1   **TMPRSS2 and TMPRSS4 mediate SARS-CoV-2 infection of human small intestinal**

2   **enterocytes**

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4   Ruochen Zang[1,2,*], Maria F.G. Castro[1,*], Broc T. McCune[3], Qiru Zeng[1], Paul W. Rothlauf[1],

5   Naomi M. Sonnek[4], Zhuoming Liu[1], Kevin F. Brulois[5,6], Xin Wang[2], Harry B. Greenberg[6,7],

6   Michael S. Diamond[1,3,8], Matthew A. Ciorba[4], Sean P.J. Whelan[1], Siyuan Ding[1]

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8   [1]Department of Molecular Microbiology, Washington University School of Medicine, St.

9   Louis, MO, USA. [2]Key Laboratory of Marine Drugs, Ministry of Education, Ocean

10   University of China, Qingdao, China. [3]Department of Medicine, Division of Infectious

11   Diseases, Washington University School of Medicine, St. Louis, MO, USA. [4]Department

12   of Medicine, Division of Gastroenterology, Washington School of Medicine, St. Louis, MO,

13   USA. [5]Department of Pathology, Stanford School of Medicine, Stanford, CA, USA. [6]VA

14   Palo Alto Health Care System, Department of Veterans Affairs, Palo Alto, CA, USA.

15   [7]Department of Medicine, Division of Gastroenterology and Hepatology, and Department

16   of Microbiology and Immunology, Stanford School of Medicine, Stanford, CA, USA.

17   [8]Department of Pathology and Immunology, Washington University School of Medicine,

18   St. Louis, MO, USA.

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20   Equal contribution: Ruochen Zang and Maria F. G. Castro

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22   Correspondence: Siyuan Ding, siyuan.ding@wustl.edu

23

24   **Abstract**

25

26   Both gastrointestinal symptoms and fecal shedding of SARS-CoV-2 RNA have been

27   frequently observed in COVID-19 patients. However, whether SARS-CoV-2 replicate in

28   the human intestine and its clinical relevance to potential fecal-oral transmission remain

29   unclear. Here, we demonstrate productive infection of SARS-CoV-2 in ACE2+ mature

30   enterocytes in human small intestinal enteroids. In addition to TMPRSS2, another

31   mucosa-specific serine protease, TMPRSS4, also enhanced SARS-CoV-2 spike

32   fusogenic activity and mediated viral entry into host cells. However, newly synthesized

33   viruses released into the intestinal lumen were rapidly inactivated by human colonic fluids

34   and no infectious virus was recovered from the stool specimens of COVID-19 patients.

35   Our results highlight the intestine as a potential site of SARS-CoV-2 replication, which

36   may contribute to local and systemic illness and overall disease progression.

37

38   **Introduction**

39

40   Coronavirus disease 2019 (COVID-19) has emerged as a new world pandemic, claiming

41   the deaths of over 160,000 people. This outbreak is caused by a novel severe acute

42   respiratory syndrome coronavirus, SARS-CoV-2 (*1, 2*), which belongs to the family of

43   *Coronaviridae*, a group of enveloped, non-segmented, positive-sense RNA viruses.

44   Currently, there are no clinically approved countermeasures available for COVID-19. The

45   SARS-CoV-2 associated disease seems to take an extra toll on the elderly, although the

46   host immunological responses to the pathogen remain largely unknown. SARS-CoV-2

47 also has a more pronounced disease time course compared to some other recent

48 respiratory viruses, raising questions about other potential modes of transmission.

49

50 The attachment of SARS-CoV-2 to the target cell is initiated by interactions between the

51 spike glycoprotein (S) and its cognate receptor, angiotensin I converting enzyme 2 (ACE2)

52 (*2-5*). Following receptor engagement, SARS-CoV-2 S is processed by a type II

53 transmembrane serine protease TMPRSS2 to gain access to the host cell cytosol (*3, 6*).

54 Interestingly, both ACE2 and TMPRSS2 are highly expressed in the gastrointestinal (GI)

55 tract, in particular by intestinal epithelial cells (IECs), the predominant target cells for

56 many human enteric viruses. In fact, multiple animal CoVs are natural enteric pathogens,

57 causing GI diseases and spreading by the fecal-oral route (*7*). Notably, GI symptoms

58 including abdominal pain and diarrhea were observed in 20-50% of COVID-19 patients

59 and sometimes preceded the development of respiratory diseases (*8-10*). Substantial

60 amounts of SARS-CoV-2 RNA have been consistently detected in stool specimens from

61 COVID-19 patients (*11-15*). In a recent study, no infectious virus was successfully

62 isolated from the feces of COVID-19 patients (*16*). However, the fecal shedding data

63 raises the possibility that SARS-CoV-2 may act like an enteric virus and could potentially

64 be transmitted via the fecal-oral route.

65

66 In the present study, we aimed to address: 1) do SARS-CoV-2 actively infect human IECs?

67 2) if so, what host factors mediate efficient replication? 3) are there replication competent

68 viruses shed in the fecal samples of COVID-19 patients? Here, we report, for the first time,

69 that SARS-CoV-2 apically infects human mature enterocytes and triggers epithelial cell

70   fusion. TMPRSS2 and TMPRSS4 serine proteases mediate this process by inducing S

71   cleavage and enhancing fusogenic activity of the virus. We also found that human colonic

72   fluids rapidly inactivate SARS-CoV-*2 in vitro* and likely in the intestinal lumen, rendering

73   viral RNA non-infectious in the stool specimens. Our findings have both mechanistic and

74   translational relevance and add to our understanding of COVID-19 pathogenesis.

75

76   **Results**

77

78   **SARS-CoV-2 infects human intestinal enteroids**

79

80   In the intestine, ACE2 functions as a chaperone for the neutral amino acid transporter

81   B^0AT1 (encoded by *SLC6A19*) on IECs and regulates microbial homeostasis ([17, 18]). In

82   fact, ACE2 expression is substantially higher in the small intestine than all the other

83   organs including the lung in both humans and mice (Fig. S1A-B). Therefore, we sought

84   to identify whether SARS-CoV-2 is capable of infecting IECs and understand the

85   implications of IEC replication for fecal-oral transmission. We performed single-cell RNA-

86   sequencing (RNA-seq) to capture the global transcriptomics in all IEC subsets in the

87   mouse small intestinal epithelia (Fig. 1A, left panel). We found that ACE2 was

88   predominantly expressed in $Cd26^+Epcam^+Cd44^-Cd45^-$ mature enterocytes ([19, 20]) (Fig.

89   1A, right panel). In addition, bulk RNA-seq results revealed that primary human ileum

90   enteroids had significantly higher mRNA levels of all known CoV receptors including

91   ACE2 than the colonic epithelial cell line HT-29 and other non-IEC human cell lines (Fig.

92   S1C). Quantitative PCR confirmed abundant ACE2 transcript levels in both human

93   duodenum and ileum derived enteroids (Fig. S1D). ACE2 protein was specifically co-

94   localized with actin at the apical plasma membrane of human enteroid monolayers (Fig.

95   1B). Higher ACE levels correlated with more mature enterocytes present in differentiated

96   enteroids (Fig. 1B), consistent with our scRNA-seq findings (Fig. 1A). We further

97   confirmed in the 3D Matrigel embedded enteroids that ACE2 had an almost perfect co-

98   localization with Villin (Fig. S1E), an IEC marker localized most strongly to the brush

99   border of the intestinal epithelium.

100

101   We next took advantage of the recently established vesicular stomatitis virus (VSV)-

102   chimera GFP reporter virus with its glycoprotein (G) genetically replaced with SARS-CoV-

103   2 S protein (In Press). This VSV-SARS-CoV-2-S-GFP virus serves as a powerful tool to

104   study viral entry and cell tropism. The levels of viral RNA increased by ~10,000 fold in

105   human duodenum enteroids over 24 hours post infection (Fig. 1C), indicative of active

106   replication. Differentiated enteroids with higher ACE2 levels (Fig. 1B) supported 39-fold

107   higher SARS-CoV-2 chimera virus replication than stem cell based cultures (Fig. 1C). We

108   could visualize GFP positive infected cells in duodenum enteroids in both traditional 3D

109   (Fig. 1D) and in flipped "inside-out" models where the apical side of the IECs was on the

110   outside of the spherical organoid (*21*) (Fig. S1F). In addition to viral RNA and protein, the

111   amount of infectious viruses increased >1,000 fold within the intestinal epithelium at 24

112   hours post infection (Fig. 1E). Robust viral replication was confirmed in human ileum and

113   colon-derived enteroids (Fig. 1F-G).

