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1 **Title: The E3 Ubiquitin Ligase Mindbomb1 controls zebrafish Planar Cell Polarity**

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12 **ABSTRACT**

13 Vertebrate Delta/Notch signaling involves multiple ligands, receptors and transcription factors. Delta
14 endocytosis – a critical event for Notch activation – is however essentially controlled by the E3 Ubiquitin
15 ligase Mindbomb1 (Mib1). Due to its position at a molecular bottleneck of the pathway, Mib1 inactivation
16 is often used to inhibit Notch signaling. However, recent findings indicate that the importance of Mib1
17 extends beyond the Notch pathway. We report an essential role of Mib1 in Planar Cell Polarity (PCP).

18 *mib1* null mutants or morphants display impaired gastrulation stage Convergence Extension (CE)
19 movements. Comparison of different *mib1* mutants and functional rescue experiments indicate that Mib1
20 controls CE independently of Notch. In contrast, Mib1-dependent CE defects can be rescued using the PCP
21 downstream mediator RhoA. Mib1 regulates CE through the RING Finger domains that have been
22 implicated in substrate ubiquitination, suggesting that Mib1 may control PCP protein trafficking.
23 Accordingly, we show that Mib1 controls the endocytosis of the PCP component Ryk and that Ryk
24 internalization is required for CE.

25 Numerous morphogenetic processes involve both Notch and PCP signaling. We show that Mib1, a known
26 Notch signaling regulator, is also an essential PCP pathway component. Care should therefore be taken
27 when interpreting Mib1 loss of function phenotypes.

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32 INTRODUCTION

33 Endocytic membrane trafficking is essential to control the abundance, localization and activity of cellular
34 signaling molecules. Depending on the biological context, the internalization of proteins from the cell
35 surface allow desensitization to extracellular stimuli, formation of endosomal signaling compartments, re-
36 secretion of signaling molecules through endosomal recycling or their lysosomal degradation (Hupalowska
37 & Miaczynska, 2012; Villaseñor et al., 2016). One key example for the importance of endosomal
38 membrane trafficking is provided by the Delta/Notch signaling pathway, where Delta ligand endocytosis
39 is required for Notch receptor activation (Chitnis, 2006; Fürthauer & González-Gaitán, 2009; Le Borgne,
40 Bardin, et al., 2005; Seib & Klein, 2021).

41 Notch receptors are single-pass transmembrane proteins that interact with Delta/Serrate/Lag2 (DSL)
42 family ligands (Bray, 2016; Hori et al., 2013). Productive ligand/receptor interactions trigger a series of
43 proteolytic cleavages that release the Notch Intracellular Domain (NICD) into the cytoplasm, allowing it
44 thereby to enter the nucleus and associate with transcriptional cofactors to activate target gene
45 expression. Studies in *Drosophila* and vertebrates revealed that Ubiquitin-dependent endocytosis of DSL
46 ligands in signal-sending cells is essential to promote Notch receptor activation in adjacent signal-receiving
47 cells (Deblandre et al., 2001; Itoh et al., 2003; Lai et al., 2001; Le Borgne & Schweisguth, 2003; Pavlopoulos
48 et al., 2001).

49 While the precise mechanism through which Delta promotes Notch activation is still under
50 investigation, current models suggest that the endocytosis of DSL ligands represents a force-generating
51 event that physically pulls on Notch receptors to promote their activation (Langridge & Struhl, 2017;
52 Meloty-Kapella et al., 2012; Seib & Klein, 2021). Notch pathway activation is therefore critically dependent
53 on Delta endocytosis, a process controlled by ligand poly-ubiquitination. Protein ubiquitination involves
54 ubiquitin-activating E1 enzymes, ubiquitin-conjugating E2 enzymes and substrate-specific E3 ubiquitin
55 ligases (Oh et al., 2018). Through their ability to recognize specific substrates, different E3 ligases control
56 the activity of various cellular signaling pathways.

57 Genetic studies revealed that two different RING (Really Interesting New Gene) finger domain E3 ligases,
58 Neuralized (Neur) and Mindbomb (Mib) control DSL ligand endocytosis in *Drosophila* (Lai et al., 2001; Le
59 Borgne, Remaud, et al., 2005; Le Borgne & Schweisguth, 2003; Pavlopoulos et al., 2001). Vertebrate
60 genomes harbor two *mib* and several *neur* homologues. However, mouse *neur1* or *neur2* single and
61 double mutants present no phenotypes indicative of defective Notch signaling (Koo et al., 2007). In
62 contrast, Notch signaling is severely impaired upon genetic inactivation or morpholino knock-down of

63 *mib1* in mice, *Xenopus* and zebrafish (Itoh et al., 2003; Koo et al., 2007; Yoon et al., 2008). In addition to
64 Mib1, its orthologue Mib2 as well as Asb11, a component of a multisubunit Cullin E3 ligase complex, have
65 been implicated in DSL ligand endocytosis (Sartori da Silva et al., 2010; Zhang, Li, & Jiang, 2007).
66 Nonetheless, mutational analysis in zebrafish failed to confirm a requirement for *mib2* in Notch signaling
67 (Mikami et al., 2015) and showed that a *mib1* inactivation is sufficient to essentially abolish the expression
68 of a transgenic Notch reporter in the central nervous system (Sharma et al., 2019). These findings identify
69 Mib1 as the major regulator of vertebrate DSL ligand endocytosis.

70 In addition to Delta ligands, Mib1 is able to interact with a number of additional protein substrates
71 (Berndt et al., 2011; Matsuda et al., 2016; Mertz et al., 2015; Tseng et al., 2014). Accordingly, functional
72 studies have implicated Mib1 in a growing number of functions that include the regulation of epithelial
73 morphogenesis (Dho et al., 2019; Matsuda et al., 2016) and cell migration (Mizoguchi et al., 2017),
74 centrosome and cilia biogenesis (Douanne et al., 2019; Joachim et al., 2017; Villumsen et al., 2013; Wang
75 et al., 2016; Čajánek et al., 2015), the control of glutamate receptor localization (Sturgeon et al., 2016) or
76 interferon production (Li et al., 2011).

77 A study in human cell culture identified the Receptor-like tyrosine kinase Ryk as a target of Mib1-
78 mediated ubiquitination (Berndt et al., 2011). Ryk is a single-pass transmembrane protein that binds Wnt
79 ligands through its extracellular Wnt Inhibitory Factor (WIF) domain. While an intracellular pseudokinase
80 domain appears devoid of functional enzymatic activity, Ryk has been suggested to regulate cell signaling
81 through scaffolding functions or the γ -secretase dependent release and nuclear translocation of its
82 intracellular domain (Roy et al., 2018). A number of studies have implicated Ryk in canonical, β -catenin
83 dependent Wnt signaling (Green et al., 2008; Lu et al., 2004; Roy et al., 2018). In this context, Mib1 has
84 been shown to promote the ubiquitination and internalization of Ryk, which appears to be required for
85 Wnt3A-mediated β -catenin stabilization/activation (Berndt et al., 2011). While experiments in *C.elegans*
86 provided evidence for genetic interactions between *mib1* and *ryk* (Berndt et al., 2011), the importance of
87 Mib1/Ryk interactions for vertebrate development or physiology has not been addressed.

88 In addition to its role in canonical Wnt signaling, several studies have linked Ryk to the non-canonical,
89 β -catenin independent, Wnt/Planar Cell Polarity (PCP) and Wnt/ Ca^{2+} pathways (Duan et al., 2017; Kim et
90 al., 2008; Lin et al., 2010; Macheda et al., 2012; Roy et al., 2018). *Ryk* mutant mice present a range of
91 diagnostic PCP phenotypes, including defects in neural tube closure and the orientation of inner ear
92 sensory hair cells (Andre et al., 2012; Macheda et al., 2012). During early fish and frog development, PCP
93 signaling regulates Wnt-dependent Convergent Extension (CE) movements that direct embryonic axis

94 extension (Butler & Wallingford, 2017; Davey & Moens, 2017; Gray et al., 2011; Tada & Heisenberg, 2012).
95 Different studies have suggested that Ryk may control PCP by acting together with Wnt-binding Frizzled
96 (Fz) receptors (Kim et al., 2008), regulating a Fz-independent parallel pathway (Lin et al., 2010) or by
97 controlling the stability of the core PCP pathway component Vangl2 (Andre et al., 2012).

98 In both vertebrates and invertebrates, the core PCP machinery is defined by three transmembrane
99 proteins Flamingo(Fmi)/CELSR, Fz and Strabismus/Vangl as well as their cytoplasmic partners Dishevelled
100 (Dvl), Prickle (Pk) and Diego (Dgo)/ANKRD6 (Butler & Wallingford, 2017; Devenport, 2014; Harrison et al.,
101 2020; Humphries & Mlodzik, 2018; Vladar et al., 2009). Polarity is established through formation of distinct
102 CELSR/Vangl/Pk and CELSR/Fz/Dvl/Dgo complexes at the opposite sides of the cell. The fact that similar
103 defects are often observed upon overexpression or inactivation of PCP pathway components suggests
104 that the levels of individual proteins need to be tightly controlled. It is therefore no surprise that factors
105 such as Dynamin, which governs endocytic vesicle scission, the early endosomal GTPase Rab5 and other
106 regulators of membrane trafficking have been shown to control the distribution of the transmembrane
107 proteins Fmi/CELSR, Fz and Vangl2 (Butler & Wallingford, 2017; Devenport et al., 2011; Mottola et al.,
108 2010; Strutt & Strutt, 2008). In addition to regulating the trafficking of core pathway components,
109 endocytosis ensures the PCP-dependent regulation of cellular adhesion molecules (Classen et al., 2005;
110 Ulrich et al., 2005).

111 While numerous studies therefore indicate a central role of membrane trafficking in PCP, it remains to
112 be established whether Ryk/PCP signaling is subject to endo-lysosomal control. In the present study, we
113 show that Mib1-mediated Ryk endocytosis is required for the PCP-dependent control of CE movements
114 during zebrafish gastrulation. The analysis of different *mib1* mutant alleles shows that the role of Mib1 in
115 PCP is separable from its function in Notch signaling. Our work thereby identifies a novel function of this
116 E3 ubiquitin ligase and establishes Mib1 as an essential regulator of the PCP pathway.

117

118 **RESULTS**

119 **Mindbomb1 regulates Convergent Extension independently of Notch**

120 Through its ability to promote Delta ligand endocytosis, the E3 Ubiquitin ligase Mindbomb1 plays an
121 essential role in vertebrate Notch receptor activation (Guo et al., 2016). In the course of experiments that
122 were initially designed to study the role of Notch signaling in the morphogenesis of the zebrafish nervous
123 system (Sharma et al., 2019), we realized that embryos injected with a *mib1* morpholino (*mib1*
124 morphants) present a reduced axial extension at the end of gastrulation that is indicative of defects in

125 embryonic Convergence Extension (CE) movements (Fig.1A). Accordingly, 2 somite stage *mib1* morphants
126 present a widening of the notochord, somites and neural plate (Fig.1B). The *mib1* exon/intron1 splice site
127 morpholino used in these experiments has been previously validated in different studies (Itoh et al., 2003;
128 Sharma et al., 2019). We further confirmed its specificity by showing that the co-injection of a WT *mib1*
129 RNA that is not targetable by the morpholino restores axis extension (Fig.1A).

130 The *mib1*^{ta52b} mutation in the C-terminal Mib1 RING finger domain (RF3, Fig.1C) disrupts the ability of
131 the protein to promote Delta ubiquitination and thereby disrupts Notch signaling (Itoh et al., 2003;
132 Sharma et al., 2019; Zhang, Li, & Jiang, 2007). Axial extension occurs however normally in *mib1*^{ta52b}
133 mutants (Fig.1D, Suppl.Fig.1A), raising the question whether Mib1 exerts a Notch-independent function
134 in CE. In accordance with this hypothesis, a constitutively activated form of Notch (NICD) that is able to
135 restore Notch-dependent defects in the nervous system (Sharma et al., 2019) fails to rescue *mib1*
136 morphant axis extension (Fig.1E, Suppl.Fig.2A).

137 Sequencing of the *mib1* cDNA in *mib1* morphants revealed a retention of intron1 that causes the
138 appearance of a premature Stop codon (Suppl.Fig.2B). As a consequence, the Mib1 morphant protein
139 comprises only the first 76 amino acids of WT Mib1 (Fig.1C). We hypothesized that this early termination
140 of the open reading frame could disrupt functions that are not affected by the *mib1*^{ta52b} point mutation.
141 Accordingly, *mib1* WT and *mib1*^{ta52b} mutant RNAs are equally capable of rescuing *mib1* morphant CE
142 phenotypes (Fig.1A,F, Suppl.Fig.2C).

