Glycosylated extracellular mucin domains protect against SARS-CoV-2 infection at the respiratory surface

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Abstract

Mucins play an essential role in protecting the respiratory tract against microbial infections but can also serve as binding sites for bacterial and viral adhesins. The heavily *O*-glycosylated gel-forming mucins MUC5AC and MUC5B eliminate pathogens by mucociliary clearance while transmembrane mucins MUC1, MUC4, and MUC16 can restrict microbial invasion at the apical surface of the epithelium. In this study, we determined the impact of host mucins and mucin glycans on SARS-CoV-2 epithelial entry. Human lung epithelial Calu-3 cells express the SARS-CoV-2 entry receptor ACE2 and high levels of glycosylated MUC1, but not MUC4 and MUC16, on their cell surface. The *O*-glycan-specific mucinase StcE specifically removed the glycosylated part of the MUC1 extracellular domain while leaving the underlying SEA domain and cytoplasmic tail intact. StcE treatment of Calu-3 cells significantly enhanced infection with SARS-CoV-2 pseudovirus and authentic virus, while removal of sialic acid and fucose from the epithelial surface did not impact viral entry. Both MUC1 and MUC16 are expressed on the surface of human air-liquid interface (ALI) differentiated airway organoids and StcE treatment led to mucin removal and increased levels of SARS-CoV-2 entry and replication. On the surface of Calu-3 cells, the transmembrane mucin MUC1 and ACE2 are often co-expressed and StcE treatment results in enhanced binding of purified spike protein and SARS-CoV-2 pseudovirus. This study points at an important role for glycosylated mucin domains as components of the host defense that can restrict SARS-CoV-2 infection.

Keywords: Mucins, transmembrane mucin, cell-bound mucin, MUC1, MUC16, *O*-linked glycans, sialic acid, fucose, SARS-CoV-2, ACE2, spike, mucin-microbe interactions

Author summary:

SARS-CoV-2, the virus that has caused the devastating COVID-19 pandemic, causes a range of symptoms in infected individuals, from mild respiratory illness to acute respiratory distress syndrome. A fundamental understanding of host factors influencing viral entry is critical to elucidate SARS-CoV-2–host interactions and identify novel therapeutic targets. In this study, we investigated the role of host mucins and mucin glycans on SARS-CoV-2 entry into the airway epithelial cells. Mucins are a family of high molecular weight *O*-glycosylated proteins that play an essential role in protecting the respiratory tract against viral and bacterial infections. The gel-forming mucins MUC5AC and MUC5B clear pathogens by mucociliary clearance while transmembrane mucins MUC1, MUC4, and MUC16 can restrict or facilitate microbial invasion at the apical surface of the epithelium. The mucin-selective protease StcE specifically cleaves the glycosylated extracellular part of the mucins without perturbing

48 the underlying domains. We show that removal of mucins from the surface of Calu-3 cells and primary
49 airway epithelial cultures with StcE mucinase increases binding of the SARS-CoV-2 spike protein to the
50 respiratory surface and greatly enhances infection. This study demonstrates the important role of
51 glycosylated extracellular mucin domains as a host defense mechanism during SARS-CoV-2 entry.
52 Future efforts should be focused on characterizing the role of specific soluble and transmembrane
53 mucins during the different stages of SARS-CoV-2 infection.

54

55 **Introduction**

56 The respiratory mucus system protects the respiratory epithelium against invading pathogens. The
57 major components of mucus are heavily *O*-glycosylated mucin glycoproteins. Soluble mucins are
58 secreted by Goblet cells and provide mucus threads for mucociliary clearance (MCC) of particles and
59 pathogens. Transmembrane mucins are expressed on the apical membrane and cilia and prevent
60 access to epithelial surface receptors. The major mucins of the respiratory system are soluble mucins
61 MUC5AC and MUC5B, and transmembrane (TM) mucins MUC1, MUC4, and MUC16 (1). MUC1 and
62 MUC4 are expressed in the upper and lower airway epithelium, whereas MUC16 expression is
63 restricted to the lower airways (2). The high molecular weight mucin glycoproteins contain domains
64 with extensive *O*-glycan structures that often terminate with charged sialic acids or hydrophobic
65 fucoses that impact their interaction with microbes (3). The expression and glycosylation profiles of
66 mucins are directly influenced by colonization and invasion by bacteria and viruses and are altered
67 during inflammation of the respiratory tract (4). Transmembrane mucins form filamentous structures
68 that extend above the apical surface of the epithelium and these mucins consist of a heavily *O*-
69 glycosylated N-terminal extracellular domain (ED), a single transmembrane domain, and a C-terminal
70 cytoplasmic domain (CT) with signaling capacity. In the lung, MUC1 primarily expresses around
71 microvilli and protrudes at least 100 nm from the cell surface whereas MUC4 (~300 nm in size), and
72 the even larger MUC16 are expressed on the surface of cilia (5). Together, the TM mucins form a
73 barrier that restricts access to the underlying epithelium, act as releasable decoy receptors, and
74 sterically hinders the binding of pathogens to underlying cellular receptors (6). MUC1 has been most
75 extensively studied and implicated in defense against respiratory infections with *Pseudomonas*
76 *aeruginosa* (7), respiratory syncytial virus (8) and Influenza A virus infection (9). SARS-CoV-2, the
77 coronavirus that is responsible for the COVID-19 pandemic, is an enveloped, single-stranded, positive-
78 sense RNA virus that belong to the β coronavirus genus within the Coronaviridae family (10, 11). SARS-
79 CoV-2 preferentially utilizes angiotensin-converting enzyme 2 (ACE2) as entry receptor by interaction
80 with its envelope-anchored spike protein (12). In addition to ACE2, SARS-CoV-2 entry requires
81 proteolytic cleavage of the spike protein that can be mediated by the transmembrane serine protease
82 2 (TMPRSS2) (13). Human coronaviruses have also been described to depend on sialic acids linked to
83 glycoproteins or gangliosides as primary attachment sites in the respiratory tract (14). Glycosylated
84 mucins can be decorated with sialic acids and therefore might provide viral binding sites, or on the
85 other hand form a barrier that restricts access to the ACE2 receptor. In this study, we investigated the
86 role of transmembrane mucins and their terminal glycans during SARS-CoV-2 entry in a respiratory
87 cell line and primary airway cultures. We show that MUC1 is abundantly expressed on the respiratory
88 Calu-3 cell line, and that both MUC1 and MUC16 are present on the surface of air-liquid interface (ALI)

89 differentiated airway organoids. Enzymatic removal of extracellular mucin domains greatly enhances
90 SARS-CoV-2 spike protein binding and viral infection. This study points towards a critical role for
91 transmembrane mucins in limiting SARS-CoV-2 infection.
92

93 ## Results

94 **MUC1 is highly expressed on the surface of ACE2-positive respiratory epithelial cells**

95 The Human Cell Atlas consortium respiratory single cell RNA-seq dataset allows analysis of gene
96 expression in the nasal cavity and proximal, intermediate, and distal respiratory tract (15). We
97 analysed this dataset to determine the expression of ACE2 and mucins in different respiratory cell
98 types present in the nasal (N), and upper and lower respiratory mucosa. ACE2-positive cells included
99 secretory, basal, suprabasal and multiciliated cells. The majority of secretory and multiciliated cells
100 expressed the major TM mucin MUC1 (Fig 1A). Next, we determined the mucin repertoire of ACE2-
101 positive cells in the nasal mucosa and lower respiratory tract. MUC1 was expressed by the majority of
102 cells to a relatively high extent, while TM mucins MUC4 and MUC16 were abundant in multiciliated
103 cells and soluble mucins MUC5AC and MUC5B were highly expressed in secretory and Goblet cells (Fig
104 1B). This analysis suggests that different respiratory cell types have unique mucin repertoires and that
105 the TM mucin MUC1 is the most abundantly expressed mucin in most types of ACE2-positive
106 respiratory cells.

107 The human respiratory Calu-3 cell line expresses ACE2 and TMPRSS2 and is highly susceptible to SARS-
108 CoV-2 spike protein-mediated entry (16, 17, 13). We first determined the expression of different
109 mucins and their glycans on Calu-3 cells by immunofluorescence confocal microscopy. Multiple Z-stack
110 images showed expression of MUC1, MUC4, and MUC5AC but only very limited expression of MUC16
111 (Fig 1C, S1A respectively). To distinguish which mucins are expressed on the extracellular cell surface,
112 we performed immunofluorescence staining without permeabilization of the Calu-3 cells. Using this
113 method, MUC1 was clearly detectable on the cell surface whereas MUC4 and MUC5AC could not be
114 stained indicating intracellular localization (Fig 1D). Next, we determined the expression of the
115 terminal mucin glycans sialic acid and fucose on Calu-3 cells. Immunofluorescence with SNA, MALII,
116 and UEA-I lectin showed the presence of α-2,6 sialic acid, α-2,3 sialic acid, and fucose on Calu-3 cells,
117 respectively (Fig 1F). The α-2,6 sialic acid and α-2,3 sialic acid signals (SNA and MALII) were more
118 prominently detected at the edge of the cell island compared to the fucose signal (UEA1) and some
119 colocalization with MUC1 could be observed. These results demonstrate that Calu-3 cells
120 endogenously express MUC1 on their surface and have abundant expression of sialic acids and fucose.
121

122 **StcE cleaves the MUC1 glycosylated ED and does not affect ACE2 expression**

123 The StcE mucinase recognizes an *O*-glycosylated serine-threonine motif that is abundant in mucins
124 and is virtually absent in non-mucin proteins (18). We previously applied this bacterial mucinase and
125 its inactive point mutant E447D to remove the MUC1 ED (19). To investigate the effect of StcE on
126 endogenous MUC1 expressed by Calu-3 cells, confocal microscopy was performed on non-treated,
127 StcE-treated and E447D-treated Calu-3 cells and stained with α-MUC1-ED antibody 214D4, α-MUC1-
128 SEA antibody 232A1, and α-MUC1-CT antibody CT2. StcE treatment efficiently removed the
129 glycosylated part of the MUC1 extracellular domain as indicated by a complete loss of α-MUC1-ED
130 214D4 staining after incubation with the enzyme (Fig 2A). The MUC1 SEA domain and CT are predicted
131 not to be digested by StcE and indeed both domains remained detectable after enzyme treatment (Fig
132 2B, 2C). We next investigated the effect of StcE, E447D, neuraminidase, and fucosidase treatment on
133 MUC1 by Western blot. Calu-3 cells were incubated with the enzymes for 3 h and then subjected to
134 Western blot analysis with the α-MUC1-ED antibody 214D4 and α-MUC1-CT antibody CT2. After
135 incubation with StcE, the glycosylated part of the extracellular domain of MUC1 (about 450 kDa) was
136 no longer detectable. The high molecular weight MUC1 band was not affected by treatment with the
137 inactive enzyme E447D or fucosidase. After neuraminidase treatment on the other hand, we did
138 observe a reduction of the MUC1 signal compared to the loading control (Fig 2D, E). However, because
139 MUC1 is still detectable on the cellular surface after neuraminidase treatment when using
140 immunofluorescence, we hypothesize that the reduced immunoblot signal could be caused by a
141 change in antibody recognition or altered protein transfer to the nitrocellulose membrane. The
142 observed banding pattern for the MUC1 cytoplasmic tail was not affected by the enzymatic treatments
143 (Fig 2F). Furthermore, we wanted to determine the effect of enzymatic treatment on ACE2 stability
144 because the ACE2 receptor itself is glycosylated (20). No change in expression of the full-length
145 glycosylated ACE2 (nearly 140 kDa) could be observed after treatment with StcE, E447D,
146 neuraminidase, or fucosidase. Interestingly, the soluble form of ACE2 (around 70 kDa) was more
147 prominently detectable after fucosidase treatment (Fig 2G). These results demonstrate that StcE
148 cleaves the glycosylated part of the MUC1 ED without affecting ACE2 expression in Calu-3 cells. To
149 investigate the effect of StcE treatment on *O*-glycosylated surface proteins other than MUC1, we
150 stained the treated and untreated Calu-3 cells with a fluorescently labelled mucin binding domain
151 derived from StcE (X409-GFP) (21). Confocal analysis of non-permeabilized and permeabilized Calu-3
152 cells demonstrated that there was limited punctate staining with X409 on the Calu-3 surface while
153 MUC1 ED staining showed a more continuous surface staining as previously observed. The MUC1
154 signal was completely lost after StcE treatment, while some staining remained for X409 (Fig. 2H). With
155 permeabilized cells, a comparable result with a higher level of remaining X409 signal was observed

