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Myosin-1b interacts with UNC45A and controls intestinal epithelial morphogenesis

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1 **Myosin-1b interacts with UNC45A and controls intestinal epithelial**
2 **morphogenesis**

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5 **Running Title: Myo1b and gut development**

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30 **Keywords:**

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33 **Summary statement (max 30 words)**

34

35

36 Myosin-1b is important for intestinal epithelium folding during zebrafish development and
37 participates in the villous atrophy clinical manifestation downstream UNC45A loss of function.

38

39

40 **Abstract**

41

42

43 Vesicle trafficking and the establishment of apico-basal polarity are essential processes in
44 epithelium morphogenesis. Myosin-1b, an actin-motor able to bind membranes, regulates
45 membrane shaping and vesicle trafficking. Here, we investigate Myosin-1b function in gut
46 morphogenesis and congenital disorders using cell line and zebrafish larvae as well as patient
47 biopsies. In a 3D Caco-2 cyst model, lumen formation is impaired in absence of Myosin-1b. In
48 zebrafish, both Morpholino knock-down and genetic mutation of *myo1b* result in intestinal bulb
49 epithelium folding defects associated with vesicle accumulation, reminiscent of a villous atrophy
50 phenotype. We show that Myosin-1b interacts with the chaperone UNC45A, genetic deletion of
51 which also results in gut folding defects in zebrafish. Loss of function mutations in *UNC45A* have
52 been reported in complex hereditary syndromes, notably exhibiting intestinal disorders associated
53 with villous atrophy. In *UNC45A*-depleted cells and in patient biopsies, Myosin-1b protein level
54 is strikingly decreased. The appearance of Myosin-1b aggregates upon proteasome inhibition in
55 cells points at a degradation mechanism of misfolded Myosin-1b in the absence of its chaperone.
56 In conclusion, Myosin-1b plays an unexpected role in the development of the intestinal epithelium
57 folds or villi downstream *UNC45A*, establishing its role in the gut defects reported in *UNC45A*
58 patients.

59

60

61 **Introduction**

62

63 The establishment of apico-basal polarity and lumen formation are two fundamental steps during
64 vertebrate intestinal epithelial morphogenesis (Chin et al., 2017). The actin cytoskeleton and the
65 vectorial vesicle trafficking play a major role in the initiation and maintenance of this process,
66 leading to a stable single layer of cells with distinct apical and basolateral domains (Lubarsky and
67 Krasnow, 2003, Martin-Belmonte and Mostov, 2008). The apical membrane of the enterocyte is
68 further organized in microvilli, plasma membrane protrusions, which are supported by bundles of
69 parallel actin filaments and interacting proteins interconnected at the basis through a network of
70 actin, spectrin, and myosins known as terminal web (Revenu et al., 2004). The interaction between
71 neighboring polarized cells is further strengthened by the formation of cadherin-based adherens
72 junctions and claudin-based tight junctions. A proper polarization of the intestinal epithelium is
73 essential to achieve its main physiological roles, such as fluids and nutrient absorption and
74 secretion. Indeed, defects in intestinal epithelial cell polarity and apical lumen formation result in
75 early onset intestinal disorders, usually appearing in the first days of life (Kwon et al., 2020).
76 Recently, loss of function (LOF) mutations in the chaperone *UNC45A* were reported in families
77 presenting complex phenotypes including congenital diarrhea and several degrees of villous
78 atrophy (Esteve et al., 2018). *UNC45A* belongs to the conserved UCS protein family (*UNC-*
79 *45/CRO1/She4p*) of myosin co-chaperones.

80 Myosins 1 are single-headed actin motors targeted to membranes. Myosin1b (*Myo1b*) was detected
81 in mouse enterocyte brush borders in a mass spectrometry analysis (Revenu et al., 2012). Studies
82 in cell cultures reported that *Myo1b* associates with organelles and regulates membrane trafficking
83 by controlling their morphology (Almeida et al., 2011). *Myo1b* can pull out membrane tubes along
84 actin bundles immobilized on a solid substrate (Yamada et al., 2014) and it controls the formation
85 of repulsive filopodia, the redistribution of actomyosin fibres driving cell repulsion (Prosperi et al.,
86 2015) as well as the formation of axons in cultured neurons by controlling actin waves (Iuliano et
87 al., 2018). Despite this progress in understanding *Myo1b* function *in vitro* and in cellular systems,
88 its function in tissue biology, especially in the intestinal epithelium where it is expressed, remains
89 unexplored. This work investigates this question in the context of gut epithelia development and
90 morphogenesis.

91

92 Here we show that *Myo1b* is one of *UNC-45A* interactors, suggesting a role for myosin1b in the
93 pathogenesis of *UNC45A* deficiency. *Myo1b* localizes at the apical brush border of intestinal
94 epithelial cells in humans and loss of *Myo1b* in enterocyte like Caco-2 cells impairs epithelial

95 morphogenesis. In zebrafish, genetic inactivation of Myo1b affects intestinal bulb fold formation
96 revealing its conserved function during normal intestinal epithelia development.

97

98 **Results**

99

100 **Myo1b is expressed in the gut epithelium and concentrates apically**

101 Myo1b gene expression and protein localization were analyzed in intestinal epithelial cells. Myo1b
102 was detected by Western Blot and immunofluorescence in the human epithelial colorectal Caco-2
103 cells (Fig. 1A-B). It accumulated apically in polarised Caco-2 cysts, as demonstrated by its
104 colocalisation with the F-actin marker phalloidin demonstrating a localisation in actin-rich area,
105 microvilli and/or the subjacent terminal web (Fig. 1B). As this model is adenocarcinoma cells, this
106 expression and localization patterns could be the result of the tumoral state. To investigate Myo1b
107 distribution *in vivo*, we looked for the homologue of *myo1b* in the zebrafish *Danio rerio*. There is
108 one single *myo1b* gene with several splicing isoforms in the current zebrafish genome assembly.
109 The corresponding Myo1b protein shares 80% identity with the *Homo sapiens* and *Mus musculus*
110 homologues (Fig. 1C). In order to determine the expression pattern of *myo1b* during zebrafish
111 development, we performed whole mount *in situ* hybridization labelling with specific antisense
112 probes. *Myo1b* transcripts were unambiguously detected at 3dpf in the digestive tract of zebrafish
113 (Fig. 1D) coinciding with the onset of gut morphogenesis (Ng et al., 2005, Wallace et al., 2005).
114 *Myo1b* transcripts were also observed at 5dpf (Fig. 1D) when the intestine becomes functional and
115 compartmentalised in bulb, mid and posterior intestines. At this stage, the transcripts were
116 restricted to the intestinal bulb, the anterior part of the gut that forms large folds. Due to the lack
117 of a zebrafish specific antibody, endogenous Myo1b sub-cellular localisation could not be assessed
118 in zebrafish larvae. However, expressing eGFP-tagged Myo1b revealed apical accumulation of the
119 protein, as previously seen in Caco-2 cysts by immunofluorescence (Fig. 1E).

120 Myo1b is thus expressed in human intestinal epithelial cells and in the developing zebrafish
121 intestinal bulb epithelium, and it preferentially localises apically in the brush border.

122

123 **Myo1b loss of function Caco-2 cysts show normal apico-basal polarization but altered** 124 **luminal development.**

125 To address the function of Myo1b in enterocyte polarisation, *myo1b* was knocked-out using
126 CRISPR/Cas9 in Caco-2 cells (*myo1b* KO, Fig. 1 A-B). The global apico-basal polarity of Caco-

127 2 cysts was not affected in *myo1b* KO cells compared to controls as shown by the correct apical
128 concentration of F-actin, pERM and villin (Fig. 2 A-B).

129 Despite the absence of major polarisation defects, *myo1b* KO Caco-2 cells were more prone than
130 controls to the formation of cysts with multiple lumen (Fig. 2). Indeed, *myo1b* KO cells showed a
131 50% drop in the percentage of well-formed cysts with single central lumen compared to controls
132 (Fig. 2C).

133

134 ***Myo1b* loss of function has no major impact on epithelial cell differentiation of zebrafish** 135 **intestinal bulb**

136 To investigate the implication of Myo1b in intestinal epithelium morphogenesis *in vivo*, we turned
137 to zebrafish as a good model for gut development. The zebrafish intestinal epithelium differentiates
138 from 3 days post fertilisation (dpf) where it is essentially a flat monolayered tube. At 5dpf,
139 epithelial folds are present, especially in the anterior most part of the gut, the intestinal bulb
140 (Wallace et al., 2005). These folds are equivalent to the mammalian villi, and although no crypts
141 are present in zebrafish, the region between folds will have a crypt-like role (Crosnier et al., 2005).
142 First, we designed a splice blocking Morpholino (*Myo1b*-MO) that is efficiently preventing proper
143 splicing of *myo1b* as determined by RT-PCR (supplemental Fig. 1A-B). At the concentration used,
144 *Myo1b* MO displayed no overt phenotype, despite occasionally a slight heart oedema
145 (supplemental Fig. 1C). To extend these results with a genetic loss of function model, we also
146 generated a mutated allele at the *myo1b* locus using the CRISPR/Cas9 system, resulting in the
147 insertion of a single base at the beginning of the open reading frame, as confirmed by sequencing
148 (supplemental Fig. 1D). This leads to a premature stop codon and to the lack of detection of the
149 protein by Western blot in gut lysates from adult homozygote mutants (*myo1b*^{-/-}, supplemental
150 Fig. 1E). As *myo1b* mRNA is maternally provided (supplemental Fig. 1F), maternal contribution
151 was suppressed by crossing *myo1b*^{-/-} mothers. As for the MO injections, the resulting maternal-
152 zygotic homozygous mutant larvae displayed no overt phenotype (supplemental Fig. 1C). In cross-
153 sections (Fig. 3A), the intestinal bulbs of *Myo1b* MO and *myo1b*^{-/-} larvae appeared smaller
154 compared to controls. A significant reduction of the number of cells per cross-section was observed
155 for both *Myo1b* MO and *myo1b*^{-/-} intestinal bulbs at 3 and 5dpf compared to their respective
156 controls (Fig. 3B). A reduction in the total cell number in the intestinal bulb could be the
157 consequence of increased apoptosis or reduced cell proliferation. No significant difference with
158 controls in the proportion of proliferative cells could be detected at 3 and 5dpf (supplemental Fig.
159 2A). A slight increase in the proportion of apoptotic cells could be detected at 5dpf but not at 3dpf
160 (supplemental Fig. 2B). This later increase in apoptosis can however not account for the reduced

