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1 **Tuning apico-basal polarity and junctional recycling in the**  
2 **hemogenic endothelium orchestrates pre-hematopoietic stem cell**  
3 **emergence complexity**

4 Léa Torcq,<sup>1,2</sup> Sara Majello,<sup>1</sup> Catherine Vivier,<sup>1</sup> and Anne A. Schmidt<sup>1,6,\*</sup>

5 <sup>1</sup> Institut Pasteur, Université Paris Cité, CNRS UMR3738, Department of  
6 Developmental and Stem Cell Biology, F-75015 Paris, France

7 <sup>2</sup> Sorbonne Université, Collège doctoral, F-75005 Paris, France

8 <sup>6</sup> Lead contact

9 \* Correspondence: [anne.schmidt@pasteur.fr](mailto:anne.schmidt@pasteur.fr)

## 10 **Abstract**

11

12 Hematopoietic stem cells emerge in the embryo from an aortic-derived tissue called the  
13 hemogenic endothelium (HE). The HE appears to give birth to cells of different nature and fate  
14 but the molecular principles underlying this complexity are largely unknown. Here we show, in  
15 the zebrafish embryo, that two cell types emerge from the aortic floor with radically different  
16 morphodynamics. With the support of live imaging, we bring evidence suggesting that the  
17 mechanics underlying the two emergence types rely, or not, on apicobasal polarity  
18 establishment. While the first type is characterized by reinforcement of apicobasal polarity and  
19 maintenance of the apical/luminal membrane until release, the second type emerges via a  
20 dynamic process reminiscent of trans-endothelial migration. Interfering with Runx1 function  
21 suggests that the balance between the two emergence types depends on tuning apicobasal  
22 polarity at the level of the HE. In addition, using new transgenic fish lines that express  
23 Junctional Adhesion Molecules and functional interference, we bring evidence for the essential  
24 role of ArhGEF11/PDZ-RhoGEF in controlling the HE-endothelial cell dynamic interface,  
25 including cell-cell intercalation, which is ultimately required for emergence completion. Overall,  
26 we highlight critical cellular and dynamic events of the endothelial-to-hematopoietic transition  
27 that support emergence complexity, with a potential impact on cell fate.

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32 **Major subject areas:** Developmental Biology and Stem cells, Cell Biology.

33 **Keywords:** hematopoiesis, stem cell, cell extrusion, endothelial-to-hematopoietic transition,  
34 apico-basal polarity, hemogenic endothelium, tight junctions, JAMs, zebrafish.

35

## 36 Introduction

37

38 Hematopoietic stem cells (HSCs) endowed with full regenerative potential in adult vertebrates  
39 are generated during a narrow-time window of few days during embryonic development. These  
40 cells, at the root of all blood and immune cells in the body, emerge from intra-embryonic aortic  
41 vessels and, more specifically, from a specialized type of vascular tissue called the hemogenic  
42 endothelium (HE, (Wu and Hirschi 2021)). A series of seminal studies have evidenced the  
43 autonomous production of repopulating HSCs within an intraembryonic region called the Aorta-  
44 Gonad-Mesonephros (AGM), before their appearance in other hematopoietic organs (A. L.  
45 Medvinsky et al. 1993; Müller et al. 1994; Garcia-Porrero, Godin, and Dieterlen-Lièvre 1995;  
46 A. Medvinsky and Dzierzak 1996; Cumano, Dieterlen-Lievre, and Godin 1996). Thereafter, the  
47 endothelial origin of HSCs was evidenced (Jaffredo et al. 1998; de Bruijn et al. 2002; T. E.  
48 North et al. 2002). The direct visualization, in real-time, of the emergence of precursors of  
49 hematopoietic stem and progenitor cells (HSPCs) from the dorsal aorta, termed the  
50 Endothelial-to-Hematopoietic Transition (EHT), was finally achieved *in vitro* (Eilken, Nishikawa,  
51 and Schroeder 2009), *ex-vivo* from mouse sections (Boisset et al. 2010), and *in vivo* in the  
52 zebrafish embryo (Bertrand et al. 2010; Kissa and Herbomel 2010), using live microscopy. This  
53 opened the way to a more detailed analysis of the characteristics of the HE at the  
54 transcriptional level, revealing its transient nature and its early hematopoietic commitment  
55 (Swiers et al. 2013).

56 The HE is characterized by inherent heterogeneity and is not only contributing to the formation  
57 of precursors to long-term HSCs (pre-HSCs) but also to more restricted progenitors (Hadland  
58 and Yoshimoto 2018). These progenitors can be born from HE sub-types that are found in  
59 extra-embryonic source such as the yolk-sac – in which case it gives rise to erythro-myeloid  
60 progenitors that will sustain erythro-myelopoiesis until birth (Frame et al. 2016) -, or both from  
61 extra and intra-embryonic sources (the yolk sac and the dorsal aorta) in which cases

62 progenitors are biased, such as for example in the mouse, toward T- and B- innate-type  
63 lymphoid progenitors (Yoshimoto et al. 2011; 2012) or toward less restricted, multipotent  
64 progenitors (Hadland and Yoshimoto 2018; Dignum et al. 2021). Importantly, while some of  
65 the non-HSC derived progenitors born during embryonic life only support the functions of the  
66 immune system during embryonic/ fetal life, others can persist in the adult to exert tissue-  
67 resident functions, as has been mainly described in the mouse (Ghosh et al. 2019). However,  
68 studies in human embryos support the idea that developmental hematopoiesis is highly similar  
69 in mice and humans (Ivanovs et al. 2011; 2017).

70 A key question regarding the issue of the capacity of the HE to give birth to cells endowed with  
71 different hematopoietic potential is whether this comes from HE cells of distinct origin (in which  
72 case the HE would be a mosaic of precursors with distinct potential) or if this results from  
73 extrinsic environmental cues that impose variability in the constantly evolving developmental  
74 context (for a discussion, see (Barone et al. 2022)).

75 To address this type of question, high-resolution experimental settings need to be developed,  
76 if possible at the single-cell resolution. Along this line, recent developments of transcriptomics,  
77 including single cell RNAseq, spatially resolved in situ transcriptomic approaches, and in situ  
78 barcoding, have been invaluable (Weijts, Yvernogeu, and Robin 2021). Among those high-  
79 resolution approaches, cell fate mapping and lineage tracing approaches that include imaging  
80 technologies have been very instrumental, particularly when performed with the zebrafish  
81 model that reproduces many aspects of developmental hematopoiesis in higher vertebrates  
82 (Orkin and Zon 2008). Recently, work performed in the zebrafish embryo has brought evidence  
83 for heterogeneity of hematopoietic stem cell precursors being born from the HE, in the ventral  
84 floor of the dorsal aorta and independently from HSCs, including a wave of transient T-  
85 lymphocytes (using temporally-spatially resolved fate-mapping, (Tian et al. 2017)) and lympho-  
86 myeloid biased progenitors born from a myeloid-lymphoid biased Spi2+ HE that appears to co-  
87 exist with an erythroid-biased HE (using single-cell RNA-sequencing, (Xia et al. 2023)).

88 While light starts to be shed on the molecular and signaling cues that appear to regulate HE  
89 sub-specification and a continuum from arterial endothelium, HE maturation, and subsequent  
90 heterogeneity in HSPCs (Zhu et al. 2020), the essential molecular and cell biological properties  
91 that support HE functional plasticity remain to be determined.

92 Here, using the zebrafish embryo as a model, we complement our previous work describing  
93 essential molecular and mechanistic features of EHT cell emergence (Lancino et al. 2018).  
94 With the support of high-resolution live imaging and image analysis, the generation of new  
95 transgenic fish lines that express functional markers of cell polarity (podocalyxin) and of inter-  
96 cellular adhesion (Junctional Adhesion Molecules - JAMs), as well as functional and genetic  
97 interference, we bring insight into additional key features of the EHT process that take their  
98 root in the differentiation and maturation of the HE.

## 99 **Results**

100

### 101 **Apicobasal polarity determines emergence types**

102 Our previous work describing the morphodynamic evolution of cells emerging from the aortic  
103 floor through the EHT (hereafter designated as “EHT cells”), in the zebrafish embryo, revealed  
104 the unusual feature of a cell extruding from the plane of a tissue while maintaining its  
105 luminal/apical membrane until the very end of the release process, thus contributing to its  
106 peculiar crescent-shaped morphology (Kissa and Herbomel 2010; Lancino et al. 2018, and  
107 see the cartoons in **Figure 1A**). However, to our knowledge, the polarity status of EHT cells  
108 has not been investigated so far and the maintenance of a *bona fide* apical domain has never  
109 been proven (with the luminal membrane enriched in apically targeted proteins and physically  
110 delimited by apical polarity factors and tight junction complexes (Rodriguez-Boulant, Müsch,  
111 and Le Bivic 2004; Buckley and St Johnston 2022)). Importantly, the fate of this apical-luminal  
112 membrane, after the release, may lead to cells potentially endowed with specific functional  
113 features. For example, this membrane surface may be directly and fully exposed to the  
114 extracellular space or released in the cytoplasm of EHT cells for recycling and/or degradation,  
115 after emergence completion (for examples of different scenarios, see **Figure 1A**). Overall, this  
116 could lead to precursors of hematopoietic stem cells that, ultimately, may be differentially fated.  
117 To address the polarity status of EHT cells, we raised transgenic fish lines that express  
118 endogenous Podocalyxin (Podxl2, (Herwig et al. 2011)), a sialomucin of the CD34 family  
119 (Nielsen and McNagny 2008). Podocalyxin was shown to take part in the formation of the  
120 preapical domain during polarization and in the regulation of its oriented organization, in tissue  
121 culture (Meder et al. 2005; Bryant et al. 2014). Its contribution to lumenization *in vivo*, in the  
122 mouse aorta, has been described and it involves negative charge repulsion induced by their  
123 glycosyl residues (Strilić et al. 2009; 2010).

124 We first attempted to express transiently, in the vascular system, the full-length Podxl2  
125 zebrafish protein fused to eGFP at its extreme N-terminus. We failed to detect the fusion  
126 protein at any time point relevant for observing easily the EHT process (a time window ranging  
127 from 48 to 60 hpf (hours post-fertilization)); therefore we designed a N-ter truncated form  
128 deleted of the mucin domain and that retains amino-acids 341-587 fused to eGFP at its N-  
129 terminus (**Figure 1B** and see **Materials and Methods**). Transient transgenesis revealed that  
130 this truncated version is detected and is targeted to the luminal membranes of EHT cells. We  
131 then raised 2 transgenic (*Tg*) fish lines that express the N-ter truncated form of Podxl2 fused  
132 to either eGFP (thereafter abbreviated eGFP-Podxl2) and under the control of the *Kdr1:Gal4*  
133 driver (*Tg(Kdr1:Gal4;UAS:RFP; 4xNR:eGFP-Podxl2)*), or to *mKate2* and under the control of  
134 the *Kdr1* promoter (*Tg(Kdr1:mKate2-Podxl2)*). We observed that eGFP-Podxl2 is enriched at  
135 the luminal side of crescent-shaped EHT undergoing cells (see **Figure 1C**, **Figure 1 - figure**  
136 **supplement 1A** and **Figure 1 – video 1** (z-stack at t=0) and their legends for the details of the  
137 luminal/apical membrane evolution through time). Thereafter, these cells will be referred to as  
138 EHT pol+ cells.

139 We also followed the cell after emergence and observed the evolution of the luminal/apical  
140 membrane appearing as internal pseudo-vacuoles. We illustrate the reduction of their volume  
141 via membrane retrieval and, ultimately, their remanence as an intracellular membrane  
142 compartment which we define as a post-EHT signature (**Figure 1- figure supplement 1A** and  
143 **Figure 1- video 2** (z-stack at t=80 min)); we propose a model for the intracellular evolution of  
144 the luminal/apical membrane which, unfortunately, cannot be traced after 2-3 hrs post-  
145 emergence because of the apparent short half-life of eGFP-Podxl2 and of the drop in activity  
146 of the *Kdr1* promoter (**Figure 1- figure supplement 1B**). Of notice, the pseudo-vacuoles are  
147 reminiscent of the cystic formations observed in EHT cells in the mouse and visualized by  
148 electron-microscopy (T. North et al. 1999; Marshall and Thrasher 2001) and also of the  
149 vacuolar structures recently described in EHT cells in avian embryos (Sato et al. 2023); in both  
150 cases, these vacuoles appear to emanate from the abluminal membrane (facing the sub-aortic

151 space) and not from the lumen. In the latter case, they disappear before EHT completion,  
152 suggesting that they contribute to exert mechanical force to initiate the detachment of the cell  
153 from the endothelial layer. In addition, their dynamics appears to depend on the activity of  
154 aquaporins and it is very possible that aquaporins are active in zebrafish too, although rather  
155 in EHT cells late in their emergence and/or in post-EHT cells, for water chase and vacuolar  
156 regression as proposed in our model (**Figure 1 – figure supplement 1B**).