156 (Fig. 2I). These results reinforce that MUC1 is highly expressed on the surface of Calu-3 cells, but also
157 demonstrate the presence of some other *O*-glycosylated mucin(-like) proteins.
158
159 **Enzymatic removal of mucin domains enhances SARS-CoV-2 entry into Calu-3 cells**
160 Next, we investigated whether the sialic acid and fucose residues on glycans or complete glycosylated
161 mucin domain can impact SARS-CoV-2 infection of respiratory cells. Surface α2,3-, α2,6-, and α2,8-
162 linked sialic acids were removed by incubation with neuraminidase and fucose by fucosidase
163 treatment and StcE was used to remove mucin extracellular domains. Neuraminidase treatment for 3
164 h removed the majority of surface-exposed α2,3- linked sialic acids as detected by MAL-II staining, and
165 α2,6-linked sialic acids detected by SNA staining (Fig S1B). Similarly, fucosidase treatment for 3 h
166 cleaved surface-exposed fucose which was detected by UEA1 staining (Fig S1C). StcE treatment was
167 effective as monitored by removal of the MUC1 glycosylated ED from the cellular surface and reduced
168 X409-GFP staining as describe above (Fig 2,3). After enzymatic treatment of the Calu-3 cells, a SARS-
169 CoV-2 pseudotyped virus carrying the spike protein and encoding a GFP reporter (SARS2-S
170 pseudotyped VSV-GFP) was added in the absence or presence of an anti-spike monoclonal antibody
171 to confirm spike mediated entry of virus and incubated for 24 hours. StcE treatment enhanced the
172 number of SARS2-S pseudotyped VSV-GFP positive cells, while E447D-treated cells did not show
173 enhanced viral entry. No obvious change in viral infection could be observed after neuraminidase or
174 fucosidase treatment (Fig 3A). In all experimental conditions, viral infection was completely blocked
175 in the presence of the monoclonal antibody against the SARS-CoV-2 spike protein demonstrating
176 spike-mediated entry in our experimental setup. The GFP signal was quantified using Image J showing
177 a significant 5.4-fold increase in Calu-3 virus infection after StcE treatment and no significant
178 difference after neuraminidase and fucosidase treatment (Fig 3B).
179 In an independent set of experiments with a luciferase pseudovirus (SARS2-S pseudotyped VSV-Luc),
180 we also observed a 4-fold increase in viral infection after StcE treatment (Fig 3C). As an additional
181 control, we performed the infection with an VSV-G pseudotyped VSV-Luc that lacks the spike protein.
182 We observed enhanced entry of VSV-G pseudotyped VSV-Luc into Calu-3 after StcE and neuraminidase
183 treatment whereas fucosidase treatment had an opposite effect. As expected, the infection could not
184 be blocked with the anti-SARS2-S mAb (Fig 3D). Confocal microscopy on Calu-3 cells infected with
185 SARS2-S pseudotyped VSV-GFP confirmed that StcE treatment increased the number of infected cells
186 (Fig 3E). Next, we investigated the effect of mucin removal on infection with the authentic SARS-CoV-
187 2 virus. Calu-3 cells were treated with the enzymes and incubated with SARS-CoV-2 virus in the
188 absence or presence of an anti-spike monoclonal antibody for 8 hours to study initial entry. In line
189 with our pseudovirus experiments, we observed a significant increase in the number of infected cells

190 when cells were treated with StcE mucinase in comparison to control. Again, neuraminidase and
191 fucosidase treatment did not significantly impact viral infection albeit we observed a trend towards
192 increased infection after neuraminidase treatment (Fig 3F). Together these data demonstrate that
193 removal of glycosylated mucin domains results in increased SARS-CoV-2 infection of lung epithelial
194 cells. No effect on viral entry was observed after removing individual glycans sialic acid and fucose.
195
196 Negatively charged molecules such as sialic acid or heparan sulphate (HS) on the cellular surface or
197 extracellular matrix proteoglycans have been described to facilitate viral entry (22, 23). Therefore, we
198 investigated if heparanase treatment to remove HS or neuraminidase treatment to remove sialic acids
199 impacted viral invasion after removal of the MUC1 glycosylated domain with StcE. Calu-3 cells were
200 first treated with StcE, followed by treatment with heparanase or neuraminidase and subsequent viral
201 infection with SARS2-S pseudotyped VSV-Luc. Confocal microscopy confirmed the removal of HS and
202 α-2,6 sialic acid from the surface of Calu-3 cells after heparanase and neuraminidase treatment,
203 respectively (Fig S2A, B). Quantification of viral infection showed that the combination treatments did
204 not significantly impact viral invasion compared to StcE only condition (Fig S2C). A small reduction of
205 viral infection was observed when the cells were treated with only heparanase in comparison to the
206 control cells without treatment. All infections in this experiment could be blocked by the mAb
207 demonstrating spike-mediated infection. This result suggests that SARS-CoV-2 entry does not depend
208 on these negatively charged molecules on the cell surface of Calu-3 cells.
209
210 **StcE treatment of human primary respiratory tissue enhances SARS-CoV-2 infection**
211 To investigate the role of mucins during SARS-CoV-2 infection in epithelial tissue that more closely
212 resembles the human respiratory surface, we performed infection experiments with human ALI-
213 differentiated airway organoids. First, we determined which mucins were expressed in the ALI
214 cultures. Differentiated cultures were prepared for immunofluorescence staining with and without
215 permeabilization and stained with MUC1, MUC4, MUC5AC and MUC16 antibodies. All mucins were
216 detectable in the permeabilized tissues (Fig 4A). In the non-permeabilized tissues transmembrane
217 mucins MUC1 and MUC16 and traces of MUC5AC networks were detectable suggesting that these
218 mucins are expressed on the cellular surface (Fig 4B). Next, we investigated the efficacy of StcE in
219 cleaving MUC1 and MUC16. After StcE treatment, MUC1 staining was still detectable on the surface
220 of the airway organoids (data not shown), but immunoblot analysis of two different donors
221 demonstrated that StcE had effectively cleaved off the glycosylated domain of MUC1 (Fig 4C). The
222 MUC16 antibody recognizes the mucin SEA domain and can therefore not be used to monitor cleavage
223 of the MUC16 glycosylated domain. The X409 domain of StcE was used to detect glycosylated mucin

224 domains and we found that it colocalized with MUC16, but not with MUC1 (Fig 4D,E). To determine
225 the effect of StcE on surface mucins in general and MUC16 in particular, X409 staining was performed
226 on control ALI cultures and after treatment with StcE and E447D inactive enzyme. A significant
227 reduction of X409 staining could be observed after StcE treatment suggesting cleavage of MUC16 (Fig
228 4F,G).
229 To determine the effect of mucin removal on SARS-CoV-2 infection, ALI-differentiated airway
230 organoids from two different donors were treated with StcE, E447D or left untreated followed by
231 infection with authentic SARS-CoV-2 virus. StcE treatment led to a significant increase in SARS-CoV-2
232 infection and replication in both donors, as measured by RNA copies and infectious virus (Fig 5A-D).
233 Nucleoprotein staining of infected tissues confirmed a high percentage of virus-infected cells in the
234 StcE condition compared to the control and E447D condition (Fig 5E,F).
235
236 **Removal of mucin domains enhances spike and virus attachment to Calu-3 cells**
237 To understand the spatial relationship between the ACE2 receptor and MUC1, the dominant TM mucin
238 in Calu-3 cells, we performed confocal microscopy on Calu-3 cells stained for ACE2 receptor and the
239 MUC1 extracellular domain with an adjusted protocol that allowed imaging of both proteins. In the
240 monolayer, MUC1- and ACE2-positive cells were observed in distinct scattered distributions (Fig 6A).
241 Some cells expressed both proteins at high levels, while other cells expressed high level of either ACE2
242 or MUC1. MUC1-negative cells most likely express other mucin-like proteins as demonstrated above
243 (Fig 2H). Next, we investigated if removal of mucin domains directly affected spike and virus
244 attachment to the cellular surface. Calu-3 cells were treated with StcE followed by incubation with
245 purified Fc tagged spike protein (SARS2-S1B-Fc) or SARS2-S pseudotyped VSV-GFP for 1 h at 4°C to
246 monitor attachment and prevent entry. The spike protein was stained without first permeabilizing to
247 prevent intracellular access. In untreated and E447D-treated cells, spike binding was observed in
248 patches along the edge of the cell island while cells treated with StcE showed extensive staining (Fig
249 6B). Quantification of the fluorescent spike signal on the edges of the cell islands using ImageJ
250 confirmed a significant increase in StcE-treated cells raw spike fluorescence values as determined by
251 integrated density/length (sum of all pixels/µm) (Fig 6C). In a similar experimental setup, we incubated
252 Calu-3 cells with SARS2-S pseudotyped VSV-GFP virus to observe attachment to the cellular surface.
253 In line with the spike-binding assays, we observed more virus attachment to Calu-3 cells after StcE
254 treatment (Fig 6D). In untreated cells, spike-positive pseudoviral particles preferentially bound to
255 areas that stained negative for MUC1 (Fig 4E). Together these results show that enzymatic removal of
256 mucin extracellular domains including the abundant MUC1 ED allows more virus attachment to cells
257 and thus increases infectivity. We propose that the glycosylated domains of transmembrane mucins

258 such as MUC1 and MUC16 on the respiratory surface form a barrier that prevents SARS-CoV-2 invasion
259 (Fig 6F).
260
261 **Discussion**
262
263 The mucosal barrier is the body's first line of defense and offers protection from infection by
264 pathogens. Mucin proteins are known for their barrier properties but can also serve as attachment
265 sites for bacterial and viral pathogens. The findings presented in this study indicate that extracellular
266 mucin domains play a substantial protective role during SARS-CoV-2 infection at the respiratory
267 surface. ACE2-positive cells in the respiratory epithelium express different combinations of mucin
268 genes including MUC1, MUC4, MUC16, MUC5AC and MUC5B of which MUC1 is the most abundantly
269 expressed mucin across different cell types. In this study, we demonstrate that human lung epithelial
270 Calu-3 cells expressed high levels of MUC1, while TM mucins MUC4 and MUC16 and secreted mucin
271 MUC5AC were barely detectable (Fig 1, 2). In airway organoids, MUC1 and MUC16 are expressed on
272 the surface and some secreted MUC5AC is detectable (Fig 4). In different SARS-CoV-2 infection studies
273 with both cell models, we demonstrate that enzymatic removal of extracellular mucin domains, but
274 not individual sialic acid or fucose sugars, enhances viral infection (Fig 3,5). Removal of glycosylated
275 mucin domains from the cellular surface increased binding of purified spike protein and virus to the
276 cellular surface (Fig 6). We propose a model in which glycosylated extracellular mucin domains form
277 a protective layer above the underlying ACE2 receptor thereby preventing access of the virus to the
278 receptor (Fig 6).
279
280 There is growing evidence that both soluble and transmembrane mucins play important roles during
281 SARS-CoV-2 infection, but whether their contributions are protective or are facilitating pathogenicity
282 at different stages of disease is still under debate. Mucus hypersecretion and accumulation most likely
283 have a negative impact on disease development due to reduced MCC and mucus plugging (24), but
284 studies into the roles of transmembrane mucins point in different directions. In the course of COVID-
285 19, elevated levels of gel-forming MUC5AC and shed MUC1 can be detected in sputum aspirated from
286 the trachea of patients (25) and high production of MUC5AC was observed in SARS-CoV-2 infected
287 primary respiratory cultures (26). The MUC5B genetic variant rs35705950 is associated with higher
288 expression of the soluble mucin MUC5B and underrepresented in COVID-19 patients compared to
289 healthy individuals, suggesting a protective role for MUC5B (27). In aged individuals, decreased mucus
290 production and weakened MCC might contribute to the higher susceptibility of SARS-CoV-2 (28). Our
291 findings on a protective role for TM mucins MUC1 and MUC16 during SARS-CoV-2 infection are in line