161 cell number per section reported from 3dpf on and could more be a readout of increased cellular
162 stress level upon prolonged absence of Myo1b, as reported after the KO of other Myosins 1 in
163 mouse and drosophila (Hegan et al., 2007, Tyska et al., 2005). Using specific markers for secreting
164 and absorptive cell lineages, defects in enterocyte differentiation could also be excluded
165 (supplemental Fig. 2 C-D). Finally, the microvilli marker Villin appeared properly localised
166 apically, lining the lumen together with F-actin (Fig. 3C), suggesting that apical polarity is not
167 affected in Myo1b MO and *myo1b*^{-/-} intestinal epithelium *in vivo*, as already shown in 3D Caco-
168 2 cell cultures (Fig. 2B).

169

170 ***Myo1b* loss of function zebrafish display MVID-like features in the intestinal bulb epithelium**

171 To analyse in 3D intestinal bulb epithelium morphogenesis in zebrafish, we used the BAC line
172 *cldn15la:cldn15la-GFP* that specifically labels the gut epithelium (Alvers et al., 2014). Both MO
173 and KO intestinal bulbs revealed single continuous lumen suggesting that early steps of lumen
174 fusion events were not affected (Alvers et al., 2014, Horne-Badovinac et al., 2001). However, in
175 Myo1b MO larvae, the intestinal bulb epithelium appeared most of the time flat at 5dpf, not
176 developing the expected folds observed in controls (Fig. 3D). Consistently with this phenotype, in
177 the KO model, we detected a significant reduction in fold length in KO versus control samples
178 (Fig. 3E). In an attempt to understand this milder phenotype in the mutant compared to the MO
179 condition, we analysed potential compensation mechanisms by other myosins 1 performing RT-
180 QPCR. On the 4 myosins 1 tested (*myo1ca*, *myo1cb*, *myo1d* and *myo1eb*), *myo1eb* showed a
181 reproducible increase of on average 60% of the WT expression level in the mutant using EF1a
182 (supplemental Fig. 1G) and RPL13a (not shown) as reference genes. *Myo1eb* is broadly expressed
183 in early developmental stages but has restricted expression patterns after 2dpf (mostly branchial
184 arches and pharynx) (Thisse and Thisse, 2004). A partial compensation of the loss of *myo1b* by an
185 upregulation of *myo1eb* could thus potentially explain the subtler and more restricted phenotype of
186 the mutant larvae compared to the knock-downs.

187 To further characterize the architecture of Myo1b-deficient intestinal bulb epithelium, a
188 histological analysis by transmission electron microscopy (TEM) was performed on 5dpf larvae.
189 It confirmed the affected folding of the intestinal bulb epithelium in MO and KO samples, and the
190 preserved apico-basal polarity of enterocytes (Fig. 4A-B). Quantifying microvilli length and
191 density did not reveal any significant defect resulting from Myo1b downregulation or absence,
192 although packing looked less regular (Fig. 4C). In contrast, a darker sub-apical band was visible
193 in Myo1b affected samples compared to controls, likely corresponding to modifications of the
194 terminal web (Fig. 4C). Moreover, this ultrastructural analysis showed an important accumulation

195 of vesicles in MO and KO samples compared to controls (Fig. 4B insets) suggesting defects in
196 membrane trafficking. These TEM observations (epithelium folding impaired, modifications of
197 the apical pole ultrastructure and trafficking defects) overall indicate that myo1b-deficient
198 enterocytes display some microvillus inclusion disease-like features.

199

200 **Myo1b is destabilized when Unc45A is affected**

201 Loss of function (LOF) mutations in the chaperone *UNC45 homolog A* have recently been
202 associated with rare human genetic syndromes notably presenting intestinal disorders, including
203 chronic diarrhea and villous atrophy of variable penetrance (Esteve et al., 2018). A pull-down
204 assay performed to detect potential Myo1b partners identified UNC45A as the most abundant
205 protein interacting with Myo1b in a mouse neuronal cell model (Supplementary table 1). This
206 interaction was confirmed by the reverse experiment using UNC45A as bait in the colorectal Caco-
207 2 cells (Duclaux-Loras *et al.*, submitted).

208 Considering this interaction, we investigated the impact of *UNC45A* depletion on Myo1b
209 expression. *UNC45A* depleted Caco-2 cells showed reduced Myo1b levels by
210 immunofluorescence (Fig. 5A). To detect aggregation-prone proteins normally sent to
211 degradation, the proteasome machinery was blocked using the proteasome inhibitor MG132.
212 MG132 induced the appearance of protein aggregates both in control and UNC45A KO Caco-2
213 cells (Fig. 5B). Myo1b staining partly co-localised with the aggresomes in the UNC45A KO
214 condition (Fig. 5B). This result suggests that Myosin1b proper folding requires the chaperone
215 UNC45A.

216 We finally looked at Myo1b expression in duodenal biopsies from control and *UNC45A* loss of
217 function patients. In control biopsies, the microvilli marker Villin was expressed apically all along
218 the epithelium and Myo1b was detected apically at the base of the villi and in crypts, partially
219 colocalising with Villin (Fig. 5C). In *UNC45A* patients, Villin was still localised apically whereas
220 Myo1b was barely detectable (Fig. 5D). In conclusion, Myo1b protein level is decreased in
221 *UNC45A*-depleted cells and in duodenal biopsies from an *UNC45A*-mutated patient.

222

223 **Discussion**

224

225 This work identifies Myo1b, an actin motor, as an unexpected player in the regulation of the
226 morphogenesis of the intestinal epithelium during gut development. In zebrafish, we report defects
227 in epithelial folding and villous atrophy when Myo1b is impaired. This phenotype is similar to the

228 ones reported in zebrafish and in human patients with loss of function mutations in *UNC45A*. We
229 show that Myo1b interacts with UNC45A and that Myo1b is destabilised in absence of UNC45A.

230

231 For this study, we analysed both *myo1b* mutant- and Morpholino-induced phenotypes. It is now
232 well established that Morpholino knock-downs often result in more severe overt phenotypes than
233 the corresponding knock-outs, at least partially due to the induction of genetic compensation
234 mechanisms in the mutants (Kok et al., 2015, Rossi et al., 2015). In the *myo1b* null case, we
235 observed a more subtle outcome than the Mopholino, which could be due to compensation
236 mechanisms (El-Brolosy and Stainier, 2017) as supported by the RT-QPCR of *myo1eb*. Here, the
237 intestinal bulb phenotypes observed with both approaches converged on reduced cell number of
238 intestinal bulb sections and impaired epithelial folding.

239

240 Up to now, myosins 1a, c, d and e had been identified in intestinal brush borders constituting the
241 apical pole of differentiated enterocytes (Benesh et al., 2010). Several class I myosins have been
242 implicated in the maintenance of intestinal epithelial differentiated state. Myo1a, which is
243 associated with the highly organised actin network of differentiated enterocytes in mammals
244 (Revenu et al., 2012, Tyska et al., 2005), but seems to lack in the zebrafish and *Drosophila*
245 genomes, is important for enterocyte polarity and participates in the structure and composition of
246 the brush border (Mazzolini et al., 2012, Tyska et al., 2005). The phenotype of the *myo1a* KO mice
247 is however mild, with reports of clear compensations by other class I myosins (Benesh et al., 2010,
248 Tyska et al., 2005). Likewise, two of the known class I myosins in *Drosophila* are also localised
249 in the apical pole of differentiated enterocytes and *Drosophila* Myo61F is necessary for the
250 stability of enterocyte apical organisation (Hegan et al., 2007). We report the expression of another
251 myosin 1 in the gut epithelium, Myo1b, and its apical localisation in enterocytes of Human
252 biopsies, of zebrafish intestinal bulb and in Caco-2 cells. Myo1b localisation in microvilli had
253 previously been reported in kidney epithelial cells (Komaba and Coluccio, 2015). In Human
254 biopsies, Myo1b is expressed at the base of the villi and in crypts suggesting a specific role in the
255 proliferative compartment and not in enterocyte differentiated state. We did not detect global
256 polarisation defects at the cell level or impaired differentiation in absence of Myo1b, whereas both
257 the Caco-2 3D model and the zebrafish model demonstrate morphogenetic defects at the tissue
258 level, respectively single lumen formation and folding.

