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## **SRSF protein kinases 1 and 2 are essential host factors for human coronaviruses including SARS-CoV-2** — [Source link](#)

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1 **The FDA-approved drug Alectinib compromises SARS-CoV-2 nucleocapsid**  
2 **phosphorylation and inhibits viral infection in vitro**

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## 52 **ABSTRACT**

53 While vaccines are vital for preventing COVID-19 infections, it is critical to develop new  
54 therapies to treat patients who become infected. Pharmacological targeting of a host  
55 factor required for viral replication can suppress viral spread with a low probability of viral  
56 mutation leading to resistance. In particular, host kinases are highly druggable targets  
57 and a number of conserved coronavirus proteins, notably the nucleoprotein (N), require  
58 phosphorylation for full functionality. In order to understand how targeting kinases could  
59 be used to compromise viral replication, we used a combination of phosphoproteomics  
60 and bioinformatics as well as genetic and pharmacological kinase inhibition to define the  
61 enzymes important for SARS-CoV-2 N protein phosphorylation and viral replication. From  
62 these data, we propose a model whereby SRPK1/2 initiates phosphorylation of the N  
63 protein, which primes for further phosphorylation by GSK-3 $\alpha/\beta$  and CK1 to achieve  
64 extensive phosphorylation of the N protein SR-rich domain. Importantly, we were able to  
65 leverage our data to identify an FDA-approved kinase inhibitor, Alectinib, that suppresses  
66 N phosphorylation by SRPK1/2 and limits SARS-CoV-2 replication. Together, these data  
67 suggest that repurposing or developing novel host-kinase directed therapies may be an  
68 efficacious strategy to prevent or treat COVID-19 and other coronavirus-mediated  
69 diseases.

70

## 71 **INTRODUCTION**

72 In December 2019, a novel human coronavirus, now known as SARS-CoV-2, emerged  
73 and began causing a human disease termed COVID-19 (1, 2). Since then, a global  
74 pandemic has infected countless numbers of people and caused more than a million

75 deaths to date. Due to the prevalence and severity of this disease, the development of  
76 therapeutic interventions is of the highest importance. Much attention has been focused  
77 on targeting viral proteins and their associated enzymatic activities. In particular, the  
78 virally encoded RNA-dependent RNA polymerase (RdRp) and the viral proteases, are  
79 attractive potential targets. Remdesivir, the only FDA-approved antiviral for SARS-CoV-  
80 2, is a nucleoside analogue which targets the viral RdRp and causes premature  
81 termination of transcription (3). Efficacy of this treatment however, unfortunately, appears  
82 limited (4).

83

84 In addition to targeting viral proteins directly, other antiviral development strategies  
85 attempt to target host factors that the virus requires to complete its lifecycle. Relative to  
86 their hosts, viruses have dramatically less coding space in their genomes and therefore  
87 utilize the host to enable means to render virus proteins to be multifunctional. The major  
88 advantages of inhibiting a virus indirectly via an essential host factor are two-fold: (1)  
89 many viruses may utilize the same host protein, therefore host-directed therapeutics have  
90 the potential to be broadly acting and (2) while direct targeting of the virus can rapidly  
91 select for resistant viral mutants, it is thought to be much more difficult for a viral mutation  
92 to overcome inhibition of a co-opted host protein. While not all host factors are easily  
93 targetable, some enzymes such as protein kinases, for which inhibitors have been  
94 developed and tested for activity against other diseases such as cancer, are of high  
95 interest for host-directed antivirals.

96

97 In this report, we focused on defining the kinases that mediate phosphorylation of the  
98 SARS-CoV-2 nucleocapsid (N) protein, specifically its SR-rich domain, due to its high  
99 level of conservation across coronaviruses, previous data showing that it is highly  
100 phosphorylated (5-10), and also the understanding that N protein phosphorylation is  
101 important for its functionality (11-16). After performing phosphoproteomics analysis on  
102 both human and monkey cells to identify the phosphorylation sites on the N protein, we  
103 utilized high-throughput kinase substrate specificity mapping and *in vitro* phosphorylation  
104 assays to define not only which host kinases can phosphorylate the N protein, but also  
105 the order of action and the specific phosphorylation sites of each kinase. We then used  
106 both genetic knockdown and pharmacological targeting of the key kinases SRPK1/2 to  
107 verify their requirement during the replication of multiple human coronaviruses including  
108 SARS-CoV-2. Finally, we showed that several SRPK1/2 inhibitors including FDA-  
109 approved kinase inhibitor Alectinib, which can inhibit SRPK1/2 (17), can compromise  
110 SARS-CoV-2 replication in multiple cell lines and primary human pneumocytes. Thus, not  
111 only are the enzymatic activities of SRPK1/2 essential for the replication of multiple  
112 human coronaviruses via phosphorylation of the N protein, but targeting these kinases  
113 may represent an immediate and effective therapeutic strategy to combat coronavirus  
114 mediated diseases, including COVID-19.

115

## 116 **RESULTS**

117 In order to first identify the phosphorylation sites on the SARS-CoV-2 nucleocapsid (N)  
118 protein with high confidence, we infected both human A549 lung epithelial cells  
119 expressing the ACE2 receptor (A549-ACE2) and African green monkey [*Chlorocebus*

120 *sabaeus*] kidney cells (Vero E6) with SARS-CoV-2, as these lines are highly susceptible  
121 to infection by SARS-CoV-2 (18). Infected and control cells were harvested in biological  
122 triplicate followed by global proteomics and phosphoproteomics analysis using LC-MS  
123 (**Figure 1A**). Analysis of the SARS-CoV-2 N protein revealed 14 phosphorylation sites in  
124 A549-ACE2 cells, 11 of them specifically in the SR-rich domain (**Table S1**). In Vero cells,  
125 26 phosphorylation sites were detected on the N protein, 15 of them in the SR-rich domain  
126 (**Table S1**). Most of the sites in the SR-rich domain were found in both cell lines in our  
127 study, as well as in 3 previous phosphoproteomics studies (8-10) (**Figure 1B**). To  
128 investigate the evolutionary conservation of the SARS-CoV-2 N protein, we next  
129 compared the nucleocapsid proteins from 82 different coronaviruses (**Table S2**) and  
130 determined the percentage conservation of each amino acid. Interestingly, we noticed  
131 that the SR-rich domain is significantly more conserved than the linker domain, and as  
132 conserved as the two known functional domains of the N protein – the N-Terminus  
133 Domain (NTD, involved in RNA binding (19-22)) and the C-Terminus Domain (CTD,  
134 involved in protein oligomerization and RNA binding (23-25) (**Figure 1B**).

135

136 Further evolutionary examination of the SR-rich domain shows that the most conserved  
137 amino acids in that domain are serines (S), threonines (T) and arginines (R) (**Figure 1C**),  
138 indicating that these are the key residues in that domain. Together with previous evidence  
139 that the phosphorylation of the SR-rich domain is important for the life cycle of  
140 coronaviruses in general (7, 26) and SARS-CoV (5, 11, 13, 15, 27) in particular, we  
141 hypothesized that the phosphorylation of the serines and threonines in this domain is  
142 important for viral life cycle of SARS-CoV-2, and that the arginines are likely to be

143 essential for the phosphorylation to occur as well. Finally, comparison of the conservation  
144 of the detected phosphorylation sites in each domain to all the other amino acids in that  
145 domain shows that only in the SR-rich domain, the phosphorylation sites are significantly  
146 more conserved relative to the rest of the region (**Figure 1D**). These data taken together  
147 suggest that not only is the N protein highly phosphorylated, but that the phosphorylation  
148 of the SR-rich domain in particular may be functionally important for SARS-CoV-2 life  
149 cycle.

150

151 We next identified the host kinase(s) that phosphorylate the serines and threonines in the  
152 SR-domain. The ability of protein kinases to phosphorylate substrates is strongly  
153 dependent on the serine/threonine phosphoacceptor's surrounding amino acid sequence.  
154 The majority of human kinases investigated show distinct preferences for or against  
155 amino acids surrounding their phosphoacceptor. This is collectively referred to as their  
156 *substrate motifs* and is useful for identifying biological substrates. To obtain the substrate  
157 motif of a kinase, ours and other laboratories have developed an unbiased approach  
158 using combinatorial peptide substrate libraries (28-31).

159

160 Previous studies have reported that the GSK-3 and SRPK families are involved in the  
161 phosphorylation of the SR-rich domain of the nucleocapsid protein of SARS-CoV (5, 15).  
162 Recent reports have suggested these kinase families to be involved in the  
163 phosphorylation of SARS-CoV-2 N protein phosphorylation as well (32-34), however an  
164 exact phosphorylation model has never been validated experimentally. To that end, we  
165 empirically characterized the biochemical substrate specificities of GSK-3 $\alpha/\beta$  and

166 SRPK1/2/3 (**Figure 2A and Figure S1**). Consistent with previous reports (35-37), GSK-  
167 3 prefers to phosphorylate serines and threonines that have an already phosphorylated  
168 serine or threonine four residues apart toward the C-terminus (designated position +4,  
169 relative to the phosphoacceptor). This phenomenon is called *phospho-priming* – in which  
170 an already phosphorylated residue promotes phosphorylation of another proximate  
171 residue. The SR-rich domain of the N protein contains three chains of serines/threonines  
172 regularly repeating every fourth residue: S206-S186, T205-S193 and S188-S176. We  
173 therefore that the C-terminal serines/threonines of these chains (the priming sites S206,  
174 T205, and S188) are phosphorylated first, which initiates a phosphorylation cascade by  
175 GSK-3, resulting in the full phosphorylation of the entire chains.

