A CRISPR/Cas9 genetically engineered organoid biobank reveals essential host factors for coronaviruses

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Abstract

Rapid identification of host genes essential for virus replication may expedite the generation of therapeutic interventions. Genetic screens are often performed in transformed cell lines that poorly represent viral target cells in vivo, leading to discoveries that may not be translated to the clinic. Intestinal organoids (IOs) are increasingly used to model human disease and are amenable to genetic engineering. To discern which host factors are reliable anti-coronavirus therapeutic targets, we generate mutant clonal IOs for 19 host genes previously implicated in coronavirus biology. We verify ACE2 and DPP4 as entry receptors for SARS-CoV/SARS-CoV-2 and MERS-CoV respectively. SARS-CoV-2 replication in IOs does not require the endosomal Cathepsin B/L proteases, but specifically depends on the cell surface protease TMPRSS2. Other TMPRSS family members were not essential. The newly emerging coronavirus variant B.1.1.7, as well as SARS-CoV and MERS-CoV similarly depended on TMPRSS2. These findings underscore the relevance of non-transformed human models for coronavirus research, identify TMPRSS2 as an attractive pan-coronavirus therapeutic target, and demonstrate that an organoid knockout biobank is a valuable tool to investigate the biology of current and future emerging coronaviruses.

Main

Three highly pathogenic coronaviruses have spread to humans in the past two decades. The Severe Acute Respiratory syndrome coronavirus (SARS-CoV) emerged in 2002 and rapidly spread between continents, but was controlled by public health interventions. Middle East respiratory syndrome virus (MERS-CoV) -discovered in 2012- causes an ongoing outbreak in the Middle East with a high case-fatality ratio of 35%, but has not attained efficient human-to-human transmission. The latest, SARS-CoV-2, emerged at the end of 2019 and is the causative agent of the COVID-19 pandemic[1]. While vaccine development has taken off at a tremendous pace, drugs that target either the virus or host factors essential for virus replication have been more difficult to develop as this requires a deep understanding of coronavirus biology.

The first step in coronavirus replication is the attachment to host cells, which is dependent on the viral spike glycoprotein[2]. Although (proteo)glycans are often involved in the initial attachment, most coronavirus spikes require a specific transmembrane protein for entry. After receptor engagement, the next step in viral entry involves proteolytic cleavage of the spike protein. This cleavage step is performed by host proteases and destabilizes the spike, causing a conformational change and the subsequent fusion of viral and host membranes. This releases the viral ribonucleoprotein complex into a host cell and initiates replication.

Most of what we know on coronavirus cell biology stems from studies on 2D transformed cell lines such as the Vero E6 kidney cell line, derived from an African green monkey[3]. Work on cell lines has identified ACE2 as the entry receptor of SARS-CoV-2 and SARS-CoV, and DPP4 as the entry receptor of MERS-CoV[4–6]. Cell lines typically consist of a homogeneous population of poorly differentiated cells, potentially limiting the translatability of findings. As a case in point, chloroquine, an endocytosis inhibitor, has been proposed as a SARS-CoV-2 antiviral drug as it blocks SARS-CoV-2 entry in several cell lines[7], yet clinical studies have failed to demonstrate efficacy in COVID-19 patients[8]. Along these lines, inhibiting protease groups in cell lines with relatively broad-acting inhibitors have revealed that spike protein cleavage can occur at the cell surface by transmembrane serine protease (e.g. TMPRSS family members) or in the endosome by cathepsins (e.g. Cathepsin B or L), depending on the cell line used. Recently published host gene loss-of-function screens in 2D cell lines have supported a role for Cathepsin L, but not TMPRSS2, in viral entry into VeroE6 cells, while the opposite was observed in a small scale CRISPR screen Calu-3 cells[9,10]. In addition, we and others have recently shown that in primary airway cells serine protease- (but not cathepsin-) inhibitors block viral entry[4,11], but these inhibitors target all TMPRSS family members. Thus, it remains unknown whether in primary cells TMPRSS2 would be a realistic therapeutic target, or whether other TMPRSS family members could

62   compensate for the loss of TMPRSS2. Similarly, several new host factors have recently been found to

63   play a role in the SARS-CoV-2 replication cycle, such as NRP1 and NDST1, but it is unknown whether

64   these genes could be used as anti-SARS-CoV-2 drug targets[12–14].

65   There may exist significant differences between individual transformed cell lines, and between

66   transformed and non-transformed cells in viral entry pathways. Intestinal organoid (IO) culture

67   systems are an attractive platform to study virus-host interactions as they are amenable to CRISPR-

68   Cas9 mediated gene editing to directly identify host proteins utilized by the virus. Their self-renewing

69   nature offers an additional advantage: biobanks of characterized mutant IO clones can be established,

70   stored and shared. Here we establish a biobank of mutant IOs in genes implied in coronavirus biology

71   and test their role in coronavirus replication to discern which host factors may represent anti-

72   coronavirus therapeutic targets. This biobank can be used as a tool to rapidly identify which genes are

73   essential for virus entry when novel SARS-CoV-2 variants or novel zoonotic (corona)viruses emerge.

74   **Results**

75   ***Transcriptomic analysis of human IOs and airway cultures reveal conserved expression of***

76   ***coronavirus host factors***

77   Multiple host factors such as entry receptors and proteases are involved in viral replication cycles[15].

78   Since organoids closely resemble the physiology of human tissues, we used IOs to assess the function

79   of individual host factors that have been implicated in the SARS-CoV-2 replication cycle, or of other

80   coronaviruses. We and others have previously shown that SARS-CoV-2 can replicate in human IOs[16–18],

81   consistent with observations of gastrointestinal symptoms in COVID-19[19,20]. Intestinal organoids are

82   readily amenable to genetic engineering by CRISPR-Cas9[21], allowing to test the role of host genes in

83   the replication of SARS-CoV-2. We reasoned that individual host factors that upon loss-of-function

84   affect coronavirus replication, represent interesting drug targets for the treatment of COVID-19. As

85   spike protein cleavage is an essential step for viral entry, we focused on the proteases TMPRSS2,

86   TMPRSS3, TMPRSS4, TMPRSS11D, TMPRSS13, Cathepsin B (CTSB), Cathepsin L (CTSL) and Furin, that

87   have previously been implicated in the entry of SARS-CoV-2 or other coronaviruses[9,22,23]. Besides

88   proteases, we included the following (putative) entry or attachment factors: the protease DPP4 (MERS-

89   CoV), peptidase ANPEP (human coronavirus 229E receptor[24], C-type lectin CLEC2B[25], structural protein

90   Vimentin[26], glycoprotein CEACAM1[27], tetraspanin CD9[28], C-type lectin receptor CD209 (DC-SIGN)[29],

91   VEGF co-receptor NRP1[13,14], MAVS which indirectly senses cytoplasmic RNA[30], heatshock protein

92   HSPA5[31], the sulfotransferase NDST1[12] and the RNA packaging protein ARC (upregulated upon SARS-

93   CoV-2 infection[17], Fig. S6E) as putative host factors involved in replication.

94 We first confirmed the expression of these host factors in human IOs using a previously generated

95 dataset (Fig. 1A, S1A)[17]. To extend the expression analysis, we performed single cell RNA sequencing

96 of expanding and differentiated IOs to assign host factors to individual cell types. The organoid atlas

97 was supplemented by a recently published intestinal tissue dataset which we reanalyzed to allow for

98 direct comparison[32]. Cells grouped into distinct stem cell, progenitor, goblet cell and enterocyte

99 clusters (Fig. S1B-D). The proteases were ubiquitously expressed among the different lineages, with

100 the exception of TMPRSS2 which displayed a slight bias towards enterocytes, and TMPRSS4 that was

101 enriched in undifferentiated cells in both tissue and organoids (Fig. 1B). These findings contrasted with

102 a recent study that used murine tissue data and identified goblet-cell enriched expression of TMPRSS2,

103 while TMPRSS4 was mostly found in enterocytes[33].

104 We additionally performed RNA sequencing of 3D nasal organoids and 2D air-liquid interface

105 differentiated airway organoids, confirming expression of all selected host genes in these epithelia,

106 while relative expression levels varied (Fig. 1A, S1A, Table S1). Of note, the airway proteases

107 TMPRSS11A and TMPRSS11D were expressed in lower airway cultures, while expressed to modest

108 levels in IOs and nasal epithelial organoids (Fig. 1A, S1A).

109 ***ACE2 is the obligate entry receptor of SARS-CoV and SARS-CoV-2***

110 Having established expression of key coronavirus host factors, we generated an extensive biobank of

111 IOs harboring a loss-of-function mutation in individual genes (Fig. 2A, S2-3). We employed transient

112 transfection of a Cas9-EGFP encoding plasmid that included a site-specific guide RNA (sgRNA).

113 Transfected cells were GFP-sorted to establish clonal lines (12-24 lines per gene), expanded and

114 sequenced to identify loss-of-function clones. We successfully established 2-6 mutated clonal IO

115 cultures for all genes with the exception of HSPA5 (0 lines) and Furin and ARC (1 line) (Fig. 2B, Table

116 S2). HSPA5 is a heatshock protein that was recently identified as an essential gene for cell survival[34].

117 We first focused on ACE2, generally accepted to be the entry receptor for SARS-CoV-2 and SARS-CoV,

118 as based on crystallographic evidence and overexpression studies[5]. Moreover, transgenic mice

119 expressing human ACE2 are susceptible to infection with the virus[35]. Multiple additional (co-)receptors

120 have been proposed for SARS-CoV-2, including CD209/DC-SIGN, and NRP1[13,14,36]. Recent findings in

121 human IOs have found no correlation between infectability of cells and their levels of ACE2 expression,

122 suggesting the potential existence of alternative entry receptors[37]. In line with these findings, we

123 observed both ACE2-positive and ACE2-negative SARS-CoV-2 infected cells in IOs (Fig. S4), although

124 absence of surface-ACE2 could also indicate downregulation after infection[37] or reflect expression

125 levels under the detection limit of immunofluorescence staining.

126 To unequivocally demonstrate that physiological levels of ACE2 are essential for SARS-CoV-2 entry into
127 non-transformed human epithelial cells, we analyzed mutant ACE2 IOs for their ability to support SARS-
128 CoV-2 replication. ACE2, located on the apical membrane of cells in wildtype IOs, was lost in mutant
129 clones (Fig. 2C). ACE2-deficient IOs were fully resistant to SARS-CoV-2 infection (Fig. 3A). Indeed, we
130 did not detect SARS-CoV-2 infected cells in ACE2-knockout organoids by immunofluorescence (Fig. 3B).
131 Similarly, infection with SARS-CoV was abrogated in ACE2-knockout organoids (Fig. S5A). We
132 concluded that ACE2 is the obligate entry receptor for SARS-CoV-2 and SARS-CoV, and that no
133 redundancy exists with other surface proteins in intestinal epithelial cells. The presence of infected
134 cells that appear ACE2-negative, implies either that surface receptors are downregulated upon
135 infection or that low levels of ACE2 proteins suffice. To confirm that viral entry occurs through the
136 apical membrane - where ACE2 is located - we attempted viral infection following our standard
137 approach in which organoids are mechanically disrupted, and using intact organoids where only the
138 basolateral surface is exposed. We could observe viral replication only in disrupted organoids,
139 supporting an obligate apical entry route for SARS-CoV-2 (Fig. S5B).

140 ***MERS-CoV infects human IOs in a DPP4-dependent manner***

141 DPP4 has been shown to be the entry receptor for MERS-CoV by spike co-immunoprecipitation and
142 overexpression in non-susceptible cells[6]. We first established that human IOs allow replication of
143 MERS-CoV (Fig. S6A). In contrast to SARS-CoV-2, MERS-CoV caused extensive cell death, killing the
144 majority of cells in organoids within 48 hours of infection (Fig. S6A). Transcriptomic analysis revealed
145 a strong upregulation of heat shock- and unfolded protein responses, while interferons were
146 effectively repressed (Fig. S6B-E, Tables S3-4). This was consistent with previous reports that MERS-
147 CoV encodes an extensive set of proteins that inhibit interferon responses[38]. We generated loss-of-
148 function DPP4-mutant IO clones (Table S2). An infection assay on two of these clones revealed that
149 MERS-CoV replication was fully blocked, while SARS-CoV-2 replicated in the DPP4-mutant organoids at
150 control levels (Fig. 3C). Immunofluorescence confirmed loss of DPP4 protein in mutant clones as well
151 as the successful replication of SARS-CoV-2 -but not of MERS-CoV- in these organoids (Fig. 3D).
152 Conversely, MERS-CoV propagated in ACE2-deficient organoids at control levels (Fig. S5A).

153 ***Loss-of-function screen of host proteases reveals essential role in viral replication for TMPRSS2 but***
154 ***not other TMPRSS family members or Cathepsins***

155 We next analyzed all IO lines that were mutant in proteases for their ability to support SARS-CoV-2
156 replication. Knockout of TMPRSS2 effectively blocked viral replication, while mutation of any of other
157 TMPRSS-genes had no effect (Fig. 4A-B). Complete depletion of TMPRSS2 protein was confirmed using

158   immunohistochemistry (Fig. S7A). These experiments were performed using a VeroE6-propagated

159   stock and recent work has pointed out that propagation on VeroE6 cells can lead to culture adaptive

160   mutations in the multibasic cleavage site[39–43]. The VeroE6 stock used in this study was deep-

161   sequenced[39] and was 64.2% wild-type in the RRAR (spike positions 682-685) multibasic cleavage site.

162   We detected another mutation adjacent to the multibasic cleavage site (S686G) at a frequency of

163   45.4%. Viruses with multibasic cleavage site cleavage site mutations, including S686G, were shown to

164   slightly increase cathepsin usage by ~20%[39], indicating that the majority of these viruses still used

165   serine protease-mediated entry.

166   We have previously shown that propagation in TMPRSS2-expessing Calu-3 cells prevents culture

167   adaptation. Using this Calu-3 stock that was completely non-adapted[39], we confirmed the dependency

168   of SARS-CoV-2 on TMPRSS2 (and ACE2) (Fig. 4C). Immunofluorescence of TMPRSS2-deficient organoids

169   showed absence of viral spread (Fig. 4D). This implied that TMPRSS2 is the main proteolytic activator

170   of the SARS-CoV-2 spike protein. In contrast to knockout screens in VeroE6 cells that showed that the

171   endocytic pathway protease Cathepsin L was essential for SARS-CoV-2 entry[9], SARS-CoV-2 replicated -

172   if anything- more efficiently in Cathepsin L-mutant than in wildtype organoids (Fig. 4A-B). Efficient

173   depletion of Cathepsin L was supported using western blot analysis (Fig. S7B). We confirmed the

174   obligate role of TMPRSS2 for SARS-CoV-2 replication in IOs derived from a different donor and from

175   another segment (duodenum) of the human intestine (Fig. 4E).

176   Since we observed differential expression of multiple proteases - including upregulation of cathepsins

177   - in differentiated organoids, we additionally assessed TMPRSS2-dependency in differentiated

178   intestinal cells (Fig. S7C). After 5 days of differentiation, both wildtype and TMPRSS2-mutant organoids

179   were infected with SARS-CoV-2. SARS-CoV-2 replicated efficiently in differentiated organoids, as we

180   reported previously[17]. Viral replication was greatly diminished in TMPRSS2-deficient organoids,

181   suggesting dependency on this protease across different intestinal cell types (Fig. S7D)

182   To assess redundancy in single TMPRSS- or cathepsin-mutant organoids, we additionally generated

183   organoids mutant for both CTSL/CTSB, or TMPRSS2/4, the most abundantly expressed cathepsins and

184   serine proteases in the intestinal epithelium. Previous work implied a role for TMPRSS4 in viral entry

185   in the intestinal epithelium[33]. We did not observe reduced replication when both cathepsins were lost.

