1 **A highly attenuated SARS-CoV-2 related pangolin coronavirus variant has a**

2 **104nt deletion at the 3'-terminus untranslated region**

3 Shanshan Lu^{1,4}, Shengdong Luo^{2,4}, Chang Liu¹, Muli Li³, Xiaoping An¹, Mengzhe Li¹,

4 Jun Hou², Huahao Fan^{1*}, Panyong Mao^{2*}, Yigang Tong^{1*}, Lihua Song^{1*}

5

6 ¹Beijing Advanced Innovation Center for Soft Matter Science and Engineering,

7 College of Life Science and Technology, Beijing University of Chemical Technology,

8 Beijing, China

9 ²Department of Infectious Diseases, Fifth Medical Center of Chinese PLA General

10 Hospital, Beijing, China

11 ³Department of Pathology, National Cancer Center/National Clinical Research Center

12 for Cancer/Cancer Hospital & Shenzhen Hospital, Chinese Academy of Medical

13 Sciences and Peking Union Medical College, Shenzhen, China

14 ⁴These authors contributed equally: Shanshan Lu, Shengdong Luo.

15

16 *Corresponding authors

17 Email: songlihua@gmail.com (LS); tong.yigang@gmail.com (YT);

18 maopy302@163.com (PM); fanhuahao@mail.buct.edu.cn (HF)

19

20

21 **Abstract**

22 SARS-CoV-2 related coronaviruses (SARS-CoV-2r) from Guangdong and Guangxi
 23 pangolins have been implicated in the emergence of SARS-CoV-2 and future
 24 pandemics. We previously reported the culture of a SARS-CoV-2r GX_P2V from
 25 Guangxi pangolins. Here we report the GX_P2V isolate rapidly adapted to Vero cells
 26 by acquiring two genomic mutations: an alanine to valine substitution in the
 27 nucleoprotein and a 104-nucleotide deletion in the hypervariable region (HVR) of the
 28 3'-terminus untranslated region (3'-UTR). We further report the characterization of the
 29 GX_P2V variant in *in vitro* and *in vivo* infection models. In cultured Vero and BGM
 30 cells, the GX_P2V variant produced minimal cell damage and small plaques. The
 31 GX_P2V variant infected golden hamsters and BALB/c mice but was highly
 32 attenuated. Golden hamsters infected intranasally had a short duration of productive
 33 infection. These productive infections induced neutralizing antibodies against
 34 pseudoviruses of GX_P2V and SARS-CoV-2. Collectively, our data show that the
 35 GX_P2V variant is highly attenuated in *in vitro* and *in vivo* infection models.
 36 Attenuation of the variant is likely due to the 104-nt deletion in the HVR in the
 37 3'-UTR. This study furthers our understanding of pangolin coronaviruses
 38 pathogenesis and provides novel insights for the design of live attenuated vaccines
 39 against SARS-CoV-2.

40

41 **Introduction**

42 The COVID-19 pandemic caused by SARS-CoV-2 (severe acute respiratory
43 syndrome coronavirus 2) is producing unprecedented damages around the globe¹⁻³. As
44 of 20th December 2021, the World Health Organization has reported over 273 million
45 cases with over 5.3 million death cases. Investigation of the origin of the virus has not
46 identified its direct ancestral viruses, but has led to the discovery of many
47 SARS-CoV-2 related coronaviruses (SARS-CoV-2r) in both bats and pangolins⁴⁻⁹.
48 Bat-derived coronaviruses found in Laos are the closest relatives of SARS-CoV-2
49 reported to date¹⁰. Despite the finding of these SARS-CoV-2r coronaviruses, very few
50 of them have been cultured, thus their biology and pathogenicity remain largely
51 unknown.

52

53 While bat viruses are the closest relative of SARS-CoV-2, pangolin coronaviruses
54 likely also play an important role in the origin and evolution of SARS-CoV-2. Two
55 SARS-CoV-2r pangolin coronaviruses GD/2019 and GX/2017 have been identified^{6,7}.
56 The GD/2019 strain has a receptor binding domain (RBD) that shares high homology
57 (97.4%) with that of SARS-CoV-2, which leads to the possibility that the origin of the
58 SARS-CoV-2 RBD is a result of recombination between bat and pangolin
59 coronaviruses⁷. The GX/2017 strain has an RBD that shares 86.8% identity with that
60 of SARS-CoV-2⁶. A recent study shows that the spike protein of GX/2017 has
61 comparable binding capabilities to human ACE2 and mediating cell entry when
62 compared to the SARS-CoV-2 spike¹¹. Because of the high similarity of RBDs of

63 pangolin CoVs binding to ACE2 receptors between humans and pangolins, pangolin
64 coronaviruses likely provide a repertoire of CoVs for future pandemics.

65

66 We previously reported the culture of a pangolin coronavirus GX_P2V⁶, which
67 shares an identical spike protein with other pangolin GX/2017 strains. The genome of
68 coronavirus GX_P2V (GenBank accession number MT072864) was determined by
69 using RNAs from the first passage of the GX_P2V sample, not a serially passaged
70 isolate⁶. This GX_P2V genome has 29,795 nucleotides, including ten unidentified
71 nucleotides and two unknown termini. Considering the similarities in genetics, cell
72 infectivity, and host tropism between this pangolin coronavirus and SARS-CoV-2, we
73 sought to determine the complete genome of the GX_P2V isolate and investigate its
74 biology and pathogenicity in animal models, which may help in understanding the
75 evolution and pathogenicity of SARS-CoV-2 related viruses.

76

77 Here, we report that, compared to the original GX_P2V sample, the GX_P2V
78 isolate is a variant with two mutations: one nonsynonymous mutation in the final
79 amino acid codon of the nucleoprotein gene and a 104-nucleotide deletion in the
80 hypervariable region (HVR) of the 3'-terminus untranslated region (3'-UTR). We then
81 describe the characterization of the GX_P2V variant's growth in two monkey cell
82 lines and two small animal models. We found that the GX_P2V variant has the
83 capability to infect but is highly attenuated in all four of the tested infection models.
84 The attenuation of this pangolin coronavirus isolate is likely due to the 104-nt deletion

85 at 3'-UTR. A short duration of productive infection in golden hamsters induced
86 neutralizing antibodies against pseudoviruses of GX_P2V and SARS-CoV-2. This
87 work advances our understanding of pangolin coronaviruses pathogenesis and
88 provides important insights for the design of live attenuated vaccines against
89 SARS-CoV-2.

90

91 **Results**

92

93 **Genomic determination of pangolin coronavirus GX_P2V isolate.** Total RNAs
94 from the eighth viral passage were used for Illumina pair-end sequencing. In total,
95 8,482,736 reads were mapped to the previous genome of GX_P2V using Bowtie 2.
96 The resulting viral reads were then assembled *de novo* by Trinity. The viral genomic
97 contig has 29,718 nucleotides including eight consecutive adenine residues at the
98 3'-terminus. The 5'-terminus of the viral genome was determined by 5'/3' RACE kits
99 (TaKaRa) and the whole genome sequence (29,729 nt) was deposited in GenBank
100 (accession number MW532698).