176

177 In order to match each site to its most likely upstream kinase based on the characterized  
178 substrate specificity matrices, we computed a favorability score (see Materials and  
179 Methods) for each characterized kinase, for every phosphorylation site in the three  
180 phosphorylation chains above (**Figure 2B and Table S2**). Our algorithm predicted S206  
181 and S188 to be phosphorylated by SRPKs (SRPK1/2/3), which have strong preference  
182 for arginine at the -3 and +3 positions, serine at the -2 and +2 positions, and proline at  
183 the +1 position (**Figure 2A and Figure S1**). Once S206 and S188 are phosphorylated  
184 and thus primed, our algorithm predicted that, as expected, a GSK-3 family member  
185 (GSK-3 $\alpha/\beta$ ) would sequentially phosphorylate the chain of serine/threonine every 4  
186 residues toward the N-terminus (S202-T198-S194-S190-S186 and S184-S180-S176,  
187 respectively).

188



189 Finally, since neither the SRPK or GSK-3 families score favorably for the third priming  
190 site (T205), we considered additional kinase(s) that might carry this out. Assuming that  
191 S202 is phosphorylated by GSK-3 as discussed above, we searched for kinases with  
192 strong preference for phosphoserine or phosphothreonine at position -3 as likely kinases  
193 to phosphorylate S205. The CK1 family is a second group of phosphoprime-dependent  
194 protein kinases that favors strongly phosphoserine or phosphothreonine at position -3 and  
195 partially select for unmodified serine at the -4 position (**Figures 2A and Figure S1**). In  
196 parallel, our algorithm predicted T205 to be a likely phosphorylation site for CK1, when  
197 primed by phosphorylation at S202 (**Figure 2B and Table S2**). Subsequent  
198 phosphorylation at S201, S197 and S193 can be carried out either by CK1 or GSK-3.  
199 **Figure 2C** summarizes our proposed model for the cluster of phosphorylation sites in the  
200 SR-rich domain of the SARS-CoV-2 nucleocapsid.

201

202 Interestingly, the amino acids that are dominant in the substrate motif of SRPK (S188:  
203 R185/S186/S190/R191; S206: R203/P207/R209) are as conserved as the  
204 phosphorylated residues and across coronaviruses are more conserved than the other  
205 amino acids in that region, suggesting that those amino acids indeed play an important  
206 role in directing the appropriate protein kinases to phosphorylate this region of the N  
207 protein (**Figure 2D**).

208

209 To test our sequential phosphorylation model, recombinant SARS-CoV-2 N protein was  
210 purified and subjected to *in vitro* phosphorylation assays with recombinant SRPK1, GSK-  
211  $3\alpha$ , and CK1 $\epsilon$ . Phos-tag gel analysis of the reactions showed an upward shift of the N

212 protein band following treatment with SRPK1, indicating stoichiometric phosphorylation  
213 at one or more sites of the N protein. Adding GSK-3 or CK1 without prior treatment with  
214 SRPK1 had only modest effects on the phos-tag shift. To determine the amount of  
215 phosphate incorporation into N protein, radioactively labeled ATP was included in the  
216 phosphorylation reactions and autoradiography of N protein was measured on SDS-  
217 PAGE. Treatment with SRPK, GSK-3 and CK1 increased phosphorylation of N protein to  
218 a greater extent than the sum of the individual kinase reactions, consistent with our model  
219 where SRPK primes the SR-region for phosphorylation by GSK-3 and CK1 (**Figure 2E**).  
220 Importantly, adding all three kinases caused a major upward shift in the Phos-tag gel and  
221 a reduced detection of protein, suggesting that the highly phosphorylated protein did not  
222 efficiently enter the gel (**Figure 2E**). The phospho-null double mutant (S188A, S206A)  
223 abolished the phos-tag shift caused by SRPK and showed reduced radioactive  
224 incorporation by SRPK, GSK-3 and CK1 (**Figure 2F**). This is consistent with our model  
225 that phosphorylation of N protein at S188/S206 by SRPK is the critical priming event for  
226 extensive phosphorylation of the SR-rich domain.

227

228 We next asked how loss of SPRK proteins would affect viral replication. It has been  
229 reported that SRPK1 is expressed at higher amounts in A549 cells relative to SRPK2; we  
230 therefore targeted SRPK1 in our ACE2-A549 cells (**Figure S3**) via RNAi (**Figure 3A**).  
231 Both the kinase RNA and protein were significantly reduced after treatment (**Figure**  
232 **3B,C**), and viral RNA levels were correspondingly suppressed (**Figure 3D**). As an  
233 orthogonal approach to validate the requirement of SRPK1 during SARS-CoV-2 infection,  
234 we utilized the SRPK inhibitor SPHINX31 to block SRPK1/2 activity (38). After treatment

235 of A549-ACE2 cells with low micromolar concentrations of SPHINX31, which inhibit both  
236 SRPK1 and SRPK2, we observed inhibition of SARS-CoV2 replication (**Figure 3E,F**). We  
237 also utilized a second SRPK1/2 inhibitor, SRPIN340 (39), and as expected, treatment  
238 decreased viral RNA and infectious viral titer in a dose dependent manner at  
239 concentrations that were well tolerated by the cells (**Figure 3G,H**).

240

241 A549-ACE2 cell lines are an artificial SARS-CoV-2 infection system; we therefore  
242 repeated the SRPK1/2 inhibitor experiments with naturally infectible Calu-3 cells (**Figure**  
243 **3I**) and primary human pneumocytes. Similar to the A549-ACE2 experiments, SARS-  
244 CoV-2 infection and replication was significantly inhibited as measured by  
245 immunofluorescence for viral replication markers, viral RNA levels, and cell free infectious  
246 viral particles (**Figure 3J-N and Figure S4**).

247

248 Finally, we were interested in determining if any FDA approved kinase inhibitors could be  
249 repurposed to target the phosphorylation of the N protein and thereby inhibit SARS-CoV-  
250 2. Although there are no drugs approved to specifically inhibit SRPK1/2, it is known that  
251 the anaplastic lymphoma kinase (ALK) inhibitor Alectinib, which is used clinically to treat  
252 non-small-cell lung cancer, also causes significant inhibition of SRPK1/2 (17). We  
253 therefore treated both A549-ACE2 and Calu-3 cells with Alectinib to investigate inhibition  
254 of SARS-CoV-2 infection. Both viral RNA and infectious titer were significantly reduced  
255 in a dose dependent manner (**Figure 4A-D**).

256

257 As part of the proteomics and phosphoproteomics experiment described in Figure 1A,  
258 biological triplicates of Alectinib pretreated, infected A549-ACE2 and Vero E6 cells were  
259 also included together with the control and infected cells (**Figure 4E**). In order to verify  
260 that Alectinib was indeed affecting the phosphorylation of the SR-rich domain of the N  
261 protein, we analyzed the protein and phosphorylation levels upon treatment, in  
262 comparison to infected untreated cells. Viral protein levels were downregulated in the  
263 treated cells (**Figure S5A, Table S3**), supporting the hypothesis that Alectinib interferes  
264 with the viral life cycle. Examination of the phosphoproteomics data revealed that the vast  
265 majority of the SR-rich domain phosphorylation sites were downregulated upon Alectinib  
266 treatment, while the phosphorylation levels of sites outside the SR-rich domain did not  
267 decrease (**Figure 4F, Figure S5B, Table S1**). Additionally, we scored all the detected  
268 (host and viral) phosphorylation sites by SRPK1/2/3 substrate specificity matrices and  
269 examined the sites that were downregulated upon Alectinib treatment. The proportion of  
270 sites which score high for the SRPK family (scoring above 90<sup>th</sup> percentile) among the  
271 downregulated phosphorylation sites is significantly greater than their proportion among  
272 all measured phosphorylation sites (**Figure 4G, Figure S5C**), further confirming that  
273 Alectinib inhibits the activity of SRPKs. To demonstrate that Alectinib is capable of  
274 inhibiting viral infection outside of immortalized cell lines, we treated our primary human  
275 pneumocyte cultures with Alectinib and again observed strong inhibition of the virus  
276 (**Figure 4H,I**). These data show that viral replication can be suppressed in some of the  
277 most vulnerable populations of lung cells affected during severe COVID-19 disease (40),  
278 at least *in vitro*, by treatment with the repurposed FDA-approved kinase inhibitor Alectinib.  
279 Since the SR-rich domains of N proteins from diverse human coronaviruses are highly

280 conserved (41, 42), we next inquired whether the requirement for SRPK1/2 activity might  
281 be broadly conserved in this family of viruses. Alectinib treatment of cells infected with  
282 the alphacoronavirus 229E (which is only distantly related to betacoronavirus SARS-CoV-  
283 2) inhibited the virus by more than 1,000-fold (**Figure 4J**). These data indicate that the  
284 requirement for SRPK1/2 activity is not restricted to SARS-CoV-2 or even  
285 betacoronaviruses.

286

## 287 **DISCUSSION**

288 Our study of N protein phosphorylation led to the identification of SRPK1 and SRPK2 as  
289 kinases that are critical for the replication of coronaviruses as divergent as 229E and  
290 SARS-CoV-2. Further, we provide evidence that the phosphorylation sites in the  
291 nucleocapsid protein SR-rich domain and their surrounding sequences are also highly  
292 conserved among bat coronaviruses, suggesting that these kinases may also be  
293 targetable for pre-pandemic coronaviruses (**Figure S6**). While SRPK1 and SRPK2 are  
294 expressed in most human tissues and have been implicated in a number of basal  
295 processes including the regulation of transcript splicing, lipid metabolism and cellular  
296 stress responses (43-55), their activity has also been previously reported as important for  
297 the replication of a number of different viruses. These viruses include: hepatitis B virus,  
298 human papillomavirus, hepatitis C virus, SARS-CoV, Ebola virus, human  
299 cytomegalovirus, and herpes simplex virus-1 (15, 56-63). While the mechanisms  
300 underlying how SRPK1/2 contribute to the replication of these viruses differ, it is clear that  
301 many viruses have evolved to take advantage of these host protein kinases.