259

260 Proliferation, apoptosis or differentiation defects do not account for the reduced cell number
261 observed on transverse sections of the intestinal bulb from 3dpf. As the sections give a 2D

262 overview of a 3D organ, this reduced cell number is thus likely the readout of the different
263 organisation in space of the epithelium. A specificity of the zebrafish intestinal bulb is the early
264 folding of the epithelium that remains pronounced to adulthood (Ng et al., 2005). The reduced
265 folding and villi formation in the mutants and morphants are clear signs of a different architecture
266 of the tissue. Also the mechanisms underlying intestinal epithelium folding are not yet fully
267 understood, the impact of tension and forces at the cell and tissue level driving compression, cell
268 intercalation and invagination through apical constriction have been investigated in other tissues
269 during development (Mammoto and Ingber, 2010). Myosins are central in the control of actin
270 cytoskeleton dynamics and in force generation (Reymann et al., 2012). In particular, Myo1b
271 deforms membranes and participates in organelle formation and trafficking (Almeida et al., 2011,
272 Coudrier and Almeida, 2011). It also remodels the actin cytoskeleton (Iuliano et al., 2018, Pernier
273 et al., 2019, Prosperi et al., 2015). Its roles in membrane traffic and in the dynamic organisation of
274 actin structures make it a plausible actor in the morphogenesis of the gut epithelium. The electron
275 microscopy data show a strong accumulation of intra-cellular vesicles in Myo1b mutant and
276 Morpholino tissues suggesting impaired trafficking, in agreement with its role in the formation of
277 post Golgi carriers and protein transport at the level of multivesicular endosomes (Almeida et al.,
278 2011, Salas-Cortes et al., 2005). Electron microscopy also reveals modifications of the terminal
279 web area, the apical actin belt linking adherens junctions in the epithelium, in agreement with its
280 role in actin dynamics. Myosin 1b function on actin dynamics and consequently on membrane
281 remodelling and membrane trafficking must impact cell and tissue mechanics (Buske et al., 2012),
282 and this way contributes to impaired intestinal epithelial folding in the absence or down-regulation
283 of *myo1b*. Myo1b restricted localisation at the base of the villi in human biopsies may indicate a
284 specific mechanical role in crypts morphogenesis.

285
286 Villous atrophy is a phenotype associated with various intestinal disorders including some rare
287 hereditary syndroms presenting congenital diarrhea like microvillous inclusion disease (MVID).
288 Mutations in MyosinVb are the main cause of MVID (Muller et al., 2008) and have notably been
289 associated with defective trafficking. A zebrafish mutant of *myoVb* develops a flat intestinal
290 epithelium (Sidhaye et al., 2016). Recently, loss of function (LOF) mutations in the chaperone
291 *UNC45A* were reported in families presenting complex phenotypes including congenital diarrhea
292 and several degrees of villous atrophy (Esteve et al., 2018). A zebrafish mutant for *UNC45A* also
293 exhibit loss of intestinal epithelium folding (Esteve et al., 2018). *UNC45A* is a chaperone
294 participating in the conformational maturation of, among others, some Myosins (Barral et al.,
295 2002, Lee et al., 2014, Lehtimaki et al., 2017). Our results in human cell lines and in patient

296 samples demonstrate a strong reduction in the protein level of Myo1b in absence of a functional
297 UNC45A variant, probably due to the degradation of misfolded and destabilised Myo1b. Myo1b
298 proper conformational folding would thus require the chaperone UNC45A. The intestinal
299 phenotypes associated with LOF mutations in *UNC45A* could hence partly be the consequence of
300 the reduced protein level of Myo1b. In conclusion, Myo1b contributes to gut morphogenesis and
301 appears as a potential player in the complex intestinal phenotype of the UNC45A LOF syndrome.

302
303

304 **Materials and Methods**

305

306 **CRISPR- Cas9 genome editing of MYO1B in Caco2 cells and 3D culture.** The lentiCRISPRv2
307 plasmid was a gift from F. Zhang (Massachusetts Institute of Technology, Boston, MA; plasmid
308 no. 98290, Addgene). The single-guide RNAs (sgRNAs) were designed using the CRISPR Design
309 Tool (Massachusetts Institute of Technology) and cloned into the BsmBI site. sgRNA sequences:
310 for-5- CACCGATCCCTACGAGATCAAGATA -3, rev-5- AAAC TA TCT TGA TCT CGT
311 AGG GAT C -3. Following production of lentiviral particles, the lentiCRISPRv2 plasmids were
312 transduced in Caco2 cells. Positively transduced cells were selected by puromycin (10µg/ml).
313 For 3D culture, CaCo2 cells were resuspended at a concentration of 10⁴ cells/mL in DMEM
314 (Gibco) with 20% FCS containing 4% Matrigel (BD Biosciences) and 2.5 10⁴ cells/well were
315 plated in 8-wells chamber slide IBIDI (Biovalley), previously precoated with 100 µL of Matrigel.
316 Cells were grown for 5 days to obtain cysts. To detect aggregation-prone proteins, the proteasome
317 inhibitor MG132 (Sigma-Aldrich) was added overnight in the culture medium at a concentration
318 of 10 µM.

319

320 **Western blot.** Caco-2 cells were lysed in RIPA buffer (Sigma) supplemented with 1X proteinase
321 inhibitor cocktail mix (Roche, Sigma). Adult zebrafish guts were dissected on ice and mechanically
322 lysed in 200µL lysis buffer (10 mM HEPES + 300 mM KCl + 5 mM MgCl₂ + 0,45% triton X100
323 + 0,05% Tween20, pH7) with 10 mM ATP and Complete protease inhibitor (Roche). 40 µg of
324 extracts in Laemmli buffer were loaded on a 4-12% polyacrylamide gradient concentration gel
325 (ThermoFisher). Primary antibodies used were mouse anti-tubulin (1:12000, Sigma), rabbit anti-
326 ratMyo1b (1,8 µg/µL, 1:500, (Salas-Cortes et al., 2005), anti GAPDH (1:1000, Cell Signaling).

327

328 **Phylogenetic analysis.** The Myo1b and Myo1a homologues in *Danio rerio*, *Homo sapiens*, *Mus*
329 *Musculus*, *Gallus gallus* and *Drosophila melanogaster* were obtained from NCBI HomoloGene.
330 Protein sequences were aligned and a phylogenetic tree was assembled using the online ‘One
331 Click’ mode at Phylogeny.fr (Dereeper et al., 2008).

332

333 **Molecular Cloning.** The *βactinhsp70:KalT4;cmc12:eGFP* construct was generated by combining
334 four plasmids using the Multisite Gateway system (Invitrogen): p5E-*βactinhsp70*, pME-KalT4,
335 p3E-polyA and pDEST-cmc12:eGFP containing Tol2 sites (Kwan et al., 2007). The *βactin*
336 promoter was cloned into the pCR-bluntII-TOPO vector (Invitrogen) and then inserted in the p5E-
337 MCS using KpnI and XhoI restriction sites. The 3’ 638bp of the zebrafish *hsp70* promoter (Dalgin
338 et al., 2011) was inserted into this p5E-*βactin* vector linearized with XhoI using the Gibson
339 Assembly Cloning Kit (New England Biolabs). The optimised Gal4 KalT4 (Distel et al., 2009)
340 was amplified and inserted in a pDONR221 using the Multisite Gateway system (Invitrogen).
341 To generate the *14UAS:ubc-eGFP-Myo1b* vector, eGFP-Rat Myo1b cDNA was amplified from a
342 previously published plasmid (Prosperi et al., 2015). It was inserted into the pT1UciMP plasmid
343 containing 14xUAS-ubc and Tol1 sites (Horstick et al., 2015) linearized with XhoI using the
344 Gibson Assembly Cloning Kit (New England Biolabs).

345

346 **Zebrafish (*Danio rerio*) husbandry.** Wild-type Tupfel long fin zebrafish strains were used and
347 raised according to standard protocols. Stable transgenic lines were generated by injection of the
348 plasmids with *tol2* or *tol1 transposase* mRNA at 25ng/μL in one-cell stage zebrafish embryos. The
349 transgenic BAC line claudin15-like-a fused to GFP (*cldn15la:cldn15la-GFP*) was kindly provided
350 by Michel Bagnat (Alvers et al., 2014).

351 For live-imaging, larvae were anaesthetised in 0.02% MS-222 and immobilised in 1% low melting
352 point agarose. Imaging was performed on a Zeiss LSM 780 confocal. All animal procedures were
353 performed in accordance with French and European Union animal welfare guidelines.

354

355 **In situ hybridization.** *In situ* hybridizations (ISH) were performed on larvae treated with 1-
356 phenyl-2-thiourea (PTU, Sigma-Aldrich) and fixed in freshly made 4% paraformaldehyde (PFA)
357 2-4h at RT and stored in 100% methanol at -20°C. After rehydration, larvae were treated with
358 proteinase K (20 μg/ml; Roche diagnostics) at RT for 1h (3dpf) or 2h (5dpf) and fixed again in 4%
359 PFA at RT for 20min. Digoxigenin-labelled antisense and sense RNA probes were synthesized by
360 *in vitro* transcription using DIG-labelled UTP according to the manufacturer's instructions (DIG
361 RNA labelling kit, Roche). Primers used were as follow: *myo1b* sense: CAA TAT GAT AGG

362 GGT AGG GGA CAT G ; antisense: TGG TTT GAA CTC AAT ATT TCC CAG C. Anti-DIG
363 antibody conjugated to alkaline phosphatase allowed detection of hybridized riboprobes according
364 to the manufacturer's instructions (Roche).

