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# The clathrin adaptor AP-1B independently controls proliferation and differentiation in the mammalian intestine

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1 **The clathrin adaptor AP-1B independently controls proliferation and differentiation in**  
2 **the mammalian intestine.**

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19 **Short title:** AP-1B plays multiple roles in small intestinal homeostasis.

20 **Keywords:** polarised sorting, AP-1B, intestine, proliferation, differentiation.

21 **Abbreviations:** AP-1B, Adaptor protein-subunit  $\mu 2/\mu 1B$ ; EGF, Epidermal Growth Factor;  
22 ISC, intestinal stem cells; mTOR, mechanistic target of rapamycin; YAP, yes-associated  
23 protein 1.

## 24 **Abstract**

25 Maintenance of the polarity of the epithelial cells facing the lumen of the small  
26 intestine is crucial to ensure the vectorial absorption of nutrients as well as the integrity of the  
27 apical brush border and the intestinal barrier. Polarized vesicular trafficking plays a key role  
28 in this process, and defective transport due to mutations in apical trafficking-related genes has  
29 been shown to affect nutrient absorption. Interestingly, it has been demonstrated that  
30 downregulation of the polarized sorting clathrin adaptor AP-1B led to both epithelial polarity  
31 and proliferation defects in the mouse intestine. This enlightened a new function of polarized  
32 trafficking in the gut epithelium and a novel link between trafficking, polarity, and  
33 proliferation. Here, using CRISPR-Cas9-mediated mutation of the AP-1B coding gene *Ap1m2*  
34 in mouse intestinal organoids, we uncovered a novel proliferation pathway controlled by AP-  
35 1B. We showed that the polarity defects induced by *Ap1m2* mutations led to a defective apical  
36 targeting of both Rab11<sup>+</sup> apical recycling endosomes and of the polarity determinant Cdc42.  
37 Moreover, we showed that these polarity defects were accompanied by an induction of YAP  
38 and EGFR/mTOR-dependent proliferation pathways. Finally, we showed that AP-1B  
39 additionally controlled a proliferation-independent differentiation pathway towards the  
40 secretory lineage. Overall, our results highlighted the pleiotropic roles played by AP-1B in the  
41 homeostasis of the gut epithelium.

## 42 **Introduction**

43           Intestinal cells lining the gut lumen are constantly renewed by the treadmilling  
44 process of new cells arising from the proliferation of Lgr5<sup>+</sup> intestinal stem cells (aka crypt-  
45 based columnar cells) and death of terminally differentiated cells at the top of the villus  
46 (Gehart and Clevers, 2019). The proliferation of intestinal stem cells (ISC) is controlled by  
47 niche factors emanating from both the neighbouring Paneth cells and mesenchymal cells  
48 secreting proliferative factors such as Wnt and the Wnt pathway agonist R-Spondin, as well as  
49 Epidermal Growth Factor (EGF) (Crosnier et al., 2006). Notably, varying exposure of cells in  
50 the transit-amplifying zone migrating towards the villus to niche gradients (e.g. Wnt, Notch)  
51 allows their differentiation into the different types of absorptive and secretory lineages of the  
52 small intestine (Beumer and Clevers, 2021). Among these cells, the major type is the  
53 enterocytes that bear a so-called brush border made of an array of microvilli increasing the  
54 membrane surface for nutrient absorption (Delacour et al., 2016).

55           The maintenance of the strong polarity of enterocytes is essential to ensure the  
56 vectorial transport of nutrients from the lumen to the blood vessels located in the underlying  
57 connective tissue. Polarized trafficking plays a major role in this maintenance and disturb  
58 trafficking to the apical membrane has been linked to major absorption defects, such as  
59 Microvillus inclusion disease (Schneeberger et al., 2018). Besides direct brush border defects,  
60 missense mutations in the gene coding a subunit of the AP-1 complex has been linked to an  
61 enteropathy through intestinal barrier defects (Klee et al., 2020). AP-1 is a clathrin adaptor  
62 tetrameric complex made of four subunits (two large subunits ( $\beta$ ,  $\gamma$ ), one medium subunit ( $\mu$ 1)  
63 and one small subunit ( $\sigma$ 1)) (Nakatsu et al., 2014). Among them, the function of the epithelia-  
64 specific  $\mu$ 1B/ $\mu$ 2 subunit has been extensively studied in the basolateral targeting of membrane  
65 proteins (Fölsch et al., 2001; Gan et al., 2002; Gravotta et al., 2007). Furthermore, we and the  
66 group of Verena Gobel showed that AP-1 also mediates the sorting of apical cargoes in *C.*

67 *C. elegans* intestinal cells (Shafaq-Zadah et al., 2012; Zhang et al., 2012), a function that has  
68 been confirmed in both cell culture and mouse models (Caceres et al., 2019; Hase et al.,  
69 2013). Notably, the polarized sorting defects induced by AP-1 subunits silencing resulted in  
70 the contralateral mislocalization of both transmembrane proteins and apical polarity  
71 determinants (e.g. Cdc42, PAR-6, PAR-3, aPKC) as well as in the apicalization of the  
72 enterocyte lateral membrane (i.e. ectopic lumen) (Hase et al., 2013; Shafaq-Zadah et al.,  
73 2012). Interestingly, it has been shown that *Ap1m2* knockout in mouse also induced a crypt  
74 hyperplasia, which enlightened a putative crosstalk between apical trafficking, cell polarity  
75 and proliferation in the gut. Here, we used CRISPR-Cas9-mediated mutation of *Ap1m2* in  
76 mouse intestinal organoids to study the relationship between cell polarity, proliferation, and  
77 differentiation in the mammalian intestine.

