From: Lindsey Pujanandez[scitranslmededitors@aaas.org]

Sent: Wed 3/25/2020 6:33:02 AM (UTC-07:00)

To: george.r.painter@emory.edu[george.r.painter@emory.edu]; mnatchu@emory.edu[mnatchu@emory.edu]; msainda@emory.edu[msainda@emory.edu]; stephanie_montgomery@med.unc.edu[stephanie_montgomery@med.unc.edu]; leist@email.unc.edu[leist@email.unc.edu]; rlgraham@email.unc.edu[rlgraham@email.unc.edu]; rbaric@email.unc.edu[rbaric@email.unc.edu]; sheahan@email.unc.edu[sheahan@email.unc.edu]; aschaefe@email.unc.edu[aschaefe@email.unc.edu]; a.kolykhalov@emory.edu[a.kolykhalov@emory.edu]; ron_swanstrom@med.unc.edu[ron_swanstrom@med.unc.edu]; gblueml@emory.edu[gblueml@emory.edu]; shuntaiz@email.unc.edu[shuntaiz@email.unc.edu]; mark.denison@vanderbilt.edu[mark.denison@vanderbilt.edu]; Sims, Amy C[amy.sims@pnnl.gov]; collinh@live.unc.edu[collinh@live.unc.edu]; maria.l.agostini.1@vumc.org[maria.l.agostini.1@vumc.org]; ardina.pruijssers@vumc.org[ardina.pruijssers@vumc.org]; ajbrown7@email.unc.edu[ajbrown7@email.unc.edu]; kdinnon@email.unc.edu[kdinnon@email.unc.edu]; zaq6@cdc.gov[zaq6@cdc.gov]; axt4@cdc.gov[axt4@cdc.gov]; nax3@cdc.gov[nax3@cdc.gov]; jim.chappell@vumc.org[jim.chappell@vumc.org]

Cc: lpujanandez@aaas.org[lpujanandez@aaas.org]

Subject: Authorship Form for your Science Translational Medicine Manuscript

Dear Author of Manuscript abb5883:

Please fill out the Authorship and License to Publish forms for Manuscript Number abb5883 "An orally bioavailable broad-spectrum antiviral inhibits SARS-CoV-2 and multiple endemic, epidemic and bat coronavirus".

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George Painter - george.r.painter@emory.edu
Michael Natchus - mnatchu@emory.edu
Manohar Saindane - msainda@emory.edu

Stephanie Montgomery - stephanie_montgomery@med.unc.edu
Sarah Leist - leist@email.unc.edu
Rachel L. Graham - rlgraham@email.unc.edu
Ralph S. Baric - rbaric@email.unc.edu
Timothy Sheahan - sheahan@email.unc.edu
Alexandra Schäfer - aschaefe@email.unc.edu
Alexander A Kolykhalov - a.kolykhalov@emory.edu
Ronald Swanstrom - ron_swanstrom@med.unc.edu
Gregory Bleumling - gblueml@emory.edu
Shuntai Zhou - shuntaiz@email.unc.edu
Mark Denison - mark.denison@vanderbilt.edu
Amy Sims - amy.sims@pnnl.gov
Collin Hill - collinh@live.unc.edu
Maria Agostini - maria.l.agostini.1@vumc.org
Andrea Pruijssers - ardina.pruijssers@vumc.org
Ariane Brown - ajbrown7@email.unc.edu
Kenneth Dinnon - kdinnon@email.unc.edu
Jennifer Harcourt - zaq6@cdc.gov
Azaibi Tamin - axt4@cdc.gov
Natalie Thornburg - nax3@cdc.gov
Jim Chappell - jim.chappell@vumc.org

Please let me know if you have any trouble logging in. Thank you for your contribution to Science Translational Medicine!

Sincerely,
The Editors
Science Translational Medicine

From: England, Erika[ee42@email.unc.edu]
Sent: Wed 3/25/2020 8:36:50 AM (UTC-07:00)
To: Sims, Amy C[amy.sims@pnnl.gov]
Subject: FW: Transfer SRA to new institution

Amy- below are the conversations I had with my CDC point of contact and CJIS. I had Eric transferred and approved on our registration within 24hours.

Hi Erika,
I just got updated by our Security Team.

- Please email CJIS at the following address: sra.cdc@leo.gov
- Indicate that name of the individual along with their present DOJ number. Also indicate the person is transferring his/her SRA-status to UNC-Chapel Hill
- Please add the person to UNC-Chapel Hill's registration via eFSAP.

Good morning Erika,
Could you please provide me the person's name and SRA ID. In the meantime, please proceed to add that individual to your registration. Once I get the person's name and ID, I will fwd to our security team. The will finalize the transition.

Thanks. Mo

Mo,

We have a new member in our group that came from the NIH and has an active SRA. We would like to add him to our registration. Now that everything is done online, what is the correct process to add him and verify his SRA without having to submit for another background check?

Morris "Mo" Blaylock, Ph.D.,
Microbiologist/Inspector
Division of Select Agents & Toxins
Center for Preparedness & Response
Centers for Disease Control & Prevention
1600 Clifton Road NE, Mailstop A-46
Atlanta, GA 30329
Office: 404-718-5970 Mobile: 470-446-7865 Fax: 404-718-2096
Email: Mblaylock@cdc.gov
https://selectagents.gov
Telework on Mondays and Fridays

Many Hands Make Light Work

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From: Holcomb, John <john.holcomb@leo.gov>
Sent: Thursday, January 24, 2019 9:55:24 AM
To: England, Erika; sra.cdc@leo.gov
Subject: Re: Transfer SRA to new institution

Erika

The SRA is valid and the person is now approved at your institution.

John

On 01/23/19 15:55, **"England, Erika"** <ee42@email.unc.edu> wrote:
CJIS,

I have a new group member who is transferring his active SRA from the NIH to UNC Chapel Hill.

Eric Lewis UIN: (b) (6) SRA date: 3/3/2017

University of North Carolina at Chapel Hill registration number (b) (6)

Please let me know if you need any other information. Thank you!

Erika England

Biosafety Specialist

Alternate Responsible Official

Environment, Health & Safety

University of North Carolina at Chapel Hill

1120 Estes Drive, Campus Box 1650

Chapel Hill, NC 27599

(919) 962-5712

--

John B. Holcomb
DSAT Security Specialist
CDC Division of Select Agents and Toxins 1600 Clifton Road MS A-46 Atlanta, GA 30333
404-718-2011
Fax: 404-718-2096

From: Sims, Amy C[amy.sims@pnnl.gov]
Sent: Wed 3/25/2020 11:50:49 AM (UTC-07:00)
To: Sheahan, Timothy Patrick[sheahan@email.unc.edu]
Subject: AAAS author things to do

HI,

Am finally in the right place on the AAAS website.

Do you want to put that I am currently at PNNL in the acknowledgements or leave it alone? I can go either way.

Also, you state that I got NIAID funding to do the 1931 work but I didn't, at least to my knowledge. That money was in Ralph's name and was a supplement on some NIH funding the Painter group had. Only Gilead gave me funds to do any drug studies.

Just letting you know. Thanks, Amy

From: Sheahan, Timothy Patrick[sheahan@email.unc.edu]
Sent: Wed 3/25/2020 12:04:35 PM (UTC-07:00)
To: Sims, Amy C[amy.sims@pnnl.gov]
Subject: Re: AAAS author things to do

I can add in your current address. That stuff about the 1931 contract to you is no longer in the paper so that's odd. That's in an older version though.

From: Amy Sims <amy.sims@pnnl.gov>
Date: Wednesday, March 25, 2020 at 2:50 PM
To: "Sheahan, Timothy Patrick" <sheahan@email.unc.edu>
Subject: AAAS author things to do

HI,

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Just letting you know. Thanks, Amy

From: Sims, Amy C[amy.sims@pnnl.gov]
Sent: Wed 3/25/2020 12:05:58 PM (UTC-07:00)
To: Sheahan, Timothy Patrick[sheahan@email.unc.edu]
Subject: Re: AAAS author things to do

I thought I opened the draft you sent out just last week. Its cool. Thank you! Sorry to bother you then. I appreciate it. Amy

---

From: "Sheahan, Timothy Patrick" <sheahan@email.unc.edu>
Date: Wednesday, March 25, 2020 at 12:04 PM
To: "Sims, Amy C" <amy.sims@pnnl.gov>
Subject: Re: AAAS author things to do

I can add in your current address. That stuff about the 1931 contract to you is no longer in the paper so that's odd. That's in an older version though.

From: Amy Sims <amy.sims@pnnl.gov>
Date: Wednesday, March 25, 2020 at 2:50 PM
To: "Sheahan, Timothy Patrick" <sheahan@email.unc.edu>
Subject: AAAS author things to do

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Just letting you know. Thanks, Amy

From: Sheahan, Timothy Patrick[sheahan@email.unc.edu]
Sent: Wed 3/25/2020 12:07:40 PM (UTC-07:00)
To: Sims, Amy C[amy.sims@pnnl.gov]
Subject: Re: AAAS author things to do

Nope. You are RIGHT. It's in an odd spot. I've deleted this. Thanks!

T

From: Amy Sims <amy.sims@pnnl.gov>
Date: Wednesday, March 25, 2020 at 3:06 PM
To: "Sheahan, Timothy Patrick" <sheahan@email.unc.edu>
Subject: Re: AAAS author things to do

I thought I opened the draft you sent out just last week. Its cool. Thank you! Sorry to bother you then. I appreciate it. Amy

---

From: "Sheahan, Timothy Patrick" <sheahan@email.unc.edu>
Date: Wednesday, March 25, 2020 at 12:04 PM
To: "Sims, Amy C" <amy.sims@pnnl.gov>
Subject: Re: AAAS author things to do

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From: Amy Sims <amy.sims@pnnl.gov>
Date: Wednesday, March 25, 2020 at 2:50 PM
To: "Sheahan, Timothy Patrick" <sheahan@email.unc.edu>
Subject: AAAS author things to do

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Just letting you know. Thanks, Amy

From: Sims, Amy C[amy.sims@pnnl.gov]
Sent: Wed 3/25/2020 12:08:09 PM (UTC-07:00)
To: Sheahan, Timothy Patrick[sheahan@email.unc.edu]
Subject: Re: AAAS author things to do

Very welcome. Thanks, Amy

From: "Sheahan, Timothy Patrick" <sheahan@email.unc.edu>
Date: Wednesday, March 25, 2020 at 12:07 PM
To: "Sims, Amy C" <amy.sims@pnnl.gov>
Subject: Re: AAAS author things to do

Nope. You are RIGHT. It's in an odd spot. I've deleted this. Thanks!

T

From: Amy Sims <amy.sims@pnnl.gov>
Date: Wednesday, March 25, 2020 at 3:06 PM
To: "Sheahan, Timothy Patrick" <sheahan@email.unc.edu>
Subject: Re: AAAS author things to do

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From: "Sheahan, Timothy Patrick" <sheahan@email.unc.edu>
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To: "Sims, Amy C" <amy.sims@pnnl.gov>
Subject: Re: AAAS author things to do

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Date: Wednesday, March 25, 2020 at 2:50 PM
To: "Sheahan, Timothy Patrick" <sheahan@email.unc.edu>
Subject: AAAS author things to do

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Just letting you know. Thanks, Amy

From: Sheahan, Timothy Patrick[sheahan@email.unc.edu]
Sent: Wed 3/25/2020 12:08:43 PM (UTC-07:00)
To: Sims, Amy C[amy.sims@pnnl.gov]
Subject: Re: AAAS author things to do

Can I have your PNNL Address, etc. in this format? [1]Department of Epidemiology, University of North Carolina at Chapel Hill, Chapel Hill, NC, 27599, USA.

From: Amy Sims <amy.sims@pnnl.gov>
Date: Wednesday, March 25, 2020 at 3:06 PM
To: "Sheahan, Timothy Patrick" <sheahan@email.unc.edu>
Subject: Re: AAAS author things to do

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From: "Sheahan, Timothy Patrick" <sheahan@email.unc.edu>
Date: Wednesday, March 25, 2020 at 12:04 PM
To: "Sims, Amy C" <amy.sims@pnnl.gov>
Subject: Re: AAAS author things to do

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From: Amy Sims <amy.sims@pnnl.gov>
Date: Wednesday, March 25, 2020 at 2:50 PM
To: "Sheahan, Timothy Patrick" <sheahan@email.unc.edu>
Subject: AAAS author things to do

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Just letting you know. Thanks, Amy

From: Sims, Amy C[amy.sims@pnnl.gov]
Sent: Wed 3/25/2020 12:11:54 PM (UTC-07:00)
To: Sheahan, Timothy Patrick[sheahan@email.unc.edu]
Subject: Re: AAAS author things to do

Yes sir.

Chemical and Biological Signature Sciences, National Security Division, Pacific Northwest National Laboratory, Richland, WA 99352, USA.

You can start with the National Security Division part if this is too long. Thank you, Amy

---

From: "Sheahan, Timothy Patrick" <sheahan@email.unc.edu>
Date: Wednesday, March 25, 2020 at 12:09 PM
To: "Sims, Amy C" <amy.sims@pnnl.gov>
Subject: Re: AAAS author things to do

Can I have your PNNL Address, etc. in this format? [1]Department of Epidemiology, University of North Carolina at Chapel Hill, Chapel Hill, NC, 27599, USA.

From: Amy Sims <amy.sims@pnnl.gov>
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To: "Sheahan, Timothy Patrick" <sheahan@email.unc.edu>
Subject: Re: AAAS author things to do

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Subject: Re: AAAS author things to do

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From: Amy Sims <amy.sims@pnnl.gov>
Date: Wednesday, March 25, 2020 at 2:50 PM
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Subject: AAAS author things to do

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Ralph's name and was a supplement on some NIH funding the Painter group had. Only Gilead gave me funds to do any drug studies.

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From: Sheahan, Timothy Patrick[sheahan@email.unc.edu]
Sent: Wed 3/25/2020 12:14:23 PM (UTC-07:00)
To: Sims, Amy C[amy.sims@pnnl.gov]
Subject: Re: AAAS author things to do

Rock on. I've put it in the paper.

T

From: Amy Sims <amy.sims@pnnl.gov>
Date: Wednesday, March 25, 2020 at 3:12 PM
To: "Sheahan, Timothy Patrick" <sheahan@email.unc.edu>
Subject: Re: AAAS author things to do

Yes sir.

Chemical and Biological Signature Sciences, National Security Division, Pacific Northwest National Laboratory, Richland, WA 99352, USA.

You can start with the National Security Division part if this is too long. Thank you, Amy

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From: "Sheahan, Timothy Patrick" <sheahan@email.unc.edu>
Date: Wednesday, March 25, 2020 at 12:09 PM
To: "Sims, Amy C" <amy.sims@pnnl.gov>
Subject: Re: AAAS author things to do

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Subject: AAAS author things to do

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Just letting you know. Thanks, Amy

From: Lu, Lu[llu@uthsc.edu]

Sent: Thur 3/26/2020 4:05:00 PM (UTC-07:00)

To: Klaus x Klaus Schughart[Klaus.Schughart@helmholtz-hzi.de]

Cc: Williams, Robert W[rwilli10@uthsc.edu]; Xu, Fuyi[fxu10@uthsc.edu]; Sims, Amy C[amy.sims@pnnl.gov]; rbaric@email.unc.edu[rbaric@email.unc.edu]; silke.bergmann@[redacted][silke.bergmann@[redacted]]; Li, Kui[kli1@uthsc.edu]

Subject: Re: [Ext] B6D2 infected RNAseq data

Attachment: Ace2_V4_260320.docx

Dear Klaus (and all others):
Here is updated version. Fuyi addressed most of your comments. Please feel free to modify it continuously.

Dear Amy and Ralph:

Some of Klaus' comment are specific to you. Could you help to reply or editing?

Thanks.

Lu Lu
Professor
Department of Genetics, Genomics and Informatics
University of Tennessee Health Science Center
71 S. Manassas St., Room 410K
Memphis, TN 38163
Tel: (901) 448-7557 (O)
Fax: (901) 448-3500 (Dept)

On Mar 26, 2020, at 1:18 PM, Schughart, Klaus <Klaus.Schughart@helmholtz-hzi.de> wrote:

Hi LuLu,
enclosed are my additional comments – now also on the discussion.
Maybe Amy has also some ideas to explain the discrepancy between lungs and HAE cells with respect to inverse up/down regulation.
Klaus

Von: Lu, Lu <llu@uthsc.edu>
Gesendet: Donnerstag, 26. März 2020 00:27
An: Schughart, Klaus <Klaus.Schughart@helmholtz-hzi.de>
Cc: Williams, Robert W <rwilli10@uthsc.edu>; Xu, Fuyi <fxu10@uthsc.edu>; amy.sims@pnnl.gov
Betreff: Re: [Ext] B6D2 infected RNAseq data

Dear Klaus:

We have accepted Rob, you and our previous editing. Here is updated version for you to check and modify continuously.

Dear Amy:

Could you check this version and address some comments that are related to you?

Thanks.

Lu Lu
Professor
Department of Genetics, Genomics and Informatics
University of Tennessee Health Science Center
71 S. Manassas St., Room 410K
Memphis, TN 38163
Tel: (901) 448-7557 (O)
Fax: (901) 448-3500 (Dept)

On Mar 25, 2020, at 3:18 PM, Xu, Fuyi <fxu10@uthsc.edu> wrote:

Dear Klaus,

Could you please send me those two images (high-resolution) in Figure 8 ? Thank you very much.

--
Fuyi Xu, PhD
Department of Genetics, Genomics, and Informatics
University of Tennessee Health Science Center

415 TRANSLATIONAL RESEARCH BUILDING
71 SOUTH MANASSAS
MEMPHIS TN 38163
Phone: (901)246-4398
E-mail: 5 U.S.C. § 552(b)(6) fxu10@uthsc.edu

From: Schughart, Klaus
Sent: Wednesday, March 25, 2020 3:04 PM
To: Lu, Lu
Cc: Xu, Fuyi; Williams, Robert W
Subject: AW: [Ext] B6D2 infected RNAseq data

Hi LuLu,
I forgot something in the Acknowledgements.
Please replace the Acknowledgements in the last version with the one in the enclosed version.
Thx,
Klaus

Von: Schughart, Klaus
Gesendet: Mittwoch, 25. März 2020 20:31
An: 'Lu, Lu' <llu@uthsc.edu>
Cc: 'Xu, Fuyi' <fxu10@uthsc.edu>; 'Williams, Robert W' <rwilli10@uthsc.edu>
Betreff: AW: [Ext] B6D2 infected RNAseq data

Dear all,
here are my comments on the revised version. I took out the previous comments that have been resolved.
Fuyi, I also enclose the data table for Figure 8A.
Once you are done with this version, I can have a look at the discussion and then you could send it off to the other co-authors.
Klaus

Von: Schughart, Klaus
Gesendet: Mittwoch, 25. März 2020 19:31
An: 'Lu, Lu' <llu@uthsc.edu>
Cc: Xu, Fuyi <fxu10@uthsc.edu>; Williams, Robert W <rwilli10@uthsc.edu>
Betreff: AW: [Ext] B6D2 infected RNAseq data

Thanks – will work it.
Klaus

Von: Lu, Lu <llu@uthsc.edu>
Gesendet: Mittwoch, 25. März 2020 19:02
An: Schughart, Klaus <Klaus.Schughart@helmholtz-hzi.de>
Cc: Xu, Fuyi <fxu10@uthsc.edu>; Williams, Robert W <rwilli10@uthsc.edu>
Betreff: Re: [Ext] B6D2 infected RNAseq data

Dear Klaus:

Fuyi and I accept all of you and rob's editing. We also clean and modified the paper, which addressed comments and answered the your questions. Please work on the updated version continuously. Hopefully we can submit this paper ASAP. Thanks.

Lu Lu
Professor
Department of Genetics, Genomics and Informatics
University of Tennessee Health Science Center
71 S. Manassas St., Room 410K
Memphis, TN 38163
Tel: (901) 448-7557 (O)
Fax: (901) 448-3500 (Dept)

> On Mar 24, 2020, at 4:36 PM, Lu, Lu <llu@uthsc.edu> wrote:
>
> Thanks, Rob. We will modify based on your comments and will send you updated version for your editing again.
>
> Lu Lu
> Professor
> Department of Genetics, Genomics and Informatics
> University of Tennessee Health Science Center
> 71 S. Manassas St., Room 410K
> Memphis, TN 38163
> Tel: (901) 448-7557 (O)
> Fax: (901) 448-3500 (Dept)

On Mar 24, 2020, at 4:14 PM, Rob Williams <rwilliams@uthsc.edu> wrote:

Dear team,

Brilliant effort,

Edited the abstract heavily (please review carefully) and built up the Methods section on BXDs a bit.

Have not gotten through all of paper, but looks great so far.

Should validate some networks and eQTLs with GEMMA.

Needs a bit more clarity to data sources—some for 43 strains, other data for parents. These details needed (imho) even in the abstract.

On Tue, Mar 24, 2020 at 2:41 PM Schughart, Klaus <Klaus.Schughart@helmholtz-hzi.de> wrote:

Hi Lulu, Fuyi,

enclosed are my comments, additions and corrections.

Looks very good!!!

Please go through and clean it up first (accept or discard changes, add information) and then I can continue to work on the cleaned-up version. Currently it is too much cluttered with all these changes and comments.

Fuyi, let me know what exactly you need in addition for the HAE data.

Klaus

Von: Schughart, Klaus
Gesendet: Freitag, 6. März 2020 19:02
An: Xu, Fuyi <fxu10@uthsc.edu>; Lu Lu [redacted] **5 U.S.C. § 552(b)(6)**
Cc: Rob Williams <rwilliams@uthsc.edu>; Jonsson, Colleen Beth (cjonsson@uthsc.edu) <cjonsson@uthsc.edu>
Betreff: AW: [Ext] B6D2 infected RNAseq data

Hi Fuyi, LuLu,

I did some extensive analyses of genes in the human HAE data set with your gene list. Enclosed are the results. Looks very good!!

For a publication, we would have to discuss which data to include and how to present the results.

Klaus

Von: Schughart, Klaus <Klaus.Schughart@helmholtz-hzi.de>
Gesendet: Donnerstag, 5. März 2020 04:45
An: Xu, Fuyi <fxu10@uthsc.edu>
Cc: Lu Lu 5 U.S.C. § 552(b)(6)
Betreff: Re: [Ext] B6D2 infected RNAseq data

Dear Fuyi,

sure I will have a look at these genes.

Klaus

Von meinem iPhone gesendet

> Am 04.03.2020 um 19:09 schrieb Xu, Fuyi <fxu10@uthsc.edu>:
>
> Dear Klaus,
>
> Lu and I are working on the Ace2 paper. We prioritized around 30 genes that are correlated with Ace2 (attached table). I am wondering if you can help me check the expression differences between infected and normal controls in the human data set last time you showed us, especially for those color highlighted. Also, is this gene list make sense from your perspective? Thank you very much.
>
> Best,
>
> --
> Fuyi Xu, PhD
> Department of Genetics, Genomics, and Informatics
> University of Tennessee Health Science Center
>
> 415 TRANSLATIONAL RESEARCH BUILDING
> 71 SOUTH MANASSAS
> MEMPHIS TN 38163
> Phone: (901)246-4398
> E-mail: **5 U.S.C. § 552(b)(6)** fxu10@uthsc.edu
>
> ---
>
> **From:** Schughart, Klaus

Sent: Friday, February 28, 2020 11:07 AM
To: Xu, Fuyi
Cc: Lu Lu
Subject: AW: [Ext] B6D2 infected RNAseq data

Hi LuLu, Fuyi,

I just remembered that I also have expression data from infected human lung slices (see enclosure).

This dataset has been published (enclosed).

Klaus

Von: Schughart, Klaus
Gesendet: Montag, 24. Februar 2020 21:00
An: 'Xu, Fuyi' <fxu10@uthsc.edu>
Cc: Lu Lu 5 U.S.C. § 552(b)(6)
Betreff: AW: [Ext] B6D2 infected RNAseq data

Dear Fuyi,

The data I sent you before (RNAseq_norm1.txt) have been normalized and log2 transformed using DESeq2 (rlogTransformation(dsDat, blind=TRUE).

Please find enclosed the raw counts for the B6/D2 infections from Wilk et al (all_mouse_counts.txt).

For the BXD data in GN:

The percent body weights are NOT the losses but the percent body weight with reference to the starting body weight (100%). So 105% means that the mouse has gained 5% of body weight relative to its starting weight when it was infected. Mice with percent body weight less than 100% have lost weight.

Klaus

Von: Xu, Fuyi <fxu10@uthsc.edu>
Gesendet: Sonntag, 23. Februar 2020 22:14
An: Schughart, Klaus <Klaus.Schughart@helmholtz-hzi.de>
Cc: Lu Lu 5 U.S.C. § 552(b)(6)
Betreff: RE: [Ext] B6D2 infected RNAseq data

Dear Dr. Klaus Schughart,

I am Lu Lu's postdoc, Fuyi Xu. Thank you for sending us the normalized RNA-seq data.

We would like to perform differential expression analysis, I am wondering if you have raw read count for this data set since the DEseq2 package depend on this kind of data matrix. By the way, is the normalized data log2 transformed ?

We also found the expression level of ACE2 across the BXD is significantly correlated with median weight loss at day 3 after infection (ID:13007), however, we found BXD65a loss 105% body weight. Is this correct? Or how should I interpret ?

<A54B14D6A4584F84B57E3AAA25DC5BBB.gif>

Best Regards,

--
Fuyi Xu, PhD
Department of Genetics, Genomics, and Informatics
University of Tennessee Health Science Center

415 TRANSLATIONAL RESEARCH BUILDING
71 SOUTH MANASSAS
MEMPHIS TN 38163
Phone: (901)246-4398
E-mail: 5 U.S.C. § 552(b)(6) fxu10@uthsc.edu

From: Lu, Lu
Sent: Monday, February 17, 2020 11:03 AM
To: Xu, Fuyi
Subject: Fwd: [Ext] B6D2 infected RNAseq data

Lu Lu
Professor
Department of Genetics, Genomics and Informatics
University of Tennessee Health Science Center
71 S. Manassas St., Room 410K
Memphis, TN 38163
Tel: (901) 448-7557 (O)
Fax: (901) 448-3500 (Dept)

Begin forwarded message:

From: "Schughart, Klaus" <Klaus.Schughart@helmholtz-hzi.de>

Subject: [Ext] B6D2 infected RNAseq data

Date: February 17, 2020 at 10:11:55 AM CST

To: "Lu, Lu" <llu@uthsc.edu>

Hi LuLu,

I checked the datasets.

GN807; RNAseq BXD infected is fine. This dataset has not been published yet.

I enclose the log2 normalized matrix for the B6/D2 infected data from Wilk et al. 2015:

RNAseq_norm1.txt -- for the mouse genes

00_influenza_lg2_RPKM_kls_280714.txt -- for the influenza gene segments

Let me know if you have any questions.

Klaus

Prof. Dr. Klaus Schughart
Head of Dept. of Infection Genetics
Helmholtz Centre for Infection Research,

University of Veterinary Medicine Hannover,

University of Tennessee Health
Science Center
Inhoffenstr. 7
D-38124 Braunschweig
Germany

E-mail : Klaus.Schughart@helmholtz-hzi.de
Phone: +49 531 6181 1100

<B59D99096FF44A7EBCC7A3BC50D960A2.png>

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Vorsitzende des Aufsichtsrates: Frau MinDir'in Prof. Dr. Veronika von Messling
Stellvertreter: MinDirig Rüdiger Eichel, Niedersächsisches Ministerium für Wissenschaft und Kultur
Geschäftsführung: Prof. Dr. Dirk Heinz; Silke Tannapfel
Gesellschaft mit beschränkter Haftung (GmbH)
Sitz der Gesellschaft: Braunschweig
Handelsregister: Amtsgericht Braunschweig, HRB 477

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Geschäftsführung: Prof. Dr. Dirk Heinz; Silke Tannapfel

Gesellschaft mit beschränkter Haftung (GmbH)
Sitz der Gesellschaft: Braunschweig
Handelsregister: Amtsgericht Braunschweig, HRB 477

<Table 1.xlsx>

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Gesellschaft mit beschränkter Haftung (GmbH)
Sitz der Gesellschaft: Braunschweig
Handelsregister: Amtsgericht Braunschweig, HRB 477

--
Rob

Robert W. Williams, Ph.D.
Chair: Department of Genetics, Genomics and Informatics
University of Tennessee Health Science Center
71 S Manassas St, Memphis TN 38103-3310, TSRB Room 405
Tele/Cell: 901 604 4752
EMAIL: rwilliams@uthsc.edu or **5 U.S.C. § 552(b)(6)**

<Ace2-Draft-03182020-V1_LuB_kls_RWW_240320_3.docx>

<8608AAAF2A14461CBDBD04DA13676A0C.png>

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Sitz der Gesellschaft: Braunschweig
Handelsregister: Amtsgericht Braunschweig, HRB 477

Genetic Dissection of the Regulatory Mechanisms of *Ace2* in the Infected Mouse Lung

Fuyi Xu[1#], Jun Gao[1, 5#], Silke Bergman[4], Amy C. Sims[7, 9], Ralph S. Baric[6,7, 8], Kui Li[4], Robert W. Williams[1], Klaus Schughart[2, 3, 4*], and Lu Lu[1*]

[1]Department of Genetics, Genomics, and Informatics, University of Tennessee Health Science Center, Memphis, TN, USA;
[2]Department of Infection Genetics, Helmholtz Centre for Infection Research, Braunschweig, Germany
[3]University of Veterinary Medicine Hannover, Hannover, Germany;
[4]Department of Microbiology, Immunology, and Biochemistry, University of Tennessee Health Science Center, Memphis, TN, USA;
[5]Institute of Animal Husbandry and Veterinary Science, Shanghai Academy of Agricultural Sciences, Shanghai, China.
[6]Department of Microbiology and Immunology, University of North Carolina at Chapel Hill, Chapel Hill, NC
[7]Department of Genetics, University of North Carolina at Chapel Hill, Chapel Hill, NC
[8]Department of Epidemiology, University of North Carolina at Chapel Hill, Chapel Hill, NC
[9]present address: Pacific Northwest National Laboratory in Richland, WA

[#] These authors contributed equally to this work.

* Correspondence:

Lu Lu: llu@uthsc.edu

Department of Genetics, Genomics and Informatics
University of Tennessee Health Science Center
Memphis, TN 38163, United States;

Klaus Schughart: klaus.schughart@helmholtz-hzi.de
Department of Infection Genetics
Helmholtz Centre for Infection Research
Braunschweig, Germany

Abstract

Acute lung injury (ALI) is an important cause of morbidity and mortality after viral infections, such as influenza virus H1N1, SARS-CoV-2, and SARS-CoV. The angiotensin 1 converting enzyme 2 (ACE2) plays a protective role in the pathogenesis of SARS virus-induced ALI. However, the contributions of *ACE2* gene variants to the post-infection heterogeneity of the host response and susceptibility and underlying mechanisms are not yet well understood. In this study, we quantified lung transcriptomes in 41 BXD recombinant inbred strains of mice plus their parental strains of C57BL/6J and DBA/2J mice after H1N1 infection. The expression of *Ace2* mRNA across strains decreased significantly and was dependent on both infection severity and time after treatment. Expression was negatively correlated with body weight loss and survival time. Data for 43 strains were analyzed using weighted gene co-expression network analysis (WGCNA). *Ace2* not only participates in virus-induced ALI by interacting with TNF, MAPK, and notch signaling pathway, but also is linked to genes that play important function in the pulmonary epithelium. Furthermore, by applying muti-layer filtration, including differential expression analysis, eQTL mapping, functional variant identification, and gene annotation, we highlighted 10 key candidate genes linked to an *Ace2* network: *Sox11*, *Tff2*, *Rnf128*, *Muc5b*, *Tmprss2*, *Ulk4*, *Cckar*, *Wdpcp*, *Ift140*, and *Ccdc40*. Of these transcripts, *Rnf128* was identified as a potential master regulator using Bayesian network analysis. The key gene set is enriched for genes known to be differentially expressed in SARS-CoV infected primary human airway epithelial cells. Collectively, these findings define an *Ace2*-associated modulatory network relevant to host response to H1N1 infection and highlight novel potential therapeutic targets for virus-induced respiratory diseases, including SARS-CoV and SARS-CoV-2. In addition, we demonstrated that BXD family of strains provide an excellent resource for dissecting host genetic regulatory mechanisms involved in the defense to viral infections and host disease.

Key words: H1N1, acute lung injury, BXD mice, *Ace2,* regulatory network

Introduction

The pandemic disease, COVID-19, is caused by a novel severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has spread with almost unprecedent speed starting in November or December 2019. As of March 25, 2020, according to preliminary estimates by World Health Organization (WHO), more than 195 countries, over 375,000 cases of COVID-19 have been reported, with more than 16,000 deaths. Since 1918, the world has experienced four influenza pandemics—1918 H1N1, 1957 H2N2, 1968 H3N2, and 2009 H1N1. The first of these resulted in at least 50 million global mortalities [1]. The main pathological features caused by both COVID-19 and 2009 influenza A (H1N1) are acute viral pneumonia, which is an important cause of acute lung injury (ALI) [2]. The known symptoms for influenza A (H1N1) and COVID-19 are nearly identical and for both infected patients can manifest in asymptomatic, mild, or severe, and that can culminate in death. Individual genetic differences almost certainly play an important role in the development and severity of the illness [3-5].

Angiotensin-converting enzyme 2 (*ACE2*), a negative regulator of the renin angiotensin system (RAS), plays a protective role in pathogenesis of ALI in several animal models of acute respiratory distress syndrome [6]. *Ace2* knockout mice exhibited severe pathology of ALI and exogenous recombinant human *ACE2* expression attenuates acute lung failure in *Ace2* knockout mice as well as in wild-type mice [7]. Studies have shown that ACE2 protein levels are significantly downregulated after infection with H1N1 virus but are dispensable for viral replication [8]. For SARS coronavirus, *ACE2* has been identified as the principal host receptor for the spike protein receptor binding domain of the virus and thereby facilitates viral entry and replication in host cells [9-11]. In *in vivo* studies, SARS-CoV infections of wild-type mice causes severe and lethal lung failure and the downregulation of *Ace2* in lungs[12]. Single-cell RNA sequencing demonstrated that 83% of *ACE2*-expressing cells are alveolar epithelial type II cells in lung and a much higher *ACE2*-expressing cell ratio is observed in Asian male donors compared to white and African American donors [13], However, the contributions of *ACE2* variation to the post-infection heterogeneity of the ALI is still poorly defined.

Susceptibility to viral infection varies greatly among mouse strains [14], especially between inbred strain C57BL/6J (B6) and DBA/2J (D2) mice [15]. B6 is relatively resistant to influenza A and often survives inoculation. In contrast, D2 loses body weight rapidly and at the same inoculum dose dies within 5–7 days. Our laboratory has generated and maintains ~120 BXD recombinant inbred (RI) strains derived from multiple independent crosses of B6 and D2 parental strains. Each of these highly diverse BXD progeny are fully inbred and each has been sequenced. Collective this family of strains segregates for ~6 million variants with minor allele frequencies above 0.3 [16], making it an ideal and well powered reference population to model the genetic susceptibility of influenza A infections [17]. Indeed, our previous genetic mapping in this population have revealed several loci and gene variants that control the host response to influenza A (H1N1) virus infection [18] and other infectious diseases [19-22]. The BXD family provides a powerful resource to dissect loci, genes, molecular networks, and even mechanisms of host genetic responses to viral infections.

Here we have analyzed the lung transcriptome by RNAseq in 41 BXD RI strains and both parental strains after infection with H1N1 influenza A virus. By using systems genetics approaches, we investigate patterns of covariation of mRNAs with induced variation in *Ace2* expression. We define networks and regulatory relations that are likely to contribute to the host defense and/or pathology of H1N1-induced ALI.

Materials and methods

Mice

The subjects for this study were female mice belonging to 41 distinct BXD recombinant inbred (RI) strains, and the parental C57BL/6J (B6) and DBA/2J (D2) strains. These BXD strains segregate for over 5 million sequence variants with minor allele frequencies greater than 0.3 [23]. They are well powered to detect major loci that contribute to genetic variation in disease susceptibility [19-22] and to define causal networks of interacting loci, genes, gene productions (mRNAs and proteins), and pathogenicity outcome measures [18, 24]. All cases were between 8 and 12 weeks of age when infected with virus. All were housed and maintained on a 12:12 light/ dark cycle, with *ad libitum* access to food and water. Procedures were approved by the UTHSC animal care and use committee.

Ethics

Protocols for the mouse experiments were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Tennessee Health Science Center.

Virus

Original stocks of mouse adapted A/Puerto Rico/8/34 (H1N1, PR8M) virus were obtained from Stefan Ludwig, University of Münster [25]. Virus stocks were propagated in the chorioallantoic cavity of ten-day-old pathogen-free embryonated chicken eggs for 48 h at 37 °C as described previously[26]. Viral titer was determined by focus-forming unit (FFU) assay as described previously [26].

Infection of mice

Females at 8–12 weeks-of-age were anesthetized by intraperitoneal injection of ketamine/xylazine (10%(v/v) of 100 mg/ml ketamine and 5%(v/v) of 20 mg/ml xylazine in 0.9% (w/v) NaCl) with a dose adjusted to the individual body weight (200 µl/20 g body weight). Infection was performed by intranasal application of virus solution in 20 µl sterile phosphate-buffered saline (PBS), with PR8M $2x10^3$ FFU.

RNA isolation and sequencing

Mice were sacrificed at 3 dpi and entire lungs were extracted. The lungs were immediately transferred to RNAlater solution (Qiagen), kept at 4 °C for one day and subsequently stored at –20 °C. RNA was isolated using Qiagen Midi Kit as described previously [27]. RNA quality was controlled on a 2100 Bioanalyzer Instrument (Agilent). 500ng of total RNA was used to prepare libraries for sequencing using the Lexogen SENSE mRNA-seq library kit for Ion Torrent. Libraries were amplified for 11 cycles as the final step of library preparation. Five-hundred nanograms of total RNA was used to prepare libraries for sequencing using the Lexogen SENSE mRNA-seq library kit for Ion Torrent. Libraries were amplified for 11 cycles as the final step of library preparation. Before sequencing, 1 µl aliquots of this material was

pooled and sequenced on an Ion Torrent PGM 314 chip. Barcode quantification data from the PGM were used to balance the barcodes for final pooling before sequencing. Following this final pooling, the library pools were sized to a target size of 260bp on a Pippin Prep instrument using the 2 % Pippin agarose gel. The sized libraries were examined on an Agilent High Sensitivity DNA chip, quantified using real-time PCR, and used to prepare spheres using a One-Touch 2 device. These spheres were then sequenced on an Ion Torrent Proton sequencer with a P1 chip. On average, 67 million reads were obtained per sample.

Read mapping and gene expression quantification

RNAseq reads were quality-trimmed using Trim Galore [28] and then mapped to the mouse mm10 reference genome or to the influenza A virus PR8M genome using STAR [29]. Read counts were summarized for genes as features using the R-package Rsubread [30], normalized and log2 transformed using the R-package DESeq2 [31], and subsequently batch-corrected using the ComBat function of the R-package sva [32, 33]. For annotations of genes, ENTREZ-ID from Rsubread were matched to RefSeq annotations using R-package biomart [34]. Duplicate RefSeq annotations were given the extension _1, _2, etc.

Correlation analysis

The correlation analysis in this study, including gene-gene and gene-phenotype were conducted with Pearson correlation coefficient in R package. Pearson correlation P-value < 0.05 was used to report statistical significance.

Weighted gene co-expression network analysis (WGCNA)

WGCNA is a method aims to find co-expressed gene networks and explore the association between gene networks and phenotypes of interest, as well as hub genes in the network. In this study, Gene co-expression networks was constructed using the WGCNA package in R [35] according to the recommended tutorials (https://horvath.genetics.ucla.edu/html/CoexpressionNetwork/Rpackages/WGCNA/Tutorials/), in which, the *Ace2* correlated genes (Pearson correlation P-value < 0.05) was used as the input. The network construction mainly includes the following four steps: (1) define the gene expression correlation matrix; (2) select the soft threshold β and transform the expression correlation matrix into an adjacency matrix; (3) convert the adjacent matrix to topologically overlapping matrix and then to the dissimilarity matrix; 4) hierarchical cluster the dissimilarity matrix to obtain a clustering tree and refine with dynamic hybrid cutting.

Gene set enrichment analysis

To understand the biological function and pathways involved in the gene set of interest, we performed Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway, and mammalian phenotype ontology (MPO) overrepresentation analysis on WebGestalt (http://www.webgestalt.org) [36] . This analysis was based on hypergeometric statistic test which generate P-values and enrich rations to evaluate whether an annotated term/category/pathway is achieved statistical enrichment in the gene set rather than by chance. In this analysis, we use the mouse genome protein coding genes as the reference background and a minimum of five genes for a category was defined.

Expression quantitative trait loci (eQTL) mapping

eQTL mapping allows the identification of chromosomal regions that specifically modulate variability in levels of mRNAs or other molecules. We performed eQTL mapping of RNA-seq expression data from lung tissues of H1N1 virus-infected BXD. Much of the analysis was done using GeneNetwork (www.genenetwork.org) [37, 38]. Likelihood ratio statistics (LRS) scores were computed using the Haley-Knott equations [39], and genome-wide significance was based on 2000 permutations. We used the BXD genotype file generated January 2017 (www.genenetwork.org/webqtl/main.py?FormID=sharinginfo&GN_AccessionId=600) were used. In addition, Genes with significant and suggestive eQTLs detected using Haley and Knott mapping were remapped using GEMMA [40] to confirm LRS scores and locations of QTLs in GeneNetwork. GEMMA used linear mixed model mapping algorithms which can account for kinship among strains.

Genetic variations between C57BL/6J and DBA/2J

Sequence differences segregating in the BXDs are from our previous work [23]. Here, we focused exclusively on protein-coding variants—nonsense, missense, and frameshift mutations.

Bayesian network construction

The Bayesian Network Webserver (compbio.uthsc.edu/BNW) [41]was used to create network models to evalute plausible causal models among the genes, transcripts, and outcome measures of interest. Model were learned from the data with default settings. Model averaging of the 1000 highest scoring networks was performed, and directed edges with weights greater than 0.5 were included in the models. Finally, each variable had a maximum of four direct parents in any network model.

RNA-seq data for time-course H1N1 infection of the BXD parental strains

This lung RNA-seq data was previously generated in our laboratory. Detailed information can be found in [15]. In brief, two strains (B6 and D2) were infected with the H1N1 virus at 10–12 weeks of age with the methods mentioned above. The RNA was isolated from lung tissues for B6 at 1, 3, 5, 8, and 14 days post infection (dpi) and for D2 at 1, 3, and 5 dpi. Three RNA samples per strain per time point were used for library preparation and sequencing. The sequence reads were mapped to the mouse reference genome mm10. The raw read counts were extracted, normalized and log transformed with DEseq2 [31] after adding 1 to all raw counts.

In this study, we extracted the *Ace2* expression levels from this dataset. Also, we performed differential expression analysis between mice at 3 dpi and mock controls using DESeq2 [31] by treating strain as a covariates. The resulting *P*-values were adjusted using the Benjamini and Hochberg's approach [42]. We reported genes with adj.p-value < 0.05 and fold change (FC) > 1.3 as differentially expressed genes (DEGs).

Microarray data for SARS-CoV infected primary human airway epithelial (HAE) cells

The experimental details for this study are described in [43] In brief, human tracheobronchial epithelial cells were obtained from airway specimens resected from patients undergoing surgery. Primary cells were expanded to generate passage 1 cells, and passage 2 cells were plated at a density of $2.5x10^5$ cells per well. Human airway epithelium (HAE) cultures were generated by provision of an air-liquid interface for 6 to 8 weeks to form well-differentiated, polarized cultures. Wild-type infectious clone-derived SARS-CoV (icSARS-CoV) and ic-SARS-CoV ORF6 were derived from the Baric laboratory's infectious clone constructs as described [43] Triplicate cultures were infected with xxxx icSARS-CoV, and icSARS-CoV ORF6 on the apical surface. Total RNA was isolated from infected cells and equivalent amounts of RNA from three biological replicates from each condition were pooled and microarray analysis was performed using Agilent 4x44K whole human gene expression microarrays. The raw data was downloaded from GEO as data matrix: GSE47963_series_matrix.txt.gz Expression values were normalized and sample descriptions quality controlled. A strong batch effect with respect to the donor from which HAE were derived was observed. Therefore, data were batch corrected for donor source using the R package "sva"[32, 33]. Rows with no gene name were deleted. Differentially expressed genes were determined using the R package Limma [45].

Data availability

The RNA-seq data from infected BXD strains has been deposited at GeneNetwork web site (www.genenetwork.org) with GN Accession number of GN807 which can be accessed through the dataset query "HZI Lung Flu Infected BXD (Nov16) RNA-Seq". RNAseq data for infection of B6 and D2 mice is available at the public expression database GEO (http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE66040. Array data from infected primary HAE cells is available at the public expression database GEO (http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE47963.

3 Results

3.1 *Ace2* expression level decreased in both C57BL/6J and DBA/2J after H1N1 infection

Previously, our group quantified lung transcriptomes with RNAseq technique for two mouse strains, B6 and D2, at 1, 3, 5, 8, and 14 dpi after H1N1 virus infection. Selection of those two strains wasd based on the following two reasons. First, B6 is a resistant strain to H1N1 infection, while D2 is a highly susceptible strain. Second, those two are parental strains for BXD RI family, which served as a genetic reference panel for systems genetics analysis of complex traits, especially for studying gene-by-environment interactions.

In this study, we extracted the *Ace2* mRNA expression levels from this dataset. Overall, the expression of *Ace2* in lung was decreased consistently in both strains after H1N1 infection (**Figure 1**). B6 mice had the lowest level on 8 dpi (Mean = 7.20, SD = 0.15) and remained unchanged on day 14 (Mean = 7.23, SD = 0.12). In addition, the expression level at 3 dpi (*P*-value = 0.026, log2FC=-0.25), 5 dpi (*P*-value = 0.008, log2FC=-0.49), 8 dpi (*P*-value = 0.003, log2FC=-0.73), and 14 dpi (*P*-value = 0.002, log2FC=-0.70) achieved statistical significance compared with 1 dpi. The infection was lethal for D2 mice at day 5 after the virus infection, accompanied with the lowest expression of *Ace2* (Mean = 7.10, SD = 0.25). Also, the

expression level on day 3 (*P*-value = 0.003, log2FC=-0.37) and 5 (*P*-value = 0.006, log2FC=-0.80) showed significantly decreasing levels compared with 1 dpi. Although expression difference of *Ace2* between B6 and D2 at 3 dpi and day 5 did not achieve statistical significance, the D2 mice consistently showed lower level of *Ace2* on 3 and 5 dpi compared with the B6 strain.

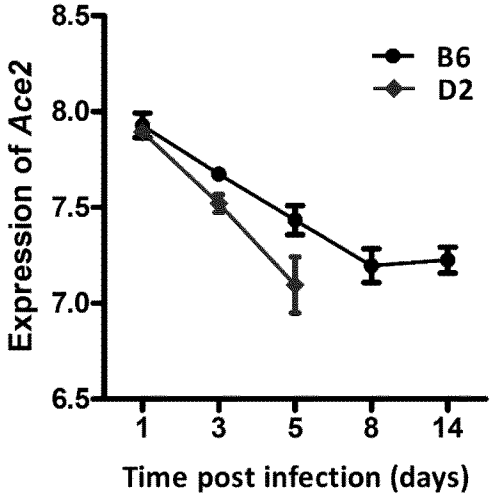


Figure 1. Line charts of the *Ace2* expression level in strains C57BL/6J and DBA/2J after H1N1 infection. Lung transcriptomes were quantified with RNA-seq for B6 and D2 strains at 1, 3, 5, 8, and 14 dpi after H1N1 infection. Three female mice (8-12 weeks of age) per strain per time point were used. The x-axis shows the time point (days) after the infection. The y-axis shows the expression level of *Ace2* with log2 transformed values.

3.2 *Ace2* mRNA level shown variability across BXD RI mice at 3 dpi after infection

D2 mice died five days after the infection. Therefore, we conducted RNAseq for a subset of BXD RI family of mice (41 BXD RI strains and two parental strains B6 and D2) at 3 dpi after the infection. As shown in **Figure 2**, *Ace2* expression shows highly variabile expression levels across BXD strains with a difference of 1.49 between extremes. The average expression of *Ace2* across all BXD strains was 8.02±0.15 (log2 scale, Mean ± SD), with the BXD77 having the highest expression of 8.36 and the BXD22 having the lowest expression of 7.78.

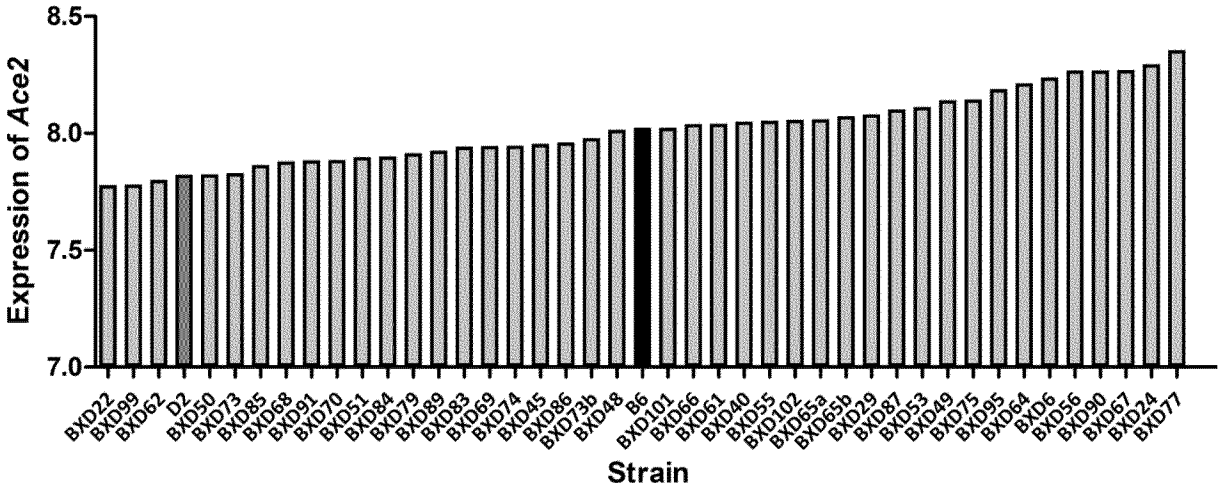


Figure 2. Bar plots of the lung *Ace2* expression levels across BXD RI mice at 3 dpi after infection. Female mice (8-12 weeks of age) were anesthetized and infected by intranasal application of H1N1 virus solution. Mice were sacrificed at 3 dpi post infection. RNA was extracted from lung and sequenced on Ion Torrent Proton sequencer (one sample per strain). The x-axis shows BXD and two parental strains. The y-axis shows the normalized log2 expression levels of *Ace2*.

3.3 *Ace2* significantly associated with survival time and virus load after infection

In order to investigate whether the *Ace2* is associated with the infection progression, we conducted Pearson correlation coefficient analysis for *Ace2* against body weight loss, mean time to death, and gene expression levels as a surrogate for viral load. Results demonstrated that *Ace2* significantly correlated with both body weight loss (r = 0.575, *P-value* = 0.008, **Figure 3A**) and median body weight loss (r = 0.468, *P-value* = 0.028, **Figure 3B**) at 3 dpi after infection. Further, *Ace2* was positively correlated to the mean time to death (r = 0.458, *P-value* = 0.032, **Figure 3C**) and negatively correlated to virus NA gene expression levels (r = 0.381, *P-value* = 0.029, **Figure 3D**).

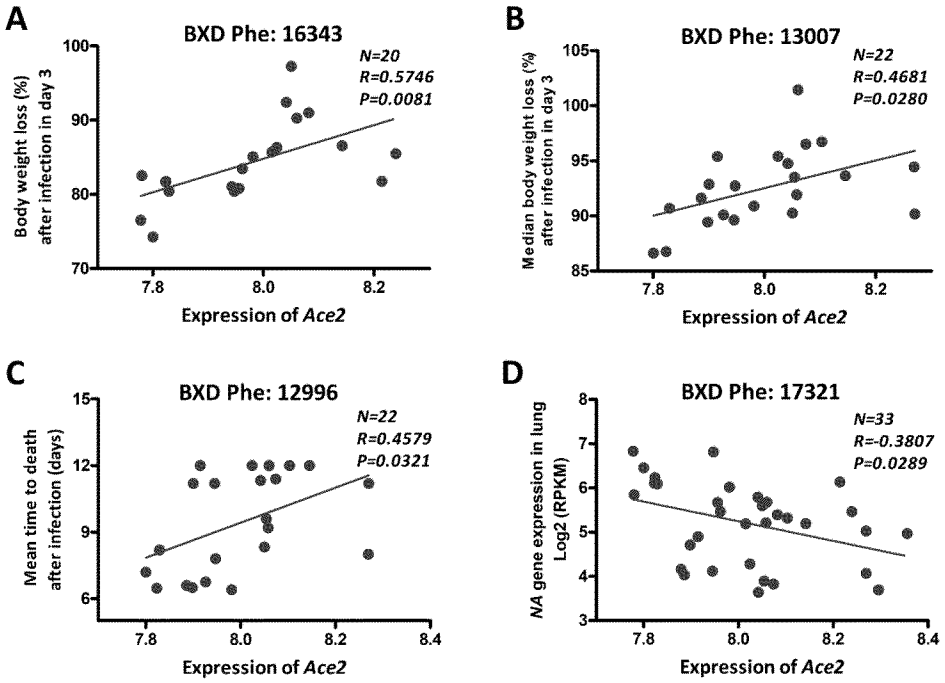


Figure 3. Scatter plots of the correlations of *Ace2* expression with body weight loss (A), median body weight loss (B), mean time to death (C), and virus *NA* gene expression level (D). Pearson correlation coefficient was used to determine the relationship. The percent body weight in **(A)** and **(B)** refer to the starting body weight (100%). Gene expression level represent normalized log2 values. The GeneNetwork accession number of the phenotypes (BXD Phe), number of samples (N), Pearson correlation R-value (R), and Pearson correlation *P*-value (P) are indicated in the figure.

3.4 *Ace2* correlated genes significantly involved in immune system-related pathways

To gain insight into the *Ace2* involved pathways and biological functions in lung after the virus infection, weconducted Pearson correlation coefficient analysis for *Ace2* against to the whole lung transcriptome on GeneNetwork. This analysis returned 5500 genes that were significantly correlated with *Ace2* (Pearson correlation *P*-value < 0.05). Next, we submitted those genes to Webgestalt (http://www.webgestalt.org/). 5118 genes were unambiguously mapped to unique entrezgene IDs. Those genes were used for an enrichment analysis. The selected genes significantly participated in TNF signaling pathway, MAPK signaling pathway, Notch signaling pathway, HTLV-I infection, and VEGF signaling pathway (**Figure 4A**). Furthermore, we also found that these genes were significantly enriched in terms related to chemokine secretion, lung morphology, and respiratory system morphology (**Figure 4B**).

For the top three enriched pathways, 48 genes were found in the TNF signaling pathway (**Supplementary Data 1**), with 35 negatively correlated with *Ace2* (Pearson *P*-value < 0.05), including the tumor necrosis factor (*Tnf,* r = -0.41, *P*-value = 0.005). There were 92 genes in the MAPK signaling pathway (**Supplementary Data 1**), with *Tnf*, transforming growth factor beta 1(*Tgfb1*, r = -0.46, *P*-value = 0.002), and other 59 genes negatively correlated with *Ace2*. In

addition, 25 genes were involved in the Notch signaling pathway (**Supplementary Data 1**), with 21 negatively correlated with *Ace2*.

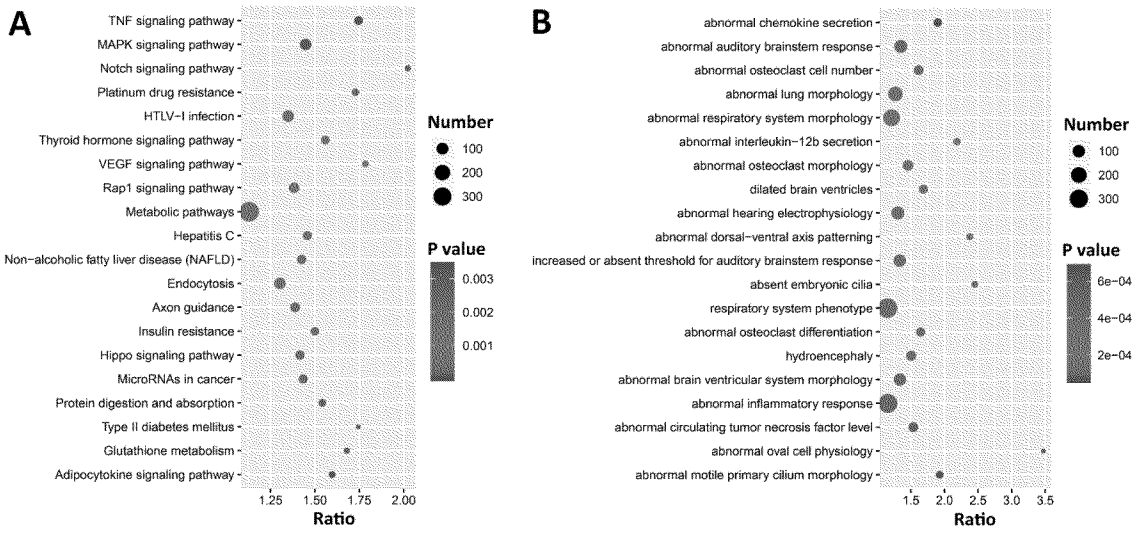


Figure 4. Bubble charts of the top 20 KEGG pathways (A) and 20 Mammalian Phenotype Ontologies (B) enriched for *Ace2* significantly correlated genes. Gene over-representation analysis for KEGG pathway and Mammalian Phenotype Ontology of the *Ace2* correlated genes (Pearson correlation *P*-value < 0.05) were performed with WebGestalt (http://www.webgestalt.org/). The x-axis represents the enrich ratio and y-axis represents enriched pathways/terms. The size of the dots indicate the number of genes and the color the *P*-value.

3.5 Co-expression network analysis narrowed down the *Ace2* correlated genes

To further identify genes that interact more directly with *Ace2*, we constructed gene co-expression network using WGCNA with the *Ace2* correlated genes (*P*-value < 0.05). The 5500 genes were parsed into 10 distinct co-expression modules using a soft-thresholding power of 8 (**Figure 5A-C**). The modules had a wide range of sizes, with the brown module containing 1673 genes and lightcyan module containing only 68 genes. *Ace2* was located in the purple module which contained 462 genes (**Figure 5C**). The genes in this module were significantly involved in regulating motile cilium physiology (**Figure 5D**)

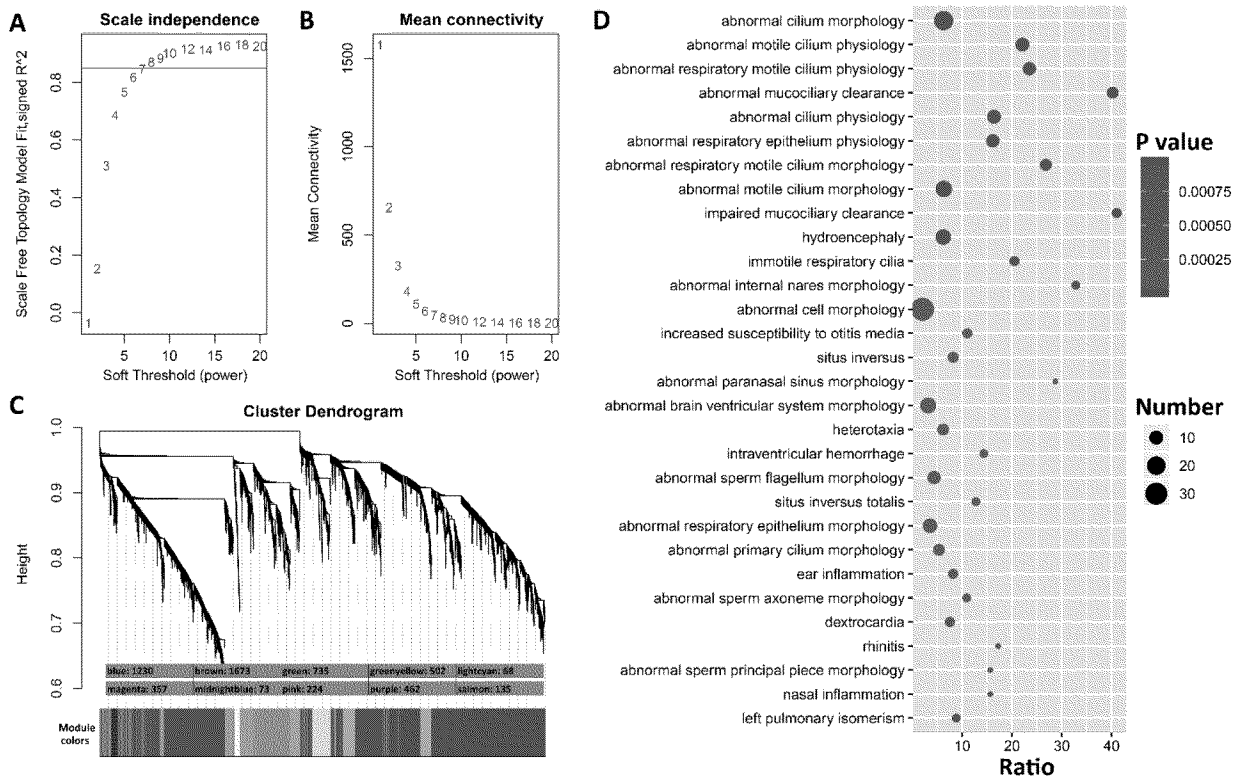


Figure 5. Weighted gene co-expression network analysis of *Ace2* correlated genes. (A) The soft thresholding index R^2 (y-axis) as a function of different powers β (x-axis) indicating the threshold of 8 chosen. **(B)** Mean connectivity (degree, y -axis) as a function of the soft-thresholding power (x -axis). **(C)** 10 co-expression modules identified from *Ace2* correlated genes. Modules are labeled with different colors. The number of genes in each module is also indicated. **(D)** Bubble charts of top 30 enriched Mammalian Phenotype Ontologies of the purple module. This analysis was performed with WebGestalt (http://www.webgestalt.org/).

Next, we focused on exploring the genes in the purple module (**Figure 6** and **Supplemental File 1**). First, we performed differential expression analysis between mice at 3 dpi after infection and mock controls by treating strain as a covariate. This identified 281 genes (61% of the module genes) that showed differential expression (adj.p-value < 0.05 and FC > 1.3). Next, we conducted eQTL mapping using WebQTL on the GeneNetwork for the whole lung transcriptome which identified 23 genes that were *cis*-regulated and 106 genes were *trans*-regulated in H1N1 infected lungs. Those QTLs were further confirmed with GEMMA in GeneNetwork. Further, we searched for the DNA sequence differences between B6 and D2, which resulted in a total of 69 genes harbor coding sequence variants (missense, nonsense, and frameshift variants). In addition, we found that there were 42 genes implicated in cilium function and regulation identified by enrichment analysis. By comparing the above four categories, 32 cilium function related genes were differentially expressed (**Table 1**). We refer to this set as 'prioritized genes'. Of these,

Sox11, *Tff2*, *Rnf128*, and *Muc5b* showed *cis*-or *trans*-regulation, and *Tmprss2*, *Ulk4*, *Cckar*, *Wdpcp*, *Ift140*, and *Ccdc40* contained functional variants. The genetic perturbations of these 10 genes (**Table 1**) may act as key regulators in this module.

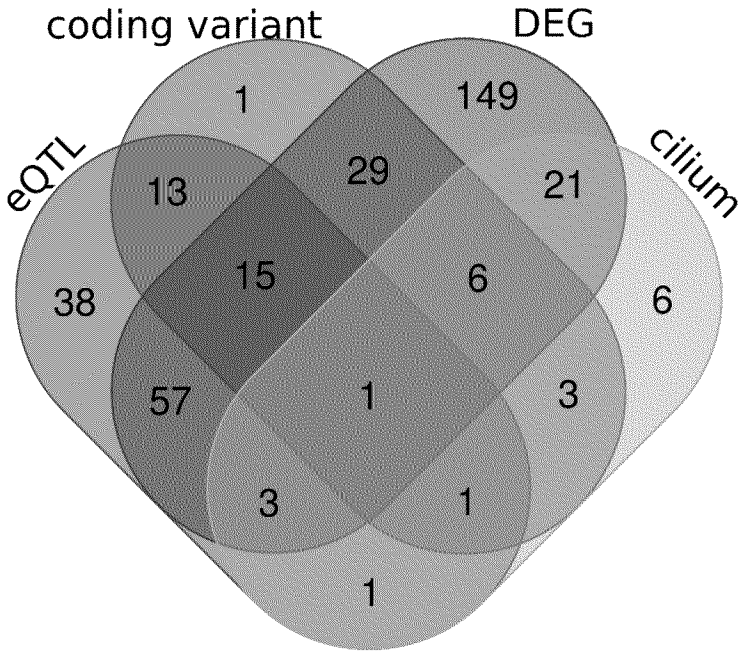


Figure 6. Venn diagrams of the purple module genes. A total of 462 genes were included in the purple module. Four criteria were applied to explore the module genes, including coding sequence variants between parental strain B6 and D2 (coding variant), eQTL (LRS > 15) regulation in H1N1 affected lung tissue (eQTL), cilium functional relevance (cilium), and differential expression (adj.p-value < 0.05 and FC > 1.3) between infected and normal controls in parental strains (DEG). The Venn diagram shows the number of overlapping genes among the four categories.

Table 1 Lists of prioritized genes in purple module.

Gene ID	Symbol	Description	Gene ID	log2FC	padj	eQTL	functional Variant
20666	**Sox11**	SRY-box 11	20666	-1.19	1.95E-12	cis	Nonsynonymous
21785	**Tff2**	trefoil factor 2 (spasmolytic protein 1)	21785	-1.01	5.38E-04	trans	NA
66889	**Rnf128**	ring finger protein 128	66889	-0.77	2.81E-05	trans	NA
74180	**Muc5b**	mucin 5, subtype B, tracheobronchial	74180	0.54	1.13E-02	trans	NA
50528	**Tmprss2**	transmembrane protease, serine 2	50528	-1.06	8.58E-10	NA	Nonsynonymous
209012	**Ulk4**	unc-51-like kinase 4 (C. elegans)	209012	-0.87	3.43E-07	NA	Nonsynonymous
12425	**Cckar**	cholecystokinin A receptor	12425	-0.73	1.91E-05	NA	Nonsynonymous
216560	**Wdpcp**	WD repeat containing planar cell polarity effector	216560	-0.69	2.36E-06	NA	Nonsynonymous
106633	**Ift140**	intraflagellar transport 140 homolog (Chlamydomonas)	106633	-0.68	3.50E-07	NA	Nonsynonymous
207607	**Ccdc40**	coiled-coil domain containing 40	207607	-0.66	1.06E-03	NA	Nonsynonymous
320752	Dpy19l2	dpy-19-like 2 (C. elegans)	320752	-1.81	2.13E-03	NA	NA
51938	Ccdc39	coiled-coil domain containing 39	51938	-1.30	1.15E-13	NA	NA
244653	Hydin	hydrocephalus inducing	244653	-1.30	8.51E-16	NA	NA

74362	Spag17	sperm associated antigen 17	74362	-1.29	9.27E-13	NA	NA
73873	Fam161a	family with sequence similarity 161, member A	73873	-1.28	1.70E-09	NA	NA
320662	Casc1	cancer susceptibility candidate 1	320662	-1.27	2.44E-08	NA	NA
66722	Spag16	sperm associated antigen 16	66722	-1.27	1.61E-10	NA	NA
74934	Armc4	armadillo repeat containing 4	74934	-1.21	8.50E-09	NA	NA
18703	Pigr	polymeric immunoglobulin receptor	18703	1.18	1.10E-09	NA	NA
69707	Iqcg	IQ motif containing G	69707	-1.18	2.97E-20	NA	NA
78801	Ak7	adenylate kinase 7	78801	-1.17	4.75E-09	NA	NA
27078	B9d1	B9 protein domain 1	27078	1.09	1.00E-02	NA	NA
68922	Dnaic1	dynein, axonemal, intermediate chain 1	68922	-1.07	3.67E-08	NA	NA
226265	Eno4	enolase 4	226265	-0.98	1.49E-09	NA	NA
71877	Efhc1	EF-hand domain (C-terminal) containing 1	71877	-0.92	3.77E-11	NA	NA
75533	Nme5	expressed in non-metastatic cells 5	75533	-0.82	5.92E-05	NA	NA
75050	Kif27	kinesin family member 27	75050	-0.71	3.05E-03	NA	NA
70008	Ace2	angiotensin I converting enzyme (peptidyl-dipeptidase A) 2	70008	-0.61	9.88E-05	NA	NA
20878	Aurka	serine/threonine kinase 6	20878	0.60	2.36E-02	NA	NA
22062	Trp73	transformation related protein 73	22062	-0.60	1.81E-03	NA	NA
77609	Ccdc151	coiled-coil domain containing 151	77609	-0.47	9.85E-02	NA	NA
252973	Grhl2	grainyhead-like 2 (Drosophila)	252973	-0.44	5.36E-04	NA	NA

Genes that were differentially expressed (adj.p-value < 0.05 and FC > 1.3) and implicated in cilium biological functions; Highlighted in bold are those 10 genes that may act as key regulators in the purple module; NA, not available.

3.6 Bayesian network analysis inferred upstream regulators of *Ace2*

In order to infer the causative relations of *Ace2* with the top 10 prioritized genes, we performed Bayesian network analysis. Results demonstrated that ring finger protein 128 (*Rnf128*) is the upstream regulator of the other 10 genes (**Figure 7**). This gene directly targets the genes intraflagellar transport 140 homolog (*Ift140*), unc-51-like kinase 4 (*Ulk4*), coiled-coil domain containing 40 (*Ccdc40*), SRY-box 11 (*Sox11*), and *Ace2*. In addition, *Ace2* is also targeted through another pathway, which begins with the expression of *Rnf128,* then the expression of *Ulk4, Wdpcp*, and, finally, to *Ace2* (**Figure 7**).

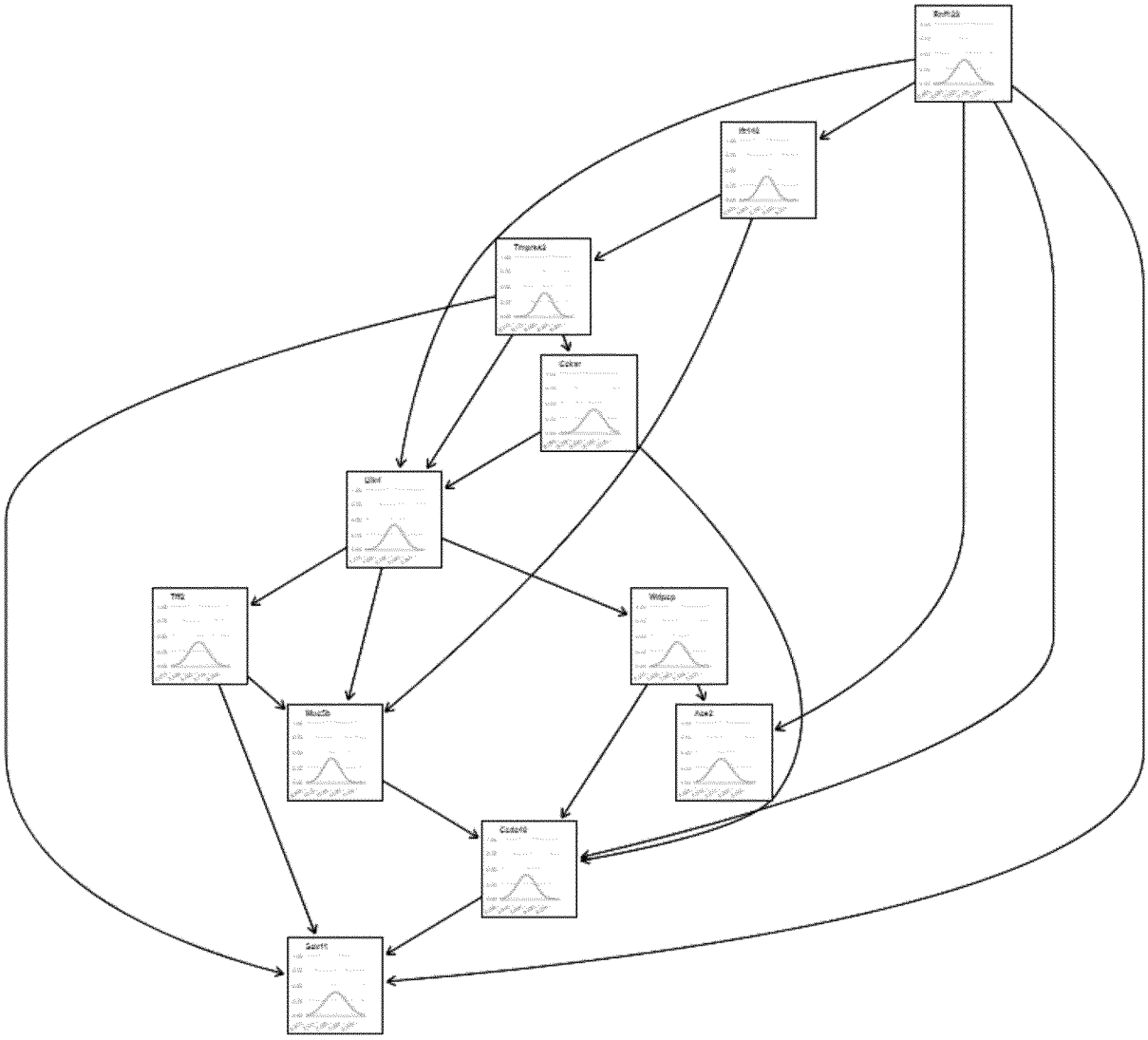


Figure 7. **Bayesian network analysis inferred upstream regulators of *Ace2***. The Bayesian network structure was constructed on Bayesian Network Webserver ((compbio.uthsc.edu/BNW) with gene expression values as the input. http://bnw.genenetwork.org/BNW/sourcecodes/layout.php?My_key=NLq

3.7 The differential expressed cilium function related genes in the purple module replicated in the SARS-CoV infected HAE cells

In order to further evaluate whether those 'prioritized genes' (**Table 1**) were also regulated in humans, especially for SARS-CoV, we downloaded a microarray data set for HAE cells infected with SARS-CoV (icSARS-CoV), ic-SARS-CoV ORF6 and BAT-SARS_CoV [43]. We found that only very few of those 'prioritized genes' were significantly regulated after infection with BAT-SARS (**Figure 8A**). However,

most of the 'prioritized genes' were differentially expressed in icSARS-CoV and ic-SARS-CoV ORF6 infected HAE cells when compared to mock controls, with 21 genes found at 96h after infection (**Figure 8A**). More importantly, those overlapping genes, except for *Ace2*, showed the same up- or down-regulating patterns in both HAE cells and our mouse model (**Table 1** and **Figure 8B**). *ACE2* was up-regulated in HAE cells whereas in whole lungs it was found to be down-regulated. This is an important observation since in the HAE cultures only airway epithelial cells are present, but no interstitial cells, endothelial cells, resident and infiltrating immune cells are present. These observations indicate that in the lung a more complex regulatory network is involved in the regulation of *ACE2* than in isolated epithelial cells and that it is indeed downregulated in all cells after infection in the whole lung. Alternatively, *ACE2* may become up-regulated only in infected cells (almost all cells in HAE cultures). In the whole lung the infected cells represent only a minority of all cells in the lung. Since the our lung RNAseq study investigated total gene expression changes of all cell types it is well conceivable that we do not detect a possible up-regulation in the few infected cells. In future studies, it would be important to analyze *Ac2* expression in infected mouse lungs to test these hypotheses.

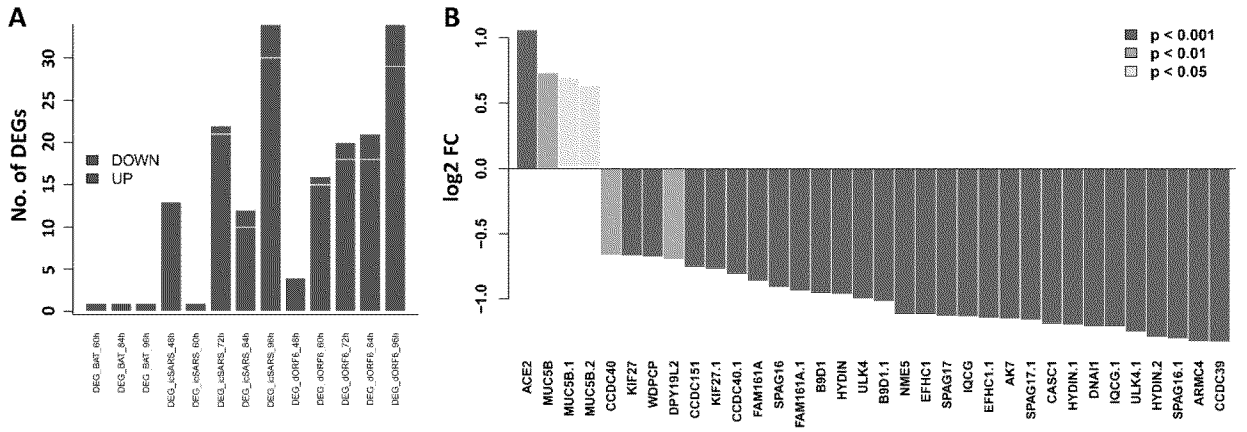


Figure 8. Bar plots of the cilium function related genes in the SARS-CoV infected HAE cells. Primary human HAE cells were infected with xxx SARS-CoV (icSARS-CoV), ic-SARS-CoV ORF6 and BAT-SARS_CoV and gene expression was analyzed by arrays. (A) Number of up-and downregulated differentially expressed genes for cilium function related genes that had orthologous genes in humans and were represented on the array (logFC > 0.58 and adjusted p-value < 0.05); y-axis: number of differentially expressed genes. (B) LogFC differences in expression values between HAE cells infected with icSARS-CoV at 96h pi and mock-treated controls for cilium function related genes that had orthologous genes in humans and were represented on the array; y-axis: differences in logFC expression levels between groups. Adjusted p-values were taken from the Limma analysis of the entire transcriptome data.

Discussion

Many studies have demonstrated that host genetic background significantly influences the susceptibility of influenza A infections in humans [46]. This phenomena was also observed in mouse models [14], especially for inbred strains B6 and D2 [15]. In this study, we systemically analyzed the potential regulatory role of *Ace2* for the host response of the H1N1 infection in a subset of BXD RI mice. Our results showed that the expression of *Ace2* in lung significantly decreased in an infection severity and time dependent manner. In addition, we also found the expression variation of *Ace2* significantly negatively correlated with the body weight loss and survival time after the infection. These results strongly suggests that *Ace2* is involved in H1N1 virus disease progression and that the lower levels of *Ace2* may play a role in virus-induced ALI. This was confirmed in *Ace2*$^{-/-}$ mice infected with influenza H7N9 [47] and respiratory syncytial virus [48] where lung pathology was more severe in mutant mice.

These observations strongly suggest that *Ace2* can mediate virus-induced ALI [47, 48]. However, the underlying mechanisms have not yet been revealed. By using 43 inbred strains, we were able to perform genetic correlation analysis and gain insight into the *Ace2* related biological pathways. We identified a total of ~5000 *Ace2*-correlated genes. These genes are significantly involved in regulating respiratory and inflammatory networks through several pathways, including TNF signaling pathway, MAPK signaling pathway, and Notch signaling pathway.

The lethal factors caused by the H1N1 mainly include virus-induced inflammatory cytokine storm and tissue destruction. Tumor necrosis factor (*TNF*) is a cytokine with multiple effects, including promoting cell growth, differentiation, apoptosis, and inducing inflammation [49, 50]. *TNF-α*, a family member of *TNF*, can activates Caspase protease, JNK, and transcription factor NF-κB, achieving its biological functions such as cytotoxicity, antiviral, and immuno-regulation. One study has demonstrated that inhibition of *Tnf-α* can protect mice from lethal H1N1 infection [51]. In the present study, we found *Ace2* was negatively correlated (r = -0.41 and *P*-value = 0.005) with the expression of *Tnf* and other 34 TNF signaling pathway related genes, suggesting that *Ace2* could reduce excessive inflammatory immune responses through downregulation of these signaling pathways and thus avoiding a hyper-inflammatory response that will lead to tissue destruction. We also found 92 genes in the MAPK signaling pathway significantly correlated with *Ace2*, including *Tgfb1* (r = -0.46 and *P*-value = 0.002). It was shown that influenza A infection can activate this pathway to support its replication [52], in which, *Tgfb1* gene acts as a pro-viral factor suppressing early immune responses during influenza A infection [53]. In addition, 25 *Ace2*-correlated genes were found in the Notch signaling pathway, with most of them (21 genes) showing negative correlations. This pathway is an important bridge between antigen-presenting cells (APCs) and T cell communication circuits and plays a central role in driving the immune system to overcome infectious disease [54].

By constructing gene co-expression network, we narrowed down the *Ace2* co-expressed genes to 462 (purple module). Enrichment analysis revealed that those genes significantly related to the cilium function, including *Ace2* itself. This finding suggests that *Ace2* not only participates in ALI by interacting with the

TNF, MAPK, and Notch signaling pathway, but also through functions in respiratory epithelium, which acts as a barrier to prevent infection and tissue injury by the secretion of mucus and the action of mucociliary clearance. In order to identify the key regulators in the purple module. We applied muti-layer filtration, including differential expression analysis, eQTL mapping, functional variant identification, and gene annotation. This resulted in 10 high priority candidates, including *Sox11*, *Tff2*, *Rnf128*, *Muc5b*, *Tmprss2*, *Ulk4*, *Cckar*, *Wdpcp*, *Ift140*, and *Ccdc40*. To further investigate the relationship of these 10 genes with *Ace2*, we performed Bayesian network analysis and identified *Rnf128* is the master regulator. This gene encodes a ring-finger-containing protein which has been shown to possess E3 ubiquitin ligase activity [55]. *RNF128* has been demonstrated involved in the induction of T cell anergic phenotype through inhibits activation-induced IL2 and IL4 cytokine production [56] and mediates T cell receptor-CD3 degradation [57]. In a recent study, Song *et al*. [58] found *RNF128* can counteract the ubiquitination of *K63* at the key kinase molecule TBK1 in the viral signaling pathway, thereby promoting the activation of *TBK1* kinase and the secretion of type I interferon, and inhibiting the replication of the virus. In vivo experiments have further confirmed that *Rnf128* deficient mice are more vulnerable infected by vesicular stomatitis virus (RNA virus) and herpes simplex virus (DNA virus). As our results shown that there are two potential gene regulatory paths for *Ace2*, which both begin at the *Rnf128*. However, the direct connection of those two genes has not yet confirmed and still need to further investigation.

Besides the master regulator *Rnf128*, other genes were also found to play significant roles in the immune and respiratory epithelium system. For instance, *MUC5B* (mucin 5, subtype B, tracheobronchial), encodes a member of the mucin family of proteins, and is significantly associated with the idiopathic pulmonary fibrosis in humans [59]. Overexpression of this gene in mice causes mucociliary dysfunction, enhances lung fibrosis [60], required for airway defense [61]. *CCDC40* (coiled-coil domain containing 40), encodes a protein that is necessary for motile cilia function. According to the OMIM (https://omim.org/) database, this gene is associated with recurrent respiratory infections (HP:0002205) and abnormal axonemal organization of respiratory motile cilia (HP:0012258). Malfunction of *Ccdc40* in mice resulted in left pulmonary isomerism and lung situs inversus [62]. *TMPRSS2* (transmembrane protease, serine 2) encodes a protein that belongs to the serine protease family. *Tmprss2* knockout mice show reduced lung inflammation with influenza A (H1N1, H3N2, or H7N9) infection and decreased susceptibility to viral infection induced morbidity/mortality [63]. Studies also demonstrated that SARS coronavirus engages *ACE2* as receptor and employs the *TMPRSS2* for Spike protein priming, which is essential for the virus entry and spread [64, 65]. Besides, *Ulk4*, *Sox11*, and *Tff2* are associated with abnormal respiratory motile cilium [66], pulmonary hypoplasia [67], and abnormal adaptive immunity [68], respectively.

The ongoing outbreak of the COVID-19 caused by SARS-CoV-2 has been emerged as a global public health threat [69-71]. The entering of SARS-CoV-2 into host cells have been demonstrated largely depends on *ACE2*, a surface receptor for SARS-CoV, MERS-CoV [72, 73], and can be blocked by a clinically approved protease inhibitor [73]. However, how *ACE2* interacts with other genes involve in the progression of SARS-CoV-2 induced respiratory diseases is still not fully elucidated. The first report based on autopsy samples demonstrated that the pathological characteristics of COVID-19 are very similar to those of SARS-CoV and MERS-CoV infected patients [74]. Therefore, we looked up those 30 prioritized genes (**Table 1**),

co-expressed with *Ace2* after H1N1 infection, in SARS-CoV infected primary human HAE cells. This resulted in 20 out of them showing consistent up/down-regulation with our H1N1 model, suggesting both viruses share common *Ace2* regulatory networks to participate in the occurrence and development of the pathology.

In summary, we have shown the expression of *Ace2* significantly decreased in the lungs of H1N1 infected mice. By using BXD RI strains, we were able to identify *Ace2* co-expressed genes with genetic correlation analysis and weighted gene co-expression network analysis. Thus, our results indicate that *Ace2* not only participates in virus-induced respiratory diseases through immune system related pathways, but also by protective functions in the respiratory epithelium. In addition, *Rnf128* was identified as the master regulator through Bayesian network analysis. Finally, 21 of our prioritized genes were found to be also differentially expressed in SARS-CoV infected HAE cells. These findings provide an *Ace2* regulatory network and several potential therapeutic targets for virus-induced (H1N1, SARS-CoV, MERS-CoV, and SARS-CoV-2) respiratory diseases. Besides, the BXD RI strains provide an excellent resource for studying the host genetic regulatory mechanism of the virus infection, such as SARS-CoV-2.

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Author Contributions

LL, KS and RSB conceived the study; SB and ACS conducted the experiments; FX and KS performed data analysis; LL, FX, KS, and JG wrote the manuscript. FX, KS, and JG prepared the figures and tables; RWW edited the manuscript; all authors read and approved the final version of the manuscript for publication.

Conflicts of Interest

The authors declare no conflicts of interests.

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From: Sims, Amy C[amy.sims@pnnl.gov]
Sent: Thur 3/26/2020 4:36:57 PM (UTC-07:00)
To: Lu, Lu[llu@uthsc.edu]; Klaus x Klaus Schughart[Klaus.Schughart@helmholtz-hzi.de]
Cc: rbaric@email.unc.edu[rbaric@email.unc.edu]
Subject: Re: [Ext] B6D2 infected RNAseq data
Attachment: Ace2_V4_260320_ACS.docx

I have added comments as requested. I am waiting on the core that generated the HAE cultures to send me an email with a blurb for the acknowledgements but otherwise I am good.

Thank you for including me. I appreciate it. I hope everyone is doing well. Best wishes, Amy

---

From: "Lu, Lu" <llu@uthsc.edu>
Date: Thursday, March 26, 2020 at 4:07 PM
To: Klaus x Klaus Schughart <Klaus.Schughart@helmholtz-hzi.de>
Cc: "Williams, Robert W" <rwilli10@uthsc.edu>, "Xu, Fuyi" <fxu10@uthsc.edu>, "Sims, Amy C" <amy.sims@pnnl.gov>, "rbaric@email.unc.edu" <rbaric@email.unc.edu>, "silke.bergmann@(b) (6) " <silke.bergmann@(b) (6) >, "Li, Kui" <kli1@uthsc.edu>
Subject: Re: [Ext] B6D2 infected RNAseq data

Dear Klaus (and all others):

Here is updated version. Fuyi addressed most of your comments. Please feel free to modify it continuously.

Dear Amy and Ralph:

Some of Klaus' comment are specific to you. Could you help to reply or editing?

Thanks.

Lu Lu
Professor
Department of Genetics, Genomics and Informatics
University of Tennessee Health Science Center
71 S. Manassas St., Room 410K
Memphis, TN 38163
Tel: (901) 448-7557 (O)
Fax: (901) 448-3500 (Dept)

> On Mar 26, 2020, at 1:18 PM, Schughart, Klaus <Klaus.Schughart@helmholtz-hzi.de> wrote:
>
> Hi LuLu,
> enclosed are my additional comments – now also on the discussion.
> Maybe Amy has also some ideas to explain the discrepancy between lungs and HAE cells with respect to inverse up/down regulation.
> Klaus
>
> ---
>
> **Von:** Lu, Lu <llu@uthsc.edu>
> **Gesendet:** Donnerstag, 26. März 2020 00:27

An: Schughart, Klaus <Klaus.Schughart@helmholtz-hzi.de>
Cc: Williams, Robert W <rwilli10@uthsc.edu>; Xu, Fuyi <fxu10@uthsc.edu>; amy.sims@pnnl.gov
Betreff: Re: [Ext] B6D2 infected RNAseq data

Dear Klaus:

We have accepted Rob, you and our previous editing. Here is updated version for you to check and modify continuously.

Dear Amy:

Could you check this version and address some comments that are related to you?

Thanks.

Lu Lu
Professor
Department of Genetics, Genomics and Informatics
University of Tennessee Health Science Center
71 S. Manassas St., Room 410K
Memphis, TN 38163
Tel: (901) 448-7557 (O)
Fax: (901) 448-3500 (Dept)

On Mar 25, 2020, at 3:18 PM, Xu, Fuyi <fxu10@uthsc.edu> wrote:

Dear Klaus,

Could you please send me those two images (high-resolution) in Figure 8 ? Thank you very much.

--
Fuyi Xu, PhD
Department of Genetics, Genomics, and Informatics
University of Tennessee Health Science Center

415 TRANSLATIONAL RESEARCH BUILDING
71 SOUTH MANASSAS
MEMPHIS TN 38163
Phone: (901)246-4398
E-mail: **5 U.S.C. § 552(b)(6)** ; fxu10@uthsc.edu

From: Schughart, Klaus
Sent: Wednesday, March 25, 2020 3:04 PM
To: Lu, Lu
Cc: Xu, Fuyi; Williams, Robert W
Subject: AW: [Ext] B6D2 infected RNAseq data

Hi LuLu,
I forgot something in the Acknowledgements.
Please replace the Acknowledgements in the last version with the one in the enclosed

version.
Thx,
Klaus

Von: Schughart, Klaus
Gesendet: Mittwoch, 25. März 2020 20:31
An: 'Lu, Lu' <llu@uthsc.edu>
Cc: 'Xu, Fuyi' <fxu10@uthsc.edu>; 'Williams, Robert W' <rwilli10@uthsc.edu>
Betreff: AW: [Ext] B6D2 infected RNAseq data

Dear all,
here are my comments on the revised version. I took out the previous comments that have been resolved.
Fuyi, I also enclose the data table for Figure 8A.
Once you are done with this version, I can have a look at the discussion and then you could send it off to the other co-authors.
Klaus

Von: Schughart, Klaus
Gesendet: Mittwoch, 25. März 2020 19:31
An: 'Lu, Lu' <llu@uthsc.edu>
Cc: Xu, Fuyi <fxu10@uthsc.edu>; Williams, Robert W <rwilli10@uthsc.edu>
Betreff: AW: [Ext] B6D2 infected RNAseq data

Thanks – will work it.
Klaus

Von: Lu, Lu <llu@uthsc.edu>
Gesendet: Mittwoch, 25. März 2020 19:02
An: Schughart, Klaus <Klaus.Schughart@helmholtz-hzi.de>
Cc: Xu, Fuyi <fxu10@uthsc.edu>; Williams, Robert W <rwilli10@uthsc.edu>
Betreff: Re: [Ext] B6D2 infected RNAseq data

Dear Klaus:

Fuyi and I accept all of you and rob's editing. We also clean and modified the paper, which addressed comments and answered the your questions. Please work on the updated version continuously. Hopefully we can submit this paper ASAP. Thanks.

Lu Lu
Professor
Department of Genetics, Genomics and Informatics
University of Tennessee Health Science Center
71 S. Manassas St., Room 410K
Memphis, TN 38163
Tel: (901) 448-7557 (O)
Fax: (901) 448-3500 (Dept)

On Mar 24, 2020, at 4:36 PM, Lu, Lu <llu@uthsc.edu> wrote:

Thanks, Rob. We will modify based on your comments and will send you updated version for your editing again.

Lu Lu
Professor
Department of Genetics, Genomics and Informatics
University of Tennessee Health Science Center
71 S. Manassas St., Room 410K
Memphis, TN 38163
Tel: (901) 448-7557 (O)
Fax: (901) 448-3500 (Dept)

On Mar 24, 2020, at 4:14 PM, Rob Williams <rwilliams@uthsc.edu> wrote:

Dear team,

Brilliant effort,

Edited the abstract heavily (please review carefully) and built up the Methods section on BXDs a bit.

Have not gotten through all of paper, but looks great so far.

Should validate some networks and eQTLs with GEMMA.

Needs a bit more clarity to data sources—some for 43 strains, other data for parents. These details needed (imho) even in the abstract.

On Tue, Mar 24, 2020 at 2:41 PM Schughart, Klaus <Klaus.Schughart@helmholtz-hzi.de> wrote:

Hi Lulu, Fuyi,

enclosed are my comments, additions and corrections.

Looks very good!!!

Please go through and clean it up first (accept or discard changes, add information) and then I can continue to work on the cleaned-up version. Currently it is too much cluttered with all these changes and comments.

Fuyi, let me know what exactly you need in addition for the HAE data.

Klaus

Von: Schughart, Klaus
Gesendet: Freitag, 6. März 2020 19:02

An: Xu, Fuyi <fxu10@uthsc.edu>; Lu Lu 5 U.S.C. § 552(b)(6)
Cc: Rob Williams <rwilliams@uthsc.edu>; Jonsson, Colleen Beth (cjonsson@uthsc.edu) <cjonsson@uthsc.edu>
Betreff: AW: [Ext] B6D2 infected RNAseq data

Hi Fuyi, LuLu,

I did some extensive analyses of genes in the human HAE data set with your gene list. Enclosed are the results. Looks very good!!

For a publication, we would have to discuss which data to include and how to present the results.

Klaus

Von: Schughart, Klaus <Klaus.Schughart@helmholtz-hzi.de>
Gesendet: Donnerstag, 5. März 2020 04:45
An: Xu, Fuyi <fxu10@uthsc.edu>
Cc: Lu Lu 5 U.S.C. § 552(b)(6)
Betreff: Re: [Ext] B6D2 infected RNAseq data

Dear Fuyi,

sure I will have a look at these genes.

Klaus

Von meinem iPhone gesendet

> Am 04.03.2020 um 19:09 schrieb Xu, Fuyi <fxu10@uthsc.edu>:
>
> Dear Klaus,
>
> Lu and I are working on the Ace2 paper. We prioritized around 30 genes that are correlated with Ace2 (attached table). I am wondering if you can help me check the expression differences between infected and normal controls in the human data set last time you showed us, especially for those color highlighted. Also, is this gene list make sense from your perspective? Thank you very much.
>
> Best,
>
> --
> Fuyi Xu, PhD
> Department of Genetics, Genomics, and Informatics
> University of Tennessee Health Science Center

415 TRANSLATIONAL RESEARCH BUILDING
71 SOUTH MANASSAS
MEMPHIS TN 38163
Phone: (901)246-4398
E-mail: 5 U.S.C. § 552(b)(6) fxu10@uthsc.edu

From: Schughart, Klaus
Sent: Friday, February 28, 2020 11:07 AM
To: Xu, Fuyi
Cc: Lu Lu
Subject: AW: [Ext] B6D2 infected RNAseq data

Hi LuLu, Fuyi,

I just remembered that I also have expression data from infected human lung slices (see enclosure).

This dataset has been published (enclosed).

Klaus

Von: Schughart, Klaus
Gesendet: Montag, 24. Februar 2020 21:00
An: 'Xu, Fuyi' <fxu10@uthsc.edu>
Cc: Lu Lu <lulu521uthsc@gmail.com>
Betreff: AW: [Ext] B6D2 infected RNAseq data

Dear Fuyi,

The data I sent you before (RNAseq_norm1.txt) have been normalized and log2 transformed using DESeq2 (rlogTransformation(dsDat, blind=TRUE).

Please find enclosed the raw counts for the B6/D2 infections from Wilk et al (all_mouse_counts.txt).

For the BXD data in GN:

The percent body weights are NOT the losses but the percent body weight with reference to the starting body weight (100%). So 105% means that the mouse has gained 5% of body weight relative to its starting weight when it was infected. Mice with percent body weight less than 100% have lost weight.

Klaus

Von: Xu, Fuyi <fxu10@uthsc.edu>
Gesendet: Sonntag, 23. Februar 2020 22:14
An: Schughart, Klaus <Klaus.Schughart@helmholtz-hzi.de>
Cc: Lu Lu [redacted]
Betreff: RE: [Ext] B6D2 infected RNAseq data

Dear Dr. Klaus Schughart,

I am Lu Lu's postdoc, Fuyi Xu. Thank you for sending us the normalized RNA-seq data.

We would like to perform differential expression analysis, I am wondering if you have raw read count for this data set since the DEseq2 package depend on this kind of data matrix. By the way, is the normalized data log2 transformed ?

We also found the expression level of ACE2 across the BXD is significantly correlated with median weight loss at day 3 after infection (ID:13007), however, we found BXD65a loss 105% body weight. Is this correct? Or how should I interpret ?

<A54B14D6A4584F84B57E3AAA25DC5BBB.gif>

Best Regards,

--
Fuyi Xu, PhD
Department of Genetics, Genomics, and Informatics
University of Tennessee Health Science Center

415 TRANSLATIONAL RESEARCH BUILDING
71 SOUTH MANASSAS
MEMPHIS TN 38163
Phone: (901)246-4398
E-mail: [redacted] fxu10@uthsc.edu

From: Lu, Lu
Sent: Monday, February 17, 2020 11:03 AM
To: Xu, Fuyi
Subject: Fwd: [Ext] B6D2 infected RNAseq data

Lu Lu

Professor
Department of Genetics, Genomics and Informatics
University of Tennessee Health Science Center
71 S. Manassas St., Room 410K
Memphis, TN 38163
Tel: (901) 448-7557 (O)
Fax: (901) 448-3500 (Dept)

Begin forwarded message:

From: "Schughart, Klaus" <Klaus.Schughart@helmholtz-hzi.de>

Subject: [Ext] B6D2 infected RNAseq data

Date: February 17, 2020 at 10:11:55 AM CST

To: "Lu, Lu" <llu@uthsc.edu>

Hi LuLu,

I checked the datasets.

GN807; RNAseq BXD infected is fine. This dataset has not been published yet.

I enclose the log2 normalized matrix for the B6/D2 infected data from Wilk et al. 2015:

RNAseq_norm1.txt -- for the mouse genes

00_influenza_lg2_RPKM_kls_280714.txt -- for the influenza gene segments

Let me know if you have any questions.

Klaus

Prof. Dr. Klaus Schughart
Head of Dept. of Infection Genetics
Helmholtz Centre for Infection Research,

University of Veterinary Medicine Hannover,

University of Tennessee Health Science Center
Inhoffenstr. 7
D-38124 Braunschweig
Germany

E-mail : Klaus.Schughart@helmholtz-hzi.de
Phone: +49 531 6181 1100

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Geschäftsführung: Prof. Dr. Dirk Heinz; Silke Tannapfel
Gesellschaft mit beschränkter Haftung (GmbH)
Sitz der Gesellschaft: Braunschweig
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477

<Table 1.xlsx>

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Gesellschaft mit beschränkter Haftung
(GmbH)
Sitz der Gesellschaft: Braunschweig
Handelsregister: Amtsgericht Braunschweig,
HRB 477

--
Rob

Robert W. Williams, Ph.D.
Chair: Department of Genetics, Genomics and Informatics
University of Tennessee Health Science Center
71 S Manassas St, Memphis TN 38103-3310, TSRB Room 405
Tele/Cell: 901 604 4752
EMAIL: rwilliams@uthsc.edu or **5 U.S.C. § 552(b)(6)**

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Genetic Dissection of the Regulatory Mechanisms of *Ace2* in the Infected Mouse Lung

Fuyi Xu[1#], Jun Gao[1, 5#], Silke Bergman[4], Amy C. Sims[7, 9], Ralph S. Baric[6,7, 8], Kui Li[4], Robert W. Williams[1], Klaus Schughart[2, 3, 4*], and Lu Lu[1*]

[1]Department of Genetics, Genomics, and Informatics, University of Tennessee Health Science Center, Memphis, TN, USA;
[2]Department of Infection Genetics, Helmholtz Centre for Infection Research, Braunschweig, Germany
[3]University of Veterinary Medicine Hannover, Hannover, Germany;
[4]Department of Microbiology, Immunology, and Biochemistry, University of Tennessee Health Science Center, Memphis, TN, USA;
[5]Institute of Animal Husbandry and Veterinary Science, Shanghai Academy of Agricultural Sciences, Shanghai, China.
[6]Department of Microbiology and Immunology, University of North Carolina at Chapel Hill, Chapel Hill, NC
[7]Department of Genetics, University of North Carolina at Chapel Hill, Chapel Hill, NC
[8]Department of Epidemiology, University of North Carolina at Chapel Hill, Chapel Hill, NC
[9]present address: Pacific Northwest National Laboratory in Richland, WA

[#] These authors contributed equally to this work.

* Correspondence:

Lu Lu: llu@uthsc.edu

Department of Genetics, Genomics and Informatics
University of Tennessee Health Science Center
Memphis, TN 38163, United States;

Klaus Schughart: klaus.schughart@helmholtz-hzi.de
Department of Infection Genetics
Helmholtz Centre for Infection Research
Braunschweig, Germany

Abstract

Acute lung injury (ALI) is an important cause of morbidity and mortality after viral infections, such as influenza virus H1N1, SARS-CoV-2, and SARS-CoV. The angiotensin 1 converting enzyme 2 (ACE2) plays a protective role in the pathogenesis of SARS virus-induced ALI. However, the contributions of *ACE2* gene variants to the post-infection heterogeneity of the host response and susceptibility and underlying mechanisms are not yet well understood. In this study, we quantified lung transcriptomes in 41 BXD recombinant inbred strains of mice plus their parental strains of C57BL/6J and DBA/2J mice after H1N1 infection. The expression of *Ace2* mRNA across strains decreased significantly and was dependent on both infection severity and time after treatment. Expression was negatively correlated with body weight loss and survival time. Data for 43 strains were analyzed using weighted gene co-expression network analysis (WGCNA). *Ace2* not only participates in virus-induced ALI by interacting with TNF, MAPK, and notch signaling pathway, but also is linked to genes that have an important function in the pulmonary epithelium. Furthermore, by applying muti-layer filtration, including differential expression analysis, eQTL mapping, functional variant identification, and gene annotation, we highlighted 10 key candidate genes linked to an *Ace2* network: *Sox11*, *Tff2*, *Rnf128*, *Muc5b*, *Tmprss2*, *Ulk4*, *Cckar*, *Wdpcp*, *Ift140*, and *Ccdc40*. Of these transcripts, *Rnf128* was identified as a potential master regulator using Bayesian network analysis. The key gene set is enriched for genes known to be differentially expressed in SARS-CoV infected primary human airway epithelial cells. Collectively, these findings define an *Ace2*-associated modulatory network relevant to host response to H1N1 infection and highlight novel potential therapeutic targets for virus-induced respiratory diseases, including SARS-CoV and SARS-CoV-2. In addition, we demonstrated that BXD family of strains provide an excellent resource for dissecting host genetic regulatory mechanisms involved in the defense to viral infections and host disease.

Key words: H1N1, acute lung injury, BXD mice, *Ace2,* regulatory network

Introduction

The pandemic disease, COVID-19, is caused by a novel severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has spread with almost unprecedent speed starting in November or December 2019. As of March 25, 2020, according to preliminary estimates by World Health Organization (WHO), more than 195 countries, over 375,000 cases of COVID-19 have been reported, with more than 16,000 deaths. Since 1918, the world has experienced four influenza pandemics—1918 H1N1, 1957 H2N2, 1968 H3N2, and 2009 H1N1. The first of these resulted in at least 50 million global mortalities [1]. The main pathological features caused by both COVID-19 and 2009 influenza A (H1N1) are acute viral pneumonia, which is an important cause of acute lung injury (ALI) [2]. The known symptoms for influenza A (H1N1) and COVID-19 are nearly identical and for both infected patients can manifest in asymptomatic, mild, or severe, and that can culminate in death. Individual genetic differences almost certainly play an important role in the development and severity of the illness [3-5].

Angiotensin-converting enzyme 2 (*ACE2*), a negative regulator of the renin angiotensin system (RAS), plays a protective role in pathogenesis of ALI in several animal models of acute respiratory distress syndrome [6]. *Ace2* knockout mice exhibited severe pathology of ALI and exogenous recombinant human *ACE2* expression attenuates acute lung failure in *Ace2* knockout mice as well as in wild-type mice [7]. Studies have shown that ACE2 protein levels are significantly downregulated after infection with H1N1 virus but are dispensable for viral replication [8]. For SARS coronavirus, *ACE2* has been identified as the principal host receptor for the spike protein receptor binding domain of the virus and thereby facilitates viral entry and replication in host cells [9-11]. In *in vivo* studies, SARS-CoV infections of wild-type mice causes severe and lethal lung failure and the downregulation of *Ace2* in lungs[12]. Single-cell RNA sequencing demonstrated that 83% of *ACE2*-expressing cells are alveolar epithelial type II cells in lung and a much higher *ACE2*-expressing cell ratio is observed in Asian male donors compared to white and African American donors [13], However, the contributions of *ACE2* variation to the post-infection heterogeneity of the ALI is still poorly defined.

Susceptibility to viral infection varies greatly among mouse strains [14], especially between inbred strain C57BL/6J (B6) and DBA/2J (D2) mice [15]. B6 is relatively resistant to influenza A and often survives inoculation. In contrast, D2 loses body weight rapidly and at the same inoculum dose dies within 5–7 days. Our laboratory has generated and maintains ~120 BXD recombinant inbred (RI) strains derived from multiple independent crosses of B6 and D2 parental strains. Each of these highly diverse BXD progeny are fully inbred and each has been sequenced. Collective this family of strains segregates for ~6 million variants with minor allele frequencies above 0.3 [16], making it an ideal and well powered reference population to model the genetic susceptibility of influenza A infections [17]. Indeed, our previous genetic mapping in this population have revealed several loci and gene variants that control the host response to influenza A (H1N1) virus infection [18] and other infectious diseases [19-22]. The BXD family provides a powerful resource to dissect loci, genes, molecular networks, and even mechanisms of host genetic responses to viral infections.

Here we have analyzed the lung transcriptome by RNAseq in 41 BXD RI strains and both parental strains after infection with H1N1 influenza A virus. By using systems genetics approaches, we investigate patterns of covariation of mRNAs with induced variation in *Ace2* expression. We define networks and regulatory relations that are likely to contribute to the host defense and/or pathology of H1N1-induced ALI.

Materials and methods

Mice

The subjects for this study were female mice belonging to 41 distinct BXD recombinant inbred (RI) strains, and the parental C57BL/6J (B6) and DBA/2J (D2) strains. These BXD strains segregate for over 5 million sequence variants with minor allele frequencies greater than 0.3 [23]. They are well powered to detect major loci that contribute to genetic variation in disease susceptibility [19-22] and to define causal networks of interacting loci, genes, gene productions (mRNAs and proteins), and pathogenicity outcome measures [18, 24]. All cases were between 8 and 12 weeks of age when infected with virus. All were housed and maintained on a 12:12 light/ dark cycle, with *ad libitum* access to food and water. Procedures were approved by the UTHSC animal care and use committee.

Ethics

Protocols for the mouse experiments were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Tennessee Health Science Center.

Virus

Original stocks of mouse adapted A/Puerto Rico/8/34 (H1N1, PR8M) virus were obtained from Stefan Ludwig, University of Münster [25]. Virus stocks were propagated in the chorioallantoic cavity of ten-day-old pathogen-free embryonated chicken eggs for 48 h at 37 °C as described previously[26]. Viral titer was determined by focus-forming unit (FFU) assay as described previously [26].

Infection of mice

Females at 8–12 weeks-of-age were anesthetized by intraperitoneal injection of ketamine/xylazine (10%(v/v) of 100 mg/ml ketamine and 5%(v/v) of 20 mg/ml xylazine in 0.9% (w/v) NaCl) with a dose adjusted to the individual body weight (200 µl/20 g body weight). Infection was performed by intranasal application of virus solution in 20 µl sterile phosphate-buffered saline (PBS), with PR8M $2x10^3$ FFU.

RNA isolation and sequencing

Mice were sacrificed at 3 dpi and entire lungs were extracted. The lungs were immediately transferred to RNAlater solution (Qiagen), kept at 4 °C for one day and subsequently stored at –20 °C. RNA was isolated using Qiagen Midi Kit as described previously [27]. RNA quality was controlled on a 2100 Bioanalyzer Instrument (Agilent). 500ng of total RNA was used to prepare libraries for sequencing using the Lexogen SENSE mRNA-seq library kit for Ion Torrent. Libraries were amplified for 11 cycles as the final step of library preparation. Five-hundred nanograms of total RNA was used to prepare libraries for sequencing using the Lexogen SENSE mRNA-seq library kit for Ion Torrent. Libraries were amplified for 11 cycles as the final step of library preparation. Before sequencing, 1 µl aliquots of this material was

pooled and sequenced on an Ion Torrent PGM 314 chip. Barcode quantification data from the PGM were used to balance the barcodes for final pooling before sequencing. Following this final pooling, the library pools were sized to a target size of 260bp on a Pippin Prep instrument using the 2 % Pippin agarose gel. The sized libraries were examined on an Agilent High Sensitivity DNA chip, quantified using real-time PCR, and used to prepare spheres using a One-Touch 2 device. These spheres were then sequenced on an Ion Torrent Proton sequencer with a P1 chip. On average, 67 million reads were obtained per sample.

Read mapping and gene expression quantification

RNAseq reads were quality-trimmed using Trim Galore [28] and then mapped to the mouse mm10 reference genome or to the influenza A virus PR8M genome using STAR [29]. Read counts were summarized for genes as features using the R-package Rsubread [30], normalized and log2 transformed using the R-package DESeq2 [31], and subsequently batch-corrected using the ComBat function of the R-package sva [32, 33]. For annotations of genes, ENTREZ-ID from Rsubread were matched to RefSeq annotations using R-package biomart [34]. Duplicate RefSeq annotations were given the extension _1, _2, etc.

Correlation analysis

The correlation analysis in this study, including gene-gene and gene-phenotype were conducted with Pearson correlation coefficient in R package. Pearson correlation P-value < 0.05 was used to report statistical significance.

Weighted gene co-expression network analysis (WGCNA)

WGCNA is a method aims to find co-expressed gene networks and explore the association between gene networks and phenotypes of interest, as well as hub genes in the network. In this study, Gene co-expression networks was constructed using the WGCNA package in R [35] according to the recommended tutorials (https://horvath.genetics.ucla.edu/html/CoexpressionNetwork/Rpackages/WGCNA/Tutorials/), in which, the *Ace2* correlated genes (Pearson correlation P-value < 0.05) was used as the input. The network construction mainly includes the following four steps: (1) define the gene expression correlation matrix; (2) select the soft threshold β and transform the expression correlation matrix into an adjacency matrix; (3) convert the adjacent matrix to topologically overlapping matrix and then to the dissimilarity matrix; 4) hierarchical cluster the dissimilarity matrix to obtain a clustering tree and refine with dynamic hybrid cutting.

Gene set enrichment analysis

To understand the biological function and pathways involved in the gene set of interest, we performed Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway, and mammalian phenotype ontology (MPO) overrepresentation analysis on WebGestalt (http://www.webgestalt.org) [36] . This analysis was based on hypergeometric statistic test which generate P-values and enrich rations to evaluate whether an annotated term/category/pathway is achieved statistical enrichment in the gene set rather than by chance. In this analysis, we use the mouse genome protein coding genes as the reference background and a minimum of five genes for a category was defined.

Expression quantitative trait loci (eQTL) mapping

eQTL mapping allows the identification of chromosomal regions that specifically modulate variability in levels of mRNAs or other molecules. We performed eQTL mapping of RNA-seq expression data from lung tissues of H1N1 virus-infected BXD. Much of the analysis was done using GeneNetwork (www.genenetwork.org) [37, 38]. Likelihood ratio statistics (LRS) scores were computed using the Haley-Knott equations [39], and genome-wide significance was based on 2000 permutations. We used the BXD genotype file generated January 2017 (www.genenetwork.org/webqtl/main.py?FormID=sharinginfo&GN_AccessionId=600) were used. In addition, Genes with significant and suggestive eQTLs detected using Haley and Knott mapping were remapped using GEMMA [40] to confirm LRS scores and locations of QTLs in GeneNetwork. GEMMA used linear mixed model mapping algorithms which can account for kinship among strains.

Genetic variations between C57BL/6J and DBA/2J

Sequence differences segregating in the BXDs are from our previous work [23]. Here, we focused exclusively on protein-coding variants—nonsense, missense, and frameshift mutations.

Bayesian network construction

The Bayesian Network Webserver (compbio.uthsc.edu/BNW) [41]was used to create network models to evalute plausible causal models among the genes, transcripts, and outcome measures of interest. Model were learned from the data with default settings. Model averaging of the 1000 highest scoring networks was performed, and directed edges with weights greater than 0.5 were included in the models. Finally, each variable had a maximum of four direct parents in any network model.

RNA-seq data for time-course H1N1 infection of the BXD parental strains

This lung RNA-seq data was previously generated in our laboratory. Detailed information can be found in [15]. In brief, two strains (B6 and D2) were infected with the H1N1 virus at 10–12 weeks of age with the methods mentioned above. The RNA was isolated from lung tissues for B6 at 1, 3, 5, 8, and 14 days post infection (dpi) and for D2 at 1, 3, and 5 dpi. Three RNA samples per strain per time point were used for library preparation and sequencing. The sequence reads were mapped to the mouse reference genome mm10. The raw read counts were extracted, normalized and log transformed with DEseq2 [31] after adding 1 to all raw counts.

In this study, we extracted the *Ace2* expression levels from this dataset. Also, we performed differential expression analysis between mice at 3 dpi and mock controls using DESeq2 [31] by treating strain as a covariates. The resulting *P*-values were adjusted using the Benjamini and Hochberg's approach [42]. We reported genes with adj.p-value < 0.05 and fold change (FC) > 1.3 as differentially expressed genes (DEGs).

Microarray data for SARS-CoV infected primary human airway epithelial (HAE) cells

The experimental details for this study are described in [43] In brief, human tracheobronchial epithelial cells were obtained from airway specimens resected from patients undergoing surgery. Primary cells were expanded to generate passage 1 cells, and passage 2 cells were plated at a density of $2.5x10^5$ cells per well. Human airway epithelium (HAE) cultures were generated by provision of an air-liquid interface for 6 to 8 weeks to form well-differentiated, polarized cultures. Wild-type infectious clone-derived SARS-CoV (icSARS-CoV) and ic-SARS-CoV ORF6 were derived from the Baric laboratory's infectious clone constructs as described [43] Bat-SARS-CoV is comprised of the HKU3 backbone and SARS-CoV receptor binding domain and was derived from the Baric laboratory's infectious clone as described in (PMID:19036930).Triplicate cultures were infected with multiplicity of infection 3 (MOI 3) icSARS-CoV, and icSARS-CoV ORF6 on the apical surface. Total RNA was isolated from infected cells and equivalent amounts of RNA from three biological replicates from each condition were pooled and microarray analysis was performed using Agilent 4x44K whole human gene expression microarrays. The raw data was downloaded from GEO as data matrix: GSE47963_series_matrix.txt.gz Expression values were normalized and sample descriptions quality controlled. A strong batch effect with respect to the donor from which HAE were derived was observed. Therefore, data were batch corrected for donor source using the R package "sva"[32, 33]. Rows with no gene name were deleted. Differentially expressed genes were determined using the R package Limma [45].

Data availability

The RNA-seq data from infected BXD strains has been deposited at GeneNetwork web site (www.genenetwork.org) with GN Accession number of GN807 which can be accessed through the dataset query "HZI Lung Flu Infected BXD (Nov16) RNA-Seq". RNAseq data for infection of B6 and D2 mice is available at the public expression database GEO (http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE66040. Array data from infected primary HAE cells is available at the public expression database GEO (http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE47963.

3 Results

3.1 *Ace2* expression level decreased in both C57BL/6J and DBA/2J after H1N1 infection

Previously, our group quantified lung transcriptomes with RNAseq technique for two mouse strains, B6 and D2, at 1, 3, 5, 8, and 14 dpi after H1N1 virus infection. Selection of those two strains was based on the following two reasons. First, B6 is a resistant strain to H1N1 infection, while D2 is a highly susceptible strain. Second, those two are parental strains for BXD RI family, which served as a genetic reference panel for systems genetics analysis of complex traits, especially for studying gene-by-environment interactions.

In this study, we extracted the *Ace2* mRNA expression levels from this dataset. Overall, the expression of *Ace2* in lung was decreased consistently in both strains after H1N1 infection (**Figure 1**). B6 mice had the lowest level on 8 dpi (Mean = 7.20, SD = 0.15) and remained unchanged on day 14 (Mean = 7.23, SD = 0.12). In addition, the expression level at 3 dpi (*P*-value = 0.026, log2FC=-0.25), 5 dpi (*P*-value = 0.008, log2FC=-0.49), 8 dpi (*P*-value = 0.003, log2FC=-0.73), and 14 dpi (*P*-value = 0.002, log2FC=-0.70) achieved statistical significance compared with 1 dpi. The infection was lethal for D2 mice at day 5 after the virus infection, accompanied with the lowest expression of *Ace2* (Mean = 7.10, SD = 0.25). Also, the

expression level on day 3 (*P*-value = 0.003, log2FC=-0.37) and 5 (*P*-value = 0.006, log2FC=-0.80) showed significantly decreasing levels compared with 1 dpi. Although expression difference of *Ace2* between B6 and D2 at 3 dpi and day 5 did not achieve statistical significance, the D2 mice consistently showed lower level of *Ace2* on 3 and 5 dpi compared with the B6 strain.

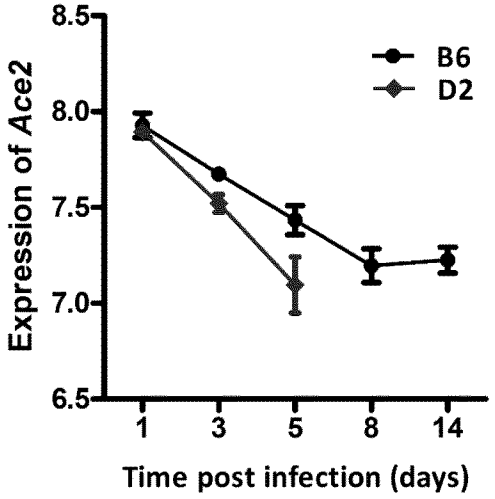


Figure 1. Line charts of the *Ace2* expression level in strains C57BL/6J and DBA/2J after H1N1 infection. Lung transcriptomes were quantified with RNA-seq for B6 and D2 strains at 1, 3, 5, 8, and 14 dpi after H1N1 infection. Three female mice (8-12 weeks of age) per strain per time point were used. The x-axis shows the time point (days) after the infection. The y-axis shows the expression level of *Ace2* with log2 transformed values.

3.2 *Ace2* mRNA level shown variability across BXD RI mice at 3 dpi after infection

D2 mice died five days after the infection. Therefore, we conducted RNAseq for a subset of BXD RI family of mice (41 BXD RI strains and two parental strains B6 and D2) at 3 dpi after the infection. As shown in **Figure 2**, *Ace2* expression shows highly variabile expression levels across BXD strains with a difference of 1.49 between extremes. The average expression of *Ace2* across all BXD strains was 8.02±0.15 (log2 scale, Mean ± SD), with the BXD77 having the highest expression of 8.36 and the BXD22 having the lowest expression of 7.78.

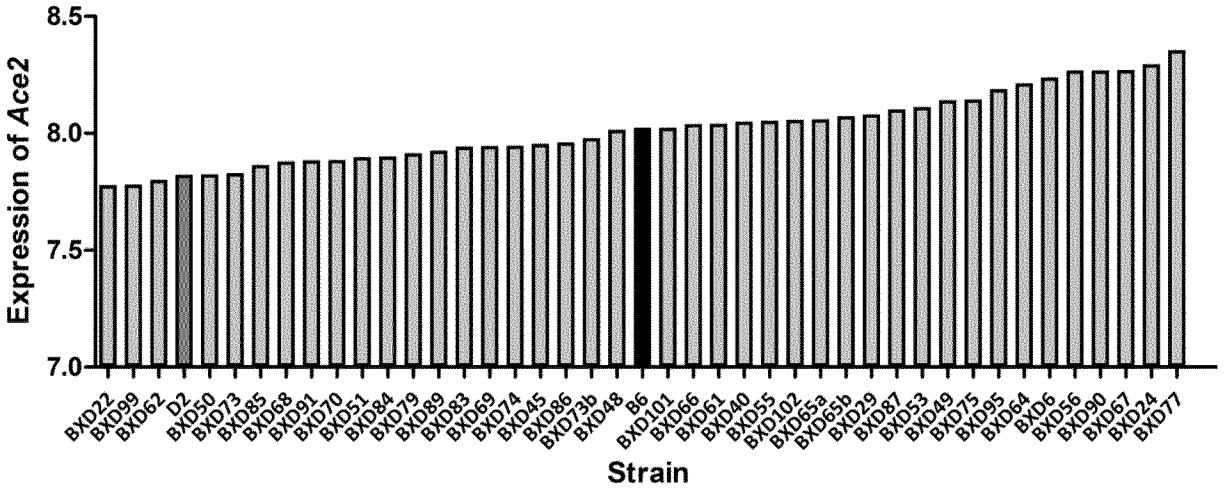


Figure 2. Bar plots of the lung *Ace2* expression levels across BXD RI mice at 3 dpi after infection. Female mice (8-12 weeks of age) were anesthetized and infected by intranasal application of H1N1 virus solution. Mice were sacrificed at 3 dpi post infection. RNA was extracted from lung and sequenced on Ion Torrent Proton sequencer (one sample per strain). The x-axis shows BXD and two parental strains. The y-axis shows the normalized log2 expression levels of *Ace2*.

3.3 *Ace2* significantly associated with survival time and virus load after infection

In order to investigate whether the *Ace2* is associated with the infection progression, we conducted Pearson correlation coefficient analysis for *Ace2* against body weight loss, mean time to death, and gene expression levels as a surrogate for viral load. Results demonstrated that *Ace2* significantly correlated with both body weight loss (r = 0.575, *P-value* = 0.008, **Figure 3A**) and median body weight loss (r = 0.468, *P-value* = 0.028, **Figure 3B**) at 3 dpi after infection. Further, *Ace2* was positively correlated to the mean time to death (r = 0.458, *P-value* = 0.032, **Figure 3C**) and negatively correlated to virus NA gene expression levels (r = 0.381, *P-value* = 0.029, **Figure 3D**).

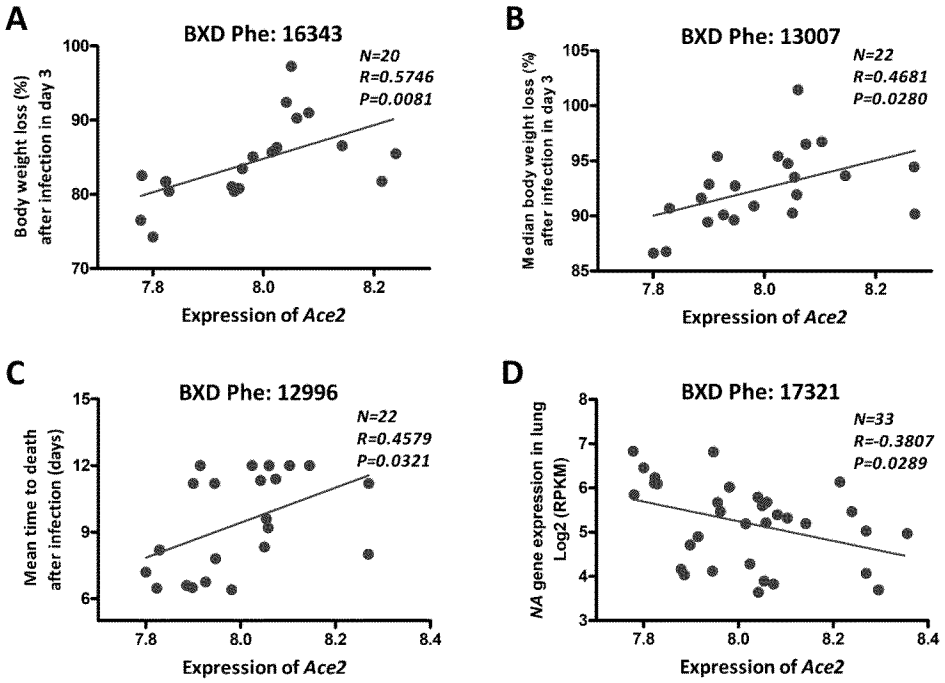


Figure 3. Scatter plots of the correlations of *Ace2* expression with body weight loss (A), median body weight loss (B), mean time to death (C), and virus *NA* gene expression level (D). Pearson correlation coefficient was used to determine the relationship. The percent body weight in **(A)** and **(B)** refer to the starting body weight (100%). Gene expression level represent normalized log2 values. The GeneNetwork accession number of the phenotypes (BXD Phe), number of samples (N), Pearson correlation R-value (R), and Pearson correlation *P*-value (P) are indicated in the figure.

3.4 *Ace2* correlated genes significantly involved in immune system-related pathways

To gain insight into the *Ace2* involved pathways and biological functions in lung after the virus infection, weconducted Pearson correlation coefficient analysis for *Ace2* against to the whole lung transcriptome on GeneNetwork. This analysis returned 5500 genes that were significantly correlated with *Ace2* (Pearson correlation *P*-value < 0.05). Next, we submitted those genes to Webgestalt (http://www.webgestalt.org/). 5118 genes were unambiguously mapped to unique entrezgene IDs. Those genes were used for an enrichment analysis. The selected genes significantly participated in TNF signaling pathway, MAPK signaling pathway, Notch signaling pathway, HTLV-I infection, and VEGF signaling pathway (**Figure 4A**). Furthermore, we also found that these genes were significantly enriched in terms related to chemokine secretion, lung morphology, and respiratory system morphology (**Figure 4B**).

For the top three enriched pathways, 48 genes were found in the TNF signaling pathway (**Supplementary Data 1**), with 35 negatively correlated with *Ace2* (Pearson *P*-value < 0.05), including the tumor necrosis factor (*Tnf,* r = -0.41, *P*-value = 0.005). There were 92 genes in the MAPK signaling pathway (**Supplementary Data 1**), with *Tnf*, transforming growth factor beta 1(*Tgfb1*, r = -0.46, *P*-value = 0.002), and other 59 genes negatively correlated with *Ace2*. In

addition, 25 genes were involved in the Notch signaling pathway (**Supplementary Data 1**), with 21 negatively correlated with *Ace2*.

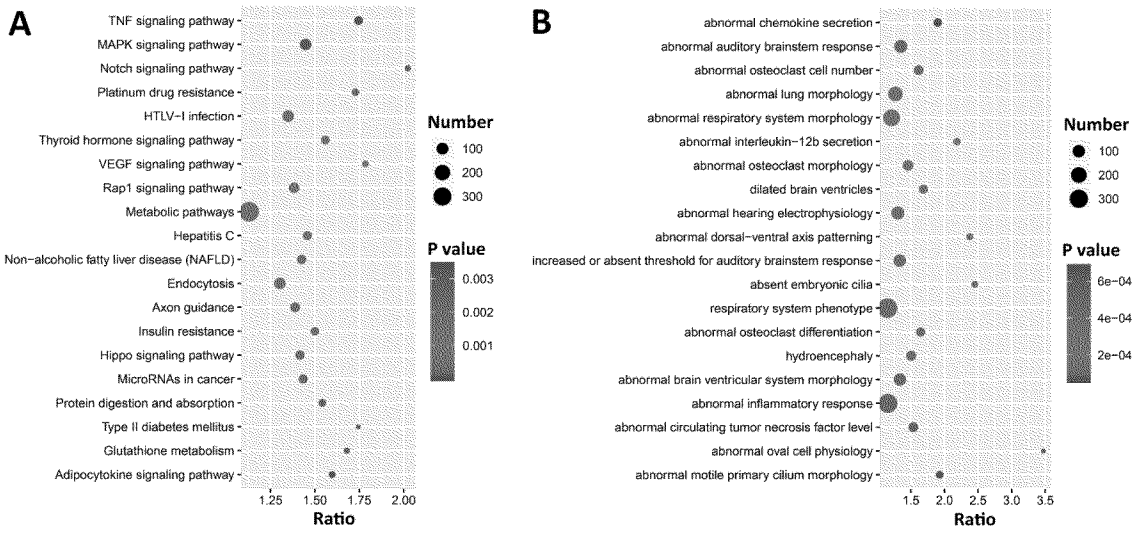


Figure 4. Bubble charts of the top 20 KEGG pathways (A) and 20 Mammalian Phenotype Ontologies (B) enriched for *Ace2* significantly correlated genes. Gene over-representation analysis for KEGG pathway and Mammalian Phenotype Ontology of the *Ace2* correlated genes (Pearson correlation *P*-value < 0.05) were performed with WebGestalt (http://www.webgestalt.org/). The x-axis represents the enrich ratio and y-axis represents enriched pathways/terms. The size of the dots indicate the number of genes and the color the *P*-value.

3.5 Co-expression network analysis narrowed down the *Ace2* correlated genes

To further identify genes that interact more directly with *Ace2*, we constructed gene co-expression network using WGCNA with the *Ace2* correlated genes (*P*-value < 0.05). The 5500 genes were parsed into 10 distinct co-expression modules using a soft-thresholding power of 8 (**Figure 5A-C**). The modules had a wide range of sizes, with the brown module containing 1673 genes and lightcyan module containing only 68 genes. *Ace2* was located in the purple module which contained 462 genes (**Figure 5C**). The genes in this module were significantly involved in regulating motile cilium physiology (**Figure 5D**)

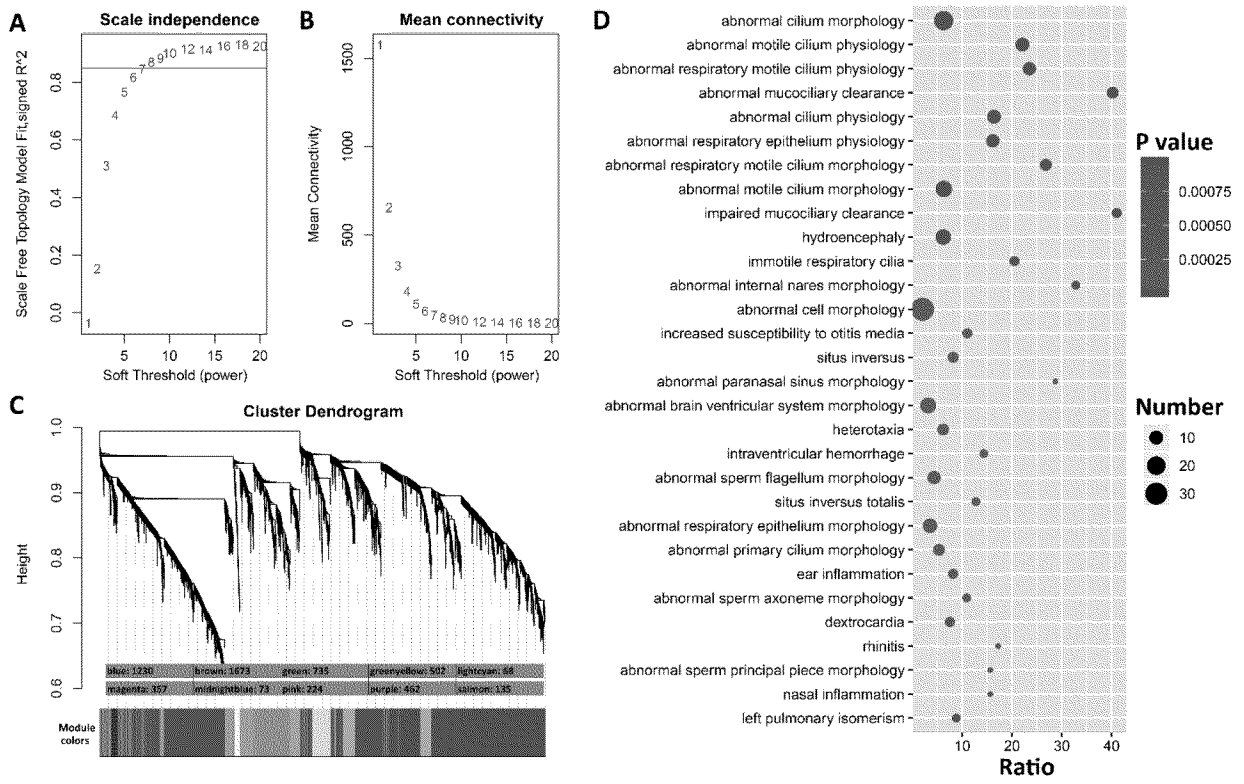


Figure 5. Weighted gene co-expression network analysis of *Ace2* correlated genes. (A) The soft thresholding index R^2 (y-axis) as a function of different powers β (x-axis) indicating the threshold of 8 chosen. **(B)** Mean connectivity (degree, y -axis) as a function of the soft-thresholding power (x -axis). **(C)** 10 co-expression modules identified from *Ace2* correlated genes. Modules are labeled with different colors. The number of genes in each module is also indicated. **(D)** Bubble charts of top 30 enriched Mammalian Phenotype Ontologies of the purple module. This analysis was performed with WebGestalt (http://www.webgestalt.org/).

Next, we focused on exploring the genes in the purple module (**Figure 6** and **Supplemental File 1**). First, we performed differential expression analysis between mice at 3 dpi after infection and mock controls by treating strain as a covariate. This identified 281 genes (61% of the module genes) that showed differential expression (adj.p-value < 0.05 and FC > 1.3). Next, we conducted eQTL mapping using WebQTL on the GeneNetwork for the whole lung transcriptome which identified 23 genes that were *cis*-regulated and 106 genes were *trans*-regulated in H1N1 infected lungs. Those QTLs were further confirmed with GEMMA in GeneNetwork. Further, we searched for the DNA sequence differences between B6 and D2, which resulted in a total of 69 genes harbor coding sequence variants (missense, nonsense, and frameshift variants). In addition, we found that there were 42 genes implicated in cilium function and regulation identified by enrichment analysis. By comparing the above four categories, 32 cilium function related genes were differentially expressed (**Table 1**). We refer to this set as 'prioritized genes'. Of these,

Sox11, *Tff2*, *Rnf128*, and *Muc5b* showed *cis*-or *trans*-regulation, and *Tmprss2*, *Ulk4*, *Cckar*, *Wdpcp*, *Ift140*, and *Ccdc40* contained functional variants. The genetic perturbations of these 10 genes (**Table 1**) may act as key regulators in this module.

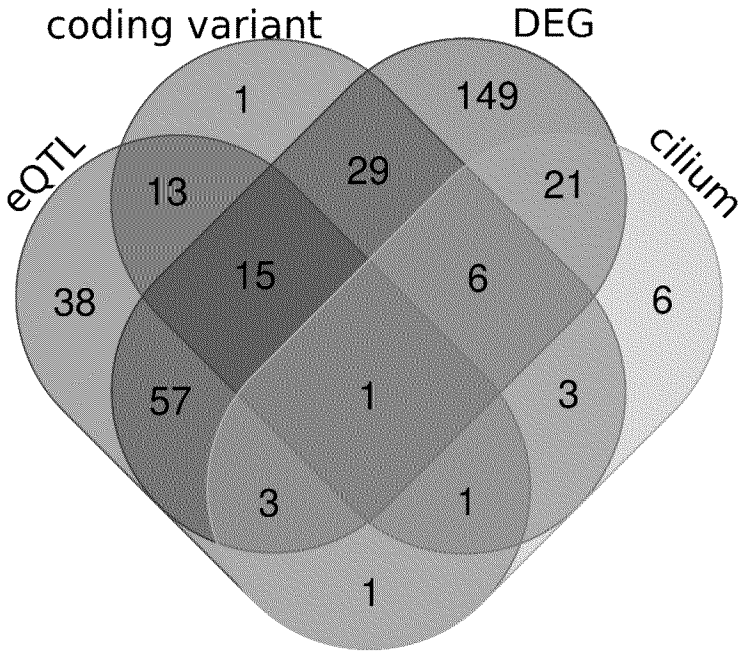


Figure 6. Venn diagrams of the purple module genes. A total of 462 genes were included in the purple module. Four criteria were applied to explore the module genes, including coding sequence variants between parental strain B6 and D2 (coding variant), eQTL (LRS > 15) regulation in H1N1 affected lung tissue (eQTL), cilium functional relevance (cilium), and differential expression (adj.p-value < 0.05 and FC > 1.3) between infected and normal controls in parental strains (DEG). The Venn diagram shows the number of overlapping genes among the four categories.

Table 1 Lists of prioritized genes in purple module.

Gene ID	Symbol	Description	Gene ID	log2FC	padj	eQTL	functional Variant
20666	**Sox11**	SRY-box 11	20666	-1.19	1.95E-12	cis	Nonsynonymous
21785	**Tff2**	trefoil factor 2 (spasmolytic protein 1)	21785	-1.01	5.38E-04	trans	NA
66889	**Rnf128**	ring finger protein 128	66889	-0.77	2.81E-05	trans	NA
74180	**Muc5b**	mucin 5, subtype B, tracheobronchial	74180	0.54	1.13E-02	trans	NA
50528	**Tmprss2**	transmembrane protease, serine 2	50528	-1.06	8.58E-10	NA	Nonsynonymous
209012	**Ulk4**	unc-51-like kinase 4 (C. elegans)	209012	-0.87	3.43E-07	NA	Nonsynonymous
12425	**Cckar**	cholecystokinin A receptor	12425	-0.73	1.91E-05	NA	Nonsynonymous
216560	**Wdpcp**	WD repeat containing planar cell polarity effector	216560	-0.69	2.36E-06	NA	Nonsynonymous
106633	**Ift140**	intraflagellar transport 140 homolog (Chlamydomonas)	106633	-0.68	3.50E-07	NA	Nonsynonymous
207607	**Ccdc40**	coiled-coil domain containing 40	207607	-0.66	1.06E-03	NA	Nonsynonymous
320752	Dpy19l2	dpy-19-like 2 (C. elegans)	320752	-1.81	2.13E-03	NA	NA
51938	Ccdc39	coiled-coil domain containing 39	51938	-1.30	1.15E-13	NA	NA
244653	Hydin	hydrocephalus inducing	244653	-1.30	8.51E-16	NA	NA

74362	Spag17	sperm associated antigen 17	74362	-1.29	9.27E-13	NA	NA
73873	Fam161a	family with sequence similarity 161, member A	73873	-1.28	1.70E-09	NA	NA
320662	Casc1	cancer susceptibility candidate 1	320662	-1.27	2.44E-08	NA	NA
66722	Spag16	sperm associated antigen 16	66722	-1.27	1.61E-10	NA	NA
74934	Armc4	armadillo repeat containing 4	74934	-1.21	8.50E-09	NA	NA
18703	Pigr	polymeric immunoglobulin receptor	18703	1.18	1.10E-09	NA	NA
69707	Iqcg	IQ motif containing G	69707	-1.18	2.97E-20	NA	NA
78801	Ak7	adenylate kinase 7	78801	-1.17	4.75E-09	NA	NA
27078	B9d1	B9 protein domain 1	27078	1.09	1.00E-02	NA	NA
68922	Dnaic1	dynein, axonemal, intermediate chain 1	68922	-1.07	3.67E-08	NA	NA
226265	Eno4	enolase 4	226265	-0.98	1.49E-09	NA	NA
71877	Efhc1	EF-hand domain (C-terminal) containing 1	71877	-0.92	3.77E-11	NA	NA
75533	Nme5	expressed in non-metastatic cells 5	75533	-0.82	5.92E-05	NA	NA
75050	Kif27	kinesin family member 27	75050	-0.71	3.05E-03	NA	NA
70008	Ace2	angiotensin I converting enzyme (peptidyl-dipeptidase A) 2	70008	-0.61	9.88E-05	NA	NA
20878	Aurka	serine/threonine kinase 6	20878	0.60	2.36E-02	NA	NA
22062	Trp73	transformation related protein 73	22062	-0.60	1.81E-03	NA	NA
77609	Ccdc151	coiled-coil domain containing 151	77609	-0.47	9.85E-02	NA	NA
252973	Grhl2	grainyhead-like 2 (Drosophila)	252973	-0.44	5.36E-04	NA	NA

Genes that were differentially expressed (adj.p-value < 0.05 and FC > 1.3) and implicated in cilium biological functions; Highlighted in bold are those 10 genes that may act as key regulators in the purple module; NA, not available.

3.6 Bayesian network analysis inferred upstream regulators of *Ace2*

In order to infer the causative relations of *Ace2* with the top 10 prioritized genes, we performed Bayesian network analysis. Results demonstrated that ring finger protein 128 (*Rnf128*) is the upstream regulator of the other 10 genes (**Figure 7**). This gene directly targets the genes intraflagellar transport 140 homolog (*Ift140*), unc-51-like kinase 4 (*Ulk4*), coiled-coil domain containing 40 (*Ccdc40*), SRY-box 11 (*Sox11*), and *Ace2*. In addition, *Ace2* is also targeted through another pathway, which begins with the expression of *Rnf128,* then the expression of *Ulk4, Wdpcp*, and, finally, to *Ace2* (**Figure 7**).

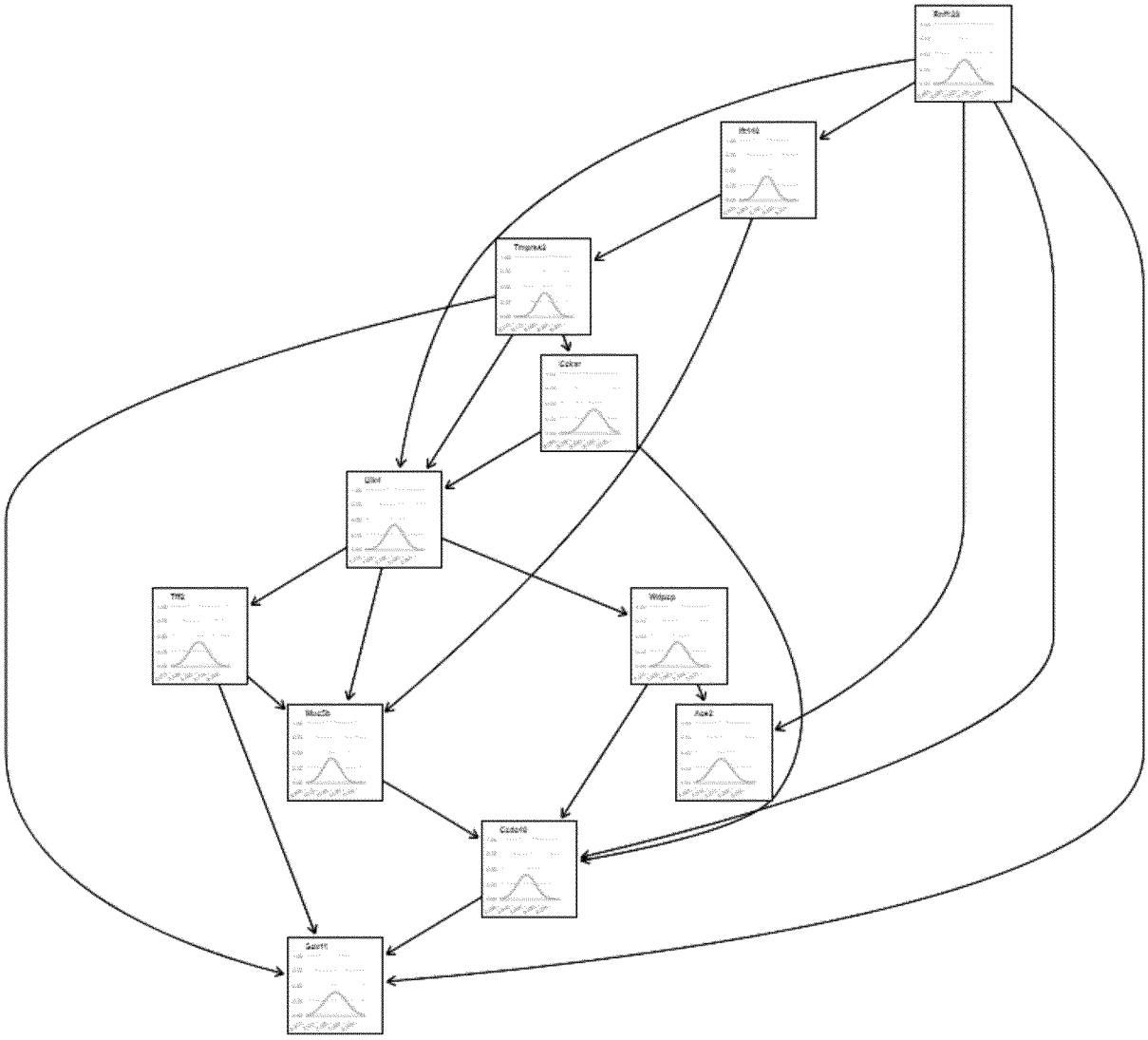


Figure 7. **Bayesian network analysis inferred upstream regulators of *Ace2***. The Bayesian network structure was constructed on Bayesian Network Webserver ((compbio.uthsc.edu/BNW) with gene expression values as the input. http://bnw.genenetwork.org/BNW/sourcecodes/layout.php?My_key=NLq

3.7 The differential expressed cilium function related genes in the purple module replicated in the SARS-CoV infected HAE cells

In order to further evaluate whether those 'prioritized genes' (**Table 1**) were also regulated in humans, especially for SARS-CoV, we downloaded a microarray data set for HAE cells infected with SARS-CoV (icSARS-CoV), ic-SARS-CoV ORF6 and BAT-SARS_CoV [43]. We found that only very few of those 'prioritized genes' were significantly regulated after infection with BAT-SARS (**Figure 8A**). However,

most of the 'prioritized genes' were differentially expressed in icSARS-CoV and ic-SARS-CoV ORF6 infected HAE cells when compared to mock controls, with 21 genes found at 96h after infection (**Figure 8A**). More importantly, those overlapping genes, except for *Ace2*, showed the same up- or down-regulating patterns in both HAE cells and our mouse model (**Table 1** and **Figure 8B**). *ACE2* was up-regulated in HAE cells whereas in whole lungs it was found to be down-regulated. This is an important observation since in the HAE cultures only airway epithelial cells are present, but no interstitial cells, endothelial cells, resident and infiltrating immune cells are present. These observations indicate that in the lung a more complex regulatory network is involved in the regulation of *ACE2* than in isolated epithelial cells and that it is indeed downregulated in all cells after infection in the whole lung. Alternatively, *ACE2* may become up-regulated only in infected cells (almost all cells in HAE cultures). In the whole lung the infected cells represent only a minority of all cells in the lung. Since the our lung RNAseq study investigated total gene expression changes of all cell types it is well conceivable that we do not detect a possible up-regulation in the few infected cells. In future studies, it would be important to analyze *Ace2* expression in infected mouse lungs to test these hypotheses.

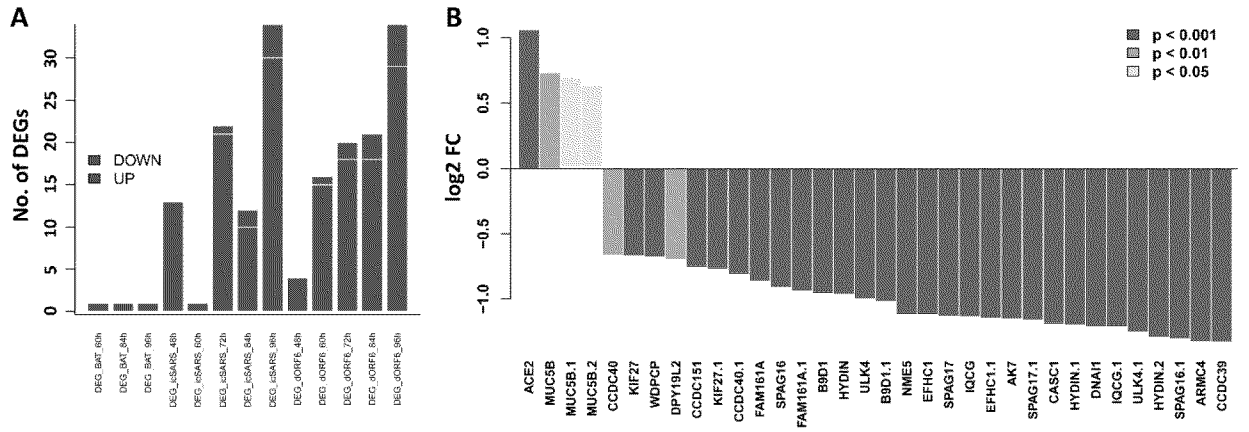


Figure 8. Bar plots of the cilium function related genes in the SARS-CoV infected HAE cells. Primary human HAE cells were infected with MOI 3 SARS-CoV (icSARS-CoV), ic-SARS-CoV ORF6 and BAT-SARS_CoV and gene expression was analyzed by arrays. (A) Number of up-and downregulated differentially expressed genes for cilium function related genes that had orthologous genes in humans and were represented on the array (logFC > 0.58 and adjusted p-value < 0.05); y-axis: number of differentially expressed genes. (B) LogFC differences in expression values between HAE cells infected with icSARS-CoV at 96h pi and mock-treated controls for cilium function related genes that had orthologous genes in humans and were represented on the array; y-axis: differences in logFC expression levels between groups. Adjusted p-values were taken from the Limma analysis of the entire transcriptome data.