157 While imaging the EHT using the Podxl2 expressing lines that clearly delimitate the luminal  
158 membrane, we unambiguously identified a second type of emergence. This second cell type  
159 is primarily characterized by a round-to-ovoid morphology (cells never bend as crescent-  
160 shaped EHT pol+ cells, see **Figure 1D** and **Figure 1 – video 3** for a time-lapse sequence).  
161 Importantly, these cells do not show any enrichment of eGFP-Podxl2 at the luminal membrane  
162 and will be referred to as EHT pol- cells. EHT pol- cells were observed in all other *Tg* fish lines  
163 that we are routinely imaging, including the *Tg(Kdrl:Gal4;UAS:RFP)* parental line that was used  
164 for transgenesis, thus excluding the possibility that these cells result from an artefact due to  
165 the expression of a deleted form of Podxl2 and/or to its overexpression.

166 Finally, we have estimated that the ratio between EHT pol+ and EHT pol- cells is of  
167 approximately 2/1, irrespective of the imaging time window and of the localization of  
168 emergence along the aortic antero-posterior axis (starting from the most anterior part of the  
169 AGM (at the limit between the balled and the elongated yolk) down to the caudal part of the  
170 aorta facing the CHT). We observed that both EHT pol+ and EHT pol- cells divide during the  
171 emergence and remain with their respective morphological characteristics after completing  
172 abscission (hence appearing as pairs of cells that exit the aortic wall sequentially, as shown  
173 **Figure 1C, D**). We also observed that both EHT pol+ and EHT pol- cells express reporters  
174 driven by the hematopoietic marker CD41 (data not shown), which indicates that they are both  
175 endowed with hematopoietic potential.

176 Altogether, our results show that hemogenic cells emerging from the aortic floor do so with  
177 heterogeneity in their morphodynamic characteristics. This suggests that this may be one of  
178 the sources underlying the complexity of pre-hematopoietic stem cell identity and of their  
179 downstream cell fate.

180

### 181 **The immature HE is not polarized**

182 EHT pol+ and EHT pol- cells appear to emerge from the hemogenic endothelium (HE), the  
183 latter constituting the virtually exclusive cell population of the aortic floor just prior to the  
184 initiation of the EHT time-window (around 28 hpf, see (Zhao et al. 2022)). In this context, we  
185 addressed the polarity status of HE cells. Surprisingly, confocal microscopy using the *eGFP-*  
186 *Podxl2* expressing fish line revealed that HE cells do not appear to be polarized, based on the  
187 absence of eGFP-Podxl2 enrichment at luminal membranes, at the initiation of the EHT time-  
188 window (at approximately 28 - 30 hpf, **Figure 2 – figure supplement 1**). Interestingly, the  
189 cytoplasm of characteristic elongated HE cells located on the aortic floor is filled with more-or-  
190 less large membrane vesicles that carry eGFP-Podxl2 (the largest vesicles reaching  
191 approximately 30 $\mu$ m in diameter). This suggests that HE cells contain a reservoir of eGFP-  
192 Podxl2 membranes that may be subjected to exocytose; as such, HE cells may be comparable  
193 to endothelial cells organizing a vascular lumen and that have been proposed to exocytose  
194 large intracellular macropinocytic-like vacuoles when cultured in 3D extracellular matrices  
195 (Bayless and Davis 2002; Davis, Bayless, and Mavila 2002) or, *in vivo*, in the zebrafish model  
196 (Kamei et al. 2006; Lagendijk, Yap, and Hogan 2014). This finding is unexpected since HE  
197 cells are assumed to possess aortic cell characteristics (i.e exhibit an apicobasal polarity) as  
198 they are supposedly integrated in the aortic wall contemporarily to aortic precursors (Jin et al.  
199 2005) and may have been taking part in the lumenization of the ancestral vascular cord, a  
200 process that takes place around 18-20 hpf. Consequently, loss of apicobasal polarity features  
201 of HE cells at 28-30 hpf may be part of the programme that initiates the EHT process.

202 Although technically difficult for long hours (because of important variations in the volume of  
203 the balled yolk that trigger drifting of embryos), we have been able to follow through time non-  
204 polarized HE cells and to visualize the evolution of their vesicular content, starting at the  
205 initiation of the EHT time-window (around 35 hpf, see **Figure 2** and **Figure 2 – figure**  
206 **supplement 2**). Interestingly, we could follow a dividing HE cell for which the vesicular content  
207 labelled with the polarity marker Podxl2 (eGFP-Podxl2) appeared to partition unequally  
208 between daughter cells (**Figure 2A**, EHT cell 1' inherits the largest macropinocytic-like vacuole  
209 and emerges unambiguously as an EHT pol+ cell). This suggests that asymmetric inheritance  
210 of cytosolic vesicles containing apical proteins may contribute, presumably after delivery to the  
211 luminal membrane, to the acquisition of apicobasal polarity by EHT pol+ cells.

212 Altogether, these results support the idea that the HE, at the initiation of the EHT time-window,  
213 is not polarized. Subsequently, HE cells establish – or not – apicobasal polarity, thus leading  
214 to the emergence of either EHT pol+ or EHT pol- cell types (see our model **Figure 2B**). In the  
215 case of EHT pol+ cells and while emergence is proceeding, apicobasal polarity is maintained  
216 (if not reinforced) until the release.

217

## 218 **Tuning of apicobasal polarity is sensitive to interfering with the Runx1 transcription** 219 **factor and with blood flow**

220 To substantiate our findings on the significance of controlling apicobasal polarity establishment  
221 on EHT sub-types, we explored the potential involvement of proteins of the Pard3 family; these  
222 proteins, recruited by transmembrane receptors via their PSD-95/Dlg/ZO-1 (PDZ) domains  
223 (Buckley and St Johnston 2022), are at the root of apicobasal polarity initiation and are  
224 essential for the maintenance of apical membrane functional properties (Román-Fernández  
225 and Bryant 2016). We anticipated that interfering directly with Pard3 proteins would hamper  
226 the development of the aorta (in addition to other functions of polarized tissues that are  
227 essential for embryonic development) and rather searched for correlative evidence for the

228 differential expression of Pard3 isoforms - including natural loss or gain of function variants -,  
229 at critical time windows of the EHT process (i.e between 30-32 hpf (the timing at which the HE,  
230 as we show, does not appear to be polarized) and 48-50 hpf (the timing at which, as we show,  
231 part of HE cells have become competent to undergo emergence)). In addition, to provide  
232 functional support to this analysis and ensure that differences relate to what is taking place in  
233 hemogenic cells, our experimental conditions also included interfering with the gradual  
234 maturation of the HE (we have estimated that HE cells represent approximately 30% of total  
235 aortic cells at 48 hpf and others have estimated that they reach up to approximately 50% of  
236 total aortic cells at 28 hpf (Zhao et al. 2022)). We altered the maturation of the HE by abrogating  
237 blood flow and, more restricted to the EHT biological process *per se*, by interfering with the  
238 function of the Runx1 transcription factor whose expression is sensitive to fluid sheer stress  
239 (Adamo et al. 2009) and whose function is essential for EHT completion (Kissa and Herbomel  
240 2010) and for regulating HSC number (Adamo et al. 2009; T. E. North et al. 2009).

241 Firstly, to reach these objectives, we sought to identify Pard3 isoforms that may be functionally  
242 relevant during EHT progression, using whole-mount *in situ* hybridization (WISH), RT-PCR  
243 and cloning. WISH performed at approximately 35 hpf allowed us to detect, in the aorta,  
244 mRNAs encoding for all Pard3 members, including Pard3ab, b, ba and bb, with prominent  
245 detection of Pard3ab and bb isoforms (see **Figure 3A** and **Materials and Methods**).

246 Messenger RNAs, in the trunk region and encoding for all four isoforms, were also detected  
247 by qRT-PCR during the 30-32 and 48-50 hpf time windows (data not shown). We also obtained  
248 and cloned partial or complete cDNAs encoding for all four members after reverse transcription  
249 of mRNAs expressed in the trunk region of 48 hpf embryos. Sequencing unveiled yet  
250 unreferenced variants and, of relevance regarding our objectives, potential loss of function  
251 proteins for Pard3ab and Pard3bb (see **Figure 3B** and **Materials and Methods**); these  
252 include, for Pard3ab, a deletion of most of the sequence but that retains the amino-terminus  
253 containing the conserved region 1 (CR1) as well as the PDZ1 domain and, for Pard3bb, a

254 carboxy-terminal truncation of the PDZ3 domain that nonetheless maintains the carboxy-  
255 terminal sequence of the protein (see **Figure 3B** and its legend for more details).

256 Secondly, to interfere with Runx1 function, we generated a *Tg* fish line that expresses a  
257 truncated form of Runx1 (dt-runx1; fish line *Tg(Kdrl:Gal4;UAS:RFP;4xNR:dt-runx1-eGFP)*  
258 thereafter abbreviated *Tg(dt-runx1)*) deleted from its transactivation domain and carboxy-  
259 terminus but retaining its DNA-binding domain (the Runt domain (aa 55-183 (Kataoka et al.  
260 2000; Burns et al. 2002; Kalev-Zylinska et al. 2002), see **Figure 3C**). Importantly, in this *Tg*  
261 fish line, dt-runx1 expression is restricted to the vascular system, hence excluding expression  
262 in the brain region that express Runx1 endogenously (see the aforementioned articles) and  
263 preventing biases owing to potential interference with neuronal functions. In addition, owing to  
264 the expression of eGFP concomitantly to dt-runx1 (eGFP is cleaved from dt-runx1 via a T2a  
265 site for endopeptidase), this fish line allows for the easy selection of embryos for imaging and  
266 for phenotypic analysis. In preliminary experiments aimed at addressing the localization of dt-  
267 runx1 as well as its stability, we expressed it transiently and measured its proper targeting to  
268 the nucleus, see **Figure 3C**).

269 To characterize further the *Tg(dt-runx1)* fish line, we performed time-lapse experiments using  
270 spinning disk confocal microscopy (with some of the experiments addressing apicobasal  
271 polarity performed with embryos obtained from outcrossing the *Tg(dt-runx1)* and  
272 *Tg(Kdrl:mKate2-Podxl2)* fish lines). Overall, we made the following observations: (i)  
273 hematopoiesis is affected, as attested by the significant increase in the number of  
274 hematopoietic cells in the thymus, at 5 dpf (days post-fertilization), in comparison to control  
275 siblings (**Figure 3- figure supplement 1**); (ii) at 30-32 hpf and contrarily to what we observed  
276 with our fish lines expressing either eGFP-Podxl2 or mKate2-Podxl2, HE cells appear to be  
277 polarized, based on enrichment of the polarity marker at luminal membranes. In addition, HE  
278 cells do not appear to contain any visible intra-cytosolic Podxl2-containing vesicles (**Figure 3-  
279 figure supplement 2**, compare with **Figure 2 and Figure 2- figure supplement 1**); (iii) at 48-

280 55 hpf, we observe an accumulation of EHT pol+ cells both in the aortic floor and in the lateral  
281 sides of the aortic wall as well as of cells of uncharacterized nature (the latter may,  
282 hypothetically, represent EHT cells at an early phase of their emergence, including cells that  
283 should have evolved as EHT pol- cells (see **Figure 3 - figure supplement 3A**)); (iv)  
284 occasionally, we observe - for EHT cells exhibiting invagination of the luminal membrane -,  
285 scattered cytosolic and sub-plasmalemmal pools of Podxl2-containing membranes and,  
286 consistently, the apparent decrease of Podxl2 enrichment at the apical/luminal membrane (see  
287 **Figure 3 - figure supplement 3B** and compare with **Figure 1C**). In addition and occasionally  
288 as well, we observe the reversion of apparent EHT pol+ cells into apparent EHT pol- cells (data  
289 not shown). These two last observations suggest that perturbing the tuning of apicobasal  
290 polarity in the HE alters the morphodynamic characteristics of emergence types, particularly in  
291 the case of EHT pol- cells whose biomechanical features appear to require turning down  
292 apicobasal polarity establishment.

293 Altogether, the results obtained upon expression of dt-runx1 show impairment of  
294 hematopoiesis and suggest that, for both EHT cell types, the progression throughout EHT is  
295 perturbed, and so until the release. The accumulation of morphologically characterized EHT  
296 pol+ like cells that we observe may result from the sustained apicobasal polarity of the HE at  
297 early time points (around 30 hpf). Consistently, this appears to be detrimental to the emergence  
298 of EHT pol- cells that we could not identify unambiguously and that may be part of the cells  
299 that are of uncharacterized morphologically. In addition, our results suggest that precursors of  
300 HE cells are polarized, as is expected to be the case for non-hemogenic aortic cells, and that  
301 Runx1 is involved in controlling the molecular events that are tuning apico-basal polarity,  
302 starting at the initiation of the EHT time-window.