365

366 ***Myo1b* zebrafish mutant generation with CRISPR/Cas9.** The sgRNA sequence (sgB:
367 CTTCTGACAAGGGCTCTAGG) was cloned into the BsaI digested pDR274 vector (Addgene).
368 The sgRNAs were synthesized by *in vitro* transcription (Megascript T7 transcription kit, Ambion).
369 After transcription, sgRNAs were purified using the RNaseasy Mini Kit (Qiagen). For injections
370 at one cell stage, the synthesized sgB was injected at 300ng/ μ L after 5-minute incubation at RT
371 with Cas9 protein (NEB) at 2 μ M final in 20mM Hepes-NaOH pH 7.5, 150mM KCl (Albadri et al.,
372 2017). Injected embryos were grown to adulthood and crossed with wild-types to identify founders.
373 Pools of 20 embryos per clutch were lysed in NaOH 50mM at 95°C for at least 30min. PCR was
374 performed on lysates to amplify the genomic region targeted by the sgB with primers forward
375 5'GGGTGTTGTTTCAGCGATGGA and reverse 5'ATAGATCTCATTGTGATCGA using
376 Phusion High-Fidelity DNA polymerase (Thermo Scientific). The amplicons were cloned in pCR-
377 bluntII-TOPO vector (Zero Blunt Topo PCR cloning kit, Invitrogen) and sequenced (GATC
378 Biotech) to identify indels and the corresponding founder fish. Sequences were analysed using
379 Geneious. After selection of the founder, genotyping of the line was performed by PCR on fin clips
380 with primers 5'AGATGAATGCAAGCAAGCCATT and
381 5'ATACGATCTGATTGTGATCGAATCGCT. The resulting product was digested with
382 restriction enzyme FspBI, the site of which is lost in the mutant, resulting in 2 fragments (208 and
383 66bp) for the WT allele and only one (275bp) for the mutated allele.

384

385 **Morpholino oligonucleotide design and injections.** *Myo1b* splice blocking morpholino was
386 designed to target the splice donor site downstream of exon 22 (*Myo1b*-MO, 5'-
387 ATGAGAAACTGTGTTTCATTACCTGG). Experiments were performed in parallel with a
388 standard control-MO (5'-CCTCCTACCTCAGTTACAATTTATA) with no target in the zebrafish
389 genome. Morpholinos (Gene Tools) diluted at 1mM in water, were injected in 1-cell stage embryos
390 at a final concentration of 0.2mM. To validate *Myo1b*-MO knock-down, RT-PCR was performed
391 on 3dpf larvae. Total RNA of 50 larvae was prepared with TRIzol and TURBO DNase-free
392 reagents (Invitrogen). mRNA (1 μ g) was retro-transcribed using oligo(dT) primers and the
393 SuperScript III First-Strand Synthesis System (Invitrogen). To amplify the region targeted by the
394 MO (supplemental Fig. 1A), PCR was performed on the cDNA with two different forward primers

395 (primer 1 in exon21 was: 5'GGCTGCGATATTCTTGCCTCC, primer 2 at the edge of exon22 and
396 the targeted intron was: 5'TCTTTCATTCGTGGATGGAAGGCC) and the reverse primer
397 5'AACCCAGGTAATGAACACAGTTTCTAT. PCR products were run on a gel (supplemental
398 Fig. 2B) and bands were gel purified (Macherey-Nagel), inserted in a pCR-bluntII-Topo vector
399 (Invitrogen) and sent for sequencing (GATC) to assess intron retention.

400

401 **Quantitative RT-PCR.** For each experiment, total RNA was prepared from 3 pools of 50 embryos
402 per phenotype with TRIzol reagent and the TURBO DNA-free kit (ThermoFisher Scientific). RNA
403 (1µg) was retro-transcribed using random primers and the SuperScript III First-Strand Synthesis
404 system (ThermoFisher Scientific). For RT-QPCR, the SYBR Green PCR Master Mix
405 (ThermoFisher Scientific) was used according to the manufacturer's instructions and PCR were
406 performed on an ABI PRISM 7900HT instrument. Experimental triplicates of each sample were
407 averaged and the relative expression level quantified with the log₂ΔCT method using EF1a and
408 RPL13a reference genes. Shown are values normalised on the wild-type samples.

409

410 **Immunohistochemistry.** Caco-2 cells and cysts were fixed in 4% PFA 30min at 37°C and washed
411 with PBS. They were permeabilised in PBS, 0.2%TX100, 1%BSA for 5min at RT and blocked in
412 PBS 3%BSA for 1-2 hours at RT. Primary antibodies used were rabbit anti-ratMyo1b (1,8 µg/µL,
413 1:100, (Salas-Cortes et al., 2005), mouse anti-Villin clone ID2C3 (1:300, (Robine et al., 1985),
414 rabbit anti-pERM (1:100, abcam ab47293). After washes, they were incubated with Alexa Fluor
415 488 secondary antibody (Molecular probes), phalloidin-Alexa Fluor 568 and Dapi. To assess
416 protein aggregation, the Proteostat Aggresome Detection Kit (ENZO, ENZ-51035) was used.
417 Briefly, after the primary antibody rabbit anti-ratMyo1b, cells were incubated for 30min at RT with
418 goat anti-rabbit-Alexa Fluor 635 antibody (1:400, Molecular probes), Proteostat 1:400 and Hoechst
419 1:800 (ENZO) in PBS 3%BSA.

420 Zebrafish larvae were fixed for 2h at room temperature in 4% PFA and incubated in 30%
421 sucrose/0.1% PBST overnight at 4°C. They were then frozen in Tissue-Tek OCT (Sakura) at -80°C
422 and sectioned using a Cryostat (Leica). Zebrafish larvae sections were incubated in blocking buffer
423 (10% serum in PBST, PBS 0.1%Tween20) and with mouse anti-Villin clone ID2C3 (1:300), mouse
424 2F11 antibody (1:100, Abcam ab71286) or 4E8 (1:100, Abcam ab73643) overnight at 4°C. After
425 washes with PBST, they were incubated with Alexa Fluor 488 secondary antibody (Molecular
426 probes), phalloidin-Alexa Fluor 568 and Dapi. To assess apoptosis, TUNEL assay was performed
427 with reaction solutions from ApopTag Red In situ Apoptosis detection kit (Millipore) according to

428 the manufacturer recommendations. To assess proliferation, larvae were injected in the yolk with
429 10mM 5-ethynyl-2'-deoxyuridine (EDU) in 1% DMSO and incubated in 100 μ M EdU, 0.4%DMSO
430 for 20 hours after injection. Animals were fixed at indicated time and processed according to the
431 Click-iT EdU Imaging Kit (Invitrogen).

432 Paraffin embedded sections of intestinal tissues from a UNC45A deficient patient and biopsies
433 from controls were obtained for diagnosis or therapeutic purposes. Duodenal biopsies were
434 routinely fixed in 4% buffered formalin for 24 hours and paraffin-embedded. Sections were heated
435 for 1hr at 65°C and paraffin was removed by two 5-min washes in xylene. Sections were then
436 hydrated with ethanol solutions of decreasing concentrations. Unmasking of the epitopes was
437 performed at 100°C for 20 min in Citrate-based Antigen Unmasking Solution (Vector
438 Laboratories). Sections were incubated for 30 min at room temperature in blocking buffer (3%
439 BSA in PBS) and then overnight at 4°C with anti Myo1b antibody (1:200, Novus Biologicals
440 NBP1-87739) in blocking. After washes with PBST, sections were incubated with goat anti rabbit
441 Alexa Fluor 488 antibody (Molecular probes), phalloidin-Alexa Fluor 568 and Dapi for 2hrs at
442 RT.

443 After extensive washes and mounting in Vectashield (Vector Lab), all stainings were imaged on a
444 LSM780 confocal microscope (Zeiss). Images were processed and numbers of cells quantified
445 using ImageJ.

446

447 **TEM analysis on zebrafish larvae.** 5dpf larvae were collected and stored at 4°C in Trump's
448 fixative. Enhanced chemical fixation was performed in a mix of 4% PFA with 2.5% glutaraldehyde
449 in 0.1 mol/L cacodylate buffer overnight at 4°C. A 1.5-hour incubation in 1% OsO₄ was followed
450 by a 1.5-hour incubation with 2% uranyl acetate at ambient temperature. Larvae were then
451 dehydrated through graded ethanol solutions, cleared in acetone, infiltrated, and embedded in
452 Epon-Araldite mix (EMS hard formula). We used adhesive frames (11560294 GENE-FRAME 65
453 μ L; Thermo Fisher Scientific) for flat-embedding, as previously described (Kolotuev et al., 2012),
454 to gain better anteroposterior orientation and sectioning. Ultrathin sections were cut on an
455 ultramicrotome (UC7; Leica Microsystems) and collected on formvar-coated slot grids (FCF2010-
456 CU, EMS). Each larva was sectioned transversally in five different places in intestinal bulb with
457 ≥ 20 μ m between each grid to examine the sample over a large region. Each grid contained at least
458 4-6 consecutive sections of 70 nm. TEM grids were observed using a JEM-1400 transmission
459 electron microscope (JEOL) operated at 120 kV, equipped with a Gatan Orius SC1000 camera
460 (Gatan) and piloted by the Digital Micrograph program. Microvilli length and density were

461 quantified using Fiji on TEM pictures of at least 50 MV from 25 enterocytes of 3 larvae per
462 condition.