Discussion

Many studies have demonstrated that host genetic background significantly influences the susceptibility of influenza A infections in humans [46]. This phenomena was also observed in mouse models [14], especially for inbred strains B6 and D2 [15]. In this study, we systemically analyzed the potential regulatory role of *Ace2* for the host response of the H1N1 infection in a subset of BXD RI mice. Our results showed that the expression of *Ace2* in lung significantly decreased in an infection severity and time dependent manner. In addition, we also found the expression variation of *Ace2* significantly negatively correlated with the body weight loss and survival time after the infection. These results strongly suggests that *Ace2* is involved in H1N1 virus disease progression and that the lower levels of *Ace2* may play a role in virus-induced ALI. This was confirmed in *Ace2*$^{-/-}$ mice infected with influenza H7N9 [47] and respiratory syncytial virus [48] where lung pathology was more severe in mutant mice.

These observations strongly suggest that *Ace2* can mediate virus-induced ALI [47, 48]. However, the underlying mechanisms have not yet been revealed. By using 43 inbred strains, we were able to perform genetic correlation analysis and gain insight into the *Ace2* related biological pathways. We identified a total of ~5000 *Ace2*-correlated genes. These genes are significantly involved in regulating respiratory and inflammatory networks through several pathways, including TNF signaling pathway, MAPK signaling pathway, and Notch signaling pathway.

The lethal factors caused by the H1N1 mainly include virus-induced inflammatory cytokine storm and tissue destruction. Tumor necrosis factor (*TNF*) is a cytokine with multiple effects, including promoting cell growth, differentiation, apoptosis, and inducing inflammation [49, 50]. *TNF-α*, a family member of *TNF*, can activates Caspase protease, JNK, and transcription factor NF-κB, achieving its biological functions such as cytotoxicity, antiviral, and immuno-regulation. One study has demonstrated that inhibition of *Tnf-α* can protect mice from lethal H1N1 infection [51]. In the present study, we found *Ace2* was negatively correlated (r = -0.41 and *P*-value = 0.005) with the expression of *Tnf* and other 34 TNF signaling pathway related genes, suggesting that *Ace2* could reduce excessive inflammatory immune responses through downregulation of these signaling pathways and thus avoiding a hyper-inflammatory response that will lead to tissue destruction. We also found 92 genes in the MAPK signaling pathway significantly correlated with *Ace2*, including *Tgfb1* (r = -0.46 and *P*-value = 0.002). It was shown that influenza A infection can activate this pathway to support its replication [52], in which, *Tgfb1* gene acts as a pro-viral factor suppressing early immune responses during influenza A infection [53]. In addition, 25 *Ace2*-correlated genes were found in the Notch signaling pathway, with most of them (21 genes) showing negative correlations. This pathway is an important bridge between antigen-presenting cells (APCs) and T cell communication circuits and plays a central role in driving the immune system to overcome infectious disease [54].

By constructing gene co-expression network, we narrowed down the *Ace2* co-expressed genes to 462 (purple module). Enrichment analysis revealed that those genes significantly related to the cilium function, including *Ace2* itself. This finding suggests that *Ace2* not only participates in ALI by interacting with the

TNF, MAPK, and Notch signaling pathway, but also through functions in respiratory epithelium, which acts as a barrier to prevent infection and tissue injury by the secretion of mucus and the action of mucociliary clearance. In order to identify the key regulators in the purple module. We applied muti-layer filtration, including differential expression analysis, eQTL mapping, functional variant identification, and gene annotation. This resulted in 10 high priority candidates, including *Sox11*, *Tff2*, *Rnf128*, *Muc5b*, *Tmprss2*, *Ulk4*, *Cckar*, *Wdpcp*, *Ift140*, and *Ccdc40*. To further investigate the relationship of these 10 genes with *Ace2*, we performed Bayesian network analysis and identified *Rnf128* is the master regulator. This gene encodes a ring-finger-containing protein which has been shown to possess E3 ubiquitin ligase activity [55]. *RNF128* has been demonstrated involved in the induction of T cell anergic phenotype through inhibits activation-induced IL2 and IL4 cytokine production [56] and mediates T cell receptor-CD3 degradation [57]. In a recent study, Song *et al*. [58] found *RNF128* can counteract the ubiquitination of *K63* at the key kinase molecule TBK1 in the viral signaling pathway, thereby promoting the activation of *TBK1* kinase and the secretion of type I interferon, and inhibiting the replication of the virus. In vivo experiments have further confirmed that *Rnf128* deficient mice are more vulnerable infected by vesicular stomatitis virus (RNA virus) and herpes simplex virus (DNA virus). As our results shown that there are two potential gene regulatory paths for *Ace2*, which both begin at the *Rnf128*. However, the direct connection of those two genes has not yet confirmed and still need to further investigation.

Besides the master regulator *Rnf128*, other genes were also found to play significant roles in the immune and respiratory epithelium system. For instance, *MUC5B* (mucin 5, subtype B, tracheobronchial), encodes a member of the mucin family of proteins, and is significantly associated with the idiopathic pulmonary fibrosis in humans [59]. Overexpression of this gene in mice causes mucociliary dysfunction, enhances lung fibrosis [60], required for airway defense [61]. *CCDC40* (coiled-coil domain containing 40), encodes a protein that is necessary for motile cilia function. According to the OMIM (https://omim.org/) database, this gene is associated with recurrent respiratory infections (HP:0002205) and abnormal axonemal organization of respiratory motile cilia (HP:0012258). Malfunction of *Ccdc40* in mice resulted in left pulmonary isomerism and lung situs inversus [62]. *TMPRSS2* (transmembrane protease, serine 2) encodes a protein that belongs to the serine protease family. *Tmprss2* knockout mice show reduced lung inflammation with influenza A (H1N1, H3N2, or H7N9) infection and decreased susceptibility to viral infection induced morbidity/mortality [63]. Studies also demonstrated that SARS coronavirus engages *ACE2* as receptor and employs the *TMPRSS2* for Spike protein priming, which is essential for the virus entry and spread [64, 65]. Besides, *Ulk4*, *Sox11*, and *Tff2* are associated with abnormal respiratory motile cilium [66], pulmonary hypoplasia [67], and abnormal adaptive immunity [68], respectively.

The ongoing outbreak of the COVID-19 caused by SARS-CoV-2 has emerged as a global public health threat [69-71]. The entering of SARS-CoV-2 into host cells have been demonstrated largely depends on *ACE2*, a surface receptor for SARS-CoV, MERS-CoV [72, 73], and can be blocked by a clinically approved protease inhibitor [73]. However, how *ACE2* interacts with other genes involve in the progression of SARS-CoV-2 induced respiratory diseases is still not fully elucidated. The first report based on autopsy samples demonstrated that the pathological characteristics of COVID-19 are very similar to those of SARS-CoV and MERS-CoV infected patients [74]. Therefore, we looked up those 30 prioritized genes (**Table 1**),

co-expressed with *Ace2* after H1N1 infection, in SARS-CoV infected primary human HAE cells. This resulted in 20 out of them showing consistent up/down-regulation with our H1N1 model, suggesting both viruses share common *Ace2* regulatory networks to participate in the occurrence and development of the pathology.

In summary, we have shown the expression of *Ace2* significantly decreased in the lungs of H1N1 infected mice. By using BXD RI strains, we were able to identify *Ace2* co-expressed genes with genetic correlation analysis and weighted gene co-expression network analysis. Thus, our results indicate that *Ace2* not only participates in virus-induced respiratory diseases through immune system related pathways, but also by protective functions in the respiratory epithelium. In addition, *Rnf128* was identified as the master regulator through Bayesian network analysis. Finally, 21 of our prioritized genes were found to be also differentially expressed in SARS-CoV infected HAE cells. These findings provide an *Ace2* regulatory network and several potential therapeutic targets for virus-induced (H1N1, SARS-CoV, MERS-CoV, and SARS-CoV-2) respiratory diseases. Besides, the BXD RI strains provide an excellent resource for studying the host genetic regulatory mechanism of the virus infection, such as SARS-CoV-2.

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Author Contributions

LL, KS and RSB conceived the study; SB and ACS conducted the experiments; FX and KS performed data analysis; LL, FX, KS, and JG wrote the manuscript. FX, KS, and JG prepared the figures and tables; RWW edited the manuscript; all authors read and approved the final version of the manuscript for publication.

Conflicts of Interest

The authors declare no conflicts of interests.

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From: Lu, Lu[llu@uthsc.edu]
Sent: Thur 3/26/2020 5:07:48 PM (UTC-07:00)
To: Sims, Amy C[amy.sims@pnnl.gov]
Cc: Klaus x Klaus Schughart[Klaus.Schughart@helmholtz-hzi.de]; rbaric@email.unc.edu[rbaric@email.unc.edu]
Subject: Re: [Ext] B6D2 infected RNAseq data

Thank you for your reply and editing.

Lu Lu
Professor
Department of Genetics, Genomics and Informatics
University of Tennessee Health Science Center
71 S. Manassas St., Room 410K
Memphis, TN 38163
Tel: (901) 448-7557 (O)
Fax: (901) 448-3500 (Dept)

On Mar 26, 2020, at 6:36 PM, Sims, Amy C <amy.sims@pnnl.gov> wrote:

I have added comments as requested. I am waiting on the core that generated the HAE cultures to send me an email with a blurb for the acknowledgements but otherwise I am good.

Thank you for including me. I appreciate it. I hope everyone is doing well. Best wishes, Amy

---

From: "Lu, Lu" <llu@uthsc.edu>
Date: Thursday, March 26, 2020 at 4:07 PM
To: Klaus x Klaus Schughart <Klaus.Schughart@helmholtz-hzi.de>
Cc: "Williams, Robert W" <rwilli10@uthsc.edu>, "Xu, Fuyi" <fxu10@uthsc.edu>, "Sims, Amy C" <amy.sims@pnnl.gov>, "rbaric@email.unc.edu" <rbaric@email.unc.edu>, "silke.bergmann@(b) (6) " <silke.bergmann@(b) (6) t>, "Li, Kui" <kli1@uthsc.edu>
Subject: Re: [Ext] B6D2 infected RNAseq data

Dear Klaus (and all others):

Here is updated version. Fuyi addressed most of your comments. Please feel free to modify it continuously.

Dear Amy and Ralph:

Some of Klaus' comment are specific to you. Could you help to reply or editing?

Thanks.

Lu Lu
Professor
Department of Genetics, Genomics and Informatics
University of Tennessee Health Science Center
71 S. Manassas St., Room 410K
Memphis, TN 38163
Tel: (901) 448-7557 (O)

On Mar 26, 2020, at 1:18 PM, Schughart, Klaus <Klaus.Schughart@helmholtz-hzi.de> wrote:

Hi LuLu,
enclosed are my additional comments – now also on the discussion.
Maybe Amy has also some ideas to explain the discrepancy between lungs and HAE cells with respect to inverse up/down regulation.
Klaus

Von: Lu, Lu <llu@uthsc.edu>
Gesendet: Donnerstag, 26. März 2020 00:27
An: Schughart, Klaus <Klaus.Schughart@helmholtz-hzi.de>
Cc: Williams, Robert W <rwilli10@uthsc.edu>; Xu, Fuyi <fxu10@uthsc.edu>; amy.sims@pnnl.gov
Betreff: Re: [Ext] B6D2 infected RNAseq data

Dear Klaus:

We have accepted Rob, you and our previous editing. Here is updated version for you to check and modify continuously.

Dear Amy:

Could you check this version and address some comments that are related to you?

Thanks.

Lu Lu
Professor
Department of Genetics, Genomics and Informatics
University of Tennessee Health Science Center
71 S. Manassas St., Room 410K
Memphis, TN 38163
Tel: (901) 448-7557 (O)
Fax: (901) 448-3500 (Dept)

On Mar 25, 2020, at 3:18 PM, Xu, Fuyi <fxu10@uthsc.edu> wrote:

Dear Klaus,

Could you please send me those two images (high-resolution) in Figure 8 ?
Thank you very much.

--
Fuyi Xu, PhD

Department of Genetics, Genomics, and Informatics
University of Tennessee Health Science Center

415 TRANSLATIONAL RESEARCH BUILDING
71 SOUTH MANASSAS
MEMPHIS TN 38163
Phone: (901)246-4398
E-mail: 5 U.S.C. § 552(b)(6) fxu10@uthsc.edu

From: Schughart, Klaus
Sent: Wednesday, March 25, 2020 3:04 PM
To: Lu, Lu
Cc: Xu, Fuyi; Williams, Robert W
Subject: AW: [Ext] B6D2 infected RNAseq data

Hi LuLu,
I forgot something in the Acknowledgements.
Please replace the Acknowledgements in the last version with the one in the enclosed version.
Thx,
Klaus

Von: Schughart, Klaus
Gesendet: Mittwoch, 25. März 2020 20:31
An: 'Lu, Lu' <llu@uthsc.edu>
Cc: 'Xu, Fuyi' <fxu10@uthsc.edu>; 'Williams, Robert W' <rwilli10@uthsc.edu>
Betreff: AW: [Ext] B6D2 infected RNAseq data

Dear all,
here are my comments on the revised version. I took out the previous comments that have been resolved.
Fuyi, I also enclose the data table for Figure 8A.
Once you are done with this version, I can have a look at the discussion and then you could send it off to the other co-authors.
Klaus

Von: Schughart, Klaus
Gesendet: Mittwoch, 25. März 2020 19:31
An: 'Lu, Lu' <llu@uthsc.edu>
Cc: Xu, Fuyi <fxu10@uthsc.edu>; Williams, Robert W <rwilli10@uthsc.edu>
Betreff: AW: [Ext] B6D2 infected RNAseq data

Thanks – will work it.
Klaus

Von: Lu, Lu <llu@uthsc.edu>
Gesendet: Mittwoch, 25. März 2020 19:02
An: Schughart, Klaus <Klaus.Schughart@helmholtz-hzi.de>
Cc: Xu, Fuyi <fxu10@uthsc.edu>; Williams, Robert W <rwilli10@uthsc.edu>
Betreff: Re: [Ext] B6D2 infected RNAseq data

Dear Klaus:

Fuyi and I accept all of you and rob's editing. We also clean and modified the paper, which addressed comments and answered the your questions. Please work on the updated version continuously. Hopefully we can submit this paper ASAP. Thanks.

Lu Lu
Professor
Department of Genetics, Genomics and Informatics
University of Tennessee Health Science Center
71 S. Manassas St., Room 410K
Memphis, TN 38163
Tel: (901) 448-7557 (O)
Fax: (901) 448-3500 (Dept)

> On Mar 24, 2020, at 4:36 PM, Lu, Lu <llu@uthsc.edu> wrote:
>
> Thanks, Rob. We will modify based on your comments and will send you updated version for your editing again.
>
> Lu Lu
> Professor
> Department of Genetics, Genomics and Informatics
> University of Tennessee Health Science Center
> 71 S. Manassas St., Room 410K
> Memphis, TN 38163
> Tel: (901) 448-7557 (O)
> Fax: (901) 448-3500 (Dept)
>
> > On Mar 24, 2020, at 4:14 PM, Rob Williams <rwilliams@uthsc.edu> wrote:
> >
> > Dear team,
> >
> > Brilliant effort,
> >
> > Edited the abstract heavily (please review carefully) and built up the Methods section on BXDs a bit.
> >
> > Have not gotten through all of paper, but looks great so far.
> >
> > Should validate some networks and eQTLs with GEMMA.
> >
> > Needs a bit more clarity to data

sources—some for 43 strains, other data for parents. These details needed (imho) even in the abstract.

On Tue, Mar 24, 2020 at 2:41 PM Schughart, Klaus <Klaus.Schughart@helmholtz-hzi.de> wrote:

Hi Lulu, Fuyi,

enclosed are my comments, additions and corrections.

Looks very good!!!

Please go through and clean it up first (accept or discard changes, add information) and then I can continue to work on the cleaned-up version. Currently it is too much cluttered with all these changes and comments.

Fuyi, let me know what exactly you need in addition for the HAE data.

Klaus

Von: Schughart, Klaus
Gesendet: Freitag, 6. März 2020 19:02
An: Xu, Fuyi <fxu10@uthsc.edu>; Lu Lu **5 U.S.C. § 552(b)(6)**
Cc: Rob Williams <rwilliams@uthsc.edu>; Jonsson, Colleen Beth (cjonsson@uthsc.edu) <cjonsson@uthsc.edu>
Betreff: AW: [Ext] B6D2 infected RNAseq data

Hi Fuyi, LuLu,

I did some extensive analyses of genes in the human HAE data set with your gene list. Enclosed are the results. Looks very good!!

For a publication, we would have to discuss which data to include and how to present the results.

Klaus

Von: Schughart, Klaus <Klaus.Schughart@helmholtz-hzi.de>
Gesendet: Donnerstag, 5. März 2020 04:45
An: Xu, Fuyi <fxu10@uthsc.edu>
Cc: Lu Lu <**5 U.S.C. § 552(b)(6)**
Betreff: Re: [Ext] B6D2 infected RNAseq data

Dear Fuyi,

sure I will have a look at these genes.

Klaus

Von meinem iPhone gesendet

> Am 04.03.2020 um 19:09 schrieb Xu, Fuyi <fxu10@uthsc.edu>:
>
> Dear Klaus,
>
> Lu and I are working on the Ace2 paper. We prioritized around 30 genes that are correlated with Ace2 (attached table). I am wondering if you can help me check the expression differences between infected and normal controls in the human data set last time you showed us, especially for those color highlighted. Also, is this gene list make sense from your perspective? Thank you very much.
>
> Best,
>
> --
> Fuyi Xu, PhD
> Department of Genetics, Genomics, and Informatics
> University of Tennessee Health Science Center
>
> 415 TRANSLATIONAL RESEARCH BUILDING
> 71 SOUTH MANASSAS
> MEMPHIS TN 38163
> Phone: (901)246-4398
> E-mail: **5 U.S.C. § 552(b)(6)** fxu10@uthsc.edu
>
> ---
> **From:** Schughart, Klaus
> **Sent:** Friday, February 28, 2020 11:07 AM
> **To:** Xu, Fuyi
> **Cc:** Lu Lu
> **Subject:** AW: [Ext] B6D2 infected RNAseq data
>
> Hi LuLu, Fuyi,
>
> I just remembered that I also have expression data from infected human

lung slices (see enclosure).

This dataset has been published (enclosed).

Klaus

Von: Schughart, Klaus
Gesendet: Montag, 24. Februar 2020 21:00
An: 'Xu, Fuyi' <fxu10@uthsc.edu>
Cc: Lu Lu 5 U.S.C. § 552(b)(6)
Betreff: AW: [Ext] B6D2 infected RNAseq data

Dear Fuyi,

The data I sent you before (RNAseq_norm1.txt) have been normalized and log2 transformed using DESeq2 (rlogTransformation(dsDat, blind=TRUE).

Please find enclosed the raw counts for the B6/D2 infections from Wilk et al (all_mouse_counts.txt).

For the BXD data in GN:

The percent body weights are NOT the losses but the percent body weight with reference to the starting body weight (100%). So 105% means that the mouse has gained 5% of body weight relative to its starting weight when it was infected. Mice with percent body weight less than 100% have lost weight.

Klaus

Von: Xu, Fuyi <fxu10@uthsc.edu>
Gesendet: Sonntag, 23. Februar 2020 22:14
An: Schughart, Klaus <Klaus.Schughart@helmholtz-hzi.de>
Cc: Lu Lu 5 U.S.C. § 552(b)(6)
Betreff: RE: [Ext] B6D2 infected RNAseq data

Dear Dr. Klaus Schughart,

I am Lu Lu's postdoc, Fuyi Xu. Thank you for sending us the normalized RNA-seq data.

We would like to perform differential expression analysis, I am wondering if you have raw read count for this data set since the DEseq2 package depend on this kind of data matrix. By the way, is the normalized data log2 transformed ?

We also found the expression level of ACE2 across the BXD is significantly correlated with median weight loss at day 3 after infection (ID:13007), however, we found BXD65a loss 105% body weight. Is this correct? Or how should I interpret ?

<A54B14D6A4584F84B57E3AAA25DC5BBB.gif>

Best Regards,

--
Fuyi Xu, PhD
Department of Genetics, Genomics, and Informatics
University of Tennessee Health Science Center

415 TRANSLATIONAL RESEARCH BUILDING
71 SOUTH MANASSAS
MEMPHIS TN 38163
Phone: (901)246-4398
E-mail: **5 U.S.C. § 552(b)(6)** fxu10@uthsc.edu

From: Lu, Lu
Sent: Monday, February 17, 2020 11:03 AM
To: Xu, Fuyi
Subject: Fwd: [Ext] B6D2 infected RNAseq data

Lu Lu

Professor
Department of Genetics, Genomics and Informatics
University of Tennessee Health Science Center
71 S. Manassas St., Room 410K
Memphis, TN 38163
Tel: (901) 448-7557 (O)
Fax: (901) 448-3500 (Dept)

Begin forwarded message:

From: "Schughart, Klaus" <Klaus.Schughart@helmholtz-hzi.de>

Subject: [Ext] B6D2 infected RNAseq data

Date: February 17, 2020 at 10:11:55 AM CST

To: "Lu, Lu" <llu@uthsc.edu>

Hi LuLu,

I checked the datasets.

GN807; RNAseq BXD infected is fine. This dataset has not been published yet.

I enclose the log2 normalized matrix for the B6/D2 infected data from Wilk et al. 2015:

RNAseq_norm1.txt -- for the mouse genes

00_influenza_lg2_RPKM_kls_280714.txt -- for the influenza gene segments

Let me know if you have any questions.

Klaus

Prof. Dr. Klaus Schughart
Head of Dept. of Infection Genetics
Helmholtz Centre for Infection Research,

University of Veterinary Medicine Hannover,

University of Tennessee Health Science Center
Inhoffenstr. 7
D-38124 Braunschweig
Germany

E-mail : Klaus.Schughart@helmholtz-hzi.de
Phone: +49 531 6181 1100

<B59D99096FF44A7EBCC7A3BC50D960A2.png>

Helmholtz-Zentrum für Infektionsforschung GmbH | Inhoffenstraße 7 | 38124 Braunschweig | www.helmholtz-hzi.de

Vorsitzende des Aufsichtsrates: Frau MinDir'in Prof. Dr. Veronika von Messling
Stellvertreter: MinDirig Rüdiger Eichel, Niedersächsisches Ministerium für Wissenschaft und Kultur

Geschäftsführung:
Prof. Dr. Dirk Heinz;
Silke Tannapfel
Gesellschaft mit
beschränkter Haftung
(GmbH)
Sitz der Gesellschaft:
Braunschweig
Handelsregister:
Amtsgericht
Braunschweig, HRB
477

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Infektionsforschung GmbH |
Inhoffenstraße 7 | 38124
Braunschweig | www.helmholtz-hzi.de

Vorsitzende des Aufsichtsrates: Frau
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Messling
Stellvertreter: MinDirig Rüdiger
Eichel, Niedersächsisches
Ministerium für Wissenschaft und
Kultur
Geschäftsführung: Prof. Dr. Dirk
Heinz; Silke Tannapfel
Gesellschaft mit beschränkter
Haftung (GmbH)
Sitz der Gesellschaft: Braunschweig
Handelsregister: Amtsgericht
Braunschweig, HRB 477

<Table 1.xlsx>

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Helmholtz-Zentrum für
Infektionsforschung GmbH |
Inhoffenstraße 7 | 38124
Braunschweig | www.helmholtz-hzi.de

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von Messling
Stellvertreter: MinDirig Rüdiger

Eichel, Niedersächsisches Ministerium für Wissenschaft und Kultur
Geschäftsführung: Prof. Dr. Dirk Heinz; Silke Tannapfel
Gesellschaft mit beschränkter Haftung (GmbH)
Sitz der Gesellschaft: Braunschweig
Handelsregister: Amtsgericht Braunschweig, HRB 477

--
Rob

Robert W. Williams, Ph.D.
Chair: Department of Genetics, Genomics and Informatics
University of Tennessee Health Science Center
71 S Manassas St, Memphis TN 38103-3310, TSRB Room 405
Tele/Cell: 901 604 4752
EMAIL: rwilliams@uthsc.edu or
5 U.S.C. § 552(b)(6)

<Ace2-Draft-03182020-V1_LuB_kls_RWW_240320_3.docx>

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Geschäftsführung: Prof. Dr. Dirk Heinz; Silke Tannapfel
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Geschäftsführung: Prof. Dr. Dirk Heinz; Silke Tannapfel
Gesellschaft mit beschränkter Haftung (GmbH)
Sitz der Gesellschaft: Braunschweig
Handelsregister: Amtsgericht Braunschweig, HRB 477

<Ace3_V3_250320_kls_260320.docx>

<Ace2_V4_260320_ACS.docx>

From: Sandoval, Norma I[Norma.Sandoval@pnnl.gov]
Sent: Fri 3/27/2020 8:21:47 AM (UTC-07:00)
To: Moore, Victoria L[victoria_moore@unc.edu]
Cc: Caldwell, Chandra[ccaldwel@email.unc.edu]; Sims, Amy C[amy.sims@pnnl.gov]
Subject: RE: Establishing subcontracts for A. Sims R21

Hi Vickie,
Following up on my email below. Can you please advise?

Also, in light of the COVID-19, do you know if NIH is providing an extension on this progress report submission? I'll be looking for guidance on the NIH website but thought to ask you as well.

Hope you are all well and staying safe.

Thank you.

Norma Sandoval
Principal Project Coordinator
Biological Sciences Division, **EBSD**

Pacific Northwest National Laboratory
902 Battelle Boulevard
P.O. Box 999, MSIN J4-18
Richland, WA 99352 USA
Tel: 509-371-6553
norma.sandoval@pnnl.gov
www.pnnl.gov

From: Sandoval, Norma I
Sent: Friday, March 20, 2020 8:44 AM
To: Moore, Victoria L <victoria_moore@unc.edu>
Cc: Caldwell, Chandra <ccaldwel@email.unc.edu>; Sandoval, Norma I <Norma.Sandoval@pnnl.gov>
Subject: RE: Establishing subcontracts for A. Sims R21

Hi Vicki,
I just wanted to follow-up on the initial email below and inquire about the Year 2 relinquishment of the R21 grant. Will UNC be submitting the request during the submission of the progress report? Or after?

Depending on the answer for the above, what will you need from Amy/PNNL?

Thx.

Norma Sandoval
Principal Project Coordinator
Biological Sciences Division, **EBSD**

Pacific Northwest National Laboratory
902 Battelle Boulevard
P.O. Box 999, MSIN J4-18
Richland, WA 99352 USA
Tel: 509-371-6553
norma.sandoval@pnnl.gov
www.pnnl.gov

From: Sims, Amy C <sims0018@email.unc.edu>
Sent: Friday, November 8, 2019 11:30 AM
To: Sandoval, Norma I <Norma.Sandoval@pnnl.gov>; Moore, Victoria L <victoria_moore@unc.edu>; Caldwell, Chandra <ccaldwel@email.unc.edu>
Subject: Establishing subcontracts for A. Sims R21
Importance: High

Norma,

Vicki Moore is the Epidemiology Business Manager and oversees the submission of the departmental grants and Chandra is her right hand woman! Please let us know if there is anyone else at PNNL that we should contact regarding this transition.

All,

I am currently principal investigator on an R21 and I need assistance making sure that there are funds at both institutions as needed through May 31, 2021.

Attached is a budget with the estimated remaining funds for January 1, 2020 through May 31, 2020 (Year 1 tab) and June 1, 2020 through May 31, 2021 (Year 2 tab). The R21 will remain with UNC during this time and I will need to establish a subcontract for my work at PNNL for this time.

In Year 2 the plan is to transition the funds to PNNL and then subcontract the necessary funds to UNC. I have spoken with my program officer and he requested that UNC submit a letter to relinquish the funds. Please let me know if I need to do anything towards generating this letter.

In the Year 2 page in the excel document I have provided estimated amounts needed at UNC and PNNL. More likely than not there will be funds to carry forward from year 1 to year 2.

I figured this would give us a good place to start the discussions as I know there is a large amount of paperwork to make this transition occur.

Thank you very much for assisting me with this process. I really appreciate it. Please let me know if I can assist. Amy

Amy Sims, Ph.D.
Associate Professor
UNC Chapel Hill
Office phone: 919-966-7991
Email: sims0018@ad.unc.edu

From: Caldwell, Chandra[ccaldwel@email.unc.edu]
Sent: Fri 3/27/2020 8:27:30 AM (UTC-07:00)
To: Sandoval, Norma I[Norma.Sandoval@pnnl.gov]
Cc: Sims, Amy C[amy.sims@pnnl.gov]
Subject: FW: NIH Update: Responding to Frequent Questions on Flexibilities Related to NIH Funding and COVID-19

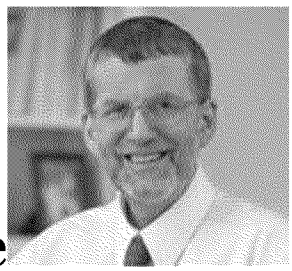
Hi Norma,

See the revised notice from NIH (sent yesterday). Vicki and I spoke yesterday and I am going to try to stick to the April 15 deadline of submitting Amy's R21 progress report. During that submission, Vicki believes the request for the transfer can be made simultaneously within the submission of the progress report. I will let Vicki expound on the subject matter with you more but that is the thought process as I look for the area within the progress report of where I can request the grant be transferred to PNNL.

Best,
Chandra

From: Update from the NIH Office of Extramural Research to the external biomedical <EXTRAMURALNEXUS@LIST.NIH.GOV> **On Behalf Of** NIH Extramural Nexus (NIH/OD)
Sent: Thursday, March 26, 2020 7:18 PM
To: EXTRAMURALNEXUS@LIST.NIH.GOV
Subject: NIH Update: Responding to Frequent Questions on Flexibilities Related to NIH Funding and COVID-19

NIH National Institutes of Health
Office of Extramural Research
Extramural Nexus



Open Mike

Responding to Frequent Questions on Flexibilities Related to NIH Funding and COVID-19

Posted on March 26, 2020 by Mike Lauer

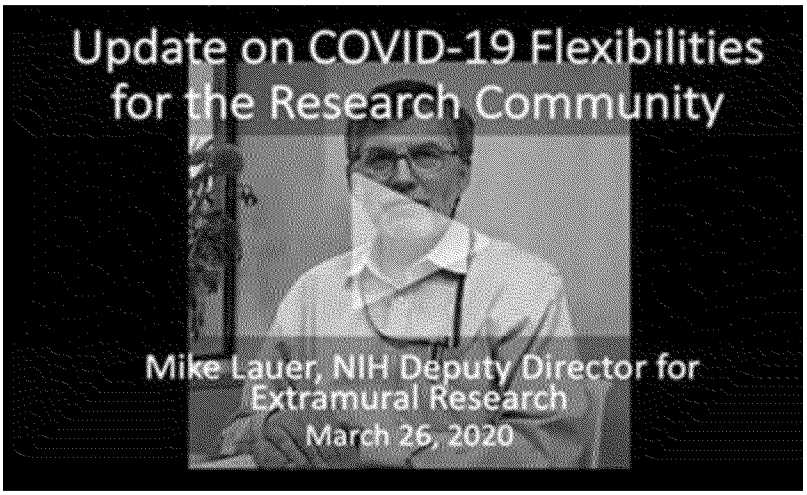
The public health emergency due to COVID-19 is causing difficulties in many aspects of our lives. My colleagues and I here at NIH are well aware of the challenges being felt in the research community as institutions are closing, people are being asked to practice social distancing, and resources and attention are justifiably focused on public health needs. We are listening to your concerns and are working quickly to develop answers to your many questions.

We recently updated our Coronavirus Disease 2019 (COVID-19): Information for NIH Applicants and Recipients website with a slew of additional FAQs, new funding opportunities, as well as the video message from me, below, where I address some of the most common questions.

Since yesterday's recording of this video, in response to community concerns about their ability to submit applications in a timely manner, we have published a notice announcing that grant applications submitted late for due dates between March 9, 2020, and May 1, 2020, will be accepted through May 1, 2020. This notice applies to all relevant funding opportunity announcements, including those that indicate no late applications will be accepted. A cover letter providing a justification is not required. NIH will be extending the expiration date of most FOAs expiring between now and May 1. Be sure to read the notice carefully for details.

Things are moving quickly. Please continue to communicate with us. We are listening.

I encourage you to monitor our website frequently. To help you identify updated content, the page now includes a link to page update history so you can easily see what's new.



From: Sandoval, Norma I[Norma.Sandoval@pnnl.gov]
Sent: Fri 3/27/2020 8:36:07 AM (UTC-07:00)
To: Caldwell, Chandra[ccaldwel@email.unc.edu]
Cc: Sims, Amy C[amy.sims@pnnl.gov]
Subject: RE: NIH Update: Responding to Frequent Questions on Flexibilities Related to NIH Funding and COVID-19

Chandra,
Thank you so much for your quick response on the deadlines. We'll ensure to get you whatever documents you need from our end before your target deadline submission, I just need verification from Vicki on what UNC needs from PNNL for the progress report.

Stay safe!

Thank you.

Norma Sandoval
Principal Project Coordinator
Biological Sciences Division, **EBSD**
Pacific Northwest National Laboratory

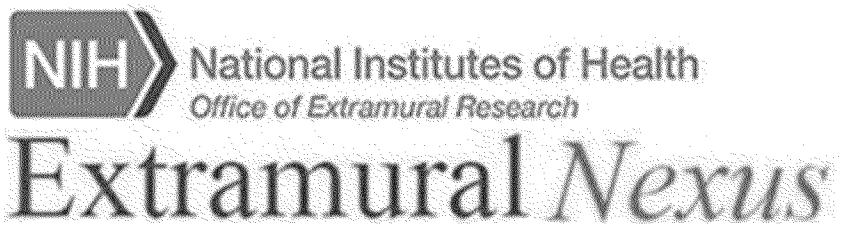
From: Caldwell, Chandra <ccaldwel@email.unc.edu>
Sent: Friday, March 27, 2020 8:28 AM
To: Sandoval, Norma I <Norma.Sandoval@pnnl.gov>
Cc: Sims, Amy C <amy.sims@pnnl.gov>
Subject: FW: NIH Update: Responding to Frequent Questions on Flexibilities Related to NIH Funding and COVID-19

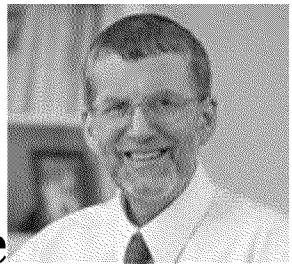
Hi Norma,

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Best,
Chandra

From: Update from the NIH Office of Extramural Research to the external biomedical <EXTRAMURALNEXUS@LIST.NIH.GOV> **On Behalf Of** NIH Extramural Nexus (NIH/OD)
Sent: Thursday, March 26, 2020 7:18 PM
To: EXTRAMURALNEXUS@LIST.NIH.GOV
Subject: NIH Update: Responding to Frequent Questions on Flexibilities Related to NIH Funding and COVID-19





Open Mike

Responding to Frequent Questions on Flexibilities Related to NIH Funding and COVID-19

Posted on **March 26, 2020** by **Mike Lauer**

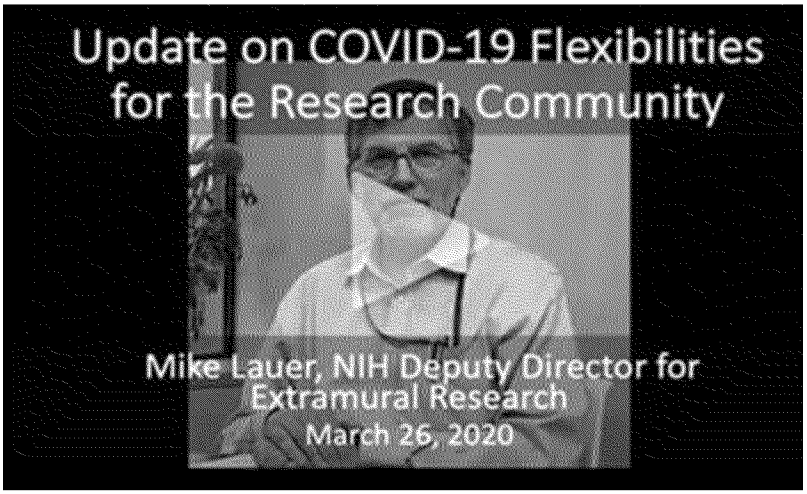
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Things are moving quickly. Please continue to communicate with us. We are listening.

I encourage you to monitor our website frequently. To help you identify updated content, the page now includes a link to page update history so you can easily see what's new.



From: Caldwell, Chandra[ccaldwel@email.unc.edu]
Sent: Fri 3/27/2020 8:50:45 AM (UTC-07:00)
To: Sandoval, Norma I[Norma.Sandoval@pnnl.gov]
Cc: Sims, Amy C[amy.sims@pnnl.gov]
Subject: RE: NIH Update: Responding to Frequent Questions on Flexibilities Related to NIH Funding and COVID-19
Attachment: 2018 Progress Report.pdf
Attachment: NIH Progress Report Questions.docx
Attachment: Example of OS with Foreign Info.pdf

Understandable, since I reminded her to make sure she emailed you back yesterday as I was asking her about relinquishing Amy's grant to PNNL. We are supposed to do a check in on Monday (if nothing unexpectedly arises today). I will remind her again if she doesn't email you and Amy directly. Should we not make the April 15 deadline, we at least have a buffer thanks to the NIH extension. If I find out anything else after speaking with her, I will let you both know.

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Chandra,
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Stay safe!

Thank you.

Norma Sandoval
Principal Project Coordinator
Biological Sciences Division, **EBSD**
Pacific Northwest National Laboratory

From: Caldwell, Chandra <ccaldwel@email.unc.edu>
Sent: Friday, March 27, 2020 8:28 AM

To: Sandoval, Norma I <Norma.Sandoval@pnnl.gov>
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Subject: FW: NIH Update: Responding to Frequent Questions on Flexibilities Related to NIH Funding and COVID-19

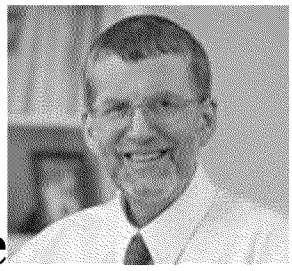
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NIH National Institutes of Health
Office of Extramural Research
Extramural Nexus



Open Mike

Responding to Frequent Questions on Flexibilities Related to NIH Funding and COVID-19

Posted on **March 26, 2020** by **Mike Lauer**

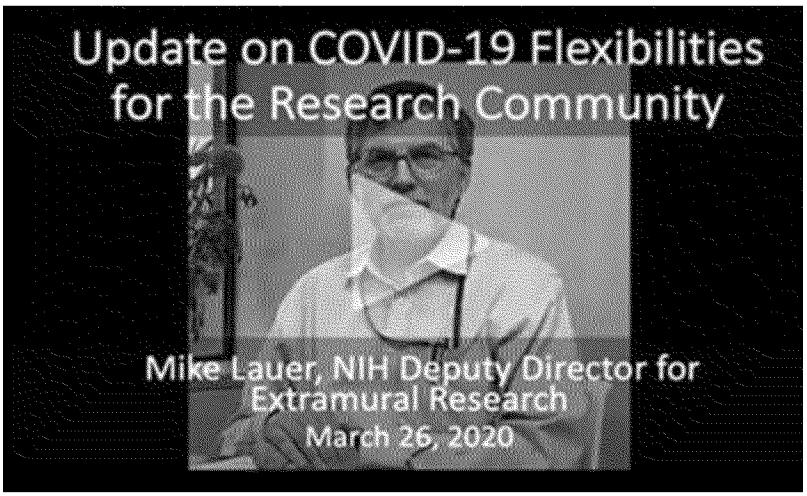
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NIH Progress Report Questions

PART B: Question #1: What are the major goals of the project?

List the major goals of the project as stated in the approved application or as approved by the agency. If the application lists milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.

Generally, the goals will not change from one reporting period to the next. However, if the awarding agency approved changes to the goals during the reporting period, list the revised goals and objectives. Also explain any significant changes in approach or methods from the agency approved application or plan.

- *Goals are equivalent to specific aims. NOTE:* ***The specific aims must be provided in the initial RPPR*** *(i.e., first progress report of the grant). In subsequent RPPRs this section will pre-populate with the aims/goals previously entered.* *****Epid faculty just send me an abbreviated version of their specific aims as a list.***

Question #2: Have the major goals changed since the initial competing award or previous report?

Select ***Yes*** *if the major goals/specific aims have changed since the initial competing award or previous report, and provide a revised description of major goals/specific aims. Remember that written prior approval from the awarding agency grants official is required for significant changes in the project or its direction.*

→The RPPR is not an appropriate vehicle to request such a change.

→The first year that an RPPR is submitted any revised goals should be entered.

→In subsequent years, if the user selects Yes the text box under B.1.a for entering revised major goals will be provided

Question #3: What was accomplished under these goals?

Describe: 1) major activities; 2) specific objectives; 3) significant results, including major findings, developments, or conclusions (both positive and negative); and 4) key outcomes or other achievements. Include a discussion of stated goals not met.

As the project progresses, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments. . (Note: If citing references in this section, please provide a full citation. Though they will be denoted in the Publications section of the progress report also.

Question #4: What opportunities for training and professional development has the project provided?

If the research is not intended to provide training and professional development opportunities or there is nothing significant to report during the reporting period, select Nothing to Report.

However, describe opportunities for training and professional development provided to anyone who worked on the project or anyone who was involved in the activities supported by the project. Training activities are those in which individuals with advanced professional skills and experience assist others in attaining greater proficiency. Training activities may include, for example, courses or one-on-one work with a mentor. Professional development activities result in increased knowledge or skill in one's area of expertise and may include workshops, conferences, seminars, study groups, and individual study. Include participation in conferences, workshops, and seminars not listed under major activities. For all projects reporting graduate students and/or postdoctoral participants as part of their paid personnel, this information is needed.

Question #5: How have results been disseminated to communities of interest?

Describe how the results have been disseminated to communities of interest. Include any outreach activities that have been undertaken to reach members of communities who are not usually aware of these research activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities. Reporting the routine dissemination of information (e.g., websites, press releases) is not required.

For awards not designed to disseminate information to the public or conduct similar outreach activities, a response is not required and the grantee should select Nothing to Report. A detailed response is only required for awards or award components that are designed to disseminate information to the public or conduct similar outreach activities

Question #6: What do you plan to do for the next reporting period to accomplish the goals?

Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.

PART C: *Question #7: Publications.*
Are there publications or manuscripts accepted for publication in a journal or other publication (e.g., book, one-time publication, monograph, preprint) during the reporting period resulting directly from the award?

If yes, let me know what publications they are so I can add them from PubMed to insure they are associated with this grant award. If you don't have any publications at this time, I can easily answer No.

Question #8: Website(s) or other internet site(s).
List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. A description is only required for awards designed to create or maintain one or more websites. For awards not designed to create or maintain one or more websites, select Nothing to Report.

Question #9: Technologies or techniques.
Identify technologies or techniques that have resulted from the research activities. Describe the technologies or techniques and how they are being shared. If there are no technologies or techniques to report select Nothing to Report

Part D: I will take care of once I receive the other support documents of each personnel
Part E: I will take care of since the answer is N/A or Nothing to Report
Part F: I will take care of since the answer is N/A or Nothing to Report
Part G: *I will have an enrollment table to update once I log in as you. The enrollment table that is currently in the progress report was extracted from your original grant submission. So if the numbers have changed, update that document and sent it to me with the Word document.*

From: Sims, Amy C[amy.sims@pnnl.gov]
Sent: Fri 3/27/2020 11:06:29 AM (UTC-07:00)
To: Caldwell, Chandra[ccaldwel@email.unc.edu]; Sandoval, Norma I[Norma.Sandoval@pnnl.gov]
Subject: Re: NIH Update: Responding to Frequent Questions on Flexibilities Related to NIH Funding and COVID-19

Thanks, Chandra. I went to the NIH website and drafted my report using those questions but it is very good to see I wasn't off base.

Thanks for the other support example. That is new for me and I managed to miss the UNC departmental meetings regarding how to do this now.

Thank you again! I hope you are doing well. Maybe COVID 19 means less people are bugging you but I doubt it! Have a good weekend. Thank you, Amy

---

From: "Caldwell, Chandra" <ccaldwel@email.unc.edu>
Date: Friday, March 27, 2020 at 8:52 AM
To: "Sandoval, Norma I" <Norma.Sandoval@pnnl.gov>
Cc: "Sims, Amy C" <amy.sims@pnnl.gov>
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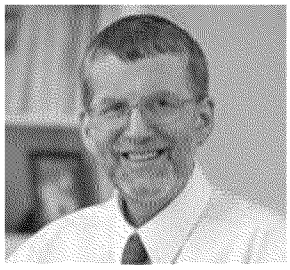
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NIH National Institutes of Health
Office of Extramural Research
Extramural Nexus

Open Mike 

Responding to Frequent Questions on Flexibilities Related to

NIH Funding and COVID-19

Posted on **March 26, 2020** by **Mike Lauer**

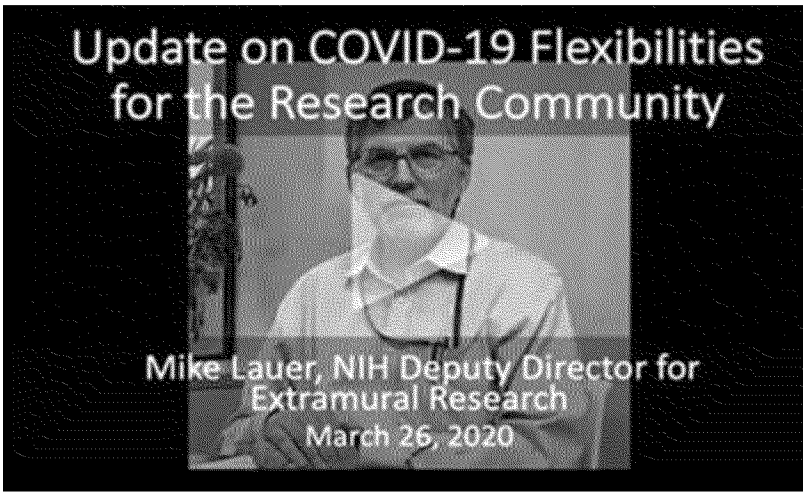
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From: Sheahan, Timothy Patrick[sheahan@email.unc.edu]

Sent: Sat 3/28/2020 11:29:00 AM (UTC-07:00)

To: Baric, Ralph S[rbaric@email.unc.edu]; Zhou, Shuntai[shuntaiz@email.unc.edu]; Sims, Amy C[amy.sims@pnnl.gov]; Hill, Collin Siva[collinh@live.unc.edu]; Leist, Sarah Rebecca[leist@email.unc.edu]; Schaefer, Alexandra[aschaefe@email.unc.edu]; Agostini, Maria L K[maria.l.agostini.1@vumc.org]; Pruijssers, Ardina[ardina.pruijssers@vumc.org]; Brown, Ariane[ajbrown7@email.unc.edu]; Greg Bluemling[gblueml@emory.edu]; Natchus, Michael[mnatchu@emory.edu]; msainda@emory.edu[msainda@emory.edu]; a.kolykhalov@emory.edu[a.kolykhalov@emory.edu]; Painter, George R.[george.r.painter@emory.edu]; Denison, Mark[mark.denison@vumc.org]; Swanstrom, Ronald I[ron_swanstrom@med.unc.edu]; Dinnon, Kenneth Harold III[kdinnon@email.unc.edu]; Graham, Rachel[rlgraham@ad.unc.edu]; Thornburg, Natalie (CDC/OID/NCIRD)[nax3@cdc.gov]; zaq6@cdc.gov[zaq6@cdc.gov]; axt4@cdc.gov[axt4@cdc.gov]; Montgomery, Stephanie Ann[stephanie_montgomery@med.unc.edu]; Chappell, Jim[jim.chappell@vumc.org]; laura.j.stevens@vumc.org[laura.j.stevens@vumc.org]; xiaotao.lu@vumc.org[xiaotao.lu@vumc.org]; tia.m.hughes@vumc.org[tia.m.hughes@vumc.org]; amelia.s.george@vumc.org[amelia.s.george@vumc.org]

Cc: adobbins@aaas.org[adobbins@aaas.org]

Subject: Your Signature Needed for Science Translational Med Paper Revision

Attachment: 2020 NHC CoV STM Paper Authorship Change Form abb5883.docx

Dear All,

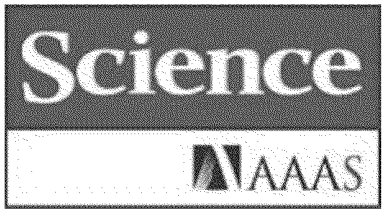
The Denison lab did a bunch of amazing work on the revised NHC coronavirus Science Translational Medicine manuscript. Four authors need to be added to the paper as a result of this.: Amelia George, Laura Stevens, Tia Hughes and Xiaotao Lu.

The journal would like each of you to sign the attached form and email it to Aneera Dobbins (adobbins@aaas.org) as soon as possible. Each can just print their name and sign their own form and send it. There's no need to have everyone on literally the same page. I've CC'd Aneera above.

Thanks!

Tim

There are changes listed in the authorship of your manuscript # ***abb5883***
Additional Authors: **Laura J. Stevens, Xiaotao Lu, Tia M Hughes , and Amelia S. George** have been added to the author list



Authorship Change Form – Science Translational Medicine

Author information has been altered on your manuscript. As stated in our Information to Contributors, written verification that any change in authorship was approved by all authors of the paper must be provided before we can publish your paper.

Approval of Authorship Change:

Printed Name	**Signature**
Printed Name	**Signature**
Printed Name	**Signature**
Printed Name	**Signature**
Printed Name	**Signature**
Printed Name	**Signature**
Printed Name	**Signature**
Printed Name	**Signature**
Printed Name	**Signature**
Printed Name	**Signature**

All signatures are required but do not have to be on the same form. If you have any questions regarding change in authorship, please contact the Editorial Department at 202-326-6550.

There are changes listed in the authorship of your manuscript # ***abb5883***
Additional Authors: **Laura J. Stevens, Xiaotao Lu, Tia M Hughes, and Amelia S. George** have been added to the author list

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Science
AAAS

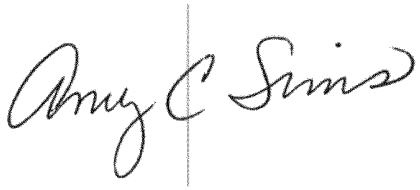
Authorship Change Form – Science Translational Medicine

Author information has been altered on your manuscript. As stated in our Information to Contributors, written verification that any change in authorship was approved by all authors of the paper must be provided before we can publish your paper.

Approval of Authorship Change:

Amy C Sims ¬

Printed Name



Signature

- ¬

Printed Name **Signature**

- ¬

Printed Name **Signature**

- ¬

Printed Name **Signature**

- ¬

Printed Name **Signature**

- ¬

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Sent: Sat 3/28/2020 3:00:52 PM (UTC-07:00)

To: Sims, Amy C[amy.sims@pnnl.gov]

Subject: Re: Your Signature Needed for Science Translational Med Paper Revision

Seems fine to me. Send it on to that lady in the email! Thanks!

> On Mar 28, 2020, at 5:16 PM, Sims, Amy C <amy.sims@pnnl.gov> wrote:
>
> Best I can do until Monday. Can you live with this? Thanks, Amy
>
> On 3/28/20, 11:43 AM, "Sheahan, Timothy Patrick" <sheahan@email.unc.edu> wrote:
>
> Dear All,
>
> The Denison lab did a bunch of amazing work on the revised NHC coronavirus Science Translational Medicine manuscript. Four authors need to be added to the paper as a result of this.: Amelia George, Laura Stevens, Tia Hughes and Xiaotao Lu.
>
> The journal would like each of you to sign the attached form and email it to Aneera Dobbins (adobbins@aaas.org) as soon as possible. Each can just print their name and sign their own form and send it. There's no need to have everyone on literally the same page. I've CC'd Aneera above.
>
> Thanks!
>
> Tim
>
>
>
>
> <SIms 2020 NHC CoV STM Paper Authorship Change Form abb5883[2].pdf>

From: Li, Kui[kli1@uthsc.edu]
Sent: Sat 3/28/2020 9:25:14 PM (UTC-07:00)
To: Lu, Lu[llu@uthsc.edu]; Klaus x Klaus Schughart[Klaus.Schughart@helmholtz-hzi.de]; Williams, Robert W[rwilli10@uthsc.edu]
Cc: Xu, Fuyi[fxu10@uthsc.edu]; Sims, Amy C[amy.sims@pnnl.gov]; rbaric@email.unc.edu[rbaric@email.unc.edu]; silke.bergmann@(b) (6) [silke.bergmann@(b) (6)]
Subject: Re: [Ext] B6D2 infected RNAseq data
Attachment: Ace2_V4_260320_Li.docx

Dear Lu, Klaus, Rob, and team,

Please find my edits of the manuscript. Use as you see fit. Thanks,

Kui

From: Lu, Lu <llu@uthsc.edu>
Sent: Thursday, March 26, 2020 6:05 PM
To: Klaus x Klaus Schughart <Klaus.Schughart@helmholtz-hzi.de>
Cc: Williams, Robert W <rwilli10@uthsc.edu>; Xu, Fuyi <fxu10@uthsc.edu>; amy.sims@pnnl.gov <amy.sims@pnnl.gov>; rbaric@email.unc.edu <rbaric@email.unc.edu>; silke.bergmann@(b) (6) <silke.bergmann@(b) (6) >; Li, Kui <kli1@uthsc.edu>
Subject: Re: [Ext] B6D2 infected RNAseq data

Dear Klaus (and all others):
Here is updated version. Fuyi addressed most of your comments. Please feel free to modify it continuously.

Dear Amy and Ralph:

Some of Klaus' comment are specific to you. Could you help to reply or editing?

Thanks.

Lu Lu
Professor
Department of Genetics, Genomics and Informatics
University of Tennessee Health Science Center
71 S. Manassas St., Room 410K
Memphis, TN 38163
Tel: (901) 448-7557 (O)
Fax: (901) 448-3500 (Dept)

On Mar 26, 2020, at 1:18 PM, Schughart, Klaus <Klaus.Schughart@helmholtz-hzi.de> wrote:

Hi LuLu,
enclosed are my additional comments – now also on the discussion.
Maybe Amy has also some ideas to explain the discrepancy between lungs and HAE cells with respect to

inverse up/down regulation.
Klaus

Von: Lu, Lu <llu@uthsc.edu>
Gesendet: Donnerstag, 26. März 2020 00:27
An: Schughart, Klaus <Klaus.Schughart@helmholtz-hzi.de>
Cc: Williams, Robert W <rwilli10@uthsc.edu>; Xu, Fuyi <fxu10@uthsc.edu>; amy.sims@pnnl.gov
Betreff: Re: [Ext] B6D2 infected RNAseq data

Dear Klaus:

We have accepted Rob, you and our previous editing. Here is updated version for you to check and modify continuously.

Dear Amy:

Could you check this version and address some comments that are related to you?

Thanks.

Lu Lu
Professor
Department of Genetics, Genomics and Informatics
University of Tennessee Health Science Center
71 S. Manassas St., Room 410K
Memphis, TN 38163
Tel: (901) 448-7557 (O)
Fax: (901) 448-3500 (Dept)

> On Mar 25, 2020, at 3:18 PM, Xu, Fuyi <fxu10@uthsc.edu> wrote:
>
> Dear Klaus,
>
> Could you please send me those two images (high-resolution) in Figure 8 ? Thank you very much.
>
> --
> Fuyi Xu, PhD
> Department of Genetics, Genomics, and Informatics
> University of Tennessee Health Science Center
>
> 415 TRANSLATIONAL RESEARCH BUILDING
> 71 SOUTH MANASSAS
> MEMPHIS TN 38163
> Phone: (901)246-4398
> E-mail: 5 U.S.C. § 552(b)(6) fxu10@uthsc.edu
>
> ---
>
> **From:** Schughart, Klaus
> **Sent:** Wednesday, March 25, 2020 3:04 PM
> **To:** Lu, Lu

Cc: Xu, Fuyi; Williams, Robert W
Subject: AW: [Ext] B6D2 infected RNAseq data

Hi LuLu,
I forgot something in the Acknowledgements.
Please replace the Acknowledgements in the last version with the one in the enclosed version.
Thx,
Klaus

Von: Schughart, Klaus
Gesendet: Mittwoch, 25. März 2020 20:31
An: 'Lu, Lu' <llu@uthsc.edu>
Cc: 'Xu, Fuyi' <fxu10@uthsc.edu>; 'Williams, Robert W' <rwilli10@uthsc.edu>
Betreff: AW: [Ext] B6D2 infected RNAseq data

Dear all,
here are my comments on the revised version. I took out the previous comments that have been resolved.
Fuyi, I also enclose the data table for Figure 8A.
Once you are done with this version, I can have a look at the discussion and then you could send it off to the other co-authors.
Klaus

Von: Schughart, Klaus
Gesendet: Mittwoch, 25. März 2020 19:31
An: 'Lu, Lu' <llu@uthsc.edu>
Cc: Xu, Fuyi <fxu10@uthsc.edu>; Williams, Robert W <rwilli10@uthsc.edu>
Betreff: AW: [Ext] B6D2 infected RNAseq data

Thanks – will work it.
Klaus

Von: Lu, Lu <llu@uthsc.edu>
Gesendet: Mittwoch, 25. März 2020 19:02
An: Schughart, Klaus <Klaus.Schughart@helmholtz-hzi.de>
Cc: Xu, Fuyi <fxu10@uthsc.edu>; Williams, Robert W <rwilli10@uthsc.edu>
Betreff: Re: [Ext] B6D2 infected RNAseq data

Dear Klaus:

Fuyi and I accept all of you and rob's editing. We also clean and modified the paper, which addressed comments and answered the your questions. Please work on the updated version continuously. Hopefully we can submit this paper ASAP. Thanks.

Lu Lu
Professor
Department of Genetics, Genomics and Informatics
University of Tennessee Health Science Center
71 S. Manassas St., Room 410K
Memphis, TN 38163
Tel: (901) 448-7557 (O)

Fax: (901) 448-3500 (Dept)

On Mar 24, 2020, at 4:36 PM, Lu, Lu <llu@uthsc.edu> wrote:

Thanks, Rob. We will modify based on your comments and will send you updated version for your editing again.

Lu Lu
Professor
Department of Genetics, Genomics and Informatics
University of Tennessee Health Science Center
71 S. Manassas St., Room 410K
Memphis, TN 38163
Tel: (901) 448-7557 (O)
Fax: (901) 448-3500 (Dept)

On Mar 24, 2020, at 4:14 PM, Rob Williams <rwilliams@uthsc.edu> wrote:

Dear team,

Brilliant effort,

Edited the abstract heavily (please review carefully) and built up the Methods section on BXDs a bit.

Have not gotten through all of paper, but looks great so far.

Should validate some networks and eQTLs with GEMMA.

Needs a bit more clarity to data sources—some for 43 strains, other data for parents. These details needed (imho) even in the abstract.

On Tue, Mar 24, 2020 at 2:41 PM Schughart, Klaus <Klaus.Schughart@helmholtz-hzi.de> wrote:

Hi Lulu, Fuyi,

enclosed are my comments, additions and corrections.

Looks very good!!!

Please go through and clean it up first (accept or discard changes,

add information) and then I can continue to work on the cleaned-up version. Currently it is too much cluttered with all these changes and comments.

Fuyi, let me know what exactly you need in addition for the HAE data.

Klaus

Von: Schughart, Klaus
Gesendet: Freitag, 6. März 2020 19:02
An: Xu, Fuyi <fxu10@uthsc.edu>; Lu Lu [5 U.S.C. § 552(b)(6)]
Cc: Rob Williams <rwilliams@uthsc.edu>; Jonsson, Colleen Beth (cjonsson@uthsc.edu) <cjonsson@uthsc.edu>
Betreff: AW: [Ext] B6D2 infected RNAseq data

Hi Fuyi, LuLu,

I did some extensive analyses of genes in the human HAE data set with your gene list. Enclosed are the results. Looks very good!!

For a publication, we would have to discuss which data to include and how to present the results.

Klaus

Von: Schughart, Klaus <Klaus.Schughart@helmholtz-hzi.de>
Gesendet: Donnerstag, 5. März 2020 04:45
An: Xu, Fuyi <fxu10@uthsc.edu>
Cc: Lu Lu [5 U.S.C. § 552(b)(6)]
Betreff: Re: [Ext] B6D2 infected RNAseq data

Dear Fuyi,

sure I will have a look at these genes.

Klaus

Von meinem iPhone gesendet

> Am 04.03.2020 um 19:09 schrieb Xu, Fuyi <fxu10@uthsc.edu>:
>
> Dear Klaus,
>
> Lu and I are working on the Ace2 paper. We prioritized around 30 genes that are correlated with Ace2 (attached table). I am wondering if you can help me check the expression differences between infected and normal controls in the human data set last time you

showed us, especially for those color highlighted. Also, is this gene list make sense from your perspective? Thank you very much.

Best,

--
Fuyi Xu, PhD
Department of Genetics, Genomics, and Informatics
University of Tennessee Health Science Center

415 TRANSLATIONAL RESEARCH BUILDING
71 SOUTH MANASSAS
MEMPHIS TN 38163
Phone: (901)246-4398
E-mail: 5 U.S.C. § 552(b)(6) fxu10@uthsc.edu

From: Schughart, Klaus
Sent: Friday, February 28, 2020 11:07 AM
To: Xu, Fuyi
Cc: Lu Lu
Subject: AW: [Ext] B6D2 infected RNAseq data

Hi LuLu, Fuyi,

I just remembered that I also have expression data from infected human lung slices (see enclosure).

This dataset has been published (enclosed).

Klaus

Von: Schughart, Klaus
Gesendet: Montag, 24. Februar 2020 21:00
An: 'Xu, Fuyi' <fxu10@uthsc.edu>
Cc: Lu Lu 5 U.S.C. § 552(b)(6)
Betreff: AW: [Ext] B6D2 infected RNAseq data

Dear Fuyi,

The data I sent you before (RNAseq_norm1.txt) have been normalized and log2 transformed using DESeq2 (rlogTransformation(dsDat, blind=TRUE).

Please find enclosed the raw counts for the B6/D2 infections from Wilk et al (all_mouse_counts.txt).

For the BXD data in GN:

The percent body weights are NOT the losses but the percent body weight with reference to the starting body weight (100%). So 105% means that the mouse has

gained 5% of body weight relative to its starting weight when it was infected. Mice with percent body weight less than 100% have lost weight.

Klaus

Von: Xu, Fuyi <fxu10@uthsc.edu>
Gesendet: Sonntag, 23. Februar 2020 22:14
An: Schughart, Klaus <Klaus.Schughart@helmholtz-hzi.de>
Cc: Lu Lu 5 U.S.C. § 552(b)(6)
Betreff: RE: [Ext] B6D2 infected RNAseq data

Dear Dr. Klaus Schughart,

I am Lu Lu's postdoc, Fuyi Xu. Thank you for sending us the normalized RNA-seq data.

We would like to perform differential expression analysis, I am wondering if you have raw read count for this data set since the DEseq2 package depend on this kind of data matrix. By the way, is the normalized data log2 transformed ?

We also found the expression level of ACE2 across the BXD is significantly correlated with median weight loss at day 3 after infection (ID:13007), however, we found BXD65a loss 105% body weight. Is this correct? Or how should I interpret ?

<A54B14D6A4584F84B57E3AAA25DC5BBB.gif>

Best Regards,

--
Fuyi Xu, PhD
Department of Genetics, Genomics, and Informatics
University of Tennessee Health Science Center

415 TRANSLATIONAL RESEARCH BUILDING
71 SOUTH MANASSAS
MEMPHIS TN 38163
Phone: (901)246-4398
E-mail: 5 U.S.C. § 552(b)(6) fxu10@uthsc.edu

From: Lu, Lu
Sent: Monday, February 17, 2020 11:03 AM
To: Xu, Fuyi
Subject: Fwd: [Ext] B6D2 infected RNAseq data

Lu Lu
Professor
Department of Genetics, Genomics and Informatics
University of Tennessee Health Science Center
71 S. Manassas St., Room 410K
Memphis, TN 38163
Tel: (901) 448-7557 (O)
Fax: (901) 448-3500 (Dept)

Begin forwarded message:

From: "Schughart, Klaus" <Klaus.Schughart@helmholtz-hzi.de>

Subject: [Ext] B6D2 infected RNAseq data

Date: February 17, 2020 at 10:11:55 AM CST

To: "Lu, Lu" <llu@uthsc.edu>

Hi LuLu,

I checked the datasets.

GN807; RNAseq BXD infected is fine. This dataset has not been published yet.

I enclose the log2 normalized matrix for the B6/D2 infected data from Wilk et al. 2015:

RNAseq_norm1.txt -- for the mouse

genes

00_influenza_lg2_RPKM_kls_280714.tx
t -- for the influenza gene segments

Let me know if you have any questions.

Klaus

Prof. Dr. Klaus Schughart
Head of Dept. of Infection Genetics
Helmholtz Centre for Infection
Research,

University of Veterinary Medicine
Hannover,

University of Tennessee Health
Science Center
Inhoffenstr. 7
D-38124 Braunschweig
Germany

E-mail : Klaus.Schughart@helmholtz-hzi.de
Phone: +49 531 6181 1100

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BC50D960A2.png>

Helmholtz-Zentrum für
Infektionsforschung GmbH |
Inhoffenstraße 7 | 38124
Braunschweig | www.helmholtz-hzi.de

Vorsitzende des Aufsichtsrates: Frau
MinDir'in Prof. Dr. Veronika von
Messling
Stellvertreter: MinDirig Rüdiger
Eichel, Niedersächsisches
Ministerium für Wissenschaft und
Kultur
Geschäftsführung: Prof. Dr. Dirk
Heinz; Silke Tannapfel
Gesellschaft mit beschränkter
Haftung (GmbH)
Sitz der Gesellschaft: Braunschweig
Handelsregister: Amtsgericht
Braunschweig, HRB 477

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Vorsitzende des Aufsichtsrates: Frau MinDir'in Prof. Dr. Veronika von Messling
Stellvertreter: MinDirig Rüdiger Eichel, Niedersächsisches Ministerium für Wissenschaft und Kultur
Geschäftsführung: Prof. Dr. Dirk Heinz; Silke Tannapfel
Gesellschaft mit beschränkter Haftung (GmbH)
Sitz der Gesellschaft: Braunschweig
Handelsregister: Amtsgericht Braunschweig, HRB 477

<Table 1.xlsx>

<B911E1AA90884DD79858B3D0CE0F7C4C.png>

Helmholtz-Zentrum für Infektionsforschung GmbH | Inhoffenstraße 7 | 38124 Braunschweig | www.helmholtz-hzi.de

Vorsitzende des Aufsichtsrates: Frau MinDir'in Prof. Dr. Veronika von Messling
Stellvertreter: MinDirig Rüdiger Eichel, Niedersächsisches Ministerium für Wissenschaft und Kultur
Geschäftsführung: Prof. Dr. Dirk Heinz; Silke Tannapfel
Gesellschaft mit beschränkter Haftung (GmbH)
Sitz der Gesellschaft: Braunschweig
Handelsregister: Amtsgericht Braunschweig, HRB 477

--
Rob

Robert W. Williams, Ph.D.
Chair: Department of Genetics, Genomics and Informatics
University of Tennessee Health Science Center
71 S Manassas St, Memphis TN 38103-3310, TSRB Room 405
Tele/Cell: 901 604 4752
EMAIL: rwilliams@uthsc.edu or 5 U.S.C. § 552(b)(6)

<Ace2-Draft-03182020-

V1_LuB_kls_RWW_240320_3.docx>

<8608AAAF2A14461CBDBD04DA13676A0C.png>

Helmholtz-Zentrum für Infektionsforschung GmbH | Inhoffenstraße 7 | 38124 Braunschweig | www.helmholtz-hzi.de

Vorsitzende des Aufsichtsrates: Frau MinDir'in Prof. Dr. Veronika von Messling
Stellvertreter: MinDirig Rüdiger Eichel, Niedersächsisches Ministerium für Wissenschaft und Kultur
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Gesellschaft mit beschränkter Haftung (GmbH)
Sitz der Gesellschaft: Braunschweig
Handelsregister: Amtsgericht Braunschweig, HRB 477

Helmholtz-Zentrum für Infektionsforschung GmbH | Inhoffenstraße 7 | 38124 Braunschweig | www.helmholtz-hzi.de

Vorsitzende des Aufsichtsrates: Frau MinDir'in Prof. Dr. Veronika von Messling
Stellvertreter: MinDirig Rüdiger Eichel, Niedersächsisches Ministerium für Wissenschaft und Kultur
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Gesellschaft mit beschränkter Haftung (GmbH)
Sitz der Gesellschaft: Braunschweig
Handelsregister: Amtsgericht Braunschweig, HRB 477

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Genetic Dissection of the Regulatory Mechanisms of *Ace2* in the Infected Mouse Lung

Fuyi Xu[1#], Jun Gao[1, 5#], Silke Bergman[4], Amy C. Sims[7, 9], Ralph S. Baric[6,7, 8], Kui Li[4], Robert W. Williams[1], Klaus Schughart[2, 3, 4*], and Lu Lu[1*]

[1]Department of Genetics, Genomics, and Informatics, University of Tennessee Health Science Center, Memphis, TN, USA;
[2]Department of Infection Genetics, Helmholtz Centre for Infection Research, Braunschweig, Germany
[3]University of Veterinary Medicine Hannover, Hannover, Germany;
[4]Department of Microbiology, Immunology, and Biochemistry, University of Tennessee Health Science Center, Memphis, TN, USA;
[5]Institute of Animal Husbandry and Veterinary Science, Shanghai Academy of Agricultural Sciences, Shanghai, China.
[6]Department of Microbiology and Immunology, University of North Carolina at Chapel Hill, Chapel Hill, NC
[7]Department of Genetics, University of North Carolina at Chapel Hill, Chapel Hill, NC
[8]Department of Epidemiology, University of North Carolina at Chapel Hill, Chapel Hill, NC
[9]present address: Pacific Northwest National Laboratory in Richland, WA

[#] These authors contributed equally to this work.

* Correspondence:

Lu Lu: llu@uthsc.edu

Department of Genetics, Genomics and Informatics
University of Tennessee Health Science Center
Memphis, TN 38163, United States;

Klaus Schughart: klaus.schughart@helmholtz-hzi.de
Department of Infection Genetics
Helmholtz Centre for Infection Research
Braunschweig, Germany

Abstract

Acute lung injury (ALI) is an important cause of morbidity and mortality after viral infections, such as influenza A virus H1N1, SARS-CoV, MERS-CoV, and SARS-CoV-2. The angiotensin 1 converting enzyme 2 (ACE2) plays a protective role in SARS-CoV-induced ALI. However, the contributions of *ACE2* gene variants to post-infection heterogeneity of the host response and susceptibility remain unclear, as do the underlying mechanisms. In this study, we quantified lung transcriptomes in 41 BXD recombinant inbred strains of mice plus their parental strains of C57BL/6J and DBA/2J mice after H1N1 infection. The expression of *Ace2* mRNA across strains decreased significantly and was associated with both disease severity and infection time. Expression was negatively correlated with body weight loss and survival time. Data from 43 strains were analyzed using weighted gene co-expression network analysis (WGCNA). *Ace2* not only participated in virus-induced ALI by interacting with TNF, MAPK, and notch signaling pathways, but also was linked to genes that have important functions in the pulmonary epithelium. Moreover, by applying multi-layer filtration, including differential expression analysis, eQTL mapping, functional variant identification, and gene annotation, we highlighted 10 key candidate genes linked to an *Ace2* network: *Sox11*, *Tff2*, *Rnf128*, *Muc5b*, *Tmprss2*, *Ulk4*, *Cckar*, *Wdpcp*, *Ift140*, and *Ccdc40*. Of these transcripts, *Rnf128* was identified as a potential master regulator using Bayesian network analysis. The key gene set is enriched for genes reported to be differentially expressed in SARS-CoV-infected primary human airway epithelial cells. Collectively, these findings define an *Ace2*-associated modulatory network relevant to host response to H1N1 infection and raise novel potential therapeutic targets for virus-induced respiratory diseases, including those caused by SARS-CoV and SARS-CoV-2. In addition, we demonstrated that BXD family of strains provide an excellent resource for dissecting host genetic regulatory mechanisms involved in the defense against viral infections and host disease.

Key words: H1N1, acute lung injury, BXD mice, *Ace2,* regulatory network

Introduction

The pandemic infectious disease, COVID-19, is caused by a novel coronavirus, termed severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Since its first emergence in November or December 2019, COVID-19 has been spreading with unprecedented speed. As of March 25, 2020, according to preliminary estimates by World Health Organization (WHO), over 375,000 cases of COVID-19 have been reported in more than 195 countries,, and the death toll has surpassed 16,000. Since 1918, the world has witnessed four influenza A virus (IAV) pandemics—1918 H1N1, 1957 H2N2, 1968 H3N2, and 2009 H1N1. The first of these resulted in at least 50 million global mortalities [1]. The main pathological features caused by COVID-19 and 2009 influenza A (H1N1) are quite similar – both can lead to acute viral pneumonia, which is an important cause of acute lung injury (ALI) [2]. In addition, the documented symptoms for influenza A (H1N1) and COVID-19 are nearly identical and for both infected patients can manifest as asymptomatic infection, or mild or severe disease, and in a minority, the outcome can be lethal. Precisely why different individuals exhibit disparate susceptibilities remains largely a mystery. Conceivably, differences in genetic makeup likely play an important part in the development and severity of the illness [3-5].

Angiotensin-converting enzyme 2 (*ACE2*), a negative regulator of the renin angiotensin system (RAS), plays a protective role in pathogenesis of ALI in several animal models of acute respiratory distress syndrome [6]. *Ace2* knockout mice exhibited severe pathology of ALI and exogenous recombinant human *ACE2* expression attenuates acute lung failure in *Ace2* knockout mice as well as in wild-type mice [7]. Studies have shown that ACE2 protein levels are significantly downregulated after infection with H1N1 virus but are dispensable for viral replication [8]. For SARS-CoV, ACE2 has been identified as the principal host receptor for the receptor-binding domain of the viral spike protein and thereby facilitates viral entry into susceptible host cells [9-11]. In *in vivo* studies, SARS-CoV infection of mice causes severe pulmonary disease and lethal lung failure, concomitant with the downregulation of *Ace2* in lungs[12]. Single-cell RNA sequencing demonstrated that 83% of *ACE2*-expressing cells in the lungs are alveolar epithelial type II cells and that a much higher *ACE2*-expressing cell ratio is observed in Asian male donors compared to white and African American donors [13], However, the contribution of *ACE2* variation to post-infection heterogeneity of the ALI is still poorly defined.

Susceptibility to viral infection varies greatly among mouse strains [14]. The difference is especially obvious between inbred strains C57BL/6J (B6) and DBA/2J (D2) mice [15]. B6 is relatively resistant to IAV and often survives inoculation. In contrast, D2 loses body weight rapidly and at the same inoculum dose dies within 5–7 days. Our laboratory has generated and maintains ~120 BXD recombinant inbred (RI) strains derived from multiple independent crosses of B6 and D2 parental strains. Each of these highly diverse BXD progeny is fully inbred and each has been fully sequenced. Collectively this family of strains segregates for ~6 million variants with minor allele frequencies above 0.3 [16], making it an ideal and well powered reference population to model the genetic susceptibility of IAV infections [17]. Indeed, our previous genetic mapping in this population have revealed several loci and gene variants that control the host response to IAV (H1N1) infection [18] and other infectious diseases [19-22]. The BXD family

provides a powerful resource to dissect loci, genes, molecular networks, and even mechanisms of host genetic responses to viral infections.

Here we have analyzed the lung transcriptome by RNAseq in 41 BXD RI strains and both parental strains after infection with H1N1 IAV. By using systems genetics approaches, we investigated patterns of covariation of mRNAs with induced variation in *Ace2* expression. We defined networks and regulatory relations that are likely to contribute to the host defense and/or pathology of H1N1-induced ALI.

Materials and methods

Mice

The subjects for this study were female mice belonging to 41 distinct BXD recombinant inbred (RI) strains, and the parental C57BL/6J (B6) and DBA/2J (D2) strains. These BXD strains segregate for over 5 million sequence variants with minor allele frequencies greater than 0.3 [23]. They are well powered to detect major loci that contribute to genetic variation in disease susceptibility [19-22] and to define causal networks of interacting loci, genes, gene productions (mRNAs and proteins), and pathogenicity outcome measures [18, 24]. All mice were between 8 and 12 weeks of age when infected with virus. All were housed and maintained on a 12:12 light/ dark cycle, with *ad libitum* access to food and water. Procedures were approved by the institutional animal care and use committee (IACUC) at the University of Tennessee Health Science Center (UTHSC).

Ethics

Protocols for the mouse experiments were approved by the UTHSC IACUC.

Virus

Original stocks of mouse adapted A/Puerto Rico/8/34 (H1N1, PR8M) virus were obtained from Stefan Ludwig, University of Münster [25]. Virus stocks were propagated in the chorioallantoic cavity of ten-day-old pathogen-free embryonated chicken eggs for 48 h at 37 °C as described previously[26]. Viral titer was determined by focus-forming unit (FFU) assay as described previously [26].

Infection of mice

Females at 8–12 weeks-of-age were anesthetized by intraperitoneal injection of ketamine/xylazine (10%(v/v) of 100 mg/ml ketamine and 5%(v/v) of 20 mg/ml xylazine in 0.9% (w/v) NaCl) with a dose adjusted to the individual body weight (200 µl/20 g body weight). Infection was performed by intranasal application of virus solution in 20 µl sterile phosphate-buffered saline (PBS), with PR8M $2x10^3$ FFU.

RNA isolation and sequencing

Mice were sacrificed at 3 dpi and entire lungs were extracted. The lungs were immediately transferred to RNAlater solution (Qiagen), kept at 4 °C for one day and subsequently stored at –20 °C. RNA was isolated using Qiagen Midi Kit as described previously [27]. RNA quality was controlled on a 2100 Bioanalyzer Instrument (Agilent). 500 ng of total RNA was used to prepare libraries for sequencing using

the Lexogen SENSE mRNA-seq library kit for Ion Torrent. Libraries were amplified for 11 cycles as the final step of library preparation. Five-hundred nanograms of total RNA was used to prepare libraries for sequencing using the Lexogen SENSE mRNA-seq library kit for Ion Torrent. Libraries were amplified for 11 cycles as the final step of library preparation. Before sequencing, 1 µl aliquots of this material was pooled and sequenced on an Ion Torrent PGM 314 chip. Barcode quantification data from the PGM were used to balance the barcodes for final pooling before sequencing. Following this final pooling, the library pools were sized to a target size of 260bp on a Pippin Prep instrument using the 2 % Pippin agarose gel. The sized libraries were examined on an Agilent High Sensitivity DNA chip, quantified using real-time PCR, and used to prepare spheres using a One-Touch 2 device. These spheres were then sequenced on an Ion Torrent Proton sequencer with a P1 chip. On average, 67 million reads were obtained per sample.

Read mapping and gene expression quantification

RNAseq reads were quality-trimmed using Trim Galore [28] and then mapped to the mouse mm10 reference genome or to the influenza A virus PR8M genome using STAR [29]. Read counts were summarized for genes as features using the R-package Rsubread [30], normalized and log2 transformed using the R-package DESeq2 [31], and subsequently batch-corrected using the ComBat function of the R-package sva [32, 33]. For annotations of genes, ENTREZ-ID from Rsubread were matched to RefSeq annotations using R-package biomart [34]. Duplicate RefSeq annotations were given the extension _1, _2, etc.

Correlation analysis

The correlation analysis in this study, including gene-gene and gene-phenotype were conducted with Pearson correlation coefficient in R package. Pearson correlation P-value < 0.05 was used to report statistical significance.

Weighted gene co-expression network analysis (WGCNA)

WGCNA is a method aims to find co-expressed gene networks and explore the association between gene networks and phenotypes of interest, as well as hub genes in the network. In this study, gene co-expression networks was constructed using the WGCNA package in R [35] according to the recommended tutorials (https://horvath.genetics.ucla.edu/html/CoexpressionNetwork/Rpackages/WGCNA/Tutorials/), in which, the *Ace2* correlated genes (Pearson correlation P-value < 0.05) was used as the input. The network construction mainly includes the following four steps: (1) define the gene expression correlation matrix; (2) select the soft threshold β and transform the expression correlation matrix into an adjacency matrix; (3) convert the adjacent matrix to topologically overlapping matrix and then to the dissimilarity matrix; 4) hierarchical cluster the dissimilarity matrix to obtain a clustering tree and refine with dynamic hybrid cutting.

Gene set enrichment analysis

To understand the biological function and pathways involved in the gene set of interest, we performed Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway, and mammalian phenotype ontology (MPO) overrepresentation analysis on WebGestalt (http://www.webgestalt.org) [36] . This analysis was

based on hypergeometric statistic test which generate *P*-values and enrich rations to evaluate whether an annotated term/category/pathway is achieved statistical enrichment in the gene set rather than by chance. In this analysis, we use the mouse genome protein coding genes as the reference background and a minimum of five genes for a category was defined.

Expression quantitative trait loci (eQTL) mapping

eQTL mapping allows the identification of chromosomal regions that specifically modulate variability in levels of mRNAs or other molecules. We performed eQTL mapping of RNA-seq expression data from lung tissues of H1N1 virus-infected BXD. Much of the analysis was done using GeneNetwork (www.genenetwork.org) [37, 38]. Likelihood ratio statistics (LRS) scores were computed using the Haley-Knott equations [39], and genome-wide significance was based on 2000 permutations. We used the BXD genotype file generated January 2017 (www.genenetwork.org/webqtl/main.py?FormID=sharinginfo&GN_AccessionId=600) were used. In addition, Genes with significant and suggestive eQTLs detected using Haley and Knott mapping were remapped using GEMMA [40] to confirm LRS scores and locations of QTLs in GeneNetwork. GEMMA used linear mixed model mapping algorithms which can account for kinship among strains.

Genetic variations between C57BL/6J and DBA/2J

Sequence differences segregating in the BXDs are from our previous work [23]. Here, we focused exclusively on protein-coding variants—nonsense, missense, and frameshift mutations.

Bayesian network construction

The Bayesian Network Webserver (compbio.uthsc.edu/BNW) [41]was used to create network models to evalute plausible causal models among the genes, transcripts, and outcome measures of interest. Model were learned from the data with default settings. Model averaging of the 1000 highest scoring networks was performed, and directed edges with weights greater than 0.5 were included in the models. Finally, each variable had a maximum of four direct parents in any network model.

RNA-seq data for time-course H1N1 infection of the BXD parental strains

This lung RNA-seq data was previously generated in our laboratory. Detailed information can be found in [15]. In brief, two strains (B6 and D2) were infected with the H1N1 virus at 10–12 weeks of age with the methods mentioned above. The RNA was isolated from lung tissues for B6 at 1, 3, 5, 8, and 14 days post infection (dpi) and for D2 at 1, 3, and 5 dpi. Three RNA samples per strain per time point were used for library preparation and sequencing. The sequence reads were mapped to the mouse reference genome mm10. The raw read counts were extracted, normalized and log transformed with DEseq2 [31] after adding 1 to all raw counts.

In this study, we extracted the *Ace2* expression levels from this dataset. Also, we performed differential expression analysis between mice at 3 dpi and mock controls using DESeq2 [31] by treating strain as a covariates. The resulting *P*-values were adjusted using the Benjamini and Hochberg's approach [42]. We

reported genes with adj.p-value < 0.05 and fold change (FC) > 1.3 as differentially expressed genes (DEGs).

Microarray data for SARS-CoV infected primary human airway epithelial (HAE) cells

The experimental details for this study are described in [43] In brief, human tracheobronchial epithelial cells were obtained from airway specimens resected from patients undergoing surgery. Primary cells were expanded to generate passage 1 cells, and passage 2 cells were plated at a density of $2.5x10^5$ cells per well. Human airway epithelium (HAE) cultures were generated by provision of an air-liquid interface for 6 to 8 weeks to form well-differentiated, polarized cultures. Wild-type infectious clone-derived SARS-CoV (icSARS-CoV) and ic-SARS-CoV ORF6 were derived from the Baric laboratory's infectious clone constructs as described [43] Triplicate cultures were infected with xxxx icSARS-CoV, and icSARS-CoV ORF6 on the apical surface. Total RNA was isolated from infected cells and equivalent amounts of RNA from three biological replicates from each condition were pooled and microarray analysis was performed using Agilent 4x44K whole human gene expression microarrays. The raw data was downloaded from GEO as data matrix: GSE47963_series_matrix.txt.gz Expression values were normalized and sample descriptions quality controlled. A strong batch effect with respect to the donor from which HAE were derived was observed. Therefore, data were batch corrected for donor source using the R package "sva"[32, 33]. Rows with no gene name were deleted. Differentially expressed genes were determined using the R package Limma [45].

Data availability

The RNA-seq data from infected BXD strains has been deposited at GeneNetwork web site (www.genenetwork.org) with GN Accession number of GN807 which can be accessed through the dataset query "HZI Lung Flu Infected BXD (Nov16) RNA-Seq". RNAseq data for infection of B6 and D2 mice is available at the public expression database GEO (http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE66040. Array data from infected primary HAE cells is available at the public expression database GEO (http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE47963.

3 Results

3.1 *Ace2* expression level decreased in both C57BL/6J and DBA/2J after H1N1 infection

Previously, our group quantified lung transcriptomes with RNAseq technique for two mouse strains, B6 and D2, at 1, 3, 5, 8, and 14 dpi after H1N1 virus infection. Selection of those two inbred strains was based on the following two reasons. First, B6 is a strain resistant to H1N1 infection, while D2 is a highly susceptible strain. Second, these two are parental strains for BXD RI family, which served as a genetic reference panel for systems genetics analysis of complex traits, especially for studying gene-by-environment interactions.

In this study, we extracted the *Ace2* mRNA expression data from this dataset. Overall, the expression of *Ace2* in lungs was decreased progressively in both strains as H1N1 infection proceeded (**Figure 1**). In B6

mice *Ace2* transcript dipped to the lowest level on 8 dpi (Mean = 7.20, SD = 0.15) and remained at that low level on day 14 (Mean = 7.23, SD = 0.12). In addition, the expression levels at 3 dpi (*P*-value = 0.026, log2FC=-0.25), 5 dpi (*P*-value = 0.008, log2FC=-0.49), 8 dpi (*P*-value = 0.003, log2FC=-0.73), and 14 dpi (*P*-value = 0.002, log2FC=-0.70) were all significantly lower than that at 1 dpi. The infection was lethal for D2 mice at 5 dpi, when mice had the lowest expression of *Ace2* (Mean = 7.10, SD = 0.25). Statistically, *Ace2* expression levels on day 3 (*P*-value = 0.003, log2FC=-0.37) and 5 (*P*-value = 0.006, log2FC=-0.80) both showed significant reduction as compared with 1 dpi. Although the differences in *Ace2* expression between B6 and D2 at 3 dpi and 5 dpi did not achieve statistical significance, D2 mice consistently showed a more rapid decline in *Ace2* expression as compared with the B6 strain.

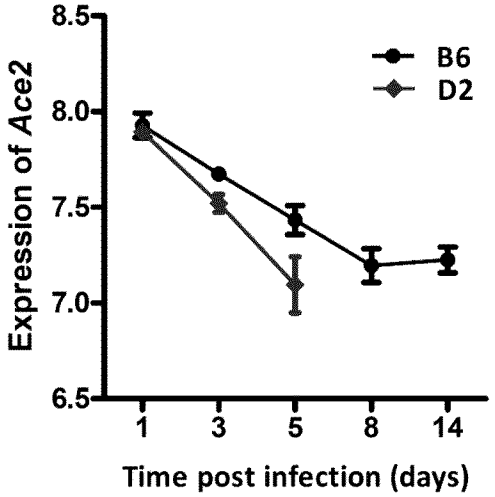


Figure 1. Line charts of the *Ace2* expression level in strains C57BL/6J and DBA/2J after H1N1 infection. Lung transcriptomes were quantified with RNA-seq for B6 and D2 strains at 1, 3, 5, 8, and 14 dpi of H1N1. Three female mice (8-12 weeks of age) per strain were used for each time point. The x-axis shows the time point (days) post infection. The y-axis shows the expression level of *Ace2* with log2 transformed values.

3.2 *Ace2* mRNA level exhibited variability across BXD RI mice at 3 dpi

All D2 mice died five days post infection. Therefore, we conducted RNAseq for a subset of BXD RI family of mice (41 BXD RI strains and two parental strains B6 and D2) at 3 dpi. As shown in **Figure 2**, *Ace2* transcript levels varied considerably across BXD strains with a difference of 1.49 between extremes. The average expression of *Ace2* across all BXD strains was 8.02±0.15 (log2 scale, Mean ± SD), with the BXD77 having the highest expression of 8.36 and the BXD22 having the lowest expression of 7.78.

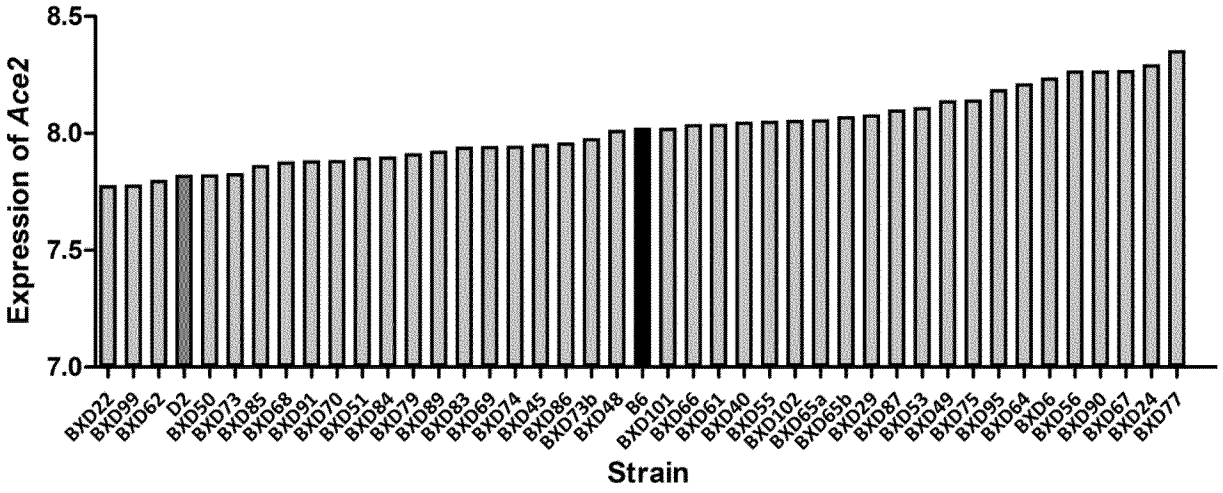


Figure 2. Bar plots of pulmonary *Ace2* expression levels across BXD RI mice at 3 dpi. Female mice (8-12 weeks of age) were anesthetized and infected by intranasal application of H1N1 virus inocula. Mice were sacrificed at 3 dpi. RNA was extracted from the lungs and sequenced on Ion Torrent Proton sequencer (one sample per strain). The x-axis shows BXD and two parental strains. The y-axis shows the normalized log2 expression levels of *Ace2*.

3.3 *Ace2* expression was associated with disease severity and viral load after infection

In order to investigate whether there is an association of *Ace2* expression with severity of disease and/or viral replication as the infection progresses, we conducted Pearson correlation coefficient analysis for *Ace2* levels against body weight loss, mean time to death, and viral RNA expression levels as a surrogate for viral load. Results demonstrated that *Ace2* expression was significantly correlated with both body weight loss ($r = 0.575$, *P-value* = 0.008, **Figure 3A**) and median body weight loss ($r = 0.468$, *P-value* = 0.028, **Figure 3B**) at 3 dpi. Further, *Ace2* was positively correlated with the mean time to death ($r = 0.458$, *P-value* = 0.032, **Figure 3C**). In contrast, *Ace2* had a negative correlation with to IAV NA gene expression levels ($r = 0.381$, *P-value* = 0.029, **Figure 3D**).

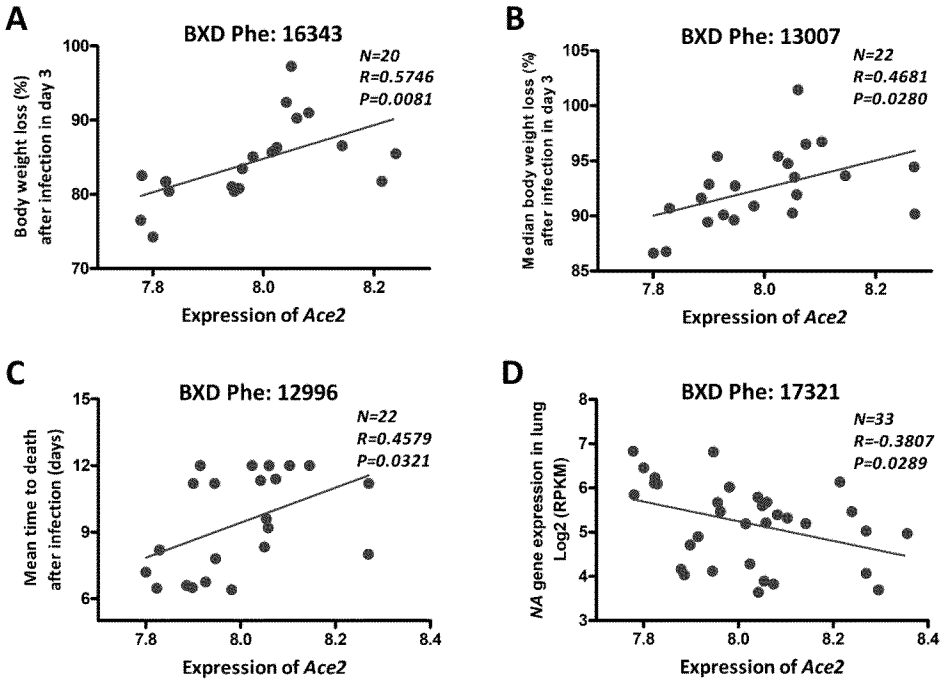


Figure 3. Scatter plots of the correlations of *Ace2* expression with body weight loss (A), median body weight loss (B), mean time to death (C), and viral *NA* gene expression level (D). Pearson correlation coefficient was used to determine the relationship. The percent body weight in **(A)** and **(B)** was relative to the starting body weight (100%). Gene expression levels represent normalized log2 values. The GeneNetwork accession number of the phenotypes (BXD Phe), number of samples (N), Pearson correlation R-value (R), and Pearson correlation *P*-value (P) are indicated in the figure.

3.4 *Ace2* correlated genes significantly involved in immune system-related pathways

To gain insight into the pulmonary pathways and biological functions that *Ace2* is involved in after the virus infection, we conducted Pearson correlation coefficient analysis for *Ace2* against to the whole lung transcriptome on GeneNetwork. This analysis returned 5500 genes that were significantly correlated with *Ace2* (Pearson correlation *P*-value < 0.05). Next, we submitted those genes to Webgestalt (http://www.webgestalt.org/). 5118 genes were unambiguously mapped to unique entrezgene IDs. Those genes were subsequently used for an enrichment analysis. The selected genes represented components of the TNF signaling pathway, MAPK signaling pathway, Notch signaling pathway, HTLV-I infection, and VEGF signaling pathway (**Figure 4A**). Furthermore, we also found that these genes were significantly enriched in terms related to chemokine secretion, lung morphology, and respiratory system morphology (**Figure 4B**).

For the top three enriched pathways, 48 genes were found in the TNF signaling pathway (**Supplementary Data 1**), with 35 negatively correlated with *Ace2* (Pearson *P*-value < 0.05). Among these included the tumor necrosis factor (*Tnf*, r = -0.41, *P*-value = 0.005). There were 92 genes in the MAPK signaling pathway (**Supplementary Data 1**), with *Tnf*, transforming growth factor beta 1(*Tgfb1*, r = -0.46, *P*-value = 0.002), and other 59 genes whose expression was

negatively correlated with *Ace2*. In addition, 25 genes were involved in the Notch signaling pathway (**Supplementary Data 1**), with 21 negatively correlated with *Ace2*.

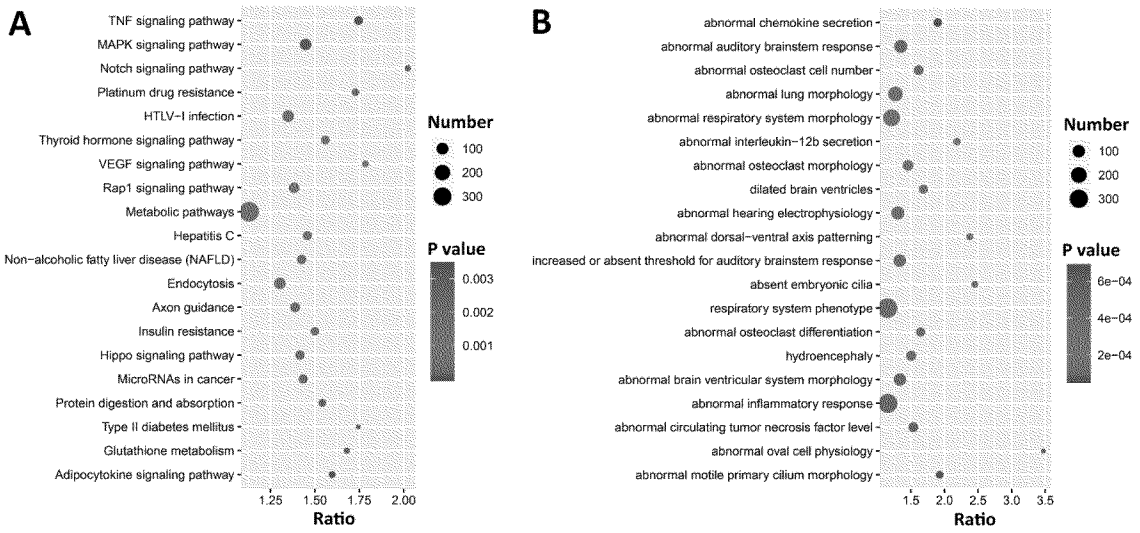


Figure 4. Bubble charts of the top 20 KEGG pathways (A) and 20 Mammalian Phenotype Ontologies (B) enriched for *Ace2* significantly correlated genes. Gene over-representation analysis for KEGG pathway and Mammalian Phenotype Ontology of the *Ace2* correlated genes (Pearson correlation *P*-value < 0.05) were performed with WebGestalt (http://www.webgestalt.org/). The x-axis represents the enrich ratio and y-axis represents enriched pathways/terms. The size of the dots denotes the number of genes and the color the *P*-value.

3.5 Co-expression network analysis narrowed down the *Ace2* correlated genes

To further identify genes that interact more directly with *Ace2*, we constructed gene co-expression network using WGCNA with the *Ace2* correlated genes (*P*-value < 0.05). The 5500 genes were parsed into 10 distinct co-expression modules using a soft-thresholding power of 8 (**Figure 5A-C**). The modules had a wide range of sizes, with the brown module containing 1673 genes and light cyan module containing only 68 genes. *Ace2* was located in the purple module that contained 462 genes (**Figure 5C**). Genes in this module were significantly involved in regulating motile cilium physiology (**Figure 5D**).

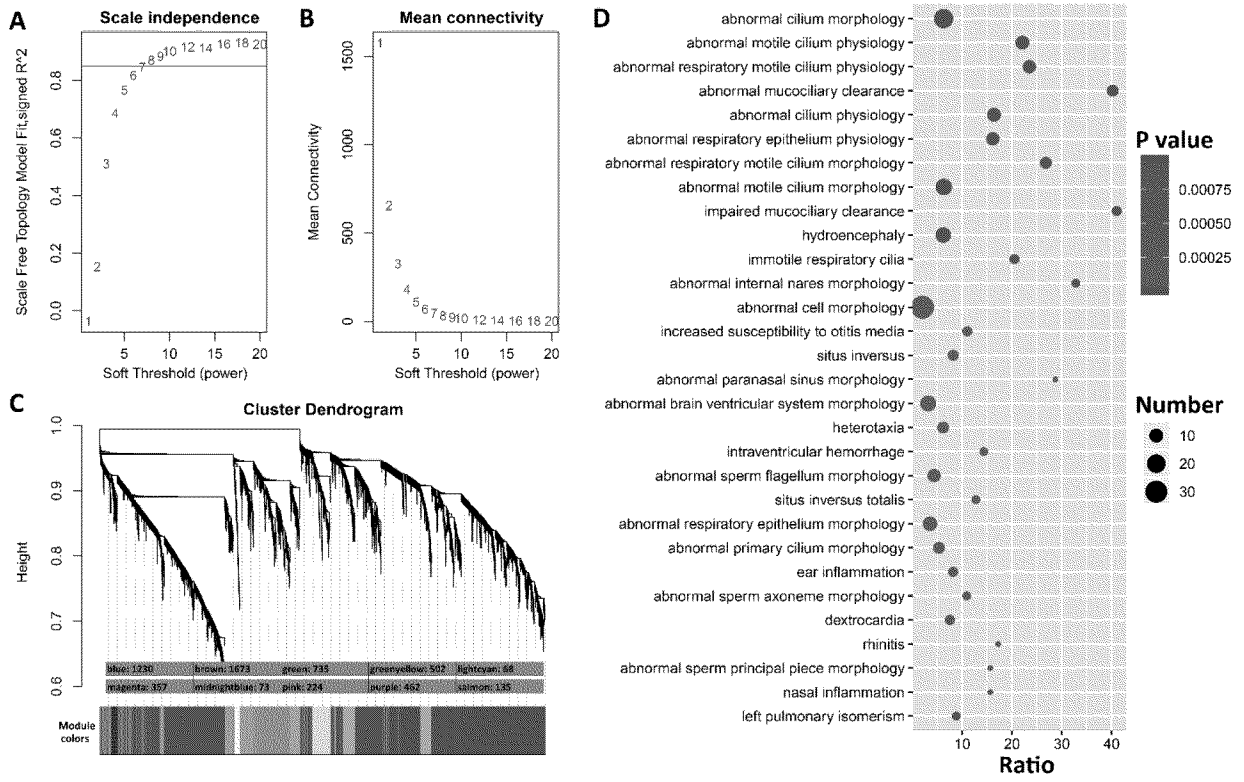


Figure 5. Weighted gene co-expression network analysis of *Ace2* correlated genes. (A) The soft thresholding index R^2 (y-axis) as a function of different powers β (x-axis) indicating the threshold of 8 chosen. **(B)** Mean connectivity (degree, y -axis) as a function of the soft-thresholding power (x -axis). **(C)** 10 co-expression modules identified from *Ace2* correlated genes. Modules are color-coded as shown. The number of genes in each module is also indicated. **(D)** Bubble charts of top 30 enriched Mammalian Phenotype Ontologies of the purple module. This analysis was performed with WebGestalt (http://www.webgestalt.org/).

Next, we focused on exploring the genes in the purple module (**Figure 6** and **Supplemental File 1**). First, we performed differential expression analysis between mice at 3 dpi and mock-infected controls by treating strain as a covariate. This led to identification of 281 genes (61% of the module genes) that showed differential expression (adj.p-value < 0.05 and FC > 1.3). Next, we conducted eQTL mapping using WebQTL on the GeneNetwork for the whole lung transcriptome which identified 23 genes that were *cis*-regulated and 106 others that were *trans*-regulated in H1N1-infected lungs. Those QTLs were further confirmed with GEMMA in GeneNetwork. Moreover, we searched for the DNA sequence differences between B6 and D2, which resulted in a total of 69 genes harbor coding sequence variants (missense, nonsense, and frameshift variants). Furthermore, we found that there were 42 genes implicated in cilium function and regulation identified by enrichment analysis. By comparing the above four categories, 32 cilium function related genes were differentially expressed (**Table 1**). We refer to this set as 'prioritized genes'.

Of these, *Sox11*, *Tff2*, *Rnf128*, and *Muc5b* showed *cis*-or *trans*-regulation, and *Tmprss2*, *Ulk4*, *Cckar*, *Wdpcp*, *Ift140*, and *Ccdc40* contained functional variants. The genetic perturbations of these 10 genes (**Table 1**) may act as key regulators in this module.

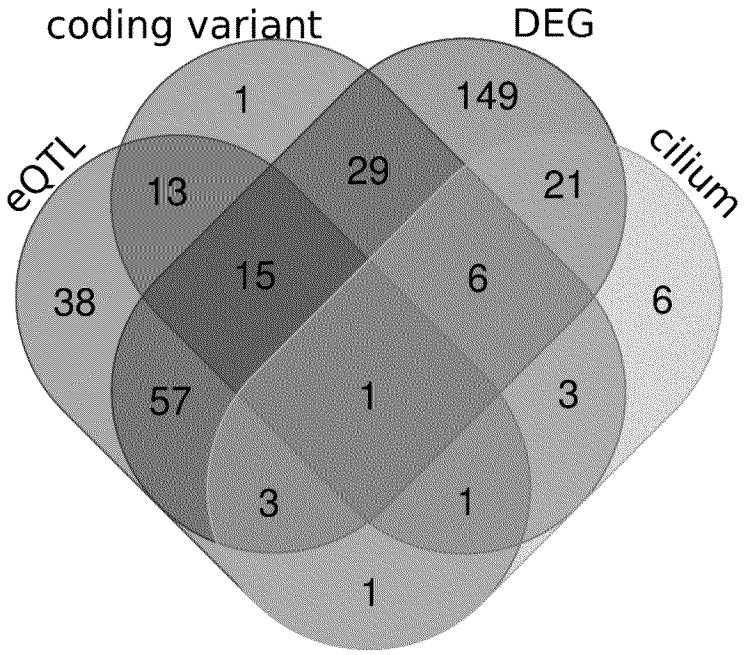


Figure 6. Venn diagrams of the purple module genes. A total of 462 genes were included in the purple module. Four criteria were applied to explore the module genes, including coding sequence variants between parental strain B6 and D2 (coding variant), eQTL (LRS > 15) regulation in H1N1 affected lung tissue (eQTL), cilium functional relevance (cilium), and differential expression (adj.p-value < 0.05 and FC > 1.3) between infected and normal controls in parental strains (DEG). The Venn diagram shows the number of overlapping genes among the four categories.

Table 1 Lists of prioritized genes in purple module.

Gene ID	Symbol	Description	Gene ID	log2FC	padj	eQTL	functional Variant
20666	**Sox11**	SRY-box 11	20666	-1.19	1.95E-12	cis	Nonsynonymous
21785	**Tff2**	trefoil factor 2 (spasmolytic protein 1)	21785	-1.01	5.38E-04	trans	NA
66889	**Rnf128**	ring finger protein 128	66889	-0.77	2.81E-05	trans	NA
74180	**Muc5b**	mucin 5, subtype B, tracheobronchial	74180	0.54	1.13E-02	trans	NA
50528	**Tmprss2**	transmembrane protease, serine 2	50528	-1.06	8.58E-10	NA	Nonsynonymous
209012	**Ulk4**	unc-51-like kinase 4 (C. elegans)	209012	-0.87	3.43E-07	NA	Nonsynonymous
12425	**Cckar**	cholecystokinin A receptor	12425	-0.73	1.91E-05	NA	Nonsynonymous
216560	**Wdpcp**	WD repeat containing planar cell polarity effector	216560	-0.69	2.36E-06	NA	Nonsynonymous
106633	**Ift140**	intraflagellar transport 140 homolog (Chlamydomonas)	106633	-0.68	3.50E-07	NA	Nonsynonymous
207607	**Ccdc40**	coiled-coil domain containing 40	207607	-0.66	1.06E-03	NA	Nonsynonymous
320752	Dpy19l2	dpy-19-like 2 (C. elegans)	320752	-1.81	2.13E-03	NA	NA
51938	Ccdc39	coiled-coil domain containing 39	51938	-1.30	1.15E-13	NA	NA
244653	Hydin	hydrocephalus inducing	244653	-1.30	8.51E-16	NA	NA

74362	Spag17	sperm associated antigen 17	74362	-1.29	9.27E-13	NA	NA
73873	Fam161a	family with sequence similarity 161, member A	73873	-1.28	1.70E-09	NA	NA
320662	Casc1	cancer susceptibility candidate 1	320662	-1.27	2.44E-08	NA	NA
66722	Spag16	sperm associated antigen 16	66722	-1.27	1.61E-10	NA	NA
74934	Armc4	armadillo repeat containing 4	74934	-1.21	8.50E-09	NA	NA
18703	Pigr	polymeric immunoglobulin receptor	18703	1.18	1.10E-09	NA	NA
69707	Iqcg	IQ motif containing G	69707	-1.18	2.97E-20	NA	NA
78801	Ak7	adenylate kinase 7	78801	-1.17	4.75E-09	NA	NA
27078	B9d1	B9 protein domain 1	27078	1.09	1.00E-02	NA	NA
68922	Dnaic1	dynein, axonemal, intermediate chain 1	68922	-1.07	3.67E-08	NA	NA
226265	Eno4	enolase 4	226265	-0.98	1.49E-09	NA	NA
71877	Efhc1	EF-hand domain (C-terminal) containing 1	71877	-0.92	3.77E-11	NA	NA
75533	Nme5	expressed in non-metastatic cells 5	75533	-0.82	5.92E-05	NA	NA
75050	Kif27	kinesin family member 27	75050	-0.71	3.05E-03	NA	NA
70008	Ace2	angiotensin I converting enzyme (peptidyl-dipeptidase A) 2	70008	-0.61	9.88E-05	NA	NA
20878	Aurka	serine/threonine kinase 6	20878	0.60	2.36E-02	NA	NA
22062	Trp73	transformation related protein 73	22062	-0.60	1.81E-03	NA	NA
77609	Ccdc151	coiled-coil domain containing 151	77609	-0.47	9.85E-02	NA	NA
252973	Grhl2	grainyhead-like 2 (Drosophila)	252973	-0.44	5.36E-04	NA	NA

Genes that were differentially expressed (adj.p-value < 0.05 and FC > 1.3) and implicated in cilium biological functions; Highlighted in bold are those 10 genes that may act as key regulators in the purple module; NA, not available.

3.6 Bayesian network analysis inferred upstream regulators of *Ace2*

In order to infer the causative relations of *Ace2* with the top 10 prioritized genes, we performed Bayesian network analysis. Ring finger protein 128 (*Rnf128*) stood out as the upstream regulator of the other 10 genes (**Figure 7**). This gene was predicted to directly target the genes intraflagellar transport 140 homolog (*Ift140*), unc-51-like kinase 4 (*Ulk4*), coiled-coil domain containing 40 (*Ccdc40*), SRY-box 11 (*Sox11*), and *Ace2*. In addition, *Ace2* was also found to be targeted through another pathway, which begins with the expression of *Rnf128,* then the expression of *Ulk4, Wdpcp*, and, finally, to *Ace2* (**Figure 7**).

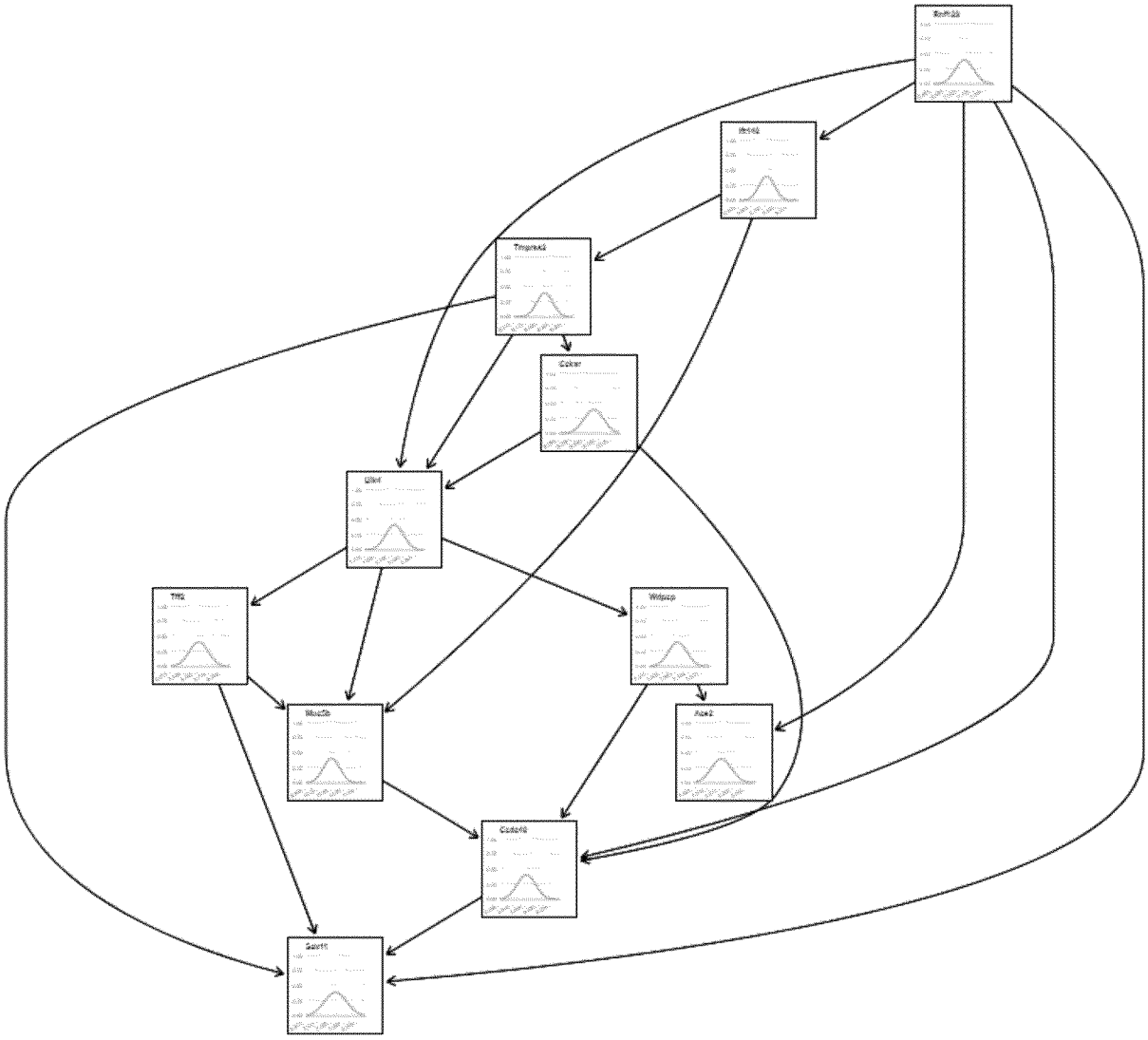


Figure 7. **Bayesian network analysis inferred upstream regulators of *Ace2***. The Bayesian network structure was constructed on Bayesian Network Webserver ((compbio.uthsc.edu/BNW) with gene expression values as the input. http://bnw.genenetwork.org/BNW/sourcecodes/layout.php?My_key=NLq

3.7 The differential expressed cilium function related genes in the purple module were replicated in SARS-CoV-infected HAE cells

In order to further evaluate whether those 'prioritized genes' (**Table 1**) were also regulated in humans, especially in the context of SARS-CoV infection, we downloaded a microarray data set for HAE cells infected with SARS-CoV (icSARS-CoV), ic-SARS-CoV ORF6 and BAT-SARS_CoV [43]. We found that very few of those 'prioritized genes' were significantly regulated after infection with BAT-SARS

(**Figure 8A**). However, most of the 'prioritized genes' were differentially expressed in icSARS-CoV and ic-SARS-CoV ORF6 infected HAE cells when compared to mock controls, with 21 genes found at 96h after infection (**Figure 8A**). More importantly, those overlapping genes, except *Ace2*, exhibited the same up- or down-regulating patterns in both HAE cultures and our mouse model (**Table 1** and **Figure 8B**). *ACE2* was up-regulated in HAE cells whereas in whole lungs it was found to be down-regulated. This is an important observation since in the HAE cultures only airway epithelial cells are present, but no interstitial cells, endothelial cells, resident and infiltrating immune cells are present. These observations indicate that in the lungs a more complex regulatory network is involved in the regulation of *ACE2* than in isolated epithelial cells and that it is indeed downregulated in all cells after infection in the whole lung. Alternatively, *ACE2* may become up-regulated only in infected cells (almost all cells in HAE cultures). In the lungs infected cells represent only a minority of all cells present. Since the our lung RNAseq study investigated total gene expression changes of all cell types it is well conceivable that we do not detect a possible up-regulation in the few infected cells. In future studies, it would be important to analyze *Ace2* expression in infected mouse lungs to test these hypotheses.

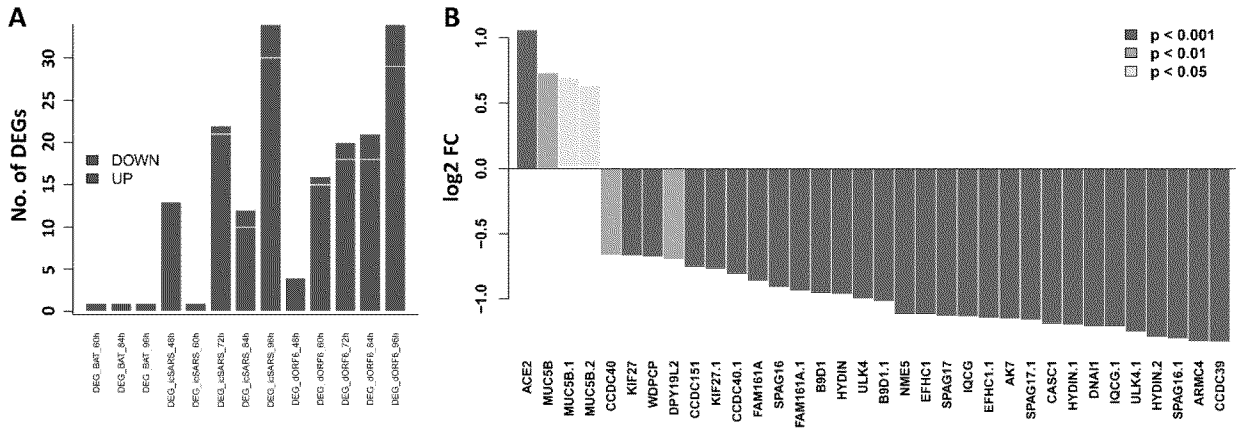


Figure 8. Bar plots of the cilium function related genes in the SARS-CoV infected HAE cells. Primary human HAE cells were infected with xxx SARS-CoV (icSARS-CoV), ic-SARS-CoV ORF6 and BAT-SARS_CoV and gene expression was analyzed by arrays. (A) Number of up-and downregulated differentially expressed genes for cilium function related genes that had orthologous genes in humans and were represented on the array (logFC > 0.58 and adjusted p-value < 0.05); y-axis: number of differentially expressed genes. (B) LogFC differences in expression values between HAE cells infected with icSARS-CoV at 96h pi and mock-treated controls for cilium function related genes that had orthologous genes in humans and were represented on the array; y-axis: differences in logFC expression levels between groups. Adjusted p-values were taken from the Limma analysis of the entire transcriptome data.

Discussion

Many studies have demonstrated that host genetic background significantly influences the susceptibility to

influenza A infections in humans [46]. This phenomenon was also observed in mouse models [14], especially for inbred strains B6 and D2 [15]. In this study, we systemically analyzed the potential regulatory role of *Ace2* for the host response to H1N1 infection in a subset of BXD RI mice. Our results showed that the expression of *Ace2* in lung significantly decreased in a disease severity- and time-dependent manner. In addition, we also found the expression variation of *Ace2* negatively correlated with the body weight loss and survival time after the infection. These results strongly suggest that *Ace2* is involved in H1N1 virus disease progression and that the lower levels of *Ace2* may play a role in virus-induced ALI. This notion was supported in studies of *Ace2*$^{-/-}$ mice infected with influenza H7N9 [47] and with respiratory syncytial virus [48] where lung pathology was more severe in mutant mice.

These observations argue strongly that *Ace2* can mediate virus-induced ALI [47, 48]. However, the underlying mechanisms remain elusive. By using 43 inbred strains, we were able to perform genetic correlation analysis and gain insight into the *Ace2*-associated biological pathways. We identified a total of ~5000 *Ace2*-correlated genes. These genes are involved in regulating respiratory and inflammatory networks through several pathways, including TNF signaling pathway, MAPK signaling pathway, and Notch signaling pathway.

The factors associated with H1N1 virus-induced lethality mainly consist of virus-induced inflammatory cytokine storm and tissue destruction. Tumor necrosis factor (*TNF*) is a cytokine with multiple effects, including promoting cell growth, differentiation, apoptosis, and inducing inflammation [49, 50]. *TNF-α*, a member of the *TNF* family, can activate caspase protease, JNK, and transcription factor NF-κB, thereby executing its biological functions such as cytotoxicity, antiviral, and immuno-regulation. A previous study has demonstrated that inhibition of *Tnf-α* can protect mice from lethal H1N1 infection [51]. In the present study, we found *Ace2* was negatively correlated (r = -0.41 and *P*-value = 0.005) with the expression of *Tnf* and other 34 TNF signaling pathway related genes, suggesting that *Ace2* could dampen excessive inflammatory immune responses through downregulating this signaling pathway and thus mitigating immune-mediated tissue destruction. We also found 92 genes in the MAPK signaling pathway significantly correlated with *Ace2*, including *Tgfb1* (r = -0.46 and *P*-value = 0.002). Of note, it was shown that IAV can activate this pathway to support its replication [52]. The authors proposed a model in which *Tgfb1* acts as a pro-viral host factor by suppressing early immune responses, creating an enviroment conducive for IAV infection [53]. Moreover, another set of 25 *Ace2*-correlated genes were involved in the Notch signaling pathway, with most of them (21 genes) showing negative correlations. This pathway is an important bridge between antigen-presenting cells (APCs) and T cell communication circuits and plays a central role in driving the immune system to overcome infectious disease [54].

By constructing gene co-expression network, we narrowed the *Ace2* co-expressed genes to 462 (purple module). Enrichment analysis revealed that those genes were significantly related to cilium function, including *Ace2* itself. This finding suggests that *Ace2* participates in ALI not only by interacting with the

TNF, MAPK, and Notch signaling pathways, but also through regulating functions of respiratory epithelium, which is a barrier to microbial infection and tissue injury by the secretion of mucus and the action of mucociliary clearance. In order to identify the key regulators in the purple module, we employed multi-layer filtration, including differential expression analysis, eQTL mapping, functional variant identification, and gene annotation. This resulted in 10 high priority candidates, i.e., *Sox11*, *Tff2*, *Rnf128*, *Muc5b*, *Tmprss2*, *Ulk4*, *Cckar*, *Wdpcp*, *Ift140*, and *Ccdc40*. To further investigate the relationship of these genes with *Ace2*, we performed Bayesian network analysis and identified *Rnf128* as the master regulator. This gene encodes a ring-finger-containing protein which has been shown to possess E3 ubiquitin ligase activity [55]. *RNF128* has been implicated in the induction of T cell anergic phenotype through inhibiting activation-induced IL2 and IL4 cytokine production [56] and mediating T cell receptor-CD3 degradation [57]. In a recent study, Song *et al*. [58] reported *RNF128* promotes lysine-63-linked ubiquitination and activation of a critical kinase, TBK1, thereby augmenting the induction of type I interferon antiviral cytokines that curb viral replication. The authors' in vivo experiments further showed that *Rnf128* deficient mice were more susceptible to vesicular stomatitis virus (an RNA virus) and herpes simplex virus (a DNA virus). Our results suggest that there are two potential gene regulatory paths for *Ace2*, both of which begin with *Rnf128*. However, direct connection of the two genes will need to be investigated experimentally in future studies.

Besides the master regulator *Rnf128*, other genes were also predicted to regulate the immune and respiratory epithelium systems. For instance, *MUC5B* (mucin 5, subtype B, tracheobronchial), encodes a member of the mucin family of proteins, and is associated with idiopathic pulmonary fibrosis in humans [59]. Overexpression of this gene in mice causes mucociliary dysfunction and enhances lung fibrosis [60]. It was reported to be required for airway defense in another study [61]. *CCDC40* (coiled-coil domain containing 40) encodes a protein that is essential for motile cilia function. According to the OMIM (https://omim.org/) database, this gene is associated with recurrent respiratory infections (HP:0002205) and abnormal axonemal organization of respiratory motile cilia (HP:0012258). Malfunction of *Ccdc40* in mice resulted in left pulmonary isomerism and lung situs inversus [62]. *TMPRSS2* (transmembrane protease, serine 2) encodes a protein that belongs to the serine protease family. *Tmprss2* knockout mice show reduced lung inflammation following IAVs (H1N1, H3N2, or H7N9) infection and exhibit decreased susceptibility to virus-induced morbidity/mortality [63]. Studies also demonstrated that SARS coronavirus engages ACE2 as the entry receptor on cell surface (Ref 9: Li W et al, Nature. 2003; 426:450-4.) and employs the TMPRSS2 for Spike protein priming, which facilitates viral entry and infection [64, 65]. Besides, *Ulk4*, *Sox11*, and *Tff2* are associated with abnormal respiratory motile cilium [66], pulmonary hypoplasia [67], and abnormal adaptive immunity [68], respectively.

The ongoing outbreak of the COVID-19 caused by SARS-CoV-2 poses a global public health threat [69-71]. The entry of SARS-CoV-2 into host cells is dependent on ACE2 [72, 73], the receptor for SARS-CoV and can be blocked by a clinically approved TMPRSS2 protease inhibitor [73]. However, how *ACE2* interacts with other genes involved in the progression of SARS-CoV-2 induced respiratory disease remains to be elucidated. The first report based on autopsy samples demonstrated that the pathological characteristics of COVID-19 are very similar to those of SARS-CoV and MERS-CoV infected patients

[74]. Therefore, we looked up those 30 prioritized genes (**Table 1**), co-expressed with *Ace2* after H1N1 infection, in SARS-CoV-infected primary human HAE cells. This resulted in 20 out of them showing consistent up/down-regulation with our H1N1 model, suggesting the two viruses share common *Ace2* regulatory networks that participate in the occurrence and development of lung pathology.

In summary, we have shown the expression of *Ace2* was significantly downregulated in the lungs of IAV H1N1-infected mice. By using BXD RI strains, we were able to identify *Ace2* co-expressed genes with genetic correlation analysis and weighted gene co-expression network analysis. Our results indicate that *Ace2* participates in virus-induced respiratory diseases not only through immune system related pathways, but also by protective functions in the respiratory epithelium. In addition, *Rnf128* was identified as the master regulator through Bayesian network analysis. Finally, 21 of our prioritized genes were found to be also differentially expressed in SARS-CoV-infected HAE cells. These findings provide an *Ace2* regulatory network and raise several potential therapeutic targets for virus-induced (H1N1, SARS-CoV, MERS-CoV, and SARS-CoV-2) respiratory diseases. They additionally illustrate that the BXD RI strains provide an excellent resource for studying the host genetic regulatory mechanisms of viral infections, such as that caused by SARS-CoV-2.

Acknowledgement

This work was supported by an intra-mural grant from the Helmholtz-Association (Program Infection and Immunity), a start-up grant from the University of Tennessee Health Science Center, a NIH/NIAID Research Grant U19-AI100625 to KS, and NIH/NIAID Research Grants U19-AI100625, U01 AI149644, and U19 AI 109761 to R.S.B. RNA sequencing has been performed by the Molecular Resource Center (MRC) at UTHSC, Memphis.

Author Contributions

LL, KS and RSB conceived the study; SB and ACS conducted the experiments; FX and KS performed data analysis; LL, FX, KS, and JG wrote the manuscript. FX, KS, and JG prepared the figures and tables; RWW and KL edited the manuscript; all authors read and approved the final version of the manuscript for publication.

Conflicts of Interest

The authors declare no conflicts of interests.

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From: Lu, Lu[llu@uthsc.edu]
Sent: Sun 3/29/2020 6:02:23 AM (UTC-07:00)
To: Li, Kui[kli1@uthsc.edu]
Cc: Klaus x Klaus Schughart[Klaus.Schughart@helmholtz-hzi.de]; Williams, Robert W[rwilli10@uthsc.edu]; Xu, Fuyi[fxu10@uthsc.edu]; Sims, Amy C[amy.sims@pnnl.gov]; rbaric@email.unc.edu[rbaric@email.unc.edu]; silke.bergmann@(b) (6) [silke.bergmann@(b) (6)]
Subject: Re: [Ext] B6D2 infected RNAseq data

Thanks, Kui. We will integrate your editing into our final version.

On Mar 28, 2020, at 11:25 PM, Li, Kui <kli1@uthsc.edu> wrote:

> Dear Lu, Klaus, Rob, and team,
>
> Please find my edits of the manuscript. Use as you see fit. Thanks,
>
> Kui

From: Lu, Lu <llu@uthsc.edu>
Sent: Thursday, March 26, 2020 6:05 PM
To: Klaus x Klaus Schughart <Klaus.Schughart@helmholtz-hzi.de>
Cc: Williams, Robert W <rwilli10@uthsc.edu>; Xu, Fuyi <fxu10@uthsc.edu>; amy.sims@pnnl.gov <amy.sims@pnnl.gov>; rbaric@email.unc.edu <rbaric@email.unc.edu>; silke.bergmann@(b) (6) <silke.bergmann@(b) (6) >; Li, Kui <kli1@uthsc.edu>
Subject: Re: [Ext] B6D2 infected RNAseq data

Dear Klaus (and all others):
Here is updated version. Fuyi addressed most of your comments. Please feel free to modify it continuously.

Dear Amy and Ralph:

Some of Klaus' comment are specific to you. Could you help to reply or editing?

Thanks.

Lu Lu
Professor
Department of Genetics, Genomics and Informatics
University of Tennessee Health Science Center
71 S. Manassas St., Room 410K
Memphis, TN 38163
Tel: (901) 448-7557 (O)
Fax: (901) 448-3500 (Dept)

On Mar 26, 2020, at 1:18 PM, Schughart, Klaus <Klaus.Schughart@helmholtz-hzi.de> wrote:

Hi LuLu,
enclosed are my additional comments – now also on the discussion.
Maybe Amy has also some ideas to explain the discrepancy between lungs and HAE cells with respect to inverse up/down regulation.
Klaus

Von: Lu, Lu <llu@uthsc.edu>
Gesendet: Donnerstag, 26. März 2020 00:27
An: Schughart, Klaus <Klaus.Schughart@helmholtz-hzi.de>
Cc: Williams, Robert W <rwilli10@uthsc.edu>; Xu, Fuyi <fxu10@uthsc.edu>; amy.sims@pnnl.gov
Betreff: Re: [Ext] B6D2 infected RNAseq data

Dear Klaus:

We have accepted Rob, you and our previous editing. Here is updated version for you to check and modify continuously.

Dear Amy:

Could you check this version and address some comments that are related to you?

Thanks.

Lu Lu
Professor
Department of Genetics, Genomics and Informatics
University of Tennessee Health Science Center
71 S. Manassas St., Room 410K
Memphis, TN 38163
Tel: (901) 448-7557 (O)
Fax: (901) 448-3500 (Dept)

> On Mar 25, 2020, at 3:18 PM, Xu, Fuyi <fxu10@uthsc.edu> wrote:
>
> Dear Klaus,
>
> Could you please send me those two images (high-resolution) in Figure 8 ?
> Thank you very much.
>
> --
> Fuyi Xu, PhD
> Department of Genetics, Genomics, and Informatics
> University of Tennessee Health Science Center
>
> 415 TRANSLATIONAL RESEARCH BUILDING
> 71 SOUTH MANASSAS

MEMPHIS TN 38163
Phone: (901)246-4398
E-mail: 5 U.S.C. § 552(b)(6) fxu10@uthsc.edu

From: Schughart, Klaus
Sent: Wednesday, March 25, 2020 3:04 PM
To: Lu, Lu
Cc: Xu, Fuyi; Williams, Robert W
Subject: AW: [Ext] B6D2 infected RNAseq data

Hi LuLu,
I forgot something in the Acknowledgements.
Please replace the Acknowledgements in the last version with the one in the enclosed version.
Thx,
Klaus

Von: Schughart, Klaus
Gesendet: Mittwoch, 25. März 2020 20:31
An: 'Lu, Lu' <llu@uthsc.edu>
Cc: 'Xu, Fuyi' <fxu10@uthsc.edu>; 'Williams, Robert W' <rwilli10@uthsc.edu>
Betreff: AW: [Ext] B6D2 infected RNAseq data

Dear all,
here are my comments on the revised version. I took out the previous comments that have been resolved.
Fuyi, I also enclose the data table for Figure 8A.
Once you are done with this version, I can have a look at the discussion and then you could send it off to the other co-authors.
Klaus

Von: Schughart, Klaus
Gesendet: Mittwoch, 25. März 2020 19:31
An: 'Lu, Lu' <llu@uthsc.edu>
Cc: Xu, Fuyi <fxu10@uthsc.edu>; Williams, Robert W <rwilli10@uthsc.edu>
Betreff: AW: [Ext] B6D2 infected RNAseq data

Thanks – will work it.
Klaus

Von: Lu, Lu <llu@uthsc.edu>
Gesendet: Mittwoch, 25. März 2020 19:02
An: Schughart, Klaus <Klaus.Schughart@helmholtz-hzi.de>
Cc: Xu, Fuyi <fxu10@uthsc.edu>; Williams, Robert W <rwilli10@uthsc.edu>
Betreff: Re: [Ext] B6D2 infected RNAseq data

Dear Klaus:

Fuyi and I accept all of you and rob's editing. We also clean and modified the paper, which addressed comments and answered the your questions. Please work on the updated version continuously. Hopefully we can submit this paper ASAP. Thanks.

Lu Lu
Professor
Department of Genetics, Genomics and Informatics
University of Tennessee Health Science Center
71 S. Manassas St., Room 410K
Memphis, TN 38163
Tel: (901) 448-7557 (O)
Fax: (901) 448-3500 (Dept)

On Mar 24, 2020, at 4:36 PM, Lu, Lu <llu@uthsc.edu> wrote:

Thanks, Rob. We will modify based on your comments and will send you updated version for your editing again.

Lu Lu
Professor
Department of Genetics, Genomics and Informatics
University of Tennessee Health Science Center
71 S. Manassas St., Room 410K
Memphis, TN 38163
Tel: (901) 448-7557 (O)
Fax: (901) 448-3500 (Dept)

On Mar 24, 2020, at 4:14 PM, Rob Williams <rwilliams@uthsc.edu> wrote:

Dear team,

Brilliant effort,

Edited the abstract heavily (please review carefully) and built up the Methods section on BXDs a bit.

Have not gotten through all of paper, but looks great so far.

Should validate some networks and eQTLs with GEMMA.

Needs a bit more clarity to data sources—some for 43 strains, other data for parents. These details needed (imho) even in the abstract.

On Tue, Mar 24, 2020 at 2:41 PM Schughart, Klaus <Klaus.Schughart@helmholtz-hzi.de> wrote:

Hi Lulu, Fuyi,

enclosed are my comments, additions and corrections.

Looks very good!!!

Please go through and clean it up first (accept or discard changes, add information) and then I can continue to work on the cleaned-up version. Currently it is too much cluttered with all these changes and comments.

Fuyi, let me know what exactly you need in addition for the HAE data.

Klaus

Von: Schughart, Klaus
Gesendet: Freitag, 6. März 2020 19:02
An: Xu, Fuyi <fxu10@uthsc.edu>; Lu Lu **5 U.S.C. § 552(b)(6)**
Cc: Rob Williams <rwilliams@uthsc.edu>; Jonsson, Colleen Beth (cjonsson@uthsc.edu) <cjonsson@uthsc.edu>
Betreff: AW: [Ext] B6D2 infected RNAseq data

Hi Fuyi, LuLu,

I did some extensive analyses of genes in the human HAE data set with your gene list. Enclosed are the results. Looks very good!!

For a publication, we would have to discuss which data to include and how to present the results.

Klaus

Von: Schughart, Klaus <Klaus.Schughart@helmholtz-hzi.de>
Gesendet: Donnerstag, 5. März 2020 04:45
An: Xu, Fuyi <fxu10@uthsc.edu>
Cc: Lu Lu **5 U.S.C. § 552(b)(6)**
Betreff: Re: [Ext] B6D2 infected RNAseq data

Dear Fuyi,

sure I will have a look at these genes.

Klaus

Von meinem iPhone gesendet

Am 04.03.2020 um 19:09 schrieb Xu, Fuyi <fxu10@uthsc.edu>:

Dear Klaus,

Lu and I are working on the Ace2 paper. We prioritized around 30 genes that are correlated with Ace2 (attached table). I am wondering if you can help me check the expression differences between infected and normal controls in the human data set last time you showed us, especially for those color highlighted. Also, is this gene list make sense from your perspective? Thank you very much.

Best,

--
Fuyi Xu, PhD
Department of Genetics, Genomics, and Informatics
University of Tennessee Health Science Center

415 TRANSLATIONAL RESEARCH BUILDING
71 SOUTH MANASSAS
MEMPHIS TN 38163
Phone: (901)246-4398
E-mail: **5 U.S.C. § 552(b)(6)** fxu10@uthsc.edu

From: Schughart, Klaus
Sent: Friday, February 28, 2020 11:07 AM
To: Xu, Fuyi
Cc: Lu Lu
Subject: AW: [Ext] B6D2 infected RNAseq data

Hi LuLu, Fuyi,

I just remembered that I also have expression data from infected human lung slices (see enclosure).

This dataset has been published (enclosed).

Klaus

Von: Schughart, Klaus
Gesendet: Montag, 24. Februar 2020 21:00
An: 'Xu, Fuyi' <fxu10@uthsc.edu>
Cc: Lu Lu; 5 U.S.C. § 552(b)(6)
Betreff: AW: [Ext] B6D2 infected RNAseq data

Dear Fuyi,

The data I sent you before (RNAseq_norm1.txt) have been normalized and log2 transformed using DESeq2 (rlogTransformation(dsDat, blind=TRUE).

Please find enclosed the raw counts for the B6/D2 infections from Wilk et al (all_mouse_counts.txt).

For the BXD data in GN:

The percent body weights are NOT the losses but the percent body weight with reference to the starting body weight (100%). So 105% means that the mouse has gained 5% of body weight relative to its starting weight when it was infected. Mice with percent body weight less than 100% have lost weight.

Klaus

Von: Xu, Fuyi <fxu10@uthsc.edu>
Gesendet: Sonntag, 23. Februar 2020 22:14
An: Schughart, Klaus <Klaus.Schughart@helmholtz-hzi.de>
Cc: Lu Lu; 5 U.S.C. § 552(b)(6)
Betreff: RE: [Ext] B6D2 infected RNAseq data

Dear Dr. Klaus Schughart,

I am Lu Lu's postdoc, Fuyi Xu.

Thank you for sending us the normalized RNA-seq data.

We would like to perform differential expression analysis, I am wondering if you have raw read count for this data set since the DEseq2 package depend on this kind of data matrix. By the way, is the normalized data log2 transformed ?

We also found the expression level of ACE2 across the BXD is significantly correlated with median weight loss at day 3 after infection (ID:13007), however, we found BXD65a loss 105% body weight. Is this correct? Or how should I interpret ?

<A54B14D6A4584F84B57E3AAA25DC5BBB.gif>

Best Regards,

--
Fuyi Xu, PhD
Department of Genetics, Genomics, and Informatics
University of Tennessee Health Science Center

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71 SOUTH MANASSAS
MEMPHIS TN 38163
Phone: (901)246-4398
E-mail: 5 U.S.C. § 552(b)(6) fxu10@uthsc.edu

From: Lu, Lu
Sent: Monday, February 17, 2020 11:03 AM
To: Xu, Fuyi
Subject: Fwd: [Ext] B6D2 infected RNAseq data

Lu Lu
Professor
Department of Genetics, Genomics and Informatics

University of Tennessee Health Science Center
71 S. Manassas St., Room 410K
Memphis, TN 38163
Tel: (901) 448-7557 (O)
Fax: (901) 448-3500 (Dept)

Begin forwarded message:

From: "Schughart, Klaus" <Klaus.Schughart@helmholtz-hzi.de>

Subject: [Ext] B6D2 infected RNAseq data

Date: February 17, 2020 at 10:11:55 AM CST

To: "Lu, Lu" <llu@uthsc.edu>

Hi LuLu,

I checked the datasets.

GN807; RNAseq BXD infected is fine. This dataset has not been published yet.

I enclose the log2 normalized matrix for the B6/D2 infected data from Wilk et al. 2015:

RNAseq_norm1.txt -- for the mouse genes

00_influenza_lg2_RPKM_kls_280714.txt -- for the influenza gene segments

Let me know if you have any questions.

Klaus

Prof. Dr. Klaus Schughart
Head of Dept. of Infection Genetics
Helmholtz Centre for Infection Research,

University of Veterinary Medicine Hannover,

University of Tennessee Health Science Center
Inhoffenstr. 7
D-38124 Braunschweig
Germany

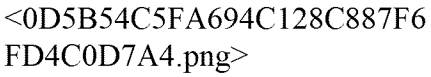
E-mail : Klaus.Schughart@helmholtz-hzi.de
Phone: +49 531 6181 1100

<B59D99096FF44A7EBCC7A3BC50D960A2.png>

Helmholtz-Zentrum für Infektionsforschung GmbH | Inhoffenstraße 7 | 38124 Braunschweig | www.helmholtz-hzi.de

Vorsitzende des Aufsichtsrates: Frau MinDir'in Prof. Dr. Veronika von Messling
Stellvertreter: MinDirig Rüdiger Eichel, Niedersächsisches Ministerium für Wissenschaft und Kultur
Geschäftsführung: Prof. Dr. Dirk Heinz;

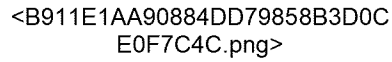
Silke Tannapfel
Gesellschaft mit
beschränkter Haftung
(GmbH)
Sitz der Gesellschaft:
Braunschweig
Handelsregister:
Amtsgericht
Braunschweig, HRB
477



Helmholtz-Zentrum für
Infektionsforschung GmbH |
Inhoffenstraße 7 | 38124
Braunschweig | www.helmholtz-
hzi.de

Vorsitzende des Aufsichtsrates: Frau
MinDir'in Prof. Dr. Veronika von
Messling
Stellvertreter: MinDirig Rüdiger
Eichel, Niedersächsisches
Ministerium für Wissenschaft und
Kultur
Geschäftsführung: Prof. Dr. Dirk
Heinz; Silke Tannapfel
Gesellschaft mit beschränkter
Haftung (GmbH)
Sitz der Gesellschaft: Braunschweig
Handelsregister: Amtsgericht
Braunschweig, HRB 477

<Table 1.xlsx>



Helmholtz-Zentrum für
Infektionsforschung GmbH |
Inhoffenstraße 7 | 38124
Braunschweig | www.helmholtz-
hzi.de

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Eichel, Niedersächsisches

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und Kultur
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Haftung (GmbH)
Sitz der Gesellschaft:
Braunschweig
Handelsregister: Amtsgericht
Braunschweig, HRB 477

--
Rob

Robert W. Williams, Ph.D.
Chair: Department of Genetics, Genomics and Informatics
University of Tennessee Health Science Center
71 S Manassas St, Memphis TN 38103-3310, TSRB Room 405
Tele/Cell: 901 604 4752
EMAIL: rwilliams@uthsc.edu or
5 U.S.C. § 552(b)(6)

<Ace2-Draft-03182020-V1_LuB_kls_RWW_240320_3.docx>

<8608AAAF2A14461CBDBD04DA13676A0C.png>

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Sitz der Gesellschaft: Braunschweig
Handelsregister: Amtsgericht Braunschweig, HRB 477

<Ace3_V3_250320_kls_260320.docx>

<Ace2_V4_260320_Li.docx>

From: Gralinski, Lisa E[lgralins@email.unc.edu]
Sent: Sun 3/29/2020 1:42:55 PM (UTC-07:00)
To: Baric, Ralph S[rbaric@email.unc.edu]; Sims, Amy C[amy.sims@pnnl.gov]
Subject: hAcd2 mice

Did we ever send the hAce2 mice to anyone other than Prof. Shi? Matt has someone from GemPharmatech offering to sell him HFH4-Ace2tg mice that are 'ready for shipping today if we get Dr. R Baric's permission'. Tim and I told him that it sounded fishy as hell.

From: Baric, Ralph S[rbaric@email.unc.edu]
Sent: Sun 3/29/2020 1:45:44 PM (UTC-07:00)
To: Gralinski, Lisa E[lgralins@email.unc.edu]; Sims, Amy C[amy.sims@pnnl.gov]
Subject: RE: hAcd2 mice

This is fishy as hell

From: Gralinski, Lisa E <lgralins@email.unc.edu>
Sent: Sunday, March 29, 2020 4:43 PM
To: Baric, Ralph S <rbaric@email.unc.edu>; Sims, Amy C <amy.sims@pnnl.gov>
Subject: hAcd2 mice

Did we ever send the hAce2 mice to anyone other than Prof. Shi? Matt has someone from GemPharmatech offering to sell him HFH4-Ace2tg mice that are 'ready for shipping today if we get Dr. R Baric's permission'. Tim and I told him that it sounded fishy as hell.

From: Baric, Ralph S[rbaric@email.unc.edu]
Sent: Sun 3/29/2020 1:45:53 PM (UTC-07:00)
To: Gralinski, Lisa E[lgralins@email.unc.edu]; Sims, Amy C[amy.sims@pnnl.gov]
Subject: RE: hAcd2 mice

And no, no one else

From: Gralinski, Lisa E <lgralins@email.unc.edu>
Sent: Sunday, March 29, 2020 4:43 PM
To: Baric, Ralph S <rbaric@email.unc.edu>; Sims, Amy C <amy.sims@pnnl.gov>
Subject: hAcd2 mice

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From: Sims, Amy C[amy.sims@pnnl.gov]
Sent: Sun 3/29/2020 3:13:48 PM (UTC-07:00)
To: Lu, Lu[llu@uthsc.edu]
Cc: Klaus x Klaus Schughart[Klaus.Schughart@helmholtz-hzi.de]; Baric, Ralph S[rbaric@email.unc.edu]
Subject: Re: [Ext] B6D2 infected RNAseq data

Dr. Lu,

Could you please add the following to the acknowledgement section for the human lung cells used in this project?

Thank you very much, Amy Sims

The following statement would be appropriate for the cells:

Primary human bronchial epithelial cells prepared as described previously (reference = PMID:23097104) were provided by Dr. Scott H. Randell (Marsico Lung Institute, Tissue Procurement and Cell Culture Core, The University of North Carolina at Chapel Hill, USA). The cells were obtained under protocol #03-1396 approved by the University of North Carolina at Chapel Hill Biomedical Institutional Review Board.

Please add the following statement in your acknowledgements.

Provision of cells by Dr. Randell was supported by grants from the Cystic Fibrosis Foundation (BOUCHE19R0 and NIH (DK065988).

From: "Lu, Lu" <llu@uthsc.edu>
Date: Sunday, March 29, 2020 at 6:02 AM
To: "Li, Kui" <kli1@uthsc.edu>
Cc: Klaus x Klaus Schughart <Klaus.Schughart@helmholtz-hzi.de>, "Williams, Robert W" <rwilli10@uthsc.edu>, "Xu, Fuyi" <fxu10@uthsc.edu>, "Sims, Amy C" <amy.sims@pnnl.gov>, "rbaric@email.unc.edu" <rbaric@email.unc.edu>, "silke.bergmann@(b) (6)" <silke.bergmann@(b) (6)>
Subject: Re: [Ext] B6D2 infected RNAseq data

Thanks, Kui. We will integrate your editing into our final version.
On Mar 28, 2020, at 11:25 PM, Li, Kui <kli1@uthsc.edu> wrote:

> Dear Lu, Klaus, Rob, and team,
>
> Please find my edits of the manuscript. Use as you see fit. Thanks,
>
> Kui

> **From:** Lu, Lu <llu@uthsc.edu>
> **Sent:** Thursday, March 26, 2020 6:05 PM
> **To:** Klaus x Klaus Schughart <Klaus.Schughart@helmholtz-hzi.de>
> **Cc:** Williams, Robert W <rwilli10@uthsc.edu>; Xu, Fuyi <fxu10@uthsc.edu>; amy.sims@pnnl.gov <amy.sims@pnnl.gov>; rbaric@email.unc.edu <rbaric@email.unc.edu>; silke.bergmann@(b) (6) <silke.bergmann@(b) (6)>; Li, Kui <kli1@uthsc.edu>
> **Subject:** Re: [Ext] B6D2 infected RNAseq data

Dear Klaus (and all others):

Here is updated version. Fuyi addressed most of your comments. Please feel free to modify it continuously.

Dear Amy and Ralph:

Some of Klaus' comment are specific to you. Could you help to reply or editing?

Thanks.

Lu Lu
Professor
Department of Genetics, Genomics and Informatics
University of Tennessee Health Science Center
71 S. Manassas St., Room 410K
Memphis, TN 38163
Tel: (901) 448-7557 (O)
Fax: (901) 448-3500 (Dept)

On Mar 26, 2020, at 1:18 PM, Schughart, Klaus <Klaus.Schughart@helmholtz-hzi.de> wrote:

Hi LuLu,
enclosed are my additional comments – now also on the discussion.
Maybe Amy has also some ideas to explain the discrepancy between lungs and HAE cells with respect to inverse up/down regulation.
Klaus

Von: Lu, Lu <llu@uthsc.edu>
Gesendet: Donnerstag, 26. März 2020 00:27
An: Schughart, Klaus <Klaus.Schughart@helmholtz-hzi.de>
Cc: Williams, Robert W <rwilli10@uthsc.edu>; Xu, Fuyi <fxu10@uthsc.edu>; amy.sims@pnnl.gov
Betreff: Re: [Ext] B6D2 infected RNAseq data

Dear Klaus:

We have accepted Rob, you and our previous editing. Here is updated version for you to check and modify continuously.

Dear Amy:

Could you check this version and address some comments that are related to you?

Thanks.

Lu Lu
Professor
Department of Genetics, Genomics and Informatics
University of Tennessee Health Science Center

71 S. Manassas St., Room 410K
Memphis, TN 38163
Tel: (901) 448-7557 (O)
Fax: (901) 448-3500 (Dept)

On Mar 25, 2020, at 3:18 PM, Xu, Fuyi <fxu10@uthsc.edu> wrote:

Dear Klaus,

Could you please send me those two images (high-resolution) in Figure 8 ?
Thank you very much.