143 As *mib1*^{ta52b} point mutants show normal CE, we further studied axis extension in *mib1* null mutants. The
144 previously reported *mib1*^{tfj91} allele causes a truncation of the Mib1 open reading frame after 59 amino
145 acids and therefore likely represents a molecular null (Fig. 1C) (Itoh et al., 2003). In contrast to *mib1*^{ta52b}
146 mutants, *mib1*^{tfj91} homozygous animals present CE defects that are statistically significant, although
147 weaker than in *mib1* morphants (Fig.1G,H, Cohen's d effect size = 0.49 for *mib1*^{tfj91}, 1.23 for MO *mib1*).
148 Similar phenotypes are observed for a new potential null allele generated in the present study (*mib1*^{nce2a},
149 Fig.1C,G, Suppl.Fig.1C,E) or in *mib1*^{tfj91/nce2a} trans-heterozygotes (Fig.1G). The *mib1*^{tfj91} and *mib1*^{nce2a}
150 mutations introduce stop codons shortly after the beginning of the *mib1* open reading frame ((Itoh et al.,
151 2003) and Suppl.Fig.1E), a mutation pattern that can cause nonsense mediated decay of mutant mRNAs
152 and could thereby trigger partial transcriptional compensation (El-Brolosy et al., 2019). Accordingly, *mib1*
153 transcript levels appear reduced in *mib1*^{tfj91} and *mib1*^{nce2a} but not in *mib1*^{ta52b} mutants (Fig.1I,
154 Suppl.Fig.1B,D).

155 Zebrafish Mib1 interacts with Epb4115 to regulate neuronal differentiation (Matsuda et al., 2016) and
156 with Catenin delta1 to control cell migration (Mizoguchi et al., 2017). Both of these activities are disrupted

157 in *mib1^{ta52b}* mutants (Matsuda et al., 2016; Mizoguchi et al., 2017). Our observation that *mib1* morphants
158 (Fig.1A) or *mib1* null mutants (Fig.1G) but not *mib1^{ta52b}* mutants (Fig.1D) present defects in gastrulation
159 stage axial extension identify thereby a novel role of Mib1 in the regulation of embryonic CE.

160

161 **Mindbomb1 RING finger domains are required for PCP**

162 Vertebrate CE requires non-canonical Wnt/PCP signaling (Butler & Wallingford, 2017; Davey & Moens,
163 2017; Gray et al., 2011; Tada & Heisenberg, 2012). To test whether Mib1 loss-of-function impairs PCP
164 pathway activity, we overexpressed the PCP downstream effector RhoA in *mib1* morphants. RhoA fully
165 restores axis extension (Fig.2A), suggesting thereby that Mib1 is required for the PCP-dependent control
166 of embryonic CE movements.

167 All Mib1 functions known to date require its E3 ubiquitin ligase activity that is dependent of the presence
168 of C-terminal RING finger domains (Guo et al., 2016). In contrast to the rescuing activity of WT Mib1
169 (Fig.1A), a truncated Mib1 variant that lacks all three RING Finger domains (Mib1^{ΔRF123}, Fig. 1C) enhances
170 the defects of *mib1* morphants as well as impairing axis extension in WT animals (Fig.2B). As RING finger
171 deficient Mib1 variants can act as substrate-sequestering dominant negatives (Guo et al., 2016), the
172 enhanced CE defects of Mib1^{ΔRF123}-injected *mib1* morphants are likely due to its capacity to interfere with
173 maternally provided Mib1 that is not targeted by the *mib1* splice morpholino. A Mib1 variant lacking only
174 the last RING finger (Mib1^{ΔRF3}, Fig.1C) produced similar results (Fig.2C).

175 Our results suggest that the substrate-ubiquitinating Mib1 RING-finger domains are required for PCP. As
176 Ubiquitin-dependent membrane trafficking is important for PCP (Butler & Wallingford, 2017; Devenport,
177 2014; Feng et al., 2021), we set out to determine whether Mib1 controls CE by regulating the trafficking
178 of a PCP pathway component.

179

180 **Convergent Extension requires Mindbomb1-dependent Ryk internalization**

181 Mammalian Mib1 has been shown to control the ubiquitin-dependent endocytic internalization of the
182 the Wnt co-receptor Receptor like tyrosine kinase Ryk (Berndt et al., 2011). Interestingly, studies in mice,
183 frogs and zebrafish have implicated Ryk in non-canonical Wnt/PCP signaling (Kim et al., 2008; Lin et al.,
184 2010; Macheda et al., 2012). To determine whether Mib1 regulates CE by controlling Ryk internalization,
185 we started by analyzing the effect of Mib1 gain or loss of function on Ryk localization. Ryk localizes to the
186 cell surface as well as intracellular compartments (Fig.3A), 70.7% of which are positive for the early
187 endosomal marker Rab5 (n=75 cells from 8 embryos, Suppl.Fig.3A) (Berndt et al., 2011; Kim et al., 2008;
188 Lin et al., 2010). In accordance with a role of Mib1 in promoting Ryk endocytosis, Mib1 overexpression

189 depleted Ryk from the cell cortex and triggered its accumulation in intracellular compartments (Fig. 3B)
190 without affecting the localization of the general plasma membrane marker GAP43-RFP (Suppl.Fig.3B,C).

191 To determine whether Mib1 specifically affects Ryk or acts as a general regulator of PCP protein
192 trafficking, we tested the effect of Mib1 overexpression on the localization of different transmembrane
193 proteins. Ryk has been shown to interact with the core PCP component Vangl2 whose endocytic trafficking
194 is crucial for PCP (Andre et al., 2012). In contrast to Ryk, Mib1 overexpression has no obvious effect on
195 Vangl2 localization (Fig. 3C,D). Similarly, we detected no impact of Mib1 overexpression on the localization
196 of Fz2 and Fz7, two Wnt receptors that have been implicated in PCP signaling (Kim et al., 2008; Lin et
197 al., 2010; Čapek et al., 2019) (Suppl.Fig.4A-D). Ryk belongs to a family of Wnt-binding Receptor Tyrosine
198 Kinases that have the particularity of harboring a most likely inactive pseudokinase domain (Roy et al.,
199 2018). Other members of this protein family include the Receptor tyrosine kinase-like Orphan Receptors
200 (ROR), among which zebrafish ROR2 has been implicated in CE (Bai et al., 2014; Mattes et al., 2018). Our
201 experiments failed to identify an effect of Mib1 overexpression on the localization of zebrafish ROR2 or
202 its orthologue ROR1 (Fig.Suppl.4E-H). Taken together, our observations suggest that Mib1 regulates CE
203 movements through the specific control of Ryk localization.

204 To determine if Mib1 function is required for Ryk endocytosis, we quantified the number of RYK-positive
205 endosomes in *mib1* morphants and *mib1*^{tf91} null mutants. In these experiments, RNAs encoding RYK-GFP
206 and Histone2B-mRFP were co-injected into *mib1* morphant or *mib1*^{tf91} mutant embryos. The Histone2B-
207 mRFP signal was used as an injection tracer to ascertain that the number of RYK-GFP positive endosomes
208 was scored in embryos that had received comparable amounts of injected material.

209 These experiments revealed a reduction in the number of Ryk-GFP positive endosomes in *mib1*
210 morphants (Fig.3E). Our observation that the CE defects of *mib1* morphants can be further enhanced by
211 the co-injection of antimorphic Mib1^{ARF123} or Mib1^{ARF3} constructs (Fig.2B,C) suggests that Mib1 activity is
212 only partially reduced in *mib1* morphants. Accordingly, increasing the amount of injected Ryk-GFP RNA
213 from 3 to 12 pg restores the number of Ryk positive endosomes in *mib1* morphants (Fig.3E). Even a high
214 dose of Ryk-GFP fails however to restore endosome number in embryos co-injected with *mib1* morpholino
215 and dominant-negative Mib1^{ARF123} (Fig. 3E,F,G, Suppl.Fig5A,B), suggesting that Ryk can no more be
216 internalized once Mib1 function is severely compromised.

217 Similar to *mib1* morphants, *mib1*^{tf91} null mutants present a reduction in the number of RYK-GFP positive
218 endosomes (Fig.3H,I,J, Suppl.Fig.5C,D). While stronger CE defects are observed in *mib1* morphants
219 compared to *mib1* mutants (Fig.1G), morphant and mutant embryos present a comparable reduction of
220 the number of RYK-GFP positive endosomes (Fig.3J, Cohen's d effect size = 3.02 for *mib1*^{tf91}, 2.99 for *mib1*

221 morphants). These observations suggest the existence of a compensatory mechanism that allows to
222 partially correct the PCP signaling defects that arise from a defect in Ryk endocytosis.

223 Why does impaired Ryk endocytosis cause CE defects? The phenotypes of Mib1-depleted embryos could
224 be either due to the loss of Ryk-positive endosomal compartments, or to the accumulation of non-
225 internalized Ryk at the cell surface. In *C.elegans*, Mib1 controls Ryk cell surface levels by promoting Ryk
226 internalization and degradation (Berndt et al., 2011). Similarly, zebrafish embryos injected with a high
227 level of Mib1 RNA present an overall loss of Ryk-GFP signal (Suppl.Fig.3D-F). If the CE phenotypes of *mib1*
228 morphants are indeed due to increased Ryk cell surface levels, then Ryk-GFP overexpression should
229 further enhance the defects of Mib1-depleted animals. The opposite prediction should however apply if
230 the CE defects of *mib1* morphants are due to the loss of Ryk-positive endosomal compartments. In this
231 case Ryk-GFP overexpression, which allows to restore the number of Ryk endosomes in *mib1* morphants
232 (Fig.3E), should also rescue the CE phenotypes of Mib1-depleted animals. In accordance with this later
233 hypothesis, Ryk-GFP overexpression restores axis extension (Fig.3K). In contrast, Vangl2 overexpression
234 did not display rescuing activity (Suppl.Fig.6).

235 If *mib1* morphant CE defects are due to a loss of Ryk-positive endosomes, *ryk* knock-down is expected
236 to further increase the severity of the observed phenotypes. To test this hypothesis, morpholinos directed
237 against *mib1* or *ryk* were injected separately or in combination. The injection of morpholino-insensitive
238 *mib1* or *ryk* RNAs allows to rescue the CE defects that are generated by their respective morpholinos,
239 validating thereby the specificity of the reagents (Fig.1A, Fig.4D, Suppl.Fig.7A). Co-injection of *mib1* and
240 *ryk* morpholinos causes CE defects that are significantly enhanced compared to single morphants (Fig.3L),
241 adding further support to our hypothesis that the CE defect of Mib1-depleted embryos are due to Ryk loss
242 of function.

243

244 ***ryk* mutants are insensitive to *mindbomb1* loss of function**

245 The abovementioned experiment shows that inhibiting Mib1 function enhances the CE defects of
246 animals that present a partial loss of Ryk activity due to morpholino knock-down (Fig.3L). If Mib1 regulates
247 CE by controlling Ryk endocytosis, Mib1 loss of function should however have no more enhancing effect
248 in animals that are not only partially, but entirely devoid of Ryk activity. To test this hypothesis, we used
249 Crispr/Cas9 mutagenesis to generate a stable *ryk* mutant line.

250 The use of a gRNA directed against exon 6 of zebrafish *ryk* led to the generation of *ryk*^{*nce4g*} mutants that
251 present an 11 base pair insertion at the target locus. The presence of the mutation in *ryk*^{*nce4g*} transcripts
252 was confirmed through sequencing of the complete *ryk* mutant cDNA (Fig.4A). The *ryk*^{*nce4g*} mutation

253 introduces a premature stop codon that truncates the extracellular domain and deletes the
254 transmembrane and cytoplasmic parts of the protein (Fig.4A). Accordingly, the *nce4g* mutation abolishes
255 the detection of an HA-tag located at C-terminus of the wild-type protein (Fig.4B,C). In contrast to wild-
256 type RNA, *ryk^{nce4g}* RNA fails to rescue *ryk* morphant CE defects (Fig.4D,E, Suppl.Fig.7A,B). While high levels
257 of WT *ryk* RNA induce severe embryonic malformations, no overexpression phenotypes were observed
258 using *ryk^{nce4g}* mutant RNA (Fig.4F).

259 Despite this evidence for functional *ryk* inactivation, our analysis failed to reveal CE defects in zygotic
260 *ryk^{nce4g}* mutants (Fig.4G, Suppl.Fig.7C). Frame-shift mutations can induce nonsense mediated degradation
261 of mutant transcripts and thereby trigger a process of transcriptional adaptation that could compensate
262 for the loss of function of the mutated protein (El-Brolosy et al., 2019). Accordingly, *ryk* transcript levels
263 are reduced in *ryk^{nce4g}* mutants (Suppl.Fig.7D).

264 Alternatively, the lack of CE phenotypes in zygotic *ryk^{nce4g}* mutants could be due to the persistence of
265 maternally deposited *ryk* RNA or protein. *ryk^{nce4g}* mutants are viable and fertile and can thereby be used
266 to generate embryos that are devoid of both maternal and zygotic Ryk function. In contrast to zygotic
267 mutants, Maternal Zygotic (MZ) *ryk^{nce4g}* mutants present highly significant CE defects compared to wild-
268 type control animals from the same genetic background (Fig.4H, see methods for details). WT *ryk* RNA
269 injection induces a significant rescue of CE, confirming thereby the specificity of the observed phenotypes
270 (Fig.4H, Suppl.Fig.7E). In further accordance with a loss of *ryk* activity in these animals, *ryk^{nce4g}* mutants
271 are insensitive to the injection of a translation-blocking *ryk* morpholino that can target both maternal and
272 zygotic RNAs (Suppl.Fig.S7F).

