The first 38 amino acids of C protein and the last 31 amino acids of E protein were retained in the replicon. Standard overlap PCR was performed to introduce the mutations in C and NS2A genes. The DNA fragments containing the mutations in C gene were cloned into pACYC-NGC FL clone through restriction enzyme sites NotI (upstream of the T7 promoter and 5'UTR of DENV-2) and SphI (at the position of 1384 in DENV-2 genome). The DNA fragments containing mutations in NS2A gene were cloned into pACYC-NGC FL or replicon through restriction enzyme sites and NheI (at the position of 2544 in DENV-2 genome) and SacI (at the position of 4342 in the DENV-2 genome).



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A mammalian expression vector, pXJ [ ADDIN EN.CITE <EndNote><Cite><Author>Xie</Author><Year>2013</Year><RecNum>85</RecNum><DisplayText>(Xie et al., 2013)</DisplayText><record><rec-number>85</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793217">85</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Xie, X.</author><author>Gayen, S.</author><author>Kang, C.</author><author>Yuan, Z.</author><author>Shi, P. Y.</author></authors></contributors><auth-address>State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Science, China.</auth-address><titles><title>Membrane topology and function of dengue virus NS2A protein</title><secondary-title>J Virol</secondary-title><alt-title>Journal of virology</alt-title></titles><periodical><full-title>J Virol</full-title></periodical><pages>4609-22</pages><volume>87</volume><number>8</number><edition>2013/02/15</edition><keywords><keyword>Animals</keyword><keyword>Cell Line</keyword><keyword>Cricetinae</keyword><keyword>Dengue Virus/*physiology</keyword><keyword>Endoplasmic Reticulum/*virology</keyword><keyword>Humans</keyword><keyword>Intracellular Membranes/*metabolism</keyword><keyword>Magnetic Resonance Spectroscopy</keyword><keyword>Models, Molecular</keyword><keyword>Protein Conformation</keyword><keyword>Viral Nonstructural Proteins/*chemistry/metabolism</keyword><keyword>*Virus Assembly</keyword></keywords><dates><year>2013</year><pub-dates><date>Apr</date></pub-dates></dates><isbn>1098-5514 (Electronic)0022-538X (Linking)</isbn><accession-num>23408612</accession-num><work-type>Research Support, Non-U.S. Gov't</work-type><urls><related-urls><url>http://www.ncbi.nlm.nih.gov/pubmed/23408612</url></related-urls></urls><custom2>3624351</custom2><electronic-resource-num>10.1128/JVI.02424-12</electronic-resource-num><language>eng</language></record></Cite></EndNote>], driven by a cytomegalovirus (CMV) promoter was used to express various viral proteins prM, E, NS2A and NS3 fusion proteins in HEK293T cells. For constructs pXJ-$C_{14}$-prM and pXJ-$C_{14}$-prM (1-130), the anchor C signal peptide ($C_{14}$) was retained at the N-terminus of prM to ensure the correct targeting on ER membrane. For construct pXJ-$M_{17}$-E, the last 17 amino acids from M protein was retained to serve as a signal peptide for translocating the E protein into the ER lumen. For constructs pXJ-SPG-$C_{16}$-NS2A-EGFP and pXJ-SPG-$C_{16}$-



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NS2A-GST, the signal peptide derived from Gaussia luciferase (SPG) [ ADDIN EN.CITE <EndNote><Cite><Author>Verhaegent</Author><Year>2002</Year><RecNum>112</RecNum><DisplayText>(Verhaegent and Christopoulos, 2002)</DisplayText><record><rec-number>112</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1561735944">112</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Verhaegent, M.</author><author>Christopoulos, T. K.</author></authors></contributors><auth-address>Department of Chemistry and Biochemistry, University of Windsor, Ontario, Canada.</auth-address><titles><title>Recombinant Gaussia luciferase. Overexpression, purification, and analytical application of a bioluminescent reporter for DNA hybridization</title><secondary-title>Anal Chem</secondary-title></titles><periodical><full-title>Anal Chem</full-title></periodical><pages>4378-85</pages><volume>74</volume><number>17</number><keywords><keyword>Animals</keyword><keyword>DNA/*analysis</keyword><keyword>DNA Probes</keyword><keyword>Genes, Reporter</keyword><keyword>*Luciferases/genetics/isolation & purification</keyword><keyword>Luminescent Proteins/genetics/isolation & purification</keyword><keyword>Plankton/*enzymology</keyword><keyword>Recombinant Proteins/genetics/isolation & purification</keyword></keywords><dates><year>2002</year><pub-dates><date>Sep 1</date></pub-dates></dates><isbn>0003-2700 (Print)0003-2700 (Linking)</isbn><accession-num>12236345</accession-num><urls><related-urls><url>https://www.ncbi.nlm.nih.gov/pubmed/12236345</url></related-urls></urls></record></Cite></EndNote>] and the last 16 amino acids of NS1 ($C_{16}$) was fused at the N-terminus of NS2A to ensure the correct processing and membrane topology of NS2A. The EGFP and GST were amplified from pEGFPluc (Clontech) and pGEX-4T-1 (Sigma-Aldrich) respectively. The Human influenza hemagglutinin (HA) tag was fused to the N-terminus of NS3 and cloned into pXJ vector, resulting the construct pXJ-HA-NS3.

pGEX-4T-1 (GE Healthcare Life Sciences) was used as the vector for expressing all N-terminally glutathione S-transferase (GST)-tagged NS2A fusion proteins. The DNA fragments containing various length of NS2A were cloned into pGEX-4T-1 through restriction enzyme sites BamHI and XhoI.



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All plasmids were propagated in *E. coli* strain Top10 (ThermoFisher Scientific). All restriction enzymes were purchased from New England BioLabs (Ipswitch, MA). The plasmids were validated by restriction enzyme digestion and Sanger DNA sequencing. All primers were synthesized from Integrated DNA Technologies (Skokie, Illinois) and are available upon request.

**RNA Transcription and Electroporation**

The NGC FL or replicon plasmids were linearized by restriction enzyme XbaI. RNA transcription and electroporation were performed as described previously [ ADDIN EN.CITE <EndNote><Cite><Author>Xie</Author><Year>2013</Year><RecNum>85</RecNum><DisplayText>(Xie et al., 2013)</DisplayText><record><rec-number>85</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793217">85</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Xie, X.</author><author>Gayen, S.</author><author>Kang, C.</author><author>Yuan, Z.</author><author>Shi, P. Y.</author></authors></contributors><auth-address>State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Science, China.</auth-address><titles><title>Membrane topology and function of dengue virus NS2A protein</title><secondary-title>J Virol</secondary-title><alt-title>Journal of virology</alt-title></titles><periodical><full-title>J Virol</full-title></periodical><pages>4609-22</pages><volume>87</volume><number>8</number><edition>2013/02/15</edition><keywords><keyword>Animals</keyword><keyword>Cell Line</keyword><keyword>Cricetinae</keyword><keyword>Dengue Virus/*physiology</keyword><keyword>Endoplasmic Reticulum/*virology</keyword><keyword>Humans</keyword><keyword>Intracellular Membranes/*metabolism</keyword><keyword>Magnetic Resonance Spectroscopy</keyword><keyword>Models, Molecular</keyword><keyword>Protein Conformation</keyword><keyword>Viral Nonstructural Proteins/*chemistry/metabolism</keyword><keyword>*Virus Assembly</keyword></keywords><dates><year>2013</year><pub-dates><date>Apr</date></pub-dates></dates><isbn>1098-5514 (Electronic)0022-538X (Linking)</isbn><accession-num>23408612</accession-num><work-type>Research Support, Non-U.S. Gov't</work-type><urls><related-



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urls><url>http://www.ncbi.nlm.nih.gov/pubmed/23408612</url></related-urls></urls><custom2>3624351</custom2><electronic-resource-num>10.1128/JVI.02424-12</electronic-resource-num><language>eng</language></record></Cite></EndNote>]. Briefly, 10 μg RNA was electroporated into 8 × $10^6$ cells in a 4-mm cuvette using the GenePulser apparatus (Bio-Rad) at settings of 0.45 kV (C6/36 cells) or 0.85 kV (BHK-21 cells) and 25 mF, pulsing three times, with 3 s intervals. After a 10-min recovery at room temperature, transfected cells were suspended in culture media and incubated at corresponding conditions: 28°C (C6/36 cells) or 37°C with 5% $CO_2$ (BHK-21).

**Virus Production and Quantification**

After electroporation, the transfected BHK-21 cells were seeded in one T-75 flask. After incubation at 37°C for 24 h, the cells were cultured in DMEM with 2% FBS at 30°C for another 4 days. At every 24 h p.t., culture fluids was collected and stored at -80°C. Viral titer was determined by crystal violet-based plaque assay using BHK-21 cells according to the protocol as described previously [ ADDIN EN.CITE <EndNote><Cite><Author>Xie</Author><Year>2013</Year><RecNum>85</RecNum><DisplayText>(Xie et al., 2013)</DisplayText><record><rec-number>85</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793217">85</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Xie, X.</author><author>Gayen, S.</author><author>Kang, C.</author><author>Yuan, Z.</author><author>Shi, P. Y.</author></authors></contributors><auth-address>State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Science, China.</auth-address><titles><title>Membrane topology and function of dengue virus NS2A protein</title><secondary-title>J Virol</secondary-title><alt-title>Journal of virology</alt-title></titles><periodical><full-title>J Virol</full-title></periodical><pages>4609-22</pages><volume>87</volume><number>8</number><edition>2013/02/15</edition><keywords><keyword>Animals</keyword><keyword>Cell Line</keyword><keyword>Cricetinae</keyword><keyword>Dengue Virus/*physiology</keyword><keyword>Endoplasmic Reticulum/*virology</keyword><keyword>Humans</keyword><keyword>Intracellular Membranes/*metabolism</keyword><keyword>Magnetic Resonance Spectroscopy</keyword><keyword>Models, Molecular</keyword><keyword>Protein Conformation</keyword><keyword>Viral Nonstructural



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Proteins/*chemistry/metabolism</keyword><keyword>*Virus Assembly</keyword></keywords><dates><year>2013</year><pub-dates><date>Apr</date></pub-dates></dates><isbn>1098-5514 (Electronic)0022-538X (Linking)</isbn><accession-num>23408612</accession-num><work-type>Research Support, Non-U.S. Gov't</work-type><urls><related-urls><url>http://www.ncbi.nlm.nih.gov/pubmed/23408612</url></related-urls></urls><custom2>3624351</custom2><electronic-resource-num>10.1128/JVI.02424-12</electronic-resource-num><language>eng</language></record></Cite></EndNote>]. Plaques were developed on day 5 post-infection.

**RNA extraction and RT-PCR**

Viral RNAs in culture fluids were extracted using a QIAamp viral RNA minikit (Qiagen). Extracted RNAs were eluted in 50 μl RNase-free water. Reverse transcription (RT)-qPCR was performed to measure the amounts of viral RNA copy in the culture medium. Viral RNAs were measured using an iScript one-Step RT-PCR kit with SYBR green (Bio-Rad) and the primer pair NGC 7764V (sequence: 5'-CGTCGAGAGAAATATGGTCACACC-3') and 7844C (sequence: 5'CCACAATAGTATGACCAGCCT-3') [ ADDIN EN.CITE <EndNote><Cite><Author>Xie</Author><Year>2013</Year><RecNum>85</RecNum><DisplayText>(Xie et al., 2013)</DisplayText><record><rec-number>85</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793217">85</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Xie, X.</author><author>Gayen, S.</author><author>Kang, C.</author><author>Yuan, Z.</author><author>Shi, P. Y.</author></authors></contributors><auth-address>State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Science, China.</auth-address><titles><title>Membrane topology and function of dengue virus NS2A protein</title><secondary-title>J Virol</secondary-title><alt-title>Journal of virology</alt-title></titles><periodical><full-title>J Virol</full-title></periodical><pages>4609-22</pages><volume>87</volume><number>8</number><edition>2013/02/15</edition><keywords><keyword>Animals</keyword><keyword>Cell Line</keyword><keyword>Cricetinae</keyword><keyword>Dengue Virus/*physiology</keyword><keyword>Endoplasmic Reticulum/*virology</keyword><keyword>Humans</keyword><keyword>Intracellular Membranes/*metabolism</keyword><keyword>Magnetic Resonance



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Spectroscopy</keyword><keyword>Models, Molecular</keyword><keyword>Protein Conformation</keyword><keyword>Viral Nonstructural Proteins/*chemistry/metabolism</keyword><keyword>*Virus Assembly</keyword></keywords><dates><year>2013</year><pub-dates><date>Apr</date></pub-dates></dates><isbn>1098-5514 (Electronic)0022-538X (Linking)</isbn><accession-num>23408612</accession-num><work-type>Research Support, Non-U.S. Gov't</work-type><urls><related-urls><url>http://www.ncbi.nlm.nih.gov/pubmed/23408612</url></related-urls></urls><custom2>3624351</custom2><electronic-resource-num>10.1128/JVI.02424-12</electronic-resource-num><language>eng</language></record></Cite></EndNote>]. Alternatively, the extracted viral RNA was reversely transcribed and amplified using SuperScript One-Step (RT)-PCR with Platinum Taq (Invitrogen). The RT-PCR products were then gel purified and subjected to Sanger DNA sequencing.

**Replicon Transient Transfection**

After electroporation of replicon RNA into BHK-21 cells, the cells were resuspended in culture medium and seeded in a 12-well plate ($2 \times 10^5$ cells/well). At various time points, the cells were washed once with phosphate-buffered saline (PBS) and lysed using lysis buffer (Promega). The plates containing the lysis buffer were sealed with Parafilm and stored at -80°C. Once samples for all time points had been collected, luciferase signals were measured in a Clarity luminescence microplate reader (BioTek) according to the manufacturer's protocol. The luciferase signals at early time points (2-6 h post-transfection) represent the translation of input viral RNA; and the luciferase signals at later points (24-46 h post-transfection) represent the stage of RNA replication.

**Immunofluorescence assay (IFA)**

Cells were seeded in an 8-well Lab-Tek II chamber slide (ThermoFisher Scientific). At given time points, cells were fixed in 4% paraformaldehyde in PBS for 20 min at room temperature. Afterwards, cells were permeabilized by 0.1% Triton X-100 (Sigma-Aldrich) for 5 min followed by 1-h incubation in blocking buffer containing 1% FBS and 0.05% Tween-20 (Sigma-Aldrich) in PBS. Cells were then incubated with primary antibodies (diluted in the blocking buffer) at room temperature for 1 h. After three PBS washes, cells were incubated with goat anti-mouse or rabbit IgG (1:1000 diluted in blocking buffer) conjugated with Alexa Fluor 488 or 594 (ThermoFisher Scientific) for 1 h in blocking buffer.



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Cells were finally washed by PBS for three times, counter-stained by Hoechst 33342 Solution (ThermoFisher Scientific) and mounted in ProLong® Diamond Antifade Mountant (ThermoFisher Scientific). Epifluorescence images were acquired by Leica DM4000 B system. Confocal fluorescence images were acquired by Zeiss LSM 5 META systems.

**Virus Revertant Selection**

For selecting revertants from G11A-transfected cells, we consecutively passaged the transfected cells for 5 rounds. In brief, after electroporation, the cells were seeded in 96-well plates and incubate at 30°C for 3 days. Afterwards, cells for each well were detached by trypsin and suspended in culture medium. About 20% of the suspended cells were transferred into the well of new 96-well plates and incubated at 37°C for one day. The cells were then incubated at 30°C for two additional days. Again, the confluent cells were detached for next passage. At every passage, the supernatants were collected. After 5 rounds of passaging, IFA was performed to determine the intracellular E protein expression by 4G2 antibody. Plaque assay was performed to examine the amounts of infectious virus particle in the culture fluids. Viral RNA from the P5 supernatants were extracted and used as template for RT-PCR. Sanger sequencing was performed to sequence the entire viral genome.

**Co-immunoprecipitation (Co-IP)**

The NS2A/prM, NS2A/E and NS2A/NS3 interactions were examined by Co-IP analysis according to the protocol as described previously[ ADDIN EN.CITE <EndNote><Cite><Author>Zou</Author><Year>2014</Year><RecNum>113</RecNum><DisplayText>(Zou et al., 2014)</DisplayText><record><rec-number>113</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1561742592">113</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Zou, J.</author><author>Xie, X.</author><author>Lee le, T.</author><author>Chandrasekaran, R.</author><author>Reynaud, A.</author><author>Yap, L.</author><author>Wang, Q. Y.</author><author>Dong, H.</author><author>Kang, C.</author><author>Yuan, Z.</author><author>Lescar, J.</author><author>Shi, P. Y.</author></authors></contributors><auth-address>State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Sciences, Wuhan, China.</auth-address><titles><title>Dimerization of flavivirus NS4B protein</title><secondary-title>J



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Virol</secondary-title></titles><periodical><full-title>J Virol</full-title></periodical><pages>3379-91</pages><volume>88</volume><number>6</number><keywords><keyword>Amino Acid Motifs</keyword><keyword>Dengue/*virology</keyword><keyword>Dengue Virus/chemistry/genetics/*metabolism</keyword><keyword>Dimerization</keyword><keyword>Humans</keyword><keyword>Viral Nonstructural Proteins/*chemistry/genetics/*metabolism</keyword><keyword>West Nile Fever/*virology</keyword><keyword>West Nile virus/chemistry/genetics/*metabolism</keyword></keywords><dates><year>2014</year><pub-dates><date>Mar</date></pub-dates></dates><isbn>1098-5514 (Electronic)0022-538X (Linking)</isbn><accession-num>24390334</accession-num><urls><related-urls><url>https://www.ncbi.nlm.nih.gov/pubmed/24390334</url></related-urls></urls><custom2>PMC3957939</custom2><electronic-resource-num>10.1128/JVI.02782-13</electronic-resource-num></record></Cite></EndNote>]. Various combinations of plasmids were transfected into HEK293T cells in 10-cm dishes using the X-tremeGENE 9 DNA transfection reagent (Roche). At 40-42 h post-transfection, cells were lysed in 1 ml immunoprecipitation (IP) buffer (20 mM Tris, pH 7.5, 100 mM NaCl, 0.5% DDM, and EDTA-free protease inhibitor cocktail [Roche]) with rotation at 4°C for 1 h. Lysates were clarified by centrifugation at 20,000 × g and 4°C for 30 min and subjected to co-IP using protein G-conjugated magnetic beads according to the manufacturer's instructions (Millipore). Briefly, immune complexes were formed at 4°C overnight by mixing 200 µl of cell lysate with 2 µg corresponding antibodies in a 500-µl reaction system containing 400 mM sodium chloride. Subsequently, the complexes were precipitated with protein G-conjugated magnetic beads at 4°C for 1 h with rotation, followed by washing extensively with phosphate-buffered saline (PBS) containing 0.1% Tween-20. Finally, proteins were eluted with 4× lithium dodecyl sulfate (LDS) sample buffer (ThermoFisher Scientific) supplemented with 100 mM 1,4-dithiothreitol (DTT), heated at 70°C for 10 min, and analyzed by Western blot.

**SDS-PAGE and Western Blot**

Protein samples were mixed with 4×LDS sample buffer (ThermoFisher Scientific). After denaturing at 70°C for 10 min, 10-30 µl samples were loaded onto to a 12% or 15% SDS-PAGE gel (Bio-Rad Laboratories). After electrophoresis, proteins were resolved and



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transferred onto a polyvinylidene difluoride (PVDF) membrane using the Bio-Rad TransBlot Turbo Blotting System. Blots were soaked for 1 hour in a blocking buffer containing TBST (10 mM Tris-HCl [pH 7.5], 150 mM NaCl, and 0.1% Tween-20) and 5% skim milk, followed by 1 h incubation with primary antibodies (diluted in blocking buffer). After three washes with TBST buffer, blots were incubated with goat anti-mouse or rabbit antibodies conjugated to HRP (1:10,000 diluted in blocking buffer). After three thorough washes with TBST buffer, blots were incubated with the ECL substrates (GE Healthcare). Chemiluminescence signals were detected in ChemiDoc System (Bio-Rad).

**Transmission Electron Microscopy**

Viral genomic RNAs were electroporated into BHK-21 cells as described above. At 48 h post-transfection, the cells were fixed for at least 1 h in a mixture of 2.5% formaldehyde (prepared from paraformaldehyde powder) and 0.1% glutaraldehyde in 0.05M cacodylate buffer (pH 7.3) to which 0.03% picric acid and 0.03% $CaCl_2$ were added. After washing in 0.1 M cacodylate buffer, the cells were scraped off and processed further as a pellet. The pellets were post-fixed in 1% OsO4 in 0.1M cacodylate buffer (pH 7.3) for 1 h, washed with distilled water and *en bloc* stained with 2% aqueous uranyl acetate for 20 min at 60°C. The pellets were dehydrated in ethanol, processed through propylene oxide and embedded in Poly/Bed 812 (Polysciences, Warrington, PA). Ultrathin sections were cut on Leica EM UC7 ultramicrotome (Leica Microsystems, Buffalo Grove, IL), stained with lead citrate and examined in a Philips 201 transmission electron microscope at 60 kV.

**Expression and purification of recombinant NS2A proteins**

The GST-tagged DENV-2 NGC NS2A and its truncates was expressed and purified by following a previously described protocol [ ADDIN EN.CITE <EndNote><Cite><Author>Zou</Author><Year>2014</Year><RecNum>113</RecNum><DisplayText>(Zou et al., 2014)</DisplayText><record><rec-number>113</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1561742592">113</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Zou, J.</author><author>Xie, X.</author><author>Lee le, T.</author><author>Chandrasekaran, R.</author><author>Reynaud, A.</author><author>Yap, L.</author><author>Wang, Q. Y.</author><author>Dong, H.</author><author>Kang, C.</author><author>Yuan, Z.</author><author>Lescar, J.</author><author>Shi, P.



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Y.</author></authors></contributors><auth-address>State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Sciences, Wuhan, China.</auth-address><titles><title>Dimerization of flavivirus NS4B protein</title><secondary-title>J Virol</secondary-title></titles><periodical><full-title>J Virol</full-title></periodical><pages>3379-91</pages><volume>88</volume><number>6</number><keywords><keyword>Amino Acid Motifs</keyword><keyword>Dengue/*virology</keyword><keyword>Dengue Virus/chemistry/genetics/*metabolism</keyword><keyword>Dimerization</keyword><keyword>Humans</keyword><keyword>Viral Nonstructural Proteins/*chemistry/genetics/*metabolism</keyword><keyword>West Nile Fever/*virology</keyword><keyword>West Nile virus/chemistry/genetics/*metabolism</keyword></keywords><dates><year>2014</year><pub-dates><date>Mar</date></pub-dates></dates><isbn>1098-5514 (Electronic)0022-538X (Linking)</isbn><accession-num>24390334</accession-num><urls><related-urls><url>https://www.ncbi.nlm.nih.gov/pubmed/24390334</url></related-urls></urls><custom2>PMC3957939</custom2><electronic-resource-num>10.1128/JVI.02782-13</electronic-resource-num></record></Cite></EndNote>] with modifications. The pGEX-4T GST-NS2A plasmids were transformed into *E. coli* strain Rosetta™ 2 (pLysS) (Novagen) cells. Bacteria culture was grown in Tefferic broth containing 50 µg/ml Kanamycin and 34 µg/ml Chloramphenicol. When the cells reached an optical density at 600 nm (OD600) of 0.8-1.0, isopropyl β-d-1-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM. After growth for 16 h at 18°C, the cells were harvested, resuspended in a lysis buffer (10 mM $Na_2HPO_4$, 1.8 mM $KH_2PO_4$, 2.7 mM KCl, 400 mM NaCl, 1 mM EDTA, 0.5× Roche protease inhibitor cocktail, pH7.5) and disrupted by sonication using a Digital Sonifier 450 (Branson). The cell debris was removed by centrifugation at 10,000 rpm for 10 min at 4°C. The supernatants were collected and pelleted down by centrifugation at 45,000 rpm for 1 h at 4°C using a Beckman type 70 Ti rotor. The pellets containing the membrane fractions were collected and solubilized in the lysis buffer supplemented with 1.5% n-Dodecyl β-D-maltoside (DDM; Anatrace) at 4°C for 2 hours. Following another centrifugation step at 45,000 rpm for 1 h, the supernatants were collected and loaded onto a GSTrap HP Fast Flow column (GE Healthcare) which was preequilibrated with buffer A (10 mM $Na_2HPO_4$, 1.8 mM $KH_2PO_4$, 2.7 mM KCl, 400 mM NaCl, 2 mM tris(2-carboxyethyl) phosphine [TCEP], 0.05% DDM, pH



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7.5). After extensively wash with buffer B (10 mM $Na_2HPO_4$, 1.8 mM $KH_2PO_4$, 2.7 mM KCl, 1M NaCl, 2 mM tris(2-carboxyethyl) phosphine [TCEP], 0.05% DDM, pH 7.5), the protein was eluted in buffer A supplied with 20 mM glutathione. The fractions containing GST-NS2A proteins were pooled, concentrated, and subjected to gel filtration using a HiLoad Superdex 200 10/300 column (GE Healthcare) in buffer C (10 mM $Na_2HPO_4$, 1.8 mM $KH_2PO_4$, 2.7 mM KCl, 150 mM NaCl, 2 mM tris(2-carboxyethyl) phosphine [TCEP], 0.05% DDM, pH 7.5) with 5% glycerol. The peak fractions containing GST-NS2A proteins were pooled and concentrated before storage at -80°C.

**RNA Pull-down Assay**

The 5'UTR-$C_{66}$-3'UTR-biotin RNA was prepared by ligating a single biotinylated nucleotide to the 3' terminus of the RNA 5'UTR-$C_{66}$-3'UTR using the Pierce™ RNA 3' End Biotinylation Kit (ThermoFisher Scientific) according to the manufacturer's instructions. The RNA 5'UTR-$C_{66}$-3'UTR was *in vitro* transcribed from a PCR template using a T7 MEGAscript kit (ThermoFisher Scientific). The PCR template contained a T7 promoter, followed by a cDNA fragment representing a DENV-2 subgenome with a deletion from nucleotides 163 to 10269 (GenBank accession number AF038403). After *in vitro* transcription, the products were passed twice through the Microspin™ G-25 Columns (GE Healthcare Life Sciences) to remove excessive UTPs. The final products were subjected to phenol-chloroform extraction followed by alcohol precipitation. The purified RNAs were resuspended in nuclease-free water prior to storage at -80°C. Other shorter RNAs 3'UTR, Δpk1, 5'UTR-$C_{66}$, Δpk(1-2), Δpk(1-3), 3'SL, pk(3-4) and pk3 were *in vitro* synthesized from corresponding PCR templates using the same method.

RNA pull-down experiments were performed using the Pierce™ Magnetic RNA-Protein Pull-Down Kit (ThermoFisher Scientific) according to the manufacturer's instructions. Briefly, 2 µg of biotin-labeled RNAs were pre-captured by 50 µl Streptavidin magnetic beads in 1 × RNA capture buffer (10mL, 20mM Tris (pH 7.5), 1M NaCl, 1mM EDTA) at room temperature for 20 min with agitation. A 28-mer biotinylated IRE RNA (5'-UCCUGCUUCAACAGUGCUUGGACGGAAC-3'-biotin) from the Pierce™ RNA 3' End Biotinylation Kit was used as a control. The RNA-bound beads were then mixed with 2 µg purified GST-tagged NS2A proteins in a 100-µl reaction system containing 20 mM Tris (pH 7.5), 200 mM NaCl, 2 mM $MgCl_2$, 0.1% Tween-20 and 15% glycerol and incubated for 45 minutes at 4°C with agitation. For RNA competition assay, the various amounts of



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competitors (tRNA and other shorter RNAs from DENV-2 genome) were immediately added to above the mixtures containing RNA-bound beads and GST-NS2A proteins. After 5 times of wash by 20 mM Tris (pH 7.5), 10 mM NaCl, 0.1% Tween-20 Detergent, the proteins were eluted in 30 µl biotin elution buffer. The eluates were mixed with 4×LDS sample buffer and heated at 70°C for 10 min. 10 µl protein samples were loaded onto a 12% SDS-PAGE gel and followed by detection by Western blot using rabbit anti-GST antibodies.

**Electrophoretic Mobility-Shift Assay**

Electrophoretic mobility-shift assay (EMSA) was performed to estimate the binding affinity of DENV-2 5'UTR-$C_{66}$-3'UTR RNA to GST-NS2A proteins. 100 pM 5'UTR-$C_{66}$-3'UTR-biotin RNA was incubated with a serial concentration of recombinant GST-NS2A protein (0-250 nM) in a 50-µl reaction system containing 50mM Tris-HCl, pH 7.5, 10 mM 2-mercaptoethanol, 2 mM $MgCl_2$, 50 mM KCl at 24°C for 15 min. Afterwards, 10 µl 6x Gel Loading Dye without SDS (New England Biolabs Inc.) were added to stop the reaction. Sample were then loaded onto a 0.4% agarose gel (prepared in 0.5× Tris/Borate/EDTA[TBE]) to resolve the RNA-protein complexes and free RNAs by electrophoresis in 1× TBE buffer (120 V for 30 min) at room temperature. After electrophoresis, RNAs were transferred from the agarose gel to a BrightStar-Plus positively charged nylon membrane (ThermoFisher Scientific) according to the procedure as described in the manual of NorthernMax Gly Kit (ThermoFisher Scientific). The nylon membrane was irradiated with 200 mJ/$cm^2$ of Ultraviolet (UV) light at 254 nm in a CX-2000 UV crosslinker (Analytic Jene, CA) with default settings. The biotinylated RNA was finally detected by the Streptavidin-horseradish peroxidase conjugate by using the Chemiluminescent Detection Module (ThermoFisher Scientific). Chemiluminescent signals were detected in the ChemiDoc™ Imaging Systems (Bio-rad).

**PAR-CLIP**

The Photoactivatable-Ribonucleoside-Enhanced Crosslinking and Immunoprecipitation (PAR-CLIP) was performed according to the protocol as described previously [ ADDIN EN.CITE ADDIN EN.CITE.DATA ] with modifications. In brief, 4-thiouridine labelled DENV-2 3'UTR RNA was prepared by adding a final concentration 0.75 mM of 4-Thio-UTP (TriLink BioTechnologies) to a 20-µl *in vitro* transcription reaction (4-Thio-UTR to UTP



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ration is 1:10) using the MEGAscript T7 transcription Kit (Thermo Fisher Scientific). After 2 hours of reaction at 37°C, the reaction was stopped and the 4-thiouridine labelled RNAs were purified and resuspended in nuclease-free water. 15 µg of 4-thiouridine labelled DENV-2 3'UTR RNA was incubated with 6 µg recombinant DENV-2 GST-NS2A protein in a 100-µl reaction system containing 50 mM Tris-HCl, pH 7.5, 2 mM $MgCl_2$, 50 mM KCl and 5% glycerol at 24°C for 15 min. The RNA-protein mixtures were then irradiated on ice with 150 mJ/cm$^2$ at UV light at 365 nm. An aliquot of the same reaction without UV irradiation was used as the uncross-linked control. Prior to immunoprecipitation, 30 µl of PureProteome™ Protein G Magnetic Beads (MilliporeSigma) were washed twice with 1 ml citrate-phosphate buffer (4.7 g/L citric acid, 9.2 g/L $Na_2HPO_4$, pH 5.0), resuspended in 100 µl citrate-phosphate buffer and followed by in addition of 1 µg of rabbit anti-GST polyclonal antibody (Abcam). The bead-antibody mixtures were incubated at 4°C for 45 min. After two washed with citrate-phosphate buffer, the antibody-bound beads were resuspended in 30 µl of citrate-phosphate buffer. Upon immunoprecipitation, the RNA-protein mixtures were mixed with antibody-bound beads and incubated at 4°C for 1 h. Afterwards, the beads were subjected to three washes in buffer I (50mM HEPES-KOH, pH 7.5, 300 mM KCl, 0.05% IGEPAL CA-630L[Sigma-Aldrich] and 0.5 mM DTT) followed by two washes in buffer II (50 mM HEPES-KOH, pH 7.5, 500 KCl, 0.05% IGEPAL CA-630 and 0.5 mM DTT). The beads were then resuspended in 100 µl Proteinase K digestion buffer (100 mM Tris, pH 7.5, 50 mM NaCl, 10 mM EDTA and 1%SDS) supplemented with proteinase K (New England BioLabs) at a final concentration of 2 mg/ml. The reaction was placed into the Eppendorf ThermoMixer® C and incubated at 37°C for 1 h with agitation at 1100 rpm. Thereafter, the RNA in the reaction was extracted by phenol-chloroform, alcohol-precipitated, and resuspended in 20 µl of nuclease-free water. The extracted RNA was subjected to reversely transcribed and followed by amplification (4 PCR-cycles) using the SuperScript® III One-Step RT-PCR System (ThermoFisher Scientific) with a pair of specific primers P1 (sequence: 5'-ACACTCTTTCCCTACACGA CGCTCTTCCGATCTAAGGCAAAACTAACATGAAAC-3'; underline indicates the sequence identical to the sequence at the 5' end of the DENV-2 3'UTR) and P2 (sequence: 5'-GTGACTGGAGTTCAGACG TGTGCTCTTCCGATCTAGAACCTGTTGATTCAACAGCAC-3'; underline indicates the sequence complimentary to the sequence at the 3' end of the DENV-2 3'UTR) targeting the DENV-2 3'UTR. Afterwards, the RT-PCR product was cleaned up using the QIAquick PCR Purification Kit (Qiagen) and subjected to another round of amplification (15 PCR-



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cycles) using the KAPA HiFi PCR Kit (Kapa Biosystems) with a pair of Illumina adapter/index primers (5' universal adaptor sequence: 5'-AATGATACGGCGAC CACCGAGATCTACACTCTTTCCCTACACGAC-3'; and 3' indexing primer sequence: 5'-CAAGCAGAAGACGGCATACGAGATnnnnnnGTGACTGGAGTTCAGACGTGT-3'. The "nnnnnn" here indicates the TruSeq index sequence). Thereafter, the PCR product was cleaned up using the QIAquick PCR Purification Kit (Qiagen) and followed by separation on 2% E-Gel™ EX Agarose Gels (ThermoFisher Scientific). The cDNAs fragment between 500 to 650 bp was excised, purified using the Zymo Research Gel DNA Recovery Kit (Zymo Research) and resuspended in nuclease-free water. The final cDNA libraries were quantified using a Qubit dsDNA Assay Kit (Invitrogen) and sequenced using a MiSeq Reagent Kit v3 (600-cycle) (illumina).

## QUANTIFICATION AND STATISTICAL ANALYSIS

### Sequence Alignment, Image Process and Densitometry Analysis

The amino acid sequences of flavivirus NS2A proteins were download from GenBank. Sequence alignment was performed with default settings using the CLC main workbench software (Qiagen). IFA images processing and cell counting was performed in software ImageJ (National Institutes of Health, NIH). At least four different IFA images were used to estimate the mean and standard deviations of percentage of viral protein staining positive-cells in Figure 2G and S2. Densitometry analysis was performed using Image lab version 6.0 (Bio-Rad Laboratories, Hercules, CA). All numerical data are presented as the mean ± SD (standard deviations). Group comparisons were performed using the unpaired parametric t-test in software Prism 8.0 (GraphPad). *$p<0.05$, significant; **$p<0.01$, very significant; ***$p<0.001$, highly significant; n.s., not significant. Images were assembled using the software Adobe illustrator CC.

### Calculating the equilibrium dissociation constant (Kd)

The intensities of bands representing the RNA/GST-NS2A complexes and free RNA in Figure 5E were quantitated by software ImageJ (NIH). The ratio of bound RNA (in the RNA/GST-NS2A complexes) to total RNA (free and bound RNA) in each lane was calculated. The ratio of bound RNA to total RNA (y-axis) versus the concentration of GST-NS2A protein (x-axis) was plotted using the software Prism 8 (Graphad). The value of Kd



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was obtained using a nonlinear regression with least-squares fitting method, where the Bmax was set as 1. All data points come from three independent experiments.

**Illumina sequencing data process and analysis**

The indexed sequencing reads of both forward and reverse strands were proceeded with the following bioinformatic pipelines: first, an initial quality filtering were conducted with Fastp [ ADDIN EN.CITE <EndNote><Cite><Author>Chen</Author><Year>2018</Year><RecNum>115</RecNum><DisplayText>(Chen et al., 2018)</DisplayText><record><rec-number>115</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1562011493">115</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Chen, S.</author><author>Zhou, Y.</author><author>Chen, Y.</author><author>Gu, J.</author></authors><translated-authors><author>Bioinformatics,</author></translated-authors></contributors><auth-address>Department of Bioinformatics, HaploX Biotechnology, Shenzhen, China.Shenzhen Institutes of Advanced Technology, Chinese Academy of Sciences, Shenzhen, China.</auth-address><titles><title>fastp: an ultra-fast all-in-one FASTQ preprocessor</title><secondary-title>Bioinformatics</secondary-title><alt-title>Bioinformatics</alt-title></titles><periodical><full-title>Bioinformatics</full-title><abbr-1>Bioinformatics</abbr-1></periodical><alt-periodical><full-title>Bioinformatics</full-title><abbr-1>Bioinformatics</abbr-1></alt-periodical><pages>i884-i890</pages><volume>34</volume><number>17</number><dates><year>2018</year><pub-dates><date>Sep 1</date></pub-dates></dates><isbn>1367-4811 (Electronic)1367-4803 (Linking)</isbn><accession-num>30423086</accession-num><urls><related-urls><url>http://www.ncbi.nlm.nih.gov/pubmed/30423086</url></related-urls></urls><custom2>6129281</custom2><electronic-resource-num>10.1093/bioinformatics/bty560</electronic-resource-num><remote-database-provider>2018 Sep 1</remote-database-provider><language>eng</language></record></Cite></EndNote>] to remove bad reads (too low in quality score, too short, too many “N”, etc.). Then, the illumina sequencing adapter sequence “AGATCGGAAGAGC” was trimmed with cutadapt [ ADDIN EN.CITE <EndNote><Cite><Author>Martin</Author><Year>2011</Year><RecNum>116</RecNum><DisplayText>(Martin, 2011)</DisplayText><record><rec-number>116</rec-



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number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1562011493">116</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Martin, Marcel</author></authors></contributors><titles><title>Cutadapt removes adapter sequences from high-throughput sequencing reads</title><secondary-title>EMBnet.journal; Vol 17, No 1: Next Generation Sequencing Data AnalysisDO - 10.14806/ej.17.1.200</secondary-title></titles><periodical><full-title>EMBnet.journal; Vol 17, No 1: Next Generation Sequencing Data AnalysisDO - 10.14806/ej.17.1.200</full-title></periodical><keywords><keyword>next generation sequencing, small RNA, microRNA, adapter removal</keyword></keywords><dates><year>2011</year><pub-dates><date>05/02/</date></pub-dates></dates><urls><related-urls><url>https://journal.embnet.org/index.php/embnetjournal/article/view/200/458</url></related-urls></urls></record></Cite></EndNote>] (command line parameters: -b AGATCGGAAGAGC -m 40). A secondary quality filter was applied with FASTX toolkit ([ HYPERLINK "http://hannonlab.cshl.edu/fastx_toolkit/index.html" ]) to remove any reads that contained more than 4% nucleotides with a PHRED score <20 (command line parameters: fastq_quality_filter -Q33 -q 20 -p 96). The remaining reads were aligned to 3'UTR of DENV genome (GenBank accession number AF038403), with Bowtie2 [ ADDIN EN.CITE <EndNote><Cite><Author>Langmead</Author><Year>2012</Year><RecNum>117</RecNum><DisplayText>(Langmead and Salzberg, 2012)</DisplayText><record><rec-number>117</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1562011493">117</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Langmead, Ben</author><author>Salzberg, Steven L.</author></authors></contributors><titles><title>Fast gapped-read alignment with Bowtie 2</title><secondary-title>Nature Methods</secondary-title></titles><periodical><full-title>Nature Methods</full-title></periodical><pages>357</pages><volume>9</volume><dates><year>2012</year><pub-dates><date>03/04/online</date></pub-dates></dates><publisher>Nature Publishing Group, a division of Macmillan Publishers Limited. All Rights Reserved.</publisher><urls><related-urls><url>https://doi.org/10.1038/nmeth.1923</url></related-urls></urls><electronic-resource-num>10.1038/nmeth.1923</electronic-resource-



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num></record></Cite></EndNote>] (command line parameters: --local). Using SAMtools [ ADDIN EN.CITE <EndNote><Cite><Author>Li</Author><RecNum>118</RecNum><DisplayText>(Li et al.)</DisplayText><record><rec-number>118</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1562011493">118</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Li, H.</author><author>Handsaker B Fau - Wysoker, Alec</author><author>Wysoker A Fau - Fennell, Tim</author><author>Fennell T Fau - Ruan, Jue</author><author>Ruan J Fau - Homer, Nils</author><author>Homer N Fau - Marth, Gabor</author><author>Marth G Fau - Abecasis, Goncalo</author><author>Abecasis G Fau - Durbin, Richard</author><author>Durbin, R.</author></authors><translated-authors><author>Bioinformatics,</author></translated-authors></contributors><auth-address>Wellcome Trust Sanger Institute, Wellcome Trust Genome Campus, Cambridge, CB10 1SA, UK, Broad Institute of MIT and Harvard, Cambridge, MA 02141, USA. FAU - Handsaker, Bob</auth-address><titles><title>The Sequence Alignment/Map format and SAMtools</title></titles><number>1367-4811 (Electronic)</number><dates></dates><call-num>1000 Genome Project Data Processing Subgroup</call-num><urls></urls><remote-database-provider>2009 Aug 15</remote-database-provider><language>eng</language></record></Cite></EndNote>], the aligned forward and reverse reads were binarily converted, merged, indexed, sorted, and mathematically noted (command line parameters: view -buS, merge, sort, index, mpileup). This is followed by extracting and calculating the T/U-to-C mutation rate at each genome position and the mutation frequencies of each of the four nucleotides.

## DATA AND SOFTWARE AVAILABILITY

Illumina sequencing data access. The raw data of illumine sequencing data can be accessed, queried and downloaded using the following link: .



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# Dengue NS2A protein orchestrates virus assembly

Xuping Xie[1]*, Jing Zou[1], Xianwen Zhang[1,2,3], Yiyang Zhou[1], Andrew Routh[1], Vsevolod L. Popov[4], Xinwen Chen[2,3,5], Qingyin Wang[6], Hong-Ping Dong[6], Pei-Yong Shi[1,7,8,9,10]*

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Running title: The role of NS2A protein in dengue virus assembly

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## SUMMARY

Dengue virus (DENV) is the most important mosquito-borne viral pathogen in humans. DENV assembly requires cleavages of viral C-prM-E polyprotein into three structural proteins (capsid, pre-membrane, and envelope), packaging of viral RNA with C protein into nucleocapsid, and budding of prM/E proteins into virions. No study has addressed how these assembly events are orchestratedThe molecular mechanisms underlying the orchestration of these complex events is unclear. Here we show that DENV-2 NS2A protein recruits viral RNA, structural proteins, and protease to the site of virion assembly site, and coordinates nucleocapsid and virus formation. The last 285 nucleotides of viral 3'UTR serve as a "packaging signal" that binds to a cytosolic loop of NS2A protein. This interaction allows NS2A to recruit nascent RNA from the replication complex to the virion assembly site. Mutations in NS2A that weaken the 3'UTR/NS2A interaction abolish virus assembly. In addition, NS2A interacts with prM, E, and NS3 to recruit C-prM-E polyprotein and NS2B/NS3 protease to the virion assembly site, leading to coordinated C-prM-E cleavage. Mature C protein assembles onto genomic RNA to form nucleocapsid, followed by prM/E envelopment and virion formation. An NS2A mutation that alters the prM/NS2A interaction reduces C-prM cleavage and abolishes virion assembly; second-site mutations in C protein that restore the C-prM cleavage rescue virion assembly. The results support a DENV assembly model that whereby NS2A recruits viral RNA to the virion assembly site where coordinated cleavage of C-prM-E ensues infections virus morphogenesis.




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## INTRODUCTION

The genus *Flavivirus* of the family *Flaviviridae* comprises over 70 viruses, many of which are important human pathogens, including dengue (DENV), yellow fever (YFV), Zika (ZIKV), West Nile (WNV), Japanese encephalitis (JEV), St. Louis encephalitis (SLE), and tick-borne encephalitis (TBEV) viruses [ ADDIN EN.CITE <EndNote><Cite><Author>Pierson</Author><Year>2013</Year><RecNum>3</RecNum><DisplayText>(Pierson and Diamond, 2013)</DisplayText><record><rec-number>3</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1536704974">3</key><key app="ENWeb" db-id="">0</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Pierson, T.C.</author><author>Diamond, M. S.</author></authors></contributors><titles><secondary-title>Flaviviruses p. 747-794. In D.M. Knipe and P.M. Howley (ed), Fields virology, 6th., vol. 1.</secondary-title></titles><periodical><full-title>Flaviviruses p. 747-794. In D.M. Knipe and P.M. Howley (ed), Fields virology, 6th., vol. 1.</full-title></periodical><dates><year>2013</year></dates><urls></urls></record></Cite></EndNote>]. The four serotypes of DENV alone cause about 390 million human infections each year, leading to dengue fever, dengue hemorrhagic fever, and dengue shock syndrome [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. ~~Because of~~Due to the huge socio~~al~~ economic burden, DENV was included in the top 10 global health threats by the World Health Organization (WHO). Moreover, ~~T~~the development of vaccines and therapeutics for DENV has ~~been~~ proven challenging [ ADDIN EN.CITE <EndNote><Cite><Author>Lim</Author><Year>2013</Year><RecNum>58</RecNum><DisplayText>(Lim et al., 2013)</DisplayText><record><rec-number>58</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv"



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64 timestamp="1560793214">58</key></foreign-keys><ref-type name="Journal
65 Article">17</ref-type><contributors><authors><author>Lim, S. P.</author><author>Wang,
66 Q. Y.</author><author>Noble, C. G.</author><author>Chen, Y. L.</author><author>Dong,
67 H.</author><author>Zou, B.</author><author>Yokokawa, F.</author><author>Nilar,
68 S.</author><author>Smith, P.</author><author>Beer, D.</author><author>Lescar,
69 J.</author><author>Shi, P. Y.</author></authors></contributors><auth-address>Novartis
70 Institute for Tropical Diseases, 10 Biopolis Road, 05-01 Chromos, Singapore 138670,
71 Singapore.</auth-address><titles><title>Ten years of dengue drug discovery: Progress
72 and prospects</title><secondary-title>Antiviral Res</secondary-title><alt-title>Antiviral
73 research</alt-title></titles><periodical><full-title>Antiviral Res</full-
74 title></periodical><pages>500-
75 19</pages><volume>100</volume><number>2</number><edition>2013/10/01</edition><
76 dates><year>2013</year><pub-dates><date>Nov</date></pub-
77 dates></dates><isbn>1872-9096 (Electronic)0166-3542
78 (Linking)</isbn><accession-num>24076358</accession-num><urls><related-
79 urls><url>http://www.ncbi.nlm.nih.gov/pubmed/24076358</url></related-
80 urls></urls><electronic-resource-num>10.1016/j.antiviral.2013.09.013</electronic-
81 resource-num><language>eng</language></record></Cite></EndNote>]. The first FDA
82 approved dengue vaccine has a low efficacy of 30-61% [ ADDIN EN.CITE ADDIN
83 EN.CITE.DATA ]. Due to the risk of vaccine-induced severe disease in children who are
84 seronegative at the time of vaccination, WHO recommended only use of the vaccine in
85 areas where the disease is highly prevalent [ ADDIN EN.CITE
86 <EndNote><Cite><Author>Whitehead</Author><Year>2016</Year><RecNum>63</RecN
87 um><DisplayText>(Whitehead, 2016)</DisplayText><record><rec-number>63</rec-
88 number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv"
89 timestamp="1560793215">63</key></foreign-keys><ref-type name="Journal



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Article">17</ref-type><contributors><authors><author>Whitehead, S. S.</author></authors></contributors><auth-address>a Laboratory of Infectious Diseases , NIAID, National Institutes of Health , Bethesda , MD , USA.</auth-address><titles><title>Development of TV003/TV005, a single dose, highly immunogenic live attenuated dengue vaccine; what makes this vaccine different from the Sanofi-Pasteur CYD vaccine?</title><secondary-title>Expert Rev Vaccines</secondary-title></titles><periodical><full-title>Expert Rev Vaccines</full-title></periodical><pages>509-17</pages><volume>15</volume><number>4</number><keywords><keyword>Dengue</keyword><keyword>live attenuated dengue vaccine</keyword><keyword>vaccine attenuation strategies</keyword><keyword>vaccine efficacy</keyword><keyword>vaccine immunogenicity</keyword><keyword>vaccine infectivity</keyword></keywords><dates><year>2016</year></dates><isbn>1744-8395 (Electronic)1476-0584 (Linking)</isbn><accession-num>26559731</accession-num><urls><related-urls><url>http://www.ncbi.nlm.nih.gov/pubmed/26559731</url></related-urls></urls><custom2>PMC4956407</custom2><electronic-resource-num>10.1586/14760584.2016.1115727</electronic-resource-num></record></Cite></EndNote>]. No clinically approved antivirals are available for flavivirus treatment, despite ~~major~~ considerable effort in the past two decades [ ADDIN EN.CITE <EndNote><Cite><Author>Zou</Author><Year>2019</Year><RecNum>64</RecNum><DisplayText>(Zou and Shi, 2019)</DisplayText><record><rec-number>64</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793215">64</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Zou, J.</author><author>Shi, P.



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Y.</author></authors></contributors><auth-address>Department of Biochemistry and Molecular Biology, University of Texas Medical Branch, Galveston, TX, USA.Department of Biochemistry and Molecular Biology, University of Texas Medical Branch, Galveston, TX, USA; Department of Phamarcology and Toxicology, University of Texas Medical Branch, Galveston, TX, USA; Sealy Center for Structural Biology and Molecular Biophysics, University of Texas Medical Branch, Galveston, TX, USA; Sealy Institute for Vaccine Sciences, University of Texas Medical Branch, Galveston, TX, USA. Electronic address: peshi@utmb.edu.</auth-address><titles><title>Strategies for Zika drug discovery</title><secondary-title>Curr Opin Virol</secondary-title></titles><periodical><full-title>Curr Opin Virol</full-title></periodical><pages>19-26</pages><volume>35</volume><dates><year>2019</year><pub-dates><date>Mar 7</date></pub-dates></dates><isbn>1879-6265 (Electronic)1879-6257 (Linking)</isbn><accession-num>30852345</accession-num><urls><related-urls><url>http://www.ncbi.nlm.nih.gov/pubmed/30852345</url></related-urls></urls><electronic-resource-num>10.1016/j.coviro.2019.01.005</electronic-resource-num></record></Cite></EndNote>]. It is therefore critical to study the molecular mechanism of flavivirus replication, which may lead to new approaches for countermeasure development.

Flaviviruses haves a plus-sense, single-stranded RNA ~~of about~~approximately 11,000 -nucleotides in length. The genomic RNA contains a 5' untranslated region (UTR), a single open-reading-frame (ORF), and a 3' UTR. The ORF ~~translates~~ encodes a polyprotein that is processed by viral and host proteases into three ~~structure~~ structural proteins ({capsid (C), pre-membrane (prM), and envelope (E)}) and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5). The structural proteins, together with genomic RNA, assemble into virions. The nonstructural proteins participate



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in viral RNA replication, virion assembly, and evasion of host immune response. Upon viral translation, a minus-sense RNA is synthesized from the genomic RNA template, forming a double-stranded RNA. The minus-strand RNA then serves as a template to make ~~many~~ multiple copies of plus-sense RNA that are packaged to form progeny virions [ ADDIN EN.CITE <EndNote><Cite><Author>Pierson</Author><Year>2013</Year><RecNum>3</RecNum><DisplayText>(Pierson and Diamond, 2013)</DisplayText><record><rec-number>3</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1536704974">3</key><key app="ENWeb" db-id="">0</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Pierson, T.C.</author><author>Diamond, M. S.</author></authors></contributors><titles><secondary-title>Flaviviruses p. 747-794. In D.M. Knipe and P.M. Howley (ed), Fields virology, 6th., vol. 1.</secondary-title></titles><periodical><full-title>Flaviviruses p. 747-794. In D.M. Knipe and P.M. Howley (ed), Fields virology, 6th., vol. 1.</full-title></periodical><dates><year>2013</year></dates><urls></urls></record></Cite></EndNote>]. Like other RNA viruses, the assembly of flavivirus is coupled with RNA replication [ ADDIN EN.CITE ADDIN EN.CITE.DATA ].

Flavivirus infection induces invaginations of the endoplasmic reticulum (ER) to form clusters of double-membrane vesicles (Ve) enclosed in the vesicle packets (VPs). Electron tomography has revealed the three-dimensional architectures of Ve and VPs formed during DENV, WNV, TBEV, and ZIKV infections [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. Ve houses the viral replication complex (including the double-stranded viral RNA, viral nonstructural proteins, and cellular proteins) and is where RNA synthesis occurs [ ADDIN EN.CITE



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166 <EndNote><Cite><Author>Junjhon</Author><Year>2014</Year><RecNum>71</RecNum
167 ><DisplayText>(Junjhon et al., 2014)</DisplayText><record><rec-number>71</rec-
168 number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv"
169 timestamp="1560793216">71</key></foreign-keys><ref-type name="Journal
170 Article">17</ref-type><contributors><authors><author>Junjhon,
171 J.</author><author>Pennington, J. G.</author><author>Edwards, T.
172 J.</author><author>Perera, R.</author><author>Lanman, J.</author><author>Kuhn, R.
173 J.</author></authors></contributors><auth-address>Markey Center for Structural Biology,
174 Department of Biological Sciences, Purdue University, West Lafayette, Indiana,
175 USA.</auth-address><titles><title>Ultrastructural characterization and three-dimensional
176 architecture of replication sites in dengue virus-infected mosquito cells</title><secondary-
177 title>J Virol</secondary-title></titles><periodical><full-title>J Virol</full-
178 title></periodical><pages>4687-
179 97</pages><volume>88</volume><number>9</number><keywords><keyword>Animals<
180 /keyword><keyword>Cell
181 Line</keyword><keyword>Culicidae/*virology</keyword><keyword>Dengue
182 Virus/*physiology/*ultrastructure</keyword><keyword>Electron Microscope
183 Tomography</keyword><keyword>Imaging, Three-
184 Dimensional</keyword><keyword>Intracellular
185 Membranes/*ultrastructure/*virology</keyword><keyword>*Virus
186 Replication</keyword></keywords><dates><year>2014</year><pub-
187 dates><date>May</date></pub-dates></dates><isbn>1098-5514 (Electronic)0022-
188 538X (Linking)</isbn><accession-num>24522909</accession-num><urls><related-
189 urls><url>http://www.ncbi.nlm.nih.gov/pubmed/24522909</url></related-
190 urls></urls><custom2>PMC3993787</custom2><electronic-resource-
191 num>10.1128/JVI.00118-14</electronic-resource-num></record></Cite></EndNote>].



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Newly synthesized plus-sense RNA is released from the Ve pore to the rough ER for new rounds of RNA translation and replication. Alternatively, the nascent RNA is assembled into virions through encapsidation (with C protein) and envelopment (with prM and E proteins) through budding into the ER lumen. The virion assembly occurs at the ER sites juxtaposed to the VPs. An initial immature virion contains 60 icosahedrally arranged prM/E heterotrimers. As the immature virion transits through the Golgi network, the acidic environment triggers the cleavage of prM by cellular furin protease, resulting in an infectious mature virion [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. Cryo-EM of flavivirus virions exhibit two important structural features: (i) The nucleocapsid core lacks organized structures and (ii) there are no observable connections between nucleocapsid and the prM/E membrane shell, suggesting that the C protein may bind to genomic RNA in a random manner without direct interaction with prM/E proteins [ ADDIN EN.CITE ADDIN EN.CITE.DATA ].

The molecular mechanism of flavivirus assembly remains largely obscure. Besides structural proteins, several nonstructural proteins, such as NS2A, NS2B, and NS3, have been shown to regulate flavivirus assembly. Different amino acids of NS2A protein ~~were~~ have been reported to play distinct roles in flavivirus RNA replication and virion assembly [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. The cleavage between C and prM is tightly modulated by NS2B/NS3 protease and cellular signalase during YFV and Murray Valley encephalitis virus assembly [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. Mutations in the NS3 helicase domain alone affect YFV assembly [ ADDIN EN.CITE <EndNote><Cite><Author>Patkar</Author><Year>2008</Year><RecNum>90</RecNum><DisplayText>(Patkar and Kuhn, 2008a)</DisplayText><record><rec-number>90</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793218">90</key></foreign-keys><ref-type name="Journal



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Article">17</ref-type><contributors><authors><author>Patkar, C. G.</author><author>Kuhn, R. J.</author></authors></contributors><auth-address>Purdue Univ, Dept Biol Sci, W Lafayette, IN 47907 USA</auth-address><titles><title>Yellow fever virus NS3 plays an essential role in virus assembly independent of its known enzymatic functions</title><secondary-title>Journal of Virology</secondary-title><alt-title>J Virol</alt-title></titles><alt-periodical><full-title>J Virol</full-title></alt-periodical><pages>3342-3352</pages><volume>82</volume><number>7</number><keywords><keyword>tick-borne encephalitis</keyword><keyword>flavivirus kunjin ns3</keyword><keyword>west-nile-virus</keyword><keyword>dengue virus</keyword><keyword>nonstructural protein</keyword><keyword>rna replication</keyword><keyword>cdna-clones</keyword><keyword>particles</keyword><keyword>ns2a</keyword><keyword>organization</keyword></keywords><dates><year>2008</year><pub-dates><date>Apr</date></pub-dates></dates><isbn>0022-538X</isbn><accession-num>WOS:000254139800015</accession-num><urls><related-urls><url><Go to ISI>://WOS:000254139800015</url></related-urls></urls><electronic-resource-num>10.1128/Jvi.02447-07</electronic-resource-num><language>English</language></record></Cite></EndNote>]. Although expression of prM/E alone is sufficient to generate empty virus-like particles, it is not known how nucleocapsid is formed and subsequently enveloped with prM/E to form infectious virions. ~~The goal of this study is~~We therefore set out to define the molecular mechanisms that orchestrate~~s~~ the three structural proteins and viral RNA to form nucleocapsid and infectious virus in a highly coordinated manner. In this study, by using~~Using~~ genetic, biochemical, and imaging approaches, we demonstrate that DENV NS2A protein choreographs virion morphogenesis by (i) interacting with viral prM and E proteins, (ii) regulating the processing of C-prM polyprotein by viral protease, and (iii) recruiting



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genomic RNA through binding to the 3'UTR. These results support a model ~~that~~ whereby DENV NS2A recruits nascent viral RNA from the replication complex (housed in Ve) to the virion assembly site (on the ER membrane juxtaposed to VPs), where ~~an~~ coordinated C-prM cleavage facilitates genomic RNA encapsidation with C protein and budding with prM and E proteins into the ER lumen, resulting in progeny virions.



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## RESULTS

**NS2A G11A mutation abolishes virion assembly**. Using DENV-2 as a model, we previously identified a panel of NS2A mutant viruses that were selectively defective in virion assembly without compromising other steps of thean infection cycle [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. Here, we performed four experiments to demonstrate that NS2A mutation G11A of DENV-2 caused a defect in virion assembly. (i) A DENV-2 luciferase replicon was used to examine the effect of NS2A G11A on viral translation and RNA synthesis. Upon transfection into BHK-21 cells, the G11A replicon produced a luciferase profile comparable to the WT replicon (Fig. 1A), suggesting that the mutation does not significantly reduce viral translation and RNA replication. (ii) When full-length viral RNA of DENV-2 was transfected into BHK-21 cells, the NS2A G11A mutant failed to release extracellular viral RNA (Fig. 1B). No infectious virus was detected from the culture medium of G11A RNA-transfected cells (Fig. 1C). (iii) In a *trans* packaging system, WT luciferase replicon could be packaged into pseudo-infectious particles (PIPs) when structural C-prM-E proteins were expressed from Semliki Forest virus replicon (Fig. S1A). The WT reporter replicon PIPs infected Vero cells and generated robust luciferase signals (Fig. S1B). In contrast, no PIPs were produced with the NS2A G11A replicon. (iv) Transmission electron microscopy revealed substantial virus-induced single-membrane vesicles (within the lumen of the rough ER) or double-membrane vesicles (closely associated with cisternae of the rough ER) in both WT and G11A RNA-transfected cells at 48 h post-transfection (Fig. 1D). A lot large number of virus particles appeared in the WT RNA-transfected cells, but no virus particles were detected in the G11A RNA-transfected cells. These results demonstrate that NS2A G11A mutation specifically abolishes virion assembly.



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**NS2A G11A mutation alters the expression and localization of viral structural proteins.** We compared the subcellular localization of viral proteins in BHK-21 cells that were transfected with WT or NS2A G11A genomic RNA. Surprisingly, the NS2A G11A mutation altered the intracellular distribution of three structural proteins. (i) C protein was disseminated in both nucleus and cytoplasm in the WT RNA-transfected cells, as previously reported [ ADDIN EN.CITE <EndNote><Cite><Author>Wang</Author><Year>2004</Year><RecNum>91</RecNum><DisplayText>(Wang et al., 2004)</DisplayText><record><rec-number>91</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793218">91</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Wang, S. H.</author><author>Syu, W. J.</author><author>Hu, S. T.</author></authors></contributors><auth-address>Institute of Microbiology and Immunology, School of Life Science, National Yang-Ming University, Taipei, Taiwan, Republic of China.</auth-address><titles><title>Identification of the homotypic interaction domain of the core protein of dengue virus type 2</title><secondary-title>J Gen Virol</secondary-title><alt-title>The Journal of general virology</alt-title></titles><periodical><full-title>J Gen Virol</full-title><abbr-1>The Journal of general virology</abbr-1></periodical><alt-periodical><full-title>J Gen Virol</full-title><abbr-1>The Journal of general virology</abbr-1></alt-periodical><pages>2307-14</pages><volume>85</volume><number>Pt 8</number><keywords><keyword>Active Transport, Cell Nucleus</keyword><keyword>Dengue Virus/*chemistry/classification</keyword><keyword>Precipitin Tests</keyword><keyword>Viral Core Proteins/*chemistry</keyword></keywords><dates><year>2004</year><pub-dates><date>Aug</date></pub-dates></dates><isbn>0022-1317 (Print)0022-1317

[ PAGE * MERGEFORMAT ]

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298 (Linking)</isbn><accession-num>15269372</accession-num><urls><related-
299 urls><url>http://www.ncbi.nlm.nih.gov/pubmed/15269372</url></related-
300 urls></urls><electronic-resource-num>10.1099/vir.0.80067-0</electronic-resource-
301 num></record></Cite></EndNote>]. However, in the G11A RNA-transfected cells, the C
302 protein was mainly restricted to the cytoplasm with rare nucleus localization (Fig. 1E). (ii)
303 prM protein was located at the ER region in the WT RNA-transfected cells, whereas the
304 prM protein was undetectable in the G11A RNA-transfected cells (Fig. 1F). (iii) E protein
305 spread broadly like an ER web in the WT RNA-transfected cells. In contrast, in the G11A
306 RNA-transfected cells, the E protein formed larger speckles at restricted subcellular areas
307 (Fig. 1G). Compared with the structural proteins, the effect of NS2A G11A on the
308 subcellular expression of viral nonstructural proteins (including NS1, NS3, NS4A, NS4B,
309 and NS5) and dsRNA was not apparent (Fig. 1E-G, *top panels* for NS3, and data not
310 shown).

Comment [RAL2]: ?

311 The distinct effect of NS2A G11A mutation on subcellular expression between the
312 viral structural and nonstructural proteins were further supported by immunofluorescent
313 co-staining experiments. NS2A G11A altered the co-staining profile of E and NS3 proteins
314 (Fig. S2). In the NS2A G11A RNA-transfected cells, 100% of NS3-expressing cells were
315 E-positive at 24 h post-transfection; however, at 48 and 72 h post-transfection, only 59%
316 and 20% of the NS3-expressing cells were E-positive, respectively (Fig. S2). In the WT
317 RNA-transfected cells, 100% of NS3-expressing cells were E-positive from 24 to 72 h
318 post-transfection (Fig. S2). The results demonstrate that NS2A G11A mutation changes
319 the expression and subcellular localization of viral structural proteins.

320 **NS2A G11A mutation impairs the cleavage of viral C-prM polyprotein.** To
321 determine the molecular mechanism of the above observations, we examined the
322 processing of viral proteins in the WT and NS2A G11A viral RNA-transfected cells at 48 h



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post-transfection by Western blot. As expected, the amounts of C, NS3, and NS5 proteins from the G11A RNA-transfected cells were 15-26% of those from the WT RNA-transfected cells (Fig. 1H-I), due to the lack of virion production or multiple rounds of infection for the G11A mutant. Notably, the amounts of prM and E proteins were further lower in the G11A RNA-transfected cells (only 1-4% of those in the WT RNA-transfected cells), suggesting an asymmetric degradation in cells. Moreover, the NS2A G11A mutation impaired the cleavage of C-prM to individual C and prM proteins: 90% of C-prM was cleaved to mature C protein in the WT RNA-transfected cells, whereas only 20% of C-prM was processed to C protein in the G11A RNA-transfected cells (Fig. 1H, *compare lanes 1 and 5 in the top panel*). The impaired C-prM cleavage could account for the altered subcellular distribution of viral structural proteins in the NS2A C11A RNA-transfected cells: Restricting the un-cleaved C-prM to the ER membrane, preventing C protein from translocating to the nucleus, and consequently blocking prM/E heterodimer formation and virion budding.

