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**Effect of mutations in the SARS-CoV-2 spike protein on protein stability,  
cleavage, and cell-cell fusion function**

Chelsea T. Barrett<sup>1</sup>, Hadley E. Neal<sup>1</sup>, Kearstin Edmonds<sup>1</sup>, Carole L. Moncman<sup>1</sup>, Rachel  
Thompson<sup>1</sup>, Jean M. Branttie<sup>1</sup>, Kerri Beth Boggs<sup>1</sup>, Cheng-Yu Wu<sup>1</sup>, Daisy W. Leung<sup>2</sup>,  
and Rebecca E. Dutch<sup>1\*</sup>

<sup>1</sup>Department of Molecular and Cellular Biochemistry, University of Kentucky, Lexington,  
Kentucky, USA

<sup>2</sup>Division of Infection Diseases, Department of Medicine, Washington University School  
of Medicine in St. Louis, St. Louis, Missouri, USA

\*Address correspondence and materials requests to Rebecca E. Dutch,  
[rdutc2@uky.edu](mailto:rdutc2@uky.edu); Tel.: +1.859.323.1795

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28

29 **Abstract**

30 The SARS-CoV-2 spike protein (S) is the sole viral protein responsible for both  
31 viral binding to a host cell and the membrane fusion event needed for cell entry. In  
32 addition to facilitating fusion needed for viral entry, S can also drive cell-cell fusion, a  
33 pathogenic effect observed in the lungs of SARS-CoV-2 infected patients. While several  
34 studies have investigated S requirements involved in viral particle entry, examination of  
35 S stability and factors involved in S cell-cell fusion remain limited. We demonstrate that  
36 S must be processed at the S1/S2 border in order to mediate cell-cell fusion, and that  
37 mutations at potential cleavage sites within the S2 subunit alter S processing at the  
38 S1/S2 border, thus preventing cell-cell fusion. We also identify residues within the  
39 internal fusion peptide and the cytoplasmic tail that modulate S cell-cell fusion.  
40 Additionally, we examine S stability and protein cleavage kinetics in a variety of  
41 mammalian cell lines, including a bat cell line related to the likely reservoir species for  
42 SARS-CoV-2, and provide evidence that proteolytic processing alters the stability of the  
43 S trimer. This work therefore offers insight into S stability, proteolytic processing, and  
44 factors that mediate S cell-cell fusion, all of which help give a more comprehensive  
45 understanding of this highly sought-after therapeutic target.

46

## 47 **Introduction**

48           Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is the causative  
49 viral agent of the ongoing coronavirus disease of 2019 (COVID-19) global pandemic.  
50 Thus far, COVID-19 has impacted over 86 million people globally, resulting in the death  
51 of more than one and a half million individuals [1]. Due to the widespread global impact  
52 of this pandemic, a concerted effort has been made to rapidly develop a vaccine or  
53 antiviral treatment.

54           The SARS-CoV-2 spike (S) protein is the major transmembrane glycoprotein  
55 studding the surface of the viral particle, and is exclusively responsible for viral  
56 attachment and cell entry, thus making it the major target of current vaccine strategies  
57 and antiviral therapeutics [2]. The S protein consists of two distinct subunits: the S1  
58 subunit, which binds to the known host receptor, angiotensin converting enzyme 2  
59 (ACE2) [3-11], and the S2 subunit that promotes the viral-to-host cell membrane fusion  
60 event needed for viral infection [2, 8, 12-18]. Most known coronavirus (CoV) S proteins  
61 undergo two post-translational proteolytic cleavage events, one at the border of the S1  
62 and S2 subunits, and one downstream within the S2 subunit (termed S2') [2, 13, 15-21].

63           Similar to several other CoVs, SARS-CoV-2 likely utilizes bats as a reservoir  
64 species, specifically *Rhinolophus affinis* or horseshoe bats [11, 22-25]. SARS-CoV-2  
65 has 96% sequence identity to a CoV found in this bat population, RaTG13, with limited  
66 differences between them [25]. One difference is the polybasic, PRRA, insertion at the  
67 S1/S2 border which gives this site the canonical sequence requirements for cleavage by  
68 the cellular proprotein convertase furin [26-29]. This change may be a key factor in the  
69 zoonotic transmission of SARS-CoV-2. The presence of a furin consensus sequence at

70 the cleavage site has been observed in other human infecting CoVs [26, 30-32],  
71 including highly pathogenic forms of influenza [33, 34] and previous studies have  
72 demonstrated its functional significance. For SARS-CoV-2, the insertion is suggested to  
73 allow for expanded cellular tropism and infectivity [13, 26, 35, 36]. For most CoVs,  
74 cleavage at a downstream S2' site may be carried out by a number of cellular  
75 proteases, including serine proteases like transmembrane serine protease 2  
76 (TMPRSS2), or endopeptidases, including members of the cathepsin family [13, 14, 19-  
77 21].

78         Following receptor binding by the S1 subunit and priming by proteolytic cleavage,  
79 the S2 subunit of S promotes the critical membrane fusion step of viral entry by  
80 undergoing dynamic conformational changes to promote merging of the viral and host  
81 cell membranes [10, 35, 37]. For entry of SARS-CoV-2, cleavage at the S1/S2 border  
82 (by furin or a similar protease), is critical for TMPRSS2 cleavage and entry at the  
83 plasma membrane. However, when S1/S2 border cleavage is blocked, viral entry can  
84 be mediated through endosomal compartments with proteolytic cleavage carried out by  
85 a member of the cathepsin family, similar to the entry pathway of SARS-CoV [10, 35,  
86 37-39]. In addition to promoting virus-cell fusion during viral particle entry, S can also  
87 promote cell-cell fusion, a pathogenic effect observed in the lungs of COVID-19 patients  
88 where neighboring cells fuse together to form large multi-nucleated cells, termed  
89 syncytia [40-45]. While the role of cellular proteases and S cleavage in viral entry is  
90 being extensively investigated, insight into the cleavage requirements for cell-cell fusion  
91 in SARS-CoV-2 remains more limited. Recent studies have suggested that S cleavage  
92 at the S1/S2 border is critical for cell-cell fusion, and TMPRSS2, while not required,

93 appears to enhance this cell-cell fusion [37, 40, 46, 47]. However, relatively little is  
94 known about the timing and efficiency of these cleavage events, and how mutations in S  
95 may affect the process.

96         Though CoVs mutate at a slower rate than most RNA viruses due to the  
97 presence of viral proofreading machinery, a meta-analysis of genomes of SARS-CoV-2  
98 strains found several mutations within S circulating in significant percentages of the  
99 analyzed populations [48, 49]. The most common mutation, now found in most of the  
100 global population, is an aspartate to glycine mutation at residue 614 (D614G) in the S1  
101 subunit. Additional mutations throughout the S1 and S2 subunits of S have been found  
102 in a smaller percentage of the viral population. Since S2 contains the fusion machinery,  
103 mutations in this region may have an impact on overall protein stability and fusion.  
104 Understanding the effects of mutations in this region will allow for a more  
105 comprehensive understanding of the overall S function.

106         We tested wild-type (wt) SARS-CoV-2 S and variants in different host cell strains  
107 to analyze the effects on stability, proteolytic processing, and cell-cell fusion. Here we  
108 demonstrate that furin cleavage of S at the S1/S2 border is required for efficient cell-cell  
109 fusion, and that the presence of TMPRSS2 in target cells enhances S mediated cell-cell  
110 fusion, consistent with previous studies [37, 46]. We also show that mutations of the  
111 cleavage sites at the S1/S2 border, S2' site, or a cathepsin L (cath L) cleavage site,  
112 conserved from SARS-CoV S, all reduce initial cleavage at the S1/S2 border during viral  
113 protein synthesis, suggesting that mutations downstream of the S1/S2 border likely alter  
114 the overall conformation of the protein. Additionally, we identify two S2 subunit  
115 residues, one in the internal fusion peptide and another in the cytoplasmic tail, that alter

116 protein fusion function when mutated without changing overall protein expression and  
117 cleavage, providing more insight into regions of the protein important for the regulation  
118 of the fusion process. Finally, we demonstrate protein turnover and cleavage kinetics in  
119 a range of host cells, as well as in the presence of several exogenous proteases,  
120 providing a more comprehensive picture of the S protein.

## 121 **Results**

### 122 *Stability and proteolytic cleavage of SARS-CoV-2 Spike in Various Cell Lines*

123 To examine the stability and cleavage patterns of SARS-CoV-2 S in a range of  
124 mammalian cell lines, the following cells were transiently transfected with pCAGGS-S:  
125 Vero, A549, MEFs, Cath L- MEFs, and LoVo cells (a human colon carcinoma line that  
126 does not express functional furin). Stability of S and the timing of proteolytic processing  
127 were determined by pulse-chase labeling and immunoprecipitation. S protein detected  
128 from immunoprecipitation was observed as two bands, a band around 150 kDa  
129 corresponding to an un-cleaved full-length species of the protein, labeled S, and a band  
130 around 97 kDa corresponding to a species of S cleaved at the border of the S1 and S2  
131 subunits, labeled S2 (Fig. 1a). After a one-hour chase, a band corresponding to S2 was  
132 observed in Vero, A549, and both MEF cell lines (Fig. 1a). In LoVo cells, a band  
133 corresponding to the S2 subunit did not appear until four hours of chase, verifying that  
134 lack of furin impedes efficient processing at S1/S2, and that the S1/S2 border can be  
135 cleaved by cellular protease other than furin (Fig. 1a) in a slower and less efficient  
136 process. Veros, A549s, MEFs, and Cath L- MEFs displayed similar cleavage patterns  
137 over time, while LoVo cells displayed significantly less cleavage at two and four hours.  
138 LoVo cells had only 2% cleavage at two hours and 18% cleavage at four hours,

139 compared to about 20-40% at two hours and 30-60% at four hours for all other cell  
140 types ( $p < 0.05$ ). However, LoVo cells reached cleavage levels similar to the other cell  
141 lines at later chase time points (Fig. 1b). Bands smaller than 90 kDa that would  
142 correspond to cleavage at the S2' site were not observed in any cell line. In the  
143 examined cell lines, expressed S remained stable through the first four hours (Fig. 1c).  
144 By 24 hours post label, only 20-30% of the original labeled protein remained for all cell  
145 lines.

