From: Munster, Vincent (NIH/NIAID) [E]
Sent: Fri, 8 Mar 2019 11:56:08 -0700
To: Peter Daszak; Kevin Olival,; Anthony, Simon J.; Letko, Michael (NIH/NIAID) [F]
Subject: FW: Reaching out to EcoHealth
Attachments: Letko_lineageB project.pdf
Importance: High

Hi guys,

I wanted to show you some prelim data from one of my post-docs (Dr. Michael Letko), which we think would be of big interest of you guys and PREDICT. Michael has recently developed a rapid and scalable platform to screen novel beta-coronaviruses related to SARS-CoV (aka, Lineage B) for host-cell entry potential. Literally hundreds of lineage B beta-coronaviruses have been discovered primarily in Rhinolophus bats in Asia. Unfortunately, like many newly-discovered viruses, little is known beyond the sequence – there is almost no information on whether or not the majority of these viruses can even infect human cells. As our approach allows us to quickly move from genotype to phenotype, we feel that this work is of high interest to the field of virus discovery and to programs like EcoHealth Alliance - PREDICT.

While we have now tested all of the variation currently published for lineage B beta-coronaviruses for their ability to enter primate cells, we are seeking additional, unpublished lineage B beta-coronavirus sequences to test in our platform. Attached is a short abstract and figure further describing our approach and demonstrating the powerful results that come from it. We kindly ask that you do not distribute this material further, however, as it is still unpublished and quite competitive.

Let me know what you guys think and whether you have any samples with lineage B sequences of S-genes or lineage B rdrp for further characterization of S. I think publishing smtg which bridges the gap between virus discovery and high-throughput initial assessment of zoonotic potential would have great interest from the scientific AND public health communities.

Cheers,

Vincent Munster, PhD
Chief, Virus Ecology Unit
Laboratory of Virology
Rocky Mountain Laboratories
NIAID/NIH

Functionally screening for zoonotic potential of the entire lineage B of betacoronaviruses

Michael Letko[1], Vincent Munster[1]

Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV) emerged in China and rapidly spread, killing over 700 of the 8000 cases. Since then, virus discovery efforts have uncovered thousands of novel coronavirus sequences across a diverse range of species and countries. While most of these viruses have never been found in humans, many are genetically similar to human CoVs within the betacoronavirus (β-CoV) genus. The β-CoVs are further divided into lineages: SARS-CoV is in lineage B, which also includes approximately 300 other novel animal viruses. Every year, additional CoV sequences are discovered. Unfortunately, little work is performed after the viral sequences are published. Therefore, it is unknown whether most of these viruses have potential to emerge in human populations and cause disease.

Cell entry is an essential component of cross-species transmission. All CoVs encode a spike protein which binds the host-cell receptor and mediates viral entry (Fig. 1A). For β-CoVs, a single region of spike called the receptor binding domain (RBD) mediates this interaction.

We have developed a platform to test cell entry of the entire lineage B of β-CoVs (Fig. 1B, C). Our sequence analysis of published genomic data revealed there are 30 unique RBDs encompassing all diversity in lineage B (Fig 1E). All 30 RBDs were synthesized and cloned into a construct we designed to make luciferase reporter viral particles. We checked each chimeric construct for expression and ability to incorporate into reporter particles (Fig. 1D). We then infected human cells with this panel of reporter particles and measured luciferase as a readout for cell entry. Only a small subset of the novel RBDs mediated entry into human cells while the majority of RBDs did not (Fig 1E). Phylogenetic analysis revealed that the RBDs with human cell entry potential form a single clade within lineage B and the other RBDs form two additional clades. Thus, by testing all spike variation within lineage B, we have elucidated clade-specific host cell entry capacity and can now predict whether or not newly published sequences in this lineage can enter human cells.

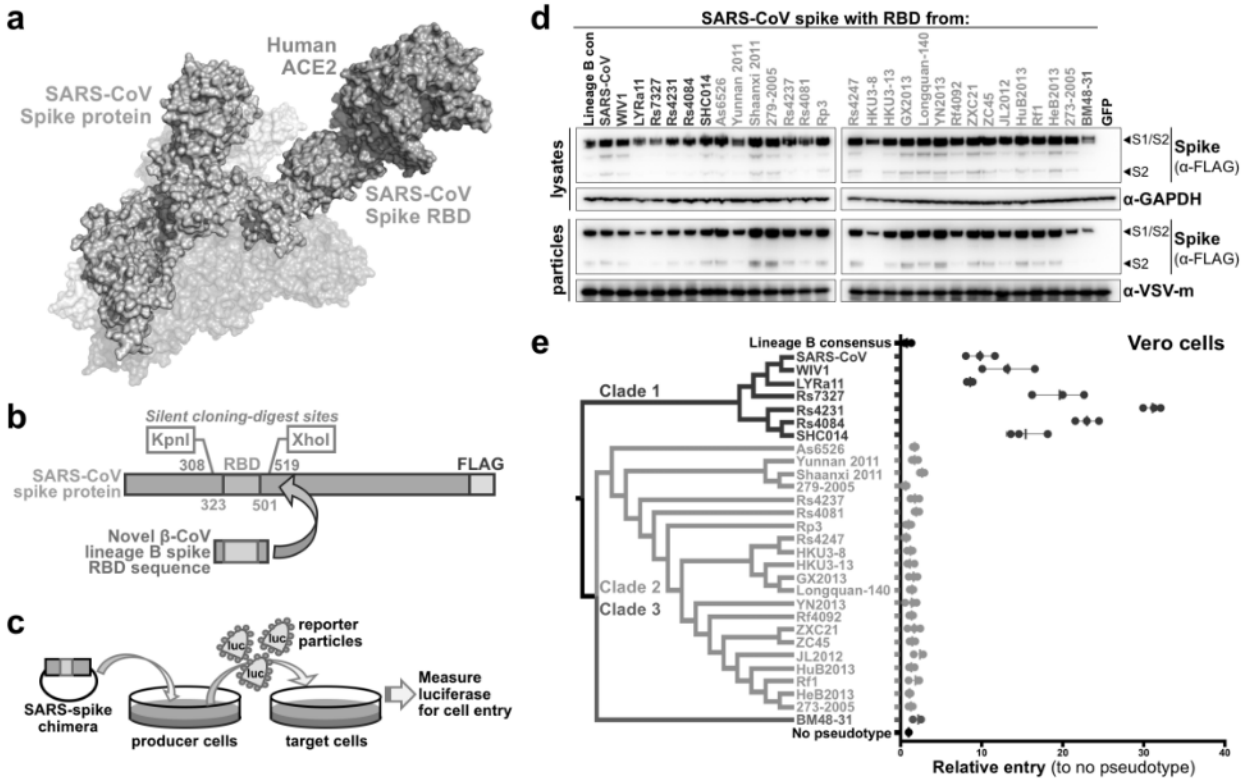
Over the past 15 years, only six viruses in lineage B have been tested for cell entry potential because these studies relied on time and labor-intensive methods. In contrast, we have tested all viruses currently known in this lineage with our platform that is cheaper, faster, and scalable - allowing us to assess zoonotic potential of novel viruses at the rate in which they are discovered. This data is invaluable to current global efforts to identify novel viruses and prepare for the next viral pandemic.

1. Virus Ecology Unit, Laboratory of Virology, Rocky Mountain Laboratories (NIAID/NIH) Hamilton, MT, 59840.

Functionally screening for zoonotic potential of the entire lineage B of betacoronaviruses

Michael Letko[1], Vincent Munster[1]

Fig. 1: β-Coronavirus lineage B entry into primate cells is clade-specific



a, All β-Coronaviruses, including SARS-CoV, interact with the host cell receptor through a short continuous Receptor Binding Domain (RBD) in spike (PDB: 5X5B, 2AJF) . **b,** SARS-CoV spike was engineered with silent mutations to facilitate cloning novel RBD sequences in place of the SARS spike RBD. SARS spike amino acid numbers are indicated for silent cloning sites and the RBD in grey and orange, respectively. **c,** outline of experimental workflow. **d,** Westernblot of producer cell lysates and concentrated reporter particles. **e,** Vero cells were infected with VSV-reporter particles pseudotyped with chimeric spikes and luciferase was measured a readout for cell entry.

1. Virus Ecology Unit, Laboratory of Virology, Rocky Mountain Laboratories (NIAID/NIH) Hamilton, MT, 59840.

From: Letko, Michael (NIH/NIAID) [F]
Sent: Fri, 5 Apr 2019 20:07:53 +0000
To: Anthony, Simon J.
Cc: Munster, Vincent (NIH/NIAID) [E]
Subject: CoV-2b sequences

Dear Simon,

I want to thank you again for agreeing to share betacoronavirus lineage B spike sequences with us. This will surely increase the impact of our story by providing a proof of principle with unpublished sequences.

We will move very quickly once we have them - these days it takes me just under 3 weeks to go from ordering synthesis of the RBD to having cell entry data.

Please let us know if you require anything else before sending the sequences.

Cheers,
-michael

--
Michael Letko, Ph.D
Postdoctoral IRTA
Dr. Vincent Munster Laboratory
Virus Ecology Unit, Laboratory of Virology
Rocky Mountain Laboratories
NIAID/NIH
PubMed
903S 4th Street
Hamilton MT 59840
(b)(6)

From: Munster, Vincent (NIH/NIAID) [E]
Sent: Fri, 29 Mar 2019 11:55:04 -0600
To: Anthony, Simon J.; Letko, Michael (NIH/NIAID) [F]
Subject: Re: Reaching out to EcoHealth

Want to do skype?

My handle is (b)(6)

Vincent Munster, PhD
Chief, Virus Ecology Unit
Laboratory of Virology
Rocky Mountain Laboratories
NIAID/NIH

From: "Anthony, Simon J." (b)(6)
Date: Wednesday, March 27, 2019 at 2:27 PM
To: Michael Letko (b)(6)
(b)(6)
Subject: Re: Reaching out to EcoHealth

Works for me!

From: "Letko, Michael (NIH/NIAID) [F]" (b)(6)
Date: Wednesday, March 27, 2019 at 4:15 PM
To: "Munster, Vincent (NIH/NIAID) [E]" (b)(6) "Anthony, Simon J."
(b)(6)
Subject: Re: Reaching out to EcoHealth

Great! How about Friday at 5:30pm (New York time).

Best,
-michael

--
Michael Letko, Ph.D
Postdoctoral IRTA
Dr. Vincent Munster Laboratory
Virus Ecology Unit, Laboratory of Virology
Rocky Mountain Laboratories
NIAID/NIH
PubMed
903S 4th Street
Hamilton MT 59840

(b)(6)

From: "Munster, Vincent (NIH/NIAID) [E]" (b)(6)
Date: Wednesday, March 27, 2019 at 2:12 PM
To: "Anthony, Simon J." (b)(6)
Cc: "Letko, Michael (NIH/NIAID) [F]" (b)(6)
Subject: Re: Reaching out to EcoHealth

Would work for me!

Cheers,

Vincent Munster, PhD
Chief, Virus Ecology Unit
Laboratory of Virology
Rocky Mountain Laboratories
NIAID/NIH

From: "Anthony, Simon J." (b)(6)
Date: Wednesday, March 27, 2019 at 2:12 PM
To: (b)(6)
Cc: Michael Letko (b)(6)
Subject: Re: Reaching out to EcoHealth

Friday? I have a meeting 3-5pm EST, but am free otherwise. Also, apologies to you, Michael! I completely forgot to respond to your follow-up message – but I wasn't (intentionally) ignoring you!

Cheers!
S.

From: "Munster, Vincent (NIH/NIAID) [E]" (b)(6)
Date: Wednesday, March 27, 2019 at 2:39 PM
To: "Anthony, Simon J." (b)(6)
Cc: "Letko, Michael (NIH/NIAID) [F]" (b)(6)
Subject: Re: Reaching out to EcoHealth

Hey Simon,

Made it back to Montana, let's pick a date/time to discuss this further,

Hope all is well,

Vincent Munster, PhD

Chief, Virus Ecology Unit
Laboratory of Virology
Rocky Mountain Laboratories
NIAID/NIH

From: "Anthony, Simon J." (b)(6)
Date: Friday, March 8, 2019 at 5:48 PM
To: (b)(6)
Subject: Re: Reaching out to EcoHealth

Hey! Looks super cool. Want to chat by phone about this? Tuesday?

From: "Munster, Vincent (NIH/NIAID) [E]" (b)(6)
Date: Friday, March 8, 2019 at 2:01 PM
To: Peter Daszak (b)(6) "Kevin Olival," (b)(6) "Anthony, Simon J." (b)(6) "Letko, Michael (NIH/NIAID) [F]" (b)(6)
Subject: FW: Reaching out to EcoHealth

Hi guys,

I wanted to show you some prelim data from one of my post-docs (Dr. Michael Letko), which we think would be of big interest of you guys and PREDICT. Michael has recently developed a rapid and scalable platform to screen novel beta-coronaviruses related to SARS-CoV (aka, Lineage B) for host-cell entry potential. Literally hundreds of lineage B beta-coronaviruses have been discovered primarily in Rhinolophus bats in Asia. Unfortunately, like many newly-discovered viruses, little is known beyond the sequence – there is almost no information on whether or not the majority of these viruses can even infect human cells. As our approach allows us to quickly move from genotype to phenotype, we feel that this work is of high interest to the field of virus discovery and to programs like EcoHealth Alliance - PREDICT.

While we have now tested all of the variation currently published for lineage B beta-coronaviruses for their ability to enter primate cells, we are seeking additional, unpublished lineage B beta-coronavirus sequences to test in our platform. Attached is a short abstract and figure further describing our approach and demonstrating the powerful results that come from it. We kindly ask that you do not distribute this material further, however, as it is still unpublished and quite competitive.

Let me know what you guys think and whether you have any samples with lineage B sequences of S-genes or lineage B rdrp for further characterization of S. I think publishing smtg which bridges the gap between virus discovery and high-throughput initial assessment of zoonotic potential would have great interest from the scientific AND public health communities.

Cheers,

Vincent Munster, PhD
Chief, Virus Ecology Unit
Laboratory of Virology
Rocky Mountain Laboratories
NIAID/NIH

From: Munster, Vincent (NIH/NIAID) [E]
Sent: Fri, 29 Mar 2019 15:28:00 -0600
To: Anthony, Simon J.
Cc: Letko, Michael (NIH/NIAID) [F]
Subject: Re: Reaching out to EcoHealth

We're ready whenever you are, we can do skype or just call

Vincent Munster, PhD
Chief, Virus Ecology Unit
Laboratory of Virology
Rocky Mountain Laboratories
NIAID/NIH

From: "Anthony, Simon J." (b)(6)
Date: Wednesday, March 27, 2019 at 2:12 PM
To: (b)(6)
Cc: Michael Letko (b)(6)
Subject: Re: Reaching out to EcoHealth

Friday? I have a meeting 3-5pm EST, but am free otherwise. Also, apologies to you, Michael! I completely forgot to respond to your follow-up message – but I wasn't (intentionally) ignoring you!

Cheers!
S.

From: "Munster, Vincent (NIH/NIAID) [E]" (b)(6)
Date: Wednesday, March 27, 2019 at 2:39 PM
To: "Anthony, Simon J." (b)(6)
Cc: "Letko, Michael (NIH/NIAID) [F]" (b)(6)
Subject: Re: Reaching out to EcoHealth

Hey Simon,

Made it back to Montana, let's pick a date/time to discuss this further,

Hope all is well,

Vincent Munster, PhD
Chief, Virus Ecology Unit
Laboratory of Virology
Rocky Mountain Laboratories
NIAID/NIH

From: "Anthony, Simon J." (b)(6)
Date: Friday, March 8, 2019 at 5:48 PM
To: (b)(6)
Subject: Re: Reaching out to EcoHealth

Hey! Looks super cool. Want to chat by phone about this? Tuesday?

From: "Munster, Vincent (NIH/NIAID) [E]" (b)(6)
Date: Friday, March 8, 2019 at 2:01 PM
To: Peter Daszak (b)(6) "Kevin Olival," (b)(6) "Anthony, Simon J." (b)(6) "Letko, Michael (NIH/NIAID) [F]" (b)(6)
Subject: FW: Reaching out to EcoHealth

Hi guys,

I wanted to show you some prelim data from one of my post-docs (Dr. Michael Letko), which we think would be of big interest of you guys and PREDICT. Michael has recently developed a rapid and scalable platform to screen novel beta-coronaviruses related to SARS-CoV (aka, Lineage B) for host-cell entry potential. Literally hundreds of lineage B beta-coronaviruses have been discovered primarily in Rhinolophus bats in Asia. Unfortunately, like many newly-discovered viruses, little is known beyond the sequence – there is almost no information on whether or not the majority of these viruses can even infect human cells. As our approach allows us to quickly move from genotype to phenotype, we feel that this work is of high interest to the field of virus discovery and to programs like EcoHealth Alliance - PREDICT.

While we have now tested all of the variation currently published for lineage B beta-coronaviruses for their ability to enter primate cells, we are seeking additional, unpublished lineage B beta-coronavirus sequences to test in our platform. Attached is a short abstract and figure further describing our approach and demonstrating the powerful results that come from it. We kindly ask that you do not distribute this material further, however, as it is still unpublished and quite competitive.

Let me know what you guys think and whether you have any samples with lineage B sequences of S-genes or lineage B rdrp for further characterization of S. I think publishing smtg which bridges the gap between virus discovery and high-throughput initial assessment of zoonotic potential would have great interest from the scientific AND public health communities.

Cheers,

Vincent Munster, PhD
Chief, Virus Ecology Unit
Laboratory of Virology
Rocky Mountain Laboratories
NIAID/NIH

From: Munster, Vincent (NIH/NIAID) [E]
Sent: Wed, 27 Mar 2019 14:51:08 -0600
To: Anthony, Simon J.; Letko, Michael (NIH/NIAID) [F]
Subject: Re: Reaching out to EcoHealth

Great!

Vincent Munster, PhD
Chief, Virus Ecology Unit
Laboratory of Virology
Rocky Mountain Laboratories
NIAID/NIH

From: "Anthony, Simon J." (b)(6)
Date: Wednesday, March 27, 2019 at 2:27 PM
To: Michael Letko (b)(6)
(b)(6)
Subject: Re: Reaching out to EcoHealth

Works for me!

From: "Letko, Michael (NIH/NIAID) [F]" (b)(6)
Date: Wednesday, March 27, 2019 at 4:15 PM
To: "Munster, Vincent (NIH/NIAID) [E]" (b)(6) "Anthony, Simon J."
(b)(6)
Subject: Re: Reaching out to EcoHealth

Great! How about Friday at 5:30pm (New York time).

Best,
-michael

--
Michael Letko, Ph.D
Postdoctoral IRTA
Dr. Vincent Munster Laboratory
Virus Ecology Unit, Laboratory of Virology
Rocky Mountain Laboratories
NIAID/NIH
PubMed
903S 4th Street
Hamilton MT 59840
(b)(6)

From: "Munster, Vincent (NIH/NIAID) [E]" (b)(6)
Date: Wednesday, March 27, 2019 at 2:12 PM
To: "Anthony, Simon J." (b)(6)
Cc: "Letko, Michael (NIH/NIAID) [F]" (b)(6)
Subject: Re: Reaching out to EcoHealth

Would work for me!

Cheers,

Vincent Munster, PhD
Chief, Virus Ecology Unit
Laboratory of Virology
Rocky Mountain Laboratories
NIAID/NIH

From: "Anthony, Simon J." (b)(6)
Date: Wednesday, March 27, 2019 at 2:12 PM
To: (b)(6)
Cc: Michael Letko (b)(6)
Subject: Re: Reaching out to EcoHealth

Friday? I have a meeting 3-5pm EST, but am free otherwise. Also, apologies to you, Michael! I completely forgot to respond to your follow-up message – but I wasn't (intentionally) ignoring you!

Cheers!
S.

From: "Munster, Vincent (NIH/NIAID) [E]" (b)(6)
Date: Wednesday, March 27, 2019 at 2:39 PM
To: "Anthony, Simon J." (b)(6)
Cc: "Letko, Michael (NIH/NIAID) [F]" (b)(6)
Subject: Re: Reaching out to EcoHealth

Hey Simon,

Made it back to Montana, let's pick a date/time to discuss this further,

Hope all is well,

Vincent Munster, PhD
Chief, Virus Ecology Unit
Laboratory of Virology

Rocky Mountain Laboratories
NIAID/NIH

From: "Anthony, Simon J." (b)(6)
Date: Friday, March 8, 2019 at 5:48 PM
To: (b)(6)
Subject: Re: Reaching out to EcoHealth

Hey! Looks super cool. Want to chat by phone about this? Tuesday?

From: "Munster, Vincent (NIH/NIAID) [E]" (b)(6)
Date: Friday, March 8, 2019 at 2:01 PM
To: Peter Daszak (b)(6) "Kevin Olival," (b)(6) "Anthony, Simon J." (b)(6) "Letko, Michael (NIH/NIAID) [F]" (b)(6)
Subject: FW: Reaching out to EcoHealth

Hi guys,

I wanted to show you some prelim data from one of my post-docs (Dr. Michael Letko), which we think would be of big interest of you guys and PREDICT. Michael has recently developed a rapid and scalable platform to screen novel beta-coronaviruses related to SARS-CoV (aka, Lineage B) for host-cell entry potential. Literally hundreds of lineage B beta-coronaviruses have been discovered primarily in Rhinolophus bats in Asia. Unfortunately, like many newly-discovered viruses, little is known beyond the sequence – there is almost no information on whether or not the majority of these viruses can even infect human cells. As our approach allows us to quickly move from genotype to phenotype, we feel that this work is of high interest to the field of virus discovery and to programs like EcoHealth Alliance - PREDICT.

While we have now tested all of the variation currently published for lineage B beta-coronaviruses for their ability to enter primate cells, we are seeking additional, unpublished lineage B beta-coronavirus sequences to test in our platform. Attached is a short abstract and figure further describing our approach and demonstrating the powerful results that come from it. We kindly ask that you do not distribute this material further, however, as it is still unpublished and quite competitive.

Let me know what you guys think and whether you have any samples with lineage B sequences of S-genes or lineage B rdrp for further characterization of S. I think publishing smtg which bridges the gap between virus discovery and high-throughput initial assessment of zoonotic potential would have great interest from the scientific AND public health communities.

Cheers,

Vincent Munster, PhD
Chief, Virus Ecology Unit

Laboratory of Virology
Rocky Mountain Laboratories
NIAID/NIH

From: Munster, Vincent (NIH/NIAID) [E]
Sent: Thu, 21 Mar 2019 03:34:39 -0700
To: Letko, Michael (NIH/NIAID) [F]
Subject: Re: Reaching out to EcoHealth

Did you get any response?

Hope work is going well,

Vincent Munster, PhD
Chief, Virus Ecology Unit
Laboratory of Virology
Rocky Mountain Laboratories
NIAID/NIH

From: Michael Letko (b)(6)
Date: Monday, March 18, 2019 at 8:47 AM
To: (b)(6) "Anthony, Simon J." (b)(6)
Subject: Re: Reaching out to EcoHealth

Dear Simon,

I am available tomorrow (Tuesday) any time after 1pm (NY time).

Does that work for you?

Best,
-michael

--
Michael Letko, Ph.D
Postdoctoral IRTA
Dr. Vincent Munster Laboratory
Virus Ecology Unit, Laboratory of Virology
Rocky Mountain Laboratories
NIAID/NIH
PubMed
903S 4th Street
Hamilton MT 59840
(b)(6)

From: "Munster, Vincent (NIH/NIAID) [E]" (b)(6)
Date: Saturday, March 9, 2019 at 10:22 AM
To: "Anthony, Simon J." (b)(6)
Cc: "Letko, Michael (NIH/NIAID) [F]" (b)(6)
Subject: Re: Reaching out to EcoHealth

Hey Simon,

I'll be travelling to Cambodia this week, would you be able to chat the week after?

I'll be in Virginia then (some state department safety training), but should hopefully be able to get a call in,

In the meantime, if you like a bit more detail feel free to chat with Michael

Vincent Munster, PhD
Chief, Virus Ecology Unit
Laboratory of Virology
Rocky Mountain Laboratories
NIAID/NIH

From: "Anthony, Simon J." (b)(6)
Date: Friday, March 8, 2019 at 5:48 PM
To: "vincent.munster@nih.gov" (b)(6)
Subject: Re: Reaching out to EcoHealth

Hey! Looks super cool. Want to chat by phone about this? Tuesday?

From: "Munster, Vincent (NIH/NIAID) [E]" (b)(6)
Date: Friday, March 8, 2019 at 2:01 PM
To: Peter Daszak (b)(6) "Kevin Olival," (b)(6) "Anthony, Simon J." (b)(6) "Letko, Michael (NIH/NIAID) [F]" (b)(6)
Subject: FW: Reaching out to EcoHealth

Hi guys,

I wanted to show you some prelim data from one of my post-docs (Dr. Michael Letko), which we think would be of big interest of you guys and PREDICT. Michael has recently developed a rapid and scalable platform to screen novel beta-coronaviruses related to SARS-CoV (aka, Lineage B) for host-cell entry potential. Literally hundreds of lineage B beta-coronaviruses have been discovered primarily in Rhinolophus bats in Asia. Unfortunately, like many newly-discovered viruses, little is known beyond the sequence – there is almost no information on whether or not the majority of these viruses can even infect human cells. As our approach allows us to quickly move from genotype to phenotype, we feel

that this work is of high interest to the field of virus discovery and to programs like EcoHealth Alliance - PREDICT.

While we have now tested all of the variation currently published for lineage B beta-coronaviruses for their ability to enter primate cells, we are seeking additional, unpublished lineage B beta-coronavirus sequences to test in our platform. Attached is a short abstract and figure further describing our approach and demonstrating the powerful results that come from it. We kindly ask that you do not distribute this material further, however, as it is still unpublished and quite competitive.

Let me know what you guys think and whether you have any samples with lineage B sequences of S-genes or lineage B rdrp for further characterization of S. I think publishing smtg which bridges the gap between virus discovery and high-throughput initial assessment of zoonotic potential would have great interest from the scientific AND public health communities.

Cheers,

Vincent Munster, PhD
Chief, Virus Ecology Unit
Laboratory of Virology
Rocky Mountain Laboratories
NIAID/NIH

From: Letko, Michael (NIH/NIAID) [F]
Sent: Fri, 8 Mar 2019 23:13:19 +0000
To: Munster, Vincent (NIH/NIAID) [E]
Subject: Another cell line to get from Muller

We want any Rhinolophus cell lines he might have. The two that are published are:

1. RhiNi = R. landeri
2. RhiLu = R. alcyone

--
Michael Letko, Ph.D
Postdoctoral IRTA
Dr. Vincent Munster Laboratory
Virus Ecology Unit, Laboratory of Virology
Rocky Mountain Laboratories
NIAID/NIH
PubMed
903S 4th Street
Hamilton MT 59840
(b)(6)

From: Letko, Michael (NIH/NIAID) [F]
Sent: Fri, 8 Mar 2019 18:09:38 +0000
To: Munster, Vincent (NIH/NIAID) [E]
Subject: RhiLu cells
Attachments: Muller 2012.pdf

We want the RhiLu cell line shown in figure 2 of the attached paper.

-michael

--
Michael Letko, Ph.D
Postdoctoral IRTA
Dr. Vincent Munster Laboratory
Virus Ecology Unit, Laboratory of Virology
Rocky Mountain Laboratories
NIAID/NIH
PubMed
903S 4th Street
Hamilton MT 59840
(b)(6)

Human Coronavirus EMC Does Not Require the SARS-Coronavirus Receptor and Maintains Broad Replicative Capability in Mammalian Cell Lines

Marcel A. Müller,[a] V. Stalin Raj,[b] Doreen Muth,[a] Benjamin Meyer,[a] Stephan Kallies,[a] Saskia L. Smits,[b] Robert Wollny,[a] Theo M. Bestebroer,[b] Sabine Specht,[c] Tasnim Suliman,[a] Katrin Zimmermann,[d] Tabea Binger,[a] Isabella Eckerle,[a] Marco Tschapka,[e] Ali M. Zaki,[f] Albert D. M. E. Osterhaus,[b] Ron A. M. Fouchier,[b] Bart L. Haagmans,[b] and Christian Drosten[a]

Institute of Virology, University of Bonn Medical Center, Bonn, Germany[a]; Viroscience Lab, Erasmus MC, Rotterdam, The Netherlands[b]; Institute of Medical Microbiology, Immunology and Parasitology, University of Bonn Medical Center, Bonn, Germany[c]; Institute of Pharmacology and Toxicology, University of Bonn Medical Center, Bonn, Germany[d]; Institute of Experimental Ecology, University of Ulm, Ulm, Germany[e]; and Virology Laboratory, Dr. Soliman Fakeeh Hospital, Jeddah, Kingdom of Saudi Arabia[f]

ABSTRACT **A new human coronavirus (hCoV-EMC) has emerged very recently in the Middle East. The clinical presentation resembled that of the severe acute respiratory syndrome (SARS) as encountered during the epidemic in 2002/2003. In both cases, acute renal failure was observed in humans. HCoV-EMC is a member of the same virus genus as SARS-CoV but constitutes a sister species. Here we investigated whether it might utilize angiotensin-converting enzyme 2 (ACE2), the SARS-CoV receptor. Knowledge of the receptor is highly critical because the restriction of the SARS receptor to deep compartments of the human respiratory tract limited the spread of SARS. In baby hamster kidney (BHK) cells, lentiviral transduction of human ACE2 (hACE2) conferred permissiveness and replication for SARS-CoV but not for hCoV-EMC. Monkey and human kidney cells (LLC-MK2, Vero, and 769-P) and swine kidney cells were permissive for both viruses, but only SARS-CoV infection could be blocked by anti-hACE2 antibody and could be neutralized by preincubation of virus with soluble ACE2. Our data show that ACE2 is neither necessary nor sufficient for hCoV-EMC replication. Moreover, hCoV-EMC, but not SARS-CoV, replicated in cell lines from *Rousettus, Rhinolophus, Pipistrellus, Myotis, and Carollia* bats, representing four major chiropteran families from both suborders. As human CoV normally cannot replicate in bat cells from different families, this suggests that hCoV-EMC might use a receptor molecule that is conserved in bats, pigs, and humans, implicating a low barrier against cross-host transmission.**

IMPORTANCE **A new human coronavirus (hCoV) emerged recently in the Middle East. The disease resembled SARS (severe acute respiratory syndrome), causing a fatal epidemic in 2002/2003. Coronaviruses have a reservoir in bats and because this novel virus is related to SARS-CoV, we investigated whether it might replicate in bat cells and use the same receptor (angiotensin-converting enzyme 2 [ACE2]). This knowledge is highly critical, because the SARS-CoV receptor influenced pathology, and its localization in the deep respiratory tract is thought to have restricted the transmissibility of SARS. Our data show that hCoV-EMC does not need the SARS-CoV receptor to infect human cells. Moreover, the virus is capable of infecting human, pig, and bat cells. This is remarkable, as human CoVs normally cannot replicate in bat cells as a consequence of host adaptation. Our results implicate that the new virus might use a receptor that is conserved between bats, pigs and humans suggesting a low barrier against cross-host transmission.**

Received 9 November 2012 **Accepted** 12 November 2012 **Published** 11 December 2012

Citation Müller MA, et al. 2012. Human coronavirus EMC does not require the SARS-coronavirus receptor and maintains broad replicative capability in mammalian cell lines. mBio 3(6):e00515-12. doi:10.1128/mBio.00515-12.

Editor Michael Buchmeier, University of California, Irvine

Copyright © 2012 Müller et al. This is an open-access article distributed under the terms of the Creative Commons Attribution-Noncommercial-Share Alike 3.0 Unported License, which permits unrestricted noncommercial use, distribution, and reproduction in any medium, provided the original author and source are credited.

Address correspondence to Bart L. Haagmans, b.haagmans@erasmusmc.nl, or Christian Drosten, drosten@virology-bonn.de.

Downloaded from http://mbio.asm.org/ on March 8, 2019 by guest

Coronaviruses (CoVs) are large enveloped plus-strand RNA viruses. Over the last decades, several animal CoVs have been identified, including major pathogens such as the bovine CoV and the avian infectious bronchitis virus. Two human CoVs (hCoVs) termed hCoV-OC43 and hCoV-229E have been known since the 1960s, causing mild respiratory disease (1). In 2002/2003, an outbreak of severe acute respiratory syndrome (SARS) leading to over 700 deaths was caused by a novel hCoV termed SARS-CoV (2, 3). In the aftermath of the SARS epidemic, two additional hCoVs termed hCoV-NL63 and -HKU1 as well as numerous novel bat-associated CoVs were described (4–7). CoVs have been reclassified into four genera designated *Alphacoronavirus* (former groups 1a and 1b), *Betacoronavirus* (groups 2a to 2d), *Gammacoronavirus* (group 3), and as recently suggested, *Deltacoronavirus* (8). A likely animal reservoir for SARS-CoV has been identified in bats (7, 9–11). Nevertheless, bat-derived CoVs have never been isolated in cell culture. Prototypic CoVs, including the SARS-CoV, are almost unculturable in bat-derived cell lines (11, 12). Double infections of bats with different CoVs are very rare (13), and bat CoVs appear to be restricted to their bat hosts at the genus level (14, 15).

These points taken together suggest that there is a tight barrier against CoV host switching, preventing humans from acquiring novel CoVs easily (1, 11).

The SARS-CoV utilizes the angiotensin-converting enzyme 2 (ACE2) as its necessary and sufficient cellular receptor (11, 16). The cellular and anatomic localization of ACE2 in the human body had an important meaning for the pathogenesis and epidemiology of SARS. The receptor is expressed on pneumocytes deep in the human lung, but hardly in the upper parts of the respiratory tract (17). The direct targeting of pneumocytes may explain why SARS-CoV infection led to a severe clinical picture with early onset of respiratory distress in affected patients. The focusing of SARS-CoV replication to the deep respiratory tract also determined a major hallmark of SARS epidemiology. Because the virus needed to reach the deep respiratory tract to seed an infection, the infectious dose was rather high. Once infected, primary replication in the deep lung rapidly caused severe clinical symptoms leading to early hospitalization and thus, isolation, before patients could develop high virus concentrations in respiratory secretions. These factors taken together are thought to have limited the spread of SARS-CoV in the human population.

In September 2012, health authorities were notified of cases of severe hCoV infection caused by a novel virus type in the Middle East region (18, 19). The disease involved severe acute respiratory failure but as an additional component affected the kidneys, causing acute renal failure (18, 19). A novel betacoronavirus was isolated from one of two patients in Jeddah, Saudi Arabia, and termed hCoV-EMC (also named hCoV-EMC/2012) (10, 20). Another case originating from Qatar was transferred to London, United Kingdom, for treatment (18, 19). Very recently, a third case was reported from Riyadh, Saudi Arabia, suggesting that more cases are to be expected (21). Availability of a virus isolate enables rapid provisional assessments of virus properties in order to guess at virus origins and project epidemic risks. Because of the taxonomic placement of hCoV-EMC and SARS-CoV as sister clades within the same virus genus, we were interested in rapidly assessing any possible overlaps in receptor usage (18, 20).

For the purpose of receptor usage studies, we have established a baby hamster kidney (BHK) cell line carrying human ACE2 (hACE2) following lentiviral transduction. BHK cells normally do not express ACE2 and are not permissive for SARS-CoV. As shown in Fig. 1A, the transduced cells termed BHK-hACE2 expressed hACE2 as demonstrable by antigen staining using an immunofluorescence assay (IFA). Surface expression of hACE2 was detected by fluorescence-activated cell sorting (FACS). SARS-CoV applied at a multiplicity of infection (MOI) of 0.5 replicated more than 10,000-fold within 40 h in BHK-hACE2 but not in the parental BHK cell line (Fig. 1B). Infection with hCoV-EMC under the same conditions showed no replication irrespective of the presence of hACE2. We concluded that hACE2 was sufficient to permit infection by SARS-CoV, but not hCoV-EMC, in BHK cells.

To exclude technical confounders due to transgenic expression of hACE2, infection studies were repeated in kidney cells naturally permissive for SARS-CoV. Next to Vero cells in which the original isolate of hCoV-EMC had been obtained, LLC-MK2 and MA104 cells are commonly used monkey kidney cell lines known to be permissive for SARS-CoV (22). As shown in Fig. 1A, LLC-MK2 and MA104 cells expressed ACE2 as demonstrated by IFA and FACS. SARS-CoV infected cells under conditions as described above replicated more than 1,000-fold within 40 h in both cell lines. In contrast, while hCoV-EMC showed strong replication in LLC-MK2 cells, it did not replicate at all in MA104 cells.

While these findings confirmed that ACE2 did not suffice for hCoV-EMC permissibility, it might still be necessary in addition to another receptor. To clarify its usage by hCoV-EMC in primate cells like LLC-MK2 and Vero cells, a polyclonal goat serum directed against hACE2 was used for receptor-blocking studies. As shown in Fig. 1C, infection of Vero cells by SARS-CoV, but not hCoV-EMC, was completely blocked by the antibody. The blocking of SARS-CoV infection occurred in a dose-dependent manner (see Fig. S1 in the supplemental material).

To exclude the remaining possibility that our antibody blocked an ACE2 epitope relevant for binding of SARS-CoV but irrelevant for hCoV-EMC, infection neutralization assays were carried out using soluble ACE2 protein in Vero cells. As shown in Fig. 1D, infection with SARS-CoV, but not with hCoV-EMC, could be competed in a dose-dependent manner by the presence of soluble ACE2. Overall, these observations led us to conclude that SARS-CoV, but not hCoV-EMC, utilizes hACE2 as a receptor for virus entry.

While it is known that SARS-related CoVs occur widely in bats, the human SARS-CoV is adapted to the human ACE2 receptor and has lost its ability to infect bat cells (11, 12). For reference, we generated kidney cells from *Rousettus aegyptiacus* bats, termed RoNi/7. As shown in Fig. 2A, lentiviral transduction and selection yielded a variant of these cells, termed RoNi/7-hACE2, which expressed hACE2. Infection of these cells with SARS-CoV resulted in high levels of virus replication, while the parental RoNi/7 cells were not susceptible (Fig. 2B; see Fig. S2 in the supplemental material). Surprisingly, these same parental RoNi/7 cells were highly permissive for hCoV-EMC. The derived RoNi/7-hACE2 cells appeared slightly more permissive for hCoV-EMC, but by performing antibody competition experiments, the possibility that this difference was due to the presence of hACE2 in RoNi/7-hACE2 cells was excluded, as SARS-CoV infection was blocked by anti-hACE2 antibody, and hCoV-EMC infection was not (Fig. 2C).

The ability of hCoV-EMC to grow in bat cells was highly remarkable, as most other prototype coronaviruses, including the human viruses and bovine, feline, or porcine CoVs, are normally unable to grow in bat cells (9, 12; our unpublished observations). Because hCoV-EMC was most closely related to bat CoV HKU5 detected in bats of the genus *Pipistrellus* (18, 20, 23), we generated two different kidney cell lines from the European bat species *Pipistrellus pipistrellus* as well as a kidney cell line from another European vespertilionid bat species, *Myotis daubentonii* (MyDauNi/2). A New World bat cell line from the species *Carollia perspicillata* (CarNi/1) was made, and a permanent cell line from *Rhinolophus landeri* lung cells was included to represent the bat genus associated with SARS-related CoV. Porcine kidney cells (PS) were tested to represent a larger range of laurasiatherian mammals, and a human kidney cancer cell line (769-P) was included to complement the tested range of primate cells. As shown in Fig. 2B, all of the bat cell lines were readily infected by hCoV-EMC (lower panel) but not by SARS-CoV (upper panel). Porcine and human kidney cell lines were infected by both coronaviruses (Fig. 2B). In summary, hCoV-EMC was able to infect cells from four major families of bats covering the whole diversity of this large mammalian order (Pteropodidae [Rousettus], Rhinolophidae [Rhinolophus], Vespertilionidae [*Pipistrellus*, *Myotis*], Phyllostomidae

Downloaded from http://mbio.asm.org/ on March 8, 2019 by guest

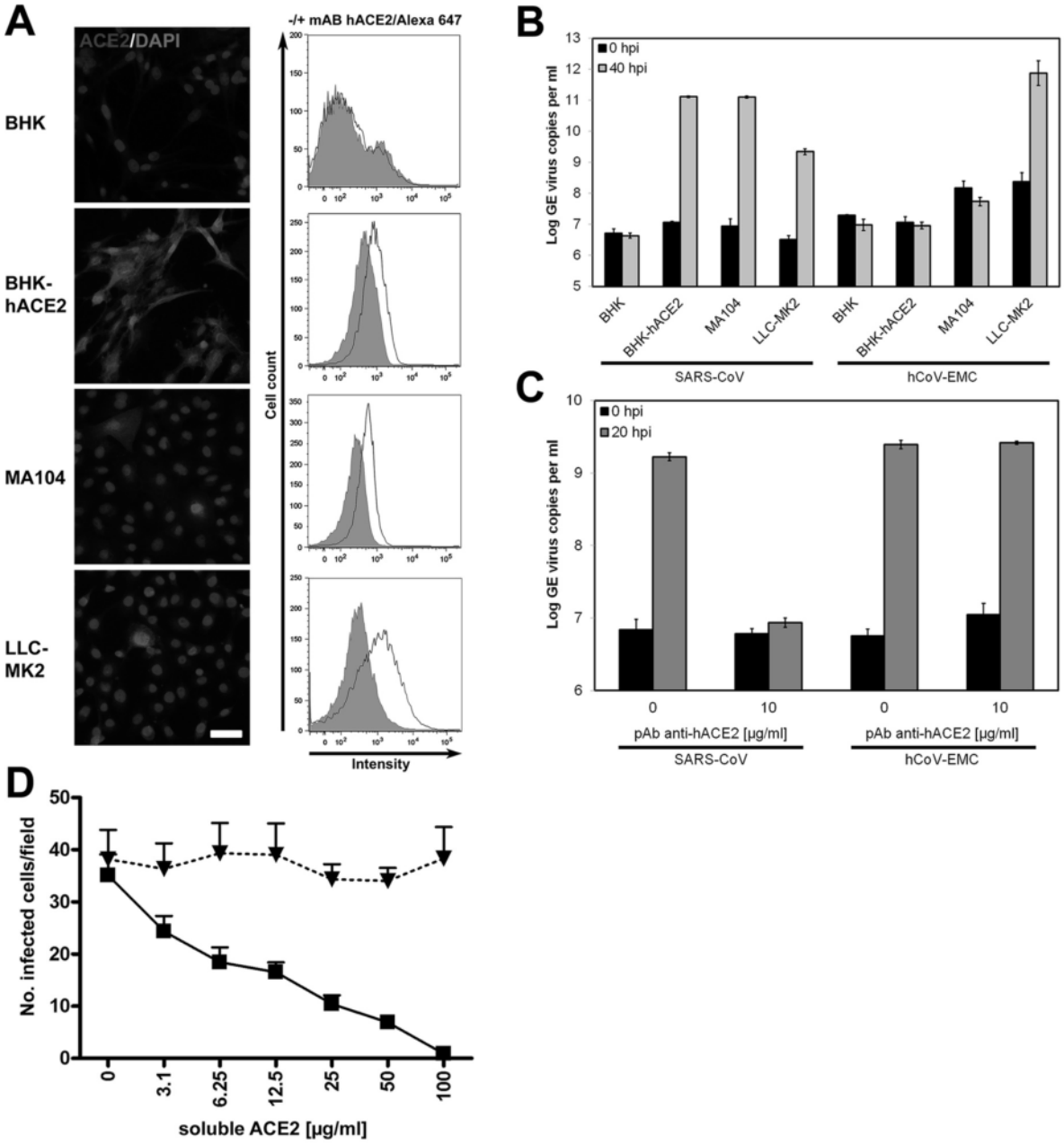


FIG 1 Human ACE2-independent entry of hCoV-EMC. (A) Baby hamster kidney (BHK) cells were transduced with hACE2-carrying lentiviruses and selected by puromycin treatment. (Left) hACE2 expression was controlled by immunofluorescence assay (IFA) by using goat anti-hACE2 immunoglobulin (Ig) (R&D Systems) followed by cyanin 3-labeled donkey anti-goat Ig (Dianova). For controls, ACE2-expressing primate cell lines (kidney cells from rhesus monkey [LLC-MK2] and African green monkey [MA104]) were stained in parallel. DAPI, 4',6'-diamidino-2-phenylindole. Bar, 50 μm. (Right) ACE2 protein surface expression was determined by FACS analysis using a mouse anti-hACE2 Ig antibody (R&D Systems) in combination with an Alexa Fluor 647-labeled goat anti-mouse Ig (Life Technologies). Data were acquired using FACS Canto and analyzed with FlowJo software. The cells were incubated with the secondary antibody only (shown in gray) for a control. mAB, monoclonal antibody. (B) The interferon-deficient BHK cells and the generated hACE2-expressing transgenic BHK cells were applied in infection studies. LLC-MK2 and MA104 primate cells known to be susceptible for SARS-CoV were infected in parallel with SARS-CoV strain Frankfurt-1 and hCoV-EMC. The cells were infected with an MOI of 0.5 for 1 h, washed twice with phosphate-buffered saline (PBS), and supplemented with medium. At time points 0 and 40 h after infection, samples from supernatants were taken in order to extract viral RNA. Real-time reverse transcription-PCR (RT-PCR) was used for absolute quantification of genome equivalents (GE) per ml of supernatant (2, 10). All experiments were performed in triplicate. hpi, hours postinfection. (C) In order to block the ACE2 receptor, Vero cells were preincubated with 10 μg/ml of polyclonal goat anti-hACE2 for 1 h at 37°C. Half of the antibody solution was stored, and cells were subsequently infected by adding viruses at an MOI of 0.01 for 30 min at 4°C. The supernatant was discarded, and medium with antibody solution was added. At time points 0 and 20 h postinfection, samples from supernatants (triplicate samples) were taken for quantification by real-time RT-PCR. pAb, polyclonal antibody. (D) Virus-neutralizing activity of soluble recombinant hACE2 (rhACE2) was tested by preincubating in triplicate different concentrations of rhACE2 (a kind gift from J. M. Penninger, Vienna, Austria) with 10^4 50% tissue culture infective dose ($TCID_{50}$)/ml hCoV-EMC (triangles) or 10^3 $TCID_{50}$/ml SARS-CoV (squares) for 1 h. The mixture was added to Vero E6 cells for 1 h, after which cells were washed and fresh medium was added. The infection was stopped after 8 h, and cells were visualized with a cross-reactive SARS-CoV serum followed by a goat anti-rabbit IgG antibody conjugated to peroxidase and stained with 3-amino-9-ethylcarbazole (AEC) substrate.

Downloaded from http://mbio.asm.org/ on March 8, 2019 by guest

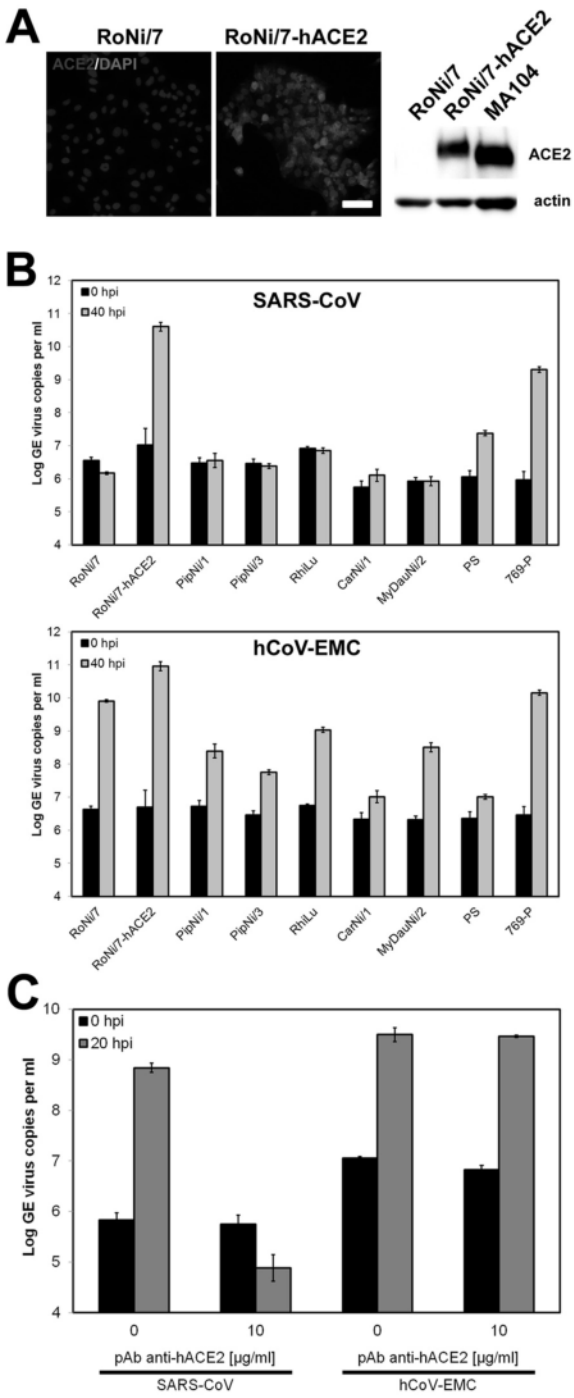


FIG 2 Bat cells allow ACE2-independent entry of hCoV-EMC. (A) Immortalized *Rousettus aegyptiacus* cells were provided with hACE2. (Left) Expression of hACE2 was controlled by immunofluorescence assay using goat anti-hACE2 Ig and cyanin 3-labeled donkey anti-goat Ig. Bar, 50 μm. (Right) Western blot analysis was done using goat anti-hACE2 Ig followed by horseradish peroxidase-labeled donkey anti-goat Ig (Dianova). For a control, a protein lysate of MA104 cells was applied to the blot. A loading control was done with a rabbit anti-pan-actin Ig (Cell Signaling). (B) Representative bat cell cultures from Yinpterochiroptera (*Rousettus aegyptiacus* kidney [RoNi/7] and *Rhinolophus landeri* lung [RhiLu]) and Yangochiroptera (*Pipistrellus pipistrellus* kidney [PipNi/1 and PipNi/3], *Carollia perspicillata* kidney [CarNi/1], and *Myotis daubentonii* kidney [MyDauNi/2]) and porcine (PS) and human kidney (769-P) cell lines were used for infection studies with SARS-CoV (top panel) and hCoV-EMC (bottom panel). For a control and for comparison, [Carollia]). Replication in porcine and primate cells suggests an even broader tropism across mammals.

Large parts of the epidemiology and pathology of SARS were related to its receptor tropism in humans (3, 11, 16). In a scenario resembling that for avian influenza viruses, the anatomical position of the receptor in the deep respiratory tract and the downregulation of its expression were postulated to aggravate the course of disease in individuals while limiting the spread of disease on the population level (24). While we are still uncertain of the epidemiology of hCoV-EMC-related infections, the importance of knowing its receptor in human cells is unquestionable (11). Our data taken together suggest that hCoV-EMC does not rely on the same receptor as SARS-CoV. Urgent further research is needed in order to identify the cellular receptor for hCoV-EMC and determine its anatomical focus of replication. Our observations regarding the cell culture tropism of this novel human virus raise an intriguing perspective. We have shown here that cells from primates, pigs, as well as bats representing four families from both chiropteran suborders, Yangochiroptera and Yinpterochiroptera, retain susceptibility for the virus. This breadth of tropism is absolutely unique among CoVs (1, 11, 12). For instance, only once have researchers succeeded in culturing any CoV in bat-derived cells, and these experiments required specifically generated bat cell cultures combined with engineered, reporter gene-expressing virus (12). The broad replicative capability of hCoV-EMC suggests that this new virus might utilize a receptor structure that bats, primates, and pigs have in common. If that receptor were expressed in mucosal surfaces in those hosts, repeated acquisition by humans would be conceivable, putting our current idea of a "tight" molecular barrier against coronaviral cross-host transmission into perspective (11, 12). Interestingly, these data provide strong support for the existence of "generalist" CoV, as recently projected based on cell culture studies (12).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00515-12/-/DCSupplemental.

Figure S1, TIF file, 0.1 MB.

Figure S2, TIF file, 0.2 MB.

ACKNOWLEDGMENTS

This study was supported by the European Union FP7 project EMPERIE (contract number 223498), ANTIGONE (contract number 278976), the German Research Foundation (DFG grants DR 772/3-1 and KA1241/18-1), as well as the German Ministry of Education and Research (BMBF SARS II).

We are grateful to Florian Gloza-Rausch (Noctalis, Bad Segeberg, Ger-

(Continued)

Figure Legend Continued

hACE2-expressing transgenic *Rousettus* bat cell cultures (RoNi/7-hACE2) were included. The cells were infected at an MOI of 0.5 for 1 h, washed twice with PBS, and supplemented with medium. At time points 0 and 40 h postinfection, samples from supernatants were taken to extract viral RNA. Real-time RT-PCR was performed as described for absolute quantification of GE. All experiments were performed in triplicate. (C) A hACE2-blocking experiment was done with RoNi/7-hACE2 cells. The cells were preincubated with 10 μg/ml of polyclonal goat anti-hACE2 for 1 h at 37°C. The cells were subsequently infected by adding viruses at an MOI of 0.01 for 30 min at 4°C. The supernatant was discarded, and medium supplemented with hACE2 antibody was added. At time points 0 and 20 h postinfection, samples from supernatants were taken to quantify GE by real-time RT-PCR. The experiment was performed in triplicate.

Downloaded from http://mbio.asm.org/ on March 8, 2019 by guest

many), Matthias Habetha, Artem Siemens, and Andrea Rasche (Institute of Virology, Bonn, Germany), and Kim Schmidt (Institute of Medical Microbiology, Immunology and Parasitology) and Elmar Endl (FACS core facility of the University of Bonn Medical Center) for technical assistance. We also thank Frank Westermann (German Cancer Research Center, Heidelberg, Germany) for providing 769-P cells.

REFERENCES

1. **Saif LJ.** 2004. Animal coronaviruses: what can they teach us about the severe acute respiratory syndrome? Rev. Sci. Tech. **23**:643–660.
2. **Drosten C, et al.** 2003. Identification of a novel coronavirus in patients with severe acute respiratory syndrome. N. Engl. J. Med. **348**:1967–1976.
3. **Peiris JS, Yuen KY, Osterhaus AD, Stöhr K.** 2003. The severe acute respiratory syndrome. N. Engl. J. Med. **349**:2431–2441.
4. **Fouchier RA, et al.** 2004. A previously undescribed coronavirus associated with respiratory disease in humans. Proc. Natl. Acad. Sci. U. S. A. **101**:6212–6216.
5. **van der Hoek L, et al.** 2004. Identification of a new human coronavirus. Nat. Med. **10**:368–373.
6. **Woo PC, et al.** 2005. Characterization and complete genome sequence of a novel coronavirus, coronavirus HKU1, from patients with pneumonia. J. Virol. **79**:884–895.
7. **Woo PC, Lau SK, Huang Y, Yuen KY.** 2009. Coronavirus diversity, phylogeny and interspecies jumping. Exp. Biol. Med. (Maywood) **234**:1117–1127.
8. **Woo PC, et al.** 2012. Discovery of seven novel mammalian and avian coronaviruses in the genus deltacoronavirus supports bat coronaviruses as the gene source of alphacoronavirus and betacoronavirus and avian coronaviruses as the gene source of gammacoronavirus and deltacoronavirus. J. Virol. **86**:3995–4008.
9. **Biesold SE, et al.** 2011. Type I interferon reaction to viral infection in interferon-competent, immortalized cell lines from the African fruit bat Eidolon helvum. PLoS One **6**:e28131.
10. **Corman V, et al.** 2012. Detection of a novel human coronavirus by real-time reverse-transcription polymerase chain reaction. Euro Surveill. **17**(39):p=20285ii.
11. **Graham RL, Baric RS.** 2010. Recombination, reservoirs, and the modular spike: mechanisms of coronavirus cross-species transmission. J. Virol. **84**:3134–3146.
12. **Huynh J, et al.** 2012. Evidence supporting a zoonotic origin of human coronavirus strain NL63. J. Virol. **86**:12816–12825.
13. **Lau SK, et al.** 2010. Coexistence of different genotypes in the same bat and serological characterization of Rousettus bat coronavirus HKU9 belonging to a novel Betacoronavirus subgroup. J. Virol. **84**:11385–11394.
14. **Drexler JF, et al.** 2010. Genomic characterization of severe acute respiratory syndrome-related coronavirus in European bats and classification of coronaviruses based on partial RNA-dependent RNA polymerase gene sequences. J. Virol. **84**:11336–11349.
15. **Gloza-Rausch F, et al.** 2008. Detection and prevalence patterns of group I coronaviruses in bats, northern Germany. Emerg. Infect. Dis. **14**:626–631.
16. **Li W, et al.** 2003. Angiotensin-converting enzyme 2 is a functional receptor for the SARS coronavirus. Nature **426**:450–454.
17. **Bertram S, et al.** 2012. Influenza and SARS-coronavirus activating proteases TMPRSS2 and HAT are expressed at multiple sites in human respiratory and gastrointestinal tracts. PLoS One **7**:e35876.
18. **Bermingham A, et al.** 2012. Severe respiratory illness caused by a novel coronavirus, in a patient transferred to the United Kingdom from the Middle East. Euro Surveill. **17**(40):p=20290ii.
19. **Pebody RG, et al.** 2012. The United Kingdom public health response to an imported laboratory confirmed case of a novel coronavirus in September 2012. Euro Surveill. **17**(40):p=20292ii.
20. **Zaki AM, van Boheemen S, Bestebroer TM, Osterhaus AD, Fouchier RA.** 2012. Isolation of a novel coronavirus from a man with pneumonia in Saudi Arabia. N. Engl. J. Med. **367**:1814–1820.
21. **Memish Z.** 2012. A new Saudi novel coronavirus case diagnosed in KSA (Kingdom of Saudi Arabia). Archive no. 20121104.1391285, Program for Monitoring Emerging Diseases (ProMedMail). International Society for Infectious Diseases, Brookline, MA.
22. **Kaye M.** 2006. SARS-associated coronavirus replication in cell lines. Emerg. Infect. Dis. **12**:128–133.
23. **Woo PC, et al.** 2006. Molecular diversity of coronaviruses in bats. Virology **351**:180–187.
24. **Kuba K, et al.** 2005. A crucial role of angiotensin converting enzyme 2 (ACE2) in SARS coronavirus-induced lung injury. Nat. Med. **11**:875–879.

Downloaded from http://mbio.asm.org/ on March 8, 2019 by guest

From: Letko, Michael Colin
Sent: Wed, 22 Jan 2020 23:38:51 +0000
To: Munster, Vincent (NIH/NIAID) [E]
Subject: Re: bioRxiv -- Manuscript Screened
Attachments: image001.jpg, image002.jpg

They responded back to my exposition request. No hard promises but they are definitely aware.

Michael Letko, Ph.D
Assistant Professor
Laboratory of Functional Viromics
Paul G. Allen School for Global Animal Health
Allen Center
Washington State University

Pullman, WA 99164

On Jan 22, 2020, at 4:38 PM, Munster, Vincent (NIH/NIAID) [E] (b)(6) wrote:

Lol, lets hope its minutes rather than hours!

Vincent Munster, PhD
Chief, Virus Ecology Section
Laboratory of Virology
Rocky Mountain Laboratories
NIAID/NIH

From: "Letko, Michael Colin" (b)(6)
Date: Wednesday, January 22, 2020 at 4:37 PM
To: (b)(6)
Subject: Fwd: bioRxiv -- Manuscript Screened

Hey ho let's go!

Michael Letko, Ph.D
Assistant Professor
Laboratory of Functional Viromics
Paul G. Allen School for Global Animal Health
Allen Center

Washington State University

Pullman, WA 99164

Begin forwarded message:

From: "biorxiv@cshlbp.org" <biorxiv@cshlbp.org>
Date: January 22, 2020 at 4:35:55 PM MST
To: "Letko, Michael Colin" (b)(6)
Cc: "biorxiv@cshlbp.org" <biorxiv@cshlbp.org>
Subject: bioRxiv -- Manuscript Screened

<image001.jpg>

MS ID#: BIORXIV/2020/915660
MS TITLE: Functional assessment of cell entry and receptor usage for lineage B β-coronaviruses, including 2019-nCoV

Dear Dr. Letko,

We are pleased to inform you that the above manuscript has passed screening and will be online shortly. Processing typically completes same or next day (occasionally longer if over a weekend or a holiday).

Once an article is published in a journal, bioRxiv will automatically update the preprint with a link to the published version. Depending on the journal, this process may take up to two weeks.

The bioRxiv team

<image002.jpg>

bioRxiv

THE PREPRINT SERVER FOR BIOLOGY



Cold Spring Harbor Laboratory

From: Munster, Vincent (NIH/NIAID) [E]
Sent: Wed, 22 Jan 2020 16:38:08 -0700
To: Letko, Michael Colin
Subject: Re: bioRxiv -- Manuscript Screened

Sweet!

Vincent Munster, PhD
Chief, Virus Ecology Section
Laboratory of Virology
Rocky Mountain Laboratories
NIAID/NIH

From: "Letko, Michael Colin" (b)(6)
Date: Wednesday, January 22, 2020 at 4:37 PM
To: (b)(6)
Subject: Re: bioRxiv -- Manuscript Screened

Oh, also, this is my new email address. Eh?

Michael Letko, Ph.D
Assistant Professor
Laboratory of Functional Viromics
Paul G. Allen School for Global Animal Health
Allen Center
Washington State University

Pullman, WA 99164

On Jan 22, 2020, at 4:36 PM, Letko, Michael Colin (b)(6) wrote:

Hey ho let's go!

Michael Letko, Ph.D
Assistant Professor

Laboratory of Functional Viromics
Paul G. Allen School for Global Animal Health
Allen Center
Washington State University

Pullman, WA 99164

Begin forwarded message:

From: "biorxiv@cshlbp.org" <biorxiv@cshlbp.org>
Date: January 22, 2020 at 4:35:55 PM MST
To: "Letko, Michael Colin" (b)(6)
Cc: "biorxiv@cshlbp.org" <biorxiv@cshlbp.org>
Subject: bioRxiv -- Manuscript Screened

<bioRxiv_Logo_BP.jpg>

MS ID#: BIORXIV/2020/915660
MS TITLE: Functional assessment of cell entry and receptor usage for lineage B β-coronaviruses, including 2019-nCoV

Dear Dr. Letko,

We are pleased to inform you that the above manuscript has passed screening and will be online shortly. Processing typically completes same or next day (occasionally longer if over a weekend or a holiday).

Once an article is published in a journal, bioRxiv will automatically update the preprint with a link to the published version. Depending on the journal, this process may take up to two weeks.

The bioRxiv team

<bioRxiv_CSH_logo_BP.jpg>

From: Munster, Vincent (NIH/NIAID) [E]
Sent: Wed, 22 Jan 2020 16:10:22 -0700
To: Clancy, Chad (NIH/NIAID) [C]
Cc: Letko, Michael (NIH/NIAID) [F]; van Doremalen, Neeltje (NIH/NIAID) [E]; Kwe Claude, Yinda (NIH/NIAID) [F]
Subject: Re: Novel coronavrus
Attachments: Letko_Munster 2020 biorXiv submission[1].pdf, Letko_Munster_2019-nCoV receptor[2].pdf

Hi Chad,

This was submitted today by us,

Pretty molecular, but first proof that the novel virus uses ACE2

Vincent Munster, PhD
Chief, Virus Ecology Section
Laboratory of Virology
Rocky Mountain Laboratories
NIAID/NIH

From: "Clancy, Chad (NIH/NIAID) [C]" (b)(6)
Date: Wednesday, January 22, 2020 at 1:52 PM
To: (b)(6)
Subject: RE: Novel coronavrus

Hi Vincent,

Do you potentially have a list of species you're specifically interested in at this point? I contacted Dan Long and he's going to start combing the tissue library to look for some control tissue sets for us.

Chad

From: Munster, Vincent (NIH/NIAID) [E] (b)(6)
Sent: Sunday, January 19, 2020 7:52 AM
To: Clancy, Chad (NIH/NIAID) [C] (b)(6) Scott, Dana (NIH/NIAID) [E] (b)(6)
Cc: van Doremalen, Neeltje (NIH/NIAID) [E] (b)(6)
Subject: Novel coronavrus

Hi chad,

Would you be interested in running some analyses with the novel coronavirus for us? We have good data to suspect that ACE2 (like SARS) is the receptor for this particular virus.

I could not find a good comparison between animal species for the distribution of ACE2 expression, but I'm in particularly interested in the complete human respiratory (an intestinal tract) in relationship to animal models like mice, ferrets, rhesus, marmosets and the Artibeus bats.

We have already set-up the staining and have done initial work with Rousettus bats (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6316779/).

For human tissues, I believe we can buy slides and for the remaining we can see what we have as control tissues in our repository.

We are also planning an Artibeus study as soon as we get the virus is,

Cheers,

Vincent Munster, PhD
Chief, Virus Ecology Section
Laboratory of Virology
Rocky Mountain Laboratories
NIAID/NIH

Functional assessment of cell entry and receptor usage for lineage B β-coronaviruses, including 2019-nCoV

Michael Letko[#] and Vincent Munster[#]

Laboratory of Virology, Division of Intramural Research, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Hamilton, MT, 59840, USA

[#] Corresponding authors:

Drs. Vincent Munster and Michael Letko

Rocky Mountain Laboratories

NIAID/NIH

903S 4th Street

Hamilton, MT 59840

Tel: (b)(6)

Email: (b)(6)

Email:

Abstract

Over the past 20 years, several coronaviruses have crossed the species barrier into humans, causing outbreaks of severe, and often fatal, respiratory illness. Since SARS-CoV was first identified in animal markets, global viromics projects have discovered thousands of coronavirus sequences in diverse animals and geographic regions. Unfortunately, there are few tools available to functionally test these novel viruses for their ability to infect humans, which has severely hampered efforts to predict the next zoonotic viral outbreak. Here we developed an approach to rapidly screen lineage B betacoronaviruses, such as SARS-CoV and the recent 2019-nCoV, for receptor usage and their ability to infect cell types from different species. We show that host protease processing during viral entry is a significant barrier for several lineage B viruses and that bypassing this barrier allows several lineage B viruses to enter human cells through an unknown receptor. We also demonstrate how different lineage B viruses can recombine to gain entry into human cells and confirm that human ACE2 is the receptor for the recently emerging 2019-nCoV.

Introduction

Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV) first emerged in humans in 2003 after transmitting from animals in open air markets in China[1,2]. Shortly thereafter, several genetically related viruses were identified in Chinese Horseshoes bats (*Rhinolophus sinicus*)[3-7]. At the same time, improvements in next generation sequencing technology lead to a boom of virus discovery, uncovering thousands of novel virus sequences in wild animal populations around the world. While most of these viruses have never been found in humans, many are genetically similar to known human viruses within the betacoronaviruses (β-CoV) genus. The β-CoVs are further divided into four lineages: lineage B, which includes SARS-CoV and the newly emerging 2019-nCoV, has approximately 200 published virus sequences whereas lineage C, which includes MERS-CoV, has over 500 viral sequences.

Every year, additional novel CoV sequences are discovered. However, there is a massive knowledge gap in the field as very little work is performed after the viral sequences are published. Therefore, it is unknown whether these novel viruses have the potential to emerge in human populations.

Current methods for studying novel β-CoVs are technically demanding. Viral isolation from field samples is rarely successful and reverse genetics recovery of recombinant virus is labor-intensive, and expensive as synthesis of a single genome can cost upwards of $15,000. These limitations are prohibitive to studying novel CoVs at the scale in which they are discovered.

Cell entry is an essential component of cross-species transmission, especially for the β-CoVs. All CoVs encode a surface glycoprotein, spike, which binds to the host-cell

receptor and mediates viral entry[8]. For β-CoVs, a single region of the spike protein called the receptor binding domain (RBD) mediates the interaction with the host cell receptor. After binding the receptor, a nearby host protease cleaves the spike, which releases the spike fusion peptide, facilitating virus entry[9-12]. Known host receptors for β-CoVs include angiotensin converting enzyme 2 (ACE2) for SARS-CoV and dipeptidyl peptidase 4 (DPP4) for MERS-CoV[13,14].

Structural studies of coronaviruses have shown that the spike RBD is capable of folding independently from the rest of the spike protein and contains all of the structural information for host receptor binding[15]. Additionally, a previous study showed that replacing the RBD of the lineage B bat virus, Rp3, allowed the virus to enter cells expressing human ACE2 (hACE2)[16]. We therefore developed a method to functionally test the RBDs from novel lineage B β-CoVs in place of the SARS-CoV spike RBD (figure 1). Synthesizing just the RBD of spike is much faster and cost-effective than conventional pseudotyping methods that rely on synthesis of the full ~4kb spike sequence for novel CoVs: a process that can take weeks and is cost-prohibitive for large panels of spike sequences. The short turnaround time for our approach allowed us to test the receptor usage of all published, unique RBD sequences in lineage B, and also rapidly confirm the ACE2 receptor usage of the 2019-nCoV spike, which emerged in China in January 2020 as our study was ongoing.

We show that lineage B RBDs divide into functionally distinct clades and that several previously-unappreciated viruses exhibit compatibility with an unknown receptor on human cells. We also show that these clades are capable of recombining to impart human host-cell entry phenotypes, and that, beyond the RDB-receptor interaction, host

protease processing is another species barrier encountered by lineage B β-CoVs during cell entry.

Methods

Cells

293T, A549, BHK, Caco-2, Huh-7.5, PK-15, and Vero cells were maintained in DMEM (Sigma) supplemented with 10% FBS, penicillin/streptomycin, and L-glutamine. RhiNi/40.1, AJ-primary, AJ*i*, HypNi, RaKSM-2.5i, RhiLu, and RhiNi cells were maintained in DMEM/F12 (Gibco) supplemented with 12% FBS, penicillin/streptomycin, non-essential amino acids, sodium pyruvate and L-glutamine. AJ-primary cells were immortalized with a lentiviral vector expressing SV40 T-antigen following the manufacturer's instructions to generate AJ*i* cells (abm; #G203). RaKSM-2.5 primary cells have been previously described and were immortalized in this study similar to AJ*i* cells[17].

Plasmids

The spike coding sequences for SARS-CoV Urbani, As6526, and BM48-31 were codon optimized for human cells, appended with a 5' kozak expression sequence (GCCACC) and 3' tetra-glycine linker followed by nucleotides encoding a FLAG tag sequence (DYKDDDDK). For SARS-CoV spike, silent mutations were introduced around codons 308 and 519 to form KpnI and XhoI digest sites. For As6526 spike, silent mutations were introduced around codons 290 and 501 to form AflII and HindIII digest sites. For BM48-31 spike, silent mutations were introduced around codons 295 and 501 to form AflII and HindIII digest sites. These engineered spike sequences were synthesized and cloned into pcDNA3.1+ (GenScript).

Spike RBDs were first codon-optimized for human cells, appended with regions of the target spike backbone to facilitate Infusion cloning and synthesized as double

stranded DNA fragments (IDT DNA). SARS-CoV, As6526 or BM48-31 engineered spike plasmids were digested with their corresponding restriction enzymes and gel purified. RBD inserts were resuspended in water and Infusion cloned into gel purified, digested spike backbone vectors (Takara).

Human ACE2 (Q9BYF1.2), DPP4 (XM_005246371.3), or APN (NP_001141.2) were synthesized and cloned into pcDNA3.1+ (GenScript). All DNA constructs were verified by Sanger sequencing (ACGT Inc.).

Receptor transfection

BHK cells were seeded in black 96-well plates and transfected the next day with 100ng plasmid DNA encoding human ACE2, DPP4, APN or empty vector, using polyethyleneimine (Polysciences). All downstream experiments were performed 24 hours post-transfection.

Pseudotype production

Pseudotypes were produced as previously described[18]. 293T cells were seeded onto 6-well plates pre-coated with poly-L-lysine (Sigma) and transfected the next day with 1200ng empty plasmid and 400ng of plasmid encoding coronavirus spike or GFP as a no pseudotype control. Twenty-four hours later, transfected cells were infected with VSVΔG particles pseudotyped with VSV-g as previously described[19]. After one hour of incubating at 37°C, cells were washed three times and incubated in 2mL DMEM supplemented with 2% FBS, penicillin/streptomycin, and L-glutamine for 48 hours. Supernatants were collected, centrifuged at 500xG for 5 minutes, aliquoted and stored at -80.