Next, we examined the role of the ubiquitin-proteasome pathway in degrading viral proteins in the G11A RNA-transfected cells. Treatment with proteasome inhibitor MG132 increased the amounts of C, prM, E, and NS5 (but not NS3) by 1-3 folds in the NS2A G11A RNA-transfected cells (Fig. 1H, *compare lanes 5 and 6*). In contrast, in the WT RNA-transfected cells, MG132 slightly reduced the amounts of C, prM, and E to different extents (Fig. 1H, *compare lanes 1 and 2*) because MG132 was known to inhibit flavivirus replication [ ADDIN EN.CITE <EndNote><Cite><Author>Bhuvanakantham</Author><Year>2013</Year><RecNum>108</RecNum><DisplayText>(Bhuvanakantham and Ng, 2013)</DisplayText><record><rec-number>108</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793288">108</key></foreign-keys><ref-type name="Journal



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348 Article">17</ref-type><contributors><authors><author>Bhuvanakantham,
349 R.</author><author>Ng, M. L.</author></authors></contributors><auth-
350 address>Flavivirology Laboratory, Department of Microbiology, Yong Loo Lin School of
351 Medicine, National University of Singapore, 5 Science Drive 2, Singapore, 117597.</auth-
352 address><titles><title>West Nile virus and dengue virus capsid protein negates the
353 antiviral activity of human Sec3 protein through the proteasome
354 pathway</title><secondary-title>Cell Microbiol</secondary-title></titles><periodical><full-
355 title>Cell Microbiol</full-title></periodical><pages>1688-
356 706</pages><volume>15</volume><number>10</number><keywords><keyword>Antivir
357 al Agents/*antagonists & inhibitors</keyword><keyword>Capsid
358 Proteins/genetics/*metabolism</keyword><keyword>Cell Line</keyword><keyword>DNA
359 Mutational Analysis</keyword><keyword>Dengue
360 Virus/*physiology</keyword><keyword>*Host-Pathogen
361 Interactions</keyword><keyword>Humans</keyword><keyword>Models,
362 Biological</keyword><keyword>Proteasome Endopeptidase
363 Complex/*metabolism</keyword><keyword>Protein Binding</keyword><keyword>Protein
364 Interaction Mapping</keyword><keyword>Proteolysis</keyword><keyword>Vesicular
365 Transport Proteins/*antagonists & inhibitors</keyword><keyword>West Nile
366 virus/*physiology</keyword></keywords><dates><year>2013</year><pub-
367 dates><date>Oct</date></pub-dates></dates><isbn>1462-5822 (Electronic)1462-
368 5814 (Linking)</isbn><accession-num>23522008</accession-num><urls><related-
369 urls><url>https://www.ncbi.nlm.nih.gov/pubmed/23522008</url></related-
370 urls></urls><electronic-resource-num>10.1111/cmi.12143</electronic-resource-
371 num></record></Cite></EndNote>]. Distinct responses to MG132 treatment among
372 different viral proteins were ~~probably~~ likely due to their subcellular distributions and
373 involvement in various biological microenvironments. Nevertheless, the results suggest



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that ubiquitin-proteasome pathway is responsible for viral protein degradation in the NS2A G11A RNA-transfected cells.

**WT NS2A rescues virion assembly of NS2A G11A mutant through restoring C-prM cleavage.** We examined if ~~*trans*~~ supply of WT NS2A in *trans* could restore virion assembly of NS2A G11A RNA and rescue C-prM protein processing (Fig.S3A). Indeed, after transfection into NS2A-EGFP BHK-21 cells ~~that~~ constitutively expressinged WT NS2A protein, the NS2A G11A RNA produced infectious virions that could infect naïve BHK-21 cells, as indicated by E-positive cells (Fig. S3B). However, f~~;~~ further passaging of culture supernatants to new BHK-21 cells did not yield any E-positive cells (Fig. S3C), ~~suggesting~~ indicating that the *trans* packaged virions could only infect cells for a single-round. Remarkably, the *trans* supplied WT NS2A protein restored the C-prM cleavage in the G11A RNA-transfected cells to WT level, exhibiting >89% C protein processing (Fig. S3D, *top panel*). Two prM bands, representing glycosylated and un-glycosylated forms of the protein (see below), were detected in both NS2A G11A and WT RNA-transfected NS2A-EGFP cells (Fig. S3D, *second panel*). The relative abundances of C, prM, and NS1 proteins were similar in the NS2A G11A and WT RNA-transfected cells (Fig. S3D). ~~Because~~ Due to~~of~~ the restored C-prM cleavage, the nucleus localization of C protein and prM-positive cells were detected in the G11A RNA-transfected NS2A-EGFP cells (Fig. S3E). These results demonstrate that WT NS2A can rescue virion assembly of NS2A G11A mutant through the restorationing ~~the~~ of C-prM cleavage.

Next, we examined the effect of NS2A on the glycosylation of prM by co-expressing NS2A and prM in HEK293T cells. As a control, when EGFP was co-expressed, only the glycosylated form of prM was detected (Fig. S3F); after PGNaseF treatment, the prM was completely deglycosylated and migrated ~~as~~ similarly to a glycosylation-knockout mutant N69Q prM (Fig. S3G). In contrast, when NS2A-EGFP ~~were~~ was co-expressed,



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both glycosylated and un-glycosylated forms of prM were detected (Fig. S3F), indicating that NS2A suppresses the glycosylation of prM. Furthermore, the G11A mutation seemed to increase the suppression effect of NS2A on prM glycosylation (Fig. 3F). These results suggest that NS2A may interact with prM, and that the G11A mutation may modulate the prM/NS2A interaction.

**Capsid R99S mutation can rescue virion assembly of NS2A G11A RNA.** We selected revertants by continuously passaging the NS2A G11A RNA-transfected BHK-21 cells for five rounds (Fig. S4A). Culture medium from each passage~~ing~~ (P1-P5) ~~were~~ was tested for ~~emergence~~ presence of infectious virus particle~~s~~ using plaque assay. The P5 culture produced infectious viruses with titers of $>10^4$ PFU/ml and plaques smaller than the WT virus (Fig. S4B). In addition, almost all P5 cells were E protein-positive (Fig. S4C). Whole genome sequencing of the P5 viruses from three independent selections consistently showed a consensus C-to-A substitution at nucleotide position 391 of viral genome, resulting in an Arg-to-Ser change at amino acid position 99 of the C protein (C_R99S; Fig. 2A). The original NS2A G11A mutation was retained in all P5 viruses.

To validate the role of C_R99S mutation in rescuing virion assembly of NS2A G11A RNA, we engineered C_R99S into a DENV-2 infectious clone [ ADDIN EN.CITE <EndNote><Cite><Author>Xie</Author><Year>2013</Year><RecNum>85</RecNum><DisplayText>(Xie et al., 2013)</DisplayText><record><rec-number>85</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793217">85</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Xie, X.</author><author>Gayen, S.</author><author>Kang, C.</author><author>Yuan, Z.</author><author>Shi, P. Y.</author></authors></contributors><auth-address>State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Science, China.</auth-



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424 address><titles><title>Membrane topology and function of dengue virus NS2A
425 protein</title><secondary-title>J Virol</secondary-title><alt-title>Journal of virology</alt-
426 title></titles><periodical><full-title>J Virol</full-title></periodical><pages>4609-
427 22</pages><volume>87</volume><number>8</number><edition>2013/02/15</edition><k
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438 538X (Linking)</isbn><accession-num>23408612</accession-num><work-type>Research
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442 12</electronic-resource-num><language>eng</language></record></Cite></EndNote>] in
443 the presence ~~or~~ and absence of NS2A G11A. BHK-21 cells were electroporated with WT,
444 NS2A G11A, C_R99S, or NS2A G11A+C_R99S viral RNA. The transfected cells were
445 analyzed for virus production and C-prM cleavage. In the NS2A G11A+C_R99S RNA-
446 transfected cells, the percentage of E-positive cells increased from 10% to 50% at 24 h
447 and 96 h post-transfection, suggesting the spread of infectious virus (Fig. 2B); indeed, the
448 transfected cells produced infectious viruses with plaques smaller than the WT virus (Fig.
449 2C); the viral titer increased over time, reaching >$10^5$ PFU/ml at 96-120 h post-transfection



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(Fig. 2D). The prM and NS1 proteins exhibited ER-like distributions in the NS2A G11A+C_R99S RNA-transfected cells, similar to that observed in the WT RNA-transfected cells (Fig. 2E). Viral C protein was detected in both cytoplasm and nucleus in the NS2A G11A+C_R99S RNA-transfected cells (Fig. 2F-G). Furthermore, 90.5% of the C-prM protein was cleaved to mature C and prM in the NS2A G11A+C_R99S RNA-transfected cells (Fig. 2G). As controls, NS2A G11A RNA showed 15.4% of C-prM cleavage and did not produce any infectious virus, whereas C_R99S RNA exhibited 99.9% of prM-C cleavage and produced a WT level of infectious virus (Fig. 2B-H). Taken together, the results demonstrate that C_R99S mutation efficiently restores C-prM cleavage and virion assembly of NS2A G11A RNA.

**Capsid R99S cannot rescue virion assembly of other NS2A mutants.** We tested ~~if~~ whether C_R99S could rescue other NS2A mutants ~~that were~~ previously shown to be defective in virion assembly, including E20A, E100A, and K188A [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. The C_R99S was engineered into those NS2A mutants, resulting in NS2A E20A+C_R99S, NS2A E100A+C_R99S, and NS2A K188A+C_R99S viral RNAs. After transfection into BHK-12 cells, none of the mutant RNAs produced increasing number of E-positive cells from 24 to 96 h (Fig. S5A). No infectious virus was detected in the culture medium from the mutant RNA-transfected cells (Fig. S5B). Viral C protein was detected in both nucleus and cytoplasm of all mutant RNA-transfected cells (Fig. S5C). In agreement with the C nucleus localization, the NS2A single mutant E20A and E100A cleaved 36% and 79% of C-prM, and the addition of C_R99S increased the C-prM cleavage to 72% and 96%, respectively. In contrast, NS2A K188A mutant cleaved 100% of C-prM in the presence or absence of C_R99S (Fig. 5D). These results demonstrate that C_R99S can only rescue the assembly defect of NS2A G11A possibly because different NS2A mutations abolish virion assembly through distinct mechanisms.



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**Capsid R99S shifts the C-prM cleavage site by two amino acids.** The above results prompted us to investigate how C_R99S rescues the virion assembly of NS2A G11A. Fig. S6 depicts the topology of viral polyprotein C-prM-E-NS1-NS2A on the ER membrane. NS2A G11 resides at the first amphipathic helix on the ER lumen side [ ADDIN EN.CITE

<EndNote><Cite><Author>Xie</Author><Year>2013</Year><RecNum>85</RecNum><DisplayText>(Xie et al., 2013)</DisplayText><record><rec-number>85</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793217">85</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Xie, X.</author><author>Gayen, S.</author><author>Kang, C.</author><author>Yuan, Z.</author><author>Shi, P. Y.</author></authors></contributors><auth-address>State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Science, China.</auth-address><titles><title>Membrane topology and function of dengue virus NS2A protein</title><secondary-title>J Virol</secondary-title><alt-title>Journal of virology</alt-title></titles><periodical><full-title>J Virol</full-title></periodical><pages>4609-22</pages><volume>87</volume><number>8</number><edition>2013/02/15</edition><keywords><keyword>Animals</keyword><keyword>Cell Line</keyword><keyword>Cricetinae</keyword><keyword>Dengue Virus/*physiology</keyword><keyword>Endoplasmic Reticulum/*virology</keyword><keyword>Humans</keyword><keyword>Intracellular Membranes/*metabolism</keyword><keyword>Magnetic Resonance Spectroscopy</keyword><keyword>Models, Molecular</keyword><keyword>Protein Conformation</keyword><keyword>Viral Nonstructural Proteins/*chemistry/metabolism</keyword><keyword>*Virus Assembly</keyword></keywords><dates><year>2013</year><pub-



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dates><date>Apr</date></pub-dates></dates><isbn>1098-5514 (Electronic)0022-538X (Linking)</isbn><accession-num>23408612</accession-num><work-type>Research Support, Non-U.S. Gov't</work-type><urls><related-urls><url>http://www.ncbi.nlm.nih.gov/pubmed/23408612</url></related-urls></urls><custom2>3624351</custom2><electronic-resource-num>10.1128/JVI.02424-12</electronic-resource-num><language>eng</language></record></Cite></EndNote>], while C_R99 is the penultimate amino acid of the C protein on the cytosolic side (Fig. S6B). Since flavivirus NS2B/NS3 protease cleaves a substrate with two basic residues (Arg or Lys) at the P1 and P2 sites and a residue with short side chain (Gly, Ser, or Ala) at the P1' site [ ADDIN EN.CITE ADDIN EN.CITE.DATA ], sequence analysis suggests that the C_R99S substitution may shift the capsid C-terminal cleavage by two amino acids from position 100 to 98 (Fig. S6B). Specifically, viral NS2B/NS3 protease cleaves the WT capsid at NRRRR↓TA and the R99S capsid at NRR↓SRTA (↓ denoting cleavage site and residue R99S underlined). To test this hypothesis, we performed a biochemical protease assay using synthetic WT peptide (NILNRRRRTAGMII) and R99S peptide (NILNRRSRTAGMII). After incubating the peptides with recombinant DENV-2 NS2B/NS3 protease, the reaction products were analyzed by liquid chromatography-mass spectrometry (LC-MS). The WT peptide was cleaved into two smaller peptides with molecular weights of 1096.73 Da and 604.77 Da, corresponding to peptides NILNRRRR and TAGMII, respectively (Fig. S6C). In contrast, the R99S peptide was cleaved into two fragments with molecular weights of 784.47 Da and 847.46 Da, corresponding to peptide NILNRR and SRTAGMII, respectively (Fig. S6D). The LC-MS also detected some unknown peptides, probably due to nonspecific degradations of the protease reaction (Fig. S6C-D). Nevertheless, the data indicate that the C_R99S mutation shifts the capsid cleavage site upstream by two amino acids.



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**NS2A G11A enhances prM/NS2A interaction.** Since NS2A G11A alters the processing of C-prM (Fig. 1H) and prM glycosylation (Fig. S3F), we hypothesized that NS2A directly interacts with prM and the G11A mutation affects the prM/NS2A interaction. To test this hypothesis, we co-transfected two plasmids into HEK293T cells. One plasmid expressed the authentic signal peptide anchorC ($C_{14}$) fused with the full-length prM. Another plasmid expressed a fusion protein containing the C-terminal 24 amino acids of the E protein, the N-terminal 50 amino acids of the NS1 protein, the C-terminal 50 amino acids of NS1, full length NS2A, and an EGFP [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]; this plasmid was previously shown to express NS2A-EGFP with a correct topology on the ER membrane [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. Co-immunoprecipitation showed that NS2A-EGFP, but not EGFP, pulled down prM (Fig. 3B, lanes 2 and 3). As controls, mouse IgG did not pull down any NS2A-EGFP or prM (Lane 1), and EGFP antibody did not pull down prM in the absence of NS2A-EGFP (Lane 6). To map the region of prM that binds to NS2A, we expressed the first 130 amino acids of prM [prM(1-130)] that lacked the C-terminal two transmembrane domains (Fig. 3A). Co-immunoprecipitation showed that prM(1-130) could not pull down NS2A-EGFP (Fig. 3B, lane 4), suggesting that (i) NS2A may bind to the transmembrane region of prM or (ii) the transmembrane region of prM may affect the conformation of its ectodomain and, consequently, abolish its interaction with NS2A.

Next, we examined if NS2A mutation G11A affects the prM/NS2A interaction. Co-immunoprecipitation experiments showed that G11A NS2A-EGFP pulled down about 2.5-fold more prM than the WT NS2A-EGFP did (Fig. 4C). Reciprocally, prM pulled down about 1.7-fold more G11A NS2A-EGFP than WT NS2A-EGFP (Fig. 4D). The results indicate that the G11A mutation enhances NS2A binding to prM.



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To further support the prM/NS2A interaction, we used confocal microscopye to examine the co-localization of prM/NS2A in cells. In this case, prM-E were co-expressed with NS2A-EGFP in HEK293T cells. As shown in Fig. 3E, NS2A-EGFP co-localized with prM. The G11A mutation did not change the co-localization of NS2A-EGFP with prM (Fig. 3E). Taken together, these results demonstrate that (i) NS2A interacts with prM and (ii) NS2A G11A significantly enhances the prM/NS2A interaction.

**The G11A mutation does not affect NS2A binding to E or NS3.** We also examined the interactions between NS2A and E or NS3 proteins that were co-expressed in HEK293T cells. For testing the E/NS2A interaction, we fused a GST tag to the C-terminus of NS2A for the co-immunoprecipitation experiment [because the EGFP tag used in NS2A-EGFP in Fig. 3B-D non-specifically interacts with E protein (data not shown)]. As shown in Fig. 3F, both WT and G11A NS2A-GST pulled down E protein at comparable efficiencies. As a control, GST alone did not pull-down E protein. The result suggests that NS2A interacts with E and the G11A mutation does not affect this interaction.

Since flavivirus NS3 is known to participate in virion assembly [ ADDIN EN.CITE ADDIN EN.CITE.DATA ], we also tested if NS2A G11A mutation affects the NS2A/NS3 interaction. When co-expressed in HEK293T cells, GST alone pulled down a trace amount of HA-tagged NS3 (NS3-HA) protein (Fig. S7, lane 1), whereas NS2A-GST pulled down 10-fold more NS3-HA (Lane 2). Equivalent amounts of NS3-HA were pulled down by the WT and G11A NS2A-GST (Compare lanes 2 and 3), suggesting that NS2A G11A does not affect NS2A/NS3 binding.

**Extension of two or three residues at the N-terminus of anchorC efficiently rescues virion assembly of NS2A G11A**. So far, our results showed that NS2A G11A mutation increases its binding to prM on the ER lumen side, leading to impaired C-prM



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574 processing and defective virion assembly. These defects can be rescued by a capsid
575 mutation R99S that shifts the C-terminal cleavage site by two amino acids. As a result of
576 this shift, the N-terminus of transmembrane anchorC is extended by two amino acids on
577 the cytosolic side of ER membrane. These data support a model of coordinated C-prM
578 processing during virion assembly (Fig. 4A) [ ADDIN EN.CITE
579 <EndNote><Cite><Author>Amberg</Author><Year>1999</Year><RecNum>295</RecNu
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584 Article">17</ref-type><contributors><authors><author>Amberg, S.
585 M.</author><author>Rice, C. M.</author></authors></contributors><auth-
586 address>Department of Molecular Microbiology, Washington University School of
587 Medicine, St. Louis, Missouri 63110-1093, USA.</auth-
588 address><titles><title>Mutagenesis of the NS2B-NS3-mediated cleavage site in the
589 flavivirus capsid protein demonstrates a requirement for coordinated
590 processing</title><secondary-title>J Virol</secondary-title><alt-title>Journal of
591 virology</alt-title></titles><periodical><full-title>J Virol</full-title><abbr-1>Journal of
592 virology</abbr-1></periodical><alt-periodical><full-title>J Virol</full-title><abbr-1>Journal
593 of virology</abbr-1></alt-periodical><pages>8083-
594 94</pages><volume>73</volume><number>10</number><keywords><keyword>Capsid/*
595 metabolism</keyword><keyword>Flavivirus/*metabolism</keyword><keyword>Mutation</
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dates><date>Oct</date></pub-dates></dates><isbn>0022-538X (Print)0022-538X (Linking)</isbn><accession-num>10482557</accession-num><urls><related-urls><url>http://www.ncbi.nlm.nih.gov/pubmed/10482557</url></related-urls></urls><custom2>112824</custom2></record></Cite></EndNote>]. During WT virus assembly, polyprotein C-anchorC-prM was first cleaved at the C-terminus of capsid protein (*i.e.*, R100) by NS2B/NS3 protease, producing mature C and anchorC-prM. The anchorC-prM was then cleaved by a host signalase at the N-terminus of prM, generating anchorC and prM. The cleaved prM coordinates with E protein for virion budding (Fig. 4A, *left panel*). In the presence of NS2A G11A mutation, NS2A binds to prM with a higher affinity, pulling anchorC (embedded in the ER membrane) towards the lumen side. The viral protease cleavage site of the "pulled anchorC" is blocked, possibly by the ER membrane (*middle panel*), leading to unprocessed C-prM. To overcome this defect, the C_R99S mutation creates a new NS2A/NS3 cleavage site at R98 of capsid protein, restoring the C-prM processing and virion assembly of NS2A G11A (*right panel*).

The key of the above model is the blockage of viral protease cleavage site at the C-terminus of capsid protein, which is caused by the NS2A G11A mutation. If the model is correct, an insertion of one or two amino acids to the N-terminus of anchorC should restore the C-prM cleavage and virion assembly in the presence of NS2A G11A mutation (Fig. 4B). To test this hypothesis, we inserted Thr, Thr-Ala, or Ser-Arg into the C-terminus of capsid protein (after R100) in the context of WT or NS2A G11A viral RNA. The inserted amino acids were selected based on the cleavage requirement for viral protease (Fig. 4B). Transfection of each viral RNA into BHK-21 cells yielded increasing number of E-positive cells from day 1 to 4 (Fig. 4C), produced infectious viruses (Fig. 4D-E), displayed cytosolic and nucleus localizations of C protein (Fig. 4F), and exhibited efficient C-prM cleavage (Fig. 4H). In the absence of NS2A G11A, the capsid insertion mutant RNAs behaved



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similarly to the WT RNA. In the presence of NS2A G11A, the capsid single-amino acid insertion (*i.e.*, NS2A G11A+C_T101) partially restored virion assembly ($1.2\times10^3$ PFU/ml; Fig. 4D-E), prM-positive cells (Fig. 4G), and C-prM cleavage (60%; Fig. 4H); while the capsid double-amino acid insertion (*i.e.*, NS2A G11A+C_TA and NS2A G11A+C_SR) almost fully restored virion assembly to the WT level ($2.3\text{-}2.5\times10^6$ PFU/ml; Fig. 4D-E) and C-prM cleavage (92-98%; Fig. 4H). These results demonstrate that addition of one or two amino acids to the N-terminus of anchorC can rescue virion assembly of NS2A G11A.

Since the single-amino acid C_T101 insertion only partially rescued virion assembly of NS2A G11A, we selected revertants for NS2A G11A+C_T101 virus. After continuous passaging of the virus on Vero cells for eight rounds, the P8 viruses were subjected to plaque assay (Fig. S8A-B) and whole-genome sequencing (Fig. S8C). The P8 viruses from all four independent selections produced viral titers (Fig. S8B) that were ≥100-fold higher than the original NS2A G11A+C_T101 virus (Fig. 4E). Among them, selection I P8 virus retained the original NS2A G11A+C_T101 mutations. In addition, an extra mutation R100S was recovered in the capsid protein. Sequence analysis suggests that the R100S could alter the protease cleavage site from the original RRRR↓T to RRR↓ST (R100S underlined) and extends the N-terminus of anchorC by one more amino acid. Selection II P8 virus also retained the original NS2A G11A+C_T101 mutations and accumulated another mutation R99S in the capsid protein. The R99S may change the protease cleavage site to RR↓SRT and extends the N-terminus of anchorC by two more amino acids. In addition, selection II P8 virus contained the R100S mutation in 27% of the viral population. Selection III P8 virus retained the NS2A G11A mutation and showed mixed sequences of R99S and/or R100S. Selection IV P8 virus exhibited WT plaque morphology; sequencing results showed that the NS2A G11A was reverted to WT Gly, but the C_T101 insertion was retained. Collectively, the results again support the conclusion



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650 that extension of the N-terminus of anchorC by two to three residues could efficiently
651 rescue virion assembly of NS2A G11A.

652 **Analysis of NS2A G11A in mosquito cells.** To test if the above findings from
653 mammalian cells could be extended to mosquito host, we analyzed NS2A G11A+capsid
654 mutant RNAs in C6/36 cells. Viral RNAs containing NS2A G11A alone, NS2A
655 G11A+C_T101, NS2A G11A+C_R99S, or NS2A G11A+C_TA were transfected into C6/36
656 cells. The transfected cells were analyzed for capsid protein-positive cells (Fig. S9A),
657 infectious virus production (Fig. S9B-C), extracellular viral RNA release (Fig. S9D), and C-
658 prM cleavage (Fig. S9E). Similar to the results obtained from mammalian cells, NS2A
659 G11A mutant was defective in C-prM processing and virion production in C6/36 cells. The
660 defects could be rescued by the capsid mutations, with the rescuing efficiency of C_TA >
661 C_R99S > C_T101 (Fig. S9). The results indicate that NS2A G11A mutation exerts the
662 same mechanism in abolishing virion assembly in mammalian and mosquito hosts.

663 **NS2A protein binds to 3'UTR RNA**. To further explore the role of DENV-2 NS2A
664 in virion assembly, we examined the ability of NS2A to bind viral genomic RNA. A series of
665 pulldown experiments were performed using biotinylated viral RNAs and NS2A protein
666 (Fig. 5A). A 616-nucleotide viral RNA (5'UTR-$C_{66}$-3'UTR) comprising the 5'UTR, the first
667 66 nucleotides from the C gene, and the 3'UTR regions of DENV-2 genome was *in vitro*
668 synthesized (Fig. S10A). The 3' end of the RNA was ligated to a biotinylated cytidine using
669 T4 RNA ligase, resulting in 5'UTR-$C_{66}$-3'UTR-biotin RNA (Fig. S10B). Two types of GST-
670 tagged NS2A protein were prepared for the pulldown experiment: One type NS2A was
671 expressed from *E. coli*, partially purified, and reconstituted in detergent DDM-containing
672 buffer (Fig. 5B); another type NS2A was from the cell lysates of HEK293T cells transiently
673 expressing the NS2A protein (Fig. 5C). As shown in Fig. 5B-C, the 5'UTR-$C_{66}$-3'UTR-biotin
674 RNA efficiently pulled down GST-tagged NS2A (Lanes 4 and 6), but not GST (Lanes 2



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and 5); in contrast, neither GST nor GST-tagged NS2A proteins was pulled down by a biotinylated non-viral RNA control (Lanes 1 and 3). To further demonstrate the specificity of NS2A/5'UTR-$C_{66}$-3'UTR RNA interaction, we performed an RNA competition experiment. Increasing amounts un-biotinylated 5'UTR-$C_{66}$-3'UTR RNA competed away the NS2A/5'UTR-$C_{66}$-3'UTR-biotin RNA interaction, whereas yeast tRNA did not (Fig. 5D). These results suggest that 5'UTR-$C_{66}$-3'UTR RNA specifically binds to NS2A protein.

We estimated the binding affinity of NS2A/viral RNA interaction. 5'UTR-$C_{66}$-3'UTR-biotin RNA was incubated with increasing amounts of recombinant GST-NS2A protein. The reactions were separated on a native polyacrylamide gel (PAGE), transferred to nylon membrane, and probed with streptavidin-HRP (Fig. 5E). Quantification of free RNA and RNA/protein complex estimated a *Kd* of 35 nM for DENV-2 NS2A biding to viral RNA.

**The last 285 nucleotides of 3'UTR RNA is responsible for binding to NS2A protein**. To map the region of viral RNA responsible for binding to NS2A protein, we performed a competition assay using a set of un-biotinylated RNA fragments from 5'UTR-$C_{66}$-3'UTR (Fig. S10C). The fragments were prepared based on the previously proposed RNA structure [ ADDIN EN.CITE <EndNote><Cite><Author>Villordo</Author><Year>2015</Year><RecNum>109</RecNum><DisplayText>(Villordo et al., 2015)</DisplayText><record><rec-number>109</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1561296709">109</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Villordo, S. M.</author><author>Filomatori, C. V.</author><author>Sanchez-Vargas, I.</author><author>Blair, C. D.</author><author>Gamarnik, A. V.</author></authors></contributors><auth-address>Fundacion Instituto Leloir-CONICET, Buenos Aires, Argentina.Arthropod-borne and Infectious Diseases Laboratory,



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701 Collins, Colorado, United States of America.</auth-address><titles><title>Dengue virus
702 RNA structure specialization facilitates host adaptation</title><secondary-title>PLoS
703 Pathog</secondary-title></titles><periodical><full-title>PLoS Pathog</full-
704 title></periodical><pages>e1004604</pages><volume>11</volume><number>1</number
705 ><keywords><keyword>3' Untranslated Regions</keyword><keyword>Adaptation,
706 Biological/genetics</keyword><keyword>Animals</keyword><keyword>Cells,
707 Cultured</keyword><keyword>Cricetinae</keyword><keyword>Culicidae</keyword><key
708 word>Dengue Virus/*genetics</keyword><keyword>Host
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710 Interactions/*genetics</keyword><keyword>Humans</keyword><keyword>Mutation</key
711 word><keyword>*Nucleic Acid Conformation</keyword><keyword>RNA,
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713 dates><date>Jan</date></pub-dates></dates><isbn>1553-7374 (Electronic)1553-
714 7366 (Linking)</isbn><accession-num>25635835</accession-num><urls><related-
715 urls><url>https://www.ncbi.nlm.nih.gov/pubmed/25635835</url></related-
716 urls></urls><custom2>PMC4311971</custom2><electronic-resource-
717 num>10.1371/journal.ppat.1004604</electronic-resource-
718 num></record></Cite></EndNote>]. As shown in Fig. 5F, 3'UTR, but not 5'UTR-$C_{66}$,
719 competed away the NS2A/5'UTR-$C_{66}$-3'UTR-biotin RNA interaction, indicating that 3'UTR
720 RNA binds to NS2A protein. To further map the region of 3'UTR for NS2A binding, we
721 deleted pseudoknot 1 (Δpk1), pseudoknots 1-2 [Δpk(1-2)], and pseudoknots 1-3 [Δpk(1-3)]
722 from the 3'UTR (Fig. S10C). Only Δpk1 and Δpk(1-2) RNAs, not Δpk(1-3) RNA, could
723 compete away the NS2A/5'UTR-$C_{66}$-3'UTR-biotin RNA interaction (Fig. 5F), suggesting
724 that Δpk(1-2) RNA, representing the last 285 nucleotides of 3'UTR RNA, is required for
725 binding to NS2A protein. This conclusion was further supported by the results that different



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fragments from Δpk(1-2) RNA, including pK(3-4), pk3, and 3'SL, did not compete away the NS2A/5'UTR-$C_{66}$-3'UTR-biotin RNA interaction (Fig. 5F).

Next, we performed a photo-activatable ribonucleoside-enhanced crosslinking (PAR-CL) experiment to define the nucleotides that may interact with NS2A protein (Fig. 5G). The 3'UTR RNA containing [ HYPERLINK "https://en.wikipedia.org/wiki/Photosensitivity" \o "Photosensitivity" ] 4-thiouridine (4-SU) was prepared through *in vitro* transcription in the presence of 4-S-UTP. After RNA/protein complex formation, the 4-thiouridine in the 3'UTR RNA was cross-linked to NS2A protein under UV light (at 365-nm wavelength). The RNA was then subjected to deep sequencing. Due to the crosslinking of 4-thiouridine to protein, a T-to-C mutation was recovered at the RNA sites that interacted with NS2A protein (Figs. 5G and S11). Fig. 5H summarizes the U-to-C mutations in the 3'UTR RNA when complexed with NS2A protein. About 67% mutations occurred within the last 285 nucleotides of 3'UTR. The result further supports the NS2A/3'UTR RNA interaction.

**Comment [RAL3]:** Ratio of 4-S-UTP to UTP?

**Comment [RAL4]:** What would the percentage be if the mutations were randomly spread across the total RNA? i.e. How significant is this enrichment?

**NS2A requires amino acids R94, K95, and K99A for binding to 3'UTR RNA and virion assembly.** To map the region of NS2A required for viral 3'UTR RNA binding, we prepared a panel of purified NS2A truncates with an N-terminal GST tag from *E. coli* (Fig. 6A). The binding efficiencies of NS2A truncates to 5'UTR-$C_{66}$-3'UTR-biotin RNA were compared using a pulldown assay. As shown in Fig. 6B, different NS2A truncates were pulled down by 5'UTR-$C_{66}$-3'UTR-biotin at varied efficiencies. The RNA pulled down NS2A 1-145, 1-168, and 1-218 (full-length) at comparable efficiencies. Compared to the full-length NS2A, the pulldown efficiencies for NS2A 1-101 and 1-71 decreased to 40% and 0%, respectively. These data suggest that amino acids 72-144 are required for NS2A binding to viral RNA.



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749 According to the membrane topology of DENV-2 NS2A [ ADDIN EN.CITE
750 <EndNote><Cite><Author>Nemesio</Author><Year>2014</Year><RecNum>82</RecNu
751 m><DisplayText>(Nemesio and Villalain, 2014)</DisplayText><record><rec-
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754 timestamp="1560793217">82</key></foreign-keys><ref-type name="Journal
755 Article">17</ref-type><contributors><authors><author>Nemesio,
756 H.</author><author>Villalain, J.</author></authors></contributors><auth-
757 address>Molecular and Cellular Biology Institute, Universitas "Miguel
758 Hernandez" , E-03202 Elche-Alicante, Spain.</auth-
759 address><titles><title>Membrane interacting regions of Dengue virus NS2A
760 protein</title><secondary-title>J Phys Chem B</secondary-title></titles><periodical><full-
761 title>J Phys Chem B</full-title></periodical><pages>10142-
762 55</pages><volume>118</volume><number>34</number><keywords><keyword>Calori
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764 Membrane/*metabolism</keyword><keyword>Fluorescence
765 Polarization</keyword><keyword>*Hydrophobic and Hydrophilic
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num>10.1021/jp504911r</electronic-resource-num></record></Cite></EndNote>], amino acids 72-144 form two transmembrane domains (72-92 and 101-118), one short cytosolic loop (93-100), and a luminal segment (118-144; Fig. 6A). Since viral RNA resides in the cytoplasm, we examined the cytosolic loop of NS2A for viral RNA binding. The cytosolic loop has three positively charged residues (R94, K95, and K99) that are conserved among the four DENV serotypes (Fig. 6C). To examine the function of these residues, we prepared a panel of recombinant DENV-2 containing Ala substitutions at single (R94A, K95A, or K99A), double (R94A+K95A), or triple residues (R94A+K95A+K99A). Upon transfection to BHK-21 cells, all mutant RNAs generated viral E protein-positive cells at day 1 (Fig. 6D). Except for the triple mutant, other RNAs produced more E-positive cells on day 4 post-transfection. Mutants R94A and K95A generated WT level of infectious viruses and plaque morphology (Fig. 6E-F). Mutants K99A and K94A+K95A generated 22- to 3-fold less viruses than the WT, respectively (Fig. 6F), and exhibited smaller plaques (Fig. 6E). Remarkably, triple mutant R94A+K95A+K99A RNA yielded almost undetectable amount of virus that was 50,000-fold lower than the WT (Fig. 6F). To examine the effect of R94A+K95A+K99A on viral RNA synthesis, we engineered the triple mutations into a luciferase replicon of DENV-2 (Fig. 6G). The R94A+K95A+K99A replicon generated a luciferase profile similar to the WT replicon, indicating that the mutations do not affect viral translation and RNA synthesis. Taken together, the results demonstrate that R94A+K95A+K99A triple mutant is defective in virion assembly.

Next, we examined the effect of R94A+K95A+K99A mutations on NS2A binding to viral RNA. Since the result from Fig. 6B indicates that NS2A fragment 72-144 is required for viral RNA binding, we expressed a short NS2A peptide 64-128 (comprising the first two NS2A transmembrane domains, the cytosolic region 93-100, and the N- and C-terminal luminal tails for correct folding of the peptide on membrane) fused with an N-terminal GST-



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tag (Fig. 6H). As shown in Fig. 6I, peptide 64-128 could be pulled down by 5'UTR-$C_{66}$-3'UTR-biotin RNA (Lane 1). Un-biotinylated 5'UTR-$C_{66}$-3'UTR or 3'UTR RNA (Lanes 2 and 4), but not 5'UTR-$C_{66}$ RNA or yeast tRNA (Lanes 3 and 5), could compete away the NS2A peptide 64-128/5'UTR-$C_{66}$-3'UTR-biotin RNA interaction. The result demonstrates that NS2A peptide 64-128 specifically binds to viral 3'UTR RNA. Importantly, mutant R94A+K95A+K99A reduced the pulldown efficiency of NS2A peptide 64-128 by over 80% (Fig. 6J). Notably, some degraded GST-tag was found to associate with the NS2A truncates in the above pulldown experiments (Figs. 6B I-J), most likely due to the dimerization property of GST_____[ ADDIN EN.CITE <EndNote><Cite><Author>Maru</Author><Year>1996</Year><RecNum>110</RecNum><DisplayText>(Maru et al., 1996)</DisplayText><record><rec-number>110</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1561302524">110</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Maru, Y.</author><author>Afar, D. E.</author><author>Witte, O. N.</author><author>Shibuya, M.</author></authors></contributors><auth-address>Department of Genetics, Institute of Medical Science, University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108, Japan.</auth-address><titles><title>The dimerization property of glutathione S-transferase partially reactivates Bcr-Abl lacking the oligomerization domain</title><secondary-title>J Biol Chem</secondary-title></titles><periodical><full-title>J Biol Chem</full-title></periodical><pages>15353-7</pages><volume>271</volume><number>26</number><keywords><keyword>*Adaptor Proteins, Signal Transducing</keyword><keyword>Animals</keyword><keyword>Bone Marrow Cells</keyword><keyword>Cell Transformation, Neoplastic</keyword><keyword>Cells, Cultured</keyword><keyword>Fusion Proteins, bcr-abl/*metabolism</keyword><keyword>GRB2 Adaptor



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826 Protein</keyword><keyword>Glutathione
827 Transferase/*metabolism</keyword><keyword>Macromolecular
828 Substances</keyword><keyword>Mice</keyword><keyword>Phosphotyrosine/metabolis
829 m</keyword><keyword>Protein Binding</keyword><keyword>Protein-Tyrosine
830 Kinases/metabolism</keyword><keyword>Proteins/metabolism</keyword><keyword>Rat
831 s</keyword><keyword>Recombinant Fusion Proteins</keyword><keyword>Structure-
832 Activity Relationship</keyword></keywords><dates><year>1996</year><pub-
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836 urls></urls><electronic-resource-num>10.1074/jbc.271.26.15353</electronic-resource-
837 num></record></Cite></EndNote>]. Overall, the results indicate that R94, K95, and K99
838 are important for NS2A binding to viral 3'UTR.

839 **DISCUSSION**

840 Flavivirus assembly consists of a tightly controlled cascade of events: export of
841 newly synthesized viral RNA from the replication complex to assembly sites, encapsidation
842 of viral RNA with C protein, and envelopment of nucleocapsid with prM and E proteins.
843 Many host and viral factors (proteins and lipids) participate in this temporal and spatial
844 process [ ADDIN EN.CITE <EndNote><Cite><Author>Apte-
845 Sengupta</Author><Year>2014</Year><RecNum>94</RecNum><DisplayText>(Apte-
846 Sengupta et al., 2014)</DisplayText><record><rec-number>94</rec-number><foreign-
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848 timestamp="1560793218">94</key></foreign-keys><ref-type name="Journal
849 Article">17</ref-type><contributors><authors><author>Apte-Sengupta,



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S.</author><author>Sirohi, D.</author><author>Kuhn, R. J.</author></authors></contributors><auth-address>Markey Center for Structural Biology, Department of Biological Sciences and Bindley Bioscience Center, Purdue University, West Lafayette, IN, USA.Markey Center for Structural Biology, Department of Biological Sciences and Bindley Bioscience Center, Purdue University, West Lafayette, IN, USA. Electronic address: kuhnr@purdue.edu.</auth-address><titles><title>Coupling of replication and assembly in flaviviruses</title><secondary-title>Curr Opin Virol</secondary-title></titles><periodical><full-title>Curr Opin Virol</full-title></periodical><pages>134-42</pages><volume>9</volume><keywords><keyword>Flavivirus/*physiology</keyword><keyword>Host-Pathogen Interactions</keyword><keyword>Humans</keyword><keyword>Lipid Metabolism</keyword><keyword>Viral Proteins/metabolism</keyword><keyword>*Virus Assembly</keyword><keyword>*Virus Replication</keyword></keywords><dates><year>2014</year><pub-dates><date>Dec</date></pub-dates></dates><isbn>1879-6265 (Electronic)1879-6257 (Linking)</isbn><accession-num>25462445</accession-num><urls><related-urls><url>http://www.ncbi.nlm.nih.gov/pubmed/25462445</url></related-urls></urls><custom2>PMC4268268</custom2><electronic-resource-num>10.1016/j.coviro.2014.09.020</electronic-resource-num></record></Cite></EndNote>]. Among them, flaviviral NS2A has emerged as a key player to orchestrate virion assembly, as evidenced by the following findings. (i) In infected cells, there are two distinct NS2A species that are required for viral RNA synthesis and virion assembly. The NS2A species required for RNA synthesis is located at the viral replication complex that could only be formed in *cis*; the other NS2A species required for virus morphogenesis is located at the virion assembly complex that could be formed in *cis*



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876 or *trans* [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. (ii) The defect in virion assembly
877 caused by DENV-2 and YFV NS2A mutations could be rescued by adaptive mutations in
878 prM, E, NS2B, and NS3 proteins, suggesting that NS2A genetically modulates other viral
879 proteins during virion assembly [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. (iii)
880 Recombinant NS2A of Kunjin virus binds to viral 3'UTR RNA [ ADDIN EN.CITE
881 <EndNote><Cite><Author>Mackenzie</Author><Year>1998</Year><RecNum>81</RecN
882 um><DisplayText>(Mackenzie et al., 1998)</DisplayText><record><rec-number>81</rec-
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884 timestamp="1560793217">81</key></foreign-keys><ref-type name="Journal
885 Article">17</ref-type><contributors><authors><author>Mackenzie, J.
886 M.</author><author>Khromykh, A. A.</author><author>Jones, M.
887 K.</author><author>Westaway, E.
888 G.</author></authors></contributors><titles><title>Subcellular localization and some
889 biochemical properties of the flavivirus Kunjin nonstructural proteins NS2A and
890 NS4A</title><secondary-title>Virology</secondary-title></titles><periodical><full-
891 title>Virology</full-title></periodical><pages>203-
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895 Viral/me [Metabolism]</keyword><keyword>Support, Non-U.S.
896 Gov't</keyword><keyword>Vero Cells</keyword><keyword>*Viral Nonstructural
897 Proteins/me [Metabolism]</keyword><keyword>Virus
898 Replication</keyword></keywords><dates><year>1998</year></dates><urls></urls></re
899 cord></Cite></EndNote>]. These studies indicate that NS2A interacts with two of the three
900 structural proteins (prM and E, not C) and viral RNA. However, how viral RNA and C
901 protein are recruited to form nucleocapsid and how nucleocapsid is enveloped with prM-E



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proteins to form virion remained unknown. The current study aimeds to address these questions.

First, we showed that DENV-2 NS2A interacted with prM to modulate virion assembly. WT NS2A directly bound to prM in the absence of other viral proteins (Fig. 3B). The biological relevance of this interaction was supported by the correlation between virion assembly and prM/NS2A interaction. Specifically, an NS2A mutation G11A, which abolished virion assembly (Fig. 1), enhanced its binding to prM by 1.7-2.5 folds (Fig. 3). Mechanistically, the enhanced interaction between the mutant NS2A and prM "over pulled" the membrane-associated anchorC towards the ER lumen, leading to a blockage of the viral protease cleavage site (most likely by the ER membrane) at the C-anchorC junction. The uncleaved C-prM resulted in a rapid degradation of all three structural proteins (Fig. 1). This is not surprising because the unprocessed C-prM is retained on the ER membrane, misfolded, and degraded via the ubiquitin-proteasome machinery (Fig. 1H). Since prM is a chaperone for E protein [ ADDIN EN.CITE <EndNote><Cite><Author>Lorenz</Author><Year>2002</Year><RecNum>96</RecNum><DisplayText>(Lorenz et al., 2002)</DisplayText><record><rec-number>96</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793218">96</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Lorenz, I. C.</author><author>Allison, S. L.</author><author>Heinz, F. X.</author><author>Helenius, A.</author></authors></contributors><auth-address>Institute of Biochemistry, Swiss Federal Institute of Technology, CH-8093 Zurich, Switzerland.</auth-address><titles><title>Folding and dimerization of tick-borne encephalitis virus envelope proteins prM and E in the endoplasmic reticulum</title><secondary-title>J Virol</secondary-title></titles><periodical><full-title>J



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933 d><keyword>Polyproteins/genetics/metabolism</keyword><keyword>*Protein
934 Folding</keyword><keyword>Protein
935 Precursors/genetics/metabolism</keyword><keyword>Recombinant Fusion
936 Proteins/genetics/metabolism</keyword><keyword>Viral Envelope
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938 dates><date>Jun</date></pub-dates></dates><isbn>0022-538X (Print)0022-538X
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940 urls><url>http://www.ncbi.nlm.nih.gov/pubmed/11991976</url></related-
941 urls></urls><custom2>PMC137023</custom2><electronic-resource-
942 num>10.1128/jvi.76.11.5480-5491.2002</electronic-resource-
943 num></record></Cite></EndNote>], the degradation of C-prM leads to the degradation of
944 E protein. These results are in agreement with a recent report that ZIKV anchorC plays an
945 important role in the stability of E protein and virion morphogenesis [ ADDIN EN.CITE
946 <EndNote><Cite><Author>Rana</Author><Year>2018</Year><RecNum>97</RecNum><
947 DisplayText>(Rana et al., 2018)</DisplayText><record><rec-number>97</rec-
948 number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv"
949 timestamp="1560793219">97</key></foreign-keys><ref-type name="Journal
950 Article">17</ref-type><contributors><authors><author>Rana, J.</author><author>Slon
951 Campos, J. L.</author><author>Leccese, G.</author><author>Francolini,
952 M.</author><author>Bestagno, M.</author><author>Poggianella,



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953 M.</author><author>Burrone, O. R.</author></authors></contributors><auth-
954 address>Molecular Immunology Group, International Centre for Genetic Engineering and
955 Biotechnology, Trieste, Italy rana@icgeb.org burrone@icgeb.org.Molecular
956 Immunology Group, International Centre for Genetic Engineering and Biotechnology,
957 Trieste, Italy.Department of Medical Biotechnology and Translational Medicine,
958 Universita degli Studi di Milano, Milan, Italy.</auth-address><titles><title>Role of Capsid
959 Anchor in the Morphogenesis of Zika Virus</title><secondary-title>J Virol</secondary-
960 title></titles><periodical><full-title>J Virol</full-
961 title></periodical><volume>92</volume><number>22</number><keywords><keyword>Zi
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964 keyword>pseudovirus</keyword><keyword>viral
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969 urls></urls><custom2>PMC6206493</custom2><electronic-resource-
970 num>10.1128/JVI.01174-18</electronic-resource-num></record></Cite></EndNote>].

971 Second, we demonstrated that the defect in virion assembly of NS2A G11A could
972 be restored by two different means. Creation of a new viral protease cleavage site by
973 extending the N-terminus of anchorC by two amino acids restored the capsid-anchorC
974 processing and, consequently, restored virion assembly. This conclusion was supported
975 by revertant analysis of the NS2A G11A mutant (Figs. 2 and S4) and amino acid insertion
976 experiment (Fig. 4). The two-amino acid extension exposes the new protease cleavage
977 site to NS2B/NS3. Alternatively, the virion assembly defect of NS2A G11A could be



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rescued by *trans* supply of WT NS2A (Fig. S3). Under this condition, the WT NS2A replaced the G11A NS2A to interact with C-prM, restored the C-prM cleavage, and ensued a productive virion morphogenesis. Collectively, our results support a previous model that the two cleavages at C-anchorC-prM junctions are coordinated in a sequential manner during flavivirus assembly. The C-anchorC junction was first cleaved by viral protease on the cytosolic side, followed by the anchorC-prM cleavage by the cellular signalase on the ER lumen side [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. Although expression of flavivirus prM-E alone could form empty virus-like particles, the coordinated C-anchorC-prM processing couples the encapsidation and envelopment events; such coupling is required for the production of infectious virus. This conclusion is also supported by ~~the~~ previous reports that separate expression of C and prM-E significantly compromised the assembly efficiency of infectious virions [ ADDIN EN.CITE ADDIN EN.CITE.DATA ].

Third, we characterized the specific interaction between DENV-2 NS2A and the 3'UTR of viral genome. Although Kunjin virus NS2A was reported to bind to the 3'UTR of viral RNA [ ADDIN EN.CITE <EndNote><Cite><Author>Mackenzie</Author><Year>1998</Year><RecNum>81</RecNum><DisplayText>(Mackenzie et al., 1998)</DisplayText><record><rec-number>81</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793217">81</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Mackenzie, J. M.</author><author>Khromykh, A. A.</author><author>Jones, M. K.</author><author>Westaway, E. G.</author></authors></contributors><titles><title>Subcellular localization and some biochemical properties of the flavivirus Kunjin nonstructural proteins NS2A and NS4A</title><secondary-title>Virology</secondary-title></titles><periodical><full-



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15</pages><volume>245</volume><number>2</number><keywords><keyword>Animal<
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Replication</keyword></keywords><dates><year>1998</year></dates><urls></urls></re
cord></Cite></EndNote>], the molecular details of the interaction has not been delineated. Using competition assay and PAR-CLIP technique, we mapped that DENV-2 NS2A bound to the last 285 nucleotides of the 3'UTR, including the pk3, pk4, and 3'SL. Interestingly, the pk3, pk3-4, or 3'SL RNA alone did not efficiently compete out the NS2A/3'UTR RNA interaction (Fig. 5F), suggesting that a complex RNA structure in the 3'UTR is required for efficient binding to NS2A protein. Since no RNA “packaging signal” has been identified for flavivirus assembly and the C protein was shown to non-specifically bind to any RNA [ADDIN EN.CITE
<EndNote><Cite><Author>Pong</Author><Year>2011</Year><RecNum>102</RecNum>
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L.</author><author>Huang, Z. S.</author><author>Teoh, P. G.</author><author>Wang,
C. C.</author><author>Wu, H. N.</author></authors></contributors><auth-
address>Institute of Molecular Biology, Academia Sinica, Taipei, Taiwan.</auth-
address><titles><title>RNA binding property and RNA chaperone activity of dengue virus
core protein and other viral RNA-interacting proteins</title><secondary-title>FEBS



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Lett</secondary-title><alt-title>FEBS letters</alt-title></titles><periodical><full-title>FEBS Lett</full-title><abbr-1>FEBS letters</abbr-1></periodical><alt-periodical><full-title>FEBS Lett</full-title><abbr-1>FEBS letters</abbr-1></alt-periodical><pages>2575-81</pages><volume>585</volume><number>16</number><keywords><keyword>Base Sequence</keyword><keyword>*Dengue Virus</keyword><keyword>Genome, Viral/genetics</keyword><keyword>Molecular Chaperones/*metabolism</keyword><keyword>Nucleic Acid Conformation</keyword><keyword>Protein Binding</keyword><keyword>Protein Engineering</keyword><keyword>RNA Helicases/metabolism</keyword><keyword>RNA, Catalytic/genetics</keyword><keyword>RNA, Viral/chemistry/genetics/*metabolism</keyword><keyword>Serine Endopeptidases/metabolism</keyword><keyword>Viral Core Proteins/*metabolism</keyword><keyword>Viral Nonstructural Proteins/metabolism</keyword></keywords><dates><year>2011</year><pub-dates><date>Aug 19</date></pub-dates></dates><isbn>1873-3468 (Electronic)0014-5793 (Linking)</isbn><accession-num>21771593</accession-num><urls><related-urls><url>http://www.ncbi.nlm.nih.gov/pubmed/21771593</url></related-urls></urls><electronic-resource-num>10.1016/j.febslet.2011.06.038</electronic-resource-num></record></Cite></EndNote>], the last 285 nucleotides of 3'UTR may serve as the "packaging signal" to recruit viral RNA to the virion assembly site through specific binding to NS2A. From the NS2A side, we have mapped the 3'UTR-binding site to a cytosolic loop (containing R94, K95, and K99) between the first and second transmembrane domains (Fig. 6). Triple mutant R94A+K95A+K99A was defective in virion assembly and lost over 80% of the NS2A binding activity to 3'UTR RNA (Fig. 6). Caution should be taken when applying the DENV-2 findings to other flaviviruses because of ~~the~~ sequence variations ~~in~~of



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NS2A proteins. The three positively charged R94, K95, and K99 of NS2A are only conserved among the four serotypes of DENV. In addition, DENV-2, YFV, and ZIKV NS2A proteins exhibit different topologies on the ER membrane [ ADDIN EN.CITE ADDIN EN.CITE.DATA ].

Our findings support a general model for flavivirus virion assembly (Fig. 7). In infected cells, NS2A molecules reside in both replication complex (housed in Ve) and virion assembly complex (located on the juxtaposed ER membrane). When plus-sense RNAs are made and released from the replication complex (through the pore on Ve), they are initially destined to new rounds of viral translation and replication on the ER. Once ~~the amounts of~~ viral RNA and proteins have accumulated over certain "thresholds", nascent RNAs are ~~switched for~~directed to virion assembly. At this time, the newly synthesized RNA exits the replication complex and binds to the NS2A located at the assembly site through the 3'UTR RNA. The NS2A molecules at the assembly site also recruits C-prM-E and NS2B/NS3 through binding to prM and NS3, respectively. ~~One~~ Single molecules of NS2A ~~molecule does~~ not ~~have to~~necessarily bind to the 3'UTR RNA, C-prM-E, and NS2B/NS3 at the same time. Rather, t~~T~~hese interactions could be sequential or through ~~different~~ mulitple NS2A molecules that oligomerize together on the ER membrane [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. Next, the C-prM-E polyprotein is sequentially cleaved by the NS2B/NS3 protease and host signalase to produce mature C, prM, and E [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. The C molecules, many of which have been previously made and stored at the nearby lipid droplets [ ADDIN EN.CITE ADDIN EN.CITE.DATA ], bind viral RNA to form nucleocapsid. The processed prM undergoes conformational change and dimerizes with E. The prM/E dimerization and oligomerization dissociate prM from NS2A. When 180 copies of prM/E oligomerize on the ER membrane, envelopment of the nucleocapsid occurs, generating spiky immature virions. During the



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above process, NS3 may also use its helicase activity to transport viral RNA from the replication complex to the assembly site and condense the RNA during encapsidation. Besides NS3, NS2A or other proteins may also contribute to the translocation of viral RNA from the replication complex to the assembly site.

The above model is centered on the multifunctional role of NS2A in flavivirus replication and virion assembly. Our results have functionally demonstrated the molecular mechanism for two important steps during DENV-2 assembly: (i) The binding of 3'UTR RNA to NS2A servers as a "packaging signal" and (ii) in the presence of a viral RNA-NS2A complex, the coordinated cleavage of C-prM-E facilitates the envelopment of nucleocapsid. The proposed model could serve as a framework for future investigations and antiviral development.

**Author contribution**

X.X., J.Z., X.Z., Y.Z., Q.W., H.-P.D. performed experiments and data analysis. X.X., J.Z., X.Z., Y.Z., A.R., V.L.P., X.W., Q.W., H.-P.D, P.-Y.S. designed the experiments and interpreted the results. X.X., P.-Y.S. wrote the manuscript.

**Acknowledgements**

We are grateful to Dr. Mariano A. Garcia-Blanco, Shelton S. Bradrick from University of Texas Medical Branch for helpful discussions during this work. P.-Y.S. was supported by NIH grants AI142759, AI[ HYPERLINK "https://public.era.nih.gov/grantfolder/piAppDetails/genericStatus.do?encryptedParam=vXeG1axw2cY.YZxjFgAisBbII6OqVj4LxDR99WdVyTI-X4FuJbCLt4g." \t "_blank" ], and AI136126, and awards from the Kleberg Foundation, John S. Dunn Foundation, Amon G. Carter



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1104 Foundation, Gilson Longenbaugh Foundation, and Summerfield Robert Foundation. X.X.
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1107 Medical Branch, respectively.



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**Figure titles and legends**

**Figure 1 Characterization of NS2A G11A mutant**. (A) Replicon transient transfection assay. Top panel depicts the DENV-2 luciferase replicon. Bottom panel shows the luciferase activities after transfecting BHK-21 cells with equal amounts (10 μg) of WT or NS2A G11A mutant replicon RNA. (B) Quantification of extracellular viral RNA. Top panel depicts the full-length genome of DENV-2. Equal amounts of WT or NS2A G11A mutant viral RNA (10 μg) were electroporated into BHK-21 cells. At different time post-transfection (p.t.), extracellular viral RNAs were quantified by RT-qPCR. The DENV-2 replicon RNA was used as a control for determining the basal level of viral RNA released from dead cells. Bottom panel shows the CT values of the RT-qPCR results. Data show the means and standard deviations from three independent experiments. (C) Plaque morphology. Culture supernatants from (B) were analyzed for virus production using plaque assay. Only WT, not NS2A G11A mutant (N.D.), produced plaques. Plaques were developed on BHK-21 cells after 5 days of infection. (D) Thin-section transmission electron microscope (TEM) images of viral RNA-transfected BHK-21 cells. At 48 h p.t., cells were fixed for TEM analysis. Ve, virus-induced vesicles; T, membrane tubes; Vi, virus particles; ER, endoplasmic reticulum. Mock transfected cells were included as a control. (E-G) Confocal microscopy analysis of subcellular localizations of C, prM, and E in viral RNA-transfected BHK-21 cells. At 48 h p.t., transfected cells were analyzed by IFA. NS3 staining (red) was used as a control. Nuclei (blue) were stained by DAPI. Scale bar, 10 μm. (H) Viral protein processing in viral RNA-transfected BHK-21 cells. At 42 h p.t., BHK-21 (Mock) or viral RNA-transfected cells were treated with 0.01% DMSO (-) or 10 μM MG132 (+). After 6 h treatment, the cells were analyzed by Western blot. (I) Densitometry analysis. The intensities of bands representing specific viral proteins from DMSO-treated cells in (H) was quantified. The amounts of viral proteins derived from WT RNA-transfected cells were set as 100%. Relative intensities are calculated by normalizing the protein intensities derived from NS2A G11A mutant RNA-transfected cells to those from WT RNA-transfected cells. Data shows the means and standard deviations from three independent experiments.

**Figure 2 Restoration of NS2A G11A virion assembly by capsid R99S mutation.** (A) Revertant mutation. BHK-21 cells were transfected with NS2A G11A viral RNA. The transfected cells were consecutively passaged for five rounds to select for revertant viruses. The P5 virus was sequenced for revertant mutations. Changes in nucleotide



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positions and amino acids are indicated. (B) IFA of viral RNA-transfected cells. WT and mutant viral RNAs were electroporated to BHK-21 cells. The transfected cells were analyzed for E protein expression at 24 and 96 h p.t. by IFA. Scale bar, 100 μm. (C) Plaque morphology. Infectious viruses, derived from the RNA-transfected cells, were analyzed by plaque assay. Plaques were developed on day 5 post-infection on BHK-21 cells. N.D., not detectable. (D) Virus production. The culture fluids from (B) were quantified for infectious viruses. Data represent the means and standard deviations from three independent measurements. Limit of detection (L.O.D.), 10 PFU/ml. (E-F) Subcellular distributions of prM, NS1, and C proteins. prM, NS1 and C proteins were detected by DM1, human IgG anti-NS1 and D2C1 antibodies, respectively. Representative IFA images of viral RNA-transfected cells are shown at 24 h post-transfection. White arrows point to nucleus with C protein speckles. (G) Quantitative analysis of C-positive cells. The C-positive cells with cytoplasm or cytoplasm & nucleus distribution from 4-5 random fields were counted and normalized to the total cell numbers. The averages and standard deviations are shown. (H) Viral protein processing in viral RNA-transfected BHK-21 cells at 48 h post-transfection. The percentages of mature C protein were calculated from the following the formula: [C intensity/(C intensity + C-prM intensity)]×100%.

**Figure 3 DENV-2 NS2A interacts with prM and E proteins**. (A) Membrane topologies of DENV-2 prM, E, and NS2A proteins. The NS2A G11 position is indicated. (B) Co-IP of WT NS2A with prM. HEK293T cells were transfected with one plasmid (encoding prM only) or two plasmids (one encoding leader-NS2A-EGFP and the other encoding full-length or the ectodomain of prM). At 40 h p.t., IP was performed using mouse anti-EGFP or control IgG antibodies. Immunoblot (IB) was performed using rabbit anti-EGFP antibody or anti-prM antibody. (C) Co-IP of WT or G11A NS2A-EGFP with prM. Mouse anti-EGFP antibody was used for IP. Rabbit anti-EGFP or anti-prM antibodies were used for IB. The intensity of prM, co-precipitated with WT NS2A-EGFP, was set as 100%. The relative efficiency of prM co-precipitated with G11A NS2A-EGFP was compared with the WT NS2A-EGFP. Statistical analysis was performed using the unpaired parametric t-test. $*p < 0.05$, significant; $**p < 0.01$, very significant. (D) Reciprocal co-IP of prM with WT or G11A NS2A-EGFP. Rabbit anti-prM antibodies were used for IP. The amounts of co-precipitated WT and G11A NS2A-EGFP were detected using mouse anti-EGFP antibody. prM in eluates were detected by rabbit anti-prM antibody followed by protein G conjugated with HRP. (E) Co-localization analysis of NS2A and prM proteins. BHK-21 cells were co-



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transfected with two plasmids: One encoding the $C_{14}$-prM-E and the other encoding SPG-$C_{16}$-NS2A-EGFP. At 24 h p.t., the cells were examined for prM and NS2A-EGFP co-localization under a confocal microscope. Scale bar, 10 μm. (F) Co-IP of NS2A with E protein. HEK293T cells were co-transfected with two plasmids: One encoding the last 17 amino acids of prM and complete E protein ($M_{17}$-E), and another encoding NS2A with a C-terminal GST tag (NS2A-GST). GST tag alone was used as a negative control. IPs were performed using rabbit anti-GST antibody. E and GST fusion proteins were detected in cell lysates and eluates by mouse anti-E monoclonal antibody (9F12) and anti-GST antibody, respectively. The relative pulldown efficiency was calculated by normalizing the intensity of E co-precipitated with G11A NS2A-GST to the intensity of E co-precipitated with the WT NS2A-GST. The means and standard deviations from three independent experiments are shown. N.S., no significance.

**Figure 4 Two-residue insertion at the capsid-anchorC junction rescues the defect of NS2A G11A**. (A) A model depicting how NS2A modulates C-anchorC-prM cleavage. On the cytosolic side, viral NS2B/NS3 protease cleaves the C-anchorC junction. On the ER lumen side, host signalase cleaves the anchorC-prM junction. Three cleavage scenarios are presented for WT, NS2A G11A, and NS2A G11A+C_R99S constructs. (B) Constructs of insertion mutants of capsid protein. One residue (C_101) or two residues (C_TA or C_SR) were inserted to the C-anchorC junction. The potential cleavage sites for viral NS2B/NS3 protease are indicated. (C) IFA of viral RNA-transfected cells. Viral RNAs containing the NS2A G11A mutation with or without capsid insertions were electroporated into BHK-21 cells. The transfected cells were analyzed for E protein expression on day 1 and 4 by IFA. Scale bar, 100 μm. (D) Plaque morphology. Viruses recovered from (C) were analyzed by plaque assay on BHK-21 cells. (E) Viral titers. Culture supernatants from (C) were measured for viral titers on day 5 post-transfection. (F) Subcellular localization of C protein in viral RNA-transfected cells. White arrows point to nucleus with C protein speckles. Scale bar, 25 μm. (G) Expression of prM and NS1 proteins in viral RNA-transfected cells. Scale bar, 50 μm. (H) C-prM processing in viral RNA-transfected cells. At 48 h p.t., WT or NS2A G11A+C_T101 RNA-transfected cells were analyzed for C, NS1, and β-actin expression by Western blot. The percentages of C-prM to mature C conversion are presented for each construct.

**Figure 5 DENV-2 NS2A protein binds to 3'UTR RNA**. (A) Diagram of pulldown assay using biotinylated RNAs. 3'-end biotinylated RNA binds to streptavidin-conjugated



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magnetic beads. After incubating the bead-bound biotinylated RNA with NS2A protein, the RNA-bound NS2A was enriched and eluted for SDS-PAGE and Western blot analysis. (B) Pulldown analysis using recombinant NS2A protein. N-terminally GST-tagged NS2A protein (GST-NS2A) was expressed in *E. coli* and affinity purified. The 5'UTR-$C_{66}$-3'UTR-biotin RNA was prepared by ligating biotinylated cytidine (bis)phosphate to the 3' end of the 5'UTR-$C_{66}$-3'UTR RNA (containing the 5'UTR, the first 66 nucleotides of C gene, and the 3'UTR). The amounts of GST-NS2A in the input and eluates were analyzed using rabbit anti-GST antibody by Western blot. (C) Pulldown analysis using HEK293T cell-expressed NS2A-GST protein. HEK293T cells were transfected with a plasmid expressing GST or C-terminally GST-tagged NS2A protein (NS2A-GST). At 42 h p.t., cells were harvested, lysed, and used for RNA pull down-assay. The pulldown assay was performed as described in (B). (D) RNA competition assay. Various amounts of unlabeled 5'UTR-$C_{66}$-3'UTR RNA were used to compete the binding of 5'UTR-$C_{66}$-3'UTR-biotin RNA to GST-NS2A protein. tRNA was used as a negative control. (E) Viral RNA/NS2A binding affinity. Electrophoretic mobility-shift assay (EMSA) was used to determine the binding affinity of DENV-2 5'UTR-$C_{66}$-3'UTR-biotin RNA with NS2A protein. 5'UTR-$C_{66}$-3'UTR-biotin RNA was incubated with increasing amounts of GST-NS2A protein. Free and bound RNAs were resolved by native agarose gel electrophoresis. The equilibrium dissociation constant (*Kd*) was determined by nonlinear regression with least-squares fitting method. The coefficient of determination ($R^2$) is shown. Data represent the means and standard deviations from three independent experiments. (F) Mapping the RNA region responsible for NS2A binding. A panel of unlabeled RNA truncates of the 5'UTR-$C_{66}$-3'UTR were used as competitors for the binding of 5'UTR-$C_{66}$-3'UTR-biotin RNA to GST-NS2A protein. The intensity of GST-NS2A (bound to the 5'UTR-$C_{66}$-3'UTR-biotin) without competitor was set as 100%. Relative GST-NS2A intensities were calculated as [(signal in the presence competitor)/(signal in the absence of competitor)]×100%. Statistical analysis was performed using the unpaired parametric t-test. (G) PAR-CLIP workflow [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. See text for detail. (H) PAR-CLIP result summary. Uridines in the 3'UTR that may participate in NS2A-binding are indicated. The dash line indicates the threshold (equal to 5.76, representing the average fold change of mutation rate of all uridines determined by Illumina sequencing). The uridine positions with fold changes of mutation rate over the threshold are indicated in the 3'UTR sequence (GenBank no. AF038403).