114

115   **SARS-CoV-2 actively replicates in ACE2+ human mature enterocytes**

116

117   To further characterize the potential SARS-CoV-2 route of infection, we employed the 2D

118   monolayer system (*22*), with a clear separation of apical and basal compartments. We

119   noted that the human IECs were preferentially (> 1,000 fold) infected by the virus from

120   the apical surface compared to the basolateral side (Fig. 2A). This data is in line with a

121   strong apical ACE2 expression (Fig. 1B and S1E). Importantly, we found that the newly

122   produced virus progenies were also predominantly released from the apical side into the

123   lumen (Fig. 2B), suggesting a possibility of fecal viral shedding in COVID-19 patients.

124

125   To definitive pinpoint the IEC subset(s) targeted by SARS-CoV-2, we infected duodenum

126   enteroids with the SARS-CoV-2 chimera virus and co-stained with different IEC markers.

127   GFP signals were exclusively found in CD26 positive mature villous absorptive

128   enterocytes and not in goblet (MUC2), enteroendocrine (CHGA), or Paneth (LYZ) cells

129   (data not shown). Using the live infectious SARS-CoV-2 virus, we validated that viral RNA

130   increased by 320-fold within the first 8 hours of infection (Fig. 2C). In addition, SARS-

131   CoV-2 infection of enteroid monolayers suggested that ACE2 positive mature enterocytes

132   were indeed the predominant target cells in the gut epithelium (Fig. 2D). Interestingly, a

133   3D reconstruction of confocal images showed that the S protein was highly concentrated

134   towards the apical surface (Fig. 2E), suggesting a potential mechanism of polarized viral

135   assembly and subsequent apical release.

136

137   While SARS-CoV induced minimal syncytia formation in the absence of exogenous

138   proteases such as trypsin (*23, 24*), SARS-CoV-2 was able to fuse cells during natural

139   infection of cultured cells (Fig. S2A). We also observed multiple events of syncytia

140   formation between IECs in both 2D monolayer (Fig. S2B) and in 3D Matrigel (Fig. S2C

141   and Video S1). The cell fusion and subsequent cytopathic effect may have important

142   implications regarding the common GI symptoms seen in COVID-19 patients (*25-29*).

143

144   **TMPRSS2, TMPRSS4 but not ST14 mediate SARS-CoV-2 entry**

145

146   Several pieces of cell line-based evidence demonstrated that the membrane-bound

147   TMPRSS2 plays a critical role in processing S cleavage and mediating SARS-CoV-2

148   entry (*3, 6, 30*). Interestingly, only two other serine proteases in the same family,

149   TMPRSS4 and matriptase (encoded by *ST14*) shared a highly specific expression pattern

150   in human IECs (Fig. 3A). Previous studies indicated that both TMPRSS4 and ST14

151   facilitate influenza A virus but neither play a role in SARS-CoV infection (*31-34*). Notably,

152   in our mouse scRNA-seq dataset, both TMPRSS4 and ST14 were found to be present in

153   mature enterocytes and had an increased co-expression pattern with ACE2 than

154   TMPRSS2 (Fig. S3A).

155

156   To mechanistically dissect the entry pathway of SARS-CoV-2 in IECs, we set up an

157   HEK293 ectopic expression system to evaluate the SARS-CoV-2 chimera virus infectivity.

158   As reported (*2, 5*), ACE2 conferred permissiveness to SARS-CoV-2 infection (Fig. 3B and

159   S3B). While TMPRSS2 alone did not mediate viral infection, co-expression of TMPRSS2

160   significantly enhanced ACE2-mediated infectivity (Fig. 3B). Importantly, we found that

161   expression of TMPRSS4 but not ST14 also resulted in a significant increase in the levels

7

162   of viral RNA and infectious virus titers in the presence of ACE2 (Fig. 3B and S3C).

163   TMPRSS4 and TMPRSS2 had an additive effect and mediated the maximal infectivity in

164   cell culture (Fig. 3B and S3C-E).

165

166   We reasoned that TMPRSS4 may function as a cell surface serine protease that

167   enhances S cleavage to promote viral entry. To test this hypothesis, we co-expressed a

168   C-terminally Strep-tagged full-length SARS-CoV-2 S protein in an HEK293 cell line that

169   stably expresses ACE2 with or without additional introduction of TMPRSS2 or TMPRSS4.

170   In mock cells, we readily observed the full-length S and a cleaved product that

171   corresponded to the size of S1 fragment, presumably cleaved by furin protease that is

172   ubiquitously expressed (*35*). Importantly, the expression of TMPRSS2 or TMPRSS4

173   enhanced S cleavage, as evidenced by the reduction of full-length S and increase of S2

174   levels (Fig. 3C).

175

176   Based on these results, we hypothesize that TMPRSS serine proteases assist virus

177   infection by inducing S cleavage and exposing the fusion peptide for efficient viral entry.

178   Indeed, TMPRSS4 expression did not affect virus binding (data not shown) whereas the

179   endocytosis was significantly enhanced (Fig. 3D). To examine S protein's fusogenic

180   activity, we examined SARS-CoV-2 S mediated cell-cell fusion in the presence of

181   absence of TMPRSS2 or TMPRSS4. Previous work with SARS-CoV and MERS-CoV

182   suggested S mediated membrane fusion takes place in a cell-type dependent manner

183   (*36*). We found that the ectopic expression of S alone was sufficient to induce syncytia

184   formation, independent of the virus infection (Fig. 3E). This process was dependent on a

8

185 concerted effort from ACE2 and TMPRSS serine proteases. TMPRSS4 expression

186 triggered S-mediated cell-cell fusion, although to a lesser extent than TMPRSS2 (Fig. 3E).

187 Collectively, we have shown that TMPRSS2 and TMPRSS4 activate SARS-CoV-2 S and

188 enhance membrane fusion and viral endocytosis into host cells.

189

190 **TMPRSS2 and TMPRSS4 promote SARS-CoV-2 infection in enteroids**

191

192 To further probe the TMPRSS mechanism of action and physiological relevance and to

193 recapitulate the IEC gene expression pattern *in vivo* (Fig. S3A), we designed an *in vitro*

194 co-culture system where ACE2 and S were expressed on target and donor cells

195 respectively. Based on the scRNA-seq data (Fig. S3A), we constructed an HEK293 stable

196 cell line that co-expresses ACE2 and TMPRSS4 to mimic mature enterocytes and another

197 HEK293 cell line that stably expresses TMPRSS2 to mimic goblet or other secretory IEC

198 types (Fig. 4A, left panel). Target cells were transfected with GFP and mixed at 1:1 ratio

199 with SARS-CoV-2 S containing donor cells that were transfected with TdTomato. We

200 found that TMPRSS4 was able to function *in cis*, i.e. on the same cells as ACE2, and

201 induced fusion of GFP positive cells (Fig. 4A, right panel), as was shown in Fig. 3E.

202 Importantly, we discovered that TMPRSS2 could act *in trans* and its expression on

203 adjacent cells markedly promoted the formation of stronger and larger cell-cell fusion (Fig.

204 4A, right panel).

205

206 Next, to determine the function of TMPRSS serine proteases in facilitating SARS-CoV-2

207 infection of primary human IECs, we used a CRISPR/Cas9-based method to genetically

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208   delete TMPRSS2 or TMPRSS4 in human duodenum enteroids. Efficient knockout was

209   confirmed by western blot (Fig. S4A). Importantly, abrogating TMPRSS4 expression led

210   to a 4-fold reduction in SARS-CoV-2 chimera virus replication in human enteroid, even

211   more significant than TMPRSS2 knockout (Fig. 4B), highlighting its importance in

212   mediating virus replication in primary cells.

213

214   In parallel to genetic depletion, we also tested the effect of pharmacological inhibition of

215   TMPRSS serine proteases on virus replication. We pre-treated enteroids with camostat

216   mesylate, a selective inhibitor of TMPRSS over other serine proteases including trypsin,

217   prostasin and matriptase (*37*). While camostat treatment significantly inhibited SARS-

218   CoV-2 chimera virus infection, soybean trypsin inhibitor (SBTI) and E-64d, a cysteine

219   protease inhibitor that blocks cathepsin activity and has excellent inhibitory activity

220   against SARS-CoV-2 *in vitro (3)*, did not had a major impact on virus replication in

221   enteroids (Fig. 4C).