Luciferase-based cell entry assay

Target cells were seeded in black 96-well plates and inoculated, in triplicate, with equivalent volumes of pseudotype stocks. For trypsin experiments, pseudotype stocks were diluted 1:1 in DMEM without FBS, trypsin was added to a final concentration of 2500µg/mL and samples were incubated at 37°C for 15 minutes. Samples were then diluted again 1:1 in cold DMEM supplemented with 2% FBS and added to cells. Inoculated plates were centrifuged at 1200xG, 4°C, for 1 hour and incubated over night at 37°C. Approximately 18-20 hours post-infection, Bright-Glo luciferase reagent (Promega) was added to each well, 1:1, without removing culture media and luciferase was measured. Relative entry was calculated by normalizing the relative light unit (RLU) for spike pseudotypes to the plate RLU average for the “no pseudotype control.”

Western blot

Producer cells (spike-transfected 293T) were lysed in 1%SDS, 150mM NaCl, 50mM Tris-HCl, 5mM EDTA and clarified by centrifugation at 14000xG for 20 minutes. Pseudotyped particles were concentrated from producer cell lysates that were overlaid a 10% OptiPrep cushion in PBS (Sigma) and centrifuged at 20,000× g for 2 hours at 4 °C. Lysates and concentrated particles were analyzed for FLAG, GAPDH and/or VSV-m expression on 10% Bis-Tris PAGE gel (ThermoFisher).

Accession numbers

Accession numbers for all spike sequences used here can be found in figure s1b.

Results

ACE2 entry is lineage B clade 1-specific

The receptor binding domain (RBD) of lineage B β-CoVs is a single, continuous domain that contains all structural information necessary to interact with the host receptor (figure 1a, b). We introduced silent mutations in the codon optimized coding sequence for SARS-CoV to facilitate replacing the SARS RBD with the RBD from other lineage B viruses (figure 1b). All lineage B sequences were downloaded from online repositories and parsed to 29 unique RBD sequences, representing all published variations of the lineage B RBD (supp. fig 1a, b). The panel of 29 RBDs phylogenetically cluster into 3 clades, as previously described[5], but these RBD clades were not apparent in phylogenetic analysis of other viral sequences, such as the RNA-dependent RNA polymerase (supp. fig 1c). All 29 RBDs were codon optimized, synthesized and cloned in place of the SARS RBD, effectively generating chimeric spike expression constructs. We then generated VSV-luciferase reporter particles pseudotyped with the chimeric spikes (figure 1c). We chose VSV over lentiviruses as our pseudotype platform because a lentiviral pseudotypes have failed to accurately reflect viral entry with novel bat coronavirus spike protein[7]. All constructs exhibited similar levels of expression in producer cells and incorporation into VSV pseudotypes, except the chimera with BM48-31 which displayed somewhat reduced expression compared to WT SARS spike (figure 1d). We then infected BHK cells expressing the receptor for SARS-CoV or empty vector (figure 1e) and observed only clade 1, which includes SARS-CoV, WIV1 and SHC014, could enter cells transfected with human ACE2 (figure 1e).

Protease enhances clade 2 entry

After binding the host receptor, host-cell protease cleaves spike, releasing the fusion peptide and allowing for host cell entry [20]. Previous studies have shown that absence of the host protease or incompatibility between the host protease and viral spike can block viral entry [21-24]. To circumvent host-cell protease incompatibility or absence, we treated our lineage B pseudotype panel and infected a wide variety of cell types from different host species (figure 2, supp. fig. 2). In the absence of exogenous protease, only clade 1 infected cells from African green monkey kidney, human gastrointestinal tract, human liver, and porcine kidney, in agreement with previous studies (figure 2; supp. fig. 2a, b). Surprisingly, exogenous protease enhanced entry of a subset of clade 2 spike chimeras in nonhuman primate, bat and human cells (figure 2). Importantly, VSV-g pseudotyped particles were able to produce luciferase signal in all cell lines tested in this study (supp. fig. 2c).

Clade 2 entry is receptor-dependent

We next tested human variants of known β-CoV receptors for their ability to mediate cell entry of clade 2 and 3 spike chimeras. We also tested human aminopeptidase N (APN), a receptor for alphacoronaviruses, which have been shown to utilize either human ACE2 or human APN for cell entry (figure 3a). Protease treatment only enhanced entry of clade 1 RBDs on cells expressing human ACE2, but not human DPP4 or APN. No entry was observed with clade 2 or 3 spikes, regardless of receptor or protease addition. Human dipeptidyl peptidase IV (DPP4), the receptor for the lineage C β-CoVs, MERS-CoV, only mediated entry of MERS-CoV (figure 2b, middle panels).

Importantly, in the absence of receptor, no entry was observed for any of the pseudotypes, suggesting that protease-mediated entry is receptor-dependent (figure 2b, right panels).

Receptor usage of 2019-nCoV

While our study was ongoing, a novel lineage B virus tentatively named 2019-nCoV was identified as the cause of a pneumonia outbreak in Hubei, China. Once the sequence was publicly available, we synthesized, cloned and tested the RBD from 2019-nCoV in our assay with human variants of known coronavirus receptors. The chimeric SARS-2019-nCoV spike protein expressed and was incorporated into particles similarly to other clade 1 chimeric spikes (figure 3c). The 2019-nCoV RBD was capable of entering cells expressing human ACE2, but not any of the other receptors tested (figure 3d; s3).

Clade determinants for ACE2 usage

Consensus sequences of the three lineage B clades showed several key differences between these groups. Only clade 1 RBDs contain all 14 residues that have been shown through crystallography, to interact with human ACE2 (figure 4a; s4). The majority of these residues are absent from clades 2 and 3, which contain additional deletions in surface exposed loops that cluster at the interface with ACE2 (figure 4 a, b). We generated a series of clade consensus RBD variants to determine the minimum number of mutations needed to impart ACE2 function on clade 2 and 3 RBDs (figure 4c). Introducing the two loop deletions from clade 1 in clade 2 results in a reduced spike expression, impaired pseudotype incorporation and loss of cell entry (figure 4c, d).

Restoring these loops in clade 2 and 3 from the loops found in clade 1 did not enhance entry with ACE2 (figure 4c; 2→1 and 3→1 version 1). Introducing all 14 ACE2 contact points in clade 2 or 3 also failed to restore ACE2 entry (figure 4c; 2→1 and 3→1 version 2). Only replacing all 14 contact points and the surrounding amino acids (also known as the receptor binding motif, RBM) lead to increased ACE2 entry with clade 2 and 3 RBDs (figure 4c; 2→1 version 3 = clade 2 residues 322-400 + clade 1 residues 400-501; 3→1 version 3 = clade 3 residues 322-385 + clade 1 residues 386-501). Taken together, these results show that the entire RBM from clade 1 is needed for ACE2 entry.

Full-spike and RBD chimeras are comparable

We next synthesized full-length clade 2 and 3 spikes to compare to our RBD chimeras. We selected the clade 2 spike, As6526, because it consistently gave strong entry signal in human cells following protease-treatment (figure 2b) as well as BM48-31, the only clade 3 spike in our panel. As we did for SARS-CoV spike, clade 2 and 3 spikes were codon optimized, FLAG-tagged and silent mutations were introduced to facilitate replacing their RBD with the consensus RBD from clade 1 (figure 5a). All chimeric constructs expressed similarly, with the exception for the SARS-BM48-31 RBD chimera, which exhibited reduced expression and incorporation (figure 5b). Protease treatment enhanced entry of both the As6526 clade 2 RBD chimera and full-length spike entry into Huh cells (figure 5c). Protease treatment had no effect on either the BM48-31 clade 3 chimera or full-length spike (figure 5c). Taken together, these findings show that SARS-lineage B RBD chimeras reflect the entry phenotype of full-length lineage B spikes.

Finally, we tested if receptor-binding and protease processing are coupled. We replaced the RBD of full-length clade 2 and 3 spike with the consensus RBD from clade 1 and tested pseudotypes on cells expressing the clade 1 receptor. The clade 1 consensus RBD efficiently facilitated entry of both As6526 and BM48-31 spike only following protease treatment. These findings show that even though BHK-hACE2 cells support full-length clade 1 spike entry, just having the RBD from a clade 1 virus is insufficient to mediate entry. As seen in our previous experiments, protease treatment did not enhance pseudotype entry in the absence of host receptor (figure 5).

Discussion

Despite significant advances in next generation sequencing technologies, which have facilitated the discovery of thousands of novel animal-derived viruses, tools for downstream functional assessment of these novel sequences are lacking. To gain traction on this ever-growing problem, we took a reductionist approach to coronavirus entry and developed a scalable, BSL2-compatible method for testing only the minimal region of the virus essential for interacting with the host receptor (figure 1, figure s1a). Because most of these viruses have never been isolated, we resorted to synthetic biology and molecular engineering to reduce the burden of gene synthesis to just a small fragment. Thus, the cost and synthesis production time for testing several spikes for entry in our system is dramatically reduced (figure s1d). In theory, this approach to functional viromics should be applicable to a wide variety of virus-host proteins and interactions.

Coronavirus entry is a multi-step process involving multiple, distinct domains in spike that mediate virus attachment to the cell surface, receptor engagement, protease processing and membrane fusion[8]. While the RBD:receptor interaction is most studied in this process, recent studies have highlighted the major role host protease processing plays as a species barrier[22,25-27]. Lineage C coronaviruses include MERS-CoV as well as distantly related viruses such as HKU4, HKU5 and PDF-2180 [28,29]. Studies have shown that HKU4 can bind human DPP4 but requires addition of exogenous trypsin to facilitate cell entry and that HKU5 and PDF-2180 spikes can enter human cells through an unidentified receptor with protease treatment[22,26]. Analogous to these earlier studies of lineage C CoVs, we observed protease-enhanced entry of lineage B CoVs (figure 2, 3, 5). While it has been shown that host proteases cleave spike, allowing for downstream

membrane fusion, other evidence suggests that protease may act on the receptor as well to activate it[30]. Addition of protease during the course of SARS-CoV infection facilitated entry in cells with low-expression of ACE2 that is normally insufficient to support virus entry[30]. Indeed, we saw evidence of residual trypsin activity on the cells after infection in our studies as the cell monolayer was loose compared to the untreated condition. Similarly, Menachery et al. also observed cell rounding during their trypsin infections[26]. Therefore, further studies are needed to assess where trypsin is enhancing entry of coronaviruses: at the level of spike, the receptor, or both.

In the absence of exogenous protease, only clade 1 RBDs entered nonhuman primate, human and porcine cell lines (figure 2a, b, s2a, b). These findings are in strong agreement with previous studies that have either isolated virus (WIV1) or rescued recombinant chimeric viruses (SHC014, Rs4231, Rs7237)[5,7,31]. However, with trypsin, a subset of genetically-similar clade 2 RBDs gained entry in these cells, suggesting their barrier is at the level of protease processing (figure 2a, b). The other spike from clade 2 and 3 did not enter the cells we tested, regardless of protease addition, suggesting an absent or incompatible receptor. Surprisingly, the protease-dependent entry phenotype was consistent in the reverse spike chimeras in which we replaced the RBD in clade 2 or 3 spike with a clade 1 RBD (figure 5d), suggesting that either the protease site between S1/S2 is not compatible with the chimeric spike backbone or the protease is not expressed in these cells (figure s5). Because clade 1 spikes enter cells expressing human ACE2 without addition of protease but clade2-clade1 chimeras require protease, our data suggests the spike protease cleavage site is adapted to the protease environment of the receptor-bound RBD (figure s5).

None of the spike pseudotypes efficiently entered *Rhinolophus* cells, which has been observed in previous studies using these cells[32,33] (figure 2c). Surprisingly, AJi cells were selectively permissive for only clade 2 entry following protease treatment, which suggests that clade 2 RBDs interact with a receptor that is distinct from clade 1 (figure 2c).

Our results show that, despite all being classified as the same virus species, most lineage B β-CoVs do not use currently known coronavirus receptors (figure 1e, 3a, b). Critically, we did not observe any pseudotype entry in the presence of protease and absence of receptor, suggesting that lineage B cell entry is still receptor-dependent following protease treatment (figure 3b). While our study was ongoing, a novel lineage B β-CoV was identified as the etiological agent behind an outbreak of pneumonia in Wuhan, Hubei, China (2019-nCoV). The RBD for 2019-nCoV has residues and motifs found in all 3 clades but forms a distinct clade, so we tested it for receptor usage and observed entry only with human ACE2 but not other known coronavirus receptors (figure 3d). Interestingly, within the backbone of SARS-CoV spike, cell entry of 2019-nCoV was similar to the other clade 1 spikes tested, including SARS-CoV. These finding suggests 2019-nCoV is capable of using human ACE2 as efficiently as SARS-CoV, which may help explain the human-to-human transmissibility of this virus. More studies are needed with the full spike sequence and, ideally, a viral isolate.

The receptor binding motif (RBM) is a small region in the C-terminal half of the RBD and contains all the residues that interface with the host receptor (figure 3a)[15]. The 14 contact points in the co-structure of the SARS-RBD bound to human ACE2 are largely absent from clade 2 and 3 RBDs, which also contain deletions compared to clade 1 RBMs (figure 4a, b, s4a). Simply mutating clade 2 and 3 to have the 14 contact points was

insufficient to impart human ACE2 usage (figure 4c). This is likely because the non-contact residues in the RBM play a supportive and structural role for these contact points, and indeed, these non-contact residues are different between the clades (figure s4).

In contrast to changing individual amino acids, our chimeric RBD constructs show that clade 2 and 3 RBD containing the clade 1 RBM are compatible with human ACE2. Coronaviruses frequently undergo recombination, gaining large swaths of genetic material at once [34,35]. Taken together with our data, it is possible that recombination with a clade 1 virus will impart compatibility with human ACE2. Interestingly, the 2019-nCoV RBD forms a clade that is distinct from the other 3 clades (figure s1c). However, the 2019-nCoV RBD contains most of the contact points with human ACE2 that are found in clade 1 as well as some amino acid variations that are unique to clade 2 and 3 (figure s4b). Taken together with our receptor assay results, it may be possible that 2019-nCoV arose from recombination between clade 1 and the other clades.

As we saw with the SARS-As6526 RBD (clade 2) spike chimera, full length As6526 spike entered cells following protease treatment, but BM48-31 (clade 3) spike did not (figure 2, 5c). These data show that the chimeric spikes generally reproduce the entry phenotypes of full-length spikes. Notably, the full length As6526 spike did not enter cells as efficiently as the SARS-As6526 chimera, suggesting that other human-cell adaptations are likely needed in As6526 spike.

The capacity to predict the zoonotic potential of newly detected viruses has been severely hindered by a lack of functional data for these novel animal virus sequences. Here, we have developed a rapid and cost-effective platform to functionally test large groups of related viruses for zoonotic potential. We found that several other lineage B

coronaviruses are capable of entering human cells through an unknown receptor and that lineage B spike proteins can recombine to gain entry with a known host-receptor. Taken together with the latest outbreak of 2019-nCoV in humans, these findings underscore the importance of continued surveillance of coronaviruses at the sequence and functional levels in order to better prepare for the next emerging virus.

Acknowledgements

We would like to thank Dr. Anthony Schountz for generously sharing primary artibeus cells, as well as Dr. Marcel Muller for providing the *Rhinolophus* cells used in this study. We would also like to acknowledge Wu,F., Zhao,S., Yu,B., Chen,Y.-M., Wang,W., Hu,Y., Song,Z.-G., Tao,Z.-W., Tian,J.-H., Pei,Y.-Y., Yuan,M.L., Zhang,Y.-L., Dai,F.-H., Liu,Y., Wang,Q.-M., Zheng,J.-J., Xu,L., Holmes,E.C. and Zhang,Y.-Z, the Wuhan Institute of Virology, Chinese Academy of Medical Sciences & Peking Union Medical College, Chinese Center for Disease Control and Prevention, and Wuhan Jinyintan Hospitar for providing the genome sequence for 2019-nCoV and other essential outbreak information to the scientific community. This work was supported by the Intramural Research Program of the National Institute of Allergy and Infectious Diseases.

Declarations of Interests

The authors declare no competing interests.

References

1 Wang, M. *et al.* SARS-CoV infection in a restaurant from palm civet. *Emerg Infect Dis* **11**, 1860-1865, doi:10.3201/eid1112.041293 (2005).

2 Kuiken, T. *et al.* Newly discovered coronavirus as the primary cause of severe acute respiratory syndrome. *Lancet* **362**, 263-270, doi:10.1016/S0140-6736(03)13967-0 (2003).

3 Li, W. *et al.* Bats are natural reservoirs of SARS-like coronaviruses. *Science* **310**, 676-679, doi:10.1126/science.1118391 (2005).

4 Ge, X. Y. *et al.* Isolation and characterization of a bat SARS-like coronavirus that uses the ACE2 receptor. *Nature* **503**, 535-538, doi:10.1038/nature12711 (2013).

5 Hu, B. *et al.* Discovery of a rich gene pool of bat SARS-related coronaviruses provides new insights into the origin of SARS coronavirus. *PLoS Pathog* **13**, e1006698, doi:10.1371/journal.ppat.1006698 (2017).

6 Lau, S. K. *et al.* Severe acute respiratory syndrome coronavirus-like virus in Chinese horseshoe bats. *Proc Natl Acad Sci U S A* **102**, 14040-14045, doi:10.1073/pnas.0506735102 (2005).

7 Menachery, V. D. *et al.* A SARS-like cluster of circulating bat coronaviruses shows potential for human emergence. *Nat Med* **21**, 1508-1513, doi:10.1038/nm.3985 (2015).

8 Li, F. Structure, Function, and Evolution of Coronavirus Spike Proteins. *Annu Rev Virol* **3**, 237-261, doi:10.1146/annurev-virology-110615-042301 (2016).

9 Simmons, G., Zmora, P., Gierer, S., Heurich, A. & Pohlmann, S. Proteolytic activation of the SARS-coronavirus spike protein: cutting enzymes at the cutting edge of antiviral research. *Antiviral Res* **100**, 605-614, doi:10.1016/j.antiviral.2013.09.028 (2013).

10 Matsuyama, S. *et al.* Efficient activation of the severe acute respiratory syndrome coronavirus spike protein by the transmembrane protease TMPRSS2. *J Virol* **84**, 12658-12664, doi:10.1128/JVI.01542-10 (2010).

11 Bertram, S. *et al.* Cleavage and activation of the severe acute respiratory syndrome coronavirus spike protein by human airway trypsin-like protease. *J Virol* **85**, 13363-13372, doi:10.1128/JVI.05300-11 (2011).

12 Belouzard, S., Chu, V. C. & Whittaker, G. R. Activation of the SARS coronavirus spike protein via sequential proteolytic cleavage at two distinct sites. *Proc Natl Acad Sci U S A* **106**, 5871-5876, doi:10.1073/pnas.0809524106 (2009).

13 Raj, V. S. *et al.* Dipeptidyl peptidase 4 is a functional receptor for the emerging human coronavirus-EMC. *Nature* **495**, 251-254, doi:10.1038/nature12005 (2013).

14 Li, W. *et al.* Angiotensin-converting enzyme 2 is a functional receptor for the SARS coronavirus. *Nature* **426**, 450-454, doi:10.1038/nature02145 (2003).

15 Li, F., Li, W., Farzan, M. & Harrison, S. C. Structure of SARS coronavirus spike receptor-binding domain complexed with receptor. *Science* **309**, 1864-1868, doi:10.1126/science.1116480 (2005).

16 Becker, M. M. *et al.* Synthetic recombinant bat SARS-like coronavirus is infectious in cultured cells and in mice. *Proc Natl Acad Sci U S A* **105**, 19944-19949, doi:10.1073/pnas.0808116105 (2008).

17 Seifert, S. N. *et al.* Rousettus aegyptiacus Bats Do Not Support Productive Nipah Virus Replication. *J Infect Dis*, doi:10.1093/infdis/jiz429 (2019).
18 Letko, M. *et al.* Adaptive Evolution of MERS-CoV to Species Variation in DPP4. *Cell Rep* **24**, 1730-1737, doi:10.1016/j.celrep.2018.07.045 (2018).
19 Takada, A. *et al.* A system for functional analysis of Ebola virus glycoprotein. *Proc Natl Acad Sci U S A* **94**, 14764-14769, doi:10.1073/pnas.94.26.14764 (1997).
20 Millet, J. K. & Whittaker, G. R. Host cell proteases: Critical determinants of coronavirus tropism and pathogenesis. *Virus Res* **202**, 120-134, doi:10.1016/j.virusres.2014.11.021 (2015).
21 Menachery, V. D. *et al.* Trypsin treatment unlocks barrier for zoonotic coronaviruses infection. *bioRxiv* (2019).
22 Yang, Y. *et al.* Receptor usage and cell entry of bat coronavirus HKU4 provide insight into bat-to-human transmission of MERS coronavirus. *Proc Natl Acad Sci U S A* **111**, 12516-12521, doi:10.1073/pnas.1405889111 (2014).
23 Matsuyama, S., Ujike, M., Morikawa, S., Tashiro, M. & Taguchi, F. Protease-mediated enhancement of severe acute respiratory syndrome coronavirus infection. *Proc Natl Acad Sci U S A* **102**, 12543-12547, doi:10.1073/pnas.0503203102 (2005).
24 Kam, Y. W. *et al.* Cleavage of the SARS coronavirus spike glycoprotein by airway proteases enhances virus entry into human bronchial epithelial cells in vitro. *PLoS One* **4**, e7870, doi:10.1371/journal.pone.0007870 (2009).
25 Yang, Y. *et al.* Two Mutations Were Critical for Bat-to-Human Transmission of Middle East Respiratory Syndrome Coronavirus. *J Virol* **89**, 9119-9123, doi:10.1128/JVI.01279-15 (2015).
26 Menachery, V. D. *et al.* Trypsin treatment unlocks barrier for zoonotic bat coronaviruses infection. *J Virol*, doi:10.1128/JVI.01774-19 (2019).
27 Beniac, D. R., deVarennes, S. L., Andonov, A., He, R. & Booth, T. F. Conformational reorganization of the SARS coronavirus spike following receptor binding: implications for membrane fusion. *PLoS One* **2**, e1082, doi:10.1371/journal.pone.0001082 (2007).
28 Woo, P. C. *et al.* Molecular diversity of coronaviruses in bats. *Virology* **351**, 180-187, doi:10.1016/j.virol.2006.02.041 (2006).
29 Anthony, S. J. *et al.* Further Evidence for Bats as the Evolutionary Source of Middle East Respiratory Syndrome Coronavirus. *mBio* **8**, doi:10.1128/mBio.00373-17 (2017).
30 Shulla, A. *et al.* A transmembrane serine protease is linked to the severe acute respiratory syndrome coronavirus receptor and activates virus entry. *J Virol* **85**, 873-882, doi:10.1128/JVI.02062-10 (2011).
31 Adney, D. R. *et al.* Replication and shedding of MERS-CoV in upper respiratory tract of inoculated dromedary camels. *Emerg Infect Dis* **20**, 1999-2005, doi:10.3201/eid2012.141280 (2014).
32 Muth, D. *et al.* Attenuation of replication by a 29 nucleotide deletion in SARS-coronavirus acquired during the early stages of human-to-human transmission. *Sci Rep* **8**, 15177, doi:10.1038/s41598-018-33487-8 (2018).
33 Hoffmann, M. *et al.* Differential sensitivity of bat cells to infection by enveloped RNA viruses: coronaviruses, paramyxoviruses, filoviruses, and influenza viruses. *PLoS One* **8**, e72942, doi:10.1371/journal.pone.0072942 (2013).

34 Liao, C. L. & Lai, M. M. RNA recombination in a coronavirus: recombination between viral genomic RNA and transfected RNA fragments. *J Virol* **66**, 6117-6124 (1992).

35 Graham, R. L. & Baric, R. S. Recombination, reservoirs, and the modular spike: mechanisms of coronavirus cross-species transmission. *J Virol* **84**, 3134-3146, doi:10.1128/JVI.01394-09 (2010).

Figures

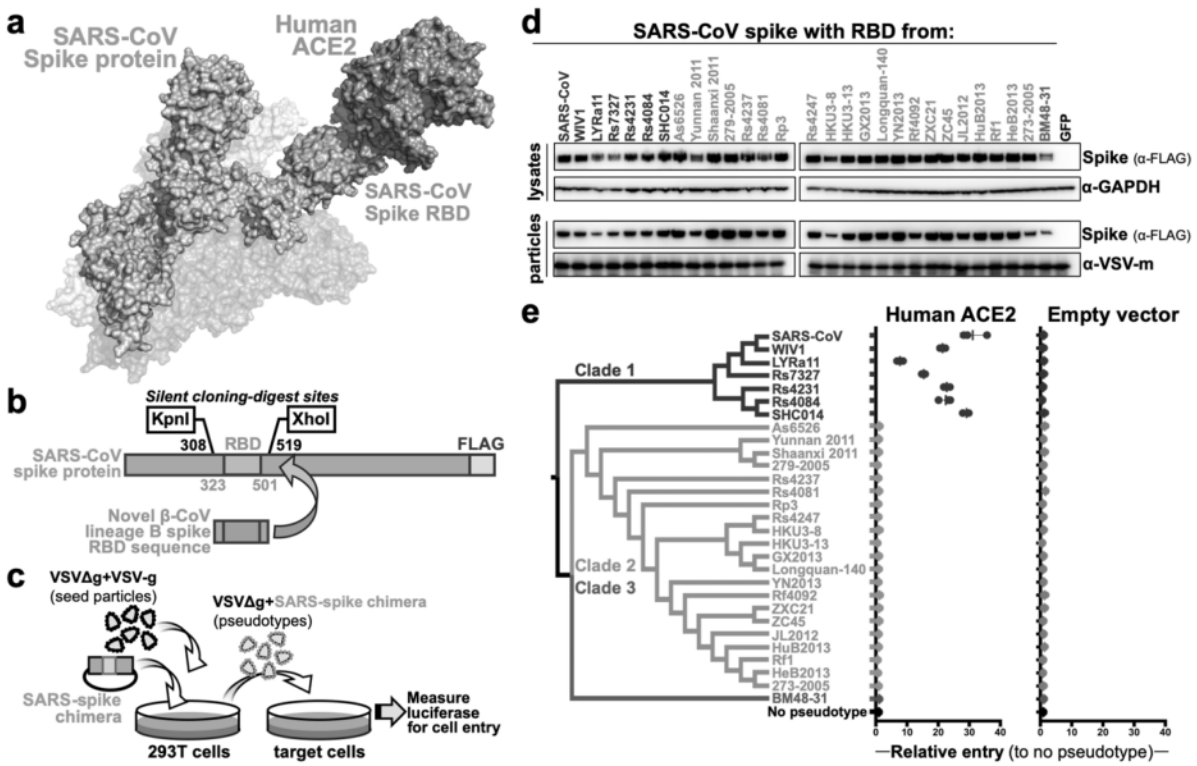


Figure 1: β-Coronavirus lineage B entry with human ACE2 is clade-specific

a, β-Coronaviruses, including SARS-CoV, interact with the host cell receptor via the Receptor Binding Domain (RBD) in spike (PDB: 5X5B, 2AJF) . **b,** SARS-CoV spike was engineered with silent mutations to facilitate cloning novel RBD sequences in place of the SARS spike RBD. SARS spike amino acid numbers are indicated for silent cloning sites and the RBD in grey and orange, respectively. **c,** outline of experimental workflow. **d,** Westernblot of producer cell lysates and concentrated reporter particles. **e,** BHK cells were transfected with either human ACE2 or empty vector, subsequently infected with VSV-reporter particles pseudotyped with chimeric spikes, luciferase was measured and normalized to no pseudotype as a readout for cell entry. Shown are the data for 3 replicates.

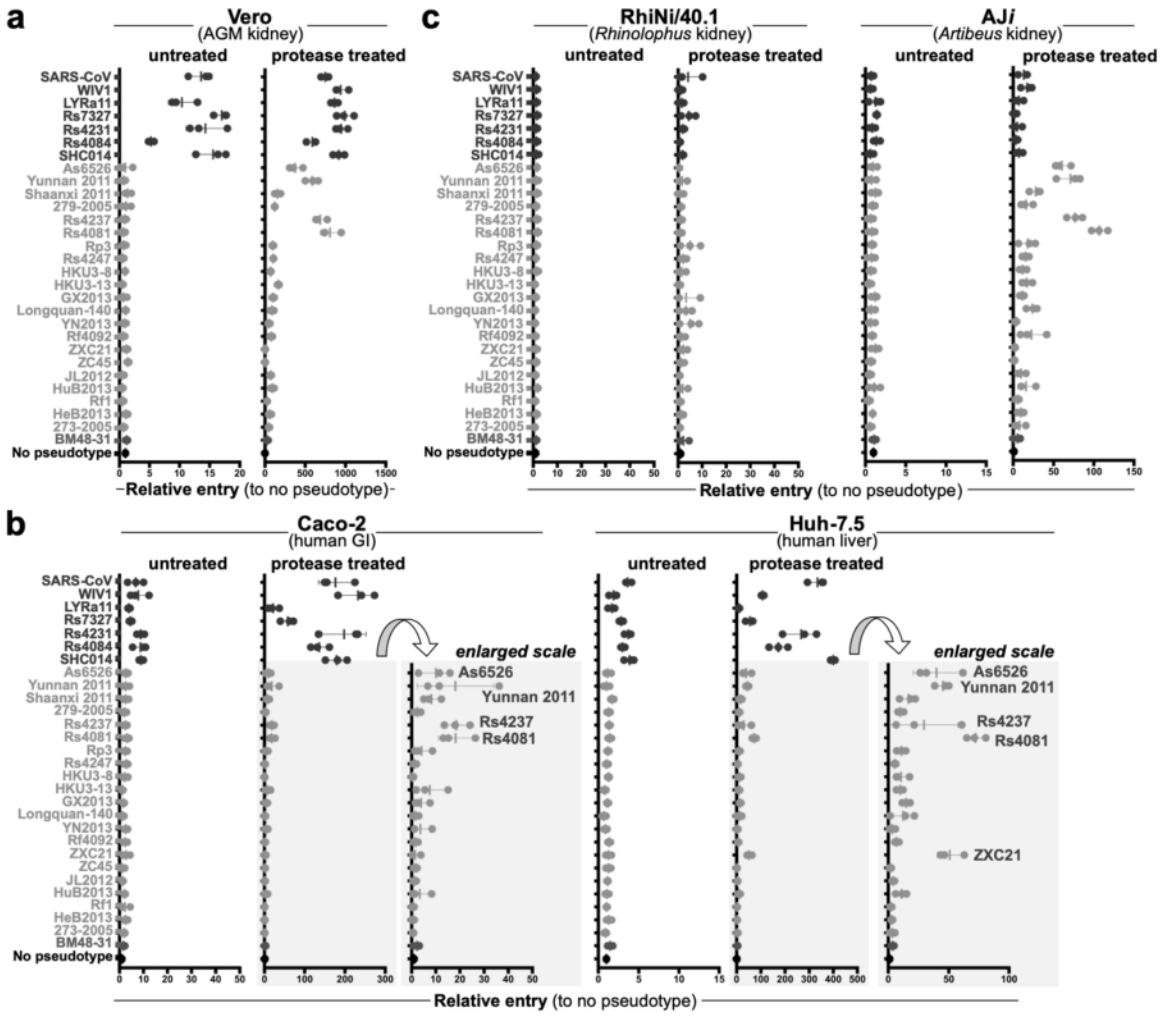


Figure 2: Trypsin enhances lineage B entry in various cell lines

a, Primate cells, **b,** human cells or **c,** bat cells were infected with VSV-particles pseudotyped with the lineage B chimeric spike panel. Pseudotypes were either left untreated or incubated with trypsin before addition to the cells. Luciferase was measured and normalized to particles produced without pseudotype. Shown are the data for 3 replicates.

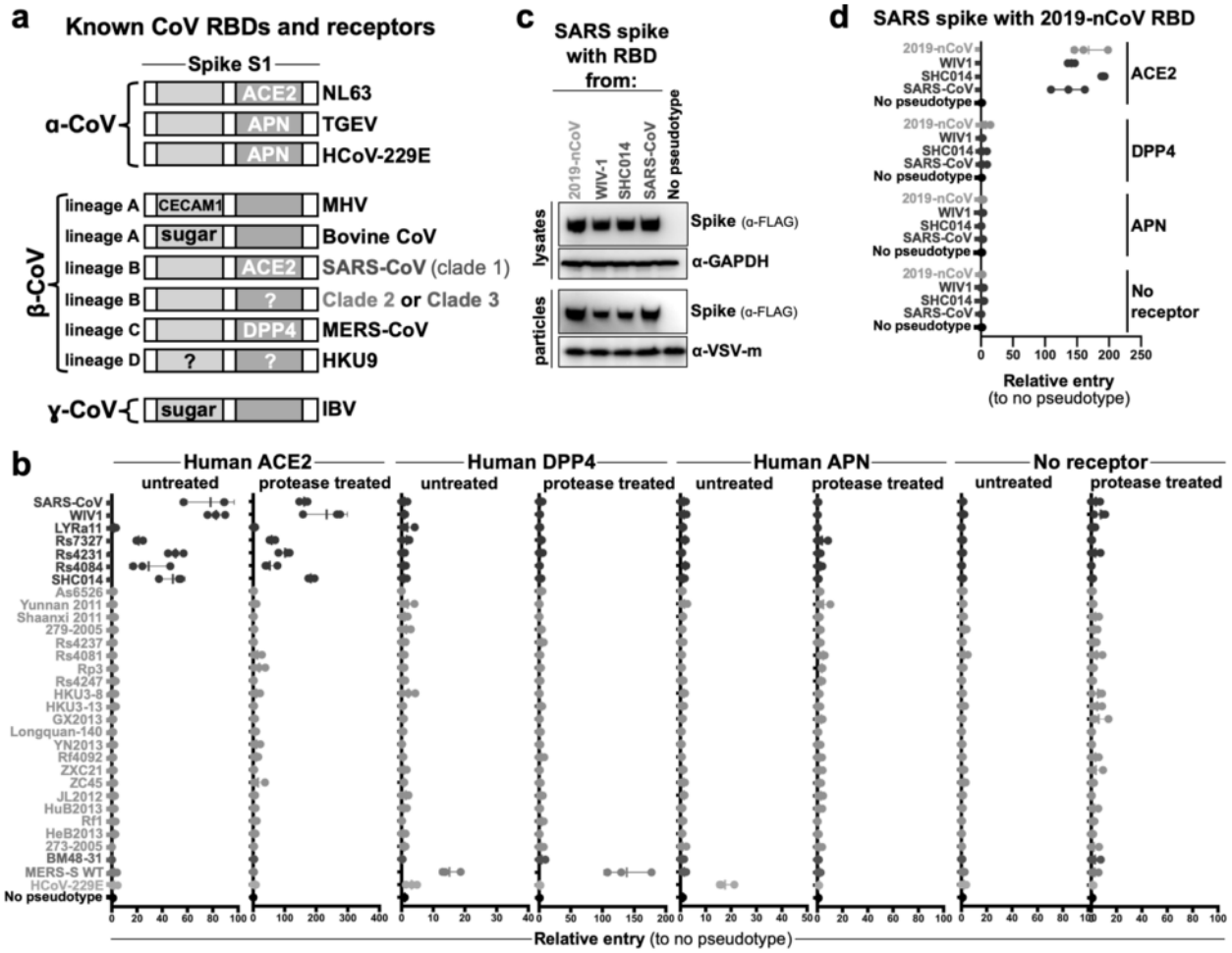


Figure 3: Lineage B entry into cells with known CoV receptors

a, Schematic of known coronavirus RBDs and their receptors. **b,** Pseudotyped particles were either left untreated and treated with trypsin and subsequently used to infect BHK cells transfected with the coronavirus receptor indicated. Shown are data from 3 replicates. **c,** Expression and pseudotype incorporation of SARS-S-2019-nCoV RBD chimeras. **d,** Pseudotypes were used to infect cells expressing hACE2, hDPP4, hAPN, or empty vector, without protease treatment.

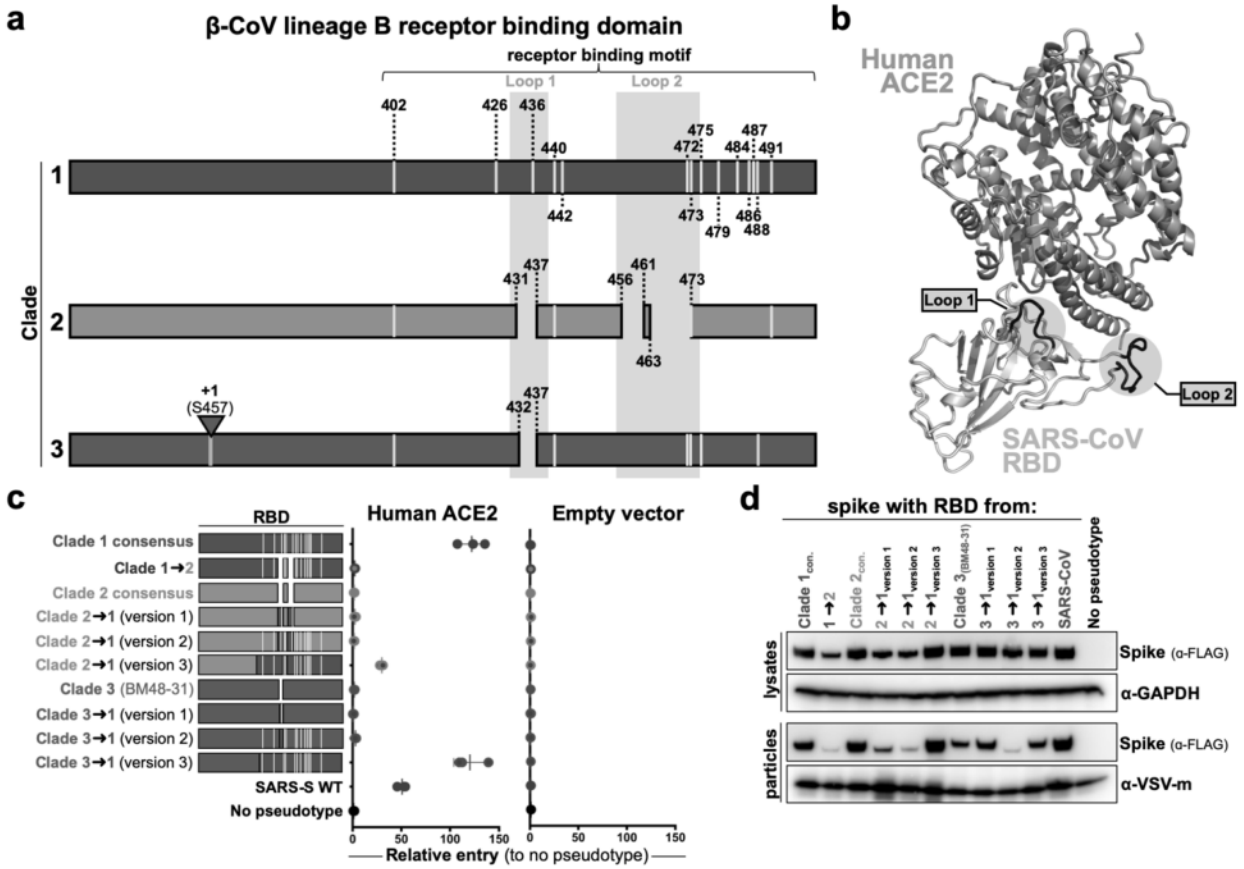


Figure 4: Lineage B clade-specific determinants for human ACE2 usage

a, Schematic overview of clade 1, 2 and 3. Highlighted in yellow are the 14 residues that contact human ACE2. Deletions in loops 1 and 2 are indicated for clades 2 and 3. **b,** Structure of human ACE2 and the SARS-S RBD (PDB: 2AJF), with loops highlighted in gray. **c,** VSV pseudotypes were generated with the indicated RBD and used to infect BHKs transfected with either human ACE2 or empty vector. Shown are data for 3 replicates. **d,** Westernblot of producer cell lysates and concentrated pseudotyped particles.

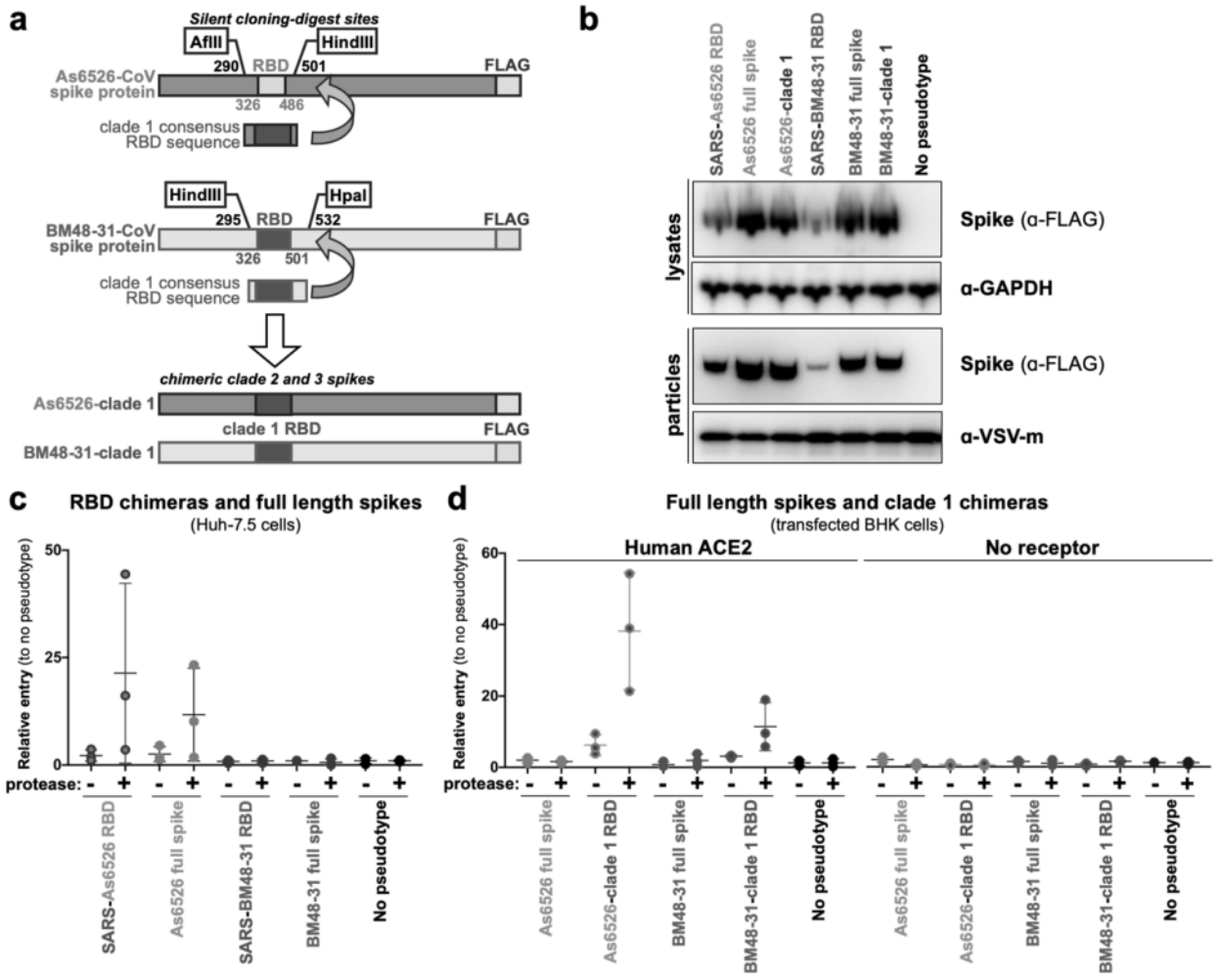


Figure 5: Comparison of chimeric and full-length clade 2 and 3 spikes

a, Full length spike sequences from As6526 (clade 2) and BM48-31 (clade 3) were codon optimized, FLAG tagged and synthesized. Silent mutations flanking the RBD facilitated replacing the native RBD with the clade 1 consensus RBD. **b,** Westernblot of producer cell lysates and concentrated pseudotypes particles. **c,** Pseudotypes with indicated spike constructs were left untreated or treated with trypsin and subsequently used to infect Huh-7.5 cells. Shown are data for 3 replicates. **d,** Pseudotypes with indicated spike constructs were left untreated or treated with trypsin and subsequently used to infect BHK cells transfected with human ACE2. Shown are data for 3 replicates.

Functional assessment of cell entry and receptor usage for lineage B β-coronaviruses, including 2019-nCoV

Michael Letko[#] and Vincent Munster[#]

Laboratory of Virology, Division of Intramural Research, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Hamilton, MT, 59840, USA

SUPPLEMENTAL INFORMATION

[#] Corresponding authors:

Drs. Vincent Munster and Michael Letko

Rocky Mountain Laboratories

NIAID/NIH

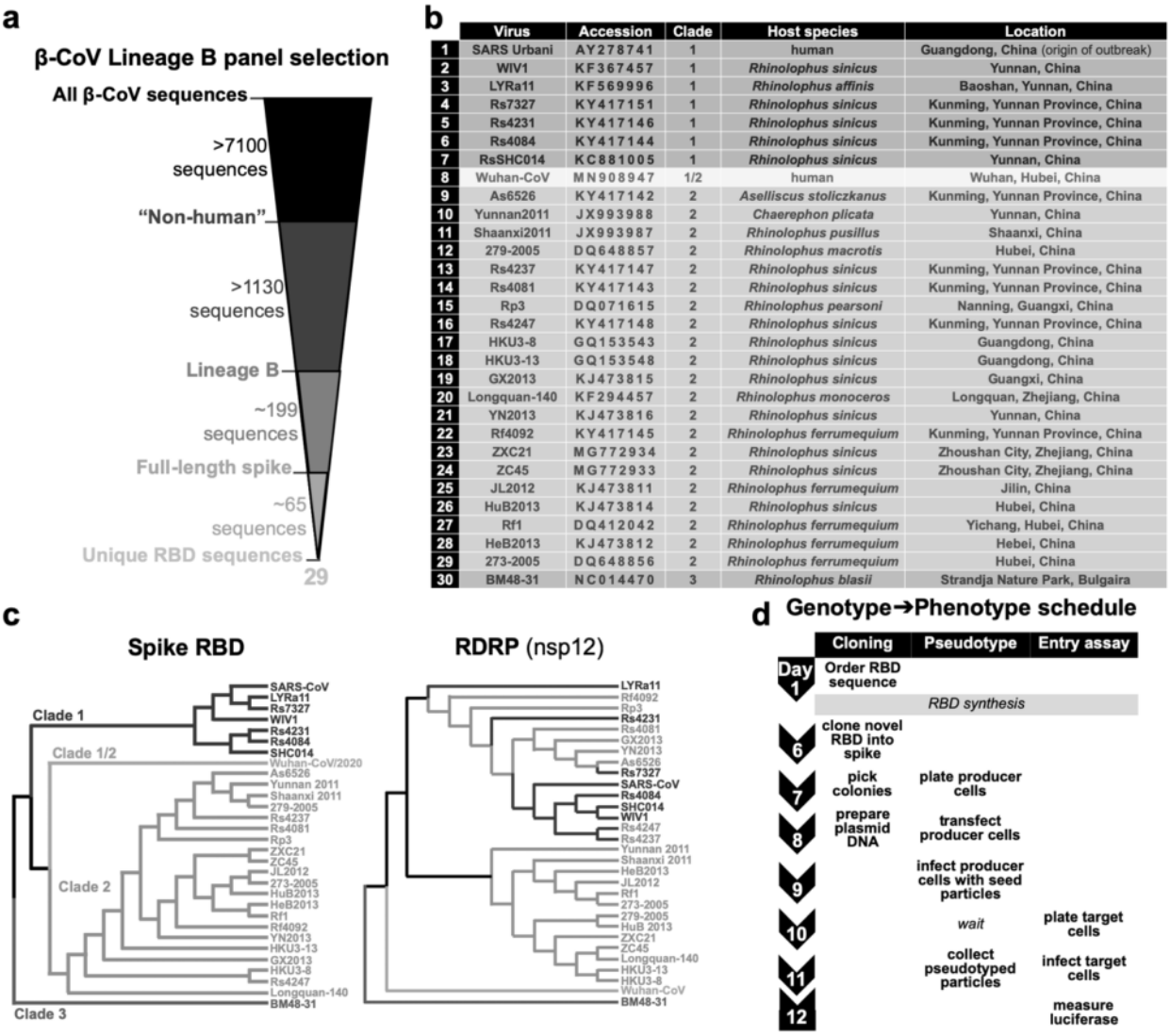
903S 4th Street

Hamilton, MT 59840

Tel: (b)(6)

Email: (b)(6)

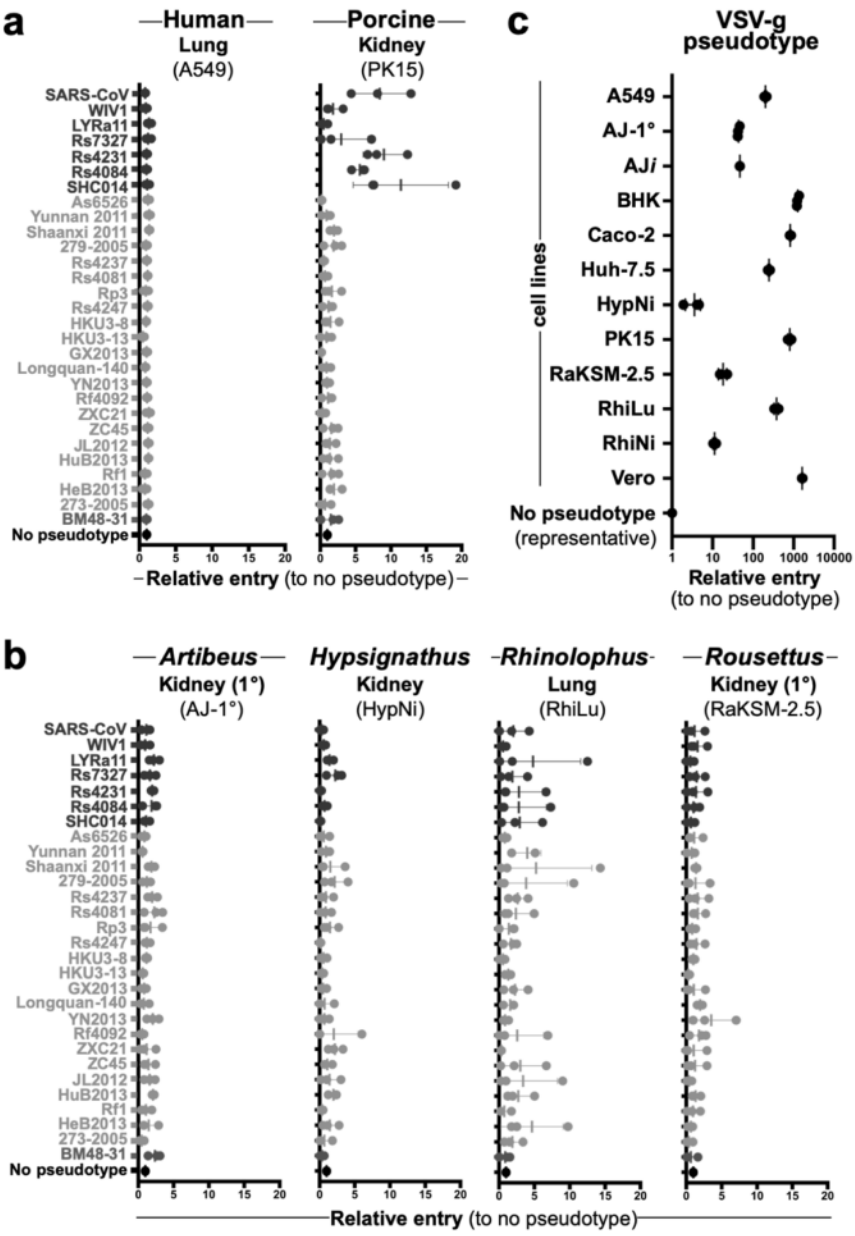
Email:



	Virus	Accession	Clade	Host species	Location
1	SARS Urbani	AY278741	1	human	Guangdong, China (origin of outbreak)
2	WIV1	KF367457	1	*Rhinolophus sinicus*	Yunnan, China
3	LYRa11	KF569996	1	*Rhinolophus affinis*	Baoshan, Yunnan, China
4	Rs7327	KY417151	1	*Rhinolophus sinicus*	Kunming, Yunnan Province, China
5	Rs4231	KY417146	1	*Rhinolophus sinicus*	Kunming, Yunnan Province, China
6	Rs4084	KY417144	1	*Rhinolophus sinicus*	Kunming, Yunnan Province, China
7	RsSHC014	KC881005	1	*Rhinolophus sinicus*	Yunnan, China
8	Wuhan-CoV	MN908947	1/2	human	Wuhan, Hubei, China
9	As6526	KY417142	2	*Aselliscus stoliczkanus*	Kunming, Yunnan Province, China
10	Yunnan2011	JX993988	2	*Chaerephon plicata*	Yunnan, China
11	Shaanxi2011	JX993987	2	*Rhinolophus pusillus*	Shaanxi, China
12	279-2005	DQ648857	2	*Rhinolophus macrotis*	Hubei, China
13	Rs4237	KY417147	2	*Rhinolophus sinicus*	Kunming, Yunnan Province, China
14	Rs4081	KY417143	2	*Rhinolophus sinicus*	Kunming, Yunnan Province, China
15	Rp3	DQ071615	2	*Rhinolophus pearsoni*	Nanning, Guangxi, China
16	Rs4247	KY417148	2	*Rhinolophus sinicus*	Kunming, Yunnan Province, China
17	HKU3-8	GQ153543	2	*Rhinolophus sinicus*	Guangdong, China
18	HKU3-13	GQ153548	2	*Rhinolophus sinicus*	Guangdong, China
19	GX2013	KJ473815	2	*Rhinolophus sinicus*	Guangxi, China
20	Longquan-140	KF294457	2	*Rhinolophus monoceros*	Longquan, Zhejiang, China
21	YN2013	KJ473816	2	*Rhinolophus sinicus*	Yunnan, China
22	Rf4092	KY417145	2	*Rhinolophus ferrumequium*	Kunming, Yunnan Province, China
23	ZXC21	MG772934	2	*Rhinolophus sinicus*	Zhoushan City, Zhejiang, China
24	ZC45	MG772933	2	*Rhinolophus sinicus*	Zhoushan City, Zhejiang, China
25	JL2012	KJ473811	2	*Rhinolophus ferrumequium*	Jilin, China
26	HuB2013	KJ473814	2	*Rhinolophus sinicus*	Hubei, China
27	Rf1	DQ412042	2	*Rhinolophus ferrumequium*	Yichang, Hubei, China
28	HeB2013	KJ473812	2	*Rhinolophus ferrumequium*	Hebei, China
29	273-2005	DQ648856	2	*Rhinolophus ferrumequium*	Hubei, China
30	BM48-31	NC014470	3	*Rhinolophus blasii*	Strandja Nature Park, Bulgaira

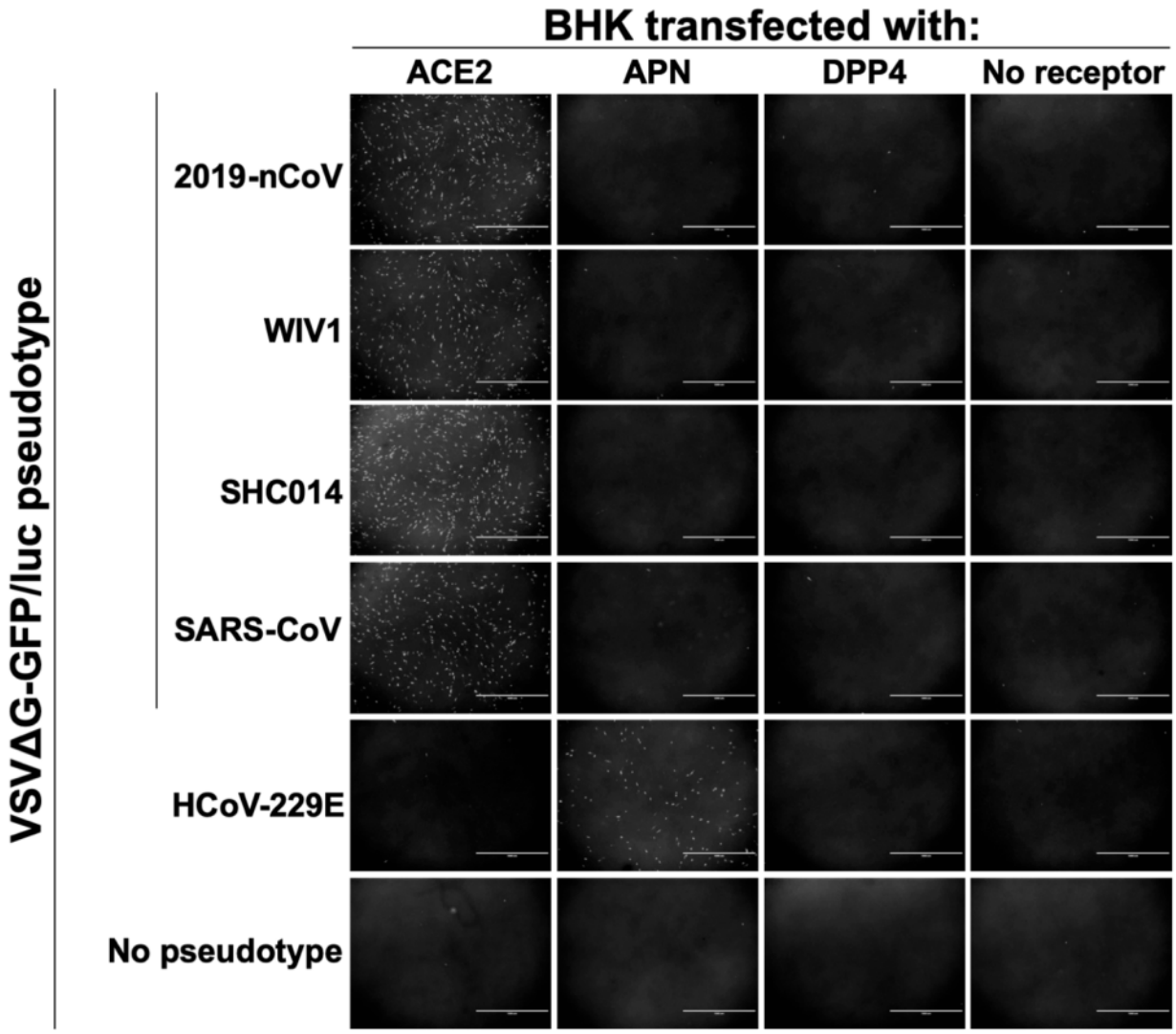
Supplemental figure 1: Lineage B RBD panel assembly and phylogenetic analysis

a, Coronavirus sequences were downloaded from NCBI and further parsed to 29 unique RBD variants. **b,** Virus isolate name, accession number, clade, host species and location of identification listed for the 29 unique lineage B RBDs used in this study. **c,** Cladograms for the spike RBD and coronavirus RNA-dependent RNA polymerase (nsp12). **d,** Overview of experimental timeline.



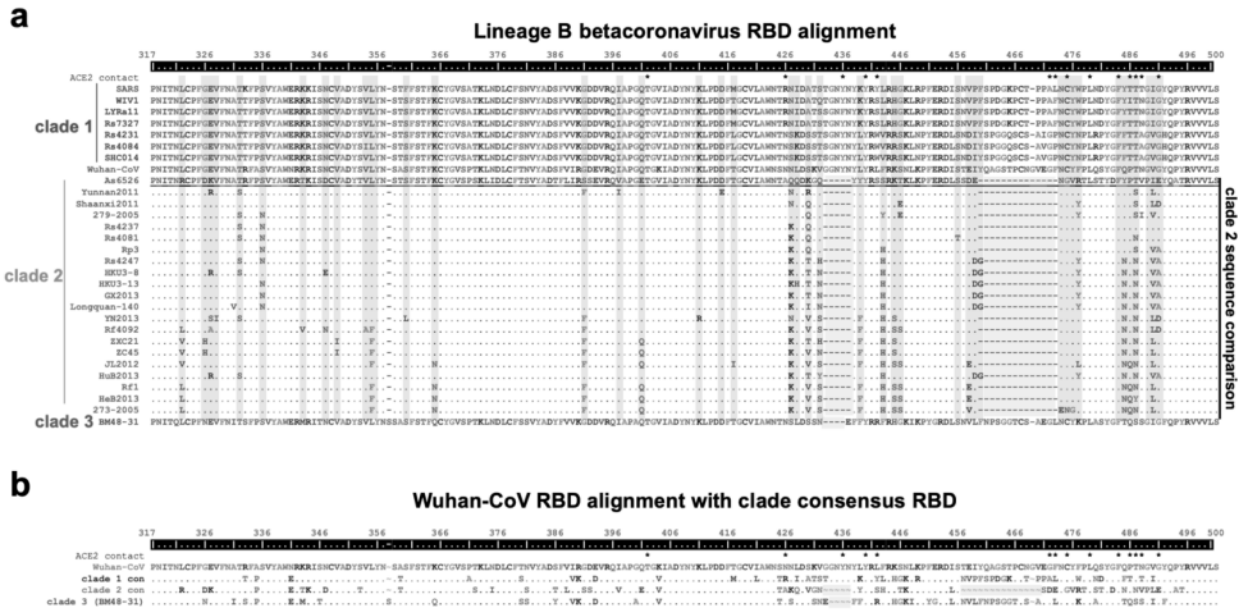
Supplemental figure 2: additional cell lines tested without protease

a, Additional human and cell lines or **b,** bat-derived cell lines derived from other species were infected with VSV-reporter particles pseudotyped with chimeric spikes and luciferase was measured a readout for cell entry. Trypsin was not used in these infections. **c,** All cell lines tested in this study supported entry and reporter expression of VSV-g pseudotyped particles. Shown are the data for 3 replicates.



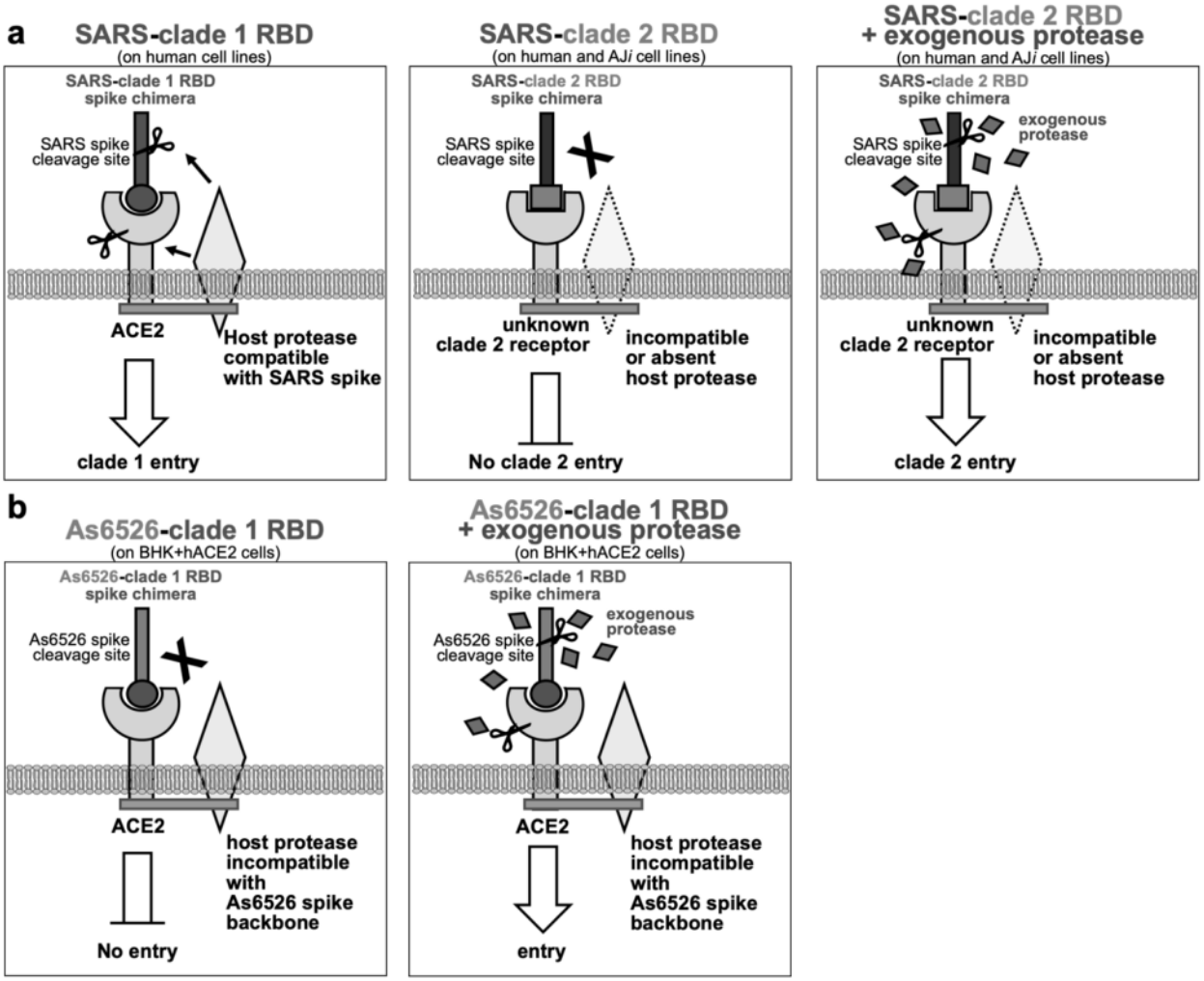
Supplemental figure 3: 2019-nCoV uses human ACE2 to enter cells

VSVΔG-GFP/luciferase particles were pseudotyped with the indicated spikes and used to infect BHKs transfected with known coronavirus receptors. Microscopy images were taken 12-20 hours-post infection.



Supplemental figure 4: Lineage B panel RBD sequence features

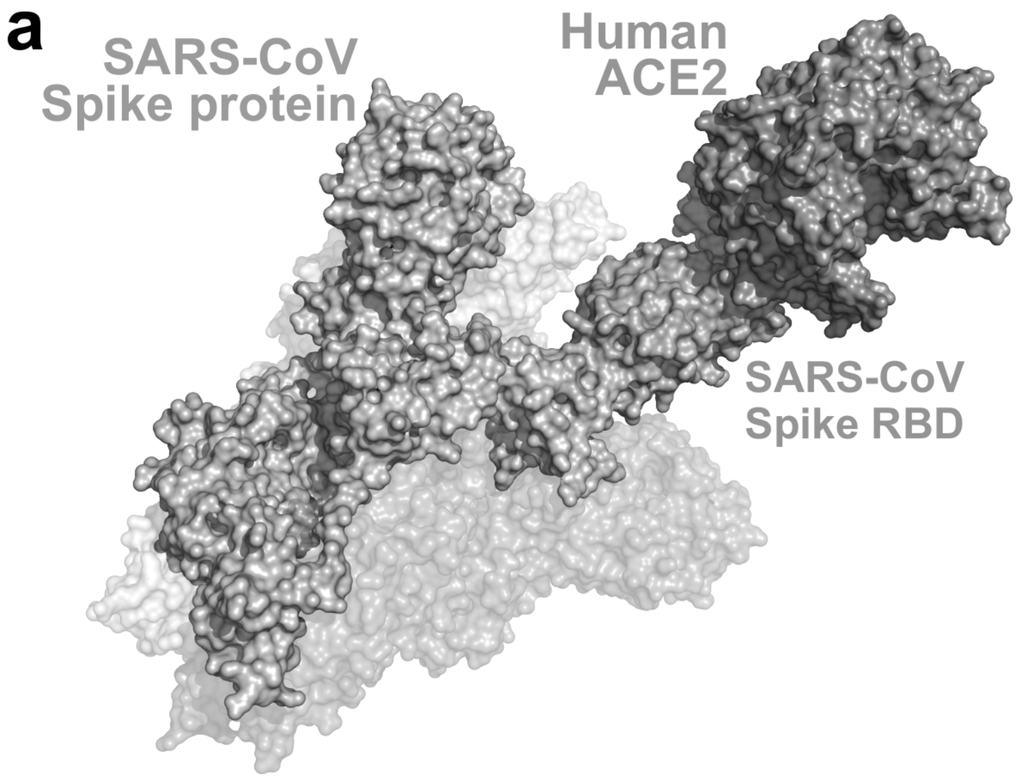
a, Amino acid sequences corresponding to SARS-spike residues 317 through 500 were aligned with ClustalW. Contact points between SARS-spike and human ACE2 are indicated with an (*). Clade 2 sequences are shown as compared to clade 2 As6526, with identical residues indicated with a (.) and sites that vary between clade 2 viruses highlighted in purple. Loop deletions are highlighted in orange. **b,** Amino acid alignment of 2019-nCoV RBD and consensus RBD sequences for clade 1 and 2 and BM48-31 (clade 3). Loop deletions are highlighted in orange.

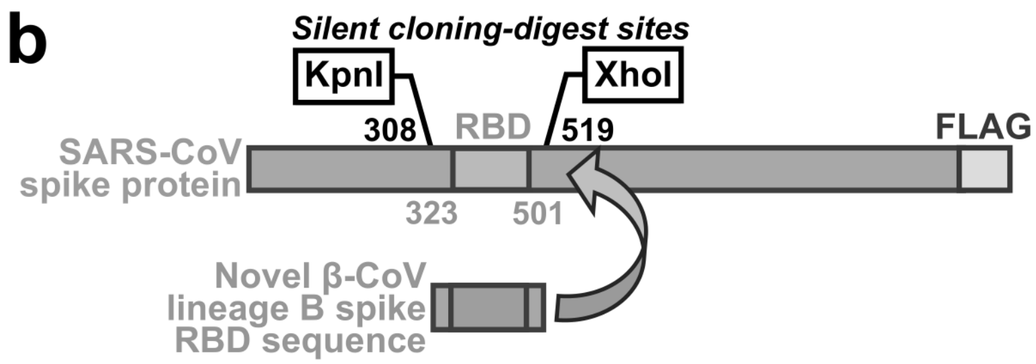


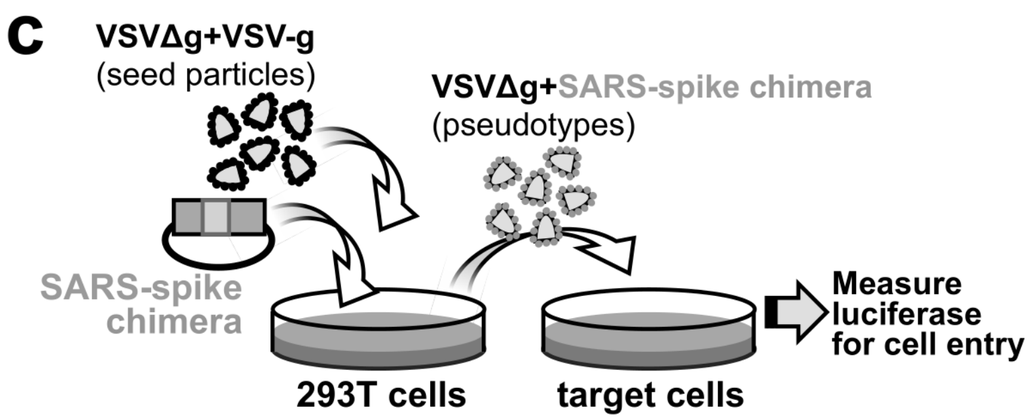
Supplemental figure 5: model of lineage B entry

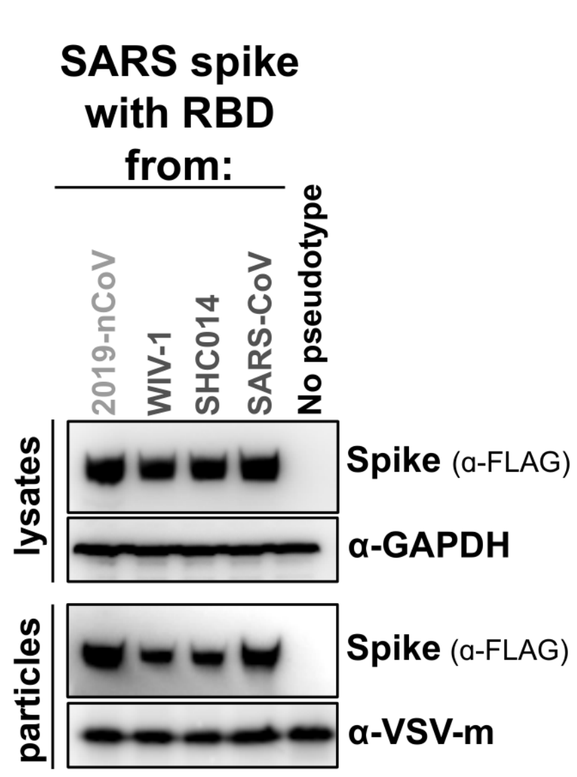
a, SARS spike with a clade 1 RBD enters cells expressing ACE2 and a host protease capable of cleaving SARS spike (left panel). While the clade 2 RBD can bind an unknown host receptor, the SARS spike backbone is incompatible with the receptor-associated protease, resulting in a lack of cleavage and entry (middle panel). The addition of exogenous protease may overcome the lack of endogenous protease cleavage of spike, resulting in receptor-dependent entry. Alternatively, the addition of exogenous protease may activate the receptor to facilitate entry. **b,** Replacing the RBD of As6526 spike with the clade 1 RBD allows for ACE2 interaction, but the As6526 spike backbone is incompatible with the ACE2-associated protease (left panel). Addition of exogenous protease overcomes protease incompatibility, allowing for ACE2-mediate entry (right panel).