## 78 **Results and discussion**

### 79 ***Ap1m2* mutations induce strong polarity defects in mouse intestinal organoids.**

80 In order to assess the role of AP-1B in intestinal cells integrity, *Ap1m2* gene, encoding  
81 the  $\mu 1B/\mu 2$  subunit, was knocked down by conditional CRISPR-Cas9-mediated mutation in  
82 organoids (Bidaud-Meynard et al., 2022; Jad et al., 2022). Sanger sequencing of the genomic  
83 DNA of CRISPR-edited organoids using Inference CRISPR Edits software (Conant et al.,  
84 2022) showed that ~82% of *Ap1m2* organoids were mutated, with half of the mutations  
85 inducing a single deletion of a guanosine located 5' of the PAM site (Fig. 1A and  
86 Supplementary Fig. 1A). AP-1B is a renown sorting factor for basolateral proteins bearing di-  
87 leucin or tyrosine-based signals (Fölsch et al., 1999) and has recently been involved in apical  
88 targeting (Caceres et al., 2019; Hase et al., 2013; Shafaq-Zadah et al., 2012; Zhang et al.,  
89 2012), making it a major player of apicobasal polarity in epithelia. To verify this in fully  
90 polarized enterocytes, the organoids culture medium was supplemented with valproic acid and  
91 inhibitor of Wnt protein 2 (IWP2), as described before (Bidaud-Meynard et al., 2022; Mosa et  
92 al., 2018). As expected, *Ap1m2* mutations induced a mispolarization of the brush border  
93 component phospho-ezrin, which accumulated at both apical and lateral membranes (Fig. 1B-  
94 C). This partial polarity inversion was also obvious by analysing the organoids by  
95 transmission electron microscopy (TEM), which revealed lateral ectopic lumen as well as  
96 regions of the enterocyte monolayer with a brush border located both at the apical and basal  
97 poles (Fig. 1D-F). In *C. elegans*, we found that these polarity defects are the consequence of  
98 the cytoplasmic dispersion of Rab11<sup>+</sup> endosomes and a mistargeting of the polarity  
99 determinant Cdc42, that has been shown to be targeted to the plasma membrane through  
100 apical trafficking (Osmani et al., 2010). Mislocalised Cdc42 consequently recruited the other  
101 members of the Cdc42/PAR apical polarity module (PAR-3, PAR-6 and PKC-3) at the lateral  
102 membrane to convert it into an apical pole (Shafaq-Zadah et al., 2012). As ectopic lumens

103 were observed upon *Ap1m2* mutation in organoids (Fig. 1E), we then assessed whether the  
104 same apical trafficking pathway is disrupted in organoids. Rab11 and Cdc42 immunostaining  
105 as well as PKC $\zeta$  Immunohistochemistry (IHC) labelling showed a decreased apical  
106 confinement of all these factors and Rab11 endosomes were often detected at the lateral  
107 membrane (Fig. 1H-L). This suggested that the mechanisms by which AP-1B controls the  
108 apical polarity of intestinal cells are conserved in mammals. Moreover, TEM also revealed  
109 dilated Trans Golgi Network and abundant associated vesicles, multivesicular bodies and  
110 lysosomes (Supplementary Fig. 1B and Supplementary Table 1). Hence, these results  
111 confirmed the strong trafficking and polarity defects in *Ap1m2* CRISPR-edited organoids.

### 112 ***Ap1m2* mutations induce an hyperproliferation in mouse intestinal organoids.**

113 In addition to apicobasal polarity, *Ap1m2* knockout in mouse additionally induced a  
114 crypt hyperplasia (Hase et al., 2013). To confirm this in our model, the proliferation of  
115 intestinal cells was measured using Ki67 staining in organoids cultured in normal  
116 EGF/Noggin/R-spondin1 (ENR) medium (i.e., presenting both crypt and villus-like regions)  
117 (Sato et al., 2011). *Ap1m2* mutations induced a 2-fold over-proliferation of intestinal cells  
118 compared to control organoids (Fig. 2A-B). This hyperplasia was correlated with a stacking of  
119 the cells which became thinner and longer (Figs 1G and 2C). The enterocyte monolayer was  
120 also dramatically disturbed, with a misalignment of nuclei and intertwined cells (Figs 1F and  
121 2C). Furthermore, we observed intercellular junctions defects with many holes between cells  
122 (Figs 1G and 2C) as well as disturb adherens and tight junctions organization (Fig. 2D), as  
123 already reported upon AP-1 complex subunits downregulation (Hase et al., 2013; Klee et al.,  
124 2020). However, while cell-cell junctions were clearly affected, we were unable to observe  
125 the dotted cytoplasmic accumulation of E-Cadherin (Hase et al., 2013). This data favoured a  
126 pathway in which E-Cadherin intracellular displacement freed  $\beta$ -catenin that accumulated  
127 intracellularly and activated downstream proliferation targets (Hase et al., 2013).

128 Furthermore, while Wnt pathway inhibition with IWP-2 completely effaced the crypts to only  
129 leave differentiated enterocytes and goblet cells in control organoids, crypt-like structures and  
130 Ki67<sup>+</sup> proliferating cells persisted in *Ap1m2*-mutated organoids (Fig. 2F-H and  
131 Supplementary Fig. 1C-D). This suggests that the activation of the  $\beta$ -catenin pathway  
132 described earlier might still rely on mesenchymal Wnt signals, which are very important for  
133 small intestine proliferation and homeostasis in the context of the organ (Valenta et al., 2016),  
134 and that additional intestine-autonomous proliferation pathway(s) may be induced upon  
135 *Ap1m2* mutations. Moreover, we observed that the polarity defects induced by *Ap1m2*  
136 mutations, such as the formation of ectopic lateral lumen and the contralateral mislocalization  
137 of the apical and basolateral proteins P-ezrin and EphrinB2, respectively, were also observed  
138 in proliferating organoids (Fig. 2I-K). Considering that both *Cdc42* and *Rab11* knockout also  
139 induced an intestinal cells hyperplasia in mice (D'Agostino et al., 2019; Melendez et al.,  
140 2013; Zhang et al., 2022), these results raised the possibility that *Ap1m2* mutations induced an  
141 additional, mesenchyme-independent, proliferative pathway that could be linked to Cdc42  
142 polarity defects.

#### 143 ***Ap1m2* mutations-induced hyperproliferation involves YAP and EGFR/mTOR.**

144 To test this hypothesis, we stained Cdc42 in organoids edited with two different  
145 sgRNAs targeting *Ap1m2*. We found that Cdc42 apical localization also decreased in  
146 normally cultured, proliferating organoids (Fig. 3A). It has recently been shown that Rab11 is  
147 involved in the targeting of the Hippo signalling factor Yes-associated protein (YAP), and  
148 that *Cdc42* silencing in mouse intestine induced an hyperplasia through YAP-EGF-mTOR  
149 signalling (Goswami et al., 2021; Zhang et al., 2022). Notably, this pathway led to an initial  
150 increase of nuclear YAP expression by inhibiting its ubiquitin-dependent proteolysis (Zhang  
151 et al., 2022). Interestingly, YAP nuclear expression in crypts increased by 1.5-fold upon  
152 *Ap1m2* mutations, which indicated that the YAP-EGFR-mTOR pathway could be enhanced