101

102 **Genomic comparison between the GX_P2V sample and the coronavirus GX_P2V**
103 **isolate revealed two mutations in the passaged viral isolate.** We next compared the
104 sequence differences between the first passage of the GX_P2V sample and the eighth
105 passage of the GX_P2V isolate. Compared to the single cell-passaged sample, the
106 multiply passaged GX_P2V isolate has complete 5'/3'-terminus sequences and two

107 mutations: a nonsynonymous mutation in the final amino acid codon of the
108 nucleoprotein gene (GCU→GUU) and a 104-nucleotide deletion in the hypervariable
109 region (HVR) of the 3'-terminus untranslated region (3'-UTR) (Fig. 1). Thus, the
110 multiply passaged GX_P2V isolate is a cell culture adapted variant. The result of the
111 single-nucleotide mutation is an alanine to valine change at the carboxyl terminal of
112 the nucleoprotein. We then asked whether these two mutations occurred in the first
113 passage of the GX_P2V in Vero cells. Among the sequencing reads of the GX_P2V
114 sample (SRR11093271), only one read contained the nucleoprotein single-nucleotide
115 mutation while no reads contained the HVR deletion. In comparison, among the
116 sequencing reads of the multiply passaged isolate, all reads covering these two
117 mutated regions contain the identified mutations. These data suggest that these two
118 identified regions have strong selection pressures in cell culture and selection
119 occurred rapidly following *in vitro* growth.

120

121 The temporal occurrence and selective factor(s) for these mutations are unknown. It
122 is plausible that both mutations increased the efficiency of viral replication. The
123 spontaneous 104-nt deletion within the viral 3'-UTR is of special interest. According
124 to the current 3'-UTR model¹², the HVR region of the GX_P2V sample is 166-nt long
125 –ranging from 29,611 to 29,776 based on the deposited sequence data (GenBank
126 accession number MT072864). The deletion region ranges from 29,617 to 29,720,
127 covering 62.7% of the entire HVR region. The HVR region is absolutely maintained
128 in all coronavirus clinical samples. Based on our knowledge, this is the first report of

129 a spontaneous deletion in the HVR region.

130

131 **Growth of the pangolin coronavirus GX_P2V variant yields mild cell damage**

132 **and small plaques in non-human primate cells.** The GX_P2V isolate was cultured

133 from a mixture of pangolin lung and small intestine samples by using Vero E6 cells⁶.

134 It has been shown that the virus only partially destroys Vero E6 cell monolayer five

135 days post-infection⁶. In cells infected with the virus for five days, the cytopathic

136 effects manifested as cell degeneration and cell lysis, with significant portion of cell

137 rounding but limited cell detachment. We next systematically investigated the growth

138 of the GX_P2V variant in two non-human primate cells: Vero and BGM. The BGM

139 cell line was selected based on the fact that BGM is susceptible to SARS-CoV and the

140 pangolin viruses GX/2017 utilize ACE2 as the cell receptor for viral attachment and

141 entry^{11,13}.

142

143 BGM and Vero cells were infected with the GX_P2V variant at a multiplicity of

144 infection (MOI) of 0.01. Growth of GX_P2V induced similar cytopathic effects in

145 both Vero and BGM cells (Fig. 2a). No or limited cell degeneration can be observed at

146 24 hours postinfection. More evident cell degeneration and cell rounding appeared at

147 48 hours postinfection. Cell lysis and detachment started around 72 hours

148 postinfection. These cytopathic effects were directly related to viral replications,

149 which was confirmed by indirect immunofluorescence (Fig. 2b). Consistent with the

150 observed slow rate of cell damages, the GX_P2V variant produced small plaques at

151 five days postinfection (Fig. 2c, d). In comparison, SARS-CoV-2 produced substantial
152 cell damages and large plaques in Vero cells at two days postinfection^{4,14}. Compared
153 to reported SARS-CoV-2 data, the GX_P2V variant is highly attenuated in causing
154 cell damage and plaque formation.

155

156 The growth kinetics of the GX_P2V variant in both BGM and Vero cells were
157 measured by quantifying the viral RNA copies and viral titers in the culture
158 supernatants by using qPCR and viral titration, respectively (Fig. 3). Cells were
159 infected with a MOI of 0.01. Supernatants were collected at indicated time points and
160 were used for quantification assays. In both cells, the viral RNA copies reached the
161 peak at 72 h.p.i. (Fig. 3a) and the peaks of viral titers were at 48 h.p.i. (Fig. 3b),
162 though only limited cell degenerations could be observed at this time point. The viral
163 titers at 48 h.p.i. were as high as 10^7 TCID₅₀/mL—a titer number that is comparable to
164 the titer of SARS-CoV-2 at this time point¹⁴. The growth curves of the GX_P2V
165 variant in both cells are similar, but do have significant differences at 24 and 120 h.p.i.
166 (Fig. 3b). Higher viral titers were detected in Vero cells at 120 h.p.i. ($p < 0.001$).
167 Consistent with this difference, Vero cells were more supportive of viral plaque assays,
168 as they have more cell lysis starting at 72 h.p.i. and produced larger viral plaques at
169 120 h.p.i. (0.36 ± 0.04 mm v.s. 0.51 ± 0.08 mm, $p < 0.0001$) (Fig. 2a, d).

170

171 **The pangolin coronavirus GX_P2V variant is highly attenuated in the golden**
172 **hamster intranasal infection model.** Studies on SARS-CoV-2 pathogenesis provide

173 animal models for investigating the pathogenicity of the GX_P2V variant¹⁵⁻¹⁷, as its
 174 spike protein has similar capabilities of binding to human ACE2¹¹. The golden
 175 hamster model is especially susceptible to SARS-CoV-2. SARS-CoV-2 administered
 176 by intranasal route can replicate efficiently in the lungs of hamsters, causing severe
 177 pathological lung lesions¹⁶. These severe lung injuries shared characteristics with
 178 SARS-CoV-2-infected human lungs, including severe, bilateral, peripherally
 179 distributed, multilobular ground glass opacity, and regions of lung consolidation^{16,17}.
 180 We next investigated the replicative ability and pathogenesis of the GX_P2V variant
 181 in golden hamsters.

182

183 Three groups of hamsters were intranasally infected with mock, 1×10^4 and 1×10^5
 184 TCID₅₀ of the GX_P2V variant, respectively. Clinical signs were recorded and
 185 samples were collected periodically throughout the 14-day experiment (Fig. 4a). The
 186 infection outcomes are clearly contrast to those described in the SARS-CoV-2
 187 infected hamsters. Hamsters infected with the GX_P2V variant had similar body
 188 weight increases to the mock infected hamsters and no apparent clinical symptoms in
 189 the two week observative period (Fig. 4b). Consistent with the absence of clinical
 190 manifestations, there was only a slight hyperemia noted on the surface of lungs
 191 following infection with the GX_P2V variant at 2 and 5 d.p.i. (Fig. 4c). Moreover, no
 192 signs of bronchopneumonia were found in the tracheas of infected hamsters (Fig. 4c).

193

194 To monitor the viral replication and pathogenesis, lungs and tracheas of hamsters

195 were collected at indicated time points. Viral RNA copies and viral titers were
196 measured. In both organs, viral RNA copies peaked at two d.p.i. and gradually
197 decreased for the remaining says (Fig. 4d, e). The RNA copy numbers at two d.p.i.
198 were larger than the original inoculum numbers, demonstratng replication in both
199 lungs and tracheas. However, viral titers were only detected in early stages
200 post-infection: at two and five d.p.i. in lungs and day two in tracheas (Fig. 4f, g). This
201 suggests a shorter duration of viral replication in both organs, but especially in
202 tracheas.

203

204 Hematoxylin and eosin staining was performed to determine the histopathological
205 changes in both lungs and tracheas. Histopathologically, the tissues from hamsters of
206 the 1×10^4 TCID₅₀ group were comparable to sham infected controls (Fig. 5a).
207 Infection with 1×10^5 TCID₅₀ of the GX_P2V variant resulted in a local infiltration
208 with very few inflammatory cells at five d.p.i., and none at other time points (Fig. 5b).
209 Compared to the mock group, no pathological lesions were found in tracheas of all
210 GX_P2V variant infected hamsters over the entire observation period (Fig. 5c). Thus,
211 the GX_P2V isolate did not induce potentially damaging inflammatory changes in
212 lungs or tracheas of either infected groups. The lack of detectable pathology and
213 short duration of viral replication in infected hamsters suggests that the GX_P2V
214 variant has a replicative deficiency in hamsters.