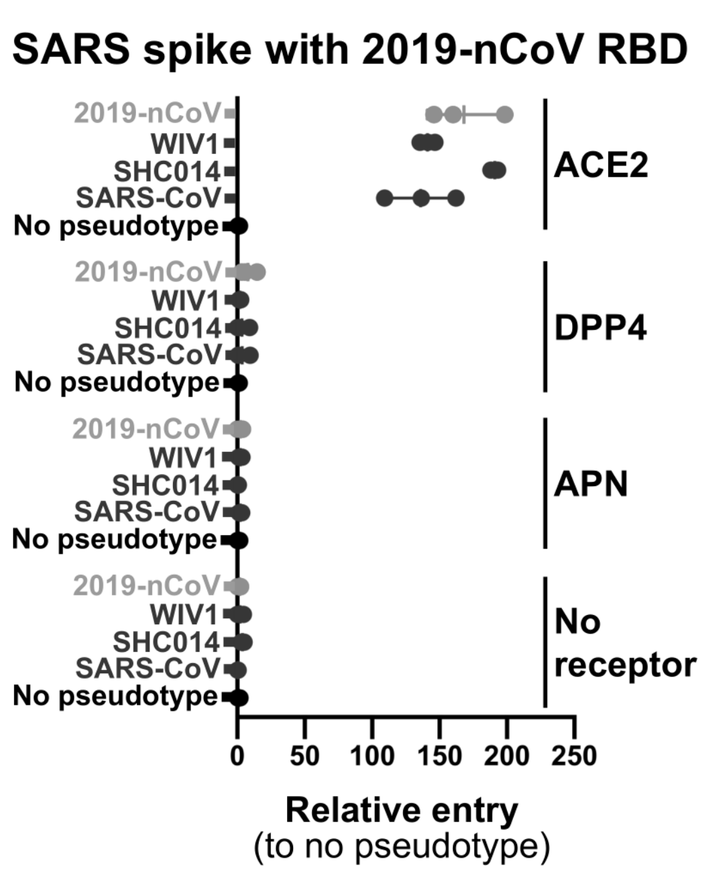
2019-nCoV enters cells expressing human ACE2











From:
Functional assessment of cell entry and receptor usage for lineage B β-coronaviruses, including 2019-nCoV
2020, *submitted*

Michael Letko[#], Vincent Munster[#]
Laboratory of Virology, Division of Intramural Research, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Hamilton, MT, 59840, USA

[#]Correspondence
michael.letko@nih.gov
vincent.munster@nih.gov

From: Munster, Vincent (NIH/NIAID) [E]
Sent: Wed, 22 Jan 2020 15:55:20 -0700
To: Pekoc, Ken (NIH/NIAID) [E]; Letko, Michael (NIH/NIAID) [F]; De wit, Emmie (NIH/NIAID) [E]
Subject: Re: ACE2 receptor usage 2019-NCoV
Attachments: Letko_Munster_2019-nCoV receptor[2].pdf

Should be either tomorrow or the day after,

Already made a figure for you, Michael is the wiz here, so any questions should be directed to him.

Vincent Munster, PhD
Chief, Virus Ecology Section
Laboratory of Virology
Rocky Mountain Laboratories
NIAID/NIH

From: Ken Pekoc (b)(6)
Date: Wednesday, January 22, 2020 at 3:53 PM
To: (b)(6) Emmie De wit (b)(6)
Subject: FW: ACE2 receptor usage 2019-NCoV

Can one of you let me know when the shared info. becomes available … NIAID wants me to provide something for social media.

From: Routh, Jennifer (NIH/NIAID) [E] (b)(6)
Sent: Wednesday, January 22, 2020 3:42 PM
To: Folkers, Greg (NIH/NIAID) [E] (b)(6) Stover, Kathy (NIH/NIAID) [E] (b)(6) Billet, Courtney (NIH/NIAID) [E] (b)(6)
Cc: Pekoc, Ken (NIH/NIAID) [E] (b)(6)
Subject: RE: ACE2 receptor usage 2019-NCoV

Noted – thanks.

Jennifer Routh [E]
News and Science Writing Branch
Office of Communications and Government Relations
National Institute of Allergy and Infectious Diseases (NIAID)
NIH/HHS
31 Center Drive Room 7A17C
Bethesda, MD 20892
Direct: (b)(6)
(b)(6)

Disclaimer: The information in this e-mail and any of its attachments is confidential and may contain sensitive information. It should not be used by anyone who is not the original intended recipient. If you have received this e-mail in error please inform the sender and delete it

from your mailbox or any other storage devices. The National Institute of Allergy and Infectious Diseases shall not accept liability for any statements made that are sender's own and not expressly made on behalf of the NIAID by one of its representatives.

From: Folkers, Greg (NIH/NIAID) [E] (b)(6)
Sent: Wednesday, January 22, 2020 5:05 PM
To: Routh, Jennifer (NIH/NIAID) [E] (b)(6) Stover, Kathy (NIH/NIAID) [E] (b)(6) Billet, Courtney (NIH/NIAID) [E] (b)(6)
Subject: FW: ACE2 receptor usage 2019-NCoV

When this hits bioRxiv might be worth a tweet

From: Munster, Vincent (NIH/NIAID) [E] (b)(6)
Sent: Wednesday, January 22, 2020 3:53 PM
To: Marston, Hilary (NIH/NIAID) [E] (b)(6) NIAID Coronavirus Response SWAT 2020 <NIAIDCoronavirusResponseSWAT2020@mail.nih.gov>
Subject: ACE2 receptor usage 2019-NCoV

First data from my lab confirming ACE2 as the receptor for 2019-NCoV,

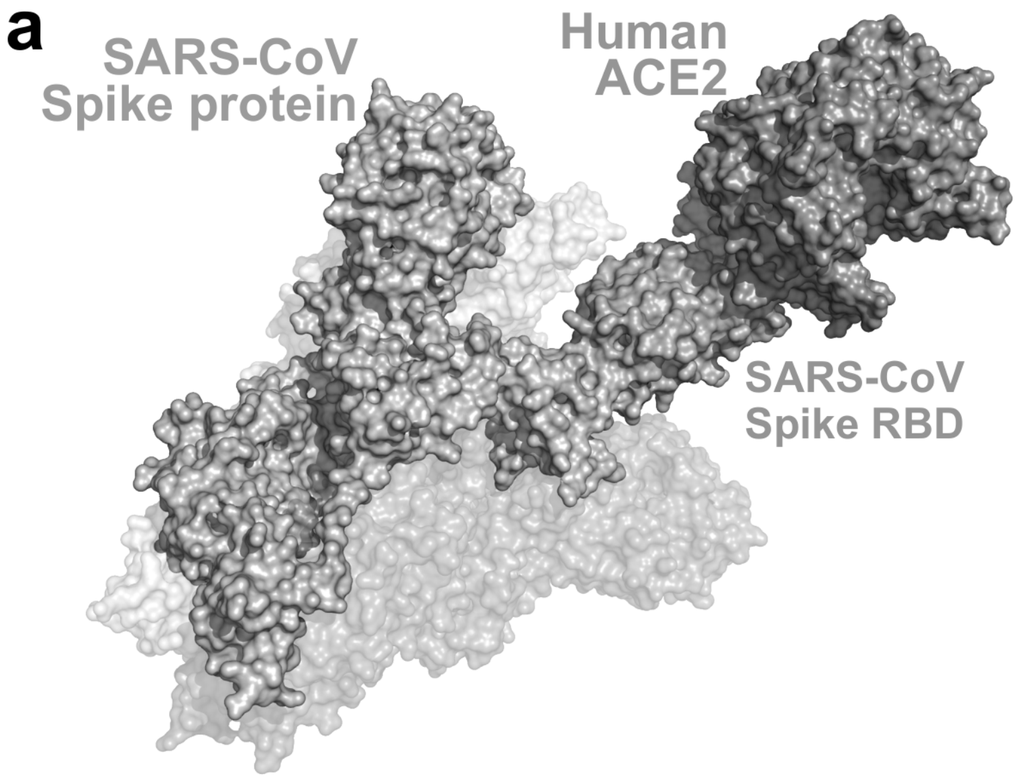
Paper submitted today: BioXRV (hopefully online in 1 – 2 days) and Nature.

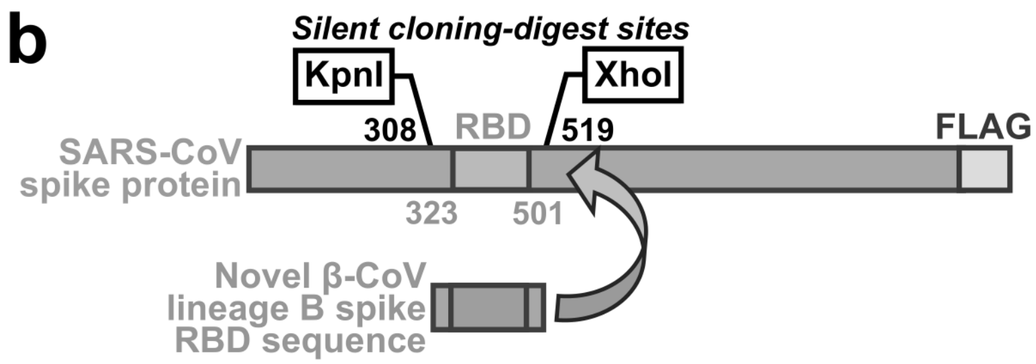
No direct public health implications, but supports more SARS-like scenario (or NL63) rather than MERS-CoV.

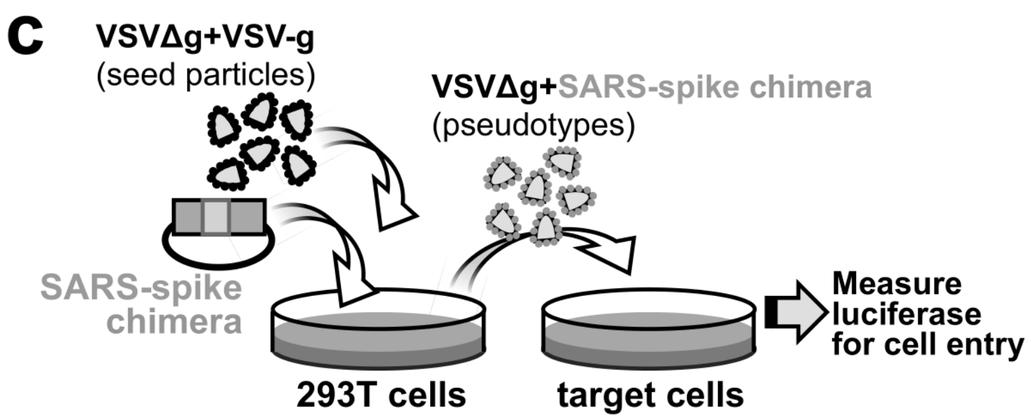
Cheers,

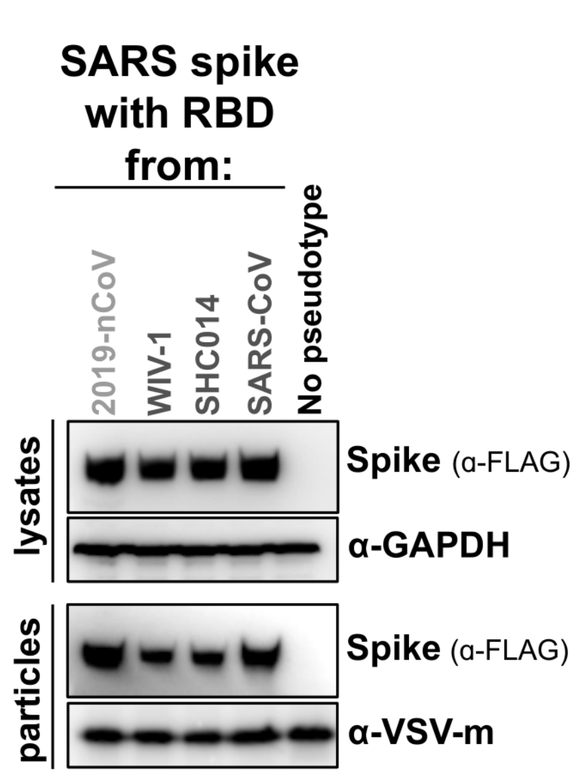
Vincent Munster, PhD
Chief, Virus Ecology Section
Laboratory of Virology
Rocky Mountain Laboratories
NIAID/NIH

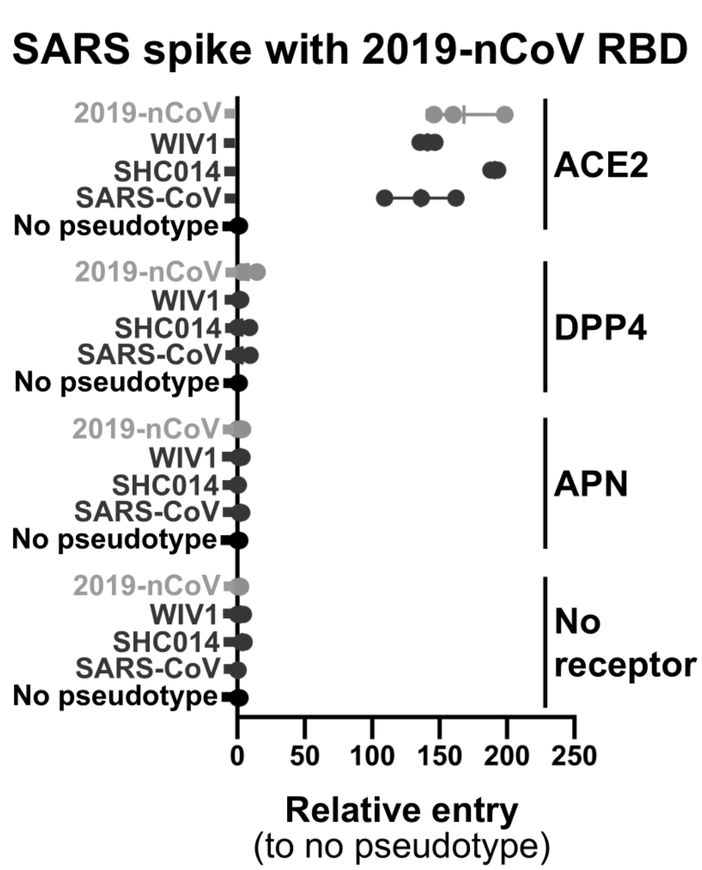
2019-nCoV enters cells expressing human ACE2











From:
Functional assessment of cell entry and receptor usage for lineage B β-coronaviruses, including 2019-nCoV
2020, *submitted*

Michael Letko[#], Vincent Munster[#]
Laboratory of Virology, Division of Intramural Research, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Hamilton, MT, 59840, USA

[#]Correspondence
michael.letko@nih.gov
vincent.munster@nih.gov

From: Munster, Vincent (NIH/NIAID) [E]
Sent: Wed, 22 Jan 2020 13:03:44 -0700
To: Letko, Michael (NIH/NIAID) [F]
Subject: Cover letter
Attachments: coverletter for linB paper ML.docx

Final review

Vincent Munster, PhD
Chief, Virus Ecology Section
Laboratory of Virology
Rocky Mountain Laboratories
NIAID/NIH

National Institute of Allergy and Infectious Diseases

ROCKY MOUNTAIN LABORATORIES

Division of Intramural Research
National Institute of Allergy and Infectious Diseases
Laboratory of Virology
Virus Ecology Unit

Dear Editors:

Herewith we would like to submit the manuscript "**Functional assessment of cell entry and receptor usage for lineage B β-coronaviruses, including 2019-nCoV**" for publication in Nature.

Since SARS-CoV emerged in humans in 2003 and was subsequently discovered in animal markets in China, there has been a massive global effort to identify and sequence all animal viruses in nature, known as the "virome". Unfortunately, most of these viruses exist only as sequences in a database and very little experimental work is performed with them. Thus, the penultimate goal of predicting the next virus that will cross the species barrier and spillover into human populations remains elusive. This is due in part to the sheer number of available sequences, which is constantly expanding, and the lack of cost-effective and scalable downstream assays to test large numbers of viruses for zoonotic potential. Because of the deficit in functional understanding for novel virus sequences, there is a growing sentiment in the field of virus discovery questioning the utility of these efforts.

Here, we present our study that attempts to address this problem by demonstrating a method to rapidly test large numbers of lineage B betacoronaviruses: the group of viruses that includes SARS-CoV and Wuhan 2019-NCoV, the recently identified virus behind the ongoing outbreak of pneumonia in China.

We feel this work is of broad interest to the scientific community and to the public health community as it provides timely and relevant information on the basic biology of 2019-nCoV Wuhan-CoV and cross-species transmission of other lineage B viruses. Our study presents several findings with broad implications:

1. **A method to rapidly test lineage B coronavirus cell entry and receptor usage**. This approach dramatically reduces the time from the sequence being published to having real *in vitro* data to under 12 days, allowing the scientific community to gain critical information on virus tropism in almost real-time with the outbreak.
    a. We effectively **demonstrate the power of this approach by confirming the receptor for Wuhan-CoV**. The sequence for this virus was only released on January 10th this year.
    b. In theory, this method should be broadly applicable to different viruses and virus-host protein interactions.
2. Evidence that several **other lineage B viruses are capable of infecting human cells** through an unknown receptor.
3. Evidence that **host protease incompatibility, not necessarily host receptor incompatibility, is a species barrier** for many lineage B betacoronaviruses. This finding is in good agreement with newly published data from other groups and will certainly go into the development of new methods to isolate novel coronaviruses.
4. Data on **how lineage B coronaviruses can recombine to gain the ability to infect cells with human ACE2** and how this is relevant for Wuhan-CoV.

903 S. 4th St., Hamilton, MT 59840
Phone: (b)(6)
Fax: 406-375-9620
Email: (b)(6)

We believe this data is of importance on our general understanding on the emergence of lineage B coronaviruses and the 2019-NCoV Wuhan virus in particular.

Sincerely yours, and on behalf of the co-authors,

Vincent Munster, PhD & Michael Letko, PhD
Virus Ecology Section
Laboratory of Virology
Rocky Mountain Laboratories
National institute of Allergy and Infectious Diseases
National Institutes of Health

From: Munster, Vincent (NIH/NIAID) [E]
Sent: Wed, 22 Jan 2020 12:17:42 -0700
To: Letko, Michael (NIH/NIAID) [F]
Subject: Re: 2019-nCoV recepter distributable

You want to add your pretty green picture?

Vincent Munster, PhD
Chief, Virus Ecology Section
Laboratory of Virology
Rocky Mountain Laboratories
NIAID/NIH

From: Michael Letko (b)(6)
Date: Wednesday, January 22, 2020 at 11:52 AM
To: (b)(6)
Subject: 2019-nCoV recepter distributable

--
Michael Letko, Ph.D
Postdoctoral IRTA
Dr. Vincent Munster Laboratory
Virus Ecology Unit, Laboratory of Virology
Rocky Mountain Laboratories
NIAID/NIH
903S 4th Street
Hamilton MT 59840
(b)(6)

From: Munster, Vincent (NIH/NIAID) [E]
Sent: Wed, 22 Jan 2020 12:17:17 -0700
To: Letko, Michael (NIH/NIAID) [F]
Subject: Re: 2019-nCoV recepter distributable
Attachments: spike manuscript 1_22_20 ML allVM.docx

This should be the right one

Vincent Munster, PhD
Chief, Virus Ecology Section
Laboratory of Virology
Rocky Mountain Laboratories
NIAID/NIH

From: Michael Letko (b)(6)
Date: Wednesday, January 22, 2020 at 11:52 AM
To: (b)(6)
Subject: 2019-nCoV recepter distributable

--
Michael Letko, Ph.D
Postdoctoral IRTA
Dr. Vincent Munster Laboratory
Virus Ecology Unit, Laboratory of Virology
Rocky Mountain Laboratories
NIAID/NIH
903S 4th Street
Hamilton MT 59840
(b)(6)

Functional assessment of cell entry and receptor usage for lineage B β-coronaviruses, including 2019-nCoV

Michael Letko[#1] and (b)(6) Vincent ~~Munster[1]~~ Munster[#]

~~1.~~ Laboratory of Virology, Division of Intramural Research, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Hamilton, MT, 59840, USA

(b)(6)

[#] Corresponding authors:

Drs. Vincent Munster and Michael Letko

Rocky Mountain Laboratories

NIAID/NIH

903S 4th Street

Hamilton, MT 59840

Tel: (b)(6)

Email: (b)(6)

Email:

Abstract

Over the past 20 years, several coronaviruses have crossed the species barrier into humans, causing outbreaks of severe, and often fatal, respiratory illness. Since SARS-CoV was first identified in animal markets, global viromics projects have discovered thousands of coronavirus sequences in diverse animals and geographic regions. Unfortunately, there are few tools available to functionally test these novel viruses for their ability to infect humans, which has severely hampered efforts to predict the next zoonotic viral outbreak. Here we developed an approach to rapidly screen lineage B betacoronaviruses, such as SARS-CoV and the recent 2019-nCoV, for receptor usage and their ability to infect cell types from different species. We show that host protease processing during viral entry is a significant barrier for several lineage B viruses and that bypassing this barrier allows several lineage B viruses to enter human cells through an unknown receptor. We also demonstrate how different lineage B viruses can recombine to gain entry into human cells and confirm that human ACE2 is the receptor for the recently emerging 2019-nCoV. ~~All together, our approach with lineage B viruses represents a step forward in predicting the next emerging zoonotic virus.~~

Introduction

Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV) first emerged in humans in 2003 after transmitting from animals in open air markets in China[1,2]. Shortly thereafter, several genetically related viruses were identified in Chinese Horseshoes bats (*Rhinolophus sinicus*)[3-7]. At the same time, improvements in next generation sequencing technology lead to a boom of virus discovery, uncovering thousands of novel virus sequences in wild animal populations around the world. While most of these viruses have never been found in humans, many are genetically similar to known human viruses within the betacoronaviruses (β-CoV) genus. The β-CoVs are further divided into four lineages: lineage B, which includes SARS-CoV and the newly ~~recently~~-emerging 2019-nCoV, has approximately 200 published virus sequences whereas lineage C, which includes MERS-CoV, has over 500 viral sequences.

Every year, additional novel CoV sequences are discovered. However, there is a massive knowledge gap in the field as very little work is performed after the viral sequences are published. Therefore, it is unknown whether these novel viruses have the potential to emerge in human populations.

Current methods for studying novel β-CoVs are technically demanding. Viral isolation from field samples is rarely successful and reverse genetics recovery of recombinant virus

is labor-intensive, and expensive as synthesis of a single genome can cost upwards of $15,000. These limitations are prohibitive to studying novel CoVs at the scale in which they are discovered.

Cell entry is an essential component of cross-species transmission, especially for the β-CoVs. All CoVs encode a surface glycoprotein, spike, which binds to the host-cell receptor and mediates viral entry[8]. For β-CoVs, a single region of the spike protein called the receptor binding domain (RBD) mediates the interaction with the host cell receptor. After binding the receptor, a nearby host protease cleaves the spike, which releases the spike fusion peptide, facilitating virus entry[9-12]. Known host receptors for β-CoVs include angiotensin converting enzyme 2 (ACE2) for SARS-CoV and dipeptidyl peptidase 4 (DPP4) for MERS-CoV[13,14].

Structural studies of coronaviruses have shown that the spike RBD is capable of folding independently from the rest of the spike protein and contains all of the structural information for host receptor binding[15]. Additionally, a previous study showed that replacing the RBD of the lineage B bat virus, Rp3, allowed the virus to enter cells expressing human ACE2 (hACE2)[16]. We therefore developed a method to functionally test the RBDs from novel lineage B β-CoVs in place of the SARS-CoV spike RBD (figure 1). Synthesizing just the RBD of spike is much faster and cost-effective than conventional pseudotyping methods that rely on synthesis of the full ~4kb spike sequence for novel CoVs: a process that can take weeks and is cost-prohibitive for large panels of spike sequences. The short turnaround time for our approach allowed us to test the receptor usage of all published, unique RBD sequences in lineage B, and also rapidly confirm the

ACE2 receptor usage of the 2019-nCoV spike, which emerged in China in January 2020 as our study was ongoing.

We show that lineage B RBDs divide into functionally distinct clades and that several previously-unappreciated viruses exhibit compatibility with an unknown receptor on human cells. We also show that these clades are capable of recombining to impart human host-cell entry phenotypes, and that, beyond the RDB-receptor interaction, host protease processing is another species barrier encountered by lineage B β-CoVs during cell entry.

Methods

Cells

293T, A549, BHK, Caco-2, Huh-7.5, PK-15, and Vero cells were maintained in DMEM (Sigma) supplemented with 10% FBS, penicillin/streptomycin, and L-glutamine. RhiNi/40.1, AJ-primary, AJ*i*, HypNi, RaKSM-2.5i, RhiLu, and RhiNi cells were maintained in DMEM/F12 (Gibco) supplemented with 12% FBS, penicillin/streptomycin, non-essential amino acids, sodium pyruvate and L-glutamine. AJ-primary cells were immortalized with a lentiviral vector expressing SV40 T-antigen following the ~~manufacturers~~manufacturer's instructions to generate AJ*i* cells (abm; #G203). RaKSM-2.5 primary cells have been previously described and were immortalized in this study similar to AJ*i* cells[17].

Plasmids

The spike coding sequences for SARS-CoV Urbani, As6526, and BM48-31 were codon optimized for human cells, appended with a 5' kozak expression sequence (GCCACC) and 3' tetra-glycine linker followed by nucleotides encoding a FLAG tag sequence (DYKDDDDK). For SARS-CoV spike, silent mutations were introduced around codons 308 and 519 to form KpnI and XhoI digest sites. For As6526 spike, silent

mutations were introduced around codons 290 and 501 to form AflII and HindIII digest sites. For BM48-31 spike, silent mutations were introduced around codons 295 and 501 to form AflII and HindIII digest sites. These engineered spike sequences were synthesized and cloned into pcDNA3.1+ (GenScript).

Spike RBDs were first codon-optimized for human cells, appended with regions of the target spike backbone to facilitate Infusion cloning and synthesized as double stranded DNA fragments (IDT DNA). SARS-CoV, As6526 or BM48-31 engineered spike plasmids were digested with their corresponding restriction enzymes and gel purified. RBD inserts were resuspended in water and Infusion cloned into gel purified, digested spike backbone vectors (Takara).

Human ACE2 (Q9BYF1.2), DPP4 (XM_005246371.3), or APN (NP_001141.2) were synthesized and cloned into pcDNA3.1+ (GenScript). All DNA constructs were verified by Sanger sequencing (ACGT Inc.).

Receptor transfection

BHK cells were seeded in black 96-well plates and transfected the next day with 100ng plasmid DNA encoding human ACE2, DPP4, APN or empty vector, using polyethyleneimine (Polysciences). All downstream experiments were performed 24 hours post-transfection.

Pseudotype production

Pseudotypes were produced as previously described[18]. 293T cells were seeded onto 6-well plates pre-coated with poly-L-lysine (Sigma) and transfected the next day with

1200ng empty plasmid and 400ng of plasmid encoding coronavirus spike or GFP as a no pseudotype control. Twenty-four hours later, transfected cells were infected with VSVΔG particles pseudotyped with VSV-g as previously described[19]. After one hour of incubating at 37°C, cells were washed three times and incubated in 2mL DMEM supplemented with 2% FBS, penicillin/streptomycin, and L-glutamine for 48 hours. Supernatants were collected, centrifuged at 500xG for 5 minutes, aliquoted and stored at -80.

Luciferase-based cell entry assay

Target cells were seeded in black 96-well plates and inoculated, in triplicate, with equivalent volumes of pseudotype stocks. For trypsin experiments, pseudotype stocks were diluted 1:1 in DMEM without FBS, trypsin was added to a final concentration of 2500µg/mL and samples were incubated at 37°C for 15 minutes. Samples were then diluted again 1:1 in cold DMEM supplemented with 2% FBS and added to cells. Inoculated plates were centrifuged at 1200xG, 4°C, for 1 hour and incubated over night at 37°C. Approximately 18-20 hours post-infection, Bright-Glo luciferase reagent (Promega) was added to each well, 1:1, without removing culture media and luciferase was measured. Relative entry was calculated by normalizing the relative light unit (RLU) for spike pseudotypes to the plate RLU average for the “no pseudotype control.”

Western blot

Producer cells (spike-transfected 293T) were lysed in 1%SDS, 150mM NaCl, 50mM Tris-HCl, 5mM EDTA and clarified by centrifugation at 14000xG for 20 minutes.

Pseudotyped particles were concentrated from producer cell lysates that were overlaid a 10% OptiPrep cushion in PBS (Sigma) and centrifuged at 20,000× g for 2 hours at 4 °C.

Lysates and concentrated particles were analyzed for FLAG, GAPDH and/or VSV-m expression on 10% Bis-Tris PAGE gel (ThermoFisher).

Results

ACE2 entry is lineage B clade 1-specific

The receptor binding domain (RBD) of lineage B β-CoVs is a single, continuous domain that contains all structural information necessary to interact with the host receptor (figure 1a, b). We introduced silent mutations in the codon optimized coding sequence for SARS-CoV to facilitate replacing the SARS RBD with the RBD from other lineage B viruses (figure 1b). All lineage B sequences were downloaded from online repositories and parsed to 29 unique RBD sequences, representing all published variations of the lineage B RBD (supp. fig 1a, b). The panel of 29 RBDs phylogenetically cluster into 3 clades, as previously described [5], but these RBD clades were not apparent in phylogenetic analysis of other viral sequences, such as the RNA-dependent RNA polymerase (supp. fig 1c). All 29 RBDs were codon optimized, synthesized and cloned in place of the SARS RBD, effectively generating chimeric spike expression constructs. We then generated VSV-luciferase reporter particles pseudotyped with the chimeric spikes (figure 1c). We chose VSV over lentiviruses as our pseudotype platform because a lentiviral pseudotypes have failed to accurately reflect viral entry with novel bat coronavirus spike protein[7]. All constructs exhibited similar levels of expression in

producer cells and incorporation into VSV pseudotypes, except the chimera with BM48-31 which displayed somewhat reduced expression compared to WT SARS spike (figure 1d). We then infected BHK cells expressing the receptor for SARS-CoV or empty vector (figure 1e) and observed only clade 1, which includes SARS-CoV, WIV1 and SHC014, could enter cells transfected with human ACE2 (figure 1e).

Protease enhances clade 2 entry

After binding the host receptor, host-cell protease cleaves spike, releasing the fusion peptide and allowing for host cell entry [20]. Previous studies have shown that absence of the host protease or incompatibility between the host protease and viral spike can block viral entry [21-24]. To circumvent host-cell protease incompatibility or absence, we treated our lineage B pseudotype panel and infected a wide variety of cell types from different host species (figure 2, supp. fig. 2). In the absence of exogenous protease, only clade 1 infected cells from African green monkey kidney, human gastrointestinal tract, human liver, and porcine kidney, in agreement with previous studies (figure 2; supp. fig. 2a, b). Surprisingly, exogenous protease enhanced entry of a subset of clade 2 spike chimeras in nonhuman primate, bat and human cells (figure 2). Importantly, VSV-g pseudotyped particles were able to produce luciferase signal in all cell lines tested in this study (supp. fig. 2c).

Clade 2 entry is receptor-dependent

We next tested human variants of known β-CoV receptors for their ability to mediate cell entry of clade 2 and 3 spike chimeras. We also tested human

aminopeptidase N (APN), a receptor for alphacoronaviruses, which have been shown to utilize either human ACE2 or human APN for cell entry (figure 3a). Protease treatment only enhanced entry of clade 1 RBDs on cells expressing human ACE2, but not human DPP4 or APN. No entry was observed with clade 2 or 3 spikes, regardless of receptor or protease addition. Human dipeptidyl peptidase IV (DPP4), the receptor for the lineage C β-CoVs, MERS-CoV, only mediated entry of MERS-CoV (figure 2b, middle panels). Importantly, in the absence of receptor, no entry was observed for any of the pseudotypes, suggesting that protease-mediated entry is receptor-dependent (figure 2b, right panels).

Receptor preference of 2019-nCoV

Commented [MV([1]: Usage?

While our study was ongoing, a novel lineage B virus tentatively named 2019-nCoV was identified as the cause of a pneumonia outbreak in Hubei, China. Once the sequence was publicly available, we synthesized, cloned and tested the RBD from 2019-nCoV in our assay with human variants of known coronavirus receptors. The chimeric SARS-2019-nCoV spike protein expressed and was incorporated into particles similarly to other clade 1 chimeric spikes (Figure 3c). The 2019-nCoV RBD was capable of entering cells expressing human ACE2, but not any of the other receptors tested (Figure 3d).

Clade determinants for ACE2 usage

Consensus sequences of the three lineage B clades showed several key differences between these groups. Only clade 1 RBDs contain all 14 residues that have been shown through crystallography, to interact with human ACE2 (figure 4a; supp. fig.

3). The majority of these residues are absent from clades 2 and 3, which contain additional deletions in surface exposed loops that cluster at the interface with ACE2 (figure 4 a, b). We generated a series of clade consensus RBD variants to determine the minimum number of mutations needed to impart ACE2 function on clade 2 and 3 RBDs (figure 4c). Introducing the two loop deletions from clade 1 in clade 2 results in a reduced spike expression, impaired pseudotype incorporation and loss of cell entry (figure 4c, d). Restoring these loops in clade 2 and 3 from the loops found in clade 1 did not enhance entry with ACE2 (figure 4c; 2→1 and 3→1 version 1). Introducing all 14 ACE2 contact points in clade 2 or 3 also failed to restore ACE2 entry (figure 4c; 2→1 and 3→1 version 2). Only replacing all 14 contact points and the surrounding amino acids (also known as the receptor binding motif, RBM) lead to increased ACE2 entry with clade 2 and 3 RBDs (figure 4c; 2→1 version 3 = clade 2 residues 322-400 + clade 1 residues 400-501; 3→1 version 3 = clade 3 residues 322-385 + clade 1 residues 386-501). Taken together, these results show that the entire RBM from clade 1 is needed for ACE2 entry.

Full-spike and RBD chimeras are comparable

We next synthesized full-length clade 2 and 3 spikes to compare to our RBD chimeras. We selected the clade 2 spike, As6526, because it consistently gave strong entry signal in human cells following protease-treatment (figure 2b) as well as BM48-31, the only clade 3 spike in our panel. As we did for SARS-CoV spike, clade 2 and 3 spikes were codon optimized, FLAG-tagged and silent mutations were introduced to facilitate replacing their RBD with the consensus RBD from clade 1 (figure 5a). All chimeric constructs expressed similarly, with the exception for the SARS-BM48-31 RBD chimera,

which exhibited reduced expression and incorporation (figure 5b). Protease treatment enhanced entry of both the As6526 clade 2 RBD chimera and full-length spike entry into Huh cells (figure 5c). Protease treatment had no effect on either the BM48-31 clade 3 chimera or full-length spike (figure 5c). Taken together, these findings show that SARS-lineage B RBD chimeras reflect the entry phenotype of full-length lineage B spikes.

Finally, we tested if receptor-binding and protease processing are coupled. We replaced the RBD of full-length clade 2 and 3 spike with the consensus RBD from clade 1 and tested pseudotypes on cells expressing the clade 1 receptor. The clade 1 consensus RBD efficiently facilitated entry of both As6526 and BM48-31 spike only following protease treatment. These findings show that even though BHK-hACE2 cells support full-length clade 1 spike entry, just having the RBD from a clade 1 virus is insufficient to mediate entry. As seen in our previous experiments, protease treatment did not enhance pseudotype entry in the absence of host receptor (figure 5).

Discussion

Despite significant advances in next generation sequencing technologies, which have facilitated the discovery of thousands of novel animal-derived viruses, tools for downstream functional assessment of these novel sequences are lacking. To gain traction on this ever-growing problem, we took a reductionist approach to coronavirus entry and developed a scalable, BSL2-compatible method for testing only the minimal region of the virus essential for interacting with the host receptor (Figure 1, figure s1a). Because most of these viruses have never been isolated, we resorted to synthetic biology and molecular engineering to reduce the burden of gene synthesis to just a small fragment. Thus, the cost and synthesis production time for testing several spikes for entry in our system is dramatically reduced (supplementary figure 1d). In theory, this approach to functional viromics should be applicable to a wide variety of virus-host proteins and interactions.

Coronavirus entry is a multi-step process involving multiple, distinct domains in spike that mediate virus attachment to the cell surface, receptor engagement, protease processing and membrane fusion[8]. While the RBD:receptor interaction is most studied in this process, recent studies have highlighted the major role host protease processing plays as a species barrier[22,25-27]. Lineage C coronaviruses include MERS-CoV as well as

distantly related viruses such as HKU4, HKU5 and PDF-2180 [28,29]. Studies have shown that HKU4 can bind human DPP4 but requires addition of exogenous trypsin to facilitate cell entry and that HKU5 and PDF-2180 spikes can enter human cells through an unidentified receptor with protease treatment[22,26]. Analogous to these earlier studies of lineage C CoVs, we observed protease-enhanced entry of lineage B CoVs (figure 2, 3, 5). While it has been shown that host proteases cleave spike, allowing for downstream membrane fusion, other evidence suggests that protease may act on the receptor as well to activate it[30]. ~~In this previous study, a~~Addition of protease during the course of SARS-CoV infection facilitated entry in cells with low-expression of ACE2 that is normally insufficient to support virus entry[30]. Indeed, we saw evidence of residual trypsin activity on the cells after infection in our studies as the cell monolayer was loose compared to the untreated condition. Similarly, Menachery et al. also observed cell rounding during their trypsin infections[26]. Therefore, further studies are needed to assess where trypsin is enhancing entry of coronaviruses: at the level of spike, the receptor, or both.

In the absence of exogenous protease, only clade 1 RBDs entered nonhuman primate, human and porcine cell lines (figure 2a, b, s2a, b). These findings are in strong agreement with previous studies that have either isolated virus (WIV1) or rescued recombinant chimeric viruses (SHC014, Rs4231, Rs7237)[5,7,31]. However, with trypsin, a subset of genetically-similar clade 2 RBDs gained entry in these cells, suggesting their barrier is at the level of protease processing (figure 2a, b). The other spike from clade 2 and 3 did not enter the cells we tested, regardless of protease addition, suggesting an absent or incompatible receptor. Surprisingly, the protease-dependent entry phenotype was consistent in the reverse spike chimeras in which we replaced the RBD in clade 2 or

3 spike with a clade 1 RBD (figure 5d), suggesting that either the protease site between S1/S2 is not compatible with the chimeric spike backbone or the protease is not expressed in these cells (figure s4). Because clade 1 spikes enter cells expressing human ACE2 without addition of protease, our data showing protease addition is needed for entry of clade 2-clade 1 spike chimera suggests the spike protease site is adapted to the protease environment of the receptor-bound RBD (figure s4).

Commented [MV([2]: Potentially delete?

None of the spike pseudotypes efficiently entered rhinolophus cells, which has been observed in previous studies using these cells[32,33] (figure 2c). Surprisingly, AJi cells were selectively permissive for only clade 2 entry following protease treatment, which suggests that clade 2 RBDs interact with a receptor that is distinct from clade 1 (figure 2c).

Our results show that, despite all being classified as the same virus species, most lineage B β-CoVs do not use currently known coronavirus receptors (figure 1e, 3a, b). Critically, we did not observe any pseudotype entry in the presence of protease and absence of receptor, suggesting that lineage B cell entry is still receptor-dependent following protease treatment (figure 3b). While our study was ongoing, a novel lineage B β-CoV was identified as the etiological agent behind an outbreak of pneumonia in Wuhan, Hubei, China (2019-nCoV). The RBD for 2019-nCoV has residues and motifs found in all 3 clades but forms a distinct clade, so we tested it for receptor usage and observed entry only with human ACE2 but not other known coronavirus receptors (figure 3d). Interestingly, within the backbone of SARS-CoV spike, cell entry of 2019-nCoV was similar to the other clade 1 spikes tested, including SARS-CoV. These finding suggests 2019-nCoV is capable of using human ACE2 as efficiently as SARS-CoV, which may help

explain the human-to-human transmissibility of this virus. More studies are needed with the full spike sequence and, ideally, a viral isolate.

The receptor binding motif (RBM) is a small region in the C-terminal half of the RBD and contains all the residues that interface with the host receptor (figure 3a)[15]. The 14 contact points in the co-structure of the SARS-RBD bound to human ACE2 are largely absent from clade 2 and 3 RBDs, which also contain deletions compared to clade 1 RBMs (figure 4a, b, s3a). Simply mutating clade 2 and 3 to have the 14 contact points was insufficient to impart human ACE2 usage (figure 4c). This is likely because the non-contact residues in the RBM play a supportive and structural role for these contact points, and indeed, these non-contact residues are different between the clades (figure s3).

In contrast to changing individual amino acids, our chimeric RBD constructs show that clade 2 and 3 RBD containing the clade 1 RBM are compatible with human ACE2. Coronaviruses frequently undergo recombination, gaining large swaths of genetic material at once [34,35]. Taken together with our data, it is possible that recombination with a clade 1 virus will impart compatibility with human ACE2. Interestingly, the 2019-nCoV RBD forms a clade that is distinct from the other 3 clades (figure s1c). However, the 2019-nCoV RBD contains most of the contact points with human ACE2 that are found in clade 1 as well as some amino acid variations that are unique to clade 2 and 3 (figure s3b). Taken together with our receptor assay results, it may be possible that 2019-nCoV arose from recombination between clade 1 and the other clades.

As we saw with the SARS-As6526 RBD (clade 2) spike chimera, full length As6526 spike entered cells following protease treatment, but BM48-31 (clade 3) spike did not (figure 2, 5c). These data show that the chimeric spikes generally reproduce the entry

phenotypes of full-length spikes. Notably, the full length As6526 spike did not enter cells as efficiently as the SARS-As6526 chimera, suggesting that other human-cell adaptations are likely needed in As6526 spike.

The capacity to predict the ~~next potential viral outbreak~~zoonotic potential of newly detected viruses ~~-~~has been severely hindered by a lack of functional data for these novel animal virus sequences. Here, we have developed a rapid and cost-effective ~~approach~~ platform to functionally test large groups of related viruses for zoonotic potential. We found that several other lineage B coronaviruses are capable of entering human cells through an unknown receptor and that lineage B spike proteins can recombine to gain entry with a known host-receptor. Taken together with the latest outbreak of 2019-nCoV in humans, these findings underscore the importance of continued surveillance of coronaviruses at the sequence and functional levels in order to better prepare for the next emerging virus.

Acknowledgements

We would like to thank Dr. Anthony Schountz for generously sharing primary artibeus cells, as well as Dr. Marcel Muller for providing the Rhinolophus cells used in this study. We would also like to acknowledge Wu,F., Zhao,S., Yu,B., Chen,Y.-M., Wang,W., Hu,Y., Song,Z.-G., Tao,Z.-W., Tian,J.-H., Pei,Y.-Y., Yuan,M.L., Zhang,Y.-L., Dai,F.-H., Liu,Y., Wang,Q.-M., Zheng,J.-J., Xu,L., Holmes,E.C. and Zhang,Y.-Z, the Wuhan Institute of Virology, Chinese Academy of Medical Sciences & Peking Union Medical College, Chinese Center for Disease Control and Prevention, and Wuhan Jinyintan Hospitar for providing the genome sequence for 2019-nCoV and other essential

outbreak information to the scientific community. This work was supported by the Intramural Research Program of the National Institute of Allergy and Infectious Diseases.

Declarations of Interests

The authors declare no competing interests.

References

1 Wang, M. *et al.* SARS-CoV infection in a restaurant from palm civet. *Emerg Infect Dis* **11**, 1860-1865, doi:10.3201/eid1112.041293 (2005).
2 Kuiken, T. *et al.* Newly discovered coronavirus as the primary cause of severe acute respiratory syndrome. *Lancet* **362**, 263-270, doi:10.1016/S0140-6736(03)13967-0 (2003).
3 Li, W. *et al.* Bats are natural reservoirs of SARS-like coronaviruses. *Science* **310**, 676-679, doi:10.1126/science.1118391 (2005).
4 Ge, X. Y. *et al.* Isolation and characterization of a bat SARS-like coronavirus that uses the ACE2 receptor. *Nature* **503**, 535-538, doi:10.1038/nature12711 (2013).
5 Hu, B. *et al.* Discovery of a rich gene pool of bat SARS-related coronaviruses provides new insights into the origin of SARS coronavirus. *PLoS Pathog* **13**, e1006698, doi:10.1371/journal.ppat.1006698 (2017).
6 Lau, S. K. *et al.* Severe acute respiratory syndrome coronavirus-like virus in Chinese horseshoe bats. *Proc Natl Acad Sci U S A* **102**, 14040-14045, doi:10.1073/pnas.0506735102 (2005).
7 Menachery, V. D. *et al.* A SARS-like cluster of circulating bat coronaviruses shows potential for human emergence. *Nat Med* **21**, 1508-1513, doi:10.1038/nm.3985 (2015).
8 Li, F. Structure, Function, and Evolution of Coronavirus Spike Proteins. *Annu Rev Virol* **3**, 237-261, doi:10.1146/annurev-virology-110615-042301 (2016).
9 Simmons, G., Zmora, P., Gierer, S., Heurich, A. & Pohlmann, S. Proteolytic activation of the SARS-coronavirus spike protein: cutting enzymes at the cutting edge of antiviral research. *Antiviral Res* **100**, 605-614, doi:10.1016/j.antiviral.2013.09.028 (2013).
10 Matsuyama, S. *et al.* Efficient activation of the severe acute respiratory syndrome coronavirus spike protein by the transmembrane protease TMPRSS2. *J Virol* **84**, 12658-12664, doi:10.1128/JVI.01542-10 (2010).
11 Bertram, S. *et al.* Cleavage and activation of the severe acute respiratory syndrome coronavirus spike protein by human airway trypsin-like protease. *J Virol* **85**, 13363-13372, doi:10.1128/JVI.05300-11 (2011).
12 Belouzard, S., Chu, V. C. & Whittaker, G. R. Activation of the SARS coronavirus spike protein via sequential proteolytic cleavage at two distinct sites. *Proc Natl Acad Sci U S A* **106**, 5871-5876, doi:10.1073/pnas.0809524106 (2009).

13 Raj, V. S. *et al.* Dipeptidyl peptidase 4 is a functional receptor for the emerging human coronavirus-EMC. *Nature* **495**, 251-254, doi:10.1038/nature12005 (2013).
14 Li, W. *et al.* Angiotensin-converting enzyme 2 is a functional receptor for the SARS coronavirus. *Nature* **426**, 450-454, doi:10.1038/nature02145 (2003).
15 Li, F., Li, W., Farzan, M. & Harrison, S. C. Structure of SARS coronavirus spike receptor-binding domain complexed with receptor. *Science* **309**, 1864-1868, doi:10.1126/science.1116480 (2005).
16 Becker, M. M. *et al.* Synthetic recombinant bat SARS-like coronavirus is infectious in cultured cells and in mice. *Proc Natl Acad Sci U S A* **105**, 19944-19949, doi:10.1073/pnas.0808116105 (2008).
17 Seifert, S. N. *et al.* Rousettus aegyptiacus Bats Do Not Support Productive Nipah Virus Replication. *J Infect Dis*, doi:10.1093/infdis/jiz429 (2019).
18 Letko, M. *et al.* Adaptive Evolution of MERS-CoV to Species Variation in DPP4. *Cell Rep* **24**, 1730-1737, doi:10.1016/j.celrep.2018.07.045 (2018).
19 Takada, A. *et al.* A system for functional analysis of Ebola virus glycoprotein. *Proc Natl Acad Sci U S A* **94**, 14764-14769, doi:10.1073/pnas.94.26.14764 (1997).
20 Millet, J. K. & Whittaker, G. R. Host cell proteases: Critical determinants of coronavirus tropism and pathogenesis. *Virus Res* **202**, 120-134, doi:10.1016/j.virusres.2014.11.021 (2015).
21 Menachery, V. D. *et al.* Trypsin treatment unlocks barrier for zoonotic coronaviruses infection. *bioRxiv* (2019).
22 Yang, Y. *et al.* Receptor usage and cell entry of bat coronavirus HKU4 provide insight into bat-to-human transmission of MERS coronavirus. *Proc Natl Acad Sci U S A* **111**, 12516-12521, doi:10.1073/pnas.1405889111 (2014).
23 Matsuyama, S., Ujike, M., Morikawa, S., Tashiro, M. & Taguchi, F. Protease-mediated enhancement of severe acute respiratory syndrome coronavirus infection. *Proc Natl Acad Sci U S A* **102**, 12543-12547, doi:10.1073/pnas.0503203102 (2005).
24 Kam, Y. W. *et al.* Cleavage of the SARS coronavirus spike glycoprotein by airway proteases enhances virus entry into human bronchial epithelial cells in vitro. *PLoS One* **4**, e7870, doi:10.1371/journal.pone.0007870 (2009).
25 Yang, Y. *et al.* Two Mutations Were Critical for Bat-to-Human Transmission of Middle East Respiratory Syndrome Coronavirus. *J Virol* **89**, 9119-9123, doi:10.1128/JVI.01279-15 (2015).
26 Menachery, V. D. *et al.* Trypsin treatment unlocks barrier for zoonotic bat coronaviruses infection. *J Virol*, doi:10.1128/JVI.01774-19 (2019).
27 Beniac, D. R., deVarennes, S. L., Andonov, A., He, R. & Booth, T. F. Conformational reorganization of the SARS coronavirus spike following receptor binding: implications for membrane fusion. *PLoS One* **2**, e1082, doi:10.1371/journal.pone.0001082 (2007).
28 Woo, P. C. *et al.* Molecular diversity of coronaviruses in bats. *Virology* **351**, 180-187, doi:10.1016/j.virol.2006.02.041 (2006).
29 Anthony, S. J. *et al.* Further Evidence for Bats as the Evolutionary Source of Middle East Respiratory Syndrome Coronavirus. *mBio* **8**, doi:10.1128/mBio.00373-17 (2017).

30 Shulla, A. *et al.* A transmembrane serine protease is linked to the severe acute respiratory syndrome coronavirus receptor and activates virus entry. *J Virol* **85**, 873-882, doi:10.1128/JVI.02062-10 (2011).
31 Adney, D. R. *et al.* Replication and shedding of MERS-CoV in upper respiratory tract of inoculated dromedary camels. *Emerg Infect Dis* **20**, 1999-2005, doi:10.3201/eid2012.141280 (2014).
32 Muth, D. *et al.* Attenuation of replication by a 29 nucleotide deletion in SARS-coronavirus acquired during the early stages of human-to-human transmission. *Sci Rep* **8**, 15177, doi:10.1038/s41598-018-33487-8 (2018).
33 Hoffmann, M. *et al.* Differential sensitivity of bat cells to infection by enveloped RNA viruses: coronaviruses, paramyxoviruses, filoviruses, and influenza viruses. *PLoS One* **8**, e72942, doi:10.1371/journal.pone.0072942 (2013).
34 Liao, C. L. & Lai, M. M. RNA recombination in a coronavirus: recombination between viral genomic RNA and transfected RNA fragments. *J Virol* **66**, 6117-6124 (1992).
35 Graham, R. L. & Baric, R. S. Recombination, reservoirs, and the modular spike: mechanisms of coronavirus cross-species transmission. *J Virol* **84**, 3134-3146, doi:10.1128/JVI.01394-09 (2010).

Figures

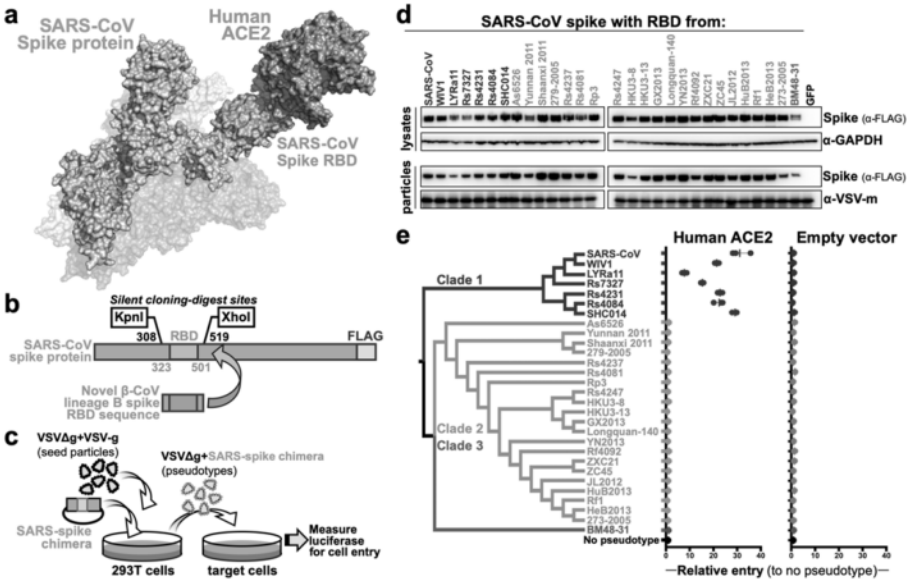


Figure 1: β-Coronavirus lineage B entry with human ACE2 is clade-specific

a, β-Coronaviruses, including SARS-CoV, interact with the host cell receptor via the Receptor Binding Domain (RBD) in spike (PDB: 5X5B, 2AJF) . **b,** SARS-CoV spike was engineered with silent mutations to facilitate cloning novel RBD sequences in place of the SARS spike RBD. SARS spike amino acid numbers are indicated for silent cloning sites and the RBD in grey and orange, respectively. **c,** outline of experimental workflow. **d,** Westernblot of producer cell lysates and concentrated reporter particles. **e,** BHK cells were transfected with either human ACE2 or empty vector, subsequently infected with VSV-reporter particles pseudotyped with chimeric spikes, luciferase was measured and normalized to no pseudotype as a readout for cell entry. Shown are the data for 3 replicates.

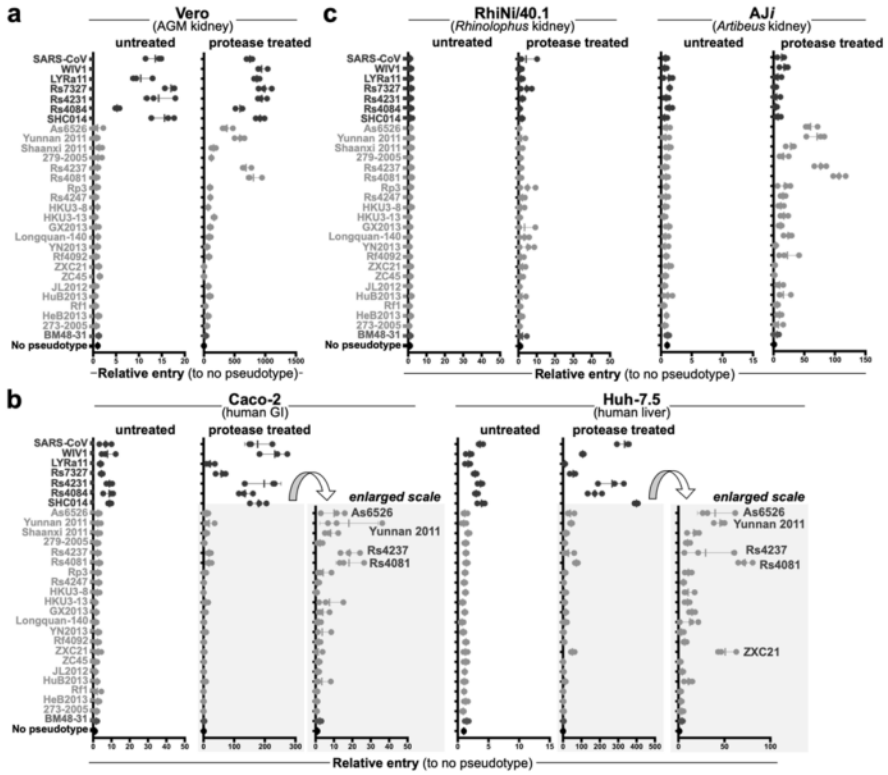


Figure 2: Trypsin enhances lineage B entry in various cell lines

a, Primate cells, **b,** human cells or **c,** bat cells were infected with VSV-particles pseudotyped with the lineage B chimeric spike panel. Pseudotypes were either left untreated or incubated with trypsin before addition to the cells. Luciferase was measured and normalized to particles produced without pseudotype. Shown are the data for 3 replicates.

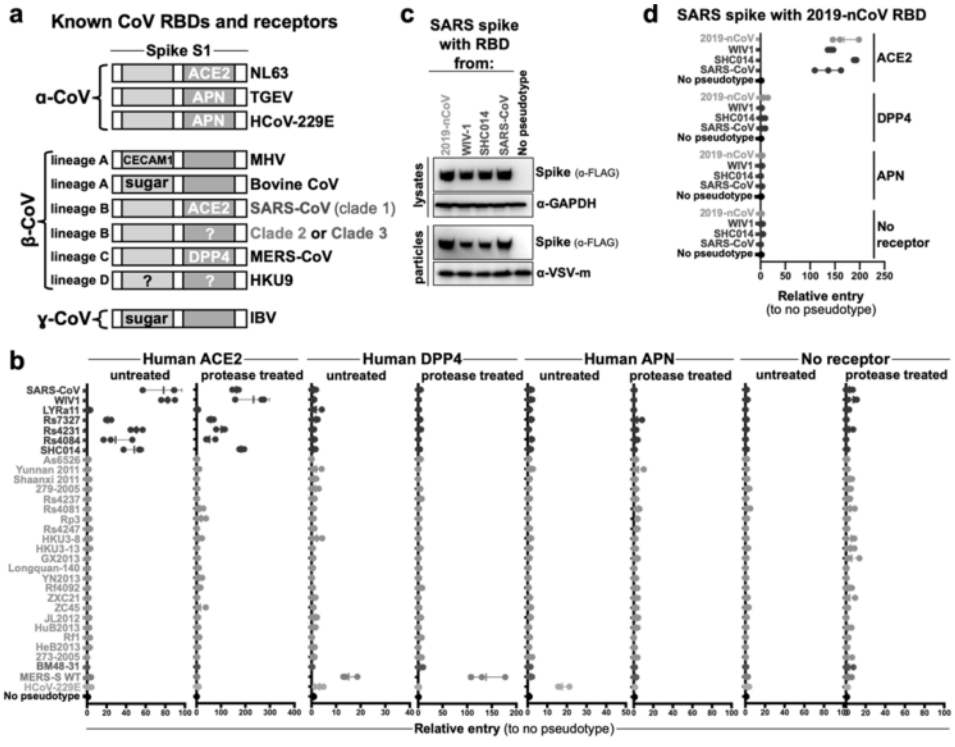


Figure 3: Lineage B entry into cells with known CoV receptors

a, Schematic of known coronavirus RBDs and their receptors. **b,** Pseudotyped particles were either left untreated and treated with trypsin and subsequently used to infect BHK cells transfected with the coronavirus receptor indicated. Shown are data from 3 replicates. **c,** Expression and pseudotype incorporation of SARS-S-2019-nCoV RBD chimeras. **d,** Pseudotypes were used to infect cells expressing hACE2, hDPP4, hAPN, or empty vector, without protease treatment.

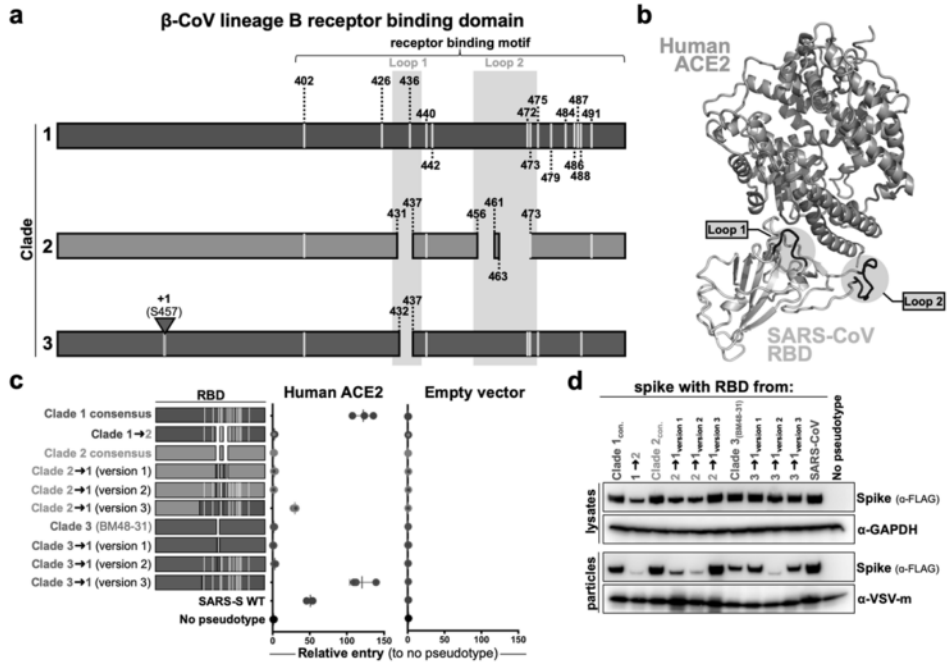


Figure 4: Lineage B clade-specific determinants for human ACE2 usage

a, Schematic overview of clade 1, 2 and 3. Highlighted in yellow are the 14 residues that contact human ACE2. Deletions in loops 1 and 2 are indicated for clades 2 and 3. **b,** Structure of human ACE2 and the SARS-S RBD (PDB: 2AJF), with loops highlighted in gray. **c,** VSV pseudotypes were generated with the indicated RBD and used to infect BHKs transfected with either human ACE2 or empty vector. Shown are data for 3 replicates. **d,** Westernblot of producer cell lysates and concentrated pseudotyped particles.

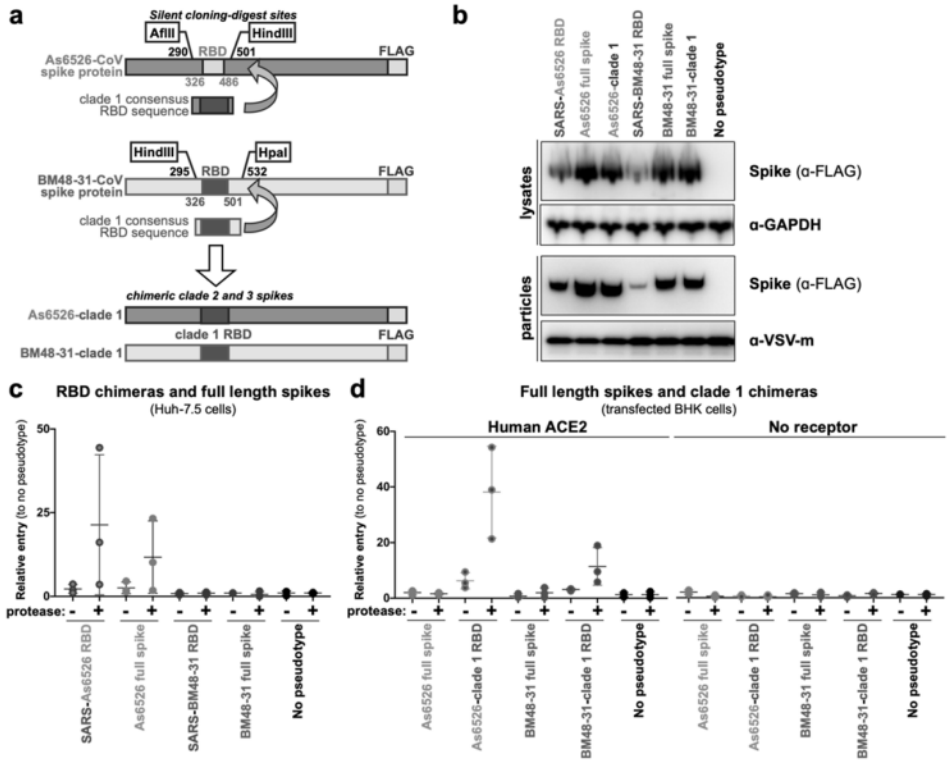
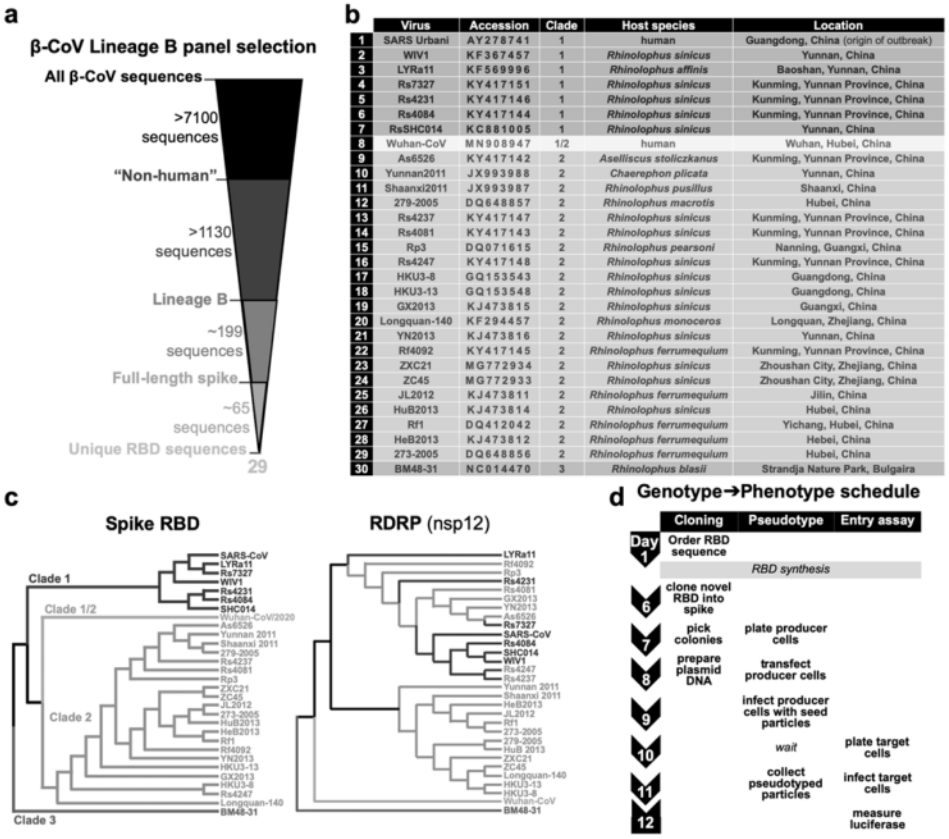


Figure 5: Comparison of chimeric and full length clade 2 and 3 spikes

a, Full length spike sequences from As6526 (clade 2) and BM48-31 (clade 3) were codon optimized, FLAG tagged and synthesized. Silent mutations flanking the RBD facilitated replacing the native RBD with the clade 1 consensus RBD. **b,** Westernblot of producer cell lysates and concentrated pseudotypes particles. **c,** Pseudotypes with indicated spike constructs were left untreated or treated with trypsin and subsequently used to infect Huh-7.5 cells. Shown are data for 3 replicates. **d,** Pseudotypes with indicated spike constructs were left untreated or treated with trypsin and subsequently used to infect BHK cells transfected with human ACE2. Shown are data for 3 replicates.

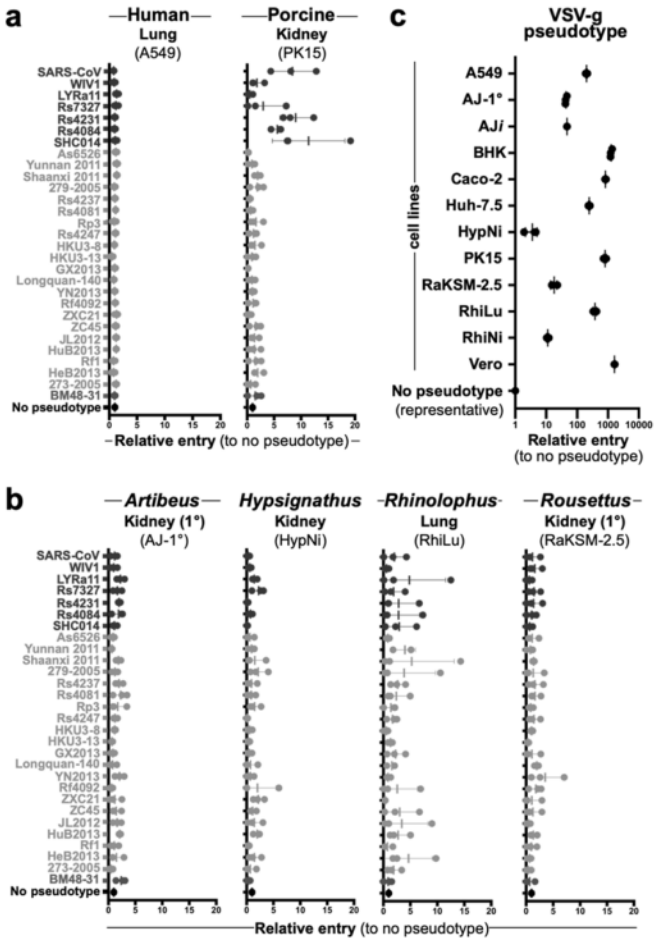
SUPPLEMENTAL INFORMATION



	Virus	Accession	Clade	Host species	Location
1	SARS Urbani	AY278741	1	human	Guangdong, China (origin of outbreak)
2	WIV1	KF367457	1	*Rhinolophus sinicus*	Yunnan, China
3	LYRa11	KF569996	1	*Rhinolophus affinis*	Baoshan, Yunnan, China
4	Rs7327	KY417151	1	*Rhinolophus sinicus*	Kunming, Yunnan Province, China
5	Rs4231	KY417146	1	*Rhinolophus sinicus*	Kunming, Yunnan Province, China
6	Rs4084	KY417144	1	*Rhinolophus sinicus*	Kunming, Yunnan Province, China
7	RsSHC014	KC881005	1	*Rhinolophus sinicus*	Yunnan, China
8	Wuhan-CoV	MN908947	1/2	human	Wuhan, Hubei, China
9	As6526	KY417142	2	*Aselliscus stoliczkanus*	Kunming, Yunnan Province, China
10	Yunnan2011	JX993988	2	*Chaerephon plicata*	Yunnan, China
11	Shaanxi2011	JX993987	2	*Rhinolophus pusillus*	Shaanxi, China
12	279-2005	DQ648857	2	*Rhinolophus macrotis*	Hubei, China
13	Rs4237	KY417147	2	*Rhinolophus sinicus*	Kunming, Yunnan Province, China
14	Rs4081	KY417143	2	*Rhinolophus sinicus*	Kunming, Yunnan Province, China
15	Rp3	DQ071615	2	*Rhinolophus pearsoni*	Nanning, Guangxi, China
16	Rs4247	KY417148	2	*Rhinolophus sinicus*	Kunming, Yunnan Province, China
17	HKU3-8	GQ153543	2	*Rhinolophus sinicus*	Guangdong, China
18	HKU3-13	GQ153548	2	*Rhinolophus sinicus*	Guangdong, China
19	GX2013	KJ473815	2	*Rhinolophus sinicus*	Guangxi, China
20	Longquan-140	KF294457	2	*Rhinolophus monoceros*	Longquan, Zhejiang, China
21	YN2013	KJ473816	2	*Rhinolophus sinicus*	Yunnan, China
22	Rf4092	KY417145	2	*Rhinolophus ferrumequlum*	Kunming, Yunnan Province, China
23	ZXC21	MG772934	2	*Rhinolophus sinicus*	Zhoushan City, Zhejiang, China
24	ZC45	MG772933	2	*Rhinolophus sinicus*	Zhoushan City, Zhejiang, China
25	JL2012	KJ473811	2	*Rhinolophus ferrumequlum*	Jilin, China
26	HuB2013	KJ473814	2	*Rhinolophus sinicus*	Hubei, China
27	Rf1	DQ412042	2	*Rhinolophus ferrumequlum*	Yichang, Hubei, China
28	HeB2013	KJ473812	2	*Rhinolophus ferrumequlum*	Hebei, China
29	273-2005	DQ648856	2	*Rhinolophus ferrumequlum*	Hubei, China
30	BM48-31	NC014470	3	*Rhinolophus blasii*	Strandja Nature Park, Bulgaira

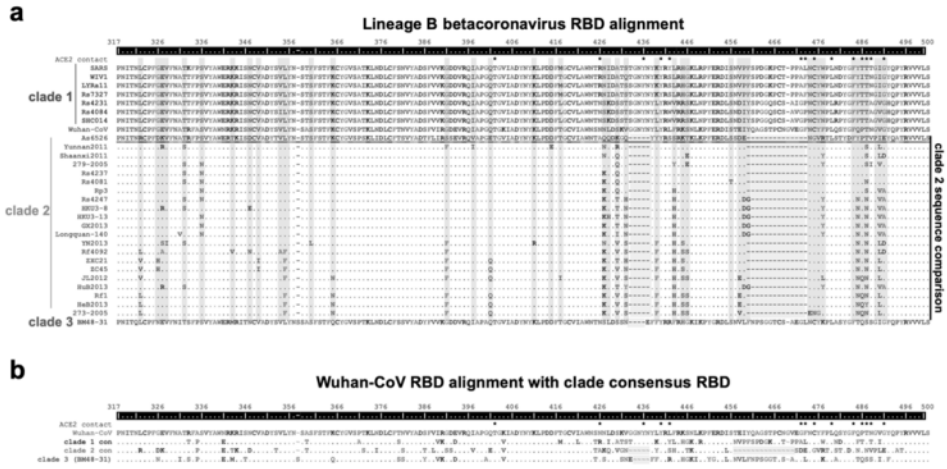
Supplemental figure 1: Lineage B RBD panel assembly and phylogenetic analysis

a, Coronavirus sequences were downloaded from NCBI and further parsed to 29 unique RBD variants. **b,** Virus isolate name, accession number, clade, host species and location of identification listed for the 29 unique lineage B RBDs used in this study. **c,** Cladograms for the spike RBD and coronavirus RNA-dependent RNA polymerase (nsp12).