--
Fuyi Xu, PhD
Department of Genetics, Genomics, and Informatics
University of Tennessee Health Science Center

415 TRANSLATIONAL RESEARCH BUILDING
71 SOUTH MANASSAS
MEMPHIS TN 38163
Phone: (901)246-4398
E-mail: 5 U.S.C. § 552(b)(6) fxu10@uthsc.edu

From: Schughart, Klaus
Sent: Wednesday, March 25, 2020 3:04 PM
To: Lu, Lu
Cc: Xu, Fuyi; Williams, Robert W
Subject: AW: [Ext] B6D2 infected RNAseq data

Hi LuLu,
I forgot something in the Acknowledgements.
Please replace the Acknowledgements in the last version with the one in the enclosed version.
Thx,
Klaus

Von: Schughart, Klaus
Gesendet: Mittwoch, 25. März 2020 20:31
An: 'Lu, Lu' <llu@uthsc.edu>
Cc: 'Xu, Fuyi' <fxu10@uthsc.edu>; 'Williams, Robert W' <rwilli10@uthsc.edu>
Betreff: AW: [Ext] B6D2 infected RNAseq data

Dear all,
here are my comments on the revised version. I took out the previous comments that have been resolved.
Fuyi, I also enclose the data table for Figure 8A.
Once you are done with this version, I can have a look at the discussion and then you could send it off to the other co-authors.
Klaus

Von: Schughart, Klaus
Gesendet: Mittwoch, 25. März 2020 19:31
An: 'Lu, Lu' <llu@uthsc.edu>
Cc: Xu, Fuyi <fxu10@uthsc.edu>; Williams, Robert W <rwilli10@uthsc.edu>
Betreff: AW: [Ext] B6D2 infected RNAseq data

Thanks – will work it.
Klaus

Von: Lu, Lu <llu@uthsc.edu>
Gesendet: Mittwoch, 25. März 2020 19:02
An: Schughart, Klaus <Klaus.Schughart@helmholtz-hzi.de>
Cc: Xu, Fuyi <fxu10@uthsc.edu>; Williams, Robert W <rwilli10@uthsc.edu>
Betreff: Re: [Ext] B6D2 infected RNAseq data

Dear Klaus:

Fuyi and I accept all of you and rob's editing. We also clean and modified the paper, which addressed comments and answered the your questions. Please work on the updated version continuously. Hopefully we can submit this paper ASAP. Thanks.

Lu Lu
Professor
Department of Genetics, Genomics and Informatics
University of Tennessee Health Science Center
71 S. Manassas St., Room 410K
Memphis, TN 38163
Tel: (901) 448-7557 (O)
Fax: (901) 448-3500 (Dept)

> On Mar 24, 2020, at 4:36 PM, Lu, Lu <llu@uthsc.edu> wrote:
>
> Thanks, Rob. We will modify based on your comments and will send you updated version for your editing again.
>
> Lu Lu
> Professor
> Department of Genetics, Genomics and Informatics
> University of Tennessee Health Science Center
> 71 S. Manassas St., Room 410K
> Memphis, TN 38163
> Tel: (901) 448-7557 (O)
> Fax: (901) 448-3500 (Dept)
>
> > On Mar 24, 2020, at 4:14 PM, Rob Williams <rwilliams@uthsc.edu> wrote:
> >
> > Dear team,

Brilliant effort,

Edited the abstract heavily (please review carefully) and built up the Methods section on BXDs a bit.

Have not gotten through all of paper, but looks great so far.

Should validate some networks and eQTLs with GEMMA.

Needs a bit more clarity to data sources—some for 43 strains, other data for parents. These details needed (imho) even in the abstract.

On Tue, Mar 24, 2020 at 2:41 PM Schughart, Klaus <Klaus.Schughart@helmholtz-hzi.de> wrote:

Hi Lulu, Fuyi,

enclosed are my comments, additions and corrections.

Looks very good!!!

Please go through and clean it up first (accept or discard changes, add information) and then I can continue to work on the cleaned-up version. Currently it is too much cluttered with all these changes and comments.

Fuyi, let me know what exactly you need in addition for the HAE data.

Klaus

Von: Schughart, Klaus
Gesendet: Freitag, 6. März 2020 19:02
An: Xu, Fuyi <fxu10@uthsc.edu>; Lu Lu
5 U.S.C. § 552(b)(6)
Cc: Rob Williams <rwilliams@uthsc.edu>; Jonsson, Colleen Beth (cjonsson@uthsc.edu) <cjonsson@uthsc.edu>
Betreff: AW: [Ext] B6D2 infected RNAseq data

Hi Fuyi, LuLu,

I did some extensive analyses of genes in the human HAE data set with your gene list. Enclosed are the results. Looks very good!!

For a publication, we would have to discuss which data

to include and how to present the results.

Klaus

Von: Schughart, Klaus <Klaus.Schughart@helmholtz-hzi.de>
Gesendet: Donnerstag, 5. März 2020 04:45
An: Xu, Fuyi <fxu10@uthsc.edu>
Cc: Lu Lu [5 U.S.C. § 552(b)(6)]
Betreff: Re: [Ext] B6D2 infected RNAseq data

Dear Fuyi,

sure I will have a look at these genes.

Klaus

Von meinem iPhone gesendet

> Am 04.03.2020 um 19:09 schrieb Xu, Fuyi <fxu10@uthsc.edu>:
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> Dear Klaus,
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> Lu and I are working on the Ace2 paper. We prioritized around 30 genes that are correlated with Ace2 (attached table). I am wondering if you can help me check the expression differences between infected and normal controls in the human data set last time you showed us, especially for those color highlighted. Also, is this gene list make sense from your perspective? Thank you very much.
>
> Best,
>
> --
> Fuyi Xu, PhD
> Department of Genetics, Genomics, and Informatics
> University of Tennessee Health Science Center
>
> 415 TRANSLATIONAL RESEARCH BUILDING
> 71 SOUTH MANASSAS
> MEMPHIS TN 38163
> Phone: (901)246-4398
> E-mail: [5 U.S.C. § 552(b)(6)] fxu10@uthsc.edu

From: Schughart, Klaus
Sent: Friday, February 28, 2020 11:07 AM
To: Xu, Fuyi
Cc: Lu Lu
Subject: AW: [Ext] B6D2 infected RNAseq data

Hi LuLu, Fuyi,

I just remembered that I also have expression data from infected human lung slices (see enclosure).

This dataset has been published (enclosed).

Klaus

Von: Schughart, Klaus
Gesendet: Montag, 24. Februar 2020 21:00
An: 'Xu, Fuyi' <fxu10@uthsc.edu>
Cc: Lu Lu **5 U.S.C. § 552(b)(6)**
Betreff: AW: [Ext] B6D2 infected RNAseq data

Dear Fuyi,

The data I sent you before (RNAseq_norm1.txt) have been normalized and log2 transformed using DESeq2 (rlogTransformation(dsDat, blind=TRUE).

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For the BXD data in GN:

The percent body weights are NOT the losses but the percent body weight with reference to the starting body weight (100%). So 105% means that the mouse has gained 5% of body weight relative to its starting weight when it was infected. Mice with percent body weight less than 100% have lost weight.

Klaus

Von: Xu, Fuyi <fxu10@uthsc.edu>
Gesendet: Sonntag, 23. Februar 2020 22:14
An: Schughart, Klaus <Klaus.Schughart@helmholtz-hzi.de>
Cc: Lu Lu 5 U.S.C. § 552(b)(6)
Betreff: RE: [Ext] B6D2 infected RNAseq data

Dear Dr. Klaus Schughart,

I am Lu Lu's postdoc, Fuyi Xu. Thank you for sending us the normalized RNA-seq data.

We would like to perform differential expression analysis, I am wondering if you have raw read count for this data set since the DEseq2 package depend on this kind of data matrix. By the way, is the normalized data log2 transformed ?

We also found the expression level of ACE2 across the BXD is significantly correlated with median weight loss at day 3 after infection (ID:13007), however, we found BXD65a loss 105% body weight. Is this correct? Or how should I interpret ?

<A54B14D6A4584F84B57E3AAA25DC5BBB.gif>

Best Regards,

--
Fuyi Xu, PhD
Department of Genetics, Genomics, and Informatics
University of Tennessee Health Science Center

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Phone: (901)246-4398

E-mail: 5 U.S.C. § 552(b)(6) fxu10@uthsc.edu

From: Lu, Lu
Sent: Monday, February 17, 2020 11:03 AM
To: Xu, Fuyi
Subject: Fwd: [Ext] B6D2 infected RNAseq data

Lu Lu
Professor
Department of Genetics, Genomics and Informatics
University of Tennessee Health Science Center
71 S. Manassas St., Room 410K
Memphis, TN 38163
Tel: (901) 448-7557 (O)
Fax: (901) 448-3500 (Dept)

Begin forwarded message:

From: "Schughart, Klaus" <Klaus.Schughart@helmholtz-hzi.de>

Subject: [Ext] B6D2 infected RNAseq data

Date: February 17, 2020 at 10:11:55 AM CST

To: "Lu, Lu" <llu@uthsc.edu>

Hi LuLu,

I checked the datasets.

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I enclose the log2 normalized matrix for the B6/D2 infected data from Wilk et al. 2015:

RNAseq_norm1.txt -- for the mouse genes

00_influenza_lg2_RPKM_kls_280714.txt -- for the influenza gene segments

Let me know if you have any questions.

Klaus

Prof. Dr. Klaus Schughart
Head of Dept. of Infection Genetics
Helmholtz Centre for Infection Research,

University of Veterinary Medicine Hannover,

University of Tennessee Health Science Center
Inhoffenstr. 7
D-38124 Braunschweig
Germany

E-mail : Klaus.Schughart@helmholtz-hzi.de
Phone: +49 531 6181 1100

<B59D99096FF44A7EBCC7A3BC50D960A2.png>

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Vorsitzende des Aufsichtsrates: Frau MinDir'in Prof. Dr. Veronika von Messling
Stellvertreter: MinDirig Rüdiger Eichel, Niedersächsisches Ministerium für Wissenschaft und Kultur
Geschäftsführung: Prof. Dr. Dirk Heinz; Silke Tannapfel
Gesellschaft mit beschränkter Haftung (GmbH)
Sitz der Gesellschaft: Braunschweig
Handelsregister: Amtsgericht Braunschweig, HRB 477

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<Table 1.xlsx>

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--
Rob

Robert W. Williams, Ph.D.
Chair: Department of Genetics, Genomics and Informatics
University of Tennessee Health Science Center
71 S Manassas St, Memphis TN 38103-3310, TSRB Room 405
Tele/Cell: 901 604 4752
EMAIL: rwilliams@uthsc.edu or
5 U.S.C. § 552(b)(6)

<Ace2-Draft-03182020-V1_LuB_kls_RWW_240320_3.docx>

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Handelsregister: Amtsgericht Braunschweig, HRB 477
<Ace3_V3_250320_kls_260320.docx>

<Ace2_V4_260320_Li.docx>

From: Lu Lu[lulu521uthsc@gmail.com]
Sent: Sun 3/29/2020 4:08:43 PM (UTC-07:00)
To: Sims, Amy C[amy.sims@pnnl.gov]
Cc: Klaus x Klaus Schughart[Klaus.Schughart@helmholtz-hzi.de]; Baric, Ralph S[rbaric@email.unc.edu]; Xu, Fuyi[fxu10@uthsc.edu]
Subject: Re: [Ext] B6D2 infected RNAseq data

Sure, we will do. We will also send final version to everyone at coming week for the approval.

Lu Lu
Professor
Department of Genetics, Genomics and Informatics
University of Tennessee Health Science Center
71 S. Manassas St., Room 410K
Memphis, TN 38163
Tel: (901) 448-7557 (O)
Fax: (901) 448-3500 (Dept)

On Mar 29, 2020, at 5:13 PM, Sims, Amy C <amy.sims@pnnl.gov> wrote:

Dr. Lu,

Could you please add the following to the acknowledgement section for the human lung cells used in this project?

Thank you very much, Amy Sims

The following statement would be appropriate for the cells:

Primary human bronchial epithelial cells prepared as described previously (reference = PMID:23097104) were provided by Dr. Scott H. Randell (Marsico Lung Institute, Tissue Procurement and Cell Culture Core, The University of North Carolina at Chapel Hill, USA). The cells were obtained under protocol #03-1396 approved by the University of North Carolina at Chapel Hill Biomedical Institutional Review Board.

Please add the following statement in your acknowledgements.

Provision of cells by Dr. Randell was supported by grants from the Cystic Fibrosis Foundation (BOUCHE19R0 and NIH (DK065988).

From: "Lu, Lu" <llu@uthsc.edu>
Date: Sunday, March 29, 2020 at 6:02 AM
To: "Li, Kui" <kli1@uthsc.edu>
Cc: Klaus x Klaus Schughart <Klaus.Schughart@helmholtz-hzi.de>, "Williams, Robert W" <rwilli10@uthsc.edu>, "Xu, Fuyi" <fxu10@uthsc.edu>, "Sims, Amy C" <amy.sims@pnnl.gov>, "rbaric@email.unc.edu" <rbaric@email.unc.edu>, "silke.bergmann@(b) (6) " <silke.bergmann@(b) (6) >
Subject: Re: [Ext] B6D2 infected RNAseq data

Thanks, Kui. We will integrate your editing into our final version.

On Mar 28, 2020, at 11:25 PM, Li, Kui <kli1@uthsc.edu> wrote:

Dear Lu, Klaus, Rob, and team,

Please find my edits of the manuscript. Use as you see fit. Thanks,

Kui

From: Lu, Lu <llu@uthsc.edu>
Sent: Thursday, March 26, 2020 6:05 PM
To: Klaus x Klaus Schughart <Klaus.Schughart@helmholtz-hzi.de>
Cc: Williams, Robert W <rwilli10@uthsc.edu>; Xu, Fuyi <fxu10@uthsc.edu>;amy.sims@pnnl.gov <amy.sims@pnnl.gov>; rbaric@email.unc.edu<rbaric@email.unc.edu>; silke.bergmann@(b) (6) <silke.bergmann@(b) (6) >; Li, Kui <kli1@uthsc.edu>
Subject: Re: [Ext] B6D2 infected RNAseq data

Dear Klaus (and all others):

Here is updated version. Fuyi addressed most of your comments. Please feel free to modify it continuously.

Dear Amy and Ralph:

Some of Klaus' comment are specific to you. Could you help to reply or editing?

Thanks.

Lu Lu
Professor
Department of Genetics, Genomics and Informatics
University of Tennessee Health Science Center
71 S. Manassas St., Room 410K
Memphis, TN 38163
Tel: (901) 448-7557 (O)
Fax: (901) 448-3500 (Dept)

On Mar 26, 2020, at 1:18 PM, Schughart, Klaus <Klaus.Schughart@helmholtz-hzi.de> wrote:

Hi LuLu,
enclosed are my additional comments – now also on the discussion.
Maybe Amy has also some ideas to explain the discrepancy between lungs and HAE cells with respect to inverse up/down regulation.
Klaus

Von: Lu, Lu <llu@uthsc.edu>

Gesendet: Donnerstag, 26. März 2020 00:27
An: Schughart, Klaus <Klaus.Schughart@helmholtz-hzi.de>
Cc: Williams, Robert W <rwilli10@uthsc.edu>; Xu, Fuyi <fxu10@uthsc.edu>; amy.sims@pnnl.gov
Betreff: Re: [Ext] B6D2 infected RNAseq data

Dear Klaus:

We have accepted Rob, you and our previous editing. Here is updated version for you to check and modify continuously.

Dear Amy:

Could you check this version and address some comments that are related to you?

Thanks.

Lu Lu
Professor
Department of Genetics, Genomics and Informatics
University of Tennessee Health Science Center
71 S. Manassas St., Room 410K
Memphis, TN 38163
Tel: (901) 448-7557 (O)
Fax: (901) 448-3500 (Dept)

> On Mar 25, 2020, at 3:18 PM, Xu, Fuyi <fxu10@uthsc.edu> wrote:
>
> Dear Klaus,
>
> Could you please send me those two images (high-resolution) in Figure 8 ? Thank you very much.
>
> --
> Fuyi Xu, PhD
> Department of Genetics, Genomics, and Informatics
> University of Tennessee Health Science Center
>
> 415 TRANSLATIONAL RESEARCH BUILDING
> 71 SOUTH MANASSAS
> MEMPHIS TN 38163
> Phone: (901)246-4398
> E-mail: **5 U.S.C. § 552(b)(6)** fxu10@uthsc.edu
>
> **From:** Schughart, Klaus
> **Sent:** Wednesday, March 25, 2020 3:04 PM
> **To:** Lu, Lu
> **Cc:** Xu, Fuyi; Williams, Robert W

Subject: AW: [Ext] B6D2 infected RNAseq data

Hi LuLu,
I forgot something in the Acknowledgements.
Please replace the Acknowledgements in the last version with the one in the enclosed version.
Thx,
Klaus

Von: Schughart, Klaus
Gesendet: Mittwoch, 25. März 2020 20:31
An: 'Lu, Lu' <llu@uthsc.edu>
Cc: 'Xu, Fuyi' <fxu10@uthsc.edu>; 'Williams, Robert W' <rwilli10@uthsc.edu>
Betreff: AW: [Ext] B6D2 infected RNAseq data

Dear all,
here are my comments on the revised version. I took out the previous comments that have been resolved.
Fuyi, I also enclose the data table for Figure 8A.
Once you are done with this version, I can have a look at the discussion and then you could send it off to the other co-authors.
Klaus

Von: Schughart, Klaus
Gesendet: Mittwoch, 25. März 2020 19:31
An: 'Lu, Lu' <llu@uthsc.edu>
Cc: Xu, Fuyi <fxu10@uthsc.edu>; Williams, Robert W <rwilli10@uthsc.edu>
Betreff: AW: [Ext] B6D2 infected RNAseq data

Thanks – will work it.
Klaus

Von: Lu, Lu <llu@uthsc.edu>
Gesendet: Mittwoch, 25. März 2020 19:02
An: Schughart, Klaus <Klaus.Schughart@helmholtz-hzi.de>
Cc: Xu, Fuyi <fxu10@uthsc.edu>; Williams, Robert W <rwilli10@uthsc.edu>
Betreff: Re: [Ext] B6D2 infected RNAseq data

Dear Klaus:

Fuyi and I accept all of you and rob's editing. We also clean and modified the paper, which addressed comments and answered the your questions. Please work on the updated version continuously. Hopefully we can submit this paper ASAP. Thanks.

Lu Lu

Professor
Department of Genetics, Genomics and Informatics
University of Tennessee Health Science Center
71 S. Manassas St., Room 410K
Memphis, TN 38163
Tel: (901) 448-7557 (O)
Fax: (901) 448-3500 (Dept)

On Mar 24, 2020, at 4:36 PM, Lu, Lu <llu@uthsc.edu> wrote:

Thanks, Rob. We will modify based on your comments and will send you updated version for your editing again.

Lu Lu
Professor
Department of Genetics, Genomics and Informatics
University of Tennessee Health Science Center
71 S. Manassas St., Room 410K
Memphis, TN 38163
Tel: (901) 448-7557 (O)
Fax: (901) 448-3500 (Dept)

On Mar 24, 2020, at 4:14 PM, Rob Williams <rwilliams@uthsc.edu> wrote:

Dear team,

Brilliant effort,

Edited the abstract heavily (please review carefully) and built up the Methods section on BXDs a bit.

Have not gotten through all of paper, but looks great so far.

Should validate some networks and eQTLs with GEMMA.

Needs a bit more clarity to data sources—some for 43 strains, other data for parents. These details needed (imho) even in the abstract.

On Tue, Mar 24, 2020 at 2:41 PM Schughart, Klaus <Klaus.Schughart@helmholtz-hzi.de> wrote:

Hi Lulu, Fuyi,

enclosed are my comments, additions and corrections.

Looks very good!!!

Please go through and clean it up first (accept or discard changes, add information) and then I can continue to work on the cleaned-up version. Currently it is too much cluttered with all these changes and comments.

Fuyi, let me know what exactly you need in addition for the HAE data.

Klaus

Von: Schughart, Klaus
Gesendet: Freitag, 6. März 2020 19:02
An: Xu, Fuyi <fxu10@uthsc.edu>; Lu Lu **5 U.S.C. § 552(b)(6)**
Cc: Rob Williams <rwilliams@uthsc.edu>; Jonsson, Colleen Beth (cjonsson@uthsc.edu) <cjonsson@uthsc.edu>
Betreff: AW: [Ext] B6D2 infected RNAseq data

Hi Fuyi, LuLu,

I did some extensive analyses of genes in the human HAE data set with your gene list. Enclosed are the results. Looks very good!!

For a publication, we would have to discuss which data to include and how to present the results.

Klaus

Von: Schughart, Klaus <Klaus.Schughart@helmholtz-hzi.de>
Gesendet: Donnerstag, 5. März 2020 04:45
An: Xu, Fuyi <fxu10@uthsc.edu>
Cc: Lu Lu **5 U.S.C. § 552(b)(6)**
Betreff: Re: [Ext] B6D2 infected RNAseq data

Dear Fuyi,

sure I will have a look at these genes.

Klaus

Von meinem iPhone gesendet

> Am 04.03.2020 um 19:09 schrieb Xu, Fuyi <fxu10@uthsc.edu>:
>
> Dear Klaus,
>
> Lu and I are working on the Ace2 paper. We prioritized around 30 genes that are correlated with Ace2 (attached table). I am wondering if you can help me check the expression differences between infected and normal controls in the human data set last time you showed us, especially for those color highlighted. Also, is this gene list make sense from your perspective? Thank you very much.
>
> Best,
>
> --
> Fuyi Xu, PhD
> Department of Genetics, Genomics, and Informatics
> University of Tennessee Health Science Center
>
> 415 TRANSLATIONAL RESEARCH BUILDING
> 71 SOUTH MANASSAS
> MEMPHIS TN 38163
> Phone: (901)246-4398
> E-mail: 5 U.S.C. § 552(b)(6)
> fxu10@uthsc.edu
>
> **From:** Schughart, Klaus

Sent: Friday, February 28, 2020 11:07 AM
To: Xu, Fuyi
Cc: Lu Lu
Subject: AW: [Ext] B6D2 infected RNAseq data

Hi LuLu, Fuyi,

I just remembered that I also have expression data from infected human lung slices (see enclosure).

This dataset has been published (enclosed).

Klaus

Von: Schughart, Klaus
Gesendet: Montag, 24. Februar 2020 21:00
An: 'Xu, Fuyi' <fxu10@uthsc.edu>
Cc: Lu Lu
5 U.S.C. § 552(b)(6)
Betreff: AW: [Ext] B6D2 infected RNAseq data

Dear Fuyi,

The data I sent you before (RNAseq_norm1.txt) have been normalized and log2 transformed using DESeq2 (rlogTransformation(dsDat, blind=TRUE).

Please find enclosed the raw counts for the B6/D2 infections from Wilk et al (all_mouse_counts.txt).

For the BXD data in GN:

The percent body weights are NOT the losses but the percent body weight with reference to the starting body weight (100%). So 105% means that the mouse has gained 5% of

body weight relative to its starting weight when it was infected. Mice with percent body weight less than 100% have lost weight.

Klaus

Von: Xu, Fuyi <fxu10@uthsc.edu>
Gesendet: Sonntag, 23. Februar 2020 22:14
An: Schughart, Klaus <Klaus.Schughart@helmholtz-hzi.de>
Cc: Lu Lu

5 U.S.C. § 552(b)(6)

Betreff: RE: [Ext] B6D2 infected RNAseq data

Dear Dr. Klaus Schughart,

I am Lu Lu's postdoc, Fuyi Xu. Thank you for sending us the normalized RNA-seq data.

We would like to perform differential expression analysis, I am wondering if you have raw read count for this data set since the DEseq2 package depend on this kind of data matrix. By the way, is the normalized data log2 transformed ?

We also found the expression level of ACE2 across the BXD is significantly correlated with median weight

loss at day 3 after infection (ID:13007), however, we found BXD65a loss 105% body weight. Is this correct? Or how should I interpret ?

<A54B14D6A4584F84B57E3AAA25DC5BBB.gif>

Best Regards,

--
Fuyi Xu, PhD
Department of Genetics, Genomics, and Informatics
University of Tennessee Health Science Center

415 TRANSLATIONAL RESEARCH BUILDING
71 SOUTH MANASSAS
MEMPHIS TN 38163
Phone: (901)246-4398
E-mail: 5 U.S.C. § 552(b)(6) fxu10@uthsc.edu

From: Lu, Lu
Sent: Monday, February 17, 2020 11:03 AM
To: Xu, Fuyi
Subject: Fwd: [Ext] B6D2 infected RNAseq data

Lu Lu
Professor
Department of Genetics, Genomics and Informatics
University of Tennessee Health Science Center
71 S. Manassas St., Room 410K
Memphis, TN 38163
Tel: (901) 448-7557 (O)
Fax: (901) 448-3500 (Dept)

Begin forwarded message:

From: "Schughart, Klaus" <Klaus.Schughart@helmholtz-hzi.de>

Subject: **[Ext] B6D2 infected RNAseq data**

Date: February 17, 2020 at 10:11:55 AM CST

To: "Lu, Lu" <llu@uthsc.edu>

Hi LuLu,

I checked the datasets.

GN807; RNAseq BXD infected is fine. This dataset has not been published yet.

I enclose the log2 normalized matrix

for the B6/D2 infected data from Wilk et al. 2015:

RNAseq_norm1.txt -- for the mouse genes

00_influenza_lg2_RPKM_kls_280714.txt -- for the influenza gene segments

Let me know if you have any questions.

Klaus

Prof. Dr. Klaus Schughart
Head of Dept. of Infection Genetics
Helmholtz Centre for Infection Research,

University of Veterinary Medicine Hannover,

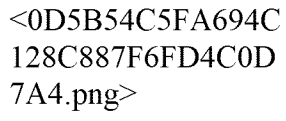
University of Tennessee Health Science Center
Inhoffens

tr. 7
D-38124
Braunsch
weig
Germany

E-mail
: Klaus.S
chughart
@helmho
ltz-hzi.de
Phone:
+49 531
6181
1100

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BC50D9
60A2.pn
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Rüdiger Eichel, Niedersächsisches Ministerium für Wissenschaft und Kultur
Geschäftsführung: Prof. Dr. Dirk Heinz; Silke Tannapfel
Gesellschaft mit beschränkter Haftung (GmbH)
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<Table 1.xlsx>

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--
Rob

Robert W. Williams, Ph.D.
Chair: Department of Genetics,
Genomics and Informatics
University of Tennessee Health Science
Center
71 S Manassas St, Memphis TN 38103-
3310, TSRB Room 405
Tele/Cell: 901 604 4752
EMAIL: rwilliams@uthsc.edu or
5 U.S.C. § 552(b)(6)

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Lu Lu

Professor
Department of Genetics, Genomics and Informatics
University of Tennessee Health Science Center
71 S. Manassas St., Room 410K
Memphis, TN 38163
Tel: (901) 448-7557 (O)
Fax: (901) 448-3500 (Dept)

From: Sheahan, Timothy Patrick[sheahan@email.unc.edu]
Sent: Sun 3/29/2020 6:45:40 PM (UTC-07:00)
To: Sims, Amy C[amy.sims@pnnl.gov]
Subject: Re: Your Signature Needed for Science Translational Med Paper Revision

Thanks dude!

On 3/29/20, 6:13 PM, "Sims, Amy C" <amy.sims@pnnl.gov> wrote:

Done. Thanks, Amy

On 3/28/20, 3:01 PM, "Sheahan, Timothy Patrick" <sheahan@email.unc.edu> wrote:

Seems fine to me. Send it on to that lady in the email! Thanks!

> On Mar 28, 2020, at 5:16 PM, Sims, Amy C <amy.sims@pnnl.gov> wrote:
>
> Best I can do until Monday. Can you live with this? Thanks, Amy
>
> On 3/28/20, 11:43 AM, "Sheahan, Timothy Patrick" <sheahan@email.unc.edu> wrote:
>
> Dear All,
>
> The Denison lab did a bunch of amazing work on the revised NHC coronavirus Science Translational Medicine manuscript. Four authors need to be added to the paper as a result of this.: Amelia George, Laura Stevens, Tia Hughes and Xiaotao Lu.
>
> The journal would like each of you to sign the attached form and email it to Aneera Dobbins (adobbins@aaas.org) as soon as possible. Each can just print their name and sign their own form and send it. There's no need to have everyone on literally the same page. I've CC'd Aneera above.
>
> Thanks!
>
> Tim
>
>
>
>
> <SIms 2020 NHC CoV STM Paper Authorship Change Form abb5883[2].pdf>

From: Sheahan, Timothy Patrick[sheahan@email.unc.edu]
Sent: Sun 3/29/2020 7:05:25 PM (UTC-07:00)
To: Sims, Amy C[amy.sims@pnnl.gov]
Subject: Scotty

Hi Amy,

Should Randell have been on the EIDD 1931 paper. I never put them on the author line thinking we just bought all the cultures for this. Is that true? Please advise.

T

On 3/29/20, 9:34 PM, "Randell, Scott H." <scott_randell@med.unc.edu> wrote:

Hi Tim, Congrats on the BioRxiv submission, should get a lot of attention. When the final paper comes around would be awesome to acknowledge the Core funding. I can provide the grant #s. We should talk about your cell needs when dust settles. Best, Scott

Sent from my iPhone

From: Sims, Amy C[amy.sims@pnnl.gov]
Sent: Sun 3/29/2020 8:00:48 PM (UTC-07:00)
To: Sheahan, Timothy Patrick[sheahan@email.unc.edu]
Subject: Re: Your Signature Needed for Science Translational Med Paper Revision

Welcome!

On 3/29/20, 6:45 PM, "Sheahan, Timothy Patrick" <sheahan@email.unc.edu> wrote:

Thanks dude!

On 3/29/20, 6:13 PM, "Sims, Amy C" <amy.sims@pnnl.gov> wrote:

Done. Thanks, Amy

On 3/28/20, 3:01 PM, "Sheahan, Timothy Patrick" <sheahan@email.unc.edu> wrote:

Seems fine to me. Send it on to that lady in the email! Thanks!

> On Mar 28, 2020, at 5:16 PM, Sims, Amy C <amy.sims@pnnl.gov> wrote:
>
> Best I can do until Monday. Can you live with this? Thanks, Amy
>
> On 3/28/20, 11:43 AM, "Sheahan, Timothy Patrick" <sheahan@email.unc.edu> wrote:
>
> Dear All,
>
> The Denison lab did a bunch of amazing work on the revised NHC coronavirus Science Translational Medicine manuscript. Four authors need to be added to the paper as a result of this.: Amelia George, Laura Stevens, Tia Hughes and Xiaotao Lu.
>
> The journal would like each of you to sign the attached form and email it to Aneera Dobbins (adobbins@aaas.org) as soon as possible. Each can just print their name and sign their own form and send it. There's no need to have everyone on literally the same page. I've CC'd Aneera above.
>
> Thanks!
>
> Tim
>
>
>
>
> <SIms 2020 NHC CoV STM Paper Authorship Change Form abb5883[2].pdf>

From: Sims, Amy C [amy.sims@pnnl.gov]
Sent: 3/29/2020 8:56:31 PM
To: Sheahan, Timothy Patrick [sheahan@email.unc.edu]
Subject: Re: Scotty

Hi. For this and all the work with Gilead and really

Get Outlook for iOS

From: Sheahan, Timothy Patrick <sheahan@email.unc.edu>
Sent: Sunday, March 29, 2020 7:05 PM
To: Sims, Amy C
Subject: Scotty

Hi Amy,

Should Randell have been on the EIDD 1931 paper. I never put them on the author line thinking we just bought all the cultures for this. Is that true? Please advise.

T

On 3/29/20, 9:34 PM, "Randell, Scott H." <scott_randell@med.unc.edu> wrote:

Hi Tim, Congrats on the BioRxiv submission, should get a lot of attention. When the final paper comes around would be awesome to acknowledge the Core funding. I can provide the grant #s. We should talk about your cell needs when dust settles. Best, Scott

Sent from my iPhone

From: Lu, Lu[llu@uthsc.edu]
Sent: Mon 3/30/2020 4:00:09 PM (UTC-07:00)
To: Williams, Robert W[rwilli10@uthsc.edu]; Sims, Amy C[amy.sims@pnnl.gov]; silke.bergmann@(b) (6) [silke.bergmann@(b) (6)]; rbaric@email.unc.edu[rbaric@email.unc.edu]; Jonsson, Colleen Beth[cjonsson@uthsc.edu]; Li, Kui[kli1@uthsc.edu]
Cc: Klaus x Klaus Schughart[Klaus.Schughart@helmholtz-hzi.de]; Xu, Fuyi[fxu10@uthsc.edu]
Subject: Ace2
Attachment: Ace2_V7_03302020B.docx

Dear all authors:
We accepted most of your editing and suggestions for *Ace2* paper. Here is the final version (except reference section. We are working on it). Please review it again and make your final editing. Please also check your name and address. Please let me know if you don't want to be co-author for this paper. Please send your updated version back to me before this Friday. We plan to submit it at this weekend.

Thank you all for your big help and contribution.

Lu Lu
Professor
Department of Genetics, Genomics and Informatics
University of Tennessee Health Science Center
71 S. Manassas St., Room 410K
Memphis, TN 38163
Tel: (901) 448-7557 (O)
Fax: (901) 448-3500 (Dept)

> On Mar 28, 2020, at 11:25 PM, Li, Kui <kli1@uthsc.edu> wrote:
>
> Dear Lu, Klaus, Rob, and team,
>
> Please find my edits of the manuscript. Use as you see fit. Thanks,
>
> Kui
>
> ---
>
> **From:** Lu, Lu <llu@uthsc.edu>
> **Sent:** Thursday, March 26, 2020 6:05 PM
> **To:** Klaus x Klaus Schughart <Klaus.Schughart@helmholtz-hzi.de>
> **Cc:** Williams, Robert W <rwilli10@uthsc.edu>; Xu, Fuyi <fxu10@uthsc.edu>; amy.sims@pnnl.gov <amy.sims@pnnl.gov>; rbaric@email.unc.edu <rbaric@email.unc.edu>; silke.bergmann@(b) (6) <silke.bergmann@(b) (6) >; Li, Kui <kli1@uthsc.edu>
> **Subject:** Re: [Ext] B6D2 infected RNAseq data
>
> Dear Klaus (and all others):
> Here is updated version. Fuyi addressed most of your comments. Please feel free to modify it continuously.
>
> Dear Amy and Ralph:
>
> Some of Klaus' comment are specific to you. Could you help to reply or editing?

Thanks.

Lu Lu
Professor
Department of Genetics, Genomics and Informatics
University of Tennessee Health Science Center
71 S. Manassas St., Room 410K
Memphis, TN 38163
Tel: (901) 448-7557 (O)
Fax: (901) 448-3500 (Dept)

On Mar 26, 2020, at 1:18 PM, Schughart, Klaus <Klaus.Schughart@helmholtz-hzi.de> wrote:

Hi LuLu,
enclosed are my additional comments – now also on the discussion.
Maybe Amy has also some ideas to explain the discrepancy between lungs and HAE cells with respect to inverse up/down regulation.
Klaus

Von: Lu, Lu <llu@uthsc.edu>
Gesendet: Donnerstag, 26. März 2020 00:27
An: Schughart, Klaus <Klaus.Schughart@helmholtz-hzi.de>
Cc: Williams, Robert W <rwilli10@uthsc.edu>; Xu, Fuyi <fxu10@uthsc.edu>; amy.sims@pnnl.gov
Betreff: Re: [Ext] B6D2 infected RNAseq data

Dear Klaus:

We have accepted Rob, you and our previous editing. Here is updated version for you to check and modify continuously.

Dear Amy:

Could you check this version and address some comments that are related to you?

Thanks.

Lu Lu
Professor
Department of Genetics, Genomics and Informatics
University of Tennessee Health Science Center
71 S. Manassas St., Room 410K
Memphis, TN 38163
Tel: (901) 448-7557 (O)
Fax: (901) 448-3500 (Dept)

On Mar 25, 2020, at 3:18 PM, Xu, Fuyi <fxu10@uthsc.edu> wrote:

Dear Klaus,

Could you please send me those two images (high-resolution) in Figure 8 ?
Thank you very much.

--
Fuyi Xu, PhD
Department of Genetics, Genomics, and Informatics
University of Tennessee Health Science Center

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71 SOUTH MANASSAS
MEMPHIS TN 38163
Phone: (901)246-4398
E-mail: 5 U.S.C. § 552(b)(6) ; fxu10@uthsc.edu

From: Schughart, Klaus
Sent: Wednesday, March 25, 2020 3:04 PM
To: Lu, Lu
Cc: Xu, Fuyi; Williams, Robert W
Subject: AW: [Ext] B6D2 infected RNAseq data

Hi LuLu,
I forgot something in the Acknowledgements.
Please replace the Acknowledgements in the last version with the one in the enclosed version.
Thx,
Klaus

Von: Schughart, Klaus
Gesendet: Mittwoch, 25. März 2020 20:31
An: 'Lu, Lu' <llu@uthsc.edu>
Cc: 'Xu, Fuyi' <fxu10@uthsc.edu>; 'Williams, Robert W' <rwilli10@uthsc.edu>
Betreff: AW: [Ext] B6D2 infected RNAseq data

Dear all,
here are my comments on the revised version. I took out the previous comments that have been resolved.
Fuyi, I also enclose the data table for Figure 8A.
Once you are done with this version, I can have a look at the discussion and then you could send it off to the other co-authors.
Klaus

Von: Schughart, Klaus
Gesendet: Mittwoch, 25. März 2020 19:31
An: 'Lu, Lu' <llu@uthsc.edu>
Cc: Xu, Fuyi <fxu10@uthsc.edu>; Williams, Robert W <rwilli10@uthsc.edu>
Betreff: AW: [Ext] B6D2 infected RNAseq data

Thanks – will work it.
Klaus

Von: Lu, Lu <llu@uthsc.edu>

Gesendet: Mittwoch, 25. März 2020 19:02
An: Schughart, Klaus <Klaus.Schughart@helmholtz-hzi.de>
Cc: Xu, Fuyi <fxu10@uthsc.edu>; Williams, Robert W <rwilli10@uthsc.edu>
Betreff: Re: [Ext] B6D2 infected RNAseq data

Dear Klaus:

Fuyi and I accept all of you and rob's editing. We also clean and modified the paper, which addressed comments and answered the your questions. Please work on the updated version continuously. Hopefully we can submit this paper ASAP. Thanks.

Lu Lu
Professor
Department of Genetics, Genomics and Informatics
University of Tennessee Health Science Center
71 S. Manassas St., Room 410K
Memphis, TN 38163
Tel: (901) 448-7557 (O)
Fax: (901) 448-3500 (Dept)

> On Mar 24, 2020, at 4:36 PM, Lu, Lu <llu@uthsc.edu> wrote:
>
> Thanks, Rob. We will modify based on your comments and will send you updated version for your editing again.
>
> Lu Lu
> Professor
> Department of Genetics, Genomics and Informatics
> University of Tennessee Health Science Center
> 71 S. Manassas St., Room 410K
> Memphis, TN 38163
> Tel: (901) 448-7557 (O)
> Fax: (901) 448-3500 (Dept)
>
> > On Mar 24, 2020, at 4:14 PM, Rob Williams <rwilliams@uthsc.edu> wrote:
> >
> > Dear team,
> >
> > Brilliant effort,
> >
> > Edited the abstract heavily (please review carefully) and built up the Methods section on BXDs a bit.

Have not gotten through all of paper, but looks great so far.

Should validate some networks and eQTLs with GEMMA.

Needs a bit more clarity to data sources—some for 43 strains, other data for parents. These details needed (imho) even in the abstract.

On Tue, Mar 24, 2020 at 2:41 PM Schughart, Klaus <Klaus.Schughart@helmholtz-hzi.de> wrote:

> Hi Lulu, Fuyi,
> enclosed are my comments, additions and corrections. Looks very good!!!
> Please go through and clean it up first (accept or discard changes, add information) and then I can continue to work on the cleaned-up version. Currently it is too much cluttered with all these changes and comments.
> Fuyi, let me know what exactly you need in addition for the HAE data.
> Klaus
>
> ---
>
> **Von:** Schughart, Klaus
> **Gesendet:** Freitag, 6. März 2020 19:02
> **An:** Xu, Fuyi <fxu10@uthsc.edu>; Lu Lu **5 U.S.C. § 552(b)(6)**
> **Cc:** Rob Williams <rwilliams@uthsc.edu>; Jonsson, Colleen Beth (cjonsson@uthsc.edu) <cjonsson@uthsc.edu>
> **Betreff:** AW: [Ext] B6D2 infected RNAseq data
>
> Hi Fuyi, LuLu,
> I did some extensive analyses of genes in the human HAE data set with your gene list. Enclosed are the results. Looks very good!!
> For a publication, we would have to discuss which data to include and how to present the results.
> Klaus
>
> ---
>
> **Von:** Schughart, Klaus <Klaus.Schughart@helmholtz-hzi.de>
> **Gesendet:** Donnerstag, 5. März 2020 04:45
> **An:** Xu, Fuyi <fxu10@uthsc.edu>
> **Cc:** Lu Lu **5 U.S.C. § 552(b)(6)**
> **Betreff:** Re: [Ext] B6D2 infected RNAseq data
>
> Dear Fuyi,
> sure I will have a look at these genes.
> Klaus
>
> Von meinem iPhone gesendet
>
> > Am 04.03.2020 um 19:09 schrieb Xu, Fuyi <fxu10@uthsc.edu>:

Dear Klaus,

Lu and I are working on the Ace2 paper. We prioritized around 30 genes that are correlated with Ace2 (attached table). I am wondering if you can help me check the expression differences between infected and normal controls in the human data set last time you showed us, especially for those color highlighted. Also, is this gene list make sense from your perspective? Thank you very much.

Best,
--
Fuyi Xu, PhD
Department of Genetics, Genomics, and Informatics
University of Tennessee Health Science Center

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71 SOUTH MANASSAS
MEMPHIS TN 38163
Phone: (901)246-4398
E-mail: 5 U.S.C. § 552(b)(6) fxu10@uthsc.edu

From: Schughart, Klaus
Sent: Friday, February 28, 2020 11:07 AM
To: Xu, Fuyi
Cc: Lu Lu
Subject: AW: [Ext] B6D2 infected RNAseq data

Hi LuLu, Fuyi,
I just remembered that I also have expression data from infected human lung slices (see enclosure).
This dataset has been published (enclosed).
Klaus

Von: Schughart, Klaus
Gesendet: Montag, 24. Februar 2020 21:00
An: 'Xu, Fuyi' <fxu10@uthsc.edu>
Cc: Lu Lu 5 U.S.C. § 552(b)(6)
Betreff: AW: [Ext] B6D2 infected RNAseq data

Dear Fuyi,
The data I sent you before (RNAseq_norm1.txt) have been normalized and log2 transformed using DESeq2 (rlogTransformation(dsDat, blind=TRUE).
Please find enclosed the raw counts for the B6/D2 infections from Wilk et al (all_mouse_counts.txt).

For the BXD data in GN:

The percent body weights are NOT the losses but the percent body weight with reference to the starting body weight (100%). So 105% means that the mouse has gained 5% of body weight relative to its starting weight when it was infected. Mice with percent body weight less than 100% have lost weight.

Klaus

Von: Xu, Fuyi <fxu10@uthsc.edu>
Gesendet: Sonntag, 23. Februar 2020 22:14
An: Schughart, Klaus <Klaus.Schughart@helmholtz-hzi.de>
Cc: Lu Lu; 5 U.S.C. § 552(b)(6)
Betreff: RE: [Ext] B6D2 infected RNAseq data

Dear Dr. Klaus Schughart,

I am Lu Lu's postdoc, Fuyi Xu.
Thank you for sending us the normalized RNA-seq data.
We would like to perform differential expression analysis, I am wondering if you have raw read count for this data set since the DEseq2 package depend on this kind of data matrix.
By the way, is the normalized data log2 transformed ?

We also found the expression level of ACE2 across the BXD is significantly correlated with median weight loss at day 3 after infection (ID:13007), however, we found BXD65a loss 105% body weight. Is this correct? Or how should I interpret ?

<A54B14D6A4584F84B57E3AAA25DC5BBB.gif>
Best Regards,
--
Fuyi Xu, PhD
Department of Genetics, Genomics, and Informatics
University of Tennessee Health Science Center

415 TRANSLATIONAL RESEARCH BUILDING
71 SOUTH MANASSAS
MEMPHIS TN 38163
Phone: (901)246-4398
E-mail: 5 U.S.C. § 552(b)(6); fxu10@uthsc.edu

From: Lu, Lu

Sent: Monday, February 17, 2020 11:03 AM
To: Xu, Fuyi
Subject: Fwd: [Ext] B6D2 infected RNAseq data

Lu Lu
Professor
Department of Genetics, Genomics and Informatics
University of Tennessee Health Science Center
71 S. Manassas St., Room 410K
Memphis, TN 38163
Tel: (901) 448-7557 (O)
Fax: (901) 448-3500 (Dept)

Begin forwarded message:

From: "Schughart, Klaus" <Klaus.Schughart@helmholtz-hzi.de>
Subject: [Ext] B6D2 infected RNAseq data
Date: February 17, 2020 at 10:11:55 AM CST
To: "Lu, Lu" <llu@uthsc.edu>

Hi LuLu,
I checked the datasets.

GN807; RNAseq BXD infected is fine. This dataset has not been published yet.

I enclose the log2 normalized matrix for the B6/D2 infected data from Wilk et al. 2015:
RNAseq_norm1.txt -- for the mouse genes
00_influenza_lg2_RPKM_kls_280714.txt -- for the influenza gene segments

Let me know if you have any questions.
Klaus

Prof. Dr. Klaus Schughart
Head of Dept. of Infection Genetics
Helmholtz Centre for Infection Research,

University of
Veterinary Medicine
Hannover,
University of
Tennessee Health
Science Center
Inhoffenstr. 7
D-38124 Braunschweig
Germany

E-mail
: Klaus.Schughart@helmholtz-hzi.de
Phone: +49 531 6181
1100

<B59D99096FF44A7EBCC7A3BC50D960A2.png>

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Stellvertreter: MinDirig Rüdiger Eichel, Niedersächsisches Ministerium für Wissenschaft und Kultur
Geschäftsführung: Prof. Dr. Dirk Heinz; Silke Tannapfel
Gesellschaft mit beschränkter Haftung (GmbH)
Sitz der Gesellschaft: Braunschweig
Handelsregister: Amtsgericht Braunschweig, HRB 477

<0D5B54C5FA694C128C887F6FD4C0D7A4.png>

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Braunschweig | www.helmholtz-hzi.de

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Geschäftsführung: Prof. Dr. Dirk Heinz; Silke Tannapfel
Gesellschaft mit beschränkter Haftung (GmbH)
Sitz der Gesellschaft: Braunschweig
Handelsregister: Amtsgericht Braunschweig, HRB 477

<Table 1.xlsx>

<B911E1AA90884DD79858B3D0CE0F7C4C.png>

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Gesellschaft mit beschränkter Haftung (GmbH)
Sitz der Gesellschaft: Braunschweig
Handelsregister: Amtsgericht Braunschweig, HRB 477

--
Rob

Robert W. Williams, Ph.D.
Chair: Department of Genetics, Genomics and Informatics
University of Tennessee Health Science Center
71 S Manassas St, Memphis TN 38103-3310, TSRB Room 405
Tele/Cell: 901 604 4752
EMAIL: rwilliams@uthsc.edu or
5 U.S.C. § 552(b)(6)

<Ace2-Draft-03182020-V1_LuB_kls_RWW_240320_3.docx>

<8608AAAF2A14461CBDBD04DA13676A0C.png>

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Stellvertreter: MinDirig Rüdiger Eichel, Niedersächsisches Ministerium für Wissenschaft und Kultur
Geschäftsführung: Prof. Dr. Dirk Heinz; Silke Tannapfel
Gesellschaft mit beschränkter Haftung (GmbH)
Sitz der Gesellschaft: Braunschweig
Handelsregister: Amtsgericht Braunschweig, HRB 477
<Ace3_V3_250320_kls_260320.docx>

<Ace2_V4_260320_Li.docx>

Genetic Dissection of the Regulatory Mechanisms of *Ace2* in the Infected Mouse Lung

Fuyi Xu[1#], Jun Gao[1, 5#], Silke Bergman[4], Amy C. Sims[7, 9], Ralph S. Baric[6,7, 8], Colleen B Jonsson[4], Kui Li[4], Robert W. Williams[1], Klaus Schughart[2, 3, 4*], and Lu Lu[1*]

[1]Department of Genetics, Genomics, and Informatics, University of Tennessee Health Science Center, Memphis, TN, USA;

[2]Department of Infection Genetics, Helmholtz Centre for Infection Research, Braunschweig, Germany

[3]University of Veterinary Medicine Hannover, Hannover, Germany;

[4]Department of Microbiology, Immunology, and Biochemistry, University of Tennessee Health Science Center, Memphis, TN, USA;

[5]Institute of Animal Husbandry and Veterinary Science, Shanghai Academy of Agricultural Sciences, Shanghai, China.

[6]Department of Microbiology and Immunology, University of North Carolina at Chapel Hill, Chapel Hill, NC

[7]Department of Genetics, University of North Carolina at Chapel Hill, Chapel Hill, NC

[8]Department of Epidemiology, University of North Carolina at Chapel Hill, Chapel Hill, NC

[9]present address: Pacific Northwest National Laboratory in Richland, WA

[#] These authors contributed equally to this work.

* Correspondence:

Lu Lu: llu@uthsc.edu

Department of Genetics, Genomics and Informatics
University of Tennessee Health Science Center
Memphis, TN 38163, United States;

Klaus Schughart: klaus.schughart@helmholtz-hzi.de
Department of Infection Genetics
Helmholtz Centre for Infection Research
Braunschweig, Germany

Abstract

Acute lung injury (ALI) is an important cause of morbidity and mortality after viral infections, such as influenza A virus H1N1, SARS-CoV, MERS-CoV, and SARS-CoV-2. The angiotensin 1 converting enzyme 2 (ACE2) plays a protective role in SARS-CoV-induced ALI. However, contributions of *ACE2* gene variants to the post-infection heterogeneity of the host response and susceptibility remain unclear, as do the underlying mechanisms. In this study, we quantified lung transcriptomes in 41 BXD recombinant inbred strains of mice plus their parental strains of C57BL/6J and DBA/2J mice after H1N1 infection. In response to infection, the expression of *Ace2* mRNA across strains decreased significantly and was associated with both disease severity and infection time. Expression was negatively correlated with body weight loss and duration of survival time. Data for all 43 genomes were analyzed using weighted gene co-expression network analysis (WGCNA). *Ace2* not only participated in virus-induced ALI by interacting with TNF, MAPK, and notch signaling pathway, but also was linked to genes that have important functions in the pulmonary epithelium. Moreover, by applying multi-layer filtration, including differential expression analysis, eQTL mapping, functional variant identification, and gene annotation, we highlighted 10 key candidate genes linked to an *Ace2* network: *Sox11*, *Tff2*, *Rnf128*, *Muc5b*, *Tmprss2*, *Ulk4*, *Cckar*, *Wdpcp*, *Ift140*, and *Ccdc40*. Of these transcripts, *Rnf128* was identified as a potential master regulator using Bayesian network analysis. The key gene set is enriched for genes reported to be differentially expressed in SARS-CoV-infected primary human airway epithelial cells. Collectively, these findings define an *Ace2*-associated modulatory network relevant to host response to H1N1 infection and raise novel potential therapeutic targets for virus-induced respiratory diseases, including those caused by SARS-CoV and SARS-CoV-2. In addition, we demonstrate that the genetically diverse BXD family provides an excellent testbed for dissecting host genetic regulatory mechanisms involved in the defense against viral infections and host disease.

Key words: H1N1, acute lung injury, BXD mice, *Ace2,* regulatory network

Introduction

The pandemic infectious disease, COVID-19, is caused by a novel coronavirus, termed severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). This pathogen has spread with almost unprecedent speed from an initial outbreak in November or December 2019. As of March 25, 2020, according to estimates by the World Health Organization (WHO), ~195 countries and ~375,000 cases of COVID-19 have been reported and linked to ~16,000 deaths.

Since 1918, the world has witnessed four influenza A virus (IAV) pandemics—1918 H1N1, 1957 H2N2, 1968 H3N2, and 2009 H1N1. The first of these resulted in at least 50 million global mortalities [1]. The primary pathological features of both COVID-19 and 2009 influenza A (H1N1) infection are acute viral pneumonia and acute lung injury (ALI) [2]. The documented symptoms of influenza A (H1N1) and COVID-19 are nearly identical. For both viral pathogens infected patients can either be asymptomatic or can have symptoms that range from mild coughing, congestion, and fever, or to severe and lethal pulmonary edema. Individual genetic differences almost certainly play an important role in the development and severity of the illness and its time course [3-5].

Angiotensin-converting enzyme 2 (*ACE2*)—a negative regulator of the renin angiotensin system (RAS)—plays a protective role in pathogenesis of ALI in several animal models of acute respiratory distress syndrome [6]. *Ace2* knockout mice exhibited severe ALI. In contrast, treatment with recombinant human *ACE2* expression attenuates acute lung failure in these *Ace2* knockouts as well as in wild-type mice [7]. Studies have shown that ACE2 protein levels are significantly downregulated after infection with H1N1 virus but are dispensable for viral replication [8]. For SARS-CoV , *ACE2* has been identified as the principal host transmembrane receptor for the receptor-binding domain of the viral spike protein and thereby facilitates endocytosis of the virus and it subsequent virion replication in host cells. In *in vivo* studies, SARS-CoV infection of mice causes severe pulmonary disease and lethal lung failure, concomitant with the downregulation of *Ace2* in lungs [12]. Single-cell RNA sequencing demonstrates that 83% of *ACE2*-expressing cells in the lungs are alveolar epithelial type II cells. It is possible that there are genetic differences in host susceptibility the depend on the numbers or ratios of *ACE2*-positive AT-2 cells in lung [13]. However, we emphasize that causal linkage of *ACE2* variation to susceptibility to infection or to post-infection heterogeneity of disease progression is still very poorly defined in humans or even mouse..

Susceptibility to viral infection varies greatly among strains of isogenic mice [14], especially between two key inbred strains—C57BL/6J (B6), the reference strain for most experimental work—and DBA/2J (D2), the oldest of inbred strains of mice [15, 16]. B6 is relatively resistant to influenza A and often survives inoculation. In contrast, D2 loses body weight rapidly and at the same inoculum dose as given to B6 dies within 5–7 days. Our laboratory has generated and maintains ~120 BXD progeny strains derived from crosses between B6 and D2 parents. Both parental strains and all of these highly diverse BXD recombinant inbred progeny has been sequenced. Collectively this family segregates for ~6 million variants with minor allele frequencies above 0.3 [16], making it an ideal and well powered reference population to model genetic susceptibility to IAV infections [17]. Indeed, in previous genetic mapping using this cohort we have

defined loci and even cloned gene variants that control the host response to IAV (H1N1) infection [18] and to other infectious diseases [19-22]. The BXD family provides a powerful resource to dissect loci, gene variants, molecular networks, mechanisms, and even therapeutic interventions in response to viral infection.

Here we have analyzed the lung transcriptome by RNAseq in 41 BXD RI strains and both parental strains after infection with H1N1 IAV. By using systems genetics approaches, we have defined patterns of covariation of mRNAs with induced variation in *Ace2* expression, as well as molecular networks and regulatory relations that are likely to contribute to the host defense and/or the pathologies of H1N1-induced ALI.

Materials and methods

Mice

The subjects for this study were females belonging to 41 distinct BXD recombinant inbred (RI) strains, and the parental C57BL/6J (B6) and DBA/2J (D2) strains. The family of strains segregates for over 6 million sequence variants with minor allele frequencies greater than 0.3 [23]. They are well powered to detect major loci that contribute to genetic variation in disease susceptibility [19-22] and to define causal networks of interacting loci, genes, gene productions (mRNAs and proteins), and pathogenicity outcome measures [18, 24]. All cases were between 8- and 12-weeks-of-age when infected with virus. All were housed and maintained on a 12:12 light/ dark cycle, with *ad libitum* access to food and water.

Ethics

Protocols for the mouse experiments were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Tennessee Health Science Center.

Virus

Original stocks of mouse adapted A/Puerto Rico/8/34 (H1N1, PR8M) virus were obtained from Stefan Ludwig, University of Münster [25]. Virus stocks were propagated in the chorioallantoic cavity of 10-day-old pathogen-free embryonated chicken eggs for 48 h at 37 °C as described previously[26]. Viral titer was determined by focus-forming unit (FFU) assay as described previously [26].

Infection of mice

Animals were anesthetized by intraperitoneal injection of ketamine/xylazine (10%(v/v) of 100 mg/ml ketamine and 5%(v/v) of 20 mg/ml xylazine in 0.9% (w/v) NaCl) with a dose adjusted to body weight (200 µl/20 g body weight). Infection was performed by intranasal application of virus solution in 20 µl sterile phosphate-buffered saline (PBS), with PR8M $2x10^3$ FFU.

RNA isolation and sequencing

Mice were sacrificed three days post infection (dpi) and entire lungs were extracted. Lungs were immediately transferred to RNAlater (Qiagen), kept at 4 °C for one day, and subsequently stored at –20 °C. RNA was isolated using Qiagen Midi kit as described previously [27]. RNA quality was controlled on

a 2100 Bioanalyzer (Agilent). 500ng of total RNA was used to prepare libraries for sequencing using the Lexogen SENSE mRNA-seq library kit for Ion Torrent. Libraries were amplified for 11 cycles as the final step of library preparation. Five-hundred nanograms of total RNA was used to prepare libraries for sequencing using the Lexogen SENSE mRNA-seq library kit for Ion Torrent. Libraries were amplified for 11 cycles as the final step of library preparation. Before sequencing, 1 µl aliquots of this material was pooled and sequenced on an Ion Torrent PGM 314 chip. Barcode quantification data from the PGM were used to balance the barcodes for final pooling before sequencing. Following this final pooling, the library pools were sized to a target size of 260bp on a Pippin Prep instrument using the 2 % Pippin agarose gel. The sized libraries were examined on an Agilent High Sensitivity DNA chip, quantified using real-time PCR, and used to prepare spheres using a One-Touch 2 device. These spheres were then sequenced on an Ion Torrent Proton sequencer with a P1 chip. On average, 67 million reads were obtained per sample. RNA sequencing has been performed by the Molecular Resource Center (MRC) at UTHSC, Memphis, USA.

Read mapping and gene expression quantification

RNAseq reads were quality-trimmed using Trim Galore [28] and then mapped to the mouse mm10 reference genome or to the influenza A virus PR8M genome using STAR [29]. Read counts were summarized for genes as features using the R-package *Rsubread* [30], normalized and log2 transformed using the R-package *DESeq2* [31], and subsequently batch-corrected using the *ComBat* function of the R-package *sva* [32, 33]. For annotations of genes, ENTREZID from *Rsubread* were matched to RefSeq annotations using R-package *biomart* [34]. Duplicate RefSeq annotations were given the extension _1, _2, etc.

Heritability estimates for *Ace2*

The heritability of *Ace2* gene expression was estimated with GEMMA which used the following formula to calculate the proportion of variance in phenotypes explained (PVE) by typed genotypes (i.e. "chip heritability"):

$$\mathrm{PVE}(\boldsymbol{\beta}, \mathbf{u}, \tau) := \frac{\mathrm{V}(\mathbf{X}\boldsymbol{\beta} + \mathbf{u})}{\mathrm{V}(\mathbf{X}\boldsymbol{\beta} + \mathbf{u}) + \tau^{-1}}$$

Where "Xβ" is the sparse effects, "u" is the random effects, and "τ^{-1}" is the variance of the residual errors. GEMMA uses Markov chain Monte Carlo to estimate β, u. BXD genotype file generated January 2017 (www.genenetwork.org/webqtl/main.py? FormID=sharinginfo&GN_AccessionId=600) and *Ace2* gene expression values across the 41 BXD RI strains were used as the inputs.

Correlation analysis

The correlation analysis in this study, including gene-gene and gene-phenotype were conducted with Pearson correlation coefficient in R package. Pearson correlation *P*-value < 0.05 was used to report statistical significance.

Weighted gene co-expression network analysis (WGCNA)

WGCNA is a method aims to find co-expressed gene networks and explore the association between gene networks and phenotypes of interest, as well as hub genes in the network. In this study, Gene co-

expression networks was constructed using the WGCNA package in R [35] according to the recommended tutorials (https://horvath.genetics.ucla.edu/html/CoexpressionNetwork/Rpackages/WGCNA/Tutorials/), in which, the *Ace2* correlated genes (Pearson correlation *P*-value < 0.05) was used as the input. The network construction mainly includes the following four steps: (1) define the gene expression correlation matrix; (2) select the soft threshold β and transform the expression correlation matrix into an adjacency matrix; (3) convert the adjacent matrix to topologically overlapping matrix and then to the dissimilarity matrix; 4) hierarchical cluster the dissimilarity matrix to obtain a clustering tree and refine with dynamic hybrid cutting.

Gene set enrichment analysis

To understand the biological function and pathways involved in the gene set of interest, we performed Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway, and mammalian phenotype ontology (MPO) overrepresentation analysis on WebGestalt (http://www.webgestalt.org) [36] . This analysis was based on hypergeometric statistic test which generate *P*-values and enrich rations to evaluate whether an annotated term/category/pathway is achieved statistical enrichment in the gene set rather than by chance. In this analysis, we use the mouse genome protein coding genes as the reference background and a minimum of five genes for a category was defined.

Expression quantitative trait loci (eQTL) mapping

eQTL mapping allows the identification of chromosomal regions that specifically modulate variability in levels of mRNAs or other molecules. We performed eQTL mapping of RNA-seq expression data from lung tissues of H1N1 virus-infected BXD. Much of the analysis was done using GeneNetwork (www.genenetwork.org) [37, 38]. Likelihood ratio statistics (LRS) scores were computed using the Haley-Knott equations [39], and genome-wide significance was based on 2000 permutations. We used the BXD genotype file generated January 2017 (www.genenetwork.org/webqtl/main.py?FormID=sharinginfo&GN_AccessionId=600). In addition, Genes with significant and suggestive eQTLs detected using Haley and Knott mapping were remapped using GEMMA [40] to confirm LRS scores and locations of QTLs in GeneNetwork. GEMMA used linear mixed model mapping algorithms which can account for kinship among strains.

Genetic variations between C57BL/6J and DBA/2J

Sequence differences segregating in the BXDs are from our previous work [23]. Here, we focused exclusively on protein-coding variants—nonsense, missense, and frameshift mutations.

Bayesian network construction

The Bayesian Network Webserver (compbio.uthsc.edu/BNW) [41]was used to create and evaluate plausible causal models among genes, transcripts, and outcome measures of interest. Model were learned from the data using the default settings. Model averaging of the 1000 highest scoring networks was performed, and directed edges with weights greater than 0.5 were included in models. Finally, each variable had a maximum of four direct parents in any model.

RNAseq time-course data for H1N1 infection of the parental strains

The lung RNA-seq data was generated previously in our laboratory (see Ref [15]). In brief, two strains (B6 and D2) were infected with the H1N1 virus at 10–12 weeks of age as described above. RNA was isolated from lung for B6 at 1, 3, 5, 8, and 14 dpi and for D2 at 1, 3, and 5 dpi. Three RNA samples per strain and per time point were used for library preparation and sequencing. The reads were mapped to the mm10 reference genome. Raw read counts were extracted, normalized, and log transformed with *DEseq2* [31] after adding 1 to all raw counts.

In this study, we extracted the *Ace2* expression levels from this dataset. Also, we performed differential expression analysis between mice at 3 dpi and mock controls using DESeq2 [31] by treating strain as a covariates. The resulting *P*-values were adjusted using the Benjamini and Hochberg's approach [42]. We reported genes with *adj.P*-value < 0.05 (also known as a *q* value), and fold change (FC) > 1.3 as differentially expressed genes (DEGs).

Microarray data for SARS-CoV infected primary human airway epithelial (HAE) cells

The experimental details for this study are described in Ref. [43] In brief, human tracheobronchial epithelial cells were obtained from airway specimens resected from patients undergoing surgery. Primary cells were expanded to generate passage 1 cells, and passage 2 cells were plated at a density of $2.5x10^5$ cells per well. Human airway epithelium (HAE) cultures were generated by provision of an air-liquid interface for 6 to 8 weeks to form well-differentiated, polarized cultures. Wild-type infectious clone-derived SARS-CoV (icSARS-CoV) and ic-SARS-CoV ORF6 were derived from infectious clone constructs as described [43] Bat-SARS-CoV is comprised of the HKU3 backbone and SARS-CoV receptor binding domain and was derived from an infectious clone as described in (PMID:19036930). Triplicate cultures were infected with multiplicity of infection 3 (MOI 3) icSARS-CoV, and icSARS-CoV ORF6 on the apical surface. Total RNA was isolated from infected cells, and equivalent amounts of RNA from three biological replicates from each condition were pooled. Transcriptome profiling was performed using Agilent 4x44K whole human gene expression arrays. Raw data were downloaded from GEO (GSE47963_series_matrix.txt.gz) Expression values were normalized and sample descriptions quality controlled. A strong batch effect with respect to the donor from which HAE were derived was corrected using the R package *sva* [32, 33]. Rows with no gene description were deleted. Differentially expressed genes were determined using the R package *Limma* [45].

Data availability

The RNA-seq data from infected BXD strains has been deposited at GeneNetwork web site (www.genenetwork.org) under the accession number GN807. These data can be accessed and downloaded through the dataset query "HZI Lung Flu Infected BXD (Nov16) RNA-Seq". RNA-seq data for infection of B6 and D2 mice is at GEO under accession number GSE66040. Array data from infected primary HAE cells are available at GEO under accession number GSE47963.

3 Results

3.1 *Ace2* expression level decreased in both C57BL/6J and DBA/2J after H1N1 infection

Previously, our group has generated lung RNAseq for the B6 and D2 at 1, 3, 5, 8, and 14 dpi with H1N1. The B6 maternal parent of the BXDs is resistant to H1N1 infection, while the D2 paternal parent is highly susceptible. Here we have exploited these parental time series data to define the *Ace2* mRNA kinetic response. Overall, expression of *Ace2* in lungs is decreased progressively in both strains as H1N1 infection proceeded (**Figure 1**). In B6 mice, *Ace2* transcript dipped to the lowest level on 8 dpi (7.20 ± 0.15 SD log2 units) and remained at that low level on 14 dpi (7.23 ± 0.12). In addition, the expression levels at 3 dpi (*P*-value = 0.026, log2FC=-0.25), 5 dpi (*P*-value = 0.008, log2FC=-0.49), 8 dpi (*P*-value = 0.003, log2FC=-0.73), and 14 dpi (*P*-value = 0.002, log2FC=-0.70) significantly lower than that at 1 dpi. The infection was lethal for D2 at 5 dpi, and this was matched with lower expression of *Ace2* (7.10 ± 0.25). Statistically, *Ace2* expression levels on 3 dpi (*P*-value = 0.003, log2FC=-0.37) and 5 (*P*-value = 0.006, log2FC=-0.80) both showed significant reduction as compared with 1 dpi. Although the differences in *Ace2* expression between B6 and D2 at 3 dpi and 5 dpi did not achieve statistical significance, D2 mice have consistently showed more rapid decline in *Ace2* expression as compared with the B6 strain.

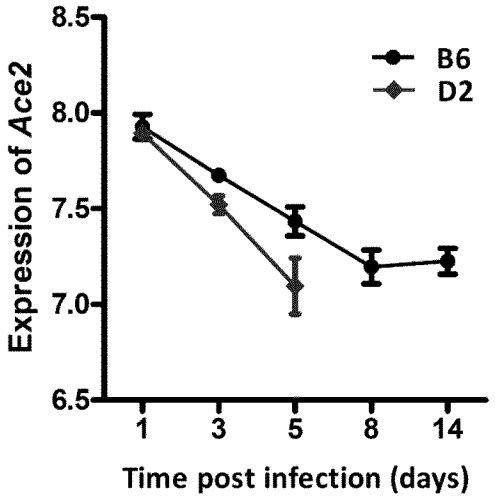


Figure 1. Line charts of the *Ace2* expression level in strains C57BL/6J and DBA/2J after H1N1 infection. Lung transcriptomes were quantified with RNA-seq for B6 and D2 strains at 1, 3, 5, 8, and 14 dpi of H1N1 infection. Three female mice (8-12 weeks of age) per strain were used for per time point. The x-axis shows the time point (days) post the infection. The y-axis shows the expression level of *Ace2* with log2 transformed values.

3.2 *Ace2* mRNA level exhibited variability across BXD RI mice at 3 dpi

All D2 mice died five days post infection. Therefore, we conducted RNAseq for a subset of BXD RI family of mice (41 BXD RI strains and two parental strains B6 and D2) at 3 dpi. As shown in **Figure 2**, *Ace2* transcript levels varied considerably across BXD family with a difference of 1.49 between extremes. The average expression of *Ace2* across all BXDs was 8.02 ± 0.15, with BXD77 having the highest expression of 8.36 and BXD22 having the lowest expression of 7.78. We also estimated the heritability of *Ace2* expression with GEMMA, which is able to calculate the so-called "chip heritability" with our RNAseq data. The resulted value was 0.181, which suggested that genetic background significantly participated in the regulation of *Ace2* mRNA level.

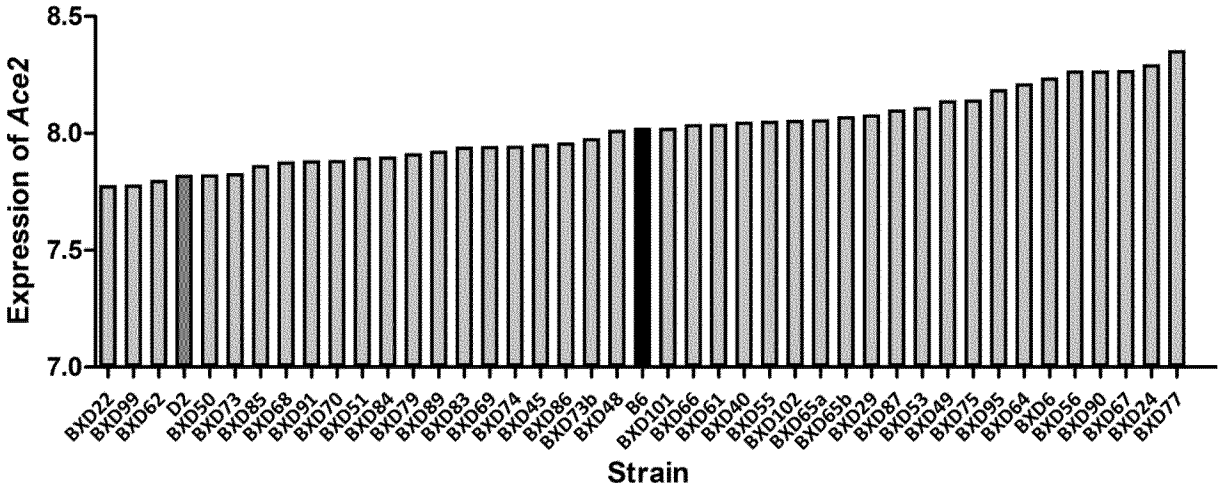


Figure 2. Bar plots of the pulmonary *Ace2* expression levels across the BXD family at 3 dpi. The x-axis shows BXDs strains and the two parental strains. The y-axis shows the normalized log2 expression levels of *Ace2*.

3.3 *Ace2* expression was associated with disease severity and viral load after infection

In order to investigate whether there is an association of *Ace2* expression with severity of disease and/or viral replication as the infection progresses, we conducted Pearson correlation coefficient analysis for *Ace2* levels against body weight loss, mean time to death, and gene expression levels as a surrogate for viral load. Results demonstrated that *Ace2* expression was significantly correlated with both body weight loss (r = 0.575, *P-value* = 0.008, **Figure 3A**) and median body weight loss (r = 0.468, *P-value* = 0.028, **Figure 3B**) at 3 dpi. Further, *Ace2* was positively correlated with the mean time to death (r = 0.458, *P-value* = 0.032, **Figure 3C**), In contrast, *Ace2* had a negative correlation with IAV NA gene expression levels (r = 0.381, *P-value* = 0.029, **Figure 3D**).

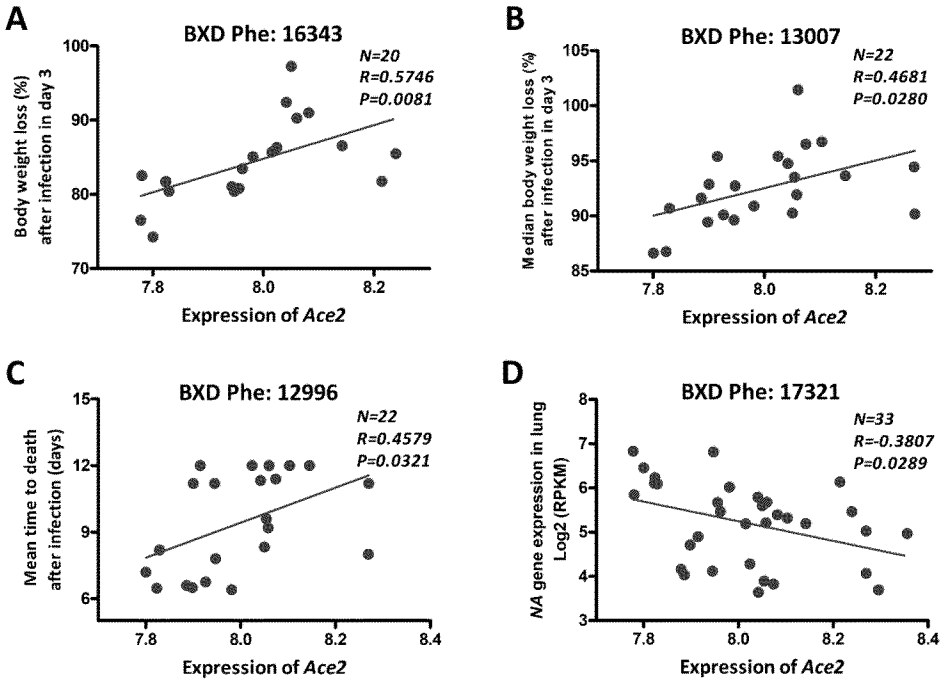


Figure 3. Scatter plots of the correlations of *Ace2* expression with body weight loss (A), median body weight loss (B), mean time to death (C), and viral *NA* gene expression level (D). Pearson correlation coefficient was used to determine the relationship. The percent body weight in **(A)** and **(B)** was relative to the starting body weight (100%). Gene expression levels represent normalized log2 values. The GeneNetwork accession number of the phenotypes (BXD Phe), number of samples (N), Pearson correlation R-value (R), and Pearson correlation *P*-value (P) are indicated in the figure.

3.4 *Ace2* correlated genes significantly involved in immune system-related pathways

To gain insight into the pulmonary pathways and biological functions that *Ace2* involved in after the virus infection, we conducted Pearson correlation coefficient analysis for *Ace2* against to the whole lung transcriptome. This analysis returned 5500 genes that were significantly correlated with *Ace2* (Pearson correlation *P*-value < 0.05). Next, we submitted those genes to Webgestalt (http://www.webgestalt.org/). 5118 genes were unambiguously mapped to unique Entrez gene IDs. Those genes were subsequently used for an enrichment analysis. The selected genes represented components of the TNF signaling pathway, MAPK signaling pathway, Notch signaling pathway, HTLV-I infection, and VEGF signaling pathway (**Figure 4A**). Furthermore, we also found that these genes were significantly enriched in terms related to chemokine secretion, lung morphology, and respiratory system morphology (**Figure 4B**).

For the top three enriched pathways, 48 genes were found in the TNF signaling pathway (**Supplementary Data 1**), with 35 negatively correlated with *Ace2* (Pearson *P*-value < 0.05), Among these included the tumor necrosis factor (*Tnf,* r = -0.41, *P*-value = 0.005). There were 92 genes in the MAPK signaling pathway (**Supplementary Data 1**), with *Tnf*, transforming growth factor beta 1(*Tgfb1*, r = -0.46, *P*-value = 0.002), and other 59 genes whose expression was negatively correlated with *Ace2*. In addition, 25 genes

were involved in the Notch signaling pathway (**Supplementary Data 1**), with 21 negatively correlated with *Ace2*.

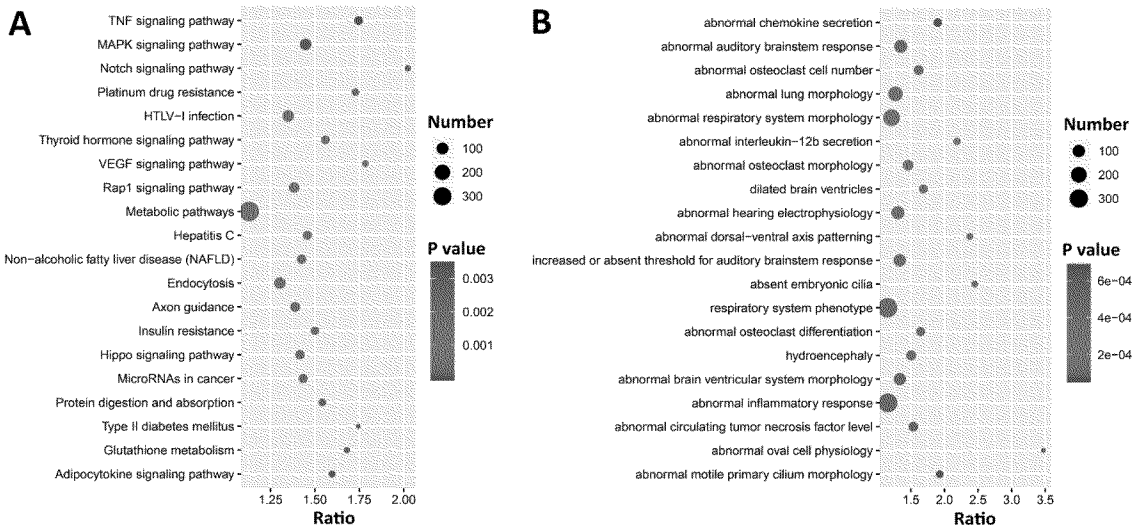


Figure 4. Bubble charts of the top 20 KEGG pathways (A) and 20 Mammalian Phenotype Ontologies (B) enriched for *Ace2* significantly correlated genes. Gene over-representation analysis for KEGG pathway and Mammalian Phenotype Ontology of the *Ace2* correlated genes (Pearson correlation *P*-value < 0.05) were performed with WebGestalt (http://www.webgestalt.org/). The x-axis represents the enrich ratio and y-axis represents enriched pathways/terms. The size of the dots denotes the number of genes and the color the *P*-value.

3.5 Co-expression network analysis narrowed down the *Ace2* correlated genes

To further identify genes that interact more directly with *Ace2*, we constructed gene co-expression network using WGCNA with the *Ace2* correlated genes (*P*-value < 0.05). The 5500 genes were parsed into 10 distinct co-expression modules using a soft-thresholding power of 8 (**Figure 5A-C**). The modules had a wide range of sizes, with the brown module containing 1673 genes and lightcyan module containing only 68 genes. *Ace2* was located in the purple module that contained 462 genes (**Figure 5C**). Genes in this module were significantly involved in regulating motile cilium physiology (**Figure 5D**)

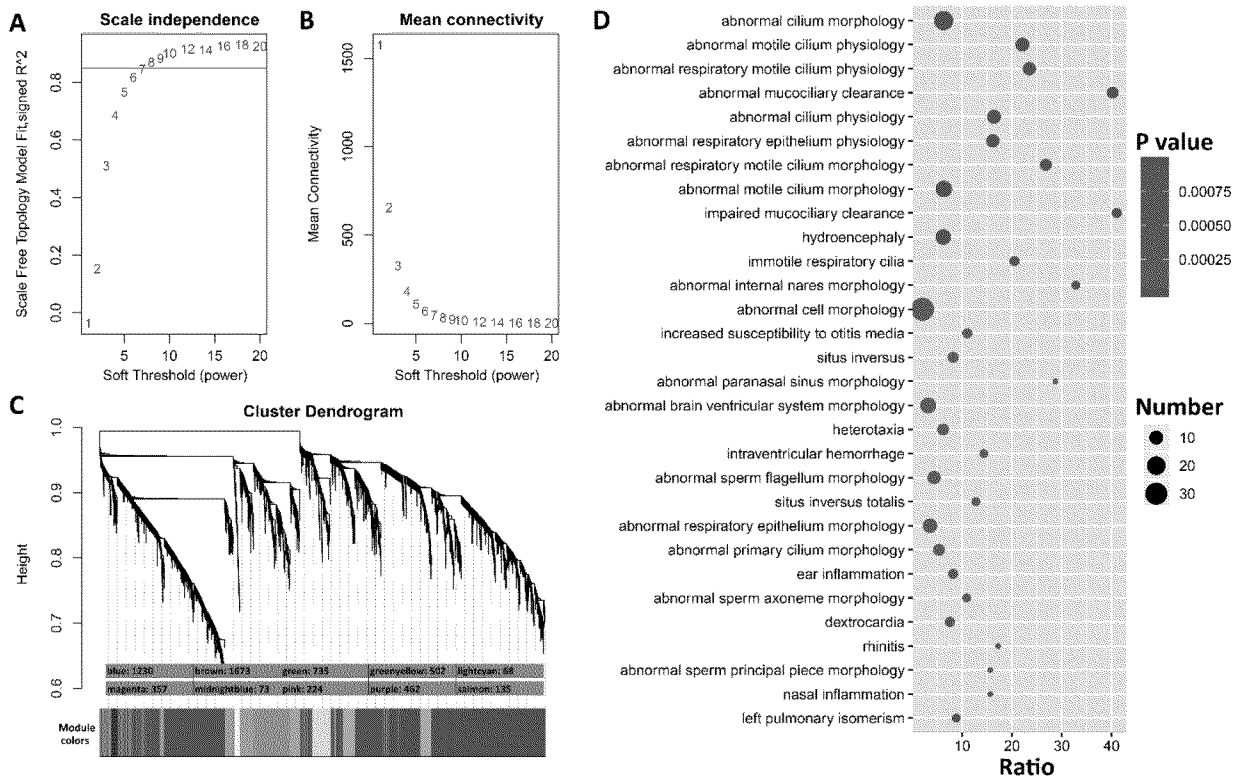


Figure 5. Weighted gene co-expression network analysis of *Ace2* correlated genes. (A) The soft thresholding index R^2 (y-axis) as a function of different powers β (x-axis) indicating the threshold of 8 chosen. **(B)** Mean connectivity (degree, y -axis) as a function of the soft-thresholding power (x -axis). **(C)** 10 co-expression modules identified from *Ace2* correlated genes. Modules are color coded as shown. The number of genes in each module is also indicated. **(D)** Bubble charts of top 30 enriched Mammalian Phenotype Ontologies of the purple module. This analysis was performed with WebGestalt (http://www.webgestalt.org/).

Next, we focused on exploring the genes in the purple module (**Figure 6** and **Supplemental File 1**). First, we performed differential expression analysis between mice at 3 dpi and mock-infected controls by treating strain as a covariate. This led to identification of 281 genes (61% of the module genes) that showed differential expression (adj.p-value < 0.05 and FC > 1.3). Next, we conducted eQTL mapping using WebQTL on the GeneNetwork for the whole lung transcriptome which identified 23 genes that were *cis*-regulated and 106 others that were *trans*-regulated in H1N1 infected lungs. Those QTLs were further confirmed with GEMMA in GeneNetwork. Moreover, we searched for the DNA sequence differences between B6 and D2, which resulted in a total of 69 genes harbor coding sequence variants (missense, nonsense, and frameshift variants). Furthermore, we found that there were 42 genes implicated in cilium function and regulation identified by enrichment analysis. By comparing the above four categories, 32 cilium function related genes were differentially expressed (**Table 1**). We refer to this set as 'prioritized genes'. Of these, *Sox11*, *Tff2*, *Rnf128*, and *Muc5b* showed *cis*-or *trans*-regulation, and *Tmprss2*, *Ulk4*,

Cckar, *Wdpcp*, *Ift140*, and *Ccdc40* contained functional variants. The genetic perturbations of these 10 genes (**Table 1**) may act as key regulators in this module.

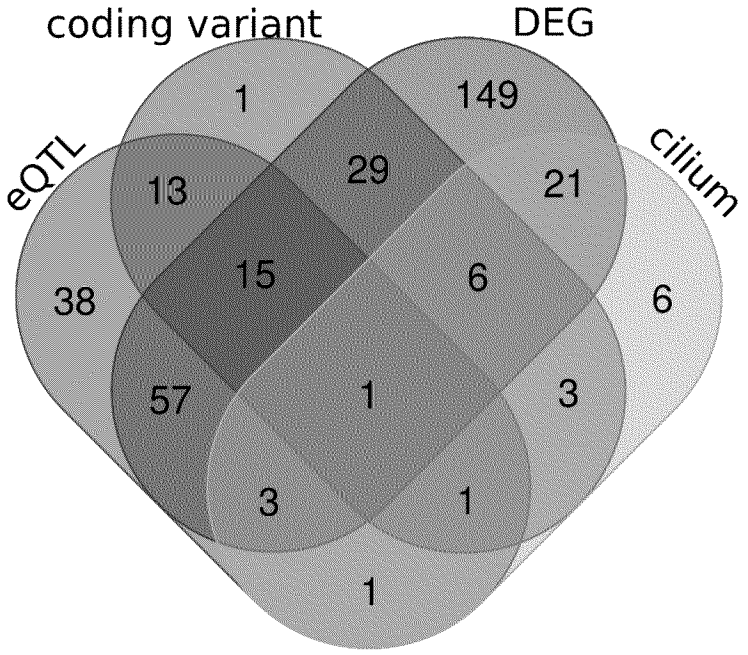


Figure 6. Venn diagrams of the purple module genes. A total of 462 genes were included in the purple module. Four criteria were applied to explore the module genes, including coding sequence variants between parental strain B6 and D2 (coding variant), eQTL (LRS > 15) regulation in H1N1 affected lung tissue (eQTL), cilium functional relevance (cilium), and differential expression (adj.p-value < 0.05 and FC > 1.3) between infected and normal controls in parental strains (DEG). The Venn diagram shows the number of overlapping genes among the four categories.

Table 1 Lists of prioritized genes in purple module.

Gene ID	Symbol	Description	Gene ID	log2FC	padj	eQTL	functional Variant
20666	**Sox11**	SRY-box 11	20666	-1.19	1.95E-12	cis	Nonsynonymous
21785	**Tff2**	trefoil factor 2 (spasmolytic protein 1)	21785	-1.01	5.38E-04	trans	NA
66889	**Rnf128**	ring finger protein 128	66889	-0.77	2.81E-05	trans	NA
74180	**Muc5b**	mucin 5, subtype B, tracheobronchial	74180	0.54	1.13E-02	trans	NA
50528	**Tmprss2**	transmembrane protease, serine 2	50528	-1.06	8.58E-10	NA	Nonsynonymous
209012	**Ulk4**	unc-51-like kinase 4 (C. elegans)	209012	-0.87	3.43E-07	NA	Nonsynonymous
12425	**Cckar**	cholecystokinin A receptor	12425	-0.73	1.91E-05	NA	Nonsynonymous
216560	**Wdpcp**	WD repeat containing planar cell polarity effector	216560	-0.69	2.36E-06	NA	Nonsynonymous
106633	**Ift140**	intraflagellar transport 140 homolog (Chlamydomonas)	106633	-0.68	3.50E-07	NA	Nonsynonymous
207607	**Ccdc40**	coiled-coil domain containing 40	207607	-0.66	1.06E-03	NA	Nonsynonymous
320752	Dpy19l2	dpy-19-like 2 (C. elegans)	320752	-1.81	2.13E-03	NA	NA
51938	Ccdc39	coiled-coil domain containing 39	51938	-1.30	1.15E-13	NA	NA
244653	Hydin	hydrocephalus inducing	244653	-1.30	8.51E-16	NA	NA
74362	Spag17	sperm associated antigen 17	74362	-1.29	9.27E-13	NA	NA
73873	Fam161a	family with sequence similarity 161, member A	73873	-1.28	1.70E-09	NA	NA

320662	Casc1	cancer susceptibility candidate 1	320662	-1.27	2.44E-08	NA	NA
66722	Spag16	sperm associated antigen 16	66722	-1.27	1.61E-10	NA	NA
74934	Armc4	armadillo repeat containing 4	74934	-1.21	8.50E-09	NA	NA
18703	Pigr	polymeric immunoglobulin receptor	18703	1.18	1.10E-09	NA	NA
69707	Iqcg	IQ motif containing G	69707	-1.18	2.97E-20	NA	NA
78801	Ak7	adenylate kinase 7	78801	-1.17	4.75E-09	NA	NA
27078	B9d1	B9 protein domain 1	27078	1.09	1.00E-02	NA	NA
68922	Dnaic1	dynein, axonemal, intermediate chain 1	68922	-1.07	3.67E-08	NA	NA
226265	Eno4	enolase 4	226265	-0.98	1.49E-09	NA	NA
71877	Efhc1	EF-hand domain (C-terminal) containing 1	71877	-0.92	3.77E-11	NA	NA
75533	Nme5	expressed in non-metastatic cells 5	75533	-0.82	5.92E-05	NA	NA
75050	Kif27	kinesin family member 27	75050	-0.71	3.05E-03	NA	NA
70008	Ace2	angiotensin I converting enzyme (peptidyl-dipeptidase A) 2	70008	-0.61	9.88E-05	NA	NA
20878	Aurka	serine/threonine kinase 6	20878	0.60	2.36E-02	NA	NA
22062	Trp73	transformation related protein 73	22062	-0.60	1.81E-03	NA	NA
77609	Ccdc151	coiled-coil domain containing 151	77609	-0.47	9.85E-02	NA	NA
252973	Grhl2	grainyhead-like 2 (Drosophila)	252973	-0.44	5.36E-04	NA	NA

Genes that were differentially expressed (adj.p-value < 0.05 and FC > 1.3) and implicated in cilium biological functions; Highlighted in bold are those 10 genes that may act as key regulators in the purple module; NA, not available.

3.6 Bayesian network analysis inferred upstream regulators of *Ace2*

In order to infer the causative relations of *Ace2* with the top 10 prioritized genes, we performed Bayesian network analysis. Ring finger protein 128 (*Rnf128*) stood out as the upstream regulator of the other 10 genes (**Figure 7**). This gene was predicted to directly target the genes intraflagellar transport 140 homolog (*Ift140*), unc-51-like kinase 4 (*Ulk4*), coiled-coil domain containing 40 (*Ccdc40*), SRY-box 11 (*Sox11*), and *Ace2*. In addition, *Ace2* is also found to be targeted through another pathway, which begins with the expression of *Rnf128,* then the expression of *Ulk4, Wdpcp*, and, finally, to *Ace2* (**Figure 7**).

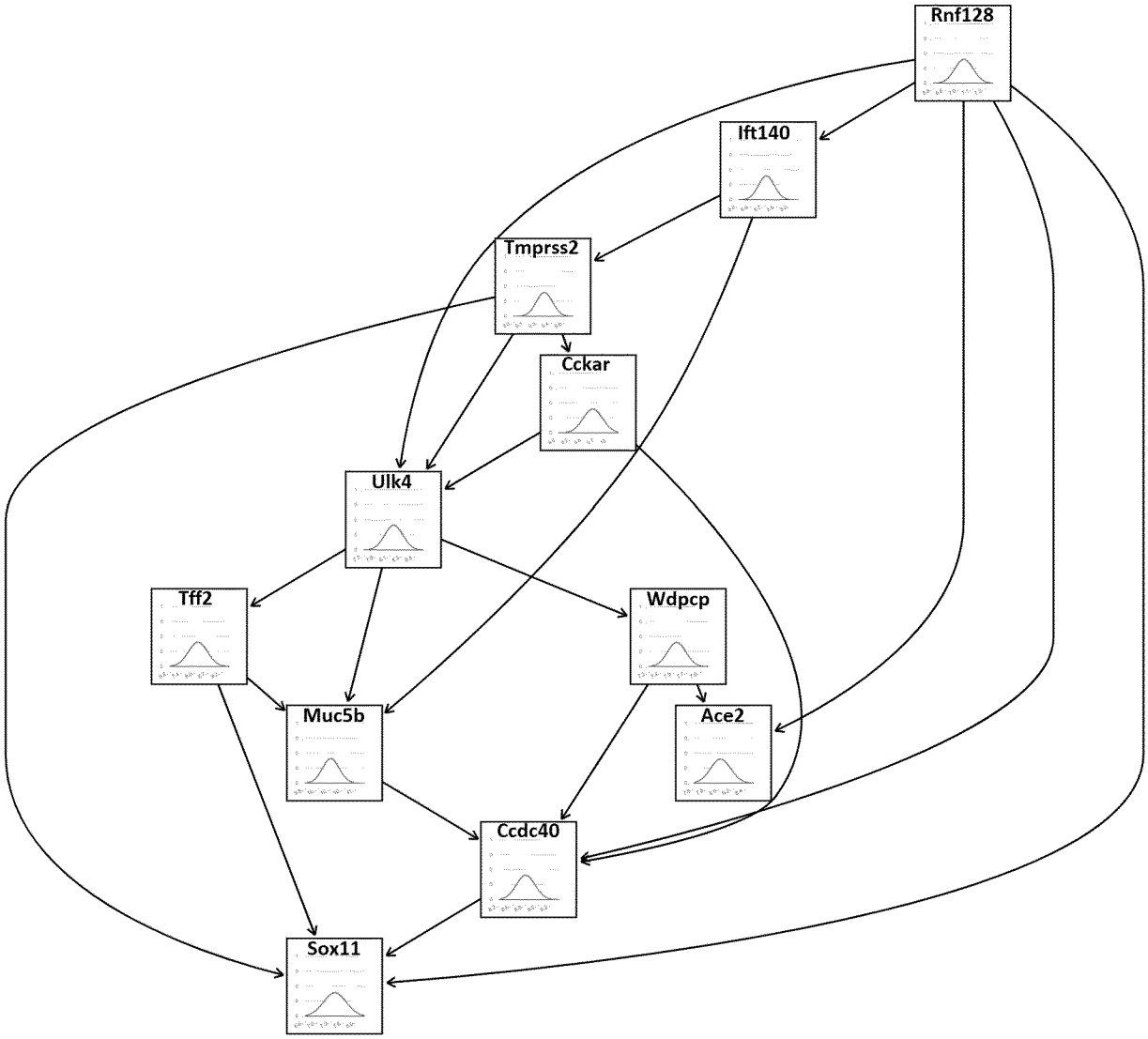


Figure 7. **Bayesian network analysis inferred upstream regulators of *Ace2***. The Bayesian network structure was constructed on Bayesian Network Webserver ((compbio.uthsc.edu/BNW) with gene expression values as the input. http://bnw.genenetwork.org/BNW/sourcecodes/layout.php?My_key=NLq

3.7 The differential expressed cilium function related genes in the purple module were replicated in SARS-CoV-infected HAE cells

In order to further evaluate whether those 'prioritized genes' (**Table 1**) were also regulated in humans, especially in the context of SARS-CoV infection, we downloaded a microarray data set for HAE cells infected with SARS-CoV (icSARS-CoV), ic-SARS-CoV ORF6 and BAT-SARS_CoV [43]. We found that very few of those 'prioritized genes' were significantly regulated after infection with BAT-SARS (**Figure 8A**). However, most of the 'prioritized genes' were differentially expressed in icSARS-CoV and ic-

SARS-CoV ORF6 infected HAE cells when compared to mock controls, with 21 genes found at 96h after infection (**Figure 8A**). More importantly, those overlapping genes, except *Ace2*, exhibited the same up- or down-regulating patterns in both HAE cells and our mouse model (**Table 1** and **Figure 8B**). *ACE2* was up-regulated in HAE cultures whereas in whole lungs it was found to be down-regulated. This is an important observation since in the HAE cultures only airway epithelial cells are present, but no interstitial cells, endothelial cells, resident and infiltrating immune cells are present. These observations indicate that in the lungs a more complex regulatory network is involved in the regulation of *ACE2* than in isolated epithelial cells and that it is indeed downregulated in all cells after infection in the whole lung. Alternatively, *ACE2* may become up-regulated only in infected cells (almost all cells in HAE cultures). In the lungs infected cells represent only a minority of all cells present. Since the our lung RNAseq study investigated total gene expression changes of all cell types it is well conceivable that we do not detect a possible up-regulation in the few infected cells. In future studies, it would be important to analyze *Ace2* expression in infected mouse lungs to test these hypotheses.

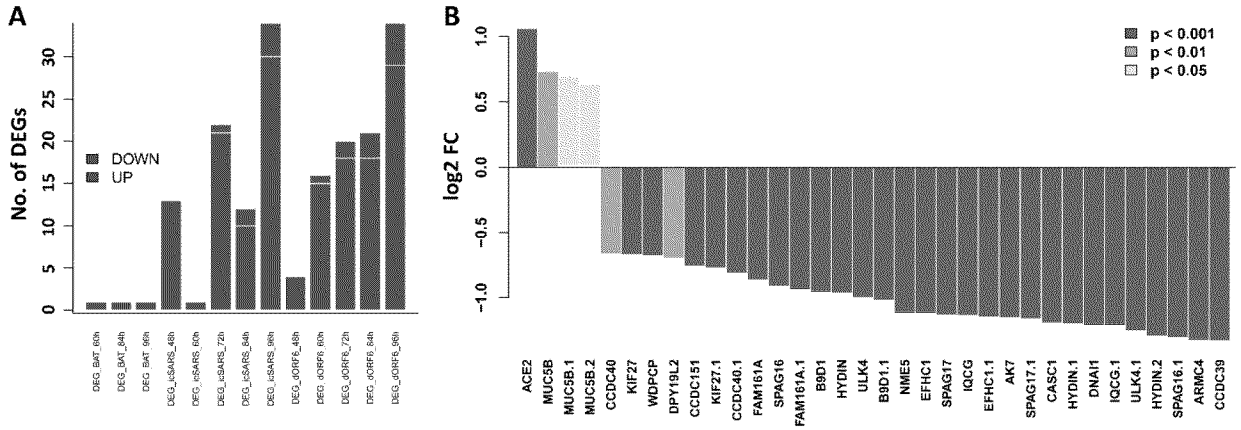


Figure 8. Bar plots of the cilium function related genes in the SARS-CoV infected HAE cells. Primary human HAE cells were infected with MOI 3 SARS-CoV (icSARS-CoV), ic-SARS-CoV ORF6 and BAT-SARS_CoV and gene expression was analyzed by arrays. (A) Number of up-and downregulated differentially expressed genes for cilium function related genes that had orthologous genes in humans and were represented on the array (logFC > 0.58 and adjusted p-value < 0.05); y-axis: number of differentially expressed genes. (B) LogFC differences in expression values between HAE cells infected with icSARS-CoV at 96h pi and mock-treated controls for cilium function related genes that had orthologous genes in humans and were represented on the array; y-axis: differences in logFC expression levels between groups. Adjusted p-values were taken from the Limma analysis of the entire transcriptome data.

Discussion

Many studies have demonstrated that host genetic background significantly influences the susceptibility to influenza A infections in humans [46]. This phenomenon was also observed in mouse models [14], especially for inbred strains B6 and D2 [15]. In this study, we systemically analyzed the potential regulatory role of *Ace2* for the host response to H1N1 infection in a subset of BXD RI mice. Our results

showed that the expression of *Ace2* in lung significantly decreased in a disease severity- and time-dependent manner. In addition, we also found the expression variation of *Ace2* negatively correlated with the body weight loss and survival time after the infection. These results strongly suggests that *Ace2* is involved in H1N1 virus disease progression and that the lower levels of *Ace2* may play a role in virus-induced ALI. This notion was supported in studies of *Ace2*$^{-/-}$ mice infected with influenza H7N9 [47] and with respiratory syncytial virus [48] where lung pathology was more severe in mutant mice.

These observations argue strongly that *Ace2* can mediate virus-induced ALI [47, 48]. However, the underlying mechanisms remain elusive. By using 43 inbred strains, we were able to perform genetic correlation analysis and gain insight into the *Ace2*-associated biological pathways. We identified a total of ~5000 *Ace2*-correlated genes. These genes are involved in regulating respiratory and inflammatory networks through several pathways, including TNF signaling pathway, MAPK signaling pathway, and Notch signaling pathway.

The factors associated with H1N1-virus-induced lethality mainly consist of virus-induced inflammatory cytokine storm and tissue destruction. Tumor necrosis factor (*TNF*) is a cytokine with multiple effects, including promoting cell growth, differentiation, apoptosis, and inducing inflammation [49, 50]. *TNF-α*, a member of the *TNF* family, can activate caspase protease, JNK, and transcription factor NF-κB, thereby executing its biological functions such as cytotoxicity, antiviral, and immuno-regulation. A previous study has demonstrated that inhibition of *Tnf-α* can protect mice from lethal H1N1 infection [51]. In the present study, we found *Ace2* was negatively correlated (r = -0.41 and *P*-value = 0.005) with the expression of *Tnf* and other 34 TNF signaling pathway related genes, suggesting that *Ace2* could dampen excessive inflammatory immune responses through downregulating of this signaling pathway and mitigating immune-mediated tissue destruction. We also found 92 genes in the MAPK signaling pathway significantly correlated with *Ace2*, including *Tgfb1* (r = -0.46 and *P*-value = 0.002). Of note, it was shown that IAV can activate this pathway to support its replication [52], The authors proposed a model in which, *Tgfb1*acts as a pro-viral host factor by suppressing early immune responses, creating an environment conducive for IAV infection [53]. Moreover, another set of 25*Ace2*-correlated genes were involved in the Notch signaling pathway, with most of them (21 genes) showing negative correlations. This pathway is an important bridge between antigen-presenting cells (APCs) and T cell communication circuits and plays a central role in driving the immune system to overcome infectious disease [54].

By constructing gene co-expression network, we narrowed the *Ace2* co-expressed genes to 462 (purple module). Enrichment analysis revealed that those genes were significantly related to cilium function, including *Ace2* itself. This finding suggests that *Ace2* participates in ALI not only by interacting with the TNF, MAPK, and Notch signaling pathways, but also through regulating functions of respiratory epithelium, which is a barrier to microbial infection and tissue injury by the secretion of mucus and the action of mucociliary clearance. In order to identify the key regulators in the purple module. We employed multi-layer filtration, including differential expression analysis, eQTL mapping, functional variant identification, and gene annotation. This resulted in 10 high priority candidates, i.e., *Sox11*, *Tff2*, *Rnf128*, *Muc5b*, *Tmprss2*, *Ulk4*, *Cckar*, *Wdpcp*, *Ift140*, and *Ccdc40*. To further investigate the relationship of these

genes with *Ace2*, we performed Bayesian network analysis and identified *Rnf128* as the master regulator. This gene encodes a ring-finger-containing protein which has been shown to possess E3 ubiquitin ligase activity [55]. *RNF128* has been implicated involved in the induction of T cell anergic phenotype through inhibiting activation-induced IL2 and IL4 cytokine production [56] and mediating T cell receptor-CD3 degradation [57]. In a recent study, Song *et al.* [58] reported *RNF128* promotes lysine-63-linked ubiquitination and activation of a critical kinase TBK1, thereby augmenting the induction of type I interferon antiviral cytokines that curb viral replication. The authors' in vivo experiments further showed that *Rnf128* deficient mice were more susceptible to vesicular stomatitis virus (an RNA virus) and herpes simplex virus (a DNA virus). Our results suggest that there are two potential gene regulatory paths for *Ace2*, both of which begin with *Rnf128*. However, direct connection of the two genes will need to be investigated experimentally in future studies.

Besides the master regulator *Rnf128*, other genes were also found to regulate the immune and respiratory epithelium system. For instance, *MUC5B* (mucin 5, subtype B, tracheobronchial), encodes a member of the mucin family of proteins, and is associated with idiopathic pulmonary fibrosis in humans [59]. Overexpression of this gene in mice causes mucociliary dysfunction, and enhances lung fibrosis [60]. It was reported to be required for airway defense in another study [61]. *CCDC40* (coiled-coil domain containing 40), encodes a protein that is essential for motile cilia function. According to the OMIM (https://omim.org/) database, this gene is associated with recurrent respiratory infections (HP:0002205) and abnormal axonemal organization of respiratory motile cilia (HP:0012258). Malfunction of *Ccdc40* in mice resulted in left pulmonary isomerism and lung situs inversus [62]. *TMPRSS2* (transmembrane protease, serine 2) encodes a protein that belongs to the serine protease family. *Tmprss2* knockout mice show reduced lung inflammation with IAVs (H1N1, H3N2, or H7N9) infection and exhibit decreased susceptibility to virus- induced morbidity/mortality [63]. Studies also demonstrated that SARS coronavirus engages *ACE2* as the entry receptor on cell surface (Ref 9: Li W et al, Nature. 2003; 426:450-4.) and employs the *TMPRSS2* for Spike protein priming, which facilitates viral entry and infection [64, 65]. Besides, *Ulk4*, *Sox11*, and *Tff2* are associated with abnormal respiratory motile cilium [66], pulmonary hypoplasia [67], and abnormal adaptive immunity [68], respectively.

The ongoing outbreak of the COVID-19 caused by SARS-CoV-2 poses a global public health threat [69-71]. The entering of SARS-CoV-2 into host cells is dependent on *ACE2*. [72, 73], the receptor for SARS-CoV and can be blocked by a clinically approved TMPRSS2 protease inhibitor [73]. However, how *ACE2* interacts with other genes involved in the progression of SARS-CoV-2 induced respiratory diseases remains to be elucidated. The first report based on autopsy samples demonstrated that the pathological characteristics of COVID-19 are very similar to those of SARS-CoV and MERS-CoV infected patients [74]. Therefore, we looked up those 30 prioritized genes (**Table 1**), co-expressed with *Ace2* after H1N1 infection, in SARS-CoV-infected primary human HAE cells. This resulted in 20 out of them showing consistent up/down-regulation with our H1N1 model, suggesting the two viruses share common *Ace2* regulatory networks that participate in the occurrence and development of lung pathology.

In summary, we have shown the expression of *Ace2* was significantly downregulated in the lungs of IAV H1N1 infected mice. By using BXD RI strains, we were able to identify *Ace2* co-expressed genes with genetic correlation analysis and weighted gene co-expression network analysis. Thus, our results indicate that *Ace2* participates in virus-induced respiratory diseases not only through immune system related pathways, but also by protective functions in the respiratory epithelium. In addition, *Rnf128* was identified as the master regulator through Bayesian network analysis. Finally, 21 of our prioritized genes were found to be also differentially expressed in SARS-CoV-infected HAE cells. These findings provide an *Ace2* regulatory network and raise several potential therapeutic targets for virus-induced (H1N1, SARS-CoV, MERS-CoV, and SARS-CoV-2) respiratory diseases. They additionally illustrate that the BXD RI strains provide an excellent resource for studying the host genetic regulatory mechanism of the viral infections, such as that caused by SARS-CoV-2.

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Author Contributions

LL, KS and RSB conceived the study; SB and ACS conducted the experiments; FX and KS performed data analysis; LL, FX, KS, and JG wrote the manuscript. FX, KS, and JG prepared the figures and tables; RWW, KL, CBJ edited the manuscript; all authors read and approved the final version of the manuscript for publication.

Conflicts of Interest

The authors declare no conflicts of interests.

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From: Purvine, Emilie[Emilie.Purvine@pnnl.gov]

Sent: Mon 3/30/2020 6:36:16 PM (UTC-07:00)

To: Amie Eisfeld[amie.eisfeld@wisc.edu]; shufang.fan@wisc.edu[shufang.fan@wisc.edu]; KEVIN B WALTERS[kevin.walters@wisc.edu]; Peter Halfmann[peter.halfmann@wisc.edu]; danielle.westhoffsmith@wisc.edu[danielle.westhoffsmith@wisc.edu]; YOSHIHIRO KAWAOKA[yoshihiro.kawaoka@wisc.edu]; Thackray, Larissa[lthackray@wustl.edu]; qtan@dom.wustl.edu[qtan@dom.wustl.edu]; Diamond, Michael[mdiamond@wustl.edu]; vmenach@UTMB.edu[vmenach@UTMB.edu]; Sims, Amy C[amy.sims@pnnl.gov]; sheahan@email.unc.edu[sheahan@email.unc.edu]; jacob.kocher@unc.edu[jacob.kocher@unc.edu]; Ralph Baric[rbaric@email.unc.edu]; Stratton, Kelly G[kelly.stratton@pnnl.gov]; Waters, Katrina M[Katrina.Waters@pnnl.gov]; Bramer, Lisa M[Lisa.Bramer@pnnl.gov]; adam_cockrell@unc.edu[adam_cockrell@unc.edu]; Heller, Natalie C[natalie.heller@pnnl.gov]

Cc: Feng, Song[song.feng@pnnl.gov]; Heath, Emily J[emily.heath@pnnl.gov]; Jefferson, Brett A[brett.jefferson@pnnl.gov]; Joslyn, Cliff A[Cliff.Joslyn@pnnl.gov]; Kvinge, Henry J[henry.kvinge@pnnl.gov]; Mcdermott, Jason E[Jason.McDermott@pnnl.gov]; Praggastis, Brenda[Brenda.Praggastis@pnnl.gov]; Mitchell, Hugh D[Hugh.Mitchell@pnnl.gov]

Subject: RE: ISMB 2020 notification for paper 230

Hello again all,

I wanted to send a quick update on this paper. The core HyperBio team (on the cc line) met last week to discuss all of the ISMB feedback and a path forward for re-submission. We decided to target a submission to BMC Bioinformatics as a Methodology article (https://bmcbioinformatics.biomedcentral.com/submission-guidelines/preparing-your-manuscript/methodology-article). We plan to make a number of revisions based on the ISMB feedback including some methodology clarifications, additional comparisons of our hypergraph methods with non-graph methods for finding important genes in host response data, and clarifying/streamlining the p-value and GSEA method of comparing results.

If any of you want to be involved in the revision process I will give you access to the Overleaf. I'm happy to have more input from the larger group! Otherwise I will send the revised version out to this distribution before submission with ample time for anybody to comment and make any final changes.

Thanks,

Emilie Purvine, Ph.D.
Senior Data Scientist and Mathematician
Pacific Northwest National Laboratory
Phone: 206-528-3461

> -----Original Message-----
> From: Purvine, Emilie
> Sent: Tuesday, March 17, 2020 4:29 PM
> To: Amie Eisfeld <amie.eisfeld@wisc.edu>; shufang.fan@wisc.edu; KEVIN B
> WALTERS <kevin.walters@wisc.edu>; Peter Halfmann
> <peter.halfmann@wisc.edu>; danielle.westhoffsmith@wisc.edu; YOSHIHIRO
> KAWAOKA <yoshihiro.kawaoka@wisc.edu>; Thackray, Larissa
> <lthackray@wustl.edu>; qtan@dom.wustl.edu; Diamond, Michael
> <mdiamond@wustl.edu>; vmenach@UTMB.edu; Sims, Amy C
> <amy.sims@pnnl.gov>; sheahan@email.unc.edu; jacob.kocher@unc.edu;
> Ralph Baric <rbaric@email.unc.edu>; Stratton, Kelly G
> <kelly.stratton@pnnl.gov>; Waters, Katrina M <Katrina.Waters@pnnl.gov>;
> Bramer, Lisa M <Lisa.Bramer@pnnl.gov>; adam_cockrell@unc.edu; Heller,
> Natalie C <natalie.heller@pnnl.gov>
> Cc: Feng, Song <song.feng@pnnl.gov>; Heath, Emily J
> <emily.heath@pnnl.gov>; Jefferson, Brett A <brett.jefferson@pnnl.gov>;
> Joslyn, Cliff A <Cliff.Joslyn@pnnl.gov>; Kvinge, Henry J
> <henry.kvinge@pnnl.gov>; Mcdermott, Jason E
> <Jason.McDermott@pnnl.gov>; Praggastis, Brenda
> <Brenda.Praggastis@pnnl.gov>; Mitchell, Hugh D <Hugh.Mitchell@pnnl.gov>

> Subject: FW: ISMB 2020 notification for paper 230
>
> Hello all,
>
> Apologies for not sending this out on Friday when I received it. Unfortunately
> the paper we submitted to ISMB was not accepted. The reviewers note a few
> things below, many of which are with respect to the p-value interpretation of
> our results. The core team here at PNNL is planning on reviewing these
> comments and revising the paper. Of course we welcome your input on that
> as well if you like. I'd also like to take this time to open up discussion among
> all of us for ways to strengthen the work and/or consider follow-on activities.
> With so many authors one zoom call is probably difficult to manage. But
> perhaps I can talk with the group from each institution separately? Or we can
> have email dialogue? I welcome suggestions!
>
> My team here is still interested in posting a version of this work on arXiv
> and/or bioRxiv but completely understand the reservations. If we address
> the reviewer comments and send out a new version, would people be
> comfortable at that point with posting it?
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> Thank you, and I look forward to a closer collaboration this time around!
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> _____
> Emilie Purvine, Ph.D.
> Senior Data Scientist and Mathematician
> Pacific Northwest National Laboratory
> Phone: 206-528-3461
>
> -----Original Message-----
> From: ismb2020@easychair.org <ismb2020@easychair.org>
> Sent: Friday, March 13, 2020 3:45 PM
> To: Purvine, Emilie <Emilie.Purvine@pnnl.gov>
> Subject: ISMB 2020 notification for paper 230
>
> Dear Emilie,
>
> Thank you for your submission to the ISMB/ECCB 2020 Proceedings Track.
> We regret to inform you that we are unable to accept your paper for
> publication. There were many strong submissions this year and only a small
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> A total of 329 papers were submitted. Of these, 64 manuscripts have been
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> Nadia El-Mabrouk and Donna Slonim
> ISMB 2020 Proceedings Chairs
>
> SUBMISSION: 230
> TITLE: Hypergraph Models of Biological Networks to Identify Genes Critical to
> Pathogenic Viral Response
>
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> AUTHORS: Song Feng, Emily Heath, Brett Jefferson, Cliff Joslyn, Henry Kvinge,
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> SCORE: -2 (reject)
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> reactions is well-accepted, even if algorithms and applications are still
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> differentially-expressed genes in a moderately-sized compendium of gene
> expression datasets related to viral pathogenesis. The authors compute the
> centrality and closeness of every node (gene) in this hypergraph, estimate
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> and compare the enrichment scores and p-values to genes ordered based on
> other criteria, including those based on graphs.
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> 2). Thus, there is very little computational innovation in this manuscript.
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> * Considering enrichment p-values is not strictly appropriate and is also an
> uninteresting way of comparing different approaches.
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> * The results do not support the authors' contention that hypergraph-based
> centrality "beats" other approaches. Considering Figure 7, the average s-
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> But this score is not appropriate for comparing different methods. For each
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> several orders of magnitude than some of the graph-based methods such as
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> than their graph counterparts because they exhibit higher GSEA scores
> (Figure 5 and Figure 7). However, it is not meaningful to compare the GSEA
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> distribution of the rank measures. To overcome this bias, it is necessary to
> compare the metrics based on the statistical significance of the enrichment
> (p-values). The comparisons based on p-values is provided in Figure 6.
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> Diamond, Ralph Baric, Katrina Waters, Yoshihiro Kawaoka and Emilie Purvine
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> SCORE: -1 (weak reject)
> ----- TEXT:
> This paper investigates the usefulness of undirected hypergraphs (a
> generalization of graphs) in prioritizing genes related to viral response across
> a broad set of gene expression datasets. The authors present a new way to
> represent this diverse gene expression data, by building a hypergraph where
> the nodes are conditions, the hyperedges are genes that are differentially
> expressed in the conditions. They then prioritize genes by hyperedge-based
> measures of closeness and centrality, which are borrowed from previous
> work, and evaluate these measures in terms of gene set enrichment for three
> "gold standard" sets of genes that are known to be involved in viral infection
> and response.
>
> This paper presents some nice ideas, and I was excited to read it. However,
> the contributions are relatively understated in this paper, and there does not
> seem to be enough rigorous evaluation and comparison of hypergraph
> measures on their dataset.
> - General statistics about the hypergraphs are not completely described. In
> Figure 4, many of the genes appear in only one condition. What should the
> reader glean from the middle panel of Figure 4? Is the pairwise intersection
> size in Figure 4 substantially different from randomized data?
> - The hypergraph is constructed by taking the absolute value of fold
> enrichment, rather than looking and over-expressed genes and under-
> expressed genes. What is the rationale for this approach, and would the
> authors find anything else if they separated these two conditions?
> - The authors choose three very general gene sets for evaluation - while they
> are from different sources, is there any further benefit for more specific GO

> terms than "immune response", for example? Do any other pathways
> appear when considering the entire ontology?
> - There seem to be some data limitations, in that an enormous amount of
> data is boiled down into a relatively small hypergraph with 179 nodes. I
> appreciate that this dual representation has benefits, but a discussion of the
> limitations and the necessarily general evaluation would help give more
> context for this application.
>
> Finally, and perhaps most importantly, the comparison to other, previously-
> published enrichment methods (including those that use graphs and those
> that do not) are missing. The authors build a hypergraph, and then compare
> centralities and other statistics of that representation to the case when the
> hypergraph becomes a graph. A major limitation is that the authors
> thresholded all the gene expression data to build the hypergraph in the first
> place. While the authors have shown that the hypergraph measures are
> better than those applied to the graph version, they have not demonstrated
> that hypergraphs achieve better enrichment than other methods that, for
> example, consider the gene expression fold change. While I think that
> hypergraph-based methods still may hold some unique power in this
> comparison, it has not been adequately described in this paper.
>
> Minor comments:
> - The authors might want to discuss the difference between hypergraphs and
> bipartite graphs to help the reader understand the benefits of hypergraphs.
> - There should be more details for the mutual information for enriching for
> important genes in the network, and the context likelihood of relatedness,
> since they are important for understanding the results.
> - The authors should consider whether singletons should be considered part
> of the hypergraph (do they contribute in any way to centrality measures?).
> Further, the size of the hypergraph shrinks considerably when removing all
> genes that appear in only one condition.
> - There is some recent work on directed hypergraphs (e.g. those that cite
> Klamt et al 2009), which might be useful to include in the discussion.
>

From: Purvine, Emilie[Emilie.Purvine@pnnl.gov]

Sent: Mon 3/30/2020 6:37:44 PM (UTC-07:00)

To: Amie Eisfeld[amie.eisfeld@wisc.edu]; shufang.fan@wisc.edu[shufang.fan@wisc.edu]; KEVIN B WALTERS[kevin.walters@wisc.edu]; Peter Halfmann[peter.halfmann@wisc.edu]; danielle.westhoffsmith@wisc.edu[danielle.westhoffsmith@wisc.edu]; YOSHIHIRO KAWAOKA[yoshihiro.kawaoka@wisc.edu]; Thackray, Larissa[lthackray@wustl.edu]; qtan@dom.wustl.edu[qtan@dom.wustl.edu]; Diamond, Michael[mdiamond@wustl.edu]; vmenach@UTMB.edu[vmenach@UTMB.edu]; Sims, Amy C[amy.sims@pnnl.gov]; sheahan@email.unc.edu[sheahan@email.unc.edu]; jacob.kocher@unc.edu[jacob.kocher@unc.edu]; Ralph Baric[rbaric@email.unc.edu]; Stratton, Kelly G[kelly.stratton@pnnl.gov]; Waters, Katrina M[Katrina.Waters@pnnl.gov]; Bramer, Lisa M[Lisa.Bramer@pnnl.gov]; adam_cockrell@unc.edu[adam_cockrell@unc.edu]; Heller, Natalie C[natalie.heller@pnnl.gov]

Cc: Feng, Song[song.feng@pnnl.gov]; Heath, Emily J[emily.heath@pnnl.gov]; Jefferson, Brett A[brett.jefferson@pnnl.gov]; Joslyn, Cliff A[Cliff.Joslyn@pnnl.gov]; Kvinge, Henry J[henry.kvinge@pnnl.gov]; Mcdermott, Jason E[Jason.McDermott@pnnl.gov]; Praggastis, Brenda[Brenda.Praggastis@pnnl.gov]; Mitchell, Hugh D[Hugh.Mitchell@pnnl.gov]

Subject: RE: ISMB 2020 notification for paper 230

Hello again all,

I wanted to send a quick update on this paper. The core HyperBio team (on the cc line) met last week to discuss all of the ISMB feedback and a path forward for re-submission. We decided to target a submission to BMC Bioinformatics as a Methodology article (https://bmcbioinformatics.biomedcentral.com/submission-guidelines/preparing-your-manuscript/methodology-article). We plan to make a number of revisions based on the ISMB feedback including some methodology clarifications, additional comparisons of our hypergraph methods with non-graph methods for finding important genes in host response data, and clarifying/streamlining the p-value and GSEA method of comparing results.

If any of you want to be involved in the revision process I will give you access to the Overleaf. I'm happy to have more input from the larger group! Otherwise I will send the revised version out to this distribution before submission with ample time for anybody to comment and make any final changes.

Thanks,

Emilie Purvine, Ph.D.
Senior Data Scientist and Mathematician
Pacific Northwest National Laboratory
Phone: 206-528-3461

> -----Original Message-----
> From: Purvine, Emilie
> Sent: Tuesday, March 17, 2020 4:29 PM
> To: Amie Eisfeld <amie.eisfeld@wisc.edu>; shufang.fan@wisc.edu; KEVIN B
> WALTERS <kevin.walters@wisc.edu>; Peter Halfmann
> <peter.halfmann@wisc.edu>; danielle.westhoffsmith@wisc.edu; YOSHIHIRO
> KAWAOKA <yoshihiro.kawaoka@wisc.edu>; Thackray, Larissa
> <lthackray@wustl.edu>; qtan@dom.wustl.edu; Diamond, Michael
> <mdiamond@wustl.edu>; vmenach@UTMB.edu; Sims, Amy C
> <amy.sims@pnnl.gov>; sheahan@email.unc.edu; jacob.kocher@unc.edu;
> Ralph Baric <rbaric@email.unc.edu>; Stratton, Kelly G
> <kelly.stratton@pnnl.gov>; Waters, Katrina M <Katrina.Waters@pnnl.gov>;
> Bramer, Lisa M <Lisa.Bramer@pnnl.gov>; adam_cockrell@unc.edu; Heller,
> Natalie C <natalie.heller@pnnl.gov>
> Cc: Feng, Song <song.feng@pnnl.gov>; Heath, Emily J
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> TITLE: Hypergraph Models of Biological Networks to Identify Genes Critical to
> Pathogenic Viral Response
> AUTHORS: Song Feng, Emily Heath, Brett Jefferson, Cliff Joslyn, Henry Kvinge,
> Jason McDermott, Hugh Mitchell, Brenda Praggastis, Amie Eisfeld, Amy Sims,
> Larissa Thackray, Shufang Fan, Kevin Walters, Peter Halfmann, Danielle
> Westhoff-Smith, Qing Tan, Vineet Menachery, Timothy Sheahan, Adam
> Cockrell, Jacob Kocher, Kelly Stratton, Natalie Heller, Lisa Bramer, Michael
> Diamond, Ralph Baric, Katrina Waters, Yoshihiro Kawaoka and Emilie Purvine
>
> ---------- Overall evaluation ----------
> SCORE: -1 (weak reject)
> ----- TEXT:
> This paper investigates the usefulness of undirected hypergraphs (a
> generalization of graphs) in prioritizing genes related to viral response across
> a broad set of gene expression datasets. The authors present a new way to
> represent this diverse gene expression data, by building a hypergraph where
> the nodes are conditions, the hyperedges are genes that are differentially
> expressed in the conditions. They then prioritize genes by hyperedge-based
> measures of closeness and centrality, which are borrowed from previous
> work, and evaluate these measures in terms of gene set enrichment for three
> "gold standard" sets of genes that are known to be involved in viral infection
> and response.
>
> This paper presents some nice ideas, and I was excited to read it. However,
> the contributions are relatively understated in this paper, and there does not
> seem to be enough rigorous evaluation and comparison of hypergraph
> measures on their dataset.
> - General statistics about the hypergraphs are not completely described. In
> Figure 4, many of the genes appear in only one condition. What should the
> reader glean from the middle panel of Figure 4? Is the pairwise intersection
> size in Figure 4 substantially different from randomized data?
> - The hypergraph is constructed by taking the absolute value of fold
> enrichment, rather than looking and over-expressed genes and under-
> expressed genes. What is the rationale for this approach, and would the
> authors find anything else if they separated these two conditions?
> - The authors choose three very general gene sets for evaluation - while they
> are from different sources, is there any further benefit for more specific GO

> terms than "immune response", for example? Do any other pathways
> appear when considering the entire ontology?
> - There seem to be some data limitations, in that an enormous amount of
> data is boiled down into a relatively small hypergraph with 179 nodes. I
> appreciate that this dual representation has benefits, but a discussion of the
> limitations and the necessarily general evaluation would help give more
> context for this application.
>
> Finally, and perhaps most importantly, the comparison to other, previously-
> published enrichment methods (including those that use graphs and those
> that do not) are missing. The authors build a hypergraph, and then compare
> centralities and other statistics of that representation to the case when the
> hypergraph becomes a graph. A major limitation is that the authors
> thresholded all the gene expression data to build the hypergraph in the first
> place. While the authors have shown that the hypergraph measures are
> better than those applied to the graph version, they have not demonstrated
> that hypergraphs achieve better enrichment than other methods that, for
> example, consider the gene expression fold change. While I think that
> hypergraph-based methods still may hold some unique power in this
> comparison, it has not been adequately described in this paper.
>
> Minor comments:
> - The authors might want to discuss the difference between hypergraphs and
> bipartite graphs to help the reader understand the benefits of hypergraphs.
> - There should be more details for the mutual information for enriching for
> important genes in the network, and the context likelihood of relatedness,
> since they are important for understanding the results.
> - The authors should consider whether singletons should be considered part
> of the hypergraph (do they contribute in any way to centrality measures?).
> Further, the size of the hypergraph shrinks considerably when removing all
> genes that appear in only one condition.
> - There is some recent work on directed hypergraphs (e.g. those that cite
> Klamt et al 2009), which might be useful to include in the discussion.
>

From: Baric, Ralph S[rbaric@email.unc.edu]
Sent: Tue 3/31/2020 4:39:22 AM (UTC-07:00)
To: Lu, Lu[llu@uthsc.edu]; Williams, Robert W[rwilli10@uthsc.edu]; Sims, Amy C[amy.sims@pnnl.gov]; silke.bergmann@(b) (6) [silke.bergmann@(b) (6)]; Jonsson, Colleen Beth[cjonsson@uthsc.edu]; Li, Kui[kli1@uthsc.edu]
Cc: Klaus x Klaus Schughart[Klaus.Schughart@helmholtz-hzi.de]; Xu, Fuyi[fxu10@uthsc.edu]
Subject: RE: Ace2
Attachment: Ace2_V7_03302020B-rsb.docx

Hi Lu Lu, Nice paper, my minor comments. Enjoyable read. ralph

From: Lu, Lu <llu@uthsc.edu>
Sent: Monday, March 30, 2020 7:00 PM
To: Williams, Robert W <rwilli10@uthsc.edu>; amy.sims@pnnl.gov; silke.bergmann@gmx.net; Baric, Ralph S <rbaric@email.unc.edu>; Jonsson, Colleen Beth <cjonsson@uthsc.edu>; Li, Kui <kli1@uthsc.edu>
Cc: Klaus x Klaus Schughart <Klaus.Schughart@helmholtz-hzi.de>; Xu, Fuyi <fxu10@uthsc.edu>
Subject: Ace2

Dear all authors:

We accepted most of your editing and suggestions for *Ace2* paper. Here is the final version (except reference section. We are working on it). Please review it again and make your final editing. Please also check your name and address. Please let me know if you don't want to be co-author for this paper. Please send your updated version back to me before this Friday. We plan to submit it at this weekend.

Thank you all for your big help and contribution.

Lu Lu
Professor
Department of Genetics, Genomics and Informatics
University of Tennessee Health Science Center
71 S. Manassas St., Room 410K
Memphis, TN 38163
Tel: (901) 448-7557 (O)
Fax: (901) 448-3500 (Dept)

> On Mar 28, 2020, at 11:25 PM, Li, Kui <kli1@uthsc.edu> wrote:
>
> Dear Lu, Klaus, Rob, and team,
>
> Please find my edits of the manuscript. Use as you see fit. Thanks,
>
> Kui

From: Lu, Lu <llu@uthsc.edu>
Sent: Thursday, March 26, 2020 6:05 PM
To: Klaus x Klaus Schughart <Klaus.Schughart@helmholtz-hzi.de>
Cc: Williams, Robert W <rwilli10@uthsc.edu>; Xu, Fuyi <fxu10@uthsc.edu>; amy.sims@pnnl.gov

<amy.sims@pnnl.gov>; rbaric@email.unc.edu <rbaric@email.unc.edu>; silke.bergmann@(b) (6)
<silke.bergmann@(b) (6)>; Li, Kui <kli1@uthsc.edu>
Subject: Re: [Ext] B6D2 infected RNAseq data

Dear Klaus (and all others):

Here is updated version. Fuyi addressed most of your comments. Please feel free to modify it continuously.

Dear Amy and Ralph:

Some of Klaus' comment are specific to you. Could you help to reply or editing?

Thanks.

Lu Lu
Professor
Department of Genetics, Genomics and Informatics
University of Tennessee Health Science Center
71 S. Manassas St., Room 410K
Memphis, TN 38163
Tel: (901) 448-7557 (O)
Fax: (901) 448-3500 (Dept)

On Mar 26, 2020, at 1:18 PM, Schughart, Klaus <Klaus.Schughart@helmholtz-hzi.de> wrote:

Hi LuLu,
enclosed are my additional comments – now also on the discussion.
Maybe Amy has also some ideas to explain the discrepancy between lungs and HAE cells with respect to inverse up/down regulation.
Klaus

Von: Lu, Lu <llu@uthsc.edu>
Gesendet: Donnerstag, 26. März 2020 00:27
An: Schughart, Klaus <Klaus.Schughart@helmholtz-hzi.de>
Cc: Williams, Robert W <rwilli10@uthsc.edu>; Xu, Fuyi <fxu10@uthsc.edu>; amy.sims@pnnl.gov
Betreff: Re: [Ext] B6D2 infected RNAseq data

Dear Klaus:

We have accepted Rob, you and our previous editing. Here is updated version for you to check and modify continuously.

Dear Amy:

Could you check this version and address some comments that are related to you?

Thanks.

Lu Lu
Professor

Department of Genetics, Genomics and Informatics
University of Tennessee Health Science Center
71 S. Manassas St., Room 410K
Memphis, TN 38163
Tel: (901) 448-7557 (O)
Fax: (901) 448-3500 (Dept)

On Mar 25, 2020, at 3:18 PM, Xu, Fuyi <fxu10@uthsc.edu> wrote:

Dear Klaus,

Could you please send me those two images (high-resolution) in Figure 8 ?
Thank you very much.

--
Fuyi Xu, PhD
Department of Genetics, Genomics, and Informatics
University of Tennessee Health Science Center

415 TRANSLATIONAL RESEARCH BUILDING
71 SOUTH MANASSAS
MEMPHIS TN 38163
Phone: (901)246-4398
E-mail: **5 U.S.C. § 552(b)(6)** ; fxu10@uthsc.edu

From: Schughart, Klaus
Sent: Wednesday, March 25, 2020 3:04 PM
To: Lu, Lu
Cc: Xu, Fuyi; Williams, Robert W
Subject: AW: [Ext] B6D2 infected RNAseq data

Hi LuLu,
I forgot something in the Acknowledgements.
Please replace the Acknowledgements in the last version with the one in the enclosed version.
Thx,
Klaus

Von: Schughart, Klaus
Gesendet: Mittwoch, 25. März 2020 20:31
An: 'Lu, Lu' <llu@uthsc.edu>
Cc: 'Xu, Fuyi' <fxu10@uthsc.edu>; 'Williams, Robert W' <rwilli10@uthsc.edu>
Betreff: AW: [Ext] B6D2 infected RNAseq data

Dear all,
here are my comments on the revised version. I took out the previous comments that have been resolved.
Fuyi, I also enclose the data table for Figure 8A.
Once you are done with this version, I can have a look at the discussion and then you could send it off to the other co-authors.

Klaus

Von: Schughart, Klaus
Gesendet: Mittwoch, 25. März 2020 19:31
An: 'Lu, Lu' <llu@uthsc.edu>
Cc: Xu, Fuyi <fxu10@uthsc.edu>; Williams, Robert W <rwilli10@uthsc.edu>
Betreff: AW: [Ext] B6D2 infected RNAseq data

Thanks – will work it.
Klaus

Von: Lu, Lu <llu@uthsc.edu>
Gesendet: Mittwoch, 25. März 2020 19:02
An: Schughart, Klaus <Klaus.Schughart@helmholtz-hzi.de>
Cc: Xu, Fuyi <fxu10@uthsc.edu>; Williams, Robert W <rwilli10@uthsc.edu>
Betreff: Re: [Ext] B6D2 infected RNAseq data

Dear Klaus:

Fuyi and I accept all of you and rob's editing. We also clean and modified the paper, which addressed comments and answered the your questions. Please work on the updated version continuously. Hopefully we can submit this paper ASAP. Thanks.

Lu Lu
Professor
Department of Genetics, Genomics and Informatics
University of Tennessee Health Science Center
71 S. Manassas St., Room 410K
Memphis, TN 38163
Tel: (901) 448-7557 (O)
Fax: (901) 448-3500 (Dept)

> On Mar 24, 2020, at 4:36 PM, Lu, Lu <llu@uthsc.edu> wrote:
>
> Thanks, Rob. We will modify based on your comments and will send you updated version for your editing again.
>
> Lu Lu
> Professor
> Department of Genetics, Genomics and Informatics
> University of Tennessee Health Science Center
> 71 S. Manassas St., Room 410K
> Memphis, TN 38163
> Tel: (901) 448-7557 (O)
> Fax: (901) 448-3500 (Dept)

On Mar 24, 2020, at 4:14 PM, Rob Williams

<rwilliams@uthsc.edu> wrote:

Dear team,

Brilliant effort,

Edited the abstract heavily (please review carefully) and built up the Methods section on BXDs a bit.

Have not gotten through all of paper, but looks great so far.

Should validate some networks and eQTLs with GEMMA.

Needs a bit more clarity to data sources—some for 43 strains, other data for parents. These details needed (imho) even in the abstract.

On Tue, Mar 24, 2020 at 2:41 PM Schughart, Klaus <Klaus.Schughart@helmholtz-hzi.de> wrote:

Hi Lulu, Fuyi,
enclosed are my comments, additions and corrections. Looks very good!!!
Please go through and clean it up first (accept or discard changes, add information) and then I can continue to work on the cleaned-up version. Currently it is too much cluttered with all these changes and comments.
Fuyi, let me know what exactly you need in addition for the HAE data.
Klaus

Von: Schughart, Klaus
Gesendet: Freitag, 6. März 2020 19:02
An: Xu, Fuyi <fxu10@uthsc.edu>; Lu Lu
5 U.S.C. § 552(b)(6)
Cc: Rob Williams <rwilliams@uthsc.edu>; Jonsson, Colleen Beth (cjonsson@uthsc.edu) <cjonsson@uthsc.edu>
Betreff: AW: [Ext] B6D2 infected RNAseq data

Hi Fuyi, LuLu,
I did some extensive analyses of genes in the human HAE data set with your gene list. Enclosed are the results. Looks very good!!
For a publication, we would have to discuss which data to include and how to present the results.
Klaus

Von: Schughart, Klaus <Klaus.Schughart@helmholtz-hzi.de>
Gesendet: Donnerstag, 5. März 2020 04:45
An: Xu, Fuyi <fxu10@uthsc.edu>
Cc: Lu Lu; **5 U.S.C. § 552(b)(6)**
Betreff: Re: [Ext] B6D2 infected RNAseq data

Dear Fuyi,
sure I will have a look at these genes.

Klaus

Von meinem iPhone gesendet

> Am 04.03.2020 um 19:09 schrieb Xu, Fuyi <fxu10@uthsc.edu>:
>
> Dear Klaus,
>
> Lu and I are working on the Ace2 paper. We prioritized around 30 genes that are correlated with Ace2 (attached table). I am wondering if you can help me check the expression differences between infected and normal controls in the human data set last time you showed us, especially for those color highlighted. Also, is this gene list make sense from your perspective? Thank you very much.
>
> Best,
> --
> Fuyi Xu, PhD
> Department of Genetics, Genomics, and Informatics
> University of Tennessee Health Science Center
>
> 415 TRANSLATIONAL RESEARCH BUILDING
> 71 SOUTH MANASSAS
> MEMPHIS TN 38163
> Phone: (901)246-4398
> E-mail: 5 U.S.C. § 552(b)(6) fxu10@uthsc.edu
>
> **From:** Schughart, Klaus
> **Sent:** Friday, February 28, 2020 11:07 AM
> **To:** Xu, Fuyi
> **Cc:** Lu Lu
> **Subject:** AW: [Ext] B6D2 infected RNAseq data
>
> Hi LuLu, Fuyi,
> I just remembered that I also have expression data from infected human lung slices (see enclosure).
> This dataset has been published (enclosed).
> Klaus
>
> **Von:** Schughart, Klaus
> **Gesendet:** Montag, 24. Februar 2020 21:00
> **An:** 'Xu, Fuyi' <fxu10@uthsc.edu>
> **Cc:** Lu Lu 5 U.S.C. § 552(b)(6)
> **Betreff:** AW: [Ext] B6D2 infected

RNAseq data

Dear Fuyi,
The data I sent you before (RNAseq_norm1.txt) have been normalized and log2 transformed using DESeq2 (rlogTransformation(dsDat, blind=TRUE).
Please find enclosed the raw counts for the B6/D2 infections from Wilk et al (all_mouse_counts.txt).

For the BXD data in GN:
The percent body weights are NOT the losses but the percent body weight with reference to the starting body weight (100%). So 105% means that the mouse has gained 5% of body weight relative to its starting weight when it was infected. Mice with percent body weight less than 100% have lost weight.

Klaus

Von: Xu, Fuyi <fxu10@uthsc.edu>
Gesendet: Sonntag, 23. Februar 2020 22:14
An: Schughart, Klaus <Klaus.Schughart@helmholtz-hzi.de>
Cc: Lu Lu [5 U.S.C. § 552(b)(6)]
Betreff: RE: [Ext] B6D2 infected RNAseq data

Dear Dr. Klaus Schughart,

I am Lu Lu's postdoc, Fuyi Xu.
Thank you for sending us the normalized RNA-seq data.
We would like to perform differential expression analysis, I am wondering if you have raw read count for this data set since the DEseq2 package depend on this kind of data matrix.
By the way, is the normalized data log2 transformed ?

We also found the expression level of ACE2 across the BXD is significantly correlated with median weight loss at day 3 after infection (ID:13007), however, we found BXD65a loss 105% body weight. Is this correct? Or how should I interpret ?

<A54B14D6A4584F84B57E3AAA25DC5BBB.gif>
Best Regards,
--
Fuyi Xu, PhD
Department of Genetics, Genomics, and

Informatics
University of Tennessee Health Science Center

415 TRANSLATIONAL RESEARCH BUILDING
71 SOUTH MANASSAS
MEMPHIS TN 38163
Phone: (901)246-4398
E-mail: 5 U.S.C. § 552(b)(6) ; fxu10@uthsc.edu

From: Lu, Lu
Sent: Monday, February 17, 2020 11:03 AM
To: Xu, Fuyi
Subject: Fwd: [Ext] B6D2 infected RNAseq data

Lu Lu
Professor
Department of Genetics, Genomics and Informatics
University of Tennessee Health Science Center
71 S. Manassas St., Room 410K
Memphis, TN 38163
Tel: (901) 448-7557 (O)
Fax: (901) 448-3500 (Dept)

Begin forwarded message:

From: "Schughart, Klaus" <Klaus.Schughart@helmholtz-hzi.de>
Subject: [Ext] B6D2 infected RNAseq data
Date: February 17, 2020 at 10:11:55 AM CST
To: "Lu, Lu" <llu@uthsc.edu>

Hi LuLu,
I checked the datasets.

GN807; RNAseq BXD infected is fine. This dataset has not been published yet.

I enclose the log2 normalized matrix for the B6/D2 infected data from Wilk et al. 2015:
RNAseq_norm1.txt -- for the mouse genes
00_influenza_lg2_RPKM_kls_280714.txt -- for the influenza gene segments

Let me know if you have any questions.
Klaus

Prof. Dr. Klaus Schughart
Head of Dept. of Infection Genetics
Helmholtz Centre for Infection Research,
University of Veterinary Medicine Hannover,
University of Tennessee Health Science Center
Inhoffenstr. 7
D-38124 Braunschweig
Germany

E-mail : Klaus.Schughart@helmholtz-hzi.de
Phone: +49 531 6181 1100

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Stellvertreter: MinDirig Rüdiger Eichel, Niedersächsisches Ministerium für Wissenschaft und Kultur
Geschäftsführung: Prof. Dr. Dirk Heinz; Silke Tannapfel
Gesellschaft mit beschränkter Haftung (GmbH)
Sitz der Gesellschaft: Braunschweig
Handelsregister: Amtsgericht Braunschweig, HRB

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Vorsitzende des Aufsichtsrates: Frau MinDir'in Prof. Dr. Veronika von Messling
Stellvertreter: MinDirig Rüdiger Eichel, Niedersächsisches Ministerium für Wissenschaft und Kultur
Geschäftsführung: Prof. Dr. Dirk Heinz; Silke Tannapfel
Gesellschaft mit beschränkter Haftung (GmbH)
Sitz der Gesellschaft: Braunschweig
Handelsregister: Amtsgericht Braunschweig, HRB 477

<Table 1.xlsx>

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Gesellschaft mit beschränkter Haftung (GmbH)
Sitz der Gesellschaft: Braunschweig
Handelsregister: Amtsgericht Braunschweig, HRB 477

--
Rob

Robert W. Williams, Ph.D.
Chair: Department of Genetics, Genomics and Informatics

University of Tennessee Health Science Center
71 S Manassas St, Memphis TN 38103-3310, TSRB
Room 405
Tele/Cell: 901 604 4752
EMAIL: rwilliams@uthsc.edu or
5 U.S.C. § 552(b)(6)

<Ace2-Draft-03182020-
V1_LuB_kls_RWW_240320_3.docx>

<8608AAAF2A14461CBDBD04DA13676A0C.png>
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Stellvertreter: MinDirig Rüdiger Eichel, Niedersächsisches Ministerium für Wissenschaft und Kultur
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Sitz der Gesellschaft: Braunschweig
Handelsregister: Amtsgericht Braunschweig, HRB 477

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Vorsitzende des Aufsichtsrates: Frau MinDir'in Prof. Dr. Veronika von Messling
Stellvertreter: MinDirig Rüdiger Eichel, Niedersächsisches Ministerium für Wissenschaft und Kultur
Geschäftsführung: Prof. Dr. Dirk Heinz; Silke Tannapfel
Gesellschaft mit beschränkter Haftung (GmbH)
Sitz der Gesellschaft: Braunschweig
Handelsregister: Amtsgericht Braunschweig, HRB 477
<Ace3_V3_250320_kls_260320.docx>

<Ace2_V4_260320_Li.docx>

Genetic Dissection of the Regulatory Mechanisms of *Ace2* in the Infected Mouse Lung

Fuyi Xu[1#], Jun Gao[1, 5#], Silke Bergman[4], Amy C. Sims[7, 9], Ralph S. Baric[6,7, 8], Colleen B Jonsson[4], Kui Li[4], Robert W. Williams[1], Klaus Schughart[2, 3, 4*], and Lu Lu[1*]

[1]Department of Genetics, Genomics, and Informatics, University of Tennessee Health Science Center, Memphis, TN, USA;

[2]Department of Infection Genetics, Helmholtz Centre for Infection Research, Braunschweig, Germany

[3]University of Veterinary Medicine Hannover, Hannover, Germany;

[4]Department of Microbiology, Immunology, and Biochemistry, University of Tennessee Health Science Center, Memphis, TN, USA;

[5]Institute of Animal Husbandry and Veterinary Science, Shanghai Academy of Agricultural Sciences, Shanghai, China.

[6]Department of Microbiology and Immunology, University of North Carolina at Chapel Hill, Chapel Hill, NC

[7]Department of Genetics, University of North Carolina at Chapel Hill, Chapel Hill, NC

[8]Department of Epidemiology, University of North Carolina at Chapel Hill, Chapel Hill, NC

[9]present address: Pacific Northwest National Laboratory in Richland, WA

[#] These authors contributed equally to this work.

* Correspondence:

Lu Lu: llu@uthsc.edu

Department of Genetics, Genomics and Informatics
University of Tennessee Health Science Center
Memphis, TN 38163, United States;

Klaus Schughart: klaus.schughart@helmholtz-hzi.de
Department of Infection Genetics
Helmholtz Centre for Infection Research
Braunschweig, Germany

Abstract

Acute lung injury (ALI) is an important cause of morbidity and mortality after viral infections, such as influenza A virus H1N1, SARS-CoV, MERS-CoV, and SARS-CoV-2. The angiotensin 1 converting enzyme 2 (ACE2) plays a protective role in SARS-CoV-induced ALI. However, contributions of *ACE2* gene variants to the post-infection heterogeneity of the host response and susceptibility remain unclear, as do the underlying mechanisms. In this study, we quantified lung transcriptomes in 41 BXD recombinant inbred strains of mice plus their parental strains of C57BL/6J and DBA/2J mice after H1N1 infection. In response to infection, the expression of *Ace2* mRNA across strains decreased significantly and was associated with both disease severity and infection time. Expression was negatively correlated with body weight loss and duration of survival time. Data for all 43 genomes were analyzed using weighted gene co-expression network analysis (WGCNA). *Ace2* not only participated in virus-induced ALI by interacting with TNF, MAPK, and notch signaling pathway, but also was linked to genes that have important functions in the pulmonary epithelium. Moreover, by applying multi-layer filtration, including differential expression analysis, eQTL mapping, functional variant identification, and gene annotation, we highlighted 10 key candidate genes linked to an *Ace2* network: *Sox11*, *Tff2*, *Rnf128*, *Muc5b*, *Tmprss2*, *Ulk4*, *Cckar*, *Wdpcp*, *Ift140*, and *Ccdc40*. Of these transcripts, *Rnf128* was identified as a potential master regulator using Bayesian network analysis. The key gene set is enriched for genes reported to be differentially expressed in SARS-CoV-infected primary human airway epithelial cells. Collectively, these findings define an *Ace2*-associated modulatory network relevant to host response to H1N1 infection and raise novel potential therapeutic targets for virus-induced respiratory diseases, including those caused by SARS-CoV and SARS-CoV-2. In addition, we demonstrate that the genetically diverse BXD family provides an excellent testbed for dissecting host genetic regulatory mechanisms involved in the defense against viral infections and host disease.

Key words: H1N1, acute lung injury, BXD mice, *Ace2,* regulatory network

Introduction

The pandemic infectious disease, COVID-19, is caused by a novel coronavirus, termed severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). This pathogen has spread with almost unprecedent speed from an initial outbreak in November or December 2019. As of March 30, 2020, according to estimates by the World Health Organization (WHO), ~195 countries and >782,000 cases of COVID-19 have been reported and linked to >37,000 deaths.

Since 1918, the world has witnessed four influenza A virus (IAV) pandemics—1918 H1N1, 1957 H2N2, 1968 H3N2, and 2009 H1N1. The first of these resulted in at least 50 million global mortalities [1]. The primary pathological features of both COVID-19 and 2009 influenza A (H1N1) infection are acute viral pneumonia and acute lung injury (ALI) [2]. The documented symptoms of influenza A (H1N1) and COVID-19 are nearly identical. For both viral pathogens infected patients can either be asymptomatic or can have symptoms that range from mild coughing, congestion, and fever, or to severe and lethal pulmonary edema. Individual genetic differences almost certainly play an important role in the development and severity of the illness and its time course [3-5].

Angiotensin-converting enzyme 2 (*ACE2*)—a negative regulator of the renin angiotensin system (RAS)—plays a protective role in pathogenesis of ALI in several animal models of acute respiratory distress syndrome [6]. *Ace2* knockout mice exhibited severe ALI. In contrast, treatment with recombinant human *ACE2* expression attenuates acute lung failure in these *Ace2* knockouts as well as in wild-type mice [7]. Studies have shown that ACE2 protein levels are significantly downregulated after infection with H1N1 virus but are dispensable for viral replication [8]. For SARS-CoV , *ACE2* has been identified as the principal host transmembrane receptor for the receptor-binding domain of the viral spike protein and thereby facilitates endocytosis of the virus and it subsequent virion replication in host cells. In *in vivo* studies, SARS-CoV infection of mice causes severe pulmonary disease and lethal lung failure, concomitant with the downregulation of *Ace2* in lungs [12]. Single-cell RNA sequencing demonstrates that 83% of *ACE2*-expressing cells in the lungs are alveolar epithelial type II cells. It is possible that there are genetic differences in host susceptibility the depend on the numbers or ratios of *ACE2*-positive AT-2 cells in lung [13]. However, we emphasize that causal linkage of *ACE2* variation to susceptibility to infection or to post-infection heterogeneity of disease progression is still very poorly defined in humans or even mouse..

Susceptibility to viral infection varies greatly among strains of isogenic mice [14], especially between two key inbred strains—C57BL/6J (B6), the reference strain for most experimental work—and DBA/2J (D2), the oldest of inbred strains of mice [15, 16]. B6 is relatively resistant to influenza A and often survives inoculation. In contrast, D2 loses body weight rapidly and at the same inoculum dose as given to B6 dies within 5–7 days. Our laboratory has generated and maintains ~120 BXD progeny strains derived from crosses between B6 and D2 parents. Both parental strains and all of these highly diverse BXD recombinant inbred progeny has been sequenced. Collectively this family segregates for ~6 million variants with minor allele frequencies above 0.3 [16], making it an ideal and well powered reference population to model genetic susceptibility to IAV infections [17]. Indeed, in previous genetic mapping using this cohort we have

defined loci and even cloned gene variants that control the host response to IAV (H1N1) infection [18] and to other infectious diseases [19-22]. The BXD family provides a powerful resource to dissect loci, gene variants, molecular networks, mechanisms, and even therapeutic interventions in response to viral infection.

Here we have analyzed the lung transcriptome by RNAseq in 41 BXD RI strains and both parental strains after infection with H1N1 IAV. By using systems genetics approaches, we have defined patterns of covariation of mRNAs with induced variation in *Ace2* expression, as well as molecular networks and regulatory relations that are likely to contribute to the host defense and/or the pathologies of H1N1-induced ALI.