215

216 **Intragastrical inoculation established limited infections in respiratory tracts but**

217 **no detectable infection in the gastrointestinal tract.** Human gastrointestinal (GI)
 218 infection by SARS-CoV-2 has been implied, as SARS-CoV-2 RNA can be frequently
 219 detected in the feces of COVID-19 patients¹⁸. In the golden hamster model, oral
 220 inoculation can establish a SARS-CoV-2 infection with an infection pattern that is
 221 consistent with mild COVID-19¹⁹. In the above described intranasal infection of
 222 golden hamsters with the GX_P2V variant, viral RNA sheddings were detected in
 223 tongues and feces only at the earliest times post inoculation (Figure. 6a), which is
 224 different than the continuous RNA sheddings in COVID-19 patients. We performed
 225 intragastrical inoculation of the GX_P2V variant in golden hamsters to further assess
 226 viral pathogenicity. Golden hamsters were intragastrically infected with 10^5 TCID₅₀ of
 227 the GX_P2V variant and samples were collected periodically (Fig. 6b). All hamsters
 228 had consecutive weight increases (Fig. 6c) and no apparent clinical signs were
 229 observed (Fig. 6d). Limited viral RNAs were detected in lungs but not in other organs
 230 or samples (stomachs, duodenum, colons, tongues, trachea, esophagus, small
 231 intestines and feces) (Fig. 6e). Thus, the virus shedding of GX_P2V in golden
 232 hamsters differs from the chronic virus shedding from the oral cavity and feces
 233 following oral inoculation of SARS-CoV-2¹⁹. Predictably, no histopathological
 234 changes were detected in lungs of intragastrically infected hamsters (Fig. 6f). Similar
 235 to previous reports with SARS-CoV-2 in golden hamsters¹⁹, we failed to detect
 236 infectious virus in fecal samples from either intranasal or intragastrical infections (Fig.
 237 6g). Thus, in golden hamsters, the GX_P2V variant has limited capability of
 238 establishing infections of the gastrointestinal tract or when introduced by the

239 intranasal route.

240

241 **The GX_P2V variant does replicate but produce no infectious virus in young and**

242 **aged BALB/c mice.** SARS-CoV-2 can rapidly adapt in aged BALB/c mice and

243 induce typical pneumonia^{20,21}. Therefore we next investigated the pathogenicity of the

244 GX_P2V variant in both young and aged BALB/c mice. BALB/c mice were

245 intranasally infected with 10⁵ TCID₅₀ of the GX_P2V variant and euthanized to

246 collect samples at specific days postinfection (Fig. 7a). Following inoculation, no

247 apparent clinical signs were observed in any inoculated mice. Notably, the body

248 weights of young BALB/c mice continued to increase (Fig. 7b) and no gross

249 pathological changes on the lung surfaces of inoculated mice were detected (Fig. 7c).

250 The viral RNAs in the lung of mice were rapidly cleared (Fig. 7d), and aged mice had

251 an even faster rate of viral clearance. This is in contrast to the slower rate of viral

252 clearance in aged mice inoculated with SARS-CoV-2²⁰. Histopathological analyses

253 suggest that lymphocytes play an important role in the rapid viral clearing (Fig. 7e, f).

254 Lungs of the inoculated young mice had local thickened alveolar septa and

255 lymphocyte aggregations at one d.p.i., then rapidly returned to normal at three d.p.i.

256 (Fig. 7e). Comparatively, the lungs of the inoculated aged mice showed multifocal

257 abundant lymphocytes and some macrophages present up to day 3 d.p.i. (Fig. 7f).

258 Furthermore, we failed to detect any virus in lungs, despite detection of high viral

259 RNA copies at the early infection time points (Fig. 7f). Altogether, our data indicate

260 that the GX_P2V variant can infect but has a replication deficiency in BALB/c mice.

261

262 **Productive infections in golden hamsters induced neutralizing antibodies against**
 263 **both GX_P2V and SARS-CoV-2.** The coronavirus GX_P2V has high genomic
 264 homology with SARS-CoV-2⁶. The spike protein and the receptor binding domain of
 265 GX_P2V share 92.4% and 86.8% amino acid identity with those of SARS-CoV-2,
 266 respectively. The attenuating nature of the GX_P2V variant, combined with its
 267 similarity to SARS-CoV-2, led us to propose that the GX_P2V variant is a potential
 268 live vaccine candidate. To address, we analyzed the humoral responses against
 269 GX_P2V in infected animals and evaluated cross-reactive antigenicity of the two
 270 spike proteins from GX_P2V and SARS-CoV-2.

271

272 Cells transfected with the full-length genes of the two spike proteins were used to
 273 perform immunofluorescence assays with sera from both convalescent COVID-19
 274 patients and intranasally infected golden hamsters (Fig. 8a). The results clearly show
 275 that these two spike proteins share strong cross-reactive antigenicity. Commercial
 276 SARS-CoV-2 S1 antibody assay kits can be used to titrate the humoral responses
 277 against GX_P2V in hamsters (Fig. 8b). The intranasally infected hamsters have a
 278 strong humoral response to the spike protein. Such strong immune responses correlate
 279 with the productive infections in intranasally infected hamsters. In contrast, low titer
 280 or no antibodies against the spike protein were detected in intragastrically infected
 281 hamsters or intranasally infected BALB/c mice (Fig. 8c).

282

283 SARS-CoV-2 neutralizing antibodies play an important role for the protection
284 against COVID-19. We analyzed the neutralizing antibodies in sera from COVID-19
285 convalescent patients, vaccinees of inactivated COVID-19, and intranasally infected
286 hamsters by using pseudoviruses of GX_P2V and SARS-CoV-2 (Fig. 8d). All tested
287 positive sera had variable titers of neutralizing antibodies against both pseudoviruses.
288 When compared to sera from convalescent patients, the hamster sera had a similar
289 titer of neutralizing antibodies against SARS-CoV-2, although the RBDs of GX_P2V
290 and SARS-CoC-2 share only 86.8% amino acid identity and share a single critical
291 receptor-binding residue⁶. This finding supports the rational for the design of
292 pan-SARS-CoV-2r vaccines.

293

294 Discussion

295

296 The devastating force of COVID-19 intensified the world's fear over SARS-CoV-2
297 and its related coronaviruses. The search and characterization of SARS-CoV-2 related
298 coronaviruses is an area of intense interest. In this study, we investigated the genome,
299 biology, pathogenicity, and cross-reactive antibody response of a SARS-CoV-2 related
300 pangolin coronavirus GX_P2V variant. We found that this GX_P2V variant is a
301 3'-UTR shortened mutant derived from the original GX_P2V sample. The GX_P2V
302 variant was highly attenuated in two cell cultures, golden hamsters, and BALB/c mice.
303 Importantly, the antibody elicited by GX_P2V isolate infection in hamsters inhibited
304 SARS-CoV-2 spike-pseudovirus infection. Attenuation and cross-neutralizing activity

305 of the GX_P2V variant provides new insights for our understanding of coronavirus
306 pathogenesis and for developing a live attenuated vaccine for SARS-CoV-2.
307 Unfortunately, the original wild type GX_P2V virus was not isolated thus it could not
308 be comparably studied. Nevertheless, this study represents the first report of
309 characterizing SARS-CoV-2 related coronavirus in animal models.