Supplemental figure 2: additional cell lines tested without protease

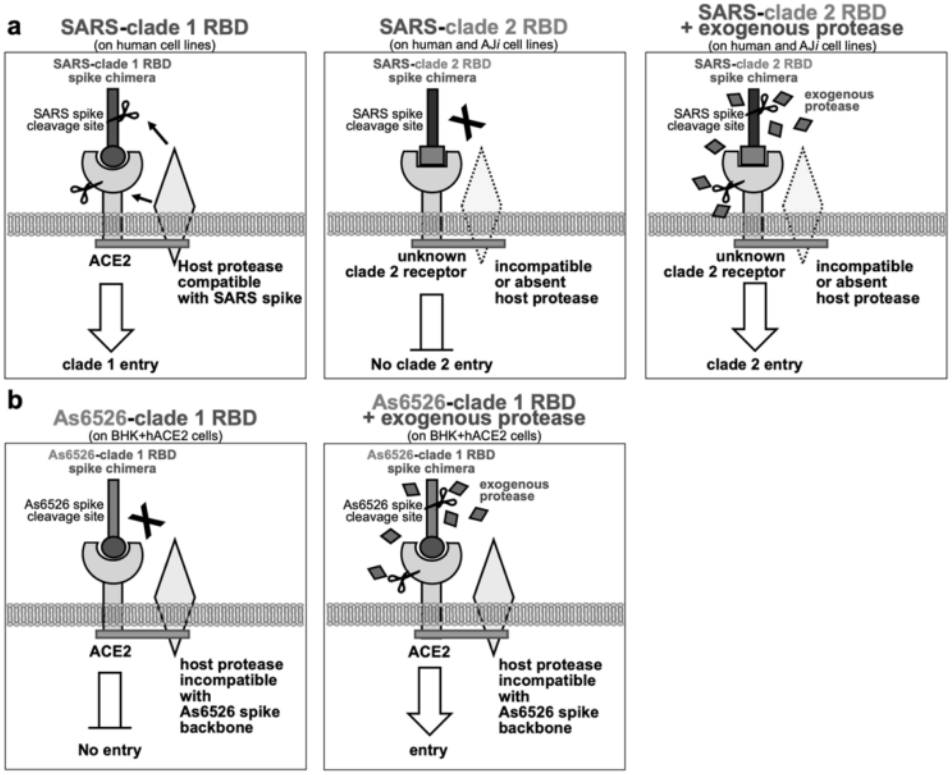
a, Additional human and cell lines or **b,** bat-derived cell lines derived from other species were infected with VSV-reporter particles pseudotyped with chimeric spikes and luciferase was measured a readout for cell entry. Trypsin was not used in these infections. **c,** All cell lines tested in this study supported entry and reporter expression of VSV-g pseudotyped particles. Shown are the data for 3 replicates.



Supplemental fig. 3: Lineage B panel RBD sequence features

a, Amino acid sequences corresponding to SARS-spike residues 317 through 500 were aligned with ClustalW. Contact points between SARS-spike and human ACE2 are

indicated with an (*). Clade 2 sequences are shown as compared to clade 2 As6526, with identical residues indicated with a (.) and sites that vary between clade 2 viruses highlighted in purple. Loop deletions are highlighted in orange. **b,** Amino acid alignment of 2019-nCoV RBD and consensus RBD sequences for clade 1 and 2 and BM48-31 (clade 3). Loop deletions are highlighted in orange.



Supplemental fig. 4: model of lineage B entry

a, SARS spike with a clade 1 RBD enters cells expressing ACE2 and a host protease capable of cleaving SARS spike (left panel). While the clade 2 RBD can bind an unknown host receptor, the SARS spike backbone is incompatible with the receptor-associated protease, resulting in a lack of cleavage and entry (middle panel). The addition of exogenous protease may overcome the lack of endogenous protease cleavage of spike, resulting in receptor-dependent entry. Alternatively, the addition of exogenous protease

may activate the receptor to facilitate entry. **b,** Replacing the RBD of As6526 spike with the clade 1 RBD allows for ACE2 interaction, but the As6526 spike backbone is incompatible with the ACE2-associated protease (left panel). Addition of exogenous protease overcomes protease incompatibility, allowing for ACE2-mediate entry (right panel).

From: Munster, Vincent (NIH/NIAID) [E]
Sent: Wed, 22 Jan 2020 12:00:52 -0700
To: Letko, Michael (NIH/NIAID) [F]
Subject: Re: Spike manuscript
Attachments: spike manuscript 1_22_20VM.docx

Here it is

Vincent Munster, PhD
Chief, Virus Ecology Section
Laboratory of Virology
Rocky Mountain Laboratories
NIAID/NIH

From: Heinrich Feldmann (b)(6)
Date: Wednesday, January 22, 2020 at 11:58 AM
To: Michael Letko (b)(6)
Cc: (b)(6)
Subject: RE: Spike manuscript

The manuscript looks great and quite convincing from the point of view of the approach – well done!

Adding the new coronavirus spike data is certainly helpful and enhances the importance and impact of this work. Having said that, you likely could use this better to sell the paper to a high-ranked journal. Here are some suggestions/thoughts:

- Title should likely be more result/conclusion-driven and not approach-driven. You may want to consider putting more emphasis on the new coronavirus
- Visually separate the work with the new coronavirus from the rest of the work by establishing new paragraphs in the different sections (Results & Discussion). At the moment it looks like an addition rather than a focus.
- You talk a lot about the “host protease” cleaving the spike protein. I should likely know but is that protease defined? If yes, why not introducing it? If no, it should be explained better.
- If the data on the new virus are supposed to be a driver for a high-ranked publication, it likely needs to be more obvious and prominent.

As usual, these are only suggestions for consideration. Please go ahead with pubclearance.

Good luck,
Heinz

From: Letko, Michael (NIH/NIAID) [F] (b)(6)
Sent: Wednesday, January 22, 2020 9:16 AM
To: Feldmann, Heinrich (NIH/NIAID) [E] (b)(6)
Cc: Munster, Vincent (NIH/NIAID) [E] (b)(6)
Subject: Spike manuscript

Dear Heinz,

We would like to submit our manuscript that shows 2019-nCoV receptor usage.

The manuscript is attached – later today I will add in the data for 2019-nCoV, but the results and discussion for this are already updated.

Let us know what you think!

Thank you,
-michael

--
Michael Letko, Ph.D
Postdoctoral IRTA
Dr. Vincent Munster Laboratory
Virus Ecology Unit, Laboratory of Virology
Rocky Mountain Laboratories
NIAID/NIH
903S 4th Street
Hamilton MT 59840
(b)(6)

Functional assessment of cell entry and receptor usage for lineage B β-coronaviruses, including 2019-nCoV

Michael Letko[#1], (b)(6) Vincent Munster[1]

1. Laboratory of Virology, Division of Intramural Research, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Hamilton, MT, 59840, USA

(b)(6)

[#] Corresponding authors:

Drs. Vincent Munster and Michael Letko

Rocky Mountain Laboratories

NIAID/NIH

903S 4th Street

Hamilton, MT 59840

Tel: (b)(6)

Email: (b)(6)

Email:

Abstract

Over the past 20 years, several coronaviruses have crossed the species barrier into humans, causing outbreaks of severe, and often fatal, respiratory illness. Since SARS-CoV was first identified in animal markets, global viromics projects have discovered thousands of coronavirus sequences in diverse animals and geographic regions. Unfortunately, there are few tools available to functionally test these novel viruses for their ability to infect humans, which has severely hampered efforts to predict the next zoonotic viral outbreak. Here we developed an approach to rapidly screen lineage B betacoronaviruses, such as SARS-CoV and the recent 2019-nCoV, for receptor usage and their ability to infect cell types from different species. We show that host protease processing during viral entry is a significant barrier for several lineage B viruses and that bypassing this barrier allows several lineage B viruses to enter human cells through an unknown receptor. We also demonstrate how different lineage B viruses can recombine to gain entry into human cells and confirm that human ACE2 is the receptor for the recently emerging 2019-nCoV. All together, our approach with lineage B viruses represents a step forward in predicting the next emerging zoonotic virus.

Introduction

Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV) first emerged in humans in 2003 after transmitting from animals in open air markets in China[1,2]. Shortly thereafter, several genetically related viruses were identified in Chinese Horseshoes bats (*Rhinolophus sinicus*)[3-7]. At the same time, improvements in next generation sequencing technology lead to a boom of virus discovery, uncovering thousands of novel virus sequences in wild animal populations around the world. While most of these viruses have never been found in humans, many are genetically similar to known human viruses within the betacoronaviruses (β-CoV) genus. The β-CoVs are further divided into four lineages: lineage B, which includes SARS-CoV and the recently-emerging 2019-nCoV, has approximately 200 published virus sequences whereas lineage C, which includes MERS-CoV, has over 500 viral sequences.

Every year, additional novel CoV sequences are discovered. However, there is a massive knowledge gap in the field as very little work is performed after the viral sequences are published. Therefore, it is unknown whether these novel viruses have the potential to emerge in human populations.

Current methods for studying novel β-CoVs are technically demanding. Viral isolation from field samples is rarely successful and reverse genetics recovery of recombinant virus is labor-intensive, and expensive as synthesis of a single genome can cost upwards of $15,000. These limitations are prohibitive to studying novel CoVs at the scale in which they are discovered.

Cell entry is an essential component of cross-species transmission, especially for the β-CoVs. All CoVs encode a glycoprotein, spike, which binds to the host-cell receptor and

mediates viral entry[8]. For β-CoVs, a single region of the spike protein called the receptor binding domain (RBD) mediates the interaction with the host cell receptor. After binding the receptor, a nearby host protease cleaves the spike, which releases the spike fusion peptide, facilitating virus entry[9-12]. Known host receptors for β-CoVs include angiotensin converting enzyme 2 (ACE2) for SARS-CoV and dipeptidyl peptidase 4 (DPP4) for MERS-CoV[13,14].

Structural studies of coronaviruses have shown that the spike RBD is capable of folding independently from the rest of the spike protein and contains all of the structural information for host receptor binding[15]. Additionally, a previous study showed that replacing the RBD of the lineage B bat virus, Rp3, allowed the virus to enter cells expressing human ACE2 (hACE2)[16]. We therefore developed a method to functionally test the RBDs from novel lineage B β-CoVs in place of the SARS-CoV spike RBD (figure 1). Synthesizing just the RBD of spike is much faster and cost-effective than conventional pseudotyping methods that rely on synthesis of the full ~4kb spike sequence for novel CoVs: a process that can take weeks and is cost-prohibitive for large panels of spike sequences. The short turnaround time for our approach allowed us to test the receptor usage of all published, unique RBD sequences in lineage B, and also rapidly confirm receptor usage of the 2019-nCoV spike, which emerged in China in January 2020 as our study was ongoing.

We show that lineage B RBDs divide into functionally distinct clades and that several previously-unappreciated viruses exhibit compatibility with an unknown receptor on human cells. We also show that these clades are capable of recombining to impart human

host-cell entry phenotypes, and that, beyond the RDB-receptor interaction, host protease processing is another species barrier encountered by lineage B β-CoVs during cell entry.

Methods

Cells

293T, A549, BHK, Caco-2, Huh-7.5, PK-15, and Vero cells were maintained in DMEM (Sigma) supplemented with 10% FBS, penicillin/streptomycin, and L-glutamine. RhiNi/40.1, AJ-primary, AJ*i*, HypNi, RaKSM-2.5, RhiLu, and RhiNi cells were maintained in DMEM/F12 (Gibco) supplemented with 12% FBS, penicillin/streptomycin, non-essential amino acids, sodium pyruvate and L-glutamine. AJ-primary cells were immortalized with a lentiviral vector expressing SV40 T-antigen following the manufacturers instructions (abm; #G203).

Plasmids

The spike coding sequences for SARS-CoV Urbani, As6526, and BM48-31 were codon optimized for human cells, appended with a 5' kozak expression sequence (GCCACC) and 3' tetra-glycine linker followed by nucleotides encoding a FLAG tag sequence (DYKDDDDK). For SARS-CoV spike, silent mutations were introduced around codons 308 and 519 to form KpnI and XhoI digest sites. For As6526 spike, silent mutations were introduced around codons 290 and 501 to form AflII and HindIII digest sites. For BM48-31 spike, silent mutations were introduced around codons 295 and 501 to form AflII and HindIII digest sites. These engineered spike sequences were synthesized and cloned into pcDNA3.1+ (GenScript).

Spike RBDs were first codon-optimized for human cells, appended with regions of the target spike backbone to facilitate Infusion cloning and synthesized as double stranded DNA fragments (IDT DNA). SARS-CoV, As6526 or BM48-31 engineered spike plasmids were digested with their corresponding restriction enzymes and gel purified. RBD inserts were resuspended in water and Infusion cloned into gel purified, digested spike backbone vectors (Takara).

Human ACE2 (Q9BYF1.2), DPP4 (XM_005246371.3), or APN (NP_001141.2) were synthesized and cloned into pcDNA3.1+ (GenScript). All DNA constructs were verified by Sanger sequencing (ACGT Inc.).

Receptor transfection

BHK cells were seeded in black 96-well plates and transfected the next day with 100ng plasmid DNA encoding human ACE2, DPP4, APN or empty vector, using polyethyleneimine (Polysciences). All downstream experiments were performed 24 hours post-transfection.

Pseudotype production

Pseudotypes were produced as previously described[17]. 293T cells were seeded onto 6-well plates pre-coated with poly-L-lysine (Sigma) and transfected the next day with 1200ng empty plasmid and 400ng of plasmid encoding coronavirus spike or GFP as a no pseudotype control. Twenty-four hours later, transfected cells were infected with VSVΔG particles pseudotyped with VSV-g as previously described[18]. After one hour of incubating at 37°C, cells were washed three times and incubated in 2mL DMEM supplemented with 2% FBS, penicillin/streptomycin, and L-glutamine for 48 hours. Supernatants were collected, centrifuged at 500xG for 5 minutes, aliquoted and stored at -80.

Luciferase-based cell entry assay

Target cells were seeded in black 96-well plates and inoculated, in triplicate, with equivalent volumes of pseudotype stocks. For trypsin experiments, pseudotype stocks were diluted 1:1 in DMEM without FBS, trypsin was added to a final concentration of 2500µg/mL and samples were incubated at 37°C for 15 minutes. Samples were then diluted again 1:1 in cold DMEM supplemented with 2% FBS and added to cells. Inoculated plates were centrifuged at 1200xG, 4°C, for 1 hour and incubated over night at 37°C. Approximately 18-20 hours post-infection, Bright-Glo luciferase reagent (Promega) was added to each well, 1:1, without removing culture media and luciferase was measured.

Relative entry was calculated by normalizing the relative light unit (RLU) for spike pseudotypes to the plate RLU average for the “no pseudotype control.”

Western blot

Producer cells (spike-transfected 293T) were lysed in 1%SDS, 150mM NaCl, 50mM Tris-HCl, 5mM EDTA and clarified by centrifugation at 14000xG for 20 minutes.

Pseudotyped particles were concentrated from producer cell lysates that were overlaid a 10% OptiPrep cushion in PBS (Sigma) and centrifuged at 20,000× g for 2 hours at 4 °C. Lysates and concentrated particles were analyzed for FLAG, GAPDH and/or VSV-m expression on 10% Bis-Tris PAGE gel (ThermoFisher).

Results

ACE2 entry is lineage B clade 1-specific

The receptor binding domain (RBD) of lineage B β-CoVs is a single, continuous domain that contains all structural information necessary to interact with the host receptor (figure 1a, b). We introduced silent mutations in the codon optimized coding sequence for SARS-CoV to facilitate replacing the SARS RBD with the RBD from other lineage B viruses (figure 1b). All lineage B sequences were downloaded from online repositories and parsed to 29 unique RBD sequences, representing all published variations of the lineage B RBD (supp. fig 1a, b). The panel of 29 RBDs phylogenetically cluster into 3 clades, as previously described [5], but these RBD clades were not apparent in phylogenetic analysis of other viral sequences, such as the RNA-dependent RNA polymerase (supp. fig 1c). All 29 RBDs were codon optimized, synthesized and cloned in place of the SARS RBD, effectively generating chimeric spike expression constructs. We then generated VSV-luciferase reporter particles pseudotyped with the chimeric spikes (figure 1c). All constructs exhibited similar levels of expression in producer cells and incorporation into VSV pseudotypes, except the chimera with BM48-31 which displayed somewhat reduced expression compared to WT SARS spike (figure 1d). We then infected BHK cells expressing the receptor for SARS-CoV or empty vector (figure 1e) and observed only clade 1, which includes SARS-CoV, WIV1 and SHC014, could enter cells transfected with human ACE2 (figure 1e).

Protease enhances clade 2 entry

After binding the host receptor, host-cell protease cleaves spike, releasing the fusion peptide and allowing for host cell entry [19]. Previous studies have shown that absence of the host protease or incompatibility between the host protease and viral spike can block viral entry [20-23]. To circumvent host-cell protease incompatibility or absence, we treated our lineage B pseudotype panel and infected a wide variety of cell types from different host species (figure 2, supp. fig. 2). In the absence of exogenous protease, only clade 1 infected cells from African green monkey kidney, human gastrointestinal tract, human liver, and porcine kidney, in agreement with previous studies (figure 2; supp. fig.

2a, b). Surprisingly, exogenous protease enhanced entry of a subset of clade 2 spike chimeras in nonhuman primate, bat and human cells (figure 2). Importantly, VSV-g pseudotyped particles were able to produce luciferase signal in all cell lines tested in this study (supp. fig. 2c).

Clade 2 entry is receptor-dependent

We next tested human variants of known β-CoV receptors for their ability to mediate cell entry of clade 2 and 3 spike chimeras. We also tested human aminopeptidase N (APN), a receptor for alphacoronaviruses, which have been shown to utilize either human ACE2 or human APN for cell entry (figure 3a). Protease treatment only enhanced entry of clade 1 RBDs on cells expressing human ACE2, but not human DPP4 or APN. No entry was observed with clade 2 or 3 spikes, regardless of receptor or protease addition. Human dipeptidyl peptidase IV (DPP4), the receptor for the lineage C β-CoVs, MERS-CoV, only mediated entry of MERS-CoV (figure 2b, middle panels). Importantly, in the absence of receptor, no entry was observed for any of the pseudotypes, suggesting that protease-mediated entry is receptor-dependent (figure 2b, right panels). While our study was ongoing, a novel lineage B virus tentatively named 2019-nCoV was identified as the cause of a pneumonia outbreak in Hubei, China. Once the sequence was publicly available, we synthesized, cloned and tested the RBD from 2019-nCoV in our assay with human variants of known coronavirus receptors. The chimeric SARS-2019-nCoV spike protein expressed and was incorporated into particles similarly to other clade 1 chimeric spikes (Figure 3c). The 2019-nCoV RBD was capable of entering cells expressing human ACE2, but not any of the other receptors tested (Figure 3d).

Clade determinants for ACE2 usage

Consensus sequences of the three lineage B clades showed several key differences between these groups. Only clade 1 RBDs contain all 14 residues that have been shown through crystallography, to interact with human ACE2 (figure 4a; supp. fig. 3). The majority of these residues are absent from clades 2 and 3, which contain additional deletions in surface exposed loops that cluster at the interface with ACE2

(figure 4 a, b). We generated a series of clade consensus RBD variants to determine the minimum number of mutations needed to impart ACE2 function on clade 2 and 3 RBDs (figure 4c). Introducing the two loop deletions from clade 1 in clade 2 results in a reduced spike expression, impaired pseudotype incorporation and loss of cell entry (figure 4c, d). Restoring these loops in clade 2 and 3 from the loops found in clade 1 did not enhance entry with ACE2 (figure 4c; 2→1 and 3→1 version 1). Introducing all 14 ACE2 contact points in clade 2 or 3 also failed to restore ACE2 entry (figure 4c; 2→1 and 3→1 version 2). Only replacing all 14 contact points and the surrounding amino acids (also known as the receptor binding motif, RBM) lead to increased ACE2 entry with clade 2 and 3 RBDs (figure 4c; 2→1 and 3→1 version 3). Taken together, these results show that the entire RBM from clade 1 is needed for ACE2 entry.

Full-spike and RBD chimeras are similar

We next synthesized full-length clade 2 and 3 spikes to compare to our RBD chimeras. We selected the clade 2 spike, As6526, because it consistently gave strong entry signal in human cells following protease-treatment (figure 2b) as well as BM48-31, the only clade 3 spike in our panel. As we did for SARS-CoV spike, clade 2 and 3 spikes were codon optimized, FLAG-tagged and silent mutations were introduced to facilitate replacing their RBD with the consensus RBD from clade 1 (figure 5a). All chimeric constructs expressed similarly, with the exception for the SARS-BM48-31 RBD chimera, which exhibited reduced expression and incorporation (figure 5b). Protease treatment enhanced entry of both the As6526 clade 2 RBD chimera and full-length spike entry into Huh cells (figure 5c). Protease treatment had no effect on either the BM48-31 clade 3 chimera or full-length spike (figure 5c). Taken together, these findings show that SARS-lineage B RBD chimeras reflect the entry phenotype of full-length lineage B spikes.

Finally, we tested if receptor-binding and protease processing are coupled. We replaced the RBD of full-length clade 2 and 3 spike with the consensus RBD from clade 1 and tested pseudotypes on cells expressing the clade 1 receptor. The clade 1 consensus RBD efficiently facilitated entry of both As6526 and BM48-31 spike only following protease treatment. These findings show that even though BHK-hACE2 cells support full-length clade 1 spike entry, just having the RBD from a clade 1 virus is

insufficient to mediate entry. As seen in our previous experiments, protease treatment did not enhance pseudotype entry in the absence of host receptor (figure 5).

Discussion

Despite significant advances in next generation sequencing technologies, which have facilitated the discovery of thousands of novel animal-derived viruses, tools for downstream functional assessment of these novel sequences are lacking. To gain traction on this ever-growing problem, we took a reductionist approach to coronavirus entry and developed a scalable method for testing only the minimal region of the virus essential for interacting with the host receptor (Figure 1, figure s1a). Because most of these viruses have never been isolated, we resorted to synthetic biology and molecular engineering to reduce the burden of gene synthesis to just a small fragment. Thus, the cost and synthesis production time for testing several spikes for entry in our system is dramatically reduced (supplementary figure 1d). In theory, **this approach to functional viromics should be applicable to a variety of virus-host proteins and interactions.**

Coronavirus entry is a multi-step process involving multiple, distinct domains in spike that mediate virus attachment to the cell surface, receptor engagement, protease processing and membrane fusion[8]. While the RBD:receptor interaction is most studied in this process, recent studies have highlighted the major role host protease processing plays as a species barrier[21,24-26]. Lineage C coronaviruses include MERS-CoV as well as distantly related viruses such as HKU4, HKU5 and PDF-2180 [27,28]. Studies have shown that HKU4 can bind human DPP4 but requires addition of exogenous trypsin to facilitate cell entry and that HKU5 and PDF-2180 spikes can enter human cells through an unidentified receptor with protease treatment[21,25]. Analogous to these earlier studies of lineage C CoVs, we observed protease-enhanced entry of lineage B CoVs (figure 2, 3, 5). While it has been shown that host proteases cleave spike, allowing for downstream membrane fusion, other evidence suggests that protease may act on the receptor as well to activate it[29]. In this previous study, addition of protease during the course of SARS-

CoV infection facilitated entry in cells with low-expression of ACE2 that is normally insufficient to support virus entry[29]. Indeed, we saw evidence of residual trypsin activity on the cells after infection in our studies as the cell monolayer was loose compared to the untreated condition. Similarly, Menachery et al. also observed **cell rounding during their trypsin** infections[25]. Therefore, further studies are needed to assess where trypsin is enhancing entry of coronaviruses: at the level of spike, the receptor, or both.

In the absence of exogenous protease, only clade 1 RBDs entered nonhuman primate, human and porcine cell lines (figure 2a, b, s2a, b). These findings are in strong agreement with previous studies that have either isolated virus (WIV1) or rescued recombinant chimeric viruses (SHC014, Rs4231, Rs7237)[5,7,30]. However, with trypsin, a subset of genetically-similar clade 2 RBDs gained entry in these cells, suggesting their barrier is at the level of protease processing (figure 2a, b). The other spike from clade 2 and 3 did not enter the cells we tested, regardless of protease addition, suggesting an absent or incompatible receptor. Surprisingly, the protease-dependent entry phenotype was consistent in the reverse spike chimeras in which we replaced the RBD in clade 2 or 3 spike with a clade 1 RBD (figure 5d), suggesting that either the protease site between S1/S2 is not compatible with the chimeric spike backbone or the protease is not expressed in these cells (figure s4). Because clade 1 spikes enter cells expressing human ACE2 without addition of protease, our data showing protease addition is needed for entry of clade 2-clade 1 spike chimera suggests **the spike protease site is adapted to the protease environment of the receptor-bound RBD** (figure s4).

None of the spike pseudotypes efficiently entered rhinolophus cells, which has been observed in previous studies using these cells[31,32] (figure 2c). Surprisingly, AJi cells were selectively permissive for only clade 2 entry following protease treatment, which suggests that **clade 2 RBDs interact with a receptor that is distinct from clade 1** (figure 2c).

Our results show that, despite all being classified as the same virus species, most lineage B β-CoVs do not use currently known coronavirus receptors (figure 1e, 3a, b). Critically, we did not observe any pseudotype entry in the presence of protease and absence of receptor, suggesting that lineage B cell entry is still receptor-dependent following protease treatment (figure 3b). While our study was ongoing, a novel lineage B β-CoV was identified as the etiological agent behind an outbreak of pneumonia in Wuhan,

Hubei, China (2019-nCoV). The **RBD for 2019-nCoV** has residues and motifs found in all 3 clades but forms a distinct clade, so we tested it for receptor usage and observed entry only with human ACE2 but not other known coronavirus receptors (figure 3d). Interestingly, within the backbone of SARS-CoV spike, cell entry of 2019-nCoV was similar to the other clade 1 spikes tested, including SARS-CoV. These finding suggests 2019-nCoV is capable of using human ACE2 as efficiently as SARS-CoV, which may help explain the human-to-human transmissibility of this virus. More studies are needed with the full spike sequence and, ideally, a viral isolate.

The receptor binding motif (RBM) is a small region in the C-terminal half of the RBD and contains all the residues that interface with the host receptor (figure 3a)[15]. The 14 contact points in the co-structure of the SARS-RBD bound to human ACE2 are largely absent from clade 2 and 3 RBDs, which also contain deletions compared to clade 1 RBMs (figure 4a, b, s3a). Simply mutating clade 2 and 3 to have the 14 contact points was insufficient to impart human ACE2 usage (figure 4c). This is likely because the non-contact residues in the RBM play a supportive and structural role for these contact points, and indeed, these non-contact residues are different between the clades (figure s3).

In contrast to changing individual amino acids, our chimeric RBD constructs show that clade 2 and 3 RBD containing the clade 1 RBM are compatible with human ACE2. Coronaviruses frequently undergo recombination, gaining large swaths of genetic material at once [33,34]. Taken together with our data, it is possible that **recombination with a clade 1 virus will impart compatibility with human ACE2**. Interestingly, the 2019-nCoV RBD forms a clade that is distinct from the other 3 clades (figure s1c). However, the 2019-nCoV RBD contains most of the contact points with human ACE2 that are found in clade 1 as well as some amino acid variations that are unique to clade 2 and 3 (figure s3b). Taken together with our receptor assay results, it **may be possible that 2019-nCoV arose from recombination between clade 1 and the other clades**.

As we saw with the SARS-As6526 RBD (clade 2) spike chimera, full length As6526 spike entered cells following protease treatment, but BM48-31 (clade 3) spike did not (figure 2, 5c). These data show that the chimeric spikes generally reproduce the entry phenotypes of full-length spikes. Notably, the full length As6526 spike did not enter cells

as efficiently as the SARS-As6526 chimera, suggesting that **other human-cell adaptations are likely needed in As6526 spike**.

The capacity to predict the next potential viral outbreak has been severely hindered by a lack of functional data for novel animal virus sequences. Here, we have developed a rapid and cost-effective approach to functionally test large groups of related viruses for zoonotic potential. We found that several other lineage B coronaviruses are capable of entering human cells through an unknown receptor and that lineage B spike proteins can recombine to gain entry with a known host-receptor. Taken together with the latest outbreak of 2019-nCoV in humans, **these findings underscore the importance of continued surveillance of coronaviruses at the sequence and functional levels in order to better prepare for the next emerging virus.**

Acknowledgements

We would like to acknowledge the National Institute for Viral Disease Control and Prevention, China CDC; Wuhan Institute of Virology, Chinese Academy of Sciences; Institute of Pathogen Biology, Chinese Academy of Medical Sciences & Peking Union Medical College; and Wuhan Jinyintan Hospital for sharing essential clinical data and the viral genome with the research community. This work was supported by the Intramural Research Program of the National Institute of Allergy and Infectious Diseases.

Declarations of Interests

The authors declare no competing interests.

References

1 Wang, M. *et al.* SARS-CoV infection in a restaurant from palm civet. *Emerg Infect Dis* **11**, 1860-1865, doi:10.3201/eid1112.041293 (2005).

2 Kuiken, T. *et al.* Newly discovered coronavirus as the primary cause of severe acute respiratory syndrome. *Lancet* **362**, 263-270, doi:10.1016/S0140-6736(03)13967-0 (2003).

3 Li, W. *et al.* Bats are natural reservoirs of SARS-like coronaviruses. *Science* **310**, 676-679, doi:10.1126/science.1118391 (2005).

4 Ge, X. Y. *et al.* Isolation and characterization of a bat SARS-like coronavirus that uses the ACE2 receptor. *Nature* **503**, 535-538, doi:10.1038/nature12711 (2013).

5 Hu, B. *et al.* Discovery of a rich gene pool of bat SARS-related coronaviruses provides new insights into the origin of SARS coronavirus. *PLoS Pathog* **13**, e1006698, doi:10.1371/journal.ppat.1006698 (2017).

6 Lau, S. K. *et al.* Severe acute respiratory syndrome coronavirus-like virus in Chinese horseshoe bats. *Proc Natl Acad Sci U S A* **102**, 14040-14045, doi:10.1073/pnas.0506735102 (2005).

7 Menachery, V. D. *et al.* A SARS-like cluster of circulating bat coronaviruses shows potential for human emergence. *Nat Med* **21**, 1508-1513, doi:10.1038/nm.3985 (2015).

8 Li, F. Structure, Function, and Evolution of Coronavirus Spike Proteins. *Annu Rev Virol* **3**, 237-261, doi:10.1146/annurev-virology-110615-042301 (2016).

9 Simmons, G., Zmora, P., Gierer, S., Heurich, A. & Pohlmann, S. Proteolytic activation of the SARS-coronavirus spike protein: cutting enzymes at the cutting edge of antiviral research. *Antiviral Res* **100**, 605-614, doi:10.1016/j.antiviral.2013.09.028 (2013).

10 Matsuyama, S. *et al.* Efficient activation of the severe acute respiratory syndrome coronavirus spike protein by the transmembrane protease TMPRSS2. *J Virol* **84**, 12658-12664, doi:10.1128/JVI.01542-10 (2010).

11 Bertram, S. *et al.* Cleavage and activation of the severe acute respiratory syndrome coronavirus spike protein by human airway trypsin-like protease. *J Virol* **85**, 13363-13372, doi:10.1128/JVI.05300-11 (2011).

12 Belouzard, S., Chu, V. C. & Whittaker, G. R. Activation of the SARS coronavirus spike protein via sequential proteolytic cleavage at two distinct sites. *Proc Natl Acad Sci U S A* **106**, 5871-5876, doi:10.1073/pnas.0809524106 (2009).

13 Raj, V. S. *et al.* Dipeptidyl peptidase 4 is a functional receptor for the emerging human coronavirus-EMC. *Nature* **495**, 251-254, doi:10.1038/nature12005 (2013).

14 Li, W. *et al.* Angiotensin-converting enzyme 2 is a functional receptor for the SARS coronavirus. *Nature* **426**, 450-454, doi:10.1038/nature02145 (2003).

15 Li, F., Li, W., Farzan, M. & Harrison, S. C. Structure of SARS coronavirus spike receptor-binding domain complexed with receptor. *Science* **309**, 1864-1868, doi:10.1126/science.1116480 (2005).
16 Becker, M. M. *et al.* Synthetic recombinant bat SARS-like coronavirus is infectious in cultured cells and in mice. *Proc Natl Acad Sci U S A* **105**, 19944-19949, doi:10.1073/pnas.0808116105 (2008).
17 Letko, M. *et al.* Adaptive Evolution of MERS-CoV to Species Variation in DPP4. *Cell Rep* **24**, 1730-1737, doi:10.1016/j.celrep.2018.07.045 (2018).
18 Takada, A. *et al.* A system for functional analysis of Ebola virus glycoprotein. *Proc Natl Acad Sci U S A* **94**, 14764-14769, doi:10.1073/pnas.94.26.14764 (1997).
19 Millet, J. K. & Whittaker, G. R. Host cell proteases: Critical determinants of coronavirus tropism and pathogenesis. *Virus Res* **202**, 120-134, doi:10.1016/j.virusres.2014.11.021 (2015).
20 Menachery, V. D. *et al.* Trypsin treatment unlocks barrier for zoonotic coronaviruses infection. *bioRxiv* (2019).
21 Yang, Y. *et al.* Receptor usage and cell entry of bat coronavirus HKU4 provide insight into bat-to-human transmission of MERS coronavirus. *Proc Natl Acad Sci U S A* **111**, 12516-12521, doi:10.1073/pnas.1405889111 (2014).
22 Matsuyama, S., Ujike, M., Morikawa, S., Tashiro, M. & Taguchi, F. Protease-mediated enhancement of severe acute respiratory syndrome coronavirus infection. *Proc Natl Acad Sci U S A* **102**, 12543-12547, doi:10.1073/pnas.0503203102 (2005).
23 Kam, Y. W. *et al.* Cleavage of the SARS coronavirus spike glycoprotein by airway proteases enhances virus entry into human bronchial epithelial cells in vitro. *PLoS One* **4**, e7870, doi:10.1371/journal.pone.0007870 (2009).
24 Yang, Y. *et al.* Two Mutations Were Critical for Bat-to-Human Transmission of Middle East Respiratory Syndrome Coronavirus. *J Virol* **89**, 9119-9123, doi:10.1128/JVI.01279-15 (2015).
25 Menachery, V. D. *et al.* Trypsin treatment unlocks barrier for zoonotic bat coronaviruses infection. *J Virol*, doi:10.1128/JVI.01774-19 (2019).
26 Beniac, D. R., deVarennes, S. L., Andonov, A., He, R. & Booth, T. F. Conformational reorganization of the SARS coronavirus spike following receptor binding: implications for membrane fusion. *PLoS One* **2**, e1082, doi:10.1371/journal.pone.0001082 (2007).
27 Woo, P. C. *et al.* Molecular diversity of coronaviruses in bats. *Virology* **351**, 180-187, doi:10.1016/j.virol.2006.02.041 (2006).
28 Anthony, S. J. *et al.* Further Evidence for Bats as the Evolutionary Source of Middle East Respiratory Syndrome Coronavirus. *mBio* **8**, doi:10.1128/mBio.00373-17 (2017).
29 Shulla, A. *et al.* A transmembrane serine protease is linked to the severe acute respiratory syndrome coronavirus receptor and activates virus entry. *J Virol* **85**, 873-882, doi:10.1128/JVI.02062-10 (2011).
30 Adney, D. R. *et al.* Replication and shedding of MERS-CoV in upper respiratory tract of inoculated dromedary camels. *Emerg Infect Dis* **20**, 1999-2005, doi:10.3201/eid2012.141280 (2014).

31 Muth, D. *et al.* Attenuation of replication by a 29 nucleotide deletion in SARS-coronavirus acquired during the early stages of human-to-human transmission. *Sci Rep* **8**, 15177, doi:10.1038/s41598-018-33487-8 (2018).

32 Hoffmann, M. *et al.* Differential sensitivity of bat cells to infection by enveloped RNA viruses: coronaviruses, paramyxoviruses, filoviruses, and influenza viruses. *PLoS One* **8**, e72942, doi:10.1371/journal.pone.0072942 (2013).

33 Liao, C. L. & Lai, M. M. RNA recombination in a coronavirus: recombination between viral genomic RNA and transfected RNA fragments. *J Virol* **66**, 6117-6124 (1992).

34 Graham, R. L. & Baric, R. S. Recombination, reservoirs, and the modular spike: mechanisms of coronavirus cross-species transmission. *J Virol* **84**, 3134-3146, doi:10.1128/JVI.01394-09 (2010).

Figures

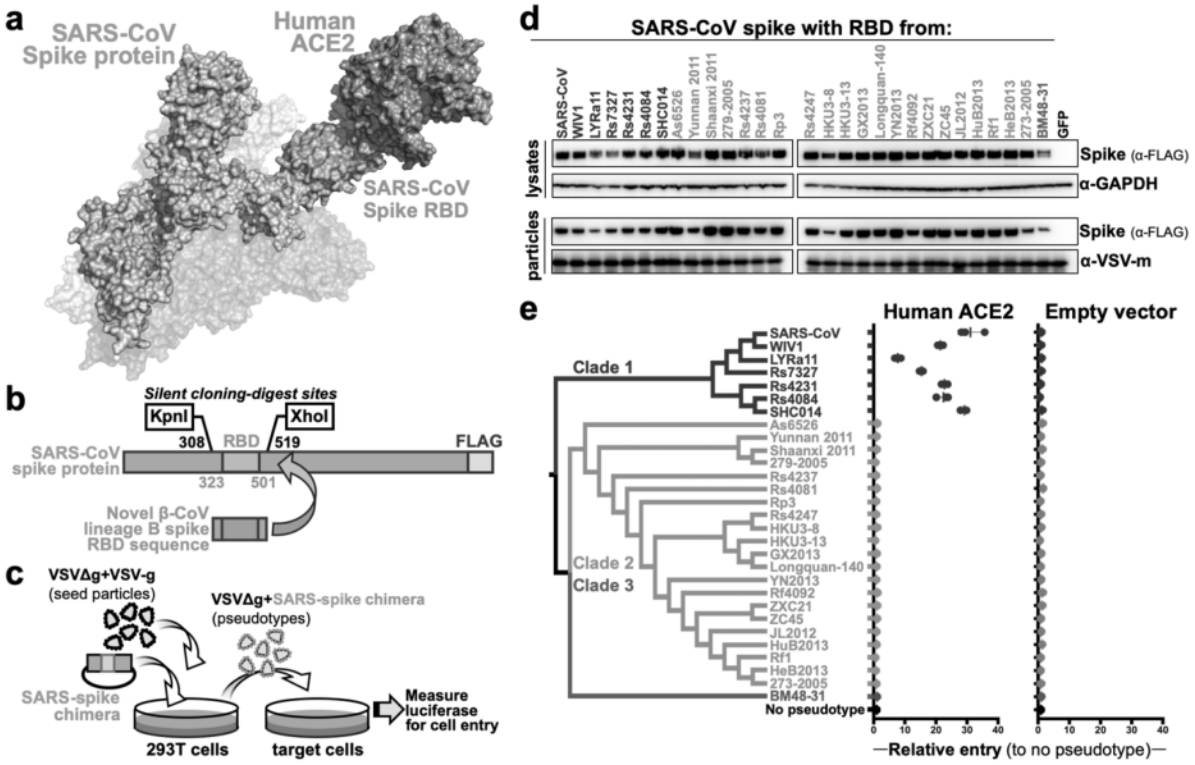


Figure 1: β-Coronavirus lineage B entry with human ACE2 is clade-specific

a, β-Coronaviruses, including SARS-CoV, interact with the host cell receptor via the Receptor Binding Domain (RBD) in spike (PDB: 5X5B, 2AJF) . **b,** SARS-CoV spike was

engineered with silent mutations to facilitate cloning novel RBD sequences in place of the SARS spike RBD. SARS spike amino acid numbers are indicated for silent cloning sites and the RBD in grey and orange, respectively. **c,** outline of experimental workflow. **d,** Westernblot of producer cell lysates and concentrated reporter particles. **e,** BHK cells were transfected with either human ACE2 or empty vector, subsequently infected with VSV-reporter particles pseudotyped with chimeric spikes, luciferase was measured and normalized to no pseudotype as a readout for cell entry. Shown are the data for 3 replicates.

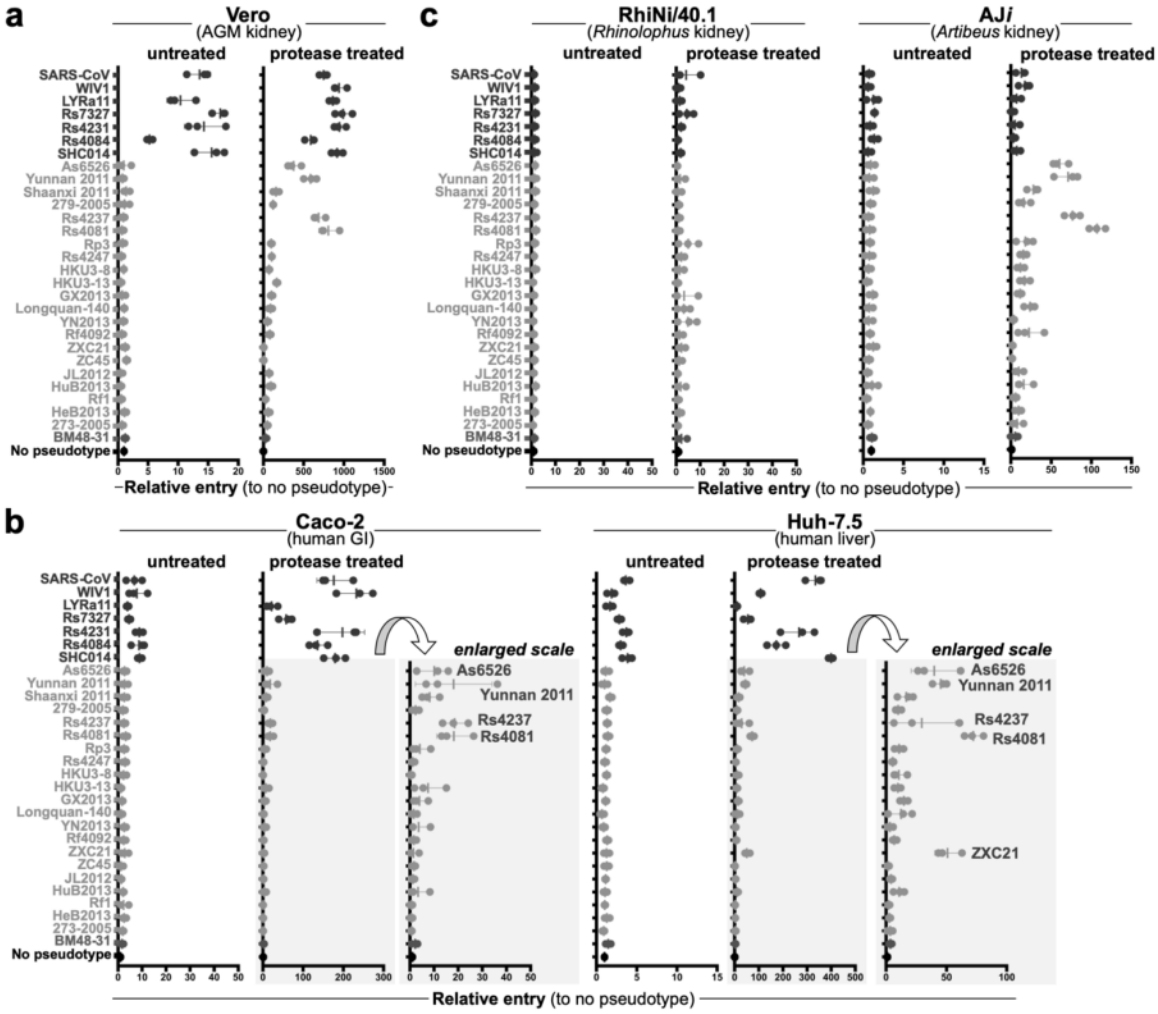


Figure 2: Trypsin enhances lineage B entry in various cell lines

a, Primate cells, **b,** human cells or **c,** bat cells were infected with VSV-particles pseudotyped with the lineage B chimeric spike panel. Pseudotypes were either left untreated or incubated with trypsin before addition to the cells. Luciferase was measured and normalized to particles produced without pseudotype. Shown are the data for 3 replicates.

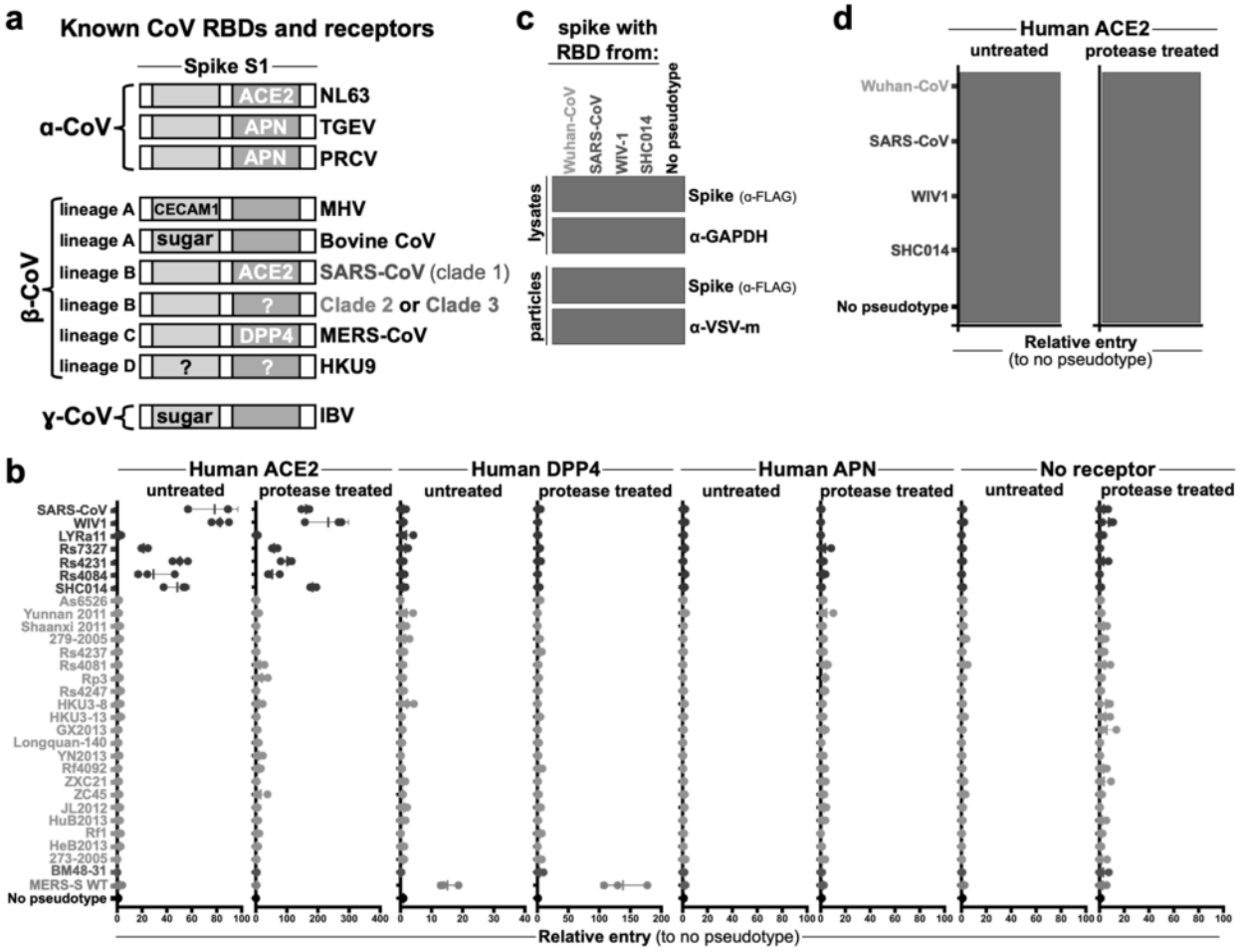


Figure 3: Lineage B entry into cells with known CoV receptors

a, Schematic of known coronavirus RBDs and their receptors. **b,** Pseudotyped particles were either left untreated and treated with trypsin and subsequently used to infect BHK cells transfected with the coronavirus receptor indicated. Shown are data from 3 replicates. **c,** Expression and pseudotype incorporation of SARS-S-2019-nCoV RBD chimeras. **d,** Pseudotypes were trypsin treated and used to infect cells expressing hACE2 or empty vector.

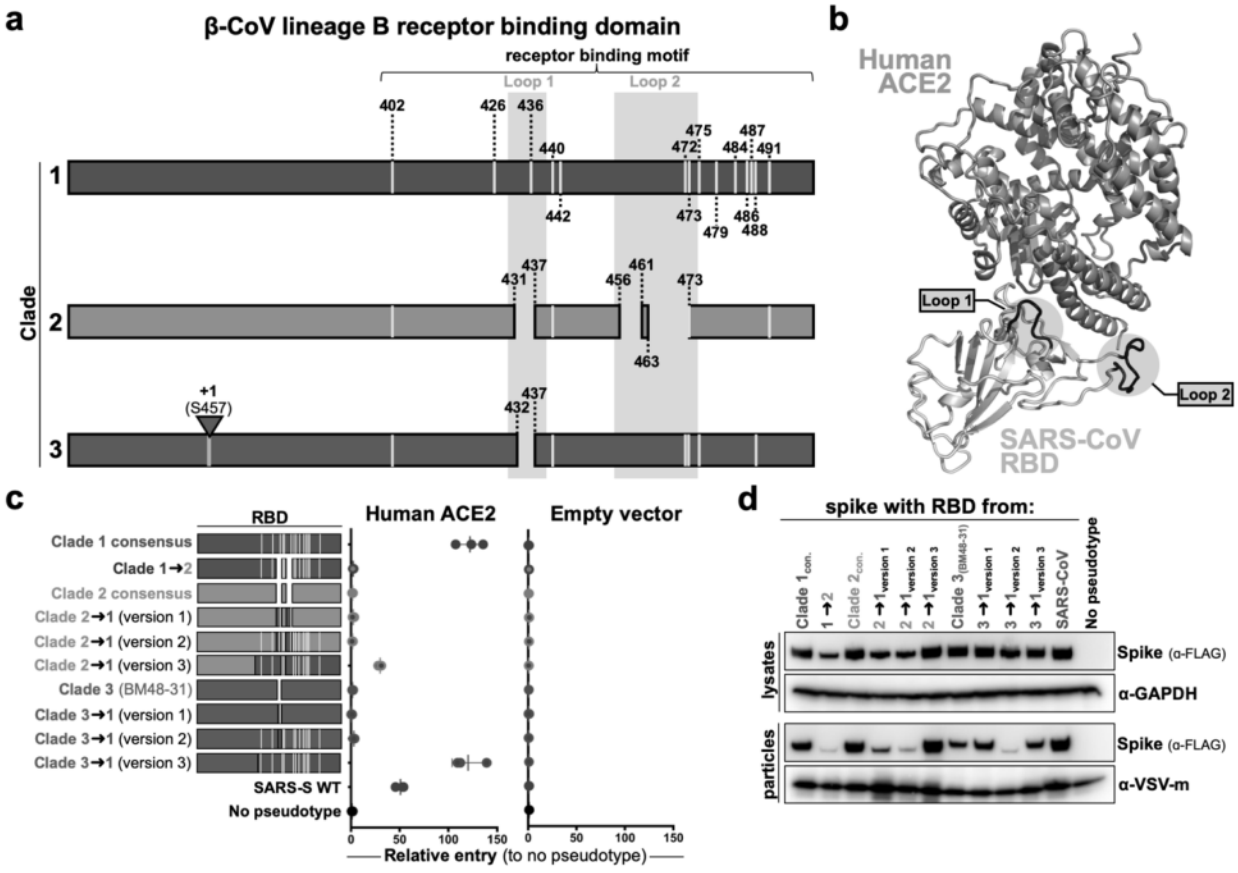


Figure 4: Lineage B clade-specific determinants for human ACE2 usage

a, Schematic overview of clade 1, 2 and 3. Highlighted in yellow are the 14 residues that contact human ACE2. Deletions in loops 1 and 2 are indicated for clades 2 and 3. **b,** Structure of human ACE2 and the SARS-S RBD (PDB: 2AJF), with loops highlighted in gray. **c,** VSV pseudotypes were generated with the indicated RBD and used to infect BHKs transfected with either human ACE2 or empty vector. Shown are data for 3 replicates. **d,** Westernblot of producer cell lysates and concentrated pseudotyped particles.

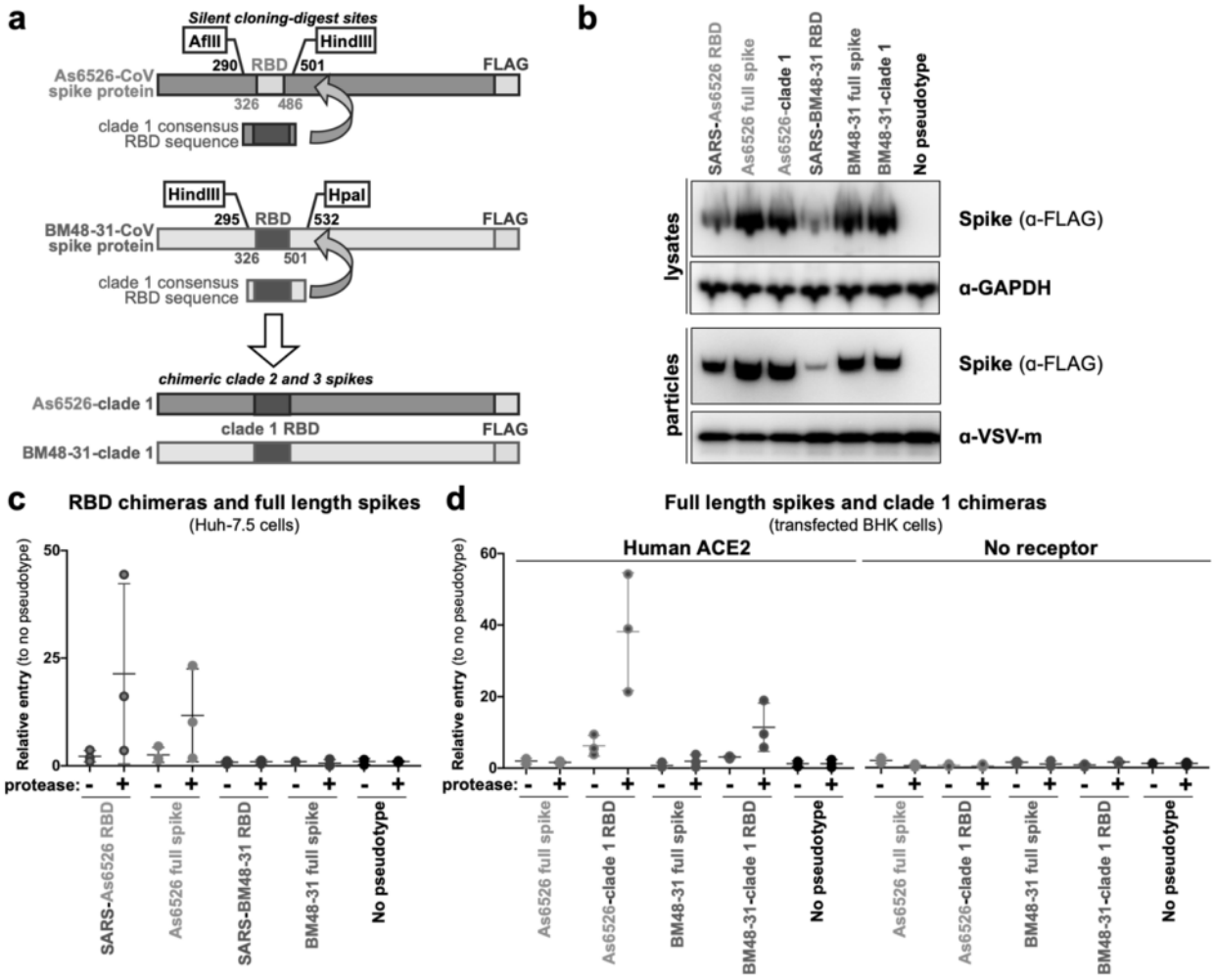
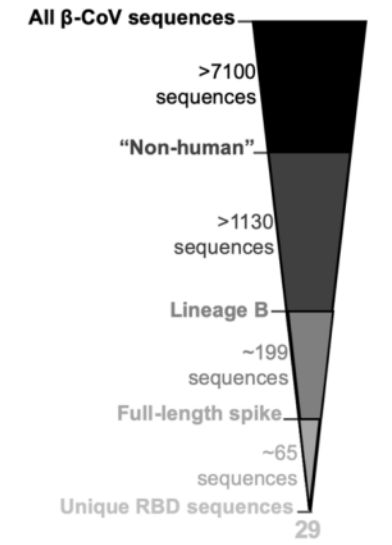


Figure 5: Comparison of chimeric and full length clade 2 and 3 spikes

a, Full length spike sequences from As6526 (clade 2) and BM48-31 (clade 3) were codon optimized, FLAG tagged and synthesized. Silent mutations flanking the RBD facilitated replacing the native RBD with the clade 1 consensus RBD. **b,** Westernblot of producer cell lysates and concentrated pseudotypes particles. **c,** Pseudotypes with indicated spike constructs were left untreated or treated with trypsin and subsequently used to infect Huh-7.5 cells. Shown are data for 3 replicates. **d,** Pseudotypes with indicated spike constructs were left untreated or treated with trypsin and subsequently used to infect BHK cells transfected with human ACE2. Shown are data for 3 replicates.

SUPPLEMENTAL INFORMATION

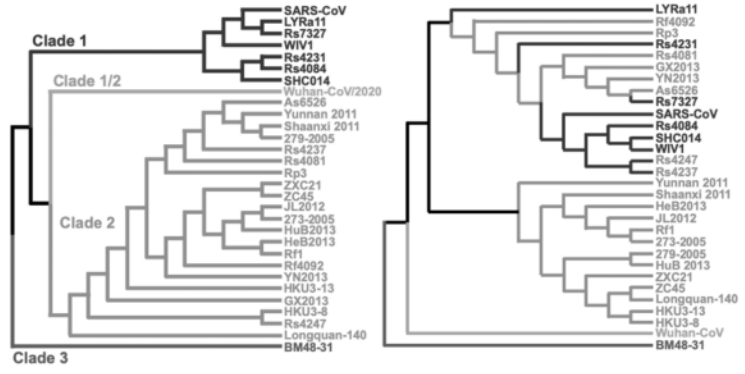
a



b

	Virus	Accession	Clade	Host species	Location
1	SARS Urbani	AY278741	1	human	Guangdong, China (origin of outbreak)
2	WIV1	KF367457	1	*Rhinolophus sinicus*	Yunnan, China
3	LYRa11	KF569996	1	*Rhinolophus affinis*	Baoshan, Yunnan, China
4	Rs7327	KY417151	1	*Rhinolophus sinicus*	Kunming, Yunnan Province, China
5	Rs4231	KY417146	1	*Rhinolophus sinicus*	Kunming, Yunnan Province, China
6	Rs4084	KY417144	1	*Rhinolophus sinicus*	Kunming, Yunnan Province, China
7	RsSHC014	KC881005	1	*Rhinolophus sinicus*	Yunnan, China
8	Wuhan-CoV	MN908947	1/2	human	Wuhan, Hubei, China
9	As6526	KY417142	2	*Aselliscus stoliczkanus*	Kunming, Yunnan Province, China
10	Yunnan2011	JX993988	2	*Chaerephon plicata*	Yunnan, China
11	Shaanxi2011	JX993987	2	*Rhinolophus pusillus*	Shaanxi, China
12	279-2005	DQ648857	2	*Rhinolophus macrotis*	Hubei, China
13	Rs4237	KY417147	2	*Rhinolophus sinicus*	Kunming, Yunnan Province, China
14	Rs4081	KY417143	2	*Rhinolophus sinicus*	Kunming, Yunnan Province, China
15	Rp3	DQ071615	2	*Rhinolophus pearsoni*	Nanning, Guangxi, China
16	Rs4247	KY417148	2	*Rhinolophus sinicus*	Kunming, Yunnan Province, China
17	HKU3-8	GQ153543	2	*Rhinolophus sinicus*	Guangdong, China
18	HKU3-13	GQ153548	2	*Rhinolophus sinicus*	Guangdong, China
19	GX2013	KJ473815	2	*Rhinolophus sinicus*	Guangxi, China
20	Longquan-140	KF294457	2	*Rhinolophus monoceros*	Longquan, Zhejiang, China
21	YN2013	KJ473816	2	*Rhinolophus sinicus*	Yunnan, China
22	Rf4092	KY417145	2	*Rhinolophus ferrumequium*	Kunming, Yunnan Province, China
23	ZXC21	MG772934	2	*Rhinolophus sinicus*	Zhoushan City, Zhejiang, China
24	ZC45	MG772933	2	*Rhinolophus sinicus*	Zhoushan City, Zhejiang, China
25	JL2012	KJ473811	2	*Rhinolophus ferrumequium*	Jilin, China
26	HuB2013	KJ473814	2	*Rhinolophus sinicus*	Hubei, China
27	Rf1	DQ412042	2	*Rhinolophus ferrumequium*	Yichang, Hubei, China
28	HeB2013	KJ473812	2	*Rhinolophus ferrumequium*	Hebei, China
29	273-2005	DQ648856	2	*Rhinolophus ferrumequium*	Hubei, China
30	BM48-31	NC014470	3	*Rhinolophus blasii*	Strandja Nature Park, Bulgaira

c

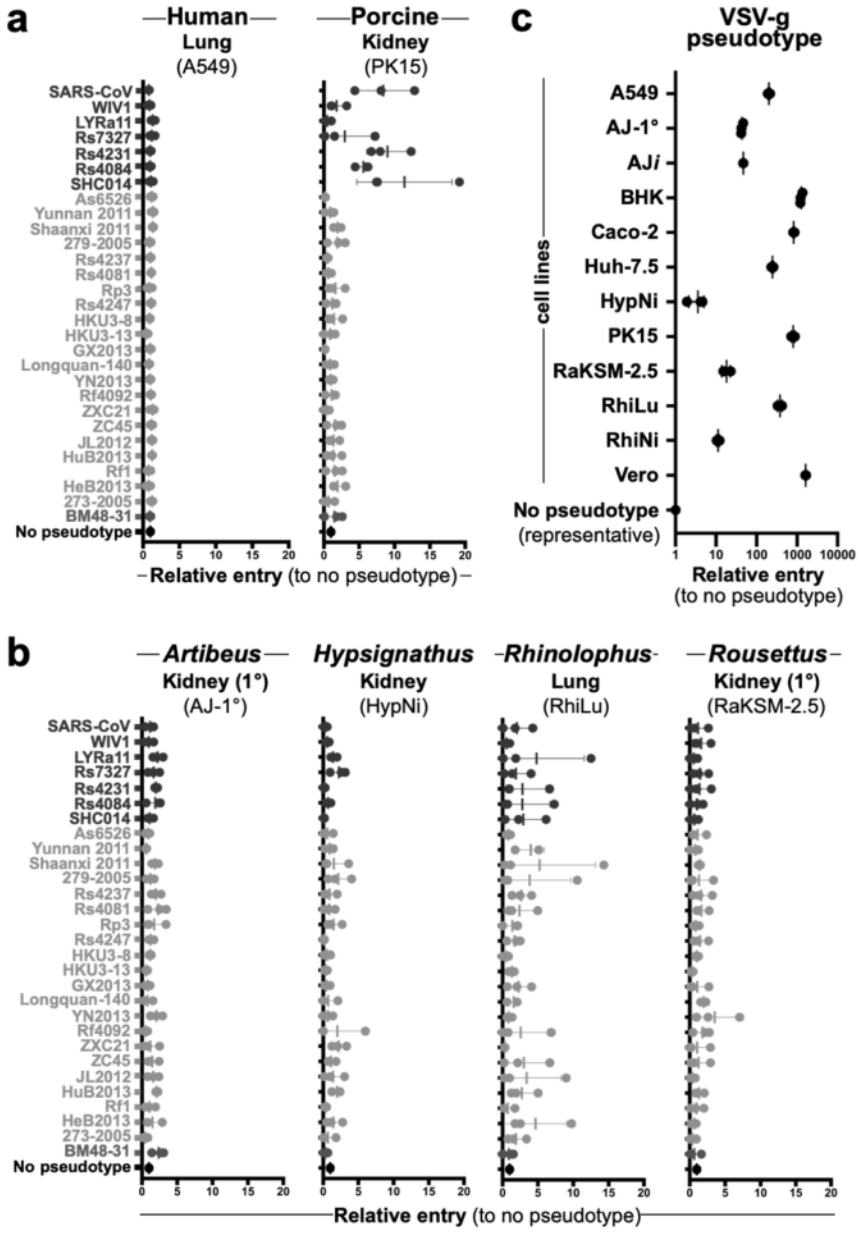


d Genotype→Phenotype schedule

	Cloning	Pseudotype	Entry assay
Day 1	Order RBD sequence		
	RBD synthesis		
6	clone novel RBD into spike		
7	pick colonies	plate producer cells	
8	prepare plasmid DNA	transfect producer cells	
9		infect producer cells with seed particles	
10		*wait*	plate target cells
11		collect pseudotyped particles	infect target cells
12			measure luciferase

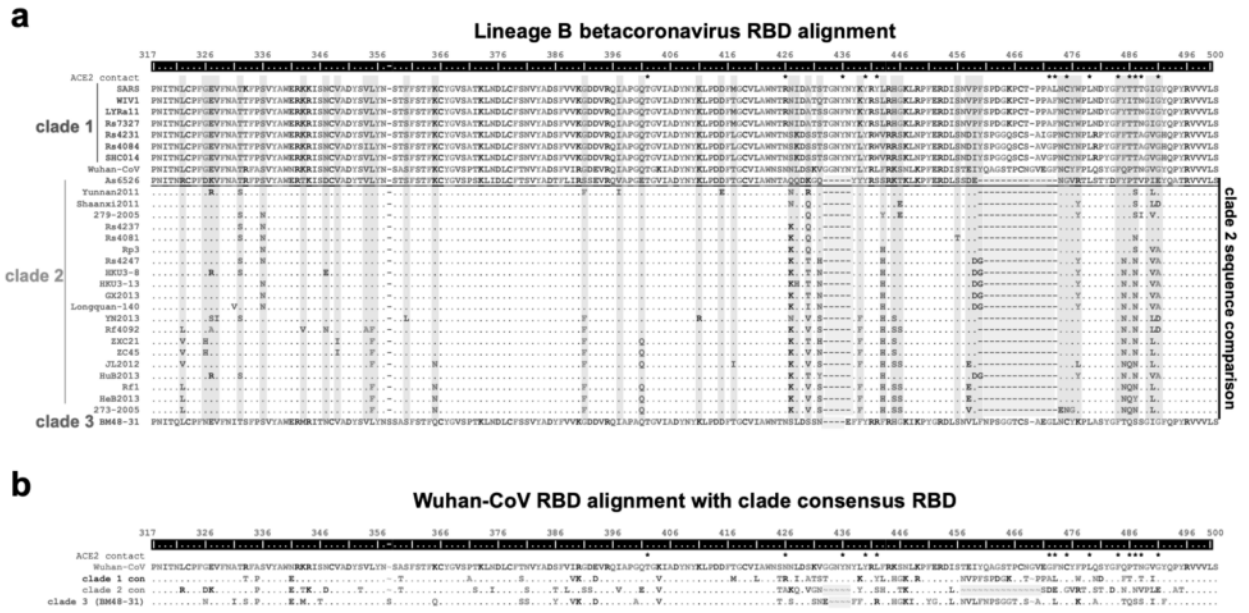
Supplemental figure 1: Lineage B RBD panel assembly and phylogenetic analysis

a, Coronavirus sequences were downloaded from NCBI and further parsed to 29 unique RBD variants. **b,** Virus isolate name, accession number, clade, host species and location of identification listed for the 29 unique lineage B RBDs used in this study. **c,** Cladograms for the spike RBD and coronavirus RNA-dependent RNA polymerase (nsp12).



