

1 **Title: SARS-CoV-2 variants of concern have acquired mutations associated with an**
2 **increased spike cleavage**

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31

32 **Abstract**

33

34 For efficient cell entry and membrane fusion, SARS-CoV-2 spike (S) protein needs to be cleaved
35 at two different sites, S1/S2 and S2' by different cellular proteases such as furin and TMPRSS2.
36 Polymorphisms in the S protein can affect cleavage, viral transmission, and pathogenesis. Here,
37 we investigated the role of arising S polymorphisms *in vitro* and *in vivo* to understand the
38 emergence of SARS-CoV-2 variants. First, we showed that the S:655Y is selected after *in vivo*
39 replication in the mink model. This mutation is present in the Gamma Variant Of Concern (VOC)
40 but it also occurred sporadically in early SARS-CoV-2 human isolates. To better understand the
41 impact of this polymorphism, we analyzed the *in vitro* properties of a panel of SARS-CoV-2
42 isolates containing S:655Y in different lineage backgrounds. Results demonstrated that this
43 mutation enhances viral replication and spike protein cleavage. Viral competition experiments
44 using hamsters infected with WA1 and WA1-655Y isolates showed that the variant with 655Y
45 became dominant in both direct infected and direct contact animals. Finally, we investigated the
46 cleavage efficiency and fusogenic properties of the spike protein of selected VOCs containing
47 different mutations in their spike proteins. Results showed that all VOCs have evolved to acquire
48 an increased spike cleavage and fusogenic capacity despite having different sets of mutations in
49 the S protein. Our study demonstrates that the S:655Y is an important adaptative mutation that
50 increases viral cell entry, transmission, and host susceptibility. Moreover, SARS-COV-2 VOCs
51 showed a convergent evolution that promotes the S protein processing.

52

53 Introduction

54

55 SARS-CoV-2 has been spreading worldwide causing millions of infections and deaths since its
56 emergence in Wuhan, China, in late 2019. Apart from humans, ferrets, cats, dogs, Syrian golden
57 hamsters, and nonhuman primates are also susceptible to SARS-CoV-2 infection and transmission
58 (1, 2). In addition, cases of viral spread in mink farms and mink-to-human cross-species
59 transmission have been reported (3, 4). The spike (S) glycoprotein of SARS-CoV-2 is the main
60 determinant of host tropism and susceptibility, and the main target of antibody responses (5).
61 Therefore, the emergence of adaptive mutations present in the spike protein can strongly affect
62 host tropism and viral transmission (6, 7). The S protein is composed of two subunits: S1 which
63 contains the receptor binding domain (RBD) that initiates infection by binding to the angiotensin
64 converting enzyme 2 (ACE2) receptor present in the host cell surface; and the S2 subunit that
65 mediates fusion between viral and cellular membranes (8, 9). To fuse with the host cell, the S
66 protein needs to be cleaved by cellular proteases at the S1/S2 and S2' sites. Importantly, the S1/S2
67 site of SARS-CoV-2 viruses contains a multibasic furin motif ($_{681}\text{PRRXR}_{685}$) absent in other beta
68 coronaviruses (10, 11) that can be processed by furin proteases, but also by transmembrane serin
69 proteases such as TMPRSS2, or by cathepsins present in the endosomes (10, 12-15). The S1/S2
70 cleavage exposes the S2' site, and a second cleavage of the S2' is needed to release an internal
71 fusion peptide that mediates membrane fusion (16).

72

73 Since 2019, several SARS-CoV-2 lineages have emerged leading to the divergence of an extensive
74 subset of more transmissible SARS-CoV-2 variants termed Variants of Concern (VOCs). This has
75 led to the natural selection of several mutations in the spike protein with different functional
76 consequences, some of them unknown. As SARS-CoV-2 variants are raising, more research is
77 needed to understand what the drivers of evolutionary changes are over time, and the potential
78 impact on the epidemiology, antigenicity, escape from neutralizing antibodies induced by previous
79 infection and vaccination and virus fitness. The first widely adaptative substitution described was
80 the S: D614G which became dominant in March 2020 and is present in most of the variants
81 currently circulating worldwide. This substitution is known to enhance viral replication in the
82 upper respiratory tract as well as *in vivo* transmission (17, 18). Several other polymorphisms
83 became dominant in late 2020. The N501Y substitution convergently evolved in early emerging
84 VOCs Alpha (B.1.1.7), Beta (B.1.351) and Gamma (P.1) variants and has been associated with an
85 enhanced spike affinity for the cellular ACE2 receptor (19, 20). This mutation is located in the
86 receptor-binding motif (RBM) of the RBD, the primary target of many neutralizing antibodies.
87 Importantly, accumulation of mutations in the RBD can decrease neutralizing antibody responses
88 elicited by infection or vaccination against ancestral SARS-CoV-2 variants (21). Similarly, the
89 later SARS-CoV-2 Kappa (B.1.617.1) and Delta (B.1.617.2) variants have also shown a
90 significantly reduced sensitivity to convalescent and immune sera (22-24). Other mutations outside
91 the RBD have also become prevalent. A clear example is the polymorphism found at position
92 S:681 in the furin cleavage site, which includes P681H and P681R in the Alpha and Kappa/Delta
93 variants, respectively. Some preliminary reports have pointed to an enhancement in virus
94 transmissibility associated with this polymorphism, perhaps due to an increase of spike cleavage
95 (25). Additionally, several other mutations have been identified at the edge of the furin cleavage
96 site. This is the case of the H655Y substitution found in the Gamma (P.1) variant. This mutation
97 was associated with changes in antigenicity by conferring escape from human monoclonal
98 antibodies (26). Moreover, it has also been found to be selected in animal models after

99 experimental infection *in vivo* (27, 28), indicating a potential role in host replication,
100 transmissibility, and pathogenicity.

101
102 Here, we characterized emerging SARS-CoV-2 spike polymorphisms *in vitro* and *in vivo* to
103 understand their impact on transmissibility, virus pathogenicity and fitness. Using the mink model
104 of COVID-19, we found that the S:H655Y substitution was acquired *in vivo* after infection with
105 the WA1 isolate (USA-WA1/2020). To investigate the advantage conferred by S:H655Y, we
106 analyzed the kinetics, spike processing by cellular proteases and syncytium formation ability of a
107 panel of SARS-CoV-2 variants harboring 655Y, including human isolates derived from patients
108 seeking care at the Mount Sinai Health System in New York (NY) City which was one of the major
109 early epicenters of COVID-19 pandemic. Our results demonstrated that the 655Y polymorphism
110 enhances spike cleavage and viral growth. Furthermore, the S:655Y substitution was transmitted
111 more efficiently than its ancestor S:655H in the hamster infection model. Finally, and in the context
112 of the current epidemiological situation, we analyzed a set of emerging SARS-CoV-2 variants to
113 investigate how different sets of mutations may impact spike processing. We demonstrated that
114 novel circulating VOCs that became more prevalent have independently acquired mutations
115 associated with a gain in spike cleavage and syncytia formation. Taken together, our study shows
116 a link between an increased spike processing and increased virus transmission due to spike
117 mutations present in SARS-CoV-2 variants that become epidemiologically more prevalent in
118 humans.

119 120 **Results**

121 122 SARS-CoV-2 VARIANTS HARBORING 655Y SHOW AN ADVANTAGE IN REPLICATION 123 AND ENHANCED SPIKE PROTEIN CLEAVAGE *IN VITRO*, AND IN TRANSMISSION *IN* 124 *VIVO*

125
126 Minks have been suggested to play a role in the initial local spread and evolution of SARS-CoV-
127 2 variants in different countries in Europe (3, 4). While minks are susceptible to SARS-CoV-2,
128 they are also capable for zoonotic transmission of SARS-CoV-2 to because of the similarity of the
129 ACE2 receptor between minks and humans. We used the mink model to investigate the replication
130 and pathogenicity of the WA1 (USA-WA1/2020) isolate of SARS-CoV-2, as a representative of
131 the first original human viruses that initiated the SARS-CoV-2 pandemic. This variant corresponds
132 to one of the first USA isolates and does not contain any changes on the S protein when compared
133 to the initial isolates from Wuhan, such as the Wuhan-1 virus. For this purpose, six minks were
134 intranasally infected with 10^6 pfu of WA1 isolate resulting in productive viral replication in the
135 upper respiratory tract with infectious virus recovered from nasal washes at days 1, 3 and 5 post-
136 infection (Supplementary Figure 1A-B). At day 4 post-inoculation, infectious virus was detected
137 by plaque assays from left cranial lung and nasal turbinates but not from any of the other tissues
138 analyzed (Supplementary Figure 1C). We then selected small and large viral plaques in the Vero-
139 E6 cell-based plaque assays from infected mink lung specimens and performed next generation
140 sequencing of the genome from the plaque-isolated viruses. As compared to the Wuhan-1 and
141 WA1 reference sequences, all mink-derived viral isolates encoded the H655Y amino acid
142 substitution within the spike (S) (Figure 1A). Additionally, the three viral isolates with the small
143 plaque phenotype encoded the T259K amino acid substitution while the three viral isolates with
144 the large plaque phenotype encoded the R682W amino acid substitution. It is known that

145 S:682W/Q substitution in the furin cleavage site region may emerge after subsequent passages in
146 VeroE6 cells (29, 30). Therefore, this mutation may have been selected during the course of the
147 VeroE6 infections and not during the infection in minks. On the other hand, S:655Y appeared
148 dominant in all the mink isolates, indicating that this mutation may confer an advantage in the
149 mink host.