302

303 Post-translational modification of viral proteins is well understood to be important for the  
304 functionality of viral proteins, with phosphorylation chief among them (7, 64). At least two  
305 recent reports have implicated different kinases including: growth factor receptor (GFR)  
306 activated kinases, casein kinase II (CK2), cyclin-dependent kinases (CDKs), and protein  
307 kinase C (PKC), as generally important for SARS-CoV-2 replication, but not necessarily  
308 directly linked to N protein phosphorylation (8-10, 34, 65, 66). Previous work with the  
309 related betacoronavirus SARS-CoV has shown that, at least *in vitro*, CDK, GSK, mitogen-  
310 activated protein kinase (MAPK), SRPK1, and CK2 can phosphorylate the SARS-CoV N  
311 protein (15, 67). In this work, while we characterize several of the kinases that are  
312 important for phosphorylation of the SARS-CoV-2 N protein SR-rich domain, it is worth  
313 noting that other kinases might also play key roles in the phosphorylation of other N  
314 protein domains and viral proteins. Understanding the relative contributions of other  
315 potential kinases to phosphorylation of different viral proteins and indeed, different  
316 domains within the same protein, remains an important area of future study.

317

318 Future work will need to establish the functional role of N protein phosphorylation in the  
319 SARS-CoV-2 life cycle. A recent study reported that the SARS-CoV-2 N protein  
320 phosphorylation affects its protein-protein and protein-RNA interactions through phase  
321 separation regulation (16). SRPK1 phosphorylation was also reported to affect the ability  
322 of the SARS-CoV N protein to multimerize and inhibit host translation, although effects  
323 on viral growth were not reported (15). Additionally, the growth of both SARS-CoV and  
324 mouse hepatitis virus (MHV) have been reported to be suppressed after treatment with  
325 GSK-3 inhibitors, presumably at least partially by affecting N protein phosphorylation. It

326 will also be important to define potential kinase redundancy and the effects on viral  
327 replication. At least for SARS-CoV-2, it appears that SRPK1/2 are central to the regulation  
328 of viral replication. It is worth noting that while our study has focused on the  
329 phosphorylation of the SR-rich domain of the viral N protein, we cannot rule out that the  
330 viral inhibition phenotype observed may be at least partially due to altered  
331 phosphorylation of host proteins or other viral proteins. Additionally, while we have  
332 provided data that SRPK1/2 inhibitors are effective in both immortalized and primary  
333 human cells, future studies will be required to determine if targeting SRPK1/2 *in vivo* will  
334 have a similar magnitude of effect. Although not definitive, favorable outcome of COVID-  
335 19 was reported in two cases of patients with non-small cell lung cancer (NSCLC)  
336 administered with Alectinib (68, 69).

337

338 In conclusion, the identification of safe and efficacious anti-COVID-19 therapeutics is of  
339 the highest importance due to the ongoing pandemic. Targeting host factors essential for  
340 viral replication represents an approach that may help limit the emergence of viral  
341 resistance. Our study characterized the activities of several kinases for which at least one  
342 FDA-approved inhibitor already exists, can be used to suppress SARS-CoV-2 infection in  
343 a variety of *in vitro* systems, and may be repurposed for treating COVID-19. Nevertheless,  
344 while Alectinib may have some clinical utility in the short term, continued development of  
345 SRPK1/2 specific inhibitors may lead to a class of broadly acting, host-directed antiviral  
346 therapeutics that could help combat the current COVID-19, and potentially future,  
347 coronavirus pandemics.

## 348 **MATERIALS AND METHODS**

### 349 Cell culture (Duke)

350 All cells were obtained from ATCC and grown at 37°C in 5% CO<sub>2</sub>. 293T and A549 and  
351 Huh7 cells were grown in DMEM with 10% FBS, Glutamax, and Penicillin/Streptomycin.  
352 For ACE2 introduction into A549 cells, both unmodified A549 cells and A549 cells  
353 harboring Cas9 were transduced and both of the resulting lines were used for  
354 experimentation. Vero E6 cells were grown in MEM media supplemented with  
355 Penicillin/Streptomycin, 10% FBS, 1 mM Pyruvate, and 1X MEM NEAA. Calu-3 cells were  
356 grown in EMEM with 10% FBS and Penicillin/Streptomycin.

357

### 358 Cell culture (ISMMS)

359 Vero E6 cells were obtained from ATCC (CRL-1586). A549 cells stably expressing the  
360 SARS-CoV-2 receptor ACE2 were previously described (71). All cells were maintained in  
361 DMEM supplemented with 10% FBS and Penicillin/Streptomycin 37°C in 5% CO<sub>2</sub>.

362

### 363 SARS-CoV-2 infections and titering (Duke)

364 A stock of BEI isolate SARS-CoV-2 USA-WA1/2020 (kind gift of Greg Sempowski) was  
365 grown on VeroE6 cells in viral growth media (MEM supplemented with 1% Pen/Strep, 2  
366 % FBS, 1 mM Sodium Pyruvate, and 1x MEM NEAA). Infection was incubated for 1 hr at  
367 37°C. After infection total volume of media was brought to 30 mL. Virus was harvested  
368 after 72 hrs of infection. To determine viral titer of stock and after drug treatment, a  
369 monolayer of VeroE6 cells was infected with serially diluted virus for 1 hr. Virus was  
370 removed and an agar overlay was added to each well (MEM, Penicillin/Streptomycin, 2%



371 FBS, 1 mM Pyruvate, 1x MEM NEAA, 0.3% Sodium Bicarbonate, Glutamax, 0.7% oxoid  
372 agar). Plaque assays were incubated for 72 hrs then stained with either 0.1% Crystal  
373 Violet in 10% Neutral Buffered Formalin or 0.05% Neutral Red in PBS. All viral infections  
374 took place in cell specific media with only 2% FBS.

375

#### 376 HCoV-229E infections and titering

377 A stock of isolate HCoV-229E VR-740 (ATCC) was grown on Huh7 cells in complete  
378 media (DMEM supplemented with 10% FBS, 1% Pen/Strep, Glutamax). Infection was  
379 incubated for 1 hr at 37°C. After infection total volume of media was brought to 20 ml.  
380 Virus was harvested after 36 hrs of infection. Viral titer of stock was determined via plaque  
381 assay on Huh7 cells. A confluent monolayer of cells was infected with serial dilutions of  
382 virus in complete media for 1 hr at 37°C. Virus was removed and an agar overlay was  
383 added to each well (DMEM, 10% FBS, 1% Pen/Strep, Glutamax, 0.5% oxoid agar).  
384 Plaque assays were incubated for 72 hrs then stained with 0.1% Crystal Violet in PBS.  
385 Viral infection assays took place in complete media (DMEM, 10% FBS, 1% Pen/Strep,  
386 Glutamax).

387

#### 388 SARS-CoV-2 infection for phosphoproteomics (ISMMS)

389 SARS-CoV-2 isolate USA-WA1/2020 (NR-52281) was deposited by the Center for  
390 Disease Control and Prevention and obtained through BEI Resources, NIAID, NIH.  
391 SARS-CoV-2 was propagated in Vero E6 cells (ATCC, CRL-1586) in DMEM  
392 supplemented with 2% FBS, 4.5 g/L D-glucose, 4 mM L-glutamine, 10 mM Non-Essential  
393 Amino Acids, 1 mM Sodium Pyruvate and 10 mM HEPES as previously described (70).

394 Virus stocks were filtered and cleared from cytokines and other contaminating host factors  
395 by centrifugation through Amicon Ultra-15 100K Centrifugal Filter Units before use.

396 For phosphoproteomic analysis,  $6 \times 10^7$  Vero E6 or  $6 \times 10^7$  A549-ACE2 cells were treated  
397 with 5  $\mu$ M Alectinib or DMSO in DMEM supplemented with 2% FBS, 4.5 g/L D-glucose, 4  
398 mM L-glutamine, 10 mM Non-Essential Amino Acids, 1 mM Sodium Pyruvate and 10 mM  
399 HEPES for 1h at 37°C prior to infection. Cells were subsequently infected with SARS-  
400 CoV-2 at an MOI of 0.5 for 24h 37°C. After two washes with PBS and removal of all cell  
401 culture media, cell monolayers were lysed in lysis buffer containing 9 M urea, 20 mM  
402 HEPES pH 8.0, with 2X phosphatase inhibitors (Cell Signaling Technology #5870).

403

#### 404 Lysis, Digestion, and Preparation for Mass Spectrometry Analysis

405 Cultured cells were rinsed with phosphate buffer saline (PBS), and scraped into lysis  
406 buffer containing 9 M urea, 20 mM HEPES pH 8.0, with 2X phosphatase inhibitors (Cell  
407 Signaling Technology #5870). Samples were probe tip sonicated, and subsequently  
408 reduced with 5 mM DTT for 50 min at 55 °C, alkylated for 30 min with 10 mM  
409 iodoacetamide, and quenched with 5 mM DTT. Samples were diluted to 2M urea with  
410 digestion dilution buffer (20 mM pH 8.5 HEPES containing 1mM CaCl<sub>2</sub>) and digested at  
411 37 °C with 20 $\mu$ g of Lysyl Endopeptidase (Wako-Chem) overnight. Samples were then  
412 diluted to 1 M urea and digested for 5 hours with 20  $\mu$ g of trypsin (Pierce). Following  
413 digestion, peptides were acidified with trifluoroacetic acid (TFA), centrifuged at 500 x g  
414 for 20 min and purified over SepPak C18 columns. Following elution, peptides were  
415 quantified with a MicroBCA assay (Thermo Fisher Scientific, San Jose, CA).