Supplemental figure 2: additional cell lines tested without protease

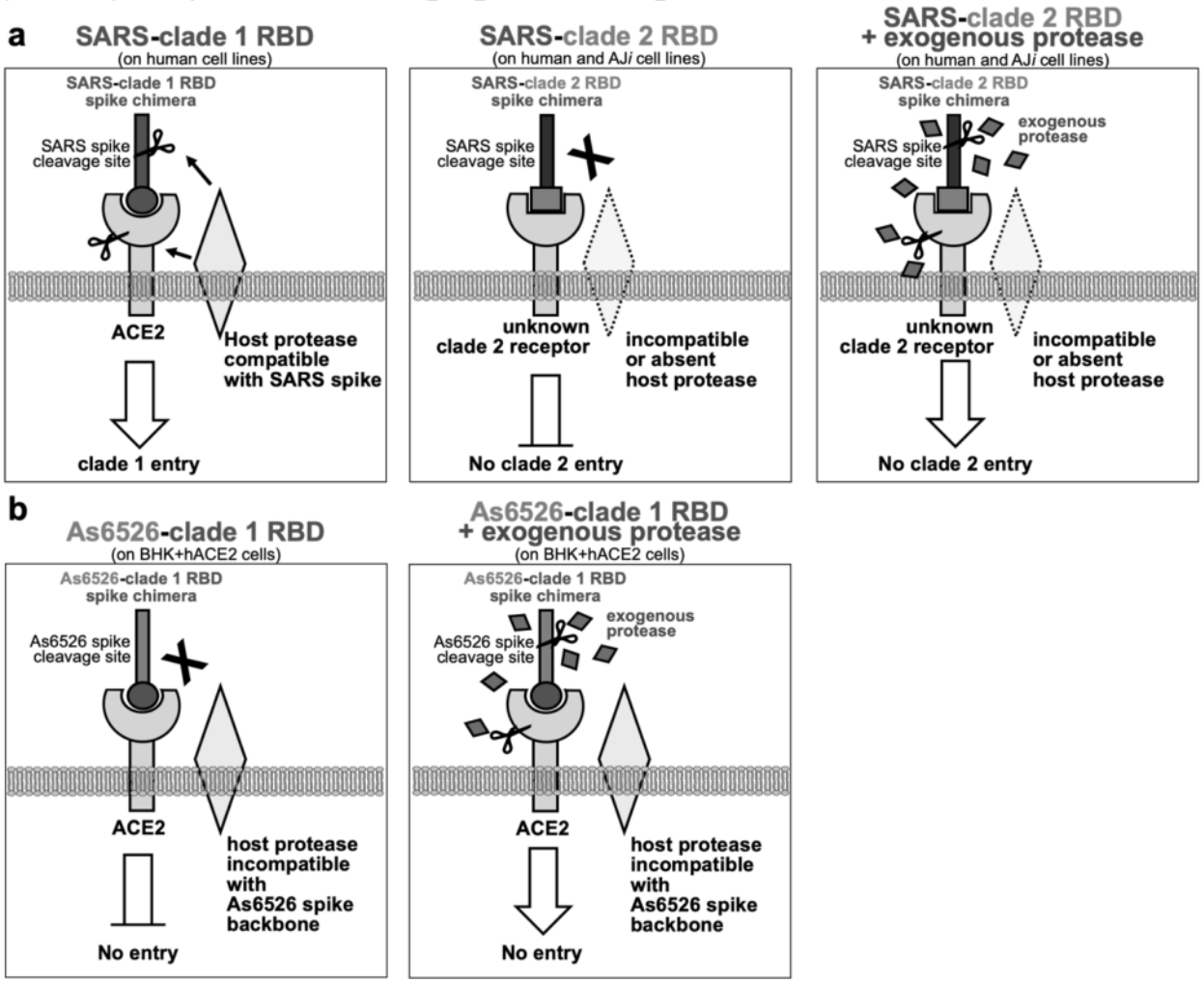
a, Additional human and cell lines or **b,** bat-derived cell lines derived from other species were infected with VSV-reporter particles pseudotyped with chimeric spikes and luciferase was measured a readout for cell entry. Trypsin was not used in these infections. **c,** All cell lines tested in this study supported entry and reporter expression of VSV-g pseudotyped particles. Shown are the data for 3 replicates.



Supplemental fig. 3: Lineage B panel RBD sequence features

a, Amino acid sequences corresponding to SARS-spike residues 317 through 500 were aligned with ClustalW. Contact points between SARS-spike and human ACE2 are

indicated with an (*). Clade 2 sequences are shown as compared to clade 2 As6526, with identical residues indicated with a (.) and sites that vary between clade 2 viruses highlighted in purple. Loop deletions are highlighted in orange. **b,** Amino acid alignment of 2019-nCoV RBD and consensus RBD sequences for clade 1 and 2 and BM48-31 (clade 3). Loop deletions are highlighted in orange.



Supplemental fig. 4: model of lineage B entry

a, SARS spike with a clade 1 RBD enters cells expressing ACE2 and a host protease capable of cleaving SARS spike (left panel). While the clade 2 RBD can bind an unknown host receptor, the SARS spike backbone is incompatible with the receptor-associated protease, resulting in a lack of cleavage and entry (middle panel). The addition of exogenous protease may overcome the lack of endogenous protease cleavage of spike, resulting in receptor-dependent entry. Alternatively, the addition of exogenous protease may activate the receptor to facilitate entry. **b,** Replacing the RBD of As6526 spike with the clade 1 RBD allows for ACE2 interaction, but the As6526 spike backbone is

incompatible with the ACE2-associated protease (left panel). Addition of exogenous protease overcomes protease incompatibility, allowing for ACE2-mediate entry (right panel).

From: Munster, Vincent (NIH/NIAID) [E]
Sent: Wed, 22 Jan 2020 08:14:13 -0700
To: Mellinger, Mary (NIH/NIAID) [C]; Gamiel, Jordyn (NIH/NIAID) [E]
Cc: Feldmann, Heinrich (NIH/NIAID) [E]; Letko, Michael (NIH/NIAID) [F]
Subject: Re: Expedited pub clear for novel coronavirus submission

Sorry, pare shoulde be pubclear. Just one

Vincent Munster, PhD
Chief, Virus Ecology Section
Laboratory of Virology
Rocky Mountain Laboratories
NIAID/NIH

From: "Mellinger, Mary (NIH/NIAID) [C]" (b)(6)
Date: Wednesday, January 22, 2020 at 8:11 AM
To: (b)(6) "Gamiel, Jordyn (NIH/NIAID) [E]" (b)(6)
Cc: Heinrich Feldmann (b)(6) Michael Letko (b)(6)
Subject: RE: Expedited pub clear for novel coronavirus submission

Plus another….a pair you said?

From: Munster, Vincent (NIH/NIAID) [E] (b)(6)
Sent: Wednesday, January 22, 2020 10:03 AM
To: Gamiel, Jordyn (NIH/NIAID) [E] (b)(6) Mellinger, Mary (NIH/NIAID) [C] (b)(6)
Cc: Feldmann, Heinrich (NIH/NIAID) [E] (b)(6) Letko, Michael (NIH/NIAID) [F] (b)(6)
Subject: Re: Expedited pub clear for novel coronavirus submission

Yes a different one. You'll be getting this one in a couple of hours. Hot of the press 😉

Vincent Munster, PhD
Chief, Virus Ecology Section
Laboratory of Virology
Rocky Mountain Laboratories
NIAID/NIH

From: "Gamiel, Jordyn (NIH/NIAID) [E]" (b)(6)
Date: Wednesday, January 22, 2020 at 8:02 AM
To: (b)(6) "Mellinger, Mary (NIH/NIAID) [C]" (b)(6)

Cc: Heinrich Feldmann (b)(6) Michael Letko (b)(6)
Subject: RE: Expedited pub clear for novel coronavirus submission

Vincent, is this one different from the one you sent yesterday? Or did you update it from what was submitted yesterday?

Thanks!
Jordyn

From: Munster, Vincent (NIH/NIAID) [E] (b)(6)
Sent: Wednesday, January 22, 2020 9:53 AM
To: Mellinger, Mary (NIH/NIAID) [C] (b)(6) Gamiel, Jordyn (NIH/NIAID) [E] (b)(6)
Cc: Feldmann, Heinrich (NIH/NIAID) [E] (b)(6) Letko, Michael (NIH/NIAID) [F] (b)(6)
Subject: Expedited pub clear for novel coronavirus submission

Hi Jordyn and Kits,

We'll have a pare coming your way later today for expedited pubclear. This paper is on the novel coronavirus receptor usage and needs to be turned around as quickly as possible. We are planning on submission today.

Regards,

Vincent Munster, PhD
Chief, Virus Ecology Section
Laboratory of Virology
Rocky Mountain Laboratories
NIAID/NIH

From: Munster, Vincent (NIH/NIAID) [E]
Sent: Wed, 22 Jan 2020 08:06:00 -0700
To: Letko, Michael (NIH/NIAID) [F]
Subject: Re: Big day

If you can get it to me I can have a good read-through again as well,

Good job! Pubclear is primed, if you send me the latest manuscript file I'll fill out the details.

Got a WHO call at 9:30 but finalize and submit afterwards

Vincent Munster, PhD
Chief, Virus Ecology Section
Laboratory of Virology
Rocky Mountain Laboratories
NIAID/NIH

From: Michael Letko (b)(6)
Date: Tuesday, January 21, 2020 at 10:42 PM
To: (b)(6)
Subject: Big day

I made a bioRXiv account tonight, so we're ready to upload tomorrow.

I will get a copy of the manuscript in its current state in Heinz's hands tomorrow morning so he can read over it for the pubclear. Maybe Heinz can even call up some Bethesda folks to ensure its expedited for pubclear?

-michael

--
Michael Letko, Ph.D
Postdoctoral IRTA
Dr. Vincent Munster Laboratory
Virus Ecology Unit, Laboratory of Virology
Rocky Mountain Laboratories
NIAID/NIH
903S 4th Street
Hamilton MT 59840
(b)(6)

From: Munster, Vincent (NIH/NIAID) [E]
Sent: Wed, 22 Jan 2020 07:51:08 -0700
To: Letko, Michael (NIH/NIAID) [F]
Subject: Re: Big day

I'll handle the pubclear asap

Vincent Munster, PhD
Chief, Virus Ecology Section
Laboratory of Virology
Rocky Mountain Laboratories
NIAID/NIH

From: Michael Letko (b)(6)
Date: Tuesday, January 21, 2020 at 10:42 PM
To: (b)(6)
Subject: Big day

I made a bioRXiv account tonight, so we're ready to upload tomorrow.

I will get a copy of the manuscript in its current state in Heinz's hands tomorrow morning so he can read over it for the pubclear. Maybe Heinz can even call up some Bethesda folks to ensure its expedited for pubclear?

-michael

--
Michael Letko, Ph.D
Postdoctoral IRTA
Dr. Vincent Munster Laboratory
Virus Ecology Unit, Laboratory of Virology
Rocky Mountain Laboratories
NIAID/NIH
903S 4th Street
Hamilton MT 59840
(b)(6)

From: Letko, Michael (NIH/NIAID) [F]
Sent: Tue, 21 Jan 2020 14:55:40 +0000
To: Munster, Vincent (NIH/NIAID) [E]
Subject: Re: Nature editorial: Stop the Wuhan virus /Vigilance, preparedness, speed, transparency and global coordination are now crucial to stopping a new infectious disease from becoming a global emergency. https://go.nature.com/2NPsQTE
Attachments: image001.jpg

Sounds good

--
Michael Letko, Ph.D
Postdoctoral IRTA
Dr. Vincent Munster Laboratory
Virus Ecology Unit, Laboratory of Virology
Rocky Mountain Laboratories
NIAID/NIH
903S 4th Street
Hamilton MT 59840
(b)(6)

On Jan 21, 2020, at 7:54 AM, Munster, Vincent (NIH/NIAID) [E] (b)(6) wrote:

Nature it is,

Lets do 2 authors and speed this up

Vincent Munster, PhD
Chief, Virus Ecology Section
Laboratory of Virology
Rocky Mountain Laboratories
NIAID/NIH

From: "Marston, Hilary (NIH/NIAID) [E]" (b)(6)
Date: Tuesday, January 21, 2020 at 7:49 AM
To: NIAID Coronavirus Response SWAT 2020 <NIAIDCoronavirusResponseSWAT2020@mail.nih.gov>
Subject: FW: Nature editorial: Stop the Wuhan virus /Vigilance, preparedness, speed, transparency and global coordination are now crucial to stopping a new infectious disease from becoming a global emergency. https://go.nature.com/2NPsQTE

From: Folkers, Greg (NIH/NIAID) [E] (b)(6)
Sent: Tuesday, January 21, 2020 9:49 AM
Subject: Nature editorial: Stop the Wuhan virus /Vigilance, preparedness, speed, transparency and global coordination are now crucial to stopping a new infectious disease from becoming a global emergency. https://go.nature.com/2NPsQTE

21 January 2020

Stop the Wuhan virus

Vigilance, preparedness, speed, transparency and global coordination are now crucial to stopping a new infectious disease from becoming a global emergency.

<image001.jpg>

The virus first surfaced at the end of 2019 in an animal and seafood market in Wuhan, central China.Credit: Noel Celis/AFP/Getty

As hundreds of millions of people in China take to the roads, railway and skies to be with their families for the new year holidays, authorities in the country and around the world have mounted an enormous operation to track and screen travellers from Wuhan in central China.

This follows the outbreak of a mysterious pneumonia-like coronavirus, first reported on the last day of December 2019, that has so far claimed six lives in China. The World Health Organization is deciding whether to declare the situation an international public-health emergency.

The virus has been spreading. On 21 January, as *Nature* went to press, there were almost 300 reported cases — seven times the figure stated five days earlier. Over the past week, authorities in South Korea, Thailand and Japan have also reported cases. Researchers at Imperial College London who have modelled the outbreak on the basis of estimates of travel out of Wuhan say the virus might have infected as many as 1,700 people.

The virus, which still lacks a formal name, is being called 2019-nCOV. It is a relative of both the deadly severe acute respiratory syndrome (SARS) and the Middle East respiratory syndrome (MERS) viruses. People with the virus report a fever along with other symptoms of lower-respiratory infection such as a cough or breathing difficulties. The first people infected in China are understood to have caught the virus in one of Wuhan's live animal and seafood markets — probably from an animal. Some 95% of the total cases, including those in Japan, South Korea and Thailand, also involved people who had been to Wuhan.

The virus has not been found in humans before and knowledge of how it is spread is still evolving. Last week, government officials and researchers in China who are tracking the virus told *Nature* they didn't think it spreads readily from human to human, at least not as fast as SARS. But this view is being revised following the intervention of SARS specialist Zhong Nanshan. After a visit to Wuhan on 20 January, Zhong, who directs the State Key Laboratory of Respiratory Disease in Guangzhou, confirmed that 14 medical workers had been infected by one virus carrier, raising concern that some people might be 'super-spreaders' of the virus. Stopping the further spread of the disease out of Wuhan, possibly by banning infected people from leaving Wuhan, has to be a top priority, he said.

China's health authorities and the government have been moving quickly. Also on 20 January, the national broadcaster reported that president Xi Jinping had ordered that the virus be "resolutely contained", and Premier Li Keqiang announced a steering group to tackle disease spread. At the

beginning of the month, local authorities in Wuhan closed and disinfected the animal market, and health authorities have reported the results of their disease surveillance efforts.

Researchers, too, have had a crucial role, in publishing and sharing genome sequences. Four different research groups sequenced the genomes of six virus samples — and analyses of all six agree that the virus is a relative of SARS. Researchers are to be commended for making sequence data available, and they should continue to do so. (Release of such data, as well as deposition of manuscripts on preprint servers, will not affect the consideration of papers submitted to *Nature*.)

As China's government has recognized, the authorities fumbled in their response to SARS, which spread globally, killing more than 770 people in 2002–03. Fifteen per cent of those infected died, a rate that seems much higher than that of the current outbreak — at least from what is known so far. In contrast to SARS, the response this time has been faster, more assured and more transparent.

But there is still much to do, and quickly. The virus's original source must be confirmed — something that is proving difficult. Researchers have found virus traces in swabs taken from the animal market. The authorities, rightly, made closing and sterilizing the market their first priority, but in their rush to do so they might have missed a chance to test the animals. In the case of SARS, we now know that bats transmitted the virus to other animals, which then passed it to humans. Other questions include confirming the method of transmission for new cases, as well as understanding the virus's ability to cause serious illness. Virus genomes from infected people will need to be sequenced continually to understand the extent to which the virus is evolving.

China's health authorities did well to act more quickly than in the past. Now, they must continue to report what they know and what more they are uncovering. The emerging situation requires global coordination and leadership from the World Health Organization, with the support of public-health agencies worldwide. Researchers must work fast, collaboratively and transparently to address the key research questions. The world has had plenty of practice with SARS and avian flu — we should know what to do.

Around 7 million people are preparing to fly from China to 400 cities in 100 countries to celebrate the Chinese New Year. Now is the time to stop this outbreak spiralling into a global health emergency.

Nature **577**, 450 (2020)
doi: 10.1038/d41586-020-00153-x

Disclaimer: Any third-party material in this email has been shared for internal use under fair use provisions of U.S. copyright law, without further verification of its accuracy/veracity. It does not necessarily represent my views nor those of NIAID, NIH, HHS, or the U.S. government.



From: Kwe Claude, Yinda (NIH/NIAID) [F]
Sent: Thu, 16 Jan 2020 22:53:44 +0000
To: Munster, Vincent (NIH/NIAID) [E]
Subject: Re: Analyses of ACE2

Hi Vincent just for SARS, WH, WIV1, SHCO14. Rs3369 was also evaluated with SHC014, so I included it sequence.



--
Kwe

From: "Munster, Vincent (NIH/NIAID) [E]" (b)(6)
Date: Thursday, January 16, 2020 at 11:26 AM
To: "Letko, Michael (NIH/NIAID) [F]" (b)(6) "Kwe Claude, Yinda (NIH/NIAID) [F]" (b)(6)
Subject: Re: Analyses of ACE2

Yeah, just looking towards smtg I can quote to be able to write the viewpoint. So if you make an alignment of the RBD and just say it is likely based on X, Y, Z than that's enough for me. At this time I'm looking for support for ACE2 as a receptor and not formal proof (makes sense)

The viewpoint will focus on transmission (public health), and not on the actual binding. Let me know what you think. I was hoping someone would have done some quick and dirty bioinformatics by now

Vincent Munster, PhD
Chief, Virus Ecology Section
Laboratory of Virology
Rocky Mountain Laboratories
NIAID/NIH

From: Michael Letko (b)(6)
Date: Thursday, January 16, 2020 at 11:22 AM
To: (b)(6) "Kwe Claude, Yinda (NIH/NIAID) [F]" (b)(6)
Subject: Re: Analyses of ACE2

That alignment is done and already part of my figures.

The computational stuff is not going to be too informative at this point, because it will force the docking to look like current ACE2-SARS co-structures.

We first need to functionally confirm that ACE2 is a receptor. In this experiment, I will also include SARS, WIV1 and SHC014 RBDs for comparison, which will be much more convincing than computational data.

-michael

--
Michael Letko, Ph.D
Postdoctoral IRTA
Dr. Vincent Munster Laboratory
Virus Ecology Unit, Laboratory of Virology
Rocky Mountain Laboratories
NIAID/NIH
903S 4th Street
Hamilton MT 59840
(b)(6)

From: "Munster, Vincent (NIH/NIAID) [E]" (b)(6)
Date: Thursday, January 16, 2020 at 11:18 AM
To: "Kwe Claude, Yinda (NIH/NIAID) [F]" (b)(6) "Letko, Michael (NIH/NIAID) [F]" (b)(6)
Subject: Analyses of ACE2

Is there any information out of the ability to bind to ACE2 yet? Even just a computational analyses?

Kwe, can you make an alignment of the residues of SARS, the Wuhan virus and WIV-1 and SCH014? I'm trying to find some information which I can use to support the hypothesis that the virus binds ACE2. I'm thinking about writing a viewpoint (so more hypothetical) on the h-to-h ability of this virus but I need to have some support for the ACE2 binding.

Vincent Munster, PhD
Chief, Virus Ecology Section
Laboratory of Virology
Rocky Mountain Laboratories
NIAID/NIH

From: Kwe Claude, Yinda (NIH/NIAID) [F]
Sent: Thu, 16 Jan 2020 20:42:04 +0000
To: Munster, Vincent (NIH/NIAID) [E]; Letko, Michael (NIH/NIAID) [F]
Subject: Re: Analyses of ACE2

Hello Vincent,

I have a quick look at the alignment of SARS, wuhan and other bats SARS-like RBD. RBM is highlighted and the underlined residues are those thought to be critical for cross species transmission.
I will make a better figure if everything is ok.

I forgot SHC014. I will add it.

_ _



Kwe Claude Yinda, PhD
Postdoc Fellow
Virus Ecology Unit, Laboratory of Virology
Rocky Mountain Laboratories, NIAID/NIH
903S 4th St. Hamilton, MT 59840
Email: (b)(6)
Tel: (b)(6)

From: "Munster, Vincent (NIH/NIAID) [E]" (b)(6)
Date: Thursday, January 16, 2020 at 11:26 AM
To: "Letko, Michael (NIH/NIAID) [F]" (b)(6) "Kwe Claude, Yinda (NIH/NIAID) [F]" (b)(6)
Subject: Re: Analyses of ACE2

Yeah, just looking towards smtg I can quote to be able to write the viewpoint. So if you make an alignment of the RBD and just say it is likely based on X, Y, Z than that's enough for me. At this time I'm looking for support for ACE2 as a receptor and not formal proof (makes sense)

The viewpoint will focus on transmission (public health), and not on the actual binding. Let me know what you think. I was hoping someone would have done some quick and dirty bioinformatics by now

Vincent Munster, PhD
Chief, Virus Ecology Section
Laboratory of Virology
Rocky Mountain Laboratories
NIAID/NIH

From: Michael Letko (b)(6)
Date: Thursday, January 16, 2020 at 11:22 AM
To: (b)(6) "Kwe Claude, Yinda (NIH/NIAID) [F]" (b)(6)
Subject: Re: Analyses of ACE2

That alignment is done and already part of my figures.

The computational stuff is not going to be too informative at this point, because it will force the docking to look like current ACE2-SARS co-structures.

We first need to functionally confirm that ACE2 is a receptor. In this experiment, I will also include SARS, WIV1 and SHC014 RBDs for comparison, which will be much more convincing than computational data.

-michael

--
Michael Letko, Ph.D
Postdoctoral IRTA
Dr. Vincent Munster Laboratory
Virus Ecology Unit, Laboratory of Virology
Rocky Mountain Laboratories
NIAID/NIH
903S 4th Street
Hamilton MT 59840
(b)(6)

From: "Munster, Vincent (NIH/NIAID) [E]" (b)(6)
Date: Thursday, January 16, 2020 at 11:18 AM
To: "Kwe Claude, Yinda (NIH/NIAID) [F]" (b)(6) "Letko, Michael (NIH/NIAID) [F]" (b)(6)
Subject: Analyses of ACE2

Is there any information out of the ability to bind to ACE2 yet? Even just a computational analyses?

Kwe, can you make an alignment of the residues of SARS, the Wuhan virus and WIV-1 and SCH014? I'm trying to find some information which I can use to support the hypothesis that the virus binds ACE2. I'm thinking about writing a viewpoint (so more hypothetical) on the h-to-h ability of this virus but I need to have some support for the ACE2 binding.

Vincent Munster, PhD
Chief, Virus Ecology Section
Laboratory of Virology

Rocky Mountain Laboratories
NIAID/NIH

From: Letko, Michael (NIH/NIAID) [F]
Sent: Thu, 16 Jan 2020 18:29:33 +0000
To: Munster, Vincent (NIH/NIAID) [E]
Subject: Re: Analyses of ACE2

What about that structure you passed around the other day?

--
Michael Letko, Ph.D
Postdoctoral IRTA
Dr. Vincent Munster Laboratory
Virus Ecology Unit, Laboratory of Virology
Rocky Mountain Laboratories
NIAID/NIH
903S 4th Street
Hamilton MT 59840
(b)(6)

From: "Munster, Vincent (NIH/NIAID) [E]" (b)(6)
Date: Thursday, January 16, 2020 at 11:26 AM
To: "Letko, Michael (NIH/NIAID) [F]" (b)(6) "Kwe Claude, Yinda (NIH/NIAID) [F]" (b)(6)
Subject: Re: Analyses of ACE2

Yeah, just looking towards smtg I can quote to be able to write the viewpoint. So if you make an alignment of the RBD and just say it is likely based on X, Y, Z than that's enough for me. At this time I'm looking for support for ACE2 as a receptor and not formal proof (makes sense)

The viewpoint will focus on transmission (public health), and not on the actual binding. Let me know what you think. I was hoping someone would have done some quick and dirty bioinformatics by now

Vincent Munster, PhD
Chief, Virus Ecology Section
Laboratory of Virology
Rocky Mountain Laboratories
NIAID/NIH

From: Michael Letko (b)(6)
Date: Thursday, January 16, 2020 at 11:22 AM
To: (b)(6) "Kwe Claude, Yinda (NIH/NIAID) [F]" (b)(6)
Subject: Re: Analyses of ACE2

That alignment is done and already part of my figures.

The computational stuff is not going to be too informative at this point, because it will force the docking to look like current ACE2-SARS co-structures.

We first need to functionally confirm that ACE2 is a receptor. In this experiment, I will also include SARS, WIV1 and SHC014 RBDs for comparison, which will be much more convincing than computational data.

-michael

--
Michael Letko, Ph.D
Postdoctoral IRTA
Dr. Vincent Munster Laboratory
Virus Ecology Unit, Laboratory of Virology
Rocky Mountain Laboratories
NIAID/NIH
903S 4th Street
Hamilton MT 59840
(b)(6)

From: "Munster, Vincent (NIH/NIAID) [E]" (b)(6)
Date: Thursday, January 16, 2020 at 11:18 AM
To: "Kwe Claude, Yinda (NIH/NIAID) [F]" (b)(6) "Letko, Michael (NIH/NIAID) [F]" (b)(6)
Subject: Analyses of ACE2

Is there any information out of the ability to bind to ACE2 yet? Even just a computational analyses?

Kwe, can you make an alignment of the residues of SARS, the Wuhan virus and WIV-1 and SCH014? I'm trying to find some information which I can use to support the hypothesis that the virus binds ACE2. I'm thinking about writing a viewpoint (so more hypothetical) on the h-to-h ability of this virus but I need to have some support for the ACE2 binding.

Vincent Munster, PhD
Chief, Virus Ecology Section
Laboratory of Virology
Rocky Mountain Laboratories
NIAID/NIH

From: Letko, Michael (NIH/NIAID) [F]
Sent: Thu, 16 Jan 2020 18:21:59 +0000
To: Munster, Vincent (NIH/NIAID) [E]; Kwe Claude, Yinda (NIH/NIAID) [F]
Subject: Re: Analyses of ACE2
Attachments: Letko 2020 alignment.png

That alignment is done and already part of my figures.

The computational stuff is not going to be too informative at this point, because it will force the docking to look like current ACE2-SARS co-structures.

We first need to functionally confirm that ACE2 is a receptor. In this experiment, I will also include SARS, WIV1 and SHC014 RBDs for comparison, which will be much more convincing than computational data.

-michael

--
Michael Letko, Ph.D
Postdoctoral IRTA
Dr. Vincent Munster Laboratory
Virus Ecology Unit, Laboratory of Virology
Rocky Mountain Laboratories
NIAID/NIH
903S 4th Street
Hamilton MT 59840
(b)(6)

From: "Munster, Vincent (NIH/NIAID) [E]" (b)(6)
Date: Thursday, January 16, 2020 at 11:18 AM
To: "Kwe Claude, Yinda (NIH/NIAID) [F]" (b)(6) "Letko, Michael (NIH/NIAID) [F]" (b)(6)
Subject: Analyses of ACE2

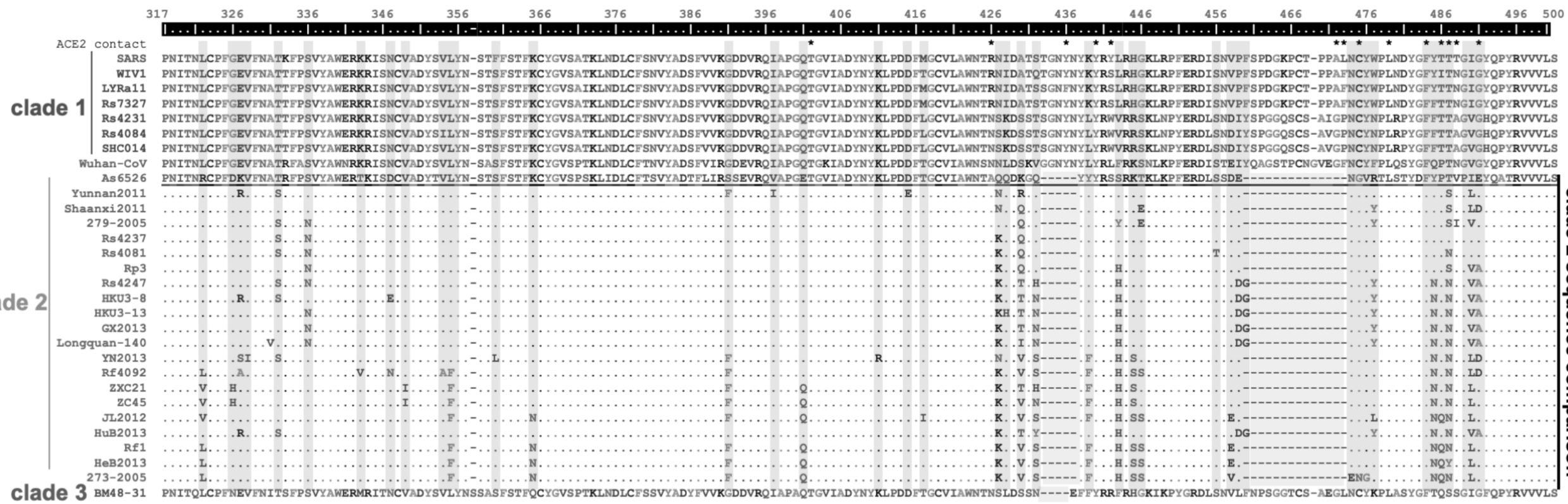
Is there any information out of the ability to bind to ACE2 yet? Even just a computational analyses?

Kwe, can you make an alignment of the residues of SARS, the Wuhan virus and WIV-1 and SCH014? I'm trying to find some information which I can use to support the hypothesis that the virus binds ACE2. I'm thinking about writing a viewpoint (so more hypothetical) on the h-to-h ability of this virus but I need to have some support for the ACE2 binding.

Vincent Munster, PhD
Chief, Virus Ecology Section
Laboratory of Virology
Rocky Mountain Laboratories
NIAID/NIH

a

Lineage B betacoronavirus RBD alignment



b

Wuhan-CoV RBD alignment with clade consensus RBD



From: Letko, Michael (NIH/NIAID) [F]
Sent: Mon, 13 Jan 2020 23:13:36 +0000
To: Munster, Vincent (NIH/NIAID) [E]; van Doremalen, Neeltje (NIH/NIAID) [E]; Avanzato, Victoria (NIH/NIAID) [F]
Subject: Re: Wuhan spike and N
Attachments: Wuhan-CoV (Jan2020) N.fas

Dear all,

Attached is an alignment of the following sequences:

1. SARS-CoV/Urbani nucleocapsid
2. Wuhan-CoV nucleocapsid (original sequence)
3. Wuhan-CoV nucleocapsid, codon optimized for homo sapiens, cloned in pCDNA3.1+ (this is what we will order from GenScript)

The N-construct is in my GenScript shopping cart. To get their ultra-rushed production will cost ~$1700 (final quote pending).

Let me know what you think!
-michael

--
Michael Letko, Ph.D
Postdoctoral IRTA
Dr. Vincent Munster Laboratory
Virus Ecology Unit, Laboratory of Virology
Rocky Mountain Laboratories
NIAID/NIH
903S 4th Street
Hamilton MT 59840
(b)(6)

From: "Munster, Vincent (NIH/NIAID) [E]" (b)(6)
Date: Saturday, January 11, 2020 at 9:40 AM
To: "Letko, Michael (NIH/NIAID) [F]" (b)(6)
Cc: "van Doremalen, Neeltje (NIH/NIAID) [E]" (b)(6)
Subject: Re: Wuhan spike and N

Lol, I did most information on the spike though

Vincent Munster, PhD
Chief, Virus Ecology Section
Laboratory of Virology

Rocky Mountain Laboratories
NIAID/NIH

From: Michael Letko (b)(6)
Date: Saturday, January 11, 2020 at 9:30 AM
To: (b)(6)
Cc: Neeltje van Doremalen (b)(6)
Subject: Re: Wuhan spike and N

Was just about to send the same figure.

Also, check my spike manuscript figures Vincent. They have lots of info too.

--
Michael Letko, Ph.D
Postdoctoral IRTA
Dr. Vincent Munster Laboratory
Virus Ecology Unit, Laboratory of Virology
Rocky Mountain Laboratories
NIAID/NIH
903S 4th Street
Hamilton MT 59840
(b)(6)

On Jan 11, 2020, at 7:55 AM, Munster, Vincent (NIH/NIAID) [E] (b)(6) wrote:

Hi Michael,

Ac you rapidly confirm that these are the ACE2 residues the SARS RBD binds too?

24	27	31	34	37	38	41	42	45	79	82	83	90	325	329	330	353	354
Q	T	K	H	E	D	Y	Q	L	L	M	Y	N	Q	E	N	K	G

Vincent Munster, PhD
Chief, Virus Ecology Section
Laboratory of Virology

Rocky Mountain Laboratories
NIAID/NIH

From: Michael Letko (b)(6)
Date: Saturday, January 11, 2020 at 12:51 AM
To: Neeltje van Doremalen (b)(6)
Cc: (b)(6)
Subject: Re: Wuhan spike and N

That last point should read:

“clade 2 and 3 only gain function with human ACE2 when given the clade 1 RBM”

--
Michael Letko, Ph.D
Postdoctoral IRTA
Dr. Vincent Munster Laboratory
Virus Ecology Unit, Laboratory of Virology
Rocky Mountain Laboratories
NIAID/NIH
903S 4th Street
Hamilton MT 59840
(b)(6)

On Jan 10, 2020, at 11:39 PM, Letko, Michael (NIH/NIAID) [F] (b)(6) wrote:

I expect to have the first cell entry results on January 26th or 27th. I think IDT can expedite synthesis of the RBD, which could save me a day or two. (We will also order full spike and N but those won’t be in until early February.)

While we wait for the RBD results, here are some of my thoughts:

1. The Wuhan spike RBD is clade 1 and retains 11 of 14 contact residues with ACE2. Some of the contacts are not identical to SARS, but they are identical to other clade 1 RBDs, so they should work. I am predicting Wuhan will enter with ACE2.

These are the three contacts points that are different from clade 1. On the left are clade 1 identities and on the right are Wuhan residues. None of these changes really jumps out as a super dramatic change from what I have already tested.

Y/S/W-442-L
N/R/S-479-Q
Y/F-484-Q

2. The other variations are kind of interesting because:
(A) Two loops found in C-terminus of clade 1 RBD but absent in the other clades are present in Wuhan spike. However, these loops do look pretty different, with variation I haven't seen before. Not sure what to make of that yet, but these loops do not form any contacts with ACE2.

(B) Other variations in the N-terminus of Wuhan RBD can be found specifically in clade 2 and 3 but not 1.

3. In light of point 2B, Wuhan might be a recombinant form of clade 2/3 (or something similar) with the receptor binding MOTIF (RBM) from clade 1. Which is neat because I already have a figure in the manuscript showing that exact thing: clade 2 and 3 only gain function when given the clade 1 RBM.

Really exciting stuff!
-michael

—
Michael Letko, Ph.D
Postdoctoral IRTA
Dr. Vincent Munster Laboratory
Virus Ecology Unit, Laboratory of Virology
Rocky Mountain Laboratories
NIAID/NIH
903S 4th Street
Hamilton MT 59840
(b)(6)

On Jan 10, 2020, at 6:30 PM, van Doremalen, Neeltje (NIH/NIAID) [E] (b)(6) wrote:

Spike:
ATGTTTGTTTTTCTTGTTTTATTGCCACTAGTCTCTAGTCAGTGTGTTAATCTTACAACCAGAACTCAATTACCCCCTG
CATACACTAATTCTTTCACACGTGGTGTTTATTACCCTGACAAAGTTTTCAGATCCTCAGTTTTACATTCAACTCAGG
ACTTGTTCTTACCTTTCTTTTCCAATGTTACTTGGTTCCATGCTATACATGTCTCTGGGACCAATGGTACTAAGAGGT
TTGATAACCCTGTCCTACCATTTAATGATGGTGTTTATTTTGCTTCCACTGAGAAGTCTAACATAATAAGAGGCTGG
ATTTTTGGTACTACTTTAGATTCGAAGACCCAGTCCCTACTTATTGTTAATAACGCTACTAATGTTGTTATTAAAGTC

TGTGAATTTCAATTTTGTAATGATCCATTTTTGGGTGTTTATTACCACAAAAACAACAAAAGTTGGATGGAAAGTGA
GTTCAGAGTTTATTCTAGTGCGAATAATTGCACTTTTGAATATGTCTCTCAGCCTTTTCTTATGGACCTTGAAGGAA
AACAGGGTAATTTCAAAAATCTTAGGGAATTTGTGTTTAAGAATATTGATGGTTATTTTAAAATATATTCTAAGCAC
ACGCCTATTAATTTAGTGCGTGATCTCCCTCAGGGTTTTTCGGCTTTAGAACCATTGGTAGATTTGCCAATAGGTAT
TAACATCACTAGGTTTCAAACTTTACTTGCTTTACATAGAAGTTATTTGACTCCTGGTGATTCTTCTTCAGGTTGGAC
AGCTGGTGCTGCAGCTTATTATGTGGGTTATCTTCAACCTAGGACTTTTCTATTAAAATATAATGAAAATGGAACCA
TTACAGATGCTGTAGACTGTGCACTTGACCCTCTCTCAGAAACAAAGTGTACGTTGAAATCCTTCACTGTAGAAAA
AGGAATCTATCAAACTTCTAACTTTAGAGTCCAACCAACAGAATCTATTGTTAGATTTCCTAATATTACAAACTTGT
GCCCTTTTGGTGAAGTTTTTAACGCCACCAGATTTGCATCTGTTTATGCTTGGAACAGGAAGAGAATCAGCAACTG
TGTTGCTGATTATTCTGTCCTATATAATTCCGCATCATTTTCCACTTTTAAGTGTTATGGAGTGTCTCCTACTAAATTA
AATGATCTCTGCTTTACTAATGTCTATGCAGATTCATTTGTAATTAGAGGTGATGAAGTCAGACAAATCGCTCCAGG
GCAAACTGGAAAGATTGCTGATTATAATTATAAATTACCAGATGATTTTACAGGCTGCGTTATAGCTTGGAATTCTA
ACAATCTTGATTCTAAGGTTGGTGGTAATTATAATTACCTGTATAGATTGTTTAGGAAGTCTAATCTCAAACCTTTT
GAGAGAGATATTTCAACTGAAATCTATCAGGCCGGTAGCACACCTTGTAATGGTGTTGAAGGTTTTAATTGTTACT
TTCCTTTACAATCATATGGTTTCCAACCCACTAATGGTGTTGGTTACCAACCATACAGAGTAGTAGTACTTTCTTTTG
AACTTCTACATGCACCAGCAACTGTTTGTGGACCTAAAAAGTCTACTAATTTGGTTAAAAACAAATGTGTCAATTTC
AACTTCAATGGTTTAACAGGCACAGGTGTTCTTACTGAGTCTAACAAAAAGTTTCTGCCTTTCCAACAATTTGGCAG
AGACATTGCTGACACTACTGATGCTGTCCGTGATCCACAGACACTTGAGATTCTTGACATTACACCATGTTCTTTTG
GTGGTGTCAGTGTTATAACACCAGGAACAAATACTTCTAACCAGGTTGCTGTTCTTTATCAGGATGTTAACTGCACA
GAAGTCCCTGTTGCTATTCATGCAGATCAACTTACTCCTACTTGGCGTGTTTATTCTACAGGTTCTAATGTTTTTCAA
ACACGTGCAGGCTGTTTAATAGGGGCTGAACATGTCAACAACTCATATGAGTGTGACATACCCATTGGTGCAGGT
ATATGCGCTAGTTATCAGACTCAGACTAATTCTCCTCGGCGGGCACGTAGTGTAGCTAGTCAATCCATCATTGCCTA
CACTATGTCACTTGGTGCAGAAAATTCAGTTGCTTACTCTAATAACTCTATTGCCATACCCACAAATTTTACTATTAG
TGTTACCACAGAAATTCTACCAGTGTCTATGACCAAGACATCAGTAGATTGTACAATGTACATTTGTGGTGATTCAA
CTGAATGCAGCAATCTTTTGTTGCAATATGGCAGTTTTTGTACACAATTAAACCGTGCTTTAACTGGAATAGCTGTT
GAACAAGACAAAAACACCCAAGAAGTTTTTGCACAAGTCAAACAAATTTACAAAACACCACCAATTAAAGATTTTG
GTGGTTTTAATTTTTCACAAATATTACCAGATCCATCAAAACCAAGCAAGAGGTCATTTATTGAAGATCTACTTTTC
AACAAAGTGACACTTGCAGATGCTGGCTTCATCAAACAATATGGTGATTGCCTTGGTGATATTGCTGCTAGAGACC
TCATTTGTGCACAAAAGTTTAACGGCCTTACTGTTTTGCCACCTTTGCTCACAGATGAAATGATTGCTCAATACACTT
CTGCACTGTTAGCGGGTACAATCACTTCTGGTTGGACCTTTGGTGCAGGTGCTGCATTACAAATACCATTTGCTATG
CAAATGGCTTATAGGTTTAATGGTATTGGAGTTACACAGAATGTTCTCTATGAGAACCAAAAATTGATTGCCAACC
AATTTAATAGTGCTATTGGCAAAATTCAAGACTCACTTTCTTCCACAGCAAGTGCACTTGGAAAACTTCAAGATGTG
GTCAACCAAAATGCACAAGCTTTAAACACGCTTGTTAAACAACTTAGCTCCAATTTTGGTGCAATTTCAAGTGTTTT
AAATGATATCCTTTCACGTCTTGACAAAGTTGAGGCTGAAGTGCAAATTGATAGGTTGATCACAGGCAGACTTCAA
AGTTTGCAGACATATGTGACTCAACAATTAATTAGAGCTGCAGAAATCAGAGCTTCTGCTAATCTTGCTGCTACTAA
AATGTCAGAGTGTGTACTTGGACAATCAAAAAGAGTTGATTTTTGTGGAAAGGGCTATCATCTTATGTCCTTCCCTC
AGTCAGCACCTCATGGTGTAGTCTTCTTGCATGTGACTTATGTCCCTGCACAAGAAAAGAACTTCACAACTGCTCCT
GCCATTTGTCATGATGGAAAAGCACACTTTCCTCGTGAAGGTGTCTTTGTTTCAAATGGCACACACTGGTTTGTAAC
ACAAAGGAATTTTTATGAACCACAAATCATTACTACAGACAACACATTTGTGTCTGGTAACTGTGATGTTGTAATAG
GAATTGTCAACAACACAGTTTATGATCCTTTGCAACCTGAATTAGACTCATTCAAGGAGGAGTTAGATAAATATTTT
AAGAATCATACATCACCAGATGTTGATTTAGGTGACATCTCTGGCATTAATGCTTCAGTTGTAAACATTCAAAAAGA
AATTGACCGCCTCAATGAGGTTGCCAAGAATTTAAATGAATCTCTCATCGATCTCCAAGAACTTGGAAAGTATGAG
CAGTATATAAAATGGCCATGGTACATTTGGCTAGGTTTTATAGCTGGCTTGATTGCCATAGTAATGGTGACAATTA
TGCTTTGCTGTATGACCAGTTGCTGTAGTTGTCTCAAGGGCTGTTGTTCTTGTGGATCCTGCTGCAAATTTGATGAA
GACGACTCTGAGCCAGTGCTCAAAGGAGTCAAATTACATTACACATAA

N:

ATGTCTGATAATGGACCCCAAAATCAGCGAAATGCACCCCGCATTACGTTTGGTGGACCCTCAGATTCAACTGGCA
GTAACCAGAATGGAGAACGCAGTGGGGCGCGATCAAAACAACGTCGGCCCCAAGGTTTACCCAATAATACTGCGT
CTTGGTTCACCGCTCTCACTCAACATGGCAAGGAAGACCTTAAATTCCCTCGAGGACAAGGCGTTCCAATTAACAC
CAATAGCAGTCCAGATGACCAAATTGGCTACTACCGAAGAGCTACCAGACGAATTCGTGGTGGTGACGGTAAAAT
GAAAGATCTCAGTCCAAGATGGTATTTCTACTACCTAGGAACTGGGCCAGAAGCTGGACTTCCCTATGGTGCTAAC
AAAGACGGCATCATATGGGTTGCAACTGAGGGAGCCTTGAATACACCAAAAGATCACATTGGCACCCGCAATCCT
GCTAACAATGCTGCAATCGTGCTACAACTTCCTCAAGGAACAACATTGCCAAAAGGCTTCTACGCAGAAGGGAGC
AGAGGCGGCAGTCAAGCCTCTTCTCGTTCCTCATCACGTAGTCGCAACAGTTCAAGAAATTCAACTCCAGGCAGCA
GTAGGGGAACTTCTCCTGCTAGAATGGCTGGCAATGGCGGTGATGCTGCTCTTGCTTTGCTGCTGCTTGACAGATT
GAACCAGCTTGAGAGCAAAATGTCTGGTAAAGGCCAACAACAACAAGGCCAAACTGTCACTAAGAAATCTGCTGC
TGAGGCTTCTAAGAAGCCTCGGCAAAAACGTACTGCCACTAAAGCATACAATGTAACACAAGCTTTCGGCAGACG
TGGTCCAGAACAAACCCAAGGAAATTTTGGGGACCAGGAACTAATCAGACAAGGAACTGATTACAAACATTGGCC
GCAAATTGCACAATTTGCCCCCAGCGCTTCAGCGTTCTTCGGAATGTCGCGCATTGGCATGGAAGTCACACCTTCG
GGAACGTGGTTGACCTACACAGCTGCCATCAAATTGGATGACAAAGATCCAAATTTCAAAGATCAAGTCATTTTGC
TGAATAAGCATATTGACGCATACAAAACATTCCCACCAACAGAGCCTAAAAAGGACAAAAAGAAGAAGGCTGATG
AAACTCAAGCCTTACCGCAGAGACAGAAGAAACAGCAAACTGTGACTCTTCTTCCTGCTGCAGATTTGGATGATTT
CTCCAAACAATTGCAACAATCCATGAGCAGTGCTGACTCAACTCAGGCCTAA

Wuhan blasts closest (86%) to MG772933, a chinese SARS-like CoV from bats.

>SARS-COV N (K062184.1:28267-29535)
~~~~~~
~~~~~~
~~~~~~
~~~~~~
~~~~~~
~~~~~~
~~~~~~
~~~~~~
~~~~~~
~~~~~~
~~~~~~
~~~~~~
~~~~~~
~~~~~~
~~~~~~
~~~~~~ATGTCTGATAATGGACCCCAATCAAACCAACGTAGTGCCCCCCGCATTACATTT
GGTGGACCCACAGATTCAACTGACAATAACCAGAATGGAGGACGCAATGGGGCAAGGCCA
AAACAGCGCCGACCCCAAGGTTTACCCAATAATACTGCGTCTTGGTTCACAGCTCTCACT
CAGCATGGCAAGGAGGAACTTAGATTCCCTCGAGGCCAGGGCGTTCCAATCAACACCAAT
AGTGGTCCAGATGACCAAATTGGCTACTACCGAAGAGCTACCCGACGAGTTCGTGGTGGT
GACGGCAAAATGAAAGAGCTCAGCCCCAGATGGTACTTCTATTACCTAGGAACTGGCCCA
GAAGCTTCACTTCCCTACGGCGCTAACAAAGAAGGCATCGTATGGGTTGCAACTGAGGGA
GCCTTGAATACACCCAAAGACCACATTGGCACCCGCAATCCTAATAACAATGCTGCCACC
GTGCTACAACTTCCTCAAGGAACAACATTGCCAAAAGGCTTCTACGCAGAGGGAAGCAGA
GGCGGCAGTCAAGCCTCTTCTCGCTCCTCATCACGTAGTCGCGGTAATTCAAGAAATTCA
ACTCCTGGCAGCAGTAGGGGAAATTCTCCTGCTCGAATGGCTAGCGGAGGTGGTGAAACT
GCCCTCGCGCTATTGCTGCTAGACAGATTGAACCAGCTTGAGAGCAAAGTTTCTGGTAAA
GGCCAACAACAACAAGGCCAAACTGTCACTAAGAAATCTGCTGCTGAGGCATCTAAAAAG
CCTCGCCAAAAACGTACTGCCACAAAACAGTACAACGTCACTCAAGCATTTGGGAGACGT
GGTCCAGAACAAACCCAAGGAAATTTCGGGGACCAAGACCTAATCAGACAAGGAACTGAT
TACAAACATTGGCCGCAAATTGCACAATTTGCTCCAAGTGCCTCTGCATTCTTTGGAATG
TCACGCATTGGCATGGAAGTCACACCTTCGGGAACATGGCTGACTTATCATGGAGCCATT
AAATTGGATGACAAAGATCCACAATTCAAAGACAACGTCATACTGCTGAACAAGCACATT
GACGCATACAAAACATTCCCACCAACAGAGCCTAAAAAGGACAAAAAGAAAAAGACTGAT
GAAGCTCAGCCTTTGCCGCAGAGACAAAAGAAGCAGCCCACTGTGACTCTTCTTCCTGCG
GCTGACATGGATGATTTCTCCAGACAACTTCAAAATTCCATGAGTGGAGCTTCTGCTGAT
TCAACTCAGGCATAA~~~~~~
~~~~~~
~~~~~~
~~~~~~
~~~~~~
~~~~~~
~~~~~~
~~~~~~
~~~~~~
~~~~~~
~~~~~~
~~~~~~
~~~~~~

```
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
~~~~~~~~~~~~
>Wuhan-CoV N (original seq)
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
~~~~~~ATGTCTGATAATGGACCCCAA~~~AATCAGCGAAATGCACCCCGCATTACGTTT
GGTGGACCCTCAGATTCAACTGGCAGTAACCAGAATGGAGAACGCAGTGGGGCGCGATCA
AAACAACGTCGGCCCCAAGGTTTACCCAATAATACTGCGTCTTGGTTCACCGCTCTCACT
CAACATGGCAAGGAAGACCTTAAATTCCCTCGAGGACAAGGCGTTCCAATTAACACCAAT
AGCAGTCCAGATGACCAAATTGGCTACTACCGAAGAGCTACCAGACGAATTCGTGGTGGT
GACGGTAAAATGAAAGATCTCAGTCCAAGATGGTATTTCTACTACCTAGGAACTGGGCCA
GAAGCTGGACTTCCCTATGGTGCTAACAAAGACGGCATCATATGGGTTGCAACTGAGGGA
GCCTTGAATACACCAAAAGATCACATTGGCACCCGCAATCCTGCTAACAATGCTGCAATC
GTGCTACAACTTCCTCAAGGAACAACATTGCCAAAAGGCTTCTACGCAGAAGGGAGCAGA
GGCGGCAGTCAAGCCTCTTCTCGTTCCTCATCACGTAGTCGCAACAGTTCAAGAAATTCA
ACTCCAGGCAGCAGTAGGGGAACTTCTCCTGCTAGAATGGCTGGCAATGGCGGTGATGCT
GCTCTTGCTTTGCTGCTGCTTGACAGATTGAACCAGCTTGAGAGCAAAATGTCTGGTAAA
GGCCAACAACAACAAGGCCAAACTGTCACTAAGAAATCTGCTGCTGAGGCTTCTAAGAAG
CCTCGGCAAAAACGTACTGCCACTAAAGCATACAATGTAACACAAGCTTTCGGCAGACGT
GGTCCAGAACAAACCCAAGGAAATTTTGGGGACCAGGAACTAATCAGACAAGGAACTGAT
TACAAACATTGGCCGCAAATTGCACAATTTGCCCCCAGCGCTTCAGCGTTCTTCGGAATG
TCGCGCATTGGCATGGAAGTCACACCTTCGGGAACGTGGTTGACCTACACAGCTGCCATC
AAATTGGATGACAAAGATCCAAATTTCAAAGATCAAGTCATTTTGCTGAATAAGCATATT
GACGCATACAAAACATTCCCACCAACAGAGCCTAAAAAGGACAAAAAGAAGAAGGCTGAT
GAAACTCAAGCCTTACCGCAGAGACAGAAGAAACAGCAAACTGTGACTCTTCTTCCTGCT
GCAGATTTGGATGATTTCTCCAAACAATTGCAACAATCCATGAGC~~~~~~AGTGCTGAC
TCAACTCAGGCCTAA~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
```

~~~~~~
~~~~~~
~~~~~~
~~~~~~
~~~~~~
~~~~~~
~~~~~~
~~~~~~
~~~~~~
~~~~~~
~~~~~~
~~~~~~
~~~~~~
~~~~~~
~~~~~~
~~~~~~
~~~~~~
~~~~~~
~~~~~~
~~~~~~
~~~~~~
~~~~~~
~~~~~~
~~~~~~~~~~~~
>Wuhan-N (hs-co-geneart) in pcDNA3.1+
GACGGATCGGGAGATCTCCCGATCCCCTATGGTGCACTCTCAGTACAATCTGCTCTGATG
CCGCATAGTTAAGCCAGTATCTGCTCCCTGCTTGTGTGTTGGAGGTCGCTGAGTAGTGCG
CGAGCAAAATTTAAGCTACAACAAGGCAAGGCTTGACCGACAATTGCATGAAGAATCTGC
TTAGGGTTAGGCGTTTTGCGCTGCTTCGCGATGTACGGGCCAGATATACGCGTTGACATT
GATTATTGACTAGTTATTAATAGTAATCAATTACGGGGTCATTAGTTCATAGCCCATATA
TGGAGTTCCGCGTTACATAACTTACGGTAAATGGCCCGCCTGGCTGACCGCCCAACGACC
CCCGCCCATTGACGTCAATAATGACGTATGTTCCCATAGTAACGCCAATAGGGACTTTCC
ATTGACGTCAATGGGTGGAGTATTTACGGTAAACTGCCCACTTGGCAGTACATCAAGTGT
ATCATATGCCAAGTACGCCCCCTATTGACGTCAATGACGGTAAATGGCCCGCCTGGCATT
ATGCCCAGTACATGACCTTATGGGACTTTCCTACTTGGCAGTACATCTACGTATTAGTCA
TCGCTATTACCATGGTGATGCGGTTTTGGCAGTACATCAATGGGCGTGGATAGCGGTTTG
ACTCACGGGGATTTCCAAGTCTCCACCCCATTGACGTCAATGGGAGTTTGTTTTGGCACC
AAAATCAACGGGACTTTCCAAAATGTCGTAACAACTCCGCCCCATTGACGCAAATGGGCG
GTAGGCGTGTACGGTGGGAGGTCTATATAAGCAGAGCTCTCTGGCTAACTAGAGAACCCA
CTGCTTACTGGCTTATCGAAATTAATACGACTCACTATAGGGAGACCCAAGCTGGCTAGC
GCCACCATGAGCGATAACGGCCCTCAG~~~AACCAGCGGAACGCCCCTAGAATCACATTT
GGCGGCCCTAGCGATAGCACCGGCAGCAATCAGAATGGCGAGAGAAGCGGCGCCAGAAGC
AAGCAGAGAAGGCCTCAAGGCCTGCCTAACAACACCGCCAGCTGGTTCACAGCCCTGACA
CAGCACGGCAAAGAGGACCTGAAGTTCCCTAGAGGACAGGGCGTGCCCATCAACACCAAC
AGCAGCCCCGATGACCAGATCGGCTACTACAGACGGGCCACCAGAAGAATCAGAGGCGGC
GACGGCAAGATGAAGGATCTGAGCCCCAGATGGTACTTCTACTACCTCGGCACAGGACCC
GAAGCCGGACTTCCTTATGGCGCCAACAAGGACGGCATCATCTGGGTTGCAACAGAAGGC
GCCCTGAACACCCCTAAGGACCACATCGGCACCAGAAATCCCGCCAACAATGCCGCCATT
GTGCTGCAGTTGCCTCAGGGCACAACACTGCCCAAGGGCTTTTACGCCGAGGGCTCTAGA
GGCGGAAGCCAGGCCTCTAGCAGAAGCAGCAGCAGATCCCGGAACTCCAGCAGAAATAGC

ACCCCTGGCAGCTCCAGAGGCACAAGCCCTGCTAGAATGGCTGGCAACGGCGGAGATGCT
GCTCTGGCACTTCTGCTGCTCGACCGGCTGAATCAGCTGGAAAGCAAGATGAGCGGCAAG
GGCCAGCAGCAACAGGGCCAGACCGTGACAAAAAAGTCTGCCGCCGAGGCCAGCAAGAAG
CCCAGACAGAAAAGAACCGCCACCAAGGCCTACAACGTGACCCAGGCCTTTGGAAGAAGA
GGCCCTGAGCAGACCCAGGGCAACTTCGGAGATCAAGAGCTGATCAGACAGGGCACCGAC
TACAAGCACTGGCCTCAGATCGCCCAGTTTGCCCCATCTGCCAGCGCCTTTTTCGGCATG
AGCCGGATCGGCATGGAAGTGACACCTAGCGGCACCTGGCTGACATACACAGCCGCCATC
AAGCTGGACGACAAGGACCCCAACTTCAAGGACCAAGTGATCCTGCTGAACAAGCACATC
GACGCCTACAAGACATTCCCTCCAACCGAGCCTAAGAAGGACAAGAAGAAGAAGGCCGAC
GAGACACAGGCCCTGCCTCAGCGCCAGAAAAAGCAGCAGACTGTGACACTGCTGCCAGCC
GCCGACCTGGACGATTTTTCTAAGCAGCTGCAGCAGAGCATGAGC~~~~~~AGCGCCGAT
TCTACACAGGCCTGAGGGCCCGTTTAAACCCGCTGATCAGCCTCGACTGTGCCTTCTAGT
TGCCAGCCATCTGTTGTTTGCCCCTCCCCCGTGCCTTCCTTGACCCTGGAAGGTGCCACT
CCCACTGTCCTTTCCTAATAAAATGAGGAAATTGCATCGCATTGTCTGAGTAGGTGTCAT
TCTATTCTGGGGGGTGGGGTGGGGCAGGACAGCAAGGGGGAGGATTGGGAAGACAATAGC
AGGCATGCTGGGGATGCGGTGGGCTCTATGGCTTCTGAGGCGGAAAGAACCAGCTGGGGC
TCTAGGGGGTATCCCCACGCGCCCTGTAGCGGCGCATTAAGCGCGGCGGGTGTGGTGGTT
ACGCGCAGCGTGACCGCTACACTTGCCAGCGCCCTAGCGCCCGCTCCTTTCGCTTTCTTC
CCTTCCTTTCTCGCCACGTTCGCCGGCTTTCCCCGTCAAGCTCTAAATCGGGGGCTCCCT
TTAGGGTTCCGATTTAGTGCTTTACGGCACCTCGACCCCAAAAAACTTGATTAGGGTGAT
GGTTCACGTAGTGGGCCATCGCCCTGATAGACGGTTTTTCGCCCTTTGACGTTGGAGTCC
ACGTTCTTTAATAGTGGACTCTTGTTCCAAACTGGAACAACACTCAACCCTATCTCGGTC
TATTCTTTTGATTTATAAGGGATTTTGCCGATTTCGGCCTATTGGTTAAAAAATGAGCTG
ATTTAACAAAAATTTAACGCGAATTAATTCTGTGGAATGTGTGTCAGTTAGGGTGTGGAA
AGTCCCCAGGCTCCCCAGCAGGCAGAAGTATGCAAAGCATGCATCTCAATTAGTCAGCAA
CCAGGTGTGGAAAGTCCCCAGGCTCCCCAGCAGGCAGAAGTATGCAAAGCATGCATCTCA
ATTAGTCAGCAACCATAGTCCCGCCCCTAACTCCGCCCATCCCGCCCCTAACTCCGCCCA
GTTCCGCCCATTCTCCGCCCCATGGCTGACTAATTTTTTTTATTTATGCAGAGGCCGAGG
CCGCCTCTGCCTCTGAGCTATTCCAGAAGTAGTGAGGAGGCTTTTTTGGAGGCCTAGGCT
TTTGCAAAAAGCTCCCGGGAGCTTGTATATCCATTTTCGGATCTGATCAAGAGACAGGAT
GAGGATCGTTTCGCATGATTGAACAAGATGGATTGCACGCAGGTTCTCCGGCCGCTTGGG
TGGAGAGGCTATTCGGCTATGACTGGGCACAACAGACAATCGGCTGCTCTGATGCCGCCG
TGTTCCGGCTGTCAGCGCAGGGGCGCCCGGTTCTTTTTGTCAAGACCGACCTGTCCGGTG
CCCTGAATGAACTGCAGGACGAGGCAGCGCGGCTATCGTGGCTGGCCACGACGGGCGTTC
CTTGCGCAGCTGTGCTCGACGTTGTCACTGAAGCGGGAAGGGACTGGCTGCTATTGGGCG
AAGTGCCGGGGCAGGATCTCCTGTCATCTCACCTTGCTCCTGCCGAGAAAGTATCCATCA
TGGCTGATGCAATGCGGCGGCTGCATACGCTTGATCCGGCTACCTGCCCATTCGACCACC
AAGCGAAACATCGCATCGAGCGAGCACGTACTCGGATGGAAGCCGGTCTTGTCGATCAGG
ATGATCTGGACGAAGAGCATCAGGGGCTCGCGCCAGCCGAACTGTTCGCCAGGCTCAAGG
CGCGCATGCCCGACGGCGAGGATCTCGTCGTGACCCATGGCGATGCCTGCTTGCCGAATA
TCATGGTGGAAAATGGCCGCTTTTCTGGATTCATCGACTGTGGCCGGCTGGGTGTGGCGG
ACCGCTATCAGGACATAGCGTTGGCTACCCGTGATATTGCTGAAGAGCTTGGCGGCGAAT
GGGCTGACCGCTTCCTCGTGCTTTACGGTATCGCCGCTCCCGATTCGCAGCGCATCGCCT
TCTATCGCCTTCTTGACGAGTTCTTCTGAGCGGGACTCTGGGGTTCGAAATGACCGACCA
AGCGACGCCCAACCTGCCATCACGAGATTTCGATTCCACCGCCGCCTTCTATGAAAGGTT
GGGCTTCGGAATCGTTTTCCGGGACGCCGGCTGGATGATCCTCCAGCGCGGGGATCTCAT
GCTGGAGTTCTTCGCCCACCCCAACTTGTTTATTGCAGCTTATAATGGTTACAAATAAAG
CAATAGCATCACAAATTTCACAAATAAAGCATTTTTTTCACTGCATTCTAGTTGTGGTTT
GTCCAAACTCATCAATGTATCTTATCATGTCTGTATACCGTCGACCTCTAGCTAGAGCTT
GGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACA

CAACATACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAGCTAACT
CACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCT
GCATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCTTCCGC
TTCCTCGCTCACTGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGCGGTATCAGCTCA
CTCAAAGGCGGTAATACGGTTATCCACAGAATCAGGGGATAACGCAGGAAAGAACATGTG
AGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGCTGGCGTTTTTCCA
TAGGCTCCGCCCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAGAGGTGGCGAAA
CCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTCC
TGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCCTTTCTCCCTTCGGGAAGCGTGGC
GCTTTCTCATAGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTTCGCTCCAAGCT
GGGCTGTGTGCACGAACCCCCCGTTCAGCCCGACCGCTGCGCCTTATCCGGTAACTATCG
TCTTGAGTCCAACCCGGTAAGACACGACTTATCGCCACTGGCAGCAGCCACTGGTAACAG
GATTAGCAGAGCGAGGTATGTAGGCGGTGCTACAGAGTTCTTGAAGTGGTGGCCTAACTA
CGGCTACACTAGAAGAACAGTATTTGGTATCTGCGCTCTGCTGAAGCCAGTTACCTTCGG
AAAAAGAGTTGGTAGCTCTTGATCCGGCAAACAAACCACCGCTGGTAGCGGTGGTTTTTT
TGTTTGCAAGCAGCAGATTACGCGCAGAAAAAAAGGATCTCAAGAAGATCCTTTGATCTT
TTCTACGGGGTCTGACGCTCAGTGGAACGAAAACTCACGTTAAGGGATTTTGGTCATGAG
ATTATCAAAAAGGATCTTCACCTAGATCCTTTTAAATTAAAAATGAAGTTTTAAATCAAT
CTAAAGTATATATGAGTAAACTTGGTCTGACAGTTACCAATGCTTAATCAGTGAGGCACC
TATCTCAGCGATCTGTCTATTTCGTTCATCCATAGTTGCCTGACTCCCCGTCGTGTAGAT
AACTACGATACGGGAGGGCTTACCATCTGGCCCCAGTGCTGCAATGATACCGCGAGACCC
ACGCTCACCGGCTCCAGATTTATCAGCAATAAACCAGCCAGCCGGAAGGGCCGAGCGCAG
AAGTGGTCCTGCAACTTTATCCGCCTCCATCCAGTCTATTAATTGTTGCCGGGAAGCTAG
AGTAAGTAGTTCGCCAGTTAATAGTTTGCGCAACGTTGTTGCCATTGCTACAGGCATCGT
GGTGTCACGCTCGTCGTTTGGTATGGCTTCATTCAGCTCCGGTTCCCAACGATCAAGGCG
AGTTACATGATCCCCCATGTTGTGCAAAAAAGCGGTTAGCTCCTTCGGTCCTCCGATCGT
TGTCAGAAGTAAGTTGGCCGCAGTGTTATCACTCATGGTTATGGCAGCACTGCATAATTC
TCTTACTGTCATGCCATCCGTAAGATGCTTTTCTGTGACTGGTGAGTACTCAACCAAGTC
ATTCTGAGAATAGTGTATGCGGCGACCGAGTTGCTCTTGCCCGGCGTCAATACGGGATAA
TACCGCGCCACATAGCAGAACTTTAAAAGTGCTCATCATTGGAAAACGTTCTTCGGGGCG
AAAACTCTCAAGGATCTTACCGCTGTTGAGATCCAGTTCGATGTAACCCACTCGTGCACC
CAACTGATCTTCAGCATCTTTTACTTTCACCAGCGTTTCTGGGTGAGCAAAAACAGGAAG
GCAAAATGCCGCAAAAAAGGGAATAAGGGCGACACGGAAATGTTGAATACTCATACTCTT
CCTTTTTCAATATTATTGAAGCATTTATCAGGGTTATTGTCTCATGAGCGGATACATATT
TGAATGTATTTAGAAAAATAAACAAATAGGGGTTCCGCGCACATTTCCCCGAAAAGTGCC
ACCTGACGTC

From: Munster, Vincent (NIH/NIAID) [E]
Sent: Sat, 11 Jan 2020 09:03:40 -0700
To: van Doremalen, Neeltje (NIH/NIAID) [E]; Letko, Michael (NIH/NIAID) [F]
Subject: Re: Wuhan spike and N

Thanks!!!!!

Vincent Munster, PhD
Chief, Virus Ecology Section
Laboratory of Virology
Rocky Mountain Laboratories
NIAID/NIH

From: Neeltje van Doremalen (b)(6)
Date: Saturday, January 11, 2020 at 9:00 AM
To: (b)(6) Michael Letko
(b)(6)
Subject: RE: Wuhan spike and N

https://science.sciencemag.org/content/309/5742/1864

The table in here has all details, also AA for palm civet, mouse, rat, and human

---

From: Munster, Vincent (NIH/NIAID) [E] (b)(6)
Sent: Saturday, January 11, 2020 8:55 AM
To: Letko, Michael (NIH/NIAID) [F] (b)(6) van Doremalen, Neeltje (NIH/NIAID) [E]
(b)(6)
Subject: Re: Wuhan spike and N
Importance: High

Hi Michael,

Ac you rapidly confirm that these are the ACE2 residues the SARS RBD binds too?

24	27	31	34	37	38	41	42	45	79	82	83	90	325	329	330	353	354
Q	T	K	H	E	D	Y	Q	L	L	M	Y	N	Q	E	N	K	G

Vincent Munster, PhD
Chief, Virus Ecology Section
Laboratory of Virology
Rocky Mountain Laboratories
NIAID/NIH

From: Michael Letko (b)(6)
Date: Saturday, January 11, 2020 at 12:51 AM
To: Neeltje van Doremalen (b)(6)
Cc: (b)(6)
Subject: Re: Wuhan spike and N

That last point should read:

"clade 2 and 3 only gain function with human ACE2 when given the clade 1 RBM"

--
Michael Letko, Ph.D
Postdoctoral IRTA
Dr. Vincent Munster Laboratory
Virus Ecology Unit, Laboratory of Virology
Rocky Mountain Laboratories
NIAID/NIH
903S 4th Street
Hamilton MT 59840
(b)(6)

On Jan 10, 2020, at 11:39 PM, Letko, Michael (NIH/NIAID) [F] (b)(6) wrote:

I expect to have the first cell entry results on January 26th or 27th. I think IDT can expedite synthesis of the RBD, which could save me a day or two. (We will also order full spike and N but those won't be in until early February.)

While we wait for the RBD results, here are some of my thoughts:

1. The Wuhan spike RBD is clade 1 and retains 11 of 14 contact residues with ACE2. Some of the contacts are not identical to SARS, but they are identical to other clade 1 RBDs, so they should work. I am predicting Wuhan will enter with ACE2.

These are the three contacts points that are different from clade 1. On the left are clade 1 identities and on the right are Wuhan residues. None of these changes really jumps out as a super dramatic change from what I have already tested.

Y/S/W-442-L
N/R/S-479-Q
Y/F-484-Q

2. The other variations are kind of interesting because:
(A) Two loops found in C-terminus of clade 1 RBD but absent in the other clades are present in Wuhan spike. However, these loops do look pretty different, with variation I haven't seen before. Not sure what to make of that yet, but these loops do not form any contacts with ACE2.

(B) Other variations in the N-terminus of Wuhan RBD can be found specifically in clade 2 and 3 but not 1.

3. In light of point 2B, Wuhan might be a recombinant form of clade 2/3 (or something similar) with the receptor binding MOTIF (RBM) from clade 1. Which is neat because I already have a figure in the manuscript showing that exact thing: clade 2 and 3 only gain function when given the clade 1 RBM.