150
151 To understand the magnitude and the spread of the 655Y polymorphism over time, we investigated
152 the frequency of S:655Y over time in sequences sampled worldwide since the initial outbreak to
153 the end of the first wave of SARS-CoV-2 (Figure 1B). For this, 7,059 sequences sampled from
154 GISAID up to September 2020 were used. Human variants harboring the 655Y mutation were
155 spread throughout the phylogenetic tree and distributed in all clades with no differences according
156 to temporal distribution, suggesting that the 655Y mutation arose independently multiple times.
157 Remarkably, the S:H655Y polymorphism was also found among the initial variants introduced in
158 New York (NY) City in March 2020. To determine the replication phenotype, we decided to
159 investigate this NY 655Y variant (NY7) together with some of its contemporaneous SARS-CoV-
160 2 isolates circulating in New York during the early pandemic outbreak (31). To this end, we
161 isolated 12 viruses based on their genotypes (31), including NY7 which carries the S:655Y
162 mutation for culture directly from nasopharyngeal specimens obtained from COVID-19 infected
163 patients. Of note, the dominant 614G spike polymorphism was present in seven (58%) of the
164 selected human SARS-CoV-2 (hCoV) NY isolates consistent with its early emergence and rapid
165 spread worldwide (17, 18). Confirmation sequencing of the isolates showed that 682W/Q
166 substitutions appeared in four (33%) viruses after initial isolation and culturing in VeroE6 cells.
167 This is consistent with *in vitro* adaptative mutations previously described (30). Moreover, a five
168 amino acid sequence (Δ 675-679) flanking the furin cleavage site was deleted in five (42%) of the
169 isolates as compared to the sequence from the original specimen. This deletion has been previously
170 reported to be a common *in vitro* mutation selected in Vero cells (29). Amino acids substitutions
171 of the S protein of these initial human isolates compared to the Wuhan-1 reference are shown
172 Figure 1C. We next studied the replication kinetics of the NY SARS-CoV-2 isolates by comparing
173 their multicycle growth curves at an MOI of 0.01 in VeroE6 and human Caco-2 cells. As expected,
174 NY2 and NY4 containing the 682Q/W showed an advantage in VeroE6, while no differences could
175 be found in Caco-2 cells (Supplementary Figure 2A-B). Remarkably, NY7 (S:655Y) showed
176 higher growth at 48 hours post-infection (p.i) in Caco-2 cells (Figure 1D, Supplementary Figure
177 2B) when compared to the rest of these early SARS-CoV-2 isolates. These results support our
178 conclusion that the 655Y polymorphism conferred a viral advantage. To investigate the spike
179 cleavage efficiency of the 655Y versus other human isolates, we performed infections in VeroE6,
180 and supernatants were analyzed by Western blot for the S2 domain of the S protein. Importantly,
181 two bands were clearly visible for the NY7 (S:655Y) (Figure 1E), corresponding to both the
182 cleaved (95 kDa) and uncleaved (180 kDa) form of the S protein. In contrast, only the uncleaved
183 S form was detected in the other early human isolates indicating that the 655Y polymorphism may
184 facilitate S protein processing.

185
186 To confirm whether the 655Y mutation was solely responsible for the increased S cleavage, we
187 analyzed the replication and cleavage efficiency of a panel of SARS-CoV-2 viruses, all bearing
188 the 655Y substitution but containing additional substitutions across the genome. We included two
189 of the isolated mink variants (MiA1 and MiA2); NY7 and another human isolate derived from a
190 COVID patient infected in February 2021 (NY13, S:614G, 655Y); and a previously published

191 WA1-655Y variant isolated after wild type WA1 infection in cats (32). Additionally, the WA1
192 reference and NY6 were used as controls since they lack the 655Y substitution. It should be noted
193 that NY6 has a five amino acid deletion before the furin cleavage site (Figure 1C and 2A). We
194 assessed differences in replication and S processing of this panel of viruses by comparing growth
195 in both VeroE6 and Vero-TMPRSS2 cells. As shown in Figure 2B, WA1-655Y infection yielded
196 higher titers in both VeroE6 and Vero-TMPRSS2 cells as compared to infection by WA1. These
197 isolates only differ in the position 655Y while the rest of the genome is isogenic, supporting that
198 655Y spike polymorphism enhances viral replication and growth. Next, viral supernatants were
199 used to analyze the plaque phenotype in VeroE6 and Vero-TMPRSS2 and to compare S protein
200 expression levels after infection. In general, all isolates showed higher plaque size in the presence
201 of TMPRSS2, consistent with enhancement of cell entry (Figure 2C). However, differences were
202 found by Western blot and only the isolates bearing 655Y showed enhanced spike cleavage in both
203 VeroE6 and Vero-TMPRSS2 (Figure 2D). Finally, and to investigate the ability to induce syncytia,
204 we infected Vero-TMPRSS2 at an MOI of 0.01 with the mink (MiA1) and human isolates (NY7,
205 NY13 and NY6) and used specific antibodies to detect the S and N protein, as well as nuclei
206 staining with DAPI after 24 p.i. by immunofluorescence microscopy. As shown in Figure 2E, the
207 isolates harboring the 655Y polymorphism showed a slight increase in syncytia formation as
208 compared to NY6 (S:Δ675-679).

209
210 Syrian golden hamsters are a recognized rodent model to investigate infection and transmission of
211 SARS-CoV-2 (33, 34). To test whether the S:655Y polymorphism enhances viral transmission,
212 five pairs of Syrian golden hamsters were placed in individual cages to perform viral competition
213 experiments. For this, one hamster of each pair was infected intranasally with 10^5 pfu of a mix of
214 WA1 and WA1-655Y viruses at a one-to-one ratio (Figure 3A). Direct infected (DI) and direct
215 contact (DC) hamsters were euthanized after day 5 and 7 post-infection, respectively, and lungs
216 and nasal turbinates were harvested for subsequent viral titer quantification. In addition, nasal
217 washes were collected on day 2 and 4 in both DI and DC, and on day 6 p.i. of DC animals. One of
218 the DI hamsters died after nasal wash collection at day 2, leaving 4 animals subjected to follow up
219 in the DI group. Hamsters were also monitored daily for body weight loss. After 2 days p.i. DC
220 hamsters exhibited a decrease in weight indicating early viral transmission from infected animals
221 (Figure 3B). This observation was further supported by detection of infectious virus in nasal
222 washes at 2 days p.i. in both DI and DC hamsters. At day 4 p.i., viral titers were detected in 3 out
223 of 4 animals and at day 6 p.i., viral replication was not detected in two of the DC nasal wash
224 samples (Figure 3C). In general, we observed a decrease over time in the infectious virus present
225 in nasal washes from DI and DC animals. We then determined the relative abundance of S:655Y
226 on the viral RNA present in the nasal washes by next-generation sequencing (Figure 3D). The
227 consensus RNA sequence from all DI hamsters contained the S:655Y polymorphism suggesting
228 that WA1-655Y virus was able to overcome the wild type WA1 isolate during infection. Similarly,
229 S:655Y was present in all the nasal washes collected from four DC hamsters indicating an
230 advantage conferred by this mutation in viral transmission. However, one DC animal (hamster 4-
231 C) showed a decrease of 655Y abundance over time. Interestingly, this hamster lost less weight
232 when compared to the rest of the animals. Next, we analyzed the viral growth in lungs and nasal
233 turbinates from DI (collected at day 5 p.i.) and DC (collected at day 7p.i.) hamsters (Figure 3E).
234 No differences were found in viral titers in the tissues from both animal groups. However, we
235 observed that three DC hamsters had lower lung titers compared to the rest of the animals. These
236 same hamsters also exhibited low viral loads in the nasal turbinates. We then sequenced the viral

237 RNA present in these tissues (Figure 3F-G). The RNA from one lung and nasal turbinate of one
238 DI and DC hamster could not be amplified by specific PCR for downstream sequencing. Figure
239 3F-G shows that all lungs and nasal turbinate tissues from DI animals analyzed had the S:655Y
240 mutation. In addition, S:655Y was present in 75% of the nasal turbinates and lungs from DC
241 animals. Taken together, our results demonstrate that 655Y polymorphism increases spike
242 cleavage and viral growth. Moreover, viral competition and transmission experiments showed that
243 S:655Y became predominant in both DI and DC animals indicating that this mutation plays a role
244 in viral transmissibility.

245

246 SARS-CoV-2 VARIANTS EVOLVE TO ACQUIRE AN INCREASED SPIKE CLEAVAGE 247 AND FUSOGENIC ABILITY

248

249 Current circulating SARS-CoV-2 VOCs bear novel spike polymorphisms that correlate with an
250 enhanced human transmission (19) and reduced antibody neutralization (22, 35). Interestingly, the
251 Gamma variant (lineage P.1) which emerged in Brazil in November 2020, harbors the amino acid
252 spike substitution H655Y. In the context of the evolving epidemiological situation, we decided to
253 investigate whether the co-emergence of different selective mutations in some representative VOC
254 had a similar phenotype to SARS-CoV-2 viruses harboring the S:655Y. We first estimated the
255 amino acid substitution frequencies around the cleavage site region (655 to 701) from globally
256 available data (2,072,987 sequences deposited in GISAID Database up to 28 June 2021). As
257 expected, the P681H/R, H655Y and A701V substitutions showed high prevalence since they are
258 harbored by the main VOCs lineages. Additionally, the less prevalent Q675H and Q677H were
259 also found to be a highly variable position and present in widely circulating variants (36). We then
260 spatially and structurally mapped these amino acid changes within and surrounding the furin
261 cleavage sequence of the S protein (Figure 4A). The 655 position was located in close proximity
262 to the furin cleavage site. Next, we performed a phylogenetic analysis of sequences sampled
263 worldwide from February 2020 to June 2021 to illustrate the temporal distribution and
264 phylogenetic relationship of the high prevalent S mutations (Figure 4B). For this, a sample set of
265 13,847 sequences deposited in GISAID up to June 2021 were analyzed. While the H665Y
266 frequency was higher in the Gamma lineage (P.1), it could be found also in 19B clade (Figure 4C),
267 in line with our identified NY7 isolate. The P681H substitution located in the furin cleavage site
268 of the spike protein was identified in the Alpha variant that emerged in September 2020.
269 Interesting, this mutation was also found in the Theta variant, first detected in February 2021
270 (Figure 4D). In contrast, Kappa and Delta variants harbor polymorphism P681R (Figure 4D).
271 Finally, the frequency of A701V mutation was higher in Beta and Iota variants which emerged in
272 October and November 2020, respectively (Figure 4E).