**Comment [RAL5]:** Need to explain different between black and blue lines (i.e. that they are replciates).



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**Figure 6 NS2A residues responsible for the NS2A/3'UTR RNA interaction**. (A) Diagram of NS2A truncates used in RNA pulldown assay. All proteins were fused with an N-terminal GST tag, expressed in *E. coli*, and affinity purified. (B) Interactions between NS2A truncates with 5'UTR-$C_{66}$-3'UTR-biotin RNA. The interactions were measured using pulldown experiments. The pulldown efficiency of full-length NS2A (1-218) was set as 100%. Relative efficiency was calculated by comparing the intensity of NS2A truncate with that of the full-length NS2A. (C) Sequence alignment of NS2A among flaviviruses. Amino acids of a cytosolic loop (amino acids 93-101) are presented. The number of viral strains used for the NS2A alignment are indicated in parentheses. Amino acid positions of NS2A are labeled according to DENV-2 strain New Guinea C. Residues R94, K95, and K99 are shaded. The conserved (:) and identical (*) residues are indicated. (D) IFA of BHK-21 cells transfected with viral RNA. WT and mutant viral RNAs were electroporated into BHK-21 cells. The cells were assayed for E protein expression. (E) Plaque morphology. Viruses derived from the transfected cells in (D) were analyzed by plaque assay. (F) Viral titers. Culture supernatants from (D) were measured for virus yields on day 5 post-transfection. (G) Replicon transient transfection assay. NS2A triple mutations (R94A+K95A+K99A) were engineered into a luciferase replicon of DENV-2. Luciferase activities were presented after the WT or mutant replicon RNAs were electroporated into BHK-21 cells. (H) Diagram of N-terminally GST-tagged NS2A peptide 64-128 [GST-NS2A (64-128)]. (I) Interaction of GST-NS2A (64-128) protein with 3'UTR RNA. GST-NS2A (64-128) was expressed in *E. coli* and affinity purified. The pulldown efficiency of GST-NS2A (64-128) by 5'UTR-$C_{66}$-3'UTR-biotin was set as 100%. The binding efficiencies of RNA variants were indicated through competition assay. In the presence of competitor RNAs, the remaining amounts of WT GST-NS2A (64-128)/5'UTR-$C_{66}$-3'UTR-biotin interaction are presented. (J) R94A+K95A+K99A mutations reduce GST-NS2A (64-128) binding to 5'UTR-$C_{66}$-3'UTR-biotin RNA. The R94A+K95A+K99A variant retained only 19.1% of the WT activity.

**Figure 7 A model depicting the role of NS2A in DENV-2 assembly.** Viral replication complex (top) and virion assembly site (bottom) are depicted. DENV-2 NS2A protein recruits viral RNA, three structural proteins, and NS2B/NS3 protease to virion assembly site, where coordinated cleavage of C-prM-E ensues infections virus formation. C molecules accumulated on lipid droplets (LD) could bind viral RNA to form nucleocapsid. See text for details.



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**Reference**

[ ADDIN EN.REFLIST ]



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## STAR METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---|---|---|
| Antibodies | | |
| Mouse IgG Anti-C (D2C1) | [ ADDIN EN.CITE ADDIN EN.CITE.DATA ] | N/A |
| Mouse IgG anti-prM (DM1) | Abcam | Cat# ab41473, RRID:AB_873758 |
| Rabbit IgG anti-prM | GeneTex | GTX128093 |
| Mouse IgG anti-E (4G2) | ATCC | Cat# HB-112, RRID:CVCL_J890 |
| Mouse IgG anti-E (9F12) | [ ADDIN EN.CITE ADDIN EN.CITE.DATA ] | N/A |
| Mouse IgG anti-NS1 | Abcam | Cat# ab41616, RRID:AB_869448 |
| Human IgG anti-NS1 | This study. Generated by Novartis Institute for Tropical Diseases in Singapore | N/A |
| Rabbit IgG anti-NS1 | GeneTex | GTX103346 |
| Rabbit IgG anti-NS3 | GeneTex | GTX124252 |
| Mouse IgG anti-NS5 | GeneTex | GTX629447 |
| Rabbit IgG anti-calnexin | SIGMA-AIDRICH | Cat# C4731, RRID:AB_476845 |
| Mouse IgG anti-EGFP | SIGMA-AIDRICH | Cat# 11814460001, RRID:AB_390913 |
| Moue IgG1 Isotype Control | SIGMA-AIDRICH | Cat# M5284, RRID:AB_1163685 |
| Rabbit IgG anti-EGFP | Abcam | Cat# ab13970, RRID:AB_300798 |
| protein G horseradish peroxidase (HRP) conjugate | Thermo Fisher Scientific | Cat# P21041 |
| Mouse IgG anti-GST | Abcam | Cat# ab19256, RRID:AB_444809 |
| Rabbit IgG anti-GST | SIGMA-AIDRICH | Cat# G7781, RRID:AB_259965 |
| Mouse IgG anti-β-actin | Abcam | Cat# ab8226, RRID:AB_306371 |
| Mouse IgG anti-HA | Abcam | Cat# ab18181, RRID:AB_444303 |
| Goat anti-mouse IgG conjugated with HRP | SIGMA-AIDRICH | Cat# A5278, RRID:AB_258232 |
| Goat anti-rabbit IgG conjugated with HRP | SIGMA-AIDRICH | Cat#AP307P, RRID:AB_92641 |
| Goat anti-mouse IgGs conjugated with Alexa 488 | Thermo Fisher Scientific | Cat#A-1100, RRID:AB_2534069 |
| Goat anti-mouse IgGs conjugated with Alexa 594 | Thermo Fisher Scientific | Cat# A-11005, RRID:AB_2534073 |
| Goat anti-rabbit IgGs conjugated with Alexa 488 | Thermo Fisher Scientific | Cat# A-11008, RRID:AB_143165 |



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| | | |
|---|---|---|
| Goat anti-rabbit IgGs conjugated with Alexa 594 | Thermo Fisher Scientific | Cat# A-11072, RRID:AB_2534116 |
| Goat anti-Human IgG (H+L) cross-adsorbed secondary antibody, Alexa Fluor 594 | Thermo Fisher Scientific | Cat# A-11014, RRID:AB_2534081 |
| Bacterial and Virus Strains | | |
| *E.* coli strain Top 10 | ThermoFisher Scientific | Cat#C404006 |
| *E.* coli strain Rosetta™ 2(DE3) pLysS | MilliporeSigma | Cat#71403-4 |



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| | | |
|---|---|---|
| DENV-2 strain New Guinea C | [ ADDIN EN.CITE <EndNote><Cite><Author>Xie</Author><Year>2013</Year><RecNum>85</RecNum><DisplayText>(Xie et al., 2013)</DisplayText><record><rec-number>85</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793217">85</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Xie, X.</author><author>Gayen, S.</author><author>Kang, C.</author><author>Yuan, Z.</author><author>Shi, P. Y.</author></authors></contributors><auth-address>State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Science, China.</auth-address><titles><title>Membrane topology and function of dengue virus NS2A protein</title><secondary-title>J Virol</secondary-title><alt-title>Journal of virology</alt-title></titles><periodical><full-title>J Virol</full-title></periodical><pages>4609-22</pages><volume>87</volume><number>8</number><edition>2013/02/15</edition><keywords><keyword>Animals</keyword><keyword>Cell Line</keyword><keyword>Cricetinae</keyword><keyword>Dengue | N.A. |



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| | | |
|---|---|---|
| Biological Samples | | |
| None | | |
| Chemicals, Peptides, and Recombinant Proteins | | |
| Peptide WT (NILNRRRRTAGMII) | GenScript | N/A |
| Peptide C_R99S (NILNRRSRTAGMII) | GenScript | N/A |
| Recombinant protein: GST | This study | N/A |
| Recombinant protein: GST-NS2A | This study | N/A |
| Recombinant protein: GST-NS2A(1-71) | This study | N/A |
| Recombinant protein: GST-NS2A(1-101) | This study | N/A |
| Recombinant protein: GST-NS2A(1-145) | This study | N/A |
| Recombinant protein: GST-NS2A(1-168) | This study | N/A |
| Recombinant protein: GST-NS2A(64-128) | This study | N/A |
| Critical Commercial Assays | | |
| iScript SYBR Green One-Step kit | Bio-Rad | Cat#170-8893 |
| QIAamp viral RNA mini kit | Qiagen | Cat#52906 |
| T7 mMessage mMachine kit | Thermo Fisher Scientific | Cat# AM1344 |
| Renilla Luciferase Assay System | Promega Corporation | Cat# E2810 |
| Pierce™ RNA 3' End Biotinylation Kit | Thermo Fisher Scientific | Cat# 20160 |
| Pierce™ Magnetic RNA-Protein Pull-Down kit | Thermo Fisher Scientific | Cat# 20164 |
| Chemiluminescent Detection Module | Thermo Fisher Scientific | Cat# 89880 |
| | | |
| Deposited Data | | |
| Raw image data for illumina sequencing | | |
| Experimental Models: Cell Lines | | |
| BHK-21 cell | ATCC | Cat#CCL-10, RRID:CVCL_1915 |
| Vero cell | ATCC | Cat#CCL-81, RRID:CVCL_0059 |
| HEK293T cell | ATCC | Cat# CRL-3216, RRID:CVCL_0063 |
| NS2A-EGFP BHK-21 cell | [ ADDIN EN.CITE ADDIN EN.CITE.DATA ] | N/A |
| | | |
| Experimental Models: Organisms/Strains | | |
| N.A. | | |
| Oligonucleotides | | |
| Primer P1(5'-ACACTCTTTCCCTACACGA CGCTCTTCCGATCTAAGGCAAAACTAACATGAA AC-3') | Integrated DNA Technologies (Skokie, Illinois) | N/A |
| Primer P2 (5'-GTGACTGGAGTTCAGACG TGTGCTCTTCCGATCTAGAACCTGTTGATTCAA CAGCAC-3') | Integrated DNA Technologies (Skokie, Illinois) | N/A |



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| | | |
|---|---|---|
| 5′ universal adaptor (5′-AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGAC-3′) | Illumine (San Diego, CA) | N/A |
| 3′ indexing primer (5′-CAAGCAGAAGACGGCATACGAGATnnnnnnGTGACTGGAGTTCAGACGTGT-3′) | Illumine (San Diego, CA) | N/A |
| Primer NGC 7764V (5′-CGTCGAGAGAAATATGGTCACACC-3′) | Integrated DNA Technologies (Skokie, Illinois) | N/A |
| Primer NGC 7844C (5′CCACAATAGTATGACCAGCCT-3′) | Integrated DNA Technologies (Skokie, Illinois) | N/A |
| Recombinant DNA | | |



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| | | |
|---|---|---|
| pACYC-NGC FL | [ ADDIN EN.CITE <EndNote><Cite><Author>Xie</Author><Year>2013</Year><RecNum>85</RecNum><DisplayText>(Xie et al., 2013)</DisplayText><record><rec-number>85</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793217">85</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Xie, X.</author><author>Gayen, S.</author><author>Kang, C.</author><author>Yuan, Z.</author><author>Shi, P. Y.</author></authors></contributors><auth-address>State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Science, China.</auth-address><titles><title>Membrane topology and function of dengue virus NS2A protein</title><secondary-title>J Virol</secondary-title><alt-title>Journal of virology</alt-title></titles><periodical><full-title>J Virol</full-title></periodical><pages>4609-22</pages><volume>87</volume><number>8</number><edition>2013/02/15</edition><keywords><keyword>Animals</keyword><keyword>Cell Line</keyword><keyword>Cricetinae</keyword><keyword>Dengue | N/A |



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| | | |
|---|---|---|
| pACYC-NGC replicon | [ ADDIN EN.CITE <EndNote><Cite><Author>Xie</Author><Year>2013</Year><RecNum>85</RecNum><DisplayText>(Xie et al., 2013)</DisplayText><record><rec-number>85</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793217">85</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Xie, X.</author><author>Gayen, S.</author><author>Kang, C.</author><author>Yuan, Z.</author><author>Shi, P. Y.</author></authors></contributors><auth-address>State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Science, China.</auth-address><titles><title>Membrane topology and function of dengue virus NS2A protein</title><secondary-title>J Virol</secondary-title><alt-title>Journal of virology</alt-title></titles><periodical><full-title>J Virol</full-title></periodical><pages>4609-22</pages><volume>87</volume><number>8</number><edition>2013/02/15</edition><keywords><keyword>Animals</keyword><keyword>Cell Line</keyword><keyword>Cricetinae</keyword><keyword>Dengue | N/A |



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| | | |
|---|---|---|
| pACYC-NGC replicon G11A | [ ADDIN EN.CITE ADDIN EN.CITE.DATA ] | N/A |
| pACYC-NGC replicon R94A+K95A+K99A | This study | N/A |
| pACYC-NGC FL C_R99S | This study | N/A |
| pACYC-NGC FL G11A | This study | N/A |
| pACYC-NGC FL G11A+C_R99S | This study | N/A |
| pACYC-NGC FL E20A | This study | N/A |
| pACYC-NGC FL E100A | This study | N/A |
| pACYC-NGC FL K188A | This study | N/A |
| pACYC-NGC FL E20A+C_R99S | This study | N/A |
| pACYC-NGC FL E100A+C_R99S | This study | N/A |
| pACYC-NGC FL K188A+C_R99S | This study | N/A |
| pACYC-NGC FL C_TA | This study | N/A |
| pACYC-NGC FL C_SR | This study | N/A |
| pACYC-NGC FL G11A+C_TA | This study | N/A |
| pACYC-NGC FL G11A+C_SR | This study | N/A |
| pACYC-NGC FL C_T101 | This study | N/A |
| pACYC-NGC FL G11A+C_T101 | This study | N/A |
| pACYC-NGC FL R94A | This study | N/A |
| pACYC-NGC FL K95A | This study | N/A |
| pACYC-NGC FL K99A | This study | N/A |
| pACYC-NGC FL R94A+K95A+K99A | This study | N/A |
| pACYC-NGC FL R94A+K95A | This study | N/A |



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| | | |
|---|---|---|
| SFV-DENV2 CprME | [ ADDIN EN.CITE <EndNote><Cite><Author>Qing</Author><Year>2010</Year><RecNum>105</RecNum><DisplayText>(Qing et al., 2010)</DisplayText><record><rec-number>105</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793220">105</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Qing, M.</author><author>Liu, W.</author><author>Yuan, Z.</author><author>Gu, F.</author><author>Shi, P. Y.</author></authors></contributors><auth-address>Novartis Institute for Tropical Diseases, 10, Biopolis Road, #05-01 Chromos, Singapore, Singapore.</auth-address><titles><title>A high-throughput assay using dengue-1 virus-like particles for drug discovery</title><secondary-title>Antiviral Res</secondary-title></titles><periodical><full-title>Antiviral Res</full-title></periodical><pages>163-71</pages><volume>86</volume><number>2</number><dates><year>2010</year><pub-dates><date>May</date></pub- [ PAGE * MERGEFORMAT ] dates></dates><accession-num>20153777</accession-num><urls><related- | N/A |

| | | |
|---|---|---|
| pXJ-SPG-$C_{16}$-NS2A-EGFP | This study | N/A |
| pXJ-SPG-$C_{16}$-NS2A(G11A)-EGFP | This study | N/A |
| pXJ-EGFP | [ ADDIN EN.CITE ADDIN EN.CITE.DATA ] | N/A |
| pXJ-$C_{14}$-prM | This study | N/A |
| pXJ-$C_{14}$-prM(1-130) | This study | N/A |
| pXJ-$C_{14}$-prM (N69Q) | This study | N/A |
| pXJ-SPG-$C_{16}$-NS2A-GST | This study | N/A |
| pXJ-SPG-$C_{16}$-NS2A(G11A)-GST | This study | N/A |
| pXJ-GST | This study | N/A |
| pXJ-HA-NS3 | This study | N/A |
| pXJ-$M_{17}$-E | This study | N/A |
| pGEX-4T-1 | GE Healthcare Life Sciences | Cat# 28954549 |
| pGEX-4T-GST-NS2A | This study | N/A |
| pGEX-4T-GST-NS2A (1-71) | This study | N/A |
| pGEX-4T-GST-NS2A (1-101) | This study | N/A |
| pGEX-4T-GST-NS2A (1-145) | This study | N/A |
| pGEX-4T-GST-NS2A (1-168) | This study | N/A |
| pGEX-4T-GST-NS2A (64-128) | This study | N/A |
| pGEX-4T-GST-NS2A (64-128) R94A+K95A+K99A | This study | N/A |
| | | |
| Software and Algorithms | | |
| Illustrator CC | Adobe | [ HYPERLINK "https://www.adobe.com/products/illustrator.html" ] |
| ImageJ | NIH | [ HYPERLINK "https://fiji.sc/" ] |
| CLC main workbench | Qiagen | [ HYPERLINK "https://www.qiagenbioinformatics.com/products/clc-main-workbench/" ] |
| Prism 8.0 software | GraphPad | [ HYPERLINK "https://www.graphpad.com/scientific-software/prism/" ] |
| | | |

## Contact for Reagent and Resource Sharing

Further information and requests for resources and reagents should be directed to and will be fulfilled by Lead Contact, Dr. Pei-Yong Shi (peshi@utmb.edu)

## Experimental Model and Subject Details



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**Cell Lines**

The Baby hamster kidney fibroblast (BHK-21) cells, African green monkey kidney epithelial (Vero) cells were purchased from the American Type Culture Collection (ATCC, Bethesda, MD) and maintained in a high-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (HyClone Laboratories, South Logan, UT) and 1% penicillin/streptomycin (P/S). The NS2A-EGFP BHK-21 cells constitutively expressing the C-terminally EGFP-tagged NS2A protein was maintained in the high-glucose DMEM medium supplemented with 10%FBS, 1% P/S and 1 mg/ml Geneticin. HEK293T cells were grown in low-glucose DMEM (Invitrogen) containing 10% FBS and 1% penicillin-streptomycin. All mammalian cell lines were grown at 37°C with 5% $CO_2$. Mosquito C6/36 cells were grown in RPMI 1640 medium (Invitrogen) with 10% FBS and 1% penicillin-streptomycin at 28°C without $CO_2$. All culture medium and antibiotics were purchased from ThermoFisher Scientific (Waltham, MA). All cell lines were tested negative for mycoplasma.

**Pseudo-infectious Particle**

Pseudo-infectious particles were produced using the a previously described protocol [ ADDIN EN.CITE <EndNote><Cite><Author>Qing</Author><Year>2010</Year><RecNum>114</RecNum><DisplayText>(Qing et al., 2010)</DisplayText><record><rec-number>114</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1561745637">114</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Qing, M.</author><author>Liu, W.</author><author>Yuan, Z.</author><author>Gu, F.</author><author>Shi, P. Y.</author></authors></contributors><auth-address>Novartis Institute for Tropical Diseases, 10, Biopolis Road, #05-01 Chromos, Singapore, Singapore.</auth-address><titles><title>A high-throughput assay using dengue-1 virus-like particles for drug discovery</title><secondary-title>Antiviral Res</secondary-title></titles><periodical><full-title>Antiviral Res</full-title></periodical><pages>163-71</pages><volume>86</volume><number>2</number><keywords><keyword>Animals</keyword><keyword>Antiviral Agents/*isolation & purification/*pharmacology</keyword><keyword>Biological Assay/methods</keyword><keyword>Cell Line</keyword><keyword>Cercopithecus



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aethiops</keyword><keyword>Cricetinae</keyword><keyword>Dengue Virus/*drug effects/genetics</keyword><keyword>Drug Discovery/*methods</keyword><keyword>Drug Evaluation, Preclinical/*methods</keyword><keyword>Electroporation</keyword><keyword>Genes, Reporter</keyword><keyword>Luciferases, Renilla/genetics/metabolism</keyword><keyword>Macaca mulatta</keyword><keyword>Viral Proteins/genetics/metabolism</keyword><keyword>Virosomes/*drug effects/genetics</keyword></keywords><dates><year>2010</year><pub-dates><date>May</date></pub-dates></dates><isbn>1872-9096 (Electronic)0166-3542 (Linking)</isbn><accession-num>20153777</accession-num><urls><related-urls><url>https://www.ncbi.nlm.nih.gov/pubmed/20153777</url></related-urls></urls><electronic-resource-num>10.1016/j.antiviral.2010.02.313</electronic-resource-num></record></Cite></EndNote>] with several modifications. 10 µg of replicon RNA was electroporated into BHK-21 cells as described above. After transfection, the cells were maintained at 37°C with 5% $CO_2$ for 24 h. Afterwards, cells were harvested and electroporated with 10 µg of a helper replicon RNA SFV-DENV2 CprME (replicon derived from Semliki forest virus that express the DENV-2 encoding the CprME). After the second electroporation, cells were cultured at 30°C with 5% $CO_2$. At 20, 28 and 48 h after the second electroporation, culture medium was collected and stored at -80 °C. Afterwards, the collected medium was used ton infection naïve Vero cells in 96-well plates. At 48 h post-infection, cells were lysed, and luciferase signals were measured using the Renilla luciferase assay system (Promega) in the Clarity luminescence microplate reader (BioTek) according to the manufacturer's protocol.

**METHOD DETAILS**

**Plasmid Construction**

The full-length cDNA infectious clone of DENV-2 strain New Guinea C (NGC), pACYC-NGC FL, and its derived replicon (pACYC-NGC replicon) clone [ ADDIN EN.CITE <EndNote><Cite><Author>Xie</Author><Year>2013</Year><RecNum>85</RecNum><DisplayText>(Xie et al., 2013)</DisplayText><record><rec-number>85</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793217">85</key></foreign-keys><ref-type name="Journal



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Article">17</ref-type><contributors><authors><author>Xie, X.</author><author>Gayen, S.</author><author>Kang, C.</author><author>Yuan, Z.</author><author>Shi, P. Y.</author></authors></contributors><auth-address>State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Science, China.</auth-address><titles><title>Membrane topology and function of dengue virus NS2A protein</title><secondary-title>J Virol</secondary-title><alt-title>Journal of virology</alt-title></titles><periodical><full-title>J Virol</full-title></periodical><pages>4609-22</pages><volume>87</volume><number>8</number><edition>2013/02/15</edition><keywords><keyword>Animals</keyword><keyword>Cell Line</keyword><keyword>Cricetinae</keyword><keyword>Dengue Virus/*physiology</keyword><keyword>Endoplasmic Reticulum/*virology</keyword><keyword>Humans</keyword><keyword>Intracellular Membranes/*metabolism</keyword><keyword>Magnetic Resonance Spectroscopy</keyword><keyword>Models, Molecular</keyword><keyword>Protein Conformation</keyword><keyword>Viral Nonstructural Proteins/*chemistry/metabolism</keyword><keyword>*Virus Assembly</keyword></keywords><dates><year>2013</year><pub-dates><date>Apr</date></pub-dates></dates><isbn>1098-5514 (Electronic)0022-538X (Linking)</isbn><accession-num>23408612</accession-num><work-type>Research Support, Non-U.S. Gov't</work-type><urls><related-urls><url>http://www.ncbi.nlm.nih.gov/pubmed/23408612</url></related-urls></urls><custom2>3624351</custom2><electronic-resource-num>10.1128/JVI.02424-12</electronic-resource-num><language>eng</language></record></Cite></EndNote>] were used as the backbones for mutagenesis study. The replicon was constructed by replacing most of the structural genes (C, prM and E) with a gene cassette (Rluc2A) encoding the Renilla luciferase and a 2A peptide from Foot-and-mouth disease virus. The first 38 amino acids of C protein and the last 31 amino acids of E protein were retained in the replicon. Standard overlap PCR was performed to introduce the mutations in C and NS2A genes. The DNA fragments containing the mutations in C gene were cloned into pACYC-NGC FL clone through restriction enzyme sites NotI (upstream of the T7 promoter and 5'UTR of DENV-2) and SphI (at the position of 1384 in DENV-2 genome). The DNA fragments containing mutations in NS2A gene were cloned into pACYC-NGC FL or replicon through restriction enzyme sites and NheI (at the position of 2544 in DENV-2 genome) and SacI (at the position of 4342 in the DENV-2 genome).



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A mammalian expression vector, pXJ [ ADDIN EN.CITE <EndNote><Cite><Author>Xie</Author><Year>2013</Year><RecNum>85</RecNum><DisplayText>(Xie et al., 2013)</DisplayText><record><rec-number>85</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793217">85</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Xie, X.</author><author>Gayen, S.</author><author>Kang, C.</author><author>Yuan, Z.</author><author>Shi, P. Y.</author></authors></contributors><auth-address>State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Science, China.</auth-address><titles><title>Membrane topology and function of dengue virus NS2A protein</title><secondary-title>J Virol</secondary-title><alt-title>Journal of virology</alt-title></titles><periodical><full-title>J Virol</full-title></periodical><pages>4609-22</pages><volume>87</volume><number>8</number><edition>2013/02/15</edition><keywords><keyword>Animals</keyword><keyword>Cell Line</keyword><keyword>Cricetinae</keyword><keyword>Dengue Virus/*physiology</keyword><keyword>Endoplasmic Reticulum/*virology</keyword><keyword>Humans</keyword><keyword>Intracellular Membranes/*metabolism</keyword><keyword>Magnetic Resonance Spectroscopy</keyword><keyword>Models, Molecular</keyword><keyword>Protein Conformation</keyword><keyword>Viral Nonstructural Proteins/*chemistry/metabolism</keyword><keyword>*Virus Assembly</keyword></keywords><dates><year>2013</year><pub-dates><date>Apr</date></pub-dates></dates><isbn>1098-5514 (Electronic)0022-538X (Linking)</isbn><accession-num>23408612</accession-num><work-type>Research Support, Non-U.S. Gov't</work-type><urls><related-urls><url>http://www.ncbi.nlm.nih.gov/pubmed/23408612</url></related-urls></urls><custom2>3624351</custom2><electronic-resource-num>10.1128/JVI.02424-12</electronic-resource-num><language>eng</language></record></Cite></EndNote>], driven by a cytomegalovirus (CMV) promoter was used to express various viral proteins prM, E, NS2A and NS3 fusion proteins in HEK293T cells. For constructs pXJ-$C_{14}$-prM and pXJ-$C_{14}$-prM (1-130), the anchor C signal peptide ($C_{14}$) was retained at the N-terminus of prM to ensure the correct targeting on ER membrane. For construct pXJ-$M_{17}$-E, the last 17 amino acids from M protein was retained to serve as a signal peptide for translocating the E protein into the ER lumen. For constructs pXJ-SPG-$C_{16}$-NS2A-EGFP and pXJ-SPG-$C_{16}$-



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NS2A-GST, the signal peptide derived from Gaussia luciferase (SPG) [ ADDIN EN.CITE <EndNote><Cite><Author>Verhaegent</Author><Year>2002</Year><RecNum>112</RecNum><DisplayText>(Verhaegent and Christopoulos, 2002)</DisplayText><record><rec-number>112</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1561735944">112</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Verhaegent, M.</author><author>Christopoulos, T. K.</author></authors></contributors><auth-address>Department of Chemistry and Biochemistry, University of Windsor, Ontario, Canada.</auth-address><titles><title>Recombinant Gaussia luciferase. Overexpression, purification, and analytical application of a bioluminescent reporter for DNA hybridization</title><secondary-title>Anal Chem</secondary-title></titles><periodical><full-title>Anal Chem</full-title></periodical><pages>4378-85</pages><volume>74</volume><number>17</number><keywords><keyword>Animals</keyword><keyword>DNA/*analysis</keyword><keyword>DNA Probes</keyword><keyword>Genes, Reporter</keyword><keyword>*Luciferases/genetics/isolation & purification</keyword><keyword>Luminescent Proteins/genetics/isolation & purification</keyword><keyword>Plankton/*enzymology</keyword><keyword>Recombinant Proteins/genetics/isolation & purification</keyword></keywords><dates><year>2002</year><pub-dates><date>Sep 1</date></pub-dates></dates><isbn>0003-2700 (Print)0003-2700 (Linking)</isbn><accession-num>12236345</accession-num><urls><related-urls><url>https://www.ncbi.nlm.nih.gov/pubmed/12236345</url></related-urls></urls></record></Cite></EndNote>] and the last 16 amino acids of NS1 ($C_{16}$) was fused at the N-terminus of NS2A to ensure the correct processing and membrane topology of NS2A. The EGFP and GST were amplified from pEGFPluc (Clontech) and pGEX-4T-1 (Sigma-Aldrich) respectively. The Human influenza hemagglutinin (HA) tag was fused to the N-terminus of NS3 and cloned into pXJ vector, resulting the construct pXJ-HA-NS3.

pGEX-4T-1 (GE Healthcare Life Sciences) was used as the vector for expressing all N-terminally glutathione S-transferase (GST)-tagged NS2A fusion proteins. The DNA fragments containing various length of NS2A were cloned into pGEX-4T-1 through restriction enzyme sites BamHI and XhoI.



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All plasmids were propagated in *E. coli* strain Top10 (ThermoFisher Scientific). All restriction enzymes were purchased from New England BioLabs (Ipswitch, MA). The plasmids were validated by restriction enzyme digestion and Sanger DNA sequencing. All primers were synthesized from Integrated DNA Technologies (Skokie, Illinois) and are available upon request.

**RNA Transcription and Electroporation**

The NGC FL or replicon plasmids were linearized by restriction enzyme XbaI. RNA transcription and electroporation were performed as described previously [ ADDIN EN.CITE <EndNote><Cite><Author>Xie</Author><Year>2013</Year><RecNum>85</RecNum><DisplayText>(Xie et al., 2013)</DisplayText><record><rec-number>85</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793217">85</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Xie, X.</author><author>Gayen, S.</author><author>Kang, C.</author><author>Yuan, Z.</author><author>Shi, P. Y.</author></authors></contributors><auth-address>State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Science, China.</auth-address><titles><title>Membrane topology and function of dengue virus NS2A protein</title><secondary-title>J Virol</secondary-title><alt-title>Journal of virology</alt-title></titles><periodical><full-title>J Virol</full-title></periodical><pages>4609-22</pages><volume>87</volume><number>8</number><edition>2013/02/15</edition><keywords><keyword>Animals</keyword><keyword>Cell Line</keyword><keyword>Cricetinae</keyword><keyword>Dengue Virus/*physiology</keyword><keyword>Endoplasmic Reticulum/*virology</keyword><keyword>Humans</keyword><keyword>Intracellular Membranes/*metabolism</keyword><keyword>Magnetic Resonance Spectroscopy</keyword><keyword>Models, Molecular</keyword><keyword>Protein Conformation</keyword><keyword>Viral Nonstructural Proteins/*chemistry/metabolism</keyword><keyword>*Virus Assembly</keyword></keywords><dates><year>2013</year><pub-dates><date>Apr</date></pub-dates></dates><isbn>1098-5514 (Electronic)0022-538X (Linking)</isbn><accession-num>23408612</accession-num><work-type>Research Support, Non-U.S. Gov't</work-type><urls><related-



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urls><url>http://www.ncbi.nlm.nih.gov/pubmed/23408612</url></related-urls></urls><custom2>3624351</custom2><electronic-resource-num>10.1128/JVI.02424-12</electronic-resource-num><language>eng</language></record></Cite></EndNote>]. Briefly, 10 μg RNA was electroporated into 8 × $10^6$ cells in a 4-mm cuvette using the GenePulser apparatus (Bio-Rad) at settings of 0.45 kV (C6/36 cells) or 0.85 kV (BHK-21 cells) and 25 mF, pulsing three times, with 3 s intervals. After a 10-min recovery at room temperature, transfected cells were suspended in culture media and incubated at corresponding conditions: 28°C (C6/36 cells) or 37°C with 5% $CO_2$ (BHK-21).

**Virus Production and Quantification**

After electroporation, the transfected BHK-21 cells were seeded in one T-75 flask. After incubation at 37°C for 24 h, the cells were cultured in DMEM with 2% FBS at 30°C for another 4 days. At every 24 h p.t., culture fluids was collected and stored at -80°C. Viral titer was determined by crystal violet-based plaque assay using BHK-21 cells according to the protocol as described previously [ ADDIN EN.CITE <EndNote><Cite><Author>Xie</Author><Year>2013</Year><RecNum>85</RecNum><DisplayText>(Xie et al., 2013)</DisplayText><record><rec-number>85</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793217">85</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Xie, X.</author><author>Gayen, S.</author><author>Kang, C.</author><author>Yuan, Z.</author><author>Shi, P. Y.</author></authors></contributors><auth-address>State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Science, China.</auth-address><titles><title>Membrane topology and function of dengue virus NS2A protein</title><secondary-title>J Virol</secondary-title><alt-title>Journal of virology</alt-title></titles><periodical><full-title>J Virol</full-title></periodical><pages>4609-22</pages><volume>87</volume><number>8</number><edition>2013/02/15</edition><keywords><keyword>Animals</keyword><keyword>Cell Line</keyword><keyword>Cricetinae</keyword><keyword>Dengue Virus/*physiology</keyword><keyword>Endoplasmic Reticulum/*virology</keyword><keyword>Humans</keyword><keyword>Intracellular Membranes/*metabolism</keyword><keyword>Magnetic Resonance Spectroscopy</keyword><keyword>Models, Molecular</keyword><keyword>Protein Conformation</keyword><keyword>Viral Nonstructural



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Proteins/*chemistry/metabolism</keyword><keyword>*Virus Assembly</keyword></keywords><dates><year>2013</year><pub-dates><date>Apr</date></pub-dates></dates><isbn>1098-5514 (Electronic)0022-538X (Linking)</isbn><accession-num>23408612</accession-num><work-type>Research Support, Non-U.S. Gov't</work-type><urls><related-urls><url>http://www.ncbi.nlm.nih.gov/pubmed/23408612</url></related-urls></urls><custom2>3624351</custom2><electronic-resource-num>10.1128/JVI.02424-12</electronic-resource-num><language>eng</language></record></Cite></EndNote>]. Plaques were developed on day 5 post-infection.

**RNA extraction and RT-PCR**

Viral RNAs in culture fluids were extracted using a QIAamp viral RNA minikit (Qiagen). Extracted RNAs were eluted in 50 µl RNase-free water. Reverse transcription (RT)-qPCR was performed to measure the amounts of viral RNA copy in the culture medium. Viral RNAs were measured using an iScript one-Step RT-PCR kit with SYBR green (Bio-Rad) and the primer pair NGC 7764V (sequence: 5'-CGTCGAGAGAAATATGGTCACACC-3') and 7844C (sequence: 5'CCACAATAGTATGACCAGCCT-3') [ ADDIN EN.CITE <EndNote><Cite><Author>Xie</Author><Year>2013</Year><RecNum>85</RecNum><DisplayText>(Xie et al., 2013)</DisplayText><record><rec-number>85</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793217">85</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Xie, X.</author><author>Gayen, S.</author><author>Kang, C.</author><author>Yuan, Z.</author><author>Shi, P. Y.</author></authors></contributors><auth-address>State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Science, China.</auth-address><titles><title>Membrane topology and function of dengue virus NS2A protein</title><secondary-title>J Virol</secondary-title><alt-title>Journal of virology</alt-title></titles><periodical><full-title>J Virol</full-title></periodical><pages>4609-22</pages><volume>87</volume><number>8</number><edition>2013/02/15</edition><keywords><keyword>Animals</keyword><keyword>Cell Line</keyword><keyword>Cricetinae</keyword><keyword>Dengue Virus/*physiology</keyword><keyword>Endoplasmic Reticulum/*virology</keyword><keyword>Humans</keyword><keyword>Intracellular Membranes/*metabolism</keyword><keyword>Magnetic Resonance



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Spectroscopy</keyword><keyword>Models, Molecular</keyword><keyword>Protein Conformation</keyword><keyword>Viral Nonstructural Proteins/*chemistry/metabolism</keyword><keyword>*Virus Assembly</keyword></keywords><dates><year>2013</year><pub-dates><date>Apr</date></pub-dates></dates><isbn>1098-5514 (Electronic)0022-538X (Linking)</isbn><accession-num>23408612</accession-num><work-type>Research Support, Non-U.S. Gov't</work-type><urls><related-urls><url>http://www.ncbi.nlm.nih.gov/pubmed/23408612</url></related-urls></urls><custom2>3624351</custom2><electronic-resource-num>10.1128/JVI.02424-12</electronic-resource-num><language>eng</language></record></Cite></EndNote>]. Alternatively, the extracted viral RNA was reversely transcribed and amplified using SuperScript One-Step (RT)-PCR with Platinum Taq (Invitrogen). The RT-PCR products were then gel purified and subjected to Sanger DNA sequencing.

**Replicon Transient Transfection**

After electroporation of replicon RNA into BHK-21 cells, the cells were resuspended in culture medium and seeded in a 12-well plate ($2 \times 10^5$ cells/well). At various time points, the cells were washed once with phosphate-buffered saline (PBS) and lysed using lysis buffer (Promega). The plates containing the lysis buffer were sealed with Parafilm and stored at -80°C. Once samples for all time points had been collected, luciferase signals were measured in a Clarity luminescence microplate reader (BioTek) according to the manufacturer's protocol. The luciferase signals at early time points (2-6 h post-transfection) represent the translation of input viral RNA; and the luciferase signals at later points (24-46 h post-transfection) represent the stage of RNA replication.

**Immunofluorescence assay (IFA)**

Cells were seeded in an 8-well Lab-Tek II chamber slide (ThermoFisher Scientific). At given time points, cells were fixed in 4% paraformaldehyde in PBS for 20 min at room temperature. Afterwards, cells were permeabilized by 0.1% Triton X-100 (Sigma-Aldrich) for 5 min followed by 1-h incubation in blocking buffer containing 1% FBS and 0.05% Tween-20 (Sigma-Aldrich) in PBS. Cells were then incubated with primary antibodies (diluted in the blocking buffer) at room temperature for 1 h. After three PBS washes, cells were incubated with goat anti-mouse or rabbit IgG (1:1000 diluted in blocking buffer) conjugated with Alexa Fluor 488 or 594 (ThermoFisher Scientific) for 1 h in blocking buffer.



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Cells were finally washed by PBS for three times, counter-stained by Hoechst 33342 Solution (ThermoFisher Scientific) and mounted in ProLong® Diamond Antifade Mountant (ThermoFisher Scientific). Epifluorescence images were acquired by Leica DM4000 B system. Confocal fluorescence images were acquired by Zeiss LSM 5 META systems.

**Virus Revertant Selection**

For selecting revertants from G11A-transfected cells, we consecutively passaged the transfected cells for 5 rounds. In brief, after electroporation, the cells were seeded in 96-well plates and incubate at 30°C for 3 days. Afterwards, cells for each well were detached by trypsin and suspended in culture medium. About 20% of the suspended cells were transferred into the well of new 96-well plates and incubated at 37°C for one day. The cells were then incubated at 30°C for two additional days. Again, the confluent cells were detached for next passage. At every passage, the supernatants were collected. After 5 rounds of passaging, IFA was performed to determine the intracellular E protein expression by 4G2 antibody. Plaque assay was performed to examine the amounts of infectious virus particle in the culture fluids. Viral RNA from the P5 supernatants were extracted and used as template for RT-PCR. Sanger sequencing was performed to sequence the entire viral genome.

**Co-immunoprecipitation (Co-IP)**

The NS2A/prM, NS2A/E and NS2A/NS3 interactions were examined by Co-IP analysis according to the protocol as described previously[ ADDIN EN.CITE <EndNote><Cite><Author>Zou</Author><Year>2014</Year><RecNum>113</RecNum><DisplayText>(Zou et al., 2014)</DisplayText><record><rec-number>113</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1561742592">113</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Zou, J.</author><author>Xie, X.</author><author>Lee le, T.</author><author>Chandrasekaran, R.</author><author>Reynaud, A.</author><author>Yap, L.</author><author>Wang, Q. Y.</author><author>Dong, H.</author><author>Kang, C.</author><author>Yuan, Z.</author><author>Lescar, J.</author><author>Shi, P. Y.</author></authors></contributors><auth-address>State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Sciences, Wuhan, China.</auth-address><titles><title>Dimerization of flavivirus NS4B protein</title><secondary-title>J



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Virol</secondary-title></titles><periodical><full-title>J Virol</full-title></periodical><pages>3379-91</pages><volume>88</volume><number>6</number><keywords><keyword>Amino Acid Motifs</keyword><keyword>Dengue/*virology</keyword><keyword>Dengue Virus/chemistry/genetics/*metabolism</keyword><keyword>Dimerization</keyword><keyword>Humans</keyword><keyword>Viral Nonstructural Proteins/*chemistry/genetics/*metabolism</keyword><keyword>West Nile Fever/*virology</keyword><keyword>West Nile virus/chemistry/genetics/*metabolism</keyword></keywords><dates><year>2014</year><pub-dates><date>Mar</date></pub-dates></dates><isbn>1098-5514 (Electronic)0022-538X (Linking)</isbn><accession-num>24390334</accession-num><urls><related-urls><url>https://www.ncbi.nlm.nih.gov/pubmed/24390334</url></related-urls></urls><custom2>PMC3957939</custom2><electronic-resource-num>10.1128/JVI.02782-13</electronic-resource-num></record></Cite></EndNote>]. Various combinations of plasmids were transfected into HEK293T cells in 10-cm dishes using the X-tremeGENE 9 DNA transfection reagent (Roche). At 40-42 h post-transfection, cells were lysed in 1 ml immunoprecipitation (IP) buffer (20 mM Tris, pH 7.5, 100 mM NaCl, 0.5% DDM, and EDTA-free protease inhibitor cocktail [Roche]) with rotation at 4°C for 1 h. Lysates were clarified by centrifugation at 20,000 × g and 4°C for 30 min and subjected to co-IP using protein G-conjugated magnetic beads according to the manufacturer's instructions (Millipore). Briefly, immune complexes were formed at 4°C overnight by mixing 200 µl of cell lysate with 2 µg corresponding antibodies in a 500-µl reaction system containing 400 mM sodium chloride. Subsequently, the complexes were precipitated with protein G-conjugated magnetic beads at 4°C for 1 h with rotation, followed by washing extensively with phosphate-buffered saline (PBS) containing 0.1% Tween-20. Finally, proteins were eluted with 4× lithium dodecyl sulfate (LDS) sample buffer (ThermoFisher Scientific) supplemented with 100 mM 1,4-dithiothreitol (DTT), heated at 70°C for 10 min, and analyzed by Western blot.

**SDS-PAGE and Western Blot**

Protein samples were mixed with 4×LDS sample buffer (ThermoFisher Scientific). After denaturing at 70°C for 10 min, 10-30 µl samples were loaded onto to a 12% or 15% SDS-PAGE gel (Bio-Rad Laboratories). After electrophoresis, proteins were resolved and



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transferred onto a polyvinylidene difluoride (PVDF) membrane using the Bio-Rad TransBlot Turbo Blotting System. Blots were soaked for 1 hour in a blocking buffer containing TBST (10 mM Tris-HCl [pH 7.5], 150 mM NaCl, and 0.1% Tween-20) and 5% skim milk, followed by 1 h incubation with primary antibodies (diluted in blocking buffer). After three washes with TBST buffer, blots were incubated with goat anti-mouse or rabbit antibodies conjugated to HRP (1:10,000 diluted in blocking buffer). After three thorough washes with TBST buffer, blots were incubated with the ECL substrates (GE Healthcare). Chemiluminescence signals were detected in ChemiDoc System (Bio-Rad).

**Transmission Electron Microscopy**

Viral genomic RNAs were electroporated into BHK-21 cells as described above. At 48 h post-transfection, the cells were fixed for at least 1 h in a mixture of 2.5% formaldehyde (prepared from paraformaldehyde powder) and 0.1% glutaraldehyde in 0.05M cacodylate buffer (pH 7.3) to which 0.03% picric acid and 0.03% $CaCl_2$ were added. After washing in 0.1 M cacodylate buffer, the cells were scraped off and processed further as a pellet. The pellets were post-fixed in 1% OsO4 in 0.1M cacodylate buffer (pH 7.3) for 1 h, washed with distilled water and *en bloc* stained with 2% aqueous uranyl acetate for 20 min at 60°C. The pellets were dehydrated in ethanol, processed through propylene oxide and embedded in Poly/Bed 812 (Polysciences, Warrington, PA). Ultrathin sections were cut on Leica EM UC7 ultramicrotome (Leica Microsystems, Buffalo Grove, IL), stained with lead citrate and examined in a Philips 201 transmission electron microscope at 60 kV.

**Expression and purification of recombinant NS2A proteins**

The GST-tagged DENV-2 NGC NS2A and its truncates was expressed and purified by following a previously described protocol [ ADDIN EN.CITE <EndNote><Cite><Author>Zou</Author><Year>2014</Year><RecNum>113</RecNum><DisplayText>(Zou et al., 2014)</DisplayText><record><rec-number>113</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1561742592">113</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Zou, J.</author><author>Xie, X.</author><author>Lee le, T.</author><author>Chandrasekaran, R.</author><author>Reynaud, A.</author><author>Yap, L.</author><author>Wang, Q. Y.</author><author>Dong, H.</author><author>Kang, C.</author><author>Yuan, Z.</author><author>Lescar, J.</author><author>Shi, P.



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Y.</author></authors></contributors><auth-address>State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Sciences, Wuhan, China.</auth-address><titles><title>Dimerization of flavivirus NS4B protein</title><secondary-title>J Virol</secondary-title></titles><periodical><full-title>J Virol</full-title></periodical><pages>3379-91</pages><volume>88</volume><number>6</number><keywords><keyword>Amino Acid Motifs</keyword><keyword>Dengue/*virology</keyword><keyword>Dengue Virus/chemistry/genetics/*metabolism</keyword><keyword>Dimerization</keyword><keyword>Humans</keyword><keyword>Viral Nonstructural Proteins/*chemistry/genetics/*metabolism</keyword><keyword>West Nile Fever/*virology</keyword><keyword>West Nile virus/chemistry/genetics/*metabolism</keyword></keywords><dates><year>2014</year><pub-dates><date>Mar</date></pub-dates></dates><isbn>1098-5514 (Electronic)0022-538X (Linking)</isbn><accession-num>24390334</accession-num><urls><related-urls><url>https://www.ncbi.nlm.nih.gov/pubmed/24390334</url></related-urls></urls><custom2>PMC3957939</custom2><electronic-resource-num>10.1128/JVI.02782-13</electronic-resource-num></record></Cite></EndNote>] with modifications. The pGEX-4T GST-NS2A plasmids were transformed into *E. coli* strain Rosetta™ 2 (pLysS) (Novagen) cells. Bacteria culture was grown in Tefferic broth containing 50 µg/ml Kanamycin and 34 µg/ml Chloramphenicol. When the cells reached an optical density at 600 nm (OD600) of 0.8-1.0, isopropyl β-d-1-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM. After growth for 16 h at 18°C, the cells were harvested, resuspended in a lysis buffer (10 mM $Na_2HPO_4$, 1.8 mM $KH_2PO_4$, 2.7 mM KCl, 400 mM NaCl, 1 mM EDTA, 0.5× Roche protease inhibitor cocktail, pH7.5) and disrupted by sonication using a Digital Sonifier 450 (Branson). The cell debris was removed by centrifugation at 10,000 rpm for 10 min at 4°C. The supernatants were collected and pelleted down by centrifugation at 45,000 rpm for 1 h at 4°C using a Beckman type 70 Ti rotor. The pellets containing the membrane fractions were collected and solubilized in the lysis buffer supplemented with 1.5% n-Dodecyl β-D-maltoside (DDM; Anatrace) at 4°C for 2 hours. Following another centrifugation step at 45,000 rpm for 1 h, the supernatants were collected and loaded onto a GSTrap HP Fast Flow column (GE Healthcare) which was preequilibrated with buffer A (10 mM $Na_2HPO_4$, 1.8 mM $KH_2PO_4$, 2.7 mM KCl, 400 mM NaCl, 2 mM tris(2-carboxyethyl) phosphine [TCEP], 0.05% DDM, pH



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7.5). After extensively wash with buffer B (10 mM $Na_2HPO_4$, 1.8 mM $KH_2PO_4$, 2.7 mM KCl, 1M NaCl, 2 mM tris(2-carboxyethyl) phosphine [TCEP], 0.05% DDM, pH 7.5), the protein was eluted in buffer A supplied with 20 mM glutathione. The fractions containing GST-NS2A proteins were pooled, concentrated, and subjected to gel filtration using a HiLoad Superdex 200 10/300 column (GE Healthcare) in buffer C (10 mM $Na_2HPO_4$, 1.8 mM $KH_2PO_4$, 2.7 mM KCl, 150 mM NaCl, 2 mM tris(2-carboxyethyl) phosphine [TCEP], 0.05% DDM, pH 7.5) with 5% glycerol. The peak fractions containing GST-NS2A proteins were pooled and concentrated before storage at -80°C.

**RNA Pull-down Assay**

The 5'UTR-$C_{66}$-3'UTR-biotin RNA was prepared by ligating a single biotinylated nucleotide to the 3' terminus of the RNA 5'UTR-$C_{66}$-3'UTR using the Pierce™ RNA 3' End Biotinylation Kit (ThermoFisher Scientific) according to the manufacturer's instructions. The RNA 5'UTR-$C_{66}$-3'UTR was *in vitro* transcribed from a PCR template using a T7 MEGAscript kit (ThermoFisher Scientific). The PCR template contained a T7 promoter, followed by a cDNA fragment representing a DENV-2 subgenome with a deletion from nucleotides 163 to 10269 (GenBank accession number AF038403). After *in vitro* transcription, the products were passed twice through the Microspin™ G-25 Columns (GE Healthcare Life Sciences) to remove excessive UTPs. The final products were subjected to phenol-chloroform extraction followed by alcohol precipitation. The purified RNAs were resuspended in nuclease-free water prior to storage at -80°C. Other shorter RNAs 3'UTR, Δpk1, 5'UTR-$C_{66}$, Δpk(1-2), Δpk(1-3), 3'SL, pk(3-4) and pk3 were *in vitro* synthesized from corresponding PCR templates using the same method.

RNA pull-down experiments were performed using the Pierce™ Magnetic RNA-Protein Pull-Down Kit (ThermoFisher Scientific) according to the manufacturer's instructions. Briefly, 2 µg of biotin-labeled RNAs were pre-captured by 50 µl Streptavidin magnetic beads in 1 × RNA capture buffer (10mL, 20mM Tris (pH 7.5), 1M NaCl, 1mM EDTA) at room temperature for 20 min with agitation. A 28-mer biotinylated IRE RNA (5'-UCCUGCUUCAACAGUGCUUGGACGGAAC-3'-biotin) from the Pierce™ RNA 3' End Biotinylation Kit was used as a control. The RNA-bound beads were then mixed with 2 µg purified GST-tagged NS2A proteins in a 100-µl reaction system containing 20 mM Tris (pH 7.5), 200 mM NaCl, 2 mM $MgCl_2$, 0.1% Tween-20 and 15% glycerol and incubated for 45 minutes at 4°C with agitation. For RNA competition assay, the various amounts of



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competitors (tRNA and other shorter RNAs from DENV-2 genome) were immediately added to above the mixtures containing RNA-bound beads and GST-NS2A proteins. After 5 times of wash by 20 mM Tris (pH 7.5), 10 mM NaCl, 0.1% Tween-20 Detergent, the proteins were eluted in 30 µl biotin elution buffer. The eluates were mixed with 4×LDS sample buffer and heated at 70°C for 10 min. 10 µl protein samples were loaded onto a 12% SDS-PAGE gel and followed by detection by Western blot using rabbit anti-GST antibodies.

**Electrophoretic Mobility-Shift Assay**

Electrophoretic mobility-shift assay (EMSA) was performed to estimate the binding affinity of DENV-2 5'UTR-$C_{66}$-3'UTR RNA to GST-NS2A proteins. 100 pM 5'UTR-$C_{66}$-3'UTR-biotin RNA was incubated with a serial concentration of recombinant GST-NS2A protein (0-250 nM) in a 50-µl reaction system containing 50mM Tris-HCl, pH 7.5, 10 mM 2-mercaptoethanol, 2 mM $MgCl_2$, 50 mM KCl at 24°C for 15 min. Afterwards, 10 µl 6x Gel Loading Dye without SDS (New England Biolabs Inc.) were added to stop the reaction. Sample were then loaded onto a 0.4% agarose gel (prepared in 0.5× Tris/Borate/EDTA[TBE]) to resolve the RNA-protein complexes and free RNAs by electrophoresis in 1× TBE buffer (120 V for 30 min) at room temperature. After electrophoresis, RNAs were transferred from the agarose gel to a BrightStar-Plus positively charged nylon membrane (ThermoFisher Scientific) according to the procedure as described in the manual of NorthernMax Gly Kit (ThermoFisher Scientific). The nylon membrane was irradiated with 200 mJ/cm$^2$ of Ultraviolet (UV) light at 254 nm in a CX-2000 UV crosslinker (Analytic Jene, CA) with default settings. The biotinylated RNA was finally detected by the Streptavidin-horseradish peroxidase conjugate by using the Chemiluminescent Detection Module (ThermoFisher Scientific). Chemiluminescent signals were detected in the ChemiDoc™ Imaging Systems (Bio-rad).

**PAR-CLIP**

The Photoactivatable-Ribonucleoside-Enhanced Crosslinking and Immunoprecipitation (PAR-CLIP) was performed according to the protocol as described previously [ ADDIN EN.CITE ADDIN EN.CITE.DATA ] with modifications. In brief, 4-thiouridine labelled DENV-2 3'UTR RNA was prepared by adding a final concentration 0.75 mM of 4-Thio-UTP (TriLink BioTechnologies) to a 20-µl *in vitro* transcription reaction (4-Thio-UTR to UTP



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ration is 1:10) using the MEGAscript T7 transcription Kit (Thermo Fisher Scientific). After 2 hours of reaction at 37°C, the reaction was stopped and the 4-thiouridine labelled RNAs were purified and resuspended in nuclease-free water. 15 µg of 4-thiouridine labelled DENV-2 3'UTR RNA was incubated with 6 µg recombinant DENV-2 GST-NS2A protein in a 100-µl reaction system containing 50 mM Tris-HCl, pH 7.5, 2 mM $MgCl_2$, 50 mM KCl and 5% glycerol at 24°C for 15 min. The RNA-protein mixtures were then irradiated on ice with 150 mJ/cm$^2$ at UV light at 365 nm. An aliquot of the same reaction without UV irradiation was used as the uncross-linked control. Prior to immunoprecipitation, 30 µl of PureProteome™ Protein G Magnetic Beads (MilliporeSigma) were washed twice with 1 ml citrate-phosphate buffer (4.7 g/L citric acid, 9.2 g/L $Na_2HPO_4$, pH 5.0), resuspended in 100 µl citrate-phosphate buffer and followed by in addition of 1 µg of rabbit anti-GST polyclonal antibody (Abcam). The bead-antibody mixtures were incubated at 4°C for 45 min. After two washed with citrate-phosphate buffer, the antibody-bound beads were resuspended in 30 µl of citrate-phosphate buffer. Upon immunoprecipitation, the RNA-protein mixtures were mixed with antibody-bound beads and incubated at 4°C for 1 h. Afterwards, the beads were subjected to three washes in buffer I (50mM HEPES-KOH, pH 7.5, 300 mM KCl, 0.05% IGEPAL CA-630L[Sigma-Aldrich] and 0.5 mM DTT) followed by two washes in buffer II (50 mM HEPES-KOH, pH 7.5, 500 KCl, 0.05% IGEPAL CA-630 and 0.5 mM DTT). The beads were then resuspended in 100 µl Proteinase K digestion buffer (100 mM Tris, pH 7.5, 50 mM NaCl, 10 mM EDTA and 1%SDS) supplemented with proteinase K (New England BioLabs) at a final concentration of 2 mg/ml. The reaction was placed into the Eppendorf ThermoMixer® C and incubated at 37°C for 1 h with agitation at 1100 rpm. Thereafter, the RNA in the reaction was extracted by phenol-chloroform, alcohol-precipitated, and resuspended in 20 µl of nuclease-free water. The extracted RNA was subjected to reversely transcriptionbed and followed by amplification (4 PCR-cycles) using the SuperScript® III One-Step RT-PCR System (ThermoFisher Scientific) with a pair of specific primers P1 (sequence: 5'-ACACTCTTTCCCTACACGA CGCTCTTCCGATCTAAGGCAAAACTAACATGAAAC-3'; underline indicates the sequence identical to the sequence at the 5' end of the DENV-2 3'UTR) and P2 (sequence: 5'-GTGACTGGAGTTCAGACG TGTGCTCTTCCGATCTAGAACCTGTTGATTCAACAGCAC-3'; underline indicates the sequence complimentary to the sequence at the 3' end of the DENV-2 3'UTR) targeting the DENV-2 3'UTR. Afterwards, the RT-PCR product was cleaned up using the QIAquick PCR Purification Kit (Qiagen) and subjected to another round of amplification (15 PCR-



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cycles) using the KAPA HiFi PCR Kit (Kapa Biosystems) with a pair of Illumina adapter/index primers (5′ universal adaptor sequence: 5′-AATGATACGGCGAC CACCGAGATCTACACTCTTTCCCTACACGAC-3′; and 3′ indexing primer sequence: 5′-CAAGCAGAAGACGGCATACGAGATnnnnnnGTGACTGGAGTTCAGACGTGT-3′. The "nnnnnn" here indicates the TruSeq index sequence). Thereafter, the PCR product was cleaned up using the QIAquick PCR Purification Kit (Qiagen) and followed by separation on 2% E-Gel™ EX Agarose Gels (ThermoFisher Scientific). The cDNAs fragment between 500 to 650 bp was excised, purified using the Zymo Research Gel DNA Recovery Kit (Zymo Research) and resuspended in nuclease-free water. The final cDNA libraries were quantified using a Qubit dsDNA Assay Kit (Invitrogen) and sequenced using a MiSeq Reagent Kit v3 (600-cycle) (illumina).

## QUANTIFICATION AND STATISTICAL ANALYSIS

### Sequence Alignment, Image Process and Densitometry Analysis

The amino acid sequences of flavivirus NS2A proteins were download from GenBank. Sequence alignment was performed with default settings using the CLC main workbench software (Qiagen). IFA images processing and cell counting was performed in software ImageJ (National Institutes of Health, NIH). At least four different IFA images were used to estimate the mean and standard deviations of percentage of viral protein staining positive-cells in Figure 2G and S2. Densitometry analysis was performed using Image lab version 6.0 (Bio-Rad Laboratories, Hercules, CA). All numerical data are presented as the mean ± SD (standard deviations). Group comparisons were performed using the unpaired parametric t-test in software Prism 8.0 (GraphPad). *$p<0.05$, significant; **$p<0.01$, very significant; ***$p<0.001$, highly significant; n.s., not significant. Images were assembled using the software Adobe illustrator CC.

### Calculating the equilibrium dissociation constant (Kd)

The intensities of bands representing the RNA/GST-NS2A complexes and free RNA in Figure 5E were quantitated by software ImageJ (NIH). The ratio of bound RNA (in the RNA/GST-NS2A complexes) to total RNA (free and bound RNA) in each lane was calculated. The ratio of bound RNA to total RNA (y-axis) versus the concentration of GST-NS2A protein (x-axis) was plotted using the software Prism 8 (Graphad). The value of Kd



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was obtained using a nonlinear regression with least-squares fitting method, where the Bmax was set as 1. All data points come from three independent experiments.

**Illumina sequencing data process and analysis**

The indexed sequencing reads of both forward and reverse strands were proceeded with the following bioinformatic pipelines: first, an initial quality filtering were conducted with Fastp [ ADDIN EN.CITE <EndNote><Cite><Author>Chen</Author><Year>2018</Year><RecNum>115</RecNum><DisplayText>(Chen et al., 2018)</DisplayText><record><rec-number>115</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1562011493">115</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Chen, S.</author><author>Zhou, Y.</author><author>Chen, Y.</author><author>Gu, J.</author></authors><translated-authors><author>Bioinformatics,</author></translated-authors></contributors><auth-address>Department of Bioinformatics, HaploX Biotechnology, Shenzhen, China.Shenzhen Institutes of Advanced Technology, Chinese Academy of Sciences, Shenzhen, China.</auth-address><titles><title>fastp: an ultra-fast all-in-one FASTQ preprocessor</title><secondary-title>Bioinformatics</secondary-title><alt-title>Bioinformatics</alt-title></titles><periodical><full-title>Bioinformatics</full-title><abbr-1>Bioinformatics</abbr-1></periodical><alt-periodical><full-title>Bioinformatics</full-title><abbr-1>Bioinformatics</abbr-1></alt-periodical><pages>i884-i890</pages><volume>34</volume><number>17</number><dates><year>2018</year><pub-dates><date>Sep 1</date></pub-dates></dates><isbn>1367-4811 (Electronic)1367-4803 (Linking)</isbn><accession-num>30423086</accession-num><urls><related-urls><url>http://www.ncbi.nlm.nih.gov/pubmed/30423086</url></related-urls></urls><custom2>6129281</custom2><electronic-resource-num>10.1093/bioinformatics/bty560</electronic-resource-num><remote-database-provider>2018 Sep 1</remote-database-provider><language>eng</language></record></Cite></EndNote>] to remove low quality ~~bad~~ reads (~~too~~ low ~~in quality~~PHRED score, too short, too many "N", etc.). Then, the illumina sequencing adapter sequence "AGATCGGAAGAGC" was trimmed with *cutadapt* [ ADDIN EN.CITE <EndNote><Cite><Author>Martin</Author><Year>2011</Year><RecNum>116</RecNum

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><DisplayText>(Martin, 2011)</DisplayText><record><rec-number>116</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1562011493">116</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Martin, Marcel</author></authors></contributors><titles><title>Cutadapt removes adapter sequences from high-throughput sequencing reads</title><secondary-title>EMBnet.journal; Vol 17, No 1: Next Generation Sequencing Data AnalysisDO - 10.14806/ej.17.1.200</secondary-title></titles><periodical><full-title>EMBnet.journal; Vol 17, No 1: Next Generation Sequencing Data AnalysisDO - 10.14806/ej.17.1.200</full-title></periodical><keywords><keyword>next generation sequencing, small RNA, microRNA, adapter removal</keyword></keywords><dates><year>2011</year><pub-dates><date>05/02/</date></pub-dates></dates><urls><related-urls><url>https://journal.embnet.org/index.php/embnetjournal/article/view/200/458</url></related-urls></urls></record></Cite></EndNote>] (command line parameters: -b AGATCGGAAGAGC -m 40). A secondary quality filter was applied with FASTX toolkit ([ HYPERLINK "http://hannonlab.cshl.edu/fastx_toolkit/index.html" ]) to remove any reads that contained more than 4% nucleotides with a PHRED score <20 (command line parameters: fastq_quality_filter -Q33 -q 20 -p 96). The remaining reads were aligned to 3'UTR of DENV genome (GenBank accession number AF038403), with Bowtie2 [ ADDIN EN.CITE <EndNote><Cite><Author>Langmead</Author><Year>2012</Year><RecNum>117</RecNum><DisplayText>(Langmead and Salzberg, 2012)</DisplayText><record><rec-number>117</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1562011493">117</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Langmead, Ben</author><author>Salzberg, Steven L.</author></authors></contributors><titles><title>Fast gapped-read alignment with Bowtie 2</title><secondary-title>Nature Methods</secondary-title></titles><periodical><full-title>Nature Methods</full-title></periodical><pages>357</pages><volume>9</volume><dates><year>2012</year><pub-dates><date>03/04/online</date></pub-dates></dates><publisher>Nature Publishing Group, a division of Macmillan Publishers Limited. All Rights Reserved.</publisher><urls><related-urls><url>https://doi.org/10.1038/nmeth.1923</url></related-urls></urls><electronic-



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resource-num>10.1038/nmeth.1923</electronic-resource-num></record></Cite></EndNote>] (command line parameters: --local). Using SAMtools [ ADDIN EN.CITE <EndNote><Cite><Author>Li</Author><RecNum>118</RecNum><DisplayText>(Li et al.)</DisplayText><record><rec-number>118</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1562011493">118</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Li, H.</author><author>Handsaker B Fau - Wysoker, Alec</author><author>Wysoker A Fau - Fennell, Tim</author><author>Fennell T Fau - Ruan, Jue</author><author>Ruan J Fau - Homer, Nils</author><author>Homer N Fau - Marth, Gabor</author><author>Marth G Fau - Abecasis, Goncalo</author><author>Abecasis G Fau - Durbin, Richard</author><author>Durbin, R.</author></authors><translated-authors><author>Bioinformatics,</author></translated-authors></contributors><auth-address>Wellcome Trust Sanger Institute, Wellcome Trust Genome Campus, Cambridge, CB10 1SA, UK, Broad Institute of MIT and Harvard, Cambridge, MA 02141, USA. FAU - Handsaker, Bob</auth-address><titles><title>The Sequence Alignment/Map format and SAMtools</title></titles><number>1367-4811 (Electronic)</number><dates></dates><call-num>1000 Genome Project Data Processing Subgroup</call-num><urls></urls><remote-database-provider>2009 Aug 15</remote-database-provider><language>eng</language></record></Cite></EndNote>], the aligned forward and reverse reads were binarily converted, merged, indexed, and sorted~~, and mathematically noted~~ (command line parameters: view -buS, merge, sort, index, mpileup). This is followed by extracting and calculating the T/U-to-C mutation rate at each genome position and the mutation frequencies of each of the four nucleotides.

## DATA AND SOFTWARE AVAILABILITY

Illumina sequencing data access. The raw data of Illuminae sequencing data can be accessed, queried and downloaded using the following link: .

**Comment [RAL6]:** Did you upload the raw data somewhere? Will probabky have to do this prior to submission. NCBI SRA is the easiest option.



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# Dengue NS2A protein orchestrates virus assembly

Xuping Xie[1]*, Jing Zou[1], Xianwen Zhang[1,2,3], Yiyang Zhou[1], Andrew Routh[1], Vsevolod L. Popov[4], Xinwen Chen[2,3,5], Qingyin Wang[6], Hong-Ping Dong[6], Pei-Yong Shi[1,7,8,9,10]*

[1]Department of Biochemistry & Molecular Biology, University of Texas Medical Branch, Galveston, Texas, USA; [2]State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Sciences, Wuhan, China. [3]University of Chinese Academy of Sciences, Beijing, China. [4]Department of Pathology, University of Texas Medical Branch, Galveston, Texas, USA; [5]Guangzhou Institute of Biomedicine and Health, Chinese Academy of Sciences, Guangzhou, China. [6]Clearbridge Health, Singapore; [7]Institute for Translational Science, University of Texas Medical Branch, Galveston, Texas, USA; [8]Sealy Institute for Vaccine Sciences, University of Texas Medical Branch, Galveston, Texas, USA; [9]Sealy Center for Structural Biology & Molecular Biophysics, University of Texas Medical Branch, Galveston, Texas, USA; [10]Institute for Human Infections & Immunity, University of Texas Medical Branch, Galveston, Texas, USA;

*Correspondence: [ HYPERLINK "mailto:xuxie@UTMB.edu" ] (X.X.), [ HYPERLINK "mailto:peshi@UTMB.edu" ] (P.-Y.S.)