416

#### 417 Total Protein Sample Preparation

418 50µg of peptides from each sample were labeled with isobaric tandem-mass-tag (TMT)  
419 11 plex reagents (Thermo Fisher Scientific, San Jose, CA) in 20 mM pH 8.5 HEPES with  
420 30% acetonitrile (v/v) with 250 ug of TMT reagent. The reaction was quenched for 15 min  
421 by adding hydroxylamine to a final concentration of 0.3% (v/v). Samples were combined,  
422 dried, purified over SepPak C18 columns, and dried again. Samples were then  
423 resuspended in 40 µL of basic reverse phase (bRP) buffer A (10 mM NH<sub>4</sub>HCO<sub>2</sub>, pH10,  
424 5% ACN) and separated on a Zorbax Extended C18 column (2.1 × 150 mm, 3.5 µm, no.  
425 763750-902, Agilent) using a gradient of 10-40% bRP buffer B (10 mM NH<sub>4</sub>HCO<sub>2</sub>, pH10,  
426 90% ACN). 96 fractions were collected and combined into 24 fractions for analysis. Each  
427 fraction was dried and desalted over a C18 stop and go extraction tip (STAGE-Tip) prior  
428 to analysis by mass spectrometry.

429

#### 430 IMAC Phosphopeptide Sample Preparation

431 High-Select™ Fe-NTA Phosphopeptide Enrichment Kits from Thermo were used to  
432 enrich phosphopeptides from 1mg of peptides for each sample. Following the elution from  
433 the IMAC column, enriched samples were dried, labeled with TMT, and bRP fractionated  
434 as described above instead with a gradient of 5-40% bRP buffer B. The resulting 24  
435 fractions were desalted over a C18 STAGE-Tip.

436

#### 437 LC-MS Analysis of Total Protein Fractions

438 Samples were analyzed on an Orbitrap Fusion Lumos mass spectrometer (Thermo Fisher  
439 Scientific, San Jose, CA) coupled with a Proxeon EASY-nLC 1200 liquid chromatography

440 (LC) pump (Thermo Fisher Scientific, San Jose, CA). Peptides were separated on a 100  
441  $\mu\text{m}$  inner diameter microcapillary column packed with  $\sim 40$  cm of Accucore150 resin (2.6  
442  $\mu\text{m}$ , 150  $\text{\AA}$ , ThermoFisher Scientific, San Jose, CA). For each analysis, we loaded  
443 approximately 1  $\mu\text{g}$  onto the column. Peptides were separated using a 2.5h gradient of  
444 6–30% acetonitrile in 0.125% formic acid with a flow rate of 550 nL/min. Each analysis  
445 used an SPS-MS3-based TMT method (72, 73), which has been shown to reduce ion  
446 interference compared to MS2 quantification (74). The scan sequence began with an MS1  
447 spectrum (Orbitrap analysis, resolution 60,000; 350-1400 m/z, automatic gain control  
448 (AGC) target  $4.0 \times 10^5$ , maximum injection time 50 ms). Precursors for MS2/MS3 analysis  
449 were selected using a Top10 method. MS2 analysis consisted of collision-induced  
450 dissociation (quadrupole ion trap; AGC  $2.0 \times 10^4$ ; normalized collision energy (NCE) 35;  
451 maximum injection time 120 ms). Following acquisition of each MS2 spectrum, we  
452 collected an MS3 spectrum using a method in which multiple MS2 fragment ions are  
453 captured in the MS3 precursor population using isolation waveforms with multiple  
454 frequency notches (73). MS3 precursors were fragmented by HCD and analyzed using  
455 the Orbitrap (NCE 65, AGC  $3.5 \times 10^5$ , maximum injection time 150 ms, isolation window  
456 1.2 Th, resolution was 50,000 at 200 Th).

457

#### 458 LC–MS Analysis of Phosphopeptide Enriched Fractions

459 For phosphopeptide analysis the same MS and HPLC instruments were used as  
460 described above. For each analysis approximately 500 ngs of enriched peptides were  
461 loaded on the column and run over a 120-minute gradient of 2-32% acetonitrile in 0.125%  
462 formic acid with a flow rate of 400 nL/min. MS1 spectra were collected in the Orbitrap at

463 a resolution of 60,000 with a scan range of 300-1500 m/z using a AGC target of 4.0e5  
464 with a maximum inject time of 25ms. Peptides for MS2 analysis were isolated using the  
465 quadrupole with an isolation window of 0.5 m/z. MS2 spectra were generated using  
466 Higher-energy collision dissociation (HCD) with a collision energy of 40%. Fragments  
467 were collected in the Orbitrap at a resolution of 50,000 with a first mass of 110m/z, an  
468 AGC target of 5.0e4 and a maximum injection time of 200ms.

469

#### 470 Total Protein Data Processing and Analysis

471 Mass Spectra were processed using a Comet-base software pipeline (75, 76). Resulting  
472 data were searched with a fully tryptic database containing both human Swissprot  
473 consensus entries plus isoforms downloaded Feb 2020, and SARS-CoV-2 pre-release  
474 entries from Uniprot (June 2020), allowing for a static modification of lysine and N-termini  
475 with TMT (229.1629 Da) and carbamidomethylation (57.0215 Da) of cysteine, along with  
476 variable oxidation (15.9949 Da) of methionine. Searches were performed using a 20 ppm  
477 precursor ion tolerance, the product ion tolerance was set to 1.0 Th. Peptide-spectrum  
478 matches (PSMs) were adjusted to a 1% false discovery rate (FDR) using previously  
479 described linear discriminant analysis (77, 78). Filtered PSMs were collapsed to a final  
480 protein-level FDR of < 1%. Protein assembly was guided by principles of parsimony to  
481 produce the smallest set of proteins necessary to account for all observed peptides (75).  
482 For TMT-based reporter ion quantitation, we extracted the summed signal-to-noise (S/N)  
483 ratio for each TMT channel and found the closest matching centroid to the expected mass  
484 of the TMT reporter ion. MS3 spectra with TMT reporter ion summed signal-to-noise ratios  
485 less than 100 were excluded from quantitation (79).

486

#### 487 Phosphorylation Data Processing and Analysis

488 When searching phosphorylation data, a variable modification for phosphorylation  
489 (79.9663 Da) was allowed on serine, threonine, and tyrosine. Searches were performed  
490 using a 20 ppm precursor ion tolerance, the product ion tolerance was set to 0.02 Th.  
491 Linear discriminant analysis (LDA) was performed to set a PSM FDR of < 1 %. Filtered  
492 PSMs were collapsed to a final protein-level FDR of < 1%. Phosphorylation sites were  
493 evaluated using an AScore method. Sites with an AScore > 13 were considered localized  
494 (80). PSMs with TMT reporter ion summed signal-to-noise ratios less than 100 were  
495 excluded from quantitation.

496

497 Quantification and normalization of TMT data. For each entry (protein or phosphorylation  
498 site), the level in each TMT channel was calculated as the log-transformation of the ratio  
499 to the median value of all 9 TMT channels (3 mock, 3 infected and 3 treated) of that entry.  
500 Then, every sample was median-normalized so the log-TMT-ratio is centered at zero.  
501 Phosphorylation levels were normalized by protein levels by subtracting the log-TMT-ratio  
502 of the corresponding protein from the log-TMT-ratio of the phosphorylation site.

503

504 Differential expression analysis. Differential expression analysis of proteins and  
505 phosphorylation sites was done using Limma v3.42 package in R (81). For protein levels,  
506 log-TMT-ratio was used as input data, and for phosphorylation sites – the protein-  
507 normalized log-TMT-ratio was used. Since the log-TMT-ratio is normally distributed, no  
508 voom-normalization was applied. Unequal variance between samples was taken into

509 account by using the “arrayWeight” function before fitting the model. P-values were  
510 computed using moderated t-test, and adjusted p-values (FDR) were calculated using the  
511 Benjamini–Hochberg (BH) correction. Significance for differential expression was  
512 determined as adjusted P-value<0.1.

513

514 Kinase substrate specificity assays. Reagents used for the peptide library experiments  
515 include: Kinase substrate library (Anaspec). Streptavidin conjugated membranes  
516 (Promega). All the recombinant kinases (SRPK1/2/3, GSK-3 $\alpha/\beta$  and CK1A/E were  
517 obtained from SignalChem.

518 To determine the substrate motifs, we performed *in vitro* phosphorylation assays with  
519 recombinant kinases on an oriented peptide array library of design:

520 Y-A-X<sub>-5</sub>-X<sub>-4</sub>-X<sub>-3</sub>-X<sub>-2</sub>-X<sub>-1</sub>-S<sub>0</sub>/T<sub>0</sub>-X<sub>1</sub>-X<sub>2</sub>-X<sub>3</sub>-X<sub>4</sub>-G-K-K-biotin

521 in the presence of ATP[ $\gamma$ -<sup>32</sup>P]. Reactions were carried out in their designated buffers plus  
522 20  $\mu$ M ATP and 0.4  $\mu$ Ci of (33 nM) [ $\gamma$ -<sup>32</sup>P]ATP) at 30°C for 90 min. The peptides were  
523 spotted onto streptavidin-coated filter sheets (Promega SAM<sup>2</sup> biotin capture membrane)  
524 and visualized by phosphorimaging on Typhoon FLA 7000. Detailed information on the  
525 protocol is provided elsewhere (30, 31).

526

527 Matrix processing and substrate scoring

528 The matrices were normalized by the sum of the 17 randomized amino acids (all amino  
529 acids expect for serine, threonine and cysteine), to yield a position specific scoring matrix.

530 The serine, threonine and cysteine columns were scaled by their median to be 1/17. For

531 scoring substrates, the values of the corresponding amino acids in the corresponding  
532 positions were multiplied and scaled by the probability of a random peptide:

533

$$534 \text{ Score}_{\text{Kin X}} = \frac{\prod_{\text{Pos}} P_{\text{Kin X}}(\text{AA}, \text{Position})}{(1/\#\text{Random AA})^{\text{length}(\text{positions})}}$$

535

536 For the percentile score of a substrate by a given kinase, we first computed the *a-priori*  
537 score distribution of that kinase by scoring all the reported S/T phosphorylation sites on  
538 PhosphoSitePlus (82) (downloaded on January 2020) by the method discussed above.  
539 The percentile score of a kinase-substrate pair is defined as the percentile ranking of the  
540 substrate within the score distribution of the given. This value is being used when  
541 analyzing all the detected phosphorylation sites (viral and host) for kinase enrichment.