153 (Fig. 3B-C). To verify this, the proliferation of intestinal cells was measured upon treatment  
154 of the organoids with the EGFR and mTOR signalling inhibitors Afatinib and Rapamycin,  
155 respectively. Remarkably, both treatments rescued the proliferation of *Ap1m2*-mutated  
156 organoids to control levels (Fig. 3D-E) which confirmed that AP-1B also controls intestinal  
157 cells proliferation through YAP, EGFR and mTOR. Surprisingly, while *Cdc42* knockout has  
158 been proposed to induce EGFR and mTOR proliferative pathways as a consequence of an  
159 increased expression of YAP (Zhang et al., 2022), the accumulation of YAP was also  
160 cancelled by Afatinib and Rapamycin treatment (Fig. 3F-G). This suggests either the  
161 existence of positive feedback loops, or that YAP acts downstream of the mTOR pathway, as  
162 proposed before (Liang et al., 2014) or, finally, that YAP and EGFR pathways are induced in  
163 parallel. Indeed, in addition to YAP, Rab11 has been shown to mediate the membrane  
164 localization of Merlin, a YAP and cortical actin regulator that has also been involved in the  
165 control of EGFR signalling (Curto et al., 2007; Goswami et al., 2021). Furthermore, while  
166 Afatinib but not Rapamycin also slightly decreased the proliferation of control organoids by  
167 1,5-fold, it dramatically inhibited *Ap1m2*-mediated overproliferation (~8.3 fold)  
168 (Supplementary Fig. 1E-F). Hence, these results suggest that *Ap1m2* mutations lead to an  
169 hyperproliferation of intestinal cells by displacing *Cdc42* from the apical membrane and  
170 activating YAP and EGF-mTOR-dependent proliferation pathway(s).

### 171 ***Ap1m2* mutations induced major and proliferation-independent differentiation defects.**

172 Besides the defects observed in the arrangement of cells within the monolayer and in  
173 the enterocytes' polarity, we observed that *Ap1m2* mutations induced severe differentiation  
174 defects. Indeed, *Olfm4*<sup>+</sup> ISCs and *Lysozyme*<sup>+</sup> Paneth cells, which are normally confined in the  
175 crypt, invaded the villus region of *Ap1m2*-mutated organoids (Fig. 4A-D). Furthermore, TEM  
176 analysis of the secretory cells revealed the presence of cells with a mixed zymogen/mucigen  
177 content - while the number of normal goblet and Paneth cells remained unchanged - upon

178 *Ap1m2* gene editing (Fig. 4E-K and Supplementary Table 1), thus demonstrating a strong  
179 defect in secretory lineage differentiation. Interestingly, this rare phenotype, together with the  
180 invasion of the villus by Paneth cells, were also observed upon *Cdc42* knockout in mouse  
181 (Melendez et al., 2013). Of note, the invasion of the villus by Paneth and ISCs was not  
182 affected by Rapamycin nor Afatinib treatment, which indicated that these differentiation  
183 defects are independent of the YAP/EGF/mTOR proliferation pathways induced by *Ap1m2*-  
184 mutations (Fig. 4L-M). These results corroborate previous data showing that inhibition of  
185 EGFR signalling affected the proliferation but not the differentiation into goblet and Paneth  
186 cells (Basak et al., 2017). Further studies would be required to understand how AP-1B  
187 controls the differentiation of the secretory lineage in the intestine. This might rely on an  
188 imbalance of Wnt and Notch signals, which are crucial in determining the fate of secretory  
189 cells (Beumer and Clevers, 2021), and whose receptors' membrane targeting is controlled by  
190 the AP-1 complex (Benhra et al., 2011; Wieffer et al., 2013). Overall, our results highlight the  
191 major roles played by AP-1B in the homeostasis of the small intestine cells, by regulating  
192 polarized trafficking, proliferation as well as differentiation (Fig. 4N) and confirm that AP-1B  
193 is essential for apical trafficking.

## 194 **Material and Methods**

### 195 **Materials**

196 Afatinib and Rapamycin were purchased from Chemietek (CT-BW2992) and APExBIO  
197 (A8167), respectively.

### 198 **Organoid culture**

199 Intestinal crypts were cultured in drops of Cultrex Reduced Growth Factor Basement  
200 Membrane Extract type 2 (BME2, 3533-010-02, Bio-technie) seeded on 12-well Greiner Cell  
201 STAR multiwell low retention plates with ENR medium (Advanced DMEM/F12 (Gibco), 10  
202 mM Hepes (15630056), 5 ml Glutamax (35050-038), 5 ml Penicillin/Streptomycin, 1X B27  
203 supplement (17504-044) (all from Life technologies), 1.25 mM N-acetylcysteine (A9165,  
204 Sigma-Aldrich), 50 ng/mL hEGF (AF-100-15, Peprotech), 5% R-Spondin1 and 10% Noggin  
205 conditioned media (produced as described (Sato et al., 2011)). Medium was changed every 2-  
206 3 days and organoids were passaged by mechanical disruption every 7 days. Gene mutation  
207 was induced 4 days after organoid passage during 6 days with 400 ng/ml doxycycline (D9891,  
208 Sigma-Aldrich). When indicated, full differentiation of the organoids was achieved by a  
209 concomitant treatment for 6 days with 1 mM valproic acid (P4543, Sigma-Aldrich), with the  
210 addition of 2.5 mg/mL inhibitor of WNT production-2 (IWP-2, I0536, Sigma-Aldrich) for the  
211 last 3 days, as described (Mosa et al., 2018). Absence of mycoplasma was checked regularly.

### 212 **Conditional gene knock out by CRISPR-Cas9**

213 Organoids were transduced in a two-step manner with Edit-R lentiviral inducible Cas9  
214 nuclease and pre-designed sgRNAs lentiviral particles (Horizon discovery, Cambridge, UK)  
215 as described earlier (Jad et al., 2022). The target sequences of the sgRNAs targeting *Ap1m2*  
216 were: 5'ACACGATGACGAAGTTGTCC3' for sgRNA1 and  
217 5'CTGATTAGCCGAAACTACAA3' for the sgRNA2. Non-targeting sgRNA#1  
218 (VSGC10215) was used to generate control organoids. Transduced organoids were selected

219 with 5 $\mu$ g/ml Blasticidin (CAS9) or 3 $\mu$ g/ml Puromycin (sgRNAs). The % of indels was  
220 calculated using the ICE software (Conant et al., 2022) after Sanger sequencing of the  
221 organoids' genomic DNA.

## 222 **Transmission Electron Microscopy**

223 Organoids were collected from BME2 and fixed in Trump's fixative at 4°C. Enhanced  
224 chemical fixation was performed in 4% paraformaldehyde/2.5% glutaraldehyde in cacodylate  
225 buffer (0.1 M, pH 7.4) overnight at 4°C. Then, organoids were incubated successively in 1%  
226 OsO<sub>4</sub> for 1.5h and 2% uranyl acetate for 1.5h, at RT. Organoids were then dehydrated  
227 through graded ethanol solutions, cleared in acetone, and flat-embedded in Epon-Araldite mix  
228 (EMS hard formula) using adhesive frames (11560294 GENE-FRAME 65  $\mu$ L, Thermo Fisher  
229 Scientific). Ultrathin 70 nm sections were cut on an ultramicrotome (UC7; Leica  
230 Microsystems) and collected on formvar-coated slot grids (FCF2010-CU, EMS). TEM grids  
231 were observed using a JEM-1400 TEM (JEOL) operated at 120 kV, equipped with a Gatan  
232 Orius SC1000 camera piloted by the Digital Micrograph 3.5 program (Gatan).

