

1 **Nucleocapsid mutations in SARS-CoV-2 augment replication and pathogenesis.**

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35 **Abstract**

36 While SARS-CoV-2 continues to adapt for human infection and transmission, genetic variation
37 outside of the spike gene remains largely unexplored. This study investigates a highly variable
38 region at residues 203-205 in the SARS-CoV-2 nucleocapsid protein. Recreating a mutation
39 found in the alpha and omicron variants in an early pandemic (WA-1) background, we find that
40 the R203K+G204R mutation is sufficient to enhance replication, fitness, and pathogenesis of
41 SARS-CoV-2. The R203K+G204R mutant corresponds with increased viral RNA and protein
42 both *in vitro* and *in vivo*. Importantly, the R203K+G204R mutation increases nucleocapsid
43 phosphorylation and confers resistance to inhibition of the GSK-3 kinase, providing a molecular
44 basis for increased virus replication. Notably, analogous alanine substitutions at positions
45 203+204 also increase SARS-CoV-2 replication and augment phosphorylation, suggesting that
46 infection is enhanced through ablation of the ancestral 'RG' motif. Overall, these results
47 demonstrate that variant mutations outside spike are key components in SARS-CoV-2's
48 continued adaptation to human infection.

49

50 **Author Summary**

51 Since its emergence, SARS-CoV-2 has continued to adapt for human infection resulting in the
52 emergence of variants with unique genetic profiles. Most studies of genetic variation have
53 focused on spike, the target of currently available vaccines, leaving the importance of variation
54 elsewhere understudied. Here, we characterize a highly variable motif at residues 203-205 in
55 nucleocapsid. Recreating the prominent nucleocapsid R203K+G204R mutation in an early
56 pandemic background, we show that this mutation is alone sufficient to enhance SARS-CoV-2
57 replication and pathogenesis. We also link augmentation of SARS-CoV-2 infection by the
58 R203K+G204R mutation to its modulation of nucleocapsid phosphorylation. Finally, we
59 characterize an analogous alanine double substitution at positions 203-204. This mutant was
60 found to mimic R203K+G204R, suggesting augmentation of infection occurs by disrupting the
61 ancestral sequence. Together, our findings illustrate that mutations outside of spike are key
62 components of SARS-CoV-2's adaptation to human infection.

63

64 Introduction

65 The emergence of severe acute respiratory syndrome coronavirus 2 (SARS)-CoV-2 is the most
66 significant infectious disease event of the 21st century (1, 2). Since its initial expansion, SARS-
67 CoV-2 has continued to adapt for human infection and transmission, resulting in several variants
68 of concern(3). While most mutations occur within a single lineage, a small number are shared
69 across multiple variants (4). Spike mutations have dominated SARS-CoV-2 variant research,
70 owing to concerns that they enhance replication, augment transmission, or allow escape from
71 immunity (4). However, less attention has been focused on mutations outside spike, despite the
72 existence of other “mutational hotspots” in the genome (4). The SARS-CoV-2 nucleocapsid (N)
73 gene is one hotspot for coding mutations, particularly at amino acid residues 203-205 within its
74 serine rich (SR) domain (5). Three prominent mutations occur in this region including
75 R203K+G204R, a double substitution (KR mt) present in the alpha, gamma, and omicron
76 variants; T205I present in the beta variant; and R203M that occurs in the kappa and delta
77 variants (6, 7). Together, this genetic variation and convergent evolution in residues 203-205
78 suggests positive selection in this motif of N.

79 Here, we utilize our reverse genetic system (8, 9) to generate the KR nucleocapsid
80 mutation in the ancestral WA-1 strain of SARS-CoV-2. This change alone was sufficient to
81 increase viral replication in respiratory cells and exhibited enhanced fitness in direct competition
82 studies with wild type (WT) SARS-CoV-2. In the hamster model, the KR mutant (mt) enhanced
83 pathogenesis and outcompeted WT in direct competition. We subsequently found that the KR
84 mt corresponds with increased viral RNA both *in vitro* and *in vivo*. Notably, we observed that the
85 KR mt resulted in augmented nucleocapsid phosphorylation relative to WT SARS-CoV-2; similar
86 increases in N phosphorylation were also seen in the alpha and kappa variants. Importantly, the
87 KR mt was more resistant to GSK-3 kinase inhibition relative to WT SARS-CoV-2, suggesting
88 that the KR mt alters interactions with N targeting kinases. Finally, an analogous alanine double
89 substitution mutant at position 203+204 (AA mt) also increased fitness and altered

90 phosphorylation relative to WT SARS-CoV-2. Together, these results suggest that disruption of
91 the ancestral “RG” motif in nucleocapsid augments infection, fitness, and pathogenesis of
92 SARS-CoV-2.

93 Results

94 Genetic Analysis of a highly variable motif of SARS-CoV-2 Nucleocapsid.

95 Using SARS-CoV-2 genomic data from the GISAID database(6), we binned each sequence by
96 month of collection and performed an *in silico* search for variation at residues 203-205 within
97 nucleocapsid (**Fig 1A**). Three prominent mutations emerged from this analysis. The first is the
98 R203K+G204R double substitution (KR mt), present in the alpha, gamma, and omicron variants
99 (**Fig 1D**). Historically, the KR mt has been the most abundant mutation in this region, emerging
100 early in the pandemic and reaching 73% of reported sequences in April 2021 (**Fig 1A, S1**
101 **Table**). The second prominent mutation, T205I, is present in the beta, eta, and mu lineages (**Fig**
102 **1D**). While also emerging early in the pandemic, T205I is a minority variant which peaked at 9%
103 in February 2021 (**Fig 1A, S1 Table**). The third prominent variant mutation is R203M, present in
104 the delta and kappa variants (**Fig 1D**). Interestingly, while the R203M mutation was first
105 detected in March 2020, it persisted as a rare (<1%) variant until April 2021 when it began
106 expanding rapidly, reaching 97% of all reported sequences by November 2021, displacing the
107 KR and T205I mutations (**Fig 1A, S1 Table**). However, with the recent emergence of the
108 omicron variant, the KR mt has regained prominence, displacing R203M as the most common
109 mutation and reaching 93% of all newly reported sequences in January 2022. Together, these
110 data reveal a complex pattern of genetic variation and convergent evolution for residues 203-
111 205 of SARS-CoV-2 N.

112 To determine if mutations in this variable motif have the potential to enhance infection,
113 we evaluated the replication kinetics of SARS-CoV-2 variants. Two cell models were selected
114 for this analysis: Vero E6 (commonly used for propagation and titration of SARS-CoV-2) and
115 Calu-3 2b4 (a respiratory cell line used to study coronavirus and influenza infection) (10, 11).
116 Briefly, Vero E6 or Calu-3 2b4 cells were inoculated at a low multiplicity of infection (MOI) of
117 0.01 plaque forming units/cell with the early pandemic Washington-1 (WA-1) strain or a SARS-

118 CoV-2 variant and replication kinetics monitored for 48 hours post infection (hpi). In Vero E6
119 cells, while the alpha and beta variants replicated to equivalent endpoint titers compared to WA-
120 1, both variants had slightly lower titers at 24 hpi (**Fig 1B, S1A Fig**). In contrast, the kappa
121 variant replicated to ~15-fold lower titer than WA-1 throughout infection (**S1A Fig**), potentially
122 due to processing mutations in SARS-CoV-2 spike shared with the delta variant (12).
123 Interestingly, in Calu-3 2b4 cells, the alpha variant replicated to a 5.6 fold higher endpoint titer
124 compared to WA-1 (**Fig 1C**). In contrast, the beta variant replicated to a lower (2.7 fold) mean
125 endpoint titer compared to WA-1 while the kappa variant showed no significant differences with
126 WA-1 (**S1B Fig**). Together, these data suggest that SARS-CoV-2 variants harboring N
127 mutations may correspond to altered replication kinetics.

128 **The KR and R203M mts alone are sufficient to increase viral replication.**

129 Because the alpha variant exhibited enhanced replication in Calu-3 2b4 cells, we selected the
130 KR mt for further examination. To study the effects of the KR mt in isolation, we utilized a
131 SARS-CoV-2 reverse genetic system to recreate the KR mt in a WA-1 background (**Fig 1E**) (8,
132 9). In addition, due to gain-of-function concerns, the accessory protein ORF7 was replaced with
133 mNeonGreen (mNG), which reduces but does not eliminate disease in golden Syrian hamsters
134 (**S2 Fig**). After recovery of recombinant virus, we evaluated the KR mt's effects on SARS-CoV-2
135 replication. Both Vero E6 and Calu-3 2b4 cells were infected at a low MOI (0.01) with either
136 SARS-CoV-2 WA-1 harboring the mNG reporter (herein referred to as WT) or the KR mt and
137 viral titer monitored for 48 hpi. Like the alpha variant, the KR mt grew to a lower titer at 24 hpi,
138 but had an equivalent endpoint titer in Vero E6 cells (**Fig 1F**). Notably, in Calu-3 2b4 cells, the
139 KR mt had increased viral titer at both 24 and 48 hpi compared to WT SARS-CoV-2 (**Fig 1G**).
140 These data suggest the KR mt in nucleocapsid alone is sufficient to enhance viral replication.

141 Next, we wanted to examine the ability of other variant mutations in the 203-205 motif of
142 nucleocapsid to enhance SARS-CoV-2 replication. Using our reverse genetic system, we

143 recreated the R203M mutation in a WA-1 mNG background and evaluated its replication in Vero
144 E6 and Calu-3 2b4 cells (**S3 Fig**). Interestingly, like the KR mt, in Vero E6 cells the R203M
145 mutant grew to lower titer at 24 hpi but had a similar endpoint titer compared to WT SARS-CoV-
146 2 (**S3B Fig**). In Calu-3 2b4 cells, the R203M mutant again mimicked the KR mt, growing to a
147 higher titer than WT at 24 and 48 hpi (**S3C Fig**). These data suggest that like the KR mt, the
148 R203M mutation alone is sufficient to enhance SARS-CoV-2 replication.

149 **The KR mt enhances SARS-CoV-2 fitness during direct competition.**

150 We next determined if the KR mt increases SARS-CoV-2 fitness using competition assays,
151 which offer increased sensitivity compared to individual culture experiments (13). WT SARS-
152 CoV-2 and the KR mt were directly competed by infecting Vero E6 and Calu-3 2b4 cells at a 1:1
153 plaque forming unit ratio. Twenty-four hpi, total cellular RNA was harvested and the ratio of WT
154 to KR mt genomes determined by next generation sequencing (NGS)(14). Consistent with the
155 kinetic data, WT outcompeted the KR mt at a ratio of ~4:1 in Vero E6 cells (**Fig 1H**). In contrast,
156 the KR mt outcompeted WT at a ratio of ~10:1 in Calu-3 2b4 cells (**Fig 1H**). These data indicate
157 that the KR mt has a fitness advantage over WT SARS-CoV-2 in Calu-3 2b4, but not Vero E6
158 cells.