542

543 Evolutionary conservation analysis. All reference proteomes of the Coronaviridae family  
544 of viruses available on Uniprot were downloaded (82 in total, see **Supplementary Table**  
545 **S3**). A BLAST search was performed over each proteome using the SARS-CoV-2  
546 nucleocapsid protein as the query; the top hit of each virus was taken to be the  
547 nucleocapsid. Each designated nucleocapsid was then individually aligned to the SARS-  
548 CoV-2 nucleocapsid using MUSCLE. Using these alignments, the percentage identity  
549 was then calculated for each position along the SARS-Cov-2 nucleocapsid. All statistical  
550 analyses between different conservation of positions were performed using the Mann-  
551 Whitney U test.

552



553 Kinase enrichment analysis. The phosphorylation sites detected in this study were scored  
554 by SRPK1/2/3 substrate specificity matrices and their ranks in the known  
555 phosphoproteome score distribution was determined as described above (percentile  
556 score). For every assessed kinase, phosphorylation sites that ranked within the top 10  
557 percentile within the score distribution were counted as biochemically favored sites for  
558 that kinase. Towards assessing SRPK kinase motif enrichment, we compared the  
559 percentage of biochemically favored sites in the downregulated peptides ( $\log_2$ Fold  
560 Change of  $\leq -0.75$  and below with an FDR of  $\geq 0.1$ ) to the percentage biochemically  
561 favored sites within the set of all detected sites in this study. Statistical significance was  
562 determined using Fisher's exact test.

563

564 Sequence logos. Normalized substrate specificity matrices were scaled to represent  
565 relative probability (each position sum up to 1), and probability sequence logos were  
566 generated using ggseqlogo v0.1 (83) package in R.

567

568 Plasmids. The ACE2-pLEX overexpression plasmid was generated by cloning a  
569 synthesized gBlock of the ACE2 ORF (Ref. seq. NM\_001371415.1) into the pLEX plasmid  
570 via HiFi DNA Assembly (NEB Cat# M5520AA) and subsequent bacterial transformation.  
571 Bacterial colonies were then selected and plasmid DNA isolated using the Genejet  
572 Plasmid Miniprep kit (Thermo Fisher Cat# K0503). Plasmids were then sequenced via  
573 Sanger sequencing to confirm successful cloning of the ACE2 ORF. Lentiviruses were  
574 packaged as per standard protocols with a VSV-G envelope protein, A549 or A549-Cas9  
575 cells were transduced and selected with puromycin at  $2\mu\text{g/mL}$ .

576

577

578 siRNA treatment of cells. A549-ACE2 cells were treated with 30 $\mu$ M siRNA (SRPK1:  
579 Horizon M-003982-02-0010; Non-targeting: Thermo 4390843) following the HiPerfect  
580 (Qiagen) Fast-Forward protocol and plated in 6 well plates. After 2 days, cells were  
581 trypsinized and replated in 24 well plates and re-transfected with siRNA (50 $\mu$ M) using the  
582 same protocol. After 2 additional days, cells were infected at MOI 0.005 for 1h in 250 $\mu$ l  
583 DMEM+2% FBS. After 1h of infection, 250 $\mu$ l DMEM+2% FBS was added to the inoculum  
584 and cells were incubated for 24h. 24h post-infection the media was removed and cells  
585 were lysed with 500 $\mu$ l TriZol. RNA was purified according to protocol below.

586

587 Quantitative Reverse Transcription PCR. Cells were resuspended in TRIzol Reagent  
588 (Thermo Fisher Cat #15596018) and total RNA was isolated by either phase separation  
589 with chloroform and isopropanol or using the Zymo Direct-zol RNA Miniprep kit (Zymo  
590 Cat #R2050). One-step qRT-PCR was performed using the Invitrogen EXPRESS One-  
591 Step Superscript qRT-PCR kit (Thermo Fisher Cat #11781200) and the commercial  
592 Taqman probe ACE2 (Hs01085333\_m1). The SARS-CoV2 primer/probe set were  
593 synthesized using IDT DNA based on the sequences provided by the CDC “Research  
594 Use Only 2019-Novel Coronavirus (2019-nCov) Real-time RT-PCR Primers and Probes”  
595 set N1. For qRT-PCR of SRPK1, commercial Taqman probe (Hs00177298\_m1 ) was  
596 used. For RNA extractions of 229E infected cells, RNA was extracted using NEB Monarch  
597 total RNA miniprep kit (T2010S). To quantify viral RNA we used commercial taqman  
598 probe CoV\_229E (Vi06439671\_s1). Reactions were cycled on the Applied Biosystems

599 QuantStudio 3 Real-Time PCR System and analyzed using QuantStudio software version  
600 v1.4.1. RNA was normalized to the endogenous 18S primer-probe set (Thermo Fisher  
601 Cat #4319413E).

602

603 Western blots. Cells were trypsinized, removed from plates, and pelleted via  
604 centrifugation (3,000g for 4 minutes). Cell pellets were washed once with PBS. Cells were  
605 lysed via resuspension in RIPA buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA pH 8.0, 1%  
606 Triton X-100, 0.1% sodium deoxycholate, 140 mM NaCl, 0.1% SDS) and incubation at 4  
607 °C for 15 minutes. Chromosomal DNA was sheared by passing lysates through an insulin  
608 syringe and cellular debris was removed via centrifugation (21,100g for 10 minutes at 4  
609 °C). Total protein concentration of cellular lysates was determined via Bradford assay and  
610 all samples were normalized to 1 µg/µL. Cellular lysates were separated by SDS-PAGE  
611 using 15-20 µg total cellular protein per lane (4-20% Mini-PROTEAN TGX gels (BioRad),  
612 120 V for 1 hour). Proteins were subsequently transferred to nitrocellulose membranes  
613 (90 V for 1 hour at 4 °C). Nitrocellulose membranes were blocked for at least 3 hours  
614 using PBST containing 5% milk. For detection of SRPK1, membranes were incubated  
615 with primary antibody (BD cat. no. 611072) at 0.025 µg/mL in PBST containing 5% milk  
616 overnight at 4 °C. For detection of GAPDH, membranes were incubated with primary  
617 antibody (Cell Signaling, cat. no. 2118S) at a dilution of 1:10,000 in PBST containing 5%  
618 milk overnight at 4 °C. Anti-mouse (Invitrogen cat. no. A16072) and anti-rabbit (Thermo  
619 Scientific cat. no. A16104) secondary antibodies were used at 1:20,000 and 1:10,000  
620 dilutions, respectively, in PBST containing 5% milk for 1 hour at room temperature. Blots  
621 were developed using Clarity Max ECL substrate (BioRad).

622

623 Crystal violet staining

624 To visualize CPE of A549 cells expressing ACE2 or not, cells were infected with a MOI  
625 of 0.1 for 1h at 37°C. After 1h incubation 0.5 ml DMEM with 2% FBS was added to each  
626 well and infection was allowed to continue for 72h. After infection, the monolayer was  
627 stained with 0.1% Crystal violet in 10% Neutral Buffered Formalin for 15 minutes. Stain  
628 was then removed and cells were rinsed with ultra-pure water and then imaged.

629

630 SRPK inhibitor treatment assays

631 Three different inhibitors of SRPK1 and SRPK2 were used to assay effects on viral  
632 replication, SRPIN340, SPHINX31, and Alectinib (MedChem Express). SRPIN340 and  
633 SPHINX were suspended in DMSO at 1000X the final concentration indicated, Alectinib  
634 at 500X. They were then diluted to the indicated concentrations in cell specific media.  
635 Cells were treated with drug 12 hours before infection. Cells were then infected for 1 hour  
636 as described above. After infection, media with 2% FBS + drug or DMSO control was  
637 added to each well (qRT-PCR and Calu-3 microscopy) or inoculum was removed and  
638 media with 2% FBS + drug or DMSO control was added to each well (infectious titer  
639 quantification).

640

641 Cytotoxicity Assays. A549-Cas9 ACE2, Calu-3, or Huh7 cells were treated with the  
642 indicated concentrations of SRPIN-340, SPHINX-31, or Alectinib in a consistent volume  
643 of vehicle (DMSO). 48 hours after treatment, samples were processed according to the

644 CellTiter-Glo (Promega) protocol and luminescence was determined using a  
645 luminometer.

646

647 Calu-3 Infection and immunofluorescence assays. For infections of Calu-3 cells for  
648 immunofluorescence assays, cells were treated with DMSO or 53  $\mu$ M SRPIN340 12h  
649 before infection. To infect Calu-3 cells media was removed and cells were washed with  
650 0.5 mL PBS. Virus was added at MOI 2.5 and cells were infected at 37°C for 1h. After  
651 infection, 200  $\mu$ L media + 2x SRPIN340 or DMSO was added to cells and incubated at  
652 37°C for 24h. To fix cells, the culture plate was submerged in 10% neutral buffered  
653 formalin for 2h. NBF was removed and cells were placed in PBS. For immunostaining  
654 cells were permeabilized with 0.1% Triton X-100 and blocked in PBS+ 5% BSA + 0.1%  
655 Tween-20. Cells were stained with either an anti-dsRNA antibody (J2, Sigma MABE1134)  
656 at a 1:125 dilution, or an anti-SARS-CoV-2 Spike RBD protein (ProSci #9087) at a 1:150  
657 dilution. Alexa fluor 488 or 594 conjugated secondary antibodies were used at a 1:1000  
658 dilution. DNA was stained with Hoechst 33342 dye at a 1:10,000 dilution in PBS. Images  
659 were taken on Bio-Rad Zoe fluorescent cell imager.

