SRSF protein kinases 1 and 2 are essential host factors for human coronaviruses including SARS-CoV-2

Brook E. Heaton*[1], Joseph D. Trimarco[1], Cait E. Hamele[1], Alfred T. Harding[1], Aleksandra Tata[2], Xinyu Zhu[1], Purushothama Rao Tata[2,3,4], Clare M. Smith[1,5], and Nicholas S. Heaton[1,3,5]*

[1] Department of Molecular Genetics and Microbiology, Duke University School of Medicine, Durham, North Carolina, USA, 27710

[2] Department of Cell Biology, Duke University School of Medicine, Durham, North Carolina, USA, 27710

[3] Duke Cancer Institute, Duke University School of Medicine, Durham, NC 27710, USA

[4] Regeneration Next, Duke University, Durham, NC 27710, USA

[5] Duke Human Vaccine Institute, Duke University School of Medicine, Durham, North Carolina, USA, 27710

*to whom correspondence should be addressed

Brook E. Heaton
Research Assistant Professor
Department of Molecular Genetics and Microbiology (MGM)
Duke University School of Medicine
213 Research Drive, 427 CARL Building, Box 3054
Durham, NC 27710
Email: brook.heaton@duke.edu

Nicholas S. Heaton
Assistant Professor
Department of Molecular Genetics and Microbiology (MGM)
Duke University School of Medicine
213 Research Drive, 426 CARL Building, Box 3054
Durham, NC 27710
Email: nicholas.heaton@duke.edu

ABSTRACT

Antiviral therapeutics against SARS-CoV-2 are needed to treat the pandemic disease

COVID-19. Pharmacological targeting of a host factor required for viral replication can

suppress viral spread with a low probability of viral mutation leading to resistance. Here,

we used a genome-wide loss of function CRISPR/Cas9 screen in human lung epithelial

cells to identify potential host therapeutic targets. Validation of our screening hits

revealed that the kinase SRPK1, together with the closely related SRPK2, were jointly

essential for SARS-CoV-2 replication; inhibition of SRPK1/2 with small molecules led to

a dramatic decrease (more than 100,000-fold) in SARS-CoV-2 virus production in

immortalized and primary human lung cells. Subsequent biochemical studies revealed

that SPRK1/2 phosphorylate the viral nucleocapsid (N) protein at sites highly conserved

across human coronaviruses and, due to this conservation, even a distantly related

coronavirus was highly sensitive to an SPRK1/2 inhibitor. Together, these data suggest

that SRPK1/2-targeted therapies may be an efficacious strategy to prevent or treat

COVID-19 and other coronavirus-mediated diseases.

INTRODUCTION

In December 2019, a novel human coronavirus, now known as SARS-CoV-2, emerged

and began causing a human disease termed COVID-19[1,2]. Since then, a global pandemic

has infected millions and caused hundreds of thousands of deaths to date. Due to the

prevalence and severity of this disease, the development of therapeutic interventions is

of the highest importance. Much attention has been focused on targeting viral proteins

and their associated enzymatic activities. In particular, the virally encoded RNA-

dependent RNA polymerase (RdRp) and the viral proteases, are attractive potential

targets. Remdesivir, the only FDA-approved antiviral for SARS-CoV-2, is a nucleoside

analogue which targets the viral RdRp and causes premature termination of

transcription[3]. Efficacy of this treatment however, unfortunately, appears limited[4].

In addition to targeting viral proteins directly, other antiviral development strategies

attempt to target host factors that the virus requires to complete its lifecycle. Relative to

their hosts, viruses have dramatically less coding space in their genomes and therefore

utilize host proteins to supplement the activities of their own proteins. The major

advantages of inhibiting a virus indirectly via an essential host factor are two-fold: (1)

many viruses may utilize the same host protein, therefore host-directed therapeutics have

the potential to be broadly acting and (2) while direct targeting of the virus can rapidly

select for resistant viral mutants, it is thought to be much more difficult for a viral mutation

to overcome inhibition of a co-opted host protein.

The development of host-directed COVID-19 therapeutics has been hindered however,

due to an incomplete understanding of the host factors required for SARS-CoV-2

replication; this is in no small part due to its recent emergence into the human population.