303 We then pursued our aim to correlate, using qRT-PCR, the expression levels of Pard3 isoforms  
304 with the apicobasal polarity status of the hemogenic tissue during the EHT critical time windows  
305 (30-32 versus 48-50 hpf). To complement interference with Runx1 function upon expression

306 of dt-Runx1, we also prevented blood flow initiation using the well characterized *silent heart*  
307 (*sih*) morpholino which inhibits the expression of cardiac troponin T (Sehnert et al. 2002).

308 In a first series of 3 independent experiments performed with cDNAs obtained from dissected  
309 trunk regions of embryos at 30-32 and 48-50 hpf (**Figure 4A**), we measured the expression  
310 levels of the four Pard3 isoforms using heterozygous *Tg(dt-runx1)* fishes that were incrossed  
311 so as to obtain embryos expressing the highest possible amounts of dt-runx1, based on eGFP  
312 fluorescence resulting from proteolytic cleavage of the dt-runx1-eGFP fusion. To validate the  
313 efficiency of our interference conditions, we also measured expression levels of *cmyb* (an early  
314 marker of hematopoiesis) and of *Scrib* (a polarity protein involved in the functional delimitation  
315 of the cellular basal domain (Buckley and St Johnston 2022)). The qRT-PCR results show, for  
316 all Pard3 isoforms, a reproducible tendency for opposite effects of dt-runx1 expression  
317 between the 30-32 and 48-50 hpf time-windows, with the significant decrease of values for  
318 Pard3ab and bb at 48-50 hpf in comparison to controls. This suggests that the expression  
319 levels of Pard3 polarity proteins are differentially controlled between the two time-windows,  
320 indicating that the expression level of Pard3 polarity proteins is positively controlled in the wild  
321 type situation when emergence is proceeding at 48-50 hpf, but that it is rather negatively  
322 controlled, and thus decreased, at early time points (during HE maturation, at 30-32 hpf).  
323 These data and conclusions are also supported by results obtained with *Scrib*, with a  
324 comparable outcome. Importantly, *cmyb* expression was significantly decreased for both time-  
325 windows, consistently with the impairment in Runx1-dependent control of hematopoiesis.

326 In a second set of 3 independent experiments, we interfered with blood flow using the  
327 *Tg(Kdrl:Gal4;UAS:RFP)* fish line after injection of the *sih* morpholino (MO) in 1 cell stage  
328 embryos. We focused on Pard3ab and Pard3bb isoforms (**Figure 4B**). We reproduced the  
329 results obtained for Pard3ab and Pard3bb upon dt-runx1 expression, with less variability and  
330 consistently with the function of blood flow in controlling Runx1 expression (Adamo et al. 2009).

331 Finally, we also investigated expression levels of the +/- PDZ2 and +/- PDZ3 variants of  
332 Pard3ab and Pard3bb, respectively (using, as for experiments reported in **Figure 4B**,  
333 incrosses of heterozygous *Tg(dt-runx1)* fishes, see **Figure 4C - left**). We found that, at 48-50  
334 hpf and for both proteins, isoforms including the full-length PDZ domains are the most  
335 decreased, hence being positively controlled in the wild type Runx1 context. This reinforces  
336 the consistency of our findings as well as the importance of controlling expression levels of  
337 fully active polarity proteins when emergence is proceeding. We also confirmed our results  
338 using *sih* MO injections, at least for the Pard3ab +/-PDZ2 variants (**Figure 4C - right**).  
339 Interestingly, the ratios between the Pard3ab +/-PDZ2 variants are opposite between the 30-  
340 32 hpf and 48-50 hpf time windows, reinforcing the idea of a fine tuning of apicobasal polarity  
341 functions mediated by aortic environmental constraints imposed by blood flow.

342

#### 343 **Junctional recycling is differentially controlled between EHT cell types**

344 To investigate the functional links between apicobasal polarity and the peculiarities of EHT  
345 pol+ and EHT pol- emergence processes, we sought to follow the dynamics of Junctional  
346 Adhesion Molecules (JAMs) that belong to tight junction complexes (Garrido-Urbani, Bradfield,  
347 and Imhof 2014). During apicobasal polarity establishment in epithelial and endothelial tissues,  
348 these molecules recruit the Pard3/aPKC complex, via a PDZ-binding peptide located at their  
349 extreme carboxy-terminus (**Figure 5A** and (Itoh et al. 2001; Ebnet et al. 2001; 2018)). In  
350 addition, JAMs are expressed in the vascular system and, in relation to this work, JAM3b is  
351 expressed in the aorta of the zebrafish embryo and promotes endothelial specification and  
352 hematopoietic development (Kobayashi et al. 2020).

353 We envisaged a scenario whereby EHT pol+ cells, whose longitudinal interface with  
354 endothelial neighbours shrinks along the X axis of the X, Y 2D-plane, may have less mobile  
355 junctional pools than EHT pol- cells whose entire junctional interface moves along the X, Y, Z  
356 3D-axes (**Figure 5 - figure supplement 1**). In the case of EHT pol- cells, the consumption of

357 the junctional interface with adjoining endothelial cells appears to result from the converging  
358 migration of endothelial neighbours crawling over the luminal membrane, based on  
359 interpretation of our time-lapse sequences (**Figure 1- video 3**). In this context, we favoured  
360 the analysis of junctional pools localized at antero-posterior sites of emerging cells as we have  
361 shown that they are enriched with tight junction components (Lancino et al., 2018). In addition  
362 and in the case of EHT pol+ cells, it is conceivable that these adhesion pools – spatially  
363 restricted owing to apicobasal polarity –, contribute to anchoring the emerging cell in the 2D-  
364 plane (**Figure 5 - figure supplement 1**).

365 To achieve our goal, we designed 2 constructs in which eGFP is introduced in the extracellular  
366 domains of the two JAM2a and JAM3b molecules (**Figure 5A**). To investigate their localization  
367 and proper targeting at junctional interfaces, these constructs were expressed transiently and  
368 ubiquitously, using the *Hsp70* heat shock promoter. We observed that the 2 fusion proteins  
369 are efficiently targeted at cellular contacts and, more specifically, at the apical side of polarized  
370 epithelia such as for example cells of the ependymal canal (**Figure 5 - figure supplement 2**).  
371 We then established Tg fish lines expressing eGFP-Jam2a and eGFP-Jam3b under the control  
372 of the vascular *kdr1* promoter (*Tg(kdr1:eGFP-Jam2a)* and *Tg(kdr1:eGFP-Jam3b)*). Using these  
373 fish lines and spinning disk confocal microscopy, we observe a remarkable efficiency of  
374 targeting to intercellular junctions, for both proteins (data not shown for eGFP-Jam2a; for  
375 eGFP-Jam3b, see **Figure 5B, C**). Deployment of the aortic wall into 2D cartographies allows  
376 to point precisely at junctional pools established at the interface between endothelial and EHT  
377 cells and emphasizes on the enrichment of eGFP-Jam3b at antero-posterior poles of EHT pol+  
378 and EHT pol- cells (**Figure 5B, C bottom**).

379 Using double transgenic *Tg(kdr1:eGFP-Jam3b; kdr1:nls-mKate2)* embryos at 48-55 hpf, we  
380 addressed the recycling capacity of junctional pools using Fluorescence Recovery After  
381 Photobleaching (FRAP). The labelling of nuclei with nls-mKate2 allowed to point at EHT pol+  
382 and EHT pol- cells unambiguously because eGFP-Jam3b is essentially targeted to junctional

383 contours and not labelling peripheral membranes, except in few cases (and nuclei of EHT pol+  
384 cells have a crescent shape, see **Figure 6A**).

385 First and to set up our protocol, we spotted bi- and tri-junctional contacts between endothelial  
386 cells (**Figure 6 – figure supplement 1**). Recycling parameters (fluorescence intensity  
387 recovery with time and maximum recovery amplitude (that addresses the mobile pool), **Figure**  
388 **6C**) showed that bi-junctional contacts are less mobile than tri-junctions, with a higher  
389 dispersion of maximum recovery amplitude values for the latter (**Figure 6E**); this introduced a  
390 clear limitation for statistical significance of the results, although clear tendencies were  
391 observed for mean fluorescence intensity recovery (**Figure 6C**) and median values for  
392 maximum recovery amplitude (**Figure 6E**). We then focused at antero-posterior sites of EHT  
393 pol+ and EHT pol- cells and more specifically in the region of tricellular junctions (shared by 1  
394 EHT cell and 2 endothelial neighbours, see **Figure 6 – figure supplement 1**) that are clearly  
395 the most enriched with eGFP-Jam3b. In each experiment, eGFP-Jam3b pools at tricellular  
396 junctions between endothelial cells were also spotted for a comparative analysis.  
397 Measurements of fluorescence recovery intensities revealed a significant increase in the  
398 mobile fraction of eGFP-Jam3b at EHT pol+ – EC versus EHT pol- – EC junctional interfaces  
399 (median values of 50% and 39% maximum recovery amplitudes after 10 min, respectively;  
400 **Figure 6E**). Differences in recovery were also measured between EHT pol+ – EC and EHT  
401 pol- – EC junctional interfaces when focusing on the earliest time points (the first 30 seconds,  
402 **Figure 6F**), although with smaller significance, with the median value of early recovery slopes  
403 for the EHT pol+ - EC versus EHT pol- – EC junctional interfaces increased by 128% (**Figure**  
404 **6G**).

405 Altogether and unexpectedly regarding our initial scenario, these results indicate that tri-  
406 junctional pools localized at antero-posterior poles of EHT cells and enriched with eGFP-  
407 Jam3b molecules are significantly more dynamic for EHT pol+ cells in comparison to EHT pol-  
408 cells. Since EHT pol+ cells - by virtue of apicobasal polarity establishment possibly assemble

409 an apical endosome -, this should favour the rapid recycling of eGFP-Jam molecules (Ebnet  
410 et al. 2003). This recycling may entertain a certain rate of delivery of Pard3 proteins at apical  
411 junctional regions and contribute to the maintenance/reinforcement of polarity throughout the  
412 entire emergence process. In addition, this turnover may be adapted to the mechanical  
413 tensions EHT pol+ cells are exposed to, in comparison to EHT pol- cells, owing to their specific  
414 morphodynamic features (ex: cellular bending); these tensions may be sensed by tri-cellular  
415 junctions which would be preferential sites for the renewal of junctional components whose  
416 half-life is reduced owing to submission to mechanical forces.

417

#### 418 **ArhGEF11/PDZ-Rho GEF plays essential functions during EHT progression**

419 Junctional maintenance and recycling are dependent on intracellular membrane trafficking,  
420 supported by sub-cortical actin remodelling and actomyosin contractility, which are controlled  
421 mainly by GTPases of the Rho family (Ridley 2006; Olayioye, Noll, and Hausser 2019). Owing  
422 to the significance of apicobasal polarity control on EHT features, as suggested by our work,  
423 we investigated which proteins may be essential for actin/actomyosin regulation and focused  
424 on regulators of Rho GTPases, in particular Rho GEFs that catalyse exchange of GDP for GTP  
425 to positively regulate their activity (Rossman, Der, and Sondek 2005). As for Pard3 proteins,  
426 several of these Rho GEFs contain one or several PDZ domain(s) that target most proteins to  
427 complexes acting at the apical side therefore interlinking actin/actomyosin regulation with cell  
428 polarity (Mack and Georgiou 2014; Ebnet and Gerke 2022). We focused on 9 PDZ-domain  
429 containing Rho GEFs, all encoded by different genes in the zebrafish (**Figure 7- figure**  
430 **supplement 1**): ArhGEF11/PDZ-RhoGEF (thereafter shortened as ArhGEF11), ArhGEF12a,  
431 ArhGEF12b, PRex1, PRex2, Tiam1a, Tiam1b, Tiam2a, Tiam2b. We first investigated their  
432 expression by Whole mount In Situ Hybridization (WISH) and found that all 9 mRNAs are  
433 detected in the aorta, and for the vast majority at 30-32 and 48-50 hpf (**Figure 7- figure**  
434 **supplement 2**). Then, using qRT-PCR (**Figure 7- figure supplement 1B**), we measured and

435 compared their expression levels in the trunk region (at 35 and 48 hpf), for dt-runx1 expressing  
436 embryos and controls. We found that, in comparison to controls, ArhGEF11, ArhGEF12b,  
437 Tiam1b and Tiam2a are significantly reduced upon dt-runx1 expression at 48 hpf (hence being  
438 positively controlled in the wild type condition) and that their expression appears to be  
439 oppositely controlled at 35 hpf; this mirrors the variations measured for polarity proteins  
440 (**Figure 4**). This is consistent with a functional link between these Rho-GEFs and apicobasal  
441 polarity control during EHT progression. We finally decided to focus on ArhGEF11 for the  
442 following reasons: (i) in comparison to Tiams that act on Rac1, ArhGEF11 and ArhGEF12  
443 (which are close relatives and can form heterodimers (Chikumi et al. 2004)), are mostly acting  
444 on RhoA which is controlling apical constriction via the RhoA-Myosin II signalling axis; as we  
445 have shown previously (Lancino et al. 2018), EHT progression requires the constriction of  
446 circumferential actomyosin; (ii) ArhGEF11 was shown to regulate the integrity of epithelial  
447 junctions by interacting directly with the scaffolding protein ZO-1, hence connecting inter-  
448 cellular adhesion with RhoA-Myosin II (Itoh et al. 2012); (iii) ArhGEF11 mediated apical  
449 constriction is essential during tissue morphogenesis such as, for example, the neural tube  
450 formation in which epithelial cells, submitted to mediolateral contractile forces, constrict at their  
451 apical side thus triggering inward bending of the neural plate which leads to the formation of  
452 the neural tube (Nishimura, Honda, and Takeichi 2012). The EHT may share features with this  
453 process, i.e the anisotropic distribution of contractile forces controlling the plane of emergence;  
454 (iv) mammalian ArhGEF11 exhibits alternative splicing in its C-terminal region that controls  
455 tight junction stability via the regulation of RhoA activity (Lee et al. 2018) as well as cell  
456 migration and invasion (Itoh et al. 2017).

