

1 **MUC13 negatively regulates tight junction proteins and intestinal epithelial barrier**  
2 **integrity via Protein Kinase C**

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23 **Short title:** MUC13 negatively regulates TJs and epithelial barrier integrity via PKC

24

25 Key words: intestinal barrier function, transmembrane mucin, cell-bound mucin, paracellular  
26 permeability, tight junctions, claudin-1, claudin-3, claudin-4, PKC $\delta$

27 **Abstract**

28 Regulation and adaptation of intestinal epithelial barrier function is essential for human health. The  
29 transmembrane mucin MUC13 is an abundant intestinal glycoprotein with important functions for  
30 mucosal maintenance that are not yet completely understood. We demonstrate that in intestinal  
31 epithelial monolayers MUC13 localized to both the apical surface and the tight junction (TJ) region  
32 on the lateral membrane. MUC13 deletion resulted in increased transepithelial resistance (TEER) and  
33 reduced translocation of small solutes. TJ proteins including claudins and occludin were highly  
34 increased in membrane fractions of MUC13 knockout cells. Removal of the MUC13 cytoplasmic tail  
35 (CT) also altered TJ composition but did not result in increased TEER. The increased buildup of TJ  
36 complexes in  $\Delta$ MUC13 and MUC13- $\Delta$ CT cells was dependent on PKC, which is in line with a predicted  
37 PKC motif in the MUC13 cytoplasmic tail. The responsible PKC member might be PKC $\delta$  based on  
38 elevated protein levels in the absence of MUC13. Our results identify MUC13 as a central player in TJ  
39 complex stability and intestinal barrier permeability.

## 40 Introduction

41 The intestinal epithelial barrier is a dynamic system that prevents bacterial invasion while at the  
42 same time allowing the transport of nutrients (1, 2). The intestinal mucosal epithelium consists of  
43 various types of enterocytes and a closely associated mucus layer in which highly glycosylated mucin  
44 proteins are the main structural component. Mucins can be categorized into soluble mucins that are  
45 secreted by goblet cells and transmembrane (TM) mucins that are cell-bound and expressed by most  
46 types of enterocytes. TM mucins expressed in the human intestinal tract include MUC1, MUC3,  
47 MUC12, MUC13, and MUC17 (3) of which MUC13 shows the most widespread expression along the  
48 different segments of the gastrointestinal tract (4). The extracellular domains of TM mucins are  
49 highly glycosylated and their cytoplasmic tails have signaling capacity (2). TM mucins are highly  
50 diverse, and the different members have been implicated in fundamental epithelial processes  
51 including the regulation of cell-cell interactions, proliferation, differentiation, apoptosis, and  
52 modulation of inflammatory responses (2, 5, 6). Dysfunction of TM mucins has been associated with  
53 the development of inflammatory bowel disease (IBD) including ulcerative colitis (UC) and Crohn's  
54 disease (CD) (7–9). Reduced intestinal barrier function and the translocation of bacterial  
55 components across the intestinal mucosal-epithelial barrier are hallmarks of IBD. The contributions  
56 of specific TM mucins to epithelial barrier integrity and development of IBD remain to be  
57 established.

58

59 MUC13 is a relatively small TM mucin that consists of a glycosylated extracellular domain (ED) that  
60 contains a Sperm protein, Enterokinase, and Agrin (SEA) domain, three epithelial growth factor  
61 (EGF)-like domains, and a cytoplasmic tail (CT) with putative phosphorylation sites. Previous studies  
62 demonstrated MUC13 expression on the apical surface of polarized epithelial cells, and cytoplasmic  
63 and nuclear localization was observed in colorectal cancer (CRC) and during metastasis (4, 10).  
64 MUC13 mRNA expression is upregulated in the inflamed colon in IBD patients (11) and a mutation in  
65 the MUC13 cytoplasmic tail was shown to be associated with the development of UC (12, 13).

66

67 The function of MUC13 seems to be multifaceted as it has been linked to different aspects of  
68 mucosal maintenance and inflammation. Overall, most MUC13-associated phenotypes can be  
69 considered pro-inflammatory and promote wound healing and tumorigenesis. MUC13 enhances the  
70 epithelial pro-inflammatory response to bacterial ligands (14) and interacts with tumor necrosis  
71 factor receptor 1 (TNFR1) thereby promoting TNF-induced NF- $\kappa$ B activation (15). Muc13-deficient  
72 mice and human intestinal MUC13 knockdown cells are more sensitive to toxin-induced apoptosis  
73 (11). Single-cell migration is enhanced in colon cancer cells with MUC13 overexpression (16). In  
74 pancreatic ductal adenocarcinoma (PDAC) cells, MUC13 interacts with HER2 resulting in activation  
75 and cytoskeletal remodeling, growth, motility, and invasive growth (17). Thus, MUC13 seems to be a  
76 key protein that is linked to several aspects of intestinal epithelial health and disease, but the  
77 underlying molecular mechanisms remain to be resolved.

78

79 Epithelial barrier integrity is critically regulated by the junction complexes that are embedded in the  
80 lateral membranes of neighboring cells. The junction complexes can be divided into adherence  
81 junctions (AJ), tight junctions (TJ), and desmosomes. Together, they form the apical junctional  
82 complex which seals the paracellular space between cells (18). TJ are large multimeric protein  
83 complexes in the lateral membrane that consist of various transmembrane proteins, including  
84 occludin and claudins (19, 20). The main function of TJs is the regulation of paracellular permeability,  
85 but they also play a role in polarization, morphogenesis, cell proliferation, and regulation of gene

86 expression (21). Intracellularly, proteins such as ZO-1 connect the TJ complex to the actin  
87 cytoskeleton and signal transduction molecules (22, 23). AJ and desmosomes are present along the  
88 full length of the lateral membrane, connecting adjacent cells, and contribute to the barrier function  
89 without sealing the paracellular space (19). The main structural protein of AJs is E-cadherin. Through  
90 its intracellular tail, E-cadherin interacts with  $\beta$ -catenin, the central regulator of the epithelial WNT  
91 pathway (24). Changes in barrier function and TJ and AJ proteins are often observed in IBD (25–28).

92

93 Multiple members of the TM mucin family have been implicated in the regulation of cell-cell  
94 interactions. MUC1, MUC4, and MUC16 all reduce the interaction between E-cadherin and  $\beta$ -catenin  
95 at the membrane, thereby promoting  $\beta$ -catenin translocation to the nucleus and subsequent  
96 activation of the Wnt signaling pathway (29–33). MUC1 and MUC16 can interact directly with  $\beta$ -  
97 catenin via the phosphorylated cytoplasmic tail (34, 35), whereas MUC13 can enhance nuclear  
98 translocation of  $\beta$ -catenin through interaction with GSK-3 $\beta$  (36). Several studies have linked MUC1,  
99 MUC16, and MUC17 with alterations in TJ proteins, thereby influencing epithelial monolayer  
100 properties, though the underlying mechanisms are not yet understood (37–40). Whether MUC13  
101 regulates TJ proteins and epithelial barrier integrity is yet unknown.

102

103 In the present study, we investigate the function of MUC13 in the regulation of barrier integrity of  
104 the intestinal epithelium. Our data identify MUC13 as a central regulator of tight junction strength  
105 and paracellular passage which has important implications for the role of this TM mucin in IBD and  
106 colorectal cancer development.

## 107 **Results**

108

### 109 **MUC13 is highly expressed in the intestinal tract and localizes to the apical and lateral membrane**

110 To determine the expression of MUC13 and other mucin genes in different segments and cell types  
111 of the gastrointestinal tract, we analyzed the gut atlas single-cell RNA-sequencing dataset  
112 (gutcellatlas.org). This dataset contains 428,000 intestinal cells from fetal, pediatric, and adult  
113 donors. We focused on the adult cells and extracted the average expression of the different  
114 transmembrane and soluble mucins from each part of the gastrointestinal tract. MUC13 was  
115 expressed in at least 50% of the cells across all locations (Fig. 1A). MUC3A was also detected in all  
116 segments but the expression was lower in the appendix and rectum. MUC1 and MUC4 expression  
117 was mainly observed in the colon and rectum, while MUC17 showed the opposite pattern with high  
118 expression in the small intestine. We then analyzed the dataset for mucin expression within different  
119 cell type lineages. As expected, high expression of the secreted mucin MUC2 was observed for  
120 goblet cells. Transmembrane mucins MUC1 and MUC4 were also highly expressed in goblet cells. A  
121 comparable expression pattern was found for MUC13 and MUC3A with high expression throughout  
122 all cell types with the highest levels in enterocytes and BEST4+ epithelial cells (Fig. 1B).

123

124 MUC13 has been reported to localize to the apical surface of differentiated intestinal epithelial  
125 tissue (4, 10). We determined the expression and localization of MUC13 in intestinal epithelial  
126 HRT18 and Caco-2 cells. Immunofluorescence confocal microscopy was performed with a MUC13  
127 antibody directed against the cytoplasmic tail. With this antibody, the majority of MUC13 was  
128 detected on the lateral membranes of both HRT18 and Caco-2 cells (Fig. 1C). By creating Z-stacks, we  
129 observed MUC13 staining from the apical side of the lateral membrane towards the middle and  
130 limited or no staining in the basal planes which depict the lower region of the lateral membrane. The  
131 tight junction protein occludin also localized to the top half of the lateral membrane, similar to  
132 MUC13 (Fig. 1D). Staining of the adherence junction protein E-cadherin was observed along the  
133 entire lateral membrane (Fig. S1). Using a previously described method for transmembrane proteins  
134 (41), we generated a novel monoclonal antibody against the extracellular domain of MUC13. This  
135 antibody stained both the apical surface and upper part of the lateral membrane in HRT18 cells (Fig.  
136 1E). These results demonstrate that different MUC13 antibodies recognize distinct MUC13  
137 populations on the apical and lateral membranes. We conclude that MUC13 localizes to the apical  
138 surface of enterocytes and the apical side of the lateral membrane in the region where tight  
139 junctions are found.

140

### 141 **Deletion of MUC13 and targeted deletion of the MUC13 cytoplasmic tail using CRISPR/Cas9**

142 To study the function of the full-length MUC13 protein and the contribution of the MUC13  
143 cytoplasmic tail, we designed CRISPR/Cas9 strategies to generate two types of HRT18 MUC13  
144 knockout cell lines. Expression of the full-length MUC13 protein was eliminated by deletion of 380  
145 base pairs in the second exon which resulted in disruption of the reading frame (Fig. 2A). As a  
146 control, HRT18 cells were transduced with an empty CRISPR plasmid without guide RNAs, and the  
147 resulting cell line was used in all the experiments as accompanying wild type (HRT18-WT). For  
148 targeted removal of the MUC13 cytoplasmic tail, we selected gRNAs that target exon 10 and were  
149 predicted to result in the removal of 121 bp that encode the majority of the MUC13 cytoplasmic tail  
150 (Fig. 2A). For all genotypes, we generated two independent cell lines resulting in two HRT18-WT (WT  
151 1 and 2), two HRT18- $\Delta$ MUC13 ( $\Delta$ MUC13 1 and 2), and two HRT18-MUC13- $\Delta$ CT cell lines (MUC13- $\Delta$ CT  
152 1 and 2). The domain structures of the MUC13 WT and  $\Delta$ CT proteins are depicted in Fig. 2B, and the

153 amino acid sequence of each domain is shown in Fig. 2C. The resulting deletion and disrupted  
154 reading frame of the different cell lines were confirmed by PCR and sequencing (Fig. 2D). The two  
155  $\Delta$ MUC13 cell lines lacked 300 and 377 bp fragments, respectively. Both MUC13- $\Delta$ CT clones had a  
156 deletion of 121 bp resulting in a stop codon three amino acids after the deletion. The predicted  
157 sequence of the remaining cytoplasmic tail is ARSNNKTKHIEEENLIDEDFQNLKLSIR\*, which lacks  
158 multiple putative phosphorylation sites and a predicted PKC motif that are present in the full-length  
159 cytoplasmic tail.

160

161 Next, we investigated the expression of MUC13 or the truncated protein in HRT18-WT,  $\Delta$ MUC13,  
162 and MUC13- $\Delta$ CT cells. Western blot analysis with the MUC13-CT antibody showed MUC13-reactive  
163 bands of 120 kDa and 130 kDa in the WT cell lines, which were absent in  $\Delta$ MUC13 and MUC13- $\Delta$ CT  
164 cells (Fig. 2E). A slightly different molecular weight was observed for MUC13 in the two WT cell lines  
165 which could be the result of differential glycosylation or processing and/or activation by  
166 (auto)proteolytic cleavage as has been reported for MUC1 (2, 42, 43). MUC13 expression in the  
167 different cell lines was also investigated by confocal microscopy. With the antibody directed against  
168 the cytoplasmic tail, we observed lateral membrane staining in HRT18-WT cells, while the signal was  
169 absent in  $\Delta$ MUC13 and MUC13- $\Delta$ CT cells (Fig. 2F). With the antibody directed against the  
170 extracellular domain, we observed apical and lateral staining in WT cells, but not in  $\Delta$ MUC13 cells.  
171 For the MUC13- $\Delta$ CT 1 cell line, we noted reduced intensity staining of the extracellular domain  
172 compared to the WT cells, while the extracellular domain was barely detectible in the MUC13- $\Delta$ CT 2  
173 cell line (Fig. 2G). These results demonstrate that while they are genetically identical, expression  
174 levels differ between the two  $\Delta$ CT cell lines which might be due to reduced stability of the truncated  
175 MUC13 protein. We conclude that our CRISPR-Cas9 strategy in the intestinal epithelial HRT18 cells  
176 was successful and resulted in MUC13 knockout cell lines and cell lines that express MUC13 without  
177 the cytoplasmic tail.