159 **The KR mt increases pathogenesis and fitness in vivo**

160 We next determined the effects of the KR mt *in vivo* using a golden Syrian hamster model of
161 SARS-CoV-2 infection (15). Three- to four-week-old male hamsters underwent intranasal
162 inoculation with either PBS (mock) or 10⁴ plaque forming units (PFU) of WT SARS-CoV-2 or the
163 KR mt and weight loss was monitored for 10 days post infection (dpi, **Fig 2A**). On days 2 and 4,
164 a cohort of five animals from each group underwent nasal washing, were euthanized, and
165 trachea and lung tissue harvested for measurement of viral loads and histopathologic analysis.
166 On day 10, surviving animals were euthanized and lung tissue harvested for histopathological

167 analysis. Strikingly, animals infected with the KR mt had increased weight loss compared to WT
168 throughout the experiment (**Fig 2B**). Curiously, weight loss changes did not correlate with
169 increased viral loads, as no significant viral titer difference in the lung or trachea was observed
170 between WT and the KR mt (**Fig 2C and D**). Furthermore, the KR mt caused a small, but
171 significant, decrease in titer in nasal washes on day 2, but not day 4 (**Fig 2E**). Contrasting the
172 titer data, histopathologic analysis of lungs revealed that the KR mt had more severe lesions
173 compared to WT SARS-CoV-2 (**S4 Fig**). Compared to mock (**S4A Fig**), both WT and the KR mt
174 had bronchiolitis and interstitial pneumonia; however, larger and more diffuse pulmonary lesions
175 were observed in the KR mt on day 4 (**S4B and C Fig**). In addition, the KR mt had cytopathic
176 alveolar pneumocytes and alveoli containing both mononuclear cells and red blood cells (**S4C**
177 **Fig**). By day 10, both WT and the KR mt showed signs of recovery, but maintained interstitial
178 pneumonia adjacent to the bronchi absent in mock-infected animals (**S4D–F Fig**). Notably, the
179 KR mt had evidence of cytopathic effect in bronchioles, perivascular edema, and immune
180 infiltration of the endothelium. Together, these data demonstrate that the KR mt increases
181 disease following SARS-CoV-2 infection *in vivo*.

182 We next evaluated the KR mt's effects on SARS-CoV-2 fitness and transmission *in vivo*.
183 Singly housed three- to four-week-old male donor hamsters were intranasally inoculated with
184 10^4 PFU of WT SARS-CoV-2 and the KR mt at a ratio of 1:1 (**Fig 2F**). On day 1 of infection,
185 each donor hamster was co-housed with a recipient for 8 hours to allow transmission. Hamsters
186 were then separated and SARS-CoV-2 present in the nasal cavities of the donors sampled by
187 nasal wash. On day 2, each recipient hamster underwent nasal washing to sample transmitted
188 SARS-CoV-2. Donor and recipient hamsters then underwent nasal washing and harvesting of
189 lung tissue on days 4 and 5, respectively, and the ratio of WT to KR mt in all samples was
190 determined by NGS(14). Neither WT nor the KR mt consistently dominated in the donor washes
191 on day 1; similarly, the day 2 nasal washes from the recipients showed no distinct advantage

192 between KR mt and WT for transmission (**Fig 2G and H**). However, at day 4 and 5 in the donor
193 and recipient, respectively, the KR mt was slightly more predominant in the nasal wash (**Fig 2G**
194 **and H**). Notably, the KR mt dominated the SARS-CoV-2 population found in lungs of both donor
195 and recipient animals on days 4 and 5. Together, these results suggest that the KR mt
196 outcompetes WT *in vivo* independent of transmission.

197 **The KR mt increases viral RNA and antigen levels.**

198 The CoV N protein has previously been shown to play a role in the transcription of viral RNA
199 (16-19). To evaluate changes in viral RNA levels during infection with the KR mt, we performed
200 RT-qPCR to measure levels of SARS-CoV-2 transcripts following infection of Calu-3 2b4 cells
201 (**S5 Fig**). Compared to WT infected cells, the KR mt had a >32-fold increase in levels of all viral
202 transcripts, demonstrating a broad increase in SARS-CoV-2 RNA levels (**Fig 3A**). This finding is
203 not surprising considering the increased viral titer observed in Calu-3 2b4 cells (**Fig 1G**). We
204 subsequently examined the levels of full-length viral RNA in the lungs of infected hamsters (**Fig**
205 **3B**), finding a significant increase at 2- and 4- dpi. In contrast, the virus lung titer at both time
206 points had no significant difference (**Fig 2C**), indicating that the KR mt increases the levels of
207 viral RNA despite not increasing titer. Further extending our analysis, we explored *in vivo*
208 SARS-CoV-2 N antigen staining in the lungs of infected animals (**Fig 3C**). KR mt infected
209 animals showed increased viral antigen staining and substantially larger lesion size compared to
210 WT. Together, these data indicate despite having no effect on lung titer, the KR mt leads to
211 increased viral RNA accumulation and greater virus spread compared to WT SARS-CoV-2.

212 **The KR mt increases phosphorylation of SARS-CoV-2 N**

213 Having confirmed a role in viral RNA transcription, we next considered how mutations in
214 residues 203-205 of nucleocapsid's SR domain might provide an advantage for SARS-CoV-2.
215 Nsp3, the multi-faceted viral protease, interacts with the SR domain to increase viral

216 transcription (18-22). Importantly, this interaction is governed by phosphorylation of the SR
217 domain, which is targeted by the SRPK, GSK-3, and Cdk1 kinases (**Fig 1D**) (5, 22-28) Given
218 the proximity to key priming residues required for GSK-3 mediated phosphorylation (**Fig 4D**)
219 (28), we hypothesized that the KR mt alters nucleocapsid phosphorylation. To overcome the
220 lack of phospho-specific antibodies for nucleocapsid, we used phosphate-affinity SDS-PAGE
221 (PA SDS-PAGE). PA SDS-PAGE utilizes a divalent Zn^{2+} compound (Phos-Tag™) within
222 acrylamide gels that selectively binds to phosphorylated serine, threonine, and tyrosine
223 residues; the bound Zn^{2+} decreases electrophoretic mobility of a protein proportionally with the
224 number of phosphorylated amino acids (29). Importantly, if a protein exhibits multiple
225 phosphorylation states, this will cause a laddering effect, with each phospho-species appearing
226 as a distinct band (**Fig 4A**).

227 To assess the KR mt's effects on N-phosphorylation, we infected Calu-3 2b4 cells at a
228 MOI of 0.01 and harvested whole cell lysates 48 hpi. Lysates then underwent PA SDS-PAGE
229 followed by western blotting with an N-specific antibody. When analyzed by PA SDS-PAGE, WT
230 SARS-CoV-2 displayed a two-band pattern consisting of a faint upper and prominent lower
231 band, corresponding to a highly phosphorylated and a less phosphorylated species, respectively
232 (**Fig 4B**, lane 1). In contrast, the KR mt displayed four dark bands of progressively slower
233 mobility, indicating a substantially different phosphorylation pattern (**Fig 4B**, lane 2). Importantly,
234 all four bands migrated more slowly than the prominent WT band, indicating an overall increase
235 in phosphorylation in the KR mt, which corresponds to increased virus replication (**Fig 1G**). We
236 next examined N phosphorylation in Vero E6 cells; cells were infected at a MOI of 0.01 and
237 whole cell lysates taken 24 hpi. In Vero E6 cells, WT SARS-CoV-2 exhibited 2-bands of equal
238 strength, indicating a relative increase in phosphorylation compared to Calu-3 2b4 cells (**Fig 4C**,
239 lane 1). In contrast, the KR mt displayed 3 dark bands with faster mobility relative to WT,
240 indicating a decrease in overall phosphorylation (**Fig 4C**, lane 2). This reduced phosphorylation

241 corresponds with the replication attenuation seen in this cell type (**Fig 1F**). Together, these data
242 indicate that the relative level of SARS-CoV-2 nucleocapsid phosphorylation plays a role in virus
243 replication.

244 Given the KR mt's effects, we next determined if SARS-CoV-2 variants had altered N-
245 phosphorylation. Calu-3 2b4 cells were infected at a MOI of 0.01 with WA-1 or the alpha, beta,
246 or kappa variants and whole cell lysates harvested at 48 hpi. When analyzed by PA SDS-
247 PAGE, WA-1 had a two-band pattern similar to WT SARS-CoV-2, while the alpha variant
248 displayed a four-band pattern with slower mobility similar to that of the KR mt (**Fig 4B**, lanes 3-
249 4). Interestingly, the mobilities of the alpha variant bands were decreased compared to the KR
250 mt indicating an even higher level of phosphorylation, potentially due to the alpha variant's
251 additional nucleocapsid mutations at D3L and S235F(6, 7). While both the beta (T205I) and
252 kappa (R203M) variants also displayed slower electrophoretic mobility compared to WA-1, the
253 beta variant displayed a two-band pattern reminiscent of WA-1 while kappa displayed a
254 ladder pattern similar to the KR mt (**Fig 4B**, lanes 5-6). Together, these data suggest variant
255 mutations at residues 203-205 result in increased N phosphorylation.

256 **The KR mt does not alter phosphorylation of virion-associated N.**

257 While CoV N proteins are hyperphosphorylated intracellularly, they are believed to lack
258 phosphorylation within the mature virion (30, 31). Nevertheless, given the ability of the KR mt to
259 augment phosphorylation, we were curious if it influenced virion-associated SARS-CoV-2 N. To
260 examine this, Calu-3 2b4 cells were infected with WT SARS-CoV-2 or the KR mt. 48 hpi, viral
261 supernatants were taken, clarified, and virions pelleted on a 20% sucrose cushion by
262 ultracentrifugation. Protein recovered from the pellets was then analyzed by PA SDS-PAGE
263 followed by western blotting with an N-specific antibody. Curiously, for both WT and the KR mt,
264 a light upper and dark lower band were detected, indicating some level of N phosphorylation is
265 present in the SARS-CoV-2 virion, albeit at a lower level than intracellular N (**S6 Fig**). However,

266 the KR mt had no effect on the banding pattern, indicating the KR mt does not affect
267 phosphorylation of mature virions.

268 **The KR mt is more resistant to GSK-3 inhibition.**

269 Our results indicate that changes in N phosphorylation correlate with differences in virus
270 replication; thus, we sought to modulate N phosphorylation using kinase inhibitors. Prior work
271 has identified two consensus sites for GSK-3 phosphorylation within the SR domain and
272 inhibition of GSK-3 has been shown to reduce SARS-CoV-2 replication (**Fig 4D**) (28).
273 Importantly, the KR mt is proximal to the priming residue of the C-terminus GSK-3 consensus
274 site, suggesting it may impact GSK-3 mediated N phosphorylation. Therefore, we examined the
275 impact of GSK-3 inhibition on both WT and the KR mt. Using kenpaullone, a GSK-3 inhibitor, we
276 showed a dose dependent inhibitory effect on both WT and KR mt titer at 48 hpi (**Fig 4E**).
277 Importantly, GSK-3 inhibition had a greater impact on WT, significantly increasing the mean titer
278 difference between WT and the KR mt from ~4-fold to ~38-fold (**S7 Fig**). This suggests that the
279 KR mt is more resistant to GSK-3 inhibition, and that the change at position 203-204 increases
280 affinity of the KR mt for GSK-3.

281 **An alanine double substitution mimics the KR mt.**

282 Given that variant mutations at residues 203-205 are diverse in sequence, we assessed the
283 importance of the specific R→K and G→R mutations to the KR mt's enhancement of infection.
284 To do so, we made a R203A+G204A double alanine substitution mutant (AA mt) in the WA-1
285 mNG background (**S8 Fig**). After recovery of recombinant SARS-CoV-2, we assessed
286 replication in Vero E6 and Calu-3 2b4 cells. In contrast with the KR mt, the AA mt had no
287 significant effect on titers in Vero E6 cells (**Fig 5A**). Nevertheless, the AA mt increased viral
288 titers over WT in Calu-3 2b4 cells throughout infection, mimicking the augmented replication of
289 the KR mt (**Fig 5B**). We next tested the fitness of the AA mt by direct competition with WT

290 SARS-CoV-2. Vero E6 and Calu-3 2b4 cells were infected with WT and the AA mt at a 1:1 ratio,
291 whole cell RNA harvested 24 hpi, and the ratio of WT to AA mt determined by NGS. In Vero E6
292 cells, the AA mt had a small but consistent fitness advantage over WT (**Fig 5C**). In contrast, the
293 AA mt outcompeted WT with a ~5:1 ratio during infection of Calu-3 2b4 cells (**Fig 5C**). Overall,
294 the similarities in replication and fitness between the KR and AA mts in Calu-3 2b4 cells suggest
295 that the KR mt enhances infection primarily by ablating the ancestral 'RG' motif.