## 233 **Immunohistochemistry**

234 Organoids were collected from BME2, fixed in 2% paraformaldehyde overnight at 4°C and  
235 washed in PBS 1X. Then, pelleted organoids were resuspended in 2 drops of Shandon  
236 Cytoblock Cell Block reagent (ThermoScientific) and embedded in paraffin wax with  
237 Excelsior ES50 during 3h. Paraffin-embedded organoid blocks were cut at 4  $\mu$ m thickness,  
238 mounted on positively charged glass slides and dried at 58°C for 60 min. The slides were then  
239 deparaffinized with xylene (3x10 min) and successive 100%-75%-50% ethanol baths (5 min  
240 each) before rehydration in water for 5 min. Immunohistochemical staining was performed on  
241 a Discovery Automated IHC Stainer. First, antigen retrieval was performed using Tris-based  
242 buffer solution CC1 at 95–100°C for 48 min. Then, endogen peroxidase was blocked with  
243 Inhibitor-D/3% H<sub>2</sub>O<sub>2</sub> for 8 min at 37°C. After rinsing, slides were incubated at 37°C for 60

244 min with anti-Cdc42 (1:50, ab64533, Abcam) and anti-phospho-ezrin (1:2000, ab47293,  
245 Abcam) primary antibodies. After rinsing, signal enhancement was performed using the  
246 Ventana DABMap (for horse anti-rabbit HRP) or OMNIMap (for goat anti-mouse/rabbit HRP  
247 or donkey anti-Goat HRP) detection kits (Ventana Medical Systems). Slides were then  
248 counterstained with haematoxylin (16 min) and bluing reagent (4 min) and rinsed before  
249 being manually dehydrated and coverslipped. Imaging was performed using an Hamatsu  
250 scanner (Nanozoomer 2.0RS/ C10730-12) and the images were analysed with NDP viewer.

### 251 **Immunostaining**

252 For immunostaining on whole organoids, they were collected from BME2 and fixed in 2%  
253 PFA/PBS overnight at 4°C. For immunostaining on organoid slices, 4 µm paraffin bloc  
254 sections were mounted on slides before deparaffination in Xylene (3x10 min) and successive  
255 100%-75%-50% Ethanol baths (5 min each). After rehydration in water for 5 min, antigen  
256 retrieval was performed with Tris-EDTA, pH8 (98°C, 40 min). Then, both organoids 4 µm  
257 slices and whole organoids in microtubes were permeabilized in PBS1X, 0,1% Tween20,  
258 0,2% Triton (20 min at room temperature under rotation) and incubated overnight at 4°C in  
259 blocking solution (PBS1X, 0.1% tween 20, 2% FBS) with primary antibodies. Whole  
260 organoids were incubated with anti-Ki67 (1:100, 14-5698-82, Invitrogen), anti-EphB2 (1:100,  
261 AF467-SP, Biotechne), anti-YAP (1:100, 14074, Cell signalling), anti-Olfm4 (1:400, 39141,  
262 Cell signalling), anti-lysozyme (1:100, A0099, Agilent). Organoid slices were incubated with  
263 anti-phospho-ezrin (1:200, ab47293, Abcam), anti-Rab11A (1:100, 71-5300, Invitrogen), anti-  
264 Cdc42 (1:50, ab64533, Abcam), and anti-E-cadherin (1:200, 24E10, Cell signalling). Then,  
265 organoids were incubated with adequate fluorescent secondary antibodies (Jackson  
266 ImmunoResearch) during 1h at room temperature before mounting in ProLong Gold Antifade  
267 reagent (P36941, Life Technologies). F-Actin was stained using Actistain (1:150,  
268 Cytoskeleton) for 1h at room temperature. Imaging was performed on a Zeiss LSM880-

269 Airyscan (Oberkochen, Germany) equipped with a 63X, 1.4 NA objective (Zen Black  
270 software) and images were analysed using Fiji.

### 271 **Quantification and statistical analysis**

272 The nuclear accumulation of Ki67 and YAP was quantified by measuring the signal on a  
273 nuclear overlay obtained after nuclear segmentation using the DAPI channel in Fiji and was  
274 divided by the signal of the same overlay in the Dapi channel to correct for signal differences  
275 due to the varying distance between the cells and the objective. The apical/cytoplasmic ratio  
276 of markers was calculated by measuring the signal intensity of a line covering the apical  
277 membrane and measuring the signal intensity of an identical ROI in the underlying cytoplasm.  
278 Gaussian distribution and homoscedasticity of data were verified by the Shapiro-Wik test and  
279 the F-test, respectively. Mann-Whitney non-parametric test was performed using GraphPad  
280 software.

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### 285 **Disclosures**

286 The authors disclose no conflicts of interest.

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- 392

393 **Figure legends**

394 **Figure 1: *Ap1m2* mutations induced polarity defects in fully differentiated organoids.**

395 (A) Analysis by Sanger sequencing of the genomic DNA of *Ap1m2* CRISPR-edited organoids  
396 (sg1 sgRNA). The histogram shows the mean  $\pm$  SD of the repartition of indels found in the  
397 organoid population (N=2 independent experiments). (B-C) Phospho-Ezrin (p-Ezrin) staining  
398 in control or *Ap1m2* organoids. (B) shows representative images and (C) the quantification of  
399 the lateral p-Ezrin grey intensity, expressed in fold change (N=4 independent experiments).  
400 (D-G) Control and *Ap1m2* organoids were analysed by TEM. The arrow and the arrowhead  
401 show brush borders at the basal and lateral membranes, respectively. Lateral membranes in  
402 (E) are highlighted in blue. (H-K) Immunofluorescence staining of Rab11 endosomes (H) and  
403 Cdc42 (I) in Control (CTL) and *Ap1m2* organoids. Inserts in (H, I) represent fire-LUT  
404 coloured magnified images of the indicated apical ROIs (arrows). The arrowhead in (H)  
405 shows the lateral localization of Rab11. Panels (J-K) show the quantification of Rab11 and  
406 Cdc42 apical/cytoplasmic ratio in fold change (from 3 and 4 independent experiments,  
407 respectively). (L) IHC analysis of PKC $\zeta$  in Control and *Ap1m2* organoids. The total number  
408 of organoids analysed is indicated in brackets, \*\*\*\*p<0.0001, Mann-Whitney test. Scale bars,  
409 10 $\mu$ m (B, H-I, L) and 5 $\mu$ m (D-G).