457 To confirm the potential function of ArhGEF11 at the junctional interface between HE/EHT and  
458 endothelial cells, we first investigated its intracellular localization. We attempted to detect the  
459 full-length form upon expression of a GFP fusion protein, in the vascular system, and failed to  
460 do so. We then generated a truncated form that retains the N-terminal fragment encompassing  
461 the PDZ and RGS domains (see **Figure 7 – figure supplement 3A**) fused with eGFP in its C-

462 terminus. Upon transient expression in the vascular system, we visualized its localization at  
463 the interface between endothelial and hemogenic cells progressing throughout the EHT, with  
464 an apparent increase in density at antero-posterior regions between adjoining EHT cells  
465 (**Figure 7 - figure supplement 3B**).

466 In line with the formerly described function of a splicing variant of ArhGEF11 in controlling tight  
467 junction integrity, particularly during egression of cells from the skin epithelium in the mouse  
468 (Lee et al. 2018), we questioned the potential role of such variant in EHT. This variant, referred  
469 to as the mesenchymal form, results from the inclusion of a peptide encoded by exon 37 or  
470 exon 38 in mouse and human, respectively (Shapiro et al. 2011; Itoh et al. 2017; Lee et al.  
471 2018). This insertion locates in the degenerated C-terminal region of the protein, which is  
472 predicted to be relatively poorly organized in its 3D structure. Upon amplifying the full-length  
473 sequence of zebrafish ArhGEF11 for investigating its localization, we cloned fragments  
474 encoding – or not – for the insertion of a peptide of 25 amino-acid residues (75 base pairs  
475 corresponding to exon 38). Although variable when compared with other species (see **Figure**  
476 **7 – figure supplement 4C, bottom panel**), this peptide is inserted in the same region as it is  
477 for mammals and may correspond to an ArhGEF11 variant functionally equivalent and involved  
478 in the regulating of junctional stability (see **Discussion**).

479 To investigate the function of ArhGEF11 on the junctional interface between HE/EHT and  
480 endothelial cells, and more specifically of the isoform containing the exon 38 encoded peptide,  
481 we used both morpholino (MO) and CRISPR-based strategies. We designed a splicing MO at  
482 the 3-prime exon/intron boundary of exon 38 that interferes with its inclusion in the encoding  
483 mRNA (**Figure 7 - figure supplement 4A**). This MO did not trigger any deleterious effect on  
484 the gross morphology of zebrafish embryos (**Figure 7 – figure supplement 4B**) and blood  
485 circulation was normal in comparison to control embryos. We attempted to generate CRISPR-  
486 based genomic deletion mutants of exon 38, both using Cpf1 and Cas9 enzymes and failed  
487 (see **Materials and Methods**). However, using CRISPR/Cas9, we obtained a deletion mutant

488 triggering a frame shift downstream of exon 38 and introducing a premature stop codon few  
489 amino-acid residues downstream thus leading to a sequence encoding for an ArhGEF11 C-  
490 terminal deleted form (see **Materials and Methods** and **Figure 7- figure supplement 4C**)).  
491 Unlike the variant skipping exon 38 induced by the splicing MO, expression of the  
492 CRISPR/Cas9 C-ter deletion mutant triggered, around 24 hpf, a severe retardation of blood  
493 circulation initiation in approximately 80% of the embryos obtained from incrossing  
494 ArhGEF11<sup>CRISPR-Cterdel/+</sup> heterozygous mutant fishes. From 24 to 48 hpf, approximately 50% of  
495 these embryos recovered a normal blood circulation (suggesting that these embryos are  
496 probably heterozygous for the mutation), approximately 35% remained with a severe  
497 phenotype (characterized by a large pericardiac oedema) and approximately 15% died, see  
498 **Figure 7 – figure supplement 4D** and **Materials and Methods**). This indicates essential  
499 functions of the C-ter region of ArhGEF11, in agreement with previously published data on the  
500 mammalian protein (Chikumi et al. 2004).

501 We then characterized more in depth the MO and CRISPR phenotypes and performed a  
502 quantitative analysis of the number and morphology of HE, EHT and adjoining endothelial  
503 cells, based on confocal images and subsequent segmentation of cell contours (using 2D  
504 deployment of the aortic cylinder).

505 For the morphants, we found approximately a double amount of hemogenic cells in the aortic  
506 floor (altogether localized on the floor and on the lateral sides of the aorta, see **Figure 7A** and  
507 **Figure 7 - figure supplement 5**); increase in the number of hemogenic cells also concerned  
508 cells oriented more perpendicularly to the blood flow axis (toward the Y-axis, **Figure 7Aa''**),  
509 consistently with variation in emergence angle, as we have shown previously (Lancino et al.  
510 2018). This is accompanied by a significant reduction in the number of morphologically  
511 characterized EHT cells (**Figure 7A** and **Figure 7 – figure supplement 5**). Similar results  
512 were obtained for the CRISPR homozygous deletion mutants, particularly regarding the  
513 accumulation of hemogenic cells, although with a smaller amplitude (**Figure 7B** and **Figure 7**

514 **- figure supplement 6**). Consistently, a significant increase in the aortic perimeter was found,  
515 at least for the morphants (**Figure 7C**). Of notice also, hemogenic cells in morphants are on  
516 average less elongated in comparison to control and to the CRISPR homozygous mutants (as  
517 well as less dispersed according to this parameter), with the accumulation of a cell population  
518 standing below the median value (**Figure 7Aa**); to some extent, this is reproduced as well  
519 when measuring hemogenic cell area). Finally, the population of small cells in the morphants  
520 are of uncharacterized morphology, which means that they appear neither as EHT pol+ nor as  
521 EHT pol- cells and may represent abortive emergences (**Figure 7A** and **Figure 7 – figure**  
522 **supplement 5** in which cellular morphology can be assessed from z-stacks).

523 Altogether, the morphants and CRISPR mutant phenotypes show that the progression  
524 throughout EHT is significantly impaired supporting the idea that ArhGEF11 exerts important  
525 functions in the process. Increase in the frequency of elongated cells for the CRISPR mutants  
526 in comparison to morphants suggests that interference triggered by the C-ter deletion of  
527 ArhGEF11 is more effective at early time points. Interference with inclusion of exon 38 in  
528 morphants triggers an increase in the number of hemogenic cells of smaller area and reduced  
529 elongation indicating a more efficient progression throughout emergence in comparison to the  
530 CRISPR mutants, albeit with impairment at later stages. This may be due to alteration in the  
531 dynamics of endothelial cell intercalation which is required for emergence completion (which  
532 is also compatible with the accumulation of pairs of cells that may be post-mitotic and that do  
533 not spread apart because this also requires aortic cell intercalation).

534

535 **ArhGEF11/PDZ-RhoGEF and its variant containing the exon 38 encoded sequence**  
536 **control junctional dynamics to support reduction of the hemogenic/endothelial**  
537 **interface and cell-cell intercalation**

538 To investigate how ArhGEF11 controls the dynamic interplay between HE/EHT and endothelial  
539 cells, we characterized further the MO and CRISPR phenotypes, at the junctional level. To do

540 so, we performed FRAP experiments using the *Tg(Kdrl:eGFP-Jam3b)* fish line that was  
541 injected at the one cell stage with the exon 38 splicing MO, or incrossing (*Kdrl:eGFP-Jam3b x*  
542 *ArhGEF11<sup>CRISPR-Cterdel/+</sup>*) heterozygous fishes to obtaining homozygous mutants and wild type  
543 alleles for control siblings.

544 FRAP experiments were focused on the hemogenic (HE)/endothelial (EC) membrane  
545 interface, with the support of images obtained after 2D deployments of aortic segments (**Figure**  
546 **8A**). Pools of *eGFP-Jam3b* molecules localized at bi-junctional (HE-EC) and at tri-junctional  
547 (HE-EC-EC and HE-HE-EC) interfaces were bleached and subsequently imaged for FRAP  
548 analysis. In the case of the MO treatment (in which case HE cells accumulate on the aortic  
549 floor (see **Figure 7A** and the 2D cartography of the aortic floor **Figure 8A**)) we measured, in  
550 comparison to control, an increase in FRAP recovery parameters for *eGFP-Jam3b* pools at  
551 the tri-junctional HE-HE-EC interface (**Figure 8B**), which is the most significant for the recovery  
552 speed at early time points (**Figure 8B, b''**). Results are different for the *ArhGEF11<sup>CRISPR-</sup>*  
553 *Cterdel/+* homozygous mutants for which we did not observe any obvious effect at HE-HE-EC tri-  
554 junctions (**Figure 8C**) but, rather, a small tendency for an increase in the mobile pool at HE-  
555 EC-EC tri-junctions (that has the particularity to concern 2 endothelial cells that contact the  
556 longitudinal membrane of one HE cell, see **Figure 8A, D**).

557 Altogether and including our results presented **Figure 7**, these results reinforce the idea that  
558 *ArhGEF11* and its variant encoded by exon 38 are involved in critical steps of EHT progression,  
559 partly by controlling the dynamics of junctional pools at the level of tri-junctions established  
560 between adjoining hemogenic and endothelial cells (see the model **Figure 8D** and its legend  
561 for a detailed analysis of mechanistic issues). The results also suggest that the +exon 38  
562 peptide encoding variant is prominently involved in controlling the HE-HE-EC interface and  
563 more specifically during intercalation, consistently with the accumulation, upon MO  
564 interference, of EHT cells having progressed throughout the emergence but appearing to be  
565 slowed down when reaching completion (cells of smaller area and less elongated, see **Figure**

566 7). Finally, ArhGEF11 is controlling RhoA and, most probably, its function in EHT progression  
567 also involves actin and acto-myosin activities that are involved in the contraction of HE and  
568 EHT cells (see Lancino et al. 2018; this is coupled with the consumption of longitudinal and  
569 transversal membrane interfaces, see also the model **Figure 8D** and its legend).

570

## 571 **Discussion**

### 572 **Characteristics of the HE and complexity of pre-hematopoietic stem cell emergence**

573 Heterogeneity in the identity and hematopoietic potential of cells emerging from the HE in the  
574 AGM has been recently shown in co-culture systems and *in vivo* in the mouse (Ganuza et al.  
575 2017; Dignum et al. 2021) and in the zebrafish model (Tian et al. 2017; Xia et al. 2023);  
576 however, knowledge on the fundamental cell biological and molecular events leading to this  
577 complexity is still lacking. Here, we highlight functional aspects that may significantly contribute  
578 to this complexity and that take root in the cellular plasticity of the HE. We bring evidence  
579 suggesting that HE cells that become competent to initiate the EHT time-window (around 28-  
580 30 hpf) tune their apicobasal polarity status which endows them with the ability to support two  
581 emergence processes with radically different morphodynamic characteristics; eventually, this  
582 would lead to differentially fated cells, which remains to be determined.