660

661 In vitro SRPK1/2 phosphorylation assays. 2  $\mu$ M recombinant N protein was incubated  
662 with recombinant kinases SRPK1 (80 nM), GSK-3A (5 nM), and/or CK1 $\epsilon$  (30 nM) in  
663 Kinase Buffer 1 (SignalChem) for 15 minutes at 30 degrees C. Reactions were terminated  
664 with the addition of SDS loading buffer. The reactions were run phos-tag gel and blotted  
665 with monoclonal anti-N protein antibody (GeneTex). For autoradiography, the reactions

666 were supplemented with  $\gamma$ [<sup>32</sup>P]-ATP and were run on an SDS-PAGE. The N protein bands  
667 (48 kDa) were excised and radioactivity was measured by Typhoon FLA 7000.

668

669 Human lung dissociation, primary type two pneumocytes purification, culturing, and  
670 immunostaining. Human lung tissues were washed with PBS containing 1% Antibiotic-  
671 Antimycotic and cut into small pieces. Then samples were digested with enzyme mixture  
672 (Collagenase type I: 1.68 mg/ml, Dispase: 5U/ml, DNase: 10U/ml) at 37°C for 1 h with  
673 rotation. The cells were filtered through a 100  $\mu$ m strainer, rinsed with 15 ml  
674 DMEM/F12+10% FBS medium and centrifuged at 450x g for 10 min. The supernatant  
675 was removed and the cell pellet was resuspended in red blood cell lysis buffer for 10min,  
676 washed with DMEM/F12 containing 10% FBS and filtered through a 40  $\mu$ m strainer. Total  
677 cells were centrifuged at 450xg for 5 min at 4°C and the cell pellet was processed for AT2  
678 purification.

679

680 For human type two pneumocytes purification, approximately 2-10 million total human  
681 lung cells were resuspended in MACS buffer and blocked with Human TruStain FcX  
682 (Biolegend cat# 422032) for 15min at 4°C followed by HTII-280 antibody (Terrace Biotech,  
683 TB-27AHT2-280) staining for 1 h at 4°C. The cells were washed twice with MACS buffer  
684 followed by incubation with anti-mouse IgM microbeads for 15 min at 4°C. The cells were  
685 loaded into the LS column and collected magnetically. AT2 cells ( $3 \times 10^3$ ) were  
686 resuspended in serum free medium, mixed with an equal amount of Matrigel (Corning  
687 Cat# 354230) and plated as drops on 6 well plates. The medium was changed every three  
688 days. Alveolospheres were passaged every 14 days. For virus infection experiments

689 human AT2 cells were seeded at  $10 \times 10^4$  cells per insert on 5% matrigel coated Transwell  
690 with 0.4- $\mu\text{m}$  pore-sized inserts (Corning). Cells were treated with DMSO or 53  $\mu\text{M}$   
691 SRPIN340 12h before infection with media on the top and bottom of transwell. To infect  
692 cells, media was removed from top of transwell and cells were washed with 200  $\mu\text{l}$  PBS.  
693 Cells were then infected at a MOI of 1 for 1h. Virus was then removed, and cells were  
694 cultured for 24h. To fix cells and remove from BSL3, plates and wells were submerged in  
695 10% Neutral buffered formalin for 2h. NBF was removed and cells were placed in PBS  
696 until staining.

697  
698 For immunostaining of the cultures, the membrane from the transwell insert was cut  
699 out with a scalpel and washed with PBS, permeabilized in PBST (0.1% Triton X-100 in  
700 PBS), and incubated with blocking buffer (1% BSA in PBST) for 1 h at room temperature.  
701 Samples were incubated with primary antibodies: Prosurfactant protein C (1:500,  
702 Milipore, cat# AB3786) and SARS-CoV-2 (1:500, Genetex cat# GTX632604) in blocking  
703 buffer at 4°C overnight. Membranes were then washed 3 times in PBST, incubated with  
704 secondary antibodies in blocking buffer for 1 h at room temperature followed by three  
705 washes with PBST and mounted using Fluor G reagent with DAPI. All confocal images  
706 were collected using Olympus Confocal Microscope FV3000 using a 40X objectives.

707

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723 School of Medicine at Mount Sinai.

724

## 725 **DISCLOSURES**

726 Duke University has filed for intellectual property protection regarding the use of SRPK  
727 inhibitors in the treatment of COVID-19. L.C.C. is a founder and member of the board of  
728 directors of Agios Pharmaceuticals and is a founder and receives research support from  
729 Petra Pharmaceuticals. L.C.C. is an inventor on patents (pending) for Combination  
730 Therapy for PI3K-associated Disease or Disorder, and The Identification of Therapeutic  
731 Interventions to Improve Response to PI3K Inhibitors for Cancer Treatment. L.C.C. is a  
732 co-founder and shareholder in Faeth Therapeutics. T.M.Y. is a stockholder and on the  
733 board of directors of DESTROKE, Inc., an early-stage start-up developing mobile  
734 technology for automated clinical stroke detection. O.E. is a founder and equity holder of



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736 board of Owkin, Freenome, Genetic Intelligence, Acuamark and Champions Oncology.  
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738 scientific advisory board of Miromatrix Inc and is a consultant and speaker for Alynlam  
739 Inc. P.R.T. serves as a consultant for Cellarity Inc. and Surrozen Inc. P.R.T. receives  
740 research support from United Therapeutics Inc. G.G. receives research funds from IBM  
741 and Pharmacyclics, and is an inventor on patent applications related to MuTect,  
742 ABSOLUTE, MutSig, MSMuTect, MSMutSig, MSIdetect, POLYSOLVER and TensorQTL.  
743 G.G. is a founder, consultant and holds privately held equity in Scorpion Therapeutics.

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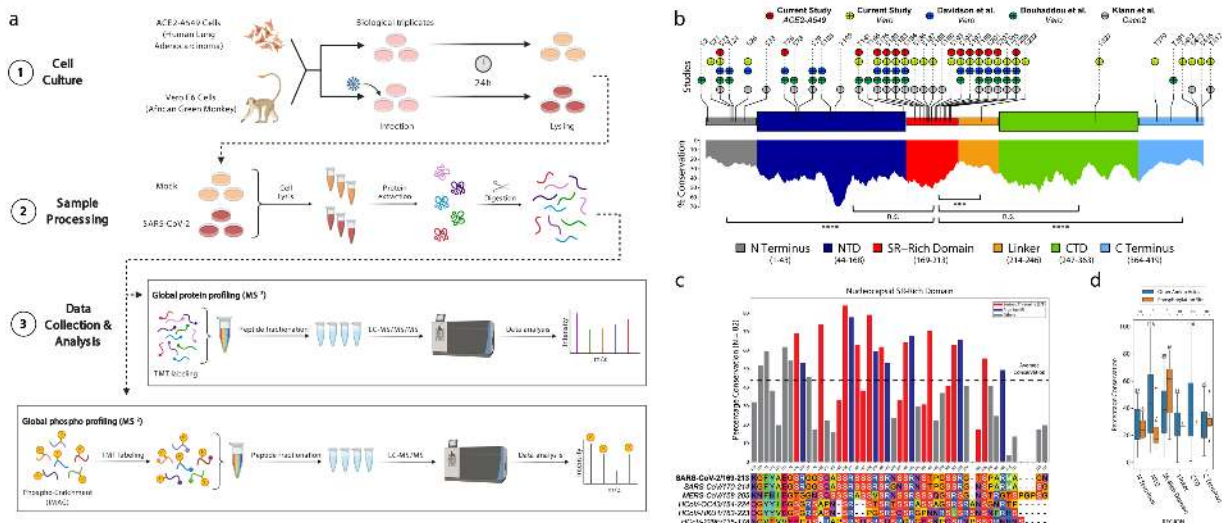
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975 **Figures and Legends**  
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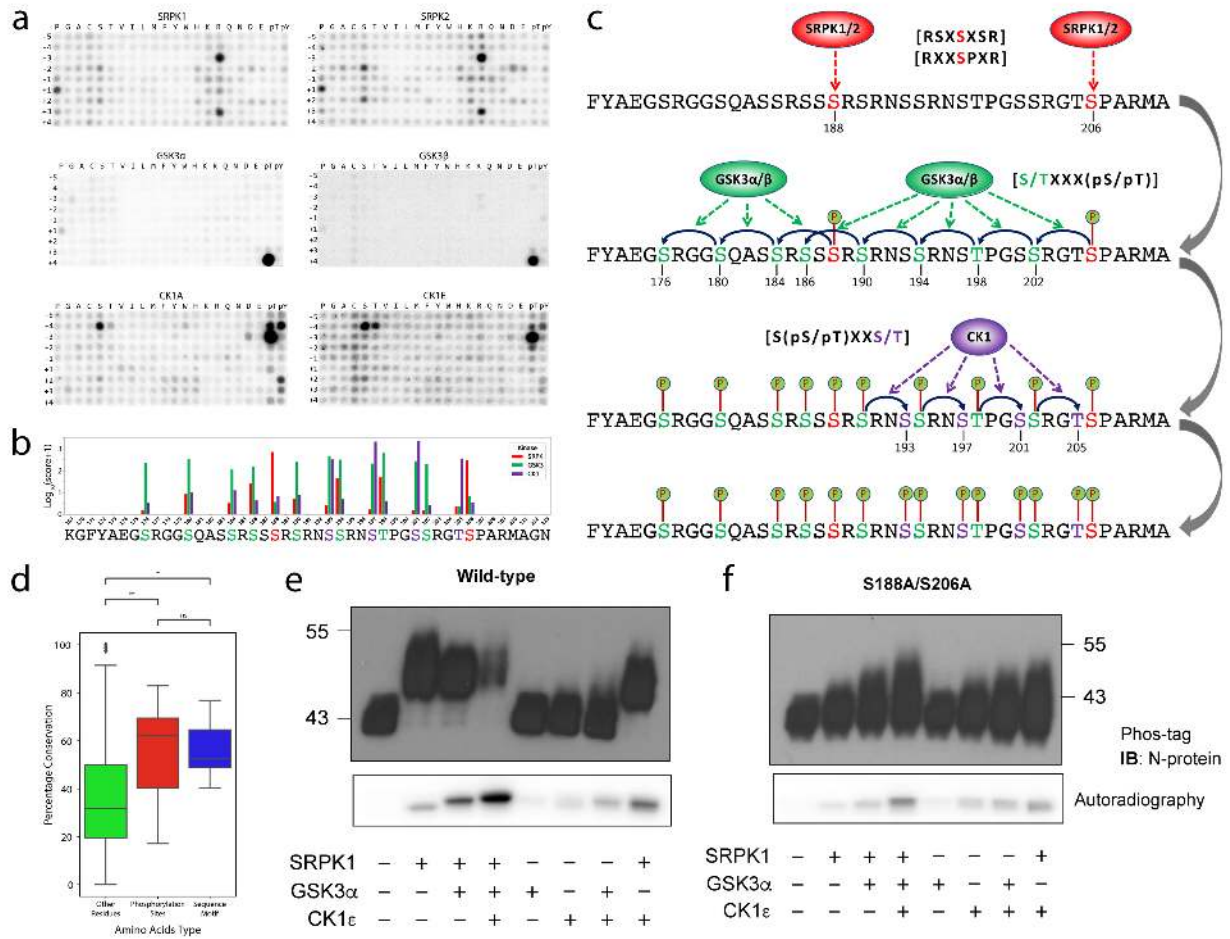