292 with a recent genome-scale CRISPR loss- and gain-of-function (GOF) study for SARS-CoV-2 entry in
293 human lung epithelial cells overexpressing TM mucins (29). In this GOF study, overexpression of TM
294 mucins MUC1, MUC4 or MUC21 reduced infection by SARS-CoV-2 compared to cells with a non-
295 targeting guide (NTG). The study also demonstrated that enzymatic removal of overexpressed MUC4
296 resulted in increased viral entry. An important role for MUC4 was also observed during SARS-CoV-1
297 infection *in vivo* where female MUC4 knockout mice that had enhanced inflammatory cytokine
298 responses and poor prognosis compared to wild type mice (30). Together with our data, these
299 different studies imply that different TM mucins might have a similar protective function during SARS-
300 CoV-2 infection.
301
302 In addition to the protective functions of mucins during SARS-CoV-2 initial, there is also emerging
303 evidence that overexpression of different mucins is correlated with severe disease. A MUC1 gene
304 variant that leads to increased expression was one of the few significant loci associated with severe
305 COVID in a large-scale GWAS study. The functional consequences of this gene variation need to be
306 addressed, but the authors suggest that mucins could have a clinically important role in the
307 development of critical illness in COVID-19 (31). This was in line with another study that found
308 increased MUC1 mRNA to be associated with critical disease (32). Single cell sequencing data of
309 COVID-19 patients demonstrated that transmembrane mucins MUC1, MUC4, MUC13 and MUC21 are
310 all highly upregulated in patients with active disease (29) and also in blood samples MUC1 and MUC2
311 mRNA expression was significantly elevated in critical and mild COVID-19 while MUC16, MUC20 and
312 MUC21 were significantly downregulated in severe COVID (33). Compound R406, the active
313 metabolite of FDA-Approved Fostamatinib that inhibits MUC1 expression is now in clinical trials for
314 hospitalized patients with advanced COVID-19 (34). At this point, we lack the critical insight to
315 conclude if transmembrane mucins in general or MUC1, MUC4 or MUC16 specifically are protective
316 or contributing to disease severity during different stages of pathogenesis within the complexity of
317 the body. It is evident that establishing the function of specific mucins during *in vivo* infection is an
318 important future challenge.
319
320 The studies that are currently available underscore the importance of extracellular domain of
321 transmembrane mucins during viral entry. Our confocal microscopy analysis indicates that ACE2-
322 positive cells in the respiratory epithelium often, but not always, express MUC1 and that both proteins
323 colocalize on the apical surface. Enzymatic removal of the MUC1 glycosylated domain did not affect
324 the underlying SEA domain or cytoplasmic tail and ACE2 expression remained detectable. As was
325 previously hypothesized, it is possible that MUC1 and ACE2 interact and/or are in the same protein

326 complex on the respiratory surface (35). Our data indicate that steric hindrance by glycosylated
327 extracellular mucin domains prevents the virus from reaching the ACE2 receptor (Fig 6). This is in line
328 with a recent study that used mucin mimetics glycopolymers that were capable of shielding surface
329 receptors (36). In a previous study, we have shown that MUC1 ED alters the cell membrane of non-
330 polarized epithelial cells to tubulated morphology and reduce β1-integrin-mediated bacterial invasion
331 (19). In the present study, we have not observed any influence of MUC1 ED on membrane architecture
332 in Calu-3 cells or airway organoids.
333
334 Different studies describe that for viral entry SARS-CoV-2 benefits from negatively charged residues
335 like sialic acid-containing glycans or membrane glycosaminoglycans such as heparan sulfate
336 proteoglycans on the cell surface (37, 38, 39). In contrast with this findings, another study reported
337 that neuraminidase treatment of Calu-3 cells only modestly increased SARS-CoV-2 infection (40). In
338 our live virus experiments we did not observe a significant increase in SARS-CoV-2 infection after
339 neuraminidase or fucosidase treatment. We addressed if the negatively charged sialic acids or heparan
340 sulfates were important for viral entry after removal of the glycosylated mucin domain. Consecutive
341 treatment with StcE and neuraminidase or heparinase was performed but did not result in a difference
342 in viral entry (Fig S2). Differences in viral dependence on negatively charged surface molecules maybe
343 be explained by levels of ACE2 and TMPRSS2 protease expression and accessibility of the receptor for
344 the viral spike protein in different cell systems. Therefore, our findings reveal that during infection of
345 human respiratory Calu-3 cells the MUC1 extracellular domain rather than individual mucin glycans
346 prevents the binding of SARS-CoV-2 to the underlying receptor.
347
348 Overproduction and excess accumulation of gel-forming mucins in the lungs of COVID-19 patients can
349 lead to airway obstruction and eventually cause life-threatening acute respiratory distress syndrome
350 (41, 42, 43). Several studies are focusing on the reduction of mucin expression overall as a therapeutic
351 strategy (44, 34). In future studies, the role of transmembrane mucins MUC1, MUC4 and MUC16 in
352 disease development should be taken into consideration. A tailored approach that boosts expression
353 of protective transmembrane mucins but reduces secretion of soluble mucins could be an attractive
354 future strategy to prevent infection with SARS-CoV-2 or other respiratory pathogens and improve
355 disease outcome.
356

357 ## Methods

358 **Single cell analysis**

359 Normalized counts and metadata from previously published single cell RNA-sequencing data of
360 healthy human airway epithelium(15) were downloaded from
361 https://www.genomique.eu/cellbrowser/HCA/. Dimensionality reduction was done using the Seurat
362 Package (45) in Rstudio (version 1.2.5019), starting with a principle component analysis. After visual
363 inspection of the principal components using and elbow plot, the first twenty components were used
364 for graph-based clustering analysis. Clusters of cells were then visualized as diffusion maps (uMAPs).
365 To determine gene expression in ACE2- and TMPRSS2-positive versus negative cells we created two
366 additional metadata slots, in which normalized transcript counts of these genes above 0 were
367 considered positive. Then, cell type assignment and normalized expression of a panel of genes of
368 interest was determined by sub-setting single or double-positive epithelial cells.
369
370 **Cell culture**
371 Calu-3 cells (ATCC Catalog # HTB-55), HEK-293T (ATCC Catalog # CRL-3216) and BHK-21 cells (ATCC
372 Catalog # CCL-10) cells were routinely grown in 25 cm^2 flasks in Dulbecco's modified Eagle's medium
373 (DMEM) containing 10% fetal calf serum (FCS) at 37°C in 5% CO_2.
374
375 **Human airway organoid culture and differentiation**
376 Adult human lung tissue was obtained from non-tumor lung tissue obtained from patients undergoing
377 lung resection. Lung tissue was obtained from residual, tumor-free, material obtained at lung
378 resection surgery for lung cancer. The Medical Ethical Committee of the Erasmus MC Rotterdam
379 granted permission for this study (METC 2012-512). Human bronchiole and bronchus stem cells were
380 isolated and maintained as described previously (46, 47), using a protocol adapted from Sachs and
381 colleagues (48). Organoids were dissociated using TrypLE express (Gibco) into single cells and plated
382 on Transwell membranes (StemCell) coated with rat tail collagen type I (Fisher Scientific) in
383 Pneumacult-ALI medium (StemCell) and airway organoid medium at a 1:1 ratio as described before
384 (46, 47). Upon confluency, cells were differentiated at an air-liquid interface in 100% Pneumacult-ALI
385 medium for 3-6 weeks. Medium was replaced every 5 days.
386
387 **Production of SARS-CoV-2 pseudotyped virus and virus neutralization assay**
388 The pseudotyped vesicular stomatitis virus (VSV) was produced by using the protocol of Whitt (49).
389 The detailed protocol of the production of pseudotyped VSV, SARS2-Spike pseudotyped VSV virus and
390 virus neutralization assay is described in the supplementary methods. The optimal working
391 concentration of SARS2-Spike pseudotyped VSV particles (SARS2-S pseudotyped VSV-GFP and SARS2-
392 S pseudotyped VSV-Luc) was determined by viral titration assay on Calu-3 cells.

393

394 **Production of authentic SARS-CoV-2 virus stock**

395 SARS-CoV-2 (isolate BetaCoV/Munich/BavPat1/2020; European Virus Archive Global #026V-03883;
396 kindly provided by Dr. C. Drosten) was propagated on Calu-3 cells in OptiMEM I (1X) + GlutaMAX
397 (Gibco), supplemented with penicillin (100 IU/mL) and streptomycin (100 IU/mL) at 37°C in a
398 humidified CO_2 incubator. Stocks were produced as described previously (50). A detailed description
399 of virus production can be found in the supplementary methods.

400

401 **Enzyme treatment of Calu-3 cells**

402 StcE and StcE-E447D were expressed and purified as described previously (19). For mucinase
403 treatment, Calu-3 cells were treated with 2.5 ug/ml of StcE or its inactive mutant E447D in 10% FCS
404 media for 3 h at 37°C and washed with DPBS. Desialylation of Calu-3 cells was achieved by incubating
405 cells grown in a 96 well plate or 24-well plate or 6 well plate with 100 U/mL α2-3,6,8,9 neuraminidase
406 A (P0722L, NEB) in 10% FCS media for 3 h at 37°C. For fucosidase treatment of Calu-3 cells, 0.4 U/ml
407 of α-(1–2,3,4,6)-L-Fucosidase (E-FUCHS; Megazyme) was added to the cells and incubated for 3 h at
408 37°C. To remove heparan sulfate (HS), 0.1 U/ml heparinase III (H8891-5UN, Sigma) was applied as
409 described for the other enzymes. After enzyme treatment, cells were washed thrice with DPBS and
410 used for subsequent experiments.

411

412 **SARS-CoV-2 infection assays on Calu-3 cells**

413 For infection experiments, Calu-3 cells were grown in 96-well plates and allowed to reach around 90%
414 confluency. Then, cells were treated with enzymes for 3 h at 37°C and 5% CO2, before they were
415 inoculated with SARS2-S pseudotyped VSV-Luc or SARS2-S pseudotyped VSV-GFP. At 20 h post-
416 infection, culture supernatants were aspirated, washed with DPBS, and cells were lysed by overnight
417 incubation with *Renilla* luciferase assay lysis buffer (Promega) at -80°C. The next day, cell lysates were
418 thawed, thoroughly resuspended, and transferred to white, opaque-walled 96-well plates and relative
419 luminescence unit (RLU) was measured. *Renilla* luciferase activity was determined using the Luciferase
420 Assay Systems (Promega) according to the manufacturer's instructions. Raw luminescence values
421 were recorded as counts per 5 seconds by Berthold Centro LB 942 plate luminometer. For SARS2-S
422 pseudotyped VSV-GFP mediated infection, GFP positive signal captured using an EVOS microscope
423 (Thermo Scientific) at 4X magnification and quantified using EVOS software. For infection experiments
424 with the authentic SARS-CoV-2 virus, Calu-3 cells were prepared as described above and inoculated
425 with approximately 200 pfu of SARS-CoV-2. At 8 h post-infection, cells were washed in PBS, fixed in
426 formalin, permeabilized in 70% ethanol and washed in PBS again. Immunofluorescent stainings were