Although a genetic screen for the host factors utilized by SARS-CoV-2 in African green

monkey cells (*Chlorocebus sp.*) was recently reported[5], we wanted to focus on the human

proteins essential for SARS-CoV-2 replication. We therefore generated a human lung

epithelial cell line (A549) modified to over-express the SARS-CoV-2 receptor ACE2 and

performed a genome wide CRISPR/Cas9 screen in BSL3 containment using the

prototype USA-WA1/2020 SARS-CoV-2 isolate. Pharmacological targeting of our top hit,

SRSF Protein Kinase 1 (SPRK1) and the highly related SRPK2, strongly inhibited the

replication of both human alpha- and beta-coronaviruses, likely due to altered

phosphorylation of the respective viral nucleocapsid proteins. Thus, not only are the

enzymatic activities of SRPK1/2 essential for the replication of multiple human

coronaviruses, but targeting these kinases may represent an effective therapeutic

approach for coronavirus mediated diseases, including COVID-19.

RESULTS

While CRISPR/Cas technology allows most known genes in the host genome to be

rapidly screened for effects on viral replication, efficient viral killing of the cells with

irrelevant genetic modifications is required.   Unfortunately, SARS-CoV-2 cytopathic

effects have been reported to be mostly restricted to cell lines derived from non-human

primates, which precludes efficient screening in human cell lines[6]. In order to overcome

this limitation (and also avoid enriching for well characterized viral entry factors), we

generated lentiviruses harboring the SARS-CoV-2 receptor ACE2, and stably transduced

a human A549 lung epithelial cell clone that had already been engineered to express

Cas9 from *Streptococcus pyogenes*. This cell line expressed ~100x more ACE2 than the

parental line, and was dramatically more permissive to SARS-CoV-2 infection.

Importantly for our screening goals, the A549-ACE2 line was efficiently killed by SARS-

CoV-2 infection as revealed by crystal violet staining (**Supplemental Figure 1**).

To perform the screen, 2×10^7 A549-Cas9-Ace2 cells (in two biological replicates) were

transduced at a multiplicity of infection (MOI) of 0.8 with lentiviruses harboring the GeCKO

V2 sgRNA library. This library contains three individual sgRNAs against most annotated

genes in the human genome[7]. Five days post-transduction, a timepoint after which

genome editing and protein turnover has occurred, half of the cells (6×10^7 cells/replicate)

were infected with SARS-CoV-2 at an MOI of 0.05 followed by multicycle viral growth for

72 hours. The remaining half of the cells were collected and used as an unselected

comparison. Most of the cells were killed by viral infection, which was expected since

most host proteins are not essential for viral replication. Those cells whose genomic

modifications rendered them refractory to viral killing however, were collected and the

sgRNAs that they harbored were sequenced via Illumina sequencing (**Figure 1A,**

Supplemental Dataset 1).

Statistical analysis of the sgRNA sequencing data, which considered sgRNA abundance,

reproducibility, and enrichment of multiple independent sgRNAs[8], indicated that the host

gene with the highest probability of being required for SARS-CoV-2 infection was the

serine/arginine-rich protein kinase, SRPK1 (**Figure 1B**). As expected from the design of

our screen, we did not enrich for ACE2, however a number of additional human genes

were also significantly enriched **(Supplemental Dataset 2)**. Rather than broadly

validating predicted screen hits, we instead chose to focus on our top hit as kinases are

generally thought to be good drug targets[9], and identifying drug targets was the initial goal

of our screen. Since a number of SARS-CoV-2 proteins have been reported to be

phosphorylated, we took a bioinformatic approach to predict potential SRPK1

phosphorylation sites[10] across the entire SARS-CoV-2 genome (**Figure 1C,**

Supplemental Table 1). The viral protein with by far the highest confidence predicted

SRPK1 sites was the viral nucleoprotein (N). Phosphoproteomic studies of SARS-CoV-

2 proteins have reported that some of the predicted SRPK1 phosphorylation sites in the

N protein are indeed phosphorylated[11-13] (**Fig 1C**). We therefore hypothesized that

SRPK1 was likely responsible for the phosphorylation of at least some sites in the N

protein.

We next wanted to define the degree to which inhibition of SRPK1 mediated N

phosphorylation would affect viral replication, especially since other non-SRPK1 kinases

have been predicted to be responsible for SARS-CoV-2 protein phosphorylation[12,13]. We

selected the SRPK1 inhibitor SPRIN340 (**Figure 1D**) as it has been reported to inhibit

SRPK1 (and SRPK2 with an ~10x higher IC50) without affecting other kinases including

the related serine/arginine-rich protein kinase family members (SRPKs)[14]. Treatment of

A549-ACE2 cells with increasing concentrations of SRPIN340 revealed significant toxicity

at a concentration of 160μM; lower concentrations however, were well tolerated (**Figure**

1E). We next infected cells treated with non-toxic concentrations of SRPIN340 with

SARS-CoV-2 and allowed multicycle viral growth for 24 hours. We observed a dramatic

SRPIN340-mediated decrease in viral RNA and infectious viral titer (up to 100,000-fold)

at the higher drug concentrations (**Figure 1F,G**).