410 **Figure 2: *Ap1m2* mutations induced an hyperproliferation in mouse SI organoids.**

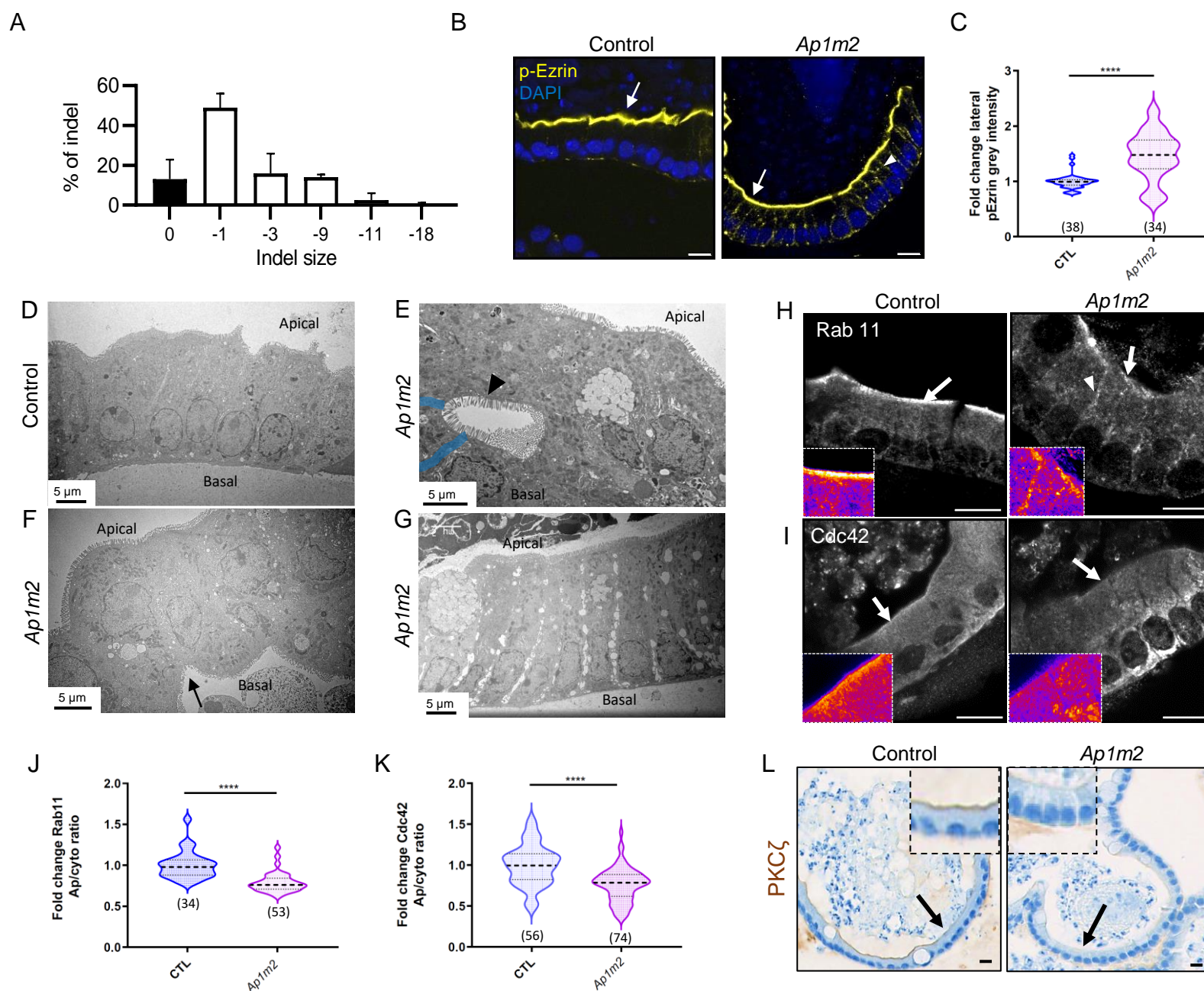
411 (A-B) Immunofluorescence staining of Ki67 in Control (CTL) and *Ap1m2* organoids. (A)  
412 shows representative z-projections and (B) the quantification of Ki67/DAPI mean grey  
413 intensity ratio, expressed in fold change (N = 11 independent experiments). (C-D) TEM  
414 images of normally grown proliferative Control and *Ap1m2* organoids. Arrowheads highlight  
415 the holes between cells. (D) shows a focus on cell-cell junctions. D, desmosome; AJ,  
416 adherence junctions; TJ, tight junctions. (E) Immunofluorescence staining of E-Cadherin  
417 (magenta), F-actin (yellow) and Dapi in Control and *Ap1m2* organoids. Arrows indicate cell-  
418 cell junctions. (F) Haematoxylin-Eosin-Saffron (HES) staining of control and *Ap1m2*  
419 organoids cultured in normal ENR or full-differentiation (ENR+VPA+IWP-2) mediums.  
420 Arrowheads show intestinal crypts. (G-H) Immunofluorescence staining of Ki67 in fully  
421 differentiated Control (CTL) and *Ap1m2* organoids. (H) shows the quantification of Ki67<sup>+</sup>  
422 cells/organoid. (I) *Ap1m2* organoids cultured in normal ENR also present ectopic lateral  
423 lumen (arrow), analysed by TEM. Lateral membranes are highlighted in blue. (J)  
424 Immunofluorescence staining of Ephrin B2 in control and *Ap1m2* organoids. Arrows and the  
425 arrowhead indicate the basolateral and the apical membranes, respectively. (K) IHC staining  
426 of p-Ezrin in control and *Ap1m2* organoids. Arrows show the apical membrane and  
427 arrowheads show the basolateral accumulation of P-ezrin. Inserts highlight the crypt and  
428 villus regions indicated by dashed lines. The total number of organoids examined is indicated  
429 in brackets, \*\*\*\*p<0.0001, Mann-Whitney test. Scale bar, 50µm (F, G, K) or 10µm (E, J).