427 performed as described for SARS-CoV-2 stock production and scanned plates were analyzed using
428 ImageQuant TL software. All work with infectious SARS-CoV-2 was performed in a Class II Biosafety
429 Cabinet under BSL-3 conditions at Erasmus Medical Center.
430
431 **SARS-CoV-2 infection assays on human airway organoid-derived ALI culture**
432 Prior to infection, ALI cells were washed three times with Advanced DMEM/F12 (Gibco) supplemented
433 with Hepes (20mM, Lonza), Glutamax (Gibco) and Primocin (200ug/ml; Invivogen) (AdDF+++). Cells
434 were pretreated for 3 hours with either 10ug/ml E447D or StcE in AdDF +++ or AdDF +++ alone. Cells
435 were washed three times with AdDF +++ and infected with an MOI of 0.01 of SARS-CoV-2 for 4 hours,
436 at which time cells were washed three times with ADdF +++ and remained on ALI for the duration of
437 the experiment. Apical washes were collected at 4, 24 and 48 hours post infection and viral loads were
438 detected in different treatment conditions. After the last collection cells were fixed in 4% formalin for
439 20 minutes, followed by 70% ethanol for 20 minutes. Plates were exported from the BSL-3 in ethanol
440 for subsequent staining.
441
442 **Human airway organoid-derived ALI infection growth curves**
443 All samples were thawed and centrifuged at 2000x g for 5 min to spin down mucus and cellular debris.
444 Supernatant was used for subsequent analysis. Virus titrations to determine pfu/ml were performed
445 as described above for determining SARS-CoV-2 titres. RNA extraction was performed by adding 60 µl
446 of sample to 90 µl MagnaPure LC Lysis buffer (Roche) for 10 minutes. Fifty µl Agencourt AMPure XP
447 beads (Beckman Coulter) were added and incubated for followed by two washes on a DynaMag-96
448 magnet (Invitrogen) and elution in 30 µl ultrapure water. All steps were performed at room
449 temperature. RNA copies were determined by qRT-PCR using primers targeting the E gene (51) and
450 comparison to a standard curve.
451
452 **Confocal Microscopy**
453 Cells were grown on coverslips up to 80% confluency were analyzed by immunofluorescent staining.
454 Cells were washed twice with DPBS and fixed with 4% paraformaldehyde in PBS (Affymetrix) for 20 min
455 at room temperature and fixation was stopped with 50 mM NH_4Cl in PBS for 10 min. The staining
456 procedure and antibody details are described in the supplementary methods.
457
458 **Human airway organoid-derived ALI fluorescent staining**
459 ALI inserts infected with SARS-CoV-2 were fixed in 4% formalin for 20 minutes followed by 70% ethanol
460 for 20 minutes and washed in PBS. Uninfected ALI inserts were either fixed and permeabilized with

461 0.1% triton-X in 10% normal goat serum (NGS) in PBS or stained live on ice. All inserts were blocked in
462 10% NGS in PBS for an hour followed by primary antibody incubation overnight or for 4 hours on live
463 cells on ice: rabbit anti-SARS-CoV-2 nucleoprotein (Sinobiological, 40143-T62, 1:1000), mouse anti-
464 MUC1 ED, mouse anti-MUC4, mouse anti-MUC5AC or mouse anti-MUC16 (source and dilution was
465 mentioned in supplementary methods). After incubation with primary antibody, live cells were fixed
466 and washed with PBS. All other inserts were washed with PBS. For secondary antibody incubation,
467 Alexa Fluor 568-conjugated goat α-mouse IgG, Alexa Fluor 488-conjugated goat α-rabbit IgG, Alexa
468 Fluor 647-conjugated goat α-mouse IgG or X409-GFP (source and dilution mentioned in
469 supplementary methods) was used. Secondary antibodies were incubated for one hour. All antibodies
470 were diluted in 10% NGS in PBS. After secondary antibody incubation cells were washed with PBS and
471 stained for nuclei using DAPI diluted in PBS. After 30 minutes incubation cells were washed in PBS and
472 mounted in Prolong Antifade (Invitrogen) mounting medium. ALI culture confocal microscopy was
473 performed on an LSM700 confocal microscope using ZEN software (Zeiss). Representative images are
474 maximum intensity projections taken from Z-stacks. Mean signal intensities of MUC1, MUC16 and
475 X409 were analysed using ZEN software.
476
477 **Western blotting**
478 Calu-3 cells were grown in 6-well plates for 7 days before enzyme treatment. Enzyme-treated cells
479 were washed thrice with cold DPBS and collected with a scraper. The cell suspension was centrifuged
480 at 5,000 rpm for 5 min at 4°C. Cell pellets were resuspended with 100 µl 1% SDS in presence of a Halt
481 protease inhibitor cocktail and 0.5 M EDTA solution (Thermo Fisher) and cells lysed mechanically by
482 scratching. Protein concentrations were measured using a BCA protein assay kit (23235#, Pierce
483 Company). For detection of the MUC1 ED, 5% mucin gels and a boric acid-Tris system were used as
484 described previously (52). α-MUC1-ED antibody 214D4 was used to detect MUC1 at a dilution of
485 1:1,000 in TSMT buffer. For detection of the CT of MUC1, 12% SDS-PAGE gel and α-MUC1-CT antibody
486 CT2 was used. For ACE2 detection, 10% SDS-PAGE gel and anti-ACE2 antibody (1:1,000, HPA000288,
487 Sigma-Aldrich) was used. Actin was detected using α-actin antibody (1:5,000; bs-0061R, Bioss).
488 Secondary antibodies used were α-mouse IgG secondary antibody (1:10,000; A2304, Sigma), α-
489 Armenian hamster IgG (1:10,000; GTX25745, Genetex) and α-rabbit IgG (1:10,000; A4914, Sigma).
490 Blots were developed with the Clarity Western ECL kit (Bio-Rad) and imaged in a Gel-Doc system (Bio-
491 Rad).
492
493 **Statistical Analysis**

494 For all experiments, at least three independent biological replicates were performed. Values are
495 expressed as the mean ± SEM of three independent experiments performed in triplicate. Repeated
496 measures one way-ANOVA with Dunnett's or Tukey's or two way-ANOVA with Tukey's post-hoc was
497 applied to test for statistical significance. P values of 0.05 or lower were considered statistically
498 significant. Symbols used are $p > 0.05$ (ns, not significant), $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***),
499 $p<0.0001$ (****). The GraphPad Prism 9 software package was used for all statistical analyses.
500

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626

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636

637 **Author Contributions**

638 MC and LZXH performed all Calu-3 characterization and pseudovirus infection experiments. AZM and
639 MML cultured the airway organoids and performed and analyzed the authentic SARS-CoV-2
640 experiments under supervision of BLH. CW and BJB provided materials and assisted with generation
641 of pseudotyped SARS-CoV-2. BW performed analysis of single cell RNA-seq data. RWW assisted with
642 confocal microscopy. JPMP reviewed the manuscript. KS supervised and helped design all aspects of
643 the study. MC and KS analyzed the results, created the figures, and wrote the manuscript. All authors
644 reviewed the final version of the manuscript.
645

646 **Competing Interests**

647 We declare no conflict of interest.
648

649 **Materials and Correspondence**

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651

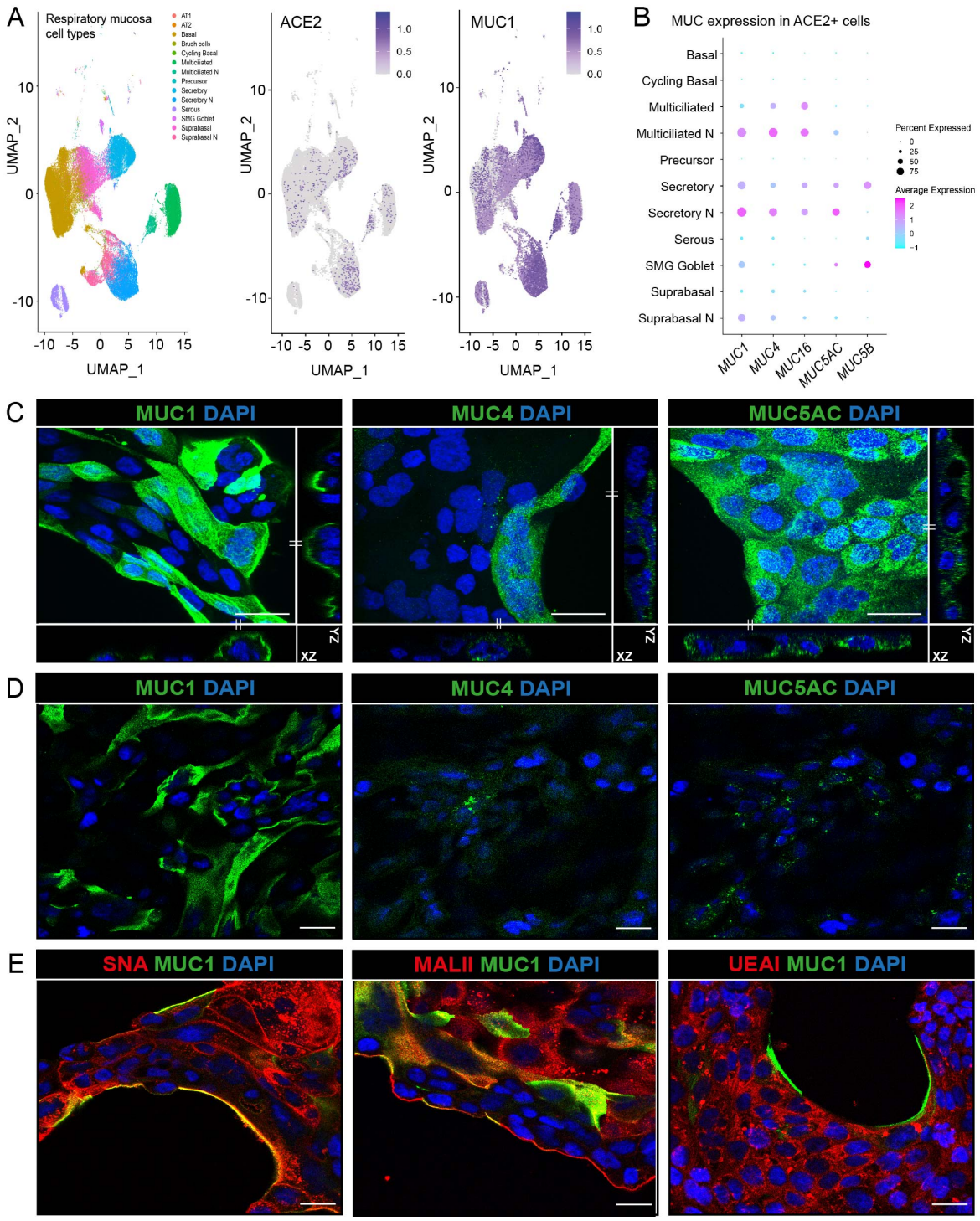

Figure 1. Expression of mucins in respiratory epithelial cells. (A) scRNA-seq analysis of ACE2 and MUC1 expression in different cell types in the respiratory mucosa. Dataset include samples from nasal cavity, upper, intermediate and lower respiratory tract(15). (B) Expression of TM mucins MUC1, MUC4 and MUC16 and gel-forming mucins MUC5AC and MUC5B in ACE2 positive cells. MUC1 is the most highly expressed mucin in ACE2-positive cells. (C) Immunofluorescence confocal microscopy images showing expression of TM mucins MUC1 (214D4, green), MUC4 (8G7, green) and gel-forming mucin MUC5AC (MUC5AC, green) in permeabilized Calu-3 cells. Maximum projections and side views of Z-stacks are shown. (D) Immunofluorescence confocal microscopy without permeabilization showing expression of MUC1 on the surface of Calu-3 cells. MUC4 and MUC5AC

663 could barely be detected suggesting intracellular localization. (E) Immunofluorescence confocal
664 microscopy imaging for α-2,6 sialic acids (SNA, red), α-2,3 sialic acids (MALII, red) and fucose (UEA1,
665 red) in combination with MUC1 (214D4 antibody, green) demonstrates high levels of sialic acid and
666 fucose in Calu-3 cells. Nuclei were stained with DAPI (blue). White scale bars represent 20 µm
667
668