Materials and methods

Mice

The subjects for this study were females belonging to 41 distinct BXD recombinant inbred (RI) strains, and the parental C57BL/6J (B6) and DBA/2J (D2) strains. The family of strains segregates for over 6 million sequence variants with minor allele frequencies greater than 0.3 [23]. They are well powered to detect major loci that contribute to genetic variation in disease susceptibility [19-22] and to define causal networks of interacting loci, genes, gene productions (mRNAs and proteins), and pathogenicity outcome measures [18, 24]. All cases were between 8- and 12-weeks-of-age when infected with virus. All were housed and maintained on a 12:12 light/ dark cycle, with *ad libitum* access to food and water.

Ethics

Protocols for the mouse experiments were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Tennessee Health Science Center.

Virus

Original stocks of mouse adapted A/Puerto Rico/8/34 (H1N1, PR8M) virus were obtained from Stefan Ludwig, University of Münster [25]. Virus stocks were propagated in the chorioallantoic cavity of 10-day-old pathogen-free embryonated chicken eggs for 48 h at 37 °C as described previously[26]. Viral titer was determined by focus-forming unit (FFU) assay as described previously [26].

Infection of mice

Animals were anesthetized by intraperitoneal injection of ketamine/xylazine (10%(v/v) of 100 mg/ml ketamine and 5%(v/v) of 20 mg/ml xylazine in 0.9% (w/v) NaCl) with a dose adjusted to body weight (200 µl/20 g body weight). Infection was performed by intranasal application of virus solution in 20 µl sterile phosphate-buffered saline (PBS), with PR8M $2x10^3$ FFU.

RNA isolation and sequencing

Mice were sacrificed three days post infection (dpi) and entire lungs were extracted. Lungs were immediately transferred to RNAlater (Qiagen), kept at 4 °C for one day, and subsequently stored at –20 °C. RNA was isolated using Qiagen Midi kit as described previously [27]. RNA quality was controlled on

a 2100 Bioanalyzer (Agilent). 500ng of total RNA was used to prepare libraries for sequencing using the Lexogen SENSE mRNA-seq library kit for Ion Torrent. Libraries were amplified for 11 cycles as the final step of library preparation. Five-hundred nanograms of total RNA was used to prepare libraries for sequencing using the Lexogen SENSE mRNA-seq library kit for Ion Torrent. Libraries were amplified for 11 cycles as the final step of library preparation. Before sequencing, 1 µl aliquots of this material was pooled and sequenced on an Ion Torrent PGM 314 chip. Barcode quantification data from the PGM were used to balance the barcodes for final pooling before sequencing. Following this final pooling, the library pools were sized to a target size of 260bp on a Pippin Prep instrument using the 2 % Pippin agarose gel. The sized libraries were examined on an Agilent High Sensitivity DNA chip, quantified using real-time PCR, and used to prepare spheres using a One-Touch 2 device. These spheres were then sequenced on an Ion Torrent Proton sequencer with a P1 chip. On average, 67 million reads were obtained per sample. RNA sequencing has been performed by the Molecular Resource Center (MRC) at UTHSC, Memphis, USA.

Read mapping and gene expression quantification

RNAseq reads were quality-trimmed using Trim Galore [28] and then mapped to the mouse mm10 reference genome or to the influenza A virus PR8M genome using STAR [29]. Read counts were summarized for genes as features using the R-package *Rsubread* [30], normalized and log2 transformed using the R-package *DESeq2* [31], and subsequently batch-corrected using the *ComBat* function of the R-package *sva* [32, 33]. For annotations of genes, ENTREZID from *Rsubread* were matched to RefSeq annotations using R-package *biomart* [34]. Duplicate RefSeq annotations were given the extension _1, _2, etc.

Heritability estimates for *Ace2*

The heritability of *Ace2* gene expression was estimated with GEMMA which used the following formula to calculate the proportion of variance in phenotypes explained (PVE) by typed genotypes (i.e. "chip heritability"):

$$\mathrm{PVE}(\boldsymbol{\beta}, \mathbf{u}, \tau) := \frac{\mathrm{V}(\mathbf{X}\boldsymbol{\beta} + \mathbf{u})}{\mathrm{V}(\mathbf{X}\boldsymbol{\beta} + \mathbf{u}) + \tau^{-1}}$$

Where "Xβ" is the sparse effects, "u" is the random effects, and "τ^{-1}" is the variance of the residual errors. GEMMA uses Markov chain Monte Carlo to estimate β, u. BXD genotype file generated January 2017 (www.genenetwork.org/webqtl/main.py? FormID=sharinginfo&GN_AccessionId=600) and *Ace2* gene expression values across the 41 BXD RI strains were used as the inputs.

Correlation analysis

The correlation analysis in this study, including gene-gene and gene-phenotype were conducted with Pearson correlation coefficient in R package. Pearson correlation *P*-value < 0.05 was used to report statistical significance.

Weighted gene co-expression network analysis (WGCNA)

WGCNA is a method aims to find co-expressed gene networks and explore the association between gene networks and phenotypes of interest, as well as hub genes in the network. In this study, Gene co-

expression networks was constructed using the WGCNA package in R [35] according to the recommended tutorials (https://horvath.genetics.ucla.edu/html/CoexpressionNetwork/Rpackages/WGCNA/Tutorials/), in which, the *Ace2* correlated genes (Pearson correlation *P*-value < 0.05) was used as the input. The network construction mainly includes the following four steps: (1) define the gene expression correlation matrix; (2) select the soft threshold β and transform the expression correlation matrix into an adjacency matrix; (3) convert the adjacent matrix to topologically overlapping matrix and then to the dissimilarity matrix; 4) hierarchical cluster the dissimilarity matrix to obtain a clustering tree and refine with dynamic hybrid cutting.

Gene set enrichment analysis

To understand the biological function and pathways involved in the gene set of interest, we performed Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway, and mammalian phenotype ontology (MPO) overrepresentation analysis on WebGestalt (http://www.webgestalt.org) [36] . This analysis was based on hypergeometric statistic test which generate *P*-values and enrich rations to evaluate whether an annotated term/category/pathway is achieved statistical enrichment in the gene set rather than by chance. In this analysis, we use the mouse genome protein coding genes as the reference background and a minimum of five genes for a category was defined.

Expression quantitative trait loci (eQTL) mapping

eQTL mapping allows the identification of chromosomal regions that specifically modulate variability in levels of mRNAs or other molecules. We performed eQTL mapping of RNA-seq expression data from lung tissues of H1N1 virus-infected BXD. Much of the analysis was done using GeneNetwork (www.genenetwork.org) [37, 38]. Likelihood ratio statistics (LRS) scores were computed using the Haley-Knott equations [39], and genome-wide significance was based on 2000 permutations. We used the BXD genotype file generated January 2017 (www.genenetwork.org/webqtl/main.py?FormID=sharinginfo&GN_AccessionId=600). In addition, Genes with significant and suggestive eQTLs detected using Haley and Knott mapping were remapped using GEMMA [40] to confirm LRS scores and locations of QTLs in GeneNetwork. GEMMA used linear mixed model mapping algorithms which can account for kinship among strains.

Genetic variations between C57BL/6J and DBA/2J

Sequence differences segregating in the BXDs are from our previous work [23]. Here, we focused exclusively on protein-coding variants—nonsense, missense, and frameshift mutations.

Bayesian network construction

The Bayesian Network Webserver (compbio.uthsc.edu/BNW) [41]was used to create and evaluate plausible causal models among genes, transcripts, and outcome measures of interest. Model were learned from the data using the default settings. Model averaging of the 1000 highest scoring networks was performed, and directed edges with weights greater than 0.5 were included in models. Finally, each variable had a maximum of four direct parents in any model.

RNAseq time-course data for H1N1 infection of the parental strains

The lung RNA-seq data was generated previously in our laboratory (see Ref [15]). In brief, two strains (B6 and D2) were infected with the H1N1 virus at 10–12 weeks of age as described above. RNA was isolated from lung for B6 at 1, 3, 5, 8, and 14 dpi and for D2 at 1, 3, and 5 dpi. Three RNA samples per strain and per time point were used for library preparation and sequencing. The reads were mapped to the mm10 reference genome. Raw read counts were extracted, normalized, and log transformed with *DEseq2* [31] after adding 1 to all raw counts.

In this study, we extracted the *Ace2* expression levels from this dataset. Also, we performed differential expression analysis between mice at 3 dpi and mock controls using DESeq2 [31] by treating strain as a covariates. The resulting *P*-values were adjusted using the Benjamini and Hochberg's approach [42]. We reported genes with *adj.P*-value < 0.05 (also known as a *q* value), and fold change (FC) > 1.3 as differentially expressed genes (DEGs).

Microarray data for SARS-CoV infected primary human airway epithelial (HAE) cells

The experimental details for this study are described in Ref. [43] In brief, human tracheobronchial epithelial cells were obtained from airway specimens resected from patients undergoing surgery. Primary cells were expanded to generate passage 1 cells, and passage 2 cells were plated at a density of $2.5x10^5$ cells per well. Human airway epithelium (HAE) cultures were generated by provision of an air-liquid interface for 6 to 8 weeks to form well-differentiated, polarized cultures. Wild-type infectious clone-derived SARS-CoV (icSARS-CoV) and ic-SARS-CoV ORF6 were derived from infectious clone constructs as described [43] Bat-SARS-CoV is comprised of the HKU3 backbone and SARS-CoV receptor binding domain and was derived from an infectious clone as described in (PMID:19036930). Triplicate cultures were infected with multiplicity of infection 3 (MOI 3) icSARS-CoV, and icSARS-CoV ORF6 on the apical surface. Total RNA was isolated from infected cells, and equivalent amounts of RNA from three biological replicates from each condition were pooled. Transcriptome profiling was performed using Agilent 4x44K whole human gene expression arrays. Raw data were downloaded from GEO (GSE47963_series_matrix.txt.gz) Expression values were normalized and sample descriptions quality controlled. A strong batch effect with respect to the donor from which HAE were derived was corrected using the R package *sva* [32, 33]. Rows with no gene description were deleted. Differentially expressed genes were determined using the R package *Limma* [45].

Data availability

The RNA-seq data from infected BXD strains has been deposited at GeneNetwork web site (www.genenetwork.org) under the accession number GN807. These data can be accessed and downloaded through the dataset query "HZI Lung Flu Infected BXD (Nov16) RNA-Seq". RNA-seq data for infection of B6 and D2 mice is at GEO under accession number GSE66040. Array data from infected primary HAE cells are available at GEO under accession number GSE47963.

3 Results

3.1 *Ace2* expression level decreased in both C57BL/6J and DBA/2J after H1N1 infection

Previously, our group has generated lung RNAseq for the B6 and D2 at 1, 3, 5, 8, and 14 dpi with H1N1. The B6 maternal parent of the BXDs is resistant to H1N1 infection, while the D2 paternal parent is highly susceptible. Here we have exploited these parental time series data to define the *Ace2* mRNA kinetic response. Overall, expression of *Ace2* in lungs is decreased progressively in both strains as H1N1 infection proceeded (**Figure 1**). In B6 mice, *Ace2* transcript dipped to the lowest level on 8 dpi (7.20 ± 0.15 SD log2 units) and remained at that low level on 14 dpi (7.23 ± 0.12). In addition, the expression levels at 3 dpi (*P*-value = 0.026, log2FC=-0.25), 5 dpi (*P*-value = 0.008, log2FC=-0.49), 8 dpi (*P*-value = 0.003, log2FC=-0.73), and 14 dpi (*P*-value = 0.002, log2FC=-0.70) significantly lower than that at 1 dpi. The infection was lethal for D2 at 5 dpi, and this was matched with lower expression of *Ace2* (7.10 ± 0.25). Statistically, *Ace2* expression levels on 3 dpi (*P*-value = 0.003, log2FC=-0.37) and 5 (*P*-value = 0.006, log2FC=-0.80) both showed significant reduction as compared with 1 dpi. Although the differences in *Ace2* expression between B6 and D2 at 3 dpi and 5 dpi did not achieve statistical significance, D2 mice have consistently showed more rapid decline in *Ace2* expression as compared with the B6 strain.

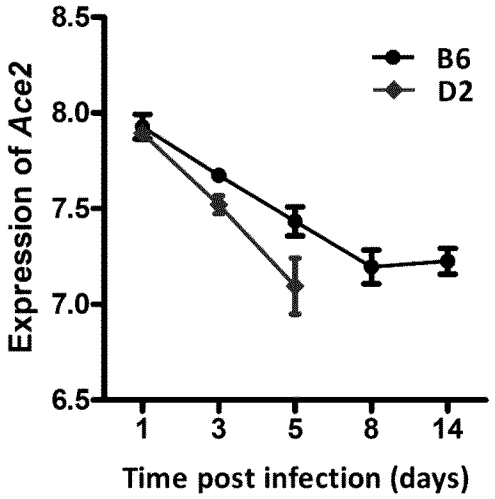


Figure 1. Line charts of the *Ace2* expression level in strains C57BL/6J and DBA/2J after H1N1 infection. Lung transcriptomes were quantified with RNA-seq for B6 and D2 strains at 1, 3, 5, 8, and 14 dpi of H1N1 infection. Three female mice (8-12 weeks of age) per strain were used for per time point. The x-axis shows the time point (days) post the infection. The y-axis shows the expression level of *Ace2* with log2 transformed values.

3.2 *Ace2* mRNA level exhibited variability across BXD RI mice at 3 dpi

All D2 mice died five days post infection. Therefore, we conducted RNAseq for a subset of BXD RI family of mice (41 BXD RI strains and two parental strains B6 and D2) at 3 dpi. As shown in **Figure 2**, *Ace2* transcript levels varied considerably across BXD family with a difference of 1.49 between extremes. The average expression of *Ace2* across all BXDs was 8.02 ± 0.15, with BXD77 having the highest expression of 8.36 and BXD22 having the lowest expression of 7.78. We also estimated the heritability of *Ace2* expression with GEMMA, which is able to calculate the so-called "chip heritability" with our RNAseq data. The resulted value was 0.181, which suggested that genetic background significantly participated in the regulation of *Ace2* mRNA level.

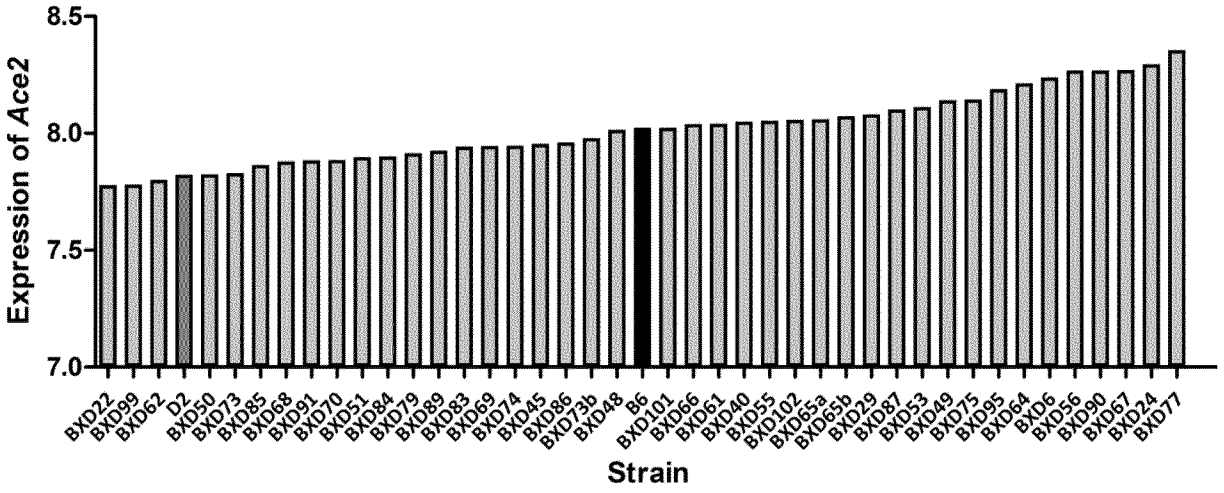


Figure 2. Bar plots of the pulmonary *Ace2* expression levels across the BXD family at 3 dpi. The x-axis shows BXDs strains and the two parental strains. The y-axis shows the normalized log2 expression levels of *Ace2*.

3.3 *Ace2* expression was associated with disease severity and viral load after infection

In order to investigate whether there is an association of *Ace2* expression with severity of disease and/or viral replication as the infection progresses, we conducted Pearson correlation coefficient analysis for *Ace2* levels against body weight loss, mean time to death, and gene expression levels as a surrogate for viral load. Results demonstrated that *Ace2* expression was significantly correlated with both body weight loss (r = 0.575, *P-value* = 0.008, **Figure 3A**) and median body weight loss (r = 0.468, *P-value* = 0.028, **Figure 3B**) at 3 dpi. Further, *Ace2* was positively correlated with the mean time to death (r = 0.458, *P-value* = 0.032, **Figure 3C**), In contrast, *Ace2* had a negative correlation with IAV NA gene expression levels (r = 0.381, *P-value* = 0.029, **Figure 3D**).

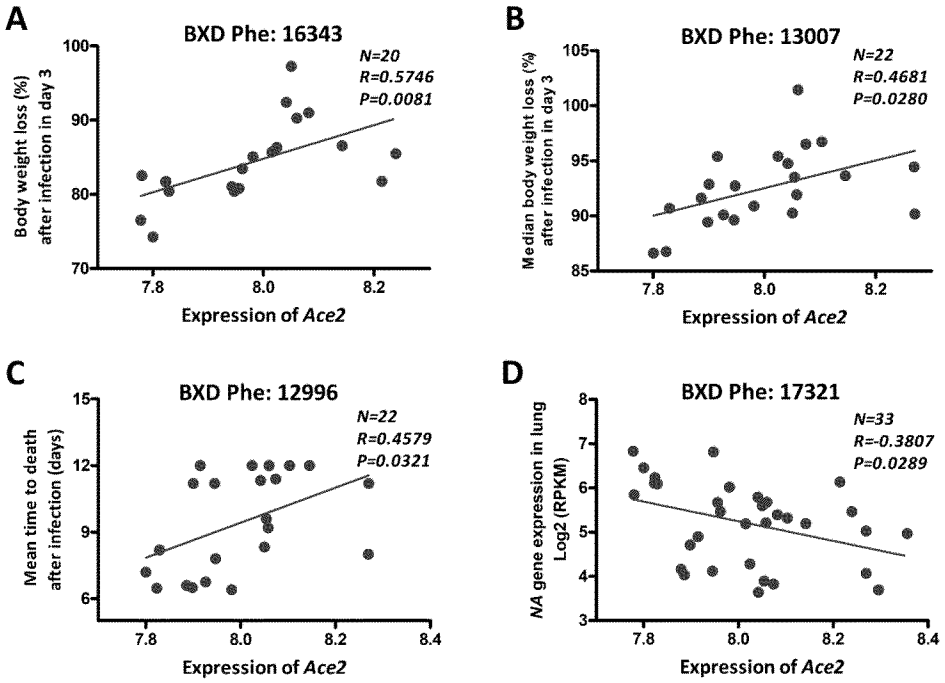


Figure 3. Scatter plots of the correlations of *Ace2* expression with body weight loss (A), median body weight loss (B), mean time to death (C), and viral *NA* gene expression level (D). Pearson correlation coefficient was used to determine the relationship. The percent body weight in **(A)** and **(B)** was relative to the starting body weight (100%). Gene expression levels represent normalized log2 values. The GeneNetwork accession number of the phenotypes (BXD Phe), number of samples (N), Pearson correlation R-value (R), and Pearson correlation *P*-value (P) are indicated in the figure.

3.4 *Ace2* correlated genes significantly involved in immune system-related pathways

To gain insight into the pulmonary pathways and biological functions that *Ace2* involved in after the virus infection, we conducted Pearson correlation coefficient analysis for *Ace2* against to the whole lung transcriptome. This analysis returned 5500 genes that were significantly correlated with *Ace2* (Pearson correlation *P*-value < 0.05). Next, we submitted those genes to Webgestalt (http://www.webgestalt.org/). 5118 genes were unambiguously mapped to unique Entrez gene IDs. Those genes were subsequently used for an enrichment analysis. The selected genes represented components of the TNF signaling pathway, MAPK signaling pathway, Notch signaling pathway, HTLV-I infection, and VEGF signaling pathway (**Figure 4A**). Furthermore, we also found that these genes were significantly enriched in terms related to chemokine secretion, lung morphology, and respiratory system morphology (**Figure 4B**).

For the top three enriched pathways, 48 genes were found in the TNF signaling pathway (**Supplementary Data 1**), with 35 negatively correlated with *Ace2* (Pearson *P*-value < 0.05), Among these included the tumor necrosis factor (*Tnf,* r = -0.41, *P*-value = 0.005). There were 92 genes in the MAPK signaling pathway (**Supplementary Data 1**), with *Tnf*, transforming growth factor beta 1(*Tgfb1*, r = -0.46, *P*-value = 0.002), and other 59 genes whose expression was negatively correlated with *Ace2*. In addition, 25 genes

were involved in the Notch signaling pathway (**Supplementary Data 1**), with 21 negatively correlated with *Ace2*.

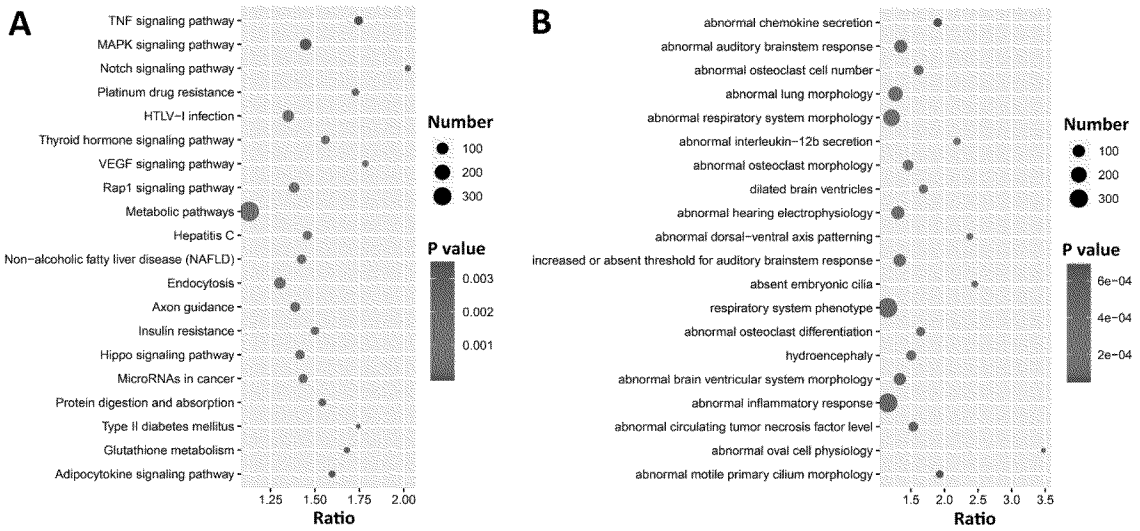


Figure 4. Bubble charts of the top 20 KEGG pathways (A) and 20 Mammalian Phenotype Ontologies (B) enriched for *Ace2* significantly correlated genes. Gene over-representation analysis for KEGG pathway and Mammalian Phenotype Ontology of the *Ace2* correlated genes (Pearson correlation *P*-value < 0.05) were performed with WebGestalt (http://www.webgestalt.org/). The x-axis represents the enrich ratio and y-axis represents enriched pathways/terms. The size of the dots denotes the number of genes and the color the *P*-value.

3.5 Co-expression network analysis narrowed down the *Ace2* correlated genes

To further identify genes that interact more directly with *Ace2*, we constructed gene co-expression network using WGCNA with the *Ace2* correlated genes (*P*-value < 0.05). The 5500 genes were parsed into 10 distinct co-expression modules using a soft-thresholding power of 8 (**Figure 5A-C**). The modules had a wide range of sizes, with the brown module containing 1673 genes and lightcyan module containing only 68 genes. *Ace2* was located in the purple module that contained 462 genes (**Figure 5C**). Genes in this module were significantly involved in regulating motile cilium physiology (**Figure 5D**)

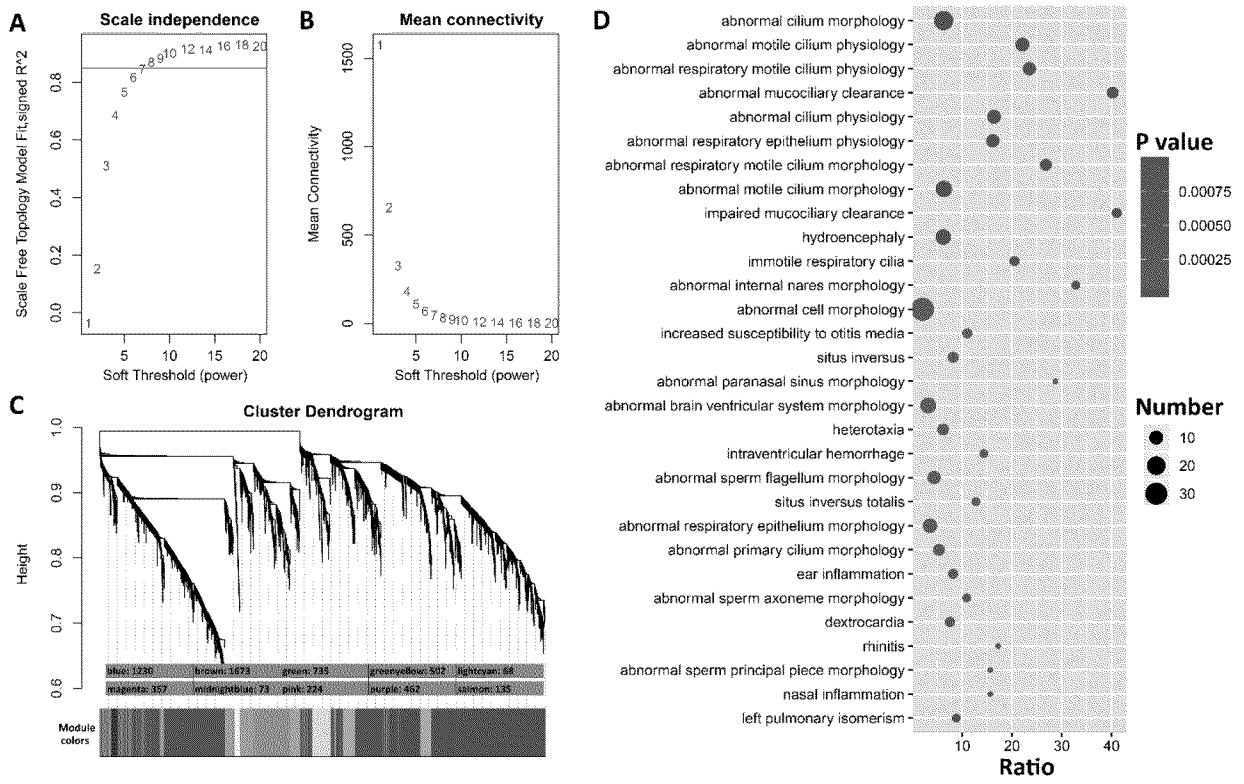


Figure 5. Weighted gene co-expression network analysis of *Ace2* correlated genes. (A) The soft thresholding index R^2 (y-axis) as a function of different powers β (x-axis) indicating the threshold of 8 chosen. **(B)** Mean connectivity (degree, y -axis) as a function of the soft-thresholding power (x -axis). **(C)** 10 co-expression modules identified from *Ace2* correlated genes. Modules are color coded as shown. The number of genes in each module is also indicated. **(D)** Bubble charts of top 30 enriched Mammalian Phenotype Ontologies of the purple module. This analysis was performed with WebGestalt (http://www.webgestalt.org/).

Next, we focused on exploring the genes in the purple module (**Figure 6** and **Supplemental File 1**). First, we performed differential expression analysis between mice at 3 dpi and mock-infected controls by treating strain as a covariate. This led to identification of 281 genes (61% of the module genes) that showed differential expression (adj.p-value < 0.05 and FC > 1.3). Next, we conducted eQTL mapping using WebQTL on the GeneNetwork for the whole lung transcriptome which identified 23 genes that were *cis*-regulated and 106 others that were *trans*-regulated in H1N1 infected lungs. Those QTLs were further confirmed with GEMMA in GeneNetwork. Moreover, we searched for the DNA sequence differences between B6 and D2, which resulted in a total of 69 genes harbor coding sequence variants (missense, nonsense, and frameshift variants). Furthermore, we found that there were 42 genes implicated in cilium function and regulation identified by enrichment analysis. By comparing the above four categories, 32 cilium function related genes were differentially expressed (**Table 1**). We refer to this set as 'prioritized genes'. Of these, *Sox11*, *Tff2*, *Rnf128*, and *Muc5b* showed *cis*-or *trans*-regulation, and *Tmprss2*, *Ulk4*,

Cckar, *Wdpcp*, *Ift140*, and *Ccdc40* contained functional variants. The genetic perturbations of these 10 genes (**Table 1**) may act as key regulators in this module.

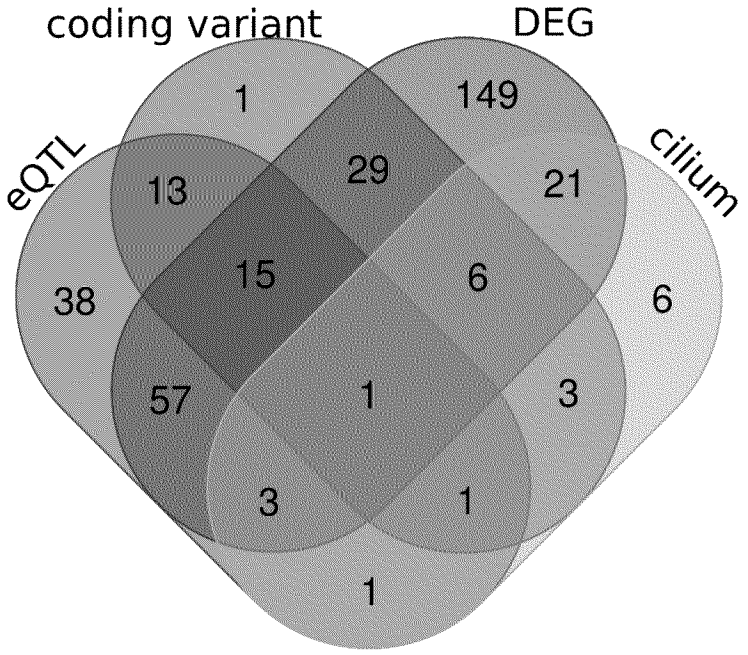


Figure 6. Venn diagrams of the purple module genes. A total of 462 genes were included in the purple module. Four criteria were applied to explore the module genes, including coding sequence variants between parental strain B6 and D2 (coding variant), eQTL (LRS > 15) regulation in H1N1 affected lung tissue (eQTL), cilium functional relevance (cilium), and differential expression (adj.p-value < 0.05 and FC > 1.3) between infected and normal controls in parental strains (DEG). The Venn diagram shows the number of overlapping genes among the four categories.

Table 1 Lists of prioritized genes in purple module.

Gene ID	Symbol	Description	Gene ID	log2FC	padj	eQTL	functional Variant
20666	**Sox11**	SRY-box 11	20666	-1.19	1.95E-12	cis	Nonsynonymous
21785	**Tff2**	trefoil factor 2 (spasmolytic protein 1)	21785	-1.01	5.38E-04	trans	NA
66889	**Rnf128**	ring finger protein 128	66889	-0.77	2.81E-05	trans	NA
74180	**Muc5b**	mucin 5, subtype B, tracheobronchial	74180	0.54	1.13E-02	trans	NA
50528	**Tmprss2**	transmembrane protease, serine 2	50528	-1.06	8.58E-10	NA	Nonsynonymous
209012	**Ulk4**	unc-51-like kinase 4 (C. elegans)	209012	-0.87	3.43E-07	NA	Nonsynonymous
12425	**Cckar**	cholecystokinin A receptor	12425	-0.73	1.91E-05	NA	Nonsynonymous
216560	**Wdpcp**	WD repeat containing planar cell polarity effector	216560	-0.69	2.36E-06	NA	Nonsynonymous
106633	**Ift140**	intraflagellar transport 140 homolog (Chlamydomonas)	106633	-0.68	3.50E-07	NA	Nonsynonymous
207607	**Ccdc40**	coiled-coil domain containing 40	207607	-0.66	1.06E-03	NA	Nonsynonymous
320752	Dpy19l2	dpy-19-like 2 (C. elegans)	320752	-1.81	2.13E-03	NA	NA
51938	Ccdc39	coiled-coil domain containing 39	51938	-1.30	1.15E-13	NA	NA
244653	Hydin	hydrocephalus inducing	244653	-1.30	8.51E-16	NA	NA
74362	Spag17	sperm associated antigen 17	74362	-1.29	9.27E-13	NA	NA
73873	Fam161a	family with sequence similarity 161, member A	73873	-1.28	1.70E-09	NA	NA

320662	Casc1	cancer susceptibility candidate 1	320662	-1.27	2.44E-08	NA	NA
66722	Spag16	sperm associated antigen 16	66722	-1.27	1.61E-10	NA	NA
74934	Armc4	armadillo repeat containing 4	74934	-1.21	8.50E-09	NA	NA
18703	Pigr	polymeric immunoglobulin receptor	18703	1.18	1.10E-09	NA	NA
69707	Iqcg	IQ motif containing G	69707	-1.18	2.97E-20	NA	NA
78801	Ak7	adenylate kinase 7	78801	-1.17	4.75E-09	NA	NA
27078	B9d1	B9 protein domain 1	27078	1.09	1.00E-02	NA	NA
68922	Dnaic1	dynein, axonemal, intermediate chain 1	68922	-1.07	3.67E-08	NA	NA
226265	Eno4	enolase 4	226265	-0.98	1.49E-09	NA	NA
71877	Efhc1	EF-hand domain (C-terminal) containing 1	71877	-0.92	3.77E-11	NA	NA
75533	Nme5	expressed in non-metastatic cells 5	75533	-0.82	5.92E-05	NA	NA
75050	Kif27	kinesin family member 27	75050	-0.71	3.05E-03	NA	NA
70008	Ace2	angiotensin I converting enzyme (peptidyl-dipeptidase A) 2	70008	-0.61	9.88E-05	NA	NA
20878	Aurka	serine/threonine kinase 6	20878	0.60	2.36E-02	NA	NA
22062	Trp73	transformation related protein 73	22062	-0.60	1.81E-03	NA	NA
77609	Ccdc151	coiled-coil domain containing 151	77609	-0.47	9.85E-02	NA	NA
252973	Grhl2	grainyhead-like 2 (Drosophila)	252973	-0.44	5.36E-04	NA	NA

Genes that were differentially expressed (adj.p-value < 0.05 and FC > 1.3) and implicated in cilium biological functions; Highlighted in bold are those 10 genes that may act as key regulators in the purple module; NA, not available.

3.6 Bayesian network analysis inferred upstream regulators of *Ace2*

In order to infer the causative relations of *Ace2* with the top 10 prioritized genes, we performed Bayesian network analysis. Ring finger protein 128 (*Rnf128*) stood out as the upstream regulator of the other 10 genes (**Figure 7**). This gene was predicted to directly target the genes intraflagellar transport 140 homolog (*Ift140*), unc-51-like kinase 4 (*Ulk4*), coiled-coil domain containing 40 (*Ccdc40*), SRY-box 11 (*Sox11*), and *Ace2*. In addition, *Ace2* is also found to be targeted through another pathway, which begins with the expression of *Rnf128,* then the expression of *Ulk4, Wdpcp*, and, finally, to *Ace2* (**Figure 7**).

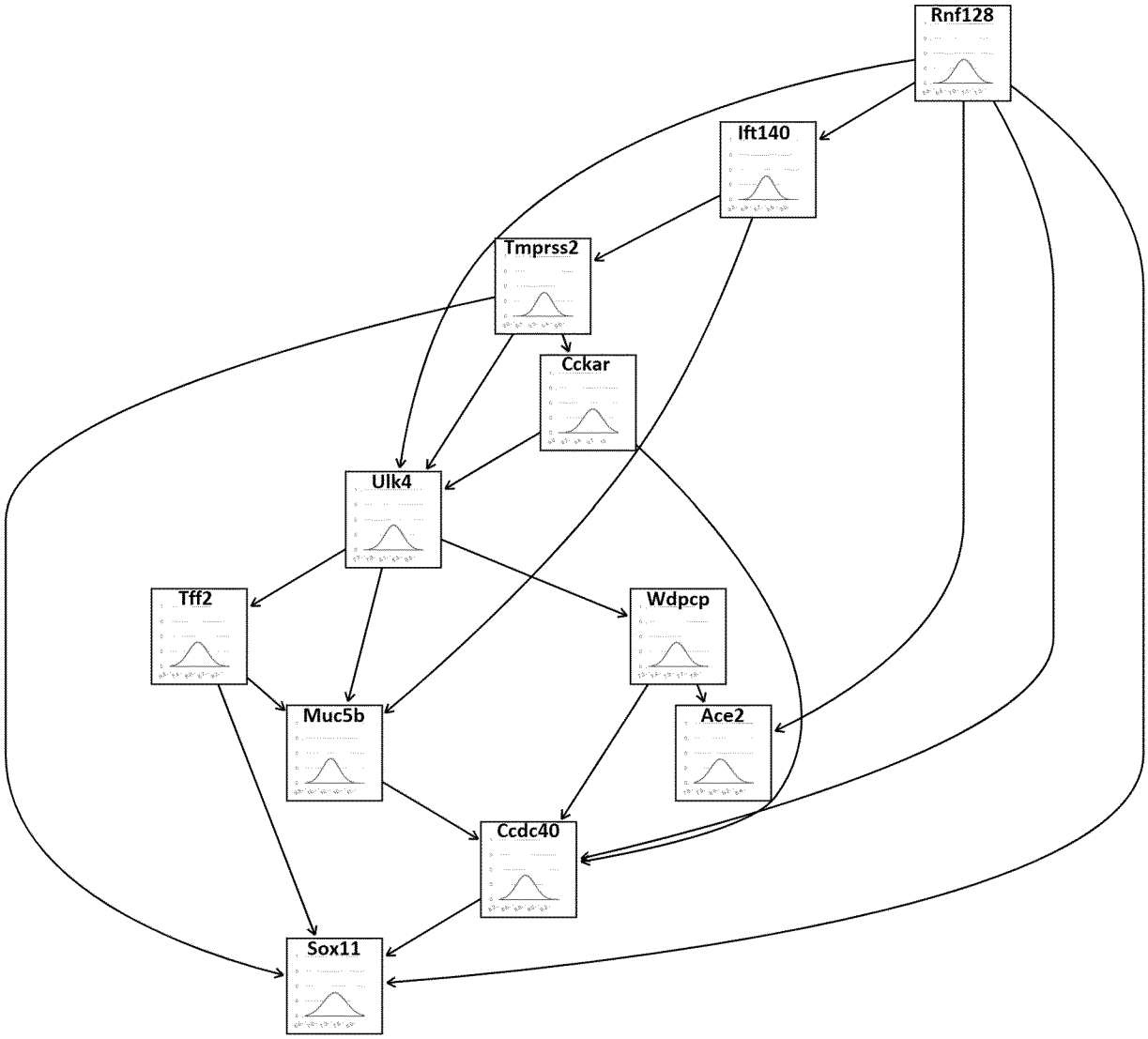


Figure 7. **Bayesian network analysis inferred upstream regulators of *Ace2***. The Bayesian network structure was constructed on Bayesian Network Webserver ((compbio.uthsc.edu/BNW) with gene expression values as the input. http://bnw.genenetwork.org/BNW/sourcecodes/layout.php?My_key=NLq

3.7 The differential expressed cilium function related genes in the purple module were replicated in SARS-CoV-infected HAE cells

In order to further evaluate whether those 'prioritized genes' (**Table 1**) were also regulated in humans, especially in the context of SARS-CoV infection, we downloaded a microarray data set for HAE cells infected with SARS-CoV (icSARS-CoV), ic-SARS-CoV ORF6 and BAT-SARS_CoV [43]. We found that very few of those 'prioritized genes' were significantly regulated after infection with BAT-SARS (**Figure 8A**). However, most of the 'prioritized genes' were differentially expressed in icSARS-CoV and ic-

SARS-CoV ORF6 infected HAE cells when compared to mock controls, with 21 genes found at 96h after infection (**Figure 8A**). More importantly, those overlapping genes, except *Ace2*, exhibited the same up- or down-regulating patterns in both HAE cells and our mouse model (**Table 1** and **Figure 8B**). *ACE2* was up-regulated in HAE cultures whereas in whole lungs it was found to be down-regulated. This is an important observation since in the HAE cultures only airway epithelial cells are present, but no interstitial cells, endothelial cells, resident and infiltrating immune cells are present. These observations indicate that in the lungs a more complex regulatory network is involved in the regulation of *ACE2* than in isolated epithelial cells and that it is indeed downregulated in all cells after infection in the whole lung. Alternatively, *ACE2* may become up-regulated only in infected cells (almost all cells in HAE cultures). In the lungs infected cells represent only a minority of all cells present. Since the our lung RNAseq study investigated total gene expression changes of all cell types it is well conceivable that we do not detect a possible up-regulation in the few infected cells. In future studies, it would be important to analyze *Ace2* expression in infected mouse lungs to test these hypotheses.

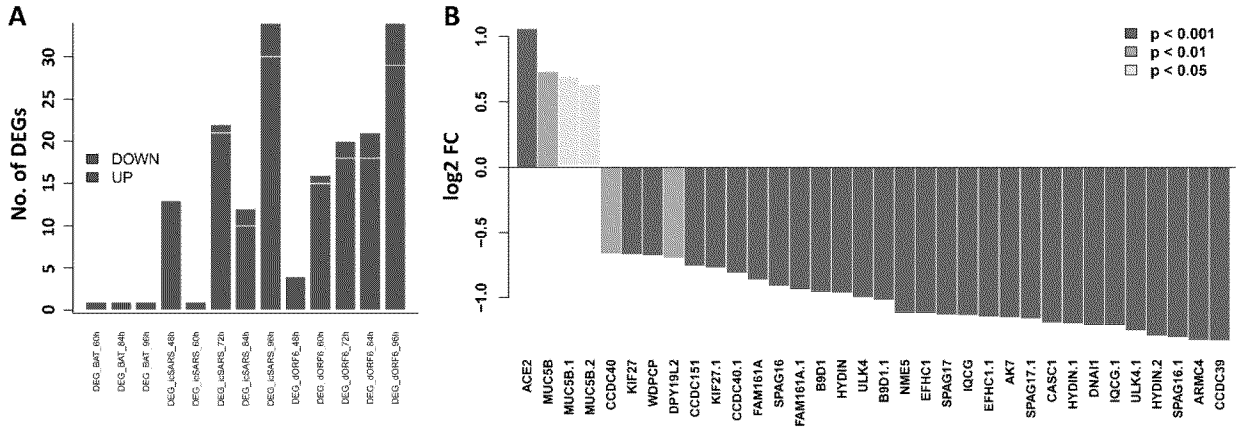


Figure 8. Bar plots of the cilium function related genes in the SARS-CoV infected HAE cells. Primary human HAE cells were infected with MOI 3 SARS-CoV (icSARS-CoV), ic-SARS-CoV ORF6 and BAT-SARS_CoV and gene expression was analyzed by arrays. (A) Number of up-and downregulated differentially expressed genes for cilium function related genes that had orthologous genes in humans and were represented on the array (logFC > 0.58 and adjusted p-value < 0.05); y-axis: number of differentially expressed genes. (B) LogFC differences in expression values between HAE cells infected with icSARS-CoV at 96h pi and mock-treated controls for cilium function related genes that had orthologous genes in humans and were represented on the array; y-axis: differences in logFC expression levels between groups. Adjusted p-values were taken from the Limma analysis of the entire transcriptome data.

Discussion

Many studies have demonstrated that host genetic background significantly influences the susceptibility to influenza A infections in humans [46]. This phenomenon was also observed in mouse models [14], especially for inbred strains B6 and D2 [15]. In this study, we systemically analyzed the potential regulatory role of *Ace2* for the host response to H1N1 infection in a subset of BXD RI mice. Our results

showed that the expression of *Ace2* in lung significantly decreased in a disease severity- and time-dependent manner. In addition, we also found the expression variation of *Ace2* negatively correlated with the body weight loss and survival time after the infection. These results strongly suggests that *Ace2* is involved in H1N1 virus disease progression and that the lower levels of *Ace2* may play a role in virus-induced ALI. This notion was supported in studies of *Ace2*$^{-/-}$ mice infected with influenza H7N9 [47] and with respiratory syncytial virus [48] where lung pathology was more severe in mutant mice.

These observations argue strongly that *Ace2* can mediate virus-induced ALI [47, 48]. However, the underlying mechanisms remain elusive. By using 43 inbred strains, we were able to perform genetic correlation analysis and gain insight into the *Ace2*-associated biological pathways. We identified a total of ~5000 *Ace2*-correlated genes. These genes are involved in regulating respiratory and inflammatory networks through several pathways, including TNF signaling pathway, MAPK signaling pathway, and Notch signaling pathway.

The factors associated with H1N1-virus-induced lethality mainly consist of virus-induced inflammatory cytokine storm and tissue destruction. Tumor necrosis factor (*TNF*) is a cytokine with multiple effects, including promoting cell growth, differentiation, apoptosis, and inducing inflammation [49, 50]. *TNF-α*, a member of the *TNF* family, can activate caspase protease, JNK, and transcription factor NF-κB, thereby executing its biological functions such as cytotoxicity, antiviral, and immuno-regulation. A previous study has demonstrated that inhibition of *Tnf-α* can protect mice from lethal H1N1 infection [51]. In the present study, we found *Ace2* was negatively correlated (r = -0.41 and *P*-value = 0.005) with the expression of *Tnf* and other 34 TNF signaling pathway related genes, suggesting that *Ace2* could dampen excessive inflammatory immune responses through downregulating of this signaling pathway and mitigating immune-mediated tissue destruction. We also found 92 genes in the MAPK signaling pathway significantly correlated with *Ace2*, including *Tgfb1* (r = -0.46 and *P*-value = 0.002). Of note, it was shown that IAV can activate this pathway to support its replication [52], The authors proposed a model in which, *Tgfb1*acts as a pro-viral host factor by suppressing early immune responses, creating an environment conducive for IAV infection [53]. Moreover, another set of 25*Ace2*-correlated genes were involved in the Notch signaling pathway, with most of them (21 genes) showing negative correlations. This pathway is an important bridge between antigen-presenting cells (APCs) and T cell communication circuits and plays a central role in driving the immune system to overcome infectious disease [54].

By constructing gene co-expression network, we narrowed the *Ace2* co-expressed genes to 462 (purple module). Enrichment analysis revealed that those genes were significantly related to cilium function, including *Ace2* itself. This finding suggests that *Ace2* participates in ALI not only by interacting with the TNF, MAPK, and Notch signaling pathways, but also through regulating functions of respiratory epithelium, which is a barrier to microbial infection and tissue injury by the secretion of mucus and the action of mucociliary clearance. In order to identify the key regulators in the purple module. We employed multi-layer filtration, including differential expression analysis, eQTL mapping, functional variant identification, and gene annotation. This resulted in 10 high priority candidates, i.e., *Sox11*, *Tff2*, *Rnf128*, *Muc5b*, *Tmprss2*, *Ulk4*, *Cckar*, *Wdpcp*, *Ift140*, and *Ccdc40*. To further investigate the relationship of these

genes with *Ace2*, we performed Bayesian network analysis and identified *Rnf128* as the master regulator. This gene encodes a ring-finger-containing protein which has been shown to possess E3 ubiquitin ligase activity [55]. *RNF128* has been implicated involved in the induction of T cell anergic phenotype through inhibiting activation-induced IL2 and IL4 cytokine production [56] and mediating T cell receptor-CD3 degradation [57]. In a recent study, Song *et al.* [58] reported *RNF128* promotes lysine-63-linked ubiquitination and activation of a critical kinase TBK1, thereby augmenting the induction of type I interferon antiviral cytokines that curb viral replication. The authors' in vivo experiments further showed that *Rnf128* deficient mice were more susceptible to vesicular stomatitis virus (an RNA virus) and herpes simplex virus (a DNA virus). Our results suggest that there are two potential gene regulatory paths for *Ace2*, both of which begin with *Rnf128*. However, direct connection of the two genes will need to be investigated experimentally in future studies.

Besides the master regulator *Rnf128*, other genes were also found to regulate the immune and respiratory epithelium system. For instance, *MUC5B* (mucin 5, subtype B, tracheobronchial), encodes a member of the mucin family of proteins, and is associated with idiopathic pulmonary fibrosis in humans [59]. Overexpression of this gene in mice causes mucociliary dysfunction, and enhances lung fibrosis [60]. It was reported to be required for airway defense in another study [61]. *CCDC40* (coiled-coil domain containing 40), encodes a protein that is essential for motile cilia function. According to the OMIM (https://omim.org/) database, this gene is associated with recurrent respiratory infections (HP:0002205) and abnormal axonemal organization of respiratory motile cilia (HP:0012258). Malfunction of *Ccdc40* in mice resulted in left pulmonary isomerism and lung situs inversus [62]. *TMPRSS2* (transmembrane protease, serine 2) encodes a protein that belongs to the serine protease family. *Tmprss2* knockout mice show reduced lung inflammation with IAVs (H1N1, H3N2, or H7N9) infection and exhibit decreased susceptibility to virus- induced morbidity/mortality [63]. Studies also demonstrated that SARS coronavirus engages *ACE2* as the entry receptor on cell surface (Ref 9: Li W et al, Nature. 2003; 426:450-4.) and employs the *TMPRSS2* for Spike protein priming, which facilitates viral entry and infection [64, 65]. Besides, *Ulk4*, *Sox11*, and *Tff2* are associated with abnormal respiratory motile cilium [66], pulmonary hypoplasia [67], and abnormal adaptive immunity [68], respectively.

The ongoing outbreak of the COVID-19 caused by SARS-CoV-2 poses a global public health threat [69-71]. The entering of SARS-CoV-2 into host cells is dependent on *ACE2*. [72, 73], the receptor for SARS-CoV and can be blocked by a clinically approved TMPRSS2 protease inhibitor [73]. However, how *ACE2* interacts with other genes involved in the progression of SARS-CoV-2 induced respiratory diseases remains to be elucidated. The first report based on autopsy samples demonstrated that the pathological characteristics of COVID-19 are very similar to those of SARS-CoV and MERS-CoV infected patients [74]. Therefore, we looked up those 30 prioritized genes (**Table 1**), co-expressed with *Ace2* after H1N1 infection, in SARS-CoV-infected primary human HAE cells. This resulted in 20 out of them showing consistent up/down-regulation with our H1N1 model, suggesting the two viruses share common *Ace2* regulatory networks that participate in the occurrence and development of lung pathology.

In summary, we have shown the expression of *Ace2* was significantly downregulated in the lungs of IAV H1N1 infected mice. By using BXD RI strains, we were able to identify *Ace2* co-expressed genes with genetic correlation analysis and weighted gene co-expression network analysis. Thus, our results indicate that *Ace2* participates in virus-induced respiratory diseases not only through immune system related pathways, but also by protective functions in the respiratory epithelium. In addition, *Rnf128* was identified as the master regulator through Bayesian network analysis. Finally, 21 of our prioritized genes were found to be also differentially expressed in SARS-CoV-infected HAE cells. These findings provide an *Ace2* regulatory network and raise several potential therapeutic targets for virus-induced (H1N1, SARS-CoV, MERS-CoV, and SARS-CoV-2) respiratory diseases. They additionally illustrate that the BXD RI strains provide an excellent resource for studying the host genetic regulatory mechanism of the viral infections, such as that caused by SARS-CoV-2.

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Author Contributions

LL, KS and RSB conceived the study; SB and ACS conducted the experiments; FX and KS performed data analysis; LL, FX, KS, and JG wrote the manuscript. FX, KS, and JG prepared the figures and tables; RWW, KL, CBJ edited the manuscript; all authors read and approved the final version of the manuscript for publication.

Conflicts of Interest

The authors declare no conflicts of interests.

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From: Lu, Lu[llu@uthsc.edu]
Sent: Tue 3/31/2020 6:39:49 AM (UTC-07:00)
To: Baric, Ralph S[rbaric@email.unc.edu]
Cc: Williams, Robert W[rwilli10@uthsc.edu]; Sims, Amy C[amy.sims@pnnl.gov]; silke.bergmann@(b) (6) [silke.bergmann@(b) (6)]; Jonsson, Colleen Beth[cjonsson@uthsc.edu]; Li, Kui[kli1@uthsc.edu]; Klaus x Klaus Schughart[Klaus.Schughart@helmholtz-hzi.de]; Xu, Fuyi[fxu10@uthsc.edu]
Subject: Re: [Ext] RE: Ace2

Hi, Ralph:
Thank you for your editing. I will integrate it into the final version.

Lu Lu
Professor
Department of Genetics, Genomics and Informatics
University of Tennessee Health Science Center
71 S. Manassas St., Room 410K
Memphis, TN 38163
Tel: (901) 448-7557 (O)
Fax: (901) 448-3500 (Dept)

On Mar 31, 2020, at 6:39 AM, Baric, Ralph S <rbaric@email.unc.edu> wrote:

Hi Lu Lu, Nice paper, my minor comments. Enjoyable read. ralph

From: Lu, Lu <llu@uthsc.edu>
Sent: Monday, March 30, 2020 7:00 PM
To: Williams, Robert W <rwilli10@uthsc.edu>; amy.sims@pnnl.gov; silke.bergmann@(b) (6) ; Baric, Ralph S <rbaric@email.unc.edu>; Jonsson, Colleen Beth <cjonsson@uthsc.edu>; Li, Kui <kli1@uthsc.edu>
Cc: Klaus x Klaus Schughart <Klaus.Schughart@helmholtz-hzi.de>; Xu, Fuyi <fxu10@uthsc.edu>
Subject: Ace2

Dear all authors:

We accepted most of your editing and suggestions for *Ace2* paper. Here is the final version (except reference section. We are working on it). Please review it again and make your final editing. Please also check your name and address. Please let me know if you don't want to be co-author for this paper. Please send your updated version back to me before this Friday. We plan to submit it at this weekend.

Thank you all for your big help and contribution.

Lu Lu
Professor
Department of Genetics, Genomics and Informatics
University of Tennessee Health Science Center
71 S. Manassas St., Room 410K
Memphis, TN 38163

Tel: (901) 448-7557 (O)
Fax: (901) 448-3500 (Dept)

On Mar 28, 2020, at 11:25 PM, Li, Kui <kli1@uthsc.edu> wrote:

Dear Lu, Klaus, Rob, and team,

Please find my edits of the manuscript. Use as you see fit. Thanks,

Kui

From: Lu, Lu <llu@uthsc.edu>
Sent: Thursday, March 26, 2020 6:05 PM
To: Klaus x Klaus Schughart <Klaus.Schughart@helmholtz-hzi.de>
Cc: Williams, Robert W <rwilli10@uthsc.edu>; Xu, Fuyi <fxu10@uthsc.edu>;amy.sims@pnnl.gov <amy.sims@pnnl.gov>; rbaric@email.unc.edu<rbaric@email.unc.edu>; silke.bergmann@(b) (6) <silke.bergmann@(b) (6) >; Li, Kui <kli1@uthsc.edu>
Subject: Re: [Ext] B6D2 infected RNAseq data

Dear Klaus (and all others):

Here is updated version. Fuyi addressed most of your comments. Please feel free to modify it continuously.

Dear Amy and Ralph:

Some of Klaus' comment are specific to you. Could you help to reply or editing?

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Cc: Williams, Robert W <rwilli10@uthsc.edu>; Xu, Fuyi <fxu10@uthsc.edu>; amy.sims@pnnl.gov
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> Could you please send me those two images (high-resolution) in Figure 8 ? Thank you very much.
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> Department of Genetics, Genomics, and Informatics
> University of Tennessee Health Science Center
>
> 415 TRANSLATIONAL RESEARCH BUILDING
> 71 SOUTH MANASSAS
> MEMPHIS TN 38163
> Phone: (901)246-4398
> E-mail: 5 U.S.C. § 552(b)(6) fxu10@uthsc.edu
>
> **From:** Schughart, Klaus
> **Sent:** Wednesday, March 25, 2020 3:04 PM

To: Lu, Lu
Cc: Xu, Fuyi; Williams, Robert W
Subject: AW: [Ext] B6D2 infected RNAseq data

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Gesendet: Mittwoch, 25. März 2020 20:31
An: 'Lu, Lu' <llu@uthsc.edu>
Cc: 'Xu, Fuyi' <fxu10@uthsc.edu>; 'Williams, Robert W' <rwilli10@uthsc.edu>
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Thanks – will work it.
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Gesendet: Mittwoch, 25. März 2020 19:02
An: Schughart, Klaus <Klaus.Schughart@helmholtz-hzi.de>
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Fax: (901) 448-3500 (Dept)

On Mar 24, 2020, at 4:36 PM, Lu, Lu <llu@uthsc.edu> wrote:

Thanks, Rob. We will modify based on your comments and will send you updated version for your editing again.

Lu Lu
Professor
Department of Genetics, Genomics and Informatics
University of Tennessee Health Science Center
71 S. Manassas St., Room 410K
Memphis, TN 38163
Tel: (901) 448-7557 (O)
Fax: (901) 448-3500 (Dept)

On Mar 24, 2020, at 4:14 PM, Rob Williams <rwilliams@uthsc.edu> wrote:

Dear team,

Brilliant effort,

Edited the abstract heavily (please review carefully) and built up the Methods section on BXDs a bit.

Have not gotten through all of paper, but looks great so far.

Should validate some networks and eQTLs with GEMMA.

Needs a bit more clarity to data sources—some for 43 strains, other data for parents. These

details needed (imho) even in the abstract.

On Tue, Mar 24, 2020 at 2:41 PM Schughart, Klaus <Klaus.Schughart@helmholtz-hzi.de> wrote:

> Hi Lulu, Fuyi,
> enclosed are my comments, additions and corrections.
> Looks very good!!!
> Please go through and clean it up first (accept or discard changes, add information) and then I can continue to work on the cleaned-up version.
> Currently it is too much cluttered with all these changes and comments.
> Fuyi, let me know what exactly you need in addition for the HAE data.
> Klaus
>
> ---
>
> **Von:** Schughart, Klaus
> **Gesendet:** Freitag, 6. März 2020 19:02
> **An:** Xu, Fuyi <fxu10@uthsc.edu>; Lu Lu 5 U.S.C. § 552(b)(6)
> **Cc:** Rob Williams <rwilliams@uthsc.edu>; Jonsson, Colleen Beth (cjonsson@uthsc.edu) <cjonsson@uthsc.edu>
> **Betreff:** AW: [Ext] B6D2 infected RNAseq data
>
> Hi Fuyi, LuLu,
> I did some extensive analyses of genes in the human HAE data set with your gene list. Enclosed are the results.
> Looks very good!!
> For a publication, we would have to discuss which data to include and how to present the results.
> Klaus
>
> ---
>
> **Von:** Schughart, Klaus <Klaus.Schughart@helmholtz-hzi.de>
> **Gesendet:** Donnerstag, 5. März 2020 04:45
> **An:** Xu, Fuyi <fxu10@uthsc.edu>
> **Cc:** Lu Lu 5 U.S.C. § 552(b)(6)
> **Betreff:** Re: [Ext] B6D2 infected RNAseq data
>
> Dear Fuyi,
> sure I will have a look at these genes.
>
> Klaus
>
> Von meinem iPhone gesendet
>
> Am 04.03.2020 um 19:09 schrieb Xu, Fuyi

<fxu10@uthsc.edu>:

Dear Klaus,

Lu and I are working on the Ace2 paper. We prioritized around 30 genes that are correlated with Ace2 (attached table). I am wondering if you can help me check the expression differences between infected and normal controls in the human data set last time you showed us, especially for those color highlighted. Also, is this gene list make sense from your perspective? Thank you very much.

Best,
--
Fuyi Xu, PhD
Department of Genetics, Genomics, and Informatics
University of Tennessee Health Science Center

415 TRANSLATIONAL RESEARCH BUILDING
71 SOUTH MANASSAS
MEMPHIS TN 38163
Phone: (901)246-4398
E-mail: 5 U.S.C. § 552(b)(6) fxu10@uthsc.edu

From: Schughart, Klaus
Sent: Friday, February 28, 2020 11:07 AM
To: Xu, Fuyi
Cc: Lu Lu
Subject: AW: [Ext] B6D2 infected RNAseq data

Hi LuLu, Fuyi,
I just remembered that I also have expression data from infected human lung slices (see enclosure).
This dataset has been published (enclosed).
Klaus

Von: Schughart, Klaus
Gesendet: Montag, 24. Februar 2020 21:00
An: 'Xu, Fuyi' <fxu10@uthsc.edu>
Cc: Lu Lu
5 U.S.C. § 552(b)(6)

Betreff: AW: [Ext] B6D2 infected RNAseq data

Dear Fuyi,
The data I sent you before (RNAseq_norm1.txt) have been normalized and log2 transformed using DESeq2 (rlogTransformation(dsDat, blind=TRUE).
Please find enclosed the raw counts for the B6/D2 infections from Wilk et al (all_mouse_counts.txt).

For the BXD data in GN: The percent body weights are NOT the losses but the percent body weight with reference to the starting body weight (100%). So 105% means that the mouse has gained 5% of body weight relative to its starting weight when it was infected. Mice with percent body weight less than 100% have lost weight.

Klaus

Von: Xu, Fuyi <fxu10@uthsc.edu>
Gesendet: Sonntag, 23. Februar 2020 22:14
An: Schughart, Klaus <Klaus.Schughart@helmholtz-hzi.de>
Cc: Lu Lu
5 U.S.C. § 552(b)(6)
Betreff: RE: [Ext] B6D2 infected RNAseq data

Dear Dr. Klaus Schughart,

I am Lu Lu's postdoc, Fuyi Xu. Thank you for sending us the normalized RNA-seq data.
We would like to perform differential expression analysis, I am wondering if you have raw read count for this data set since

the DEseq2 package depend on this kind of data matrix. By the way, is the normalized data log2 transformed ?

We also found the expression level of ACE2 across the BXD is significantly correlated with median weight loss at day 3 after infection (ID:13007), however, we found BXD65a loss 105% body weight. Is this correct? Or how should I interpret ?

<A54B14D6A4584F84B57E3AAA25DC5BBB.gif>
Best Regards,
--
Fuyi Xu, PhD
Department of Genetics, Genomics, and Informatics
University of Tennessee Health Science Center

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71 SOUTH MANASSAS
MEMPHIS TN 38163
Phone: (901)246-4398
E-mail: 5 U.S.C. § 552(b)(6) fxu10@uthsc.edu

From: Lu, Lu
Sent: Monday, February 17, 2020 11:03 AM
To: Xu, Fuyi
Subject: Fwd: [Ext] B6D2 infected RNAseq data

Lu Lu
Professor
Department of Genetics, Genomics and Informatics
University of Tennessee Health Science Center
71 S. Manassas St., Room 410K
Memphis, TN 38163
Tel: (901) 448-7557 (O)
Fax: (901) 448-3500 (Dept)

Begin forwarded message:

From: "Schughart, Klaus" <Klaus.Schughart@helmholtz-hzi.de>
Subject: **[Ext] B6D2 infected RNAseq data**
Date: February 17, 2020 at 10:11:55 AM CST
To: "Lu, Lu" <llu@uthsc.edu>

Hi LuLu,
I checked the datasets.

GN807; RNAseq BXD infected is fine. This dataset has not been published yet.

I enclose the log2 normalized matrix for the B6/D2 infected data from Wilk et al. 2015: RNAseq_norm1.txt -- for the mouse genes 00_influenza_lg2_RPKM_kls_

280714.txt -- for the influenza gene segments

Let me know if you have any questions.
Klaus

Prof. Dr. Klaus Schughart
Head of Dept. of Infection Genetics
Helmholtz Centre for Infection Research,
University of Veterinary Medicine Hannover,
University of Tennessee Health Science Center
Inhoffenstr. 7
D-38124 Braunschweig
Germany

E-mail: Klaus.Schughart@helmholtz-hzi.de
Phone: +49 531 6181 1100

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BC50D9
60A2.pn
g>

Helmholtz-Zentrum für Infektionsforschung GmbH | Inhoffenstraße 7 | 38124 Braunschweig | www.helmholtz-hzi.de

Vorsitzende des Aufsichtsrates: Frau MinDir'in Prof. Dr. Veronika von Messling
Stellvertreter: MinDirig Rüdiger Eichel, Niedersächsisches Ministerium für Wissenschaft und Kultur
Geschäftsführung: Prof. Dr. Dirk Heinz; Silke Tannapfel
Gesellschaft mit beschränkter Haftung (GmbH)
Sitz der Gesellschaft:

Braunschweig Handelsregister: Amtsgericht Braunschweig, HRB 477

<0D5B54C5FA694C128C887F6FD4C0D7A4.png>

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<Table 1.xlsx>

<B911E1AA90884DD79858B3D0CE0F7C4C.png>

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--
Rob

Robert W. Williams, Ph.D.
Chair: Department of Genetics, Genomics and Informatics
University of Tennessee Health Science Center
71 S Manassas St, Memphis TN 38103-3310, TSRB Room 405
Tele/Cell: 901 604 4752
EMAIL: rwilliams@uthsc.edu or
5 U.S.C. § 552(b)(6)

<Ace2-Draft-03182020-V1_LuB_kls_RWW_240320_3.docx>

<8608AAAF2A14461CBDBD04DA13676A0C.png>

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<Ace3_V3_250320_kls_260320.docx>

<Ace2_V4_260320_Li.docx>

<Ace2_V7_03302020B-rsb.docx>

From: Schaefer, Alexandra[aschaefe@email.unc.edu]
Sent: Tue 3/31/2020 9:14:35 AM (UTC-07:00)
To: Sims, Amy C[amy.sims@pnnl.gov]
Subject: Re: FB primers for epigenetic R21?

Hi Amy,

based on the differential ISG expression in FBs at 24 hpi, the following ISGs will be target genes for ChiP-PCR:
up-regulated:
ARHGAP27
MX1
IFIT1
IFIT2
HES4

down-regulated:
APOBEC3G
GMPR
APOL3

Hope that helps,
Alex

From: Sims, Amy C <amy.sims@pnnl.gov>
Sent: Monday, March 30, 2020 6:37 PM
To: Schaefer, Alexandra <aschaefe@email.unc.edu>
Subject: FB primers for epigenetic R21?

Hi Alex,

I am putting together a table for the R21 progress report and was going to add the FB gene list and primer set of you've gotten that far.

I hope you are doing well. I will send you a draft of the report asap.

Thank you, Amy

From: Schaefer, Alexandra[aschaefe@email.unc.edu]
Sent: Tue 3/31/2020 9:20:06 AM (UTC-07:00)
To: Sims, Amy C[amy.sims@pnnl.gov]
Subject: Re: FB primers for epigenetic R21?

no worries, thought you wanted to put it into the report

From: Sims, Amy C <amy.sims@pnnl.gov>
Sent: Tuesday, March 31, 2020 12:18 PM
To: Schaefer, Alexandra <aschaefe@email.unc.edu>
Subject: Re: FB primers for epigenetic R21?

I was just curious if you had the primers designed yet. Not a big deal. Thank you, Amy

---

From: "Schaefer, Alexandra" <aschaefe@email.unc.edu>
Date: Tuesday, March 31, 2020 at 9:15 AM
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To: Lu, Lu[llu@uthsc.edu]; Williams, Robert W[rwilli10@uthsc.edu]; Sims, Amy C[amy.sims@pnnl.gov]; silke.bergmann@(b) (6) [silke.bergmann@(b) (6)]; rbaric@email.unc.edu[rbaric@email.unc.edu]; Jonsson, Colleen Beth[cjonsson@uthsc.edu]; Li, Kui[kli1@uthsc.edu]
Cc: Xu, Fuyi[fxu10@uthsc.edu]
Subject: AW: Ace2
Attachment: Ace2_V7_03302020BLuLu_kls.docx

Hi LuLu,
enclosed a few minor last corrections.
Klaus

Von: Lu, Lu <llu@uthsc.edu>
Gesendet: Dienstag, 31. März 2020 01:00
An: Williams, Robert W <rwilli10@uthsc.edu>; amy.sims@pnnl.gov; silke.bergmann@(b) (6) ; rbaric@email.unc.edu; Jonsson, Colleen Beth <cjonsson@uthsc.edu>; Li, Kui <kli1@uthsc.edu>
Cc: Schughart, Klaus <Klaus.Schughart@helmholtz-hzi.de>; Xu, Fuyi <fxu10@uthsc.edu>
Betreff: Ace2

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Thank you all for your big help and contribution.

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University of Tennessee Health Science Center

415 TRANSLATIONAL RESEARCH BUILDING
71 SOUTH MANASSAS
MEMPHIS TN 38163
Phone: (901)246-4398
E-mail: 5 U.S.C. § 552(b)(6) fxu10@uthsc.edu

From: Schughart, Klaus
Sent: Wednesday, March 25, 2020 3:04 PM
To: Lu, Lu
Cc: Xu, Fuyi; Williams, Robert W
Subject: AW: [Ext] B6D2 infected RNAseq data

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Von: Schughart, Klaus
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An: 'Lu, Lu' <llu@uthsc.edu>
Cc: 'Xu, Fuyi' <fxu10@uthsc.edu>; 'Williams, Robert W' <rwilli10@uthsc.edu>
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Betreff: AW: [Ext] B6D2 infected RNAseq data

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Von: Lu, Lu <llu@uthsc.edu>
Gesendet: Mittwoch, 25. März 2020 19:02
An: Schughart, Klaus <Klaus.Schughart@helmholtz-hzi.de>
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Memphis, TN 38163
Tel: (901) 448-7557 (O)
Fax: (901) 448-3500 (Dept)

> On Mar 24, 2020, at 4:36 PM, Lu, Lu <llu@uthsc.edu> wrote:
>
> Thanks, Rob. We will modify based on your comments and will send you updated version for your editing again.
>
> Lu Lu
> Professor
> Department of Genetics, Genomics and Informatics
> University of Tennessee Health Science Center
> 71 S. Manassas St., Room 410K
> Memphis, TN 38163
> Tel: (901) 448-7557 (O)
> Fax: (901) 448-3500 (Dept)

On Mar 24, 2020, at 4:14 PM, Rob Williams <rwilliams@uthsc.edu> wrote:

Dear team,

Brilliant effort,

Edited the abstract heavily (please review carefully) and built up the Methods section on BXDs a bit.

Have not gotten through all of paper, but looks great so far.

Should validate some networks and eQTLs with GEMMA.

Needs a bit more clarity to data sources—some for 43 strains, other data for parents. These details needed (imho) even in the abstract.

On Tue, Mar 24, 2020 at 2:41 PM Schughart, Klaus <Klaus.Schughart@helmholtz-hzi.de> wrote:

Hi Lulu, Fuyi,
enclosed are my comments, additions and corrections. Looks very good!!!
Please go through and clean it up first (accept or discard changes, add information) and then I can continue to work on the cleaned-up version. Currently it is too much cluttered with all these changes and comments.
Fuyi, let me know what exactly you need in addition for the HAE data.
Klaus

Von: Schughart, Klaus
Gesendet: Freitag, 6. März 2020 19:02
An: Xu, Fuyi <fxu10@uthsc.edu>; Lu Lu
5 U.S.C. § 552(b)(6)
Cc: Rob Williams <rwilliams@uthsc.edu>; Jonsson, Colleen Beth (cjonsson@uthsc.edu) <cjonsson@uthsc.edu>
Betreff: AW: [Ext] B6D2 infected RNAseq data

Hi Fuyi, LuLu,
I did some extensive analyses of genes in the human HAE data set with your gene list. Enclosed are the results. Looks very good!!
For a publication, we would have to discuss which data to include and how to present the results.
Klaus

Von: Schughart, Klaus <Klaus.Schughart@helmholtz-hzi.de>
Gesendet: Donnerstag, 5. März 2020 04:45
An: Xu, Fuyi <fxu10@uthsc.edu>

Cc: Lu Lu 5 U.S.C. § 552(b)(6)
Betreff: Re: [Ext] B6D2 infected RNAseq data

Dear Fuyi,
sure I will have a look at these genes.

Klaus

Von meinem iPhone gesendet

> Am 04.03.2020 um 19:09 schrieb Xu, Fuyi <fxu10@uthsc.edu>:
>
> Dear Klaus,
>
> Lu and I are working on the Ace2 paper. We prioritized around 30 genes that are correlated with Ace2 (attached table). I am wondering if you can help me check the expression differences between infected and normal controls in the human data set last time you showed us, especially for those color highlighted. Also, is this gene list make sense from your perspective? Thank you very much.
>
> Best,
> --
> Fuyi Xu, PhD
> Department of Genetics, Genomics, and Informatics
> University of Tennessee Health Science Center
>
> 415 TRANSLATIONAL RESEARCH BUILDING
> 71 SOUTH MANASSAS
> MEMPHIS TN 38163
> Phone: (901)246-4398
> E-mail: 5 U.S.C. § 552(b)(6) ; fxu10@uthsc.edu
>
> **From:** Schughart, Klaus
> **Sent:** Friday, February 28, 2020 11:07 AM
> **To:** Xu, Fuyi
> **Cc:** Lu Lu
> **Subject:** AW: [Ext] B6D2 infected RNAseq data
>
> Hi LuLu, Fuyi,
> I just remembered that I also have expression data from infected human lung slices (see enclosure).
> This dataset has been published (enclosed).
> Klaus
>
> **Von:** Schughart, Klaus
> **Gesendet:** Montag, 24. Februar 2020 21:00
> **An:** 'Xu, Fuyi' <fxu10@uthsc.edu>

Cc: Lu Lu 5 U.S.C. § 552(b)(6)
Betreff: AW: [Ext] B6D2 infected RNAseq data

Dear Fuyi,
The data I sent you before (RNAseq_norm1.txt) have been normalized and log2 transformed using DESeq2 (rlogTransformation(dsDat, blind=TRUE).
Please find enclosed the raw counts for the B6/D2 infections from Wilk et al (all_mouse_counts.txt).

For the BXD data in GN:
The percent body weights are NOT the losses but the percent body weight with reference to the starting body weight (100%). So 105% means that the mouse has gained 5% of body weight relative to its starting weight when it was infected. Mice with percent body weight less than 100% have lost weight.

Klaus

Von: Xu, Fuyi <fxu10@uthsc.edu>
Gesendet: Sonntag, 23. Februar 2020 22:14
An: Schughart, Klaus <Klaus.Schughart@helmholtz-hzi.de>
Cc: Lu Lu 5 U.S.C. § 552(b)(6)
Betreff: RE: [Ext] B6D2 infected RNAseq data

Dear Dr. Klaus Schughart,

I am Lu Lu's postdoc, Fuyi Xu.
Thank you for sending us the normalized RNA-seq data.
We would like to perform differential expression analysis, I am wondering if you have raw read count for this data set since the DEseq2 package depend on this kind of data matrix.
By the way, is the normalized data log2 transformed ?

We also found the expression level of ACE2 across the BXD is significantly correlated with median weight loss at day 3 after infection (ID:13007), however, we found BXD65a loss 105% body weight. Is this correct? Or how should I interpret ?

Best Regards,
--

Fuyi Xu, PhD
Department of Genetics, Genomics, and Informatics
University of Tennessee Health Science Center

415 TRANSLATIONAL RESEARCH BUILDING
71 SOUTH MANASSAS
MEMPHIS TN 38163
Phone: (901)246-4398
E-mail: 5 U.S.C. § 552(b)(6) fxu10@uthsc.edu

From: Lu, Lu
Sent: Monday, February 17, 2020 11:03 AM
To: Xu, Fuyi
Subject: Fwd: [Ext] B6D2 infected RNAseq data

Lu Lu
Professor
Department of Genetics, Genomics and Informatics
University of Tennessee Health Science Center
71 S. Manassas St., Room 410K
Memphis, TN 38163
Tel: (901) 448-7557 (O)
Fax: (901) 448-3500 (Dept)

Begin forwarded message:

From: "Schughart, Klaus" <Klaus.Schughart@helmholtz-hzi.de>
Subject: [Ext] B6D2 infected RNAseq data
Date: February 17, 2020 at 10:11:55 AM CST
To: "Lu, Lu" <llu@uthsc.edu>

Hi LuLu,
I checked the datasets.

GN807; RNAseq BXD infected is fine. This dataset has not been published yet.

I enclose the log2 normalized matrix for the B6/D2 infected data from Wilk et al. 2015: RNAseq_norm1.txt -- for the mouse genes

00_influenza_lg2_RPKM_kls_280714.txt -- for the influenza gene segments

Let me know if you have any questions.
Klaus

Prof. Dr. Klaus Schughart
Head of Dept. of Infection Genetics
Helmholtz Centre for Infection Research,
University of Veterinary Medicine Hannover,
University of Tennessee Health Science Center
Inhoffenstr. 7
D-38124 Braunschweig
Germany

E-mail : Klaus.Schughart@helmholtz-hzi.de
Phone: +49 531 6181 1100

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Helmholtz-Zentrum für Infektionsforschung GmbH | Inhoffenstraße 7 | 38124 Braunschweig | www.helmholtz-hzi.de

Vorsitzende des Aufsichtsrates: Frau MinDir'in Prof. Dr. Veronika von Messling
Stellvertreter: MinDirig Rüdiger Eichel, Niedersächsisches Ministerium für Wissenschaft und Kultur
Geschäftsführung: Prof. Dr. Dirk Heinz; Silke Tannapfel
Gesellschaft mit beschränkter Haftung (GmbH)
Sitz der Gesellschaft: Braunschweig

Handelsregister:
Amtsgericht
Braunschweig, HRB
477

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Infektionsforschung GmbH |
Inhoffenstraße 7 | 38124
Braunschweig | www.helmholtz-
hzi.de

Vorsitzende des Aufsichtsrates: Frau
MinDir'in Prof. Dr. Veronika von
Messling
Stellvertreter: MinDirig Rüdiger
Eichel, Niedersächsisches
Ministerium für Wissenschaft und
Kultur
Geschäftsführung: Prof. Dr. Dirk
Heinz; Silke Tannapfel
Gesellschaft mit beschränkter
Haftung (GmbH)
Sitz der Gesellschaft: Braunschweig
Handelsregister: Amtsgericht
Braunschweig, HRB 477

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Helmholtz-Zentrum für
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Inhoffenstraße 7 | 38124
Braunschweig | www.helmholtz-
hzi.de

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Sitz der Gesellschaft:
Braunschweig
Handelsregister: Amtsgericht
Braunschweig, HRB 477

--
Rob

Robert W. Williams, Ph.D.
Chair: Department of Genetics, Genomics and Informatics
University of Tennessee Health Science Center
71 S Manassas St, Memphis TN 38103-3310, TSRB Room 405
Tele/Cell: 901 604 4752
EMAIL: rwilliams@uthsc.edu or
5 U.S.C. § 552(b)(6)

<Ace2-Draft-03182020-V1_LuB_kls_RWW_240320_3.docx>

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Gesellschaft mit beschränkter Haftung (GmbH)
Sitz der Gesellschaft: Braunschweig
Handelsregister: Amtsgericht Braunschweig, HRB 477
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Vorsitzende des Aufsichtsrates: Frau MinDir'in Prof. Dr. Veronika von Messling
Stellvertreter: MinDirig Rüdiger Eichel, Niedersächsisches Ministerium für Wissenschaft und Kultur
Geschäftsführung: Prof. Dr. Dirk Heinz; Silke Tannapfel
Gesellschaft mit beschränkter Haftung (GmbH)
Sitz der Gesellschaft: Braunschweig
Handelsregister: Amtsgericht Braunschweig, HRB 477

Genetic Dissection of the Regulatory Mechanisms of *Ace2* in the Infected Mouse Lung

Fuyi Xu[1#], Jun Gao[1, 5#], Silke Bergman[4], Amy C. Sims[7, 9], Ralph S. Baric[6,7, 8], Colleen B Jonsson[4], Kui Li[4], Robert W. Williams[1], Klaus Schughart[2, 3, 4*], and Lu Lu[1*]

[1]Department of Genetics, Genomics, and Informatics, University of Tennessee Health Science Center, Memphis, TN, USA;

[2]Department of Infection Genetics, Helmholtz Centre for Infection Research, Braunschweig, Germany

[3]University of Veterinary Medicine Hannover, Hannover, Germany;

[4]Department of Microbiology, Immunology, and Biochemistry, University of Tennessee Health Science Center, Memphis, TN, USA;

[5]Institute of Animal Husbandry and Veterinary Science, Shanghai Academy of Agricultural Sciences, Shanghai, China.

[6]Department of Microbiology and Immunology, University of North Carolina at Chapel Hill, Chapel Hill, NC

[7]Department of Genetics, University of North Carolina at Chapel Hill, Chapel Hill, NC

[8]Department of Epidemiology, University of North Carolina at Chapel Hill, Chapel Hill, NC

[9]present address: Pacific Northwest National Laboratory in Richland, WA

[#] These authors contributed equally to this work.

* Correspondence:

Lu Lu: llu@uthsc.edu

Department of Genetics, Genomics and Informatics
University of Tennessee Health Science Center
Memphis, TN 38163, United States;

Klaus Schughart: klaus.schughart@helmholtz-hzi.de
Department of Infection Genetics
Helmholtz Centre for Infection Research
Braunschweig, Germany

Abstract

Acute lung injury (ALI) is an important cause of morbidity and mortality after viral infections, such as influenza A virus H1N1, SARS-CoV, MERS-CoV, and SARS-CoV-2. The angiotensin 1 converting enzyme 2 (ACE2) plays a protective role in SARS-CoV-induced ALI. However, contributions of *ACE2* gene variants to the post-infection heterogeneity of the host response and susceptibility remain unclear, as do the underlying mechanisms. In this study, we quantified lung transcriptomes in 41 BXD recombinant inbred strains of mice plus their parental strains of C57BL/6J and DBA/2J mice after H1N1 infection. In response to infection, the expression of *Ace2* mRNA across strains decreased significantly and was associated with both disease severity and infection time. Expression was negatively correlated with body weight loss and duration of survival time. Data for all 43 genomes were analyzed using weighted gene co-expression network analysis (WGCNA). *Ace2* not only participated in virus-induced ALI by interacting with TNF, MAPK, and notch signaling pathway, but also was linked to genes that have important functions in the pulmonary epithelium. Moreover, by applying multi-layer filtration, including differential expression analysis, eQTL mapping, functional variant identification, and gene annotation, we highlighted 10 key candidate genes linked to an *Ace2* network: *Sox11*, *Tff2*, *Rnf128*, *Muc5b*, *Tmprss2*, *Ulk4*, *Cckar*, *Wdpcp*, *Ift140*, and *Ccdc40*. Of these transcripts, *Rnf128* was identified as a potential master regulator using Bayesian network analysis. The key gene set is enriched for genes reported to be differentially expressed in SARS-CoV-infected primary human airway epithelial cells. Collectively, these findings define an *Ace2*-associated modulatory network relevant to host response to H1N1 infection and raise novel potential therapeutic targets for virus-induced respiratory diseases, including those caused by SARS-CoV and SARS-CoV-2. In addition, we demonstrate that the genetically diverse BXD family provides an excellent testbed for dissecting host genetic regulatory mechanisms involved in the defense against viral infections and host disease.

Key words: H1N1, acute lung injury, BXD mice, *Ace2,* regulatory network

Introduction

The pandemic infectious disease, COVID-19, is caused by a novel coronavirus, termed severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). This pathogen has spread with almost unprecedent speed from an initial outbreak in November or December 2019. As of March 25, 2020, according to estimates by the World Health Organization (WHO), ~195 countries and ~375,000 cases of COVID-19 have been reported and linked to ~16,000 deaths.

Since 1918, the world has witnessed four influenza A virus (IAV) pandemics—1918 H1N1, 1957 H2N2, 1968 H3N2, and 2009 H1N1. The first of these resulted in at least 50 million global mortalities [1]. The primary pathological features of both COVID-19 and 2009 influenza A (H1N1) infection are acute viral pneumonia and acute lung injury (ALI) [2]. The documented symptoms of influenza A (H1N1) and COVID-19 are nearly identical. For both viral pathogens infected patients can either be asymptomatic or can have symptoms that range from mild coughing, congestion, and fever, or to severe and lethal pulmonary edema. Individual genetic differences almost certainly play an important role in the development and severity of the illness and its time course [3-5].

Angiotensin-converting enzyme 2 (*ACE2*)—a negative regulator of the renin angiotensin system (RAS)—plays a protective role in pathogenesis of ALI in several animal models of acute respiratory distress syndrome [6]. *Ace2* knockout mice exhibited severe ALI. In contrast, treatment with recombinant human *ACE2* expression attenuates acute lung failure in these *Ace2* knockouts as well as in wild-type mice [7]. Studies have shown that ACE2 protein levels are significantly downregulated after infection with H1N1 virus but are dispensable for viral replication [8]. For SARS-CoV , *ACE2* has been identified as the principal host transmembrane receptor for the receptor-binding domain of the viral spike protein and thereby facilitates endocytosis of the virus and it subsequent virion replication in host cells. In *in vivo* studies, SARS-CoV infection of mice causes severe pulmonary disease and lethal lung failure, concomitant with the downregulation of *Ace2* in lungs [12]. Single-cell RNA sequencing demonstrates that 83% of *ACE2*-expressing cells in the lungs are alveolar epithelial type II cells. It is possible that there are genetic differences in host susceptibility the depend on the numbers or ratios of *ACE2*-positive AT-2 cells in lung [13]. However, we emphasize that causal linkage of *ACE2* variation to susceptibility to infection or to post-infection heterogeneity of disease progression is still very poorly defined in humans or even mouse..

Susceptibility to viral infection varies greatly among strains of isogenic mice [14], especially between two key inbred strains—C57BL/6J (B6), the reference strain for most experimental work—and DBA/2J (D2), the oldest of inbred strains of mice [15, 16]. B6 is relatively resistant to influenza A and often survives inoculation. In contrast, D2 loses body weight rapidly and at the same inoculum dose as given to B6 dies within 5–7 days. Our laboratory has generated and maintains ~120 BXD progeny strains derived from crosses between B6 and D2 parents. Both parental strains and all of these highly diverse BXD recombinant inbred progeny has been sequenced. Collectively this family segregates for ~6 million variants with minor allele frequencies above 0.3 [16], making it an ideal and well powered reference population to model genetic susceptibility to IAV infections [17]. Indeed, in previous genetic mapping using this cohort we have

defined loci and even cloned gene variants that control the host response to IAV (H1N1) infection [18] and to other infectious diseases [19-22]. The BXD family provides a powerful resource to dissect loci, gene variants, molecular networks, mechanisms, and even therapeutic interventions in response to viral infection.

Here we have analyzed the lung transcriptome by RNAseq in 41 BXD RI strains and both parental strains after infection with H1N1 IAV. By using systems genetics approaches, we have defined patterns of covariation of mRNAs with induced variation in *Ace2* expression, as well as molecular networks and regulatory relations that are likely to contribute to the host defense and/or the pathologies of H1N1-induced ALI.

Materials and methods

Mice

The subjects for this study were females belonging to 41 distinct BXD recombinant inbred (RI) strains, and the parental C57BL/6J (B6) and DBA/2J (D2) strains. The family of strains segregates for over 6 million sequence variants with minor allele frequencies greater than 0.3 [23]. They are well powered to detect major loci that contribute to genetic variation in disease susceptibility [19-22] and to define causal networks of interacting loci, genes, gene productions (mRNAs and proteins), and pathogenicity outcome measures [18, 24]. All cases were between 8- and 12-weeks-of-age when infected with virus. All were housed and maintained on a 12:12 light/ dark cycle, with *ad libitum* access to food and water.

Ethics

Protocols for the mouse experiments were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Tennessee Health Science Center.

Virus

Original stocks of mouse adapted A/Puerto Rico/8/34 (H1N1, PR8M) virus were obtained from Stefan Ludwig, University of Münster [25]. Virus stocks were propagated in the chorioallantoic cavity of 10-day-old pathogen-free embryonated chicken eggs for 48 h at 37 °C as described previously[26]. Viral titer was determined by focus-forming unit (FFU) assay as described previously [26].

Infection of mice

Animals were anesthetized by intraperitoneal injection of ketamine/xylazine (10 %(v/v) of 100 mg/ml ketamine and 5 %(v/v) of 20 mg/ml xylazine in 0.9 % (w/v) NaCl) with a dose adjusted to body weight (200 µl/20 g body weight). Infection was performed by intranasal application of virus solution in 20 µl sterile phosphate-buffered saline (PBS), with PR8M $2x10^3$ FFU.

RNA isolation and sequencing

Mice were sacrificed three days post infection (dpi) and entire lungs were extracted. Lungs were immediately transferred to RNAlater (Qiagen), kept at 4 °C for one day, and subsequently stored at –20 °C. RNA was isolated using Qiagen Midi kit as described previously [27]. RNA quality was controlled on

a 2100 Bioanalyzer (Agilent). 500 ng of total RNA was used to prepare libraries for sequencing using the Lexogen SENSE mRNA-seq library kit for Ion Torrent. Libraries were amplified for 11 cycles as the final step of library preparation. Five-hundred nanograms of total RNA was used to prepare libraries for sequencing using the Lexogen SENSE mRNA-seq library kit for Ion Torrent. Libraries were amplified for 11 cycles as the final step of library preparation. Before sequencing, 1 µl aliquots of this material was pooled and sequenced on an Ion Torrent PGM 314 chip. Barcode quantification data from the PGM were used to balance the barcodes for final pooling before sequencing. Following this final pooling, the library pools were sized to a target size of 260 bp on a Pippin Prep instrument using the 2 % Pippin agarose gel. The sized libraries were examined on an Agilent High Sensitivity DNA chip, quantified using real-time PCR, and used to prepare spheres using a One-Touch 2 device. These spheres were then sequenced on an Ion Torrent Proton sequencer with a P1 chip. On average, 67 million reads were obtained per sample. RNA sequencing has been performed by the Molecular Resource Center (MRC) at UTHSC, Memphis, USA.

Read mapping and gene expression quantification

RNAseq reads were quality-trimmed using Trim Galore [28] and then mapped to the mouse mm10 reference genome or to the influenza A virus PR8M genome using STAR [29]. Read counts were summarized for genes as features using the R-package *Rsubread* [30], normalized and log2 transformed using the R-package *DESeq2* [31], and subsequently batch-corrected using the *ComBat* function of the R-package *sva* [32, 33]. For annotations of genes, ENTREZID from *Rsubread* were matched to RefSeq annotations using R-package *biomart* [34]. Duplicate RefSeq annotations were given the extension _1, _2, etc.

Heritability estimates for *Ace2*

The heritability of *Ace2* gene expression was estimated with GEMMA which used the following formula to calculate the proportion of variance in phenotypes explained (PVE) by typed genotypes (i.e. "chip heritability"):

$$\mathrm{PVE}(\boldsymbol{\beta}, \mathbf{u}, \tau) := \frac{\mathrm{V}(\mathbf{X}\boldsymbol{\beta} + \mathbf{u})}{\mathrm{V}(\mathbf{X}\boldsymbol{\beta} + \mathbf{u}) + \tau^{-1}}$$

Where "Xβ" is the sparse effects, "u" is the random effects, and "τ^{-1}" is the variance of the residual errors. GEMMA uses Markov chain Monte Carlo to estimate β, u. BXD genotype file generated January 2017 (www.genenetwork.org/webqtl/main.py? FormID=sharinginfo&GN_AccessionId=600) and *Ace2* gene expression values across the 41 BXD RI strains were used as the inputs.

Correlation analysis

The correlation analysis in this study, including gene-gene and gene-phenotype were conducted with Pearson correlation coefficient in R. Pearson correlation *P*-value < 0.05 was used to report statistical significance.

Weighted gene co-expression network analysis (WGCNA)

WGCNA is a method aims to find co-expressed gene networks and explore the association between gene networks and phenotypes of interest, as well as hub genes in the network. In this study, Gene co-

expression networks was constructed using the WGCNA package in R [35] according to the recommended tutorials (https://horvath.genetics.ucla.edu/html/CoexpressionNetwork/Rpackages/WGCNA/Tutorials/), in which, the *Ace2* correlated genes (Pearson correlation *P*-value < 0.05) was used as the input. The network construction mainly includes the following four steps: (1) define the gene expression correlation matrix; (2) select the soft threshold β and transform the expression correlation matrix into an adjacency matrix; (3) convert the adjacent matrix to topologically overlapping matrix and then to the dissimilarity matrix; 4) hierarchical cluster the dissimilarity matrix to obtain a clustering tree and refine with dynamic hybrid cutting.

Gene set enrichment analysis

To understand the biological function and pathways involved in the gene set of interest, we performed Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway, and mammalian phenotype ontology (MPO) overrepresentation analysis on WebGestalt (http://www.webgestalt.org) [36] . This analysis was based on hypergeometric statistic test which generate *P*-values and enrich rations to evaluate whether an annotated term/category/pathway is achieved statistical enrichment in the gene set rather than by chance. In this analysis, we use the mouse genome protein coding genes as the reference background and a minimum of five genes for a category was defined.

Expression quantitative trait loci (eQTL) mapping

eQTL mapping allows the identification of chromosomal regions that specifically modulate variability in levels of mRNAs or other molecules. We performed eQTL mapping of RNA-seq expression data from lung tissues of H1N1 virus-infected BXD. Much of the analysis was done using GeneNetwork (www.genenetwork.org) [37, 38]. Likelihood ratio statistics (LRS) scores were computed using the Haley-Knott equations [39], and genome-wide significance was based on 2000 permutations. We used the BXD genotype file generated January 2017 (www.genenetwork.org/webqtl/main.py?FormID=sharinginfo&GN_AccessionId=600). In addition, Genes with significant and suggestive eQTLs detected using Haley and Knott mapping were remapped using GEMMA [40] to confirm LRS scores and locations of QTLs in GeneNetwork. GEMMA used linear mixed model mapping algorithms which can account for kinship among strains.

Genetic variations between C57BL/6J and DBA/2J

Sequence differences segregating in the BXDs are from our previous work [23]. Here, we focused exclusively on protein-coding variants—nonsense, missense, and frameshift mutations.

Bayesian network construction

The Bayesian Network Webserver (compbio.uthsc.edu/BNW) [41]was used to create and evaluate plausible causal models among genes, transcripts, and outcome measures of interest. Model were learned from the data using the default settings. Model averaging of the 1000 highest scoring networks was performed, and directed edges with weights greater than 0.5 were included in models. Finally, each variable had a maximum of four direct parents in any model.

RNAseq time-course data for H1N1 infection of the parental strains

The lung RNA-seq data was generated previously in our laboratory (see Ref [15]). In brief, two strains (B6 and D2) were infected with the H1N1 virus at 10–12 weeks of age as described above. RNA was isolated from lung for B6 at 1, 3, 5, 8, and 14 dpi and for D2 at 1, 3, and 5 dpi. Three RNA samples per strain and per time point were used for library preparation and sequencing. The reads were mapped to the mm10 reference genome. Raw read counts were extracted, normalized, and log transformed with *DEseq2* [31] after adding 1 to all raw counts.

In this study, we extracted the *Ace2* expression levels from this dataset. Also, we performed differential expression analysis between mice at 3 dpi and mock controls using DESeq2 [31] by treating strain as a covariates. The resulting *P*-values were adjusted using the Benjamini and Hochberg's approach [42]. We reported genes with *adj.P*-value < 0.05 (also known as a *q* value), and fold change (FC) > 1.3 as differentially expressed genes (DEGs).

Microarray data for SARS-CoV infected primary human airway epithelial (HAE) cells

The experimental details for this study are described in Ref. [43] In brief, human tracheobronchial epithelial cells were obtained from airway specimens resected from patients undergoing surgery. Primary cells were expanded to generate passage 1 cells, and passage 2 cells were plated at a density of $2.5x10^5$ cells per well. Human airway epithelium (HAE) cultures were generated by provision of an air-liquid interface for 6 to 8 weeks to form well-differentiated, polarized cultures. Wild-type infectious clone-derived SARS-CoV (icSARS-CoV) and ic-SARS-CoV ORF6 were derived from infectious clone constructs as described [43] Bat-SARS-CoV is comprised of the HKU3 backbone and SARS-CoV receptor binding domain and was derived from an infectious clone as described in (PMID:19036930). Triplicate cultures were infected with multiplicity of infection 3 (MOI 3) icSARS-CoV, and icSARS-CoV ORF6 on the apical surface. Total RNA was isolated from infected cells, and equivalent amounts of RNA from three biological replicates from each condition were pooled. Transcriptome profiling was performed using Agilent 4x44K whole human gene expression arrays. Raw data were downloaded from GEO (GSE47963_series_matrix.txt.gz) Expression values were normalized and sample descriptions quality controlled. A strong batch effect with respect to the donor from which HAE were derived was corrected using the R package *sva* [32, 33]. Rows with no gene description were deleted. Differentially expressed genes were determined using the R package *Limma* [45].

Data availability

The RNA-seq data from infected BXD strains has been deposited at GeneNetwork web site (www.genenetwork.org) under the accession number GN807. These data can be accessed and downloaded through the dataset query "HZI Lung Flu Infected BXD (Nov16) RNA-Seq". RNA-seq data for infection of B6 and D2 mice is at GEO under accession number GSE66040. Array data from infected primary HAE cells are available at GEO under accession number GSE47963.

3 Results

3.1 *Ace2* expression level decreased in both C57BL/6J and DBA/2J after H1N1 infection

Previously, our group has generated lung RNAseq for the B6 and D2 at 1, 3, 5, 8, and 14 dpi with H1N1. The B6 maternal parent of the BXDs is resistant to H1N1 infection, while the D2 paternal parent is highly susceptible. Here we have exploited these parental time series data to define the *Ace2* mRNA kinetic response. Overall, expression of *Ace2* in lungs is decreased progressively in both strains as H1N1 infection proceeded (**Figure 1**). In B6 mice, *Ace2* transcript dipped to the lowest level on 8 dpi (7.20 ± 0.15 SD log2 units) and remained at that low level on 14 dpi (7.23 ± 0.12). In addition, the expression levels at 3 dpi (*P*-value = 0.026, log2FC=-0.25), 5 dpi (*P*-value = 0.008, log2FC=-0.49), 8 dpi (*P*-value = 0.003, log2FC=-0.73), and 14 dpi (*P*-value = 0.002, log2FC=-0.70) significantly lower than that at 1 dpi. The infection was lethal for D2 at 5 dpi, and this was matched with lower expression of *Ace2* (7.10 ± 0.25). Statistically, *Ace2* expression levels on 3 dpi (*P*-value = 0.003, log2FC=-0.37) and 5 (*P*-value = 0.006, log2FC=-0.80) both showed significant reduction as compared with 1 dpi. Although the differences in *Ace2* expression between B6 and D2 at 3 dpi and 5 dpi did not achieve statistical significance, D2 mice have consistently showed more rapid decline in *Ace2* expression as compared with the B6 strain.

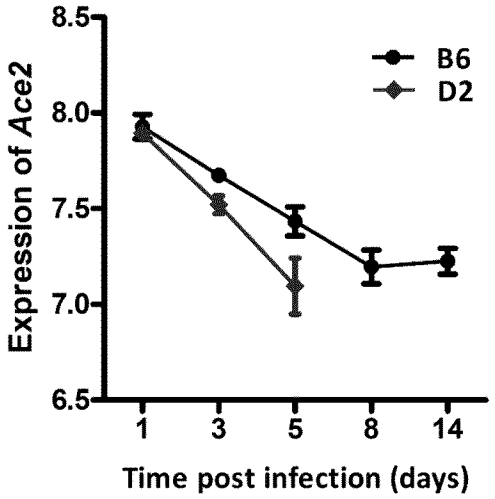


Figure 1. Line charts of the *Ace2* expression level in strains C57BL/6J and DBA/2J after H1N1 infection. Lung transcriptomes were quantified with RNA-seq for B6 and D2 strains at 1, 3, 5, 8, and 14 dpi of H1N1 infection. Three female mice (8-12 weeks of age) per strain were used for per time point. The x-axis shows the time point (days) post the infection. The y-axis shows the expression level of *Ace2* with log2 transformed values.

3.2 *Ace2* mRNA level exhibited variability across BXD RI mice at 3 dpi

All D2 mice died five days post infection. Therefore, we conducted RNAseq for a subset of BXD RI family of mice (41 BXD RI strains and two parental strains B6 and D2) at 3 dpi. As shown in **Figure 2**, *Ace2* transcript levels varied considerably across BXD family with a difference of 1.49 between extremes. The average expression of *Ace2* across all BXDs was 8.02 ± 0.15, with BXD77 having the highest expression of 8.36 and BXD22 having the lowest expression of 7.78. We also estimated the heritability of *Ace2* expression with GEMMA, which is able to calculate the so-called "chip heritability" with our RNAseq data. The resulted value was 0.181, which suggested that genetic background significantly participated in the regulation of *Ace2* mRNA level.

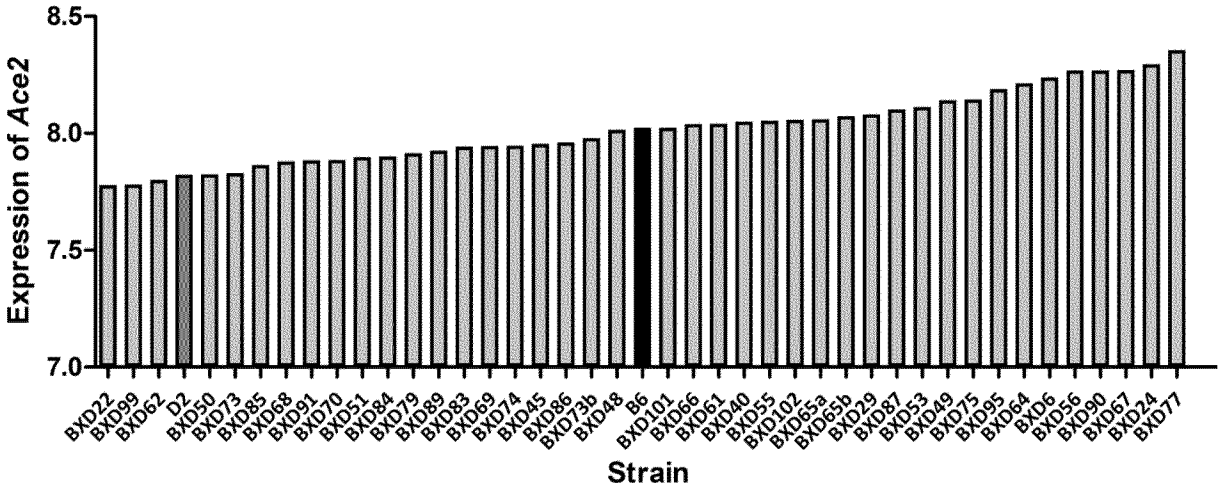


Figure 2. Bar plots of the pulmonary *Ace2* expression levels across the BXD family at 3 dpi. The x-axis shows BXDs strains and the two parental strains. The y-axis shows the normalized log2 expression levels of *Ace2*.

3.3 *Ace2* expression was associated with disease severity and viral load after infection

In order to investigate whether there is an association of *Ace2* expression with severity of disease and/or viral replication as the infection progresses, we conducted Pearson correlation coefficient analysis for *Ace2* levels against body weight loss, mean time to death, and gene expression levels as a surrogate for viral load. Results demonstrated that *Ace2* expression was significantly correlated with both body weight loss (r = 0.575, *P-value* = 0.008, **Figure 3A**) and median body weight loss (r = 0.468, *P-value* = 0.028, **Figure 3B**) at 3 dpi. Further, *Ace2* was positively correlated with the mean time to death (r = 0.458, *P-value* = 0.032, **Figure 3C**), In contrast, *Ace2* had a negative correlation with IAV NA gene expression levels (r = 0.381, *P-value* = 0.029, **Figure 3D**).

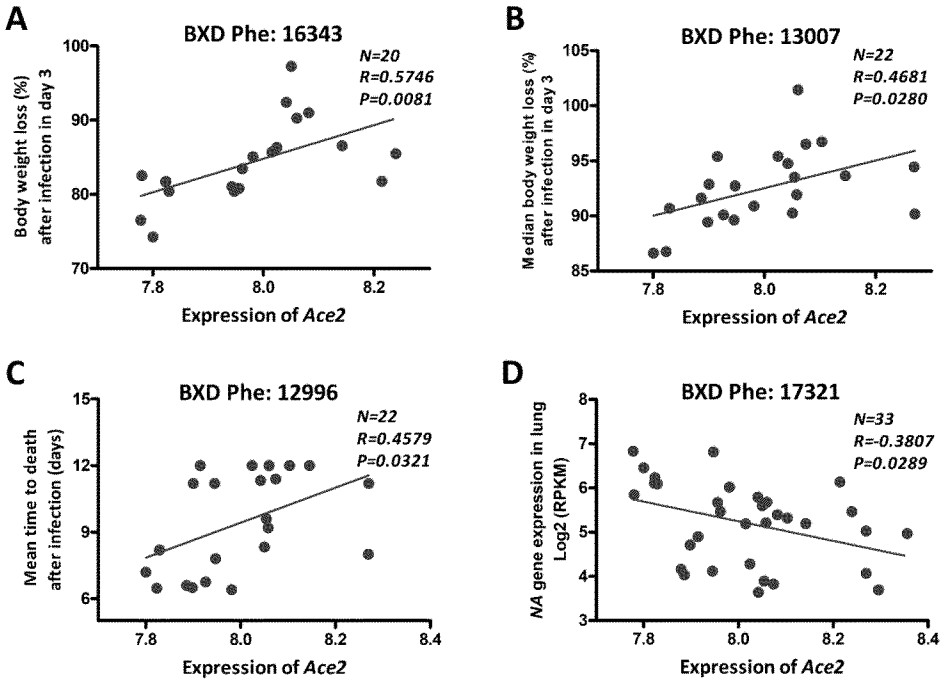


Figure 3. Scatter plots of the correlations of *Ace2* expression with body weight loss (A), median body weight loss (B), mean time to death (C), and viral *NA* gene expression level (D). Pearson correlation coefficient was used to determine the relationship. The percent body weight in **(A)** and **(B)** was relative to the starting body weight (100 %). Gene expression levels represent normalized log2 values. The GeneNetwork accession number of the phenotypes (BXD Phe), number of samples (N), Pearson correlation R-value (R), and Pearson correlation *P*-value (P) are indicated in the figure.

3.4 *Ace2* correlated genes significantly involved in immune system-related pathways

To gain insight into the pulmonary pathways and biological functions that *Ace2* involved in after the virus infection, we conducted Pearson correlation coefficient analysis for *Ace2* against to the whole lung transcriptome. This analysis returned 5500 genes that were significantly correlated with *Ace2* (Pearson correlation *P*-value < 0.05). Next, we submitted those genes to Webgestalt (http://www.webgestalt.org/). 5118 genes were unambiguously mapped to unique Entrez gene IDs. Those genes were subsequently used for an enrichment analysis. The selected genes represented components of the TNF signaling pathway, MAPK signaling pathway, Notch signaling pathway, HTLV-I infection, and VEGF signaling pathway (**Figure 4A**). Furthermore, we also found that these genes were significantly enriched in terms related to chemokine secretion, lung morphology, and respiratory system morphology (**Figure 4B**).

For the top three enriched pathways, 48 genes were found in the TNF signaling pathway (**Supplementary Data 1**), with 35 negatively correlated with *Ace2* (Pearson *P*-value < 0.05), Among these included the tumor necrosis factor (*Tnf,* r = -0.41, *P*-value = 0.005). There were 92 genes in the MAPK signaling pathway (**Supplementary Data 1**), with *Tnf*, transforming growth factor beta 1(*Tgfb1*, r = -0.46, *P*-value = 0.002), and other 59 genes whose expression was negatively correlated with *Ace2*. In addition, 25 genes

were involved in the Notch signaling pathway (**Supplementary Data 1**), with 21 negatively correlated with *Ace2*.

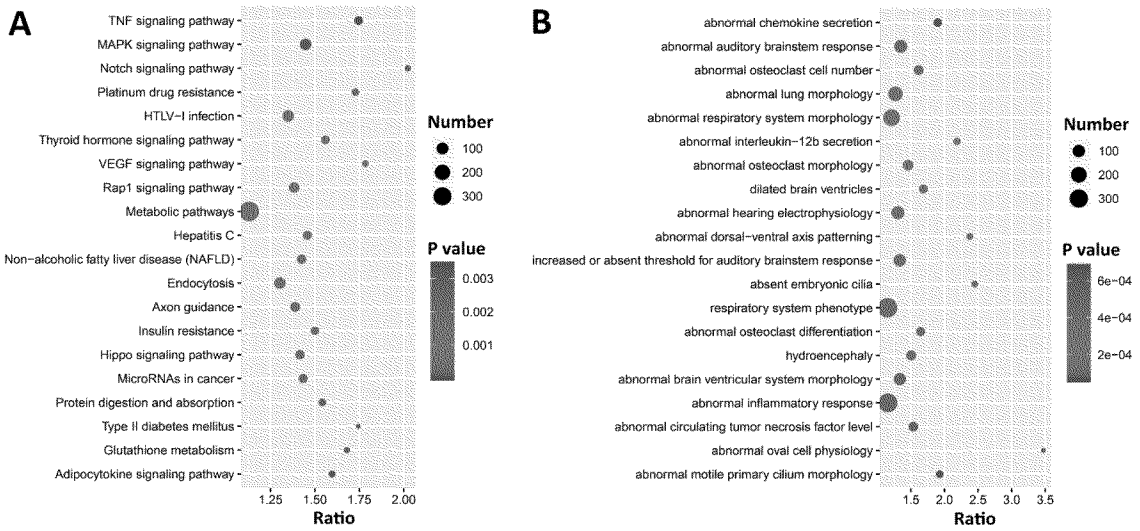


Figure 4. Bubble charts of the top 20 KEGG pathways (A) and 20 Mammalian Phenotype Ontologies (B) enriched for *Ace2* significantly correlated genes. Gene over-representation analysis for KEGG pathway and Mammalian Phenotype Ontology of the *Ace2* correlated genes (Pearson correlation *P*-value < 0.05) were performed with WebGestalt (http://www.webgestalt.org/). The x-axis represents the enrich ratio and y-axis represents enriched pathways/terms. The size of the dots denotes the number of genes and the color the *P*-value.

3.5 Co-expression network analysis narrowed down the *Ace2* correlated genes

To further identify genes that interact more directly with *Ace2*, we constructed gene co-expression network using WGCNA with the *Ace2* correlated genes (*P*-value < 0.05). The 5500 genes were parsed into 10 distinct co-expression modules using a soft-thresholding power of 8 (**Figure 5A-C**). The modules had a wide range of sizes, with the brown module containing 1673 genes and lightcyan module containing only 68 genes. *Ace2* was located in the purple module that contained 462 genes (**Figure 5C**). Genes in this module were significantly involved in regulating motile cilium physiology (**Figure 5D**)

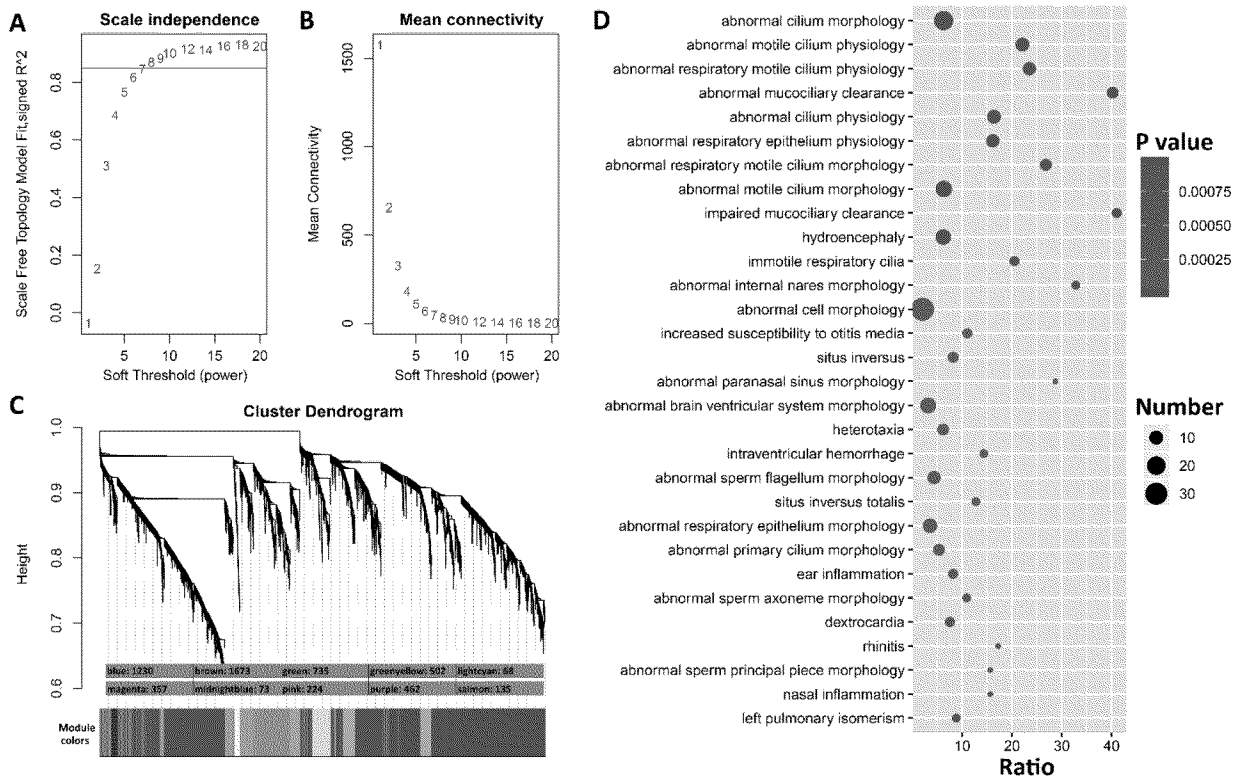


Figure 5. Weighted gene co-expression network analysis of *Ace2* correlated genes. (A) The soft thresholding index R^2 (y-axis) as a function of different powers β (x-axis) indicating the threshold of 8 chosen. **(B)** Mean connectivity (degree, y -axis) as a function of the soft-thresholding power (x -axis). **(C)** 10 co-expression modules identified from *Ace2* correlated genes. Modules are color coded as shown. The number of genes in each module is also indicated. **(D)** Bubble charts of top 30 enriched Mammalian Phenotype Ontologies of the purple module. This analysis was performed with WebGestalt (http://www.webgestalt.org/).

Next, we focused on exploring the genes in the purple module (**Figure 6** and **Supplemental File 1**). First, we performed differential expression analysis between mice at 3 dpi and mock-infected controls by treating strain as a covariate. This led to identification of 281 genes (61 % of the module genes) that showed differential expression (adj.p-value < 0.05 and FC > 1.3). Next, we conducted eQTL mapping using WebQTL on the GeneNetwork for the whole lung transcriptome which identified 23 genes that were *cis*-regulated and 106 others that were *trans*-regulated in H1N1 infected lungs. Those QTLs were further confirmed with GEMMA in GeneNetwork. Moreover, we searched for the DNA sequence differences between B6 and D2, which resulted in a total of 69 genes harbor coding sequence variants (missense, nonsense, and frameshift variants). Furthermore, we found that there were 42 genes implicated in cilium function and regulation identified by enrichment analysis. By comparing the above four categories, 32 cilium function related genes were differentially expressed (**Table 1**). We refer to this set as 'prioritized genes'. Of these, *Sox11*, *Tff2*, *Rnf128*, and *Muc5b* showed *cis*-or *trans*-regulation, and *Tmprss2*, *Ulk4*,

Cckar, *Wdpcp*, *Ift140*, and *Ccdc40* contained functional variants. The genetic perturbations of these 10 genes (**Table 1**) may act as key regulators in this module.

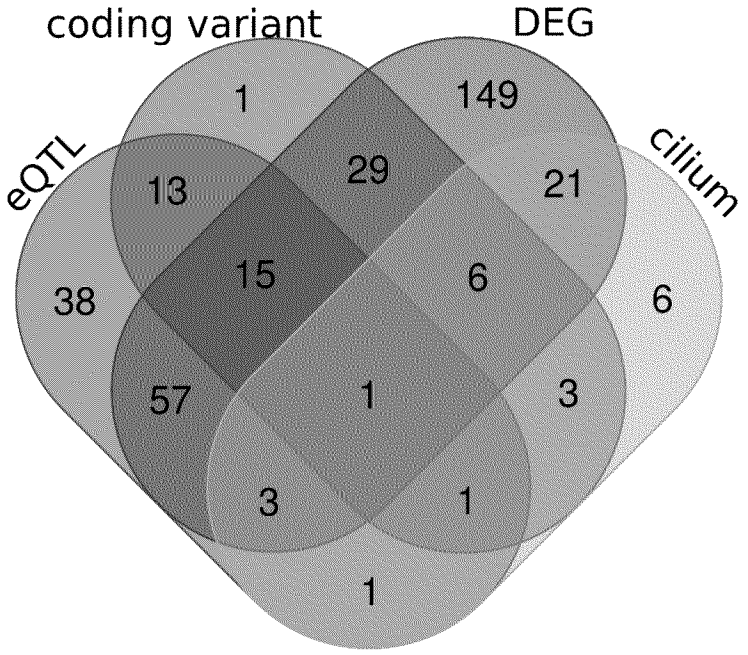


Figure 6. Venn diagrams of the purple module genes. A total of 462 genes were included in the purple module. Four criteria were applied to explore the module genes, including coding sequence variants between parental strain B6 and D2 (coding variant), eQTL (LRS > 15) regulation in H1N1 affected lung tissue (eQTL), cilium functional relevance (cilium), and differential expression (adj.p-value < 0.05 and FC > 1.3) between infected and normal controls in parental strains (DEG). The Venn diagram shows the number of overlapping genes among the four categories.

Table 1 Lists of prioritized genes in purple module.

Gene ID	Symbol	Description	Gene ID	log2FC	padj	eQTL	functional Variant
20666	**Sox11**	SRY-box 11	20666	-1.19	1.95E-12	cis	Nonsynonymous
21785	**Tff2**	trefoil factor 2 (spasmolytic protein 1)	21785	-1.01	5.38E-04	trans	NA
66889	**Rnf128**	ring finger protein 128	66889	-0.77	2.81E-05	trans	NA
74180	**Muc5b**	mucin 5, subtype B, tracheobronchial	74180	0.54	1.13E-02	trans	NA
50528	**Tmprss2**	transmembrane protease, serine 2	50528	-1.06	8.58E-10	NA	Nonsynonymous
209012	**Ulk4**	unc-51-like kinase 4 (C. elegans)	209012	-0.87	3.43E-07	NA	Nonsynonymous
12425	**Cckar**	cholecystokinin A receptor	12425	-0.73	1.91E-05	NA	Nonsynonymous
216560	**Wdpcp**	WD repeat containing planar cell polarity effector	216560	-0.69	2.36E-06	NA	Nonsynonymous
106633	**Ift140**	intraflagellar transport 140 homolog (Chlamydomonas)	106633	-0.68	3.50E-07	NA	Nonsynonymous
207607	**Ccdc40**	coiled-coil domain containing 40	207607	-0.66	1.06E-03	NA	Nonsynonymous
320752	Dpy19l2	dpy-19-like 2 (C. elegans)	320752	-1.81	2.13E-03	NA	NA
51938	Ccdc39	coiled-coil domain containing 39	51938	-1.30	1.15E-13	NA	NA
244653	Hydin	hydrocephalus inducing	244653	-1.30	8.51E-16	NA	NA
74362	Spag17	sperm associated antigen 17	74362	-1.29	9.27E-13	NA	NA
73873	Fam161a	family with sequence similarity 161, member A	73873	-1.28	1.70E-09	NA	NA

320662	Casc1	cancer susceptibility candidate 1	320662	-1.27	2.44E-08	NA	NA
66722	Spag16	sperm associated antigen 16	66722	-1.27	1.61E-10	NA	NA
74934	Armc4	armadillo repeat containing 4	74934	-1.21	8.50E-09	NA	NA
18703	Pigr	polymeric immunoglobulin receptor	18703	1.18	1.10E-09	NA	NA
69707	Iqcg	IQ motif containing G	69707	-1.18	2.97E-20	NA	NA
78801	Ak7	adenylate kinase 7	78801	-1.17	4.75E-09	NA	NA
27078	B9d1	B9 protein domain 1	27078	1.09	1.00E-02	NA	NA
68922	Dnaic1	dynein, axonemal, intermediate chain 1	68922	-1.07	3.67E-08	NA	NA
226265	Eno4	enolase 4	226265	-0.98	1.49E-09	NA	NA
71877	Efhc1	EF-hand domain (C-terminal) containing 1	71877	-0.92	3.77E-11	NA	NA
75533	Nme5	expressed in non-metastatic cells 5	75533	-0.82	5.92E-05	NA	NA
75050	Kif27	kinesin family member 27	75050	-0.71	3.05E-03	NA	NA
70008	Ace2	angiotensin I converting enzyme (peptidyl-dipeptidase A) 2	70008	-0.61	9.88E-05	NA	NA
20878	Aurka	serine/threonine kinase 6	20878	0.60	2.36E-02	NA	NA
22062	Trp73	transformation related protein 73	22062	-0.60	1.81E-03	NA	NA
77609	Ccdc151	coiled-coil domain containing 151	77609	-0.47	9.85E-02	NA	NA
252973	Grhl2	grainyhead-like 2 (Drosophila)	252973	-0.44	5.36E-04	NA	NA

Genes that were differentially expressed (adj.p-value < 0.05 and FC > 1.3) and implicated in cilium biological functions; Highlighted in bold are those 10 genes that may act as key regulators in the purple module; NA, not available.

3.6 Bayesian network analysis inferred upstream regulators of *Ace2*

In order to infer the causative relations of *Ace2* with the top 10 prioritized genes, we performed Bayesian network analysis. Ring finger protein 128 (*Rnf128*) stood out as the upstream regulator of the other 10 genes (**Figure 7**). This gene was predicted to directly target the genes intraflagellar transport 140 homolog (*Ift140*), unc-51-like kinase 4 (*Ulk4*), coiled-coil domain containing 40 (*Ccdc40*), SRY-box 11 (*Sox11*), and *Ace2*. In addition, *Ace2* is also found to be targeted through another pathway, which begins with the expression of *Rnf128,* then the expression of *Ulk4, Wdpcp*, and, finally, to *Ace2* (**Figure 7**).

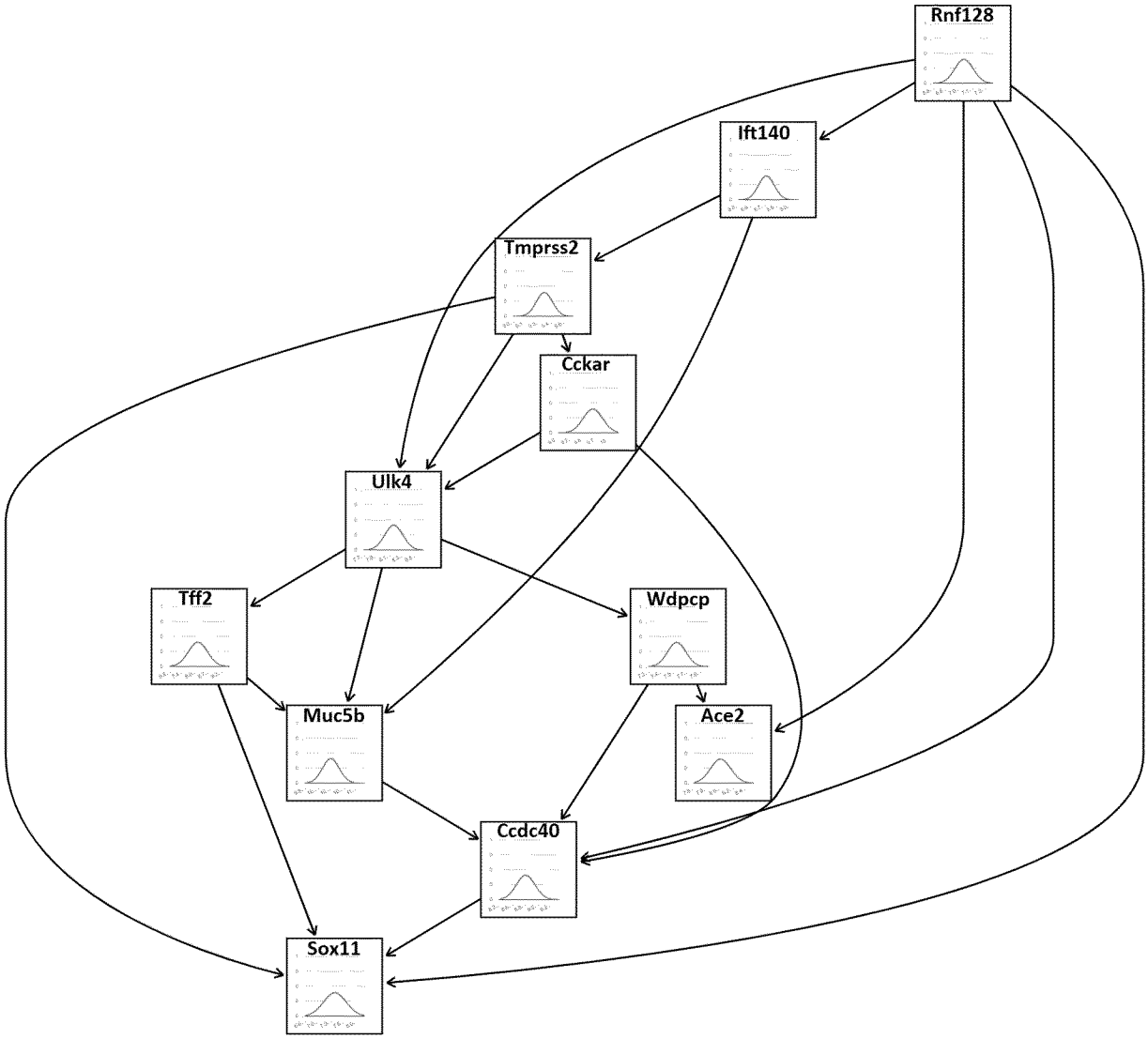


Figure 7. **Bayesian network analysis inferred upstream regulators of *Ace2***. The Bayesian network structure was constructed on Bayesian Network Webserver ((compbio.uthsc.edu/BNW) with gene expression values as the input. http://bnw.genenetwork.org/BNW/sourcecodes/layout.php?My_key=NLq

3.7 The differential expressed cilium function related genes in the purple module were replicated in SARS-CoV-infected HAE cells

In order to further evaluate whether those 'prioritized genes' (**Table 1**) were also regulated in humans, especially in the context of SARS-CoV infection, we downloaded a microarray data set for HAE cells infected with SARS-CoV (icSARS-CoV), ic-SARS-CoV ORF6 and BAT-SARS_CoV [43]. We found that very few of those 'prioritized genes' were significantly regulated after infection with BAT-SARS (**Figure 8A**). However, most of the 'prioritized genes' were differentially expressed in icSARS-CoV and ic-

SARS-CoV ORF6 infected HAE cells when compared to mock controls, with 21 genes found at 96 h after infection (**Figure 8A**). More importantly, those overlapping genes, except *Ace2*, exhibited the same up- or down-regulating patterns in both HAE cells and our mouse model (**Table 1** and **Figure 8B**). *ACE2* was up-regulated in HAE cultures whereas in whole lungs it was found to be down-regulated. This is an important observation since in the HAE cultures only airway epithelial cells are present, but no interstitial cells, endothelial cells, resident and infiltrating immune cells are present. These observations indicate that in the lungs a more complex regulatory network is involved in the regulation of *ACE2* than in isolated epithelial cells and that it is indeed downregulated in all cells after infection in the whole lung. Alternatively, *ACE2* may become up-regulated only in infected cells. In the lungs, infected cells represent only a minority of all cells present. Since our lung RNAseq study investigated total gene expression changes of all cell types it is well conceivable that we do not detect a possible up-regulation in these infected cells. In future studies, it will be important to analyze *Ace2* expression in infected mouse lungs by immunohistochemistry or *in situ* hybridization to test these hypotheses.

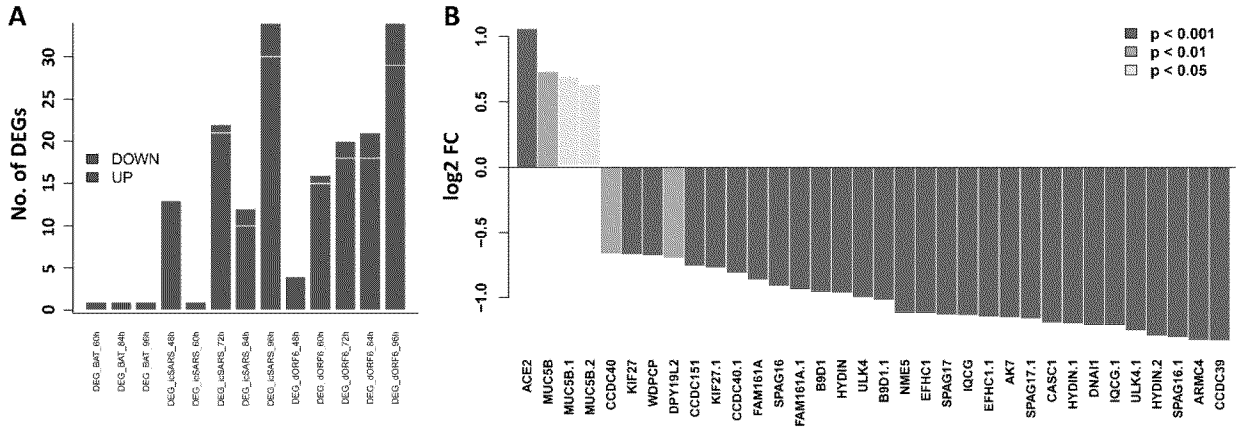


Figure 8. Bar plots of the cilium function related genes in the SARS-CoV infected HAE cells. Primary human HAE cells were infected with MOI 3 SARS-CoV (icSARS-CoV), ic-SARS-CoV ORF6 and BAT-SARS_CoV and gene expression was analyzed by arrays. (A) Number of up-and downregulated differentially expressed genes for cilium function related genes that had orthologous genes in humans and were represented on the array (logFC > 0.58 and adjusted p-value < 0.05); y-axis: number of differentially expressed genes. (B) LogFC differences in expression values between HAE cells infected with icSARS-CoV at 96h pi and mock-treated controls for cilium function related genes that had orthologous genes in humans and were represented on the array; y-axis: differences in log2 fold change expression levels between groups. Adjusted p-values were taken from the Limma analysis of the entire transcriptome data.

Discussion

Many studies have demonstrated that host genetic background significantly influences the susceptibility to influenza A infections in humans [46]. This phenomenon was also observed in mouse models [14], especially for inbred strains B6 and D2 [15]. In this study, we systemically analyzed the potential regulatory role of *Ace2* for the host response to H1N1 infection in a subset of BXD RI mice. Our results

showed that the expression of *Ace2* in lung significantly decreased in a disease severity- and time-dependent manner. In addition, we also found the expression variation of *Ace2* negatively correlated with the body weight loss and survival time after the infection. These results strongly suggests that *Ace2* is involved in H1N1 virus disease progression and that the lower levels of *Ace2* may play a role in virus-induced ALI. This notion was supported in studies of *Ace2*$^{-/-}$ mice infected with influenza H7N9 [47] and with respiratory syncytial virus [48] where lung pathology was more severe in mutant mice.

These observations argue strongly that *Ace2* can mediate virus-induced ALI [47, 48]. However, the underlying mechanisms remain elusive. By using 43 inbred strains, we were able to perform genetic correlation analysis and gain insight into the *Ace2*-associated biological pathways. We identified a total of ~5000 *Ace2*-correlated genes. These genes are involved in regulating respiratory and inflammatory networks through several pathways, including TNF signaling pathway, MAPK signaling pathway, and Notch signaling pathway.

The factors associated with H1N1-virus-induced lethality mainly consist of virus-induced inflammatory cytokine storm and tissue destruction. Tumor necrosis factor (*TNF*) is a cytokine with multiple effects, including promoting cell growth, differentiation, apoptosis, and inducing inflammation [49, 50]. *TNF-α*, a member of the *TNF* family, can activate caspase protease, JNK, and transcription factor NF-κB, thereby executing its biological functions such as cytotoxicity, antiviral, and immuno-regulation. A previous study has demonstrated that inhibition of *Tnf-α* can protect mice from lethal H1N1 infection [51]. In the present study, we found *Ace2* was negatively correlated (r = -0.41 and *P*-value = 0.005) with the expression of *Tnf* and other 34 TNF signaling pathway related genes, suggesting that *Ace2* could dampen excessive inflammatory immune responses through downregulating of this signaling pathway and mitigating immune-mediated tissue destruction. We also found 92 genes in the MAPK signaling pathway significantly correlated with *Ace2*, including *Tgfb1* (r = -0.46 and *P*-value = 0.002). Of note, it was shown that IAV can activate this pathway to support its replication [52], The authors proposed a model in which, *Tgfb1*acts as a pro-viral host factor by suppressing early immune responses, creating an environment conducive for IAV infection [53]. Moreover, another set of 25*Ace2*-correlated genes were involved in the Notch signaling pathway, with most of them (21 genes) showing negative correlations. This pathway is an important bridge between antigen-presenting cells (APCs) and T cell communication circuits and plays a central role in driving the immune system to overcome infectious disease [54].

By constructing gene co-expression network, we narrowed the *Ace2* co-expressed genes to 462 (purple module). Enrichment analysis revealed that those genes were significantly related to cilium function, including *Ace2* itself. This finding suggests that *Ace2* participates in ALI not only by interacting with the TNF, MAPK, and Notch signaling pathways, but also through regulating functions of respiratory epithelium, which is a barrier to microbial infection and tissue injury by the secretion of mucus and the action of mucociliary clearance. In order to identify the key regulators in the purple module. We employed multi-layer filtration, including differential expression analysis, eQTL mapping, functional variant identification, and gene annotation. This resulted in 10 high priority candidates, i.e., *Sox11*, *Tff2*, *Rnf128*, *Muc5b*, *Tmprss2*, *Ulk4*, *Cckar*, *Wdpcp*, *Ift140*, and *Ccdc40*. To further investigate the relationship of these

genes with *Ace2*, we performed Bayesian network analysis and identified *Rnf128* as the master regulator. This gene encodes a ring-finger-containing protein which has been shown to possess E3 ubiquitin ligase activity [55]. *RNF128* has been implicated involved in the induction of T cell anergic phenotype through inhibiting activation-induced IL2 and IL4 cytokine production [56] and mediating T cell receptor-CD3 degradation [57]. In a recent study, Song *et al.* [58] reported *RNF128* promotes lysine-63-linked ubiquitination and activation of a critical kinase TBK1, thereby augmenting the induction of type I interferon antiviral cytokines that curb viral replication. The authors' in vivo experiments further showed that *Rnf128* deficient mice were more susceptible to vesicular stomatitis virus (an RNA virus) and herpes simplex virus (a DNA virus). Our results suggest that there are two potential gene regulatory paths for *Ace2*, both of which begin with *Rnf128*. However, direct connection of the two genes will need to be investigated experimentally in future studies.

Besides the master regulator *Rnf128*, other genes were also found to regulate the immune and respiratory epithelium system. For instance, *MUC5B* (mucin 5, subtype B, tracheobronchial), encodes a member of the mucin family of proteins, and is associated with idiopathic pulmonary fibrosis in humans [59]. Overexpression of this gene in mice causes mucociliary dysfunction, and enhances lung fibrosis [60]. It was reported to be required for airway defense in another study [61]. *CCDC40* (coiled-coil domain containing 40), encodes a protein that is essential for motile cilia function. According to the OMIM (https://omim.org/) database, this gene is associated with recurrent respiratory infections (HP:0002205) and abnormal axonemal organization of respiratory motile cilia (HP:0012258). Malfunction of *Ccdc40* in mice resulted in left pulmonary isomerism and lung situs inversus [62]. *TMPRSS2* (transmembrane protease, serine 2) encodes a protein that belongs to the serine protease family. *Tmprss2* knockout mice show reduced lung inflammation with IAVs (H1N1, H3N2, or H7N9) infection and exhibit decreased susceptibility to virus- induced morbidity/mortality [63]. Studies also demonstrated that SARS coronavirus engages *ACE2* as the entry receptor on cell surface (Ref 9: Li W et al, Nature. 2003; 426:450-4.) and employs the *TMPRSS2* for Spike protein priming, which facilitates viral entry and infection [64, 65]. Besides, *Ulk4*, *Sox11*, and *Tff2* are associated with abnormal respiratory motile cilium [66], pulmonary hypoplasia [67], and abnormal adaptive immunity [68], respectively.

The ongoing outbreak of the COVID-19 caused by SARS-CoV-2 poses a global public health threat [69-71]. The entering of SARS-CoV-2 into host cells is dependent on *ACE2*. [72, 73], the receptor for SARS-CoV and can be blocked by a clinically approved TMPRSS2 protease inhibitor [73]. However, how *ACE2* interacts with other genes involved in the progression of SARS-CoV-2 induced respiratory diseases remains to be elucidated. The first report based on autopsy samples demonstrated that the pathological characteristics of COVID-19 are very similar to those of SARS-CoV and MERS-CoV infected patients [74]. Therefore, we looked up those 30 prioritized genes (**Table 1**), co-expressed with *Ace2* after H1N1 infection, in SARS-CoV-infected primary human HAE cells. This resulted in 20 out of them showing consistent up/down-regulation with our H1N1 model, suggesting the two viruses share common *Ace2* regulatory networks that participate in the occurrence and development of lung pathology.

In summary, we have shown the expression of *Ace2* was significantly downregulated in the lungs of IAV H1N1 infected mice. By using BXD RI strains, we were able to identify *Ace2* co-expressed genes with genetic correlation analysis and weighted gene co-expression network analysis. Thus, our results indicate that *Ace2* participates in virus-induced respiratory diseases not only through immune system related pathways, but also by protective functions in the respiratory epithelium. In addition, *Rnf128* was identified as the master regulator through Bayesian network analysis. Finally, 21 of our prioritized genes were found to be also differentially expressed in SARS-CoV-infected HAE cells. These findings provide an *Ace2* regulatory network and raise several potential therapeutic targets for virus-induced (H1N1, SARS-CoV, MERS-CoV, and SARS-CoV-2) respiratory diseases. They additionally illustrate that the BXD RI strains provide an excellent resource for studying the host genetic regulatory mechanism of the viral infections, such as that caused by SARS-CoV-2.

Acknowledgement

Primary human bronchial epithelial cells prepared as described previously (reference = PMID:23097104) were provided by Dr. Scott H. Randell (Marsico Lung Institute, Tissue Procurement and Cell Culture Core, The University of North Carolina at Chapel Hill, USA). The cells were obtained under protocol #03-1396 approved by the University of North Carolina at Chapel Hill Biomedical Institutional Review Board.

This work was supported by an intra-mural grant from the Helmholtz-Association (Program Infection and Immunity), a start-up grant from the University of Tennessee Health Science Center, a NIH/NIAID Research Grant U19-AI100625 to KS, and NIH/NIAID Research Grants U19-AI100625, U01 AI149644, and U19 AI 109761 to R.S.B. Provision of cells by Dr. Randell was supported by grants from the Cystic Fibrosis Foundation (BOUCHE19R0 and NIH (DK065988).

Author Contributions

LL, KS and RSB conceived the study; SB and ACS conducted the experiments; FX and KS performed data analysis; LL, FX, KS, and JG wrote the manuscript. FX, KS, and JG prepared the figures and tables; RWW, KL, CBJ edited the manuscript; all authors read and approved the final version of the manuscript for publication.

Conflicts of Interest

The authors declare no conflicts of interests.

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From: Caldwell, Chandra[ccaldwel@email.unc.edu]
Sent: Wed 4/1/2020 2:07:47 PM (UTC-07:00)
To: Sims, Amy C[amy.sims@pnnl.gov]
Subject: RE: Do you have 15 minutes to chat tomorrow?

Hey there,

It would be ideal for us to talk tomorrow but Vicki is dragging her feet in sending me the info she found on how your grant transfer can be requested at the same time your progress report is submitted. I have asked each day this week to send it to me and she keeps forgetting. I have a call with her tomorrow at noon. So I will email you and let you know if 3pm or 4pm EST will work for you to have a call. I can be reached at the number you have for me since we have been taking weekly turns "babysitting" your former colleagues!

Thanks,
Chandra

PS: If I can keep my parents from going to the grocery store every other day and to restock on canned goods and get V to stay on top of email, things would be easy breezy as I could take things in stride! But I will just continue to have wishful thinking. Hope you are doing good amongst the chaos in adapting to all your new surroundings.

From: Sims, Amy C <amy.sims@pnnl.gov>
Sent: Wednesday, April 1, 2020 4:22 PM
To: Caldwell, Chandra <ccaldwel@email.unc.edu>
Subject: Do you have 15 minutes to chat tomorrow?

Hi,

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I want to make sure I get you all the information you need for the progress report and I am trying to figure out how much of my changing jobs/work sites needs to be documented in the progress report.

Thank you!! Amy

PS. How are you??

From: Caldwell, Chandra[ccaldwel@email.unc.edu]
Sent: Thur 4/2/2020 10:51:15 AM (UTC-07:00)
To: Sims, Amy C[amy.sims@pnnl.gov]
Subject: RE: Do you have 15 minutes to chat tomorrow?

Hey Amy,

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Thanks,
C.

From: Sims, Amy C <amy.sims@pnnl.gov>
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To: Caldwell, Chandra <ccaldwel@email.unc.edu>
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That sounds perfect. Right now I don't have anything in the afternoon so I think I will block off a couple of hours to make sure I don't have anything else.

Thank you, Amy

From: "Caldwell, Chandra" <ccaldwel@email.unc.edu>
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To: Sims, Amy C[amy.sims@pnnl.gov]
Subject: RE: Do you have 15 minutes to chat tomorrow?

Hey there,

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To: Caldwell, Chandra <ccaldwel@email.unc.edu>
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Do you want me to hold the 4 to 5pm slot tomorrow and then we can move the time as needed? Thank you, Amy

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Sent: Thur 4/2/2020 11:00:24 AM (UTC-07:00)
To: Sims, Amy C[amy.sims@pnnl.gov]
Subject: RE: Do you have 15 minutes to chat tomorrow?

Trust me…you are fine! It's trying to get the info I've been requesting for over 2 weeks now from V that is the problem!!

From: Sims, Amy C <amy.sims@pnnl.gov>
Sent: Thursday, April 2, 2020 1:59 PM
To: Caldwell, Chandra <ccaldwel@email.unc.edu>
Subject: Re: Do you have 15 minutes to chat tomorrow?

Awesome. Thank you. Hopefully I am not adding to your daily stress but I wanted to make sure all the things I was supposed to capture are in the progress report. Obviously the NIH knows I moved but I wasn't sure how much needed to be documented.

Thank you very much! Amy

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From: Sims, Amy C <amy.sims@pnnl.gov>
Sent: Wednesday, April 1, 2020 6:13 PM
To: Caldwell, Chandra <ccaldwel@email.unc.edu>
Subject: Re: Do you have 15 minutes to chat tomorrow?

That sounds perfect. Right now I don't have anything in the afternoon so I think I will block off a couple of hours to make sure I don't have anything else.

Thank you, Amy

From: "Caldwell, Chandra" <ccaldwel@email.unc.edu>
Date: Wednesday, April 1, 2020 at 2:08 PM
To: "Sims, Amy C" <amy.sims@pnnl.gov>
Subject: RE: Do you have 15 minutes to chat tomorrow?

Hey there,

It would be ideal for us to talk tomorrow but Vicki is dragging her feet in sending me the info she found on how your grant transfer can be requested at the same time your progress report is submitted. I have asked each day this week to send it to me and she keeps forgetting. I have a call with her tomorrow at noon. So I will email you and let you know if 3pm or 4pm EST will work for you to have a call. I can be reached at the number you have for me since we have been taking weekly turns "babysitting" your former colleagues!

Thanks,
Chandra

PS: If I can keep my parents from going to the grocery store every other day and to restock on canned goods and get V to stay on top of email, things would be easy breezy as I could take things in stride! But I will just continue to have wishful thinking. Hope you are doing good amongst the chaos in adapting to all your new surroundings.

From: Sims, Amy C <amy.sims@pnnl.gov>
Sent: Wednesday, April 1, 2020 4:22 PM
To: Caldwell, Chandra <ccaldwel@email.unc.edu>
Subject: Do you have 15 minutes to chat tomorrow?

Hi,

Do you have any time to talk Thursday or Friday of this week? Best times for me are going to be Noon to whenever you leave each day.

I want to make sure I get you all the information you need for the progress report and I am trying to figure out how much of my changing jobs/work sites needs to be documented in the progress report.

Thank you!! Amy

PS. How are you??

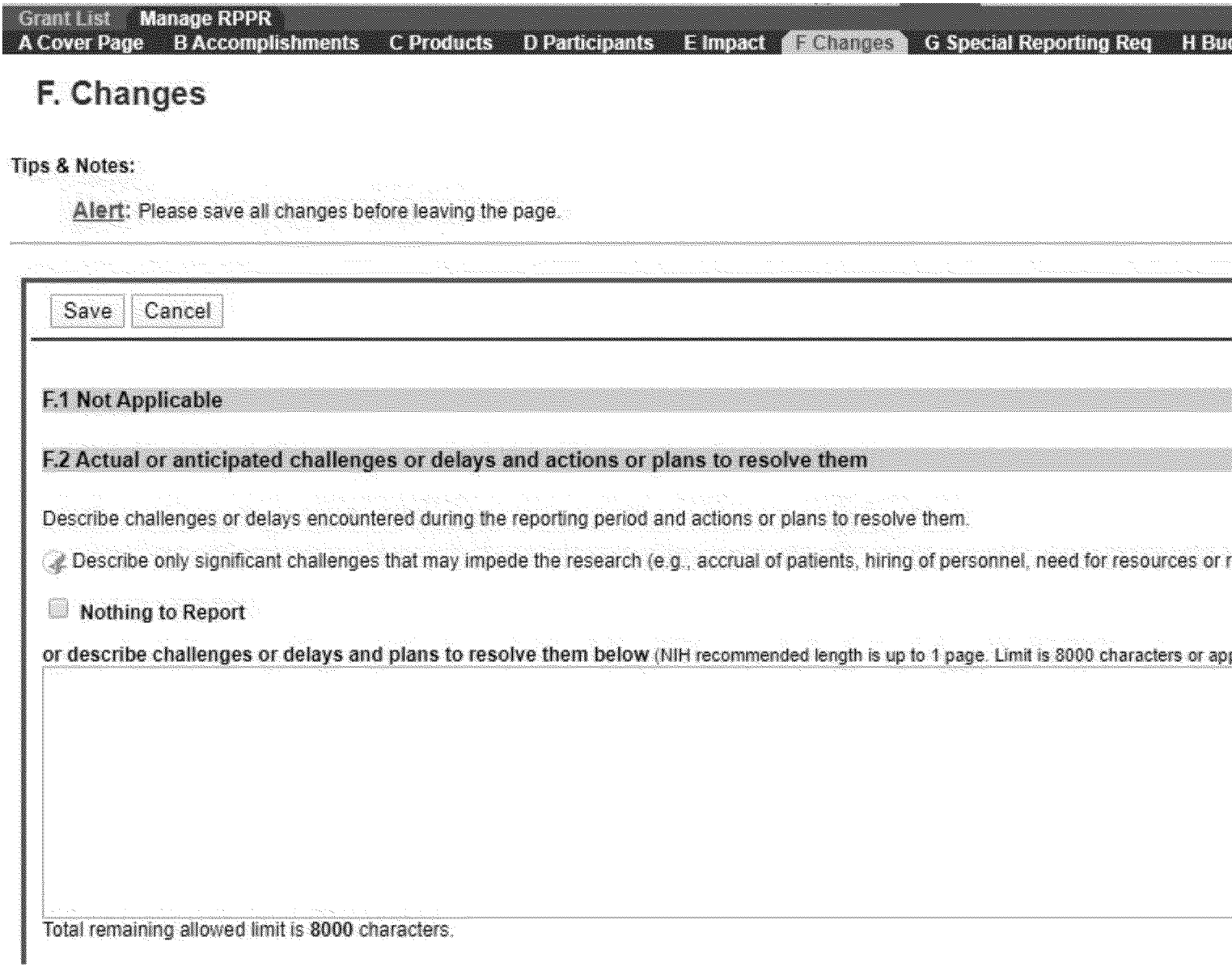
From: Sims, Amy C[amy.sims@pnnl.gov]
Sent: Fri 4/3/2020 11:23:56 AM (UTC-07:00)
To: Caldwell, Chandra[ccaldwel@email.unc.edu]
Subject: What time do you want to talk today??

I hope the call earlier went well. Thank you, Amy

From: Caldwell, Chandra[ccaldwel@email.unc.edu]
Sent: Fri 4/3/2020 11:52:12 AM (UTC-07:00)
To: Sims, Amy C[amy.sims@pnnl.gov]
Subject: RE: What time do you want to talk today??

It was rescheduled because the PO double booked conference calls...ugh!! So we will deal with your progress report and relinquish statement in two separate manners. I will get the progress report submitted and once that is in the eRA Commons que – we will do the relinquish statement before your next budget period begins June 1, 2020. I can tell that V is getting annoyed at me asking so I will let her breathe over the weekend, as I begin again next week.

As for you noting your departure, per the question template I sent you – you can mention it in section B under accomplishments (very briefly...no more than 2-3 sentences) . I would then recommend me putting a paragraph in the changes section (section F) about your new position, the date it became effective and stating after the submission of this RPPR, the grant will be transferred to PNNL before the start of the new budget year.



From: Sims, Amy C <amy.sims@pnnl.gov>
Sent: Friday, April 3, 2020 2:24 PM
To: Caldwell, Chandra <ccaldwel@email.unc.edu>

Subject: What time do you want to talk today??