146         Several studies have examined the cellular proteases involved in the cleavage of  
147 S. Furin and TMPRSS2 appear to play key roles in cleavage at the S1/S2 border and  
148 S2' site, respectively [26, 35, 50-52]. Additionally, lysosomal proteases such as cath L/B  
149 can be utilized for viral entry in TMPRSS2 deficient cells [10, 38, 46]. To examine how  
150 higher expression levels of these proteases affect S stability and cleavage, Vero and  
151 A549 cells were transiently transfected with S alone or S with TMPRSS2, furin, or cath  
152 L. Pulse-chase analysis demonstrated that the transient expression of TMPRSS2 or  
153 cath L did not affect the cleavage pattern of S (Fig. 1d and 1e, S1b), and a band  
154 corresponding to S2' cleavage was not observed in either Veros or A549s. However,  
155 transient over-expression of furin increased the cleavage observed at the S1/S2 border  
156 in Veros at four and eight hours of chase ( $p < 0.05$ ) and at all times after zero for A549s  
157 ( $p < 0.01$  for one- and eight-hour chase,  $p < 0.0001$  for two- and four-hour chase times)  
158 (Fig. 1e and 1f). This suggests that the normal levels of cellular furin can eventually  
159 promote maximal levels of S1/S2 cleavage in both Veros and A549s, but over-  
160 expression of furin facilitates more rapid cleavage of the S1/S2 border. Interestingly, in  
161 both experiments (Fig. 1a and 1d) some un-cleaved S remains even after 24 hours,

162 indicating that a small portion of the S population is not cleaved by furin or other  
163 endogenous proteases in these cell lines. Finally, overall protein stability was not  
164 affected by co-expression of any tested proteases (Fig. S1b).

### 165 *Spike Mediated Cell-Cell Fusion*

166 The S2 subunit of S mediates both viral-cell fusion and cell-cell fusion [40-42],  
167 with cell-cell fusion readily observed both in a laboratory setting and in the lungs of  
168 SARS-CoV-2 infected patients [40-45]. To better understand the requirements and  
169 contribution of cellular proteases to S2 mediated cell-cell fusion, we performed syncytia  
170 and reporter gene assays. For syncytia analysis, a small number of syncytia, were  
171 observed at 24 hpt in all samples (Fig. 2a). At 48 hpt, similar numbers of large syncytia  
172 were observed with S alone or S co-expressed with TMPRSS2 or cath L (Fig. 2b).  
173 However, co-expression of S with furin resulted in increased syncytia formation. The  
174 cells exhibited nearly complete fusion, suggesting that the presence of exogenous furin  
175 further increases S mediated cell-cell fusion (Fig. 2b, panel 3).

176 To quantitate S mediated cell-cell fusion, luciferase reporter gene fusion assays  
177 were performed (Fig. S2a), using a nine hour overlay that was determined to be optimal  
178 (Fig. S2b). To characterize the role of cellular proteases in the hACE2 expressing target  
179 cells, S-expressing effector cells were overlaid with target cells containing hACE2 alone  
180 or hACE2 with TMPRSS2, furin, or cath L. The amount of plasmid transfected was kept  
181 constant by supplementing with a plasmid encoding an empty expression vector (EV).  
182 When Vero cells were used as the S-expressing effector cell and TMPRSS2 was  
183 present in the target cells, a significant increase in fusion was observed. This is  
184 consistent with the concept that TMPRSS2 plays a role in fusion after or during the



185 hACE2 (receptor) binding step in the fusion cascade (Fig. 2c) [10, 12, 32, 37, 46],  
186 although the presence of TMPRSS2 in these target cells also appeared to process  
187 hACE2 (Fig. S2c, also observed in[40]). In samples with cath L or furin in the target  
188 cells, fusion levels were similar to hACE2+EV (Fig. 2c). When A549 cells were used as  
189 the S-expressing effector cell, none of the conditions produced statistically significant  
190 differences from background levels (Fig. 2c), so Vero cells were used as the effector  
191 cells for the remainder of the experiments performed.

192         Having analyzed the function of proteases in the target cells, we were also  
193 interested in the role of proteases present in the S-expressing effector cells. To test  
194 this, EV, TMPRSS2, cath L, or furin were co-expressed with S and samples were  
195 overlaid with target cells expressing hACE2. Similar to what we observed in syncytia  
196 assays, only co-expression of S and furin produced a statistically significant increase in  
197 fusion. This increase is likely due to the increase in the amount of cleaved protein  
198 present when S is co-expressed with furin (Fig. 1e).

199         Neuropilin-1 has been suggested as a co-receptor for SARS-CoV-2 S and may  
200 be important for the viral infection infiltrating the neuronal network [53-55]. To assess  
201 the contribution of neuropilin in cell-cell fusion, effector cells were transfected with S and  
202 either EV, furin, neuropilin, or furin and neuropilin (F+N). Target cells were transfected  
203 with EV, hACE2, neuropilin, or hACE2 and neuropilin. However, no significant increase  
204 in fusion was observed when neuropilin was present in either the target or effector cells  
205 (Fig. 2e), suggesting that neuropilin does not appear to play a significant role in cell-cell  
206 mediated fusion. Interestingly, when neuropilin is co-expressed in S containing effector  
207 cells, there is no difference observed in fusion compared to samples with S+EV,

208 suggesting that neuropilin also does not have an inhibitory effect (Fig. 2e). Additionally,  
209 when neuropilin alone is expressed in the target cells, fusion levels above background  
210 levels are not observed. This indicates that in cell-cell fusion, S binding hACE2 appears  
211 to be the major interaction during the receptor attachment function.

### 212 *Importance of CoV-2 cleavage sites*

213 Early protein sequence analysis of CoV-2 S protein demonstrated the presence  
214 of three potential cleavage sites [26]: a putative furin cleavage site at the S1/S2 border;  
215 a conserved site 10 residues downstream from the S1/S2 border, shown to be cleaved  
216 by cath L in SARS-CoV; and the S2' site which is potentially cleaved by TMPRSS2 [26].  
217 To functionally understand the role of each cleavage site in S cell-cell fusion, a series of  
218 mutants were made. Alanine mutations of all the residues within each potential  
219 cleavage site (S1/S2 AAAAA, Cath L AAAA, S2' AA), and single alanine mutations at  
220 the terminal arginine of the S1/S2 border and S2' site (S1/S2 PRRAA, S2' KA) were  
221 created. Finally, a mutant with residues (PRRA) upstream of the S1/S2 border deleted  
222 (del. PRRA), leaving a single R residue at this site, was made, creating an S1/S2 border  
223 similar to SARS-CoV S (Fig. 3A). Pulse-chase analysis (Fig. 3b) showed that all  
224 mutants had similar protein turnover compared to wt S in Veros. However, in A549s  
225 several mutants demonstrated more rapid protein turnover than wt S at later chase time  
226 points. Surprisingly, mutations at all three sites led to either a complete loss or  
227 significant delay in the proteolytic processing of the S protein at the S1/S2 border,  
228 indicated by the lack of a band corresponding to the S2 subunit. This suggests that  
229 mutations at distal sites can strongly influence cleavage at S1/S2. After an eight-hour  
230 chase, no cleavage at the S1/S2 border was observed for the mutants del. PRRA and

231 S1/S2 AAAAA, confirming that deletion or mutation of the furin consensus prevents  
232 cleavage at this site. For all other mutants, cleavage at the S1/S2 border reached 30-  
233 50% of wt levels in both Vero and A549 cells the eight-hour time point (Fig. 3c and 3d).  
234 Accurate analysis of protein cleavage was not possible by the 24-hour time point, since  
235 only 20-30% of protein remained (Fig. S1b). Finally, surface biotinylation showed that  
236 both total and cell surface expression of all mutants were similar to wt S levels (Fig. 3e,  
237 f, and g).

238 To assess the effects of the mutations on cell-cell fusion, syncytia formation  
239 assays in Vero cells were performed. While syncytia were readily observed in all  
240 samples containing wt S, none of the mutants exhibited syncytia formation at 24 or 48  
241 hpt when expressed alone (Fig. S3, panel 2). Addition of TMPRSS2 did not recover  
242 syncytia formation in any mutant (Fig. S3, panel 3), and the addition of furin only  
243 recovered syncytia formation in the S1/S2 PRRAA mutant (Fig. S3, panel 4, syncytia  
244 denoted with black arrows). To analyze this result, cells were lysed following the 48-  
245 hour imaging and protein levels examined by western blot. Results showed that co-  
246 expression of furin with the S1/S2 PRRAA mutant restored cleavage at the S1/S2  
247 border, while all other mutants did not show cleavage at this site (data not shown). This  
248 suggests that cleavage at the S1/S2 border is critical for cell-cell fusion, and that the  
249 double R motif in the PRRAA mutant can be cleaved by over-expressed furin.

250 Luciferase reporter gene analysis of fusion in Veros transfected with wt S or each  
251 mutant showed similar results to the syncytia assays, with none of the mutants showing  
252 fusion levels above background (Fig. 3h). Interestingly, the S2' AA mutant displayed  
253 high background levels, suggesting this mutant may have a conformational change, or

254 characteristics that increase receptor binding or alter S2 trimeric association, leading to  
255 higher background signals. Reporter gene assays were also carried out with addition of  
256 transiently expressed furin in the S-expressing effector cells, but no significant  
257 increases in fusion were observed. Since all cleavage mutants created reduced  
258 cleavage at the S1/S2 subunit border, the reductions in cell-cell fusion may be  
259 attributable to loss of cleavage at this site.