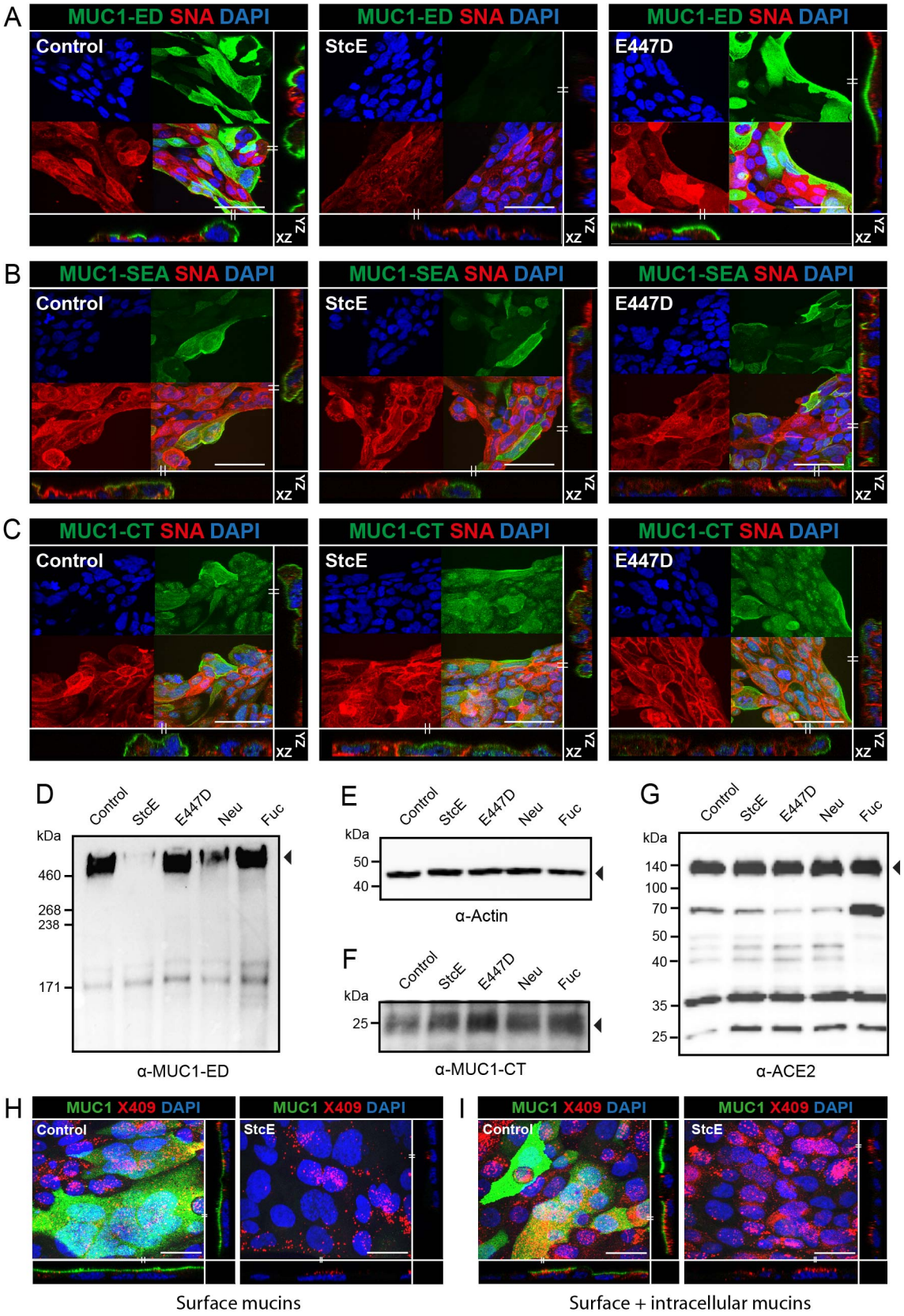

Figure 2. StcE specifically cleaves the glycosylated MUC1 ED and does not affect ACE2 expression. (A) Immunofluorescence confocal microscopy images showing Calu-3 cells treated with StcE or E447D stained for the glycosylated part of the MUC1 extracellular domain (214D4, green) and α-2,6-linked sialic acids (SNA, red). Complete loss of 214D4 signal was observed after treatment with StcE. (B,C)

675 Immunofluorescence confocal microscopy images of Calu-3 cells as above stained for the MUC1 SEA
676 domain (α-MUC1-SEA antibody 232A1, green) or cytoplasmic tail of MUC1 (α-MUC1-CT antibody CT2,
677 green) in combination with α-2,6-linked sialic acids (SNA, red). The SEA domain and CT were not
678 affected by StcE treatment. Nuclei were stained with DAPI (blue). White scale bars represent 20 µm.
679 Western blot analysis of 7-day grown Calu-3 cells incubated with indicated enzymes for 3 h at 37°C
680 stained with α-MUC1-ED antibody 214D4 (D), β-actin loading control (E), the MUC1 cytoplasmic tail
681 with α-MUC1-CT antibody CT2 (F), and ACE2 (G). StcE treatment removes the MUC1 ED but does not
682 affect the MUC1 CT or ACE2 receptor. (H) Immunofluorescence confocal microscopy images showing
683 Calu-3 cells treated with StcE stained for the glycosylated part of the MUC1 extracellular domain
684 (214D4, green) and fluorescently labelled mucin binding domain derived from StcE (X409-GFP) (X409,
685 red). More continuous surface staining for MUC1 ED and limited punctate staining with X409 on the
686 non-permeabilized cells Calu-3 cells. The MUC1 signal was completely removed after StcE treatment,
687 while some staining remained for X409. (I) Immunofluorescence confocal microscopy images showing,
688 a comparable result with a higher level of remaining X409 signal with permeabilized cells. White scale
689 bars represent 20 µm.

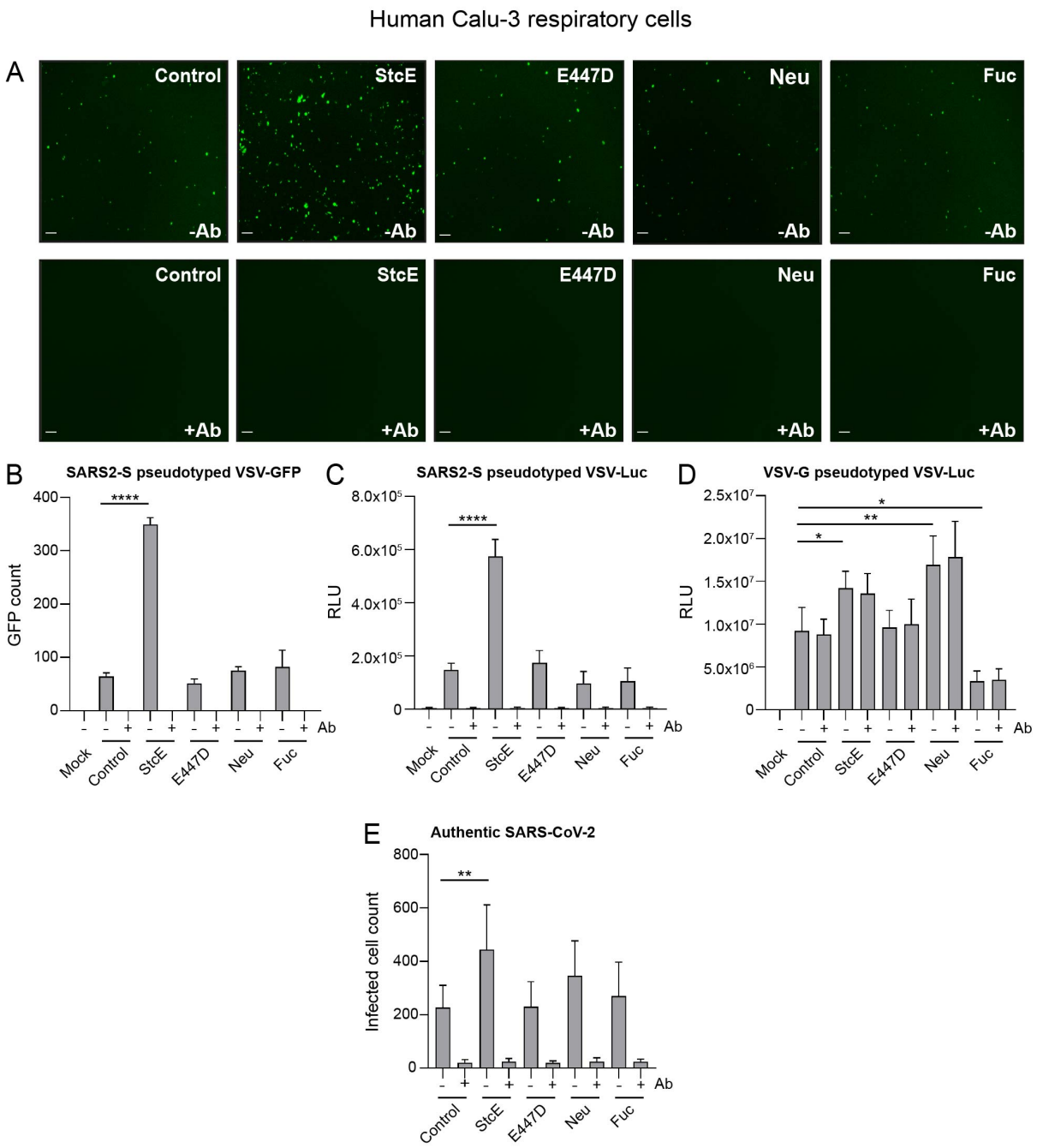

Figure 3. Removal of the glycosylated MUC1 extracellular domain enhances SARS-CoV-2 entry. (A) Microscopy images of Calu-3 cells treated with StcE, E447D, neuraminidase or fucosidase infected with SARS2-S pseudotyped VSV-GFP without or with neutralizing monoclonal antibody (mAb) against SARS2-Spike. White scale bars represent 200 µm. (B) Quantification of SARS2-S pseudotyped VSV-GFP signal in Calu-3 cells using EVOS software. StcE treatment resulted in a 5.4-fold increase in infection. (C) Quantification of luciferase signal (RLU) in Calu-3 cells after treatment with indicated enzymes and infection with SARS2-S pseudotyped VSV-Luc in the absence or presence of mAb against spike. A 4-fold increase in RLU value was observed when cells were treated with StcE. (D) Quantification of Calu-3 cell infection with VSV-G pseudotyped VSV-Luc lacking the spike protein. Infection was not blocked by the anti- spike mAb. (E) Infection of Calu-3 cells with authentic SARS-CoV-2 after treatment with indicated enzymes. StcE treatment resulted in a 2-fold increase in infected cell count. Neuraminidase and fucosidase treatment did not significantly impact viral entry. Represented values are the mean ± SEM of three biological replicates performed in triplicate. Statistical analysis was performed by

repeated measures one way-ANOVA with Dunnett's post-hoc test. $p > 0.05$ [ns, not significant], $p<0.05$ [*], $p<0.01$ [**], $p<0.001$ [***], $p<0.0001$ [****].