I hope the call earlier went well. Thank you, Amy

From: Lu, Lu[llu@uthsc.edu]

Sent: Sat 4/4/2020 8:29:34 AM (UTC-07:00)

To: Sims, Amy C[amy.sims@pnnl.gov]; silke.bergmann@(b) (6) [silke.bergmann@(b) (6)]; rbaric@email.unc.edu[rbaric@email.unc.edu]; Jonsson, Colleen Beth[cjonsson@uthsc.edu]; Li, Kui[kli1@uthsc.edu]

Cc: Xu, Fuyi[fxu10@uthsc.edu]; Klaus x Klaus Schughart[Klaus.Schughart@helmholtz-hzi.de]; Williams, Robert W[rwilli10@uthsc.edu]

Subject: Ace2

Attachment: Ace2_V9_04042020B.docx

Dear all authors:
We updated manuscript based on the second editing from part of authors. We plan to submit this version at coming week. Please make your final check and let us know before end of coming Monday if you have change. Thanks.

Lu Lu
Professor
Department of Genetics, Genomics and Informatics
University of Tennessee Health Science Center
71 S. Manassas St., Room 410K
Memphis, TN 38163
Tel: (901) 448-7557 (O)
Fax: (901) 448-3500 (Dept)

Genetic Dissection of the Regulatory Mechanisms of *Ace2* in the Infected Mouse Lung

Fuyi Xu[1#], Jun Gao[1, 5#], Silke Bergman[4], Amy C. Sims[7, 9], Ralph S. Baric[6,7, 8], Colleen B Jonsson[4], Kui Li[4], Robert W. Williams[1*], Klaus Schughart[2, 3, 4*], and Lu Lu[1*]

[1]Department of Genetics, Genomics, and Informatics, University of Tennessee Health Science Center, Memphis, TN, USA;
[2]Department of Infection Genetics, Helmholtz Centre for Infection Research, Braunschweig, Germany
[3]University of Veterinary Medicine Hannover, Hannover, Germany;
[4]Department of Microbiology, Immunology, and Biochemistry, University of Tennessee Health Science Center, Memphis, TN, USA;
[5]Institute of Animal Husbandry and Veterinary Science, Shanghai Academy of Agricultural Sciences, Shanghai, China.
[6]Department of Microbiology and Immunology, University of North Carolina at Chapel Hill, Chapel Hill, NC
[7]Department of Genetics, University of North Carolina at Chapel Hill, Chapel Hill, NC
[8]Department of Epidemiology, University of North Carolina at Chapel Hill, Chapel Hill, NC
[9]present address: Pacific Northwest National Laboratory in Richland, WA

[#] These authors contributed equally to this work.

* Correspondence:

Lu Lu: llu@uthsc.edu
Department of Genetics, Genomics and Informatics
University of Tennessee Health Science Center
Memphis, TN 38163, United States

Klaus Schughart: klaus.schughart@helmholtz-hzi.de
Department of Infection Genetics
Helmholtz Centre for Infection Research
Braunschweig, Germany

Robert W. Williams: rwilliams@uthsc.edu
Department of Genetics, Genomics and Informatics
University of Tennessee Health Science Center
Memphis, TN 38163, United States

Abstract

Acute lung injury (ALI) is an important cause of morbidity and mortality after viral infections, such as influenza A virus H1N1, SARS-CoV, MERS-CoV, and SARS-CoV-2. The angiotensin 1 converting enzyme 2 (*ACE2*) plays a protective role in SARS-CoV-induced ALI. However, contributions of *ACE2* gene variants to the post-infection heterogeneity of the host response and susceptibility remain unclear, as do underlying mechanisms. In this study, we quantified H1NI-infected lung transcriptomes of 41 BXD recombinant inbred strains of mice and both parental strains—C57BL/6J and DBA/2J. In response to infection, the expression of *Ace2* mRNA across strains decreased significantly and was associated with both disease severity and infection time. Expression was negatively correlated with body weight loss and duration of survival. Data for all 43 genomes were analyzed using weighted gene co-expression network analysis (WGCNA). *Ace2* not only participated in virus-induced ALI by interacting with TNF, MAPK, and NOTCH signaling pathways, but also was linked to gene products that have important functions in the pulmonary epithelium. Moreover, by applying multi-layer filtration, including differential expression analysis, eQTL mapping, functional variant identification, and gene annotation, we highlighted 11 key candidate genes linked to *Ace2* mRNA networks: *Sox11*, *Tff2*, *Rnf128*, *Muc5b*, *Tmprss2*, *Ulk4*, *Cckar*, *Wdpcp*, *Ift140*, *Ccdc40,* and *Elmod1*. Of these transcripts, *Rnf128* was identified as a potential master regulator using Bayesian network analysis. This gene set is enriched for genes also shown here to be differentially expressed in SARS-CoV-infected primary human airway epithelial cells. Collectively, these findings define an *Ace2*-associated modulatory network relevant to host response to H1N1 infection and define new therapeutic targets for virus-induced respiratory diseases, including those caused by SARS-CoV and SARS-CoV-2. In addition, we demonstrate that the genetically diverse BXD family of strains provides an excellent testbed for dissecting host genetic mechanisms involved in the defense against viral infections and host disease.

Key words: H1N1, acute lung injury, BXD mice, *Ace2,* regulatory network

Introduction

The pandemic infectious disease, COVID-19, is caused by a novel coronavirus, termed severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). This pathogen has spread with almost unprecedent speed from an initial outbreak in November or December 2019. As of March 30, 2020, according to estimates by the World Health Organization (WHO), ~195 countries and >782,000 cases of COVID-19 have been reported and linked to >37,000 deaths.

Since 1918, the world has witnessed four influenza A virus (IAV) pandemics—1918 H1N1, 1957 H2N2, 1968 H3N2, and 2009 H1N1. The first of these resulted in at least 50 million global mortalities [1]. The primary pathological features of both COVID-19 and 2009 influenza A (H1N1) infection are acute viral pneumonia and acute lung injury (ALI) [2]. The well documented symptoms of influenza A (H1N1) and COVID-19 have substantial overlap. For both pathogens, infected patients can either be asymptomatic or can have symptoms ranging from mild coughing, congestion, and fever, to severe and lethal pulmonary edema. Individual genetic differences almost certainly play an important role in the development and severity of the illness and its time course [3-5].

Angiotensin 1 converting enzyme 2 (*ACE2*)—a negative regulator of the renin angiotensin system (RAS)—plays a protective role in pathogenesis of ALI in several animal models of acute respiratory distress syndrome [6]. *Ace2* knockouts exhibit severe ALI. In contrast, treatment with recombinant human *ACE2* attenuates acute lung failure in both *Ace2* knockouts and wildtype control [7]. Studies have shown that ACE2 protein levels are significantly downregulated after infection with H1N1 virus, but thereafter ACE2 is dispensable for viral replication [8]. For SARS-CoV, ACE2 is the principal host transmembrane receptor in binding of the viral spike protein and thereby greatly facilitates endocytosis. In *in vivo* studies, SARS-CoV infection of mice causes severe pulmonary disease and lethal lung failure, concomitant with the downregulation of *Ace2* in lungs [9]. In addition, single-cell RNA sequencing demonstrates that most of *ACE2*-expressing cells in the human lungs are in the small airway in alveolar epithelial type II cells (AT2) [10].Collectively, these findings are of considerable importance because steady-stage expression of *ACE2* in human lung varies nearly 20-fold among a large sample of generally health human subjects [11] (https://genenetwork.org/show_trait?trait_id=ENSG00000130234&dataset=GTEXv8_Lung_tpm_0220)
It is possible, even likely that there are genetic differences in host susceptibility depending on the numbers or ratios of *ACE2*-positive AT2 cells and epithelial cells in lung [10], and the level of expression by these cells of ACE2. However, we emphasize that causal linkage of variation in *ACE2* expression level or sequence variants to susceptibility of infection or to post-infection disease progression is still very poorly defined in humans or even in mouse.

Susceptibility to IAV (such as H1N1 and H7N7) infection varies greatly among strains of isogenic mice [12], especially between two key inbred strains—C57BL/6J (B6), the reference strain for most experimental work—and DBA/2J (D2), the oldest of inbred strains of mice [13]. B6 is relatively resistant to IAV and often survives infection. In contrast, D2 loses body weight rapidly and at the same inoculum dose as given to B6 dies within 5–7 days. Our laboratory has generated and maintains approximately 120 BXD progeny strains derived from crosses between B6 and D2 parents. Both parental strains and all of

these highly diverse BXD recombinant inbred (RI) progeny have been sequenced. Collectively this family segregates for ~6 million variants with minor allele frequencies above 0.3 [14], making it an ideal and well powered reference population to model genetic susceptibility to IAV infections [15]. Indeed, in previous genetic mapping using this cohort we have defined loci and even cloned gene variants that control the host response to IAV (H1N1) infection [16] and to other infectious diseases [17-20]. The BXD family thus provides a powerful resource to dissect loci, gene variants, molecular networks, mechanisms, and even therapeutic interventions in response to viral infection.

Here we have analyzed the lung transcriptome by RNA-seq in 41 BXD RI strains and both parental strains after infection with IAV (H1N1). By using systems genetics approaches, we have defined patterns of covariation of mRNAs with induced variation in *Ace2* expression, as well as molecular networks and regulatory relations that are likely to contribute to the host defense and/or the pathologies of H1N1 and possibly SARS-CoV-induced ALI.

Materials and methods

Mice

The subjects for this study were females belonging to 41 distinct BXD RI strains, and the parental B6 and D2 strains. The family of strains segregates for ~6 million sequence variants with minor allele frequencies greater than 0.3 [21]. They are well powered to detect major loci that contribute to genetic variation in disease susceptibility [17-20] and to define causal networks of interacting loci, genes, gene productions (mRNAs and proteins), and pathogenicity outcome measures [16, 22]. All cases were between 8 and 12 weeks of age when infected with virus. All were housed and maintained on a 12:12 light/ dark cycle, with *ad libitum* access to food and water.

Ethics

Protocols for the mouse experiments were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Tennessee Health Science Center.

Virus

Original stocks of mouse adapted A/Puerto Rico/8/34 (H1N1, PR8M) virus were obtained from Stefan Ludwig, University of Münster [23]. Virus stocks were propagated in the chorioallantoic cavity of 10-day-old pathogen-free embryonated chicken eggs for 48 h at 37 °C as described previously[24]. Viral titer was determined by focus-forming unit (FFU) assay as described previously [24].

Infection of mice

Animals were anesthetized by intraperitoneal injection of ketamine/xylazine (10 % (v/v) of 100 mg/ml ketamine and 5 % (v/v) of 20 mg/ml xylazine in 0.9 % (w/v) NaCl) with a dose adjusted to body weight (200 µl/20 g body weight). Infection was performed by intranasal application of virus solution in 20 µl sterile phosphate-buffered saline (PBS), with PR8M 2×10^3 FFU.

RNA isolation and sequencing

Mice were sacrificed three days post infection (dpi) and entire lungs were extracted. Lungs were immediately transferred to RNAlater (Qiagen), kept at 4 °C for one day, and subsequently stored at –20 °C. RNA was isolated using Qiagen Midi kit as described previously [25]. RNA quality was controlled on a 2100 Bioanalyzer (Agilent). Five-hundred nanograms of total RNA was used to prepare libraries for sequencing using the Lexogen SENSE RNA-seq library kit for Ion Torrent. Libraries were amplified for 11 cycles as the final step of library preparation. Before sequencing, 1 µl aliquots of this material was pooled and sequenced on an Ion Torrent PGM 314 chip. Barcode quantification data from the PGM were used to balance the barcodes for final pooling before sequencing. Following this final pooling, the library pools were sized to a target size of 260 bp on a Pippin Prep instrument using the 2 % Pippin agarose gel. The sized libraries were examined on an Agilent High Sensitivity DNA chip, quantified using real-time PCR, and used to prepare spheres using a One-Touch 2 device. These spheres were then sequenced on an Ion Torrent Proton sequencer with a P1 chip. On average, 67 million reads were obtained per sample. RNA sequencing has been performed by the Molecular Resource Center (MRC) at UTHSC, Memphis, USA.

Read mapping and gene expression quantification

RNA-seq reads were quality-trimmed using Trim Galore [26] and then mapped to the mouse mm10 reference genome or to the influenza A virus PR8M genome using STAR [27]. Read counts were summarized for genes as features using the R-package *Rsubread* [28], normalized and log2 transformed using the R-package *DESeq2* [29], and subsequently batch-corrected using the *ComBat* function of the R-package *sva* [30, 31]. For annotations of genes, ENTREZID from *Rsubread* were matched to RefSeq annotations using R-package *biomart* [32]. Duplicate RefSeq annotations were given the extension _1, _2, etc.

Heritability estimates for *Ace2*

The heritability of *Ace2* gene expression was estimated with GEMMA [33] which used the following formula to calculate the proportion of variance in phenotypes explained (PVE) by typed genotypes (also known informally as *chip heritability*):

$$\mathrm{PVE}(\boldsymbol{\beta}, \mathbf{u}, \tau) := \frac{\mathrm{V}(\mathbf{X}\boldsymbol{\beta} + \mathbf{u})}{\mathrm{V}(\mathbf{X}\boldsymbol{\beta} + \mathbf{u}) + \tau^{-1}}$$

Where "Xβ" is the sparse effects, "u" is the random effects, and "τ^{-1}" is the variance of the residual errors. GEMMA uses a Markov chain Monte Carlo method to estimate β, u. We used the BXD genotype file generated January 2017 (www.genenetwork.org/webqtl/main.py?FormID=sharinginfo&GN_AccessionId=600) and *Ace2* gene expression values across the 41 BXD family were used as input.

Correlation analysis

The correlation analysis in this study, including gene-gene and gene-phenotype were conducted with Pearson correlation coefficient in *R* [34]. Pearson correlation $p < 0.05$ was used to report statistical significance.

Weighted gene co-expression network analysis (WGCNA)

WGCNA is a method that defines modules of co-expression that is often used to explore the association between variation in mRNA levels and phenotypes of interest. It can be used to define hub genes in molecular or genetic network. In this study, gene co-expression networks were constructed using the *WGCNA package* [35] following standard methods (see tutorials (https://horvath.genetics.ucla.edu/html/CoexpressionNetwork/Rpackages/WGCNA/Tutorials/). Modules were generated using the subset of 5500 H1N1 infected lung mRNAs that covaried well with *Ace2* expression (Pearson r) at a nominal $p < 0.05$. We used the standard WGCNA beta weighing parameter of 8 to achieve close to a scale-free distribution.

Gene set enrichment analysis

To understand the biological function and pathways involved in WGCNA modules, we compared set membership to Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways, and to the mammalian phenotype ontology (MPO) using WebGestalt (http://www.webgestalt.org) [36]. The analyses uses a hypergeometric statistic test to generate p values and enrichment ratios. In this analysis, we used onlhy protein coding mouse genes as the reference background and a threshold minimum of five genes per category.

Expression quantitative trait loci (eQTL) mapping

eQTL mapping defines chromosomal loci that specifically modulate variability in levels of mRNAs or other molecules; in our case gene-level summarized RNA-seq data from infected lung tissues. Much of the analysis was done using the tools in GeneNetwork (www.genenetwork.org) [37, 38]. Likelihood ratio statistics (LRS) scores were computed initially using Haley-Knott equations [39], and genome-wide significance was based on 2000 permutations. We used the BXD genotype file generated January 2017 (www.genenetwork.org/webqtl/main.py? FormID=sharinginfo&GN_AccessionId=600). Transcripts associated with significant and suggestive eQTLs using the Haley and Knott method were remapped using GEMMA [33] to verify linkage statistics and locations of eQTLs in GeneNetwork. GEMMA uses a linear mixed model mapping algorithm that can corrects for kinship among BXD strains.

Genetic variations between C57BL/6J and DBA/2J

Sequence differences segregating in the BXDs are from our previous work [21]. Here, we focused exclusively on protein-coding variants—nonsense, missense, and frameshift mutations.

Bayesian network construction

The Bayesian Network Webserver (compbio.uthsc.edu/BNW) [40] was used to create and evaluate plausible causal models among genes, transcripts, and outcome measures of interest. Model was learned from the data using the default settings. Model averaging of the 1000 highest scoring networks was performed, and directed edges with weights greater than 0.5 were included in models. Finally, each variable had a maximum of four direct parents in any model.

RNA-seq time-course data for H1N1 infection of the parental strains

The lung RNA-seq data was generated previously in our laboratory (see Ref [13]). In brief, two strains (B6 and D2) were infected with the H1N1 virus at 10–12 weeks of age as described above. RNA was isolated from lung for B6 at 1, 3, 5, 8, and 14 dpi and for D2 at 1, 3, and 5 dpi. Three RNA samples per strain and per time point were used for library preparation and sequencing. The reads were mapped to the mm10 reference genome. Raw read counts were extracted, normalized, and log transformed with *DEseq2* [29] after adding 1 to all raw counts. In this study, we extracted the *Ace2* expression levels from this dataset. Also, we performed differential expression analysis between mice at 3 dpi and mock controls using *DESeq2* [29] by treating strain as a covariates. The resulting p values were adjusted using the Benjamini and Hochberg's approach [41]. We reported genes with adj.$p < 0.05$ (also known as a q value), and fold change (FC) > 1.3 as differentially expressed genes (DEGs).

Microarray data for SARS-CoV infected primary human airway epithelial (HAE) cells

The experimental details for this study are described in Ref. [42] In brief, human tracheobronchial epithelial cells were obtained from airway specimens resected from patients undergoing surgery. Primary cells were expanded to generate passage 1 cells, and passage 2 cells were plated at a density of 2.5×10^5 cells per well. Human airway epithelium (HAE) cultures were generated by provision of an air-liquid interface for 6 to 8 weeks to form well-differentiated, polarized cultures. Wild-type infectious clone-derived SARS-CoV (icSARS-CoV) and ic-SARS-CoV ORF6 were derived from infectious clone constructs as described [42] Bat-SARS-CoV is comprised of the HKU3 backbone and SARS-CoV receptor binding domain and was derived from an infectious clone as described in [43]. Triplicate cultures were infected with multiplicity of infection 3 (MOI 3) icSARS-CoV, and icSARS-CoV ORF6 on the apical surface. Total RNA was isolated from infected cells, and equivalent amounts of RNA from three biological replicates from each condition were pooled. Transcriptome profiling was performed using Agilent 4x44K whole human gene expression arrays. Raw data were downloaded from GEO (GSE47963_series_matrix.txt.gz). Expression values were normalized and sample descriptions quality controlled. A strong batch effect with respect to the donor from which HAE were derived was corrected using the R package *sva* [30, 31]. Rows with no gene description were deleted. Differentially expressed genes were determined using the R package *Limma* [44].

Data availability

The RNA-seq data from infected BXD strains has been deposited at GeneNetwork.org under the accession ID GN807. The summarized RNA-seq data as entered in GN can be accessed and downloaded at http://gn1.genenetwork.org/webqtl/main.py?FormID=sharinginfo&GN_AccessionId=807&InfoPageName=HZI_LungBXD_RNA-Seq_1116. RNA-seq data for infected B6 and D2 parents are available at NCBI GEO under the accession number GSE66040. Array data from infected primary HAE cells are available at GEO under accession number GSE47963.

3 Results

3.1 *Ace2* expression level decreased in both C57BL/6J and DBA/2J after H1N1 infection

We have previously generated H1N1 inflected lung RNA-seq for B6 and D2 parental strains at 1, 3, 5, 8, and 14 dpi. The B6 maternal parent of the BXDs is resistant to H1N1 infection, whereas the D2 paternal

parent is highly susceptible. Here we have exploited these parental time-series data to define the *Ace2* mRNA post-infection dynamic response. Overall, expression of *Ace2* in lungs drops progressively in both strains after infection (**Figure 1**). In B6, *Ace2* transcript reaches its nadir on 8 dpi (7.20 ± 0.15 SD log2 units) and remains at a low level through to 14 dpi (7.23 ± 0.12). Expression relative to 1 dpi is significantly lower at following timepoints: 3 dpi (p = 0.026, log2FC = –0.25), 5 dpi (p = 0.008, log2FC = –0.49), 8 dpi (p = 0.003, log2FC = –0.73), and 14 dpi (p = 0.002, log2FC = –0.70). In comparison, the same infection titer is lethal for D2 at 5 dpi, and this is matched with lower expression of *Ace2* (7.10 ± 0.25) at 1 dpi. Statistically, *Ace2* expression 3 dpi (p = 0.003, log2FC = –0.37) and 5 dpi (p = 0.006, log2FC = –0.80) are significant reduced compared to 1 dpi. Although the differences in *Ace2* expression between B6 and D2 at 3 dpi and 5 dpi is not appreciable, D2 cases have a consistently more rapid decline in expression compared to B6.

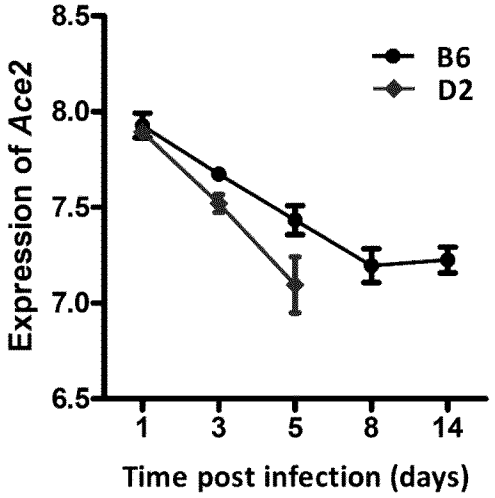


Figure 1. Line charts of the *Ace2* expression level in strains C57BL/6J (B6) and DBA/2J (D2) after H1N1 infection. Lung transcriptomes were quantified with RNA-seq for B6 and D2 strains at 1, 3, 5, 8, and 14 dpi of H1N1 infection. Three female mice (8–12 weeks of age) per strain were used for per time point. The x-axis shows the time point (days) post the infection. The y-axis shows the expression level of *Ace2* on a log2 transformed y-axis expression scale.

3.2 *Ace2* expression exhibited variability across BXD strains at 3 dpi and controlled by genetic loci

All D2 mice died at 5 dpi. We therefore generated RNA-seq data for BXDs at 3 dpi. Changes in *Ace2* transcript level after infection varied considerably across the family with a 1.5-fold range (**Figure 2A**, and see genenetwork.org/show_trait?trait_id=ENSMUSG00000015405&dataset=HZI_LungBXD_RNA-Seq_1116). Mean expression of *Ace2* across all BXDs was 8.02 ± 0.15 SD. BXD22 had the lowest expression of 7.78. and BXD77 had the highest expression of 8.36. The distribution of values is weakly bimodal. The estimated heritability of *Ace2* expression variation is ~0.18. We identified four potential genomic loci (–log(p) > 3, **Figure 2B**) that modulate *Ace2* expression by performing eQTL mapping with GEMMA. Suggestive and significant eQTLs map to Chr 1: 91–94.4 Mb, Chr 4: 105.1–114.7 Mb, Chr 9: 49.2–54.8 Mb, and Chr 11: 42.7–46.4 Mb, respectively. All loci are far from the *Ace2* gene itself (Chr X:

164.14 Mb) —in other words all loci are putative trans-acting eQTLs [45, 46]. These four loci overlaps at total of ~406 genes, and a subset of 36 covary well with *Ace2* expression, have altered expression after infection, and have known roles in immune system, including *Twist2* and *Pdcd1* in the Chr 1 locus; *Gpx7*, *Lexm*, *Acot11*, and *Cdkn2c* in the Chr 4 locus; *Elmod1*, *Bco2*, *Acat1*, *Sik2*, *Cul5*, *and Dmxl2* in the Chr 9 locus; and *Havcr2*, *Il12b*, *Ebf1*, *Nipal4*, and *Itk* in the Chr 11 locus.

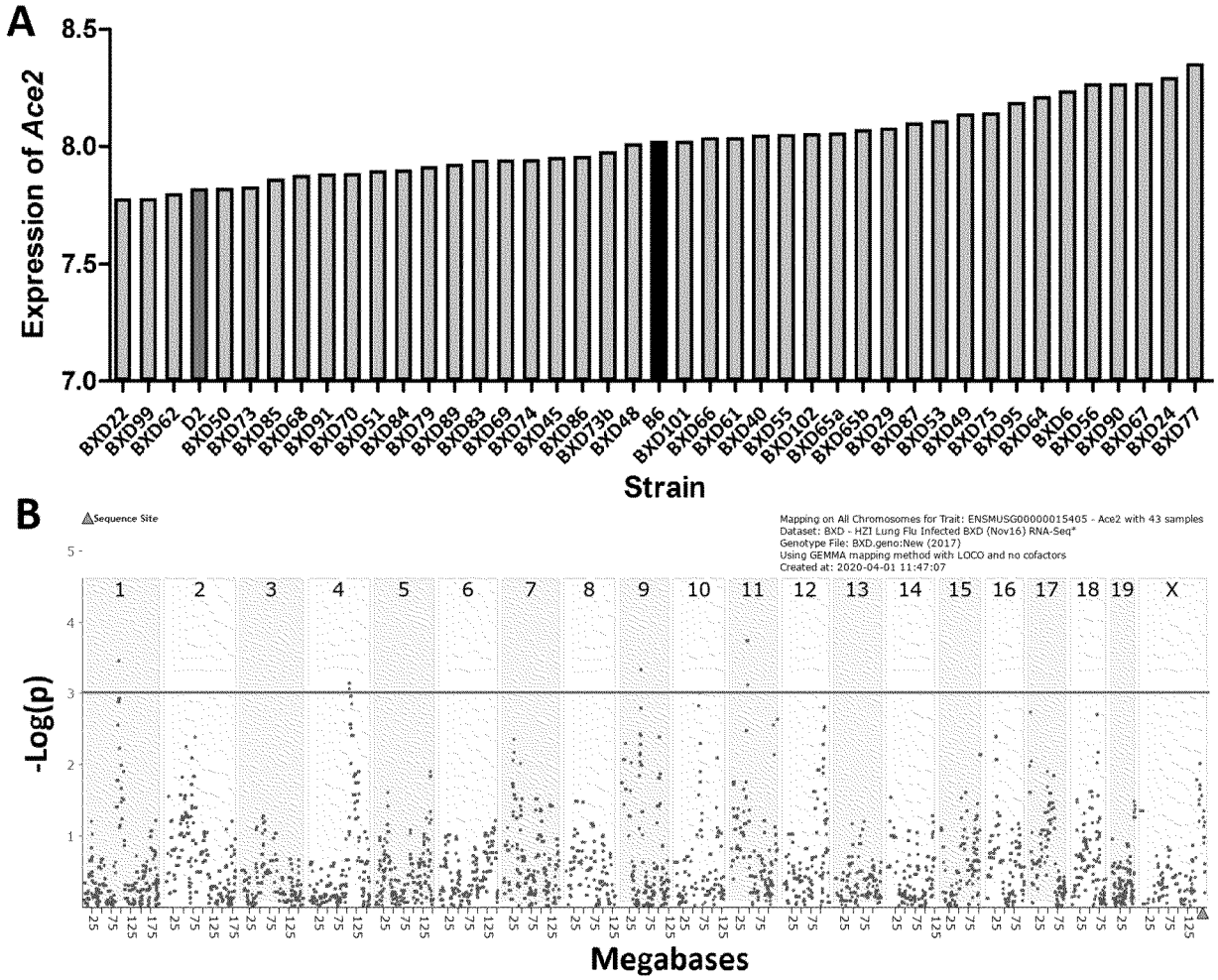


Figure 2. Expression and eQTL mapping of *Ace2* in BXD family. (A) Bar plots of the pulmonary *Ace2* expression levels across the BXD family at 3 dpi. The x-axis shows BXDs strains and the two parental strains. The y-axis shows the normalized log2 expression levels of *Ace2*. **(B)** Manhattan plot of linkage to *Ace2* expresssion after H1N1 infection. The x-axis is chromosome position on a megabases (Mb) scale, while the y-axis gives the –log(p) of linkage. The purple triangle on the x-axis on ChrX indicates the genomic position of *Ace2*. The red line indicates approximate suggestive threshold for a genome-wide scan at a–log(p) of 3. Map was computed with GEMMA using the LOCO option.

3.3 *Ace2* expression was associated with disease severity and viral load after infection

In order to investigate whether there is an association of *Ace2* expression with severity of disease and/or viral replication as infection progresses, we computed correlations of *Ace2* levels against body weight loss,

mean time to death, and viral gene expression levels as a surrogate for viral load. *Ace2* expression was significantly correlated with both body weight loss (r = 0.575, p = 0.008, **Figure 3A**) and median body weight loss (r = 0.468, p = 0.028, **Figure 3B**) at 3 dpi. Further, *Ace2* was positively correlated with the mean time to death (r = 0.458, p = 0.032, **Figure 3C**), In contrast, *Ace2* had a negative correlation with virus NA gene expression levels (r = 0.381, p = 0.029, **Figure 3D**).

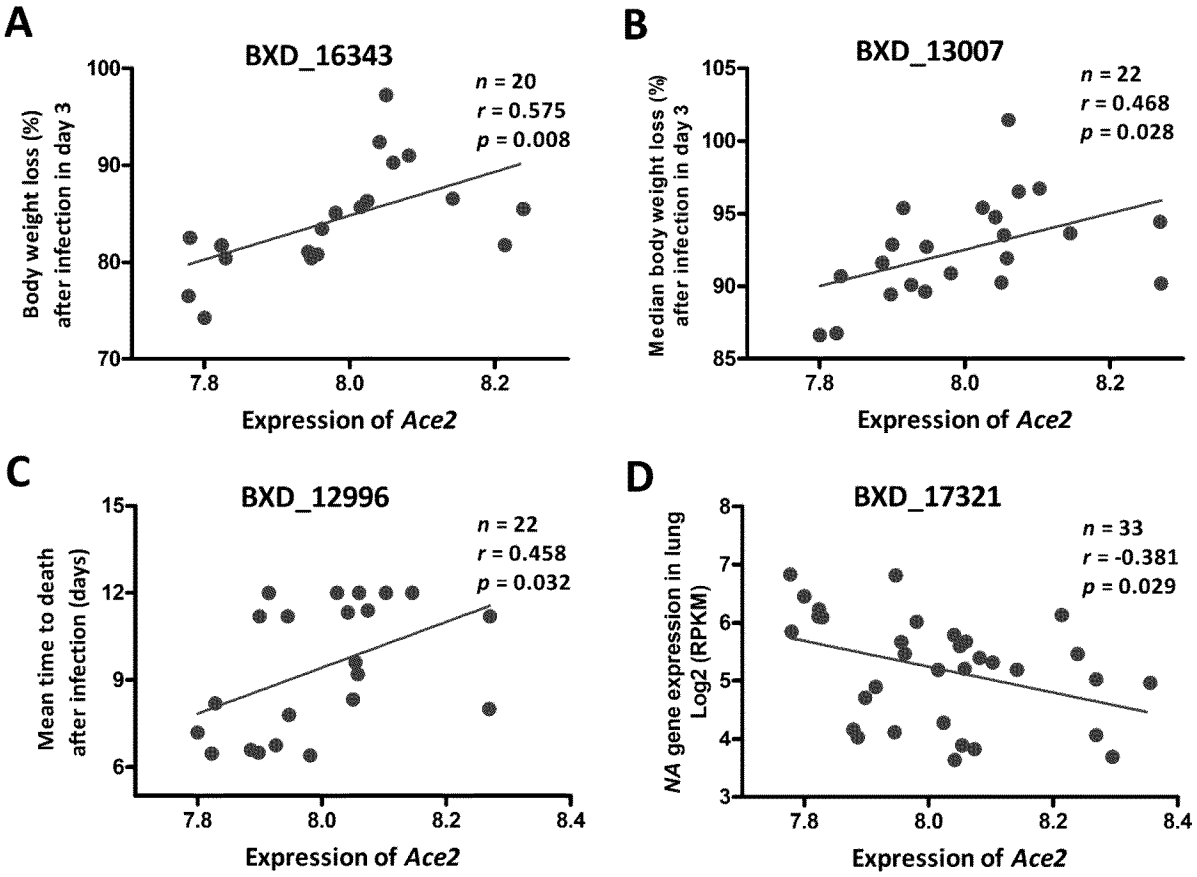


Figure 3. Scatter plots of the correlations of *Ace2* expression with body weight loss (A), median body weight loss (B), mean time to death (C), and viral *NA* gene expression level (D). Pearson correlation coefficient was used to determine the relationship. Gene expression levels represent normalized log2 values. GeneNetwork BXD phenotype identifiers (e.g. ID "BXD_16343") at the top of each plot.

3.4 *Ace2* correlated transcripts significantly involved in immune system-related pathways

To gain insight into the pulmonary pathways and biological functions that *Ace2* is involved in after viral infection, we conducted Pearson correlation coefficient analysis against to the whole lung transcriptome. This analysis returned 5,500 transcripts that are significantly correlated with *Ace2* expression ($p < 0.05$). Next, we submitted the corresponding genes to WebGestalt (http://www.webgestalt.org/) for gene ontology analysis. 5,118 transcripts were unambiguously mapped to unique Entrez gene IDs. Those genes were subsequently used for an enrichment analysis. The selected genes represented components of the TNF

signaling pathway, MAPK signaling pathway, Notch signaling pathway, HTLV-I infection, and VEGF signaling pathway (**Figure 4A**). Furthermore, we also found that these genes were significantly enriched in terms related to chemokine secretion, lung morphology, and respiratory system morphology (**Figure 4B**).

For the top three enriched pathways, 48 genes were found in the TNF signaling pathway (**Supplementary Data 1**), with 35 negatively correlated with *Ace2* ($p < 0.05$), Among these included the tumor necrosis factor (*Tnf,* $r = –0.414$, $p = 0.005$). There were 92 genes in the MAPK signaling pathway (**Supplementary Data 1**), with *Tnf*, transforming growth factor beta 1(*Tgfb1*, $r = –0.459$, $p = 0.002$), and other 59 genes whose expression was negatively correlated with *Ace2*. In addition, 25 genes were involved in the Notch signaling pathway (**Supplementary Data 1**), with 21 negatively correlated with *Ace2*.

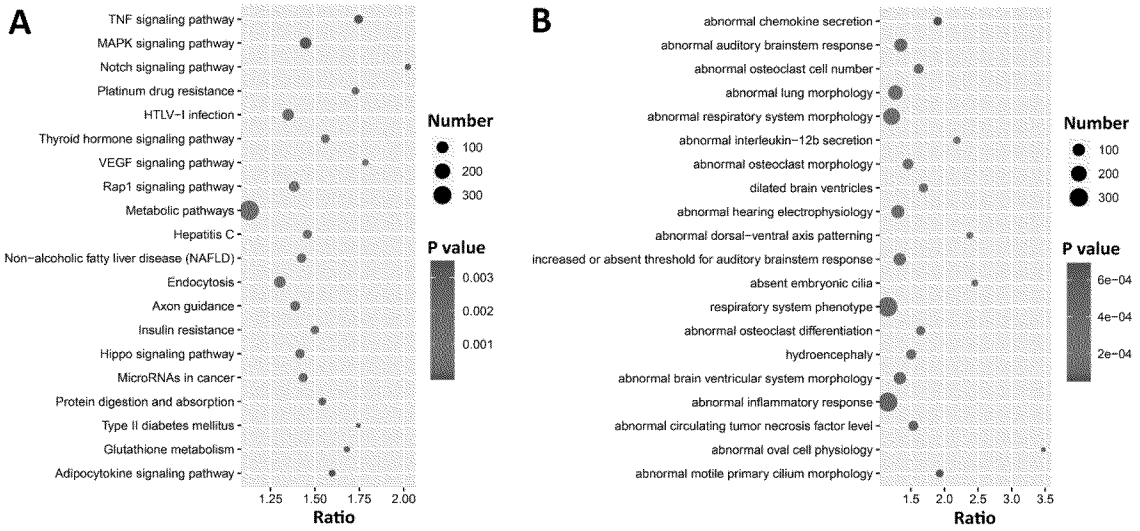


Figure 4. Bubble charts of the top 20 KEGG pathways (A) and 20 Mammalian Phenotype Ontologies (B) enriched for *Ace2* significantly correlated genes. Gene over-representation analysis for KEGG pathway and Mammalian Phenotype Ontology of the *Ace2* correlated transcripts ($p < 0.05$) were performed with WebGestalt (www.webgestalt.org). The x-axis represents the enrich ratio and y-axis represents enriched pathways/terms. The size of the dots denotes the number of genes and the color the *p* value.

3.5 Co-expression network analysis narrowed down the *Ace2* correlated transcripts

To identify genes that interact more directly with *Ace2*, we constructed co-expression modules using WGCNA with *Ace2* correlated transcripts. The 5,500 transcripts were parsed into 10 co-expression modules using a soft-thresholding power of 8 (**Figure 5A-C**). Modules have a wide range of sizes, with the *brown* module containing 1,673 transcripts and *lightcyan* containing only 68 transcripts. *Ace2* was located in the *purple* module with 461 other genes/transcripts (**Figure 5C**). Genes in this module are significantly involved in cilium function (**Figure 5D**)

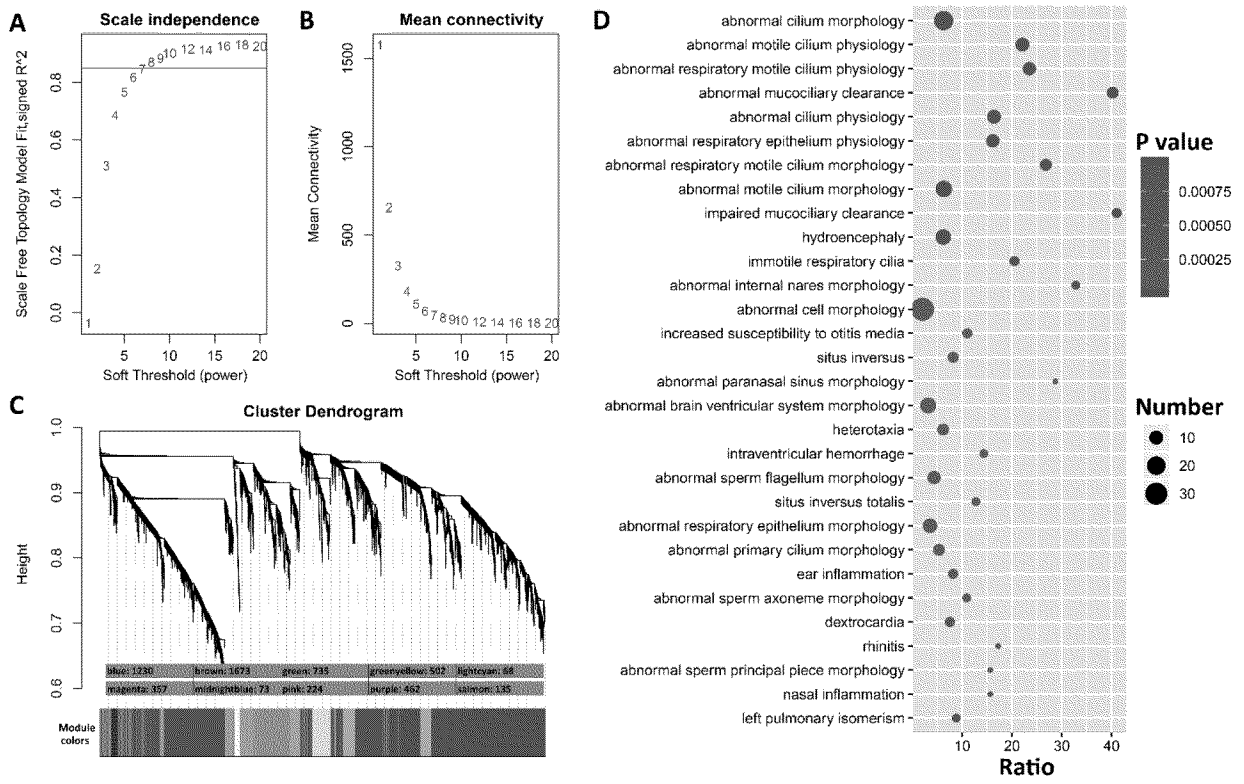


Figure 5. WGCNA modules associated with *Ace2* expression in H1N1 infected lung. (A) The soft thresholding index R^2 (y-axis) as a function of different powers β (x-axis) indicating the threshold of 8 chosen. **(B)** Mean connectivity (degree, y -axis) as a function of the soft-thresholding power (x -axis). **(C)** 10 co-expression modules identified from *Ace2* correlated genes. Modules are color coded as shown. The number of genes in each module is also indicated. **(D)** Bubble charts of top 30 enriched Mammalian Phenotype Ontologies of the purple module. This analysis was performed with WebGestalt (http://www.webgestalt.org/).

Next, we focused on exploring the genes in the purple module (**Figure 6** and **Supplemental File 1**). First, we performed differential expression analysis between mice at 3 dpi and mock-infected controls by treating strain as a covariate. This led to identification of 281 genes (61% of the module genes) that showed differential expression (adj.p < 0.05 and FC > 1.3). Next, we conducted eQTL mapping using WebQTL on the GeneNetwork for the whole lung transcriptome which identified 23 genes that were *cis*-regulated and 106 others that were *trans*-regulated in H1N1 infected lungs. Those QTLs were further confirmed with GEMMA in GeneNetwork. Moreover, we searched for the DNA sequence differences between B6 and D2, which resulted in a total of 69 genes harbor coding sequence variants (missense, nonsense, and frameshift variants). Furthermore, we found that there were 42 genes implicated in cilium function and regulation identified by enrichment analysis. By comparing the above four categories, 32 cilium function related genes were differentially expressed (**Table 1**). We refer to this set as 'prioritized genes'. Of these, *Sox11*, *Tff2*, *Rnf128*, and *Muc5b* showed *cis*- or *trans*-regulation, and *Tmprss2*, *Ulk4*, *Cckar*, *Wdpcp*, *Ift140*, and *Ccdc40* contained functional variants (**Table 1**). In addition, we also found seven candidate genes at *Ace2*

regulating QTL regions are located in the purple module, of which, *Elmod1* (ELMO Domain Containing 1 shown strong positive correlation (r = 0.595, p = 1.5E-05) with *Ace2*, has significant differential expression (log2FC = –0.702, adj.p = *0.00017*) between mice at 3 dpi and mock-infected controls and was also in involved in immune regulation. Taken together, we considered the genetic perturbations of the top 10 'prioritized genes' (**Table 1**) and *Elmod1* may act as key regulators in this module.

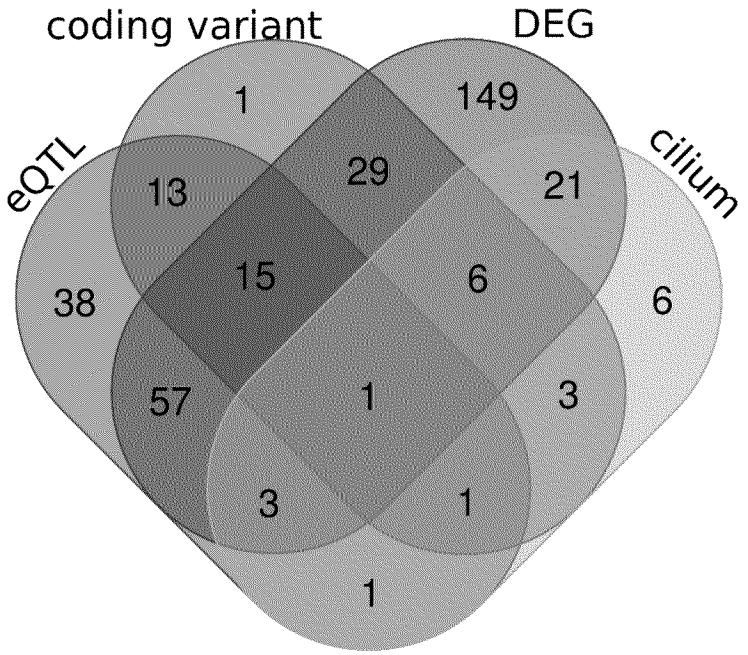


Figure 6. Venn diagrams of the purple module genes. A total of 462 genes were included in the purple module. Four criteria were applied to explore the module genes, including coding sequence variants between parental strain B6 and D2 (**coding variant**), eQTL (LRS > 15) regulation in H1N1 affected lung tissue (**eQTL**), cilium functional relevance (**cilium**), and differential expression (adj.p < 0.05 and FC > 1.3) between infected and normal controls in parental strains (**DEG**). The Venn diagram shows the number of overlapping genes among the four categories.

Table 1 Lists of prioritized genes in purple module that were differentially expressed (adj.p < 0.05 and FC > 1.3) and implicated in cilium biological functions.

Gene ID	Symbol	Description	Gene ID	log2FC	padj	eQTL	functional Variant
20666	**Sox11**	SRY-box 11	20666	-1.19	1.95E-12	cis	Nonsynonymous
21785	**Tff2**	trefoil factor 2 (spasmolytic protein 1)	21785	-1.01	5.38E-04	trans	NA
66889	**Rnf128**	ring finger protein 128	66889	-0.77	2.81E-05	trans	NA
74180	**Muc5b**	mucin 5, subtype B, tracheobronchial	74180	0.54	1.13E-02	trans	NA
50528	**Tmprss2**	transmembrane protease, serine 2	50528	-1.06	8.58E-10	NA	Nonsynonymous
209012	**Ulk4**	unc-51-like kinase 4 (C. elegans)	209012	-0.87	3.43E-07	NA	Nonsynonymous
12425	**Cckar**	cholecystokinin A receptor	12425	-0.73	1.91E-05	NA	Nonsynonymous
216560	**Wdpcp**	WD repeat containing planar cell polarity effector	216560	-0.69	2.36E-06	NA	Nonsynonymous
106633	**Ift140**	intraflagellar transport 140 homolog (Chlamydomonas)	106633	-0.68	3.50E-07	NA	Nonsynonymous

207607	**Ccdc40**	coiled-coil domain containing 40	207607	-0.66	1.06E-03	NA	Nonsynonymous
320752	Dpy19l2	dpy-19-like 2 (C. elegans)	320752	-1.81	2.13E-03	NA	NA
51938	Ccdc39	coiled-coil domain containing 39	51938	-1.30	1.15E-13	NA	NA
244653	Hydin	hydrocephalus inducing	244653	-1.30	8.51E-16	NA	NA
74362	Spag17	sperm associated antigen 17	74362	-1.29	9.27E-13	NA	NA
73873	Fam161a	family with sequence similarity 161, member A	73873	-1.28	1.70E-09	NA	NA
320662	Casc1	cancer susceptibility candidate 1	320662	-1.27	2.44E-08	NA	NA
66722	Spag16	sperm associated antigen 16	66722	-1.27	1.61E-10	NA	NA
74934	Armc4	armadillo repeat containing 4	74934	-1.21	8.50E-09	NA	NA
18703	Pigr	polymeric immunoglobulin receptor	18703	1.18	1.10E-09	NA	NA
69707	Iqcg	IQ motif containing G	69707	-1.18	2.97E-20	NA	NA
78801	Ak7	adenylate kinase 7	78801	-1.17	4.75E-09	NA	NA
27078	B9d1	B9 protein domain 1	27078	1.09	1.00E-02	NA	NA
68922	Dnaic1	dynein, axonemal, intermediate chain 1	68922	-1.07	3.67E-08	NA	NA
226265	Eno4	enolase 4	226265	-0.98	1.49E-09	NA	NA
71877	Efhc1	EF-hand domain (C-terminal) containing 1	71877	-0.92	3.77E-11	NA	NA
75533	Nme5	expressed in non-metastatic cells 5	75533	-0.82	5.92E-05	NA	NA
75050	Kif27	kinesin family member 27	75050	-0.71	3.05E-03	NA	NA
70008	Ace2	angiotensin I converting enzyme (peptidyl-dipeptidase A) 2	70008	-0.61	9.88E-05	NA	NA
20878	Aurka	serine/threonine kinase 6	20878	0.60	2.36E-02	NA	NA
22062	Trp73	transformation related protein 73	22062	-0.60	1.81E-03	NA	NA
77609	Ccdc151	coiled-coil domain containing 151	77609	-0.47	9.85E-02	NA	NA
252973	Grhl2	grainyhead-like 2 (Drosophila)	252973	-0.44	5.36E-04	NA	NA

Note: Genes highlighted in bold are those ten genes that may act as key regulators in the purple module; NA, not available.

3.6 Bayesian network analysis inferred upstream modulators of *Ace2*

In order to infer the causative relations of *Ace2* with those key regulators, we performed Bayesian network analysis. Ring finger protein 128 (*Rnf128*) stood out as the upstream regulator of the other 11 genes (**Figure 7**). This gene was predicted to directly target the genes *Elmod1*, *Ift140* (intraflagellar transport 140 homolog), *Ccdc40* (coiled-coil domain containing 40), and *Sox11* (SRY-box 11). *Ace2* was found to be targeted by *Rnf128* through two paths, which both begins with the expression of *Rnf128,* then the expression of *Elmod1* or *Ift140*, and finally, to *Ace2* (**Figure 7**).

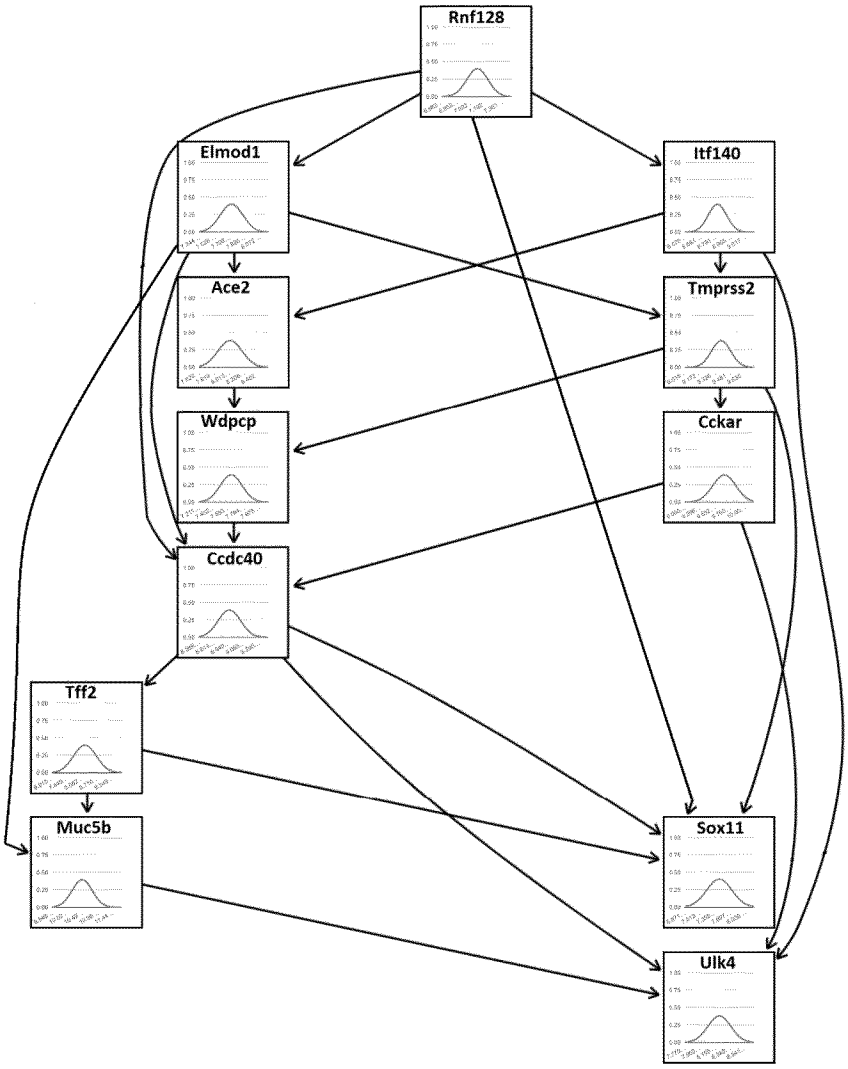


Figure 7. **Bayesian network analysis inferred upstream regulators of *Ace2***. The Bayesian network structure was constructed on Bayesian Network Webserver (compbio.uthsc.edu/BNW) with gene expression values as the input.

3.7 The differentially expressed cilium function related genes in the purple module were replicated in SARS-CoV-infected HAE cells

In order to further evaluate whether those 'prioritized genes' (**Table 1**) were also regulated in humans, especially in the context of SARS-CoV infection, we downloaded a microarray data set for HAE cells infected with SARS-CoV (icSARS-CoV), ic-SARS-CoV ORF6 and BAT-SARS_CoV [42]. We found that very few of those 'prioritized genes' were significantly regulated after infection with BAT-SARS (**Figure 8A**). However, most of the 'prioritized genes' were differentially expressed in icSARS-CoV and ic-SARS-CoV ORF6 infected HAE cells when compared to mock controls, with 21 genes found at 96_h after infection (**Figure 8A**). More importantly, those overlapping genes, except *Ace2*, exhibited the same up- or down-regulating patterns in both HAE cells and our mouse model (**Table 1** and **Figure 8B**). *ACE2* was up-regulated in HAE cultures whereas in whole lungs it was found to be down-regulated. This is an important

observation since in the HAE cultures only airway epithelial cells are present, but no interstitial cells, endothelial cells, resident and infiltrating immune cells are present. These observations indicate that in lungs a more complex regulatory network is involved in the regulation of *ACE2* than in isolated epithelial cells and that it may indeed be downregulated in all cells after infection in the whole lung. Alternatively, *ACE2* may become up-regulated only in infected cells [42, 47]. In the lungs, infected cells represent only a minority of all cells present. Since our lung RNA-seq study investigated total gene expression changes of all cell types, it is conceivable that we do not detect a possible up-regulation in this lower number of infected cells. In future studies, it will be important to analyze *Ace2* expression in infected mouse lungs by immunohistochemistry or *in situ* hybridization to test these hypotheses.

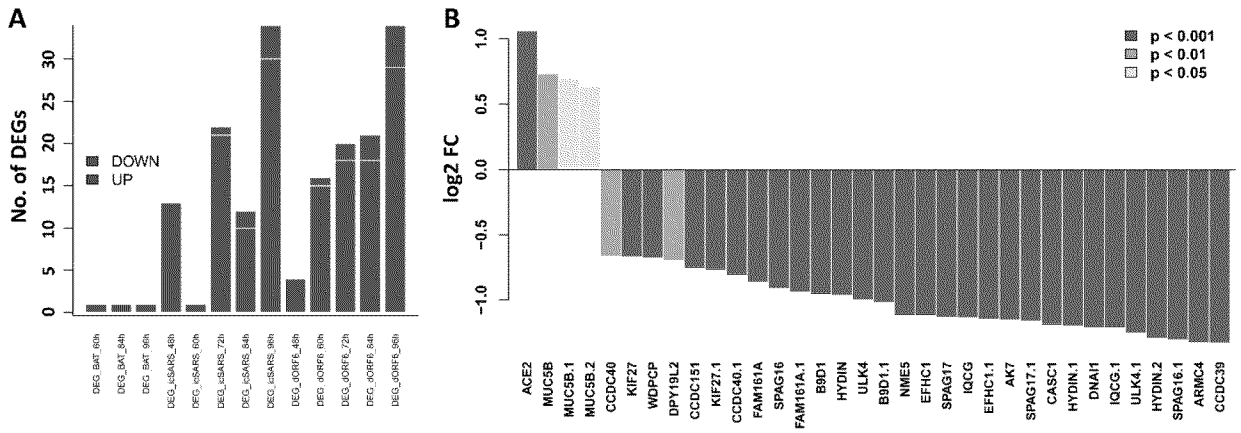


Figure 8. Bar plots of the cilium function related genes in the SARS-CoV infected HAE cells. Primary human HAE cells were infected with MOI 3 SARS-CoV (icSARS-CoV), ic-SARS-CoV ORF6 and BAT-SARS_CoV and gene expression was analyzed by arrays. (**A**) Number of up-and downregulated differentially expressed genes for cilium function related genes that had orthologous genes in humans and were represented on the array (adj.$p < 0.05$ and log2 FC > 0.58); y-axis: number of differentially expressed genes. (**B**) Log2 FC differences in expression values between HAE cells infected with icSARS-CoV at 96_h pi and mock-treated controls for cilium function related genes that had orthologous genes in humans and were represented on the array; y-axis: differences in log2 FC expression levels between groups. Adjusted p-values were taken from the Limma analysis of the entire transcriptome data.

Discussion

Many studies have shown that host genetic background significantly influences the susceptibility to IAV infections in humans [48]. This phenomenon was also observed in mouse models [12], especially for inbred strains B6 and D2 [13]. In this study, we systemically analyzed the potential regulatory role of *Ace2* for the host response to H1N1 infection in a subset of BXD RI mice. Our results showed that the expression of *Ace2* in lung significantly decreased in a disease severity- and time-dependent manner. In addition, we also found the expression variation of *Ace2* negatively correlated with the body weight loss and survival time after the infection. These results strongly suggest that *Ace2* is involved in H1N1 virus disease progression

and that the lower levels of *Ace2* may play a role in virus-induced ALI. This notion was supported in studies of *Ace2*$^{-/-}$ mice infected with influenza H7N9 [49] and with respiratory syncytial virus [50] where lung pathology was more severe in mutant mice.

These observations argue strongly that *Ace2* can mediate virus-induced ALI [49, 50]. However, the underlying mechanisms remain elusive. By using 43 inbred strains, we were able to perform genetic correlation analysis and gain insight into the *Ace2*-associated biological pathways. We identified a total of ~5000 *Ace2*-correlated genes. These genes are involved in regulating respiratory and inflammatory networks through several pathways, including TNF signaling pathway, MAPK signaling pathway, and Notch signaling pathway.

The factors associated with H1N1-virus-induced lethality mainly consist of virus-induced inflammatory cytokine storm and tissue destruction. Tumor necrosis factor (*TNF*) is a cytokine with multiple effects, including promoting cell growth, differentiation, apoptosis, and inducing inflammation [51, 52]. *TNF-α*, a member of the *TNF* family, can activate caspase protease, JNK, and transcription factor NF-κB, thereby executing its biological functions such as cytotoxicity, antiviral, and immuno-regulation. A previous study reported that inhibition of *Tnf-α* can protect mice from lethal H1N1 infection [53]. In the present study, we found *Ace2* was negatively correlated ($r = –0.414$, $p = 0.005$) with the expression of *Tnf* and other 34 TNF signaling pathway related genes, suggesting that *Ace2* could dampen excessive inflammatory immune responses through downregulating of this signaling pathway and mitigating immune-mediated tissue destruction. We also found 92 genes in the MAPK signaling pathway significantly correlated with *Ace2*, including *Tgfb1* ($r = –0.459$, $p = 0.002$). Of note, it was shown that IAV can activate this pathway to support its replication [54], The authors proposed a model in which, *Tgfb1* acts as a pro-viral host factor by suppressing early immune responses, creating an environment conducive for IAV infection [55]. Moreover, another set of 25 *Ace2*-correlated genes were involved in the Notch signaling pathway, with most of them (21 genes) showing negative correlations. This pathway is an important bridge between antigen-presenting cells (APCs) and T cell communication circuits and plays a central role in driving the immune system to overcome infectious disease [56].

By constructing gene co-expression network, we narrowed the *Ace2* co-expressed genes to 462 (purple module). Enrichment analysis revealed that those genes were significantly related to cilium function, including *Ace2* itself. This finding suggests that *Ace2* participates in ALI not only by interacting with the TNF, MAPK, and Notch signaling pathways, but also through regulating functions of respiratory epithelium, which is a barrier to microbial infection and tissue injury by the secretion of mucus and the action of mucociliary clearance. In order to identify the key regulators in the purple module. We employed multi-layer filtration, including differential expression analysis, eQTL mapping, functional variant identification, and gene annotation. This resulted in ten high priority candidates, i.e., *Sox11*, *Tff2*, *Rnf128*, *Muc5b*, *Tmprss2*, *Ulk4*, *Cckar*, *Wdpcp*, *Ift140*, and *Ccdc40*. To further investigate the relationship of these genes with *Ace2*, we performed Bayesian network analysis and identified *Rnf128* as the master regulator. This gene encodes a ring-finger-containing protein which has been shown to possess E3 ubiquitin ligase activity [57]. *RNF128* has been implicated involved in the induction of T cell anergic phenotype through

inhibiting activation-induced IL2 and IL4 cytokine production [58] and mediating T cell receptor-CD3 degradation [59]. In a recent study, Song *et al.* [60] reported *RNF128* promotes lysine-63-linked ubiquitination and activation of a critical kinase TBK1, thereby augmenting the induction of type I interferon antiviral cytokines that curb viral replication. The authors' *in vivo* experiments further showed that *Rnf128* deficient mice were more susceptible to vesicular stomatitis virus (an RNA virus) and herpes simplex virus (a DNA virus).

Our results suggest that there are two potential gene regulatory paths for *Ace2*, both of which begin with *Rnf128*, then *Elmod1* or *Ift140*, and finally, to *Ace2*. It is worth noting that *Elmod1* was also identified as a candidate up-regulator by our eQTL mapping. This gene was significantly correlated (r = 0.595, p = 1.5E-05) with *Ace2* and downregulated (log2FC = –0.702, adj.p = *0.00017*) on 3 dpi. Malfunction of *Elmod1* in mice shown decreased B cell, lymphocyte cell, monocyte cell, and leukocyte cell numbers [61]. Leukocytes have chemotaxis and act as wandering sentinels of the innate immune system. Mammalian ELMO-family proteins are important mediators of small G protein activity, regulating actin-mediated processes such as chemotaxis and engulfment [62]. The association between Elmo proteins and chemotaxis of leukocytes haven't been fully elucidate. Moreover, stereocilia of inner hair cells become greatly elongated and fused in *Elmod1* deficient mice, which establishes a link between the Ras superfamily of small regulatory GTPases and the actin cytoskeleton dynamics of hair cell stereocilia [63]. Whether ELMOD1 involved in regulating of respiratory epithelium system remains unclear. Moreover, direct connection of those genes will need to be investigated experimentally in future studies.

Besides the master regulator *Rnf128*, other genes were also found to regulate the immune and respiratory epithelium system. For instance, *MUC5B* (mucin 5, subtype B, tracheobronchial), encodes a member of the mucin family of proteins, is associated with idiopathic pulmonary fibrosis in humans [64]. Overexpression of this gene in mice causes mucociliary dysfunction, and enhances lung fibrosis [65]. It was reported to be required for airway defense in another study [66]. *CCDC40* (coiled-coil domain containing 40) encodes a protein that is essential for motile cilia function. According to the OMIM (https://omim.org/) database, this gene is associated with recurrent respiratory infections (HP:0002205) and abnormal axonemal organization of respiratory motile cilia (HP:0012258). Malfunction of *Ccdc40* in mice resulted in left pulmonary isomerism and lung situs inversus [67]. *TMPRSS2* (transmembrane protease, serine 2) encodes a protein that belongs to the serine protease family. *Tmprss2* knockout mice show reduced lung inflammation with IAVs (H1N1, H3N2, or H7N9) infection and exhibit decreased susceptibility to virus-induced morbidity/mortality [68]. Studies also demonstrated that SARS coronavirus engages *ACE2* as the entry receptor on cell surface [9, 69] and employs the *TMPRSS2* for Spike protein priming, which facilitates viral entry and infection [70, 71]. Besides, *Ulk4*, *Sox11*, and *Tff2* are associated with abnormal respiratory motile cilium [72], pulmonary hypoplasia [73], and abnormal adaptive immunity [74], respectively.

The ongoing outbreak of the COVID-19 caused by SARS-CoV-2 poses a global public health threat [75-77]. The entering of SARS-CoV-2 into host cells is dependent on *ACE2* [9, 69], the receptor for SARS-CoV and can be blocked by a clinically approved TMPRSS2 protease inhibitor [71]. However, how *ACE2* interacts with other genes involved in the progression of SARS-CoV-2 induced respiratory diseases

remains to be elucidated. The first report based on autopsy samples demonstrated that the pathological characteristics of COVID-19 are very similar to those of SARS-CoV and MERS-CoV infected patients [78]. Therefore, we looked up those 30 prioritized genes (**Table 1**), co-expressed with *Ace2* after H1N1 infection, in SARS-CoV-infected primary human HAE cells. This resulted in 20 out of them showing consistent up/down-regulation with our H1N1 model, suggesting the two viruses share common *Ace2* regulatory networks that participate in the occurrence and development of lung pathology.

In summary, we have shown the expression of *Ace2* was significantly downregulated in the lungs of IAV H1N1 infected mice. By using BXD RI strains, we were able to map *Ace2* genetic regulate loci and identify *Ace2* co-expressed genes with genetic correlation analysis and weighted gene co-expression network analysis. Thus, our results indicate that *Ace2* participates in virus-induced respiratory diseases not only through immune system related pathways, but also by protective functions in the respiratory epithelium. In addition, *Rnf128* was identified as the master regulator through Bayesian network analysis. Finally, 21 of our prioritized genes were found to be also differentially expressed in SARS-CoV-infected HAE cells. These findings provide an *Ace2* regulatory network and raise several potential therapeutic targets for virus-induced (H1N1, SARS-CoV, MERS-CoV, and SARS-CoV-2) respiratory diseases. They additionally illustrate that the BXD RI strains provide an excellent resource for studying the host genetic regulatory mechanism of the viral infections, such as that caused by SARS-CoV-2.

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Author Contributions

LL, KS and RSB conceived the study; SB and ACS conducted the experiments; FX, KS and RWW performed data analysis; LL, FX, KS, RWW, and JG wrote the manuscript. FX, KS, and JG prepared the figures and tables; RWW, KL, CBJ edited the manuscript; all authors read and approved the final version of the manuscript for publication.

Conflicts of Interest

The authors declare no conflicts of interests.

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From: Gralinski, Lisa E[lgralins@email.unc.edu]
Sent: Sun 4/5/2020 8:01:00 PM (UTC-07:00)
To: Sims, Amy C[amy.sims@pnnl.gov]
Subject: mers paper edits
Attachment: Apoptosis Sci Trans 3_25_2020 final draft - LG.docx
Attachment: sm126 histo figure - updated.pdf

Hey Amy,
I made just a few tiny text edits to your MERS paper, I think it reads really well. I also updated histology figure 8 to use the same color scheme as figure 7. Hopefully everyone else has already responded, let me know if I should give anyone on this end a nudge. The histo figure is too big for email, hence the PDF. I assume I'll need to get you the powerpoint w/ embedded images for official manuscript upload, maybe dropbox or something will work for that. Let me know if you have a preference.
Thanks and stay safe!
Lisa

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(123/135 or less characters including spaces) Title: Inhibition of the Unfolded Protein Response Reduces Middle East Respiratory Syndrome Coronavirus Induced Acute Lung Injury

Authors: Amy C. Sims[1*]†, Hugh Mitchell[2]†, Lisa E. Gralinski[1], Jennifer E. Kyle[2], Kristin E. Burnum-Johnson[2], Mariam Lam[3], M. Leslie Fulcher[3], Ande West[1], Richard D. Smith[2], Scott Randell[3], Thomas O. Metz[2], Timothy P. Sheahan[1]‡, Katrina Waters[2]‡, Ralph S. Baric[1]‡

Affiliations:
[1]Department of Epidemiology, University of North Carolina at Chapel Hill, Chapel Hill, NC.

[2]Biological Sciences Division, Pacific Northwest National Laboratories, Richland, WA.

[3]University of North Carolina Tissue Procurement and Cell Culture Core, University of North Carolina at Chapel Hill, Chapel Hill, NC.

*To whom correspondence should be addressed: Amy C. Sims amy.sims@pnnl.gov current address: National Security Division, Pacific Northwest National Laboratory Richland, WA.

†contributed equally to manuscript.

‡ co-senior author

(148/150 or less characters including spaces) **One Sentence Summary:** Inhibition of the unfolded protein response reduced acute lung injury signatures following Middle East Respiratory Syndrome Coronavirus infection.

Abstract: Currently, there are no approved treatment options for Middle East Respiratory Syndrome Coronavirus (MERS-CoV) induced pneumonia and acute respiratory distress syndrome (ARDS) and little is understood about how individual primary human lung cell types

contribute to overall disease outcomes. MERS-CoV replicates equivalently in primary human lung microvascular endothelial cells (MVE) and fibroblasts (FB) but only MVE demonstrate virus induced cytopathic effect. To explore potential mechanisms leading to reduced MVE viability, three sets of donor-matched human lung MVE and FB were infected, and samples collected for transcriptomic, proteomic, and lipidomic analysis over time. Functional enrichment analysis results were validated and demonstrated that MERS-CoV-infected MVE were likely dying via an unfolded protein response (UPR) mediated apoptotic mechanism. Pharmacologic manipulation of the UPR in MERS-CoV infected MVE, FB confirmed its importance in cell death, virus replication and infection outcomes. In infected mice, treatment resulted in improved respiratory function and reduced weight loss, with accompanying reductions in the pathologic signatures of acute lung injury and improved times to recovery. Bioinformatic analysis of multi-omics systems biology datasets indicate that infection and death of MVE likely triggers loss of endothelial barrier integrity and vascular leakage into the airway, contributing to the ARDS and progressive end stage lung disease phenotypes commonly seen in emerging coronavirus infected patients.

[Main Text:]

Introduction

Coronaviruses (CoV) are important emerging pathogens associated with severe disease outcomes in humans and animals causing significant global morbidity and mortality. In 2003, severe acute respiratory coronavirus (SARS-CoV) emerged from closely related bat CoV strains, spread to 26 nations, and caused approximately 8,000 human cases and 800 deaths, worldwide (*1, 2*). In 2012, Middle East respiratory syndrome CoV (MERS-CoV) emerged, causing approximately 2,400 cases with a ~35% mortality rate in 27 countries and the outbreak is still

ongoing (*3*). In 2020, the causative agent of the coronavirus infectious disease 2019 (COVID-19) pandemic was identified as severe acute respiratory syndrome coronavirus 2 (SARS-CoV 2) and to date well more than 1,000,000 cases have been confirmed with a morality rate of ~4% in >160 countries with ongoing virus transmission resulting in significant economic losses and global public health concerns (WHO COVID 19 website). SARS-CoV 2, SARS-CoV, and MERS-CoV disease severity are strongly influenced by aging and other co-morbidities (e.g., diabetes, obesity) and mortality rates approach 15% or exceed 50% in aged individuals, respectively (>60 years)(*4*). These data underscore the highly pathogenic potential of emerging CoVs and reinforce the critical need for comprehensive experimental approaches that define the viral and host pathogenic programs which could be targeted by countermeasures to reverse severe disease outcomes.

Acute Respiratory Distress Syndrome (ARDS) is a severe end stage lung disease characterized by rapid onset respiratory failure resulting from diffuse alveolar damage, hyaline membrane formation, vascular leakage into the airways and pulmonary fibrosis (*5-8*). ARDS is a complex syndrome with multiple etiologies, which complicates diagnosis and treatment. While not the most prevalent cause(*9*), severe respiratory infections can progress to ARDS including those by highly pathogenic avian influenza(*10*) (H5N1) and SARS-CoV, SARS-CoV 2 and MERS-CoV(*2, 11-14*). Dissecting the mechanisms of MERS-CoV-induced ARDS has been hampered human genetic variation, variable co-morbidities and limited human sample availability (*11, 15*). Highly pathogenic human coronaviruses oftentimes cause programmed cell death within in vitro and in vivo models of viral pathogenesis as well as in patient autopsy tissues (*4, 16-23*). Because MERS-CoV replicates efficiently in multiple cell types essential for lung physiology (e.g. lung fibroblasts, epithelial and endothelial cells) (*24*), primary culture systems provide a biologically relevant

platform to better understand virus and virus-mediated- tissue specific host interactions that may drive severe lung pathology.

Here we report a kinetic multi-omics analysis of matched MERS-CoV infected primary human lung fibroblasts (FB) and microvascular endothelial (MVE) cells from three different human donors. While MERS-CoV replication kinetics were similar among FB and MVE, the host responses, as determined by multi-omic transcriptomic, proteomic, and lipidomic analyses differed significantly by cell type and suggested that MVE cells were dying via apoptotic mechanisms initiated by the unfolded protein response (UPR), which helps monitor and maintain homeostasis in the endoplasmic reticulum (ER) following a stress response. Pharmacologic manipulation of UPR demonstrated that active/dimerized PERK was critical for viral replication in vitro. More importantly, mice infected with MERS-CoV and treated with a PERK specific inhibitor demonstrated reduced weight loss, pneumonia and acute lung injury leading to improved respiratory function and time to recovery. These data not only articulate an important role for the epithelial-endothelial cell barrier in MERS-CoV pathogenesis and acute lung injury, but also reveal new strategies for the treatment of highly pathogenic emerging coronavirus infections in humans and animals.

Results

MERS-CoV induces cytopathic effect in primary human lung endothelial cells but not fibroblasts

Understanding MERS-CoV pathogenic mechanisms requires a detailed understanding of the critical virus-host interactions in infected primary human lung cells that provide the vital structural and physiological requirements for lung function. As clinical samples for MERS-CoV are scarce, using matched primary human lung cells from transplant recipients provides a novel opportunity to elucidate tissue specific changes that may explain disease phenotypes in the intact organ after

infection. To characterize MERS-CoV infection in primary human lung microvascular endothelial cells (MVE) and primary human lung fibroblasts (FB), we infected cells from three matched human donors and obtained virologic and multi-omics samples (transcriptomic, proteomic, and lipidomic) at 0, 12, 24, 36 and 48 hours post infection. Viral growth kinetics and peak titers (~10^7 plaque forming units (PFU)/mL) were nearly identical regardless of donor background (Figure 1A-B). We also determined infection frequency via microscopy by comparing the total number of cells (i.e. nuclei) to the number of MERS-CoV infected cells per high powered field (Figure 1C and D and Supplemental Figure 1). To simplify this process, we utilized a MERS-CoV expressing the red fluorescent protein (MERS-RFP) (Figure 1C and D). Unlike infection frequency in FB which increased over time, the numbers of total cells per field (Figure 1C) as well infection frequency decreased over time in MVE (Figure 1C-D). Since the number of cells per field in mock-infected MVE cultures did not diminish over time, virus induced cytopathic effect (CPE) was likely mediating the loss of cells in MERS-CoV infected MVE (Figure 1C and D). To quantitate CPE in MERS-CoV infected MVE and FB over time, we performed a cell viability assay (CellTiter-Glo) which estimates the number of metabolically active viable cells by measuring the levels of adenosine triphosphate (ATP) in each well. Unlike FB which had similar cell viability estimates in mock- and MERS-CoV-infected cultures for most time points (Figure 1E), infected MVE viability decreased significantly over time as compared to mock (Figure 1F), supporting a loss in cell viability. Together, these data demonstrate that both primary MVE and FB are similarly susceptible to MERS-CoV infection but MVE cell viability is significantly reduced over time, yet FB are relatively unaffected.

Cross-donor consensus transcriptomic, proteomic and lipidomic responses

To gain insight into the divergent outcomes in cell viability following MERS-CoV infection of FB and MVE, we analyzed donor matched kinetic multi-omic datasets. Functional enrichment was performed on transcriptomic and proteomic data across both cell types, which showed distinct differences in the modulation of the transcriptome and proteome among infected FB and MVE (Figure 2). We found that pathways related to apoptosis (transcription, Figure 2A) and the unfolded protein response (UPR, transcripts and proteins, Figure 2A and 2B), were significantly enriched in MERS-CoV infected MVE at multiple timepoints which was not observed in similarly infected FB. Interestingly, increased hemoglobin protein expression levels in both FB and MVE were detected at 48 hours post infection (arrow Figure 2B), which has been demonstrated to regulate endothelial and epithelial cell communication as well as to regulate the durability of vascular walls (*25-27*). We then investigated if mothers against decapentaplegic homolog 7 (SMAD7) and/or fibroblast growth factor 2 (FGF2) transcripts were differentially expressed in MERS-CoV infected MVE or FB as previous studies indicated that upregulated expression of SMAD7 and FGF2 in MERS-CoV infected immortalized kidney and lung epithelial cells led to cellular apoptosis (*28*). Neither SMAD7 nor FGF2 expression levels were distinct following infection and did not appear to explain the differences in cytopathic effect seen in the current studies (Supplemental Figure S2). Finally, we sought to determine if MERS-CoV infection would alter the lipidome in FB or MVE. We classified lipids into broad categories for enrichment analysis, which revealed differential expression of ceramides and triglycerides in FB and MVE (Figure 3A). When analyzed at a per-lipid-species level, we found dramatic and significant differences in specific species of ceramides (Figure 3B, upper panel) and triglycerides (Figure 3B, lower panel) both of which are involved in activating apoptotic processes (*29*).

MERS-CoV activates Caspases 3 and 7 in infected MVE

To validate the complementary multi-omics observations suggesting that MERS-CoV infection induces apoptosis in MVE, we simultaneously measured death effector caspases 3 and 7 and cell viability in mock or MERS-CoV infected FB and MVE using the Promega Apotox Triplex kit. We included staurosporine (induces caspase 3/7 activation) or ionomycin (induces necrotic cell death with no caspase activation) treatment as controls. In addition, we added UV-inactivated MERS-CoV virions in order to control for effects on cultures independent of virus replication (i.e. entry, uncoating, etc.). Uninfected MVE and FB treated with staurosporine significantly induced caspase 3/7 activation as compared to mock and cells treated with UV-inactivated MERS-CoV (Figure 4A-B). As expected, ionomycin treatment did not induce caspase 3/7 activation. With MERS-CoV infection, significant caspase 3/7 activation was observed at both 24 and 48 hours post infection but only in MVE (Figure 4A) not in FB (Figure 4B). Caspase activation was not observed with UV-inactivated MERS-CoV treatment or in mock-infected cells for either cell type (Figure 4A-B). When measuring cell viability within the same assay, neither mock infection nor treatment with UV-inactivated virions appreciably affected cell viability (Figure 4C-D). Importantly, treatment of both FB and MVE with control compounds (e.g. staurosporine or ionomycin) diminished cell viability but MERS-CoV infection only diminished cell viability in MVE (Figure 4C-D). Death of MERS-CoV infected MVE could lead to permeability at the epithelial/endothelial cell barrier as one of the early steps of ARDS while viable MERS-CoV infected FB would continue to produce high levels of infectious virus; both of which would contribute to the overall disease burden in the infected patient.

Expression of proteins indicative of the UPR pathway are increased in MERS-CoV infected MVE

Both systems data and confirmatory experimental validation studies suggested that MERS-CoV infected MVE were dying via apoptotic pathways. To better understand the initiation of this

process, we re-analyzed our systems biology data focusing on specific mediators of apoptosis and UPR. We found increased protein expression of three well-established UPR markers, glucose regulated protein 78 (GRP78 also known as binding immunoglobulin protein (BiP)), heat shock protein 90kDa beta member 1 (HSP90B1 also known as GRP94) and calnexin (CANX) in MERS-CoV infected MVE but not infected FB (Figure 5A-C). GRP78/BiP is a master regulatory protein for the UPR that binds to regulatory enzymes within the UPR pathway keeping them inactive unless misfolded proteins accumulate triggering dissociation and enzyme activation. HSP90B1/GRP94 and CANX are ER protein chaperones that facilitate nascent protein folding. In support of the viral titer data, the expression of MERS-CoV structural proteins, spike glycoprotein and membrane protein as well as the accessory open reading frame protein 4a (ORF4a) were expressed to similar levels in both cell types for all donors by 12 hours post infection (Figure 5D-F). Cumulatively, these data suggest that MERS-CoV modulation of the UPR is specific to a particular cellular environment (MVE) and may not be broadly applicable to all infected cells and tissues.

PERK activation is critical for MERS-CoV replication in primary human lung MVE and FB

To further validate the results thus far, which suggested that apoptosis of MVE was associated with the UPR, we pharmacologically perturbed the UPR in MERS-CoV infected MVE and FB using an inhibitor specific to the first enzyme activated following misfolded protein accumulation, protein kinase R-like endoplasmic reticulum kinase (PERK, AMG44). Acti vated PERK regulates phosphorylation of the key translation mediator, elongation initiation factor 2alpha (eIF2alpha). Following activation of the UPR and PERK, host translational shutoff serves to reestablish ER homeostasis and proper protein folding, which if not achieved, results in the activation of apoptotic pathways. Dose response studies were performed in mock and MERS-CoV infected

MVE and FB with PERK inhibitor (AMG 44 at 100 to 2 μM) to determine if pharmacologic perturbation of the PERK arm of the UPR would affect MERS-CoV replication and/or cell death (*30*). We utilized a MERS-CoV nanoluciferase reporter virus (MERS nanoluc) to increase accuracy and throughput of the assay (*31*). As shown in Figure 6, inhibition of PERK diminished MERS-CoV replication in both FB (Figure 6A and B) and MVE (Figure 6C and D) in a dose dependent manner when assayed at either 24 (Figure 6A and C) or 48 hours post infection (Figure 6B and D). In addition to monitoring changes in virus replication in the context of PERK inhibition, we also monitored cytotoxicity in drug treated uninfected cultures via CellTiter-Glo cell viability assay. Unlike FB in which we did not observe cytotoxicity at either timepoint (Figure 6A and B), in MVE we observed a dose dependent increase in cytotoxicity with PERK inhibition that was most notable at 48hpi (Figure 6D). Importantly, we found that at 24 hours post infection MERS-CoV replication was inhibited at multiple drug doses that did not exhibit cytotoxicity. These results suggest that primary human lung MVE are more sensitive to inhibition of the UPR in the absence of infection in contrast to the more tolerant primary lung FB. To determine if PERK inhibition modifies induction of apoptosis following MERS-CoV infection, we performed similar dose response assays as described in Figure 6A-D but then assayed for caspase 3 and 7 activation as in Figure 4 using similar UV inactivated and small molecule controls (i.e. staurosporin, ionomycin). No significant dose responsive change in caspase 3/7 activation above untreated infected cells was detected in either primary human lung MVE or FB following MERS-CoV infection and treatment with PERK inhibitor (Supplemental Figure S3).

PERK inhibition improves pulmonary function and diminishes MERS-CoV pathogenesis and ALI. Because *in vitro* PERK inhibition resulted in a significant decrease in MERS-CoV replication in both cell types, we sought to determine if *in vivo* inhibition would result in a change

in viral replication and/or pathogenesis. To address this, we utilized a transgenic mouse model where the murine ortholog of the human MERS-CoV receptor, dipeptidyl peptidase 4 (DPP4), was humanized at residues 288 and 330 (hDPP4) facilitating high titer virus replication localized to the respiratory tract and lung pathology similar to that observed in humans(*32, 33*). We treated hDPP4 mice with AMG44 or vehicle at 24 hours prior to and post MERS-CoV infection. By three days post MERS-CoV infection, vehicle treated mice lost significantly (p value <0.05) more weight than AMG44 treated mice. Importantly, AMG44 treated mice also displayed an improved time to recovery over the remainder of the study (Figure 7A). Interestingly, virus titer in the lung on day 3 post infection was similar regardless of treatment (Figure 7B). "Pulmonary hemorrhage", a gross pathological phenotype that increases coincident with MERS-CoV pathogenesis, was significantly diminished in AMG44 treated mice as compared to those receiving vehicle treatment (Figure 7C). To determine if PERK inhibition through AMG44 treatment would affect pulmonary function, we performed whole body plethysmography (WBP) daily. Concordant with weight loss and pulmonary hemorrhage, AMG44 treatment improved pulmonary function as compared to those receiving vehicle. For three WPB metrics typically induced following MERS-CoV infection (*32-34*), Pause, Penh and EF50, AMG44 treated mice had significantly improved values (p value <0.05) than vehicle treated animals at multiple timepoints (Figure 7D-F). Pause, PenH and EF50 are all measures of airway constriction or obstruction and indicate that AMG44 treatment is relieving or preventing lung pathology that negatively affects lung function. To quantitatively assess the impact of AMG44 treatment on MERS-CoV infected mice, histological lung sections were assessed using the American Thoracic Society (ATS) scoring system designed to more closely relate data from small animal models of acute lung injury (ALI) to infected patient outcomes as previously described (*35, 36*). Hematoxylin and eosin

stained fields of lung tissue were blindly scored for the presence of neutrophils in the alveolar and interstitial space, hyaline membranes, proteinaceous debris filling the airway spaces, and thickening of the alveolar septal (*35*). ATS lung injury scores were significantly reduced in male but not female MERS-CoV infected AMG44 treated mice (Figure 8A-E). Together with the wide variety of supportive data described above, the *in vivo* studies with AMG44 directly demonstrate the importance of PERK signaling in promoting MERS-CoV pathogenesis in mice. Our unbiased systems biology-based approach identified a pathway differentially modulated during MERS-CoV infection of primary human cells, validated the importance of this pathway for MERS-CoV replication and cell death *in vitro* and then demonstrated its importance in regulating viral pathogenic outcomes *in vivo*, leading to a targeted antiviral therapy.

Discussion

Viral pathogenesis is a multifactorial process driven by virus and host factors, responses in infected and neighboring cells, innate and adaptive immunity, host and viral genetics, and responses at the cellular, tissue, organ and organismal level. Due to this inherent complexity, defining the relative contribution of each of these factors to the disease state and the underlying cellular mechanisms that contribute to the progression towards severe disease is complicated. Thus, new models are needed to define cell and tissue specific host response patterns that play critical roles in viral pathogenesis. SARS-CoV, SARS-CoV2 and MERS-CoV all cause severe respiratory disease with replication and pathogenesis primarily limited to the lung. As shown in a recent pathologic evaluation of human tissue from a fatal case of MERS-CoV by Ng et al., diffuse alveolar damage, the pathological hallmark of acute lung injury (ALI), was noted along with viral antigen primarily found in alveolar pneumocytes with no extrapulmonary viral antigen detected (*4*). Similar findings have been reported following severe SARS-CoV and SARS-CoV2

infections, suggesting common mechanisms of coronavirus-induced ALI (*14, 37*). While the few post-mortem analyses of tissue samples from MERS-CoV patients have provided insight into the types of cells targeted by virus and the degree and severity of pathology in the lung, many basic questions related to the kinetics of virus replication, cell targeting, and the host response remain unknown. Primary cell cultures offer an opportunity to decrease the complexity of the intact organ to study how virus host interactions determine the fate of specific cell types following infection. While most studies with pneumotropic viruses have focused on airway epithelial cells or type II pneumocytes, MVE and FB play critical roles in maintaining lung architecture, compartmentalization and function (*38*). FB play a central role in the homeostasis of the extracellular matrix and are effector cells during injury repair, while the microvascular endothelium regulates vascular homeostasis, as vascular leakage can result in inefficient gas exchange in the lungs (*39*). In addition to MERS-CoV, H5N1 and other highly pathogenic influenza viruses also replicate efficiently in endothelial cells, providing diverse model systems to investigate virus-host interactions and identify novel tissue specific response patterns that can be used to attenuate virus replication and/or disease (*24, 40, 41*). Understanding the fate of these cells after infection provides a unique opportunity to understand how MERS-CoV induced disease phenotypes manifest despite limited clinical sample availability. Death of MERS-CoV infected MVE and epithelial cells (*4, 16*) could result in the breakdown of the epithelial-endothelial cell barrier and allow vascular leakage of fluids into the lung, leading to alveolar flooding, a hallmark feature of ALI and ARDS often seen in MERS-CoV, SARS-CoV and SARS-CoV2 patients and mouse models of human disease (*11, 32, 42, 43*). These findings support a more thorough examination of MERS-CoV lung cell tropism as clinical samples become available. The current findings also highlight that even within the same organ cell types can respond to

infection with very different outcomes and emphasize the value of studying primary cell types in culture.

During viral infection, viral glycoproteins, unfolded and misfolded host and viral proteins accumulate in the endoplasmic reticulum (ER) disrupting ER homeostasis. ER stress is sensed by several ER chaperone proteins (GRP78, etc.), allowing the cells to activate pro-survival responses that are mediated in part by UPR signaling through PERK, the transmembrane protein inositol -requiring protein-1 (IRE1α), and the activating transcription factor-6 (ATF6) (*44, 45*). If the UPR response fails to restore ER homoeostasis, it promotes cell death through the induction of the specific proapoptotic genes like CHOP and various caspases (*46*). To date the impact of highly pathogenic human CoV infection on different lung cell types has been poorly studied in primary human lung cells. Analysis of systems biology datasets provides an unbiased approach to define host pathways that are crucial for virus replication and cell fate outcomes following infection, providing novel insights into different tissue and cell specific responses to viral infection. Functional enrichment analysis of transcriptomic, proteomic and lipidomic datasets suggested that activation of the UPR promoted apoptotic cell death in MERS-CoV infected MVE, as compared with FB cells that displayed a minimal UPR response. Analysis of a single dataset would have hinted at either activated stress response or specific cell death pathways in MERS-CoV infected MVE. However, concordant findings across transcriptomic, proteomic and lipidomic datasets in matched cell types across multiple patient donors suggested key indicators of activated UPR and apoptotic pathways in MERS-CoV infected MVE cultures supporting the hypothesis that prolonged UPR signaling led to ER stress, PERK activation and programmed apoptotic cell death. As virus titers and viral protein expression are equivalent in both cell types, the robust activation of the UPR and apoptotic responses in MVE, but not in FB cells derived

from the same individual is puzzling. UPR is activated when the influx of nascent, unfolded polypeptides exceeds the folding capacity of the ER, which may differ between MVE and FB cells, yet viral structural and accessory protein levels were similar in both cell types. We hypothesize that lung FB can maintain ER homeostasis longer than infected MVE due to higher endogenous levels of ER folding chaperone proteins or increased overall ER volume, allowing continued cell viability after MERS-CoV infection. Future studies will probe how primary lung FB survive MERS-CoV infection. Importantly, ceremide and triglyceride are also potent stimulators of ER stress and apoptosis and these lipids are also highly upregulated following MERS-CoV infection of MVE, but much less so in FB cells. Increased expression of multiple triglyceride and ceramide species (*47, 48*) (products of sphingomyelinase pathways) is strongly correlative with virus induced CPE in mouse models demonstrating the *in vivo* relevance of these markers as well (*49*). Future studies will need to focus on evaluating the cell type specific UPR responses to various biochemical stimuli, after contemporary coronavirus and other respiratory virus infections, and identifying which ceramides and triglycerides regulate the UPR and pro-apoptotic response in primary lung MVE and FB.

AMG44 is a selective and potent PERK inhibitor and treatment efficiently blocked MERS-CoV replication in FB and MVE cells *in vitro* (*30*). These studies validate the importance of PERK activation and the UPR response as being critical for MERS-CoV replication fitness early in infection, especially in MVE cells. The most likely mechanism for AMG44 inhibition of MERS-CoV growth is that the PERK signaling pathway and the UPR response promote increased membrane reorganizations and suppressed host translation that optimizes an environmental milieu for improved virus replication, maturation and egress. AMG44 would promote ER stress and translation of host proteins, potentially disrupting virus replication complexes, budding and

release. Coronavirus infection is associated with extensive membrane reorganization (*50, 51*). In fact, coronavirus membrane-associated proteins nonstructural protein 3 (nsp3) and nsp4 have intrinsic membrane remodeling properties and usurp ER membranes to generate double membrane vesicles (DMVs) and other convoluted membrane structures that likely function as prominent sites of coronavirus replication (*50, 52-54*). Moreover, coronavirus egress occurs in a rER/Golgi intermediate compartment where coronavirus glycoproteins can induce ER stress and activate the UPR response (*17, 55-62*). Other coronaviruses respond to the PERK signaling cascade activation in a variety of ways including increased virus induced apoptosis (*56, 57*) or decreased viral titers (*60-62*). PERK activation was detected following overexpression of both SARS-CoV spike and ORF3a but the effects on replication were not determined (*17, 58, 59*). In contrast, the SARS-CoV E glycoprotein protected from UPR stress mediated by the IRE1 pathway (*63*). The role of MERS-CoV structural and nonstructural proteins are less certain although ORF4a has been reported to prevent activation of the stress response pathway via protein kinase R (PKR) antagonism (*62*). AMG44 prophylactic treatment protected MERS-CoV infected mice from clinical disease as evidenced by reduced weight loss, lung hemorrhage, and ALI signatures, leading to improved respiratory function and time to recovery. It is noteworthy that AMG44 treatment did not alter viral titers at day 3 post infection, perhaps reflecting the differential drug susceptibilities of the multiple cell types (e.g., airway epithelium, alveolar epithelial cells, FB and MVE) that are infected by MERS-CoV *in vivo*. In mice, PERK activation may not be pro-viral in all MERS-CoV susceptible cell types and/or the dosing regimen may not have been equivalent in all infected cell types. These results also suggest that PERK may play a role in a damaging pathogenic process *in vivo* where the individual cell types are part of a larger organ-based physiology and that treatment with the PERK inhibitor actually improves pulmonary function.

Interestingly, a previous study in Huh7 cells that explored the temporal kinase following MERS-CoV infection did not identify PERK as important for MERS-CoV infection, emphasizing the importance of using the most relevant primary human cells for global analysis studies (*64*).

Currently there are no approved therapeutics to treat highly pathogenic human coronavirus infections associated with the ongoing MERS-CoV epidemic or the 2019 SARS-CoV 2 pandemic, so methods to identify novel viral and host targets are recognized as a high priority by the WHO (*65*). Despite considerable progress, no therapy is presently available to reverse the widespread vascular leak that is observed during ARDS. Additional treatment studies with AMG44 in the MERS-CoV challenge model are needed to resolve and expand upon the panel of disease-attenuating drug candidates. SARS-CoV, SARS-CoV2 and MERS-CoV cause more serious disease in males, so it is interesting that prophylactically treated male, but not female mice had a significant reduction in hallmark ALI disease phenotypes after MERS-CoV infection. Adding additional days of treatment or performing twice daily treatment may improve the outcomes noted in the current study, using AMG44 in combination with robust antivirals (*31, 36*) **doi:** https://doi.org/10.1101/2020.03.19.997890), and determining the therapeutic window for AMG44 efficacy against MERS-CoV and SARS-CoV2 are also critical research priorities of the future. Nevertheless, the comprehensive systems biology approach described herein led to identification and validation of critical host pathways that mediated MVE cell death, leading to potential protein kinase based therapeutic treatment options that were successful in limiting disease phenotypes in mouse models of MERS-CoV induced ALI. When coupled with the data presented here, modulating UPR responses provides a robust strategy to develop novel therapeutics to control infection mediated vascular permeability, inflammation and ALI, especially when linked with potent direct acting antiviral inhibitors of virus replication *in vivo* (*31, 36*).

Materials and Methods

Primary human lung cells and immortalized cells. Primary human lung cells were isolated from distal lung tissue and processed as described (*24*). Human lung microvascular endothelial cells (MVE) were cultured in Vasculife VEGF-MVE Endothelial Media (Lifeline Cell Tech). Human lung fibroblasts (FB) were cultured in DMEM-H Basal Medium (Corning), 1X penicillin/streptomycin (Sigma), 10% fetal bovine serum (Gemini Bio). Media fetal bovine serum concentrations were reduced to 4% prior to infection. Vero 81 cells were cultured in Dulbecco's modified essential media (DMEM Gibco) with 10% fetal clone II (Hyclone) and 1X antibiotic/antimycotic (Gibco).

Viruses and viral titration Wild-type MERS-CoV (EMC 2012 strain), MERS-CoV expressing nano luciferase (MERS nanoluc) and MERS-CoV expressing the red fluorescent protein (MERS-RFP) were rescued from infectious clones as previously described (*24, 31*). Vero C81 cells were used to generate viral stocks and to quantitate viral titers following infection (*24, 31*).

Infections to collect transcriptomic, proteomic, and lipidomic samples. Donor matched MVE and FB from three different human tissue donors were infected and samples harvested for systems biology analysis. For transcriptomic analysis, five replicate wells of MVE or FB were either mock-infected or infected with wild type MERS-CoV at multiplicity of infection (MOI) 5. At 0, 12, 24, 36, and 48 hours post infection, 100uL of media/supernatant was collected for viral titration assays, media removed and then 1mL of Trizol (Invitrogen/ThermoFisher) added per well. Total RNA samples were sent to Arraystar for downstream analysis. In parallel, an additional five replicate wells were subjected to chloroform/methanol (2:1 mix) precipitation as previously

described (*66, 67*). All proteomic and lipidomic samples were desiccated and frozen prior to shipment to Pacific Northwest National Laboratories for downstream analysis.

Transcriptomic Analysis Scanned images were analyzed using Agilent Feature Extraction Software (v11.0.1.1). The limma package for R (available on Bioconductor) was used to perform background correction, quantile normalization (normalizeBetweenArrays), and summarization (avereps) to derive a single normalized intensity value per probe. Outlier samples were detected using PCA and by visual inspection of heatmaps, and all data was re-processed after removing outlier samples. All data processing for each of the biological replicates was performed independently of the other.

Proteomic Analysis The algorithm RMD-PAV (*68*) was used to identify any outlier biological samples, which was confirmed via Pearson correlation. Peptides with inadequate data for either qualitative or quantitative statistical tests were also removed from the dataset (*69*). The SPANS algorithm (*69*) was used to identify the best normalization method for each dataset. Peptides were evaluated with Analysis of Variance (ANOVA) with a Dunnett test correction and a Bonferroni-corrected g-test to compare each virus to the associated mock within each time point. To perform signature-based protein quantification, BP-Quant (*70*), each peptide was categorized as a vector of length equal to the number of viruses being evaluated. If all comparisons for all time points are 0 for a specific virus it is considered as non-changing and given a value of 0. If there are more time points with an increase in virus to mock than decreasing it is categorized as a +1 and the contrary -1 is given for the decrease in virus to mock. BP-Quant was run with a default parameter of 0.9. All proteins were then analyzed using the same methodology as for the peptides; ANOVA with a Dunnett test correction and a Bonferroni-corrected g-test to compare each virus to the associated mock within each time point.

Lipidomic Analysis The algorithm RMD-PAV (*68*) was used to identify any outlier biological samples, which was confirmed via Pearson correlation Lipids with inadequate data for either qualitative or quantitative statistical tests were also removed from the dataset (*69*). Median centering was used for normalization. Lipids were evaluated with a standard two sample t-test to compare each infected condition to the associated mock within each time point.

Functional Enrichment We identified significantly changed features (transcripts, proteins, lipids) by using an adjusted p-value of 0.05 as a threshold. For each experimental condition, perturbed features were divided into one list each of up- and down-regulated items. Each list was tested against the Gene Ontology (GO) database of gene sets using the EASE-adjusted one-sided Fisher exact test (*71*), such that each condition is tested for up- and down-regulation of each function/pathway in the ontology database. Results across multiple conditions were visualized in heatmaps using the log10 p-values of the significance tests, colored to indicate the direction of change. For lipids, feature sets were generated by categorizing the lipids observed in our experiments according to broad lipid classifications; these classifications were then used as "gene sets" and the enrichment analysis was then performed in the same manner as for transcripts and proteins.

Cell viability and percent infection assays To assess numbers of viable cells following MERS-CoV infection of FB and MVE, we used the CellTiter-Glo Luminescent Cell Viability Assay (Promega) at 24 and 48 hours post infection/treatment according to manufacturer's instructions and relative light units were determined by Spectromax plate reader (Molecular Devices), averaged and plotted. Percent infection was determined using MVE and FB infected with MERS-RFP (MOI 5) or mock-infected and counting nuclei (Horcht stain diluted 1:5,000 in media) in replicate wells at 0, 12, 24, 36, and 48 hours post infection. Cell numbers were determined using the Fiji (*72*) cell

counter function and total number of cells versus number of red fluorescent cells were determined for at least three wells per condition per cell type. Statistical significance was determined by Manfield Whitney unpaired t test (performed in GraphPad Prism).

Apoptosis Assays Infected FB and MVE were assayed using either the Caspase-Glo 3/7 Kit (Promega) to measure caspase activation or using the ApoTox-Glo Triplex Assay (Promega) to assess cell viability, cytotoxicity and caspase 3/7 activation in a single set of samples at 24 and 48 hours post infection according to manufacturer's instructions. Control wells were treated with UV inactivated virus stocks, staurosporin (Sigma, 8uM for MVE or 10uM for FB) or ionomycin (ThermoFisher, 40uM for MVE and 60uM for FB). Relative light units or emission excitation wavelengths were determined by Spectromax plate reader (Molecular Devices). Statistical significance was determined by Manfield Whitney unpaired t test (performed in GraphPad Prism).

Inhibitor studies MERS-CoV infected MVE and FB were tested at 24 and 48 hours post infection following treatment with a dilution series PERK inhibitor (AMG 44(*30, 73*) 100 uM to 2 uM) and was evaluated for cytotoxicity with drug treatment alone (cell viability assay, no virus), activation of the death caspases 3/7 (wild type MERS-CoV) and viral replication (detection of luciferase as a surrogate for replication, MERS nanoluc virus). All assay results were read by SpectraMax (Molecular Devices). Results are graphed as percent inhibition and toxicity (effective and cytotoxic concentration 50) as determined by GraphPad Prism.

Mouse studies 288-330$^{+/+}$ mice (*32*) were housed and bred in accordance with the University of North Carolina Department of Comparative Medicine, AALAC #329. For a single group of mice, dosing occurred once a day 24 hours prior to infection and a second single dose was administered 24 hours post infection via the intraperitoneal route (12mg/kg of PERK AMG'44 suspended in water or with a sham control(*30, 73*)). Immediately prior to infection, mice were anesthetized

with 50 uL of ketamine/xylazine mixture via intraperitoneal injection. 16-20 week old mice were intranasally infected with 10^4 PFU of mouse adapted MERS-CoV maM35c4 diluted in OPTIMEM (*33*), a total volume of 50 uL was given. Mice were weighed daily following infection and had their respiratory function measured one day prior to infection and at days two through six post infection using Buxco Whole Body Plethysmography (Data Sciences International) (*34*). Briefly, mice were allowed to acclimate for 30 minutes in the plethysmography chamber located within the biological safety cabinet after which a 5 minute measurement was taken. Readings were collected every 2 seconds for a total of 150 measurements per mouse per day. At days three and seven post infection mice were euthanized via isoflurane overdose after which lung tissue was harvested to determine viral replication titers. Gross pulmonary hemorrhage was observed and scored from zero (none) to four (severe and total) at the time of dissection.

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Figures and Figure Legends

Figure 1. Viral growth kinetics, percent infected cells and cell viability following MERS-CoV infection of primary human lung fibroblasts and microvascular endothelial cells. A-B. Viral replication: Donor matched microvascular endothelial cells (MVE) and fibroblasts (FB) were infected with wild type MERS-CoV (MOI of 5) and supernatants collected at the indicated times and viral titers determined by plaque assay. Results are shown as plaque forming units (pfu) per mL over time. Each data point represents averaged data from supernatant collected from 10

different wells (5 wells harvested for RNA and 5 wells fractionated for proteins, metabolites and lipids). The graphs in A and B show levels of replication detected for all three tissue donors in both cell types. Error bars indicate standard deviation from the mean. Open and filled blue circles and blue triangles represent data from MERS-CoV infected FB, while open and filled red circles and red triangles represent MERS-CoV infected MVE. **C-D.** Percent MERS-CoV infected cells: Donor matched MVE and FB were mock-infected or infected with MERS-CoV expressing the red fluorescent protein (MERS-RFP), nuclei were stained with Hoechst stain, and images captured at the indicated times post infection. Total numbers of cells (shown for all conditions in **C**) and total numbers of infected (contained red fluorescence) cells were assessed for at least three fields per condition and total percent of infected cells determined and plotted (**D**). Representative data is shown for one of the three donors. Error bars indicate standard deviation from the mean. No statistical significance (ns) was found when comparing the percentage of infected FB and MVE at any specific timepoint. Statistical analysis performed in GraphPad determined by Manfield Whitney unpaired t test. Open blue bars- mock-infected FB, open red bars- mock-infected MVE, filled blue bars- MERS-RFP infected FB, filled red bars- MERS-CoV RFP infected MVE. **E-F.** Cell viability following MERS-CoV infection: Donor matched MVE and FB were infected with wild type MERS-CoV and assessed for cell viability using the CellTiter-Glo kit according to manufacturer's instructions (Promega) at the indicated times post infection. Relative light units were graphed over time and error bars indicate standard deviation from the mean. Statistical analysis performed in GraphPad determined by Manfield Whitney unpaired t test. Gray bars- mock infected cells, red bars- MERS-CoV infected MVE, blue bars- MERS-CoV infected FB, **** $p = <0.0001$, ** $p = <0.002$, ns not statistically significant.

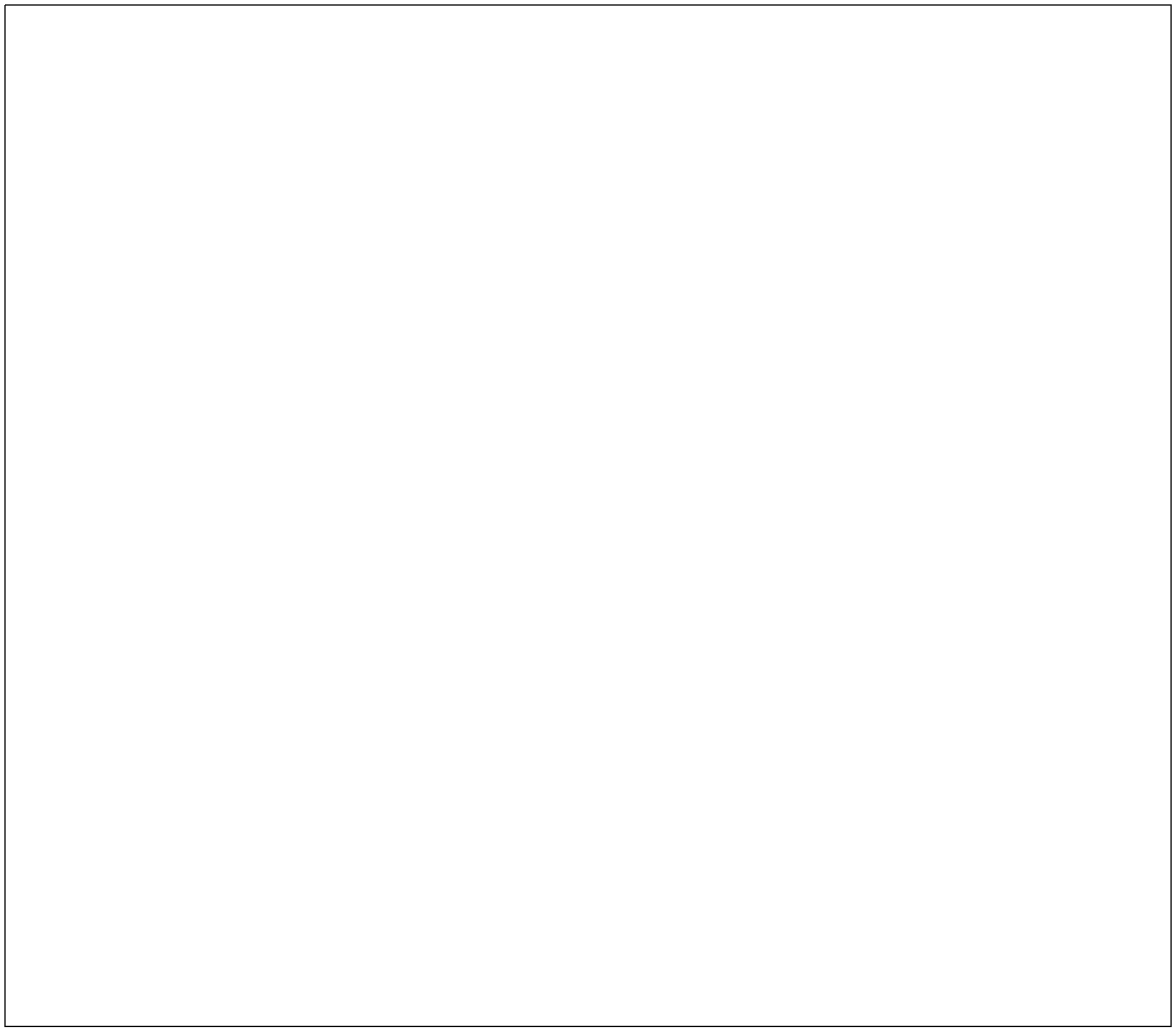


Figure 2. Transcriptomics and proteomics suggest activation of apoptotic pathways. A. Functional enrichment was performed on transcriptomic data from all three donor samples in both cell types, and results were only retained that were present in all three donors, keeping only the least significant score. In this way a true consensus response is represented by all indicated functions. **B.** Protein expression for proteins associated with the GO term "positive regulation of protein insertion into mitochondrial membrane involved in apoptotic signaling pathway". All apoptosis-associated proteins are displayed across the three donors for fibroblasts and MVE. The blue arrow highlights proteins in the hemoglobin complex and red asterisks highlight apoptotic proteins from the endoplasmic reticulum.

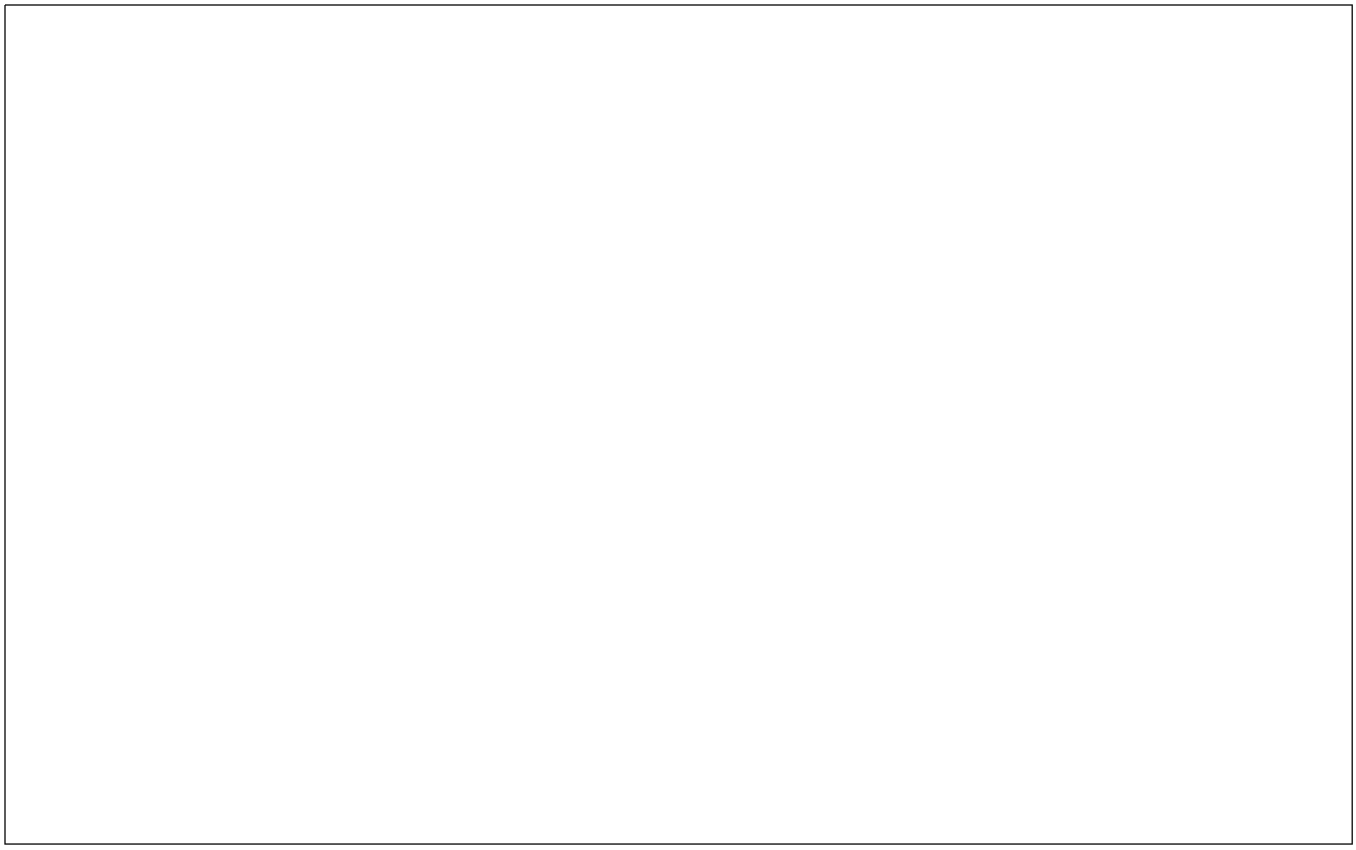


Figure 3. Lipid profiles suggest activation of apoptotic pathways. A. Enrichment analysis using lipid classes as enrichment sets. Cer-ceramide, TG-triglycerides, PC-phosphatidylcholine, PE-phosphatidylethanolamine, PS-phosphatidylserine, SM-sphingomyelin. **B.** Abundance of individual lipid species.

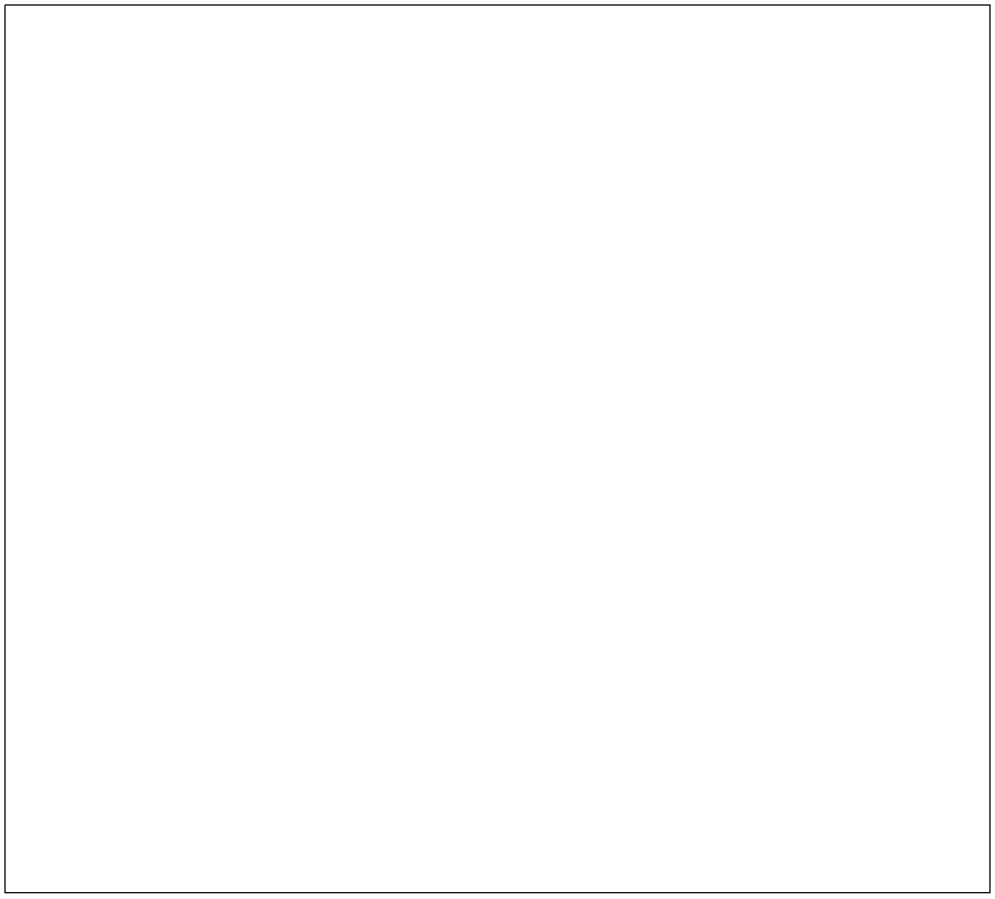


Figure 4 Caspases 3/7 are activated following MERS-CoV infection of primary human lung MVE but not primary lung FB. A-D. Donor matched MVE and FB were plated and mock-infected or infected with UV-inactivated or wild type MERS-CoV and at 24 and 48 hours post infection caspase 3/7 activation or cell viability was determined according to manufacturer's instructions using the Apotox Triplex kit (each well was measured for both parameters). Uninfected cells were treated with staurosporin (8 uM MVE/10 uM FB) to determine the maximal amount of caspase 3/7 activation. Uninfected cells were treated with ionomycin (50 uM MVE/60 uM FB) to demonstrate loss of cell viability via non-apoptotic pathway. Results are graphed as relative light units and error bars indicate standard deviation from the mean. Statistical analysis performed in GraphPad determined by Manfield Whitney unpaired t test. **A-B** Results

from caspase 3/7 activation. **C-D.** Results from cell viability assay. Solid red bars- MVE 24 hour samples, open red bars- MVE 48 hour samples, solid blue bars- FB 24 hour samples, open blue bars- FB 48 hour samples. **** p=<0.0001, *** p= <0.0002, ns- not statistically significant.

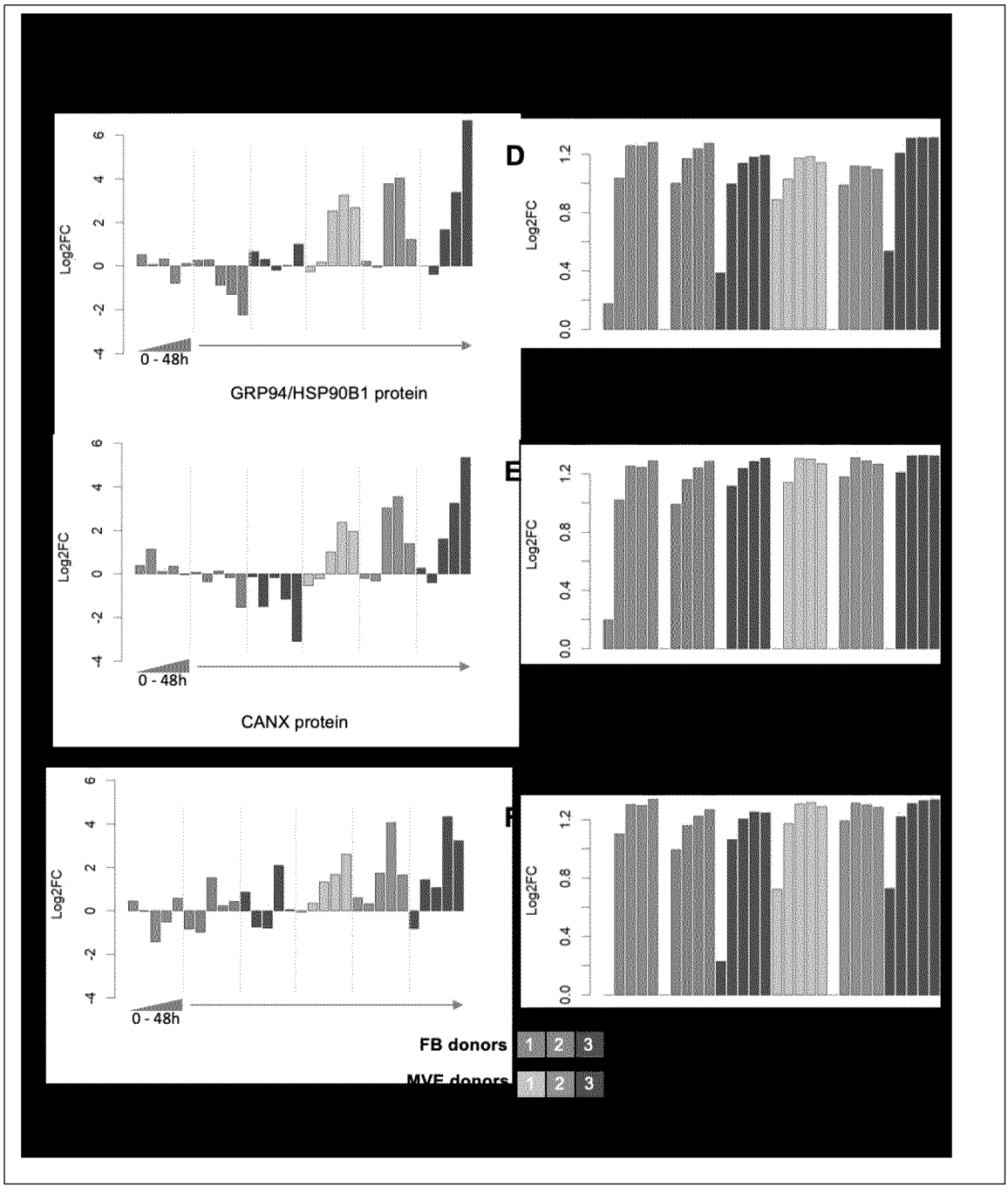


Figure 5 Protein markers suggest activation of unfolded protein response (UPR). A-C. Protein expression of markers of the unfolded protein response. Each figure shows the expression behavior of a UPR marker in FB (blues) and MVE (reds), with individual donors represented separately as individual color hues. For each donor, a time course of expression is shown that includes samples taken at 0, 12, 24, 36 and 48 hours post infection. Values are expressed as the log10 p-value of the change between the infection and control conditions, with sign assigned according to the direction of fold change. **D-F.** Viral protein expression. MERS-CoV spike, open reading frame 4a (ORF4a) and membrane protein abundances are represented as the ratio of each protein to the average abundance of all other proteins detected in each respective sample.

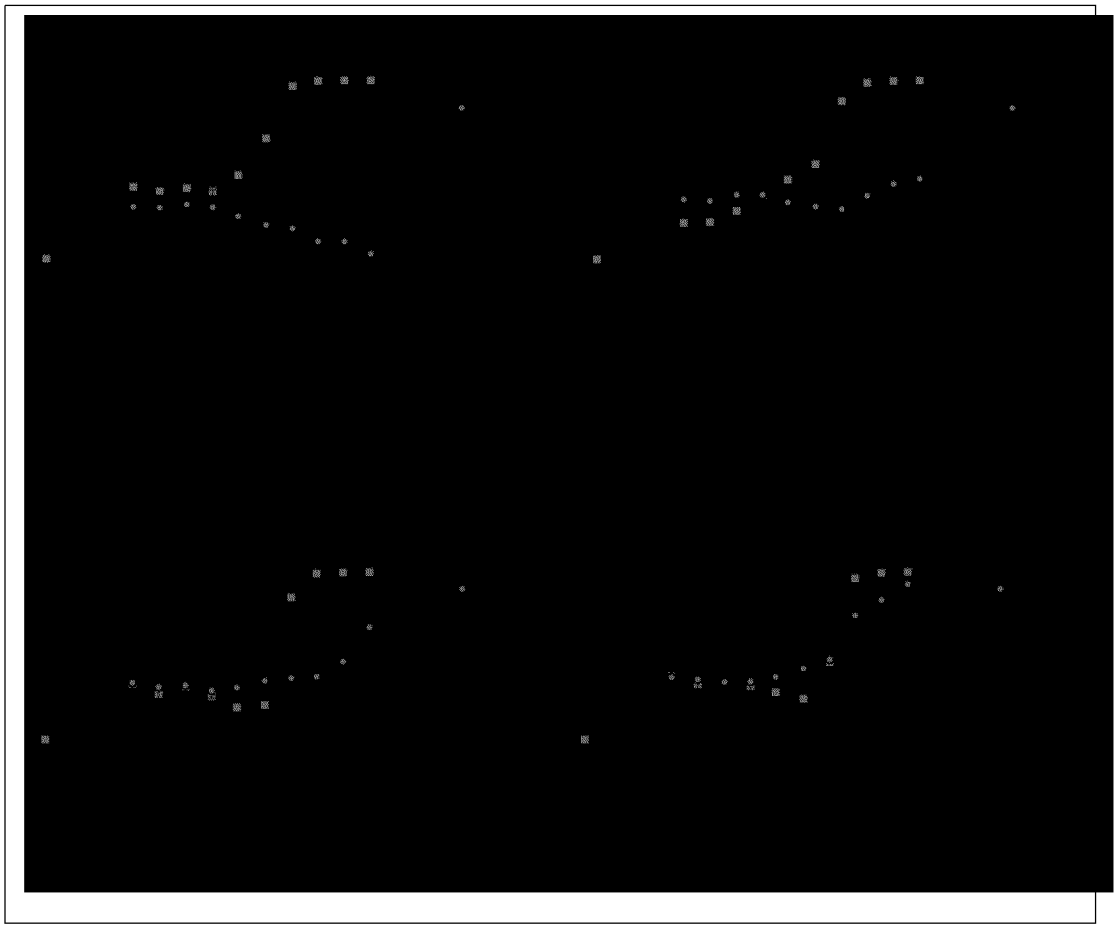


Figure 6 PERK inhibitor reduces MERS-CoV replication in infected primary human lung MVE and FB. A-D. Donor matched uninfected FB (circles, A, B) and MVE (circles, C, D) were treated with serial dilutions of PERK inhibitor (100 uM to 2 uM) and cell viability assessed at 24 (A, C) and 48 (B, D) hours post treatment using Promega's CellTiter Glo kit according to manufacturer's instructions. Each circle represents mean values from two experiments and are graphed as percent toxicity. Error bars indicate standard deviation from the mean. In parallel, the same donor matched FB (squares, A, B) and MVE (squares, C, D) were infected with MERS-nanoluc and simultaneously treated with the same serial dilutions of PERK inhibitor. Nanoluciferase expression was assayed at 24 (squares, A, C) and 48 (squares, B, D) hours post infection using Promega's NanoGlo kit according to manufacturer's instructions. Control wells were either treated with drug diluent and UV-inactivated virus or infected with MERS-nanoluc and treated with only drug diluent. Each square represents mean values from two experiments and are graphed as percent inhibition. Error bars indicate standard deviation from the mean. Blue squares- MERS-nanoluc infected FB treated with PERK inhibitor serial dilutions, blue circles- uninfected FB treated with PERK inhibitor serial dilutions, red squares- MERS-nanoluc infected MVE treated with PERK inhibitor serial dilutions, red circles- uninfected MVE treated with PERK inhibitor serial dilutions. PERK protein kinase R-like ER kinase, MVE primary human lung microvascular endothelial cell, FB primary human lung fibroblast, MERS-nanoluc MERS-CoV expressing nanoluciferase

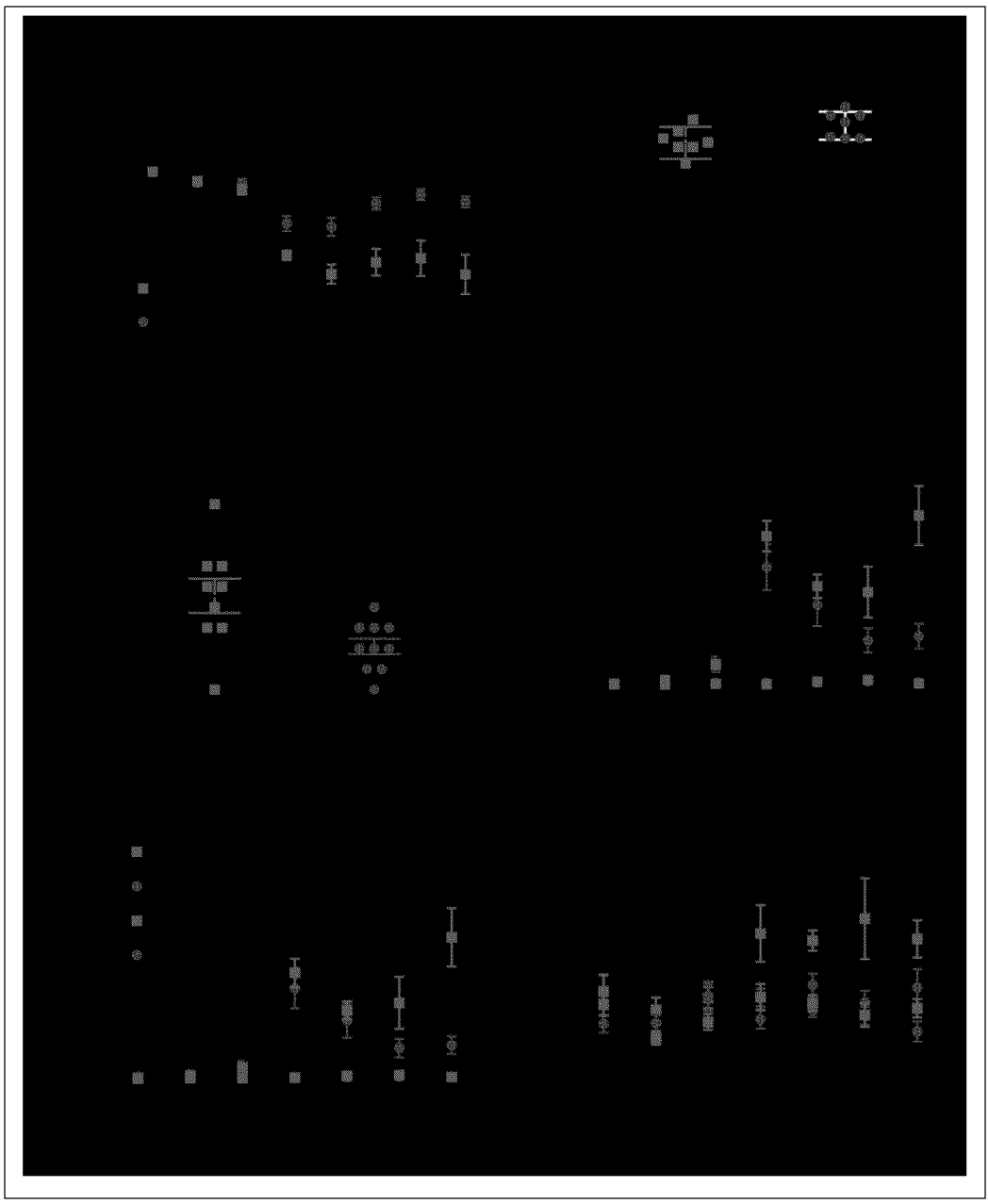


Figure 7 PERK inhibitor AMG44 treatment decreases MERS-CoV pathogenesis

A-F. 288/330+/+ mice were treated with PERK AMG'44 or sham control and infected with $5x10^4$ PFU of MERS-CoV MAm35c4 or PBS control. Weight loss was observed over the course of a seven day infection (A) and viral titers (B) and pulmonary hemorrhage was scored at the time of harvest (C). Daily respiratory function measurements (D-F) were taken via whole body

plethysmography. Error bars indicate standard error of the mean, # indicates a p value of < 0.1 and * indicates a p value of < 0.05.



Figure 8 AMG44 reduces clinical signs of acute lung injury in MERS-CoV challenge model

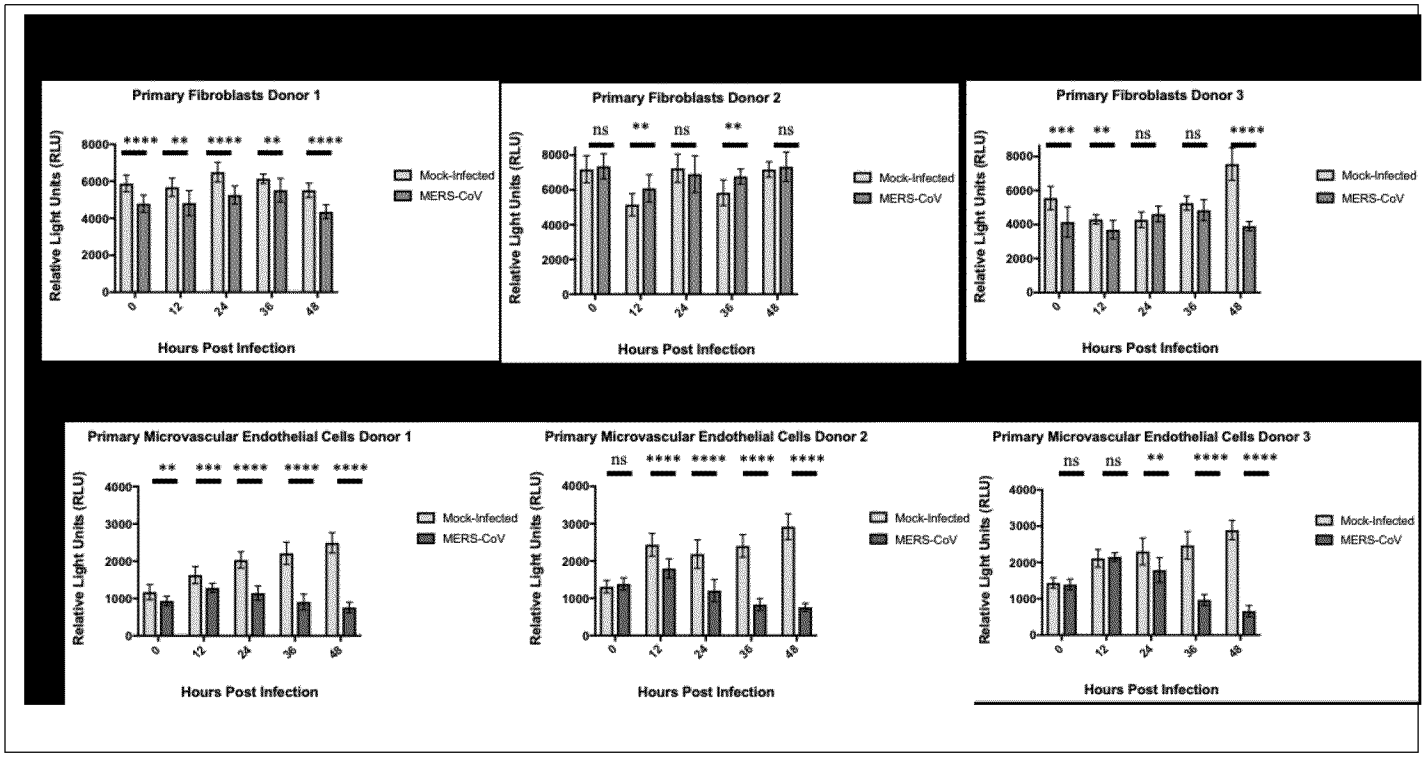
A-E. The histological features of acute lung injury were blindly scored using the American Thoracic Society Lung Injury Scoring system by Matute-Bello, creating an aggregate score for: neutrophils in the alveolar and interstitial space, hyaline membranes, proteinaceous debris filling the air spaces, and alveolar septal thickening. **A-B.** Scoring for ALI and diffuse alveolar damage in male and female mice, respectively. Representative lung pathology for C) mock-infected, D) MERS-CoV infected and sham treated and E) MERS-CoV infected and AMG44 treated male mice at 7 days post infection. * indicates a p value of <0.001.

Supplementary Materials

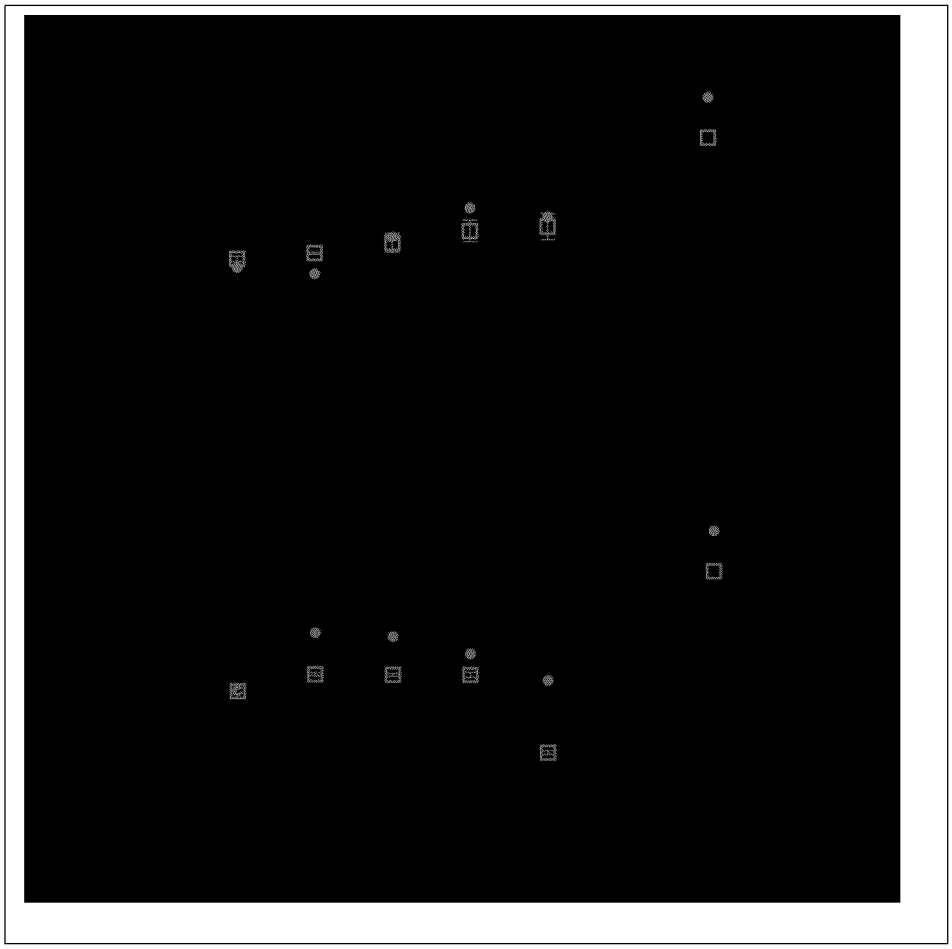
Supplementary Materials and Methods

All methodologies are described in the Materials and Methods section above.

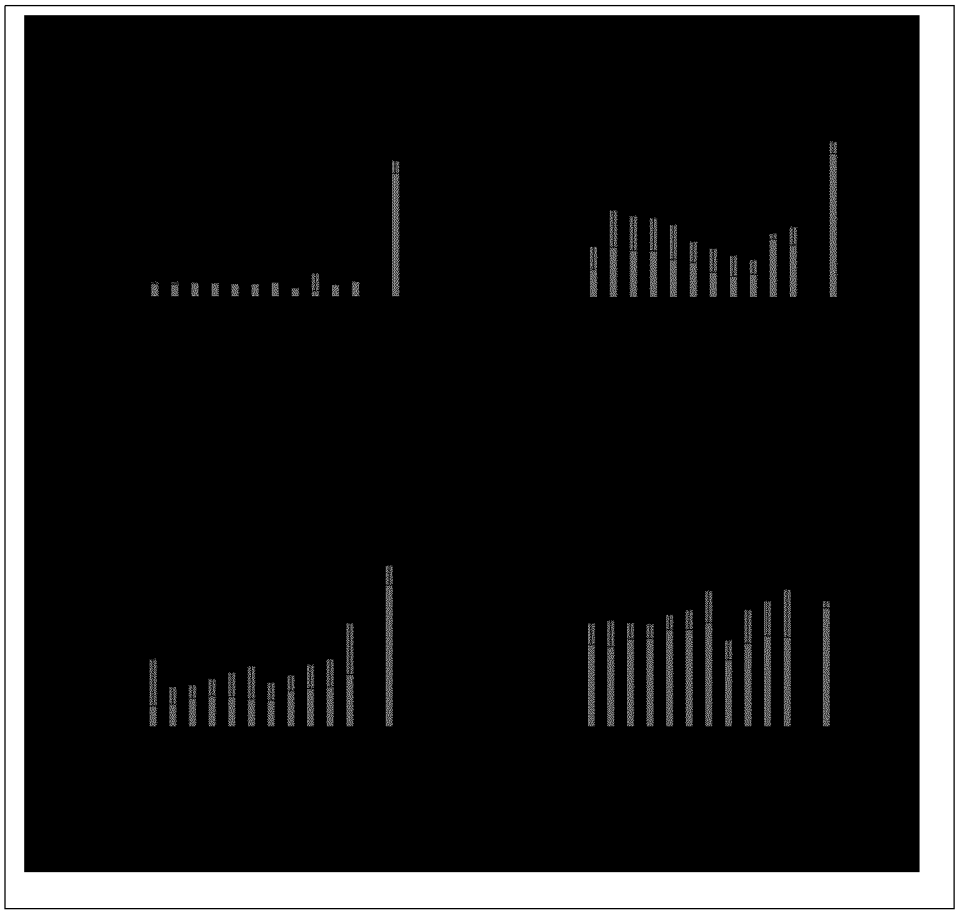
Supplementary Figures and Tables



Supplementary Fig. S1 MERS-CoV reduces cell viability for all three donors in MVE but not FB. Donor matched MVE and FB were infected with wild type MERS-CoV and assessed for cell viability using the CellTiter-Glo kit according to manufacturer's instructions (Promega) at the indicated times post infection. Relative light units (RLU) were graphed over time and error bars indicate standard deviation from the mean. Statistical analysis performed in GraphPad determined by Manfield Whitney unpaired t test. Gray bars- mock infected cells, red bars- MERS-CoV infected MVE, blue bars- MERS-CoV infected FB, **** p= <0.0001, ** p= <0.002, ns not statistically significant.



Supplementary Fig. S2 Neither SMAD7 nor FGF2 expression contribute to MERS-CoV induced cytopathic effect in primary human lung cells. A-B. Graphs show transcript expression levels for SMAD7 and FGF2 in MERS-CoV infected MVE and FB over the 48 hour time course (derived from microarray analysis and are from the same datasets used in Figure 2). Values graphed are fold change (log_2) and error bars are standard deviations from the mean. mothers against decapentaplegic homolog 7 (SMAD7); fibroblast growth factor 2 (FGF2)



Supplementary Fig. S3 Inhibition of PERK by AMG44 does not alter caspase 3/7 activation following MERS-CoV infection. A-D. Donor matched FB (A, B) and MVE (C, D) were plated and infected with UV-inactivated or wild type MERS-CoV and at 24 (A, C) and 48 (B, D) hours post infection caspase 3/7 activation was determined according to manufacturer's instructions

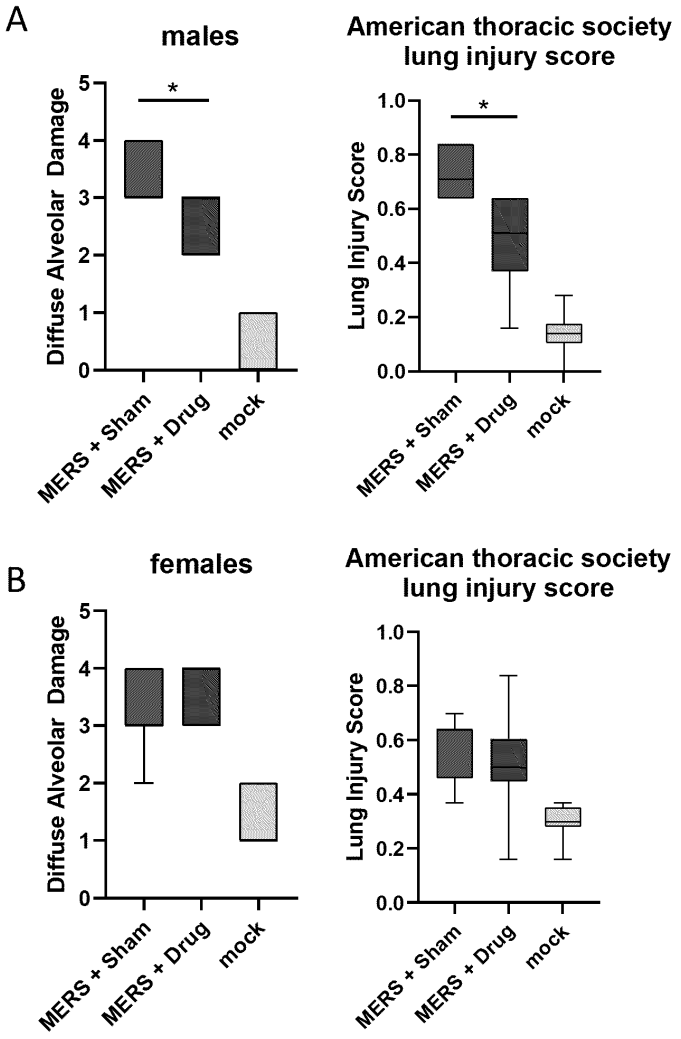
using the Apotox Triplex kit. Control uninfected cells were treated with staurosporin (8 uM MVE/10 uM FB, activates caspase 3/7) or ionomycin (50 uM MVE/60 uM FB, does not activate caspase 3/7). Results are graphed as relative light units and error bars indicate standard deviation from the mean. Statistical analysis performed in GraphPad determined by Manfield Whitney unpaired t test.

Supplementary Table 1 Pathways contained within categories found by functional enrichment analysis of transcriptomic data in Figure 2

Please see separate Excel spreadsheet.

Supplementary Table 2 Pathways contained within categories found by functional enrichment analysis of proteomic data in Figure 2

Please see separate Excel spreadsheet.


A
males
Diffuse Alveolar Damage
American thoracic society lung injury score
Lung Injury Score
MERS + Sham
MERS + Drug
mock
B
females
American thoracic society lung injury score
Diffuse Alveolar Damage
Lung Injury Score
MERS + Sham
MERS + Drug
mock

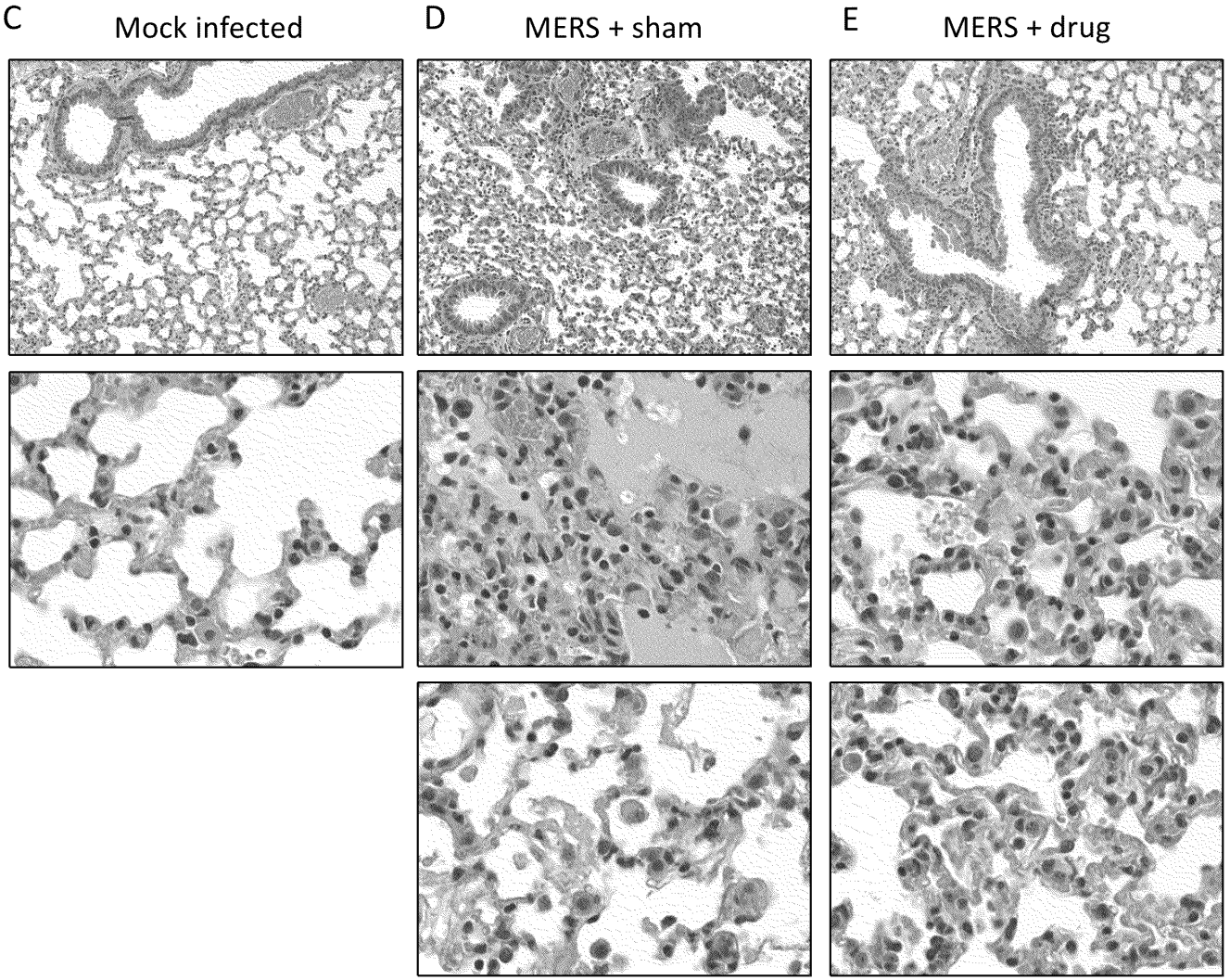

C
Mock infected
D
MERS + sham
E
MERS + drug

Figure Legend. The histological features of acute lung injury were blindly scored using the American Thoracic Society Lung Injury Scoring system by Batute-Bello, creating an aggregate score for: neutrophils in the alveolar and interstitial space, hyaline membranes, proteinaceous debris filling the air spaces, and alveolar septal thickening. A) and B) Scoring for acute lung injury and diffuse alveolar damage in male and female mice, respectively. Representative lung pathology for C) mock infected, D) MERS-CoV infected and sham treated and E) MERS-CoV infected and PERK'AMG treated male mice at 7 dpi. * indicates a p value of <0.001.

From: Caldwell, Chandra[ccaldwel@email.unc.edu]
Sent: Mon 4/6/2020 12:36:44 PM (UTC-07:00)
To: Parrish, Kalyn[klparrish@unc.edu]
Cc: Sims, Amy C[amy.sims@pnnl.gov]
Subject: Relinquish Statement for Amy Sims R21 grant

Hi Kalyn,

After speaking to Vicki this afternoon, we are going to move forward in getting UNC to process the relinquish statement for Amy Sim's R21 grant (project id: 5113910, *How MERS-CoV Regulates Innate Immunity in Primary Human Lung Cells*). Before making the request to OSR, Vicki wants to make sure Battelle/PNNL has sent all their subcontract invoices through March 31, 2020 and can provide you with an estimate of how much they have left for the remaining two months (April and May) of their subcontract (to insure the amounts match with what UNC has on file and will denote on the relinquish statement). Once this info is confirmed, Vicki and/or Carol will contact OSR to get moving on this request.

Thanks,
Chandra

From: Schaefer, Alexandra[aschaefe@email.unc.edu]
Sent: Mon 4/6/2020 2:01:50 PM (UTC-07:00)
To: Sims, Amy C[amy.sims@pnnl.gov]; Sheahan, Timothy Patrick[sheahan@email.unc.edu]
Subject: Re: Draft progress report for R21
Attachment: science draft R21 Year 1 progress report 4_2_2020_AS.docx

Hi Amy,

Hope all is well!

attached are my comments.
The data looks nice
and it all sounds good to me

Stay safe,
Alex

From: Sims, Amy C <amy.sims@pnnl.gov>
Sent: Thursday, April 2, 2020 1:47 PM
To: Sheahan, Timothy Patrick <sheahan@email.unc.edu>; Schaefer, Alexandra <aschaefe@email.unc.edu>
Subject: Draft progress report for R21

Hi,

Here is a draft of the progress report for the R21.

Alex, I would greatly appreciate your feedback. I think we are limited to two pages. The new data in Figure 3 is shown as just fold change now I thought that would be okay.

Tim, you do not need to make comments but as your name will go with it I wanted to make sure you had a chance to read it.