186   Moreover, TMPRSS4 knock-out in a TMPRSS2-mutant background did not further decrease infectivity

187   (Fig. S7E). In line with this, the broad serine protease inhibitor camostat did not affect replication in

188   TMPRSS2-deficient IOs (Fig. S7F). We concluded that the cathepsins and TMPRSS4 do not play a role

189   in viral entry in the intestinal epithelium.

190   To confirm that the endocytic pathway is dispensable for viral entry, we treated IOs with 1) the
191   endosomal pathway inhibitor chloroquine, the cathepsin protease inhibitor E64D, or the broad serine
192   protease inhibitor camostat. These drugs were well-tolerated with no growth impairment at the
193   concentrations used (Fig. S8A-B). As published previously, chloroquine was effective in VeroE6 cells
194   (Fig. S8C)[7]. While camostat effectively inhibited viral replication, chloroquine and E64D did not affect
195   replication in IOs (Fig. 4F). E64D-treated organoids displayed a trend towards more efficient viral
196   replication (Fig. 4F), consistent with observations in the Cathepsin L-mutant organoids (Fig. 4A-B). We
197   concluded that Cathepsin-mediated entry through the endosomal route may be the central port of
198   viral entry in cell lines, but not in IOs, in which SARS-CoV-2 enters through the activity of TMPRSS2 (Fig
199   4A-B). These observations may also explain why (hydro)-chloroquine has emerged from cell line
200   studies but has proven ineffective in the clinic.

201   ***Redundancy for non-protease host factors in viral replication***

202   We further tested IOs mutant in a range of non-protease host factors to assess their role in coronavirus
203   replication, of which some have already been linked to the SARS-CoV-2 replication cycle. NRP1 recently
204   attracted attention as a novel co-receptor for SARS-CoV-2 in two separate studies that used Hela,
205   HEK293T and the colorectal cancer cell line Caco-2[13,14]. These findings were substantiated by x-ray
206   crystallography data supporting binding of the viral spike protein to NRP1 SARS-CoV-2 infection was
207   significantly inhibited by NRP1-blocking antibodies[13,14]. Additionally, CD209 was recently identified as
208   potential SARS-CoV-2 receptor, and facilitated viral entry in HEK-293 cells when overexpressed[36]. A
209   recent study found that SARS-CoV-2 can bind heparan sulfate on the cell surface through its spike
210   protein. When enzymes involved in the sulfation of heparan sulfate, including NDST1, were knocked
211   out in Hep3B cells, viral replication was almost entirely abolished[12]. None of these, nor the additional
212   host factors we assessed, significantly impacted on viral replication when mutated in IOs (Fig. 5A, Fig.
213   S9). We conclude that all of these proteins would therefore not be viable drug targets for the treatment
214   of COVID-19 (Fig. 5B). Further studies may assess whether loss of these factors influence the cellular
215   response to coronaviruses in any other way than replication efficiency.

216   ***TMPRSS2-dependency in SARS-CoV-2 B.1.1.7, SARS-CoV and MERS-CoV***

217   The mutant host factor KO biobank can readily be employed when new coronaviruses or viral strains
218   appear, to assess the dependency on host factors and identify druggable targets. We first tested
219   whether the same TMPRSS2-dependency exists for the other two coronaviruses. SARS-CoV replication
220   was strongly diminished upon TMPRSS2 loss, while MERS-CoV replication was reduced more modestly
221   (Fig. 6A). The latter potential redundancy may be explained by the presence of two functional

222   multibasic cleavage sites in the MERS-CoV spike, whereas SARS-CoV-2 and SARS-CoV possess one and

223   none, respectively[22]. Both viruses could replicate in the absence of Cathepsin L, suggesting that

224   coronaviruses generally do not use the endosomal entry route in primary epithelial cells as present in

225   organoids (Fig. 6A).

226   Recently, a novel SARS-CoV-2 variant (clade B.1.1.7 or British variant) emerged and is rapidly replacing

227   endemic viruses globally. Epidemiological data suggest that this variant is 1.35-2 fold more

228   transmissible than the ancestral lineage and is associated with higher viral loads[44–46]. Interestingly, this

229   variant contains a mutation (P681H) directly N-terminal from the RRAR multibasic cleavage site that

230   adds another basic residue to the multibasic cleavage site, creating an HRRAR motif. A similar mutation

231   (P681R) was detected in the Indian variant (clade B.1.617). As the multibasic cleavage site facilitates

232   serine protease-mediated entry[11], mutations in or near this site may alter protease usage for S2′

233   cleavage, which directly triggers fusion and entry. We found that the British variant replicated

234   efficiently in wildtype and cathepsin mutant organoids, but not in TMPRSS2-deficient cells (Fig. 4E),

235   indicating that the British variant did not broaden its protease usage. These experiments provide a

236   proof-of-concept on how emerging viral strains could be screened against mutant IOs.

237   **Discussion**

238   The current COVID-19 pandemic has exposed weaknesses in our preparedness for coronavirus

239   pandemics. No effective coronavirus antivirals are approved for use in humans and all completed large-

240   scale COVID-19 drug trials have failed to show efficacy to this date, including (hydroxy)chloroquine and

241   remdesivir[8,47]. The disappointing clinical effects of (hydroxy)chloroquine in humans in particular

242   highlights gaps in the understanding of fundamental coronavirus biology. (Hydroxy)chloroquine, an

243   inhibitor of the endosomal acidification was identified as a potent inhibitor of SARS-CoV[48] and SARS-

244   CoV-2[7] viral entry in cell line-based assay, confirmed here. In agreement with this, recent whole

245   genome CRISPR/Cas9 genetic screens in transformed cell lines again suggested that endosomal entry

246   factors, such as cathepsin L, are crucial for SARS-CoV-2 entry[9,49,50].

247   Here, we use human intestinal organoids as a non-transformed model to study genes implicated in

248   coronavirus biology. We have chosen to use only IOs since it is currently not possible to efficiently

249   genetically engineer airway organoids due to limited clonal outgrowth of these cells. Nevertheless, IOs

250   express the majority of host factors assessed, including proteases, to a similar level as the airways (Fig.

251   1). We confirmed that in this model ACE2 is the obligate entry receptor for SARS-CoV-2 and SARS-CoV,

252   while DPP4 is the entry receptor for MERS-CoV, indicating that accessory receptors may not play crucial

253   roles for these viruses. Indeed, knockout of NRP1, recently proposed as a SARS-CoV-2 (co-)receptor in

254   Hela and Caco-2 cells, did not affect SARS-CoV-2 entry[13,14]. Furthermore, we demonstrate that

255   Cathepsin L and B are not involved in SARS-CoV-2 entry in IOs. In accordance with this, a cathepsin

256   inhibitor (E64D) and chloroquine did not inhibit SARS-CoV-2 in these IOs, while the serine protease

257   inhibitor Camostat effectively blocked viral propagation. A similar anti-SARS-CoV-2 effect of camostat

258   was observed in organoid-derived airway cells[11]. The broad activity of Camostat does not allow to

259   pinpoint which serine protease mediates entry.

260   TMPRSS2-deficiency in IOs strongly decreased SARS-CoV-2 replication and spread, indicating that

261   TMPRSS2 is the main priming protease. Other related TMPRSS genes have previously also been linked

262   to SARS-CoV-2 replication, including TMPRSS4 in the intestine[33]. Overexpression of TMPRSS11D and

263   TMPRSS13 promoted viral entry into the hamster kidney cell line BHK-21[51], while TMPRSS4

264   overexpression facilitated viral entry in HEK293 cells[33]. Like TMPRSS2, TMPRSS4 is highly expressed in

265   intestinal tissue and IOs, yet TMPRSS4 does not appear to rescue loss of TMPRSS2. This discrepancy

266   with previous work may reflect the fact that our study relies on physiological expression of these

267   proteases, rather than on overexpression. Importantly, intestinal organoids express similar TMPRSS

268   family members compared to airway tissue, including high levels of TMPRSS2, TMPRSS3, TMPRSS4 and

269   TMPRSS13. Only the TMPRSS-11A and –11D are relatively enriched in airway- versus intestinal

270   epithelium[52]. Although we cannot exclude the possibility that these TMPRSS family members function

271   in activation of SARS-CoV-2 spike in the airways, their expression levels are much lower than that of

272   TMPRSS2 in airway organoids (Fig. 1A) and lung tissue[52].

273   Altogether, these findings indicate that multiple TMPRSS genes may be able to mediate entry when

274   overexpressed, but -at physiological levels in IOs- only TMPRSS2 plays an essential role, which may

275   inspire the development of high-specificity TMPRSS2 inhibitors. The high TMPRSS-2 dependency of

276   SARS-CoV (this study) indicates that such inhibitors may well be effective against future SARS-like

277   coronavirus pandemics. The observation that TMPRSS2-null mice do not display a visible phenotype[53]

278   implies that such inhibitors may be well-tolerated. Our findings match with observations that SARS-

279   CoV and to a lesser extent MERS-CoV replication and dissemination was reduced in TMPRSS2-deficient

280   mice[54].

281   In conclusion, our findings underscore the relevance of non-transformed human models for

282   (corona)virus research and identify TMPRSS2 as an attractive therapeutic target in contrast to many

283   other genes (e.g. cathepsin L, cathepsin B, NRP1, NDST1 etc) that -as deduced from our observations-

284   unlikely to be of clinical value. Future emerging viruses could be readily screened against our IO host

285   factor knockout biobank to rapidly identify therapeutic targets.

Author contributions

J.B., M.H.G. and M.M.L. designed the study and performed the experiments. J.P. and J.V. analyzed RNA-sequencing data. B.H. and H.C. supervised the study.

Declaration of interest

H.C. is inventor on several patents related to organoid technology; his full disclosure is given at https://www.uu.nl/staff/JCClevers/.

Figure legends

Figure 1 Expression levels of host genes potentially involved in SARS-CoV-2 biology in lung and intestinal organoids and tissue

a) Graphs depicting the transcript counts (logarithmic scale) determined by RNA sequencing of host factors and proteases in organoids derived from different parts of the airway and intestine. Airway organoids (trachea, bronchus and bronchiole) were differentiated as 2D ALI-cultures.

b) t-SNE maps and violin plots displaying expression of host factors in the human intestinal organoid cell and human intestinal tissue atlases (in vivo atlas derived from[32]. Bars in t-SNE maps display color-coded normalized unique transcript expression (logarithmic scale).

Figure 2 Generation of a coronavirus host gene knockout biobank

a) Overview of the workflow of generation of gene knockouts.

b) Overview of the number of clones that were amplified and biobanked in this study.

c) Immunofluorescent staining of WT and ACE2 KO organoids. ACE2 locates to the apical membrane and is absent in mutant organoids. Scale bars are 50 μm. Contrast was enhanced in unzoomed images for visualization purposes.

Figure 3 ACE2 and DPP4 are the obligate entry receptors for SARS-CoV-2 and MERS-CoV respectively

311 a) qPCR analysis targeting the E gene to quantify viral replication of wildtype (WT) and ACE2 knockout
312 (KO) organoids. Error bars represent SEM. Each data point represents the mean of 3 replicates.

313 b) Immunofluorescent staining of WT and ACE2 KO organoids, infected with SARS-CoV-2 visualized by
314 nucleocapsid protein and dsRNA. ACE2 locates to the apical membrane and is absent in mutant
315 organoids.

316 c) qPCR analysis targeting the E gene (SARS-CoV-2) or upE region (MERS-CoV) to quantify viral
317 replication in WT and DPP4 KO organoids. Each data point represents the mean of 3 replicates.

318 d) Immunofluorescent staining of MERS-CoV (visualized by dsRNA) and SARS-CoV-2 (visualized by
319 nucleocapsid protein) infected organoids. DPP4 locates to the apical membrane and is absent in
320 mutant organoids. Scale bars are 50 μm.

321 **Figure 4 Loss-of-function screen of host proteases reveals essential role for TMPRSS2 but not**
322 **Cathepsins in viral replication**

323 a) Violin plot displaying the Log10 ratio between the viral titer at 48 hours compared to 2 hours post
324 infection (p.i.) quantified by qPCR targeting the E gene. The dotted line indicates a fold change of 1.
325 Dotted lines within the violins indicate the median and quartiles. Data from replication curves in Fig.
326 3A and 3C is included. N ≥ 3.

327 b) qPCR analysis targeting the E gene to quantify viral replication of wildtype organoids and cells
328 harboring a loss-of-function of a single host factor. Error bars represent SEM. Each data point
329 represents the mean of 3 replicates.

330 c) Violin plot displaying the Log10 ratio between the viral titer at 48 hours compared to 2 hours post
331 infection (p.i.) quantified by qPCR targeting the E gene in organoids infected with SARS-CoV-2
332 propagated on Calu-3 cells. The dotted line indicates a fold change of 1. Dotted lines within the violins
333 indicate the median and quartiles. N ≥ 3.

334 d) Immunofluorescent staining of SARS-CoV-2 infected WT and CTSL and TMPRSS2 KO organoids. Virus
335 is visualized by nucleocapsid protein. TMPRSS2 KO organoids do not facilitate viral infection, although
336 very rare, infected cells can be observed. CTSL KO organoids display abundant viral infection. Scale bars
337 are 50 μm.

338 e) Violin plot displaying the Log10 ratio between the viral titer at 48 hours compared to 2 hours post
339 infection (p.i.) quantified by qPCR targeting the E gene in organoids of the human duodenum. The

340   dotted line indicates a fold change of 1. Dotted lines within the violins indicate the median and

341   quartiles. N ≥ 3.

342   f) qPCR analysis targeting the E gene to quantify viral replication of organoids treated with the serine

343   protease inhibitor Camostat, chloroquine or cysteine protease inhibitor E64D. Error bars represent

344   SEM. Each data point represents the mean of 3 replicates.

345   P < 0.05 *; P<0.01** ; P<0.001*** ; P<0.0001****.

346   **Figure 5 Replication of SARS-CoV-2 in additional proposed host factors**

347   a)) Violin plot displaying the Log10 ratio between the viral titer at 48 hours compared to 2 hours post

348   infection (p.i.) quantified by qPCR targeting the E gene in wildtype organoids and cells harboring a loss-

349   of-function of a single host factor. Graphs display the ratio between viral titer at 48 hours compared

350   to 2 hours post infection (p.i.). The dotted line indicates a fold change of 1. Dotted lines within the

351   violins indicate the median and quartiles. N ≥ 2. The WT data is redispayed from Fig. 4A.

352   b) Model of host entry receptors and proteases involved in the entry of SARS-CoV-2. Essential and non-

353   essential host factors based on the phenotypes in IO mutants are marked

354   **Figure 6 Host protease dependency in the SARS-CoV-2 strain B.1.1.7 and other coronaviruses**

355   a)) Violin plot displaying the Log10 ratio between the viral titer at 48 hours compared to 2 hours post

356   infection (p.i.) quantified by qPCR targeting the the N gene (SARS-CoV) or upE region (MERS-CoV) to

357   measure viral replication in WT and TMPRSS2 KO organoids. DPP4 and ACE2 data include replication

358   curves in Fig. 3C and S4A. The dotted line indicates a fold change of 1. Dotted lines within the violins

359   indicate the median and quartiles. N ≥ 3.