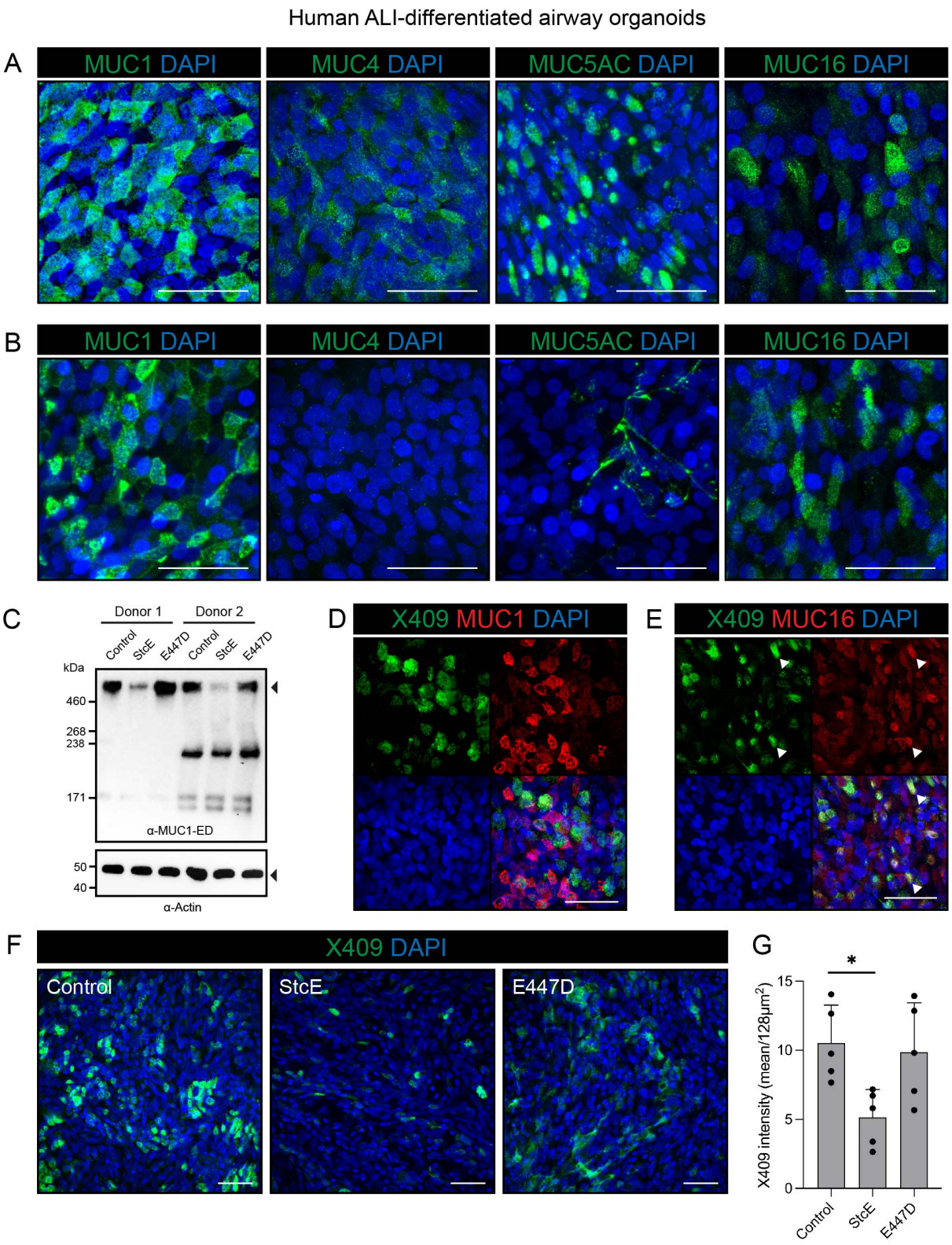

Figure 4. MUC1 and MUC16 are expressed on the surface of human airway organoid-derived air-liquid interface cultures and decreases upon StcE treatment. (A) Microscopy of permeabilized human airway organoid-derived air-liquid interface cultures showing combined extracellular and intracellular staining of MUC1, MUC4, MUC5AC and MUC16. (B) Microscopy of live stained air-liquid culture for MUC1, MUC4, MUC5AC and MUC16 without permeabilization. MUC1 and MUC16 are detectable demonstrating expression on the cell surface, whereas MUC4 staining is negative and MUC5AC only

713 stains positive in occasional mucus strands on top of the cells. (C) Immunoblot analysis of MUC1 levels
714 in human airway organoid-derived air-liquid interface cultures from donor 1 and donor 2 treated with
715 StcE, E447D or no treatment. The high molecular weight MUC1 is removed upon StcE treatment. (D,
716 E) Microscopy of MUC1 (D) and MUC16 (E) in permeabilized air-liquid cultures along with *O*-glycan
717 probe X409-GFP. Arrows indicate co-localization of X409 with MUC16, but not MUC1. (F) Microscopy
718 of surface binding of X409 on untreated, 10ug/ml StcE and 10ug/ml E447D treated air-liquid cultures.
719 All white scale bars indicate 50 μm. (G) Quantification of X409 signal intensity per imaged field from
720 experiment performed in E. Statistical analysis was performed by repeated measures one way-ANOVA
721 with Tukey's post-hoc test. $p<0.05$ [*].

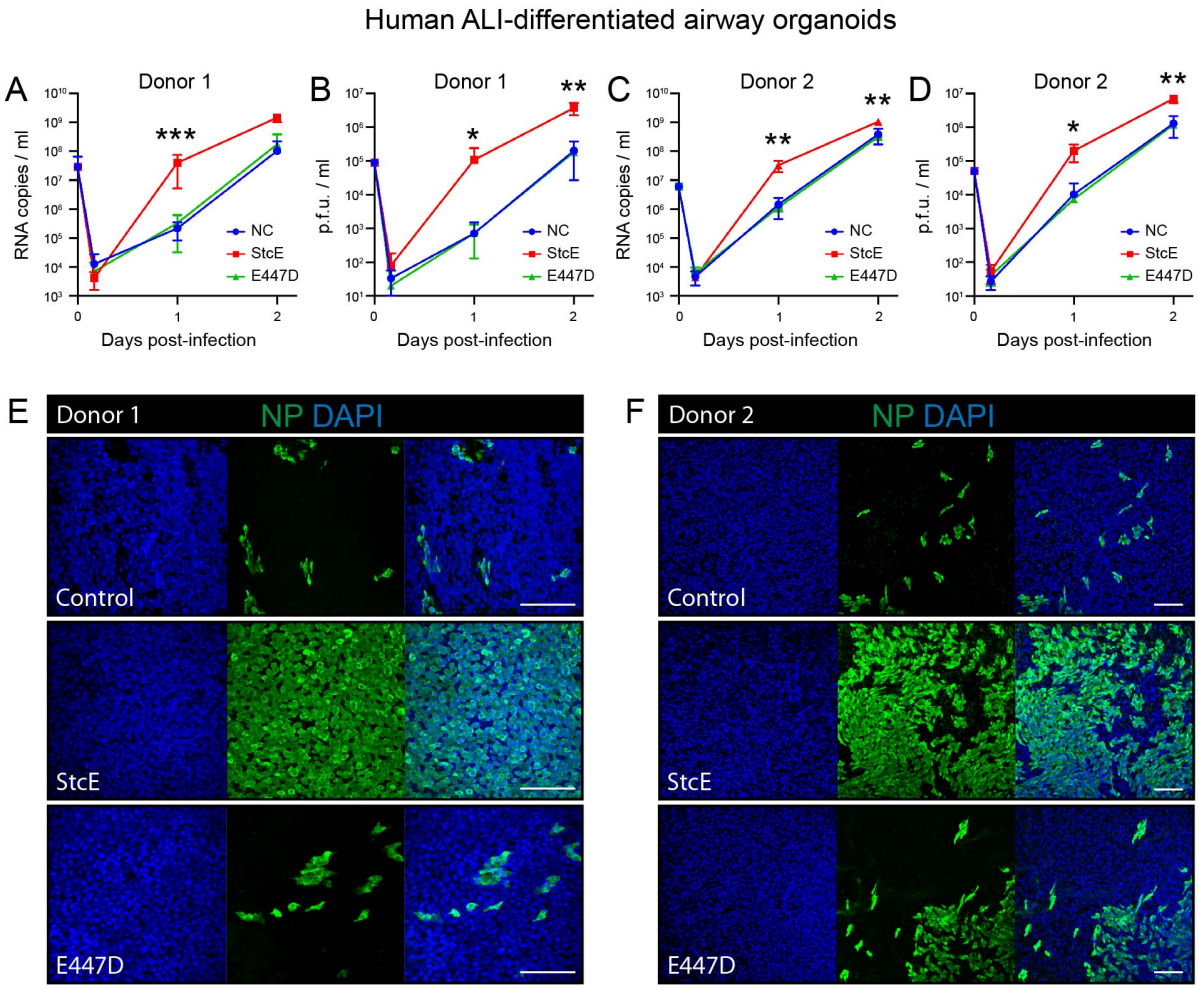

Figure 5. StcE treatment of human airway organoid-derived air-liquid interface cultures increases SARS-CoV-2 replication. (A-D) Replication kinetics of SARS-CoV-2 in air-liquid interface cultures in terms of RNA copies (A, C) and infectious virus (B, D) in two donors. Represented values are the mean ± SD of three replicates. Statistical analysis was performed for donor 2 by repeated measures two way-ANOVA with Tukey's post-hoc test. $p<0.05$ [*], $p<0.01$ [**], $p<0.001$ [***], $p<0.0001$ [****]. $p<0.01$ was found between NC and StcE and E447D and StcE treated cells at 1 day post infection and at 2 days post infection between E447D and StcE treated cells (C). $p<0.05$ was found between NC and StcE and E447D and StcE treated cells at 2 days post infection (D). (E-F) Microscopy images of untreated, 10ug/ml E447D or 10ug/ml StcE treated cells from donor 1 (E) or donor 2 (F), infected with SARS-CoV-2 at two days post-infection. White scale bars represent 100 μm. NP=nucleoprotein.

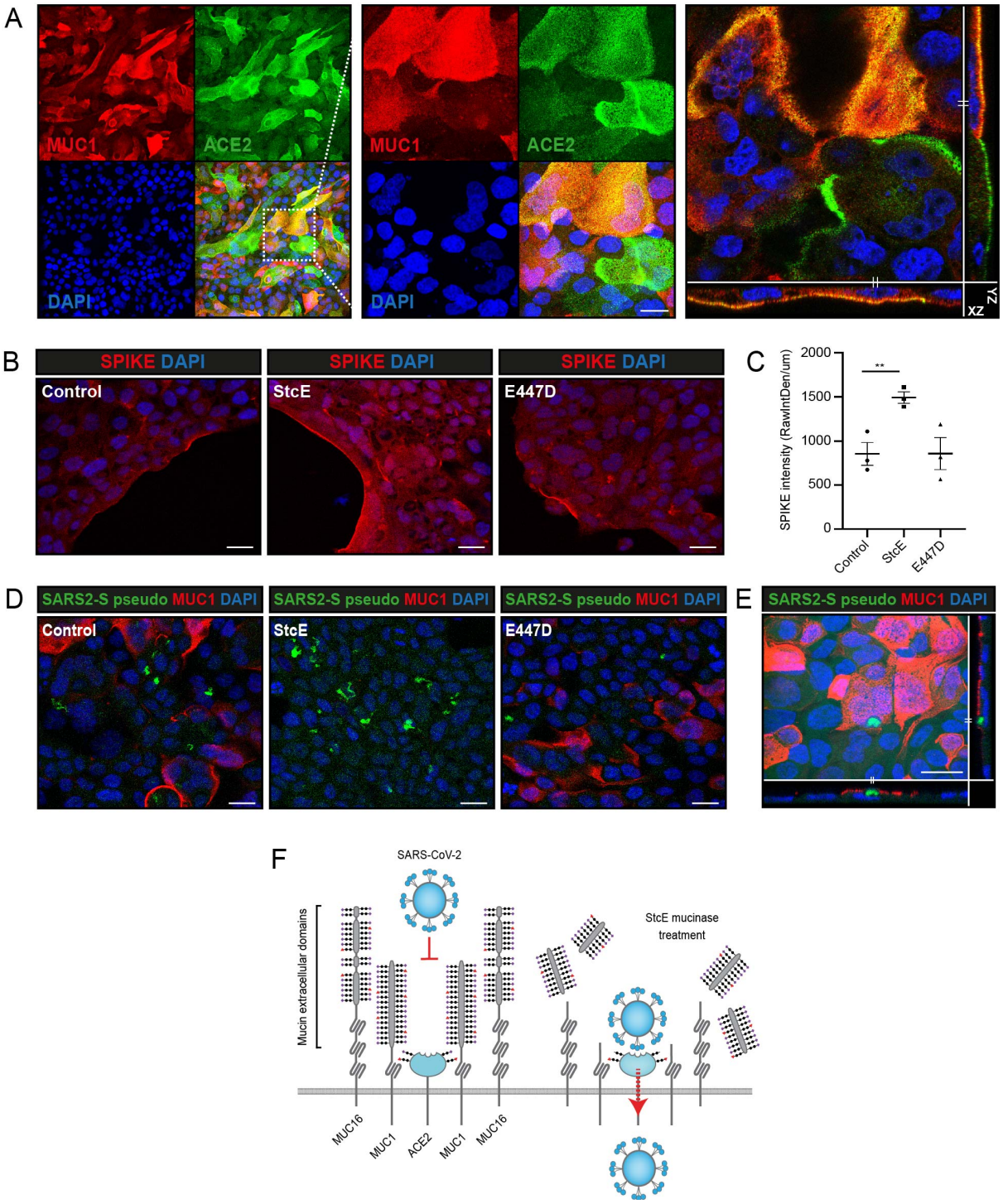

Figure 6. Removal of the MUC1 extracellular domain increases spike and virus attachment. (A) Immunofluorescence confocal microscopy analysis of expression and localization of ACE2 (green) and TM mucin MUC1 (214D4, red) in Calu-3 cells. (B) Immunofluorescence confocal microscopy of Calu-3 cells incubated with 2.5 ug/ml SARS-CoV-2 spike (Fc-tagged SARS2-S1B-Fc, red) at 4°C for 1 h. Spike was stained without permeabilization. Increased spike binding and higher spike signal intensity was observed after treatment with StcE in comparison to E447D treatment and control. (C) Quantification of spike fluorescence signal as depicted in B. Fluorescence intensity along the edge of cell island was determined in control, StcE- and E447D-treated cells using ImageJ. Mean ± SEM raw integrated density/length from three random fields from three independent experiments are plotted. The area of spike binding was significantly higher in StcE-treated cells. (D) Immunofluorescence