273 To determine the effect of *mib1* loss of function in embryos that are totally devoid of Ryk activity, we
274 introduced the *mib1^{tf191}* mutation in the MZ *ryk^{nce4g}* mutant genetic background. MZ *ryk^{nce4g};mib1^{tf191}*
275 double mutants display no significant difference in CE compared to MZ *ryk^{nce4g}* single mutants (Fig.4I). This
276 observation is in agreement with our model that the genetic inactivation of *mib1* results in a specific
277 impairment of Ryk activity. Accordingly, Mib1 loss of function has no further consequences on CE in
278 animals that are already devoid of Ryk.

279

280 **DISCUSSION**

281 Vertebrate Notch signaling involves multiple ligands, receptors and downstream transcription factors,
282 but the internalization of Delta ligands that triggers Notch receptor activation is regulated essentially by
283 Mib1 (Koo et al., 2007; Mikami et al., 2015). For this reason, numerous studies have turned to the
284 functional inactivation of *mib1* to inhibit Notch signaling in different biological contexts. A number of

285 recent studies have however shown that the role of Mib1 extends beyond the Notch pathway (Li et al.,
286 2011; Mizoguchi et al., 2017; Sturgeon et al., 2016; Villumsen et al., 2013; Čajánek et al., 2015). In the
287 present study, we have identified a novel Notch-independent function of Mib1 in the regulation of PCP-
288 dependent CE movements during zebrafish gastrulation.

289 We show that two potential *mib1* null alleles, *mib1^{tf91}* and the newly generated *mib1^{nce2a}* (which retain
290 only the first 59 or 57 amino acids of the 1130 residue wild type protein), cause defects in gastrulation
291 stage axis extension movements (Fig.1G). Similar defects are observed in *mib1* morphants (Fig.1A) but not
292 in *mib1^{ta52b}* mutants that present a missense mutation in the C-terminal RF domain (Fig.1D). In the context
293 of Delta/Notch signaling, the Mib1^{ta52b} mutant protein exerts a dominant-negative activity and thereby
294 causes Notch loss of function phenotypes that are even more severe than the ones observed for *mib1^{tf91}*
295 null mutants (Zhang, Li, Lim, et al., 2007). The fact that *mib1^{ta52b}* mutants present no defects in embryonic
296 CE suggests therefore that the role of Mib1 in CE is distinct from its function in Notch signaling. This
297 hypothesis is further substantiated by the finding that the CE phenotypes of *mib1*-depleted animals can
298 be rescued using the PCP pathway component RhoA (Fig.2A), but not with constitutively active Notch
299 (Fig.1E).

300 All known Mib1 functions require the C-terminal RF domains that are key for the protein's E3 ligase
301 activity. In accordance with a similar mode of action, a Mib1 variant that lacks all three RF domains is
302 unable to support proper CE (Fig.2B). While axis extension occurs normally in *mib1^{ta52b}* RF3 point mutants,
303 a Mib1 RF3 deletion variant is unable to support CE (Fig.2C). Previous studies revealed that the N-terminal
304 part of Mib1 interacts with specific protein substrates (Berndt et al., 2011; Guo et al., 2016). Our
305 observations show that the function of Mib1 in specific signaling pathways can also be altered through
306 alterations in RF3. As the RF domains interact with ubiquitin-conjugating E2 enzymes (Guo et al., 2016), it
307 is tempting to speculate that Mib1 might regulate Notch and PCP signaling using different E2 enzymes.

308 Mib1 promotes Ryk endocytosis in mammalian cell culture (Berndt et al., 2011), but the functional
309 relevance of this interaction for vertebrate development has not been addressed. Studies in *Xenopus* and
310 zebrafish identified Ryk functions in PCP-dependent morphogenetic processes (Kim et al., 2008; Lin et al.,
311 2010; Macheda et al., 2012). Our experiments in *mib1* null mutants and *mib1* morphants identify Mib1 as
312 an essential regulator of Ryk endocytosis and Ryk-dependent CE (Fig.3J,K).

313 Both *mib1* mutants and *mib1* morphants present a partial impairment of Ryk endocytosis (Fig.3J). Due
314 to this situation, we were able to restore the number of Ryk-positive endosomes by Ryk overexpression
315 (Fig.3E). The observation that Ryk overexpression not only rescues the number of Ryk-positive endosomes

316 but also the CE movements of *mib1*-depleted animals (Fig.3K) suggests that Ryk-positive endosomal
317 compartments are required for PCP signaling.

318 The partial loss of Ryk-positive endosomes that is observed in *mib1* mutants is most likely due to the
319 perdurance of maternally deposited products. Accordingly, Ryk endocytosis is nearly completely abolished
320 in embryos that have been co-injected with *mib1* splice morpholino (that blocks zygotic *mib1* production)
321 and dominant-negative Mib1^{ARF123} (that blocks maternal protein). The observation that under these
322 conditions neither the number of Ryk-positive endosomes (Fig.3E), nor embryonic CE (Fig.3K) can be
323 restored by Ryk overexpression suggests that Ryk endocytosis is essentially dependent on Mib1.

324 As a growing number of Mib1 substrates are being identified (Matsuda et al., 2016; Mertz et al., 2015;
325 Mizoguchi et al., 2017; Tseng et al., 2014), the question arises whether Mib1 could regulate the ubiquitin-
326 dependent trafficking of additional PCP pathway components. Our observations do not support this
327 hypothesis: First, Mib1 overexpression specifically promotes Ryk internalization, while having no
328 discernable effect on the localization of other PCP-related transmembrane proteins (Fig.3A-D,
329 Suppl.Fig.4). Second, our analysis of *mib1;ryk* double mutants shows that the CE defects that are already
330 observed in animals that are entirely devoid of Ryk are not further enhanced by the loss of Mib1 (Fig.4I).

331 Despite the fact that *mib1* null mutants and *mib1* morphants present a similar reduction in the number
332 of Ryk-positive endosomes (Fig.3J), CE defects are more severe in morphants than mutants (Fig.1G). The
333 *mib1^{tf91}* and *mib1^{ncc2a}* alleles used in our study introduce early stop codons in the *mib1* open reading
334 frame, a mutation pattern that has been reported to induce degradation of mutant transcripts and
335 upregulation of compensatory genes (El-Brolosy et al., 2019). In accordance with such a scenario, *mib1*
336 transcript levels are reduced in these *mib1* mutants (Fig.1I, Suppl.Fig.1D). While compensation could
337 potentially occur through a mechanism that promotes Mib1-independent Ryk endocytosis, our data do
338 not support such a hypothesis (Fig.3J). Instead, our observations indicate a mechanism of PCP pathway
339 resilience that allows to partially correct the defects that arise from a failure in Ryk endocytosis.

340 Taken together, our findings identify the E3 ubiquitin ligase Mib1 as an essential novel PCP regulator.
341 Mib1 regulates CE movements through the control of Ryk endocytosis, independent of its role in
342 Delta/Notch signaling. As processes such as the morphogenesis of the vertebrate neural tube involve both
343 Notch and PCP signaling, our data suggest that great care should be taken for the interpretation of Mib1
344 loss of function phenotypes.

345 **MATERIALS AND METHODS**

346 **Crispr/Cas mutagenesis:**

347 **Generation of a *mib1*^{n_{ce2a}} mutants**

348 Crispr/Cas9 mutagenesis of zebrafish *mib1* was performed using the reverse strand exon 1 target site
349 5'- GGAGCAGCGGTAATTGGCGG**CGG**-3' (bold lettering indicates the PAM motif). gRNA design and *in vitro*
350 transcription were performed according to reported protocols (Hruscha et al., 2013; Jao et al., 2013). A
351 pre-assembled complex of purified Cas9 protein (NEB) and gRNA was injected and the efficiency of
352 Crispr/Cas9-induced mutagenesis in the F0 generation monitored at 24 hpf using a T7 endonuclease assay
353 (Jao et al., 2013) on a PCR amplicon comprising the Crispr target region (Forward primer: 5'-
354 TGA**CT**GGAAAGTGGGGGAAGC-3', Reverse primer: 5'- TGCAGTATTAGAAACGCGTG-3'). Direct sequencing of
355 the same PCR amplicon was used to identify induced mutations in the F1 generation. This procedure led
356 to the identification of the *mib1*^{n_{ce2a}} mutant allele which introduces a frame shift after amino acid 57 and
357 induces the appearance of a premature stop codon after residue 69.

358

359 **Generation of a *ryk*^{n_{ce4g}} mutants**

360 Crispr/Cas9 mutagenesis of zebrafish *ryk* was performed using the reverse strand exon 5 target site 5'-
361 GGCAGAGTTTTGGGGGCTCT**TGG**-3' using the strategy mentioned above. The T7 endonuclease assay in
362 the F0 generation and mutation identification in F1 were performed using the same PCR amplicon
363 (Forward primer: 5'-GTGATGTTAGACTTGCATAC-3', Reverse primer: 5'-GAAGGTTTACAAGGGCAGAATG-
364 3'). The *ryk*^{n_{ce4g}} mutation introduces an 11 bp insertion that causes a frame shift after amino acid 196 and
365 induces the appearance of a premature stop codon after residue 214.

366

367

368

369 **Fish strains and molecular genotyping**

370 Unless otherwise specified, experiments were performed in embryos derived from an ABTÜ hybrid wild-
371 type strain. Mutant strains included *mib1*^{ta52b} (Itoh et al., 2003), *mib1*^{tfi91} (Itoh et al., 2003), *mib1*^{nce2a} (this
372 study) and *ryk*^{nce4g} (this study).

373 Depending on the experiment, *mib1* homozygous mutants were identified using molecular genotyping
374 (see below) or through the identification of the characteristic white tail phenotype that can easily be
375 identified by 36 hpf.

376 For the genotyping of *mib1*^{ta52b} mutants a 4-primer-PCR was used to identify WT and mutant alleles in
377 a single PCR. The primers used were: 5'-ACAGTAACTAAGGAGGGC-3' (generic forward primer), 5'-
378 AGATCGGGCACTCGCTCA-3' (specific WT reverse primer), 5'-TCAGCTGTGTGGAGACCGCAG-3' (specific
379 forward primer for the *mib1*^{ta52b} allele), and 5'-CTTACCATGCTCTACAC-3' (generic reverse primer). WT
380 and *mib1*^{ta52b} mutant alleles respectively yield 303 bp and 402 bp amplification fragments. As some
381 zebrafish strains present polymorphic *mib1* WT alleles, it is important to validate the applicability of this
382 protocol before use in a given genetic background.

383 For the genotyping of *mib1*^{tfi91} mutants two separate allele-specific PCRs were used to identify WT and
384 mutant alleles. The primers used were: 5'-TAACGGCACCGCCGCAATTAC-3' and 5'-
385 GCGACCCCAGATTAATAAAGGG-3' (WT allele), 5'-ATGACCACCGGCAGGAATAACC-3' and 5'-
386 ACATCATAAGCCCCGAGCAGCGC-3' (mutant allele).

387 For the genotyping of *mib1*^{nce2a} mutants two separate allele-specific PCRs were used to identify WT and
388 mutant alleles. The primers used were: 5'-GCAGGAATAACCGAGTGATG-3' and 5'-
389 AGCAGCGGTAATTGGCGG-3' (WT allele), 5'-GCAGGAATAACCGAGTGATG-3' and 5'-
390 GAGCAGCGGTAATTGAATA-3' (mutant allele).

391 For the genotyping of *ryk*^{nce4g} mutants a single PCR reaction was used to amplify the mutation-carrying
392 region (Forward primer 5'-GTGATGTTAGACTTGCATAC-3', Reverse primer 5'-

393 GAAGGTTTACAAGGGCAGAATG-3'). Due to the presence of an 11 bp insertion, mutant and WT alleles can
394 be distinguished on a 2.5% agarose gel.

395 To avoid issues related to variations among genetic backgrounds, the different adult fish used in the
396 course of our analysis of *ryk^{nce4g}* single and *ryk^{nce4g}; mib1^{tji91}* double mutants (Fig.4G,H,I, Suppl.Fig.7C,E,F)
397 were all derived from a single incross of *ryk^{nce4g/+}; mib1^{tji91/+}* parents.

398 For DNA extraction embryos were lysed 20 min at 95°C in 28.5 µl 50 mM NaOH, and then neutralized
399 by adding 1.5 µl Tris-HCl pH 7.5. PCR amplifications were carried out using GoTaq G2 polymerase
400 (Promega) at 1.5 mM MgCl₂ using the following cycling parameters: 2 min 95°C - 10 cycles [30 sec 95°C –
401 30 sec 65 to 55°C – 60 sec 72°C] – 25 cycles [30 sec 95°C – 30 sec 55°C – 60 sec 72°C] – 5 min 72°C.