430 **Figure 3. *Ap1m2* mutations induced a Cdc42, YAP and EGFR/mTOR-dependent**  
431 **hyperproliferation.**

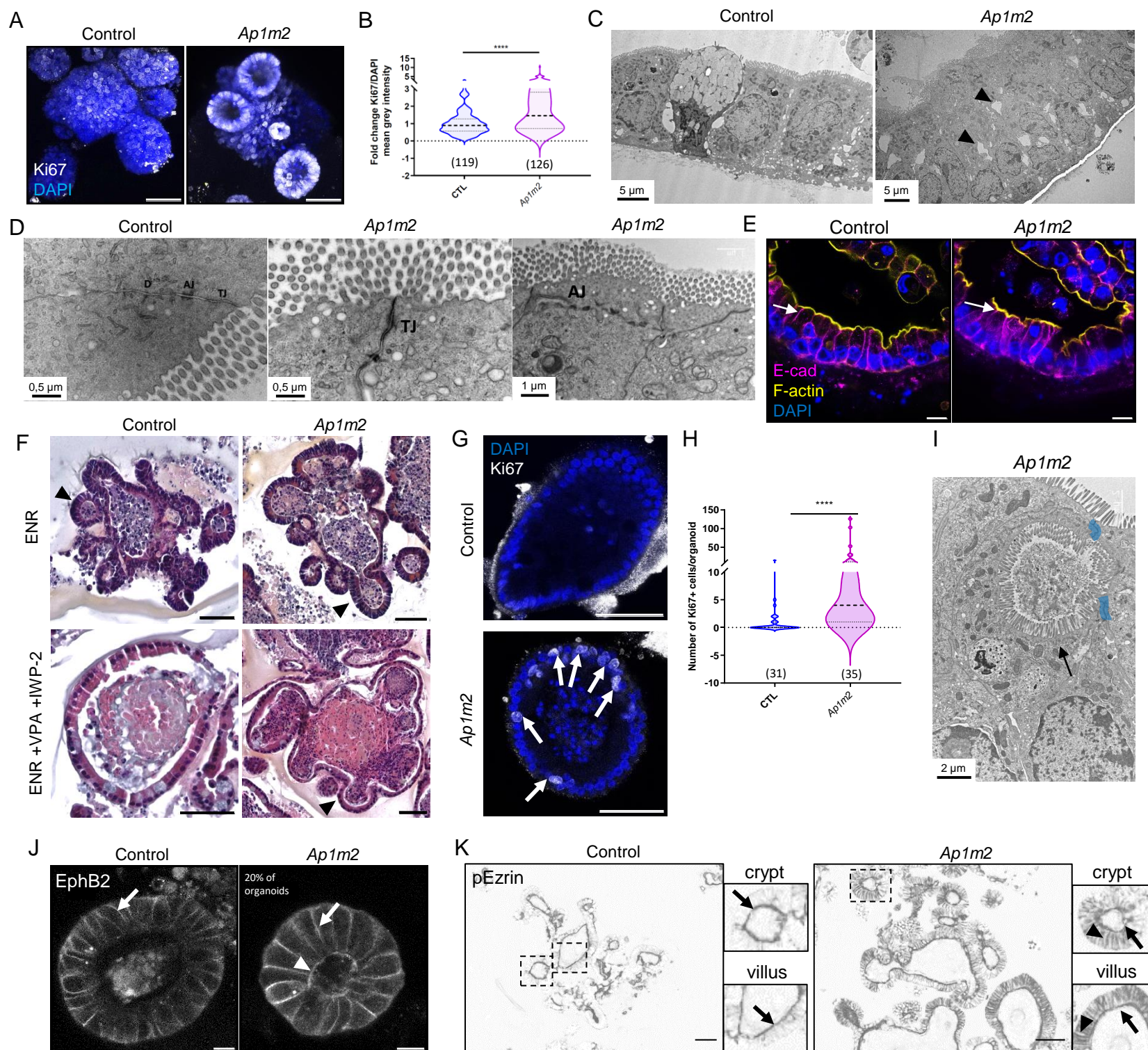
432 (A) IHC staining of Cdc42 in control and *Ap1m2*-mutated organoids. sg1 and sg2 indicate  
433 organoids where *Ap1m2* has been edited with two different sgRNAs. The arrowheads show  
434 the apical membrane in the inserts, that are magnified images of the indicated ROIs. (B-C)  
435 Immunofluorescence staining of YAP in control (CTL) and *Ap1m2* organoids. (B) is a  
436 representative z-projection and (C) is the quantification of the nuclear YAP/DAPI mean grey  
437 intensity ratio expressed in fold change (N=7 independent experiments). (D-G) Quantification  
438 of Ki67/DAPI (D-E) and YAP/DAPI (F-G) ratios in control (CTL) and *Ap1m2* organoids  
439 treated with 200nM Afatinib (afa) (N=6 independent experiments) or 1  $\mu$ M Rapamycin (rapa)  
440 for 3 days (N=7 independent experiments). The total number of organoids analysed is  
441 indicated in brackets, \*p<0,05, \*\*p<0,01; \*\*\*p<0,001; \*\*\*\*p<0,0001, unpaired Mann-  
442 Whitney test. Scale bar, 50 $\mu$ m.

443 **Figure 4: *Ap1m2* mutations affect progenitors' distribution and secretory lineage**  
444 **differentiation.**

445 (A) Immunofluorescence staining of *Olfm4* in Control (CTL) and *Ap1m2* organoids. (A)  
446 shows representative images and (B) the quantification of the % of organoids displaying  
447 *Olfm4*<sup>+</sup> cells outside the crypts (N = 4 independent experiments). (C-D) Immunofluorescence  
448 staining of Lysozyme in control (CTL) and *Ap1m2* organoids. (C) shows representative  
449 images and (D) the quantification of the % of organoids displaying Lysozyme<sup>+</sup> cells (N = 9  
450 independent experiments). In (A, C) crypts and villus-like regions are delineated in white and  
451 magenta, respectively. (E-H) Secretory cells were observed by TEM, showing Paneth (E,  
452 arrow), Goblet (E, arrows) as well as intermediate mixed cells (G, H). Arrowheads show the  
453 mixed zymogen/mucigen content of intermediate cells. (I-K) Quantification of the number of  
454 Goblet (I), Paneth (J) et intermediate mixed cells (K) per organoid from 3 control (CTL) and 4  
455 *Ap1m2* organoids, from TEM images. (L-M) Quantification of organoids displaying ectopic  
456 Paneth cells in control (CTL) or *Ap1m2* organoids treated with 200nM Afatinib (afa) (N=3  
457 independent experiments) or 1 $\mu$ M Rapamycin (rapa) for 3 days (N=3 independent  
458 experiments). The total number of organoids analysed is indicated in brackets, \*p<0,05,  
459 \*\*p<0,01; \*\*\*p<0,001; \*\*\*\*p<0,0001, unpaired Mann-Whitney test. (N) Model. We propose  
460 that AP-1 controls the apical organisation of Rab11 endosomes that allow the apical targeting  
461 of Cdc42, as well as inhibits the YAP and EGFR/mTOR proliferation pathways.  
462 Independently, AP-1 positively regulates the differentiation of intestinal cells towards the  
463 secretory lineage. Scale bar, 50 $\mu$ m.