Running title: The role of NS2A protein in dengue virus assembly

**Comment [RAL1]:** Please add affiliation: Sealy Centre for Structural Biology and Molecular Biophysics, University of Texas Medical Branch, Galveston, Texas, USA.



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## SUMMARY

Dengue virus (DENV) is the most important mosquito-borne viral pathogen in humans. DENV assembly requires cleavages of viral C-prM-E polyprotein into three structural proteins (capsid, pre-membrane, and envelope), packaging of viral RNA with C protein into nucleocapsid, and budding of prM/E proteins into virions. No study has addressed how these assembly events are orchestratedThe molecular mechanisms underlying the orchestration of these complex events is unclear. Here we show that DENV-2 NS2A protein recruits viral RNA, structural proteins, and protease to the site of virion assembly site, and coordinates nucleocapsid and virus formation. The last 285 nucleotides of viral 3'UTR serve as a "packaging signal" that binds to a cytosolic loop of NS2A protein. This interaction allows NS2A to recruit nascent RNA from the replication complex to the virion assembly site. Mutations in NS2A that weaken the 3'UTR/NS2A interaction abolish virus assembly. In addition, NS2A interacts with prM, E, and NS3 to recruit C-prM-E polyprotein and NS2B/NS3 protease to the virion assembly site, leading to coordinated C-prM-E cleavage. Mature C protein assembles onto genomic RNA to form nucleocapsid, followed by prM/E envelopment and virion formation. An NS2A mutation that alters the prM/NS2A interaction reduces C-prM cleavage and abolishes virion assembly; second-site mutations in C protein that restore the C-prM cleavage rescue virion assembly. The results support a DENV assembly model that whereby NS2A recruits viral RNA to the virion assembly site where coordinated cleavage of C-prM-E ensues infections virus morphogenesis.




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## INTRODUCTION

The genus *Flavivirus* of the family *Flaviviridae* comprises over 70 viruses, many of which are important human pathogens, including dengue (DENV), yellow fever (YFV), Zika (ZIKV), West Nile (WNV), Japanese encephalitis (JEV), St. Louis encephalitis (SLE), and tick-borne encephalitis (TBEV) viruses [ ADDIN EN.CITE <EndNote><Cite><Author>Pierson</Author><Year>2013</Year><RecNum>3</RecNum><DisplayText>(Pierson and Diamond, 2013)</DisplayText><record><rec-number>3</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1536704974">3</key><key app="ENWeb" db-id="">0</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Pierson, T.C.</author><author>Diamond, M. S.</author></authors></contributors><titles><secondary-title>Flaviviruses p. 747-794. In D.M. Knipe and P.M. Howley (ed), Fields virology, 6th., vol. 1.</secondary-title></titles><periodical><full-title>Flaviviruses p. 747-794. In D.M. Knipe and P.M. Howley (ed), Fields virology, 6th., vol. 1.</full-title></periodical><dates><year>2013</year></dates><urls></urls></record></Cite></EndNote>]. The four serotypes of DENV alone cause about 390 million human infections each year, leading to dengue fever, dengue hemorrhagic fever, and dengue shock syndrome [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. ~~Because of~~Due to the huge socio~~al~~ economic burden, DENV was included in the top 10 global health threats by the World Health Organization (WHO). Moreover, ~~T~~the development of vaccines and therapeutics for DENV has ~~been~~ proven challenging [ ADDIN EN.CITE <EndNote><Cite><Author>Lim</Author><Year>2013</Year><RecNum>58</RecNum><DisplayText>(Lim et al., 2013)</DisplayText><record><rec-number>58</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv"



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64 timestamp="1560793214">58</key></foreign-keys><ref-type name="Journal
65 Article">17</ref-type><contributors><authors><author>Lim, S. P.</author><author>Wang,
66 Q. Y.</author><author>Noble, C. G.</author><author>Chen, Y. L.</author><author>Dong,
67 H.</author><author>Zou, B.</author><author>Yokokawa, F.</author><author>Nilar,
68 S.</author><author>Smith, P.</author><author>Beer, D.</author><author>Lescar,
69 J.</author><author>Shi, P. Y.</author></authors></contributors><auth-address>Novartis
70 Institute for Tropical Diseases, 10 Biopolis Road, 05-01 Chromos, Singapore 138670,
71 Singapore.</auth-address><titles><title>Ten years of dengue drug discovery: Progress
72 and prospects</title><secondary-title>Antiviral Res</secondary-title><alt-title>Antiviral
73 research</alt-title></titles><periodical><full-title>Antiviral Res</full-
74 title></periodical><pages>500-
75 19</pages><volume>100</volume><number>2</number><edition>2013/10/01</edition><
76 dates><year>2013</year><pub-dates><date>Nov</date></pub-
77 dates></dates><isbn>1872-9096 (Electronic)0166-3542
78 (Linking)</isbn><accession-num>24076358</accession-num><urls><related-
79 urls><url>http://www.ncbi.nlm.nih.gov/pubmed/24076358</url></related-
80 urls></urls><electronic-resource-num>10.1016/j.antiviral.2013.09.013</electronic-
81 resource-num><language>eng</language></record></Cite></EndNote>]. The first FDA
82 approved dengue vaccine has a low efficacy of 30-61% [ ADDIN EN.CITE ADDIN
83 EN.CITE.DATA ]. Due to the risk of vaccine-induced severe disease in children who are
84 seronegative at the time of vaccination, WHO recommended only use of the vaccine in
85 areas where the disease is highly prevalent [ ADDIN EN.CITE
86 <EndNote><Cite><Author>Whitehead</Author><Year>2016</Year><RecNum>63</RecN
87 um><DisplayText>(Whitehead, 2016)</DisplayText><record><rec-number>63</rec-
88 number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv"
89 timestamp="1560793215">63</key></foreign-keys><ref-type name="Journal



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Article">17</ref-type><contributors><authors><author>Whitehead, S. S.</author></authors></contributors><auth-address>a Laboratory of Infectious Diseases , NIAID, National Institutes of Health , Bethesda , MD , USA.</auth-address><titles><title>Development of TV003/TV005, a single dose, highly immunogenic live attenuated dengue vaccine; what makes this vaccine different from the Sanofi-Pasteur CYD vaccine?</title><secondary-title>Expert Rev Vaccines</secondary-title></titles><periodical><full-title>Expert Rev Vaccines</full-title></periodical><pages>509-17</pages><volume>15</volume><number>4</number><keywords><keyword>Dengue</keyword><keyword>live attenuated dengue vaccine</keyword><keyword>vaccine attenuation strategies</keyword><keyword>vaccine efficacy</keyword><keyword>vaccine immunogenicity</keyword><keyword>vaccine infectivity</keyword></keywords><dates><year>2016</year></dates><isbn>1744-8395 (Electronic)1476-0584 (Linking)</isbn><accession-num>26559731</accession-num><urls><related-urls><url>http://www.ncbi.nlm.nih.gov/pubmed/26559731</url></related-urls></urls><custom2>PMC4956407</custom2><electronic-resource-num>10.1586/14760584.2016.1115727</electronic-resource-num></record></Cite></EndNote>]. No clinically approved antivirals are available for flavivirus treatment, despite ~~major~~ considerable effort in the past two decades [ ADDIN EN.CITE <EndNote><Cite><Author>Zou</Author><Year>2019</Year><RecNum>64</RecNum><DisplayText>(Zou and Shi, 2019)</DisplayText><record><rec-number>64</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793215">64</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Zou, J.</author><author>Shi, P.



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116 Y.</author></authors></contributors><auth-address>Department of Biochemistry and
117 Molecular Biology, University of Texas Medical Branch, Galveston, TX,
118 USA.Department of Biochemistry and Molecular Biology, University of Texas
119 Medical Branch, Galveston, TX, USA; Department of Phamarcology and Toxicology,
120 University of Texas Medical Branch, Galveston, TX, USA; Sealy Center for Structural
121 Biology and Molecular Biophysics, University of Texas Medical Branch, Galveston, TX,
122 USA; Sealy Institute for Vaccine Sciences, University of Texas Medical Branch, Galveston,
123 TX, USA. Electronic address: peshi@utmb.edu.</auth-address><titles><title>Strategies
124 for Zika drug discovery</title><secondary-title>Curr Opin Virol</secondary-
125 title></titles><periodical><full-title>Curr Opin Virol</full-title></periodical><pages>19-
126 26</pages><volume>35</volume><dates><year>2019</year><pub-dates><date>Mar
127 7</date></pub-dates></dates><isbn>1879-6265 (Electronic)1879-6257
128 (Linking)</isbn><accession-num>30852345</accession-num><urls><related-
129 urls><url>http://www.ncbi.nlm.nih.gov/pubmed/30852345</url></related-
130 urls></urls><electronic-resource-num>10.1016/j.coviro.2019.01.005</electronic-resource-
131 num></record></Cite></EndNote>]. It is therefore critical to study the molecular
132 mechanism of flavivirus replication, which may lead to new approaches for
133 countermeasure development.

134 Flaviviruses haves a plus-sense, single-stranded RNA ~~of about~~approximately
135 11,000 -nucleotides in length. The genomic RNA contains a 5' untranslated region (UTR),
136 a single open-reading-frame (ORF), and a 3' UTR. The ORF ~~translates~~ encodes a
137 polyprotein that is processed by viral and host proteases into three ~~structure~~ structural
138 proteins ({capsid (C), pre-membrane (prM), and envelope (E)}) and seven nonstructural
139 proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5). The structural proteins, ~~,~~
140 together with genomic RNA, ~~,~~ assemble into virions. The nonstructural proteins participate



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in viral RNA replication, virion assembly, and evasion of host immune response. Upon viral translation, a minus-sense RNA is synthesized from the genomic RNA template, forming a double-stranded RNA. The minus-strand RNA then serves as a template to make ~~many~~ multiple copies of plus-sense RNA that are packaged to form progeny virions [ ADDIN EN.CITE <EndNote><Cite><Author>Pierson</Author><Year>2013</Year><RecNum>3</RecNum><DisplayText>(Pierson and Diamond, 2013)</DisplayText><record><rec-number>3</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1536704974">3</key><key app="ENWeb" db-id="">0</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Pierson, T.C.</author><author>Diamond, M. S.</author></authors></contributors><titles><secondary-title>Flaviviruses p. 747-794. In D.M. Knipe and P.M. Howley (ed), Fields virology, 6th., vol. 1.</secondary-title></titles><periodical><full-title>Flaviviruses p. 747-794. In D.M. Knipe and P.M. Howley (ed), Fields virology, 6th., vol. 1.</full-title></periodical><dates><year>2013</year></dates><urls></urls></record></Cite></EndNote>]. Like other RNA viruses, the assembly of flavivirus is coupled with RNA replication [ ADDIN EN.CITE ADDIN EN.CITE.DATA ].

Flavivirus infection induces invaginations of the endoplasmic reticulum (ER) to form clusters of double-membrane vesicles (Ve) enclosed in the vesicle packets (VPs). Electron tomography has revealed the three-dimensional architectures of Ve and VPs formed during DENV, WNV, TBEV, and ZIKV infections [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. Ve houses the viral replication complex (including the double-stranded viral RNA, viral nonstructural proteins, and cellular proteins) and is where RNA synthesis occurs [ ADDIN EN.CITE



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166 <EndNote><Cite><Author>Junjhon</Author><Year>2014</Year><RecNum>71</RecNum
167 ><DisplayText>(Junjhon et al., 2014)</DisplayText><record><rec-number>71</rec-
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170 Article">17</ref-type><contributors><authors><author>Junjhon,
171 J.</author><author>Pennington, J. G.</author><author>Edwards, T.
172 J.</author><author>Perera, R.</author><author>Lanman, J.</author><author>Kuhn, R.
173 J.</author></authors></contributors><auth-address>Markey Center for Structural Biology,
174 Department of Biological Sciences, Purdue University, West Lafayette, Indiana,
175 USA.</auth-address><titles><title>Ultrastructural characterization and three-dimensional
176 architecture of replication sites in dengue virus-infected mosquito cells</title><secondary-
177 title>J Virol</secondary-title></titles><periodical><full-title>J Virol</full-
178 title></periodical><pages>4687-
179 97</pages><volume>88</volume><number>9</number><keywords><keyword>Animals<
180 /keyword><keyword>Cell
181 Line</keyword><keyword>Culicidae/*virology</keyword><keyword>Dengue
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184 Dimensional</keyword><keyword>Intracellular
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187 dates><date>May</date></pub-dates></dates><isbn>1098-5514 (Electronic)0022-
188 538X (Linking)</isbn><accession-num>24522909</accession-num><urls><related-
189 urls><url>http://www.ncbi.nlm.nih.gov/pubmed/24522909</url></related-
190 urls></urls><custom2>PMC3993787</custom2><electronic-resource-
191 num>10.1128/JVI.00118-14</electronic-resource-num></record></Cite></EndNote>].



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Newly synthesized plus-sense RNA is released from the Ve pore to the rough ER for new rounds of RNA translation and replication. Alternatively, the nascent RNA is assembled into virions through encapsidation (with C protein) and envelopment (with prM and E proteins) through budding into the ER lumen. The virion assembly occurs at the ER sites juxtaposed to the VPs. An initial immature virion contains 60 icosahedrally arranged prM/E heterotrimers. As the immature virion transits through the Golgi network, the acidic environment triggers the cleavage of prM by cellular furin protease, resulting in an infectious mature virion [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. Cryo-EM of flavivirus virions exhibit two important structural features: (i) The nucleocapsid core lacks organized structures and (ii) there are no observable connections between nucleocapsid and the prM/E membrane shell, suggesting that the C protein may bind to genomic RNA in a random manner without direct interaction with prM/E proteins [ ADDIN EN.CITE ADDIN EN.CITE.DATA ].

The molecular mechanism of flavivirus assembly remains largely obscure. Besides structural proteins, several nonstructural proteins, such as NS2A, NS2B, and NS3, have been shown to regulate flavivirus assembly. Different amino acids of NS2A protein ~~were~~ have been reported to play distinct roles in flavivirus RNA replication and virion assembly [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. The cleavage between C and prM is tightly modulated by NS2B/NS3 protease and cellular signalase during YFV and Murray Valley encephalitis virus assembly [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. Mutations in the NS3 helicase domain alone affect YFV assembly [ ADDIN EN.CITE <EndNote><Cite><Author>Patkar</Author><Year>2008</Year><RecNum>90</RecNum><DisplayText>(Patkar and Kuhn, 2008a)</DisplayText><record><rec-number>90</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793218">90</key></foreign-keys><ref-type name="Journal



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Article">17</ref-type><contributors><authors><author>Patkar, C. G.</author><author>Kuhn, R. J.</author></authors></contributors><auth-address>Purdue Univ, Dept Biol Sci, W Lafayette, IN 47907 USA</auth-address><titles><title>Yellow fever virus NS3 plays an essential role in virus assembly independent of its known enzymatic functions</title><secondary-title>Journal of Virology</secondary-title><alt-title>J Virol</alt-title></titles><alt-periodical><full-title>J Virol</full-title></alt-periodical><pages>3342-3352</pages><volume>82</volume><number>7</number><keywords><keyword>tick-borne encephalitis</keyword><keyword>flavivirus kunjin ns3</keyword><keyword>west-nile-virus</keyword><keyword>dengue virus</keyword><keyword>nonstructural protein</keyword><keyword>rna replication</keyword><keyword>cdna-clones</keyword><keyword>particles</keyword><keyword>ns2a</keyword><keyword>organization</keyword></keywords><dates><year>2008</year><pub-dates><date>Apr</date></pub-dates></dates><isbn>0022-538X</isbn><accession-num>WOS:000254139800015</accession-num><urls><related-urls><url><Go to ISI>://WOS:000254139800015</url></related-urls></urls><electronic-resource-num>10.1128/Jvi.02447-07</electronic-resource-num><language>English</language></record></Cite></EndNote>]. Although expression of prM/E alone is sufficient to generate empty virus-like particles, it is not known how nucleocapsid is formed and subsequently enveloped with prM/E to form infectious virions. ~~The goal of this study is~~We therefore set out to define the molecular mechanisms that orchestrate~~s~~ the three structural proteins and viral RNA to form nucleocapsid and infectious virus in a highly coordinated manner. In this study, by using~~Using~~ genetic, biochemical, and imaging approaches, we demonstrate that DENV NS2A protein choreographs virion morphogenesis by (i) interacting with viral prM and E proteins, (ii) regulating the processing of C-prM polyprotein by viral protease, and (iii) recruiting



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genomic RNA through binding to the 3'UTR. These results support a model ~~that~~ whereby DENV NS2A recruits nascent viral RNA from the replication complex (housed in Ve) to the virion assembly site (on the ER membrane juxtaposed to VPs), where ~~an~~ coordinated C-prM cleavage facilitates genomic RNA encapsidation with C protein and budding with prM and E proteins into the ER lumen, resulting in progeny virions.



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## RESULTS

**NS2A G11A mutation abolishes virion assembly**. Using DENV-2 as a model, we previously identified a panel of NS2A mutant viruses that were selectively defective in virion assembly without compromising other steps of thean infection cycle [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. Here, we performed four experiments to demonstrate that NS2A mutation G11A of DENV-2 caused a defect in virion assembly. (i) A DENV-2 luciferase replicon was used to examine the effect of NS2A G11A on viral translation and RNA synthesis. Upon transfection into BHK-21 cells, the G11A replicon produced a luciferase profile comparable to the WT replicon (Fig. 1A), suggesting that the mutation does not significantly reduce viral translation and RNA replication. (ii) When full-length viral RNA of DENV-2 was transfected into BHK-21 cells, the NS2A G11A mutant failed to release extracellular viral RNA (Fig. 1B). No infectious virus was detected from the culture medium of G11A RNA-transfected cells (Fig. 1C). (iii) In a *trans* packaging system, WT luciferase replicon could be packaged into pseudo-infectious particles (PIPs) when structural C-prM-E proteins were expressed from Semliki Forest virus replicon (Fig. S1A). The WT reporter replicon PIPs infected Vero cells and generated robust luciferase signals (Fig. S1B). In contrast, no PIPs were produced with the NS2A G11A replicon. (iv) Transmission electron microscopy revealed substantial virus-induced single-membrane vesicles (within the lumen of the rough ER) or double-membrane vesicles (closely associated with cisternae of the rough ER) in both WT and G11A RNA-transfected cells at 48 h post-transfection (Fig. 1D). A lot large number of virus particles appeared in the WT RNA-transfected cells, but no virus particles were detected in the G11A RNA-transfected cells. These results demonstrate that NS2A G11A mutation specifically abolishes virion assembly.



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**NS2A G11A mutation alters the expression and localization of viral structural proteins.** We compared the subcellular localization of viral proteins in BHK-21 cells that were transfected with WT or NS2A G11A genomic RNA. Surprisingly, the NS2A G11A mutation altered the intracellular distribution of three structural proteins. (i) C protein was disseminated in both nucleus and cytoplasm in the WT RNA-transfected cells, as previously reported [ ADDIN EN.CITE <EndNote><Cite><Author>Wang</Author><Year>2004</Year><RecNum>91</RecNum><DisplayText>(Wang et al., 2004)</DisplayText><record><rec-number>91</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793218">91</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Wang, S. H.</author><author>Syu, W. J.</author><author>Hu, S. T.</author></authors></contributors><auth-address>Institute of Microbiology and Immunology, School of Life Science, National Yang-Ming University, Taipei, Taiwan, Republic of China.</auth-address><titles><title>Identification of the homotypic interaction domain of the core protein of dengue virus type 2</title><secondary-title>J Gen Virol</secondary-title><alt-title>The Journal of general virology</alt-title></titles><periodical><full-title>J Gen Virol</full-title><abbr-1>The Journal of general virology</abbr-1></periodical><alt-periodical><full-title>J Gen Virol</full-title><abbr-1>The Journal of general virology</abbr-1></alt-periodical><pages>2307-14</pages><volume>85</volume><number>Pt 8</number><keywords><keyword>Active Transport, Cell Nucleus</keyword><keyword>Dengue Virus/*chemistry/classification</keyword><keyword>Precipitin Tests</keyword><keyword>Viral Core Proteins/*chemistry</keyword></keywords><dates><year>2004</year><pub-dates><date>Aug</date></pub-dates></dates><isbn>0022-1317 (Print)0022-1317



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(Linking)</isbn><accession-num>15269372</accession-num><urls><related-urls><url>http://www.ncbi.nlm.nih.gov/pubmed/15269372</url></related-urls></urls><electronic-resource-num>10.1099/vir.0.80067-0</electronic-resource-num></record></Cite></EndNote>]. However, in the G11A RNA-transfected cells, the C protein was mainly restricted to the cytoplasm with rare nucleus localization (Fig. 1E). (ii) prM protein was located at the ER region in the WT RNA-transfected cells, whereas the prM protein was undetectable in the G11A RNA-transfected cells (Fig. 1F). (iii) E protein spread broadly like an ER web in the WT RNA-transfected cells. In contrast, in the G11A RNA-transfected cells, the E protein formed larger speckles at restricted subcellular areas (Fig. 1G). Compared with the structural proteins, the effect of NS2A G11A on the subcellular expression of viral nonstructural proteins (including NS1, NS3, NS4A, NS4B, and NS5) and dsRNA was not apparent (Fig. 1E-G, *top panels* for NS3, and data not shown).

Comment [RAL2]: ?

The distinct effect of NS2A G11A mutation on subcellular expression between the viral structural and nonstructural proteins were further supported by immunofluorescent co-staining experiments. NS2A G11A altered the co-staining profile of E and NS3 proteins (Fig. S2). In the NS2A G11A RNA-transfected cells, 100% of NS3-expressing cells were E-positive at 24 h post-transfection; however, at 48 and 72 h post-transfection, only 59% and 20% of the NS3-expressing cells were E-positive, respectively (Fig. S2). In the WT RNA-transfected cells, 100% of NS3-expressing cells were E-positive from 24 to 72 h post-transfection (Fig. S2). The results demonstrate that NS2A G11A mutation changes the expression and subcellular localization of viral structural proteins.

**NS2A G11A mutation impairs the cleavage of viral C-prM polyprotein.** To determine the molecular mechanism of the above observations, we examined the processing of viral proteins in the WT and NS2A G11A viral RNA-transfected cells at 48 h



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post-transfection by Western blot. As expected, the amounts of C, NS3, and NS5 proteins from the G11A RNA-transfected cells were 15-26% of those from the WT RNA-transfected cells (Fig. 1H-I), due to the lack of virion production or multiple rounds of infection for the G11A mutant. Notably, the amounts of prM and E proteins were further lower in the G11A RNA-transfected cells (only 1-4% of those in the WT RNA-transfected cells), suggesting an asymmetric degradation in cells. Moreover, the NS2A G11A mutation impaired the cleavage of C-prM to individual C and prM proteins: 90% of C-prM was cleaved to mature C protein in the WT RNA-transfected cells, whereas only 20% of C-prM was processed to C protein in the G11A RNA-transfected cells (Fig. 1H, *compare lanes 1 and 5 in the top panel*). The impaired C-prM cleavage could account for the altered subcellular distribution of viral structural proteins in the NS2A C11A RNA-transfected cells: Restricting the un-cleaved C-prM to the ER membrane, preventing C protein from translocating to the nucleus, and consequently blocking prM/E heterodimer formation and virion budding.

Next, we examined the role of the ubiquitin-proteasome pathway in degrading viral proteins in the G11A RNA-transfected cells. Treatment with proteasome inhibitor MG132 increased the amounts of C, prM, E, and NS5 (but not NS3) by 1-3 folds in the NS2A G11A RNA-transfected cells (Fig. 1H, *compare lanes 5 and 6*). In contrast, in the WT RNA-transfected cells, MG132 slightly reduced the amounts of C, prM, and E to different extents (Fig. 1H, *compare lanes 1 and 2*) because MG132 was known to inhibit flavivirus replication [ ADDIN EN.CITE <EndNote><Cite><Author>Bhuvanakantham</Author><Year>2013</Year><RecNum>108</RecNum><DisplayText>(Bhuvanakantham and Ng, 2013)</DisplayText><record><rec-number>108</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793288">108</key></foreign-keys><ref-type name="Journal



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Article">17</ref-type><contributors><authors><author>Bhuvanakantham, R.</author><author>Ng, M. L.</author></authors></contributors><auth-address>Flavivirology Laboratory, Department of Microbiology, Yong Loo Lin School of Medicine, National University of Singapore, 5 Science Drive 2, Singapore, 117597.</auth-address><titles><title>West Nile virus and dengue virus capsid protein negates the antiviral activity of human Sec3 protein through the proteasome pathway</title><secondary-title>Cell Microbiol</secondary-title></titles><periodical><full-title>Cell Microbiol</full-title></periodical><pages>1688-706</pages><volume>15</volume><number>10</number><keywords><keyword>Antiviral Agents/*antagonists & inhibitors</keyword><keyword>Capsid Proteins/genetics/*metabolism</keyword><keyword>Cell Line</keyword><keyword>DNA Mutational Analysis</keyword><keyword>Dengue Virus/*physiology</keyword><keyword>*Host-Pathogen Interactions</keyword><keyword>Humans</keyword><keyword>Models, Biological</keyword><keyword>Proteasome Endopeptidase Complex/*metabolism</keyword><keyword>Protein Binding</keyword><keyword>Protein Interaction Mapping</keyword><keyword>Proteolysis</keyword><keyword>Vesicular Transport Proteins/*antagonists & inhibitors</keyword><keyword>West Nile virus/*physiology</keyword></keywords><dates><year>2013</year><pub-dates><date>Oct</date></pub-dates></dates><isbn>1462-5822 (Electronic)1462-5814 (Linking)</isbn><accession-num>23522008</accession-num><urls><related-urls><url>https://www.ncbi.nlm.nih.gov/pubmed/23522008</url></related-urls></urls><electronic-resource-num>10.1111/cmi.12143</electronic-resource-num></record></Cite></EndNote>]. Distinct responses to MG132 treatment among different viral proteins were ~~probably~~ likely due to their subcellular distributions and involvement in various biological microenvironments. Nevertheless, the results suggest



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that ubiquitin-proteasome pathway is responsible for viral protein degradation in the NS2A G11A RNA-transfected cells.

**WT NS2A rescues virion assembly of NS2A G11A mutant through restoring C-prM cleavage.** We examined if ~~*trans*~~ supply of WT NS2A in *trans* could restore virion assembly of NS2A G11A RNA and rescue C-prM protein processing (Fig.S3A). Indeed, after transfection into NS2A-EGFP BHK-21 cells ~~that~~ constitutively expressinged WT NS2A protein, the NS2A G11A RNA produced infectious virions that could infect naïve BHK-21 cells, as indicated by E-positive cells (Fig. S3B). However, f~~;~~ further passaging of culture supernatants to new BHK-21 cells did not yield any E-positive cells (Fig. S3C), ~~suggesting~~ indicating that the *trans* packaged virions could only infect cells for a single-round. Remarkably, the *trans* supplied WT NS2A protein restored the C-prM cleavage in the G11A RNA-transfected cells to WT level, exhibiting >89% C protein processing (Fig. S3D, *top panel*). Two prM bands, representing glycosylated and un-glycosylated forms of the protein (see below), were detected in both NS2A G11A and WT RNA-transfected NS2A-EGFP cells (Fig. S3D, *second panel*). The relative abundances of C, prM, and NS1 proteins were similar in the NS2A G11A and WT RNA-transfected cells (Fig. S3D). ~~Because~~ Due to~~of~~ the restored C-prM cleavage, the nucleus localization of C protein and prM-positive cells were detected in the G11A RNA-transfected NS2A-EGFP cells (Fig. S3E). These results demonstrate that WT NS2A can rescue virion assembly of NS2A G11A mutant through the restorationing ~~the~~ of C-prM cleavage.

Next, we examined the effect of NS2A on the glycosylation of prM by co-expressing NS2A and prM in HEK293T cells. As a control, when EGFP was co-expressed, only the glycosylated form of prM was detected (Fig. S3F); after PGNaseF treatment, the prM was completely deglycosylated and migrated ~~as~~ similarly to a glycosylation-knockout mutant N69Q prM (Fig. S3G). In contrast, when NS2A-EGFP ~~were~~ was co-expressed,



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399 both glycosylated and un-glycosylated forms of prM were detected (Fig. S3F), indicating
400 that NS2A suppresses the glycosylation of prM. Furthermore, the G11A mutation seemed
401 to increase the suppression effect of NS2A on prM glycosylation (Fig. 3F). These results
402 suggest that NS2A may interact with prM, and that the G11A mutation may modulate the
403 prM/NS2A interaction.

404 **Capsid R99S mutation can rescue virion assembly of NS2A G11A RNA.** We
405 selected revertants by continuously passaging the NS2A G11A RNA-transfected BHK-21
406 cells for five rounds (Fig. S4A). Culture medium from each passage~~ing~~ (P1-P5) ~~were~~ was
407 tested for ~~emergence~~ presence of infectious virus particle~~s~~s using plaque assay. The P5
408 culture produced infectious viruses with titers of $>10^4$ PFU/ml and plaques smaller than the
409 WT virus (Fig. S4B). In addition, almost all P5 cells were E protein-positive (Fig. S4C).
410 Whole genome sequencing of the P5 viruses from three independent selections
411 consistently showed a consensus C-to-A substitution at nucleotide position 391 of viral
412 genome, resulting in an Arg-to-Ser change at amino acid position 99 of the C protein
413 (C_R99S; Fig. 2A). The original NS2A G11A mutation was retained in all P5 viruses.

414 To validate the role of C_R99S mutation in rescuing virion assembly of NS2A
415 G11A RNA, we engineered C_R99S into a DENV-2 infectious clone [ ADDIN EN.CITE
416 <EndNote><Cite><Author>Xie</Author><Year>2013</Year><RecNum>85</RecNum><Di
417 splayText>(Xie et al., 2013)</DisplayText><record><rec-number>85</rec-
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420 Article">17</ref-type><contributors><authors><author>Xie, X.</author><author>Gayen,
421 S.</author><author>Kang, C.</author><author>Yuan, Z.</author><author>Shi, P.
422 Y.</author></authors></contributors><auth-address>State Key Laboratory of Virology,
423 Wuhan Institute of Virology, Chinese Academy of Science, China.</auth-



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address><titles><title>Membrane topology and function of dengue virus NS2A protein</title><secondary-title>J Virol</secondary-title><alt-title>Journal of virology</alt-title></titles><periodical><full-title>J Virol</full-title></periodical><pages>4609-22</pages><volume>87</volume><number>8</number><edition>2013/02/15</edition><keywords><keyword>Animals</keyword><keyword>Cell Line</keyword><keyword>Cricetinae</keyword><keyword>Dengue Virus/*physiology</keyword><keyword>Endoplasmic Reticulum/*virology</keyword><keyword>Humans</keyword><keyword>Intracellular Membranes/*metabolism</keyword><keyword>Magnetic Resonance Spectroscopy</keyword><keyword>Models, Molecular</keyword><keyword>Protein Conformation</keyword><keyword>Viral Nonstructural Proteins/*chemistry/metabolism</keyword><keyword>*Virus Assembly</keyword></keywords><dates><year>2013</year><pub-dates><date>Apr</date></pub-dates></dates><isbn>1098-5514 (Electronic)0022-538X (Linking)</isbn><accession-num>23408612</accession-num><work-type>Research Support, Non-U.S. Gov't</work-type><urls><related-urls><url>http://www.ncbi.nlm.nih.gov/pubmed/23408612</url></related-urls></urls><custom2>3624351</custom2><electronic-resource-num>10.1128/JVI.02424-12</electronic-resource-num><language>eng</language></record></Cite></EndNote>] in the presence ~~or~~ and absence of NS2A G11A. BHK-21 cells were electroporated with WT, NS2A G11A, C_R99S, or NS2A G11A+C_R99S viral RNA. The transfected cells were analyzed for virus production and C-prM cleavage. In the NS2A G11A+C_R99S RNA-transfected cells, the percentage of E-positive cells increased from 10% to 50% at 24 h and 96 h post-transfection, suggesting the spread of infectious virus (Fig. 2B); indeed, the transfected cells produced infectious viruses with plaques smaller than the WT virus (Fig. 2C); the viral titer increased over time, reaching >$10^5$ PFU/ml at 96-120 h post-transfection



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(Fig. 2D). The prM and NS1 proteins exhibited ER-like distributions in the NS2A G11A+C_R99S RNA-transfected cells, similar to that observed in the WT RNA-transfected cells (Fig. 2E). Viral C protein was detected in both cytoplasm and nucleus in the NS2A G11A+C_R99S RNA-transfected cells (Fig. 2F-G). Furthermore, 90.5% of the C-prM protein was cleaved to mature C and prM in the NS2A G11A+C_R99S RNA-transfected cells (Fig. 2G). As controls, NS2A G11A RNA showed 15.4% of C-prM cleavage and did not produce any infectious virus, whereas C_R99S RNA exhibited 99.9% of prM-C cleavage and produced a WT level of infectious virus (Fig. 2B-H). Taken together, the results demonstrate that C_R99S mutation efficiently restores C-prM cleavage and virion assembly of NS2A G11A RNA.

**Capsid R99S cannot rescue virion assembly of other NS2A mutants.** We tested ~~if~~ whether C_R99S could rescue other NS2A mutants ~~that were~~ previously shown to be defective in virion assembly, including E20A, E100A, and K188A [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. The C_R99S was engineered into those NS2A mutants, resulting in NS2A E20A+C_R99S, NS2A E100A+C_R99S, and NS2A K188A+C_R99S viral RNAs. After transfection into BHK-12 cells, none of the mutant RNAs produced increasing number of E-positive cells from 24 to 96 h (Fig. S5A). No infectious virus was detected in the culture medium from the mutant RNA-transfected cells (Fig. S5B). Viral C protein was detected in both nucleus and cytoplasm of all mutant RNA-transfected cells (Fig. S5C). In agreement with the C nucleus localization, the NS2A single mutant E20A and E100A cleaved 36% and 79% of C-prM, and the addition of C_R99S increased the C-prM cleavage to 72% and 96%, respectively. In contrast, NS2A K188A mutant cleaved 100% of C-prM in the presence or absence of C_R99S (Fig. 5D). These results demonstrate that C_R99S can only rescue the assembly defect of NS2A G11A possibly because different NS2A mutations abolish virion assembly through distinct mechanisms.



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**Capsid R99S shifts the C-prM cleavage site by two amino acids.** The above results prompted us to investigate how C_R99S rescues the virion assembly of NS2A G11A. Fig. S6 depicts the topology of viral polyprotein C-prM-E-NS1-NS2A on the ER membrane. NS2A G11 resides at the first amphipathic helix on the ER lumen side [ ADDIN EN.CITE

<EndNote><Cite><Author>Xie</Author><Year>2013</Year><RecNum>85</RecNum><DisplayText>(Xie et al., 2013)</DisplayText><record><rec-number>85</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793217">85</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Xie, X.</author><author>Gayen, S.</author><author>Kang, C.</author><author>Yuan, Z.</author><author>Shi, P. Y.</author></authors></contributors><auth-address>State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Science, China.</auth-address><titles><title>Membrane topology and function of dengue virus NS2A protein</title><secondary-title>J Virol</secondary-title><alt-title>Journal of virology</alt-title></titles><periodical><full-title>J Virol</full-title></periodical><pages>4609-22</pages><volume>87</volume><number>8</number><edition>2013/02/15</edition><keywords><keyword>Animals</keyword><keyword>Cell Line</keyword><keyword>Cricetinae</keyword><keyword>Dengue Virus/*physiology</keyword><keyword>Endoplasmic Reticulum/*virology</keyword><keyword>Humans</keyword><keyword>Intracellular Membranes/*metabolism</keyword><keyword>Magnetic Resonance Spectroscopy</keyword><keyword>Models, Molecular</keyword><keyword>Protein Conformation</keyword><keyword>Viral Nonstructural Proteins/*chemistry/metabolism</keyword><keyword>*Virus Assembly</keyword></keywords><dates><year>2013</year><pub-



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dates><date>Apr</date></pub-dates></dates><isbn>1098-5514 (Electronic)0022-538X (Linking)</isbn><accession-num>23408612</accession-num><work-type>Research Support, Non-U.S. Gov't</work-type><urls><related-urls><url>http://www.ncbi.nlm.nih.gov/pubmed/23408612</url></related-urls></urls><custom2>3624351</custom2><electronic-resource-num>10.1128/JVI.02424-12</electronic-resource-num><language>eng</language></record></Cite></EndNote>], while C_R99 is the penultimate amino acid of the C protein on the cytosolic side (Fig. S6B). Since flavivirus NS2B/NS3 protease cleaves a substrate with two basic residues (Arg or Lys) at the P1 and P2 sites and a residue with short side chain (Gly, Ser, or Ala) at the P1' site [ ADDIN EN.CITE ADDIN EN.CITE.DATA ], sequence analysis suggests that the C_R99S substitution may shift the capsid C-terminal cleavage by two amino acids from position 100 to 98 (Fig. S6B). Specifically, viral NS2B/NS3 protease cleaves the WT capsid at NRRRR↓TA and the R99S capsid at NRR↓SRTA (↓ denoting cleavage site and residue R99S underlined). To test this hypothesis, we performed a biochemical protease assay using synthetic WT peptide (NILNRRRRTAGMII) and R99S peptide (NILNRRSRTAGMII). After incubating the peptides with recombinant DENV-2 NS2B/NS3 protease, the reaction products were analyzed by liquid chromatography-mass spectrometry (LC-MS). The WT peptide was cleaved into two smaller peptides with molecular weights of 1096.73 Da and 604.77 Da, corresponding to peptides NILNRRRR and TAGMII, respectively (Fig. S6C). In contrast, the R99S peptide was cleaved into two fragments with molecular weights of 784.47 Da and 847.46 Da, corresponding to peptide NILNRR and SRTAGMII, respectively (Fig. S6D). The LC-MS also detected some unknown peptides, probably due to nonspecific degradations of the protease reaction (Fig. S6C-D). Nevertheless, the data indicate that the C_R99S mutation shifts the capsid cleavage site upstream by two amino acids.



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**NS2A G11A enhances prM/NS2A interaction.** Since NS2A G11A alters the processing of C-prM (Fig. 1H) and prM glycosylation (Fig. S3F), we hypothesized that NS2A directly interacts with prM and the G11A mutation affects the prM/NS2A interaction. To test this hypothesis, we co-transfected two plasmids into HEK293T cells. One plasmid expressed the authentic signal peptide anchorC ($C_{14}$) fused with the full-length prM. Another plasmid expressed a fusion protein containing the C-terminal 24 amino acids of the E protein, the N-terminal 50 amino acids of the NS1 protein, the C-terminal 50 amino acids of NS1, full length NS2A, and an EGFP [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]; this plasmid was previously shown to express NS2A-EGFP with a correct topology on the ER membrane [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. Co-immunoprecipitation showed that NS2A-EGFP, but not EGFP, pulled down prM (Fig. 3B, lanes 2 and 3). As controls, mouse IgG did not pull down any NS2A-EGFP or prM (Lane 1), and EGFP antibody did not pull down prM in the absence of NS2A-EGFP (Lane 6). To map the region of prM that binds to NS2A, we expressed the first 130 amino acids of prM [prM(1-130)] that lacked the C-terminal two transmembrane domains (Fig. 3A). Co-immunoprecipitation showed that prM(1-130) could not pull down NS2A-EGFP (Fig. 3B, lane 4), suggesting that (i) NS2A may bind to the transmembrane region of prM or (ii) the transmembrane region of prM may affect the conformation of its ectodomain and, consequently, abolish its interaction with NS2A.

Next, we examined if NS2A mutation G11A affects the prM/NS2A interaction. Co-immunoprecipitation experiments showed that G11A NS2A-EGFP pulled down about 2.5-fold more prM than the WT NS2A-EGFP did (Fig. 4C). Reciprocally, prM pulled down about 1.7-fold more G11A NS2A-EGFP than WT NS2A-EGFP (Fig. 4D). The results indicate that the G11A mutation enhances NS2A binding to prM.



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To further support the prM/NS2A interaction, we used confocal microscopye to examine the co-localization of prM/NS2A in cells. In this case, prM-E were co-expressed with NS2A-EGFP in HEK293T cells. As shown in Fig. 3E, NS2A-EGFP co-localized with prM. The G11A mutation did not change the co-localization of NS2A-EGFP with prM (Fig. 3E). Taken together, these results demonstrate that (i) NS2A interacts with prM and (ii) NS2A G11A significantly enhances the prM/NS2A interaction.

**The G11A mutation does not affect NS2A binding to E or NS3.** We also examined the interactions between NS2A and E or NS3 proteins that were co-expressed in HEK293T cells. For testing the E/NS2A interaction, we fused a GST tag to the C-terminus of NS2A for the co-immunoprecipitation experiment [because the EGFP tag used in NS2A-EGFP in Fig. 3B-D non-specifically interacts with E protein (data not shown)]. As shown in Fig. 3F, both WT and G11A NS2A-GST pulled down E protein at comparable efficiencies. As a control, GST alone did not pull-down E protein. The result suggests that NS2A interacts with E and the G11A mutation does not affect this interaction.

Since flavivirus NS3 is known to participate in virion assembly [ ADDIN EN.CITE ADDIN EN.CITE.DATA ], we also tested if NS2A G11A mutation affects the NS2A/NS3 interaction. When co-expressed in HEK293T cells, GST alone pulled down a trace amount of HA-tagged NS3 (NS3-HA) protein (Fig. S7, lane 1), whereas NS2A-GST pulled down 10-fold more NS3-HA (Lane 2). Equivalent amounts of NS3-HA were pulled down by the WT and G11A NS2A-GST (Compare lanes 2 and 3), suggesting that NS2A G11A does not affect NS2A/NS3 binding.

**Extension of two or three residues at the N-terminus of anchorC efficiently rescues virion assembly of NS2A G11A**. So far, our results showed that NS2A G11A mutation increases its binding to prM on the ER lumen side, leading to impaired C-prM



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574 processing and defective virion assembly. These defects can be rescued by a capsid
575 mutation R99S that shifts the C-terminal cleavage site by two amino acids. As a result of
576 this shift, the N-terminus of transmembrane anchorC is extended by two amino acids on
577 the cytosolic side of ER membrane. These data support a model of coordinated C-prM
578 processing during virion assembly (Fig. 4A) [ ADDIN EN.CITE
579 <EndNote><Cite><Author>Amberg</Author><Year>1999</Year><RecNum>295</RecNu
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585 M.</author><author>Rice, C. M.</author></authors></contributors><auth-
586 address>Department of Molecular Microbiology, Washington University School of
587 Medicine, St. Louis, Missouri 63110-1093, USA.</auth-
588 address><titles><title>Mutagenesis of the NS2B-NS3-mediated cleavage site in the
589 flavivirus capsid protein demonstrates a requirement for coordinated
590 processing</title><secondary-title>J Virol</secondary-title><alt-title>Journal of
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599 Proteins/genetics/*metabolism</keyword></keywords><dates><year>1999</year><pub-



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dates><date>Oct</date></pub-dates></dates><isbn>0022-538X (Print)0022-538X (Linking)</isbn><accession-num>10482557</accession-num><urls><related-urls><url>http://www.ncbi.nlm.nih.gov/pubmed/10482557</url></related-urls></urls><custom2>112824</custom2></record></Cite></EndNote>]. During WT virus assembly, polyprotein C-anchorC-prM was first cleaved at the C-terminus of capsid protein (*i.e.*, R100) by NS2B/NS3 protease, producing mature C and anchorC-prM. The anchorC-prM was then cleaved by a host signalase at the N-terminus of prM, generating anchorC and prM. The cleaved prM coordinates with E protein for virion budding (Fig. 4A, *left panel*). In the presence of NS2A G11A mutation, NS2A binds to prM with a higher affinity, pulling anchorC (embedded in the ER membrane) towards the lumen side. The viral protease cleavage site of the "pulled anchorC" is blocked, possibly by the ER membrane (*middle panel*), leading to unprocessed C-prM. To overcome this defect, the C_R99S mutation creates a new NS2A/NS3 cleavage site at R98 of capsid protein, restoring the C-prM processing and virion assembly of NS2A G11A (*right panel*).

The key of the above model is the blockage of viral protease cleavage site at the C-terminus of capsid protein, which is caused by the NS2A G11A mutation. If the model is correct, an insertion of one or two amino acids to the N-terminus of anchorC should restore the C-prM cleavage and virion assembly in the presence of NS2A G11A mutation (Fig. 4B). To test this hypothesis, we inserted Thr, Thr-Ala, or Ser-Arg into the C-terminus of capsid protein (after R100) in the context of WT or NS2A G11A viral RNA. The inserted amino acids were selected based on the cleavage requirement for viral protease (Fig. 4B). Transfection of each viral RNA into BHK-21 cells yielded increasing number of E-positive cells from day 1 to 4 (Fig. 4C), produced infectious viruses (Fig. 4D-E), displayed cytosolic and nucleus localizations of C protein (Fig. 4F), and exhibited efficient C-prM cleavage (Fig. 4H). In the absence of NS2A G11A, the capsid insertion mutant RNAs behaved



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similarly to the WT RNA. In the presence of NS2A G11A, the capsid single-amino acid insertion (*i.e.*, NS2A G11A+C_T101) partially restored virion assembly ($1.2\times10^3$ PFU/ml; Fig. 4D-E), prM-positive cells (Fig. 4G), and C-prM cleavage (60%; Fig. 4H); while the capsid double-amino acid insertion (*i.e.*, NS2A G11A+C_TA and NS2A G11A+C_SR) almost fully restored virion assembly to the WT level ($2.3\text{-}2.5\times10^6$ PFU/ml; Fig. 4D-E) and C-prM cleavage (92-98%; Fig. 4H). These results demonstrate that addition of one or two amino acids to the N-terminus of anchorC can rescue virion assembly of NS2A G11A.

Since the single-amino acid C_T101 insertion only partially rescued virion assembly of NS2A G11A, we selected revertants for NS2A G11A+C_T101 virus. After continuous passaging of the virus on Vero cells for eight rounds, the P8 viruses were subjected to plaque assay (Fig. S8A-B) and whole-genome sequencing (Fig. S8C). The P8 viruses from all four independent selections produced viral titers (Fig. S8B) that were ≥100-fold higher than the original NS2A G11A+C_T101 virus (Fig. 4E). Among them, selection I P8 virus retained the original NS2A G11A+C_T101 mutations. In addition, an extra mutation R100S was recovered in the capsid protein. Sequence analysis suggests that the R100S could alter the protease cleavage site from the original RRRR↓T to RRR↓ST (R100S underlined) and extends the N-terminus of anchorC by one more amino acid. Selection II P8 virus also retained the original NS2A G11A+C_T101 mutations and accumulated another mutation R99S in the capsid protein. The R99S may change the protease cleavage site to RR↓SRT and extends the N-terminus of anchorC by two more amino acids. In addition, selection II P8 virus contained the R100S mutation in 27% of the viral population. Selection III P8 virus retained the NS2A G11A mutation and showed mixed sequences of R99S and/or R100S. Selection IV P8 virus exhibited WT plaque morphology; sequencing results showed that the NS2A G11A was reverted to WT Gly, but the C_T101 insertion was retained. Collectively, the results again support the conclusion



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650 that extension of the N-terminus of anchorC by two to three residues could efficiently
651 rescue virion assembly of NS2A G11A.

652 **Analysis of NS2A G11A in mosquito cells.** To test if the above findings from
653 mammalian cells could be extended to mosquito host, we analyzed NS2A G11A+capsid
654 mutant RNAs in C6/36 cells. Viral RNAs containing NS2A G11A alone, NS2A
655 G11A+C_T101, NS2A G11A+C_R99S, or NS2A G11A+C_TA were transfected into C6/36
656 cells. The transfected cells were analyzed for capsid protein-positive cells (Fig. S9A),
657 infectious virus production (Fig. S9B-C), extracellular viral RNA release (Fig. S9D), and C-
658 prM cleavage (Fig. S9E). Similar to the results obtained from mammalian cells, NS2A
659 G11A mutant was defective in C-prM processing and virion production in C6/36 cells. The
660 defects could be rescued by the capsid mutations, with the rescuing efficiency of C_TA >
661 C_R99S > C_T101 (Fig. S9). The results indicate that NS2A G11A mutation exerts the
662 same mechanism in abolishing virion assembly in mammalian and mosquito hosts.

663 **NS2A protein binds to 3'UTR RNA**. To further explore the role of DENV-2 NS2A
664 in virion assembly, we examined the ability of NS2A to bind viral genomic RNA. A series of
665 pulldown experiments were performed using biotinylated viral RNAs and NS2A protein
666 (Fig. 5A). A 616-nucleotide viral RNA (5'UTR-$C_{66}$-3'UTR) comprising the 5'UTR, the first
667 66 nucleotides from the C gene, and the 3'UTR regions of DENV-2 genome was *in vitro*
668 synthesized (Fig. S10A). The 3' end of the RNA was ligated to a biotinylated cytidine using
669 T4 RNA ligase, resulting in 5'UTR-$C_{66}$-3'UTR-biotin RNA (Fig. S10B). Two types of GST-
670 tagged NS2A protein were prepared for the pulldown experiment: One type NS2A was
671 expressed from *E. coli*, partially purified, and reconstituted in detergent DDM-containing
672 buffer (Fig. 5B); another type NS2A was from the cell lysates of HEK293T cells transiently
673 expressing the NS2A protein (Fig. 5C). As shown in Fig. 5B-C, the 5'UTR-$C_{66}$-3'UTR-biotin
674 RNA efficiently pulled down GST-tagged NS2A (Lanes 4 and 6), but not GST (Lanes 2



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and 5); in contrast, neither GST nor GST-tagged NS2A proteins was pulled down by a biotinylated non-viral RNA control (Lanes 1 and 3). To further demonstrate the specificity of NS2A/5'UTR-$C_{66}$-3'UTR RNA interaction, we performed an RNA competition experiment. Increasing amounts un-biotinylated 5'UTR-$C_{66}$-3'UTR RNA competed away the NS2A/5'UTR-$C_{66}$-3'UTR-biotin RNA interaction, whereas yeast tRNA did not (Fig. 5D). These results suggest that 5'UTR-$C_{66}$-3'UTR RNA specifically binds to NS2A protein.

We estimated the binding affinity of NS2A/viral RNA interaction. 5'UTR-$C_{66}$-3'UTR-biotin RNA was incubated with increasing amounts of recombinant GST-NS2A protein. The reactions were separated on a native polyacrylamide gel (PAGE), transferred to nylon membrane, and probed with streptavidin-HRP (Fig. 5E). Quantification of free RNA and RNA/protein complex estimated a *Kd* of 35 nM for DENV-2 NS2A biding to viral RNA.

**The last 285 nucleotides of 3'UTR RNA is responsible for binding to NS2A protein**. To map the region of viral RNA responsible for binding to NS2A protein, we performed a competition assay using a set of un-biotinylated RNA fragments from 5'UTR-$C_{66}$-3'UTR (Fig. S10C). The fragments were prepared based on the previously proposed RNA structure [ ADDIN EN.CITE <EndNote><Cite><Author>Villordo</Author><Year>2015</Year><RecNum>109</RecNum><DisplayText>(Villordo et al., 2015)</DisplayText><record><rec-number>109</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1561296709">109</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Villordo, S. M.</author><author>Filomatori, C. V.</author><author>Sanchez-Vargas, I.</author><author>Blair, C. D.</author><author>Gamarnik, A. V.</author></authors></contributors><auth-address>Fundacion Instituto Leloir-CONICET, Buenos Aires, Argentina.Arthropod-borne and Infectious Diseases Laboratory,



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700 Department of Microbiology, Immunology and Pathology, Colorado State University, Fort
701 Collins, Colorado, United States of America.</auth-address><titles><title>Dengue virus
702 RNA structure specialization facilitates host adaptation</title><secondary-title>PLoS
703 Pathog</secondary-title></titles><periodical><full-title>PLoS Pathog</full-
704 title></periodical><pages>e1004604</pages><volume>11</volume><number>1</number
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707 Cultured</keyword><keyword>Cricetinae</keyword><keyword>Culicidae</keyword><key
708 word>Dengue Virus/*genetics</keyword><keyword>Host
709 Specificity/genetics</keyword><keyword>Host-Pathogen
710 Interactions/*genetics</keyword><keyword>Humans</keyword><keyword>Mutation</key
711 word><keyword>*Nucleic Acid Conformation</keyword><keyword>RNA,
712 Viral/*chemistry/genetics</keyword></keywords><dates><year>2015</year><pub-
713 dates><date>Jan</date></pub-dates></dates><isbn>1553-7374 (Electronic)1553-
714 7366 (Linking)</isbn><accession-num>25635835</accession-num><urls><related-
715 urls><url>https://www.ncbi.nlm.nih.gov/pubmed/25635835</url></related-
716 urls></urls><custom2>PMC4311971</custom2><electronic-resource-
717 num>10.1371/journal.ppat.1004604</electronic-resource-
718 num></record></Cite></EndNote>]. As shown in Fig. 5F, 3'UTR, but not 5'UTR-$C_{66}$,
719 competed away the NS2A/5'UTR-$C_{66}$-3'UTR-biotin RNA interaction, indicating that 3'UTR
720 RNA binds to NS2A protein. To further map the region of 3'UTR for NS2A binding, we
721 deleted pseudoknot 1 (Δpk1), pseudoknots 1-2 [Δpk(1-2)], and pseudoknots 1-3 [Δpk(1-3)]
722 from the 3'UTR (Fig. S10C). Only Δpk1 and Δpk(1-2) RNAs, not Δpk(1-3) RNA, could
723 compete away the NS2A/5'UTR-$C_{66}$-3'UTR-biotin RNA interaction (Fig. 5F), suggesting
724 that Δpk(1-2) RNA, representing the last 285 nucleotides of 3'UTR RNA, is required for
725 binding to NS2A protein. This conclusion was further supported by the results that different



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fragments from Δpk(1-2) RNA, including pK(3-4), pk3, and 3'SL, did not compete away the NS2A/5'UTR-$C_{66}$-3'UTR-biotin RNA interaction (Fig. 5F).

Next, we performed a photo-activatable ribonucleoside-enhanced crosslinking (PAR-CL) experiment to define the nucleotides that may interact with NS2A protein (Fig. 5G). The 3'UTR RNA containing [ HYPERLINK "https://en.wikipedia.org/wiki/Photosensitivity" \o "Photosensitivity" ] 4-thiouridine (4-SU) was prepared through *in vitro* transcription in the presence of 4-S-UTP. After RNA/protein complex formation, the 4-thiouridine in the 3'UTR RNA was cross-linked to NS2A protein under UV light (at 365-nm wavelength). The RNA was then subjected to deep sequencing. Due to the crosslinking of 4-thiouridine to protein, a T-to-C mutation was recovered at the RNA sites that interacted with NS2A protein (Figs. 5G and S11). Fig. 5H summarizes the U-to-C mutations in the 3'UTR RNA when complexed with NS2A protein. About 67% mutations occurred within the last 285 nucleotides of 3'UTR. The result further supports the NS2A/3'UTR RNA interaction.

**Comment [RAL3]:** Ratio of 4-S-UTP to UTP?

**Comment [RAL4]:** What would the percentage be if the mutations were randomly spread across the total RNA? i.e. How significant is this enrichment?

**NS2A requires amino acids R94, K95, and K99A for binding to 3'UTR RNA and virion assembly.** To map the region of NS2A required for viral 3'UTR RNA binding, we prepared a panel of purified NS2A truncates with an N-terminal GST tag from *E. coli* (Fig. 6A). The binding efficiencies of NS2A truncates to 5'UTR-$C_{66}$-3'UTR-biotin RNA were compared using a pulldown assay. As shown in Fig. 6B, different NS2A truncates were pulled down by 5'UTR-$C_{66}$-3'UTR-biotin at varied efficiencies. The RNA pulled down NS2A 1-145, 1-168, and 1-218 (full-length) at comparable efficiencies. Compared to the full-length NS2A, the pulldown efficiencies for NS2A 1-101 and 1-71 decreased to 40% and 0%, respectively. These data suggest that amino acids 72-144 are required for NS2A binding to viral RNA.



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According to the membrane topology of DENV-2 NS2A [ ADDIN EN.CITE <EndNote><Cite><Author>Nemesio</Author><Year>2014</Year><RecNum>82</RecNum><DisplayText>(Nemesio and Villalain, 2014)</DisplayText><record><rec-number>82</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793217">82</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Nemesio, H.</author><author>Villalain, J.</author></authors></contributors><auth-address>Molecular and Cellular Biology Institute, Universitas "Miguel Hernandez" , E-03202 Elche-Alicante, Spain.</auth-address><titles><title>Membrane interacting regions of Dengue virus NS2A protein</title><secondary-title>J Phys Chem B</secondary-title></titles><periodical><full-title>J Phys Chem B</full-title></periodical><pages>10142-55</pages><volume>118</volume><number>34</number><keywords><keyword>Calorimetry, Differential Scanning</keyword><keyword>Cell Membrane/*metabolism</keyword><keyword>Fluorescence Polarization</keyword><keyword>*Hydrophobic and Hydrophilic Interactions</keyword><keyword>Peptide Library</keyword><keyword>Spectrophotometry, Infrared</keyword><keyword>Viral Nonstructural Proteins/*chemistry/*metabolism</keyword></keywords><dates><year>2014</year><pub-dates><date>Aug 28</date></pub-dates></dates><isbn>1520-5207 (Electronic)1520-5207 (Linking)</isbn><accession-num>25119664</accession-num><urls><related-urls><url>http://www.ncbi.nlm.nih.gov/pubmed/25119664</url></related-urls></urls><custom2>PMC4148155</custom2><electronic-resource-



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num>10.1021/jp504911r</electronic-resource-num></record></Cite></EndNote>], amino acids 72-144 form two transmembrane domains (72-92 and 101-118), one short cytosolic loop (93-100), and a luminal segment (118-144; Fig. 6A). Since viral RNA resides in the cytoplasm, we examined the cytosolic loop of NS2A for viral RNA binding. The cytosolic loop has three positively charged residues (R94, K95, and K99) that are conserved among the four DENV serotypes (Fig. 6C). To examine the function of these residues, we prepared a panel of recombinant DENV-2 containing Ala substitutions at single (R94A, K95A, or K99A), double (R94A+K95A), or triple residues (R94A+K95A+K99A). Upon transfection to BHK-21 cells, all mutant RNAs generated viral E protein-positive cells at day 1 (Fig. 6D). Except for the triple mutant, other RNAs produced more E-positive cells on day 4 post-transfection. Mutants R94A and K95A generated WT level of infectious viruses and plaque morphology (Fig. 6E-F). Mutants K99A and K94A+K95A generated 22- to 3-fold less viruses than the WT, respectively (Fig. 6F), and exhibited smaller plaques (Fig. 6E). Remarkably, triple mutant R94A+K95A+K99A RNA yielded almost undetectable amount of virus that was 50,000-fold lower than the WT (Fig. 6F). To examine the effect of R94A+K95A+K99A on viral RNA synthesis, we engineered the triple mutations into a luciferase replicon of DENV-2 (Fig. 6G). The R94A+K95A+K99A replicon generated a luciferase profile similar to the WT replicon, indicating that the mutations do not affect viral translation and RNA synthesis. Taken together, the results demonstrate that R94A+K95A+K99A triple mutant is defective in virion assembly.

Next, we examined the effect of R94A+K95A+K99A mutations on NS2A binding to viral RNA. Since the result from Fig. 6B indicates that NS2A fragment 72-144 is required for viral RNA binding, we expressed a short NS2A peptide 64-128 (comprising the first two NS2A transmembrane domains, the cytosolic region 93-100, and the N- and C-terminal luminal tails for correct folding of the peptide on membrane) fused with an N-terminal GST-



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tag (Fig. 6H). As shown in Fig. 6I, peptide 64-128 could be pulled down by 5'UTR-$C_{66}$-3'UTR-biotin RNA (Lane 1). Un-biotinylated 5'UTR-$C_{66}$-3'UTR or 3'UTR RNA (Lanes 2 and 4), but not 5'UTR-$C_{66}$ RNA or yeast tRNA (Lanes 3 and 5), could compete away the NS2A peptide 64-128/5'UTR-$C_{66}$-3'UTR-biotin RNA interaction. The result demonstrates that NS2A peptide 64-128 specifically binds to viral 3'UTR RNA. Importantly, mutant R94A+K95A+K99A reduced the pulldown efficiency of NS2A peptide 64-128 by over 80% (Fig. 6J). Notably, some degraded GST-tag was found to associate with the NS2A truncates in the above pulldown experiments (Figs. 6B I-J), most likely due to the dimerization property of GST_____[ ADDIN EN.CITE <EndNote><Cite><Author>Maru</Author><Year>1996</Year><RecNum>110</RecNum><DisplayText>(Maru et al., 1996)</DisplayText><record><rec-number>110</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1561302524">110</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Maru, Y.</author><author>Afar, D. E.</author><author>Witte, O. N.</author><author>Shibuya, M.</author></authors></contributors><auth-address>Department of Genetics, Institute of Medical Science, University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108, Japan.</auth-address><titles><title>The dimerization property of glutathione S-transferase partially reactivates Bcr-Abl lacking the oligomerization domain</title><secondary-title>J Biol Chem</secondary-title></titles><periodical><full-title>J Biol Chem</full-title></periodical><pages>15353-7</pages><volume>271</volume><number>26</number><keywords><keyword>*Adaptor Proteins, Signal Transducing</keyword><keyword>Animals</keyword><keyword>Bone Marrow Cells</keyword><keyword>Cell Transformation, Neoplastic</keyword><keyword>Cells, Cultured</keyword><keyword>Fusion Proteins, bcr-abl/*metabolism</keyword><keyword>GRB2 Adaptor



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826 Protein</keyword><keyword>Glutathione
827 Transferase/*metabolism</keyword><keyword>Macromolecular
828 Substances</keyword><keyword>Mice</keyword><keyword>Phosphotyrosine/metabolis
829 m</keyword><keyword>Protein Binding</keyword><keyword>Protein-Tyrosine
830 Kinases/metabolism</keyword><keyword>Proteins/metabolism</keyword><keyword>Rat
831 s</keyword><keyword>Recombinant Fusion Proteins</keyword><keyword>Structure-
832 Activity Relationship</keyword></keywords><dates><year>1996</year><pub-
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836 urls></urls><electronic-resource-num>10.1074/jbc.271.26.15353</electronic-resource-
837 num></record></Cite></EndNote>]. Overall, the results indicate that R94, K95, and K99
838 are important for NS2A binding to viral 3'UTR.

839 **DISCUSSION**

840 Flavivirus assembly consists of a tightly controlled cascade of events: export of
841 newly synthesized viral RNA from the replication complex to assembly sites, encapsidation
842 of viral RNA with C protein, and envelopment of nucleocapsid with prM and E proteins.
843 Many host and viral factors (proteins and lipids) participate in this temporal and spatial
844 process [ ADDIN EN.CITE <EndNote><Cite><Author>Apte-
845 Sengupta</Author><Year>2014</Year><RecNum>94</RecNum><DisplayText>(Apte-
846 Sengupta et al., 2014)</DisplayText><record><rec-number>94</rec-number><foreign-
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849 Article">17</ref-type><contributors><authors><author>Apte-Sengupta,



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851 J.</author></authors></contributors><auth-address>Markey Center for Structural Biology,
852 Department of Biological Sciences and Bindley Bioscience Center, Purdue University,
853 West Lafayette, IN, USA.Markey Center for Structural Biology, Department of
854 Biological Sciences and Bindley Bioscience Center, Purdue University, West Lafayette, IN,
855 USA. Electronic address: kuhnr@purdue.edu.</auth-address><titles><title>Coupling of
856 replication and assembly in flaviviruses</title><secondary-title>Curr Opin
857 Virol</secondary-title></titles><periodical><full-title>Curr Opin Virol</full-
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859 42</pages><volume>9</volume><keywords><keyword>Flavivirus/*physiology</keyword>
860 <keyword>Host-Pathogen
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863 Assembly</keyword><keyword>*Virus
864 Replication</keyword></keywords><dates><year>2014</year><pub-
865 dates><date>Dec</date></pub-dates></dates><isbn>1879-6265 (Electronic)1879-
866 6257 (Linking)</isbn><accession-num>25462445</accession-num><urls><related-
867 urls><url>http://www.ncbi.nlm.nih.gov/pubmed/25462445</url></related-
868 urls></urls><custom2>PMC4268268</custom2><electronic-resource-
869 num>10.1016/j.coviro.2014.09.020</electronic-resource-
870 num></record></Cite></EndNote>]. Among them, flaviviral NS2A has emerged as a key
871 player to orchestrate virion assembly, as evidenced by the following findings. (i) In infected
872 cells, there are two distinct NS2A species that are required for viral RNA synthesis and
873 virion assembly. The NS2A species required for RNA synthesis is located at the viral
874 replication complex that could only be formed in *cis*; the other NS2A species required for
875 virus morphogenesis is located at the virion assembly complex that could be formed in *cis*



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or *trans* [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. (ii) The defect in virion assembly caused by DENV-2 and YFV NS2A mutations could be rescued by adaptive mutations in prM, E, NS2B, and NS3 proteins, suggesting that NS2A genetically modulates other viral proteins during virion assembly [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. (iii) Recombinant NS2A of Kunjin virus binds to viral 3'UTR RNA [ ADDIN EN.CITE <EndNote><Cite><Author>Mackenzie</Author><Year>1998</Year><RecNum>81</RecNum><DisplayText>(Mackenzie et al., 1998)</DisplayText><record><rec-number>81</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793217">81</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Mackenzie, J. M.</author><author>Khromykh, A. A.</author><author>Jones, M. K.</author><author>Westaway, E. G.</author></authors></contributors><titles><title>Subcellular localization and some biochemical properties of the flavivirus Kunjin nonstructural proteins NS2A and NS4A</title><secondary-title>Virology</secondary-title></titles><periodical><full-title>Virology</full-title></periodical><pages>203-15</pages><volume>245</volume><number>2</number><keywords><keyword>Animal</keyword><keyword>Cercopithecus aethiops</keyword><keyword>*Flavivirus/ph [Physiology]</keyword><keyword>Immunohistochemistry</keyword><keyword>RNA, Viral/me [Metabolism]</keyword><keyword>Support, Non-U.S. Gov't</keyword><keyword>Vero Cells</keyword><keyword>*Viral Nonstructural Proteins/me [Metabolism]</keyword><keyword>Virus Replication</keyword></keywords><dates><year>1998</year></dates><urls></urls></record></Cite></EndNote>]. These studies indicate that NS2A interacts with two of the three structural proteins (prM and E, not C) and viral RNA. However, how viral RNA and C protein are recruited to form nucleocapsid and how nucleocapsid is enveloped with prM-E



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proteins to form virion remained unknown. The current study aimeds to address these questions.