If you can get this back to me tomorrow I would really appreciate it. Thank you, Amy

6.1 Section A Cover page

6.2 Section B-Accomplishments

B.1 What are the major goals of the project?

The major goals of the project are to determine if primary human lung cells infected with Middle East respiratory syndrome coronavirus (MERS-CoV) use epigenetic mechanisms to regulate the expression of interferon stimulated genes (ISG). Specific Aim 1: Does histone methylation status coordinate with ISG expression patterns in MERS-CoV infected MVE, HAE, and/or FB? Aim 1 focuses on defining cell type specific type I and III ISG for primary human lung airway epithelial cell cultures (HAE), microvascular endothelial cells (MVE) and fibroblasts (FB), identifying if transcription factors mediate any regulation of ISG expression, and determining if MERS-CoV infection affects the methylation state of lysine 4 or 27 on cellular histones which determine whether chromatin in in an open state and accessible for transcription or in a closed state preventing transcription from occurring. Specific Aim 2: Exploring modulation of host chromatin remodeling enzymes following MERS-COV infection of primary human lung MVE, HAE, FB. Aim 2 seeks to define the DNA/histone methyltransfersae and deaceytylatse enzymes MERS-CoV may be modulating to regulate ISG expression. Assays will determine if host chromatin remodeling enzymes are active or inhibited following MERS-CoV replication and whether MERS-CoV replication and/or ISG expression are affected by inhibition of methyltransferase/deaceytlse enzyme activity. Finally, we will use novel protein-protein interaction technologies to determine MERS-CoV proteins that may mediate the epigenetic regulation of ISG expression.

B1.1a. have the major goals changed since the initial competing award or previous report?

No, the major goals have not changed however there may be delays in completing experiments due to the shelter in place requirements for CoVID-19.

B.2 What was accomplished under these goals?

The ongoing MERS-CoV epidemic and recent identification of SARS-CoV-2 as the causative agent of coronavirus infectious disease 2019 (COVID-19) highlight the need for a better understanding of how highly pathogenic human coronaviruses manipulate the host immune response. Previous studies demonstrated that MERS-CoV infects an expanded range of human primary cell types including microvascular endothelial cells and lung fibroblasts which are not permissive for the 2002 SARS-CoV strain. In immortalized human lung epithelial cells, MERS-CoV downregulates the expression of several interferon stimulated genes (ISG) restricting the host innate immune response and previous studies suggest that this downregulation is mediated by epigenetic mechanisms that regulate the methylation state of histones keeping the chromatin in an inaccessible or closed state and upregulating the methylation of host DNA leading to a decrease in the expression of major histocompatibility complex genes. The goal of these studies was to determine if MERS-CoV uses epigenetic mechanisms to regulate the innate immune response in primary human lung endothelial cells (MVE) and fibroblasts (FB). Initial studies evaluated levels of infection in more than 20 donor tissues (all cell types collected for each donor) to determine which were best for the MERS-CoV epigenetic studies. Three donors were selected, and the University of North Carolina at Chapel Hill Tissue Procurement Core earmarked these frozen cell aliquots for these studies and sufficient numbers of cells are available for all studies within this funding application (**Figure 1**).

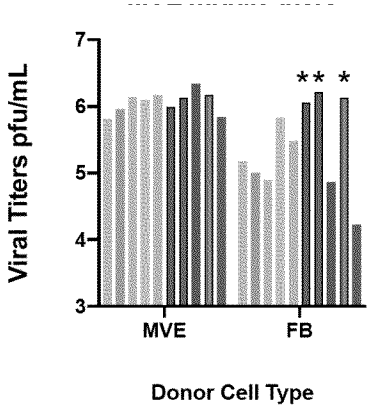


Figure 1. Representative MERS-CoV titers from tissue donor candidates for epigenetic experiments. MVE and FB from 20 donors were plated and infected with MERS-CoV for 24 hours and supernatants assessed by plaque assay to determine levels of viral replication. Three stars indicate the donors (only FB shown) used for downstream epigenetic testing. * highlight chosen donors.

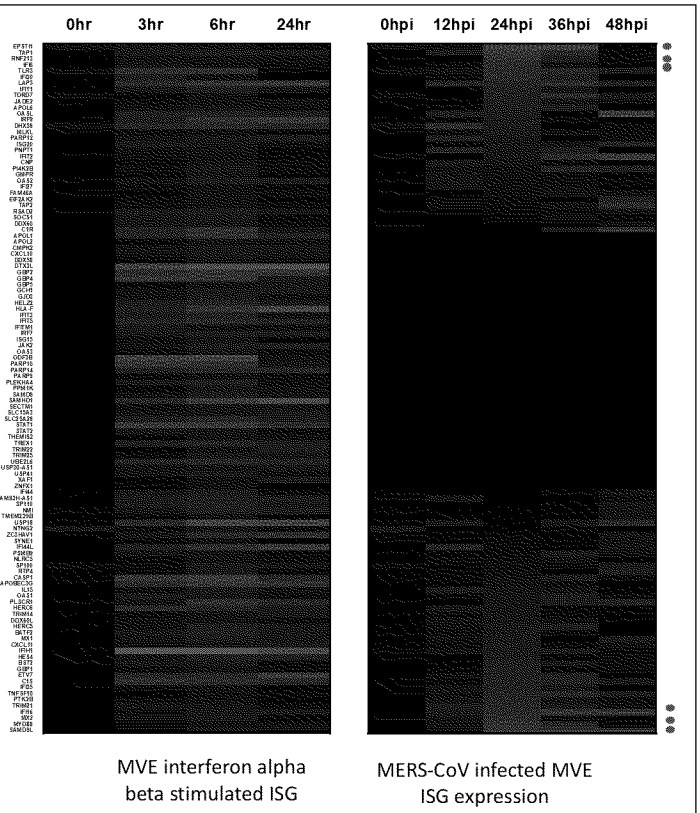


Figure 2 MERS-CoV regulates the innate immune response in primary human lung MVE. Left panel: Human lung MVE were treated with interferon alpha beta for the indicated times and ISG identified following microarray analysis. Right panel: ISG list identified in left panel following MERS-CoV infection of MVE. Red dots indicate up-regulated ISG used for ChIP-PCR studies; green dots indicate down-regulated ISG used for ChIP-PCR studies

Cell type specific lists of ISG from MVE and FB were generated following treatment of MVE and FB with interferon alpha/beta and the top six upregulated and downregulated (12 genes total per cell type) for each cell type were identified (**Table** and **Figure 2**). Triplicate samples of MVE and FB for all three donors were infected with MERS-CoV, protein and DNA crosslinked by formaldehyde, samples were safety tested and removed from biosafety level three. Due to the number of total samples to process analysis of one cell type at a time will be performed. Batch one analysis was performed on MERS-CoV infected MVE cells and has been completed. We anticipate that batch two analysis on MERS-CoV

infected FB will be completed by the end of Year 1/May 2020, but this work may be delayed until the shelter in place requirement has been removed. Formalin treated lysates from MERS-CoV infected MVE were assayed by chromatin immunoprecipitation-PCR (ChIP-PCR) using antisera specific for the open, transcriptionally active form of histone bound chromatin (trimethylated lysine 4 in histone 3 (H3K4me3)) or for the closed transcriptionally inactive form of histone bound chromatin (trimethylated lysine 27 in histone 3 (H3K27me3)) to determine if histone modifications could explain the differential expression of specific ISG. PCR is used to amplify the promoter regions of the ISG following immunoprecipitation to determine if the chromatin precipitated is in the active or inactive state following MERS-CoV infection (**Table**). Representative results are shown in **Figure 3** for two ISG with increased differential expression (interferon gamma inducible protein 6 (IFI 6) and ring finger protein 213 (RNF213)) or decreased expression (myeloid differentiation primary response protein 88 (MyD88) and sterile alpha motif domain containing 9L (SMAD9L)). H3 histones bound to the promoter regions of ISG that had upregulated expression (IFI 6 and RNF213) following MERS-CoV infection of MVE were predominantly trimethylated on lysine 4 and in the active confirmation for all three tissue donors (Figure 3 top panels) with little if any associate with histones in the closed confirmation. In contrast, H3 histones bound to ISG promoter regions with decreased expression levels (SMAD9L and MyD88) were predominantly trimethylated on lysine 27 and in the closed confirmation with little associate with histones in the active confirmation. This data suggests that epigenetic regulation may play a role in down regulation of the innate immune response to MERS-CoV in primary human lung microvascular endothelial cells.

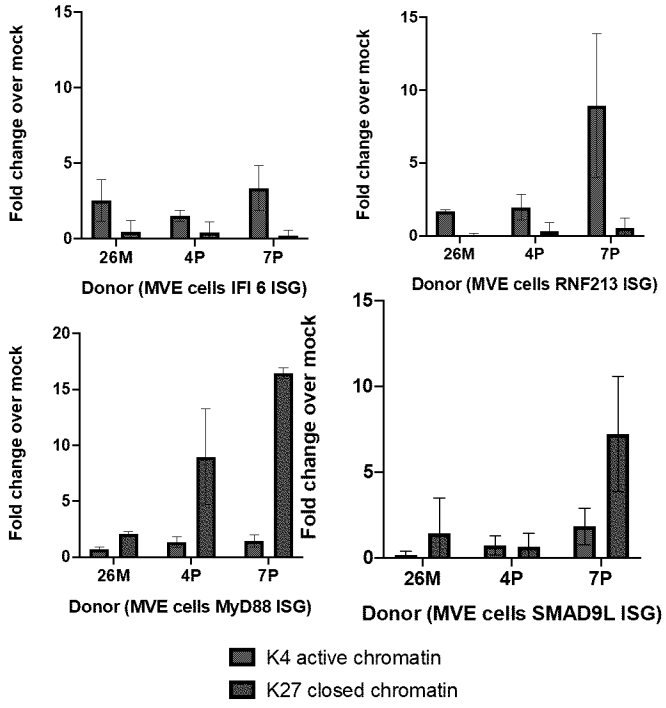


Figure 3 Altered histone methylation patterns suggest mechanism for ISG expression following MERS-CoV infection in primary human lung MVE. ChIP-PCR was performed on MERS-CoV infected MVE lysate for three donors. Antibodies specific for histones with lysine 4 methylation (shown in red) or for histones with lysine 27 methylation (shown in green) were used to precipitate chromatin and assayed by real time PCR. Top panels are ISG with increased expression following MERS-CoV infection and bottom panels are ISG with decreased expression following infection.

Cell Type	Gene Name	Differential Gene Expression	Primer Pairs	Analysis as Predicted
MVE	Sterile alpha motif domain containing 9L (SMAD9L)	Down	cagcctcattttcgtagccctcat ggccatctacaagccaaggagaga	Yes
MVE	Interferon gamma inducible protein 16 (IFI 16)	Down	tcatagcagacatttccccaacca gcttcttctcccctcctttcctgt	Mixed between donors
MVE	Myeloid differentiation primary response 88 (MyD88)	Down	Ccctcctcacaggttcagtccta gagcatggcacaaccaagaaaaga	Yes
MVE	Epithelial stromal interaction 1 (EPSTI 1)	Up	ccaggttggagtacagtggcatga tacaacaattagctgggcgtggtg	Yes
MVE	Interferon alpha inducible protein 6 (IFI 6)	Up	cagcaggcagcacacaaatgttc agccaatccctgtcggagtttcta	Yes
MVE	Ring finger protein 213 (RNF213)	Up	ggatgaaggggtgagaggagaagg gtgtgaccagggacctccgtttta	Yes

We are excited by the results from MERS-CoV infected MVE that suggest epigenetic regulation controls ISG expression in endothelial as well as epithelial lung cells. We anticipate similar findings from MERS-CoV infected fibroblasts and look forward to expanding our understanding of which enzymes that modify chromatin structure contribute to the regulation of the innate immune response following MERS-CoV infection.

B.3 Competitive Revisions/Administrative Supplements
Not applicable

B.4 What opportunities for training and professional development had the project provided?
Nothing to report

B.5 How have results been disseminated to communities of interest?
No

B.6 What do you plan to do for the next reporting period to accomplish the goals?
Prior to the end of the funding period we anticipate completing the following. Experiments to determine if histone modification plays a role in ISG differential expression in MERS-CoV infected FB will begin as soon as the shelter in place bans have been removed as all samples have been harvested and are in the freezer awaiting analysis. Existing lysate (from both MVE and FB) will also be used to explore if transcription factors contribute to regulation of ISG expression following MERS-CoV infection. Experiments to determine if the genes responsible for modifying chromatin structure are differentially expressed following MERS-CoV infection of primary human lung MVE and FB will be performed as described in Aim 2 using BioRad's PrimerPCR Pathway arrays. To determine if inhibition of enzymes that mediate chromatin structure rearrangement alters coronavirus replication and or changes the innate immune response to virus, drug efficacy studies will be performed, and infected treated samples will be assayed to determine levels of viral replication and cellular viability. If changes are detected in levels of viral replication additional samples will be generated to determine how ISG expression has been altered. We anticipate obtaining sufficient preliminary data for the submission of an RO1 by fall of 2021.

6.3 Section C -Products

C.1 Publications
Nothing to report

C.2 Websites or other internet sites
Nothing to report

C.3 technologies or techniques
Nothing to report

C.4 inventions, patent applications, and/or licenses
Nothing to report

C.5 Other products and resources
C.5.a Other products
Nothing to report

C.5.b Resource Sharing
Nothing to report

6.4 Section D Participants

D.1 What individuals have worked on the project?

Program Director/Principal Investigator (Last, First, Middle):

ALL PERSONNEL REPORT

Place this form at the end of the signed original copy of the application. Do not duplicate.

GRANT NUMBER

Always list the PD/PI(s). In addition, list all other personnel who participated in the project during the current budget period for at least one person month or more, regardless of the source of compensation (a person month equals approximately 160 hours or 8.3% of annualized effort). Use the following abbreviated categories for describing Role on Project:

- PD/PI*
- Co-Investigator
- Faculty
- Postdoctoral (scholar, fellow, or other postdoctoral position)*
- Technician
- Staff Scientist (doctoral level)
- Statistician
- Graduate Student (research assistant)
- Non-student Research Assistant
- Undergraduate Student
- High School Student
- Consultant
- Other (please specify)

If personnel are supported by a Reentry or Diversity Supplement please indicate such after the Role on Project, using the following abbreviations: RS - Reentry Supplement; DS - Diversity Supplement.

***Commons ID required for any personnel holding this Role on Project and for all individuals supported by a Reenty or Diversity Supplement. The Commons ID will be required in the future for all individuals with a graduate student, or undergraduate role. The Commons ID is strongly encouraged, but not required, for all other Project Personnel.**

Use Cal (calendar), Acad, or Summer to enter months devoted to project.

Commons ID*	Name	Degree(s)	SSN (last 4 digits)	Role on Project	DoB (MM /YY)	Cal	Acad	Summer

From: Sims, Amy C [amy.sims@pnnl.gov]
Sent: 4/9/2020 2:13:18 PM
To: Caldwell, Chandra [ccaldwel@email.unc.edu]
Subject: Re: Science for R21 progress report

For this year it is just me. It took so long to get things transitioned that it seemed like a safer bet.

Thank you very much, Amy

From: "Caldwell, Chandra" <ccaldwel@email.unc.edu>
Date: Thursday, April 9, 2020 at 12:25 PM
To: "Sims, Amy C" <amy.sims@pnnl.gov>
Subject: RE: Science for R21 progress report

One more thing, are you the only one being paid from these funds at PNNL/Battelle? If yes, I will just wait to receive your other support next week to denote the amount of effort you are contributing on the project. If the answer is no, I will need the attached personnel form completed to account for everyone outside of you.

Thanks,
C.

From: Caldwell, Chandra
Sent: Thursday, April 9, 2020 3:12 PM
To: Sims, Amy C <amy.sims@pnnl.gov>
Subject: RE: Science for R21 progress report

Thanks for checking on it!

From: Sims, Amy C <amy.sims@pnnl.gov>
Sent: Thursday, April 9, 2020 3:08 PM
To: Caldwell, Chandra <ccaldwel@email.unc.edu>
Subject: Re: Science for R21 progress report

Hi,

I generated one but sent it to my administrative people at their request.

I will make sure you get it! Thank you, Amy

From: "Caldwell, Chandra" <ccaldwel@email.unc.edu>
Date: Thursday, April 9, 2020 at 12:03 PM
To: "Sims, Amy C" <amy.sims@pnnl.gov>
Subject: RE: Science for R21 progress report

Thanks for sending the science, etc. and the only thing missing is your other support document!

From: Sims, Amy C <amy.sims@pnnl.gov>
Sent: Monday, April 6, 2020 2:27 PM
To: Caldwell, Chandra <ccaldwel@email.unc.edu>

Cc: Sheahan, Timothy Patrick <sheahan@email.unc.edu>; Sandoval, Norma I <Norma.Sandoval@pnnl.gov>
Subject: Science for R21 progress report

Hi Chandra,

I typed out a template and used it for the progress report. If this means I missed any of your sections please let me know. I know you will.

I have included the science accomplishments as a separate document as you requested.

Tim, right now I have the direct dollars broken up for year 2 as follows.

UNC direct dollars 5 U.S.C. § 552(b)(4)

PNNL direct dollars

For now this is FYI only as Chandra is going to wait until after the progress report gets done to work on Year 2 budget issues.

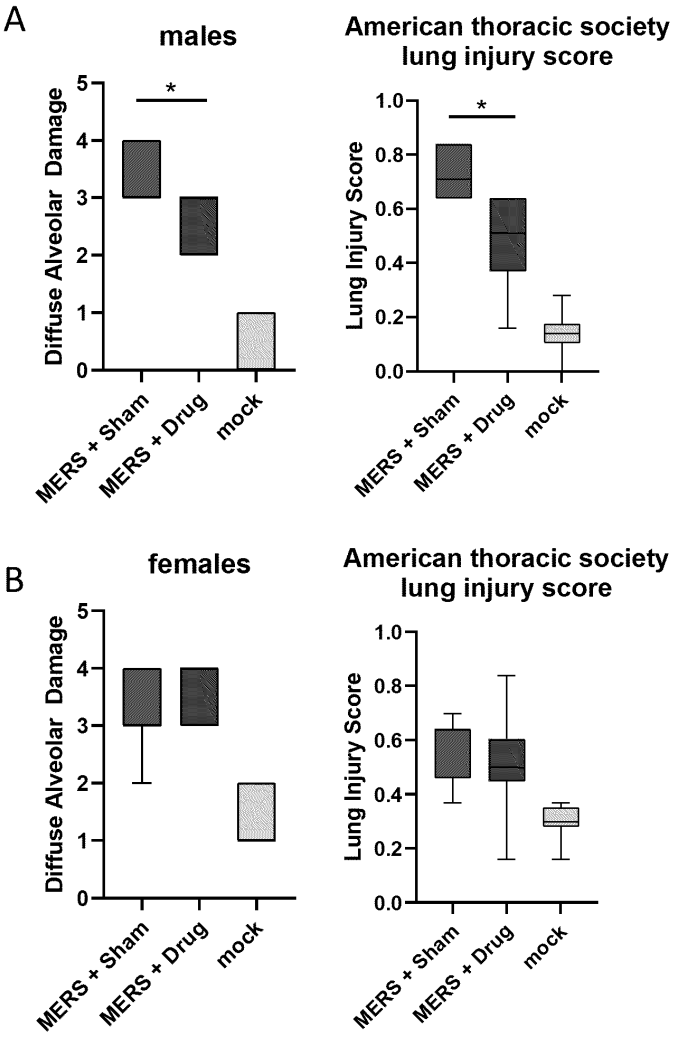
Please let me know if you have any questions or if I missed anything.

Thank you very much, Amy

From: Gralinski, Lisa E[lgralins@email.unc.edu]
Sent: Thur 4/9/2020 3:34:04 PM (UTC-07:00)
To: Sims, Amy C[amy.sims@pnnl.gov]
Subject: figure with scale bar
Attachment: sm126 histo figure w scale bar.pdf

Scale bar added now! Let me know if you need anything else.
thanks,
Lisa

Lisa Gralinski
Assistant Professor
Department of Epidemiology
University of North Carolina
(b) (6)


A
males
Diffuse Alveolar Damage
American thoracic society lung injury score
Lung Injury Score
MERS + Sham
MERS + Drug
mock
B
females
American thoracic society lung injury score
Diffuse Alveolar Damage
Lung Injury Score
MERS + Sham
MERS + Drug
mock

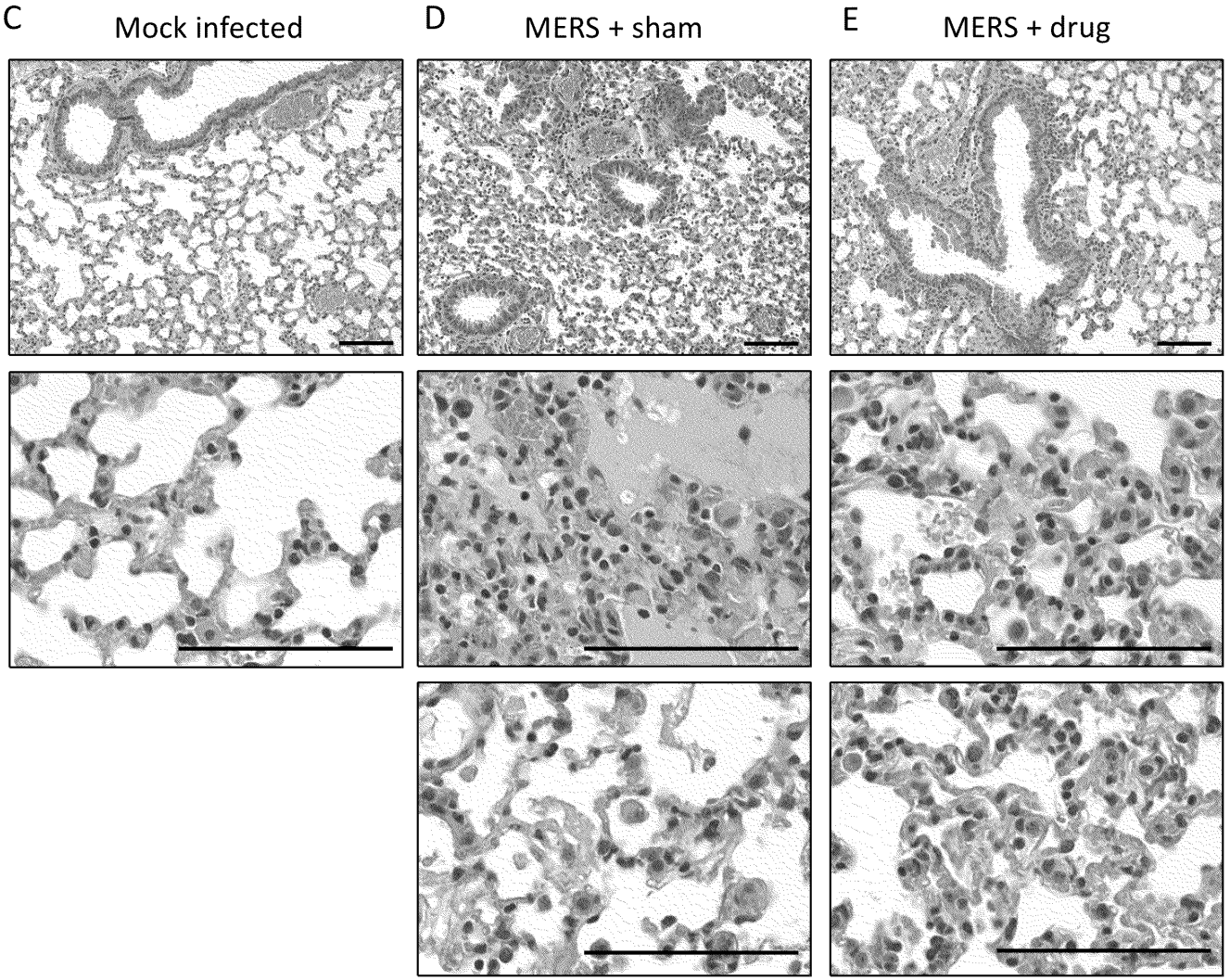

C
Mock infected
D
MERS + sham
E
MERS + drug

Figure Legend. The histological features of acute lung injury were blindly scored using the American Thoracic Society Lung Injury Scoring system by Batute-Bello, creating an aggregate score for: neutrophils in the alveolar and interstitial space, hyaline membranes, proteinaceous debris filling the air spaces, and alveolar septal thickening. A) and B) Scoring for acute lung injury and diffuse alveolar damage in male and female mice, respectively. Representative lung pathology for C) mock infected, D) MERS-CoV infected and sham treated and E) MERS-CoV infected and PERK'AMG treated male mice at 7 dpi. Scale bar indicates 100μm * indicates a p value of <0.001.

From: Sims, Amy C [amy.sims@pnnl.gov]
Sent: 4/9/2020 3:37:00 PM
To: Gralinski, Lisa E [lgralins@email.unc.edu]
Subject: Re: figure with scale bar

Awesome! Thank you so much. Katrina just asked me if I submitted the paper! Thank you again, Amy

From: "Gralinski, Lisa E" <lgralins@email.unc.edu>
Date: Thursday, April 9, 2020 at 3:34 PM
To: "Sims, Amy C" <amy.sims@pnnl.gov>
Subject: figure with scale bar

Scale bar added now! Let me know if you need anything else.
thanks,
Lisa

Lisa Gralinski
Assistant Professor
Department of Epidemiology
University of North Carolina

5 U.S.C. § 552(b)(6)

From: Sandoval, Norma I[Norma.Sandoval@pnnl.gov]
Sent: Thur 4/9/2020 3:55:13 PM (UTC-07:00)
To: Caldwell, Chandra[ccaldwel@email.unc.edu]
Cc: Sims, Amy C[amy.sims@pnnl.gov]; Sandoval, Norma I[Norma.Sandoval@pnnl.gov]
Subject: PNNL - Other Support (A. Sims) - 75881
Attachment: Sims_Other Support_April_2020.docx

Hi Chandra,
Attached is the other support document for Amy. Note this only includes Amy's other support. When the subcontract paperwork was submitted to you/UNC we included Katrina Waters as Key Personnel (0.0 CM), however she will not be listed as part of Year 2. Do you need us to provide her 'Other support'?

Also, I can confirm that Amy's effort on the grant (via the subcontract at PNNL) is estimated to be ~1.7 CM. Including her effort at UNC prior to transition to PNNL I believe her effort combined meets the proposed calendar months. I am not sure how you want to list this in the progress report, so I will leave this up to you.

Please let me know if you need anything else from PNNL for the progress report.

Thx.

Norma Sandoval
Principal Project Coordinator
Biological Sciences Division, **EBSD**

Pacific Northwest National Laboratory
902 Battelle Boulevard
P.O. Box 999, MSIN J4-18
Richland, WA 99352 USA
Tel: 509-371-6553
norma.sandoval@pnnl.gov
www.pnnl.gov

From: Sims, Amy C [amy.sims@pnnl.gov]
Sent: 4/9/2020 4:01:14 PM
To: Sandoval, Norma I [Norma.Sandoval@pnnl.gov]; Caldwell, Chandra [ccaldwel@email.unc.edu]
Subject: Re: PNNL - Other Support (A. Sims) - 75881

Thank you, Norma. I really appreciate it.

Norma and Chandra if I don't email you tomorrow Happy Easter! Amy

---

From: "Sandoval, Norma I" <Norma.Sandoval@pnnl.gov>
Date: Thursday, April 9, 2020 at 3:55 PM
To: "Caldwell, Chandra" <ccaldwel@email.unc.edu>
Cc: "Sims, Amy C" <amy.sims@pnnl.gov>, "Sandoval, Norma I" <Norma.Sandoval@pnnl.gov>
Subject: PNNL - Other Support (A. Sims) - 75881

Hi Chandra,
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Please let me know if you need anything else from PNNL for the progress report.

Thx.

Norma Sandoval
Principal Project Coordinator
Biological Sciences Division, **EBSD**

Pacific Northwest National Laboratory
902 Battelle Boulevard
P.O. Box 999, MSIN J4-18
Richland, WA 99352 USA
Tel: 509-371-6553
norma.sandoval@pnnl.gov
www.pnnl.gov

From: deBruin, Christine[cdebruin@atcc.org]
Sent: Fri 4/10/2020 5:21:32 AM (UTC-07:00)
To: Rashid, Sujatha[srashid@atcc.org]; Sheahan, Timothy Patrick[sheahan@email.unc.edu]
Cc: Sims, Amy C[amy.sims@pnnl.gov]; Baric, Ralph S[rbaric@email.unc.edu]
Subject: RE: Greetings from BEI Resources!

Hello Tim,

I hope you are doing well. I was checking in to see if there are any update to characterizing the clone. Thanks!

ATCC

Christine deBruin, M.B.A., PMP®
Project Coordinator

10801 University Boulevard
Manassas, VA 20110-2209
Tel: (703) 365-2700 ext. 2438
Fax: (703) 365-2701
Email: cdebruin@atcc.org

From: Rashid, Sujatha <srashid@atcc.org>
Sent: Friday, March 20, 2020 12:04 PM
To: Sheahan, Timothy Patrick <sheahan@email.unc.edu>
Cc: Sims, Amy C <amy.sims@pnnl.gov>; Baric, Ralph S <rbaric@email.unc.edu>; deBruin, Christine <cdebruin@atcc.org>
Subject: RE: Greetings from BEI Resources!

Thank you, Amy!

I do recall meeting you, Tim. Thank you for the update, I will pass this information on to NIAID.

Best regards,
Sujatha

Sujatha Rashid, MS, PhD, PMP®
Program Manager, ATCC Federal Solutions
Virology Team Lead, BEI Resources

10801 University Boulevard
Manassas, VA 20110-2209
Tel: (703) 365-2700 ext. 2660

From: Sheahan, Timothy Patrick <sheahan@email.unc.edu>
Sent: Friday, March 20, 2020 11:54 AM
To: Rashid, Sujatha <srashid@atcc.org>
Cc: Sims, Amy C <amy.sims@pnnl.gov>; Baric, Ralph S <rbaric@email.unc.edu>
Subject: Re: Greetings from BEI Resources!

Hi Sujatha,

I think we met at ASM Biothreats. I can be your contact for this. Right now, we are still characterizing this clone. When we are sure of how this reagent behaves, we can talk about deposition at BEI. Talk soon,

Tim

From: Amy Sims <amy.sims@pnnl.gov>
Date: Friday, March 20, 2020 at 11:38 AM
To: "Baric, Ralph S" <rbaric@email.unc.edu>, "Sheahan, Timothy Patrick" <sheahan@email.unc.edu>
Subject: FW: Greetings from BEI Resources!

Please the email below from BEI Resources. Sujatha is really great to work with! I can provide examples of the paperwork to assist with what level of details are needed if you are going to provide the reagent to them for distribute.

Tim suggested that Ben would be someone likely to assist with this but I don't I remember his last name or have his email address now that I don't have access to my UNC account.

Hope things are going well. Thank you, Amy

From: "Rashid, Sujatha" <srashid@atcc.org>
Date: Tuesday, March 17, 2020 at 7:18 AM
To: "Sims, Amy C" <amy.sims@pnnl.gov>
Cc: "deBruin, Christine" <cdebruin@atcc.org>
Subject: RE: Greetings from BEI Resources!

Hi Amy
Dr. Baric was supposed to give us an ic of Wuhan isolate according to NIAID program offoicer. Can you give another lab contact? so I can work on it like I used to keep pinging you 😊
Thanks
Best regards,
Sujatha

Sujatha Rashid, MS, PhD, PMP®
Program Manager, ATCC Federal Solutions
Virology Team Lead, BEI Resources

10801 University Boulevard
Manassas, VA 20110-2209
Tel: (703) 365-2700 ext. 2660

From: Sheahan, Timothy Patrick[sheahan@email.unc.edu]
Sent: Fri 4/10/2020 6:06:29 AM (UTC-07:00)
To: deBruin, Christine[cdebruin@atcc.org]; Rashid, Sujatha[srashid@atcc.org]
Cc: Sims, Amy C[amy.sims@pnnl.gov]; Baric, Ralph S[rbaric@email.unc.edu]
Subject: Re: Greetings from BEI Resources!

Hi Christine,

We are trying to finalize a manuscript for this paper. I would defer to Ralph as to when we would transfer reagents to BEI.
Thanks!

Tim

From: "deBruin, Christine" <cdebruin@atcc.org>
Date: Friday, April 10, 2020 at 8:21 AM
To: "Rashid, Sujatha" <srashid@atcc.org>, "Sheahan, Timothy Patrick" <sheahan@email.unc.edu>
Cc: Amy Sims <amy.sims@pnnl.gov>, "Baric, Ralph S" <rbaric@email.unc.edu>
Subject: RE: Greetings from BEI Resources!

Hello Tim,

I hope you are doing well. I was checking in to see if there are any update to characterizing the clone. Thanks!

ATCC

Christine deBruin, M.B.A., PMP®
Project Coordinator

10801 University Boulevard
Manassas, VA 20110-2209
Tel: (703) 365-2700 ext. 2438
Fax: (703) 365-2701
Email: cdebruin@atcc.org

From: Rashid, Sujatha <srashid@atcc.org>
Sent: Friday, March 20, 2020 12:04 PM
To: Sheahan, Timothy Patrick <sheahan@email.unc.edu>
Cc: Sims, Amy C <amy.sims@pnnl.gov>; Baric, Ralph S <rbaric@email.unc.edu>; deBruin, Christine <cdebruin@atcc.org>
Subject: RE: Greetings from BEI Resources!

Thank you, Amy!

I do recall meeting you, Tim. Thank you for the update, I will pass this information on to NIAID.

Best regards,
Sujatha

Sujatha Rashid, MS, PhD, PMP®
Program Manager, ATCC Federal Solutions
Virology Team Lead, BEI Resources

10801 University Boulevard

Manassas, VA 20110-2209
Tel: (703) 365-2700 ext. 2660

From: Sheahan, Timothy Patrick <sheahan@email.unc.edu>
Sent: Friday, March 20, 2020 11:54 AM
To: Rashid, Sujatha <srashid@atcc.org>
Cc: Sims, Amy C <amy.sims@pnnl.gov>; Baric, Ralph S <rbaric@email.unc.edu>
Subject: Re: Greetings from BEI Resources!

Hi Sujatha,

I think we met at ASM Biothreats. I can be your contact for this. Right now, we are still characterizing this clone. When we are sure of how this reagent behaves, we can talk about deposition at BEI. Talk soon,

Tim

From: Amy Sims <amy.sims@pnnl.gov>
Date: Friday, March 20, 2020 at 11:38 AM
To: "Baric, Ralph S" <rbaric@email.unc.edu>, "Sheahan, Timothy Patrick" <sheahan@email.unc.edu>
Subject: FW: Greetings from BEI Resources!

Please the email below from BEI Resources. Sujatha is really great to work with! I can provide examples of the paperwork to assist with what level of details are needed if you are going to provide the reagent to them for distribute.

Tim suggested that Ben would be someone likely to assist with this but I don't I remember his last name or have his email address now that I don't have access to my UNC account.

Hope things are going well. Thank you, Amy

From: "Rashid, Sujatha" <srashid@atcc.org>
Date: Tuesday, March 17, 2020 at 7:18 AM
To: "Sims, Amy C" <amy.sims@pnnl.gov>
Cc: "deBruin, Christine" <cdebruin@atcc.org>
Subject: RE: Greetings from BEI Resources!

Hi Amy
Dr. Baric was supposed to give us an ic of Wuhan isolate according to NIAID program offoicer. Can you give another lab contact? so I can work on it like I used to keep pinging you 😊
Thanks
Best regards,
Sujatha

Sujatha Rashid, MS, PhD, PMP®
Program Manager, ATCC Federal Solutions
Virology Team Lead, BEI Resources

10801 University Boulevard
Manassas, VA 20110-2209
Tel: (703) 365-2700 ext. 2660

From: Sheahan, Timothy Patrick[sheahan@email.unc.edu]

Sent: Mon 4/13/2020 1:53:15 PM (UTC-07:00)

To: Sims, Amy C[amy.sims@pnnl.gov]

Subject: FW: STM Editors Inbox: Acceptance of your Science Translational Medicine Manuscript abb5883

Get another spot open on your office wall because we got the cover again!

On 4/13/20, 1:48 PM, "Lindsey Pujanandez" <lpujanandez@aaas.org> wrote:

Thanks for the note!

We're working on it __.

Also, you will be getting galley proofs at the end of this week for the fully copyedited paper that will appear in an upcoming issue. With the cover.

On 4/3/20, 4:46 PM, "Science Translational Medicine Editors" <scitranslmededitors@aaas.org> wrote:

FYI

-----Original Message-----
From: Sheahan, Timothy Patrick <sheahan@email.unc.edu>
Sent: Thursday, April 02, 2020 10:20 PM
To: Science Translational Medicine Editors <scitranslmededitors@aaas.org>
Subject: Re: Acceptance of your Science Translational Medicine Manuscript abb5883

Thank you Dr. P.
Another great paper. Thank you for your editorial expertise. You are clearly good at this. Perhaps we can get the cover again!? Ha! Our first one is hung up in my office. Thanks again

Tim

> On Apr 2, 2020, at 10:00 PM, Lindsey Pujanandez <scitranslmededitors@aaas.org> wrote:
>

From: Sims, Amy C [amy.sims@pnnl.gov]
Sent: 4/13/2020 1:55:03 PM
To: Sheahan, Timothy Patrick [sheahan@email.unc.edu]
Subject: Re: STM Editors Inbox: Acceptance of your Science Translational Medicine Manuscript abb5883

Ha! Congratulations! That is fantastic news Dr. Sheahan. Thanks for making time to share!

On 4/13/20, 1:53 PM, "Sheahan, Timothy Patrick" <sheahan@email.unc.edu> wrote:

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>

From: Sheahan, Timothy Patrick[sheahan@email.unc.edu]
Sent: Mon 4/13/2020 1:58:33 PM (UTC-07:00)
To: Sims, Amy C[amy.sims@pnnl.gov]
Subject: Re: STM Editors Inbox: Acceptance of your Science Translational Medicine Manuscript abb5883

I'll make it up like I did the last time and send you a pic to print. Although I am busy, I like to think I always have time for you but I guess that's not true since I don't have enough time for my kids apparently!

On 4/13/20, 4:55 PM, "Sims, Amy C" <amy.sims@pnnl.gov> wrote:

Ha! Congratulations! That is fantastic news Dr. Sheahan. Thanks for making time to share!

On 4/13/20, 1:53 PM, "Sheahan, Timothy Patrick" <sheahan@email.unc.edu> wrote:

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Tim

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>

From: Sheahan, Timothy Patrick[sheahan@email.unc.edu]

Sent: Mon 4/13/2020 2:03:09 PM (UTC-07:00)

To: Sims, Amy C[amy.sims@pnnl.gov]

Subject: Re: STM Editors Inbox: Acceptance of your Science Translational Medicine Manuscript abb5883

Hi Aims,

This was not a problem for the EIDD paper. I remember it being one for our first one though. Their website says something about what is included but I don't remember. For a first submission, I would not really go crazy about length of the body of the paper. The abstract and title does have to be in the limits because you paste them into a box in the submission site.

T

On 4/13/20, 4:57 PM, "Sims, Amy C" <amy.sims@pnnl.gov> wrote:

Stupid question. What do you count for STM when you do the 10,000 word limit? If you do the initials in the acknowledgments like this A.C.S. it is three words!

Going nuts. Thank you, Amy

On 4/13/20, 1:53 PM, "Sheahan, Timothy Patrick" <sheahan@email.unc.edu> wrote:

Get another spot open on your office wall because we got the cover again!

On 4/13/20, 1:48 PM, "Lindsey Pujanandez" <lpujanandez@aaas.org> wrote:

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> On Apr 2, 2020, at 10:00 PM, Lindsey Pujanandez <scitranslmededitors@aaas.org> wrote:
>

From: deBruin, Christine[cdebruin@atcc.org]
Sent: Mon 4/20/2020 8:19:42 AM (UTC-07:00)
To: Sheahan, Timothy Patrick[sheahan@email.unc.edu]; Rashid, Sujatha[srashid@atcc.org]
Cc: Sims, Amy C[amy.sims@pnnl.gov]; Baric, Ralph S[rbaric@email.unc.edu]
Subject: RE: Greetings from BEI Resources!

Hello Dr. Baric,

I hope this email finds you well. I saw checking to see if you would still be willing to deposit the Wuhan isolate with BEI resources. Please let me know and I can arrange the paperwork. Thanks!

ATCC

Christine deBruin, M.B.A., PMP®
Project Coordinator

10801 University Boulevard
Manassas, VA 20110-2209
Tel: (703) 365-2700 ext. 2438
Fax: (703) 365-2701
Email: cdebruin@atcc.org

From: Sheahan, Timothy Patrick <sheahan@email.unc.edu>
Sent: Friday, April 10, 2020 9:06 AM
To: deBruin, Christine <cdebruin@atcc.org>; Rashid, Sujatha <srashid@atcc.org>
Cc: Sims, Amy C <amy.sims@pnnl.gov>; Baric, Ralph S <rbaric@email.unc.edu>
Subject: Re: Greetings from BEI Resources!

Hi Christine,

We are trying to finalize a manuscript for this paper. I would defer to Ralph as to when we would transfer reagents to BEI.
Thanks!

Tim

From: "deBruin, Christine" <cdebruin@atcc.org>
Date: Friday, April 10, 2020 at 8:21 AM
To: "Rashid, Sujatha" <srashid@atcc.org>, "Sheahan, Timothy Patrick" <sheahan@email.unc.edu>
Cc: Amy Sims <amy.sims@pnnl.gov>, "Baric, Ralph S" <rbaric@email.unc.edu>
Subject: RE: Greetings from BEI Resources!

Hello Tim,

I hope you are doing well. I was checking in to see if there are any update to characterizing the clone. Thanks!

ATCC

Christine deBruin, M.B.A., PMP®
Project Coordinator

10801 University Boulevard
Manassas, VA 20110-2209
Tel: (703) 365-2700 ext. 2438
Fax: (703) 365-2701
Email: cdebruin@atcc.org

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To: Sheahan, Timothy Patrick <sheahan@email.unc.edu>
Cc: Sims, Amy C <amy.sims@pnnl.gov>; Baric, Ralph S <rbaric@email.unc.edu>; deBruin, Christine <cdebruin@atcc.org>
Subject: RE: Greetings from BEI Resources!

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Sujatha

Sujatha Rashid, MS, PhD, PMP®
Program Manager, ATCC Federal Solutions
Virology Team Lead, BEI Resources

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Tel: (703) 365-2700 ext. 2660

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To: Rashid, Sujatha <srashid@atcc.org>
Cc: Sims, Amy C <amy.sims@pnnl.gov>; Baric, Ralph S <rbaric@email.unc.edu>
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From: Amy Sims <amy.sims@pnnl.gov>
Date: Friday, March 20, 2020 at 11:38 AM
To: "Baric, Ralph S" <rbaric@email.unc.edu>, "Sheahan, Timothy Patrick" <sheahan@email.unc.edu>
Subject: FW: Greetings from BEI Resources!

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Tim suggested that Ben would be someone likely to assist with this but I don't I remember his last name or have his email address now that I don't have access to my UNC account.

Hope things are going well. Thank you, Amy

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Date: Tuesday, March 17, 2020 at 7:18 AM
To: "Sims, Amy C" <amy.sims@pnnl.gov>

Cc: "deBruin, Christine" <cdebruin@atcc.org>
Subject: RE: Greetings from BEI Resources!

Hi Amy
Dr. Baric was supposed to give us an ic of Wuhan isolate according to NIAID program offoicer. Can you give another lab contact? so I can work on it like I used to keep pinging you 😊
Thanks
Best regards,
Sujatha

Sujatha Rashid, MS, PhD, PMP®
Program Manager, ATCC Federal Solutions
Virology Team Lead, BEI Resources

10801 University Boulevard
Manassas, VA 20110-2209
Tel: (703) 365-2700 ext. 2660

From: McManus, Julie Clarke[jnoel@email.unc.edu]
Sent: Mon 4/20/2020 11:53:53 AM (UTC-07:00)
To: Sims, Amy C[amy.sims@pnnl.gov]
Subject: Delivery today

Hi Amy,

This was just delivered for you. Please let me know where you'd like it left for you?

Thanks,

Julie McManus
Office of Planning and Construction
Gillings School of Global Public Health
The University of North Carolina at Chapel Hill
204 Rosenau Hall, CB #7400
Chapel Hill, NC 27599-7400

Phone: 919-843-7872
https://sph.unc.edu/SPH-facilities/

From: Baric, Ralph S[rbaric@email.unc.edu]
Sent: Tue 4/21/2020 1:29:09 PM (UTC-07:00)
To: Sims, Amy C[amy.sims@pnnl.gov]; Baric, Toni C[antoinette_baric@med.unc.edu]
Subject: RE: Time to talk

Hi Amy, I look forward to chatting. Ralph

From: Sims, Amy C <amy.sims@pnnl.gov>
Sent: Tuesday, April 21, 2020 4:28 PM
To: Baric, Ralph S <rbaric@email.unc.edu>; Baric, Toni C <antoinette_baric@med.unc.edu>
Subject: Time to talk

Ralph,

Can you and Toni find an hour in your crazy schedule so that we can chat, please?

If you could give me some time windows I will do my best to make them work.

Thank you very much, Amy

From: Baric, Toni C[antoinette_baric@med.unc.edu]
Sent: Tue 4/21/2020 1:30:54 PM (UTC-07:00)
To: Sims, Amy C[amy.sims@pnnl.gov]; Baric, Ralph S[rbaric@email.unc.edu]
Subject: RE: Time to talk

Amy,
Ralph has time on Thursday 4/23 between 10:30-1 pm ET. Does that work for you?
Toni

From: Sims, Amy C <amy.sims@pnnl.gov>
Sent: Tuesday, April 21, 2020 4:28 PM
To: Baric, Ralph S <rbaric@email.unc.edu>; Baric, Toni C <antoinette_baric@med.unc.edu>
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Thank you very much, Amy

From: Baric, Toni C[antoinette_baric@med.unc.edu]
Sent: Tue 4/21/2020 1:43:47 PM (UTC-07:00)
To: Sims, Amy C[amy.sims@pnnl.gov]; Baric, Ralph S[rbaric@email.unc.edu]
Subject: RE: Time to talk

Please call cell. 5 U.S.C. § 552(b)(6)

From: Sims, Amy C <amy.sims@pnnl.gov>
Sent: Tuesday, April 21, 2020 4:42 PM
To: Baric, Toni C <antoinette_baric@med.unc.edu>; Baric, Ralph S <rbaric@email.unc.edu>
Subject: Re: Time to talk

Many thanks for the rapid responses.

I can talk from 11am to noon eastern, 8am to 9am pacific. Thank you again! Amy

Amy PNNL/Skype number 509-372-6496 please call this number or I can call your office if you prefer

---

From: "Baric, Toni C" <antoinette_baric@med.unc.edu>
Date: Tuesday, April 21, 2020 at 1:31 PM
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Thank you very much, Amy

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Sent: 4/21/2020 1:49:09 PM
To: Baric, Toni C [antoinette_baric@med.unc.edu]; Baric, Ralph S [rbaric@email.unc.edu]
Subject: Re: Time to talk

Ah, I keep forgetting. I will call his cell instead.

Thank you, Amy

From: "Baric, Toni C" <antoinette_baric@med.unc.edu>
Date: Tuesday, April 21, 2020 at 1:44 PM
To: "Sims, Amy C" <amy.sims@pnnl.gov>, "Baric, Ralph S" <rbaric@email.unc.edu>
Subject: RE: Time to talk

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If you could give me some time windows I will do my best to make them work.

Thank you very much, Amy

From: Sims, Amy C[amy.sims@pnnl.gov]
Sent: Thur 4/23/2020 2:33:14 PM (UTC-07:00)
To: Gralinski, Lisa E[lgralins@email.unc.edu]
Subject: Iacuc protocol number for the inhibitor studies please

Hi Lisa,

Can you please tell me the IACUC protocol number for the work you did for the MVE apoptosis manuscript??

Thank you very much! Amy

PS. Ralph mentioned you might have time to try the inhibitor in some in vivo SARS-COV 2 studies. That would be amazing. I hope you, Mike and kitty are doing well.

From: Gralinski, Lisa E[lgralins@email.unc.edu]
Sent: Sat 4/25/2020 2:04:07 PM (UTC-07:00)
To: Sims, Amy C[amy.sims@pnnl.gov]
Subject: RE: Iacuc protocol number for the inhibitor studies please

Hey Amy,
The IACUC protocol was 17-294. I ordered some more inhibitor but am actually not sure if it has come in yet or not. I should check on that!
thanks,
Lisa

From: Sims, Amy C <amy.sims@pnnl.gov>
Sent: Thursday, April 23, 2020 5:33 PM
To: Gralinski, Lisa E <lgralins@email.unc.edu>
Subject: Iacuc protocol number for the inhibitor studies please

Hi Lisa,

Can you please tell me the IACUC protocol number for the work you did for the MVE apoptosis manuscript??

Thank you very much! Amy

PS. Ralph mentioned you might have time to try the inhibitor in some in vivo SARS-COV 2 studies. That would be amazing. I hope you, Mike and kitty are doing well.

From: Sims, Amy C[amy.sims@pnnl.gov]
Sent: Mon 4/27/2020 4:37:41 PM (UTC-07:00)
To: Caldwell, Chandra[ccaldwel@email.unc.edu]
Subject: FW: 76382 Relinquish NIH Grant status

Chandra,

Just keeping you in the loop. I sent an email last week and no one responded. Any idea of when this might move forward would be great. Thank you, Amy

---

From: "Hays, Barbara A" <barb.hays@pnnl.gov>
Date: Monday, April 27, 2020 at 4:34 PM
To: "Moore, Victoria L" <victoria_moore@unc.edu>, "Parrish, Kalyn" <klparrish@unc.edu>
Cc: "Hays, Barbara A" <barb.hays@pnnl.gov>, "Sims, Amy C" <amy.sims@pnnl.gov>, "Sandoval, Norma I" <Norma.Sandoval@pnnl.gov>
Subject: 76382 Relinquish NIH Grant status

Kalyn and Vicki, I'll be your contracts point of contact to support Amy and Norma with the transfer of Amy's NIH Grant from UNC to PNNL.

- What is your planned timeline for the UNC relinquishment request to NIH?
- What do you need from us and when?

I assume I will receive a request for proposal from NIH at some point? Any guidance you can give us to get our proposal documents ready would be much appreciated. We look forward to hearing from you.
Thank you

Barb Hays
Senior Contracts Specialist
Pacific Northwest National Laboratory
902 Battelle Boulevard
P.O. Box 999
Richland, WA 99352 USA
Tel: 509-372-4008

barb.hays@pnnl.gov

From: Sims, Amy C[amy.sims@pnnl.gov]
Sent: Mon 4/27/2020 6:39:40 PM (UTC-07:00)
To: Caldwell, Chandra[ccaldwel@email.unc.edu]
Subject: Re: 76382 Relinquish NIH Grant status

You rock!! I never know when to include Carol.

Thank you very much. Amy

Get Outlook for iOS

From: Caldwell, Chandra <ccaldwel@email.unc.edu>
Sent: Monday, April 27, 2020 5:44 PM
To: Sims, Amy C
Subject: Re: 76382 Relinquish NIH Grant status

Hey Amy,

So as luck would have it, I gave Carol a heads up that she would need to connect with Vicki to get this grant relinquished ASAP since you need to submit a admin supplement as the PI. She didn't say anything about my email. I didn't speak to V today since she was busy getting ready for a conference call with Ralph, Tim and Til. If you don't hear anything by tomorrow, I would send a reminder (including Carol on the email) because Carol can do this task - unlike Kelly, she just won't do it without V giving the green light....why I do not know. If I speak to V tomorrow (or this week), I will remind her as well since I don't know what the delay is when it is just the click of a few buttons in eRA Commons to submit the relinquish request.

Thanks,
C.

From: Sims, Amy C <amy.sims@pnnl.gov>
Sent: Monday, April 27, 2020 7:37:41 PM
To: Caldwell, Chandra <ccaldwel@email.unc.edu>
Subject: FW: 76382 Relinquish NIH Grant status

Chandra,

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---

From: "Hays, Barbara A" <barb.hays@pnnl.gov>
Date: Monday, April 27, 2020 at 4:34 PM
To: "Moore, Victoria L" <victoria_moore@unc.edu>, "Parrish, Kalyn" <klparrish@unc.edu>
Cc: "Hays, Barbara A" <barb.hays@pnnl.gov>, "Sims, Amy C" <amy.sims@pnnl.gov>, "Sandoval, Norma I" <Norma.Sandoval@pnnl.gov>
Subject: 76382 Relinquish NIH Grant status

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Senior Contracts Specialist
Pacific Northwest National Laboratory
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P.O. Box 999
Richland, WA 99352 USA
Tel: 509-372-4008

barb.hays@pnnl.gov

From: Baric, Ralph S[rbaric@email.unc.edu]
Sent: Tue 4/28/2020 7:50:16 AM (UTC-07:00)
To: Sims, Amy C[amy.sims@pnnl.gov]
Cc: Sheahan, Timothy Patrick[sheahan@email.unc.edu]
Subject: FW: Coronavirus / host kinase paper

Amy, I think Cell is interested in your paper! Go for it! Ralph

---

From: Rennekamp, Andrew (ELS-CMA) <arennekamp@cell.com>
Sent: Tuesday, April 28, 2020 10:10 AM
To: Baric, Ralph S <rbaric@email.unc.edu>
Subject: Coronavirus / host kinase paper

Hi Ralph,

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Best,
-Andrew

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Andrew J. Rennekamp, Ph.D.
Scientific Editor, *Cell*

From: Sheahan, Timothy Patrick[sheahan@email.unc.edu]
Sent: Tue 4/28/2020 7:51:01 AM (UTC-07:00)
To: Baric, Ralph S[rbaric@email.unc.edu]; Sims, Amy C[amy.sims@pnnl.gov]
Subject: Re: Coronavirus / host kinase paper

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Date: Tuesday, April 28, 2020 at 10:50 AM
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Sent: Tue 4/28/2020 8:00:06 AM (UTC-07:00)
To: Sheahan, Timothy Patrick[sheahan@email.unc.edu]; Sims, Amy C[amy.sims@pnnl.gov]
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Sent: Tue 4/28/2020 8:08:33 AM (UTC-07:00)
To: Baric, Ralph S[rbaric@email.unc.edu]; Sheahan, Timothy Patrick[sheahan@email.unc.edu]
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Sent: Tue 4/28/2020 8:17:11 AM (UTC-07:00)
To: Sims, Amy C[amy.sims@pnnl.gov]; Sheahan, Timothy Patrick[sheahan@email.unc.edu]
Cc: Hou, Yixuan Jacob[y.jacob.hou@unc.edu]
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To: Baric, Ralph S[rbaric@email.unc.edu]; Sims, Amy C[amy.sims@pnnl.gov]; Sheahan, Timothy Patrick[sheahan@email.unc.edu]
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Hi all,

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Their policy for a research article is:

Title: no more than 85 characters
Summary: no more than 150 words
Total words: no more than 45,000 characters (including space and main Fig legends)
Main figure: no more than 7

You may find more details in the following link:
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Date: Tuesday, April 28, 2020 at 8:31 AM
To: "Baric, Ralph S" <rbaric@email.unc.edu>, "Sims, Amy C" <amy.sims@pnnl.gov>, "Sheahan, Timothy Patrick" <sheahan@email.unc.edu>
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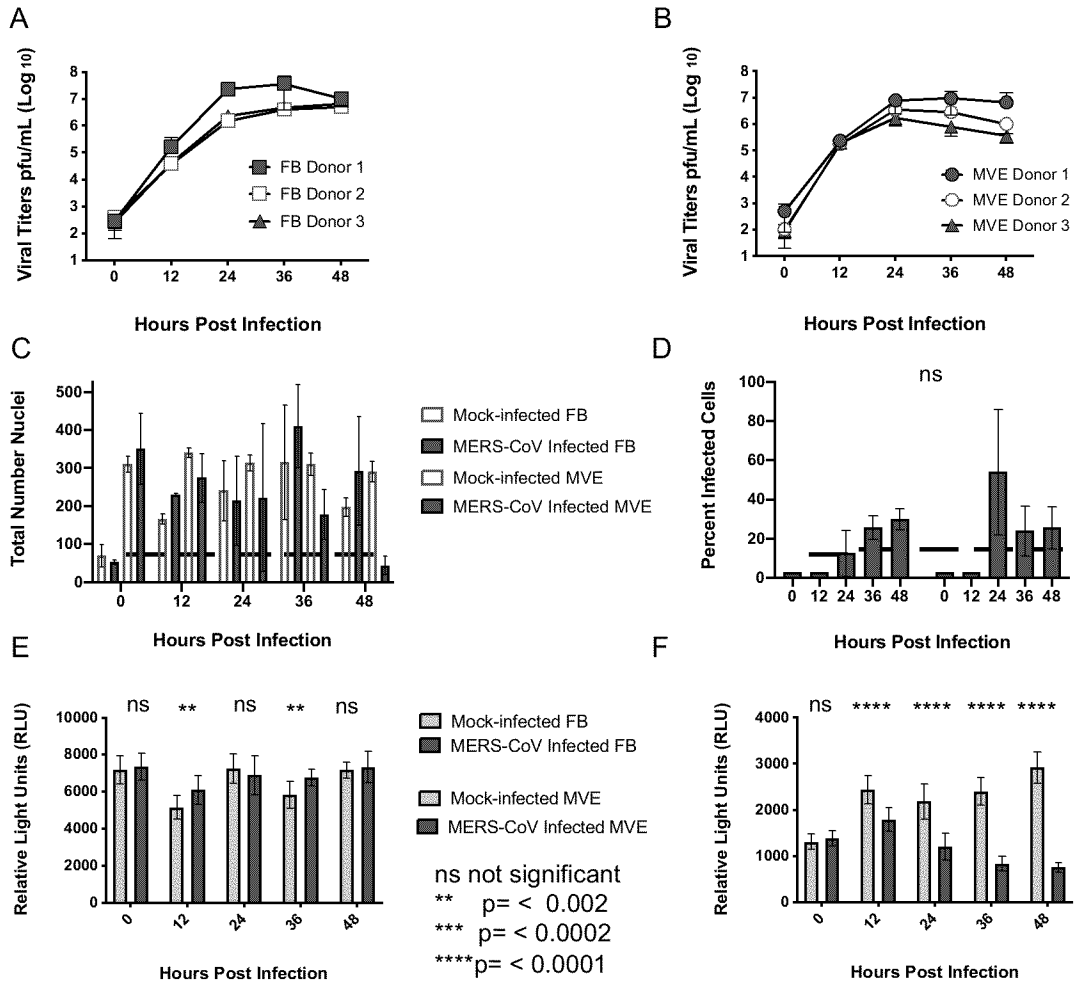


Figure 1. Viral growth kinetics, percent infected cells and cell viability following MERS-CoV infection of primary human lung fibroblasts and microvascular endothelial cells. A-B. Viral replication: Donor matched microvascular endothelial cells (MVE) and fibroblasts (FB) were infected with wild type MERSCoV (MOI of 5) and supernatants collected at the indicated times and viral titers determined by plaque assay. Results are shown as plaque forming units (pfu) per mL over time. Each data point represents averaged data from supernatant collected from 10 different wells (5 wells harvested for RNA and 5 wells fractionated for proteins, metabolites and lipids). The graphs in A and B show levels of replication detected for all three tissue donors in both cell types. Error bars indicate standard deviation from the mean. Open and filled blue circles and blue triangles represent data from MERSCoV infected FB, while open and filled red circles and red triangles represent MERS-CoV infected MVE. **C-D.** Percent MERS-CoV infected cells: Donor matched MVE and FB were mock-infected or infected with MERS-CoV expressing the red fluorescent protein (MERS-RFP), nuclei were stained with Hoechst stain, and images captured at the indicated times post infection. Total numbers of cells (shown for all conditions in **C**) and total numbers of infected (contained red fluorescence) cells were assessed for at least three fields per condition and total percent of infected cells determined and plotted (**D**). Representative data is shown for one of the three donors. Error bars indicate standard deviation from the mean. No statistical significance (ns) was found when comparing the percentage of infected FB and MVE at any specific timepoint. Statistical analysis performed in GraphPad determined by Mann-Whitney U test. Open blue bars- mock-infected FB, open red bars- mock-infected MVE, filled blue bars- MERS-RFP infected FB, filled red bars- MERS-CoV RFP infected MVE. **E-F.** Cell viability following MERS-CoV infection: Donor matched MVE and FB were infected with wild type MERS-CoV and assessed for cell viability using the CellTiter-Glo kit according to manufacturer's instructions (Promega) at the indicated times post infection. Relative light units were graphed over time and error bars indicate standard deviation from the mean. Statistical analysis performed in GraphPad determined by Mann-Whitney U test. Gray bars- mock infected cells, red bars- MERS-CoV infected MVE, blue bars- MERS-CoV infected FB, **** p= <0.0001, ** p= <0.002, ns not statistically significant.

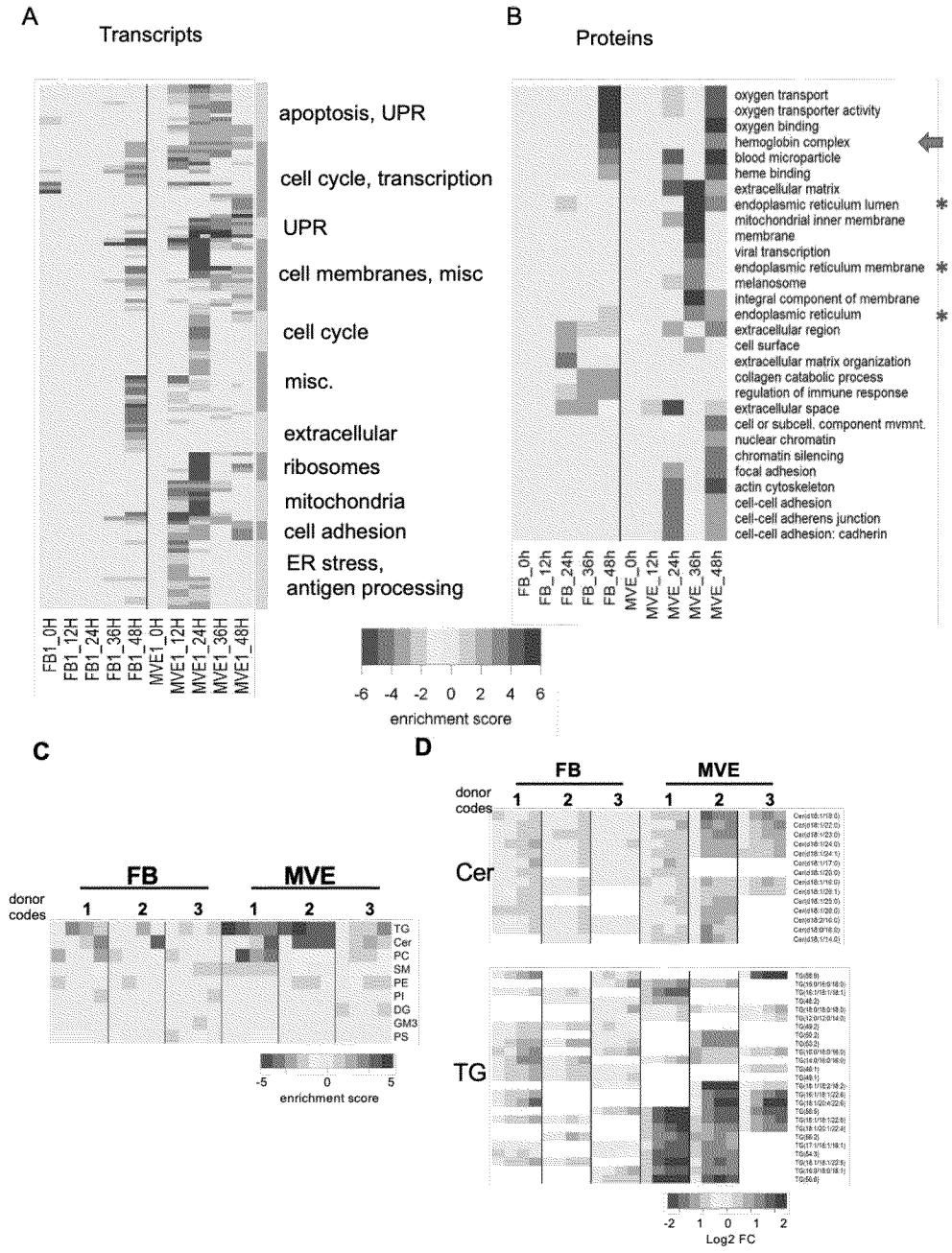


Figure 2. Transcriptomics, proteomics, and lipidomics suggest activation of apoptotic pathways. A. Functional enrichment was performed on transcriptomic data from all three donor samples in both cell types, and results were only retained that were present in all three donors, keeping only the least significant score In this way a true consensus response is represented by all indicated functions. **B.** Protein expression for proteins associated with the GO term "positive regulation of protein insertion into mitochondrial membrane involved in apoptotic signaling pathway". All apoptosis-associated proteins are displayed across the three donors for fibroblasts and MVE. The blue arrow highlights proteins in the hemoglobin complex and red asterisks highlight apoptotic proteins from the endoplasmic reticulum. **C.** Enrichment analysis using lipid classes as enrichment sets. Cer-ceramide, TG-triglycerides, PC-phosphatidylcholine, PE-phosphatidylethanolamine, PS-phosphatidylserine, SM-sphingomyelin. **D.** Abundance of individual lipid species.

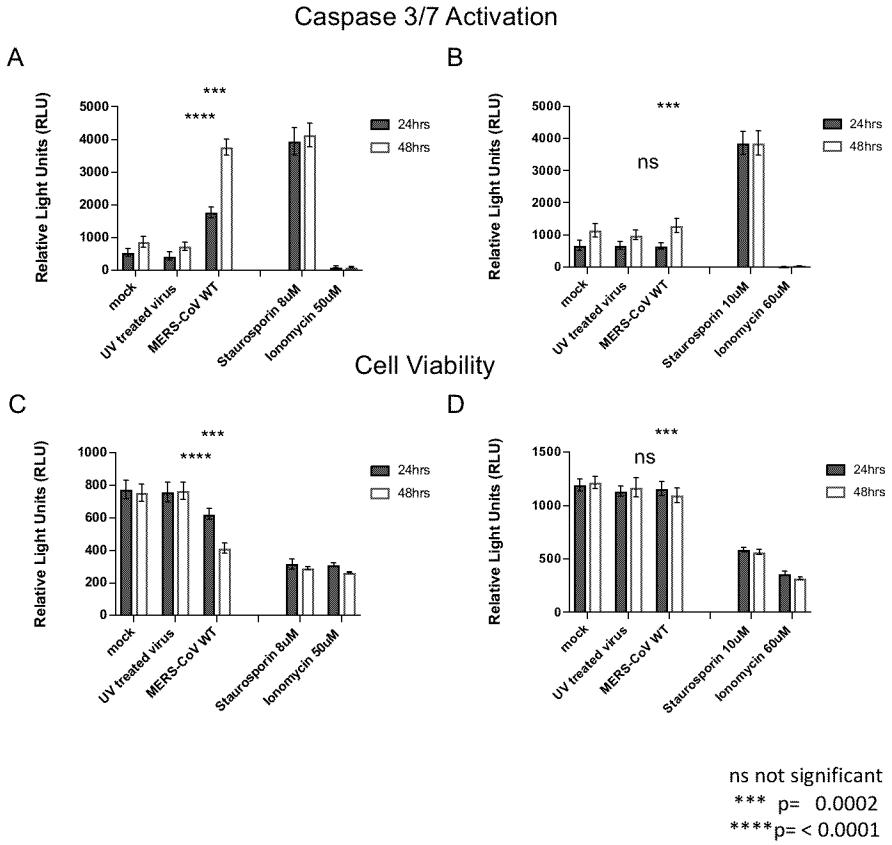


Figure 3 Caspases 3/7 are activated following MERS-CoV infection of primary human lung MVE but not primary lung FB. A-D. Donor matched MVE and FB were plated and mock-infected or infected with UV-inactivated or wild type MERS-CoV and at 24 and 48 hours post infection caspase 3/7 activation or cell viability was determined according to manufacturer's instructions using the Apotox Triplex kit (each well was measured for both parameters). Uninfected cells were treated with staurosporin (8 uM MVE/10 uM FB) to determine the maximal amount of caspase 3/7 activation. Uninfected cells were treated with ionomycin (50 uM MVE/60 uM FB) to demonstrate loss of cell viability via non-apoptotic pathway. Results are graphed as relative light units and error bars indicate standard deviation from the mean. Statistical analysis performed in GraphPad determined by Mann-Whitney U test. **A-B** Results from caspase 3/7 activation. **C-D.** Results from cell viability assay. Solid red bars- MVE 24 hour samples, open red bars- MVE 48 hour samples, solid blue bars- FB 24 hour samples, open blue bars- FB 48 hour samples. **** p=<0.0001, *** p= <0.0002, ns- not statistically significant.

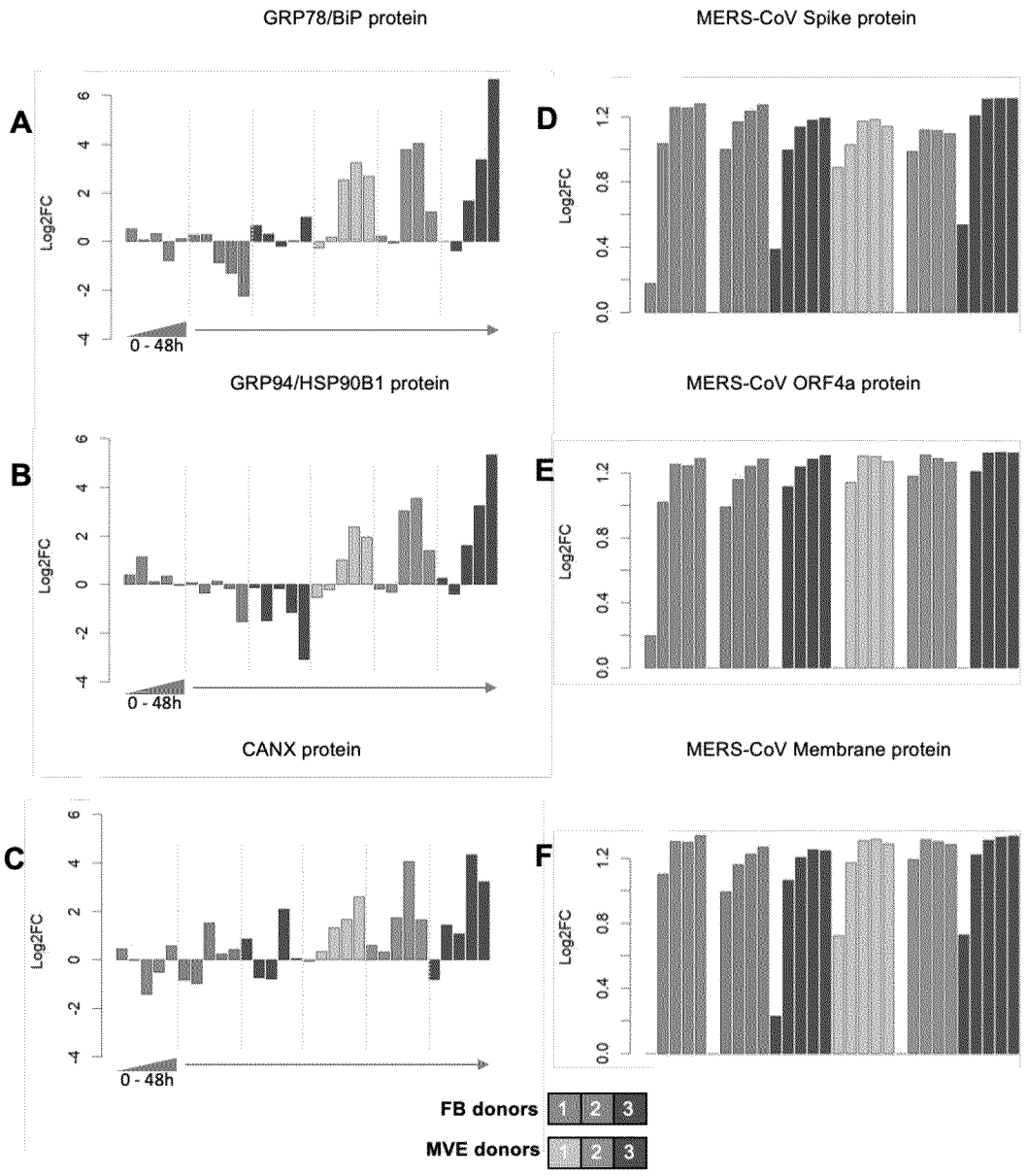


Figure 4 Protein markers suggest activation of unfolded protein response (UPR). A-C. Protein expression of markers of the unfolded protein response. Each figure shows the expression behavior of a UPR marker inFB (blues) and MVE (reds), with individual donors represented separately as individual color hues. For each donor, a time course of expression is shown that includes samples taken at 0, 12, 24, 36 and 48 hours post infection. Values are expressed as the log10 p-value of the change between the infection and control conditions, with sign assigned according to the direction of fold change. **D-F.** Viral protein expression. MERS-CoV spike, open reading frame 4a (ORF4a) and membrane protein abundances are represented as the ratio of each protein to the average abundance of all other proteins detected in each respective sample.

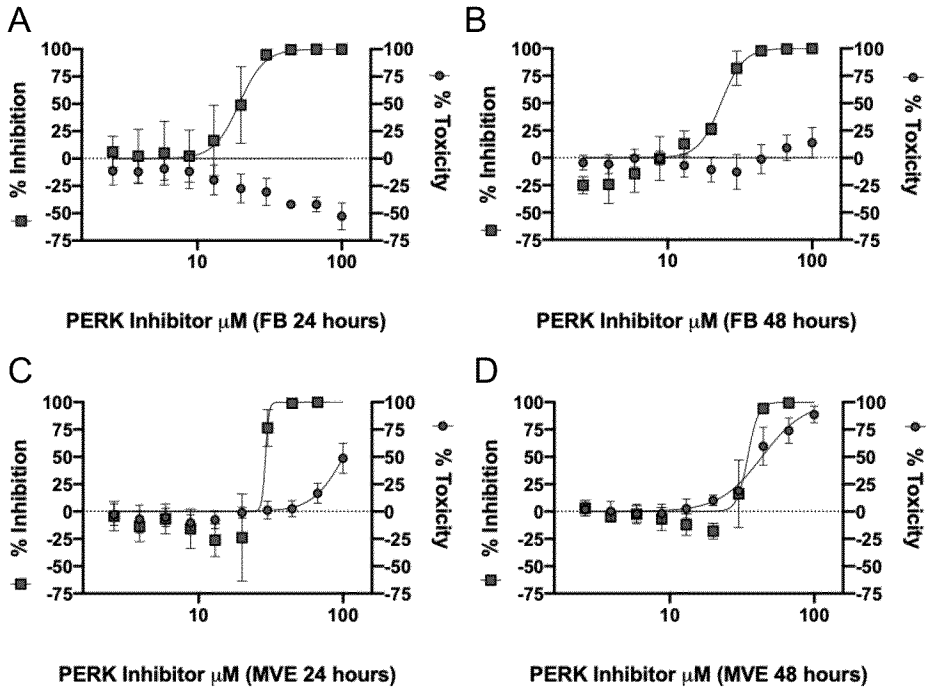


Figure 5 PERK inhibitor reduces MERS-CoV replication in infected primary human lung MVE and FB. A-D. Donor matched uninfected FB (circles, A, B) and MVE (circles, C, D) were treated with serial dilutions of PERK inhibitor (100 uM to 2 uM) and cell viability assessed at 24 (A, C) and 48 (B, D) hours post treatment using Promega's CellTiter Glo kit according to manufacturer's instructions. Each circle represents mean values from two experiments and are graphed as percent toxicity. Error bars indicate standard deviation from the mean. In parallel, the same donor matched FB (squares, A, B) and MVE (squares, C, D) were infected with MERS-nanoluc and simultaneously treated with the same serial dilutions of PERK inhibitor. Nanoluciferase expression was assayed at 24 (squares, A, C) and 48 (squares, B, D) hours post infection using Promega's NanoGlo kit according to manufacturer's instructions. Control wells were either treated with drug diluent and UV-inactivated virus or infected with MERS-nanoluc and treated with only drug diluent. Each square represents mean values from two experiments and are graphed as percent inhibition. Error bars indicate standard deviation from the mean. Blue squares- MERS-nanoluc infected FB treated with PERK inhibitor serial dilutions, blue circles- uninfected FB treated with PERK inhibitor serial dilutions, red squares- MERS-nanoluc infected MVE treated with PERK inhibitor serial dilutions, red circles- uninfected MVE treated with PERK inhibitor serial dilutions. PERK protein kinase R-like ER kinase, MVE primary human lung microvascular endothelial cell, FB primary human lung fibroblast, MERS-nanoluc MERS-CoV expressing nanoluciferase

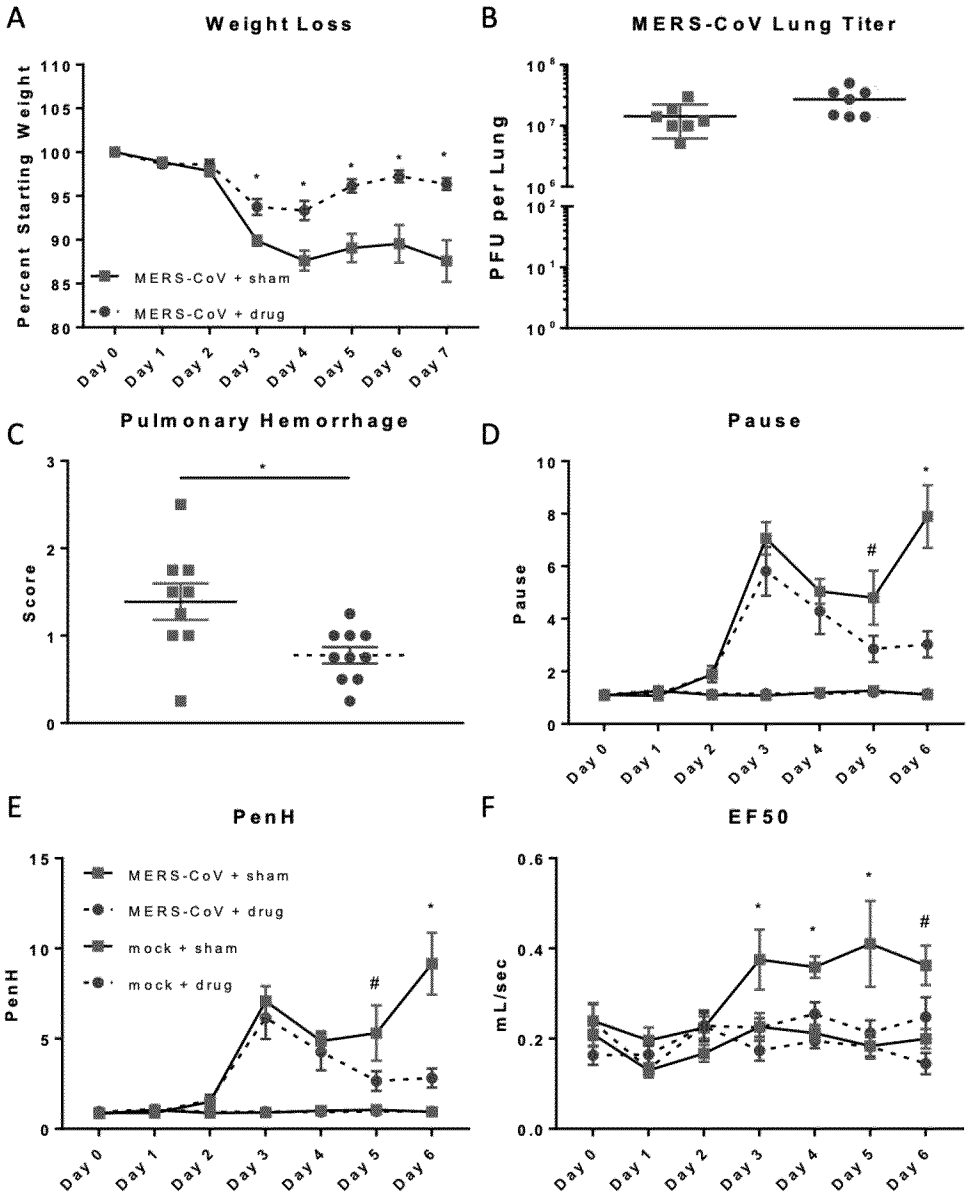


Figure 6 PERK inhibitor AMG44 treatment decreases MERS-CoV pathogenesis
A-F. hDPP4 mice were treated with PERK AMG'44 or sham control and infected with $5x10^4$ PFU of MERS-CoV MAm35c4 or PBS control. Weight loss was observed over the course of a seven day infection (A) and viral titers (B) and pulmonary hemorrhage was scored at the time of harvest (C). Daily respiratory function measurements (D-F) were taken via whole body plethysmography. Error bars indicate standard error of the mean, # indicates a p value of < 0.1 and * indicates a p value of < 0.05.

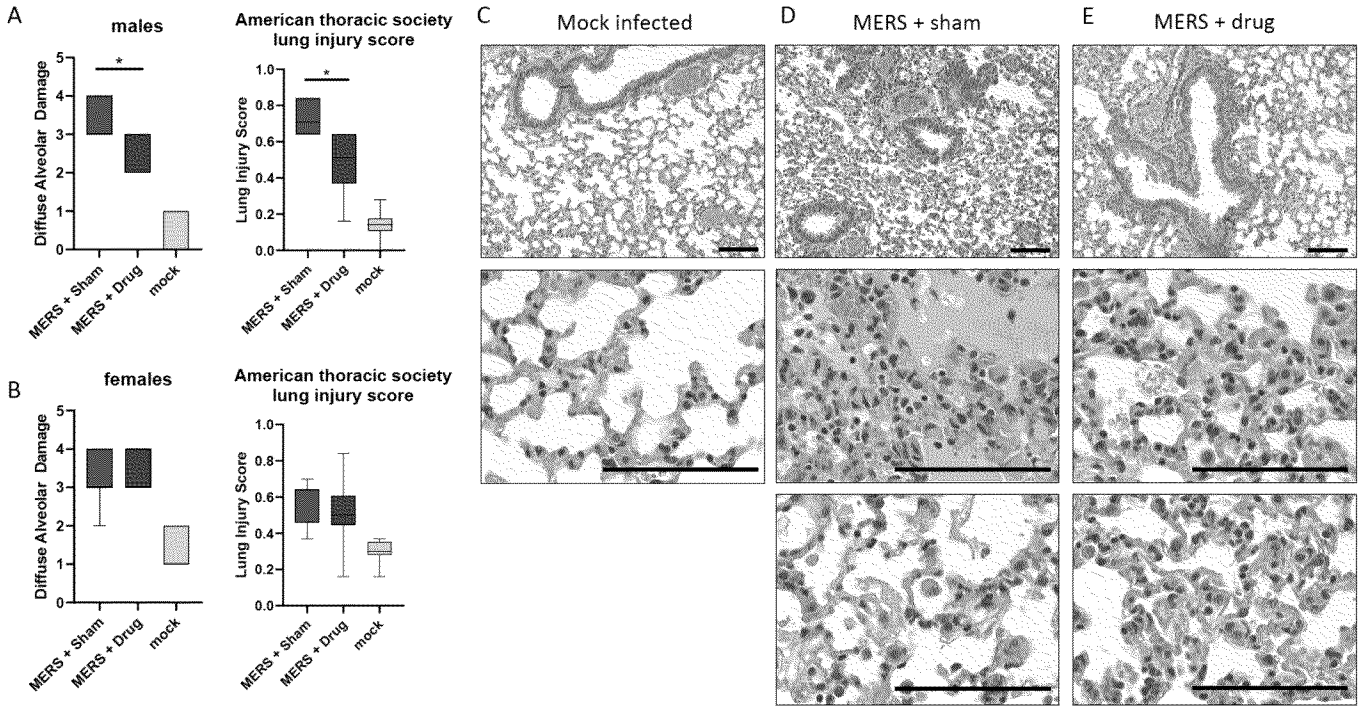


Figure 7 AMG44 reduces clinical signs of acute lung injury in MERS-CoV challenge model

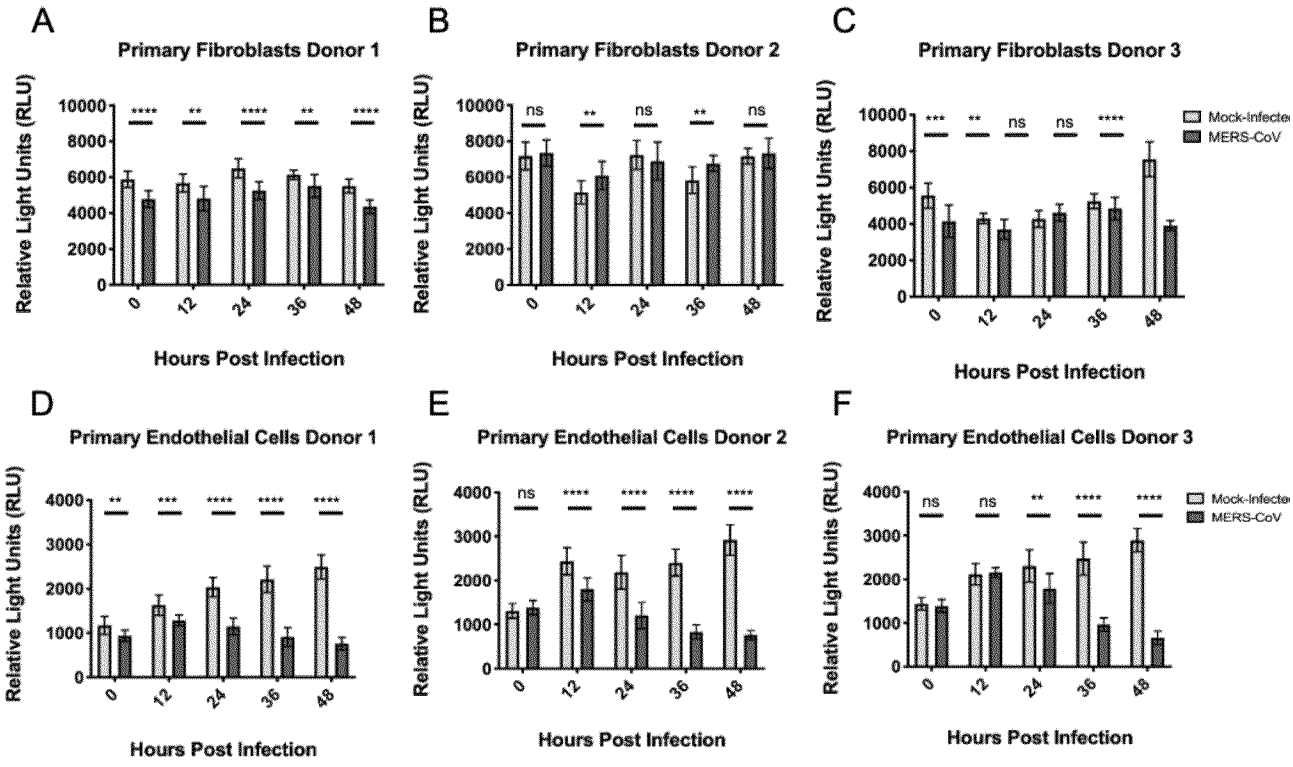
A-E. The histological features of acute lung injury were blindly scored using the American Thoracic Society Lung Injury Scoring system by Matute-Bello, creating an aggregate score for: neutrophils in the alveolar and interstitial space, hyaline membranes, proteinaceous debris filling the air spaces, and alveolar septal thickening. **A-B.** Scoring for ALI and diffuse alveolar damage in male and female mice, respectively. Representative lung pathology for C) mock-infected, D) MERS-CoV infected and sham treated and E) MERS-CoV infected and AMG44 treated male mice at 7 days post infection. * indicates a p value of <0.001.

Supplementary Materials

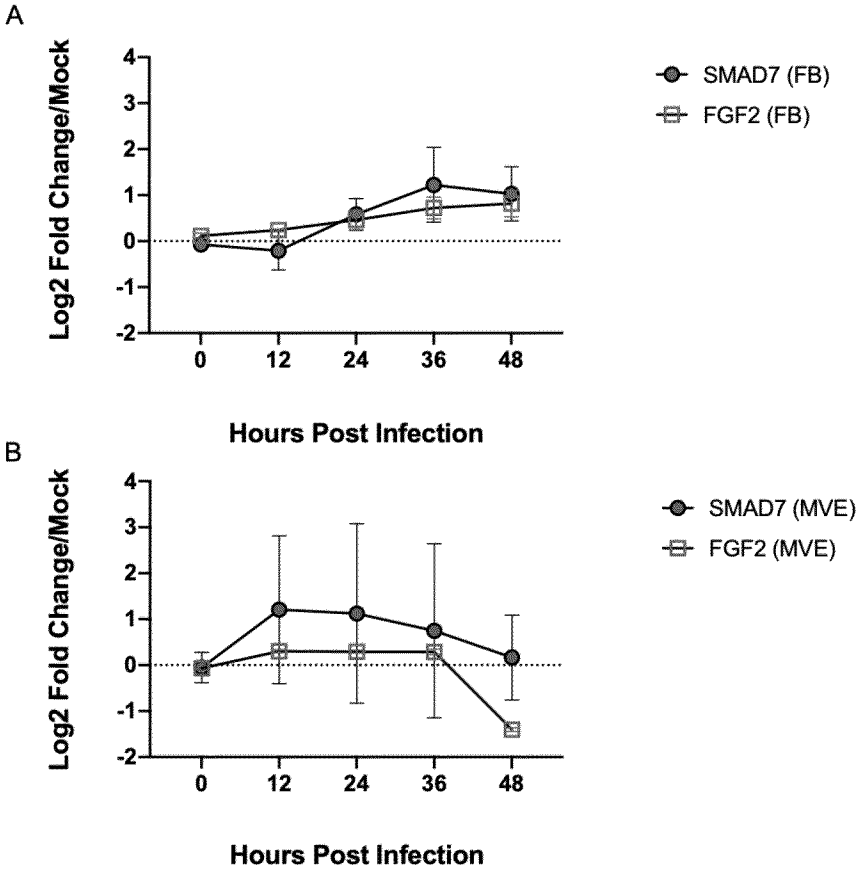
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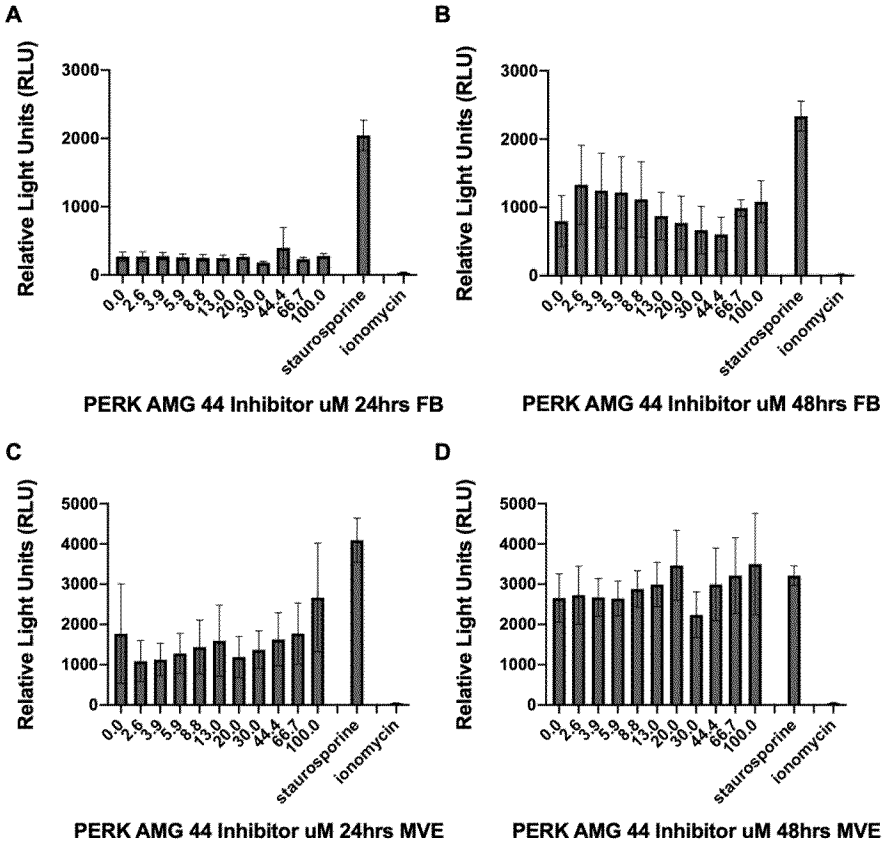
Supplementary Figures and Tables



Supplementary Fig. S1 MERS-CoV reduces cell viability for all three donors in MVE but not FB. Donor matched MVE and FB were infected with wild type MERS-CoV and assessed for cell viability using the CellTiter-Glo kit according to manufacturer's instructions (Promega) at the indicated times post infection. Relative light units (RLU) were graphed over time and error bars indicate standard deviation from the mean. Statistical analysis performed in GraphPad determined by Mann-Whitney U test. Gray bars- mock infected cells, red bars- MERS-CoV infected MVE, blue bars- MERS-CoV infected FB, **** p= <0.0001, ** p= <0.002, ns not statistically significant.



Supplementary Fig. S2 Neither SMAD7 nor FGF2 expression contribute to MERS-CoV induced cytopathic effect in primary human lung cells. A-B. Graphs show transcript expression levels for SMAD7 and FGF2 in MERS-CoV infected MVE and FB over the 48 hour time course (derived from microarray analysis and are from the same datasets used in Figure 2). Values graphed are fold change ($\log_2$) and error bars are standard deviations from the mean. mothers against decapentaplegic homolog 7 (SMAD7); fibroblast growth factor 2 (FGF2)



Supplementary Fig. S3 Inhibition of PERK by AMG44 does not alter caspase 3/7 activation following MERS-CoV infection. A-D. Donor matched FB (A, B) and MVE (C, D) were plated and infected with UV-inactivated or wild type MERS-CoV and at 24 (A, C) and 48 (B, D) hours post infection caspase 3/7 activation was determined according to manufacturer's instructions using the Apotox Triplex kit. Control uninfected cells were treated with staurosporin (8 uM MVE/10 uM FB, activates caspase 3/7) or ionomycin (50 uM MVE/60 uM FB, does not activate caspase 3/7). Results are graphed as relative light units and error bars indicate standard deviation from the mean. Statistical analysis performed in GraphPad determined by Mann-Whitney U test.

Supplementary Table 1 Enrichment results from transcriptomic data represented in Figure 2

Please see separate Excel spreadsheet.

Supplementary Table 2 Enrichment results from proteomic data represented in Figure 2

Please see separate Excel spreadsheet.

From: Mitchell, Hugh D[Hugh.Mitchell@pnnl.gov]
Sent: Tue 4/28/2020 4:48:31 PM (UTC-07:00)
To: Sims, Amy C[amy.sims@pnnl.gov]; Baric, Ralph S[rbaric@email.unc.edu]; Waters, Katrina M[Katrina.Waters@pnnl.gov]
Cc: Sheahan, Timothy Patrick[sheahan@email.unc.edu]
Subject: RE: Coronavirus / host kinase paper

Short and sweet. Looks good to me! -Hugh

From: Sims, Amy C <amy.sims@pnnl.gov>
Sent: Tuesday, April 28, 2020 4:43 PM
To: Baric, Ralph S <rbaric@email.unc.edu>; Mitchell, Hugh D <Hugh.Mitchell@pnnl.gov>; Waters, Katrina M <Katrina.Waters@pnnl.gov>
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Thank you, Amy

From: "Baric, Ralph S" <rbaric@email.unc.edu>
Date: Tuesday, April 28, 2020 at 7:50 AM
To: "Sims, Amy C" <amy.sims@pnnl.gov>
Cc: "Sheahan, Timothy Patrick" <sheahan@email.unc.edu>
Subject: FW: Coronavirus / host kinase paper

Amy, I think Cell is interested in your paper! Go for it! Ralph

---

From: Rennekamp, Andrew (ELS-CMA) <arennekamp@cell.com>
Sent: Tuesday, April 28, 2020 10:10 AM
To: Baric, Ralph S <rbaric@email.unc.edu>
Subject: Coronavirus / host kinase paper

Hi Ralph,

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Best,
-Andrew

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Andrew J. Rennekamp, Ph.D.
Scientific Editor, *Cell*

From: Sheahan, Timothy Patrick[sheahan@email.unc.edu]
Sent: Tue 4/28/2020 6:12:29 PM (UTC-07:00)
To: Sims, Amy C[amy.sims@pnnl.gov]
Subject: Re: Coronavirus / host kinase paper

Go. For. It!

From: Amy Sims <amy.sims@pnnl.gov>
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Sent: Tue 4/28/2020 6:18:52 PM (UTC-07:00)
To: Mitchell, Hugh D[Hugh.Mitchell@pnnl.gov]; Sims, Amy C[amy.sims@pnnl.gov]; Baric, Ralph S[rbaric@email.unc.edu]
Cc: Sheahan, Timothy Patrick[sheahan@email.unc.edu]
Subject: RE: Coronavirus / host kinase paper

Go for it!

Katrina Waters
Tel: 509-375-3907
Biological Sciences Division

From: Mitchell, Hugh D <Hugh.Mitchell@pnnl.gov>
Sent: Tuesday, April 28, 2020 4:49 PM
To: Sims, Amy C <amy.sims@pnnl.gov>; Baric, Ralph S <rbaric@email.unc.edu>; Waters, Katrina M <Katrina.Waters@pnnl.gov>
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Sent: Tue 4/28/2020 7:01:18 PM (UTC-07:00)
To: Sims, Amy C[amy.sims@pnnl.gov]
Subject: RE: Coronavirus / host kinase paper
Attachment: CELL Apoptosis 4_28_2020 final draft_2-rsb.docx
Attachment: Cell cover letter April 2020-rsb.docx

Hi Amy, beautiful story. Some minor comments designed to link the role of the vascular endothelium to other emerging cov like sars-2. Trying to build alittle more impact and breadth. Take a look and make the call. Submit when ready! Congrats! Ralph

---

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Scientific Editor, *Cell*

Title:

Unfolded Protein Response Inhibition Reduces MERS-CoV Induced Acute Lung Injury

Authors:

Amy C. Sims[1*]†, Hugh Mitchell[2]†, Lisa E. Gralinski[1], Jennifer E. Kyle[2], Kristin E. Burnum-Johnson[2], Mariam Lam[3], M. Leslie Fulcher[3], Ande West[1], Richard D. Smith[2], Scott H Randell[3,4], Thomas O. Metz[2], Timothy P. Sheahan[1]‡, Katrina M. Waters[2]‡, Ralph S. Baric[1]‡

Affiliations:

[1]Department of Epidemiology, University of North Carolina at Chapel Hill, Chapel Hill, NC, 27599; United States of America.

[2]Biological Sciences Division, Pacific Northwest National Laboratories, Richland, WA, 99352; United States of America.

[3]Marsico Lung Institute Tissue Procurement and Cell Culture Core, University of North Carolina at Chapel Hill, Chapel Hill, NC, 27599; United States of America.

[4]Department of Cell Biology and Physiology, University of North Carolina at Chapel Hill, Chapel Hill, NC, 27599; United States of America.

*To whom correspondence should be addressed: Amy C. Sims amy.sims@pnnl.gov current address: National Security Directorate, Pacific Northwest National Laboratory Richland, WA.

†authors contributed equally to manuscript.

‡ co-senior author

Summary (150 words):

Currently, there are no approved therapeutics for emerging coronavirus infections and little is understood about how lung cell types, like endothelial cells, contribute to disease outcomes. MERS-CoV replicates equivalently in primary human lung microvascular endothelial cells (MVE) and fibroblasts (FB) but only MVE demonstrate virus induced cytopathic effect. To explore mechanisms leading to reduced MVE viability, donor-matched human lung MVE and FB were infected, and their transcriptomes, proteomes, and lipidomes followed over time. Validated functional enrichment analysis demonstrated that MERS-CoV-infected MVE were dying via an unfolded protein response (UPR) mediated apoptosis. Pharmacologic manipulation of the UPR in MERS-CoV infected mice improved respiratory function with accompanying reductions in weight loss, pathologic signatures of acute lung injury and times to recovery. Bioinformatic analysis of systems biology datasets indicate that MERS-CoV infection and death of MVE triggers loss of endothelial barrier integrity and vascular leakage, contributing to progressive end stage lung disease.

Keywords

MERS-CoV, acute lung injury, primary human lung cells, microvascular endothelial cells, fibroblasts, apoptosis, unfolded protein response

Introduction

Coronaviruses (CoV) are important emerging pathogens associated with severe disease outcomes in humans and animals causing significant global morbidity and mortality. In 2003, severe acute respiratory coronavirus (SARS-CoV) emerged from closely related bat CoV strains, spread to 26 nations, and caused approximately 8,000 human cases and 800 deaths, worldwide (Graham et al., 2013; Peiris et al., 2004). In 2012, Middle East respiratory syndrome CoV (MERS-CoV) emerged, causing approximately 2,519 cases with a ~35% mortality rate in 27 countries and the outbreak is still ongoing (Zaki et al., 2012). In 2020, the causative agent of coronavirus infectious disease 2019 (COVID 19) was identified as severe acute respiratory syndrome coronavirus 2 (SARS-CoV 2) and to date >3 million cases have been confirmed with a morality rate of ~7% in >200 countries/territories with ongoing virus transmission resulting in significant economic losses and global public health concerns (WHO COVID 19 website). SARS-CoV 2, SARS-CoV, and MERS-CoV disease severity are strongly influenced by aging and other co-morbidities (e.g., diabetes, obesity) and mortality rates approach 15% or exceed 50% in aged individuals, respectively (>60 years)(Ng et al., 2016). These data underscore the highly pathogenic potential of emerging CoVs and reinforce the critical need for comprehensive experimental approaches that define the viral and host pathogenic programs which could be targeted by countermeasures to reverse severe disease outcomes.

Acute Respiratory Distress Syndrome (ARDS) is a severe end stage lung disease characterized by rapid onset respiratory failure resulting from diffuse alveolar damage, hyaline membrane formation, vascular leakage into the airways and pulmonary fibrosis (Blondonnet et al., 2016; Force et al., 2012; Husain and Kumar, 2010; Ware and Matthay, 2000). ARDS is a complex syndrome with multiple etiologies, complicating diagnosis and treatment. The pulmonary endothelium is involved in lung homoeostasis and many of its functions are disrupted in ARDS ().

While not the most prevalent cause (Luyt et al., 2011), severe avian influenza (Ramsey and Kumar, 2011) (H5N1) and SARS-CoV, SARS-CoV 2 and MERS-CoV (Arabi et al., 2017; Huang et al., 2020; Peiris et al., 2003; Peiris et al., 2004; Smits et al., 2011) respiratory infections can progress to ARDS. Dissecting the mechanisms of MERS-CoV-induced ARDS has been hampered by human genetic variation, variable co-morbidities and limited human sample availability (Arabi et al., 2017; Beigel et al., 2019). Evidence of programmed cell death has been detected in models of coronavirus pathogenesis and in-patient autopsy tissues (Chan et al., 2013; Chu et al., 2016; de Wilde et al., 2013; Hocke et al., 2013; Ng et al., 2016; Tao et al., 2013; Yan et al., 2004; Zhang et al., 2003; Zhou et al., 2017). Because MERS-CoV replicates efficiently in multiple cell types essential for lung physiology (e.g. lung fibroblasts, epithelial and endothelial cells) (Scobey et al., 2013), primary culture systems provide a platform to better understand virus and virus-mediated- tissue specific host interactions that may drive severe lung pathology.

Here we report a kinetic multi-omics analysis of matched MERS-CoV infected primary human lung fibroblasts (FB) and microvascular endothelial (MVE) cells from three different human donors. While MERS-CoV replication kinetics were similar among FB and MVE, the host responses, as determined by multi-omic transcriptomic, proteomic, and lipidomic analyses differed significantly by cell type and suggested that MVE cells were dying via apoptotic mechanisms initiated by the unfolded protein response (UPR), which helps monitor and maintain homeostasis in the endoplasmic reticulum (ER) following a stress response. Pharmacologic manipulation of UPR demonstrated that active/dimerized PERK was critical for viral replication *in vitro*. More importantly, mice infected with MERS-CoV and treated with a PERK specific inhibitor demonstrated reduced weight loss, pneumonia and acute lung injury leading to improved respiratory function and time to recovery. These data not only articulate an important role for the

epithelial-endothelial cell barrier in MERS-CoV pathogenesis and acute lung injury, but also reveal new strategies for treatment of emerging coronavirus infections.

Results:

MERS-CoV induces cytopathic effect in primary human lung endothelial cells but not fibroblasts

Understanding MERS-CoV pathogenic mechanisms requires understanding critical virus-host interactions in infected primary human lung cells that provide the vital structural and physiological requirements for lung function. As clinical samples for MERS-CoV are scarce, using matched primary human lung cells from previously healthy lung donors provides a novel opportunity to elucidate tissue specific changes that may explain disease phenotypes. To characterize MERS-CoV infection in primary human lung microvascular endothelial cells (MVE) and primary human lung fibroblasts (FB), we infected cells from three matched human donors and conducted virologic and multi-omic (transcriptomic, proteomic, and lipidomic) analyses at 0, 12, 24, 36 and 48 hours post infection. Viral growth kinetics and peak titers (~10^7 plaque forming units (PFU)/mL) were nearly identical regardless of donor background (Figure 1A-B). We also determined infection frequency via microscopy by comparing the total number of cells (i.e. nuclei) to the number of MERS-CoV infected cells per high powered field (Figure 1C and D and Supplemental Figure 1). To simplify this process, we utilized a MERS-CoV expressing the red fluorescent protein (MERS-RFP) (Figure 1C and D). Unlike infection frequency in FB which increased over time, the numbers of total cells per field (Figure 1C) as well infection frequency decreased over time in MVE (Figure 1C-D). Since the number of cells per field in mock-infected MVE cultures did not diminish over time, virus induced cytopathic effect (CPE) was likely mediating the loss of cells in MERS-CoV infected MVE (Figure 1C and D). To quantitate CPE

in MERS-CoV infected MVE and FB over time, we performed a cell viability assay (CellTiter-Glo) which estimates the number of metabolically active viable cells by measuring the levels of adenosine triphosphate (ATP) in each well. Unlike FB which had similar cell viability estimates in mock- and MERS-CoV-infected cultures for most time points (Figure 1E), infected MVE viability decreased significantly over time as compared to mock (Figure 1F), supporting a loss in cell viability. Together, these data demonstrate that both primary MVE and FB are similarly susceptible to MERS-CoV infection. While MVE cell viability is significantly reduced over time, FB viability is relatively unaffected.

Cross-donor consensus transcriptomic, proteomic and lipidomic responses

To gain insight into the divergent outcomes in FB and MVE viability following MERS-CoV infection, functional enrichment was performed on donor matched kinetic transcriptomic and proteomic data across both cell types. We found distinct differences in the modulation of the transcriptome and proteome among infected FB and MVE (Figure 2), revealing pathways related to apoptosis (transcription, Figure 2A) and the unfolded protein response (UPR, transcripts and proteins, Figure 2A and 2B), were significantly enriched in MERS-CoV infected MVE at multiple timepoints, but not observed in similarly infected FB. Interestingly, increased hemoglobin protein expression levels in both FB and MVE were detected at 48 hours post infection (arrow Figure 2B), which has been demonstrated to regulate endothelial and epithelial cell communication as well as to regulate the durability of vascular walls (Lechauve et al., 2018; Straub et al., 2012; Straub et al., 2014). We then investigated if mothers against decapentaplegic homolog 7 (SMAD7) and/or fibroblast growth factor 2 (FGF2) transcripts were differentially expressed in MERS-CoV infected MVE or FB, as previous studies indicated that upregulated expression of SMAD7 and FGF2 in MERS-CoV infected immortalized kidney and lung epithelial cells led to

cellular apoptosis (Yeung et al., 2016). Neither SMAD7 nor FGF2 expression levels were distinct following infection and did not appear to explain the differences in cytopathic effect seen in the current studies (Supplemental Figure S2). Finally, we sought to determine if MERS-CoV infection alters the lipidome in FB or MVE. We classified lipids into broad categories for enrichment analysis, which revealed differential expression of ceramides and triglycerides in FB and MVE (Figure 2C). When analyzed at a per-lipid-species level, we found dramatic and significant differences in specific species of ceramides (Figure 2D, upper panel) and triglycerides (Figure 2D, lower panel) in MERS-CoV infected MVE, both of which are involved in activating apoptotic processes (Dart and Chin-Dusting, 1999).

MERS-CoV activates Caspases 3 and 7 in infected MVE

To validate the complementary multi-omics observations suggesting that MERS-CoV infection induces apoptosis in MVE, we simultaneously measured death effector caspases 3 and 7 and cell viability in mock or MERS-CoV infected FB and MVE using the Promega Apotox Triplex kit. We included staurosporine (induces caspase 3/7 activation) or ionomycin (induces necrotic cell death with no caspase activation) treatment as controls. In addition, we added UV-inactivated MERS-CoV virions in order to control for effects on cultures independent of virus replication (i.e. entry, uncoating, etc.). Uninfected MVE and FB treated with staurosporine significantly induced caspase 3/7 activation as compared to mock and cells treated with UV-inactivated MERS-CoV (Figure 3A-B). As expected, ionomycin treatment did not induce caspase 3/7 activation. With MERS-CoV infection, significant caspase 3/7 activation was observed at both 24 and 48 hours post infection, but only in MVE (Figure 3A) not in FB (Figure 3B). Caspase activation was not observed with UV-inactivated MERS-CoV treatment or in mock-infected cells for either cell type (Figure 3A-B). When measuring cell viability within the same assay, neither mock infection

nor treatment with UV-inactivated virions appreciably affected cell viability (Figure 3C-D). Importantly, treatment of both FB and MVE with control compounds (e.g. staurosporine or ionomycin) diminished cell viability, but MERS-CoV infection only diminished cell viability in MVE (Figure 3C-D). Death of MERS-CoV infected MVE could lead to permeability at the epithelial/endothelial cell barrier as one of the early steps of ARDS while viable MERS-CoV infected FB would continue to produce high levels of infectious virus both of which would contribute to the overall disease burden in the infected patient.

Expression of proteins indicative of the UPR pathway are increased in MERS-CoV infected MVE

Both systems data and confirmatory experimental validation studies suggested that MERS-CoV infected MVE were dying via apoptotic pathways. To better understand the initiation of this process, we re-analyzed our systems biology data focusing on specific mediators of apoptosis and UPR. We found increased protein expression of three well-established UPR markers, glucose regulated protein 78 (GRP78; also known as binding immunoglobulin protein (BiP)), heat shock protein 90kDa beta member 1 (HSP90B1; also known as GRP94) and calnexin (CANX) in MERS-CoV infected MVE, but not infected FB (Figure 4A-C). GRP78/BiP is a master regulatory protein for the UPR that binds to regulatory enzymes within the UPR pathway keeping them inactive unless misfolded proteins accumulate triggering dissociation and enzyme activation. HSP90B1/GRP94 and CANX are ER protein chaperones that facilitate nascent protein folding. In support of the viral titer data, the expression of MERS-CoV structural proteins, spike glycoprotein and membrane protein as well as the accessory open reading frame protein 4a (ORF4a) were expressed to similar levels in both cell types for all donors by 12 hours post infection (Figure 4D-F). Cumulatively, these data suggest that MERS-CoV modulation of the

UPR is specific to a particular cellular environment (MVE) and may not be broadly applicable to all infected cells and tissues.

PERK activation is critical for MERS-CoV replication in primary human lung MVE and FB

To further validate the results thus far, which suggested that apoptosis of MVE was associated with the UPR, we pharmacologically perturbed the UPR in MERS-CoV infected MVE and FB using an inhibitor specific to the first enzyme activated following misfolded protein accumulation, protein kinase R-like endoplasmic reticulum kinase (PERK, AMG44). Activated PERK regulates phosphorylation of the key translation mediator, elongation initiation factor 2alpha (eIF2alpha). Following activation of the UPR and PERK, host translational shutoff serves to reestablish ER homeostasis and proper protein folding, which if not achieved, results in the activation of apoptotic pathways. Dose response studies were performed in mock and MERS-CoV infected MVE and FB with PERK inhibitor (AMG 44 at 100 to 2 μM) to determine if pharmacologic perturbation of the PERK arm of the UPR would affect MERS-CoV replication and/or cell death (Smith et al., 2015). We utilized a MERS-CoV nanoluciferase reporter virus (MERS nanoluc) to increase accuracy and throughput of the assay (Sheahan et al., 2017). Inhibition of PERK diminished MERS-CoV replication in both FB (Figure 5A and B) and MVE (Figure 5C and D) in a dose dependent manner when assayed at either 24 (Figure 5A and C) or 48 hours post infection (Figure 5B and D). In addition to monitoring changes in virus replication in the context of PERK inhibition, we also monitored cytotoxicity in drug treated uninfected cultures via CellTiter-Glo cell viability assay. Unlike FB in which we did not observe cytotoxicity at either timepoint (Figure 5A and B), in MVE we observed a dose dependent increase in cytotoxicity with PERK inhibition that was most notable at 48hpi (Figure 5D). Importantly, we found that at 24 hours post infection MERS-CoV replication was inhibited at multiple drug doses that did not exhibit cytotoxicity.

These results suggest that primary human lung MVE are more sensitive to inhibition of the UPR in the absence of infection in contrast to the more tolerant primary lung FB. To determine if PERK inhibition modifies induction of apoptosis following MERS-CoV infection, we performed similar dose response assays as described in Figure 5A-D but then assayed for caspase 3 and 7 activation as in Figure 3 using similar UV inactivated and small molecule controls (i.e. staurosporin, ionomycin). No significant dose responsive change in caspase 3/7 activation above untreated infected cells was detected in either primary human lung MVE or FB following MERS-CoV infection and treatment with PERK inhibitor (Supplemental Figure S3).

PERK inhibition improves pulmonary function and diminishes MERS-CoV pathogenesis and ALI. Because *in vitro* PERK inhibition resulted in a significant decrease in MERS-CoV replication in both cell types, we sought to determine if *in vivo* inhibition would result in a change in viral replication and/or pathogenesis. To address this, we utilized a transgenic mouse model where the murine ortholog of the human MERS-CoV receptor, dipeptidyl peptidase 4 (DPP4), was humanized at residues 288 and 330 (hDPP4) facilitating high titer virus replication localized to the respiratory tract and lung pathology similar to that observed in humans(Cockrell et al., 2016; Douglas et al., 2018). We treated hDPP4 mice with AMG44 or vehicle at 24 hours prior to and post MERS-CoV infection. By three days post MERS-CoV infection, vehicle treated mice lost significantly (p value <0.05) more weight than AMG44 treated mice. Importantly, AMG44 treated mice also displayed an improved time to recovery over the remainder of the study (Figure 6A). Interestingly, virus titer in the lung on day 3 post infection was similar regardless of treatment (Figure 6B). "Pulmonary hemorrhage", a gross pathological phenotype that increases coincident with MERS-CoV pathogenesis, was significantly diminished in AMG44 treated mice as compared to those receiving vehicle treatment (Figure 6C). To determine if PERK inhibition

through AMG44 treatment would affect pulmonary function, we performed whole body plethysmography (WBP) daily. Concordant with weight loss and pulmonary hemorrhage, AMG44 treatment improved pulmonary function as compared to those receiving vehicle. For three WPB metrics typically induced following MERS-CoV infection (Cockrell et al., 2016; Douglas et al., 2018; Menachery et al., 2015), Pause, Penh and EF50, AMG44 treated mice had significantly improved values (p value <0.05) than vehicle treated animals at multiple timepoints (Figure 6D-F). Pause, PenH and EF50 are all measures of airway constriction or obstruction and indicate that AMG44 treatment is relieving or preventing lung pathology that negatively affects lung function. To quantitatively assess the impact of AMG44 treatment on MERS-CoV infected mice, histological lung sections were assessed using the American Thoracic Society (ATS) scoring system designed to more closely relate data from small animal models of acute lung injury (ALI) to infected patient outcomes as previously described (Matute-Bello et al., 2011; Sheahan et al., 2020a). Hematoxylin and eosin stained fields of lung tissue were blindly scored for the presence of neutrophils in the alveolar and interstitial space, hyaline membranes, proteinaceous debris filling the airway spaces, and thickening of the alveolar septa (Matute-Bello et al., 2011). ATS lung injury scores were significantly reduced in male but not female MERS-CoV infected AMG44 treated mice (Figure 7A-E). Together with the wide variety of supportive data described above, the *in vivo* studies with AMG44 directly demonstrate the importance of PERK signaling in promoting MERS-CoV pathogenesis in mice. Our unbiased systems biology-based approach identified a pathway differentially modulated during MERS-CoV infection of primary human cells, validated the importance of this pathway for MERS-CoV replication and cell death *in vitro* and then demonstrated its importance in regulating viral pathogenic outcomes *in vivo*, leading to a targeted antiviral therapy.

Discussion:

Viral pathogenesis is a multifactorial process driven by virus and host factors, responses in infected and neighboring cells, innate and adaptive immunity, host and viral genetics, and responses at the cellular, tissue, organ and organismal level. Due to this inherent complexity, defining the relative contribution of each of these factors to the disease state and the underlying cellular mechanisms that contribute to the progression towards severe disease is complicated. Thus, new models are needed to define cell and tissue specific host response patterns that play critical roles in viral pathogenesis. SARS-CoV, SARS-CoV 2 and MERS-CoV all cause severe respiratory disease with replication and pathogenesis primarily limited to the airways and lung. All three emerging coronavirus infections are thought to disrupt the endothelial vascular, which modulates leucocyte diapedesis, intravascular coagulation, vasomotor tone, and solute and the regulation of barrier permeability (). As shown in a recent pathologic evaluation of human tissue from a fatal case of MERS-CoV, diffuse alveolar damage, the pathological hallmark of acute lung injury (ALI), was noted along with viral antigen primarily found in alveolar pneumocytes with no extrapulmonary viral antigen detected (Ng et al., 2016). Similar findings have been reported following severe SARS-CoV and SARS-CoV2 infections, suggesting common mechanisms of coronavirus-induced ALI (Huang et al., 2020; Liu et al., 2020). While the few post-mortem analyses of tissue samples from MERS-CoV patients have provided insight into the types of cells targeted by virus and the degree and severity of pathology in the lung, many basic questions related to the kinetics of virus replication, cell targeting, and the host response remain unknown. Primary cell cultures offer an opportunity to decrease the complexity of the intact organ to study how virus host interactions determine the fate of specific cell types following infection. While most studies with pneumotropic viruses have focused on airway epithelial cells or type II

pneumocytes, MVE and FB play critical roles in maintaining lung architecture, compartmentalization and function (White, 2015). FB play a central role in the homeostasis of the extracellular matrix and are effector cells during injury repair, while the microvascular endothelium regulates vascular homeostasis, as vascular leakage can result in inefficient gas exchange in the lungs (Aman et al., 2016). In addition to MERS-CoV, H5N1 and other highly pathogenic influenza viruses also replicate efficiently in endothelial cells, providing diverse model systems to investigate virus-host interactions and identify novel tissue specific response patterns that can be used to attenuate virus replication and/or disease (Scobey et al., 2013; Tundup et al., 2017; Zeng et al., 2012). Understanding the fate of these cells after infection provides a unique opportunity to understand how MERS-CoV induced disease phenotypes manifest despite limited clinical sample availability. Death of MERS-CoV infected MVE and epithelial cells (Chan et al., 2013; Ng et al., 2016) could result in the breakdown of the epithelial-endothelial cell barrier and allow vascular leakage of fluids into the lung, leading to alveolar flooding, a hallmark feature of ALI and ARDS often seen in MERS-CoV, SARS-CoV and SARS-CoV2 patients and mouse models of human disease (Arabi et al., 2017; Cockrell et al., 2016; Das et al., 2016; Roberts et al., 2007). These findings support a more thorough examination of MERS-CoV lung cell tropism as clinical samples become available. The current findings also highlight that even within the same organ, cell types can respond to infection with very different outcomes and emphasize the value of studying primary cell types in culture.

During viral infection, unfolded and misfolded host and viral proteins accumulate in the endoplasmic reticulum (ER) disrupting ER homeostasis. ER stress is sensed by several ER chaperone proteins (GRP78, etc.), allowing the cells to activate pro-survival responses that are mediated in part by UPR signaling through PERK, the transmembrane protein inositol-requiring

protein-1 (IRE1α), and the activating transcription factor-6 (ATF6) (Dufey et al., 2014; Malhi and Kaufman, 2011). If the UPR response fails to restore ER homoeostasis, it promotes cell death through the induction of the specific proapoptotic genes like CHOP and various caspases (Logue et al., 2013). To date the impact of highly pathogenic human CoV infection on different lung cell types has been poorly studied in primary human lung cells. Analysis of systems biology datasets provides an unbiased approach to define host pathways that are crucial for virus replication and cell fate outcomes following infection, providing novel insights into different tissue and cell specific responses to viral infection. Functional enrichment analysis of transcriptomic, proteomic and lipidomic datasets suggested that activation of the UPR promoted apoptotic cell death in MERS-CoV infected MVE, as compared with FB cells that displayed a minimal UPR response. Analysis of a single dataset would have hinted at either activated stress response or specific cell death pathways in MERS-CoV infected MVE. However, concordant findings across transcriptomic, proteomic and lipidomic datasets in matched cell types across multiple patient donors suggested key indicators of activated UPR and apoptotic pathways in MERS-CoV infected MVE cultures supporting the hypothesis that prolonged UPR signaling led to ER stress, PERK activation and programmed apoptotic cell death. As virus titers and viral protein expression are equivalent in both cell types, the robust activation of the UPR and apoptotic responses in MVE, but not in FB cells derived from the same individual is puzzling. UPR is activated when the influx of nascent, unfolded polypeptides exceeds the folding capacity of the ER, which may differ between MVE and FB cells, yet viral structural and accessory protein levels were similar in both cell types. We hypothesize that lung FB can maintain ER homeostasis longer than infected MVE due to higher endogenous levels of ER folding chaperone proteins or increased overall ER volume, allowing continued cell viability after MERS-CoV infection. Future

studies will probe how primary lung FB survive MERS-CoV infection. Importantly, ceremide and triglyceride are also potent stimulators of ER stress and apoptosis and these lipids are also highly upregulated following MERS-CoV infection of MVE, but much less so in FB cells. Increased expression of multiple triglyceride and ceramide species (Aflaki et al., 2012; Kim et al., 2019) (products of sphingomyelinase pathways) is strongly correlative with virus induced CPE in mouse models demonstrating the *in vivo* relevance of these markers as well (Dautel et al., 2017). Future studies will need to focus on evaluating the cell type specific UPR responses to various biochemical stimuli, after contemporary coronavirus and other respiratory virus infections, and identifying which ceramides and triglycerides regulate the UPR and pro-apoptotic response in primary lung MVE and FB. The datasets described herein also provide a key resource for comparing the impact of different respiratory virus infections on MVE and FB function.

AMG44 is a selective and potent PERK inhibitor and treatment efficiently blocked MERS-CoV replication in FB and MVE cells *in vitro* (Smith et al., 2015). These studies validate the importance of PERK activation and the UPR response as being critical for MERS-CoV replication fitness early in infection, especially in MVE cells. The most likely mechanism for AMG44 inhibition of MERS-CoV growth is that the PERK signaling pathway and the UPR response promote increased membrane reorganization and suppressed host translation that optimizes an environmental milieu for improved virus replication, maturation and egress. AMG44 would promote ER stress and translation of host proteins, potentially disrupting virus replication complexes, budding and release. Coronavirus infection is associated with extensive membrane reorganization (Knoops et al., 2008; Zhou et al., 2010). In fact, coronavirus membrane-associated proteins nonstructural protein 3 (nsp3) and nsp4 have intrinsic membrane remodeling properties and usurp ER membranes to generate double membrane vesicles (DMVs) and other convoluted

membrane structures that likely function as prominent sites of coronavirus replication (Angelini et al., 2013; Knoops et al., 2008; Oudshoorn et al., 2017; Romero-Brey and Bartenschlager, 2016). Moreover, coronavirus egress occurs in a rER/Golgi intermediate compartment where coronavirus glycoproteins can induce ER stress and activate the UPR response (Bechill et al., 2008; Chu et al., 2016; Fung and Liu, 2014; Liao et al., 2013; Minakshi et al., 2009; Perrier et al., 2019; Rabouw et al., 2016; Sung et al., 2009; Wang et al., 2014; Xue et al., 2018). Other coronaviruses respond to the PERK signaling cascade activation in a variety of ways including increased virus induced apoptosis (Fung and Liu, 2014; Liao et al., 2013) or decreased viral titers (Rabouw et al., 2016; Wang et al., 2014; Xue et al., 2018). PERK activation was detected following overexpression of both SARS-CoV spike and ORF3a but the effects on replication were not determined (Chu et al., 2016; Minakshi et al., 2009; Sung et al., 2009). In contrast, the SARS-CoV E glycoprotein protected from UPR stress mediated by the IRE1 pathway (DeDiego et al., 2011). The role of MERS-CoV structural and nonstructural proteins are less certain although ORF4a has been reported to prevent activation of the stress response pathway via protein kinase R (PKR) antagonism (Rabouw et al., 2016). Interestingly, a previous study in Huh7 cells that explored the temporal kinome did not identify PERK following MERS-CoV infection, emphasizing the importance of using the most relevant primary human cells (Kindrachuk et al., 2015)

AMG44 prophylactic treatment protected MERS-CoV infected mice from clinical disease as evidenced by reduced weight loss, lung hemorrhage, and ALI signatures, leading to improved respiratory function and time to recovery. It is noteworthy that AMG44 treatment did not alter viral titers at day 3 post infection, perhaps reflecting the differential drug susceptibilities of the multiple cell types (e.g., airway epithelium, alveolar epithelial cells, FB and MVE) that are

infected by MERS-CoV *in vivo*. In mice, PERK activation may not be pro-viral in all MERS-CoV susceptible cell types and/or the dosing regimen may not have been equivalent in all infected cell types. These results also suggest that PERK may play a role in a damaging pathogenic process *in vivo* where the individual cell types are part of a larger organ-based physiology and that treatment with the PERK inhibitor actually improves pulmonary function.

Currently there are no approved therapeutics to treat highly pathogenic human coronavirus infections associated with the ongoing MERS-CoV epidemic or the 2019 SARS-CoV 2 pandemic, so methods to identify novel viral and host targets are recognized as a high priority by the WHO (Asokan et al., 2019). Despite considerable progress, no therapy is presently available to reverse the widespread vascular leak that is observed during ARDS. Additional treatment studies with AMG44 in the MERS-CoV challenge model are needed to resolve and expand upon the panel of disease-attenuating drug candidates. SARS-CoV, SARS-CoV2 and MERS-CoV cause more serious disease in males, so it is interesting that prophylactically treated male, but not female mice had a significant reduction in hallmark ALI disease pathologic phenotypes after MERS-CoV infection. Gender specific effects on ER stress have previously reported increased disease severity in males (). Adding additional days of treatment or performing twice daily treatment may improve the outcomes noted in the current study, using AMG44 in combination with robust antivirals (Sheahan et al., 2017; Sheahan et al., 2020a; Sheahan et al., 2020b), and determining the therapeutic window for AMG44 efficacy against MERS-CoV and SARS-CoV2 are also critical research priorities of the future. Nevertheless, the comprehensive systems biology approach described herein led to identification and validation of critical host pathways that mediated MVE cell death, leading to potential protein kinase based therapeutic treatment options that were successful in limiting disease phenotypes in mouse models of MERS-

CoV induced ALI. When coupled with the data presented here, modulating UPR responses provides a robust strategy to develop novel therapeutics to control infection mediated vascular permeability, inflammation and ALI, especially when linked with potent direct acting antiviral inhibitors of virus replication *in vivo* (Sheahan et al., 2017; Sheahan et al., 2020a). Given the critical importance of the vascular endothelium in ARDS, our data suggests a critical need to evaluate endothelial cell function in SARS-CoV-2 patients and animal models of human diseases, as a potential therapeutic target.

Materials and Methods:

Primary human lung cells and immortalized cells. Primary human lung cells were isolated from distal lung tissue and processed as described (Scobey et al., 2013). The lung cells were obtained under protocol #03-1396 approved by the University of North Carolina at Chapel Hill Institutional Review Board. Human lung microvascular endothelial cells (MVE) were cultured in Vasculife VEGF-MVE Endothelial Media (Lifeline Cell Tech). Human lung fibroblasts (FB) were cultured in DMEM-H Basal Medium (Corning), 1X penicillin/streptomycin (Sigma), 10% fetal bovine serum (Gemini Bio). Media fetal bovine serum concentrations were reduced to 4% prior to infection. Vero 81 cells were cultured in Dulbecco's modified essential media (DMEM Gibco) with 10% fetal clone II (Hyclone) and 1X antibiotic/antimycotic (Gibco).

Viruses and viral titration Wild-type MERS-CoV (EMC 2012 strain), MERS-CoV expressing nano luciferase (MERS nanoluc) and MERS-CoV expressing the red fluorescent protein (MERS-RFP) were rescued from infectious clones as previously described (Scobey et al., 2013; Sheahan et al., 2017). Vero C81 cells were used to generate viral stocks and to quantitate viral titers following infection (Scobey et al., 2013; Sheahan et al., 2017).

Infections to collect transcriptomic, proteomic, and lipidomic samples. Donor matched MVE and FB from three different human tissue donors were infected and samples harvested for systems biology analysis. For transcriptomic analysis, five replicate wells of MVE or FB were either mock-infected or infected with wild type MERS-CoV at multiplicity of infection (MOI) 5. At 0, 12, 24, 36, and 48 hours post infection, 100uL of media/supernatant was collected for viral titration assays, media removed and then 1mL of Trizol (Invitrogen/ThermoFisher) added per well. Total RNA samples were sent to Arraystar for downstream analysis. In parallel, an additional five replicate wells were subjected to the MPLEx protocol (chloroform/methanol, 2:1 mix) precipitation as previously described (Burnum-Johnson et al., 2017; Nakayasu et al., 2016), which results in simultaneous extraction of proteins, metabolities, and lipids with concomitant inactivation of virus. All proteomic and lipidomic samples were desiccated and frozen prior to shipment to Pacific Northwest National Laboratories for downstream analysis.

Transcriptomic Analysis Scanned images were analyzed using Agilent Feature Extraction Software (v11.0.1.1). The limma package for R (available on Bioconductor) was used to perform background correction, quantile normalization (normalizeBetweenArrays), and summarization (avereps) to derive a single normalized intensity value per probe. Outlier samples were detected using PCA and by visual inspection of heatmaps, and all data was re-processed after removing outlier samples. All data processing for each of the biological replicates was performed independently of the other.

Proteomic Analysis Samples for proteomics analysis were prepared for and analyzed using liquid chromatography-mass spectrometry and the Accurate Mass and Time Tag approach, as described previously (Sims et al., 2013). The RMD-PAV algorithm (Matzke et al., 2011) was used to identify any outlier biological samples and was confirmed via Pearson correlation. Peptides with

inadequate data for either qualitative or quantitative statistical tests were also removed from the dataset (Webb-Robertson et al., 2010). The SPANS algorithm (Webb-Robertson et al., 2010) was used to identify the best normalization method for each dataset. Peptides were evaluated with Analysis of Variance (ANOVA) with a Dunnett test correction and a Bonferroni-corrected g-test to compare each virus to the associated mock within each time point. To perform signature-based protein quantification, BP-Quant (Webb-Robertson et al., 2014), each peptide was categorized as a vector of length equal to the number of viruses being evaluated. If all comparisons for all time points are 0 for a specific virus it is considered as non-changing and given a value of 0. If there are more time points with an increase in virus to mock than decreasing it is categorized as a +1 and the contrary -1 is given for the decrease in virus to mock. BP-Quant was run with a default parameter of 0.9. All proteins were then analyzed using the same methodology as for the peptides; ANOVA with a Dunnett test correction and a Bonferroni-corrected g-test to compare each virus to the associated mock within each time point.

Lipidomic Analysis Samples for lipidomics analysis were prepared for and analyzed using liquid chromatography-tandem mass spectrometry, as described previously (Dautel et al., 2017). Lipids were identified using the in-house tool LIQUID (Kyle et al., 2017), and their quantitative data were extracted using MZmine 2.0 as previously described (Eisfeld et al., 2017). The RMD-PAV algorithm (Matzke et al., 2011) was also used to identify any outlier biological samples and was confirmed via Pearson correlation. Lipids with inadequate data for either qualitative or quantitative statistical tests were also removed from the dataset (Webb-Robertson et al., 2010). Median centering was used for normalization. Lipids were evaluated with a standard two sample t-test to compare each infected condition to the associated mock within each time point.

Functional Enrichment We identified significantly changed features (transcripts, proteins, lipids) by using an adjusted p-value of 0.05 as a threshold. For each experimental condition, perturbed features were divided into one list each of up- and down-regulated items. Each list was tested against the Gene Ontology (GO) database of gene sets using the EASE-adjusted one-sided Fisher exact test (Hosack et al., 2003), such that each condition is tested for up- and down-regulation of each function/pathway in the ontology database. Results across multiple conditions were visualized in heatmaps using the log10 p-values of the significance tests, colored to indicate the direction of change. For lipids, feature sets were generated by categorizing the lipids observed in our experiments according to broad lipid classifications; these classifications were then used as "gene sets" and the enrichment analysis was then performed in the same manner as for transcripts and proteins.

Cell viability and percent infection assays To assess numbers of viable cells following MERS-CoV infection of FB and MVE, we used the CellTiter-Glo Luminescent Cell Viability Assay (Promega) at 24 and 48 hours post infection/treatment according to manufacturer's instructions and relative light units were determined by Spectromax plate reader (Molecular Devices), averaged and plotted. Percent infection was determined using MVE and FB infected with MERS-RFP (MOI 5) or mock-infected and counting nuclei (Horcht stain diluted 1:5,000 in media) in replicate wells at 0, 12, 24, 36, and 48 hours post infection. Cell numbers were determined using the Fiji (Schindelin et al., 2012) cell counter function and total number of cells versus number of red fluorescent cells were determined for at least three wells per condition per cell type. Statistical significance was determined by Mann-Whitney U test (performed in GraphPad Prism).

Apoptosis Assays Infected FB and MVE were assayed using either the Caspase-Glo 3/7 Kit (Promega) to measure caspase activation or using the ApoTox-Glo Triplex Assay (Promega) to

assess cell viability, cytotoxicity and caspase 3/7 activation in a single set of samples at 24 and 48 hours post infection according to manufacturer's instructions. Control wells were treated with UV inactivated virus stocks, staurosporin (Sigma, 8uM for MVE or 10uM for FB) or ionomycin (ThermoFisher, 40uM for MVE and 60uM for FB). Relative light units or emission excitation wavelengths were determined by Spectromax plate reader (Molecular Devices). Statistical significance was determined by Mann-Whitney U test (performed in GraphPad Prism).

Inhibitor studies MERS-CoV infected MVE and FB were tested at 24 and 48 hours post infection following treatment with a dilution series PERK inhibitor (AMG 44(Rojas-Rivera et al., 2017; Smith et al., 2015) 100 uM to 2 uM) and was evaluated for cytotoxicity with drug treatment alone (cell viability assay, no virus), activation of the death caspases 3/7 (wild type MERS-CoV) and viral replication (detection of luciferase as a surrogate for replication, MERS nanoluc virus). All assay results were read by SpectraMax (Molecular Devices). Results are graphed as percent inhibition and toxicity (effective and cytotoxic concentration 50) as determined by GraphPad Prism.

Mouse studies hDPP4 mice (Cockrell et al., 2016) were housed and bred in accordance with the University of North Carolina Department of Comparative Medicine, AALAC #329. For a single group of mice, dosing occurred once a day 24 hours prior to infection and a second single dose was administered 24 hours post infection via the intraperitoneal route (12mg/kg of PERK AMG'44 suspended in water or with a sham control(Rojas-Rivera et al., 2017; Smith et al., 2015)). Immediately prior to infection, mice were anesthetized with 50 uL of ketamine/xylazine mixture via intraperitoneal injection. 16-20 week old mice were intranasally infected with 10^4 PFU of mouse adapted MERS-CoV maM35c4 diluted in OPTIMEM (Douglas et al., 2018), a total volume of 50 uL was given. Mice were weighed daily following infection and had their

respiratory function measured one day prior to infection and at days two through six post infection using Buxco Whole Body Plethysmography (Data Sciences International) (Menachery et al., 2015). Briefly, mice were allowed to acclimate for 30 minutes in the plethysmography chamber located within the biological safety cabinet after which measurements were taken for 5 minutes. Readings were collected every 2 seconds for a total of 150 measurements per mouse per day. At days three and seven post infection mice were euthanized via isoflurane overdose after which lung tissue was harvested to determine viral replication titers. Gross pulmonary hemorrhage was observed and scored from zero (none) to four (severe and total) at the time of dissection.

Statistical analysis All statistical data analyses were performed in Graphpad Prism 8. Statistical significance for each endpoint was determined with specific statistical tests. For each test, a p-value <0.05 was considered significant. Specific tests are noted in each figure legend.

Ethical use of laboratory animals Efficacy studies were performed under biosafety level three containment in a facility approved for the use of rodents at University of North Carolina at Chapel Hill (UNC). All work was conducted with protocols approved by the UNC Institutional Animal Care and Use Committee (Protocol #17-294) according to guidelines sets by the Association for the Assessment and Accreditation of Laboratory Animal Care and the United States Department of Agriculture.

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Author contributions ACS, SR, TOM, KW, RSB- designed *in vitro* systems biology experiments; ML, MLF, SR-harvested, isolated and characterized primary human lung cells; ACS, AW- performed *in vitro* systems biology experiment and phenotypic characterization; HM, JEK, KEBJ, RDS, TOM, KW-processed and analyzed systems biology samples/datasets; ACS, TPS, HM, RSB-designed cell viability and caspase assays; ACS- performed cell viability, caspase assays, PERK inhibitor assays; HM, JEK-functional enrichment analysis or transcript, protein and lipid datasets; LEG, TPS, ACS, RSB designed *in vivo* PERK inhibitor study; LEG performed PERK inhibitor study *in vivo*; ACS, TPS, RSB, HM, KW wrote the manuscript. **Competing interests**: UNC and PNNL are pursuing IP protection for AMG44 treatment of coronavirus infection. **Data and materials availability:** All datasets used for these analyses can be found at data.pnnl.gov under the following numbers. MERS-CoV infected fibroblasts-13095 (FB donor 1), 13096 (FB donor 2), 13097 (FB donor 3), MERS-CoV infected microvascular endothelial cells 13102 (MVE donor 1), 13103 (MVE donor 2), and 13104 (MVE donor 3).

Figure Legends

Figure 1. Viral growth kinetics, percent infected cells and cell viability following MERS-CoV infection of primary human lung fibroblasts and microvascular endothelial cells. A-B. Viral replication: Donor matched microvascular endothelial cells (MVE) and fibroblasts (FB) were infected with wild type MERS-CoV (MOI of 5) and supernatants collected at the indicated times and viral titers determined by plaque assay. Results are shown as plaque forming units (pfu) per mL over time. Each data point represents averaged data from supernatant collected from 10

different wells (5 wells harvested for RNA and 5 wells fractionated for proteins, metabolites and lipids). The graphs in A and B show levels of replication detected for all three tissue donors in both cell types. Error bars indicate standard deviation from the mean. Open and filled blue circles and blue triangles represent data from MERS-CoV infected FB, while open and filled red circles and red triangles represent MERS-CoV infected MVE. **C-D.** Percent MERS-CoV infected cells: Donor matched MVE and FB were mock-infected or infected with MERS-CoV expressing the red fluorescent protein (MERS-RFP), nuclei were stained with Hoechst stain, and images captured at the indicated times post infection. Total numbers of cells (shown for all conditions in **C**) and total numbers of infected (contained red fluorescence) cells were assessed for at least three fields per condition and total percent of infected cells determined and plotted (**D**). Representative data is shown for one of the three donors. Error bars indicate standard deviation from the mean. No statistical significance (ns) was found when comparing the percentage of infected FB and MVE at any specific timepoint. Statistical analysis performed in GraphPad determined by Mann-Whitney U test. Open blue bars- mock-infected FB, open red bars- mock-infected MVE, filled blue bars- MERS-RFP infected FB, filled red bars- MERS-CoV RFP infected MVE. **E-F.** Cell viability following MERS-CoV infection: Donor matched MVE and FB were infected with wild type MERS-CoV and assessed for cell viability using the CellTiter-Glo kit according to manufacturer's instructions (Promega) at the indicated times post infection. Relative light units were graphed over time and error bars indicate standard deviation from the mean. Statistical analysis performed in GraphPad determined by Mann-Whitney U test. Gray bars- mock infected cells, red bars- MERS-CoV infected MVE, blue bars- MERS-CoV infected FB, **** $p= <0.0001$, ** $p= <0.002$, ns not statistically significant.

Figure 2. Transcriptomics, proteomics, and lipidomics suggest activation of apoptotic pathways. A. Functional enrichment was performed on transcriptomic data from all three donor samples in both cell types, and results were only retained that were present in all three donors, keeping only the least significant score. In this way a true consensus response is represented by all indicated functions. **B.** Protein expression for proteins associated with the GO term "positive regulation of protein insertion into mitochondrial membrane involved in apoptotic signaling pathway". All apoptosis-associated proteins are displayed across the three donors for fibroblasts and MVE. The blue arrow highlights proteins in the hemoglobin complex and red asterisks highlight apoptotic proteins from the endoplasmic reticulum. **C.** Enrichment analysis using lipid classes as enrichment sets. Cer-ceramide, TG-triglycerides, PC-phosphatidylcholine, PE-phosphatidylethanolamine, PS-phosphatidylserine, SM-sphingomyelin. **D.** Abundance of individual lipid species.

Figure 3 Caspases 3/7 are activated following MERS-CoV infection of primary human lung MVE but not primary lung FB. A-D. Donor matched MVE and FB were plated and mock-infected or infected with UV-inactivated or wild type MERS-CoV and at 24 and 48 hours post infection caspase 3/7 activation or cell viability was determined according to manufacturer's instructions using the Apotox Triplex kit (each well was measured for both parameters). Uninfected cells were treated with staurosporin (8 uM MVE/10 uM FB) to determine the maximal amount of caspase 3/7 activation. Uninfected cells were treated with ionomycin (50 uM MVE/60 uM FB) to demonstrate loss of cell viability via non-apoptotic pathway. Results are graphed as relative light units and error bars indicate standard deviation from the mean. Statistical analysis performed in GraphPad determined by Mann-Whitney U test. **A-B** Results from caspase 3/7 activation. **C-D.** Results from cell viability assay. Solid red bars- MVE 24 hour samples, open

red bars- MVE 48 hour samples, solid blue bars- FB 24 hour samples, open blue bars- FB 48 hour samples. **** p=<0.0001, *** p= <0.0002, ns- not statistically significant.

Figure 4 Protein markers suggest activation of unfolded protein response (UPR). A-C. Protein expression of markers of the unfolded protein response. Each figure shows the expression behavior of a UPR marker in FB (blues) and MVE (reds), with individual donors represented separately as individual color hues. For each donor, a time course of expression is shown that includes samples taken at 0, 12, 24, 36 and 48 hours post infection. Values are expressed as the log10 p-value of the change between the infection and control conditions, with sign assigned according to the direction of fold change. **D-F.** Viral protein expression. MERS-CoV spike, open reading frame 4a (ORF4a) and membrane protein abundances are represented as the ratio of each protein to the average abundance of all other proteins detected in each respective sample.

Figure 5 PERK inhibitor reduces MERS-CoV replication in infected primary human lung MVE and FB. A-D. Donor matched uninfected FB (circles, A, B) and MVE (circles, C, D) were treated with serial dilutions of PERK inhibitor (100 uM to 2 uM) and cell viability assessed at 24 (A, C) and 48 (B, D) hours post treatment using Promega's CellTiter Glo kit according to manufacturer's instructions. Each circle represents mean values from two experiments and are graphed as percent toxicity. Error bars indicate standard deviation from the mean. In parallel, the same donor matched FB (squares, A, B) and MVE (squares, C, D) were infected with MERS-nanoluc and simultaneously treated with the same serial dilutions of PERK inhibitor. Nanoluciferase expression was assayed at 24 (squares, A, C) and 48 (squares, B, D) hours post infection using Promega's NanoGlo kit according to manufacturer's instructions. Control wells were either treated with drug diluent and UV-inactivated virus or infected with MERS-nanoluc and treated with only drug diluent. Each square represents mean values from two experiments

and are graphed as percent inhibition. Error bars indicate standard deviation from the mean. Blue squares- MERS-nanoluc infected FB treated with PERK inhibitor serial dilutions, blue circles- uninfected FB treated with PERK inhibitor serial dilutions, red squares- MERS-nanoluc infected MVE treated with PERK inhibitor serial dilutions, red circles- uninfected MVE treated with PERK inhibitor serial dilutions. PERK protein kinase R-like ER kinase, MVE primary human lung microvascular endothelial cell, FB primary human lung fibroblast, MERS-nanoluc MERS-CoV expressing nanoluciferase

Figure 6 PERK inhibitor AMG44 treatment decreases MERS-CoV pathogenesis

A-F. hDPP4 mice were treated with PERK AMG'44 or sham control and infected with $5x10^4$ PFU of MERS-CoV MAm35c4 or PBS control. Weight loss was observed over the course of a seven day infection (A) and viral titers (B) and pulmonary hemorrhage was scored at the time of harvest (C). Daily respiratory function measurements (D-F) were taken via whole body plethysmography. Error bars indicate standard error of the mean, # indicates a p value of < 0.1 and * indicates a p value of < 0.05.

Figure 7 AMG44 reduces clinical signs of acute lung injury in MERS-CoV challenge model

A-E. The histological features of acute lung injury were blindly scored using the American Thoracic Society Lung Injury Scoring system by Matute-Bello, creating an aggregate score for: neutrophils in the alveolar and interstitial space, hyaline membranes, proteinaceous debris filling the air spaces, and alveolar septal thickening. **A-B.** Scoring for ALI and diffuse alveolar damage in male and female mice, respectively. Representative lung pathology for C) mock-infected, D) MERS-CoV infected and sham treated and E) MERS-CoV infected and AMG44 treated male mice at 7 days post infection. * indicates a p value of <0.001.

Supplementary Materials

Supplementary Materials and Methods

All methodologies are described in the Materials and Methods section above.

Supplementary Figures and Tables Legends

Supplementary Fig. S1 MERS-CoV reduces cell viability for all three donors in MVE but not FB. Donor matched MVE and FB were infected with wild type MERS-CoV and assessed for cell viability using the CellTiter-Glo kit according to manufacturer's instructions (Promega) at the indicated times post infection. Relative light units (RLU) were graphed over time and error bars indicate standard deviation from the mean. Statistical analysis performed in GraphPad determined by Mann-Whitney U test. Gray bars- mock infected cells, red bars- MERS-CoV infected MVE, blue bars- MERS-CoV infected FB, **** p= <0.0001, ** p= <0.002, ns not statistically significant.

Supplementary Fig. S2 Neither SMAD7 nor FGF2 expression contribute to MERS-CoV induced cytopathic effect in primary human lung cells. A-B. Graphs show transcript expression levels for SMAD7 and FGF2 in MERS-CoV infected MVE and FB over the 48 hour time course (derived from microarray analysis and are from the same datasets used in Figure 2). Values graphed are fold change (log_2) and error bars are standard deviations from the mean. mothers against decapentaplegic homolog 7 (SMAD7); fibroblast growth factor 2 (FGF2)

Supplementary Fig. S3 Inhibition of PERK by AMG44 does not alter caspase 3/7 activation following MERS-CoV infection. A-D. Donor matched FB (A, B) and MVE (C, D) were plated and infected with UV-inactivated or wild type MERS-CoV and at 24 (A, C) and 48 (B, D) hours

post infection caspase 3/7 activation was determined according to manufacturer's instructions using the Apotox Triplex kit. Control uninfected cells were treated with staurosporin (8 uM MVE/10 uM FB, activates caspase 3/7) or ionomycin (50 uM MVE/60 uM FB, does not activate caspase 3/7). Results are graphed as relative light units and error bars indicate standard deviation from the mean. Statistical analysis performed in GraphPad determined by Mann-Whitney U test.

Supplementary Table 1 Enrichment results from transcriptomic data represented in Figure 2

Please see separate Excel spreadsheet.

Supplementary Table 2 Enrichment results from proteomic data represented in Figure 2

Please see separate Excel spreadsheet.