463

464 **Pull Down assay.** 10^6 N1E115 cells were transfected with pEGFP Myo1b (Salas-Cortes et al.,
465 2005) and lysed in TRIS 150mM, NaCl 150mM, EDTA 1mM, EGTA 1mM, ATP 10 mM, 10%
466 glycerol, 1mM DTT, 0,5% triton and protease inhibitor 24 hours after transfection. The lysate was
467 then incubated with 15 ml of GFP trap Beads (Chromotek) overnight. After washing the beads
468 were resuspended in water and treated for mass spectrometry analysis.

469

470 **Statistical analysis.** The numbers of cells reported are coming from manual counting. No
471 sample was excluded from the analysis, except for the total cell number per section where
472 we made sure to analyse samples displaying single cell layers through the whole gut cross-
473 sections and not the side of some villi. The sample size (n=) is defined as the number of larvae
474 analysed (one section per larva). For statistical analysis, we applied the non-parametric
475 Wilcoxon-Mann Whitney test.

476

477

478

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486

487 **Competing Interests**

488 No competing interests declared.

489

490 **Contributions**

491 MP generated the KO cells. JS and CR generated the *Myo1b* null allele in zebrafish. MR
492 generated the stable zebrafish transgenic lines. CR performed immunofluorescence
493 stainings. KD, JV and MR performed ISH. MP, MR, CR and RDL performed WB. KD and CR did

494 the RT-qPCR analysis. JS and JV genotyped the mutants. ON and GM did the TEM and analysis.
495 MP, RDL and CL prepared cell culture samples. PL generated preliminary data with
496 Morpholinos. MTP and EC performed the pull-down assay. CR analysed the results and wrote
497 the paper. FDB, EC and NCB supervised the work. MR, MP, GM, EC and FDB edited the
498 manuscript.

499

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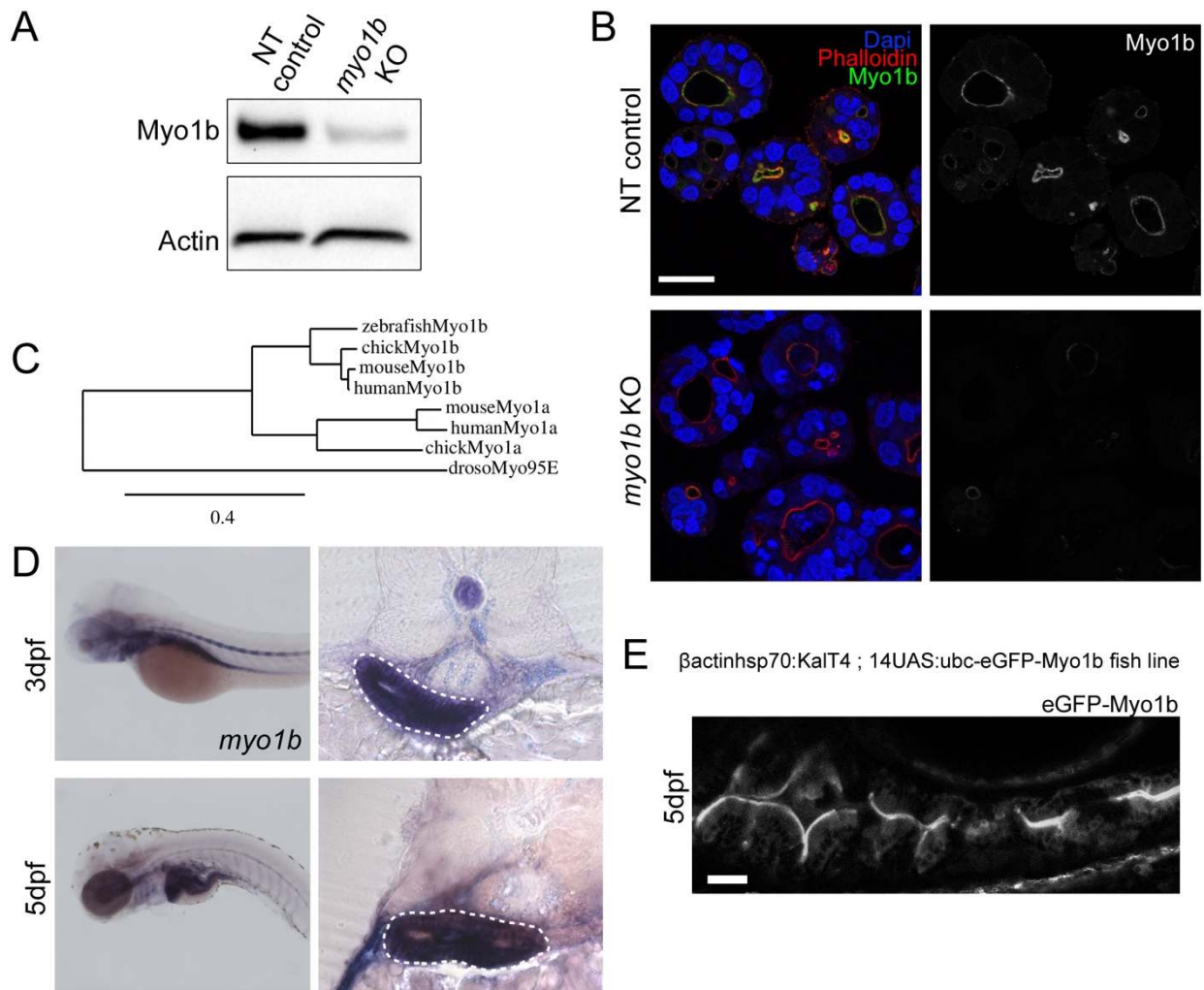
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636

637



Revenu et al., Figure 1

638

639 **Figure 1**

640 **Myo1b expression and apical localisation in gut epithelial cells.** **A-** Western blot analysis of
641 Myo1b expression in extracts from non-targeted (NT) control and *myo1b* targeted Caco-2 cells
642 (KO) using CRISPR/Cas9. **B-** Confocal sections of Caco-2 3D cultures stained for Myo1b, F-actin
643 (phalloidin) and nuclei (Dapi). **C-** Phylogenetic tree based on protein sequence of zebrafish, chick,
644 human and mouse Myo1b and Myo1a and drosophila Myosin95E. **D-** In situ hybridization for
645 *myo1b* transcripts on 3 and 5 dpf zebrafish larvae whole mounts (left panel) and cross-sections at
646 the level of the intestinal bulb (right panels). On sections, the forming intestinal bulb is circled
647 with white dashed lines. **E-** Live, longitudinal (antero-posterior axis) confocal section of the
648 intestinal bulb of a 5 dpf zebrafish larva expressing the transcription activator KalT4 driving the
649 expression of the eGFP-Myo1b transgene under the control of an upstream activating sequence
650 (UAS). The precise construction of the transgenes is annotated in the panel. Scale bars 30 μ m.