296 Given that the AA mt mimicked the KR mt's enhancement of *in vitro* infection, we next
297 determined if they employed the same mechanism. Using RT-qPCR, we examined viral RNA
298 expression in the AA mt (**Fig 5D**). Consistent with the KR mt, at 24 hpi the AA mt increased viral
299 transcript levels compared to WT in Calu-3 2b4 cells, although the magnitude of change in the
300 AA mt was less than the KR mt (16 fold vs 32 fold). We next examined changes in N
301 phosphorylation. Calu-3 2b4 cells were infected at a MOI of 0.01 with WT SARS-CoV-2 or the
302 AA mt and whole cell lysates were harvested 48 hpi. Lysates then underwent PA SDS-PAGE,
303 followed by Western blotting for nucleocapsid. Interestingly, the AA mt exhibited several dark
304 bands with ladder mobility absent in WT, suggesting augmented phosphorylation similar to
305 the KR mt. However, contrasting with the KR mt, in the AA mt the electrophoretic mobility of the
306 lowest band was similar to WT (**Fig 5D**). These data suggest that while the KR and AA mts both
307 alter nucleocapsid phosphorylation, their effects are not identical. Nevertheless, the changes in
308 phosphorylation induced by the KR and AA mt are both sufficient to enhance SARS-CoV-2
309 replication. Finally, we examined GSK-3 inhibition comparing WT and AA mt (**Fig 5F**). Similar
310 to the KR mt, the AA mt was less sensitive to GSK-3 inhibition than WT, suggesting that
311 disrupting the original RG motif resulted in increased affinity for the kinase. Overall, these data
312 demonstrate that mutations at positions 203-204 disrupt an ancestral motif that interferes with
313 GSK-3 kinase activity.

314

315 **Discussion**

316 In this manuscript, we investigate a highly variable motif in SARS-CoV-2 nucleocapsid by
317 characterizing the effects of the R203K+G204R double substitution mutation (KR mt), present in
318 the alpha and omicron variants. Inserting the KR mt in the WA-1 background demonstrated that
319 the KR mt alone is sufficient to increase titer and fitness in respiratory cells. Similarly, the KR mt
320 is sufficient to increase disease in infected hamsters and enhance fitness in the lung during
321 direct competition studies. Importantly, we demonstrate that the KR mt augmented nucleocapsid
322 phosphorylation, which correlated with increased virus replication. We also demonstrate the KR
323 mt is resistant to inhibition of the GSK-3 kinase, mechanistically linking N phosphorylation with
324 phenotypic changes. SARS-CoV-2 variants harboring analogous mutations in the 203-205 motif
325 also exhibit augmented nucleocapsid phosphorylation, suggesting that increasing N
326 phosphorylation is a common mechanism driving variant emergence. Notably, an analogous
327 double alanine substitution (AA mt) also showed increased replication, fitness, altered
328 nucleocapsid phosphorylation, and similar resistance to kinase inhibition. Together, these data
329 suggest that the KR mt and similar mutations enhance SARS-CoV-2 infection by increasing N
330 phosphorylation through disruption of the ancestral “RG” motif.

331 The KR mt occurs within the serine-rich (SR) domain of nucleocapsid, which has a
332 complex role during SARS-CoV-2 infection. This region of N is hyper-phosphorylated
333 intracellularly, but hypo-phosphorylated within the mature virion (30, 31). Several studies
334 suggest that phosphorylation of the SR domain acts as a biophysical switch, regulating
335 nucleocapsid function through phase separation (27, 32, 33). In the proposed model,
336 unphosphorylated nucleocapsid forms gel-like condensates with viral RNA and the SARS-CoV-
337 2 membrane (M) protein to facilitate genome packaging and virus assembly. In contrast,
338 phosphorylated nucleocapsid forms distinct liquid-like condensates to promote N binding to
339 SARS-CoV-2 nsp3 (27), G3P1 in stress granules (32), and (presumably) other nucleocapsid
340 functions (34). While not tested in the context of infection, this model is consistent with studies

341 demonstrating interactions between N and M(35, 36), G3BP1 and G3BP2 within stress
342 granules(37-39), and phosphorylated N and nsp3 to promote the synthesis of viral RNA(18-22,
343 30, 40). Within this context, the KR mt may optimize this biomolecular switch for human
344 infection, increasing the amount of phosphorylated nucleocapsid and shifting the overall function
345 of N during infection. Alternatively, the KR mt may impact the interaction between nucleocapsid
346 and host 14-3-3 proteins which bind the SR domain in a phosphorylation dependent
347 manner(41). This interaction is required for cytoplasmic localization of nucleocapsid in SARS-
348 CoV (42). Notably, one 14-3-3 binding site encompasses the 203-205 motif examined in this
349 study,(41) suggesting that the KR mt may enhance infection by altering this interaction.

350 Overall, in this study we establish that the KR mt enhances SARS-CoV-2 infection
351 relative to WT, increasing viral fitness *in vitro* and *in vivo*, which along with the N501Y
352 mutation(43), likely selected for the emergence of the alpha variant. We also find that the KR mt
353 increases nucleocapsid phosphorylation; coupled with increased resistance to GSK-3 inhibition,
354 these results provide a molecular basis for the KR mt's effects. Importantly, we show that other
355 variant mutations in this motif also increase nucleocapsid phosphorylation, which may have
356 aided in the emergence of their respective lineages. When taken with the prevalence of
357 mutations in residues 203-205 of the SR domain among circulating variants, these data suggest
358 that mutations increasing nucleocapsid phosphorylation represent a positive selection event for
359 SARS-CoV-2 during adaptation to human infection. Importantly, our work highlights that
360 mutations outside of SARS-CoV-2 spike have significant effects on infection, and must not be
361 overlooked while characterizing mechanisms of variant emergence.

362

363 **Materials and Methods**

364 **Construction of Recombinant SARS-CoV-2**

365 The sequence of recombinant wild-type (WT) SARS-CoV-2 is based on the USA-WA1/2020
366 strain provided by the World Reference Center for Emerging Viruses and Arboviruses
367 (WRCEVA) and originally isolated by the USA Centers for Disease Control and Prevention(10).
368 Recombinant WT SARS-CoV-2 and mutant viruses were created using a cDNA clone and
369 standard cloning techniques as described previously (8, 9). The recombinant SARS-CoV-2
370 alpha variant (B.1.1.7) was created by introducing twenty-three individual mutations to the WT
371 SARS-CoV-2 infectious clone(12). Construction of WT SARS-CoV-2 and mutant viruses were
372 approved by the University of Texas Medical Branch Biosafety Committee.

373 **Cell Culture**

374 Vero E6 cells were grown in DMEM (Gibco #11965-092) supplemented with 10% fetal bovine
375 serum (FBS) and 1% antibiotic/antimitotic (Gibco #5240062). Calu-3 2b4 cells were grown in
376 DMEM supplemented with 10% FBS, 1% antibiotic/antimitotic, and 1 mg/ml sodium pyruvate.

377 **In vitro SARS-CoV-2 infection**

378 Vero E6 and Calu3 2b4 cells were infected with SARS-CoV-2 according to standard protocols
379 described previously (9, 44). Briefly, growth medium was removed, and cells were subsequently
380 infected with SARS-CoV-2 at a multiplicity of infection (MOI) of 0.01 diluted in 100 µl of PBS.
381 Cells were then incubated for 45 minutes at 37°C and 5% CO₂. After incubation, the inoculum
382 was removed, cells washed three times with PBS, and fresh growth medium returned. For
383 inhibitor experiments, Calu-3 cells were pretreated with 1-100 µM kenpaullone (Sigma-Aldrich
384 #422000) in growth media for 1 hour at 37°C. Cells were then infected with SARS-CoV-2 at an
385 MOI of 0.01 for 45 min at 37°C and 5% CO₂. Inoculum was then removed, cells were then
386 washed three times with PBS, and fresh growth media with kenpaullone added.

387 **Focus forming assays**

388 For viral titrations, a focus-forming assay (FFA) was developed by adapting a protocol for a
389 SARS-CoV-2 focus reduction neutralization test (FRNT) published elsewhere (45). Briefly,
390 culture supernatants, nasal washes, or homogenized tissue containing SARS-CoV-2 underwent
391 five 10-fold serial dilutions. 20 μ l of the raw sample or each dilution was then used to infect
392 individual wells of 96-well plates containing Vero E6 cells and incubated for 45 minutes at 37°C
393 and 5% CO₂. After incubation, the inoculum was removed and 100 μ l of 0.85% methylcellulose
394 (Sigma# M0512) overlay added to each well and cells incubated for 24 hours at 37°C and 5%
395 CO₂. After incubation, the overlay was removed, cells washed three times with PBS, and cells
396 fixed with 10% formalin for 30 minutes to inactivate SARS-CoV-2. Cells were then
397 permeabilized by incubating in 0.1% saponin/0.1% bovine serum albumin in PBS for 30 minutes
398 followed by treatment with a nucleocapsid specific antibody (provided by S. Makino). After
399 overnight incubation at 4°C, cells were washed three times with PBS and incubated with a
400 horseradish peroxidase (HRP)-conjugated anti-rabbit secondary antibody (Cell Signaling #7074)
401 for 1 hour at room temperature. The secondary antibody was then removed by washing three
402 times with PBS and foci developed by the application of TruBlue HRP Substrate (Seracare Life
403 Sciences #55100030). Images were then taken with a Cytation 7 cell imaging multi-mode reader
404 (BioTek) and foci counted manually. Prior to inactivation with 10% formalin, all procedures
405 involving the use of infectious SARS-CoV-2 were performed in Biosafety Level 3 (BSL3) or
406 Animal Biosafety Level 3 (ABSL3) facilities at the University of Texas Medical Branch
407 (Galveston, TX).

408 **In vitro competition assays**

409 Vero E6 or Calu-3 2b4 cells were infected at a 1:1 ratio of WT to mutant SARS-CoV-2, as
410 determined by stock titers. Twenty-four hours post infection, whole cell RNA was harvested by
411 the addition of Triazol reagent (Invitrogen #15596026) and RNA extracted with the RNA
412 Miniprep Plus kit (Zymo Research #R2072) per the manufacturer's instructions.

413 **Library preparation, sequencing, and analysis**

414 Extracted RNA samples were prepared for Tiled-ClickSeq libraries(14), with a pool of 396
415 unique primers. A modified pre-RT annealing protocol was applied in this study. Briefly, a
416 mixture of template RNA, AzDNT/dNTP, and primer pools were heated at 95°C for 2 min;
417 ramped down to 65°C at 1°C/s; incubated at 65°C for 30s; ramped down to 50°C at 0.1°C/s; the
418 rest of RT components were added to libraries while samples were held at 50°C. All subsequent
419 steps followed the previously described method(14), and the final libraries comprising 300-700
420 bps fragments were pooled and sequenced on a Illumina NextSeq platform with paired-end
421 sequencing.

422 The raw Illumina data of the Tiled-ClickSeq libraries were processed with previously established
423 bioinformatics pipelines (14). One modification is the introduction of ten wild cards (“N”) covering
424 the KR and AA mutations in the reference genome to allow *bowtie2*(46) to align reads to wild
425 type or variant genomes without bias. PCR duplications were removed using *UMI-tools*(47), and
426 the number of unique reads representing wild type, KR and AA variants were counted
427 thereafter. All raw sequencing data are available in the NCBI Small Read Archive with
428 BioProject ID: PRJNA773399.

429 **Analysis of publicly available genomic data**

430 SARS-CoV-2 sequences were accessed from the GISAID database on February 14, 2022(6).
431 Sequences were binned by the month during which the sample was collected. The number of
432 R203M, R203K+G204R, T205I, and all other non-wild type options at positions 203-205 were
433 recorded and subtracted from the total number of sequences, with the balance assumed wild
434 type at positions 203-205. Data was then graphed as a percent of total sequences collected in
435 that month.