The stronger viral inhibitory phenotype at higher concentrations of SRPIN340 suggested

that SRPK1 and SRPK2 (which are known to have similar substrate specificities[14,15]) likely

need to be inhibited simultaneously for full suppression of viral replication. To further test

that hypothesis, we made use of another small molecule (SPHINX31) that targets SRPK1

without appreciably affecting SRPK2[16]. Consistent with our previous data, while we could

significantly inhibit SARS-CoV-2 replication by targeting SRPK1 alone, the magnitude of

the effect was smaller than with SRPIN340 (**Supplemental Figure 2**). Finally, in order

to provide direct evidence that both SRPK1 and SRPK2 could indeed phosphorylate the

SARS-CoV-2 N protein, we performed *in vitro* phosphorylation assays with purified N,

SRPK1 or SRPK2, and γ 32-P ATP. In a dose dependent manner, increasing

concentrations of the kinases led to increased N protein phosphorylation (**Figure 1H,I**).

Together, these data indicate that while SRPK1 is the dominant kinase (and thus most

enriched in the screen), SRPK2 likely provides at least partial redundancy for N

phosphorylation.

Since all of our experiments had been performed in our artificially generated A549-ACE2

cell lines, we next repeated the SRPIN340 inhibitor experiments in Calu-3 cells. Calu-3

is a human lung adenocarcinoma line that is naturally permissive to SARS-CoV-2

infection (**Figure 2A**). Toxicity of SRPIN340 in Calu-3 cells was similar to A549-ACE2

cells, and as expected, SARS-CoV-2 was significantly inhibited at the higher

concentrations of SRPIN340 (**Figure 2B-E**). After SRPIN340 treatment, the viral

replication intermediate double stranded RNA (dsRNA) became un-detectable,

expression of the viral S protein was significantly reduced, and viral RNA and cell free

infectious viral particles were reduced by more than three orders of magnitude (**Figure

2C-E, Supplemental Figure 3**).

N proteins of diverse human coronaviruses have highly conserved domains[17,18], including

the predicted SRPK1 phosphorylation sites (**Figure 2F, Supplemental Table 2**). We

therefore wanted to determine if the viral requirement for SRPK1/2 activity might be

broadly conserved as well.   SRPIN340 treatment of cells infected with the

alphacoronavirus 229E (which is only distantly related to betacoronavirus SARS-CoV-2)

inhibited the virus by more than 1,000-fold at non-toxic concentrations of the drug (**Figure**

2G-H). These data indicate that the requirement for SRPK1/2 activity is not restricted to

SARS-CoV-2.

Finally, we wanted to understand the potential for targeting SARS-CoV-2 via inhibition of

SRPK1/2 in primary human lung cells.   Type-2 pneumocytes isolated from four

independent healthy human donor lungs were first expanded in organoids embedded in

Matrigel (**Figure 2I**). For viral infection studies, pneumocytes were then cultured on

transwell membranes as monolayers. These distal alveolar cells were then treated with

drug or vehicle followed by infection with SARS-CoV-2. Viral replication (as measured by

viral protein staining) was strongly inhibited and reduced almost to the level of detection

(**Figure 2J,K**). Thus, the viral requirement for SRPK1/2 is not restricted to immortalized

cell lines and their kinase activities are essential for viral replication in some of the most

vulnerable populations of lung cells affected during severe COVID-19 disease[19].

DISCUSSION

Our screen for human host targets that could be pharmacologically inhibited to treat

COVID-19 disease identified SRPK1 and SRPK2 as kinases that are critical for the

replication of coronaviruses as divergent as 229E and SARS-CoV-2. SRPK1 and 2 are

expressed in most human tissues and have been implicated in a number of basal

processes including the regulation of transcript splicing and cellular stress responses[20,21].

Further, SRPK1 is upregulated in multiple cancers including prostate, breast, lung, and

glioma[22]. In particular, it is thought that its regulation of vascular endothelial growth factor

(VEGF) is central to its importance for a number of different diseases[23]. As such, a

number of different inhibitors of SRPK1 have been developed and used in various pre-

clinical disease models, however none of these compounds have yet been FDA

approved. In addition to its normal physiological roles and potential roles in cancer,

SRPK1/2 kinase activity has been previously reported as important for the replication of

a number of different viruses.   These viruses include: hepatitis B virus, human

papillomavirus, hepatitis C virus, SARS-CoV, Ebola virus, human cytomegalovirus, and

herpes simplex virus-1[24-32]. While the mechanisms underlying how SRPK1/2 contribute

to the replication of these viruses differ, it is clear that many viruses have evolved to take

advantage of these host kinases.