First, we showed that DENV-2 NS2A interacted with prM to modulate virion assembly. WT NS2A directly bound to prM in the absence of other viral proteins (Fig. 3B). The biological relevance of this interaction was supported by the correlation between virion assembly and prM/NS2A interaction. Specifically, an NS2A mutation G11A, which abolished virion assembly (Fig. 1), enhanced its binding to prM by 1.7-2.5 folds (Fig. 3). Mechanistically, the enhanced interaction between the mutant NS2A and prM "over pulled" the membrane-associated anchorC towards the ER lumen, leading to a blockage of the viral protease cleavage site (most likely by the ER membrane) at the C-anchorC junction. The uncleaved C-prM resulted in a rapid degradation of all three structural proteins (Fig. 1). This is not surprising because the unprocessed C-prM is retained on the ER membrane, misfolded, and degraded via the ubiquitin-proteasome machinery (Fig. 1H). Since prM is a chaperone for E protein [ ADDIN EN.CITE <EndNote><Cite><Author>Lorenz</Author><Year>2002</Year><RecNum>96</RecNum><DisplayText>(Lorenz et al., 2002)</DisplayText><record><rec-number>96</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793218">96</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Lorenz, I. C.</author><author>Allison, S. L.</author><author>Heinz, F. X.</author><author>Helenius, A.</author></authors></contributors><auth-address>Institute of Biochemistry, Swiss Federal Institute of Technology, CH-8093 Zurich, Switzerland.</auth-address><titles><title>Folding and dimerization of tick-borne encephalitis virus envelope proteins prM and E in the endoplasmic reticulum</title><secondary-title>J Virol</secondary-title></titles><periodical><full-title>J



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927 Virol</full-title></periodical><pages>5480-
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942 num>10.1128/jvi.76.11.5480-5491.2002</electronic-resource-
943 num></record></Cite></EndNote>], the degradation of C-prM leads to the degradation of
944 E protein. These results are in agreement with a recent report that ZIKV anchorC plays an
945 important role in the stability of E protein and virion morphogenesis [ ADDIN EN.CITE
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950 Article">17</ref-type><contributors><authors><author>Rana, J.</author><author>Slon
951 Campos, J. L.</author><author>Leccese, G.</author><author>Francolini,
952 M.</author><author>Bestagno, M.</author><author>Poggianella,



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956 Immunology Group, International Centre for Genetic Engineering and Biotechnology,
957 Trieste, Italy.Department of Medical Biotechnology and Translational Medicine,
958 Universita degli Studi di Milano, Milan, Italy.</auth-address><titles><title>Role of Capsid
959 Anchor in the Morphogenesis of Zika Virus</title><secondary-title>J Virol</secondary-
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969 urls></urls><custom2>PMC6206493</custom2><electronic-resource-
970 num>10.1128/JVI.01174-18</electronic-resource-num></record></Cite></EndNote>].

971 Second, we demonstrated that the defect in virion assembly of NS2A G11A could
972 be restored by two different means. Creation of a new viral protease cleavage site by
973 extending the N-terminus of anchorC by two amino acids restored the capsid-anchorC
974 processing and, consequently, restored virion assembly. This conclusion was supported
975 by revertant analysis of the NS2A G11A mutant (Figs. 2 and S4) and amino acid insertion
976 experiment (Fig. 4). The two-amino acid extension exposes the new protease cleavage
977 site to NS2B/NS3. Alternatively, the virion assembly defect of NS2A G11A could be



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978 rescued by *trans* supply of WT NS2A (Fig. S3). Under this condition, the WT NS2A
979 replaced the G11A NS2A to interact with C-prM, restored the C-prM cleavage, and ensued
980 a productive virion morphogenesis. Collectively, our results support a previous model that
981 the two cleavages at C-anchorC-prM junctions are coordinated in a sequential manner
982 during flavivirus assembly. The C-anchorC junction was first cleaved by viral protease on
983 the cytosolic side, followed by the anchorC-prM cleavage by the cellular signalase on the
984 ER lumen side [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. Although expression of
985 flavivirus prM-E alone could form empty virus-like particles, the coordinated C-anchorC-
986 prM processing couples the encapsidation and envelopment events; such coupling is
987 required for the production of infectious virus. This conclusion is also supported by ~~the~~
988 previous reports that separate expression of C and prM-E significantly compromised the
989 assembly efficiency of infectious virions [ ADDIN EN.CITE ADDIN EN.CITE.DATA ].

990 Third, we characterized the specific interaction between DENV-2 NS2A and the
991 3'UTR of viral genome. Although Kunjin virus NS2A was reported to bind to the 3'UTR of
992 viral RNA [ ADDIN EN.CITE
993 <EndNote><Cite><Author>Mackenzie</Author><Year>1998</Year><RecNum>81</RecN
994 um><DisplayText>(Mackenzie et al., 1998)</DisplayText><record><rec-number>81</rec-
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997 Article">17</ref-type><contributors><authors><author>Mackenzie, J.
998 M.</author><author>Khromykh, A. A.</author><author>Jones, M.
999 K.</author><author>Westaway, E.
1000 G.</author></authors></contributors><titles><title>Subcellular localization and some
1001 biochemical properties of the flavivirus Kunjin nonstructural proteins NS2A and
1002 NS4A</title><secondary-title>Virology</secondary-title></titles><periodical><full-



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Replication</keyword></keywords><dates><year>1998</year></dates><urls></urls></re
cord></Cite></EndNote>], the molecular details of the interaction has not been delineated. Using competition assay and PAR-CLIP technique, we mapped that DENV-2 NS2A bound to the last 285 nucleotides of the 3'UTR, including the pk3, pk4, and 3'SL. Interestingly, the pk3, pk3-4, or 3'SL RNA alone did not efficiently compete out the NS2A/3'UTR RNA interaction (Fig. 5F), suggesting that a complex RNA structure in the 3'UTR is required for efficient binding to NS2A protein. Since no RNA “packaging signal” has been identified for flavivirus assembly and the C protein was shown to non-specifically bind to any RNA [ADDIN EN.CITE
<EndNote><Cite><Author>Pong</Author><Year>2011</Year><RecNum>102</RecNum>
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L.</author><author>Huang, Z. S.</author><author>Teoh, P. G.</author><author>Wang,
C. C.</author><author>Wu, H. N.</author></authors></contributors><auth-
address>Institute of Molecular Biology, Academia Sinica, Taipei, Taiwan.</auth-
address><titles><title>RNA binding property and RNA chaperone activity of dengue virus
core protein and other viral RNA-interacting proteins</title><secondary-title>FEBS



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NS2A proteins. The three positively charged R94, K95, and K99 of NS2A are only conserved among the four serotypes of DENV. In addition, DENV-2, YFV, and ZIKV NS2A proteins exhibit different topologies on the ER membrane [ ADDIN EN.CITE ADDIN EN.CITE.DATA ].

Our findings support a general model for flavivirus virion assembly (Fig. 7). In infected cells, NS2A molecules reside in both replication complex (housed in Ve) and virion assembly complex (located on the juxtaposed ER membrane). When plus-sense RNAs are made and released from the replication complex (through the pore on Ve), they are initially destined to new rounds of viral translation and replication on the ER. Once ~~the amounts of~~ viral RNA and proteins have accumulated over certain "thresholds", nascent RNAs are ~~switched for~~directed to virion assembly. At this time, the newly synthesized RNA exits the replication complex and binds to the NS2A located at the assembly site through the 3'UTR RNA. The NS2A molecules at the assembly site also recruits C-prM-E and NS2B/NS3 through binding to prM and NS3, respectively. ~~One~~ Single molecules of NS2A ~~molecule does~~ not ~~have to~~necessarily bind to the 3'UTR RNA, C-prM-E, and NS2B/NS3 at the same time. Rather, t~~T~~hese interactions could be sequential or through ~~different~~ mulitple NS2A molecules that oligomerize together on the ER membrane [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. Next, the C-prM-E polyprotein is sequentially cleaved by the NS2B/NS3 protease and host signalase to produce mature C, prM, and E [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. The C molecules, many of which have been previously made and stored at the nearby lipid droplets [ ADDIN EN.CITE ADDIN EN.CITE.DATA ], bind viral RNA to form nucleocapsid. The processed prM undergoes conformational change and dimerizes with E. The prM/E dimerization and oligomerization dissociate prM from NS2A. When 180 copies of prM/E oligomerize on the ER membrane, envelopment of the nucleocapsid occurs, generating spiky immature virions. During the



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above process, NS3 may also use its helicase activity to transport viral RNA from the replication complex to the assembly site and condense the RNA during encapsidation. Besides NS3, NS2A or other proteins may also contribute to the translocation of viral RNA from the replication complex to the assembly site.

The above model is centered on the multifunctional role of NS2A in flavivirus replication and virion assembly. Our results have functionally demonstrated the molecular mechanism for two important steps during DENV-2 assembly: (i) The binding of 3'UTR RNA to NS2A servers as a "packaging signal" and (ii) in the presence of a viral RNA-NS2A complex, the coordinated cleavage of C-prM-E facilitates the envelopment of nucleocapsid. The proposed model could serve as a framework for future investigations and antiviral development.

**Author contribution**

X.X., J.Z., X.Z., Y.Z., Q.W., H.-P.D. performed experiments and data analysis. X.X., J.Z., X.Z., Y.Z., A.R., V.L.P., X.W., Q.W., H.-P.D, P.-Y.S. designed the experiments and interpreted the results. X.X., P.-Y.S. wrote the manuscript.

**Acknowledgements**

We are grateful to Dr. Mariano A. Garcia-Blanco, Shelton S. Bradrick from University of Texas Medical Branch for helpful discussions during this work. P.-Y.S. was supported by NIH grants AI142759, AI[ HYPERLINK "https://public.era.nih.gov/grantfolder/piAppDetails/genericStatus.do?encryptedParam=vXeG1axw2cY.YZxjFgAisBbII6OqVj4LxDR99WdVyTI-X4FuJbCLt4g." \t "_blank" ], and AI136126, and awards from the Kleberg Foundation, John S. Dunn Foundation, Amon G. Carter



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Foundation, Gilson Longenbaugh Foundation, and Summerfield Robert Foundation. X.X. and J.Z. were awarded with postdoctoral fellowships from the Novartis Institutes for Biomedical Research (NIBR) and the McLaughlin Endowment at University of Texas Medical Branch, respectively.



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## Figure titles and legends

**Figure 1 Characterization of NS2A G11A mutant**. (A) Replicon transient transfection assay. Top panel depicts the DENV-2 luciferase replicon. Bottom panel shows the luciferase activities after transfecting BHK-21 cells with equal amounts (10 μg) of WT or NS2A G11A mutant replicon RNA. (B) Quantification of extracellular viral RNA. Top panel depicts the full-length genome of DENV-2. Equal amounts of WT or NS2A G11A mutant viral RNA (10 μg) were electroporated into BHK-21 cells. At different time post-transfection (p.t.), extracellular viral RNAs were quantified by RT-qPCR. The DENV-2 replicon RNA was used as a control for determining the basal level of viral RNA released from dead cells. Bottom panel shows the CT values of the RT-qPCR results. Data show the means and standard deviations from three independent experiments. (C) Plaque morphology. Culture supernatants from (B) were analyzed for virus production using plaque assay. Only WT, not NS2A G11A mutant (N.D.), produced plaques. Plaques were developed on BHK-21 cells after 5 days of infection. (D) Thin-section transmission electron microscope (TEM) images of viral RNA-transfected BHK-21 cells. At 48 h p.t., cells were fixed for TEM analysis. Ve, virus-induced vesicles; T, membrane tubes; Vi, virus particles; ER, endoplasmic reticulum. Mock transfected cells were included as a control. (E-G) Confocal microscopy analysis of subcellular localizations of C, prM, and E in viral RNA-transfected BHK-21 cells. At 48 h p.t., transfected cells were analyzed by IFA. NS3 staining (red) was used as a control. Nuclei (blue) were stained by DAPI. Scale bar, 10 μm. (H) Viral protein processing in viral RNA-transfected BHK-21 cells. At 42 h p.t., BHK-21 (Mock) or viral RNA-transfected cells were treated with 0.01% DMSO (-) or 10 μM MG132 (+). After 6 h treatment, the cells were analyzed by Western blot. (I) Densitometry analysis. The intensities of bands representing specific viral proteins from DMSO-treated cells in (H) was quantified. The amounts of viral proteins derived from WT RNA-transfected cells were set as 100%. Relative intensities are calculated by normalizing the protein intensities derived from NS2A G11A mutant RNA-transfected cells to those from WT RNA-transfected cells. Data shows the means and standard deviations from three independent experiments.

**Figure 2 Restoration of NS2A G11A virion assembly by capsid R99S mutation.** (A) Revertant mutation. BHK-21 cells were transfected with NS2A G11A viral RNA. The transfected cells were consecutively passaged for five rounds to select for revertant viruses. The P5 virus was sequenced for revertant mutations. Changes in nucleotide



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positions and amino acids are indicated. (B) IFA of viral RNA-transfected cells. WT and mutant viral RNAs were electroporated to BHK-21 cells. The transfected cells were analyzed for E protein expression at 24 and 96 h p.t. by IFA. Scale bar, 100 μm. (C) Plaque morphology. Infectious viruses, derived from the RNA-transfected cells, were analyzed by plaque assay. Plaques were developed on day 5 post-infection on BHK-21 cells. N.D., not detectable. (D) Virus production. The culture fluids from (B) were quantified for infectious viruses. Data represent the means and standard deviations from three independent measurements. Limit of detection (L.O.D.), 10 PFU/ml. (E-F) Subcellular distributions of prM, NS1, and C proteins. prM, NS1 and C proteins were detected by DM1, human IgG anti-NS1 and D2C1 antibodies, respectively. Representative IFA images of viral RNA-transfected cells are shown at 24 h post-transfection. White arrows point to nucleus with C protein speckles. (G) Quantitative analysis of C-positive cells. The C-positive cells with cytoplasm or cytoplasm & nucleus distribution from 4-5 random fields were counted and normalized to the total cell numbers. The averages and standard deviations are shown. (H) Viral protein processing in viral RNA-transfected BHK-21 cells at 48 h post-transfection. The percentages of mature C protein were calculated from the following the formula: [C intensity/(C intensity + C-prM intensity)]×100%.

**Figure 3 DENV-2 NS2A interacts with prM and E proteins**. (A) Membrane topologies of DENV-2 prM, E, and NS2A proteins. The NS2A G11 position is indicated. (B) Co-IP of WT NS2A with prM. HEK293T cells were transfected with one plasmid (encoding prM only) or two plasmids (one encoding leader-NS2A-EGFP and the other encoding full-length or the ectodomain of prM). At 40 h p.t., IP was performed using mouse anti-EGFP or control IgG antibodies. Immunoblot (IB) was performed using rabbit anti-EGFP antibody or anti-prM antibody. (C) Co-IP of WT or G11A NS2A-EGFP with prM. Mouse anti-EGFP antibody was used for IP. Rabbit anti-EGFP or anti-prM antibodies were used for IB. The intensity of prM, co-precipitated with WT NS2A-EGFP, was set as 100%. The relative efficiency of prM co-precipitated with G11A NS2A-EGFP was compared with the WT NS2A-EGFP. Statistical analysis was performed using the unpaired parametric t-test. $*p < 0.05$, significant; $**p < 0.01$, very significant. (D) Reciprocal co-IP of prM with WT or G11A NS2A-EGFP. Rabbit anti-prM antibodies were used for IP. The amounts of co-precipitated WT and G11A NS2A-EGFP were detected using mouse anti-EGFP antibody. prM in eluates were detected by rabbit anti-prM antibody followed by protein G conjugated with HRP. (E) Co-localization analysis of NS2A and prM proteins. BHK-21 cells were co-



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transfected with two plasmids: One encoding the $C_{14}$-prM-E and the other encoding SPG-$C_{16}$-NS2A-EGFP. At 24 h p.t., the cells were examined for prM and NS2A-EGFP co-localization under a confocal microscope. Scale bar, 10 μm. (F) Co-IP of NS2A with E protein. HEK293T cells were co-transfected with two plasmids: One encoding the last 17 amino acids of prM and complete E protein ($M_{17}$-E), and another encoding NS2A with a C-terminal GST tag (NS2A-GST). GST tag alone was used as a negative control. IPs were performed using rabbit anti-GST antibody. E and GST fusion proteins were detected in cell lysates and eluates by mouse anti-E monoclonal antibody (9F12) and anti-GST antibody, respectively. The relative pulldown efficiency was calculated by normalizing the intensity of E co-precipitated with G11A NS2A-GST to the intensity of E co-precipitated with the WT NS2A-GST. The means and standard deviations from three independent experiments are shown. N.S., no significance.

**Figure 4 Two-residue insertion at the capsid-anchorC junction rescues the defect of NS2A G11A**. (A) A model depicting how NS2A modulates C-anchorC-prM cleavage. On the cytosolic side, viral NS2B/NS3 protease cleaves the C-anchorC junction. On the ER lumen side, host signalase cleaves the anchorC-prM junction. Three cleavage scenarios are presented for WT, NS2A G11A, and NS2A G11A+C_R99S constructs. (B) Constructs of insertion mutants of capsid protein. One residue (C_101) or two residues (C_TA or C_SR) were inserted to the C-anchorC junction. The potential cleavage sites for viral NS2B/NS3 protease are indicated. (C) IFA of viral RNA-transfected cells. Viral RNAs containing the NS2A G11A mutation with or without capsid insertions were electroporated into BHK-21 cells. The transfected cells were analyzed for E protein expression on day 1 and 4 by IFA. Scale bar, 100 μm. (D) Plaque morphology. Viruses recovered from (C) were analyzed by plaque assay on BHK-21 cells. (E) Viral titers. Culture supernatants from (C) were measured for viral titers on day 5 post-transfection. (F) Subcellular localization of C protein in viral RNA-transfected cells. White arrows point to nucleus with C protein speckles. Scale bar, 25 μm. (G) Expression of prM and NS1 proteins in viral RNA-transfected cells. Scale bar, 50 μm. (H) C-prM processing in viral RNA-transfected cells. At 48 h p.t., WT or NS2A G11A+C_T101 RNA-transfected cells were analyzed for C, NS1, and β-actin expression by Western blot. The percentages of C-prM to mature C conversion are presented for each construct.

**Figure 5 DENV-2 NS2A protein binds to 3'UTR RNA**. (A) Diagram of pulldown assay using biotinylated RNAs. 3'-end biotinylated RNA binds to streptavidin-conjugated



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magnetic beads. After incubating the bead-bound biotinylated RNA with NS2A protein, the RNA-bound NS2A was enriched and eluted for SDS-PAGE and Western blot analysis. (B) Pulldown analysis using recombinant NS2A protein. N-terminally GST-tagged NS2A protein (GST-NS2A) was expressed in *E. coli* and affinity purified. The 5'UTR-$C_{66}$-3'UTR-biotin RNA was prepared by ligating biotinylated cytidine (bis)phosphate to the 3' end of the 5'UTR-$C_{66}$-3'UTR RNA (containing the 5'UTR, the first 66 nucleotides of C gene, and the 3'UTR). The amounts of GST-NS2A in the input and eluates were analyzed using rabbit anti-GST antibody by Western blot. (C) Pulldown analysis using HEK293T cell-expressed NS2A-GST protein. HEK293T cells were transfected with a plasmid expressing GST or C-terminally GST-tagged NS2A protein (NS2A-GST). At 42 h p.t., cells were harvested, lysed, and used for RNA pull down-assay. The pulldown assay was performed as described in (B). (D) RNA competition assay. Various amounts of unlabeled 5'UTR-$C_{66}$-3'UTR RNA were used to compete the binding of 5'UTR-$C_{66}$-3'UTR-biotin RNA to GST-NS2A protein. tRNA was used as a negative control. (E) Viral RNA/NS2A binding affinity. Electrophoretic mobility-shift assay (EMSA) was used to determine the binding affinity of DENV-2 5'UTR-$C_{66}$-3'UTR-biotin RNA with NS2A protein. 5'UTR-$C_{66}$-3'UTR-biotin RNA was incubated with increasing amounts of GST-NS2A protein. Free and bound RNAs were resolved by native agarose gel electrophoresis. The equilibrium dissociation constant (*Kd*) was determined by nonlinear regression with least-squares fitting method. The coefficient of determination ($R^2$) is shown. Data represent the means and standard deviations from three independent experiments. (F) Mapping the RNA region responsible for NS2A binding. A panel of unlabeled RNA truncates of the 5'UTR-$C_{66}$-3'UTR were used as competitors for the binding of 5'UTR-$C_{66}$-3'UTR-biotin RNA to GST-NS2A protein. The intensity of GST-NS2A (bound to the 5'UTR-$C_{66}$-3'UTR-biotin) without competitor was set as 100%. Relative GST-NS2A intensities were calculated as [(signal in the presence competitor)/(signal in the absence of competitor)]×100%. Statistical analysis was performed using the unpaired parametric t-test. (G) PAR-CLIP workflow [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. See text for detail. (H) PAR-CLIP result summary. Uridines in the 3'UTR that may participate in NS2A-binding are indicated. The dash line indicates the threshold (equal to 5.76, representing the average fold change of mutation rate of all uridines determined by Illumina sequencing). The uridine positions with fold changes of mutation rate over the threshold are indicated in the 3'UTR sequence (GenBank no. AF038403).

**Comment [RAL5]:** Need to explain different between black and blue lines (i.e. that they are replciates).



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**Figure 6 NS2A residues responsible for the NS2A/3'UTR RNA interaction**. (A) Diagram of NS2A truncates used in RNA pulldown assay. All proteins were fused with an N-terminal GST tag, expressed in *E. coli*, and affinity purified. (B) Interactions between NS2A truncates with 5'UTR-$C_{66}$-3'UTR-biotin RNA. The interactions were measured using pulldown experiments. The pulldown efficiency of full-length NS2A (1-218) was set as 100%. Relative efficiency was calculated by comparing the intensity of NS2A truncate with that of the full-length NS2A. (C) Sequence alignment of NS2A among flaviviruses. Amino acids of a cytosolic loop (amino acids 93-101) are presented. The number of viral strains used for the NS2A alignment are indicated in parentheses. Amino acid positions of NS2A are labeled according to DENV-2 strain New Guinea C. Residues R94, K95, and K99 are shaded. The conserved (:) and identical (*) residues are indicated. (D) IFA of BHK-21 cells transfected with viral RNA. WT and mutant viral RNAs were electroporated into BHK-21 cells. The cells were assayed for E protein expression. (E) Plaque morphology. Viruses derived from the transfected cells in (D) were analyzed by plaque assay. (F) Viral titers. Culture supernatants from (D) were measured for virus yields on day 5 post-transfection. (G) Replicon transient transfection assay. NS2A triple mutations (R94A+K95A+K99A) were engineered into a luciferase replicon of DENV-2. Luciferase activities were presented after the WT or mutant replicon RNAs were electroporated into BHK-21 cells. (H) Diagram of N-terminally GST-tagged NS2A peptide 64-128 [GST-NS2A (64-128)]. (I) Interaction of GST-NS2A (64-128) protein with 3'UTR RNA. GST-NS2A (64-128) was expressed in *E. coli* and affinity purified. The pulldown efficiency of GST-NS2A (64-128) by 5'UTR-$C_{66}$-3'UTR-biotin was set as 100%. The binding efficiencies of RNA variants were indicated through competition assay. In the presence of competitor RNAs, the remaining amounts of WT GST-NS2A (64-128)/5'UTR-$C_{66}$-3'UTR-biotin interaction are presented. (J) R94A+K95A+K99A mutations reduce GST-NS2A (64-128) binding to 5'UTR-$C_{66}$-3'UTR-biotin RNA. The R94A+K95A+K99A variant retained only 19.1% of the WT activity.

**Figure 7 A model depicting the role of NS2A in DENV-2 assembly.** Viral replication complex (top) and virion assembly site (bottom) are depicted. DENV-2 NS2A protein recruits viral RNA, three structural proteins, and NS2B/NS3 protease to virion assembly site, where coordinated cleavage of C-prM-E ensues infections virus formation. C molecules accumulated on lipid droplets (LD) could bind viral RNA to form nucleocapsid. See text for details.



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**Reference**

[ ADDIN EN.REFLIST ]



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## STAR METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---|---|---|
| Antibodies | | |
| Mouse IgG Anti-C (D2C1) | [ ADDIN EN.CITE ADDIN EN.CITE.DATA ] | N/A |
| Mouse IgG anti-prM (DM1) | Abcam | Cat# ab41473, RRID:AB_873758 |
| Rabbit IgG anti-prM | GeneTex | GTX128093 |
| Mouse IgG anti-E (4G2) | ATCC | Cat# HB-112, RRID:CVCL_J890 |
| Mouse IgG anti-E (9F12) | [ ADDIN EN.CITE ADDIN EN.CITE.DATA ] | N/A |
| Mouse IgG anti-NS1 | Abcam | Cat# ab41616, RRID:AB_869448 |
| Human IgG anti-NS1 | This study. Generated by Novartis Institute for Tropical Diseases in Singapore | N/A |
| Rabbit IgG anti-NS1 | GeneTex | GTX103346 |
| Rabbit IgG anti-NS3 | GeneTex | GTX124252 |
| Mouse IgG anti-NS5 | GeneTex | GTX629447 |
| Rabbit IgG anti-calnexin | SIGMA-AIDRICH | Cat# C4731, RRID:AB_476845 |
| Mouse IgG anti-EGFP | SIGMA-AIDRICH | Cat# 11814460001, RRID:AB_390913 |
| Moue IgG1 Isotype Control | SIGMA-AIDRICH | Cat# M5284, RRID:AB_1163685 |
| Rabbit IgG anti-EGFP | Abcam | Cat# ab13970, RRID:AB_300798 |
| protein G horseradish peroxidase (HRP) conjugate | Thermo Fisher Scientific | Cat# P21041 |
| Mouse IgG anti-GST | Abcam | Cat# ab19256, RRID:AB_444809 |
| Rabbit IgG anti-GST | SIGMA-AIDRICH | Cat# G7781, RRID:AB_259965 |
| Mouse IgG anti-β-actin | Abcam | Cat# ab8226, RRID:AB_306371 |
| Mouse IgG anti-HA | Abcam | Cat# ab18181, RRID:AB_444303 |
| Goat anti-mouse IgG conjugated with HRP | SIGMA-AIDRICH | Cat# A5278, RRID:AB_258232 |
| Goat anti-rabbit IgG conjugated with HRP | SIGMA-AIDRICH | Cat#AP307P, RRID:AB_92641 |
| Goat anti-mouse IgGs conjugated with Alexa 488 | Thermo Fisher Scientific | Cat#A-1100, RRID:AB_2534069 |
| Goat anti-mouse IgGs conjugated with Alexa 594 | Thermo Fisher Scientific | Cat# A-11005, RRID:AB_2534073 |
| Goat anti-rabbit IgGs conjugated with Alexa 488 | Thermo Fisher Scientific | Cat# A-11008, RRID:AB_143165 |



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| | | |
|---|---|---|
| Goat anti-rabbit IgGs conjugated with Alexa 594 | Thermo Fisher Scientific | Cat# A-11072, RRID:AB_2534116 |
| Goat anti-Human IgG (H+L) cross-adsorbed secondary antibody, Alexa Fluor 594 | Thermo Fisher Scientific | Cat# A-11014, RRID:AB_2534081 |
| Bacterial and Virus Strains | | |
| *E.* coli strain Top 10 | ThermoFisher Scientific | Cat#C404006 |
| *E.* coli strain Rosetta™ 2(DE3) pLysS | MilliporeSigma | Cat#71403-4 |



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|---|---|---|
| DENV-2 strain New Guinea C | [ ADDIN EN.CITE <EndNote><Cite><Author>Xie</Author><Year>2013</Year><RecNum>85</RecNum><DisplayText>(Xie et al., 2013)</DisplayText><record><rec-number>85</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793217">85</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Xie, X.</author><author>Gayen, S.</author><author>Kang, C.</author><author>Yuan, Z.</author><author>Shi, P. Y.</author></authors></contributors><auth-address>State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Science, China.</auth-address><titles><title>Membrane topology and function of dengue virus NS2A protein</title><secondary-title>J Virol</secondary-title><alt-title>Journal of virology</alt-title></titles><periodical><full-title>J Virol</full-title></periodical><pages>4609-22</pages><volume>87</volume><number>8</number><edition>2013/02/15</edition><keywords><keyword>Animals</keyword><keyword>Cell Line</keyword><keyword>Cricetinae</keyword><keyword>Dengue | N.A. |



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| | | |
|---|---|---|
| Biological Samples | | |
| None | | |
| Chemicals, Peptides, and Recombinant Proteins | | |
| Peptide WT (NILNRRRRTAGMII) | GenScript | N/A |
| Peptide C_R99S (NILNRRSRTAGMII) | GenScript | N/A |
| Recombinant protein: GST | This study | N/A |
| Recombinant protein: GST-NS2A | This study | N/A |
| Recombinant protein: GST-NS2A(1-71) | This study | N/A |
| Recombinant protein: GST-NS2A(1-101) | This study | N/A |
| Recombinant protein: GST-NS2A(1-145) | This study | N/A |
| Recombinant protein: GST-NS2A(1-168) | This study | N/A |
| Recombinant protein: GST-NS2A(64-128) | This study | N/A |
| Critical Commercial Assays | | |
| iScript SYBR Green One-Step kit | Bio-Rad | Cat#170-8893 |
| QIAamp viral RNA mini kit | Qiagen | Cat#52906 |
| T7 mMessage mMachine kit | Thermo Fisher Scientific | Cat# AM1344 |
| Renilla Luciferase Assay System | Promega Corporation | Cat# E2810 |
| Pierce™ RNA 3' End Biotinylation Kit | Thermo Fisher Scientific | Cat# 20160 |
| Pierce™ Magnetic RNA-Protein Pull-Down kit | Thermo Fisher Scientific | Cat# 20164 |
| Chemiluminescent Detection Module | Thermo Fisher Scientific | Cat# 89880 |
| | | |
| Deposited Data | | |
| Raw image data for illumina sequencing | | |
| Experimental Models: Cell Lines | | |
| BHK-21 cell | ATCC | Cat#CCL-10, RRID:CVCL_1915 |
| Vero cell | ATCC | Cat#CCL-81, RRID:CVCL_0059 |
| HEK293T cell | ATCC | Cat# CRL-3216, RRID:CVCL_0063 |
| NS2A-EGFP BHK-21 cell | [ ADDIN EN.CITE ADDIN EN.CITE.DATA ] | N/A |
| | | |
| Experimental Models: Organisms/Strains | | |
| N.A. | | |
| Oligonucleotides | | |
| Primer P1(5'-ACACTCTTTCCCTACACGA CGCTCTTCCGATCTAAGGCAAAACTAACATGAA AC-3') | Integrated DNA Technologies (Skokie, Illinois) | N/A |
| Primer P2 (5'-GTGACTGGAGTTCAGACG TGTGCTCTTCCGATCTAGAACCTGTTGATTCAA CAGCAC-3') | Integrated DNA Technologies (Skokie, Illinois) | N/A |



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|---|---|---|
| 5′ universal adaptor (5′-AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGAC-3′) | Illumine (San Diego, CA) | N/A |
| 3′ indexing primer (5′-CAAGCAGAAGACGGCATACGAGATnnnnnnGTGACTGGAGTTCAGACGTGT-3′) | Illumine (San Diego, CA) | N/A |
| Primer NGC 7764V (5′-CGTCGAGAGAAATATGGTCACACC-3′) | Integrated DNA Technologies (Skokie, Illinois) | N/A |
| Primer NGC 7844C (5′CCACAATAGTATGACCAGCCT-3′) | Integrated DNA Technologies (Skokie, Illinois) | N/A |
| Recombinant DNA | | |



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|---|---|---|
| pACYC-NGC FL | [ ADDIN EN.CITE <EndNote><Cite><Author>Xie</Author><Year>2013</Year><RecNum>85</RecNum><DisplayText>(Xie et al., 2013)</DisplayText><record><rec-number>85</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793217">85</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Xie, X.</author><author>Gayen, S.</author><author>Kang, C.</author><author>Yuan, Z.</author><author>Shi, P. Y.</author></authors></contributors><auth-address>State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Science, China.</auth-address><titles><title>Membrane topology and function of dengue virus NS2A protein</title><secondary-title>J Virol</secondary-title><alt-title>Journal of virology</alt-title></titles><periodical><full-title>J Virol</full-title></periodical><pages>4609-22</pages><volume>87</volume><number>8</number><edition>2013/02/15</edition><keywords><keyword>Animals</keyword><keyword>Cell Line</keyword><keyword>Cricetinae</keyword><keyword>Dengue | N/A |



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| | | |
|---|---|---|
| pACYC-NGC replicon | [ ADDIN EN.CITE <EndNote><Cite><Author>Xie</Author><Year>2013</Year><RecNum>85</RecNum><DisplayText>(Xie et al., 2013)</DisplayText><record><rec-number>85</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793217">85</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Xie, X.</author><author>Gayen, S.</author><author>Kang, C.</author><author>Yuan, Z.</author><author>Shi, P. Y.</author></authors></contributors><auth-address>State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Science, China.</auth-address><titles><title>Membrane topology and function of dengue virus NS2A protein</title><secondary-title>J Virol</secondary-title><alt-title>Journal of virology</alt-title></titles><periodical><full-title>J Virol</full-title></periodical><pages>4609-22</pages><volume>87</volume><number>8</number><edition>2013/02/15</edition><keywords><keyword>Animals</keyword><keyword>Cell Line</keyword><keyword>Cricetinae</keyword><keyword>Dengue | N/A |



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| | | |
|---|---|---|
| pACYC-NGC replicon G11A | [ ADDIN EN.CITE ADDIN EN.CITE.DATA ] | N/A |
| pACYC-NGC replicon R94A+K95A+K99A | This study | N/A |
| pACYC-NGC FL C_R99S | This study | N/A |
| pACYC-NGC FL G11A | This study | N/A |
| pACYC-NGC FL G11A+C_R99S | This study | N/A |
| pACYC-NGC FL E20A | This study | N/A |
| pACYC-NGC FL E100A | This study | N/A |
| pACYC-NGC FL K188A | This study | N/A |
| pACYC-NGC FL E20A+C_R99S | This study | N/A |
| pACYC-NGC FL E100A+C_R99S | This study | N/A |
| pACYC-NGC FL K188A+C_R99S | This study | N/A |
| pACYC-NGC FL C_TA | This study | N/A |
| pACYC-NGC FL C_SR | This study | N/A |
| pACYC-NGC FL G11A+C_TA | This study | N/A |
| pACYC-NGC FL G11A+C_SR | This study | N/A |
| pACYC-NGC FL C_T101 | This study | N/A |
| pACYC-NGC FL G11A+C_T101 | This study | N/A |
| pACYC-NGC FL R94A | This study | N/A |
| pACYC-NGC FL K95A | This study | N/A |
| pACYC-NGC FL K99A | This study | N/A |
| pACYC-NGC FL R94A+K95A+K99A | This study | N/A |
| pACYC-NGC FL R94A+K95A | This study | N/A |



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| | | |
|---|---|---|
| SFV-DENV2 CprME | [ ADDIN EN.CITE <EndNote><Cite><Author>Qing</Author><Year>2010</Year><RecNum>105</RecNum><DisplayText>(Qing et al., 2010)</DisplayText><record><rec-number>105</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793220">105</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Qing, M.</author><author>Liu, W.</author><author>Yuan, Z.</author><author>Gu, F.</author><author>Shi, P. Y.</author></authors></contributors><auth-address>Novartis Institute for Tropical Diseases, 10, Biopolis Road, #05-01 Chromos, Singapore, Singapore.</auth-address><titles><title>A high-throughput assay using dengue-1 virus-like particles for drug discovery</title><secondary-title>Antiviral Res</secondary-title></titles><periodical><full-title>Antiviral Res</full-title></periodical><pages>163-71</pages><volume>86</volume><number>2</number><dates><year>2010</year><pub-dates><date>May</date></pub- [ PAGE * MERGEFORMAT ] dates></dates><accession-num>20153777</accession-num><urls><related- | N/A |

| | | |
|---|---|---|
| pXJ-SPG-$C_{16}$-NS2A-EGFP | This study | N/A |
| pXJ-SPG-$C_{16}$-NS2A(G11A)-EGFP | This study | N/A |
| pXJ-EGFP | [ ADDIN EN.CITE ADDIN EN.CITE.DATA ] | N/A |
| pXJ-$C_{14}$-prM | This study | N/A |
| pXJ-$C_{14}$-prM(1-130) | This study | N/A |
| pXJ-$C_{14}$-prM (N69Q) | This study | N/A |
| pXJ-SPG-$C_{16}$-NS2A-GST | This study | N/A |
| pXJ-SPG-$C_{16}$-NS2A(G11A)-GST | This study | N/A |
| pXJ-GST | This study | N/A |
| pXJ-HA-NS3 | This study | N/A |
| pXJ-$M_{17}$-E | This study | N/A |
| pGEX-4T-1 | GE Healthcare Life Sciences | Cat# 28954549 |
| pGEX-4T-GST-NS2A | This study | N/A |
| pGEX-4T-GST-NS2A (1-71) | This study | N/A |
| pGEX-4T-GST-NS2A (1-101) | This study | N/A |
| pGEX-4T-GST-NS2A (1-145) | This study | N/A |
| pGEX-4T-GST-NS2A (1-168) | This study | N/A |
| pGEX-4T-GST-NS2A (64-128) | This study | N/A |
| pGEX-4T-GST-NS2A (64-128) R94A+K95A+K99A | This study | N/A |
| | | |
| Software and Algorithms | | |
| Illustrator CC | Adobe | [ HYPERLINK "https://www.adobe.com/products/illustrator.html" ] |
| ImageJ | NIH | [ HYPERLINK "https://fiji.sc/" ] |
| CLC main workbench | Qiagen | [ HYPERLINK "https://www.qiagenbioinformatics.com/products/clc-main-workbench/" ] |
| Prism 8.0 software | GraphPad | [ HYPERLINK "https://www.graphpad.com/scientific-software/prism/" ] |
| | | |

## Contact for Reagent and Resource Sharing

Further information and requests for resources and reagents should be directed to and will be fulfilled by Lead Contact, Dr. Pei-Yong Shi (peshi@utmb.edu)

## Experimental Model and Subject Details



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**Cell Lines**

The Baby hamster kidney fibroblast (BHK-21) cells, African green monkey kidney epithelial (Vero) cells were purchased from the American Type Culture Collection (ATCC, Bethesda, MD) and maintained in a high-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (HyClone Laboratories, South Logan, UT) and 1% penicillin/streptomycin (P/S). The NS2A-EGFP BHK-21 cells constitutively expressing the C-terminally EGFP-tagged NS2A protein was maintained in the high-glucose DMEM medium supplemented with 10%FBS, 1% P/S and 1 mg/ml Geneticin. HEK293T cells were grown in low-glucose DMEM (Invitrogen) containing 10% FBS and 1% penicillin-streptomycin. All mammalian cell lines were grown at 37°C with 5% $CO_2$. Mosquito C6/36 cells were grown in RPMI 1640 medium (Invitrogen) with 10% FBS and 1% penicillin-streptomycin at 28°C without $CO_2$. All culture medium and antibiotics were purchased from ThermoFisher Scientific (Waltham, MA). All cell lines were tested negative for mycoplasma.

**Pseudo-infectious Particle**

Pseudo-infectious particles were produced using the a previously described protocol [ ADDIN EN.CITE <EndNote><Cite><Author>Qing</Author><Year>2010</Year><RecNum>114</RecNum><DisplayText>(Qing et al., 2010)</DisplayText><record><rec-number>114</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1561745637">114</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Qing, M.</author><author>Liu, W.</author><author>Yuan, Z.</author><author>Gu, F.</author><author>Shi, P. Y.</author></authors></contributors><auth-address>Novartis Institute for Tropical Diseases, 10, Biopolis Road, #05-01 Chromos, Singapore, Singapore.</auth-address><titles><title>A high-throughput assay using dengue-1 virus-like particles for drug discovery</title><secondary-title>Antiviral Res</secondary-title></titles><periodical><full-title>Antiviral Res</full-title></periodical><pages>163-71</pages><volume>86</volume><number>2</number><keywords><keyword>Animals</keyword><keyword>Antiviral Agents/*isolation & purification/*pharmacology</keyword><keyword>Biological Assay/methods</keyword><keyword>Cell Line</keyword><keyword>Cercopithecus



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aethiops</keyword><keyword>Cricetinae</keyword><keyword>Dengue Virus/*drug effects/genetics</keyword><keyword>Drug Discovery/*methods</keyword><keyword>Drug Evaluation, Preclinical/*methods</keyword><keyword>Electroporation</keyword><keyword>Genes, Reporter</keyword><keyword>Luciferases, Renilla/genetics/metabolism</keyword><keyword>Macaca mulatta</keyword><keyword>Viral Proteins/genetics/metabolism</keyword><keyword>Virosomes/*drug effects/genetics</keyword></keywords><dates><year>2010</year><pub-dates><date>May</date></pub-dates></dates><isbn>1872-9096 (Electronic)0166-3542 (Linking)</isbn><accession-num>20153777</accession-num><urls><related-urls><url>https://www.ncbi.nlm.nih.gov/pubmed/20153777</url></related-urls></urls><electronic-resource-num>10.1016/j.antiviral.2010.02.313</electronic-resource-num></record></Cite></EndNote>] with several modifications. 10 µg of replicon RNA was electroporated into BHK-21 cells as described above. After transfection, the cells were maintained at 37°C with 5% $CO_2$ for 24 h. Afterwards, cells were harvested and electroporated with 10 µg of a helper replicon RNA SFV-DENV2 CprME (replicon derived from Semliki forest virus that express the DENV-2 encoding the CprME). After the second electroporation, cells were cultured at 30°C with 5% $CO_2$. At 20, 28 and 48 h after the second electroporation, culture medium was collected and stored at -80 °C. Afterwards, the collected medium was used ton infection naïve Vero cells in 96-well plates. At 48 h post-infection, cells were lysed, and luciferase signals were measured using the Renilla luciferase assay system (Promega) in the Clarity luminescence microplate reader (BioTek) according to the manufacturer's protocol.

**METHOD DETAILS**

**Plasmid Construction**

The full-length cDNA infectious clone of DENV-2 strain New Guinea C (NGC), pACYC-NGC FL, and its derived replicon (pACYC-NGC replicon) clone [ ADDIN EN.CITE <EndNote><Cite><Author>Xie</Author><Year>2013</Year><RecNum>85</RecNum><DisplayText>(Xie et al., 2013)</DisplayText><record><rec-number>85</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793217">85</key></foreign-keys><ref-type name="Journal



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Article">17</ref-type><contributors><authors><author>Xie, X.</author><author>Gayen, S.</author><author>Kang, C.</author><author>Yuan, Z.</author><author>Shi, P. Y.</author></authors></contributors><auth-address>State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Science, China.</auth-address><titles><title>Membrane topology and function of dengue virus NS2A protein</title><secondary-title>J Virol</secondary-title><alt-title>Journal of virology</alt-title></titles><periodical><full-title>J Virol</full-title></periodical><pages>4609-22</pages><volume>87</volume><number>8</number><edition>2013/02/15</edition><keywords><keyword>Animals</keyword><keyword>Cell Line</keyword><keyword>Cricetinae</keyword><keyword>Dengue Virus/*physiology</keyword><keyword>Endoplasmic Reticulum/*virology</keyword><keyword>Humans</keyword><keyword>Intracellular Membranes/*metabolism</keyword><keyword>Magnetic Resonance Spectroscopy</keyword><keyword>Models, Molecular</keyword><keyword>Protein Conformation</keyword><keyword>Viral Nonstructural Proteins/*chemistry/metabolism</keyword><keyword>*Virus Assembly</keyword></keywords><dates><year>2013</year><pub-dates><date>Apr</date></pub-dates></dates><isbn>1098-5514 (Electronic)0022-538X (Linking)</isbn><accession-num>23408612</accession-num><work-type>Research Support, Non-U.S. Gov't</work-type><urls><related-urls><url>http://www.ncbi.nlm.nih.gov/pubmed/23408612</url></related-urls></urls><custom2>3624351</custom2><electronic-resource-num>10.1128/JVI.02424-12</electronic-resource-num><language>eng</language></record></Cite></EndNote>]
were used as the backbones for mutagenesis study. The replicon was constructed by replacing most of the structural genes (C, prM and E) with a gene cassette (Rluc2A) encoding the Renilla luciferase and a 2A peptide from Foot-and-mouth disease virus. The first 38 amino acids of C protein and the last 31 amino acids of E protein were retained in the replicon. Standard overlap PCR was performed to introduce the mutations in C and NS2A genes. The DNA fragments containing the mutations in C gene were cloned into pACYC-NGC FL clone through restriction enzyme sites NotI (upstream of the T7 promoter and 5'UTR of DENV-2) and SphI (at the position of 1384 in DENV-2 genome). The DNA fragments containing mutations in NS2A gene were cloned into pACYC-NGC FL or replicon through restriction enzyme sites and NheI (at the position of 2544 in DENV-2 genome) and SacI (at the position of 4342 in the DENV-2 genome).



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A mammalian expression vector, pXJ [ ADDIN EN.CITE <EndNote><Cite><Author>Xie</Author><Year>2013</Year><RecNum>85</RecNum><DisplayText>(Xie et al., 2013)</DisplayText><record><rec-number>85</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793217">85</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Xie, X.</author><author>Gayen, S.</author><author>Kang, C.</author><author>Yuan, Z.</author><author>Shi, P. Y.</author></authors></contributors><auth-address>State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Science, China.</auth-address><titles><title>Membrane topology and function of dengue virus NS2A protein</title><secondary-title>J Virol</secondary-title><alt-title>Journal of virology</alt-title></titles><periodical><full-title>J Virol</full-title></periodical><pages>4609-22</pages><volume>87</volume><number>8</number><edition>2013/02/15</edition><keywords><keyword>Animals</keyword><keyword>Cell Line</keyword><keyword>Cricetinae</keyword><keyword>Dengue Virus/*physiology</keyword><keyword>Endoplasmic Reticulum/*virology</keyword><keyword>Humans</keyword><keyword>Intracellular Membranes/*metabolism</keyword><keyword>Magnetic Resonance Spectroscopy</keyword><keyword>Models, Molecular</keyword><keyword>Protein Conformation</keyword><keyword>Viral Nonstructural Proteins/*chemistry/metabolism</keyword><keyword>*Virus Assembly</keyword></keywords><dates><year>2013</year><pub-dates><date>Apr</date></pub-dates></dates><isbn>1098-5514 (Electronic)0022-538X (Linking)</isbn><accession-num>23408612</accession-num><work-type>Research Support, Non-U.S. Gov't</work-type><urls><related-urls><url>http://www.ncbi.nlm.nih.gov/pubmed/23408612</url></related-urls></urls><custom2>3624351</custom2><electronic-resource-num>10.1128/JVI.02424-12</electronic-resource-num><language>eng</language></record></Cite></EndNote>], driven by a cytomegalovirus (CMV) promoter was used to express various viral proteins prM, E, NS2A and NS3 fusion proteins in HEK293T cells. For constructs pXJ-$C_{14}$-prM and pXJ-$C_{14}$-prM (1-130), the anchor C signal peptide ($C_{14}$) was retained at the N-terminus of prM to ensure the correct targeting on ER membrane. For construct pXJ-$M_{17}$-E, the last 17 amino acids from M protein was retained to serve as a signal peptide for translocating the E protein into the ER lumen. For constructs pXJ-SPG-$C_{16}$-NS2A-EGFP and pXJ-SPG-$C_{16}$-



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NS2A-GST, the signal peptide derived from Gaussia luciferase (SPG) [ ADDIN EN.CITE <EndNote><Cite><Author>Verhaegent</Author><Year>2002</Year><RecNum>112</RecNum><DisplayText>(Verhaegent and Christopoulos, 2002)</DisplayText><record><rec-number>112</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1561735944">112</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Verhaegent, M.</author><author>Christopoulos, T. K.</author></authors></contributors><auth-address>Department of Chemistry and Biochemistry, University of Windsor, Ontario, Canada.</auth-address><titles><title>Recombinant Gaussia luciferase. Overexpression, purification, and analytical application of a bioluminescent reporter for DNA hybridization</title><secondary-title>Anal Chem</secondary-title></titles><periodical><full-title>Anal Chem</full-title></periodical><pages>4378-85</pages><volume>74</volume><number>17</number><keywords><keyword>Animals</keyword><keyword>DNA/*analysis</keyword><keyword>DNA Probes</keyword><keyword>Genes, Reporter</keyword><keyword>*Luciferases/genetics/isolation & purification</keyword><keyword>Luminescent Proteins/genetics/isolation & purification</keyword><keyword>Plankton/*enzymology</keyword><keyword>Recombinant Proteins/genetics/isolation & purification</keyword></keywords><dates><year>2002</year><pub-dates><date>Sep 1</date></pub-dates></dates><isbn>0003-2700 (Print)0003-2700 (Linking)</isbn><accession-num>12236345</accession-num><urls><related-urls><url>https://www.ncbi.nlm.nih.gov/pubmed/12236345</url></related-urls></urls></record></Cite></EndNote>] and the last 16 amino acids of NS1 ($C_{16}$) was fused at the N-terminus of NS2A to ensure the correct processing and membrane topology of NS2A. The EGFP and GST were amplified from pEGFPluc (Clontech) and pGEX-4T-1 (Sigma-Aldrich) respectively. The Human influenza hemagglutinin (HA) tag was fused to the N-terminus of NS3 and cloned into pXJ vector, resulting the construct pXJ-HA-NS3.

pGEX-4T-1 (GE Healthcare Life Sciences) was used as the vector for expressing all N-terminally glutathione S-transferase (GST)-tagged NS2A fusion proteins. The DNA fragments containing various length of NS2A were cloned into pGEX-4T-1 through restriction enzyme sites BamHI and XhoI.



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All plasmids were propagated in *E. coli* strain Top10 (ThermoFisher Scientific). All restriction enzymes were purchased from New England BioLabs (Ipswitch, MA). The plasmids were validated by restriction enzyme digestion and Sanger DNA sequencing. All primers were synthesized from Integrated DNA Technologies (Skokie, Illinois) and are available upon request.

**RNA Transcription and Electroporation**

The NGC FL or replicon plasmids were linearized by restriction enzyme XbaI. RNA transcription and electroporation were performed as described previously [ ADDIN EN.CITE <EndNote><Cite><Author>Xie</Author><Year>2013</Year><RecNum>85</RecNum><DisplayText>(Xie et al., 2013)</DisplayText><record><rec-number>85</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793217">85</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Xie, X.</author><author>Gayen, S.</author><author>Kang, C.</author><author>Yuan, Z.</author><author>Shi, P. Y.</author></authors></contributors><auth-address>State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Science, China.</auth-address><titles><title>Membrane topology and function of dengue virus NS2A protein</title><secondary-title>J Virol</secondary-title><alt-title>Journal of virology</alt-title></titles><periodical><full-title>J Virol</full-title></periodical><pages>4609-22</pages><volume>87</volume><number>8</number><edition>2013/02/15</edition><keywords><keyword>Animals</keyword><keyword>Cell Line</keyword><keyword>Cricetinae</keyword><keyword>Dengue Virus/*physiology</keyword><keyword>Endoplasmic Reticulum/*virology</keyword><keyword>Humans</keyword><keyword>Intracellular Membranes/*metabolism</keyword><keyword>Magnetic Resonance Spectroscopy</keyword><keyword>Models, Molecular</keyword><keyword>Protein Conformation</keyword><keyword>Viral Nonstructural Proteins/*chemistry/metabolism</keyword><keyword>*Virus Assembly</keyword></keywords><dates><year>2013</year><pub-dates><date>Apr</date></pub-dates></dates><isbn>1098-5514 (Electronic)0022-538X (Linking)</isbn><accession-num>23408612</accession-num><work-type>Research Support, Non-U.S. Gov't</work-type><urls><related-



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urls><url>http://www.ncbi.nlm.nih.gov/pubmed/23408612</url></related-urls></urls><custom2>3624351</custom2><electronic-resource-num>10.1128/JVI.02424-12</electronic-resource-num><language>eng</language></record></Cite></EndNote>]. Briefly, 10 μg RNA was electroporated into 8 × $10^6$ cells in a 4-mm cuvette using the GenePulser apparatus (Bio-Rad) at settings of 0.45 kV (C6/36 cells) or 0.85 kV (BHK-21 cells) and 25 mF, pulsing three times, with 3 s intervals. After a 10-min recovery at room temperature, transfected cells were suspended in culture media and incubated at corresponding conditions: 28°C (C6/36 cells) or 37°C with 5% $CO_2$ (BHK-21).

**Virus Production and Quantification**

After electroporation, the transfected BHK-21 cells were seeded in one T-75 flask. After incubation at 37°C for 24 h, the cells were cultured in DMEM with 2% FBS at 30°C for another 4 days. At every 24 h p.t., culture fluids was collected and stored at -80°C. Viral titer was determined by crystal violet-based plaque assay using BHK-21 cells according to the protocol as described previously [ ADDIN EN.CITE <EndNote><Cite><Author>Xie</Author><Year>2013</Year><RecNum>85</RecNum><DisplayText>(Xie et al., 2013)</DisplayText><record><rec-number>85</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793217">85</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Xie, X.</author><author>Gayen, S.</author><author>Kang, C.</author><author>Yuan, Z.</author><author>Shi, P. Y.</author></authors></contributors><auth-address>State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Science, China.</auth-address><titles><title>Membrane topology and function of dengue virus NS2A protein</title><secondary-title>J Virol</secondary-title><alt-title>Journal of virology</alt-title></titles><periodical><full-title>J Virol</full-title></periodical><pages>4609-22</pages><volume>87</volume><number>8</number><edition>2013/02/15</edition><keywords><keyword>Animals</keyword><keyword>Cell Line</keyword><keyword>Cricetinae</keyword><keyword>Dengue Virus/*physiology</keyword><keyword>Endoplasmic Reticulum/*virology</keyword><keyword>Humans</keyword><keyword>Intracellular Membranes/*metabolism</keyword><keyword>Magnetic Resonance Spectroscopy</keyword><keyword>Models, Molecular</keyword><keyword>Protein Conformation</keyword><keyword>Viral Nonstructural



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Proteins/*chemistry/metabolism</keyword><keyword>*Virus Assembly</keyword></keywords><dates><year>2013</year><pub-dates><date>Apr</date></pub-dates></dates><isbn>1098-5514 (Electronic)0022-538X (Linking)</isbn><accession-num>23408612</accession-num><work-type>Research Support, Non-U.S. Gov't</work-type><urls><related-urls><url>http://www.ncbi.nlm.nih.gov/pubmed/23408612</url></related-urls></urls><custom2>3624351</custom2><electronic-resource-num>10.1128/JVI.02424-12</electronic-resource-num><language>eng</language></record></Cite></EndNote>]. Plaques were developed on day 5 post-infection.

**RNA extraction and RT-PCR**

Viral RNAs in culture fluids were extracted using a QIAamp viral RNA minikit (Qiagen). Extracted RNAs were eluted in 50 µl RNase-free water. Reverse transcription (RT)-qPCR was performed to measure the amounts of viral RNA copy in the culture medium. Viral RNAs were measured using an iScript one-Step RT-PCR kit with SYBR green (Bio-Rad) and the primer pair NGC 7764V (sequence: 5'-CGTCGAGAGAAATATGGTCACACC-3') and 7844C (sequence: 5'CCACAATAGTATGACCAGCCT-3') [ ADDIN EN.CITE <EndNote><Cite><Author>Xie</Author><Year>2013</Year><RecNum>85</RecNum><DisplayText>(Xie et al., 2013)</DisplayText><record><rec-number>85</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793217">85</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Xie, X.</author><author>Gayen, S.</author><author>Kang, C.</author><author>Yuan, Z.</author><author>Shi, P. Y.</author></authors></contributors><auth-address>State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Science, China.</auth-address><titles><title>Membrane topology and function of dengue virus NS2A protein</title><secondary-title>J Virol</secondary-title><alt-title>Journal of virology</alt-title></titles><periodical><full-title>J Virol</full-title></periodical><pages>4609-22</pages><volume>87</volume><number>8</number><edition>2013/02/15</edition><keywords><keyword>Animals</keyword><keyword>Cell Line</keyword><keyword>Cricetinae</keyword><keyword>Dengue Virus/*physiology</keyword><keyword>Endoplasmic Reticulum/*virology</keyword><keyword>Humans</keyword><keyword>Intracellular Membranes/*metabolism</keyword><keyword>Magnetic Resonance



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Spectroscopy</keyword><keyword>Models, Molecular</keyword><keyword>Protein Conformation</keyword><keyword>Viral Nonstructural Proteins/*chemistry/metabolism</keyword><keyword>*Virus Assembly</keyword></keywords><dates><year>2013</year><pub-dates><date>Apr</date></pub-dates></dates><isbn>1098-5514 (Electronic)0022-538X (Linking)</isbn><accession-num>23408612</accession-num><work-type>Research Support, Non-U.S. Gov't</work-type><urls><related-urls><url>http://www.ncbi.nlm.nih.gov/pubmed/23408612</url></related-urls></urls><custom2>3624351</custom2><electronic-resource-num>10.1128/JVI.02424-12</electronic-resource-num><language>eng</language></record></Cite></EndNote>]. Alternatively, the extracted viral RNA was reversely transcribed and amplified using SuperScript One-Step (RT)-PCR with Platinum Taq (Invitrogen). The RT-PCR products were then gel purified and subjected to Sanger DNA sequencing.

**Replicon Transient Transfection**

After electroporation of replicon RNA into BHK-21 cells, the cells were resuspended in culture medium and seeded in a 12-well plate ($2 \times 10^5$ cells/well). At various time points, the cells were washed once with phosphate-buffered saline (PBS) and lysed using lysis buffer (Promega). The plates containing the lysis buffer were sealed with Parafilm and stored at -80°C. Once samples for all time points had been collected, luciferase signals were measured in a Clarity luminescence microplate reader (BioTek) according to the manufacturer's protocol. The luciferase signals at early time points (2-6 h post-transfection) represent the translation of input viral RNA; and the luciferase signals at later points (24-46 h post-transfection) represent the stage of RNA replication.

**Immunofluorescence assay (IFA)**

Cells were seeded in an 8-well Lab-Tek II chamber slide (ThermoFisher Scientific). At given time points, cells were fixed in 4% paraformaldehyde in PBS for 20 min at room temperature. Afterwards, cells were permeabilized by 0.1% Triton X-100 (Sigma-Aldrich) for 5 min followed by 1-h incubation in blocking buffer containing 1% FBS and 0.05% Tween-20 (Sigma-Aldrich) in PBS. Cells were then incubated with primary antibodies (diluted in the blocking buffer) at room temperature for 1 h. After three PBS washes, cells were incubated with goat anti-mouse or rabbit IgG (1:1000 diluted in blocking buffer) conjugated with Alexa Fluor 488 or 594 (ThermoFisher Scientific) for 1 h in blocking buffer.



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Cells were finally washed by PBS for three times, counter-stained by Hoechst 33342 Solution (ThermoFisher Scientific) and mounted in ProLong® Diamond Antifade Mountant (ThermoFisher Scientific). Epifluorescence images were acquired by Leica DM4000 B system. Confocal fluorescence images were acquired by Zeiss LSM 5 META systems.

**Virus Revertant Selection**

For selecting revertants from G11A-transfected cells, we consecutively passaged the transfected cells for 5 rounds. In brief, after electroporation, the cells were seeded in 96-well plates and incubate at 30°C for 3 days. Afterwards, cells for each well were detached by trypsin and suspended in culture medium. About 20% of the suspended cells were transferred into the well of new 96-well plates and incubated at 37°C for one day. The cells were then incubated at 30°C for two additional days. Again, the confluent cells were detached for next passage. At every passage, the supernatants were collected. After 5 rounds of passaging, IFA was performed to determine the intracellular E protein expression by 4G2 antibody. Plaque assay was performed to examine the amounts of infectious virus particle in the culture fluids. Viral RNA from the P5 supernatants were extracted and used as template for RT-PCR. Sanger sequencing was performed to sequence the entire viral genome.

**Co-immunoprecipitation (Co-IP)**

The NS2A/prM, NS2A/E and NS2A/NS3 interactions were examined by Co-IP analysis according to the protocol as described previously[ ADDIN EN.CITE <EndNote><Cite><Author>Zou</Author><Year>2014</Year><RecNum>113</RecNum><DisplayText>(Zou et al., 2014)</DisplayText><record><rec-number>113</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1561742592">113</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Zou, J.</author><author>Xie, X.</author><author>Lee le, T.</author><author>Chandrasekaran, R.</author><author>Reynaud, A.</author><author>Yap, L.</author><author>Wang, Q. Y.</author><author>Dong, H.</author><author>Kang, C.</author><author>Yuan, Z.</author><author>Lescar, J.</author><author>Shi, P. Y.</author></authors></contributors><auth-address>State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Sciences, Wuhan, China.</auth-address><titles><title>Dimerization of flavivirus NS4B protein</title><secondary-title>J



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Virol</secondary-title></titles><periodical><full-title>J Virol</full-title></periodical><pages>3379-91</pages><volume>88</volume><number>6</number><keywords><keyword>Amino Acid Motifs</keyword><keyword>Dengue/*virology</keyword><keyword>Dengue Virus/chemistry/genetics/*metabolism</keyword><keyword>Dimerization</keyword><keyword>Humans</keyword><keyword>Viral Nonstructural Proteins/*chemistry/genetics/*metabolism</keyword><keyword>West Nile Fever/*virology</keyword><keyword>West Nile virus/chemistry/genetics/*metabolism</keyword></keywords><dates><year>2014</year><pub-dates><date>Mar</date></pub-dates></dates><isbn>1098-5514 (Electronic)0022-538X (Linking)</isbn><accession-num>24390334</accession-num><urls><related-urls><url>https://www.ncbi.nlm.nih.gov/pubmed/24390334</url></related-urls></urls><custom2>PMC3957939</custom2><electronic-resource-num>10.1128/JVI.02782-13</electronic-resource-num></record></Cite></EndNote>]. Various combinations of plasmids were transfected into HEK293T cells in 10-cm dishes using the X-tremeGENE 9 DNA transfection reagent (Roche). At 40-42 h post-transfection, cells were lysed in 1 ml immunoprecipitation (IP) buffer (20 mM Tris, pH 7.5, 100 mM NaCl, 0.5% DDM, and EDTA-free protease inhibitor cocktail [Roche]) with rotation at 4°C for 1 h. Lysates were clarified by centrifugation at 20,000 × g and 4°C for 30 min and subjected to co-IP using protein G-conjugated magnetic beads according to the manufacturer's instructions (Millipore). Briefly, immune complexes were formed at 4°C overnight by mixing 200 µl of cell lysate with 2 µg corresponding antibodies in a 500-µl reaction system containing 400 mM sodium chloride. Subsequently, the complexes were precipitated with protein G-conjugated magnetic beads at 4°C for 1 h with rotation, followed by washing extensively with phosphate-buffered saline (PBS) containing 0.1% Tween-20. Finally, proteins were eluted with 4× lithium dodecyl sulfate (LDS) sample buffer (ThermoFisher Scientific) supplemented with 100 mM 1,4-dithiothreitol (DTT), heated at 70°C for 10 min, and analyzed by Western blot.

**SDS-PAGE and Western Blot**

Protein samples were mixed with 4×LDS sample buffer (ThermoFisher Scientific). After denaturing at 70°C for 10 min, 10-30 µl samples were loaded onto to a 12% or 15% SDS-PAGE gel (Bio-Rad Laboratories). After electrophoresis, proteins were resolved and



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transferred onto a polyvinylidene difluoride (PVDF) membrane using the Bio-Rad TransBlot Turbo Blotting System. Blots were soaked for 1 hour in a blocking buffer containing TBST (10 mM Tris-HCl [pH 7.5], 150 mM NaCl, and 0.1% Tween-20) and 5% skim milk, followed by 1 h incubation with primary antibodies (diluted in blocking buffer). After three washes with TBST buffer, blots were incubated with goat anti-mouse or rabbit antibodies conjugated to HRP (1:10,000 diluted in blocking buffer). After three thorough washes with TBST buffer, blots were incubated with the ECL substrates (GE Healthcare). Chemiluminescence signals were detected in ChemiDoc System (Bio-Rad).

**Transmission Electron Microscopy**

Viral genomic RNAs were electroporated into BHK-21 cells as described above. At 48 h post-transfection, the cells were fixed for at least 1 h in a mixture of 2.5% formaldehyde (prepared from paraformaldehyde powder) and 0.1% glutaraldehyde in 0.05M cacodylate buffer (pH 7.3) to which 0.03% picric acid and 0.03% $CaCl_2$ were added. After washing in 0.1 M cacodylate buffer, the cells were scraped off and processed further as a pellet. The pellets were post-fixed in 1% OsO4 in 0.1M cacodylate buffer (pH 7.3) for 1 h, washed with distilled water and *en bloc* stained with 2% aqueous uranyl acetate for 20 min at 60°C. The pellets were dehydrated in ethanol, processed through propylene oxide and embedded in Poly/Bed 812 (Polysciences, Warrington, PA). Ultrathin sections were cut on Leica EM UC7 ultramicrotome (Leica Microsystems, Buffalo Grove, IL), stained with lead citrate and examined in a Philips 201 transmission electron microscope at 60 kV.

**Expression and purification of recombinant NS2A proteins**

The GST-tagged DENV-2 NGC NS2A and its truncates was expressed and purified by following a previously described protocol [ ADDIN EN.CITE <EndNote><Cite><Author>Zou</Author><Year>2014</Year><RecNum>113</RecNum><DisplayText>(Zou et al., 2014)</DisplayText><record><rec-number>113</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1561742592">113</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Zou, J.</author><author>Xie, X.</author><author>Lee le, T.</author><author>Chandrasekaran, R.</author><author>Reynaud, A.</author><author>Yap, L.</author><author>Wang, Q. Y.</author><author>Dong, H.</author><author>Kang, C.</author><author>Yuan, Z.</author><author>Lescar, J.</author><author>Shi, P.