### 260 *Effect of Circulating S Mutations on Protein Stability, Cleavage, and Fusion*

261 An early examination revealed several mutations in the S protein gene in  
262 circulating viral strains [48, 49], including the D614G substitution now found in most of  
263 the global SARS-CoV-2 strains [48, 56-62]. The D614G mutation lies in the S1 subunit  
264 of the protein, just downstream of the receptor binding domain, and is proposed to play  
265 a critical role in receptor binding by alteration of the positioning of the receptor binding  
266 domain. Other mutations in circulating strains were found throughout the S2 subunit  
267 [49]. To assess the effect(s) of these mutations, we created the mutants D614G,  
268 A831V, D839Y/N/E, S943P, and P1263L (Fig. 4a). Pulse-chase analysis in Veros and  
269 A549s (Fig. 4b, c) demonstrated that all circulating mutants tested exhibited protein  
270 turnover at similar rates as wt S in both cell lines (Fig. S1d). Surface biotinylation  
271 confirmed that all tested mutants displayed total protein and surface protein levels  
272 comparable to wt S, suggesting that none of the mutants caused major defects or  
273 enhancement of protein trafficking to the cell surface (Fig. 4d, e). Syncytia formation  
274 and evaluation of protein location by immunofluorescence were similar between all  
275 mutants and wt S (Fig. S4). Interestingly, cellular extensions containing the S protein  
276 were observed for the wt and each of the mutants (Fig. S4, white arrows) [63]. Finally,

277 luciferase reporter gene assays were performed. While most of the mutants displayed  
278 fusion levels similar to wt S, three mutants exhibited significant changes (Fig. 4f).  
279 D839Y and D839N displayed significantly reduced levels of fusion compared to wt  
280 ( $p < 0.01$  and  $p < 0.05$ , respectively), and P1263L showed a significant increase in fusion  
281 compared to wt ( $p < 0.05$ ). These changes in fusion cannot be attributed to differences in  
282 cell surface protein expression or cleavage levels, suggesting that residues near the  
283 internal fusion peptide, where D839 is located, and residues in the cytoplasmic tail,  
284 where P1263 is located, may play an important role in controlling the fusion cascade.

#### 285 *Trypsin accessibility and protein-protein association in select Spike mutants*

286         Since all the S cleavage site mutants exhibited defects in cleavage at the S1/S2  
287 border, we evaluated the accessibility of this site using a trypsin treatment assay to  
288 determine if the lack of cleavage was due to misfolding in the S1/S2 border region.  
289 Veros or A549s were transfected with wt S or each cleavage mutant and metabolically  
290 labeled. Cell surface proteins were biotinylated and then cells were either left untreated  
291 or treated with 0.3  $\mu\text{g}/\mu\text{l}$  of TPCK-Trypsin prior to lysis. When treated with exogenous  
292 TPCK-Trypsin, both the del. PRRA and S1/S2 PRRAA mutants were efficiently cleaved  
293 at the S1/S2 border, shown by the appearance of a band corresponding to S2 in the  
294 lanes treated with trypsin (Fig. 5a, quantified in Fig. 5b). This suggests that the  
295 observed defects in cleavage at the S1/S2 border are not due to inaccessibility at the  
296 site, but rather to the removal of the furin consensus sequence. Interestingly, mutations  
297 at the downstream cath L or S2' potential cleavage sites also render defects in protein  
298 cleavage at the S1/S2 border site. However, treatment with exogenous trypsin did not

299 significantly affect the amount of cleavage observed, a result consistent with a change  
300 in conformation that renders the S1/S2 border cleavage site inaccessible.

301         CoV S proteins associate as homo-trimers shortly after synthesis and remain in  
302 this trimeric form throughout the fusion cascade [12, 15]. To determine if proteolytic  
303 processing affects the stability of S trimer association, Veros or A549s transfected with  
304 wt S or mutants D614G, S1/S2 AAAAA, S2'AA, or wt S plus additional furin, were  
305 metabolically labeled. After lysis and immunoprecipitation, samples were then treated at  
306 50°C or 100°C prior to separation on non-reducing SDS-PAGE. When wt S was  
307 incubated at 50°C prior to separation, species that correspond to a full-length S  
308 monomer, dimer, and trimer were observed (Fig. 5c). Interestingly, species that fall in  
309 between sizes corresponding to a monomer, dimer, and trimer (Fig. 5c, red and purple  
310 \*) were also observed. These intermediate species may be the result of dimers or  
311 trimers made up of a mixture of full-length S protomers and cleaved S protomers.  
312 When wt S was incubated at 100°C prior to separation, bands corresponding only to full  
313 length S monomer, dimer, trimers, and cleaved S2 monomers were apparent. Similar  
314 results were also observed in D614G samples, suggesting that species containing  
315 cleaved protomers may be less stable. Consistent with this data, the S1/S2 AAAAA  
316 mutant, which cannot undergo cleavage at the S1/S2 border site, migrated primarily as  
317 a trimeric species after 50°C incubation, with little monomer or dimer observed.  
318 Additionally, when wt S was co-expressed with furin (shown to increase S cleavage in  
319 Fig. 1e and 1f), the predominant observed species was monomeric, after both 50°C and  
320 100°C incubation. Overall, these results suggest that cleavage at the S1/S2 border  
321 alters the stability of S trimeric association.

322 *Furin or furin-like proteases in bat cells can cleave the S1/S2 border of SARS-CoV-2*

323 *Spike*

324 *Rhinolophus affinis* horseshoe bats have been identified as the likely reservoir  
325 species for the novel SARS-CoV-2 [25]. To understand the proteolytic processing,  
326 expression, and stability of CoV-2 S in a cell line closely related to its reservoir host, we  
327 utilized *Pteropus alecto* fetus (pt. fetus) or lung (pt. lung) cells [64] that have a furin  
328 enzyme with ~90% sequence homology to bats in the *Rhinolophus* family. Our previous  
329 studies on paramyxovirus virus fusion protein cleavage have shown that efficient furin  
330 and cathepsin cleavage occurs in these cells, although the furin cleavage occurs with  
331 delayed kinetics compared to Veros or A549s [65].

332 Surface biotinylation demonstrated that wt S and the del. PRRA mutant were  
333 readily expressed at the surface at similar levels in both cell lines, with cleavage at the  
334 S1/S2 border only observed for wt S and not for the del. PRRA mutant (Fig. 6a and 6b).  
335 Pulse-chase analysis showed that S expressed in both pt. lung and pt. fetus cells was  
336 cleaved at the S1/S2 border by one hour, with cleavage extent reaching approximately  
337 40% at eight-hours, and 60% at 24 hours (Fig. 6c and 6d). Thus, furin or other  
338 proteases in *P. alecto* cells are able to process S, although this processing occurred  
339 more slowly than in other mammalian cell lines (compare to Fig. 1b). Interestingly,  
340 some cleavage was also observed in both pt. lung and pt. fetus cells for the del. PRRA  
341 mutation (Fig. 6c and 6d). Additionally, the wt S and del. PRRA mutant were slightly  
342 less stable in the *P. alecto* cells, demonstrating about 30-50% protein remaining at eight  
343 hours, and about 20% at 24 hours (Fig. 6e). In contrast, previously used mammalian

344 cells lines showed 60-90% of wt S remained at eight hours, with 30-50% at 24 hours of  
345 chase (Fig. 1c).

## 346 **Discussion**

347 In this study, we present a detailed characterization of the cleavage patterns,  
348 protein stability, and cell-cell fusion function of the SARS-CoV-2 S protein, as well as  
349 analysis of mutations within the S2 subunit that may affect these important protein  
350 properties. Consistent with recently published work [26, 35, 47, 50, 51, 66], our analysis  
351 confirms that S is readily cleaved at the S1/S2 border in a variety of mammalian cell  
352 lines. Additionally, we show for the first time, that cleavage occurs in a bat cell line  
353 similar to the SARS-CoV-2 reservoir species. While cleavage appears to be primarily  
354 carried about by the cellular protease furin, the sequence at this border does have the  
355 ability to be cleaved by other members of the pro-protein convertase family when furin is  
356 not present [47], and this likely accounts for the small amount of cleavage we observed  
357 in furin-negative LoVo cells.

358 Additionally, we carefully assessed the role different proteases play in cell-cell  
359 fusion, finding that furin increases cell-cell fusion when present in the same cell as S,  
360 and TMPRSS2 increases cell-cell fusion when present in a target cell, consistent with  
361 previous studies [37, 46]. Interestingly, when cell-cell fusion assays were performed  
362 using A549 cells as the effector cell (Fig. 2c), high background fusion levels were  
363 observed. This could be due to high endogenous levels of TMPRSS2 in this cell line  
364 compared to Veros, that were ultimately used for this experiment (Fig. S2c). High  
365 TMPRSS2 expression or exogenous treatment with trypsin has been shown to restore  
366 cell-cell fusion in low ACE2 receptor expression environments for SARS-CoV S [67, 68].



367 It is also worth noting that the presence of TMPRSS2 in the target (BSR/T7) cells also  
368 appears to process hACE2 (Fig. S2c, [40]). Therefore, we cannot exclude the possibility  
369 that the increase in fusion observed when TMPRSS2 is present in these cells is due to  
370 an effect on hACE2. In addition to the effect of proteases on cell-cell fusion, we also  
371 assessed the effect of Neuropilin-1, which has been suggested to be a co-receptor for  
372 SARS-CoV-2 viral entry and may be key for SARS-CoV-2 infiltration of the neuronal  
373 network [53-55]. We show that the presence of Neuropilin-1 with hACE2 in target cells  
374 does not impact S mediated cell-cell fusion (Fig. 2e). Additionally, co-expressing  
375 Neuropilin-1 with S in effector cells did not have an inhibitory effect on cell-cell fusion.  
376 While reports suggest Neuropilin-1 plays a role in viral entry of SARS-CoV-2, this  
377 indicates it does not play a significant role in S cell-cell fusion in our assay, although this  
378 was not investigated in neuronal cells.