Andrew J. Rennekamp, Ph.D.
Scientific Editor, *Cell*
areneekamp@cell.com

April 28, 2020

Dear Dr. Rennekamp,

Please find included our research article entitled "Unfolded protein response inhibition reduces MERS-CoV induced acute lung injury". Currently, two newly emerged human coronaviruses are causing disease globally, SARS-CoV-2 and the MERS-CoV. The overall goal of our work is to use bioinformatic analysis of systems biology datasets derived from MERS-CoV infected primary human lung cells to understand how infection of each cell type may contribute to disease outcomes in the intact lung and to define host pathways that can be targeted for therapeutic intervention. In contrast to SARS-CoV, MERS-CoV infects primary human lung endothelial cells and fibroblasts in addition to epithelial cells. Here we demonstrate that MERS-CoV replicates equivalently in primary human lung fibroblasts and endothelial cells, but cytopathic effect was noted exclusively in the infected endothelial cells. To explore mechanisms leading to reduced MVE viability, donor-matched human lung MVE and FB were infected, and their transcriptomes, proteomes, and lipidomes followed over time. Validated functional enrichment analysis demonstrated that MERS-CoV-infected MVE were dying via an unfolded protein response (UPR) mediated apoptosis. Pharmacologic manipulation of the UPR in MERS-CoV infected primary hung lung cells reduced viral replication and treatment of infected mice improved respiratory function and reduced virus induced weight loss. Most importantly, UPR inhibition led to a significant reduction in pathologic signatures of acute lung injury and times to recovery in treated infected mice. Our comprehensive systems biology approach led to identification and validation of critical host pathways that mediated endothelial cell death and to potential protein kinase based therapeutic treatment options that were successful in limiting disease phenotypes in mouse models of MERS-CoV induced acute lung injury. Modulating activation of the UPR provides a robust strategy to identify therapeutics to control infection mediated vascular permeability, inflammation and acute lung injury, especially when linked with potent direct acting antiviral inhibitors of virus replication *in vivo*. *Using emerging coronaviruses as models, our study design provides a novel approach to identify host kinases and networks that can be targeted with inhibitors to reverse disease signatures in animal models of human disease.*

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From: Amy Sims <amy.sims@pnnl.gov>
Date: Wednesday, April 29, 2020 at 2:27 PM
To: "Baric, Ralph S" <rbaric@email.unc.edu>, "Waters, Katrina M" <Katrina.Waters@pnnl.gov>, "Mitchell, Hugh D" <Hugh.Mitchell@pnnl.gov>, "Sheahan, Timothy Patrick" <sheahan@email.unc.edu>
Subject: FW: Confirmation for your Cell submission

It's in the hands of Cell. Thank you all, Amy

From: <em.cell.0.6af207.651cd697@editorialmanager.com> on behalf of Cell Editorial Office <em@editorialmanager.com>
Reply-To: Cell Editorial Office <cellms@cell.com>
Date: Wednesday, April 29, 2020 at 11:25 AM
To: "Sims, Amy C" <amy.sims@pnnl.gov>
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Dear Dr. Sims,

Thank you for your submission to Cell. The manuscript entitled "Unfolded Protein Response Inhibition Reduces MERS-CoV Induced Acute Lung Injury" has been received by our editorial office and will be given a manuscript number once an Editor has been assigned.

You are welcome to login to Editorial Manager at https://www.editorialmanager.com/cell/ at any time for a status update.

Best,

Jennifer Estrompa
Editorial Operations Associate, Cell
50 Hampshire Street
Cambridge, MA 02139
(617) 397-2825
cellms@cell.com

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From: Sheahan, Timothy Patrick[sheahan@email.unc.edu]
Sent: Wed 4/29/2020 11:31:58 AM (UTC-07:00)
To: Sims, Amy C[amy.sims@pnnl.gov]
Subject: Re: Confirmation for your Cell submission

Well. High five. Now we wait!

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Date: Wednesday, April 29, 2020 at 2:31 PM
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Sent: Wed 4/29/2020 11:50:54 AM (UTC-07:00)
To: Sims, Amy C[amy.sims@pnnl.gov]
Subject: Re: Confirmation for your Cell submission

Yea. We are ok. Hard to know how the summer is going to go. If camps are cancelled, we are going to have a work-life balance problem! It's bad enough the kids are in school for another month but at home. Work is crazy. Blah blah blah. Lots of papers to write and get out. So happy to have your paper out! What's next for you?

From: Amy Sims <amy.sims@pnnl.gov>
Date: Wednesday, April 29, 2020 at 2:35 PM
To: "Sheahan, Timothy Patrick" <sheahan@email.unc.edu>
Subject: Re: Confirmation for your Cell submission

Hope you are doing well. I know you have to be crazy stupid busy. Miss seeing the ladies Sheahan. And you too of course!

From: "Sheahan, Timothy Patrick" <sheahan@email.unc.edu>
Date: Wednesday, April 29, 2020 at 11:33 AM
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Subject: Re: Confirmation for your Cell submission

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From: Sims, Amy C[amy.sims@pnnl.gov]
Sent: Thur 4/30/2020 10:23:33 AM (UTC-07:00)
To: Caldwell, Chandra[ccaldwel@email.unc.edu]
Subject: Re: 76382 Relinquish NIH Grant status

Wow. Thank you very much! I really appreciate the head's up. Amy

---

From: "Caldwell, Chandra" <ccaldwel@email.unc.edu>
Date: Thursday, April 30, 2020 at 10:19 AM
To: "Sims, Amy C" <amy.sims@pnnl.gov>
Subject: FW: 76382 Relinquish NIH Grant status

FYI: Progress is finally being made….now it goes to OSR to move it along once Vicki reviews and signs it. I know she will be online in the AM since she is doing a series of Zoom calls back to back this afternoon.

C.

From: Burkhart, Carol J. <carol_burkhart@unc.edu>
Sent: Thursday, April 30, 2020 12:00 PM
To: Moore, Victoria L <victoria_moore@unc.edu>
Cc: Caldwell, Chandra <ccaldwel@email.unc.edu>
Subject: FW: 76382 Relinquish NIH Grant status

Hi Vicki,

Attached is Tim's relinquishing statement and prior approval form. Ronda will need to initiate and submit the request in eRA Commons. Please let me know if any changes are needed.
If not, please sign the prior approval form and return to me.

Thanks,

Carol

From: Caldwell, Chandra <ccaldwel@email.unc.edu>
Sent: Wednesday, April 29, 2020 5:48 PM
To: Burkhart, Carol J. <carol_burkhart@unc.edu>
Cc: Moore, Victoria L <victoria_moore@unc.edu>
Subject: RE: 76382 Relinquish NIH Grant status

Hi Carol,

Below is Tim Sheahan's eRA info after submitting the RPPR for Amy's R21 grant earlier this month.

Per my discussion with Vicki this afternoon, please move forward in initiating the relinquishing statement for grant #: R21 AI146872, *How MERS-CoV Regulates Innate Immunity in Primary Human Lung Cells*. Vicki would like to review the relinquishing statement before it is submitted.

Thanks,
Chandra

From: Moore, Victoria L <victoria_moore@unc.edu>
Sent: Wednesday, April 29, 2020 5:01 PM
To: Caldwell, Chandra <ccaldwel@email.unc.edu>
Subject: FW: 76382 Relinquish NIH Grant status

From: Hays, Barbara A <barb.hays@pnnl.gov>
Sent: Monday, April 27, 2020 7:35 PM
To: Moore, Victoria L <victoria_moore@unc.edu>; Parrish, Kalyn <klparrish@unc.edu>
Cc: Hays, Barbara A <barb.hays@pnnl.gov>; Sims, Amy C <amy.sims@pnnl.gov>; Sandoval, Norma I <Norma.Sandoval@pnnl.gov>
Subject: 76382 Relinquish NIH Grant status

Kalyn and Vicki, I'll be your contracts point of contact to support Amy and Norma with the transfer of Amy's NIH Grant from UNC to PNNL.

- What is your planned timeline for the UNC relinquishment request to NIH?
- What do you need from us and when?

I assume I will receive a request for proposal from NIH at some point? Any guidance you can give us to get our proposal documents ready would be much appreciated. We look forward to hearing from you.
Thank you

Barb Hays
Senior Contracts Specialist
Pacific Northwest National Laboratory
902 Battelle Boulevard
P.O. Box 999
Richland, WA 99352 USA
Tel: 509-372-4008

barb.hays@pnnl.gov

From: Baric, Ralph S[rbaric@email.unc.edu]
Sent: Thur 5/7/2020 9:39:17 AM (UTC-07:00)
To: Sims, Amy C[amy.sims@pnnl.gov]
Subject: RE: Confirmation for your Cell submission

Hi Amy, Did the paper go out for review? Ralph

From: Sims, Amy C <amy.sims@pnnl.gov>
Sent: Wednesday, April 29, 2020 2:30 PM
To: Baric, Ralph S <rbaric@email.unc.edu>
Subject: Re: Confirmation for your Cell submission

Thank you for reading it again. I felt much better about submitting it today. Thank you again, Amy

---

From: "Baric, Ralph S" <rbaric@email.unc.edu>
Date: Wednesday, April 29, 2020 at 11:28 AM
To: "Sims, Amy C" <amy.sims@pnnl.gov>
Subject: RE: Confirmation for your Cell submission

Awesome! congrats

From: Sims, Amy C <amy.sims@pnnl.gov>
Sent: Wednesday, April 29, 2020 2:27 PM
To: Baric, Ralph S <rbaric@email.unc.edu>; Waters, Katrina M <Katrina.Waters@pnnl.gov>; Mitchell, Hugh D <Hugh.Mitchell@pnnl.gov>; Sheahan, Timothy Patrick <sheahan@email.unc.edu>
Subject: FW: Confirmation for your Cell submission

It's in the hands of Cell. Thank you all, Amy

From: <em.cell.0.6af207.651cd697@editorialmanager.com> on behalf of Cell Editorial Office <em@editorialmanager.com>
Reply-To: Cell Editorial Office <cellms@cell.com>
Date: Wednesday, April 29, 2020 at 11:25 AM
To: "Sims, Amy C" <amy.sims@pnnl.gov>
Subject: Confirmation for your Cell submission

Dear Dr. Sims,

Thank you for your submission to Cell. The manuscript entitled "Unfolded Protein Response Inhibition Reduces MERS-CoV Induced Acute Lung Injury" has been received by our editorial office and will be given a manuscript number once an Editor has been assigned.

You are welcome to login to Editorial Manager at https://www.editorialmanager.com/cell/ at any time for a status update.

Best,

Jennifer Estrompa
Editorial Operations Associate, Cell
50 Hampshire Street

Cambridge, MA 02139
(617) 397-2825
cellms@cell.com

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From: Sims, Amy C[amy.sims@pnnl.gov]
Sent: Thur 5/7/2020 10:22:16 AM (UTC-07:00)
To: Baric, Ralph S[rbaric@email.unc.edu]
Subject: Re: Confirmation for your Cell submission

Actually I looked yesterday and it said "with the editor". I pulled it up now and it says "under review".

I am guessing that means yes it has progressed. Hope you are doing well.

Yesterday was Lisa's birthday in case you missed it! Thank you, Amy

---

From: "Baric, Ralph S" <rbaric@email.unc.edu>
Date: Thursday, May 7, 2020 at 9:39 AM
To: "Sims, Amy C" <amy.sims@pnnl.gov>
Subject: RE: Confirmation for your Cell submission

Hi Amy, Did the paper go out for review? Ralph

From: Sims, Amy C <amy.sims@pnnl.gov>
Sent: Wednesday, April 29, 2020 2:30 PM
To: Baric, Ralph S <rbaric@email.unc.edu>
Subject: Re: Confirmation for your Cell submission

Thank you for reading it again. I felt much better about submitting it today. Thank you again, Amy

---

From: "Baric, Ralph S" <rbaric@email.unc.edu>
Date: Wednesday, April 29, 2020 at 11:28 AM
To: "Sims, Amy C" <amy.sims@pnnl.gov>
Subject: RE: Confirmation for your Cell submission

Awesome! congrats

From: Sims, Amy C <amy.sims@pnnl.gov>
Sent: Wednesday, April 29, 2020 2:27 PM
To: Baric, Ralph S <rbaric@email.unc.edu>; Waters, Katrina M <Katrina.Waters@pnnl.gov>; Mitchell, Hugh D <Hugh.Mitchell@pnnl.gov>; Sheahan, Timothy Patrick <sheahan@email.unc.edu>
Subject: FW: Confirmation for your Cell submission

It's in the hands of Cell. Thank you all, Amy

From: <em.cell.0.6af207.651cd697@editorialmanager.com> on behalf of Cell Editorial Office <em@editorialmanager.com>
Reply-To: Cell Editorial Office <cellms@cell.com>
Date: Wednesday, April 29, 2020 at 11:25 AM
To: "Sims, Amy C" <amy.sims@pnnl.gov>
Subject: Confirmation for your Cell submission

Dear Dr. Sims,

Thank you for your submission to Cell. The manuscript entitled "Unfolded Protein Response Inhibition Reduces MERS-CoV Induced Acute Lung Injury" has been received by our editorial office and will be given a manuscript number once an Editor has been assigned.

You are welcome to login to Editorial Manager at https://www.editorialmanager.com/cell/ at any time for a status update.

Best,

Jennifer Estrompa
Editorial Operations Associate, Cell
50 Hampshire Street
Cambridge, MA 02139
(617) 397-2825
cellms@cell.com

Sneak Peek 2.0: Now it's even easier to preview Cell Press papers under review

Exciting science needs to be shared, and fast. That's why we launched Cell Press Sneak Peek, an author opt-in preview of the papers under review in our primary research journals. Sneak Peek makes papers discoverable earlier in the publication process—so authors can surface their research quickly and readers can build on their work. Now hosted on SSRN, Sneak Peek 2.0 has improved search and easy access to abstracts. DOI registration and single article links means papers posted to Sneak Peek can be cited.
Go on, satisfy your curiosity! Visit Sneak Peek.

In compliance with data protection regulations, you may request that we remove your personal registration details at any time. (Remove my information/details). Please contact the publication office if you have any questions.

From: Sims, Amy C[amy.sims@pnnl.gov]

Sent: Thur 5/7/2020 10:31:26 AM (UTC-07:00)

To: Mitchell, Hugh D[Hugh.Mitchell@pnnl.gov]; Gralinski, Lisa E[lgralins@email.unc.edu]; Kyle, Jennifer E[Jennifer.Kyle@pnnl.gov]; Burnum-Johnson, Kristin E[Kristin.Burnum-Johnson@pnnl.gov]; Lam, Mariam[mariam_lam@med.unc.edu]; Fulcher, Leslie[leslie_fulcher@med.unc.edu]; Smith, Richard D[dick.smith@pnnl.gov]; Metz, Thomas O[Thomas.Metz@pnnl.gov]; Sheahan, Timothy Patrick[sheahan@email.unc.edu]; Waters, Katrina M[Katrina.Waters@pnnl.gov]; Baric, Ralph S[rbaric@email.unc.edu]; westande@email.unc.edu[westande@email.unc.edu]; scott_randell@med.unc.edu[scott_randell@med.unc.edu]

Subject: Re: Apoptosis paper

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From: Gralinski, Lisa E[lgralins@email.unc.edu]

Sent: Thur 5/7/2020 10:34:13 AM (UTC-07:00)

To: Sims, Amy C[amy.sims@pnnl.gov]; Mitchell, Hugh D[Hugh.Mitchell@pnnl.gov]; Kyle, Jennifer E[Jennifer.Kyle@pnnl.gov]; Burnum-Johnson, Kristin E[Kristin.Burnum-Johnson@pnnl.gov]; Lam, Mariam[mariam_lam@med.unc.edu]; Fulcher, Leslie[leslie_fulcher@med.unc.edu]; Smith, Richard D[dick.smith@pnnl.gov]; Metz, Thomas O[Thomas.Metz@pnnl.gov]; Sheahan, Timothy Patrick[sheahan@email.unc.edu]; Waters, Katrina M[Katrina.Waters@pnnl.gov]; Baric, Ralph S[rbaric@email.unc.edu]; West, Ande[westande@email.unc.edu]; Randell, Scott H.[scott_randell@med.unc.edu]

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From: Waters, Katrina M[Katrina.Waters@pnnl.gov]

Sent: Thur 5/7/2020 10:36:27 AM (UTC-07:00)

To: Gralinski, Lisa E[lgralins@email.unc.edu]; Sims, Amy C[amy.sims@pnnl.gov]; Mitchell, Hugh D[Hugh.Mitchell@pnnl.gov]; Kyle, Jennifer E[Jennifer.Kyle@pnnl.gov]; Burnum-Johnson, Kristin E[Kristin.Burnum-Johnson@pnnl.gov]; Lam, Mariam[mariam_lam@med.unc.edu]; Fulcher, Leslie[leslie_fulcher@med.unc.edu]; Smith, Richard D[dick.smith@pnnl.gov]; Metz, Thomas O[Thomas.Metz@pnnl.gov]; Sheahan, Timothy Patrick[sheahan@email.unc.edu]; Baric, Ralph S[rbaric@email.unc.edu]; West, Ande[westande@email.unc.edu]; Randell, Scott H.[scott_randell@med.unc.edu]

Subject: RE: Apoptosis paper

Thanks for the update Amy!

Katrina Waters
Tel: 509-375-3907
Biological Sciences Division

From: Gralinski, Lisa E <lgralins@email.unc.edu>
Sent: Thursday, May 7, 2020 10:34 AM
To: Sims, Amy C <amy.sims@pnnl.gov>; Mitchell, Hugh D <Hugh.Mitchell@pnnl.gov>; Kyle, Jennifer E <Jennifer.Kyle@pnnl.gov>; Burnum-Johnson, Kristin E <Kristin.Burnum-Johnson@pnnl.gov>; Lam, Mariam <mariam_lam@med.unc.edu>; Fulcher, Leslie <leslie_fulcher@med.unc.edu>; Smith, Richard D <dick.smith@pnnl.gov>; Metz, Thomas O <Thomas.Metz@pnnl.gov>; Sheahan, Timothy Patrick <sheahan@email.unc.edu>; Waters, Katrina M <Katrina.Waters@pnnl.gov>; Baric, Ralph S <rbaric@email.unc.edu>; West, Ande <westande@email.unc.edu>; Randell, Scott H. <scott_randell@med.unc.edu>
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To: "Mitchell, Hugh D" <Hugh.Mitchell@pnnl.gov>, "Gralinski, Lisa E" <lgralins@email.unc.edu>, "Kyle, Jennifer E" <Jennifer.Kyle@pnnl.gov>, "Burnum-Johnson, Kristin E" <Kristin.Burnum-Johnson@pnnl.gov>, "Lam, Mariam" <mariam_lam@med.unc.edu>, "Fulcher, Leslie" <leslie_fulcher@med.unc.edu>, "Smith,

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Sent: Thur 5/7/2020 10:37:04 AM (UTC-07:00)

To: Sims, Amy C[amy.sims@pnnl.gov]; Gralinski, Lisa E[lgralins@email.unc.edu]; Kyle, Jennifer E[Jennifer.Kyle@pnnl.gov]; Burnum-Johnson, Kristin E[Kristin.Burnum-Johnson@pnnl.gov]; Lam, Mariam[mariam_lam@med.unc.edu]; Fulcher, Leslie[leslie_fulcher@med.unc.edu]; Smith, Richard D[dick.smith@pnnl.gov]; Metz, Thomas O[Thomas.Metz@pnnl.gov]; Sheahan, Timothy Patrick[sheahan@email.unc.edu]; Waters, Katrina M[Katrina.Waters@pnnl.gov]; Baric, Ralph S[rbaric@email.unc.edu]; westande@email.unc.edu[westande@email.unc.edu]; scott_randell@med.unc.edu[scott_randell@med.unc.edu]

Subject: RE: Apoptosis paper

Yeah that's great news!

From: Sims, Amy C <amy.sims@pnnl.gov>
Sent: Thursday, May 07, 2020 10:31 AM
To: Mitchell, Hugh D <Hugh.Mitchell@pnnl.gov>; Gralinski, Lisa E <lgralins@email.unc.edu>; Kyle, Jennifer E <Jennifer.Kyle@pnnl.gov>; Burnum-Johnson, Kristin E <Kristin.Burnum-Johnson@pnnl.gov>; Lam, Mariam <mariam_lam@med.unc.edu>; Fulcher, Leslie <leslie_fulcher@med.unc.edu>; Smith, Richard D <dick.smith@pnnl.gov>; Metz, Thomas O <Thomas.Metz@pnnl.gov>; Sheahan, Timothy Patrick <sheahan@email.unc.edu>; Waters, Katrina M <Katrina.Waters@pnnl.gov>; Baric, Ralph S <rbaric@email.unc.edu>; westande@email.unc.edu; scott_randell@med.unc.edu
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From: Lam, Mariam[mariam_lam@med.unc.edu]

Sent: Thur 5/7/2020 10:38:35 AM (UTC-07:00)

To: Sims, Amy C[amy.sims@pnnl.gov]; Mitchell, Hugh D[Hugh.Mitchell@pnnl.gov]; Gralinski, Lisa E[lgralins@email.unc.edu]; Kyle, Jennifer E[Jennifer.Kyle@pnnl.gov]; Burnum-Johnson, Kristin E[Kristin.Burnum-Johnson@pnnl.gov]; Fulcher, Leslie[leslie_fulcher@med.unc.edu]; Smith, Richard D[dick.smith@pnnl.gov]; Metz, Thomas O[Thomas.Metz@pnnl.gov]; Sheahan, Timothy Patrick[sheahan@email.unc.edu]; Waters, Katrina M[Katrina.Waters@pnnl.gov]; Baric, Ralph S[rbaric@email.unc.edu]; West, Ande[westande@email.unc.edu]; Randell, Scott H.[scott_randell@med.unc.edu]

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Very exciting!! Thank you for sharing!

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Sent: Thursday, May 7, 2020 1:31 PM
To: Mitchell, Hugh D <Hugh.Mitchell@pnnl.gov>; Gralinski, Lisa E <lgralins@email.unc.edu>; Kyle, Jennifer E <Jennifer.Kyle@pnnl.gov>; Burnum-Johnson, Kristin E <Kristin.Burnum-Johnson@pnnl.gov>; Lam, Mariam <mariam_lam@med.unc.edu>; Fulcher, Leslie <leslie_fulcher@med.unc.edu>; Smith, Richard D <dick.smith@pnnl.gov>; Metz, Thomas O <Thomas.Metz@pnnl.gov>; Sheahan, Timothy Patrick <sheahan@email.unc.edu>; Waters, Katrina M <Katrina.Waters@pnnl.gov>; Baric, Ralph S <rbaric@email.unc.edu>; West, Ande <westande@email.unc.edu>; Randell, Scott H. <scott_randell@med.unc.edu>
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Sent: Thur 5/7/2020 10:39:15 AM (UTC-07:00)

To: Sims, Amy C[amy.sims@pnnl.gov]; Mitchell, Hugh D[Hugh.Mitchell@pnnl.gov]; Gralinski, Lisa E[lgralins@email.unc.edu]; Kyle, Jennifer E[Jennifer.Kyle@pnnl.gov]; Burnum-Johnson, Kristin E[Kristin.Burnum-Johnson@pnnl.gov]; Lam, Mariam[mariam_lam@med.unc.edu]; Fulcher, Leslie[leslie_fulcher@med.unc.edu]; Smith, Richard D[dick.smith@pnnl.gov]; Sheahan, Timothy Patrick[sheahan@email.unc.edu]; Waters, Katrina M[Katrina.Waters@pnnl.gov]; Baric, Ralph S[rbaric@email.unc.edu]; westande@email.unc.edu[westande@email.unc.edu]; scott_randell@med.unc.edu[scott_randell@med.unc.edu]

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Great news!

Tom

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Sent: Thur 5/7/2020 10:55:24 AM (UTC-07:00)

To: Metz, Thomas O[Thomas.Metz@pnnl.gov]; Sims, Amy C[amy.sims@pnnl.gov]; Mitchell, Hugh D[Hugh.Mitchell@pnnl.gov]; Gralinski, Lisa E[lgralins@email.unc.edu]; Kyle, Jennifer E[Jennifer.Kyle@pnnl.gov]; Burnum-Johnson, Kristin E[Kristin.Burnum-Johnson@pnnl.gov]; Lam, Mariam[mariam_lam@med.unc.edu]; Fulcher, Leslie[leslie_fulcher@med.unc.edu]; Smith, Richard D[dick.smith@pnnl.gov]; Waters, Katrina M[Katrina.Waters@pnnl.gov]; Baric, Ralph S[rbaric@email.unc.edu]; West, Ande[westande@email.unc.edu]; Randell, Scott H.[scott_randell@med.unc.edu]

Subject: Re: Apoptosis paper

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T

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Many thanks to all who submitted comments (earlier and this round). I believe I have them all incorporated now. I got an email from Ralph and Tim this morning and apparently the editor at Cell would like to review the paper. I have made the necessary modifications to meet the journal requirements.
If anyone has any objections please let me know.

Thank you very much. Amy

PS. I hope everyone is doing well.

From: Sims, Amy C[amy.sims@pnnl.gov]
Sent: Thur 5/7/2020 10:59:31 AM (UTC-07:00)
To: Sheahan, Timothy Patrick[sheahan@email.unc.edu]
Subject: Re: Apoptosis paper

Fingers crossed. Would be good to start pondering the graphical abstract just in case.

Thank you! Amy

From: "Sheahan, Timothy Patrick" <sheahan@email.unc.edu>
Date: Thursday, May 7, 2020 at 10:55 AM
To: "Metz, Thomas O" <Thomas.Metz@pnnl.gov>, "Sims, Amy C" <amy.sims@pnnl.gov>, "Mitchell, Hugh D" <Hugh.Mitchell@pnnl.gov>, "Gralinski, Lisa E" <lgralins@email.unc.edu>, "Kyle, Jennifer E" <Jennifer.Kyle@pnnl.gov>, "Burnum-Johnson, Kristin E" <Kristin.Burnum-Johnson@pnnl.gov>, "Lam, Mariam" <mariam_lam@med.unc.edu>, "Fulcher, Leslie" <leslie_fulcher@med.unc.edu>, "Smith, Richard D" <dick.smith@pnnl.gov>, "Waters, Katrina M" <Katrina.Waters@pnnl.gov>, "Baric, Ralph S" <rbaric@email.unc.edu>, "West, Ande" <westande@email.unc.edu>, "Randell, Scott H." <scott_randell@med.unc.edu>
Subject: Re: Apoptosis paper

Super! Let's hope they like it.

T

From: "Metz, Thomas O" <Thomas.Metz@pnnl.gov>
Date: Thursday, May 7, 2020 at 1:39 PM
To: Amy Sims <amy.sims@pnnl.gov>, "Mitchell, Hugh D" <Hugh.Mitchell@pnnl.gov>, "Gralinski, Lisa E" <lgralins@email.unc.edu>, "Kyle, Jennifer E" <Jennifer.Kyle@pnnl.gov>, "Burnum-Johnson, Kristin E" <Kristin.Burnum-Johnson@pnnl.gov>, "Lam, Mariam" <mariam_lam@med.unc.edu>, "Fulcher, Leslie" <leslie_fulcher@med.unc.edu>, "Smith, Richard D" <dick.smith@pnnl.gov>, "Sheahan, Timothy Patrick" <sheahan@email.unc.edu>, "Waters, Katrina M" <Katrina.Waters@pnnl.gov>, "Baric, Ralph S" <rbaric@email.unc.edu>, "West, Ande" <westande@email.unc.edu>, "Randell, Scott H." <scott_randell@med.unc.edu>
Subject: RE: Apoptosis paper

Great news!

Tom

From: Sims, Amy C <amy.sims@pnnl.gov>
Sent: Thursday, May 7, 2020 10:31 AM
To: Mitchell, Hugh D <Hugh.Mitchell@pnnl.gov>; Gralinski, Lisa E <lgralins@email.unc.edu>; Kyle, Jennifer E <Jennifer.Kyle@pnnl.gov>; Burnum-Johnson, Kristin E <Kristin.Burnum-Johnson@pnnl.gov>; Lam, Mariam <mariam_lam@med.unc.edu>; Fulcher, Leslie <leslie_fulcher@med.unc.edu>; Smith, Richard D <dick.smith@pnnl.gov>; Metz, Thomas O <Thomas.Metz@pnnl.gov>; Sheahan, Timothy Patrick <sheahan@email.unc.edu>; Waters, Katrina M <Katrina.Waters@pnnl.gov>; Baric, Ralph S <rbaric@email.unc.edu>; westande@email.unc.edu; scott_randell@med.unc.edu
Subject: Re: Apoptosis paper

All,

I didn't want to email again until it was officially under review but the manuscript has moved from with the editor to under review at Cell.

We shall see what happens next. I hope everyone is doing well. Thank you, Amy

---

From: "Sims, Amy C" <amy.sims@pnnl.gov>
Date: Tuesday, April 28, 2020 at 4:46 PM
To: "Mitchell, Hugh D" <Hugh.Mitchell@pnnl.gov>, "Gralinski, Lisa E" <lgralins@email.unc.edu>, "Kyle, Jennifer E" <Jennifer.Kyle@pnnl.gov>, "Burnum-Johnson, Kristin E" <Kristin.Burnum-Johnson@pnnl.gov>, "Lam, Mariam" <mariam_lam@med.unc.edu>, "Fulcher, Leslie" <leslie_fulcher@med.unc.edu>, "Smith, Richard D" <dick.smith@pnnl.gov>, "Metz, Thomas O" <Thomas.Metz@pnnl.gov>, "Sheahan, Timothy Patrick" <sheahan@email.unc.edu>, "Waters, Katrina M" <Katrina.Waters@pnnl.gov>, "Baric, Ralph S" <rbaric@email.unc.edu>, "westande@email.unc.edu" <westande@email.unc.edu>, "scott_randell@med.unc.edu" <scott_randell@med.unc.edu>
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If anyone has any objections please let me know.

Thank you very much. Amy

PS. I hope everyone is doing well.

From:	Sims, Amy C[amy.sims@pnnl.gov]
Attendees:	Schaefer, Alexandra
Location:	Please call Amy at 509 372 6496
Subject:	R21 Year 2 discussion
Importance:	Normal
Start Time:	Wed 5/13/2020 2:00:00 PM (UTC-07:00)
End Time:	Wed 5/13/2020 3:00:00 PM (UTC-07:00)
Required Attendees:	Schaefer, Alexandra

Alex,

Do you have access to Microsoft Teams? I wasn't sure. We can use that for the conference call if it helps.

Thank you, Amy

From: Caldwell, Chandra [ccaldwel@email.unc.edu]
Sent: 5/11/2020 9:22:12 AM
To: Sims, Amy C [amy.sims@pnnl.gov]
Subject: FW: RAMSeS: Award Received, Proposal No. 20-4513
Attachments: AAN20-4513_NIH_NOA_5R21AI146872-02.PDF

FYI: Just to let you know the notice of award was issued for year 2. Hopefully, the relinquish statement is being processed.

Best,
Chandra

From: No Reply <no-reply@unc.edu>
Sent: Monday, May 11, 2020 10:35 AM
To: Sheahan, Timothy Patrick <sheahan@email.unc.edu>; Caldwell, Chandra <ccaldwel@email.unc.edu>; Moore, Victoria L <victoria_moore@unc.edu>; Sondrini, Christina Marie <csondrini@unc.edu>; Farrell, Ronda Lee <farrelrl@email.unc.edu>
Cc: Moore, Victoria L <victoria_moore@unc.edu>; Burkhart, Carol J. <carol_burkhart@unc.edu>
Subject: RAMSeS: Award Received, Proposal No. 20-4513

Proposal No.: 20-4513 (click to access proposal)
Lead PI: Timothy Sheahan
Proposal Title: How MERS-CoV Regulates Innate Immunity in Primary Human Lung Cells (CHANGE GRANTEE INSTITUTION)
Sponsor: National Institutes of Health (NIH)
Prime Sponsor (if applicable):
Award Dept: Epidemiology Operations
Primary Award Contact: Victoria L Moore

This email is to inform you that the Office of Sponsored Research (OSR) has received an award notification for the above-referenced proposal (see attached) and has begun negotiations on behalf of the institution. OSR has assigned a Sponsored Projects Specialist (SPS), Ronda Lee Farrell to set up this award.

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Please note that effective October 16, 2019, all documentation and information pertaining to subagreement requests must be submitted to OSR via the Subagreement Initiation Portal (SIP).

Subagreements will not be drafted until the prime award has been executed and entered in ConnectCarolina.

*Subrecipient(s) currently identified on this IPF:

- **Battelle**

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Thank you.

As applicable to NIH Training awards: https://grants.nih.gov/grants/guide/pa-files/PA-16-152.html (See Section 3 - Additional Information on Eligibility for Trainee Eligibility Requirements).

From: Sims, Amy C[amy.sims@pnnl.gov]
Sent: Mon 5/11/2020 9:31:17 AM (UTC-07:00)
To: Caldwell, Chandra[ccaldwel@email.unc.edu]
Subject: Re: RAMSeS: Award Received, Proposal No. 20-4513

Thank you very much for letting me know. Durr. Now the NIH has to revise it. I am following up with Kalyn to limit bugging you. Last week she told me the letter has been sent to OSR. Now OSR just has to get it out and then we wait on the NIH. Patience has never been my strong suit. Thank you again. I hope you had a good weekend. Are you back to work on a regular schedule now? I wasn't sure what UNC was doing now that NC is open again. Amy

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From: "Caldwell, Chandra" <ccaldwel@email.unc.edu>
Date: Monday, May 11, 2020 at 9:23 AM
To: "Sims, Amy C" <amy.sims@pnnl.gov>
Subject: FW: RAMSeS: Award Received, Proposal No. 20-4513

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From: No Reply <no-reply@unc.edu>
Sent: Monday, May 11, 2020 10:35 AM
To: Sheahan, Timothy Patrick <sheahan@email.unc.edu>; Caldwell, Chandra <ccaldwel@email.unc.edu>; Moore, Victoria L <victoria_moore@unc.edu>; Sondrini, Christina Marie <csondrini@unc.edu>; Farrell, Ronda Lee <farrelrl@email.unc.edu>
Cc: Moore, Victoria L <victoria_moore@unc.edu>; Burkhart, Carol J. <carol_burkhart@unc.edu>
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Thank you.

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From: Caldwell, Chandra[ccaldwel@email.unc.edu]
Sent: Mon 5/11/2020 9:36:42 AM (UTC-07:00)
To: Sims, Amy C[amy.sims@pnnl.gov]
Subject: RE: RAMSeS: Award Received, Proposal No. 20-4513

Ok, hopefully the saga will end this week and we are still working remotely since the university is under reduced operations.

From: Sims, Amy C <amy.sims@pnnl.gov>
Sent: Monday, May 11, 2020 12:31 PM
To: Caldwell, Chandra <ccaldwel@email.unc.edu>
Subject: Re: RAMSeS: Award Received, Proposal No. 20-4513

Thank you very much for letting me know. Durr. Now the NIH has to revise it. I am following up with Kalyn to limit bugging you. Last week she told me the letter has been sent to OSR. Now OSR just has to get it out and then we wait on the NIH. Patience has never been my strong suit. Thank you again. I hope you had a good weekend. Are you back to work on a regular schedule now? I wasn't sure what UNC was doing now that NC is open again. Amy

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Sent: Monday, May 11, 2020 10:35 AM
To: Sheahan, Timothy Patrick <sheahan@email.unc.edu>; Caldwell, Chandra <ccaldwel@email.unc.edu>; Moore, Victoria L <victoria_moore@unc.edu>; Sondrini, Christina Marie <csondrini@unc.edu>; Farrell, Ronda Lee <farrelrl@email.unc.edu>
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From: Sims, Amy C[amy.sims@pnnl.gov]
Sent: Mon 5/11/2020 9:38:48 AM (UTC-07:00)
To: Caldwell, Chandra[ccaldwel@email.unc.edu]
Subject: Re: RAMSeS: Award Received, Proposal No. 20-4513

Got it. I was just curious. Thank you again for letting me know. I really appreciate it. Amy

---

From: "Caldwell, Chandra" <ccaldwel@email.unc.edu>
Date: Monday, May 11, 2020 at 9:37 AM
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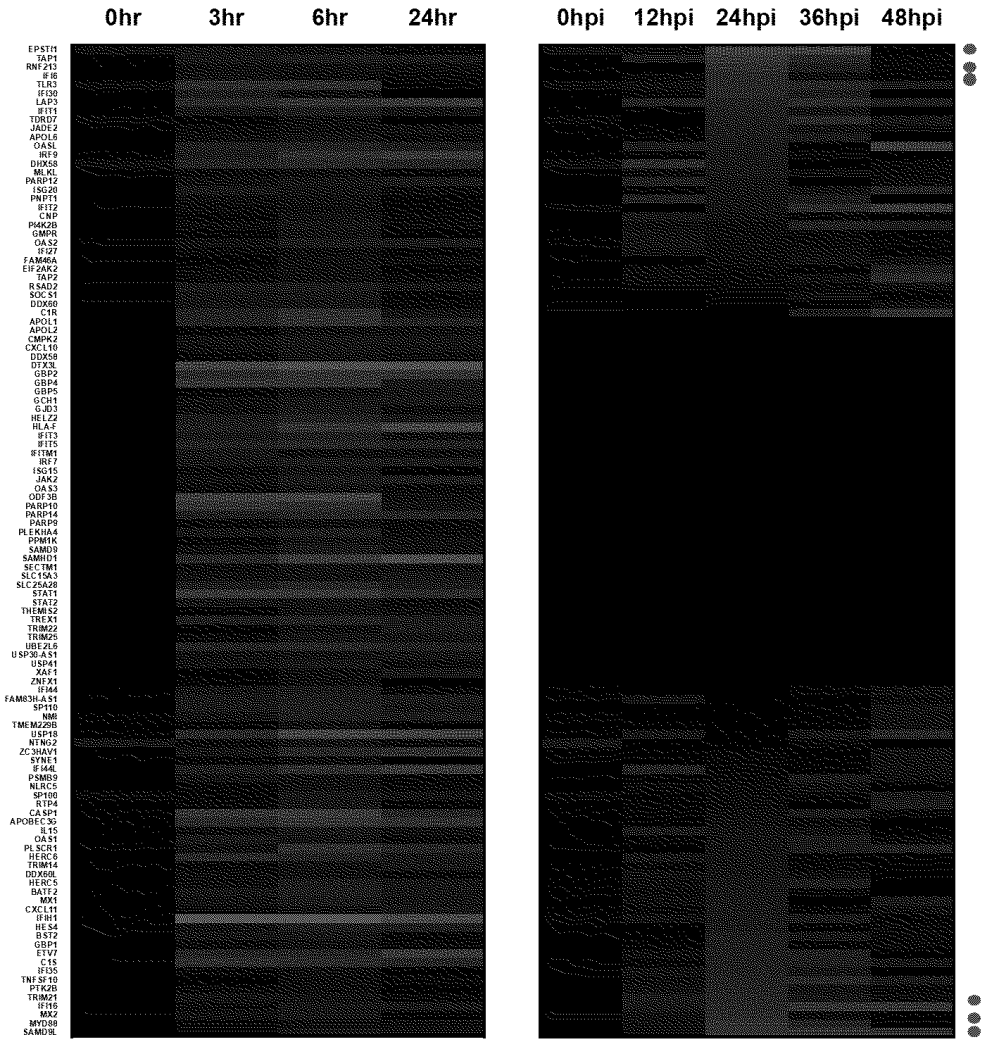
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From: Sims, Amy C[amy.sims@pnnl.gov]
Sent: Wed 5/13/2020 1:58:34 PM (UTC-07:00)
To: Schaefer, Alexandra[aschaefe@email.unc.edu]
Subject: Need 3 minutes then ready for call

From: Sims, Amy C[amy.sims@pnnl.gov]
Sent: Wed 5/13/2020 2:05:18 PM (UTC-07:00)
To: Schaefer, Alexandra[aschaefe@email.unc.edu]
Subject: <no subject>
Attachment: R21 chat with Alex 5_13_2020.pptx

MERS-CoV Induced ISG Expression in Primary Human Lung MVE



MVE interferon alpha beta stimulated ISG

MERS-CoV infected MVE ISG expression

Chromatin Accessibility Tracks with ISG Expression Level in MERS-CoV Infected MVE

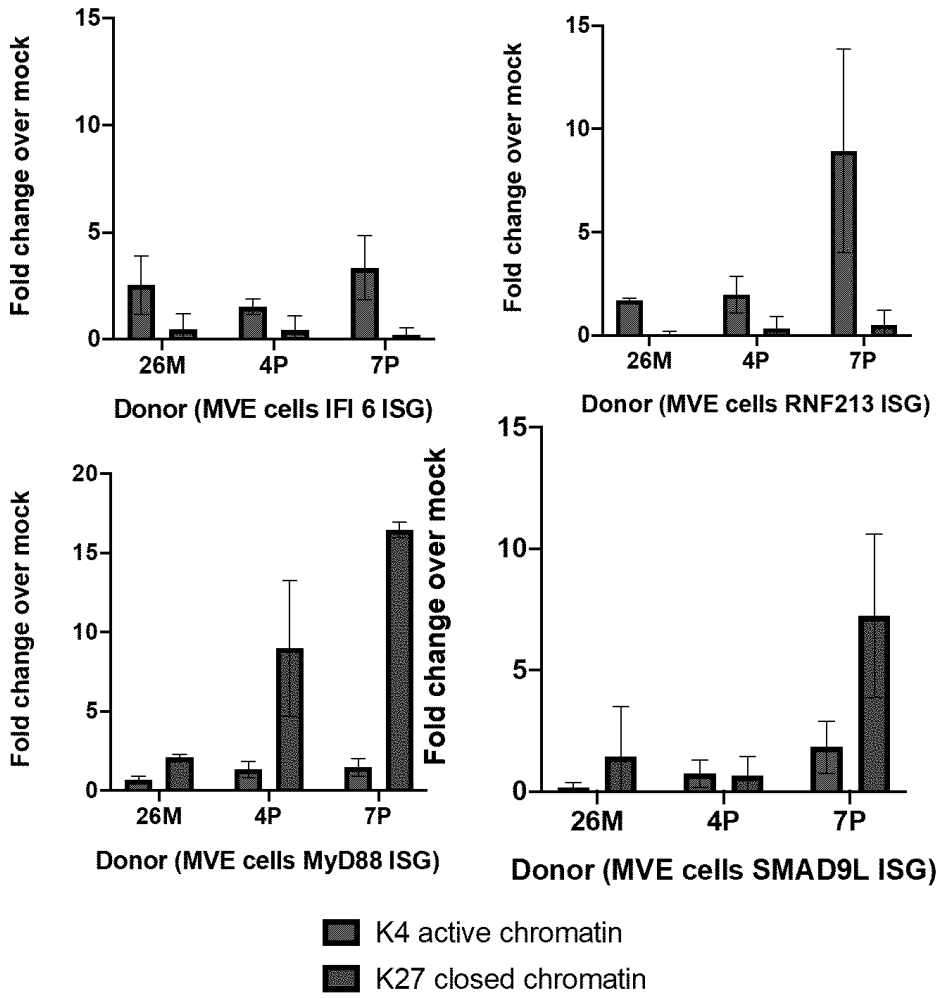
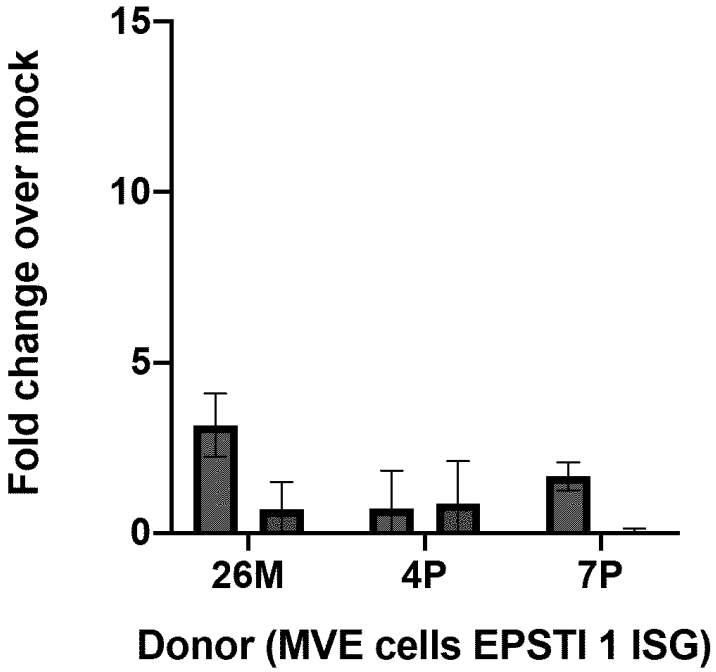
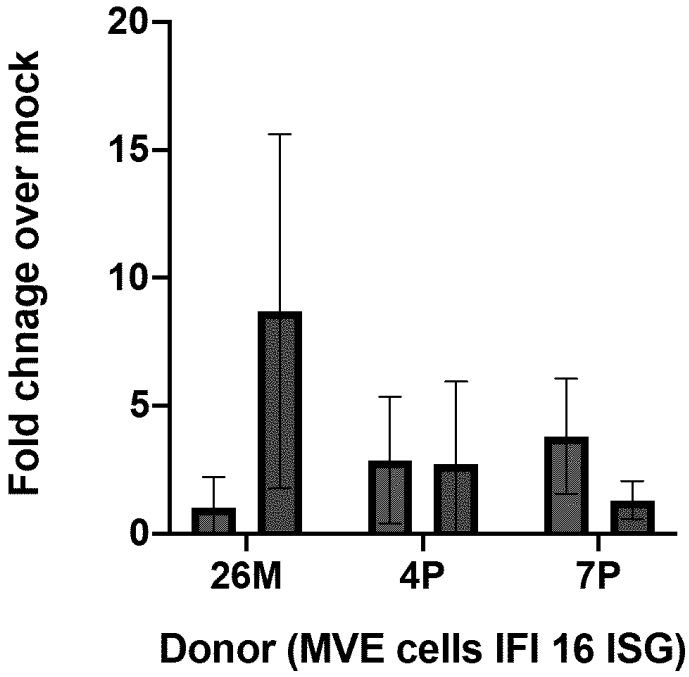


Figure used for progress report

Chromatin Accessibility Tracks with ISG Expression Level in MERS-CoV Infected MVE



Ones I did not show



Chromatin Accessibility Tracks with ISG Expression Level in MERS-CoV Infected MVE

Cell Type	Gene Name	Differential Gene Expression	Primer Pairs	Analysis as Predicted
MVE	Sterile alpha motif domain containing 9L (SMAD9L)	Down	cagcctcattttcgtagccctcat ggccatctacaagccaaggagaga	Yes
MVE	Interferon gamma inducible protein 16 (IFI 16)	Down	tcatagcagacatttccccaacca gcttcttctcccctcctttcctgt	Mixed between donors
MVE	Myeloid differentiation primary response 88 (MyD88)	Down	Cccctcctcacaggttcagtccta gagcatggcacaaccaagaaaaga	Yes
MVE	Epithelial stromal interaction 1 (EPSTI 1)	Up	ccaggttggagtacagtggcatga tacaacaattagctgggcgtggtg	Yes
MVE	Interferon alpha inducible protein 6 (IFI 6)	Up	cagcaggcagcacacaaatgttc agccaatccctgtcggagtttcta	Yes
MVE	Ring finger protein 213 (RNF213)	Up	ggatgaaggggtgagaggagaagg gtgtgaccagggacctccgtttta	Yes

R21 Aim 1 Experiments left to do

- FB ChIP PCR
  - Reagents ordered finished by end of month
  - I am still concerned that FB may need more time to show the same results as MVE
- Transcription Factor IP
- Interferon gamma treatment of MVE and FB
  - Do we want to do this?

R21 Aim 2 experiments

- RNA Seq on MERS-CoV infected MVE/FB single time point all three donors
- Additional BioRad Primer PCR Pathway arrays on MERS-CoV infected MVE and FB
- Abcam epigenetic enzyme activation assays
- Drug inhibitor studies for enzymes that mediate chromatin structure rearrangement and DNA methylation etc.
- Can Ethan still do BioID? He did not respond to my email.

Points to discuss

- How to divide up experiments
- Direction for manuscript
- Establish monthly call to discuss progress and plans

From: Sims, Amy C[amy.sims@pnnl.gov]
Sent: Wed 5/13/2020 3:32:17 PM (UTC-07:00)
To: Caldwell, Chandra[ccaldwel@email.unc.edu]
Subject: Question

Hiya,

I hope you are doing well. I have a question after chatting with my colleagues in the Baric laboratory.

What is UNC's policy on who can or more specifically cannot serve as PI of a subcontract? Is this role limited to tenure and research track faculty or can EHRA non faculty also be PIs?

Thank you very much for letting me know! I appreciate it. Have a great rest of the week and weekend! Amy

From: Caldwell, Chandra[ccaldwel@email.unc.edu]
Sent: Wed 5/13/2020 3:36:38 PM (UTC-07:00)
To: Sims, Amy C[amy.sims@pnnl.gov]
Subject: RE: Question

Hey there,

Your answer is highlighted below and your former colleagues need to be working on getting me a budget instead of chit-chatting since the word "NO" is not in their vocabulary these days!!

All is well and hopefully things are looking up in Washington as we ease back into a limited normal routine of the some retails stores starting to reopen with reduced hours.

Take care,
Chandra

From: Sims, Amy C <amy.sims@pnnl.gov>
Sent: Wednesday, May 13, 2020 6:32 PM
To: Caldwell, Chandra <ccaldwel@email.unc.edu>
Subject: Question

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From: Sims, Amy C[amy.sims@pnnl.gov]
Sent: Wed 5/13/2020 3:51:45 PM (UTC-07:00)
To: Caldwell, Chandra[ccaldwel@email.unc.edu]
Subject: Re: Question

Many thanks! I appreciate it and that is what I figured. I was asked directly and so I had no choice but to inquire and at least this makes it official. I can assist with their budget if need be.

Very glad to hear that all is well. We are still on lock down until the end of May but PNNL is trying to slowly add back some lab work to get things moving again. We shall see how it goes.

Thank you for the rapid reply! If you see or talk to Vicki/Carol later today or most likely tomorrow if someone could reply to the email from Barb Hays at PNNL it would be really great. Kalyn answered my email but PNNL would prefer to hear from the powers that be! Ha ha! Have a good evening. Thank you again, Amy

---

From: "Caldwell, Chandra" <ccaldwel@email.unc.edu>
Date: Wednesday, May 13, 2020 at 3:37 PM
To: "Sims, Amy C" <amy.sims@pnnl.gov>
Subject: RE: Question

Hey there,

Your answer is highlighted below and your former colleagues need to be working on getting me a budget instead of chit-chatting since the word "NO" is not in their vocabulary these days!!

All is well and hopefully things are looking up in Washington as we ease back into a limited normal routine of the some retails stores starting to reopen with reduced hours.

Take care,
Chandra

From: Sims, Amy C <amy.sims@pnnl.gov>
Sent: Wednesday, May 13, 2020 6:32 PM
To: Caldwell, Chandra <ccaldwel@email.unc.edu>
Subject: Question

Hiya,

I hope you are doing well. I have a question after chatting with my colleagues in the Baric laboratory.

What is UNC's policy on who can or more specifically cannot serve as PI of a subcontract? Is this role limited to **tenure and research track faculty** or can EHRA non faculty also be PIs?

Thank you very much for letting me know! I appreciate it. Have a great rest of the week and weekend! Amy

From: Caldwell, Chandra [ccaldwel@email.unc.edu]
Sent: 5/13/2020 3:54:58 PM
To: Sims, Amy C [amy.sims@pnnl.gov]
Subject: RE: Question

Funny you should mentioned Vicki since I spoke to her before your email arrived. I asked her if she had submitted your relinquish statement, her response " I have to go back and add the year 2 funding since the award notice was issued and change the start to be that of the first day for the year 2 funding." Should I speak to her tomorrow, I will remind her again to get it done ASAP since Carol could at least give an update on the status of things to not keep your team wondering.

From: Sims, Amy C <amy.sims@pnnl.gov>
Sent: Wednesday, May 13, 2020 6:52 PM
To: Caldwell, Chandra <ccaldwel@email.unc.edu>
Subject: Re: Question

Many thanks! I appreciate it and that is what I figured. I was asked directly and so I had no choice but to inquire and at least this makes it official. I can assist with their budget if need be.

Very glad to hear that all is well. We are still on lock down until the end of May but PNNL is trying to slowly add back some lab work to get things moving again. We shall see how it goes.

Thank you for the rapid reply! If you see or talk to Vicki/Carol later today or most likely tomorrow if someone could reply to the email from Barb Hays at PNNL it would be really great. Kalyn answered my email but PNNL would prefer to hear from the powers that be! Ha ha! Have a good evening. Thank you again, Amy

---

From: "Caldwell, Chandra" <ccaldwel@email.unc.edu>
Date: Wednesday, May 13, 2020 at 3:37 PM
To: "Sims, Amy C" <amy.sims@pnnl.gov>
Subject: RE: Question

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Thank you very much for letting me know! I appreciate it. Have a great rest of the week and weekend! Amy

From: Sims, Amy C[amy.sims@pnnl.gov]
Sent: Wed 5/13/2020 4:00:51 PM (UTC-07:00)
To: Caldwell, Chandra[ccaldwel@email.unc.edu]
Subject: Re: Question

Okay. Thank you for letting me know. I really appreciate it. Amy

---

From: "Caldwell, Chandra" <ccaldwel@email.unc.edu>
Date: Wednesday, May 13, 2020 at 3:55 PM
To: "Sims, Amy C" <amy.sims@pnnl.gov>
Subject: RE: Question

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From: Fulcher, Leslie[leslie_fulcher@med.unc.edu]
Sent: Thur 5/14/2020 9:53:55 AM (UTC-07:00)
To: Hou, Yixuan Jacob[y.jacob.hou@unc.edu]; Sims, Amy C[amy.sims@pnnl.gov]
Cc: Lam, Mariam[mariam_lam@med.unc.edu]; Williams, Allison Blair[ablair05@email.unc.edu]
Subject: RE: Primary cell question

Jacob,
This should not be a problem. We will be in touch later next week with a plan for the transfer of cells. Thank you,
leslie

From: Hou, Yixuan Jacob <y.jacob.hou@unc.edu>
Sent: Thursday, May 14, 2020 11:55 AM
To: Sims, Amy C <amy.sims@pnnl.gov>
Cc: Fulcher, Leslie <leslie_fulcher@med.unc.edu>
Subject: Re: Primary cell question

Hey Amy,

Thank you so much, I'll do a pilot experiment first before a large scale one. Hope things are going well in your new lab.

Hey Leslie,

If the 72M and 34M of MVE and fibroblasts available, can you plate both of them into two plates in the below format?

72M	72M	72M
34M	34M	34M

72M	72M	72M
34M	34M	34M

Thank you,
Jacob

From: Sims, Amy C <amy.sims@pnnl.gov>
Sent: Thursday, May 14, 2020 11:33 AM
To: Hou, Yixuan Jacob <y.jacob.hou@unc.edu>
Cc: Fulcher, Leslie <leslie_fulcher@med.unc.edu>
Subject: Re: Primary cell question

Jacob,

I would suggest trying a pilot infection with the following two codes if Leslie says they are still available. Start with MERS-CoV wild type before testing your new virus as there was an issue with the number of cells plating in the pilot experiment I did last year.

I would test codes 72M and 34M.

Leslie,

I believe everything you and Mariam gave me to test last year were all DD codes. If possible can you leave 68K and the other two codes used for the omics experiments ear marked in my name? We will need them for future experiments. If that is not possible please let me know. I can try to find the other two numbers but I am spacing on them now. I think maybe 63K and 4L??

If you need any more information please let me know that too.

Thank you, Amy

From: "Hou, Yixuan Jacob" <y.jacob.hou@unc.edu>
Date: Thursday, May 14, 2020 at 5:34 AM
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Thanks,
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Cc: Lam, Mariam[mariam_lam@med.unc.edu]; Williams, Allison Blair[ablair05@email.unc.edu]
Subject: Re: Primary cell question

Great, thank you so much!

Jacob

From: Fulcher, Leslie <leslie_fulcher@med.unc.edu>
Sent: Thursday, May 14, 2020 12:53 PM
To: Hou, Yixuan Jacob <y.jacob.hou@unc.edu>; Sims, Amy C <amy.sims@pnnl.gov>
Cc: Lam, Mariam <mariam_lam@med.unc.edu>; Williams, Allison Blair <ablair05@email.unc.edu>
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If you need any more information please let me know that too.

Thank you, Amy

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Thanks,
Jacob

From: Sims, Amy C[amy.sims@pnnl.gov]
Sent: Tue 5/19/2020 3:03:27 PM (UTC-07:00)
To: McGlaughon, Ben[benmcg@email.unc.edu]; Baric, Ralph S[rbaric@email.unc.edu]
Subject: MTA for Amy at PNNL
Attachment: Amy Sims UNC MTA April 27 2020.docx

Ralph,

As we discussed earlier attached is a list of items I would like to request from the Baric lab and require an MTA.

I believe that PNNL should send something to OTD to initiate this request. I am not sure if Ben is helping with these or not but I am cc'ing him in case he is.

I hope you both are doing well. Please let me know if you have any questions or concerns.

For the paperwork/submission in Blue, please just list my email address (amy.sims@pnnl.gov) and 5 U.S.C. § 552(b)(6) 5 U.S.C. § 552(b)(6) for the contact information. My employer is the Pacific Northwest National Laboratory.

Thank you for assisting me with getting this paperwork in place.

Best wishes, Amy

Amy Sims, new hire at PNNL) would like to request an MTA to Ralph Baric's laboratory at University of North Carolina at Chapel Hill. I would like to obtain multiple recombinant virus strains (replication competent virus, no infectious clones) that his laboratory has generated. These viruses will be used to characterize coronavirus replication, virus-host interactions, virus induced cytopathic effect and pathogenesis.

1. wild type infectious clone derived Middle East Respiratory syndrome coronavirus (MERS-CoV)
2. MERS-CoV expressing the red fluorescent protein gene
3. MERS-CoV expressing the nanoluciferase protein gene
4. MERS-CoV delta open reading frame 3 to 5 (MERS-CoV delta 3-5)
5. Wild type infectious clone derived severe acute respiratory syndrome coronavirus 2002-2003 outbreak** select agent (SARS-CoV)
6. Wild type infectious clone derived SARS-CoV 2
7. SARS-CoV 2 expressing the green fluorescent protein gene
8. SARS-CoV 2 expressing the nanoluciferase protein gene
9. 2 vials of Vero E6/USAMRIID cells
10. 2 vials of Vero WHO/CC81 cells
11. 2 vials 2B4 Calu3 cells
12. 2 vials MDCK cells
13. 2 vials DBT cells

From: McGlaughon, Ben[benmcg@email.unc.edu]
Sent: Tue 5/19/2020 3:29:35 PM (UTC-07:00)
To: Sims, Amy C[amy.sims@pnnl.gov]
Cc: Baric, Ralph S[rbaric@email.unc.edu]
Subject: Re: MTA for Amy at PNNL

Should I submit each of these separately, or can I combine them into one under the research description you have in the attachment?

Ben

On May 19, 2020, at 6:03 PM, Sims, Amy C <amy.sims@pnnl.gov> wrote:

Ralph,

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I believe that PNNL should send something to OTD to initiate this request. I am not sure if Ben is helping with these or not but I am cc'ing him in case he is.

I hope you both are doing well. Please let me know if you have any questions or concerns.

For the paperwork/submission in Blue, please just list my email address (amy.sims@pnnl.gov) and office number 509 372 6496 for the contact information. My employer is the Pacific Northwest National Laboratory.

Thank you for assisting me with getting this paperwork in place.

Best wishes, Amy

<Amy Sims UNC MTA April 27 2020.docx>

From: Sims, Amy C[amy.sims@pnnl.gov]
Sent: Tue 5/19/2020 3:33:12 PM (UTC-07:00)
To: McGlaughon, Ben[benmcg@email.unc.edu]; Baric, Ralph S[rbaric@email.unc.edu]
Cc: Lindesmith, Lisa C[lisal@unc.edu]
Subject: FW: 529913_MTA_Battelle_University of North Carolina
Attachment: 529913_MTA 051920_Battelle_University of North Carolina.doc

Ralph and Ben,

Here is the email I mentioned earlier. Please let me know what I can do to assist. Thank you very much, Amy

---

From: "Belsito, Erica L" <Erica.Belsito@pnnl.gov>
Date: Tuesday, May 19, 2020 at 3:28 PM
To: "rbaric@email.unc.edu" <rbaric@email.unc.edu>
Cc: ^PNNL NDA Specialist <ndaspecialist@pnnl.gov>, "Sims, Amy C" <amy.sims@pnnl.gov>, "Greeter, Jeremy S" <jeremy.greeter@pnnl.gov>
Subject: 529913_MTA_Battelle_University of North Carolina

Dear Mr. Ralph Baric:

At the request of staff at the Pacific Northwest National Laboratory, attached is our Material Transfer Agreement for exchanges of information between the parties.

If acceptable please have the agreement signed and dated by an authorized representative and return it to my attention – scan or fax is acceptable. I'll have the agreement signed for Battelle and return a copy to you for your records.

If you have any questions please contact me at your earliest convenience.
If no response is received within 6 weeks of this e-mail Battelle will terminate the MTA request.

Erica Belsito
NDA Specialist
Intellectual Property Legal Services
Pacific Northwest National Laboratory
902 Battelle Boulevard
P.O. Box 999, MSIN K1-53
Richland, WA 99354
Tel: 509-375-6518
Fax: 509-375-4487
erica.belsito@pnnl.gov

From: Sims, Amy C[amy.sims@pnnl.gov]
Sent: Tue 5/19/2020 3:39:09 PM (UTC-07:00)
To: McGlaughon, Ben[benmcg@email.unc.edu]
Cc: Baric, Ralph S[rbaric@email.unc.edu]
Subject: Re: MTA for Amy at PNNL

Please make it one single MTA. I just forwarded a second email to you that has the draft MTA from PNNL ready to go. Hopefully this will speed things up.

You can upload the document into Blue as part of the submission process. Let me know if you have any questions. Thank you very much! Amy

---

From: "McGlaughon, Ben" <benmcg@email.unc.edu>
Date: Tuesday, May 19, 2020 at 3:34 PM
To: "Sims, Amy C" <amy.sims@pnnl.gov>
Cc: "Baric, Ralph S" <rbaric@email.unc.edu>
Subject: Re: MTA for Amy at PNNL

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> Thank you for assisting me with getting this paperwork in place.
>
> Best wishes, Amy
>
> <Amy Sims UNC MTA April 27 2020.docx>

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Sent: Tue 5/19/2020 3:40:25 PM (UTC-07:00)
To: McGlaughon, Ben[benmcg@email.unc.edu]
Cc: Baric, Ralph S[rbaric@email.unc.edu]; Lindesmith, Lisa C[lisal@unc.edu]
Subject: Re: 529913_MTA_Battelle_University of North Carolina

Much appreciated. Thank you! Amy

From: "McGlaughon, Ben" <benmcg@email.unc.edu>
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To: "Sims, Amy C" <amy.sims@pnnl.gov>
Cc: "Baric, Ralph S" <rbaric@email.unc.edu>, "Lindesmith, Lisa C" <lisal@unc.edu>
Subject: Re: 529913_MTA_Battelle_University of North Carolina

Ah, that's easy then. I'll submit this into BLUE tonight.

Ben

> On May 19, 2020, at 6:33 PM, Sims, Amy C <amy.sims@pnnl.gov> wrote:
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> Ralph and Ben,
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> Here is the email I mentioned earlier. Please let me know what I can do to assist. Thank you very much, Amy
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>
> ---
>
> **Erica Belsito**
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> 902 Battelle Boulevard
> P.O. Box 999, MSIN K1-53

Richland, WA 99354
Tel: 509-375-6518
Fax: 509-375-4487
erica.belsito@pnnl.gov

<529913_MTA 051920_Battelle_University of North Carolina.doc>

From: UNC Office of Tech Dev MTA [mta@unc.edu]
Sent: 5/20/2020 5:52:25 AM
To: Sims, Amy C [amy.sims@pnnl.gov]
CC: Baric, Ralph S [rbaric@email.unc.edu]; UNC Office of Tech Dev MTA [mta@unc.edu]; McGlaughon, Ben [benmcg@email.unc.edu]
Subject: MO-16244 BARIC UNC-PNNL MTA
Attachments: MO-16244 BARIC UNC-PNL MTA Draft.docx

Dear Dr. Sims,

I am part of the MTA team in the technology transfer office at UNC Chapel Hill. On behalf of UNC investigator Ralph Baric, please find attached a draft MTA for review by your institution. Let us know if there are any questions or comments.

Best,
Nate

Nathan Whitman, PhD
Licensing Fellow

THE UNIVERSITY OF NORTH CAROLINA AT CHAPEL HILL
INNOVATION, ENTREPRENEURSHIP AND ECONOMIC DEVELOPMENT – INNOVATE CAROLINA
Office of Technology Commercialization
Campus Box 4105
109 Church Street | Chapel Hill, NC 27516
T: 919.962.6369 | F: 919.962.0646
Email: nathan.whitman@unc.edu | Twitter: @innovateunc | Facebook: @InnovateUNC
Web: otc.unc.edu | innovate.unc.edu

From: Sims, Amy C[amy.sims@pnnl.gov]
Sent: Wed 5/20/2020 7:57:28 AM (UTC-07:00)
To: UNC Office of Tech Dev MTA[mta@unc.edu]
Cc: Baric, Ralph S[rbaric@email.unc.edu]; McGlaughon, Ben[benmcg@email.unc.edu]
Subject: Re: MO-16244 BARIC UNC-PNNL MTA

Nate,

Thank you very much for the rapid turn-around. Best wishes, Amy

---

From: UNC Office of Tech Dev MTA <mta@unc.edu>
Date: Wednesday, May 20, 2020 at 5:54 AM
To: "Sims, Amy C" <amy.sims@pnnl.gov>
Cc: "Baric, Ralph S" <rbaric@email.unc.edu>, UNC Office of Tech Dev MTA <mta@unc.edu>, "McGlaughon, Ben" <benmcg@email.unc.edu>
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T: 919.962.6369 | F: 919.962.0646
Email: nathan.whitman@unc.edu | Twitter: @innovateunc | Facebook: @InnovateUNC
Web: otc.unc.edu | innovate.unc.edu

From: Belsito, Erica L[Erica.Belsito@pnnl.gov]
Sent: Wed 5/20/2020 9:23:18 AM (UTC-07:00)
To: mta@unc.edu[mta@unc.edu]; rbaric@email.unc.edu[rbaric@email.unc.edu]; nathan.whitman@unc.edu[nathan.whitman@unc.edu]
Cc: Sims, Amy C[amy.sims@pnnl.gov]; Greeter, Jeremy S[jeremy.greeter@pnnl.gov]; ^PNNL NDA Specialist[ndaspecialist@pnnl.gov]
Subject: RE: MO-16244 BARIC UNC-PNNL MTA / 529913_MTA_Battelle_UNC
Attachment: 529913_MTA 052020_Battelle(REV1)_University of North Carolina.docx

Good morning Dr. Whitman,

At the request of staff at the Pacific Northwest National Laboratory, attached is your Material Transfer Agreement with Battelle's suggested edits. Some of these revisions are required by Battelle's contract with the U.S. Department Energy (DOE) for the management and operation of the Pacific Northwest National Laboratory, and some are required by Battelle policy.

If acceptable please provide a "clean" version for Battelle signature. If you have any questions please contact me at your earliest convenience.

Erica Belsito
IP Specialist
Intellectual Property Legal Services
Pacific Northwest National Laboratory
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Web: otc.unc.edu | innovate.unc.edu

From: UNC Office of Tech Dev MTA[mta@unc.edu]
Sent: Wed 5/20/2020 10:48:34 AM (UTC-07:00)
To: Belsito, Erica L[Erica.Belsito@pnnl.gov]
Cc: Sims, Amy C[amy.sims@pnnl.gov]; Greeter, Jeremy S[jeremy.greeter@pnnl.gov]; ^PNNL NDA Specialist[ndaspecialist@pnnl.gov]; UNC Office of Tech Dev MTA[mta@unc.edu]; Baric, Ralph S[rbaric@email.unc.edu]
Subject: RE: MO-16244 BARIC UNC-PNNL MTA / 529913_MTA_Battelle_UNC
Attachment: 529913_MTA 052020_Battelle(REV1)_University of North Carolina_CLEAN.docx

Hi Erica,

Thanks for your revisions and explanations. We have accepted all of the proposed changes and decided to remain silent on governing law. Please find attached a clean copy of the agreement for signature. Once signed, please return a copy to mta@unc.edu for countersignature.

Thanks,
Nate

Nathan Whitman, PhD
Licensing Fellow

THE UNIVERSITY OF NORTH CAROLINA AT CHAPEL HILL
INNOVATION, ENTREPRENEURSHIP AND ECONOMIC DEVELOPMENT – INNOVATE CAROLINA
Office of Technology Commercialization
Campus Box 4105
109 Church Street | Chapel Hill, NC 27516
T: 919.962.6369 | F: 919.962.0646
Email: nathan.whitman@unc.edu | Twitter: @innovateunc | Facebook: @InnovateUNC
Web: otc.unc.edu | innovate.unc.edu

From: Belsito, Erica L <Erica.Belsito@pnnl.gov>
Sent: Wednesday, May 20, 2020 12:23 PM
To: UNC Office of Tech Dev MTA <mta@unc.edu>; Baric, Ralph S <rbaric@email.unc.edu>; Whitman, Nathan Ashbrook <nathan.whitman@unc.edu>
Cc: Sims, Amy C <amy.sims@pnnl.gov>; Greeter, Jeremy S <jeremy.greeter@pnnl.gov>; ^PNNL NDA Specialist <ndaspecialist@pnnl.gov>
Subject: RE: MO-16244 BARIC UNC-PNNL MTA / 529913_MTA_Battelle_UNC

Good morning Dr. Whitman,

At the request of staff at the Pacific Northwest National Laboratory, attached is your Material Transfer Agreement with Battelle's suggested edits. Some of these revisions are required by Battelle's contract with the U.S. Department Energy (DOE) for the management and operation of the Pacific Northwest National Laboratory, and some are required by Battelle policy.

If acceptable please provide a "clean" version for Battelle signature. If you have any questions please contact me at your earliest convenience.

Erica Belsito
IP Specialist
Intellectual Property Legal Services
Pacific Northwest National Laboratory
902 Battelle Boulevard
P.O. Box 999, MSIN K1-53
Richland, WA 99354
Tel: 509-375-6518

Fax: 509-375-4487
erica.belsito@pnnl.gov

From: UNC Office of Tech Dev MTA <mta@unc.edu>
Date: Wednesday, May 20, 2020 at 5:54 AM
To: "Sims, Amy C" <amy.sims@pnnl.gov>
Cc: "Baric, Ralph S" <rbaric@email.unc.edu>, UNC Office of Tech Dev MTA <mta@unc.edu>, "McGlaughon, Ben" <benmcg@email.unc.edu>
Subject: MO-16244 BARIC UNC-PNNL MTA

Dear Dr. Sims,

I am part of the MTA team in the technology transfer office at UNC Chapel Hill. On behalf of UNC investigator Ralph Baric, please find attached a draft MTA for review by your institution. Let us know if there are any questions or comments.

Best,
Nate

Nathan Whitman, PhD
Licensing Fellow

THE UNIVERSITY OF NORTH CAROLINA AT CHAPEL HILL
INNOVATION, ENTREPRENEURSHIP AND ECONOMIC DEVELOPMENT – INNOVATE CAROLINA
Office of Technology Commercialization
Campus Box 4105
109 Church Street | Chapel Hill, NC 27516
T: 919.962.6369 | F: 919.962.0646
Email: nathan.whitman@unc.edu | Twitter: @innovateunc | Facebook: @InnovateUNC
Web: otc.unc.edu | innovate.unc.edu

From: Sims, Amy C[amy.sims@pnnl.gov]
Sent: Thur 5/21/2020 4:34:02 PM (UTC-07:00)
To: Baric, Ralph S[rbaric@email.unc.edu]; McGlaughon, Ben[benmcg@email.unc.edu]
Cc: Lindesmith, Lisa C[lisal@unc.edu]
Subject: Re: 529913_MTA_Battelle_University of North Carolina

I am doing well. This is almost done and should be coming your way for signature very soon. Might see it as early as tomorrow.

Really good to hear from you! Hope you are hanging in there and getting a little rest. Thank you again! Amy

PS Ben, I will need a little longer before I am ready for the shipment. I will be in touch about that once the paperwork gets settled.

From: "Baric, Ralph S" <rbaric@email.unc.edu>
Date: Thursday, May 21, 2020 at 4:31 PM
To: "Sims, Amy C" <amy.sims@pnnl.gov>, "McGlaughon, Ben" <benmcg@email.unc.edu>
Cc: "Lindesmith, Lisa C" <lisal@unc.edu>
Subject: RE: 529913_MTA_Battelle_University of North Carolina

Hi Ben, thanks for getting this moving. Amy, hope your doing well. Ralph

---

From: Sims, Amy C <amy.sims@pnnl.gov>
Sent: Tuesday, May 19, 2020 6:40 PM
To: McGlaughon, Ben <benmcg@email.unc.edu>
Cc: Baric, Ralph S <rbaric@email.unc.edu>; Lindesmith, Lisa C <lisal@unc.edu>
Subject: Re: 529913_MTA_Battelle_University of North Carolina

Much appreciated. Thank you! Amy

From: "McGlaughon, Ben" <benmcg@email.unc.edu>
Date: Tuesday, May 19, 2020 at 3:39 PM
To: "Sims, Amy C" <amy.sims@pnnl.gov>
Cc: "Baric, Ralph S" <rbaric@email.unc.edu>, "Lindesmith, Lisa C" <lisal@unc.edu>
Subject: Re: 529913_MTA_Battelle_University of North Carolina

Ah, that's easy then. I'll submit this into BLUE tonight.

Ben

> On May 19, 2020, at 6:33 PM, Sims, Amy C <amy.sims@pnnl.gov> wrote:
>
> Ralph and Ben,
>
> Here is the email I mentioned earlier. Please let me know what I can do to assist. Thank you very much, Amy
>
> ---
>
> **From:** "Belsito, Erica L" <Erica.Belsito@pnnl.gov>
> **Date:** Tuesday, May 19, 2020 at 3:28 PM
> **To:** "rbaric@email.unc.edu" <rbaric@email.unc.edu>

Cc: ^PNNL NDA Specialist <ndaspecialist@pnnl.gov>, "Sims, Amy C" <amy.sims@pnnl.gov>, "Greeter, Jeremy S" <jeremy.greeter@pnnl.gov>
Subject: 529913_MTA_Battelle_University of North Carolina

Dear Mr. Ralph Baric:

At the request of staff at the Pacific Northwest National Laboratory, attached is our Material Transfer Agreement for exchanges of information between the parties.

If acceptable please have the agreement signed and dated by an authorized representative and return it to my attention – scan or fax is acceptable. I'll have the agreement signed for Battelle and return a copy to you for your records.

If you have any questions please contact me at your earliest convenience.
If no response is received within 6 weeks of this e-mail Battelle will terminate the MTA request.

Erica Belsito
NDA Specialist
Intellectual Property Legal Services
Pacific Northwest National Laboratory
902 Battelle Boulevard
P.O. Box 999, MSIN K1-53
Richland, WA 99354
Tel: 509-375-6518
Fax: 509-375-4487
erica.belsito@pnnl.gov

<529913_MTA 051920_Battelle_University of North Carolina.doc>

From: Baric, Ralph S[rbaric@email.unc.edu]
Sent: Thur 5/21/2020 4:36:57 PM (UTC-07:00)
To: Sims, Amy C[amy.sims@pnnl.gov]
Subject: RE: MTA for Amy at PNNL

Ok-no problem-sorry for the delay. Ben can certainly help with getting this MTA paperwork together. Obviously, the select agent will be the potential bottleneck. hear anything from the Cell paper? Do you have the list of differentially expressed proteins and RNA from the MVE and FB cells that we could use? Thanks, ralph

---

From: Sims, Amy C <amy.sims@pnnl.gov>
Sent: Tuesday, May 19, 2020 6:03 PM
To: McGlaughon, Ben <benmcg@email.unc.edu>; Baric, Ralph S <rbaric@email.unc.edu>
Subject: MTA for Amy at PNNL

Ralph,

As we discussed earlier attached is a list of items I would like to request from the Baric lab and require an MTA.

I believe that PNNL should send something to OTD to initiate this request. I am not sure if Ben is helping with these or not but I am cc'ing him in case he is.

I hope you both are doing well. Please let me know if you have any questions or concerns.

For the paperwork/submission in Blue, please just list my email address (amy.sims@pnnl.gov) and office number 509 372 6496 for the contact information. My employer is the Pacific Northwest National Laboratory.

Thank you for assisting me with getting this paperwork in place.

Best wishes, Amy

From: Sims, Amy C[amy.sims@pnnl.gov]
Sent: Thur 5/21/2020 4:50:45 PM (UTC-07:00)
To: Baric, Ralph S[rbaric@email.unc.edu]
Subject: Re: MTA for Amy at PNNL

There haven't been any delays. Ben initiated this immediately and the documents should be to you for signatures next week. No worries.

Yes, I know the select agent transfer will take the longest but it seemed like a good idea to just complete one MTA for all of the virus strains and cell lines at once.

I have checked several times on the status of the Cell paper and it still says under review. I know people are really slammed right now so I suspect it might take a little while.

Tim Sheahan has a hard drive with all of the omics datasets on his desk. He should be able to get you anything you want. If that doesn't work let me know and I will get you what you need.

Hang in there. Thanks, Amy

From: "Baric, Ralph S" <rbaric@email.unc.edu>
Date: Thursday, May 21, 2020 at 4:37 PM
To: "Sims, Amy C" <amy.sims@pnnl.gov>
Subject: RE: MTA for Amy at PNNL

Ok-no problem-sorry for the delay. Ben can certainly help with getting this MTA paperwork together. Obviously, the select agent will be the potential bottleneck. hear anything from the Cell paper? Do you have the list of differentially expressed proteins and RNA from the MVE and FB cells that we could use? Thanks, ralph

---

From: Sims, Amy C <amy.sims@pnnl.gov>
Sent: Tuesday, May 19, 2020 6:03 PM
To: McGlaughon, Ben <benmcg@email.unc.edu>; Baric, Ralph S <rbaric@email.unc.edu>
Subject: MTA for Amy at PNNL

Ralph,

As we discussed earlier attached is a list of items I would like to request from the Baric lab and require an MTA.

I believe that PNNL should send something to OTD to initiate this request. I am not sure if Ben is helping with these or not but I am cc'ing him in case he is.

I hope you both are doing well. Please let me know if you have any questions or concerns.

For the paperwork/submission in Blue, please just list my email address (amy.sims@pnnl.gov) and

office number 509 372 6496 for the contact information. My employer is the Pacific Northwest National Laboratory.

Thank you for assisting me with getting this paperwork in place.

Best wishes, Amy

From: Sims, Amy C[amy.sims@pnnl.gov]
Sent: Thur 5/21/2020 4:52:03 PM (UTC-07:00)
To: McGlaughon, Ben[benmcg@email.unc.edu]
Cc: Baric, Ralph S[rbaric@email.unc.edu]; Lindesmith, Lisa C[lisal@unc.edu]
Subject: Re: 529913_MTA_Battelle_University of North Carolina

Got it. Figured we would take it one step at a time. Thank you so much for the assistance. I know how hard it is to coordinate many things at once. Thank you again, Amy

---

From: "McGlaughon, Ben" <benmcg@email.unc.edu>
Date: Thursday, May 21, 2020 at 4:50 PM
To: "Sims, Amy C" <amy.sims@pnnl.gov>
Cc: "Baric, Ralph S" <rbaric@email.unc.edu>, "Lindesmith, Lisa C" <lisal@unc.edu>
Subject: Re: 529913_MTA_Battelle_University of North Carolina

Good, because I'm nowhere near ready to send all this! Just keep me posted on a timeline and we'll make it happen.

Ben

> On May 21, 2020, at 7:34 PM, Sims, Amy C <amy.sims@pnnl.gov> wrote:
>
> I am doing well. This is almost done and should be coming your way for signature very soon. Might see it as early as tomorrow.
>
> Really good to hear from you! Hope you are hanging in there and getting a little rest. Thank you again! Amy
>
> PS Ben, I will need a little longer before I am ready for the shipment. I will be in touch about that once the paperwork gets settled.
>
> ---
>
> **From:** "Baric, Ralph S" <rbaric@email.unc.edu>
> **Date:** Thursday, May 21, 2020 at 4:31 PM
> **To:** "Sims, Amy C" <amy.sims@pnnl.gov>, "McGlaughon, Ben" <benmcg@email.unc.edu>
> **Cc:** "Lindesmith, Lisa C" <lisal@unc.edu>
> **Subject:** RE: 529913_MTA_Battelle_University of North Carolina
>
> Hi Ben, thanks for getting this moving. Amy, hope your doing well. Ralph
>
> ---
>
> **From:** Sims, Amy C <amy.sims@pnnl.gov>
> **Sent:** Tuesday, May 19, 2020 6:40 PM
> **To:** McGlaughon, Ben <benmcg@email.unc.edu>
> **Cc:** Baric, Ralph S <rbaric@email.unc.edu>; Lindesmith, Lisa C <lisal@unc.edu>
> **Subject:** Re: 529913_MTA_Battelle_University of North Carolina
>
> Much appreciated. Thank you! Amy
>
> ---
>
> **From:** "McGlaughon, Ben" <benmcg@email.unc.edu>
> **Date:** Tuesday, May 19, 2020 at 3:39 PM
> **To:** "Sims, Amy C" <amy.sims@pnnl.gov>
> **Cc:** "Baric, Ralph S" <rbaric@email.unc.edu>, "Lindesmith, Lisa C" <lisal@unc.edu>

Subject: Re: 529913_MTA_Battelle_University of North Carolina

Ah, that's easy then. I'll submit this into BLUE tonight.

Ben

On May 19, 2020, at 6:33 PM, Sims, Amy C <amy.sims@pnnl.gov> wrote:

Ralph and Ben,

Here is the email I mentioned earlier. Please let me know what I can do to assist. Thank you very much, Amy

---

From: "Belsito, Erica L" <Erica.Belsito@pnnl.gov>
Date: Tuesday, May 19, 2020 at 3:28 PM
To: "rbaric@email.unc.edu" <rbaric@email.unc.edu>
Cc: ^PNNL NDA Specialist <ndaspecialist@pnnl.gov>, "Sims, Amy C" <amy.sims@pnnl.gov>, "Greeter, Jeremy S" <jeremy.greeter@pnnl.gov>
Subject: 529913_MTA_Battelle_University of North Carolina

Dear Mr. Ralph Baric:

At the request of staff at the Pacific Northwest National Laboratory, attached is our Material Transfer Agreement for exchanges of information between the parties.

If acceptable please have the agreement signed and dated by an authorized representative and return it to my attention – scan or fax is acceptable. I'll have the agreement signed for Battelle and return a copy to you for your records.

If you have any questions please contact me at your earliest convenience.
If no response is received within 6 weeks of this e-mail Battelle will terminate the MTA request.

Erica Belsito
NDA Specialist
Intellectual Property Legal Services
Pacific Northwest National Laboratory
902 Battelle Boulevard
P.O. Box 999, MSIN K1-53
Richland, WA 99354
Tel: 509-375-6518
Fax: 509-375-4487
erica.belsito@pnnl.gov

<529913_MTA 051920_Battelle_University of North Carolina.doc>

From: EcoHealth[onbehalfof@manuscriptcentral.com]

Sent: Sun 5/24/2020 6:57:40 PM (UTC-07:00)

To: sims0018@email.unc.edu[sims0018@email.unc.edu]; Sims, Amy C[amy.sims@pnnl.gov]

Subject: E-Mail address change for EcoHealth

Dear Dr. Amy Sims:

This e-mail address associated with your ScholarOne Manuscripts account has been successfully changed from sims0018@email.unc.edu to amy.sims@pnnl.gov. A copy of this email has been sent to both accounts as a security measure.

If you believe this change was made in error, please contact your administrator for assistance

Sincerely,

ScholarOne Manuscripts

From: Graham, Rachel[rlgraham@ad.unc.edu]

Sent: Tue 5/26/2020 1:04:33 PM (UTC-07:00)

To: Baric, Tommy Joseph[tjbaric@email.unc.edu]; West, Ande[westande@email.unc.edu]; Tse, Long Ping Victor[victor_tse@med.unc.edu]; Jensen, Kara[kara_jensen@med.unc.edu]; Leist, Sarah Rebecca[leist@email.unc.edu]; Brewer-Jensen, Paul D[pbj@email.unc.edu]; Yount, Boyd L Jr[byount@email.unc.edu]; Brown, Ariane[ajbrown7@email.unc.edu]; Fritch, Ethan James[ejfritch@email.unc.edu]; Gralinski, Lisa E[lgralins@email.unc.edu]; Mallory, Michael L[mlmallor@live.unc.edu]; Steininger, Kirsten[kirdy@live.unc.edu]; Gully, Kendra[klgully@email.unc.edu]; Sims, Amy C[amy.sims@pnnl.gov]; Dinnon, Kenneth Harold III[kdinnon@email.unc.edu]; Schaefer, Alexandra[aschaefe@email.unc.edu]; Lindesmith, Lisa C[lisal@unc.edu]; Scobey, Trevor[trevor.scobey@unc.edu]; Swanstrom, Jesica[jesica_swanstrom@med.unc.edu]; Edwards, Caitlin E[caitedw@unc.edu]; Sheahan, Timothy Patrick[sheahan@email.unc.edu]; Young, Ellen F[ellen_young@med.unc.edu]; Graham, Rachel[rlgraham@ad.unc.edu]; Martinez, David Rafael[davidmar@email.unc.edu]; Baric, Ralph S[rbaric@email.unc.edu]

Subject: Fwd: Re: Transportation & Parking Questions

Hi all,

Here is the response to the parking question I received from Transporation and Parking. Looks like tomorrow will be the day.

~~~~~~~~~~~~
Rachel Graham, Ph.D.
Assistant Professor
Baric Lab | UNC-Chapel Hill
(919) 966-4689

---------- Forwarded message ----------
From: Jenkins, Karen E <jenkinsc@psafety.unc.edu>
Date: May 26, 2020, 4:02 PM -0400
To: Graham, Rachel <rlgraham@ad.unc.edu>
Subject: Re: Transportation & Parking Questions

Rachel,
The Transportation and Parking website has not launched the summer parking processes at this time. It is anticipated to occur no later than Wednesday May 27. There will be a format to request your parking zone options when the website has been updated at https://move.unc.edu/

Thank you,
UNC Transportation and Parking

From: Graham, Rachel <rlgraham@ad.unc.edu>
Sent: Tuesday, May 26, 2020 12:27 PM
To: Jenkins, Karen E <jenkinsc@psafety.unc.edu>
Subject: Transportation & Parking Questions

Name:
Rachel Graham
PID Number (if available)
(b) (6)
~~~~~~~~~~~~

Affiliation:

University Faculty/Staff

Email:

rlgraham@ad.unc.edu

Phone:

(919) 966-4689

Question:

Hello, I am a researcher and faculty member in a coronavirus lab on campus. We have been working through the COVID-19 shutdown and have been parking on campus while parking regulations have been suspended. However, we were told (through a contact with another department) that parking regulations would resume on June 1 but that there would be an opportunity to purchase a summer-only on-campus parking permit. Because our research is coronavirus-related and we are concerned that the limitations on bus transport will be significant, we would like to take advantage of this option but have received no other guidance (we were told the link to purchase permits would go live today at noon but have heard no other information). Could you please advise me if this information is correct, and if so, where this link will be/is available? Thanks, Rachel

From: Caldwell, Chandra [ccaldwel@email.unc.edu]
Sent: 5/27/2020 10:12:12 AM
To: Sims, Amy C [amy.sims@pnnl.gov]
Subject: Incoming Mail for Sims
Attachments: Sims Mail.pdf

Hi Amy,

The attached mail was in out departmental mailbox this morning. This is the first time I am seeing something for you from Missouri so I don't know if the first attempt was delivered to someone in the Baric lab on your behalf.

Thanks,
Chandra

From: Sims, Amy C[amy.sims@pnnl.gov]
Sent: Wed 5/27/2020 1:25:11 PM (UTC-07:00)
To: Caldwell, Chandra[ccaldwel@email.unc.edu]
Subject: Re: Incoming Mail for Sims

Thank you for sending this. This is for an undergraduate who worked with us for a brief time. She seems to generate a lot of these requests. I will do what I can to respond.

Thank you again for getting this to me. I really appreciate it. Amy

---

From: "Caldwell, Chandra" <ccaldwel@email.unc.edu>
Date: Wednesday, May 27, 2020 at 10:14 AM
To: "Sims, Amy C" <amy.sims@pnnl.gov>
Subject: Incoming Mail for Sims

Hi Amy,

The attached mail was in out departmental mailbox this morning. This is the first time I am seeing something for you from Missouri so I don't know if the first attempt was delivered to someone in the Baric lab on your behalf.

Thanks,
Chandra

From: Sims, Amy C[amy.sims@pnnl.gov]
Sent: Thur 5/28/2020 2:13:07 PM (UTC-07:00)
To: Baric, Ralph S[rbaric@email.unc.edu]; Sheahan, Timothy Patrick[sheahan@email.unc.edu]; Waters, Katrina M[Katrina.Waters@pnnl.gov]; Mitchell, Hugh D[Hugh.Mitchell@pnnl.gov]
Subject: FW: Cell Editorial Decision CELL-D-20-01383

All,

We have officially been rejected from Cell. This is annoying given our long wait.

Okay, do we go back to the original plan of Science Translational Medicine or consider another Cell journal?

I am interested in your feedback. Bad way to end the week. Thanks, Amy

---

From: <em.cell.0.6b95c1.abe328dd@editorialmanager.com> on behalf of Cell Editorial Office <em@editorialmanager.com>
Reply-To: Cell Editorial Office <cellms@cell.com>
Date: Thursday, May 28, 2020 at 1:59 PM
To: "Sims, Amy C" <amy.sims@pnnl.gov>
Subject: Cell Editorial Decision CELL-D-20-01383

Dr. Amy C Sims
902 Battelle Boulevard
Richland, WA 99352
UNITED STATES

CELL-D-20-01383
Unfolded Protein Response Inhibition Reduces MERS-CoV Induced Acute Lung Injury

May 28, 2020

Dear Dr. Sims,

I am enclosing the comments that the reviewers made on your paper. Unfortunately, the consensus recommendation is against publication in *Cell*. The reviewers refer to the potential timliness of the topic and interest in the approach, but cite that significant further work would be necessary, especially to demonstrate the specificity of the compound and its proposed mechanism of action in vivo. Given their comments, and the unclear relevance to non-MERS-CoV driven coronavirus pathology, we unfortunately cannot proceed further with this manuscript at Cell.

I am sorry that the outcome for this manuscript was not more positive. I hope, however, that the reviewers' comments are clear and constructive and that you will continue to consider *Cell* for future submissions.

Although we cannot offer to proceed further with this particular paper for *Cell*, the manuscript may be a stronger candidate for another journal within the Cell Press manuscript transfer system. If you are interested in learning more about transfer as an option, please contact the relevant journal directly, as each journal is editorially independent. The link below will bring you to a page where you can select the journal to which you would like to transfer your paper (you can review each journal's aims and scopes before choosing your target).

Agree to Transfer

After your files have been transferred, you will be given an opportunity to update your materials before they are delivered to the editors at your new journal. You will be able to make any desired revisions or additions at this stage, so feel free to select a transfer option now and work on your revisions after that. For more details on the transfer process, please click here or contact the editors at your new target journal.

Please note that the above link will expire Jun 27, 2020. However, if you still wish to transfer after that period, you may submit directly to the journal of your choice and reference your original manuscript number in the cover letter.

We hope this option may be helpful for you, however if you decide not to transfer this manuscript, please click here:

Decline to Transfer

Declining to transfer will officially close out the manuscript in our system. If you do not accept or decline by Jun 27, 2020, the system will automatically decline on your behalf. If you prefer, you do have the choice of submitting this manuscript or a revised version of it to another Cell Press journal as a regular new submission, in which case you can decline the transfer.

Best wishes,
Andrew

Andrew J. Rennekamp, Ph.D.
Scientific Editor, *Cell*

Reviewers' Comments:

Reviewer #1:

This manuscript by Sims and co-workers describes the results of an unbiased, systems biology-based approach that identifies the unfolded protein response (UPR) as differentially modulated during MERS-CoV infection of primary cells. They used donor matched primary human lung microvascular endothelial cells (MVEs) and fibroblasts (FB), and found that only MVEs exhibited cytopathic effect in response to infection with MERS-CoV. To determine if a specific pathway was responsible for the differential outcome in MVEs versus FBs, they compared the transcriptomes, proteomes, and lipidomes of MERS-CoV-infected cells over time, and repeated this experiment using 3 different donors of the cells. This analysis revealed that the MERS-CoV-infected MVEs were dying via an Unfolded Protein Response (UPR) mediated apoptosis. They validated the importance of the UPR pathway for MERS-CoV replication-induced cell death in MEVs by addition of UPR pathway inhibitor (AMG44), which reduced replication and cell death. Importantly, they also demonstrated that drug targeting of this pathway ameliorated the outcome of MERS-CoV infection in mice (reduced weight loss and improved respiratory function). Overall, this systems biology approach allowed them to identifying a targeted antiviral therapy that reduced morbidity and mortality in MERS-CoV-infected mice. The manuscript is well written and the experiments are clearly presented. Appropriate statistical analysis is provided for each experiment. The results of this study document an approach for identifying targeted antiviral therapy for coronavirus infection. This study has important implications for developing therapies for the current pandemic.
Comments for the authors' consideration (minor issues):
1. Legend to Fig 2. Is it least significant score or most significant score?
2. Legend to Fig 3. MERS-CoV MOI?
3. Title to Fig 4: Protein markers suggest activation of UPR in MVEs.

4. Legend to Fig 5. MVEs were infected with MERS-nanoluc (MOI=?)
5. Line 344: cite Gosert et al., 2002 for identification of DMVs
6. Lines 486-493: Was this MERS-nanoluc virus?
7. Lines 486-493: AMG44 is referenced to two other papers, but better to name the commercial source.
8. Do your results implicate other UPR modulators such as GSK2606414 and KIRA6 (Mahameed at al., Cell Death and Disease, 2019) as potential modulators of coronavirus replication and disease? Discussion of UPR modulators as potential therapeutics would be appropriate (line 377).
9. Line 377, start new paragraph to discuss mortality in male and female mice
10. Line 132: mothers?
11. Line 349: add recent paper Volk, Hackbart et al., 2020 J. Virol for activation of the UPR in macrophages in response to coronavirus replication.
12. Line 392: add drug names remdesivir and EIDD-2801 here

Reviewer #2: Coronavirus infection and replication is well known for its ability to stimulate an intracellular unfolded protein response (UPR). In this paper these authors take an unbiased omics-based approach to examine pathways stimulated by MERS-CoV. They identify an induction of UPR gene signature - particularly in microvascular endothelial cells (MVE) - more than fibroblasts. They find that the use of a PERK inhibitor reduces MERS-CoV replication in infected primary human lung MVE and FB and treatment with the same PERK inhibitor AMG144 in an in vivo mouse model of MERS lung disease leads to some clinical improvement. They show that it reduces lung disease and other features of disease pathogenesis - though they fail to show a reduction in MERS-CoV viral replication in infected cells. It should also be noted that PERK inhibitors are wonderfully pleiotropic.

The experiments seem to lack a certain logic? They emphasise the importance of maintaining the epithelial-endothelial cell barrier in MERS-CoV pathogenesis and I would agree that this is a critical aspect of coronavirus lung disease. They speculate that the PERK signaling pathway and the UPR response promote increased membrane reorganization and suppress host translation that helps optimise an environmental milieu for improved virus replication, maturation and egress. However, the virus is thought to enter the cells through the respiratory tissue - most likely the type II alveolar cell. What then is the rationale of looking at the effect of virus on fibroblasts and microvascular endothelial cells when the virus presumably enters through DPP4 + lung epithelial cells in the alveolus? Isn't this the cell type they should be looking at - as this is the likely cell to initiate the UPR - what is the point of looking at endothelial cells. It would seem that once the virus has infected the endothelial cell, there has already been a breakdown of the epithelial-endothelial barrier. Is there evidence of clinically important levels of viral replication in humans in endothelial cells?

Other comments

They perform transcriptomics/proteomics/lipidomics at various time points. The % cell infection is shown FIG 1D, and varies between ~1% and 30%. So the omics studies are all performed on cells at different time points with different % infected cells i.e. the number of infected fibroblasts range from ~1 - 30% max at 48hrs and the number of infected MVE cells range from ~1 - 50% max at 24hrs. From what I could gather they haven't performed any enrichment of infected cells - so the % infected cells was always low and varied at different timepoints, making an assessment and comparison of the data somewhat arbitrary.

Figure 5 Confusing presentation of data. It would be helpful to show the number of cells infected - rather than inhibition of infection - or maybe show both e.g. it's unclear to the reader if this is 10% infected or 70%?
At 48hrs with MVE there is no effect.
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The in vivo mouse data shows a modest effect on weight loss - but no effect on viral titre - so I'm not sure they know why the drug is having a mild beneficial effect.

The most one could say here is that UPR inhibition has a modest improvement in disease outcome in this mouse model. Not sure one can make further conclusions - how this relates to their findings is also unclear.

Reviewer #3: Summary
In this study, Sims et al. report an important finding that a strain of coronavirus, MERS-CoV, leads to different cytopathic effects in primary human lung microvascular endothelial cells and in lung fibroblasts (MVE and FB, respectively). While MERS-CoV display similar viral replication in both cell lineages, the cytopathic effect of unfolded protein response (UPR)-mediated apoptosis is limited to MVE, but not FB. The authors subsequently tested a pharmacologic PERK kinase inhibitor compound, namely AMG 44, and further demonstrated that AMG 44 exhibits cytoprotective effect in vitro on MVE cells. Furthermore, prophylactic treatment with AMG 44 protected MERS-CoV infected mice from clinical manifestation of lung damage typically seen after MERS-CoV infection. Taken together, the exhibited phenotypes are highly interesting and potentially significant for translational use of UPR pharmacologic compounds as a potential therapy against coronavirus-mediated human diseases, which warrants investment and further investigation of underlying molecular mechanisms.
However, although the authors demonstrated in the current manuscript that MERS-CoV replication kinetics were similar among FB and MVE, and MVE cells' death is likely a result from apoptotic mechanisms accompanied by UPR activation in these primary cells, there is insufficient evidence to underpin PERK branch of the UPR as a culprit for MERS-CoV associated cell death, neither in vitro nor in vivo. Furthermore, as authors correctly pointed out that "Understanding MERS-CoV pathogenic mechanisms requires understanding critical virus-host interactions", the in vivo protective effect mediated by prophylactic AMG 44 treatment may not be as simple as a PERK-kinase inhibition effect. Usage of genetic UPR mouse models to rigorously examine the hypothesis is recommended. For consideration of publication at Cell, at least a different compound targeting a downstream node of the same PERK pathways is necessary to cross-validate the conclusion that PERK branch of the UPR promotes cell apoptosis, which is largely dependent on one "PERK inhibitor (AMG 44)" at an obviously supra-pharmacological dosage used in this manuscript.

Main Critique:
Strength:
The authors, apparently pioneered in the coronavirus research, carried out important and urgently-needed studies in an effort to find a cure for coronavirus induced human diseases, including MERS-CoV induced human lung disease. In this study, the authors infected primary lung fibroblasts derived from three matched human donors and conducted both virologic and thorough multi-omics analysis of these cells, examining the in vitro effects of MERS-CoV infection. This aspect of the manuscript is definitely both novel and necessary to promote the related research urgently needed to help address the current challenges brought by the COVID19 pandemic.
Weakness:
There is insufficient evidence to support the main conclusion that MERS-CoV induced MVE cell death is mediated by the UPR, specifically via the PERK branch, leading to cellular apoptosis. In addition, there is lack

of consideration of the human immune component in the disease models in vitro by cell culture. Thirdly, the in vivo effect from prophylactic treatment with AMG 44 cannot be unequivocally attributed to PERK kinase inhibition. All drugs have off target effects.
To address the above concerns, genetic modulations of the PERK pathway signaling molecules is needed. At a minimum, other compounds targeting ER stress and/or downstream of the PERK pathway signaling molecules are absolutely necessary to support authors' main conclusion underpinning PERK kinase activity as a culprit for cell death.

Specific Comments:
1. Comparing results in Fig. 1 and 2 suggest that the preferential induction of the UPR in MVE cells is not a result of viral replication, since no difference was detected between FB and MVE in virus production (Fig. 1 A vs. B) and infection (Fig. 1D).
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¬ The authors should identify which UPR arm causes the difference between MVE and FB cells

3. On the usage of the "PERK inhibitor" - AMG 44:
It is known that AMG 44 is a "Potent and selective PERK inhibitor (IC50 = 6 nM), which "exhibits >160-fold selectivity for PERK over B-Raf and GCN2, as well as a panel of 387 other kinases. Orally bioavailable." (For more information, see https://www.tocris.com/products/amg-perk-44_5517).
¬ Given the fact that authors needed to use at least > 10 μM of AMG 44 to achieve a MERS-CoV inhibition effect (Fig. 5), this reviewer considers whether AMG 44 inhibited other kinases in authors' experimental

system. For example, two other "PERK inhibitors", GSK2606414 and GSK2656157 exhibit potent inhibitory effect on RIPK1 (doi: 10.1038/cdd.2017.58.) and AMPK.
¬ To address this concern, another PERK inhibitor such as the GSK compound should be used, at the specific "PERK inhibiting" dosage (<10 nM), to confirm PERK inhibition conferred cyto-protective effect on MVEs.
¬ MVE cells are apparently more susceptible to high doses of AMG 44 than FB, without MERS-CoV, creating a safety concern for administering AMG 44 to human patients. This safety issue should not to be taken lightly, as another PERK inhibitor, GSK2656157, exerted serious adverse effects on mouse pancreas (doi: 10.1158/0008-5472.CAN-12-3109), another grave safety concern.
¬ For a review on the toxicity profile of PERK inhibitors and their impact on physiology, please refer to the following article - Protein kinase R(PKR)-like endoplasmic reticulum kinase (PERK) inhibitors: a patent review (2010-2015) https://doi.org/10.1080/13543776.2017.1238072

4. The successful prophylactic treatment with AMG 44 in mouse models of MERS-CoV induced lung disease is the highlight of this manuscript. Although it is impossible to contribute the AMG 44 effect is specific to PERK kinase inhibition in MVE cells in vivo, the interesting preclinical observations surely hold promise for translational use of AMG 44 as a COVID19 therapy.
¬ The observation that AMG 44 does not confer protection to female mice infected with MERS-CoV is intriguing, as reviewer does not expect a sex-specific PERK difference in female mice.
¬ Could the above observation be explained by "off-target" effect of the "PERK kinase inhibitors", such as RIPK1? (doi: 10.1038/cdd.2017.58.)
¬ Given the importance of RIPK1 in mediating host versus pathogen immune response downstream of TNFR1, the therapeutic effect of AMG 44 on murine immune components should be examined in this context, for this mouse model.
¬ Nonetheless, this reviewer agrees the AMG 44 induced whole animal protection effect is both obvious and significant, regardless of the mechanism.

Notice of confidentiality: This email and any information and files transmitted with it are confidential and intended solely for the private use of the individual or entity to whom they are addressed. Public posting or distribution of all or part of this communication without the express permission of the sender is not permitted. If you are not the intended recipient, any use of this communication is unlawful. If you have received this transmission in error, please immediately notify us by return e-mail and destroy the original message.

In compliance with data protection regulations, you may request that we remove your personal registration details at any time. (Remove my information/details). Please contact the publication office if you have any questions.

From: Sims, Amy C[amy.sims@pnnl.gov]
Sent: Thur 5/28/2020 2:57:55 PM (UTC-07:00)
To: Caldwell, Chandra[ccaldwel@email.unc.edu]
Subject: A little help please

Hi Chandra,
Is it possible for you to tell me exactly how much carry forward UNC reported to the NIH for the R21 that is being relinquished? We are working on that paperwork and I am not sure of the exact dollar amount that was left over.

If it is easier and contained within the progress report you can just send me that!

I hope you are doing well and that life is getting a little easier at UNC. I hope you got to do something fun over the long weekend. Thank you very much! Amy

From: Caldwell, Chandra [ccaldwel@email.unc.edu]
Sent: 5/28/2020 3:03:12 PM
To: Sims, Amy C [amy.sims@pnnl.gov]
Subject: RE: A little help please
Attachments: 2020 Progress Report_RV.pdf

Hi Amy,

Attached is the progress report. However, you can't go by the amount that is on the progress report since the notice of award was issued to UNC before the relinquish statement was submitted. Therefore, they had to include the amount for your year 2 award along with the remaining balance from year 1.

You will have to ask Carol for the info since she completed the relinquish statement and shared it with Vicki to get her approval before sending it to OSR to submit and process.

Thanks,
Chandra

From: Sims, Amy C <amy.sims@pnnl.gov>
Sent: Thursday, May 28, 2020 5:58 PM
To: Caldwell, Chandra <ccaldwel@email.unc.edu>
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Sent: Thur 5/28/2020 3:04:48 PM (UTC-07:00)
To: Caldwell, Chandra[ccaldwel@email.unc.edu]
Subject: Re: A little help please

Got it. Thank you very much! Amy

From: "Caldwell, Chandra" <ccaldwel@email.unc.edu>
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From: Mitchell, Hugh D [Hugh.Mitchell@pnnl.gov]
Sent: 5/29/2020 9:30:32 AM
To: Sims, Amy C [amy.sims@pnnl.gov]; Baric, Ralph S [rbaric@email.unc.edu]; Sheahan, Timothy Patrick [sheahan@email.unc.edu]; Waters, Katrina M [Katrina.Waters@pnnl.gov]
Subject: RE: Cell Editorial Decision CELL-D-20-01383

Dang, that's a shame. I think there are some good points here. There IS a bit of a disconnect between the effect in vitro and in vivo, and we are well above the IC50 of the PERK inhibitor. I'm fine with going back to Science Translational Medicine. Hard to get a feel for how much of a foot in the door we have now with Cell journals. I would definitely defer to the rest of you all though.
-Hugh

From: Sims, Amy C <amy.sims@pnnl.gov>
Sent: Thursday, May 28, 2020 2:13 PM
To: Baric, Ralph S <rbaric@email.unc.edu>; Sheahan, Timothy Patrick <sheahan@email.unc.edu>; Waters, Katrina M <Katrina.Waters@pnnl.gov>; Mitchell, Hugh D <Hugh.Mitchell@pnnl.gov>
Subject: FW: Cell Editorial Decision CELL-D-20-01383

All,

We have officially been rejected from Cell. This is annoying given our long wait.

Okay, do we go back to the original plan of Science Translational Medicine or consider another Cell journal?

I am interested in your feedback. Bad way to end the week. Thanks, Amy

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From: <em.cell.0.6b95c1.abe328dd@editorialmanager.com> on behalf of Cell Editorial Office <em@editorialmanager.com>
Reply-To: Cell Editorial Office <cellms@cell.com>
Date: Thursday, May 28, 2020 at 1:59 PM
To: "Sims, Amy C" <amy.sims@pnnl.gov>
Subject: Cell Editorial Decision CELL-D-20-01383

Dr. Amy C Sims
902 Battelle Boulevard
Richland, WA 99352
UNITED STATES

CELL-D-20-01383
Unfolded Protein Response Inhibition Reduces MERS-CoV Induced Acute Lung Injury

May 28, 2020

Dear Dr. Sims,

I am enclosing the comments that the reviewers made on your paper. Unfortunately, the consensus recommendation is against publication in *Cell*. The reviewers refer to the potential timliness of the topic and interest in the approach, but cite that significant further work would be necessary, especially to demonstrate the

specificity of the compound and its proposed mechanism of action in vivo. Given their comments, and the unclear relevance to non-MERS-CoV driven coronavirus pathology, we unfortunately cannot proceed further with this manuscript at Cell.

I am sorry that the outcome for this manuscript was not more positive. I hope, however, that the reviewers' comments are clear and constructive and that you will continue to consider *Cell* for future submissions.

Although we cannot offer to proceed further with this particular paper for *Cell*, the manuscript may be a stronger candidate for another journal within the Cell Press manuscript transfer system. If you are interested in learning more about transfer as an option, please contact the relevant journal directly, as each journal is editorially independent. The link below will bring you to a page where you can select the journal to which you would like to transfer your paper (you can review each journal's aims and scopes before choosing your target).

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After your files have been transferred, you will be given an opportunity to update your materials before they are delivered to the editors at your new journal. You will be able to make any desired revisions or additions at this stage, so feel free to select a transfer option now and work on your revisions after that. For more details on the transfer process, please click here or contact the editors at your new target journal.

Please note that the above link will expire Jun 27, 2020. However, if you still wish to transfer after that period, you may submit directly to the journal of your choice and reference your original manuscript number in the cover letter.

We hope this option may be helpful for you, however if you decide not to transfer this manuscript, please click here:

Decline to Transfer

Declining to transfer will officially close out the manuscript in our system. If you do not accept or decline by Jun 27, 2020, the system will automatically decline on your behalf. If you prefer, you do have the choice of submitting this manuscript or a revised version of it to another Cell Press journal as a regular new submission, in which case you can decline the transfer.

Best wishes,
Andrew

Andrew J. Rennekamp, Ph.D.
Scientific Editor, *Cell*

Reviewers' Comments:

Reviewer #1:

This manuscript by Sims and co-workers describes the results of an unbiased, systems biology-based approach that identifies the unfolded protein response (UPR) as differentially modulated during MERS-CoV infection of primary cells. They used donor matched primary human lung microvascular endothelial cells (MVEs) and fibroblasts (FB), and found that only MVEs exhibited cytopathic effect in response to infection with MERS-CoV. To determine if a specific pathway was responsible for the differential outcome in MVEs versus FBs, they compared the transcriptomes, proteomes, and lipidomes of MERS-CoV-infected cells over time, and repeated this experiment using 3 different donors of the cells. This analysis revealed that the MERS-CoV-infected MVEs

were dying via an Unfolded Protein Response (UPR) mediated apoptosis. They validated the importance of the UPR pathway for MERS-CoV replication-induced cell death in MEVs by addition of UPR pathway inhibitor (AMG44), which reduced replication and cell death. Importantly, they also demonstrated that drug targeting of this pathway ameliorated the outcome of MERS-CoV infection in mice (reduced weight loss and improved respiratory function). Overall, this systems biology approach allowed them to identifying a targeted antiviral therapy that reduced morbidity and mortality in MERS-CoV-infected mice. The manuscript is well written and the experiments are clearly presented. Appropriate statistical analysis is provided for each experiment. The results of this study document an approach for identifying targeted antiviral therapy for coronavirus infection. This study has important implications for developing therapies for the current pandemic.
Comments for the authors' consideration (minor issues):
1. Legend to Fig 2. Is it least significant score or most significant score?
2. Legend to Fig 3. MERS-CoV MOI?
3. Title to Fig 4: Protein markers suggest activation of UPR in MVEs.
4. Legend to Fig 5. MVEs were infected with MERS-nanoluc (MOI=?)
5. Line 344: cite Gosert et al., 2002 for identification of DMVs
6. Lines 486-493: Was this MERS-nanoluc virus?
7. Lines 486-493: AMG44 is referenced to two other papers, but better to name the commercial source.
8. Do your results implicate other UPR modulators such as GSK2606414 and KIRA6 (Mahameed at al., Cell Death and Disease, 2019) as potential modulators of coronavirus replication and disease? Discussion of UPR modulators as potential therapeutics would be appropriate (line 377).
9. Line 377, start new paragraph to discuss mortality in male and female mice
10. Line 132: mothers?
11. Line 349: add recent paper Volk, Hackbart et al., 2020 J. Virol for activation of the UPR in macrophages in response to coronavirus replication.
12. Line 392: add drug names remdesivir and EIDD-2801 here

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3. On the usage of the "PERK inhibitor" - AMG 44:
It is known that AMG 44 is a "Potent and selective PERK inhibitor (IC50 = 6 nM), which "exhibits >160-fold selectivity for PERK over B-Raf and GCN2, as well as a panel of 387 other kinases. Orally bioavailable." (For more information, see https://www.tocris.com/products/amg-perk-44_5517).
¬ Given the fact that authors needed to use at least > 10 μM of AMG 44 to achieve a MERS-CoV inhibition effect (Fig. 5), this reviewer considers whether AMG 44 inhibited other kinases in authors' experimental system. For example, two other "PERK inhibitors", GSK2606414 and GSK2656157 exhibit potent inhibitory effect on RIPK1 (doi: 10.1038/cdd.2017.58.) and AMPK.
¬ To address this concern, another PERK inhibitor such as the GSK compound should be used, at the specific "PERK inhibiting" dosage (<10 nM), to confirm PERK inhibition conferred cyto-protective effect on MVEs.
¬ MVE cells are apparently more susceptible to high doses of AMG 44 than FB, without MERS-CoV, creating a safety concern for administering AMG 44 to human patients. This safety issue should not to be taken lightly, as another PERK inhibitor, GSK2656157, exerted serious adverse effects on mouse pancreas (doi: 10.1158/0008-5472.CAN-12-3109), another grave safety concern.
¬ For a review on the toxicity profile of PERK inhibitors and their impact on physiology, please refer to the following article - Protein kinase R(PKR)-like endoplasmic reticulum kinase (PERK) inhibitors: a patent review (2010-2015) https://doi.org/10.1080/13543776.2017.1238072

4. The successful prophylactic treatment with AMG 44 in mouse models of MERS-CoV induced lung disease is the highlight of this manuscript. Although it is impossible to contribute the AMG 44 effect is specific to PERK kinase inhibition in MVE cells in vivo, the interesting preclinical observations surely hold promise for translational use of AMG 44 as a COVID19 therapy.
¬ The observation that AMG 44 does not confer protection to female mice infected with MERS-CoV is intriguing, as reviewer does not expect a sex-specific PERK difference in female mice.
¬ Could the above observation be explained by "off-target" effect of the "PERK kinase inhibitors", such as RIPK1? (doi: 10.1038/cdd.2017.58.)
¬ Given the importance of RIPK1 in mediating host versus pathogen immune response downstream of TNFR1, the therapeutic effect of AMG 44 on murine immune components should be examined in this context, for this mouse model.
¬ Nonetheless, this reviewer agrees the AMG 44 induced whole animal protection effect is both obvious and significant, regardless of the mechanism.

Notice of confidentiality: This email and any information and files transmitted with it are confidential and intended solely for the private use of the individual or entity to whom they are addressed. Public posting or distribution of all or part of this communication without the express permission of the sender is not permitted. If you are not the intended recipient, any use of this communication is unlawful. If you have received this transmission in error, please immediately notify us by return e-mail and destroy the original message.

In compliance with data protection regulations, you may request that we remove your personal registration details at any time. (Remove my information/details). Please contact the publication office if you have any questions.

From: Sims, Amy C [amy.sims@pnnl.gov]
Sent: 5/29/2020 9:41:26 AM
To: daszak@ecohealthalliance.org
CC: chmura@ecohealth.net; andre@ecohealth.net
Subject: Re: Review Editor for Manuscript ID ECH-20-0041

Peter and Aleksei,

I am terribly sorry but I cannot seem to figure out how to get to the editor section of the EcoHealth Journal. Is there a link or more information you can provide? I have never been an editor and I am very confused.

Thank you very much! I really appreciate your assistance. Also, did the authors recommend any potential reviewers or should I just find them myself?

Thank you again, Amy

On 5/24/20, 6:59 PM, "EcoHealth" <onbehalfof@manuscriptcentral.com> wrote:

24-May-2020

Dear Dr. Sims:

Manuscript ID ECH-20-0041 entitled "AVIAN CORONAVIRUSES IN WILD BIRDS ALONG BRAZILIAN SEGMENT OF ATLANTIC FLYWAY," with Dr. Barbosa as submitting author, has been submitted to EcoHealth. I have just assigned this manuscript to you to act as Review Editor.

Please assess this manuscript and determine whether is acceptable to send out for review. If so, select, invite, and assign reviewers as soon as possible.

Please contact Aleksei at if you are having difficulties finding reviewers and/or have any questions about the manuscript or the Manuscript Central Review Editor process. I look forward to reading your decision.

Thank you,

Peter Daszak
Editor-in-Chief, EcoHealth

5 U.S.C. § 552(b)(6)

Visit our blog at: https://protect2.fireeye.com/v1/url?k=efb0aba0-b3059419-efb081b5-0cc47adc5fce-168ff613f7c21a24&q=1&e=7da4dcbb-ea67-4c64-bc1c-18c14274750f&u=http%3A%2F%2Fwww.ecohealthalliance.org%2Fblog

EcoHealth Alliance leads cutting-edge research into the critical connections between human and wildlife health and delicate ecosystems. With this science we develop solutions that promote conservation and prevent pandemics.

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For information on how to install Adobe Reader and configure your viewing environment please see http://www.adobe.com/go/pdf_forms_configure.

BIOGRAPHICAL SKETCH

Provide the following information for the Senior/key personnel and other significant contributors.
Follow this format for each person. **DO NOT EXCEED FIVE PAGES.**

NAME: Sheahan, Timothy Patrick

eRA COMMONS USER NAME (credential, e.g., agency login): tsheahan

POSITION TITLE: Assistant Professor

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, include postdoctoral training and residency training if applicable. Add/delete rows as necessary.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	Completion Date MM/YYYY	FIELD OF STUDY
University of New Hampshire	B.S.	06/1999	Microbiology/Water Resources
University of North Carolina at Chapel Hill	Ph.D.	05/2008	Virology
The Rockefeller University	Postdoctoral	03/2013	Systems Virology

A. Personal Statement

I have over a fifteen years of experience performing translational research focused on evaluating antiviral therapeutics and elucidating virus and host factor targets for antiviral development. Through my academic and industrial training, I have acquired a broad skillset necessary to lead this program and meet our milestones. I gained extensive knowledge of coronavirus (CoV) molecular biology, pathogenesis, vaccinology, and animal model development within which to evaluate therapeutics through my graduate research at UNC Chapel Hill with Dr. Ralph S. Baric. The goals of my graduate research were to gain a better understand of the molecular mechanisms guiding CoV zoonotic emergence and to evaluate the efficacy of vaccines and antibodies against epidemic CoV and zoonotic CoV. During my graduate career I published extensively on zoonotic CoV and therapeutics (15 publications) and the skills gained executing these studies continue to be of use today. Under the guidance of Dr. Charles M. Rice at The Rockefeller University, my postdoctoral research focused on the creation of single cell systems within which to better understand the molecular mechanisms guiding hepatitis C virus (HCV) chronic infection. During my tenure at Rockefeller, I was awarded an NIH F32 fellowship through which I gained my first experience independently guiding milestone driven research. To carry out proposed grant aims, I developed a systems virology approach coupling primary human hepatocyte cultures and laser-capture microdissection facilitating the isolation and transcriptional profiling of HCV infected cells at a resolution approaching that of a single cell. This work was also featured on Dr. Vincent Racaniello's popular podcast "This Week in Virology." After my postdoctoral fellowship, I became an investigator at the Antiviral Discovery Performance Unit at GlaxoSmithKline (GSK) based in Research Triangle Park. At GSK, I was part of several programs focused on developing and evaluating host targeting small molecules as antivirals. Through this work, I gained expertise in whole genome siRNA screens and triage of hits, antiviral assay development, and design of in vivo efficacy studies. Importantly, I became fluent in the language of preclinical drug development through interactions with experts in drug metabolism, pharmacokinetics, drug safety and toxicology. I also led a three-way public private partnership between GSK, Perkin Elmer and the University of Wisconsin at Madison to develop in vivo imaging technology that facilitated the imaging of virus replication and pulmonary inflammation in live animals. Not only did this program result in a publication on in vivo imaging techniques for influenza virus, but I also gained expertise in managing research collaborations between academia and industry which continues to be of great value. In July of 2015, I became faculty at UNC Chapel Hill in the Department of Epidemiology focusing on broad-spectrum therapeutic approaches targeting CoV with the long-term goal of developing vaccines, therapeutic antibodies and small molecules to prevent future pandemics. Through a Partnership R01 (PI Sheahan/Baric) awarded in 2016, we have been working with Gilead Sciences and Vanderbilt School of Medicine to

accelerate the preclinical development of the broad-spectrum antiviral, remdesivir, to treat MERS-CoV and related CoVs. The publications resulting from this program (see below) have in part facilitated the initiation of a clinical trial for remdesivir in China to treat nCoV-2019. My training and experience make me well suited to support this program to accelerate broad-spectrum antiviral approaches to combat emerging CoV infections.

B. Positions and Honors

Positions and Employment

1999-2001	Laboratory Technician, Harvard Gene Therapy Initiative, Harvard Medical School, Boston, MA.
2001-2003	Laboratory Technician, Tissue Engineering Laboratory of Joseph Vacanti. Massachusetts General Hospital, Boston, MA.
2003-2008	Graduate Student, Laboratory of Ralph S. Baric, University of North Carolina, Chapel Hill, NC.
2008-2014	Postdoctoral Fellow, Laboratory of Charles M. Rice, The Rockefeller University, NY, NY.
2014-2015	Investigator, Antiviral Discovery Performance Unit, GlaxoSmithKline, RTP, NC.
2015-	Assistant Professor, Department of Epidemiology, University of North Carolina, Chapel Hill, NC.

Other Experience and Professional Memberships

2002-	Member, American Society for Microbiology
2007-	Member, American Society for Virology
2019-	Member, International Society for Antiviral Research

Honors

1998	Gordon Byers Scholarship for an Outstanding Water Resources Student.
2002	Partners in Excellence Award, Massachusetts General Hospital.
2009	Ruth L. Kirschstein National Research Service Award (Postdoctoral Fellowship).
2015	Third Place Regional GSK Beautiful Biology Award. *"In vivo imaging: A new platform to accelerate drug discovery at the host/pathogen interface".*
2015	Second Place Global GSK Beautiful Biology Award. *"In vivo imaging: A new platform to accelerate drug discovery at the host/pathogen interface".*

C. Contributions to Science

1. My early work focused on the molecular mechanisms guiding zoonotic CoV emergence and viral/host determinants of SARS-CoV pathogenesis. We elucidated mutations in the SARS-CoV required to increase pathogenesis in mice and shift zoonotic CoV host range to infect human cells. The mouse models created through these studies continue to be of use today as we evaluate new therapies targeting CoV. Moreover, these studies provided the technical foundation for synthetic genome design and recovery of recombinant zoonotic CoV, which has since been repeated multiple times by the Baric Lab.
    a. Roberts A, Deming D, Paddock CD, Cheng A, Yount B, Vogel L, Herman BD, **Sheahan T**, Heise M, Genrich GL, Zaki SR, Baric R, Subbarao K. A mouse-adapted SARS-coronavirus causes disease and mortality in BALB/c mice. *PLoS Pathogens.* 2007 Jan;3(1):e5.
    b. **Sheahan T**, Rockx B, Donaldson E, Sims A, Pickles R, Corti D, Baric R. Mechanisms of Zoonotic SARS-CoV Host Range Expansion in Human Airway Epithelium. *Journal of Virology.* 2008 Mar;82(5):2274-85.
    c. **Sheahan T**, Rockx B, Donaldson E, Corti D, Baric R. Pathways of Cross Species Transmission of Synthetically Reconstructed Zoonotic SARS-CoV. *Journal of Virology.* 2008 Sep;82(17):8721-32.
    d. **Sheahan T**, Morrison T, Funkhouser W, Akira S, Heise M, Baric R. MyD88 is required for protection from lethal infection with a mouse adapted SARS-CoV. *PLoS Pathogens.* 2008 Dec;4(12):e1000240.
2. Throughout my career, I have placed a special emphasis on pursuing and performing translational research, which is summarized in select publications below. My work on HCV in primary human

hepatocytes demonstrated that specific interferon lambda alleles within hepatocytes were associated with permissiveness to infection corroborating phenotypes seen clinically. For the first time, these data showed that genetic defects in hepatocytes likely guide the development of chronic HCV infection. More recently, I have been focusing on developing broad-spectrum antivirals for emerging coronavirus. We have published multiple high impact papers describing the preclinical development of the nucleoside analog, remdesivir, which are noted below. Together, these studies highlight my commitment to translational research and the development of therapeutics for emerging viral diseases.

a. **Sheahan TP**, Imanaka N, Marukian S, Dorner M, Liu P, Ploss A, Rice CM. Interferon Lambda Alleles Predict Innate Antiviral Immune Responses and Hepatitis C Virus Permissiveness. *Cell Host and Microbe.* 2014 Feb 12;15(2):190-202.
b. **Sheahan TP**, Sims AC, Graham RL, Menachery VD, Gralinski LE, Case JB, Leist SR, Pyrc K, Feng JY, Trantcheva I, Bannister R, Park Y, Babusis D, Clarke MO, Mackman RL, Spahn JE, Palmiotti CA, Siegel D, Ray AS, Cihlar T, Jordan R, Denison MR, Baric RS. Broad-spectrum antiviral GS-5734 inhibits both epidemic and zoonotic coronaviruses. Sci Transl Med. 2017 Jun 28;9(396).
c. Brown AJ, Won JJ, Graham RL, Dinnon KH 3rd, Sims AC, Feng JY, Cihlar T, Denison MR, Baric RS, **Sheahan TP**. Broad spectrum antiviral remdesivir inhibits human endemic and zoonotic deltacoronaviruses with a highly divergent RNA dependent RNA polymerase. Antiviral Res. 2019 Jun 21;169:104541
d. **Sheahan TP**, Sims AC, Leist SR, Schäfer A, Won J, Brown AJ, Montgomery SA, Hogg A, Babusis D, Clarke MO, Spahn JE, Bauer L, Sellers S, Porter D, Feng JY, Cihlar T, Jordan R, Denison MR, Baric RS. Comparative therapeutic efficacy of remdesivir and combination lopinavir, ritonavir, and interferon beta against MERS-CoV. Nat Commun. 2020 Jan 10;11(1):222.

3. New technologies facilitate advances in science by accelerating the pace of discovery and increasing observational resolution. The work below highlights how new technologies can refine and accelerate translational research. With in vivo bioluminescent imaging, virus replication can be visualized in live animals. When applied to drug efficacy studies, inhibition of virus replication can be observed instantaneously in a single animal over time thus eliminating the need for large cohorts and sacrifice of mice over time to measure virus replication. Antivirals often inhibit virus replication. Single molecule RNA fluorescence in situ hybridization (smRNA FISH) techniques facilitate the simultaneous quantitation of viral RNAs and cellular RNAs at the single cell level. When applied to drug development as in the publication below, this technique can be applied to define the stage of replication inhibited by an antiviral and help refine the mechanism of action. Lastly, we established readily translatable metrics like complete blood count at BSL3 for both SARS and MERS-CoV.
    a. Tran V, Poole DS, Jeffery JJ, **Sheahan TP**, Creech D, Yevtodiyenko A, Peat AJ, Francis KP, You S, Mehle A. Multi-Modal Imaging with a Toolbox of Influenza A Reporter Viruses. Viruses. 2015 Oct 13;7(10):5319-27.
    b. Ramanan V, Trehan K, Ong ML, Luna JM, Hoffmann HH, Espiritu C, **Sheahan TP**, Chandrasekar H, Schwartz RE, Christine KS, Rice CM, van Oudenaarden A, Bhatia SN. Viral genome imaging of hepatitis C virus to probe heterogeneous viral infection and responses to antiviral therapies. Virology. 2016 Apr 26;494:236-247. doi: 10.1016/j.virol.2016.04.020. PMID: 27128351
    c. Leist SR, Jensen KL, Baric RS, **Sheahan TP**. Increasing the translation of mouse models of MERS coronavirus pathogenesis through kinetic hematological analysis. PLoS One. 2019 Jul 24;14(7):e0220126.

Complete List of Published Work in NCBI MyBibliography:
http://www.ncbi.nlm.nih.gov/myncbi/browse/collection/40066608/?sort=date&direction=descending

D. Additional Information: Research Support and/or Scholastic Performance

Ongoing Research Support

U19 AI142759 CETR (PI: Whitley) 03/01/19-02/28/24
UAB/NIH/NIAID
Antiviral Drug Discovery and Development Center

The specific aims of the proposal will identify small molecule inhibitors of CoV replication, define their mechanism of action, and determine their efficacy against SARS-CoV, MERS-CoV and across CoV families using in vivo mouse models of acute and persistent CoV disease. Role: Investigator

R01 AI131688-01 (PI: Rice) 03/15/17-02/281/22
Rockefeller/NIH
Analysis of immunity, viral adaptation and pathogenesis in a new mouse model of HCV-related rodent hepacivirus infection
Mechanisms that contribute to the persistence of hepatotropic viruses, such as HCV, are not well understood. We have recently established the first immune-competent mouse model of an HCV-related virus. With this new model, we propose to systematically study immunity and host-virus interactions during a hepatotropic RNA virus infection in vivo. The specific aims of this proposal are to 1) characterize the innate and adaptive responses to NrHV infection in WT mice, 2) create new NrHV pathogenesis models through the adaptation of virus in WT and immune deficient mice and through the screening of CC mouse strains for increased susceptibility. Loci driving enhanced pathogenesis and resistance will be mapped, and 3) develop new models with mouse adapted virus to better model the effects of age, obesity and alcohol on the development of liver disease.
Role: Subcontract PI

R01 AI132178-01 (MPI: Sheahan/Baric) 08/09/17-07/31/22
NIH
Broad-spectrum antiviral GS-5734 to treat MERS-CoV and related emerging CoV
In partnership with Gilead Sciences, we aim to accelerate the preclinical development of GS-5734 and promote IND licensure. We define the pharmacokinetics, pharmacodynamics, resistance profile, efficacy breadth and mechanism of action of GS-5734 against MERS-CoV and related emerging CoV.

R01AI108197-06 (MPI: Baric/Denison) 03/01/18-02/28/23
Vanderbilt/NIH
Determinants of Coronavirus Fidelity in Replication and Pathogenesis
To identify common and unique determinants of CoV nsp14-ExoN functions CoV replication, fidelity and IFN sensitivity across CoVs; To determine pathways of adaptation to loss of nsp14-ExoN activity in vitro and in vivo; and to define mechanisms of ExoN-regulated CoV sensitivity to the innate antiviral immune response.
Role: Co-Investigator

U01AI149644 (PI: Baric) 04/19/19-03/31/24
NIH
Respiratory Virus Vaccine and Adjuvant Exploration
Vaccination is one of the most effective public health measures for protecting against infectious disease, and the proposed studies will identify adjuvants and adjuvant combinations that safely elicit long lived protective immunity against emerging pathogens in at risk populations.
Role: Investigator

Statement of Work (SOW)
Univ. of North Carolina at Chapel Hill Subcontract with Battelle-PNWD, Operator of PNNL for US DOE

Project Title: How MERS-CoV Regulates Innate Immunity in Primary Human Lung Cells
Period of Performance: June 1, 2020 – May 31, 2021

Background:
The molecular mechanisms regulating emerging highly pathogenic human respiratory coronavirus pathogenesis are complex and include dampening of the host innate immune response allowing higher viral titers immediately following entry. Previous studies tracking the expression of interferon stimulated gene (ISG) expression in Middle East respiratory syndrome coronavirus (MERS-CoV) infected continuous human lung epithelial cells demonstrated that specific ISG expression patterns could be detected and these patterns could be used to determine how CoV were modulating the innate immune response. Epigenetic/chromatin remodeling mechanisms were found to be important regulators of host chromatin remodeling that altered host expression ISG patterns. This proposal will build on these foundational studies performed in continuous human lung cell lines and define ways that MERS-CoV regulates innate immunity in primary human lung fibroblasts and microvascular endothelial cells.

Scope:
Will analyze the fibroblast ChIP PCR samples collected in 2019 and discuss data with PNNL to determine next steps. Perform ChIP Seq analysis of MERS-CoV infected microvascular endothelial cells at a single time point for three donors. Will perform BioRad Primer PCR Pathway Arrays on MERS-CoV infected microvascular endothelial cells and fibroblasts. Drs Sheahan and Schaefer will work in close collaboration with PNNL to analyze datasets to determine how MERS-CoV regulates ISG expression in human lung microvascular endothelial cells and fibroblasts.

Deliverables:
- Evaluate project results and direction, in collaboration with PNNL
- Collaborate on experiment direction and identify alternatives for experiments
- Participate in meetings to discuss project status/progress, findings, and trouble shoot as needed
- Write and submit progress report and manuscript draft in collaboration with PNNL

From: Sims, Amy C[amy.sims@pnnl.gov]
Sent: Mon 6/1/2020 5:27:16 PM (UTC-07:00)
To: Schaefer, Alexandra[aschaefe@email.unc.edu]
Subject: biosketch

Hi Alex,

Can you please send me your latest biosketch? I need it to get UNC's subcontract set up for the R21.

I hope you are doing well. Thank you, Amy

From: Powell, Tamia (NIH/NIAID) [E][tamia.powell@nih.gov]
Sent: Wed 6/3/2020 10:59:51 AM (UTC-07:00)
To: Sims, Amy C[amy.sims@pnnl.gov]; Sandoval, Norma I[Norma.Sandoval@pnnl.gov]; Hays, Barbara A[barb.hays@pnnl.gov]
Cc: kati_chipps@unc.edu[kati_chipps@unc.edu]; sheahan@email.unc.edu[sheahan@email.unc.edu]
Subject: RE: Change of Institution - Anniversary/Mid-Year Research and Career Awards - 5R21AI146872-02
Attachment: PHS3734 Relinquishing Statement.pdf

Hi Amy,

My response is two-fold and will require UNC-Chapel Hill to resubmit their relinquishing statement.

As the Year 2 award was already issued to UNC, the transfer action will be taken on the Year 2 and only the Year 2 dollars should reflected in PNNL budget. However, the attached relinquishing statement submitted by UNC exceeds the total dollars allocated in Year 2 an appears to include both funds unspent from Year 1 and funds allocated for Year 2.

UNC – please resubmit the relinquishing statement as a non-fillable pdf to indicate only the Year 2 dollars that UNC is relinquishing to PNNL.

Thanks,
Tamia

From: Sims, Amy C <amy.sims@pnnl.gov>
Sent: Wednesday, June 3, 2020 1:28 PM
To: Powell, Tamia (NIH/NIAID) [E] <tamia.powell@nih.gov>
Cc: Sandoval, Norma I <Norma.Sandoval@pnnl.gov>; Hays, Barbara A <barb.hays@pnnl.gov>
Subject: Change of Institution - Anniversary/Mid-Year Research and Career Awards - 5R21AI146872-02

Dear Dr. Powell,

PNNL's administrative team has a question which is stated below. Can you please advise us on how to proceed? Thank you in advance for your assistance.

Best wishes, Amy Sims

Amy Sims
Biomedical Scientist
Pacific Northwest National Laboratory (PNNL)
Email amy.sims@pnnl.gov
Office 509 372 6496

From: "Sandoval, Norma I" <Norma.Sandoval@pnnl.gov>
Date: Wednesday, June 3, 2020 at 9:06 AM
To: "Sims, Amy C" <amy.sims@pnnl.gov>
Cc: "Hays, Barbara A" <barb.hays@pnnl.gov>
Subject: RE: Change of Institution - Anniversary/Mid-Year Research and Career Awards - 5R21AI146872-02

Hi Amy,
Can you send the below question to Tamia?

Good Morning Ms. Powell,
I have received the transfer instructions and we, PNNL, are working on our internal documents for submission.

We do have a budget inquiry though, my understanding is that the current grantee institution (UNC) had Year 1 funds remaining and have provided us the total amount remaining. Should this cost be included in our budget request? If so, how should we account for it in our budget?

Thank you.

Norma Sandoval
Principal Project Coordinator
Biological Sciences Division, **EBSD**
Pacific Northwest National Laboratory

From: Sims, Amy C <amy.sims@pnnl.gov>
Sent: Tuesday, May 26, 2020 8:25 AM
To: Sandoval, Norma I <Norma.Sandoval@pnnl.gov>; Hays, Barbara A <barb.hays@pnnl.gov>
Subject: FW: Change of Institution - Anniversary/Mid-Year Research and Career Awards - 5R21AI146872-02

Dear Norma and Barb,

I just received this email. Please let me know what I need to do. Thank you very much! Amy

From: "Ranellone, Ashley (NIH/NIAID) [E]" <ranelloneac@niaid.nih.gov>
Date: Tuesday, May 26, 2020 at 7:56 AM
To: "sheahan@email.unc.edu" <sheahan@email.unc.edu>
Cc: "Powell, Tamia (NIH/NIAID) [E]" <tamia.powell@nih.gov>, "Chipps, Kati" <kati_chipps@unc.edu>, "Sims, Amy C" <amy.sims@pnnl.gov>
Subject: Change of Institution - Anniversary/Mid-Year Research and Career Awards - 5R21AI146872-02

Good Morning,

A relinquishing statement was received for the above referenced grant award. Please following the instructions as noted below. The specialist assigned to the transfer is Tamia Powell, also cc: to this correspondence. All future inquiries should be forwarded to her.

From the Current Grantee Institution (University of North Carolina Chapel Hill)

Form PHS 3734, Official Statement Relinquishing Interests and Rights in a PHS Research Grant submitted via eRA Commons. The transfer application cannot be processed until this form has been received by the NIAID. This form provides the effective date of relinquishment, estimated unexpended Direct and Facilities and Administrative (F&A) Costs, and a list of equipment costing $5,000 or more transferring with the project. Note: the grantee should not relinquish more than the 'Total Federal Award Amount' shown on the most recent NoA. **Received from University of North Carolina Chapel Hill on May 22nd.**

Final Invention Statement and Certification (HHS 568) - is required for all active grants being transferred but is not due until 90 days after the grant is terminated at the old institution as part of

the required closeout.

Final Federal Financial Report (FFR, Standard Form 425) – is required for all active grants being transferred but not due until 90 days after the grant is terminated at the old institution.

From the Proposed New Institution (Battelle Centers for Public Health Research and Evaluation)
Transfer Application – the proposed new institution should prepare and submit its transfer application before the investigator transfers. Form pages below are required to submit the transfer application electronically. For detailed instructions on how to complete and submit an electronic Change of Grantee Institution application please visit PA-18-590.

The electronic application from the proposed grantee institution should include, at a minimum, the following:
SF 424 (R&R) Cover Component
SF 424 (R&R) Project/Performance Site Location(s)
SF 424 (R&R) Other Project information:

- Certification of IRB/IACUC approval, including OHRP and OLAW assurance numbers, if applicable.
- Facilities and Other Resources, including probable effect of the move on the project.
- Detailed list of any equipment purchased with grant funds to be transferred to the new organization (inclusion of this list in the transfer application from the new organization indicates its acceptance of title to that equipment).

SF 424 Research & Related Senior/Key Person Profile:

- Updated biographical sketches for the PD/PI and existing senior/key personnel and biographical sketches for any proposed new senior/key personnel and updated "other support" page(s) as necessary.

Budget pages applicable for activity code (current and future years). NOTE: Even if the budget for the original award was submitted in a modular format, applicants must use the R&R Detailed Budget form.

- Applicants may complete all the fields in the R&R Detailed Budget as appropriate, or
- Complete only the costs for the PD/PI (Section A) to reflect a modular budget and include the remainder of the direct costs under Section F (Other Direct Costs) Item 8, and Section H (Indirect Costs).
- For awards resulting from modular applications, include narrative budget information for the current budget period, including total direct cost and the basis for computing F&A costs and, if applicable, future budget periods.
- Budget requests should not exceed the direct costs previously recommended for any budget period.

PHS 398 Research Plan

- If transferring on the anniversary date, include the progress report for the current year including a statement regarding the goals for the upcoming year. For all transfer applications include also a statement indicating whether the overall research plans/aims have changed from the original submission, and, if so, provide updated information.

- PHS 398 Cover Page Supplement

- PHS 398 Checklist

Note also that a change of recipient request normally will be permitted only when all of the permanent benefits attributable to the original grant can be transferred, including equipment purchased in whole or in part with grant funds. In reviewing a request to transfer a grant, NIH will consider whether there is a continued need for the grant-supported project or activity and the impact of any proposed changes in the scope of the project. NIH will also consider the length of time, the percentage of funds, and the amount of work remaining in the project period. Transfers will take longer to complete if the proposed organization needs any of the assurances and IRB or IACUC approvals.

The deadline for the transfer application is June 9th, 2020.

Thanks,

Ashley Ranellone
Grants Management Specialist
DHHS, NIH, NIAID, DEA, GMP
5601 Fishers Lane, Rm 4G55
Bethesda, MD 20892
(301) 594-8541



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  /ASCII85EncodePages false
  /AllowTransparency false
  /AutoPositionEPSFiles true
  /AutoRotatePages /None
  /Binding /Left
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  /CalCMYKProfile (U.S. Web Coated \050SWOP\051 v2)
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  /CompatibilityLevel 1.4
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  /DefaultRenderingIntent /Default
  /DetectBlends true
  /DetectCurves 0.0000
  /ColorConversionStrategy /CMYK
  /DoThumbnails false
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From: Sims, Amy C[amy.sims@pnnl.gov]
Sent: Wed 6/3/2020 11:06:27 AM (UTC-07:00)
To: Moore, Victoria L[victoria_moore@unc.edu]; Burkhart, Carol J.[carol_burkhart@unc.edu]
Cc: Caldwell, Chandra[ccaldwel@email.unc.edu]; Sandoval, Norma I[Norma.Sandoval@pnnl.gov]; Hays, Barbara A[barb.hays@pnnl.gov]
Subject: FW: Change of Institution - Anniversary/Mid-Year Research and Career Awards - 5R21AI146872-02
Attachment: PHS3734 Relinquishing Statement.pdf

All,

Please see the request below from the NIH. They need a revised version of the relinquishment letter. Thank you very much! Amy

---

From: "Powell, Tamia (NIH/NIAID) [E]" <tamia.powell@nih.gov>
Date: Wednesday, June 3, 2020 at 11:04 AM
To: "Sims, Amy C" <amy.sims@pnnl.gov>, "Sandoval, Norma I" <Norma.Sandoval@pnnl.gov>, "Hays, Barbara A" <barb.hays@pnnl.gov>
Cc: "kati_chipps@unc.edu" <kati_chipps@unc.edu>, "sheahan@email.unc.edu" <sheahan@email.unc.edu>
Subject: RE: Change of Institution - Anniversary/Mid-Year Research and Career Awards - 5R21AI146872-02

Hi Amy,

My response is two-fold and will require UNC-Chapel Hill to resubmit their relinquishing statement.

As the Year 2 award was already issued to UNC, the transfer action will be taken on the Year 2 and only the Year 2 dollars should reflected in PNNL budget. However, the attached relinquishing statement submitted by UNC exceeds the total dollars allocated in Year 2 an appears to include both funds unspent from Year 1 and funds allocated for Year 2.

UNC – please resubmit the relinquishing statement as a non-fillable pdf to indicate only the Year 2 dollars that UNC is relinquishing to PNNL.

Thanks,
Tamia

From: Sims, Amy C <amy.sims@pnnl.gov>
Sent: Wednesday, June 3, 2020 1:28 PM
To: Powell, Tamia (NIH/NIAID) [E] <tamia.powell@nih.gov>
Cc: Sandoval, Norma I <Norma.Sandoval@pnnl.gov>; Hays, Barbara A <barb.hays@pnnl.gov>
Subject: Change of Institution - Anniversary/Mid-Year Research and Career Awards - 5R21AI146872-02

Dear Dr. Powell,

PNNL's administrative team has a question which is stated below. Can you please advise us on how to proceed? Thank you in advance for your assistance.

Best wishes, Amy Sims

Amy Sims
Biomedical Scientist

Pacific Northwest National Laboratory (PNNL)
Email amy.sims@pnnl.gov
Office 509 372 6496

From: "Sandoval, Norma I" <Norma.Sandoval@pnnl.gov>
Date: Wednesday, June 3, 2020 at 9:06 AM
To: "Sims, Amy C" <amy.sims@pnnl.gov>
Cc: "Hays, Barbara A" <barb.hays@pnnl.gov>
Subject: RE: Change of Institution - Anniversary/Mid-Year Research and Career Awards - 5R21AI146872-02

Hi Amy,
Can you send the below question to Tamia?

Good Morning Ms. Powell,
I have received the transfer instructions and we, PNNL, are working on our internal documents for submission.

We do have a budget inquiry though, my understanding is that the current grantee institution (UNC) had Year 1 funds remaining and have provided us the total amount remaining. Should this cost be included in our budget request? If so, how should we account for it in our budget?

Thank you.

Norma Sandoval
Principal Project Coordinator
Biological Sciences Division, **EBSD**
Pacific Northwest National Laboratory

From: Sims, Amy C <amy.sims@pnnl.gov>
Sent: Tuesday, May 26, 2020 8:25 AM
To: Sandoval, Norma I <Norma.Sandoval@pnnl.gov>; Hays, Barbara A <barb.hays@pnnl.gov>
Subject: FW: Change of Institution - Anniversary/Mid-Year Research and Career Awards - 5R21AI146872-02

Dear Norma and Barb,

I just received this email. Please let me know what I need to do. Thank you very much! Amy

---

From: "Ranellone, Ashley (NIH/NIAID) [E]" <ranelloneac@niaid.nih.gov>
Date: Tuesday, May 26, 2020 at 7:56 AM
To: "sheahan@email.unc.edu" <sheahan@email.unc.edu>
Cc: "Powell, Tamia (NIH/NIAID) [E]" <tamia.powell@nih.gov>, "Chipps, Kati" <kati_chipps@unc.edu>, "Sims, Amy C" <amy.sims@pnnl.gov>
Subject: Change of Institution - Anniversary/Mid-Year Research and Career Awards - 5R21AI146872-02

Good Morning,

A relinquishing statement was received for the above referenced grant award. Please following the instructions as noted below. The specialist assigned to the transfer is Tamia Powell, also cc: to this correspondence. All future inquiries should be forwarded to her.

From the Current Grantee Institution (University of North Carolina Chapel Hill)

Form PHS 3734, Official Statement Relinquishing Interests and Rights in a PHS Research Grant submitted via eRA Commons. The transfer application cannot be processed until this form has been received by the NIAID. This form provides the effective date of relinquishment, estimated unexpended Direct and Facilities and Administrative (F&A) Costs, and a list of equipment costing $5,000 or more transferring with the project. Note: the grantee should not relinquish more than the 'Total Federal Award Amount' shown on the most recent NoA. **Received from University of North Carolina Chapel Hill on May 22nd.**

Final Invention Statement and Certification (HHS 568) - is required for all active grants being transferred but is not due until 90 days after the grant is terminated at the old institution as part of the required closeout.

Final Federal Financial Report (FFR, Standard Form 425) – is required for all active grants being transferred but not due until 90 days after the grant is terminated at the old institution.

From the Proposed New Institution (Battelle Centers for Public Health Research and Evaluation)
Transfer Application – the proposed new institution should prepare and submit its transfer application before the investigator transfers. Form pages below are required to submit the transfer application electronically. For detailed instructions on how to complete and submit an electronic Change of Grantee Institution application please visit PA-18-590.

The electronic application from the proposed grantee institution should include, at a minimum, the following:
SF 424 (R&R) Cover Component
SF 424 (R&R) Project/Performance Site Location(s)
SF 424 (R&R) Other Project information:

- Certification of IRB/IACUC approval, including OHRP and OLAW assurance numbers, if applicable.
- Facilities and Other Resources, including probable effect of the move on the project.
- Detailed list of any equipment purchased with grant funds to be transferred to the new organization (inclusion of this list in the transfer application from the new organization indicates its acceptance of title to that equipment).

SF 424 Research & Related Senior/Key Person Profile:

- Updated biographical sketches for the PD/PI and existing senior/key personnel and biographical sketches for any proposed new senior/key personnel and updated "other support" page(s) as necessary.

Budget pages applicable for activity code (current and future years). NOTE: Even if the budget for the original award was submitted in a modular format, applicants must use the R&R Detailed Budget form.

- Applicants may complete all the fields in the R&R Detailed Budget as appropriate, or
- Complete only the costs for the PD/PI (Section A) to reflect a modular budget and include the remainder of the direct costs under Section F (Other Direct Costs) Item 8, and Section H (Indirect Costs).
- For awards resulting from modular applications, include narrative budget information for the current budget period, including total direct cost and the basis for computing F&A costs and, if applicable, future budget periods.
- Budget requests should not exceed the direct costs previously recommended for any budget

period.

PHS 398 Research Plan

- If transferring on the anniversary date, include the progress report for the current year including a statement regarding the goals for the upcoming year. For all transfer applications include also a statement indicating whether the overall research plans/aims have changed from the original submission, and, if so, provide updated information.

- PHS 398 Cover Page Supplement

- PHS 398 Checklist

Note also that a change of recipient request normally will be permitted only when all of the permanent benefits attributable to the original grant can be transferred, including equipment purchased in whole or in part with grant funds. In reviewing a request to transfer a grant, NIH will consider whether there is a continued need for the grant-supported project or activity and the impact of any proposed changes in the scope of the project. NIH will also consider the length of time, the percentage of funds, and the amount of work remaining in the project period. Transfers will take longer to complete if the proposed organization needs any of the assurances and IRB or IACUC approvals.

The deadline for the transfer application is June 9th, 2020.

Thanks,

Ashley Ranellone
Grants Management Specialist
DHHS, NIH, NIAID, DEA, GMP
5601 Fishers Lane, Rm 4G55
Bethesda, MD 20892
(301) 594-8541

National Institute of Allergy and Infectious Diseases

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  /PageSize [612.000 792.000]
>> setpagedevice
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Sent: Wed 6/3/2020 2:22:29 PM (UTC-07:00)
To: Powell, Tamia (NIH/NIAID) [E][tamia.powell@nih.gov]; Sims, Amy C[amy.sims@pnnl.gov]; Sandoval, Norma I[Norma.Sandoval@pnnl.gov]; Hays, Barbara A[barb.hays@pnnl.gov]
Cc: Sheahan, Timothy Patrick[sheahan@email.unc.edu]; Farrell, Ronda Lee[farrelrl@email.unc.edu]; Moore, Victoria L[victoria_moore@unc.edu]
Subject: Re: Change of Institution - Anniversary/Mid-Year Research and Career Awards - 5R21AI146872-02
Attachment: PHS 3734 Official Statement Relinquishing .pdf

Good evening,

Thank you. Please find attached, the revised phs3734 Relinquishing Statement reflective of YR 2 funds only.

best,
Kati

Kati Chipps
Research Administrative Manager
Team 3 - School of Public Health
Office of Sponsored Research
The University of North Carolina at Chapel Hill

104 Airport Drive, Suite 2200, Campus Box 1350
Chapel Hill, NC 27599-1350
(919) 962-4665
kati_chipps@unc.edu
Pronouns: She/Her/Hers

The Office of Sponsored Research is currently working remotely and remains committed to timely responses and providing excellent support. During this time, we will receive voicemail messages by email and will actively respond.

From: Powell, Tamia (NIH/NIAID) [E] <tamia.powell@nih.gov>
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Best wishes, Amy Sims

Amy Sims
Biomedical Scientist
Pacific Northwest National Laboratory (PNNL)
Email amy.sims@pnnl.gov
Office 509 372 6496

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Date: Wednesday, June 3, 2020 at 9:06 AM
To: "Sims, Amy C" <amy.sims@pnnl.gov>
Cc: "Hays, Barbara A" <barb.hays@pnnl.gov>
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Cc: "Powell, Tamia (NIH/NIAID) [E]" <tamia.powell@nih.gov>, "Chipps, Kati" <kati_chipps@unc.edu>, "Sims, Amy C" <amy.sims@pnnl.gov>
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The deadline for the transfer application is June 9th, 2020.