402

403 **mRNA and morpholino injections**

404 Microinjections into dechorionated embryos were carried out using a pressure microinjector
405 (Eppendorf FemtoJet). Capped mRNAs were synthesized using the SP6 mMessage mMachine kit
406 (Ambion). RNA and morpholinos were injected together with 0.2% Phenol Red.

407 Morpholinos were injected at 500 (*mib1* 5'-GCAGCCTCACCTGTAGGCGCACTGT-3', (Itoh et al., 2003)) or
408 62.5 µM (*ryk* 5'-GGCAGAAACATCACAGCCCACCGTC-3').

409 RNA microinjection was performed using the following constructs and quantities: *Mib1^{ta52b}-pCS2+* (125
410 pg) and *Mib1^{ΔRF123}* (125 pg) (Zhang, Li, & Jiang, 2007). *Mib1-pCS2+* (12.5 pg unless otherwise indicated)
411 and *Mib^{ΔRF3}-pCS2+* (125 pg) (this study). *Ryk-GFP-pCS2+* (25 pg) and *Flag-Myc-Ryk-pCS2+* (50 pg) (Lin et
412 al., 2010). *Ryk-pCS2+* (0.75-25 pg), *Ryk^{nce4g}-pCS2+* (1.5-25 pg), *Ryk-HA-pCS2+* (25 pg), *Ryk^{nce4g}-HA-pCS2+*
413 (25 pg), *Ryk-GFP-pCS2+* (3-12 pg) (this study, all constructs have been engineered to abolish *ryk*
414 morpholino binding without altering the *Ryk* protein sequence). *GFP-Vangl2-pCS2+* (Mahuzier et al.,
415 2012). *Fz2-mCherry-pCS2+* (50 pg) (Lin et al., 2010). *Fz7-YFP-pCS2+* (25 pg) (Witzel et al., 2006). *ROR1-*
416 *GFP-pCS2+* (25 pg, this study). *ROR2-mCherry-pCS2+* (25 pg) (Mattes et al., 2018). *GFP-Rab5c-pCS2+* (50

417 pg, this study). RhoA-pCS2+ (25 pg) (Castanon et al., 2013). NICD-pCS2+ (37.5 pg) (Takke & Campos-
418 Ortega, 1999). GAP43-RFP-pCS2+ (25 pg). Histone2B-mRFP-pCS2+ (12.5 pg) (Gong et al., 2004). Histone2B-
419 GFP (6 pg) and Histone2B-tagBFP (1.5 pg) (this study).

420

421 **RNA *in situ* hybridization**

422 Whole mount RNA *in situ* hybridizations were performed as previously described (Thisse & Thisse,
423 2008). The *dlx3* probe has been previously described (Kilian et al., 2003). *ryk* antisense RNA was
424 transcribed from *ryk*-pBSK (this study). For *papc* and *mib1* *in situ* probes were transcribed from PCR
425 products that contained a T7 promoter sequence at their 3' end. The *papc* region amplified from genomic
426 DNA extended from 5'-TCCTTCTGCAGCTCGTCCGACTGGAAG-3' (forward strand) to 5'-
427 GGTAACCACCCACAGTTGAC-3' (reverse). The *mib1* probe region amplified from *Mib1*-pCS2+ extended
428 from 5'-CCCGAGTGCCATGCGTGTGCTGC-3' (forward) to 5'-CGCCGAATCCTGCTTTAC-3' (reverse).

429

430 **Immunocytochemistry**

431 Dechorionated embryos were fixed overnight at 4°C in PEM (80 mM Sodium-Pipes, 5 mM EGTA, 1 mM
432 MgCl₂) - 4% PFA - 0.04% TritonX100. After washing 2 x 5 min in PEMT (PEM - 0.2% TritonX100), 10 min in
433 PEM - 50 mM NH₄Cl, 2 x 5 min in PEMT and blocking in PEMT - 5% NGS, embryos were incubated 2 hrs at
434 room temperature with primary antibodies. Following incubation, embryos were washed during 5, 10, 15
435 and 20 min in PEMT, blocked in PEMT - 5% NGS, and incubated again with secondary antibodies for 2 hrs.
436 Embryos were again washed during 5, 10, 15 and 20 min in PEMT. The following primary antibodies were
437 used: Rat@HA (Roche 11 867 423 001, 1:500). Mouse@c-Myc9E10 (Santa Cruz sc-40, 1:250). Secondary
438 antibodies Goat@Rat-Alexa488 (Invitrogen) and Goat@Mouse-Cy5 (Jackson Immunoresearch) were used
439 at a dilution of 1:500.

440

441 **Microscopy and image analysis**

442 For confocal imaging, embryos were mounted in 0.75% low melting agarose (Sigma) in glass bottom
443 dishes (Mattek). Embryos were imaged on Spinning disk (Andor) or Laser scanning confocal microscopes
444 (Zeiss LSM710, 780 and 880) using 40x Water or 60x Oil immersion objectives. Bud stage axis extension
445 and *in situ* gene expression patterns were documented on Leica M205FA-Fluocombi or Leica MZ-FLIII
446 stereomicroscopes coupled to Lumenera color CCDs. Image analysis was performed using ImageJ
447 (<http://rbs.info.nih.gov/ij/>). Quantifications were performed blindfolded without knowledge of the
448 sample genotype.

449

450 **Statistical analysis**

451 Statistical analysis was performed using R. Data normality and variance were analyzed using Shapiro-Wilk
452 and Levene's tests and statistical tests chosen accordingly. Complete informations about sample sizes,
453 numerical values, and tests statistics for all experiments are provided in the Supplementary Material.

454

455 **Use of research animals**

456 Animal experiments were performed in the iBV Zebrafish facility (authorization #B-06-088-17) in
457 accordance with the guidelines of the ethics committee Ciepál Azur and the iBV animal welfare committee
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459

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461

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471

472 **AUTHOR CONTRIBUTIONS**

473 VMS, PS, AJK, SP and MF performed experiments and analyzed data. MP generated reagents used in the
474 study. MF designed the study and wrote the manuscript.

475

476 **COMPETING INTERESTS**

477 The authors declare no competing interests.

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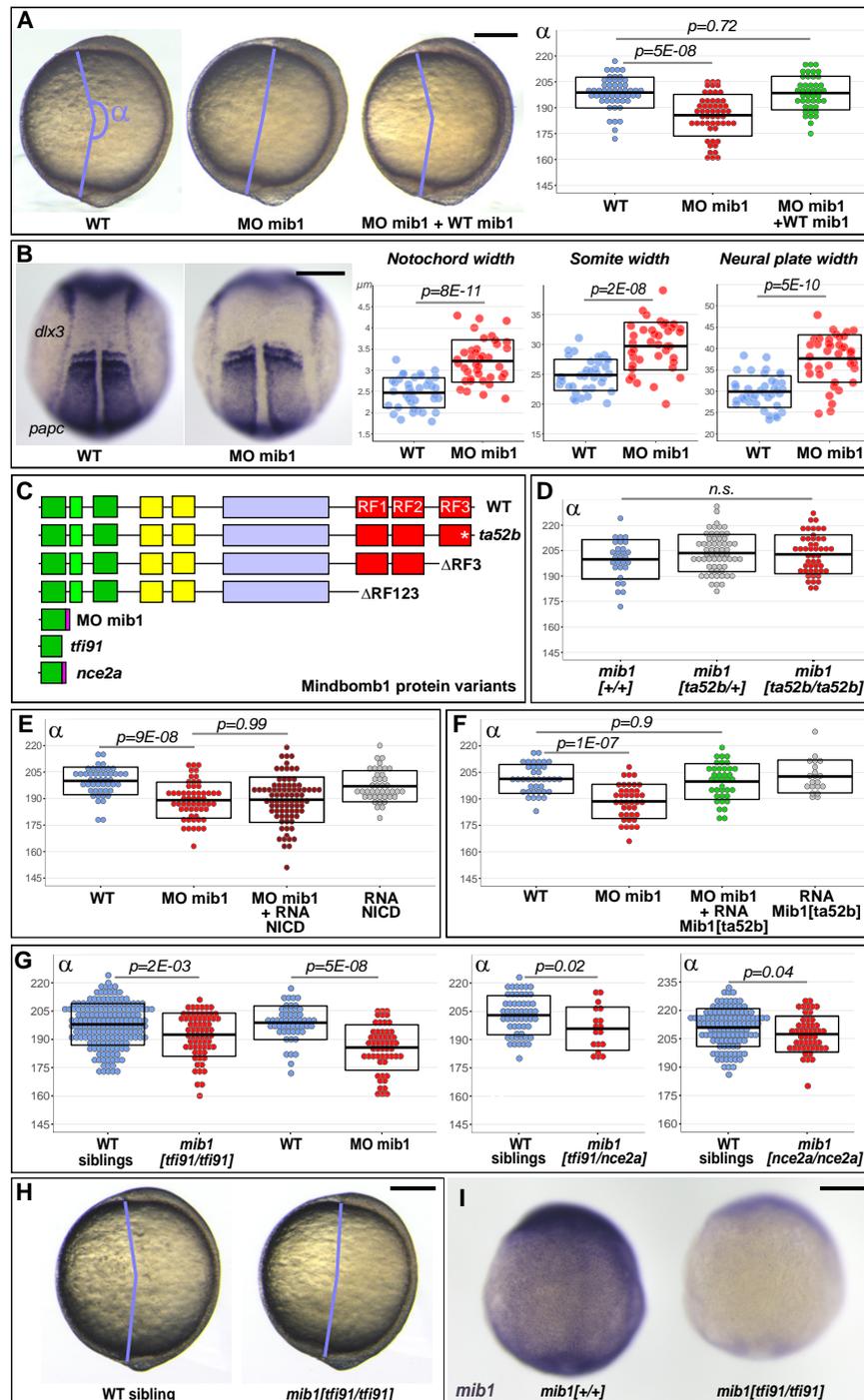


Figure 1: Mib1 regulates PCP-dependent convergent extension movements independently of Notch

(A) Axis extension was quantified at bud stage by measuring the axis extension angle α . Axis extension is reduced in *mib1* morphants but restored upon coinjection of WT *mib1* RNA. Lateral views of bud stage embryos, anterior up, dorsal to the right. (B) *mib1* morphants present a widening of the notochord, somites and neural plate. Dorsal views of 2 somite stage embryos, anterior up. *dlx3* *in situ* hybridization outlines the neural plate, *papc* the somites and the adaxial cells lining the notochord. Widths indicated in microns. (C) Mib1 protein variants used in the study. (D) The *mib1^{ta52b}* mutation has no effect on axis extension. (E) Constitutively activated Notch (NICD) fails to restore *mib1* morphant axis extension. (F) *mib1^{ta52b}* RNA injection restores *mib1* morphant axis extension. (G,H) Axis extension is impaired in *mib1^{tfi91}* or *mib1^{nce2a}* null mutants. On the left panel the *mib1* morphant data from Fig. 1A are included for comparison. (I) *In situ* hybridization reveals reduced *mib1* transcript levels in n=27 *mib1^{tfi91}* mutant embryos. Dorsal views of bud stage embryos, anterior up. To warrant identical acquisition conditions, two embryos were photographed on a single picture. Scalebars: 200 μ m. Boxes in (A,B, D-G) represent mean values \pm SD. See supplementary material for complete statistical information.

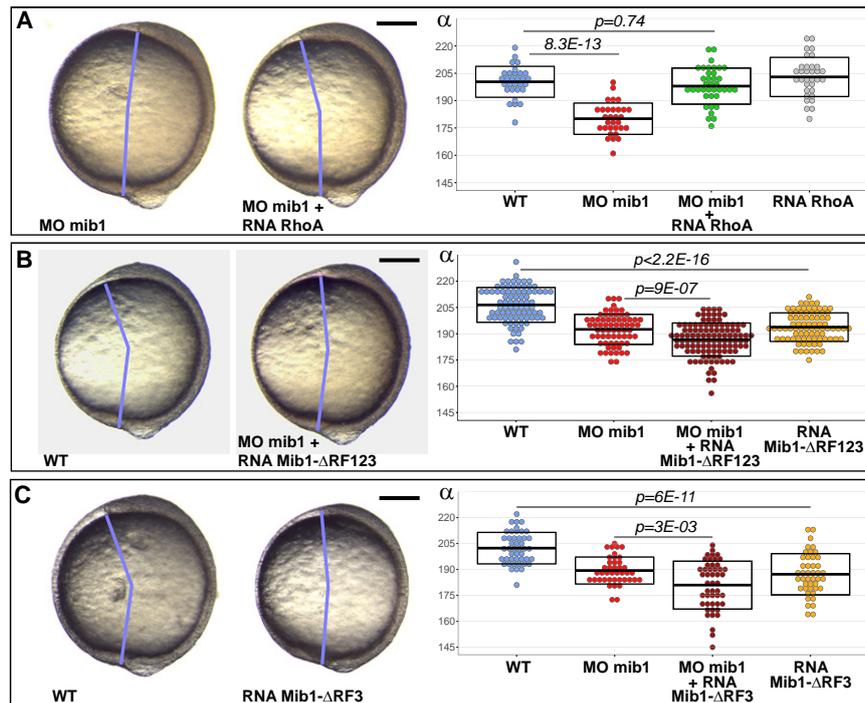


Figure 2: Mib1 controls PCP through its RING finger domains

(A) RhoA overexpression rescues mib1 morphant axis extension. **(B,C)** Mib1 proteins lacking all (Mib1 Δ RF123, B) or only the last (Mib1 Δ RF3, C) RING finger impair axis extension in mib1 morphant or WT embryos. Lateral views of bud stage embryos, anterior up, dorsal to the right. Scalebars: 200 μ m. Boxes represent mean values \pm SD. See supplementary material for complete statistical information.