379 The viral entry and cell-cell fusion pathways of SARS-CoV, MERS-CoV, and  
380 SARS-CoV-2 have several noteworthy commonalities, but do have marked differences.  
381 They all share the ability to facilitate entry through endosomal pathways, with S  
382 proteolytic activation mediated by endosomal/lysosomal proteases [10, 19, 35, 37-39,  
383 69-72]. Additionally, they all can utilize cell surface (such as TMPRSS2) or extracellular  
384 proteases (trypsin) for S activation and subsequent viral entry [10, 37, 38, 47, 67, 72-  
385 78]. SARS-CoV-2 and MERS-CoV S differ from SARS-CoV S in that their S1/S2 border  
386 harbors a canonical furin cleavage motif [26, 27, 32], resulting in S pre-activation by  
387 furin during synthesis and cellular trafficking, prior to reaching the cell surface or being  
388 incorporated into viral particles [19, 35, 37, 39, 75]. This pre-activation by furin likely  
389 enhances the ability of SARS-CoV-2 and MERS-CoV S to participate in cell-cell

390 mediated fusion without over-expression of cell surface or extracellular proteases [37,  
391 46]. Addition of this cleavage sequence in SARS-CoV S allows SARS-S to facilitate cell-  
392 cell fusion without exogenous proteases [37, 79]. We show an increase in both syncytia  
393 formation and luciferase reporter gene assay fusion when cleavage at the S1/S2 border  
394 is enhanced by overexpression of furin (Fig. 2b and 2c), confirming that furin cleavage  
395 of SARS-CoV-2 S plays a critical role in cell-cell fusion. Interestingly, furin cleavage is  
396 not required for SARS-CoV-2 infection [10, 35, 37, 47], although removal of the site or  
397 inhibition of furin does appear to attenuate the virus [35, 39, 47] and reduce cellular  
398 tropism [46].

399         The presence of a furin consensus sequence is not only a marked difference  
400 between SARS-CoV and SARS-CoV-2, but it is also one of the differences between  
401 SARS-CoV-2 and a similar CoV circulating in a bat population [25]. Analysis of SARS-  
402 CoV-2 wt S in *P. alecto* cells demonstrates that this motif can be recognized and  
403 cleaved by furin in these cells (Fig. 6c and 6d), although the kinetics of this cleavage are  
404 noticeably slower than in other mammalian cell lines (compare to Fig. 1b). Previous  
405 work has shown that the fusion proteins of Hendra virus, processed by cathepsins, and  
406 parainfluenza virus 5, processed by furin, are also cleaved in *P. alecto* cells [65]. Pulse-  
407 chase analysis in this prior study demonstrated an increase in processing kinetics,  
408 although this kinetic difference can be accounted for by differences in protease  
409 expression levels between different bat cell lines (pt. kidney cells in [65], and pt. lung  
410 and pt. fetus cells in our work), suggesting there may be cellular differences in protein  
411 trafficking or furin activity. Intriguingly, a CoV-2 S mutant with a deletion of the inserted  
412 PRRA residues still demonstrated some cleavage in both utilized bat cell lines (Fig. 6c

413 and 6d), while not showing any in Veros or A549s (Fig. 3c and 3d). Earlier work on  
414 MERS-CoV S showed that furin or other proprotein convertases in bat cells can process  
415 MERS S S1/S2 border without the presence of a canonical recognition motif [80]. Taken  
416 together, these results suggest that mutations in circulating bat CoVs that allow for  
417 human protease recognition at critical cleavage sites may be an important factor for  
418 zoonotic transmission of several CoVs.

419 Two other potential cleavage sites have been identified in work with other CoVs.  
420 The S2' site is essential for both SARS and MERS infection [12, 32, 81-83] while a cath  
421 L activated site play a critical role for SARS-CoV S [13, 20, 84, 85]. Interestingly,  
422 mutations made at the S2' site of SARS-CoV-2 S significantly reduce S1/S2 border  
423 cleavage, both in our study and others (Fig. 3b-d, [46, 86]), even though the sites are  
424 distal from each other. A similar reduction in cleavage is observed when the conserved  
425 cathepsin site is mutated (Fig. 3b-d). Our analysis of the published structures [3, 4, 87,  
426 88] indicates that a full alanine mutation of this site may simply collapse the exposed  
427 S1/S2 loop. Our finding that exogenous trypsin treatment of cells expressing the S2' or  
428 cathepsin site mutants does not restore cleavage at the S1/S2 border (Fig. 5a and 5b)  
429 suggests that these mutations result in proteins with altered furin loop structure [87],  
430 rendering it inaccessible. However, these mutants are still synthesized and trafficked to  
431 the surface despite not being cleaved (Fig. 3e-g), thus this change in conformation is  
432 unlikely to have drastically misfolded the protein. These results suggest that there may  
433 be a dynamic interaction between the S1/S2 border and S2' cleavage sites in SARS-  
434 CoV-2 S needed to facilitate viral entry and cell-cell fusion. This dynamic control could

435 also be regulated by S receptor binding exposing cryptic protease sites, although  
436 studies analyzing this in SARS and MERS S conflict on this topic [19, 70, 77, 89, 90].

437 We also assessed the effect on protein stability, cleavage, and cell-cell fusion  
438 function of a series of mutations in other regions of S. The D614G mutation emerged  
439 during 2020, and is now found in most circulating strains globally [48]. D614G has been  
440 shown to increase S incorporation into viral particles [91], increase receptor binding [92,  
441 93], and reduce S1 subunit shedding and particle infectivity [94]. Importantly, the  
442 D614G mutant shifts S to favor a “heads up” conformation of the receptor binding  
443 domain [93, 95, 96]. In our study, the D614G mutation did not impact the cell-cell fusion  
444 function (Fig. 4f), expression, or stability of the protein (Fig. 4d/e, and Fig. S1),  
445 consistent with one previous study [86]. Our fusion results however conflict with two  
446 previous studies that demonstrated D614G increases cell-cell fusion, measured by cell  
447 depletion in flow cytometry [92], and syncytia formation in 293T and Hela cells stably  
448 expressing hACE2 [97]. These discrepancies may be due to differences in  
449 experimental conditions or cell types utilized. We are, however, the first to date to  
450 utilize a luciferase reporter gene assay to quantitate cell-cell fusion of a D614G S  
451 mutant. Using this assay, we also show that mutations found at two other residues  
452 (discovered in small, non-dominant population subsets [49]) alter the cell-cell fusion  
453 activity of S (Fig. 4f) without changing the overall protein expression or stability levels  
454 (Fig. 4d-e, Fig. S1d). Mutations at D839, a residue within the internal fusion peptide, to  
455 the polar amino acids, tyrosine or asparagine, significantly reduce fusion. Interestingly,  
456 a mutation at this residue that conserves the negative charge, D839E, has no effect on  
457 fusion activity. The negative charge at this residue may play a role in the regulation of S

458 mediated fusion due to its location in the internal fusion peptide. Alternatively, this  
459 residue is in close proximity to C840, which may participate in a disulfide bond, so  
460 mutations at D839 may disrupt this disulfide bond, destabilizing the protein and  
461 changing fusion activity. Additionally, mutation of residue P1263 to a leucine  
462 significantly increases S mediated cell-cell fusion, suggesting that residues in the  
463 cytoplasmic tail may play a role in the S-promoted cell-cell fusion process. Notably, a  
464 study that removed the entire S cytoplasmic tail still observed syncytia formation at  
465 levels similar to wt S [86], indicating that regulation by the cytoplasmic tail may be  
466 complex or that the role of the cytoplasmic tail in fusion is not regulation, but interaction  
467 with cellular host factors [98].

468 In this work, we also provide critical insight into the kinetics of protein cleavage  
469 and overall stability of CoV-2 S. S protein processing at the S1/S2 border occurs within  
470 two hours of synthesis (Fig. 1a and 1b; one hour of label, one hour of chase) in several  
471 mammalian cell lines (Vero, MEF, A549), and continues to increase over time, reaching  
472 60-80% protein cleavage by eight hours of chase time, depending on the cell type.  
473 Overexpression of furin increased the efficiency of S1/S2 border cleavage (Fig. 1d-f),  
474 and this increase in cleavage may account for the increase in cell-cell fusion observed  
475 when furin is co-expressed with S (Fig. 2a-c, [37, 46]). Additionally, we show that  
476 transiently transfected S is stable in several mammalian cells for 4-5 hours post-protein  
477 synthesis with demonstrable turnover after this point, (Fig. 1c, Fig. S1). This protein  
478 turnover is similar to turnover rates seen in PIV5 fusion protein, also activated by  
479 cellular furin [99], and slightly slower turnover than Hendra fusion protein, activated by  
480 cellular cathepsins [100, 101]. Over-expression of cellular proteases that may process

481 S did not affect these protein turnover rates. Interestingly, analysis of S in non-reducing  
482 conditions found that cleavage of the S1/S2 border appears to destabilize trimeric  
483 interactions (Fig. 5b). In these non-reducing conditions, no differences were observed  
484 in oligomeric stability between wt S and the D614G S mutations, despite the D614G  
485 favoring a 'heads up' conformation [93, 95, 96] and Vero cells having sufficient levels of  
486 endogenous ACE2 to facilitate syncytia formation (Fig. S2c), suggesting that changes in  
487 receptor binding do not alter overall protein trimeric association. Notably, in these non-  
488 reducing conditions after a 50°C treatment for wt S, the D614G mutant, and wt S+furin,  
489 bands between monomer, dimer, and trimer species are observed (Fig. 5b, indicated  
490 with \*). These intermediate species are not observed after treatment at 100°C. These  
491 may represent protein oligomers that are not identically cleaved and are therefore  
492 partially destabilized, a phenomenon proposed for MERS-CoV S [32], and murine  
493 hepatitis virus CoV S, [102]. Protein oligomers with differential proteolytic processing  
494 may also account for the small population of un-cleaved protein we observed at the cell  
495 surface in our experiments (Fig. 3e, Fig. 4d, Fig. 5a, and Fig. 6a).

496 Through biochemical and cell biological analysis of the SARS-CoV-2 S protein,  
497 we have provided important observations about the stability, proteolytic processing, and  
498 requirements for cell-cell fusion of this highly sought-after therapeutic target. This  
499 information may be helpful in directing treatments that inhibit S protein fusion, or for  
500 discerning methods to stabilize CoV-2 S in therapeutic development. Additional studies  
501 are needed to understand the potential interplay between S cleavage sites and how that  
502 may contribute to S protein function, as well as to further investigate spike S2 subunit  
503 regions that are critical for protein function.