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**Figure 1. SARS-CoV-2 nucleocapsid protein is heavily phosphorylated at the SR-rich domain.**

- 980 A. Diagram of the proteomics and phosphoproteomics workflow for cells infected with  
981 SARS-CoV-2. A549-ACE2 and Vero E6 cells were infected with SARS-CoV-2 (MOI 0.5) and  
982 mock infected for 24h (in biological triplicates each). Then, cells were harvested and lysed,  
983 and proteins were cleaved into peptides using trypsin. Global protein profiling was carried  
984 out using liquid chromatography mass spectrometry (LC-MS) on an aliquot of the peptides  
985 from each sample. The rest of the peptides were enriched for phosphorylation using  
986 IMAC, and were analyzed by LC-MS as well. Peptides and phosphorylation sites were  
987 mapped to the human proteome and the SARS-CoV-2 proteins.
- 988 B. Phosphorylation sites on the SARS-CoV-2 N protein identified in 5 different  
989 phosphoproteomics analyses (two in the current study and three in previously published  
990 studies). Most of the sites at the SR-rich domain were detected in at least two  
991 independent studies (top). Evolutionary conservation analysis of the different domains of  
992 the N protein across 82 different coronaviruses shows that the SR-rich domain is more  
993 conserved than the linker domain, and is as conserved as the other two functional  
994 domains of the N protein – NTD and CTD (bottom). Mann-Whitney U test: n.s. – not  
995 significant, \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$ .
- 996 C. Alignment of the SR-rich domain of the N protein across 6 different human coronaviruses  
997 (bottom), and the percentage identity of each amino acid across 82 coronaviruses in  
998 multiple species (top). The most conserved residues are serines, threonines and arginines.  
999
- 1000 D. The conservation of the phosphorylation sites in each domain was compared to the  
1001 conservation of the other amino acids in that domain. Only in the SR-rich domain the  
1002 phosphorylation sites are significantly more conserved than the rest of the residues. The  
1003 numbers of amino acids compared in each domain are annotated above the boxes. Mann-  
1004 Whitney U test: n.s. – not significant, \* $p < 0.05$ .

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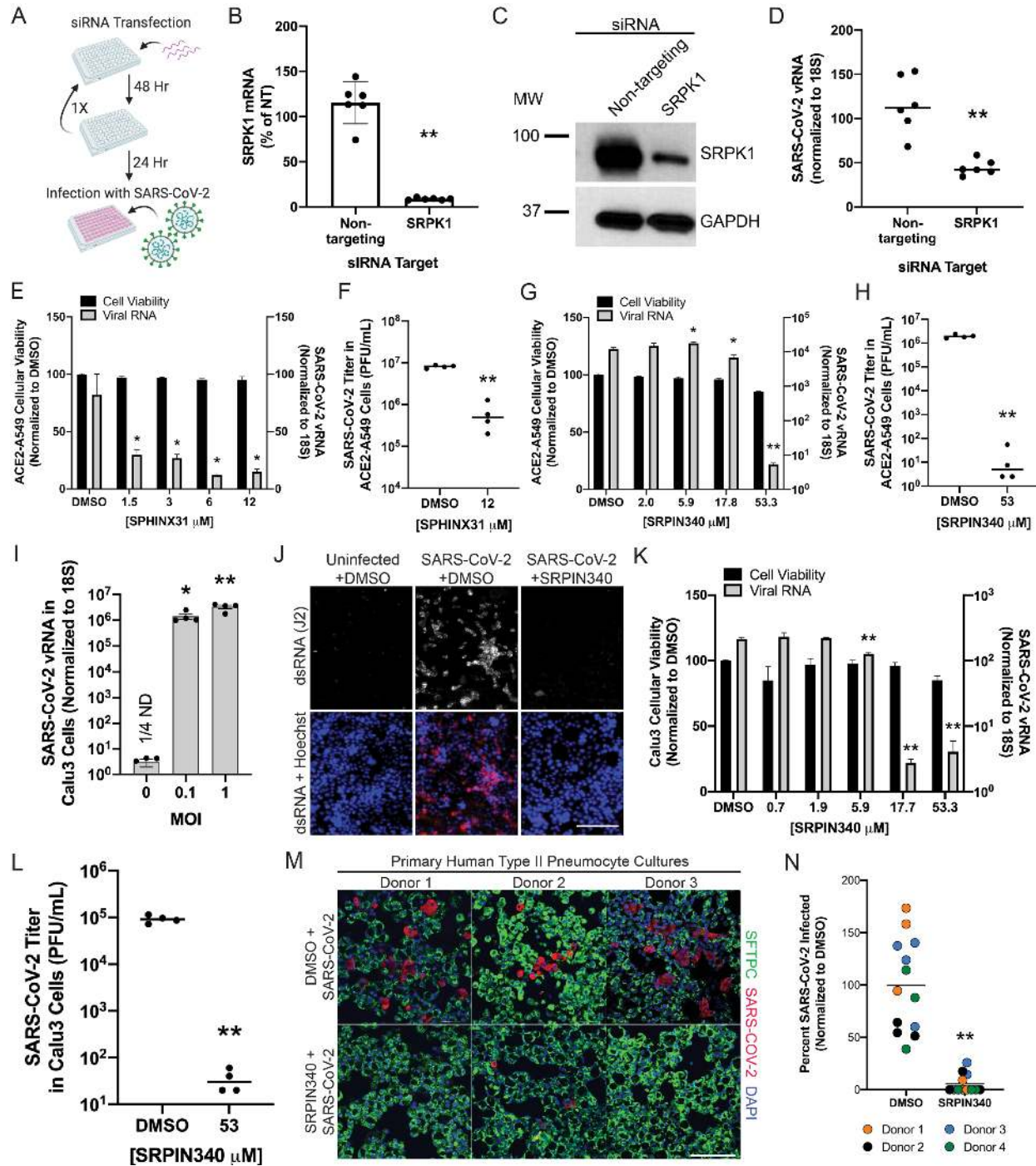


**Figure 2. Phosphorylation model of the N protein SR-rich domain by SRPK, GSK-3 and CK1.**

- A. Biochemical substrate specificity of SRPK1/2 (top), GSK-3 $\alpha/\beta$  (middle) and CK1A/G (bottom). SRPKs are selective for arginines at the -3 and +3 positions, serines at the -2 and +2 positions, and proline at the +1 position. GSK-3s are selective for phosphoserine or phosphothreonine at position +4. CK1s are selective for phosphoserine and phosphothreonine at position -3 and for serine at position -4. (see Supplementary Figure 1 for substrate specificities of additional kinases from these families).
- B. Favorability scores for the different phosphorylation sites at the SR-rich domain according to the SRPK (SRPK1/2/3), GSK-3 (GSK-3 $\alpha/\beta$ ) and CK1 (CK1A/D/ $\epsilon$ /G1) families. SRPK family scores the highest for S206 and S188, and once phosphorylated, those sites prime GSK-3 family to phosphorylate every serine or threonine located 4 positions toward the N terminus (phosphorylation chains). Finally, CK1 family scores the highest for T205 where both GSK-3 and CK1 families score high for the other sites in its corresponding phosphorylation chain.
- C. Proposed scheme for the multisite phosphorylation of the nucleocapsid SR-rich domain. Simplified substrate consensus motifs are shown here in parenthesis, whereas detailed logos are provided in Figure S2.