360   b) Violin plot displaying the Log10 ratio between the viral titer at 48 hours compared to 2 hours post

361   infection (p.i.) quantified by qPCR targeting the E gene to measure viral replication of the SARS-CoV-2

362   B.1.1.7 strain in intestinal organoid cells harboring a loss-of-function mutation in the TMPRSS2 and

363   CTSL proteases, or ACE2. The dotted line indicates a fold change of 1. Dotted lines within the violins

364   indicate the median and quartiles. N ≥ 3.

365   P < 0.05 *; P<0.01** ; P<0.001*** ; P<0.0001****.

366   **Supplementary figure 1 Bulk and single cell RNA sequencing reveals intestinal expression of host**

367   **proteases involved in viral entry**

368   a) Heatmaps depicting expression of host genes (top) and proteases (bottom) in intestinal, nose and

369   airway organoids. Nose organoids were cultured in expansion (EM) or differentiation (DM) medium.

370   Colored bar represents Z-score of log2 transformed values.

371   b) t-SNE maps displaying a newly generated human organoid single cell RNA sequencing atlas (left),

372   and a dataset reanalyzed from[32] (right). Colors indicate different cell types.

373   c) t-SNE maps displaying the human organoid single cell sequencing atlas. Color codes indicate cells

374   derived from expansion or differentiation medium.

375   d) t-SNE maps and violin plots displaying expression of host factors in the human intestinal organoid

376   cell atlas. Bars in t-SNE maps display color-coded normalized unique transcript expression (logarithmic

377   scale).

378   e) t-SNE maps and violin plots displaying expression of host factors in the human intestinal tissue atlas.

379   Bars in t-SNE maps display color-coded normalized unique transcript expression (logarithmic scale).

380

381   **Supplementary figure 2 Generation of a host factor loss-of-function organoid biobank**

382   Overview of the genetic alterations causing frameshifts in the different host factors. Green boxes

383   indicate PAM sequence, red dashes or bases indicate respectively deletions and insertions.

384   **Supplementary figure 3 Organoid genotyping by sanger sequencing and *in silico* ICE analysis.**

385   Sanger traces and subsequent *in silico* sanger deconvolution by ICE v2 for the first clone of ACE2,

386   TMPRS2, DDP4 and CTSL indicating out-of-frame indel induction at the target site. Green boxes

387   indicate PAM sequence.

388   **Supplementary figure 4 SARS-CoV-2 infected cells contain varying degrees of membranous ACE2**

389   **protein**

390   Immunofluorescent staining of SARS-CoV-2 infected organoids. Virus is visualized by dsRNA. Some

391   infected cells are devoid of visible ACE2 on the outer membrane. Scale bars are 50 μm.

392   **Supplementary figure 5 SARS-CoV but not MERS-CoV replication depends on ACE2**

393   a) qPCR analysis targeting the N gene (SARS-CoV) or upE region (MERS-CoV) to quantify viral replication

394   in WT and ACE2 KO organoids. Error bars represent SEM. Each data point represents the mean of 3

395   replicates.

396   b) qPCR analysis targeting the E gene to quantify viral replication of the SARS-CoV-2 in mechanically

397   disrupted or intact organoids, where virus can only access the basolateral side. Graphs display the ratio

398   between viral titer at 48 hours compared to 2 hours post infection (p.i.). Each data point represents

399   the mean of 3 replicates. Error bars represent SEM.

400   **Supplementary figure 6 Establishment of MERS infection model in human intestinal organoids**

401   a) Immunofluorescent staining of organoids 48 hours after MERS-CoV infection. Virus is visualized by

402   staining for the spike protein. The majority of the infected organoids display massive cell death. Scale

403   bars are 50 µm.

404   b) Graphs depicting the transcript counts determined by RNA sequencing of different genes upon

405   MERS-CoV infection. Different numbers indicate timepoints (hours) after infection

406   c) Graph depicting the transcript counts mapping to human and MERS genomes in MERS-CoV infected

407   organoids. MERS reads increase over time, but drop again at 48 hours potentially due to cell death of

408   infected cells. For all other analyses, MERS reads were removed from analyses for normalization

409   purposes.

410   d) Go term enrichment analysis for biological processes of the 60 most significantly upregulated genes

411   upon MERS-CoV infection in organoids.

412   e) Heatmap depicting the expression profile of the 25 genes with strongest upregulation upon SARS-

413   CoV-2 infection[17]; right heatmaps) and the same genes upon MERS-CoV infection (left heatmaps). The

414   top heatmaps show the most prominently upregulated genes after 60 hours of SARS-CoV-2 infection,

415   the lower heatmaps after 24 hours. In contrast to SARS-CoV-2, MERS-CoV does not induce expression

416   of ISGs. Colored bar represent Z-score of log2 transformed values.

417   **Supplementary figure 7 Lack of redundancy in cathepsins and serine proteases in viral entry**

418   a) Immunohistochemical staining of TMPRSS2 in wildtype and TMPRSS2-knock out (KO) organoids.

419   TMPRSS2 locates mostly to the apical membrane in wildtype cells, and is absent in mutant organoids.

420   Scale bars are 50 µm.

421   b) Western blotting for CTSL and Integrin B4 (ITGB4, loading control) in wildtype, TMPRSS2- and CTSL-
422   KO organoids. CTSL protein is completely lost in corresponding mutant organoids

423   c) Brightfield images of expanding and 5-day differentiated organoids that were infected with SARS-
424   CoV-2. Scale bars are 400 μm.

425   d) Graph displaying the ratio between the viral titer at 48 hours compared to 2 hours post infection
426   (p.i.) quantified by qPCR targeting the E gene to measure viral replication of SARS-CoV-2 in expanding
427   and 5-day differentiated intestinal organoid cells harboring a loss-of-function mutation in the TMPRSS2
428   gene. Error bars represent SEM. N=2.

429   e) qPCR analysis targeting the E gene to quantify viral replication of the SARS-CoV-2 in organoids
430   harboring different single and double mutants in host proteases. Graphs display the ratio between
431   viral titer at 48 hours compared to 2 hours post infection (p.i.). Error bars represent SEM. The dotted
432   line indicates a fold change of 1. N=2.

433   f) qPCR analysis targeting the E gene to quantify viral replication of the SARS-CoV-2 in TMPRSS2-
434   deficient organoids treated with the broad serine protease inhibitor Camostat. Graphs display the ratio
435   between viral titer at 48 hours compared to 2 hours post infection (p.i.). Error bars represent SEM.
436   N=2.

437   **Supplementary figure 8 Inhibition of serine proteases but not chloroquine inhibits viral replication**
438   **in organoids**

439   a) Brightfield images of organoids treated for 48 hours with serine protease inhibitor Camostat,
440   chloroquine or cysteine protease inhibitor E64D.

441   b) Violin plot of average sizes in organoids from Fig. 6E. The diameter was measured in at least n=50
442   organoids per treatment. Organoid size was not significantly changed in any of the treatments,
443   indicating similar growth. Dotted lines within the violins indicate the median and quartiles.

444   c) Quantification of viral entry in Vero E6 cells upon treatment with chloroquine using immunostaining
445   8 hours after infection. Error bars represent SEM.

446   **Supplementary figure 9 SARS-CoV-2 replication in Furin- en ARC-mutant IOs**

447   Graph displaying the ratio between the viral titer at 48 hours compared to 2 hours post infection (p.i.)
448   quantified by qPCR targeting the E gene to measure viral replication of SARS-CoV-2 in wildtype, and

449   ARC- and Furin- mutant organoids. Experiment was performed with n=1 biological replicate. The WT

450   data is redispayed from Fig. 4A.

Supplementary table 1 Normalized transcript counts in intestinal and airway organoids

452   Table shows normalized transcript counts determined by RNA sequencing of duplicate organoids from

453   the respective regions, and intestinal organoids infected with SARS-CoV-2. The intestinal organoid

454   dataset was obtained from[17].

Supplementary table 2 Overview of genetically modified organoids generated in this study

Supplementary table 3 Normalized transcript counts in MERS-CoV infected organoids

457   Table shows normalized transcript counts determined by RNA sequencing of a duplicate control

458   treatment (NC), and 16, 24 and 48 hours after MERS-CoV infection.

Supplementary table 4 Differentially regulated genes upon MERS-CoV infection in organoids

460   Fold change in gene expression versus control after 48 hours of MERS-CoV infection.

Supplementary table 5 Oligos used in this study as gRNAs and sequencing primers

Methods

Cell culture of human intestinal organoids and human airway

464   Human small intestinal tissue was obtained from the UMC Utrecht with informed consent of the

465   patient. The patient was operated for a colorectal tumor, and a sample from non-transformed, normal

466   mucosa was taken for this study. The study was approved by the UMC Utrecht (Utrecht, The

467   Netherlands) ethical committee and was in accordance with the Declaration of Helsinki and according

468   to Dutch law. This study is compliant with all relevant ethical regulations regarding research involving

469   human participants.

470   Nasal inferior turbinate brushes were obtained from the Hadassah Medical Center, Jerusalem, with

471   informed consent of the patient. Patients were diagnosed with primary ciliary dyskinesia, and tissue

472   was obtained from healthy donors as a comparison. Healthy material was used for the RNA sequencing

473   in this work. The study was approved by the ethical committee and was in accordance with the

474   Declaration of Helsinki and according to Israeli law under IRB approval number 075-16 HMO. This study

475   is compliant with all relevant ethical regulations regarding research involving human participants.

476   Adult lung tissue was obtained from residual, tumor-free, material obtained at lung resection surgery

477   for lung cancer. The Medical Ethical Committee of the Erasmus MC Rotterdam granted permission for

478   this study (METC 2012-512).

479   Human small intestinal cells were isolated, processed and cultured as described previously[55,56]. Wnt

480   surrogate was used (0,15nM, U-Protein Express) instead of Wnt conditioned media. Differentiation of

481   intestinal organoids was achieved as described previously[56].

482   Nose tissue was dissociated and cultured as described previously[57]. Differentiation towards ciliated

483   cells was performed by activating BMP signaling and inhibiting Notch signaling for 10 days (Van der

484   Vaart et al., under review)

485   Isolation of human bronchial airway stem cells was performed using a protocol similar to Sachs and

486   colleagues[57]. Small airway stem cells were isolated from distal human lung parenchyma as described

487   before[57]. Tracheal stem cells were collected from tracheal aspirates of intubated preterm infants (28

488   weeks gestational age). Organoids were cultured as described before[57]. To obtain differentiated

489   organoid-derived cultures, organoids were dissociated into single cells using TrypLE express (Gibco;

490   #12604013). Cells were seeded on Transwell membranes (Corning) coated with rat tail collagen type I

491   (Fisher Scientific). Single cells were seeded in AO growth medium : complete base medium (CBM;

492   Stemcell Pneumacult-ALI; #05001) at a 1:1 ratio. After 2-4 days, confluent monolayers were cultured

493   at air-liquid interphase in CBM. Medium was changed every 5 days for 8 weeks.

494   **Transfection of organoids for CRISPR-Cas9 experiments**

495   sgRNAs targeting loci of interest were cloned into a SpCas9-EGFP vector (addgene plasmid #48138)

496   using a protocol described before[58]. sgRNAs were designed using WTSI website

497   (https://www.sanger.ac.uk/htgt/wge/). A full list of gRNAs and primers to generate SpCas9-EGFP

498   expressing plasmids can be found in supplementary table 5. To generate homozygous frameshift

499   mutations in genes of interest, organoids were transfected with SpCas9-EGFP containing the locus-

500   specific sgRNA. Transient transfection using a NEPA21 electroporator was performed as described

501   before[59]. 3-7 days after transfection, organoids were dissociated using TryplE (TryplE Express; Life

502   Technologies) and sorted on a FACS-ARIA (BD Biosciences) for GFP positivity. After sorting, Rho kinase

503   inhibitor (Y-27632 dihydrochloride; 10µM, Abmole) was added for 1 week to support single cell

504   outgrowth.

505   **Generation of stable genetically modified organoid lines**

506   To generate clonal organoid lines with genotypes of preference, organoids were picked 2 weeks after

507   sorting. Manually picked organoids were dissociated using TryplE (TryplE Express; Life Technologies)

508   and plated in BME in pre-warmed cell culture plates. After two weeks, single cells grew into organoids

509   and were split again to verify actively dividing stem cells. After the second split, 20μL of organoid-BME

510   suspension was directly taken from the plate and DNA was extracted from the organoids using the

511   Zymogen Quick-DNA microprep kit according to protocol. Regions around sgRNA target sites were

512   amplified using Q5 high fidelity polymerase (NEB) according to manufacturer's protocol. CRISPR/Cas9-

513   mediated indel formation was confirmed by sanger sequencing of these amplicons (Macrogen).

514   Subsequently, sanger trace deconvolution was performed with the use of ICE v2 CRISPR analysis tool

515   (synthego website) to call clonal organoid lines with homozygous frameshift mutations at the target

516   site. Knockout clones were further expanded for viral infection experiments. Primers used for

517   amplification and sanger sequencing can be found in supplementary table 5. For the generation of

518   TMPRSS2/TMPRSS4 double mutants, TMPRSS4 was knocked out in TMPRSS2-clone 9. For the

519   generation of CTSL/CTSB double mutants CTSL was knocked out in CTSB clone 3.

520   **Viruses and cell lines**

521   Vero and VeroE6 cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco)

522   supplemented with 10% fetal calf serum (FCS), HEPES, sodium bicabonate, penicillin (100 IU/mL) and

523   streptomycin (100 IU/mL) at 37°C in a humidified CO_2 incubator. Calu-3 cells were maintained in Opti-

524   MEM I (1X) + GlutaMAX (Gibco)(Gibco) supplemented with 10% FCS, penicillin (100 IU/mL) and

525   streptomycin (100 IU/mL) at 37°C in a humidified CO_2 incubator. SARS-CoV (isolate HKU 39849,

526   genbank accession no. AY278491), SARS-CoV-2 (isolate Bavpat-1; European Virus Archive Global

527   #026V-03883; kindly provided by Dr. C. Drosten) were propagated on VeroE6 cells in Opti-MEM I (1X)

528   + GlutaMAX (Gibco), supplemented with penicillin (100 IU/mL) and streptomycin (100 IU/mL) at 37°C

529   in a humidified CO_2 incubator. MERS-CoV (isolate EMC, genbank accession no. NC019843) was

530   propagated on Vero cells in the same medium. Non-adapted SARS-CoV-2 Bavpat-1 and B.1.1.7

531   (genbank accession no. MW947280) were propagated in Calu-3 cells as described before[39]. Stocks were

532   produced by infecting cells at a multiplicity of infection (MOI) of 0.01 and incubating the cells for 72

533   hours. The culture supernatant was cleared by centrifugation and stored in aliquots at −80°C. Stock

534   titers were determined by preparing 10-fold serial dilutions in Opti-MEM I (1X) + GlutaMAX. Aliquots

535   of each dilution were added to monolayers of 2 × 104 VeroE6 (for SARS-CoV and SARS-CoV-2) or Vero

536   cells (for MERS-CoV) in the same medium in a 96-well plate. Plates were incubated at 37°C for 5 days

537   and then examined for cytopathic effect. The TCID50 was calculated according to the method of

538   Spearman & Kärber. All work with infectious SARS-CoV, SARS-CoV-2 and MERS-CoV was performed in

539   a Class II Biosafety Cabinet under BSL-3 conditions at Erasmus Medical Center.