310

311 In cell cultures, attenuation of the GX_P2V variant caused mild cell damage and
312 small plaques. The attenuation is likely independent of viral infectivity, as the spike
313 proteins of GX_P2V and SARS-CoV-2 have comparable capabilities of mediating cell
314 attachment and entry¹¹. It is also likely viral replication and egress were unaffected, as
315 titers of live GX_P2V in the supernatants reach similar levels at 48 h.p.i. as
316 SARS-CoV-2^{14,22}. How could GX_P2V produce these attenuating phenotypes while
317 having growth capabilities similar to those of SARS-CoV-2? Certain mutations in the
318 spike protein have been linked with viral plaque sizes in SARS-CoV-2²². Therefore, it
319 is possible the spike protein of GX_P2V is less effective in host cell interactions than
320 that of SARS-CoV-2.

321

322 The attenuation of the GX_P2V variant in animal models can be explained by two
323 aspects of our findings. The first is the 104-nucleotide deletion in the HVR of the
324 3'-UTR of the GX_P2V variant. In the mouse hepatitis virus (MHV) model, the
325 association between HVR mutations and viral pathogenicity has been
326 comprehensively investigated²³. MHV with a deletion of the entire HVR had minimal

change on viral growth in cell cultures, but was highly attenuated in mice, causing no signs of clinical disease²³. The HVR of GX_P2V likely also plays a significant role in viral pathogenesis. Another aspect is the lower inner shell disorder in GX_P2V as predicted by measuring the percentage of protein intrinsic disorder of the nucleoprotein²⁴. Goh et al²⁴ predicted that the wild type GX_P2V virus was an attenuated SARS-CoV-2 vaccine strain even though the virus was initially identified in dead pangolins.

The golden hamsters can be infected by both SARS-CoV and SARS-CoV-2, but disease outcomes varied by different strains and different infection routes^{15,19,25}. Hamsters intranasally inoculated with a SARS-CoV Urbani strain had robust viral replication, with significant pathology in the respiratory tract, but without weight loss or evidence of disease²⁵. A follow-up study reported a lethal SARS-CoV Frk-1 strain, which differs from the Urbani strain by an L1148F substitution in the S2 domain²⁶. Hamsters intranasally infected with SARS-CoV-2 were found to resemble human infections by multiple groups^{15,16,27}. Overall, hamsters intranasally infected with either SARS-CoV or SARS-CoV-2 consistently had similar viral durations and significant pathologies in the lungs. While having similar viral loads and similar duration of viral titers in lungs, the GX_P2V variant in hamsters caused no pathology or clinical signs of disease. We surmise the absence of pathology is likely in part due to the low virulence nature of the GX_P2V spike protein.

349 Live attenuated vaccines are attractive because they can stimulate a strong and
350 durable immune response with both humoral and cellular immunity. The challenge of
351 constructing live attenuated vaccines is the difficulty of adopting acceptable
352 attenuation strategies. Currently, two live attenuated SARS-CoV-2 vaccine candidates
353 generated by using genome recoding have been reported^{28,29}. Our data suggest that
354 shortening the 3'-UTR is a novel strategy for making a live attenuated SARS-CoV-2
355 variant that warrants further investigations.

356

357 **EXPERIMENTAL MODEL AND SUBJECT DETAILS**

358 Vero and BGM cells, obtained from the American Type Culture Collection (ATCC)
359 (Manassas, USA), were grown in Roswell Park Memorial Institute (RMPI) 1640
360 medium (Gibco, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco,
361 USA) and 100 units/mL penicillin-streptomycin (Gibco, USA) at 37°C in 5% CO₂.
362 Stable hACE2-293T cells were kindly provided by Pro. Zheng Zhang (Shenzhen
363 Third People's Hospital) and were cultured in DMEM (Gibco, USA) with 10% FBS
364 and 100 units/mL penicillin-streptomycin.

365

366 The pangolin coronavirus GX_P2V, originally cultured from the lung-intestine
367 mixed samples of a pangolin captured in anti-smuggling operations in 2017⁶, was
368 passaged in Vero cells. Cell supernatants were harvested and centrifugated (10,000 ×
369 g) for five minutes, then supernatants were aliquoted and stored at -80°C. Virus titers
370 were determined by using a standard 50% tissue culture infection dose (TCID₅₀)

371 assay.

372

373 Human serum samples were collected from healthy donors, COVID-19 patients,
374 and vaccinees of two doses of inactivated SARS-CoV-2 vaccine BBIBP-CorV at the
375 Fifth Medical Center of General Hospital of Chinese PLA. All participants signed
376 informed consent forms. The blood serum samples from COVID-19 patients and
377 vaccinees were collected at 21 to 28 days after symptom onset, and two weeks after
378 receiving the second vaccination, respectively. All serum samples were stored at
379 -80°C. This study was approved by the institutional review board of the Fifth Medical
380 Center, General Hospital of Chinese PLA (approval number: 2020027D).

381

382 Six-week-old male hamsters, six-week-old male BALB/c and 48-week-old female
383 BALB/c were used in this study. The procedure of animal experiments was approved
384 by the Institutional Animal Care and Use Committee of Fifth Medical Center, General
385 Hospital of Chinese PLA (IACUC-2018-0020).

386

387 **METHOD DETAILS**

388 **Next-generation sequencing.** The eighth passage of the pangolin coronavirus
389 GX_P2V was partially purified from cell culture supernatants by ultra-centrifugation.
390 Total RNAs were extracted by using the AxyPrep™ body fluid viral DNA/RNA
391 Miniprep kit (Axygen, Cat No. AP-MN-BF-VNA-250, Hangzhou, Zhejiang, China)
392 according to the manufacturer's instructions. Whole-genome sequencing was

performed using next-generation sequencing (NGS) methodology. The cDNA libraries were constructed using a TruSeq RNA library prep kit (Illumina) according to the manufacturer's instructions and sequenced on an Illumina MiSeq system. Sequence data was deposited in the China National Microbiological Data Center (project accession number NMDC10017729 and sequence accession number: NMDC40002427).

399

Assembly and characterization of the coronavirus genome. Raw reads were adaptor- and quality- trimmed with the Fastp program³⁰. The clean reads were then mapped to the near complete genome of pangolin coronavirus GX_P2V (GenBank accession number MT072864) using Bowtie 2³¹. The reads that were mapped to the GX_P2V genome were then assembled *de novo* using Trinity with default settings³². The assembled contig had a complete 3'-terminus. Then, the 5'-terminus of the viral genome was determined by 5'/3' RACE kits (TaKaRa). The resulting whole genome sequence of the GX_P2V variant was deposited in GenBank (accession number MW532698). To characterize and map the mutations of the GX_P2V isolate, the viral genomic sequences of the GX_P2V variant and the GX_P2V sample were aligned using ClustalW³³.

411

Plaque assay and cytopathic effect (CPE). The plaque assay using methylcellulose, a low-viscosity, semi-solid overlay, was performed to determine the plaque phenotype and infectious titers as previously described³⁴. Confluent monolayers of BGM and

415 Vero cells seeded in 6-well plates were incubated with ten-fold serially diluted viral
416 stocks ranging from 10^{-1} to 10^{-6} for 2 h at 37°C with gentle rocking every 15 minutes.
417 After viral adsorption, cells were washed twice with phosphate buffer saline (PBS)
418 and overlaid with Minimum Essential Medium (MEM) (Gibco, USA) containing 2%
419 FBS, 1% methylcellulose, and HEPES (20 mM) and incubated at 37°C in 5% CO₂
420 incubator. Cytopathic effect (CPE) was observed and photographed daily for 5 days
421 under a light microscopy. At 5 days post-infection (d.p.i.), cells were fixed with 4%
422 paraformaldehyde for 2 h at room temperature, and then stained with 0.2% crystal
423 violet for 20 min. They were gently rinsed under tap water and dried prior to analysis
424 of the plaque phenotype and calculation of virus titers.

425

426 **Viral growth kinetics.** Confluent monolayers of BGM and Vero cells in 6-well plates
427 were incubated with viral stocks at a multiplicity of infection (MOI) of 0.01 for 2 h at
428 37°C followed by washing twice with PBS and maintained in RMPI 1640 medium
429 with 2% FBS and HEPES (20 mM) at 37°C in a 5% CO₂ incubator. Culture
430 supernatants were collected at the indicated time points and stored at -80°C for
431 subsequent viral growth kinetics assay by quantifying the viral RNA copies and viral
432 titers by using qPCR and 50% tissue culture infectious dose (TCID₅₀), respectively.

433

434 **TCID₅₀ assay.** BGM cells (2.5×10^4 cells/well) in 96-well plates were incubated
435 together with ten-fold serial dilutions of the cell culture supernatants at 37°C in a 5%
436 CO₂ incubator. Cytopathic effect was observed at 5 d.p.i. Viral titers were calculated

437 and were shown as TCID₅₀/mL.

438

439 **RNA extraction and qRT-PCR analysis.** Total viral RNA from cell culture
440 supernatants was extracted by using the EasyPure® Viral DNA/RNA Kit (TransGen,
441 Cat No. ER201-01 Beijing, China) according to the manufacturer's instructions.
442 Reverse transcription was performed to produce complementary DNA (cDNA) using
443 Hifair □ 1st Strand cDNA Synthesis Kit (Yeasten Biotech, Cat No. 11123ES60,
444 Shanghai, China), and then 2 µL cDNA was used as template for subsequent qPCR.
445 The target N gene of GX_P2V isolate was amplified by qPCR using FastFire qPCR
446 PreMix (Probe) (TIANGEN, Cat No. FP208-02, Beijing, China) according to the
447 manufacturer's instructions. Sequences of qRT-PCR primers are as follow: P2VF,
448 TCTTCCTGCTGCAGATTTGGAT; P2VR, ATTCTGCACAAGAGTAGACTATGTA
449 TCGT; and Probe, FAM-TGCAGACCACACAAGGCAGATGGGC-TAMRA. In
450 addition, a standard plasmid was constructed through inserting a fragment amplified
451 using P2VF and P2VR into a cloning vector pEASY-T1 (TransGen, Cat No.
452 CT101-01 Beijing, China). Plasmids in a range of 10² to 10⁸ DNA copies were used
453 as template to generate a standard curve.

454

455 **Immunofluorescence assay (IFA) for viral growth.** Confluent monolayers of BGM
456 cells grown on coverslips in 12-well plates were incubated with GX_P2V at an MOI
457 of 0.01 for two hours at 37°C in 5% CO₂, then washed twice with PBS and cultured in
458 RMPI 1640 with 2% FBS and HEPES (20 mM) at 37°C in 5% CO₂. At indicated time

459 points, the cells were washed twice with PBS and fixed with 4% paraformaldehyde
460 for 20 min at room temperature, permeabilized by using 0.2% (vol/vol) Triton X-100
461 in PBS for 10 min, then blocked overnight with 3% bovine serum albumin (BSA) at
462 4°C. Then, cells were incubated with anti-sera (1:200, prepared in our laboratory)
463 from GX_P2V-infected hamsters for two hours at room temperature. After being
464 washed five times with PBS, cells were stained with a fluorescein isothiocyanate
465 (FITC)-labeled goat anti-mouse IgG antibody (1:300, ZSGB-BIO, Cat No. ZF-0312,
466 Beijing, China) for one hour at room temperature. Cells were washed five times and
467 stained with 4',6-Diamidino-2-phenylindole (DAPI) (Sigma, Cat No. 10236276001,
468 Germany) for 20 min. Finally, after being washed five times, cells on the coverslips
469 were scanned and photographed using a fluorescence microscopy (Nikon, Japan).

470

471 **Golden hamster model of both intranasal and intragastrical infection.** Specific
472 pathogen-free, 6-week-old male golden hamsters were purchased from Charles River
473 and maintained under specific-pathogen-free conditions. For intranasal infection,
474 hamsters were infected with 10^4 or 10^5 TCID₅₀ of GX_P2V in 50 µL RPMI 1640
475 containing 2% FBS after deep anesthesia with pentobarbital sodium. Equal volume of
476 RPMI 1640 containing 2% FBS was set as mock-control group. The mock-infected
477 group (n = 3) was monitored and sacrificed at 7 d.p.i. In the GX_P2V-infected group
478 (n = 12 per group), three hamsters were sacrificed at each indicated time points (2, 5,
479 7 and 14 d.p.i.). For intragastrical infection, hamsters were infected with 10^5 TCID₅₀
480 of GX_P2V in 100 µL RPMI 1640 containing 2% FBS. Equal volume of PBS was

481 used to mock-infect the control group. Mock-infected hamsters (n = 4) were sacrificed
482 at 14 d.p.i., while four hamsters of GX_P2V-infected (n = 20) were sacrificed at each
483 indicated time points (2, 5, 7, 9 and 14 d.p.i).

484

485 After viral infection, various indexes were collected during 14-day experimental
486 period. The body weight changes and clinical symptoms³⁵ were recorded before the
487 hamsters were humanly euthanized. Left lungs and bottom tracheas were placed in 4%
488 paraformaldehyde and were stored at room temperature at least two days for
489 observation of the pathological changes. The rest of the lungs and other organ tissues
490 (stomach, duodenum, colon, tongue, trachea, esophagus, small intestine, feces) and
491 sera samples were collected and frozen directly at -40°C for viral detection.

492

493 **Intranasal infection in both young and aged BALB/c mice.** Specific pathogen-free,
494 6-week-old male young BALB/c mice and 48-week-old male aged BALB/c mice
495 were obtained from SiPeiFu (Beijing). Young and old mice were randomly divided
496 into two groups, respectively. Mice were anesthetized intraperitoneally (i.p.) with
497 pentobarbital sodium and then infected intranasally with 10⁵ TCID₅₀ of GX_P2V in
498 30 µL RPMI 1640 containing 2% FBS or PBS as control group. Mock-infected young
499 (n = 5) or aged (n = 3) mice were sacrificed at 14 d.p.i., while five young or three
500 aged GX_P2V-infected mice (n = 25 or 15, respectively) were sacrificed at each of the
501 indicated time points (1, 3, 6, 9 and 14 d.p.i). Body weight and clinical symptoms
502 were assessed periodically. Blood samples and lungs were collected regularly. Left

503 lungs were fixed with 4% paraformaldehyde for determining histopathological
504 changes. Right lungs were frozen directly at -40°C for analyzing the viral loads and
505 titers.

506

507 **Measurement of viral RNA copies and virus titers in lungs and tracheas.** Lung,
508 trachea, stomach, duodenum, colon, tongue, esophagus, small intestine, and feces
509 specimens were homogenized in 10 µL PBS per milligram of tissue for 60 s at 60 Hz
510 4 times using a TissueLyse-24 system (Jingxin, Shanghai). Tissue homogenates were
511 centrifuged at $5000 \times g$ at 4°C for 5 min. Total viral RNAs from tissue supernatants
512 were exacted by using EasyPure® Viral DNA/RNA Kit (TransGen, Cat No.
513 ER201-01, Beijing, China) for qRT-PCR analysis as noted above. Infectious titers in
514 tissue supernatants were detected and calculated by TCID₅₀ assay as mentioned above.
515 Virus titers were indicated as TCID₅₀/mL.

516

517 **Histopathology of lungs and tracheas.** The lungs and tracheas were grossly
518 examined and photographed before freezing and fixing. The left lungs and bottom
519 tracheas were fixed in 4% paraformaldehyde and embedded in paraffin. The paraffin
520 sections were prepared for H&E staining. The histopathology of the lungs and
521 tracheas tissues was detected by microscopy (Nikon, Japan). The histopathological
522 results were analyzed by professional pathologists.

523

524 **Anti-sera preparation from hamsters.** Hamsters were intranasally challenged with

525 two doses (1 d.p.i and 21 d.p.i) of 10^5 TCID₅₀ GX_P2V. Sera were collected at 42
526 d.p.i for analysis of antibody responses by immunofluorescence assay (IFA), ELISA,
527 and pseudovirus neutralization titer assay (pVNT). Anti-sera were heated at 56°C for
528 30 min before use.

529

530 **Immunofluorescence assay for cross-immunity responses.** The full-length spike
531 protein gene of SARS-CoV-2 (accession number NC_045512.2) and GX_P2V
532 (accession number MW532698) were codon optimized, synthesized, and cloned into
533 pcDNA3.1 vector by Beijing BioMed Gene Technology. Confluent monolayers of
534 293T cells grown on coverslips in 12-well plates overnight were transfected with
535 plasmids containing codon-optimized spike genes of SARS-CoV-2 and GX_P2V by
536 using liposome (TransGen, Cat No. FT201-02, Beijing, China) according to the
537 instructions. After five hours, the cells were washed twice with PBS and cultured in
538 DMEM with 2% FBS at 37°C in 5% CO₂. After 36 h post-transfection, cells were
539 fixed, permeabilized, and blocked. Then, cells were incubated with sera from
540 GX_P2V-infected hamsters (1:200), convalescent COVID-19 patients (1:200),
541 mock-infected hamsters, or healthy donors. After being washed five times with PBS,
542 cells were stained with secondary antibodies: fluorescein isothiocyanate
543 (FITC)-labeled goat anti-mouse IgG (1:300, ZSGB-BIO, Cat No. ZF-0312, Beijing,
544 China) or goat anti-human IgG (1:300, ZSGB-BIO, Cat No. ZF-0308, Beijing, China),
545 accordingly. Following five washes with PBS, cells were stained with
546 4',6-Diamidino-2-phenylindole (DAPI) (Sigma, Cat No. 10236276001, Germany) for

10 min. Finally, after being washed five times, cells on the coverslips were analyzed using fluorescence microscopy (Nikon, Japan).

ELISA binding assay. SARS-CoV-2 RBD, S1 and S1+S2 (ECD) antibody titer assay kits (Sino Biological, Cat No. KIT002, KIT003, KIT004, respectively, Beijing, China) were purchased. Experiments were carried out following the manufacturer's instructions with minor modifications. All sera were diluted with dilution buffer at a ratio of 1:100. Patient's sera were tested according to the instructions. For testing sera from hamsters and BALB/c mice, the secondary antibodies were replaced by HRP-goat anti-mouse IgG (1:5000, ZSGB-BIO). Plates were read at 450 nm wavelength on a synergy H4 hybrid reader (Biotek, USA).

Pseudotyped virus neutralization assay. The VSV-based SARS-CoV-2 pseudovirus was kindly provided by Prof. Youchun Wang (National Institutes for Food and Drug Control). The SARS-CoV-2 pseudovirus was used as seed stock and combined with the GX_P2V spike protein expression plasmid to generate VSV-based GX_P2V pseudovirus in 293T-hACE2 cells. The pseudovirus based neutralization assay procedure was conducted as previously described with minor modifications³⁶. Briefly, sera samples were diluted in five three-fold serial dilutions and incubated with 1000 TCID₅₀ of pseudovirus in 96-well plates, making the initial dilution 1:30. After one h of incubation at 37°C, cells (2.5×10^4 cells/well) were added and incubated for another 24 hours at 37°C in a CO₂ incubator. Cells were lysed by adding luciferase substrate

(PerkinElmer, USA). Luciferase activity was then measured using a synergy H4 hybrid reader (Biotek, USA). The 50% neutralization titer (NT₅₀) was calculated for each individual serum sample by using the Reed-Muench method.

572

Statistical analysis. The statistical analyses were performed with GraphPad Prism version 9.0 software. The differences of two groups were analyzed by two-tailed Student *t*-test. The *p*-values of less than 0.05 (*p* < 0.05) were considered statistically significant.

577

578 **References**

- 579 1 Zhu, N. *et al.* A Novel Coronavirus from Patients with Pneumonia in China, 2019. *N. Engl. J. Med.* **382**, 727-733, doi:10.1056/NEJMoa2001017 (2020).
- 580
- 581 2 Lu, R. *et al.* Genomic characterisation and epidemiology of 2019 novel coronavirus: implications for virus origins and receptor binding. *Lancet* **395**, 565-574, doi:10.1016/s0140-6736(20)30251-8 (2020).
- 582
- 583
- 584 3 Wu, F. *et al.* A new coronavirus associated with human respiratory disease in China. *Nature* **579**, 265-269, doi:10.1038/s41586-020-2008-3 (2020).
- 585
- 586 4 Zhou, P. *et al.* A pneumonia outbreak associated with a new coronavirus of probable bat origin. *Nature* **579**, 270-273, doi:10.1038/s41586-020-2012-7 (2020).
- 587
- 588
- 589 5 Zhou, H. *et al.* A Novel Bat Coronavirus Closely Related to SARS-CoV-2 Contains Natural Insertions at the S1/S2 Cleavage Site of the Spike Protein.
- 590

591 *Curr. Biol.* **30**, 2196-2203.e2193, doi:10.1016/j.cub.2020.05.023 (2020).

592 6 Lam, T. T. *et al.* Identifying SARS-CoV-2-related coronaviruses in Malayan
593 pangolins. *Nature* **583**, 282-285, doi:10.1038/s41586-020-2169-0 (2020).

594 7 Xiao, K. *et al.* Isolation of SARS-CoV-2-related coronavirus from Malayan
595 pangolins. *Nature* **583**, 286-289, doi:10.1038/s41586-020-2313-x (2020).

596 8 Murakami, S. *et al.* Detection and Characterization of Bat Sarbecovirus
597 Phylogenetically Related to SARS-CoV-2, Japan. *Emerg. Infect. Dis.* **26**,
598 3025-3029, doi:10.3201/eid2612.203386 (2020).

599 9 Zhou, H. *et al.* Identification of novel bat coronaviruses sheds light on the
600 evolutionary origins of SARS-CoV-2 and related viruses. *Cell* **184**,
601 4380-4391.e4314, doi:10.1016/j.cell.2021.06.008 (2021).

602 10 Sarah, T. *et al.* Coronaviruses with a SARS-CoV-2-like receptor-binding
603 domain allowing ACE2-mediated entry into human cells isolated from bats of
604 Indochinese peninsula. *Nature Portfolio*, doi:10.21203/rs.3.rs-871965/v1
605 (2021).

606 11 Zhang, S. *et al.* Bat and pangolin coronavirus spike glycoprotein structures
607 provide insights into SARS-CoV-2 evolution. *Nat. Commun.* **12**, 1607,
608 doi:10.1038/s41467-021-21767-3 (2021).

609 12 Züst, R., Miller, T. B., Goebel, S. J., Thiel, V. & Masters, P. S. Genetic
610 interactions between an essential 3' cis-acting RNA pseudoknot, replicase gene
611 products, and the extreme 3' end of the mouse coronavirus genome. *J. Virol.*
612 **82**, 1214-1228, doi:10.1128/jvi.01690-07 (2008).

613 13 Kaye, M. SARS-associated coronavirus replication in cell lines. *Emerging*
614 *infectious diseases* **12**, 128-133, doi:10.3201/eid1201.050496 (2006).

615 14 Xie, X. *et al.* An Infectious cDNA Clone of SARS-CoV-2. *Cell. Host. Microbe.*
616 **27**, 841-848.e843, doi:10.1016/j.chom.2020.04.004 (2020).

617 15 Sia, S. F. *et al.* Pathogenesis and transmission of SARS-CoV-2 in golden
618 hamsters. *Nature* **583**, 834-838, doi:10.1038/s41586-020-2342-5 (2020).

619 16 Imai, M. *et al.* Syrian hamsters as a small animal model for SARS-CoV-2
620 infection and countermeasure development. *Proc. Natl. Acad. Sci. USA.* **117**,
621 16587-16595, doi:10.1073/pnas.2009799117 (2020).

622 17 Chan, J. F. *et al.* Simulation of the Clinical and Pathological Manifestations of
623 Coronavirus Disease 2019 (COVID-19) in a Golden Syrian Hamster Model:
624 Implications for Disease Pathogenesis and Transmissibility. *Clin. Infect. Dis.*
625 **71**, 2428-2446, doi:10.1093/cid/ciaa325 (2020).

626 18 Rokkas, T. Gastrointestinal involvement in COVID-19: a systematic review
627 and meta-analysis. *Ann. Gastroenterol.* **33**, 355-365,
628 doi:10.20524/aog.2020.0506 (2020).

629 19 Lee, A. C. *et al.* Oral SARS-CoV-2 Inoculation Establishes Subclinical
630 Respiratory Infection with Virus Shedding in Golden Syrian Hamsters. *Cell.*
631 *Rep. Med.* **1**, 100121, doi:10.1016/j.xcrm.2020.100121 (2020).

632 20 Zhang, Y. *et al.* SARS-CoV-2 rapidly adapts in aged BALB/c mice and
633 induces typical pneumonia. *J. Virol.* **95**, doi:10.1128/jvi.02477-20 (2021).

634 21 Gu, H. *et al.* Adaptation of SARS-CoV-2 in BALB/c mice for testing vaccine

635 efficacy. *Science* **369**, 1603-1607, doi:10.1126/science.abc4730 (2020).

636 22 Ogando, N. S. *et al.* SARS-coronavirus-2 replication in Vero E6 cells:
637 replication kinetics, rapid adaptation and cytopathology. *J. Gen. Virol.* **101**,
638 925-940, doi:10.1099/jgv.0.001453 (2020).

639 23 Goebel, S. J., Miller, T. B., Bennett, C. J., Bernard, K. A. & Masters, P. S. A
640 hypervariable region within the 3' cis-acting element of the murine
641 coronavirus genome is nonessential for RNA synthesis but affects
642 pathogenesis. *J. Gen. Virol.* **81**, 1274-1287, doi:10.1128/jvi.00803-06 (2007).

643 24 Goh, G. K., Dunker, A. K., Foster, J. A. & Uversky, V. N. Shell Disorder
644 Analysis Suggests That Pangolins Offered a Window for a Silent Spread of an
645 Attenuated SARS-CoV-2 Precursor among Humans. *J. Proteome. Res.* **19**,
646 4543-4552, doi:10.1021/acs.jproteome.0c00460 (2020).

647 25 Roberts, A. *et al.* Severe acute respiratory syndrome coronavirus infection of
648 golden Syrian hamsters. *J. Virol.* **79**, 503-511,
649 doi:10.1128/jvi.79.1.503-511.2005 (2005).

650 26 Roberts, A. *et al.* Animal models and vaccines for SARS-CoV infection. *Virus.*
651 *Res.* **133**, 20-32, doi:10.1016/j.virusres.2007.03.025 (2008).

652 27 Song, Z. *et al.* SARS-CoV-2 Causes a Systemically Multiple Organs Damages
653 and Dissemination in Hamsters. *Front. Microbiol.* **11**, 618891,
654 doi:10.3389/fmicb.2020.618891 (2020).

655 28 Wang, Y. *et al.* Scalable live-attenuated SARS-CoV-2 vaccine candidate
656 demonstrates preclinical safety and efficacy. *Proc. Natl. Acad. Sci. USA.* **118**,

657 doi:10.1073/pnas.2102775118 (2021).

658 29 Trimper, J. *et al.* Development of safe and highly protective live-attenuated
659 SARS-CoV-2 vaccine candidates by genome recoding. *Cell. Rep.* **36**, 109493,
660 doi:<https://doi.org/10.1016/j.celrep.2021.109493> (2021).

661 30 Chen, S., Zhou, Y., Chen, Y. & Gu, J. fastp: an ultra-fast all-in-one FASTQ
662 preprocessor. *Bioinformatics* **34**, i884-i890, doi:10.1093/bioinformatics/bty560
663 (2018).

664 31 Langmead, B. & Salzberg, S. L. Fast gapped-read alignment with Bowtie 2.
665 *Nat. Methods.* **9**, 357-359, doi:10.1038/nmeth.1923 (2012).

666 32 Grabherr, M. G. *et al.* Full-length transcriptome assembly from RNA-Seq data
667 without a reference genome. *Nat. Biotechnol.* **29**, 644-652,
668 doi:10.1038/nbt.1883 (2011).

669 33 Hung, J. H. & Weng, Z. Sequence Alignment and Homology Search with
670 BLAST and ClustalW. *Cold. Spring. Harb. Protoc.* **2016**,
671 doi:10.1101/pdb.prot093088 (2016).

672 34 Honko, A. N., Storm, N., Bean, D. J., Vasquez, J. H. & Griffiths, A. Rapid
673 Quantification and Neutralization Assays for Novel Coronavirus SARS-CoV-2
674 Using Avicel RC-591 Semi-Solid Overlay. *Preprint*,
675 <https://doi.org/10.20944/preprints202005.0264.v1> (2020).

676 35 Seitz, M. *et al.* A novel intranasal mouse model for mucosal colonization by
677 *Streptococcus suis* serotype 2. *J. Med. Microbiol.* **61**, 1311-1318,
678 doi:10.1099/jmm.0.043885-0 (2012).

679 36 Nie, J. *et al.* Quantification of SARS-CoV-2 neutralizing antibody by a
680 pseudotyped virus-based assay. *Nat Protoc.* **15**, 3699-3715,
681 doi:10.1038/s41596-020-0394-5 (2020).

682

683 **Acknowledgements**

684 We thank Harlan D. Caldwell for critical review of the manuscript. This research was
685 supported by NSFC-MFST projects (China-Mongolia) (No. 3211101856 and
686 31961143024), Inner Mongolia Key Research and Development Program (No.
687 2019ZD006), Funds for First-class Discipline Construction (No. XK1805 and
688 XK1803-06), National Key Research and Development Program of China (No.
689 2018YFA0903000, 2020YFC2005405, 2020YFA0712100, 2020YFC0840805,
690 19SWAQ06, 20SWAQX27 and 20SWAQK22), Fundamental Research Funds for
691 Central Universities (No. BUCTRC201917 and BUCTZY2022).

692

693 **Author contributions**

694 We contributed to the work as follows. L.S., Y.T. and P.M. designed and supervised
695 research. S. Lu, S. Luo, C.L., M.L., J.L., X.A., Z.L., Y.Y., J.H., and H.F. conducted
696 experiments. P.M. and L.S. performed analyses. S. Lu, S. Luo and L.S. wrote the
697 paper. All of us reviewed and approved the manuscript.

698

699 **Competing interests**

700 We declare no competing financial interests.

701

702 **Figure legends**

703

A

SARS-CoV-2:	UUG CAA CAA UCC AUG AGC AGU GCU GAC UCA ACU CAG GCC UAA
GX_P2V sample:	UU ...
GX_P2V isolate:	UUU ...

B

SARS-CoV-2:	UUAAUCUCACAUAGCAAUCUUUAAUCAGUGUGUAACAUAUAGGGAGGACUUGAAAG
GX_P2V sample:	
GX_P2V isolate:	

SARS-CoV-2:	AGCCACCACAUUUUCACCGAGGCCACGCGGAGUACGAUCGAGUGUACAGUGAACA
GX_P2V sample:	G.....U.
GX_P2V isolate:	-----

SARS-CoV-2:	AUGCUAGGGAGAGCUGCCUAUAUGGAAGAG
GX_P2V sample:	
GX_P2V isolate:	-----

704

705 Fig. 1 Sequence comparisons between SARS-CoV-2, the original GX_P2V sample
706 and the pangolin coronavirus GX_P2V isolate. The GX_P2V isolate is a variant with
707 two interesting mutations: one nonsynonymous mutation (C to U) in the final amino
708 acid codon of the nucleoprotein and one 104-nt deletion in the 3'-UTR. **a** Alignment
709 of the 3'-terminus sequences of the nucleoprotein genes. **b** Alignment of the partial
710 3'-UTR sequences. Dots represent residues that are identical to those in SARS-CoV-2
711 and hyphens represent nucleotide deletions.

712

713

714 Fig. 2 Growth of the pangolin coronavirus GX_P2V variant in two non-human

715 primate cells: BGM and Vero. **a** BGM and Vero cells infected with the GX_P2V

716 variant show similar cytopathic effects: limited cell degeneration at 24 h postinfection,

717 and more evident cell degeneration and cell rounding after 48 h postinfection. Scale
718 bars, 100 μ m. **b** The replication of the GX_P2V variant in BGM cells at different time
719 points was detected by immunofluorescence assays with anti-sera from
720 GX_P2V-infected hamsters. Nuclei were stained with DAPI (blue). Scale bars, 50 μ m.
721 **c** The GX_P2V variant forms small plaques in BGM and Vero cells five days
722 postinfection. The small white dots represent plaques. **d** The diameter of plaques (blue
723 circle) was measured by using Image Pro plus 6.0 software. The GX_P2V variant
724 forms bigger plaques in Vero than in BGM ($p < 0.0001$).

725

726

727 Fig. 3 Replication kinetics of the GX_P2V variant in BGM cells (solid line) and Vero
728 cells (dotted line). Results are expressed as the mean of three biological replicates. **a**
729 Viral RNA copies were determined by qRT-PCR. **b** Viral titers were titrated by using a
730 standard TCID₅₀ assay. The two cells appear to produce different viral titers at 24 and
731 120 h postinfection (** $p < 0.001$).

732

Fig. 4 Intranasal infection of golden hamsters shows the GX_P2V variant had a short duration of viral replication and caused no gross pathology in the respiratory tracts. **a**

736 Scheme of intranasal infection. **b** Both mock and GX_P2V-infected hamsters had
 737 normal body weight increases and no apparent clinical symptoms in the two-week
 738 observational period. Individual data points represent percent weight changes
 739 compared to day zero, or cumulative clinical score. **c** Representative images of lungs
 740 and tracheas from mock hamsters, 10^4 , and 10^5 TCID₅₀ infected hamsters at indicated
 741 time points. All organs had no gross pathology. **d-g** Viral RNA loads (**d, e**) and viral
 742 titers (**f, g**) in homogenate supernatants of lungs (**d, f**) and tracheas (**e, g**) of
 743 GX_P2V-infected hamsters at different days post-inoculation were determined by
 744 qRT-PCR and the TCID₅₀ assay, respectively. The detection limit is shown by the
 745 dotted line. Error bars represent means $\pm$ SD.

746

747

748 Fig. 5 Histopathological analyses of lung and trachea tissues from intranasally

749 infected hamsters. H&E 400 × are the magnifications of the region in the

750 corresponding red box in H&E 100 ×. **a** 10^4 TCID₅₀ infected group (n = 3), no

751 pathological changes were observed in the lungs. **b** 10^5 TCID₅₀ infected group (n = 3),

752 lungs had no tissue lesions. Few inflammatory cells of lymphocytes and neutrophils

753 associated with blood vessels were observed at five days postinfection but not at other
754 time points. **c** Representative images show that tracheas of both infection dose groups
755 had no significant histopathological changes at all time points post-infection.
756

757
758 Fig. 6 Intra-gastric inoculation of the GX_P2V variant in golden hamsters
759 established limited infection in the respiratory tract. **a** Viral RNAs were detected in
760 samples of tongues and feces from the above golden hamsters with intranasal
761 infection, suggesting established infections in the gastrointestinal tract. **b** Scheme of
762 intra-gastric infection. Samples of tongues and feces were collected at indicated time
763 points. **c** Body weights of all golden hamsters had continuous increases. **d** No clinical
764 symptoms of infection were identified. **e** Limited viral RNAs in the lungs, but not in
765 the stomach, colon or faeces were detected by qPCR. **f** No histopathological changes
766 were identified in lung tissues of mock and GX_P2V-inoculated golden hamsters. **g**
767 Viruses were not detected in feces from golden hamsters following intranasal or

768 intragastric inoculation.

769

770

771 Fig. 7 Intranasal inoculation of the GX_P2V variant in both young and aged BALB/c

772 mice. **a** Scheme of intranasal infection. Young and aged BALB/c mice were

773 intranasally infected with mock or 10⁵ TCID₅₀ of the GX_P2V variant. Various

774 infection outcomes and tissue samples were collected at indicated time points. **b**

775 Young mice had constant body weight increases after infection. **c** Intranasal
 776 inoculation of GX_P2V in young and aged BALB/c mice produce no detectable gross
 777 pathology. **d** Viral RNA loads in infected lungs of both young and aged BALB/c mice
 778 were determined by qPCR. **e-f** Representative H&E images of infected lungs from
 779 young (**e**) and aged (**f**) BALB/c mice. **g** No viral titers were detected in infected lungs
 780 from both young and aged BALB/c mice.

781

782

783 Fig. 8 Serological cross-reactivity and cross-neutralization between GX_P2V and

784 SARS-CoV-2. **a** Immunofluorescence assays with 293T cell expressing spike proteins

785 (green) of SARS-CoV-2 and GX_P2V, using sera from convalescent COVID patients,

786 healthy donors, and GX_P2V-infected golden hamsters. Nuclei were stained with

787 DAPI (blue). **b** Three commercial ELISA kits for detecting antibodies against

788 SARS-CoV-2 S1+S2 (ECD), S1, and RBD were used for measuring antibodies in sera

789 from healthy donors (n = 10), convalescent COVID patients (n = 16), vaccinees of
790 inactivated COVID-19 vaccines (n = 8), mock-infected golden hamsters (n = 3), and
791 GX_P2V-infected golden hamsters (n = 16), respectively. **c** Cross-reacting antibodies
792 against SARS-CoV-2 S1 and RBD in sera from GX_P2V-infected golden hamsters
793 and BALB/c mice were measured by using the aforementioned ELISA kits. No
794 cross-reacting antibodies against SARS-CoV-2 S1 and RBD were detected in sera
795 from intragastrically infected golden hamsters or intranasally infected BALB/c mice.
796 **d** Neutralizing antibodies against two pseudoviruses, SARS-CoV-2 and GX_P2V,
797 were detected in sera as described in figure 8b. The error bars indicate means $\pm$ SD.
798