746 confocal microscopy of Calu-3 cells incubated with SARS2-S pseudotyped VSV-GFP (green) at 4°C for 1
747 h. Complete disappearance of MUC1 ED signal (214D4, red) and increased virus attachment (green)
748 and was observed in StcE-treated cells. Nuclei were stained with DAPI (blue). (E) Confocal microscopy
749 image showing spike-positive pseudoviral particles preferentially attach to a MUC1-negative surface
750 before invasion. White scale bars represent 20 µm. (F) Working model describing the protective
751 functions of the extracellular domains of transmembrane mucins MUC1 and MUC16 during SARS-CoV-
752 2 infection. The extended glycosylated extracellular domains prevent access of the virus to the ACE2
753 receptor (left). Enzymatic removal of the glycosylated part of the extracellular domains with the StcE
754 mucinase allows the viral spike protein to connect with the ACE2 receptor resulting in viral entry into
755 lung epithelial cells (right).

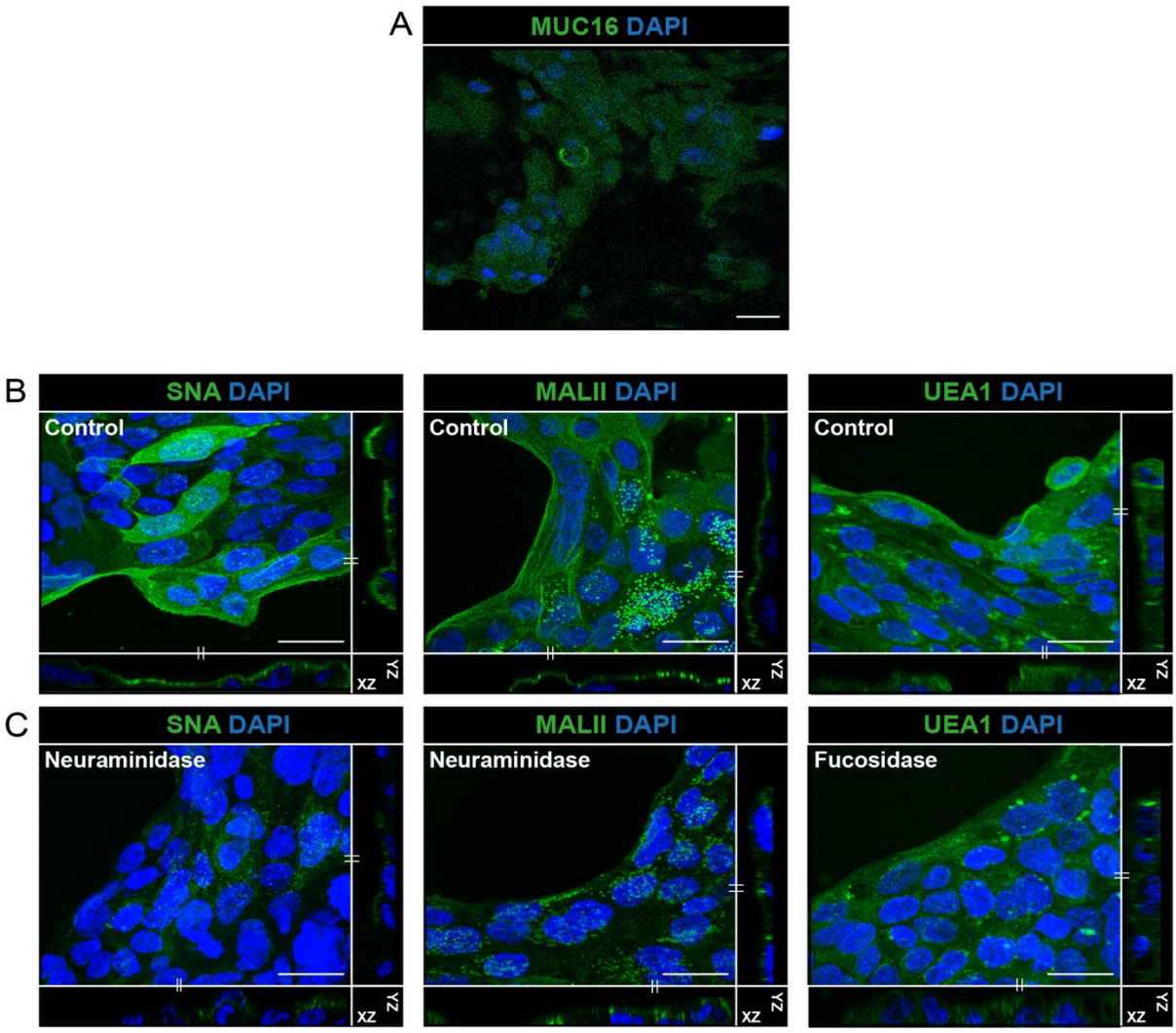

Figure S1. Characterization of expression of mucins and mucin glycans on Calu-3 cells. (A) Immunofluorescence confocal microscopy of 4-days grown Calu-3 cells revealed very limited expression of MUC16 (α-MUC16 ED, green). (B) Immunofluorescence confocal microscopy images for α-2,6 sialic acid (SNA, green) and α-2,3 sialic acid (MALII, green) levels after neuraminidase treatment and fucose (UEA1, green) after fucosidase treatment of Calu-3 cells. Nuclei were stained with DAPI (blue). White scale bars represent 20 µm.

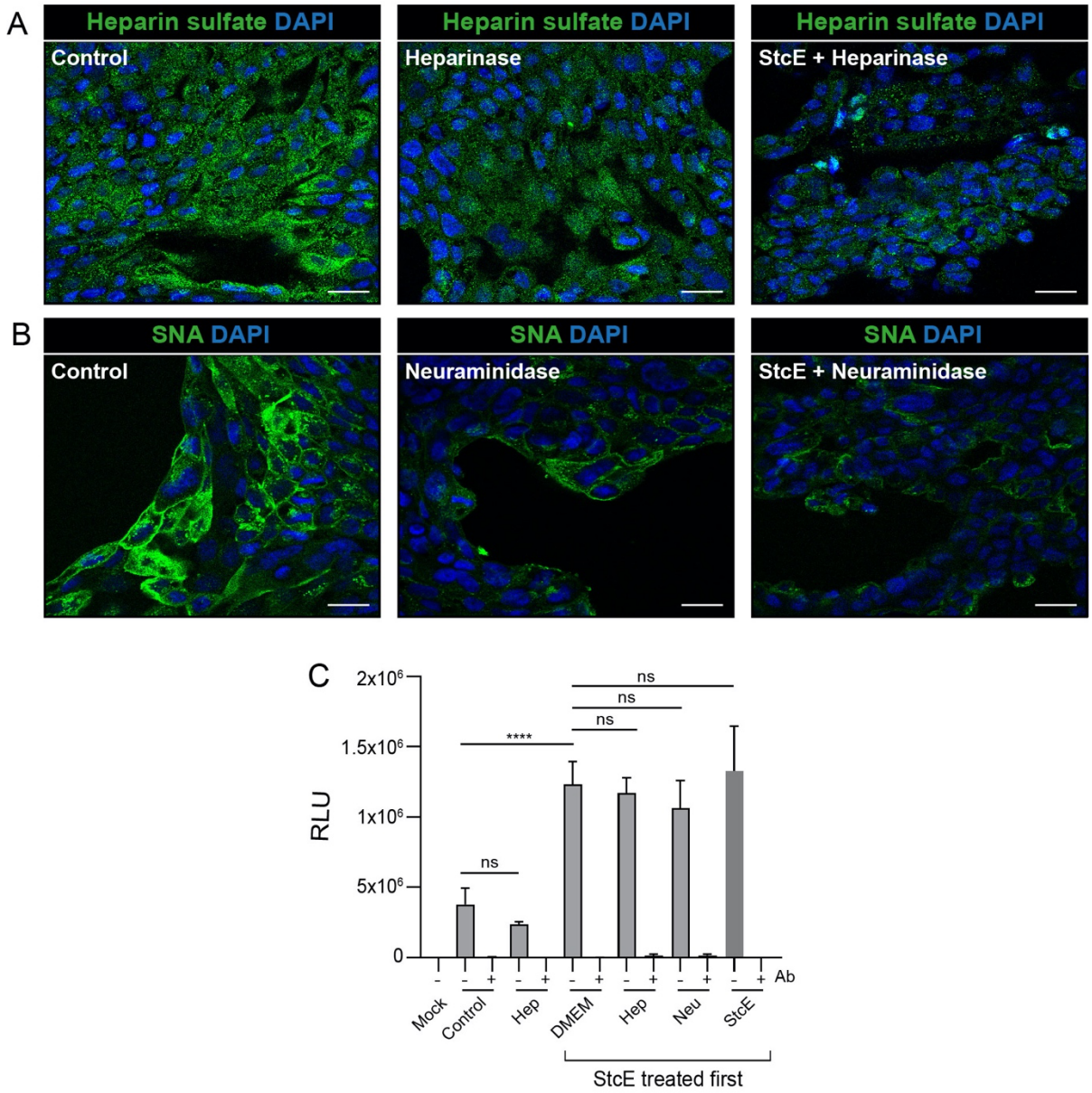

Figure S2. Heparinase or neuraminidase treatment in combination with mucinase does not affect SARS-CoV-2 entry.

(A) Confocal microscopy image showing levels of heparin sulfate (F69-3G10, green) in control, heparinase-treated and StcE/heparinase-treated Calu-3 cells. (B) Confocal microscopy image showing levels of α-2,6 sialic acid (SNA, green) control, neuraminidase-treated and StcE/neuraminidase-treated Calu-3 cells. Nuclei were stained with DAPI (blue). White scale bars represent 20 µm. (C) Luciferase quantification of viral infection of Calu-3 cells were treated with StcE for 3 h at 37°C followed by heparinase or neuraminidase for an additional 3 h at 37°C and infection with VSVΔG-Rluc*SARS2-Spike without or with monoclonal antibody (mAb) against SARS2-Spike. No significant changes in RLU values were observed in any of the cases. Represented values are the mean ± SEM of three biological replicates performed in triplicate. Statistical analysis was performed by repeated measures one way-ANOVA with Dunnett's post-hoc test. $p > 0.05$ [ns, not significant], $p<0.05$ [*], $p<0.01$ [**], $p<0.001$ [***], $p<0.0001$ [****].