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Y.</author></authors></contributors><auth-address>State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Sciences, Wuhan, China.</auth-address><titles><title>Dimerization of flavivirus NS4B protein</title><secondary-title>J Virol</secondary-title></titles><periodical><full-title>J Virol</full-title></periodical><pages>3379-91</pages><volume>88</volume><number>6</number><keywords><keyword>Amino Acid Motifs</keyword><keyword>Dengue/*virology</keyword><keyword>Dengue Virus/chemistry/genetics/*metabolism</keyword><keyword>Dimerization</keyword><keyword>Humans</keyword><keyword>Viral Nonstructural Proteins/*chemistry/genetics/*metabolism</keyword><keyword>West Nile Fever/*virology</keyword><keyword>West Nile virus/chemistry/genetics/*metabolism</keyword></keywords><dates><year>2014</year><pub-dates><date>Mar</date></pub-dates></dates><isbn>1098-5514 (Electronic)0022-538X (Linking)</isbn><accession-num>24390334</accession-num><urls><related-urls><url>https://www.ncbi.nlm.nih.gov/pubmed/24390334</url></related-urls></urls><custom2>PMC3957939</custom2><electronic-resource-num>10.1128/JVI.02782-13</electronic-resource-num></record></Cite></EndNote>] with modifications. The pGEX-4T GST-NS2A plasmids were transformed into *E. coli* strain Rosetta™ 2 (pLysS) (Novagen) cells. Bacteria culture was grown in Tefferic broth containing 50 µg/ml Kanamycin and 34 µg/ml Chloramphenicol. When the cells reached an optical density at 600 nm (OD600) of 0.8-1.0, isopropyl β-d-1-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM. After growth for 16 h at 18°C, the cells were harvested, resuspended in a lysis buffer (10 mM $Na_2HPO_4$, 1.8 mM $KH_2PO_4$, 2.7 mM KCl, 400 mM NaCl, 1 mM EDTA, 0.5× Roche protease inhibitor cocktail, pH7.5) and disrupted by sonication using a Digital Sonifier 450 (Branson). The cell debris was removed by centrifugation at 10,000 rpm for 10 min at 4°C. The supernatants were collected and pelleted down by centrifugation at 45,000 rpm for 1 h at 4°C using a Beckman type 70 Ti rotor. The pellets containing the membrane fractions were collected and solubilized in the lysis buffer supplemented with 1.5% n-Dodecyl β-D-maltoside (DDM; Anatrace) at 4°C for 2 hours. Following another centrifugation step at 45,000 rpm for 1 h, the supernatants were collected and loaded onto a GSTrap HP Fast Flow column (GE Healthcare) which was preequilibrated with buffer A (10 mM $Na_2HPO_4$, 1.8 mM $KH_2PO_4$, 2.7 mM KCl, 400 mM NaCl, 2 mM tris(2-carboxyethyl) phosphine [TCEP], 0.05% DDM, pH



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7.5). After extensively wash with buffer B (10 mM $Na_2HPO_4$, 1.8 mM $KH_2PO_4$, 2.7 mM KCl, 1M NaCl, 2 mM tris(2-carboxyethyl) phosphine [TCEP], 0.05% DDM, pH 7.5), the protein was eluted in buffer A supplied with 20 mM glutathione. The fractions containing GST-NS2A proteins were pooled, concentrated, and subjected to gel filtration using a HiLoad Superdex 200 10/300 column (GE Healthcare) in buffer C (10 mM $Na_2HPO_4$, 1.8 mM $KH_2PO_4$, 2.7 mM KCl, 150 mM NaCl, 2 mM tris(2-carboxyethyl) phosphine [TCEP], 0.05% DDM, pH 7.5) with 5% glycerol. The peak fractions containing GST-NS2A proteins were pooled and concentrated before storage at -80°C.

**RNA Pull-down Assay**

The 5'UTR-$C_{66}$-3'UTR-biotin RNA was prepared by ligating a single biotinylated nucleotide to the 3' terminus of the RNA 5'UTR-$C_{66}$-3'UTR using the Pierce™ RNA 3' End Biotinylation Kit (ThermoFisher Scientific) according to the manufacturer's instructions. The RNA 5'UTR-$C_{66}$-3'UTR was *in vitro* transcribed from a PCR template using a T7 MEGAscript kit (ThermoFisher Scientific). The PCR template contained a T7 promoter, followed by a cDNA fragment representing a DENV-2 subgenome with a deletion from nucleotides 163 to 10269 (GenBank accession number AF038403). After *in vitro* transcription, the products were passed twice through the Microspin™ G-25 Columns (GE Healthcare Life Sciences) to remove excessive UTPs. The final products were subjected to phenol-chloroform extraction followed by alcohol precipitation. The purified RNAs were resuspended in nuclease-free water prior to storage at -80°C. Other shorter RNAs 3'UTR, Δpk1, 5'UTR-$C_{66}$, Δpk(1-2), Δpk(1-3), 3'SL, pk(3-4) and pk3 were *in vitro* synthesized from corresponding PCR templates using the same method.

RNA pull-down experiments were performed using the Pierce™ Magnetic RNA-Protein Pull-Down Kit (ThermoFisher Scientific) according to the manufacturer's instructions. Briefly, 2 µg of biotin-labeled RNAs were pre-captured by 50 µl Streptavidin magnetic beads in 1 × RNA capture buffer (10mL, 20mM Tris (pH 7.5), 1M NaCl, 1mM EDTA) at room temperature for 20 min with agitation. A 28-mer biotinylated IRE RNA (5'-UCCUGCUUCAACAGUGCUUGGACGGAAC-3'-biotin) from the Pierce™ RNA 3' End Biotinylation Kit was used as a control. The RNA-bound beads were then mixed with 2 µg purified GST-tagged NS2A proteins in a 100-µl reaction system containing 20 mM Tris (pH 7.5), 200 mM NaCl, 2 mM $MgCl_2$, 0.1% Tween-20 and 15% glycerol and incubated for 45 minutes at 4°C with agitation. For RNA competition assay, the various amounts of



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competitors (tRNA and other shorter RNAs from DENV-2 genome) were immediately added to above the mixtures containing RNA-bound beads and GST-NS2A proteins. After 5 times of wash by 20 mM Tris (pH 7.5), 10 mM NaCl, 0.1% Tween-20 Detergent, the proteins were eluted in 30 µl biotin elution buffer. The eluates were mixed with 4×LDS sample buffer and heated at 70°C for 10 min. 10 µl protein samples were loaded onto a 12% SDS-PAGE gel and followed by detection by Western blot using rabbit anti-GST antibodies.

**Electrophoretic Mobility-Shift Assay**

Electrophoretic mobility-shift assay (EMSA) was performed to estimate the binding affinity of DENV-2 5'UTR-$C_{66}$-3'UTR RNA to GST-NS2A proteins. 100 pM 5'UTR-$C_{66}$-3'UTR-biotin RNA was incubated with a serial concentration of recombinant GST-NS2A protein (0-250 nM) in a 50-µl reaction system containing 50mM Tris-HCl, pH 7.5, 10 mM 2-mercaptoethanol, 2 mM $MgCl_2$, 50 mM KCl at 24°C for 15 min. Afterwards, 10 µl 6x Gel Loading Dye without SDS (New England Biolabs Inc.) were added to stop the reaction. Sample were then loaded onto a 0.4% agarose gel (prepared in 0.5× Tris/Borate/EDTA[TBE]) to resolve the RNA-protein complexes and free RNAs by electrophoresis in 1× TBE buffer (120 V for 30 min) at room temperature. After electrophoresis, RNAs were transferred from the agarose gel to a BrightStar-Plus positively charged nylon membrane (ThermoFisher Scientific) according to the procedure as described in the manual of NorthernMax Gly Kit (ThermoFisher Scientific). The nylon membrane was irradiated with 200 mJ/cm$^2$ of Ultraviolet (UV) light at 254 nm in a CX-2000 UV crosslinker (Analytic Jene, CA) with default settings. The biotinylated RNA was finally detected by the Streptavidin-horseradish peroxidase conjugate by using the Chemiluminescent Detection Module (ThermoFisher Scientific). Chemiluminescent signals were detected in the ChemiDoc™ Imaging Systems (Bio-rad).

**PAR-CLIP**

The Photoactivatable-Ribonucleoside-Enhanced Crosslinking and Immunoprecipitation (PAR-CLIP) was performed according to the protocol as described previously [ ADDIN EN.CITE ADDIN EN.CITE.DATA ] with modifications. In brief, 4-thiouridine labelled DENV-2 3'UTR RNA was prepared by adding a final concentration 0.75 mM of 4-Thio-UTP (TriLink BioTechnologies) to a 20-µl *in vitro* transcription reaction (4-Thio-UTR to UTP



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ration is 1:10) using the MEGAscript T7 transcription Kit (Thermo Fisher Scientific). After 2 hours of reaction at 37°C, the reaction was stopped and the 4-thiouridine labelled RNAs were purified and resuspended in nuclease-free water. 15 µg of 4-thiouridine labelled DENV-2 3'UTR RNA was incubated with 6 µg recombinant DENV-2 GST-NS2A protein in a 100-µl reaction system containing 50 mM Tris-HCl, pH 7.5, 2 mM $MgCl_2$, 50 mM KCl and 5% glycerol at 24°C for 15 min. The RNA-protein mixtures were then irradiated on ice with 150 mJ/cm$^2$ at UV light at 365 nm. An aliquot of the same reaction without UV irradiation was used as the uncross-linked control. Prior to immunoprecipitation, 30 µl of PureProteome™ Protein G Magnetic Beads (MilliporeSigma) were washed twice with 1 ml citrate-phosphate buffer (4.7 g/L citric acid, 9.2 g/L $Na_2HPO_4$, pH 5.0), resuspended in 100 µl citrate-phosphate buffer and followed by in addition of 1 µg of rabbit anti-GST polyclonal antibody (Abcam). The bead-antibody mixtures were incubated at 4°C for 45 min. After two washed with citrate-phosphate buffer, the antibody-bound beads were resuspended in 30 µl of citrate-phosphate buffer. Upon immunoprecipitation, the RNA-protein mixtures were mixed with antibody-bound beads and incubated at 4°C for 1 h. Afterwards, the beads were subjected to three washes in buffer I (50mM HEPES-KOH, pH 7.5, 300 mM KCl, 0.05% IGEPAL CA-630L[Sigma-Aldrich] and 0.5 mM DTT) followed by two washes in buffer II (50 mM HEPES-KOH, pH 7.5, 500 KCl, 0.05% IGEPAL CA-630 and 0.5 mM DTT). The beads were then resuspended in 100 µl Proteinase K digestion buffer (100 mM Tris, pH 7.5, 50 mM NaCl, 10 mM EDTA and 1%SDS) supplemented with proteinase K (New England BioLabs) at a final concentration of 2 mg/ml. The reaction was placed into the Eppendorf ThermoMixer® C and incubated at 37°C for 1 h with agitation at 1100 rpm. Thereafter, the RNA in the reaction was extracted by phenol-chloroform, alcohol-precipitated, and resuspended in 20 µl of nuclease-free water. The extracted RNA was subjected to reversely transcriptionbed and followed by amplification (4 PCR-cycles) using the SuperScript® III One-Step RT-PCR System (ThermoFisher Scientific) with a pair of specific primers P1 (sequence: 5'-ACACTCTTTCCCTACACGA CGCTCTTCCGATCTAAGGCAAAACTAACATGAAAC-3'; underline indicates the sequence identical to the sequence at the 5' end of the DENV-2 3'UTR) and P2 (sequence: 5'-GTGACTGGAGTTCAGACG TGTGCTCTTCCGATCTAGAACCTGTTGATTCAACAGCAC-3'; underline indicates the sequence complimentary to the sequence at the 3' end of the DENV-2 3'UTR) targeting the DENV-2 3'UTR. Afterwards, the RT-PCR product was cleaned up using the QIAquick PCR Purification Kit (Qiagen) and subjected to another round of amplification (15 PCR-



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cycles) using the KAPA HiFi PCR Kit (Kapa Biosystems) with a pair of Illumina adapter/index primers (5′ universal adaptor sequence: 5′-AATGATACGGCGAC CACCGAGATCTACACTCTTTCCCTACACGAC-3′; and 3′ indexing primer sequence: 5′-CAAGCAGAAGACGGCATACGAGATnnnnnnGTGACTGGAGTTCAGACGTGT-3′. The "nnnnnn" here indicates the TruSeq index sequence). Thereafter, the PCR product was cleaned up using the QIAquick PCR Purification Kit (Qiagen) and followed by separation on 2% E-Gel™ EX Agarose Gels (ThermoFisher Scientific). The cDNAs fragment between 500 to 650 bp was excised, purified using the Zymo Research Gel DNA Recovery Kit (Zymo Research) and resuspended in nuclease-free water. The final cDNA libraries were quantified using a Qubit dsDNA Assay Kit (Invitrogen) and sequenced using a MiSeq Reagent Kit v3 (600-cycle) (illumina).

## QUANTIFICATION AND STATISTICAL ANALYSIS

### Sequence Alignment, Image Process and Densitometry Analysis

The amino acid sequences of flavivirus NS2A proteins were download from GenBank. Sequence alignment was performed with default settings using the CLC main workbench software (Qiagen). IFA images processing and cell counting was performed in software ImageJ (National Institutes of Health, NIH). At least four different IFA images were used to estimate the mean and standard deviations of percentage of viral protein staining positive-cells in Figure 2G and S2. Densitometry analysis was performed using Image lab version 6.0 (Bio-Rad Laboratories, Hercules, CA). All numerical data are presented as the mean ± SD (standard deviations). Group comparisons were performed using the unpaired parametric t-test in software Prism 8.0 (GraphPad). *$p<0.05$, significant; **$p<0.01$, very significant; ***$p<0.001$, highly significant; n.s., not significant. Images were assembled using the software Adobe illustrator CC.

### Calculating the equilibrium dissociation constant (Kd)

The intensities of bands representing the RNA/GST-NS2A complexes and free RNA in Figure 5E were quantitated by software ImageJ (NIH). The ratio of bound RNA (in the RNA/GST-NS2A complexes) to total RNA (free and bound RNA) in each lane was calculated. The ratio of bound RNA to total RNA (y-axis) versus the concentration of GST-NS2A protein (x-axis) was plotted using the software Prism 8 (Graphad). The value of Kd



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was obtained using a nonlinear regression with least-squares fitting method, where the Bmax was set as 1. All data points come from three independent experiments.

**Illumina sequencing data process and analysis**

The indexed sequencing reads of both forward and reverse strands were proceeded with the following bioinformatic pipelines: first, an initial quality filtering were conducted with Fastp [ ADDIN EN.CITE <EndNote><Cite><Author>Chen</Author><Year>2018</Year><RecNum>115</RecNum><DisplayText>(Chen et al., 2018)</DisplayText><record><rec-number>115</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1562011493">115</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Chen, S.</author><author>Zhou, Y.</author><author>Chen, Y.</author><author>Gu, J.</author></authors><translated-authors><author>Bioinformatics,</author></translated-authors></contributors><auth-address>Department of Bioinformatics, HaploX Biotechnology, Shenzhen, China.Shenzhen Institutes of Advanced Technology, Chinese Academy of Sciences, Shenzhen, China.</auth-address><titles><title>fastp: an ultra-fast all-in-one FASTQ preprocessor</title><secondary-title>Bioinformatics</secondary-title><alt-title>Bioinformatics</alt-title></titles><periodical><full-title>Bioinformatics</full-title><abbr-1>Bioinformatics</abbr-1></periodical><alt-periodical><full-title>Bioinformatics</full-title><abbr-1>Bioinformatics</abbr-1></alt-periodical><pages>i884-i890</pages><volume>34</volume><number>17</number><dates><year>2018</year><pub-dates><date>Sep 1</date></pub-dates></dates><isbn>1367-4811 (Electronic)1367-4803 (Linking)</isbn><accession-num>30423086</accession-num><urls><related-urls><url>http://www.ncbi.nlm.nih.gov/pubmed/30423086</url></related-urls></urls><custom2>6129281</custom2><electronic-resource-num>10.1093/bioinformatics/bty560</electronic-resource-num><remote-database-provider>2018 Sep 1</remote-database-provider><language>eng</language></record></Cite></EndNote>] to remove low quality ~~bad~~ reads (~~too~~ low ~~in quality~~PHRED score, too short, too many "N", etc.). Then, the illumina sequencing adapter sequence "AGATCGGAAGAGC" was trimmed with *cutadapt* [ ADDIN EN.CITE <EndNote><Cite><Author>Martin</Author><Year>2011</Year><RecNum>116</RecNum

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><DisplayText>(Martin, 2011)</DisplayText><record><rec-number>116</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1562011493">116</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Martin, Marcel</author></authors></contributors><titles><title>Cutadapt removes adapter sequences from high-throughput sequencing reads</title><secondary-title>EMBnet.journal; Vol 17, No 1: Next Generation Sequencing Data AnalysisDO - 10.14806/ej.17.1.200</secondary-title></titles><periodical><full-title>EMBnet.journal; Vol 17, No 1: Next Generation Sequencing Data AnalysisDO - 10.14806/ej.17.1.200</full-title></periodical><keywords><keyword>next generation sequencing, small RNA, microRNA, adapter removal</keyword></keywords><dates><year>2011</year><pub-dates><date>05/02/</date></pub-dates></dates><urls><related-urls><url>https://journal.embnet.org/index.php/embnetjournal/article/view/200/458</url></related-urls></urls></record></Cite></EndNote>] (command line parameters: -b AGATCGGAAGAGC -m 40). A secondary quality filter was applied with FASTX toolkit ([ HYPERLINK "http://hannonlab.cshl.edu/fastx_toolkit/index.html" ]) to remove any reads that contained more than 4% nucleotides with a PHRED score <20 (command line parameters: fastq_quality_filter -Q33 -q 20 -p 96). The remaining reads were aligned to 3'UTR of DENV genome (GenBank accession number AF038403), with Bowtie2 [ ADDIN EN.CITE <EndNote><Cite><Author>Langmead</Author><Year>2012</Year><RecNum>117</RecNum><DisplayText>(Langmead and Salzberg, 2012)</DisplayText><record><rec-number>117</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1562011493">117</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Langmead, Ben</author><author>Salzberg, Steven L.</author></authors></contributors><titles><title>Fast gapped-read alignment with Bowtie 2</title><secondary-title>Nature Methods</secondary-title></titles><periodical><full-title>Nature Methods</full-title></periodical><pages>357</pages><volume>9</volume><dates><year>2012</year><pub-dates><date>03/04/online</date></pub-dates></dates><publisher>Nature Publishing Group, a division of Macmillan Publishers Limited. All Rights Reserved.</publisher><urls><related-urls><url>https://doi.org/10.1038/nmeth.1923</url></related-urls></urls><electronic-



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resource-num>10.1038/nmeth.1923</electronic-resource-num></record></Cite></EndNote>] (command line parameters: --local). Using SAMtools [ ADDIN EN.CITE <EndNote><Cite><Author>Li</Author><RecNum>118</RecNum><DisplayText>(Li et al.)</DisplayText><record><rec-number>118</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1562011493">118</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Li, H.</author><author>Handsaker B Fau - Wysoker, Alec</author><author>Wysoker A Fau - Fennell, Tim</author><author>Fennell T Fau - Ruan, Jue</author><author>Ruan J Fau - Homer, Nils</author><author>Homer N Fau - Marth, Gabor</author><author>Marth G Fau - Abecasis, Goncalo</author><author>Abecasis G Fau - Durbin, Richard</author><author>Durbin, R.</author></authors><translated-authors><author>Bioinformatics,</author></translated-authors></contributors><auth-address>Wellcome Trust Sanger Institute, Wellcome Trust Genome Campus, Cambridge, CB10 1SA, UK, Broad Institute of MIT and Harvard, Cambridge, MA 02141, USA. FAU - Handsaker, Bob</auth-address><titles><title>The Sequence Alignment/Map format and SAMtools</title></titles><number>1367-4811 (Electronic)</number><dates></dates><call-num>1000 Genome Project Data Processing Subgroup</call-num><urls></urls><remote-database-provider>2009 Aug 15</remote-database-provider><language>eng</language></record></Cite></EndNote>], the aligned forward and reverse reads were binarily converted, merged, indexed, and sorted~~, and mathematically noted~~ (command line parameters: view -buS, merge, sort, index, mpileup). This is followed by extracting and calculating the T/U-to-C mutation rate at each genome position and the mutation frequencies of each of the four nucleotides.

## DATA AND SOFTWARE AVAILABILITY

Illumina sequencing data access. The raw data of Illuminae sequencing data can be accessed, queried and downloaded using the following link: .

**Comment [RAL6]:** Did you upload the raw data somewhere? Will probabky have to do this prior to submission. NCBI SRA is the easiest option.



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# Dengue NS2A protein orchestrates virus assembly

Xuping Xie[1]*, Jing Zou[1], Xianwen Zhang[1,2,3], Yiyang Zhou[1], Andrew Routh[1], Vsevolod L. Popov[4], Xinwen Chen[2,3,5], Qingyin Wang[6], Hong-Ping Dong[6], Pei-Yong Shi[1,7,8,9,10]*

[1]Department of Biochemistry & Molecular Biology, University of Texas Medical Branch, Galveston, Texas, USA; [2]State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Sciences, Wuhan, China. [3]University of Chinese Academy of Sciences, Beijing, China. [4]Department of Pathology, University of Texas Medical Branch, Galveston, Texas, USA; [5]Guangzhou Institute of Biomedicine and Health, Chinese Academy of Sciences, Guangzhou, China. [6]Clearbridge Health, Singapore; [7]Institute for Translational Science, University of Texas Medical Branch, Galveston, Texas, USA; [8]Sealy Institute for Vaccine Sciences, University of Texas Medical Branch, Galveston, Texas, USA; [9]Sealy Center for Structural Biology & Molecular Biophysics, University of Texas Medical Branch, Galveston, Texas, USA; [10]Institute for Human Infections & Immunity, University of Texas Medical Branch, Galveston, Texas, USA;

*Correspondence: [ HYPERLINK "mailto:xuxie@UTMB.edu" ] (X.X.), [ HYPERLINK "mailto:peshi@UTMB.edu" ] (P.-Y.S.)

Running title: The role of NS2A protein in dengue virus assembly

**Comment [RAL1]:** Please add affiliation: Sealy Centre for Structural Biology and Molecular Biophysics, University of Texas Medical Branch, Galveston, Texas, USA.



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## SUMMARY

Dengue virus (DENV) is the most important mosquito-borne viral pathogen in humans. DENV assembly requires cleavages of viral C-prM-E polyprotein into three structural proteins (capsid, pre-membrane, and envelope), packaging of viral RNA with C protein into nucleocapsid, and budding of prM/E proteins into virions. No study has addressed how these assembly events are orchestratedThe molecular mechanisms underlying the orchestration of these complex events is unclear. Here we show that DENV-2 NS2A protein recruits viral RNA, structural proteins, and protease to the site of virion assembly site, and coordinates nucleocapsid and virus formation. The last 285 nucleotides of viral 3'UTR serve as a "packaging signal" that binds to a cytosolic loop of NS2A protein. This interaction allows NS2A to recruit nascent RNA from the replication complex to the virion assembly site. Mutations in NS2A that weaken the 3'UTR/NS2A interaction abolish virus assembly. In addition, NS2A interacts with prM, E, and NS3 to recruit C-prM-E polyprotein and NS2B/NS3 protease to the virion assembly site, leading to coordinated C-prM-E cleavage. Mature C protein assembles onto genomic RNA to form nucleocapsid, followed by prM/E envelopment and virion formation. An NS2A mutation that alters the prM/NS2A interaction reduces C-prM cleavage and abolishes virion assembly; second-site mutations in C protein that restore the C-prM cleavage rescue virion assembly. The results support a DENV assembly model that whereby NS2A recruits viral RNA to the virion assembly site where coordinated cleavage of C-prM-E ensues infections virus morphogenesis.




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## INTRODUCTION

The genus *Flavivirus* of the family *Flaviviridae* comprises over 70 viruses, many of which are important human pathogens, including dengue (DENV), yellow fever (YFV), Zika (ZIKV), West Nile (WNV), Japanese encephalitis (JEV), St. Louis encephalitis (SLE), and tick-borne encephalitis (TBEV) viruses [ ADDIN EN.CITE <EndNote><Cite><Author>Pierson</Author><Year>2013</Year><RecNum>3</RecNum><DisplayText>(Pierson and Diamond, 2013)</DisplayText><record><rec-number>3</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1536704974">3</key><key app="ENWeb" db-id="">0</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Pierson, T.C.</author><author>Diamond, M. S.</author></authors></contributors><titles><secondary-title>Flaviviruses p. 747-794. In D.M. Knipe and P.M. Howley (ed), Fields virology, 6th., vol. 1.</secondary-title></titles><periodical><full-title>Flaviviruses p. 747-794. In D.M. Knipe and P.M. Howley (ed), Fields virology, 6th., vol. 1.</full-title></periodical><dates><year>2013</year></dates><urls></urls></record></Cite></EndNote>]. The four serotypes of DENV alone cause about 390 million human infections each year, leading to dengue fever, dengue hemorrhagic fever, and dengue shock syndrome [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. ~~Because of~~Due to the huge socio~~al~~ economic burden, DENV was included in the top 10 global health threats by the World Health Organization (WHO). Moreover, ~~T~~the development of vaccines and therapeutics for DENV has ~~been~~ proven challenging [ ADDIN EN.CITE <EndNote><Cite><Author>Lim</Author><Year>2013</Year><RecNum>58</RecNum><DisplayText>(Lim et al., 2013)</DisplayText><record><rec-number>58</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv"



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64 timestamp="1560793214">58</key></foreign-keys><ref-type name="Journal
65 Article">17</ref-type><contributors><authors><author>Lim, S. P.</author><author>Wang,
66 Q. Y.</author><author>Noble, C. G.</author><author>Chen, Y. L.</author><author>Dong,
67 H.</author><author>Zou, B.</author><author>Yokokawa, F.</author><author>Nilar,
68 S.</author><author>Smith, P.</author><author>Beer, D.</author><author>Lescar,
69 J.</author><author>Shi, P. Y.</author></authors></contributors><auth-address>Novartis
70 Institute for Tropical Diseases, 10 Biopolis Road, 05-01 Chromos, Singapore 138670,
71 Singapore.</auth-address><titles><title>Ten years of dengue drug discovery: Progress
72 and prospects</title><secondary-title>Antiviral Res</secondary-title><alt-title>Antiviral
73 research</alt-title></titles><periodical><full-title>Antiviral Res</full-
74 title></periodical><pages>500-
75 19</pages><volume>100</volume><number>2</number><edition>2013/10/01</edition><
76 dates><year>2013</year><pub-dates><date>Nov</date></pub-
77 dates></dates><isbn>1872-9096 (Electronic)0166-3542
78 (Linking)</isbn><accession-num>24076358</accession-num><urls><related-
79 urls><url>http://www.ncbi.nlm.nih.gov/pubmed/24076358</url></related-
80 urls></urls><electronic-resource-num>10.1016/j.antiviral.2013.09.013</electronic-
81 resource-num><language>eng</language></record></Cite></EndNote>]. The first FDA
82 approved dengue vaccine has a low efficacy of 30-61% [ ADDIN EN.CITE ADDIN
83 EN.CITE.DATA ]. Due to the risk of vaccine-induced severe disease in children who are
84 seronegative at the time of vaccination, WHO recommended only use of the vaccine in
85 areas where the disease is highly prevalent [ ADDIN EN.CITE
86 <EndNote><Cite><Author>Whitehead</Author><Year>2016</Year><RecNum>63</RecN
87 um><DisplayText>(Whitehead, 2016)</DisplayText><record><rec-number>63</rec-
88 number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv"
89 timestamp="1560793215">63</key></foreign-keys><ref-type name="Journal



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Article">17</ref-type><contributors><authors><author>Whitehead, S. S.</author></authors></contributors><auth-address>a Laboratory of Infectious Diseases , NIAID, National Institutes of Health , Bethesda , MD , USA.</auth-address><titles><title>Development of TV003/TV005, a single dose, highly immunogenic live attenuated dengue vaccine; what makes this vaccine different from the Sanofi-Pasteur CYD vaccine?</title><secondary-title>Expert Rev Vaccines</secondary-title></titles><periodical><full-title>Expert Rev Vaccines</full-title></periodical><pages>509-17</pages><volume>15</volume><number>4</number><keywords><keyword>Dengue</keyword><keyword>live attenuated dengue vaccine</keyword><keyword>vaccine attenuation strategies</keyword><keyword>vaccine efficacy</keyword><keyword>vaccine immunogenicity</keyword><keyword>vaccine infectivity</keyword></keywords><dates><year>2016</year></dates><isbn>1744-8395 (Electronic)1476-0584 (Linking)</isbn><accession-num>26559731</accession-num><urls><related-urls><url>http://www.ncbi.nlm.nih.gov/pubmed/26559731</url></related-urls></urls><custom2>PMC4956407</custom2><electronic-resource-num>10.1586/14760584.2016.1115727</electronic-resource-num></record></Cite></EndNote>]. No clinically approved antivirals are available for flavivirus treatment, despite ~~major~~ considerable effort in the past two decades [ ADDIN EN.CITE <EndNote><Cite><Author>Zou</Author><Year>2019</Year><RecNum>64</RecNum><DisplayText>(Zou and Shi, 2019)</DisplayText><record><rec-number>64</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793215">64</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Zou, J.</author><author>Shi, P.



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Y.</author></authors></contributors><auth-address>Department of Biochemistry and Molecular Biology, University of Texas Medical Branch, Galveston, TX, USA.Department of Biochemistry and Molecular Biology, University of Texas Medical Branch, Galveston, TX, USA; Department of Phamarcology and Toxicology, University of Texas Medical Branch, Galveston, TX, USA; Sealy Center for Structural Biology and Molecular Biophysics, University of Texas Medical Branch, Galveston, TX, USA; Sealy Institute for Vaccine Sciences, University of Texas Medical Branch, Galveston, TX, USA. Electronic address: peshi@utmb.edu.</auth-address><titles><title>Strategies for Zika drug discovery</title><secondary-title>Curr Opin Virol</secondary-title></titles><periodical><full-title>Curr Opin Virol</full-title></periodical><pages>19-26</pages><volume>35</volume><dates><year>2019</year><pub-dates><date>Mar 7</date></pub-dates></dates><isbn>1879-6265 (Electronic)1879-6257 (Linking)</isbn><accession-num>30852345</accession-num><urls><related-urls><url>http://www.ncbi.nlm.nih.gov/pubmed/30852345</url></related-urls></urls><electronic-resource-num>10.1016/j.coviro.2019.01.005</electronic-resource-num></record></Cite></EndNote>]. It is therefore critical to study the molecular mechanism of flavivirus replication, which may lead to new approaches for countermeasure development.

Flaviviruses haves a plus-sense, single-stranded RNA ~~of about~~approximately 11,000 -nucleotides in length. The genomic RNA contains a 5' untranslated region (UTR), a single open-reading-frame (ORF), and a 3' UTR. The ORF ~~translates~~ encodes a polyprotein that is processed by viral and host proteases into three ~~structure~~ structural proteins ({capsid (C), pre-membrane (prM), and envelope (E)}) and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5). The structural proteins, together with genomic RNA, assemble into virions. The nonstructural proteins participate



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in viral RNA replication, virion assembly, and evasion of host immune response. Upon viral translation, a minus-sense RNA is synthesized from the genomic RNA template, forming a double-stranded RNA. The minus-strand RNA then serves as a template to make ~~many~~ multiple copies of plus-sense RNA that are packaged to form progeny virions [ ADDIN EN.CITE <EndNote><Cite><Author>Pierson</Author><Year>2013</Year><RecNum>3</RecNum><DisplayText>(Pierson and Diamond, 2013)</DisplayText><record><rec-number>3</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1536704974">3</key><key app="ENWeb" db-id="">0</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Pierson, T.C.</author><author>Diamond, M. S.</author></authors></contributors><titles><secondary-title>Flaviviruses p. 747-794. In D.M. Knipe and P.M. Howley (ed), Fields virology, 6th., vol. 1.</secondary-title></titles><periodical><full-title>Flaviviruses p. 747-794. In D.M. Knipe and P.M. Howley (ed), Fields virology, 6th., vol. 1.</full-title></periodical><dates><year>2013</year></dates><urls></urls></record></Cite></EndNote>]. Like other RNA viruses, the assembly of flavivirus is coupled with RNA replication [ ADDIN EN.CITE ADDIN EN.CITE.DATA ].

Flavivirus infection induces invaginations of the endoplasmic reticulum (ER) to form clusters of double-membrane vesicles (Ve) enclosed in the vesicle packets (VPs). Electron tomography has revealed the three-dimensional architectures of Ve and VPs formed during DENV, WNV, TBEV, and ZIKV infections [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. Ve houses the viral replication complex (including the double-stranded viral RNA, viral nonstructural proteins, and cellular proteins) and is where RNA synthesis occurs [ ADDIN EN.CITE



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166 <EndNote><Cite><Author>Junjhon</Author><Year>2014</Year><RecNum>71</RecNum
167 ><DisplayText>(Junjhon et al., 2014)</DisplayText><record><rec-number>71</rec-
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170 Article">17</ref-type><contributors><authors><author>Junjhon,
171 J.</author><author>Pennington, J. G.</author><author>Edwards, T.
172 J.</author><author>Perera, R.</author><author>Lanman, J.</author><author>Kuhn, R.
173 J.</author></authors></contributors><auth-address>Markey Center for Structural Biology,
174 Department of Biological Sciences, Purdue University, West Lafayette, Indiana,
175 USA.</auth-address><titles><title>Ultrastructural characterization and three-dimensional
176 architecture of replication sites in dengue virus-infected mosquito cells</title><secondary-
177 title>J Virol</secondary-title></titles><periodical><full-title>J Virol</full-
178 title></periodical><pages>4687-
179 97</pages><volume>88</volume><number>9</number><keywords><keyword>Animals<
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187 dates><date>May</date></pub-dates></dates><isbn>1098-5514 (Electronic)0022-
188 538X (Linking)</isbn><accession-num>24522909</accession-num><urls><related-
189 urls><url>http://www.ncbi.nlm.nih.gov/pubmed/24522909</url></related-
190 urls></urls><custom2>PMC3993787</custom2><electronic-resource-
191 num>10.1128/JVI.00118-14</electronic-resource-num></record></Cite></EndNote>].



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Newly synthesized plus-sense RNA is released from the Ve pore to the rough ER for new rounds of RNA translation and replication. Alternatively, the nascent RNA is assembled into virions through encapsidation (with C protein) and envelopment (with prM and E proteins) through budding into the ER lumen. The virion assembly occurs at the ER sites juxtaposed to the VPs. An initial immature virion contains 60 icosahedrally arranged prM/E heterotrimers. As the immature virion transits through the Golgi network, the acidic environment triggers the cleavage of prM by cellular furin protease, resulting in an infectious mature virion [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. Cryo-EM of flavivirus virions exhibit two important structural features: (i) The nucleocapsid core lacks organized structures and (ii) there are no observable connections between nucleocapsid and the prM/E membrane shell, suggesting that the C protein may bind to genomic RNA in a random manner without direct interaction with prM/E proteins [ ADDIN EN.CITE ADDIN EN.CITE.DATA ].

The molecular mechanism of flavivirus assembly remains largely obscure. Besides structural proteins, several nonstructural proteins, such as NS2A, NS2B, and NS3, have been shown to regulate flavivirus assembly. Different amino acids of NS2A protein ~~were~~ have been reported to play distinct roles in flavivirus RNA replication and virion assembly [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. The cleavage between C and prM is tightly modulated by NS2B/NS3 protease and cellular signalase during YFV and Murray Valley encephalitis virus assembly [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. Mutations in the NS3 helicase domain alone affect YFV assembly [ ADDIN EN.CITE <EndNote><Cite><Author>Patkar</Author><Year>2008</Year><RecNum>90</RecNum><DisplayText>(Patkar and Kuhn, 2008a)</DisplayText><record><rec-number>90</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793218">90</key></foreign-keys><ref-type name="Journal



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Article">17</ref-type><contributors><authors><author>Patkar, C. G.</author><author>Kuhn, R. J.</author></authors></contributors><auth-address>Purdue Univ, Dept Biol Sci, W Lafayette, IN 47907 USA</auth-address><titles><title>Yellow fever virus NS3 plays an essential role in virus assembly independent of its known enzymatic functions</title><secondary-title>Journal of Virology</secondary-title><alt-title>J Virol</alt-title></titles><alt-periodical><full-title>J Virol</full-title></alt-periodical><pages>3342-3352</pages><volume>82</volume><number>7</number><keywords><keyword>tick-borne encephalitis</keyword><keyword>flavivirus kunjin ns3</keyword><keyword>west-nile-virus</keyword><keyword>dengue virus</keyword><keyword>nonstructural protein</keyword><keyword>rna replication</keyword><keyword>cdna-clones</keyword><keyword>particles</keyword><keyword>ns2a</keyword><keyword>organization</keyword></keywords><dates><year>2008</year><pub-dates><date>Apr</date></pub-dates></dates><isbn>0022-538X</isbn><accession-num>WOS:000254139800015</accession-num><urls><related-urls><url><Go to ISI>://WOS:000254139800015</url></related-urls></urls><electronic-resource-num>10.1128/Jvi.02447-07</electronic-resource-num><language>English</language></record></Cite></EndNote>]. Although expression of prM/E alone is sufficient to generate empty virus-like particles, it is not known how nucleocapsid is formed and subsequently enveloped with prM/E to form infectious virions. ~~The goal of this study is~~We therefore set out to define the molecular mechanisms that orchestrate~~s~~ the three structural proteins and viral RNA to form nucleocapsid and infectious virus in a highly coordinated manner. In this study, by using~~Using~~ genetic, biochemical, and imaging approaches, we demonstrate that DENV NS2A protein choreographs virion morphogenesis by (i) interacting with viral prM and E proteins, (ii) regulating the processing of C-prM polyprotein by viral protease, and (iii) recruiting



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genomic RNA through binding to the 3'UTR. These results support a model ~~that~~ whereby DENV NS2A recruits nascent viral RNA from the replication complex (housed in Ve) to the virion assembly site (on the ER membrane juxtaposed to VPs), where ~~an~~ coordinated C-prM cleavage facilitates genomic RNA encapsidation with C protein and budding with prM and E proteins into the ER lumen, resulting in progeny virions.



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## RESULTS

**NS2A G11A mutation abolishes virion assembly**. Using DENV-2 as a model, we previously identified a panel of NS2A mutant viruses that were selectively defective in virion assembly without compromising other steps of thean infection cycle [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. Here, we performed four experiments to demonstrate that NS2A mutation G11A of DENV-2 caused a defect in virion assembly. (i) A DENV-2 luciferase replicon was used to examine the effect of NS2A G11A on viral translation and RNA synthesis. Upon transfection into BHK-21 cells, the G11A replicon produced a luciferase profile comparable to the WT replicon (Fig. 1A), suggesting that the mutation does not significantly reduce viral translation and RNA replication. (ii) When full-length viral RNA of DENV-2 was transfected into BHK-21 cells, the NS2A G11A mutant failed to release extracellular viral RNA (Fig. 1B). No infectious virus was detected from the culture medium of G11A RNA-transfected cells (Fig. 1C). (iii) In a *trans* packaging system, WT luciferase replicon could be packaged into pseudo-infectious particles (PIPs) when structural C-prM-E proteins were expressed from Semliki Forest virus replicon (Fig. S1A). The WT reporter replicon PIPs infected Vero cells and generated robust luciferase signals (Fig. S1B). In contrast, no PIPs were produced with the NS2A G11A replicon. (iv) Transmission electron microscopy revealed substantial virus-induced single-membrane vesicles (within the lumen of the rough ER) or double-membrane vesicles (closely associated with cisternae of the rough ER) in both WT and G11A RNA-transfected cells at 48 h post-transfection (Fig. 1D). A lot large number of virus particles appeared in the WT RNA-transfected cells, but no virus particles were detected in the G11A RNA-transfected cells. These results demonstrate that NS2A G11A mutation specifically abolishes virion assembly.



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**NS2A G11A mutation alters the expression and localization of viral structural proteins.** We compared the subcellular localization of viral proteins in BHK-21 cells that were transfected with WT or NS2A G11A genomic RNA. Surprisingly, the NS2A G11A mutation altered the intracellular distribution of three structural proteins. (i) C protein was disseminated in both nucleus and cytoplasm in the WT RNA-transfected cells, as previously reported [ ADDIN EN.CITE <EndNote><Cite><Author>Wang</Author><Year>2004</Year><RecNum>91</RecNum><DisplayText>(Wang et al., 2004)</DisplayText><record><rec-number>91</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793218">91</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Wang, S. H.</author><author>Syu, W. J.</author><author>Hu, S. T.</author></authors></contributors><auth-address>Institute of Microbiology and Immunology, School of Life Science, National Yang-Ming University, Taipei, Taiwan, Republic of China.</auth-address><titles><title>Identification of the homotypic interaction domain of the core protein of dengue virus type 2</title><secondary-title>J Gen Virol</secondary-title><alt-title>The Journal of general virology</alt-title></titles><periodical><full-title>J Gen Virol</full-title><abbr-1>The Journal of general virology</abbr-1></periodical><alt-periodical><full-title>J Gen Virol</full-title><abbr-1>The Journal of general virology</abbr-1></alt-periodical><pages>2307-14</pages><volume>85</volume><number>Pt 8</number><keywords><keyword>Active Transport, Cell Nucleus</keyword><keyword>Dengue Virus/*chemistry/classification</keyword><keyword>Precipitin Tests</keyword><keyword>Viral Core Proteins/*chemistry</keyword></keywords><dates><year>2004</year><pub-dates><date>Aug</date></pub-dates></dates><isbn>0022-1317 (Print)0022-1317



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298 (Linking)</isbn><accession-num>15269372</accession-num><urls><related-
299 urls><url>http://www.ncbi.nlm.nih.gov/pubmed/15269372</url></related-
300 urls></urls><electronic-resource-num>10.1099/vir.0.80067-0</electronic-resource-
301 num></record></Cite></EndNote>]. However, in the G11A RNA-transfected cells, the C
302 protein was mainly restricted to the cytoplasm with rare nucleus localization (Fig. 1E). (ii)
303 prM protein was located at the ER region in the WT RNA-transfected cells, whereas the
304 prM protein was undetectable in the G11A RNA-transfected cells (Fig. 1F). (iii) E protein
305 spread broadly like an ER web in the WT RNA-transfected cells. In contrast, in the G11A
306 RNA-transfected cells, the E protein formed larger speckles at restricted subcellular areas
307 (Fig. 1G). Compared with the structural proteins, the effect of NS2A G11A on the
308 subcellular expression of viral nonstructural proteins (including NS1, NS3, NS4A, NS4B,
309 and NS5) and dsRNA was not apparent (Fig. 1E-G, *top panels* for NS3, and data not
310 shown).

Comment [RAL2]: ?

311 The distinct effect of NS2A G11A mutation on subcellular expression between the
312 viral structural and nonstructural proteins were further supported by immunofluorescent
313 co-staining experiments. NS2A G11A altered the co-staining profile of E and NS3 proteins
314 (Fig. S2). In the NS2A G11A RNA-transfected cells, 100% of NS3-expressing cells were
315 E-positive at 24 h post-transfection; however, at 48 and 72 h post-transfection, only 59%
316 and 20% of the NS3-expressing cells were E-positive, respectively (Fig. S2). In the WT
317 RNA-transfected cells, 100% of NS3-expressing cells were E-positive from 24 to 72 h
318 post-transfection (Fig. S2). The results demonstrate that NS2A G11A mutation changes
319 the expression and subcellular localization of viral structural proteins.

320 **NS2A G11A mutation impairs the cleavage of viral C-prM polyprotein.** To
321 determine the molecular mechanism of the above observations, we examined the
322 processing of viral proteins in the WT and NS2A G11A viral RNA-transfected cells at 48 h



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post-transfection by Western blot. As expected, the amounts of C, NS3, and NS5 proteins from the G11A RNA-transfected cells were 15-26% of those from the WT RNA-transfected cells (Fig. 1H-I), due to the lack of virion production or multiple rounds of infection for the G11A mutant. Notably, the amounts of prM and E proteins were further lower in the G11A RNA-transfected cells (only 1-4% of those in the WT RNA-transfected cells), suggesting an asymmetric degradation in cells. Moreover, the NS2A G11A mutation impaired the cleavage of C-prM to individual C and prM proteins: 90% of C-prM was cleaved to mature C protein in the WT RNA-transfected cells, whereas only 20% of C-prM was processed to C protein in the G11A RNA-transfected cells (Fig. 1H, *compare lanes 1 and 5 in the top panel*). The impaired C-prM cleavage could account for the altered subcellular distribution of viral structural proteins in the NS2A C11A RNA-transfected cells: Restricting the un-cleaved C-prM to the ER membrane, preventing C protein from translocating to the nucleus, and consequently blocking prM/E heterodimer formation and virion budding.

Next, we examined the role of the ubiquitin-proteasome pathway in degrading viral proteins in the G11A RNA-transfected cells. Treatment with proteasome inhibitor MG132 increased the amounts of C, prM, E, and NS5 (but not NS3) by 1-3 folds in the NS2A G11A RNA-transfected cells (Fig. 1H, *compare lanes 5 and 6*). In contrast, in the WT RNA-transfected cells, MG132 slightly reduced the amounts of C, prM, and E to different extents (Fig. 1H, *compare lanes 1 and 2*) because MG132 was known to inhibit flavivirus replication [ ADDIN EN.CITE <EndNote><Cite><Author>Bhuvanakantham</Author><Year>2013</Year><RecNum>108</RecNum><DisplayText>(Bhuvanakantham and Ng, 2013)</DisplayText><record><rec-number>108</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793288">108</key></foreign-keys><ref-type name="Journal



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Article">17</ref-type><contributors><authors><author>Bhuvanakantham, R.</author><author>Ng, M. L.</author></authors></contributors><auth-address>Flavivirology Laboratory, Department of Microbiology, Yong Loo Lin School of Medicine, National University of Singapore, 5 Science Drive 2, Singapore, 117597.</auth-address><titles><title>West Nile virus and dengue virus capsid protein negates the antiviral activity of human Sec3 protein through the proteasome pathway</title><secondary-title>Cell Microbiol</secondary-title></titles><periodical><full-title>Cell Microbiol</full-title></periodical><pages>1688-706</pages><volume>15</volume><number>10</number><keywords><keyword>Antiviral Agents/*antagonists & inhibitors</keyword><keyword>Capsid Proteins/genetics/*metabolism</keyword><keyword>Cell Line</keyword><keyword>DNA Mutational Analysis</keyword><keyword>Dengue Virus/*physiology</keyword><keyword>*Host-Pathogen Interactions</keyword><keyword>Humans</keyword><keyword>Models, Biological</keyword><keyword>Proteasome Endopeptidase Complex/*metabolism</keyword><keyword>Protein Binding</keyword><keyword>Protein Interaction Mapping</keyword><keyword>Proteolysis</keyword><keyword>Vesicular Transport Proteins/*antagonists & inhibitors</keyword><keyword>West Nile virus/*physiology</keyword></keywords><dates><year>2013</year><pub-dates><date>Oct</date></pub-dates></dates><isbn>1462-5822 (Electronic)1462-5814 (Linking)</isbn><accession-num>23522008</accession-num><urls><related-urls><url>https://www.ncbi.nlm.nih.gov/pubmed/23522008</url></related-urls></urls><electronic-resource-num>10.1111/cmi.12143</electronic-resource-num></record></Cite></EndNote>]. Distinct responses to MG132 treatment among different viral proteins were ~~probably~~ likely due to their subcellular distributions and involvement in various biological microenvironments. Nevertheless, the results suggest



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that ubiquitin-proteasome pathway is responsible for viral protein degradation in the NS2A G11A RNA-transfected cells.

**WT NS2A rescues virion assembly of NS2A G11A mutant through restoring C-prM cleavage.** We examined if ~~*trans*~~ supply of WT NS2A in *trans* could restore virion assembly of NS2A G11A RNA and rescue C-prM protein processing (Fig.S3A). Indeed, after transfection into NS2A-EGFP BHK-21 cells ~~that~~ constitutively expressinged WT NS2A protein, the NS2A G11A RNA produced infectious virions that could infect naïve BHK-21 cells, as indicated by E-positive cells (Fig. S3B). However, f~~;~~ further passaging of culture supernatants to new BHK-21 cells did not yield any E-positive cells (Fig. S3C), ~~suggesting~~ indicating that the *trans* packaged virions could only infect cells for a single-round. Remarkably, the *trans* supplied WT NS2A protein restored the C-prM cleavage in the G11A RNA-transfected cells to WT level, exhibiting >89% C protein processing (Fig. S3D, *top panel*). Two prM bands, representing glycosylated and un-glycosylated forms of the protein (see below), were detected in both NS2A G11A and WT RNA-transfected NS2A-EGFP cells (Fig. S3D, *second panel*). The relative abundances of C, prM, and NS1 proteins were similar in the NS2A G11A and WT RNA-transfected cells (Fig. S3D). ~~Because~~ Due to~~of~~ the restored C-prM cleavage, the nucleus localization of C protein and prM-positive cells were detected in the G11A RNA-transfected NS2A-EGFP cells (Fig. S3E). These results demonstrate that WT NS2A can rescue virion assembly of NS2A G11A mutant through the restorationing ~~the~~ of C-prM cleavage.

Next, we examined the effect of NS2A on the glycosylation of prM by co-expressing NS2A and prM in HEK293T cells. As a control, when EGFP was co-expressed, only the glycosylated form of prM was detected (Fig. S3F); after PGNaseF treatment, the prM was completely deglycosylated and migrated ~~as~~ similarly to a glycosylation-knockout mutant N69Q prM (Fig. S3G). In contrast, when NS2A-EGFP ~~were~~ was co-expressed,



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both glycosylated and un-glycosylated forms of prM were detected (Fig. S3F), indicating that NS2A suppresses the glycosylation of prM. Furthermore, the G11A mutation seemed to increase the suppression effect of NS2A on prM glycosylation (Fig. 3F). These results suggest that NS2A may interact with prM, and that the G11A mutation may modulate the prM/NS2A interaction.

**Capsid R99S mutation can rescue virion assembly of NS2A G11A RNA.** We selected revertants by continuously passaging the NS2A G11A RNA-transfected BHK-21 cells for five rounds (Fig. S4A). Culture medium from each passage~~ing~~ (P1-P5) ~~were~~ was tested for ~~emergence~~ presence of infectious virus particle~~s~~ using plaque assay. The P5 culture produced infectious viruses with titers of $>10^4$ PFU/ml and plaques smaller than the WT virus (Fig. S4B). In addition, almost all P5 cells were E protein-positive (Fig. S4C). Whole genome sequencing of the P5 viruses from three independent selections consistently showed a consensus C-to-A substitution at nucleotide position 391 of viral genome, resulting in an Arg-to-Ser change at amino acid position 99 of the C protein (C_R99S; Fig. 2A). The original NS2A G11A mutation was retained in all P5 viruses.

To validate the role of C_R99S mutation in rescuing virion assembly of NS2A G11A RNA, we engineered C_R99S into a DENV-2 infectious clone [ ADDIN EN.CITE <EndNote><Cite><Author>Xie</Author><Year>2013</Year><RecNum>85</RecNum><DisplayText>(Xie et al., 2013)</DisplayText><record><rec-number>85</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793217">85</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Xie, X.</author><author>Gayen, S.</author><author>Kang, C.</author><author>Yuan, Z.</author><author>Shi, P. Y.</author></authors></contributors><auth-address>State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Science, China.</auth-



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424 address><titles><title>Membrane topology and function of dengue virus NS2A
425 protein</title><secondary-title>J Virol</secondary-title><alt-title>Journal of virology</alt-
426 title></titles><periodical><full-title>J Virol</full-title></periodical><pages>4609-
427 22</pages><volume>87</volume><number>8</number><edition>2013/02/15</edition><k
428 eywords><keyword>Animals</keyword><keyword>Cell
429 Line</keyword><keyword>Cricetinae</keyword><keyword>Dengue
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431 Reticulum/*virology</keyword><keyword>Humans</keyword><keyword>Intracellular
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438 538X (Linking)</isbn><accession-num>23408612</accession-num><work-type>Research
439 Support, Non-U.S. Gov't</work-type><urls><related-
440 urls><url>http://www.ncbi.nlm.nih.gov/pubmed/23408612</url></related-
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442 12</electronic-resource-num><language>eng</language></record></Cite></EndNote>] in
443 the presence ~~or~~ and absence of NS2A G11A. BHK-21 cells were electroporated with WT,
444 NS2A G11A, C_R99S, or NS2A G11A+C_R99S viral RNA. The transfected cells were
445 analyzed for virus production and C-prM cleavage. In the NS2A G11A+C_R99S RNA-
446 transfected cells, the percentage of E-positive cells increased from 10% to 50% at 24 h
447 and 96 h post-transfection, suggesting the spread of infectious virus (Fig. 2B); indeed, the
448 transfected cells produced infectious viruses with plaques smaller than the WT virus (Fig.
449 2C); the viral titer increased over time, reaching >$10^5$ PFU/ml at 96-120 h post-transfection



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(Fig. 2D). The prM and NS1 proteins exhibited ER-like distributions in the NS2A G11A+C_R99S RNA-transfected cells, similar to that observed in the WT RNA-transfected cells (Fig. 2E). Viral C protein was detected in both cytoplasm and nucleus in the NS2A G11A+C_R99S RNA-transfected cells (Fig. 2F-G). Furthermore, 90.5% of the C-prM protein was cleaved to mature C and prM in the NS2A G11A+C_R99S RNA-transfected cells (Fig. 2G). As controls, NS2A G11A RNA showed 15.4% of C-prM cleavage and did not produce any infectious virus, whereas C_R99S RNA exhibited 99.9% of prM-C cleavage and produced a WT level of infectious virus (Fig. 2B-H). Taken together, the results demonstrate that C_R99S mutation efficiently restores C-prM cleavage and virion assembly of NS2A G11A RNA.

**Capsid R99S cannot rescue virion assembly of other NS2A mutants.** We tested ~~if~~ whether C_R99S could rescue other NS2A mutants ~~that were~~ previously shown to be defective in virion assembly, including E20A, E100A, and K188A [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. The C_R99S was engineered into those NS2A mutants, resulting in NS2A E20A+C_R99S, NS2A E100A+C_R99S, and NS2A K188A+C_R99S viral RNAs. After transfection into BHK-12 cells, none of the mutant RNAs produced increasing number of E-positive cells from 24 to 96 h (Fig. S5A). No infectious virus was detected in the culture medium from the mutant RNA-transfected cells (Fig. S5B). Viral C protein was detected in both nucleus and cytoplasm of all mutant RNA-transfected cells (Fig. S5C). In agreement with the C nucleus localization, the NS2A single mutant E20A and E100A cleaved 36% and 79% of C-prM, and the addition of C_R99S increased the C-prM cleavage to 72% and 96%, respectively. In contrast, NS2A K188A mutant cleaved 100% of C-prM in the presence or absence of C_R99S (Fig. 5D). These results demonstrate that C_R99S can only rescue the assembly defect of NS2A G11A possibly because different NS2A mutations abolish virion assembly through distinct mechanisms.



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**Capsid R99S shifts the C-prM cleavage site by two amino acids.** The above results prompted us to investigate how C_R99S rescues the virion assembly of NS2A G11A. Fig. S6 depicts the topology of viral polyprotein C-prM-E-NS1-NS2A on the ER membrane. NS2A G11 resides at the first amphipathic helix on the ER lumen side [ ADDIN EN.CITE

<EndNote><Cite><Author>Xie</Author><Year>2013</Year><RecNum>85</RecNum><DisplayText>(Xie et al., 2013)</DisplayText><record><rec-number>85</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793217">85</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Xie, X.</author><author>Gayen, S.</author><author>Kang, C.</author><author>Yuan, Z.</author><author>Shi, P. Y.</author></authors></contributors><auth-address>State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Science, China.</auth-address><titles><title>Membrane topology and function of dengue virus NS2A protein</title><secondary-title>J Virol</secondary-title><alt-title>Journal of virology</alt-title></titles><periodical><full-title>J Virol</full-title></periodical><pages>4609-22</pages><volume>87</volume><number>8</number><edition>2013/02/15</edition><keywords><keyword>Animals</keyword><keyword>Cell Line</keyword><keyword>Cricetinae</keyword><keyword>Dengue Virus/*physiology</keyword><keyword>Endoplasmic Reticulum/*virology</keyword><keyword>Humans</keyword><keyword>Intracellular Membranes/*metabolism</keyword><keyword>Magnetic Resonance Spectroscopy</keyword><keyword>Models, Molecular</keyword><keyword>Protein Conformation</keyword><keyword>Viral Nonstructural Proteins/*chemistry/metabolism</keyword><keyword>*Virus Assembly</keyword></keywords><dates><year>2013</year><pub-



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dates><date>Apr</date></pub-dates></dates><isbn>1098-5514 (Electronic)0022-538X (Linking)</isbn><accession-num>23408612</accession-num><work-type>Research Support, Non-U.S. Gov't</work-type><urls><related-urls><url>http://www.ncbi.nlm.nih.gov/pubmed/23408612</url></related-urls></urls><custom2>3624351</custom2><electronic-resource-num>10.1128/JVI.02424-12</electronic-resource-num><language>eng</language></record></Cite></EndNote>], while C_R99 is the penultimate amino acid of the C protein on the cytosolic side (Fig. S6B). Since flavivirus NS2B/NS3 protease cleaves a substrate with two basic residues (Arg or Lys) at the P1 and P2 sites and a residue with short side chain (Gly, Ser, or Ala) at the P1' site [ ADDIN EN.CITE ADDIN EN.CITE.DATA ], sequence analysis suggests that the C_R99S substitution may shift the capsid C-terminal cleavage by two amino acids from position 100 to 98 (Fig. S6B). Specifically, viral NS2B/NS3 protease cleaves the WT capsid at NRRRR↓TA and the R99S capsid at NRR↓SRTA (↓ denoting cleavage site and residue R99S underlined). To test this hypothesis, we performed a biochemical protease assay using synthetic WT peptide (NILNRRRRTAGMII) and R99S peptide (NILNRRSRTAGMII). After incubating the peptides with recombinant DENV-2 NS2B/NS3 protease, the reaction products were analyzed by liquid chromatography-mass spectrometry (LC-MS). The WT peptide was cleaved into two smaller peptides with molecular weights of 1096.73 Da and 604.77 Da, corresponding to peptides NILNRRRR and TAGMII, respectively (Fig. S6C). In contrast, the R99S peptide was cleaved into two fragments with molecular weights of 784.47 Da and 847.46 Da, corresponding to peptide NILNRR and SRTAGMII, respectively (Fig. S6D). The LC-MS also detected some unknown peptides, probably due to nonspecific degradations of the protease reaction (Fig. S6C-D). Nevertheless, the data indicate that the C_R99S mutation shifts the capsid cleavage site upstream by two amino acids.



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**NS2A G11A enhances prM/NS2A interaction.** Since NS2A G11A alters the processing of C-prM (Fig. 1H) and prM glycosylation (Fig. S3F), we hypothesized that NS2A directly interacts with prM and the G11A mutation affects the prM/NS2A interaction. To test this hypothesis, we co-transfected two plasmids into HEK293T cells. One plasmid expressed the authentic signal peptide anchorC ($C_{14}$) fused with the full-length prM. Another plasmid expressed a fusion protein containing the C-terminal 24 amino acids of the E protein, the N-terminal 50 amino acids of the NS1 protein, the C-terminal 50 amino acids of NS1, full length NS2A, and an EGFP [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]; this plasmid was previously shown to express NS2A-EGFP with a correct topology on the ER membrane [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. Co-immunoprecipitation showed that NS2A-EGFP, but not EGFP, pulled down prM (Fig. 3B, lanes 2 and 3). As controls, mouse IgG did not pull down any NS2A-EGFP or prM (Lane 1), and EGFP antibody did not pull down prM in the absence of NS2A-EGFP (Lane 6). To map the region of prM that binds to NS2A, we expressed the first 130 amino acids of prM [prM(1-130)] that lacked the C-terminal two transmembrane domains (Fig. 3A). Co-immunoprecipitation showed that prM(1-130) could not pull down NS2A-EGFP (Fig. 3B, lane 4), suggesting that (i) NS2A may bind to the transmembrane region of prM or (ii) the transmembrane region of prM may affect the conformation of its ectodomain and, consequently, abolish its interaction with NS2A.

Next, we examined if NS2A mutation G11A affects the prM/NS2A interaction. Co-immunoprecipitation experiments showed that G11A NS2A-EGFP pulled down about 2.5-fold more prM than the WT NS2A-EGFP did (Fig. 4C). Reciprocally, prM pulled down about 1.7-fold more G11A NS2A-EGFP than WT NS2A-EGFP (Fig. 4D). The results indicate that the G11A mutation enhances NS2A binding to prM.



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To further support the prM/NS2A interaction, we used confocal microscopye to examine the co-localization of prM/NS2A in cells. In this case, prM-E were co-expressed with NS2A-EGFP in HEK293T cells. As shown in Fig. 3E, NS2A-EGFP co-localized with prM. The G11A mutation did not change the co-localization of NS2A-EGFP with prM (Fig. 3E). Taken together, these results demonstrate that (i) NS2A interacts with prM and (ii) NS2A G11A significantly enhances the prM/NS2A interaction.

**The G11A mutation does not affect NS2A binding to E or NS3.** We also examined the interactions between NS2A and E or NS3 proteins that were co-expressed in HEK293T cells. For testing the E/NS2A interaction, we fused a GST tag to the C-terminus of NS2A for the co-immunoprecipitation experiment [because the EGFP tag used in NS2A-EGFP in Fig. 3B-D non-specifically interacts with E protein (data not shown)]. As shown in Fig. 3F, both WT and G11A NS2A-GST pulled down E protein at comparable efficiencies. As a control, GST alone did not pull-down E protein. The result suggests that NS2A interacts with E and the G11A mutation does not affect this interaction.

Since flavivirus NS3 is known to participate in virion assembly [ ADDIN EN.CITE ADDIN EN.CITE.DATA ], we also tested if NS2A G11A mutation affects the NS2A/NS3 interaction. When co-expressed in HEK293T cells, GST alone pulled down a trace amount of HA-tagged NS3 (NS3-HA) protein (Fig. S7, lane 1), whereas NS2A-GST pulled down 10-fold more NS3-HA (Lane 2). Equivalent amounts of NS3-HA were pulled down by the WT and G11A NS2A-GST (Compare lanes 2 and 3), suggesting that NS2A G11A does not affect NS2A/NS3 binding.

**Extension of two or three residues at the N-terminus of anchorC efficiently rescues virion assembly of NS2A G11A**. So far, our results showed that NS2A G11A mutation increases its binding to prM on the ER lumen side, leading to impaired C-prM



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574 processing and defective virion assembly. These defects can be rescued by a capsid
575 mutation R99S that shifts the C-terminal cleavage site by two amino acids. As a result of
576 this shift, the N-terminus of transmembrane anchorC is extended by two amino acids on
577 the cytosolic side of ER membrane. These data support a model of coordinated C-prM
578 processing during virion assembly (Fig. 4A) [ ADDIN EN.CITE
579 <EndNote><Cite><Author>Amberg</Author><Year>1999</Year><RecNum>295</RecNu
580 m><DisplayText>(Amberg and Rice, 1999a)</DisplayText><record><rec-
581 number>295</rec-number><foreign-keys><key app="EN" db-
582 id="etas5sxzr5s5d2edf05vas5e0zt9epvrvepr"
583 timestamp="1443861966">295</key></foreign-keys><ref-type name="Journal
584 Article">17</ref-type><contributors><authors><author>Amberg, S.
585 M.</author><author>Rice, C. M.</author></authors></contributors><auth-
586 address>Department of Molecular Microbiology, Washington University School of
587 Medicine, St. Louis, Missouri 63110-1093, USA.</auth-
588 address><titles><title>Mutagenesis of the NS2B-NS3-mediated cleavage site in the
589 flavivirus capsid protein demonstrates a requirement for coordinated
590 processing</title><secondary-title>J Virol</secondary-title><alt-title>Journal of
591 virology</alt-title></titles><periodical><full-title>J Virol</full-title><abbr-1>Journal of
592 virology</abbr-1></periodical><alt-periodical><full-title>J Virol</full-title><abbr-1>Journal
593 of virology</abbr-1></alt-periodical><pages>8083-
594 94</pages><volume>73</volume><number>10</number><keywords><keyword>Capsid/*
595 metabolism</keyword><keyword>Flavivirus/*metabolism</keyword><keyword>Mutation</
596 keyword><keyword>Protein Processing, Post-Translational</keyword><keyword>RNA
597 Helicases</keyword><keyword>Serine Endopeptidases</keyword><keyword>Viral
598 Nonstructural
599 Proteins/genetics/*metabolism</keyword></keywords><dates><year>1999</year><pub-



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dates><date>Oct</date></pub-dates></dates><isbn>0022-538X (Print)0022-538X (Linking)</isbn><accession-num>10482557</accession-num><urls><related-urls><url>http://www.ncbi.nlm.nih.gov/pubmed/10482557</url></related-urls></urls><custom2>112824</custom2></record></Cite></EndNote>]. During WT virus assembly, polyprotein C-anchorC-prM was first cleaved at the C-terminus of capsid protein (*i.e.*, R100) by NS2B/NS3 protease, producing mature C and anchorC-prM. The anchorC-prM was then cleaved by a host signalase at the N-terminus of prM, generating anchorC and prM. The cleaved prM coordinates with E protein for virion budding (Fig. 4A, *left panel*). In the presence of NS2A G11A mutation, NS2A binds to prM with a higher affinity, pulling anchorC (embedded in the ER membrane) towards the lumen side. The viral protease cleavage site of the "pulled anchorC" is blocked, possibly by the ER membrane (*middle panel*), leading to unprocessed C-prM. To overcome this defect, the C_R99S mutation creates a new NS2A/NS3 cleavage site at R98 of capsid protein, restoring the C-prM processing and virion assembly of NS2A G11A (*right panel*).