**Figure 1**



**Figure 2**

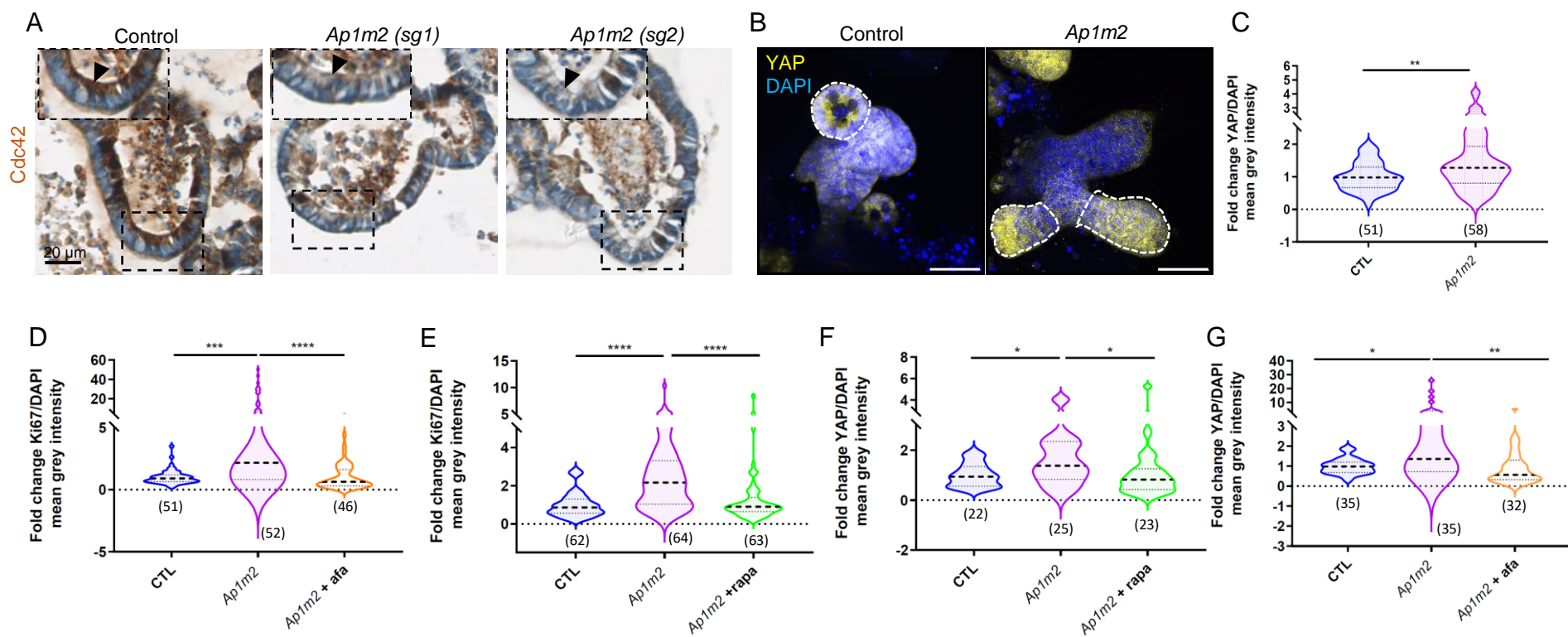
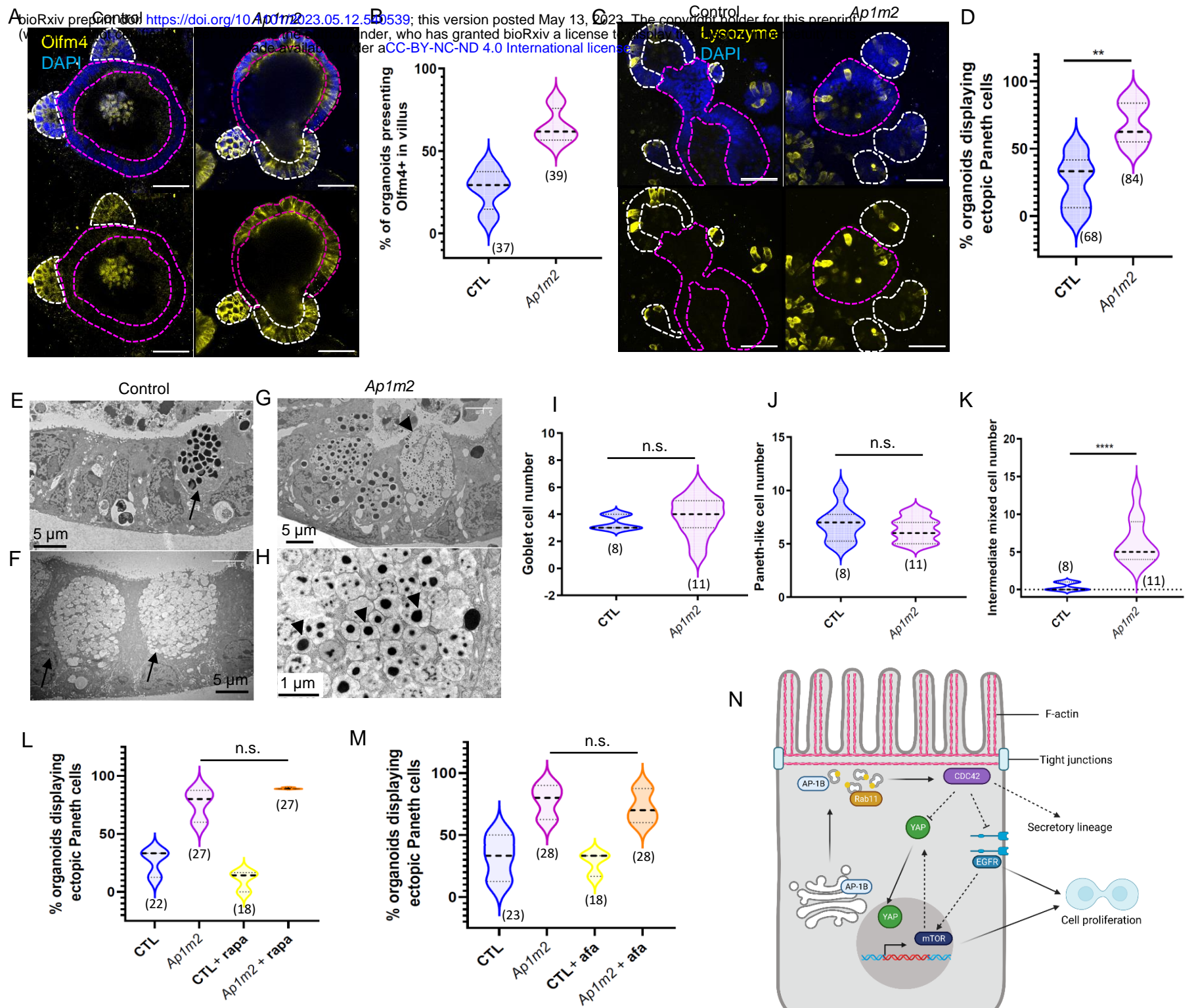
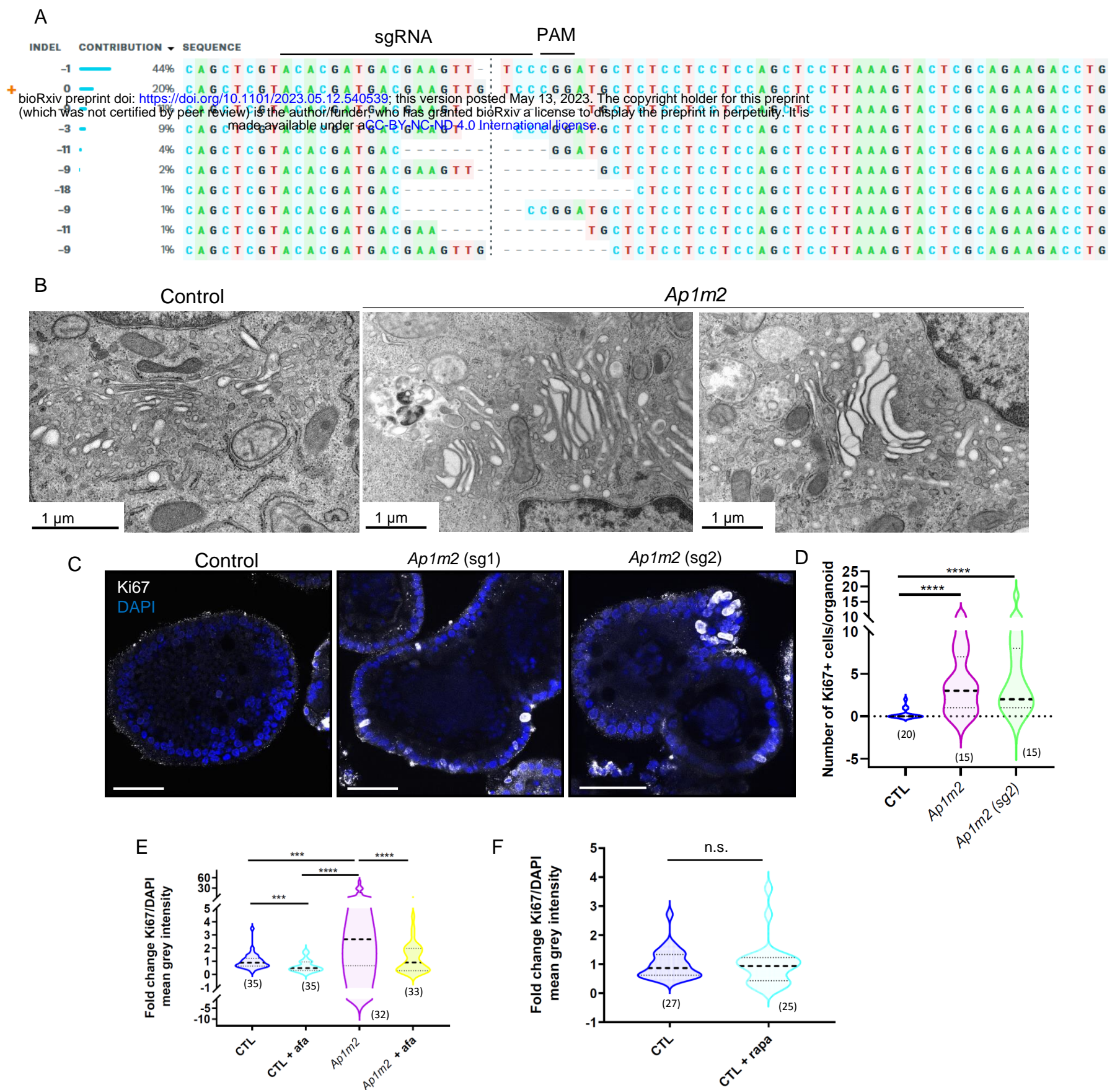


