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## SARS-coronavirus-2 replication in Vero E6 cells: replication kinetics, rapid adaptation and cytopathology

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*This paper is dedicated to the loving memory of*

*José Manuel Ogando Fernandes Pereira (72) and María de los Ángeles Martín (93)*

*who succumbed to SARS-CoV-2 infection on March 27 and 28, 2020.*

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**Keywords:** plaque phenotype, evolution, RNA synthesis, antisera, furin cleavage site, antiviral drugs

**Abbreviations:** SARS-CoV, severe acute respiratory syndrome coronavirus; CoV, Coronavirus; CPE, cytopathic effect; HCoV, human coronavirus; MERS-CoV, Middle East respiratory syndrome coronavirus;; nsp, non-structural protein; S protein, spike protein; ACE2, angiotensin-converting enzyme 2; NGS, next-generation sequencing; RO, replication organelle; DMV, Double-membrane vesicle; PEG-IFN- $\alpha$ , pegylated interferon alpha; UTR, untranslated region.

35 **ABSTRACT**

36

37 The sudden emergence of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)  
38 at the end of 2019 from the Chinese province of Hubei and its subsequent pandemic spread  
39 highlight the importance of understanding the full molecular details of coronavirus infection  
40 and pathogenesis. Here, we compared a variety of replication features of SARS-CoV-2 and  
41 SARS-CoV and analysed the cytopathology caused by the two closely related viruses in the  
42 commonly used Vero E6 cell line. Compared to SARS-CoV, SARS-CoV-2 generated higher  
43 levels of intracellular viral RNA, but strikingly about 50-fold less infectious viral progeny was  
44 recovered from the culture medium. Immunofluorescence microscopy of SARS-CoV-2-  
45 infected cells established extensive cross-reactivity of antisera previously raised against a  
46 variety of nonstructural proteins, membrane and nucleocapsid protein of SARS-CoV. Electron  
47 microscopy revealed that the ultrastructural changes induced by the two SARS viruses are  
48 very similar and occur within comparable time frames after infection. Furthermore, we  
49 determined that the sensitivity of the two viruses to three established inhibitors of coronavirus  
50 replication (Remdesivir, Alisporivir and chloroquine) is very similar, but that SARS-CoV-2  
51 infection was substantially more sensitive to pre-treatment of cells with pegylated interferon  
52 alpha. An important difference between the two viruses is the fact that - upon passaging in  
53 Vero E6 cells - SARS-CoV-2 apparently is under strong selection pressure to acquire adaptive  
54 mutations in its spike protein gene. These mutations change or delete a putative 'furin-like  
55 cleavage site' in the region connecting the S1 and S2 domains and result in a very prominent  
56 phenotypic change in plaque assays.

57

## 58 INTRODUCTION

59

60 For the first time in a century, societies and economies worldwide have come to a near-  
61 complete standstill due to a pandemic outbreak of a single RNA virus. This virus, the severe  
62 acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (1) belongs to the coronavirus (CoV)  
63 family, which is thought to have given rise to zoonotic introductions on multiple previous  
64 occasions during the past centuries. Coronaviruses are abundantly present in mammalian  
65 reservoir species, including bats (2), and should now be recognized definitively as a  
66 continuous zoonotic threat with the ability to cause severe human disease and explosive  
67 pandemic transmission.

68 To date, seven CoVs that can infect humans have been identified, which segregate into two  
69 classes. On the one hand, there are four endemic human CoVs (HCoVs), the first of which  
70 were identified in the 1960's, annually causing a substantial number of common colds (3, 4).  
71 On the other hand, we now know of (at least) three zoonotic CoVs that have caused outbreaks  
72 in the human population recently: severe acute respiratory syndrome coronavirus (SARS-  
73 CoV) (5, 6) in 2002-2003, Middle East respiratory syndrome-coronavirus (MERS-CoV) (7, 8)  
74 since 2012 (and probably earlier) and the currently pandemic SARS-CoV-2 (9, 10). The latter  
75 agent emerged near Wuhan (People's Republic of China) in the fall of 2019 and its animal  
76 source is currently under investigation (11-13). Transmission to humans of SARS-CoV and  
77 MERS-CoV was attributed to civet cats (14) and dromedary camels (15), respectively,  
78 although both species may have served merely as an intermediate host due to their close  
79 contact with humans. All three zoonotic CoVs belong to the genus *betacoronavirus* (beta-  
80 CoV), which is abundantly represented among the CoVs that circulate in the many bat species  
81 on this planet (2, 16-19). The genetic diversity of bat CoVs and their phylogenetic relationships  
82 with the four known endemic HCoVs (OC43, HKU1, 229E and NL63; the latter two being  
83 alpha-CoVs) suggests that also these may have their evolutionary origins in bat hosts, for  
84 most of them probably centuries ago (20). The potential of multiple CoVs from different genera  
85 to cross-species barriers had been predicted and documented previously (2, 16-19, 21, 22),

86 but regrettably was not taken seriously enough to invest more extensively in prophylactic and  
87 therapeutic solutions that could have contributed to rapidly containing an outbreak of the  
88 current magnitude.

89 Compared to other RNA viruses, CoVs possess an unusually large positive-sense RNA  
90 genome with a size ranging from 26 to 34 kilobases (23). The CoV genome is single-stranded  
91 and its 5'-proximal two-thirds encode for the large and partially overlapping replicase  
92 polyproteins pp1a and pp1ab (4,000-4,500 and 6,700-7,200 amino acids long, respectively),  
93 with the latter being a C-terminally extended version of the former that results from ribosomal  
94 frameshifting. The replicase polyproteins are processed into 16 cleavage products (non-  
95 structural proteins, nsps) by two internal proteases, the papain-like protease (PL<sup>pro</sup>) in nsp3  
96 and the 3C-like or 'main' protease (M<sup>pro</sup>) in nsp5 (24). Specific trans-membrane nsps (nsp3, 4  
97 and 6) then cooperate to transform intracellular membranes into a viral replication organelle  
98 (RO) (25) that serves to organize and execute CoV RNA synthesis, which entails genome  
99 replication and the synthesis of an extensive nested set of subgenomic (sg) mRNAs. The latter  
100 are used to express the genes present in the 3'-proximal third of the genome, which encode  
101 the four common CoV structural proteins (spike (S), envelope (E), membrane (M) and  
102 nucleocapsid (N) protein) and the 'so-called' accessory protein genes, most of which are  
103 thought to be involved in the modulation of host responses to CoV infection (26). The CoV  
104 proteome includes a variety of potential targets for drug repurposing or *de novo* development  
105 of specific inhibitors of e.g. viral entry (S protein) or RNA synthesis (27). The latter process  
106 depends on a set of enzymatic activities (24) including an RNA-dependent RNA polymerase  
107 (RdRp; in nsp12), RNA helicase (in nsp13), two methyltransferases involved in mRNA capping  
108 (a guanine-N7-methyltransferase in nsp14 and a nucleoside-2'-O-methyltransferase in nsp16)  
109 and a unique exoribonuclease (ExoN, in nsp14) that promotes the fidelity of the replication of  
110 the large CoV genome (28). Other potential drug targets are the transmembrane proteins that  
111 direct the formation of the viral RO, several less well characterised enzymatic activities and a

112 set of smaller nsps (nsp7-10) that mainly appear to serve as cofactors/modulators of other  
113 nsps.

114 The newly emerged SARS-CoV-2 was rapidly identified as a CoV that is relatively closely  
115 related to the 2003 SARS-CoV (9, 29, 30). The two genome sequences are about ~80%  
116 identical and the organization of open reading frames is essentially the same. The overall level  
117 of amino acid sequence identity of viral proteins ranges from about 65% in the least conserved  
118 parts of the S protein to about 95% in the most conserved replicative enzyme domains,  
119 prompting the coronavirus study group of the International Committee on the Taxonomy of  
120 Viruses to classify the new agent within the species *Severe acute respiratory syndrome-*  
121 *related coronavirus*, which also includes the 2003 SARS-CoV (1). The close phylogenetic  
122 relationship also implies that much of our knowledge of SARS-CoV molecular biology,  
123 accumulated over the past 17 years, can probably be translated to SARS-CoV-2. Many reports  
124 posted over the past months have described such similarities, including the common affinity  
125 of the two viruses for the angiotensin-converting enzyme 2 (ACE2) receptor (9, 31). This  
126 receptor is abundantly expressed in Vero cells (African green monkey kidney cells). Since  
127 2003, Vero cells have been used extensively for SARS-CoV research in cell culture-based  
128 infection models by many laboratories, including our own.

129 We set out to establish the basic features of SARS-CoV-2 replication in Vero cells and  
130 compare it to the Frankfurt-1 SARS-CoV isolate from 2003 (32, 33) . When requesting virus  
131 isolates (February 2020), and in spite of the rapidly emerging public health crisis, we were  
132 confronted - not for the first time - with administrative hurdles and discussions regarding the  
133 alleged 'ownership' of virus isolates cultured from (anonymous) clinical samples. From a  
134 biological and evolutionary point of view, this would seem a strangely anthropocentric  
135 consideration, but it ultimately forced us to reach out across the globe to Australian colleagues  
136 in Melbourne. After checking our credentials and completing a basic material transfer  
137 agreement, they provided us (within one week) with their first SARS-CoV-2 isolate (originally  
138 named 2019-nCoV/Victoria/1/2020 and subsequently renamed

139 BetaCoV/Australia/VIC01/2020; (34), which will be used throughout this study. Until now, this  
140 isolate has been provided to 17 other laboratories worldwide to promote the rapid  
141 characterization of SARS-CoV-2, in this critical time of lockdowns and other preventive  
142 measures to avoid a collapse of public health systems.