The key of the above model is the blockage of viral protease cleavage site at the C-terminus of capsid protein, which is caused by the NS2A G11A mutation. If the model is correct, an insertion of one or two amino acids to the N-terminus of anchorC should restore the C-prM cleavage and virion assembly in the presence of NS2A G11A mutation (Fig. 4B). To test this hypothesis, we inserted Thr, Thr-Ala, or Ser-Arg into the C-terminus of capsid protein (after R100) in the context of WT or NS2A G11A viral RNA. The inserted amino acids were selected based on the cleavage requirement for viral protease (Fig. 4B). Transfection of each viral RNA into BHK-21 cells yielded increasing number of E-positive cells from day 1 to 4 (Fig. 4C), produced infectious viruses (Fig. 4D-E), displayed cytosolic and nucleus localizations of C protein (Fig. 4F), and exhibited efficient C-prM cleavage (Fig. 4H). In the absence of NS2A G11A, the capsid insertion mutant RNAs behaved



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similarly to the WT RNA. In the presence of NS2A G11A, the capsid single-amino acid insertion (*i.e.*, NS2A G11A+C_T101) partially restored virion assembly ($1.2\times10^3$ PFU/ml; Fig. 4D-E), prM-positive cells (Fig. 4G), and C-prM cleavage (60%; Fig. 4H); while the capsid double-amino acid insertion (*i.e.*, NS2A G11A+C_TA and NS2A G11A+C_SR) almost fully restored virion assembly to the WT level ($2.3\text{-}2.5\times10^6$ PFU/ml; Fig. 4D-E) and C-prM cleavage (92-98%; Fig. 4H). These results demonstrate that addition of one or two amino acids to the N-terminus of anchorC can rescue virion assembly of NS2A G11A.

Since the single-amino acid C_T101 insertion only partially rescued virion assembly of NS2A G11A, we selected revertants for NS2A G11A+C_T101 virus. After continuous passaging of the virus on Vero cells for eight rounds, the P8 viruses were subjected to plaque assay (Fig. S8A-B) and whole-genome sequencing (Fig. S8C). The P8 viruses from all four independent selections produced viral titers (Fig. S8B) that were ≥100-fold higher than the original NS2A G11A+C_T101 virus (Fig. 4E). Among them, selection I P8 virus retained the original NS2A G11A+C_T101 mutations. In addition, an extra mutation R100S was recovered in the capsid protein. Sequence analysis suggests that the R100S could alter the protease cleavage site from the original RRRR↓T to RRR↓ST (R100S underlined) and extends the N-terminus of anchorC by one more amino acid. Selection II P8 virus also retained the original NS2A G11A+C_T101 mutations and accumulated another mutation R99S in the capsid protein. The R99S may change the protease cleavage site to RR↓SRT and extends the N-terminus of anchorC by two more amino acids. In addition, selection II P8 virus contained the R100S mutation in 27% of the viral population. Selection III P8 virus retained the NS2A G11A mutation and showed mixed sequences of R99S and/or R100S. Selection IV P8 virus exhibited WT plaque morphology; sequencing results showed that the NS2A G11A was reverted to WT Gly, but the C_T101 insertion was retained. Collectively, the results again support the conclusion



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650 that extension of the N-terminus of anchorC by two to three residues could efficiently
651 rescue virion assembly of NS2A G11A.

652 **Analysis of NS2A G11A in mosquito cells.** To test if the above findings from
653 mammalian cells could be extended to mosquito host, we analyzed NS2A G11A+capsid
654 mutant RNAs in C6/36 cells. Viral RNAs containing NS2A G11A alone, NS2A
655 G11A+C_T101, NS2A G11A+C_R99S, or NS2A G11A+C_TA were transfected into C6/36
656 cells. The transfected cells were analyzed for capsid protein-positive cells (Fig. S9A),
657 infectious virus production (Fig. S9B-C), extracellular viral RNA release (Fig. S9D), and C-
658 prM cleavage (Fig. S9E). Similar to the results obtained from mammalian cells, NS2A
659 G11A mutant was defective in C-prM processing and virion production in C6/36 cells. The
660 defects could be rescued by the capsid mutations, with the rescuing efficiency of C_TA >
661 C_R99S > C_T101 (Fig. S9). The results indicate that NS2A G11A mutation exerts the
662 same mechanism in abolishing virion assembly in mammalian and mosquito hosts.

663 **NS2A protein binds to 3'UTR RNA**. To further explore the role of DENV-2 NS2A
664 in virion assembly, we examined the ability of NS2A to bind viral genomic RNA. A series of
665 pulldown experiments were performed using biotinylated viral RNAs and NS2A protein
666 (Fig. 5A). A 616-nucleotide viral RNA (5'UTR-$C_{66}$-3'UTR) comprising the 5'UTR, the first
667 66 nucleotides from the C gene, and the 3'UTR regions of DENV-2 genome was *in vitro*
668 synthesized (Fig. S10A). The 3' end of the RNA was ligated to a biotinylated cytidine using
669 T4 RNA ligase, resulting in 5'UTR-$C_{66}$-3'UTR-biotin RNA (Fig. S10B). Two types of GST-
670 tagged NS2A protein were prepared for the pulldown experiment: One type NS2A was
671 expressed from *E. coli*, partially purified, and reconstituted in detergent DDM-containing
672 buffer (Fig. 5B); another type NS2A was from the cell lysates of HEK293T cells transiently
673 expressing the NS2A protein (Fig. 5C). As shown in Fig. 5B-C, the 5'UTR-$C_{66}$-3'UTR-biotin
674 RNA efficiently pulled down GST-tagged NS2A (Lanes 4 and 6), but not GST (Lanes 2



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and 5); in contrast, neither GST nor GST-tagged NS2A proteins was pulled down by a biotinylated non-viral RNA control (Lanes 1 and 3). To further demonstrate the specificity of NS2A/5'UTR-$C_{66}$-3'UTR RNA interaction, we performed an RNA competition experiment. Increasing amounts un-biotinylated 5'UTR-$C_{66}$-3'UTR RNA competed away the NS2A/5'UTR-$C_{66}$-3'UTR-biotin RNA interaction, whereas yeast tRNA did not (Fig. 5D). These results suggest that 5'UTR-$C_{66}$-3'UTR RNA specifically binds to NS2A protein.

We estimated the binding affinity of NS2A/viral RNA interaction. 5'UTR-$C_{66}$-3'UTR-biotin RNA was incubated with increasing amounts of recombinant GST-NS2A protein. The reactions were separated on a native polyacrylamide gel (PAGE), transferred to nylon membrane, and probed with streptavidin-HRP (Fig. 5E). Quantification of free RNA and RNA/protein complex estimated a *Kd* of 35 nM for DENV-2 NS2A biding to viral RNA.

**The last 285 nucleotides of 3'UTR RNA is responsible for binding to NS2A protein**. To map the region of viral RNA responsible for binding to NS2A protein, we performed a competition assay using a set of un-biotinylated RNA fragments from 5'UTR-$C_{66}$-3'UTR (Fig. S10C). The fragments were prepared based on the previously proposed RNA structure [ ADDIN EN.CITE <EndNote><Cite><Author>Villordo</Author><Year>2015</Year><RecNum>109</RecNum><DisplayText>(Villordo et al., 2015)</DisplayText><record><rec-number>109</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1561296709">109</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Villordo, S. M.</author><author>Filomatori, C. V.</author><author>Sanchez-Vargas, I.</author><author>Blair, C. D.</author><author>Gamarnik, A. V.</author></authors></contributors><auth-address>Fundacion Instituto Leloir-CONICET, Buenos Aires, Argentina.Arthropod-borne and Infectious Diseases Laboratory,



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700 Department of Microbiology, Immunology and Pathology, Colorado State University, Fort
701 Collins, Colorado, United States of America.</auth-address><titles><title>Dengue virus
702 RNA structure specialization facilitates host adaptation</title><secondary-title>PLoS
703 Pathog</secondary-title></titles><periodical><full-title>PLoS Pathog</full-
704 title></periodical><pages>e1004604</pages><volume>11</volume><number>1</number
705 ><keywords><keyword>3' Untranslated Regions</keyword><keyword>Adaptation,
706 Biological/genetics</keyword><keyword>Animals</keyword><keyword>Cells,
707 Cultured</keyword><keyword>Cricetinae</keyword><keyword>Culicidae</keyword><key
708 word>Dengue Virus/*genetics</keyword><keyword>Host
709 Specificity/genetics</keyword><keyword>Host-Pathogen
710 Interactions/*genetics</keyword><keyword>Humans</keyword><keyword>Mutation</key
711 word><keyword>*Nucleic Acid Conformation</keyword><keyword>RNA,
712 Viral/*chemistry/genetics</keyword></keywords><dates><year>2015</year><pub-
713 dates><date>Jan</date></pub-dates></dates><isbn>1553-7374 (Electronic)1553-
714 7366 (Linking)</isbn><accession-num>25635835</accession-num><urls><related-
715 urls><url>https://www.ncbi.nlm.nih.gov/pubmed/25635835</url></related-
716 urls></urls><custom2>PMC4311971</custom2><electronic-resource-
717 num>10.1371/journal.ppat.1004604</electronic-resource-
718 num></record></Cite></EndNote>]. As shown in Fig. 5F, 3'UTR, but not 5'UTR-$C_{66}$,
719 competed away the NS2A/5'UTR-$C_{66}$-3'UTR-biotin RNA interaction, indicating that 3'UTR
720 RNA binds to NS2A protein. To further map the region of 3'UTR for NS2A binding, we
721 deleted pseudoknot 1 (Δpk1), pseudoknots 1-2 [Δpk(1-2)], and pseudoknots 1-3 [Δpk(1-3)]
722 from the 3'UTR (Fig. S10C). Only Δpk1 and Δpk(1-2) RNAs, not Δpk(1-3) RNA, could
723 compete away the NS2A/5'UTR-$C_{66}$-3'UTR-biotin RNA interaction (Fig. 5F), suggesting
724 that Δpk(1-2) RNA, representing the last 285 nucleotides of 3'UTR RNA, is required for
725 binding to NS2A protein. This conclusion was further supported by the results that different



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fragments from Δpk(1-2) RNA, including pK(3-4), pk3, and 3'SL, did not compete away the NS2A/5'UTR-$C_{66}$-3'UTR-biotin RNA interaction (Fig. 5F).

Next, we performed a photo-activatable ribonucleoside-enhanced crosslinking (PAR-CL) experiment to define the nucleotides that may interact with NS2A protein (Fig. 5G). The 3'UTR RNA containing [ HYPERLINK "https://en.wikipedia.org/wiki/Photosensitivity" \o "Photosensitivity" ] 4-thiouridine (4-SU) was prepared through *in vitro* transcription in the presence of 4-S-UTP. After RNA/protein complex formation, the 4-thiouridine in the 3'UTR RNA was cross-linked to NS2A protein under UV light (at 365-nm wavelength). The RNA was then subjected to deep sequencing. Due to the crosslinking of 4-thiouridine to protein, a T-to-C mutation was recovered at the RNA sites that interacted with NS2A protein (Figs. 5G and S11). Fig. 5H summarizes the U-to-C mutations in the 3'UTR RNA when complexed with NS2A protein. About 67% mutations occurred within the last 285 nucleotides of 3'UTR. The result further supports the NS2A/3'UTR RNA interaction.

**Comment [RAL3]:** Ratio of 4-S-UTP to UTP?

**Comment [RAL4]:** What would the percentage be if the mutations were randomly spread across the total RNA? i.e. How significant is this enrichment?

**NS2A requires amino acids R94, K95, and K99A for binding to 3'UTR RNA and virion assembly.** To map the region of NS2A required for viral 3'UTR RNA binding, we prepared a panel of purified NS2A truncates with an N-terminal GST tag from *E. coli* (Fig. 6A). The binding efficiencies of NS2A truncates to 5'UTR-$C_{66}$-3'UTR-biotin RNA were compared using a pulldown assay. As shown in Fig. 6B, different NS2A truncates were pulled down by 5'UTR-$C_{66}$-3'UTR-biotin at varied efficiencies. The RNA pulled down NS2A 1-145, 1-168, and 1-218 (full-length) at comparable efficiencies. Compared to the full-length NS2A, the pulldown efficiencies for NS2A 1-101 and 1-71 decreased to 40% and 0%, respectively. These data suggest that amino acids 72-144 are required for NS2A binding to viral RNA.



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749 According to the membrane topology of DENV-2 NS2A [ ADDIN EN.CITE
750 <EndNote><Cite><Author>Nemesio</Author><Year>2014</Year><RecNum>82</RecNu
751 m><DisplayText>(Nemesio and Villalain, 2014)</DisplayText><record><rec-
752 number>82</rec-number><foreign-keys><key app="EN" db-
753 id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv"
754 timestamp="1560793217">82</key></foreign-keys><ref-type name="Journal
755 Article">17</ref-type><contributors><authors><author>Nemesio,
756 H.</author><author>Villalain, J.</author></authors></contributors><auth-
757 address>Molecular and Cellular Biology Institute, Universitas "Miguel
758 Hernandez" , E-03202 Elche-Alicante, Spain.</auth-
759 address><titles><title>Membrane interacting regions of Dengue virus NS2A
760 protein</title><secondary-title>J Phys Chem B</secondary-title></titles><periodical><full-
761 title>J Phys Chem B</full-title></periodical><pages>10142-
762 55</pages><volume>118</volume><number>34</number><keywords><keyword>Calori
763 metry, Differential Scanning</keyword><keyword>Cell
764 Membrane/*metabolism</keyword><keyword>Fluorescence
765 Polarization</keyword><keyword>*Hydrophobic and Hydrophilic
766 Interactions</keyword><keyword>Peptide
767 Library</keyword><keyword>Spectrophotometry, Infrared</keyword><keyword>Viral
768 Nonstructural
769 Proteins/*chemistry/*metabolism</keyword></keywords><dates><year>2014</year><pub
770 -dates><date>Aug 28</date></pub-dates></dates><isbn>1520-5207
771 (Electronic)1520-5207 (Linking)</isbn><accession-num>25119664</accession-
772 num><urls><related-
773 urls><url>http://www.ncbi.nlm.nih.gov/pubmed/25119664</url></related-
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num>10.1021/jp504911r</electronic-resource-num></record></Cite></EndNote>], amino acids 72-144 form two transmembrane domains (72-92 and 101-118), one short cytosolic loop (93-100), and a luminal segment (118-144; Fig. 6A). Since viral RNA resides in the cytoplasm, we examined the cytosolic loop of NS2A for viral RNA binding. The cytosolic loop has three positively charged residues (R94, K95, and K99) that are conserved among the four DENV serotypes (Fig. 6C). To examine the function of these residues, we prepared a panel of recombinant DENV-2 containing Ala substitutions at single (R94A, K95A, or K99A), double (R94A+K95A), or triple residues (R94A+K95A+K99A). Upon transfection to BHK-21 cells, all mutant RNAs generated viral E protein-positive cells at day 1 (Fig. 6D). Except for the triple mutant, other RNAs produced more E-positive cells on day 4 post-transfection. Mutants R94A and K95A generated WT level of infectious viruses and plaque morphology (Fig. 6E-F). Mutants K99A and K94A+K95A generated 22- to 3-fold less viruses than the WT, respectively (Fig. 6F), and exhibited smaller plaques (Fig. 6E). Remarkably, triple mutant R94A+K95A+K99A RNA yielded almost undetectable amount of virus that was 50,000-fold lower than the WT (Fig. 6F). To examine the effect of R94A+K95A+K99A on viral RNA synthesis, we engineered the triple mutations into a luciferase replicon of DENV-2 (Fig. 6G). The R94A+K95A+K99A replicon generated a luciferase profile similar to the WT replicon, indicating that the mutations do not affect viral translation and RNA synthesis. Taken together, the results demonstrate that R94A+K95A+K99A triple mutant is defective in virion assembly.

Next, we examined the effect of R94A+K95A+K99A mutations on NS2A binding to viral RNA. Since the result from Fig. 6B indicates that NS2A fragment 72-144 is required for viral RNA binding, we expressed a short NS2A peptide 64-128 (comprising the first two NS2A transmembrane domains, the cytosolic region 93-100, and the N- and C-terminal luminal tails for correct folding of the peptide on membrane) fused with an N-terminal GST-



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tag (Fig. 6H). As shown in Fig. 6I, peptide 64-128 could be pulled down by 5'UTR-$C_{66}$-3'UTR-biotin RNA (Lane 1). Un-biotinylated 5'UTR-$C_{66}$-3'UTR or 3'UTR RNA (Lanes 2 and 4), but not 5'UTR-$C_{66}$ RNA or yeast tRNA (Lanes 3 and 5), could compete away the NS2A peptide 64-128/5'UTR-$C_{66}$-3'UTR-biotin RNA interaction. The result demonstrates that NS2A peptide 64-128 specifically binds to viral 3'UTR RNA. Importantly, mutant R94A+K95A+K99A reduced the pulldown efficiency of NS2A peptide 64-128 by over 80% (Fig. 6J). Notably, some degraded GST-tag was found to associate with the NS2A truncates in the above pulldown experiments (Figs. 6B I-J), most likely due to the dimerization property of GST_____[ ADDIN EN.CITE <EndNote><Cite><Author>Maru</Author><Year>1996</Year><RecNum>110</RecNum><DisplayText>(Maru et al., 1996)</DisplayText><record><rec-number>110</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1561302524">110</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Maru, Y.</author><author>Afar, D. E.</author><author>Witte, O. N.</author><author>Shibuya, M.</author></authors></contributors><auth-address>Department of Genetics, Institute of Medical Science, University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108, Japan.</auth-address><titles><title>The dimerization property of glutathione S-transferase partially reactivates Bcr-Abl lacking the oligomerization domain</title><secondary-title>J Biol Chem</secondary-title></titles><periodical><full-title>J Biol Chem</full-title></periodical><pages>15353-7</pages><volume>271</volume><number>26</number><keywords><keyword>*Adaptor Proteins, Signal Transducing</keyword><keyword>Animals</keyword><keyword>Bone Marrow Cells</keyword><keyword>Cell Transformation, Neoplastic</keyword><keyword>Cells, Cultured</keyword><keyword>Fusion Proteins, bcr-abl/*metabolism</keyword><keyword>GRB2 Adaptor



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826 Protein</keyword><keyword>Glutathione
827 Transferase/*metabolism</keyword><keyword>Macromolecular
828 Substances</keyword><keyword>Mice</keyword><keyword>Phosphotyrosine/metabolis
829 m</keyword><keyword>Protein Binding</keyword><keyword>Protein-Tyrosine
830 Kinases/metabolism</keyword><keyword>Proteins/metabolism</keyword><keyword>Rat
831 s</keyword><keyword>Recombinant Fusion Proteins</keyword><keyword>Structure-
832 Activity Relationship</keyword></keywords><dates><year>1996</year><pub-
833 dates><date>Jun 28</date></pub-dates></dates><isbn>0021-9258 (Print)0021-
834 9258 (Linking)</isbn><accession-num>8663064</accession-num><urls><related-
835 urls><url>https://www.ncbi.nlm.nih.gov/pubmed/8663064</url></related-
836 urls></urls><electronic-resource-num>10.1074/jbc.271.26.15353</electronic-resource-
837 num></record></Cite></EndNote>]. Overall, the results indicate that R94, K95, and K99
838 are important for NS2A binding to viral 3'UTR.

839 **DISCUSSION**

840 Flavivirus assembly consists of a tightly controlled cascade of events: export of
841 newly synthesized viral RNA from the replication complex to assembly sites, encapsidation
842 of viral RNA with C protein, and envelopment of nucleocapsid with prM and E proteins.
843 Many host and viral factors (proteins and lipids) participate in this temporal and spatial
844 process [ ADDIN EN.CITE <EndNote><Cite><Author>Apte-
845 Sengupta</Author><Year>2014</Year><RecNum>94</RecNum><DisplayText>(Apte-
846 Sengupta et al., 2014)</DisplayText><record><rec-number>94</rec-number><foreign-
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848 timestamp="1560793218">94</key></foreign-keys><ref-type name="Journal
849 Article">17</ref-type><contributors><authors><author>Apte-Sengupta,



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S.</author><author>Sirohi, D.</author><author>Kuhn, R. J.</author></authors></contributors><auth-address>Markey Center for Structural Biology, Department of Biological Sciences and Bindley Bioscience Center, Purdue University, West Lafayette, IN, USA.Markey Center for Structural Biology, Department of Biological Sciences and Bindley Bioscience Center, Purdue University, West Lafayette, IN, USA. Electronic address: kuhnr@purdue.edu.</auth-address><titles><title>Coupling of replication and assembly in flaviviruses</title><secondary-title>Curr Opin Virol</secondary-title></titles><periodical><full-title>Curr Opin Virol</full-title></periodical><pages>134-42</pages><volume>9</volume><keywords><keyword>Flavivirus/*physiology</keyword><keyword>Host-Pathogen Interactions</keyword><keyword>Humans</keyword><keyword>Lipid Metabolism</keyword><keyword>Viral Proteins/metabolism</keyword><keyword>*Virus Assembly</keyword><keyword>*Virus Replication</keyword></keywords><dates><year>2014</year><pub-dates><date>Dec</date></pub-dates></dates><isbn>1879-6265 (Electronic)1879-6257 (Linking)</isbn><accession-num>25462445</accession-num><urls><related-urls><url>http://www.ncbi.nlm.nih.gov/pubmed/25462445</url></related-urls></urls><custom2>PMC4268268</custom2><electronic-resource-num>10.1016/j.coviro.2014.09.020</electronic-resource-num></record></Cite></EndNote>]. Among them, flaviviral NS2A has emerged as a key player to orchestrate virion assembly, as evidenced by the following findings. (i) In infected cells, there are two distinct NS2A species that are required for viral RNA synthesis and virion assembly. The NS2A species required for RNA synthesis is located at the viral replication complex that could only be formed in *cis*; the other NS2A species required for virus morphogenesis is located at the virion assembly complex that could be formed in *cis*



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876 or *trans* [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. (ii) The defect in virion assembly
877 caused by DENV-2 and YFV NS2A mutations could be rescued by adaptive mutations in
878 prM, E, NS2B, and NS3 proteins, suggesting that NS2A genetically modulates other viral
879 proteins during virion assembly [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. (iii)
880 Recombinant NS2A of Kunjin virus binds to viral 3'UTR RNA [ ADDIN EN.CITE
881 <EndNote><Cite><Author>Mackenzie</Author><Year>1998</Year><RecNum>81</RecN
882 um><DisplayText>(Mackenzie et al., 1998)</DisplayText><record><rec-number>81</rec-
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886 M.</author><author>Khromykh, A. A.</author><author>Jones, M.
887 K.</author><author>Westaway, E.
888 G.</author></authors></contributors><titles><title>Subcellular localization and some
889 biochemical properties of the flavivirus Kunjin nonstructural proteins NS2A and
890 NS4A</title><secondary-title>Virology</secondary-title></titles><periodical><full-
891 title>Virology</full-title></periodical><pages>203-
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893 /keyword><keyword>Cercopithecus aethiops</keyword><keyword>*Flavivirus/ph
894 [Physiology]</keyword><keyword>Immunohistochemistry</keyword><keyword>RNA,
895 Viral/me [Metabolism]</keyword><keyword>Support, Non-U.S.
896 Gov't</keyword><keyword>Vero Cells</keyword><keyword>*Viral Nonstructural
897 Proteins/me [Metabolism]</keyword><keyword>Virus
898 Replication</keyword></keywords><dates><year>1998</year></dates><urls></urls></re
899 cord></Cite></EndNote>]. These studies indicate that NS2A interacts with two of the three
900 structural proteins (prM and E, not C) and viral RNA. However, how viral RNA and C
901 protein are recruited to form nucleocapsid and how nucleocapsid is enveloped with prM-E



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proteins to form virion remained unknown. The current study aimeds to address these questions.

First, we showed that DENV-2 NS2A interacted with prM to modulate virion assembly. WT NS2A directly bound to prM in the absence of other viral proteins (Fig. 3B). The biological relevance of this interaction was supported by the correlation between virion assembly and prM/NS2A interaction. Specifically, an NS2A mutation G11A, which abolished virion assembly (Fig. 1), enhanced its binding to prM by 1.7-2.5 folds (Fig. 3). Mechanistically, the enhanced interaction between the mutant NS2A and prM "over pulled" the membrane-associated anchorC towards the ER lumen, leading to a blockage of the viral protease cleavage site (most likely by the ER membrane) at the C-anchorC junction. The uncleaved C-prM resulted in a rapid degradation of all three structural proteins (Fig. 1). This is not surprising because the unprocessed C-prM is retained on the ER membrane, misfolded, and degraded via the ubiquitin-proteasome machinery (Fig. 1H). Since prM is a chaperone for E protein [ ADDIN EN.CITE <EndNote><Cite><Author>Lorenz</Author><Year>2002</Year><RecNum>96</RecNum><DisplayText>(Lorenz et al., 2002)</DisplayText><record><rec-number>96</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793218">96</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Lorenz, I. C.</author><author>Allison, S. L.</author><author>Heinz, F. X.</author><author>Helenius, A.</author></authors></contributors><auth-address>Institute of Biochemistry, Swiss Federal Institute of Technology, CH-8093 Zurich, Switzerland.</auth-address><titles><title>Folding and dimerization of tick-borne encephalitis virus envelope proteins prM and E in the endoplasmic reticulum</title><secondary-title>J Virol</secondary-title></titles><periodical><full-title>J



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942 num>10.1128/jvi.76.11.5480-5491.2002</electronic-resource-
943 num></record></Cite></EndNote>], the degradation of C-prM leads to the degradation of
944 E protein. These results are in agreement with a recent report that ZIKV anchorC plays an
945 important role in the stability of E protein and virion morphogenesis [ ADDIN EN.CITE
946 <EndNote><Cite><Author>Rana</Author><Year>2018</Year><RecNum>97</RecNum><
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951 Campos, J. L.</author><author>Leccese, G.</author><author>Francolini,
952 M.</author><author>Bestagno, M.</author><author>Poggianella,



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955 Biotechnology, Trieste, Italy rana@icgeb.org burrone@icgeb.org.Molecular
956 Immunology Group, International Centre for Genetic Engineering and Biotechnology,
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958 Universita degli Studi di Milano, Milan, Italy.</auth-address><titles><title>Role of Capsid
959 Anchor in the Morphogenesis of Zika Virus</title><secondary-title>J Virol</secondary-
960 title></titles><periodical><full-title>J Virol</full-
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964 keyword>pseudovirus</keyword><keyword>viral
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970 num>10.1128/JVI.01174-18</electronic-resource-num></record></Cite></EndNote>].

971 Second, we demonstrated that the defect in virion assembly of NS2A G11A could
972 be restored by two different means. Creation of a new viral protease cleavage site by
973 extending the N-terminus of anchorC by two amino acids restored the capsid-anchorC
974 processing and, consequently, restored virion assembly. This conclusion was supported
975 by revertant analysis of the NS2A G11A mutant (Figs. 2 and S4) and amino acid insertion
976 experiment (Fig. 4). The two-amino acid extension exposes the new protease cleavage
977 site to NS2B/NS3. Alternatively, the virion assembly defect of NS2A G11A could be



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978 rescued by *trans* supply of WT NS2A (Fig. S3). Under this condition, the WT NS2A
979 replaced the G11A NS2A to interact with C-prM, restored the C-prM cleavage, and ensued
980 a productive virion morphogenesis. Collectively, our results support a previous model that
981 the two cleavages at C-anchorC-prM junctions are coordinated in a sequential manner
982 during flavivirus assembly. The C-anchorC junction was first cleaved by viral protease on
983 the cytosolic side, followed by the anchorC-prM cleavage by the cellular signalase on the
984 ER lumen side [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. Although expression of
985 flavivirus prM-E alone could form empty virus-like particles, the coordinated C-anchorC-
986 prM processing couples the encapsidation and envelopment events; such coupling is
987 required for the production of infectious virus. This conclusion is also supported by ~~the~~
988 previous reports that separate expression of C and prM-E significantly compromised the
989 assembly efficiency of infectious virions [ ADDIN EN.CITE ADDIN EN.CITE.DATA ].

990 Third, we characterized the specific interaction between DENV-2 NS2A and the
991 3'UTR of viral genome. Although Kunjin virus NS2A was reported to bind to the 3'UTR of
992 viral RNA [ ADDIN EN.CITE
993 <EndNote><Cite><Author>Mackenzie</Author><Year>1998</Year><RecNum>81</RecN
994 um><DisplayText>(Mackenzie et al., 1998)</DisplayText><record><rec-number>81</rec-
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996 timestamp="1560793217">81</key></foreign-keys><ref-type name="Journal
997 Article">17</ref-type><contributors><authors><author>Mackenzie, J.
998 M.</author><author>Khromykh, A. A.</author><author>Jones, M.
999 K.</author><author>Westaway, E.
1000 G.</author></authors></contributors><titles><title>Subcellular localization and some
1001 biochemical properties of the flavivirus Kunjin nonstructural proteins NS2A and
1002 NS4A</title><secondary-title>Virology</secondary-title></titles><periodical><full-



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cord></Cite></EndNote>], the molecular details of the interaction has not been delineated. Using competition assay and PAR-CLIP technique, we mapped that DENV-2 NS2A bound to the last 285 nucleotides of the 3'UTR, including the pk3, pk4, and 3'SL. Interestingly, the pk3, pk3-4, or 3'SL RNA alone did not efficiently compete out the NS2A/3'UTR RNA interaction (Fig. 5F), suggesting that a complex RNA structure in the 3'UTR is required for efficient binding to NS2A protein. Since no RNA “packaging signal” has been identified for flavivirus assembly and the C protein was shown to non-specifically bind to any RNA [ADDIN EN.CITE
<EndNote><Cite><Author>Pong</Author><Year>2011</Year><RecNum>102</RecNum>
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L.</author><author>Huang, Z. S.</author><author>Teoh, P. G.</author><author>Wang,
C. C.</author><author>Wu, H. N.</author></authors></contributors><auth-
address>Institute of Molecular Biology, Academia Sinica, Taipei, Taiwan.</auth-
address><titles><title>RNA binding property and RNA chaperone activity of dengue virus
core protein and other viral RNA-interacting proteins</title><secondary-title>FEBS



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1047 urls></urls><electronic-resource-num>10.1016/j.febslet.2011.06.038</electronic-resource-
1048 num></record></Cite></EndNote>], the last 285 nucleotides of 3'UTR may serve as the
1049 “packaging signal” to recruit viral RNA to the virion assembly site through specific binding
1050 to NS2A. From the NS2A side, we have mapped the 3'UTR-binding site to a cytosolic loop
1051 (containing R94, K95, and K99) between the first and second transmembrane domains
1052 (Fig. 6). Triple mutant R94A+K95A+K99A was defective in virion assembly and lost over
1053 80% of the NS2A binding activity to 3'UTR RNA (Fig. 6). Caution should be taken when
1054 applying the DENV-2 findings to other flaviviruses because of ~~the~~ sequence variations ~~in~~of



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NS2A proteins. The three positively charged R94, K95, and K99 of NS2A are only conserved among the four serotypes of DENV. In addition, DENV-2, YFV, and ZIKV NS2A proteins exhibit different topologies on the ER membrane [ ADDIN EN.CITE ADDIN EN.CITE.DATA ].

Our findings support a general model for flavivirus virion assembly (Fig. 7). In infected cells, NS2A molecules reside in both replication complex (housed in Ve) and virion assembly complex (located on the juxtaposed ER membrane). When plus-sense RNAs are made and released from the replication complex (through the pore on Ve), they are initially destined to new rounds of viral translation and replication on the ER. Once ~~the amounts of~~ viral RNA and proteins have accumulated over certain "thresholds", nascent RNAs are ~~switched for~~directed to virion assembly. At this time, the newly synthesized RNA exits the replication complex and binds to the NS2A located at the assembly site through the 3'UTR RNA. The NS2A molecules at the assembly site also recruits C-prM-E and NS2B/NS3 through binding to prM and NS3, respectively. ~~One~~ Single molecules of NS2A ~~molecule does~~ not ~~have to~~necessarily bind to the 3'UTR RNA, C-prM-E, and NS2B/NS3 at the same time. Rather, t~~T~~hese interactions could be sequential or through ~~different~~ mulitple NS2A molecules that oligomerize together on the ER membrane [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. Next, the C-prM-E polyprotein is sequentially cleaved by the NS2B/NS3 protease and host signalase to produce mature C, prM, and E [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. The C molecules, many of which have been previously made and stored at the nearby lipid droplets [ ADDIN EN.CITE ADDIN EN.CITE.DATA ], bind viral RNA to form nucleocapsid. The processed prM undergoes conformational change and dimerizes with E. The prM/E dimerization and oligomerization dissociate prM from NS2A. When 180 copies of prM/E oligomerize on the ER membrane, envelopment of the nucleocapsid occurs, generating spiky immature virions. During the



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above process, NS3 may also use its helicase activity to transport viral RNA from the replication complex to the assembly site and condense the RNA during encapsidation. Besides NS3, NS2A or other proteins may also contribute to the translocation of viral RNA from the replication complex to the assembly site.

The above model is centered on the multifunctional role of NS2A in flavivirus replication and virion assembly. Our results have functionally demonstrated the molecular mechanism for two important steps during DENV-2 assembly: (i) The binding of 3'UTR RNA to NS2A servers as a "packaging signal" and (ii) in the presence of a viral RNA-NS2A complex, the coordinated cleavage of C-prM-E facilitates the envelopment of nucleocapsid. The proposed model could serve as a framework for future investigations and antiviral development.

**Author contribution**

X.X., J.Z., X.Z., Y.Z., Q.W., H.-P.D. performed experiments and data analysis. X.X., J.Z., X.Z., Y.Z., A.R., V.L.P., X.W., Q.W., H.-P.D, P.-Y.S. designed the experiments and interpreted the results. X.X., P.-Y.S. wrote the manuscript.

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1106 Biomedical Research (NIBR) and the McLaughlin Endowment at University of Texas
1107 Medical Branch, respectively.



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## Figure titles and legends

**Figure 1 Characterization of NS2A G11A mutant**. (A) Replicon transient transfection assay. Top panel depicts the DENV-2 luciferase replicon. Bottom panel shows the luciferase activities after transfecting BHK-21 cells with equal amounts (10 μg) of WT or NS2A G11A mutant replicon RNA. (B) Quantification of extracellular viral RNA. Top panel depicts the full-length genome of DENV-2. Equal amounts of WT or NS2A G11A mutant viral RNA (10 μg) were electroporated into BHK-21 cells. At different time post-transfection (p.t.), extracellular viral RNAs were quantified by RT-qPCR. The DENV-2 replicon RNA was used as a control for determining the basal level of viral RNA released from dead cells. Bottom panel shows the CT values of the RT-qPCR results. Data show the means and standard deviations from three independent experiments. (C) Plaque morphology. Culture supernatants from (B) were analyzed for virus production using plaque assay. Only WT, not NS2A G11A mutant (N.D.), produced plaques. Plaques were developed on BHK-21 cells after 5 days of infection. (D) Thin-section transmission electron microscope (TEM) images of viral RNA-transfected BHK-21 cells. At 48 h p.t., cells were fixed for TEM analysis. Ve, virus-induced vesicles; T, membrane tubes; Vi, virus particles; ER, endoplasmic reticulum. Mock transfected cells were included as a control. (E-G) Confocal microscopy analysis of subcellular localizations of C, prM, and E in viral RNA-transfected BHK-21 cells. At 48 h p.t., transfected cells were analyzed by IFA. NS3 staining (red) was used as a control. Nuclei (blue) were stained by DAPI. Scale bar, 10 μm. (H) Viral protein processing in viral RNA-transfected BHK-21 cells. At 42 h p.t., BHK-21 (Mock) or viral RNA-transfected cells were treated with 0.01% DMSO (-) or 10 μM MG132 (+). After 6 h treatment, the cells were analyzed by Western blot. (I) Densitometry analysis. The intensities of bands representing specific viral proteins from DMSO-treated cells in (H) was quantified. The amounts of viral proteins derived from WT RNA-transfected cells were set as 100%. Relative intensities are calculated by normalizing the protein intensities derived from NS2A G11A mutant RNA-transfected cells to those from WT RNA-transfected cells. Data shows the means and standard deviations from three independent experiments.

**Figure 2 Restoration of NS2A G11A virion assembly by capsid R99S mutation.** (A) Revertant mutation. BHK-21 cells were transfected with NS2A G11A viral RNA. The transfected cells were consecutively passaged for five rounds to select for revertant viruses. The P5 virus was sequenced for revertant mutations. Changes in nucleotide



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positions and amino acids are indicated. (B) IFA of viral RNA-transfected cells. WT and mutant viral RNAs were electroporated to BHK-21 cells. The transfected cells were analyzed for E protein expression at 24 and 96 h p.t. by IFA. Scale bar, 100 μm. (C) Plaque morphology. Infectious viruses, derived from the RNA-transfected cells, were analyzed by plaque assay. Plaques were developed on day 5 post-infection on BHK-21 cells. N.D., not detectable. (D) Virus production. The culture fluids from (B) were quantified for infectious viruses. Data represent the means and standard deviations from three independent measurements. Limit of detection (L.O.D.), 10 PFU/ml. (E-F) Subcellular distributions of prM, NS1, and C proteins. prM, NS1 and C proteins were detected by DM1, human IgG anti-NS1 and D2C1 antibodies, respectively. Representative IFA images of viral RNA-transfected cells are shown at 24 h post-transfection. White arrows point to nucleus with C protein speckles. (G) Quantitative analysis of C-positive cells. The C-positive cells with cytoplasm or cytoplasm & nucleus distribution from 4-5 random fields were counted and normalized to the total cell numbers. The averages and standard deviations are shown. (H) Viral protein processing in viral RNA-transfected BHK-21 cells at 48 h post-transfection. The percentages of mature C protein were calculated from the following the formula: [C intensity/(C intensity + C-prM intensity)]×100%.

**Figure 3 DENV-2 NS2A interacts with prM and E proteins**. (A) Membrane topologies of DENV-2 prM, E, and NS2A proteins. The NS2A G11 position is indicated. (B) Co-IP of WT NS2A with prM. HEK293T cells were transfected with one plasmid (encoding prM only) or two plasmids (one encoding leader-NS2A-EGFP and the other encoding full-length or the ectodomain of prM). At 40 h p.t., IP was performed using mouse anti-EGFP or control IgG antibodies. Immunoblot (IB) was performed using rabbit anti-EGFP antibody or anti-prM antibody. (C) Co-IP of WT or G11A NS2A-EGFP with prM. Mouse anti-EGFP antibody was used for IP. Rabbit anti-EGFP or anti-prM antibodies were used for IB. The intensity of prM, co-precipitated with WT NS2A-EGFP, was set as 100%. The relative efficiency of prM co-precipitated with G11A NS2A-EGFP was compared with the WT NS2A-EGFP. Statistical analysis was performed using the unpaired parametric t-test. $*p < 0.05$, significant; $**p < 0.01$, very significant. (D) Reciprocal co-IP of prM with WT or G11A NS2A-EGFP. Rabbit anti-prM antibodies were used for IP. The amounts of co-precipitated WT and G11A NS2A-EGFP were detected using mouse anti-EGFP antibody. prM in eluates were detected by rabbit anti-prM antibody followed by protein G conjugated with HRP. (E) Co-localization analysis of NS2A and prM proteins. BHK-21 cells were co-



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transfected with two plasmids: One encoding the $C_{14}$-prM-E and the other encoding SPG-$C_{16}$-NS2A-EGFP. At 24 h p.t., the cells were examined for prM and NS2A-EGFP co-localization under a confocal microscope. Scale bar, 10 μm. (F) Co-IP of NS2A with E protein. HEK293T cells were co-transfected with two plasmids: One encoding the last 17 amino acids of prM and complete E protein ($M_{17}$-E), and another encoding NS2A with a C-terminal GST tag (NS2A-GST). GST tag alone was used as a negative control. IPs were performed using rabbit anti-GST antibody. E and GST fusion proteins were detected in cell lysates and eluates by mouse anti-E monoclonal antibody (9F12) and anti-GST antibody, respectively. The relative pulldown efficiency was calculated by normalizing the intensity of E co-precipitated with G11A NS2A-GST to the intensity of E co-precipitated with the WT NS2A-GST. The means and standard deviations from three independent experiments are shown. N.S., no significance.

**Figure 4 Two-residue insertion at the capsid-anchorC junction rescues the defect of NS2A G11A**. (A) A model depicting how NS2A modulates C-anchorC-prM cleavage. On the cytosolic side, viral NS2B/NS3 protease cleaves the C-anchorC junction. On the ER lumen side, host signalase cleaves the anchorC-prM junction. Three cleavage scenarios are presented for WT, NS2A G11A, and NS2A G11A+C_R99S constructs. (B) Constructs of insertion mutants of capsid protein. One residue (C_101) or two residues (C_TA or C_SR) were inserted to the C-anchorC junction. The potential cleavage sites for viral NS2B/NS3 protease are indicated. (C) IFA of viral RNA-transfected cells. Viral RNAs containing the NS2A G11A mutation with or without capsid insertions were electroporated into BHK-21 cells. The transfected cells were analyzed for E protein expression on day 1 and 4 by IFA. Scale bar, 100 μm. (D) Plaque morphology. Viruses recovered from (C) were analyzed by plaque assay on BHK-21 cells. (E) Viral titers. Culture supernatants from (C) were measured for viral titers on day 5 post-transfection. (F) Subcellular localization of C protein in viral RNA-transfected cells. White arrows point to nucleus with C protein speckles. Scale bar, 25 μm. (G) Expression of prM and NS1 proteins in viral RNA-transfected cells. Scale bar, 50 μm. (H) C-prM processing in viral RNA-transfected cells. At 48 h p.t., WT or NS2A G11A+C_T101 RNA-transfected cells were analyzed for C, NS1, and β-actin expression by Western blot. The percentages of C-prM to mature C conversion are presented for each construct.

**Figure 5 DENV-2 NS2A protein binds to 3'UTR RNA**. (A) Diagram of pulldown assay using biotinylated RNAs. 3'-end biotinylated RNA binds to streptavidin-conjugated



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magnetic beads. After incubating the bead-bound biotinylated RNA with NS2A protein, the RNA-bound NS2A was enriched and eluted for SDS-PAGE and Western blot analysis. (B) Pulldown analysis using recombinant NS2A protein. N-terminally GST-tagged NS2A protein (GST-NS2A) was expressed in *E. coli* and affinity purified. The 5'UTR-$C_{66}$-3'UTR-biotin RNA was prepared by ligating biotinylated cytidine (bis)phosphate to the 3' end of the 5'UTR-$C_{66}$-3'UTR RNA (containing the 5'UTR, the first 66 nucleotides of C gene, and the 3'UTR). The amounts of GST-NS2A in the input and eluates were analyzed using rabbit anti-GST antibody by Western blot. (C) Pulldown analysis using HEK293T cell-expressed NS2A-GST protein. HEK293T cells were transfected with a plasmid expressing GST or C-terminally GST-tagged NS2A protein (NS2A-GST). At 42 h p.t., cells were harvested, lysed, and used for RNA pull down-assay. The pulldown assay was performed as described in (B). (D) RNA competition assay. Various amounts of unlabeled 5'UTR-$C_{66}$-3'UTR RNA were used to compete the binding of 5'UTR-$C_{66}$-3'UTR-biotin RNA to GST-NS2A protein. tRNA was used as a negative control. (E) Viral RNA/NS2A binding affinity. Electrophoretic mobility-shift assay (EMSA) was used to determine the binding affinity of DENV-2 5'UTR-$C_{66}$-3'UTR-biotin RNA with NS2A protein. 5'UTR-$C_{66}$-3'UTR-biotin RNA was incubated with increasing amounts of GST-NS2A protein. Free and bound RNAs were resolved by native agarose gel electrophoresis. The equilibrium dissociation constant (*Kd*) was determined by nonlinear regression with least-squares fitting method. The coefficient of determination ($R^2$) is shown. Data represent the means and standard deviations from three independent experiments. (F) Mapping the RNA region responsible for NS2A binding. A panel of unlabeled RNA truncates of the 5'UTR-$C_{66}$-3'UTR were used as competitors for the binding of 5'UTR-$C_{66}$-3'UTR-biotin RNA to GST-NS2A protein. The intensity of GST-NS2A (bound to the 5'UTR-$C_{66}$-3'UTR-biotin) without competitor was set as 100%. Relative GST-NS2A intensities were calculated as [(signal in the presence competitor)/(signal in the absence of competitor)]×100%. Statistical analysis was performed using the unpaired parametric t-test. (G) PAR-CLIP workflow [ ADDIN EN.CITE ADDIN EN.CITE.DATA ]. See text for detail. (H) PAR-CLIP result summary. Uridines in the 3'UTR that may participate in NS2A-binding are indicated. The dash line indicates the threshold (equal to 5.76, representing the average fold change of mutation rate of all uridines determined by Illumina sequencing). The uridine positions with fold changes of mutation rate over the threshold are indicated in the 3'UTR sequence (GenBank no. AF038403).

**Comment [RAL5]:** Need to explain different between black and blue lines (i.e. that they are replciates).



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**Figure 6 NS2A residues responsible for the NS2A/3'UTR RNA interaction**. (A) Diagram of NS2A truncates used in RNA pulldown assay. All proteins were fused with an N-terminal GST tag, expressed in *E. coli*, and affinity purified. (B) Interactions between NS2A truncates with 5'UTR-$C_{66}$-3'UTR-biotin RNA. The interactions were measured using pulldown experiments. The pulldown efficiency of full-length NS2A (1-218) was set as 100%. Relative efficiency was calculated by comparing the intensity of NS2A truncate with that of the full-length NS2A. (C) Sequence alignment of NS2A among flaviviruses. Amino acids of a cytosolic loop (amino acids 93-101) are presented. The number of viral strains used for the NS2A alignment are indicated in parentheses. Amino acid positions of NS2A are labeled according to DENV-2 strain New Guinea C. Residues R94, K95, and K99 are shaded. The conserved (:) and identical (*) residues are indicated. (D) IFA of BHK-21 cells transfected with viral RNA. WT and mutant viral RNAs were electroporated into BHK-21 cells. The cells were assayed for E protein expression. (E) Plaque morphology. Viruses derived from the transfected cells in (D) were analyzed by plaque assay. (F) Viral titers. Culture supernatants from (D) were measured for virus yields on day 5 post-transfection. (G) Replicon transient transfection assay. NS2A triple mutations (R94A+K95A+K99A) were engineered into a luciferase replicon of DENV-2. Luciferase activities were presented after the WT or mutant replicon RNAs were electroporated into BHK-21 cells. (H) Diagram of N-terminally GST-tagged NS2A peptide 64-128 [GST-NS2A (64-128)]. (I) Interaction of GST-NS2A (64-128) protein with 3'UTR RNA. GST-NS2A (64-128) was expressed in *E. coli* and affinity purified. The pulldown efficiency of GST-NS2A (64-128) by 5'UTR-$C_{66}$-3'UTR-biotin was set as 100%. The binding efficiencies of RNA variants were indicated through competition assay. In the presence of competitor RNAs, the remaining amounts of WT GST-NS2A (64-128)/5'UTR-$C_{66}$-3'UTR-biotin interaction are presented. (J) R94A+K95A+K99A mutations reduce GST-NS2A (64-128) binding to 5'UTR-$C_{66}$-3'UTR-biotin RNA. The R94A+K95A+K99A variant retained only 19.1% of the WT activity.

**Figure 7 A model depicting the role of NS2A in DENV-2 assembly.** Viral replication complex (top) and virion assembly site (bottom) are depicted. DENV-2 NS2A protein recruits viral RNA, three structural proteins, and NS2B/NS3 protease to virion assembly site, where coordinated cleavage of C-prM-E ensues infections virus formation. C molecules accumulated on lipid droplets (LD) could bind viral RNA to form nucleocapsid. See text for details.



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**Reference**

[ ADDIN EN.REFLIST ]



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## STAR METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---|---|---|
| Antibodies | | |
| Mouse IgG Anti-C (D2C1) | [ ADDIN EN.CITE ADDIN EN.CITE.DATA ] | N/A |
| Mouse IgG anti-prM (DM1) | Abcam | Cat# ab41473, RRID:AB_873758 |
| Rabbit IgG anti-prM | GeneTex | GTX128093 |
| Mouse IgG anti-E (4G2) | ATCC | Cat# HB-112, RRID:CVCL_J890 |
| Mouse IgG anti-E (9F12) | [ ADDIN EN.CITE ADDIN EN.CITE.DATA ] | N/A |
| Mouse IgG anti-NS1 | Abcam | Cat# ab41616, RRID:AB_869448 |
| Human IgG anti-NS1 | This study. Generated by Novartis Institute for Tropical Diseases in Singapore | N/A |
| Rabbit IgG anti-NS1 | GeneTex | GTX103346 |
| Rabbit IgG anti-NS3 | GeneTex | GTX124252 |
| Mouse IgG anti-NS5 | GeneTex | GTX629447 |
| Rabbit IgG anti-calnexin | SIGMA-AIDRICH | Cat# C4731, RRID:AB_476845 |
| Mouse IgG anti-EGFP | SIGMA-AIDRICH | Cat# 11814460001, RRID:AB_390913 |
| Moue IgG1 Isotype Control | SIGMA-AIDRICH | Cat# M5284, RRID:AB_1163685 |
| Rabbit IgG anti-EGFP | Abcam | Cat# ab13970, RRID:AB_300798 |
| protein G horseradish peroxidase (HRP) conjugate | Thermo Fisher Scientific | Cat# P21041 |
| Mouse IgG anti-GST | Abcam | Cat# ab19256, RRID:AB_444809 |
| Rabbit IgG anti-GST | SIGMA-AIDRICH | Cat# G7781, RRID:AB_259965 |
| Mouse IgG anti-β-actin | Abcam | Cat# ab8226, RRID:AB_306371 |
| Mouse IgG anti-HA | Abcam | Cat# ab18181, RRID:AB_444303 |
| Goat anti-mouse IgG conjugated with HRP | SIGMA-AIDRICH | Cat# A5278, RRID:AB_258232 |
| Goat anti-rabbit IgG conjugated with HRP | SIGMA-AIDRICH | Cat#AP307P, RRID:AB_92641 |
| Goat anti-mouse IgGs conjugated with Alexa 488 | Thermo Fisher Scientific | Cat#A-1100, RRID:AB_2534069 |
| Goat anti-mouse IgGs conjugated with Alexa 594 | Thermo Fisher Scientific | Cat# A-11005, RRID:AB_2534073 |
| Goat anti-rabbit IgGs conjugated with Alexa 488 | Thermo Fisher Scientific | Cat# A-11008, RRID:AB_143165 |



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| | | |
|---|---|---|
| Goat anti-rabbit IgGs conjugated with Alexa 594 | Thermo Fisher Scientific | Cat# A-11072, RRID:AB_2534116 |
| Goat anti-Human IgG (H+L) cross-adsorbed secondary antibody, Alexa Fluor 594 | Thermo Fisher Scientific | Cat# A-11014, RRID:AB_2534081 |
| Bacterial and Virus Strains | | |
| *E.* coli strain Top 10 | ThermoFisher Scientific | Cat#C404006 |
| *E.* coli strain Rosetta™ 2(DE3) pLysS | MilliporeSigma | Cat#71403-4 |



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| | | |
|---|---|---|
| DENV-2 strain New Guinea C | [ ADDIN EN.CITE <EndNote><Cite><Author>Xie</Author><Year>2013</Year><RecNum>85</RecNum><DisplayText>(Xie et al., 2013)</DisplayText><record><rec-number>85</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793217">85</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Xie, X.</author><author>Gayen, S.</author><author>Kang, C.</author><author>Yuan, Z.</author><author>Shi, P. Y.</author></authors></contributors><auth-address>State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Science, China.</auth-address><titles><title>Membrane topology and function of dengue virus NS2A protein</title><secondary-title>J Virol</secondary-title><alt-title>Journal of virology</alt-title></titles><periodical><full-title>J Virol</full-title></periodical><pages>4609-22</pages><volume>87</volume><number>8</number><edition>2013/02/15</edition><keywords><keyword>Animals</keyword><keyword>Cell Line</keyword><keyword>Cricetinae</keyword><keyword>Dengue | N.A. |



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| | | |
|---|---|---|
| Biological Samples | | |
| None | | |
| Chemicals, Peptides, and Recombinant Proteins | | |
| Peptide WT (NILNRRRRTAGMII) | GenScript | N/A |
| Peptide C_R99S (NILNRRSRTAGMII) | GenScript | N/A |
| Recombinant protein: GST | This study | N/A |
| Recombinant protein: GST-NS2A | This study | N/A |
| Recombinant protein: GST-NS2A(1-71) | This study | N/A |
| Recombinant protein: GST-NS2A(1-101) | This study | N/A |
| Recombinant protein: GST-NS2A(1-145) | This study | N/A |
| Recombinant protein: GST-NS2A(1-168) | This study | N/A |
| Recombinant protein: GST-NS2A(64-128) | This study | N/A |
| Critical Commercial Assays | | |
| iScript SYBR Green One-Step kit | Bio-Rad | Cat#170-8893 |
| QIAamp viral RNA mini kit | Qiagen | Cat#52906 |
| T7 mMessage mMachine kit | Thermo Fisher Scientific | Cat# AM1344 |
| Renilla Luciferase Assay System | Promega Corporation | Cat# E2810 |
| Pierce™ RNA 3' End Biotinylation Kit | Thermo Fisher Scientific | Cat# 20160 |
| Pierce™ Magnetic RNA-Protein Pull-Down kit | Thermo Fisher Scientific | Cat# 20164 |
| Chemiluminescent Detection Module | Thermo Fisher Scientific | Cat# 89880 |
| | | |
| Deposited Data | | |
| Raw image data for illumina sequencing | | |
| Experimental Models: Cell Lines | | |
| BHK-21 cell | ATCC | Cat#CCL-10, RRID:CVCL_1915 |
| Vero cell | ATCC | Cat#CCL-81, RRID:CVCL_0059 |
| HEK293T cell | ATCC | Cat# CRL-3216, RRID:CVCL_0063 |
| NS2A-EGFP BHK-21 cell | [ ADDIN EN.CITE ADDIN EN.CITE.DATA ] | N/A |
| | | |
| Experimental Models: Organisms/Strains | | |
| N.A. | | |
| Oligonucleotides | | |
| Primer P1(5'-ACACTCTTTCCCTACACGA CGCTCTTCCGATCTAAGGCAAAACTAACATGAA AC-3') | Integrated DNA Technologies (Skokie, Illinois) | N/A |
| Primer P2 (5'-GTGACTGGAGTTCAGACG TGTGCTCTTCCGATCTAGAACCTGTTGATTCAA CAGCAC-3') | Integrated DNA Technologies (Skokie, Illinois) | N/A |



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| | | |
|---|---|---|
| 5′ universal adaptor (5′-AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGAC-3′) | Illumine (San Diego, CA) | N/A |
| 3′ indexing primer (5′-CAAGCAGAAGACGGCATACGAGATnnnnnnGTGACTGGAGTTCAGACGTGT-3′) | Illumine (San Diego, CA) | N/A |
| Primer NGC 7764V (5′-CGTCGAGAGAAATATGGTCACACC-3′) | Integrated DNA Technologies (Skokie, Illinois) | N/A |
| Primer NGC 7844C (5′CCACAATAGTATGACCAGCCT-3′) | Integrated DNA Technologies (Skokie, Illinois) | N/A |
| Recombinant DNA | | |



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|---|---|---|
| pACYC-NGC FL | [ ADDIN EN.CITE <EndNote><Cite><Author>Xie</Author><Year>2013</Year><RecNum>85</RecNum><DisplayText>(Xie et al., 2013)</DisplayText><record><rec-number>85</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793217">85</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Xie, X.</author><author>Gayen, S.</author><author>Kang, C.</author><author>Yuan, Z.</author><author>Shi, P. Y.</author></authors></contributors><auth-address>State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Science, China.</auth-address><titles><title>Membrane topology and function of dengue virus NS2A protein</title><secondary-title>J Virol</secondary-title><alt-title>Journal of virology</alt-title></titles><periodical><full-title>J Virol</full-title></periodical><pages>4609-22</pages><volume>87</volume><number>8</number><edition>2013/02/15</edition><keywords><keyword>Animals</keyword><keyword>Cell Line</keyword><keyword>Cricetinae</keyword><keyword>Dengue | N/A |



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| | | |
|---|---|---|
| pACYC-NGC replicon | [ ADDIN EN.CITE <EndNote><Cite><Author>Xie</Author><Year>2013</Year><RecNum>85</RecNum><DisplayText>(Xie et al., 2013)</DisplayText><record><rec-number>85</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793217">85</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Xie, X.</author><author>Gayen, S.</author><author>Kang, C.</author><author>Yuan, Z.</author><author>Shi, P. Y.</author></authors></contributors><auth-address>State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Science, China.</auth-address><titles><title>Membrane topology and function of dengue virus NS2A protein</title><secondary-title>J Virol</secondary-title><alt-title>Journal of virology</alt-title></titles><periodical><full-title>J Virol</full-title></periodical><pages>4609-22</pages><volume>87</volume><number>8</number><edition>2013/02/15</edition><keywords><keyword>Animals</keyword><keyword>Cell Line</keyword><keyword>Cricetinae</keyword><keyword>Dengue | N/A |



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| | | |
|---|---|---|
| pACYC-NGC replicon G11A | [ ADDIN EN.CITE ADDIN EN.CITE.DATA ] | N/A |
| pACYC-NGC replicon R94A+K95A+K99A | This study | N/A |
| pACYC-NGC FL C_R99S | This study | N/A |
| pACYC-NGC FL G11A | This study | N/A |
| pACYC-NGC FL G11A+C_R99S | This study | N/A |
| pACYC-NGC FL E20A | This study | N/A |
| pACYC-NGC FL E100A | This study | N/A |
| pACYC-NGC FL K188A | This study | N/A |
| pACYC-NGC FL E20A+C_R99S | This study | N/A |
| pACYC-NGC FL E100A+C_R99S | This study | N/A |
| pACYC-NGC FL K188A+C_R99S | This study | N/A |
| pACYC-NGC FL C_TA | This study | N/A |
| pACYC-NGC FL C_SR | This study | N/A |
| pACYC-NGC FL G11A+C_TA | This study | N/A |
| pACYC-NGC FL G11A+C_SR | This study | N/A |
| pACYC-NGC FL C_T101 | This study | N/A |
| pACYC-NGC FL G11A+C_T101 | This study | N/A |
| pACYC-NGC FL R94A | This study | N/A |
| pACYC-NGC FL K95A | This study | N/A |
| pACYC-NGC FL K99A | This study | N/A |
| pACYC-NGC FL R94A+K95A+K99A | This study | N/A |
| pACYC-NGC FL R94A+K95A | This study | N/A |



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| | | |
|---|---|---|
| SFV-DENV2 CprME | [ ADDIN EN.CITE <EndNote><Cite><Author>Qing</Author><Year>2010</Year><RecNum>105</RecNum><DisplayText>(Qing et al., 2010)</DisplayText><record><rec-number>105</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793220">105</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Qing, M.</author><author>Liu, W.</author><author>Yuan, Z.</author><author>Gu, F.</author><author>Shi, P. Y.</author></authors></contributors><auth-address>Novartis Institute for Tropical Diseases, 10, Biopolis Road, #05-01 Chromos, Singapore, Singapore.</auth-address><titles><title>A high-throughput assay using dengue-1 virus-like particles for drug discovery</title><secondary-title>Antiviral Res</secondary-title></titles><periodical><full-title>Antiviral Res</full-title></periodical><pages>163-71</pages><volume>86</volume><number>2</number><dates><year>2010</year><pub-dates><date>May</date></pub- [ PAGE * MERGEFORMAT ] dates></dates><accession-num>20153777</accession-num><urls><related- | N/A |

| | | |
|---|---|---|
| pXJ-SPG-$C_{16}$-NS2A-EGFP | This study | N/A |
| pXJ-SPG-$C_{16}$-NS2A(G11A)-EGFP | This study | N/A |
| pXJ-EGFP | [ ADDIN EN.CITE ADDIN EN.CITE.DATA ] | N/A |
| pXJ-$C_{14}$-prM | This study | N/A |
| pXJ-$C_{14}$-prM(1-130) | This study | N/A |
| pXJ-$C_{14}$-prM (N69Q) | This study | N/A |
| pXJ-SPG-$C_{16}$-NS2A-GST | This study | N/A |
| pXJ-SPG-$C_{16}$-NS2A(G11A)-GST | This study | N/A |
| pXJ-GST | This study | N/A |
| pXJ-HA-NS3 | This study | N/A |
| pXJ-$M_{17}$-E | This study | N/A |
| pGEX-4T-1 | GE Healthcare Life Sciences | Cat# 28954549 |
| pGEX-4T-GST-NS2A | This study | N/A |
| pGEX-4T-GST-NS2A (1-71) | This study | N/A |
| pGEX-4T-GST-NS2A (1-101) | This study | N/A |
| pGEX-4T-GST-NS2A (1-145) | This study | N/A |
| pGEX-4T-GST-NS2A (1-168) | This study | N/A |
| pGEX-4T-GST-NS2A (64-128) | This study | N/A |
| pGEX-4T-GST-NS2A (64-128) R94A+K95A+K99A | This study | N/A |
| | | |
| Software and Algorithms | | |
| Illustrator CC | Adobe | [ HYPERLINK "https://www.adobe.com/products/illustrator.html" ] |
| ImageJ | NIH | [ HYPERLINK "https://fiji.sc/" ] |
| CLC main workbench | Qiagen | [ HYPERLINK "https://www.qiagenbioinformatics.com/products/clc-main-workbench/" ] |
| Prism 8.0 software | GraphPad | [ HYPERLINK "https://www.graphpad.com/scientific-software/prism/" ] |
| | | |

## Contact for Reagent and Resource Sharing

Further information and requests for resources and reagents should be directed to and will be fulfilled by Lead Contact, Dr. Pei-Yong Shi (peshi@utmb.edu)

## Experimental Model and Subject Details



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**Cell Lines**

The Baby hamster kidney fibroblast (BHK-21) cells, African green monkey kidney epithelial (Vero) cells were purchased from the American Type Culture Collection (ATCC, Bethesda, MD) and maintained in a high-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (HyClone Laboratories, South Logan, UT) and 1% penicillin/streptomycin (P/S). The NS2A-EGFP BHK-21 cells constitutively expressing the C-terminally EGFP-tagged NS2A protein was maintained in the high-glucose DMEM medium supplemented with 10%FBS, 1% P/S and 1 mg/ml Geneticin. HEK293T cells were grown in low-glucose DMEM (Invitrogen) containing 10% FBS and 1% penicillin-streptomycin. All mammalian cell lines were grown at 37°C with 5% $CO_2$. Mosquito C6/36 cells were grown in RPMI 1640 medium (Invitrogen) with 10% FBS and 1% penicillin-streptomycin at 28°C without $CO_2$. All culture medium and antibiotics were purchased from ThermoFisher Scientific (Waltham, MA). All cell lines were tested negative for mycoplasma.

**Pseudo-infectious Particle**

Pseudo-infectious particles were produced using the a previously described protocol [ ADDIN EN.CITE <EndNote><Cite><Author>Qing</Author><Year>2010</Year><RecNum>114</RecNum><DisplayText>(Qing et al., 2010)</DisplayText><record><rec-number>114</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1561745637">114</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Qing, M.</author><author>Liu, W.</author><author>Yuan, Z.</author><author>Gu, F.</author><author>Shi, P. Y.</author></authors></contributors><auth-address>Novartis Institute for Tropical Diseases, 10, Biopolis Road, #05-01 Chromos, Singapore, Singapore.</auth-address><titles><title>A high-throughput assay using dengue-1 virus-like particles for drug discovery</title><secondary-title>Antiviral Res</secondary-title></titles><periodical><full-title>Antiviral Res</full-title></periodical><pages>163-71</pages><volume>86</volume><number>2</number><keywords><keyword>Animals</keyword><keyword>Antiviral Agents/*isolation & purification/*pharmacology</keyword><keyword>Biological Assay/methods</keyword><keyword>Cell Line</keyword><keyword>Cercopithecus



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aethiops</keyword><keyword>Cricetinae</keyword><keyword>Dengue Virus/*drug effects/genetics</keyword><keyword>Drug Discovery/*methods</keyword><keyword>Drug Evaluation, Preclinical/*methods</keyword><keyword>Electroporation</keyword><keyword>Genes, Reporter</keyword><keyword>Luciferases, Renilla/genetics/metabolism</keyword><keyword>Macaca mulatta</keyword><keyword>Viral Proteins/genetics/metabolism</keyword><keyword>Virosomes/*drug effects/genetics</keyword></keywords><dates><year>2010</year><pub-dates><date>May</date></pub-dates></dates><isbn>1872-9096 (Electronic)0166-3542 (Linking)</isbn><accession-num>20153777</accession-num><urls><related-urls><url>https://www.ncbi.nlm.nih.gov/pubmed/20153777</url></related-urls></urls><electronic-resource-num>10.1016/j.antiviral.2010.02.313</electronic-resource-num></record></Cite></EndNote>] with several modifications. 10 µg of replicon RNA was electroporated into BHK-21 cells as described above. After transfection, the cells were maintained at 37°C with 5% $CO_2$ for 24 h. Afterwards, cells were harvested and electroporated with 10 µg of a helper replicon RNA SFV-DENV2 CprME (replicon derived from Semliki forest virus that express the DENV-2 encoding the CprME). After the second electroporation, cells were cultured at 30°C with 5% $CO_2$. At 20, 28 and 48 h after the second electroporation, culture medium was collected and stored at -80 °C. Afterwards, the collected medium was used ton infection naïve Vero cells in 96-well plates. At 48 h post-infection, cells were lysed, and luciferase signals were measured using the Renilla luciferase assay system (Promega) in the Clarity luminescence microplate reader (BioTek) according to the manufacturer's protocol.