273

274 Next, we analyzed the *in vitro* phenotype of some of the most prevalent SARS-CoV-2 VOCs.
275 Multiple protein sequence alignment of the VOCs used are shown in Figure 5A. In contrast to the
276 NY isolates previously analyzed, VOCs showed a considerable number of unique changes across
277 the whole spike protein. However, all of them showed similar replication kinetics and plaque
278 phenotypes in Vero-TMPRSS2 cell monolayers. Conversely, clear differences were observed
279 when replication efficiency was determined in VeroE6 cells at 24 hours post-infection (p.i.)
280 (Figure 5B). Additionally, viral supernatants were titrated on both VeroE6 and Vero-TMRPSS2
281 cells (Figure 5C). Substantial differences in plaque phenotypes were observed, especially for the
282 Kappa (B.1.617.1) and Delta (B.1.617.2) variants, when TMRPSS2 was present. Thus, these late

283 VOCs that emerged late in the COVID-19 pandemic might strongly depend on the presence of
284 TMRPSS2 to establish optimal infection *in vitro*. Last, we investigated the extent of the spike
285 cleavage of these VOCs. For this, infections were performed in VeroE6 and Vero-TMPRSS2 at
286 an MOI of 0.01 and viral supernatants were analyzed after 48 hours post-infection (p.i.) by Western
287 blot (Figure 5D). N protein was used as a control for viral replication and loading. Similarly, WA1
288 was included as a reference since no selective mutations are found in the S protein. Figure 5D
289 shows similar cleaved and uncleaved S protein levels for all the SARS-CoV-2 VOCs in the
290 presence of TMRPSS2 expressed in Vero-TMPRSS2 cells. In contrast, only Beta (B.1.351) and
291 Gamma (P.1), exhibited an increased spike cleavage when the infections were performed in wild
292 type VeroE6 cells. Interestingly, the spike and nucleocapsid expression of Kappa (B.1.617.1) and
293 Delta (B.1.617.2) variants was not detectable by Western blot analysis of VeroE6 supernatants.
294 The canonical cleavage at the S1/S2 site occurs at the last arginine (R) of the multibasic PRRAR
295 motif and is performed by furin proteases at this specific residue. Thus, we next quantified the
296 abundance of furin-cleaved peptide of VOCs in Vero-TMPRSS2 cell supernatants by targeted
297 mass spectrometry. Vero-TMPRSS2 cells were infected at an MOI of 0.1 with the indicated VOCs
298 and NY7 (S:H655Y) and WA1-655Y isolates. WA1 and NY6 were used as controls. Cell extracts
299 were collected after 24 hours post-infection and samples were prepared. The abundance of the C-
300 terminal peptide resulting from endogenous furin cleavage at the terminal arginine (PRRAR \
301 SVASQSIAYTMSLGAE) was quantified as a proxy of cleavage efficiency since this peptide is
302 common for all SARS-CoV-2 VOCs, except for the Beta that contained a V instead of A at the
303 end of the peptide (SVASQSIAYTMSLGVE). Fold change peptide-level abundance for each
304 variant compared to WA1 control was calculated and plotted in Figure 5E. Isolates WA1-655Y,
305 NY7 and Gamma, all of them harboring the 655Y mutation, and Beta harboring 701V, showed the
306 higher abundance of furin-cleaved peptide. Conversely, lower levels of C-terminal cleaved peptide
307 were found for the VOCs harboring the 681H/R amino acid change suggesting that an introduction
308 of an amino acid change at this position might modify the canonical cleavage residue at the last R
309 of the furin cleavage site. Nonetheless, when we assessed the fusogenic capacity of the S protein
310 of VOCs by immunofluorescence microscopy of infected Vero-TMPRSS2 cells, we found strong
311 syncytium formation induced by all the variants (Figure 5F). Interestingly, extensive fusogenic
312 capacity was also exhibited by Delta and Kappa variants (Figure 5F-G) consistent with abundant
313 cleaved S form found by Western blot (Figure 5D). Because cleavage at the multibasic furin motif
314 is believed to be required for optimal syncytium formation (10), we finally compared the ability
315 to induce cell fusion by the Kappa variant and a mutated form lacking amino acids at the furin
316 cleavage site (S: Δ 678-682). This Δ 678-682 Kappa was obtained after consecutive passage and
317 culturing in VeroE6 cells. As shown in Figure 5G, a loss of fusogenic activity was observed when
318 compared to the intact Kappa VOC. Altogether, our results are consistent with the notion that
319 current highly transmissible circulating VOCs have evolved independently to acquire mutations
320 associated with increased spike protein processing and transmission.

321

322 Discussion

323

324 Emerging SARS-CoV-2 VOCs contain novel spike polymorphisms with unclear functional
325 consequences on epidemiology, viral fitness, and antigenicity. In this study, we evaluated the
326 impact of different spike mutations on viral infection, pathogenicity, and *in vivo* transmission. We
327 found that in the mink animal model the 655Y spike substitution is selected after infection with
328 the WA1 isolate. Phylogenetic analysis of genome sequences collected worldwide showed an early

329 sporadic appearance of S:655Y during the first pandemic wave in New York in March 2020, and
330 the presence of this mutation in several posterior lineages, including SARS-CoV-2 Gamma
331 variant, pointing to a potential role in adaptation and evolution. To better understand the impact of
332 this polymorphism, we isolated and *in vitro* characterized a panel of SARS-CoV-2 viruses bearing
333 the 655Y spike mutation. Our results demonstrated that S:655Y enhances the viral growth and the
334 spike protein processing required for optimal cell entry and viral-host membrane fusion. In
335 addition, we performed viral competition and transmission experiments in the hamster animal
336 model and showed that S:655Y became predominant in both direct infected and direct contact
337 animals. Finally, we showed that VOCs converge to gain spike cleavage efficiency and fusogenic
338 potential.

339
340 Here, we demonstrate that viruses containing the H655Y polymorphism confer a growth advantage
341 in both VeroE6 and human-like Vero-TMPRSS2 cells. Interestingly, the early human isolate NY7
342 harboring the 655Y mutation also showed higher replication in human Caco-2 cells. However, it
343 is known that other mutations outside of the S gene could be impacting viral replication and
344 infection (37, 38). Therefore, we confirmed the S:655Y mutation alone was responsible for the
345 enhanced growth and spike cleavage phenotype when comparing WA1 wild type and WA1-655Y
346 isolates. These variants have the same viral protein amino acid sequence except for the amino acid
347 present at position 655 of the spike. Since most of the isolates used in this study contain a
348 constellation of mutations across the genome that could increase viral fitness, comparison of both
349 viruses in parallel allowed to detect differences in growth and spike cleavage that can be attributed
350 only to 655Y polymorphism. S:655Y is present in the S1 spike domain outside of the RBD and
351 has been associated with a decrease of the neutralizing activity when targeted by some monoclonal
352 antibodies(26). However, H655Y has been also naturally selected in cats and mice suggesting a
353 beneficial impact of this substitution in widen viral host range and susceptibility (27, 28). Our data
354 further supports this argument because we also found that S:655Y is selected after replication in
355 minks, a natural host for SARS-CoV-2. Besides, when we assessed the viral transmission
356 efficiency of 655Y versus the ancestor 655H in competition experiments in the hamster model, we
357 also found that 655Y becomes more prevalent, as the bulk of infectious viruses recovered from the
358 infected animals harbored this mutation, except for one hamster. This indicates that S:655Y can
359 overcome S:655H *in vivo*.

360
361 Intense worldwide surveillance has established that SARS-CoV-2 variants are constantly
362 emerging. In particular, the spike protein has shown high plasticity (6). Most of the spike mutations
363 associated with a decrease in neutralization by antibodies against earlier viruses are located in the
364 RBD or N-terminal domain (NTD), which are critical for binding and interacting with the ACE2
365 cellular receptor. While mutations at these domains may impact SARS-CoV-2 vaccine efficacy, it
366 is also vital to characterize other mutations that might explain the gain in transmissibility observed
367 for the VOCs. Since the Gamma variant that emerged in November 2020 also harbors the 655Y
368 polymorphism (Figure 5A), we decided to investigate its phenotype *in vitro*. Similar to the earlier
369 S:655Y isolates, this variant also exhibited an increase in spike processing efficiency. More
370 importantly, this phenotype was also confirmed in all emerging VOCs analyzed when infections
371 were performed in the Vero-TMPRSS2 cells indicating that additional mutations within S confer
372 this advantage. Most likely, the spike mutations P681H in Alpha variant -first identified in United
373 Kingdom- and P681R harbored by Kappa and Delta variants -first emerged in India- allowed this

374 enhanced S cleavage. Interestingly, for these variants, optimal cleavage appeared to be dependent
375 on TMPRSS2 protease activity (Figure 5D).

376
377 To confirm the cleavage at the putative furin cleavage site, we determined the relative abundance
378 of the furin cleaved peptide produced after the 685-terminal arginine. We observed higher amount
379 of cleavage at this position as compared to the previous circulating viruses, although lower
380 amounts were detected in Alpha, Kappa and Delta variants as compared to the viruses harboring
381 the 655Y mutation. This suggests that a change in residue 681 may introduce an additional
382 cleavage site, perhaps recognized by TMPRSS2 protease that enhances spike cleavage of these
383 variants and produces an additional cleavage peptide different in size and amino acid sequences.
384 Further research is needed to confirm the existence of a recognition site for additional proteases
385 different than furin in the amino acid motif SH/RRRAR when the P681S/H mutation is present. In
386 any case, all the VOCs analyzed proved to be strong syncytia inducers which could potentially
387 indicate a role in pathogenesis and lung damage mediated by TMPRSS2 activity after infection in
388 humans (39). On the other hand, the Beta variant, which was first identified in South Africa in
389 October 2020, does not contain a change in the furin cleavage site or in the spike position 655, but
390 instead a change in the residue found at position 701. Although this residue is found around 20
391 amino acids away from the furin cleavage motif, we found similar results when the extent of the
392 spike processing was investigated (Figure 4A-E; 5D-G). It is important to note that the VOCs
393 investigated in here independently acquired S mutations around the furin cleavage site that became
394 epidemiologically more prevalent in humans. When we investigated the spatial distribution by
395 superimposition of the crystal structure of the S protein, we found that these highly prevalent
396 polymorphisms were all located in close proximity to the furin site loop (Figure 4A). Any
397 substitution in this protein domain is likely to have an effect on the structural integrity and
398 dynamics, potentially impacting the accessibility of the polybasic site to the relevant protease and
399 likely facilitating the recognition by furin.

400
401 In summary, our study demonstrates that the 655Y spike polymorphism, present in the Gamma
402 VOC, is a key determinant of SARS-CoV-2 infection and transmission. The selection and
403 increasing frequency of S:655Y in the human population and following SARS-CoV-2 infection of
404 different animal models such as cats, mice and minks suggests this mutation is associated with an
405 improvement of viral fitness and adaptation to diverse hosts through an increased cleavage of the
406 spike protein. Additionally, we provide evidence of adaptative mutations that SARS-CoV-2 VOCs
407 have been acquired and are associated with an increased spike protein processing. This has
408 significant implications in the understanding of the viral determinants that can impact viral
409 transmissibility, viral evolution, and possibly SARS-CoV-2 antigenicity and pathogenicity.

410
411
412
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417
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453
454
455 **Author contributions:** TA and AGS conceived, designed, and supervised the study. TA provided
456 training to AE. AE performed most of the experiments including growth of viral stocks, viral
457 infections and titration of growth curves, plaque phenotype analysis, sample preparation for
458 western blot and mass spectrometry analysis, western blot experiments, RT-qPCR and infectivity
459 analysis, immunofluorescence with the help of TA (viral isolation, plaque assay and plaque
460 purification of viruses, immunofluorescence imaging acquisition), SA (titration by plaque assay
461 analysis) and ML (immunofluorescence staining). AGR and AVG performed experiments for
462 whole-genome and spike-specific sequencing. AGR performed genome assembly, variant calling,
463 global phylogenetic and variant-prevalence analyses. IM provided Gamma VOC viral stock and
464 performed the hamster competition experiment with the help of RP, ML and AE. RA and RP
465 performed the mink infections, titration by plaque assay and mink-adapted S:655Y (MiA) viral

466 purification and isolation experiments. AF performed the targeted proteomics and analyzed the
467 targeted proteomics data. RR provided Alpha and Beta VOCs viral stocks. TK provided mink
468 selected (MiA1 and MiA2) viral stocks. DAM, VB, CM, and JAR isolated and provided the WA1-
469 655Y cat selected-variant. VS and EMS provided human nasopharyngeal swabs from SARS-CoV-
470 2 infected individuals. HA isolated SARS-CoV-2 viruses from human nasopharyngeal swabs. GB
471 provided the spike structural analysis figure. MB, LZA, NK and HVB provided methods and
472 expertise. AE and TA analyzed data, wrote the manuscript, and prepared the figures. All the
473 authors reviewed and edited the manuscript.

474
475 **Competing interests:** The A.G.-S. laboratory has received research support from Pfizer, Senhwa
476 Biosciences, Kenall Manufacturing, Avimex, Johnson & Johnson, Dynavax, 7Hills Pharma, N-
477 fold LLC, Pharmamar, ImmunityBio, Accurius, Nanocomposix, Hexamer and Merck, outside of
478 the reported work. A.G.-S. has consulting agreements for the following companies involving cash
479 and/or stock: Vivaldi Biosciences, Contrafect, 7Hills Pharma, Avimex, Vaxalto, Pagoda,
480 Accurius, Esperovax, Farmak, Applied Biological Laboratories and Pfizer, outside of the reported
481 work. A.G.-S. is inventor on patents and patent applications on the use of antivirals and vaccines
482 for the treatment and prevention of virus infections, owned by the Icahn School of Medicine at
483 Mount Sinai, New York. The Icahn School of Medicine at Mount Sinai has filed a patent
484 application relating to SARS-CoV-2 serological assays, which lists Viviana Simon as co-inventor.
485 Mount Sinai has spun out a company, Kantaro, to market serological tests for SARS-CoV-2.

486
487 **Data availability:** All data is available in the manuscript or the supplementary materials. The
488 targeted mass spectrometry data including raw files, Skyline document and resulting transition
489 lists via ProteomeXchange with identifier PXD027641. Reagents used are almost exclusively
490 commercially available and non-proprietary.

491

492 **Figure 1. Mink and human SARS-CoV-2 variants bearing the 655Y polymorphism.** A)
493 Multiple sequence alignment of the spike (S) protein from SARS-CoV2 viruses isolated after
494 infection in minks with WA1 isolate. Diagram shows the corresponding S amino acid substitutions
495 mapped to the S gene. B) Time-calibrated phylogenetic analysis of the global distribution of
496 H655Y substitution during the early SARS-CoV-2 outbreak. The phylogenetic tree was generated
497 with Nextstrain with 7059 genomes sampled for representation of the H655Y substitution over
498 time of worldwide data deposited in the GISAID database from December 2019 to September
499 2020. C) Multiple sequence alignment of the S protein from SARS-CoV2 viruses isolated from
500 nasal swabs collected during the first pandemic wave in NY. Diagram shows the corresponding S
501 amino acid substitutions mapped to the S gene. D) Viral growth of the NY7 containing the 655Y
502 (red) versus its ancestors 655H (grey) in VeroE6 and Caco-2 cells. Cells were infected at an MOI
503 of 0.01 and supernatants were titrated at the indicated hours post-infection (p.i.) and expressed as
504 plaque forming units per milliliter (PFU). Means and SD are shown for the NY isolates containing
505 655H. ANOVA test was performed to compare mean differences within each group at different
506 time points. Statistical significance was considered when $p \leq 0.05$ (****, $p < 0.0001$). E) Western
507 blotting of spike protein cleavage from supernatants of VeroE6 infected cells. Infections were
508 performed at an MOI of 0.01 and supernatants were collected at 48 hours p.i. Full length (FL)
509 spike protein (180 kDa), S2 cleaved spike (95 kDa) and Nucleocapsid (N, 50 kDa) were detected
510 using specific antibodies. Levels of N protein were used as loading control.

511
512 **Figure 2. The H655Y amino acid substitution enhances spike cleavage and viral growth.** A)
513 Multiple alignment of the amino acid sequences around the 655 position and S1/S2 and S2'
514 cleavage sites from a panel of mink-adapted, cat, early human SARS-CoV-2 New York (NY)
515 variants and WA1 virus. Wuhan1 is included as a reference. Red arrows indicate the location of
516 S1/S2 and S2' cleavage sites. Spike polymorphisms present across the S gene for each SARS-
517 CoV-2 isolate are also shown. B) Replication kinetics of early SARS-CoV-2 viruses in Vero and
518 Vero-TMPRSS2 cells. Infection was performed at an MOI of 0.01. Viral titers were determined
519 by plaque assay at the indicated hours post-infection and expressed as PFU per milliliter. Color
520 codes relate to the isolates shown in A. C) Plaque phenotype according to TMPRSS2 expression
521 in Vero cells. The same viral supernatant was used to infect Vero and Vero-TMPRSS2 cells.
522 Plaques were developed by immunostaining. D) Western blotting of S protein from supernatants
523 of Vero and Vero-TMPRSS2 infected cells. Infections were performed at an MOI of 0.01 and viral
524 supernatants were collected at 48 hours post-infection (p.i.). Full length (FL) spike protein (180
525 kDa), S2 cleaved spike (95 kDa) and Nucleocapsid (N, 50 kDa) were detected using specific
526 antibodies. Levels of N protein were used as loading control. E) Immunofluorescence of SARS-
527 CoV-2 S and N protein localization in Vero-TMPRSS2 infected cells at an MOI of 0.01 and 24
528 hours p.i. Spike protein was detected using a specific monoclonal antibody 3AD7 (green), N
529 protein was detected using a polyclonal antiserum (red) and 4',6-diamidino-2-phenylindole
530 (DAPI) was used to stain the nucleus.

531
532 **Figure 3. The 655Y polymorphism prevails over the 655H in the transmission *in vivo* model.**
533 A) Ten 3-weeks-old female Syrian hamsters were placed in pairs. Only one hamster per cage was
534 infected intranasally with a total of 10^5 pfu of SARS-CoV-2 WA1 and WA1-655Y isolates in a
535 one-to-one ratio. Nasal washes were collected at day 2, 4 and 6 post-infection (p.i.). Lungs and
536 nasal turbinates were harvested from direct-infected (DI) and direct-contact (DC) hamsters at day
537 5 and 7 p.i., respectively. B) Body weight change of individual hamsters over time. C) Viral titers

538 of nasal washes expressed as PFU per milliliter. Error bars indicate SDs. D) Relative abundance
539 of 655Y mutation in the RNA from nasal washes in the DI and DC hamsters. The y axis shows the
540 percentage of 655Y polymorphism in the total good quality sequencing reads from each biological
541 RNA sample and the x axis indicates the day p.i. samples were collected. E) Viral titers of lungs
542 and nasal turbinates expressed as PFU per gram of tissue. Error bars indicate SDs. Titters of DI
543 and DC hamsters are shown at day 5 and 7 p.i., respectively. F-G) Proportion of hamsters with
544 655Y (blue) and H (green) in the nasal turbinates and lungs from DI and DC as confirmed by next
545 generation sequencing.

546
547 **Figure 4. Global epidemiology of SARS-CoV-2 variants of concern (VOCs).** The amino acid
548 substitution frequencies around the cleavage site region (655 to 701) from globally available data
549 (2,072,987 sequences deposited in GISAID Database as of 28 June 2021) was estimated. A) Shows
550 the high prevalent mutations identified mapped onto the structure of the S glycoprotein. The model
551 was generated by superposition of PDB 6M0J and 7C2L (40, 41). One RBD in the up conformation
552 (red) is bound with ACE2 receptor (pink). The NTD is colored blue, the amino-acid substitutions
553 are shown as gold spheres and the furin cleavage loop (disordered and therefore missing in most
554 atomic models) is flanked with cyan spheres. One spike protomer is shown in bold colors while
555 the other two are colored white. A zoomed-in image of the region of interest and the sequence of
556 the furin site loop is also shown. Amino-acid residues of interest are highlighted in gold. B) Time-
557 calibrated phylogenetic tree of SARS-CoV-2 circulating variants illustrating the temporal
558 distribution and phylogenetic relationships of the most prevalent S mutations along the S1/S2
559 region (highlighted in color). The phylogenetic tree was generated using NextStrain and analysis
560 was performed using a sample of 13,847 genomes focused on the most prevalent substitutions
561 between S:655 and S:701 between February 2020 and June 2021 from GISAID database. C-E)
562 Frequency per clade of H655Y, P681H/R and A701V spike polymorphisms.

563
564 **Figure 5. SARS-CoV-2 VOCs evolve to a convergent phenotype associated to an increase on**
565 **S cleavage and fusogenicity.** A) Multiple alignments of the S protein of the indicated SARS-
566 CoV-2 VOCs. Diagram shows the corresponding S amino acid substitutions mapped to the S gene.
567 B) Viral growth of SARS-CoV-2 variants in Vero and Vero-TMPRSS2 cells. Infections were
568 performed at an MOI of 0.01. Viral titers were determined by plaque assay at the indicated hours
569 post-infection and expressed as PFU per milliliter. C) Plaque phenotype of VOCs according to
570 TMPRSS2 expression. Same viral supernatant was used to infect Vero and Vero-TMPRSS2 cells.
571 Plaques were developed by immunostaining. D) Western blotting of spike cleavage in supernatants
572 from Vero and Vero-TMPRSS2 infected cells at an MOI of 0.01. Viral supernatants were collected
573 at 48 hours post-infection. Full length (FL) S protein (180 kDa), S2 cleaved spike (95 kDa) and
574 Nucleocapsid (N, 50 kDa) were detected using specific antibodies. Levels of N protein were used
575 as loading control. E) Quantification of the cleavage efficiency by mass spectrometry. Vero-
576 TMPRSS2 cells were infected at an MOI of 0.1 with the indicated VOCs and NY7 (S:H655Y) and
577 WA1-655Y isolates. WA1 and NY6 were used as controls. Cells extracts were collected after 24
578 hours post-infection. Cleavage efficiency was determined by measuring the abundance of the
579 resulting peptide (SVASQSIIAYTMSLGAE) after cleavage at the terminal arginine of the furin
580 cleavage site. The y axis shows the log₂ of fold change between cleaved peptide abundance for
581 each variant normalized by WA1 control. F-G) Immunofluorescence of SARS-CoV-2 S and N
582 protein in Vero-TMPRSS2 infected cells at an MOI of 0.01 and 24 hours p.i. for the indicated
583 VOCs. Spike protein was detected using a specific monoclonal antibody 3AD7 (green), N protein

584 was detected using a polyclonal antiserum (red) and 4',6-diamidino-2-phenylindole (DAPI) was
585 used to stain the nucleus.
586

587 **Materials and Methods**

588

589 **Cell lines:** VeroE6 and Caco-2 cell lines were originally purchased from the American Type
590 Culture Collection (ATCC). VeroE6-TMPRSS2 cell line was purchased from BPS Bioscience. A
591 master cell bank was created for each cell line and early-passage cells were thawed in every
592 experimental step. VeroE6 and Caco-2 cell lines were maintained in Dulbecco's modified Eagle's
593 medium (DMEM) with glucose, L-glutamine, and sodium pyruvate (Corning) supplemented with
594 10% fetal bovine serum (FBS, Gibco), non-essential amino acids, penicillin (100 UI/mL),
595 streptomycin (100 UI/mL) (Corning) and normocin (100 ug/mL) (InvivoGen) to prevent
596 mycoplasma infection. VeroE6-TMPRSS2 cell line was cultured in the same growth media
597 described above and further supplemented with sodium pyruvate (Corning) and puromycin (3
598 ug/mL) (InvivoGen). All cell lines were grown at 37°C in 5% CO₂.

599

600 **Viruses:** All experiments in this study were performed in the BSL-3 facility following Icahn
601 School of Medicine biosafety guidelines. Human SARS-CoV-2: nasopharyngeal swab specimens
602 were collected as part of the routine SARS-CoV-2 surveillance conducted by the Mount Sinai
603 Pathogen Surveillance program (IRB approved, HS#13-00981). Specimens were selected for viral
604 culture on Vero-E6 cells based on the complete viral genome sequence information (31). Human
605 isolates NY1 to NY12 were obtained from the nasopharyngeal swabs of patients infected with
606 SARS-CoV-2 in March 2020 while NY13 (PV28021) was cultured from the nasopharyngeal swab
607 of a patient infected in February 2021. All nasal swabs were kindly provided by Dr. Viviana
608 Simon. GISAID accession numbers of these isolates are shown in Supplementary Table 1. Viral
609 transport media was incubated with VeroE6 cells until cytopathic effect was observed and
610 supernatants from infected cells were used for plaque purification of clonal population as
611 previously described (42). SARS-CoV-2 isolates USA-WA1/2020 (NR-52281), hCoV-
612 19/England/204820464/2020 (NR-54000, Alpha) and hCoV-19/Japan/TY7-503/2021 (NR-54982,
613 Gamma) were obtained from BEI resources. hCoV-19/USA/MD-HP01542/2021 JHU (Beta
614 variant) was a gift from Dr. Andy Pekosz. Kappa and Delta variants were kindly provided by Dr.
615 Mehul S. Suthar and Dr. Viviana Simon, respectively. Animal SARS-CoV-2: Mink SARS-CoV-
616 2 variants (MiA-1 and MiA-2) were isolated during the mink experiment described below. After
617 plaque assay analysis of the left cranial lung collected from a WA1 infected mink at 4 days p.i.,
618 two plaque phenotypes were observed. Only the small plaque phenotype viruses were grown and
619 used in this study. USA-WA1/2020-H655Y (WA1-655Y) was kindly provided by Dr. Jurgen
620 Richt. This variant was isolated after a WA1 cat infection. All the viral stocks were produced by
621 infecting VeroE6 or VeroE6-TMPRSS2 cells at a MOI of 0.01. Infected cells were maintained in
622 infection media (DMEM with glucose, L-glutamine, and sodium pyruvate supplemented with 2%
623 FBS, non-essential amino acids, HEPES, penicillin (100 UI/mL) and streptomycin (100 UI/mL))
624 at 37°C in 5% CO₂. Infected cells were monitored by microscopy and cell-infected supernatants
625 were collected at day 2 post-infection when cytopathic effect was observed. Viral supernatants
626 were clarified of cell debris by spin down followed by centrifugation at 2000 x g for 20 min in
627 Amicon Ultra-15 centrifugal filters (Sigma, 100 kDa cutoff) to concentrate the viral stocks.
628 Aliquots were stored at -80°C until titration by plaque assay. All SARS-CoV-2 variants were
629 sequence-confirmed before performing the experiments.

630

631 **Infection of cell cultures:** Approximately 3.2×10^5 VeroE6 or VeroE6-TMPRSS2 or Caco-2 were
632 seeded in a 12 well-plate and cultured at 37°C in 5% CO₂ for 16 hours. Cells were infected with

633 the corresponding SARS-CoV-2 isolate at an MOI of 0.01. Cells were incubated with the virus for
634 1 hour and then, cells were washed with PBS to ensure removal of non-attached virus. After
635 infection, cells were maintained in infection media. Supernatants were collected at and the
636 indicated time points and stored at -80°C for plaque assay analysis and virus quantification.
637

638 **Western blotting:** VeroE6 or VeroE6-TMPRSS2 cells were infected with the indicated SARS-
639 CoV-2 isolates, similar to the description above. Viral supernatants were collected at 24- and 48-
640 hours post-infection. Supernatants were clarified by low-speed spin. Viral supernatants and cell
641 extracts were mixed with RIPA buffer (Sigma Aldrich) containing EDTA-free protease inhibitor
642 cocktail (Roche) and 10% SDS (Invitrogen) to a final concentration of 1%. Then, samples were
643 boiled for 10 minutes at 100°C and centrifuged for 10 minutes at 4°C and maximum speed. Viral
644 supernatants were subjected to SDS-PAGE protein electrophoresis using precast 10% TGX gels
645 (Bio-Rad). Gels were run at 120 V and subsequently transferred to polyvinylidene fluoride (PVDF)
646 membranes (BioRad) using BIO-RAD semi-dry transfer system. Then, membranes were fixed
647 with 100% methanol for 1 minute and blocked with 5 % non-fat dry milk-containing Tris-buffered
648 saline with Tween-20 (TBST) with 0.1% Tween-20 for 1 hour in shaking and room temperature
649 (RT). Next, membranes were incubated with primary antibodies overnight at 4°C followed by
650 incubation with secondary antibodies in a 3% milk diluted in TBST for 1 hour at RT. Primary
651 antibodies against SARS-CoV-2 Spike S2 protein (Abcam; ab6823) and nucleocapsid (Novus
652 Biologicals; NB100-56576) were purchased from the indicated suppliers and used at a dilution of
653 1:3000 and 1:2000 respectively. Anti-mouse secondary IgG-HRP antibody (abcam, 6823) was
654 used at a dilution 1:5000 to detect SARS-CoV-2 Spike protein and anti-rabbit secondary IgG-HRP
655 antibody (Kindle Biosciences, R1006) at 1:2000 to detect SARS-CoV-2 nucleocapsid.
656

657 **Plaque assay:** To determine viral titers, 3.2×10^5 VeroE6 or VeroE6-TMPRSS2 were seeded in a
658 12 well-plate the day before plaque assay was performed. Briefly, ten-fold serial dilutions were
659 performed in infection media for SARS-CoV-2 and inoculated onto confluent VeroE6 or VeroE6-
660 TMPRSS2 cell monolayer. After one-hour adsorption, supernatants were removed, and cells
661 monolayers were overlaid with minimum essential media (MEM) containing 2% FBS and purified
662 agar (OXOID) at a final concentration of 0.7%. Cells were then incubated 3 days at 37°C. Cells
663 were fixed overnight with 10% formaldehyde for inactivation of potential SARS-CoV-2 virus.
664 Overlay was removed and cells were washed once with PBS. Plaques were visualized by
665 immunostaining. Briefly, cells were blocked in 5 % milk diluted in TBST. After 1-hour, anti-
666 mouse SARS-CoV-2- NP antibody (1C7C7, kindly provided by Dr. Moran) was added at a dilution
667 of 1:1000 in 1% milk-TBST and incubated for 1 hour at RT. Then, cells were washed two times
668 in PBS and stained with goat anti-mouse secondary IgG-HRP antibody (abcam, 6823) at a dilution
669 of 1:5000 in 1% milk-TBST and incubated for 1 hour at RT. Finally, cells were washed three times
670 and the plaques were developed with TrueBlue substrate (KPL-Seracare). Viral titers were
671 calculated as plaque forming units (PFU)/ml.
672

673 **RT-qPCR for viral infectivity analysis:** To quantify the levels of SARS-CoV-2 RNA after
674 infection in VeroE6 and Caco-2 cells, we used the CDC 2019-nCoV real-time RT-qPCR protocol,
675 with modifications. Primers and probes were purchased from the indicated supplier (Integrated
676 DNA Technologies, 10006713, RUO Kit) and consisted of two 2019-nCoV-specific sets (N1, N2).
677 Names and sequences of the primers used are shown in Supplementary Table 3. Assays were run
678 in a 384-well format using the QuantiFast Pathogen RT-PCR + IC Kit (QIAGEN; 211454).

679 USA/WA-1/2020 SARS-CoV-2 RNA (20,000 genome copies per reaction) and nuclease-free
680 water were included as controls. Reactions were performed in duplicate using the following
681 cycling conditions on the Roche LightCycler 480 Instrument II (Roche Molecular Systems;
682 05015243001): 50 °C for 20 min, 95 °C for 1 s, 95 °C for 5 min, followed by 40 cycles of 95 °C
683 for 15 s and 60 °C for 45 s. To determine the limit of detection for SARS-CoV-2, we used a
684 commercially available plasmid control (Integrated DNA Technologies;10006625). Infectivity
685 was calculated as a ratio between genomic RNA calculated by RT-qPCR and PFU values
686 determined by plaque assay analysis.

687
688 **Immunofluorescence:** VeroE6-TMPRSS2 cells were seeded at a concentration of 3.2×10^5 cells
689 per well in a 12 well glass-bottom plate and cultured at 37°C in 5% CO₂ for 16 hours. Cells were
690 then infected with the corresponding SARS-CoV-2 variant at an MOI of 0.01 and maintained in
691 infection media. After 24 post-infection, cells were fixed with 10% methanol-free formaldehyde
692 and incubated with primary antibodies against spike KL-S-3A7 (43) and nucleoprotein polyclonal
693 anti-serum (44) diluted in 3% bovine serum albumin (BSA) for 1 hour at RT. Then, cells were
694 washed and stained with secondary antibodies anti-mouse Alexa Fluor-488 (ThermoFisher;
695 A21202) and anti- Rabbit Alexa Fluor 568 (ThermoFisher; A11011) in 5% BSA for 1h at RT.
696 DAPI (4',6-diamidino-2-phenylindole) was used to visualize the nucleus.

697
698 **Hamster infections:** Ten female golden Syrian hamster of approximately 4-weeks-old were
699 placed in pairs in five different cages. Only one hamster per cage was inoculated intranasally with
700 a total of 10^5 pfu of a mix of WA1 and WA1-655 SARS-CoV-2 viruses in a one-to-one ratio
701 administered in 100 ul of PBS. Animals were monitored daily for body weight loss. On days 2, 4,
702 6 post-infection, animals were anesthetized with 100 mg/kg Ketamine and 20 mg/kg Xylazine and
703 nasal washes were collected in 200 ul PBS. Directly infected (DI) and direct contact (DC)
704 hamsters were humanely euthanized for collection of lungs and nasal turbinates on day 5 and 7
705 post-infection, respectively. Anesthetized hamsters were euthanized by intracardiac injection of
706 sodium pentobarbital (Sleepaway - Zoetis) euthanasia solution. Samples were collected for viral
707 quantification by plaque assay and next-generation sequencing. All animal studies were approved
708 by the Institutional Animal Care and Use Committee (IACUC) of Icahn School of Medicine at
709 Mount Sinai (ISMMS).

710
711 **Mink infection:** Nine female American Mink (Neovison vison) of approximately 6-months-old
712 were sourced by Triple F Farms (Gillett, PA). All mink were individually housed, given ad libitum
713 access to food and water, and maintained on a 12-hour light/dark cycle. Six minks were infected
714 with an infectious dose of 10^6 pfu of WA-1 isolate administered intranasally in a 1 mL volume.
715 Three minks were mock-infected to serve as healthy controls. Minks were anesthetized by
716 intramuscular administration of 30 mg/kg Ketamine and 2 mg/kg Xylazine prior to intranasal
717 infection, collection of specimens, or euthanasia. Nasal washes, rectal swabs, and oropharyngeal
718 swabs were collected on days 1, 3, and 5 post-infection. On days 4 and 7 post-infection, three mink
719 per day were humanely euthanized for collection of tissue specimens for viral quantification by
720 plaque assay and sequencing. Body weights of mink were collected days 0, 1, 3, 4, 5 and 7 post-
721 infection. Anesthetized minks were euthanized by intracardiac injection of sodium pentobarbital
722 (Sleepaway - Zoetis) euthanasia solution. The Institutional Animal Care and Use Committee
723 (IACUC) of the Icahn School of Medicine at Mount Sinai (ISMMS) reviewed and approved the

724 mink model of COVID-19. Experiments with infected SARS-CoV-2 mink were performed in an
725 ABSL-3 facility.

726

727 **Sample preparation for targeted proteomics:** VeroE6-TMPRSS2 cells were seeded in a 6 well-
728 plate and infected with the corresponding SARS-CoV-2 variants at an MOI of 0.1. Cells were
729 lysated after 24 hours with RIPA buffer containing EDTA-free protease inhibitor cocktail and 10%
730 SDS to a final concentration of 1%. Then, samples were boiled for 10 minutes at 100°C. Cells were
731 centrifuged at 12k rpm on a tabletop centrifuge at RT for 20 minutes to remove insoluble debris
732 and separated into three samples to assess technical reproducibility. 50 ul for each sample were
733 loaded in a 1:4 ratio (v/v) with urea buffer (8M urea, 100 mM AmBic pH 8.1) on a Microcon 30
734 kDa MWCO (Millipore, Sigma) and centrifuged to dryness at 9500 rpm for 15 minutes at RT, until
735 all sample was loaded. The filters were washed three times with 200 ul of urea buffer using similar
736 centrifugation parameter as the sample loading. 100 ul of reduction buffer (8 M urea, 100 mM
737 AmBic pH 8.1, 5 mM TCEP) was added and the samples were incubated at 37°C for 20 minutes
738 to reduce the cysteines. Chloroacetamide (CAA) was added to 10 mM final concentration and the
739 samples were incubated for 30 minutes in the dark at RT. The filters were washed 3 times with
740 200 ul of urea buffer and 3 times with 200 ul of digestion buffer (50 mM AmBiC). GluC was
741 added to samples in a 1:100 ratio (w/w) and the filters were incubated on a shaker for 16 hours at
742 37°C and 450 rpm. Peptides were collected by centrifugation and the filters were washed twice
743 with 100 ul of LC-MS grade water. Desalting was done using the Nest group microspin C18
744 columns. Activation of the resin was done with 1 column volume (CV) of MeCN and the columns
745 were equilibrated with 2 CV of 0.1% FA in water. Samples were loaded and flowthrough was
746 loaded again before washing the columns with 3 CV of 0.1% FA in water. Peptide elution was
747 done with 2 CV of 50% MeCN in 0.1% FA and 1 CV of 80% MeCN in 0.1% FA. Following
748 collection, the peptides were dried under the vacuum. Samples were resuspended at 1 ug/ul in
749 0.1% FA and approximately 1 ug was injected into the mass spectrometer.

750

751 **Mass spectrometry:** All samples were acquired on a Thermo Q Exactive (Thermo Fisher)
752 connected to a nanoLC easy 1200 (Thermo Fisher). Samples containing mutation in the region of
753 interest (furin cleavage) as well as one representative sample having conservation in the furin
754 cleavage site were analyzed by Data Dependent Acquisition (DDA) MS to obtain fragments library
755 to design the targeted assays. For DDA the peptides were separated in 120 minutes with the
756 following gradient: 4% B (0.1% FA in MeCN) to 18% B for 65 minutes, followed by another
757 linear gradient from 18% to 34% of B and lastly the organic solvent was increased to 95% in 5
758 minutes and kept for 5 minutes to wash the column. The mass spectrometer was operated in
759 positive mode, each MS1 scan was performed with a resolution of 70000 at 200 m/z. Peptide ions
760 were accumulated for 100 ms or until the ion population reached an AGC of 3e6. The top 15 most
761 abundant precursors were fragmented using high-collisional-induced dissociation (HCD) with a
762 normalized energy of 27 using an isolation window of 2.2 Da and a resolution of 17500 (200 m/z).
763 For targeted analysis, the samples were separated in 62 minutes to concentrate the analytes in
764 narrower peaks and increase signal. The gradient employed was from 3% B to 34% in 40 minutes
765 then B was increased to 42% in 10 minutes and then finally to 95% in 5 minutes. As for the DDA
766 the column was washed for 5 minutes at 95% B before the next run. The mass spectrometer was
767 operated in positive mode and targeted acquisition. Specifically, one MS1 scan (70000 resolution,
768 1e6 AGC, 100 ms IT) and seven unscheduled targeted MS2 scan were performed per cycle. Each

769 MS2 was acquired at 35000 resolution, with a AGC of 2e5 a maximum IT of 110 ms and an
770 isolation window of 2.0 m/z. Isolated ions were fragmented using HCD at 27 NCE.

771
772 **Illumina sequencing:** Viral RNA nasal washes from hamsters was extracted using Omega
773 E.Z.N.A Viral RNA kit (R6874) following manufacturer's instructions. Viral RNA from hamster
774 nasal turbinates and lungs was isolated Direct-zol RNA Miniprep kit (R2050) using
775 manufacturer's instructions. Samples for sequencing were prepared using whole-genome
776 amplification with custom designed tiling primers (45) and the Artic Consortium protocol
777 (<https://artic.network/ncov-2019>), with modifications. Briefly, cDNA synthesis was performed
778 with random hexamers and ProtoScript II (New England Biolabs, cat. E6560) using 7 μ L of RNA
779 according to manufacturer's recommendations. The RT reaction was incubated for 30 minutes at
780 48°C, followed by enzyme inactivation at 85°C for 5 minutes. Targeted amplification was
781 performed as previously described(31). Next, amplicons were visualized on a 2% agarose gel and
782 cleaned with Ampure XT beads. Amplicon libraries were prepared using the Nextera XT DNA
783 Sample Preparation kit (Illumina, cat. FC-131-1096), as recommended by the manufacturer.
784 Finally, to assembly SARS-CoV-2 genomes a custom reference-based analysis pipeline was used
785 (https://github.com/mjsull/COVID_pipe⁴³). For the hamster samples and the inoculum, in addition
786 to whole genome sequencing, the same workflow was used to sequence a 2,223 bp amplicon
787 targeting the S1 to S1/S2 region (nucleotide positions 21,386 to 23,609) to quantify the variant
788 frequency at the position S:655 position.

789
790 **Oxford Nanopore sequencing:** The frequency of variants at position S:655 in the hamster samples
791 was further confirmed with Oxford Nanopore (ONT) sequencing in a MinION Mk1C instrument.
792 The same cDNA used for Illumina sequencing was used to amplify a fragment of 356bp along the
793 spike region of interest (positions 23,468 to 23,821) using the Artic primer pair nCoV-2019_78
794 V3 (<https://artic.network/ncov-2019>). The purified amplicons were barcoded with the Native
795 Barcode expansion kit (Oxford Nanopore, cat. EXP-NBD196) and PCR-free libraries were
796 prepared using the ligation sequencing (Oxford Nanopore, cat. SQK-LSK109). A total of 20 fmol
797 of the multiplexed library was sequenced on a Flongle flowcell (Oxford Nanopore, cat. FLO-
798 FLG001) in a single 8-hour run. Sequencing data acquisition was done with the ONT software
799 MinKNOW v4.3.7. Basecalling and demultiplexing was done with Guppy 5.0.12 in high accuracy
800 basecalling mode. The Genome assembly was done with the Artic pipeline ([artic-network/artic-
ncov2019](https://artic.network/ncov2019)) with default parameters, where reads were aligned to the reference genome Wuhan-
801 Hu-1 (MN908947.3) using minimap2 (2.17-r941), consensus variants were called with Nanopolish
802 (0.13.2). Final read coverage for the targeted region ranged from 80 to 3251x, (median coverage
803 of 1578x).

804 805 806 **Data Analysis**

807
808 Multiple alignment of the spike protein of mink and human NY variants (Figure 1) was performed
809 using COBALT multiple alignment tool (46). Spike amino acid sequence of SARS-CoV-2
810 ancestor (MN908947.3) was used to compare and identify the spike mutation of these variants.
811 For the analysis of high prevalent amino acid changes within and surrounding the furin cleavage
812 of the spike protein of current circulating VOCs, amino acid substitution frequencies around the
813 cleavage site region (655 to 701) were estimated from globally available data (Available from
814 GISAID [PMID: 31565258] as of 2021-06-28 with 2,072,987 records). The downloaded data was

815 processed through the augur pipeline with Nextstrain v7 for SARS-CoV-2
816 (<https://github.com/nextstrain/ncov>) for sequence alignment and curation with default parameters
817 (47). Multiple sequence alignment of the spike amino acid sequences was parsed and sliced for the
818 region of interest in Biophyton v.75 with BioAlignIO package. In order to determine the more
819 prevalent substitutions, sequences with ambiguous consensus calls were removed, and residues
820 with substitutions along the 655-701 region present in frequencies of 0.05% or less across the
821 entire dataset were masked. The 5% cutoff value was chosen based on the frequency distribution
822 of substitutions per site on a histogram that revealed that variants in most positions occur in
823 frequencies of less than 0.2%. The final list of most variable positions included residues 655, 675,
824 677, 681 and 701. The relative proportion of their occurrence time and Nextstrain clade
825 (<https://nextstrain.org/blog/2021-01-06-updated-SARS-CoV-2-clade-naming>) was plotted in R
826 with ggplot2 (48) v3.3.5.