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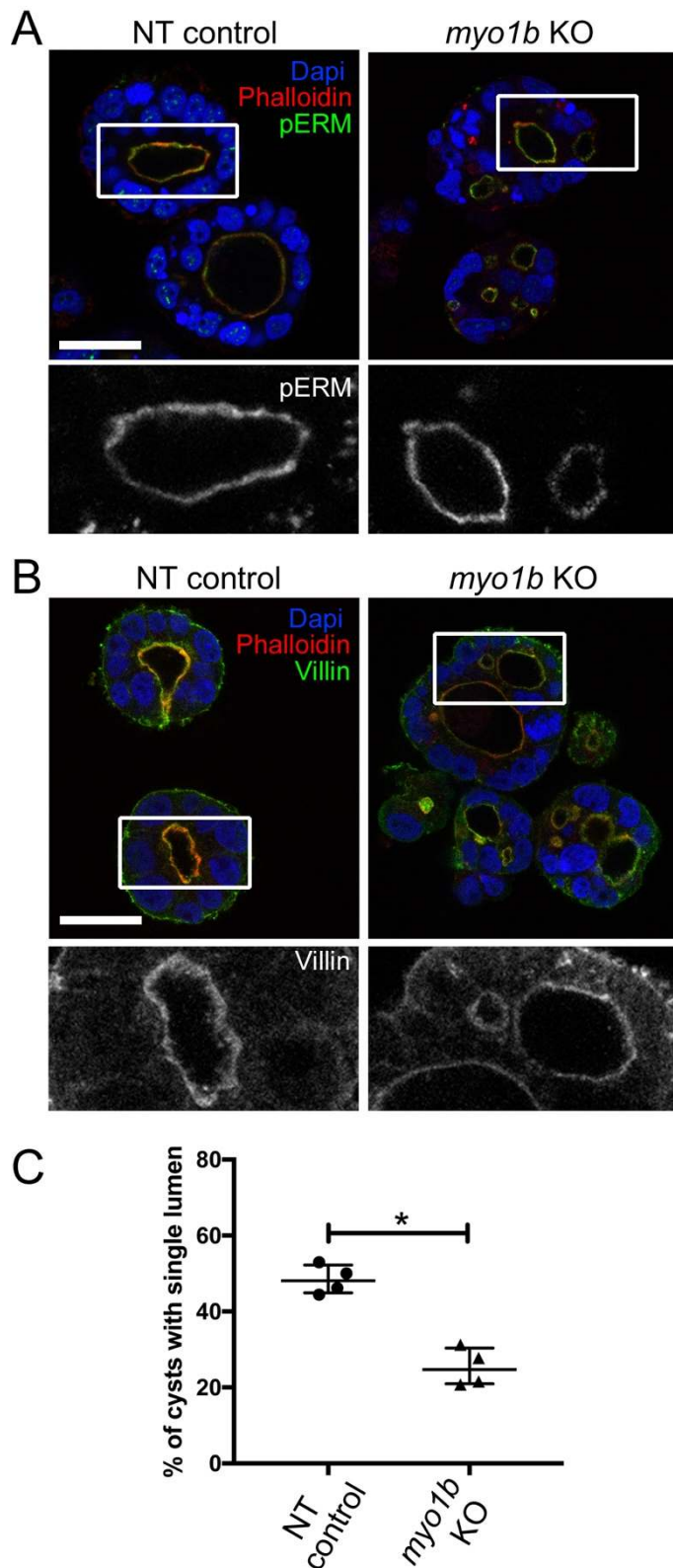
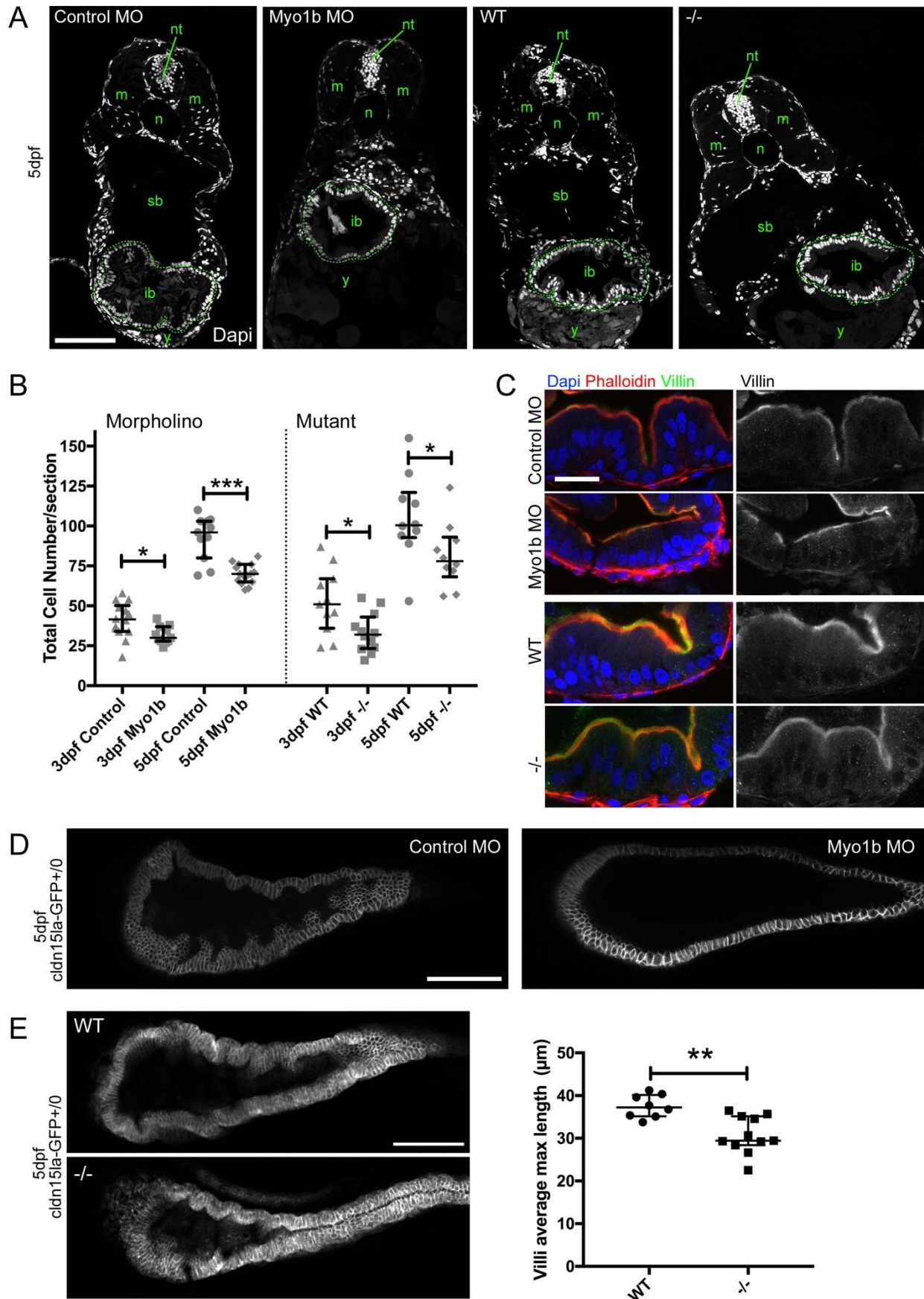


Figure 2

Enterocyte 3D cyst organization is affected in the absence of Myo1b despite normal apico-basal polarization. Confocal sections of NT control and *myo1b* KO Caco-2 3D cultures stained for the apical and microvilli markers phospho-Ezrin (pERM, **A**) and Villin (**B**). F-actin (phalloidin) and nuclei (Dapi) are stained, scale bars 30 μ m, boxed areas showed in insets are enlarged 2.5x. **C**-Quantification of the percentage of well-formed cysts with a single central lumen in NT control and *myo1b* KO Caco-2 3D cultures. Data represented are median and interquartile range from n=4 replicates, Wilcoxon test, *p<0.05.

Revenu et al., Figure 2

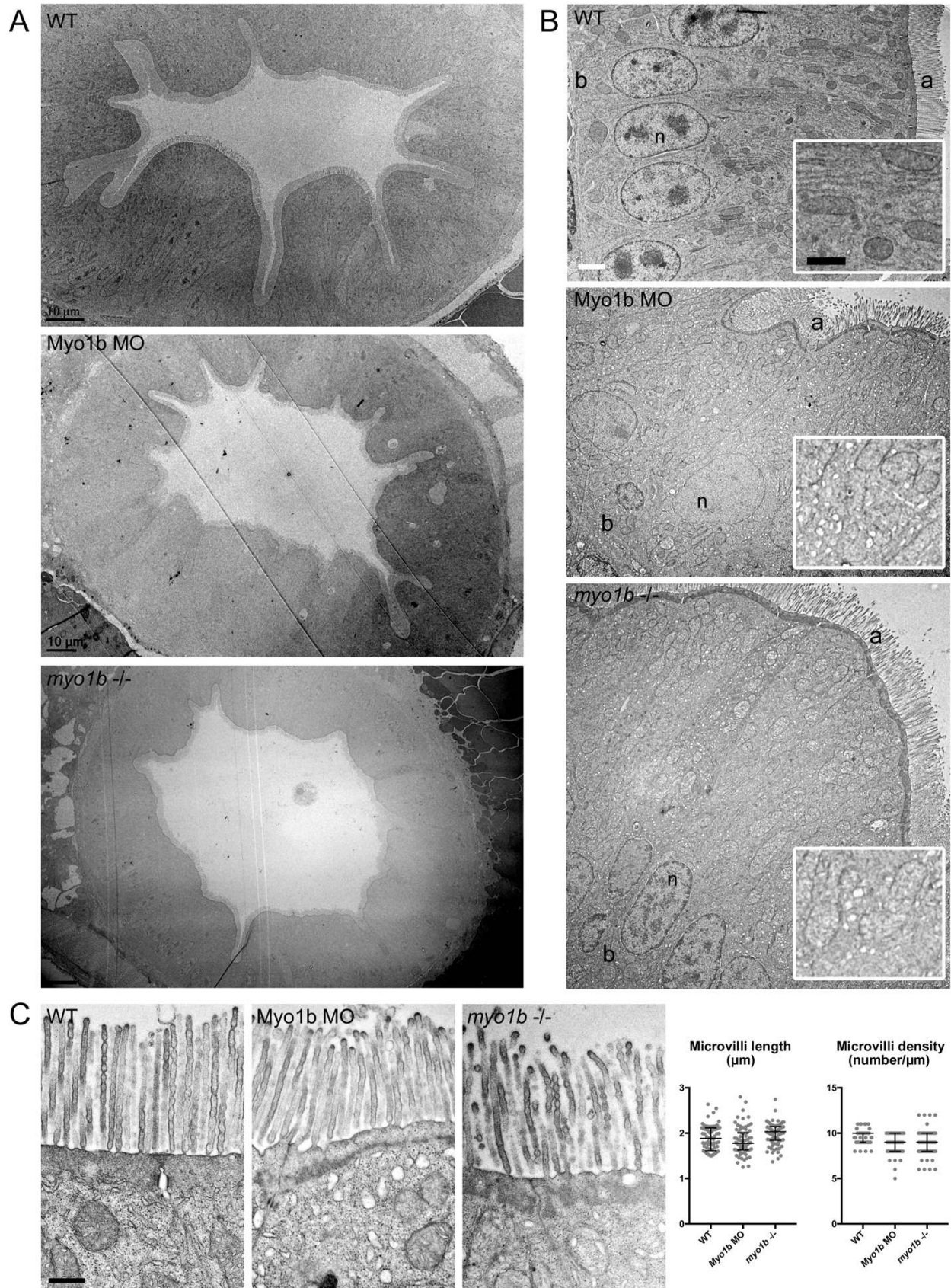


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Revenu et al., Figure 3

676 **Figure 3**

677 **Myo1b knock-down and knock-out impair intestinal bulb fold development.** **A-** Confocal
678 single optical sections stained with nuclear labelling (Dapi) of 5dpf larvae injected with control
679 and Myo1b Morpholinos (MO), and of 5dpf wild-type (WT) and *myo1b*^{-/-} (-/-) larvae. ib intestinal
680 bulb (circled with dashed lines), m muscles, n notochord, nt neural tube, sb swim bladder, y yolk.
681 Scale bar=100µm. **B-** Quantifications from Dapi stained sections of the total number of cells per
682 section at 3 and 5dpf in the four conditions. Data represented are median and interquartile range,
683 n=10 to 15, Wilcoxon test, *p<0.05, ***p<0.001. **C-** Confocal optical sections of the intestinal
684 bulb of 5dpf larvae in the four conditions stained for the microvilli marker Villin, F-actin
685 (phalloidin) and nuclei (Dapi) showing the preserved apico-basal polarity of enterocytes when
686 Myo1b is affected. Scale bar 20µm. **D-** Single confocal planes of live 5dpf larvae expressing
687 Cldn15la-GFP injected with control MO (left) and Myo1b MO (right). Note the flat epithelium in
688 the Myo1b MO condition. Scale bar=100µm. **E-** Single confocal planes of live 5dpf WT and
689 *myo1b*^{-/-} larvae expressing Cldn15la-GFP and quantification of the average length of the 3 longest
690 folds per intestinal bulb analysed. Scale bar=100µm. Data presented are median and interquartile
691 range, n_{WT}=8 and n_{-/-}=11, Wilcoxon test, **p<0.01.
692