540   **SARS-CoV, SARS-CoV-2 and MERS-CoV infection**

541   Infections were performed using a protocol similar to[17]. Briefly, organoids were harvested in cold

542   Advanced DMEM (including HEPES, Glutamax and antibiotics, termed AdDF+++[17]), washed once in

543   AdDF+++, and sheared using a flamed Pasteur pipette in AdDF+++. Differentiated organoids were

544   broken using a 5-minute incubation with TrypLE (TrypLE Express; Life Technologies). After shearing,

545   organoids were washed once in AdDF+++ before infection was performed in expansion medium. For

546   the experiment in Figure S5B, organoids were gently harvested using a wide pipet tip to avoid shearing

547   organoids. A multiplicity of infection (MOI) of 0.1 was used for SARS-CoV and SARS-CoV-2 and an MOI

548   of 0.2 was used for MERS-CoV. After 2 hours of virus adsorption at 37°C 5% CO_2, cultures were washed

549   four times with 4 ml AdDF+++ to remove unbound virus. Organoids were re-embedded into 30 μL BME

550   in 48-well tissue culture plates and cultured in 250 μL expansion or differentiation medium at 37°C

551   with 5% CO_2. Each well contained ~200,000 cells per well. At indicated time points cells were harvested

552   by resuspending the BME droplet containing organoids into 200 μL AdDF+++. Samples were stored at

553   -80°C, a process which lysed the organoids, releasing their contents into the medium upon thawing.

554   For testing the antiviral activity of chloroquine diphosphate (Sigma), camostat mesylate (Sigma) and

555   E64D (Medchemexpress) in intestinal organoids, sheared organoids were preincubated with these

556   compounds in AdDF+++ at the indicated concentrations for 30 min at 37°C 5% CO_2 before infection at

557   an MOI of 0.1 with SARS-CoV-2. After virus adsorption for 2 hours at 37°C 5% CO_2, organoids were

558   washed and re-embedded in BME as indicated above. Medium containing the inhibitors was added to

559   the wells for the duration of the experiment. Cells were harvested at indicated time points as described

560   above and stored at -80°C.

561   **SARS-CoV-2 entry inhibition assay by chloroquine in VeroE6 cells**

562   Chloroquine was two-fold serially diluted in Opti-MEM I (1X) + GlutaMAX starting from a concentration

563   of 100 μg/mL. 100 μl of each dilution was added to a confluent 96-well plate of VeroE6 cells and pre-

564   incubated at 37°C 5% CO_2 for 30 minutes. Next, cells were incubated with 400 plaque-forming units of

565   virus in the same concentration range of chloroquine at 37°C 5% CO_2. After 8 hours incubation, cells

566   were fixed with formalin, permeabilized with 70% ethanol and stained with polyclonal rabbit anti-

567   SARS-CoV nucleoprotein antibody (1:1000; 40588-T62, Sino Biological) followed by secondary

568   Alexa488 conjugated goat-anti-rabbit antibody (Invitrogen). Plates were scanned on the Amersham™

569 Typhoon™ Biomolecular Imager (channel Cy2; resolution 10µm; GE Healthcare). Data was analyzed

570 using ImageQuant TL 8.2 image analysis software (GE Healthcare).

Determination of virus titer using qRT-PCR

572 For determining the viral titer using qPCR, samples were thawed and centrifuged at 2,000 g for 5 min.

573 Sixty µL supernatant was lysed in 90 µL MagnaPure LC Lysis buffer (Roche) at RT for 10 min. RNA was

574 extracted by incubating samples with 50 µL Agencourt AMPure XP beads (Beckman Coulter) for 15 min

575 at RT, washing beads twice with 70% ethanol on a DynaMag-96 magnet (Invitrogen) and eluting in 30

576 µL MagnaPure LC elution buffer (Roche). Viral titers (TCID50 equivalents per mL) were determined by

577 qRT-PCR using primer-probe sets described previously[60–62] and comparing the Ct values to a standard

578 curve derived from a titrated virus stock.

Immunostainings and western blot

580 Organoids were stained as described before[55]. Whole organoids were collected by gently dissolving

581 the BME in ice-cold PBS, and subsequently fixed overnight at 4°C in 4% paraformaldehyde (Sigma).

582 Next, organoids were permeabilized and blocked in PBS containing 0,5% Triton X-100 (Sigma) and 2%

583 normal donkey serum (Jackson ImunoResearch) for 30 min at room temperature. All stainings were

584 performed in blocking buffer (2% normal donkey serum in PBS). For immunofluorescence, primary

585 antibodies used were mouse anti-nucleoprotein (1:200; 40143-MM05, Sino Biological), mouse anti-

586 dsRNA (1:200; Scicons), goat anti-ACE (1:100; R&D Systems, AF933), goat anti-DPP-4 (1:200; R&D

587 systems, AF1180) and rabbit anti-MERS S1 (1:200; 40069-T52, Sino Biological). For

588 immunofluorescence, organoids were incubated with the corresponding secondary antibodies

589 Alexa488-, 568- and 647-conjugated anti-rabbit and anti-goat (1:1,000; Molecular Probes) or

590 Phalloidin-Alexa488 (Thermofisher Scientific) in blocking buffer containing 4',6-diamidino-2-

591 phenylindole (DAPI; 1;1,000, Invitrogen). After staining, organoids were transfected to a glass slide

592 embedded in Vectashield (Vector labs). Stained organoids were imaged using a SP8 confocal

593 microscope (Leica) or a Zeiss LSM700, and image analysis and presentation was performed using

594 ImageJ software.

595 Immunohistochemistry was performed as described before[63]. Antigen retrieval for TMPRSS2 staining

596 was achieved by boiling for 20 minutes in citrate buffer. Primary antibody used was rabbit anti-

597 TMPRSS2 (1:100; Abcam, ab109131) followed by anti-rabbit conjugated to horseradish peroxidase

598 (Powervision, Leica)

599   For Western blot of CTSL, organoid proteins were solubilized using a standard RIPA buffer for 30 min

600   on ice in the presence of protease inhibitors. Samples were run on a 4-15% PAA gel (BioRad) under

601   reducing conditions. Proteins were electrophoresed to PVDF membranes from Immobilon.Both

602   primary antibodies, mouse anti-CTSL (± 25 kDa) and mouse anti-ITGB4 (± 200 kDa), were incubated

603   O/N at 4C in PBS/10% milk protein/0.1% Tween20. The secondary rabbit anti-mouse HRP-conjugate

604   (Dako) was incubated for 2hrs at 4C in the same buffer. The mouse IgG2a antibody against ITGB4,

605   58XB4, was a gift from A. Sonnenberg (NKI, Amsterdam, The Netherlands). HRP activity was visualized

606   with ECL (GE-Healthcare).

607

608   **Bulk RNA sequencing**

609   Single Cell Discoveries (Utrecht, The Netherlands) performed library preparation, using an adapted

610   version of the CEL-seq protocol, as we have done previously[17]. After library generation, paired-end

611   sequencing was performed on the Illumina Nextseq500 platform using barcoded 1 x 75 nt read setup.

612   Read 1 was used to identify the Illumina library index and CEL-Seq sample barcode. Read 2 was aligned

613   to the CRCh38 human RefSeq transcriptome, with the addition SARS-CoV-2 (Ref-SKU: 026V-03883) or

614   MERS (NC_038294.1) genomes, using BWA using standard settings[64]. Reads that mapped equally well

615   to multiple locations were discarded. Mapping and generation of count tables was performed using

616   the in-house MapAndGo script, filtering to exclude reads with identical library- and molecule-barcodes.

617   RNA sequencing data from expanding and differentiated human intestinal organoids, infected with

618   SARS-CoV-2, was used from a previous publication[17]. Normalization using the median of ratios method

619   and differential gene expression analysis was performed using the DESeq2 package[64]. SARS- and MERS-

620   mapping reads were removed before normalization to avoid biasing organoid transcript counts. To

621   generate heatmaps, row z-scores of selected genes were calculated from the samples selected.

622

623   **Organoid preparation for single cell sequencing analysis**

624   Human ileal organoids were differentiated as previously described[56]. A control condition was kept

625   inhuman organoid expansion medium to obtain stem- and progenitor cells for comparison.

626   Dissociation of organoids to single cells was performed by a 10-minute incubation with TrypLE (TrypLE

627   Express; Life Technologies) supported by repeated mechanical disruption using a narrowed glass

628   pipette. Viable cells were sorted using a BD FACS Aria (BD Biosciences) using DAPI exclusion. Individual

629   cells were collected in 384-well plates with ERCC spike-ins (Agilent), reverse transcription primers and

630   dNTPs (both Promega). Single cell sequencing was performed according to the Sort-seq method[65].

631 Sequencing libraries were generated with TruSeq small RNA primers (Illumina) and sequenced paired-

632 end at 60 and 26 bp read length, respectively, on the Illumina NextSeq.

633 **Single cell RNA sequencing analysis from intestinal organoids and tissue**

634 Reads derived from 1344 cells (192 expansion medium, 1152 differentiation medium) were mapped

635 to the human GRCh37 genome assembly. Sort-seq read counts were filtered to exclude reads with

636 identical library-, cell- and molecule barcodes. UMI counts were adjusted using Poisson counting

637 statistics[65]. Cells with fewer than 1,000 unique transcripts were excluded from further analysis. This

638 resulted in 944 remaining cells (126 from expansion, 818 from differentiation medium)

639 Subsequently, RaceID3 was used for k-medoids-based clustering (knn = 5; cln = 20) of cells and

640 differential gene expression analysis between clusters using the standard settings described at

641 https://github.com/dgrun/RaceID3_StemID2_package. Cell types were annotated by cluster based on

642 the expression of marker genes (OLFM4 for stem cells, FABP1 for enterocytes, FCGBP for goblet cells.

643 Lack of these and expression of cell cycle markers including PCNA defined proliferating progenitors

644 cells).

645 For comparison with tissue-derived cells, we reanalyzed a previously published dataset[32] of primary

646 human ileal cell types. To compare with the organoid data set, cells were required to be annotated as

647 stem cell, progenitor cell, goblet cell or enterocyte and exhibit more than 3,000 unique transcripts.

648 This resulted in 2137 included cells, which were subsequently clustered using the standard settings of

649 RaceID3 (cln = 16). Cells were assigned an identity based on their annotation from[32].

650 **Quantification and statistics**

651 No statistical methods were used to predetermine sample size. The experiments were not randomized

652 and the investigators were not blinded to the sample allocation during experiments and outcome

653 assessment. All data are presented as mean ± standard error of the mean (SEM), unless stated

654 otherwise. Value of n is always displayed in the figure as individual data points, and in the legends.

655 Statistical analysis was performed with the GraphPad Prism 9 software. We compared differences in

656 virus replication and organoid growth by one-way ANOVA followed by a multiple-comparison test

657 (Original FDR method of Benjamini and Hochberg; Q = 0.05) on log10 transformed values. Statistics

658 were applied if $N \geq 3$.

659 **Data availability statement**

660   All bulk and single cell RNA sequencing data of this study has been uploaded to the Gene Expression

661   Omnibus (GEO), and will be publically available upon publication (GEO accession number is pending).

662   **References**

663   1.   Zhou, P. *et al.* A pneumonia outbreak associated with a new coronavirus of probable bat

664   origin. *Nature* **579**, 270–273 (2020).

665   2.   Hulswit, R. J. G., de Haan, C. A. M. & Bosch, B. J. Coronavirus Spike Protein and Tropism

666   Changes. in *Advances in Virus Research* **96**, 29–57 (2016).

667   3.   Kaye, M. *et al.* SARS-associated Coronavirus Replication in Cell Lines. *Emerg. Infect. Dis.*

668   (2006). doi:10.3201/eid1201.050496

669   4.   Hoffmann, M. *et al.* SARS-CoV-2 Cell Entry Depends on ACE2 and TMPRSS2 and Is Blocked by a

670   Clinically Proven Protease Inhibitor. *Cell* **181**, 271-280.e8 (2020).

671   5.   Li, W. *et al.* Angiotensin-converting enzyme 2 is a functional receptor for the SARS

672   coronavirus. *Nature* (2003). doi:10.1038/nature02145

673   6.   Raj, V. S. *et al.* Dipeptidyl peptidase 4 is a functional receptor for the emerging human

674   coronavirus-EMC. *Nature* (2013). doi:10.1038/nature12005

675   7.   Wang, M. *et al.* Remdesivir and chloroquine effectively inhibit the recently emerged novel

676   coronavirus (2019-nCoV) in vitro. *Cell Research* (2020). doi:10.1038/s41422-020-0282-0

677   8.   Boulware, D. R. *et al.* A Randomized Trial of Hydroxychloroquine as Postexposure Prophylaxis

678   for Covid-19. *N. Engl. J. Med.* (2020). doi:10.1056/NEJMoa2016638

679   9.   Wei, J. *et al.* Genome-wide CRISPR Screens Reveal Host Factors Critical for SARS-CoV-2

680   Infection. *Cell* (2020). doi:10.1016/j.cell.2020.10.028

681   10.   Zhu, Y. *et al.* A genome-wide CRISPR screen identifies host factors that regulate SARS-CoV-2

682   entry. *Nat. Commun.* (2021). doi:10.1038/s41467-021-21213-4

683   11.   Mykytyn, A. Z. *et al.* The SARS-CoV-2 multibasic cleavage site facilitates early serine protease-

684   mediated entry into organoid-derived human airway cells 2 3. *bioRxiv* (2020).

685   12.   Clausen, T. M. *et al.* SARS-CoV-2 Infection Depends on Cellular Heparan Sulfate and ACE2. *Cell*

686   (2020). doi:10.1016/j.cell.2020.09.033

687   13.   Cantuti-Castelvetri, L. *et al.* Neuropilin-1 facilitates SARS-CoV-2 cell entry and infectivity.

688   *Science (80-.).* **370**, (2020).

689   14.   Daly, J. L. *et al.* Neuropilin-1 is a host factor for SARS-CoV-2 infection. *Science* **370**, 861–865
690   (2020).

691   15.   Shang, J. *et al.* Cell entry mechanisms of SARS-CoV-2. *Proc. Natl. Acad. Sci. U. S. A.* (2020).
692   doi:10.1073/pnas.2003138117

693   16.   Yang, L. *et al.* A Human Pluripotent Stem Cell-based Platform to Study SARS-CoV-2 Tropism
694   and Model Virus Infection in Human Cells and Organoids. *Cell Stem Cell* (2020).
695   doi:10.1016/j.stem.2020.06.015

696   17.   Lamers, M. M. *et al.* SARS-CoV-2 productively infects human gut enterocytes. *Science* (2020).
697   doi:10.1126/science.abc1669

698   18.   Zhou, J. *et al.* Infection of bat and human intestinal organoids by SARS-CoV-2. *Nat. Med.*
699   (2020). doi:10.1038/s41591-020-0912-6

700   19.   Cholankeril, G. *et al.* High Prevalence of Concurrent Gastrointestinal Manifestations in
701   Patients With Severe Acute Respiratory Syndrome Coronavirus 2: Early Experience From
702   California. *Gastroenterology* (2020). doi:10.1053/j.gastro.2020.04.008

703   20.   Gu, J., Han, B. & Wang, J. COVID-19: Gastrointestinal Manifestations and Potential Fecal–Oral
704   Transmission. *Gastroenterology* (2020). doi:10.1053/j.gastro.2020.02.054

705   21.   Drost, J. *et al.* Sequential cancer mutations in cultured human intestinal stem cells. *Nature*
706   **521**, 43–47 (2015).