827
828 Phylogenetic analysis was performed using the same dataset from above to build a time-calibrated
829 phylogenetic tree with Nextstrain, to visualize the distribution of H655Y (up to 2020-09-30) and
830 other prevalent substitutions along the 655-701 region (up to 2021-06-23). A global subsampling
831 scheme with a focus on the variable residues was done to ensure their representation by
832 geographical region and over time. The final builds contained 7,059 (early) and 13,847 (late)
833 sequences including the early New York isolates.

834
835 For the mass spectrometry analysis DDA data was searched with MSFragger (49) using a FASTA
836 file combining the human proteome, the SARS-Cov2 proteome, all the variants and the C-term
837 and N-term cleaved Spike protein entry for each variant. The Speclib workflow was used to
838 generate a library which was imported into Skyline (50) for selection of peptides and internal
839 controls. Overall, 3 versions of the C-term furin cleaved peptide (SVASQSIHAYTMSLGAE) with
840 two charge states (2+ and 3+) and the oxidated methionine were used. 4 other peptides were
841 included as controls: 2 from the C-term spike fragment to be used as proxy for total spike quantity
842 and 2 from Orf3a and N protein to be used as internal standard to normalize across variants.
843 Following acquisition, the PRM data was imported into the Skyline document with the following
844 transition settings: MS1 filtering was enabled, and MS/MS filtering was changed to targeted using
845 Orbitrap as mass analyzer (35000 resolution) and high selectivity extraction. A minimum of 6
846 transitions and a maximum of 18 having $m/z >$ precursors were selected for data analysis. After
847 manual peak boundaries selection and elimination of interferences the transition results were
848 exported. Transitions where the signal/background ratio was less than 5 were removed to ensure
849 robust quantitative accuracy. The transitions were summed within the same charge state and the
850 2+ unmodified SVASQSIHAYTMSLGAE was used for quantification. The data was normalized
851 using median centering of the other Spike peptide (ILPVSMTKTSVD) as internal standard.
852 Following normalization, \log_2 fold change was calculated by averaging the intensities for the furin-
853 cleaved peptide per variant and divide them by the one from the WA1 variant used here as control
854 sample. Resulting ratios were logged and used for visualization and statistical analysis.

855
856 Statistical analysis was performed using GraphPad Prism. Two-way ANOVA with Holm-
857 Šídák posttest was used for multiple comparisons. Statistical significance was established at a P
858 value of <0.05 .

859
860

861 **References**

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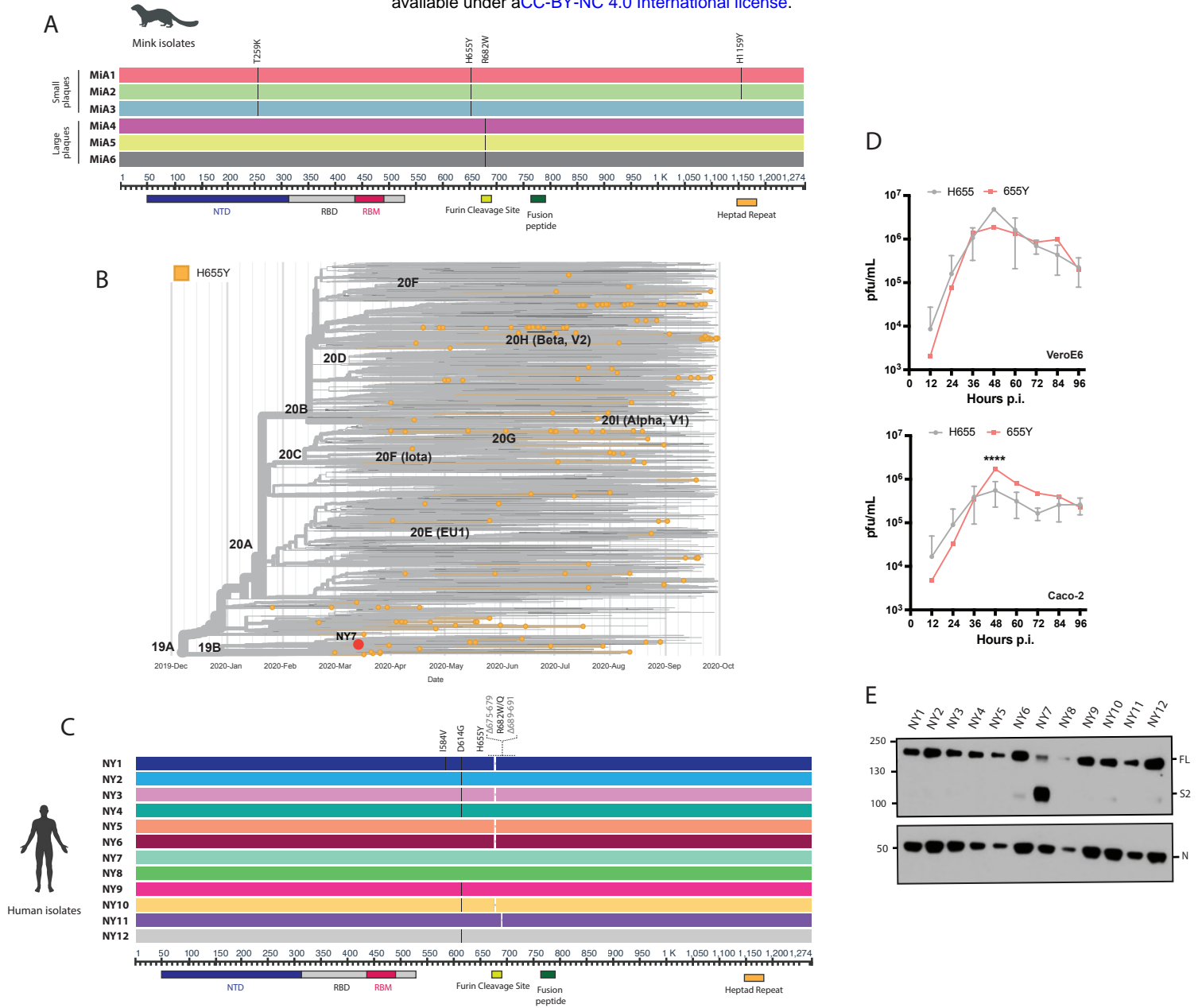


Figure 1.

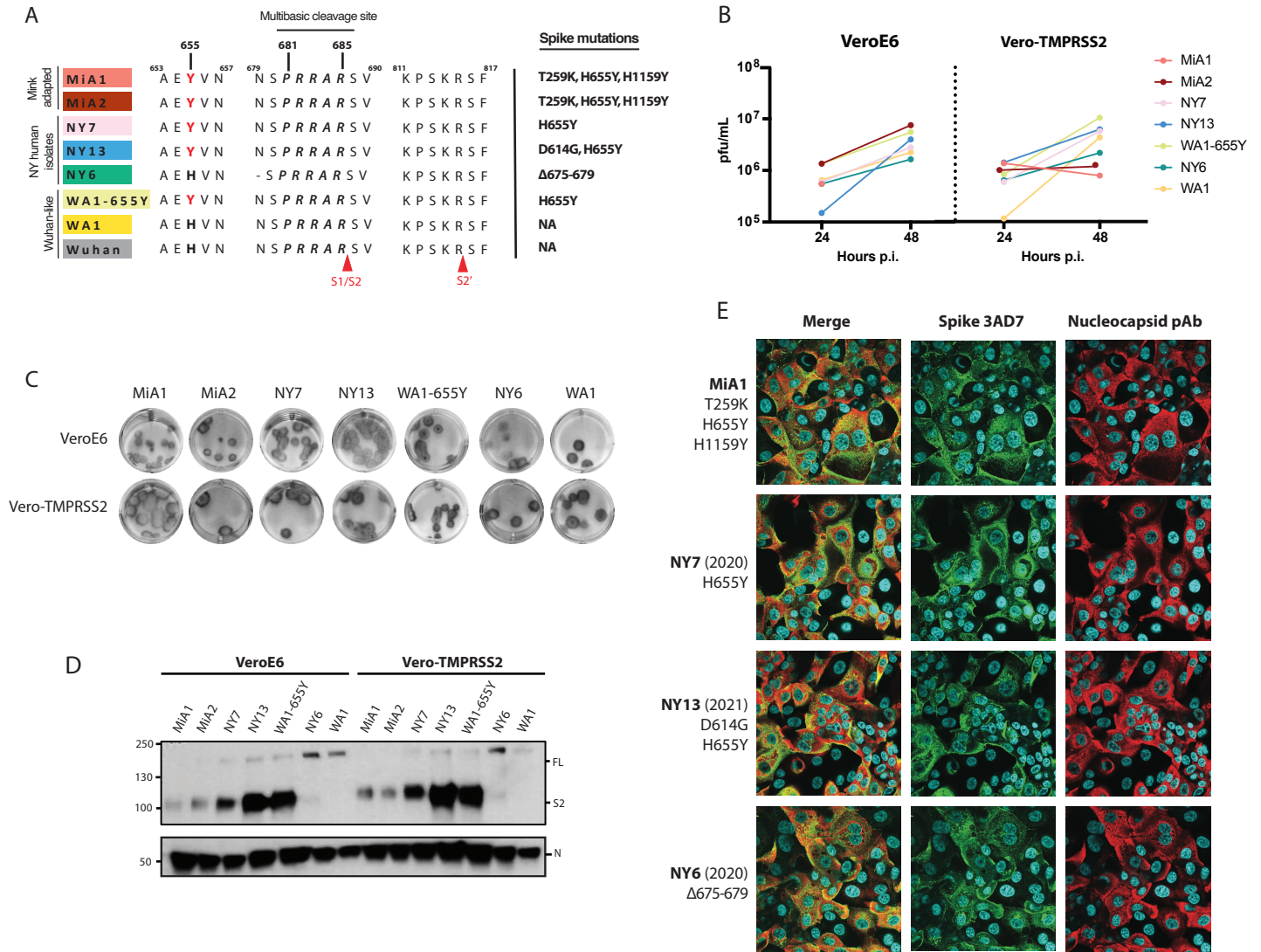


Figure 2.