222

223   Taken together, our data support a model where SARS-CoV-2, at least in human IECs,

224   seems to be more reliant on the membrane-bound TMPRSS serine proteases (Fig. 4A,

225   left panel), as compared to SARS-CoV and other CoVs that can efficiently utilize both

226   TMPRSS2 and endosome-localized cathepsins as alternative routes of entry (*38, 39*).

227

228   **SARS-CoV-2 is rapidly inactivated in the human GI tract**

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230   Our current results strongly suggest that SARS-CoV-2 viruses are able to infect human

231   IECs apically and are released via the apical route into the lumen (Fig. 2). Human enteric

232   viruses that spread via the fecal-oral route typically withstand the harsh environment in

233   the GI tract, including the low pH of gastric fluids, bile and digestive enzymes in the small

234   intestine, dehydration and exposure to multiple bacterial by-products in the colon. We

235   sought to investigate the stability of SARS-CoV and SARS-CoV-2 chimera viruses in

236   different human gastric and intestinal fluids. Compared to rotavirus, a prototypic human

237   enteric virus known to successfully transmit fecal-orally (40), both CoV chimera viruses

238   quickly lost infectivity in a low pH condition (Fig. S5A). However, SARS-CoV-2-S exhibited

239   greater stability than SARS-CoV-S in human small intestinal fluids that contain biological

240   surfactants including taurocholic acid sodium salt and lecithin (Fig. 5A). Interestingly,

241   SARS-CoV-2-S chimera virus was not resistant to certain components in the human

242   colonic fluids (Fig. 5A). The virus titers decreased by 100-fold within 1 hour and no

243   infectious virus was detectable at the 24 hour time point (Fig. 5A). In contrast, rotavirus

244   remained stable in all gastric and enteric fluids tested (Fig. S5A-B).

245

246   Combined with the human enteroid results (Fig. 1-2), we hypothesize that the SARS-CoV-

247   2 has the potential to and likely does replicate in human IECs but then get quickly

248   inactivated the GI tract. We collected stool specimens from a small group of COVID-19

249   patients. From 3 out of 5 fecal samples, we detected high RNA copy numbers of SARS-

250   CoV-2 viral genome (Fig. 5B). However, we were unable to recover any infectious virus

251   using a highly sensitive cell-based assay (6).

252

11

Discussion

In this study we set out to address an important basic and clinically relevant question: Is the persistent viral RNA seen in COVID-19 patients' stool infectious and transmissible? We showed that despite SARS-CoV-2's ability to establish robust infection and replication in human IECs (Fig. 1-2), the virus is rapidly inactivated by colonic fluids and we were unable to detect infectious virus in fecal samples (Fig. 5). Thus, the large quantities of viral RNA that transit through the GI tract and shed into the feces are likely not infectious in nature. Due to the limitation of sample size, we cannot definitely conclude that fecal-oral transmission of COVID-19 does not exist. However, our data is consistent with the previous reports from SARS-CoV and MERS-CoV publications which concluded that despite long duration of viral RNA shedding, no infectious virus could be recovered from the patients' feces (*41, 42*).

Intriguingly, the small intestinal fluids, which contain taurocholic acid sodium salt and lecithin, important surfactant components of the bile, did not inactivate virus as expected (Fig. 5A). In addition, we also observed that the virus titers did not drop in the presence of 0.03% bovine bile (data not shown), the highest concentration that did not induce cytotoxicity, contradicting the known fragility of lipid bilayers and sensitivity to bile salts which serve as detergents in gut. Recent studies showed a heavy glycosylation of SARS-CoV-2 S protein (*43*) and it is possible the sugar coating conferred some stability against the enzymatic digestion and bile salt solubilization. Two recent publications did demonstrate that SARS-CoV-2 has unique stability in the environment, at least in a

12

276   laboratory setting (*44, 45*). Further work is needed to determine which bioactive(s) in the

277   colonic fluids that are absent from small intestinal fluids function to potentially disrupt viral

278   lipid membranes *in vivo*.

279

280   Mechanistically, recent work from multiple groups showed a key role of TMPRSS2 in

281   cleaving SARS-CoV-2 S protein. Here, we provide data that besides TMPRSS2,

282   TMPRSS4 also increases SARS-CoV-2 infectivity, at least in gut epithelial cells (Fig. 3-

283   4). We observed an intriguing additive effect of the two enzymes, as both target single

284   arginine or lysine residues (R/K↓). One may speculate that this synergy stems from a

285   distinct subcellular localization in neighboring cells (Fig. 4A). Future work with

286   recombinant proteins and enzymatic digestion may help address whether S cleavage is

287   sequential and whether the two enzymes act on different sites on the S protein.

288

289   Finally, we observed strong syncytia formation in human enteroids (Fig. S2B-C). These

290   results may have great implications for virus cell-cell spread and evasion from antibody

291   neutralization (*46*). It is unclear at this point whether the fused IECs will still undergo

292   anoikis or an alternative accelerated cell death, which may cause a breach in barrier

293   integrity and give rise to GI pathology seen in COVID-19 patients. It is possible that in the

294   small intestine, whereas SARS-CoV-2 is relatively stable, additional proteases such as

295   trypsin likely enhance viral pathogenesis by triggering more robust IEC fusion (Fig. S2A).

296   In this sense, although viruses are not released into the basolateral compartment (Fig.

297   2B), one can imagine that a leaky gut will allow the virus to disseminate to other systemic

298   organs including the lung and liver. This hypothesis is consistent with the clinical

299   observation that in some COVID-19 patients, the GI symptoms preceded the respiratory

300   illness (*8, 9*). Whether the gut serves as a primary site of infection and whether it

301   contributes to systemic diseases in individual patients require further studies using

302   appropriate animal models.

303

304

305   **Materials and Methods**

306

307   **Plasmids, Cells, Reagents, and Viruses**

308   Plasmids: Human ACE2, DPP4, ANPEP, TMPRSS2, TMPRSS4, and ST14 were cloned

309   into a pcDNA3.1/nV5-DEST vector with an N-terminal V5 tag and a neomycin selection

310   marker. Human TMPRSS2 and TMPRSS4 were also cloned into a pLX304 lentiviral

311   vector with a C-terminal V5 tag and a blasticidin selection marker. pEGFP-N1 and pCMV-

312   TdTomato were commercially purchased from Clontech. Codon-optimized SARS-CoV-2

313   S was a kind gift from Dr. Nevan J. Krogan at UCSF (*47*).

314

315   Cells: African Green Monkey kidney epithelial cell lines MA104 (CRL-2378.1) and human

316   embryonic kidney cell line HEK293 (CRL-1573) were originally obtained from American

317   Type Culture Collection (ATCC) and cultured in complete M199 medium and complete

318   DMEM medium, respectively. HEK293 cells stably expressing human ACE2 or TMPRSS2

319   were selected under 500 µg/ml G418. HEK293 cells stably expressing human ACE2 and

320   TMPRSS2 were selected under 500 µg/ml G418 and 5 µg/ml blasticidin.

321

322 Reagents: Human gastric fluids (Fasted State Simulated Gastric Fluid/FaSSGF, pH 1.6),

323 small intestinal fluids (Fasted State Simulated Intestinal Fluid/FaSSIF-V2, pH 6.5), colonic

324 fluids (Fasted State Simulated Colonic Fluid/FaSSCoF, pH 7.8) were purchased from

325 BioRelevant, UK and reconstituted based on the manufacturer's instructions. Soybean

326 trypsin inhibitor (SBTI), camostat mesylate, and E-64d were purchased from Selleckchem.

327 Primary antibodies used in this study included: GAPDH (631402, Biolegend); GFP

328 (2555S, Cell Signaling); SARS-CoV-2-S (S1) (PA5-81795, Thermo Fisher); TMPRSS2

329 (sc-515727, Santa Cruz); TMPRSS4 (sc-376415, Santa Cruz); and V5 (13202S, Cell

330 Signaling).

331

332 Viruses: Human rotavirus WI61 strain was propagated as described before (*48*). VSV-

333 SARS-CoV-2-S-GFP and VSV-SARS-CoV-S-GFP were propagated in MA104 cells in

334 T175 flasks and virus sequences can be found in Table S1. Plaque assays were

335 performed in MA104 cells seeded in 6-well plates using an adapted version of the

336 rotavirus plaque assay protocol (*19*). TCID50 assays were performed in MA104 cells

337 seeded in flat-bottom 96-well plates using serial dilutions. Virus infections were performed

338 with the initial inoculum removed at 1 hour post adsorption.