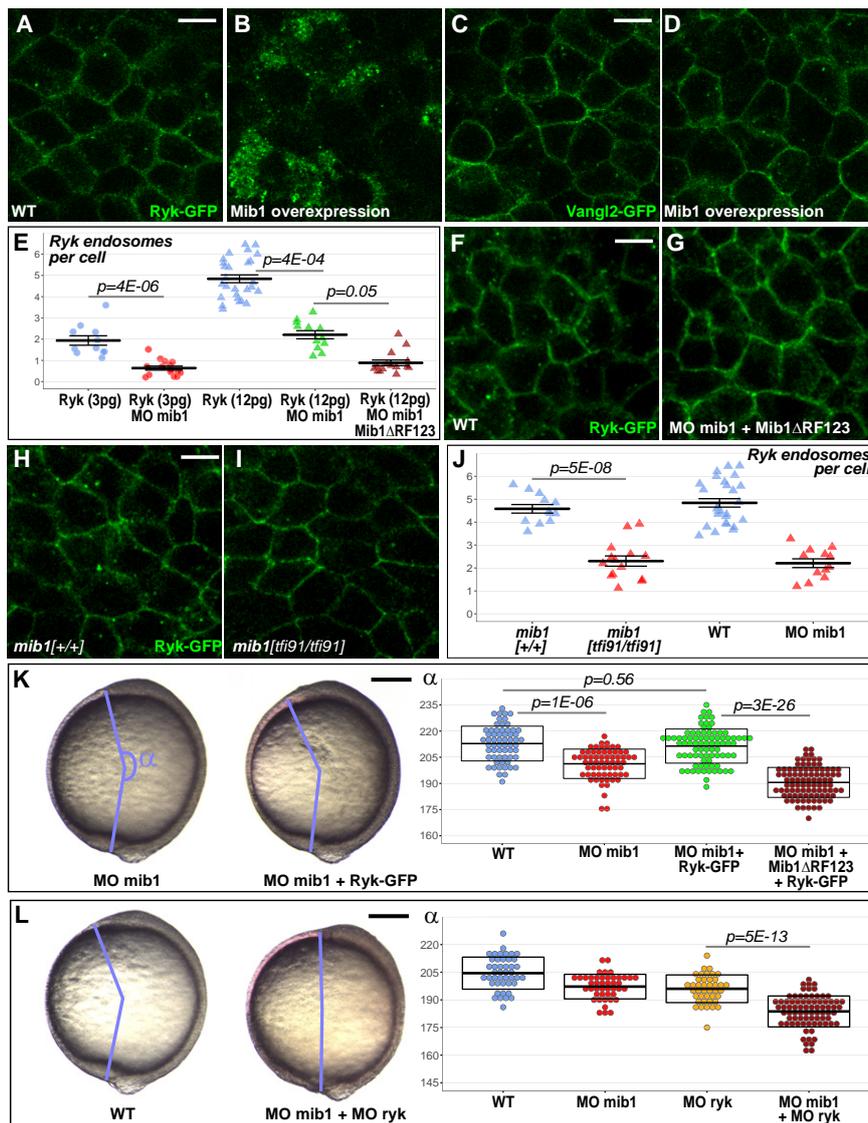


Figure 3: Mib1-mediated Ryk endocytosis controls Convergent Extension movements

(A-D) WT *mib1* RNA injection triggers Ryk internalization in 20/21 embryos (B) but has no effect on Vangl2 localization (D, n=23). (E-G) *Mib1* morpholino injection reduces the number of Ryk endosomes that are present upon injection of Ryk-GFP RNA. Increasing the dose of Ryk-GFP RNA restores endosome number in *mib1* morphants but not in embryos coinjected with *Mib1* Δ RF123. (H-J) The number of Ryk endosomes that are present upon injection of Ryk-GFP RNA (12 pg) is reduced in *mib1* null mutants. *mib1* morphant data from panel E are shown again for comparison. (K) Ryk-GFP RNA (12 pg) rescues axis extension in *mib1* morphants but not in embryos coinjected with *Mib1* Δ RF123. (L) Ryk morpholino injection aggravates *mib1* morphant axis extension phenotypes. (A-D,F,G,H,I) dorsal views of 90% epiboly stage embryos, anterior up, scalebars 10 μ m. (K,L) Lateral views of bud stage embryos, anterior up, scalebars 200 μ m. In (E,J) each data point represents the mean number of endosomes for 20 cells from a single embryo. For comparison J again includes the *mib1* morphant E. Bars represent mean values \pm SEM. In (K,L) boxes represent mean values \pm SD. See supplementary material for complete statistical information.

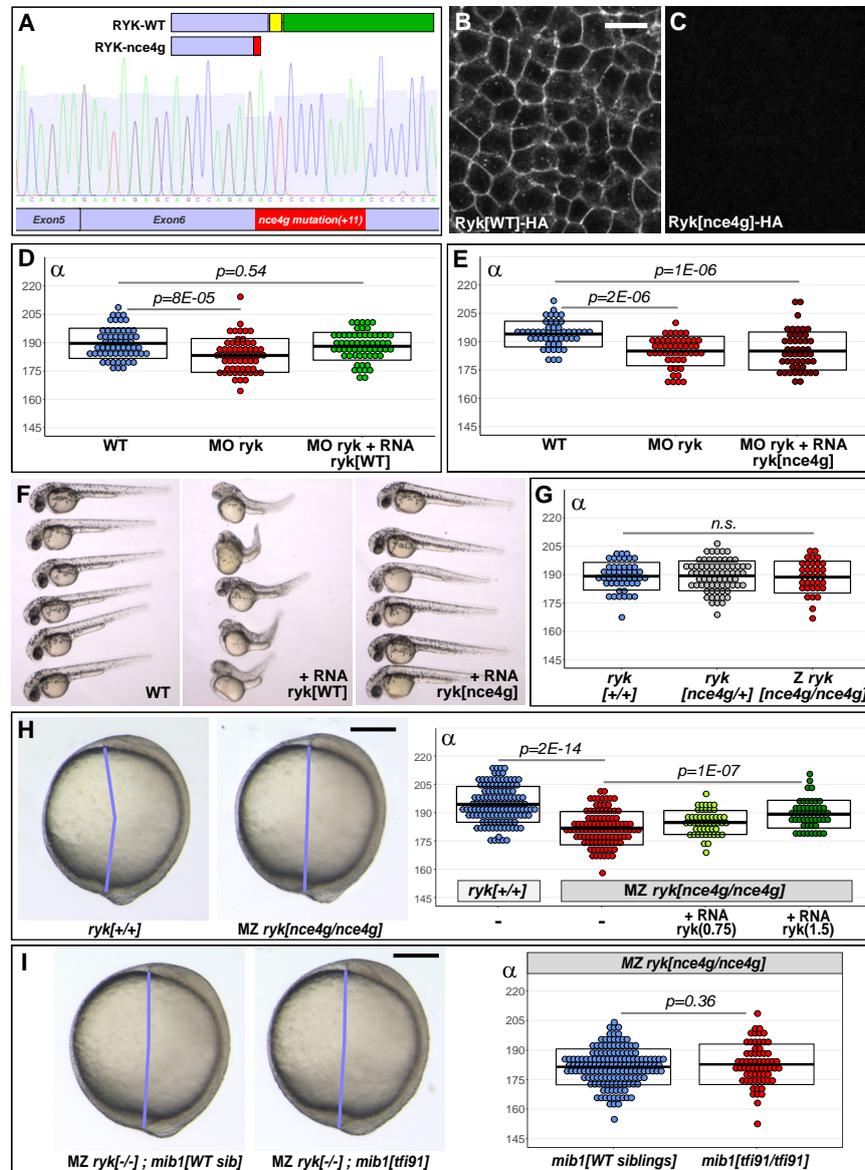
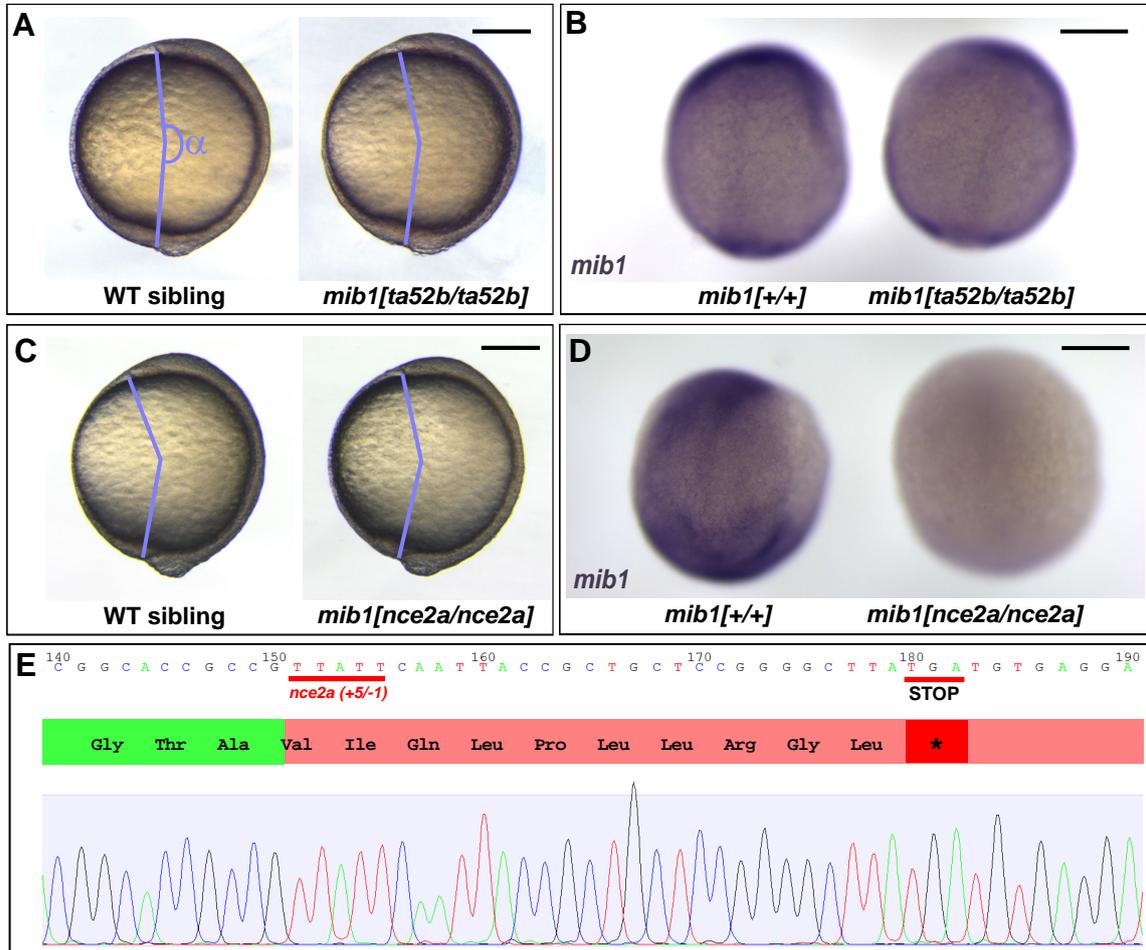
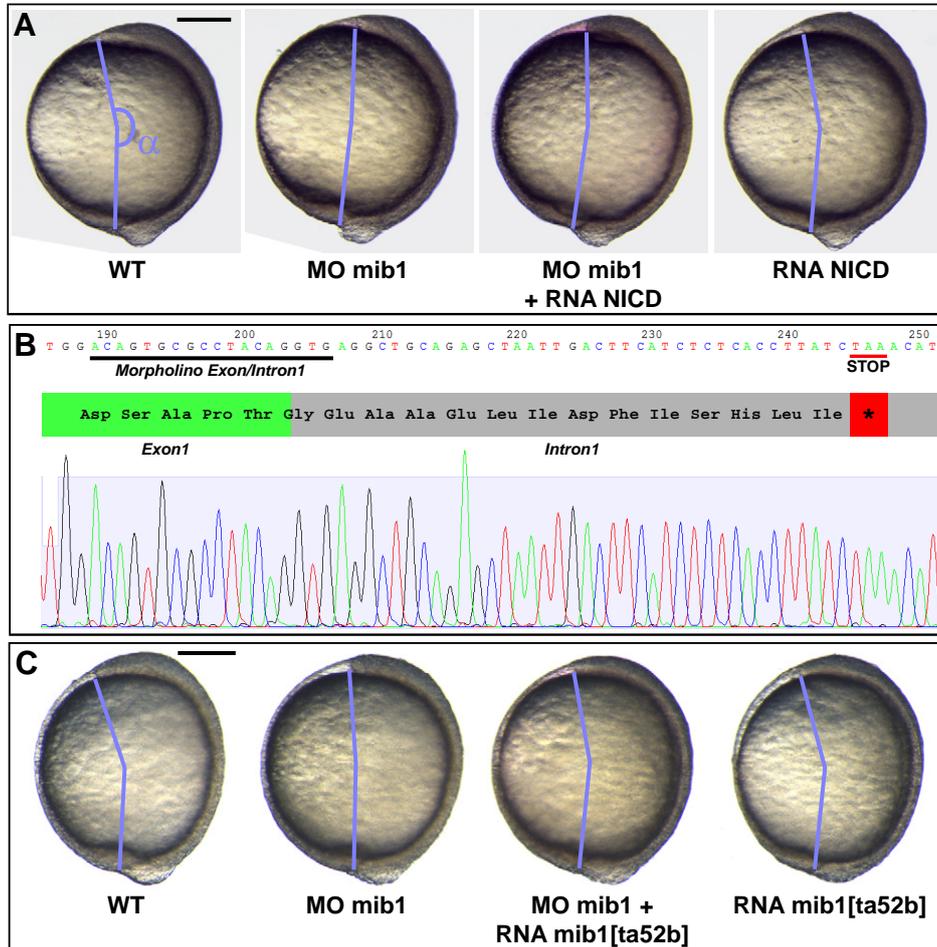