One major question raised by our work is the relative contributions of different kinases to

SARS-CoV-2 viral protein phosphorylation. At least two recent reports have implicated

different kinases including: growth factor receptor (GFR) activated kinases, casein kinase

II (CK2), cyclin-dependent kinase (CDK), and protein kinase C (PKC) as generally

important, but not necessarily directly linked to N protein phosphorylation[12,13]. Previous

work with the related betacoronavirus SARS-CoV has shown that, at least in vitro, CDK,

glycogen synthase kinase (GSK), mitogen-activated protein kinase, SRPK1, and CK2 can

all phosphorylate the SARS-CoV N protein[32,33]. SRPK1 phosphorylation was reported to

affect ability of the SARS-CoV N protein to multimerize and inhibits host translation,

however any requirement for SRPK2, as well as effects on viral growth were not

reported[32]. Additionally, the growth of both SARS-CoV and mouse hepatitis virus (MHV)

have been reported to suppressed after treatment with GSK-3 inhibitors, presumably by

affecting N protein phosphorylation[34]. Further studies will be required to understand how

viral phosphorylation of SARS-CoV-2 protein affects their functions, as well as

experimentally define which kinases phosphorylate which viral proteins at which specific

locations. It will also be important to define potential kinase redundancy and the effects

on viral replication. At least for SARS-CoV-2, it appears that SRPK1/2 are central to the

regulation of viral infection, but it is worth noting that although our study has focused on

the phosphorylation of the N protein, we cannot rule out that the viral inhibition phenotype

observed is also or instead due to altered phosphorylation of other viral, or indeed host,

proteins.

Additionally, while we have provided data that SRPK1/2 inhibitors are effective in both

immortalized and primary cells, it remains unclear if targeting SRPK1/2 *in vivo* will have

a similar magnitude of effect. In support of potentially using SRPK1/2 inhibitors to treat

COVID-19 disease, several reports have utilized inhibitors of SRPK1 in animal models

and shown efficacy in suppressing phenotypes such as neovascular eye disease and

tumor growth, without apparent toxicity[16,35]. In conclusion, the development of safe and

efficacious anti-COVID-19 therapeutics is of the highest importance due to the ongoing

pandemic. Targeting of host factors essential for viral replication represents an approach

which may help to limit the emergence of viral resistance. Our study identified two host

kinases, against which inhibitors have already been developed and for which the

magnitude of inhibition of viral replication is on par with direct targeting of the viral

polymerase[36]. Continued development, optimization, and use of these kinase inhibitors

may lead to a class of host-directed antiviral therapeutics that could help combat the

current, and potentially future, coronavirus pandemics.

MATERIALS AND METHODS

<u>Cell culture.</u> All cells were obtained from ATCC and grown at 37°C in 5% CO_2. 293T and

A549 and Huh7 cells were grown in DMEM with 10% FBS, Glutamax, and

Penicillin/Streptomycin. VeroE6 cells were grown in MEM media supplemented with

Penicillin/Streptomycin, 10% FBS, 1 mM Pyruvate, and 1X MEM NEAA. Calu-3 cells

were grown in EMEM with 10% FBS and Penicillin/Streptomycin. Cells were selected in

0.5μg/ml Puromycin and 1μg/ml Blasticidin.

<u>Plasmids.</u> The ACE2-pLEX overexpression plasmid was generated by cloning a

synthesized gBlock of the ACE2 ORF (Ref. seq. NM_001371415.1) into the pLEX plasmid

via HiFi DNA Assembly (NEB Cat# M5520AA) and subsequent bacterial transformation.

Bacterial colonies were then selected and plasmid DNA isolated using the Genejet

Plasmid Miniprep kit (Thermo Fisher Cat# K0503). Plasmids were then sequenced via

Sanger sequencing to confirm successful cloning of the ACE2 ORF. Lentiviruses were

packaged as per standard protocols with a VSV-G envelope protein, A549-Cas9 cells

were transduced and selected with puromycin at 2μg/mL.

<u>SARS-CoV-2 infections and titering</u>

A stock of BEI isolate SARS-CoV-2 USA-WA1/2020 (kind gift of Greg Sempowski) was

grown on VeroE6 cells in viral growth media (MEM supplemented with 1% Pen/Strep, 2

% FBS, 1 mM Sodium Pyruvate, and 1x MEM NEAA). Infection was incubated for 1 hr

at 37℃. After infection total volume of media was brought to 30 mL. Virus was harvested

after 72 hrs of infection. To determine viral titer of stock and after drug treatment, a

monolayer of VeroE6 cells was infected with serially diluted virus for 1 hr. Virus was

removed and an agar overlay was added to each well (MEM, Penicillin/Streptomycin, 2%

FBS, 1 mM Pyruvate, 1x MEM NEAA, 0.3% Sodium Bicarbonate, Glutamax, 0.7% oxoid

agar). Plaque assays were incubated for 72 hrs then stained with either 0.1% Crystal

Violet in 10% Neutral Buffered Formalin or 0.05% Neutral Red. All viral infections took

place in cell specific media with only 2% FBS.

<u>HCoV-229E infections and titering</u>

A stock of isolate HCoV-229E VR-740 (ATCC) was grown on Huh7 cells in complete

media (DMEM supplemented with 10% FBS, 1% Pen/Strep, Glutamax). Infection was

incubated for 1hr at 37°C. After infection total volume of media was brought to 20 ml.

Virus was harvested after 36 hrs of infection. Viral titer of stock was determined via plaque

assay on Huh7 cells. A confluent monolayer of cells was infected with serial dilutions of

virus in complete media for 1 hr at 37°C. Virus was removed and an agar overlay was

added to each well (DMEM, 10% FBS, 1% Pen/Strep, Glutamax, 0.5% oxoid agar).

Plaque assays were incubated for 72 hrs then stained with 0.1% Crystal Violet in PBS.

Viral infection assays took place in complete media (DMEM, 10% FBS, 1% Pen/Strep,

Glutamax).

Quantitative Reverse Transcription PCR. Cells were resuspended in TRIzol Reagent

(Thermo Fisher Cat #15596018) and total RNA was isolated by either phase separation

with chloroform and isopropanol or using the Zymo Direct-zol RNA Miniprep kit (Zymo

Cat #R2050). One-step qRT-PCR was performed using the Invitrogen EXPRESS One-

Step Superscript qRT-PCR kit (Thermo Fisher Cat #11781200) and the commercial

Taqman probe ACE2 (Hs01085333_m1). The SARS-CoV2 primer/probe set were

synthesized using IDT DNA based on the sequences provided by the CDC "Research

Use Only 2019-Novel Coronavirus (2019-nCov) Real-time RT-PCR Primers and Probes"

set N1. For RNA extractions of 229E infected cells, RNA was extracted using NEB

Monarch total RNA miniprep kit (T2010S). To quantify viral RNA we used commercial

taqman probe CoV_229E (Vi06439671_s1). Reactions were cycled on the Applied

Biosystems QuantStudio 3 Real-Time PCR System and analyzed using QuantStudio

software version v1.4.1. RNA was normalized to the endogenous 18S primer-probe set

(Thermo Fisher Cat #4319413E).

Crystal violet staining

To visualize CPE of A549 cells expressing Ace2 or not, cells were infected with a MOI of

0.1 for 1 hr at 37℃. After 1 hr incubation 0.5 ml DMEM with 2% FBS was added to each

well and infection was allowed to continue for 72 hrs. After infection, the monolayer was

stained with 0.1% Crystal violet in 10% Neutral Buffered Formalin for 15 minutes. Stain

was then removed and cells were rinsed with ultra-pure water and then imaged.

<u>CRISPR Screening and analysis</u>

For the CRISPR screen, 2×10^7 cells per replicate were transduced with CRISPR GeCKO

Library A lenti virus at a MOI of 0.8 in 7.5 ml DMEM with DEAE dextran. After 2 days,

cells were expanded and then seeded on day 4 post-transduction in 8 T75 plates per

replicate. Cells were infected at a MOI of 0.05 for 1 hr at 37℃. 10 ml of 2% DMEM was

added to the infection and cells were placed back in the incubator for 72 hrs. After

infection, cells were trypsinized, and centrifuged for 5 minutes at 500xg. Cells were

resuspended in DMEM with 10% FBS and replated in 4 T75 flasks. Cells grew for 48hrs

and then were expanded to 8 T75 flasks. The next day, 4 flasks of cells were collected

and the pellet was resuspended in DNA/RNA shield (Zymo Research). Genomic DNA

was purified using QiaAmp Blood Maxi (Qiagen). PCR was performed using Ex Taq DNA

Polymerase (Takara Cat #RR001) on 18.5 μg of genomic DNA. PCR bands were gel

purified using the GeneJet gel extraction kit (Thermo Scientific Cat #K0832). PCR

amplicons were quantified using an Agilent Bioanalyzer and samples were then

sequenced on an Illumina MiSeq. Raw reads were extracted and analyzed using the

MAGeCK pipeline[8]. Genes were ranked by the p-value assigned by MAGeCK.

<u>SRPK inhibitor treatment assays</u>

Two different inhibitors of SRPK1 and SRPK2 were used to assay effects on viral

replication, SRPIN340 and SPHINX31 (MedChem Express). Both drugs were suspended

in DMSO at 1000X the final concentration indicated. They were then diluted to the

indicated concentrations in cell specific media. Cells were treated with drug 12 hours

before infection. Cells were then infected for 1 hour as described above. After infection,

media with 2% FBS + drug or DMSO control was added to each well (qRT-PCR and Calu-

3 microscopy) or inoculum was removed and media with 2% FBS + drug or DMSO control

was added to each well (infectious titer quantification).

<u>Cytotoxicity Assays.</u> A549-Cas9 ACE2, Calu-3, or Huh7 cells were treated with the

indicated concentrations of SRPIN-340 or SPHINX-31 in a consistent volume of vehicle

(DMSO). 48 hours after treatment, samples were processed according to the CellTiter-

Glo (Promega) protocol and luminescence was determined using a luminometer.

<u>Calu-3 Infection and immunofluorescence assays.</u> For infections of Calu-3 cells for

immunofluorescence assays, cells were treated with DMSO or 53 μM SRPIN340 12 hrs

before infection. To infect Calu-3 cells media was removed and cells were washed with

0.5 mL PBS. Virus was added at MOI 2.5 and cells were infected at 37$°$C for 1 hr. After

infection, 200 μL media + 2x SRPIN340 or DMSO was added to cells and incubated at

37$°$C for 24 hrs. To fix cells, the culture plate was submerged in 10% neutral buffered

formalin for 2 hrs. NBF was removed and cells were placed in PBS. For immunostaining

cells were permeabilized with 0.1% Triton X-100 and blocked in PBS+ 5% BSA + 0.1%

Tween-20. Cells were stained with either an anti-dsRNA antibody (J2, Sigma

MABE1134) at a 1:125 dilution or an anti-SARS-CoV-2 Spike RBD protein (ProSci #9087)

at a 1:150 dilution. Alexa fluor 488 or 594 conjugated secondary antibodies were used at

a 1:1000 dilution. DNA was stained with Hoechst 33342 dye at a 1:10,000 dilution in

PBS. Images were taken on Bio-Rad Zoe fluorescent cell imager.

<u>In vitro SRPK1/2 phosphorylation assays.</u> To produce SARS-CoV-2 N protein, a 10 cm

plate of 293T cells were transfected with 15 μg of the pLVX-EF1alpha-nCOV2019-N-

2xStrep-IRES-Puro plasmid[12] using 45 μl Trans-IT LT1. 48 hrs post-transfection, cells

were lysed with 2 ml lysis buffer (50mM Tris pH 7.4, 150mM NaCl, 1% Triton X-100, 10%

Glycerol, cOmplete, EDTA-free protease inhibitor cocktail (Roche), PhosSTOP (Roche)

phosphatase inhibitor (1 tab per 10ml buffer)). Cellular debris was removed by

centrifugation 16,000xg for 5 minutes, and lysate was transferred to 2x 2ml tubes each

with 100 μl of Dynabeads Streptavidin M-280 (ThermoFisher) that were washed 1 time

with lysis buffer. Lysates rotated for 4 hrs at 4°C then beads were collected with a

magnet. The lysate was removed, and the beads were washed 3 times with 500 μl TBS

(50mM Tris pH 7.4, 150mM NaCl, EDTA-free protease inhibitor cocktail (Roche)). The 2

tubes of beads were combined and resuspended in 300μl TBS.

For kinase assays, N-bound beads were collected, and buffer was removed. The beads

were resuspended in 300 μl Kinase buffer (50 mM Tris pH7.4, 50 mM KCl, 5 mM $MgCl_2$,

1mM DTT) and 10 μl of beads+N or beads only were placed in 1.5 ml screw cap tubes.