339

340 **Human intestinal enteroids**

341 One duodenum (#CD94), five ileum (#14-75, #211D, #262D-2, #265D, #251D-2), and

342 one colon (#235A) enteroids were used in this study. All enteroids were derived from de-

343 identified tissue from healthy, non-IBD subjects undergoing colonoscopy and provided

344 informed consent at Stanford University and Washington University in St. Louis. In brief,

15

345 enteroids were cultured in maintenance media (advanced DMEM/F12 media

346 supplemented with L-WRN-conditioned media that contains Wnt-3a, R-spondin 3, and

347 Noggin) in 3D Matrigel in 24-well plates (*49*). For passing enteroids, Y27632 and

348 CHIR99021 were added. For differentiation, conditioned media was replaced with 50%

349 Noggin in the form of recombinant proteins. Enteroids, when required, were digested into

350 single cells using TrypLE and seeded into collagen-coated transwells (0.33cm^2, 0.4μm

351 pore size, Polycarbonate, Fisher #07-200-147) in ROCK Inhibitor Y-27632 supplemented

352 maintenance media in 24-well plates. TEER measurement was performed using the

353 Millicell ERS-2 Voltohmmeter (Millipore). Enteroids in monolayers were used for virus

354 infection if the cultures had TEER greater than 1000 Ω.cm2 at 5 days post seeding. For

355 CRISPR/Cas9 knockout, enteroids in 3D Matrigel were digested with 5 mM EDTA and

356 transduced with lentiviral vectors encoding Cas9 and single-guide RNA against

357 TMPRSS2 or TMPRSS4 (see Table S1) in the presence of polybrene (8 μg/ml). At 48

358 hours post transduction, puromycin (2 μg/ml) was added to the maintenance media.

359 Puromycin was adjusted to 1 μg/ml upon the death of untransduced control enteroids.

360

361 **RNA extraction and quantitative PCR**

362 Total RNA was extracted from cells using RNeasy Mini kit (Qiagen) and reverse

363 transcription was performed with High Capacity RT kit and random hexamers as

364 previously described (*50*). QPCR was performed using the AriaMX (Agilent) with a 25 μl

365 reaction, composed of 50 ng of cDNA, 12.5 μl of Power SYBR Green master mix (Applied

366 Biosystems), and 200 nM both forward and reverse primers. All SYBR Green primers and

367 Taqman probes used in this study are listed in Table S1.

368

Bright-field and immunofluorescence microscopy

370 For brightfield and epifluorescence, cultured cells or human enteroids were cultured in

371 24-well plates and images were taken by REVOLVE4 microscope (ECHO) with a 10X

372 objective. For confocal microscopy, enteroids were seeded into 8-well Nunc chamber

373 slides (Thermo Fisher), cultured under maintenance or differentiation conditioned,

374 infected with SARS-CoV-2 chimera virus, and fixed with 4% paraformaldehyde as

375 previously described (48). Samples were then stained with the following primary

376 antibodies or fluorescent dyes: ACE2 (sc-390851 AF594, Santa Cruz), DAPI (P36962,

377 Thermo Fisher), SARS-CoV-2 S (CR3022 human monoclonal antibody (51)), Villin (sc-

378 58897 AF488, Santa Cruz), and phalloidin (Alexa 647-conjugated, Thermo Fisher).

379 Stained cells were washed with PBS, whole mounted with Antifade Mountant, and imaged

380 with Zeiss LSM880 Confocal Microscope at the Molecular Microbiology imaging core

381 facility at Washington University in St. Louis. Z-stack was applied for imaging 3D human

382 enteroids. Images were analyzed by Volocity v6.3 (PerkinElmer) and quantification was

383 determined by CellProfiler (Broad Institute).

384

Single-cell RNA-sequencing analysis

386 Small intestinal IEC isolation by EDTA-DTT extraction method and sorting were

387 performed as previously described (19, 52). Cells were stained with LIVE/DEAD Aqua

388 Dead Cell Stain Kit (Thermo Fisher) and live cells were sorted using a BD FACSAria II

389 flow cytometer. Preparation of single cell suspensions was performed at the Stanford

390 Genome Sequencing Service Center (GSSC). Cell suspensions were processed for

17

391 single-cell RNA-sequencing using Chromium Single Cell 3' Library and Gel Bead Kit v2

392 (10X Genomics, PN-120237) according to 10X Genomics guidelines. Libraries were

393 sequenced on an Illumnia NextSeq 500 using 150 cycles high output V2 kit (Read 1-26,

394 Read2-98 and Index 1-8 bases). The Cell Ranger package (v3.0.2) was used to align

395 high quality reads to the mouse genome (mm10). Normalized log expression values were

396 calculated using the scran package (*53*). Highly variable genes were identified using the

397 FindVariableGenes function (Seurat, v2.1) as described (*54*). Batch effects from technical

398 replicates were removed using the MNN algorithm as implemented in the batchelor

399 package's (v1.0.1) fastMNN function. Imputed expression values were calculated using a

400 customized implementation (https://github.com/kbrulois/magicBatch) of the MAGIC

401 (Markov Affinity-based Graph Imputation of Cells) algorithm (*55*) and optimized

402 parameters (t = 2, k = 9, ka = 3). Cells were classified as dividing or resting using a pooled

403 expression value for cell cycle genes (Satija Lab Website: regev_lab_cell_cycle_genes).

404 For UMAP and tSpace embeddings, cell cycle effects were removed by splitting the data

405 into dividing and resting cells and using the fastMNN function to align the dividing cells

406 with their resting counterparts. Dimensionality reduction was performed using the UMAP

407 algorithm and nearest neighbor alignments for trajectory inference and vascular modeling

408 were calculated using the tSpace algorithm. Pooled expression of all viral genes was

409 calculated using the AddModuleScore function from the Seurat package.

410

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417

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425   Center for their help with the single-cell RNA-seq experiments.

426

427   **Figure legends**

428   **Fig. 1. SARS-CoV-2 S chimera virus infects human small intestinal enteroids**

429   (A) Mouse small intestinal cells were analyzed by single-cell RNA-sequencing and

430   resolved into 20 clusters based on gene expression profiles (left panel). Transcript

431   levels of Cd26, Epcam, Cd44, Cd45, and Ace2 were indicated for different

432   intestinal cell subsets. Each dot represents a single cell. Note that Ace2^{high} cells

433   are also positive for Cd26 and Epcam but negative for Cd44 and Cd45.

434   (B) Human duodenum enteroids were cultured in the transwell monolayer system

435   using maintenance (MAINT) or differentiation (DIFF) conditions for 3 days.

19

436   Monolayers were stained for ACE2 (red) and actin (phalloidin, white). Scale bar:

437   32 µm.

438   (C) Human duodenum enteroids in monolayer, cultured in either maintenance (MAINT)

439   or differentiation (DIFF) conditions, were apically infected with $1.5X10^5$ plaque

440   forming units (PFUs) of SARS-CoV-2 chimera virus (MOI=0.3) for 24 hours. The

441   expression of VSV-N was measured by RT-qPCR and normalized to that of

442   GAPDH.

443   (D) Human duodenum enteroids in 3D Matrigel were cultured in maintenance (MAINT)

444   media or differentiation (DIFF) media for 3 days and infected with $2.2X10^5$ PFUs

445   of SARS-CoV-2 chimera virus for 18 hours. Enteroids were stained for virus

446   (green), actin (phalloidin, white), and nucleus (DAPI, blue). Scale bar: 50 µm.

447   (E) Same as (C) except that virus titers were measured using an TCID50 assay

448   instead of viral RNA levels by QPCR.

449   (F) Same as (D) except that human ileum enteroids were used instead. Scale bar: 80

450   µm.

451   (G) Same as (D) except that human ileum enteroids were used instead. Scale bar: 80

452   µm.

453

454   **Fig. 2. SARS-CoV-2 actively replicates in ACE2+ human mature enterocytes**

455   (A) Human duodenum enteroids in monolayer, cultured in either maintenance (MAINT)

456   or differentiation (DIFF) conditions, were apically or basolaterally infected with

457    $1.5X10^5$ PFUs of SARS-CoV-2 chimera virus for 24 hours. The expression of VSV-

458   N was measured by RT-qPCR and normalized to that of GAPDH.

459   (B) Supernatants in both apical and basal chambers were collected from (A) and were

460   subjected to an TCID50 assay to measure the amount of infectious viruses.

461   (C) Differentiated duodenum enteroids in monolayer were apically infected with

462        $2.5X10^5$ PFUs of infectious SARS-CoV-2 virus (MOI=0.5) for 8 hours. The

463   expression of SARS-CoV-2 N was measured by RT-qPCR using a Taqman assay

464   and normalized to that of GAPDH.