Figure 4: *mindbomb1* loss of function has no effect on convergent extension in maternal zygotic *ryk* mutants
(A) *ryk^{nce4g}* mutants present an 11 base pair insertion in exon 6. The RYK-nce4g mutant protein comprises only a part of the extracellular (blue) and lacks the entire transmembrane (yellow) and intracellular (green) domains. **(B,C)** Accordingly, a C-terminal HA tag that allows to localize WT Ryk (B, n=12) becomes undetectable upon introduction of the *ryk^{nce4g}* mutation (C, n=14). Dorsal views of 90% epiboly stage embryos, anterior up. Scalebar 20 μ m. **(D,E)** The Convergent Extension (CE) phenotypes of *ryk* morphant animals can be rescued using 1.5 pg WT *ryk* (D) but not *ryk^{nce4g}* mutant (E) RNA. **(F)** Overexpressing high levels (25 pg) WT *ryk* RNA causes severe embryonic malformations while no effect is observed using *ryk^{nce4g}* mutant RNA. 32 hpf embryos, anterior to the left, dorsal up (n=24 embryos/condition). **(G)** Zygotic (Z) *ryk* loss of function does not impair CE. **(H)** Maternal Zygotic (MZ) *ryk* mutants present defects in CE. *ryk* WT RNA injection allows a significant rescue of the observed phenotypes. **(I)** Similar CE defects are observed in MZ *ryk* single mutants and MZ *ryk*; *mib1* double mutants. **(H,I)** Lateral views of bud stage embryos, anterior up, dorsal to the right. Scalebars 200 μ m. In (D,E,G,H,I) boxes represent mean values +/- SD. See supplementary material for complete statistical information.



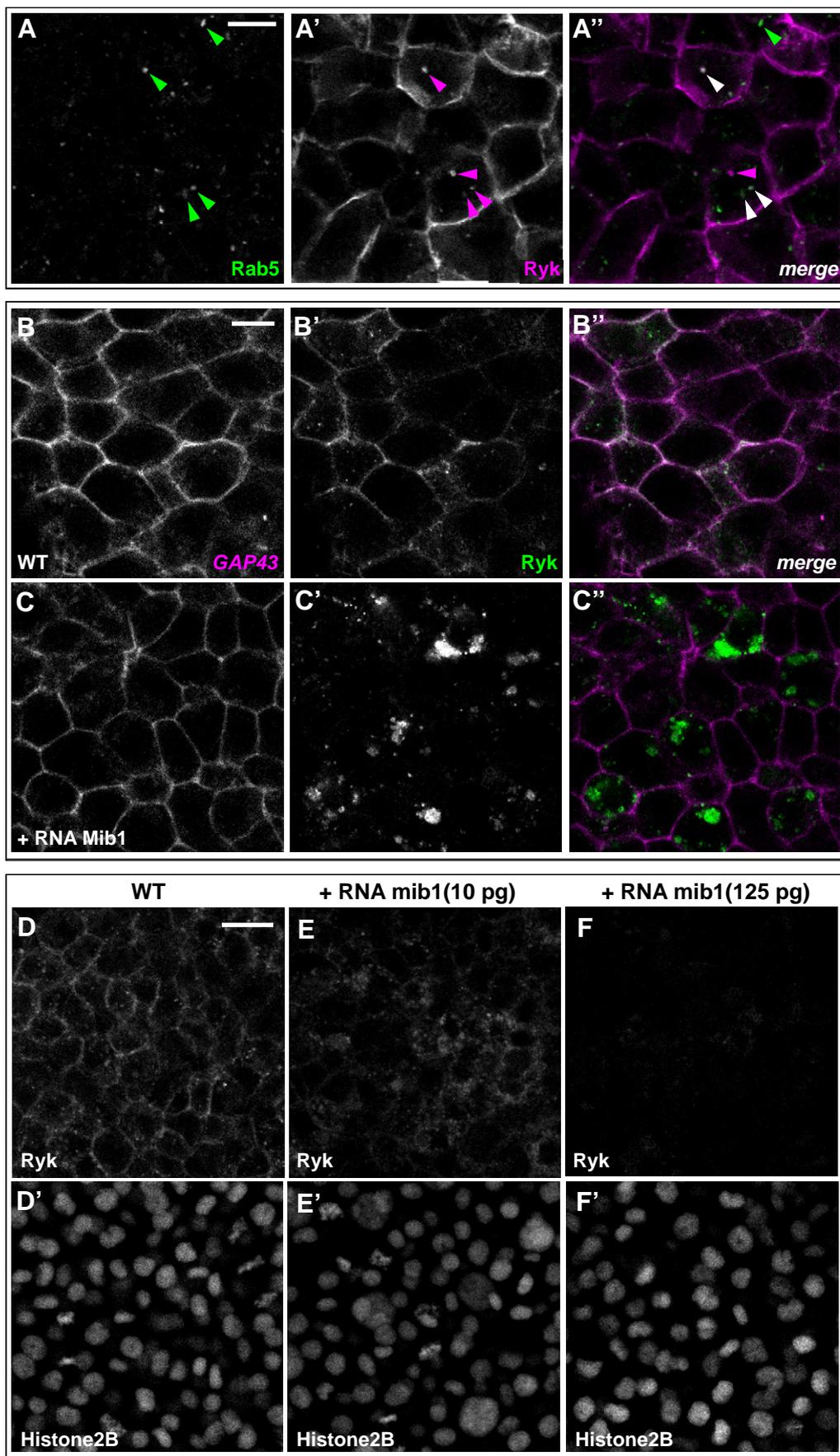
Supplementary Figure 1: Axis extension and RNA expression in *mib1^{ta52b}* and *mib1^{nce2a}* mutants

(A,B) Analysis of bud stage axis extension (A) and *mib1* *in situ* hybridization (B) show that the *mib1^{ta52b}* mutation does not impair Convergent Extension (CE) or *mib1* transcript abundance (B, n=4 mutant embryos analyzed). (C,D) In contrast *mib1^{nce2a}* mutants present a weak reduction of CE (C) and a loss of *mib1* transcripts (D, n=6 mutant embryos). (E) *mib1^{nce2a}* mutants present a CrisprCas-induced InDel that introduces a frame shift and leads to premature protein truncation. (A,C) depict lateral views of bud stage embryos, anterior up, dorsal to the right. A quantitative analysis of the corresponding data sets is provided in Fig.1D,G. (B,D) Represent dorsal views of bud stage embryos, anterior up. To warrant identical acquisition conditions, two embryos were photographed on a single picture. Scalebars: 200 μ m.



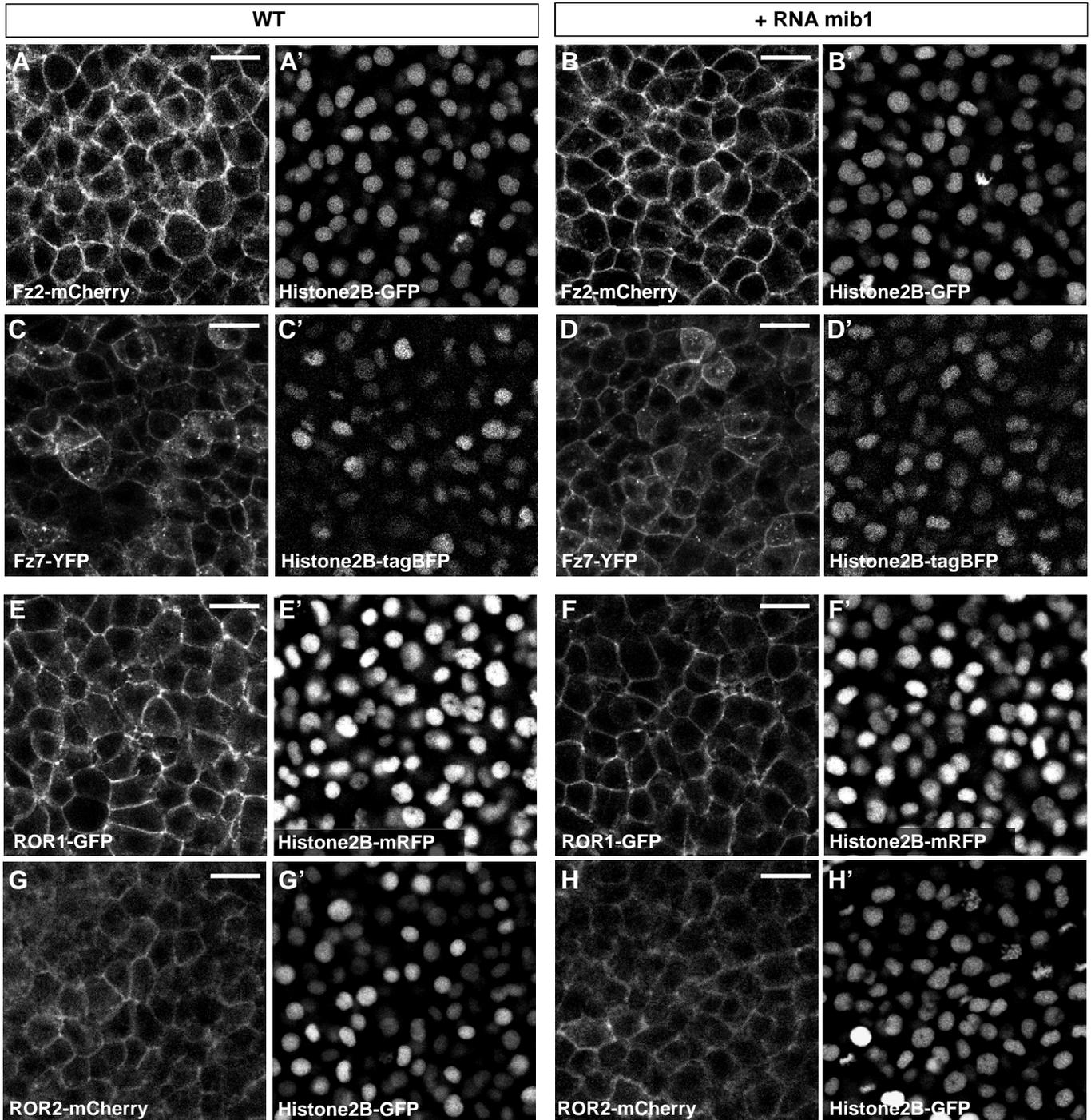
Supplementary Figure 2: Mindbomb1 regulates convergent extension independently of Notch

(A) Mib1 morphants display reduced convergent extension (quantified through the measure of the axis extension level α) that is not rescued by constitutively activated Notch (NICD). **(B)** cDNA sequence from wild-type embryos injected with mib1 exon/intron1 splice morpholino. Morpholino injection causes a retention of intron 1. As a consequence, the Mib1 morphant proteins comprises only the first 76 amino acids of WT Mib1 followed by 14 intronically encoded residues and a premature Stop codon. **(C)** Mib1 morphant axis extension can be restored through the overexpression of Notch-signaling deficient mib1^{ta52b}. (A,C) depict lateral views of bud stage embryos, anterior up, dorsal to the right. A quantitative analysis of the corresponding data sets is provided in Fig.1E,F. Scalebars: 200 μ m.