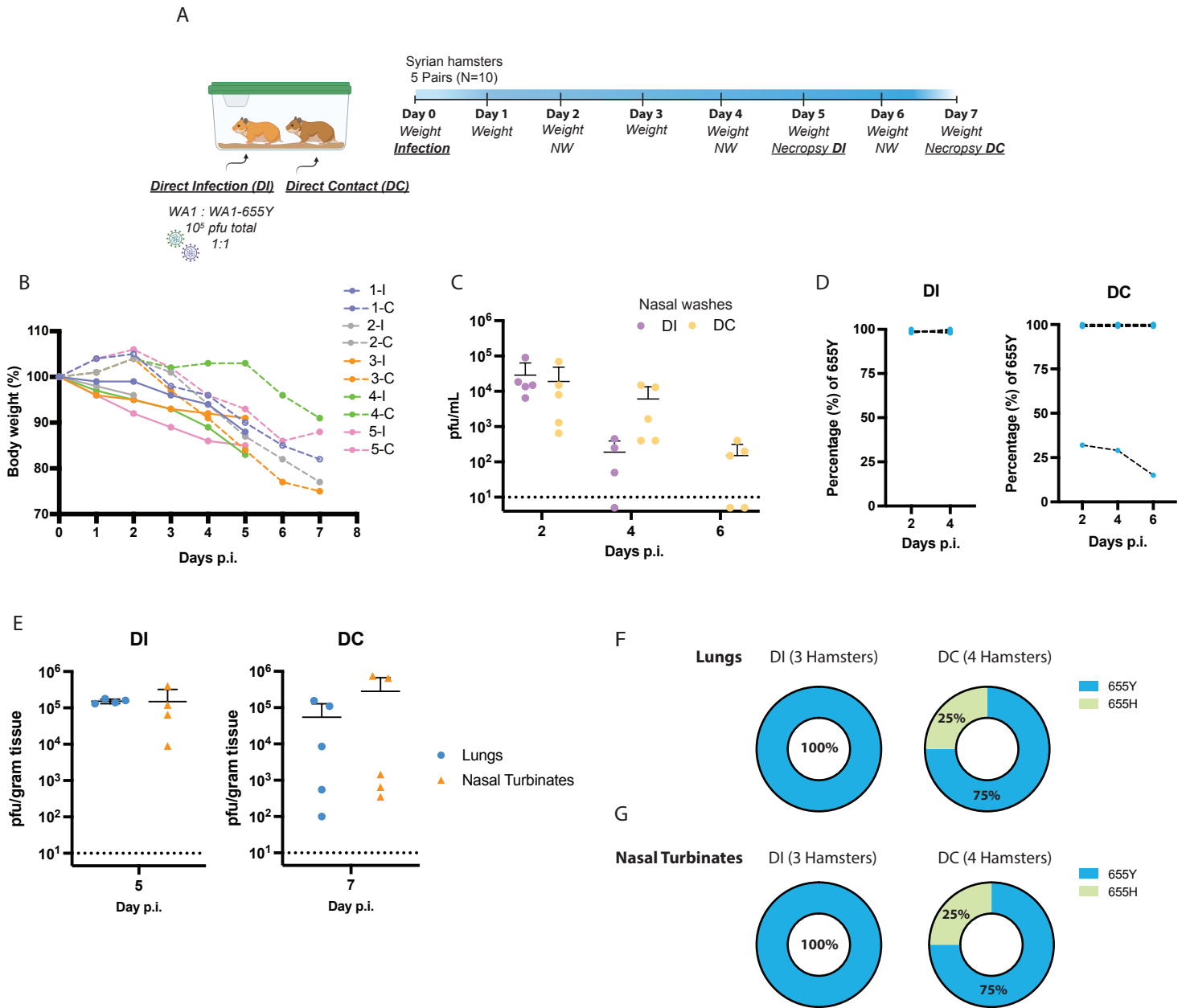
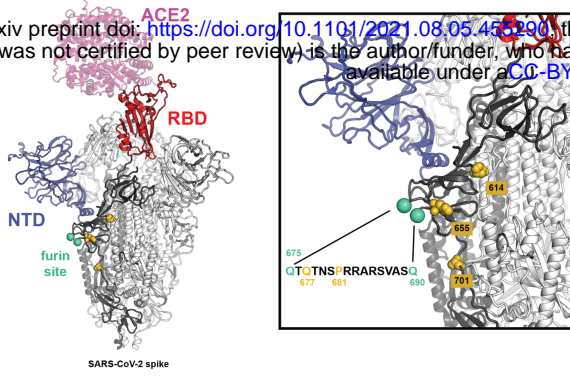


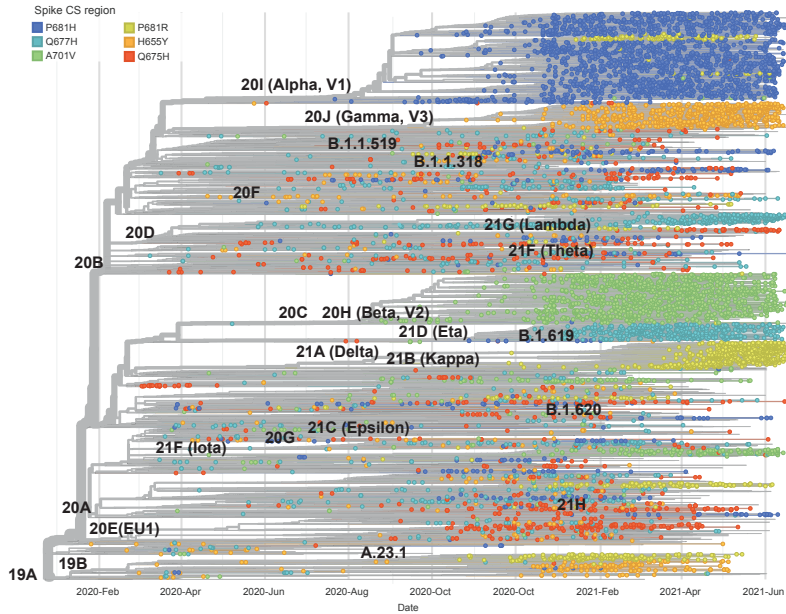
Figure 3.

A

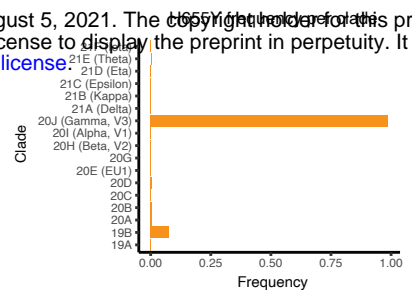
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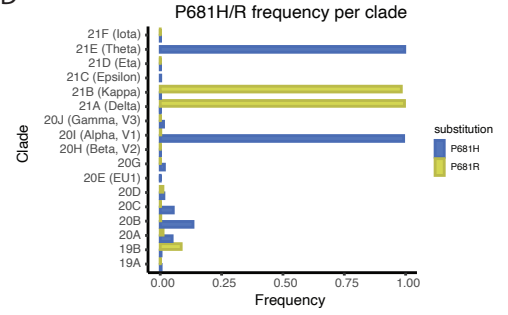
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C



D



E

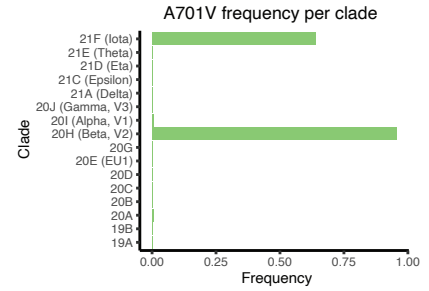


Figure 4.

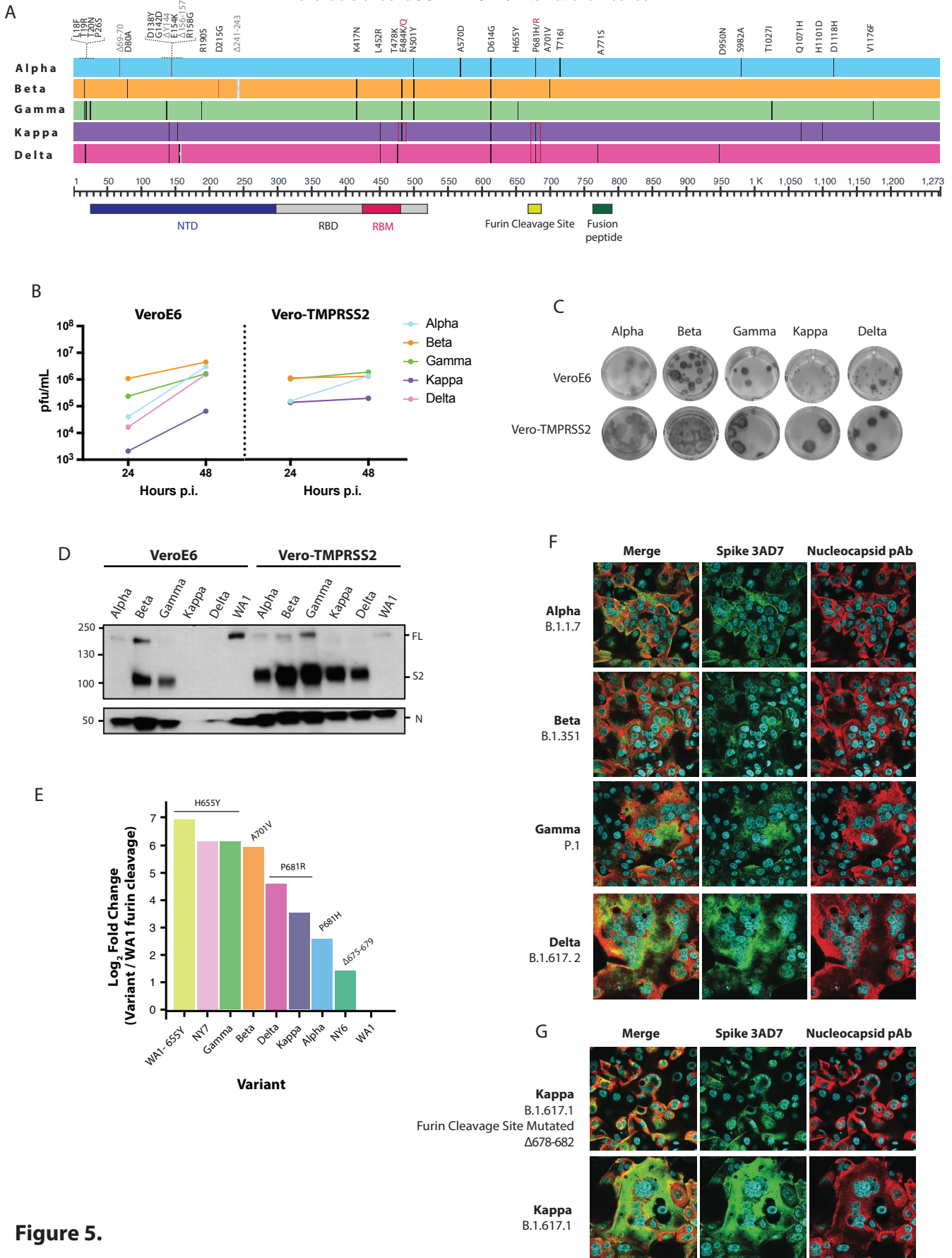


Figure 5.