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- 1025 D. Evolutionary conservation across 82 different coronaviruses from multiple species shows  
1026 that the amino acids predicted to be essential for substrate specificity of the priming sites  
1027 are as conserved as the phosphorylation sites themselves, and are more conserved than  
1028 the other amino acids in the SR-rich domain. Mann-Whitney U test: n.s. – not significant,  
1029 \* $p < 0.05$ , \*\* $p < 0.01$ .
- 1030 E. Top: Immunoblot of recombinant N protein on Phos-tag gel after treatment with  
1031 recombinant kinases SRPK1, GSK-3 $\alpha$ , and/or CK1 $\epsilon$ . Bottom: SDS-PAGE/autoradiography  
1032 of recombinant N protein after treatment with SRPK1, GSK-3 $\alpha$ , and/or CK1 $\epsilon$ , in the  
1033 presence of ATP[ $\gamma$ -<sup>32</sup>P].
- 1034 F. Removing S188 and S206 reduced the phosphorylation level of the N protein. Phos-tag  
1035 gel analysis (top) and autoradiography (bottom) of recombinant N protein with the  
1036 priming phosphorylation sites mutated (S188A, S206A), performed as described in (E). For  
1037 comparison, autoradiography of the mutant and WT N proteins was measured together.  
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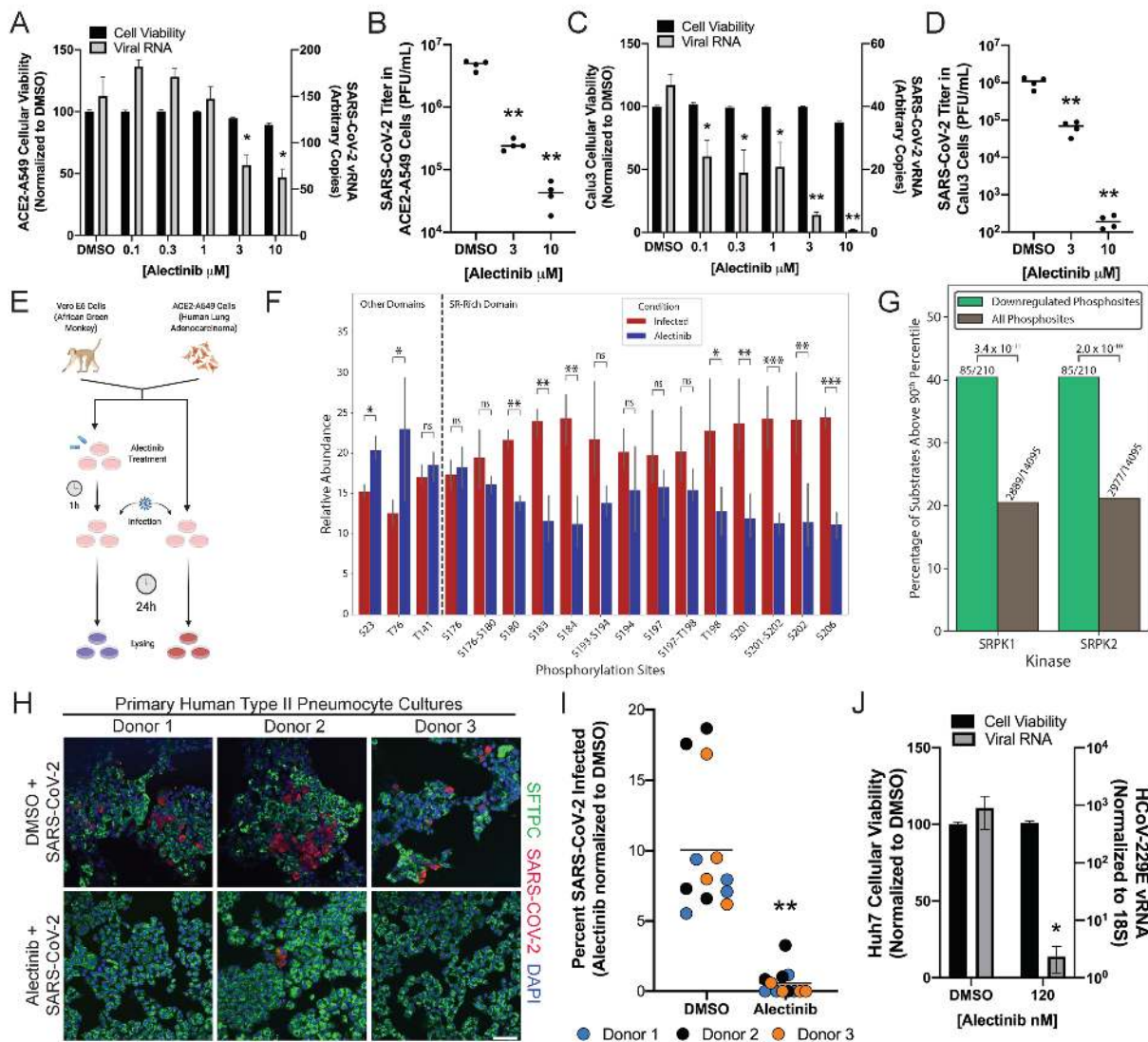


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**Figure 3. SRPK1/2 inhibitors can suppress SARS-CoV-2 infection.**

- A. Diagram of siRNA transfection. Cells were plated and treated with siRNA on day 0. After 48hrs of transfection, cells were trypsinized again and replated in 24 well plates and transfected with siRNA once again. 48 hours later, cells were infected with SARS-CoV-2.
- B. qRT-PCR shows SRPK1 mRNA in cells treated with Non-targeting or SRPK1 siRNA transfected A549-ACE2 cells.
- C. Western Blot of cells that have been treated with Non-targeting or SRPK1 siRNA.

- 1048 D. SARS-CoV-2 viral RNA was measured by qRT-PCR 24h after infection of Non-targeting or  
1049 SRPK1 siRNA transfected A549-ACE2 cells.
- 1050 E. Cellular viability was measured after A549-ACE2 cells were treated with varying  
1051 concentrations of SPHINX31 (left axis) and SARS-CoV-2 viral RNA was measured by qRT-  
1052 PCR 24h after infection of cells that had been treated with varying concentrations of  
1053 SPHINX31 (right axis).
- 1054 F. Infectious titer was measured by plaque assay of supernatants of cells that were treated  
1055 with SPHINX31 and infected for 48h.
- 1056 G. Cellular viability was measured after A549-ACE2 cells were treated with varying  
1057 concentrations of SRPIN340 (left axis) and SARS-CoV-2 viral RNA was measured by qRT-  
1058 PCR 24h after infection of cells that had been treated with varying concentrations of  
1059 SRPIN340 (right axis).
- 1060 H. Infectious titer was measured by plaque assay of supernatants of cells that were treated  
1061 with SRPIN340 and infected for 48h.
- 1062 I. Calu3 cells were infected with varying MOIs and SARS-CoV-2 viral RNA was quantified with  
1063 qRT-PCR after 24hrs of infection.
- 1064 J. Calu 3 cells treated with DMSO control or SRPIN340 were infected and after 24h post-  
1065 infection were fixed and stained for DNA with Hoechst and dsRNA using the J2 antibody.  
1066 Scale bar=150  $\mu$ m.
- 1067 K. Cellular viability was measured after Calu3 cells were treated with varying concentrations  
1068 of SRPIN340 (left axis) and SARS-CoV-2 viral RNA was measured by qRT-PCR 24h after  
1069 infection of cells that had been treated with varying concentrations of SRPIN340 (right  
1070 axis).
- 1071 L. Infectious titer was measured by plaque assay of supernatants of Calu3 cells that were  
1072 treated with SRPIN340 and infected for 48h.
- 1073 M. Primary Human Type II Pneumocyte cultures were treated with SRPIN340 for 12h pre-  
1074 infection, then infected for 24h. Cells were then fixed and stained for SARS-CoV-2, DNA  
1075 with DAPI, and Surfactant Protein C. Scale bar=100  $\mu$ m.
- 1076 N. The percent of cells SARS-CoV-2 infected were quantified using 3 Images from 4 different  
1077 human donors. For all panels \* $p$ <0.05 \*\* $p$ <0.001 by an unpaired, student's t-test.



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1080 **Figure 4. Alectinib can be repurposed to inhibit SARS-CoV-2 infection and reduce N**  
1081 **phosphorylation.**

- 1082 A. Cell viability was measured after A549-ACE2 cells were treated with varying  
1083 concentrations of Alectinib (left axis) and SARS-CoV-2 viral RNA was measured by qRT-  
1084 PCR 24h after infection of cells that had been treated with varying concentrations of  
1085 Alectinib (right axis).
- 1086 B. Infectious titer was measured by plaque assay of supernatants of cells that were treated  
1087 with Alectinib and infected for 48h.
- 1088 C. Cell viability was measured after Calu3 cells were treated with varying concentrations of  
1089 Alectinib (left axis) and SARS-CoV-2 viral RNA was measured by qRT-PCR 24h after  
1090 infection of cells that had been treated with varying concentrations of Alectinib (right  
1091 axis).

- 1092 D. Infectious titer was measured by plaque assay of supernatants of Calu3 cells that were  
1093 treated with Alectinib and infected for 48h.
- 1094 H. Diagram of Alectinib treatment and infection for proteomics and phosphoproteomics  
1095 analysis. A549-ACE2 and Vero E6 cells were pre-treated with Alectinib (5 $\mu$ M) for 1 h, then  
1096 infected with SARS-CoV-2 (MOI 0.5) and mock infected for 24h (in biological triplicates  
1097 each). Proteomics and phosphoproteomics analysis were carried out similarly to the  
1098 workflow described in Fig. 1A.
- 1099 I. Phosphorylation abundance (normalized by protein level) of the different sites on the N  
1100 protein, with and without Alectinib treatment in A549-ACE2 cells. The phosphorylation  
1101 levels of most of the SR-rich domain sites decrease upon Alectinib treatment, while  
1102 phosphorylation sites outside that domain do not change (or even increase).  
1103 P-values were computed by moderated t-test and adjusted using Benjamini-Hochberg  
1104 (BH) correction. n.s. – not significant, \*FDR<0.1, \*\*FDR <0.05, \*\*\*FDR<0.01.
- 1105 J. The subset of downregulated sites upon Alectinib treatment is enriched for sites that  
1106 score high for SRPK1 and SRPK2 (above 90<sup>th</sup> percentile) compared to their abundance  
1107 among all the detected cellular phosphorylation sites in A549-ACE2 cells. Denoted  
1108 p-values were computed using Fisher's exact test.
- 1109 K. Primary Human Type II Pneumocyte cultures were treated with Alectinib for 12h before  
1110 infection, then infected for 24h. Cells were then fixed and stained for SARS-CoV-2, DNA  
1111 with DAPI, and Surfactant Protein C. Scale bar=50 $\mu$ m.
- 1112 L. The percent of cells SARS-CoV-2 infected were quantified using 4 Images from 3 different  
1113 human donors.
- 1114 M. Cell viability was measured after HuH7 cells were treated with Alectinib (left axis) and  
1115 229E viral RNA was measured by qRT-PCR 24h after infection of cells that had been  
1116 treated with Alectinib (right axis). For all panels \*p<0.05 \*\*p<0.001 by an unpaired,  
1117 student's t-test, unless otherwise stated.