777 ## Supplementary methods

778 **Production of pseudotyped vesicular stomatitis virus (VSV) and SARS2-Spike pseudotyped VSV virus**
779 BHK-21 cells were co-transfected with the plasmids pVSVΔG-GFP (green fluorescent protein reporter)
780 or pVSVΔG-Rluc (*Renilla reniformis* luciferase reporter) and the four assembly plasmids encoding the
781 VSV-N, -P, -G and -L proteins. The VSVΔG-GFP and Rluc virus stocks were harvested and added to BHK-
782 21 cells that were transfected with pCAGGS expression vector encoding VSV-G glycoprotein for the
783 production of VSV-G pseudotyped VSV-GFP and VSV-G pseudotyped VSV-Luc virus. After 48 h,
784 supernatant containing VSV-G pseudotyped VSV-GFP or VSV-G pseudotyped VSV-Luc viral particles
785 were harvested and used for the production of SARS2-Spike pseudotyped VSV-GFP or SARS2-Spike
786 pseudotyped VSV-Luc respectively according a previously described method (53).
787 For the production of SARS-CoV-2 pseudotyped virus, HEK-293T cells were transfected with a pCAGGS
788 expression vector encoding SARS-CoV-2 Spike carrying an 18-a.a. cytoplasmic tail truncation (SARS2-
789 S-d18-Flag). Two days post-transfection, cells were infected with the VSV-G pseudotyped VSV-GFP or
790 VSV-G pseudotyped VSV-Luc at a multiplicity of infection (MOI) of 1. After 4 h of infection, infection
791 medium was removed and washed thoroughly to remove surface-attached VSV pseudovirus. Twenty-
792 four hours later, supernatants containing SARS2-Spike pseudotyped VSV particles (SARS2-S
793 pseudotyped VSV-GFP and SARS2-S pseudotyped VSV-Luc) were harvested and stored at -80°C until
794 use. For the virus neutralization assay, monoclonal antibody (mAb) REGN10933 against SARS2-Spike
795 (developed in the group of Berend Jan Bosch) was used at a final concentration of 1 μg/ml in DMEM
796 supplemented with 1% FCS (Gibco), 100 U/ml Penicillin and 100 μg/ml Streptomycin. Diluted mAbs
797 were incubated with an equal volume of SARS2-S pseudotyped VSV-GFP or SARS2-S pseudotyped VSV-
798 Luc viral particles for 1 hour at room temperature, followed by inoculation on confluent Calu-3 cells in
799 a 96-well plate and incubation at 37°C for 18-20 h. Luciferase activity was measured on a Berthold
800 Centro LB 942 plate luminometer using the *Renilla* luciferase substrate coelenterazine (Promega). The
801 percentage of infectivity was determined as a ratio of luciferase signal in the absence of mAbs
802 normalized to luciferase signal in the presence of mAb.
803
804 **Production of authentic SARS-CoV-2 virus stock**
805 Briefly, cells were infected at a MOI of 0.01 and incubated for 48-72 h. Collected supernatant was
806 cleared by centrifugation before being additionally cleared using a 0.45 μM low protein binding filter
807 (Millipore) to remove mucus debris produced by the cells. The medium was exchanged three times
808 with Opti-MEM I (1X) + GlutaMAX (Gibco) using an Amicon Ultra-15 column (100 kDa cutoff). After
809 three exchanges, the purified virus was transferred to a new 50 ml tube and the Amicon Ultra-15
810 column was washed with 1 ml Opti-MEM I (1X) + GlutaMAX (Gibco), adding each wash to the tube

811 containing the purified virus preparation until the volume in the purified virus stock was equal to the
812 original volume of culture supernatant. Purified virus was stored at −80°C in aliquots. Stock titers were
813 determined by plaque assay. Briefly, 10-fold serial dilutions were performed in 2 ml Opti-MEM I (1X)
814 + GlutaMAX (Gibco). One ml of each virus dilution was added to monolayers of Calu-3 cells in the same
815 medium in a 12 well plate. Cells were incubated at 37°C for 1 hr and then overlaid with 1.2% Avicel
816 (FMC biopolymers) in Opti-MEM I (1X) + GlutaMAX (Gibco) for 72 hours. Next, cells were washed once
817 in PBS, fixed in formalin, permeabilized in 70% ethanol and washed in PBS again. Cells were blocked
818 in 3% BSA (bovine serum albumin; Sigma) in PBS, stained with mouse anti-nucleocapsid (Sino
819 biological; 1:1000) in PBS containing 0.1% BSA, washed three times in PBS, then stained with goat anti-
820 mouse Alexa Fluor 488 (Invitrogen; 1:2000) in PBS containing 0.1% BSA and then washed three times
821 in PBS. All staining steps were performed at room temperature for one hour. Plates were scanned on
822 the Amersham Typhoon Biomolecular Imager (channel Cy2; resolution 10 µm; GE Healthcare). All
823 work with infectious SARS-CoV-2 was performed in a Class II Biosafety Cabinet under BSL-3 conditions
824 at Erasmus Medical Center.
825
826 **Confocal microscopy**
827 For staining of the α-MUC1 ED, α-MUC1 SEA, α-MUC4 ED, α-MUC16 ED, α-MUC5AC, SNA, MALII, UEA1,
828 ACE2, and Spike, fixed cells on coverslips were permeabilized in binding buffer containing 0.1%
829 saponin (Sigma) and 0.2% BSA (Sigma) in DPBS for 30 min. After permeabilization, cells were washed
830 two times with DPBS and incubated onto 50-µl drops containing 214D4 antibody (CD227, Nordic
831 MUbio) for α-MUC1 ED, 232A1 antibody (a kind gift from Dr. John Hilkens, AVL Amsterdam) for α-
832 MUC1 SEA, α-MUC4 ED (8G7; sc-53945, Santa Cruz Biotechnology), α-MUC5AC (ab198294, Abcam),
833 α-MUC16 ED (a kind gift from Ulla Mandel, University of Copenhagen, Denmark), biotinylated-SNA (B-
834 1305-2; Vector Lab), biotinylated-MALII (B-1265-1; Vector Lab), biotinylated-UEA1 (B-1065-2; Vector
835 Lab), ACE2 (ab272690, Abcam), Heparin sulphate (370260-S; Amsbio) diluted 1:100 and Fc-tagged
836 SARS2-S1B-Fc (a kind gift from Berend Jan Bosch, Utrecht University) diluted 1:164 in the binding
837 buffer on parafilm for 1 h at RT. For staining of the MUC1 CT domain, cells on coverslips were
838 permeabilized in DPBS containing 0.2% Triton X-100 (Merck) for 10 min. After permeabilization, cells
839 were blocked with 1% BSA and 22.5 mg/ml glycine in PBST (DPBS + 0.1% Tween 20 [Sigma]) for 30 min
840 and washed three times with DPBS. Cells were incubated onto 50-µl drops of the α-MUC1-CT antibody
841 (ab80952, Abcam) diluted 1:100 with 1% BSA in PBST on parafilm for 1 h at RT. For non-
842 permeabilization microscopy, the cell permeabilization step was omitted and primary antibody was
843 used in 0.2% BSA in DPBS for 1 h at RT. After removing the primary antibody, 3 washing steps were
844 performed. The coverslips were further incubated with the secondary antibodies Alexa Fluor 488-

845 conjugated goat α-mouse IgG (1:100; A11029, Thermo Fisher), Alexa Fluor 568-conjugated goat α-
846 mouse IgG (1:100; A11031, Thermo Fisher), Alexa Fluor 647-conjugated goat α-mouse IgG (1:100;
847 ab150115, Abcam), Alexa Fluor 488-conjugated goat α-rabbit IgG (1:100; A11034, Thermo Fisher),
848 Alexa Fluor 488-conjugated goat α-Armenian hamster IgG (1:100, ab173003, Abcam), Alexa Fluor 594-
849 conjugated goat α-human IgG (1:400; A-11014, Thermo Fisher), Streptavidin-488 (1:100; A6374,
850 Thermo Fisher) and 568 (1:100; S11226, Thermo Fisher) and DAPI at 2 µg/ml (D21490, Invitrogen) for
851 1 h. Coverslips were washed 3 times with DPBS, washed a final time with MilliQ, dried, embedded in
852 Prolong diamond mounting solution (Thermo Fisher), and allowed to solidify overnight at RT. For initial
853 attachment of spike and virus study, 2.5 ug/ml purified Fc-tagged spike protein of SARS-CoV-2 (SARS2-
854 S1B-Fc) and VSVΔG-GFP*SARS2-Spike was added to Calu-3 cells at 4°C for 1 h after StcE and E447D
855 treatment. Cells were washed thoroughly to remove unbound spike/virus before fixation and followed
856 the non-permeabilization protocol for staining. For the 20 h infection study, VSVΔG-GFP*SARS2-Spike
857 was added to Calu-3 cells after StcE and E447D treatment and incubated at 37°C for 20 h. Cells were
858 fixed after three washes and the permeabilization protocol was followed.
859 For costaining of the α-MUC1 ED and ACE2, fixed cells on coverslips were permeabilized in 70%
860 absolute ethanol (Merck) for 10 min. After permeabilization, cells were washed two times with DPBS
861 and then blocked with 0.6% BSA in DPBS for 30 min. Cells were incubated onto 75-µl drops containing
862 214D4 antibody (CD227, Nordic MUbio) diluted 1:100 for MUC1-ED and ACE2 antibody (AF933, R&D
863 Systems) diluted 1:200 with 0.6% BSA in DPBS on parafilm overnight at 4°C. Afterwards three washing
864 steps were performed. The coverslips were further incubated with the secondary antibodies Alexa
865 Fluor 488-conjugated rabbit α-goat IgG (1:500; A11078, Invitrogen), Alexa Fluor 568-conjugated
866 donkey α-mouse IgG (1:100; A10037, Thermo Fisher) and DAPI at 2 µg/ml (D21490, Invitrogen) for 1
867 h. Coverslips were washed 3 times with DPBS, washed a final time with MilliQ, dried, embedded in
868 Prolong diamond mounting solution (Thermo Fisher), and allowed to solidify overnight at RT.
869 A Leica SPE-II confocal microscope (Leica Microsystems, Wetzlar, Germany) was used to acquire single
870 plane images with a 40X objective (NA 1.3, HCX PLANAPO oil) controlled by Leica LAS AF software with
871 default factory settings. A quad band dichroic was used, allowing diode laser wavelengths 405, 488,
872 561 nm lines to pass and fluorescent signal to enter the prism to sequentially detect DAPI, Alexa Fluor
873 488, Alexa Fluor 568/594, Alexa Fluor 647. Images stacks were collected using a 63X oil immersion
874 objective in bidirectional mode (average 16). Sequential laser illumination through a quadband
875 dichroic (405, 488, 561, and 647) was used in combination with emission factor default spectral
876 detection of DAPI, Alexa Fluor 488, Alexa Fluor 568/594, and Alexa Fluor 647, respectively. XZ and YZ
877 Orthogonal views of 30-pixel thickness are shown generated in either Imaris (Oxford Instruments
878 version 8.2, Belfast, UK). Acquired images were processed in Leica Application Suite X (LAS X; Leica).

879 Maximum intensity projections are shown, as well as representative slices from the image series. Final
880 outlining of the figures was performed in Adobe Illustrator (Adobe Inc., San Jose, USA).
881 Fluorescent spike signal was quantified by ImageJ software (54). A segmented line (line width=10) was
882 outlined along the edge of the cell island to select the region of interest (ROI) in control, StcE and
883 E447D treated Calu-3 cells. The sum of all pixels in the ROI was measured by Raw Integrated Density
884 (RawIntDen) and the bar diagram was represented as RawIntDen/length (µm). Three random regions
885 were selected, and three independent experiments were performed.
886 Spinning disk imaging was performed on an Olympus SpinSR10 system equipped with a Yokogawa W1-
887 SoRa spinning disk mounted on a IX83 stand with an ORCA Flash 4.0 camera (Olympus, Leidendorp,
888 the Netherlands). The system was run in confocal mode using a 60 x Uplan Apo OHR objective (NA 1.5)
889 and multiband dichroic to sequentially illuminate 405, 488 and 561 nm laserlines, emission filter
890 wheels set to detect 447/60, 525/50 and 617/73 bandwidths for DAPI, Alexa488 and Alexa568
891 respectively. Images were deconvolved using maximum likelihood estimation in 5 iterations in
892 CellSense Dimension (Olympus) and intensity adjusted.