465   (D) Same as (C) except that enteroids were fixed and stained for SARS-CoV-2 S

466   (green), ACE2 (red), and nucleus (DAPI, blue). Scale bar: 32 µm. SARS-CoV-2

467   infected ACE2 positive cells are enlarged in the inset (yellow box).

468   (E) SARS-CoV-2 infected duodenum monolayers were imaged along the z-stacks and

469   sectioned for YZ planes (top panel) and reconstructed for 3D images (bottom

470   panel).

471

472 **Fig. 3. TMPRSS2, TMPRSS4 but not ST14 mediate SARS-CoV-2 entry**

473   (A) Bulk RNA-sequencing results of intestine-specific serine protease expression in

474   HEK293, Huh7.5, H1-Hela, HT-29 cells and human ileum enteroids.

475   (B) HEK293 cells were transfected with pcDNA3.1-V5-ACE2, DDP4, or ANPEP for 24

476   hours (left panel), or transfected with indicated plasmid combination for 24 hours

477   (right panel), and infected with $1.5X10^5$ PFUs of SARS-CoV-2 chimera virus for 24

478   hours. The expression of VSV-N was measured by RT-qPCR and normalized to

479   that of GAPDH.

480   (C) HEK293 cells stably expressing human ACE2 were transfected with C-terminally

481   tagged SARS-CoV-2 S and TMPRSS2 or TMPRSS4 or 48 hours. The levels of S

482   and GAPDH were measured by western blot. The intensity of bands were

483   quantified by ImageJ and shown as percentage of the top band in lane 1.

484   (D) HEK293 cells stably expressing human ACE2 were transfected with TMPRSS2 or

485   TMPRSS4 for 24 hours, incubated with 5.8×10^5 PFUs of SARS-CoV-2 chimera

486   virus on ice for 1 hour, washed with cold PBS for 3 times, and shifted to 37 ºC for

487   another hour. The expression of VSV-N was measured by RT-qPCR and

488   normalized to that of GAPDH.

489   (E) Wild-type or human ACE2 expressing HEK293 cells were transfected with SARS-

490   CoV-2 S and GFP, with or without TMPRSS2 or TMPRSS4 or 24 hours. The red

491   arrows highlight the formation of large syncytia.

492

493 **Fig. 4. TMPRSS2 and TMPRSS4 promote SARS-CoV-2 infection in enteroids**

494   (A) Schematic diagram of SARS-CoV-2 infection of human mature enterocytes (left

495   panel). An HEK293 stable cell line expressing ACE2 and TMPRSS4 were

496   transfected with GFP and an HEK293 stable cell line expressing TMPRSS2 were

497   transfected with SARS-CoV-2 S and TdTomato for 24 hours. These two cell lines

498   were then mixed at 1:1 ratio and cultured for another 24 hours. Note the formation

499   of cell-cell fusion (yellow), highlighted by black arrows.

500   (B) Human duodenum enteroids in 3D Matrigel were transduced with lentiviruses

501   encoding Cas9 and sgRNA against TMPRSS2 or TMPRSS4 (oligonucleotide

502   information in Table S1). Gene knockout enteroids were seeded into monolayers

503   and infected with 1.5×10^5 PFUs of SARS-CoV-2 chimera virus for 24 hours. The

504   expression of VSV-N was measured by RT-qPCR and normalized to that of

505   GAPDH.

506   (C) Human duodenum enteroids seeded into collagen-coated 96-well plates were

507   differentiated for 3 days, pre-treated with 50 µg/ml of soybean trypsin inhibitor

508   (SBTI), 10 µM of camostat mesylate, or 10 µM of E-64d for 30 minutes, and

509   infected with $1.5X10^5$ PFUs of SARS-CoV-2 chimera virus for 24 hours. The

510   expression of VSV-N was measured by RT-qPCR and normalized to that of

511   GAPDH.

512

513   **Fig. 5. SARS-CoV-2 rapidly lose infectivity in the human GI tract**

514   (A) $2.9X10^5$ PFUs of SARS-CoV-2 and SARS-CoV chimera viruses were incubated

515   with M199 media, human small intestinal (SI) fluids, or human large intestinal (LI)

516   fluids for indicated time points at 37 ºC. The infectivity of viruses was subsequently

517   determined by a standard TCID50 cell culture based assay.

518   (B) Stool specimens from five COVID-19 patients were collected and subjected to

519   QPCR experiments to quantify the absolute levels of SARS-CoV-2 N gene.

520

521   **Fig. S1. ACE2 is highly expressed in the human intestine**

522   (A) ACE2 expression in different human tissues and organs from human protein atlas

523   (www.proteinatlas.org/).

524   (B) Ace2 expression in different mouse tissues and cell types from BioGPS

525   (biogps.org).

23

526   (C) Bulk RNA-sequencing results of ACE2 and other CoV receptor expression in

527   HEK293, Huh7.5, H1-Hela, HT-29 cells and human ileum enteroids.

528   (D) ACE2 expression level in HEK293, HT-29 cells, duodenum enteroids and ileum

529   enteroids were measured by RT-qPCR and normalized to that of GAPDH.

530   (E) Human duodenum enteroids in 3D Matrigel were cultured in differentiation media

531   for 3 days and stained for ACE2 (red), villin (green), and nucleus (DAPI, blue).

532   Scale bar: 50 μm.

533   (F) Differentiated human duodenum enteroids were allowed to flip inside out, infected

534   with $1.5\text{X}10^5$ PFUs of SARS-CoV-2 chimera virus for 24 hours. Enteroids were

535   stained for virus (green), actin (phalloidin, grey), and nucleus (DAPI, blue). Scale

536   bar: 32 μm.

537

538   **Fig. S2. SARS-CoV-2 chimera virus induces syncytia formation in cell culture and**

539   **human intestinal cells**

540   (A) MA104 cells in serum-free media were infected with SARS-CoV and SARS-CoV-

541   2 chimera viruses (MOI=0.1) with or without trypsin (0.5 μg/ml) for 48 hours.

542   (B) Human ileum enteroids seeded into collagen-coated 96-well plates were apically

543   infected with SARS-CoV-2 chimera virus (MOI=0.1) for 24 hours.

544   (C) Human ileum enteroids in 3D Matrigel were infected with SARS-CoV-2 chimera

545   virus (MOI=0.1) for 24 hours and stained for virus (green), ACE2 (red), and

546   nucleus (DAPI, blue). Also see Supplementary Video 1 for 3D view.

547

548   **Fig. S3. TMPRSS2 and TMPRSS4 enhance SARS-CoV-2 infectivity**

549   (A) Transcript levels of Ace2, Tmprss2, Tmprss4, and St14 were indicated for different

550   intestinal cell subsets. Each dot represents a single cell.

551   (B) HEK293 cells were transfected with pcDNA3.1-V5-ACE2, DDP4, or ANPEP for 24

552   hours (left panel), or transfected with indicated plasmid combination for 24 hours

553   (right panel). The levels of V5 and GAPDH were measured by western blot.

554   (C) HEK293 cells were transfected with indicated plasmid combination for 24 hours

555   (right panel), and infected with 1.5×10^5 PFUs of SARS-CoV-2 chimera virus for 24

556   hours. The amount of infectious viruses was measured using an TCID50 assay.

557   (D) Same as (C) except that virus-infected cells (GFP) were imaged.

558   (E) Same as (C) except that the levels of V5, GFP, and GAPDH were measured by

559   western blot.

560

561 **Fig. S4. Validation of CRISPR/Cas9 mediated knockout of TMPRSS2 and TMPRSS4**

562 **in human enteroids**

563   (A) Human duodenum enteroids were transduced with lentiviral vectors encoding

564   Cas9 and single-guide RNA targeting TMPRSS2 or TMPRSS4, selected under

565   puromycin (2 µg/ml) for 7 days, allowed for expansion, and harvested for western

566   blot examining the protein levels of TMPRSS2, TMPRSS4 and GAPDH.

567

568 **Fig. S5. Rotavirus remains infectious in human gastric and intestinal fluids**

569   (A) 2.9×10^5 PFUs of SARS-CoV-2, SARS-CoV chimera viruses and rotavirus were

570   incubated with M199 media or human gastric fluids for indicated time points. The

571   amount of infectious viruses was determined by a TCID50 assay.

572   (B) Same as (A) except that rotavirus and human small intestinal (SI) and large

573   intestinal (LI) fluids were used instead.