178

#### 179 **Generation of MUC13-GFP overexpression and complementation cell lines**

180 To complement MUC13 in the knockout cell lines, we cloned a doxycycline-inducible MUC13-GFP  
181 plasmid with a codon-optimized MUC13 DNA sequence that left the amino acid sequence unaltered  
182 but allowed cloning and expression. Lentiviral transduction was used to introduce the MUC13-GFP  
183 construct into HRT18 wild type and  $\Delta$ MUC13 cells resulting in overexpression WT+pMUC13 and  
184 complemented  $\Delta$ MUC13+pMUC13 cell lines. Doxycycline induction resulted in MUC13-GFP  
185 expression in at least 50% of the total cell populations (Fig. 2H).

186

#### 187 **Deletion of MUC13 alters epithelial barrier properties**

188 To investigate the contribution of MUC13 to epithelial barrier properties, we grew the HRT18 cell  
189 lines for two weeks to allow the buildup of cell junctions. Cells reached full confluency on day 3. To  
190 determine the monolayer architecture, we performed immunofluorescence microscopy and stained  
191 for occludin, E-cadherin, and nuclei (DAPI). All cell lines formed confluent monolayers with  
192 comparable occludin and E-cadherin staining.  $\Delta$ MUC13 cells did show a less rounded cell  
193 morphology compared to WT and MUC13- $\Delta$ CT cells (Fig. 3A). Next, confluent monolayers were  
194 grown on membranes in Transwell plates and differentiated for 14 days. Transepithelial electrical  
195 resistance (TEER), a measure of TJ strength based on electrical resistance, was determined over time  
196 for all cell lines. The TEER of  $\Delta$ MUC13 clones was, on average, three-times higher compared to WT  
197 cells while the TEER of the MUC13- $\Delta$ CT cells was comparable to WT cells (Fig. 3B, C). To rule out the  
198 possibility that differences in TEER were caused by differences in cell numbers, we counted the

199 number of nuclei per plane after 14 days of differentiation. The numbers of nuclei were comparable  
200 between the cell lines, indicating that the difference in TEER was not a result of differences in  
201 proliferation (Fig. 3D). Next, we determined the buildup of TEER in the MUC13 overexpression and  
202 complementation cell lines WT+pMUC13 and  $\Delta$ MUC13+pMUC13. Overexpression of MUC13-GFP in  
203 the  $\Delta$ MUC13 background led to a significant reduction of TEER buildup over time, while  
204 overexpression in the wild type background did not affect TEER (Fig. 3E, F). Together, these data  
205 indicate that MUC13 negatively regulates TEER buildup.

206

#### 207 **MUC13 deletion leads to decreased paracellular passage of small molecules**

208 TEER reflects the conductance of small ions via the paracellular pathway, which represents the  
209 passage of molecules through the intercellular spaces between adjacent epithelial cells. The flux of  
210 larger molecules through the paracellular pathways can be addressed using organic tracers, such as  
211 Lucifer Yellow CH and fluoresceinated (FITC)-dextran particles. We seeded our cell lines on Transwell  
212 membranes as before and the transfer of compounds from the apical compartment to the  
213 basolateral side was determined. WT,  $\Delta$ MUC13, and MUC13- $\Delta$ CT cells were all highly restrictive for  
214 the passage of 4 and 70 kDa FITC-dextran particles. For the smaller 520 Da Lucifer Yellow tracer,  
215  $\Delta$ MUC13, and MUC13- $\Delta$ CT monolayers were restrictive while WT cells were permeable (Fig. 3G).  
216 Because translocation of bacterial endotoxin lipopolysaccharide (LPS) across the intestinal barrier is  
217 an important hallmark of intestinal barrier dysfunction, we determined the passage of *Escherichia*  
218 *coli* 0111:B4 lipopolysaccharide (LPS-EB). A maximum of  $\sim$ 300 ng/ $\mu$ L LPS reached the basal  
219 compartment after 24 hours incubation for the control wells, and less than 0.1 ng/ $\mu$ L LPS passage for  
220 the different HRT18 cell lines. LPS passage was comparable between WT,  $\Delta$ MUC13, and MUC13- $\Delta$ CT  
221 cell lines indicating the restrictiveness of these cells to the passage of larger particles (Fig. 3H). In  
222 summary, we observed that deletion of MUC13 results in a higher buildup of TEER and lower  
223 paracellular passage of the small organic solute Lucifer Yellow compared to WT. The TEER of the  
224 MUC13- $\Delta$ CT cell line was comparable to WT, but a significant restriction of Lucifer Yellow passage  
225 compared to WT was observed. We conclude that the paracellular pathway is altered in both MUC13  
226 deletion cell lines.

227

#### 228 **Epithelial barrier strengthening by *Lactobacillus plantarum* is independent of MUC13**

229 To investigate the role of MUC13 in TEER regulation, we made use of a probiotic bacterium known to  
230 enhance TEER formation. *Lactobacillus plantarum* (LP) is a commensal of the large intestine that can  
231 enhance intestinal barrier function by activating Toll-like receptor 2 (TLR2) signaling which triggers  
232 the translocation of occludin and ZO-1 to the TJ region in Caco-2 cells (44, 45). LP was added to 14  
233 days-differentiated WT and  $\Delta$ MUC13 cell monolayers at a multiplicity of infection (MOI) of 50. TEER  
234 was measured every 12 h for two days and the largest increase was observed 42 h after the addition  
235 of the bacteria. The incubation with LP resulted in a comparable increase in TEER in WT and  $\Delta$ MUC13  
236 cells by 2.2-fold and 2.8-fold, respectively (Fig. 3I). To confirm that the increase in TEER was not due  
237 to increased cell counts we confirmed that the number of nuclei per plane at 42 h post-infection was  
238 comparable between the different cell lines and conditions (Fig. 3J). These data show that the  
239 increase in epithelial barrier properties in response to LP is not dependent on MUC13. The function  
240 of MUC13 in epithelial barrier regulation seems therefore not linked to the pathway of TLR-  
241 mediated increase of ZOs and occludin proteins induced by LP.

242

#### 243 **Tight junction proteins are highly upregulated in the absence of MUC13**



244 Based on the TEER and translocation data, we hypothesized that  $\Delta$ MUC13 cells might have stronger  
245 tight junctions that reduce the paracellular translocation of ions and small particles. The functional  
246 fraction of junction proteins localizes to the plasma membrane. To be able to detect this functional  
247 fraction with increased sensitivity, we developed a fractionation protocol to enrich for the  
248 membrane fractions of WT,  $\Delta$ MUC13, and MUC13- $\Delta$ CT monolayers (Fig. 4A). We verified the  
249 fractionation strategy by Western blot with Na<sup>+</sup>/K<sup>+</sup>-ATPase (as membrane marker), Histone-H3 (as  
250 nuclear marker), and  $\beta$ -actin (as cytoplasmic marker). Membrane fractions showed a successful  
251 enrichment of membrane proteins and were free of nuclear contamination (Fig. 4B). Next to the  
252 validation by Western blotting, we further analyzed the membrane fractions by mass spectrometry.  
253 From three technical replicates, a total of 4.054 proteins were identified, of which 3.916 proteins  
254 were quantifiable in at least two out of three replicates.

255

256 Within this group of identified proteins, 1.189 proteins had at least one membrane annotation,  
257 suggesting that the ultracentrifugation significantly enriched the membrane fractions and was  
258 important in increasing the coverage of plasma membrane and plasma membrane-recruited  
259 proteins. Upon data normalization, the intensity of the marker protein Na<sup>+</sup>/K<sup>+</sup>-ATPase was  
260 consistent between samples and biological replicates, demonstrating stringent and reproducible  
261 membrane profiling across different MUC13 mutant lines. By quantitative comparison of WT,  
262  $\Delta$ MUC13, and MUC13- $\Delta$ CT membranes by mass spectrometry, we observed a striking increase in  
263 junction proteins, as depicted in Fig. 4C. The TJ proteins occludin, tricellulin, ZO-1, ZO-2, ZO-3, and  
264 several claudins (claudins-1, -2, -3, -4, -7, and -12) were found at higher levels in  $\Delta$ MUC13  
265 membranes compared to WT membranes. We also noted an upregulation of the AJ proteins E-  
266 cadherin,  $\beta$ -catenin, and Cadherin-17 in  $\Delta$ MUC13 membranes compared to WT membranes, though  
267 this difference was less pronounced than the upregulation of TJ proteins. In the membranes of the  
268 MUC13- $\Delta$ CT cell lines, ZO-1 and claudins-1 and -2 were consistently more abundant compared to WT  
269 membranes. The identified major tight junctions' alterations in the  $\Delta$ MUC13 and MUC13- $\Delta$ CT cell  
270 lines could underly the observed restriction of the paracellular pathway upon deletion of MUC13 or  
271 removal of its cytoplasmic tail.

272

### 273 **The degradation rate of tight junction proteins is not affected by MUC13**

274 TJs are dynamic complexes in which proteins can be added and removed at different rates and  
275 quantities via vesicular transport (20). Internalized proteins are transported to early endosomes,  
276 followed by either trafficking to recycling endosomes to end up back at the TJ, or into late  
277 endosomes for degradation (46, 47). To assess the turnover of TJ proteins, monolayers were  
278 incubated with sulfo-NHS SS-biotin to label all extracellularly exposed proteins, including tight  
279 junction proteins. Cells were harvested after 1 h, 1 day, and 3 days of incubation. Biotinylated  
280 proteins were isolated from whole-cell lysates with streptavidin beads and analyzed by Western blot  
281 with specific antibodies. As before, the TJ proteins were more abundant in the  $\Delta$ MUC13 cells than in  
282 WT and MUC13- $\Delta$ CT cells. In WT and MUC13- $\Delta$ CT cells, biotinylated occludin was lost after 1 day  
283 while it was still detectable in  $\Delta$ MUC13 cells (Fig. 5A). Also, claudin-1 and claudin-4 were detectable  
284 for a longer period in  $\Delta$ MUC13 cells compared to WT. However, quantification demonstrated that  
285 the possibility to detect proteins at day 1 was caused by the higher starting concentration, since an  
286 equal degradation rate was seen for the TJ proteins occludin, claudins-1, and claudin-4, as well as of  
287 the AJ protein E-cadherin in WT,  $\Delta$ MUC13, and MUC13- $\Delta$ CT cells (Fig. 5B). These data indicate that  
288 the rate of degradation of tight junction proteins is comparable between the different cell lines, and  
289 that the increased levels of junction proteins are not due to reduced degradation.



290

### 291 **Total tight junction protein abundance is increased in the absence of MUC13**

292 To investigate whether these changes result from increased total expression or selective recruitment  
293 to the plasma membrane, we analyzed the abundance of selected junction proteins in the total  
294 lysates of the different cell lines by immunoblot. An upregulation of occludin, ZO-1, claudin-1, and  
295 claudin-4 was detected in  $\Delta$ MUC13 lysates, whereas MUC13- $\Delta$ CT lysates displayed increased levels  
296 of occludin and claudins-1 and -4 compared to WT (Fig. 5C). Taken together, these results indicate  
297 that the increased accumulation of TJ proteins at the membrane of  $\Delta$ MUC13 and MUC13- $\Delta$ CT cell  
298 lines are the result of higher total protein abundance.

299

### 300 **TEER buildup in the absence of MUC13 is dependent on MLCK, ROCK, and PKC kinases**

301 The assembly, disassembly, and maintenance of TJs are known to be regulated by the kinases  
302 Myosin Light Chain Kinase (MLCK), Rho-associated protein kinase (ROCK), and members of the  
303 protein kinase C (PKC) family (48). MLCK, ROCK, and PKCs are all involved in the phosphorylation of  
304 MLC2, a key protein in the contraction and relaxation of the perijunctional actomyosin ring, a  
305 mechanism needed for TEER formation. In addition, PKC members phosphorylate different TJ  
306 proteins resulting in enhanced stability (49–51). For inhibition of the three kinases, we selected  
307 inhibitors ML-7 (MLCK), Y-27632 (ROCK), and GF-109293X (PKC). WT,  $\Delta$ MUC13, and MUC13- $\Delta$ CT cell  
308 lines were grown for 14 days in the presence of inhibitors added on days 3, 6, and 9. The inhibitors  
309 did not have a significant effect on the TEER of WT or MUC13- $\Delta$ CT cells (Fig. 5D, F). In  $\Delta$ MUC13 cells,  
310 on the other hand, the enhanced TEER buildup was not taking place in the presence of any of the  
311 three inhibitors (Fig. 5E). These results suggest that all three kinases are essential for the increased  
312 TEER buildup in the absence of the full-length MUC13.

313

### 314 **The effect of MUC13 on TJs is mediated by PKC proteins**

315 We used the NetPhos-3.1 software to predict putative phosphorylation sites in the MUC13 tail and  
316 found two putative PKC binding motifs. The first motif (-VTARS-) has been previously suggested (4).  
317 Because this motif is situated directly adjacent to the MUC13 transmembrane domain, accessibility  
318 for PKC binding seems unlikely. The second putative PKC site -RITASRDSQ- is further removed from  
319 the transmembrane domain (amino acid residues 488-496). The truncated MUC13- $\Delta$ CT still contains  
320 the -VTARS- motif, but the -RITASRDSQ- motif is absent due to the cytoplasmic tail deletion (Fig. 6A).

321

322 To further investigate the contribution of PKC to the MUC13-related TJ phenotype, we investigated  
323 the protein expression levels of PKC isotypes PKC $\alpha$  and PKC $\delta$ . PKC $\alpha$  expression was comparable  
324 between the cell lines, but PKC $\delta$  levels were increased in the  $\Delta$ MUC13 cell line (Fig. 6B). These data  
325 suggest a functional link between MUC13 and PKC $\delta$  but do not conclusively establish a connection  
326 with the PKC motif in the cytoplasmic tail. We next collected membrane fractions from WT,  
327  $\Delta$ MUC13, and MUC13- $\Delta$ CT monolayers differentiated in the absence and presence of the PKC  
328 inhibitor GF-109293X. PKC inhibition clearly resulted in reduced expression of barrier-forming  
329 claudin-1, claudin-3, and claudin-4 in the  $\Delta$ MUC13 membrane fractions, and some reductions were  
330 observed in WT and MUC13- $\Delta$ CT cells (Fig. 6C). Quantification of claudin-1, claudin-3, and claudin-4  
331 in three independent experiments demonstrated a significant reduction of claudin-3 and claudin-4  
332 in membrane fractions of the  $\Delta$ MUC13 monolayers, while the other differences were not significantly  
333 reduced. These data demonstrate that deletion of MUC13 promotes TEER buildup through increased  
334 synthesis and accumulation of TJ proteins in a PKC-dependent manner.

335

336 **Discussion**

337 MUC13 is one of the most ubiquitously expressed transmembrane mucins in the intestinal tract, but  
338 the role of MUC13 in intestinal health and disease is not fully understood. This study explores the  
339 contribution of MUC13 to the development of intestinal epithelial barrier integrity. We provide  
340 evidence that MUC13 negatively regulates the assembly of TJ complexes and regulates the  
341 paracellular transport of small solutes. The increase in TEER observed in MUC13 knockout cells  
342 requires the signaling molecule PKC.

343

344 One of the main functions of mucins is to protect the mucosal epithelium and underlying tissues  
345 against luminal agents. In addition, the modulation of cell-cell interactions seems to be a general  
346 trait of transmembrane mucins. Several TM mucins have been implicated in the regulation of AJ  
347 proteins E-cadherin and  $\beta$ -catenin, namely MUC1 (29, 34), MUC4 (31, 32), MUC13 (36), and MUC16  
348 (30). Additionally, mucin knockdown studies demonstrated the roles of several TM mucins in the  
349 regulation of TJ proteins. Silencing of MUC1 in human bronchial epithelial cells BEAS-2B led to  
350 reduced levels of occludin and claudin-1 (37). MUC16 knockdown in human corneal cells resulted in  
351 disruption of ZO-1 and occludin proteins, decreased TEER, and increased dye and bacterial  
352 penetration (38, 39). MUC17 silencing in Caco-2 and HT29-19A cells resulted in a profound reduction  
353 of occludin and ZO-1 levels and an increase in paracellular permeability after infection with  
354 enteroinvasive *Escherichia coli* (EIEC) when compared to wild type cells (40). In contrast to MUC1,  
355 MUC16, and MUC17 which positively regulate tight junction proteins, our study shows, for the first  
356 time, a transmembrane mucin (MUC13) that negatively regulates TJs and epithelial barrier integrity.

357

358 One of the most important functions of the intestinal epithelium is to transport nutrients and water  
359 to the mucosal tissues, while preventing the diffusion of toxins, allergens, and inflammatory  
360 molecules, such as LPS (52). The overall tightness or leakiness of a cell layer depends on the tight  
361 junction composition within the membrane (53, 54). We discovered that the removal of MUC13  
362 causes an increased accumulation of claudins at the cell membrane, including claudins-1, -2, -3, -4, -  
363 7, and -12 (Fig. 4C). This group contains one pore-forming claudin (claudin-2), one claudin with yet  
364 unknown barrier effect (claudin-12), and is dominated by the barrier-forming claudins-1, -3, -4, and -  
365 7 (20), together resulting in the phenotypic buildup of TEER in  $\Delta$ MUC13 cells compared to WT cells  
366 (Fig. 3B, C). Deletion of just the MUC13 cytoplasmic tail was sufficient to increase the accumulation  
367 of claudins-1, -2, -3, and -4. Besides ions and water, TJ proteins can regulate the paracellular flux of  
368 bigger particles through the “leak pathway”. Occludin and tricellulin have been shown to regulate  
369 the transepithelial flux of particles of various sizes (55–57). In our study, all three tested intestinal  
370 cell lines were highly restrictive for the passage of large particles, including LPS and FITC-dextran of 4  
371 kDa and 70 kDa, but WT cells were permeable to the 520 Da Lucifer Yellow tracer while MUC13  
372 knockout cells were not (Fig. 3G, H). Together, our results show that, in the absence of a fully  
373 functional MUC13, there is a reduction in the passage of ions and small-sized particles to deeper  
374 layers of the intestinal tissue. Alterations in ion fluxes through the paracellular channels have been  
375 described to lead to a dysfunctional intestinal barrier, causing diarrhea and malabsorption of  
376 nutrients (58, 59). Our observations about the link between MUC13 and TJs shed a new light on  
377 previous reports showing alterations of TJ proteins in IBD patients. The main TJ proteins occludin  
378 and tricellulin, together with sealing claudins (such as claudins-3, -4, -5, -7, and -8) are  
379 downregulated in colonic and rectal tissue of IBD patients, while claudin-1 and the pore-forming  
380 claudin-2 are upregulated leading to reduced barrier function (28, 60–63). The negative regulation of  
381 intestinal barrier MUC13 that we observe in our model may in part explain the loss of intestinal

382 integrity in IBD patients.

383

384 The effect of MUC13 deletion on claudin expression at the membrane does not depend on altered  
385 turnover of TJ proteins (Fig. 5A-B), but rather requires kinases known to be involved in the buildup of  
386 TJs. We found that MLCK and ROCK are necessary to build up the TJ complexes and TEER in  $\Delta$ MUC13  
387 cells (Fig. 5E). These kinases are known to control the contraction of the perijunctional actomyosin  
388 ring and subsequent paracellular permeability (48, 64). Moreover, members of the PKC family have  
389 been implicated in the regulation of many different TJ proteins as they are responsible for the  
390 phosphorylation of claudins (Franke et al., 2010; Yoo et al., 2003; Banan et al., 2005; González-  
391 Mariscal et al., 2008, 2010), occludin (51, 68, 69), and ZO-1 (49, 70). Our study demonstrates that  
392 PKC activity is needed to accumulate high levels of claudins at the membrane of all three cell lines  
393 but is especially important in  $\Delta$ MUC13 cells (Fig. 6C-D). The MUC13 cytoplasmic tail contains several  
394 potential phosphorylation sites (8 serine and 2 tyrosine residues) (2, 4) and two putative PKC motifs  
395 (Fig. 6A). We observed that in the absence of the full-length MUC13, the total levels of PKC $\delta$  are  
396 increased but deletion of the cytoplasmic tail alone did not evidently increase PKC $\delta$  levels (Fig. 6B).  
397 On the other hand, PKC inhibition did reduce the expression of some TJ proteins in the MUC13- $\Delta$ CT  
398 cells, but these changes were not significant between three experiments (Fig. 6C, D). This might  
399 suggest that deletion of the MUC13 tail only can enhance PKC activity to stabilize TJ proteins. A role  
400 for PKC $\delta$  in the regulation of TJs is in line with previously reported results linking PKC $\delta$  to  
401 upregulation of claudin-1 and claudin-7 protein levels in different epithelial cell lines (51, 71, 72). In  
402 future studies, we intend to address the link between MUC13 and PKC $\delta$  during TJ regulation in more  
403 detail. Based on our current data, we propose a model in which MUC13 negatively impacts claudin  
404 build up at the membrane by regulating the levels and/or activity of PKC $\delta$  (Fig. 7A-C).

405

406 Confirmation of the MUC13 cytoplasmic tail deletion cell lines with MUC13-targeted antibodies  
407 indicated that both MUC13- $\Delta$ CT cell lines lacked the cytoplasmic tail. The MUC13 extracellular  
408 domain was detectable in the MUC13- $\Delta$ CT 1 cell line but expression in the MUC13- $\Delta$ CT 2 cell line was  
409 very limited (Fig. 2F, G). The reduced percentage of MUC13- $\Delta$ CT cells expressing MUC13 on the  
410 surface compared to WT cells might indicate reduced stability of the MUC13 protein lacking the  
411 cytoplasmic tail. Despite the possible differences in MUC13 stability or antibody binding, the  
412 phenotypic characterization of both MUC13- $\Delta$ CT cells was very similar and neither cell line  
413 phenocopied the effect of the full MUC13 knockout on TEER and TJ. Therefore, we are confident that  
414 the targeted removal of the MUC13 cytoplasmic tail was successful, and the results obtained with  
415 these cell lines are reliable. Removal of the MUC13 cytoplasmic tail led to a partial phenotype,  
416 where ZO-1 and claudins-1, -2, -3, and -4 were upregulated in the membrane, although to a lower  
417 extent compared to the full knockout. This was accompanied by a slight increase in TEER and  
418 reduced paracellular permeability to Lucifer Yellow substrate compared to WT cells. Also in the PKC  
419 experiments, the MUC13- $\Delta$ CT cells showed a partial phenotype but did demonstrate a dependency  
420 on PKC activity for elevated expression of TJ proteins. Together, these results point to a pivotal role  
421 for both the MUC13 extracellular domain and cytoplasmic tail in TJ buildup and underline the  
422 challenge of studying the functions of different TM mucin domains.

423

424 In healthy conditions, the transmembrane mucin MUC13 is involved in important biological  
425 processes, including cell growth and maintenance (16, 17), protection of cells from toxin-induced  
426 damage (11), and formation of a physical barrier to reduce bacterial contact (2). MUC13 is  
427 upregulated during IBD (11, 73) and CRC (10, 74), correlating with increased pro-inflammatory

428 responses (75), cell growth, and migration (16, 17). Our results demonstrate that MUC13 is a  
429 negative regulator of PKC-mediated TJ protein assembly at the membrane. Overexpression of  
430 MUC13, as observed in IBD, may lead to a reduction in TJ proteins, such as occludin, claudins, and  
431 ZO<sub>s</sub>, and increased paracellular permeability to water, ions, and organic solutes. Opening of TJ  
432 complexes is essential to allow sampling of luminal bacteria by immune cells but decreased barrier  
433 function can also contribute to the development of chronic intestinal inflammation. It is interesting  
434 to speculate that MUC13 with its complex extracellular domain, could play a role in sensing the  
435 inflammatory state of the intestine and can respond by regulating TJs through its cytoplasmic tail.  
436 Our study brings to light that the transmembrane mucin MUC13 plays a unique role in the intestinal  
437 epithelium and emphasizes the need for further studies into the functions of specific mucins.

438

439

## 440 **Materials and Methods**

441

### 442 **Cell lines, bacteria, and culture conditions**

443 The human intestinal epithelial cell lines HRT18 (ATCC-CCL-244), Caco-2 (ATCC-HTB-37), and  
444 CRISPR/Cas9 knockout derivative cell lines used in this study, as well as HEK293T cells (CRL-3216,  
445 ATCC) were routinely grown in 25 cm<sup>2</sup> flasks in Dulbecco's modified Eagle's medium (DMEM) +  
446 glutamax (Life technologies, 31966047) containing 10% fetal calf serum (FCS) (Sigma, F7524) at 37°C  
447 in 10% CO<sub>2</sub>. HEK-Blue<sup>TM</sup> Null and HEK-Blue<sup>TM</sup> hTLR4 cells were purchased from InvivoGen (hkb-hTLR4)  
448 and cultured in DMEM containing 10% heat-inactivated FCS, penicillin/streptomycin (BioConnect,  
449 ML-105L), and antibiotics from Invivogen (Zeozin (ant-zn) and Normocin (ant-nr) for HEK-Blue<sup>TM</sup> Null  
450 cells; Zeozin, Normocin, Blasticidin (ant-bl), and Hygromycin (ant-hg) for HEK-Blue<sup>TM</sup> hTLR4 at 37°C in  
451 5% CO<sub>2</sub>. Cells were detached with 0.25% trypsin (ThermoFisher, 25200-072), passaged twice a week  
452 in a 1:10 dilution and split before they reached 80% confluency. *Lactobacillus plantarum* (ATCC,  
453 14917) was grown in MRS medium in aerobic conditions.

454

### 455 **Antibodies and reagents**

456 For Western blotting, antibodies against claudin-1 (ThermoFisher, 51-9000), claudin-3  
457 (ThermoFisher, 34-1700), claudin-4 (ThermoFisher, 32-9400), occludin (Invitrogen, 33-1500), Zonula  
458 Occludens-1 (ZO-1) (Abcam, ab216880), E-cadherin (Abcam, ab1416), PKC $\alpha$  (ab32376), PKC $\delta$   
459 (ab182126), b-actin (Bioss, bs-0061R), MUC13 (Abcam, ab235450), MUC13 hybridoma supernatant  
460 (in house), Na<sup>+</sup>/K<sup>+</sup>-ATPase (Abcam, ab76020), and acetyl-Histone H3K9 (Merck, 07-352) were used.  
461 Secondary antibodies used for immunoblotting were goat anti-mouse-HRP (Sigma, A2304) and goat  
462 anti-rabbit-HRP (Sigma, A4914). Secondary antibodies used for immunofluorescence were goat anti-  
463 mouse-Alexa488 (ThermoFisher, A11029), goat anti-mouse-Alexa568 (ThermoFisher, A11031), goat  
464 anti-rabbit-Alexa488 (ThermoFisher, A11034), goat anti-mouse-Alexa568 (ThermoFisher, A11036),  
465 and DAPI (D21490, Invitrogen).

466 For permeability assays, 4 kDa (46944) and 70 kDa (46945) Fluorescein isothiocyanate-dextran  
467 (FITC), and Lucifer Yellow CH dipotassium salt (L0144) were purchased from Sigma. Ultrapure LPS  
468 from *Escherichia coli* was purchased from InvivoGen (tlrl-3pelps). For biotinylation assays, Pierce<sup>TM</sup>  
469 Premium Grade sulfo-NHS SS-biotin was acquired from ThermoFisher (PG82077).

470

### 471 **Bioinformatics Single Cell Studies**

472 Single cell gene expression from intestinal epithelial cells was analyzed using a public single cell RNA-  
473 sequencing dataset (76). The H5AD file containing data from all epithelial cells was downloaded

474 from <https://www.gutcellatlas.org> and further analyzed in Rstudio using the packages “Seurat”,  
475 “SeuratData”, and “SeuratDisk”. Cells from healthy adult subjects were selected, and low-quality  
476 cells (less than 2000 genes or >20% of counts mapping to mitochondrial genes) were removed. Data  
477 from the remaining 37,325 cells were then normalized using the SCTransform algorithm and dotplots  
478 showing the expression by cell type or by intestinal zone were made. Rare cell types (less than 100 in  
479 the dataset) are not shown in the plots.

480

#### 481 **Generation of HRT18 $\Delta$ MUC13 and MUC13- $\Delta$ CT cell lines using CRISPR/Cas9**

482 To generate  $\Delta$ MUC13 cells, we used the pCRISPR-hCas9-2xgRNA-Puro plasmid (Langereis *et al.*,  
483 2015) that encodes Cas9 with two MUC13-specific guide RNAs to generate a 380 bp deletion in the  
484 second exon of the *MUC13* gene. The pCRISPR plasmid was digested with *SapI* and simultaneously  
485 dephosphorylated with alkaline phosphatase (FastAP; ThermoFisher). Guide RNA primer sets A (KS40  
486 5'- ACCGACCACAGAAACTGCGACTAG -3' and KS41 5'- AACCTAGTCGAGTTTCTGTGGTC-3') and B  
487 (KS42 5'-CCGTCCCACTGGCACCGCTTTATG-3' and KS43 5'-AAACATAAAGCGGTGCCAGTGGGA-3') were  
488 phosphorylated with T4 polynucleotide kinase (ThermoFisher) at 37°C for 30 min and annealed by  
489 cooling down from 85°C to 25°C at 0.1°C/sec. Annealed primer sets were ligated into the *SapI*-  
490 digested pCRISPR plasmid and confirmed by sequencing with primers KS46 5'-  
491 GTTCACGTAGTGCCAAGGTCG-3' and KS47 5'-GAGTCAGTGAGCGAGGAAGC-3', resulting in plasmid  
492 pCR4. Two-day grown HRT18 cells were trypsinized from a 25 cm<sup>2</sup> flask and transfected in  
493 suspension with 2  $\mu$ g of pCR4, pCRISPR-empty, or no plasmid using Fugene (Promega) according to  
494 the manufacturer's instructions. Cells were cultured in DMEM + 10% FCS for two days, after which 5  
495  $\mu$ g/ml puromycin (Life Technologies) was added to the medium to select for positively transfected  
496 cells. Cells were maintained in medium with puromycin until all negative control cells had died.  
497 Single-cell cloning was performed by serial dilution and single-cell clones were tested for the MUC13  
498 deletion by PCR with primers KS126 5'-CCAGGGGTTTATGACCAATCTAGG-3' and KS127 5'-  
499 TGCACAGCTAGCAAATAACTTGAGG-3'. The deletion in the MUC13 was confirmed by sequencing and  
500 the clones were named HRT18- $\Delta$ MUC13 clones 1 and 2. The cells transfected with the empty CRISPR  
501 plasmid served as control in all experiments and were renamed wild type (WT) for clarity of the  
502 figures. The absence of MUC13 protein in the knockout cell lines was confirmed by immunoblot with  
503 anti-MUC13 antibody. To generate the MUC13- $\Delta$ CT cell line, a similar protocol as described above  
504 was followed with guide RNA primer sets A (CSP5 5'- ACCGAATCTAAAACCTGCGGTCGAC -3' and CSP6  
505 5'- AACGTCGACCGCAGTTTATGATTCC -3') and B (CSP7 5'- CCGGCACTGACTCACCTAATAGTCG -3' and  
506 CSP8 5'- AAACGACTATTAGGTGAGTCAGTGC -3') to generate a deletion of 121 bp in the tenth exon of  
507 the *MUC13* gene. The resulting single clones after transfection were confirmed with primers CSP96  
508 5'-TCAAGTGATCTGCCACCACGG-3' and CSP97 5'-TCTGCCCTGGTGCATTCCTCC-3'.

509

#### 510 **Overexpression of MUC13 in HRT18-WT and HRT18- $\Delta$ MUC13 cells**

511 Cloning of the original MUC13 gene sequence in *E. coli* DH5a was problematic. The Softberry  
512 promoter prediction algorithm (77) was used to analyze the MUC13 sequence which revealed a  
513 multitude of predicted Sigma70 binding sites. We altered the MUC13 sequence with synonymous  
514 mutations to remove the predicted binding sites. The optimized MUC13 sequence was ordered from  
515 Thermo Fisher. To generate doxycycline-inducible expression of the MUC13-opt constructs, the  
516 plasmid pInducer 20-extended MCS (pKSU59) (78) was used as a vector. First, restriction sites were  
517 added via PCR amplification with primers XL14-Fwd (5'  
518 CCGCTCGAGGCCACCATGGAAGCCATCATCTTACTCTTC 3') and XL13-Rev (5'  
519 TATGGCGCGCCCATAGAGCCACCGCATC 3') to obtain the insert fragment (MUC13opt-GFP) from



520 plasmid pDS2 (pcDNA3.1-MUC13opt-GFP). Then, both insert and vector were digested with Ascl-FD  
521 and XhoI-FD ligated together to generate plasmid pJSU002 (pInducer20-MUC13opt-GFP). This  
522 plasmid was subsequently used to generate inducible overexpression of MUC13 in HRT18-WT and  
523 HRT18- $\Delta$ MUC13 cells using lentiviral transduction. For lentiviral production, HEK293T cells were  
524 seeded at 70% confluence in 6-well tissue culture plates 24 h before transfection. Lipofectamine™  
525 3000 (Invitrogen, L3000001) was used as the transfection reagent according to the manufacture's  
526 protocol. Cells were incubated with the transfection mix for 6 h, media was replaced with fresh  
527 DMEM/10% FCS and grown for 48 h. The subsequent steps for lentivirus transduction on HRT18-WT  
528 and HRT18- $\Delta$ MUC13 cells were performed as previously described (78). The resulting cells were  
529 called HRT18-WT+pMUC13 and HRT18- $\Delta$ MUC13+pMUC13 cells. Cells were also transfected with  
530 empty pInducer plasmid as controls, resulting in HRT18-WT Ctr and HRT18- $\Delta$ MUC13 Ctr cell lines. To  
531 validate the expression of MUC13opt-GFP constructs, cells were induced with 20 ng/ml of  
532 doxycycline (Sigma, D3072) for 24 h and observed under a fluorescent microscope for GFP signal.

533

#### 534 **Immunofluorescence and confocal microscopy**

535 For immunofluorescence, cells were grown on coverslips in 24-well plates for 14 days. Monolayers  
536 were washed twice with Dulbecco's Phosphate Buffered Saline (DPBS, Sigma, D8537) and fixed with  
537 4% cold paraformaldehyde in PBS (VWR, J19943) for 30 min at room temperature (RT). The fixation  
538 was stopped by incubation with 50 mM NH<sub>4</sub>Cl in PBS for 10 minutes. Cells were washed twice with  
539 DPBS before they were incubated with primary antibodies (MUC13 at 1:100 dilution, occludin at  
540 1:50, and E-cadherin at 1:100) in binding buffer (0.2% Triton-X100 (Sigma, X100), 2.2% gelatin  
541 (Sigma, CM135a), and 0.2% BSA (Sigma, A7030) in DPBS for 1h at RT. Coverslips were washed 3  
542 times with binding buffer followed by incubation with secondary antibodies (1:200) and DAPI (1:500)  
543 for 1h at RT. Coverslips were washed 3 times with DPBS, once with MilliQ, and embedded in Prolong  
544 diamond mounting solution (ThermoFisher, P36961). Images were collected on a Leica SPE-II  
545 confocal microscope in combination with Leica LAS AF software. Image analysis was performed using  
546 Fiji/ImageJ.

547

#### 548 **Transepithelial electrical resistance (TEER) measurements**

549 Cells were seeded in 12-well Transwell plates with 12 mm inserts and 0.4 mM membrane pore size  
550 (Costar, 3401) at 30%, 40%, and 60% confluency, respectively. Wells without cells were taken along  
551 as negative control. Transepithelial electrical resistance was determined with a Millicell ERS-2  
552 Voltohmmeter (Millipore). All measurements were performed on three individual wells. TEER  
553 measurements were taken every 2-3 days for 2 weeks. TEER W/cm<sup>2</sup> values were calculated by  
554 subtracting the average negative control value from the measurement and multiplying it by the well  
555 surface (1,12 cm<sup>2</sup>). For the MUC13 overexpression experiments, HRT18-WT Ctr, HRT18-  
556 WT+pMUC13, HRT18- $\Delta$ MUC13 Ctr, and HRT18- $\Delta$ MUC13+pMUC13 cells were seeded in 24 Transwell  
557 plates with 6.5 mm inserts and 0.4 mM membrane pore size (Costar, 3470) at 30% (WT) and 60%  
558 ( $\Delta$ MUC13) confluency. TEER was measured every 2-3 days for 2 weeks. At day 14, doxycycline was  
559 added to the top compartment at a concentration of 0.2  $\mu$ g/mL. To study the effect of MLCK, ROCK,  
560 and PKC on TEER build up over time, ML-7 (50 mM), Y-27632 (50 mM), or GF-109203X (20 mM)  
561 inhibitors were added to the upper compartment, respectively, at days 3, 6, and 9. TEER  
562 measurements were taken every 1-2 days for 2 weeks.

563

#### 564 **Epithelial permeability assays with Lucifer Yellow, FITC-Dextran and LPS**

565 Epithelial paracellular permeability for particles was assessed by measuring the flux of 0.5 kDa  
566 Lucifer Yellow CH dipotassium salt and 4 and 70 kDa FITC-Dextran particles across confluent  
567 monolayers. Cells were grown for 2 weeks in 12-well Transwell plates with 12 mm inserts and 0.4  
568 mM membrane pore size (Costar, 3401). To minimize interference from the media when measuring  
569 FITC, media from the bottom wells was changed to DMEM without red phenol + 10% FCS.  
570 Subsequently, 500 mL of 4 or 70 kDa FITC-Dextran dissolved to 1 mg/mL in DMEM without red  
571 phenol or 500 mL of 400 mg/mL LY was added to the top compartments. After 2 h incubation with LY  
572 or 6 h with FITC-Dextran particles, 100 mL aliquots were taken from the bottom wells and the  
573 fluorescent intensity was measured with a FLUOstar Omega Microplate Reader (BMG Labtech). The  
574 excitation and emission wavelengths were 492 nm and 520 nm for FITC-Dextran, and 428 and 540  
575 nm for LY. The percentage of permeability was calculated by comparing the fluorescence intensity to  
576 that of membrane-only wells.

577

#### 578 **LPS translocation assays**

579 Cells were grown for 2 weeks in 12-well Transwell plates with 12 mm inserts and 0.4 mM membrane  
580 pore size (Costar, 3401). The media from the bottom compartment was changed to 500 mL DMEM  
581 without FCS. 5 mg of Ultrapure *E. coli* LPS diluted in DMEM without FCS was added to the top wells  
582 and incubated at 37°C for 24 h. To determine the maximum amount of LPS that could be  
583 translocated, 5 mg of LPS was added to wells without cells (membrane only). The next day, the  
584 bottom compartments were frozen at -20°C until further use. For quantitative detection of LPS, HEK-  
585 Blue™ hTLR4 were used with HEK-Blue™ Null cells as negative control. 2.5x10<sup>4</sup> HEK-Blue™ Null cells  
586 and 3.5x10<sup>4</sup> HEK-Blue™ hTLR4 cells (due to slightly slower growth of the Null cells) were seeded in  
587 96-well flat-bottomed tissue culture plates and incubated at 37°C for 24 h. Then, cells were  
588 stimulated with 100 mL of media from the Transwell bottom of the LPS translocation experiment.  
589 For quantification of the LPS concentration, 10-fold dilutions of LPS from 100 ng/mL to 0.1 ng/mL in  
590 100 mL were used. HEK-Blue™ hTLR4 and HEK-Blue™ Null cells were stimulated with the LPS-  
591 containing fractions for 24 h at 37°C. Relative NF-κB activity as a result of TLR4 stimulation was  
592 determined by quantifying the secreted alkaline phosphatase (SEAP) activity. Twenty mL of HEK-  
593 Blue™ supernatants were transferred to a 96-well plate containing 180 mL pre-warmed (37°C)  
594 QUANTI-Blue™ (the substrate for SEAP, InvivoGen, rep-qbs). Reactions were developed at 37°C for  
595 50-90 min and measured at 630 nm using FLUOstar Omega Microplate Reader (BMG Lactech). Three  
596 wells with DMEM only were used as blanks and subtracted from the other measurements.

597

#### 598 **Epithelial barrier experiments with *Lactobacillus plantarum***

599 Cells were grown for 2 weeks in 12-well Transwell plates with 12 mm inserts and 0.4 mM membrane  
600 pore size (Costar, 3401). An overnight culture of *L. plantarum* was added at MOI 50 at the apical side  
601 in a final volume of 500 mL in DMEM without FCS. Media in the basolateral compartment was  
602 replaced with fresh DMEM without FCS. TEER was measured at multiple time points until 42 h. All  
603 measurements were performed on three individual wells and in three independent biological  
604 replicates. TEER W/cm<sup>2</sup> values were calculated by subtracting the average negative control value  
605 from the measurement and multiplying it by the well surface (1.12 cm<sup>2</sup>).

606

#### 607 **Subcellular fractionation**

608 For subcellular fractionation of epithelial monolayers, a protocol from Abcam  
609 (<https://www.abcam.com/protocols/subcellular-fractionation-protocol>) was used with some  
610 modifications. Cells were grown in 10 cm<sup>2</sup> culture dishes for 2 weeks, washed twice with ice-cold PBS



611 and scrapped with a cell scraper in 500 mL fractionation buffer (20 mM HEPES pH 7.4, 10 mM KCl, 2  
612 mM MgCl<sub>2</sub>, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and 1x protease and phosphatase inhibitors) and  
613 transferred to an Eppendorf tube. Cell suspensions were passed 10 times through a 26G needle and  
614 centrifuged at 300 x g for 5 min. The supernatant containing cytoplasm, membranes and  
615 mitochondria was transferred to a new Eppendorf tube and kept on ice. To maximize cell membrane  
616 rupture, these steps were repeated: resuspension of the pellet in buffer, lysis by a needle, and  
617 centrifugation. The recovered supernatants were centrifuged at 10,000 x g for 10 min to separate  
618 the mitochondria (pellet) from the cytoplasm and membranes (supernatant). Supernatants were  
619 transferred to 1.5 mL microcentrifuge tubes (Beckman Coulter) and centrifuged in an ultracentrifuge  
620 at 100,000 x g for 1 h at 4°C. Supernatants containing the cytosolic fraction were transferred to a  
621 Spin-X UF 10 kDa Centrifugal Concentrator (Corning) and concentrated by centrifugation to a final  
622 volume of 100 mL. The pellet of the ultracentrifugation step containing the membrane fraction was  
623 taken up in 500 mL of fractionation buffer and re-centrifuged at 100,000 x g for 1 h at 4°C for  
624 increased purity. The pellet was resuspended in 100 mL TBS (50 mM Tris, 150 mM NaCl, 1% SDS).  
625 Protein concentrations in all fractions were determined with Pierce™ BCA Protein Assay kit  
626 (ThermoFisher, 23225) and equal amounts were loaded into SDS-PAGE gels to confirm the efficiency  
627 of the protocol. Na<sup>+</sup>/K<sup>+</sup>-ATPase protein was chosen as a control for the membrane fraction, b-actin  
628 for the cytosolic fraction, and acetyl-Histone H3K9 to exclude nuclear contamination.

629

#### 630 **Immunoblotting**

631 Cell pellets were taken up in 1% SDS in PBS and lysed by mechanical lysis through a 26G needle.  
632 Protein concentration was determined using a Pierce™ BCA Protein Assay kit and equal amounts of  
633 protein were prepared in Laemmli sample buffer and boiled for 5 minutes at 96°C. For  
634 immunoblotting of MUC13, protein lysates were loaded onto 8% SDS-PAGE gel and transferred to a  
635 PVDF membrane using a semi-dry transfer system (Biorad) for 10 min at 2.5 amperes. The  
636 membranes were blocked with 5% skimmed milk powder in PBS-Tween for 1 h at RT. Subsequently,  
637 the membranes were incubated with MUC13 antibody directed against CT domain (Abcam) at 1:250  
638 dilution in PBS-Tween containing 5% skimmed milk powder o/n at 4°C. The next day, the membranes  
639 were washed 4 times with PBS-Tween (10 minutes each) and incubated with secondary antibody  
640 diluted 1:5,000 in PBS-Tween containing 5% skimmed milk powder for 1 h at RT. For immunoblotting  
641 of other proteins, protein lysates were loaded onto 8-12% SDS-PAGE gels and transferred to PVDF  
642 membranes. Blocking was done o/n at 4°C in 5% BSA-TSMT (20 mM Tris, 150 mM NaCl, 1 mM CaCl<sub>2</sub>,  
643 2 mM MgCl<sub>2</sub> adjusted to pH 7 with HCl and 0.1% Tween 20). Antibodies were diluted in 1% BSA-  
644 TSMT and incubated for 1 h at RT. Antibodies were used at 1:1,000 dilution, except for claudin  
645 antibodies which were used at 1:500 dilution and b-actin antibody at 1:2,000. For visualization, blots  
646 were incubated with Clarity Western ECL solution (Biorad) and imaged in a Bio-Rad Gel-Doc system.

647

#### 648 **Cell-surface biotinylation to determine recycling of TJ proteins**

649 Cells were grown for 10 days in 6-well Transwell plates with 24 mm inserts and 0.4 mM membrane  
650 pore size (Costar, 3412). 1 mg/mL of sulfo-NHS SS-biotin dissolved in PBS was added to the upper  
651 and basal compartments and incubated for 1 h at 4°C. Free biotin was washed away twice with cold  
652 sulfo-NHS SS-biotin blocking solution (50 mM NH<sub>4</sub>Cl in PBS, 1 mM MgCl<sub>2</sub>, 0.1 mM CaCl<sub>2</sub>). Five  
653 hundred mL Lysis Buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% SDS, 1 mM PMSF, and EDTA-free  
654 protease inhibitor cocktail from Roche, dissolved in PBS) was added and cells were harvested from  
655 the Transwell membrane using a disposable cell scraper and transferred to an Eppendorf tube.  
656 Samples were lysed for 45 min at RT by mechanical lysis. These samples were labeled as Day 0

657 (maximum amount of labeled proteins). Fresh DMEM + 10% FCS + Pen/Strep was added to the other  
658 wells and incubated at 37 °C for 1 day or 3 days, after which cells were harvested and lysed as  
659 described above. After incubation with Lysis Buffer, lysates were cleared of insoluble debris by  
660 centrifugation at 16,000 x g for 10 min. A small fraction of all cleared lysates was saved in another  
661 tube for the total protein sample. Per sample, 60 mL of Pierce Streptavidin Agarose Beads  
662 (ThermoScientific) were washed with 1 mL Lysis Buffer in a 2 mL microcentrifuge tube, and  
663 centrifuged for 2 min at 4,500 x g. After a second wash, beads were resuspended in Lysis Buffer  
664 equivalent to 60 mL/sample. Samples (20 mL) were loaded onto SDS-PAGE gels and immunoblotting  
665 was performed using claudin-1 and -4, occludin, and E-cadherin antibodies. Band intensities in each  
666 blot were analyzed with Image Lab Software 5.0.

667

#### 668 **Sample Preparation for Mass Spectrometry**

669 After fractionation, proteins in the membrane fraction were reduced in 10 mM dithiothreitol (DTT)  
670 at 20°C for 1 h and then alkylated with 20 mM iodoacetamide (IAA) at 20°C for 30 min in the dark.  
671 Excess IAA was quenched with an additional 10 mM DTT. Lys-C (Wako, Japan) was added at an  
672 enzyme/protein ratio of 1/75 and incubated for 4 h at 37 °C. Then, the solution was diluted with 50  
673 mM ammonium bicarbonate to reach a 2 M final concentration of urea, and trypsin was added  
674 (Sigma, USA) at an enzyme/protein ratio of 1/75 and digested overnight at 37 °C. The digested  
675 samples were quenched with 2% formic acid on the second day and desalted with Sep-Pak C18 1 cc  
676 Vac cartridge (Waters, USA). Desalted samples were dried by vacuum centrifugation and stored at -  
677 80 °C for further use.

678

#### 679 **LC-MS/MS**

680 Peptides were reconstituted in 2% formic acid and analysed in triplicates. LC-MS/MS was performed  
681 using an Orbitrap Exploris 480 mass spectrometer (Thermo Scientific) coupled with an UltiMate 3000  
682 UHPLC system (Thermo Scientific) fitted with a  $\mu$ -precolumn (C18 PepMap100, 5  $\mu$ m, 100 Å, 5 mm  $\times$   
683 300  $\mu$ m; Thermo Scientific), and an analytical column (120 EC-C18, 2.7  $\mu$ m, 50 cm  $\times$  75  $\mu$ m; Agilent  
684 Poroshell). Peptides were loaded in solvent A (0.1% formic acid in water) with a flow rate of 30  
685  $\mu$ l/min and then separated by using a 115-min linear gradient at a flow rate of 0.3  $\mu$ l/min. The  
686 gradient was as follows: 9% solvent B (0.1% formic acid in 80% acetonitrile, 20% water) for 1 min, 9–  
687 10% in 1 min, 10–36% in 95 min, 36–99% in 3 min, 99% for 4 min, 99-9% in 1 min, and finally the  
688 system equilibrated with 9% B for 10 min. Electrospray ionization was performed by using 1.9 kV  
689 spray voltage; the temperature of the ion transfer tube was set to 275 °C, and the RF lens voltage  
690 was set to 40%. MS data were acquired in data-dependent acquisition mode. Full scan MS spectra  
691 were acquired accumulating to 'Standard' pre-set automated gain control (AGC) target, at a  
692 resolution of 60,000 within the m/z range of 375-1600. Multiply charged precursor ions starting  
693 from m/z 120 were selected for further fragmentation. Higher energy collision dissociation (HCD)  
694 was performed with 28% normalized collision energy (NCE), at an orbitrap resolution of 30,000.  
695 Dynamic exclusion time was set to 16 s and 1.4 m/z isolation window was used for fragmentation.

696

#### 697 **Data analysis for MS**

698 MaxQuant software (version 1.6.10.0) was used for raw data analysis. The database search was  
699 performed against on human UniProt database (version April 22, 2021) by using the integrated  
700 Andromeda search engine. Protein N-terminal acetylation and methionine oxidation were added as  
701 variable modifications; cysteine carbamidomethylation was added as a fixed modification. Digestion  
702 was defined as Trypsin/P and up to 2 miscleavages were allowed. Label-free quantification (LFQ) and

703 the match-between-runs feature were applied for identification. 1% false discovery rate (FDR) was  
704 applied for both peptide and protein identification.

705 Quantitative data filtering was performed in the Perseus software (version 1.6.10.0). Potential  
706 contaminants and reverse peptides were removed, and all the LFQ intensities were normalized with  
707 log<sub>2</sub> transformation. Proteins quantifiable in at least two out of three replicates were retained.  
708 Imputation was performed based on a normal distribution. A two-sided paired Student's t-test was  
709 performed with permutation-based FDR (*q*-values) from 250 randomizations. Proteins were  
710 considered significant if *q*-values were 0.05 or less.

711

712 **MS data availability:** All the proteomics raw data were deposited to the ProteomeXchange  
713 Consortium with the dataset identifier PXD029606.

714

### 715 **Statistical analysis**

716 Statistical analysis was performed in IBM SPSS Statistics version 27 and depicted by Graph Pad Prism  
717 7 software. Kolmogorov-Smirnov test was used to assess normality of the data, and log  
718 transformation was used when the data was not normally distributed. Statistical differences in data  
719 including TEER development over time, FITC Dextran, Lucifer Yellow, and LPS translocation were  
720 analyzed using one-way ANOVA (analysis of variance) with Tukey's HSD *post hoc* test. TEER build up  
721 in the presence of inhibitors was analyzed using two-way ANOVA with Dunnett's *post hoc* test. The  
722 effect of *L. plantarum* on TEER was determined by calculating the fold change (42 h vs 0 h) and  
723 analyzing statistical differences using an independent t-test. All graphs depict the mean and  
724 standard error of the mean (SEM) of at least three independent experiments. Results of all  
725 performed statistical tests are depicted in the figures. A *p*-value of <0.05 was considered significant.  
726 \*, *p*<0.05; \*\* *p*<0.01; \*\*\* *p*<0.001.

727

728

### 729 **Acknowledgments**

730 We thank Dr. Richard Wubbolts and Ing. Esther van 't Veld from the Center for Cell Imaging of  
731 Utrecht University for assistance with microscopy.

732

733 **Funding:** C. Segui-Perez is supported by a ZonMW TOP grant that was awarded to K. Strijbis and T.  
734 Geijtenbeek (grant number 91218017). K. Strijbis has received funding from the European Research  
735 Council (ERC) under the European Union's Horizon 2020 research and innovation program (ERC-  
736 2019-STG 852452) by which D.A.C. Stapels and J. Su are supported.

737

738 **Author contributions:** Conceptualization: CSP, DS, KS. Methodology: CSP, DS, BW, WW, KS.  
739 Investigation: CSP, DS, ZM, JS, BW, WW, KS. Visualization: CSP, KS. Supervision: KS, JVP. Writing  
740 original draft: CSP, KS. Review & editing: CSP, DS, ZM, JS, BW, WW, JVP, KS.

741

742 **Conflicting interests:** The authors declare that they have no conflict of interest.

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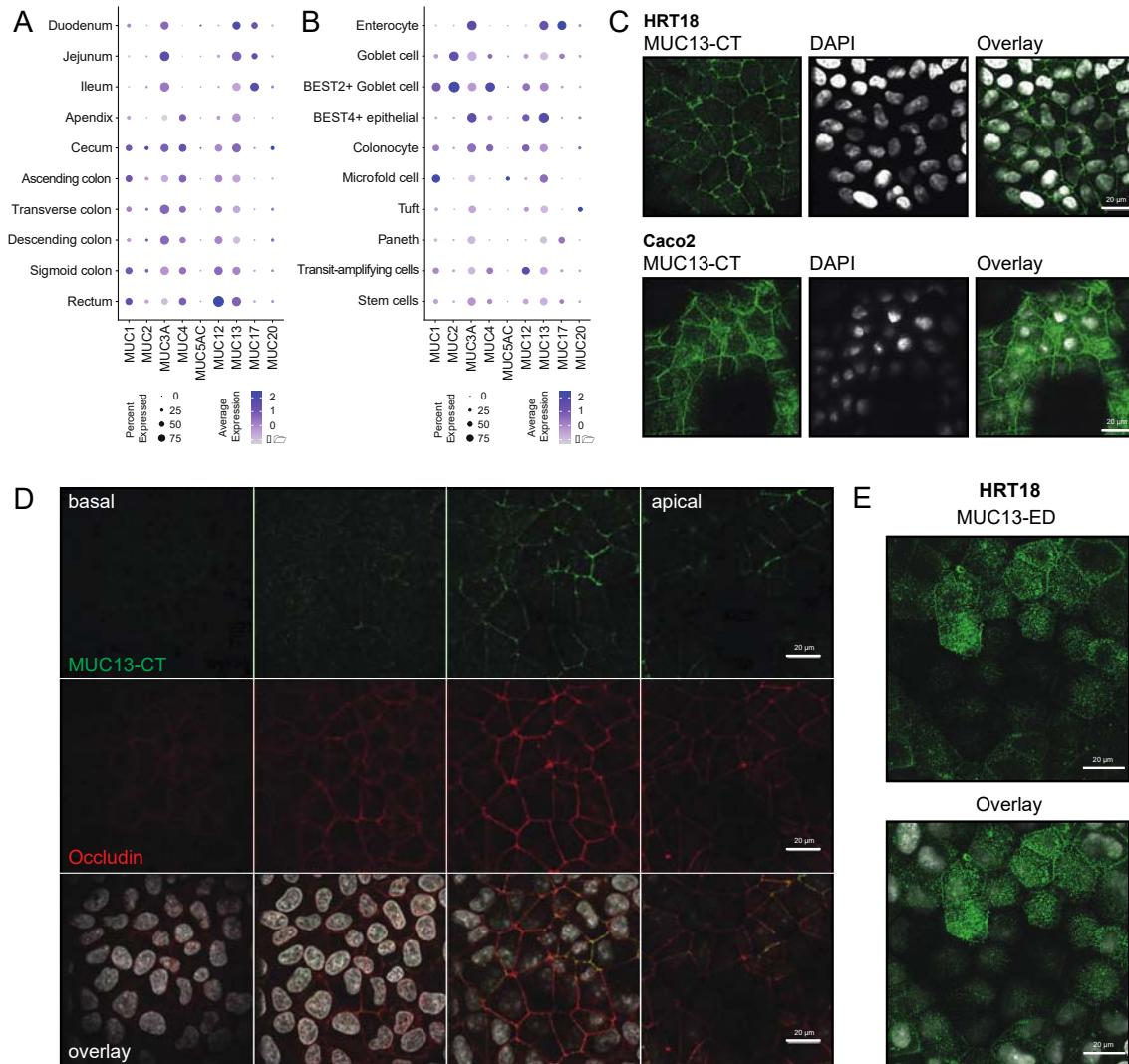
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## 973 **Figures**

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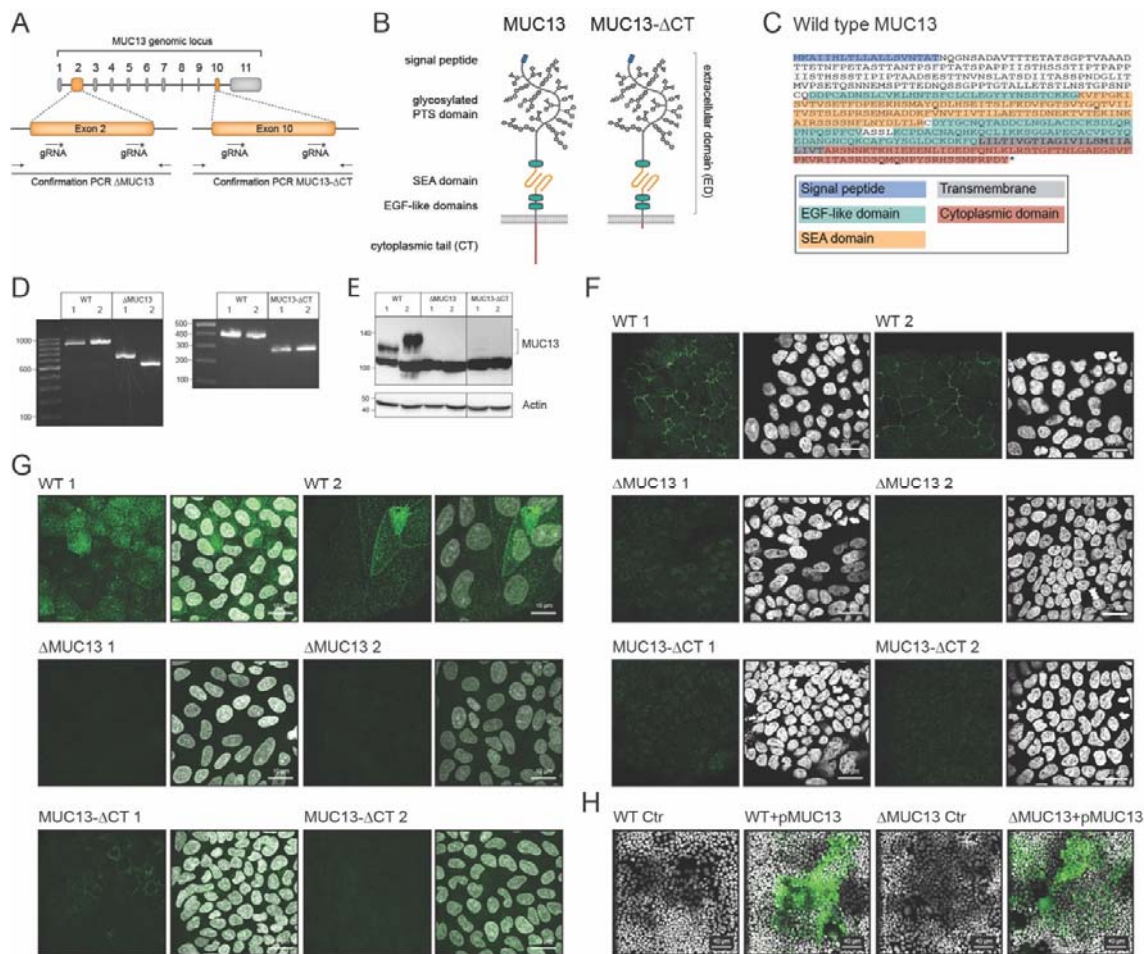
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**Fig. 1. MUC13 is highly expressed in the intestinal epithelium and localizes at the apical and lateral membrane.** (A-B) Single-cell RNA-sequencing data of adult donors showing expression levels of mucin genes along each section of the intestinal tract (A) and by different cell types (B). (C) Immunofluorescence microscopy of HRT18 and Caco-2 intestinal cells stained for MUC13 cytoplasmic tail (MUC13-CT) (green) and nuclei (white). (D) Immunofluorescence microscopy of HRT18 cells with antibodies against MUC13-CT and occludin, in combination with DAPI from basal to lateral Z planes. (E) Immunofluorescence microscopy of HRT18 cells with monoclonal MUC13 antibody against the extracellular domain. White scale bars represent 20  $\mu$ m.



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988 **Fig. 2. Generation of MUC13 knockout and MUC13-GFP overexpression cell lines.** (A) CRISPR/Cas9

989 targeting strategy using two guide RNAs directed against exon 2 or exon 10 of MUC13. (B) Schematic

990 representation of WT and MUC13- $\Delta$ CT MUC13 domain structure. (C) Wild type MUC13 protein

991 sequence with domains color-coded as in Fig. 2B. (D) Confirmation PCR of WT and  $\Delta$ MUC13 cell lines

992 (left), and WT and MUC13- $\Delta$ CT cell lines (right). (E) Immunoblot of WT,  $\Delta$ MUC13, and MUC13- $\Delta$ CT

993 cell lines with anti-MUC13-CT antibody and actin loading control. Molecular mass standards (kDa)

994 are indicated on the left. (F) Immunofluorescence confocal image of WT,  $\Delta$ MUC13, and MUC13- $\Delta$ CT

995 cells stained for MUC13-CT (green) and nuclei (white). White scale bars represent 20 mM. (G)

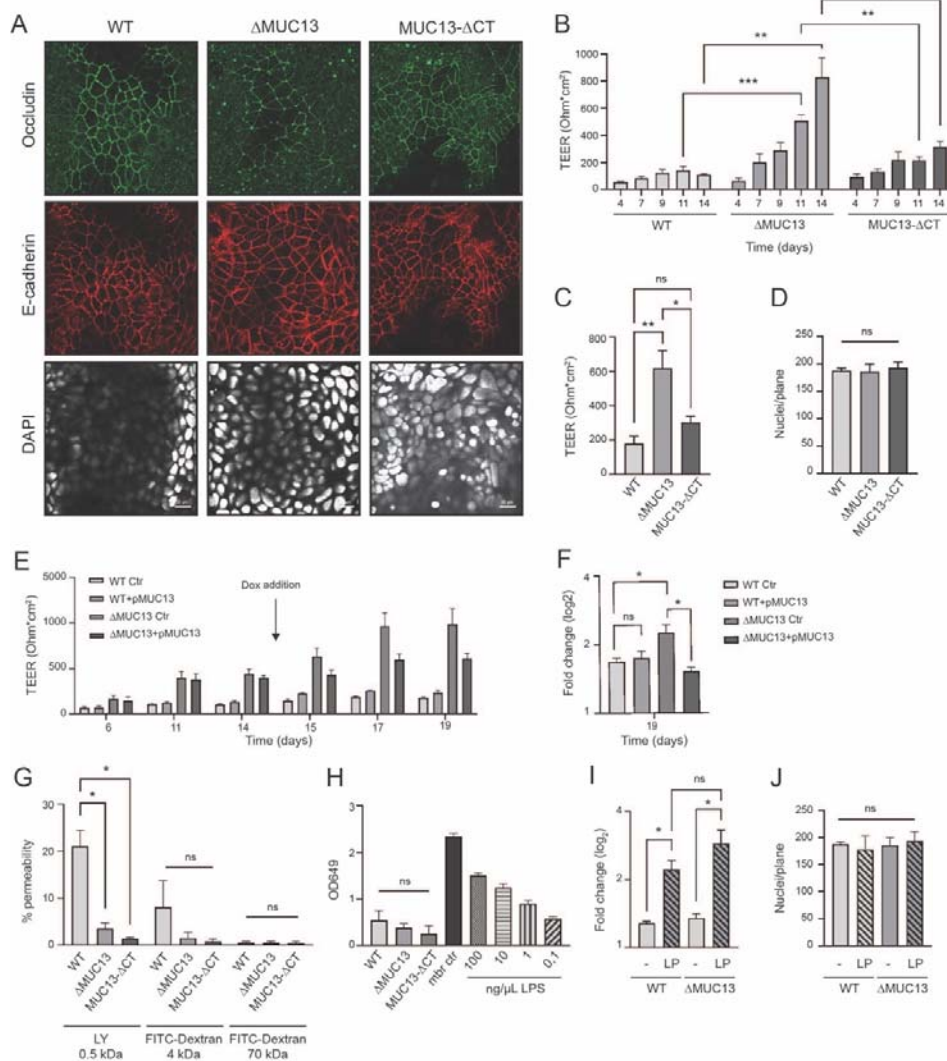
996 Immunofluorescence confocal images of WT,  $\Delta$ MUC13, and MUC13- $\Delta$ CT cells stained for MUC13-ED

997 (green) and nuclei (white). White scale bars represent 10 mM. (H) Immunofluorescence confocal

998 image of WT Ctr (empty plasmid), WT+pMUC13 (with inducible MUC13-GFP construct),  $\Delta$ MUC13 Ctr,

999 and  $\Delta$ MUC13+pMUC13 complementation cell lines after doxycycline induction for 24h. MUC13-GFP

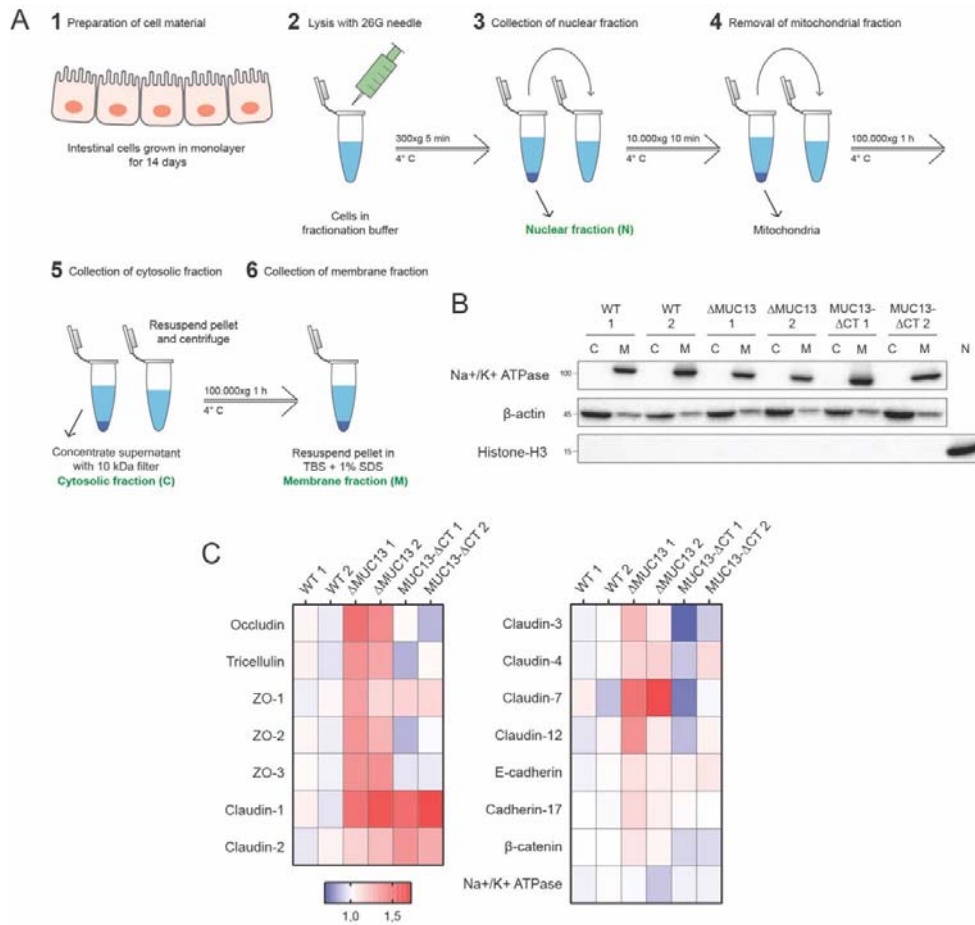
is depicted in green, and nuclei are shown in white. White scale bars represent 40 mM.



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1002 **Fig. 3. Deletion of MUC13 alters epithelial barrier properties.** (A) Immunofluorescence confocal  
1003 image of WT, ΔMUC13, and MUC13-ΔCT cell monolayers showing occludin (green), E-cadherin (red),  
1004 and nuclei (DAPI; white) staining. White scale bars represent 20 μm. (B) Transepithelial electrical  
1005 resistance (TEER) buildup in cell monolayers grown for up to 14 days. (C) TEER buildup in 2-weeks-  
1006 differentiated monolayers. (D) Quantification of cell nuclei per plane by confocal microscopy (DAPI)  
1007 in cell monolayers after 14 days of differentiation. (E) TEER buildup in the MUC13 overexpression  
1008 and complementation WT+pMUC13 and ΔMUC13+pMUC13 cell lines. Doxycycline was added on day  
1009 14 as indicated by an arrow. (F) Fold change (log<sub>2</sub>) of TEER increase in WT+pMUC13 and  
1010 ΔMUC13+pMUC13 cells on day 19 compared to day 14 before the addition of doxycycline. (G)  
1011 Paracellular passage of Lucifer Yellow CH substrate and FITC-dextran particles in 14-days-  
1012 differentiated cell monolayers. (H) Paracellular permeability assay with LPS from *Escherichia coli*  
1013 O111:B4 in 14-days differentiated monolayers. (I) Fold change (log<sub>2</sub>) compared to time 0 of TEER  
1014 increase in 14 days-differentiated WT and ΔMUC13 cell monolayers after addition of *Lactobacillus*  
1015 *plantarum* (LP) for 42 h at MOI 50. (J) Quantification of cell nuclei per plane by confocal microscopy  
1016 (DAPI) in WT and ΔMUC13 cell monolayers after 42 h incubation with LP. All graphs represent the  
1017 average and SEM of three independent experiments. ns, non-significant; \*, p<0.05; \*\* p<0.01; \*\*\*  
1018 p<0.001.





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1021 **Fig. 4. Tight junction proteins are highly upregulated in the absence of MUC13.** (A) Subcellular

1022 fractionation protocol for the enrichment of the membrane fraction from epithelial monolayers. 1)

1023 Intestinal epithelial cell lines were grown for 2 weeks in 10 cm<sup>2</sup> culture dishes. 2) Monolayers were

1024 lysed by passing through a needle in hyperosmotic fractionation buffer. 3) Nuclei (and unbroken

1025 cells) were pelleted by centrifugation and stored as the nuclear fraction (N). 4) The supernatant was

1026 collected and centrifuged again to pellet mitochondria. 5) Supernatant was again collected, and

1027 membranes were pelleted by ultracentrifugation. 6) The supernatant containing the cytosolic

1028 fraction (C) was stored. The pellet was washed and resuspended in fractionation buffer and pelleted

1029 by ultracentrifugation a second time to increase purity. 6) The resulting pellet was resuspended in

1030 TSB + 1% SDS buffer and stored as the membrane fraction (M). (B) Immunoblot analysis of

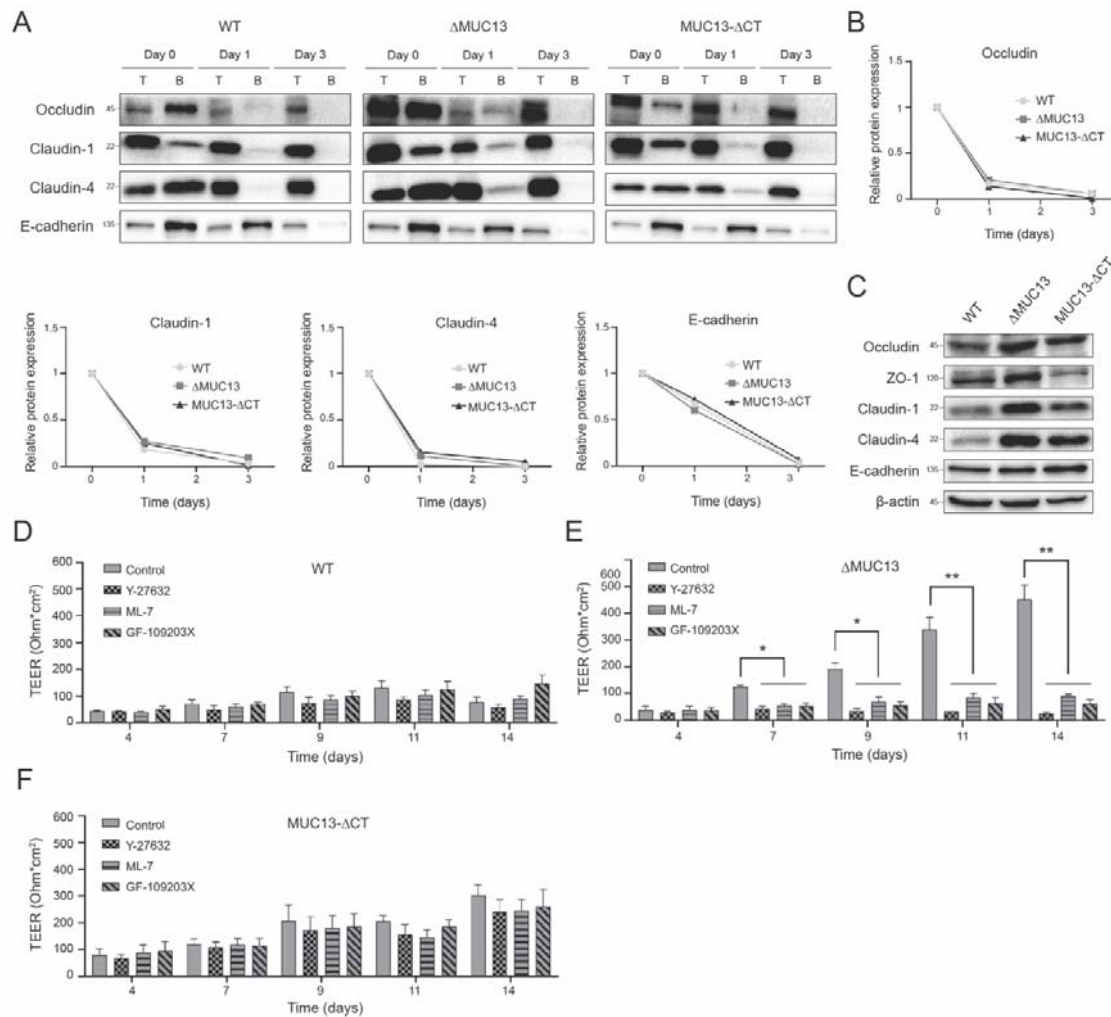
1031 subcellular fractionation of two WT, ΔMUC13, and MUC13-ΔCT cell lines using Na<sup>+</sup>/K<sup>+</sup>-ATPase

1032 (membrane marker), Histone-H3 (nuclear marker), and β-actin (cytoplasmic marker). C (cytosolic

1033 fraction), M (membrane fraction), N (nuclear fraction). Molecular mass standards (kDa) are indicated

1034 on the left. (C) Relative abundance of cell junction proteins identified by mass spectrometry in

membrane fractions of WT, ΔMUC13, and MUC13-ΔCT monolayers grown for 2 weeks.



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1038 **Fig. 5. TEER buildup in the absence of MUC13 is dependent on MLCK, ROCK, and PKC kinases. (A)**

1039 Degradation rates of biotinylated occludin, claudin-1, claudin-4, and E-cadherin analyzed by

1040 immunoblot in cell monolayers. Cells were incubated with biotin-NHS on day 0 and the presence of

1041 biotinylated proteins was determined on days 0, 1, and 3. T (total lysate), B (elution from

1042 streptavidin beads). The assay was performed at least three times and representative images are

1043 shown. Molecular mass standards (kDa) are indicated on the left. **(B)** Relative protein abundance of

1044 biotinylated occludin, claudin-1, claudin-4, and E-cadherin proteins on days 0, 1, and 3. **(C)**

1045 Immunoblot of occludin, ZO-1, claudin-1, claudin-4, E-cadherin, and  $\beta$ -actin in total lysates of

1046 monolayers grown for 2 weeks. The assay was performed at least three times and representative

1047 images are shown. Molecular mass standards (kDa) are indicated on the left. **(D-F)** TEER buildup of

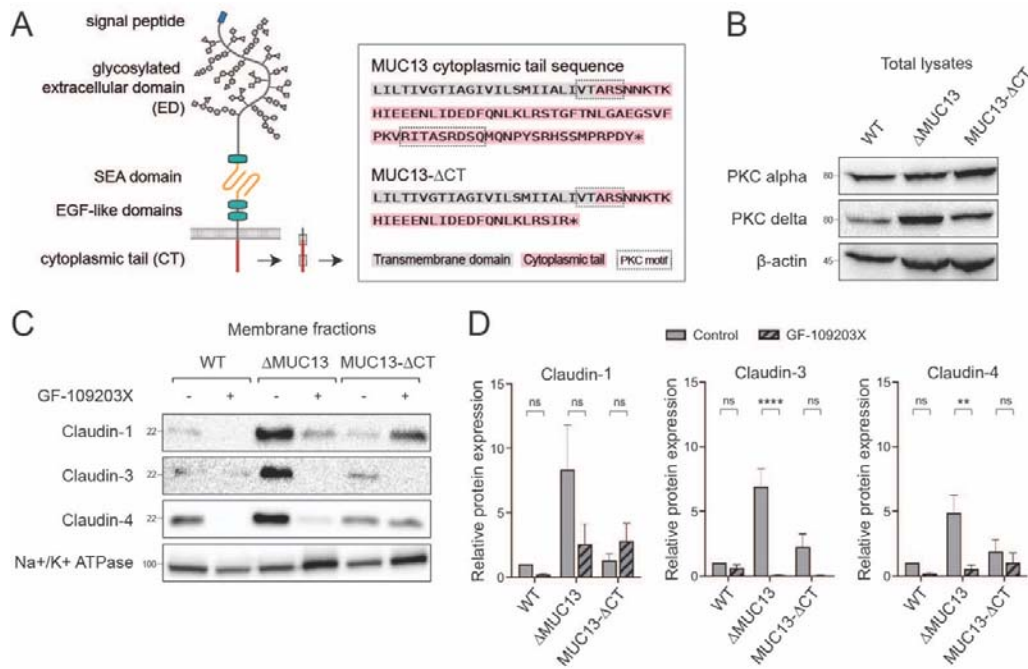
1048 WT **(D)**,  $\Delta$ MUC13 **(E)**, and MUC13- $\Delta$ CT **(F)** cell lines over time in the presence of kinase inhibitors ML-

1049 7 (MLCK), Y-27632 (ROCK), and GF-109203X (PKC). Inhibitors were added on days 3, 6, and 9 at a

1050 concentration of 50  $\mu$ M (ML-7 and Y-27632) and 20  $\mu$ M (GF-109203X). One representative clone for

1051 each cell line was used in these experiments. Bars represent the average and SEM of three

independent experiments. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ .



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1055 **Fig. 6. PKCs are involved in TJ regulation in the absence of MUC13.** (A) Schematic representation of

1056 WT MUC13 domain structure (left) and protein sequence (right). The transmembrane domain (grey),

1057 the cytoplasmic tail (red), and two predicted PKC binding motifs (black boxes) are marked. (B)

1058 Immunoblot analysis of PKCα, PKCδ, and β-actin in total lysates of monolayers grown for 2 weeks.

1059 Molecular mass standards (kDa) are indicated on the left. (C) Immunoblot analysis of isolated

1060 membrane fractions from monolayers grown for 2 weeks in the presence/absence of 20 mM PKC

1061 inhibitor (GF-109203X) added every 3 days. Claudin-1, claudin-3, claudin-4, and the control protein

1062 Na<sup>+</sup>/K<sup>+</sup>-ATPase are shown. Molecular mass standards (kDa) are indicated on the left. (D)

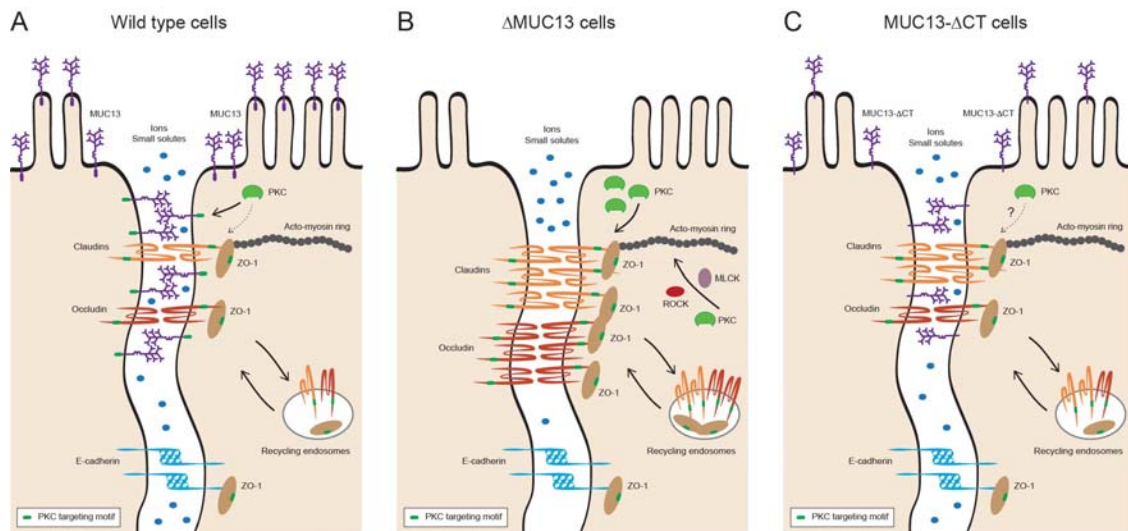
1063 Quantification of relative protein expression of claudin-1, claudin-3, and claudin-4 in isolated

1064 fractions of cells grown in the presence/absence of GF-109203X as depicted in C. All assays were

1065 performed at least three times and representative images are shown. One representative clone for

1066 each cell line was used in these experiments. Bars represent average and SEM of three independent

experiments. ns, non-significant; \*\* p<0.01; \*\*\* p<0.001.



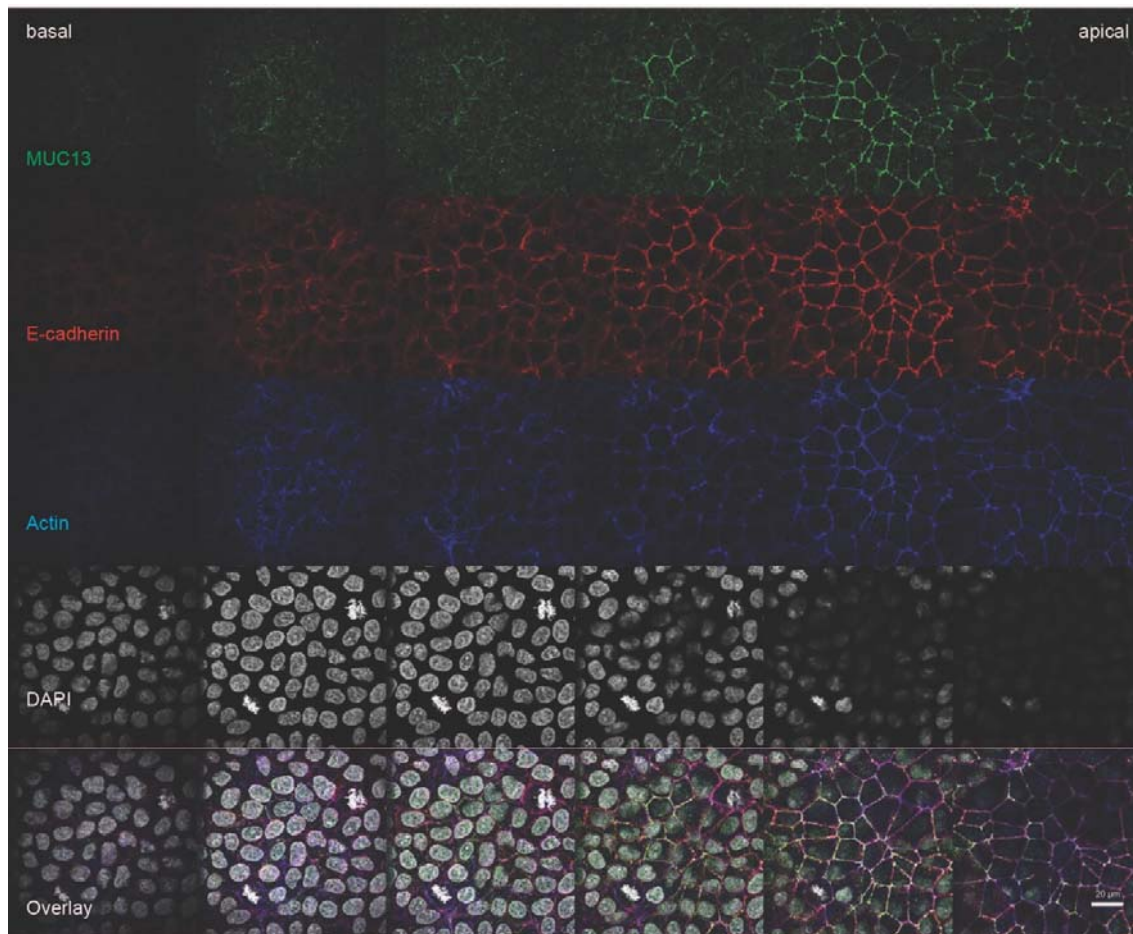
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1069 **Fig. 7. Tight junction regulation by MUC13.** (A) In wild type cells, MUC13 localizes to both the apical  
1070 surface and tight junction (TJ) region of the lateral membrane. Cell junction complexes that contain  
1071 claudins, occludin, ZOs, and E-cadherin, are assembled along the lateral membrane. Under normal  
1072 conditions, there is some paracellular passage of ions and small solutes, a process that is controlled  
1073 by the TJ proteins claudins and occludin. MUC13 cytoplasmic tail has a putative PKC binding motif,  
1074 which may play a role in recruiting PKC and controlling its activity and/or stability. Cell junction  
1075 proteins such as claudins, occludin, and ZO-1 also can be targeted by PKCs. (B) In the absence of the  
1076 complete MUC13 protein, TJ proteins (occludin, claudins, and ZO-1) are accumulating at the  
1077 membrane over time, causing increased transepithelial resistance (TEER) and lower paracellular  
1078 passage of small solutes. The TEER buildup in  $\Delta$ MUC13 cells is dependent on MLCK, ROCK and PKC  
1079 kinases. The accumulation of claudins at the membrane in  $\Delta$ MUC13 cells is PKC-dependent and is  
1080 not caused by slower degradation rates of TJ proteins through recycling endosomes. (C) Removal of  
1081 the MUC13 cytoplasmic tail leads to an intermediate phenotype with some accumulation of claudin-  
1082 1, -3, -4, and ZO-1 at the membrane, but to a lower extent compared to the full knockout. The role  
1083 of PKC in this cell line remains to be determined. MUC13- $\Delta$ CT cells are less permeable to small  
1084 solutes but do not show a significant increase in TEER when compared to WT cells. The degradation  
1085 rate of TJ proteins in MUC13- $\Delta$ CT cells is comparable to WT and  $\Delta$ MUC13.



1086 **Supplementary Materials**

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1090 **Fig. S1. MUC13 localizes at the upper side of the lateral membrane.** (A) Immunofluorescence of  
1091 HRT18 intestinal cells stained for MUC13 cytoplasmic tail (MUC13-CT) (green), E-cadherin (red),  $\beta$ -  
1092 actin (blue), and DAPI (white). White scale bars represent 20  $\mu$ m. Pictures were taken at different  
1093 heights in the epithelial monolayer (Z).