504 **Experimental procedures**

505 **Cell lines and culture.** Vero (ATCC), BSR T7/5 cells (provided by Karl-Klaus  
506 Conzelmann, Pettenkofer Institut), mouse embryonic fibroblasts (MEFs) from cathepsin  
507 L knockout mice (Cath L- MEFs) (a gift from Terence Dermody, University of  
508 Pittsburgh), and *P. alecto* bat cells harvested from fetus (pt. fetus) and lung (pt. lung) (a  
509 gift from Linfa Wang, Duke-NUS) [64] were all maintained in Dulbecco's modified  
510 Eagle's medium (DMEM, GE Healthcare), with 10% fetal bovine serum (FBS) and 1%  
511 penicillin/streptomycin. Every third passage, 0.5mg/ml of G-418 (Invitrogen) was added  
512 to the culture media of BSR T7/5 cells to select for the expression of the T7 polymerase.  
513 A549 and human colon carcinoma LoVo cells (both purchased from ATCC) were  
514 cultured in F12 Kaighns Modification media (GE Healthcare) with 10% FBS and 1%  
515 penicillin/streptomycin.

516 **Plasmids, Antibodies, and Mutagenesis.** pCAGGS-SARS-CoV-2 spike was obtained  
517 from BEI Resources. pcDNA3.1(+)-hACE2 and pcDNA3.1(+)-TMPRSS2 were provided  
518 by Gaya Amarasinghe (Washington University). Human Neuropilin-1 was expressed  
519 with an exogenous PTP $\alpha$  signal sequence from the pLEXm vector (from Craig Vander  
520 Kooi, University of Kentucky). SARS-CoV-2 S was subcloned into pUC57 and all S  
521 mutants were created in pUC57 using the QuikChange site-directed mutagenesis kit  
522 (Stratagene) with primers purchased from Eurofins. Constructs were then subcloned  
523 back into the pCAGGS expression vector. Other plasmids utilized include pSG5-  
524 Cathepsin L (from Terence Dermody, University of Pittsburgh), pCAGGS-furin  
525 (Promega), and T7 promoted-luciferase (Promega). Antibodies anti-SARS spike

526 glycoprotein (ab252690) and anti-hACE2 (ab15348) were purchased from Abcam, and  
527 anti-TMPRSS2 (H-4) was purchased from Santa Cruz Biotechnology, Inc.

528 **Gel electrophoresis and western blotting.** Proteins were separated on a 10% sodium  
529 dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). For western blot  
530 analysis, proteins were transferred to a polyvinylidene difluoride (PVDF) membrane  
531 (Fisher Scientific) at 60V for 100 minutes. After blocking with 5% milk in tris-buffered  
532 saline + Tween-20 (tTBS) for 1 hour, membranes were incubated with respective  
533 antibodies (anti-SARS S 1:5000 dilution, anti-TMPRSS2 1:1000 dilution, anti-hACE2  
534 1:1000 dilution) at 4°C overnight. Membranes were then washed with tTBS and  
535 incubated with (Li-Cor) secondary antibodies at 1:10000 dilution in 5% milk solution for  
536 1 hour. Membranes were washed again with tTBS and diH<sub>2</sub>O, before being imaged on  
537 the Odyssey Image Analyzer (Li-Cor).

538 **Syncytia Assay.** Cells (Vero or A549s) in 6 well plates were transiently transfected with  
539 2µg of either wild-type or mutant SARS-CoV-2 S protein plasmid with Lipofectamine  
540 3000 (Invitrogen) at a ratio of 1:2:2 DNA: P3000: Lipofectamine 3000. For experiments  
541 with the addition of proteases, the total DNA transfected was kept constant at 2µg, in  
542 those cases we used 1µg of S and 1µg of the indicated protease. Syncytia formation  
543 was imaged at 24 and 48 hours post transfection on a Nikon Ti2 at 20X magnification.

544 **Luciferase Reporter Gene Assay** Effector cells (Vero or A549s) were plated in 12-well  
545 plates at 70-90% confluency and transfected with 1µg of total DNA (0.4µg of a T7  
546 promoted luciferase plasmid, 0.6µg of wild-type (wt) or mutant S protein or S protein  
547 with additional proteases). At the same time BSR cells (constitutively expressing a T7  
548 promoter) seeded in 6-well plates were transfected with 2µg either empty pCAGGS or



549 pcDNA3.1(+)-hACE2. Eighteen to twenty-four hours post transfection BSR cells were  
550 lifted using trypsin, centrifuged for five minutes at 1500 rpm, resuspended in normal  
551 DMEM+10% FBS, and overlaid onto the S expressing cells at a ratio of 1:1. Overlaid  
552 samples were then incubated at 37°C for 9 hours (or as described in the text). Samples  
553 were lysed in 100µL of Reporter Gene Lysis buffer (Promega) and frozen overnight.  
554 Plates were then scraped on ice, lysates were vortexed for 10 seconds, centrifuged at  
555 13,000 rpm for 1 minute at 4°C, and 20µL of the supernatant was added to an opaque  
556 96 well plate. Luciferase activity was measured on a SpectraMax iD3 (Molecular  
557 Devices) using a Luciferase Assay System (Promega). Background values were  
558 subtracted (empty pCAGGS in BSRs and effector cells) and luciferase activity was  
559 expressed as a percentage of wt S (effector cells) and hACE2 (BSR cells).

560 **Surface Biotinylation.** Two µg of wt or mutant S protein was transfected into Vero or  
561 A549 cells using the Lipofectamine 3000 system (Invitrogen; ratios described above).  
562 Eighteen to twenty-four hours post transfection, cells were starved in Cys<sup>-</sup>/Met<sup>-</sup> media  
563 (Gibco) for 45 minutes, and metabolically labelled for six hours using 50µCi of S<sup>35</sup>  
564 (PerkinElmer) incorporated into Cys and Met (S<sup>35</sup> Cys/Met). After the label, cells were  
565 washed once with PBS (pH 8) and incubated with 1 mg/ml of EZ-link Sulfo-NHS-biotin  
566 (Thermo Fisher) in PBS (pH 8) at 4°C for 35 minutes, and then at room temperature for  
567 15 minutes. Next the cells were lysed in 500µl of RIPA buffer (100 mM Tris-HCl [pH  
568 7.4], 0.1% SDS, 1% Triton X-100, 1% deoxycholic acid) containing 150 mM NaCl,  
569 protease inhibitors (1 U aprotinin, 1mM PMSF, [both from Sigma-Aldrich]), 5 mM  
570 iodoacetamide, and cComplete EDTA-free Protease Inhibitor Cocktail Tablets (all from  
571 Sigma-Aldrich). Cell lysates were centrifuged at 55,000 rpm for 10 minutes, and the

572 supernatant was incubated with anti-SARS S polyclonal antibody at 4°C for three hours.  
573 Following incubation, Protein A conjugated to Sepharose beads (Cytiva) were added to  
574 the samples, and incubated at 4°C for an additional 30 minutes. Post-incubation  
575 samples were washed two times with each RIPA Buffer+0.3M NaCl, RIPA Buffer+0.15M  
576 NaCl, and SDS-Wash II buffer (50mM Tris-HCl [pH 7.4], 150mM NaCl, and 2.5mM  
577 EDTA). After buffer aspiration and addition of 10% SDS, samples were boiled for 10  
578 minutes. The supernatant was removed to a separate tube. 15µl of supernatant was  
579 removed and added to an equal portion of 2X SDS loading buffer and labeled "TOTAL".  
580 Biotinylation buffer (20 mM Tris [pH 8], 150mM NaCl, 5mM EDTA, 1% Triton X-100, and  
581 0.2% BSA) and Streptavidin conjugated beads were added to the remaining  
582 supernatant, and this was incubated at 4°C for one hour. Samples were again washed  
583 as described above and 2X SDS loading buffer was added following the washes.  
584 Samples were boiled for 15 minutes and run on a 10% SDS-PAGE gel. Gels were dried  
585 and exposed on a phosphoscreen for two to four days, then visualized using a Typhoon  
586 Imaging System (GE Healthcare). Bands were quantified using band densitometry  
587 using the ImageQuant software (GE Healthcare).

588 **Time Course Immunoprecipitation.** 2µg of wt or mutant S was transfected into Vero  
589 or A549 cells using the Lipofectamine 3000 system (Invitrogen; ratios described above).  
590 Eighteen to twenty-four hours post transfection, cells were starved in Cys<sup>-</sup>/Met<sup>-</sup> media  
591 (Gibco) for 45 minutes, and metabolically label for one hour using 50µCi of S<sup>35</sup> Cys/Met.  
592 After the one-hour label, cells were washed once with PBS and normal DMEM + 10%  
593 FBS was added for indicated times. Cells were then lysed in 500µl of RIPA lysis buffer.  
594 Anti-SARS S polyclonal antibodies were used to immunoprecipitate the CoV-2 S protein

595 as previously described and the protein was analyzed on a 10% SDS-PAGE gel. Gels  
596 were dried and exposed on a phosphoscreen for 2-4 days and visualized using a  
597 Typhoon Imaging System (GE Healthcare). Bands were quantified using band  
598 densitometry using the ImageQuant software (GE Healthcare).

599 **Non-reducing Gel Electrophoresis.** Two  $\mu\text{g}$  of wild-type or mutant S was transfected  
600 into Vero or A549 cells using the Lipofectamine 3000 system (Invitrogen; ratios  
601 described above). Eighteen to twenty-four hours post transfection, cells were starved in  
602 Cys/Met media (Gibco) for 45 minutes, and metabolically labeled for six hours using  
603  $50\mu\text{Ci}$  of  $\text{S}^{35}$  Cys/Met. Lysed cells were immunoprecipitated as described above,  
604 however after the washing steps,  $30\mu\text{l}$  of 2X SDS loading buffer without dithiothreitol  
605 (DTT) was added to each sample. Samples were treated at  $50^\circ\text{C}$  or  $100^\circ\text{C}$ , as  
606 indicated, for 20 minutes and analyzed on a 3.5% acrylamide gel under non-reducing  
607 conditions. The gel was dried, exposed, and imaged as described for surface  
608 biotinylation.