583 While the ability of the HE to regulate its polarity features may be inherited from molecular  
584 cues involved in its upstream intra-aortic specification (such as for example NOTCH (Robert-  
585 Moreno et al. 2008; Gama-Norton et al. 2015; Bonkhofer et al. 2019), TGF $\beta$  (Monteiro et al.  
586 2016), Gata2 (Butko et al. 2015; Daniel et al. 2019)), or any other of the more upstream factors  
587 involved in hemogenic specification (Zhao et al. 2022)), our results suggest that Runx1 is  
588 taking part in this regulation, consistently with its hematopoietic and EHT-inducing activity  
589 (North et al. 1999; Kalev-Zylinska et al. 2002; Kissa and Herbomel 2010; Lancrin et al. 2012).  
590 Our experiments using an interfering form of Runx1 (dt-Runx1) that produces a relatively weak  
591 an subtle phenotype in comparison to morpholino treatment that prevents aortic cells to convert

592 into HE cells (Bonkhofer et al. 2019) or genetic mutation and gene knockout abrogating  
593 hematopoiesis (T. E. North et al. 2009; Sood et al. 2010; Gao et al. 2018), provided the  
594 opportunity to observe that, at 30 hpf, elongated HE cells on the aortic floor appear to be  
595 maintained and to exhibit apicobasal polarity, with the absence of apparent cytosolic vacuolar  
596 structures (as has been described in the mouse in Runx1 interfering condition (T. North et al.  
597 1999)), which correlated with the subsequent accumulation of EHT pol+ cells that appeared to  
598 be at the expense of EHT pol- cells. This is in favour of the idea that the two cell types arise  
599 from a common precursor capable of evolving toward the one or the other, and that has lost  
600 apicobasal polarity (see the model **Figure 2B**). Recent work that has identified Runx1 targets  
601 in the zebrafish embryo at 29 hpf does not point at any of the conventional apicobasal polarity  
602 organizers as being direct targets although Pard3 and Pard6 mRNAs are clearly  
603 downregulated in the HE in comparison to aortic roof cells (Bonkhofer et al. 2019) which is  
604 consistent with our results at early time points of the EHT time-window.

605 A key question arising from our observations is what would control the balance orienting toward  
606 an EHT pol+ or an EHT pol- type of emergence. Cell division may be orienting toward EHT  
607 pol- emergence type (which would also be the reason why these cells have a round-shape  
608 morphology in comparison to EHT pol + cells). The frequency of EHT pol+ versus EHT pol-  
609 cells that we have estimated and that is of approximately 2/1 is not incompatible with this  
610 possibility since divisions take place in the HE at a relatively high frequency. Indeed, it has  
611 been estimated that 50% of HE cells that constitute the aortic floor at 28 hpf will divide and will  
612 undergo emergence thereafter during the 28-72 hpf time window (Zhao et al. 2022). However,  
613 we observe that both EHT pol+ and EHT pol- cells can divide during the emergence process  
614 (see also our former work Lancino et al. 2018 for EHT pol+ cells), which is followed by the full-  
615 recovery of their respective morphodynamic characteristics (not shown). Hence, this excludes  
616 the possibility of a short term and purely mechanical effect of cell division on emergence  
617 morphodynamics. In link with cell division however, there exist the possibility that the pool of

618 large vesicular structures potentially awaiting for exocytosis in HE cells having lost their  
619 apicobasal polarity may become asymmetrically inherited after division. This would endow the  
620 cell remaining with the largest pool to undergo EHT pol+ emergence after exocytosis and  
621 expansion of the apical/luminal membrane. Our results **Figure 2** would support this possibility  
622 but what controls this asymmetry would remain to be established.

623 Still, regarding the control of the balance orienting toward an EHT pol+ or an EHT pol- type of  
624 emergence, the intrinsic nature of the HE, i.e. its acquaintance with the aortic differentiation  
625 programme (Bonkhofer et al. 2019), raises the possibility of a partial recovery of an aortic cell  
626 phenotype in the case of EHT pol+ emergence, thus supporting its very unique  
627 mechanobiology. Therefore, the regulation of local signalling between aortic and HE cells may  
628 tune the balance toward the one or the other fate; this could be the case, for example, for  
629 adjoining HE cells in comparison to isolated ones that would then receive unequal signalling  
630 cues owing to the difference in homotypic versus heterotypic contacting surfaces. In this  
631 scenario, NOTCH signalling which is determinant for aortic and HE specification (Gama-Norton  
632 et al. 2015) may play a significant role, particularly via the ratio of the Jag1/Dll4 ligands that is  
633 fundamentally involved in HE versus aortic specification (Robert-Moreno et al. 2008;  
634 Bonkhofer et al. 2019).

635 Finally, we cannot totally exclude that EHT pol+ and EHT pol- cells would originate from  
636 different precursors. On this line, it has been shown that the aortic wall can home a transient  
637 venous-fated progenitor cell population that will lead to the underlying vein via selective  
638 sprouting from 21 to 23 hpf (Herbert et al. 2009). This option would require a massive  
639 expansion of a minor fraction of the cell population remaining in the aortic floor since it is  
640 expected that most of it has been moving out toward the sub-aortic region, which is not in  
641 favour of this possibility. In addition, recent scRNAseq analysis of floor and roof aortic cells at  
642 21 and 28 hpf has led, for both timing points, to two well defined clusters defined only by aortic  
643 and hematopoietic signatures (Zhao et al. 2022).

644

645 **The complexity of pre-HSC emergence and heterogeneity of hematopoietic stem cell**  
646 **and progenitor populations**

647 Currently, we do not know what would be the features that would endow the two cell types to  
648 behave differently after their release. Since post-EHT cells have to migrate in transient  
649 developmental niches (i.e the CHT, the thymus, the pronephric region), these features may  
650 provide them with more or less ability to migrate in the sub-aortic space and pass throughout  
651 the wall of the underlying vein to conquest more distant niches (Murayama et al. 2006), or to  
652 remain in a local AGM niche and be exposed to specific signalling there, in the proximity to the  
653 aorta as it is the case for hematopoietic clusters in mammals and avians (Ciau-Uitz et al. 2014;  
654 Jaffredo and Yvernogeu 2014). Beside their potential differences in colonizing different niches  
655 which would impact on their subsequent fate, these cells may also contribute to more or less  
656 transient waves of hematopoietic stem cells and progenitors that would be specific to the  
657 developmental period such as, for example, a restricted sub-set of T-lymphocytes that was  
658 described before (Tian et al. 2017). In the specific case of the Tian et al. work, the AGM was  
659 shown to produce transient T-lymphocytes proposed to be independent of long-lived HSCs  
660 according to a gradient that increases from the anterior to the posterior part of the trunk region,  
661 as well as from the aorta in the tail (the posterior blood island in the tail region that was  
662 proposed to give rise to hematopoietic precursors related to mammalian erythro-myeloid  
663 progenitors, (Bertrand et al. 2007)). Since the EHT pol<sup>+</sup> and EHT pol<sup>-</sup> cells that we describe in  
664 our work are equally produced from the aorta in the trunk and in the tail regions (thus  
665 irrespective of the antero-posterior axis of the aorta), the one or the other of the two EHT types  
666 most probably does not directly relate to the first wave of T-lymphopoiesis arising from the  
667 aortic endothelium described in Tian et al 2017.

668

669 **The cell biology behind emergence control and mechanics**

670 The intrinsic capacity of the HE, that we show here to be able to support two highly distinct  
671 emergence processes, in the zebrafish embryo, is very unique. Indeed, not only are they taking  
672 place contemporarily, but also can the two cell types proceed throughout emergence at a  
673 distance shorter than 50  $\mu\text{m}$ , which accommodates the intercalation of a single adjoining  
674 endothelial cell. This may reflect the remarkable properties of the vascular system that needs  
675 to allow cellular extrusion and transmigration. The latter is particularly relevant in the context  
676 of developmental hematopoiesis since hematopoietic precursors that travel via blood  
677 circulation need to pass throughout the aortic wall to conquest their distant niches.

678 In the present work, we provide some of the mechanistic insights into the specificities of the  
679 two emergence types and that rely on two fundamental aspects of cellular biology, i.e  
680 apicobasal polarity establishment and junctional mobility. The establishment and maintenance  
681 of apicobasal polarity in a cell extruding from its environment, as is the case for EHT pol+ cells,  
682 is at odds with the fundamental mechanisms of cell extrusion irrespective of the context into  
683 which the process is taking place (Nieto et al. 2016; Gudipaty and Rosenblatt 2017; Pei et al.  
684 2019; Staneva and Levayer 2023); this places the emergence of EHT pol+ cells as an extreme  
685 case of extrusion and it is somehow unexpected to observe this type of emergence when the  
686 aortic wall can extrude EHT pol- cells that appear to emerge contemporarily, apparently  
687 according to a more conventional mode. During the EHT time-window, Runx1 is directly  
688 controlling the emergence programme by regulating Rho-dependent cytoskeletal functions in  
689 HE cells derived from mouse ES lines (Lie-A-Ling et al. 2014) and inducing the expression of  
690 Gfi1ab in the zebrafish (Bonkhofer et al. 2019) that belongs to the family of transcriptional  
691 repressors of the arterial programme expressed in the HE (Gfi1 and Gfi1b in the mouse,  
692 (Lancrin et al. 2012)). This programme downregulates key molecules involved in the  
693 maintenance of the aortic endothelium among which Cdh5 (VE-Cadh) and the downregulation  
694 of cadherins is a hallmark of extrusion. Thus, for EHT cells, and particularly EHT pol+ cells,  
695 downregulation of VE-Cadh should be compensated by other mechanisms that allow for the

696 maintenance of aortic integrity. In this context of adhesion downregulation, the JAMs (that are  
697 expressed endogenously in the vascular system and that were used in this study) appear to  
698 be a well-suited substitute. As part of tight junction complexes, they can strengthen adhesion  
699 and, in addition, since they are also involved in cell migration (in particular leukocyte trans-  
700 endothelial migration, see (Ebnet 2017)), they could in theory support the extrusion of EHT  
701 pol- cells that do so according to a seemingly migration-type emergence mode (inferred from  
702 our time-lapse sequences) more or less related to trans-endothelial migration. In the case of  
703 EHT pol+ cells, we propose that JAMs may significantly contribute to the re-establishment of  
704 apicobasal polarity and to the strengthening of adhesion while EHT pol+ cells should undergo  
705 strong mechanical tension (owing to cellular bending). Interestingly, Jam3b appears to be a  
706 direct and positively regulated target of Runx1 (Bonkhofer et al. 2019), which is supporting its  
707 aforementioned potential functions during EHT.

708 Finally, regarding inter-cellular junctions and the control of their dynamics all along the EHT  
709 process, we also addressed the dynamic interface between aortic and HE cells. As for EHT  
710 cells, we focused more specifically on tri-junctional interfaces that have been proposed to  
711 sense tension and transmit the information to other types of junctional complexes (Bosveld,  
712 Wang, and Bellaïche 2018); in the EHT context, the contracting hemogenic endothelium is  
713 additionally exposed to high mechanical tension owing to wall shear stress imposed by the  
714 blood flow and that was proposed to regulate emergence efficiency (Lundin et al. 2020;  
715 Campinho et al. 2020; Chalin et al. 2021). In our work, we highlight one essential aspect of  
716 controlling junctional recycling which is during cell-cell intercalation which concerns either  
717 contacting HE cells lying on the aortic floor or HE/EHT cells after cytokinesis (as mentioned  
718 before, cell division is rather frequent during the EHT time window (Zhao et al. 2022)). In this  
719 context, we addressed the function of ArhGEF11/PDZ-RhoGEF which provides a functional  
720 link between cell polarity (with its PDZ-binding motif and its RGS domain that couples to G-  
721 protein coupled receptor signalling to regulate planar cell polarity (Nishimura, Honda, and  
722 Takeichi 2012)), cell adhesion (it binds to the tight junction associated regulatory protein ZO-

723 1, (Itoh et al. 2012)), and cytoskeletal regulation/contraction (Itoh et al. 2012; Itoh 2013), all  
724 three essential mechanistic components of morphogenetic movements and tissue remodelling.  
725 Since morphodynamic events leading to cell extrusion and to the control of cell migration do  
726 not only rely on the regulation of mRNAs levels but also on alternative splicing (Pradella et al.  
727 2017) our interest for investigating the function of ArhGEF11 in EHT was raised by the fact that  
728 the protein undergoes changes in its biological properties via the alternative splicing of a small  
729 exon which leads to a modification of its C-terminus. We identified a splicing event in this region  
730 (concerning exon 38) for a potential zebrafish isoform ortholog. However, the precise function  
731 of this variant for the zebrafish protein, that was shown in the case of the mammalian isoform  
732 to induce cell migration and motility (Itoh et al. 2017) and to bind to the Pak4 kinase thus  
733 leading to the subsequent destabilization of tight junction complexes via loss of RhoA activation  
734 (Lee et al. 2018), remains to be established. On this line, our results consistently indicate that  
735 preventing exon 38 insertion interferes with EHT progression (as well as deleting the C-  
736 terminus of the protein as shown with our CRISPR mutant) and appears to impair intercalation  
737 of aortic cells between adjoining HE cells. Hence, the +exon 38 isoform, by regulating the  
738 dynamic interplay between HE and endothelial cells, would favour one of the essential steps  
739 of the EHT process leading to its completion and requiring the complete isolation of EHT cells  
740 to ensure the ultimate sealing of the aortic floor (see our former work (Lancino et al. 2018)). It  
741 remains to be established, as our measurements indicate, why the ArhGEF11 +exon 38  
742 isoform appears to slow down junctional recycling which is counterintuitive with increasing the  
743 HE/endothelial dynamic interface. However, upon interference, we have been following Jam3b  
744 recycling which is only a sub-population of tight junction components that should be at play in  
745 the system. Finally, the EHT process on its whole may be submitted to a complex regime of  
746 alternating contraction and stabilization phases as we have shown previously in the context of  
747 EHT pol+ emergence and that may reflect the necessity to adapt to mechanical constraints  
748 imposed by the environment as well as the rearrangements of the HE/EHT/aortic cellular  
749 interface (Lancino et al. 2018). In this context, back and forth regulatory mechanisms,

750 particularly involved in the control of the RhoA-Myosin II signalling axis partially regulated by  
751 ArhGEF11, may locally and at specific timing points change the turnover of junctional  
752 molecules, thus blurring the correlation between gross phenotype (here the organization of the  
753 HE/aortic interface) and very local and dynamic molecular events (for a complement to the  
754 discussion, see also the legend of the model presented **Figure 8C**).