693 Revenu et al., Figure 4

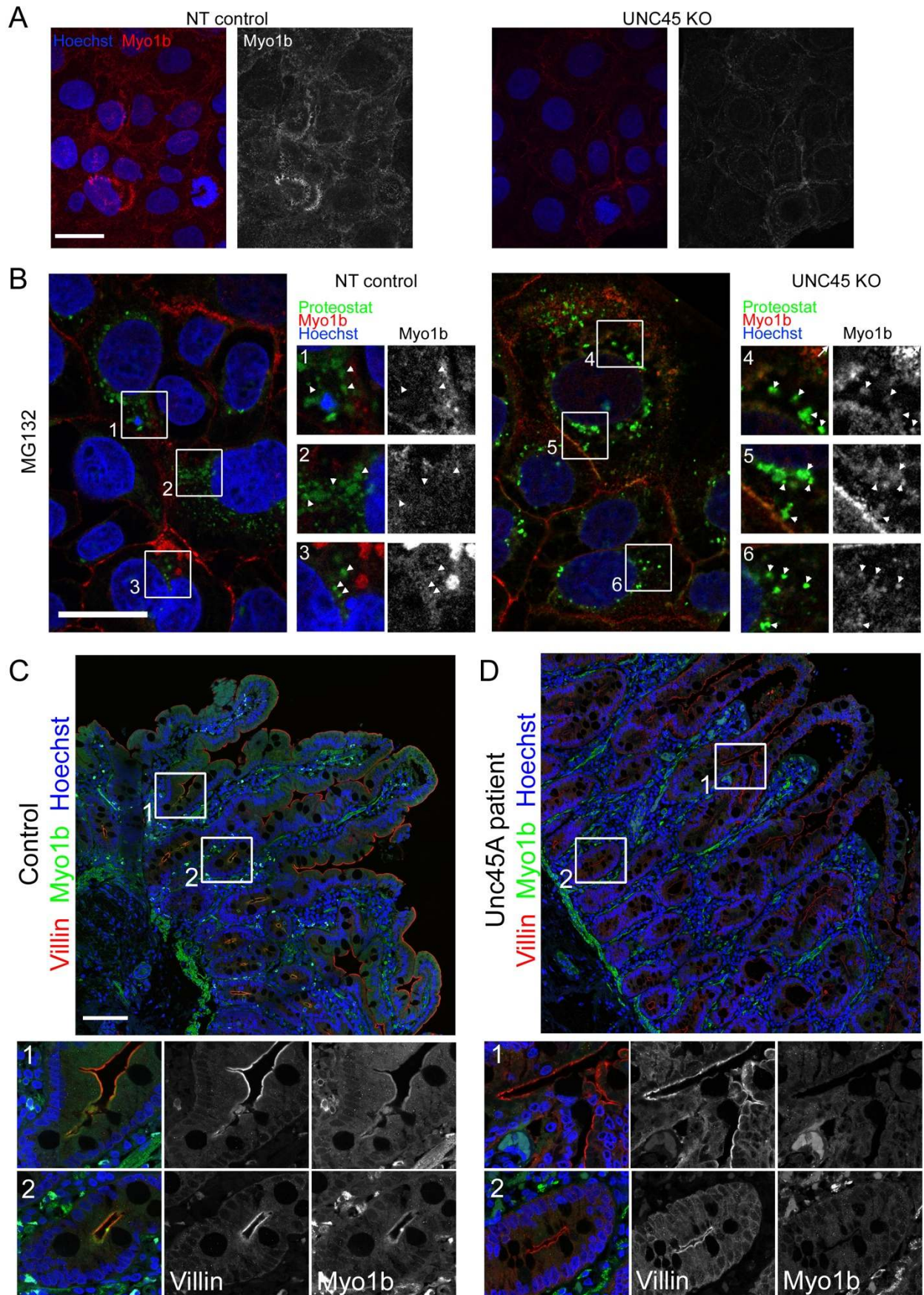
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696 **Figure 4**

697 **Electron Microscopy confirms folding defects and shows affected trafficking.** Transmission
698 electron micrographs of sections of intestinal bulbs from WT, Myo1b MO and *myo1b*^{-/-} 5dpf
699 larvae presenting a general view of the folds of the epithelium (**A**, scale bars 10µm) and of the
700 apico-basally polarized enterocytes (**B**, scale bar 2µm ; b basal, a apical, n nuclei). Insets in **B**,
701 show higher magnifications of the cytoplasm region to highlight the accumulation of vesicles in
702 Myo1b MO and *myo1b*^{-/-} samples, scale bar 1µm. **C**- Transmission electron micrographs of
703 sections of intestinal bulbs from WT, Myo1b MO and *myo1b*^{-/-} 5dpf larvae illustrating the
704 organization of the brush border, and quantifications of the average length and density of the
705 intestinal microvilli in the different conditions. Data presented are median and interquartile range,
706 $n_{\text{length}}=77$, $n_{\text{density}}=75$ per condition. Scale bar 500 nm.

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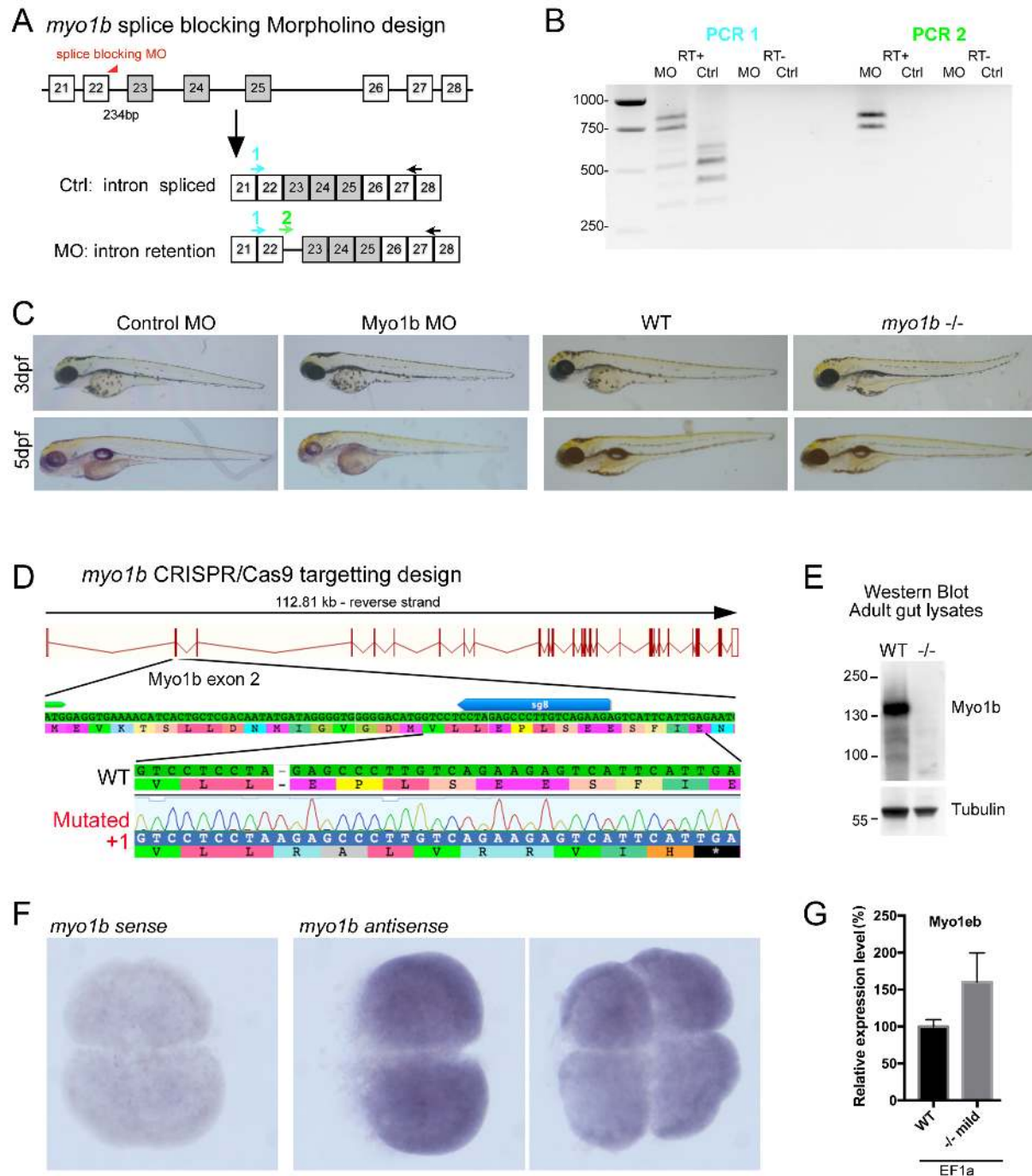
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Revenu et al., Figure 5

710 **Figure 5**

711 **Myo1b expression is destabilized in UNC45A depleted cells and in biopsies from UNC45A**
712 **mutated patients. A-** Immunohistochemistry analyses of Myo1b in non-targeted (NT) control and
713 UNC45A deficient (KO) Caco-2 cells show decreased Myo1b levels. Pictures are maximal
714 projections of confocal stacks, Hoechst labels nuclei, scale bar 30 μ m. **B-** Confocal sections of NT
715 control and UNC45A KO Caco-2 cells treated with the proteasome inhibitor MG132 and stained
716 for Myo1b and the aggresome probe Proteostat. Hoechst labels nuclei, scale bar 30 μ m. Boxed
717 areas showed in insets are enlarged 2x, arrowheads point at Proteostat-labelled protein aggregates
718 and highlight colocalisation with Myo1b proteins in UNC45A KO cells. **C,D-** Confocal sections
719 of a human biopsy from a healthy patient (control, C) and from a UNC45A LOF patient (D)
720 immuno-labelled for the microvilli marker Villin and for Myo1b ; Hoechst labels nuclei, scale bar
721 100 μ m. Boxed areas showed in insets are enlarged 3x, and highlight the apical localisation of
722 Myo1b in control tissue (C) at the base of the villi (1) and in crypts (2), which is essentially lost in
723 the UNC45A LOF tissue (D).

Supplementary Figures and Table

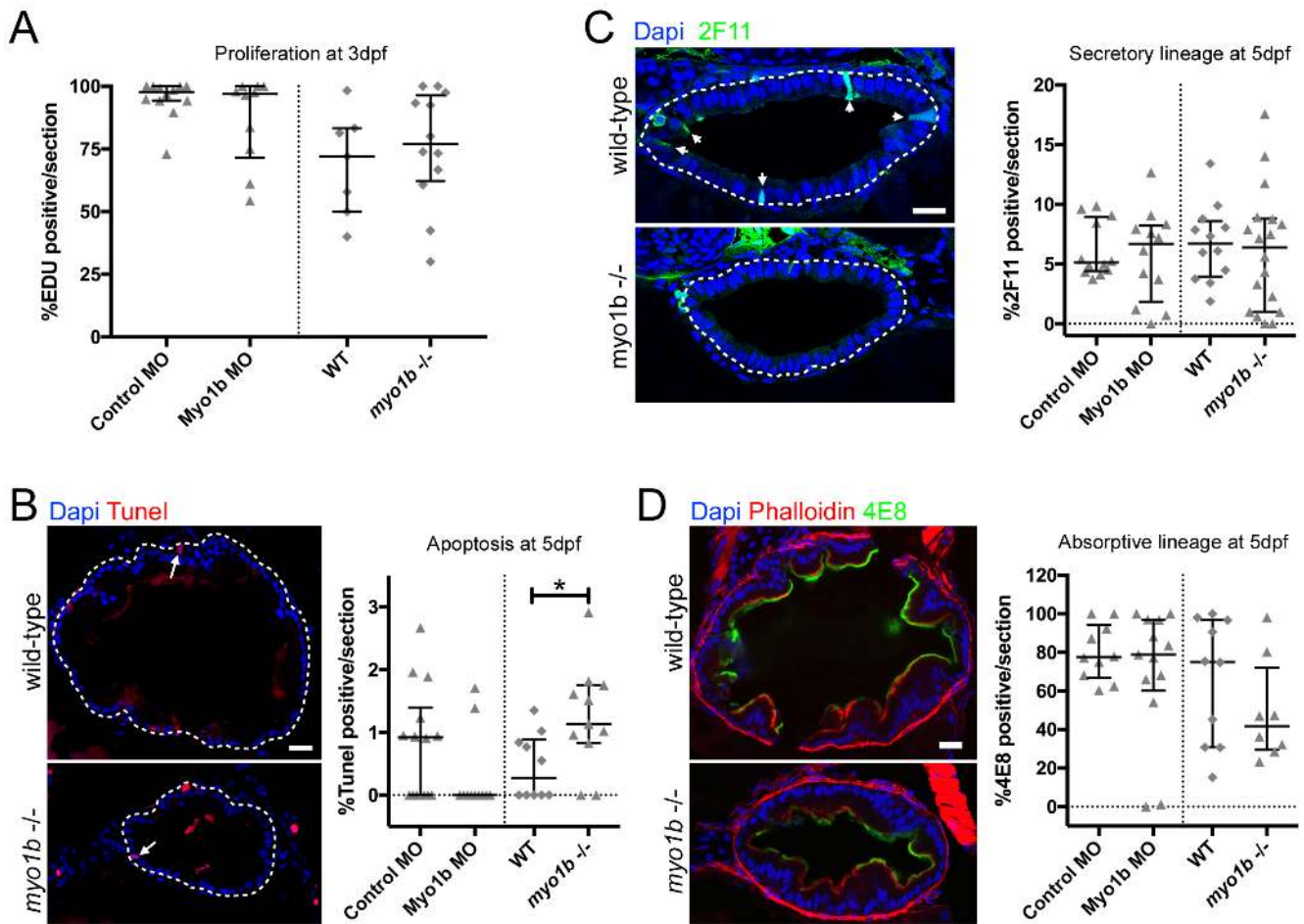


Revenu et al., Figure S1

Figure S1

Myo1b Morpholino and CRISPR mutant design and validation. **A-** Schematics of the design and **B-** DNA gel of the RT-PCR performed to control Myo1b-MO knock-down efficiency. Higher bands amplified in PCR1 MO compared to control and bands amplified in PCR2 MO correspond to Myo1b cDNA retaining the intron targeted by Myo1b-sMO, as verified by sequencing. In B, the multiple bands amplified, both in control and MO conditions, correspond to expected splicing variants of exons 23, 24 and 25 (highlighted in grey)

as checked by sequencing. RT- is the control RT without superscript compared to RT+. **C-** Bright field pictures of 3 and 5dpf larvae presenting the phenotypes of control and Myo1b Morpholinos, WT and *myo1b*^{-/-} larvae. **D-** Schematics of CRISPR/Cas9-mediated gene disruption at the *myo1b* genomic locus. The sgRNA (sgB, blue arrow) was targeting exon 2 downstream the start codon (ATG, green arrow). Compared to the WT sequence, the mutated allele displayed an insertion of 1bp generating a frame shift from amino-acid 21 and a premature STOP codon after 29 amino-acids. **E-** Western Blot with antibodies against Myo1b and Tubulin on lysates of dissected guts from WT and ^{-/-} adults. **F-** Negative control with a sense probe and in situ hybridisation with a *myo1b* anti-sense probe on wild-type embryos at 2 and 4 cell-stages showing maternal contribution for *myo1b* mRNA. **G-** RT-QPCR of *myo1eb* expression at 3dpf with EF1a used as reference gene, normalised on expression of the WT samples. Shown are mean and sem (WT=100.0±9.2, ^{-/-} =160.1±39.5 , n=6).



Revenu et al., Figure S2

Figure S2

Proliferation, apoptosis and differentiation are essentially unaffected in Myo1b MO and mutant conditions. **A-** Quantifications from EDU and Dapi stained sections of the proportion of cells in S-phase at 3dpf do not reveal significant differences in the proliferative rate of Control (n=12) vs Myo1b MO (n=10) and of WT (n=7) vs *myo1b*^{-/-} (n=12) samples. **B-** Confocal sections of intestinal bulbs stained for apoptosis (Tunel, red) at 5dpf in WT and *myo1b*^{-/-} samples and quantifications of the proportion of apoptotic cells in the four conditions (Control n=14, Myo1b MO n=11; WT n=10 ; *myo1b*^{-/-} n=11). **C, D-** Confocal sections of intestinal bulbs stained for differentiation markers in WT and *myo1b*^{-/-} samples and quantifications of the proportion of differentiated cells in the four conditions. At 5dpf, neither differentiation of the secretory lineage (**C** - 2F11, Control n=12, Myo1b MO n=12; WT n=12 ; *myo1b*^{-/-} n=18) nor differentiation of the absorptive lineage (**D** - 4E8, Control n=10, Myo1b MO n=13; WT n=10 ; *myo1b*^{-/-} n=8) are significantly altered. For all quantifications, data represented are median and interquartile range, Wilcoxon test, *p<0.05. For confocal images, nuclei are counterstained with Dapi (blue), bars=20µm.

Rank	Accession	Description	MW [kDa]	Coverage %	#Unique Peptides	#PSMs
1	P46735	Unconventional myosin-Ib OS=Mus musculus [MYO1B MOUSE]	128,483	65,31	102	633
2	Q99KD5	Protein unc-45 homolog A OS=Mus musculus [UN45A MOUSE]	103,3818	22,14	24	53

Table S1

Mass spectrometry result of the GFP-Myo1b pull down assay. UNC45A ranks second, directly after Myo1b. MW, molecular weight; #Unique Peptides, number of distinct peptide sequences identified; #PSMs (peptide spectrum matches) total number of identified peptide sequences for the protein.