609 **Immunofluorescence experiments.** Sub-confluent cells on coverslips in 6 well plates  
610 were transfected with  $2\mu\text{g}$  of DNA using the Lipofectamine 3000 transfection system  
611 (Invitrogen). Eighteen to twenty-four hours post transfection cells were fixed with 4%  
612 PFA for 15 minutes at room temperature. Cells were permeabilized in a solution of 1%  
613 Triton X-100 in PBS+0.02% Sodium Azide (PBSN) for 15 minutes at  $4^\circ\text{C}$ . After  
614 permeabilization, coverslips were moved to a humidity chamber and blocked with 1%  
615 normal goat serum (NGS) in PBSN for 1 hour at  $4^\circ\text{C}$ . Cells were labeled with anti-  
616 SARS S antibody (1:2000 dilution) in blocking buffer overnight at  $4^\circ\text{C}$  or for three to five  
617 hours at room temperature. Samples were washed with PBSN+0.01% Tween-20 seven

618 times and incubated for 1 hour at 4°C with goat anti-rabbit FITC (1:2000 dilution).

619 Samples were again washed with PBSN+0.01% Tween seven times and mounted onto  
620 slides using Vectashield mounting media (Vector Laboratories). Slides were imaged on  
621 an Axiovert 200M (Zeiss) at 63x magnification using Metamorph to collect Z-stacks and  
622 processed using Nikon NIS Elements.

623 **Statistical analysis** Statistical analysis was performed using Prism 7 for Windows  
624 (GraphPad). A *p* value of <0.05 was considered statistically significant. Multiple  
625 comparison tests were generated using one-way or two-way analysis of variance  
626 (ANOVA) with Dunnett's multiple comparison test. \*:  $p < 0.05$ , \*\*:  $p < 0.01$ , \*\*\*:  $p < 0.0005$ ,  
627 \*\*\*\*:  $p < 0.0001$

628

#### 629 **Data availability**

630 The datasets generated during and/or analyzed during the current study are available  
631 upon request from the corresponding author, Rebecca Dutch ([rdu2c2@uky.edu](mailto:rdu2c2@uky.edu)), on  
632 reasonable request.

633

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636 Neuropilin-1 expression plasmid and for providing structural insight. We would also like  
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638 providing the TMPRSS2 and hACE2 expression plasmids, and providing feedback  
639 regarding experimental design.

640

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645

646 **Conflict of interest**

647 The authors declare that they have no conflicts of interest with the contents of this  
648 article.

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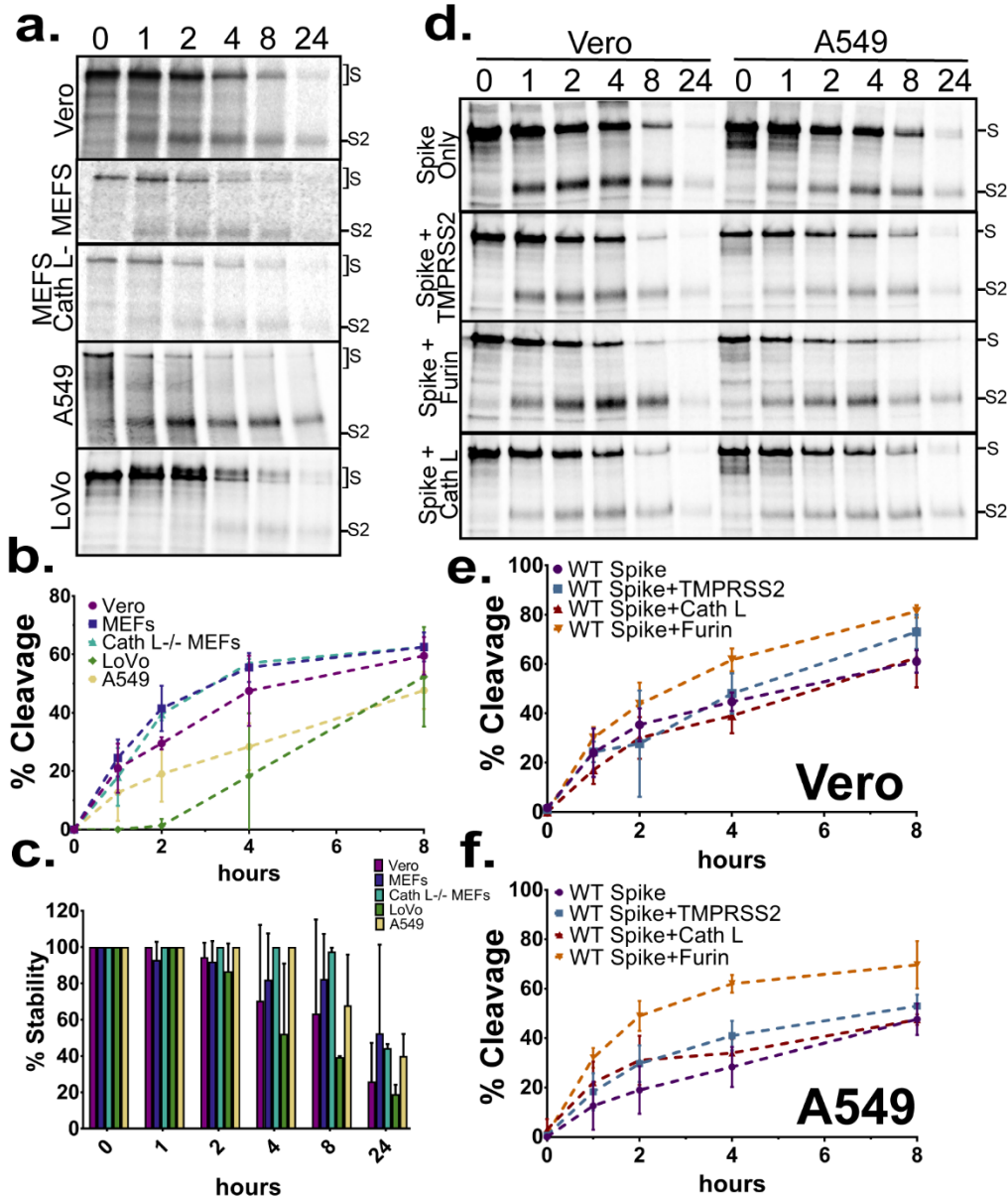
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664 **Figures**

665



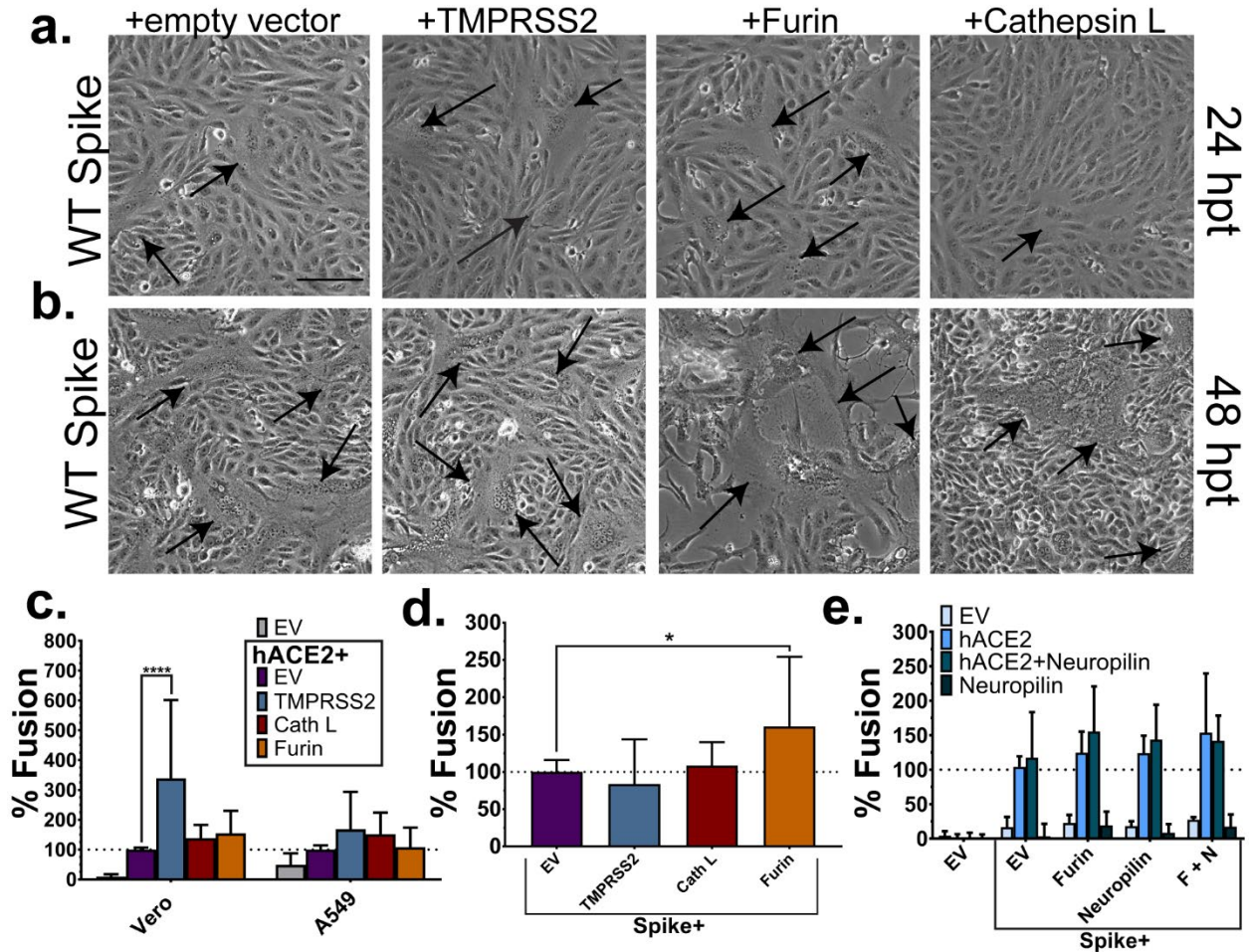
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667 **Figure 1: SARS-CoV-2 Spike is cleaved at the S1/S2 subunit border in a variety of cell**  
 668 **lines.** a) The indicated cell types transiently expressing S were metabolically labeled for one  
 669 one hour, and chased for times indicated (hours). Band densitometry was used to quantify bands  
 670 representing full length S or S cleaved at the S1/S2 border (S2) (b) Percent cleavage [S2  
 671 divided by S plus S2] and (b) Overall protein stability [Total S, S plus S2, for each time point,  
 672 normalized to time point 0] were calculated for spike in each cell line (n=3). d) S alone, or S  
 673 with proteases transiently expressed in Vero and A549 cells, cells were metabolically labeled,  
 674 and chased for the times indicated (hours). Percent cleavage was measured using band

675 densitometry in both (e) Vero and (f) A549 cells (b, c, e, f are represented as the average  $\pm$  SD  
676 for 3 independent experiments).