Really exciting stuff!
-michael

—
Michael Letko, Ph.D
Postdoctoral IRTA
Dr. Vincent Munster Laboratory
Virus Ecology Unit, Laboratory of Virology
Rocky Mountain Laboratories
NIAID/NIH
903S 4th Street
Hamilton MT 59840
(b)(6)

On Jan 10, 2020, at 6:30 PM, van Doremalen, Neeltje (NIH/NIAID) [E] (b)(6) wrote:

Spike:
ATGTTTGTTTTTCTTGTTTTATTGCCACTAGTCTCTAGTCAGTGTGTTAATCTTACAACCAGAACTCAATTACCCCCTG
CATACACTAATTCTTTCACACGTGGTGTTTATTACCCTGACAAAGTTTTCAGATCCTCAGTTTTACATTCAACTCAGG
ACTTGTTCTTACCTTTCTTTTCCAATGTTACTTGGTTCCATGCTATACATGTCTCTGGGACCAATGGTACTAAGAGGT
TTGATAACCCTGTCCTACCATTTAATGATGGTGTTTATTTTGCTTCCACTGAGAAGTCTAACATAATAAGAGGCTGG
ATTTTTGGTACTACTTTAGATTCGAAGACCCAGTCCCTACTTATTGTTAATAACGCTACTAATGTTGTTATTAAAGTC
TGTGAATTTCAATTTTGTAATGATCCATTTTTGGGTGTTTATTACCACAAAAACAACAAAAGTTGGATGGAAAGTGA
GTTCAGAGTTTATTCTAGTGCGAATAATTGCACTTTTGAATATGTCTCTCAGCCTTTTCTTATGGACCTTGAAGGAA
AACAGGGTAATTTCAAAAATCTTAGGGAATTTGTGTTTAAGAATATTGATGGTTATTTTAAAATATATTCTAAGCAC
ACGCCTATTAATTTAGTGCGTGATCTCCCTCAGGGTTTTTCGGCTTTAGAACCATTGGTAGATTTGCCAATAGGTAT
TAACATCACTAGGTTTCAAACTTTACTTGCTTTACATAGAAGTTATTTGACTCCTGGTGATTCTTCTTCAGGTTGGAC
AGCTGGTGCTGCAGCTTATTATGTGGGTTATCTTCAACCTAGGACTTTTCTATTAAAATATAATGAAAATGGAACCA
TTACAGATGCTGTAGACTGTGCACTTGACCCTCTCTCAGAAACAAAGTGTACGTTGAAATCCTTCACTGTAGAAAA
AGGAATCTATCAAACTTCTAACTTTAGAGTCCAACCAACAGAATCTATTGTTAGATTTCCTAATATTACAAACTTGT

GCCCTTTTGGTGAAGTTTTTAACGCCACCAGATTTGCATCTGTTTATGCTTGGAACAGGAAGAGAATCAGCAACTG
TGTTGCTGATTATTCTGTCCTATATAATTCCGCATCATTTTCCACTTTTAAGTGTTATGGAGTGTCTCCTACTAAATTA
AATGATCTCTGCTTTACTAATGTCTATGCAGATTCATTTGTAATTAGAGGTGATGAAGTCAGACAAATCGCTCCAGG
GCAAACTGGAAAGATTGCTGATTATAATTATAAATTACCAGATGATTTTACAGGCTGCGTTATAGCTTGGAATTCTA
ACAATCTTGATTCTAAGGTTGGTGGTAATTATAATTACCTGTATAGATTGTTTAGGAAGTCTAATCTCAAACCTTTT
GAGAGAGATATTTCAACTGAAATCTATCAGGCCGGTAGCACACCTTGTAATGGTGTTGAAGGTTTTAATTGTTACT
TTCCTTTACAATCATATGGTTTCCAACCCACTAATGGTGTTGGTTACCAACCATACAGAGTAGTAGTACTTTCTTTTG
AACTTCTACATGCACCAGCAACTGTTTGTGGACCTAAAAAGTCTACTAATTTGGTTAAAAACAAATGTGTCAATTTC
AACTTCAATGGTTTAACAGGCACAGGTGTTCTTACTGAGTCTAACAAAAAGTTTCTGCCTTTCCAACAATTTGGCAG
AGACATTGCTGACACTACTGATGCTGTCCGTGATCCACAGACACTTGAGATTCTTGACATTACACCATGTTCTTTTG
GTGGTGTCAGTGTTATAACACCAGGAACAAATACTTCTAACCAGGTTGCTGTTCTTTATCAGGATGTTAACTGCACA
GAAGTCCCTGTTGCTATTCATGCAGATCAACTTACTCCTACTTGGCGTGTTTATTCTACAGGTTCTAATGTTTTTCAA
ACACGTGCAGGCTGTTTAATAGGGGCTGAACATGTCAACAACTCATATGAGTGTGACATACCCATTGGTGCAGGT
ATATGCGCTAGTTATCAGACTCAGACTAATTCTCCTCGGCGGGCACGTAGTGTAGCTAGTCAATCCATCATTGCCTA
CACTATGTCACTTGGTGCAGAAAATTCAGTTGCTTACTCTAATAACTCTATTGCCATACCCACAAATTTTACTATTAG
TGTTACCACAGAAATTCTACCAGTGTCTATGACCAAGACATCAGTAGATTGTACAATGTACATTTGTGGTGATTCAA
CTGAATGCAGCAATCTTTTGTTGCAATATGGCAGTTTTTGTACACAATTAAACCGTGCTTTAACTGGAATAGCTGTT
GAACAAGACAAAAACACCCAAGAAGTTTTTGCACAAGTCAAACAAATTTACAAAACACCACCAATTAAAGATTTTG
GTGGTTTTAATTTTTCACAAATATTACCAGATCCATCAAAACCAAGCAAGAGGTCATTTATTGAAGATCTACTTTTC
AACAAAGTGACACTTGCAGATGCTGGCTTCATCAAACAATATGGTGATTGCCTTGGTGATATTGCTGCTAGAGACC
TCATTTGTGCACAAAAGTTTAACGGCCTTACTGTTTTGCCACCTTTGCTCACAGATGAAATGATTGCTCAATACACTT
CTGCACTGTTAGCGGGTACAATCACTTCTGGTTGGACCTTTGGTGCAGGTGCTGCATTACAAATACCATTTGCTATG
CAAATGGCTTATAGGTTTAATGGTATTGGAGTTACACAGAATGTTCTCTATGAGAACCAAAAATTGATTGCCAACC
AATTTAATAGTGCTATTGGCAAAATTCAAGACTCACTTTCTTCCACAGCAAGTGCACTTGGAAAACTTCAAGATGTG
GTCAACCAAAATGCACAAGCTTTAAACACGCTTGTTAAACAACTTAGCTCCAATTTTGGTGCAATTTCAAGTGTTTT
AAATGATATCCTTTCACGTCTTGACAAAGTTGAGGCTGAAGTGCAAATTGATAGGTTGATCACAGGCAGACTTCAA
AGTTTGCAGACATATGTGACTCAACAATTAATTAGAGCTGCAGAAATCAGAGCTTCTGCTAATCTTGCTGCTACTAA
AATGTCAGAGTGTGTACTTGGACAATCAAAAAGAGTTGATTTTTGTGGAAAGGGCTATCATCTTATGTCCTTCCCTC
AGTCAGCACCTCATGGTGTAGTCTTCTTGCATGTGACTTATGTCCCTGCACAAGAAAAGAACTTCACAACTGCTCCT
GCCATTTGTCATGATGGAAAAGCACACTTTCCTCGTGAAGGTGTCTTTGTTTCAAATGGCACACACTGGTTTGTAAC
ACAAAGGAATTTTTATGAACCACAAATCATTACTACAGACAACACATTTGTGTCTGGTAACTGTGATGTTGTAATAG
GAATTGTCAACAACACAGTTTATGATCCTTTGCAACCTGAATTAGACTCATTCAAGGAGGAGTTAGATAAATATTTT
AAGAATCATACATCACCAGATGTTGATTTAGGTGACATCTCTGGCATTAATGCTTCAGTTGTAAACATTCAAAAAGA
AATTGACCGCCTCAATGAGGTTGCCAAGAATTTAAATGAATCTCTCATCGATCTCCAAGAACTTGGAAAGTATGAG
CAGTATATAAAATGGCCATGGTACATTTGGCTAGGTTTTATAGCTGGCTTGATTGCCATAGTAATGGTGACAATTA
TGCTTTGCTGTATGACCAGTTGCTGTAGTTGTCTCAAGGGCTGTTGTTCTTGTGGATCCTGCTGCAAATTTGATGAA
GACGACTCTGAGCCAGTGCTCAAAGGAGTCAAATTACATTACACATAA

N:
ATGTCTGATAATGGACCCCAAAATCAGCGAAATGCACCCCGCATTACGTTTGGTGGACCCTCAGATTCAACTGGCA
GTAACCAGAATGGAGAACGCAGTGGGGCGCGATCAAAACAACGTCGGCCCCAAGGTTTACCCAATAATACTGCGT
CTTGGTTCACCGCTCTCACTCAACATGGCAAGGAAGACCTTAAATTCCCTCGAGGACAAGGCGTTCCAATTAACAC
CAATAGCAGTCCAGATGACCAAATTGGCTACTACCGAAGAGCTACCAGACGAATTCGTGGTGGTGACGGTAAAAT
GAAAGATCTCAGTCCAAGATGGTATTTCTACTACCTAGGAACTGGGCCAGAAGCTGGACTTCCCTATGGTGCTAAC
AAAGACGGCATCATATGGGTTGCAACTGAGGGAGCCTTGAATACACCAAAAGATCACATTGGCACCCGCAATCCT
GCTAACAATGCTGCAATCGTGCTACAACTTCCTCAAGGAACAACATTGCCAAAAGGCTTCTACGCAGAAGGGAGC
AGAGGCGGCAGTCAAGCCTCTTCTCGTTCCTCATCACGTAGTCGCAACAGTTCAAGAAATTCAACTCCAGGCAGCA
GTAGGGGAACTTCTCCTGCTAGAATGGCTGGCAATGGCGGTGATGCTGCTCTTGCTTTGCTGCTGCTTGACAGATT

GAACCAGCTTGAGAGCAAAATGTCTGGTAAAGGCCAACAACAACAAGGCCAAACTGTCACTAAGAAATCTGCTGC
TGAGGCTTCTAAGAAGCCTCGGCAAAAACGTACTGCCACTAAAGCATACAATGTAACACAAGCTTTCGGCAGACG
TGGTCCAGAACAAACCCAAGGAAATTTTGGGGACCAGGAACTAATCAGACAAGGAACTGATTACAAACATTGGCC
GCAAATTGCACAATTTGCCCCCAGCGCTTCAGCGTTCTTCGGAATGTCGCGCATTGGCATGGAAGTCACACCTTCG
GGAACGTGGTTGACCTACACAGCTGCCATCAAATTGGATGACAAAGATCCAAATTTCAAAGATCAAGTCATTTTGC
TGAATAAGCATATTGACGCATACAAAACATTCCCACCAACAGAGCCTAAAAAGGACAAAAAGAAGAAGGCTGATG
AAACTCAAGCCTTACCGCAGAGACAGAAGAAACAGCAAACTGTGACTCTTCTTCCTGCTGCAGATTTGGATGATTT
CTCCAAACAATTGCAACAATCCATGAGCAGTGCTGACTCAACTCAGGCCTAA

Wuhan blasts closest (86%) to MG772933, a chinese SARS-like CoV from bats.

From: Munster, Vincent (NIH/NIAID) [E]
Sent: Sat, 11 Jan 2020 08:22:32 -0700
To: van Doremalen, Neeltje (NIH/NIAID) [E]; Letko, Michael (NIH/NIAID) [F]
Subject: Re: Wuhan spike and N

I contacted Jason McClellan, see whether he can use Jun's ACE2 sequences and make some inferences on the animal model and host breadth based on modelling rather than experimental,

We should be able to set this up ASAP to confirm potentially with pseudotypes, but I think we could sell without

Vincent Munster, PhD
Chief, Virus Ecology Section
Laboratory of Virology
Rocky Mountain Laboratories
NIAID/NIH

From: Neeltje van Doremalen (b)(6)
Date: Saturday, January 11, 2020 at 6:44 AM
To: Michael Letko (b)(6)
Cc: (b)(6)
Subject: Re: Wuhan spike and N

Cool Michael,

I came to the same conclusion with how many contact residues are different, and so did Vineet. We can assume that every coronavirus expert out there will try to show ACE2 usage of spike, but if you would get results so quickly we could have a competitive edge. Likely the Chinese will come out with an ACE2 paper with live virus. However - maybe we can do modelling with Jun's DPP4s and show entry for interesting candidates? Which DPP4s do we have in the lab, and could be tested for entry with your assay using Wuhan RBM? Are you planning on submitting your paper as soon as the Wuhan data is available?

Super exciting.

I still find the lack of H2H suspicious. People were infected over a 2 week period, which could of course all be animal to human but usually that's not super efficient, even with MERS the majority of cases still comes from H2H. With SARS, again H2H played a major role. If it truly was just animals at the market, they must have been shedding a ton of virus. I also have heard rumours there are two families involved.

Vincent - once we have the virus do you want bob/Trent to do stability studies? Include silver and/or copper.

The patient who passed was male, 61, and had chronic liver disease and abdominal tumours. He got severe pneumonia, went in coma, and died from multiple organ failure and cirrhosis. This is from an unofficial Chinese translation, I'm sure WHO will confirm to some extend, they have so far with all news

from that source. This means that a) the virus can be lethal b) severity of disease could be linked to age and underlying disease, potentially gender. Obviously very hard to conclude anything based on one case.

Neeltje

From: "Letko, Michael (NIH/NIAID) [F]" (b)(6)
Date: Saturday, January 11, 2020 at 12:51:51 AM
To: "van Doremalen, Neeltje (NIH/NIAID) [E]" (b)(6)
Cc: "Munster, Vincent (NIH/NIAID) [E]" (b)(6)
Subject: Re: Wuhan spike and N

That last point should read:

“clade 2 and 3 only gain function with human ACE2 when given the clade 1 RBM”

--
Michael Letko, Ph.D
Postdoctoral IRTA
Dr. Vincent Munster Laboratory
Virus Ecology Unit, Laboratory of Virology
Rocky Mountain Laboratories
NIAID/NIH
903S 4th Street
Hamilton MT 59840
(b)(6)

On Jan 10, 2020, at 11:39 PM, Letko, Michael (NIH/NIAID) [F] (b)(6) wrote:

I expect to have the first cell entry results on January 26th or 27th. I think IDT can expedite synthesis of the RBD, which could save me a day or two. (We will also order full spike and N but those won’t be in until early February.)

While we wait for the RBD results, here are some of my thoughts:

1. The Wuhan spike RBD is clade 1 and retains 11 of 14 contact residues with ACE2. Some of the contacts are not identical to SARS, but they are identical to other clade 1 RBDs, so they should work. I am predicting Wuhan will enter with ACE2.

These are the three contacts points that are different from clade 1. On the left are clade 1 identities and on the right are Wuhan residues. None of these changes really jumps out as a super dramatic change from what I have already tested.

Y/S/W-442-L
N/R/S-479-Q
Y/F-484-Q

2. The other variations are kind of interesting because:
(A) Two loops found in C-terminus of clade 1 RBD but absent in the other clades are present in Wuhan spike. However, these loops do look pretty different, with variation I haven't seen before. Not sure what to make of that yet, but these loops do not form any contacts with ACE2.

(B) Other variations in the N-terminus of Wuhan RBD can be found specifically in clade 2 and 3 but not 1.

3. In light of point 2B, Wuhan might be a recombinant form of clade 2/3 (or something similar) with the receptor binding MOTIF (RBM) from clade 1. Which is neat because I already have a figure in the manuscript showing that exact thing: clade 2 and 3 only gain function when given the clade 1 RBM.

Really exciting stuff!
-michael

—
Michael Letko, Ph.D
Postdoctoral IRTA
Dr. Vincent Munster Laboratory
Virus Ecology Unit, Laboratory of Virology
Rocky Mountain Laboratories
NIAID/NIH
903S 4th Street
Hamilton MT 59840
(b)(6)

On Jan 10, 2020, at 6:30 PM, van Doremalen, Neeltje (NIH/NIAID) [E] (b)(6) wrote:

Spike:
ATGTTTGTTTTTCTTGTTTTATTGCCACTAGTCTCTAGTCAGTGTGTTAATCTTACAACCAGAACTCAATTACCCCCTG
CATACACTAATTCTTTCACACGTGGTGTTTATTACCCTGACAAAGTTTTCAGATCCTCAGTTTTACATTCAACTCAGG
ACTTGTTCTTACCTTTCTTTTCCAATGTTACTTGGTTCCATGCTATACATGTCTCTGGGACCAATGGTACTAAGAGGT
TTGATAACCCTGTCCTACCATTTAATGATGGTGTTTATTTTGCTTCCACTGAGAAGTCTAACATAATAAGAGGCTGG

ATTTTTGGTACTACTTTAGATTCGAAGACCCAGTCCCTACTTATTGTTAATAACGCTACTAATGTTGTTATTAAAGTC
TGTGAATTTCAATTTTGTAATGATCCATTTTTGGGTGTTTATTACCACAAAAACAACAAAAGTTGGATGGAAAGTGA
GTTCAGAGTTTATTCTAGTGCGAATAATTGCACTTTTGAATATGTCTCTCAGCCTTTTCTTATGGACCTTGAAGGAA
AACAGGGTAATTTCAAAAATCTTAGGGAATTTGTGTTTAAGAATATTGATGGTTATTTTAAAATATATTCTAAGCAC
ACGCCTATTAATTTAGTGCGTGATCTCCCTCAGGGTTTTTCGGCTTTAGAACCATTGGTAGATTTGCCAATAGGTAT
TAACATCACTAGGTTTCAAACTTTACTTGCTTTACATAGAAGTTATTTGACTCCTGGTGATTCTTCTTCAGGTTGGAC
AGCTGGTGCTGCAGCTTATTATGTGGGTTATCTTCAACCTAGGACTTTTCTATTAAAATATAATGAAAATGGAACCA
TTACAGATGCTGTAGACTGTGCACTTGACCCTCTCTCAGAAACAAAGTGTACGTTGAAATCCTTCACTGTAGAAAA
AGGAATCTATCAAACTTCTAACTTTAGAGTCCAACCAACAGAATCTATTGTTAGATTTCCTAATATTACAAACTTGT
GCCCTTTTGGTGAAGTTTTTAACGCCACCAGATTTGCATCTGTTTATGCTTGGAACAGGAAGAGAATCAGCAACTG
TGTTGCTGATTATTCTGTCCTATATAATTCCGCATCATTTTCCACTTTTAAGTGTTATGGAGTGTCTCCTACTAAATTA
AATGATCTCTGCTTTACTAATGTCTATGCAGATTCATTTGTAATTAGAGGTGATGAAGTCAGACAAATCGCTCCAGG
GCAAACTGGAAAGATTGCTGATTATAATTATAAATTACCAGATGATTTTACAGGCTGCGTTATAGCTTGGAATTCTA
ACAATCTTGATTCTAAGGTTGGTGGTAATTATAATTACCTGTATAGATTGTTTAGGAAGTCTAATCTCAAACCTTTT
GAGAGAGATATTTCAACTGAAATCTATCAGGCCGGTAGCACACCTTGTAATGGTGTTGAAGGTTTTAATTGTTACT
TTCCTTTACAATCATATGGTTTCCAACCCACTAATGGTGTTGGTTACCAACCATACAGAGTAGTAGTACTTTCTTTTG
AACTTCTACATGCACCAGCAACTGTTTGTGGACCTAAAAAGTCTACTAATTTGGTTAAAAACAAATGTGTCAATTTC
AACTTCAATGGTTTAACAGGCACAGGTGTTCTTACTGAGTCTAACAAAAAGTTTCTGCCTTTCCAACAATTTGGCAG
AGACATTGCTGACACTACTGATGCTGTCCGTGATCCACAGACACTTGAGATTCTTGACATTACACCATGTTCTTTTG
GTGGTGTCAGTGTTATAACACCAGGAACAAATACTTCTAACCAGGTTGCTGTTCTTTATCAGGATGTTAACTGCACA
GAAGTCCCTGTTGCTATTCATGCAGATCAACTTACTCCTACTTGGCGTGTTTATTCTACAGGTTCTAATGTTTTTCAA
ACACGTGCAGGCTGTTTAATAGGGGCTGAACATGTCAACAACTCATATGAGTGTGACATACCCATTGGTGCAGGT
ATATGCGCTAGTTATCAGACTCAGACTAATTCTCCTCGGCGGGCACGTAGTGTAGCTAGTCAATCCATCATTGCCTA
CACTATGTCACTTGGTGCAGAAAATTCAGTTGCTTACTCTAATAACTCTATTGCCATACCCACAAATTTTACTATTAG
TGTTACCACAGAAATTCTACCAGTGTCTATGACCAAGACATCAGTAGATTGTACAATGTACATTTGTGGTGATTCAA
CTGAATGCAGCAATCTTTTGTTGCAATATGGCAGTTTTTGTACACAATTAAACCGTGCTTTAACTGGAATAGCTGTT
GAACAAGACAAAAACACCCAAGAAGTTTTTGCACAAGTCAAACAAATTTACAAAACACCACCAATTAAAGATTTTG
GTGGTTTTAATTTTTCACAAATATTACCAGATCCATCAAAACCAAGCAAGAGGTCATTTATTGAAGATCTACTTTTC
AACAAAGTGACACTTGCAGATGCTGGCTTCATCAAACAATATGGTGATTGCCTTGGTGATATTGCTGCTAGAGACC
TCATTTGTGCACAAAAGTTTAACGGCCTTACTGTTTTGCCACCTTTGCTCACAGATGAAATGATTGCTCAATACACTT
CTGCACTGTTAGCGGGTACAATCACTTCTGGTTGGACCTTTGGTGCAGGTGCTGCATTACAAATACCATTTGCTATG
CAAATGGCTTATAGGTTTAATGGTATTGGAGTTACACAGAATGTTCTCTATGAGAACCAAAAATTGATTGCCAACC
AATTTAATAGTGCTATTGGCAAAATTCAAGACTCACTTTCTTCCACAGCAAGTGCACTTGGAAAACTTCAAGATGTG
GTCAACCAAAATGCACAAGCTTTAAACACGCTTGTTAAACAACTTAGCTCCAATTTTGGTGCAATTTCAAGTGTTTT
AAATGATATCCTTTCACGTCTTGACAAAGTTGAGGCTGAAGTGCAAATTGATAGGTTGATCACAGGCAGACTTCAA
AGTTTGCAGACATATGTGACTCAACAATTAATTAGAGCTGCAGAAATCAGAGCTTCTGCTAATCTTGCTGCTACTAA
AATGTCAGAGTGTGTACTTGGACAATCAAAAAGAGTTGATTTTTGTGGAAAGGGCTATCATCTTATGTCCTTCCCTC
AGTCAGCACCTCATGGTGTAGTCTTCTTGCATGTGACTTATGTCCCTGCACAAGAAAAGAACTTCACAACTGCTCCT
GCCATTTGTCATGATGGAAAAGCACACTTTCCTCGTGAAGGTGTCTTTGTTTCAAATGGCACACACTGGTTTGTAAC
ACAAAGGAATTTTTATGAACCACAAATCATTACTACAGACAACACATTTGTGTCTGGTAACTGTGATGTTGTAATAG
GAATTGTCAACAACACAGTTTATGATCCTTTGCAACCTGAATTAGACTCATTCAAGGAGGAGTTAGATAAATATTTT
AAGAATCATACATCACCAGATGTTGATTTAGGTGACATCTCTGGCATTAATGCTTCAGTTGTAAACATTCAAAAAGA
AATTGACCGCCTCAATGAGGTTGCCAAGAATTTAAATGAATCTCTCATCGATCTCCAAGAACTTGGAAAGTATGAG
CAGTATATAAAATGGCCATGGTACATTTGGCTAGGTTTTATAGCTGGCTTGATTGCCATAGTAATGGTGACAATTA
TGCTTTGCTGTATGACCAGTTGCTGTAGTTGTCTCAAGGGCTGTTGTTCTTGTGGATCCTGCTGCAAATTTGATGAA
GACGACTCTGAGCCAGTGCTCAAAGGAGTCAAATTACATTACACATAA

N:

ATGTCTGATAATGGACCCCAAAATCAGCGAAATGCACCCCGCATTACGTTTGGTGGACCCTCAGATTCAACTGGCA
GTAACCAGAATGGAGAACGCAGTGGGGCGCGATCAAAACAACGTCGGCCCCAAGGTTTACCCAATAATACTGCGT
CTTGGTTCACCGCTCTCACTCAACATGGCAAGGAAGACCTTAAATTCCCTCGAGGACAAGGCGTTCCAATTAACAC
CAATAGCAGTCCAGATGACCAAATTGGCTACTACCGAAGAGCTACCAGACGAATTCGTGGTGGTGACGGTAAAAT
GAAAGATCTCAGTCCAAGATGGTATTTCTACTACCTAGGAACTGGGCCAGAAGCTGGACTTCCCTATGGTGCTAAC
AAAGACGGCATCATATGGGTTGCAACTGAGGGAGCCTTGAATACACCAAAAGATCACATTGGCACCCGCAATCCT
GCTAACAATGCTGCAATCGTGCTACAACTTCCTCAAGGAACAACATTGCCAAAAGGCTTCTACGCAGAAGGGAGC
AGAGGCGGCAGTCAAGCCTCTTCTCGTTCCTCATCACGTAGTCGCAACAGTTCAAGAAATTCAACTCCAGGCAGCA
GTAGGGGAACTTCTCCTGCTAGAATGGCTGGCAATGGCGGTGATGCTGCTCTTGCTTTGCTGCTGCTTGACAGATT
GAACCAGCTTGAGAGCAAAATGTCTGGTAAAGGCCAACAACAACAAGGCCAAACTGTCACTAAGAAATCTGCTGC
TGAGGCTTCTAAGAAGCCTCGGCAAAAACGTACTGCCACTAAAGCATACAATGTAACACAAGCTTTCGGCAGACG
TGGTCCAGAACAAACCCAAGGAAATTTTGGGGACCAGGAACTAATCAGACAAGGAACTGATTACAAACATTGGCC
GCAAATTGCACAATTTGCCCCCAGCGCTTCAGCGTTCTTCGGAATGTCGCGCATTGGCATGGAAGTCACACCTTCG
GGAACGTGGTTGACCTACACAGCTGCCATCAAATTGGATGACAAAGATCCAAATTTCAAAGATCAAGTCATTTTGC
TGAATAAGCATATTGACGCATACAAAACATTCCCACCAACAGAGCCTAAAAAGGACAAAAAGAAGAAGGCTGATG
AAACTCAAGCCTTACCGCAGAGACAGAAGAAACAGCAAACTGTGACTCTTCTTCCTGCTGCAGATTTGGATGATTT
CTCCAAACAATTGCAACAATCCATGAGCAGTGCTGACTCAACTCAGGCCTAA

Wuhan blasts closest (86%) to MG772933, a chinese SARS-like CoV from bats.

From: Munster, Vincent (NIH/NIAID) [E]
Sent: Sun, 06 Oct 2019 09:34:34 -0600
To: Letko, Michael (NIH/NIAID) [F]
Subject: Re: Definitely on the right track

Could be a nice computational analyses of all available spikes:

https://www.nature.com/articles/srep00261

you can do this with trypsin sites as well,

Vincent Munster, PhD
Chief, Virus Ecology Section
Laboratory of Virology
Rocky Mountain Laboratories
NIAID/NIH

From: Michael Letko (b)(6)
Date: Friday, October 4, 2019 at 4:01 PM
To: (b)(6)
Subject: Definitely on the right track

Vineet and Baric just put a paper on BioRXIV showing that trypsin treatment facilitates entry of both PDF2810-CoV and HKU5 into human cells.

See attachment.

Seems like more groups will be converging on this idea soon…

-michael

--
Michael Letko, Ph.D
Postdoctoral IRTA
Dr. Vincent Munster Laboratory
Virus Ecology Unit, Laboratory of Virology
Rocky Mountain Laboratories
NIAID/NIH
903S 4th Street
Hamilton MT 59840
(b)(6)

From: Munster, Vincent (NIH/NIAID) [E]
Sent: Sun, 06 Oct 2019 09:31:26 -0600
To: Letko, Michael (NIH/NIAID) [F]
Subject: Re: Definitely on the right track

So they focus on S rather than the receptor, but your work suggests more receptor activation, right?

You should be able to make S with a more broader cleavage signature (like furin-like) to enhance infectivity. You can include a motif like this R-X-[K/R]-R↓ Might be cool to get some synthesized

Are you using caco-2 cells as well, I think so but can't remember out of the top of my head

Vincent Munster, PhD
Chief, Virus Ecology Section
Laboratory of Virology
Rocky Mountain Laboratories
NIAID/NIH

From: Michael Letko (b)(6)
Date: Friday, October 4, 2019 at 4:01 PM
To: (b)(6)
Subject: Definitely on the right track

Vineet and Baric just put a paper on BioRXIV showing that trypsin treatment facilitates entry of both PDF2810-CoV and HKU5 into human cells.

See attachment.

Seems like more groups will be converging on this idea soon…

-michael

--
Michael Letko, Ph.D
Postdoctoral IRTA
Dr. Vincent Munster Laboratory
Virus Ecology Unit, Laboratory of Virology
Rocky Mountain Laboratories
NIAID/NIH
903S 4th Street
Hamilton MT 59840
(b)(6)

From: Munster, Vincent (NIH/NIAID) [E]
Sent: Fri, 04 Oct 2019 16:39:58 -0600
To: Letko, Michael (NIH/NIAID) [F]
Subject: Re: Definitely on the right track

Better get your stuff wrapped up!

Maybe smtg on trypsin rich environments on mucosal surfaces?

Vincent Munster, PhD
Chief, Virus Ecology Section
Laboratory of Virology
Rocky Mountain Laboratories
NIAID/NIH

From: Michael Letko (b)(6)
Date: Friday, October 4, 2019 at 4:01 PM
To: (b)(6)
Subject: Definitely on the right track

Vineet and Baric just put a paper on BioRXIV showing that trypsin treatment facilitates entry of both PDF2810-CoV and HKU5 into human cells.

See attachment.

Seems like more groups will be converging on this idea soon…

-michael

--
Michael Letko, Ph.D
Postdoctoral IRTA
Dr. Vincent Munster Laboratory
Virus Ecology Unit, Laboratory of Virology
Rocky Mountain Laboratories
NIAID/NIH
903S 4th Street
Hamilton MT 59840
(b)(6)

From: Müller, Marcel Alexander
Sent: Thu, 1 Aug 2019 09:08:54 +0000
To: Letko, Michael (NIH/NIAID) [F]
Cc: Munster, Vincent (NIH/NIAID) [E]
Subject: Re: [ext] FW: FedEx Shipment 437560785326: Scheduled delivery update
Attachments: Hoffmann.final journal.pone.0072942.pdf

Dear Michael,

that´s great news. When you thaw the cells, please use a small flask first (25 cm2) or even a 6-well. The cells do not like to be expanded rapidly at the beginning.
The cells were immortalized with large T antigen of SV40 so they should grow properly after a while. We use the cells max 25 passages as we saw that the properties like IFN competence changes over time.
Our current research focuses on bat innate immunity, autophagy and stress response. Please feel free to contact me if you want to collaborate in these fields.

Regarding your entry approaches, I think we should discuss things with (b)(6) (b)(6) I told Vincent already that we have quite a huge unpublished data set on (b)(4) (b)(4) (first results were already published long ago, see Hoffmann paper attached). After that first publication, (b)(6) (b)(4) (b)(4)

Good luck and let´s keep in contact!

Best, Marcel

—

PD Dr. Marcel A. Müller

Group leader, Institute of Virology
Charite - Universitätsmedizin Berlin

Campus Charite Mitte
Chariteplatz 1
D-10117 Berlin
Germany

Tel. (b)(6)
Fax +49 30 450 7525907

Email: (b)(6)

http://virologie-ccm.charite.de/

_____

Visiting/Postal address:
Charite - Universitätsmedizin Berlin
Institute of Virology (Helmut Ruska Haus)
Chariteplatz 1 (Rahel Hirsch Weg 3)
D-10117 Berlin
Germany

Von: "Letko, Michael (NIH/NIAID) [F]" (b)(6)
Datum: Mittwoch, 31. Juli 2019 um 20:27
An: "Müller, Marcel Alexander" (b)(6)
Cc: Vincent Munster (b)(6)
Betreff: Re: [ext] FW: FedEx Shipment 437560785326: Scheduled delivery update

Dear Dr. Müller,

The two vials of cells arrived today! I just transferred them into our liquid nitrogen tanks and will begin working with them next week. We will keep you posted with what we find.

Thank you again for sharing such a precious reagent with us.

Best,
-michael

--
Michael Letko, Ph.D
Postdoctoral IRTA
Dr. Vincent Munster Laboratory
Virus Ecology Unit, Laboratory of Virology
Rocky Mountain Laboratories
NIAID/NIH
903S 4th Street
Hamilton MT 59840
(b)(6)

From: "Müller, Marcel Alexander" (b)(6)
Date: Wednesday, July 31, 2019 at 1:39 AM
To: "Letko, Michael (NIH/NIAID) [F]" (b)(6)
Subject: Re: [ext] FW: FedEx Shipment 437560785326: Scheduled delivery update

Dear Micheal,

it is all on the Certificate of analysis that accompanies the cells.

It is standard DMEM with 10% FBS as described in Biesold et al 2011 Plos One. The FBS should not have too much endotoxins, otherwise the cells will die.
In case you encounter problems please contact me.

Best, Marcel

—
PD Dr. Marcel A. Müller

Group leader, Institute of Virology
Charite - Universitätsmedizin Berlin

Campus Charite Mitte
Chariteplatz 1
D-10117 Berlin
Germany

Tel. (b)(6)
Fax +49 30 450 7525907

Email: (b)(6)
http://virologie-ccm.charite.de/

Visiting/Postal address:
Charite - Universitätsmedizin Berlin
Institute of Virology (Helmut Ruska Haus)
Chariteplatz 1 (Rahel Hirsch Weg 3)
D-10117 Berlin
Germany

Von: "Letko, Michael (NIH/NIAID) [F]" (b)(6)
Datum: Dienstag, 30. Juli 2019 um 22:08
An: "Müller, Marcel Alexander" (b)(6)
Betreff: Re: [ext] FW: FedEx Shipment 437560785326: Scheduled delivery update

No problem!

Do you have any details on culturing these cells? Ie, media type, splitting density, etc.

Again, thank you so much,
-michael

--
Michael Letko, Ph.D
Postdoctoral IRTA
Dr. Vincent Munster Laboratory
Virus Ecology Unit, Laboratory of Virology
Rocky Mountain Laboratories

NIAID/NIH
903S 4th Street
Hamilton MT 59840
(b)(6)

From: "Müller, Marcel Alexander" (b)(6)
Date: Tuesday, July 30, 2019 at 3:03 AM
To: "Letko, Michael (NIH/NIAID) [F]" (b)(6)
Cc: "Munster, Vincent (NIH/NIAID) [E]" (b)(6) "Menk, Kay (NIH/NIAID) [E]" (b)(6)
Subject: Re: [ext] FW: FedEx Shipment 437560785326: Scheduled delivery update

Hi,

the FedEx no. is (b)(6)
Please keep in mind that most of our bat cell lines were generated under field conditions.
Although we previously tested the cells for mycoplasms, I would recommend to re-test them before experiments.
In general, I would also recommend to genotype them again after a few passages (@Vincent: the RE06 story has made me cautious 😉).
Species determination was not always very easy in the field.
If you discover discrepancies, please contact me immediately.

Best, Marcel

—
PD Dr. Marcel A. Müller

Group leader, Institute of Virology
Charite - Universitätsmedizin Berlin

Campus Charite Mitte
Chariteplatz 1
D-10117 Berlin
Germany

Tel. (b)(6)
Fax +49 30 450 7525907

Email: (b)(6)
http://virologie-ccm.charite.de/

Visiting/Postal address:
Charite - Universitätsmedizin Berlin

Institute of Virology (Helmut Ruska Haus)
Chariteplatz 1 (Rahel Hirsch Weg 3)
D-10117 Berlin
Germany

Von: "Letko, Michael (NIH/NIAID) [F]" (b)(6)
Datum: Montag, 29. Juli 2019 um 22:14
An: "Müller, Marcel Alexander" (b)(6)
Cc: Vincent Munster (b)(6) "Menk, Kay (NIH/NIAID) [E]"
(b)(6)
Betreff: Re: [ext] FW: FedEx Shipment 437560785326: Scheduled delivery update

This is great news!

Thank you again for all of your help and for the cells.

We will, of course, keep you updated.

Best,
-michael

—
Michael Letko, Ph.D
Postdoctoral IRTA
Dr. Vincent Munster Laboratory
Virus Ecology Unit, Laboratory of Virology
Rocky Mountain Laboratories
NIAID/NIH
903S 4th Street
Hamilton MT 59840
(b)(6)

On Jul 29, 2019, at 2:10 PM, Müller, Marcel Alexander (b)(6) wrote:

Hi Michael,
The cells were shipped today. I will pass on the tracking no tomorrow.
Best Marcel

—
PD Dr. Marcel A. Müller

Group leader, Institute of Virology
Charite - Universitätsmedizin Berlin

Campus Charite Mitte
Chariteplatz 1
D-10117 Berlin
Germany

Tel. (b)(6)
Fax +49 30 450 7525907

Email: (b)(6)
http://virologie-ccm.charite.de/

Am 29.07.2019 um 19:37 schrieb Letko, Michael (NIH/NIAID) [F] (b)(6)

Dear Dr. Mueller,

Do you require anything else from us to send the Rhinolophus cell lines?

Thank you again, we really appreciate it.

Best,
-michael

--
Michael Letko, Ph.D
Postdoctoral IRTA
Dr. Vincent Munster Laboratory
Virus Ecology Unit, Laboratory of Virology
Rocky Mountain Laboratories
NIAID/NIH
903S 4th Street
Hamilton MT 59840
(b)(6)

From: "Letko, Michael (NIH/NIAID) [F]" (b)(6)
Date: Wednesday, July 17, 2019 at 10:37 AM
To: (b)(6)
Cc: "Munster, Vincent (NIH/NIAID) [E]" (b)(6) "Menk, Kay (NIH/NIAID) [E]" (b)(6)
Subject: Re: [ext] FW: FedEx Shipment 437560785326: Scheduled delivery update

Dear Dr. Mueller,

I would like to thank you again for sharing your Rhinolophus cell lines.

Below is our lab's FedEx account number and address.

FedEx account #: (b)(6)
Attn: Michael Letko and Trent Bushmaker
Laboratory of Virology
Rocky Mountain Laboratories
NIAID/NIH
903S 4th street
Hamilton, MT 59840
(b)(6)

Please let me know if you need anything else.

Thank you,
-michael

--
Michael Letko, Ph.D
Postdoctoral IRTA
Dr. Vincent Munster Laboratory
Virus Ecology Unit, Laboratory of Virology
Rocky Mountain Laboratories
NIAID/NIH
903S 4th Street
Hamilton MT 59840
(b)(6)

From: "Munster, Vincent (NIH/NIAID) [E]" (b)(6)
Date: Friday, July 12, 2019 at 9:13 AM
To: "Thruston, Jeffrey (NIH/NIAID) [E]" (b)(6) "Müller, Marcel Alexander" (b)(6)
Cc: "Letko, Michael (NIH/NIAID) [F]" (b)(6)
Subject: Re: [ext] FW: FedEx Shipment 437560785326: Scheduled delivery update

Great job guys,

Marcel, as soon as your ready to ship discuss the logistics with Michael. They can be shipped using our fedex account,

Cheers,

Vincent Munster, PhD
Chief, Virus Ecology Unit
Laboratory of Virology
Rocky Mountain Laboratories
NIAID/NIH

From: "Thruston, Jeffrey (NIH/NIAID) [E]" (b)(6)
Date: Friday, July 12, 2019 at 6:13 AM
To: "Müller, Marcel Alexander" (b)(6)
Cc: (b)(6)
Subject: RE: [ext] FW: FedEx Shipment 437560785326: Scheduled delivery update

Dear Dr. Müller,

Thank you for the email. No, we have everything we need. The signed phsyical copies of the MTA are en route to you as we speak. You shold receive them on Monday, 15 July. NIAID can accept digital copies, so a signed PDF will suffice for our records. If you have any additional questions, please let me know.

Sincerely,

Jeff Thruston

Jeffrey T. Thruston, J.D., M.S.
Technology Transfer and Patent Specialist
Immunology & Emerging Infections Branch
TTIPO/NIAID/NIH
5601 Fishers Lane, Suite 2G46, MSC 9804
Rockville, MD 20892-9804
(b)(6)

The information in this e-mail and any of its attachments is confidential and may contain sensitive information. It should not be used by anyone who is not the original intended recipient. If you have received this e-mail in error, please inform the sender and delete it from your mailbox or any other storage devices. National Institute of Allergy and Infectious Diseases shall not accept liability for any statements made that are sender's own and not expressly made on behalf of the NIAID by one of its representatives.

From: Müller, Marcel Alexander (b)(6)
Sent: Friday, July 12, 2019 7:00 AM
To: Thruston, Jeffrey (NIH/NIAID) [E] (b)(6)
Cc: Munster, Vincent (NIH/NIAID) [E] (b)(6)
Subject: Re: [ext] FW: FedEx Shipment 437560785326: Scheduled delivery update

Dear Mr Thruston,

do you need any other statements from me?

Best, Marcel Müller

—
PD Dr. Marcel A. Müller

Group leader, Institute of Virology
Charite - Universitätsmedizin Berlin

Campus Charite Mitte
Charitеplatz 1
D-10117 Berlin
Germany

Tel. (b)(6)
Fax +49 30 450 7525907

Email: (b)(6)
http://virologie-ccm.charite.de/

Visiting/Postal address:
Charite - Universitätsmedizin Berlin
Institute of Virology (Helmut Ruska Haus)
Charitеplatz 1 (Rahel Hirsch Weg 3)
D-10117 Berlin
Germany

Von: Vincent Munster (b)(6)
Datum: Dienstag, 25. Juni 2019 um 13:29
An: "Müller, Marcel Alexander" (b)(6) "Thruston, Jeffrey (NIH/NIAID) [E]" (b)(6)
Betreff: Re: [ext] FW: FedEx Shipment 437560785326: Scheduled delivery update

Hi Marcel,

Absolutely no problem, no host interaction / genomic analyses are planned. But if this might change in the future we will discuss this with you and wait for you to approve.

Btw, if you would like to have some of our bsl4 pathogens done on these cells, just let me know. Very easy to do and then ship you the RNA.

Cheers,

Vincent Munster, PhD
Chief Virus Ecology Section
Rocky Mountain Laboratories, NIAID, NIH
903S 4th street, Hamilton, MT, USA
(b)(6)

From: "Müller, Marcel Alexander" (b)(6)
Date: Monday, June 24, 2019 at 4:14 PM
To: "Thruston, Jeffrey (NIH/NIAID) [E]" (b)(6)
Cc: (b)(6)
Subject: Re: [ext] FW: FedEx Shipment 437560785326: Scheduled delivery update

Dear Mr Thruston,

I included an additional sentence:
“Characterization of the designated cell lines by -omics approaches will not be done without prior consultation with the Provider.”

Is that OK Vincent? Sorry for being a little cautious here but better be safe than sorry, right?
Regarding the testing of the different spike proteins I talked to (b)(6) and he seems to be open for a collaboration.
Talk later.

Best, Marcel

—
PD Dr. Marcel A. Müller

Group leader, Institute of Virology
Charite - Universitätsmedizin Berlin

Campus Charite Mitte
Chariteplatz 1
D-10117 Berlin
Germany

Tel. (b)(6)
Fax +49 30 450 7525907

Email: (b)(6)
http://virologie-ccm.charite.de/

_____
Visiting/Postal address:
Charite - Universitätsmedizin Berlin
Institute of Virology (Helmut Ruska Haus)
Chariteplatz 1 (Rahel Hirsch Weg 3)
D-10117 Berlin
Germany

Von: "Thruston, Jeffrey (NIH/NIAID) [E]" (b)(6)
Datum: Freitag, 21. Juni 2019 um 22:14
An: "Müller, Marcel Alexander" (b)(6)

Cc: Vincent Munster (b)(6)
Betreff: FW: [ext] FW: FedEx Shipment 437560785326: Scheduled delivery update

Dear Dr. Müller,

I hope this email finds you well. I am writing to follow up on the attached, updated MTA redline with new virus list, per your request. Have you had an opportunity to review the attached document? If you have any questions or concerns, please let me know. Otherwise, I will start the signature process once I get your approval.

Sincerely,

Jeff Thruston

Jeffrey T. Thruston, J.D., M.S.
Technology Transfer and Patent Specialist
Immunology & Emerging Infections Branch
TTIPO/NIAID/NIH
5601 Fishers Lane, Suite 2G46, MSC 9804
Rockville, MD 20892-9804
(b)(6)

The information in this e-mail and any of its attachments is confidential and may contain sensitive information. It should not be used by anyone who is not the original intended recipient. If you have received this e-mail in error, please inform the sender and delete it from your mailbox or any other storage devices. National Institute of Allergy and Infectious Diseases shall not accept liability for any statements made that are sender's own and not expressly made on behalf of the NIAID by one of its representatives.

From: Thruston, Jeffrey (NIH/NIAID) [E]
Sent: Tuesday, June 4, 2019 4:04 PM
To: 'Müller, Marcel Alexander' (b)(6)
Subject: FW: [ext] FW: FedEx Shipment 437560785326: Scheduled delivery update
Importance: High

Dear Dr. Müller,

I hope this email finds you well. I am writing to follow up on the attached MTA redline, which has been updated to include the virus list you requested. If everything appears acceptable, I will initiate the signature process as soon as possible. If there is anything else that I may do to expedite the MTA, please let me know.

Sincerely,

Jeff Thruston

Jeffrey T. Thruston, J.D., M.S.
Technology Transfer and Patent Specialist
Immunology & Emerging Infections Branch
TTIPO/NIAID/NIH
5601 Fishers Lane, Suite 2G46, MSC 9804

Rockville, MD 20892-9804
(b)(6)

The information in this e-mail and any of its attachments is confidential and may contain sensitive information. It should not be used by anyone who is not the original intended recipient. If you have received this e-mail in error, please inform the sender and delete it from your mailbox or any other storage devices. National Institute of Allergy and Infectious Diseases shall not accept liability for any statements made that are sender's own and not expressly made on behalf of the NIAID by one of its representatives.

From: Thruston, Jeffrey (NIH/NIAID) [E]
Sent: Friday, May 24, 2019 9:44 AM
To: 'Müller, Marcel Alexander' (b)(6)
Subject: FW: [ext] FW: FedEx Shipment 437560785326: Scheduled delivery update

Dear Dr. Müller,

I hope this email finds you well. Have you had an opportunity to review the updated research plan in the MTA? The list of viruses has been updated, per your request. If you have any questions or concerns, please let me know.

Sincerely,

Jeff Thruston

Jeffrey T. Thruston, J.D., M.S.
Technology Transfer and Patent Specialist
Immunology & Emerging Infections Branch
TTIPO/NIAID/NIH
5601 Fishers Lane, Suite 2G46, MSC 9804
Rockville, MD 20892-9804
(b)(6)

The information in this e-mail and any of its attachments is confidential and may contain sensitive information. It should not be used by anyone who is not the original intended recipient. If you have received this e-mail in error, please inform the sender and delete it from your mailbox or any other storage devices. National Institute of Allergy and Infectious Diseases shall not accept liability for any statements made that are sender's own and not expressly made on behalf of the NIAID by one of its representatives.

From: Thruston, Jeffrey (NIH/NIAID) [E]
Sent: Friday, May 10, 2019 4:16 PM
To: Müller, Marcel Alexander (b)(6)
Subject: RE: [ext] FW: FedEx Shipment 437560785326: Scheduled delivery update

Dear Dr. Müller,

Thank you for the email. Attached, please find the updated MTA with complete list of viruses. Should you have any other questions, please let me know.

Sincerely,

Jeff Thruston

Jeffrey T. Thruston, J.D., M.S.
Technology Transfer and Patent Specialist
Immunology & Emerging Infections Branch
TTIPO/NIAID/NIH
5601 Fishers Lane, Suite 2G46, MSC 9804
Rockville, MD 20892-9804
(b)(6)

The information in this e-mail and any of its attachments is confidential and may contain sensitive information. It should not be used by anyone who is not the original intended recipient. If you have received this e-mail in error, please inform the sender and delete it from your mailbox or any other storage devices. National Institute of Allergy and Infectious Diseases shall not accept liability for any statements made that are sender's own and not expressly made on behalf of the NIAID by one of its representatives.

From: Müller, Marcel Alexander (b)(6)
Sent: Monday, May 6, 2019 10:14 AM
To: Thruston, Jeffrey (NIH/NIAID) [E] (b)(6)
Subject: Re: [ext] FW: FedEx Shipment 437560785326: Scheduled delivery update

Dear Mr Thruston,

please see attached the modifications in the Annex. Please include which viruses will be used for infection if possible.

The other MTA was signed and will be sent back to you this week.

Best,

Marcel Müller

—

PD Dr. Marcel A. Müller

Group leader, Institute of Virology
Charite - Universitätsmedizin Berlin

Campus Charite Mitte
Chariteplatz 1
D-10117 Berlin
Germany

Tel. (b)(6)
Fax +49 30 450 7525907

Email: (b)(6)
http://virologie-ccm.charite.de/

_____

Visiting/Postal address:
Charite - Universitätsmedizin Berlin
Institute of Virology (Helmut Ruska Haus)
Chariteplatz 1 (Rahel Hirsch Weg 3)
D-10117 Berlin
Germany

Von: "Thruston, Jeffrey (NIH/NIAID) [E]" (b)(6)
Datum: Donnerstag, 2. Mai 2019 um 16:07
An: "Müller, Marcel Alexander" (b)(6)
Betreff: FW: [ext] FW: FedEx Shipment 437560785326: Scheduled delivery update

Dear Dr. Müller,

I hope this email finds you well. Did you receive the partially executed MTA for (b)(6) Have you had an opportunity to review the attached MTA redline for Dr. Munster? If there is any information that you need from me, please let me know.

Sincerely,

Jeff Thruston

Jeffrey T. Thruston, J.D., M.S.
Technology Transfer and Patent Specialist
Immunology & Emerging Infections Branch
TTIPO/NIAID/NIH
5601 Fishers Lane, Suite 2G46, MSC 9804
Rockville, MD 20892-9804
(b)(6)

The information in this e-mail and any of its attachments is confidential and may contain sensitive information. It should not be used by anyone who is not the original intended recipient. If you have received this e-mail in error, please inform the sender and delete it from your mailbox or any other storage devices. National Institute of Allergy and Infectious Diseases shall not accept liability for any statements made that are sender's own and not expressly made on behalf of the NIAID by one of its representatives.

From: Thruston, Jeffrey (NIH/NIAID) [E]
Sent: Wednesday, April 24, 2019 12:30 PM
To: 'Müller, Marcel Alexander' (b)(6)
Subject: FW: [ext] FW: FedEx Shipment 437560785326: Scheduled delivery update

Dear Dr. Müller,

I hope this email finds you well. I am writing to follow up on two matters:

1. The attached MTA redline for Dr. Vincent Munster, and
2. The physical MTA for (b)(6) which arrived at Charite last week.

Have you received the physical MTA for signature? Have you had an opportunity to review the attached MTA redline? If you have any questions or concerns, please let me know.

Sincerely,

Jeff Thruston

Jeffrey T. Thruston, J.D., M.S.
Technology Transfer and Patent Specialist
Immunology & Emerging Infections Branch
TTIPO/NIAID/NIH
5601 Fishers Lane, Suite 2G46, MSC 9804
Rockville, MD 20892-9804
(b)(6)

The information in this e-mail and any of its attachments is confidential and may contain sensitive information. It should not be used by anyone who is not the original intended recipient. If you have received this e-mail in error, please inform the sender and delete it from your mailbox or any other storage devices. National Institute of Allergy and Infectious Diseases shall not accept liability for any statements made that are sender's own and not expressly made on behalf of the NIAID by one of its representatives.

From: Thruston, Jeffrey (NIH/NIAID) [E]
Sent: Wednesday, April 17, 2019 11:58 AM
To: Müller, Marcel Alexander (b)(6)
Subject: RE: [ext] FW: FedEx Shipment 437560785326: Scheduled delivery update

Dear Dr. Müller,

Thank you for the email. The MTA should arrive to you soon (FedEx has had some internal issues).

In parallel, I received a second MTA request through Charite covering bat cells for Dr. Vincent Munster – I have used the previous MTA as a template and updated the research description. However, I am not sure what the materials are, specifically. Please let me know if the attached is acceptable, and if you could provide a material description, that would be very helpful.

Sincerely,

Jeff Thruston

Jeffrey T. Thruston, J.D., M.S.
Technology Transfer and Patent Specialist
Immunology & Emerging Infections Branch
TTIPO/NIAID/NIH
5601 Fishers Lane, Suite 2G46, MSC 9804
Rockville, MD 20892-9804
(b)(6)

The information in this e-mail and any of its attachments is confidential and may contain sensitive information. It should not be used by anyone who is not the original intended recipient. If you have received this e-mail in error, please inform the sender and delete it from your mailbox or any other storage devices. National Institute of Allergy and Infectious Diseases shall not accept liability for any statements made that are sender's own and not expressly made on behalf of the NIAID by one of its representatives.

From: Müller, Marcel Alexander (b)(6)
Sent: Monday, April 15, 2019 9:12 AM
To: Thruston, Jeffrey (NIH/NIAID) [E] (b)(6)
Subject: Re: [ext] FW: FedEx Shipment 437560785326: Scheduled delivery update

Dear Mr Thruston,

you can use my address if you want to:

Charite - Universitätsmedizin Berlin
Institute of Virology (Helmut Ruska Haus)
PD Dr. Marcel A. Müller
Chariteplatz 1
Campus address: Rahel Hirsch Weg 3
D-10117 Berlin
Germany

Best, Marcel

—

Von: "Thruston, Jeffrey (NIH/NIAID) [E]" (b)(6)
Datum: Montag, 15. April 2019 um 14:45
An: "Müller, Marcel Alexander" (b)(6)
Betreff: [ext] FW: FedEx Shipment 437560785326: Scheduled delivery update

Dear Dr. Muller,

I just received the present update from FedEx regarding the status of the two original MTA we sent. It appears that the address within the MTA (and listed as the delivery address below) has been deemed incorrect by FedEx. How would you like for me to proceed?

Sincerely,

Jeff Thruston

From: TrackingUpdates@fedex.com <TrackingUpdates@fedex.com>
Sent: Monday, April 15, 2019 6:04 AM
To: Morgan, Cheri (NIH/NIAID) [C] (b)(6)
Subject: FedEx Shipment 437560785326: Scheduled delivery update

<image001.jpg>

Your delivery schedule has been updated to , .

See "Preparing for Delivery" for helpful tips

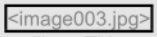
Tracking # 437560785326

Ship date:	<image002.jpg>	Scheduled delivery:
Thu, 4/11/2019	Delivery exception	Pending

FREIGHT FORWARDING SECTION NIH/TRANSPORTATION MANAGEMENT DIV. BETHESDA, MD 20892 US	(b)(6) CHARITE UNIVERSITATMEDIZINE BE CHARITEPLATZ 1 TECHNOLOGIENTRANSFER STELLE BERLIN, 10117 DE

Shipment Facts

Tracking number:	437560785326
Reference:	687104
Service type:	FedEx International Priority®
Packaging type:	FedEx® Envelope
Number of pieces:	1
Weight:	1.00 lb.
Special handling/Services:	Deliver Weekday
Standard transit:	4/15/2019 by 12:00 pm



Please do not respond to this message. This email was sent from an unattended mailbox. This report was generated at approximately 5:04 AM CDT on 04/15/2019.

All weights are estimated.

The shipment is scheduled for delivery on or before the scheduled delivery displayed above. FedEx does not determine money-back guarantee or delay claim requests based on the scheduled delivery. Please see the FedEx Service Guide for terms and conditions of service, including the FedEx Money-Back Guarantee, or contact your FedEx customer support representative.

To track the latest status of your shipment, click on the tracking number above.

Standard transit is the date and time the package is scheduled to be delivered by, based on the selected service, destination and ship date. Limitations and exceptions may apply. Please see the FedEx Service Guide for terms and conditions of service, including the FedEx Money-Back Guarantee, or contact your FedEx Customer Support representative.

© 2019 Federal Express Corporation. The content of this message is protected by copyright and trademark laws under U.S. and international law. Review our privacy policy. All rights reserved.

Thank you for your business.

PLOS ONE

Differential Sensitivity of Bat Cells to Infection by Enveloped RNA Viruses: Coronaviruses, Paramyxoviruses, Filoviruses, and Influenza Viruses

Markus Hoffmann[1], Marcel Alexander Müller[2], Jan Felix Drexler[2], Jörg Glende[1], Meike Erdt[1], Tim Gützkow[1], Christoph Losemann[1], Tabea Binger[2], Hongkui Deng[4], Christel Schwegmann-Weßels[1], Karl-Heinz Esser[3], Christian Drosten[2], Georg Herrler[1]*

1 Institute of Virology, University of Veterinary Medicine Hannover, Hannover, Germany, **2** Institute of Virology, University of Bonn Medical Centre, Bonn, Germany, **3** Institute of Zoology, University of Veterinary Medicine Hannover, Hannover, Germany, **4** Department of Cell Biology and Genetics, College of Life Sciences, Peking University, Beijing, P. R. China

Abstract

Bats (*Chiroptera*) host major human pathogenic viruses including corona-, paramyxo, rhabdo- and filoviruses. We analyzed six different cell lines from either *Yinpterochiroptera* (including African flying foxes and a rhinolophid bat) or *Yangochiroptera* (genera *Carollia* and *Tadarida*) for susceptibility to infection by different enveloped RNA viruses. None of the cells were sensitive to infection by transmissible gastroenteritis virus (TGEV), a porcine coronavirus, or to infection mediated by the Spike (S) protein of SARS-coronavirus (SARS-CoV) incorporated into pseudotypes based on vesicular stomatitis virus (VSV). The resistance to infection was overcome if cells were transfected to express the respective cellular receptor, porcine aminopeptidase N for TGEV or angiotensin-converting enzyme 2 for SARS-CoV. VSV pseudotypes containing the S proteins of two bat SARS-related CoV (Bg08 and Rp3) were unable to infect any of the six tested bat cell lines. By contrast, viral pseudotypes containing the surface protein GP of Marburg virus from the family *Filoviridae* infected all six cell lines though at different efficiency. Notably, all cells were sensitive to infection by two paramyxoviruses (Sendai virus and bovine respiratory syncytial virus) and three influenza viruses from different subtypes. These results indicate that bat cells are more resistant to infection by coronaviruses than to infection by paramyxoviruses, filoviruses and influenza viruses. Furthermore, these results show a receptor-dependent restriction of the infection of bat cells by CoV. The implications for the isolation of coronaviruses from bats are discussed.

Citation: Hoffmann M, Müller MA, Drexler JF, Glende J, Erdt M, et al. (2013) Differential Sensitivity of Bat Cells to Infection by Enveloped RNA Viruses: Coronaviruses, Paramyxoviruses, Filoviruses, and Influenza Viruses. PLoS ONE 8(8): e72942. doi:10.1371/journal.pone.0072942

Editor: Volker Thiel, Kantonal Hospital St. Gallen, Switzerland

Received January 21, 2013; **Accepted** July 16, 2013; **Published** August 30, 2013

Copyright: © 2013 Hoffmann et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This work was supported by grants to GH from Deutsche Forschungsgemeinschaft (SFB621 TP B7, SFB587 TP A1) and Bundesministerium für Bildung und Forschung (Ecology and Pathogenesis of SARS, an Archetypical Zoonosis, project code 01KI1005B and FluResearchNet, project code 01KI1006D). CD was supported by a grant from Bundesministerium für Bildung und Forschung (Ecology and Pathogenesis of SARS, an Archetypical Zoonosis, project code 01KIO701). 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.

* E-mail: georg.herrler@tiho-hannover.de

Introduction

Coronaviruses (order *Nidovirales*, family *Coronaviridae*) are enveloped positive-stranded RNA viruses that may be pathogenic for mammals and birds. According to a proposal to the International Committee of Taxonomy of Viruses (ICTV) this group of viruses is classified into three genera (*Alpha-, Beta-, and Gammacoronavirus*) [1]. *A* novel genus, *Deltacoronavirus*, has recently been accepted [2]. Alpha- and betacoronaviruses infect mammals, whereas gamma- and deltacoronaviruses have been detected first and foremost in birds. In the search for the origin of the severe acute respiratory syndrome (SARS) coronavirus (SARS-CoV), fecal samples of many different bats were found to contain coronaviral genomic RNA [3–9]. Bats have been hypothesized to act as the principal reservoir hosts for alpha- and betacoronaviruses [8,10]. Sequence analysis suggested that coronaviruses have succeeded to cross the species barrier to different mammalian species several times so that for example the different human coronaviruses OC43, 229E, SARS-CoV and the recently identified MERS-CoV (formerly designated HCoV-EMC) are the result of distinct interspecies transmission events that may be separated from each other by hundreds of years [11,12]. An enigma in the analysis of the spread of coronaviruses from its reservoir host to other species is the failure of all attempts so far to isolate an infectious virus from bats [3,4,6,7,11,13–17]. The reason for this is not clear. However, the surface protein Spike (S) appears to be responsible at least for the inability of bat coronaviruses to replicate in non-bat cells. A synthetic recombinant bat SARS-related coronavirus (SARSr-CoV) was able to infect primate or murine cells expressing the receptor for SARS-CoV, human angiotensin-converting enzyme 2 (hACE2) provided that the receptor binding domain in the bat S protein was replaced by that of the S protein of SARS-CoV [18].

The S protein is the largest glycoprotein of coronaviruses projecting from the viral envelope into the environmental space

[19]. Thus, it is responsible not only for the corona-like appearance of the surface projections when viewed under the electron microscope but also for the initial interaction of the virus with target cells. The S protein mediates the binding to the cellular receptor. Apart from the above mentioned hACE2 [20,21], other proteins that have been identified as receptors for coronaviruses are aminopeptidase N [22,23] for the alphacoronaviruses transmissible gastroenteritis virus (TGEV), feline enteric coronavirus (FECV), and HCoV-229E, and the carcinoembryonic antigen-related cell adhesion molecule 1a (CEACAM1a) for mouse hepatitis virus (MHV) [24]. In addition to binding to a defined protein receptor, some coronaviruses have a sialic acid-binding activity. For viruses like TGEV or avian infectious bronchitis virus (IBV) binding to sialylated macromolecules may not be sufficient for initiation of infection, however, it may increase the binding and infection efficiency [25–27]. Some coronaviruses like bovine coronavirus (BCoV) resemble influenza C virus by using N-acetyl-9-O-acetyl neuraminic acid ($Neu5,9Ac_2$) as a receptor determinant on cell surface macromolecules for binding to and infection of target cells [28,29]. Furthermore, they contain an acetylesterase activity that releases the 9-O-acetyl group from $Neu5,9Ac_2$ and thus is able to inactivate the receptor determinant [30]. This so-called receptor-destroying enzyme may – in analogy to influenza viruses – help to avoid binding events that do not result in infection, e.g. virus aggregation or binding to infected cells, and thus increase the spread of infection in the host.

Following binding to cell surface receptors, coronaviruses enter host cells by a fusion event that is also mediated by the S protein. With some coronaviruses, e.g. IBV and MHV, efficient fusion activity depends on proteolytic cleavage of the S protein into the subunits S1 and S2 by furin-like enzymes [31]. Other coronaviruses, e.g. SARS-CoV, TGEV, and HCoV-229E, contain the S protein on the viral surface in an uncleaved form. However, for these viruses, proteolytic activation may also be required because inhibitors of cathepsin L prevent the S protein from mediating infection [32]. This protease may act on viruses during virus entry, e.g. in the endosomal compartment. Recently, a human airway trypsin-like protease has also been implicated in the entry of SARS-CoV into respiratory epithelial cells [33].

Apart from coronaviruses, bats (order *Chiroptera*) have been shown to host a variety of emerging viruses [34,35], comprising different viral families like *Orthomyxoviridae* [36–38], *Rhabdoviridae* (especially the genus *Lyssavirus*) [39–41], *Paramyxoviridae* [42–46], *Filoviridae* [47–49], and others (see [34] for more information).

In order to isolate infectious coronaviruses from bats it is necessary to use appropriate cells, i.e. cells that are susceptible to infection. To identify such cells we applied a pseudotype approach to analyze the ability of two different S proteins from SARSr-CoV to mediate infection. These two SARSr-CoV termed Bg08 and Rp3 were identified previously by us and others in Europe and China, representing two distinct virus lineages within this CoV species [9,11]. A major species barrier for infection of bat cells was found to be at the level of cell surface receptors. Whereas bat cells were easily infected by paramyxoviruses and influenza viruses as well as by pseudotypes containing the glycoproteins of Marburg virus, the S proteins of SARS-CoV and TGEV were able to mediate infection only when the respective cellular receptor, human ACE2 or porcine APN, was expressed on the cell surface. Two S proteins of bat coronaviruses were unable to mediate infection of either of the bat cell lines analyzed.

Results

Infection of bat cells mediated by the S proteins of SARS-CoV or TGEV

Infection by coronaviruses is usually restricted to cells of the respective host or cells from related species. A major species barrier is the virus receptor on the surface of the target cell, e.g. hACE2, the receptor for SARS-CoV, and pAPN, the receptor for TGEV. We assessed whether this restriction is also valid for bat cells. For this purpose, a number of bat cells were analyzed whether they are susceptible to infection mediated by the S proteins of SARS-CoV or TGEV. The S protein of SARS-CoV was investigated with the help of the pseudotype system based on vesicular stomatitis virus (VSV). VSV pseudotypes containing SARS-CoV S protein are efficient in infecting Vero E6 cells [50–52]. As shown in Figure 1, out of six cell lines derived from the kidney (Ni) or lung (Lu) of *Yinpterochiroptera* (genera, *Rousettus* (Ro), *Hypsignathus* (Hyp), *Epomops* (Epo), or *Rhinolophus* (Rhi)) or *Yangochiroptera* (genera *Carollia* (Cp), *Tadarida* (Tb)), none was susceptible to SARS-CoV S-mediated infection (Figure 1). When the cells were transfeced with a plasmid for expression of hACE2, all cell lines became susceptible to infection as indicated by the GFP expression. Large differences were observed in the transfection efficiency as indicated by the percentage of hACE2-expressing cells which ranged from 5% (CpLu) to 50% (HypNi/1.1 and Tb 1 Lu). Among the hACE2-positive cells, about 10% were infected by pseudotypes containing the SARS-CoV S protein. The S protein of a porcine coronavirus, TGEV, was included in our analysis (Figure 2). Here, cells were not infected by pseudotypes but by the virus itself. Again, none of the bat cell lines was sensitive to infection. However, they became susceptible when pAPN was expressed on the cell surface. Infection was detected by staining for the presence of TGEV S protein. Interestingly, the staining pattern varied to a large extent depending on the cell line used. Bright staining distributed all over the cell was observed with HypNi/1.1 cells, while only a few fluorescent spots were detected in TGEV-infected EpoNi/22.1 cells expressing pAPN. This result shows that (i) TGEV infection of bat cells is restricted at the level of the cellular receptor, and (ii) there are large differences in the efficiency of the post-entry steps of the TGEV infection.

Infection mediated by the S proteins of bat coronaviruses

Having shown that infection of bat cells by human and porcine coronaviruses is restricted at the entry stage, we wanted to know whether such restrictions are also observed when S proteins of bat coronaviruses are analyzed for the ability to mediate infection. As no replication-competent bat coronavirus is available up to now, we used the VSV pseudotype system to investigate whether the S proteins of the bat-derived SARSr-CoV Bg08 and Rp3 are able to infect any of the bat cells. The S proteins of these two viruses were highly distinct from each other (75% amino acid identity) and about equally distinct from the corresponding protein in SARS-CoV (SARSr-CoV Rp3 S: 79% vs. SARSr-CoV Bg08 S: 75% amino acid identity). It was shown previously, that the RBD of the European SARSr-CoV Bg08 is more related to that of SARS-CoV than that of the Chinese virus Rp3, which in turn is more related to SARS-CoV in most other genomic regions [9,11]. In our comparative analysis, VSV G protein and the SARS-CoV S protein served as positive or negative controls, respectively. Pseudotypes containing the VSV G protein infected all cell lines, though at different efficiency (Figure 3). The low values determined in CpLu cells are due to the less efficient transfection

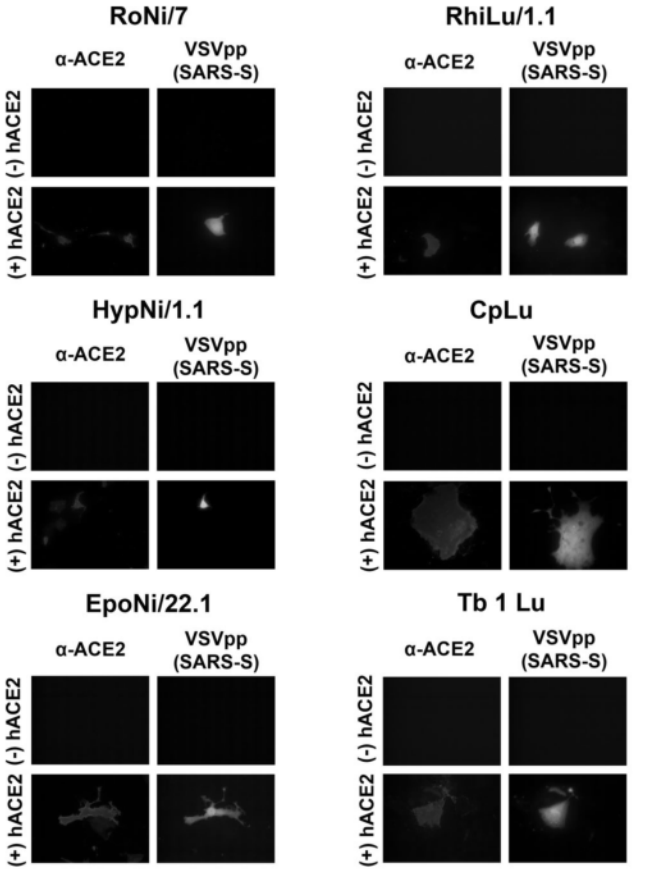


Figure 1. Sensitivity of bat cells to infection by VSV pseudotypes containing the S protein of SARS-CoV. Bat cells (RoNi/7, HypNi/1.1, EpoNi/22.1, RhiLu1.1, CpLu, Tb 1 Lu) were tranfected either with control plasmid (−hACE2) or with an expression plasmid for the human ACE2, the cellular receptor of SARS-CoV (+hACE2). At 24 h post transfection, the cells were infected with VSV pseutotyped with SARS-CoV SΔ18. Expression of hACE2 on the cell surface was detected by antibody staining, whereas VSV pseudotype infection was monitored by EGFP expression. All experiments were performed in triplicates and repeated three times.
doi:10.1371/journal.pone.0072942.g001

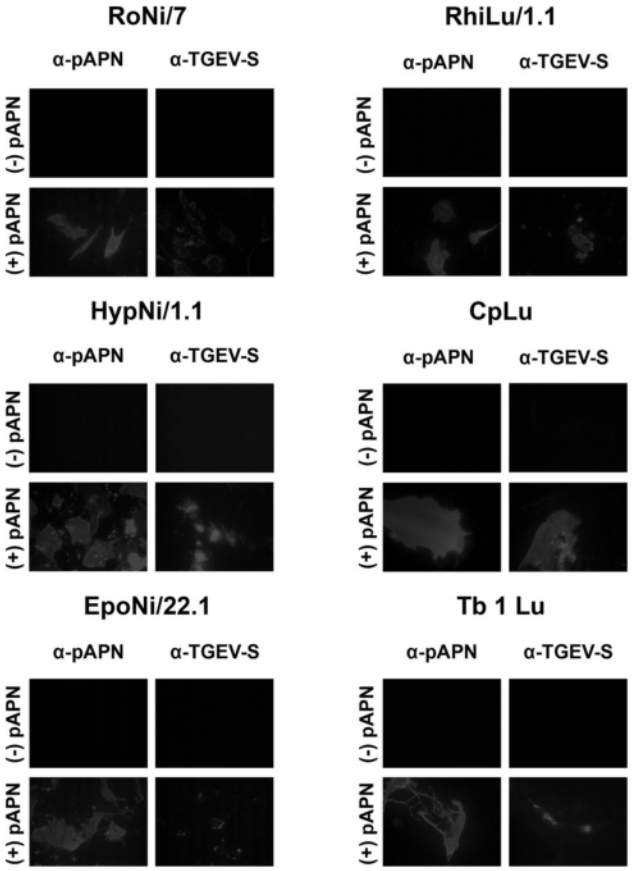


Figure 2. Sensitivity of bat cells to infection by TGEV. Bat cells (RoNi/7, HypNi/1.1, EpoNi/22.1, RhiLu/1.1, CpLu, Tb 1 Lu) were tranfected either with control plasmid (−pAPN) or with an expression plasmid for the porcine APN, the cellular receptor of TGEV (+pAPN). At 24 h post transfection, the cells were infected with TGEV. Expression of pAPN on the cell surface as well as intracellular TGEV antigen was detected by antibody staining. All tests were performed in triplicates and repeated three times.
doi:10.1371/journal.pone.0072942.g002

and the slower growth of these cells. On the other hand, the S protein of SARS-CoV was only able to mediate infection of Vero E6 cells whereas in all bat cells only background signals were observed. The S proteins of Bg08 and Rp3 were also found to be unable to infect either of the bat cells (Figure 3).

Infection mediated by the G protein of Marburg virus

A general restriction for virus entry can be ruled out as some of the applied bat cell lines (EpoNi/22.1 and HypNi/1.1) could be infected by VSV pseudotypes carrying Ebola virus glycoprotein [53]. As Marburg virus (MARV) was previously shown to be hosted by *Rousettus aegyptiacus* [48,54] we extended the previous study by analyzing the surface protein GP of the related Marburg virus with a larger panel of bat cells. As shown in Figure 4, the filovirus glycoprotein mediated infection of all cells analyzed. Infection efficiency varied but this variation was also observed with the VSV G protein. In general, titres determined with the MARV GP were comparable to those obtained with the VSV G protein. Thus, in contrast to surface proteins of coronaviruses, the filovirus surface glycoprotein is able to infect bat cells in the context of a VSV pseudotype system.