436 **Hamster infection studies**

437 For all experiments, golden Syrian hamsters (male, three- to four-week old) were purchased
438 from Envigo. All studies were carried out in accordance with a protocol approved by the UTMB
439 Institutional Animal Care and Use Committee and complied with USDA guidelines in a
440 laboratory accredited by the Association for Assessment and Accreditation of Laboratory Animal
441 Care. Procedures involving infectious SARS-CoV-2 were performed in the Galveston National
442 Laboratory ABSL3 facility.

443 For pathogenesis studies, animals were housed in groups of five and intranasally inoculated
444 with 10^4 PFU of WT or KR mt SARS-CoV-2. For competition/transmission studies, animals were
445 intranasally inoculated with 10^4 PFU of SARS-CoV-2 comprising both WT and the KR mt at a
446 1:1 ratio based on stock titer. During competition/transmission studies, animals were singly
447 housed throughout the experiment, except during the 8-hour transmission period, when they
448 were housed in groups of two (1 donor and 1 recipient). For all experiments, animals were
449 monitored daily for weight loss and development of clinical disease until the termination of the
450 experiment. Inoculations and other animal manipulations (except weighing) were carried out
451 under anesthesia with isoflurane (Henry Schein Animal Health).

452 **SDS-PAGE and western blotting**

453 Vero E6 or Calu-3 2b4 cells were infected with SARS-CoV-2 at an MOI of 0.01 and incubated
454 for 24 or 48 hours, respectively. Virus was then inactivated and whole cell lysates taken by the
455 addition of 2× Laemmli buffer (Bio-Rad #161073) + 2-mercaptoethanol (Bio-Rad #1610710)
456 directly to the cell monolayer followed by incubation at 95°C for 15 minutes. For standard SDS-
457 PAGE, 4–20% Mini-PROTEAN TGX Gels (Bio-Rad #4561093) were used for electrophoresis.
458 For phosphate-affinity SDS-PAGE, 7.5% SuperSep™ Phos-Tag™ gels (Wako Chemical #198-
459 17981) were used for electrophoresis. For all gels, protein was transferred to polyvinylidene
460 difluoride (PVDF) membranes and blotted with a SARS-CoV nucleocapsid specific antibody
461 (Novus Biologicals #NB100-56576) followed by horseradish peroxidase (HRP)-conjugated anti-
462 rabbit secondary antibody (Cell Signaling Technology #7074). Images were developed by

463 treating blots for 5 minutes with Clarity Western ECL substrate (Bio-Rad #1705060) followed by
464 imaging on a ChemiDoc MP System (Bio-Rad #12003154). Images were processed with
465 ImageLab 6.0.1 (Bio-Rad #2012931).

466 **Virion Purification**

467 Calu-3 2b4 cells were infected with WT or the KR mt at an MOI of 0.01. Forty-eight hours post
468 infection; the supernatants were collected and clarified by centrifugation at 1200 rpm. SARS-
469 CoV-2 virions were then pelleted by ultracentrifugation through a 20% sucrose cushion at
470 26,000 rpm for 3 hours using a Beckman SW32 Ti rotor. Virion pellets were then inactivated in
471 2X Laemmli buffer (Bio-Rad #161073). N phosphorylation was then analyzed as described in
472 'SDS-PAGE and western blotting.'

473 **Histology**

474 Left lungs (Days 2 and 4) or both lungs (Day 10) were harvested from hamsters and fixed in
475 10% buffered formalin solution for at least 7 days. After buffer exchange, fixed tissue was then
476 embedded in paraffin, cut into 5 μ M sections, and stained with hematoxylin and eosin (H&E) on
477 a SAKURA VIP6 processor by the University of Texas Medical Branch Histology Laboratory.
478 Briefly, fixed tissues were submerged twice in 10% formalin baths, followed by repeated
479 submersion in a series of alcohol baths ranging from 65-100% alcohol. Tissues were then
480 submerged in xylene three times before embedding in paraffin at 60°C. Sections were then cut
481 and mounted on slides, deparaffinized by repeated washing with xylene and alcohol, and then
482 stained with hematoxylin and counterstained with eosin. Alternatively, after mounting slides
483 were deparaffinized and antigen stained in house with a SARS-CoV-2 N specific antibody (Sino
484 Biologicals #40143-R001) at a dilution of 1:30,000 followed by goat anti-rabbit secondary
485 (Vector Labs #BA1000). Signal was developed with ImmPact NovaRED HRP substrate (Vector
486 Labs # SK-4805).

487 **Real-time Quantitative PCR**

488 For determination of transcript levels in *in vitro* samples, cells were infected as described in 'In
489 vitro SARS-CoV-2 infection.' 24 hours post infection, supernatants were discarded and whole
490 cell RNA collected by the addition of Triazol reagent (Invitrogen #15596026). For *in vivo*
491 samples, hamsters were infected as described in 'hamster infection studies' and lung lobes
492 placed in RNAlater (Sigma-Aldrich #R0901) and stored at -80°C. Lung lobes were then placed
493 in 1 milliliter Triazol and homogenized with zirconia beads with a MagNA Lyser instrument
494 (Roche Life Science).

495 RNA from all sources was extracted from Triazol using the Direct-zol RNA Miniprep Plus kit
496 (Zymo Research #R2072) by the manufacturer's instructions. cDNA was then generated from
497 each RNA sample with the iScript cDNA Synthesis kit (Bio-Rad #1708891). RT-qPCR was
498 performed with Luna Universal qPCR Master Mix (New England Biolabs #M3003) per the
499 manufacturer's instructions on a CFX Connect instrument (Bio-Rad #1855200). All experiments
500 used an annealing temperature of 51°C. For the analysis of *in vitro* samples, the relative full-
501 length and subgenomic transcript levels between mutants and WT were determined by the
502 delta-delta CT method, with 18S ribosomal RNA serving as an internal control. For *in vivo*
503 samples, the levels of full-length SARS-CoV-2 genomes were quantitated with an 8-point
504 standard curve (1×10^1 to 1×10^8 copies per μ l).

505 A common forward primer binding upstream of the transcription regulatory sequence (TRS)
506 leader region was used for all transcripts (ACCAACCAACTTTCGATCTCT). For determination
507 of full-length SARS-CoV-2 genomes, a reverse primer targeting downstream of the TRS leader
508 region was used (CTCGTGTCCTGTCAACGACA). For each sub-genomic (sg) transcript,
509 reverse primers downstream of each TRS body sequence were used: sg2
510 (TGCAGGGGGTAATTGAGTTCT), sg3 (GCGCGAACAAAATCTGAAGGA), sg4
511 (AGCAAGAATACCACGAAAGCA), sg5 (ACCGTTGGAATCTGCCATGG), sg6
512 (GCCAATCCTGTAGCGACTGT), sg7 [mNeonGreen] (TGCCCTCGTATGTTCCAGAAG), sg8
513 (ACATTCTTGGTGAAATGCAGCT), and sg9 (CCCCTGCGTTCTCCATTCT). The 18S

514 targeting primers used were forward (CCGGTACAGTGAAACTGCGAATG) and reverse
515 (GTTATCCAAGTAGGAGAGGAGCGAG).

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643 **Author contributions:**

644 Conceptualization: BAJ, VDM; Formal analysis: BAJ, YZ, JAP, ALR; Funding acquisition: BAJ,
645 YZ, SCW, P-YS, ALR, VDM; Investigation: BAJ, YZ, KGL, MNV, NB, BK, CS, PACV, YL, DS,
646 JAP, XX, DW, KSP; Methodology: BAJ, YZ, PACV, JAP, ALR, SCW, VDM; Project
647 Administration: BAJ, VDM; Supervision: PA, SCW, P-YS, KSP, ALR, VDM, Visualization: BAJ,
648 JAP, DW, Writing – original draft: BAJ, VDM; Writing – review and editing: BAJ, P-YS, PACV,
649 JAP, YL, XX, SCW, DW, ALR, CS, KSP, VDM

650 **Data Reporting:** All raw sequencing data are available in the NCBI Small Read Archive
651 Bioproject ID: PRJNA773399. Raw data available from the corresponding author upon request.

652 **Correspondence and requests for materials** should be addressed to V.D.M.

653

654

Figure Captions

655

Fig 1. The KR mt enhances SARS-CoV-2 replication. (A) Amino acid frequencies for nucleocapsid residues 203-205 in SARS-CoV-2 sequences reported to the GISAID, binned by month of collection and graphed as percent of total sequences reported during that period. (B-C) Viral titer from Vero E6 (B) or Calu-3 2b4 cells (C) infected with WA-1 (black) or the alpha variant (red) at an MOI of 0.01 ($n \geq 6$). (D). Schematic of the SR domain of SARS-CoV-2 nucleocapsid. Variable residues are displayed as red text within the sequence of their corresponding lineages. Phosphorylated residues are indicated by a †. (E) Schematic of the SARS-CoV-2 genome, showing the creation of the KR mutation and the replacement of ORF7 with the mNG reporter protein. (F-G) Viral titer of Vero E6 (F) or Calu-3 2b4 (G) cells infected with WT (black) or the KR mt (red) at a MOI of 0.01 ($n=9$). (H) Competition assay between WT (gray) and KR mt (red) in Vero E6 or Calu-3 2b4 cells infected at a 1:1 input ratio with an MOI of 0.01 ($n=6$). Titer data are the mean \pm s.d. For competition, individual replicates are graphed as points, while the mean percentage of each virus is displayed as a bar graph. Statistical significance was determined by two-tailed student's T-test with $p \leq 0.05$ (*), $p \leq 0.01$ (**), and $p \leq 0.001$ (***). Grey dotted lines are equal to LOD.

670

Fig 2. The KR mt enhances SARS-CoV-2 pathogenesis and fitness. (A) Schematic of the infection of hamsters with SARS-CoV-2. (B-E) Three- to four-week-old male hamsters were mock-infected (gray) or inoculated with 10^4 PFU of WT SARS-CoV-2 (black) or the KR mt (red). Animals were then monitored for weight loss (B). On days 2 and 4 post infection, viral titers in the lung (C), trachea (D), and from nasal washes (E) were determined. (F) Schematic of competition/transmission experiment. (G-H) Three- to four-week-old male donor hamsters were inoculated with 10^4 PFU of WT SARS-CoV-2 and the KR mt at a 1:1 ratio. On day 1 of the experiment, donor and recipient hamsters were co-housed for 8 hours, then separated, and the donor hamsters underwent nasal washing. On day 2, recipient hamsters were nasal washed. Hamsters were then monitored and nasal washes and lung tissue harvested on days 4 (donors)

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680 and 5 (recipients). The ratio of WT (gray) to KR mt (red) was then determined by NGS of all
681 donor (G) and recipient (H) samples. For weight loss data, mean percent weight loss was
682 graphed \pm s.e.m. For titer data, individuals were graphed with means \pm s.d. indicated by lines.
683 For competition studies, individual replicates are graphed as points, while bars represent the
684 mean. Statistical significance was between WT and the KR mt was determined by student's T-
685 Test with $p \leq 0.05$ (*), $p \leq 0.01$ (**), and $p \leq 0.001$ (***). Grey dotted lines are equal to LOD.

686 **Fig 3. The KR mt increases levels of viral RNA and antigen.** (A) Full-length and subgenomic
687 transcript levels 24 hours post infection from Calu-3 2b4 cells infected at an MOI of 0.01 with
688 WT SARS-CoV-2 or the KR mt (n=3). Transcript levels were normalized to 18S ribosomal RNA
689 and graphed as the fold change in the KR mt relative to WT (B-C) Three- to four-week old male
690 hamsters were inoculated with PBS (mock) or 10^4 PFU of WT or the KR mt. On days 2 and 4
691 post infection, lung tissue was harvested. The levels of full-length SARS-CoV-2 RNA in WT and
692 KR mt infected animals (n=5) (B). Representative SARS-CoV-2 antigen staining (anti-
693 Nucleocapsid) of lung tissue from mock, WT, or KR mt infected animals (n=5) (C). For *in vitro*
694 transcripts, bars are mean titer \pm s.d. For *in vivo* RNA, individual replicates are graphed with
695 means \pm s.d. indicated by lines. Significance was determined by student's T-Test with $p \leq 0.05$ (*)
696 and $p \leq 0.01$ (**).

697 **Fig 4. The KR mt increases N phosphorylation to enhance infection.** (A) Schematic of
698 phosphate-affinity (PA) SDS-PAGE. (B) Whole cell lysates from Calu-3 2b4 cells infected with
699 SARS-CoV-2 WA-1-mNG (WT), the KR mt, WA-1, alpha, beta, and kappa variants were
700 analyzed by PA SDS-Page (top) or standard SDS-PAGE (bottom) followed by blotting with an
701 N-specific antibody (n=3). (C) Whole cell lysates from Vero E6 cells infected with WT or the KR
702 mt and analyzed by PA-SDS-Page (top) or standard SDS-Page (bottom) followed by blotting
703 with an N-specific antibody (n=3). (D) Schematic of phosphorylation by GSK-3 of the SR domain
704 of SARS-CoV-2 N. Residues targeted by GSK-3 are indicated with arrows and priming residues
705 designated by a '*'. (E) Viral titer 48 hours post infection from Calu-3 2b4 cells infected with WT

706 SARS-CoV-2 (gray) or the KR mt (red) at an MOI of 0.01. Cells were treated with the indicated
707 concentrations of kenpaullone prior to and during infection. Bars are the mean titer \pm s.d. (n=4).
708 Significance indicates a change in mean titer difference and was determined by student's T-Test
709 with $p \leq 0.05$ (*) and $p \leq 0.01$ (**).

710 **Fig 5. The AA mt mimics the KR mt's enhancement of SARS-CoV-2 infection. (A-B).** Viral
711 titer from Vero E6 (A) or Calu-3 2b4 (B) cells infected at an MOI of 0.01 with WT (black) or the
712 AA mt (green) (n=9). **(C)** Competition assay between WT (gray) and the AA mt (green) in Vero
713 E6 and Calu-3 2b4 cells at a 1:1 input ratio and an MOI of 0.01 (n=6). **(D)** Full-length and
714 subgenomic transcript levels 24 hours post infection from Calu-3 2b4 cells infected with WT or
715 the AA mt. Transcripts were normalized to 18S ribosomal RNA and graphed as fold change in
716 the AA mt relative to WT (n=3). **(E)** Whole cell lysates from Calu-3 2b4 cells infected with WT or
717 the AA mt and analyzed by PA SDS-Page (top) and standard SDS-Page (bottom) followed by
718 blotting with an N-specific antibody (n=3). **(F)** Viral titer 48 hours post infection from Calu-3 2b4
719 cells infected with WT (gray) or the AA mt (green) at an MOI of 0.01. Cells were treated with the
720 indicated concentrations of kenpaullone prior to and during infection (n=4). Graphs represent
721 mean titer \pm s.d. Significance was determined by two-tailed student's t-test with $p \leq 0.01$ (**) and
722 $p \leq 0.001$ (***). Grey dotted lines are equal to LOD.

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Figures

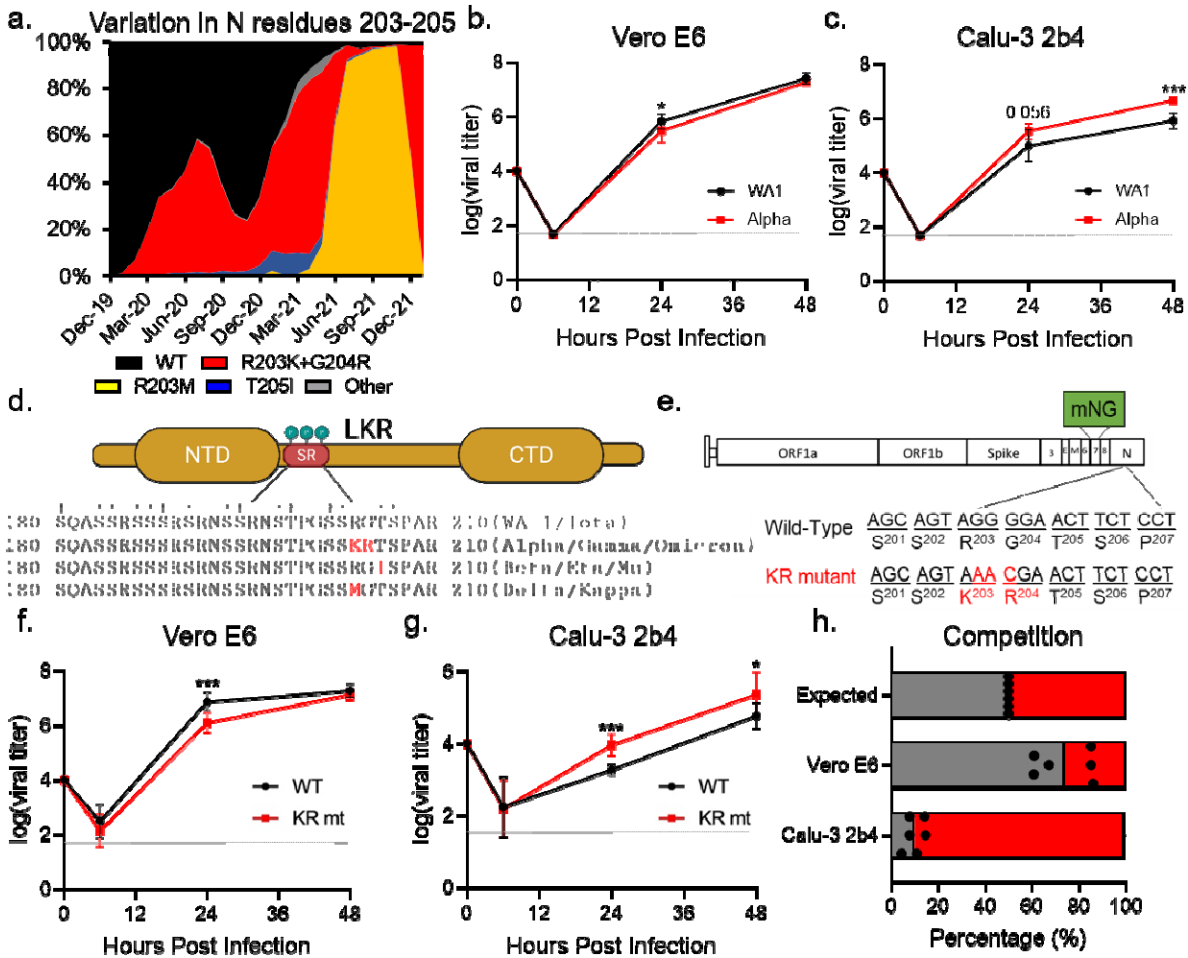
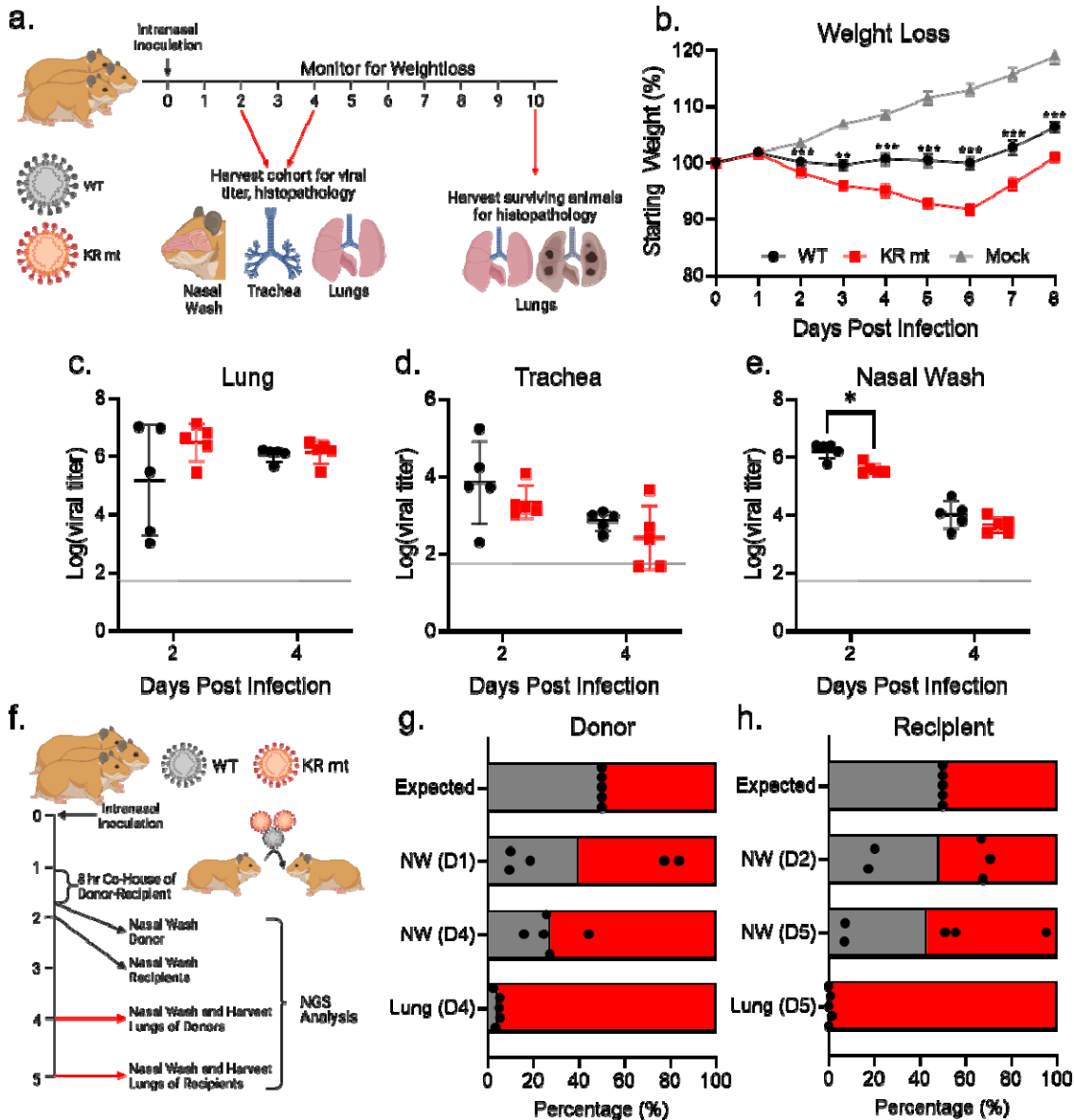


Fig 1. The KR mt enhances SARS-CoV-2 replication. (A) Amino acid frequencies for nucleocapsid residues 203-205 in SARS-CoV-2 sequences reported to the GISAID, binned by month of collection and graphed as percent of total sequences reported during that period. (B-C) Viral titer from Vero E6 (B) or Calu-3 2b4 cells (C) infected with WA-1 (black) or the alpha variant (red) at an MOI of 0.01 ($n \geq 6$). (D). Schematic of the SR domain of SARS-CoV-2 nucleocapsid. Variable residues are displayed as red text within the sequence of their corresponding lineages. Phosphorylated residues are indicated by a †. (E) Schematic of the SARS-CoV-2 genome, showing the creation of the KR mutation and the replacement of ORF7 with the mNG reporter protein. (F-G) Viral titer of Vero E6 (F) or Calu-3 2b4 (G) cells infected with WT (black) or the KR mt (red) at a MOI of 0.01 ($n=9$). (H) Competition assay between WT (gray) and KR mt (red) in Vero E6 or Calu-3 2b4 cells infected at a 1:1 input ratio with an MOI of 0.01 ($n=6$). Titer data are the mean \pm s.d. For competition, individual replicates are graphed as points, while the mean percentage of each virus is displayed as a bar graph. Statistical significance was determined by two-tailed student's T-test with $p \leq 0.05$ (*), $p \leq 0.01$ (**), and $p \leq 0.001$ (***). Grey dotted lines are equal to LOD.

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739 **Fig 2. The KR mt enhances SARS-CoV-2 pathogenesis and fitness.** (A) Schematic of the infection of hamsters
 740 with SARS-CoV-2. (B-E) Three- to four-week-old male hamsters were mock-infected (gray) or inoculated with 10^4
 741 PFU of WT SARS-CoV-2 (black) or the KR mt (red). Animals were then monitored for weight loss (B). On days 2 and
 742 4 post infection, viral titers in the lung (C), trachea (D), and from nasal washes (E) were determined. (F) Schematic of
 743 competition/transmission experiment. (G-H) Three- to four-week-old male donor hamsters were inoculated with 10^4
 744 PFU of WT SARS-CoV-2 and the KR mt at a 1:1 ratio. On day 1 of the experiment, donor and recipient hamsters
 745 were co-housed for 8 hours, then separated, and the donor hamsters underwent nasal washing. On day 2, recipient
 746 hamsters were nasal washed. Hamsters were then monitored and nasal washes and lung tissue harvested on days 4
 747 (donors) and 5 (recipients). The ratio of WT (gray) to KR mt (red) was then determined by NGS of all donor (G) and
 748 recipient (H) samples. For weight loss data, mean percent weight loss was graphed \pm s.e.m. For titer data, individuals
 749 were graphed with means \pm s.d. indicated by lines. For competition studies, individual replicates are graphed as
 750 points, while bars represent the mean. Statistical significance was between WT and the KR mt was determined by
 751 student's T-Test with $p \leq 0.05$ (*), $p \leq 0.01$ (**), and $p \leq 0.001$ (***). Grey dotted lines are equal to LOD.
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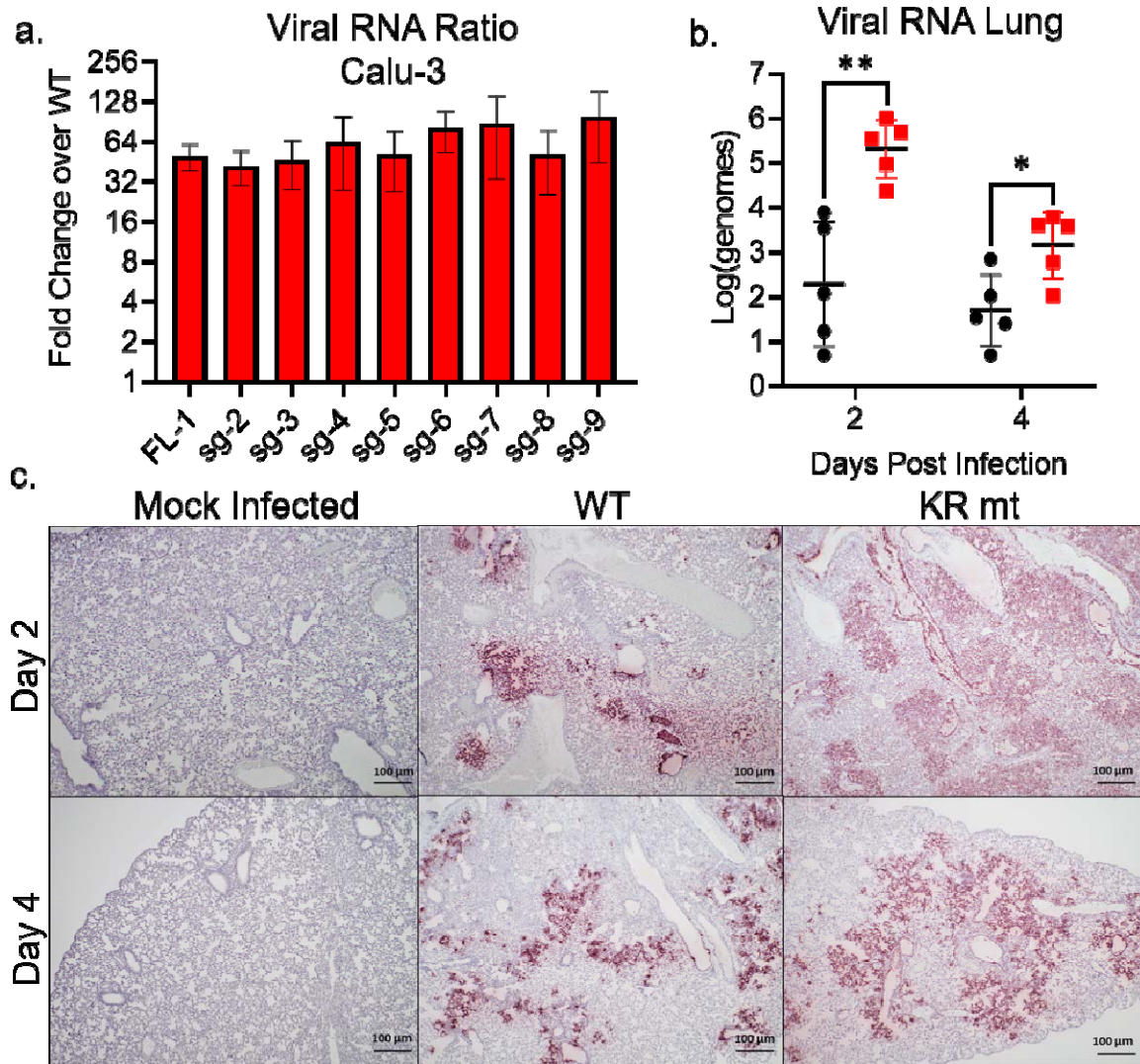


Fig 3. The KR mt increases levels of viral RNA and antigen. (A) Full-length and subgenomic transcript levels 24 hours post infection from Calu-3 2b4 cells infected at an MOI of 0.01 with WT SARS-CoV-2 or the KR mt (n=3). Transcript levels were normalized to 18S ribosomal RNA and graphed as the fold change in the KR mt relative to WT **(B-C)** Three- to four-week old male hamsters were inoculated with PBS (mock) or 10^4 PFU of WT or the KR mt. On days 2 and 4 post infection, lung tissue was harvested. The levels of full-length SARS-CoV-2 RNA in WT and KR mt infected animals (n=5) **(B)**. Representative SARS-CoV-2 antigen staining (anti-Nucleocapsid) of lung tissue from mock, WT, or KR mt infected animals (n=5) **(C)**. For *in vitro* transcripts, bars are mean titer \pm s.d. For *in vivo* RNA, individual replicates are graphed with means \pm s.d. indicated by lines. Significance was determined by student's T-Test with $p \leq 0.05$ (*) and $p \leq 0.01$ (**).

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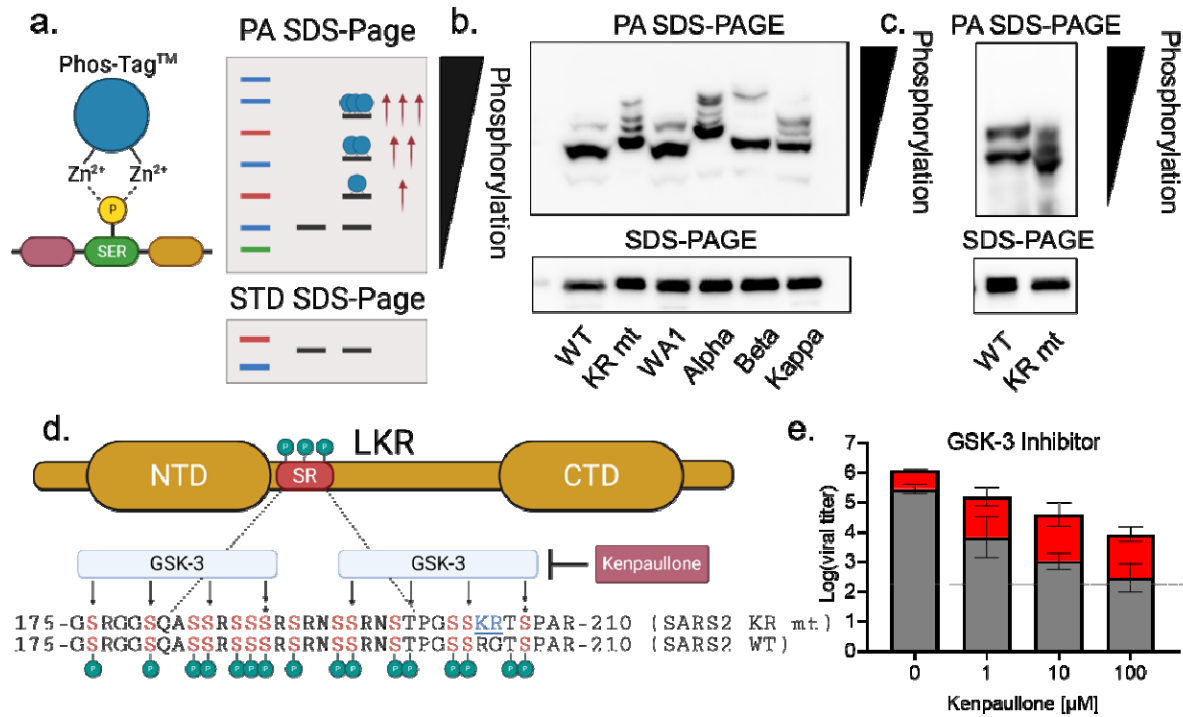
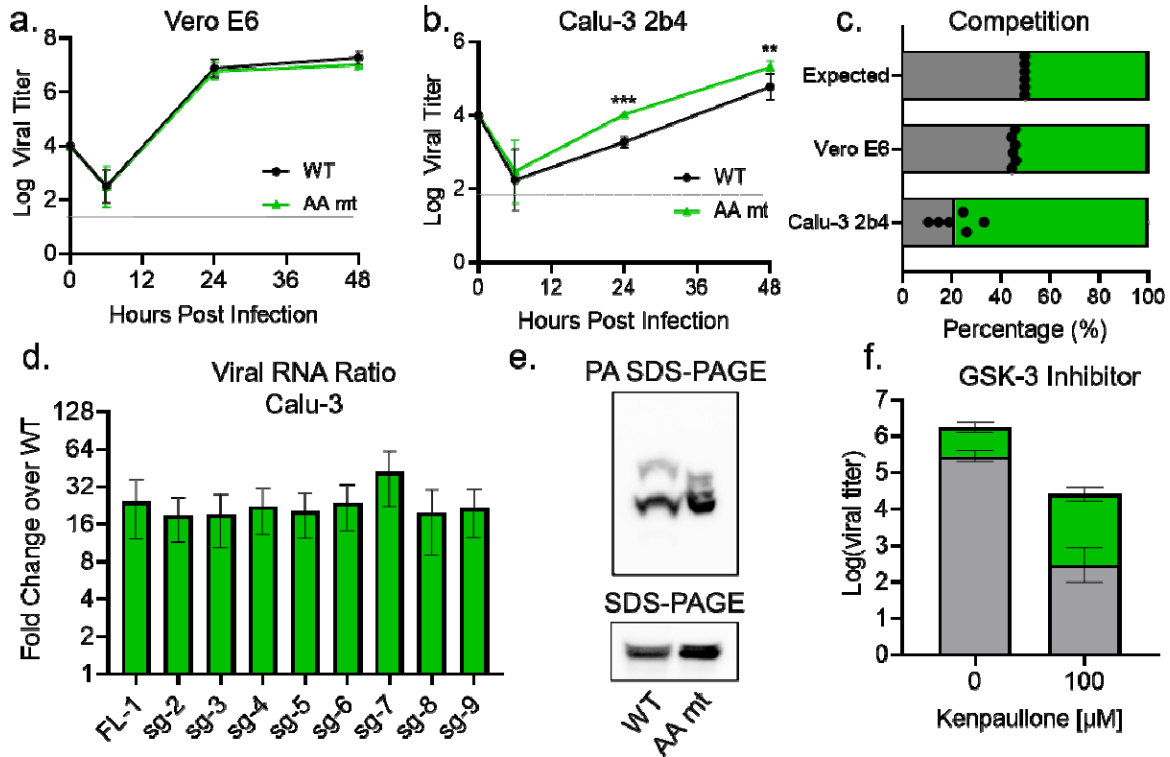


Fig 4. The KR mt increases N phosphorylation to enhance infection. (A) Schematic of phosphate-affinity (PA) SDS-PAGE. (B) Whole cell lysates from Calu-3 2b4 cells infected with SARS-CoV-2 WA-1-mNG (WT), the KR mt, WA-1, alpha, beta, and kappa variants were analyzed by PA SDS-Page (top) or standard SDS-PAGE (bottom) followed by blotting with an N-specific antibody (n=3). (C) Whole cell lysates from Vero E6 cells infected with WT or the KR mt and analyzed by PA-SDS-Page (top) or standard SDS-Page (bottom) followed by blotting with an N-specific antibody (n=3). (D) Schematic of phosphorylation by GSK-3 of the SR domain of SARS-CoV-2 N. Residues targeted by GSK-3 are indicated with arrows and priming residues designated by a '*'. (E) Viral titer 48 hours post infection from Calu-3 2b4 cells infected with WT SARS-CoV-2 (gray) or the KR mt (red) at an MOI of 0.01. Cells were treated with the indicated concentrations of kenpaullone prior to and during infection. Bars are the mean titer ± s.d. (n=4). Significance indicates a change in mean titer difference and was determined by student's T-Test with p≤0.05 (*) and p≤0.01 (**).

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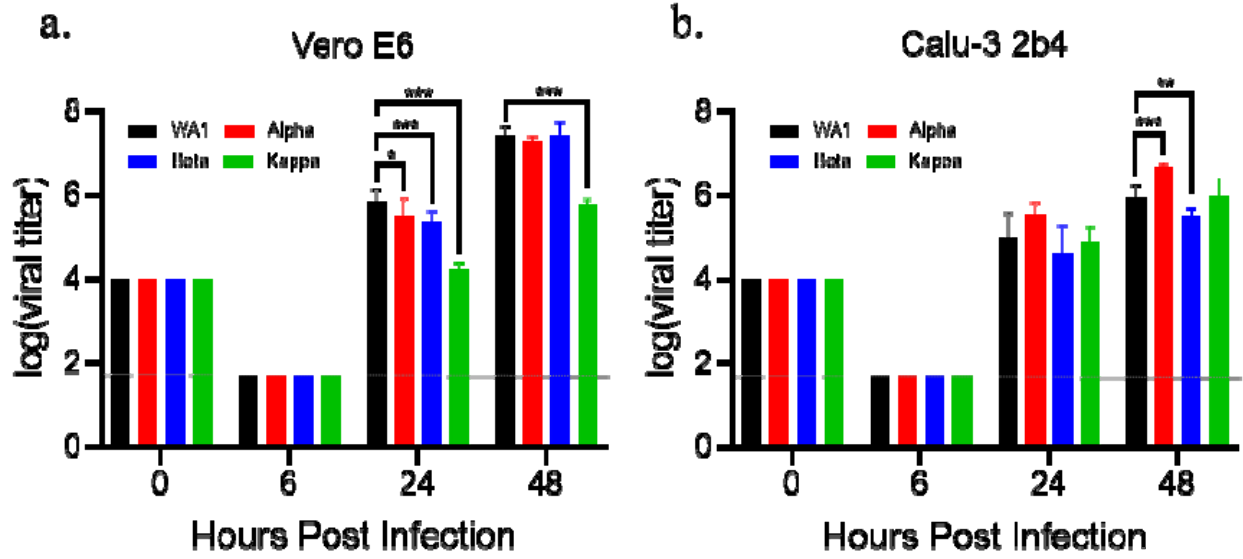
779 **Fig 5. The AA mt mimics the KR mt's enhancement of SARS-CoV-2 infection.** (A-B). Viral titer from Vero E6 (A) or Calu-3 2b4
 780 (B) cells infected at an MOI of 0.01 with WT (black) or the AA mt (green) (n=9). (C) Competition assay between WT (gray) and the
 781 AA mt (green) in Vero E6 and Calu-3 2b4 cells at a 1:1 input ratio and an MOI of 0.01 (n=6). (D) Full-length and subgenomic
 782 transcript levels 24 hours post infection from Calu-3 2b4 cells infected with WT or the AA mt. Transcripts were normalized to 18S
 783 ribosomal RNA and graphed as fold change in the AA mt relative to WT (n=3). (E) Whole cell lysates from Calu-3 2b4 cells infected
 784 with WT or the AA mt and analyzed by PA SDS-Page (top) and standard SDS-Page (bottom) followed by blotting with an N-specific
 785 antibody (n=3). (F) Viral titer 48 hours post infection from Calu-3 2b4 cells infected with WT (gray) or the AA mt (green) at an MOI of
 786 0.01. Cells were treated with the indicated concentrations of kenpaulone prior to and during infection (n=4). Graphs represent mean
 787 titer ± s.d. Significance was determined by two-tailed student's t-test with p<0.01 (**) and p< 0.001 (***). Grey dotted lines are equal
 788 to LOD.

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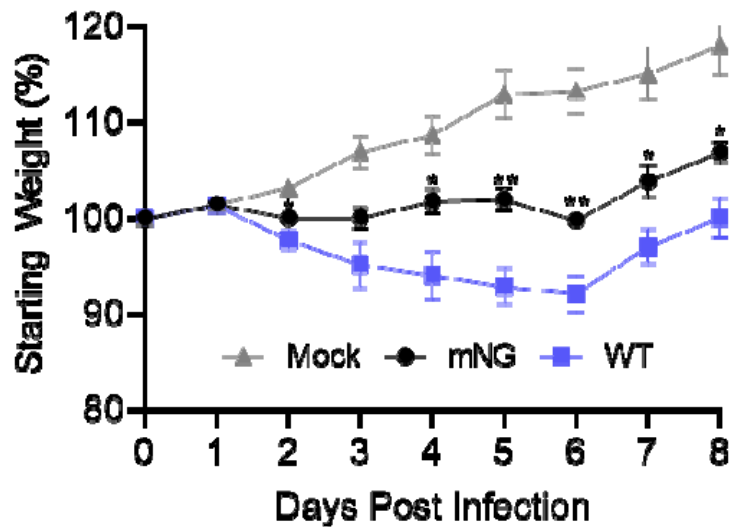
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Supporting Information



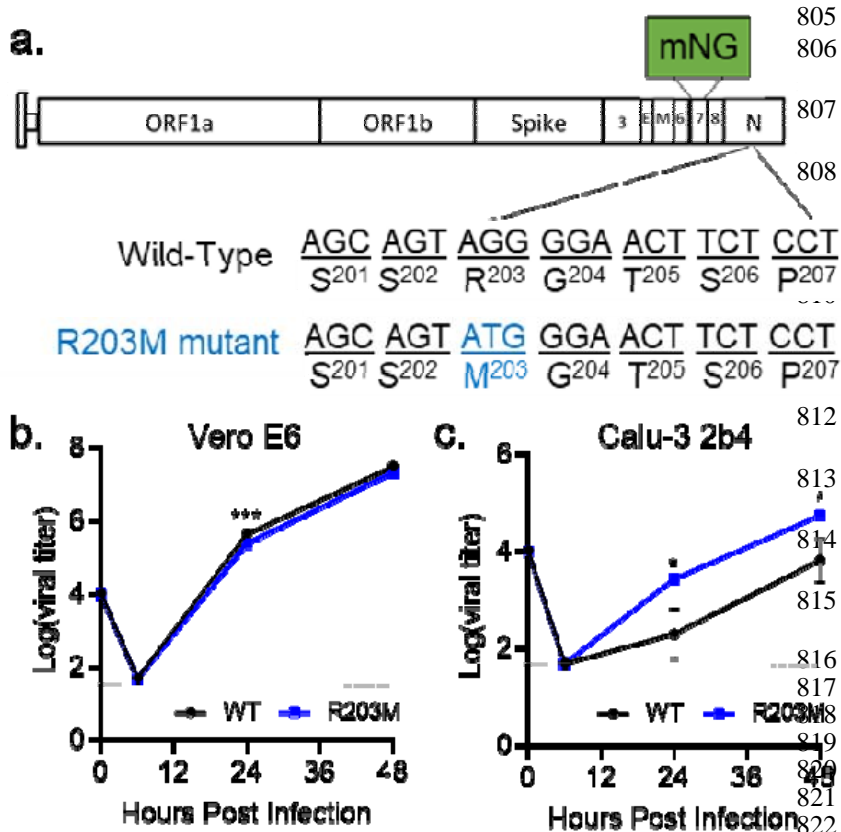
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794 **S1 Fig. Replication of SARS-CoV-2 variants.** Viral titer from Vero E6 (A) or Calu-3 2b4 cells (B) inoculated with
795 SARS-CoV-2 WA-1 (black) or the alpha (red), beta (blue) or kappa (green) variants at a MOI of 0.01. Graphed data
796 represent the mean \pm s.d. Statistical significance was determined by two-tailed student's T-test with $p \leq 0.05$ (*),
797 $p \leq 0.01$ (**), and $p \leq 0.001$ (***). Grey dotted lines are equal to LOD.
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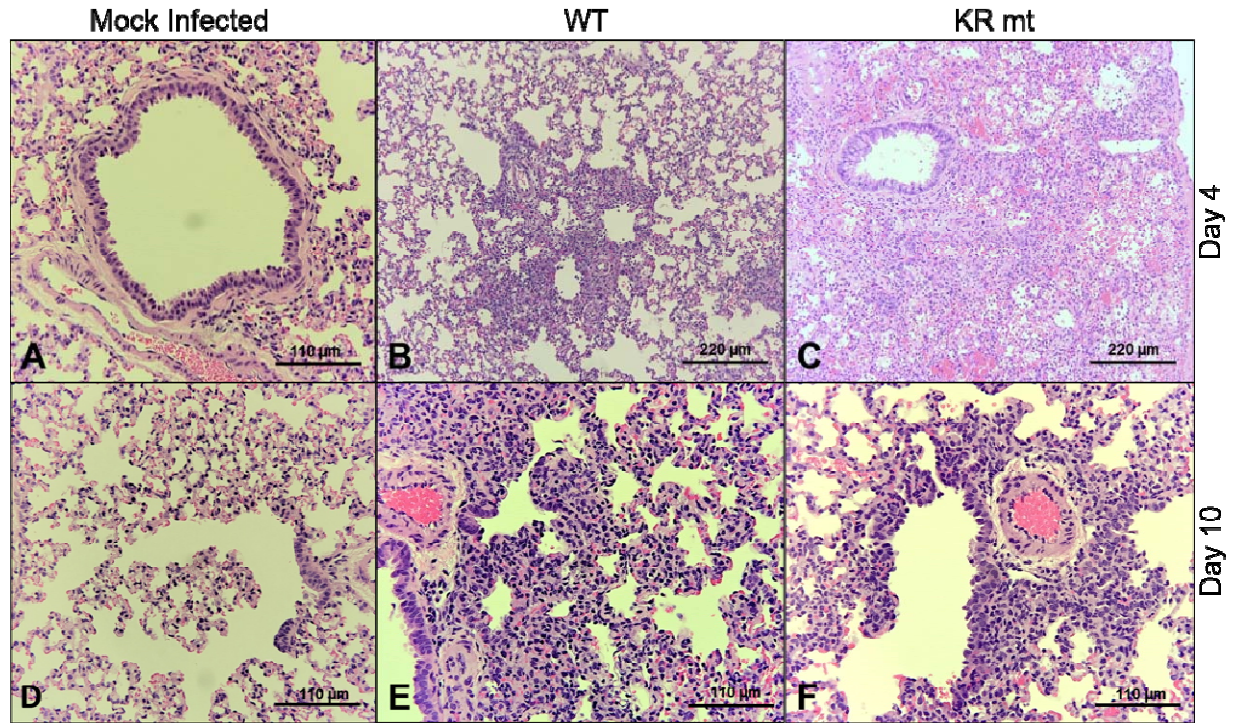
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S2 Fig. *In vivo* attenuation of the SARS-CoV-2 mNeonGreen reporter virus. Three- to four-week-old Golden Syrian hamsters were intranasally inoculated with PBS alone (gray) or 10^4 PFU of WA-1 SARS-CoV-2 (blue) or mNG SARS-CoV-2 (black). Graphed data represent the mean weight loss \pm s.e.m ($n \geq 5$). Statistical significance between WT and mNG determined by two-tailed students T-test with $p \leq 0.05$ (*) and $p \leq 0.01$ (**).



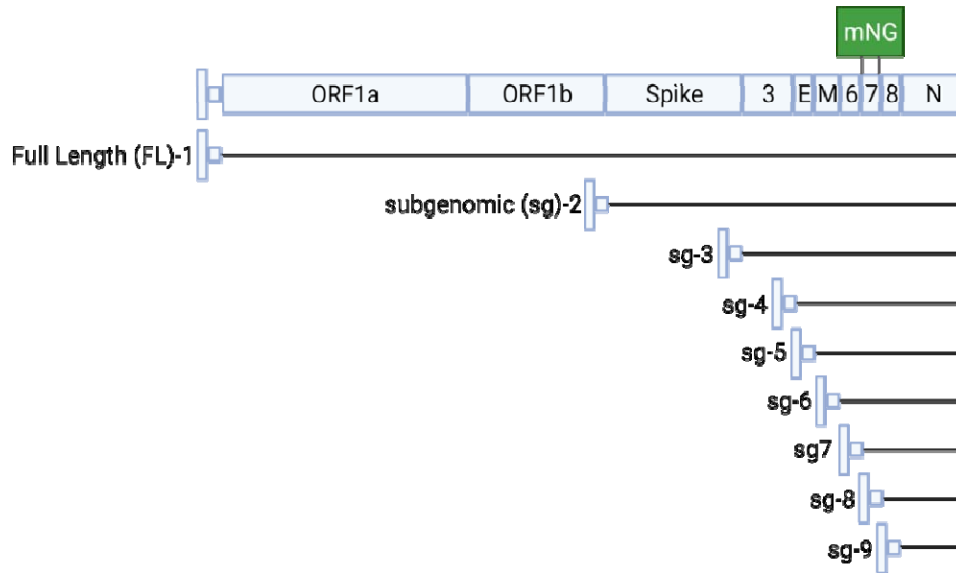
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S3 Fig. The R203M mutation enhances SARS-CoV-2 replication. (A) Schematic of the SARS-CoV-2 genome, showing the creation of the R203M mutation and the replacement of ORF7 with the mNG reporter protein. (B-C) Viral titers from Vero E6 (B) or Calu-3 2b4 (C) infected with WT or R203M SARS-CoV-2 at an MOI of 0.01. Graphed data represent mean \pm s.d. (n=3). Statistical significance was determined by two-tailed student's T-test with $p \leq 0.05$ (*) and $p \leq 0.001$ (**). Grey dotted lines are equal to LOD.



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832 **S4 Fig. Lung histopathology in hamsters infected with WT and KR mt SARS-CoV-2.** Lung tissue was harvested,
833 fixed, and 5 μm sections cut from mock, WT SARS-CoV-2, or KR mt-infected hamsters and stained with hematoxylin
834 and eosin. (A) Normal bronchus, pulmonary artery, and alveoli in mock infection on day 4 (20X). (B) Bronchiolitis,
835 peribronchiolitis, interstitial pneumonia, and edema surrounding branch of the pulmonary artery at day 4 in hamsters
836 infected with WT virus (10X). (C) Severe bronchiolar cytopathic effect, interstitial pneumonia, cytopathic alveolar
837 pneumocytes, alveoli containing mononuclear cells and red blood cells at day 4 in hamsters infected with KR mt. This
838 lesion extended over numerous fields (10X). (D) Normal respiratory bronchiole, alveolar ducts, and alveolar sacs in
839 mock infection on day 10 (20X). (E) Interstitial pneumonia adjacent to a bronchus at day 10 in hamsters infected with
840 WT (20X). (F) Bronchiolar epithelial cytopathic effect, peribronchiolitis, focal interstitial pneumonia, branch of
841 pulmonary artery with surrounding edema and mononuclear cell infiltration of endothelium at day 10 in a hamster
842 infected with the KR mt (20X). Shown are representative images typical of data gathered from 5 animals from each
843 group.

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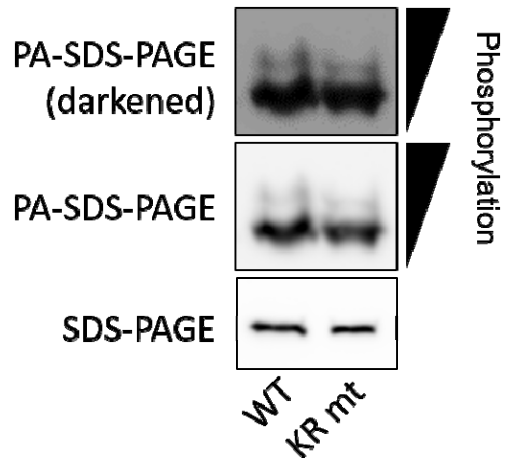
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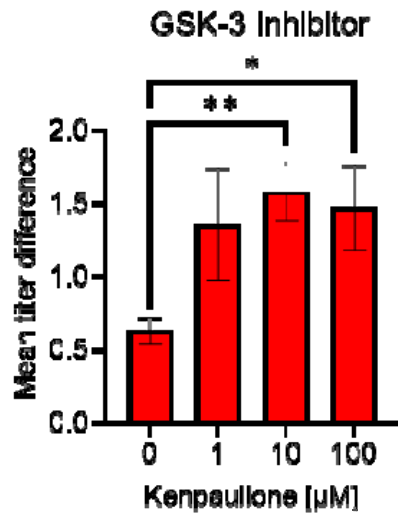
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S5 Fig. Schematic of SARS-CoV-2 RNAs. Illustration of full length (FL) and subgenomic (sg) RNAs produced during SARS-CoV-2 infection.



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S6 Fig. The KR mt has no effect on phosphorylation in virions. Calu-3 2b4 cells were infected at an MOI of 0.01 with WT or KR mt SARS-CoV-2. Forty-eight hours post infection, viral supernatants were taken. Virions were then purified from supernatants by ultracentrifugation on a 20% sucrose cushion, inactivated, and N levels analyzed by both phospho-affinity and standard SDS-Page. Results are representative of two independent experiments.



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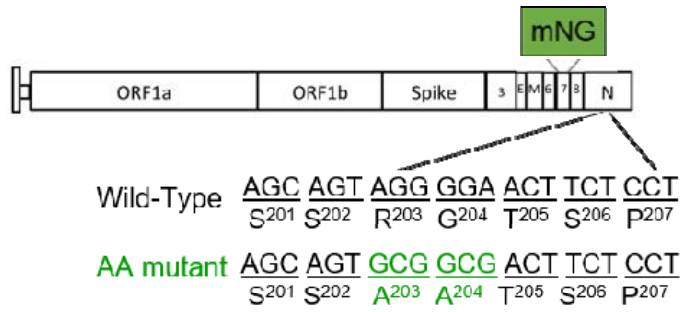
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S7 Fig. Mean titer differences between WT and KR mutant during GSK-3 inhibition. Mean difference in viral titer between WT SARS-CoV-2 and the KR mutant when treated with kenpauillone at the indicated concentrations (n=4). Error bars are \pm s.e.m. Significance by student T-test with $p \leq 0.05$ (*) and $p \leq 0.01$ (**).

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S8 Fig. Schematic representation of the AA mt. Schematic shows the creation of the AA mutation within the SARS-CoV-2 genome and the replacement of ORF7 with the mNeonGreen reporter.

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a. # Of Sequences

Collection Period	Total # of Sequences	Wild Type	R203M	R203K+G204R	T205I	Other
Dec-19	24	24	0	0	0	0
Jan-20	636	627	0	5	4	0
Feb-20	1,776	1,654	0	118	4	0
Mar-20	51,789	41,743	34	9,849	110	53
Apr-20	52,515	34,698	17	17,492	225	83
May-20	26,722	16,797	12	9,478	365	70
Jun-20	30,279	16,755	13	12,992	438	81
Jul-20	33,232	13,702	51	18,630	574	175
Aug-20	37,256	16,805	28	19,578	484	301
Sep-20	39,886	24,574	20	14,203	871	218
Oct-20	65,901	48,270	18	16,184	1,117	312
Nov-20	95,020	72,554	53	19,843	2,262	458
Dec-20	130,225	86,181	67	37,771	5,709	497
Jan-21	231,857	124,432	5,180	132,295	19,616	0
Feb-21	253,458	86,343	650	135,877	23,757	6,831
Mar-21	400,236	71,259	3,225	269,755	36,535	9,458
Apr-21	395,799	47,657	12,634	291,466	25,294	8,748
May-21	290,394	22,032	39,441	200,985	11,291	16,641
Jun-21	217,989	9,358	137,295	64,233	5,220	1,882
Jul-21	193,146	3,828	178,242	11,290	1,786	0
Aug-21	845,586	26,686	797,037	17,209	4,308	346
Sep-21	693,944	15,524	674,257	3,613	1,986	564
Oct-21	649,702	15,444	631,301	1,216	1,294	446
Nov-21	784,122	11,516	767,155	3,336	1,285	810
Dec-21	918,035	17,155	905,448	392,943	1,214	1,375
Jan-22	807,414	15,544	34,728	756,328	123	691

b. % Of Sequences

Collection Period	Total # of Sequences	Wild Type	R203M	R203K+G204R	T205I	Other
Dec-19	N/A	100.00	0.00	0.00	0.00	0.00
Jan-20	N/A	98.58	0.00	0.79	0.63	0.00
Feb-20	N/A	93.13	0.00	6.64	0.23	0.00
Mar-20	N/A	80.60	0.07	19.02	0.21	0.10
Apr-20	N/A	66.07	0.03	33.31	0.43	0.16
May-20	N/A	62.86	0.04	35.47	1.37	0.26
Jun-20	N/A	55.34	0.04	42.91	1.45	0.27
Jul-20	N/A	41.36	0.15	56.23	1.73	0.53
Aug-20	N/A	45.18	0.08	52.63	1.30	0.81
Sep-20	N/A	61.61	0.05	35.61	2.18	0.55
Oct-20	N/A	73.25	0.03	24.56	1.69	0.47
Nov-20	N/A	76.32	0.06	20.87	2.27	0.48
Dec-20	N/A	66.18	0.05	29.00	4.38	0.38
Jan-21	N/A	45.04	2.36	44.12	8.47	0.00
Feb-21	N/A	34.07	0.26	55.61	9.37	2.70
Mar-21	N/A	17.80	0.81	67.40	9.13	4.86
Apr-21	N/A	12.04	3.19	75.64	6.39	4.74
May-21	N/A	7.59	13.58	69.21	3.89	5.73
Jun-21	N/A	4.29	62.98	29.47	2.39	0.86
Jul-21	N/A	1.96	91.34	5.79	0.92	0.00
Aug-21	N/A	3.16	94.25	2.04	0.51	0.04
Sep-21	N/A	2.23	96.88	0.52	0.29	0.08
Oct-21	N/A	2.38	97.17	0.19	0.20	0.07
Nov-21	N/A	1.47	97.84	0.43	0.16	0.10
Dec-21	N/A	1.87	55.05	42.80	0.12	0.15
Jan-22	N/A	1.93	4.30	93.67	0.02	0.09

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S1 Table. Frequency of mutations in residues 203-205 in SARS-CoV-2 nucleocapsid. (A-B) Frequency of WT, R203M, R203K+G204R, T205I, or all other genotypes binned by month of collection, represented as the raw totals (A) or as a percentage of total sequences in a given month (B)..