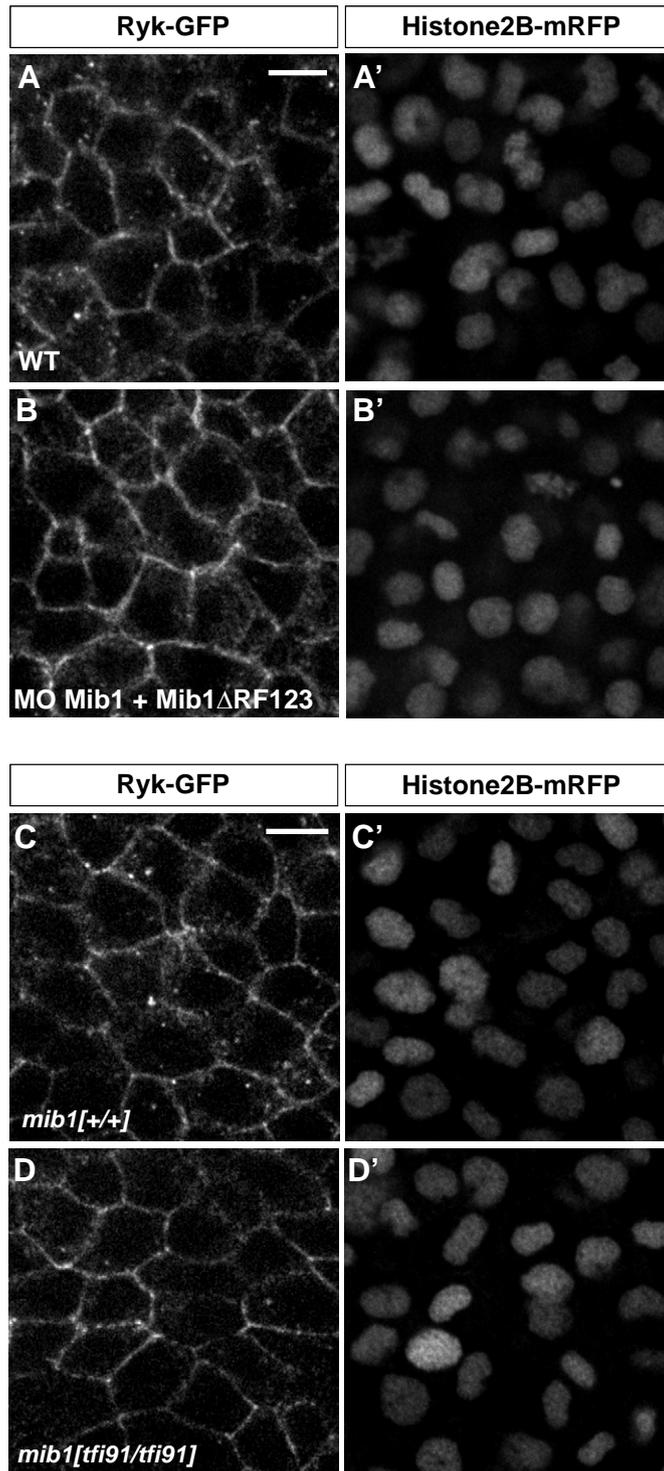
Supplementary Figure 3: Mindbomb1 promotes Ryk internalization and degradation

(A) Coinjection of RNAs encoding Rab5-GFP and Flag-Ryk-Myc reveals that many Ryk-expressing intracellular compartments are positive for the early endosomal marker Rab5. Arrowheads in A'' indicate compartments that are positive for Rab5 only (green), Ryk only (magenta), or present both markers (white). (B,C) Mib1 overexpression promotes the internalization of Ryk-GFP but not the one of the plasma membrane marker GAP43-RFP (n=4). (D-F) RNAs encoding Ryk-GFP and Histone2B-mRFP were injected with increasing amounts of mib1 RNA. While a low dose of Mib1 relocates Ryk from the plasma membrane to intracellular compartments (n=6), high amounts of Mib1 cause an overall loss of Ryk signal (n=6). D'-F', The Histone2B-mRFP signal was used to ascertain that embryos had received a comparable amount of injected material. D-F and D'-F' are sum projections of 3 consecutive slices from confocal stacks. All pictures depict dorsal views of 90% epiboly stage embryos, anterior up. Scalebars: 10 μ m in A-C, 20- μ m in D-F.



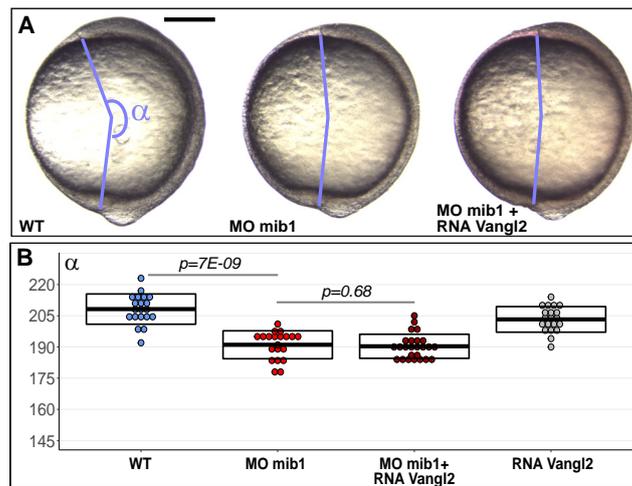
Supplementary Figure 4: Mindbomb1 overexpression does not affect Frizzled/Ror localization

(A-D) Mib1 overexpression has no effect on the localization of the Wnt receptors Frizzled2 (Fz2, n=6) or Frizzled7 (Fz7, n=8). (E-H) Mib1 overexpression has no effect on the localization of the Wnt-binding receptor tyrosine kinases ROR1 (n=7) or ROR2 (n=10). All pictures depict dorsal views of 90% epiboly stage embryos, anterior up. G,H are sum projections of three consecutive confocal slices. A'-H' Display the signal for fluorescently tagged Histone2B constructs that were coinjected to ascertain that control and mib1-expressing embryos had received a comparable amounts of injected material. Scalebars: 20- μ m.



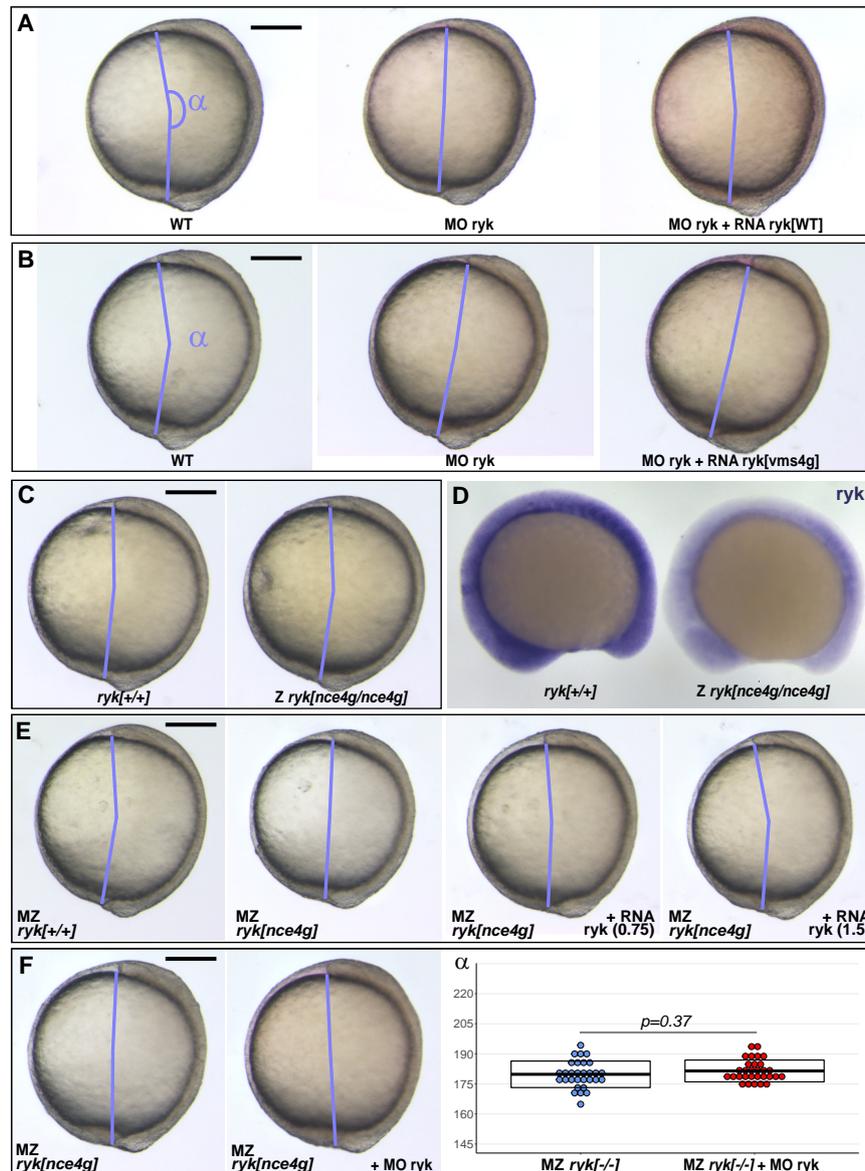
Supplementary Figure 5: Mindbomb1 loss of function impairs Ryk endocytosis

(A,B) Coinjecting *mib1* morpholino (MO *mib1*) and RNA encoding dominant-negative Mib1 (*mib1* Δ RF123) impairs Ryk endocytosis. (C,D) Ryk endocytosis is reduced in *mib1*^{*tfi91*} mutant embryos. All pictures depict dorsal views of 90% epiboly stage embryos, anterior up. A'-D', The Histone2B-mRFP signal was used to ascertain that control and *mib1*-depleted embryos had received a comparable amount of injected material. Embryos depicted in A-D were injected with 12 pg Ryk-GFP RNA. A-D correspond to the display items also shown in Fig.3F-I. Scalebars: 10 μ m.



Supplementary Figure 6: mindbomb1 morphant defects are not rescued upon vangl2 overexpression

(A,B) vangl2 RNA injection does not rescue axis extension in mib1 morphants. (A) Lateral views of bud stage embryos, anterior up, dorsal to the right. Scalebar 200 μ m. In (B) boxes represent mean values \pm SD. See supplementary material for complete statistical information.



Supplementary Figure 7: Maternal zygotic *ryk^{nce4g}* mutants present Convergent Extension defects

(A,B) *ryk* morphants present Convergent Extension (CE) defects that can be rescued by WT *ryk* (A) but not *ryk^{nce4g}* mutant (B) RNA. (C) CE is similar in Zygotic (Z) *ryk^{nce4g}* mutants and their WT siblings. (D) *In situ* hybridization reveals that *ryk* transcript levels are reduced in Z *ryk^{nce4g}* mutants (n=24) compared to WT siblings (n=24). 12 somite stage embryos, anterior to the left, dorsal up. To warrant identical acquisition conditions, two embryos were photographed on a single picture. (E) In contrast to Z *ryk^{nce4g}* mutants, Maternal Zygotic (MZ) *ryk^{nce4g}* mutants present CE defects. To exclude any defects due to genetic background variation, the parental fish used to obtain the embryos for this experiment were *ryk*[+/+] and *ryk*[nce4g/nce4g] siblings obtained from the same incross. *ryk* WT RNA injection allows to rescue MZ *ryk^{nce4g}* mutant CE defects. (F) *ryk* morpholino injection has no effect in MZ *ryk^{nce4g}* mutants. (A,B,C,E,F) Lateral views of bud stage embryos, anterior up, dorsal to the right. Scalebar 200 μ m. In (F) boxes represent mean values +/- SD. Quantitative analysis of the data sets displayed in (A,B,C,E) is provided in Fig.4D,E,G,H respectively. See supplementary material for complete statistical information.

SUPPLEMENTARY INFORMATION

Complete statistical information for the experiments reported in different display items

Fig.1A: Axis extension angle in mib1 morphants

	Mean value	Standard deviation	Number of embryos
WT	198.8	8.9	53
MO mib1	185.7	12.1	54
MO mib1 + RNA WT mib1	198.5	9.7	43
<i>Test statistics for Kruskal-Wallis test</i>			
F = 38.7	p = 3.9E-09		
<i>Adjusted p-values for pairwise comparisons (Dunn post-hoc test, Holm correction)</i>			
	MO mib1	MO mib1 + RNA WT mib1	
WT	5.3E-08	0.72	
MO mib1		1.32E-06	

Fig.1B: Notochord, somite & neural plate width in WT and mib1 morphants

	Mean notochord width	Standard deviation	Number of embryos
WT	2.47	0.35	40
MO mib1	3.22	0.50	39
<i>Welch's t-test:</i>			
p = 8.2E-11			

	Mean somite width	Standard deviation	Number of embryos
WT	24.9	2.62	40
MO mib1	29.7	3.99	39
<i>Welch's t-test:</i>			
p = 2.3E-08			

	Mean neural plate width	Standard deviation	Number of embryos
WT	29.9	3.71	40
MO mib1	37.6	5.56	39
<i>Welch's t-test:</i>			
p = 5.4E-10			

Fig.1D: Axis extension angle in *mib1^{ta52b}*

	Mean value	Standard deviation	Number of embryos
<i>mib1</i> [+/+]	199.9	11.6	29
<i>mib1</i> [<i>ta52b</i> /+]	203.5	10.6	62
<i>mib1</i> [<i>ta52b/ta52b</i>]	202.8	11.5	48
<i>Test statistics for One Way Anova</i>			
F = 1.1	p = 0.34		

Fig.1E: Axis extension angle in *mib1* morphants injected with NICD RNA

	<i>Mean value</i>	<i>Standard deviation</i>	<i>Number of embryos</i>
WT	201.0	7.8	47
MO <i>mib1</i>	189.1	10.2	57
MO <i>mib1</i> + RNA NICD	189.3	12.8	83
RNA NICD	197.0	8.8	41
<i>Test statistics for Welch's Anova</i>			
F = 18.4	p = 7.4E-10		
<i>Adjusted p-values for pairwise comparisons (Games Howell Test)</i>			
	MO <i>mib1</i>	MO <i>mib1</i> + RNA NICD	RNA NICD
WT	8.6E-08	2.0E-07	0.34
MO <i>mib1</i>		0.99	5.2E-04
MO <i>mib1</i> + RNA NICD			1.0E-03

Fig.1F: Axis extension angle in *mib1* morphants injected with *mib1*[*ta52b*] RNA

	<i>Mean value</i>	<i>Standard deviation</i>	<i>Number of embryos</i>
WT	201.3	8.1	38
MO <i>mib1</i>	188.6	9.7	40
MO <i>mib1</i> + RNA <i>mib1</i> [<i>ta52b</i>]	199.8	10.1	38
RNA <i>mib1</i> [<i>ta52b</i>]	202.7	9.3	20
<i>Test statistics for One way Anova</i>			
F = 17.0	p = 2.2E-09		
<i>Adjusted p-values for pairwise comparisons (Tukey HSD test)</i>			
	MO <i>mib1</i>	MO <i>mib1</i> + RNA <i>mib1</i> [<i>ta52b</i>]	RNA <i>mib1</i> [<i>ta52b</i>]
WT	1.1E-07	0.90	0.95
MO <i>mib1</i>		2.8E-06	1.1E-06
MO <i>mib1</i> + RNA <i>mib1</i> [<i>ta52b</i>]			0.69

Fig.1G: Axis extension angle in *mib1*^{*tfi91*}

	<i>Mean value</i>	<i>Standard deviation</i>	<i>Number of embryos</i>
WT siblings	197.9	11.0	175
<i>mib1</i> [<i>tfi91</i> / <i>tfi91</i>]	192.4	11.4	67
<i>Wilcoxon test:</i>			
p = 1.9E-03			

Fig.1G: Axis extension angle in *mib1*^{*tfi91/nce2a*}

	<i>Mean value</i>	<i>Standard deviation</i>	<i>Number of embryos</i>
WT siblings	202.9	10.4	55
<i>mib1</i> [<i>tfi91/nce2a</i>]	195.8	11.5	10
<i>t-test:</i>			
p = 1.6E-02			

Fig.1G: Axis extension angle in *mib1^{nce2a}*

	<i>Mean value</i>	<i>Standard deviation</i>	<i>Number of embryos</i>
WT siblings	210.9	10.0	114
<i>mib1[nce2a/nce2a]</i>	207.4	9.5	50
<i>Welch's t-test:</i>			
p = 3.9E-02			

Fig.2A: Axis extension angle in *mib1* morphants injected with RhoA RNA

	<i>Mean value</i>	<i>Standard deviation</i>	<i>Number of embryos</i>
WT	200.3	8.5	30
MO <i>mib1</i>	180.1	8.6	31
MO <i>mib1</i> + RNA RhoA	197.9	9.9	38
RNA RhoA	203.0	10.7	33
<i>Test statistics for One way Anova</i>			
F = 37.2	p = 2.3E-17		
<i>Adjusted p-values for pairwise comparisons (Tukey HSD test)</i>			
	MO <i>mib1</i>	MO <i>mib1</i> + RNA RhoA	RNA RhoA
WT	8.2E-13	0.74	0.68
MO <i>mib1</i>		1.5E-11	3.0E-14
MO <i>mib1</i> + RNA RhoA			0.12

Fig.2B: Axis extension angle in *mib1* morphants injected with Mib1- Δ RF123 RNA

	<i>Mean value</i>	<i>Standard deviation</i>	<i>Number of embryos</i>
WT	206.4	9.9	84
MO <i>mib1</i>	192.5	8.6	68
MO <i>mib1</i> + RNA Mib1- Δ RF123	186.7	9.5	111
RNA Mib1- Δ RF123	193.8	8.2	84
<i>Test statistics for One way Anova</i>			
F = 76.6	p = 6.4E-38		
<i>Adjusted p-values for pairwise comparisons (Tukey HSD Test)</i>			
	MO <i>mib1</i>	MO <i>mib1</i> + RNA Mib1- Δ RF123	RNA Mib1- Δ RF123
WT	<2.2E-16	<2.2E-16	<2.2E-16
MO <i>mib1</i>		8.7E-07	0.83
MO <i>mib1</i> + RNA Mib1- Δ RF123			2.5E-04

Fig.2C: Axis extension angle in mib1 morphants injected with Mib1-ΔRF3 RNA

	Mean value	Standard deviation	Number of embryos
WT	202.3	9.1	45
MO mib1	189.4	7.8	41
MO mib1 + RNA Mib1-ΔRF3	180.9	13.8	48
RNA Mib1-ΔRF3	187.2	11.9	44
<i>Test statistics for Welch's Anova</i>			
F = 32.9	p = 1.1E-14		
<i>Adjusted p-values for pairwise comparisons (Games Howell Test)</i>			
	MO mib1	MO mib1 + RNA Mib1-ΔRF3	RNA Mib1-ΔRF3
WT	2.4E-09	5.7E-11	1.6E-08
MO mib1		3.1E-03	0.75
MO mib1 + RNA Mib1-ΔRF3			0.09

Fig.3E: Number of Ryk endosomes in mib1 morphants injected with 3 pg Ryk-GFP RNA

	Mean value	Standard error	Sample size
WT	1.94	0.22	11 embryos – 20 cells/embryo
MO mib1	0.64	0.09	15 embryos – 20 cells/embryo
<i>t-test:</i>			
p = 4.1E-06			

Fig.3E: Ryk endosomes in MO mib1 + Mib1-ΔRF123 injected with 12 pg Ryk-GFP RNA

	Mean value	Standard error	Number of embryos
WT	4.84	0.18	27 embryos 20 cells/embryo
MO mib1	2.21	0.19	12 embryos 20 cells/embryo
MO mib1 + RNA Mib1-ΔRF123	0.88	0.13	15 embryos 20 cells/embryo
<i>Test statistics for Kruskal-Wallis test</i>			
Chi-squared = 43.5	p = 3.5E-10		
<i>Adjusted p-values for pairwise comparisons (Dunn post-hoc test, Holm correction)</i>			
	MO mib1	MO mib1 + RNA Mib1-ΔRF123	
WT	3.7E-04	5.7E-10	
MO mib1		0.05	

Fig.3J: Number of Ryk endosomes in *mib1^{tf91}* mutants injected with 12 pg Ryk-GFP RNA

	Mean value	Standard error	Sample size
<i>mib1</i> [+/-]	4.58	0.19	12 embryos – 20 cells/embryo
<i>mib1</i> [<i>tf91/</i> <i>tf91</i>]	2.30	0.22	14 embryos – 20 cells/embryo
<i>t-test:</i>			
p = 4.8E-08			

Fig.3K: Axis extension angle in mib1 morphants injected with Ryk-GFP

	Mean value	Standard deviation	Number of embryos
WT	212.9	10.0	61
MO mib1	201.2	8.4	65
MO mib1 + RNA Ryk-GFP	211.4	9.8	82
MO mib1 + RNA Mib1- Δ RF123 + RNA Ryk-GFP	190.6	8.6	94
<i>Test statistics for Kruskal-Wallis test</i>			
Chi-squared = 160	p = 1.7E-34		
<i>Adjusted p-values for pairwise comparisons (Dunn post-hoc test, Holm correction)</i>			
	MO mib1	MO mib1 + RNA Ryk-GFP	MO mib1 + RNA Mib1- Δ RF123 + RNA Ryk-GFP
WT	1.2E-06	0.56	4.5E-25
MO mib1		2.5E-06	1.4E-06
MO mib1 + RNA Ryk-GFP			2.7E-27

Fig.3L: Axis extension angle in mib1 ryk double morphants

	Mean value	Standard deviation	Number of embryos
WT	204.5	8.7	44
MO mib1	197.2	6.7	44
MO ryk	196.0	7.5	41
MO mib1 + MO ryk	183.7	8.4	78
<i>Test statistics for One way Anova</i>			
F = 32.9	p = 1.1E-14		
<i>Adjusted p-values for pairwise comparisons (Tukey HSD Test)</i>			
	MO mib1	MO ryk	MO mib1 + MO ryk
WT	1.6E-04	1.1E-05	<2.2E-16
MO mib1		0.90	<2.2E-16
MO ryk			5E-13

Fig.4D: Axis extension angle in MO ryk + RNA ryk[WT]

	Mean value	Standard deviation	Number of embryos
WT	189.7	7.8	60
MO ryk	183.2	9.0	60
MO ryk + RNA ryk[WT]	188.1	7.4	60
<i>Test statistics for One way Anova:</i>			
F = 10.2	p = 6.7E-05		
<i>Adjusted p-values for pairwise comparisons (Dunn post-hoc test, Holm correction)</i>			
	MO ryk	MO ryk + RNA ryk[WT]	
WT	7.6E-05	0.54	
MO ryk		3.7E-03	

Fig.4E: Axis extension angle in MO ryk + RNA ryk[nce4g]

	Mean value	Standard deviation	Number of embryos
WT	194.0	6.8	49
MO ryk	185.0	7.8	49
MO ryk + RNA ryk[nce4g]	185.0	10.1	49
<i>Test statistics for Kruskal-Wallis test:</i>			
Chi-squared = 33.5	p = 5.2E-08		
<i>Adjusted p-values for pairwise comparisons (Dunn post-hoc test, Holm correction)</i>			
	MO ryk	MO ryk + RNA ryk[nce4g]	
WT	1.7E-06	1.0E-06	
MO ryk		0.86	

Fig.4G: Axis extension angle in Zygotic ryk^{nce4g} mutants

	Mean value	Standard deviation	Number of embryos
ryk[+/+]	189.1	7.3	49
ryk[nce4g/+]	189.4	7.9	71
Z ryk[nce4g/nce4g]	188.7	8.4	35
<i>Test statistics for One Way Anova</i>			
F = 0.09	p = 0.91		

Fig.4H: Axis extension angle in Maternal Zygotic ryk^{nce4g} + ryk RNA

	Mean value	Standard deviation	Number of embryos
MZ ryk[+/+]	194.4	9.5	125
MZ ryk[nce4g/nce4g]	181.8	8.8	102
MZ ryk[nce4g/nce4g] + RNA ryk (0.75 pg)	184.8	6.3	50
MZ ryk[nce4g/nce4g] + RNA ryk (1.5 pg)	189.2	7.3	50
<i>Test statistics for Welch's Anova</i>			
F = 39.8	p = 6.9E-19		
<i>Adjusted p-values for pairwise comparisons (Tukey HSD Test)</i>			
	MZ ryk[nce4g/nce4g]	MZ ryk[nce4g/nce4g] + RNA ryk (0.75 pg)	MZ ryk[nce4g/nce4g] + RNA ryk (1.5 pg)
MZ ryk[+/+]	1.9E-14	1.1E-11	9.8E-04
MZ ryk[nce4g/nce4g]		0.08	1.7E-06
MZ ryk[nce4g/nce4g] + RNA ryk (0.75 pg)			9.8E-03

Fig.4I: Axis extension angle in ryk ; mib1 double mutants

	Mean value	Standard deviation	Sample size
MZ ryk[vms4g/vms4g] ; mib1[WT sibs]	181.4	9.1	151
MZ ryk[vms4g/vms4g] ; mib1[tfi91/tfi91]	182.7	10.3	64
<i>t-test:</i>			
p = 0.36			

Fig.S6: Axis extension angle in mib1 morphants injected with Vangl2 RNA

	<i>Mean value</i>	<i>Standard deviation</i>	<i>Number of embryos</i>
WT	208.2	7.3	20
MO mib1	191.1	6.7	20
MO mib1 + RNA Vangl2	190.3	5.8	25
RNA Vangl2	203.3	6.1	20
<i>Test statistics for Kruskal-Wallis test:</i>			
Chi-squared = 51.6	p = 3.7E-11		
<i>Adjusted p-values for pairwise comparisons (Dunn post-hoc test, Holm correction)</i>			
	MO mib1	MO mib1 + RNA Vangl2	RNA Vangl2
WT	6.9E-07	1.4E-08	0.40
MO mib1		0.68	1.9E-04
MO mib1 + RNA Vangl2			1.5E-05

Fig.S7F: Axis extension angle in Maternal Zygotic *ryk^{nce4g}* + MO *ryk*

	<i>Mean value</i>	<i>Standard deviation</i>	<i>Sample size</i>
MZ <i>ryk[vms4g/vms4g]</i>	179.8	6.6	30
MZ <i>ryk[vms4g/vms4g]</i> + MO <i>ryk</i>	181.5	5.4	30
<i>Wilcoxon test:</i>			
p = 0.37			