755 Overall, our work highlights the complexity of pre-hematopoietic stem cell emergence as well  
756 as some of the essential molecular and mechanistic aspects involved. We show that the aorta,  
757 in the zebrafish embryo, produces two fundamentally different types of EHT cells and propose  
758 that this results from specific functional features of the HE regulated by the transcription factor  
759 Runx1. This would support the production of cell types potentially endowed with different cell  
760 fate potential, subsequently reinforced by the type of niche into which these cells will establish  
761 homing. It now remains to be established if the different cell types that we describe are indeed  
762 leading to cells that will eventually hold specific hematopoietic fate and properties.

## 763 Materials and Methods

### 764 Key Resources Table

765 For the exhaustive list of all primers, see Materials and Methods - Supplementary Table.

Reagent type (species) or resource	Designation	Source or reference	Identifiers	Additional information
strain, strain background (zebrafish <i>Danio rerio</i> )	AB	Zebrafish International Resource Center (ZIRC)	ZFIN: ZDB-GENO-960809-7	
strain, strain background (zebrafish <i>Danio rerio</i> )	Tg(kdrl:HsHRAS - mCherry): s916Tg	(Chi et al. 2008)	ZFIN ID: ZDB-ALT-090506-2	referred to as Tg(kdrl:Ras-mCherry)
strain, strain background (zebrafish <i>Danio rerio</i> )	Tg(kdrl:nls-mKate2)	N/A	N/A	
strain, strain background (zebrafish <i>Danio rerio</i> )	Tg(kdrl:Gal4;UAS:RFP)	N/A	N/A	
strain, strain background (zebrafish <i>Danio rerio</i> )	Tg(kdrl:Gal4;UAS:RFP;4xNR:eGFP-Podxl2)	This paper	N/A	
strain, strain background (zebrafish <i>Danio rerio</i> )	Tg(kdrl:mKate2-Podxl2)	This paper	N/A	
strain, strain background (zebrafish <i>Danio rerio</i> )	Tg(kdrl:eGFP-Jam2a)	This paper	N/A	
strain, strain background (zebrafish <i>Danio rerio</i> )	Tg(kdrl:eGFP-Jam3b)	This paper	N/A	
strain, strain background (zebrafish <i>Danio rerio</i> )	Tg(kdrl:Gal4;UAS:RFP;4xNR:dt-runx1-eGFP)	This paper	N/A	
strain, strain background (zebrafish <i>Danio rerio</i> )	Tg(ArhGEF11 <sup>CRISPR-Cterdel-/+</sup> )	This paper	N/A	
recombinant DNA reagent	pG1-flk1-MCS-toI2	(Jin et al. 2005)	N/A	
recombinant DNA reagent	pEGFP-C1	Clontech	N/A	
recombinant DNA reagent	Transposase pCS-zT2TP	(Suster et al. 2011)	N/A	

Reagent type (species) or resource	Designation	Source or reference	Identifiers	Additional information
recombinant DNA reagent	pmKate2-f-mem	Evrogen	Cat# FP186	
antibody	goat anti-rabbit Alexa Fluor 488	Invitrogen	Cat# A11070	1/400
antibody	rabbit anti-GFP	MBL	Cat# 598	1/300
antibody	mouse anti-HA	Sigma	Cat# 12ca5	1/50
antibody	goat anti-mouse HRP-conjugated	Thermo Fisher Scientific	Cat# G-21040	1/300
sequence-based reagent	Morpholino sih	Sehnert et al., 2002	ZDB-MRPHLNO-060317-4	CATGTTTGCTCTG ATCTGACACGCA
sequence-based reagent	Morpholino ArhGEF11 exon 38	This paper	N/A	GAAATAAATGAAG CCCCACCTCCGT
sequence-based reagent	Oligo for CRISPR: Alt-R® CRISPR-Cas12a (Cpf1) crRNAs -1	This paper	N/A	TATCACACACACA TCACCTTCTA
sequence-based reagent	Oligo for CRISPR: Alt-R® CRISPR-Cas12a (Cpf1) crRNAs -2	This paper	N/A	TTTCTCAGCGCTC CTGACAGATG
sequence-based reagent	Oligo for CRISPR: Alt-R® CRISPR-Cas9 crRNAs	This paper	N/A	AGCCAATCGTCTG AGGACGG
sequence-based reagent	Oligo for CRISPR: Alt-R® tracrRNA	IDT	Cat# 1072532	
software, algorithm	CRISPRscan	(Moreno-Mateos et al. 2015)		N/A
software, algorithm	Zen Pro2 software	Zeiss	<a href="https://www.zeiss.com/microscopy/en/products/software/zeiss-zen.html">https://www.zeiss.com/microscopy/en/products/software/zeiss-zen.html</a>	N/A
software, algorithm	QUANTSTUDIO™ DESIGN & ANALYSIS 2	Thermofisher	<a href="https://apps.thermofisher.com/apps/da2/">https://apps.thermofisher.com/apps/da2/</a>	N/A
software, algorithm	MetaMorph software	Molecular Devices	<a href="https://www.moleculardevices.com/products/cellular-imaging-systems/acquisition-and-analysis-software/metamorph-microscopy">https://www.moleculardevices.com/products/cellular-imaging-systems/acquisition-and-analysis-software/metamorph-microscopy</a>	N/A
software, algorithm	Acquisition-Imaging analysis-LAS X	Leica	<a href="http://www.leica-microsystems.com">http://www.leica-microsystems.com</a>	N/A
software, algorithm	Image Analysis-Fiji	NIH	<a href="https://imagej.net/Fiji">https://imagej.net/Fiji</a>	N/A

Reagent type (species) or resource	Designation	Source or reference	Identifiers	Additional information
software, algorithm	Image Analysis Icy	(de Chaumont et al., 2012)	<a href="http://icy.bioimaginganalysis.org/">http://icy.bioimaginganalysis.org/</a>	N/A
software, algorithm	Image Analysis TubeSkinner plugin for Icy	(Lancino et al. 2018)	<a href="https://icy.bioimaginganalysis.org/plugin/tubeskinner/">https://icy.bioimaginganalysis.org/plugin/tubeskinner/</a>	N/A
software, algorithm	Figures-Illustrator CC 2017.1.1	Adobe	<a href="http://www.adobe.com/cn/">http://www.adobe.com/cn/</a>	N/A
software, algorithm	Active contour plugin	(Dufour et al. 2010)	<a href="https://icy.bioimaginganalysis.org/plugin/active-contours/">https://icy.bioimaginganalysis.org/plugin/active-contours/</a>	N/A
software, algorithm	NIS software	Nikon	<a href="https://www.micromoscope.healthcare.nikon.com/en_EU/products/software/nis-elements">https://www.micromoscope.healthcare.nikon.com/en_EU/products/software/nis-elements</a>	N/A
software, algorithm	Rstudio	RStudio Team (2020)	<a href="http://www.rstudio.com/">http://www.rstudio.com/</a>	N/A
software, algorithm	ggplot2	(Wickham 2016)	<a href="https://ggplot2.tidyverse.org">https://ggplot2.tidyverse.org</a> .	N/A
software, algorithm	readr	(Wickham, Hester, and Bryan 2022)	<a href="https://github.com/tidyverse/readr">https://github.com/tidyverse/readr</a>	N/A
software, algorithm	cowplot	(Wilke 2020)	<a href="https://wilkelab.org/cowplot/">https://wilkelab.org/cowplot/</a>	N/A
software, algorithm	dplyr	(Wickham et al. 2023)	<a href="https://github.com/tidyverse/dplyr">https://github.com/tidyverse/dplyr</a>	N/A
software, algorithm	wesanderson	(Ram and Wickham 2018)	<a href="https://github.com/karthik/wesanderson">https://github.com/karthik/wesanderson</a>	N/A
software, algorithm	matrixStats	(Ram and Wickham 2018)	<a href="https://github.com/HenrikBengtsson/matrixStats">https://github.com/HenrikBengtsson/matrixStats</a>	N/A
software, algorithm	stringr	(Wickham 2022)	<a href="https://github.com/tidyverse/stringr">https://github.com/tidyverse/stringr</a>	N/A
software, algorithm	ggsci	(Xiao N et al. 2023)	<a href="https://github.com/nanxstats/ggsci">https://github.com/nanxstats/ggsci</a> .	N/A
software, algorithm	ggpubr	(Kassambara et al. 2020)	<a href="https://rpkgs.datanovia.com/ggpubr/">https://rpkgs.datanovia.com/ggpubr/</a>	N/A
software, algorithm	ggstatsplot	(Patil 2022)	<a href="https://indrajeetpatil.github.io/ggstatsplot/index.html">https://indrajeetpatil.github.io/ggstatsplot/index.html</a>	N/A
software, algorithm	ggbeeswarm	(Clarke and Sherrill-Mix 2017)	<a href="https://github.com/eclarke/ggbeeswarm">https://github.com/eclarke/ggbeeswarm</a>	N/A
software, algorithm	DT	(Xie, Cheng, and Tan 2022)	<a href="https://github.com/rstudio/DT">https://github.com/rstudio/DT</a>	N/A

Reagent type (species) or resource	Designation	Source or reference	Identifiers	Additional information
software, algorithm	SciViews	(Grosjean 2019)	<a href="https://www.sciviews.org/SciViews/">https://www.sciviews.org/SciViews/</a>	N/A
software, algorithm	nls.multstart	(Padfield and Matheson 2020)	<a href="https://github.com/padpadpadpad/nls.multstart">https://github.com/padpadpadpad/nls.multstart</a>	N/A

766

767

## 768 **Contact for reagent and resource sharing**

769 Further information and requests for resources and reagents should be directed to and will be  
770 fulfilled by the corresponding Author, Anne A. Schmidt ([anne.schmidt@pasteur.fr](mailto:anne.schmidt@pasteur.fr)).

## 771 **Zebrafish husbandry**

772 Zebrafish (*Danio rerio*) of the AB background and transgenic fish carrying the following  
773 transgenes *Tg(kdrl:ras-mCherry)* (Chi et al. 2008); *Tg(kdrl:nls-mKate2)*;  
774 *Tg(kdrl:Gal4;UAS:RFP)*; and the fish lines generated in this study:  
775 *Tg(kdrl:Gal4;UAS:RFP;4xNR:eGFP-Podxl2)*; *Tg(kdrl:mKate2-Podxl2)*; *Tg(kdrl:eGFP-Jam2a)*;  
776 *Tg(kdrl:eGFP-Jam3b)*; *Tg(kdrl:Gal4;UAS:RFP;4xNR:dt-runx1-eGFP)*; *Tg(ArhGEF11<sup>CRISPR-Cterdel/+</sup>)*  
777 were raised and staged as previously described (Kimmel et al. 1995). Adult fish lines  
778 were maintained on a 14 hr light/10-hr dark cycle. Embryos were collected and raised at either  
779 28.5 or 24°C in N-Phenylthiourea (PTU, Sigma Aldrich, Cat# P7629)/Volvic source water  
780 (0.003% final) to prevent pigmentation complemented with 280 µg/L methylene blue (Sigma  
781 Aldrich, Cat# M4159). Embryos used for imaging, extracting mRNA, or for WISH ranged from  
782 developmental stages 28-to-60 hpf and larvae used for imaging were of 3-5 dpf, precluding  
783 sex determination of the animals. The fish maintenance at the Pasteur Institute follows the  
784 regulations of the 2010/63 UE European directives and is supervised by the veterinarian office  
785 of Myriam Mattei.