143 In this report, we describe a comparative study of the basic replication features of SARS-CoV  
144 and SARS-CoV-2 in Vero E6 cells, including growth kinetics, virus titres, plaque phenotype  
145 and an analysis of intracellular viral RNA and protein synthesis. Additionally, we analysed  
146 infected cells by light and electron microscopy, and demonstrated cross-reactivity of 13  
147 available SARS-CoV-specific antisera (recognising 10 different viral proteins) with their SARS-  
148 CoV-2 counterparts. Finally, we established the conditions for a medium-throughput assay to  
149 evaluate basic antiviral activity and assessed the impact of some known CoV inhibitors on  
150 SARS-CoV-2 replication. In addition to many anticipated similarities, our results also  
151 established some remarkable differences between the two viruses that warrant further  
152 investigation. One of them is the rapid evolution - during virus passaging in Vero cells - of a  
153 specific region of the SARS-CoV-2 S protein that contains the so-called 'furin-like cleavage  
154 site'.

155

156

## 157 **METHODS**

158

### 159 **Cell and virus culture**

160 Vero E6 cells and HuH7 cells were grown as described previously (35). SARS-CoV-2 isolate  
161 Australia/VIC01/2020 (GeneBank ID: MT007544.1; (34)) was derived from a positively-testing  
162 nasopharyngeal swab in Melbourne, Australia, and was propagated twice in Vero/hSLAM  
163 cells, before being shared with other laboratories. In Leiden, the virus was passaged two more  
164 times at low multiplicity of infection (m.o.i.) in Vero E6 cells to obtain a working stock (p2 stock).  
165 SARS-CoV isolate Frankfurt 1 (36) was used to compare growth kinetics and other features

166 with SARS-CoV-2. Infection of Vero E6 cells was carried out in phosphate-buffered saline  
167 (PBS) containing 50 µg/ml DEAE-dextran and 2% fetal calf serum (FCS; Bodinco). The  
168 inoculum was added to the cells for 1 h at 37°C, after which cells were washed twice with PBS  
169 and maintained in Eagle's minimal essential medium (EMEM; Lonza) with 2% FCS, 2mM L-  
170 glutamine (PAA) and antibiotics (Sigma). Viral titres were determined by plaque assay in Vero  
171 E6 cells as described previously (37). For plaque picking, plaque assays were performed using  
172 our p1 stock, while using an overlay containing 1% of agarose instead of Avicel (RC-581; FMC  
173 Biopolymer). Following neutral red staining, small and large plaques were picked and used to  
174 inoculate a 9.6-cm<sup>2</sup> dish of Vero E6 cells containing 2 ml of EMEM-2%FCS medium, yielding  
175 p1 virus. After 48 h, 200 µl of the culture supernatant was used to infect the next dish of cells  
176 (p2), a step that was repeated one more time to obtain p3 virus. All work with live SARS-CoV  
177 and SARS-CoV-2 was performed in biosafety laboratory level 3 facilities at Leiden University  
178 Medical Center, the Netherlands.

179

#### 180 **Analysis of intracellular viral RNA and protein synthesis**

181 Isolation of intracellular RNA was performed by lysing infected cell monolayers with TriPure  
182 isolation reagent (Roche Applied Science) according to the manufacturer's instructions. After  
183 purification and ethanol precipitation, intracellular RNA samples were loaded onto a 1.5%  
184 agarose gel containing 2.2 M formaldehyde, which was run overnight at low voltage overnight  
185 in MOPS buffer (10 mM MOPS (sodium salt) (pH 7), 5 mM sodium acetate, 1 mM EDTA).  
186 Dried agarose gels were used for direct detection of viral mRNAs by hybridization with a <sup>32</sup>P-  
187 labeled oligonucleotide probe (5'-CACATGGGGATAGCACTAC-3') that is complementary to  
188 a fully conserved sequence located 30 nucleotides upstream of the 3' end of the genome and  
189 all subgenomic mRNAs produced by SARS-CoV-2 and SARS-CoV. After hybridization, RNA  
190 bands were visualised and quantified by phosphorimaging using a Typhoon-9410 variable  
191 mode scanner (GE Healthcare) and ImageQuant TL software (GE Healthcare). In order to  
192 verify the amount of RNA loaded, a second hybridization was performed using a <sup>32</sup>P-labeled  
193 oligonucleotide probe recognizing 18S ribosomal RNA (5'-GATCCGAGGGCCTCACTAAAC-

194 3'). Protein lysates were obtained by lysing infected cell monolayers in 4x Laemmli sample  
195 buffer and were analysed by semi-dry Western blotting onto Hybond 0.2 $\mu$ M polyvinylidene  
196 difluoride (PVDF) membrane (GE Healthcare). Membranes were incubated with rabbit  
197 antisera diluted in PBS with 0.05% Tween-20 containing 5% dry milk (Campina). Primary  
198 antibodies were detected with a horseradish peroxidase-conjugated swine anti-rabbit IgG  
199 antibody (Dako) and protein bands were visualised using Clarity Western Blot substrate  
200 (Biorad) and detected using an Advanced Q9 Alliance imager (Uvitec Cambridge).

201

### 202 **Next-generation sequencing and bioinformatics analysis**

203 SARS-CoV-2 genomic RNA was isolated from cell culture supernatants using TriPure isolation  
204 reagent (Roche Applied Science) and purified according to manufacturer's instructions. The  
205 total amount of RNA in samples was measured using a Qubit fluorometer and RNA High  
206 Sensitivity kit (Thermo Fisher Scientific). For next-generation sequencing (NGS) library  
207 preparation, RNA (25-100 ng) was mixed with random oligonucleotide primers using the  
208 NEBNext® First Strand Synthesis Module kit for Illumina® (NEB) and incubated for 10 min at  
209 94°C. NGS of samples was performed by a commercial service provider (GenomeScan,  
210 Leiden, the Netherlands) while including appropriate quality controls after each step of the  
211 procedure. Sequencing was performed using a NovaSeq 6000 Sequencing System (Illumina).  
212 Subsequently, sequencing reads were screened for the presence of human (GRCh37.75),  
213 mouse (GRCm38.p4), E. coli MG1655 (EMBL U00096.2), phiX (RefSeq NC\_001422.1) and  
214 common vector sequences (UniVec and ChISab1.1). Prior to alignment, reads were trimmed  
215 to remove adapter sequences and filtered for sequence quality. The remaining reads were  
216 mapped to the SARS-CoV-2 GenBank reference sequence (NC\_045512.2; (38)). Data  
217 analysis was performed using Bowtie 2 (39). Raw NGS data sets for each virus sample  
218 analysed in this study are deposited in NCBI Bioproject and available under the following  
219 links: ---. Only SARS-CoV-2-specific reads were included in these data files.



220 To study evolution/adaptation of the S protein gene, we performed an in-depth analysis of  
221 reads covering the S1/S2 region of the S protein gene. This was done for the p2 stock and for  
222 the four virus samples of the plaque picking experiment shown in Fig. 1a. First, all reads  
223 spanning nt 23,576 to 23,665 of the SARS-CoV genome were selected. Next, reads  
224 constituting less than 1% of the total number of selected reads were excluded for further  
225 analysis. The remaining number of reads were 3,860 (p2 stock), 1,924 (S5p1), 2,263 (S5p2),  
226 4,049 (S5p3) and 3,323 (L8p1). These reads were translated in the S protein open reading  
227 frame and the resulting amino acid sequences were aligned, grouped on the basis of  
228 containing the same mutations/deletions in the S1/S2 region and ranked by frequency of  
229 occurrence (Fig. 1b).

230

### 231 **Antisera and immunofluorescence microscopy**

232 The SARS-CoV-specific rabbit or mouse antisera/antibodies used in this study are listed in  
233 Table 1. Most antisera were described previously (see references in Table 1), with the  
234 exception of three rabbit antisera recognizing SARS-CoV nsps 8, 9 and 15. These were raised  
235 using full-length (His)<sub>6</sub>-tagged bacterial expression products (nsp8 and nsp15) or a synthetic  
236 peptide (nsp9, aa 4209-4230 of SARS-CoV pp1a), which were used to immunize New Zealand  
237 white rabbits as described previously (40, 41). Cross-reactivity of antisera to SARS-CoV-2  
238 targets was evaluated microscopically by immunofluorescence assay (IFA) and for some  
239 antisera (nsp3 and N protein) also by Western blot analysis. Double-stranded RNA was  
240 detected using mouse monoclonal antibody J2 from Scicons (42).

241 Cells were grown on glass coverslips and infected as described above (43). At 12, 24, 48 or  
242 72 h p.i., cells were fixed overnight at 4°C using 3% paraformaldehyde in PBS (pH 7.4). Cells  
243 were washed with PBS containing 10 mM glycine and permeabilized with 0.1% Triton X-100  
244 in PBS. Cells were incubated with antisera diluted in PBS containing 5% FCS. Secondary  
245 antibodies used were an Alexa488-conjugated goat anti-rabbit IgG antibody (Invitrogen), a  
246 Cy3-conjugated donkey anti-rabbit IgG antibody (Jackson ImmunoResearch Laboratories)

247 and an Alexa488-conjugated goat anti-mouse IgG antibody (Invitrogen). Nuclei were stained  
248 with 1 µg/ml Hoechst 33258 (ThermoFischer). Samples were embedded using Prolong Gold  
249 (Life Technologies) and analysed with a Leica DM6B fluorescence microscope using LASX  
250 software.

251

## 252 **Electron microscopy**

253 Vero E6 cells were grown on TC treated Cell Star dishes (Greiner Bio-One) and infected at an  
254 m.o.i. of 3, or mock-infected. Cells were fixed after 6, 8 and 10 h p.i. for 30 min at room  
255 temperature with freshly prepared 2% (vol/vol) glutaraldehyde in 0.1 M cacodylate buffer (pH  
256 7.4) and then stored overnight in the fixative at 4°C. The samples were then washed with 0.1  
257 M cacodylate buffer, treated for 1 hour with 1% (wt/vol) OsO<sub>4</sub> at 4°C, washed with 0.1 M  
258 cacodylate buffer and Milli-Q water, and stained with 1% (wt/vol) uranyl acetate in Mili-Q water.  
259 After a new washing step, samples were dehydrated in increasing concentrations of ethanol  
260 (70%, 80%, 90%,100%), embedded in epoxy resin (LX-112, Ladd Research) and polymerized  
261 at 60°C. Sections (100 nm thick) were collected on mesh-100 copper EM grids covered with  
262 a carbon-coated Pioloform layer and post-stained with 7% (wt/vol) uranyl acetate and  
263 Reynold's lead citrate. The samples were examined in a Twin transmission electron  
264 microscope (Thermo Fisher Scientific (formerly FEI)) operated at 120 kV and images were  
265 collected with a OneView 4k high-frame rate CMOS camera (Gatan).

266

## 267 **Compounds and antiviral screening assay**

268 A 10-mM stock of Remdesivir (HY-104077; MedChemexpress) was dissolved in DMSO and  
269 stored in aliquots for single use at -80°C. Alisporivir was kindly provided by DebioPharm (Dr.  
270 Grégoire Vuagniaux, Lausanne, Switzerland; (44)) and a 20-mM stock was dissolved in 96%  
271 ethanol and stored in aliquots for single use at -20°C. A 20-mM chloroquine stock (C6628;  
272 Sigma) was dissolved in PBS and stored in aliquots for single use at -20°C. Pegylated  
273 interferon alpha-2a (PEG-IFN-α; Pegasys, 90 mcg, Roche) was aliquoted and stored at room  
274 temperature until further use. Vero E6 cells were seeded in a 96-well flat bottom plates in 100

275  $\mu$ l at a density of 10,000 cells/well and grown overnight at 37°C. Two-fold serial dilutions of  
276 compounds were prepared in EMEM with 2% FCS and 50  $\mu$ l was added to the cells 30 min  
277 prior to infection. Subsequently, half of the wells were infected with 300 PFU each of SARS-  
278 CoV or SARS-CoV-2 in order to evaluate inhibition of infection, while the other wells were  
279 used to in parallel monitor the (potential) cytotoxicity of compounds. Each compound  
280 concentration was tested in quadruplicate and each assay plate contained the following  
281 controls: no cells (background control), cells only treated with medium (mock infection for  
282 normalization), infected/untreated cells and infected/solvent-treated cells (infection control). At  
283 3 days p.i., 20  $\mu$ L/well of CellTiter 96 Aqueous Non-Radioactive Cell Proliferation reagent  
284 (Promega) was added and plates were incubated for 2 h at 37°C. Reactions were stopped  
285 and virus inactivated by adding 30  $\mu$ l of 37% formaldehyde. Absorbance was measured using  
286 a monochromatic filter in a multimode plate reader (Envision; Perkin Elmer). Data was  
287 normalized to the mock-infected control, after which EC<sub>50</sub> and CC<sub>50</sub> values were calculated  
288 with Graph-Pad Prism 7.

289

290

## 291 **RESULTS**

292

### 293 **Rapid adaptation of SARS-CoV-2 BetaCoV/Australia/VIC01/2020 during passaging in** 294 **Vero E6 cells**

295 SARS-CoV-2 isolate BetaCoV/Australia/VIC01/2020 was received as a stock derived from two  
296 consecutive passages in Vero/hSLAM cells (34). The virus was then propagated two more  
297 times at low MOI in Vero E6 cells, in which it caused a severe cytopathic effect (CPE). We  
298 also attempted propagation in HuH7 cells, using the same amount of virus or a ten-fold larger  
299 inoculum, but did not observe any cytopathology after 72 h (data not shown). At 24 h p.i.,  
300 immunofluorescence microscopy (see below) revealed infection of only a small percentage of  
301 the HuH7 cells, without any clear spread to other cells occurring in the next 48 h. We therefore

302 conclude that infection of HuH7 cells does not lead to a productive SARS-CoV-2 infection and  
303 deemed this cell line unsuitable for further SARS-CoV-2 studies.

304 The infectivity titre of the Leiden-p2 stock grown in Vero E6 cells was analysed by plaque  
305 assay, after which we noticed a mixed plaque phenotype (~1:3 ratio of small versus large  
306 (plaques; data not shown) while a virus titre of  $7 \times 10^6$  PFU/ml was calculated. To verify the  
307 identity and genome sequence of the SARS-CoV-2/p2 virus stock, we isolated genomic RNA  
308 from culture supernatant and applied next-generation sequencing (NGS; see methods for  
309 details). The resulting consensus sequence was found to be identical to the sequence  
310 previously deposited in GenBank (accession number MT007544.1) (34), with one exception  
311 (see below). Compared to the SARS-CoV-2 GenBank reference sequence (NC\_045512.3)  
312 (38) and other field isolates (29), isolate BetaCoV/Australia/VIC01/2020 exhibits >99.9%  
313 sequence identity. In addition to synonymous mutations in the nsp14-coding sequence  
314 (U19065 to C) and S protein gene (U22303 to G), ORF3a contains a single non-synonymous  
315 mutation (G26144 to U). Strikingly, the 3' untranslated region (UTR) contains a 10-nt deletion  
316 (nt 29750-29759; CGAUCGAGUG) located 120 nt upstream of the genomic 3' end, which is  
317 not present in other SARS-CoV-2 isolates thus far (>670 SARS-CoV2 sequences present in  
318 GenBank on April 17, 2020).

319 In about 71% of the 95,173 p2 NGS reads covering this position, we noticed a G23607 to A  
320 mutation encoding an Arg682 to Gln substitution near the so-called S1/S2 cleavage site of the  
321 viral S protein (see Discussion), with the other 29% of the reads being wild-type sequence. As  
322 this ratio approximated the observed ratio between large and small plaques, we performed a  
323 plaque assay on the p1 virus stock (Fig. 1a, leftmost well) and picked multiple plaques of each  
324 size, which were passaged three times in Vero E6 cells while monitoring their plaque  
325 phenotype. Interestingly, for several of the small-plaque virus clones (like S5; Fig. 1a) we  
326 observed rapid conversion to a mixed or large-plaque phenotype during these three passages,  
327 while large-plaque virus clones (like L8) stably retained their plaque phenotype (Fig. 1a). NGS  
328 analysis of the genome of a large-plaque p1 virus (L8p1) revealed that >99% of the reads in

329 the S1/S2 cleavage site region contained the G23607 to A mutation described above. No other  
330 mutations were detected in the genome, thus clearly linking the Arg682 to Gln substitution in  
331 the S protein to the large-plaque phenotype observed for the L8p1 virus.

332 Next, we also analysed the genomes of the p1, p2 and p3 viruses derived from a small-plaque  
333 (S5) that was picked. This virus clone retained its small-plaque phenotype during the first  
334 passage (Fig. 1a; S5p1), but began to yield an increasing proportion of large(r) plaques during  
335 subsequent passages. Sequencing of S5p2 (Fig 1b) revealed a variety of low-frequency reads  
336 with mutations near the S1/S2 cleavage site motif (aa 681-687; PRRAR↓SV), with G23607 to  
337 A (specifying the Arg682 to Gln substitution) again being the dominant one (in ~0.9% of the  
338 reads covering nt 23,576 to 23,665 of the genome). At lower frequencies single-nucleotide  
339 changes specifying Arg682 to Trp and Arg683 to Leu substitutions were also detected.  
340 Furthermore, a 10-aa deletion (residues 679-688) that erases the S1/S2 cleavage site region  
341 was discovered, as well as a 5-aa deletion (residues 675-679) immediately preceding that  
342 region. The amount of large plaques increased substantially upon the next passage, with NGS  
343 revealing the prominent emergence of the mutants containing the 10-aa deletion or the Arg682  
344 to Gln point mutation (~22% and ~12% of the reads, respectively), and yet other minor variants  
345 with mutations in the PRRAR↓SV sequence being discovered. Taken together these data  
346 clearly link the large-plaque phenotype of SARS-CoV-2 to the acquisition of mutations in this  
347 particular region of the S protein, which apparently provides a strong selective advantage  
348 during passaging in Vero E6 cells.

349

### 350 **Comparative kinetics of SARS-CoV and SARS-CoV-2 replication in Vero E6 cells**

351 To our knowledge, a detailed comparison of SARS-CoV-2 and SARS-CoV replication kinetics  
352 in cell culture has not been reported so far. Therefore, we infected Vero E6 cells with the  
353 SARS-CoV-2/p2 virus stock at high m.o.i. to analyse viral RNA synthesis, protein expression  
354 and the release of infectious viral progeny (Fig. 2a). This experiment was performed using 4  
355 replicates per time point and for comparison we included the SARS-CoV Frankfurt-1 isolate

356 (Drosten, Gunther et al. 2003), which has been used in our laboratory since 2003. During the  
357 early stages of infection (until 8 h p.i.), the growth curves of the two viruses were similar, but  
358 subsequently cells infected with SARS-CoV clearly produced more infectious progeny (about  
359 50-fold more) than SARS-CoV-2-infected cells, with both viruses reaching their plateau by  
360 about 14 h p.i. As shown in Fig. 2b, despite its transition to a mainly large-plaque phenotype,  
361 the largest SARS-CoV-2/p3 plaques were still substantially smaller than those obtained with  
362 SARS-CoV Frankfurt-1.

363 In parallel, we analysed the kinetics of viral RNA synthesis by isolating intracellular viral RNA,  
364 subjecting it to agarose gel electrophoresis and visualizing the various viral mRNA species by  
365 in-gel hybridization with a <sup>32</sup>P-labeled oligonucleotide probe recognizing a fully conserved 19-  
366 nt sequence located 30 nt upstream of the 3' end of both viral genomes (Fig. 3a). This revealed  
367 the anticipated presence of the genomic RNA and eight subgenomic mRNAs, together forming  
368 the well-known 5'- and 3'- coterminal nested set of transcripts required for full CoV genome  
369 expression.

370 In general, for both viruses, the accumulation of viral RNAs followed the growth curves  
371 depicted in Fig. 2a. The relative abundance of the individual RNAs was determined using the  
372 12, 14 and 24 h p.i. samples (averages presented in Fig. 3b) and found to be largely similar,  
373 with the exception of SARS-CoV-2 mRNAs 7 and 8, which accumulated to about 4 and 2 times  
374 higher levels, respectively. Strikingly, in spite of the ultimately lower yield of infectious viral  
375 progeny, SARS-CoV-2 RNA synthesis was detected earlier and reached an overall level  
376 exceeding that of SARS-CoV.

377 We also monitored viral protein production by Western blot analysis using antisera targeting  
378 a non-structural (nsp3) and structural (N) protein. As expected from the RNA analysis, the  
379 accumulation of both viral proteins increased with time, and was detected somewhat earlier  
380 for SARS-CoV-2 than for SARS-CoV (data not shown). Overall, we conclude that in Vero E6  
381 cells, SARS-CoV-2 produces levels of intracellular RNA and proteins that are at least

382 comparable to those of SARS-CoV, although this does not translate into the release of equal  
383 amounts of infectious viral progeny (Fig. 2a).

384

### 385 **Cross-reactivity of antisera previously raised against SARS-CoV targets**

386 To be able to follow virus replication in SARS-CoV-2-infected cells more closely, we explored  
387 cross-reactivity of a variety of antisera previously raised against SARS-CoV targets, in  
388 particular a variety of nsps. In an earlier study, many of those were found to cross-react also  
389 with the corresponding MERS-CoV targets (35), despite the relatively large evolutionary  
390 distance between MERS-CoV and SARS-CoV. Based on the much closer relationship with  
391 SARS-CoV-2, similar or better cross-reactivity of these SARS-CoV reagents was expected,  
392 which was explored using immunofluorescence microscopy.

393 Indeed, most antisera recognizing SARS-CoV nsps that were tested (nsp3, nsp4, nsp5, nsp8,  
394 nsp9, nsp13, nsp15) strongly cross-reacted with the corresponding SARS-CoV-2 target (Fig.  
395 4 and Table 1), the exception being a polyclonal nsp6 rabbit antiserum. Likewise, both a  
396 polyclonal rabbit antiserum and mouse monoclonal antibody recognizing the N protein cross-  
397 reacted strongly (Fig. 4b and Table 1). The same was true for a rabbit antiserum raised against  
398 a C-terminal peptide of the SARS-CoV M protein (Fig 4e). Labelling patterns were essentially  
399 identical to those previously documented for SARS-CoV (Stertz, Reichelt et al. 2007, Knoops,  
400 Kikkert et al. 2008), with nsps accumulating in the perinuclear region of infected cells, where  
401 the elaborate membrane structures of the viral ROs are formed (Fig. 4a, c, d). Punctate  
402 structures in the same area of the cell were labelled using an antibody recognizing double-  
403 stranded RNA (dsRNA), which presumably recognizes replicative intermediates of viral RNA  
404 synthesis (45, 46). The N protein signal was diffusely cytosolic (Fig. 4b), whereas the M protein  
405 labelling predominantly showed the expected localization to the Golgi complex (Fig. 4e), where  
406 the protein is known to accumulate (47).

407

408

## 409 **Ultrastructural characterisation of SARS-CoV-2-infected cells**

410 We next used electron microscopy to investigate the ultrastructural changes that SARS-CoV-  
411 2 induces in infected cells, and focused on the membranous replication organelles (ROs) that  
412 supports viral RNA synthesis and on the assembly and release of new virions (Fig. 5).  
413 Compared to mock-infected control cells (Fig. 5a-b), various distinct membrane alterations  
414 were observed in cells infected with either SARS-CoV or SARS-CoV-2 (Fig. 5c-j). At 6 h p.i.,  
415 larger regions with membrane alterations were found particularly in cells infected with SARS-  
416 CoV-2 (data not shown), which may align with the somewhat faster onset of intracellular RNA  
417 synthesis in SARS-CoV2-infected Vero E6 cells (Fig. 3a). From 8 h p.i onwards, SARS-CoV-  
418 and SARS-CoV-2-infected cells appeared more similar (Fig. 5c-f and 5g-j). Double-membrane  
419 vesicles (DMVs) were the most prominent membrane alteration up to this stage (Fig. 5d-e and  
420 and 5h-i, asterisks). In addition, convoluted membranes (45) were readily detected in SARS-  
421 CoV-infected cells, while zippered ER (25, 48, 49) appeared to be the predominant structure  
422 in SARS-CoV-2-infected cells (Fig. 5e and 5i, white arrowheads). As previously described for  
423 SARS-CoV (45), also SARS-CoV-2-induced DMV appeared to fuse through their outer  
424 membrane, giving rise to vesicles packets that increased in numbers as infection progressed  
425 (Fig 5f and 5k, white asterisks). Virus budding near the Golgi apparatus, presumably into  
426 smooth membranes of the ER-Golgi intermediate compartment (ERGIC) (50-52), was  
427 frequently observed at 8 h p.i. (Fig. 5k-l and 5o-p). This step is followed by transport to the  
428 plasma membrane and release of virus particles into extracellular space. By 10 h p.i., released  
429 progeny virions were abundantly detected around all infected cells (Fig. 5m-n and 5q-r).  
430 Interestingly, whereas spikes were clearly present on SARS-CoV progeny virions, a relatively  
431 large proportion of SARS-CoV-2 particles seemed to carry few or no visible spike projections  
432 on their surface, perhaps suggesting a relatively inefficient incorporation of spike proteins into  
433 SARS-CoV-2 virions. This could potentially reduce the yield of infectious particles and may  
434 contribute to the lower progeny titres obtained for this virus (Fig. 2a).

435

436



437 **Establishing a CPE-based assay to screen compounds for anti-SARS-CoV-2 activity**

438 In order to establish and validate a CPE-based assay to identify potential inhibitors of SARS-  
439 CoV-2 replication, we selected four previously identified inhibitors of CoV replication:  
440 Remdesivir (53, 54), chloroquine (55, 56), Alisporivir (57, 58) and pegylated interferon alpha  
441 (PEG-IFN- $\alpha$ ) (35, 59). Cells were infected at low MOI to allow for multiple cycles of replication.  
442 After three days, a colorimetric cell viability assay (60) was used to measure drug toxicity and  
443 inhibition of virus replication in mock- and virus-infected cells, respectively. With the exception  
444 of PEG-IFN- $\alpha$ , the inhibition of virus replication by compounds tested and the calculated half-  
445 maximal effective concentrations ( $EC_{50}$ ) were similar for SARS-CoV and SARS-CoV-2. For  
446 Remdesivir, we obtained higher  $EC_{50}$  values for SARS-CoV-2 and SARS-CoV (4.4 and 4.5  
447  $\mu$ M, respectively; Fig. 6a) than previously reported by others, but this may be explained by  
448 technical differences like a longer assay incubation time (72 h instead of 48 h) and the use of  
449 a different read-out (cell viability instead of qRT-PCR or viral load). Based on the obtained  
450  $CC_{50}$  values of  $>100 \mu$ M, a selectivity index  $>22.5$  was calculated. Chloroquine potently  
451 blocked virus infection at low-micromolar concentrations, with an  $EC_{50}$  value of 2.3  $\mu$ M for both  
452 viruses ( $CC_{50} >100 \mu$ M, SI  $>45.5$ ; Fig. 6b). Alisporivir, a known inhibitor of different groups of  
453 RNA viruses, was previously found to effectively reduce the production of CoV progeny. In  
454 this study, we measured  $EC_{50}$  values of 4.9 and 4.3  $\mu$ M for SARS-CoV-2 and SARS-CoV,  
455 respectively (Fig. 6c;  $CC_{50} >100 \mu$ M, SI  $>20$ ). Treatment with PEG-IFN- $\alpha$  completely inhibited  
456 replication of SARS-CoV-2, even at the lowest dose of 7.8 ng/ml (Fig. 6d). In line with previous  
457 results (35, 59), SARS-CoV was much less sensitive to PEG-IFN- $\alpha$  treatment, yielding only  
458 partial inhibition at all concentrations tested (from 7.8 to 1000 ng/ml). Overall, we conclude  
459 that Vero E6 cells provide a suitable basis to perform antiviral compound screening and select  
460 the most promising hits for in-depth mechanistic studies and further development.

461

## 462 **Discussion**

463

464 In this report, we describe a comparative analysis of the replication features of SARS-CoV-2  
465 and SARS-CoV in Vero E6 cells, one of the most commonly used cell lines for studying these  
466 two viruses. However, in contrast to the stable phenotype exhibited by SARS-CoV during our  
467 17 years of working with this virus in these cells, SARS-CoV-2 began to exhibit remarkable  
468 phenotypic variation in plaque assays within a few passages after its isolation from clinical  
469 samples (Fig. 1a). In addition to the BetaCoV/Australia/VIC01/2020 isolate used in this study,  
470 similar observations were made for a variety of other clinical isolates (data not shown). To  
471 establish the genetic basis for the observed plaque size heterogeneity, small and large  
472 plaques were picked and the resulting virus clones were passaged repeatedly and analysed  
473 using NGS. The consensus sequences obtained for S5p1 and L8p1, which differed by a single  
474 nucleotide substitution in the S protein gene, clearly established that a single S protein  
475 mutation (Arg682 to Gln) was responsible for the observed plaque size difference. This  
476 mutation is localized near the so-called 'furin-like' S1/S2 cleavage site (Fig. 1b) (61) in the S  
477 protein (62). This sequence constitutes a (potential) processing site that is present in a subset  
478 of CoVs (including SARS-CoV-2 and MERS-CoV) but is lacking in others, like SARS-CoV and  
479 certain bat CoVs (61, 63). This polybasic motif (PRRAR↓SV, in SARS-CoV-2) can be  
480 recognized by intracellular furin-like proteases during viral egress and its cleavage is thought  
481 to prime the S protein for fusion and entry (64), which also requires a second cleavage event  
482 to occur at the downstream S2' cleavage site (61). In general, the presence of the furin-like  
483 cleavage site does not appear to be critical for successful CoV infection. Using pseudotyped  
484 virions carrying mutant S proteins of SARS-CoV (65) or SARS-CoV-2 (66), it was shown that  
485 its presence minimally impacts S protein functionality. In the SARS-CoV S protein, an adjacent  
486 sequence that is conserved across CoVs can be cleaved by other host proteases like  
487 cathepsin L or TMPRSS2 (67-69), thus providing an alternative pathway to trigger viral entry.  
488 Possibly, this pathway is also employed by our Vero E6-cell adapted SARS-CoV-2 mutants  
489 that have lost the furin-like cleavage site, like clone L8p1 and multiple variants encountered in

490 S5p3 (Fig. 1a). These variants contain either single point mutations or deletions of 5 to 10 aa  
491 (Fig. 1b), resembling variants recently reported by other laboratories (30, 70, 71). Interestingly  
492 similar changes were also observed in some clinical SARS-CoV-2 isolates that had not been  
493 passaged in cell culture (70). It is currently being investigated why mutations that inactivate  
494 the furin-like cleavage site provide such a major selective advantage during SARS-CoV-2  
495 passaging in Vero E6 cells and how this translates into the striking large-plaque phenotype  
496 documented in this paper.

497 An additional remarkable feature confirmed by our re-sequencing of the  
498 BetaCoV/Australia/VIC01/2020 isolate of SARS-CoV-2 is the presence of a 10-nt deletion in  
499 the 3' UTR of the genome (34). Screening of other available SARS-CoV-2 genome sequences  
500 indicated that the presence of this deletion apparently is unique for this particular isolate, and  
501 likely represents an additional adaptation acquired during cell culture passaging. This deletion  
502 maps to a previously described "hypervariable region" in the otherwise conserved 3' UTR, and  
503 in particular to the so-called s2m motif (72) that is conserved among CoVs and also found in  
504 several other virus groups (73, 74). The s2m element has been implicated in the binding of  
505 host factors to viral RNAs, but its exact function has remained enigmatic thus far. Strikingly,  
506 for the mouse hepatitis coronavirus the entire hypervariable region (including s2m) was found  
507 to be dispensable for replication in cell culture, but highly relevant for viral pathogenesis in  
508 mice (72). Although the impact of this deletion for SARS-CoV-2 remains to be studied in more  
509 detail, these previous data suggest that this mutation need not have a major impact on SARS-  
510 CoV-2 replication in Vero E6 cells. This notion is also supported by the fact that the results of  
511 our antiviral screening assays (Fig. 6) correlate well with similar studies performed with other  
512 SARS-CoV-2 isolates (54, 75, 76). Clearly, this could be different for *in vivo* studies, for which  
513 it would probably be better to rely on SARS-CoV-2 isolates not carrying this deletion in their  
514 3' UTR.

515 Vero E6 cells are commonly used to isolate, propagate, and study SARS-CoV-like viruses as  
516 they support viral replication to high titres (77-81). This may be due to a high expression level

517 of the ACE-2 receptor (82) that is used by both SARS-CoV-2 and SARS-CoV (9) and/or the  
518 fact that they lack the ability to produce interferon (83, 84). It will be interesting to evaluate  
519 whether there is a similarly strong selection pressure to adapt the S1/S2 region of the S protein  
520 when SARS-CoV-2 is passaged in other cell types. Such studies are currently in progress in  
521 our laboratory and already established that HuH7 cells may be a poor choice, despite the fact  
522 that they were used for virus propagation (9, 85) and antiviral screening in other studies (54,  
523 86). Immunolabelling of infected HuH7 cells (data not shown) revealed non-productive  
524 infection of only a small fraction of the cells and a general lack of cytopathology. While other  
525 cell lines are being evaluated, as illustrated above, the monitoring of the plaque phenotype  
526 (plaque size and homogeneity) may provide a quick and convenient method to assess the  
527 composition of SARS-CoV-2 stocks propagated in Vero E6 cells, at least where it concerns  
528 the evolution of the S1/S2 region of the S protein.

529 Given the ongoing SARS-CoV-2 pandemic, the detailed characterization of its replication cycle  
530 is an important step in understanding the molecular biology of the virus and defining potential  
531 targets for inhibitors of replication. The cross-reacting antisera described in this study (Table  
532 1) will be a useful tool during such studies. In general, the subcellular localization of viral nsps  
533 and structural proteins (Fig. 4) and the ultrastructural changes associated with RO formation  
534 (Fig. 5) were very similar for the two viruses. We also observed comparable replication kinetics  
535 for SARS-CoV-2 and SARS-CoV in Vero E6 cells, although clearly lower final infectivity titres  
536 were measured for SARS-CoV-2 (~50-fold lower; Fig. 2). Nevertheless, RNA synthesis could  
537 be detected somewhat earlier for SARS-CoV-2 and the overall amount of viral RNA produced  
538 exceeded that produced by SARS-CoV (Fig. 3). This may be indicative of certain assembly or  
539 maturation problems or of virus-host interactions that are different in the case SARS-CoV-2.  
540 These possibilities merit further investigation, in particular since our preliminary EM studies  
541 suggested intriguing differences with SARS-CoV where it concerns the presence of spikes on  
542 the surface of freshly released SARS-CoV-2 particles (Fig. 5n and 5r).

543 Our analysis of SARS-CoV-2 subgenomic mRNA synthesis revealed the increased relative  
544 abundance of mRNAs 7 and 8 (~4- and ~2-fold, respectively) when SARS-CoV-2 was  
545 compared to SARS-CoV. Mechanistically, these differences do not appear to be caused by  
546 extended base pairing possibilities of the transcription regulatory sequences that direct the  
547 synthesis of these two mRNAs (24). As in SARS-CoV, mRNA7 of SARS-CoV-2 encodes for  
548 two proteins, the ORF7a and ORF7b proteins, with the latter presumably being expressed  
549 following leaky ribosomal scanning (32). Upon its ectopic expression, the ORF7a protein has  
550 been reported to induce apoptosis via a caspase-dependent pathway (87) and/or to be  
551 involved in cell cycle arrest (88). The ORF7b product is a poorly studied integral membrane  
552 protein that has (also) been detected in virions (89). When ORF7a/b or ORF7a were deleted  
553 from the SARS-CoV genome, there was a minimal impact on the kinetics of virus replication  
554 *in vitro* in different cell lines, including Vero cells, and *in vivo* using mice. In another study,  
555 however, partial deletion of SARS-CoV ORF7b was reported to provide a replicative  
556 advantage in CaCo-2 and HuH7 cells, but not in Vero cells (90).

557 The SARS-CoV ORF8 protein is membrane-associated and able to induce endoplasmic  
558 reticulum stress (91, 92), although it has not been characterised in great detail in the context  
559 of viral infection. Soon after the emergence of SARS-CoV in 2003, a conspicuous 29-nt (out-  
560 of-frame) deletion in ORF8 was noticed in late(r) human isolates, but not in early human  
561 isolates and SARS-like viruses obtained from animal sources (93-95). Consequently, loss of  
562 ORF8 function was postulated to reflect an adaptation to the human host. The re-engineering  
563 of an intact ORF8, using a reverse genetics system for the SARS-CoV Frankfurt-1 isolate,  
564 yielded a virus with strikingly enhanced (up to 23-fold) replication properties in multiple  
565 systems (96). Clearly, it remains to be established that the increased synthesis of mRNAs 7  
566 and 8 is a general feature of SARS-CoV-2 isolates, and that this indeed also translates into  
567 higher expression levels of the accessory proteins encoded by ORFs 7a, 7b and 8. If  
568 confirmed, these differences definitely warrant an in-depth follow-up analysis as CoV  
569 accessory proteins in general have been shown to be important determinants of virulence.

570 They may thus be relevant for our understanding of the wide spectrum of respiratory disease  
571 symptoms observed in COVID-19 patients (97).

572 Based on the close ancestral relationship between SARS-CoV-2 and SARS-CoV (98), one  
573 might expect that the patterns and modes of interaction with host antiviral defence  
574 mechanisms would be similar. However, our experiments with type I interferon treatment of  
575 Vero E6 cells (Fig. 6) revealed a clear difference, with SARS-CoV-2 being considerably more  
576 sensitive than SARS-CoV, as also observed by other laboratories (76). Essentially, SARS-  
577 CoV-2 replication could be inhibited by similarly low concentrations of PEG-IFN-alpha-2a that  
578 inhibit MERS-CoV replication in cell culture (35). Taken together, our data suggest that SARS-  
579 CoV-2 is less able to counter a primed type I IFN response than SARS-CoV (76, 99).

580 Previously identified inhibitors of CoV replication were used to further validate our cell-based  
581 assay for SARS-CoV-2 inhibitor screening. These compounds inhibited replication at similar  
582 low-micromolar concentrations and in a similar dose-dependent manner as observed for  
583 SARS-CoV (Fig. 6). Remdesivir is a prodrug of an adenosine analogue developed by Gilead  
584 Sciences. It was demonstrated to target the CoV RNA polymerase and act as a chain  
585 terminator (100-102). The clinical efficacy of Remdesivir is still being evaluated and, after  
586 some first encouraging results (103), worldwide compassionate use trials are now being  
587 conducted. Likewise, hydroxychloroquine and chloroquine have been labelled as potential  
588 “game changers” and are being evaluated for treatment of severe COVID-19 patients (104).  
589 Both compounds have been used to treat malaria and amebiasis (105), until drug-resistant  
590 *Plasmodium* strains emerged (106). These compounds can be incorporated into endosomes  
591 and lysosomes, raising the pH inside these intracellular compartments, which in turn may lead  
592 to defects in protein degradation and intracellular trafficking (68, 107). An alternative  
593 hypothesis to explain their anti-SARS-CoV activity is based on their impact on glycosylation  
594 of the ACE2 receptor that is used by SARS-CoV (56). Finally, as expected, the non-  
595 immunosuppressive cyclosporin A analogue Alisporivir inhibited SARS-CoV-2 replication, as  
596 demonstrated previously for SARS-CoV and MERS-CoV (58). Although the exact mode of

597 action of this inhibitor it is unclear, it is thought to modulate CoV interactions with members of  
598 the cyclophilin family (108). Unfortunately, all of these *in vitro* antiviral activities should  
599 probably be classified as modest, emphasizing the urgency of large-scale drug repurposing  
600 and discovery programmes that target SARS-CoV-2 and coronaviruses at large.

601 **Authors and contributors**

602 NO, JD, MK, MB, IS and ES conceptualised the study. NO, TD, JZ, RL, YM and LC performed  
603 experimental work and contributed to analysis of the results and preparation of figures. NO,  
604 LC, JD, JV, IS and ES performed NGS and were involved in the bioinformatics analysis of the  
605 data. NO and ES wrote the manuscript, with input from all authors.

606

607

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609 The authors declare that there are no conflicts of interest.

610

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936

937 **Figure legends**

938

939 **Fig. 1. Rapid evolution of SARS-CoV-2 during passaging in Vero E6 cells.** (a) Outline of  
940 a plaque picking experiment that was initiated when the p2 stock of SARS-CoV-2  
941 Australia/VIC01/2020 showed remarkable plaque heterogeneity on Vero E6 cells (leftmost  
942 well). Following a plaque assay of the p1 virus stock, small and large plaques were picked and  
943 these virus clones were passaged three times in Vero E6 cells, while their plaque phenotype  
944 was monitored. In contrast to the large plaque viruses (example L8; bottom row), the plaque  
945 phenotype of the small plaque viruses (example S5; top row) rapidly evolved within these 3  
946 passages. (b) Evolution/adaptation of the S protein gene during Vero E6 passaging. Overview  
947 of NGS data obtained for the p2 stock, S5p1/p2/p3 and S8p1 in the S1/S2 region of the SARS-  
948 CoV-2 S protein gene that encodes the so-called 'furin-like cleavage site. The analysis was  
949 based on NGS reads spanning nt 23,576 to 23,665 of the SARS-CoV genome (see Methods  
950 for details) and their translation in the S protein open reading frame. Deletions are indicated  
951 with  $\Delta$  followed by the affected amino acid residues.

952

953 **Fig. 2. Comparison of SARS-CoV-2 and SARS-CoV replication kinetics in Vero E6 cells.**

954 (a) Growth curve showing the release of infectious viral progeny into the medium of infected  
955 Vero E6 cells (m.o.i. 3), as determined by plaque assay (n = 4; mean  $\pm$  sd is presented). (b) .  
956 Comparison of SARS-CoV-2 Australia/VIC01/2020 and SARS-CoV Frankfurt-1 plaque  
957 phenotype in Vero E6 cells.

958

959 **Fig. 3. Kinetics of SARS-CoV-2 and SARS-CoV RNA synthesis in infected Vero E6 cells.**

960 (a) Hybridization analysis of viral mRNAs isolated from SARS-CoV-2- and SARS-CoV-infected  
961 Vero E6 cells, separated in an agarose gel and probed with a radiolabelled oligonucleotide  
962 recognizing the genome and subgenomic mRNAs of both viruses. Subsequently, the gel was  
963 re-hybridized to a probe specific for 18S ribosomal RNA, which was used as a loading control.

964 (b) Analysis of the relative abundance of each of the SARS-CoV-2 and SARS-CoV transcripts.  
965 Phosphorimager quantification was performed for the bands of the samples isolated at 12, 14  
966 and 24 h p.i., which yielded essentially identical relative abundances. The table shows the  
967 average of these three measurements. SARS-CoV-2 mRNA sizes were calculated on the  
968 basis of the position of the leader and body transcription-regulatory sequences (ACGAAC) in  
969 the viral genome (Sawicki and Sawicki 1995, Xu, Hu et al. 2003).

970

971 **Fig. 4. Cross-reactivity of antisera raised against SARS-CoV structural and non-**  
972 **structural proteins.**

973 Selected antisera previously raised against SARS-CoV nsps and structural proteins cross-  
974 react with corresponding SARS-CoV-2 proteins. SARS-CoV-2-infected Vero E6 cells (m.o.i.  
975 of 0.3) were fixed at 12 or 24 h p.i. For immunofluorescence microscopy, cells were  
976 (double)labelled with (a) a rabbit antiserum recognising nsp4 and a mouse mAb recognising  
977 dsRNA; (b) anti-nsp4 rabbit serum and a mouse mAb directed against the N protein; (c-e)  
978 rabbit antisera recognising against nsp3, nsp13 and the M protein, respectively. Nuclear DNA  
979 was stained with Hoechst 33258. Bar, 20  $\mu$ m.

980

981 **Fig. 5. Visualisation of SARS-CoV-2 and SARS-CoV infection by electron microscopy.**

982 Electron micrographs of Vero E6 cells infected with either SARS-CoV-2 or SARS-CoV at the  
983 indicated time points (c-r). Images from a mock-infected cell are included for comparison (a-  
984 b). (c-j) RRegions containing viral replication organelles. These virus-induced structures  
985 accumulated in large clusters in the perinuclear region by 8 h p.i. (c, g, boxed regions enlarged  
986 in d and h, respectively). These regions primarily contained DMVs (d-e, h-i, black asterisks).  
987 Additionally, virus-induced convoluted membranes (e, white arrowhead) were observed in  
988 SARS-CoV infection, whereas zippered ER (i, white arrowheads) appeared to be more  
989 common in SARS-CoV-2-infected cells. At 10 h p.i., vesicle packets (f, j, white asterisks),  
990 which seem to arise by fusion of two or more DMVs through their outer membrane, became  
991 abundant in the RO regions. (k-r) Examples of virion assembly and release in infected cells.



992 Virus particles budding into membranes of the ERGIC (k-l, o-p, arrowheads). The black  
993 arrowheads in the boxed areas highlight captured budding events, enlarged in l and p.  
994 Subsequently, virus particles are transported to the plasma membrane which, at 10 h p.i., is  
995 surrounded by a large number of released virions (m, q, boxed areas enlarged in n and r,  
996 respectively). N, nucleus; m, mitochondria; G, Golgi apparatus. Scale bars: 1  $\mu\text{m}$  (a, c, g); 500  
997 nm (b, d-f, h-j, k, m, o, q); 100 nm (l, n, p, r).

998

999 **Fig. 6. Assay to screen for compounds that inhibit SARS-CoV-2 replication.**

1000 Inhibition of SARS-CoV-2 replication (coloured bars) was tested in Vero E6 cells by developing  
1001 a CPE-reduction assay and evaluating several previously identified inhibitors of SARS-CoV,  
1002 which was included for comparison (grey bars). For each compound a two-fold serial dilution  
1003 series in the low-micromolar range was tested; (a) Remdesivir, (b) chloroquine, (c) Alisporivir  
1004 and (d) pegylated interferon alpha-2. Cell viability was assayed using the CellTiter 96®  
1005 Aqueous One Solution cell proliferation assay (MTS assay). Compound toxicity (solid line)  
1006 was evaluated in parallel using mock-infected, compound-treated cells. The graphs show the  
1007 results of 3 independent experiments, each performed using quadruplicate samples (mean  $\pm$   
1008 SD are shown).

1009 **Table 1.** SARS-CoV-specific antisera used and their cross-reactivity with corresponding  
 1010 SARS-CoV-2 targets.

<b>SARS-CoV antiserum</b>	<b>function of target</b>	<b>antigen type</b>	<b>antibody type</b>	<b>IFA signal*</b>	<b>reference</b>
nsp3 (DGD7)	transmembrane replicase protein, containing PL <sup>pro</sup>	bacterial expression product	rabbit polyclonal	++	(47)
nsp4 (FGQ4)	transmembrane replicase protein	synthetic peptide	rabbit polyclonal	++	(109)
nsp5 (DUE5)	M <sup>pro</sup>	bacterial expression product	rabbit polyclonal	+	(47)
nsp6 (GBZ7)	transmembrane replicase protein	synthetic peptide	rabbit polyclonal	-	(109)
nsp8 (DUK4)	RNA polymerase co-factor	bacterial expression product	rabbit polyclonal	++	(47)
nsp8 (39-12)	RNA polymerase co-factor	bacterial expression product	mouse monoclonal	++	unpublished
nsp9 (HLJ5)	RNA-binding protein	synthetic peptide	rabbit polyclonal	++	unpublished
nsp13 (CQS2)	RNA helicase	synthetic peptide	rabbit polyclonal	++	(47)
nsp15 (HLT5)	endoribonuclease	bacterial expression product	rabbit polyclonal	+	unpublished
nsp15 (BGU6)	endoribonuclease	synthetic peptide	rabbit polyclonal	+	(47)
M (EKU9)	membrane protein	synthetic peptide	rabbit polyclonal	+	(47)
N (JUC3)	nucleocapsid protein	bacterial expression product	rabbit polyclonal	+	(35)
N (46-4)	nucleocapsid protein	bacterial expression product	mouse monoclonal	++	(41)

1011 \* ++, strongly positive; +, positive; -, negative.

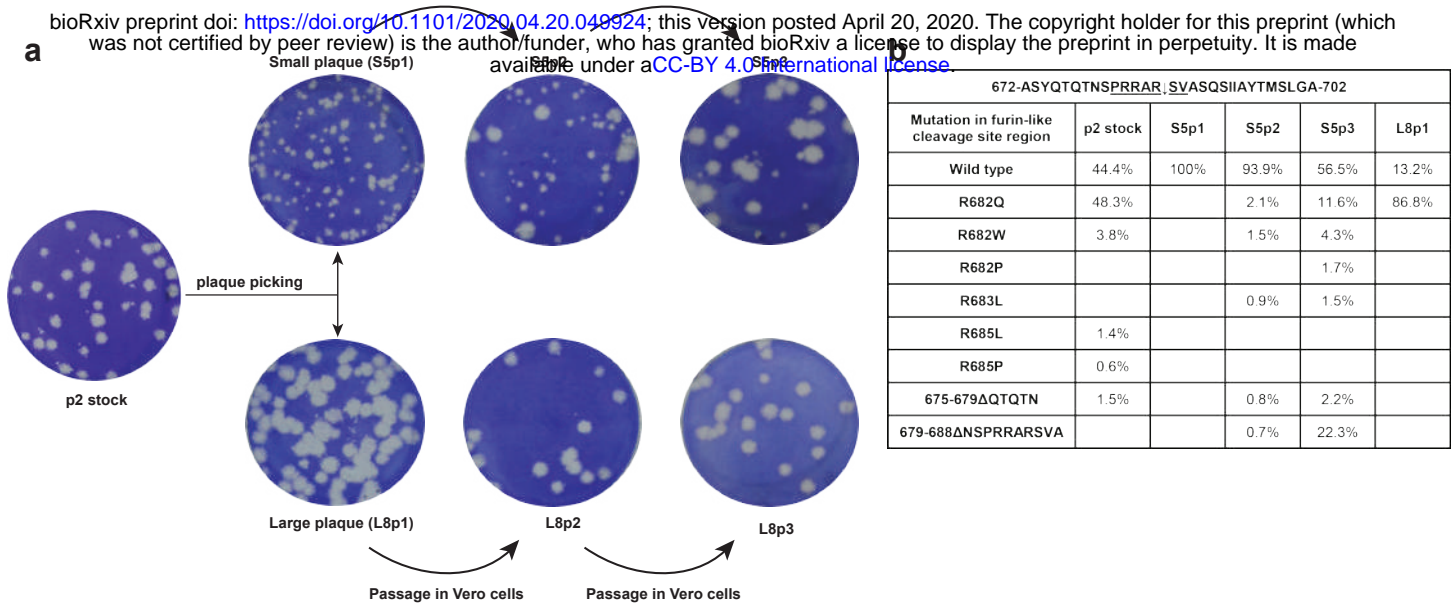


Figure 1

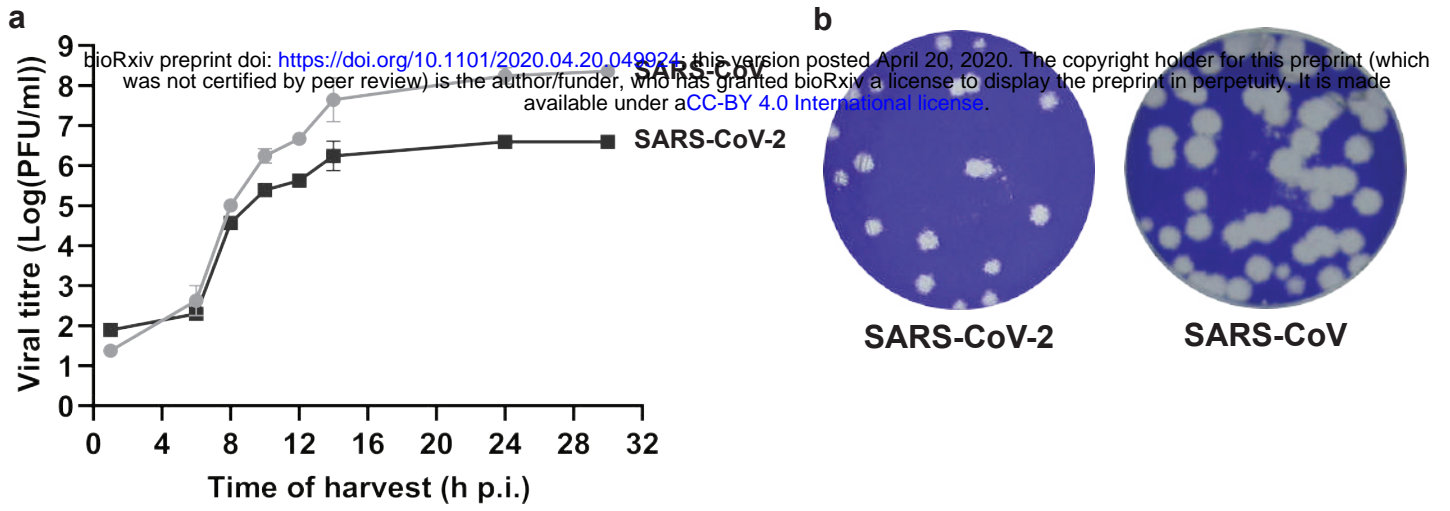
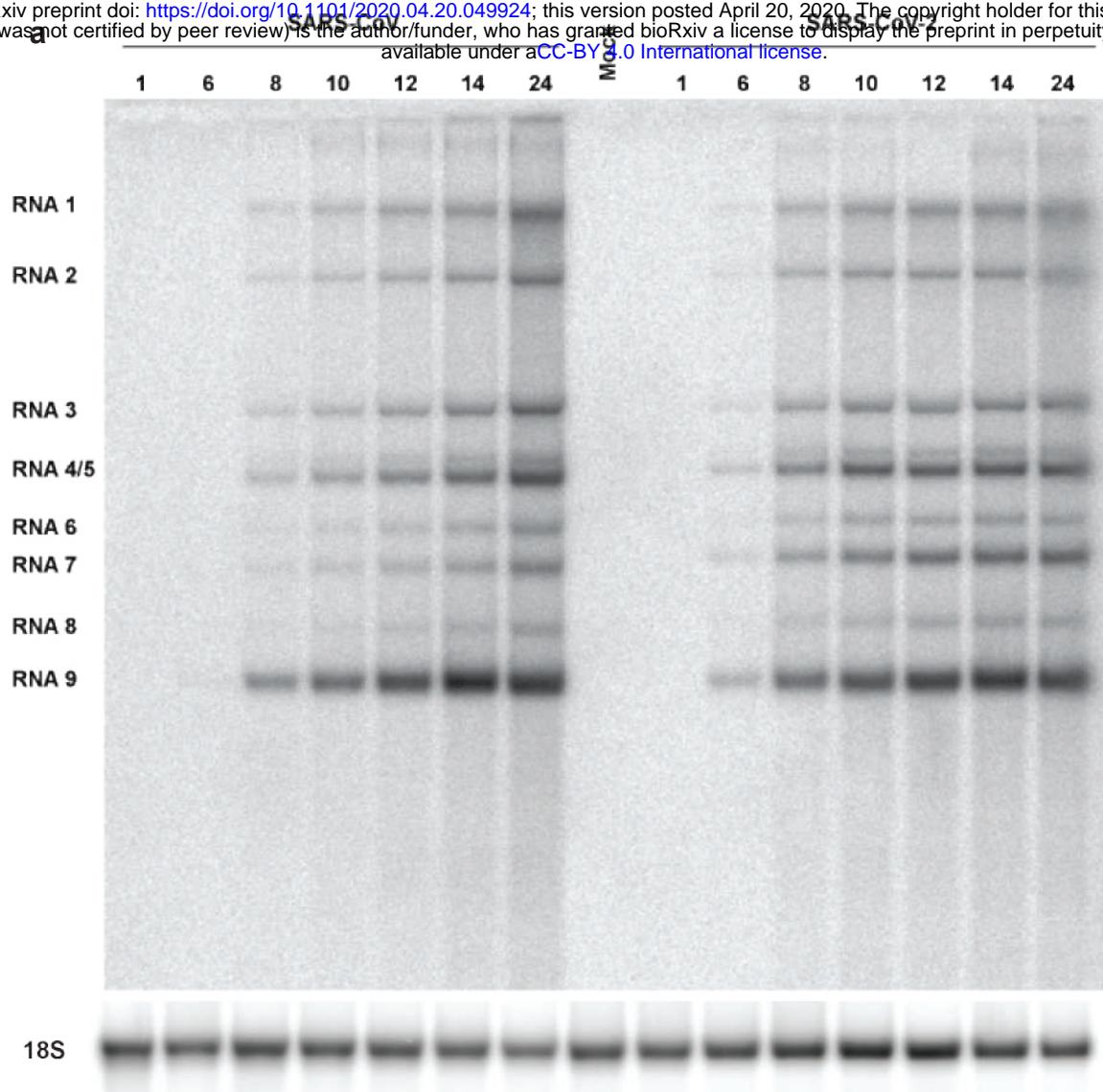


Figure 2



**b**

RNA	Size (kb) of SARS-CoV-2 mRNA	Relative abundance (%)	
		SARS-CoV	SARS-CoV-2
1	29.9	6.0	5.1
2	8.4	4.0	3.5
3	4.5	7.2	6.9
4	3.7	1.9	2.0
5	3.4	14.2	18.0
6	2.9	2.0	2.5
7	2.5	3.5	13.7
8	2.0	1.1	2.3
9	1.7	60.0	45.9

Figure 3

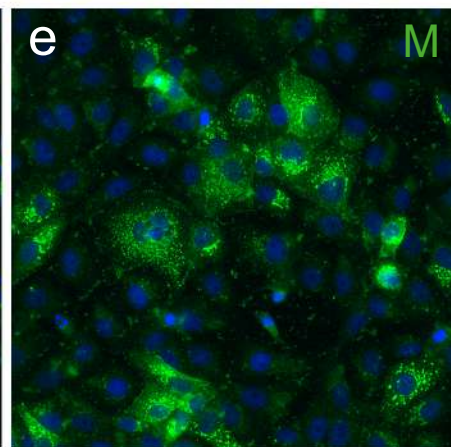
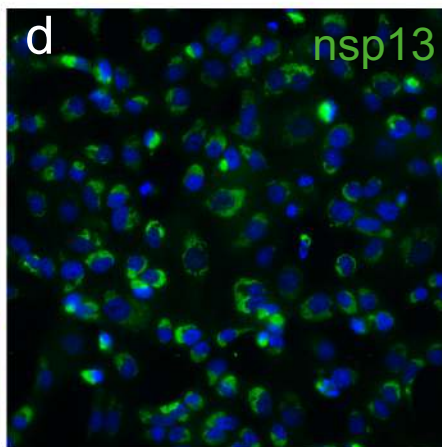
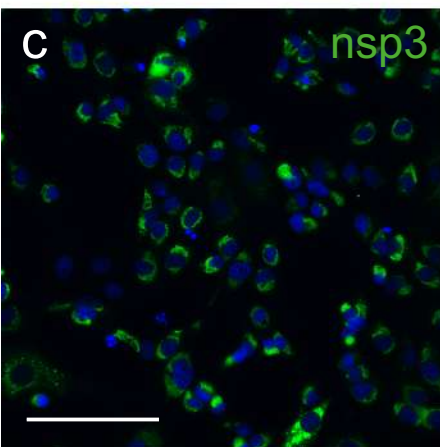
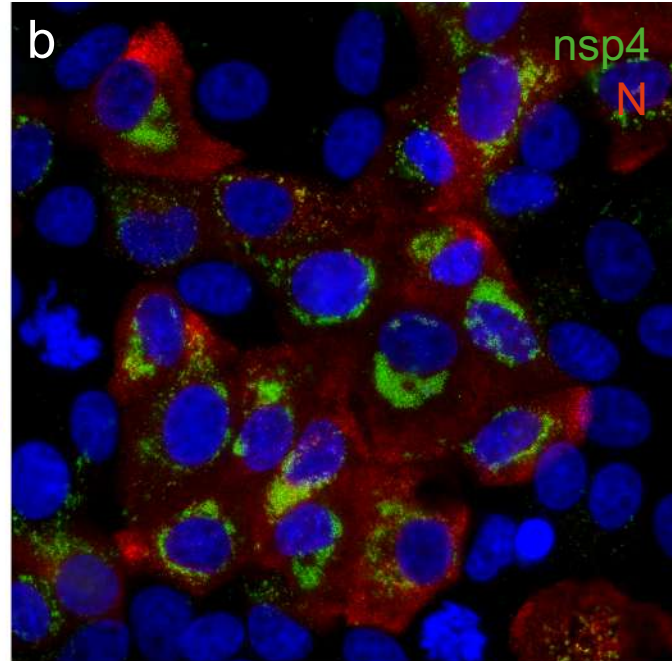
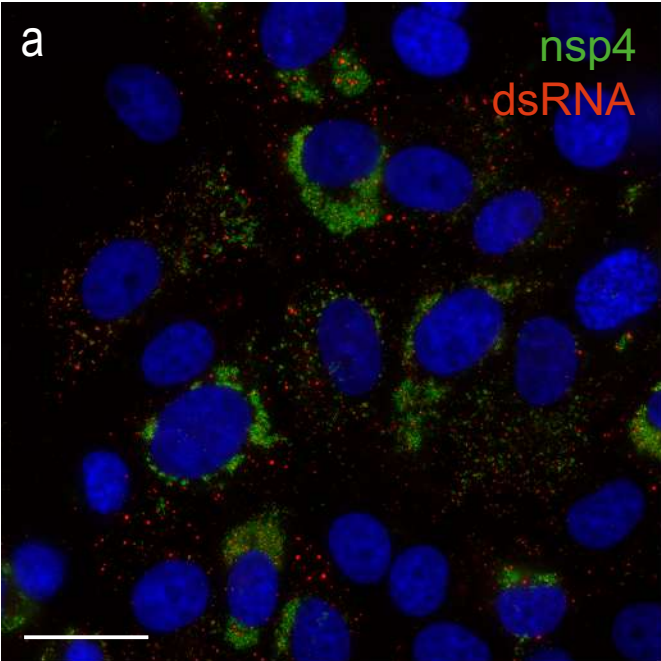


Figure 4

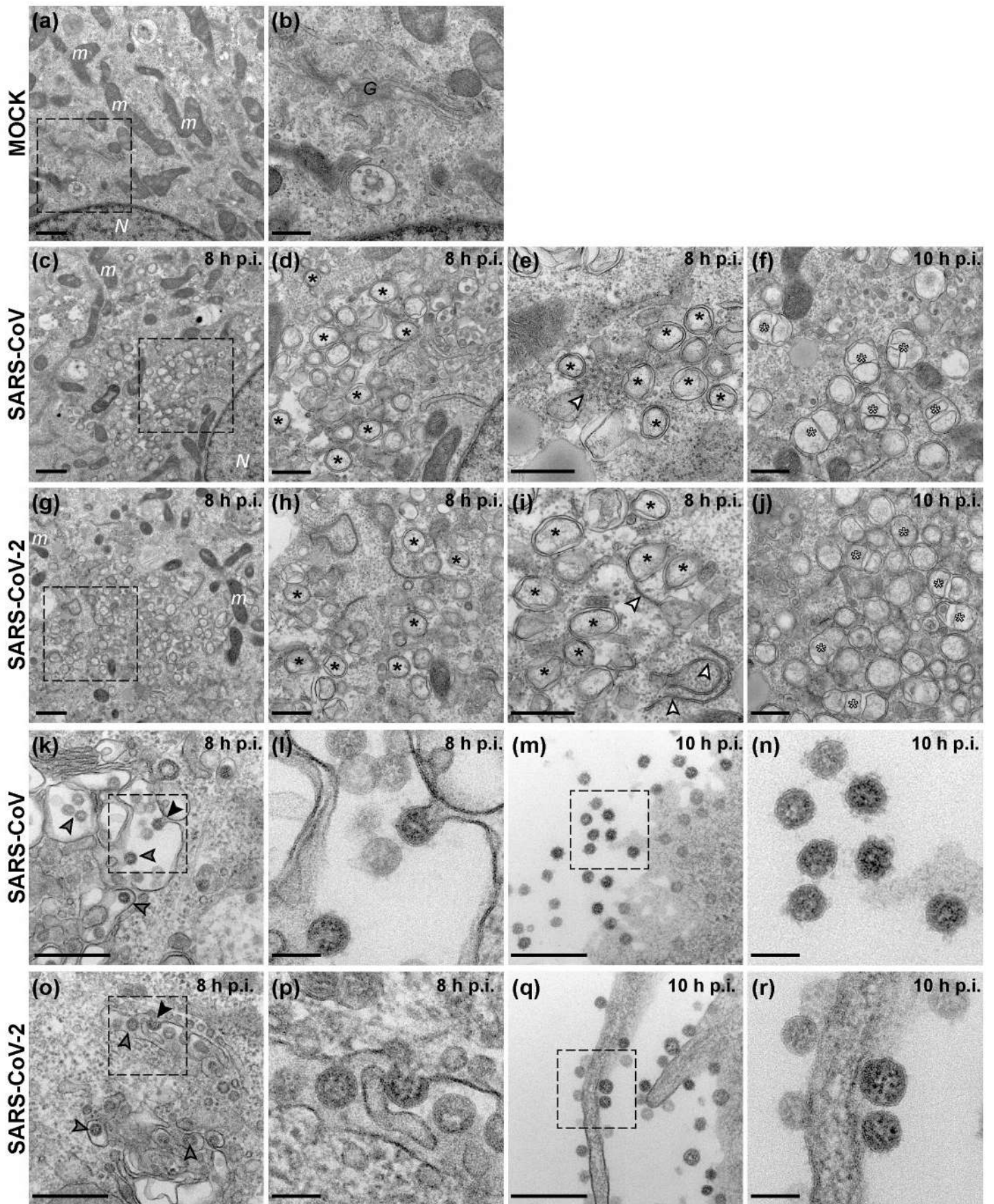


Figure 5

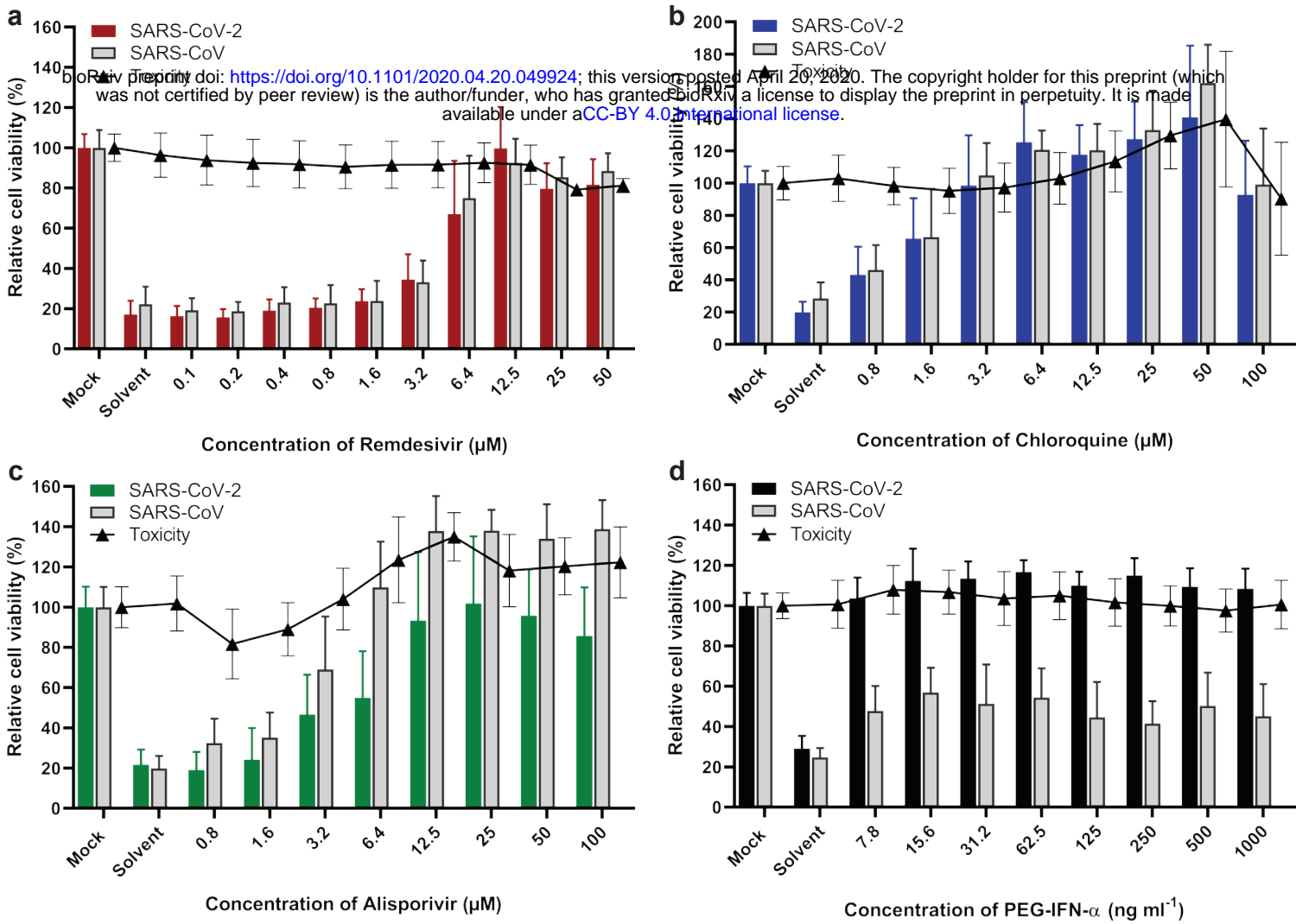


Figure 6