1 ng, 10 ng, or 100 ng of SRPK1 or SRPK2 (ThermoFisher) were added to reactions as

well as Kinase buffer to bring total volume to 95 μl. Finally, ATP, [γ-32P]- 6000Ci/mmol

10mCi/ml was diluted 1:250 in Kinase buffer and 5μl of the diluted hot ATP (0.2 μCi) was

added to each reaction. Each reaction was done in triplicate and the reactions were

incubated at 30 degrees for 60 minutes with shaking. The samples were washed 3 x with

1 ml PBS (Gibco) and radioactivity incorporated was counted on a Beckman LS 6500

scintillation counter. The samples were then resuspended in loading dye and run on

BioRad Mini-PROTEAN TGX 4-20% stain-free gels. The gels were dried and radioactivity

visualized using Carestream BIOMAX MS film. A second gel was stained with

Coomassie Brilliant Blue to visualize bead bound proteins.

<u>Bioinformatic prediction of SRPK1 phosphorylation sites.</u> Sites predicted to be

phosphorylated by SRPK1 were identified using Group-based Prediction System v5.0[10]

and the following filter: Phosphorylation/Serine/threonine kinase/CMGC/SRPK/SRPK1.

Amino acid sequences from annotated ORFs in the following genbank accession

numbers (MN985325.1, NC_004718.3, JX869059.2, KF686340.1, MN306036.1,

KF530114.1, KF514433.1) were entered individually and a threshold of "high" was

applied. Only phosphorylation sites with a prediction score greater than the threshold of

71.312 were included in further analyses.

<u>Human lung dissociation, primary type two pneumocytes purification, culturing, and</u>

<u>immunostaining.</u> Human lung tissues were washed with PBS containing 1% Antibiotic-

Antimycotic and cut into small pieces. Then samples were digested with enzyme mixture

(Collagenase type I: 1.68 mg/ml, Dispase: 5U/ml, DNase: 10U/ml) at 37°C for 1h with

rotation. The cells were filtered through a 100 μm strainer, rinsed with 15 ml

DMEM/F12+10% FBS medium and centrifuged at 450x g for 10 min. The supernatant

was removed and the cell pellet was resuspended in red blood cell lysis buffer for 10min,

washed with DMEM/F12 containing 10% FBS and filtered through a 40 µm strainer. Total

cells were centrifuged at 450xg for 5 min at 4ºC and the cell pellet was processed for AT2

purification.

For human type two pneumocytes purification, approximately 2-10 million total human

lung cells were resuspended in MACS buffer and blocked with Human TruStain FcX

(Biolegend cat# 422032) for 15min at 4ºC followed by HTII-280 antibody (Terrace Biotech,

TB-27AHT2-280) staining for 1h at 4ºC. The cells were washed twice with MACS buffer

followed by incubation with anti-mouse IgM microbeads for 15 min at 4ºC. The cells were

loaded into the LS column and collected magnetically. AT2 cells ($3x10^3$) were

resuspended in serum free medium, mixed with an equal amount of Matrigel (Corning

Cat# 354230) and plated as drops on 6 well plates. The medium was changed every three

days. Alveolospheres were passaged every 14 days. For virus infection experiments

human AT2 cells were seeded at 10×10^4 cells per insert on 5% matrigel coated Transwell

with 0.4-µm pore-sized inserts (Corning). Cells were treated with DMSO or 53 µM

SRPIN340 12 hrs before infection with media on the top and bottom of transwell. To

infect cells, media was removed from top of transwell and cells were washed with 200 µl

PBS. Cells were then infected at a MOI of 1 for 1 hr. Virus was then removed, and cells

were cultured for 24 hrs. To fix cells and remove from BSL3, plates and wells were

submerged in 10% Neutral buffered formalin for 2 hrs. NBF was removed and cells were

placed in PBS until staining.

For immunostaining of the cultures, the membrane from the transwell insert was cut

out with a scalpel and washed with PBS, permeabilized in PBST (0.1% Triton X-100 in

PBS), and incubated with blocking buffer (1% BSA in PBST) for 1h at room temperature.

Samples were incubated with primary antibodies: Prosurfactant protein C (1:500,

Milipore, cat# AB3786) and SARS-CoV-2 (1:500, Genetex cat# GTX632604) in blocking

buffer at 4°C overnight. Membranes were then washed 3 times in PBST, incubated with

secondary antibodies in blocking buffer for 1h at room temperature followed be three

washes with PBST and mounted using Fluor G reagent with DAPI. All confocal images

were collected using Olympus Confocal Microscope FV3000 using a 40X objectives.