## 786 **mRNA extraction and cDNA synthesis**

787 Total RNA was extracted from whole 48 hpf embryos for cDNA cloning or from pooled trunks  
788 at the desired developmental stages (30-32 hpf, 48-50 hpf time windows for qRT-PCR  
789 experiments) (~30 individuals per tube). Briefly, embryos were anesthetized using balneation  
790 in embryo medium supplemented with tricaine methanesulfonate (MS-222, Sigma-Aldrich Cat#  
791 A5040), at a final concentration of 160  $\mu\text{g/ml}$ . RNA was extracted via organic extraction using  
792 TRIzol reagent (Invitrogen Cat# 15596026) according to the manufacturer's guideline. gDNA  
793 contaminant was removed using TURBO™ DNase (Invitrogen Cat# AM2238) treatment  
794 according to the manufacturer's guideline. Total RNA was stored at  $-80^{\circ}\text{C}$ . Reverse  
795 transcription of mRNA was performed using SuperScriptIV (Invitrogen Cat# 18090010) with  
796 OligodT primer. cDNA samples were stored at  $-20^{\circ}\text{C}$  until further processing (for quantitative  
797 real-time PCR, PCR, cloning and sequencing).

#### 798 **Transient and stable transgenesis**

799 The *kdr1* promoter (*flk*) (Jin et al. 2005) was used to drive endothelial expression of mKate2-  
800 Podxl2, eGFP-Jam2a and eGFP-Jam3b, with eGFP (Clontech) and mKate2 (Evrogen) cDNAs  
801 amplified using overlapping primers for Gibson cloning. eGFP-Podxl2, ArhGEF11(PDZ-PRD-  
802 RGS-eGFP) and dt-runx1-eGFP were amplified for cloning into a pG1-4XNR vector (built from  
803 the 4XNR (non-repetitive) 4X UAS sequence less susceptible to methylation than the 14X UAS  
804 described in (Akitake et al. 2011)). For the sequence of all the designed cloning primers, see  
805 the Key Resources Table.

806 Podocalyxin-like 2 (Podxl2), ArhGEF11/PDZ-RhoGEF, JAM2a and JAM3b full length  
807 sequences were amplified from pools of 48 hpf whole embryo cDNA (see before for mRNA  
808 extraction and cDNA synthesis methodologies). All constructs generated in this study were  
809 obtained using the Gibson assembly assay (NEB, Cat # E2611S).

810 For eGFP-Jam2a and eGFP-Jam3b cloning, we used the JAM2a signaling peptide sequence  
811 (included in the 5-prime amplification primers and encoding for the signal peptide:  
812 mlvcvsliliihsvpvpvtvssr) and, for both constructs, the eGFP sequence was inserted in frame

813 upstream of the sequence encoding for the transmembrane domain; for the eGFP-Jam2a  
814 construct, the eGFP was inserted upstream of amino-acid D221 (DLNVAA) of the NCBI  
815 Reference Sequence: NP\_001091734.1; for the eGFP-Jam3b construct, the eGFP was  
816 inserted upstream of amino-acid D243 (DINIAG) of the NCBI Reference Sequence:  
817 NP\_001076332.2. For transient ubiquitous and inducible expression, the eGFP-Jam2a and  
818 eGFP-Jam3b fusion constructs were inserted into a KpnI pre-digested pG1 plasmid containing  
819 tol2 sites and the HSP70-4 promoter. For stable transgenesis using the *kdr1* (*flk1*) promoter,  
820 constructs cloned into the pG1-HSP70 plasmids were extracted using XhoI/NotI restriction  
821 enzymes and inserted into the XhoI/NotI pre-digested pG1-flk1 vector using DNA T4 ligation  
822 (NEB, Cat # M0202S).

823 For the Podxl2 N-ter deletion mutant that has been used for establishing stable *Tg* fish lines,  
824 the cDNA encoding for the peptide sequence starting with amino-acid G341 (GGTEYL;  
825 fragment 341-587) of the NCBI Reference Sequence: XP\_692207.6 was fused with either  
826 eGFP or mKate2 cDNAs. The sequence encoding for the signal peptide of the human CD8  
827 (malpvtallplalllhaarpsqfrvs) was introduced upstream (5-prime) of the eGFP or mKate2  
828 sequences (included in the 5-prime primers designed for the Gibson cloning strategy). For the  
829 construct designed to obtain the *Tg(kdr1:Gal4;UAS:RFP;4xNR:eGFP-Podxl2)* fish line, 2  
830 overlapping fragments were amplified for cloning into a pG1-tol2\_4xNR vector after digestion  
831 with NcoI, with the following couples of primers: 4XNR\_CD8-eGFP\_fw and  
832 delPodxl2\_eGFP\_rev; delPodxl2\_fw and Podxl2\_pG1-4XNR\_rev. For the construct designed  
833 to obtain the *Tg(kdr1:mKate2-Podxl2)* fish line 2 overlapping fragments were amplified for  
834 cloning into a pG1-flk1 vector after digestion with EcoR1, with the following couples of primers:  
835 pG1-flk1\_CD8\_mKate2\_fw and delPodxL2-mKate2\_rev; mKate2-delPodxL2\_fw and  
836 Podxl2\_PG1-flk1\_rev.

837 For the ArhGEF11(PDZ-PRD-RGS-eGFP) construct, the cDNA encoding for the peptide  
838 ending upstream of the DH domain at Valine 709 of the amino-acid sequence (GenBank:  
839 AY295347.1; PALDEDV; fragment 1-709) was fused upstream of the sequence encoding for

840 eGFP, for expression using the pG1-4xNR vector. The cDNA was amplified using the  
841 GB\_4xUAS-gef11\_fw; GB\_eGFP-RGS\_rev couple of primers.

842 For the construct designed to obtain the *Tg(kdrl:Gal4;UAS:RFP;4xNR:dt-runx1-eGFP)* fish  
843 line, the Runx1 sequence amplified omits the ATG (replaced by an nls) and ends downstream  
844 of the Runt domain (ending at amino-acid sequence PAHSQIP). The construct allows for the  
845 expression of the dt-runx1 protein under the control of the 4xNR driver using the same strategy  
846 as for the above PodxL2 constructs. The dt-runx1 cDNA construct was amplified from the  
847 zebrafish Runx1 cDNA sequence (Kalev-Zylinska et al. 2002) using the couple of primers GB-  
848 4xnr-nlsRunx1\_fw; GB-eGFP-T2A-2xHA-delRunx1\_rev. The sequence of these primers each  
849 allowed for the introduction, in the protein sequence, of a nls (encoded by the fw primer) and  
850 of a 2xHA-T2A peptide (encoded by the rev primer that overlaps with the 5-prime sequence of  
851 eGFP).

852 For all constructs, cloning reactions were used to transform NEB 5-alpha Competent E. coli  
853 (NEB, Cat # C2987) and resistant clones then used for miniprep plasmid preparations  
854 (Nucleospin plasmid, Macherey-Nagel, Cat # 740588.50), followed by sequencing. Plasmid  
855 DNA from clones containing correct insertions were then used to transform E. coli TOP10  
856 competent cells (Invitrogen, Cat # C404010) and purified using the endotoxin free NucleoBond  
857 Xtra Midi kit (Macherey Nagel, Cat# 740420.10). Transgenesis was then performed by co-  
858 injecting 1 nl of plasmid (at 25 ng/ $\mu$ l) mixed with tol2 transposase mRNA (at 25 ng/ $\mu$ l)  
859 transcribed from linearized pCS-zT2TP plasmid using the mMACHINE SP6 kit  
860 (Ambion, Cat# AM1340). For stable transgenesis, embryos were screened for fluorescence  
861 between 24 hpf and 48 hpf and raised to adulthood. Founders with germline integration were  
862 then isolated by screening for fluorescence after outcrossing with AB adults and for  
863 establishment of the F1 generation.

864

## 865 **CRISPR methodology and GEF11 mutant transgenic line screening**

866 *CRISPR/Cas9 mutagenesis and isolation of founders*

867 A sgRNA was designed against the splice donor site at the end of exon 38 with the aim to  
868 interfere with the production of the splicing variant including its encoding sequence, using the  
869 CRISPRscan web tool (Moreno-Mateos et al. 2015). The sgRNA was obtained by annealing  
870 for 5 min at 95°C equal volumes of 100 $\mu$ M specific Alt-R® crRNAs (IDT, Cat# sequence:  
871 AGCCAATCGTCTGAGGACGG) with 100 $\mu$ M Alt-R® generic tracrRNA (IDT, Cat# 1072532).  
872 Cas9 Nuclease Reaction Buffer (NEB Cat# B0386A) was added to obtain a final 45 $\mu$ M sgRNA  
873 stock solution. For the generation of the sgRNAs–Cas9 complex, a mix containing 18 $\mu$ M  
874 sgRNA and 12 $\mu$ M Cas9 protein (EnGen™ Spy Cas9-NLS *S. pyogenes*, NEB Cat# M0646T)  
875 was incubated for 10 min at room temperature. For mutagenesis, 1 nl of the sgRNA-Cas9  
876 complex was injected into one-cell stage embryos, that were subsequently raised until 48 hpf.  
877 Bulk genomic DNA (gDNA) was extracted from 60 injected and 60 non-injected control  
878 embryos. gDNA extraction proceeded as follows: pooled samples were incubated for 3 hrs at  
879 55°C in lysis buffer (100mM NaCl, 20mM Tris-HCl pH 8, 25mM EDTA, 0.5% SDS and 2  $\mu$ g/ $\mu$ l  
880 proteinase K), purified using phenol:chloroform. Precipitation was done in 0.1 volume of 3M  
881 NaAc and 2.5 volume of 100% ethanol for 20 min at -20°C. Samples were centrifuged for 30  
882 min at 4°C before washing with 70% ethanol and resuspension in water. gDNA was used for  
883 PCR, using DNA polymerase Platinum™ SuperFi™ (Invitrogen Cat# 12351010) and the  
884 SM31-SM32bis couple of primers (TTTCACTTTCTCTCTGCCTCTTACA;  
885 AACTCTGTCCAGATGATTGAGGAGC) flanking the targeted region. PCR products were run  
886 on gel electrophoresis before gel extraction of bands of interest (with sizes corresponding to  
887 either a wild-type allele or a deleted allele) (Nucleospin gel and PCR clean-up, Macherey-  
888 Nagel Cat# 740609.50). Fragments were cloned into blunt-end TOPO vector (Invitrogen Cat#  
889 45024), transformed into *E. coli* TOP10 competent cells and grown on selective medium. 12  
890 colonies for each PCR product were sent for sequencing. Mutant sequences contained either  
891 a 7 nucleotides deletion, or a mix of 1,4 or 7 nucleotides deletions located at the same site (at  
892 the end of exon 38, see **Figure 7 - figure supplement 4A** for the 7 nucleotides deletion),  
893 attesting of the proper functioning of our guide.

894 A stable homogeneous mutant line was generated by injecting the sgRNA:Cas9 complex into  
895 one-cell stage *Tg(kdrl:Gal4;UAS:RFP)* embryos similarly to what was described above. 100  
896 embryos were raised until adulthood. Adult founders were identified by crossing adult F0 with  
897 AB fishes, and single embryo genotyping was performed on their progeny (10 embryos per  
898 adult fish). Briefly, single embryos were incubated in 40 $\mu$ l 25mM NaOH 0.2mM EDTA and  
899 heated for 10 min at 95°C, before cooling for 10 min at 4°C and addition of 40 $\mu$ l 40mM Tris-  
900 HCl pH5 to stop the reaction. PCR was performed using 5 $\mu$ l of template and specific primers  
901 (couple SM31-SM34 –TTTCACTTTCTCTCTGCCTCTTACA;  
902 ATAAATGAAGCCCCACCTCCGTCC – for wild-type (WT) allele and couple SM31-SM40 –  
903 TTTCACTTTCTCTCTGCCTCTTACA; ATGAAGCCCCACCTCAGACGATTGGC– for mutant  
904 allele). Presence of WT or mutant allele for each single embryo was assessed by the  
905 presence/absence of amplification band on gel electrophoresis. Four F0 founders were  
906 isolated, that had a transmission rate in the germline ranging from 20% to 80%. Progeny of the  
907 F0 founders and subsequent generations were raised separately until adulthood, and  
908 genotyped. After complete anesthesia (in fish water supplemented with 160 $\mu$ g/ml tricaine  
909 methanesulfonate), a small tissue sample was collected from the caudal fin, placed into DNA  
910 extraction buffer (10mM Tris pH8.2, 10mM EDTA, 200mMNaCl, 0.5% SDS, 200 $\mu$ g/ml  
911 proteinase K) and incubated 3 hrs at 50°C. DNA precipitation and resuspension was performed  
912 as described above. 7 $\mu$ L gDNA was used as template for PCR amplification using the primer  
913 couple SM31-SM32bis. Sequenced PCR products showed that all founders harbored the same  
914 7 nucleotides deletion (see **Figure 7 - figure supplement 4A**). All experiments relative to  
915 **Figure 7 and Figure 8** were performed using the progenies of one heterozygous mutant F3  
916 couple. For these experiments, genotyping on single embryos was performed on gDNA as  
917 mentioned above. Only WT homozygous and mutant homozygous embryos were kept for  
918 analysis.