**METHOD DETAILS**

**Plasmid Construction**

The full-length cDNA infectious clone of DENV-2 strain New Guinea C (NGC), pACYC-NGC FL, and its derived replicon (pACYC-NGC replicon) clone [ ADDIN EN.CITE <EndNote><Cite><Author>Xie</Author><Year>2013</Year><RecNum>85</RecNum><DisplayText>(Xie et al., 2013)</DisplayText><record><rec-number>85</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793217">85</key></foreign-keys><ref-type name="Journal



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Article">17</ref-type><contributors><authors><author>Xie, X.</author><author>Gayen, S.</author><author>Kang, C.</author><author>Yuan, Z.</author><author>Shi, P. Y.</author></authors></contributors><auth-address>State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Science, China.</auth-address><titles><title>Membrane topology and function of dengue virus NS2A protein</title><secondary-title>J Virol</secondary-title><alt-title>Journal of virology</alt-title></titles><periodical><full-title>J Virol</full-title></periodical><pages>4609-22</pages><volume>87</volume><number>8</number><edition>2013/02/15</edition><keywords><keyword>Animals</keyword><keyword>Cell Line</keyword><keyword>Cricetinae</keyword><keyword>Dengue Virus/*physiology</keyword><keyword>Endoplasmic Reticulum/*virology</keyword><keyword>Humans</keyword><keyword>Intracellular Membranes/*metabolism</keyword><keyword>Magnetic Resonance Spectroscopy</keyword><keyword>Models, Molecular</keyword><keyword>Protein Conformation</keyword><keyword>Viral Nonstructural Proteins/*chemistry/metabolism</keyword><keyword>*Virus Assembly</keyword></keywords><dates><year>2013</year><pub-dates><date>Apr</date></pub-dates></dates><isbn>1098-5514 (Electronic)0022-538X (Linking)</isbn><accession-num>23408612</accession-num><work-type>Research Support, Non-U.S. Gov't</work-type><urls><related-urls><url>http://www.ncbi.nlm.nih.gov/pubmed/23408612</url></related-urls></urls><custom2>3624351</custom2><electronic-resource-num>10.1128/JVI.02424-12</electronic-resource-num><language>eng</language></record></Cite></EndNote>] were used as the backbones for mutagenesis study. The replicon was constructed by replacing most of the structural genes (C, prM and E) with a gene cassette (Rluc2A) encoding the Renilla luciferase and a 2A peptide from Foot-and-mouth disease virus. The first 38 amino acids of C protein and the last 31 amino acids of E protein were retained in the replicon. Standard overlap PCR was performed to introduce the mutations in C and NS2A genes. The DNA fragments containing the mutations in C gene were cloned into pACYC-NGC FL clone through restriction enzyme sites NotI (upstream of the T7 promoter and 5'UTR of DENV-2) and SphI (at the position of 1384 in DENV-2 genome). The DNA fragments containing mutations in NS2A gene were cloned into pACYC-NGC FL or replicon through restriction enzyme sites and NheI (at the position of 2544 in DENV-2 genome) and SacI (at the position of 4342 in the DENV-2 genome).



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A mammalian expression vector, pXJ [ ADDIN EN.CITE <EndNote><Cite><Author>Xie</Author><Year>2013</Year><RecNum>85</RecNum><DisplayText>(Xie et al., 2013)</DisplayText><record><rec-number>85</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793217">85</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Xie, X.</author><author>Gayen, S.</author><author>Kang, C.</author><author>Yuan, Z.</author><author>Shi, P. Y.</author></authors></contributors><auth-address>State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Science, China.</auth-address><titles><title>Membrane topology and function of dengue virus NS2A protein</title><secondary-title>J Virol</secondary-title><alt-title>Journal of virology</alt-title></titles><periodical><full-title>J Virol</full-title></periodical><pages>4609-22</pages><volume>87</volume><number>8</number><edition>2013/02/15</edition><keywords><keyword>Animals</keyword><keyword>Cell Line</keyword><keyword>Cricetinae</keyword><keyword>Dengue Virus/*physiology</keyword><keyword>Endoplasmic Reticulum/*virology</keyword><keyword>Humans</keyword><keyword>Intracellular Membranes/*metabolism</keyword><keyword>Magnetic Resonance Spectroscopy</keyword><keyword>Models, Molecular</keyword><keyword>Protein Conformation</keyword><keyword>Viral Nonstructural Proteins/*chemistry/metabolism</keyword><keyword>*Virus Assembly</keyword></keywords><dates><year>2013</year><pub-dates><date>Apr</date></pub-dates></dates><isbn>1098-5514 (Electronic)0022-538X (Linking)</isbn><accession-num>23408612</accession-num><work-type>Research Support, Non-U.S. Gov't</work-type><urls><related-urls><url>http://www.ncbi.nlm.nih.gov/pubmed/23408612</url></related-urls></urls><custom2>3624351</custom2><electronic-resource-num>10.1128/JVI.02424-12</electronic-resource-num><language>eng</language></record></Cite></EndNote>], driven by a cytomegalovirus (CMV) promoter was used to express various viral proteins prM, E, NS2A and NS3 fusion proteins in HEK293T cells. For constructs pXJ-$C_{14}$-prM and pXJ-$C_{14}$-prM (1-130), the anchor C signal peptide ($C_{14}$) was retained at the N-terminus of prM to ensure the correct targeting on ER membrane. For construct pXJ-$M_{17}$-E, the last 17 amino acids from M protein was retained to serve as a signal peptide for translocating the E protein into the ER lumen. For constructs pXJ-SPG-$C_{16}$-NS2A-EGFP and pXJ-SPG-$C_{16}$-



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NS2A-GST, the signal peptide derived from Gaussia luciferase (SPG) [ ADDIN EN.CITE <EndNote><Cite><Author>Verhaegent</Author><Year>2002</Year><RecNum>112</RecNum><DisplayText>(Verhaegent and Christopoulos, 2002)</DisplayText><record><rec-number>112</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1561735944">112</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Verhaegent, M.</author><author>Christopoulos, T. K.</author></authors></contributors><auth-address>Department of Chemistry and Biochemistry, University of Windsor, Ontario, Canada.</auth-address><titles><title>Recombinant Gaussia luciferase. Overexpression, purification, and analytical application of a bioluminescent reporter for DNA hybridization</title><secondary-title>Anal Chem</secondary-title></titles><periodical><full-title>Anal Chem</full-title></periodical><pages>4378-85</pages><volume>74</volume><number>17</number><keywords><keyword>Animals</keyword><keyword>DNA/*analysis</keyword><keyword>DNA Probes</keyword><keyword>Genes, Reporter</keyword><keyword>*Luciferases/genetics/isolation & purification</keyword><keyword>Luminescent Proteins/genetics/isolation & purification</keyword><keyword>Plankton/*enzymology</keyword><keyword>Recombinant Proteins/genetics/isolation & purification</keyword></keywords><dates><year>2002</year><pub-dates><date>Sep 1</date></pub-dates></dates><isbn>0003-2700 (Print)0003-2700 (Linking)</isbn><accession-num>12236345</accession-num><urls><related-urls><url>https://www.ncbi.nlm.nih.gov/pubmed/12236345</url></related-urls></urls></record></Cite></EndNote>] and the last 16 amino acids of NS1 ($C_{16}$) was fused at the N-terminus of NS2A to ensure the correct processing and membrane topology of NS2A. The EGFP and GST were amplified from pEGFPluc (Clontech) and pGEX-4T-1 (Sigma-Aldrich) respectively. The Human influenza hemagglutinin (HA) tag was fused to the N-terminus of NS3 and cloned into pXJ vector, resulting the construct pXJ-HA-NS3.

pGEX-4T-1 (GE Healthcare Life Sciences) was used as the vector for expressing all N-terminally glutathione S-transferase (GST)-tagged NS2A fusion proteins. The DNA fragments containing various length of NS2A were cloned into pGEX-4T-1 through restriction enzyme sites BamHI and XhoI.



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All plasmids were propagated in *E. coli* strain Top10 (ThermoFisher Scientific). All restriction enzymes were purchased from New England BioLabs (Ipswitch, MA). The plasmids were validated by restriction enzyme digestion and Sanger DNA sequencing. All primers were synthesized from Integrated DNA Technologies (Skokie, Illinois) and are available upon request.

**RNA Transcription and Electroporation**

The NGC FL or replicon plasmids were linearized by restriction enzyme XbaI. RNA transcription and electroporation were performed as described previously [ ADDIN EN.CITE <EndNote><Cite><Author>Xie</Author><Year>2013</Year><RecNum>85</RecNum><DisplayText>(Xie et al., 2013)</DisplayText><record><rec-number>85</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793217">85</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Xie, X.</author><author>Gayen, S.</author><author>Kang, C.</author><author>Yuan, Z.</author><author>Shi, P. Y.</author></authors></contributors><auth-address>State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Science, China.</auth-address><titles><title>Membrane topology and function of dengue virus NS2A protein</title><secondary-title>J Virol</secondary-title><alt-title>Journal of virology</alt-title></titles><periodical><full-title>J Virol</full-title></periodical><pages>4609-22</pages><volume>87</volume><number>8</number><edition>2013/02/15</edition><keywords><keyword>Animals</keyword><keyword>Cell Line</keyword><keyword>Cricetinae</keyword><keyword>Dengue Virus/*physiology</keyword><keyword>Endoplasmic Reticulum/*virology</keyword><keyword>Humans</keyword><keyword>Intracellular Membranes/*metabolism</keyword><keyword>Magnetic Resonance Spectroscopy</keyword><keyword>Models, Molecular</keyword><keyword>Protein Conformation</keyword><keyword>Viral Nonstructural Proteins/*chemistry/metabolism</keyword><keyword>*Virus Assembly</keyword></keywords><dates><year>2013</year><pub-dates><date>Apr</date></pub-dates></dates><isbn>1098-5514 (Electronic)0022-538X (Linking)</isbn><accession-num>23408612</accession-num><work-type>Research Support, Non-U.S. Gov't</work-type><urls><related-



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urls><url>http://www.ncbi.nlm.nih.gov/pubmed/23408612</url></related-urls></urls><custom2>3624351</custom2><electronic-resource-num>10.1128/JVI.02424-12</electronic-resource-num><language>eng</language></record></Cite></EndNote>]. Briefly, 10 μg RNA was electroporated into 8 × $10^6$ cells in a 4-mm cuvette using the GenePulser apparatus (Bio-Rad) at settings of 0.45 kV (C6/36 cells) or 0.85 kV (BHK-21 cells) and 25 mF, pulsing three times, with 3 s intervals. After a 10-min recovery at room temperature, transfected cells were suspended in culture media and incubated at corresponding conditions: 28°C (C6/36 cells) or 37°C with 5% $CO_2$ (BHK-21).

**Virus Production and Quantification**

After electroporation, the transfected BHK-21 cells were seeded in one T-75 flask. After incubation at 37°C for 24 h, the cells were cultured in DMEM with 2% FBS at 30°C for another 4 days. At every 24 h p.t., culture fluids was collected and stored at -80°C. Viral titer was determined by crystal violet-based plaque assay using BHK-21 cells according to the protocol as described previously [ ADDIN EN.CITE <EndNote><Cite><Author>Xie</Author><Year>2013</Year><RecNum>85</RecNum><DisplayText>(Xie et al., 2013)</DisplayText><record><rec-number>85</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1560793217">85</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Xie, X.</author><author>Gayen, S.</author><author>Kang, C.</author><author>Yuan, Z.</author><author>Shi, P. Y.</author></authors></contributors><auth-address>State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Science, China.</auth-address><titles><title>Membrane topology and function of dengue virus NS2A protein</title><secondary-title>J Virol</secondary-title><alt-title>Journal of virology</alt-title></titles><periodical><full-title>J Virol</full-title></periodical><pages>4609-22</pages><volume>87</volume><number>8</number><edition>2013/02/15</edition><keywords><keyword>Animals</keyword><keyword>Cell Line</keyword><keyword>Cricetinae</keyword><keyword>Dengue Virus/*physiology</keyword><keyword>Endoplasmic Reticulum/*virology</keyword><keyword>Humans</keyword><keyword>Intracellular Membranes/*metabolism</keyword><keyword>Magnetic Resonance Spectroscopy</keyword><keyword>Models, Molecular</keyword><keyword>Protein Conformation</keyword><keyword>Viral Nonstructural



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1513 Proteins/*chemistry/metabolism</keyword><keyword>*Virus
1514 Assembly</keyword></keywords><dates><year>2013</year><pub-
1515 dates><date>Apr</date></pub-dates></dates><isbn>1098-5514 (Electronic)0022-
1516 538X (Linking)</isbn><accession-num>23408612</accession-num><work-type>Research
1517 Support, Non-U.S. Gov't</work-type><urls><related-
1518 urls><url>http://www.ncbi.nlm.nih.gov/pubmed/23408612</url></related-
1519 urls></urls><custom2>3624351</custom2><electronic-resource-num>10.1128/JVI.02424-
1520 12</electronic-resource-num><language>eng</language></record></Cite></EndNote>].
1521 Plaques were developed on day 5 post-infection.

1522 **RNA extraction and RT-PCR**

1523 Viral RNAs in culture fluids were extracted using a QIAamp viral RNA minikit (Qiagen).
1524 Extracted RNAs were eluted in 50 µl RNase-free water. Reverse transcription (RT)-qPCR
1525 was performed to measure the amounts of viral RNA copy in the culture medium. Viral
1526 RNAs were measured using an iScript one-Step RT-PCR kit with SYBR green (Bio-Rad)
1527 and the primer pair NGC 7764V (sequence: 5'-CGTCGAGAGAAATATGGTCACACC-3')
1528 and 7844C (sequence: 5'CCACAATAGTATGACCAGCCT-3') [ ADDIN EN.CITE
1529 <EndNote><Cite><Author>Xie</Author><Year>2013</Year><RecNum>85</RecNum><Di
1530 splayText>(Xie et al., 2013)</DisplayText><record><rec-number>85</rec-
1531 number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv"
1532 timestamp="1560793217">85</key></foreign-keys><ref-type name="Journal
1533 Article">17</ref-type><contributors><authors><author>Xie, X.</author><author>Gayen,
1534 S.</author><author>Kang, C.</author><author>Yuan, Z.</author><author>Shi, P.
1535 Y.</author></authors></contributors><auth-address>State Key Laboratory of Virology,
1536 Wuhan Institute of Virology, Chinese Academy of Science, China.</auth-
1537 address><titles><title>Membrane topology and function of dengue virus NS2A
1538 protein</title><secondary-title>J Virol</secondary-title><alt-title>Journal of virology</alt-
1539 title></titles><periodical><full-title>J Virol</full-title></periodical><pages>4609-
1540 22</pages><volume>87</volume><number>8</number><edition>2013/02/15</edition><k
1541 eywords><keyword>Animals</keyword><keyword>Cell
1542 Line</keyword><keyword>Cricetinae</keyword><keyword>Dengue
1543 Virus/*physiology</keyword><keyword>Endoplasmic
1544 Reticulum/*virology</keyword><keyword>Humans</keyword><keyword>Intracellular
1545 Membranes/*metabolism</keyword><keyword>Magnetic Resonance



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Spectroscopy</keyword><keyword>Models, Molecular</keyword><keyword>Protein Conformation</keyword><keyword>Viral Nonstructural Proteins/*chemistry/metabolism</keyword><keyword>*Virus Assembly</keyword></keywords><dates><year>2013</year><pub-dates><date>Apr</date></pub-dates></dates><isbn>1098-5514 (Electronic)0022-538X (Linking)</isbn><accession-num>23408612</accession-num><work-type>Research Support, Non-U.S. Gov't</work-type><urls><related-urls><url>http://www.ncbi.nlm.nih.gov/pubmed/23408612</url></related-urls></urls><custom2>3624351</custom2><electronic-resource-num>10.1128/JVI.02424-12</electronic-resource-num><language>eng</language></record></Cite></EndNote>]. Alternatively, the extracted viral RNA was reversely transcribed and amplified using SuperScript One-Step (RT)-PCR with Platinum Taq (Invitrogen). The RT-PCR products were then gel purified and subjected to Sanger DNA sequencing.

**Replicon Transient Transfection**

After electroporation of replicon RNA into BHK-21 cells, the cells were resuspended in culture medium and seeded in a 12-well plate ($2 \times 10^5$ cells/well). At various time points, the cells were washed once with phosphate-buffered saline (PBS) and lysed using lysis buffer (Promega). The plates containing the lysis buffer were sealed with Parafilm and stored at -80°C. Once samples for all time points had been collected, luciferase signals were measured in a Clarity luminescence microplate reader (BioTek) according to the manufacturer's protocol. The luciferase signals at early time points (2-6 h post-transfection) represent the translation of input viral RNA; and the luciferase signals at later points (24-46 h post-transfection) represent the stage of RNA replication.

**Immunofluorescence assay (IFA)**

Cells were seeded in an 8-well Lab-Tek II chamber slide (ThermoFisher Scientific). At given time points, cells were fixed in 4% paraformaldehyde in PBS for 20 min at room temperature. Afterwards, cells were permeabilized by 0.1% Triton X-100 (Sigma-Aldrich) for 5 min followed by 1-h incubation in blocking buffer containing 1% FBS and 0.05% Tween-20 (Sigma-Aldrich) in PBS. Cells were then incubated with primary antibodies (diluted in the blocking buffer) at room temperature for 1 h. After three PBS washes, cells were incubated with goat anti-mouse or rabbit IgG (1:1000 diluted in blocking buffer) conjugated with Alexa Fluor 488 or 594 (ThermoFisher Scientific) for 1 h in blocking buffer.



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Cells were finally washed by PBS for three times, counter-stained by Hoechst 33342 Solution (ThermoFisher Scientific) and mounted in ProLong® Diamond Antifade Mountant (ThermoFisher Scientific). Epifluorescence images were acquired by Leica DM4000 B system. Confocal fluorescence images were acquired by Zeiss LSM 5 META systems.

**Virus Revertant Selection**

For selecting revertants from G11A-transfected cells, we consecutively passaged the transfected cells for 5 rounds. In brief, after electroporation, the cells were seeded in 96-well plates and incubate at 30°C for 3 days. Afterwards, cells for each well were detached by trypsin and suspended in culture medium. About 20% of the suspended cells were transferred into the well of new 96-well plates and incubated at 37°C for one day. The cells were then incubated at 30°C for two additional days. Again, the confluent cells were detached for next passage. At every passage, the supernatants were collected. After 5 rounds of passaging, IFA was performed to determine the intracellular E protein expression by 4G2 antibody. Plaque assay was performed to examine the amounts of infectious virus particle in the culture fluids. Viral RNA from the P5 supernatants were extracted and used as template for RT-PCR. Sanger sequencing was performed to sequence the entire viral genome.

**Co-immunoprecipitation (Co-IP)**

The NS2A/prM, NS2A/E and NS2A/NS3 interactions were examined by Co-IP analysis according to the protocol as described previously[ ADDIN EN.CITE <EndNote><Cite><Author>Zou</Author><Year>2014</Year><RecNum>113</RecNum><DisplayText>(Zou et al., 2014)</DisplayText><record><rec-number>113</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1561742592">113</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Zou, J.</author><author>Xie, X.</author><author>Lee le, T.</author><author>Chandrasekaran, R.</author><author>Reynaud, A.</author><author>Yap, L.</author><author>Wang, Q. Y.</author><author>Dong, H.</author><author>Kang, C.</author><author>Yuan, Z.</author><author>Lescar, J.</author><author>Shi, P. Y.</author></authors></contributors><auth-address>State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Sciences, Wuhan, China.</auth-address><titles><title>Dimerization of flavivirus NS4B protein</title><secondary-title>J



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Virol</secondary-title></titles><periodical><full-title>J Virol</full-title></periodical><pages>3379-91</pages><volume>88</volume><number>6</number><keywords><keyword>Amino Acid Motifs</keyword><keyword>Dengue/*virology</keyword><keyword>Dengue Virus/chemistry/genetics/*metabolism</keyword><keyword>Dimerization</keyword><keyword>Humans</keyword><keyword>Viral Nonstructural Proteins/*chemistry/genetics/*metabolism</keyword><keyword>West Nile Fever/*virology</keyword><keyword>West Nile virus/chemistry/genetics/*metabolism</keyword></keywords><dates><year>2014</year><pub-dates><date>Mar</date></pub-dates></dates><isbn>1098-5514 (Electronic)0022-538X (Linking)</isbn><accession-num>24390334</accession-num><urls><related-urls><url>https://www.ncbi.nlm.nih.gov/pubmed/24390334</url></related-urls></urls><custom2>PMC3957939</custom2><electronic-resource-num>10.1128/JVI.02782-13</electronic-resource-num></record></Cite></EndNote>]. Various combinations of plasmids were transfected into HEK293T cells in 10-cm dishes using the X-tremeGENE 9 DNA transfection reagent (Roche). At 40-42 h post-transfection, cells were lysed in 1 ml immunoprecipitation (IP) buffer (20 mM Tris, pH 7.5, 100 mM NaCl, 0.5% DDM, and EDTA-free protease inhibitor cocktail [Roche]) with rotation at 4°C for 1 h. Lysates were clarified by centrifugation at 20,000 × g and 4°C for 30 min and subjected to co-IP using protein G-conjugated magnetic beads according to the manufacturer's instructions (Millipore). Briefly, immune complexes were formed at 4°C overnight by mixing 200 µl of cell lysate with 2 µg corresponding antibodies in a 500-µl reaction system containing 400 mM sodium chloride. Subsequently, the complexes were precipitated with protein G-conjugated magnetic beads at 4°C for 1 h with rotation, followed by washing extensively with phosphate-buffered saline (PBS) containing 0.1% Tween-20. Finally, proteins were eluted with 4× lithium dodecyl sulfate (LDS) sample buffer (ThermoFisher Scientific) supplemented with 100 mM 1,4-dithiothreitol (DTT), heated at 70°C for 10 min, and analyzed by Western blot.

**SDS-PAGE and Western Blot**

Protein samples were mixed with 4×LDS sample buffer (ThermoFisher Scientific). After denaturing at 70°C for 10 min, 10-30 µl samples were loaded onto to a 12% or 15% SDS-PAGE gel (Bio-Rad Laboratories). After electrophoresis, proteins were resolved and



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transferred onto a polyvinylidene difluoride (PVDF) membrane using the Bio-Rad TransBlot Turbo Blotting System. Blots were soaked for 1 hour in a blocking buffer containing TBST (10 mM Tris-HCl [pH 7.5], 150 mM NaCl, and 0.1% Tween-20) and 5% skim milk, followed by 1 h incubation with primary antibodies (diluted in blocking buffer). After three washes with TBST buffer, blots were incubated with goat anti-mouse or rabbit antibodies conjugated to HRP (1:10,000 diluted in blocking buffer). After three thorough washes with TBST buffer, blots were incubated with the ECL substrates (GE Healthcare). Chemiluminescence signals were detected in ChemiDoc System (Bio-Rad).

**Transmission Electron Microscopy**

Viral genomic RNAs were electroporated into BHK-21 cells as described above. At 48 h post-transfection, the cells were fixed for at least 1 h in a mixture of 2.5% formaldehyde (prepared from paraformaldehyde powder) and 0.1% glutaraldehyde in 0.05M cacodylate buffer (pH 7.3) to which 0.03% picric acid and 0.03% $CaCl_2$ were added. After washing in 0.1 M cacodylate buffer, the cells were scraped off and processed further as a pellet. The pellets were post-fixed in 1% OsO4 in 0.1M cacodylate buffer (pH 7.3) for 1 h, washed with distilled water and *en bloc* stained with 2% aqueous uranyl acetate for 20 min at 60°C. The pellets were dehydrated in ethanol, processed through propylene oxide and embedded in Poly/Bed 812 (Polysciences, Warrington, PA). Ultrathin sections were cut on Leica EM UC7 ultramicrotome (Leica Microsystems, Buffalo Grove, IL), stained with lead citrate and examined in a Philips 201 transmission electron microscope at 60 kV.

**Expression and purification of recombinant NS2A proteins**

The GST-tagged DENV-2 NGC NS2A and its truncates was expressed and purified by following a previously described protocol [ ADDIN EN.CITE <EndNote><Cite><Author>Zou</Author><Year>2014</Year><RecNum>113</RecNum><DisplayText>(Zou et al., 2014)</DisplayText><record><rec-number>113</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1561742592">113</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Zou, J.</author><author>Xie, X.</author><author>Lee le, T.</author><author>Chandrasekaran, R.</author><author>Reynaud, A.</author><author>Yap, L.</author><author>Wang, Q. Y.</author><author>Dong, H.</author><author>Kang, C.</author><author>Yuan, Z.</author><author>Lescar, J.</author><author>Shi, P.



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Y.</author></authors></contributors><auth-address>State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Sciences, Wuhan, China.</auth-address><titles><title>Dimerization of flavivirus NS4B protein</title><secondary-title>J Virol</secondary-title></titles><periodical><full-title>J Virol</full-title></periodical><pages>3379-91</pages><volume>88</volume><number>6</number><keywords><keyword>Amino Acid Motifs</keyword><keyword>Dengue/*virology</keyword><keyword>Dengue Virus/chemistry/genetics/*metabolism</keyword><keyword>Dimerization</keyword><keyword>Humans</keyword><keyword>Viral Nonstructural Proteins/*chemistry/genetics/*metabolism</keyword><keyword>West Nile Fever/*virology</keyword><keyword>West Nile virus/chemistry/genetics/*metabolism</keyword></keywords><dates><year>2014</year><pub-dates><date>Mar</date></pub-dates></dates><isbn>1098-5514 (Electronic)0022-538X (Linking)</isbn><accession-num>24390334</accession-num><urls><related-urls><url>https://www.ncbi.nlm.nih.gov/pubmed/24390334</url></related-urls></urls><custom2>PMC3957939</custom2><electronic-resource-num>10.1128/JVI.02782-13</electronic-resource-num></record></Cite></EndNote>] with modifications. The pGEX-4T GST-NS2A plasmids were transformed into *E. coli* strain Rosetta™ 2 (pLysS) (Novagen) cells. Bacteria culture was grown in Tefferic broth containing 50 µg/ml Kanamycin and 34 µg/ml Chloramphenicol. When the cells reached an optical density at 600 nm (OD600) of 0.8-1.0, isopropyl β-d-1-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM. After growth for 16 h at 18°C, the cells were harvested, resuspended in a lysis buffer (10 mM $Na_2HPO_4$, 1.8 mM $KH_2PO_4$, 2.7 mM KCl, 400 mM NaCl, 1 mM EDTA, 0.5× Roche protease inhibitor cocktail, pH7.5) and disrupted by sonication using a Digital Sonifier 450 (Branson). The cell debris was removed by centrifugation at 10,000 rpm for 10 min at 4°C. The supernatants were collected and pelleted down by centrifugation at 45,000 rpm for 1 h at 4°C using a Beckman type 70 Ti rotor. The pellets containing the membrane fractions were collected and solubilized in the lysis buffer supplemented with 1.5% n-Dodecyl β-D-maltoside (DDM; Anatrace) at 4°C for 2 hours. Following another centrifugation step at 45,000 rpm for 1 h, the supernatants were collected and loaded onto a GSTrap HP Fast Flow column (GE Healthcare) which was preequilibrated with buffer A (10 mM $Na_2HPO_4$, 1.8 mM $KH_2PO_4$, 2.7 mM KCl, 400 mM NaCl, 2 mM tris(2-carboxyethyl) phosphine [TCEP], 0.05% DDM, pH



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7.5). After extensively wash with buffer B (10 mM $Na_2HPO_4$, 1.8 mM $KH_2PO_4$, 2.7 mM KCl, 1M NaCl, 2 mM tris(2-carboxyethyl) phosphine [TCEP], 0.05% DDM, pH 7.5), the protein was eluted in buffer A supplied with 20 mM glutathione. The fractions containing GST-NS2A proteins were pooled, concentrated, and subjected to gel filtration using a HiLoad Superdex 200 10/300 column (GE Healthcare) in buffer C (10 mM $Na_2HPO_4$, 1.8 mM $KH_2PO_4$, 2.7 mM KCl, 150 mM NaCl, 2 mM tris(2-carboxyethyl) phosphine [TCEP], 0.05% DDM, pH 7.5) with 5% glycerol. The peak fractions containing GST-NS2A proteins were pooled and concentrated before storage at -80°C.

**RNA Pull-down Assay**

The 5'UTR-$C_{66}$-3'UTR-biotin RNA was prepared by ligating a single biotinylated nucleotide to the 3' terminus of the RNA 5'UTR-$C_{66}$-3'UTR using the Pierce™ RNA 3' End Biotinylation Kit (ThermoFisher Scientific) according to the manufacturer's instructions. The RNA 5'UTR-$C_{66}$-3'UTR was *in vitro* transcribed from a PCR template using a T7 MEGAscript kit (ThermoFisher Scientific). The PCR template contained a T7 promoter, followed by a cDNA fragment representing a DENV-2 subgenome with a deletion from nucleotides 163 to 10269 (GenBank accession number AF038403). After *in vitro* transcription, the products were passed twice through the Microspin™ G-25 Columns (GE Healthcare Life Sciences) to remove excessive UTPs. The final products were subjected to phenol-chloroform extraction followed by alcohol precipitation. The purified RNAs were resuspended in nuclease-free water prior to storage at -80°C. Other shorter RNAs 3'UTR, Δpk1, 5'UTR-$C_{66}$, Δpk(1-2), Δpk(1-3), 3'SL, pk(3-4) and pk3 were *in vitro* synthesized from corresponding PCR templates using the same method.

RNA pull-down experiments were performed using the Pierce™ Magnetic RNA-Protein Pull-Down Kit (ThermoFisher Scientific) according to the manufacturer's instructions. Briefly, 2 µg of biotin-labeled RNAs were pre-captured by 50 µl Streptavidin magnetic beads in 1 × RNA capture buffer (10mL, 20mM Tris (pH 7.5), 1M NaCl, 1mM EDTA) at room temperature for 20 min with agitation. A 28-mer biotinylated IRE RNA (5'-UCCUGCUUCAACAGUGCUUGGACGGAAC-3'-biotin) from the Pierce™ RNA 3' End Biotinylation Kit was used as a control. The RNA-bound beads were then mixed with 2 µg purified GST-tagged NS2A proteins in a 100-µl reaction system containing 20 mM Tris (pH 7.5), 200 mM NaCl, 2 mM $MgCl_2$, 0.1% Tween-20 and 15% glycerol and incubated for 45 minutes at 4°C with agitation. For RNA competition assay, the various amounts of



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competitors (tRNA and other shorter RNAs from DENV-2 genome) were immediately added to above the mixtures containing RNA-bound beads and GST-NS2A proteins. After 5 times of wash by 20 mM Tris (pH 7.5), 10 mM NaCl, 0.1% Tween-20 Detergent, the proteins were eluted in 30 µl biotin elution buffer. The eluates were mixed with 4×LDS sample buffer and heated at 70°C for 10 min. 10 µl protein samples were loaded onto a 12% SDS-PAGE gel and followed by detection by Western blot using rabbit anti-GST antibodies.

**Electrophoretic Mobility-Shift Assay**

Electrophoretic mobility-shift assay (EMSA) was performed to estimate the binding affinity of DENV-2 5'UTR-$C_{66}$-3'UTR RNA to GST-NS2A proteins. 100 pM 5'UTR-$C_{66}$-3'UTR-biotin RNA was incubated with a serial concentration of recombinant GST-NS2A protein (0-250 nM) in a 50-µl reaction system containing 50mM Tris-HCl, pH 7.5, 10 mM 2-mercaptoethanol, 2 mM $MgCl_2$, 50 mM KCl at 24°C for 15 min. Afterwards, 10 µl 6x Gel Loading Dye without SDS (New England Biolabs Inc.) were added to stop the reaction. Sample were then loaded onto a 0.4% agarose gel (prepared in 0.5× Tris/Borate/EDTA[TBE]) to resolve the RNA-protein complexes and free RNAs by electrophoresis in 1× TBE buffer (120 V for 30 min) at room temperature. After electrophoresis, RNAs were transferred from the agarose gel to a BrightStar-Plus positively charged nylon membrane (ThermoFisher Scientific) according to the procedure as described in the manual of NorthernMax Gly Kit (ThermoFisher Scientific). The nylon membrane was irradiated with 200 mJ/cm$^2$ of Ultraviolet (UV) light at 254 nm in a CX-2000 UV crosslinker (Analytic Jene, CA) with default settings. The biotinylated RNA was finally detected by the Streptavidin-horseradish peroxidase conjugate by using the Chemiluminescent Detection Module (ThermoFisher Scientific). Chemiluminescent signals were detected in the ChemiDoc™ Imaging Systems (Bio-rad).

**PAR-CLIP**

The Photoactivatable-Ribonucleoside-Enhanced Crosslinking and Immunoprecipitation (PAR-CLIP) was performed according to the protocol as described previously [ ADDIN EN.CITE ADDIN EN.CITE.DATA ] with modifications. In brief, 4-thiouridine labelled DENV-2 3'UTR RNA was prepared by adding a final concentration 0.75 mM of 4-Thio-UTP (TriLink BioTechnologies) to a 20-µl *in vitro* transcription reaction (4-Thio-UTR to UTP



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ration is 1:10) using the MEGAscript T7 transcription Kit (Thermo Fisher Scientific). After 2 hours of reaction at 37°C, the reaction was stopped and the 4-thiouridine labelled RNAs were purified and resuspended in nuclease-free water. 15 µg of 4-thiouridine labelled DENV-2 3'UTR RNA was incubated with 6 µg recombinant DENV-2 GST-NS2A protein in a 100-µl reaction system containing 50 mM Tris-HCl, pH 7.5, 2 mM $MgCl_2$, 50 mM KCl and 5% glycerol at 24°C for 15 min. The RNA-protein mixtures were then irradiated on ice with 150 mJ/cm$^2$ at UV light at 365 nm. An aliquot of the same reaction without UV irradiation was used as the uncross-linked control. Prior to immunoprecipitation, 30 µl of PureProteome™ Protein G Magnetic Beads (MilliporeSigma) were washed twice with 1 ml citrate-phosphate buffer (4.7 g/L citric acid, 9.2 g/L $Na_2HPO_4$, pH 5.0), resuspended in 100 µl citrate-phosphate buffer and followed by in addition of 1 µg of rabbit anti-GST polyclonal antibody (Abcam). The bead-antibody mixtures were incubated at 4°C for 45 min. After two washed with citrate-phosphate buffer, the antibody-bound beads were resuspended in 30 µl of citrate-phosphate buffer. Upon immunoprecipitation, the RNA-protein mixtures were mixed with antibody-bound beads and incubated at 4°C for 1 h. Afterwards, the beads were subjected to three washes in buffer I (50mM HEPES-KOH, pH 7.5, 300 mM KCl, 0.05% IGEPAL CA-630L[Sigma-Aldrich] and 0.5 mM DTT) followed by two washes in buffer II (50 mM HEPES-KOH, pH 7.5, 500 KCl, 0.05% IGEPAL CA-630 and 0.5 mM DTT). The beads were then resuspended in 100 µl Proteinase K digestion buffer (100 mM Tris, pH 7.5, 50 mM NaCl, 10 mM EDTA and 1%SDS) supplemented with proteinase K (New England BioLabs) at a final concentration of 2 mg/ml. The reaction was placed into the Eppendorf ThermoMixer® C and incubated at 37°C for 1 h with agitation at 1100 rpm. Thereafter, the RNA in the reaction was extracted by phenol-chloroform, alcohol-precipitated, and resuspended in 20 µl of nuclease-free water. The extracted RNA was subjected to reversely transcriptionbed and followed by amplification (4 PCR-cycles) using the SuperScript® III One-Step RT-PCR System (ThermoFisher Scientific) with a pair of specific primers P1 (sequence: 5'-ACACTCTTTCCCTACACGA CGCTCTTCCGATCTAAGGCAAAACTAACATGAAAC-3'; underline indicates the sequence identical to the sequence at the 5' end of the DENV-2 3'UTR) and P2 (sequence: 5'-GTGACTGGAGTTCAGACG TGTGCTCTTCCGATCTAGAACCTGTTGATTCAACAGCAC-3'; underline indicates the sequence complimentary to the sequence at the 3' end of the DENV-2 3'UTR) targeting the DENV-2 3'UTR. Afterwards, the RT-PCR product was cleaned up using the QIAquick PCR Purification Kit (Qiagen) and subjected to another round of amplification (15 PCR-



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cycles) using the KAPA HiFi PCR Kit (Kapa Biosystems) with a pair of Illumina adapter/index primers (5′ universal adaptor sequence: 5′-AATGATACGGCGAC CACCGAGATCTACACTCTTTCCCTACACGAC-3′; and 3′ indexing primer sequence: 5′-CAAGCAGAAGACGGCATACGAGATnnnnnnGTGACTGGAGTTCAGACGTGT-3′. The "nnnnnn" here indicates the TruSeq index sequence). Thereafter, the PCR product was cleaned up using the QIAquick PCR Purification Kit (Qiagen) and followed by separation on 2% E-Gel™ EX Agarose Gels (ThermoFisher Scientific). The cDNAs fragment between 500 to 650 bp was excised, purified using the Zymo Research Gel DNA Recovery Kit (Zymo Research) and resuspended in nuclease-free water. The final cDNA libraries were quantified using a Qubit dsDNA Assay Kit (Invitrogen) and sequenced using a MiSeq Reagent Kit v3 (600-cycle) (illumina).

## QUANTIFICATION AND STATISTICAL ANALYSIS

### Sequence Alignment, Image Process and Densitometry Analysis

The amino acid sequences of flavivirus NS2A proteins were download from GenBank. Sequence alignment was performed with default settings using the CLC main workbench software (Qiagen). IFA images processing and cell counting was performed in software ImageJ (National Institutes of Health, NIH). At least four different IFA images were used to estimate the mean and standard deviations of percentage of viral protein staining positive-cells in Figure 2G and S2. Densitometry analysis was performed using Image lab version 6.0 (Bio-Rad Laboratories, Hercules, CA). All numerical data are presented as the mean ± SD (standard deviations). Group comparisons were performed using the unpaired parametric t-test in software Prism 8.0 (GraphPad). *$p<0.05$, significant; **$p<0.01$, very significant; ***$p<0.001$, highly significant; n.s., not significant. Images were assembled using the software Adobe illustrator CC.

### Calculating the equilibrium dissociation constant (Kd)

The intensities of bands representing the RNA/GST-NS2A complexes and free RNA in Figure 5E were quantitated by software ImageJ (NIH). The ratio of bound RNA (in the RNA/GST-NS2A complexes) to total RNA (free and bound RNA) in each lane was calculated. The ratio of bound RNA to total RNA (y-axis) versus the concentration of GST-NS2A protein (x-axis) was plotted using the software Prism 8 (Graphad). The value of Kd



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was obtained using a nonlinear regression with least-squares fitting method, where the Bmax was set as 1. All data points come from three independent experiments.

**Illumina sequencing data process and analysis**

The indexed sequencing reads of both forward and reverse strands were proceeded with the following bioinformatic pipelines: first, an initial quality filtering were conducted with Fastp [ ADDIN EN.CITE <EndNote><Cite><Author>Chen</Author><Year>2018</Year><RecNum>115</RecNum><DisplayText>(Chen et al., 2018)</DisplayText><record><rec-number>115</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1562011493">115</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Chen, S.</author><author>Zhou, Y.</author><author>Chen, Y.</author><author>Gu, J.</author></authors><translated-authors><author>Bioinformatics,</author></translated-authors></contributors><auth-address>Department of Bioinformatics, HaploX Biotechnology, Shenzhen, China.Shenzhen Institutes of Advanced Technology, Chinese Academy of Sciences, Shenzhen, China.</auth-address><titles><title>fastp: an ultra-fast all-in-one FASTQ preprocessor</title><secondary-title>Bioinformatics</secondary-title><alt-title>Bioinformatics</alt-title></titles><periodical><full-title>Bioinformatics</full-title><abbr-1>Bioinformatics</abbr-1></periodical><alt-periodical><full-title>Bioinformatics</full-title><abbr-1>Bioinformatics</abbr-1></alt-periodical><pages>i884-i890</pages><volume>34</volume><number>17</number><dates><year>2018</year><pub-dates><date>Sep 1</date></pub-dates></dates><isbn>1367-4811 (Electronic)1367-4803 (Linking)</isbn><accession-num>30423086</accession-num><urls><related-urls><url>http://www.ncbi.nlm.nih.gov/pubmed/30423086</url></related-urls></urls><custom2>6129281</custom2><electronic-resource-num>10.1093/bioinformatics/bty560</electronic-resource-num><remote-database-provider>2018 Sep 1</remote-database-provider><language>eng</language></record></Cite></EndNote>] to remove low quality ~~bad~~ reads (~~too~~ low ~~in quality~~PHRED score, too short, too many "N", etc.). Then, the illumina sequencing adapter sequence "AGATCGGAAGAGC" was trimmed with *cutadapt* [ ADDIN EN.CITE <EndNote><Cite><Author>Martin</Author><Year>2011</Year><RecNum>116</RecNum

Formatted: Font: Italic



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1869 ><DisplayText>(Martin, 2011)</DisplayText><record><rec-number>116</rec-
1870 number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv"
1871 timestamp="1562011493">116</key></foreign-keys><ref-type name="Journal
1872 Article">17</ref-type><contributors><authors><author>Martin,
1873 Marcel</author></authors></contributors><titles><title>Cutadapt removes adapter
1874 sequences from high-throughput sequencing reads</title><secondary-title>EMBnet.journal;
1875 Vol 17, No 1: Next Generation Sequencing Data AnalysisDO -
1876 10.14806/ej.17.1.200</secondary-title></titles><periodical><full-title>EMBnet.journal; Vol
1877 17, No 1: Next Generation Sequencing Data AnalysisDO - 10.14806/ej.17.1.200</full-
1878 title></periodical><keywords><keyword>next generation sequencing, small RNA,
1879 microRNA, adapter removal</keyword></keywords><dates><year>2011</year><pub-
1880 dates><date>05/02/</date></pub-dates></dates><urls><related-
1881 urls><url>https://journal.embnet.org/index.php/embnetjournal/article/view/200/458</url></r
1882 elated-urls></urls></record></Cite></EndNote>] (command line parameters: -b
1883 AGATCGGAAGAGC -m 40). A secondary quality filter was applied with FASTX toolkit
1884 ([ HYPERLINK "http://hannonlab.cshl.edu/fastx_toolkit/index.html" ]) to remove any reads that
1885 contained more than 4% nucleotides with a PHRED score <20 (command line parameters:
1886 fastq_quality_filter -Q33 -q 20 -p 96). The remaining reads were aligned to 3'UTR of DENV
1887 genome (GenBank accession number AF038403), with Bowtie2 [ ADDIN EN.CITE
1888 <EndNote><Cite><Author>Langmead</Author><Year>2012</Year><RecNum>117</Rec
1889 Num><DisplayText>(Langmead and Salzberg, 2012)</DisplayText><record><rec-
1890 number>117</rec-number><foreign-keys><key app="EN" db-
1891 id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv"
1892 timestamp="1562011493">117</key></foreign-keys><ref-type name="Journal
1893 Article">17</ref-type><contributors><authors><author>Langmead,
1894 Ben</author><author>Salzberg, Steven
1895 L.</author></authors></contributors><titles><title>Fast gapped-read alignment with
1896 Bowtie 2</title><secondary-title>Nature Methods</secondary-
1897 title></titles><periodical><full-title>Nature Methods</full-
1898 title></periodical><pages>357</pages><volume>9</volume><dates><year>2012</year>
1899 <pub-dates><date>03/04/online</date></pub-dates></dates><publisher>Nature
1900 Publishing Group, a division of Macmillan Publishers Limited. All Rights
1901 Reserved.</publisher><urls><related-
1902 urls><url>https://doi.org/10.1038/nmeth.1923</url></related-urls></urls><electronic-



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resource-num>10.1038/nmeth.1923</electronic-resource-num></record></Cite></EndNote>] (command line parameters: --local). Using SAMtools [ ADDIN EN.CITE <EndNote><Cite><Author>Li</Author><RecNum>118</RecNum><DisplayText>(Li et al.)</DisplayText><record><rec-number>118</rec-number><foreign-keys><key app="EN" db-id="txv2awx0sz9dfle59pjpwxxqrzxw25sssvrv" timestamp="1562011493">118</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Li, H.</author><author>Handsaker B Fau - Wysoker, Alec</author><author>Wysoker A Fau - Fennell, Tim</author><author>Fennell T Fau - Ruan, Jue</author><author>Ruan J Fau - Homer, Nils</author><author>Homer N Fau - Marth, Gabor</author><author>Marth G Fau - Abecasis, Goncalo</author><author>Abecasis G Fau - Durbin, Richard</author><author>Durbin, R.</author></authors><translated-authors><author>Bioinformatics,</author></translated-authors></contributors><auth-address>Wellcome Trust Sanger Institute, Wellcome Trust Genome Campus, Cambridge, CB10 1SA, UK, Broad Institute of MIT and Harvard, Cambridge, MA 02141, USA. FAU - Handsaker, Bob</auth-address><titles><title>The Sequence Alignment/Map format and SAMtools</title></titles><number>1367-4811 (Electronic)</number><dates></dates><call-num>1000 Genome Project Data Processing Subgroup</call-num><urls></urls><remote-database-provider>2009 Aug 15</remote-database-provider><language>eng</language></record></Cite></EndNote>], the aligned forward and reverse reads were binarily converted, merged, indexed, and sorted~~, and mathematically noted~~ (command line parameters: view -buS, merge, sort, index, mpileup). This is followed by extracting and calculating the T/U-to-C mutation rate at each genome position and the mutation frequencies of each of the four nucleotides.

## DATA AND SOFTWARE AVAILABILITY

Illumina sequencing data access. The raw data of Illuminae sequencing data can be accessed, queried and downloaded using the following link: .

**Comment [RAL6]:** Did you upload the raw data somewhere? Will probabky have to do this prior to submission. NCBI SRA is the easiest option.



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**From:** LeDuc, James W.
**Sent:** Tuesday, July 16, 2019 2:20 PM
**To:** Rusek, Benjamin;Dave Franz (davidrfranz@gmail.com);Shi, Pei yong
**Cc:** Lowenthal, Micah
**Subject:** RE: Letter to CAS re dialogue meeting with in Wuhan
**Attachments:** Letter to CAS from Griffin and Hamburg v2-jwl.docx

Hi Ben,

Thanks for preparing the draft letter. I made a couple of minor edits on the attached for your consideration. Specifically, a suggestion that the next meeting be held in the "vicinity of Wuhan" as we may wish to go to a more leisure site since we have been in Wuhan a couple of times already. This was suggested by Zhiming I believe when he last spoke to Pei-Yong.

The larger question is, Is now the right time to be asking to set up a formal MOU and standing committee for this initiative? Given the sour political environment and the fact that Kunming has already bowed out, I'm a little concerned that requesting to formalize things at this time may just provide the CAS with an excuse to bump up a decision to higher political levels and thereby delay a decision, or worse, result in a negative reply. I wonder if we should ask Zhiming or George Gao their opinion before finalizing the letter.

Maybe we can have a quick conference call to discuss?

Thanks, Jim

**From:** Rusek, Benjamin <BRusek@nas.edu>
**Sent:** Tuesday, July 16, 2019 1:40 PM
**To:** LeDuc, James W. <jwleduc@UTMB.EDU>; Dave Franz (davidrfranz@gmail.com) <davidrfranz@gmail.com>; Shi, Pei yong <peshi@UTMB.EDU>
**Cc:** Lowenthal, Micah <mlowenth@nas.edu>
**Subject:** Letter to CAS re dialogue meeting with in Wuhan
**Importance:** High

**WARNING:** This email originated from outside of UTMB's email system. Do not click links or open attachments unless you recognize the sender and know the content is safe.

Hi Jim, Dave and Pei-yong,

Here is the draft letter to seek approval to hold the next U.S. China bio dialogue meeting with NIV in Wuhan. The letter would be signed by Peggy Hamburg and Diane Griffin (and I will reach out to them in a separate email) but I wanted to send it to you for final review first. I have been told by our leadership that Chunli Bai is no longer the president of CAS, Pei-yong could you ask Zhiming who this should go to after it gets signed?

Note that the letter also includes a request to formalize the dialogue (via memorandum of understanding and the appointment of a standing organizing committee) as a regular, ongoing activity between NASEM and CAS. It makes sense to do this now for several reasons. Was thinking that the standing committee could include Americans and the Chinese scientists/leaders we have been working with but happy to hear your thoughts on this.

Kind regards,



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Ben

Benjamin Rusek
The U.S. National Academy of Sciences
1-202-334-3975



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**RE: Proposed Chinese-U.S. dialogue meeting on the Challenges of Emerging Infections, Laboratory Safety & Global Health Security in Wuhan, China**

~~Dr. Bai Chunli~~
President, Academician
Chinese Academy of Sciences

**We are writing to formally request your permission and support to hold the next in a series of China-U.S. meetings on emerging infections, laboratory safety, and global health security** co-convened by the National Academies of Sciences, Engineering, and Medicine (NASEM) and the Wuhan Institute of Virology, Chinese Academy of Sciences (CAS) in Wuhan, China in late 2019.

Over the past several years, Dr. David Franz and Dr. James Le Duc have worked with Dr. Yuan Zhiming, Professor and Deputy Director, Wuhan Institute of Virology, CAS, and other Chinese officials to organize a series of meetings, a dialogue between experts designated by the CAS, China Center for Disease Control (CCDC), the Chinese Academy of Agricultural Sciences (CAAS), and the Chinese Academy of Medical Sciences and the NASEM. These dialogue meetings have been designed to allow scientists from China and the United States to share research findings, best practices, and lessons learned and to create collaborative partnerships in areas identified during the workshops. Meetings have been held in Beijing, Wuhan, Galveston, TX and most recently the CAAS Veterinary Institute in Harbin, China. In addition to ourselves, participants have included esteemed senior Chinese and American Scientists, such as Gao Fu (CAS, CCDC, NAM), Yi Shi (CAS), Linda Saif (NAS), David Relman (NAM), Bu Zhigao, (CAAS), and many others.

During the meeting in Harbin in January 2019, Dr. Le Duc and Dr. Yuan discussed holding a second dialogue meeting in the vicinity of Wuhan. Topics that are proposed for discussion at the meeting include: medical countermeasure development, especially advanced development of vaccines, therapeutics, and diagnostics; development and use of animal models of high-consequence diseases in biocontainment; host-pathogen interactions; the special challenges associated with the conduct of regulated studies in biocontainment; response to disease outbreaks; risk assessment of gene editing and other advanced biotechnologies in pathogen research; appropriate institutional oversight of biosafety and biosecurity training and facilities operations; and vector-borne disease research.

With your approval, NASEM and CAS will establish an informal joint organizing committee to plan the next dialogue meeting, invite workshop speakers and participants, and make workshop logistical arrangements. NASEM will pay the air, lodging, and ancillary costs of all the American participants and can contribute financial support for meeting costs in China, if necessary. **We hope that we can use a portion of this meeting to formalize, via memorandum of understanding and the appointment of a standing organizing committee of Chinese and American experts, the existence of the China-U.S. dialogue on the challenges of emerging infections, laboratory safety, and global health security as a regular, ongoing activity between our two organizations.**

China and the United States are world leaders in life science research and development, have large populations that may be susceptible to emerging infections, and are world leaders in public health and response infectious disease. Both of our countries operate complex high biological containment laboratories that are critical to responding to outbreaks of especially dangerous pathogens. It is important to cooperate and discusses these and other key areas of interest and concern for our two countries and as the life sciences advance, continue to exchange best practices to ensure the safe and secure conduct of the life science enterprise going forward. We hope that you approve and will support this activity.

**Commented [RB1]:** No longer the president of CAS

**Deleted:** We hope that you approve and will support this activity.

**Deleted:** combating

**Deleted:** ¶

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We look forward to your thoughts on the organization, content, and substance of the proposed dialogue meeting, your thoughts on the proposal for formalize the dialogue and any other issues that you and your colleagues wish to address.

Sincerely,

Diane E. Griffin
Vice President, NAS

Margaret Hamburg
Foreign secretary, NAM

...

...

Additional points of contact:

James Le Duc Ph.D.
Director, Galveston National Laboratory
jwleduc@utmb.edu, 1.409.266.6500

Benjamin J. Rusek, Senior Program Officer
U.S. National Academy of Sciences
brusek@nas.edu 1.202.334.3975

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**From:** Dave Franz <davidrfranz@gmail.com>
**Sent:** Wednesday, July 24, 2019 4:35 PM
**To:** Benjamin Rusek
**Cc:** LeDuc, James W.;Micah Lowenthal;Shi, Pei yong
**Subject:** Re: Letter to CAS to hold next dialogue meeting in Wuhan
**Attachments:** drfLetter to CAS from Griffin and Hamburg for review.docx

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Thanks Ben, I've made a few comments in the draft email (in red) and some comments in the text of the letter (using track changes). Use what's helpful; I will value Jim and Pei-Yong's thoughts. Best, dave

***

Greetings Diane and Peggy,

I have attached a letter detailing a request to hold a dialogue meeting in Wuhan, China between NASEM and the Chinese Academy of Science (CAS) in cooperation with the Wuhan National Institute of Virology (NIV) of the CAS. Yuan Zhiming, Professor and Deputy Director of the NIV, is very supportive but has let us know that we need to send a formal letter to the President of CAS (as we did before the 2017 meeting in Wuhan) to get approval for the meeting. Zhiming says that the president is still Bai Chunli but this may have changed recently.

More broadly (as Diane knows) we had hoped to hold a dialogue meeting with the of Kunming Institute of Medical Sciences, Chinese Academy of Medical Sciences (CAMS) but the Kunming Institute leadership is unwilling to seek approval from CAMS to hold a bilateral with NASEM at this time.

The letter includes details about the recent dialogue meetings and outlines the topics we plan to address at the next dialogue meeting. It also includes a proposal noting (?) that this dialogue meeting could be used to formalize the dialogue as a regular, ongoing activity between NASEM and CAS. I think it's the right time to propose this because it has taken many years to get to this point, after a long period of struggling unsuccessfully to organize meetings between CISAC and the Chinese Biological Scientists Group. We have now held several useful dialogue meetings between NASEM and CAS (with CCDC, CAAS and other institutions involved) and could use those to justify a more formal relationship. We are working with a committed group of Chinese scientists and leaders who recognize the importance of dialogue and communication that are tied formally to CAS (Zhiming, George Gao, others) who could serve on a Chinese organizing committee. The current political relationship between the U.S. and China has started to affect ad hoc activities like these as well as the willingness of American scientists to travel to China and of Chinese scientists to travel to the U.S. without more formal sanction. We believe this climate only adds to the value of these technical dialogues and frank and open communication among expert colleageus from both sides. I should add that Jim and Dave did mention some some concern about this being the right time to seek a formal agreement establishing a formal dialogue. Therefore, I have written



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the draft text so that the CAS leadership could decline this part of the request if they do not think it is the right time to do this.

We look forward to hearing your thoughts on the letter, the meeting and the proposal to formalize the dialogue under NASEM and CAS.

Kind regards,

Ben

Benjamin Rusek
The U.S. National Academy of Sciences
1-202-334-3975

<Letter to CAS from Griffin and Hamburg for review.docx>



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**RE: Proposed Chinese-U.S. dialogue meeting on the Challenges of Emerging Infections, Laboratory Safety & Global Health Security in Wuhan, China**

Dr. Bai Chunli
President, Academician
Chinese Academy of Sciences

**We are writing to formally request your permission and support to hold the next in a series of China-U.S. meetings on emerging infections, laboratory safety, and global health security** co-convened by the National Academies of Sciences, Engineering, and Medicine (NASEM) and the Wuhan Institute of Virology, Chinese Academy of Sciences (CAS) in Wuhan, China in late 2019.

Over the past several years, Dr. David Franz and Dr. James Le Duc have worked with Dr. Yuan Zhiming, Professor and Deputy Director, Wuhan Institute of Virology, CAS, and other Chinese officials to organize a series of meetings, now an informal dialogue, between experts designated by the CAS, China Center for Disease Control (CCDC), the Chinese Academy of Agricultural Sciences (CAAS), and the Chinese Academy of Medical Sciences and the NASEM. These dialogue meetings have been designed to allow scientists from China and the United States to share research findings, best practices, and lessons learned and to create collaborative partnerships in areas identified during the workshops. Meetings have been held in Beijing, Wuhan, Galveston, Texas and most recently the CAAS Veterinary Institute in Harbin, China. In addition to ourselves, participants have included esteemed senior Chinese and American Scientists, such as Gao Fu (CAS, CCDC, NAM), Yi Shi (CAS), Linda Saif (NAS), David Relman (NAM), Bu Zhigao, (CAAS), and many others.

During the meeting in Harbin in January 2019, Dr. Le Duc and Dr. Yuan discussed holding a second dialogue meeting in the vicinity of Wuhan. Topics that are proposed for discussion at the meeting include: medical countermeasure development, especially advanced development of vaccines, therapeutics, and diagnostics; development and use of animal models of high-consequence diseases in biocontainment; host-pathogen interactions; the special challenges associated with the conduct of regulated studies in biocontainment; response to disease outbreaks; risk assessment of gene editing and other advanced biotechnologies in pathogen research; appropriate institutional oversight of biosafety and biosecurity training and facilities operations; and vector-borne disease research.

With your approval, NASEM and CAS will establish an informal joint organizing committee to plan the next dialogue meeting, invite workshop speakers and participants, and make workshop logistical arrangements. NASEM will pay the air, lodging, and ancillary costs of all the American participants and can contribute financial support for meeting costs in China, if necessary.

**This excellent series of meetings could also be used to formalize, via memorandum of understanding and the appointment of a standing organizing committee of Chinese and American experts as *The China-U.S. dialogue on the challenges of emerging infections, laboratory safety, and global health security* as a regular, ongoing activity between our two organizations.**

China and the United States are world leaders in life science research and development, have large populations that may be susceptible to emerging infections, and are world leaders in public health and response to infectious disease. Both of our countries operate complex high biological containment laboratories that are critical to responding to outbreaks of especially dangerous pathogens. It is important to cooperate and discuss these and other key areas of interest and concern for our two countries and as the life sciences advance, continue to exchange best practices to ensure the safe and secure conduct of the ~~life science~~ enterprise going forward. ~~We hope that you approve and will support this activity.~~

**Commented [drf1]:** ...or we just thinking about Kunming then?

**Commented [drf2]:** Many of these topics were put forward for the Kunming meeting, where they are actually more appropriate, e.g. advanced development of MCMs....probably less relevant in Wuhan.

**Commented [drf3]:** ???? See what Pei-Yong thinks...

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We look forward to your thoughts on the organization, content, and substance of the dialogue meeting, your ~~thoughts on~~ the proposal to formalize the dialogue and any other issues that you and your colleagues wish to address.

Sincerely,

Diane E. Griffin
Vice President, NAS

Margaret Hamburg
Foreign secretary, NAM

...

...

Additional points of contact:

James Le Duc Ph.D.
Director, Galveston National Laboratory
jwleduc@utmb.edu, 1.409.266.6500

Benjamin J. Rusek, Senior Program Officer
U.S. National Academy of Sciences
brusek@nas.edu 1.202.334.3975

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**From:** Dave Franz <davidrfranz@gmail.com>
**Sent:** Wednesday, July 24, 2019 4:39 PM
**To:** LeDuc, James W.
**Cc:** Benjamin Rusek;Micah Lowenthal;Shi, Pei yong
**Subject:** Re: Letter to CAS to hold next dialogue meeting in Wuhan

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I will not attend Tom's meeting. Do we have a list of Chinese attendees?

Oh, and Jim, I used your name in vein on a comment in the draft email to Diane and Peggy. Maybe it's not appropriate—and you can take Jim's name off Ben, if you think appropriate but Jim doesn't want to. I just thought it was worth mentioning to them that we are aware and thinking about the greater political climate. Dave

On Jul 24, 2019, at 4:52 PM, LeDuc, James W. <jwleduc@UTMB.EDU> wrote:

Thanks Ben. I made a couple of minor comments in the attached, but I think it's fine. RE the title of the standing committee, is "global health security" an acceptable phrase in this group?

Will you or Dave be attending Tom Inglesby's US-China meeting on synthetic biology in DC on Friday? I will be there and scheduled to speak in the morning session.

Thanks, Jim

**From:** Rusek, Benjamin <BRusek@nas.edu>
**Sent:** Wednesday, July 24, 2019 3:41 PM
**To:** LeDuc, James W. <jwleduc@UTMB.EDU>; Dave Franz (davidrfranz@gmail.com) <davidrfranz@gmail.com>
**Cc:** Lowenthal, Micah <mlowenth@nas.edu>; Shi, Pei yong <peshi@UTMB.EDU>
**Subject:** Letter to CAS to hold next dialogue meeting in Wuhan
**Importance:** High

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Dave and Jim,

Sorry for the delay, was out of town for other work last week, I have attached a note for Diane and Peggy along with a revised latter.

I wanted to make sure you are ok with the language in the letter and in the note before I send it to them for signature. I understand your rational for caution but would still like to try and formalize the



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relationship (reasons in the note) but toned down the sentence about it in the letter. Let me know what you think.

Kind regards,

Ben

***

Greetings Diane and Peggy,

I have attached a letter detailing a request to hold a dialogue meeting in Wuhan, China between NASEM and the Chinese Academy of Science (CAS) in cooperation with the Wuhan National Institute of Virology (NIV) of the CAS. Yuan Zhiming, Professor and Deputy Director of the NIV, is very supportive but has let us know that we need to send a formal letter to the President of CAS (as we done before the 2017 meeting in Wuhan) to get approval for the meeting. Zhiming says that the president is still Bai Chunli but this may have changed recently.

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The letter includes details about the recent dialogue meetings and outlines the topics we plan to address at the next dialogue meeting. It also includes a proposal nothing that this dialogue meeting could be used to formalize the dialogue as a regular, ongoing activity between NASEM and CAS. I think it's the right time to propose this because it has taken many years to get to the point, after a long period of struggling unsuccessfully to organize meetings between CISAC and the Chinese Biological Scientists Group, we have now held several useful dialogue meetings between NASEM and CAS (with CCDC, CAAS and other institutions involved) and could use those to justify a more formal relationship, we are working with a committed group of Chinese scientists and leaders who recognize the importance of dialogue and communication that are tied formally to CAS (Zhiming, George Gao, others) who could serve on a Chinese organizing committee, the political relationship between the U.S. and China has started to affect ad hoc activities like these as well as the willingness of American scientists to travel to China and of Chinese scientists to travel to the U.S. without more formal sanction. I wrote the text so that the CAS leadership could decline this part of the request if they do not think it is the right time to do this.

We look forward to hearing your thoughts on the letter, the meeting and the proposal to formalize the dialogue under NASEM and CAS.

Kind regards,

Ben

Benjamin Rusek
The U.S. National Academy of Sciences
1-202-334-3975

<Letter to CAS from Griffin and Hamburg for review-jwl.docx>



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**From:** LeDuc, James W.
**Sent:** Wednesday, July 24, 2019 5:17 PM
**To:** Dave Franz
**Cc:** Benjamin Rusek;Micah Lowenthal;Shi, Pei yong
**Subject:** Re: Letter to CAS to hold next dialogue meeting in Wuhan

No problem in using my name if you think it would be helpful

Sent from my iPhone

On Jul 24, 2019, at 4:38 PM, Dave Franz <davidrfranz@gmail.com> wrote:

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I will not attend Tom's meeting. Do we have a list of Chinese attendees?

Oh, and Jim, I used your name in vein on a comment in the draft email to Diane and Peggy. Maybe it's not appropriate—and you can take Jim's name off Ben, if you think appropriate but Jim doesn't want to. I just thought it was worth mentioning to them that we are aware and thinking about the greater political climate. Dave

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Thanks, Jim

**From:** Rusek, Benjamin <BRusek@nas.edu>
**Sent:** Wednesday, July 24, 2019 3:41 PM
**To:** LeDuc, James W. <jwleduc@UTMB.EDU>; Dave Franz (davidrfranz@gmail.com) <davidrfranz@gmail.com>
**Cc:** Lowenthal, Micah <mlowenth@nas.edu>; Shi, Pei yong <peshi@UTMB.EDU>
**Subject:** Letter to CAS to hold next dialogue meeting in Wuhan
**Importance:** High

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Dave and Jim,



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Sorry for the delay, was out of town for other work last week, I have attached a note for Diane and Peggy along with a revised latter.

I wanted to make sure you are ok with the language in the letter and in the note before I send it to them for signature. I understand your rational for caution but would still like to try and formalize the relationship (reasons in the note) but toned down the sentence about it in the letter. Let me know what you think.

Kind regards,

Ben

***

Greetings Diane and Peggy,

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We look forward to hearing your thoughts on the letter, the meeting and the proposal to formalize the dialogue under NASEM and CAS.

Kind regards,

Ben



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Benjamin Rusek
The U.S. National Academy of Sciences
1-202-334-3975

<Letter to CAS from Griffin and Hamburg for review-jwl.docx>



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| | |
|---|---|
| **From:** | Rusek, Benjamin <BRusek@nas.edu> |
| **Sent:** | Wednesday, July 24, 2019 8:11 PM |
| **To:** | Diane Griffin (dgriffi6@jhu.edu);Hamburg, Margaret |
| **Cc:** | LeDuc, James W.;Dave Franz (davidrfranz@gmail.com);Shi, Pei yong;Lowenthal, Micah;Boright, John |
| **Subject:** | Letter to CAS to hold next dialogue meeting in Wuhan? |
| **Attachments:** | Letter to CAS from Griffin and Hamburg for review final.docx |

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Greetings Diane and Peggy,

I have attached a letter detailing a request to hold a dialogue meeting in Wuhan, China between NASEM and the Chinese Academy of Science (CAS) in cooperation with the Wuhan National Institute of Virology (NIV) of the CAS. Yuan Zhiming, Professor and Deputy Director of the NIV, is very supportive but has let us know that we need to send a formal letter to the President of CAS (as we did before the 2017 meeting in Wuhan) to get approval for the meeting. (Zhiming says that the president is still Bai Chunli but this may have changed recently.)

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Kind regards,

Ben

Benjamin Rusek
The U.S. National Academy of Sciences
1-202-334-3975



FOIA Confidential Treatment Requested by Univ of Texas System UTSystem_008999

**From:** Diane Griffin <dgriffi6@jhmi.edu>
**Sent:** Thursday, July 25, 2019 5:53 AM
**To:** Rusek, Benjamin;Hamburg, Margaret
**Cc:** LeDuc, James W.;Dave Franz (davidrfranz@gmail.com);Shi, Pei yong;Lowenthal, Micah;Boright, John
**Subject:** Re: Letter to CAS to hold next dialogue meeting in Wuhan?

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Ben - I think this is a good idea and the letter looks fine to me.
Depending on the rapidity of the response, it may be tough to get something organized by the end of the year, but it's worth a try.

Diane

---

**From:** Rusek, Benjamin <BRusek@nas.edu>
**Sent:** Wednesday, July 24, 2019 9:10:30 PM
**To:** Diane Griffin; Hamburg, Margaret
**Cc:** jwleduc@UTMB.EDU; Dave Franz (davidrfranz@gmail.com); Shi, Pei yong (peshi@UTMB.EDU); Lowenthal, Micah; Boright, John
**Subject:** Letter to CAS to hold next dialogue meeting in Wuhan?

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Kind regards,

Ben

Benjamin Rusek
The U.S. National Academy of Sciences
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