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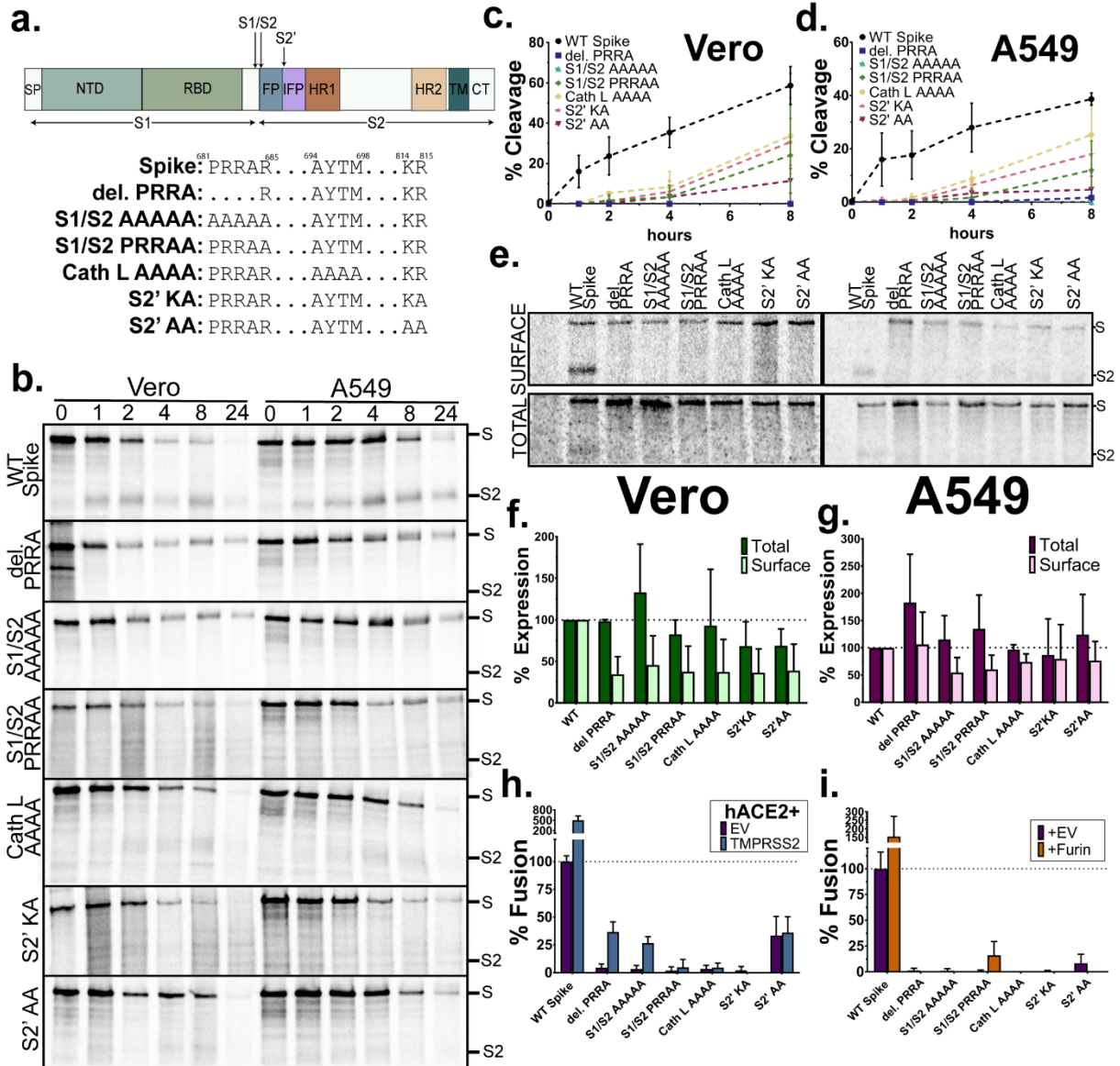


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679 **Figure 2: CoV-2 spike alone mediates cell-cell fusion.** Veros expressing S and TMPRSS2,  
 680 furin, or cathepsin L were imaged at 24 (a) and 48 (b) hpt for syncytia formation (black arrows).  
 681 Magnification bar is 100 $\mu$ M. c) A luciferase reporter gene assay was performed with target cells  
 682 (BSR/T7s expressing hACE2 and additional proteases) overlaid onto effector cells (Vero or  
 683 A549s expressing S) for 9 hours. d) Luciferase reporter gene experiment was performed with  
 684 additional proteases co-expressed with S in Veros and overlain with target cells expressing  
 685 hACE2. e) The effect of Neuropilin in both target and effector (Vero) cells was examined with a  
 686 luciferase reporter gene assay. Effector cells expression is listed along the x-axis. Target cell  
 687 expression is listed in the graph legend. Results expressed as the percent fusion normalized to  
 688 samples with S in the effector cells, and hACE2 only in the target cells (c-e are average  $\pm$  SD for  
 689 3 independent experiments, performed in duplicate). Significance was determined by two-way  
 690 ANOVA. \*:  $p < 0.05$ , \*\*\*\*:  $p < 0.0001$

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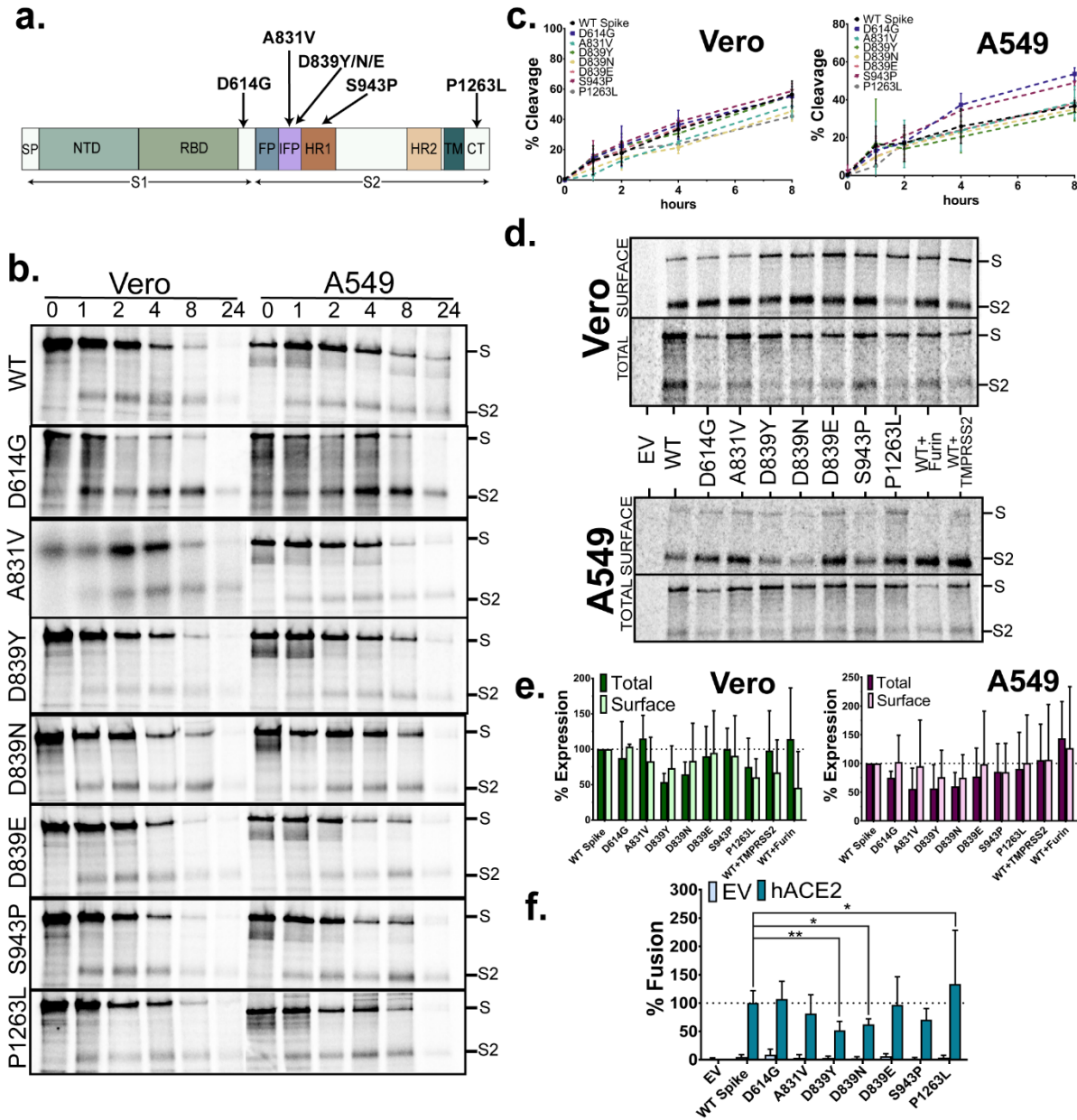
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694 **Figure 3: Mutations at all three potential spike cleavage sites reduce cleavage at the**  
 695 **S1/S2 subunit border.** a) Full or partial alanine substitution mutations were made at each of  
 696 the three potential cleavage sites. b) Plasmids expressing wt S or mutants were transfected into  
 697 Veros and A549s, cells were metabolically labeled for one hour, and chased for the times  
 698 indicated. Percent cleavage was determined in (c) Veros and (d) A549s (average  $\pm$  SD for 3  
 699 independent experiments) e) Surface biotinylation was performed on cells expressing wt S and  
 700 each mutant. Cells were radiolabeled for 6 hours. Protein expression in (f) Vero and (g) A549  
 701 cells, results are normalized to wt S, and error bars represent the standard deviation (average  $\pm$   
 702 SD for 3 independent experiments). h) A luciferase reporter gene assay was performed using  
 703 target cells expressing hACE2 and EV or TMPRSS2, and effector (Vero) cells with wt S or  
 704 each mutant. i) Luciferase reporter gene analysis with cells expressing hACE2 and effector

705 (Vero) cells transfected with S or S mutants and EV or furin expressing plasmids. Results of  
706 both reporter gene assays are shown normalized to samples with wt S in the effector with  
707 hACE2 in target cells (average  $\pm$  SD for 3 independent experiments, performed in duplicate).