707   22.   Millet, J. K. & Whittaker, G. R. Host cell proteases: Critical determinants of coronavirus
708   tropism and pathogenesis. *Virus Res.* (2015). doi:10.1016/j.virusres.2014.11.021

709   23.   Simmons, G., Zmora, P., Gierer, S., Heurich, A. & Pöhlmann, S. Proteolytic activation of the
710   SARS-coronavirus spike protein: Cutting enzymes at the cutting edge of antiviral research.
711   *Antiviral Research* **100**, 605–614 (2013).

712   24.   Nomura, R. *et al.* Human Coronavirus 229E Binds to CD13 in Rafts and Enters the Cell through
713   Caveolae. *J. Virol.* **78**, 8701–8708 (2004).

714   25.   Sung, P. S. & Hsieh, S. L. CLEC2 and CLEC5A: Pathogenic Host Factors in Acute Viral Infections.
715   *Frontiers in Immunology* **10**, (2019).

716   26.   Yu, Y. T. C. *et al.* Surface vimentin is critical for the cell entry of SARS-CoV. *J. Biomed. Sci.* **23**,
717   (2016).

718   27.   Miura, H. S., Nakagaki, K. & Taguchi, F. N-Terminal Domain of the Murine Coronavirus

719   Receptor CEACAM1 Is Responsible for Fusogenic Activation and Conformational Changes of

720   the Spike Protein. *J. Virol.* (2004). doi:10.1128/jvi.78.1.216-223.2004

721   28.   Earnest, J. T. *et al.* The tetraspanin CD9 facilitates MERS-coronavirus entry by scaffolding host

722   cell receptors and proteases. *PLoS Pathog.* **13**, (2017).

723   29.   Marzi, A. *et al.* DC-SIGN and DC-SIGNR Interact with the Glycoprotein of Marburg Virus and

724   the S Protein of Severe Acute Respiratory Syndrome Coronavirus. *J. Virol.* **78**, 12090–12095

725   (2004).

726   30.   Shi, C.-S. *et al.* SARS-Coronavirus Open Reading Frame-9b Suppresses Innate Immunity by

727   Targeting Mitochondria and the MAVS/TRAF3/TRAF6 Signalosome. *J. Immunol.* **193**, 3080–

728   3089 (2014).

729   31.   Chu, H. *et al.* Middle East respiratory syndrome coronavirus and bat coronavirus HKU9 both

730   can utilize GRP78 for attachment onto host cells. *J. Biol. Chem.* (2018).

731   doi:10.1074/jbc.RA118.001897

732   32.   Wang, Y. *et al.* Single-cell transcriptome analysis reveals differential nutrient absorption

733   functions in human intestine. *J. Exp. Med.* (2020). doi:10.1084/jem.20191130

734   33.   Zang, R. *et al.* TMPRSS2 and TMPRSS4 promote SARS-CoV-2 infection of human small

735   intestinal enterocytes. *Sci. Immunol.* (2020). doi:10.1126/sciimmunol.abc3582

736   34.   Wang, T. *et al.* Identification and characterization of essential genes in the human genome.

737   *Science (80-.).* **350**, 1096–1101 (2015).

738   35.   Winkler, E. S. *et al.* SARS-CoV-2 infection of human ACE2-transgenic mice causes severe lung

739   inflammation and impaired function. *Nat. Immunol.* (2020). doi:10.1038/s41590-020-0778-2

740   36.   Amraie, R. *et al.* CD209L/L-SIGN and CD209/DC-SIGN act as receptors for SARS-CoV-2 and are

741   differentially expressed in lung and kidney epithelial and endothelial cells. *bioRxiv Prepr. Serv.*

742   *Biol.* (2020). doi:10.1101/2020.06.22.165803

743   37.   Triana, S. *et al.* Single-cell analyses reveal SARS-CoV-2 interference with intrinsic immune

744   response in the human gut. *Mol. Syst. Biol.* **17**, e10232 (2021).

745   38.   Kindler, E., Thiel, V. & Weber, F. Interaction of SARS and MERS Coronaviruses with the

746   Antiviral Interferon Response. in *Advances in Virus Research* (2016).

747   doi:10.1016/bs.aivir.2016.08.006

748   39.   Lamers, M. M. *et al.* Human airway cells prevent SARS-CoV-2 multibasic cleavage site cell
749   culture adaptation. *Elife* (2021). doi:10.7554/elife.66815

750   40.   Klimstra, W. B. *et al.* SARS-CoV-2 growth, furin-cleavage-site adaptation and neutralization
751   using serum from acutely infected hospitalized COVID-19 patients. *J. Gen. Virol.* (2020).
752   doi:10.1099/jgv.0.001481

753   41.   Ogando, N. S. *et al.* SARS-coronavirus-2 replication in Vero E6 cells: Replication kinetics, rapid
754   adaptation and cytopathology. *J. Gen. Virol.* (2020). doi:10.1099/jgv.0.001453

755   42.   Davidson, A. D. *et al.* Characterisation of the transcriptome and proteome of SARS-CoV-2
756   reveals a cell passage induced in-frame deletion of the furin-like cleavage site from the spike
757   glycoprotein. *Genome Med.* (2020). doi:10.1186/s13073-020-00763-0

758   43.   Peacock, T. P. *et al.* The furin cleavage site in the SARS-CoV-2 spike protein is required for
759   transmission in ferrets. *Nat. Microbiol.* (2021). doi:10.1038/s41564-021-00908-w

760   44.   Graham, M. S. *et al.* Changes in symptomatology, reinfection, and transmissibility associated
761   with the SARS-CoV-2 variant B.1.1.7: an ecological study. *Lancet Public Heal.* (2021).
762   doi:10.1016/s2468-2667(21)00055-4

763   45.   Frampton, D. *et al.* Genomic characteristics and clinical effect of the emergent SARS-CoV-2
764   B.1.1.7 lineage in London, UK: a whole-genome sequencing and hospital-based cohort study.
765   *Lancet Infect. Dis.* (2021). doi:10.1016/s1473-3099(21)00170-5

766   46.   Volz, E. *et al.* Assessing transmissibility of SARS-CoV-2 lineage B.1.1.7 in England. *Nature*
767   (2021). doi:10.1038/s41586-021-03470-x

768   47.   WHO Solidarity Trial Consortium *et al.* Repurposed Antiviral Drugs for Covid-19 - Interim WHO
769   Solidarity Trial Results. *N. Engl. J. Med.* (2020). doi:10.1056/NEJMoa2023184

770   48.   Vincent, M. J. *et al.* Chloroquine is a potent inhibitor of SARS coronavirus infection and
771   spread. *Virol. J.* **2**, (2005).

772   49.   Daniloski, Z. *et al.* Identification of Required Host Factors for SARS-CoV-2 Infection in Human
773   Cells. *Cell* (2020). doi:10.1016/j.cell.2020.10.030

774   50.   Schneider, W. M. *et al.* Genome-scale identification of SARS-CoV-2 and pan-coronavirus host
775   factor networks. *Cell* (2020). doi:10.1016/j.cell.2020.12.006

776   51.   Hoffmann, M. *et al.* Camostat mesylate inhibits SARS-CoV-2 activation by TMPRSS2-related
777   proteases and its metabolite GBPA exerts antiviral activity. *EBioMedicine* (2021).

778   doi:10.1016/j.ebiom.2021.103255

779   52. Singh, M., Bansal, V. & Feschotte, C. A Single-Cell RNA Expression Map of Human Coronavirus
780   Entry Factors. *Cell Rep.* (2020). doi:10.1016/j.celrep.2020.108175

781   53. Kim, T. S., Heinlein, C., Hackman, R. C. & Nelson, P. S. Phenotypic Analysis of Mice Lacking the
782   Tmprss2-Encoded Protease. *Mol. Cell. Biol.* **26**, 965–975 (2006).

783   54. Iwata-Yoshikawa, N. *et al.* TMPRSS2 Contributes to Virus Spread and Immunopathology in the
784   Airways of Murine Models after Coronavirus Infection. *J. Virol.* **93**, (2019).

785   55. Beumer, J. *et al.* Enteroendocrine cells switch hormone expression along the crypt-to-villus
786   BMP signalling gradient. *Nature Cell Biology* **20**, 909–916 (2018).

787   56. Sato, T. *et al.* Long-term expansion of epithelial organoids from human colon, adenoma,
788   adenocarcinoma, and Barrett's epithelium. *Gastroenterology* **141**, 1762–1772 (2011).

789   57. Sachs, N. *et al.* Long-term expanding human airway organoids for disease modeling. *EMBO J.*
790   **38**, (2019).

791   58. Ran, F. A. *et al.* Genome engineering using the CRISPR-Cas9 system. *Nat Protoc.* **8**, (2013).

792   59. Fujii, M., Matano, M., Nanki, K. & Sato, T. Efficient genetic engineering of human intestinal
793   organoids using electroporation. *Nat. Protoc.* **10**, 1474–1485 (2015).

794   60. Corman, V. M. *et al.* Detection of a novel human coronavirus by real-time reverse-
795   transcription polymerase chain reaction. *Eurosurveillance* (2012).
796   doi:10.2807/ese.17.39.20285-en

797   61. Corman, V. M. *et al.* Detection of 2019 novel coronavirus (2019-nCoV) by real-time RT-PCR.
798   *Eurosurveillance* (2020). doi:10.2807/1560-7917.ES.2020.25.3.2000045

799   62. Kuiken, T. *et al.* Newly discovered coronavirus as the primary cause of severe acute
800   respiratory syndrome. *Lancet* **362**, 263–270 (2003).

801   63. Muncan, V. *et al.* Rapid Loss of Intestinal Crypts upon Conditional Deletion of the Wnt/Tcf-4
802   Target Gene c-Myc. *Mol. Cell. Biol.* (2006). doi:10.1128/mcb.00821-06

803   64. Love, M. I., Huber, W. & Anders, S. Moderated estimation of fold change and dispersion for
804   RNA-seq data with DESeq2. *Genome Biol.* **15**, 550 (2014).

805   65. Muraro, M. J. *et al.* A Single-Cell Transcriptome Atlas of the Human Pancreas. *Cell Syst.* **3**, 385–
806   394 (2016).

807

Figure 1 Expression levels of potential host genes and proteases in lung and intestinal organoids and tissue

Figure 2 Generation of a coronavirus host gene knockout biobank
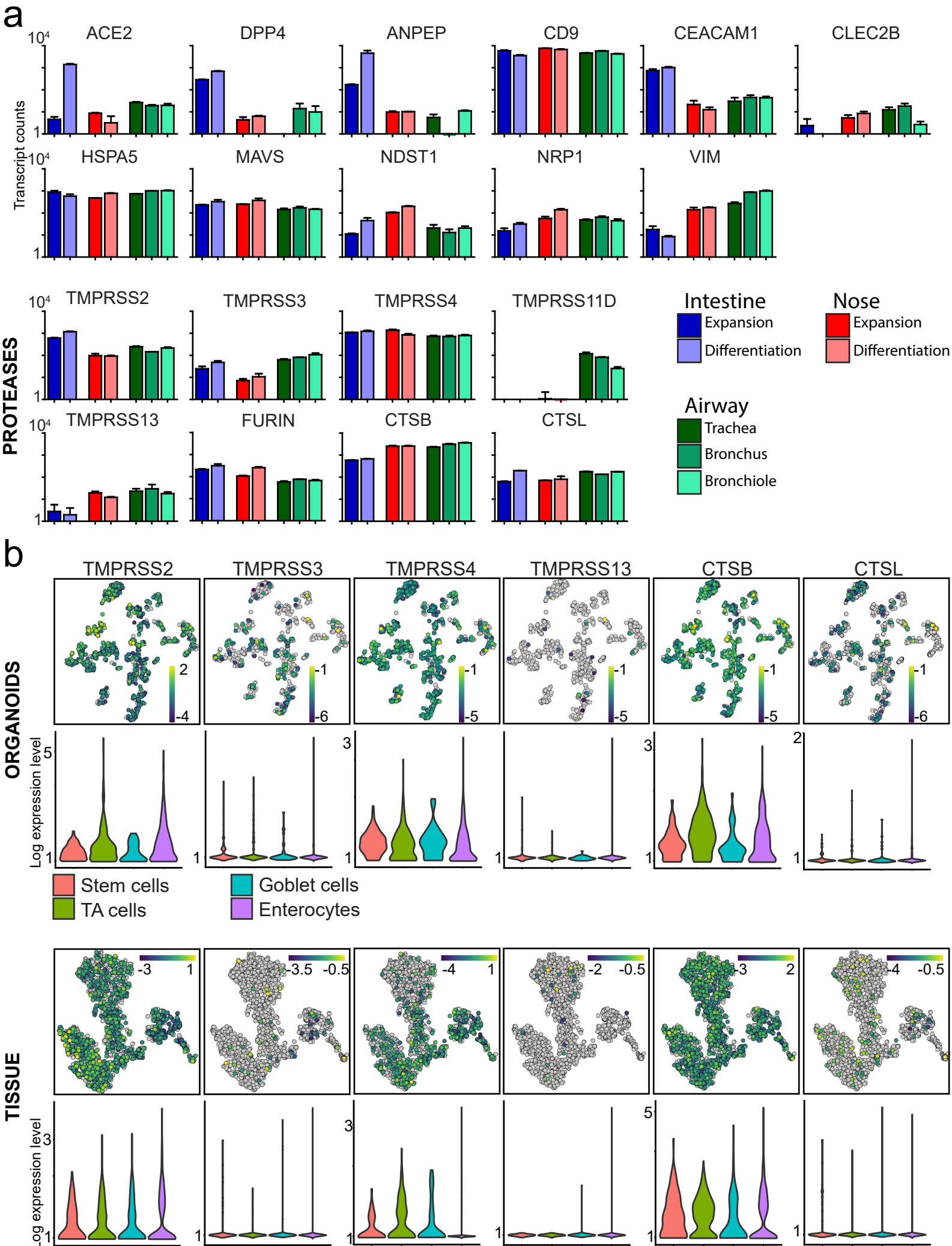
a
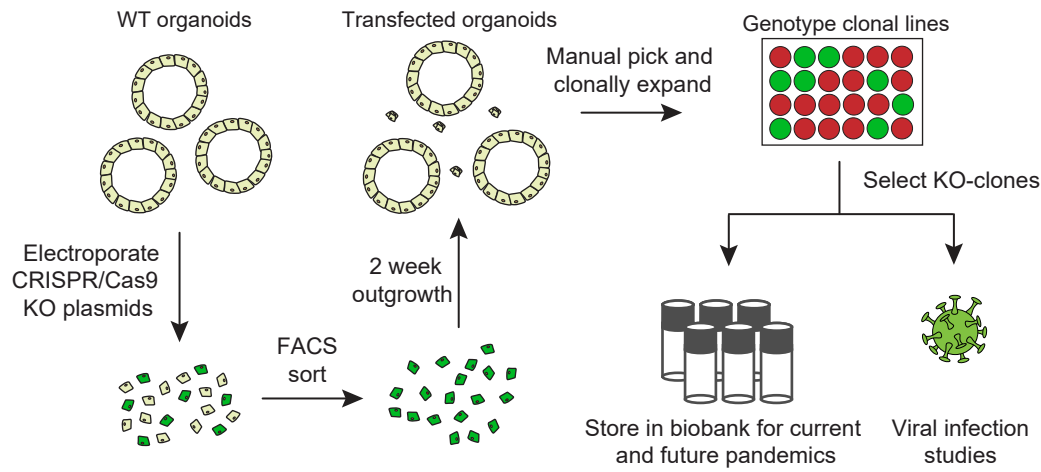
b