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MAIN FIGURES AND LEGENDS

Figure 1. CRISPR screening identifies SRPK1/2 as kinases that mediate phosphorylation of the SARS-CoV-2 N protein. (a) Diagram of CRISPR GeCKO screen in A549-Cas9-Ace2 cells with SARS-CoV-2. (b) Representation of predicted hits from the CRISPR screen. Genes are arranged on x-axis alphabetically and y-axis score is inverse of the p-value for enriched genes. Genes with p-values of less than 10^{-4} were

represented with larger dots and the gene name. (c) Map of the SARS-CoV-2 genome

with potential SRPK1 phosphorylation sites indicated in graph on the top. Zoom-in of

amino acids 176-204 of the N protein that contain multiple potential SRPK1

phosphorylation sites.   # above the amino acids indicate predicted SRPK1

phosphorylation sites while the yellow circles bellow the amino acids indicate the serines

reported to be phosphorylated by both Davidson et al. and Bouhaddou et al.[11,12]. (d)

Chemical structure of SRPIN340. (e) CellTiter-GLO assay in A549-Cas9-Ace2 cells (n=6)

determining the toxicity of SRPIN340 at multiple concentrations of drug.   (f) After

treatment of A549-Cas9-Ace2 cells with different concentrations of SRPIN 340, SARS-

CoV-2 mRNA (n=4) was quantified by qRT-PCR. (g) Infectious virus was quantified (n=4)

by plaque assay 48 hrs after infection of A549-Cas9-Ace2 cells that were treated with

DMSO or 53 μM SRPIN340. (h) *In vitro* phosphorylation assay using purified SRPK1

protein and SARS-CoV-2 N protein on streptavidin beads or streptavidin beads only as

substrate. On the left CPM were quantified after phosphorylation reaction, n=3. On the

right top panel radiograph of phosphorylated N protein, on the bottom panel Coomassie

stained gel showing equal loading of N protein. (i) *In vitro* phosphorylation assay using

purified SRPK2 protein and SARS-CoV-2 N protein on streptavidin beads or streptavidin

beads only as substrate. On the left CPM were quantified after phosphorylation reaction,

n=3. On the right top panel radiograph of phosphorylated N protein, on the bottom panel

Coomassie stained gel showing equal loading of N protein.   All experiments are

representative of at least two independent biological replicates. In all panels, error bars

represent SEM, *p<0.05 **p<0.001 by an unpaired, student's t-test

Figure 2. Pharmacological inhibition of SRPK1/2 compromises the replication of multiple human coronaviruses. (a) Quantification of SARS-CoV-2 mRNA by qRT-PCR after infection of Calu-3 cells at different MOIs n=4. (b) CellTiter-Glo assay in Calu-3 cells determining the toxicity of SRPIN340 at multiple concentrations of drug n=3. (c) Calu-3 cells were uninfected or infected with SARS-CoV-2, MOI=2.5 after treatment with DMSO control or 53 μM SRPIN340. DNA was stained with Hoechst (blue) and dsRNA stained with J2 antibody (red). Scale bar represents 150 μm. (d) Calu-3 cells were treated with DMSO control or different concentrations of SRPIN340 then infected with MOI 0.005.

SARS-CoV-2 mRNA was quantified by qRT-PCR n=4. (e) Calu-3 cells were treated with

DMSO control or 53 μM SRPIN340 then infected with MOI 0.01 for 48 hrs. Infectious

virus was quantified by plaque assay n=4. (f) Diagram of the N protein of multiple

coronaviruses. Red lines within protein indicate predicted SRPK1 phosphorylation sites.

(g) Huh7 cells were treated with different concentrations of SRPIN340 and cytotoxicity

was assayed with the CellTiter-Glo assay n=4. (h) Huh7 cells were treated with DMSO

or 17 μM SRPIN340 then infected with 229E MOI 0.01. Viral RNA was quantified with

qRT-PCR n=4. (i) Diagram of primary type II pneumocyte cell culturing and infections.

(j) Primary type II pneumocytes were isolated from different donors, cultured on transwell

membranes, treated with DMSO or 53 μM SRPIN340 and then infected at MOI=1. After

24 hrs cells were fixed and stained for type II marker SFTPC (green), DAPI (blue) or

SARS-CoV-2 (red). Scale bar= 100 μm. (k) Microscopy images from (j) were quantified

and % positive cells from four independent donors were plotted, n$\geq$3. For all panels, error

bars represent SEM and *$p<0.05$ **$p<0.001$ by an unpaired, student's t-test.