Thanks,

Ashley Ranellone
Grants Management Specialist
DHHS, NIH, NIAID, DEA, GMP
5601 Fishers Lane, Rm 4G55

Bethesda, MD 20892
(301) 594-8541

National Institute of Allergy and Infectious Diseases

Disclaimer: The information in this email and any of its attachments is confidential and may contain sensitive information. It should not be used by anyone who is not the originally intended recipient. If you have received this email 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 the sender's own and not expressly made on behalf of NIAID by one of its representatives.

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Research Administrative Manager

Team 3 - School of Public Health

Office of Sponsored Research

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(919) 962-4665

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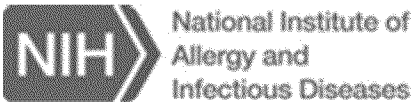
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From: Aleksei Chmura [chmura@ecohealth.net]
Sent: 6/3/2020 11:32:56 PM
To: Sims, Amy C [amy.sims@pnnl.gov]
CC: daszak@ecohealthalliance.org; andre@ecohealth.net
Subject: Re: Review Editor for Manuscript ID ECH-20-0041

No worries, Amy!

If you have time Friday, we can do a quick walk/through in Manuscript Central.

Cheers!

-Aleksei

Aleksei Chmura, PhD
Managing Editor, *EcoHealth*
460 West 34th Street, 17th Floor
New York, NY 10001
5 U.S.C. § 552(b)(6)
Skype: Aleksei MacDurian

Website: www.ecohealth.net
Submissions and Author/Reviewer Log-in: https://mc.manuscriptcentral.com/ecohealth
Author Instructions: http://www.ecohealth.net/submit.php

On May 29, 2020, at 12:41, Sims, Amy C <amy.sims@pnnl.gov> wrote:

Peter and Aleksei,

I am terribly sorry but I cannot seem to figure out how to get to the editor section of the EcoHealth Journal. Is there a link or more information you can provide? I have never been an editor and I am very confused.

Thank you very much! I really appreciate your assistance. Also, did the authors recommend any potential reviewers or should I just find them myself?

Thank you again, Amy

On 5/24/20, 6:59 PM, "EcoHealth" <onbehalfof@manuscriptcentral.com> wrote:

24-May-2020

Dear Dr. Sims:

Manuscript ID ECH-20-0041 entitled "AVIAN CORONAVIRUSES IN WILD BIRDS ALONG BRAZILIAN SEGMENT OF ATLANTIC FLYWAY," with Dr. Barbosa as submitting

author, has been submitted to EcoHealth. I have just assigned this manuscript to you to act as Review Editor.

Please assess this manuscript and determine whether is acceptable to send out for review. If so, select, invite, and assign reviewers as soon as possible.

Please contact Aleksei at if you are having difficulties finding reviewers and/or have any questions about the manuscript or the Manuscript Central Review Editor process. I look forward to reading your decision.

Thank you,

Peter Daszak
Editor-in-Chief, EcoHealth

c
l

5 U.S.C. § 552(b)(6)

Visit our blog at: https://protect2.fireeye.com/v1/url?k=efb0aba0-b3059419-efb081b5-0cc47adc5fce-168ff613f7c21a24&q=1&e=7da4dcbb-ea67-4c64-bc1c-18c14274750f&u=http%3A%2F%2Fwww.ecohealthalliance.org%2Fblog

EcoHealth Alliance leads cutting-edge research into the critical connections between human and wildlife health and delicate ecosystems. With this science we develop solutions that promote conservation and prevent pandemics.

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Thank you. I am sure we can find a time that will work. I hope you get some rest today!

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Aleksei Chmura, PhD
Managing Editor, *EcoHealth*

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Website: www.ecohealth.net
Submissions and Author/Reviewer Log-in: https://mc.manuscriptcentral.com/ecohealth
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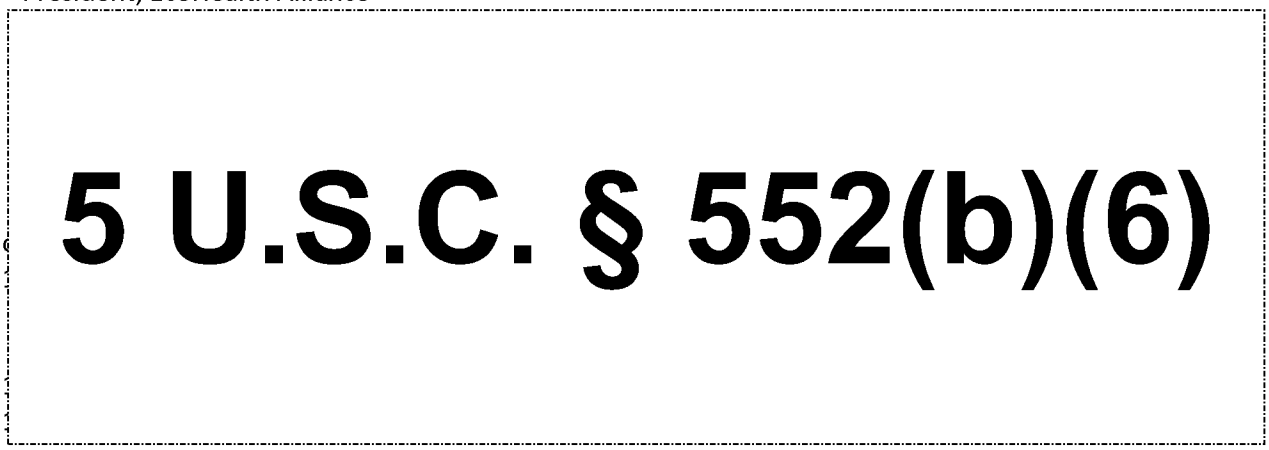
Please assess this manuscript and determine whether is acceptable to send out for review. If so, select, invite, and assign reviewers as soon as possible.

Please contact Aleksei at if you are having difficulties finding reviewers and/or have any questions about the manuscript or the Manuscript Central Review Editor process. I look forward to reading your decision.

Thank you,

Peter Daszak
Editor-in-Chief, EcoHealth

President, EcoHealth Alliance



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From: Sims, Amy C[amy.sims@pnnl.gov]
Sent: Thur 6/4/2020 8:27:48 AM (UTC-07:00)
To: Baric, Ralph S[rbaric@email.unc.edu]; Baric, Toni C[antoinette_baric@med.unc.edu]
Subject: FW: Cell Editorial Decision CELL-D-20-01383

Ralph,

Do you have time to discuss next steps soon? I suspect this will take no more than 15 minutes.

Thank you, Amy

From: <em.cell.0.6b95c1.abe328dd@editorialmanager.com> on behalf of Cell Editorial Office <em@editorialmanager.com>
Reply-To: Cell Editorial Office <cellms@cell.com>
Date: Thursday, May 28, 2020 at 1:59 PM
To: "Sims, Amy C" <amy.sims@pnnl.gov>
Subject: Cell Editorial Decision CELL-D-20-01383

Dr. Amy C Sims
902 Battelle Boulevard
Richland, WA 99352
UNITED STATES

CELL-D-20-01383
Unfolded Protein Response Inhibition Reduces MERS-CoV Induced Acute Lung Injury

May 28, 2020

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Please note that the above link will expire Jun 27, 2020. However, if you still wish to transfer after that period, you may submit directly to the journal of your choice and reference your original manuscript number in the cover letter.

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Declining to transfer will officially close out the manuscript in our system. If you do not accept or decline by Jun 27, 2020, the system will automatically decline on your behalf. If you prefer, you do have the choice of submitting this manuscript or a revised version of it to another Cell Press journal as a regular new submission, in which case you can decline the transfer.

Best wishes,
Andrew

Andrew J. Rennekamp, Ph.D.
Scientific Editor, *Cell*

Reviewers' Comments:

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Other comments

They perform transcriptomics/proteomics/lipidomics at various time points. The % cell infection is shown FIG 1D, and varies between ~1% and 30%. So the omics studies are all performed on cells at different time points with different % infected cells i.e. the number of infected fibroblasts range from ~1 - 30% max at 48hrs and the number of infected MVE cells range from ~1 - 50% max at 24hrs. From what I could gather they haven't performed any enrichment of infected cells - so the % infected cells was always low and varied at different timepoints, making an assessment and comparison of the data somewhat arbitrary.

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barrier as one of the early steps of ARDS while viable MERS-CoV infected FB would continue to produce high levels of infectious virus both of which would contribute to the overall disease burden in the infected patient."

The in vivo mouse data shows a modest effect on weight loss - but no effect on viral titre - so I'm not sure they know why the drug is having a mild beneficial effect.

The most one could say here is that UPR inhibition has a modest improvement in disease outcome in this mouse model. Not sure one can make further conclusions - how this relates to their findings is also unclear.

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However, although the authors demonstrated in the current manuscript that MERS-CoV replication kinetics were similar among FB and MVE, and MVE cells' death is likely a result from apoptotic mechanisms accompanied by UPR activation in these primary cells, there is insufficient evidence to underpin PERK branch of the UPR as a culprit for MERS-CoV associated cell death, neither in vitro nor in vivo. Furthermore, as authors correctly pointed out that "Understanding MERS-CoV pathogenic mechanisms requires understanding critical virus-host interactions", the in vivo protective effect mediated by prophylactic AMG 44 treatment may not be as simple as a PERK-kinase inhibition effect. Usage of genetic UPR mouse models to rigorously examine the hypothesis is recommended. For consideration of publication at Cell, at least a different compound targeting a downstream node of the same PERK pathways is necessary to cross-validate the conclusion that PERK branch of the UPR promotes cell apoptosis, which is largely dependent on one "PERK inhibitor (AMG 44)" at an obviously supra-pharmacological dosage used in this manuscript.

Main Critique:
Strength:
The authors, apparently pioneered in the coronavirus research, carried out important and urgently-needed studies in an effort to find a cure for coronavirus induced human diseases, including MERS-CoV induced human lung disease. In this study, the authors infected primary lung fibroblasts derived from three matched human donors and conducted both virologic and thorough multi-omics analysis of these cells, examining the in vitro effects of MERS-CoV infection. This aspect of the manuscript is definitely both novel and necessary to promote the related research urgently needed to help address the current challenges brought by the COVID19 pandemic.
Weakness:
There is insufficient evidence to support the main conclusion that MERS-CoV induced MVE cell death is mediated by the UPR, specifically via the PERK branch, leading to cellular apoptosis. In addition, there is lack of consideration of the human immune component in the disease models in vitro by cell culture. Thirdly, the in vivo effect from prophylactic treatment with AMG 44 cannot be unequivocally attributed to PERK kinase inhibition. All drugs have off target effects.

To address the above concerns, genetic modulations of the PERK pathway signaling molecules is needed. At a minimum, other compounds targeting ER stress and/or downstream of the PERK pathway signaling molecules are absolutely necessary to support authors' main conclusion underpinning PERK kinase activity as a culprit for cell death.

Specific Comments:
1. Comparing results in Fig. 1 and 2 suggest that the preferential induction of the UPR in MVE cells is not a result of viral replication, since no difference was detected between FB and MVE in virus production (Fig. 1 A vs. B) and infection (Fig. 1D).
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¬ A western blot quantification of ER-translated viral proteins (such as S-glycoprotein) is needed to confirm the intracellular expression of MERS-CoV component and envelop proteins. Proteomics analysis is not an ideal substitute in Fig. 4 D to F.
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¬ Can differences in growth rate account for difference between the cell types? There should also be a direct readout on mitochondrial function and not simple cellular ATP.

2. In Fig. 4, reinforcing the impression by results shown in Fig. 1, there is no difference in MERS-CoV Spike protein, ORF4a protein nor the membrane protein abundance. However, in contrast to FB cells, MVE cells displayed a distinctive pattern of greatly induced ER chaperone proteins, such as BiP, GRP94 and CANX.
¬ Can you knock down PERK and show resistance to AMG44?
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¬ Instead, the data suggest an alternative explanation - FB cells can fold the MERS-CoV envelop proteins more efficiently than MVE cells, and therefore require less ER chaperones to mitigate ER stress. Could there be a difference in protein glycosylation in these two cell types? S-proteins are heavily glycosylated.
¬ Chemical chaperones, namely Tauroursodeoxycholic acid (TUDCA) and 4-phenylbutyric acid (4-PBA), should be considered in these experiments to distinguish the interesting phenotypes displayed by MVE to see whether MVE cells are indeed prone to protein misfolding-induced ER stress.
¬ The authors should identify which UPR arm causes the difference between MVE and FB cells

3. On the usage of the "PERK inhibitor" - AMG 44:
It is known that AMG 44 is a "Potent and selective PERK inhibitor (IC50 = 6 nM), which "exhibits >160-fold selectivity for PERK over B-Raf and GCN2, as well as a panel of 387 other kinases. Orally bioavailable." (For more information, see https://www.tocris.com/products/amg-perk-44_5517).
¬ Given the fact that authors needed to use at least > 10 μM of AMG 44 to achieve a MERS-CoV inhibition effect (Fig. 5), this reviewer considers whether AMG 44 inhibited other kinases in authors' experimental system. For example, two other "PERK inhibitors", GSK2606414 and GSK2656157 exhibit potent inhibitory effect on RIPK1 (doi: 10.1038/cdd.2017.58.) and AMPK.
¬ To address this concern, another PERK inhibitor such as the GSK compound should be used, at the specific

"PERK inhibiting" dosage (<10 nM), to confirm PERK inhibition conferred cyto-protective effect on MVEs.
¬ MVE cells are apparently more susceptible to high doses of AMG 44 than FB, without MERS-CoV, creating a safety concern for administering AMG 44 to human patients. This safety issue should not to be taken lightly, as another PERK inhibitor, GSK2656157, exerted serious adverse effects on mouse pancreas (doi: 10.1158/0008-5472.CAN-12-3109), another grave safety concern.
¬ For a review on the toxicity profile of PERK inhibitors and their impact on physiology, please refer to the following article - Protein kinase R(PKR)-like endoplasmic reticulum kinase (PERK) inhibitors: a patent review (2010-2015) https://doi.org/10.1080/13543776.2017.1238072

4. The successful prophylactic treatment with AMG 44 in mouse models of MERS-CoV induced lung disease is the highlight of this manuscript. Although it is impossible to contribute the AMG 44 effect is specific to PERK kinase inhibition in MVE cells in vivo, the interesting preclinical observations surely hold promise for translational use of AMG 44 as a COVID19 therapy.
¬ The observation that AMG 44 does not confer protection to female mice infected with MERS-CoV is intriguing, as reviewer does not expect a sex-specific PERK difference in female mice.
¬ Could the above observation be explained by "off-target" effect of the "PERK kinase inhibitors", such as RIPK1? (doi: 10.1038/cdd.2017.58.)
¬ Given the importance of RIPK1 in mediating host versus pathogen immune response downstream of TNFR1, the therapeutic effect of AMG 44 on murine immune components should be examined in this context, for this mouse model.
¬ Nonetheless, this reviewer agrees the AMG 44 induced whole animal protection effect is both obvious and significant, regardless of the mechanism.

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From: Baric, Ralph S[rbaric@email.unc.edu]
Sent: Thur 6/4/2020 8:34:18 AM (UTC-07:00)
To: Sims, Amy C[amy.sims@pnnl.gov]
Subject: RE: Cell Editorial Decision CELL-D-20-01383

Hi Amy, I was sulking regarding the review. I guess the criticisms requiring the use of another drug tareting a downstream kinase is what Nat has suggested. I can't remember the status of that data or whether we tried it in vivo or in vitro. I'll be glad to talk. Maaybe Friday around 6PM EST? ralph

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From: Sims, Amy C <amy.sims@pnnl.gov>
Sent: Thursday, June 4, 2020 11:28 AM
To: Baric, Ralph S <rbaric@email.unc.edu>; Baric, Toni C <antoinette_baric@med.unc.edu>
Subject: FW: Cell Editorial Decision CELL-D-20-01383

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¬ Instead, the data suggest an alternative explanation - FB cells can fold the MERS-CoV envelop proteins more efficiently than MVE cells, and therefore require less ER chaperones to mitigate ER stress. Could there be a difference in protein glycosylation in these two cell types? S-proteins are heavily glycosylated.

¬ Chemical chaperones, namely Tauroursodeoxycholic acid (TUDCA) and 4-phenylbutyric acid (4-PBA), should be considered in these experiments to distinguish the interesting phenotypes displayed by MVE to see whether MVE cells are indeed prone to protein misfolding-induced ER stress.

¬ The authors should identify which UPR arm causes the difference between MVE and FB cells

3. On the usage of the "PERK inhibitor" - AMG 44:
It is known that AMG 44 is a "Potent and selective PERK inhibitor (IC50 = 6 nM), which "exhibits >160-fold selectivity for PERK over B-Raf and GCN2, as well as a panel of 387 other kinases. Orally bioavailable." (For more information, see https://www.tocris.com/products/amg-perk-44_5517).
¬ Given the fact that authors needed to use at least > 10 μM of AMG 44 to achieve a MERS-CoV inhibition effect (Fig. 5), this reviewer considers whether AMG 44 inhibited other kinases in authors' experimental system. For example, two other "PERK inhibitors", GSK2606414 and GSK2656157 exhibit potent inhibitory effect on RIPK1 (doi: 10.1038/cdd.2017.58.) and AMPK.
¬ To address this concern, another PERK inhibitor such as the GSK compound should be used, at the specific "PERK inhibiting" dosage (<10 nM), to confirm PERK inhibition conferred cyto-protective effect on MVEs.
¬ MVE cells are apparently more susceptible to high doses of AMG 44 than FB, without MERS-CoV, creating a safety concern for administering AMG 44 to human patients. This safety issue should not to be taken lightly, as another PERK inhibitor, GSK2656157, exerted serious adverse effects on mouse pancreas (doi: 10.1158/0008-5472.CAN-12-3109), another grave safety concern.
¬ For a review on the toxicity profile of PERK inhibitors and their impact on physiology, please refer to the following article - Protein kinase R(PKR)-like endoplasmic reticulum kinase (PERK) inhibitors: a patent review (2010-2015) https://doi.org/10.1080/13543776.2017.1238072

4. The successful prophylactic treatment with AMG 44 in mouse models of MERS-CoV induced lung disease is the highlight of this manuscript. Although it is impossible to contribute the AMG 44 effect is specific to PERK kinase inhibition in MVE cells in vivo, the interesting preclinical observations surely hold promise for translational use of AMG 44 as a COVID19 therapy.
¬ The observation that AMG 44 does not confer protection to female mice infected with MERS-CoV is intriguing, as reviewer does not expect a sex-specific PERK difference in female mice.
¬ Could the above observation be explained by "off-target" effect of the "PERK kinase inhibitors", such as RIPK1? (doi: 10.1038/cdd.2017.58.)
¬ Given the importance of RIPK1 in mediating host versus pathogen immune response downstream of TNFR1, the therapeutic effect of AMG 44 on murine immune components should be examined in this context, for this mouse model.
¬ Nonetheless, this reviewer agrees the AMG 44 induced whole animal protection effect is both obvious and significant, regardless of the mechanism.

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In compliance with data protection regulations, you may request that we remove your personal registration details at any time. (Remove my information/details). Please contact the publication office if you have any questions.

From: Sims, Amy C[amy.sims@pnnl.gov]
Sent: Thur 6/4/2020 8:51:26 AM (UTC-07:00)
To: Baric, Ralph S[rbaric@email.unc.edu]
Subject: Re: Cell Editorial Decision CELL-D-20-01383

Ha, okay. I didn't think you would be depressed! It was a long shot at least to me. I did try another drug (Nat's drug whose name is escaping me at the moment) that should have the same effect as the PERK inhibitor (preventing eIF2alpha phosphorylation) but by acting on a different part of the pathway. We did not try that one in vivo. To streamline the paper we removed that data. The second drug did not inhibit replication but did have some effect on cell viability.

I have been busy with grant writing and haven't had time to think about this too much. I have another call at 6pm eastern tomorrow. Can you do 7pm or should we try next week? I just want to make sure we are on the same page as far as next steps go.

Thank you for the rapid response. I appreciate it. I hope you are doing well otherwise. Amy

---

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Date: Thursday, June 4, 2020 at 8:35 AM
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Sent: Thursday, June 4, 2020 11:28 AM
To: Baric, Ralph S <rbaric@email.unc.edu>; Baric, Toni C <antoinette_baric@med.unc.edu>
Subject: FW: Cell Editorial Decision CELL-D-20-01383

Ralph,

Do you have time to discuss next steps soon? I suspect this will take no more than 15 minutes.

Thank you, Amy

From: <em.cell.0.6b95c1.abe328dd@editorialmanager.com> on behalf of Cell Editorial Office <em@editorialmanager.com>
Reply-To: Cell Editorial Office <cellms@cell.com>
Date: Thursday, May 28, 2020 at 1:59 PM
To: "Sims, Amy C" <amy.sims@pnnl.gov>
Subject: Cell Editorial Decision CELL-D-20-01383

Dr. Amy C Sims
902 Battelle Boulevard
Richland, WA 99352
UNITED STATES

CELL-D-20-01383
Unfolded Protein Response Inhibition Reduces MERS-CoV Induced Acute Lung Injury

May 28, 2020

Dear Dr. Sims,

I am enclosing the comments that the reviewers made on your paper. Unfortunately, the consensus recommendation is against publication in *Cell*. The reviewers refer to the potential timliness of the topic and interest in the approach, but cite that significant further work would be necessary, especially to demonstrate the specificity of the compound and its proposed mechanism of action in vivo. Given their comments, and the unclear relevance to non-MERS-CoV driven coronavirus pathology, we unfortunately cannot proceed further with this manuscript at Cell.

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Although we cannot offer to proceed further with this particular paper for *Cell*, the manuscript may be a stronger candidate for another journal within the Cell Press manuscript transfer system. If you are interested in learning more about transfer as an option, please contact the relevant journal directly, as each journal is editorially independent. The link below will bring you to a page where you can select the journal to which you would like to transfer your paper (you can review each journal's aims and scopes before choosing your target).

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Please note that the above link will expire Jun 27, 2020. However, if you still wish to transfer after that period, you may submit directly to the journal of your choice and reference your original manuscript number in the cover letter.

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Best wishes,
Andrew

Andrew J. Rennekamp, Ph.D.
Scientific Editor, *Cell*

Reviewers' Comments:

Reviewer #1:

This manuscript by Sims and co-workers describes the results of an unbiased, systems biology-based approach that identifies the unfolded protein response (UPR) as differentially modulated during MERS-CoV infection of primary cells. They used donor matched primary human lung microvascular endothelial cells (MVEs) and fibroblasts (FB), and found that only MVEs exhibited cytopathic effect in response to infection with MERS-CoV. To determine if a specific pathway was responsible for the differential outcome in MVEs versus FBs, they compared the transcriptomes, proteomes, and lipidomes of MERS-CoV-infected cells over time, and repeated this experiment using 3 different donors of the cells. This analysis revealed that the MERS-CoV-infected MVEs were dying via an Unfolded Protein Response (UPR) mediated apoptosis. They validated the importance of the UPR pathway for MERS-CoV replication-induced cell death in MEVs by addition of UPR pathway inhibitor (AMG44), which reduced replication and cell death. Importantly, they also demonstrated that drug targeting of this pathway ameliorated the outcome of MERS-CoV infection in mice (reduced weight loss and improved respiratory function). Overall, this systems biology approach allowed them to identifying a targeted antiviral therapy that reduced morbidity and mortality in MERS-CoV-infected mice. The manuscript is well written and the experiments are clearly presented. Appropriate statistical analysis is provided for each experiment. The results of this study document an approach for identifying targeted antiviral therapy for coronavirus infection. This study has important implications for developing therapies for the current pandemic.
Comments for the authors' consideration (minor issues):
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6. Lines 486-493: Was this MERS-nanoluc virus?
7. Lines 486-493: AMG44 is referenced to two other papers, but better to name the commercial source.
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9. Line 377, start new paragraph to discuss mortality in male and female mice
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11. Line 349: add recent paper Volk, Hackbart et al., 2020 J. Virol for activation of the UPR in macrophages in response to coronavirus replication.
12. Line 392: add drug names remdesivir and EIDD-2801 here

Reviewer #2: Coronavirus infection and replication is well known for its ability to stimulate an intracellular unfolded protein response (UPR). In this paper these authors take an unbiased omics-based approach to examine pathways stimulated by MERS-CoV. They identify an induction of UPR gene signature - particularly in microvascular endothelial cells (MVE) - more than fibroblasts. They find that the use of a PERK inhibitor reduces MERS-CoV replication in infected primary human lung MVE and FB and treatment with the same PERK inhibitor AMG144 in an in vivo mouse model of MERS lung disease leads to some clinical improvement. They show that it reduces lung disease and other features of disease pathogenesis - though they fail to show a reduction in MERS-CoV viral replication in infected cells. It should also be noted that PERK inhibitors are wonderfully pleiotropic.

The experiments seem to lack a certain logic? They emphasise the importance of maintaining the epithelial-endothelial cell barrier in MERS-CoV pathogenesis and I would agree that this is a critical aspect of

coronavirus lung disease. They speculate that the PERK signaling pathway and the UPR response promote increased membrane reorganization and suppress host translation that helps optimise an environmental milieu for improved virus replication, maturation and egress. However, the virus is thought to enter the cells through the respiratory tissue - most likely the type II alveolar cell. What then is the rationale of looking at the effect of virus on fibroblasts and microvascular endothelial cells when the virus presumably enters through DPP4 + lung epithelial cells in the alveolus? Isn't this the cell type they should be looking at - as this is the likely cell to initiate the UPR - what is the point of looking at endothelial cells. It would seem that once the virus has infected the endothelial cell, there has already been a breakdown of the epithelial-endothelial barrier. Is there evidence of clinically important levels of viral replication in humans in endothelial cells?

Other comments

They perform transcriptomics/proteomics/lipidomics at various time points. The % cell infection is shown FIG 1D, and varies between ~1% and 30%. So the omics studies are all performed on cells at different time points with different % infected cells i.e. the number of infected fibroblasts range from ~1 - 30% max at 48hrs and the number of infected MVE cells range from ~1 - 50% max at 24hrs. From what I could gather they haven't performed any enrichment of infected cells - so the % infected cells was always low and varied at different timepoints, making an assessment and comparison of the data somewhat arbitrary.

Figure 5 Confusing presentation of data. It would be helpful to show the number of cells infected - rather than inhibition of infection - or maybe show both e.g. it's unclear to the reader if this is 10% infected or 70%?
At 48hrs with MVE there is no effect.
At 24hrs the effect is binary - no dose response curve - why is this - can this be repeated with more dose response points in the critical range?
What is negative toxicity with fibroblasts?

Line 163 "Death of MERS-CoV infected MVE could lead to permeability at the epithelial/endothelial cell barrier as one of the early steps of ARDS while viable MERS-CoV infected FB would continue to produce high levels of infectious virus both of which would contribute to the overall disease burden in the infected patient."

The in vivo mouse data shows a modest effect on weight loss - but no effect on viral titre - so I'm not sure they know why the drug is having a mild beneficial effect.

The most one could say here is that UPR inhibition has a modest improvement in disease outcome in this mouse model. Not sure one can make further conclusions - how this relates to their findings is also unclear.

Reviewer #3: Summary
In this study, Sims et al. report an important finding that a strain of coronavirus, MERS-CoV, leads to different cytopathic effects in primary human lung microvascular endothelial cells and in lung fibroblasts (MVE and FB, respectively). While MERS-CoV display similar viral replication in both cell lineages, the cytopathic effect of unfolded protein response (UPR)-mediated apoptosis is limited to MVE, but not FB. The authors subsequently tested a pharmacologic PERK kinase inhibitor compound, namely AMG 44, and further demonstrated that AMG 44 exhibits cytoprotective effect in vitro on MVE cells. Furthermore, prophylactic treatment with AMG 44 protected MERS-CoV infected mice from clinical manifestation of lung damage typically seen after MERS-CoV infection. Taken together, the exhibited phenotypes are highly interesting and potentially significant for translational use of UPR pharmacologic compounds as a potential therapy against coronavirus-mediated human diseases, which warrants investment and further investigation of underlying molecular mechanisms.

However, although the authors demonstrated in the current manuscript that MERS-CoV replication kinetics were similar among FB and MVE, and MVE cells' death is likely a result from apoptotic mechanisms accompanied by UPR activation in these primary cells, there is insufficient evidence to underpin PERK branch of the UPR as a culprit for MERS-CoV associated cell death, neither in vitro nor in vivo. Furthermore, as authors correctly pointed out that "Understanding MERS-CoV pathogenic mechanisms requires understanding critical virus-host interactions", the in vivo protective effect mediated by prophylactic AMG 44 treatment may not be as simple as a PERK-kinase inhibition effect. Usage of genetic UPR mouse models to rigorously examine the hypothesis is recommended. For consideration of publication at Cell, at least a different compound targeting a downstream node of the same PERK pathways is necessary to cross-validate the conclusion that PERK branch of the UPR promotes cell apoptosis, which is largely dependent on one "PERK inhibitor (AMG 44)" at an obviously supra-pharmacological dosage used in this manuscript.

Main Critique:

Strength:

The authors, apparently pioneered in the coronavirus research, carried out important and urgently-needed studies in an effort to find a cure for coronavirus induced human diseases, including MERS-CoV induced human lung disease. In this study, the authors infected primary lung fibroblasts derived from three matched human donors and conducted both virologic and thorough multi-omics analysis of these cells, examining the in vitro effects of MERS-CoV infection. This aspect of the manuscript is definitely both novel and necessary to promote the related research urgently needed to help address the current challenges brought by the COVID19 pandemic.

Weakness:

There is insufficient evidence to support the main conclusion that MERS-CoV induced MVE cell death is mediated by the UPR, specifically via the PERK branch, leading to cellular apoptosis. In addition, there is lack of consideration of the human immune component in the disease models in vitro by cell culture. Thirdly, the in vivo effect from prophylactic treatment with AMG 44 cannot be unequivocally attributed to PERK kinase inhibition. All drugs have off target effects.

To address the above concerns, genetic modulations of the PERK pathway signaling molecules is needed. At a minimum, other compounds targeting ER stress and/or downstream of the PERK pathway signaling molecules are absolutely necessary to support authors' main conclusion underpinning PERK kinase activity as a culprit for cell death.

Specific Comments:

1. Comparing results in Fig. 1 and 2 suggest that the preferential induction of the UPR in MVE cells is not a result of viral replication, since no difference was detected between FB and MVE in virus production (Fig. 1 A vs. B) and infection (Fig. 1D).

¬ What is rationale for studying infection in MVE and EB?

¬ This observation begs the explanation of UPR difference, since it is unlikely to be caused by an exacerbated viral protein load effect in the ER of MVE cells.

¬ A western blot quantification of ER-translated viral proteins (such as S-glycoprotein) is needed to confirm the intracellular expression of MERS-CoV component and envelop proteins. Proteomics analysis is not an ideal substitute in Fig. 4 D to F.

¬ Related to the same comment, there is an upregulation of various ER associated proteins in the proteomics data set (Fig. 2B, by red asterisks), specifically for MVEs. These proteins are usually indicative of an ER compartment expansion, but not necessarily as a result of dysregulated UPR signaling. At the same time, there are also other "membrane" components observed in the same dataset, such as "mitochondrial inner membrane" and (plasma?) "membrane". Read out of UPR should look at more proximal readouts of UPR sensor activation.

¬ The upregulation of intracellular lipids in MVEs is interesting (Fig. 2C), which may suggest either an inhibition or disruption of normal cellular lipid secretion machinery, or an upregulation of lipid de novo synthesis. It is important to distinguish the two possibilities through labeling the lipid precursor metabolites.

¬ Can differences in growth rate account for difference between the cell types? There should also be a direct readout on mitochondrial function and not simple cellular ATP.

2. In Fig. 4, reinforcing the impression by results shown in Fig. 1, there is no difference in MERS-CoV Spike protein, ORF4a protein nor the membrane protein abundance. However, in contrast to FB cells, MVE cells displayed a distinctive pattern of greatly induced ER chaperone proteins, such as BiP, GRP94 and CANX.
¬ Can you knock down PERK and show resistance to AMG44?
¬ It is important to realize that the upregulation of ER chaperones are usually cyto-protective, in response to increased ER protein synthesis burden, and should not be taken as an evidence that UPR is disproportionally activated in the MVE cells.
¬ Instead, the data suggest an alternative explanation - FB cells can fold the MERS-CoV envelop proteins more efficiently than MVE cells, and therefore require less ER chaperones to mitigate ER stress. Could there be a difference in protein glycosylation in these two cell types? S-proteins are heavily glycosylated.
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Sent: Fri 6/5/2020 8:14:13 AM (UTC-07:00)
To: Baric, Ralph S[rbaric@email.unc.edu]
Subject: TALK TO AMY?? Re: Cell Editorial Decision CELL-D-20-01383

Hi Ralph,

I wasn't sure if your calendar exploded today or not. Do you have time later to talk? I can try to push my 6pm eastern meeting back if that is still the time that works best for you. Thank you, Amy

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Dr. Amy C Sims
902 Battelle Boulevard
Richland, WA 99352
UNITED STATES

CELL-D-20-01383
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Best wishes,
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Andrew J. Rennekamp, Ph.D.
Scientific Editor, *Cell*

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The experiments seem to lack a certain logic? They emphasise the importance of maintaining the epithelial-endothelial cell barrier in MERS-CoV pathogenesis and I would agree that this is a critical aspect of coronavirus lung disease. They speculate that the PERK signaling pathway and the UPR response promote increased membrane reorganization and suppress host translation that helps optimise an environmental milieu for improved virus replication, maturation and egress. However, the virus is thought to enter the cells through the respiratory tissue - most likely the type II alveolar cell. What then is the rationale of looking at the effect of virus on fibroblasts and microvascular endothelial cells when the virus presumably enters through DPP4 + lung epithelial cells in the alveolus? Isn't this the cell type they should be looking at - as this is the likely cell to initiate the UPR - what is the point of looking at endothelial cells. It would seem that once the virus has infected the endothelial cell, there has already been a breakdown of the epithelial-endothelial barrier. Is there evidence of clinically important levels of viral replication in humans in endothelial cells?

Other comments

They perform transcriptomics/proteomics/lipidomics at various time points. The % cell infection is shown FIG 1D, and varies between ~1% and 30%. So the omics studies are all performed on cells at different time points with different % infected cells i.e. the number of infected fibroblasts range from ~1 - 30% max at 48hrs and the number of infected MVE cells range from ~1 - 50% max at 24hrs. From what I could gather they haven't performed any enrichment of infected cells - so the % infected cells was always low and varied at different timepoints, making an assessment and comparison of the data somewhat arbitrary.

Figure 5 Confusing presentation of data. It would be helpful to show the number of cells infected - rather than inhibition of infection - or maybe show both e.g. it's unclear to the reader if this is 10% infected or 70%?
At 48hrs with MVE there is no effect.
At 24hrs the effect is binary - no dose response curve - why is this - can this be repeated with more dose response points in the critical range?
What is negative toxicity with fibroblasts?

Line 163 "Death of MERS-CoV infected MVE could lead to permeability at the epithelial/endothelial cell barrier as one of the early steps of ARDS while viable MERS-CoV infected FB would continue to produce high levels of infectious virus both of which would contribute to the overall disease burden in the infected patient."

The in vivo mouse data shows a modest effect on weight loss - but no effect on viral titre - so I'm not sure they know why the drug is having a mild beneficial effect.

The most one could say here is that UPR inhibition has a modest improvement in disease outcome in this mouse model. Not sure one can make further conclusions - how this relates to their findings is also unclear.

Reviewer #3: Summary
In this study, Sims et al. report an important finding that a strain of coronavirus, MERS-CoV, leads to different cytopathic effects in primary human lung microvascular endothelial cells and in lung fibroblasts (MVE and FB, respectively). While MERS-CoV display similar viral replication in both cell lineages, the cytopathic effect of unfolded protein response (UPR)-mediated apoptosis is limited to MVE, but not FB. The authors subsequently tested a pharmacologic PERK kinase inhibitor compound, namely AMG 44, and further demonstrated that AMG 44 exhibits cytoprotective effect in vitro on MVE cells. Furthermore, prophylactic treatment with AMG 44 protected MERS-CoV infected mice from clinical manifestation of lung damage typically seen after MERS-CoV infection. Taken together, the exhibited phenotypes are highly interesting and potentially significant for translational use of UPR pharmacologic compounds as a potential therapy against coronavirus-mediated human diseases, which warrants investment and further investigation of underlying molecular mechanisms.
However, although the authors demonstrated in the current manuscript that MERS-CoV replication kinetics were similar among FB and MVE, and MVE cells' death is likely a result from apoptotic mechanisms accompanied by UPR activation in these primary cells, there is insufficient evidence to underpin PERK branch of the UPR as a culprit for MERS-CoV associated cell death, neither in vitro nor in vivo. Furthermore, as authors correctly pointed out that "Understanding MERS-CoV pathogenic mechanisms requires understanding critical virus-host interactions", the in vivo protective effect mediated by prophylactic AMG 44 treatment may not be as simple as a PERK-kinase inhibition effect. Usage of genetic UPR mouse models to rigorously examine the hypothesis is recommended. For consideration of publication at Cell, at least a different compound targeting a downstream node of the same PERK pathways is necessary to cross-validate the

conclusion that PERK branch of the UPR promotes cell apoptosis, which is largely dependent on one "PERK inhibitor (AMG 44)" at an obviously supra-pharmacological dosage used in this manuscript.

Main Critique:
Strength:
The authors, apparently pioneered in the coronavirus research, carried out important and urgently-needed studies in an effort to find a cure for coronavirus induced human diseases, including MERS-CoV induced human lung disease. In this study, the authors infected primary lung fibroblasts derived from three matched human donors and conducted both virologic and thorough multi-omics analysis of these cells, examining the in vitro effects of MERS-CoV infection. This aspect of the manuscript is definitely both novel and necessary to promote the related research urgently needed to help address the current challenges brought by the COVID19 pandemic.
Weakness:
There is insufficient evidence to support the main conclusion that MERS-CoV induced MVE cell death is mediated by the UPR, specifically via the PERK branch, leading to cellular apoptosis. In addition, there is lack of consideration of the human immune component in the disease models in vitro by cell culture. Thirdly, the in vivo effect from prophylactic treatment with AMG 44 cannot be unequivocally attributed to PERK kinase inhibition. All drugs have off target effects.
To address the above concerns, genetic modulations of the PERK pathway signaling molecules is needed. At a minimum, other compounds targeting ER stress and/or downstream of the PERK pathway signaling molecules are absolutely necessary to support authors' main conclusion underpinning PERK kinase activity as a culprit for cell death.

Specific Comments:
1. Comparing results in Fig. 1 and 2 suggest that the preferential induction of the UPR in MVE cells is not a result of viral replication, since no difference was detected between FB and MVE in virus production (Fig. 1 A vs. B) and infection (Fig. 1D).
¬ What is rationale for studying infection in MVE and EB?
¬ This observation begs the explanation of UPR difference, since it is unlikely to be caused by an exacerbated viral protein load effect in the ER of MVE cells.
¬ A western blot quantification of ER-translated viral proteins (such as S-glycoprotein) is needed to confirm the intracellular expression of MERS-CoV component and envelop proteins. Proteomics analysis is not an ideal substitute in Fig. 4 D to F.
¬ Related to the same comment, there is an upregulation of various ER associated proteins in the proteomics data set (Fig. 2B, by red asterisks), specifically for MVEs. These proteins are usually indicative of an ER compartment expansion, but not necessarily as a result of dysregulated UPR signaling. At the same time, there are also other "membrane" components observed in the same dataset, such as "mitochondrial inner membrane" and (plasma?) "membrane". Read out of UPR should look at more proximal readouts of UPR sensor activation.
¬ The upregulation of intracellular lipids in MVEs is interesting (Fig. 2C), which may suggest either an inhibition or disruption of normal cellular lipid secretion machinery, or an upregulation of lipid de novo synthesis. It is important to distinguish the two possibilities through labeling the lipid precursor metabolites.
¬ Can differences in growth rate account for difference between the cell types? There should also be a direct readout on mitochondrial function and not simple cellular ATP.

2. In Fig. 4, reinforcing the impression by results shown in Fig. 1, there is no difference in MERS-CoV Spike protein, ORF4a protein nor the membrane protein abundance. However, in contrast to FB cells, MVE cells displayed a distinctive pattern of greatly induced ER chaperone proteins, such as BiP, GRP94 and CANX.
¬ Can you knock down PERK and show resistance to AMG44?
¬ It is important to realize that the upregulation of ER chaperones are usually cyto-protective, in response to

increased ER protein synthesis burden, and should not be taken as an evidence that UPR is disproportionally activated in the MVE cells.
¬ Instead, the data suggest an alternative explanation - FB cells can fold the MERS-CoV envelop proteins more efficiently than MVE cells, and therefore require less ER chaperones to mitigate ER stress. Could there be a difference in protein glycosylation in these two cell types? S-proteins are heavily glycosylated.
¬ Chemical chaperones, namely Tauroursodeoxycholic acid (TUDCA) and 4-phenylbutyric acid (4-PBA), should be considered in these experiments to distinguish the interesting phenotypes displayed by MVE to see whether MVE cells are indeed prone to protein misfolding-induced ER stress.
¬ The authors should identify which UPR arm causes the difference between MVE and FB cells

3. On the usage of the "PERK inhibitor" - AMG 44:
It is known that AMG 44 is a "Potent and selective PERK inhibitor (IC50 = 6 nM), which "exhibits >160-fold selectivity for PERK over B-Raf and GCN2, as well as a panel of 387 other kinases. Orally bioavailable." (For more information, see https://www.tocris.com/products/amg-perk-44_5517).
¬ Given the fact that authors needed to use at least > 10 μM of AMG 44 to achieve a MERS-CoV inhibition effect (Fig. 5), this reviewer considers whether AMG 44 inhibited other kinases in authors' experimental system. For example, two other "PERK inhibitors", GSK2606414 and GSK2656157 exhibit potent inhibitory effect on RIPK1 (doi: 10.1038/cdd.2017.58.) and AMPK.
¬ To address this concern, another PERK inhibitor such as the GSK compound should be used, at the specific "PERK inhibiting" dosage (<10 nM), to confirm PERK inhibition conferred cyto-protective effect on MVEs.
¬ MVE cells are apparently more susceptible to high doses of AMG 44 than FB, without MERS-CoV, creating a safety concern for administering AMG 44 to human patients. This safety issue should not to be taken lightly, as another PERK inhibitor, GSK2656157, exerted serious adverse effects on mouse pancreas (doi: 10.1158/0008-5472.CAN-12-3109), another grave safety concern.
¬ For a review on the toxicity profile of PERK inhibitors and their impact on physiology, please refer to the following article - Protein kinase R(PKR)-like endoplasmic reticulum kinase (PERK) inhibitors: a patent review (2010-2015) https://doi.org/10.1080/13543776.2017.1238072

4. The successful prophylactic treatment with AMG 44 in mouse models of MERS-CoV induced lung disease is the highlight of this manuscript. Although it is impossible to contribute the AMG 44 effect is specific to PERK kinase inhibition in MVE cells in vivo, the interesting preclinical observations surely hold promise for translational use of AMG 44 as a COVID19 therapy.
¬ The observation that AMG 44 does not confer protection to female mice infected with MERS-CoV is intriguing, as reviewer does not expect a sex-specific PERK difference in female mice.
¬ Could the above observation be explained by "off-target" effect of the "PERK kinase inhibitors", such as RIPK1? (doi: 10.1038/cdd.2017.58.)
¬ Given the importance of RIPK1 in mediating host versus pathogen immune response downstream of TNFR1, the therapeutic effect of AMG 44 on murine immune components should be examined in this context, for this mouse model.
¬ Nonetheless, this reviewer agrees the AMG 44 induced whole animal protection effect is both obvious and significant, regardless of the mechanism.

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this communication is unlawful. If you have received this transmission in error, please immediately notify us by return e-mail and destroy the original message.

In compliance with data protection regulations, you may request that we remove your personal registration details at any time. (Remove my information/details). Please contact the publication office if you have any questions.

From: Baric, Ralph S[rbaric@email.unc.edu]
Sent: Fri 6/5/2020 8:20:34 AM (UTC-07:00)
To: Sims, Amy C[amy.sims@pnnl.gov]
Subject: RE: TALK TO AMY?? Re: Cell Editorial Decision CELL-D-20-01383

6PM or 7PM EST is best I think

From: Sims, Amy C <amy.sims@pnnl.gov>
Sent: Friday, June 5, 2020 11:14 AM
To: Baric, Ralph S <rbaric@email.unc.edu>
Subject: TALK TO AMY?? Re: Cell Editorial Decision CELL-D-20-01383

Hi Ralph,

I wasn't sure if your calendar exploded today or not. Do you have time later to talk? I can try to push my 6pm eastern meeting back if that is still the time that works best for you. Thank you, Amy

---

From: "Baric, Ralph S" <rbaric@email.unc.edu>
Date: Thursday, June 4, 2020 at 8:35 AM
To: "Sims, Amy C" <amy.sims@pnnl.gov>
Subject: RE: Cell Editorial Decision CELL-D-20-01383

Hi Amy, I was sulking regarding the review. I guess the criticisms requiring the use of another drug tareting a downstream kinase is what Nat has suggested. I can't remember the status of that data or whether we tried it in vivo or in vitro. I'll be glad to talk. Maaybe Friday around 6PM EST? ralph

---

From: Sims, Amy C <amy.sims@pnnl.gov>
Sent: Thursday, June 4, 2020 11:28 AM
To: Baric, Ralph S <rbaric@email.unc.edu>; Baric, Toni C <antoinette_baric@med.unc.edu>
Subject: FW: Cell Editorial Decision CELL-D-20-01383

Ralph,

Do you have time to discuss next steps soon? I suspect this will take no more than 15 minutes.

Thank you, Amy

From: <em.cell.0.6b95c1.abe328dd@editorialmanager.com> on behalf of Cell Editorial Office <em@editorialmanager.com>
Reply-To: Cell Editorial Office <cellms@cell.com>
Date: Thursday, May 28, 2020 at 1:59 PM
To: "Sims, Amy C" <amy.sims@pnnl.gov>
Subject: Cell Editorial Decision CELL-D-20-01383

Dr. Amy C Sims
902 Battelle Boulevard
Richland, WA 99352
UNITED STATES

CELL-D-20-01383
Unfolded Protein Response Inhibition Reduces MERS-CoV Induced Acute Lung Injury

May 28, 2020

Dear Dr. Sims,

I am enclosing the comments that the reviewers made on your paper. Unfortunately, the consensus recommendation is against publication in *Cell*. The reviewers refer to the potential timliness of the topic and interest in the approach, but cite that significant further work would be necessary, especially to demonstrate the specificity of the compound and its proposed mechanism of action in vivo. Given their comments, and the unclear relevance to non-MERS-CoV driven coronavirus pathology, we unfortunately cannot proceed further with this manuscript at Cell.

I am sorry that the outcome for this manuscript was not more positive. I hope, however, that the reviewers' comments are clear and constructive and that you will continue to consider *Cell* for future submissions.

Although we cannot offer to proceed further with this particular paper for *Cell*, the manuscript may be a stronger candidate for another journal within the Cell Press manuscript transfer system. If you are interested in learning more about transfer as an option, please contact the relevant journal directly, as each journal is editorially independent. The link below will bring you to a page where you can select the journal to which you would like to transfer your paper (you can review each journal's aims and scopes before choosing your target).

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Declining to transfer will officially close out the manuscript in our system. If you do not accept or decline by Jun 27, 2020, the system will automatically decline on your behalf. If you prefer, you do have the choice of submitting this manuscript or a revised version of it to another Cell Press journal as a regular new submission, in which case you can decline the transfer.

Best wishes,
Andrew

Andrew J. Rennekamp, Ph.D.
Scientific Editor, *Cell*

Reviewers' Comments:

Reviewer #1:

This manuscript by Sims and co-workers describes the results of an unbiased, systems biology-based approach that identifies the unfolded protein response (UPR) as differentially modulated during MERS-CoV infection of primary cells. They used donor matched primary human lung microvascular endothelial cells (MVEs) and fibroblasts (FB), and found that only MVEs exhibited cytopathic effect in response to infection with MERS-CoV. To determine if a specific pathway was responsible for the differential outcome in MVEs versus FBs, they compared the transcriptomes, proteomes, and lipidomes of MERS-CoV-infected cells over time, and repeated this experiment using 3 different donors of the cells. This analysis revealed that the MERS-CoV-infected MVEs were dying via an Unfolded Protein Response (UPR) mediated apoptosis. They validated the importance of the UPR pathway for MERS-CoV replication-induced cell death in MEVs by addition of UPR pathway inhibitor (AMG44), which reduced replication and cell death. Importantly, they also demonstrated that drug targeting of this pathway ameliorated the outcome of MERS-CoV infection in mice (reduced weight loss and improved respiratory function). Overall, this systems biology approach allowed them to identifying a targeted antiviral therapy that reduced morbidity and mortality in MERS-CoV-infected mice. The manuscript is well written and the experiments are clearly presented. Appropriate statistical analysis is provided for each experiment. The results of this study document an approach for identifying targeted antiviral therapy for coronavirus infection. This study has important implications for developing therapies for the current pandemic.
Comments for the authors' consideration (minor issues):
1. Legend to Fig 2. Is it least significant score or most significant score?
2. Legend to Fig 3. MERS-CoV MOI?
3. Title to Fig 4: Protein markers suggest activation of UPR in MVEs.
4. Legend to Fig 5. MVEs were infected with MERS-nanoluc (MOI=?)
5. Line 344: cite Gosert et al., 2002 for identification of DMVs
6. Lines 486-493: Was this MERS-nanoluc virus?
7. Lines 486-493: AMG44 is referenced to two other papers, but better to name the commercial source.
8. Do your results implicate other UPR modulators such as GSK2606414 and KIRA6 (Mahameed at al., Cell Death and Disease, 2019) as potential modulators of coronavirus replication and disease? Discussion of UPR modulators as potential therapeutics would be appropriate (line 377).
9. Line 377, start new paragraph to discuss mortality in male and female mice
10. Line 132: mothers?
11. Line 349: add recent paper Volk, Hackbart et al., 2020 J. Virol for activation of the UPR in macrophages in response to coronavirus replication.
12. Line 392: add drug names remdesivir and EIDD-2801 here

Reviewer #2: Coronavirus infection and replication is well known for its ability to stimulate an intracellular unfolded protein response (UPR). In this paper these authors take an unbiased omics-based approach to examine pathways stimulated by MERS-CoV. They identify an induction of UPR gene signature - particularly in microvascular endothelial cells (MVE) - more than fibroblasts. They find that the use of a PERK inhibitor reduces MERS-CoV replication in infected primary human lung MVE and FB and treatment with the same PERK inhibitor AMG144 in an in vivo mouse model of MERS lung disease leads to some clinical improvement. They show that it reduces lung disease and other features of disease pathogenesis - though they fail to show a reduction in MERS-CoV viral replication in infected cells. It should also be noted that PERK inhibitors are wonderfully pleiotropic.

The experiments seem to lack a certain logic? They emphasise the importance of maintaining the epithelial-endothelial cell barrier in MERS-CoV pathogenesis and I would agree that this is a critical aspect of coronavirus lung disease. They speculate that the PERK signaling pathway and the UPR response promote increased membrane reorganization and suppress host translation that helps optimise an environmental milieu

for improved virus replication, maturation and egress. However, the virus is thought to enter the cells through the respiratory tissue - most likely the type II alveolar cell. What then is the rationale of looking at the effect of virus on fibroblasts and microvascular endothelial cells when the virus presumably enters through DPP4 + lung epithelial cells in the alveolus? Isn't this the cell type they should be looking at - as this is the likely cell to initiate the UPR - what is the point of looking at endothelial cells. It would seem that once the virus has infected the endothelial cell, there has already been a breakdown of the epithelial-endothelial barrier. Is there evidence of clinically important levels of viral replication in humans in endothelial cells?

Other comments

They perform transcriptomics/proteomics/lipidomics at various time points. The % cell infection is shown FIG 1D, and varies between ~1% and 30%. So the omics studies are all performed on cells at different time points with different % infected cells i.e. the number of infected fibroblasts range from ~1 - 30% max at 48hrs and the number of infected MVE cells range from ~1 - 50% max at 24hrs. From what I could gather they haven't performed any enrichment of infected cells - so the % infected cells was always low and varied at different timepoints, making an assessment and comparison of the data somewhat arbitrary.

Figure 5 Confusing presentation of data. It would be helpful to show the number of cells infected - rather than inhibition of infection - or maybe show both e.g. it's unclear to the reader if this is 10% infected or 70%?
At 48hrs with MVE there is no effect.
At 24hrs the effect is binary - no dose response curve - why is this - can this be repeated with more dose response points in the critical range?
What is negative toxicity with fibroblasts?

Line 163 "Death of MERS-CoV infected MVE could lead to permeability at the epithelial/endothelial cell barrier as one of the early steps of ARDS while viable MERS-CoV infected FB would continue to produce high levels of infectious virus both of which would contribute to the overall disease burden in the infected patient."

The in vivo mouse data shows a modest effect on weight loss - but no effect on viral titre - so I'm not sure they know why the drug is having a mild beneficial effect.

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However, although the authors demonstrated in the current manuscript that MERS-CoV replication kinetics were similar among FB and MVE, and MVE cells' death is likely a result from apoptotic mechanisms

accompanied by UPR activation in these primary cells, there is insufficient evidence to underpin PERK branch of the UPR as a culprit for MERS-CoV associated cell death, neither in vitro nor in vivo. Furthermore, as authors correctly pointed out that "Understanding MERS-CoV pathogenic mechanisms requires understanding critical virus-host interactions", the in vivo protective effect mediated by prophylactic AMG 44 treatment may not be as simple as a PERK-kinase inhibition effect. Usage of genetic UPR mouse models to rigorously examine the hypothesis is recommended. For consideration of publication at Cell, at least a different compound targeting a downstream node of the same PERK pathways is necessary to cross-validate the conclusion that PERK branch of the UPR promotes cell apoptosis, which is largely dependent on one "PERK inhibitor (AMG 44)" at an obviously supra-pharmacological dosage used in this manuscript.

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Strength:
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Weakness:
There is insufficient evidence to support the main conclusion that MERS-CoV induced MVE cell death is mediated by the UPR, specifically via the PERK branch, leading to cellular apoptosis. In addition, there is lack of consideration of the human immune component in the disease models in vitro by cell culture. Thirdly, the in vivo effect from prophylactic treatment with AMG 44 cannot be unequivocally attributed to PERK kinase inhibition. All drugs have off target effects.
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Specific Comments:
1. Comparing results in Fig. 1 and 2 suggest that the preferential induction of the UPR in MVE cells is not a result of viral replication, since no difference was detected between FB and MVE in virus production (Fig. 1 A vs. B) and infection (Fig. 1D).
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¬ A western blot quantification of ER-translated viral proteins (such as S-glycoprotein) is needed to confirm the intracellular expression of MERS-CoV component and envelop proteins. Proteomics analysis is not an ideal substitute in Fig. 4 D to F.
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¬ The authors should identify which UPR arm causes the difference between MVE and FB cells

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It is known that AMG 44 is a "Potent and selective PERK inhibitor (IC50 = 6 nM), which "exhibits >160-fold selectivity for PERK over B-Raf and GCN2, as well as a panel of 387 other kinases. Orally bioavailable." (For more information, see https://www.tocris.com/products/amg-perk-44_5517).
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In compliance with data protection regulations, you may request that we remove your personal registration details at any time. (Remove my information/details). Please contact the publication office if you have any questions.

From: Sims, Amy C[amy.sims@pnnl.gov]
Sent: Fri 6/5/2020 8:23:59 AM (UTC-07:00)
To: Baric, Ralph S[rbaric@email.unc.edu]
Subject: Re: TALK TO AMY?? Re: Cell Editorial Decision CELL-D-20-01383

If you are willing to talk at 7pm eastern that would be great for me! Can you add that to your calendar please? Thank you very much! Amy

I am also going to send you a calendar invite if that helps.

From: "Baric, Ralph S" <rbaric@email.unc.edu>
Date: Friday, June 5, 2020 at 8:22 AM
To: "Sims, Amy C" <amy.sims@pnnl.gov>
Subject: RE: TALK TO AMY?? Re: Cell Editorial Decision CELL-D-20-01383

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Date: Thursday, June 4, 2020 at 8:35 AM
To: "Sims, Amy C" <amy.sims@pnnl.gov>
Subject: RE: Cell Editorial Decision CELL-D-20-01383

Hi Amy, I was sulking regarding the review. I guess the criticisms requiring the use of another drug tareting a downstream kinase is what Nat has suggested. I can't remember the status of that data or whether we tried it in vivo or in vitro. I'll be glad to talk. Maaybe Friday around 6PM EST? ralph

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From: Sims, Amy C <amy.sims@pnnl.gov>
Sent: Thursday, June 4, 2020 11:28 AM
To: Baric, Ralph S <rbaric@email.unc.edu>; Baric, Toni C <antoinette_baric@med.unc.edu>
Subject: FW: Cell Editorial Decision CELL-D-20-01383

Ralph,

Do you have time to discuss next steps soon? I suspect this will take no more than 15 minutes.

Thank you, Amy

From: <em.cell.0.6b95c1.abe328dd@editorialmanager.com> on behalf of Cell Editorial Office <em@editorialmanager.com>
Reply-To: Cell Editorial Office <cellms@cell.com>
Date: Thursday, May 28, 2020 at 1:59 PM

To: "Sims, Amy C" <amy.sims@pnnl.gov>
Subject: Cell Editorial Decision CELL-D-20-01383

Dr. Amy C Sims
902 Battelle Boulevard
Richland, WA 99352
UNITED STATES

CELL-D-20-01383
Unfolded Protein Response Inhibition Reduces MERS-CoV Induced Acute Lung Injury

May 28, 2020

Dear Dr. Sims,

I am enclosing the comments that the reviewers made on your paper. Unfortunately, the consensus recommendation is against publication in *Cell*. The reviewers refer to the potential timliness of the topic and interest in the approach, but cite that significant further work would be necessary, especially to demonstrate the specificity of the compound and its proposed mechanism of action in vivo. Given their comments, and the unclear relevance to non-MERS-CoV driven coronavirus pathology, we unfortunately cannot proceed further with this manuscript at Cell.

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Although we cannot offer to proceed further with this particular paper for *Cell*, the manuscript may be a stronger candidate for another journal within the Cell Press manuscript transfer system. If you are interested in learning more about transfer as an option, please contact the relevant journal directly, as each journal is editorially independent. The link below will bring you to a page where you can select the journal to which you would like to transfer your paper (you can review each journal's aims and scopes before choosing your target).

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After your files have been transferred, you will be given an opportunity to update your materials before they are delivered to the editors at your new journal. You will be able to make any desired revisions or additions at this stage, so feel free to select a transfer option now and work on your revisions after that. For more details on the transfer process, please click here or contact the editors at your new target journal.

Please note that the above link will expire Jun 27, 2020. However, if you still wish to transfer after that period, you may submit directly to the journal of your choice and reference your original manuscript number in the cover letter.

We hope this option may be helpful for you, however if you decide not to transfer this manuscript, please click here:

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submitting this manuscript or a revised version of it to another Cell Press journal as a regular new submission, in which case you can decline the transfer.

Best wishes,
Andrew

Andrew J. Rennekamp, Ph.D.
Scientific Editor, *Cell*

Reviewers' Comments:

Reviewer #1:

This manuscript by Sims and co-workers describes the results of an unbiased, systems biology-based approach that identifies the unfolded protein response (UPR) as differentially modulated during MERS-CoV infection of primary cells. They used donor matched primary human lung microvascular endothelial cells (MVEs) and fibroblasts (FB), and found that only MVEs exhibited cytopathic effect in response to infection with MERS-CoV. To determine if a specific pathway was responsible for the differential outcome in MVEs versus FBs, they compared the transcriptomes, proteomes, and lipidomes of MERS-CoV-infected cells over time, and repeated this experiment using 3 different donors of the cells. This analysis revealed that the MERS-CoV-infected MVEs were dying via an Unfolded Protein Response (UPR) mediated apoptosis. They validated the importance of the UPR pathway for MERS-CoV replication-induced cell death in MEVs by addition of UPR pathway inhibitor (AMG44), which reduced replication and cell death. Importantly, they also demonstrated that drug targeting of this pathway ameliorated the outcome of MERS-CoV infection in mice (reduced weight loss and improved respiratory function). Overall, this systems biology approach allowed them to identifying a targeted antiviral therapy that reduced morbidity and mortality in MERS-CoV-infected mice. The manuscript is well written and the experiments are clearly presented. Appropriate statistical analysis is provided for each experiment. The results of this study document an approach for identifying targeted antiviral therapy for coronavirus infection. This study has important implications for developing therapies for the current pandemic.
Comments for the authors' consideration (minor issues):
1. Legend to Fig 2. Is it least significant score or most significant score?
2. Legend to Fig 3. MERS-CoV MOI?
3. Title to Fig 4: Protein markers suggest activation of UPR in MVEs.
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5. Line 344: cite Gosert et al., 2002 for identification of DMVs
6. Lines 486-493: Was this MERS-nanoluc virus?
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8. Do your results implicate other UPR modulators such as GSK2606414 and KIRA6 (Mahameed at al., Cell Death and Disease, 2019) as potential modulators of coronavirus replication and disease? Discussion of UPR modulators as potential therapeutics would be appropriate (line 377).
9. Line 377, start new paragraph to discuss mortality in male and female mice
10. Line 132: mothers?
11. Line 349: add recent paper Volk, Hackbart et al., 2020 J. Virol for activation of the UPR in macrophages in response to coronavirus replication.
12. Line 392: add drug names remdesivir and EIDD-2801 here

Reviewer #2: Coronavirus infection and replication is well known for its ability to stimulate an intracellular unfolded protein response (UPR). In this paper these authors take an unbiased omics-based approach to examine pathways stimulated by MERS-CoV. They identify an induction of UPR gene signature - particularly in microvascular endothelial cells (MVE) - more than fibroblasts. They find that the use of a PERK inhibitor

reduces MERS-CoV replication in infected primary human lung MVE and FB and treatment with the same PERK inhibitor AMG144 in an in vivo mouse model of MERS lung disease leads to some clinical improvement. They show that it reduces lung disease and other features of disease pathogenesis - though they fail to show a reduction in MERS-CoV viral replication in infected cells. It should also be noted that PERK inhibitors are wonderfully pleiotropic.

The experiments seem to lack a certain logic? They emphasise the importance of maintaining the epithelial-endothelial cell barrier in MERS-CoV pathogenesis and I would agree that this is a critical aspect of coronavirus lung disease. They speculate that the PERK signaling pathway and the UPR response promote increased membrane reorganization and suppress host translation that helps optimise an environmental milieu for improved virus replication, maturation and egress. However, the virus is thought to enter the cells through the respiratory tissue - most likely the type II alveolar cell. What then is the rationale of looking at the effect of virus on fibroblasts and microvascular endothelial cells when the virus presumably enters through DPP4 + lung epithelial cells in the alveolus? Isn't this the cell type they should be looking at - as this is the likely cell to initiate the UPR - what is the point of looking at endothelial cells. It would seem that once the virus has infected the endothelial cell, there has already been a breakdown of the epithelial-endothelial barrier. Is there evidence of clinically important levels of viral replication in humans in endothelial cells?

Other comments

They perform transcriptomics/proteomics/lipidomics at various time points. The % cell infection is shown FIG 1D, and varies between ~1% and 30%. So the omics studies are all performed on cells at different time points with different % infected cells i.e. the number of infected fibroblasts range from ~1 - 30% max at 48hrs and the number of infected MVE cells range from ~1 - 50% max at 24hrs. From what I could gather they haven't performed any enrichment of infected cells - so the % infected cells was always low and varied at different timepoints, making an assessment and comparison of the data somewhat arbitrary.

Figure 5 Confusing presentation of data. It would be helpful to show the number of cells infected - rather than inhibition of infection - or maybe show both e.g. it's unclear to the reader if this is 10% infected or 70%?
At 48hrs with MVE there is no effect.
At 24hrs the effect is binary - no dose response curve - why is this - can this be repeated with more dose response points in the critical range?
What is negative toxicity with fibroblasts?

Line 163 "Death of MERS-CoV infected MVE could lead to permeability at the epithelial/endothelial cell barrier as one of the early steps of ARDS while viable MERS-CoV infected FB would continue to produce high levels of infectious virus both of which would contribute to the overall disease burden in the infected patient."

The in vivo mouse data shows a modest effect on weight loss - but no effect on viral titre - so I'm not sure they know why the drug is having a mild beneficial effect.

The most one could say here is that UPR inhibition has a modest improvement in disease outcome in this mouse model. Not sure one can make further conclusions - how this relates to their findings is also unclear.

Reviewer #3: Summary
In this study, Sims et al. report an important finding that a strain of coronavirus, MERS-CoV, leads to different cytopathic effects in primary human lung microvascular endothelial cells and in lung fibroblasts (MVE and FB, respectively). While MERS-CoV display similar viral replication in both cell lineages, the cytopathic

effect of unfolded protein response (UPR)-mediated apoptosis is limited to MVE, but not FB. The authors subsequently tested a pharmacologic PERK kinase inhibitor compound, namely AMG 44, and further demonstrated that AMG 44 exhibits cytoprotective effect in vitro on MVE cells. Furthermore, prophylactic treatment with AMG 44 protected MERS-CoV infected mice from clinical manifestation of lung damage typically seen after MERS-CoV infection. Taken together, the exhibited phenotypes are highly interesting and potentially significant for translational use of UPR pharmacologic compounds as a potential therapy against coronavirus-mediated human diseases, which warrants investment and further investigation of underlying molecular mechanisms.
However, although the authors demonstrated in the current manuscript that MERS-CoV replication kinetics were similar among FB and MVE, and MVE cells' death is likely a result from apoptotic mechanisms accompanied by UPR activation in these primary cells, there is insufficient evidence to underpin PERK branch of the UPR as a culprit for MERS-CoV associated cell death, neither in vitro nor in vivo. Furthermore, as authors correctly pointed out that "Understanding MERS-CoV pathogenic mechanisms requires understanding critical virus-host interactions", the in vivo protective effect mediated by prophylactic AMG 44 treatment may not be as simple as a PERK-kinase inhibition effect. Usage of genetic UPR mouse models to rigorously examine the hypothesis is recommended. For consideration of publication at Cell, at least a different compound targeting a downstream node of the same PERK pathways is necessary to cross-validate the conclusion that PERK branch of the UPR promotes cell apoptosis, which is largely dependent on one "PERK inhibitor (AMG 44)" at an obviously supra-pharmacological dosage used in this manuscript.

Main Critique:
Strength:
The authors, apparently pioneered in the coronavirus research, carried out important and urgently-needed studies in an effort to find a cure for coronavirus induced human diseases, including MERS-CoV induced human lung disease. In this study, the authors infected primary lung fibroblasts derived from three matched human donors and conducted both virologic and thorough multi-omics analysis of these cells, examining the in vitro effects of MERS-CoV infection. This aspect of the manuscript is definitely both novel and necessary to promote the related research urgently needed to help address the current challenges brought by the COVID19 pandemic.
Weakness:
There is insufficient evidence to support the main conclusion that MERS-CoV induced MVE cell death is mediated by the UPR, specifically via the PERK branch, leading to cellular apoptosis. In addition, there is lack of consideration of the human immune component in the disease models in vitro by cell culture. Thirdly, the in vivo effect from prophylactic treatment with AMG 44 cannot be unequivocally attributed to PERK kinase inhibition. All drugs have off target effects.
To address the above concerns, genetic modulations of the PERK pathway signaling molecules is needed. At a minimum, other compounds targeting ER stress and/or downstream of the PERK pathway signaling molecules are absolutely necessary to support authors' main conclusion underpinning PERK kinase activity as a culprit for cell death.

Specific Comments:
1. Comparing results in Fig. 1 and 2 suggest that the preferential induction of the UPR in MVE cells is not a result of viral replication, since no difference was detected between FB and MVE in virus production (Fig. 1 A vs. B) and infection (Fig. 1D).
¬ What is rationale for studying infection in MVE and EB?
¬ This observation begs the explanation of UPR difference, since it is unlikely to be caused by an exacerbated viral protein load effect in the ER of MVE cells.
¬ A western blot quantification of ER-translated viral proteins (such as S-glycoprotein) is needed to confirm the intracellular expression of MERS-CoV component and envelop proteins. Proteomics analysis is not an ideal substitute in Fig. 4 D to F.

¬ Related to the same comment, there is an upregulation of various ER associated proteins in the proteomics data set (Fig. 2B, by red asterisks), specifically for MVEs. These proteins are usually indicative of an ER compartment expansion, but not necessarily as a result of dysregulated UPR signaling. At the same time, there are also other "membrane" components observed in the same dataset, such as "mitochondrial inner membrane" and (plasma?) "membrane". Read out of UPR should look at more proximal readouts of UPR sensor activation.
¬ The upregulation of intracellular lipids in MVEs is interesting (Fig. 2C), which may suggest either an inhibition or disruption of normal cellular lipid secretion machinery, or an upregulation of lipid de novo synthesis. It is important to distinguish the two possibilities through labeling the lipid precursor metabolites.
¬ Can differences in growth rate account for difference between the cell types? There should also be a direct readout on mitochondrial function and not simple cellular ATP.

2. In Fig. 4, reinforcing the impression by results shown in Fig. 1, there is no difference in MERS-CoV Spike protein, ORF4a protein nor the membrane protein abundance. However, in contrast to FB cells, MVE cells displayed a distinctive pattern of greatly induced ER chaperone proteins, such as BiP, GRP94 and CANX.
¬ Can you knock down PERK and show resistance to AMG44?
¬ It is important to realize that the upregulation of ER chaperones are usually cyto-protective, in response to increased ER protein synthesis burden, and should not be taken as an evidence that UPR is disproportionally activated in the MVE cells.
¬ Instead, the data suggest an alternative explanation - FB cells can fold the MERS-CoV envelop proteins more efficiently than MVE cells, and therefore require less ER chaperones to mitigate ER stress. Could there be a difference in protein glycosylation in these two cell types? S-proteins are heavily glycosylated.
¬ Chemical chaperones, namely Tauroursodeoxycholic acid (TUDCA) and 4-phenylbutyric acid (4-PBA), should be considered in these experiments to distinguish the interesting phenotypes displayed by MVE to see whether MVE cells are indeed prone to protein misfolding-induced ER stress.
¬ The authors should identify which UPR arm causes the difference between MVE and FB cells

3. On the usage of the "PERK inhibitor" - AMG 44:
It is known that AMG 44 is a "Potent and selective PERK inhibitor (IC50 = 6 nM), which "exhibits >160-fold selectivity for PERK over B-Raf and GCN2, as well as a panel of 387 other kinases. Orally bioavailable." (For more information, see https://www.tocris.com/products/amg-perk-44_5517).
¬ Given the fact that authors needed to use at least > 10 μM of AMG 44 to achieve a MERS-CoV inhibition effect (Fig. 5), this reviewer considers whether AMG 44 inhibited other kinases in authors' experimental system. For example, two other "PERK inhibitors", GSK2606414 and GSK2656157 exhibit potent inhibitory effect on RIPK1 (doi: 10.1038/cdd.2017.58.) and AMPK.
¬ To address this concern, another PERK inhibitor such as the GSK compound should be used, at the specific "PERK inhibiting" dosage (<10 nM), to confirm PERK inhibition conferred cyto-protective effect on MVEs.
¬ MVE cells are apparently more susceptible to high doses of AMG 44 than FB, without MERS-CoV, creating a safety concern for administering AMG 44 to human patients. This safety issue should not to be taken lightly, as another PERK inhibitor, GSK2656157, exerted serious adverse effects on mouse pancreas (doi: 10.1158/0008-5472.CAN-12-3109), another grave safety concern.
¬ For a review on the toxicity profile of PERK inhibitors and their impact on physiology, please refer to the following article - Protein kinase R(PKR)-like endoplasmic reticulum kinase (PERK) inhibitors: a patent review (2010-2015) https://doi.org/10.1080/13543776.2017.1238072

4. The successful prophylactic treatment with AMG 44 in mouse models of MERS-CoV induced lung disease is the highlight of this manuscript. Although it is impossible to contribute the AMG 44 effect is specific to PERK kinase inhibition in MVE cells in vivo, the interesting preclinical observations surely hold promise for translational use of AMG 44 as a COVID19 therapy.
¬ The observation that AMG 44 does not confer protection to female mice infected with MERS-CoV is intriguing, as reviewer does not expect a sex-specific PERK difference in female mice.

¬ Could the above observation be explained by "off-target" effect of the "PERK kinase inhibitors", such as RIPK1? (doi: 10.1038/cdd.2017.58.)
¬ Given the importance of RIPK1 in mediating host versus pathogen immune response downstream of TNFR1, the therapeutic effect of AMG 44 on murine immune components should be examined in this context, for this mouse model.
¬ Nonetheless, this reviewer agrees the AMG 44 induced whole animal protection effect is both obvious and significant, regardless of the mechanism.

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In compliance with data protection regulations, you may request that we remove your personal registration details at any time. (Remove my information/details). Please contact the publication office if you have any questions.

From: Baric, Ralph S[rbaric@email.unc.edu]
Sent: Fri 6/5/2020 8:28:43 AM (UTC-07:00)
To: Sims, Amy C[amy.sims@pnnl.gov]
Subject: RE: TALK TO AMY?? Re: Cell Editorial Decision CELL-D-20-01383

Ok, send an invite

From: Sims, Amy C <amy.sims@pnnl.gov>
Sent: Friday, June 5, 2020 11:24 AM
To: Baric, Ralph S <rbaric@email.unc.edu>
Subject: Re: TALK TO AMY?? Re: Cell Editorial Decision CELL-D-20-01383

If you are willing to talk at 7pm eastern that would be great for me! Can you add that to your calendar please? Thank you very much! Amy

I am also going to send you a calendar invite if that helps.

From: "Baric, Ralph S" <rbaric@email.unc.edu>
Date: Friday, June 5, 2020 at 8:22 AM
To: "Sims, Amy C" <amy.sims@pnnl.gov>
Subject: RE: TALK TO AMY?? Re: Cell Editorial Decision CELL-D-20-01383

6PM or 7PM EST is best I think

From: Sims, Amy C <amy.sims@pnnl.gov>
Sent: Friday, June 5, 2020 11:14 AM
To: Baric, Ralph S <rbaric@email.unc.edu>
Subject: TALK TO AMY?? Re: Cell Editorial Decision CELL-D-20-01383

Hi Ralph,

I wasn't sure if your calendar exploded today or not. Do you have time later to talk? I can try to push my 6pm eastern meeting back if that is still the time that works best for you. Thank you, Amy

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Date: Thursday, June 4, 2020 at 8:35 AM
To: "Sims, Amy C" <amy.sims@pnnl.gov>
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Scientific Editor, *Cell*

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Other comments

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The in vivo mouse data shows a modest effect on weight loss - but no effect on viral titre - so I'm not sure they know why the drug is having a mild beneficial effect.

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Reviewer #3: Summary

In this study, Sims et al. report an important finding that a strain of coronavirus, MERS-CoV, leads to different cytopathic effects in primary human lung microvascular endothelial cells and in lung fibroblasts (MVE and FB, respectively). While MERS-CoV display similar viral replication in both cell lineages, the cytopathic effect of unfolded protein response (UPR)-mediated apoptosis is limited to MVE, but not FB. The authors subsequently tested a pharmacologic PERK kinase inhibitor compound, namely AMG 44, and further demonstrated that AMG 44 exhibits cytoprotective effect in vitro on MVE cells. Furthermore, prophylactic treatment with AMG 44 protected MERS-CoV infected mice from clinical manifestation of lung damage typically seen after MERS-CoV infection. Taken together, the exhibited phenotypes are highly interesting and potentially significant for translational use of UPR pharmacologic compounds as a potential therapy against coronavirus-mediated human diseases, which warrants investment and further investigation of underlying molecular mechanisms.

However, although the authors demonstrated in the current manuscript that MERS-CoV replication kinetics were similar among FB and MVE, and MVE cells' death is likely a result from apoptotic mechanisms accompanied by UPR activation in these primary cells, there is insufficient evidence to underpin PERK branch of the UPR as a culprit for MERS-CoV associated cell death, neither in vitro nor in vivo. Furthermore, as authors correctly pointed out that "Understanding MERS-CoV pathogenic mechanisms requires understanding critical virus-host interactions", the in vivo protective effect mediated by prophylactic AMG 44 treatment may not be as simple as a PERK-kinase inhibition effect. Usage of genetic UPR mouse models to rigorously examine the hypothesis is recommended. For consideration of publication at Cell, at least a different compound targeting a downstream node of the same PERK pathways is necessary to cross-validate the conclusion that PERK branch of the UPR promotes cell apoptosis, which is largely dependent on one "PERK inhibitor (AMG 44)" at an obviously supra-pharmacological dosage used in this manuscript.

Main Critique:

Strength:

The authors, apparently pioneered in the coronavirus research, carried out important and urgently-needed studies in an effort to find a cure for coronavirus induced human diseases, including MERS-CoV induced human lung disease. In this study, the authors infected primary lung fibroblasts derived from three matched human donors and conducted both virologic and thorough multi-omics analysis of these cells, examining the in vitro effects of MERS-CoV infection. This aspect of the manuscript is definitely both novel and necessary to promote the related research urgently needed to help address the current challenges brought by the COVID19 pandemic.

Weakness:

There is insufficient evidence to support the main conclusion that MERS-CoV induced MVE cell death is mediated by the UPR, specifically via the PERK branch, leading to cellular apoptosis. In addition, there is lack of consideration of the human immune component in the disease models in vitro by cell culture. Thirdly, the in vivo effect from prophylactic treatment with AMG 44 cannot be unequivocally attributed to PERK kinase inhibition. All drugs have off target effects.

To address the above concerns, genetic modulations of the PERK pathway signaling molecules is needed. At a minimum, other compounds targeting ER stress and/or downstream of the PERK pathway signaling molecules are absolutely necessary to support authors' main conclusion underpinning PERK kinase activity as a culprit for cell death.

Specific Comments:

1. Comparing results in Fig. 1 and 2 suggest that the preferential induction of the UPR in MVE cells is not a result of viral replication, since no difference was detected between FB and MVE in virus production (Fig. 1 A vs. B) and infection (Fig. 1D).

¬ What is rationale for studying infection in MVE and EB?
¬ This observation begs the explanation of UPR difference, since it is unlikely to be caused by an exacerbated viral protein load effect in the ER of MVE cells.
¬ A western blot quantification of ER-translated viral proteins (such as S-glycoprotein) is needed to confirm the intracellular expression of MERS-CoV component and envelop proteins. Proteomics analysis is not an ideal substitute in Fig. 4 D to F.
¬ Related to the same comment, there is an upregulation of various ER associated proteins in the proteomics data set (Fig. 2B, by red asterisks), specifically for MVEs. These proteins are usually indicative of an ER compartment expansion, but not necessarily as a result of dysregulated UPR signaling. At the same time, there are also other "membrane" components observed in the same dataset, such as "mitochondrial inner membrane" and (plasma?) "membrane". Read out of UPR should look at more proximal readouts of UPR sensor activation.
¬ The upregulation of intracellular lipids in MVEs is interesting (Fig. 2C), which may suggest either an inhibition or disruption of normal cellular lipid secretion machinery, or an upregulation of lipid de novo synthesis. It is important to distinguish the two possibilities through labeling the lipid precursor metabolites.
¬ Can differences in growth rate account for difference between the cell types? There should also be a direct readout on mitochondrial function and not simple cellular ATP.

2. In Fig. 4, reinforcing the impression by results shown in Fig. 1, there is no difference in MERS-CoV Spike protein, ORF4a protein nor the membrane protein abundance. However, in contrast to FB cells, MVE cells displayed a distinctive pattern of greatly induced ER chaperone proteins, such as BiP, GRP94 and CANX.
¬ Can you knock down PERK and show resistance to AMG44?
¬ It is important to realize that the upregulation of ER chaperones are usually cyto-protective, in response to increased ER protein synthesis burden, and should not be taken as an evidence that UPR is disproportionally activated in the MVE cells.
¬ Instead, the data suggest an alternative explanation - FB cells can fold the MERS-CoV envelop proteins more efficiently than MVE cells, and therefore require less ER chaperones to mitigate ER stress. Could there be a difference in protein glycosylation in these two cell types? S-proteins are heavily glycosylated.
¬ Chemical chaperones, namely Tauroursodeoxycholic acid (TUDCA) and 4-phenylbutyric acid (4-PBA), should be considered in these experiments to distinguish the interesting phenotypes displayed by MVE to see whether MVE cells are indeed prone to protein misfolding-induced ER stress.
¬ The authors should identify which UPR arm causes the difference between MVE and FB cells

3. On the usage of the "PERK inhibitor" - AMG 44:
It is known that AMG 44 is a "Potent and selective PERK inhibitor (IC50 = 6 nM), which "exhibits >160-fold selectivity for PERK over B-Raf and GCN2, as well as a panel of 387 other kinases. Orally bioavailable." (For more information, see https://www.tocris.com/products/amg-perk-44_5517).
¬ Given the fact that authors needed to use at least > 10 μM of AMG 44 to achieve a MERS-CoV inhibition effect (Fig. 5), this reviewer considers whether AMG 44 inhibited other kinases in authors' experimental system. For example, two other "PERK inhibitors", GSK2606414 and GSK2656157 exhibit potent inhibitory effect on RIPK1 (doi: 10.1038/cdd.2017.58.) and AMPK.
¬ To address this concern, another PERK inhibitor such as the GSK compound should be used, at the specific "PERK inhibiting" dosage (<10 nM), to confirm PERK inhibition conferred cyto-protective effect on MVEs.
¬ MVE cells are apparently more susceptible to high doses of AMG 44 than FB, without MERS-CoV, creating a safety concern for administering AMG 44 to human patients. This safety issue should not to be taken lightly, as another PERK inhibitor, GSK2656157, exerted serious adverse effects on mouse pancreas (doi: 10.1158/0008-5472.CAN-12-3109), another grave safety concern.
¬ For a review on the toxicity profile of PERK inhibitors and their impact on physiology, please refer to the following article - Protein kinase R(PKR)-like endoplasmic reticulum kinase (PERK) inhibitors: a patent review (2010-2015) https://doi.org/10.1080/13543776.2017.1238072

4. The successful prophylactic treatment with AMG 44 in mouse models of MERS-CoV induced lung disease is the highlight of this manuscript. Although it is impossible to contribute the AMG 44 effect is specific to PERK kinase inhibition in MVE cells in vivo, the interesting preclinical observations surely hold promise for translational use of AMG 44 as a COVID19 therapy.

¬ The observation that AMG 44 does not confer protection to female mice infected with MERS-CoV is intriguing, as reviewer does not expect a sex-specific PERK difference in female mice.

¬ Could the above observation be explained by "off-target" effect of the "PERK kinase inhibitors", such as RIPK1? (doi: 10.1038/cdd.2017.58.)

¬ Given the importance of RIPK1 in mediating host versus pathogen immune response downstream of TNFR1, the therapeutic effect of AMG 44 on murine immune components should be examined in this context, for this mouse model.

¬ Nonetheless, this reviewer agrees the AMG 44 induced whole animal protection effect is both obvious and significant, regardless of the mechanism.

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In compliance with data protection regulations, you may request that we remove your personal registration details at any time. (Remove my information/details). Please contact the publication office if you have any questions.

From: Sims, Amy C[amy.sims@pnnl.gov]
Sent: Fri 6/5/2020 8:29:57 AM (UTC-07:00)
To: Baric, Ralph S[rbaric@email.unc.edu]
Subject: Re: TALK TO AMY?? Re: Cell Editorial Decision CELL-D-20-01383

You should have it now. Let me know if I need to try again. Thank you, Amy

---

From: "Baric, Ralph S" <rbaric@email.unc.edu>
Date: Friday, June 5, 2020 at 8:29 AM
To: "Sims, Amy C" <amy.sims@pnnl.gov>
Subject: RE: TALK TO AMY?? Re: Cell Editorial Decision CELL-D-20-01383

Ok, send an invite

From: Sims, Amy C <amy.sims@pnnl.gov>
Sent: Friday, June 5, 2020 11:24 AM
To: Baric, Ralph S <rbaric@email.unc.edu>
Subject: Re: TALK TO AMY?? Re: Cell Editorial Decision CELL-D-20-01383

If you are willing to talk at 7pm eastern that would be great for me! Can you add that to your calendar please? Thank you very much! Amy

I am also going to send you a calendar invite if that helps.

From: "Baric, Ralph S" <rbaric@email.unc.edu>
Date: Friday, June 5, 2020 at 8:22 AM
To: "Sims, Amy C" <amy.sims@pnnl.gov>
Subject: RE: TALK TO AMY?? Re: Cell Editorial Decision CELL-D-20-01383

6PM or 7PM EST is best I think

From: Sims, Amy C <amy.sims@pnnl.gov>
Sent: Friday, June 5, 2020 11:14 AM
To: Baric, Ralph S <rbaric@email.unc.edu>
Subject: TALK TO AMY?? Re: Cell Editorial Decision CELL-D-20-01383

Hi Ralph,

I wasn't sure if your calendar exploded today or not. Do you have time later to talk? I can try to push my 6pm eastern meeting back if that is still the time that works best for you. Thank you, Amy

---

From: "Baric, Ralph S" <rbaric@email.unc.edu>
Date: Thursday, June 4, 2020 at 8:35 AM
To: "Sims, Amy C" <amy.sims@pnnl.gov>
Subject: RE: Cell Editorial Decision CELL-D-20-01383

Hi Amy, I was sulking regarding the review. I guess the criticisms requiring the use of another drug tareting a downstream kinase is what Nat has suggested. I can't remember the status of that data or whether we tried it in vivo or in vitro. I'll be glad to talk. Maaybe Friday around 6PM EST? ralph

---

From: Sims, Amy C <amy.sims@pnnl.gov>

Sent: Thursday, June 4, 2020 11:28 AM
To: Baric, Ralph S <rbaric@email.unc.edu>; Baric, Toni C <antoinette_baric@med.unc.edu>
Subject: FW: Cell Editorial Decision CELL-D-20-01383

Ralph,

Do you have time to discuss next steps soon? I suspect this will take no more than 15 minutes.

Thank you, Amy

From: <em.cell.0.6b95c1.abe328dd@editorialmanager.com> on behalf of Cell Editorial Office <em@editorialmanager.com>
Reply-To: Cell Editorial Office <cellms@cell.com>
Date: Thursday, May 28, 2020 at 1:59 PM
To: "Sims, Amy C" <amy.sims@pnnl.gov>
Subject: Cell Editorial Decision CELL-D-20-01383

Dr. Amy C Sims
902 Battelle Boulevard
Richland, WA 99352
UNITED STATES

CELL-D-20-01383
Unfolded Protein Response Inhibition Reduces MERS-CoV Induced Acute Lung Injury

May 28, 2020

Dear Dr. Sims,

I am enclosing the comments that the reviewers made on your paper. Unfortunately, the consensus recommendation is against publication in *Cell*. The reviewers refer to the potential timliness of the topic and interest in the approach, but cite that significant further work would be necessary, especially to demonstrate the specificity of the compound and its proposed mechanism of action in vivo. Given their comments, and the unclear relevance to non-MERS-CoV driven coronavirus pathology, we unfortunately cannot proceed further with this manuscript at Cell.

I am sorry that the outcome for this manuscript was not more positive. I hope, however, that the reviewers' comments are clear and constructive and that you will continue to consider *Cell* for future submissions.

Although we cannot offer to proceed further with this particular paper for *Cell*, the manuscript may be a stronger candidate for another journal within the Cell Press manuscript transfer system. If you are interested in learning more about transfer as an option, please contact the relevant journal directly, as each journal is editorially independent. The link below will bring you to a page where you can select the journal to which you would like to transfer your paper (you can review each journal's aims and scopes before choosing your target).

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are delivered to the editors at your new journal. You will be able to make any desired revisions or additions at this stage, so feel free to select a transfer option now and work on your revisions after that. For more details on the transfer process, please click here or contact the editors at your new target journal.

Please note that the above link will expire Jun 27, 2020. However, if you still wish to transfer after that period, you may submit directly to the journal of your choice and reference your original manuscript number in the cover letter.

We hope this option may be helpful for you, however if you decide not to transfer this manuscript, please click here:

Decline to Transfer

Declining to transfer will officially close out the manuscript in our system. If you do not accept or decline by Jun 27, 2020, the system will automatically decline on your behalf. If you prefer, you do have the choice of submitting this manuscript or a revised version of it to another Cell Press journal as a regular new submission, in which case you can decline the transfer.

Best wishes,
Andrew

Andrew J. Rennekamp, Ph.D.
Scientific Editor, *Cell*

Reviewers' Comments:

Reviewer #1:

This manuscript by Sims and co-workers describes the results of an unbiased, systems biology-based approach that identifies the unfolded protein response (UPR) as differentially modulated during MERS-CoV infection of primary cells. They used donor matched primary human lung microvascular endothelial cells (MVEs) and fibroblasts (FB), and found that only MVEs exhibited cytopathic effect in response to infection with MERS-CoV. To determine if a specific pathway was responsible for the differential outcome in MVEs versus FBs, they compared the transcriptomes, proteomes, and lipidomes of MERS-CoV-infected cells over time, and repeated this experiment using 3 different donors of the cells. This analysis revealed that the MERS-CoV-infected MVEs were dying via an Unfolded Protein Response (UPR) mediated apoptosis. They validated the importance of the UPR pathway for MERS-CoV replication-induced cell death in MEVs by addition of UPR pathway inhibitor (AMG44), which reduced replication and cell death. Importantly, they also demonstrated that drug targeting of this pathway ameliorated the outcome of MERS-CoV infection in mice (reduced weight loss and improved respiratory function). Overall, this systems biology approach allowed them to identifying a targeted antiviral therapy that reduced morbidity and mortality in MERS-CoV-infected mice. The manuscript is well written and the experiments are clearly presented. Appropriate statistical analysis is provided for each experiment. The results of this study document an approach for identifying targeted antiviral therapy for coronavirus infection. This study has important implications for developing therapies for the current pandemic.
Comments for the authors' consideration (minor issues):
1. Legend to Fig 2. Is it least significant score or most significant score?
2. Legend to Fig 3. MERS-CoV MOI?
3. Title to Fig 4: Protein markers suggest activation of UPR in MVEs.
4. Legend to Fig 5. MVEs were infected with MERS-nanoluc (MOI=?)
5. Line 344: cite Gosert et al., 2002 for identification of DMVs
6. Lines 486-493: Was this MERS-nanoluc virus?
7. Lines 486-493: AMG44 is referenced to two other papers, but better to name the commercial source.

8. Do your results implicate other UPR modulators such as GSK2606414 and KIRA6 (Mahameed at al., Cell Death and Disease, 2019) as potential modulators of coronavirus replication and disease? Discussion of UPR modulators as potential therapeutics would be appropriate (line 377).
9. Line 377, start new paragraph to discuss mortality in male and female mice
10. Line 132: mothers?
11. Line 349: add recent paper Volk, Hackbart et al., 2020 J. Virol for activation of the UPR in macrophages in response to coronavirus replication.
12. Line 392: add drug names remdesivir and EIDD-2801 here

Reviewer #2: Coronavirus infection and replication is well known for its ability to stimulate an intracellular unfolded protein response (UPR). In this paper these authors take an unbiased omics-based approach to examine pathways stimulated by MERS-CoV. They identify an induction of UPR gene signature - particularly in microvascular endothelial cells (MVE) - more than fibroblasts. They find that the use of a PERK inhibitor reduces MERS-CoV replication in infected primary human lung MVE and FB and treatment with the same PERK inhibitor AMG144 in an in vivo mouse model of MERS lung disease leads to some clinical improvement. They show that it reduces lung disease and other features of disease pathogenesis - though they fail to show a reduction in MERS-CoV viral replication in infected cells. It should also be noted that PERK inhibitors are wonderfully pleiotropic.

The experiments seem to lack a certain logic? They emphasise the importance of maintaining the epithelial-endothelial cell barrier in MERS-CoV pathogenesis and I would agree that this is a critical aspect of coronavirus lung disease. They speculate that the PERK signaling pathway and the UPR response promote increased membrane reorganization and suppress host translation that helps optimise an environmental milieu for improved virus replication, maturation and egress. However, the virus is thought to enter the cells through the respiratory tissue - most likely the type II alveolar cell. What then is the rationale of looking at the effect of virus on fibroblasts and microvascular endothelial cells when the virus presumably enters through DPP4 + lung epithelial cells in the alveolus? Isn't this the cell type they should be looking at - as this is the likely cell to initiate the UPR - what is the point of looking at endothelial cells. It would seem that once the virus has infected the endothelial cell, there has already been a breakdown of the epithelial-endothelial barrier. Is there evidence of clinically important levels of viral replication in humans in endothelial cells?

Other comments

They perform transcriptomics/proteomics/lipidomics at various time points. The % cell infection is shown FIG 1D, and varies between ~1% and 30%. So the omics studies are all performed on cells at different time points with different % infected cells i.e. the number of infected fibroblasts range from ~1 - 30% max at 48hrs and the number of infected MVE cells range from ~1 - 50% max at 24hrs. From what I could gather they haven't performed any enrichment of infected cells - so the % infected cells was always low and varied at different timepoints, making an assessment and comparison of the data somewhat arbitrary.

Figure 5 Confusing presentation of data. It would be helpful to show the number of cells infected - rather than inhibition of infection - or maybe show both e.g. it's unclear to the reader if this is 10% infected or 70%?
At 48hrs with MVE there is no effect.
At 24hrs the effect is binary - no dose response curve - why is this - can this be repeated with more dose response points in the critical range?
What is negative toxicity with fibroblasts?

Line 163 "Death of MERS-CoV infected MVE could lead to permeability at the epithelial/endothelial cell barrier as one of the early steps of ARDS while viable MERS-CoV infected FB would continue to produce

high levels of infectious virus both of which would contribute to the overall disease burden in the infected patient."

The in vivo mouse data shows a modest effect on weight loss - but no effect on viral titre - so I'm not sure they know why the drug is having a mild beneficial effect.

The most one could say here is that UPR inhibition has a modest improvement in disease outcome in this mouse model. Not sure one can make further conclusions - how this relates to their findings is also unclear.

Reviewer #3: Summary
In this study, Sims et al. report an important finding that a strain of coronavirus, MERS-CoV, leads to different cytopathic effects in primary human lung microvascular endothelial cells and in lung fibroblasts (MVE and FB, respectively). While MERS-CoV display similar viral replication in both cell lineages, the cytopathic effect of unfolded protein response (UPR)-mediated apoptosis is limited to MVE, but not FB. The authors subsequently tested a pharmacologic PERK kinase inhibitor compound, namely AMG 44, and further demonstrated that AMG 44 exhibits cytoprotective effect in vitro on MVE cells. Furthermore, prophylactic treatment with AMG 44 protected MERS-CoV infected mice from clinical manifestation of lung damage typically seen after MERS-CoV infection. Taken together, the exhibited phenotypes are highly interesting and potentially significant for translational use of UPR pharmacologic compounds as a potential therapy against coronavirus-mediated human diseases, which warrants investment and further investigation of underlying molecular mechanisms.
However, although the authors demonstrated in the current manuscript that MERS-CoV replication kinetics were similar among FB and MVE, and MVE cells' death is likely a result from apoptotic mechanisms accompanied by UPR activation in these primary cells, there is insufficient evidence to underpin PERK branch of the UPR as a culprit for MERS-CoV associated cell death, neither in vitro nor in vivo. Furthermore, as authors correctly pointed out that "Understanding MERS-CoV pathogenic mechanisms requires understanding critical virus-host interactions", the in vivo protective effect mediated by prophylactic AMG 44 treatment may not be as simple as a PERK-kinase inhibition effect. Usage of genetic UPR mouse models to rigorously examine the hypothesis is recommended. For consideration of publication at Cell, at least a different compound targeting a downstream node of the same PERK pathways is necessary to cross-validate the conclusion that PERK branch of the UPR promotes cell apoptosis, which is largely dependent on one "PERK inhibitor (AMG 44)" at an obviously supra-pharmacological dosage used in this manuscript.

Main Critique:
Strength:
The authors, apparently pioneered in the coronavirus research, carried out important and urgently-needed studies in an effort to find a cure for coronavirus induced human diseases, including MERS-CoV induced human lung disease. In this study, the authors infected primary lung fibroblasts derived from three matched human donors and conducted both virologic and thorough multi-omics analysis of these cells, examining the in vitro effects of MERS-CoV infection. This aspect of the manuscript is definitely both novel and necessary to promote the related research urgently needed to help address the current challenges brought by the COVID19 pandemic.
Weakness:
There is insufficient evidence to support the main conclusion that MERS-CoV induced MVE cell death is mediated by the UPR, specifically via the PERK branch, leading to cellular apoptosis. In addition, there is lack of consideration of the human immune component in the disease models in vitro by cell culture. Thirdly, the in vivo effect from prophylactic treatment with AMG 44 cannot be unequivocally attributed to PERK kinase inhibition. All drugs have off target effects.
To address the above concerns, genetic modulations of the PERK pathway signaling molecules is needed. At a

minimum, other compounds targeting ER stress and/or downstream of the PERK pathway signaling molecules are absolutely necessary to support authors' main conclusion underpinning PERK kinase activity as a culprit for cell death.

Specific Comments:
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¬ Can you knock down PERK and show resistance to AMG44?
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4. The successful prophylactic treatment with AMG 44 in mouse models of MERS-CoV induced lung disease is the highlight of this manuscript. Although it is impossible to contribute the AMG 44 effect is specific to PERK kinase inhibition in MVE cells in vivo, the interesting preclinical observations surely hold promise for translational use of AMG 44 as a COVID19 therapy.
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¬ Nonetheless, this reviewer agrees the AMG 44 induced whole animal protection effect is both obvious and significant, regardless of the mechanism.

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In compliance with data protection regulations, you may request that we remove your personal registration details at any time. (Remove my information/details). Please contact the publication office if you have any questions.

From: Schaefer, Alexandra[aschaefe@email.unc.edu]
Sent: Fri 6/5/2020 10:45:56 AM (UTC-07:00)
To: Sims, Amy C[amy.sims@pnnl.gov]
Subject: Re: biosketch
Attachment: biosketch-AS_v4.0.docx

Hi Amy,

sorry, I thought I already answered you....
attached is my Biosketch

Also, today I finished the STAT1- ChIP for the MVEs and I am starting to ran the PCRs- as soon as there are done, I'll send you the result

However, we are having big issues with the Qiagen EpiTect ChIP OneDay kit...
if you search for the kit online, the item comes up without any problem, but if Cait wants to order them through ePro it looks like they are not available.
We were just both sitting at her computer and no matter how we search for them in the system, we can't find them.
She also has emailed our Qiagen Rep several times, but he appears to be very unresponsive.

Do you have a Rep in your area who might be able to contact?
I am not sure, how to proceed with this--- except for getting a different kit..... which is fine, it just makes a comparison between the MVE histone and FB histone results impossible

What are you thinking,
Alex

From: Sims, Amy C <amy.sims@pnnl.gov>
Sent: Monday, June 1, 2020 8:27 PM
To: Schaefer, Alexandra <aschaefe@email.unc.edu>
Subject: biosketch

Hi Alex,

Can you please send me your latest biosketch? I need it to get UNC's subcontract set up for the R21.

I hope you are doing well. Thank you, Amy

OMB No. 0925-0001 and 0925-0002 (Rev. 09/17 Approved Through 03/31/2020)

BIOGRAPHICAL SKETCH

Provide the following information for the Senior/key personnel and other significant contributors.
Follow this format for each person. **DO NOT EXCEED FIVE PAGES.**

NAME: Alexandra Schaefer

eRA COMMONS USER NAME (credential, e.g., agency login): aschaefe

POSITION TITLE: Research Associate

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, include postdoctoral training and residency training if applicable. Add/delete rows as necessary.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	Completion Date MM/YYYY	FIELD OF STUDY
Johannes Gutenberg University, Mainz, Germany	BS	04/1996	Biology
Johannes Gutenberg University, Mainz, Germany	MS	08/1999	Biology
Friedrich-Alexander University, Erlangen-Nuremberg, Germany	PhD	07/2003	Virology
Duke University Medical Center, Durham, NC	Postdoctoral	03/2007	Virology
Carolina Vaccine Institute, University of North Carolina, Chapel Hill, NC	Postdoctoral	01/2013	Virology

A. Personal Statement: My research in the Baric Laboratory focuses on virus-host interaction, viral pathogenesis, viral immunity, and the role of host genetics in viral infection during coronaviruses (SARS-CoV and MERS-CoV), influenza virus (H5N1, 2009 H1N1, and 1918 H1N1), Henipaviruses (NiV and HeV), and filovirus (EBOV and MARV) infection.
In my current position as a Senior Scientist in the Baric Laboratory my research efforts focus on SARS-CoV/MERS-CoV/SARS-CoV-2 pathogenesis host susceptibility, immunity, and vaccine/drug efficacy using genetic diverse, as well as aged mouse model systems. Our main approaches include the usage of several diverse model and systems platforms, including systems genetics (Collaborative Cross, multiparent panel of recombinant inbred (RI) mice), transcriptomics, proteomics, and high throughput sequencing to identify host genetic factors that determine resistance or susceptibility to Coronaviruses. Over the last years, under my lead in the Baric Laboratory and in collaboration with the Rocky Mountain Laboratories, NIAID, NIH, Hamilton, MT, we expanded our system genetics approaches to study host genetics during infection with EBOV and MARV.

1. Noll KE, Whitmore AC, West A, McCarthy MK, Morrison CR, Plante KS, Hampton BK, Kollmus H, Pilzner C Leist SR, Gralinski LE, Menachery VD, **Schäfer A**, Miller D, Shaw G, Mooney M, McWeeney, S, Pardo-Manuel de Villena F, Schughart K, Morrison TE, Baric RS, Ferris MT, Heise MT. Complex Genetic Architecture Underlies Regulation of Influenza-A-Virus-Specific Antibody Responses in the Collaborative Cross. (2020). Cell Rep. 31(4):10758
2. Sheahan TP, Sims AC, Leist SR, **Schäfer A**, Won J, Brown AJ, Montgomery SA, Hogg A, Babusis D, Clarke MO, Spahn JE, Bauer L, Sellers S, Porter D, Feng JY, Cihlar T, Jordan R, Denison MR, Baric RS. Comparative Therapeutic Efficacy of Remdesivir and Combination Lopinavir, Ritonavir, and Interferon Beta Against MERS-CoV. (2020). Nat. Commun. 11(1):222
3. Graham JB, Swarts JL, Menachery VD, Gralinski LE, **Schäfer A**, Plante KS, Morrison CR, Voss KM, Green R, Choonoo G, Jeng S, Miller DR, Mooney MA, McWeeney SK, Ferris MT, Pardo-Manuel de

Villena F, Gale M, Heise MT, Baric RS, Lund JM. Immune Predictors of Mortality After Ribonucleic Acid Virus Infection. (2020). J. Infect. Dis. 221(6):882-889

4. van Doremalen N, **Schäfer A**, Menachery VD, Letko M, Bushmaker T, Fischer RJ, Figueroa DM, Hanley PW, Saturday G, Baric RS, Munster VJ. SARS-Like Coronavirus WIV-1 Does Not Replicate in Egyptian Fruit Bats (*Rousettus aegyptiacus*). (2018) Viruses. Dec 19;10(12)
5. Gralinski L, Menachery VD, Beall A, Kocher J, Morgan AP, Plante J, Shostak C, Totura AL, **Schäfer A**, Pardo-Manuel de Villena F, Ferris MT, Baric RS. Allelic Variation in the Toll-Like Receptor Adaptor Protein *Ticam2* Contributes to SARS-Coronavirus Pathogenesis in Mices. (2017). G3 (Bethesda). 7(6): 1653-63

B. Positions and Honors:

Positions and Employment

2013- present Research Associate, Department of Epidemiology, UNC-Chapel Hill
2015- present Guest Researcher, Laboratory of Virology, Rocky Mountain Laboratories, NIAID, NIH, Hamilton, MT

Honors

1998- 1999 Stipend for excellent performance as a graduate student, Johannes-Gutenberg University Mainz, Germany
1999- 2003 Graduate Student Stipend, SFB466, DFG-Germany, 'Lymphproliferation und virale Immundefizienz
2003- 2007 Postdoctoral Research Fellow, HHMI, Duke University Medical Center
2006 Young Investigator Travel Award, 13th Conference on Retroviruses and Opportunistic Infections,
2012 Invitation as 'Early Career Investigator', Nature Medicine: Herrenhausen Symposium on Viral Pathogenesis

Professional Memberships

1999- present German Society for Virology (Deutsche Gesellschaft fuer Virologie, GfV)
2004- present American Society for Virology (ASV)
2004- present American Society for Microbiology (ASM)
2018- present International Mammalian Genome Society (IMGS)

Professional Services

2011- present Reviewer, Virology
2016- present Reviewer, Viruses
2019- present Reviewer, Scientific Reports, JCP
2020- present Reviewer for the German Federal Ministry of Education and Research (BMBF), Study Section 'Research on COVID-19 in the wake of the SARS-CoV-2 outbreak'

C. Contributions to Virology: My research efforts in several fields of virology (retroviruses, herpesviruses, arboviruses, coronaviruses, influenza viruses, and filoviruses) have made significant contributions in our understanding of many aspects of virology, including:

i) My research project during my PhD was focused on studying the function of orf73/LANA (latency-associated nuclear antigen) of Herpesvirus saimiri during virus infection. I showed that orf73/LANA mediates inhibition of the switch from viral latency to lytic replication. Since then, this exact mechanism has been described for other γ–herpesviruses, such as RRV (Rhesus rhadinovirus) and KSHV (Kaposi's sarcoma associated virus).

- **Schäfer A**, Lengenfelder D, Grillhösl C, Wieser C, Fleckenstein B, Ensser A. (2003). The latency-associated nuclear antigen homolog of herpesvirus saimiri inhibits lytic virus replication. *J. Virol.* 77: 5911-25

ii) During my postdoctoral years at Duke University Medical Center, I studied the function and the packaging of various APOBEC3 proteins during HIV-1 infection; APOBEC3 proteins belong to a family of intracellular restriction factors essential for species specificity and replication inhibition of retroviruses and retrotransposons. The packaging of APOBEC3 proteins into the virus particles is the foundation of inhibition of retrovirus relication. My studies identified the nucleocapsid and viral RNA as the main mediator of

APOBEC3G, and other APOBEC3 proteins, like mAPOEBC3 and APOBEC3B, for virion incorporation. Our identification of APOBEC3 cytidine deaminases as strong inhibitors of retrotransposition indicated that the ABPOBEC3 family are general restriction factors for endogenous transposable elements and exogenous retroviruses.

- Doehle BP, **Schäfer A**, Cullen BR. (2005). Human APOBEC3B is a potent inhibitor of HIV-1 infectivity and is resistant to HIV-1 Vif. *Virology* 339: 281-8
- Dutko JA, **Schäfer A**, Kenny AE, Cullen BR, Curcio MJ. (2005). Inhibition of a yeast LTR retrotransposon by human APOBEC3 cytidine deaminases. *Current Biology* 15: 661-6
- Doehle BP, **Schäfer A**, Wiegand HL, Bogerd HP, Cullen BR. (2005). Differential sensitivity of murine leukemia virus to APOBEC3-mediated inhibition is governed by virion exclusion. *J. Virol.* 79: 8201-7
- Russell RA, Wiegand HL, Moore MD, **Schäfer A**, McClure MO, Cullen BR. (2005). Foamy virus Bet proteins function as novel inhibitors of the APOBEC3 family of innate antiretroviral defense factors. *J. Virol.* 79: 8724-31
- **Schäfer A**, Bogerd HP, Cullen BR. (2004). Specific packaging of APOBEC3G into HIV-1 virions is mediated by the nucleocapsid domain of the gag polyprotein precursor. *Virology* 328: 163-8

iii) A second research focus during my postdoctoral studies at Duke University Medical Center were focused on the identification of miRNAs expressed by herpesviruses during latency. I both lead and was involved in studies that pioneered the identification, cloning and expression strategies of miRNAs encoded in EBV (Epstein Barr virus) and RRV (Rhesus Rhadinovirus).

- **Schäfer A**, Cai X, Bilello JP, Desrosiers RC, Cullen BR. (2007). Cloning and analysis of miRNAs encoded by the primate gamma-herpesvirus rhesus monkey rhadinovirus. *Virology* 364: 21-7
- Cai X, **Schäfer A**, Lu S, Bilello JP, Desrosiers RC, Edwards R, Raab-Traub N, Cullen BR. (2006). Epstein-Barr Virus microRNAs are Evolutionary Conserved and Differentially Expressed in Infected Cells. *PLoS Pathogens* 2(3): e23

iv) During my second postdoctoral appointment at the Carolina Vaccine Institute at the University of North Carolina at Chapel Hill, I studied the mechanism of infection of the central nervous system (CNS) and neuroinvasion of Venezuelan equine encephalitis virus. I established and characterized a reductionist encephalitis model in mice by using Venezuelan equine encephalitis virus (VEEV) replicon partilces (VRPs), which allowed the investigation of innate and inflammatory response after VEEV infection on a cellular- and tissue-specific level. I also established that peripheral VEE enters the CNS in a two-step process where VEE virus initially enters the CNS through the olfactory pathways and initiates viral replication in the brain, which induces the opening of the blood brain barrier, allowing a second wave of invading virus from the periphery to enter the brain.

- **Schäfer A,** Brooke CC, Whitmore AC, Johnston RE. (2011). The role of the blood-brain barrier during Venezuelan equine encephalitis virus CNS infection. *J. Virol.* 85: 10682-90
- **Schäfer A**, Whitmore AC, Konopka JL, Johnston RE. (2009) Replicon particles of Venezuelan equine encephalitis virus as a reductionist murine model for encephalitis. *J. Virol.* 83: 1045-57
- Brooke CC, **Schäfer A,** Whitmore AC, Johnston RE. (2012). Early interaction with the host complement system determine disease outcome following Venezuelan equine encephalitis virus infection. *J. Gen. Virol.* 93: 797-806

v) In the Baric laboratory I have established the ChIP method (Chomatin Immunoprecipitation)-Seq and MeDIP (methylated DNA immunoprecipitation)-Seq in Calu3 cells (human epithelia fibroblast cell line) during MERS-CoV, SARS-CoV, H5N1 IAV, and H1N1 infection. These studies are among the first ones in the field showing that pathogeneic respiratory viruses, like corona- and influenza viruses, use chromatin modulation to antagonize the host's innate and adaptive immune response.

- **Schäfer A***, Menachery VD*, Burnum-Johnson KE, Mitchell HM, Eisfeld AJ, Sims AC, Walters KB, Nicora CD, Purvine SO, Casey CP, Monroe ME, Weitz KK, Stratton KG, Webb-Robertson B-JM,. Gralinski LE, Metz TO, Smith RD, Waters KM, Kawaoka Y, Baric RS. MERS-CoV and H5N1 influenza virus antagonize antigen presentation by altering the epigenetic landscape. (2018) Proc Natl Acad Sci U S A. 30;115(5): E1012-E1021
- **Schäfer A**, Baric RS. Epigenetic Landscape during Coronavirus Infection. (2017). Pathogens. 15;6(1): 10.3390/pathogens6010008.
- Menachery VD, Eisfeld AJ, **Schäfer A**, Josset L, Sims AC, Proll S, Fan S, Li C, Neumann G, Tilton SC, Chang J, Gralinski LE, Long CE, Green R, Williams CM, Matzke MM, Webb_Robertson B-JM,

Shukula KM, Metz TO, Smith RD, Waters KM, Katze MG, Kawaoka Y, Baric RS. Pathogenic Influenza and coronaviruses utilize similar and contrasting approaches to control the interferon stimulated gene response (2014). *MBio.* 5(3): e01174-14

- Matthews K, **Schäfer A**, Pham A, Frieman M. The SARS Coronavirus Papain Like Protease can inhibit IRF3 at a post activation step that requires deubiquitination activity (2014). *Virol J*. 2014 11: 209
- Sims AC, Tilton SC, Menachery VD, Gralinski LE, **Schäfer A**, Matzke MM, Webb-Robertson B-JM, Chang J, Luna ML, Long CE, Shukula AK, Bankhead III AR, Burkett SE, Zornetzer G, Kent Tseng C-T, Metz TO, Pickles R, McWeeney S, Smith RD, Katze MG, Waters KM, Baric RS (2013). Release of SARS-CoV Nuclear Import Block Enhances Host Transcription in Human Lung Cells. *J. Virol.* 87: 3885-902

D. Research Support:

U19 A1 AI100625 (PI Baric/Heise), 'Symstems Immunogenetics of Biodefense in the Collaborative Cross', September 2017 – July 2022

R21 AI146872-01, 'How MERS-CoV Regulates Innate Immunity in Primary Human Lung Cells', July 2019 – June 2021

From: Sims, Amy C[amy.sims@pnnl.gov]
Sent: Fri 6/5/2020 11:36:55 AM (UTC-07:00)
To: Schaefer, Alexandra[aschaefe@email.unc.edu]
Subject: Re: biosketch

Hi Alex,

Thank you for the biosketch. I appreciate it.

I am very excited to see what happens with the STAT1 ChIP for the MVE. Please do keep me posted. I appreciate you finding time to do these experiments . I can't imagine how busy the Baric lab is right now.

I am sorry you are continuing to struggle with ordering the kits from Qiagen.

I will be happy to see what I can do on this end but it will be next week before I can work on it. I will keep you posted.

Thank you and I hope you get some down time this weekend. Best wishes, Amy

---

From: "Schaefer, Alexandra" <aschaefe@email.unc.edu>
Date: Friday, June 5, 2020 at 10:48 AM
To: "Sims, Amy C" <amy.sims@pnnl.gov>
Subject: Re: biosketch

Hi Amy,

sorry, I thought I already answered you....
attached is my Biosketch

Also, today I finished the STAT1- ChIP for the MVEs and I am starting to ran the PCRs- as soon as there are done, I'll send you the result

However, we are having big issues with the Qiagen EpiTect ChIP OneDay kit...
if you search for the kit online, the item comes up without any problem, but if Cait wants to order them through ePro it looks like they are not available.
We were just both sitting at her computer and no matter how we search for them in the system, we can't find them.
She also has emailed our Qiagen Rep several times, but he appears to be very unresponsive.

Do you have a Rep in your area who might be able to contact?
I am not sure, how to proceed with this--- except for getting a different kit..... which is fine, it just makes a comparison between the MVE histone and FB histone results impossible

What are you thinking,
Alex

From: Sims, Amy C <amy.sims@pnnl.gov>
Sent: Monday, June 1, 2020 8:27 PM
To: Schaefer, Alexandra <aschaefe@email.unc.edu>

Subject: biosketch

Hi Alex,

Can you please send me your latest biosketch? I need it to get UNC's subcontract set up for the R21.

I hope you are doing well. Thank you, Amy

Please wait...

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Statement of Work (SOW)
Univ. of North Carolina at Chapel Hill Subcontract with Battelle-PNWD, Operator of PNNL for US DOE

Project Title: How MERS-CoV Regulates Innate Immunity in Primary Human Lung Cells
Period of Performance: June 1, 2020 – May 31, 2021

Background:
The molecular mechanisms regulating emerging highly pathogenic human respiratory coronavirus pathogenesis are complex and include dampening of the host innate immune response allowing higher viral titers immediately following entry. Previous studies tracking the expression of interferon stimulated gene (ISG) expression in Middle East respiratory syndrome coronavirus (MERS-CoV) infected continuous human lung epithelial cells demonstrated that specific ISG expression patterns could be detected and these patterns could be used to determine how CoV were modulating the innate immune response. Epigenetic/chromatin remodeling mechanisms were found to be important regulators of host chromatin remodeling that altered host expression ISG patterns. This proposal will build on these foundational studies performed in continuous human lung cell lines and define ways that MERS-CoV regulates innate immunity in primary human lung fibroblasts and microvascular endothelial cells.

Scope:
Will analyze the fibroblast ChIP PCR samples collected in 2019 and discuss data with PNNL to determine next steps. Perform ChIP Seq analysis of MERS-CoV infected microvascular endothelial cells at a single time point for three donors. Will perform BioRad Primer PCR Pathway Arrays on MERS-CoV infected microvascular endothelial cells and fibroblasts. Drs Sheahan and Schaefer will work in close collaboration with PNNL to analyze datasets to determine how MERS-CoV regulates ISG expression in human lung microvascular endothelial cells and fibroblasts.

Deliverables:
- Evaluate project results and direction, in collaboration with PNNL
- Collaborate on experiment direction and identify alternatives for experiments
- Participate in meetings to discuss project status/progress, findings, and trouble shoot as needed
- Write and submit progress report and manuscript draft in collaboration with PNNL

From: Baric, Ralph S[rbaric@email.unc.edu]
Sent: Fri 6/5/2020 4:00:34 PM (UTC-07:00)
To: Sims, Amy C[amy.sims@pnnl.gov]
Subject: RE: TALK TO AMY?? Re: Cell Editorial Decision CELL-D-20-01383

? I didn't get any invite?

From: Sims, Amy C <amy.sims@pnnl.gov>
Sent: Friday, June 5, 2020 11:30 AM
To: Baric, Ralph S <rbaric@email.unc.edu>
Subject: Re: TALK TO AMY?? Re: Cell Editorial Decision CELL-D-20-01383

You should have it now. Let me know if I need to try again. Thank you, Amy

---

From: "Baric, Ralph S" <rbaric@email.unc.edu>
Date: Friday, June 5, 2020 at 8:29 AM
To: "Sims, Amy C" <amy.sims@pnnl.gov>
Subject: RE: TALK TO AMY?? Re: Cell Editorial Decision CELL-D-20-01383

Ok, send an invite

From: Sims, Amy C <amy.sims@pnnl.gov>
Sent: Friday, June 5, 2020 11:24 AM
To: Baric, Ralph S <rbaric@email.unc.edu>
Subject: Re: TALK TO AMY?? Re: Cell Editorial Decision CELL-D-20-01383

If you are willing to talk at 7pm eastern that would be great for me! Can you add that to your calendar please? Thank you very much! Amy

I am also going to send you a calendar invite if that helps.

From: "Baric, Ralph S" <rbaric@email.unc.edu>
Date: Friday, June 5, 2020 at 8:22 AM
To: "Sims, Amy C" <amy.sims@pnnl.gov>
Subject: RE: TALK TO AMY?? Re: Cell Editorial Decision CELL-D-20-01383

6PM or 7PM EST is best I think

From: Sims, Amy C <amy.sims@pnnl.gov>
Sent: Friday, June 5, 2020 11:14 AM
To: Baric, Ralph S <rbaric@email.unc.edu>
Subject: TALK TO AMY?? Re: Cell Editorial Decision CELL-D-20-01383

Hi Ralph,

I wasn't sure if your calendar exploded today or not. Do you have time later to talk? I can try to push my 6pm eastern meeting back if that is still the time that works best for you. Thank you, Amy

---

From: "Baric, Ralph S" <rbaric@email.unc.edu>
Date: Thursday, June 4, 2020 at 8:35 AM
To: "Sims, Amy C" <amy.sims@pnnl.gov>

Subject: RE: Cell Editorial Decision CELL-D-20-01383

Hi Amy, I was sulking regarding the review. I guess the criticisms requiring the use of another drug tareting a downstream kinase is what Nat has suggested. I can't remember the status of that data or whether we tried it in vivo or in vitro. I'll be glad to talk. Maaybe Friday around 6PM EST? ralph

---

From: Sims, Amy C <amy.sims@pnnl.gov>
Sent: Thursday, June 4, 2020 11:28 AM
To: Baric, Ralph S <rbaric@email.unc.edu>; Baric, Toni C <antoinette_baric@med.unc.edu>
Subject: FW: Cell Editorial Decision CELL-D-20-01383

Ralph,

Do you have time to discuss next steps soon? I suspect this will take no more than 15 minutes.

Thank you, Amy

From: <em.cell.0.6b95c1.abe328dd@editorialmanager.com> on behalf of Cell Editorial Office <em@editorialmanager.com>
Reply-To: Cell Editorial Office <cellms@cell.com>
Date: Thursday, May 28, 2020 at 1:59 PM
To: "Sims, Amy C" <amy.sims@pnnl.gov>
Subject: Cell Editorial Decision CELL-D-20-01383

Dr. Amy C Sims
902 Battelle Boulevard
Richland, WA 99352
UNITED STATES

CELL-D-20-01383
Unfolded Protein Response Inhibition Reduces MERS-CoV Induced Acute Lung Injury

May 28, 2020

Dear Dr. Sims,

I am enclosing the comments that the reviewers made on your paper. Unfortunately, the consensus recommendation is against publication in *Cell*. The reviewers refer to the potential timliness of the topic and interest in the approach, but cite that significant further work would be necessary, especially to demonstrate the specificity of the compound and its proposed mechanism of action in vivo. Given their comments, and the unclear relevance to non-MERS-CoV driven coronavirus pathology, we unfortunately cannot proceed further with this manuscript at Cell.

I am sorry that the outcome for this manuscript was not more positive. I hope, however, that the reviewers' comments are clear and constructive and that you will continue to consider *Cell* for future submissions.

Although we cannot offer to proceed further with this particular paper for *Cell*, the manuscript may be a stronger candidate for another journal within the Cell Press manuscript transfer system. If you are interested in learning more about transfer as an option, please contact the relevant journal directly, as each journal is

editorially independent. The link below will bring you to a page where you can select the journal to which you would like to transfer your paper (you can review each journal's aims and scopes before choosing your target).

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After your files have been transferred, you will be given an opportunity to update your materials before they are delivered to the editors at your new journal. You will be able to make any desired revisions or additions at this stage, so feel free to select a transfer option now and work on your revisions after that. For more details on the transfer process, please click here or contact the editors at your new target journal.

Please note that the above link will expire Jun 27, 2020. However, if you still wish to transfer after that period, you may submit directly to the journal of your choice and reference your original manuscript number in the cover letter.

We hope this option may be helpful for you, however if you decide not to transfer this manuscript, please click here:

Decline to Transfer

Declining to transfer will officially close out the manuscript in our system. If you do not accept or decline by Jun 27, 2020, the system will automatically decline on your behalf. If you prefer, you do have the choice of submitting this manuscript or a revised version of it to another Cell Press journal as a regular new submission, in which case you can decline the transfer.

Best wishes,
Andrew

Andrew J. Rennekamp, Ph.D.
Scientific Editor, *Cell*

Reviewers' Comments:

Reviewer #1:

This manuscript by Sims and co-workers describes the results of an unbiased, systems biology-based approach that identifies the unfolded protein response (UPR) as differentially modulated during MERS-CoV infection of primary cells. They used donor matched primary human lung microvascular endothelial cells (MVEs) and fibroblasts (FB), and found that only MVEs exhibited cytopathic effect in response to infection with MERS-CoV. To determine if a specific pathway was responsible for the differential outcome in MVEs versus FBs, they compared the transcriptomes, proteomes, and lipidomes of MERS-CoV-infected cells over time, and repeated this experiment using 3 different donors of the cells. This analysis revealed that the MERS-CoV-infected MVEs were dying via an Unfolded Protein Response (UPR) mediated apoptosis. They validated the importance of the UPR pathway for MERS-CoV replication-induced cell death in MEVs by addition of UPR pathway inhibitor (AMG44), which reduced replication and cell death. Importantly, they also demonstrated that drug targeting of this pathway ameliorated the outcome of MERS-CoV infection in mice (reduced weight loss and improved respiratory function). Overall, this systems biology approach allowed them to identifying a targeted antiviral therapy that reduced morbidity and mortality in MERS-CoV-infected mice. The manuscript is well written and the experiments are clearly presented. Appropriate statistical analysis is provided for each experiment. The results of this study document an approach for identifying targeted antiviral therapy for coronavirus infection. This study has important implications for developing therapies for the current pandemic. Comments for the authors' consideration (minor issues):

1. Legend to Fig 2. Is it least significant score or most significant score?
2. Legend to Fig 3. MERS-CoV MOI?
3. Title to Fig 4: Protein markers suggest activation of UPR in MVEs.
4. Legend to Fig 5. MVEs were infected with MERS-nanoluc (MOI=?)
5. Line 344: cite Gosert et al., 2002 for identification of DMVs
6. Lines 486-493: Was this MERS-nanoluc virus?
7. Lines 486-493: AMG44 is referenced to two other papers, but better to name the commercial source.
8. Do your results implicate other UPR modulators such as GSK2606414 and KIRA6 (Mahameed at al., Cell Death and Disease, 2019) as potential modulators of coronavirus replication and disease? Discussion of UPR modulators as potential therapeutics would be appropriate (line 377).
9. Line 377, start new paragraph to discuss mortality in male and female mice
10. Line 132: mothers?
11. Line 349: add recent paper Volk, Hackbart et al., 2020 J. Virol for activation of the UPR in macrophages in response to coronavirus replication.
12. Line 392: add drug names remdesivir and EIDD-2801 here

Reviewer #2: Coronavirus infection and replication is well known for its ability to stimulate an intracellular unfolded protein response (UPR). In this paper these authors take an unbiased omics-based approach to examine pathways stimulated by MERS-CoV. They identify an induction of UPR gene signature - particularly in microvascular endothelial cells (MVE) - more than fibroblasts. They find that the use of a PERK inhibitor reduces MERS-CoV replication in infected primary human lung MVE and FB and treatment with the same PERK inhibitor AMG144 in an in vivo mouse model of MERS lung disease leads to some clinical improvement. They show that it reduces lung disease and other features of disease pathogenesis - though they fail to show a reduction in MERS-CoV viral replication in infected cells. It should also be noted that PERK inhibitors are wonderfully pleiotropic.

The experiments seem to lack a certain logic? They emphasise the importance of maintaining the epithelial-endothelial cell barrier in MERS-CoV pathogenesis and I would agree that this is a critical aspect of coronavirus lung disease. They speculate that the PERK signaling pathway and the UPR response promote increased membrane reorganization and suppress host translation that helps optimise an environmental milieu for improved virus replication, maturation and egress. However, the virus is thought to enter the cells through the respiratory tissue - most likely the type II alveolar cell. What then is the rationale of looking at the effect of virus on fibroblasts and microvascular endothelial cells when the virus presumably enters through DPP4 + lung epithelial cells in the alveolus? Isn't this the cell type they should be looking at - as this is the likely cell to initiate the UPR - what is the point of looking at endothelial cells. It would seem that once the virus has infected the endothelial cell, there has already been a breakdown of the epithelial-endothelial barrier. Is there evidence of clinically important levels of viral replication in humans in endothelial cells?

Other comments

They perform transcriptomics/proteomics/lipidomics at various time points. The % cell infection is shown FIG 1D, and varies between ~1% and 30%. So the omics studies are all performed on cells at different time points with different % infected cells i.e. the number of infected fibroblasts range from ~1 - 30% max at 48hrs and the number of infected MVE cells range from ~1 - 50% max at 24hrs. From what I could gather they haven't performed any enrichment of infected cells - so the % infected cells was always low and varied at different timepoints, making an assessment and comparison of the data somewhat arbitrary.

Figure 5 Confusing presentation of data. It would be helpful to show the number of cells infected - rather than inhibition of infection - or maybe show both e.g. it's unclear to the reader if this is 10% infected or 70%?

At 48hrs with MVE there is no effect.
At 24hrs the effect is binary - no dose response curve - why is this - can this be repeated with more dose response points in the critical range?
What is negative toxicity with fibroblasts?

Line 163 "Death of MERS-CoV infected MVE could lead to permeability at the epithelial/endothelial cell barrier as one of the early steps of ARDS while viable MERS-CoV infected FB would continue to produce high levels of infectious virus both of which would contribute to the overall disease burden in the infected patient."

The in vivo mouse data shows a modest effect on weight loss - but no effect on viral titre - so I'm not sure they know why the drug is having a mild beneficial effect.

The most one could say here is that UPR inhibition has a modest improvement in disease outcome in this mouse model. Not sure one can make further conclusions - how this relates to their findings is also unclear.

Reviewer #3: Summary
In this study, Sims et al. report an important finding that a strain of coronavirus, MERS-CoV, leads to different cytopathic effects in primary human lung microvascular endothelial cells and in lung fibroblasts (MVE and FB, respectively). While MERS-CoV display similar viral replication in both cell lineages, the cytopathic effect of unfolded protein response (UPR)-mediated apoptosis is limited to MVE, but not FB. The authors subsequently tested a pharmacologic PERK kinase inhibitor compound, namely AMG 44, and further demonstrated that AMG 44 exhibits cytoprotective effect in vitro on MVE cells. Furthermore, prophylactic treatment with AMG 44 protected MERS-CoV infected mice from clinical manifestation of lung damage typically seen after MERS-CoV infection. Taken together, the exhibited phenotypes are highly interesting and potentially significant for translational use of UPR pharmacologic compounds as a potential therapy against coronavirus-mediated human diseases, which warrants investment and further investigation of underlying molecular mechanisms.
However, although the authors demonstrated in the current manuscript that MERS-CoV replication kinetics were similar among FB and MVE, and MVE cells' death is likely a result from apoptotic mechanisms accompanied by UPR activation in these primary cells, there is insufficient evidence to underpin PERK branch of the UPR as a culprit for MERS-CoV associated cell death, neither in vitro nor in vivo. Furthermore, as authors correctly pointed out that "Understanding MERS-CoV pathogenic mechanisms requires understanding critical virus-host interactions", the in vivo protective effect mediated by prophylactic AMG 44 treatment may not be as simple as a PERK-kinase inhibition effect. Usage of genetic UPR mouse models to rigorously examine the hypothesis is recommended. For consideration of publication at Cell, at least a different compound targeting a downstream node of the same PERK pathways is necessary to cross-validate the conclusion that PERK branch of the UPR promotes cell apoptosis, which is largely dependent on one "PERK inhibitor (AMG 44)" at an obviously supra-pharmacological dosage used in this manuscript.

Main Critique:
Strength:
The authors, apparently pioneered in the coronavirus research, carried out important and urgently-needed studies in an effort to find a cure for coronavirus induced human diseases, including MERS-CoV induced human lung disease. In this study, the authors infected primary lung fibroblasts derived from three matched human donors and conducted both virologic and thorough multi-omics analysis of these cells, examining the in vitro effects of MERS-CoV infection. This aspect of the manuscript is definitely both novel and necessary to promote the related research urgently needed to help address the current challenges brought by the COVID19 pandemic.

Weakness:
There is insufficient evidence to support the main conclusion that MERS-CoV induced MVE cell death is mediated by the UPR, specifically via the PERK branch, leading to cellular apoptosis. In addition, there is lack of consideration of the human immune component in the disease models in vitro by cell culture. Thirdly, the in vivo effect from prophylactic treatment with AMG 44 cannot be unequivocally attributed to PERK kinase inhibition. All drugs have off target effects.
To address the above concerns, genetic modulations of the PERK pathway signaling molecules is needed. At a minimum, other compounds targeting ER stress and/or downstream of the PERK pathway signaling molecules are absolutely necessary to support authors' main conclusion underpinning PERK kinase activity as a culprit for cell death.

Specific Comments:
1. Comparing results in Fig. 1 and 2 suggest that the preferential induction of the UPR in MVE cells is not a result of viral replication, since no difference was detected between FB and MVE in virus production (Fig. 1 A vs. B) and infection (Fig. 1D).
¬ What is rationale for studying infection in MVE and EB?
¬ This observation begs the explanation of UPR difference, since it is unlikely to be caused by an exacerbated viral protein load effect in the ER of MVE cells.
¬ A western blot quantification of ER-translated viral proteins (such as S-glycoprotein) is needed to confirm the intracellular expression of MERS-CoV component and envelop proteins. Proteomics analysis is not an ideal substitute in Fig. 4 D to F.
¬ Related to the same comment, there is an upregulation of various ER associated proteins in the proteomics data set (Fig. 2B, by red asterisks), specifically for MVEs. These proteins are usually indicative of an ER compartment expansion, but not necessarily as a result of dysregulated UPR signaling. At the same time, there are also other "membrane" components observed in the same dataset, such as "mitochondrial inner membrane" and (plasma?) "membrane". Read out of UPR should look at more proximal readouts of UPR sensor activation.
¬ The upregulation of intracellular lipids in MVEs is interesting (Fig. 2C), which may suggest either an inhibition or disruption of normal cellular lipid secretion machinery, or an upregulation of lipid de novo synthesis. It is important to distinguish the two possibilities through labeling the lipid precursor metabolites.
¬ Can differences in growth rate account for difference between the cell types? There should also be a direct readout on mitochondrial function and not simple cellular ATP.

2. In Fig. 4, reinforcing the impression by results shown in Fig. 1, there is no difference in MERS-CoV Spike protein, ORF4a protein nor the membrane protein abundance. However, in contrast to FB cells, MVE cells displayed a distinctive pattern of greatly induced ER chaperone proteins, such as BiP, GRP94 and CANX.
¬ Can you knock down PERK and show resistance to AMG44?
¬ It is important to realize that the upregulation of ER chaperones are usually cyto-protective, in response to increased ER protein synthesis burden, and should not be taken as an evidence that UPR is disproportionally activated in the MVE cells.
¬ Instead, the data suggest an alternative explanation - FB cells can fold the MERS-CoV envelop proteins more efficiently than MVE cells, and therefore require less ER chaperones to mitigate ER stress. Could there be a difference in protein glycosylation in these two cell types? S-proteins are heavily glycosylated.
¬ Chemical chaperones, namely Tauroursodeoxycholic acid (TUDCA) and 4-phenylbutyric acid (4-PBA), should be considered in these experiments to distinguish the interesting phenotypes displayed by MVE to see whether MVE cells are indeed prone to protein misfolding-induced ER stress.
¬ The authors should identify which UPR arm causes the difference between MVE and FB cells

3. On the usage of the "PERK inhibitor" - AMG 44:
It is known that AMG 44 is a "Potent and selective PERK inhibitor (IC50 = 6 nM), which "exhibits >160-fold selectivity for PERK over B-Raf and GCN2, as well as a panel of 387 other kinases. Orally bioavailable."

(For more information, see https://www.tocris.com/products/amg-perk-44_5517).
¬ Given the fact that authors needed to use at least > 10 μM of AMG 44 to achieve a MERS-CoV inhibition effect (Fig. 5), this reviewer considers whether AMG 44 inhibited other kinases in authors' experimental system. For example, two other "PERK inhibitors", GSK2606414 and GSK2656157 exhibit potent inhibitory effect on RIPK1 (doi: 10.1038/cdd.2017.58.) and AMPK.
¬ To address this concern, another PERK inhibitor such as the GSK compound should be used, at the specific "PERK inhibiting" dosage (<10 nM), to confirm PERK inhibition conferred cyto-protective effect on MVEs.
¬ MVE cells are apparently more susceptible to high doses of AMG 44 than FB, without MERS-CoV, creating a safety concern for administering AMG 44 to human patients. This safety issue should not to be taken lightly, as another PERK inhibitor, GSK2656157, exerted serious adverse effects on mouse pancreas (doi: 10.1158/0008-5472.CAN-12-3109), another grave safety concern.
¬ For a review on the toxicity profile of PERK inhibitors and their impact on physiology, please refer to the following article - Protein kinase R(PKR)-like endoplasmic reticulum kinase (PERK) inhibitors: a patent review (2010-2015) https://doi.org/10.1080/13543776.2017.1238072

4. The successful prophylactic treatment with AMG 44 in mouse models of MERS-CoV induced lung disease is the highlight of this manuscript. Although it is impossible to contribute the AMG 44 effect is specific to PERK kinase inhibition in MVE cells in vivo, the interesting preclinical observations surely hold promise for translational use of AMG 44 as a COVID19 therapy.
¬ The observation that AMG 44 does not confer protection to female mice infected with MERS-CoV is intriguing, as reviewer does not expect a sex-specific PERK difference in female mice.
¬ Could the above observation be explained by "off-target" effect of the "PERK kinase inhibitors", such as RIPK1? (doi: 10.1038/cdd.2017.58.)
¬ Given the importance of RIPK1 in mediating host versus pathogen immune response downstream of TNFR1, the therapeutic effect of AMG 44 on murine immune components should be examined in this context, for this mouse model.
¬ Nonetheless, this reviewer agrees the AMG 44 induced whole animal protection effect is both obvious and significant, regardless of the mechanism.

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From: Sheahan, Timothy Patrick[sheahan@email.unc.edu]
Sent: Sun 6/7/2020 4:36:52 AM (UTC-07:00)
To: Sims, Amy C[amy.sims@pnnl.gov]
Subject: Sketch for R21 Year 2
Attachment: 2020 Sheahan CoV Biosketch CoV Host Range.docx

Hi Amy,

See attached!
Thanks!
T

OMB No. 0925-0001 and 0925-0002 (Rev. 09/17 Approved Through 03/31/2020)

BIOGRAPHICAL SKETCH

Provide the following information for the Senior/key personnel and other significant contributors.
Follow this format for each person. **DO NOT EXCEED FIVE PAGES.**

NAME: Sheahan, Timothy Patrick

eRA COMMONS USER NAME (credential, e.g., agency login): tsheahan

POSITION TITLE: Assistant Professor

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, include postdoctoral training and residency training if applicable. Add/delete rows as necessary.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	Completion Date MM/YYYY	FIELD OF STUDY
University of New Hampshire	B.S.	06/1999	Microbiology/Water Resources
University of North Carolina at Chapel Hill	Ph.D.	05/2008	Virology
The Rockefeller University	Postdoctoral	03/2013	Systems Virology

A. Personal Statement

I have over a fifteen years of experience performing translational research focused on evaluating antiviral therapeutics and elucidating virus and host factor targets for antiviral development. Through my academic and industrial training, I have acquired a broad skillset necessary to lead this program and meet our milestones. I gained extensive knowledge of coronavirus (CoV) molecular biology, pathogenesis, vaccinology, and animal model development within which to evaluate therapeutics through my graduate research at UNC Chapel Hill with Dr. Ralph S. Baric. The goals of my graduate research were to gain a better understand of the molecular mechanisms guiding CoV zoonotic emergence and to evaluate the efficacy of vaccines and antibodies against epidemic CoV and zoonotic CoV. During my graduate career I published extensively on zoonotic CoV and therapeutics (15 publications) and the skills gained executing these studies continue to be of use today. Under the guidance of Dr. Charles M. Rice at The Rockefeller University, my postdoctoral research focused on the creation of single cell systems within which to better understand the molecular mechanisms guiding hepatitis C virus (HCV) chronic infection. During my tenure at Rockefeller, I was awarded an NIH F32 fellowship through which I gained my first experience independently guiding milestone driven research. To carry out proposed grant aims, I developed a systems virology approach coupling primary human hepatocyte cultures and laser-capture microdissection facilitating the isolation and transcriptional profiling of HCV infected cells at a resolution approaching that of a single cell. This work was also featured on Dr. Vincent Racaniello's popular podcast "This Week in Virology." After my postdoctoral fellowship, I became an investigator at the Antiviral Discovery Performance Unit at GlaxoSmithKline (GSK) based in Research Triangle Park. At GSK, I was part of several programs focused on developing and evaluating host targeting small molecules as antivirals. Through this work, I gained expertise in whole genome siRNA screens and triage of hits, antiviral assay development, and design of in vivo efficacy studies. Importantly, I became fluent in the language of preclinical drug development through interactions with experts in drug metabolism, pharmacokinetics, drug safety and toxicology. I also led a three-way public private partnership between GSK, Perkin Elmer and the University of Wisconsin at Madison to develop in vivo imaging technology that facilitated the imaging of virus replication and pulmonary inflammation in live animals. Not only did this program result in a publication on in vivo imaging techniques for influenza virus, but I also gained expertise in managing research collaborations between academia and industry which continues to be of great value. In July of 2015, I became faculty at UNC Chapel Hill in the Department of Epidemiology focusing on broad-spectrum therapeutic approaches targeting CoV with the long-term goal of developing vaccines, therapeutic antibodies and small molecules to prevent future pandemics. Through a Partnership R01 (PI Sheahan/Baric)

awarded in 2016, we have been working with Gilead Sciences and Vanderbilt School of Medicine to accelerate the preclinical development of the broad-spectrum antiviral, remdesivir, to treat MERS-CoV and related CoVs. The publications resulting from this program (see below) have in part facilitated the initiation of a clinical trial for remdesivir in China to treat nCoV-2019. My training and experience make me well suited to support this program to better understand coronavirus host range and pathogenesis.

B. Positions and Honors

Positions and Employment

1999-2001	Laboratory Technician, Harvard Gene Therapy Initiative, Harvard Medical School, Boston, MA.
2001-2003	Laboratory Technician, Tissue Engineering Laboratory of Joseph Vacanti. Massachusetts General Hospital, Boston, MA.
2003-2008	Graduate Student, Laboratory of Ralph S. Baric, University of North Carolina, Chapel Hill, NC.
2008-2014	Postdoctoral Fellow, Laboratory of Charles M. Rice, The Rockefeller University, NY, NY.
2014-2015	Investigator, Antiviral Discovery Performance Unit, GlaxoSmithKline, RTP, NC.
2015-	Assistant Professor, Department of Epidemiology, University of North Carolina, Chapel Hill, NC.

Other Experience and Professional Memberships

2002-	Member, American Society for Microbiology
2007-	Member, American Society for Virology
2019-	Member, International Society for Antiviral Research

Honors

1998	Gordon Byers Scholarship for an Outstanding Water Resources Student.
2002	Partners in Excellence Award, Massachusetts General Hospital.
2009	Ruth L. Kirschstein National Research Service Award (Postdoctoral Fellowship).
2015	Third Place Regional GSK Beautiful Biology Award. *"In vivo imaging: A new platform to accelerate drug discovery at the host/pathogen interface".*
2015	Second Place Global GSK Beautiful Biology Award. *"In vivo imaging: A new platform to accelerate drug discovery at the host/pathogen interface".*

C. Contributions to Science

1. My early work focused on the molecular mechanisms guiding zoonotic CoV emergence and viral/host determinants of SARS-CoV pathogenesis. We elucidated mutations in the SARS-CoV required to increase pathogenesis in mice and shift zoonotic CoV host range to infect human cells. The mouse models created through these studies continue to be of use today as we evaluate new therapies targeting CoV. Moreover, these studies provided the technical foundation for synthetic genome design and recovery of recombinant zoonotic CoV, which has since been repeated multiple times by the Baric Lab.
    a. Roberts A, Deming D, Paddock CD, Cheng A, Yount B, Vogel L, Herman BD, **Sheahan T**, Heise M, Genrich GL, Zaki SR, Baric R, Subbarao K. A mouse-adapted SARS-coronavirus causes disease and mortality in BALB/c mice. *PLoS Pathogens.* 2007 Jan;3(1):e5.
    b. **Sheahan T**, Rockx B, Donaldson E, Sims A, Pickles R, Corti D, Baric R. Mechanisms of Zoonotic SARS-CoV Host Range Expansion in Human Airway Epithelium. *Journal of Virology.* 2008 Mar;82(5):2274-85.
    c. **Sheahan T**, Rockx B, Donaldson E, Corti D, Baric R. Pathways of Cross Species Transmission of Synthetically Reconstructed Zoonotic SARS-CoV. *Journal of Virology.* 2008 Sep;82(17):8721-32.
    d. **Sheahan T**, Morrison T, Funkhouser W, Akira S, Heise M, Baric R. MyD88 is required for protection from lethal infection with a mouse adapted SARS-CoV. *PLoS Pathogens.* 2008 Dec;4(12):e1000240.

2. Throughout my career, I have placed a special emphasis on pursuing and performing translational research, which is summarized in select publications below. My work on HCV in primary human hepatocytes demonstrated that specific interferon lambda alleles within hepatocytes were associated with permissiveness to infection corroborating phenotypes seen clinically. For the first time, these data showed that genetic defects in hepatocytes likely guide the development of chronic HCV infection. More recently, I have been focusing on developing broad-spectrum antivirals for emerging coronavirus. We have published multiple high impact papers describing the preclinical development of the nucleoside analog, remdesivir, which are noted below. Together, these studies highlight my commitment to translational research and the development of therapeutics for emerging viral diseases.
    a. **Sheahan TP**, Imanaka N, Marukian S, Dorner M, Liu P, Ploss A, Rice CM. Interferon Lambda Alleles Predict Innate Antiviral Immune Responses and Hepatitis C Virus Permissiveness. *Cell Host and Microbe.* 2014 Feb 12;15(2):190-202.
    b. **Sheahan TP**, Sims AC, Graham RL, Menachery VD, Gralinski LE, Case JB, Leist SR, Pyrc K, Feng JY, Trantcheva I, Bannister R, Park Y, Babusis D, Clarke MO, Mackman RL, Spahn JE, Palmiotti CA, Siegel D, Ray AS, Cihlar T, Jordan R, Denison MR, Baric RS. Broad-spectrum antiviral GS-5734 inhibits both epidemic and zoonotic coronaviruses. Sci Transl Med. 2017 Jun 28;9(396).
    c. Brown AJ, Won JJ, Graham RL, Dinnon KH 3rd, Sims AC, Feng JY, Cihlar T, Denison MR, Baric RS, **Sheahan TP**. Broad spectrum antiviral remdesivir inhibits human endemic and zoonotic deltacoronaviruses with a highly divergent RNA dependent RNA polymerase. Antiviral Res. 2019 Jun 21;169:104541
    d. **Sheahan TP**, Sims AC, Leist SR, Schäfer A, Won J, Brown AJ, Montgomery SA, Hogg A, Babusis D, Clarke MO, Spahn JE, Bauer L, Sellers S, Porter D, Feng JY, Cihlar T, Jordan R, Denison MR, Baric RS. Comparative therapeutic efficacy of remdesivir and combination lopinavir, ritonavir, and interferon beta against MERS-CoV. Nat Commun. 2020 Jan 10;11(1):222.

3. New technologies facilitate advances in science by accelerating the pace of discovery and increasing observational resolution. The work below highlights how new technologies can refine and accelerate translational research. With in vivo bioluminescent imaging, virus replication can be visualized in live animals. When applied to drug efficacy studies, inhibition of virus replication can be observed instantaneously in a single animal over time thus eliminating the need for large cohorts and sacrifice of mice over time to measure virus replication. Antivirals often inhibit virus replication. Single molecule RNA fluorescence in situ hybridization (smRNA FISH) techniques facilitate the simultaneous quantitation of viral RNAs and cellular RNAs at the single cell level. When applied to drug development as in the publication below, this technique can be applied to define the stage of replication inhibited by an antiviral and help refine the mechanism of action. Lastly, we established readily translatable metrics like complete blood count at BSL3 for both SARS and MERS-CoV.
    a. Tran V, Poole DS, Jeffery JJ, **Sheahan TP**, Creech D, Yevtodiyenko A, Peat AJ, Francis KP, You S, Mehle A. Multi-Modal Imaging with a Toolbox of Influenza A Reporter Viruses. Viruses. 2015 Oct 13;7(10):5319-27.
    b. Ramanan V, Trehan K, Ong ML, Luna JM, Hoffmann HH, Espiritu C, **Sheahan TP**, Chandrasekar H, Schwartz RE, Christine KS, Rice CM, van Oudenaarden A, Bhatia SN. Viral genome imaging of hepatitis C virus to probe heterogeneous viral infection and responses to antiviral therapies. Virology. 2016 Apr 26;494:236-247. doi: 10.1016/j.virol.2016.04.020. PMID: 27128351
    c. Leist SR, Jensen KL, Baric RS, **Sheahan TP**. Increasing the translation of mouse models of MERS coronavirus pathogenesis through kinetic hematological analysis. PLoS One. 2019 Jul 24;14(7):e0220126.

Complete List of Published Work in NCBI MyBibliography:
http://www.ncbi.nlm.nih.gov/myncbi/browse/collection/40066608/?sort=date&direction=descending

D. Additional Information: Research Support and/or Scholastic Performance

Ongoing Research Support

ACTIVE:

U19 AI142759 CETR (PI: Whitley) 03/01/19-02/28/24
UAB/NIH/NIAID
Antiviral Drug Discovery and Development Center
The specific aims of the proposal will identify small molecule inhibitors of CoV replication, define their mechanism of action, and determine their efficacy against SARS-CoV, MERS-CoV and across CoV families using in vivo mouse models of acute and persistent CoV disease.
Role: Investigator

R01 AI131688-01 (PI: Rice) 03/15/17-02/281/22
Rockefeller/NIH
Analysis of immunity, viral adaptation and pathogenesis in a new mouse model of HCV-related rodent hepacivirus infection
Mechanisms that contribute to the persistence of hepatotropic viruses, such as HCV, are not well understood. We have recently established the first immune-competent mouse model of an HCV-related virus. With this new model, we propose to systematically study immunity and host-virus interactions during a hepatotropic RNA virus infection in vivo. The specific aims of this proposal are to 1) characterize the innate and adaptive responses to NrHV infection in WT mice, 2) create new NrHV pathogenesis models through the adaptation of virus in WT and immune deficient mice and through the screening of CC mouse strains for increased susceptibility. Loci driving enhanced pathogenesis and resistance will be mapped, and 3) develop new models with mouse adapted virus to better model the effects of age, obesity and alcohol on the development of liver disease.
Role: Subcontract PI

R01 AI132178-01 (MPI: Sheahan/Baric) 08/09/17-07/31/22
NIH
Broad-spectrum antiviral GS-5734 to treat MERS-CoV and related emerging CoV
In partnership with Gilead Sciences, we aim to accelerate the preclinical development of GS-5734 and promote IND licensure. We define the pharmacokinetics, pharmacodynamics, resistance profile, efficacy breadth and mechanism of action of GS-5734 against MERS-CoV and related emerging CoV.

R01AI108197-06 (MPI: Baric/Denison) 03/01/18-02/28/23
Vanderbilt/NIH
Determinants of Coronavirus Fidelity in Replication and Pathogenesis
To identify common and unique determinants of CoV nsp14-ExoN functions CoV replication, fidelity and IFN sensitivity across CoVs; To determine pathways of adaptation to loss of nsp14-ExoN activity in vitro and in vivo; and to define mechanisms of ExoN-regulated CoV sensitivity to the innate antiviral immune response.
Role: Co-Investigator

U01AI149644 (PI: Baric) 04/19/19-03/31/24
NIH
Respiratory Virus Vaccine and Adjuvant Exploration
Vaccination is one of the most effective public health measures for protecting against infectious disease, and the proposed studies will identify adjuvants and adjuvant combinations that safely elicit long lived protective immunity against emerging pathogens in at risk populations.
Role: Investigator

Completed Research Support
F32 AI 084448 Sheahan (PI) 9/1/2009 – 9/31/2012
Hepatitis C virus host interactions in micropatterned hepatocyte co-cultures. The goal of this study was to develop technology facilitating the transcriptional profiling of HCV infected primary human hepatocytes.
Role: PI