Interaction of coronaviral S proteins with human and chiropteran ACE2

After having shown that the inability of the SARS-CoV S and the TGEV S protein to mediate infection of chiropteran cells can be overcome when the respective receptor (hACE2 or pAPN, respectively) is expressed, we analysed whether expression of bat ACE2 renders cells susceptible to infection mediated by the S protein of coronaviruses. For this purpose, we compared the ability of VSVpp harboring the S proteins of SARS-CoV or either of the two SARSr-CoV, Bg08 and Rp3, to utilize human or rhinolophid ACE2 for initiating infection. The ACE2 coding sequence of the RhiLu/1.1 cell line, obtained from *Rhinolophus alcyone*, was analyzed for its ability to enable the entry of VSVpp. VSV pseudotyped with VSV G or MARV GP were able to infect BHK-21 cell, irrespective of ACE2 expression. SARS-CoV S mediated VSVpp infection of BHK-21 cells expressing hACE2. Interestingly, *Rhinolophus alcyone* ACE2 (RhiLu/1.1_ACE2) was very efficiently used for SARS-CoV S-driven pseudotype entry (Fig. 5). The infectivity mediated by RhiLu/1.1_ACE2 was almost as efficient as in the case of BHK-21 cells expressing hACE2. The S proteins of Bg08 and Rp3 were unable to mediate infection of cells expressing either hACE2 or bat ACE2.

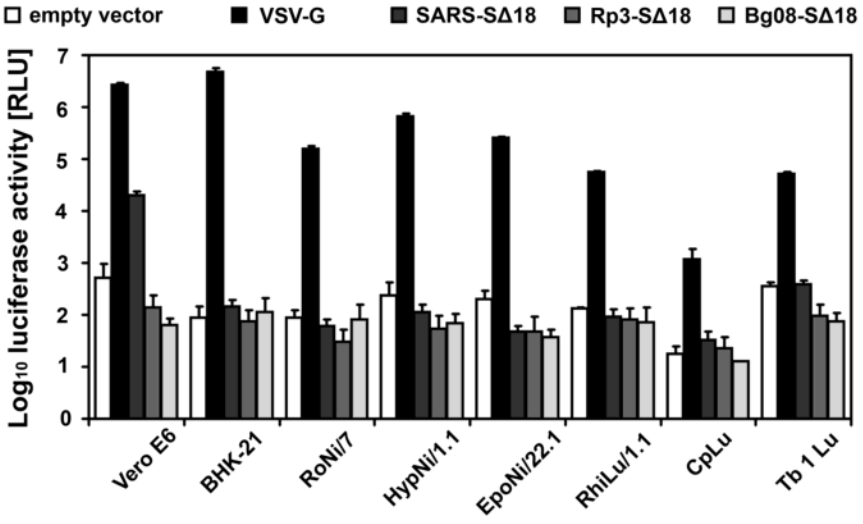


Figure 3. Susceptibility of bat cell lines to infection mediated by the S proteins of two bat-derived SARSr-CoVs, Rp3 and Bg08. VSV pseudotyped with either SARS-CoV SΔ18 (SARS SΔ18), SARSr-CoV Rp3 SΔ18 (Rp3 SΔ18), or SARSr-CoV Bg08 SΔ18 (Bg08 SΔ18) were applied to confluent bat cells and infection efficiency was determined by measuring the luciferase activity 18 h p.i.. VSV pseudotypes generated with VSV G (VSV G) or with an empty pCG1 vector alone (empty vector) served as positive and negative controls, respectively. All tests were performed in quadruplicates and the data shown are the result of three independent experiments.
doi:10.1371/journal.pone.0072942.g003

To address the question whether the SARSr-CoV S protein is functional in a virus-free assay or can achieve functional activity after protease treatment, a finding that has been described for SARS-CoV S [33,55–60], we performed a cell-based fusion assay, in which BHK-21 cells were co-transfected with combinations of expression plasmids for CoV S with a carboxyterminal DsRed-tag and different ACE2s. After transfection, cells were treated with trypsin. The presence of the two proteins was verified by fluorescence microscopy following immunostaining (ACE2). Trypsin-treated SARS-CoV S is able to induce fusion of the S-expressing cells with ACE2 expressing cells resulting in the formation of syncytia [55,56]. We observed that SARS-CoV S was able to mediate fusion following trypsin-treatment, only with cells expressing hACE2 or RhiLu/1.1_ACE2 (Fig. 6a), as indicated by the detection for multinucleated cells that were positive for both, SARS-CoV S and the respective ACE2. In contrast, neither untreated nor trypsin-treated SARSr-CoV Bg08 S resulted in the formation of syncytia when co-expressed with either of the ACE2 proteins (Fig. 6b). Control experiments with cells expressing ACE2 proteins only did not reveal any syncytia formation (data not shown).

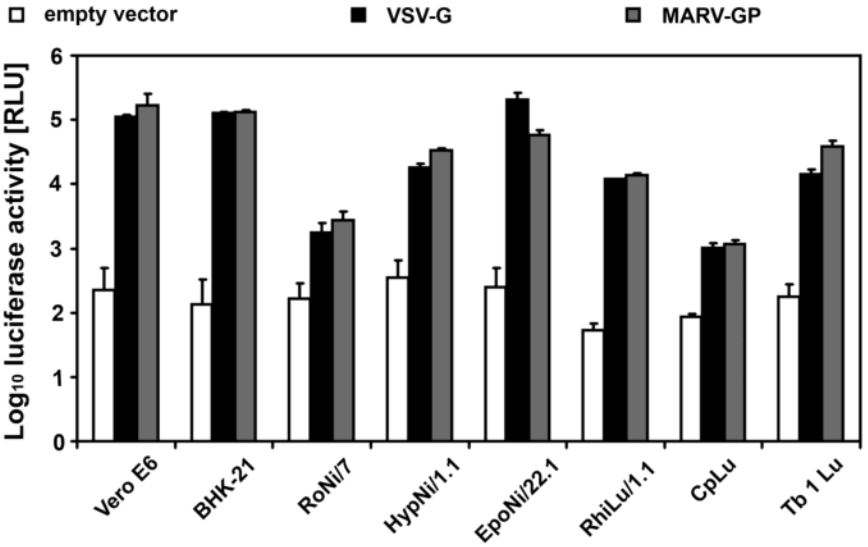


Figure 4. Sensitivity of bat cell lines to infection mediated by the GP of MARV. VSV pseudotypes containing VSV G (VSV G), MARV GP (MARV GP), or empty pCG1 vector alone (empty vector) were used to infect confluent bat cells. Infection was evaluated at 18 h p.i. by measuring the luciferase acticvity. Data shown are the result of three independent experiments.
doi:10.1371/journal.pone.0072942.g004

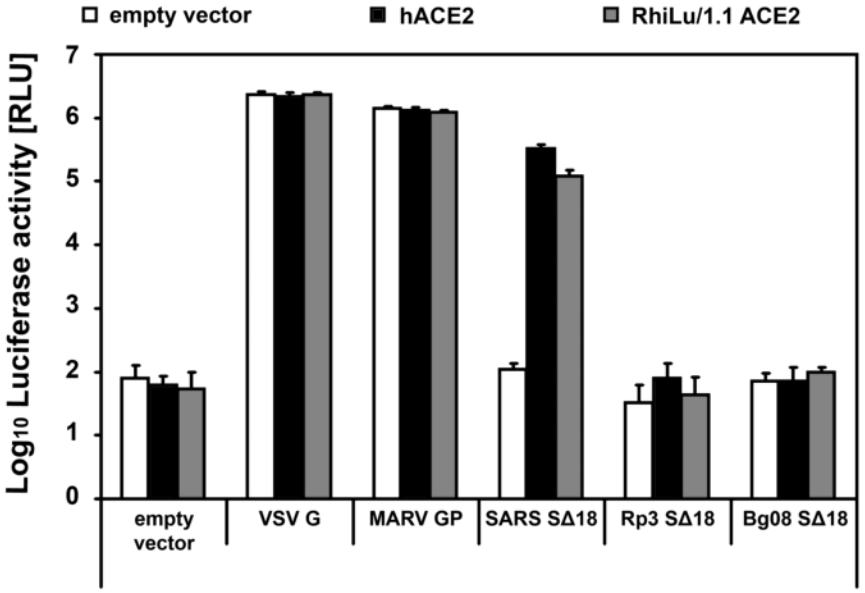


Figure 5. Analysis of the ability of human or RhiLu/1.1_ACE2 to serve as an entry receptor for VSV pseudotypes harboring SARS-CoV S, SARSr-CoV Rp3 S, or SARSr-CoV Bg08 S. BHK-21 cells, grown in white, opaque-walled 96well plates were transfected with expression plasmids for human (hACE2), *Rhinolophus alcyone* ACE2 (RhiLu/1.1_ACE2), or empty pCG1 vector alone (empty vector) prior to infection with VSV pseudotypes harboring either VSV G (VSV G), MARV GP (MARV GP), SARS-CoV SΔ18 (SARS SΔ18), SARSr-CoV Rp3 S Δ18 (Rp3 SΔ18), or SARSr-CoV Bg08 SΔ18 (Bg08 SΔ18). VSV pseudotypes generated with empty pCG1 vector alone (empty vector) served as a negative control. All tests were performed in quadruplicates and the data shown were obtained from three independent experiments.
doi:10.1371/journal.pone.0072942.g005

Infection of bat cells by paramyxoviruses and influenza viruses

Having shown that bat cells are susceptible to infection by VSV if an appropriate surface glycoprotein is incorporated into the viral envelope we analyzed whether other enveloped RNA viruses are able to infect bat cells. For this purpose we chose paramyxoviruses and influenza viruses. Recent data suggest that bats may also serve as a natural reservoir for paramyxoviruses [46]. As shown in Figure 7, the paramyxoviruses used in our study, bovine respiratory syncytial virus and Sendai virus, efficiently infected all six bat cell lines (RoNi/7, EpoNi/22.1, HypNi/1.1, RhiLu/1.1, CpLu, and Tb 1 Lu). Influenza viruses were also included in our study. A recent report demonstrated the presence of an influenza A virus in bats captured in Guatemala [37]. For our infection study with influenza viruses, we chose two low-pathogenic avian strains belonging to the subtypes H7N7 and H9N2 and a porcine H1N1 virus. These viruses differ in their recognition of sialic acid with the H7N7 virus having a preference for α2,3-linked sialic acid and the H1N1 virus showing preferential binding to α2,6-linked sialic acid. The H9N2 virus recognizes both linkage types. Despite the difference in the sialic acid binding activity, all three influenza viruses showed a similar infection pattern (Figure 7). RoNi/7, HypNi/1.1, EpoNi/22.1, and CpLu cells were highly sensitive to infection with almost all cells of the monolayer showing viral antigen. RhiLu/1.1 and Tb 1 Lu cells were also infected by influenza viruses, but less efficiently as indicated by the lower number of cells showing viral antigen. This result indicates that the general resistance of bat cells to infection by coronaviruses is not observed when influenza and paramyxoviruses are analyzed.

Discussion

We have shown that the S proteins of two SARSr-CoV are unable to mediate infection of bat cells derived from different species of *Yinptero-* and *Yangochiroptera*. The resistance of the bat cells is not due to the pseudotype system used because (i) pseudotypes containing the VSV G protein or the G protein of Marburg virus were found to be quite efficient in infecting the different bat cells and (ii) in the case of the S protein of SARS-CoV, the resistance of the bat cells to infection by pseudotypes was overcome after the cells had been treated to express the receptor for SARS-CoV, hACE2. One might argue that the lack of infection in the case of the bat CoV S protein may be related to an inefficient particle production or to a functional impairment of the S protein due to the deletion of the carboxyterminal amino acids of the cytoplamic tail. This deletion was found to be favourable for the function of the S proteins of SARS-CoV and TGEV in VSV and lentiviral pseudotype systems [52,61–63]. We do not know what is the effect of such a deletion in the case of the S proteins of bat CoV but the above experiments were also performed with native S proteins and the result was the same, i.e the S protein of SARS-CoV infected hACE2-expressing cells whereas the S proteins of the two bat SARSr-CoV were unable to mediate infection (not shown). As far as particle production is concerned, Western blot analysis of pseudotype particles in the cell supernatant indicated that the nucleoprotein N of VSV was present in similar amounts in pseudotype preparations irrespective of the source of the S protein (not shown). A receptor-dependent resistance to infection was also observed when the cells were infected with an infectious coronavirus, TGEV. Susceptibility of bat cells to infection by TGEV was dependent on the expression of porcine APN on the surface of the bat cells. These results suggest that the failure of the two S proteins of bat CoV to mediate infection in the context of VSV pseudotypes reflects a property of the viral glycoproteins rather than a problem of the pseudotype system. The inability of the Rp3 S protein to mediate the entry process has also been reported by others [15] and may be explained by a strict species specificity of bat coronaviruses. There

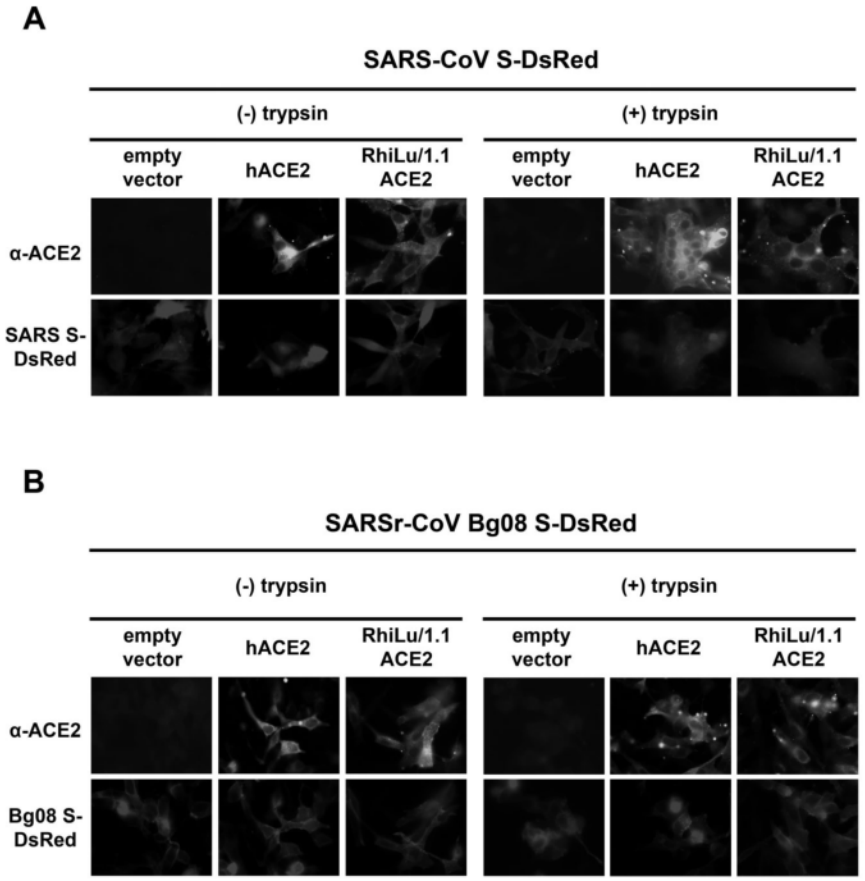


Figure 6. Effect of trypsin-treatment on the ability of SARS-CoV S and SARSr-CoV Bg08 S to induce syncytia formation when co-expressed with human or RhiLu/1.1_ACE2. BHK-21 cells, grown on coverslips were co-transfected with different combinations of expression plasmids for either (a) SARS-CoV S (SARS-CoV S-DsRed), or (b) SARSr-CoV Bg08 S (SARSr-CoV Bg08 S-DsRed), and ACE2 molecules of human (hACE2) or chiropteran (*Rhinolophus alcyone*, RhiLu/1.1_ACE2) origin. In both cases, empty pCG1 vector served as controls (pCG1). At 24 h post transfection, cells were either treated with medium containing trypsin (+ trypsin) to enable proteolytic activation of the CoV S or were left untreated (- trypsin). Subsequently, ACE2 was stained by antibody incubation (α-ACE2) and screened for syncytia formation by fluorescence microscopy. All tests were performed in triplicates and repeated three times.
doi:10.1371/journal.pone.0072942.g006

are ecological studies suggesting host-restricted distribution of bat coronaviruses at the species level or at the genus level [3,7,11]. It is not known which step in the replication cycle is responsible for the host-restriction. If virus entry is the limiting step, our results would suggest that the S proteins interact only with receptors from cells of the same species but not of the same genus. The S proteins used were derived from *R. blasii* and *R. sinicus*, respectively. Cells of horseshoe bats, in our study, were derived from the species *R. alcyone* and they were resistant to S-mediated infection. An alternative explanation for the inability of the S proteins of bat coronaviruses to mediate infection may be the lack or insufficient expression of the cellular receptor on the surface of the bat cells. In this context it is interesting that, recently, SARS-CoV has been reported to use the ACE2 of different bats as a receptor for virus entry into Hela cells [64] which is confirmed by our results shown in Fig. 5. At present, it is not known whether bat coronaviruses use ACE2 or another protein as a receptor for infection. Immunostaining did not reveal detectable amounts of endogenous ACE2 expression in the bat cells analysed here. Transfected cells expressing ACE2 of RhiLu1.1 cells were also resistant to infection mediated by the S proteins of bat coronaviruses. As long as the identity of the cellular receptor for bat coronaviruses is not known, inefficient expression of this receptor would also explain why the two S proteins failed to mediate infection. This explanation may be applied also to other cellular factors required for virus entry, e.g post-adsorption (fusion) receptors or proteases required for induction of the fusion activity of the S proteins. The recently identified coronavirus MERS-CoV appears to differ from other human coronaviruses by a less restricted cell tropism [65]. It remains to be shown whether this property has arisen during transmission to human hosts and only applies to the virus of the diseased patients or whether it is also a characteristic of related bat viruses.

Our findings and the above mentioned explanations have important implications. They may explain why up to now all attempts to isolate infectious bat coronaviruses have failed [3,4,6,7,11,13–17]. As a consequence, future attempts to isolate such viruses should consider that not all available bat cells are suitable host cells. Pseudotypes containing the S protein of bat coronaviruses should be helpful to identify cells susceptible to infection. Five of the cell lines used in this study were immortalized by SV40 large T antigen. CpLu cells were spontaneously immortalized. In the future, it may by worthwhile, to include primary cells in the analysis. As the respiratory and intestinal epithelium is a common target for many coronaviruses, it should be interesting to find out whether differentiated airway or

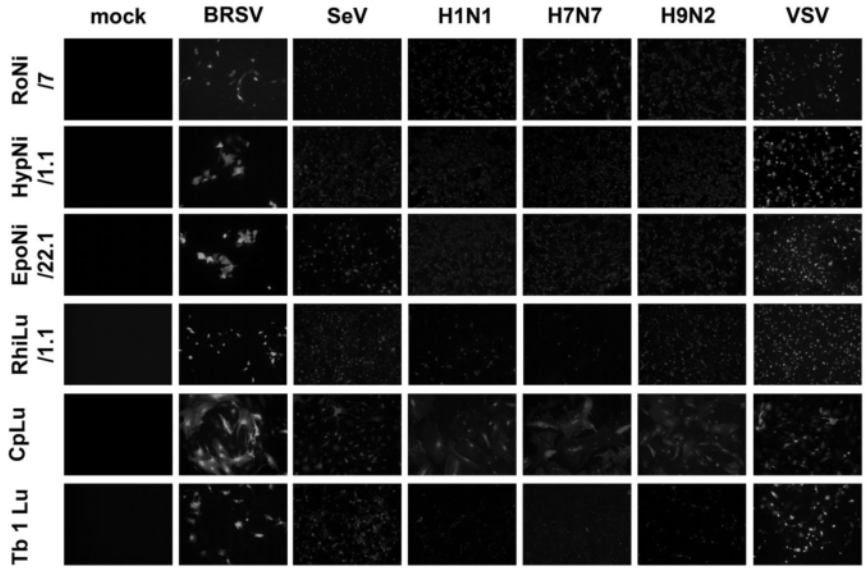


Figure 7. Sensitivity of bat cells to infection by paramyxoviruses and influenza viruses. Bat cells were grown on coverslips and subjected to infection by either BRSV(GFP) (BRSV), SeV(DsRed) (SeV), influenza A viruses of the subtypes H1N1 (H1N1), H7N7 (H7N7), and H9N2 (H9N2), as well as VSV(GFP) (VSV). Infection was monitored at 24 h or in case of BRSV(GFP) at 48 h p.i. via fluorescence microscopy either directly (BRSV(GFP), SeV(dsRed), VSV(GFP)) or after antibody staining of viral antigen (H1N1, H7N7, H9N2). In all cases, uninfected cells served as controls (mock). All tests were performed in triplicates and repeated three times.
doi:10.1371/journal.pone.0072942.g007

intestinal epithelial cells are susceptible to S-mediated infection. This would indicate that the receptor is expressed upon differentiation of the cells. Alternatively, cell surface expression may be age-dependent which might be one explanation why genomic coronavirus RNA was detected more frequently in fecal samples from young animals than from adult bats [3].

The choice of cells to be used for isolation of bat coronaviruses is not only dependent on the presence of appropriate receptors. Intracellular factors may also be critical for a productive replication cycle. This is evident from the infection by TGEV. The expression of porcine APN was sufficient for virus entry, but the subsequent course of infection was quite different. In some cells viral antigen was distributed all over the cytoplasm, whereas in others expression of viral proteins was restricted to dot-like structures.

Infection of bat cells by filoviruses, paramyxoviruses, and influenza viruses appears not to be as restricted as infection by coronaviruses. This finding is interesting because for filoviruses and paramyxoviruses recent data suggest that bats may serve as a natural reservoir [46,49]. Bats may also be a host for influenza viruses as indicated by a recent report where a virus strain – from bats captured in Central America – was identified and has been tentatively assigned to a new subtype, H17 [37]. Our results suggest that transmission of filoviruses, paramyxoviruses and influenza viruses from bats to new hosts appears to be less restricted at the level of cellular receptors than is transmission of coronaviruses. Infection of bat cells from different species by Ebola virus has been demonstrated [53] and our pseudotype experminents with the G protein of Marburg virus confirm and extend these results. Evidently, the receptor interaction of filoviruses is not species-specific. The reason for the different infection efficiency observed among the cells analyzed is not known. Future studies have to find out whether this difference is accounted for by a species-dependent recognition of the receptor or by the expression level of the cellular receptor for these viruses. In the case of influenza viruses and paramyxoviruses, infection was observed with all cells analyzed. For the different influenza viruses and for Sendai virus, successful infection can be explained by their use of sialic acid as a receptor determinant for infection of cells. Evidently, both α2,3-linked and α2,6-linked sialic acids are present on the surface of the cells.

Taken together, our results show that many bat cell lines are resistant to infection mediated by the S proteins of SARSr-CoV; viral pseudotypes can be used to identify susceptible cells and thus may help to isolate infectious SARSr-CoV from bats.

Materials and Methods

Cells and viruses

BHK-21 cells were maintained in Eagle's minimum essential medium (EMEM; Gibco) supplemented with 5% fetal calf serum (FCS; Biochrom) and 1% penicillin/streptomycin (PAA), Vero E6 and all bat cell lines were maintained in Dulbecco's minimal essential medium (DMEM; Gibco) supplemented with 10% FCS and 1% penicillin/streptomycin. The bat cell lines additionally were supplemented with 1 mM sodium pyruvate (PAA). All cells were cultivated in 75 cm^2 tissue culture flasks (Greiner Bio-One) at 37°C and 5% CO_2, and passaged 1:4 (CpLu, Tb 1 Lu) or 1:10 when they had reached 90% confluency. Bat cell lines from the following species were used in our studies: *Rousettus aegyptiacus* (kidney-derived; RoNi/7), *Hypsignathus monstrosus* (kidney-derived; HypNi/1.1), *Epomops buettikoferi* (kidney-derived; EpoNi/22.1), *Rhinolophus alcyone* (lung-derived; RhiLu/1.1, subcloned cell line), *Carollia perspicillata* (lung-derived; CpLu), and *Tadarida brasiliensis* (lung-derived; Tb 1 Lu; provided by Friedrich-Loeffler-Institut, Insel Riems, Germany). RoNi/7 (mixed cell culture), HypNi/1.1, EpoNi/22.1 (both subcloned cell lines) were generated from mechanically and enzymatically separated organ samples as described previously [53,66], RhiLu/1.1, and CpLu cell lines were prepared accordingly. Apart from CpLu cells, all other bat cell lines were immortalized by the SV40 large T antigen [67]. RoNi/7, HypNi/1.1, EpoNi/22.1, and RhiLu/1.1 cell lines were

generated from bats caught in Ghana. For all capturing, sacrificing, and sampling, permission was obtained from the Wildlife Division, Forestry Commission, Accra, Ghana. Samples were exported under a state contract between the Republic of Ghana and the Federal Republic of Germany, and under an additional export permission from the Veterinary Services of the Ghana Ministry of Food and Agriculture (permit no. CHRPE49/09; A04957). Under the auspices of Ghana authorities bats were caught with mist nets, anaesthetized with a Ketamine/Xylazine mixture and euthanized to perform organ preparations (permit no. CHRPE49/09; A04957). The CpLu cell line was generated from a *C. perspicillata* breeding colony maintained at the institute of zoology (University of Veterinary Medicine Hannover), for research purposes. The keeping and breeding of *Carollia perspicillata* was approved by the Landeshauptstadt Hannover, Fachbereich Recht und Ordnung, Gewerbe und Veterinärangelegenheiten (No. 42500/1H). The corresponding cell line is derived from postmortem tissue of bats sacrificed for other purposes. Animals were sacrificed (permit No. 11/0435) by cervical dislocation in deep inhalation anaesthesia (halothane).

In the present study we employed a vesicular stomatitis virus (VSV) pseudotype system that is based on a replication-deficient VSV replicon in which the open reading frame (ORF) of the VSV glycoprotein (VSV G) has been replaced by two individual ORFs for an enhanced green fluorescent protein (EGFP) and a firefly luciferase (Luc). This replicon (VSV*ΔG-Luc) has been constructed in collaboration with G. Zimmer [68] and has already been used in other studies [68–70].

Replication-competent viruses used for infection of bat cell lines were recombinant VSV that codes for an EGFP in an additional ORF between the VSV G and VSV L genes (VSV(GFP)), provided by G. Zimmer. Recombinant bovine respiratory syncytial virus that codes for an EGFP, BRSV(GFP), has been described recently [71]. Recombinant Sendai virus encoding dsRed, SeV(dsRed), has been described by Zimmer et al. [72]. The three influenza viruses used in this study comprise one porcine strain of the subtype H1N1 (A/swine/Potsdam/15/81) and two avian strains of the subtypes H7N7 (A/duck/Potsdam/15/80) and H9N2 (A/chicken/Saudi Arabia/CP7/98). The Purdue strain of TGEV was provided by Luis Enjuanes.

Expression plasmids

The expression plasmid for the cellular receptor of SARS-CoV, human angiotensin converting enzyme 2 (ACE2, NM_021804.2, pCG1-hACE2) was constructed from the pcDNA3.1-hACE2 expression plasmid which was kindly provided by E. Snijder, and cloned into the pCG1 vector (kindly provided by R. Cattaneo) using *Xba*I and *Sal*I restriction sites. Additionally, we succesfully isolated and cloned the ACE2 coding sequence of the RhiLu/1.1 cell line, derived from *Rhinolophus alcyone* (as mentioned in the next section). The sequence will be submitted to GenBank. The coding sequence of the cellular receptor of TGEV, porcine aminopeptidase N (pAPN) was obtained by *reverse-transcription* (RT-) PCR of mRNA from ST (swine testicular) cells followed by primer-specific PCR. The PCR product was cloned into the pCG1 vector using *BamH*I and *Sal*I restriction sites. The sequence will be submitted to GenBank. The pcDNA3.1-VSV G as a positive control for VSV pseudotype preparation was provided by G. Zimmer; the pCAGGS-MARV GP construct was provided by S. Becker. For production of VSV pseudotypes harboring SARS-CoV and SARSr-CoV S we generated expression plasmids based on the pCG1 vector (pCG1-SARS CoV SΔ18, pCG1-SARSr-CoV Rp3 SΔ18, and pCG1-SARSr-CoV Bg08 SΔ18) using *BamH*I and *Xba*I restriction sites. In these CoV S proteins, the cytoplasmic tail regions were truncated by 18 amino acids, a feature that has been shown previously to result in an increased incoporation of CoV S into VSV pseudotypes [52]. The pCG1-SARS-CoV SΔ18, pCG1-SARSr-CoV Rp3 SΔ18 (from *Rhinolophus sinicus*) possess a tissue plasminogen activator signal sequence instead of the original signal peptide to promote CoV S cell surface expression [73,74]. The pCG1-SARSr-CoV Bg08 SΔ18 was generated based on SARSr-CoV RNA that has been detected in fecal samples of a horseshoe bat (*Rhinolophus blasii*, BtCoV/BM48-31/Bulgaria/2008) from Bulgaria [11]. Additionally, DsRed-linked constructs of full-lenght SARS-CoV S (Frankfurt-1 isolate, AY291315.1, pCG1-SARS-CoV S-DsRed) and SARSr-CoV Bg08 S (pCG1-SARSr-CoV Bg08 S-DsRed) were generated for the cell-based fusion assay. Here, the respective CoV S was carboxyterminally fused to a non-flexible linker sequence (amino acid sequence: GPDPPVAT) and the coding sequence for a DsRed protein (EU827527.1).

Cloning of chiropteran ACE2 from permanent bat cell lines

RhiLu/1.1 cells were used for RNA extraction using the RNeasy extraction kit (Qiagen) according to the manifecture's protocol. The concentration of the extracted RNA was quantified by an Eppendorf BioPhotometer Plus (Eppendorf) and directly used for RT-PCR using the SuperScript III First-Strand Synthesis System (Life Technologies). The synthesis of cDNA was performed following the instructions for an oligo dT-based RT-PCR as mentioned in the manifacture's protocol. Subsequently, 5 µl of the cDNA were used for an ACE2-specific PCR using the Phusion polymerase (Thermo Scientific). The primers for this PCR were designed on the basis of Hou *et al.* [64] (forward: CTTTCTAGAATGTCAGGCTCTTYCTGG/reverse: CCGGTCGACCTAAAABGAVGTCTGAACATCATC). The PCR was performed under the following conditions: 2 min at 98°C/40× (20 sec at 98°C/20 sec at 52°C/3 min at 72°C)/10 min at 72°C/storage until further use at 4°C. The success of the cloning was determined by gel electrophoresis. A band of the calculated size of ACE2 was obtained. Next, the DNA fragment was cloned into the pCG1 vector using the *BamH*I and *Sal*I restriction sites. To exclude mutations that were generated during the cloning process, at least 4 different clones were sequenced.

Antibodies and reagents

Transfection of BHK-21 cells was performed using *Lipofectamine2000 reagent (Life Technologies), while* transfection of bat cell lines was carried out with the help of *Lipofectamine2000* LTX (*Life Technologies*) and addition of PLUS reagent (*Life Technologies*), both according to the manifacturer's protocol.

For detection of TGEV S and pAPN we used antibodies raised in mice (anti-S 6A.C3 kindly provided by L. Enjuanes; 1:250; anti-pAPN G43 kindly provided by H. Laude; 1:250). For the detection of ACE2 a polyclonal goat antibody (R&D Systems, 1:250) was used. Infection of bat cell lines by influenza A viruses was analyzed using a monoclonal antibody directed against the viral nucleoprotein (NP) (Serotec; 1:750). For fluorescence-labeling, Cy3-linked anti-mouse (Sigma Aldrich, 1:750), Cy3-linked anti-goat (Sigma Aldrich, 1:750), and FITC-labeled anti-goat (Sigma Aldrich, 1:750) antibodies were used. All antibodies were diluted in phosphate buffered saline (PBS) containing 1% bovine serum albumin.

Generation of replication-incompetent VSV pseudotypes

To investigate whether CoV S proteins are able to mediate infection of bat cells we used a VSV pseudotype system that has been described elsewhere [70] with slight modifications.

BHK-21 cells were seeded in Ø 10 cm cell culture dishes (Greiner Bio-One). As soon as they had reached 75% confluency they were transfected with 10 µg of pCG1-SARS-CoV SΔ18, pCG1-SARSr-CoV Rp3 SΔ18, pCG1-SARSr-CoV Bg08 SΔ18, pCG1-MARV GP, pcDNA3.1-VSV G (positive control), or pCG1 (negative control). At 16 h post transfection the supernatant was removed, cells were washed three times with PBS, and infected by VSV pseudotypes that were transcomplemented with VSV G, VSV*ΔG-Luc, at an MOI of 3 for 1 h. Subsequently, the inoculum was removed, cells were washed three times with PBS, and neutralization of residual input virus was carried out using a polyclonal anti-VSV serum raised in rabbit at a dilution of 1:1,000. After incubation for 1 h at 37°C and 5% CO_2, the neutralization medium was removed, cells were washed three times with PBS, and EMEM supplemented with 3% FCS was added. The VSVpp were harvested 16–20 h later, by collecting the supernatant followed by centrifugation (3.500× g) to remove cell debris, and kept at 4°C for up to one week.

Susceptibility of bat cell lines to virus infection

The different bat cell lines were seeded in 24-well plates (Greiner Bio-One) containing coverslips (Roth, immunofluorescence analysis) or white, opaque-walled 96-well plates (Greiner Bio-One, luciferase assay) and grown to 75% confluency before they were subjected to infection. To investigate the importance of the surface expression of different ACE2s or pAPN for infection by VSVpp or TGEV, respectively, cells were grown to 50% confluency and transfection with either pCG1-hACE, pCG1-RhiLu/1.1_ACE2, pCG1-pAPN, or empty pCG1 vector (negative control) was performed 24 h prior to infection. Cells grown in 96well plates were transfected with 0.3 µg, while cells grown on coverslips where transfected with 1 µg of the respective expression plasmid.

For infection, the medium was removed by aspiration, cells were washed three times with PBS, and infected with 1×10^5 ffu/ml of TGEV, BRSV(GFP), SeV(dsRed), H1N1, H7N7, H9N2, or VSV(GFP). Alternatively, cells were infected with undiluted VSVpp harboring either VSV G, MARV GP, SARS-CoV SΔ18, SARSr-CoV Rp3 SΔ18, SARSr-CoV Bg08 SΔ18, or no glycoprotein at all (pCG1, empty vector). After 1 h of incubation at 37°C and 5% CO_2 under slight agitation, the inoculum was removed, the cells were washed three times with PBS and EMEM containing 2% FCS and 1% methylcellulose (Sigma Aldrich) was applied to reduce spread of the replication-competent viruses. In the case of the replication-deficient VSVpp, DMEM containing 2% FCS was used for further incubation.

Detection of infection by immunofluorescence analysis (IFA) and fluorescence microscopy

At 24 h (in case of BRSV(GFP) at 48 h) post infection (p.i.), the cell culture supernatant was removed by aspiration and the cells were washed three times with PBS before they were fixed by incubation with 3% paraformaldehyde in PBS for 20 min at room temperature (RT). After fixation, the cells were washed three times with PBS and residual paraformaldehyde was quenched by incubation with 0.1 M glycine in PBS for 30 min at RT. For intracellular detection of TGEV S and influenza virus NP, cells were permeabilized with methanol/acetone (1:1, v/v) for 30 sec at RT, followed by washing with PBS. Antibody incubation was carried out in humidity chambers with one drop of antibody solution between a strip of parafilm and the cells on the coverslip. After 1 h of incubation at RT, the coverslips were transferred back to 24-well plates, washed three times with PBS and incubated with a fluorescent dye-labeled secondary antibody under the same conditions. If the cells were transfected to express hACE2 or pAPN for studying the infection of VSVpp(SARS-CoV S Δ18) or TGEV, additional antibody staining was performed with antibodies directed against the respective receptor molecules under the same conditions. Subsequently, the cells were washed and the nuclei were stained by incubation with DAPI (Roth, 1 µg/ml ethanol) for 10 min at 37°C. Subsequently the cells were washed one time with PBS, 3 times with *aqua dest.*, and mounted with mowiol. Fluorencence microscopy was performed using the Nikon Eclipse Ti and the NIS Elements AR software (Nikon).

Quantification of VSVpp infection

Quantification of the infection of bat cell lines by the different VSVpp was carried out (i) by observation of EGFP-positive cells under the fluorescence microscope or (ii) by determining the luciferase activity:

(i) VSVpp infected cells were fixed 18 h p.i. and representative fluorescence microscopical pictures were taken (data not shown). (ii) The cell culture supernatant was removed at 18 h p.i. by aspiration and the cells were lysed by incubation with luciferase cell culture lysis reagent (Promega) for 30 min at RT on an orbital shaker. Subsequently, freshly prepared luciferase substrate from the luciferase assay system (Promega) was added and the emitted light signal was measured after 1 min of incubation using a Chemiluminometer (TECAN).

Cell-based fusion assay

BHK-21 cells were seeded in 24-well plates containing coverslips to reach 50% confluency the next day. Then, the cells were co-transfected with combinations of pCG1-SARS-CoV S-DsRed, pCG1-SARSr-CoV Bg08 S-DsRed, or empty pCG1 vector (negative control) and different ACE2s of human (pCG1-hACE2) or chiropteran (pCG1-RhiLu/1.1_ACE2) origin. At 24 h post transfection, the cells were washed 3 times with serum-free medium and either incubated with fusion medium (DMEM +2 µg/ml acetylated trypsin, Sigma Aldrich) or non-fusion medium (DMEM) for 4 h, before the cells were washed again three times and further incubated for 16–18 h with DMEM containing 3% FCS. At this point, the cells were fixed, permeabilized (as described before), and ACE2 expression was confirmed by antibody staining. The screening for the formation of syncytia was performed using the Nikon Eclipse Ti fluorescence microscope (Nikon) and representative pictures were taken.

Acknowledgments

Part of this work was performed by M.H. in partial fulfillment of the requirements for a Dr. rer. nat. degree from Leibniz Universität Hannover.

Author Contributions

Conceived and designed the experiments: MH GH. Performed the experiments: MH MAM JFD JG ME TG CL TB. Analyzed the data: MH MAM JFD HD CSW KHE CD GH. Contributed reagents/materials/analysis tools: MAM JFD HD CSW KHE CD. Wrote the paper: MH GH.

References

1. de Groot RJ, Baker SC, Baric R, Enjuanes L, Gorbalenya AE, et al. (2011) Family Coronaviridae. In: King AMQ, Lefkowitz E, Adams MJ, Carstens EB, editors. Virus Taxonomy: Ninth Report of the International Committee on Taxonomy of Viruses: Elsevier Science. pp. 806–828.
2. Adams MJ, Carstens EB (2012) Ratification vote on taxonomic proposals to the International Committee on Taxonomy of Viruses (2012). Arch Virol 157: 1411–1422.
3. Gloza-Rausch F, Ipsen A, Seebens A, Gottsche M, Panning M, et al. (2008) Detection and prevalence patterns of group I coronaviruses in bats, northern Germany. Emerg Infect Dis 14: 626–631.
4. Lau SK, Woo PC, Li KS, Huang Y, Tsoi HW, et al. (2005) Severe acute respiratory syndrome coronavirus-like virus in Chinese horseshoe bats. Proc Natl Acad Sci U S A 102: 14040–14045.
5. Pfefferle S, Oppong S, Drexler JF, Gloza-Rausch F, Ipsen A, et al. (2009) Distant relatives of severe acute respiratory syndrome coronavirus and close relatives of human coronavirus 229E in bats, Ghana. Emerg Infect Dis 15: 1377–1384.
6. Poon LL, Chu DK, Chan KH, Wong OK, Ellis TM, et al. (2005) Identification of a novel coronavirus in bats. J Virol 79: 2001–2009.
7. Tang XC, Zhang JX, Zhang SY, Wang P, Fan XH, et al. (2006) Prevalence and genetic diversity of coronaviruses in bats from China. J Virol 80: 7481–7490.
8. Vijaykrishna D, Smith GJ, Zhang JX, Peiris JS, Chen H, et al. (2007) Evolutionary insights into the ecology of coronaviruses. J Virol 81: 4012–4020.
9. Li W, Shi Z, Yu M, Ren W, Smith C, et al. (2005) Bats are natural reservoirs of SARS-like coronaviruses. Science 310: 676–679.
10. Woo PC, Lau SK, Huang Y, Yuen KY (2009) Coronavirus diversity, phylogeny and interspecies jumping. Exp Biol Med (Maywood) 234: 1117–1127.
11. Drexler JF, Gloza-Rausch F, Glende J, Corman VM, Muth D, et al. (2010) Genomic characterization of severe acute respiratory syndrome-related coronavirus in European bats and classification of coronaviruses based on partial RNA-dependent RNA polymerase gene sequences. J Virol 84: 11336–11349.
12. Annan A, Baldwin HJ, Corman VM, Klose SM, Owusu M, et al. (2013) Human betacoronavirus 2c EMC/2012-related viruses in bats, Ghana and Europe. Emerg Infect Dis 19: 456–459.
13. Chu DK, Poon LL, Chan KH, Chen H, Guan Y, et al. (2006) Coronaviruses in bent-winged bats (Miniopterus spp.). J Gen Virol 87: 2461–2466.
14. Woo PC, Wang M, Lau SK, Xu H, Poon RW, et al. (2007) Comparative analysis of twelve genomes of three novel group 2c and group 2d coronaviruses reveals unique group and subgroup features. J Virol 81: 1574–1585.
15. Ren W, Qu X, Li W, Han Z, Yu M, et al. (2008) Difference in receptor usage between severe acute respiratory syndrome (SARS) coronavirus and SARS-like coronavirus of bat origin. J Virol 82: 1899–1907.
16. Watanabe S, Masangkay JS, Nagata N, Morikawa S, Mizutani T, et al. (2010) Bat coronaviruses and experimental infection of bats, the Philippines. Emerg Infect Dis 16: 1217–1223.
17. Gouilh MA, Puechmaille SJ, Gonzalez JP, Teeling E, Kittayapong P, et al. (2011) SARS-Coronavirus ancestor's foot-prints in South-East Asian bat colonies and the refuge theory. Infect Genet Evol 11: 1690–1702.
18. Becker MM, Graham RL, Donaldson EF, Rockx B, Sims AC, et al. (2008) Synthetic recombinant bat SARS-like coronavirus is infectious in cultured cells and in mice. Proc Natl Acad Sci U S A 105: 19944–19949.
19. Du L, He Y, Zhou Y, Liu S, Zheng BJ, et al. (2009) The spike protein of SARS-CoV–a target for vaccine and therapeutic development. Nat Rev Microbiol 7: 226–236.
20. Li W, Moore MJ, Vasilieva N, Sui J, Wong SK, et al. (2003) Angiotensin-converting enzyme 2 is a functional receptor for the SARS coronavirus. Nature 426: 450–454.
21. Wang P, Chen J, Zheng A, Nie Y, Shi X, et al. (2004) Expression cloning of functional receptor used by SARS coronavirus. Biochem Biophys Res Commun 315: 439–444.
22. Delmas B, Gelfi J, L'Haridon R, Vogel LK, Sjostrom H, et al. (1992) Aminopeptidase N is a major receptor for the entero-pathogenic coronavirus TGEV. Nature 357: 417–420.
23. Yeager CL, Ashmun RA, Williams RK, Cardellichio CB, Shapiro LH, et al. (1992) Human aminopeptidase N is a receptor for human coronavirus 229E. Nature 357: 420–422.
24. Williams RK, Jiang GS, Holmes KV (1991) Receptor for mouse hepatitis virus is a member of the carcinoembryonic antigen family of glycoproteins. Proc Natl Acad Sci U S A 88: 5533–5536.
25. Schultze B, Zimmer G, Herrler G (1996) Virus entry into a polarized epithelial cell line (MDCK): similarities and dissimilarities between influenza C virus and bovine coronavirus. J Gen Virol 77 (Pt 10): 2507–2514.
26. Winter C, Schwegmann-Wessels C, Cavanagh D, Neumann U, Herrler G (2006) Sialic acid is a receptor determinant for infection of cells by avian Infectious bronchitis virus. J Gen Virol 87: 1209–1216.
27. Schwegmann-Wessels C, Bauer S, Winter C, Enjuanes L, Laude H, et al. (2011) The sialic acid binding activity of the S protein facilitates infection by porcine transmissible gastroenteritis coronavirus. Virol J 8: 435.
28. Herrler G, Rott R, Klenk HD (1985) Neuraminic acid is involved in the binding of influenza C virus to erythrocytes. Virology 141: 144–147.
29. Schultze B, Herrler G (1992) Bovine coronavirus uses N-acetyl-9-O-acetylneuraminic acid as a receptor determinant to initiate the infection of cultured cells. J Gen Virol 73 (Pt 4): 901–906.
30. Vlasak R, Luytjes W, Leider J, Spaan W, Palese P (1988) The E3 protein of bovine coronavirus is a receptor-destroying enzyme with acetylesterase activity. J Virol 62: 4686–4690.
31. Sturman LS, Ricard CS, Holmes KV (1985) Proteolytic cleavage of the E2 glycoprotein of murine coronavirus: activation of cell-fusing activity of virions by trypsin and separation of two different 90K cleavage fragments. J Virol 56: 904–911.
32. Simmons G, Gosalia DN, Rennekamp AJ, Reeves JD, Diamond SL, et al. (2005) Inhibitors of cathepsin L prevent severe acute respiratory syndrome coronavirus entry. Proc Natl Acad Sci U S A 102: 11876–11881.
33. Bertram S, Glowacka I, Muller MA, Lavender H, Gnirss K, et al. (2011) Cleavage and activation of the severe acute respiratory syndrome coronavirus spike protein by human airway trypsin-like protease. J Virol 85: 13363–13372.
34. Calisher CH, Childs JE, Field HE, Holmes KV, Schountz T (2006) Bats: important reservoir hosts of emerging viruses. Clin Microbiol Rev 19: 531–545.
35. Halpin K, Hyatt AD, Plowright RK, Epstein JH, Daszak P, et al. (2007) Emerging viruses: coming in on a wrinkled wing and a prayer. Clin Infect Dis 44: 711–717.
36. Kelkar SD, Kadam SS, Banerjee K (1981) Haemagglutination inhibition antibodies against influenza virus in bats. Indian J Med Res 74: 147–152.
37. Tong S, Li Y, Rivailler P, Conrardy C, Castillo DA, et al. (2012) A distinct lineage of influenza A virus from bats. Proc Natl Acad Sci U S A 109: 4269–4274.
38. L'Vov D K, Easterday B, Hinshow W, Dandurov Iu V, Arkhipov PN (1979) [Isolation of strains of the Hong Kong complex (H3N2) influenza virus from Nyctalus noctula bats in Kazakhstan]. Vopr Virusol: 338–341.
39. Fraser GC, Hooper PT, Lunt RA, Gould AR, Gleeson LJ, et al. (1996) Encephalitis caused by a Lyssavirus in fruit bats in Australia. Emerg Infect Dis 2: 327–331.
40. Mackenzie JS, Field HE (2004) Emerging encephalitogenic viruses: lyssaviruses and henipaviruses transmitted by frugivorous bats. Arch Virol Suppl: 97–111.
41. Harris SL, Mansfield K, Marston DA, Johnson N, Pajamo K, et al. (2007) Isolation of European bat lyssavirus type 2 from a Daubenton's bat (Myotis daubentonii) in Shropshire. Vet Rec 161: 384–386.
42. Young PL, Halpin K, Selleck PW, Field H, Gravel JL, et al. (1996) Serologic evidence for the presence in Pteropus bats of a paramyxovirus related to equine morbillivirus. Emerg Infect Dis 2: 239–240.
43. Halpin K, Young PL, Field HE, Mackenzie JS (2000) Isolation of Hendra virus from pteropid bats: a natural reservoir of Hendra virus. J Gen Virol 81: 1927–1932.
44. Philbey AW, Kirkland PD, Ross AD, Davis RJ, Gleeson AB, et al. (1998) An apparently new virus (family Paramyxoviridae) infectious for pigs, humans, and fruit bats. Emerg Infect Dis 4: 269–271.
45. Chua KB, Koh CL, Hooi PS, Wee KF, Khong JH, et al. (2002) Isolation of Nipah virus from Malaysian Island flying-foxes. Microbes Infect 4: 145–151.
46. Drexler JF, Corman VM, Muller MA, Maganga GD, Vallo P, et al. (2012) Bats host major mammalian paramyxoviruses. Nat Commun 3: 796.
47. Towner JS, Amman BR, Sealy TK, Carroll SA, Comer JA, et al. (2009) Isolation of genetically diverse Marburg viruses from Egyptian fruit bats. PLoS Pathog 5: e1000536.
48. Towner JS, Pourrut X, Albarino CG, Nkogue CN, Bird BH, et al. (2007) Marburg virus infection detected in a common African bat. PLoS One 2: e764.
49. Leroy EM, Kumulungui B, Pourrut X, Rouquet P, Hassanin A, et al. (2005) Fruit bats as reservoirs of Ebola virus. Nature 438: 575–576.
50. Ren X, Glende J, Al-Falah M, de Vries V, Schwegmann-Wessels C, et al. (2006) Analysis of ACE2 in polarized epithelial cells: surface expression and function as receptor for severe acute respiratory syndrome-associated coronavirus. J Gen Virol 87: 1691–1695.
51. Glende J, Schwegmann-Wessels C, Al-Falah M, Pfefferle S, Qu X, et al. (2008) Importance of cholesterol-rich membrane microdomains in the interaction of the S protein of SARS-coronavirus with the cellular receptor angiotensin-converting enzyme 2. Virology 381: 215–221.
52. Schwegmann-Wessels C, Glende J, Ren X, Qu X, Deng H, et al. (2009) Comparison of vesicular stomatitis virus pseudotyped with the S proteins from a porcine and a human coronavirus. J Gen Virol 90: 1724–1729.
53. Kuhl A, Hoffmann M, Muller MA, Munster VJ, Gnirss K, et al. (2011) Comparative analysis of Ebola virus glycoprotein interactions with human and bat cells. J Infect Dis 204 Suppl 3: S840–849.
54. Swanepoel R, Smit SB, Rollin PE, Formenty P, Leman PA, et al. (2007) Studies of reservoir hosts for Marburg virus. Emerg Infect Dis 13: 1847–1851.
55. Simmons G, Reeves JD, Rennekamp AJ, Amberg SM, Piefer AJ, et al. (2004) Characterization of severe acute respiratory syndrome-associated coronavirus (SARS-CoV) spike glycoprotein-mediated viral entry. Proc Natl Acad Sci U S A 101: 4240–4245.
56. Matsuyama S, Ujike M, Morikawa S, Tashiro M, Taguchi F (2005) Protease-mediated enhancement of severe acute respiratory syndrome coronavirus infection. Proc Natl Acad Sci U S A 102: 12543–12547.

57. Matsuyama S, Nagata N, Shirato K, Kawase M, Takeda M, et al. (2010) Efficient activation of the severe acute respiratory syndrome coronavirus spike protein by the transmembrane protease TMPRSS2. J Virol 84: 12658–12664.
58. Simmons G, Bertram S, Glowacka I, Steffen I, Chaipan C, et al. (2011) Different host cell proteases activate the SARS-coronavirus spike-protein for cell-cell and virus-cell fusion. Virology 413: 265–274.
59. Belouzard S, Madu I, Whittaker GR (2010) Elastase-mediated activation of the severe acute respiratory syndrome coronavirus spike protein at discrete sites within the S2 domain. J Biol Chem 285: 22758–22763.
60. Belouzard S, Chu VC, Whittaker GR (2009) Activation of the SARS coronavirus spike protein via sequential proteolytic cleavage at two distinct sites. Proc Natl Acad Sci U S A 106: 5871–5876.
61. Giroglou T, Cinatl J, Jr., Rabenau H, Drosten C, Schwalbe H, et al. (2004) Retroviral vectors pseudotyped with severe acute respiratory syndrome coronavirus S protein. J Virol 78: 9007–9015.
62. Fukushi S, Mizutani T, Saijo M, Matsuyama S, Miyajima N, et al. (2005) Vesicular stomatitis virus pseudotyped with severe acute respiratory syndrome coronavirus spike protein. J Gen Virol 86: 2269–2274.
63. Watanabe R, Matsuyama S, Shirato K, Maejima M, Fukushi S, et al. (2008) Entry from the cell surface of severe acute respiratory syndrome coronavirus with cleaved S protein as revealed by pseudotype virus bearing cleaved S protein. J Virol 82: 11985–11991.
64. Hou Y, Peng C, Yu M, Li Y, Han Z, et al. (2010) Angiotensin-converting enzyme 2 (ACE2) proteins of different bat species confer variable susceptibility to SARS-CoV entry. Arch Virol 155: 1563–1569.
65. Muller MA, Raj VS, Muth D, Meyer B, Kallies S, et al. (2012) Human Coronavirus EMC Does Not Require the SARS-Coronavirus Receptor and Maintains Broad Replicative Capability in Mammalian Cell Lines. MBio 3.
66. Biesold SE, Ritz D, Gloza-Rausch F, Wollny R, Drexler JF, et al. (2011) Type I interferon reaction to viral infection in interferon-competent, immortalized cell lines from the African fruit bat Eidolon helvum. PLoS One 6: e28131.
67. Hofmann A, Kessler B, Ewerling S, Kabermann A, Brem G, et al. (2006) Epigenetic regulation of lentiviral transgene vectors in a large animal model. Mol Ther 13: 59–66.
68. Berger Rentsch M, Zimmer G (2011) A vesicular stomatitis virus replicon-based bioassay for the rapid and sensitive determination of multi-species type I interferon. PLoS One 6: e25858.
69. Frentzen A, Huging K, Bitzegeio J, Friesland M, Haid S, et al. (2011) Completion of hepatitis C virus replication cycle in heterokaryons excludes dominant restrictions in human non-liver and mouse liver cell lines. PLoS Pathog 7: e1002029.
70. Hanika A, Larisch B, Steinmann E, Schwegmann-Wessels C, Herrler G, et al. (2005) Use of influenza C virus glycoprotein HEF for generation of vesicular stomatitis virus pseudotypes. J Gen Virol 86: 1455–1465.
71. Goris K, Uhlenbruck S, Schwegmann-Wessels C, Kohl W, Niedorf F, et al. (2009) Differential sensitivity of differentiated epithelial cells to respiratory viruses reveals different viral strategies of host infection. J Virol 83: 1962–1968.
72. Zimmer G, Bossow S, Kolesnikova L, Hinz M, Neubert WJ, et al. (2005) A chimeric respiratory syncytial virus fusion protein functionally replaces the F and HN glycoproteins in recombinant Sendai virus. J Virol 79: 10467–10477.
73. Nie Y, Wang P, Shi X, Wang G, Chen J, et al. (2004) Highly infectious SARS-CoV pseudotyped virus reveals the cell tropism and its correlation with receptor expression. Biochem Biophys Res Commun 321: 994–1000.
74. Zhang H, Wang G, Li J, Nie Y, Shi X, et al. (2004) Identification of an antigenic determinant on the S2 domain of the severe acute respiratory syndrome coronavirus spike glycoprotein capable of inducing neutralizing antibodies. J Virol 78: 6938–6945.

From: Müller, Marcel Alexander
Sent: Mon, 29 Jul 2019 20:07:49 +0000
To: Letko, Michael (NIH/NIAID) [F]
Cc: Munster, Vincent (NIH/NIAID) [E]; Menk, Kay (NIH/NIAID) [E]
Subject: Re: [ext] FW: FedEx Shipment 437560785326: Scheduled delivery update
Attachments: image001.jpg, image002.jpg, image003.jpg

Hi Michael,
The cells were shipped today. I will pass on the tracking no tomorrow.
Best Marcel

—

PD Dr. Marcel A. Müller

Group leader, Institute of Virology
Charite - Universitätsmedizin Berlin

Campus Charite Mitte
Chariteplatz 1
D-10117 Berlin
Germany

Tel. (b)(6)
Fax +49 30 450 7525907

Email: (b)(6)
http://virologie-ccm.charite.de/

Am 29.07.2019 um 19:37 schrieb Letko, Michael (NIH/NIAID) [F] (b)(6)

Dear Dr. Mueller,

Do you require anything else from us to send the Rhinolophus cell lines?

Thank you again, we really appreciate it.

Best,
-michael

--
Michael Letko, Ph.D
Postdoctoral IRTA
Dr. Vincent Munster Laboratory
Virus Ecology Unit, Laboratory of Virology
Rocky Mountain Laboratories

NIAID/NIH
903S 4th Street
Hamilton MT 59840
(b)(6)

From: "Letko, Michael (NIH/NIAID) [F]" (b)(6)
Date: Wednesday, July 17, 2019 at 10:37 AM
To: (b)(6)
Cc: "Munster, Vincent (NIH/NIAID) [E]" (b)(6) "Menk, Kay (NIH/NIAID) [E]" (b)(6)
Subject: Re: [ext] FW: FedEx Shipment 437560785326: Scheduled delivery update

Dear Dr. Mueller,

I would like to thank you again for sharing your Rhinolophus cell lines.

Below is our lab's FedEx account number and address.

FedEx account #: (b)(6)
Attn: Michael Letko and Trent Bushmaker
Laboratory of Virology
Rocky Mountain Laboratories
NIAID/NIH
903S 4th street
Hamilton, MT 59840
(b)(6)

Please let me know if you need anything else.

Thank you,
-michael

--
Michael Letko, Ph.D
Postdoctoral IRTA
Dr. Vincent Munster Laboratory
Virus Ecology Unit, Laboratory of Virology
Rocky Mountain Laboratories
NIAID/NIH
903S 4th Street
Hamilton MT 59840

(b)(6)

From: "Munster, Vincent (NIH/NIAID) [E]" (b)(6)
Date: Friday, July 12, 2019 at 9:13 AM
To: "Thruston, Jeffrey (NIH/NIAID) [E]" (b)(6) "Müller, Marcel Alexander" (b)(6)
Cc: "Letko, Michael (NIH/NIAID) [F]" (b)(6)
Subject: Re: [ext] FW: FedEx Shipment 437560785326: Scheduled delivery update

Great job guys,

Marcel, as soon as your ready to ship discuss the logistics with Michael. They can be shipped using our fedex account,

Cheers,

Vincent Munster, PhD
Chief, Virus Ecology Unit
Laboratory of Virology
Rocky Mountain Laboratories
NIAID/NIH

From: "Thruston, Jeffrey (NIH/NIAID) [E]" (b)(6)
Date: Friday, July 12, 2019 at 6:13 AM
To: "Müller, Marcel Alexander" (b)(6)
Cc: (b)(6)
Subject: RE: [ext] FW: FedEx Shipment 437560785326: Scheduled delivery update

Dear Dr. Müller,

Thank you for the email. No, we have everything we need. The signed phsyical copies of the MTA are en route to you as we speak. You shold receive them on Monday, 15 July. NIAID can accept digital copies, so a signed PDF will suffice for our records. If you have any additional questions, please let me know.

Sincerely,

Jeff Thruston

Jeffrey T. Thruston, J.D., M.S.
Technology Transfer and Patent Specialist
Immunology & Emerging Infections Branch
TTIPO/NIAID/NIH
5601 Fishers Lane, Suite 2G46, MSC 9804
Rockville, MD 20892-9804
(b)(6)

The information in this e-mail and any of its attachments is confidential and may contain sensitive information. It should not be used by anyone who is not the original intended recipient. If you have received this e-mail in error, please inform the sender and delete it from your mailbox or any other storage devices. National Institute of Allergy and Infectious Diseases shall not accept liability for any statements made that are sender's own and not expressly made on behalf of the NIAID by one of its representatives.

From: Müller, Marcel Alexander (b)(6)
Sent: Friday, July 12, 2019 7:00 AM
To: Thruston, Jeffrey (NIH/NIAID) [E] (b)(6)
Cc: Munster, Vincent (NIH/NIAID) [E] (b)(6)
Subject: Re: [ext] FW: FedEx Shipment 437560785326: Scheduled delivery update

Dear Mr Thruston,

do you need any other statements from me?

Best, Marcel Müller

—
PD Dr. Marcel A. Müller

Group leader, Institute of Virology
Charite - Universitätsmedizin Berlin

Campus Charite Mitte
Chariteplatz 1
D-10117 Berlin
Germany

Tel. (b)(6)
Fax +49 30 450 7525907

Email: (b)(6)
http://virologie-ccm.charite.de/

Visiting/Postal address:
Charite - Universitätsmedizin Berlin
Institute of Virology (Helmut Ruska Haus)
Chariteplatz 1 (Rahel Hirsch Weg 3)
D-10117 Berlin
Germany

Von: Vincent Munster (b)(6)
Datum: Dienstag, 25. Juni 2019 um 13:29
An: "Müller, Marcel Alexander" (b)(6) "Thruston, Jeffrey (NIH/NIAID) [E]" (b)(6)
Betreff: Re: [ext] FW: FedEx Shipment 437560785326: Scheduled delivery update

Hi Marcel,

Absolutely no problem, no host interaction / genomic analyses are planned. But if this might change in the future we will discuss this with you and wait for you to approve.

Btw, if you would like to have some of our bsl4 pathogens done on these cells, just let me know. Very easy to do and then ship you the RNA.

Cheers,

Vincent Munster, PhD
Chief Virus Ecology Section
Rocky Mountain Laboratories, NIAID, NIH
903S 4th street, Hamilton, MT, USA
(b)(6)

From: "Müller, Marcel Alexander" (b)(6)
Date: Monday, June 24, 2019 at 4:14 PM
To: "Thruston, Jeffrey (NIH/NIAID) [E]" (b)(6)
Cc: (b)(6)
Subject: Re: [ext] FW: FedEx Shipment 437560785326: Scheduled delivery update

Dear Mr Thruston,

I included an additional sentence:
“Characterization of the designated cell lines by -omics approaches will not be done without prior consultation with the Provider.”

Is that OK Vincent? Sorry for being a little cautious here but better be safe than sorry, right?
Regarding the testing of the different spike proteins I talked to (b)(6) and he seems to be open for a collaboration.
Talk later.

Best, Marcel

—

PD Dr. Marcel A. Müller

Group leader, Institute of Virology
Charite - Universitätsmedizin Berlin

Campus Charite Mitte
Chariteplatz 1
D-10117 Berlin
Germany

Tel. (b)(6)
Fax +49 30 450 7525907

Email: (b)(6)
http://virologie-ccm.charite.de/

_____
Visiting/Postal address:
Charite - Universitätsmedizin Berlin
Institute of Virology (Helmut Ruska Haus)
Chariteplatz 1 (Rahel Hirsch Weg 3)
D-10117 Berlin
Germany

Von: "Thruston, Jeffrey (NIH/NIAID) [E]" (b)(6)
Datum: Freitag, 21. Juni 2019 um 22:14
An: "Müller, Marcel Alexander" (b)(6)
Cc: Vincent Munster (b)(6)
Betreff: FW: [ext] FW: FedEx Shipment 437560785326: Scheduled delivery update

Dear Dr. Müller,

I hope this email finds you well. I am writing to follow up on the attached, updated MTA redline with new virus list, per your request. Have you had an opportunity to review the attached document? If you have any questions or concerns, please let me know. Otherwise, I will start the signature process once I get your approval.

Sincerely,

Jeff Thruston

Jeffrey T. Thruston, J.D., M.S.
Technology Transfer and Patent Specialist
Immunology & Emerging Infections Branch
TTIPO/NIAID/NIH
5601 Fishers Lane, Suite 2G46, MSC 9804
Rockville, MD 20892-9804
(b)(6)

The information in this e-mail and any of its attachments is confidential and may contain sensitive information. It should not be used by anyone who is not the original intended recipient. If you have received this e-mail in error, please inform the sender and delete it from your mailbox or any other storage devices. National Institute of Allergy and Infectious Diseases shall not accept liability for any statements made that are sender's own and not expressly made on behalf of the NIAID by one of its representatives.

From: Thruston, Jeffrey (NIH/NIAID) [E]
Sent: Tuesday, June 4, 2019 4:04 PM
To: 'Müller, Marcel Alexander' (b)(6)
Subject: FW: [ext] FW: FedEx Shipment 437560785326: Scheduled delivery update
Importance: High

Dear Dr. Müller,

I hope this email finds you well. I am writing to follow up on the attached MTA redline, which has been updated to include the virus list you requested. If everything appears acceptable, I will initiate the signature process as soon as possible. If there is anything else that I may do to expedite the MTA, please let me know.

Sincerely,

Jeff Thruston

Jeffrey T. Thruston, J.D., M.S.
Technology Transfer and Patent Specialist
Immunology & Emerging Infections Branch
TTIPO/NIAID/NIH
5601 Fishers Lane, Suite 2G46, MSC 9804
Rockville, MD 20892-9804
(b)(6)

The information in this e-mail and any of its attachments is confidential and may contain sensitive information. It should not be used by anyone who is not the original intended recipient. If you have received this e-mail in error, please inform the sender and delete it from your mailbox or any other storage devices. National Institute of Allergy and Infectious Diseases shall not accept liability for any statements made that are sender's own and not expressly made on behalf of the NIAID by one of its representatives.

From: Thruston, Jeffrey (NIH/NIAID) [E]
Sent: Friday, May 24, 2019 9:44 AM
To: 'Müller, Marcel Alexander' (b)(6)
Subject: FW: [ext] FW: FedEx Shipment 437560785326: Scheduled delivery update

Dear Dr. Müller,

I hope this email finds you well. Have you had an opportunity to review the updated research plan in the MTA? The list of viruses has been updated, per your request. If you have any questions or concerns, please let me know.

Sincerely,

Jeff Thruston

Jeffrey T. Thruston, J.D., M.S.
Technology Transfer and Patent Specialist
Immunology & Emerging Infections Branch
TTIPO/NIAID/NIH
5601 Fishers Lane, Suite 2G46, MSC 9804
Rockville, MD 20892-9804
(b)(6)

The information in this e-mail and any of its attachments is confidential and may contain sensitive information. It should not be used by anyone who is not the original intended recipient. If you have received this e-mail in error, please inform the sender and delete it from your mailbox or any other storage devices. National Institute of Allergy and Infectious Diseases shall not accept liability for any statements made that are sender's own and not expressly made on behalf of the NIAID by one of its representatives.

From: Thruston, Jeffrey (NIH/NIAID) [E]
Sent: Friday, May 10, 2019 4:16 PM
To: Müller, Marcel Alexander (b)(6)
Subject: RE: [ext] FW: FedEx Shipment 437560785326: Scheduled delivery update

Dear Dr. Müller,

Thank you for the email. Attached, please find the updated MTA with complete list of viruses. Should you have any other questions, please let me know.

Sincerely,

Jeff Thruston

Jeffrey T. Thruston, J.D., M.S.
Technology Transfer and Patent Specialist
Immunology & Emerging Infections Branch
TTIPO/NIAID/NIH
5601 Fishers Lane, Suite 2G46, MSC 9804
Rockville, MD 20892-9804
(b)(6)

The information in this e-mail and any of its attachments is confidential and may contain sensitive information. It should not be used by anyone who is not the original intended recipient. If you have received this e-mail in error, please inform the sender and delete it from your mailbox or any other storage devices. National Institute of Allergy and Infectious Diseases shall not accept liability for any statements made that are sender's own and not expressly made on behalf of the NIAID by one of its representatives.

From: Müller, Marcel Alexander (b)(6)
Sent: Monday, May 6, 2019 10:14 AM
To: Thruston, Jeffrey (NIH/NIAID) [E] (b)(6)
Subject: Re: [ext] FW: FedEx Shipment 437560785326: Scheduled delivery update

Dear Mr Thruston,

please see attached the modifications in the Annex. Please include which viruses will be used for infection if possible.

The other MTA was signed and will be sent back to you this week.

Best,

Marcel Müller

—
PD Dr. Marcel A. Müller

Group leader, Institute of Virology
Charite - Universitätsmedizin Berlin

Campus Charite Mitte
Chariteplatz 1
D-10117 Berlin
Germany

Tel. (b)(6)
Fax +49 30 450 7525907

Email: (b)(6)
http://virologie-ccm.charite.de/

_____
Visiting/Postal address:
Charite - Universitätsmedizin Berlin
Institute of Virology (Helmut Ruska Haus)
Chariteplatz 1 (Rahel Hirsch Weg 3)
D-10117 Berlin
Germany

Von: "Thruston, Jeffrey (NIH/NIAID) [E]" (b)(6)
Datum: Donnerstag, 2. Mai 2019 um 16:07
An: "Müller, Marcel Alexander" (b)(6)
Betreff: FW: [ext] FW: FedEx Shipment 437560785326: Scheduled delivery update

Dear Dr. Müller,

I hope this email finds you well. Did you receive the partially executed MTA for (b)(6) Have you had an opportunity to review the attached MTA redline for Dr. Munster? If there is any information that you need from me, please let me know.

Sincerely,

Jeff Thruston

Jeffrey T. Thruston, J.D., M.S.
Technology Transfer and Patent Specialist
Immunology & Emerging Infections Branch
TTIPO/NIAID/NIH
5601 Fishers Lane, Suite 2G46, MSC 9804
Rockville, MD 20892-9804
(b)(6)

The information in this e-mail and any of its attachments is confidential and may contain sensitive information. It should not be used by anyone who is not the original intended recipient. If you have received this e-mail in error, please inform the sender and delete it from your mailbox or any other storage devices. National Institute of Allergy and Infectious Diseases shall not accept liability for any statements made that are sender's own and not expressly made on behalf of the NIAID by one of its representatives.

From: Thruston, Jeffrey (NIH/NIAID) [E]
Sent: Wednesday, April 24, 2019 12:30 PM
To: 'Müller, Marcel Alexander' (b)(6)
Subject: FW: [ext] FW: FedEx Shipment 437560785326: Scheduled delivery update

Dear Dr. Müller,

I hope this email finds you well. I am writing to follow up on two matters:

1. The attached MTA redline for Dr. Vincent Munster, and
2. The physical MTA for (b)(6) which arrived at Charite last week.

Have you received the physical MTA for signature? Have you had an opportunity to review the attached MTA redline? If you have any questions or concerns, please let me know.

Sincerely,

Jeff Thruston

Jeffrey T. Thruston, J.D., M.S.
Technology Transfer and Patent Specialist
Immunology & Emerging Infections Branch
TTIPO/NIAID/NIH
5601 Fishers Lane, Suite 2G46, MSC 9804
Rockville, MD 20892-9804
(b)(6)

The information in this e-mail and any of its attachments is confidential and may contain sensitive information. It should not be used by anyone who is not the original intended recipient. If you have received this e-mail in error, please inform the sender and delete it from your mailbox or any other storage devices. National Institute of Allergy and Infectious Diseases shall not accept liability for any statements made that are sender's own and not expressly made on behalf of the NIAID by one of its representatives.

From: Thruston, Jeffrey (NIH/NIAID) [E]
Sent: Wednesday, April 17, 2019 11:58 AM
To: Müller, Marcel Alexander (b)(6)
Subject: RE: [ext] FW: FedEx Shipment 437560785326: Scheduled delivery update

Dear Dr. Müller,

Thank you for the email. The MTA should arrive to you soon (FedEx has had some internal issues).

In parallel, I received a second MTA request through Charite covering bat cells for Dr. Vincent Munster – I have used the previous MTA as a template and updated the research description. However, I am not sure what the materials are, specifically. Please let me know if the attached is acceptable, and if you could provide a material description, that would be very helpful.

Sincerely,

Jeff Thruston

Jeffrey T. Thruston, J.D., M.S.
Technology Transfer and Patent Specialist
Immunology & Emerging Infections Branch
TTIPO/NIAID/NIH

5601 Fishers Lane, Suite 2G46, MSC 9804
Rockville, MD 20892-9804
(b)(6)

The information in this e-mail and any of its attachments is confidential and may contain sensitive information. It should not be used by anyone who is not the original intended recipient. If you have received this e-mail in error, please inform the sender and delete it from your mailbox or any other storage devices. National Institute of Allergy and Infectious Diseases shall not accept liability for any statements made that are sender's own and not expressly made on behalf of the NIAID by one of its representatives.