Ileum		Duodenum	
	Number of KO clones		*Number of KO clones*
Wildtype	4	Wildtype	2
ACE2	4	ACE2	4
DPP4	4		
NRP1	4		
ANPEP	3		
DC-SIGN	4		
CD9	2		
TMPRSS2	4	TMPRSS2	4
TMPRSS3	4		
TMPRSS4	4		
TMPRSS11D	5		
TMPRSS13	6		
FURIN	1		
CTSB	6		
CTSL	2		
ARC	1		
CLEC2B	2		
MAVS	2		
VIMENTIN	2		
HSPA5	0		
NDST1	2		
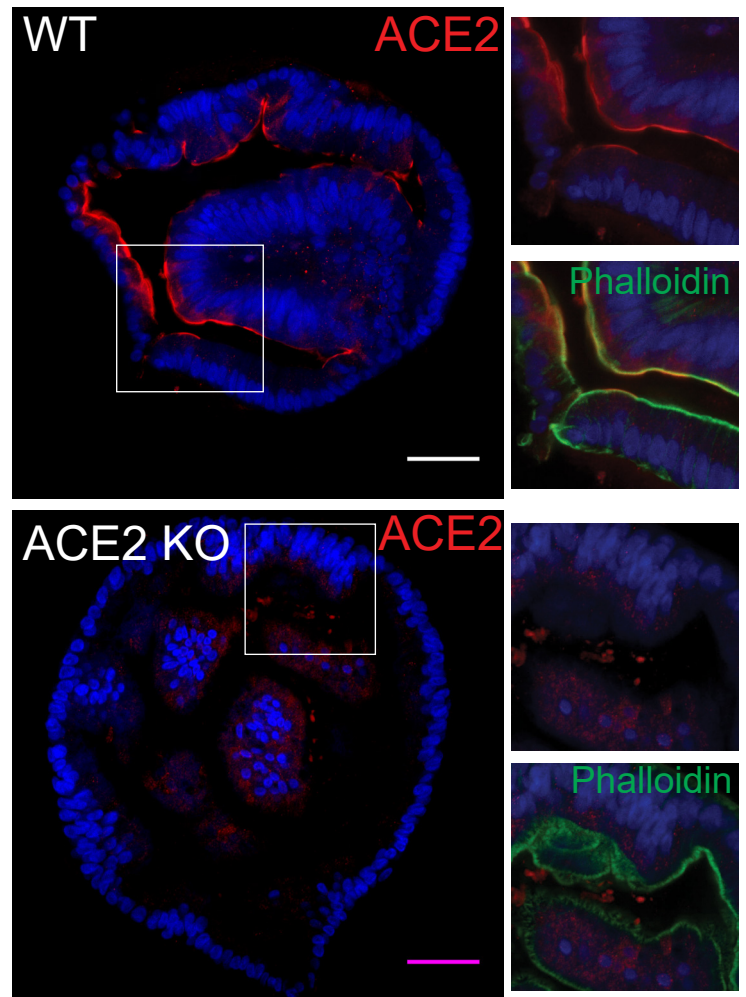
(RECEPTORS: ACE2, DPP4, NRP1, ANPEP, DC-SIGN, CD9; PROTEASES: TMPRSS2, TMPRSS3, TMPRSS4, TMPRSS11D, TMPRSS13, FURIN, CTSB, CTSL; OTHER: ARC, CLEC2B, MAVS, VIMENTIN, HSPA5, NDST1)

c

Figure 3 ACE2 and DPP4 are the obligate entry receptors for SARS-CoV-2 and MERS-CoV respectively
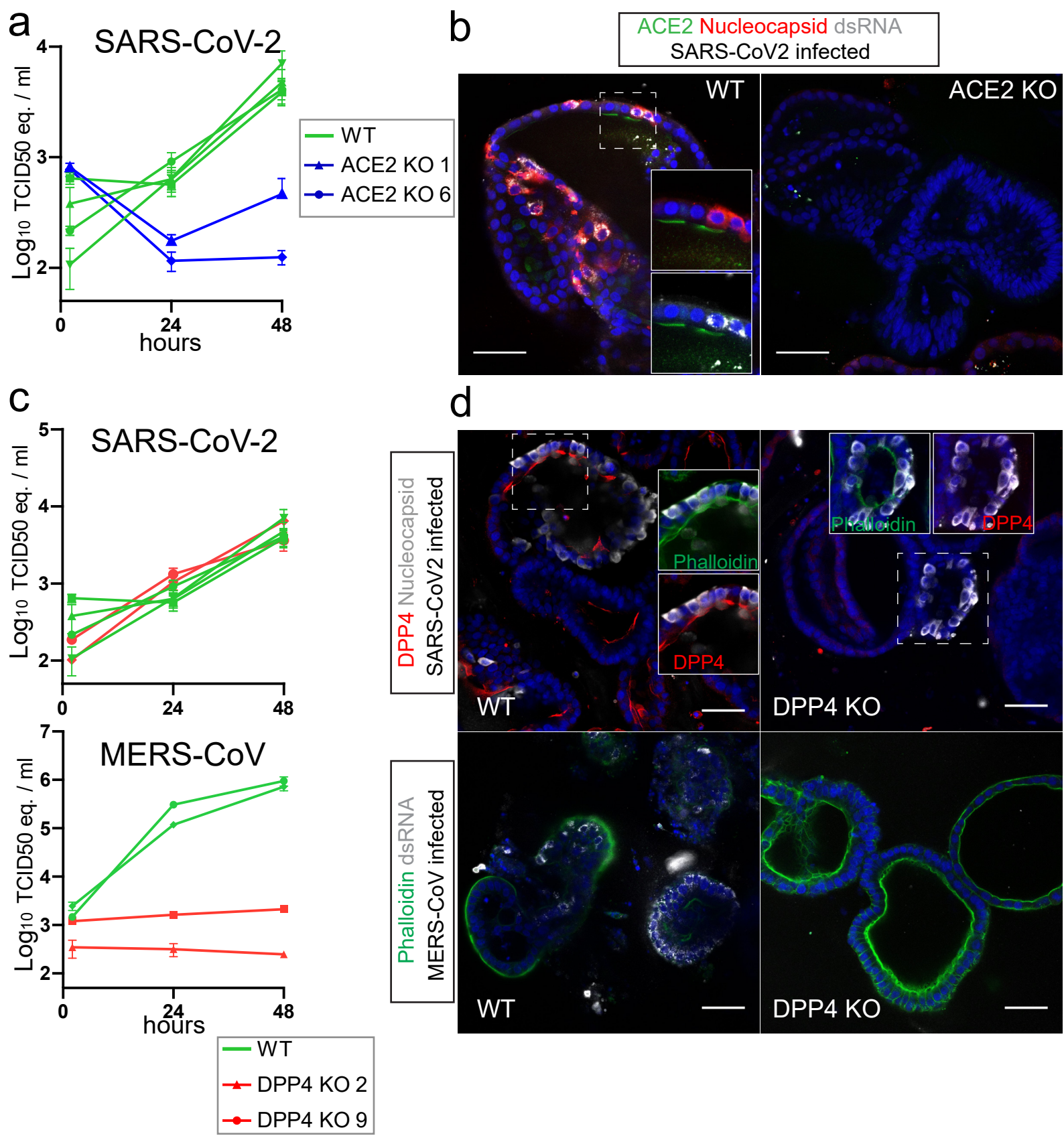
Figure 4 Loss-of-function screen of host proteases reveals essential role for TMPRSS2 but not Cathepsins in viral replication
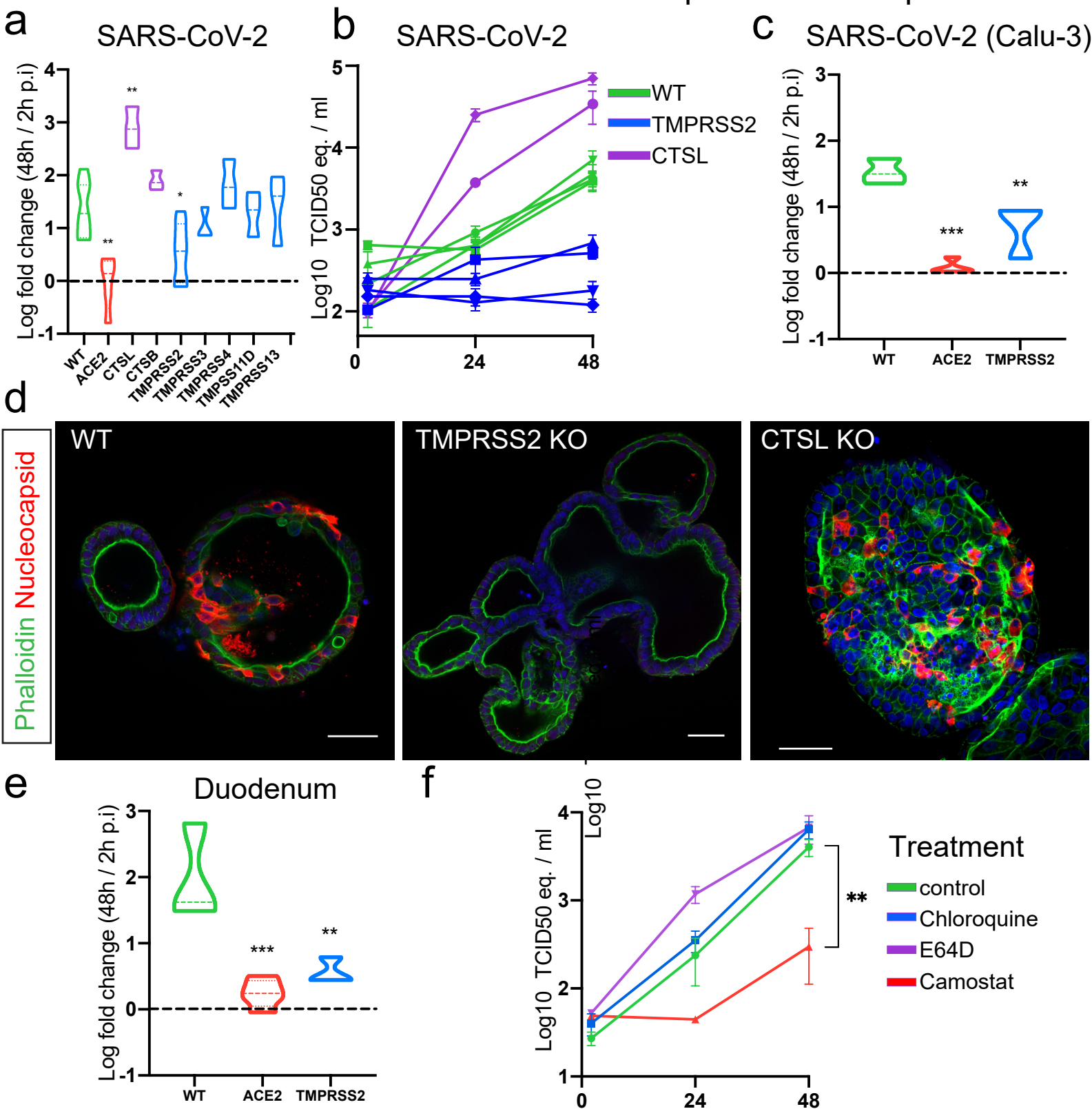
Figure 5 Replication of SARS-CoV-2 is not altered by loss of different host factors

Figure 6 Host protease dependency in the SARS-CoV-2 strain B.1.1.7 and other coronaviruses

Bulk and single cell RNA sequencing reveals intestinal expression of host proteases involved in viral entry

Supplementary figure 2 Generation of a host factor loss-of-function organoid biobank