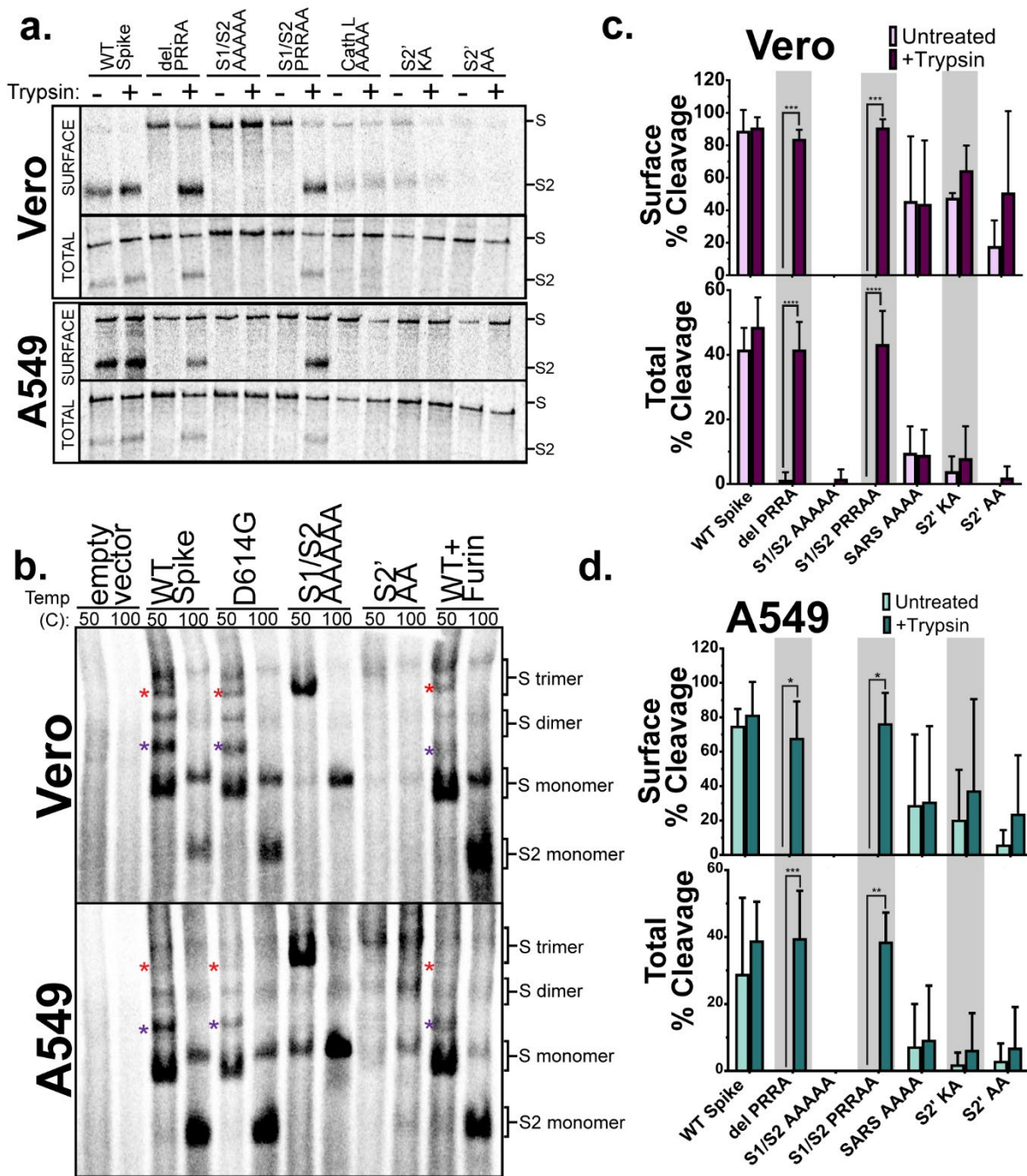
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710 **Figure 4: Spike S2 subunit mutations found in circulating strains variably affect spike**  
 711 **mediated cell-cell fusion.** a) Mutations in the S2 subunit of S identified in circulating SARS-  
 712 CoV-2 strains, b) Wt S or the mutants were transfected into Veros and A549s, metabolically  
 713 labeled for one hour, and chased for the times indicated. Percent cleavage was determined in  
 714 (c) Veros and A549s (average  $\pm$  SD for 3 independent experiments). d) Surface biotinylation on  
 715 cells expressing wt S or each mutant. e) Total and surface protein expression normalized to wt  
 716 S (average  $\pm$  SD for 3 independent experiments). f) A luciferase reporter gene assay was  
 717 performed using target cells expressing EV or hACE2, overlaid onto effector cells transfected  
 718 with wt S or each mutant. Results are normalized to samples with wt S in the effector cells and  
 719 hACE2 in target cells (average  $\pm$  SD for 3 independent experiments, performed in duplicate).  
 720 Significance was determined by two-way ANOVA, \*:  $p < 0.05$ , \*\*:  $p < 0.01$ .

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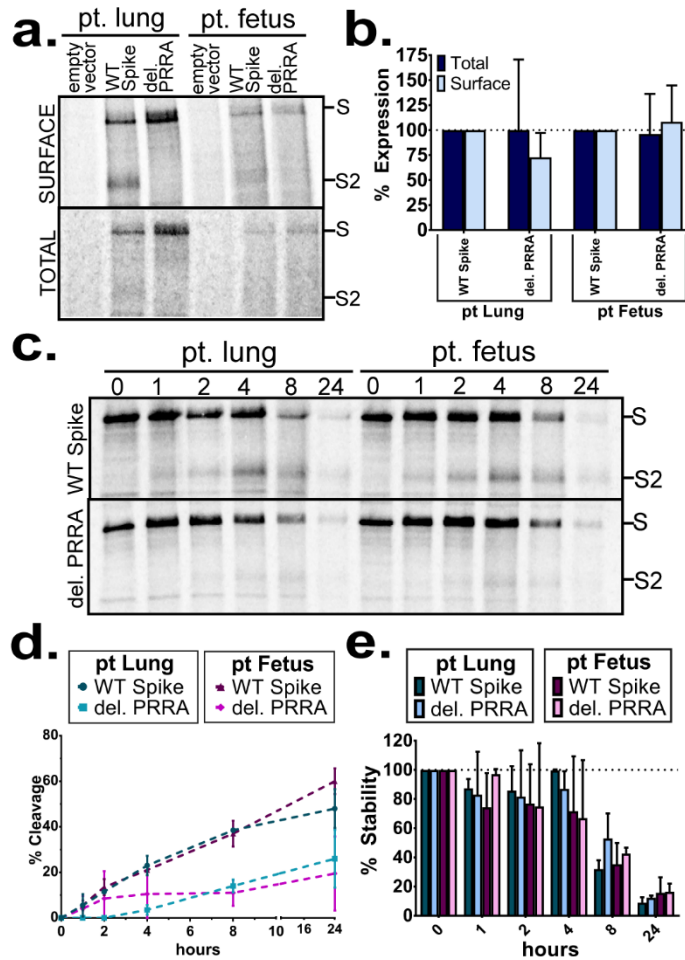
723 **Figure 5: Mutations at downstream potential cleavage sites render the S1/S2 border**  
 724 **cleavage site less accessible to proteases.** a) Veros or A549s expressing wt S or S  
 725 cleavage mutants were metabolically labeled for 6 hours. Surface proteins were biotinylated,  
 726 and samples were either treated for 10 minutes with TPCK-Trypsin or left as untreated controls  
 727 (as indicated). b) Veros or A549s expressing indicated proteins were metabolically labeled for 6  
 728 hours. Samples were treated at the indicated temperatures before separation on a nonreducing  
 729 SDS-PAGE. Oligomers are labeled on the right based on size, and colored \* represents

730 potential intermediate species (n=3). Using band densitometry to quantify the bands in (a),  
731 percent cleavage was measured in (c) Vero and (d) A549 cells for both the surface (top graphs)  
732 and total (bottom graphs) populations (average  $\pm$  SD for 3 independent experiments).  
733 Significance was determined by two-way ANOVA, \*: p<0.05, \*\*: p<0.01, \*\*\*: p<0.0005, \*\*\*\*:  
734 p<0.0001.

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738 **Figure 6: Furin or furin-like proteases in pteropus bat cells can cleave the S1/S2 border**  
739 **site of SARS-CoV-2 Spike.** a) Surface biotinylation was performed on pteropus lung and  
740 pteropus fetus cells transfected with wt S or the del. PRRA mutant. b) Surface or total protein  
741 expression levels were quantified using band densitometry and normalized to wt S levels. c) pt.  
742 lung and pt. fetus cells were transfected with wt S or del. PRRA mutant, metabolically labeled  
743 for one hour, and chased for the times indicated. Again, using band densitometry to quantify  
744 bands results were expressed as (d) protein cleavage and (e) protein stability over time. (b,d,e  
745 average  $\pm$  SD for 3 independent experiments)

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