From: Müller, Marcel Alexander (b)(6)
Sent: Monday, April 15, 2019 9:12 AM
To: Thruston, Jeffrey (NIH/NIAID) [E] (b)(6)
Subject: Re: [ext] FW: FedEx Shipment 437560785326: Scheduled delivery update

Dear Mr Thruston,

you can use my address if you want to:

Charite - Universitätsmedizin Berlin
Institute of Virology (Helmut Ruska Haus)
PD Dr. Marcel A. Müller
Chariteplatz 1
Campus address: Rahel Hirsch Weg 3
D-10117 Berlin
Germany

Best, Marcel

—

Von: "Thruston, Jeffrey (NIH/NIAID) [E]" (b)(6)
Datum: Montag, 15. April 2019 um 14:45
An: "Müller, Marcel Alexander" (b)(6)
Betreff: [ext] FW: FedEx Shipment 437560785326: Scheduled delivery update

Dear Dr. Muller,

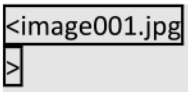
I just received the present update from FedEx regarding the status of the two original MTA we sent. It appears that the address within the MTA (and listed as the delivery address below) has been deemed incorrect by FedEx. How would you like for me to proceed?

Sincerely,

Jeff Thruston

From: TrackingUpdates@fedex.com <TrackingUpdates@fedex.com>
Sent: Monday, April 15, 2019 6:04 AM

To: Morgan, Cheri (NIH/NIAID) [C] (b)(6)
Subject: FedEx Shipment 437560785326: Scheduled delivery update



Your delivery schedule has been updated to , .

See "Preparing for Delivery" for helpful tips

Tracking # 437560785326

Ship date:
Thu, 4/11/2019

FREIGHT FORWARDING SECTION
NIH/TRANSPORTATION MANAGEMENT DIV.
BETHESDA, MD 20892
US

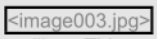

Delivery exception

Scheduled delivery:
Pending

(b)(6)
CHARITE
UNIVERSITATMEDIZINE BE
CHARITEPLATZ 1
TECHNOLOGIENTRANSFER STELLE
BERLIN, 10117
DE

Shipment Facts

Tracking number:	437560785326
Reference:	687104
Service type:	FedEx International Priority®
Packaging type:	FedEx® Envelope
Number of pieces:	1
Weight:	1.00 lb.
Special handling/Services:	Deliver Weekday
Standard transit:	4/15/2019 by 12:00 pm

 Please do not respond to this message. This email was sent from an unattended mailbox. This report was generated at approximately 5:04 AM CDT on 04/15/2019.

All weights are estimated.

The shipment is scheduled for delivery on or before the scheduled delivery displayed above. FedEx does not determine money-back guarantee or delay claim requests based on the scheduled delivery. Please see the FedEx Service Guide for terms and conditions of service, including the FedEx Money-Back Guarantee, or contact your FedEx customer support representative.

To track the latest status of your shipment, click on the tracking number above.

Standard transit is the date and time the package is scheduled to be delivered by, based on the selected service, destination and ship date. Limitations and exceptions may apply. Please see the FedEx Service Guide for terms and conditions of service, including the FedEx Money-Back Guarantee, or contact your FedEx Customer Support representative.

© 2019 Federal Express Corporation. The content of this message is protected by copyright and trademark laws under U.S. and international law. Review our privacy policy. All rights reserved.

Thank you for your business.

From: Letko, Michael (NIH/NIAID) [F]
Sent: Mon, 29 Jul 2019 15:19:57 +0000
To: Munster, Vincent (NIH/NIAID) [E]
Subject: Re: [ext] FW: FedEx Shipment 437560785326: Scheduled delivery update

Hi Vincent,

I have not heard anything back from Marcel yet. Should we poke him soom?

-michael

--
Michael Letko, Ph.D
Postdoctoral IRTA
Dr. Vincent Munster Laboratory
Virus Ecology Unit, Laboratory of Virology
Rocky Mountain Laboratories
NIAID/NIH
903S 4th Street
Hamilton MT 59840
(b)(6)

From: "Letko, Michael (NIH/NIAID) [F]" (b)(6)
Date: Wednesday, July 17, 2019 at 10:37 AM
To: (b)(6)
Cc: "Munster, Vincent (NIH/NIAID) [E]" (b)(6) "Menk, Kay (NIH/NIAID) [E]" (b)(6)
Subject: Re: [ext] FW: FedEx Shipment 437560785326: Scheduled delivery update

Dear Dr. Mueller,

I would like to thank you again for sharing your Rhinolophus cell lines.

Below is our lab's FedEx account number and address.

FedEx account #: (b)(6)
Attn: Michael Letko and Trent Bushmaker
Laboratory of Virology
Rocky Mountain Laboratories
NIAID/NIH
903S 4th street
Hamilton, MT 59840
(b)(6)

Please let me know if you need anything else.

Thank you,
-michael

--
Michael Letko, Ph.D
Postdoctoral IRTA
Dr. Vincent Munster Laboratory
Virus Ecology Unit, Laboratory of Virology
Rocky Mountain Laboratories
NIAID/NIH
903S 4th Street
Hamilton MT 59840
(b)(6)

From: "Munster, Vincent (NIH/NIAID) [E]" (b)(6)
Date: Friday, July 12, 2019 at 9:13 AM
To: "Thruston, Jeffrey (NIH/NIAID) [E]" (b)(6) "Müller, Marcel Alexander" (b)(6)
Cc: "Letko, Michael (NIH/NIAID) [F]" (b)(6)
Subject: Re: [ext] FW: FedEx Shipment 437560785326: Scheduled delivery update

Great job guys,

Marcel, as soon as your ready to ship discuss the logistics with Michael. They can be shipped using our fedex account,

Cheers,

Vincent Munster, PhD
Chief, Virus Ecology Unit
Laboratory of Virology
Rocky Mountain Laboratories
NIAID/NIH

From: "Thruston, Jeffrey (NIH/NIAID) [E]" (b)(6)
Date: Friday, July 12, 2019 at 6:13 AM
To: "Müller, Marcel Alexander" (b)(6)

Cc: (b)(6)
Subject: RE: [ext] FW: FedEx Shipment 437560785326: Scheduled delivery update

Dear Dr. Müller,

Thank you for the email. No, we have everything we need. The signed phsyical copies of the MTA are en route to you as we speak. You shold receive them on Monday, 15 July. NIAID can accept digital copies, so a signed PDF will suffice for our records. If you have any additional questions, please let me know.

Sincerely,

Jeff Thruston

Jeffrey T. Thruston, J.D., M.S.
Technology Transfer and Patent Specialist
Immunology & Emerging Infections Branch
TTIPO/NIAID/NIH
5601 Fishers Lane, Suite 2G46, MSC 9804
Rockville, MD 20892-9804
(b)(6)

The information in this e-mail and any of its attachments is confidential and may contain sensitive information. It should not be used by anyone who is not the original intended recipient. If you have received this e-mail in error, please inform the sender and delete it from your mailbox or any other storage devices. National Institute of Allergy and Infectious Diseases shall not accept liability for any statements made that are sender's own and not expressly made on behalf of the NIAID by one of its representatives.

From: Müller, Marcel Alexander (b)(6)
Sent: Friday, July 12, 2019 7:00 AM
To: Thruston, Jeffrey (NIH/NIAID) [E] (b)(6)
Cc: Munster, Vincent (NIH/NIAID) [E] (b)(6)
Subject: Re: [ext] FW: FedEx Shipment 437560785326: Scheduled delivery update

Dear Mr Thruston,

do you need any other statements from me?

Best, Marcel Müller

—

PD Dr. Marcel A. Müller

Group leader, Institute of Virology
Charite - Universitätsmedizin Berlin

Campus Charite Mitte
Chariteplatz 1
D-10117 Berlin
Germany

Tel. (b)(6)
Fax +49 30 450 7525907

Email: (b)(6)
http://virologie-ccm.charite.de/

_____
Visiting/Postal address:
Charite - Universitätsmedizin Berlin
Institute of Virology (Helmut Ruska Haus)
Chariteplatz 1 (Rahel Hirsch Weg 3)
D-10117 Berlin
Germany

Von: Vincent Munster (b)(6)
Datum: Dienstag, 25. Juni 2019 um 13:29
An: "Müller, Marcel Alexander" (b)(6) "Thruston, Jeffrey (NIH/NIAID) [E]" (b)(6)
Betreff: Re: [ext] FW: FedEx Shipment 437560785326: Scheduled delivery update

Hi Marcel,

Absolutely no problem, no host interaction / genomic analyses are planned. But if this might change in the future we will discuss this with you and wait for you to approve.

Btw, if you would like to have some of our bsl4 pathogens done on these cells, just let me know. Very easy to do and then ship you the RNA.

Cheers,

Vincent Munster, PhD
Chief Virus Ecology Section
Rocky Mountain Laboratories, NIAID, NIH
903S 4th street, Hamilton, MT, USA
(b)(6)

From: "Müller, Marcel Alexander" (b)(6)
Date: Monday, June 24, 2019 at 4:14 PM
To: "Thruston, Jeffrey (NIH/NIAID) [E]" (b)(6)
Cc: (b)(6)
Subject: Re: [ext] FW: FedEx Shipment 437560785326: Scheduled delivery update

Dear Mr Thruston,

I included an additional sentence:

“Characterization of the designated cell lines by -omics approaches will not be done without prior consultation with the Provider.”

Is that OK Vincent? Sorry for being a little cautious here but better be safe than sorry, right?
Regarding the testing of the different spike proteins I talked to (b)(6) and he seems to be open for a collaboration.
Talk later.

Best, Marcel

—
PD Dr. Marcel A. Müller

Group leader, Institute of Virology
Charite - Universitätsmedizin Berlin

Campus Charite Mitte
Chariteplatz 1
D-10117 Berlin
Germany

Tel. (b)(6)
Fax +49 30 450 7525907

Email: (b)(6)
http://virologie-ccm.charite.de/

Visiting/Postal address:
Charite - Universitätsmedizin Berlin
Institute of Virology (Helmut Ruska Haus)
Chariteplatz 1 (Rahel Hirsch Weg 3)
D-10117 Berlin
Germany

Von: "Thruston, Jeffrey (NIH/NIAID) [E]" (b)(6)
Datum: Freitag, 21. Juni 2019 um 22:14
An: "Müller, Marcel Alexander" (b)(6)
Cc: Vincent Munster (b)(6)
Betreff: FW: [ext] FW: FedEx Shipment 437560785326: Scheduled delivery update

Dear Dr. Müller,

I hope this email finds you well. I am writing to follow up on the attached, updated MTA redline with new virus list, per your request. Have you had an opportunity to review the attached document? If you have any questions or concerns, please let me know. Otherwise, I will start the signature process once I get your approval.

Sincerely,

Jeff Thruston

Jeffrey T. Thruston, J.D., M.S.
Technology Transfer and Patent Specialist
Immunology & Emerging Infections Branch
TTIPO/NIAID/NIH
5601 Fishers Lane, Suite 2G46, MSC 9804
Rockville, MD 20892-9804
(b)(6)

The information in this e-mail and any of its attachments is confidential and may contain sensitive information. It should not be used by anyone who is not the original intended recipient. If you have received this e-mail in error, please inform the sender and delete it from your mailbox or any other storage devices. National Institute of Allergy and Infectious Diseases shall not accept liability for any statements made that are sender's own and not expressly made on behalf of the NIAID by one of its representatives.

From: Thruston, Jeffrey (NIH/NIAID) [E]
Sent: Tuesday, June 4, 2019 4:04 PM
To: 'Müller, Marcel Alexander' (b)(6)
Subject: FW: [ext] FW: FedEx Shipment 437560785326: Scheduled delivery update
Importance: High

Dear Dr. Müller,

I hope this email finds you well. I am writing to follow up on the attached MTA redline, which has been updated to include the virus list you requested. If everything appears acceptable, I will initiate the signature process as soon as possible. If there is anything else that I may do to expedite the MTA, please let me know.

Sincerely,

Jeff Thruston

Jeffrey T. Thruston, J.D., M.S.
Technology Transfer and Patent Specialist
Immunology & Emerging Infections Branch
TTIPO/NIAID/NIH
5601 Fishers Lane, Suite 2G46, MSC 9804
Rockville, MD 20892-9804
(b)(6)

The information in this e-mail and any of its attachments is confidential and may contain sensitive information. It should not be used by anyone who is not the original intended recipient. If you have received this e-mail in error, please inform the sender and delete it from your mailbox or any other storage devices. National Institute of Allergy and Infectious Diseases shall not accept liability for any statements made that are sender's own and not expressly made on behalf of the NIAID by one of its representatives.

From: Thruston, Jeffrey (NIH/NIAID) [E]
Sent: Friday, May 24, 2019 9:44 AM

To: 'Müller, Marcel Alexander' (b)(6)
Subject: FW: [ext] FW: FedEx Shipment 437560785326: Scheduled delivery update

Dear Dr. Müller,

I hope this email finds you well. Have you had an opportunity to review the updated research plan in the MTA? The list of viruses has been updated, per your request. If you have any questions or concerns, please let me know.

Sincerely,

Jeff Thruston

Jeffrey T. Thruston, J.D., M.S.
Technology Transfer and Patent Specialist
Immunology & Emerging Infections Branch
TTIPO/NIAID/NIH
5601 Fishers Lane, Suite 2G46, MSC 9804
Rockville, MD 20892-9804
(b)(6)

The information in this e-mail and any of its attachments is confidential and may contain sensitive information. It should not be used by anyone who is not the original intended recipient. If you have received this e-mail in error, please inform the sender and delete it from your mailbox or any other storage devices. National Institute of Allergy and Infectious Diseases shall not accept liability for any statements made that are sender's own and not expressly made on behalf of the NIAID by one of its representatives.

From: Thruston, Jeffrey (NIH/NIAID) [E]
Sent: Friday, May 10, 2019 4:16 PM
To: Müller, Marcel Alexander (b)(6)
Subject: RE: [ext] FW: FedEx Shipment 437560785326: Scheduled delivery update

Dear Dr. Müller,

Thank you for the email. Attached, please find the updated MTA with complete list of viruses. Should you have any other questions, please let me know.

Sincerely,

Jeff Thruston

Jeffrey T. Thruston, J.D., M.S.
Technology Transfer and Patent Specialist
Immunology & Emerging Infections Branch
TTIPO/NIAID/NIH
5601 Fishers Lane, Suite 2G46, MSC 9804
Rockville, MD 20892-9804
(b)(6)

The information in this e-mail and any of its attachments is confidential and may contain sensitive information. It should not be used by anyone who is not the original intended recipient. If you have received this e-mail in error, please inform the sender and delete it from your mailbox or

any other storage devices. National Institute of Allergy and Infectious Diseases shall not accept liability for any statements made that are sender's own and not expressly made on behalf of the NIAID by one of its representatives.

From: Müller, Marcel Alexander (b)(6)
Sent: Monday, May 6, 2019 10:14 AM
To: Thruston, Jeffrey (NIH/NIAID) [E] (b)(6)
Subject: Re: [ext] FW: FedEx Shipment 437560785326: Scheduled delivery update

Dear Mr Thruston,

please see attached the modifications in the Annex. Please include which viruses will be used for infection if possible.

The other MTA was signed and will be sent back to you this week.

Best,

Marcel Müller

—
PD Dr. Marcel A. Müller

Group leader, Institute of Virology
Charite - Universitätsmedizin Berlin

Campus Charite Mitte
Chariteplatz 1
D-10117 Berlin
Germany

Tel. (b)(6)
Fax +49 30 450 7525907

Email: (b)(6)
http://virologie-ccm.charite.de/

Visiting/Postal address:
Charite - Universitätsmedizin Berlin
Institute of Virology (Helmut Ruska Haus)
Chariteplatz 1 (Rahel Hirsch Weg 3)
D-10117 Berlin
Germany

Von: "Thruston, Jeffrey (NIH/NIAID) [E]" (b)(6)
Datum: Donnerstag, 2. Mai 2019 um 16:07
An: "Müller, Marcel Alexander" (b)(6)
Betreff: FW: [ext] FW: FedEx Shipment 437560785326: Scheduled delivery update

Dear Dr. Müller,

I hope this email finds you well. Did you receive the partially executed MTA for (b)(6) Have you had an opportunity to review the attached MTA redline for Dr. Munster? If there is any information that you need from me, please let me know.

Sincerely,

Jeff Thruston

Jeffrey T. Thruston, J.D., M.S.
Technology Transfer and Patent Specialist
Immunology & Emerging Infections Branch
TTIPO/NIAID/NIH
5601 Fishers Lane, Suite 2G46, MSC 9804
Rockville, MD 20892-9804
(b)(6)

The information in this e-mail and any of its attachments is confidential and may contain sensitive information. It should not be used by anyone who is not the original intended recipient. If you have received this e-mail in error, please inform the sender and delete it from your mailbox or any other storage devices. National Institute of Allergy and Infectious Diseases shall not accept liability for any statements made that are sender's own and not expressly made on behalf of the NIAID by one of its representatives.

From: Thruston, Jeffrey (NIH/NIAID) [E]
Sent: Wednesday, April 24, 2019 12:30 PM
To: 'Müller, Marcel Alexander' (b)(6)
Subject: FW: [ext] FW: FedEx Shipment 437560785326: Scheduled delivery update

Dear Dr. Müller,

I hope this email finds you well. I am writing to follow up on two matters:

1. The attached MTA redline for Dr. Vincent Munster, and
2. The physical MTA for (b)(6) which arrived at Charite last week.

Have you received the physical MTA for signature? Have you had an opportunity to review the attached MTA redline? If you have any questions or concerns, please let me know.

Sincerely,

Jeff Thruston

Jeffrey T. Thruston, J.D., M.S.
Technology Transfer and Patent Specialist
Immunology & Emerging Infections Branch
TTIPO/NIAID/NIH
5601 Fishers Lane, Suite 2G46, MSC 9804
Rockville, MD 20892-9804
(b)(6)

The information in this e-mail and any of its attachments is confidential and may contain sensitive information. It should not be used by anyone who is not the original intended recipient. If you have received this e-mail in error, please inform the sender and delete it from your mailbox or any other storage devices. National Institute of Allergy and Infectious Diseases shall not accept liability for any statements made that are sender's own and not expressly made on behalf of the NIAID by one of its representatives.

From: Thruston, Jeffrey (NIH/NIAID) [E]
Sent: Wednesday, April 17, 2019 11:58 AM
To: Müller, Marcel Alexander (b)(6)
Subject: RE: [ext] FW: FedEx Shipment 437560785326: Scheduled delivery update

Dear Dr. Müller,

Thank you for the email. The MTA should arrive to you soon (FedEx has had some internal issues).

In parallel, I received a second MTA request through Charite covering bat cells for Dr. Vincent Munster – I have used the previous MTA as a template and updated the research description. However, I am not sure what the materials are, specifically. Please let me know if the attached is acceptable, and if you could provide a material description, that would be very helpful.

Sincerely,

Jeff Thruston

Jeffrey T. Thruston, J.D., M.S.
Technology Transfer and Patent Specialist
Immunology & Emerging Infections Branch
TTIPO/NIAID/NIH
5601 Fishers Lane, Suite 2G46, MSC 9804
Rockville, MD 20892-9804
(b)(6)

The information in this e-mail and any of its attachments is confidential and may contain sensitive information. It should not be used by anyone who is not the original intended recipient. If you have received this e-mail in error, please inform the sender and delete it from your mailbox or any other storage devices. National Institute of Allergy and Infectious Diseases shall not accept liability for any statements made that are sender's own and not expressly made on behalf of the NIAID by one of its representatives.

From: Müller, Marcel Alexander (b)(6)
Sent: Monday, April 15, 2019 9:12 AM
To: Thruston, Jeffrey (NIH/NIAID) [E] (b)(6)
Subject: Re: [ext] FW: FedEx Shipment 437560785326: Scheduled delivery update

Dear Mr Thruston,

you can use my address if you want to:

Charite - Universitätsmedizin Berlin
Institute of Virology (Helmut Ruska Haus)
PD Dr. Marcel A. Müller
Chariteplatz 1
Campus address: Rahel Hirsch Weg 3

D-10117 Berlin
Germany

Best, Marcel

—

Von: "Thruston, Jeffrey (NIH/NIAID) [E]" (b)(6)
Datum: Montag, 15. April 2019 um 14:45
An: "Müller, Marcel Alexander" (b)(6)
Betreff: [ext] FW: FedEx Shipment 437560785326: Scheduled delivery update

Dear Dr. Muller,

I just received the present update from FedEx regarding the status of the two original MTA we sent. It appears that the address within the MTA (and listed as the delivery address below) has been deemed incorrect by FedEx. How would you like for me to proceed?

Sincerely,

Jeff Thruston

From: TrackingUpdates@fedex.com <TrackingUpdates@fedex.com>
Sent: Monday, April 15, 2019 6:04 AM
To: Morgan, Cheri (NIH/NIAID) [C] (b)(6)
Subject: FedEx Shipment 437560785326: Scheduled delivery update

Your delivery schedule has been updated to , .

See "Preparing for Delivery" for helpful tips

Tracking # 437560785326

Ship date:
Thu, 4/11/2019

FREIGHT FORWARDING SECTION
NIH/TRANSPORTATION MANAGEMENT DIV.
BETHESDA, MD 20892
US

Delivery exception

Scheduled delivery:
Pending

(b)(6)
CHARITE
UNIVERSITATMEDIZINE BE
CHARITEPLATZ 1
TECHNOLOGIENTRANSFERSTELLE
BERLIN, 10117
DE

Shipment Facts

Tracking number:	437560785326
Reference:	687104
Service type:	FedEx International Priority®
Packaging type:	FedEx® Envelope
Number of pieces:	1
Weight:	1.00 lb.
Special handling/Services:	Deliver Weekday
Standard transit:	4/15/2019 by 12:00 pm

Please do not respond to this message. This email was sent from an unattended mailbox. This report was generated at approximately 5:04 AM CDT on 04/15/2019.

All weights are estimated.

The shipment is scheduled for delivery on or before the scheduled delivery displayed above. FedEx does not determine money-back guarantee or delay claim requests based on the scheduled delivery. Please see the FedEx Service Guide for terms and conditions of service, including the FedEx Money-Back Guarantee, or contact your FedEx customer support representative.

To track the latest status of your shipment, click on the tracking number above.

Standard transit is the date and time the package is scheduled to be delivered by, based on the selected service, destination and ship date. Limitations and exceptions may apply. Please see the FedEx Service Guide for terms and conditions of service, including the FedEx Money-Back Guarantee, or contact your FedEx Customer Support representative.

© 2019 Federal Express Corporation. The content of this message is protected by copyright and trademark laws under U.S. and international law. Review our privacy policy. All rights reserved.

Thank you for your business.

From: Munster, Vincent (NIH/NIAID) [E]
Sent: Thu, 23 May 2019 09:33:32 -0600
To: Müller, Marcel Alexander
Cc: Letko, Michael (NIH/NIAID) [F]
Subject: Short project description
Attachments: short summary B.pdf

Hi Marcel,

Was great talking to you yesterday. We have attached the outline of the project we are working on with the coronavirus spikes.

Let me know what your think,

Vincent Munster, PhD
Chief, Virus Ecology Unit
Laboratory of Virology
Rocky Mountain Laboratories
NIAID/NIH

Functionally screening for zoonotic potential of lineage B betacoronaviruses

Michael Letko[1], Vincent Munster[1]

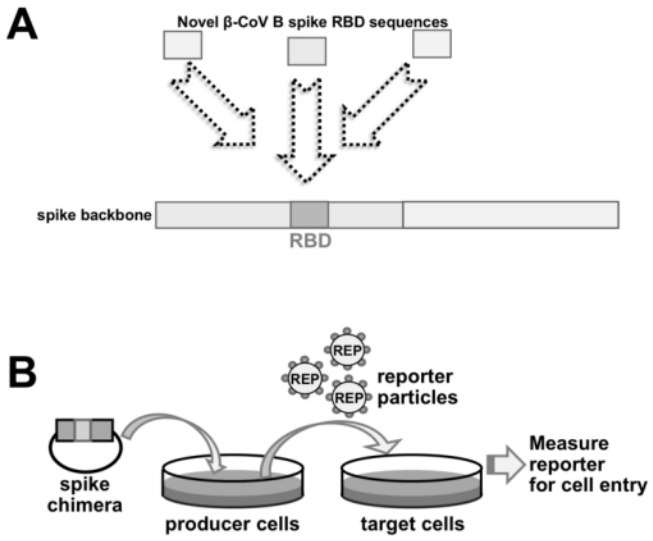
1. Virus Ecology Unit, Laboratory of Virology, Rocky Mountain Laboratories (NIAID/NIH) Hamilton, MT, 59840.

Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV) emerged in China and rapidly spread, killing over 700 of the 8000 cases. Since then, virus discovery efforts have uncovered thousands of novel coronavirus sequences across a diverse range of species and countries. While most of these viruses have never been found in humans, many are genetically similar to human CoVs within the betacoronavirus (β-CoV) genus. The β-CoVs are further divided into lineages: SARS-CoV is in lineage B, which also includes approximately 300 other novel animal viruses. Every year, additional CoV sequences are discovered. Unfortunately, little work is performed after the viral sequences are published. Therefore, it is unknown whether most of these viruses have potential to emerge in human populations and cause disease.

Cell entry is an essential component of cross-species transmission. All CoVs encode a spike protein which binds the host-cell receptor and mediates viral entry. For β-CoVs, a single region of spike called the receptor binding domain (RBD) mediates this interaction.

We have developed a scalable platform to test cell entry β-CoV lineage B viruses (Fig. 1). In this platform, we identify unique RBD sequences from published data. We then generate pseudotyped reporter particles in which we have replaced the RBD of a generic spike backbone with novel RBD sequences (Fig. 1). These chimeric-spike pseudotypes are then used to infect a variety of cell lines to elucidate host-receptor compatibility and species tropism.

Over the past 15 years, only six viruses in lineage B have been tested for cell entry potential because these studies relied on time and labor-intensive methods. In contrast, we have tested nearly every virus currently known in this lineage with our platform that is cheaper, faster, and scalable - allowing us to assess zoonotic potential of novel viruses at the rate in which they are discovered. This data is invaluable to current global efforts to identify novel viruses and prepare for the next viral pandemic.



CONFIDENTIAL

From: Letko, Michael (NIH/NIAID) [F]
Sent: Wed, 22 May 2019 17:52:40 +0000
To: Munster, Vincent (NIH/NIAID) [E]
Subject: Re: Short blurb
Attachments: short summary B.pdf

Took out a few more key details.

Now it's nice and generic.

--
Michael Letko, Ph.D
Postdoctoral IRTA
Dr. Vincent Munster Laboratory
Virus Ecology Unit, Laboratory of Virology
Rocky Mountain Laboratories
NIAID/NIH
903S 4th Street
Hamilton MT 59840
(b)(6)

From: "Letko, Michael (NIH/NIAID) [F]" (b)(6)
Date: Wednesday, May 22, 2019 at 11:40 AM
To: "Munster, Vincent (NIH/NIAID) [E]" (b)(6)
Subject: Short blurb

Take a look and let me know if you think it gives too much detail (…or too little).

--
Michael Letko, Ph.D
Postdoctoral IRTA
Dr. Vincent Munster Laboratory
Virus Ecology Unit, Laboratory of Virology
Rocky Mountain Laboratories
NIAID/NIH
903S 4th Street
Hamilton MT 59840
(b)(6)

Functionally screening for zoonotic potential of lineage B betacoronaviruses

Michael Letko[1], Vincent Munster[1]

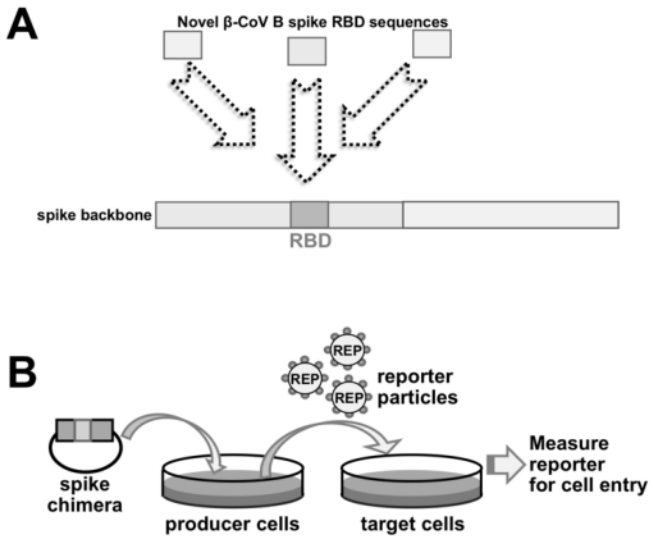
1. Virus Ecology Unit, Laboratory of Virology, Rocky Mountain Laboratories (NIAID/NIH) Hamilton, MT, 59840.

Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV) emerged in China and rapidly spread, killing over 700 of the 8000 cases. Since then, virus discovery efforts have uncovered thousands of novel coronavirus sequences across a diverse range of species and countries. While most of these viruses have never been found in humans, many are genetically similar to human CoVs within the betacoronavirus (β-CoV) genus. The β-CoVs are further divided into lineages: SARS-CoV is in lineage B, which also includes approximately 300 other novel animal viruses. Every year, additional CoV sequences are discovered. Unfortunately, little work is performed after the viral sequences are published. Therefore, it is unknown whether most of these viruses have potential to emerge in human populations and cause disease.

Cell entry is an essential component of cross-species transmission. All CoVs encode a spike protein which binds the host-cell receptor and mediates viral entry. For β-CoVs, a single region of spike called the receptor binding domain (RBD) mediates this interaction.

We have developed a scalable platform to test cell entry β-CoV lineage B viruses (Fig. 1). In this platform, we identify unique RBD sequences from published data. We then generate pseudotyped reporter particles in which we have replaced the RBD of a generic spike backbone with novel RBD sequences (Fig. 1). These chimeric-spike pseudotypes are then used to infect a variety of cell lines to elucidate host-receptor compatibility and species tropism.

Over the past 15 years, only six viruses in lineage B have been tested for cell entry potential because these studies relied on time and labor-intensive methods. In contrast, we have tested nearly every virus currently known in this lineage with our platform that is cheaper, faster, and scalable - allowing us to assess zoonotic potential of novel viruses at the rate in which they are discovered. This data is invaluable to current global efforts to identify novel viruses and prepare for the next viral pandemic.



CONFIDENTIAL

From: Letko, Michael (NIH/NIAID) [F]
Sent: Wed, 22 May 2019 17:40:57 +0000
To: Munster, Vincent (NIH/NIAID) [E]
Subject: Short blurb
Attachments: short summary.pdf

Take a look and let me know if you think it gives too much detail (…or too little).

--
Michael Letko, Ph.D
Postdoctoral IRTA
Dr. Vincent Munster Laboratory
Virus Ecology Unit, Laboratory of Virology
Rocky Mountain Laboratories
NIAID/NIH
903S 4th Street
Hamilton MT 59840
(b)(6)

Functionally screening for zoonotic potential of the entire lineage B of betacoronaviruses

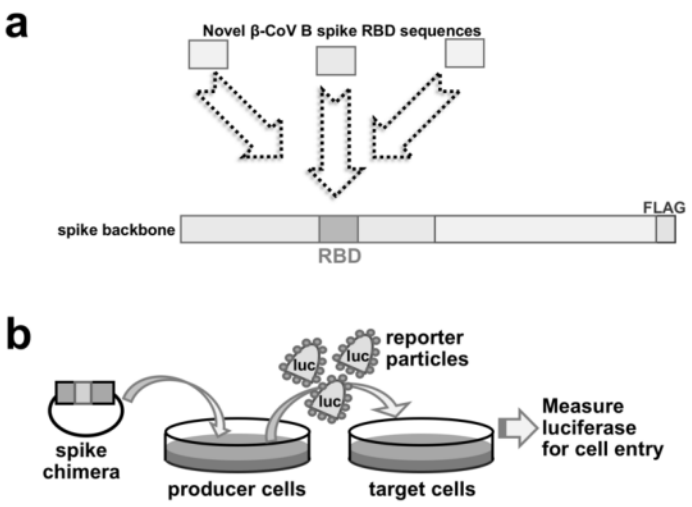
Michael Letko[1], Vincent Munster[1]

Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV) emerged in China and rapidly spread, killing over 700 of the 8000 cases. Since then, virus discovery efforts have uncovered thousands of novel coronavirus sequences across a diverse range of species and countries. While most of these viruses have never been found in humans, many are genetically similar to human CoVs within the betacoronavirus (β-CoV) genus. The β-CoVs are further divided into lineages: SARS-CoV is in lineage B, which also includes approximately 300 other novel animal viruses. Every year, additional CoV sequences are discovered. Unfortunately, little work is performed after the viral sequences are published. Therefore, it is unknown whether most of these viruses have potential to emerge in human populations and cause disease.

Cell entry is an essential component of cross-species transmission. All CoVs encode a spike protein which binds the host-cell receptor and mediates viral entry. For β-CoVs, a single region of spike called the receptor binding domain (RBD) mediates this interaction.

We have developed a scalable platform to test cell entry β-CoV lineage B viruses (Fig. 1). In this platform, we identify unique RBD sequences from published data. We then generate pseudotyped reporter particles in which we have replaced the RBD of a generic spike backbone with novel RBD sequences (Fig. 1). These chimeric-spike pseudotypes are then used to infect a variety of cell lines to elucidate host-receptor compatibility and species tropism.

Over the past 15 years, only six viruses in lineage B have been tested for cell entry potential because these studies relied on time and labor-intensive methods. In contrast, we have tested nearly every virus currently known in this lineage with our platform that is cheaper, faster, and scalable - allowing us to assess zoonotic potential of novel viruses at the rate in which they are discovered. This data is invaluable to current global efforts to identify novel viruses and prepare for the next viral pandemic.



CONFIDENTIAL

1. Virus Ecology Unit, Laboratory of Virology, Rocky Mountain Laboratories (NIAID/NIH) Hamilton, MT, 59840.

From: Munster, Vincent (NIH/NIAID) [E]
Sent: Fri, 10 May 2019 09:17:45 -0600
To: Thruston, Jeffrey (NIH/NIAID) [E]
Subject: Re: Charite University MTA Question

Hi Jeff:

(b)(4)

WIV-1 coronavirus
MERS-CoV
SARS-CoV

(b)(4)

Let me know if you need anything else,

Cheers,

Vincent Munster, PhD
Chief, Virus Ecology Unit
Laboratory of Virology
Rocky Mountain Laboratories
NIAID/NIH

From: "Thruston, Jeffrey (NIH/NIAID) [E]" (b)(6)
Date: Wednesday, May 8, 2019 at 10:43 AM
To: (b)(6)
Subject: RE: Charite University MTA Question

Hi Dr. Munster,

I believe Charite is wanting a list of specific viruses. The statement you initially provided is below. Charite pointed to the highlighted "infect" below and questioned which viruses. So, it appears they want to know which coronaviruses specifically. Do you have a specific group of proteins that you are also looking at?

Study bat coronaviruses and their protein interactions with host cells. As Rhinolophus sp. are a host reservoir for a variety of bat coronaviruses, Recipient will try to infect cells from these species in order to model stages of infection which more closely resemble what may occur in natural hosts.

Please let me know if you have any questions.

Sincerely,

Jeff Thruston

Jeffrey T. Thruston, J.D., M.S.
Technology Transfer and Patent Specialist
Immunology & Emerging Infections Branch
TTIPO/NIAID/NIH
5601 Fishers Lane, Suite 2G46, MSC 9804
Rockville, MD 20892-9804
(b)(6)

The information in this e-mail and any of its attachments is confidential and may contain sensitive information. It should not be used by anyone who is not the original intended recipient. If you have received this e-mail in error, please inform the sender and delete it from your mailbox or any other storage devices. National Institute of Allergy and Infectious Diseases shall not accept liability for any statements made that are sender's own and not expressly made on behalf of the NIAID by one of its representatives.

From: Munster, Vincent (NIH/NIAID) [E]
Sent: Wednesday, May 8, 2019 11:51 AM
To: Thruston, Jeffrey (NIH/NIAID) [E] (b)(6)
Subject: Re: Charite University MTA Question

Hi Jeff,

We will be using the cells for filoviruses, henipaviruses and cororonaviruses (bat cornonaviruses and human coronaviruses).

Will his be sufficient,

Just got back from travel, but will got through all the emails and see what is still open in trems of CRADAs and signing of paperwork,

Cheers,

Vincent Munster, PhD
Chief, Virus Ecology Unit
Laboratory of Virology
Rocky Mountain Laboratories
NIAID/NIH

From: "Thruston, Jeffrey (NIH/NIAID) [E]" (b)(6)
Date: Tuesday, May 7, 2019 at 7:30 AM
To: (b)(6)
Subject: Charite University MTA Question

ctions with host cells. As Rhinolophus sp. are a host reservoir for a variety of bat coronaviruses, Recipient will try to infect cells from these species in order to model stages of infection which more closely resemble what may occur i

From: Munster, Vincent (NIH/NIAID) [E]
Sent: Sat, 4 May 2019 18:15:19 +0000
To: Anthony, Simon J.
Cc: Letko, Michael (NIH/NIAID) [F]
Subject: Re: CoV-2b sequences

Absolutely stellar! Will keep you posted on the results!

On May 4, 2019, at 18:36, Anthony, Simon J. (b)(6) wrote:

Wonderful. I've attached the sequences here.

- PREDICT_CoV-51 (PDF-0663) was found in Malaysia and is SUPER divergent. Could be really interesting.
- PREDICT_CoV-43 was found in both Uganda and Rwanda. I've included two sequences for this (PDF-2370 and PRD—0038) because there are some substitutions that could be interesting.

Let me know if you have any questions!!
S. ☺

From: "Letko, Michael (NIH/NIAID) [F]" (b)(6)
Date: Thursday, May 2, 2019 at 10:43 AM
To: "Anthony, Simon J." (b)(6)
Subject: Re: CoV-2b sequences

Awesome! I really appreciate how flexible you are willing to be with everything. It does not matter to me if there are only one or two new sequences because they can still be quite interesting. So for that, I am fine with adding co-authors if we use the sequences.

I would really like to see the sequences and then decide whether or not we should use them.

Thank you again,
-michael

--
Michael Letko, Ph.D
Postdoctoral IRTA
Dr. Vincent Munster Laboratory
Virus Ecology Unit, Laboratory of Virology
Rocky Mountain Laboratories
NIAID/NIH
903S 4th Street
Hamilton MT 59840
(b)(6)

From: "Anthony, Simon J." (b)(6)
Date: Thursday, May 2, 2019 at 8:27 AM
To: "Letko, Michael (NIH/NIAID) [F]" (b)(6)
Subject: Re: CoV-2b sequences

I did, yes. We were all together in Vancouver this week, so I had a chance to touch base with the relevant partners. I also asked Heather from my team to double-check what sequences we have, and we don't actually have that many 2b spike sequences. Sorry, I thought we had more. We did detect around 5 or 6 novel 2b viruses, but we've only managed to get spikes for 2 of them so far. The first comes from Malaysia, and the second from Uganda and Rwanda. Apologies that this is not quite the treasure trove of 2bs you'd hoped for but we'll happily share others as we discover them and get them sequenced.

Here comes the trickier part: Neither of these sequences have been published yet, meaning that your publication would be the first report of these viruses in the literature. We generally adopt a very inclusive authorship philosophy, so would need to include several people as co-authors on your paper. I recognize that these sequences only add incrementally to your study – but it would be super important that our in-country partners and PREDICT global team are included. It is COMPLETELY up to you if you would like to proceed, or wait until the sequences are published (then you are free from us, haha). I now have agreement to send you the sequences, but you might not consider it worthwhile if you have to add a bunch of co-authors. (FYI - Middle authorship for all concerned would be completely acceptable). What do you think? No hard feelings either way. I'm also happy to send you the sequences anyway for evaluation – then you can decide if they are interesting enough to include.

What do you think?

From: "Letko, Michael (NIH/NIAID) [F]" (b)(6)
Date: Tuesday, April 30, 2019 at 12:14 PM
To: "Anthony, Simon J." (b)(6)
Subject: Re: CoV-2b sequences

Hi Simon,

Did you have a chance to meet with the group responsible for the betacoronavirus lineage B spike sequences?

Cheers,
-michael

--
Michael Letko, Ph.D

Postdoctoral IRTA
Dr. Vincent Munster Laboratory
Virus Ecology Unit, Laboratory of Virology
Rocky Mountain Laboratories
NIAID/NIH
903S 4th Street
Hamilton MT 59840
(b)(6)

From: "Anthony, Simon J." (b)(6)
Date: Saturday, April 6, 2019 at 1:20 PM
To: "Letko, Michael (NIH/NIAID) [F]" (b)(6)
Cc: "Munster, Vincent (NIH/NIAID) [E]" (b)(6)
Subject: Re: CoV-2b sequences

Thanks for reminder, Michael. ☺ Meeting with Heather on Monday – sorry for the delay.

From: "Letko, Michael (NIH/NIAID) [F]" (b)(6)
Date: Friday, April 5, 2019 at 4:08 PM
To: "Anthony, Simon J." (b)(6)
Cc: "Munster, Vincent (NIH/NIAID) [E]" (b)(6)
Subject: CoV-2b sequences

Dear Simon,

I want to thank you again for agreeing to share betacoronavirus lineage B spike sequences with us. This will surely increase the impact of our story by providing a proof of principle with unpublished sequences.

We will move very quickly once we have them - these days it takes me just under 3 weeks to go from ordering synthesis of the RBD to having cell entry data.

Please let us know if you require anything else before sending the sequences.

Cheers,
-michael

--
Michael Letko, Ph.D
Postdoctoral IRTA
Dr. Vincent Munster Laboratory
Virus Ecology Unit, Laboratory of Virology
Rocky Mountain Laboratories

NIAID/NIH
PubMed
903S 4th Street
Hamilton MT 59840
(b)(6)

<Spike sequences for Michael Letko.txt>

From: Thruston, Jeffrey (NIH/NIAID) [E]
Sent: Mon, 22 Apr 2019 14:40:59 +0000
To: Munster, Vincent (NIH/NIAID) [E]
Cc: Letko, Michael (NIH/NIAID) [F]
Subject: RE: [ext] Cel lines

Hi Vincent,

Dr. Mueller has the MTA markup (identical terms to a previously executed MTA) for review. Once he gives the "ok" regarding the terms, I can route the agreement for signatures. Of note, he requires wet, original signatures, so I will FedEx you physical copies for signature when the time comes.

Sincerely,

Jeff Thruston

From: Munster, Vincent (NIH/NIAID) [E]
Sent: Sunday, April 21, 2019 11:58 AM
To: Thruston, Jeffrey (NIH/NIAID) [E] (b)(6)
Cc: Letko, Michael (NIH/NIAID) [F] (b)(6)
Subject: Re: [ext] Cel lines

Any luck with this MTA yet?

Cheers,

Vincent Munster, PhD
Chief, Virus Ecology Unit
Laboratory of Virology
Rocky Mountain Laboratories
NIAID/NIH

From: (b)(6)
Date: Monday, April 1, 2019 at 4:32 PM
To: "Thruston, Jeffrey (NIH/NIAID) [E]" (b)(6)
Cc: Michael Letko (b)(6) "Müller, Marcel Alexander" (b)(6)
Subject: FW: [ext] Cel lines

Hi Jeff,

Another MTA. This one is for acquiring bat cell lines from a collaborator (prof. Marcel Muller) in Germany,

“The purpose is to study bat coronaviruses and their protein interactions with host cells. As Rhinolophus sp. are a host reservoir for a variety of bat coronaviruses, our group will try to infect cells from these species in order to model stages of infection which more closely resemble what may occur in natural hosts.”

Let me know if you need any further information,

Vincent Munster, PhD
Chief, Virus Ecology Unit
Laboratory of Virology
Rocky Mountain Laboratories
NIAID/NIH

From: "Müller, Marcel Alexander" (b)(6)
Date: Tuesday, March 12, 2019 at 11:36 AM
To: (b)(6) Michael Letko (b)(6)
Subject: Re: [ext] Cel lines

Hi Vincent,

yes, I am in Berlin and happy about it! It took a while to get settled but now everything works fine.
I will not be in Rotterdam unfortunately.
I really try to focus on bat immunology (innate immune and stress response) now!
So what are your plans? Anything we can do together?
Attached an MTA that I will need for the exchange of cells to make them eligible within our EU EVAg infrastructure project.
Hope it will work with your legal office. (b)(6)

Best, Marcel

—

PD Dr. Marcel A. Müller

Group leader, Institute of Virology
Charite - Universitätsmedizin Berlin

Campus Charite Mitte
Chariteplatz 1
D-10117 Berlin
Germany

Tel. (b)(6)
Fax +49 30 450 7525907

Email: (b)(6)
http://virologie-ccm.charite.de/

_____
Visiting/Postal address:
Charite - Universitätsmedizin Berlin
Institute of Virology (Helmut Ruska Haus)
Chariteplatz 1 (Rahel Hirsch Weg 3)
D-10117 Berlin
Germany

Von: Vincent Munster (b)(6)
Datum: Samstag, 9. März 2019 um 00:21
An: "Müller, Marcel Alexander" (b)(6) "Letko, Michael (NIH/NIAID) [F]" (b)(6)
Betreff: [ext] Cel lines

Hi Marcel,

I hope life in Berlin is treating you well? I saw that you established a nice research group at charite, very cool.

Are you by chance going to the ECV meeting in Rotterdam in April?

I was wondering whether you would be willing to share two of your bat cellines with us?

We are looking for:

1. RhiNi = R. landeri
2. RhiLu = R. alcyone

Just let me know, My post-doc Dr. Michael Letko is in charge of all of the arrangements,

Cheers en hartelijke groet uit Montana,

Vincent Munster, PhD
Chief, Virus Ecology Unit
Laboratory of Virology
Rocky Mountain Laboratories
NIAID/NIH

From: Thruston, Jeffrey (NIH/NIAID) [E]
Sent: Tue, 2 Apr 2019 15:00:27 +0000
To: Munster, Vincent (NIH/NIAID) [E]
Cc: Letko, Michael (NIH/NIAID) [F]
Subject: RE: [ext] Cel lines

Hi Dr. Munster,

Thank you for the email. I will review the MTA and provide comments, if any, asap.

Sincerely,

Jeff Thruston

Jeffrey T. Thruston, J.D., M.S.
Technology Transfer and Patent Specialist
Immunology & Emerging Infections Branch
TTIPO/NIAID/NIH
5601 Fishers Lane, Suite 2G46, MSC 9804
Rockville, MD 20892-9804
(b)(6)

The information in this e-mail and any of its attachments is confidential and may contain sensitive information. It should not be used by anyone who is not the original intended recipient. If you have received this e-mail in error, please inform the sender and delete it from your mailbox or any other storage devices. National Institute of Allergy and Infectious Diseases shall not accept liability for any statements made that are sender's own and not expressly made on behalf of the NIAID by one of its representatives.

From: Munster, Vincent (NIH/NIAID) [E]
Sent: Monday, April 1, 2019 6:29 PM
To: Thruston, Jeffrey (NIH/NIAID) [E] (b)(6)
Cc: Letko, Michael (NIH/NIAID) [F] (b)(6) Müller, Marcel Alexander (b)(6)
Subject: FW: [ext] Cel lines

Hi Jeff,

Another MTA. This one is for acquiring bat cell lines from a collaborator (prof. Marcel Muller) in Germany,

“The purpose is to study bat coronaviruses and their protein interactions with host cells. As Rhinolophus sp. are a host reservoir for a variety of bat coronaviruses, our group will try to infect cells from these species in order to model stages of infection which more closely resemble what may occur in natural hosts.”

Let me know if you need any further information,

Vincent Munster, PhD
Chief, Virus Ecology Unit
Laboratory of Virology

Rocky Mountain Laboratories
NIAID/NIH

From: "Müller, Marcel Alexander" (b)(6)
Date: Tuesday, March 12, 2019 at 11:36 AM
To: (b)(6) Michael Letko
(b)(6)
Subject: Re: [ext] Cel lines

Hi Vincent,

yes, I am in Berlin and happy about it! It took a while to get settled but now everything works fine.
I will not be in Rotterdam unfortunately.
I really try to focus on bat immunology (innate immune and stress response) now!
So what are your plans? Anything we can do together?
Attached an MTA that I will need for the exchange of cells to make them eligible within our EU EVAg infrastructure project.
Hope it will work with your legal office.(b)(6)

Best, Marcel

—

PD Dr. Marcel A. Müller

Group leader, Institute of Virology
Charite - Universitätsmedizin Berlin

Campus Charite Mitte
Chariteplatz 1
D-10117 Berlin
Germany

Tel.(b)(6)
Fax +49 30 450 7525907

Email:(b)(6)
http://virologie-ccm.charite.de/

Visiting/Postal address:
Charite - Universitätsmedizin Berlin
Institute of Virology (Helmut Ruska Haus)
Chariteplatz 1 (Rahel Hirsch Weg 3)
D-10117 Berlin
Germany

Von: Vincent Munster (b)(6)
Datum: Samstag, 9. März 2019 um 00:21
An: "Müller, Marcel Alexander" (b)(6) "Letko, Michael (NIH/NIAID) [F]" (b)(6)
Betreff: [ext] Cel lines

Hi Marcel,

I hope life in Berlin is treating you well? I saw that you established a nice research group at charite, very cool.

Are you by chance going to the ECV meeting in Rotterdam in April?

I was wondering whether you would be willing to share two of your bat cellines with us?

We are looking for:

1. RhiNi = R. landeri
2. RhiLu = R. alcyone

Just let me know, My post-doc Dr. Michael Letko is in charge of all of the arrangements,

Cheers en hartelijke groet uit Montana,

Vincent Munster, PhD
Chief, Virus Ecology Unit
Laboratory of Virology
Rocky Mountain Laboratories
NIAID/NIH

From: Munster, Vincent (NIH/NIAID) [E]
Sent: Mon, 1 Apr 2019 16:28:55 -0600
To: Thruston, Jeffrey (NIH/NIAID) [E]
Cc: Letko, Michael (NIH/NIAID) [F]; Müller, Marcel Alexander
Subject: FW: [ext] Cel lines
Attachments: MTA_Charite Müller_template.doc

Hi Jeff,

Another MTA. This one is for acquiring bat cell lines from a collaborator (prof. Marcel Muller) in Germany,

“The purpose is to study bat coronaviruses and their protein interactions with host cells. As Rhinolophus sp. are a host reservoir for a variety of bat coronaviruses, our group will try to infect cells from these species in order to model stages of infection which more closely resemble what may occur in natural hosts.”

Let me know if you need any further information,

Vincent Munster, PhD
Chief, Virus Ecology Unit
Laboratory of Virology
Rocky Mountain Laboratories
NIAID/NIH

From: "Müller, Marcel Alexander" (b)(6)
Date: Tuesday, March 12, 2019 at 11:36 AM
To: (b)(6) Michael Letko
(b)(6)
Subject: Re: [ext] Cel lines

Hi Vincent,

yes, I am in Berlin and happy about it! It took a while to get settled but now everything works fine.
I will not be in Rotterdam unfortunately.
I really try to focus on bat immunology (innate immune and stress response) now!
So what are your plans? Anything we can do together?
Attached an MTA that I will need for the exchange of cells to make them eligible within our EU EVAg infrastructure project.
Hope it will work with your legal office. (b)(6)

Best, Marcel

—
PD Dr. Marcel A. Müller

Group leader, Institute of Virology
Charite - Universitätsmedizin Berlin

Campus Charite Mitte
Chariteplatz 1
D-10117 Berlin
Germany

Tel. (b)(6)
Fax +49 30 450 7525907

Email: (b)(6)
http://virologie-ccm.charite.de/

_____
Visiting/Postal address:
Charite - Universitätsmedizin Berlin
Institute of Virology (Helmut Ruska Haus)
Chariteplatz 1 (Rahel Hirsch Weg 3)
D-10117 Berlin
Germany

Von: Vincent Munster (b)(6)
Datum: Samstag, 9. März 2019 um 00:21
An: "Müller, Marcel Alexander" (b)(6) "Letko, Michael (NIH/NIAID) [F]" (b)(6)
Betreff: [ext] Cel lines

Hi Marcel,

I hope life in Berlin is treating you well? I saw that you established a nice research group at charite, very cool.

Are you by chance going to the ECV meeting in Rotterdam in April?

I was wondering whether you would be willing to share two of your bat cellines with us?

We are looking for:

1. RhiNi = R. landeri
2. RhiLu = R. alcyone

Just let me know, My post-doc Dr. Michael Letko is in charge of all of the arrangements,

Cheers en hartelijke groet uit Montana,

Vincent Munster, PhD
Chief, Virus Ecology Unit
Laboratory of Virology
Rocky Mountain Laboratories
NIAID/NIH

(b)(4)

Material Transfer Agreement

between

CHARITÉ – Universitätsmedizin Berlin,
Charitéplatz 1, D-10117 Berlin

supported by the

(b)(4)

(hereinafter referred to as "SUPPLIER")

and

Company/Institution
(hereinafter referred to as "RECIPIENT")

MATERIAL: described in Annex A

RECIPIENT'S CONTACT:

SUPPLIER'S CONTACT:

(b)(6)
Charité – Universitätsmedizin Berlin
(b)(6)
Charitéplatz 1
10117 Berlin
Germany

1. **Background**.
RECIPIENT desires to obtain the material and/or information described in **Appendix A** (together with all fragments and mixtures "MATERIAL") from SUPPLIER for use by RECIPIENT solely for non-human experiments described in **Appendix A** (the "TESTS")

under the terms and conditions of this Agreement. The obligations of RECIPIENT herein described will apply to any biological MATERIAL that incorporates the MATERIAL or any recombinant version thereof.

2. The MATERIAL and the TESTS.

RECIPIENT acknowledges that SUPPLIER owns the MATERIAL. SUPPLIER will use commercially reasonable efforts to provide RECIPIENT with the quantity of the MATERIAL described in Appendix A. RECIPIENT will use the MATERIAL solely for the TESTS and for no other purpose. RECIPIENT agrees not to analyze the MATERIAL for the purpose of determining the structure nor amino acid sequence thereof. Furthermore, the use for any commercial purpose, such as for production is prohibited under this Agreement. In particular, no rights are provided to use the MATERIAL or any related patents for profit-making or commercial provision of a service to a third party in exchange for consideration. RECIPIENT will not use the MATERIAL for testing in or treatment of human subjects. RECIPIENT acknowledges that the MATERIAL is experimental and will comply with all laws and regulations applicable to its handling and use. Any MATERIAL remaining upon completion of the Research either will be returned to SUPPLIER or discarded, consistent with SUPPLIER'S instructions. RECIPIENT agrees to not distribute or release the MATERIAL to any other person or entity, except laboratory personnel of RECIPIENT. RECIPIENT shall ensure that no one will be allowed to take or send the MATERIAL to any other location than mentioned above, unless written permission is obtained from SUPPLIER.

3. Confidentiality.

RECIPIENT shall treat in confidence, for a period of five (5) years from the date of its disclosure, any written information pertaining to the MATERIAL provided to RECIPIENT by SUPPLIER or SUPPLIER'S Scientist(s). Excluded from this obligation shall be any information

(a) that was previously known to RECIPIENT prior to receipt of information from SUPPLIER;

(b) that lawfully is, or becomes publicly available during said five (5) year period through no fault of RECIPIENT;

(c) which is disclosed to RECIPIENT without confidentiality obligations by a third party having the right to make such disclosure; or

(d) which is independently developed by RECIPIENT without the use of or reference to any information received from SUPPLIER.

If RECIPIENT is suspected to breach this Agreement, RECIPIENT has to prove that the matter of suspected violation is information in compliance with 3 a - d.

4. Research Results.

RECIPIENT will conduct TESTS with the MATERIAL and gather research results. RECIPIENT will inform SUPPLIER of Research results related to the MATERIAL, by personal communication and by providing SUPPLIER with written information describing the results of such Research. In case of joint inventions, SUPPLIER and Recipient shall conclude in good faith a separate agreement concerning the use, patenting and

commercialization of those joint inventions. Recipient shall grant to SUPPLIER an irrevocable non-exclusive royalty-free license to practise the invention, improvement or modification for scientific research purposes.

5. Publication and Acknowledgement.

The RECIPIENT shall have the right, consistent with academic standards, to publish or present the results of the research work performed in accordance with this Agreement. The RECIPIENT shall disclose planned publications to SUPPLIER at least thirty (30) days prior to submission for publication. If SUPPLIER does not send an objection during this thirty (30) days, RECIPEINT will be free to submit for publication without delay.
In presentations or publications concerning the use of the Material, the RECIPIENT will acknowledge the SUPPLIER and the named SUPPLIER´s contact as the source of the MATERIAL. When research work is performed in collaboration, co- authorship of SUPPLIER is required.

6. No Warranty.

RECIPIENT acknowledges that any MATERIAL delivered to it under this agreement is experimental in nature. SUPPLIER makes no representations nor extends any warranties of any kind, either expressed or implied, with respect to the MATERIAL. There are no express or implied warranties of merchantability or fitness for a particular purpose, nor does SUPPLIER represent that the use of the MATERIAL will not infringe any patent, copyright, trade secret, trademark or other rights of third parties.

7. Indemnification.

SUPPLIER shall not be liable to RECIPIENT for any loss, claim, or demand made by RECIPIENT, or made against RECIPIENT by any other party, due to, or arising from, the use of the MATERIAL by the RECIPIENT. To the extent permitted by law, RECIPIENT shall indemnify, defend and hold harmless SUPPLIER, its trustees, assignees, agents and employees from any claim asserted against them arising from the negligence or wilful misconduct in the use of the MATERIAL by RECIPIENT or its agents or employees.

8. No obligations.

No rights or licenses to trademarks, inventions, copyrights or patents are implied or granted under this Agreement.

9. Final Agreement.

This Agreement and Appendix A attached hereto and hereby incorporated herein, contains the final, complete and exclusive agreement of the parties relative to the subject matter hereof and supersedes all prior and contemporaneous understandings and agreements relating to its subject matter. This Agreement may not be changed, modified, amended or supplemented except by a written instrument signed by both parties.

10. Start and Termination.
This Agreement starts with the date of signature of RECIPIENT. Either party may terminate this Agreement upon thirty (30) days' prior written notice to the other party. Upon termination, RECIPIENT will immediately return to SUPPLIER its Confidential Information, and any unused samples of the MATERIAL, and all of RECIPIENT's rights to use the MATERIAL will end. Following termination, neither party will have any further obligations under this Agreement, except that Sections 2, 3, 5 and 6 will survive.

11. Miscellaneous.
This Agreement shall be governed by the laws of Germany, excluding its conflicts of laws principles. Place of jurisdiction is Berlin, Germany.

If you agree to accept the MATERIAL under the above conditions, please have this Agreement signed by an authorized representative of RECIPIENT and return two originals to:

Charité – Universitätsmedizin Berlin
(b)(6)
Charitéplatz 1
D-10117 Berlin

The MATERIAL will be sent to you as soon as possible after the receipt of the signed Agreement.

RECIPIENT ORGANIZATION APPROVAL:

***Company*, Date:** _____

By:_____

_____
Print Name of Authorized Official

_____
Position

AGREED AND ACCEPTED BY RECIPIENT SCIENTIST

Date: _____

By:_____

_____
Print Name of Recipient Scientist

_____
Position

Charité – Universitätsmedizin Berlin

Date: _____

By:_____

(b)(6)

Date: _____

By: (b)(6)

(b)(6)

CHARITÉ – Universitätsmedizin Berlin,
Charitéplatz 1, D-10117 Berlin

Appendix A

MATERIAL:

EXAMPLE: RoNi/7.1

Comment: Please always use the cell designations exactly as stated in this MTA.

Short description of work:

TESTS:

From: Letko, Michael (NIH/NIAID) [F]
Sent: Mon, 1 Apr 2019 22:23:49 +0000
To: Munster, Vincent (NIH/NIAID) [E]
Subject: Re: [ext] Cel lines

The Virus Ecology Unit is currently studying bat coronaviruses and their protein interactions with host cells. As *Rhinolophus sp.* are a host reservoir for a variety of bat coronaviruses, our group will try to infect cells from these species in order to model stages of infection which more closely resemble what may occur in natural hosts.

--
Michael Letko, Ph.D
Postdoctoral IRTA
Dr. Vincent Munster Laboratory
Virus Ecology Unit, Laboratory of Virology
Rocky Mountain Laboratories
NIAID/NIH
PubMed
903S 4th Street
Hamilton MT 59840
(b)(6)

From: "Munster, Vincent (NIH/NIAID) [E]" (b)(6)
Date: Monday, April 1, 2019 at 4:13 PM
To: "Müller, Marcel Alexander" (b)(6) "Letko, Michael (NIH/NIAID) [F]" (b)(6)
Subject: Re: [ext] Cel lines

Ha, always thought that bat innate would be a good match for you! Do you have your own group now, independent from Christian?

Working on bat infection models, filo's and henipa's. Would definitely be interested in some more extensive collaboration (should have you over again, last time is already 8 (!) years ago!

I have forwarded the MTA to our legal people, we'll see. They are typically a pain in rear,

Cheers!

Vincent Munster, PhD
Chief, Virus Ecology Unit
Laboratory of Virology
Rocky Mountain Laboratories
NIAID/NIH

From: "Müller, Marcel Alexander" (b)(6)
Date: Tuesday, March 12, 2019 at 11:36 AM
To: (b)(6) Michael Letko
(b)(6)
Subject: Re: [ext] Cel lines

Hi Vincent,

yes, I am in Berlin and happy about it! It took a while to get settled but now everything works fine.
I will not be in Rotterdam unfortunately.
I really try to focus on bat immunology (innate immune and stress response) now!
So what are your plans? Anything we can do together?
Attached an MTA that I will need for the exchange of cells to make them eligible within our EU EVAg infrastructure project.
Hope it will work with your legal office. (b)(6)

Best, Marcel

—

PD Dr. Marcel A. Müller

Group leader, Institute of Virology
Charite - Universitätsmedizin Berlin

Campus Charite Mitte
Chariteplatz 1
D-10117 Berlin
Germany

Tel. (b)(6)
Fax +49 30 450 7525907

Email: (b)(6)
http://virologie-ccm.charite.de/

Visiting/Postal address:
Charite - Universitätsmedizin Berlin
Institute of Virology (Helmut Ruska Haus)
Chariteplatz 1 (Rahel Hirsch Weg 3)
D-10117 Berlin
Germany

Von: Vincent Munster (b)(6)
Datum: Samstag, 9. März 2019 um 00:21
An: "Müller, Marcel Alexander" (b)(6) "Letko, Michael (NIH/NIAID) [F]" (b)(6)
Betreff: [ext] Cel lines

Hi Marcel,

I hope life in Berlin is treating you well? I saw that you established a nice research group at charite, very cool.

Are you by chance going to the ECV meeting in Rotterdam in April?

I was wondering whether you would be willing to share two of your bat cellines with us?

We are looking for:

1. RhiNi = R. landeri
2. RhiLu = R. alcyone

Just let me know, My post-doc Dr. Michael Letko is in charge of all of the arrangements,

Cheers en hartelijke groet uit Montana,

Vincent Munster, PhD
Chief, Virus Ecology Unit
Laboratory of Virology
Rocky Mountain Laboratories
NIAID/NIH

From: Letko, Michael (NIH/NIAID) [F]
Sent: Mon, 25 Mar 2019 17:02:57 +0000
To: Munster, Vincent (NIH/NIAID) [E]
Subject: FW: [ext] Cel lines
Attachments: MTA_Charite Müller_template.doc

Do you need anything from me to get this going?

-michael

--
Michael Letko, Ph.D
Postdoctoral IRTA
Dr. Vincent Munster Laboratory
Virus Ecology Unit, Laboratory of Virology
Rocky Mountain Laboratories
NIAID/NIH
PubMed
903S 4th Street
Hamilton MT 59840
(b)(6)

From: "Müller, Marcel Alexander" (b)(6)
Date: Tuesday, March 12, 2019 at 11:36 AM
To: "Munster, Vincent (NIH/NIAID) [E]" (b)(6) "Letko, Michael (NIH/NIAID) [F]" (b)(6)
Subject: Re: [ext] Cel lines

Hi Vincent,

yes, I am in Berlin and happy about it! It took a while to get settled but now everything works fine.
I will not be in Rotterdam unfortunately.
I really try to focus on bat immunology (innate immune and stress response) now!
So what are your plans? Anything we can do together?
Attached an MTA that I will need for the exchange of cells to make them eligible within our EU EVAg infrastructure project.
Hope it will work with your legal office.(b)(6)

Best, Marcel

—
PD Dr. Marcel A. Müller

Group leader, Institute of Virology
Charite - Universitätsmedizin Berlin

Campus Charite Mitte
Chariteplatz 1
D-10117 Berlin
Germany

Tel. (b)(6)
Fax +49 30 450 7525907

Email:(b)(6)
http://virologie-ccm.charite.de/

_____
Visiting/Postal address:
Charite - Universitätsmedizin Berlin
Institute of Virology (Helmut Ruska Haus)
Chariteplatz 1 (Rahel Hirsch Weg 3)
D-10117 Berlin
Germany

Von: Vincent Munster (b)(6)
Datum: Samstag, 9. März 2019 um 00:21
An: "Müller, Marcel Alexander" (b)(6) "Letko, Michael (NIH/NIAID) [F]" (b)(6)
Betreff: [ext] Cel lines

Hi Marcel,

I hope life in Berlin is treating you well? I saw that you established a nice research group at charite, very cool.

Are you by chance going to the ECV meeting in Rotterdam in April?

I was wondering whether you would be willing to share two of your bat cellines with us?

We are looking for:

1. RhiNi = R. landeri
2. RhiLu = R. alcyone

Just let me know, My post-doc Dr. Michael Letko is in charge of all of the arrangements,

Cheers en hartelijke groet uit Montana,

Vincent Munster, PhD

Chief, Virus Ecology Unit
Laboratory of Virology
Rocky Mountain Laboratories
NIAID/NIH

(b)(4)

Material Transfer Agreement

between

CHARITÉ – Universitätsmedizin Berlin,
Charitéplatz 1, D-10117 Berlin

supported by the

(b)(4)

(hereinafter referred to as "SUPPLIER")

and

Company/Institution
(hereinafter referred to as "RECIPIENT")

MATERIAL: described in Annex A

RECIPIENT'S CONTACT:

SUPPLIER'S CONTACT:

(b)(6)
Charité – Universitätsmedizin Berlin
(b)(6)
Charitéplatz 1
10117 Berlin
Germany

1. **Background**.
RECIPIENT desires to obtain the material and/or information described in **Appendix A** (together with all fragments and mixtures "MATERIAL") from SUPPLIER for use by RECIPIENT solely for non-human experiments described in **Appendix A** (the "TESTS")

under the terms and conditions of this Agreement. The obligations of RECIPIENT herein described will apply to any biological MATERIAL that incorporates the MATERIAL or any recombinant version thereof.

2. The MATERIAL and the TESTS.

RECIPIENT acknowledges that SUPPLIER owns the MATERIAL. SUPPLIER will use commercially reasonable efforts to provide RECIPIENT with the quantity of the MATERIAL described in Appendix A. RECIPIENT will use the MATERIAL solely for the TESTS and for no other purpose. RECIPIENT agrees not to analyze the MATERIAL for the purpose of determining the structure nor amino acid sequence thereof. Furthermore, the use for any commercial purpose, such as for production is prohibited under this Agreement. In particular, no rights are provided to use the MATERIAL or any related patents for profit-making or commercial provision of a service to a third party in exchange for consideration. RECIPIENT will not use the MATERIAL for testing in or treatment of human subjects. RECIPIENT acknowledges that the MATERIAL is experimental and will comply with all laws and regulations applicable to its handling and use. Any MATERIAL remaining upon completion of the Research either will be returned to SUPPLIER or discarded, consistent with SUPPLIER'S instructions. RECIPIENT agrees to not distribute or release the MATERIAL to any other person or entity, except laboratory personnel of RECIPIENT. RECIPIENT shall ensure that no one will be allowed to take or send the MATERIAL to any other location than mentioned above, unless written permission is obtained from SUPPLIER.

3. Confidentiality.

RECIPIENT shall treat in confidence, for a period of five (5) years from the date of its disclosure, any written information pertaining to the MATERIAL provided to RECIPIENT by SUPPLIER or SUPPLIER'S Scientist(s). Excluded from this obligation shall be any information

(a) that was previously known to RECIPIENT prior to receipt of information from SUPPLIER;

(b) that lawfully is, or becomes publicly available during said five (5) year period through no fault of RECIPIENT;

(c) which is disclosed to RECIPIENT without confidentiality obligations by a third party having the right to make such disclosure; or

(d) which is independently developed by RECIPIENT without the use of or reference to any information received from SUPPLIER.

If RECIPIENT is suspected to breach this Agreement, RECIPIENT has to prove that the matter of suspected violation is information in compliance with 3 a - d.

4. Research Results.

RECIPIENT will conduct TESTS with the MATERIAL and gather research results. RECIPIENT will inform SUPPLIER of Research results related to the MATERIAL, by personal communication and by providing SUPPLIER with written information describing the results of such Research. In case of joint inventions, SUPPLIER and Recipient shall conclude in good faith a separate agreement concerning the use, patenting and

commercialization of those joint inventions. Recipient shall grant to SUPPLIER an irrevocable non-exclusive royalty-free license to practise the invention, improvement or modification for scientific research purposes.

5. Publication and Acknowledgement.

The RECIPIENT shall have the right, consistent with academic standards, to publish or present the results of the research work performed in accordance with this Agreement. The RECIPIENT shall disclose planned publications to SUPPLIER at least thirty (30) days prior to submission for publication. If SUPPLIER does not send an objection during this thirty (30) days, RECIPEINT will be free to submit for publication without delay.
In presentations or publications concerning the use of the Material, the RECIPIENT will acknowledge the SUPPLIER and the named SUPPLIER´s contact as the source of the MATERIAL. When research work is performed in collaboration, co- authorship of SUPPLIER is required.

6. No Warranty.

RECIPIENT acknowledges that any MATERIAL delivered to it under this agreement is experimental in nature. SUPPLIER makes no representations nor extends any warranties of any kind, either expressed or implied, with respect to the MATERIAL. There are no express or implied warranties of merchantability or fitness for a particular purpose, nor does SUPPLIER represent that the use of the MATERIAL will not infringe any patent, copyright, trade secret, trademark or other rights of third parties.

7. Indemnification.

SUPPLIER shall not be liable to RECIPIENT for any loss, claim, or demand made by RECIPIENT, or made against RECIPIENT by any other party, due to, or arising from, the use of the MATERIAL by the RECIPIENT. To the extent permitted by law, RECIPIENT shall indemnify, defend and hold harmless SUPPLIER, its trustees, assignees, agents and employees from any claim asserted against them arising from the negligence or wilful misconduct in the use of the MATERIAL by RECIPIENT or its agents or employees.

8. No obligations.

No rights or licenses to trademarks, inventions, copyrights or patents are implied or granted under this Agreement.

9. Final Agreement.

This Agreement and Appendix A attached hereto and hereby incorporated herein, contains the final, complete and exclusive agreement of the parties relative to the subject matter hereof and supersedes all prior and contemporaneous understandings and agreements relating to its subject matter. This Agreement may not be changed, modified, amended or supplemented except by a written instrument signed by both parties.

10. Start and Termination.
This Agreement starts with the date of signature of RECIPIENT. Either party may terminate this Agreement upon thirty (30) days' prior written notice to the other party. Upon termination, RECIPIENT will immediately return to SUPPLIER its Confidential Information, and any unused samples of the MATERIAL, and all of RECIPIENT's rights to use the MATERIAL will end. Following termination, neither party will have any further obligations under this Agreement, except that Sections 2, 3, 5 and 6 will survive.

11. Miscellaneous.
This Agreement shall be governed by the laws of Germany, excluding its conflicts of laws principles. Place of jurisdiction is Berlin, Germany.

If you agree to accept the MATERIAL under the above conditions, please have this Agreement signed by an authorized representative of RECIPIENT and return two originals to:

Charité – Universitätsmedizin Berlin
(b)(6)
Charitéplatz 1
D-10117 Berlin

The MATERIAL will be sent to you as soon as possible after the receipt of the signed Agreement.

RECIPIENT ORGANIZATION APPROVAL:

***Company*, Date:** _____

By:_____

_____
Print Name of Authorized Official

_____
Position

AGREED AND ACCEPTED BY RECIPIENT SCIENTIST

Date: _____

By:_____

_____
Print Name of Recipient Scientist

_____
Position

Charité – Universitätsmedizin Berlin

Date: _____

By:_____

(b)(6)

Date: _____

By: (b)(6)

(b)(6)

CHARITÉ – Universitätsmedizin Berlin,
Charitéplatz 1, D-10117 Berlin

Appendix A

MATERIAL:

EXAMPLE: RoNi/7.1

Comment: Please always use the cell designations exactly as stated in this MTA.

Short description of work:

TESTS: