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1 **Title: A CRISPR-screen in intestinal epithelial cells identifies novel factors for polarity**  
2 **and apical transport**

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16

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42 **Abstract**

43 **Epithelial polarization and polarized cargo transport are highly coordinated and**  
44 **interdependent processes. In our search for novel regulators of epithelial polarization**  
45 **and protein secretion, we used a genome-wide CRISPR/Cas9 screen and combined it**  
46 **with an assay based on fluorescence-activated cell sorting (FACS) to measure the**  
47 **secretion of the apical brush border hydrolase dipeptidyl peptidase 4 (DPP4). In this**  
48 **way, we performed the first CRISPR screen to date in human polarized epithelial cells.**  
49 **Using high-resolution microscopy, we detected polarization defects and mislocalization**  
50 **of DPP4 to late endosomes/lysosomes after knock-down of TM9SF4, anoctamin 8, and**  
51 **ARHGAP33, confirming the identification of novel factors for epithelial polarization and**  
52 **apical cargo secretion. Thus, we provide a powerful tool suitable for studying**  
53 **polarization and cargo secretion in epithelial cells. In addition, we provide a dataset that**  
54 **serves as a resource for the study of novel mechanisms for epithelial polarization and**  
55 **polarized transport and facilitates the investigation of novel congenital diseases**  
56 **associated with these processes.**

57

## 58 Introduction

59 Epithelia are highly specialized tissues that line inner and outer surfaces of various organs of  
60 metazoans, performing absorption, secretion, and barrier functions. During polarization  
61 epithelial cells assume their characteristic shape, by building specialized apical- and  
62 basolateral plasma membrane (PM) domains (Rodriguez-Boulán and Macara, 2014; Apodaca  
63 et al., 2012), which are separated by junctional complexes and characterized by a specific  
64 composition of lipids and proteins (Martin-Belmonte et al., 2007). The asymmetric distribution  
65 of polarity complexes and the mutual exclusion of proteins from one domain, by proteins from  
66 the other domain are critical for the maintenance of apico- basolateral domains at the cell  
67 cortex (Rodriguez-Boulán and Macara, 2014; Román-Fernández and Bryant, 2016).  
68 Additionally, tightly orchestrated transport mechanisms and machineries, as Rab-GTPases,  
69 motor proteins, soluble NSF attachment receptor (SNARE)-proteins and specific adapter  
70 proteins, ensure the establishment and maintenance of specialized membrane domains  
71 (Gaisano et al., 1996; Low et al., 1996; Weimbs et al., 1997; Li et al., 2002).

72 Defects in polarization and polarized traffic often cause diseases, such as congenital diarrhea  
73 and enteropathies (Thiagarajah et al., 2018; Canani et al., 2010; Apodaca et al., 2012).  
74 Microvillus inclusion disease is an autosomal-recessive enteropathy (Cutz et al., 1989),  
75 characterized by intractable diarrhea in neonates (Cutz et al., 1989; Ruemmele et al., 2006).  
76 Enterocytes of MVID-patients show loss of brush-border microvilli, formation of so-called  
77 microvillus-inclusions and subapical accumulation of so-called “secretory granules” (Cutz et  
78 al., 1989; Phillips et al., 2000). Our studies identified mutations in *MYO5B*, *STX3* and *STXBP2*  
79 to be causative for MVID (Müller et al., 2008; Ruemmele et al., 2010; Wiegerinck et al., 2014;  
80 Vogel et al., 2017b); they revealed that a molecular transport machinery involving myosin Vb  
81 (*myo5b*), the small Rab-GTPases Rab11a and Rab8a, the t-SNARE syntaxin3 (*stx3*) and the  
82 v-SNAREs *slp4a* and *vamp7* is essential for apical cargo delivery (Vogel et al., 2015b, 2017b).  
83 This cascade is required for the delivery of apical transmembrane transporters that are  
84 important for proper physiological function of enterocytes, such as sodium-hydrogen  
85 exchanger 3 (NHE3), glucose transporter 5 (GLUT5) and cystic fibrosis transmembrane  
86 conductance receptor (CFTR), but not for dipeptidyl-peptidase-4 (DPP4), sucrase-isomaltase  
87 (SI) and amino-peptidase-N (APN). This suggests the presence of additional trafficking routes  
88 and transport mechanisms for these apical cargos.

89 Since molecular signals for apical cargo sorting and transport are believed to be highly diverse,  
90 various mechanisms have been proposed to underly epithelial protein secretion (Levic and  
91 Bagnat, 2021). A common, characteristic feature of apical cargo is the presence of post-  
92 translational modifications, such as N- and O-linked glycosylations that are recognized by  
93 specific lectins, as well as GPI-anchors that allow sorting into cholesterol-rich lipid

94 microdomains (Weisz and Rodriguez-Boulan, 2009; Zurzolo and Simons, 2016). Additionally,  
95 recent studies have proposed that protein oligomerization coincides with sorting into  
96 specialized membrane domains in the *trans*-Golgi network (TGN), which depends on the pH-  
97 regulation of the TGN lumen (Levic and Bagnat, 2021; Levic et al., 2020).

98 To uncover protein functions for a wide range of cellular processes, genome-wide clustered  
99 regularly interspaced short palindromic repeats (CRISPR) mediated screens have advanced  
100 to a state-of-the-art strategy (Shalem et al., 2014, 2015; Kampmann, 2018). In addition to their  
101 application to understanding the regulation of tumor biology, viral infection, or miRNA  
102 processing, CRISPR-mediated screening approaches have recently proven highly effective in  
103 discovering novel factors for intracellular protein trafficking and secretion (He et al., 2021; Zhu  
104 et al., 2021; Hutter et al., 2020; Stewart et al., 2017; Popa et al., 2020; Bassaganyas et al.,  
105 2019). Additionally, the CRISPR-Cas9 technology has been successfully used in madine-  
106 darby canine kidney (MDCK) cells with the generation of a collection of Rab-GTPase  
107 knockouts, which has provided great value for phenotypic analyses of Rab-KOs in epithelial  
108 cells (Homma et al., 2019).

109 In this study, we employed the CRISPR-screening technology as an unbiased experimental  
110 strategy to uncover novel regulators of epithelial cell polarization and trafficking by  
111 investigating factors required for the apical delivery of DPP4. The brush-border hydrolase  
112 DPP4 is a type II transmembrane protein. It is heavily modified with N- and O-linked glycans  
113 in its extracellular domain (Misumi et al., 1992; Baricault et al., 1995; Fan et al., 1997), which  
114 have been suggested to be critical apical sorting determinants of DPP4 (Alfalah et al., 2002).  
115 Even though several studies have suggested diverse trafficking routes for DPP4, the  
116 mechanisms and protein-machineries underlying these processes remain enigmatic so far  
117 (Casanova et al., 1991; Baricault et al., 1993; Low et al., 1992; Sobajima et al., 2015).

118 Here, we conducted the first CRISPR-screen in polarized human epithelial cells to date. We  
119 present an experimental strategy for applying the CRISPR screening system in polarized  
120 epithelial cells to study novel protein functions. We have developed an easy to use and  
121 adaptable, FACS-based assay to measure the efficiency of protein secretion in polarized  
122 epithelial cells after genome editing. In combination with a detailed characterization of selected  
123 proteins by immunofluorescence and cryo-based electron microscopy, we have identified  
124 novel factors required for proper apico-basolateral polarization and secretion of apical cargo.  
125 Therefore, our dataset serves as a foundation for future studies aimed at deciphering novel  
126 mechanisms underlying epithelial polarization and polarized cargo transport. In addition, it  
127 provides a powerful resource for the investigation and validation of new congenital disease  
128 genes to be identified.



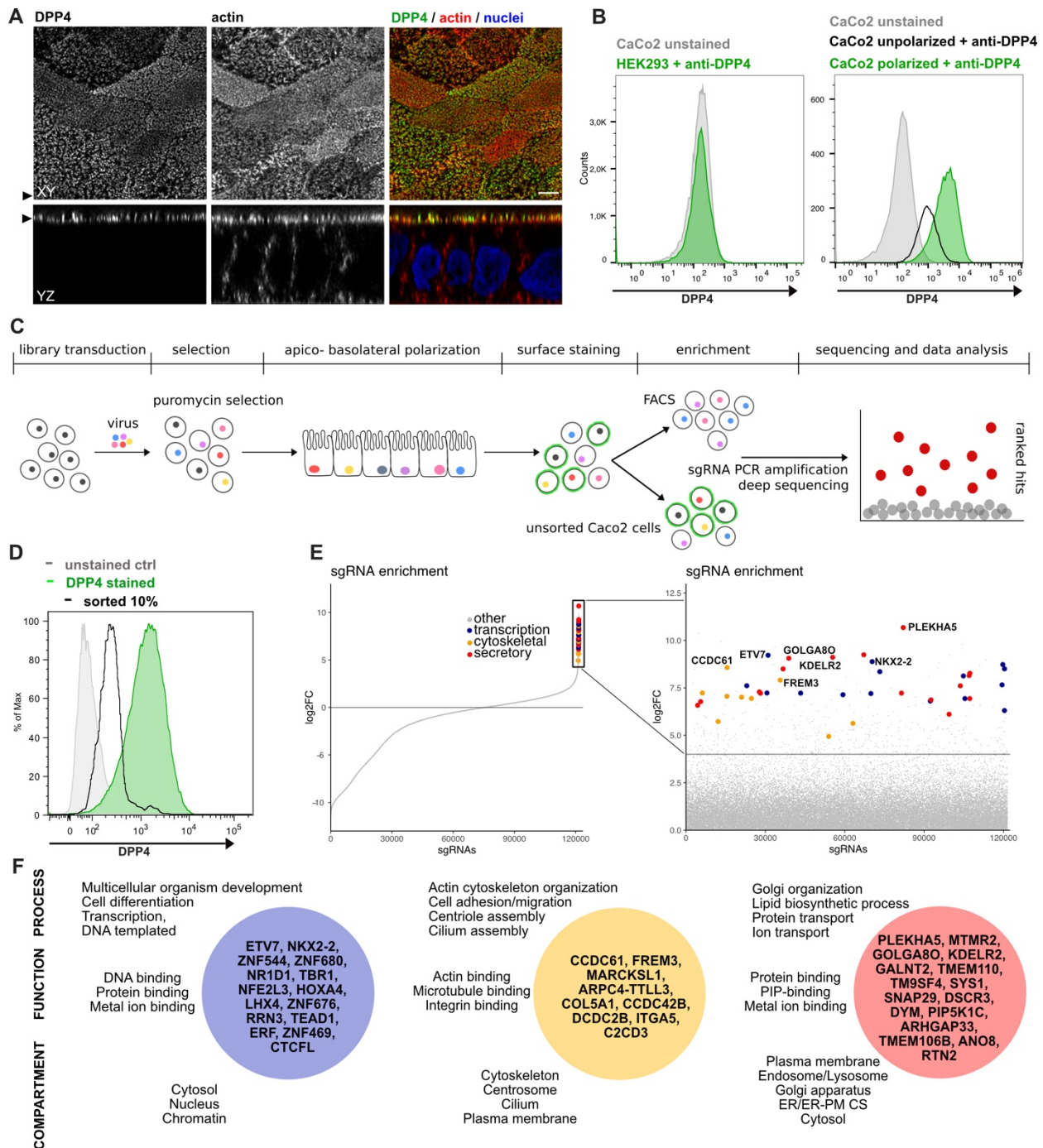
130 **Results**

131 ***Development of a genome-wide CRISPR-screen to identify factors required for plasma***  
132 ***membrane localization of the apical cargo DPP4***

133 We established an unbiased CRISPR-Cas9-loss of function screen to define factors involved  
134 in surface targeting of the apical model cargo DPP4 in the enterocyte like colon carcinoma cell  
135 line, CaCo2 (Fig. 1). DPP4 is a type 2 transmembrane protein, that can be detected with  
136 antibodies binding to the extracellular C-terminus of the protein (Fig. 1 A). We made use of this  
137 feature to read out the efficiency of endogenous DPP4 surface delivery by fluorescence  
138 activated cell sorting (FACS) in CaCo2 cells after epithelial polarization. Here, we used a period  
139 of 18 to 21 days, during which surface DPP4 signal is significantly increased in the course of  
140 cell surface expansion and specialized polarized trafficking processes (Fig. 1 B). In this  
141 context, we aimed to define factors required for apical membrane differentiation and cargo  
142 trafficking, thereby leading to a strong reduction of DPP4 after surface polarization. First, we  
143 generated Cas9-expressing CaCo2 cells and then transduced two biological replicates at a  
144 low multiplicity of infection (MOI) (0.2) using the human lentiviral GeCKOv2 CRISPR-library,  
145 selecting for successful viral integration with antibiotic treatment with Puromycin. We then  
146 seeded the infected CaCo2 cultures at high density and allowed the confluent monolayers to  
147 further polarize and differentiate for 18 days. Next, polarized cells were detached, stained for  
148 endogenous DPP4, and subjected to FACS, separating those cells with only 10% of surface  
149 signal left, due to CRISPR-targeting (Fig. 1 C and D). To determine the abundance of gRNAs  
150 in sorted versus unsorted cell populations, genomic DNA was isolated and read counts were  
151 determined by next generation sequencing. Subsequent analysis using GenePattern and  
152 Galaxy analysis tools enabled the identification of 89 gRNAs significantly enriched in the sorted  
153 cell population ( $p < 0.05$ ) and represented genes whose downregulation had resulted in reduced  
154 DPP4 surface release (Fig. 1 D and E; Table S1 A and B).

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157 **Figure 1. A CRISPR mediated loss of function screen in polarized enterocytes. A.**

158 Dipeptidylpeptidase 4 (DPP4) localizes to the apical brush-border of polarized enterocytes and

159 can be detected with a specific antibody, at its extracellular stalk domain. Top-view (XZ) and

160 lateral view (YZ) of a polarized CaCo2 monolayer. Scale = 5 μm **B.** During polarization, apical

161 DPP4 is increased due to polarized traffic and surface expansion, which can be measured by

162 flow cytometry (right panel, CaCo2 unpolarized versus polarized). HEK293T cells, not

163 expressing DPP4, serve as quality control for staining specificity. **C.** CaCo2-Cas9 cells are

164 transduced with the lentiGuide-Puro library and selected with Puromycin. After selection

165 CaCo2 cells are seeded to confluent monolayers and cultured for apico-basolateral

166 polarization. Subsequently cells are detached, stained, and subjected to fluorescence

167 activated cell sorting (FACS). Sorted and unsorted control cells are processed for gDNA  
168 extraction and genomically integrated CRISPR-constructs are amplified by PCR. Finally, PCR  
169 products of sorted and unsorted cell populations are analyzed by next generation sequencing  
170 and sgRNAs are ranked by their enrichment in the sorted vs. unsorted cell population. **D.**  
171 Sorting was performed for 10% of the cells, with lowest surface-signal intensity, thereby  
172 enriching for the cell population that had lost 90% of surface DPP4 signal, due to efficient  
173 CRISPR targeting. **E.** 89 single guide RNAs were significantly enriched in the sorted cell  
174 population. **F.** Factors enriched in the sorted cell population could functionally be associated  
175 with secretory traffic, cytoskeletal architecture or transcription, in a manual gene-ontology  
176 analysis.

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178 ***A genome-wide CRISPR-screen in polarized enterocytes identifies factors associated***  
179 ***with secretory traffic***

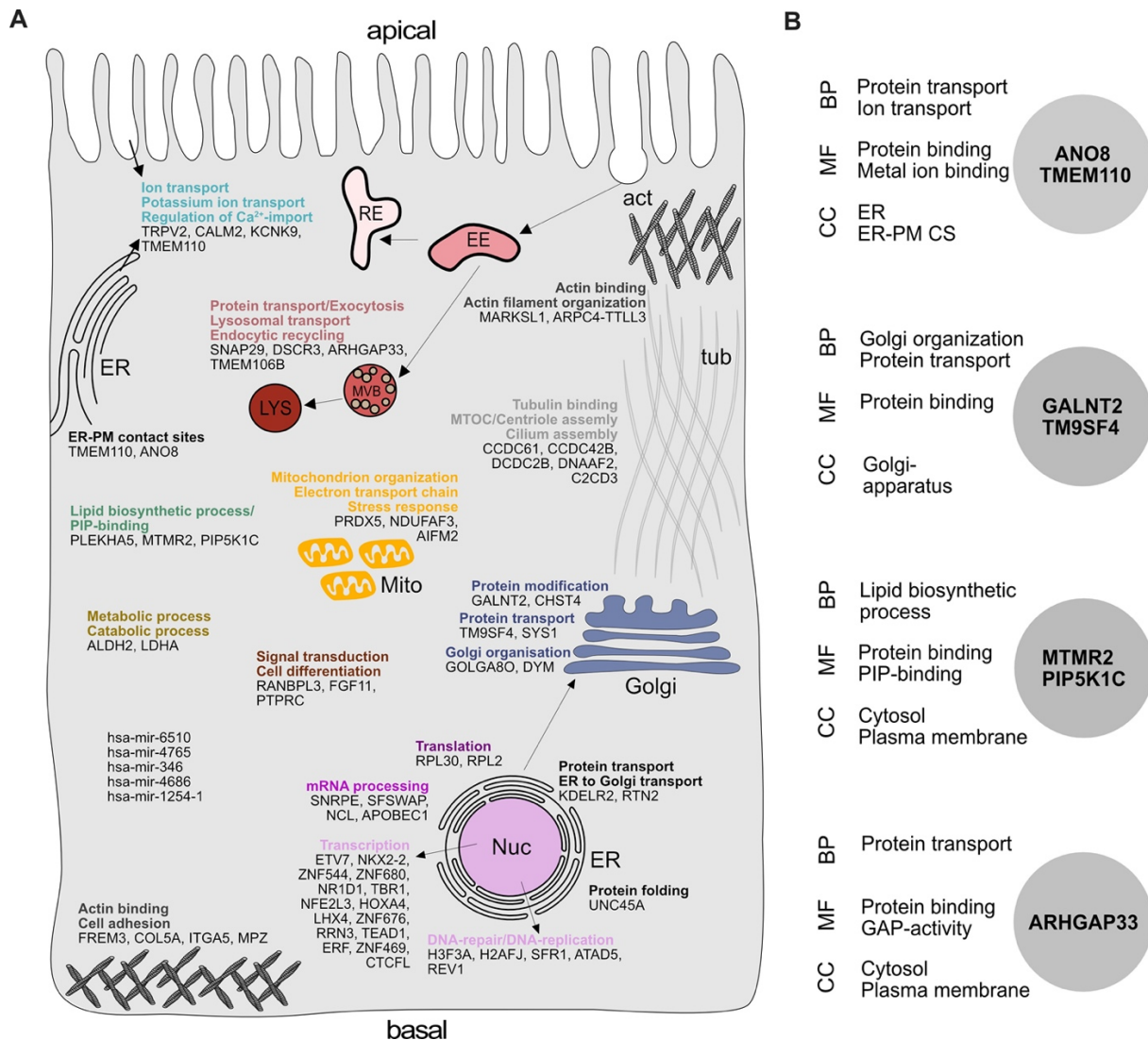
180 Next, we wanted to get a comprehensive overview on the gene classes represented in our list  
181 of enriched gRNAs. However, automated KEGG pathway and gene enrichment analyses of  
182 our results were insufficient. Hence, we manually analyzed the 89 identified genes for common  
183 gene ontology (GO)-terms and grouped them accordingly. We listed three GO-terms from each  
184 category (biological process, molecular function, cellular compartment) for each hit, including  
185 the most common GO-terms captured by the QuickGO-search database, focusing on including  
186 GO-terms that indicate a role in the secretory pathway (Table S1 C).

187 Our analysis highlighted several genes, with functions related to the organization of the  
188 secretory pathway (Fig. 1 E and F; Fig. 2 A), including general organization and maintenance  
189 of organelles such as the endoplasmic reticulum (ER), the Golgi apparatus or protein transport  
190 at early steps of the secretory pathway (e.g., *KDEL2*, *RTN2*, *GOLGA80*). Further, identified  
191 hits were related to protein modification and transport at *cis*- and *trans*-Golgi compartments  
192 (*GALNT2*, *SYS1*), lipid-biosynthesis (*MTMR2*, *PIP5K1C*), vesicle fusion and endocytic  
193 recycling (*SNAP29*, *DSCR3*). Interestingly, we identified two genes associated with ER-plasma  
194 membrane (ER-PM) contact sites (*TMEM110*, *ANO8*). Furthermore, we found several factors  
195 required for various aspects of cytoskeletal organization such actin-filament  
196 organization/polymerization (e.g., *MARCKSL1*, *ARPC4-TTLL3*), cell adhesion (e.g., *ITGA5*,  
197 *FREM3*, *MPZ*) but also microtubule organizing centre (MTOC)/centriole- and cilium assembly  
198 and association (e.g., *CCDC61*, *CCDC42B*, *C2CD3*). Finally, we found numerous factors with  
199 functions related to DNA-templated transcription and cell-differentiation (e.g., *ETV7*, *NKX2-2*,  
200 *ERF*), as well as mRNA-processing/RNA-splicing (e.g., *SNRPE*, *SFSWAP*), translation (e.g.,  
201 *RPL30*, *RPL2*) and DNA-repair/DNA-replication (e.g., *SFR1*, *ATAD5*, *REV1*) (Fig. 1 E and F;  
202 Fig. 2 A).

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203 Overall, in a CRISPR-mediated loss-of-function screen, we identified a variety of factors that  
 204 affect surface transmission of an apical model cargo protein, DPP4, at different cellular levels.  
 205 This underscores the value of our dataset and approach to identify novel factors for secretory  
 206 membrane trafficking in polarized epithelial cells.

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209 **Figure 2. Gene-ontology analysis of hits obtained in a CRISPR-mediated loss of function**  
 210 **screen in polarized CaCo2 cells. A.** Schematic representation of significantly enriched genes  
 211 obtained from a CRISPR-screening approach, grouped and organized according to their  
 212 associated gene ontology (GO) terms. **B.** GO-association analysis of the 7 factors, that were  
 213 chosen for CRISPR-screen readout-validation and further characterization. CC = cellular  
 214 compartment, MF = molecular function, BP = biological process.

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## 217 ***Novel factors for surface localization of the apical cargo DPP4***

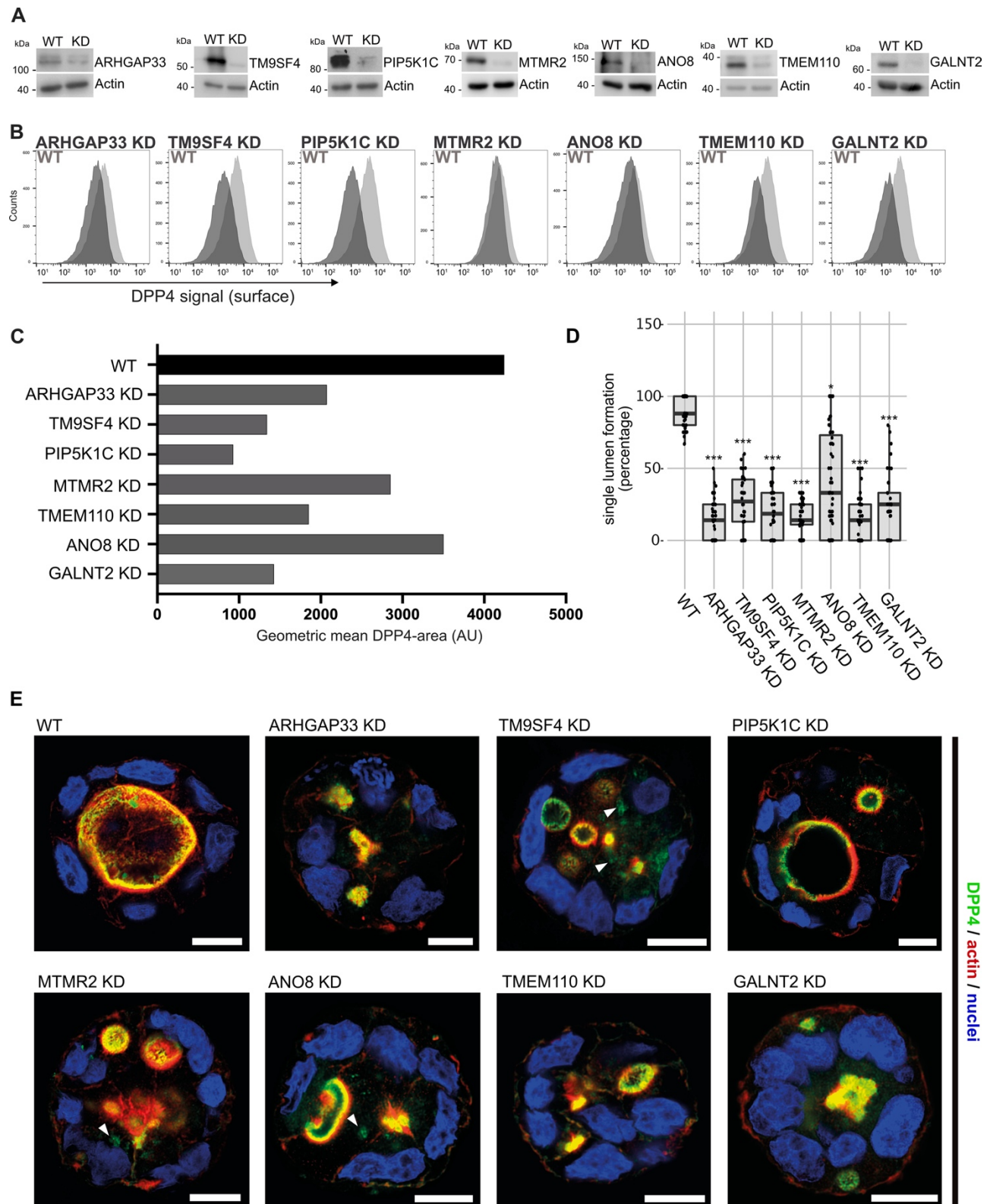
218 After setting up a CRISPR-mediated screening platform in polarized CaCo2 cells, we validated  
219 our screening approach by further characterizing potentially novel factors for apical cargo  
220 traffic and membrane polarization. Since we had identified several genes with functions related  
221 to secretory trafficking (Fig. 2 A), we chose 7 promising candidates for further analyses (Fig. 2  
222 B). These factors function on various levels of the endomembrane system: the anoctamin  
223 family member anoctamin 8 (ANO8) and the stromal interaction molecule (STIM) enhancing  
224 tethering protein STIMATE (TMEM110) are required for establishment and maintenance of  
225 ER-PM contact sites, which in turn, have recently been shown to be required for apical PM-  
226 establishment in bile-canaliculi (Jha et al., 2019; Quintana et al., 2015; Chung et al., 2020).

227 The nonaspanin-family member TM9SF4 has been suggested to be required for  
228 transmembrane domain sorting in early steps of the secretory pathway but also in the  
229 generation of specialized membrane domains in the early *cis*- Golgi compartment (Perrin et  
230 al., 2015; Vernay et al., 2018; Yamaji et al., 2019). Polypeptide N-  
231 acetylgalactosaminyltransferase 2 (GALNT2) regulates O-linked glycosylation of  
232 transmembrane proteins in the Golgi, and was chosen as a candidate for screen validation,  
233 with a potentially global effect on secretory traffic (Wandall et al., 1997; Moremen et al., 2012).  
234 Sorting nexin 26 (SNX26/ARHGAP33) was included since it has been described as a GTPase  
235 activating protein for Cdc42, a major player in apical domain differentiation (Kim et al., 2013).  
236 Finally, we chose the lipid kinase subunit phosphatidylinositol 4-phosphate 5-kinase type-1  
237 gamma (PIP5K1C) and the lipid phosphatase myotubularin-related protein 2 (MTMR2) for  
238 screen validation and further analysis- since they are known regulators of apical  
239 phosphatidylinositolphosphate (PIP)-pools (Xu et al., 2019; Román-Fernández et al., 2018).

240 We generated clonal knock-down (KD) cell lines of those candidates, using the CRISPR-  
241 technology and those gRNAs that had proven to efficiently target in our CRISPR-screen (Fig.  
242 3 A). We then analyzed KD cell lines for surface localization of DPP4 by flow cytometry, using  
243 the previously described polarization assay from our CRISPR-screen (Fig. 3 B). These  
244 measurements showed that targeting of the selected candidates indeed leads to reduced  
245 surface localization of DPP4, but to varying degrees (Fig. 3 C). The strongest effect on DPP4  
246 surface localization was caused by interference with PIP5K1C (~75% reduction), followed by  
247 TM9SF4, TMEM110 and GALNT2 (~50% reduction). Interestingly, ANO8-, MTMR2- and  
248 ARHGAP33-KDs showed the mildest phenotype (~30% reduction), (Fig. 3 B and C).

249 By growing KD cell lines of selected candidate genes and reanalyzing them for the effects of  
250 CRISPR targeting on PM localization of DPP4, we validated our primary CRISPR loss-of-  
251 function screen and thereby identified new players for surface localization of the apical cargo  
252 protein DPP4.

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254 **Figure 3. Validation of selected candidates, identified in a CRISPR-mediated loss of**  
 255 **function screen. A.** Generation of clonal knock-down (KD) cell lines of 7 candidates, chosen  
 256 for primary CRISPR-screen validation and further analysis. Residual protein levels, if left, after  
 257 CRISPR-targeting in the KD-clones were determined by Western-blotting by comparison to  
 258 wildtype (WT) cells. Beta-actin was used as loading control. Molecular size markers are  
 259 depicted in kDa. **B.** The effect of the respective KDs on DPP4 surface transport, in clonal KD

260 cell lines. Cell lines were polarized for 18 days and then subjected to flow cytometry, to  
261 determine the KD-effect on DPP4 surface targeting. **C.** Geometric means of DPP4-area (DPP4  
262 intensity on the cellular surface) were determined of respective cell lines. All KD cell lines show  
263 varying extents of DPP4-surface reduction, with PIP5K1C-KD displaying the strongest and  
264 ANO8-KD the mildest effect. **D.** 3D cyst assay were performed with WT and KD cultures. Single  
265 central lumen formation was quantified. The percentage of cysts with a single central lumen is  
266 substantially decreased in the respective KD-clonal cells lines (dot box plot, Mann–Whitney U  
267 test \*\*\* $p < 0.001$ ). **E.** Immunofluorescence micrographs of 3D-cysts generated from WT and  
268 KD cell lines. All knockdown cell lines display the formation of multiple lumina or no lumina.  
269 DPP4 localizes to actin-rich structures in all KD cell lines and additionally, to intracellular, actin-  
270 negative compartments in TM9SF4-, MTMR2- and ANO8-KD clones (white arrowheads).  
271 Scale = 10  $\mu\text{m}$ .

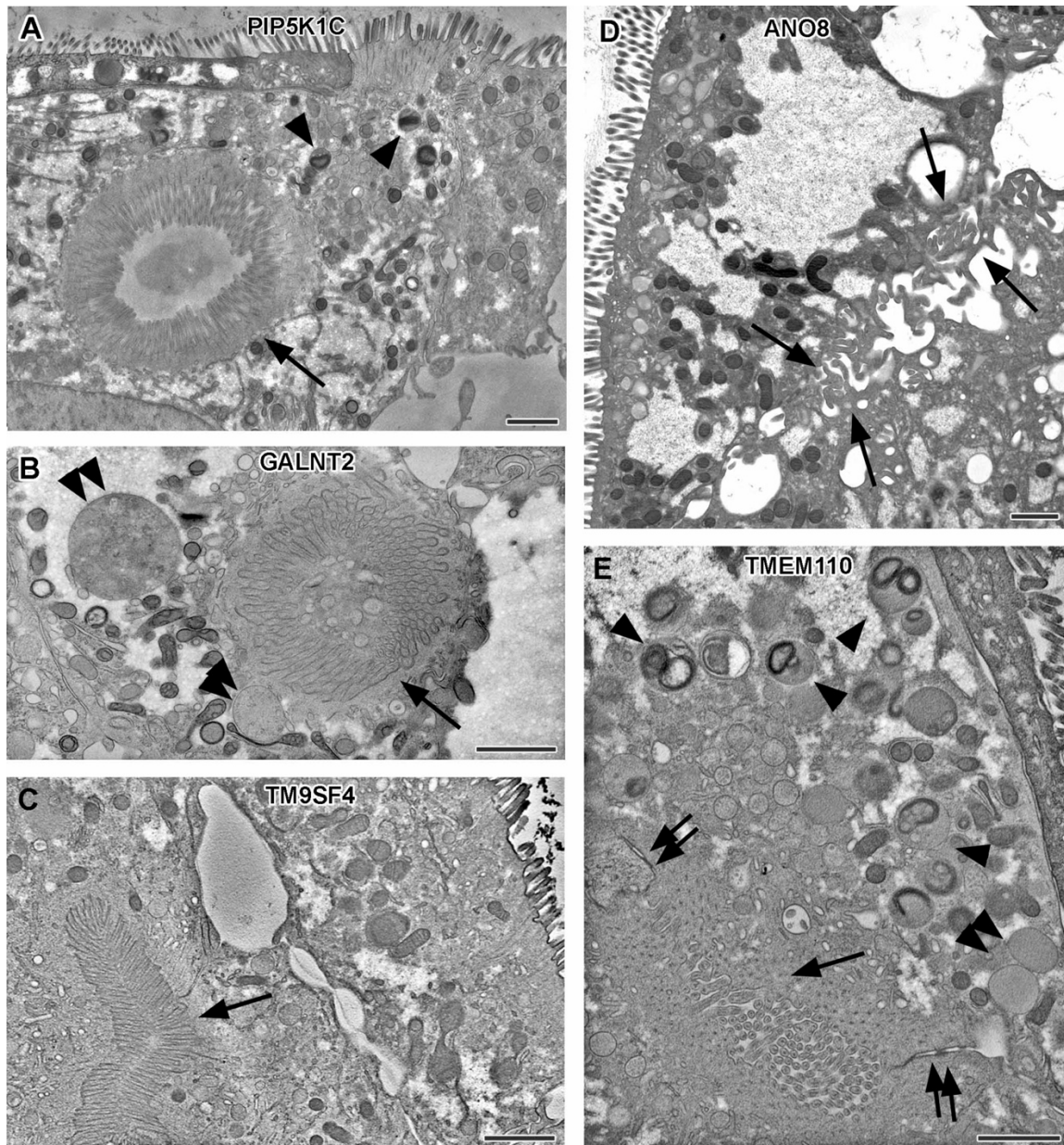
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### 273 ***3D cyst models and high-resolution microscopy reveal novel factors for proper*** 274 ***epithelial polarization***

275 Because apical transport and the correct establishment of epithelial polarity are closely linked,  
276 we investigated the relevance of the newly identified factors for polarization. Therefore, we  
277 performed 3D cyst assays using WT and the corresponding KD cell lines (Fig. 3 D and E).  
278 Cysts were analyzed by immunofluorescence microscopy (IF) to determine the targeting of  
279 DPP4 to apical membrane domains (Fig. 3 E). We found that all KD cell lines had severe  
280 defects in the formation of a single, central lumen, but rather established multiple or no lumina  
281 (Fig. 3 D and E). Although DPP4 was localized in the apical PM domains in all KD-cell lines,  
282 TM9SF4-, MTMR2-, and ANO8-KD-cell lines additionally displayed aberrant intracellular  
283 accumulation of DPP4 (Fig. 3 E).

284 To characterize the involvement of the selected candidates in apico-basolateral polarization  
285 and apical transport in greater detail, we complemented fluorescence microscopy with cryo-  
286 based electron microscopy and investigated the ultrastructural phenotype and the subcellular  
287 distribution of selected marker molecules in the respective cell lines. For this, TM9SF4-, ANO8-  
288 , ARHGAP33-, TMEM110-, MTMR2-, PIP5K1C- and GALNT2-KD cell lines were grown for 18-  
289 21 days on permeable filter membranes to obtain fully polarized, differentiated 2D cell  
290 monolayers. Samples were then subjected to rapid cryo-fixation (high-pressure freezing and  
291 freeze-substitution) for transmission electron microscopy (TEM) or to conventional aldehyde  
292 fixation for scanning EM (SEM) and immunogold-TEM.





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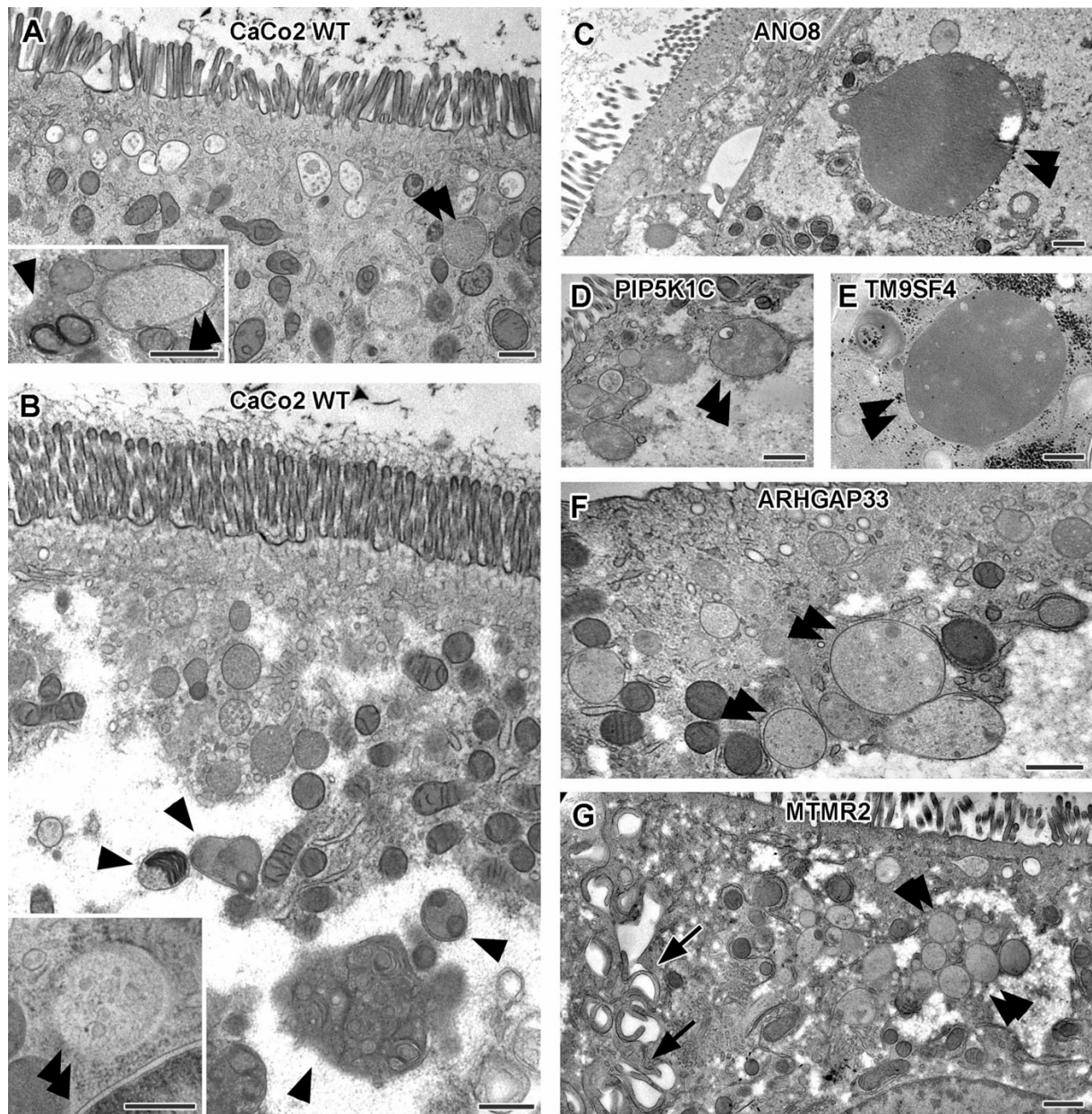
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**Figure 4. Electron micrographs with various configurations of abundant ectopic microvilli reflect polarity defects in 18-day-old 2D cultures of selected CaCo2 knock-down (KD) cell lines. Scale = 1  $\mu$ m. A.** Distinct intracellular lumen, lined by ectopic brush border and adjacent terminal web, thus, a typical microvillus inclusion (arrow) inside a PIP5K1C-KD cell. Arrow-heads mark lysosomes. **B.** Spherical intracellular mass of tangled microvilli associated with ectopic terminal web (arrow) inside a GALNT2-KD cell. Double arrow-heads mark enlarged late endocytic organelles. **C.** Paracellular, basolateral spot of densely packed microvilli plus associated ectopic terminal web (arrow) in polarized TM9SF4-KD-cell culture. **D.** Numerous, slightly bent microvilli facing widened intercellular space (arrows) in polarized ANO8-KD cell culture. **E.** Paracellular microvillar spot with adjacent ectopic terminal web (arrow) and associated cell junctions (double arrows) in polarized TMEM110-KD cell culture. Arrow-heads mark lysosomes, double arrow head marks a poorly structured late endocytic organelle.



307 In contrast to CaCo2 WT cells, all KD-cell lines had conspicuous clusters of ectopic microvilli  
308 (Fig. 4 A-E, Fig. S1 A, B). They appeared either as well-organized ectopic brush border and  
309 adjacent ectopic terminal web, lining distinct intracellular lumina (i.e., typical microvillus  
310 inclusions) (Fig. 4 A) or as less complex inclusions filled with intertwined masses of long,  
311 curved microvilli (Fig. 4 B, Fig. S1 B). Similar configurations occurred basolaterally as  
312 paracellular spots lined by densely packed microvilli (Fig. 4 C, E), frequently associated with  
313 ectopic tight- and adherens-junctions (Fig. 4 E). In addition, numerous long, curved microvilli  
314 were found facing the basolateral intercellular space (Fig. 4 D, Fig. S1 A), often forming  
315 conspicuous cell interdigitations. All these patterns together confirm the highly disturbed  
316 polarity of all KD cell lines studied here.

317 Notably, remarkable ultrastructural alterations also involved late endocytic and catabolic  
318 organelles (Fig. 5). In WT CaCo2 cells the different types and/or maturation stages of  
319 multivesicular bodies (MVBs) and lysosomes, as well as some autophagic organelles,  
320 appeared normal in size, abundance and morphology (Fig. 5 A and B) and resembled patterns  
321 previously mapped for other cryo-fixed mammalian cell lines (e.g., MEF, HeLa: (Vogel et al.,  
322 2015a; Yordanov et al., 2019; Hess and Huber, 2021)). However, in all KD-lines, the late  
323 endosomal/lysosomal endomembrane system was dominated by partly huge, spherical  
324 organelles (in addition to normal MVBs) – at the expense of normal lysosomes (Fig. 4 B and  
325 E; Fig. 5 C-G: double arrow-heads). These large compartments had either weakly stained,  
326 homogeneous granular contents with few intraluminal elements (Fig. 4 B and E; Fig. 5 C-G),  
327 or different amounts of partially degraded material. According to ultrastructural criteria, we  
328 tentatively interpreted these poorly structured, faint compartments as types of peculiar  
329 endolysosomes or (autophago)lysosomes (Bright et al., 2016; Fujita et al., 1990; Remis et al.,  
330 2014). Their size and frequency varied throughout the different KD-lines. Especially in  
331 TM9SF4-, ANO8- and ARHGAP33-KD they reached dimensions of up to 2x6  $\mu\text{m}$  in diameter  
332 (e.g., Fig. 5 C), in other KD cell lines only diameters of approx. 500nm. In CaCo2 WT cells we  
333 sporadically found this type of organelle as well, but here they had rather normal dimensions  
334 (Fig. 5 A and B: double arrow-heads).



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**Figure 5. Ultrastructure of late endocytic and lysosomal organelles in CaCo2 wildtype (WT) cells versus selected knock-down (KD) cell lines of cryo-fixed 18-day-old polarized filter cultures under steady state conditions. Scale = 500 nm. A,B. WT CaCo2 cells with normal endosomes and lysosomes: multi-vesicular bodies (MVBs) with varying contents (regarding intraluminal vesicle (ILV) number, size and staining patterns), different types of (autophago)lysosomes (arrow-heads), all filled with clearly stained, finely granular material plus membrane remnants (i.e., multilamellar bodies) and/or opaque, amorphous residues (i.e., dense-core lysosomes). Double arrow-heads mark rare examples of inconspicuous spherical organelles with weakly stained, quite homogeneous granular contents harboring only sporadic ILVs and/or other structured components, interpreted as kind of endolysosome (Bright et al., 2016). C. Large, poorly structured putative endolysosome (double arrow-head), practically the only type of late endocytic and/or catabolic organelles in ANO8 KD under steady state. D.**

348 Moderately sized and slightly enlarged putative endolysosomes (double arrow heads)  
349 occurring together with normal lysosomes in PIP5K1C KD cells. **E.** Enlarged putative  
350 endolysosome (arrow-head), the predominant type of late endocytic/catabolic organelles in  
351 TM9SF4 KD cells. **F.** Moderately enlarged putative endolysosomes (double arrow heads)  
352 occurring together with a few normal lysosomes in ARHGAP33 KD cells. **G.** Moderately  
353 enlarged putative endolysosomes (double arrow heads) occurring together with a few normal  
354 lysosomes in MTMR2 KD cells. Arrows mark lateral microvilli.

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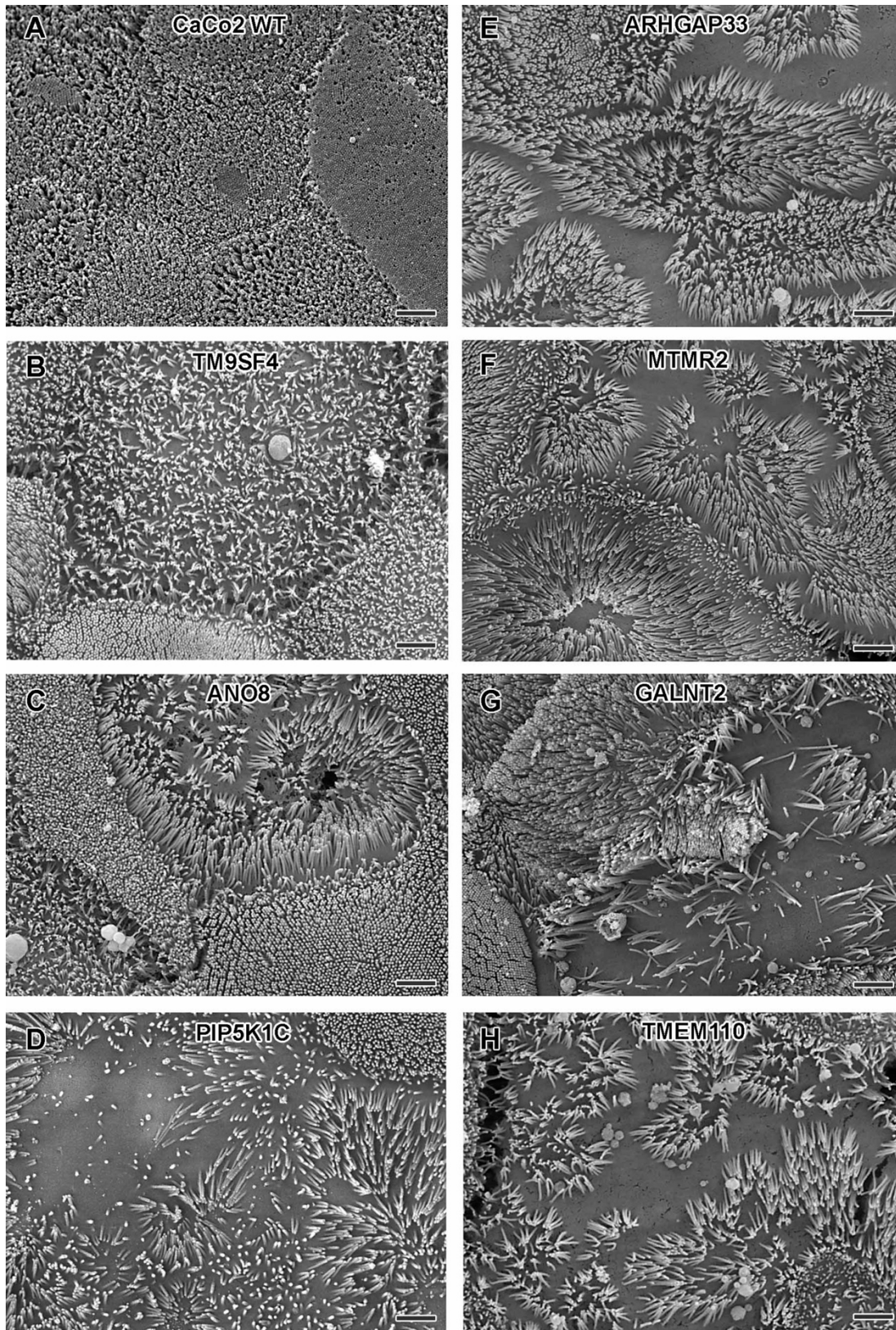
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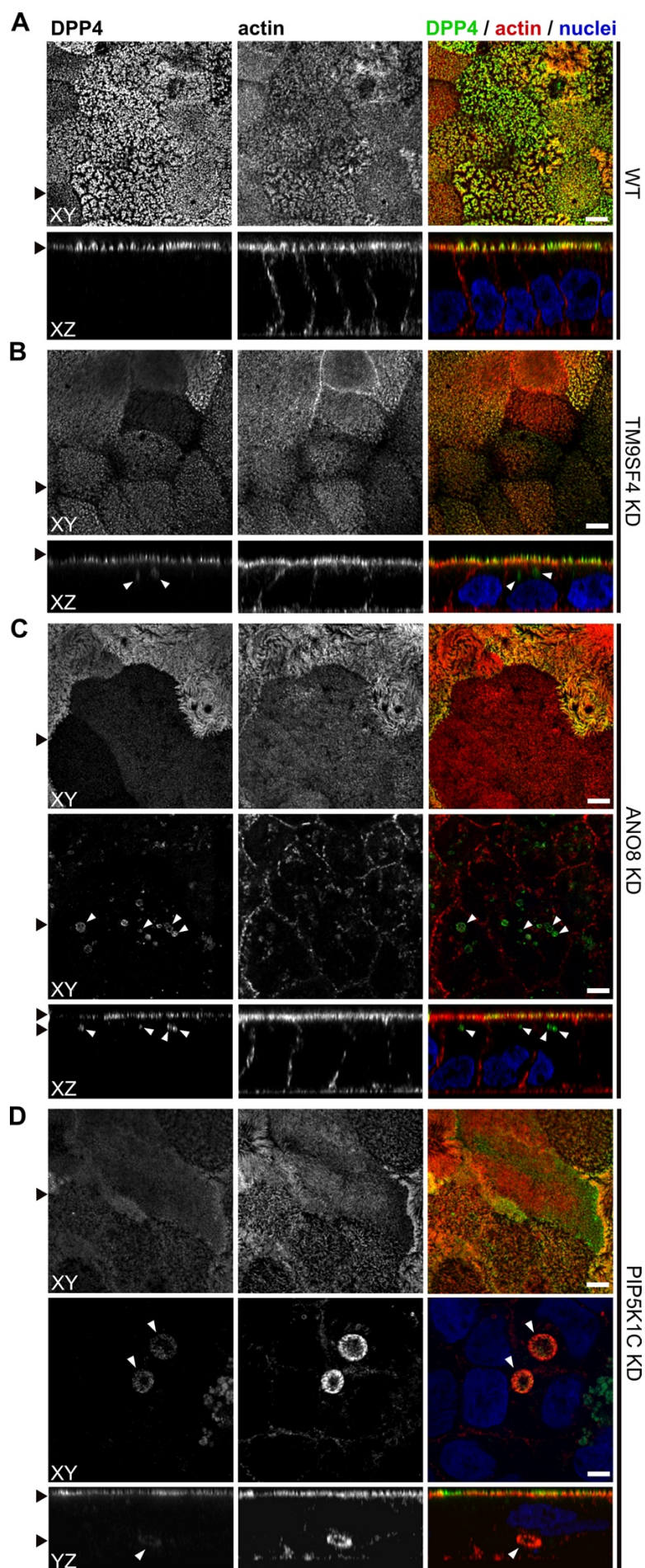
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364 **Figure 6. Scanning EM surface views on apical microvilli of 18 day-old polarized CaCo2**  
365 **wildtype (WT) cells versus knock-down (KD) cells. Scale = 2  $\mu$ m. A.** CaCo2 WT cells with  
366 dense, quite uniform brush border. **B-H.** Patchy distribution of partly abnormal microvilli  
367 characterize the apical surface of all KD cell lines specified here.

368 Regarding the general architecture of the brush border, scanning electron microscopy (SEM)  
369 and actin fluorescence microscopy revealed more or less severe irregularities in all KD-cell  
370 lines studied here (Fig. 6-9). They included patchy distribution or complete absence of apical  
371 microvilli, together with the occurrence of extremely long microvilli (Fig. 6 B to H). We then  
372 combined actin labeling with immunofluorescence microscopy using antibodies against the  
373 apical components DPP4 and stx3 (Fig. 7 A-D; Fig. 8 A-D; Fig. 9 A-H). At first glance, we  
374 detected DPP4 in most CaCo2-KD monolayers predominantly at the apical plasma membrane.  
375 However, more detailed analysis of confocal stacks revealed that DPP4 was also mislocalized  
376 to intracellular structures in ARHGAP33-, TM9SF4-, PIP5K1C-, MTMR2- and ANO8-KD-cell  
377 lines (Fig. 7 B-D; Fig. 9 A and B), although to varying degrees. While DPP4 was mislocalized  
378 to subapical compartments in ARHGAP33-, TM9SF4- and ANO8-KD cell lines, PIP5K1C- and  
379 MTMR2-KDs displayed DPP4 localization to large, actin-rich, basolateral structures,  
380 reminiscent of microvillus inclusions, observed by EM. Consistent with these observations,  
381 stx3 was detected at the apical brush-border microvilli in all KD cell lines (Fig. 8 B-D; Fig. 9 E-  
382 H). However, this was accompanied by additional ectopic localization of stx3 in TM9SF4-,  
383 ARHGAP33-, MTMR2- and PIP5K1C-KDs, with MTMR2- and PIP5K1C-KDs exhibiting stx3-  
384 positive, basolateral inclusion-like compartments (Fig. 8 D and Fig. 9 F), whereas stx3-positive  
385 structures were seen in apical regions in TM9SF4- and ARHGAP33-KD cells (Fig. 8 B and Fig.  
386 9 E). To investigate whether KD of each candidate also affects the junctions and differentiation  
387 of basolateral domains, we stained all cell lines for the apical tight-junction protein claudin-3,  
388 and the basolateral adherens junction protein E-Cadherin (Fig. 10 A-H). Our analyses revealed  
389 a generally normal distribution of those markers in all KD-cell lines. Claudin-3 showed the  
390 characteristic localization pattern, with an enrichment towards the apical domain and locally  
391 also lateral membrane distribution and E-Cadherin marking basolateral membrane domains  
392 (Fig. 10 A-H). Interestingly, transepithelial electric resistance (TEER) measurements of filter-  
393 grown, polarized 2D monolayers of WT and KD-cell lines revealed an increase of TEER after  
394 measurement-day 7 upon KD of ARHGAP33, while TEER of the other KD-cell lines remained  
395 comparable to those of WT cells (Fig. 10 I).



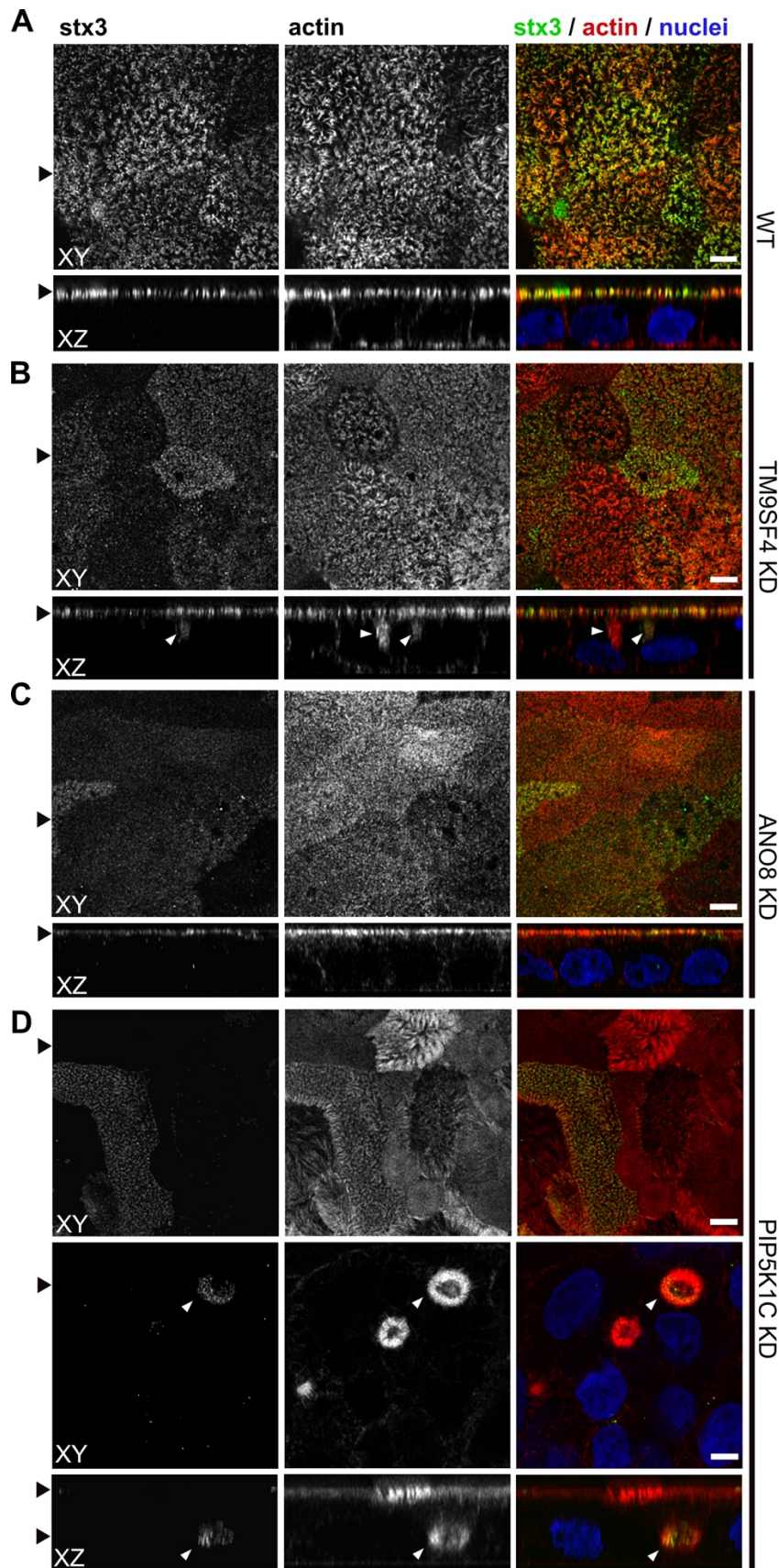
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397 **Figure 7. Confocal micrographs of DPP4 and actin immunofluorescence staining from**  
398 **wildtype (WT) and respective knock-down (KD) cell lines. A.** DPP4 localization is restricted  
399 to the actin-rich microvillus brush-border in WT cells. **B, C.** DPP4 can still be targeted to the  
400 apical plasma membrane, but also mislocalizes to subapical compartments in TM9SF4- (B)  
401 and ANO8-KD (C) cells (white arrowheads). **D.** PIP5K1C-KD cell lines display large, DPP4-  
402 and actin-positive, basolateral compartments (white arrowheads). XY = top view of polarized  
403 monolayer; XZ/YZ = lateral view of polarized monolayer. Scale = 5 $\mu$ m.

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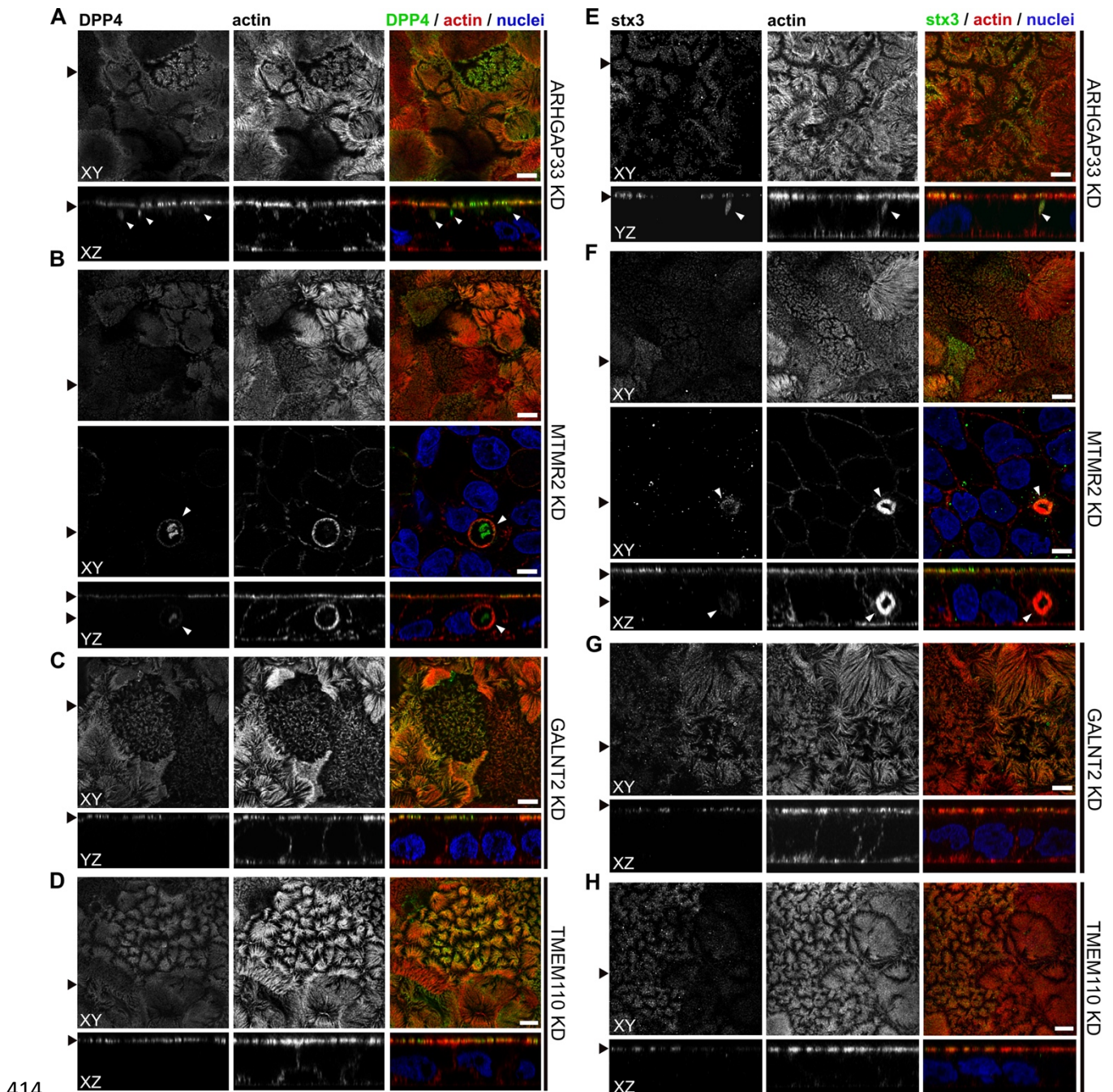
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406 **Figure 8. Confocal micrographs of syntaxin-3 (stx3) and actin immunofluorescence**  
407 **staining from wildtype (WT) and respective knock-down (KD) cell lines. A. stx3 localizes**  
408 **strictly to the apical plasma membrane in WT cells. B. stx3 aberrantly localizes to a subapical**



409 compartment in TM9SF4-KD cells. **C.** ANO8-KD cell lines display apical localization of stx3. **D.**  
410 stx3 mislocalizes to large, basolateral, actin-rich compartments in PIP5K1C-KD cells,  
411 reminiscent of microvillus inclusions. XY = top view of polarized monolayer; XZ/YZ = lateral  
412 view of polarized monolayer. Scale = 5µm.

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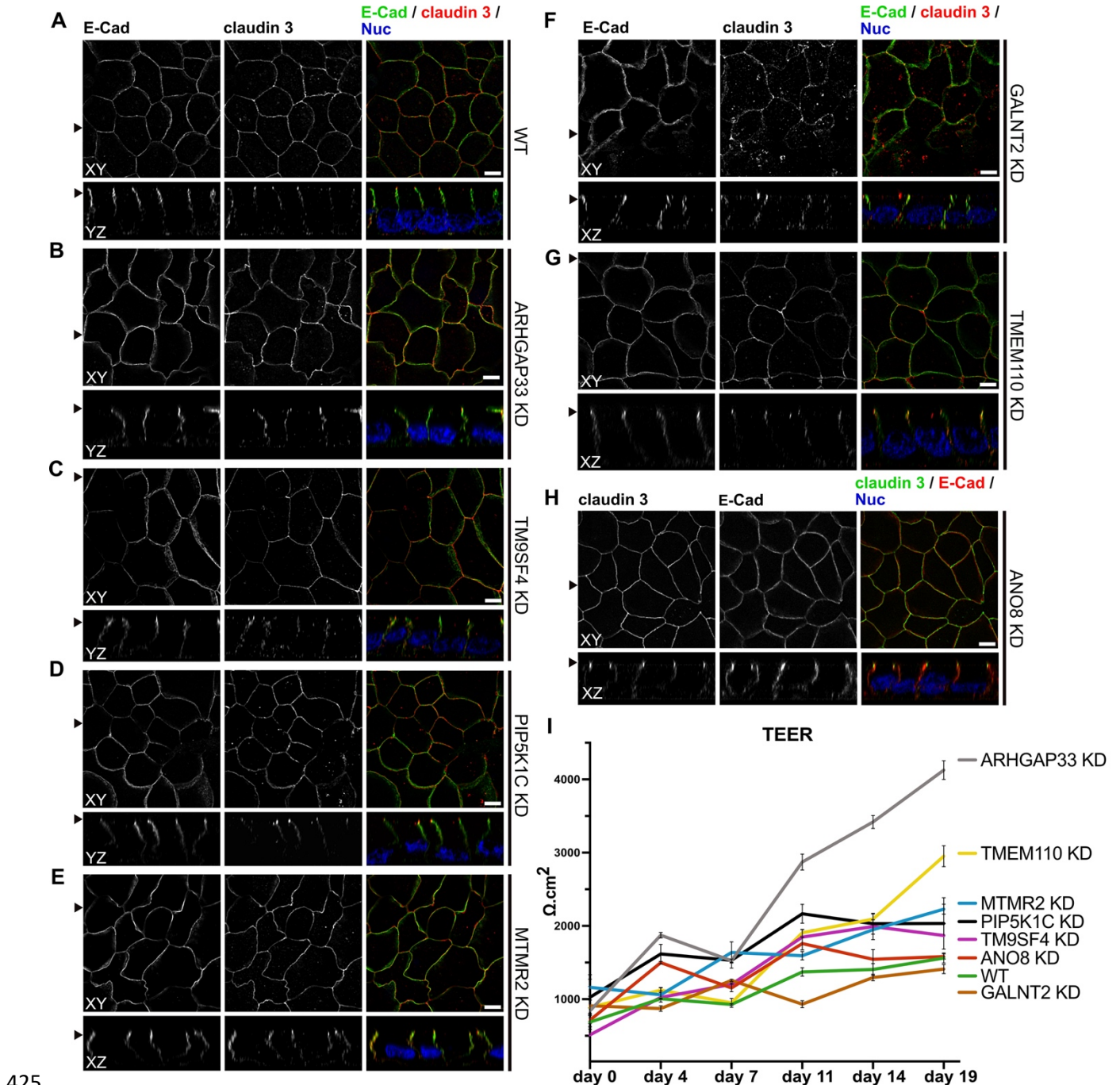


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415 **Figure 9. Confocal micrographs of DPP4 (A-D) and stx3 (E-H) together with actin**  
416 **immunofluorescence staining of knock-down (KD) cell lines. A.** DPP4 mislocalizes to  
417 subapical compartments in ARHGAP33-KD cells (white arrowheads). **B.** MTMR2-KD cell lines  
418 display large, actin rich basolateral compartments, that also show DPP4 (white arrowheads). **C-**  
419 **D.** In GALNT2- and TMEM110-KD cells, DPP4 localizes strictly to the apical plasma membrane.

420 **E.** stx3 localizes to a subapical compartment in ARHGAP33-KD cells. **F.** stx3 mislocalizes to  
 421 large, basolateral actin-rich compartments in MTMR2-KD cells. **G-H.** GALNT2- and TMEM110-  
 422 KD cell lines display apical localization of stx3. XY = top view of polarized monolayer; XZ/YZ =  
 423 lateral view of polarized monolayer. Scale = 5µm.

424



425

426 **Figure 10. Confocal micrographs of E-Cadherin and claudin 3 immunofluorescence**  
 427 **staining from wildtype (WT) and respective knock-down (KD) cell lines. A.** Localization of  
 428 E-Cadherin and claudin 3 in WT cells, where E-Cadherin is distributed over the basolateral  
 429 plasma membrane and claudin 3 is enriched at apical domains, but can also be found at



430 basolateral membrane regions. **B-H.** ARHGAP33-, TM9SF4-, PIP5K1C-, MTMR2-, GALNT2-,  
431 TMEM110- and ANO8-KD cell lines show basolateral E-Cadherin localization as well as  
432 claudin 3 enrichment at apical and lateral domains, similar to WT cells (A). Scale = 5  $\mu$ m. **I.**  
433 TEER-measurements of WT and respective KD clones. TEER of ARHGAP33-KD cells  
434 increases substantially around day 7 of the measurement. Measurements are depicted as  
435 means with standard-deviation. XY= top view of polarized monolayer; XZ/YZ= lateral view of  
436 polarized monolayer

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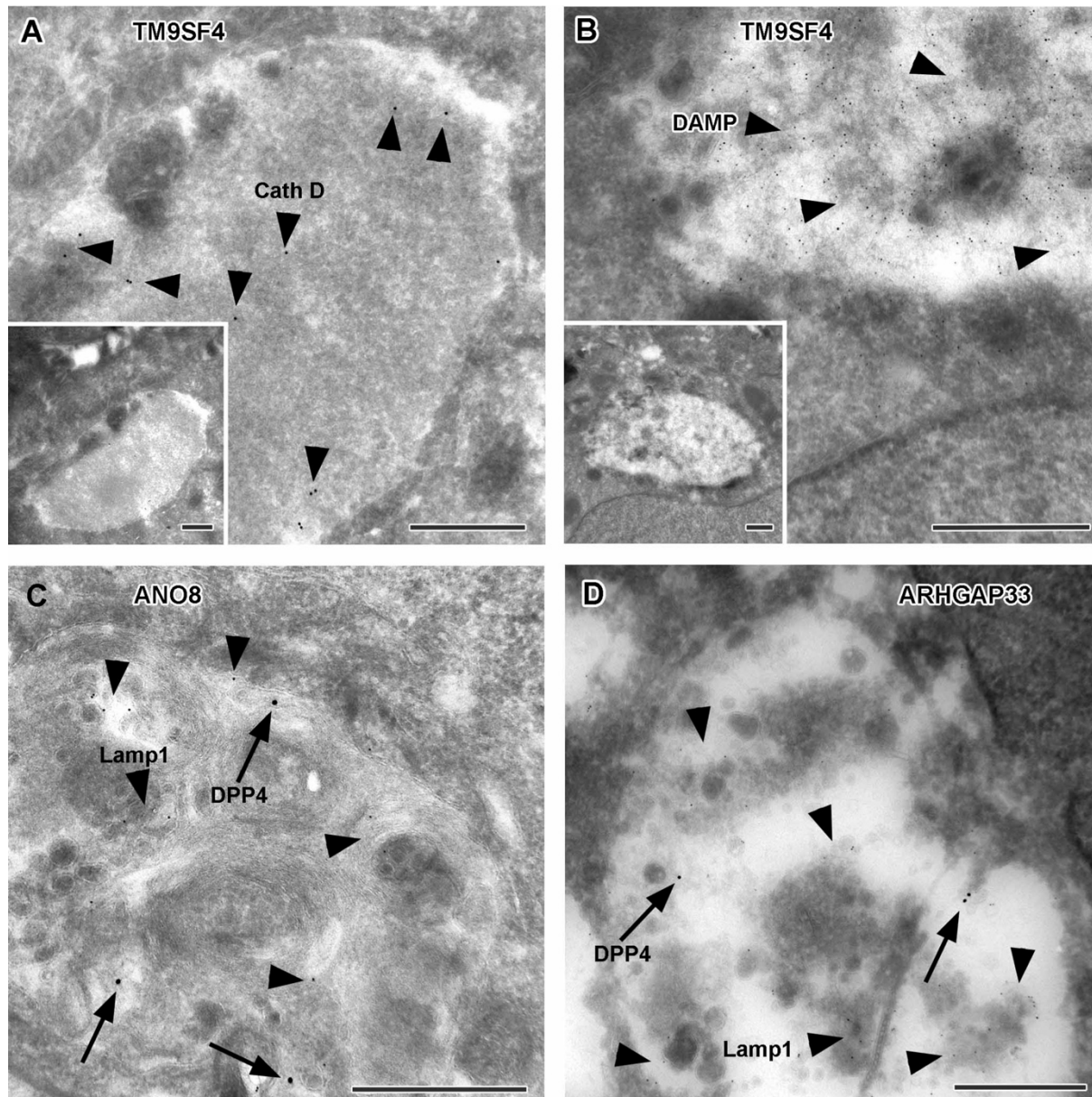
439 Taken together, our analyses have so far provided several indications of polarity defects after  
440 elimination of the selected candidates (Table 1): In a 3D-polarization assay, all KD-cell lines  
441 showed severe defects in forming normal cysts with a single, central lumen but rather  
442 generated multiple lumina or no lumina at all. Additionally, TM9SF4-, ANO8- and MTMR2-KD-  
443 cell lines showed intracellular mislocalization of the apical marker DPP4 in 3D cysts.  
444 Transmission EM of polarized 2D monolayers revealed that KD of all candidates induced  
445 ectopic intra- and paracellular clusters of microvilli, reminiscent of typical microvillus inclusions,  
446 and SEM complemented these findings with observed alterations in apical brush-border  
447 formation in all generated KD cell lines. Immunofluorescence micrographs finally confirmed  
448 these observations and additionally indicated partial mislocalization of apical proteins (Fig. 13  
449 B).

450 ***Apical markers mislocalize in enlarged, degradative compartments of TM9SF4-,***  
451 ***ARHGAP33- and ANO8-KD cells***

452 To further complement these results, we used single and double immunogold labeling primarily  
453 to characterize the abnormally large, usually poorly structured organelles in TM9SF4, ANO8,  
454 and ARHGAP33 KDs and to evaluate their possible association with the abnormal intracellular  
455 DPP4 staining in immunofluorescence (Fig. 11 A-G). Membrane or contents of those  
456 compartments showed distinct Lamp1, Lamp3 or CathepsinD immunogold label, respectively,  
457 in all 3 KD-cell lines (Fig. 11 A, C and D). These findings were also consistent with Lamp1  
458 immunofluorescence micrographs (Fig. 12 A-D). Successful immunogold detection of the  
459 previously internalized acidotropic reagent DAMP provided further evidence for the clearly  
460 acidic nature of those organelles, justifying their classification as species of modified  
461 endolysosomes/(autophago)lysosomes (Fig. 11 B, Fig. S1 C and D). They regularly contained  
462 mislocalized DPP4 (Fig. 11 C and D; Fig. S1 C, D). Moderate, but distinct, aberrant stx3 label  
463 that was not detectable by immunofluorescence was also observed in these organelles – in  
464 addition to normal apical localization and ectopic localization of stx3 and DPP4 at microvillus  
465 inclusions and paracellular microvillar spots (Fig. S1 I and J). Further insight into the late

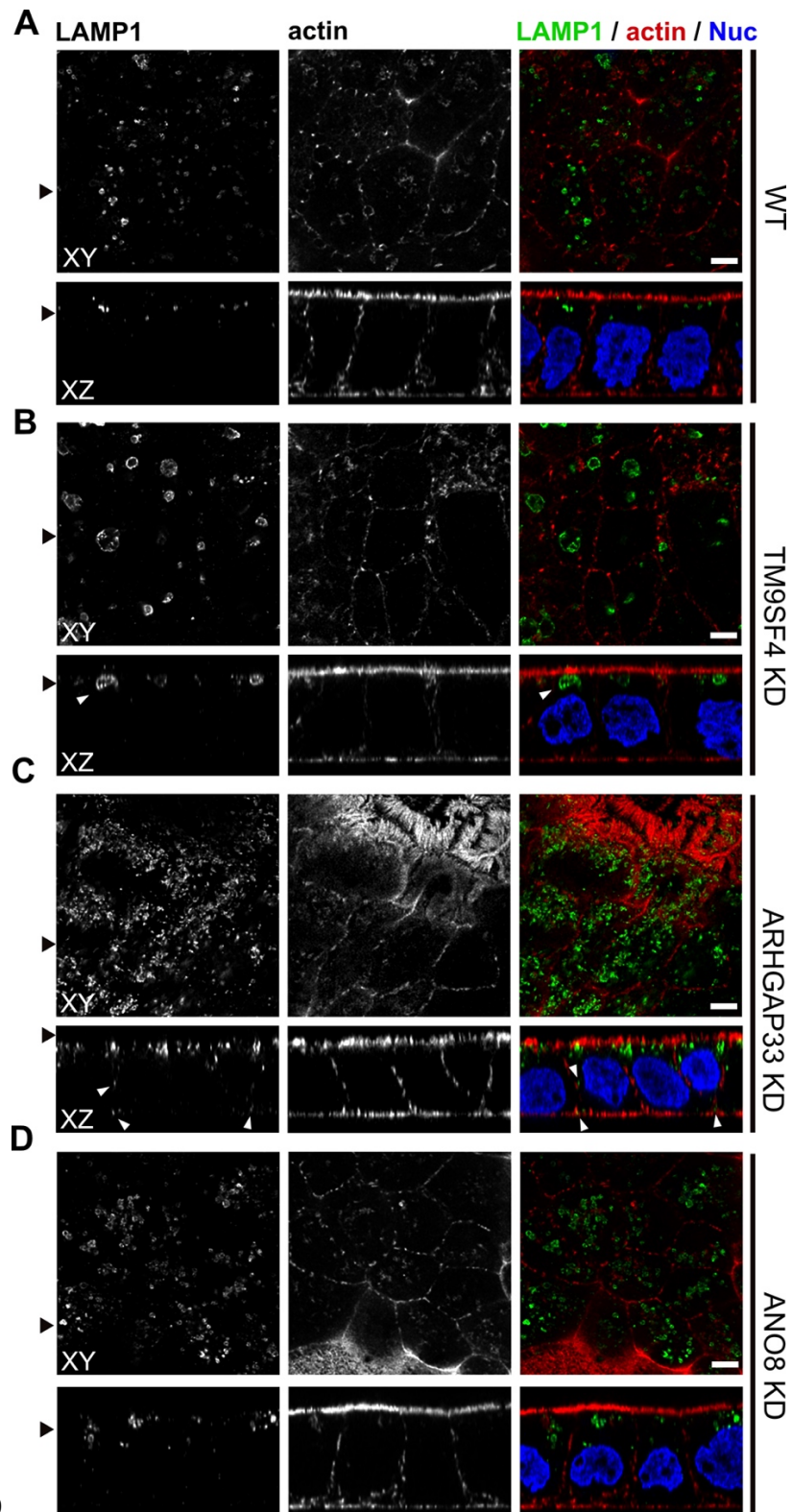
466 endocytic/catabolic endomembrane system of TM9SF4-, ANO8- and ARHGAP33-KD cells  
467 was obtained from starvation experiments. After serum deprivation overnight almost all the  
468 giant, poorly structured, faint compartments had disappeared, likely due to autophagic  
469 removal, and reformed lysosomes of normal size and morphology were regularly observed  
470 (Fig. S1 E-H).

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473 **Figure 11. Immunoelectron microscopy of late endocytic and catabolic organelles in**  
474 **selected CaCo2 knock-down (KD) cells. Scale = 500 nm. A,B.** Enlarged, poorly structured  
475 endolysosomes in TM9SF4 KD cells showing distinct cathepsin D and DAMP-immunogold  
476 label (arrow heads) indicative of their acidic contents. Inserts show respective overviews of the  
477 depicted organelles. **C,D.** Mislocalized DPP4 (arrows) colocalizing with LAMP1 (arrow heads)  
478 in enlarged compartments in ANO8 and ARHGAP33-KD cells.



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**Figure 12. Confocal micrographs of Lamp1 immunofluorescence staining from wildtype (WT) and respective knock-down (KD) cell lines. A-D.** The Lamp1-positive compartments appear enlarged upon KD of TM9SF4 (B) and localize to basolateral regions upon KD of ARHGAP33 (C) (white arrowheads). Scale = 5  $\mu$ m.

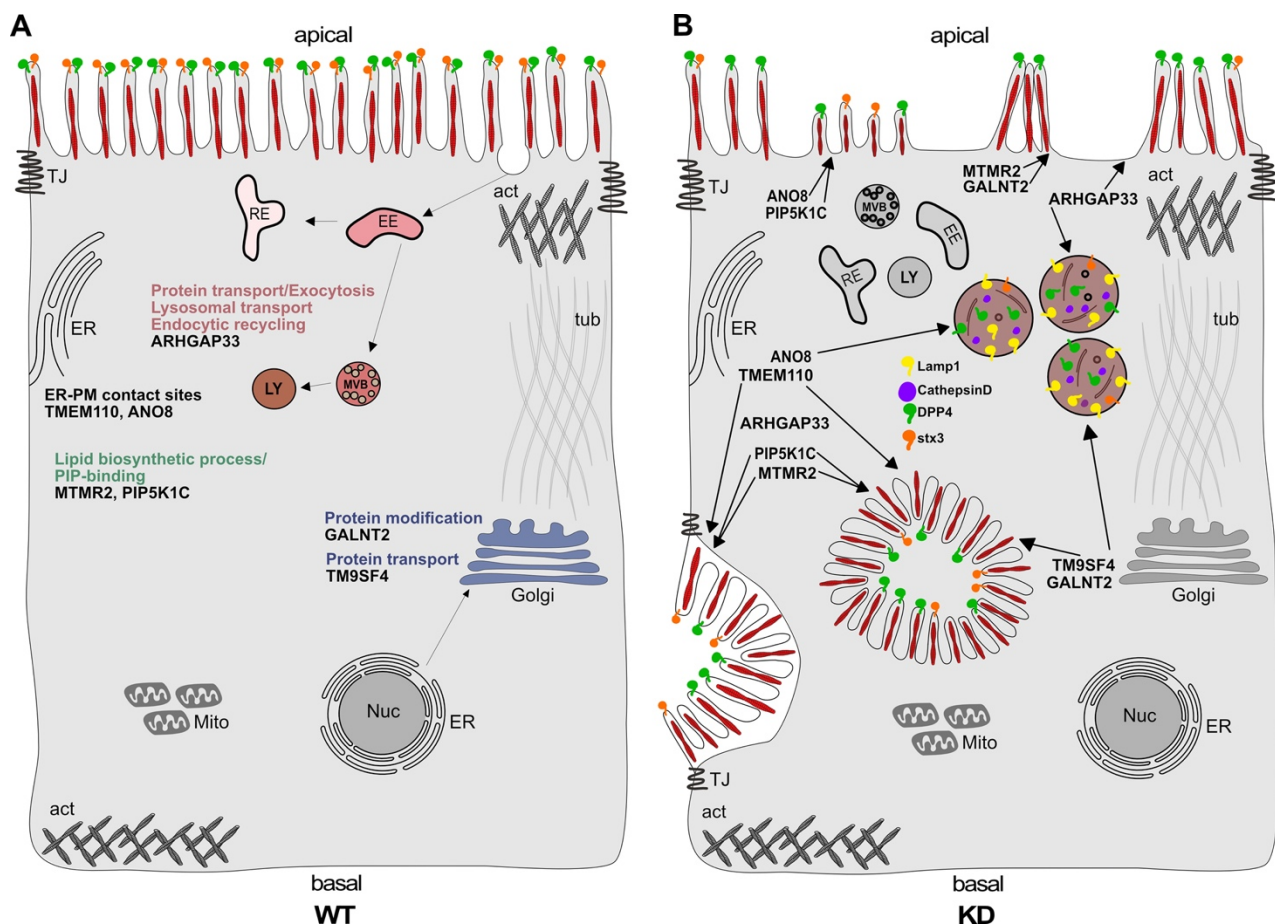


485 Finally, ZO-1 and E-cadherin immunogold labeling allowed us to verify our ultrastructural  
486 observations of ectopic tight and adherens junctions associated with paracellular clusters of  
487 microvilli, that were not seen in lower resolution immunofluorescence micrographs (Fig. S1 K  
488 and L).

489 In summary, our analyses showed that in addition to defects in cell polarization, KDs of all  
490 candidates lead to basolateral mislocalization of apical cargo (Table 1). In particular, we  
491 observed localization of DPP4 and stx3, to enlarged, endolysosomal/lysosomal compartments,  
492 as shown by immunogold labeling of Lamp1, Lamp3, and CathepsinD (Fig. 13 B; Table 1).  
493 Moreover, these enlarged compartments were always capable of acidification, and apparently  
494 also autophagic degradation and lysosomal reformation. Thus, our observations suggest that  
495 KD of the factors studied, TM9SF4, ANO8 and ARHGAP33, leads to aberrant traffic of apical  
496 cargo proteins.

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500 **Figure 13. Simplified scheme of the phenotypes observed upon knock-down (KD) of**  
501 **ARHGAP33, TM9SF4, PIP5K1C, MTMR2, GALNT2, ANO8 and TMEM110. A.** Scheme of a  
502 healthy enterocyte. The investigated factors for screen validation and further phenotypic  
503 characterization are displayed together with their associated GO-terms. **B.** KDs of ARHGAP33,  
504 TM9SF4, PIP5K1C, MTMR2, GALNT2, ANO8 and TMEM110 lead to the formation of

505 microvillus inclusions and lateral pseudo-apical domains with microvilli. KD of ARHGAP33,  
 506 TM9SF4 and ANO8 leads to the formation of enlarged late endosomal/lysosomal  
 507 compartments positive for Lamp1 and Cathepsin-D and that contain the apical markers DPP4  
 508 and stx3. All KDs additionally lead to aberrant, “tipi-like” assemblies of apical microvilli.

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512 **Table 1. Synopsis of phenotype characteristics of CaCo-WT versus TM9SF4-, ANO8-,**  
 513 **PIP5K1C-, ARHGAP333-, MTMR2-, TMEM110-KD cells**

**Phenotype characteristics of CaCo-WT versus TM9SF4-, ANO8-, PIP5K1C-, ARHGAP333-, MTMR2-, TMEM110-KD cells**

		CaCo2-WT	TM9SF4-KD	ANO8-KD	PIP5K1C-KD	ARHGAP33-KD	MTMR2-KD	GALNT2-KD	TMEM110-KD
FACS	DPP4 intensity	100%	50%	70%	25%	70%	70%	50%	50%
IF: 3D	Cyst lumen	single	multiple	multiple	multiple	multiple	multiple	multiple	multiple
IF: 3D	DPP4	Brush border (BB)	BB, intracellular dots	BB, intracellular dots	BB	BB	BB	BB, intracellular dots	BB
IF: 2D	DPP4	BB	BB, subapical dots	BB, subapical dots	BB, basolateral MVI/par MV	BB, subapical dots	BB, basolateral MVI/par MV	BB	BB
IF: 2D	stx3	BB	BB, subapical dots	BB	BB, basolateral MVI/par MV	BB, subapical dots	BB, basolateral MVI/par MV	BB	BB
IF: 2D	LAMP1	normal subapical organelles	huge subapical organelles	large subapical organelles	<i>n.d.</i>	subapical & basolateral organelles	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>
SEM: 2D	Apical microvilli	dense BB, uniform	± patchy BB, partly irregular size/shape	± patchy BB, partly irregular size/shape	± patchy BB, partly irregular size/shape	± patchy BB, partly irregular size/shape	± patchy BB, partly irregular size/shape	± patchy BB, partly irregular size/shape	± patchy BB, partly irregular size/shape
TEM: 2D	Ectopic microvilli	negligible	MVI, par MV, lat MV	MVI, par MV, lat MV	MVI, par MV, lat MV	MVI, par MV, lat MV	MVI, par MV, lat MV	MVI, par MV, lat MV	MVI, par MV, lat MV
IEM: 2D	Ectopic junctions: ZO-1, E-cadherin	<i>n.d.</i>	par MV	par MV	<i>n.d.</i>	par MV	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>
TEM: 2D	Lysosomes (LY)	typical, predominant	typical, very rare	typical, very rare	typical, frequent	typical, rare	typical, moderate	typical, predominant	typical, predominant
TEM: 2D	Endolysosomes (ELY)	normal-sized, rare; Lamp1 & 3, CathD, DAMP	huge, abundant; Lamp1 & 3, CathD, DAMP	huge, abundant; Lamp1 & 3, CathD, DAMP	enlarged, frequent; <i>IEM: n.d.</i>	enlarged, frequent; Lamp1 & 3, CathD, DAMP	enlarged, frequent; <i>IEM: n.d.</i>	normal-sized, moderate; <i>IEM: n.d.</i>	normal-sized, moderate; <i>IEM: n.d.</i>
IEM: 2D	DPP4	BB	BB, ELY, MVI, par MV	BB, ELY, MVI, par MV	<i>n.d.</i>	BB, ELY, MVI, par MV	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>
IEM: 2D	stx3	BB	BB, MVI, par MV (ELY)	BB, MVI, par MV (ELY)	<i>n.d.</i>	BB, MVI, par MV (ELY)	<i>n.d.</i>	<i>n.d.</i>	<i>n.d.</i>

**Abbreviations:** BB= brush border, CathD=Cathepsin D, ELY=endolysosomes, LY=lysosomes, MVI=microvillus inclusions, lat MV= lateral microvillar assemblies, par MV=paracellular microvillar clusters, n.d.=not done, IEM=immuno-EM, SEM=Scanning EM, TEM=Transmission EM

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## 523 **Discussion**

524 Coordination of molecular polarization and transport machineries in concert with polarized  
525 cargo sorting mechanisms is key to epithelial tissue homeostasis. Numerous studies have  
526 contributed to our understanding of these processes in the past (Bryant et al., 2010; Levic and  
527 Bagnat, 2021; Apodaca et al., 2012). Our previous findings on the mechanisms underlying  
528 MVID have elucidated the role of a myo5b-stx3-munc-18-2-dependent trafficking cascade for  
529 apical, actin-based cargo delivery; they also suggest the presence of additional trafficking  
530 routes and transport mechanisms that direct protein secretion to the apical cortex (Vogel et al.,  
531 2015b, 2017a; b), which have not been elucidated to date. However, technical advances in  
532 CRISPR-technology, particularly the development of CRISPR-screening strategies, have  
533 paved the way for the discovery of protein functions for a wide range of cellular processes  
534 (Popa et al., 2020; Hutter et al., 2020; Zhu et al., 2021). In particular, CRISPR-mediated loss  
535 of function screens have proven to be highly efficient in discovering novel factors for  
536 intracellular protein transport and secretory trafficking (Stewart et al., 2017; Bassaganyas et  
537 al., 2019).

538 Here we performed the first CRISPR-Cas9 loss of function screen in polarized human epithelial  
539 cells to identify novel regulators of epithelial polarization and polarized membrane trafficking.  
540 We developed a FACS-based assay for the detection of endogenous plasma membrane  
541 cargo, which is easy to apply and can be adapted to a variety of transmembrane proteins,  
542 given that specific antibodies are available. For our purposes, we used this assay in  
543 combination with the highly efficient CRISPR screening system to study genes involved in  
544 plasma membrane targeting of the apical model cargo DPP4. Furthermore, it seems obvious  
545 and feasible to adapt this FACS-based assay for other types of apical transmembrane cargo,  
546 thus expanding the range of data that can be obtained with whole-genome screenings. Our  
547 CRISPR-screen in polarized enterocytes identified 89 genes, critically involved in apical  
548 targeting of our model cargo, DPP4. This rather moderate number of enriched genes resulted  
549 from the high stringency in the screening assay, namely sorting for cells with a quite drastic  
550 reduction of surface DPP4 (90%). Even though this allowed to enrich for cells with a high gRNA  
551 targeting efficiency and increased the specificity of our screen, we thereby also limited our  
552 approach in terms of quantity and diversity of the identified hits.

553 Our experimental approach, combined with GO analysis of the 89 hits, highlighted several  
554 genes with functions associated to the secretory pathway. To demonstrate the validity of our  
555 dataset, we selected 7 factors for phenotypic and morphological characterization, focusing,  
556 mainly on organelles associated with protein transport.

557 We demonstrated that KD of all selected candidates causes disturbed epithelial polarization.  
558 This was demonstrated by 3D cyst assays and EM analyses of filter-grown, polarized 2D



559 monolayers, where we detected the formation of ectopic, intracellular and paracellular clusters  
560 of microvilli. This phenotype was particularly pronounced in KD cells of PIP5K1C and MTMR2,  
561 where the localization of the apical components DPP4, stx3 but also ZO-1 in the intracellular  
562 and paracellular microvillar clusters indicated the formation of ectopic neo-/pseudo-apical  
563 domains. This highlights yet uncharacterized regulators for epithelial polarization and proposes  
564 potentially novel mechanisms for this process. PIP5K1C and MTMR2 are involved in the  
565 regulation of PIP-pools accounting for apical PM composition and hence we propose  
566 imbalances in cellular PIP-pools as basis for the observed phenotypes. Interestingly, MTMR2  
567 and PIP5K1C mRNAs were shown to be expressed mostly in polarized 2D-cultures, and are  
568 downregulated in 3D cysts of MDCK cells, suggesting differential PIP-regulation in 2D  
569 polarized monolayers and polarization 'de-novo' (Román-Fernández et al., 2018). In contrast,  
570 our data suggests a role for both enzymes in polarization of 3D cultures as well, as we observe  
571 aberrant lumen formation upon KDs.

572 Furthermore, the diversity of signals and determinants that coordinate the formation of  
573 specialized membrane domains is illustrated by the different functions with which the  
574 candidates selected here are associated. The ER-PM contact site proteins ANO8 and  
575 TMEM110 might regulate polarization via the control of Ca<sup>2+</sup> influx and signaling (Jha et al.,  
576 2019; Quintana et al., 2015), while TM9SF4, which has been implicated in the regulation of  
577 glycolipids in the Golgi apparatus (Perrin et al., 2015) and VH-ATPase assembly (Lozupone  
578 et al., 2015), possibly controls polarization through generation of lipid microdomains and pH-  
579 regulation. GALNT2 might contribute to establishing polarity via its role as O-glycosylating  
580 enzyme in the Golgi apparatus and ARHGAP33, as a GAP-protein for the small GTPase  
581 Cdc42, might itself be critically involved in polarization and polarized traffic as well (Nakazawa  
582 et al., 2016). Here, elevated TEER-levels upon KD of ARHGAP33 are further evidence of  
583 fundamentally disturbed epithelial polarity, supporting this hypothesis.

584 Features indicating disturbed polarization, were accompanied by the formation of conspicuous,  
585 enlarged Lamp1, Lamp3 and Cathepsin-D-positive endolysosomal/lysosomal structures, upon  
586 KD of all cell lines, that were additionally positive for DPP4 and stx3 in ANO8-, ARHGAP33-  
587 and TM9SF4-KDs. Notably, these compartments showed functional acidification and could  
588 undergo autophagic degradation followed by lysosomal reformation. Therefore, we propose  
589 that defective polarization upon disruption of one of these genes may be associated with  
590 altered cargo transport and/or sorting of apical cargo into lysosomal compartments by various  
591 cellular mechanisms. Little is known about a potential role for ARHGAP33 in epithelial  
592 polarization, however, it is arguable that a role in modulating Cdc42 might account for the  
593 observed polarity defect and the mislocalization/mistrafficking of apical cargo to late-  
594 endosomes/lysosomes (Nakazawa et al., 2016; Schuster et al., 2015). The Golgi apparatus is  
595 believed to be a major hub for sorting events of secreted cargo proteins and many signals and

596 mechanisms have been proposed to be major Golgi-associated sorting determinants (Weisz  
597 and Rodriguez-Boulan, 2009; Rodriguez-Boulan et al., 2005). Ca<sup>2+</sup> levels in the Golgi  
598 apparatus, for example, were shown to regulate apical sorting of GPI-anchored proteins in  
599 polarized epithelial cells (Lebreton et al., 2021). Thus, mislocalization of DPP4 in ANO8-KDs  
600 to endosomal/lysosomal compartments could result from aberrant, Ca<sup>2+</sup> dependent sorting in  
601 the Golgi apparatus. Finally, lipid microdomains and pH-regulation represent major sorting  
602 determinants of apical cargo in the Golgi (Hallermann, 2014; Medina et al., 2015; De Araujo et  
603 al., 2017; Schuck and Simons, 2004). Therefore, it is plausible to assume that the observed  
604 mistargeting of apical cargo into lysosomal compartments may coincide with defects in  
605 glycosphingolipid synthesis and/or V-ATPase-mediated pH-regulation, caused by TM9SF4-KD  
606 (Lozupone et al., 2015; Levic et al., 2020; Levic and Bagnat, 2021).

607 It seems noteworthy that cellular Ca<sup>2+</sup> homeostasis, the regulation of intracellular pH, as well  
608 as the synthesis PIP-species and lipid microdomains regulate a variety of processes related  
609 to endocytic recycling of membrane cargo, autophagy, and lysosomal biogenesis (Medina et  
610 al., 2015; Sbano et al., 2017; Hallermann, 2014). Defects in either of these processes could  
611 therefore be responsible for the observed lysosome-related phenotypes, leading either  
612 primarily or secondarily to defective epithelial polarization/secretory traffic.

613 Taken together, our data suggests that the generation of specialized membrane domains and  
614 specialized membrane traffic are regulated by the interplay of several determinants of  
615 polarization, trafficking, and cargo sorting, such as the synthesis of PIP species and  
616 glycosphingolipids, N-/O-linked glycosylations, and changes in cellular Ca<sup>2+</sup> levels or pH  
617 regulation.

618 Because several diseases characterized in the past have been associated with defects in  
619 polarized trafficking and protein missorting, we also screened our dataset for all possible genes  
620 that have been associated with congenital enteropathies. Apart from the association of Unc-  
621 45 Myosin Chaperone A (UNC45A) with syndromal diarrhea and cholestasis, no other genes  
622 identified in our CRISPR screen have been published in this context to date. (Esteve et al.,  
623 2018; Li et al., 2022; Dulcaux-Loras et al., 2022). However, MVID caused by mutations of  
624 *myo5b*, *stx3*, *stxbp2*, or *unc45a* is a prominent example for pathological accumulation of  
625 considerably enlarged autophagosomal and/or lysosomal organelles in the cell periphery.  
626 Despite their abundance, those catabolic organelles apparently suffer from some degree of  
627 overload due to their inability to remove misdirected excess cargo (i.e., "secretory granules")  
628 and efficiently degrade the ectopic apical domains/microvillar structures. Moreover, abnormal  
629 late endosomes/lysosomes with close resemblance to the respective faint, poorly structured  
630 lysosomal compartments in the phenotypes we describe here had recently also been  
631 implicated in another neonatal intestinal disorder, namely, human mucopolidosis type IV. In

632 newly born mice the absence of mucolipin-1 and 3 caused aberrant swelling of those  
633 organelles in enterocytes, diminished apical endocytosis from the intestinal lumen, diarrhea,  
634 and delayed growth (Remis et al., 2014). In our opinion, all those examples underline the  
635 crucial role of proper establishment and maturation of the highly complex system of (late)  
636 endosomal and lysosomal organelles in the small intestine, for example, during the early  
637 neonatal period of mammals, especially the transient, so-called "giant lysosomes" during the  
638 suckling period (Wilson et al., 1991; Fujita et al., 1990). In addition to neonates, this seemingly  
639 broad spectrum of overload and/or accumulation of enlarged catabolic organelles with  
640 enhanced - but insufficient - autophagy and lysosomal inefficiency has also been described  
641 from intestinal disorders of adults, e.g., in patients with necrotizing enterocolitis (Yamoto et al.,  
642 2020).

643 Finally, it should be noted that several genes identified in our screening have been associated  
644 with neuropathies or myopathies (Charcot-Marie-Tooth syndrome (MTMR2); Dyggve-  
645 Melchior-Clausen syndrome (DYM)) (Denais et al., 2011; Wang et al., 2019). Thus, it would  
646 be worthwhile to examine our dataset in the context of other cellular systems, as defective  
647 secretory transport and cellular polarization provide a mechanistic basis for a spectrum of  
648 pathologies in many tissues and organs.

649 In summary, this genome-wide CRIPR/Cas9 screen, together with the extensively described  
650 and illustrated representation of cellular organelle pathologies, provides a very valuable  
651 resource for future investigations aimed at unraveling the complexity and diversity of  
652 mechanisms underlying epithelial polarization and polarized cargo transport. In addition, this  
653 study can contribute to the understanding of many yet-to-be-discovered pathologies  
654 associated with impaired epithelial differentiation, polarization, and integrity and will therefore  
655 serve as a powerful resource for the investigation and characterization of congenital diseases.  
656 (Thiagarajah et al., 2018; Canani et al., 2010).

657

## 658 **Material and Methods**

### 659 *Antibodies and reagents*

660 The following antibodies were commercially obtained and used as stated:

661 Anoctamin 8 (WB 1:500, #HPA049206, Atlas Antibodies), ARHGAP33 (Western-blotting (WB)  
662 1:500, #HPA030117, Atlas Antibodies), beta-Actin (WB 1:2000, #A2228, Sigma-Aldrich),  
663 cathepsin D (EM 1:50, #219361, Calbiochem), CD63/Lamp3 (EM 1:20, #M1544, Sanquin),  
664 claudin 3 (IF 1:200, #SAB4500435, Sigma-Aldrich), DNP (EM 1:6-1:30, #71-3500, Invitrogen),  
665 DPP4 (IF/FACS 1:100, #HBB3/775/42, DSHB; EM 1:10, #AF1180, R&D Systems), E-Cadherin  
666 (IF 1:200, EM 1:30, #610181, BD Biosciences), GALNT2 (WB 1:1000, #AF7507, Novus  
667 Biologicals), Lamp1 (IF 1:200, EM 1:10, #1D4B, DSHB), MTMR2 (WB 1:500, #sc-365184,  
668 Santa Cruz Biotechnology), PIP5K1C (WB 1:1000, #3296S, Cell Signaling Technology),  
669 syntaxin3 (IF 1:100, EM 1:10, #133750, Abcam), TM9SF4 (WB 1:1000, #sc-374473, Santa  
670 Cruz Biotechnology), TMEM110 (WB 1:1000, #NBP1-69238, Novus Biologicals), ZO-1 (EM  
671 1:50, #61-7300 Zymed);

672 Secondary horse-radish peroxidase-coupled (HRP) goat anti-mouse and goat anti-rabbit  
673 (1:5000, Sigma-Aldrich) were used for WB and secondary Alexa Fluor-conjugated (Alexa Fluor  
674 488 and 568) goat anti-mouse (1:1000, Life Technologies), goat anti-rabbit (1:1000, Life  
675 Technologies) were used for IF labelling. For labelling of actin filaments, we used phalloidin-  
676 Alexa Fluor 568 (1:500, Life Technologies) and for nuclear staining we used Hoechst 3342  
677 (1:10,000, Thermo Fisher Scientific). Secondary antibodies conjugated to 5, 6, 10 or 15nm  
678 colloidal gold particles diluted to 1:50-150 for EM were from British Biocell Intl. and Aurion.

### 679 *Plasmids and lentivirus production*

680 For CRISPR/Cas9-mediated depletion, guide RNA (gRNA) targeting sequences for  
681 ARHGAP33 (5'-TCCACCGGTGCATATTTGAC-3'), TM9SF4 (5'-  
682 GCCCAGCAAGATAACCTACA-3'), Pip5k1c (5'- GAAGTTGGGCCATCGAGGTG -3'), Mtmr2  
683 (5'- AGTCGAGGTGAAAATTCTTA -3'), Tmem110 (5'-GAGCAAGGTCCGCTACCGGA-3'),  
684 ANO8 (5'-CCGATGACCACACGCTGCTA-3') and Galnt2 (5'-ACTGCGAGTGTAATGAGCAC-  
685 3') were selected from the Geckov2-CRISPR library, according to their targeting efficiency in  
686 the primary CRISPR-screen (CRISPR Design; Zhang lab, Massachusetts Institute of  
687 Technology; Hsu et al., 2013). gRNAs were cloned into a lentiCRISPRv2 vector via BsmBI  
688 restriction enzyme sites. lentiCRISPRv2 was a gift from F. Zhang (Massachusetts Institute of  
689 Technology, Cambridge, MA; Addgene plasmid 52961; Sanjana et al., 2014). For the  
690 generation of knock-down cell lines, lentiviral plasmids were cotransfected using Lipofectamine  
691 LTX (Invitrogen)-transfection reagent together with pVSV-G and psPAX2 in the Hek293LTV  
692 producer cell line. Virus containing supernatants were collected after 48 and 72 h after

693 transfection and directly used for CaCo2 cell infection. 6 days after infection, cells were  
694 selected with 10 µg/ml puromycin (Sigma-Aldrich) or 20 µg/ml blasticidin S (Invitrogen).  
695 Depletion efficiency was verified via WB.

#### 696 *Cell culture*

697 Hek293LTV, CaCo2 WT, and KI cells were cultured in DMEM (Sigma-Aldrich) containing high  
698 glucose, sodium pyruvate, 100 U/ml penicillin (Sigma-Aldrich), 100 µg/ml streptomycin (Sigma-  
699 Aldrich), 5% nonessential amino acids (Gibco), and 10% FBS (Gibco) in a humidified  
700 atmosphere with 5% CO<sub>2</sub> at 37°C. For experiments requiring fully polarized growth conditions,  
701 CaCo2 cells were seeded on 24-mm or 75-mm filters (Costar Transwell; pore size of 0.4 µm;  
702 Corning) and cultured for 14–28 d. For 3D cyst assays, CaCo2 cells were cultivated and  
703 processed as described previously (Vogel et al., 2015b; Jaffe et al., 2008). For this purpose,  
704 1-2x10<sup>4</sup>/mL single cells were embedded in Matrigel (BD Biosciences, #356231) per well of an  
705 8x chamber slide (Lab-Tek®-chamber slide, Sigma) and grown for 7 days (Román-Fernández  
706 et al., 2018). Three biological and three technical replicates were performed.

#### 707 *TEER measurements*

708 Transepithelial electric resistance (TEER) measurements were performed in CaCo2 wildtype  
709 and the generated KD-cell lines. TEER was measured using an STX2 electrode together with  
710 the EVOM epithelial volt-ohmmeter from World precision instruments. TEER measurements  
711 were performed on days 0, 4, 7, 11, 14, and 19 after cells were seeded on transwell filters at  
712 confluence for polarization. The measurements were performed at three different areas on the  
713 filter inserts and calculated as described previously (Klee et al., 2020). Two biological and one  
714 technical replicate were performed.

#### 715 *Genome-wide CRISPR- screen*

716 For the CRISPR-screen in polarized CaCo2 cells, we used the 2-vector system (lenti-guide  
717 Puro; Addgene #1000000049; Feng Zhang Lab, (Sanjana et al., 2014)). For the generation of  
718 the target CaCo2 cell line, we introduced the vector encoding Cas9 (lentiCas9-Blast) for stable  
719 expression with a lentivirus to CaCo2 cells.

720 For virus production containing the sgRNA library, HEK293T cells seeded to 150mm dishes  
721 were transfected with 21 µg of the human gRNA pooled library in lentiGuide-Puro (Addgene  
722 #1000000049), 15.75 µg of pSPAX2 and 5.25 µg of pVSV-G plasmids. Viral supernatants were  
723 collected after 36 h and 50 h and concentrated with Amicon ultra-15 centrifugation tubes  
724 (Merck). For storage and further usage samples were snap frozen in liquid nitrogen.

725 The CRISPR/Cas9 screen was performed as described in previous studies by (Hutter et al.,  
726 2020; Shalem et al., 2014). One biological and two technical replicates were performed. For



727 each replicate  $2 \times 10^8$  CaCo2-Cas9 screen cells were transduced with a virus preparation  
728 containing 16  $\mu\text{g}/\text{mL}$  polybrene at an MOI of 0.2 and seeded at a low density ( $10^7$  cells/150  
729 mm plate) to 20 150mm culture dishes. 72 hours after infection, selection with 10  $\mu\text{g}$  Puromycin  
730 was started and cells were selected for 7 days. After 7 days of selection, surviving screen cells  
731 were pooled and evenly distributed to 100 mm culture dishes, to obtain confluent monolayers.  
732 The cells were then cultivated for polarization as confluent monolayers for 18 days. After 18  
733 days, cells were detached with StemPro™ Accutase™ Cell Dissociation Reagent (Thermo  
734 Fisher Scientific, #A1110501) and stained for FACS. Thereby cell suspensions were washed  
735 after detachment 2x with ice cold PBS and subsequently incubated in PBS containing 1% FBS  
736 and anti-DPP4 antibody (1:100) on ice for 20 minutes. After incubation cells were washed 2x  
737 in ice cold PBS and then incubated with PBS containing 1% FBS and a secondary anti-mouse  
738 AlexaFluor-488 antibody (1:1000) on ice for 20 minutes. After the incubation, cells were  
739 washed 2x in ice cold PBS, resuspended in PBS containing 2% FBS and subjected to FACS  
740 sorting using an ARIA III (Becton Dickinson). For each replicate, approx. 1.5 Mio cells  
741 corresponding to the lowest 10% Alexa-488 (A-488-negative) of the total cell population was  
742 sorted. Unsorted cells were saved and used as control. Genomic DNA (gDNA) was isolated  
743 from sorted and unsorted cells using the Nucleospin Tissue Mini Kit (Macherey-Nagel) and  
744 sgRNA sequences were retrieved by a nested PCR approach that pre-amplified sgRNAs in a  
745 first round with primers specific to the lentiGuide-Puro construct (5'-  
746 AGAGGGCCTATTTCCCATGA-3') and added stagger bases, specific barcodes and the  
747 Illumina adapters in the second round. The PCR products were separated on a 1% agarose  
748 gel, purified by gel extraction, quantified and then pooled before sequencing on a Hiseq4000  
749 (Illumina) in collaboration with the Biomedical Sequencing Facility (BSF, Vienna, Austria).  
750 Sequencing data were analyzed with the publically available online tools GenePattern  
751 (Chapman et al., 2006) and Galaxy (Afgan et al., 2018). Reads were first demultiplexed and  
752 trimmed followed by alignment of the sgRNA sequences to a reference using Bowtie2  
753 (Langmead and Salzberg, 2012). SgRNAs enriched in the sorted A-488-negative populations  
754 were identified using the edgeR shRNaseq tool (Table CRISPR screen Analysis for Enriched  
755 sgRNAs in the A-488-negative; (Ritchie et al., 2014)).

#### 756 *Flow cytometry*

757 To measure DPP4 levels at the surface of WT and respective KD cell lines, cells were seeded  
758 as confluent monolayers and polarized for 18 days on 6 well cell culture dishes. Subsequently,  
759 cells were detached and stained as mentioned above (*Genome-wide CRISPR-Screen*). After  
760 staining, cells were subjected to measurements at an Attune NxT Flow Cytometer (Invitrogen)  
761 and Data was analyzed with FloJo software (Becton Dickinson). Two biological and one  
762 technical replicate were performed.

763 *Immunoblots*

764 Total cell lysates were prepared and westernblot analysis was performed as described  
765 previously (Cattelani et al., 2021). Cells were washed in 1x cold PBS, scraped from respective  
766 culture-plates and pelleted with 1500xg for 5 min at 4°C. Cell pellets were resuspended in  
767 Lysis buffer (50 mM HEPES pH 8.0, 150 mM NaCl, 5 mM EDTA pH 8.0, 0.5 % NP-40, 50 mM  
768 NaF, 10 µg/mL Leupeptin, 0.4 mM Pefablock, 1 µ/mL Pepstatin, 10 µg/mL Aprotinin, 0.5 mM  
769 PMSF, 1 mM N3VO4) and lysed for 60 minutes on ice. Followingly, lysates were centrifuged  
770 at 13.000xg for 15 minutes and cleared lysate was obtained. Lysates were separated by SDS-  
771 Polyacrylamide Gel Electrophoresis (PAGE). Polyacrylamide gels were prepared consisting  
772 of stacking (125 mM Tris pH 6.8, 4% Acrylamide/Bis solution (37:5:1), 6% glycerol, 0.1% SDS,  
773 0.075% APS, and 0.1% TEMED) and resolving gels (0.375 mM Tris pH 8.8, 7–15%  
774 Acrylamide/Bis solution (37:5:1), 0.1% SDS, 0.05% APS, and 0.05% TEMED). All SDS PAGE  
775 gels were run in 192 mM glycine, 25 mM Trisma Base, 0.1% SDS. After separation, the  
776 proteins were wet transferred onto 'Amersham™ Protran™0.2 µm NC' nitrocellulose  
777 membranes (GE10600002 Sigma-Aldrich Handels GmbH, Vienna, Austria) at constant 80 V  
778 for 1,5 h. The wet transfer buffer contained 25 mM Tris, 192 mM glycine, 0.1% SDS, and 20%  
779 methanol (vol/vol), adjusted to pH 8.3. Membranes were subsequently blocked in 3% BSA  
780 (fraction V), 1 mM EDTA, 0.05% Tween20, and 0.02% NaN3, and probed with the respective  
781 antibodies.

782 *Immunofluorescence microscopy*

783 Immunofluorescence stainings on cells grown and polarized on glass-coverslips or of 3D cyst  
784 cultures were performed as described previously (Vogel et al., 2017b, 2015b). Briefly, for  
785 stainings of polarized 2D monolayers, cells grown on glass coverslips were fixed with  
786 4%formaldehyde (made from paraformaldehyde) at room temperature for 3 hours or 100%  
787 methanol at -20°C for 5 minutes, respectively. Cells stained with anti-DPP4, anti-stx3, anti- HA  
788 and phalloidin, were fixed with formaldehyde, while for stainings with anti-ZO-1, anti-  
789 ECadherin, anti-clau3, anti-NaK-ATPase and anti-Moesin, cells were fixed with methanol.  
790 CaCo2 cysts were prepared for IF microscopy as described previously (Jaffe et al., 2008).  
791 Confocal stacks from monolayers/cysts mounted in Mowiol were taken on confocal  
792 fluorescence microscopes (SP5 and SP8; Leica) using a glycerol 63 x lense with a numerical  
793 aperture of 1.3 (Leica) on a Leica SP5 microscope and a glycerol 93 x lense with a numerical  
794 aperture of 1.3 on the Leica SP8 microscope, at room temperature. As recording softwares  
795 LASAF 2.7.3. (Leica) and LAS X (Leica) were used. All images were deconvolved using  
796 Huygens Professional Deconvolution and Analysis Software (Scientific Volume Imaging) and  
797 exported using Imaris 3D rendering (Bitplane) and finally adjusted for brightness and contrast  
798 using Fiji-ImageJ software.

799 *Electron microscopy*

800 Transmission EM for morphology of filter-grown, polarized monolayers included rapid cryo-  
801 immobilization through means of high-pressure freezing, followed by freeze-substitution and  
802 epoxy resin embedding as described previously (Vogel et al., 2015b; Ruemmele et al., 2010).  
803 Immunogold EM was described previously; in brief, polarized monolayers grown for 14 days  
804 in petri dishes were fixed with 4% formaldehyde or 4% formaldehyde plus 0.1% glutaraldehyde  
805 and processed for Tokuyasu-ultracryotomy (Vogel et al., 2017a; Tokuyasu, 1973). Optionally,  
806 cells were subjected to serum-stravation overnight, followed by incubation for 2 hours with  
807 DAMP ((Orci et al., 1986); 3-(2,4-Dinitroanilino)-3'-amino-N-methylpropyl-amine, #D1552 from  
808 Molecular Probes; 30µM/l dissolved in serum-free medium) prior to aldehyde fixation. Analysis  
809 of thin sections was performed with a Philips CM120 (now ThermoFisher Scientific), equipped  
810 with a MORADA digital camera and ITEM-software (EMSIS, Münster Germany). Image  
811 contrast, brightness, greyscale and sharpness were adjusted with Photoshop CS6 (Adobe,  
812 San José, CA, USA). Two biological and three technical replicates were performed.

813 Scanning EM of filter-grown polarized monolayers was performed with a DSM 982 Gemini  
814 (ZEISS, Oberkochen, Germany) as described previously (Ruemmele et al., 2010); briefly,  
815 sample processing included chemical fixation, dehydration, critical point drying and sputter  
816 coating. Two biological and three technical replicates were performed.

817 *GO-term analysis*

818 For manual GO-term analysis, each of the 89 significantly enriched genes from out CRISPR-  
819 screen were subjected to a manual GO-term search using <https://www.uniprot.org/> and  
820 <https://www.ebi.ac.uk/QuickGO/annotations>. For each gene, 3 GO-terms were listed,  
821 prioritizing most common GO-terms suggested by the QuickGO-Database and GO-terms  
822 indicating a relation to the secretory pathway, for each of the three categories, biological  
823 process (BP), molecular function (MF), cellular compartment (CC). According to commonalities  
824 in the individuals sets of GO-terms, genes were then grouped and graphically visualized using  
825 Affinity Designer.

826 *Statistics and Software*

827 The software used, if not already specified, were Affinity Designer (Version 1.9.3), Fiji/ImageJ  
828 (Version 2.1.0/1.53c), GraphPad Prism 9 (Version 9.1.0), Serial Cloner (Version 2.6); For the  
829 analysis and visualization of FACS-Data we used Flowjo (Becton Dickinson). Dot box plot  
830 graphs were generated and the unpaired Mann-Whitney U test was calculated using R (R  
831 Core Team (2021). R: A language and environment for statistical Computing. R Foundation for  
832 Statistical Computing, Vienna, Austria. URL <https://www.R-project.org>) and ggplot2 package.

833 *Data availability*



834 Next generation sequencing data was made available at  
835 <https://doi.org/10.5061/dryad.m0cfxpp62>.

836

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1112 **Abbreviations**

1113 CaCo2 caucasian colorectal adenocarcinoma 2

1114 CODE congenital diarrhea and enteropathy

1115 DAMP 3-(2,4-Dinitroanilino)-3'amino-N-methylpropyl-amine

1116 DPP4 Dipeptidylpeptidase 4

1117 GO gene ontology

1118 KD knock-down

1119 KEGG Kyoto Encyclopedia of Genes and Genomes

1120 MDCK madine darby canine kidney

1121 MVI microvillus inclusion

1122 MVID microvillus inclusion disease

1123 TEER transepithelial electric resistance

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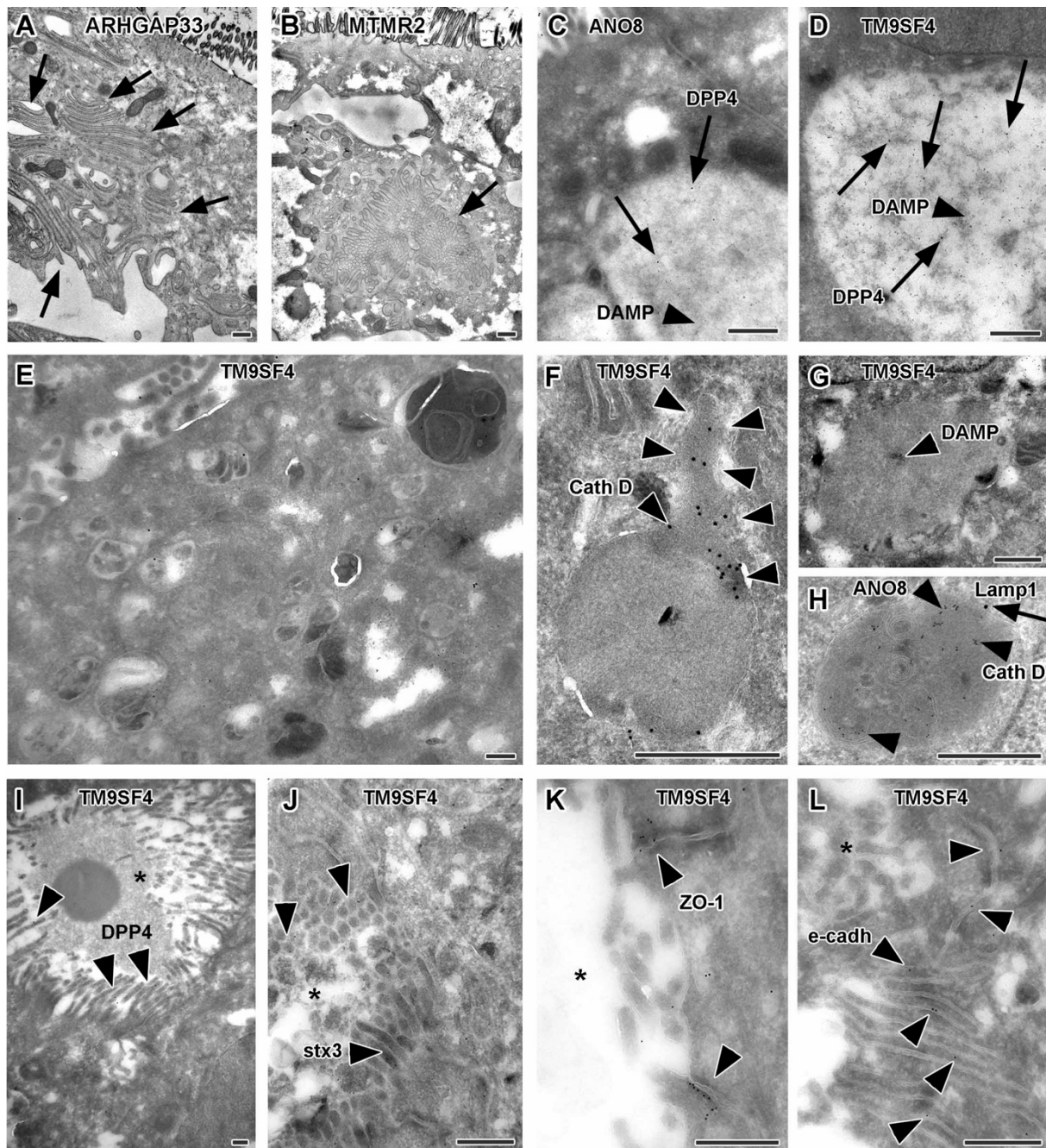
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1136 **Figure S1. Complementary ultrastructure and immunoelectron microscopy on the**  
1137 **phenotype of selected, polarized KD cells. Scale = 500 nm. A.** Paracellular microvilli a/o  
1138 numerous interdigitations in cryo-fixed ARHGAP33 KD cells. **B.** Intracellular microvillar cluster  
1139 in cryo-fixed MTMR2 KD cell. **C.** Mislocalized DPP4 (arrows) in a huge, acidic (i.e., DAMP-  
1140 positive: arrow head) compartment in ANO8 KD cell. **D.** Mislocalized DPP4 (arrows) in a huge  
1141 DAMP-positive (arrow head) compartment in TM9SF4 KD cell. **E-H.** Normal late endosomal  
1142 and lysosomal organelles as observed after serum starvation overnight. **E.** Overview on  
1143 TM9SF4 KD cell. **F.** Reformed lysosome with cathepsin D immunogold label concentrated at  
1144 its tubular part (outlined by arrow heads) in TM9SF4 KD cell. **G.** Weak DAMP-immunogold  
1145 label (arrow head) within newly formed lysosome/protolysosome in TM9SF4 KD cell. **H.** Lamp1  
1146 (arrow) and cathepsin D (arrow heads) immunogold label in normal lysosome in ANO8 KD cell.



1147 **I.** Ectopic DPP4 immunogold label (arrow heads) at basolateral, paracellular microvilli in  
1148 TM9SF4 KD cell. **J.** Ectopic stx3 immunogold label (arrow heads) at basolateral, paracellular  
1149 microvilli TM9SF4 KD cell. **K.** ZO1 immunogold label (arrow heads) at ectopic, basolateral tight  
1150 junctions associated with paracellular microvillar spot (asterisk marks intercellular space) in  
1151 TM9SF4 KD cell culture. **L.** E-cadherin immunogold label (arrow heads) at basolateral  
1152 adherens junctions adjacent to a paracellular microvillar spot (asterisk marks intercellular  
1153 space) in TM9SF4 KD cell culture.

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