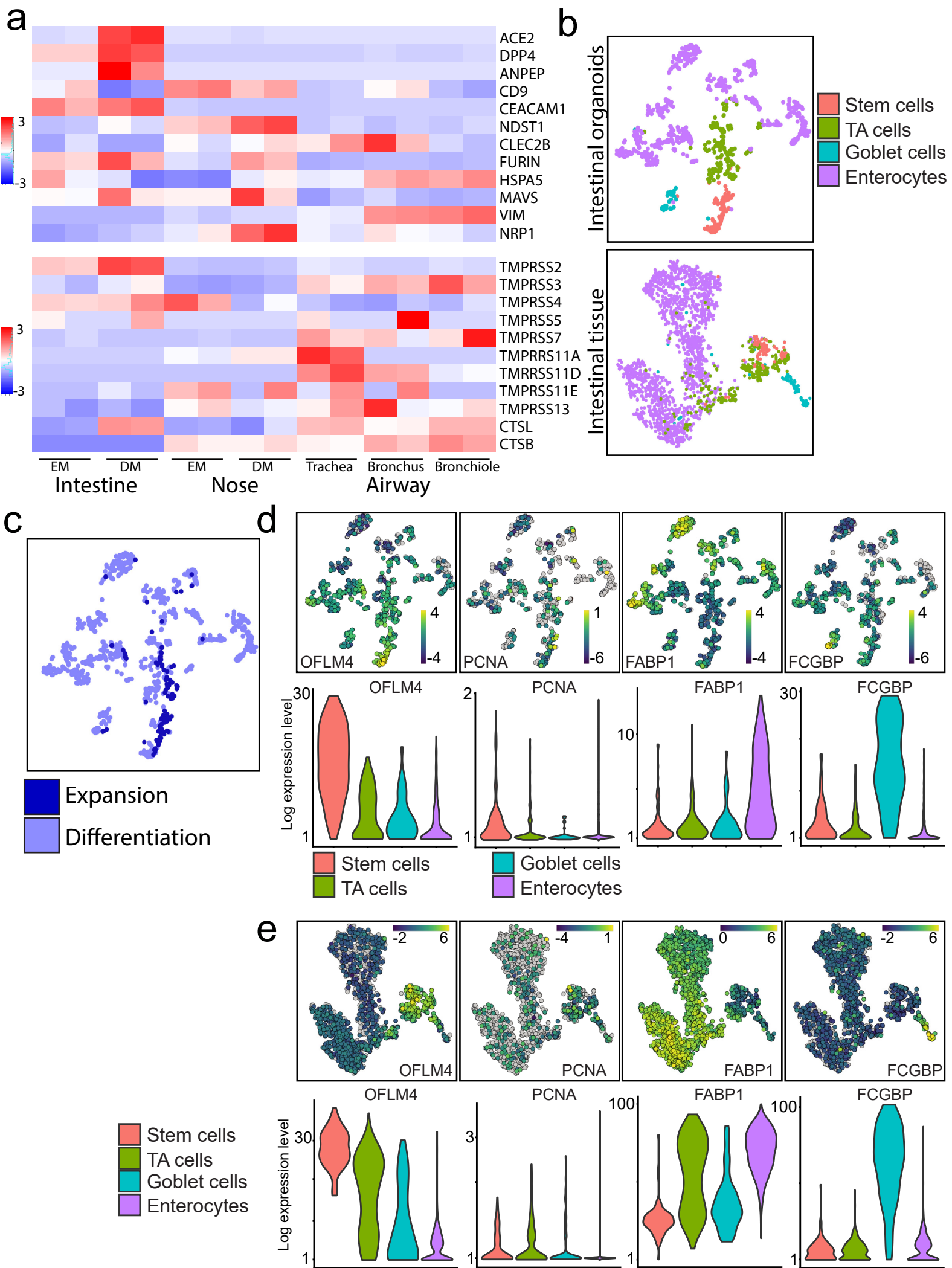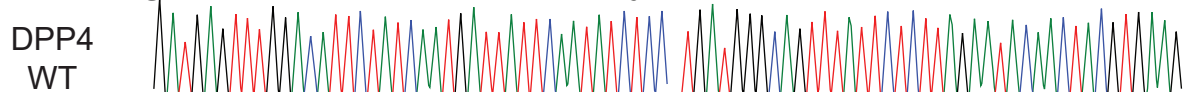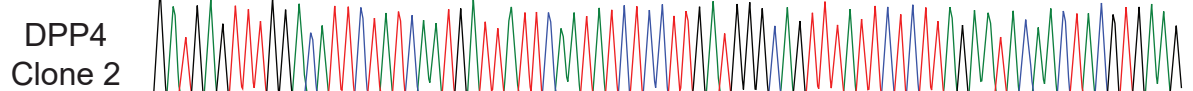| Gene / Clone	Allele	Sequence
ACE2 Clone 1	Wild-type	GCCAGTTGATTGAAGATGTGGAACATACCTTTGAA
	Allele 1 del4	GCCAGTTGATT----ATGTGGAACATACCTTTGAA
	Allele 2 X	x
ACE2 Clone 6	Wild-type	GCCAGTTGATTGAAGATGTGGAACATACCTTTGAA
	Allele 1 ins2	GCCAGTTGATTGAAGGATTGTGGAACATACCTTTG
	Allele 2 X	x
TMPRSS2 Clone 1	Wild-type	GCAGCCCAAATCCCCATCCGGGACAGTGTGCACCT
	Allele 1 del4	GCAGCCCAAATCCCC----GGGACAGTGTGCACCT
	Allele 2 ins1	GCAGCCCAAATCCCTCATCCGGGACAGTGTGCACC
TMPRSS2 Clone 4	Wild-type	GCAGCCCAAATCCCCATCCGGGACAGTGTGCACCT
	Allele 1 del1	GCAGCCCAAATCCC-ATCGGGACAGTGTGCACCT
	Allele 2 del1	GCAGCCCAAATCCC-ATCGGGACAGTGTGCACCT
TMPRSS2 Clone 6	Wild-type	GCAGCCCAAATCCCCATCCGGGACAGTGTGCACCT
	Allele 1 ins1	GCAGCCCAAATCCCCCATCCGGGACAGTGTGCAC
	Allele 2 ins2	GCAGCCCAAATCCCCCCATCCGGGACAGTGTGCA
TMPRSS2 Clone 9	Wild-type	GCAGCCCAAATCCCCATCCGGGACAGTGTGCACCT
	Allele 1 ins1	GCAGCCCAAATCCCCCATCCGGGACAGTGTGCAC
	Allele 2 del1	GCAGCCCAAATCCC-ATCGGGACAGTGTGCACCT
CTSL Clone 15	Wild-type	AATCAGGAATACAGGGAAGGGAAACACAGCTTCAC
	Allele 1 ins1	AATCAGGAATTACAGGGAAGGGAAACACAGCTTCA
	Allele 2 ins1	AATCAGGAATGACAGGGAAGGGAAACACAGCTTCA
CTSL Clone 16	Wild-type	AATCAGGAATACAGGGAAGGGAAACACAGCTTCAC
	Allele 1 del26	AATCAGG--------------------------AC
	Allele 2 del26	AATCAGG--------------------------AC
MAVS Clone 3	Wild-type	TACTTCATTGCGGCACTGAGGGGCTGTGAGCTAGT
	Allele 1 del2	TACTTCATTGCGGCA--GAGGGGCTGTGAGCTAGT
	Allele 2 ins5	TACTTCATTGCG-CAACTTACTGAGGGGCTGTGAG
MAVS Clone 6	Wild-type	TACTTCATTGCGGCACTGAGGGGCTGTGAGCTAGT
	Allele 1 ins1	TACTTCATTGCGGCACTTGAGGGGCTGTGAGCTAG
	Allele 2 ins1	TACTTCATTGCGGCACTTGAGGGGCTGTGAGCTAG
ANPEP Clone 10	Wild-type	TCACGCTTATCCACCCCAAGGACCTGACAGCCCTG
	Allele 1 del4	TCACGCTTAT----CCCAAGGACCTGACAGCCCTG
	Allele 2 del4	TCACGCTTAT----CCCAAGGACCTGACAGCCCTG
ANPEP Clone 11	Wild-type	TCACGCTTATCCACCCCAAGGACCTGACAGCCCTG
	Allele 1 del1	TCACGCTTATCCA-CCCCAAGGACCTGACAGCCCTG
	Allele 2 del1	TCACGCTTATCCA-CCCCAAGGACCTGACAGCCCTG
CLEC2B Clone 1	Wild-type	AGAGTTTATGCCCCTATGATTGGATTGGTTTCCAAA
	Allele 1 ins1	AGAGTTTATGCCCCTATTGATTGGATTGGTTTCCAA
	Allele 2 ins1	AGAGTTTATGCCCCTATTGATTGGATTGGTTTCCAA
CLEC2B Clone 9	Wild-type	AGAGTTTATGCCCCTATGATTGGATTGGTTTCCAAA
	Allele 1 del2	AGAGTTTATGCCCCTA--ATTGGATTGGTTTCCAAA
	Allele 2 del1	AGAGTTTATGCCCCTA-GATTGGATTGGTTTCCAAA
VIM Clone 1	Wild-type	AGGGAGATGCTTCAGAGAGAGGAAGCCGAAAACAC
	Allele 1 ins1	AGGGAGATGCTTCAGAGAAGAGGAAGCCGAAAACA
	Allele 2 ins1	AGGGAGATGCTTCAGAGAGGAGGAAGCCGAAAACA
VIM Clone 9	Wild-type	AGGGAGATGCTTCAGAGAGAGGAAGCCGAAAACAC
	Allele 1 del4	AGGGAGATGCTTC----AGAGGAAGCCGAAAACAC
	Allele 2 del2	AGGGAGATGCTTCA--GAGGAAGCCGAAAACAC
CD9 Clone 2	Wild-type	GATTGCTGTCCTTGCCATTGGACTATGGCTCCGAT
	Allele 1 del1	GATTGCTGTCCTTGCCATT-GACTATGGCTCCGAT
	Allele 2 ins1	GATTGCTGTCCTTGCCATT-GACTATGGCTCCGAT
CD9 Clone 4	Wild-type	GATTGCTGTCCTTGCCATTGGACTATGGCTCCGAT
	Allele 1 del1	GATTGCTGTCCTTGCCATT-GACTATGGCTCCGAT
	Allele 2 ins1	GATTGCTGTCCTTGCCATTGCGACTATGGCTCCGA
ARC Clone 18	Wild-type	TGCTGGAGCACGTGCGGCGGACGCACCGGCACCT
	Allele 1 del1	TGCTGGAGCACG-GCGGCGGACGCACCGGCACCT
	Allele 2 ins1	TGCTGGAGCACGTGGCGGCGGACGCACCGGCACC
CAECAM1 Clone 2	Wild-type	CCATTCAATGTTGCAGAGGGGAAGGAGGTTCTTCT
	Allele 1 ins1	CCATTCAATGTTGCAAGAGGGGAAGGAGGTTCTTC
	Allele 2 ins1	CCATTCAATGTTGCAAGAGGGGAAGGAGGTTCTTC
CAECAM1 Clone 3	Wild-type	CCATTCAATGTTGCAGAGGGGAAGGAGGTTCTTCT
	Allele 1 ins1	CCATTCAATGTTGCAAGAGGGGAAGGAGGTTCTTC
	Allele 2 ins1	CCATTCAATGTTGCAAGAGGGGAAGGAGGTTCTTC
CAECAM1 Clone 5	Wild-type	CCATTCAATGTTGCAGAGGGGAAGGAGGTTCTTCT
	Allele 1 ins1	CCATTCAATGTTGCAAGAGGGGAAGGAGGTTCTTC
	Allele 2 ins1	CCATTCAATGTTGCAAGAGGGGAAGGAGGTTCTTC
CTSB Clone 3	Wild-type	CACGGGAACAATGGCCACAGTGTCCCACCATCAAA
	Allele 1 ins1	CACGGGAACAATGGCCACAGTTGTCCCACCATCAA
	Allele 2 ins1	CACGGGAACAATGGCCACAGTTGTCCCACCATCAA
CTSB Clone 4	Wild-type	CACGGGAACAATGGCCACAGTGTCCCACCATCAAA
	Allele 1 del2	CACGGGAACAATGGCCACA--GTCCCACCATCAAA
	Allele 2 del2	CACGGGAACAATGGCCACA--GTCCCACCATCAAA
CTSB Clone 7	Wild-type	CACGGGAACAATGGCCACAGTGTCCCACCATCAAA
	Allele 1 ins1	CACGGGAACAATGGCCACAGTTGTCCCACCATCAA
	Allele 2 ins1	CACGGGAACAATGGCCACAGTTGTCCCACCATCAA
Furin Clone 9	Wild-type	TGGTGTAGGTGTGGCCTACAACGCCCGCATTGGA
	Allele 1 del1	TGGTGTAGGTGTGGCCT-CAACGCCCGCATTGGA
	Allele 2 del1	TGGTGTAGGTGTGGCCT-CAACGCCCGCATTGGA
DPP4 Clone 2	Wild-type	GATTATTCAATATCTCCTGATGGGCAGTTTATTCTCT
	Allele 1 ins1	GATTATTCAATATCTCCTTGATGGGCAGTTTATTCTC
	Allele 2 ins1	GATTATTCAATATCTCCTTGATGGGCAGTTTATTCTC
DPP4 Clone 9	Wild-type	GATTATTCAATATCTCCTGATGGGCAGTTTATTCTCT
	Allele 1 ins1	GATTATTCAATATCTCCTTGATGGGCAGTTTATTCTC
	Allele 2 del5	GATTATTCAATAT-----GATGGGCAGTTTATTCTCT
NDST1 Clone 2	Wild-type	CAACCCCAAGTCCCCGCTGCTCTACGTGACGCGGAC
	Allele 1 ins1	CAACCCCAAGTCCCCGCTTGCTCTACGTGACGCGGA
	Allele 2 ins1	CAACCCCAAGTCCCCGCTTGCTCTACGTGACGCGGA
NDST1 Clone 3	Wild-type	CAACCCCAAGTCCCCGCTGCTCTACGTGACGCGGAC
	Allele 1 ins1	CAACCCCAAGTCCCCGCTTGCTCTACGTGACGCGGA
	Allele 2 ins1	CAACCCCAAGTCCCCGCTTGCTCTACGTGACGCGGA
NRP1 Clone 2	Wild-type	ACAGCGCGATAGCAAAAGAGGTTTCTCAGCAAACT
	Allele 1 ins1	ACAGCGCGAATAGCAAAAGAAGGTTTCTCAGCAAAC
	Allele 2 ins1	ACAGCGCGAGTAGCAAAAGAAGGTTTCTCAGCAAAC
NRP1 Clone 11	Wild-type	ACAGCGCGATAGCAAAAGAGGTTTCTCAGCAAACT
	Allele 1 ins1	ACAGCGCGAATAGCAAAAGAAGGTTTCTCAGCAAAC
	Allele 2 ins1	ACAGCGCGAATAGCAAAAGAAGGTTTCTCAGCAAAC
DC-SIGN Clone 6	Wild-type	TGGATTCCGACAGACTCGAGGATACAAGAGCTTAGC
	Allele 1 del5	TGGATTCCGACAGA-----GGATACAAGAGCTTAGC
	Allele 2 del1	TGGATTCCGACAGAG-CTCGAGGATACAAGAGCTTAG
DC-SIGN Clone 11	Wild-type	TGGATTCCGACAGACTCGAGGATACAAGAGCTTAGC
	Allele 1 del1	TGGATTCCGACAG-CTCGAGGATACAAGAGCTTAGC
	Allele 2 del1	TGGATTCCGACAG-CTCGAGGATACAAGAGCTTAGC
TMPRSS4 Clone 7	Wild-type	TGGCAGGTCAGCATCCAGTACGACAAACAGCACGT
	Allele 1 ins1	TGGCAGGTCAGCATCCAGTATCGACAAACAGCACG
	Allele 2 ins1	TGGCAGGTCAGCATCCAGTATCGACAAACAGCACG
TMPRSS4 Clone 9	Wild-type	TGGCAGGTCAGCATCCAGTACGACAAACAGCACGT
	Allele 1 ins2	TGGCAGGTCAGCATCCAGTAAACGACAAACAGCAC
	Allele 2 ins2	TGGCAGGTCAGCATCCAGTAAACGACAAACAGCAC
TMPRSS4 Clone 11	Wild-type	TGGCAGGTCAGCATCCAGTACGACAAACAGCACGT
	Allele 1 del1	TGGCAGGTCAGCATCCAGTA-GACAAACAGCACGT
	Allele 2 del1	TGGCAGGTCAGCATCCAGTA-GACAAACAGCACGT
TMPRSS3 Clone 1	Wild-type	GAGTGAGCTCGCTGGAGGGGCAGTTCCGGGAGG
	Allele 1 ins1	GAGTGAGCTCGCTGAGAGGGGCAGTTCCGGGAG
	Allele 2 ins1	GAGTGAGCTCGCTGTGAGGGGCAGTTCCGGGAG
TMPRSS3 Clone 4	Wild-type	GAGTGAGCTCGCTGGAGGGGCAGTTCCGGGAGG
	Allele 1 ins2	GAGTGAGCTCGCTGTGGAGGGGCAGTTCCGGGA
	Allele 2 ins1	GAGTGAGCTCGCTGTGAGGGGCAGTTCCGGGAG
TMPRSS3 Clone 7	Wild-type	GAGTGAGCTCGCTGGAGGGGCAGTTCCGGGAGG
	Allele 1 del1	GAGTGAGCTCGCTG-AGGGGCAGTTCCGGGAGG
	Allele 2 del1	GAGTGAGCTCGCTG-AGGGGCAGTTCCGGGAGG
TMPRSS11D Clone 7	Wild-type	GGAATACAGGACTTTGAGTGGAAGAATTGAATCTC
	Allele 1 del4	GGAATACAGGACTTTT----GAGTGGAAGAATTGAATCT
	Allele 2 del2	GGAATACAGGACTTTT--GAGTGGAAGAATTGAATCT
TMPRSS11D Clone 8	Wild-type	GGAATACAGGACTTTGAGTGGAAGAATTGAATCTC
	Allele 1 ins1	GGAATACAGGACTTTTGAGTGGAAGAATTGAATCT
	Allele 2 del5	GGAATACAGG-----GAGTGGAAGAATTGAATCTC
TMPRSS11D Clone 9	Wild-type	GGAATACAGGACTTTGAGTGGAAGAATTGAATCTC
	Allele 1 del1	GGAATACAGGACTTT-GAGTGGAAGAATTGAATCT
	Allele 2 ins1	GGAATACAGGACTTTTGAGTGGAAGAATTGAATCT
TMPRSS13 Clone 2	Wild-type	AAGACCTGCCAGCAGCTGGGTTTCGAGAGGTAAC
	Allele 1 ins1	AAGACCTGCCAGCAAGCTGGGTTTCGAGAGGTAA
	Allele 2 ins1	AAGACCTGCCAGCAAGCTGGGTTTCGAGAGGTAA
TMPRSS13 Clone 11	Wild-type	AAGACCTGCCAGCAGCTGGGTTTCGAGAGGTAAC
	Allele 1 ins1	AAGACCTGCCAGCAAGCTGGGTTTCGAGAGGTAA
	Allele 2 ins1	AAGACCTGCCAGCAAGCTGGGTTTCGAGAGGTAA
TMPRSS13 Clone 12	Wild-type	AAGACCTGCCAGCAGCTGGGTTTCGAGAGGTAAC
	Allele 1 ins1	AAGACCTGCCAGCAAGCTGGGTTTCGAGAGGTAA
	Allele 2 ins1	AAGACCTGCCAGCAAGCTGGGTTTCGAGAGGTAA
Duo TMPRSS2 Clone 9	Wild-type	GCAGCCCAAATCCCCATCCGGGACAGTGTGCACCT
	Allele 1 del26	GCA--------------------------GCACCT
	Allele 2 del26	GCA--------------------------GCACCT
Duo ACE2 Clone 4	Wild-type	GGCCAGTTGATTGAAGATGTGGAACATACCTTTGAA
	Allele 1 del4	GGCCAGTTGATT----ATGTGGAACATACCTTTGAA
	Allele 2 del4	GGCCAGTTGATT----ATGTGGAACATACCTTTGAA
Duo ACE2 Clone 12	Wild-type	GCGGCCAGTTGATTGAAGATGTGGAACATACCTTTG
	Allele 1 del11	GCGGCCAGTTGATTG-----------CATACCTTTG
	Allele 2 del25	GC-------------------------ATACCTTTG
T2/T4 Clone 1	Wild-type	TGGCAGGTCAGCATCCAGTACGACAAACAGCACGT
	Allele 1 del19	TGGCAGGT-------------------CAGCAC
	Allele 2 del19	TGGCAGGT-------------------CAGCAC
CTSL/CTSB Clone 1	Wild-type	AATCAGGAATACAGGGAAGGGAAACACAGCTTCAC
	Allele 1 ins1	AATCAGGAATTACAGGGAAGGGAAACACAGCTTCA
	Allele 2 ins1	AATCAGGAATTACAGGGAAGGGAAACACAGCTTCA
CTSL/CTSB Clone 9	Wild-type	AATCAGGAATACAGGGAAGGGAAACACAGCTTCAC
	Allele 1 del4	AATC----ATACAGGGAAGGGAAACACAGCTTCAC
	Allele 2 del4	AATC----ATACAGGGAAGGGAAACACAGCTTCAC

Supplementary figure 3 Organoid genotyping by sanger sequencing and in silico ICE analysis.
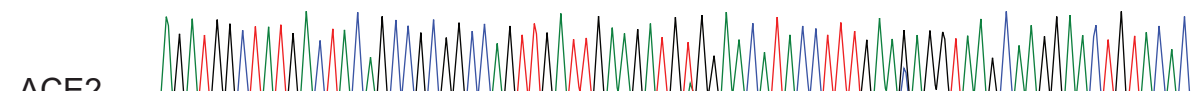

DPP4 WT

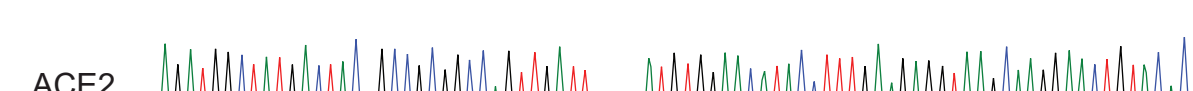GATGAGTTTGGACATTCTATCAATGATTATTCAATATCTCC-TGATGGGCAGTTTATTCTCTTAGAATACAACTACGTGAAG

DPP4 Clone 2

GATGAGTTTGGACATTCTATCAATGATTATTCAATATCTCCTTGATGGGCAGTTTATTCTCTTAGAATACAACTACGTGAAG

INDEL	CONTRIBUTION ▾	SEQUENCE
+1	99%	TATCAATGATTATTCAATATCTCCT ┊ NGATGGGCAGTTTATTCTCTTAGA

ACE2 WT

AGATGGCTATGACTACAGCCGCGGCCAGTTGATTGAAGATGTGGAACATACCTTTGAAGAGGTAAGCAAGGAACTGTACAC

ACE2 Clone 1

AGATGGCTATGACTACAGCCGCGGCCAGTTGATT----ATGTGGAACATACCTTTGAAGAGGTAAGCAAGGAACTGTACAC

INDEL	CONTRIBUTION ▾	SEQUENCE
−4	89%	TACAGCCGCGGCCAGTTGATT----┊ATGTGGAACATACCTTTGAAGAGGTAA
−4	3%	TACAGCCGCGGCCAGTTGATTG---┊-TGTGGAACATACCTTTGAAGAGGTAA
−4	1%	TACAGCCGCGGCCAGTTGATTGAA-┊---TGGAACATACCTTTGAAGAGGTAA

TMPRSS2 WT
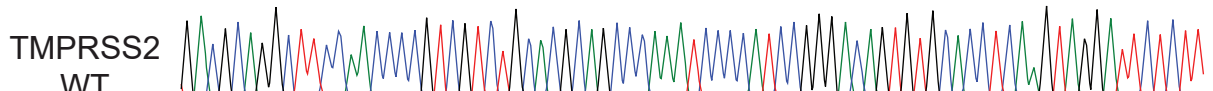
GACGCAGGCTTCCAACCCCGTCGTCTGCACGCAGCCCAAATCCCCATCCGGGACAGTGTGCACCTCAAGTAGGATTCTCTT

TMPRSS2 Clone 1
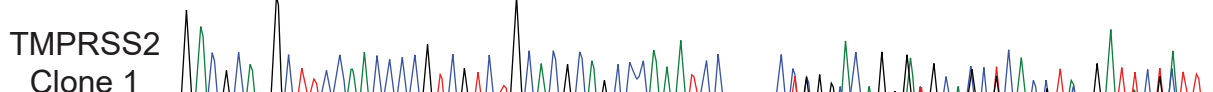
GACGCAGGCTTCCAACCCCGTCGTCTGCACGCAGCCCAAATCC----CCCGGACAGGGCGCAGCTCACCTAAGATTCTATT

INDEL	CONTRIBUTION ▾	SEQUENCE
−4	57%	TCGTCTGCACGCAGCCCAAATCCCC┊----GGGACAGTGTGCACCTCAAG
+1	35%	TCGTCTGCACGCAGCCCAAATCCCC┊NATCCGGGACAGTGTGCACCTCAA

CTSL WT
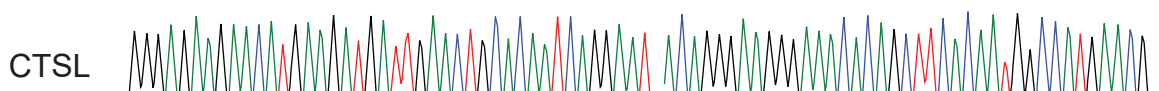
GGGAGAAGAACATGAAGATGATTGAACTGCACAATCAGGAAT-ACAGGGAAGGGAAACACAGCTTCACAATGGCCATGAACG

CTSL Clone 15
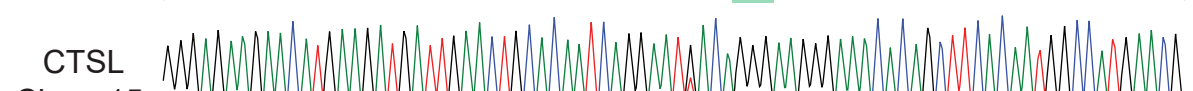
GGGAGAAGAACATGAAGATGATTGAACTGCACAATCAGGAATGACAGGGAAGGGAAACACAGCTTCACAATGGCCATGAACG

INDEL	CONTRIBUTION ▾	SEQUENCE
+1	100%	GTGAAGCTGTGTTTCCCTTCCCTGT┊NATTCCTGATTGTGCAGTTCAATC

Supplementary figure 4 SARS-CoV-2 infected cells contain varying degrees of membranous ACE2 protein

ACE2 dsRNA (SARS-CoV-2) Phalloidin DAPI

Supplementary figure 5 SARS-CoV but not MERS-CoV replication depends on ACE2

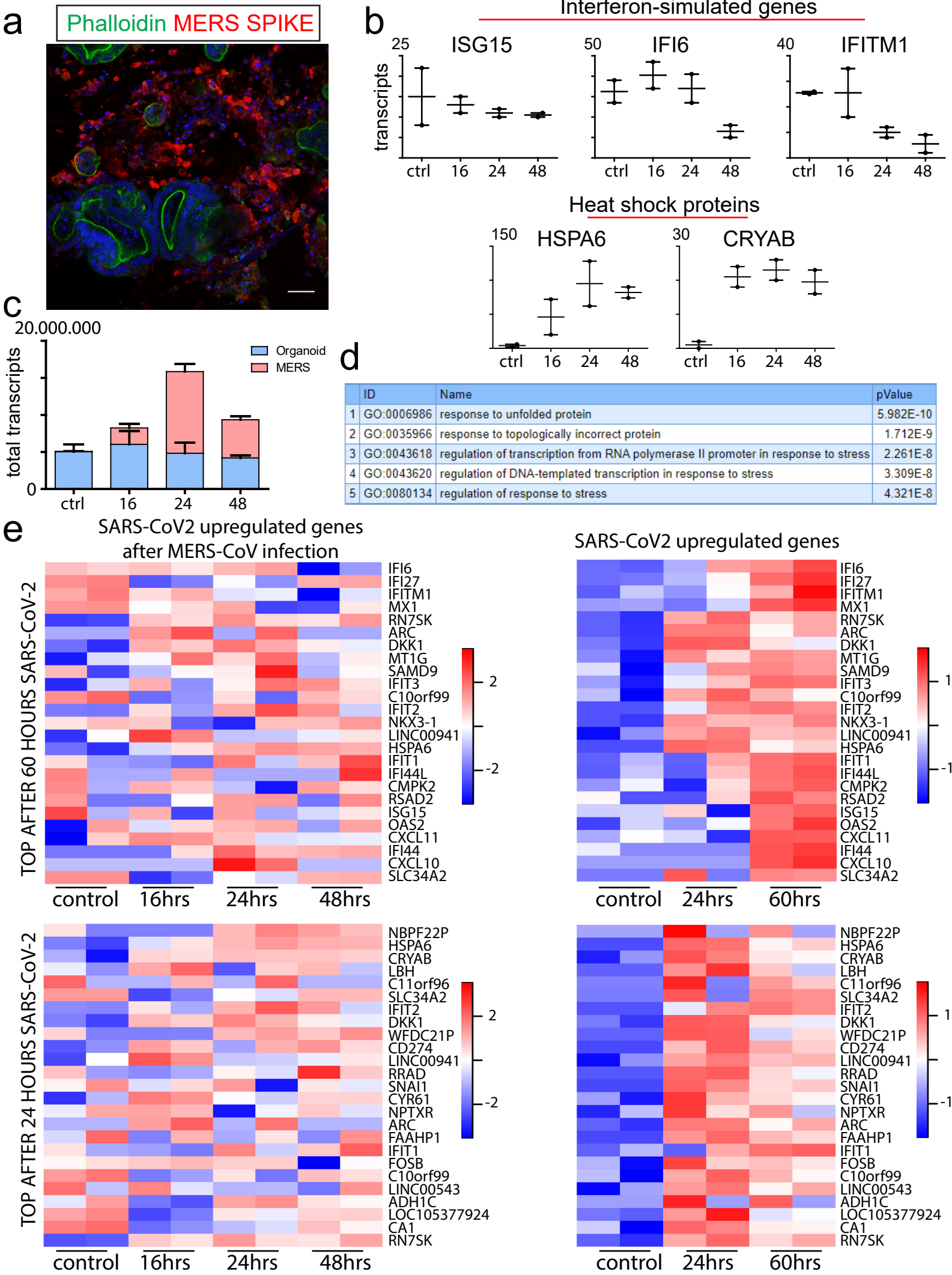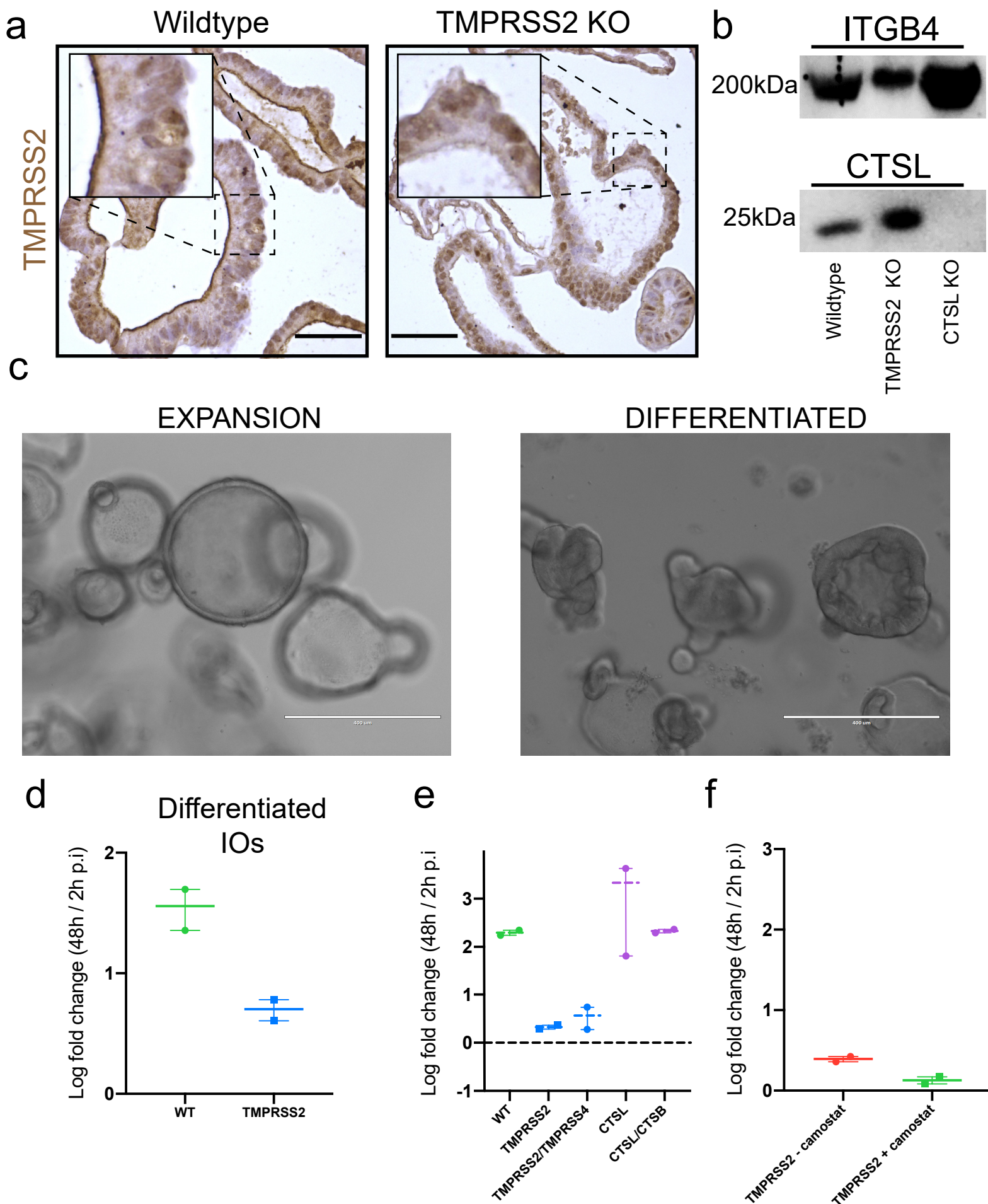# Supplementary figure - Lack of redudancy in cathepsins and serine proteases in viral entry

Supplementary figure 8 Inhibition of serine proteases but not chloroquine inhibits viral replication in organoids

Supplementary figure 9 SARS-CoV-2 replication in Furin- en ARC-mutant IOs