574

575   Video S1. Formation of syncytia in 3D human duodenum enteroids infected by SARS-

576   CoV-2 GFP chimera virus

577

578   Table S1. QPCR and CRISPR deletion oligonucleotide information

579

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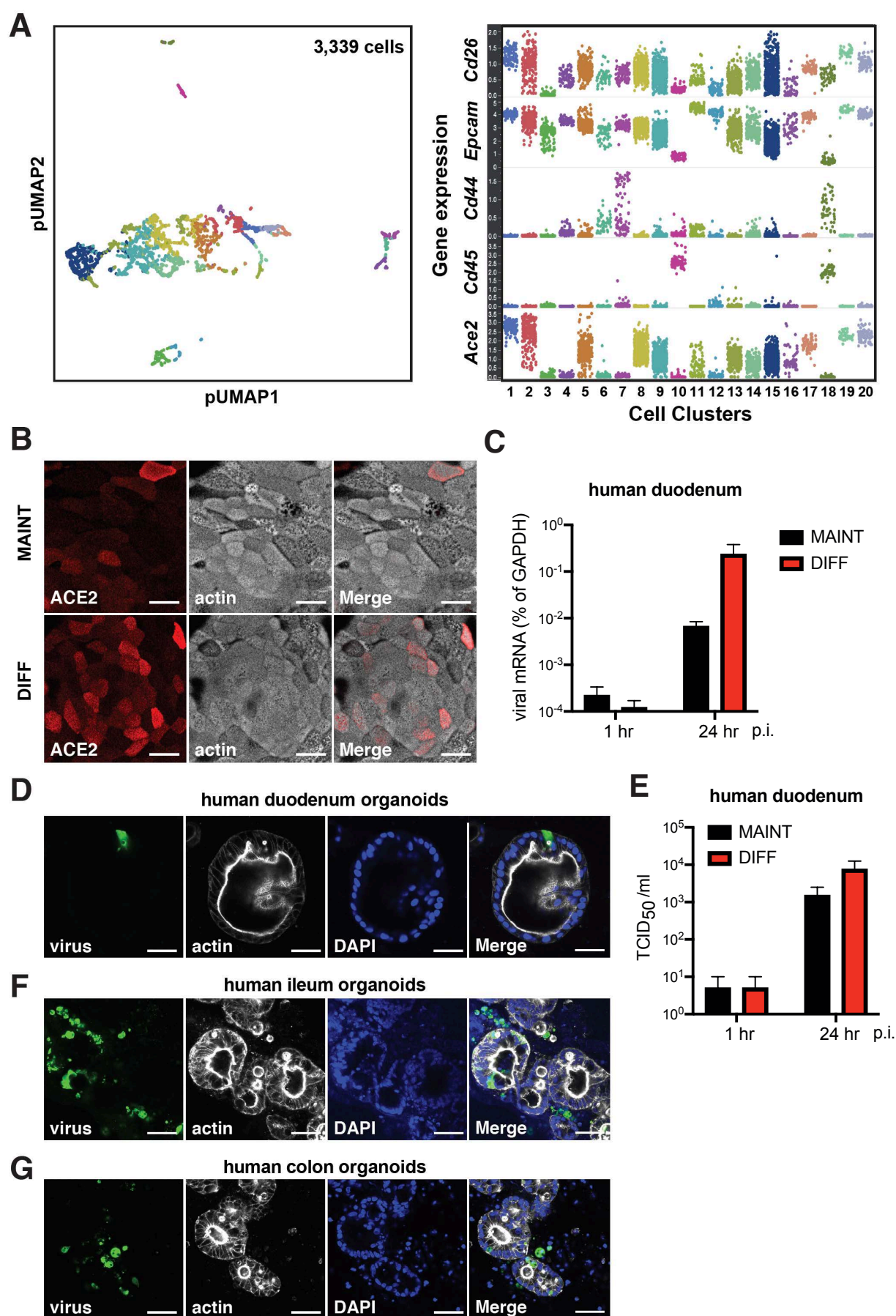724   Data Diffusion. *Cell* **174**, 716-729 e727 (2018).
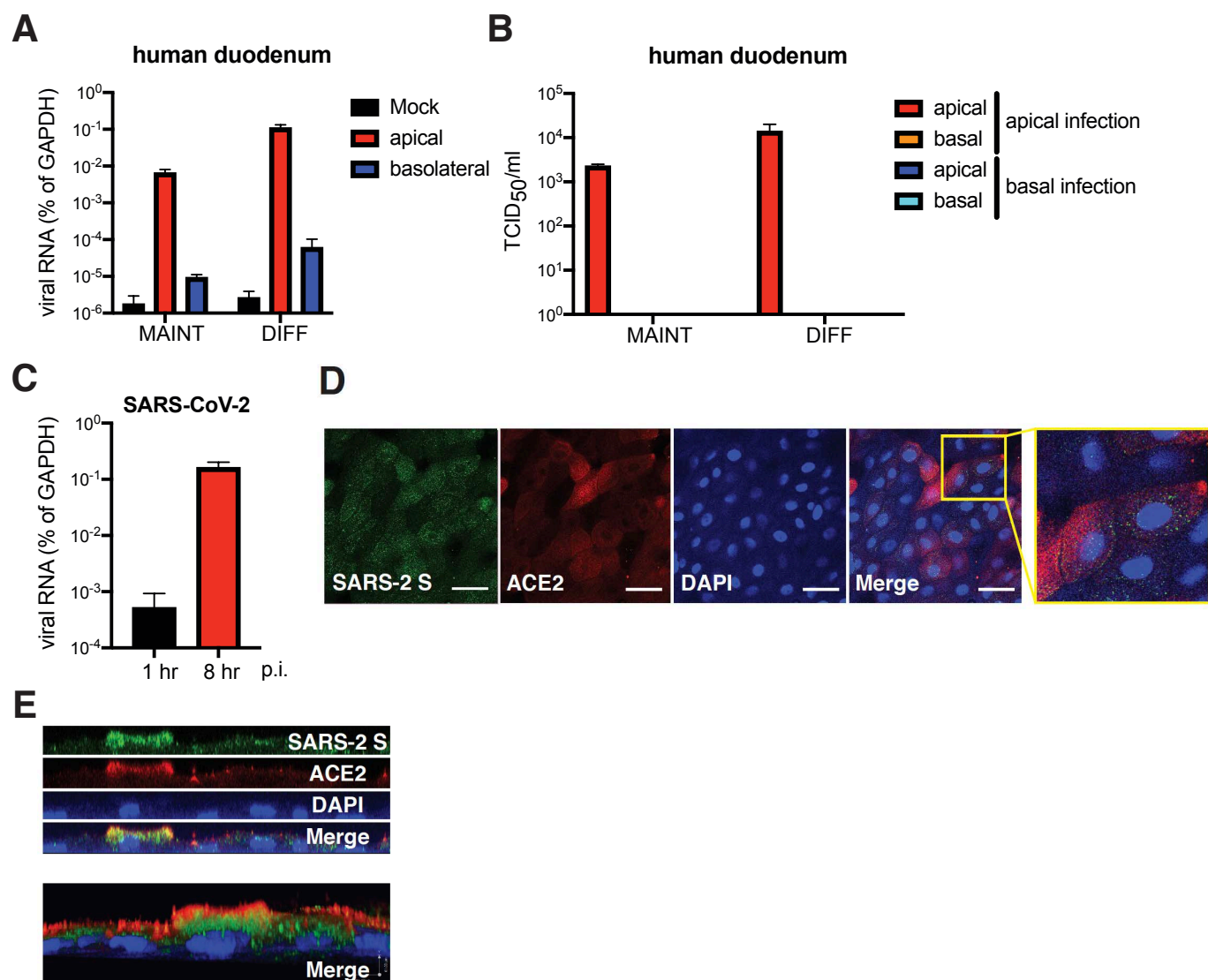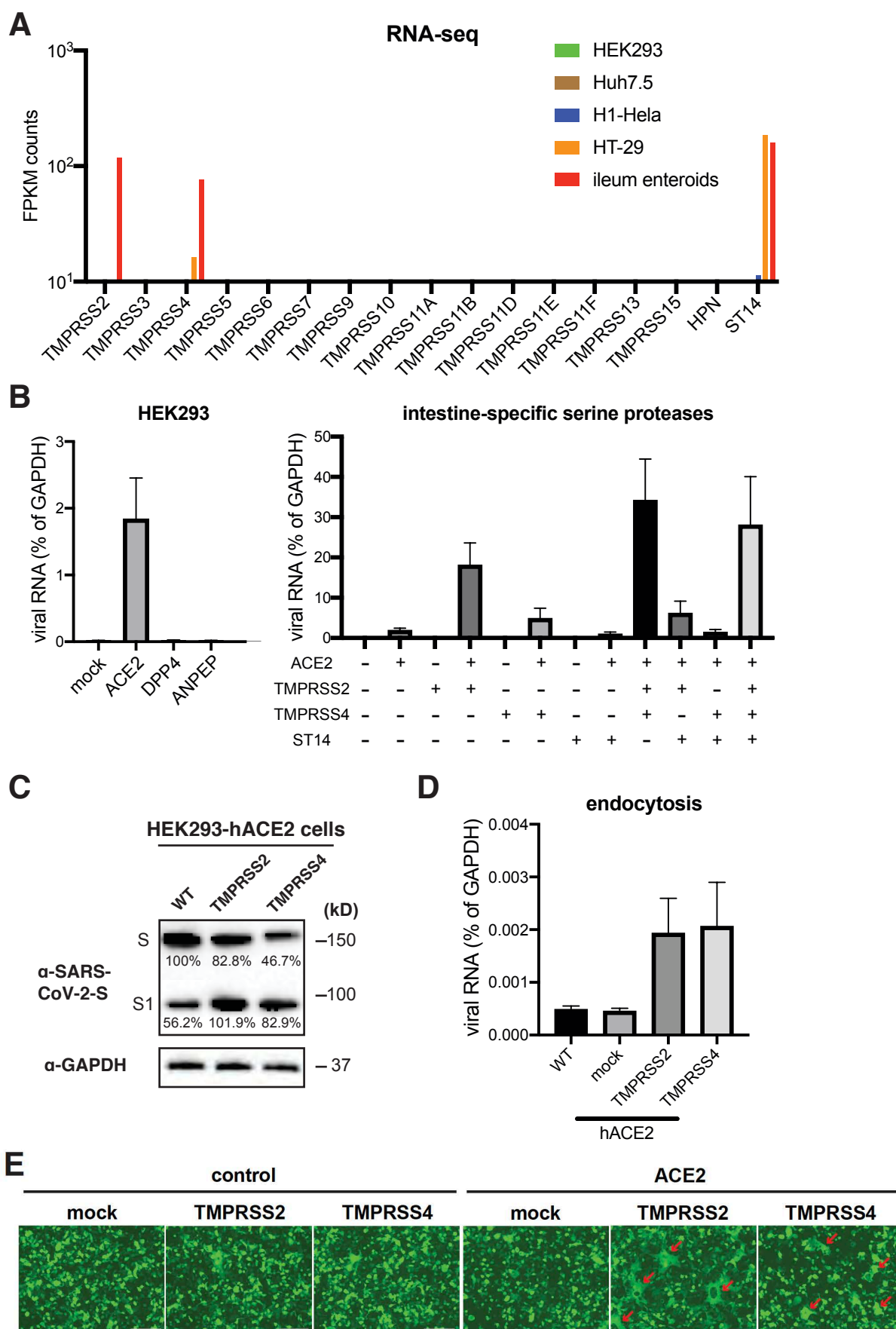
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Figure 1

Figure 2

A

human duodenum

B

human duodenum
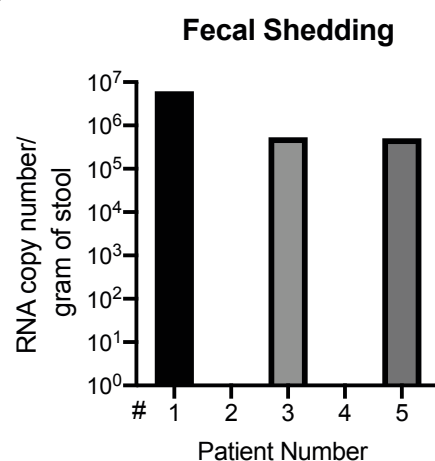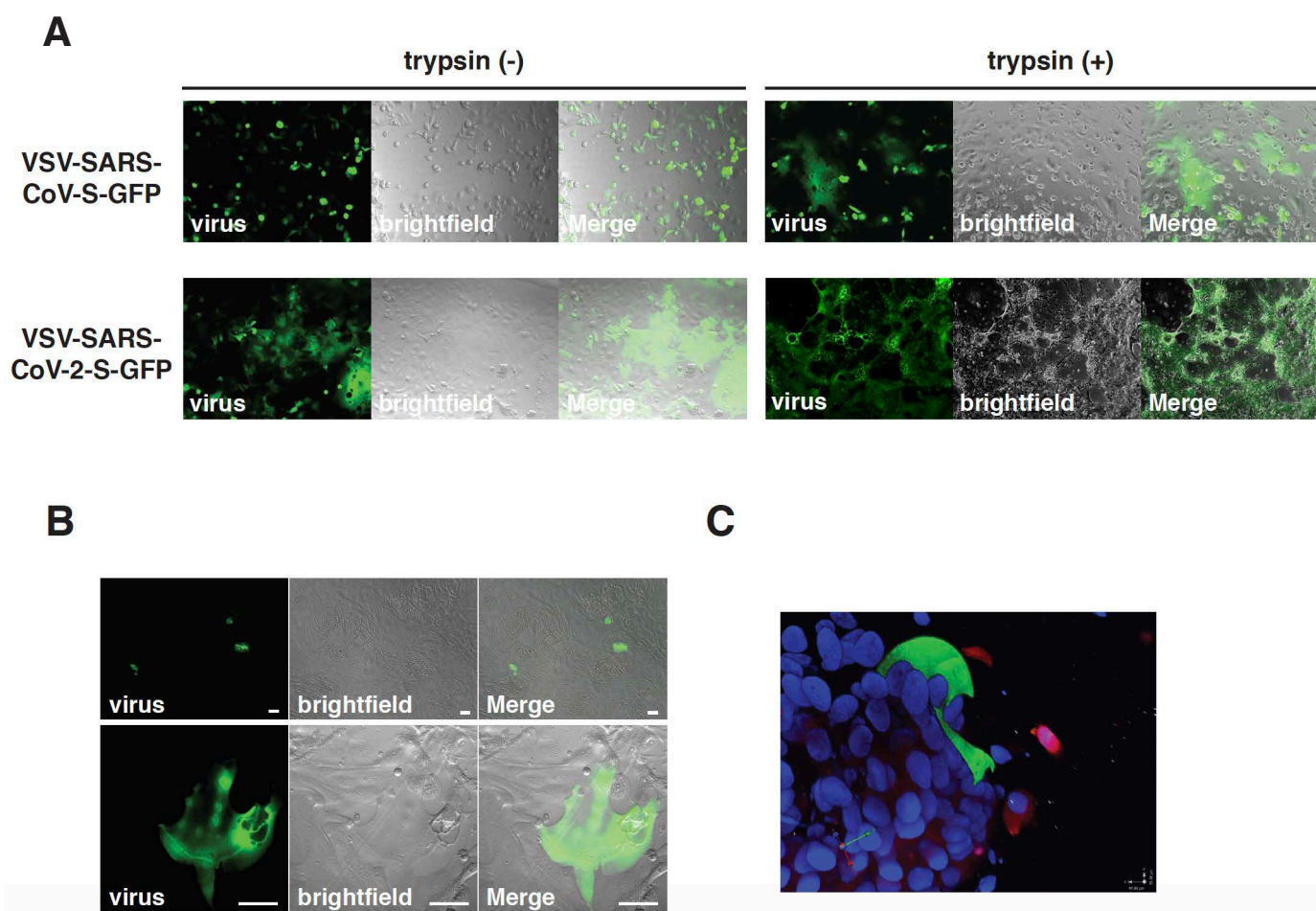
C