Figure 3





**Figure 4**



**Supplementary Figure 1.** (A) ICE analysis of a genomic DNA sequencing of CRISPR-edited organoids. The sgRNA sequence (sg1) targeting *Ap1m2* gene and the PAM site are indicated. (B) TEM analysis of the Golgi apparatus in control and *Ap1m2* organoids. (C-D) Immunofluorescence staining of Ki67 in fully-differentiated control (CTL), *Ap1m2* (sg1) and *Ap1m2* (sg2) organoids. Scale bars, 50 μm. (D) shows the quantification of Ki67+ cells/organoid. (E-F) Quantification of Ki67/DAPI ratio in organoids treated with 200 nM Afatinib (afa) during (N=3 independent experiments) or 1 μM Rapamycin (rapa) during (N=3 independent experiments). The total number of organoids analysed is indicated in brackets. \* $p < 0,05$ , \*\* $p < 0,01$ ; \*\*\* $p < 0,001$ ; \*\*\*\* $p < 0,0001$ , unpaired Mann-Whitney test.

Sample	Control	<i>Ap1m2</i>
<b>Number of samples</b>	5	6
<b>Ultrastructural variability</b>	+	++
<b>Brush border</b>	Normal	Normal
<b>Subapical vesicular structures</b>	No	No
<b>Enlarged vacuolar structures/Mixed organelles</b>	No	<b>Enlarged degradative vacuoles (100%)</b> Various size, shape and electron density
<b>Lysosomes</b>	Small and normal (50%)	<b>Increased number of lysosomes and presence of giant lysosomes (100%)</b> Heterogeneous in size and content
<b>Ectopic lumen</b>	No	<b>Lateral lumen with microvilli-like structures, separated by ectopic junctions (100%)</b>
<b>Intercellular spaces</b>	No	<b>Loose connection between enterocytes (100%)</b>
<b>Apical junctions</b>	Normal	<b>Affected</b>
<b>Intermediate mixed cells</b>	No	<b>Presence of intermediate cells (100%)</b>
<b>Polarity</b>	Normal	<b>Inverted polarity (50%)</b>

**Supplementary Table 1.** Phenotypic analysis of Control and *Ap1m2* fully-differentiated organoids by TEM. The percentages correspond to the percentage of organoids displaying the indicated phenotype.