SARS-CoV-2

D

E
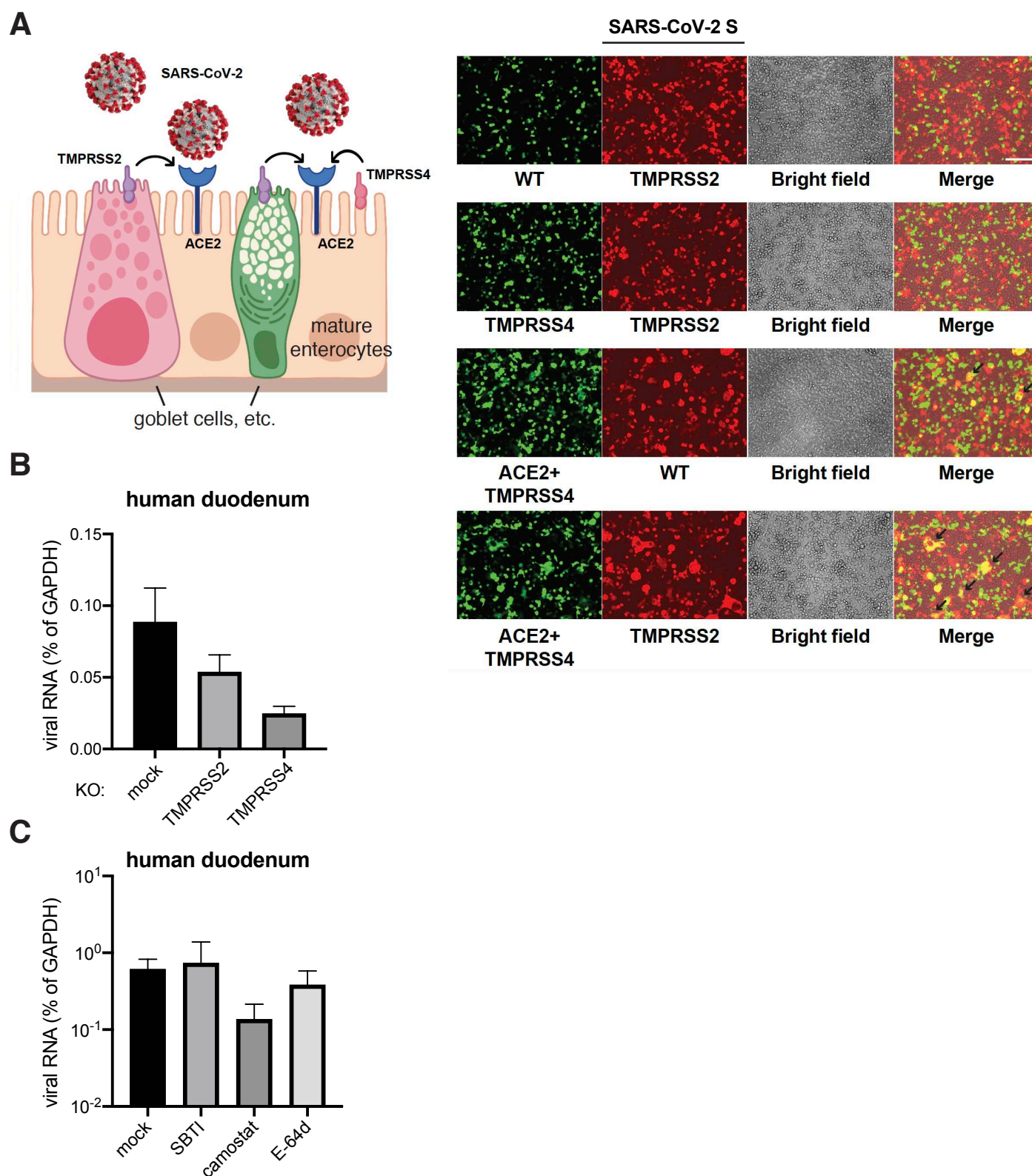
Figure 3

Figure 4

Figure 5

A
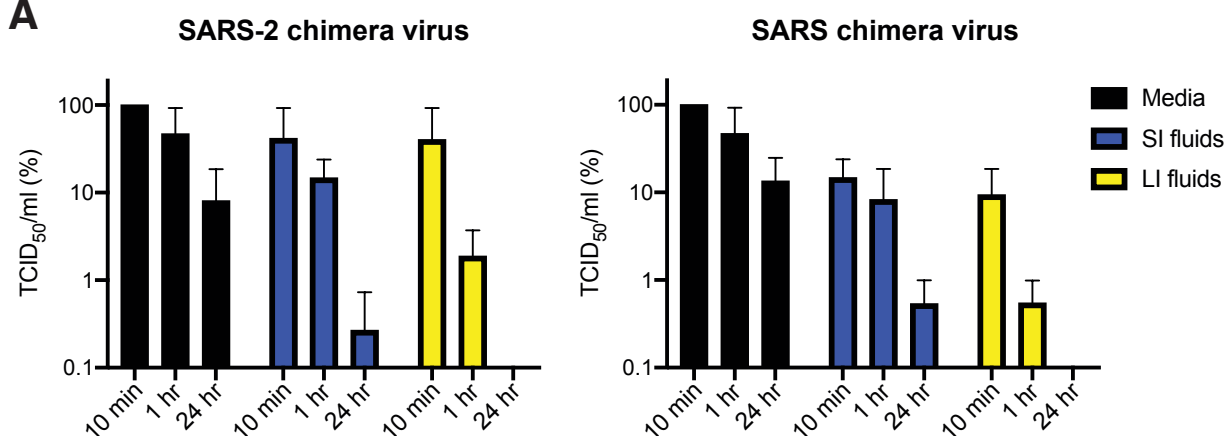
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Figure S1
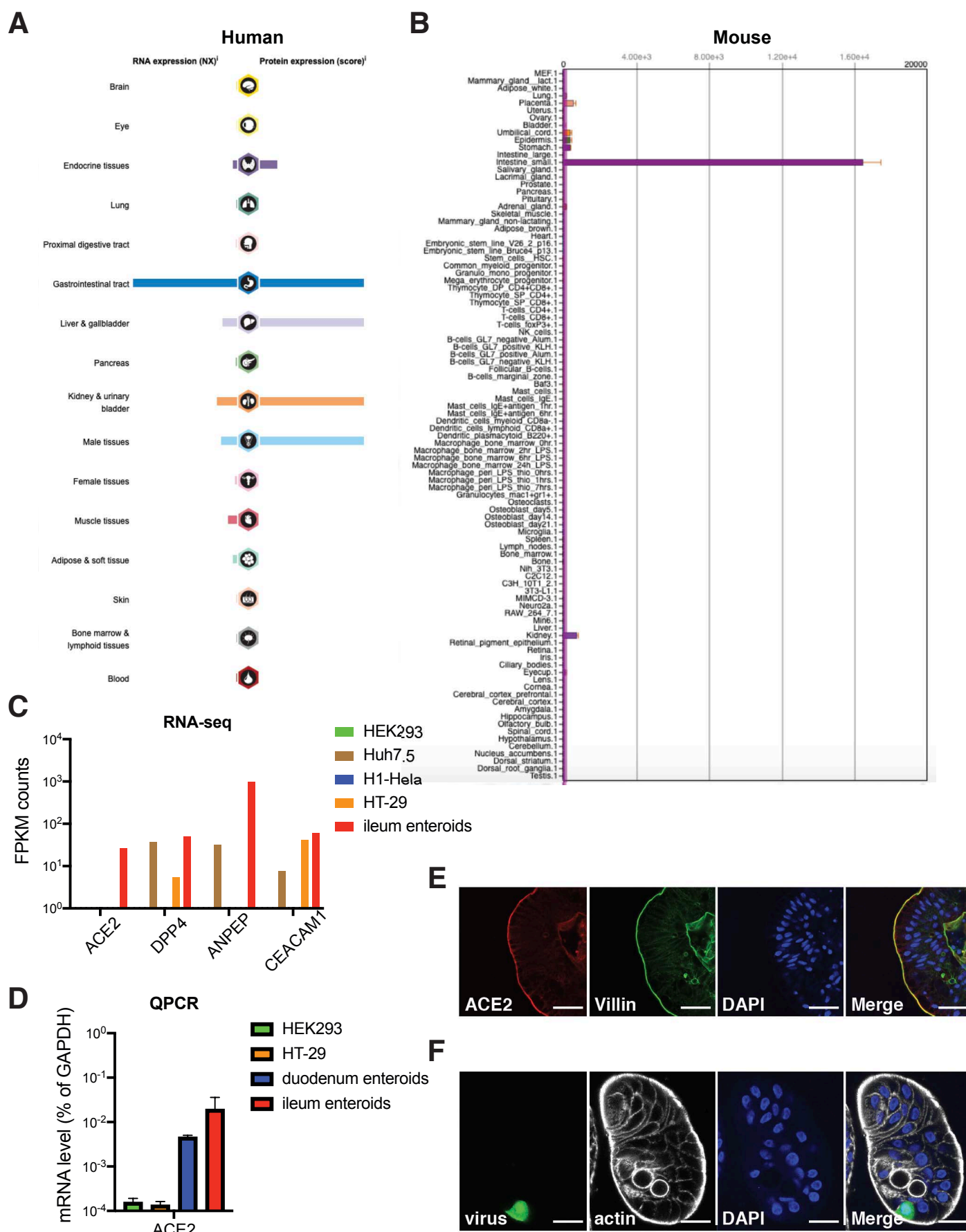
Figure S2

Figure S3

Figure S4

A

Figure S5