919 *Alternative Cpf1 approach*

920 As an alternative approach to the CRISPR/Cas9 deletion, we attempted to generate a second  
921 mutant line, using to CRISPR/Cpf1(Cas12a) system to delete a 315 nucleotides region  
922 encompassing exon 38 (with specific guides located in the introns before and after exon 38).  
923 The sequence of guides that have been tested are: TATCACACACACATCACCTTCTA and  
924 TTTCTCAGCGCTCCTGACAGATG. However, due to the structure of the intronic regions  
925 (repeat rich) and the necessary presence of specific PAM sequence, we only generated off-  
926 target deletions of intronic regions, without deletions of exon 38, leading us to focus on the  
927 CRISPR/Cas9 approach.

928

### 929 **Morpholinos and injections**

930 Morpholinos were obtained from GeneTools (see Key Resources Table for sequences). The  
931 *sih* Tnnt2 translation start codon and flanking 5-prime sequence MO (Sehnert et al. 2002) as  
932 well as the ArhGEF11 exon 38 splice blocking MO (overlapping the exon 38/intron 38-39  
933 boundary) were resuspended in ddH<sub>2</sub>O to obtain stock solutions at 2mM and 1mM,  
934 respectively. The *sih* MO (1.5ng) and ArhGEF11 exon 38 MO (3ng) were injected into 1-cell  
935 stage zebrafish embryos, after dilution in ddH<sub>2</sub>O. For *sih* morphants, embryos that were used  
936 in the experiments were checked for absence of heart beating 24 hrs after injection as well as  
937 before being used for dissection and RNA extractions at the 30-32 and 48-50 hpf time-  
938 windows. For measuring the efficiency of the ArhGEF11 exon 38 MO, total RNA was extracted  
939 from pools of injected and control embryos followed by mRNA reverse transcription. PCR on  
940 cDNA flanking the exon 38 was performed using DNA polymerase Platinum™ SuperFi™  
941 (Invitrogen Cat# 12351010) and analyzed on gel electrophoresis before extraction, cloning and  
942 sequencing to verify the absence of the DNA sequence encoded by exon 38 (see **Figure 7 -**  
943 **figure supplement 4A**).

944

### 945 **Whole mount in situ hybridization**

946 For the sequence of all the primers used to amplify probes, see the Key Resources Table. The  
947 probes for WISH concern Pard3ab, Pard3bb (**Figure 3**) and PDZ-RhoGEFs (**Figure 7 – figure**  
948 **supplement 2**).

949 Whole-mount chromogenic in situ hybridization was performed as described in Lancino et al  
950 2018. Probes were synthesized using the manufacturer recommendation (T7 RNA polymerase,  
951 Promega, Cat# P2075, DIG-nucleotides, Jena Bioscience, Cat# NU-803-DIGX).

952 For Pard3ab (GenBank: AY524776.2), a probe of 717bp (nucleotides 1258-1974)  
953 encompassing the nucleotide sequence starting in the 3-prime of the sequence encoding for  
954 PDZ1 and ending between PDZ2 and PDZ3 was amplified with the couple of primers  
955 pard3ab\_WISH\_Nter\_fw and pard3ab\_WISH\_Nter\_T7\_rev. For Pard3bb (NCBI Reference  
956 sequence: XM\_021478865.1), a probe of 747bp (nucleotides 468-1214) encompassing the  
957 nucleotide sequence starting in the 5-prime of the sequence encoding for the N-ter of the  
958 protein and ending at the 3-prime of PDZ1 was amplified with the couple of primers  
959 pard3bb\_WISH\_Nter\_fw and pard3bb\_WISH\_Nter\_T7\_rev (for the position of the primers  
960 respectively to the 2 pard3 proteins sequences and domains, see Figure 3B).

961 Images were captured with the Zeiss Axio ZOOM V16 microscope with the Zen Pro2 software,  
962 with a brightfield transmitted light optics. Post-processing steps were performed using the  
963 Extended-Depth Focus method to combine in focus regions from multiple z-planes and convert  
964 into in a transmitted light z-stack to generate a unique in-focus image.

965

#### 966 **Immunofluorescence detection of the HA-tagged dt-runx1 deletion mutant**

967 Whole mount immunostaining was performed on hand-dechorionated 48 hpf *Tg(dt-runx1)*  
968 embryos to assess the localization of the 2XHA-tagged dt-runx1 protein in aortic cells. Briefly,  
969 embryos were fixed in 4% methanol free PFA (Polysciences Cat# 040181) for 3 hrs at room  
970 temperature (RT) and washed in PBS/0.1% tween20 (PBT). Embryos were treated in  
971 successive baths of milliQ water supplemented with 0.1% tween20, then in cold acetone for  
972 10 min at -20°C and again in milliQ water plus 0.1% tween20. They were then washed in 1X

973 HBSS (Invitrogen Cat# 14025), and permeabilized for 45 min at RT in 1X HBSS, 0.1%  
974 tween20, 5mM CaCl<sub>2</sub> and 0.1mg/ml collagenase (Sigma-Aldrich Cat# C9891). Embryos were  
975 then rinsed in PBSDT and incubated in blocking solution 1x sheep serum (SS, Sigma Cat#  
976 S2263) for at least 4 hrs at RT. They were then incubated overnight at 4°C with primary  
977 antibodies in blocking solution: rabbit anti-GFP (MBL Cat# 598; 1/300) and mouse anti-HA  
978 (Sigma, Cat# 12ca5; 1/50). On the next day, embryos were washed several times in PBSDT  
979 and endogenous peroxidase activity was inactivated by treatment with 6%H<sub>2</sub>O<sub>2</sub> (Sigma-  
980 Aldrich, Cat# H1009) for 1 hr at 4°C. Embryos were then incubated in the NGS for at least 4  
981 hrs and incubated overnight at 4°C with secondary antibodies: goat anti-rabbit Alexa Fluor 488  
982 (Invitrogen Cat# A11070; 1/400) and goat anti-mouse HRP-conjugated (Thermo Fisher  
983 Scientific Cat# G-21040; 1/300). Finally, embryos were rinsed several times first in PBSDT and  
984 after in PBT before HRP fluorescent revelation. Embryos were incubated for 45 min at RT in  
985 the dark in imidazole buffer (0.1 M imidazole (Sigma-Aldrich, Cat# I5513) in PBT supplemented  
986 with 1% H<sub>2</sub>O<sub>2</sub>) with Cy3 Tyramide Reagent (Cy3 NHS sigma Cat# PA13101, tyramide sigma  
987 Cat# T-2879, dimethyl formamide sigma Cat# T-8654 and triethylamine sigma Cat# T-0886).  
988 Final washes in PBT and in 6% H<sub>2</sub>O<sub>2</sub> for POD inactivation were performed before embryos  
989 were mounted in low-melting agarose in 1X PBT for fluorescence confocal imaging.

990

## 991 **Quantitative real-time PCR**

### 992 *Preparation of samples*

993 To determine the impact of runx1 interference and blood flow on pard3 family genes and splice  
994 variants expression, total RNA from dissected trunk regions was extracted (as described  
995 previously) from 30-32 hpf and 48-50 hpf embryos from either incross of the Tg(kdrl:dt-  
996 runx1(eGFP)) fish line, outcross of the Tg(kdrl:dt-runx1(eGFP)) and Tg(kdrl:gal4;UAS:RFP)  
997 fish lines - with eGFP positive embryos as the interfering condition and eGFP negative  
998 embryos as sibling controls -, or Tg(kdrl:gal4;UAS:RFP) embryos injected at the single cell  
999 stage with sih morpholino. Analysis was carried on 3 biological replicates.

1000 *Technical procedure*

1001 For the sequence of all the designed qRT-PCR primers, see the Key Resources Table.

1002 qRT-PCR was performed using Takyon Rox SYBR 2x Master mix blue dTTP kit (Eurogentec  
1003 Cat# UF-RSMT-B0701), with starting concentrations of template of around 10ng/ $\mu$ l and primer  
1004 concentrations of 0.5 $\mu$ M, on a QuantStudio3 system (Applied Biosystems). Each reaction was  
1005 performed in technical triplicates and in at least 3 biological replicates. Ct was determined  
1006 automatically by the QUANTSTUDIO™ DESIGN & ANALYSIS 2 software on the Thermofisher  
1007 cloud and exported to be analyzed manually. Standard deviation was calculated within the  
1008 technical triplicates, and when superior to 0.3, obvious outliers were removed (Dixon's Q test).  
1009 The delta-delta-Ct method (double normalization to endogenous control — zebrafish  
1010 elongation factor ef1 $\alpha$  — and to control sample) was used to compare gene expression in  
1011 control and altered conditions.

1012

1013 **In vivo confocal imaging**

1014 Embryos, dechorionated manually, were anesthetized using tricaine (Sigma Aldrich, Cat#  
1015 A5040). They were then embedded on the side position in 1% low melting agarose (Promega,  
1016 Cat# V2111) in a glass bottom 60 $\mu$ -Dish (35mm high; Ibidi, Cat# 81156). To avoid movements  
1017 and pigmentation during image acquisitions, 1x tricaine /1x PTU Volvic water was incorporated  
1018 to the low melting agarose and, after solidification, 1ml of 1x tricaine /1x PTU Volvic water was  
1019 added before sealing the dish.

1020 Embryos were imaged with 2 confocal microscope systems. For the results presented Figure  
1021 1, z-stacks and time-lapse sequences were obtained using a Leica TCS SP8 inverted confocal  
1022 microscope as described in Lancino et al 2018.

1023 For all the other confocal microscopy results, embryos were imaged using an Andor (Oxford  
1024 Instruments) spinning disk confocal system (CSU-W1 Dual camera with 50 $\mu$ m Disk pattern  
1025 and single laser input (445/488/561/642nm), LD Quad 405/488/561/640 and Tripl 445/561/640  
1026 dichroic mirrors), equipped with a Leica DMI8 fluorescence inverted microscope and CMOS

1027 cameras (Orca Flash 4.0 V2+ (Hamamatsu)). Imaging was performed using a 40x water  
1028 immersion objective (HC PL APO 40x/1.10 Water CORR CS2), a LEDs light source (CoolLED  
1029 pE-4000 16 wavelength LED fluorescence system), and the acquisitions were piloted with the  
1030 support of the MetaMorph software.

1031

### 1032 **Morphological and morphometric analysis of aortic and hemogenic cells**

1033 For the morphometric analysis of the aorta and aortic cells, large z-stack of 48-55 hpf  
1034 Tg(kdrl:eGFP-JAM3b; kdrl:nls-mKate2) or Tg(kdrl:eGFP-JAM2a; kdrl:nls-mKate2)  
1035 encompassing the whole aortic depth were acquired with optimal z-resolution (0.3 $\mu$ m z-step).  
1036 For each embryo, 3 contiguous z-stacks of 330 $\mu$ m width were acquired, allowing us to image  
1037 the entirety of the AGM region, from the anterior to the posterior end of the elongated yolk.

#### 1038 *Samples*

1039 For ArhGEF11 splicing morpholino experiment, 3 splicing morpholino injected embryos and 2  
1040 control non injected siblings were kept for the analysis.

1041 For ArhGEF11 CRISPR Cter deletion and when performing the experiments, imaging was  
1042 done, for putative mutants, on embryos that had shown a delay in initiation of circulation at 24  
1043 hpf, regained circulation at 48 hpf albeit with a clearly visible oedema in the cardiac region  
1044 (non-altered embryos were kept as sibling controls). All imaged embryos whose signal allowed  
1045 us to generate segmentable 2D-cartography from the whole length of the aorta were kept and  
1046 processed further for genotyping. Genotyping confirmed that the circulation problems at 24 hpf  
1047 and the remaining oedema at 48 hpf corresponded to embryos bearing the mutation.

1048 For CRISPR mutant phenotype analyses, 2 mutant embryos and 2 wild type siblings were kept,  
1049 after genotyping.

#### 1050 *Image analysis*

1051 The following image analysis was performed on Icy software.

1052 For each segment, we generated a 2D-cartography of the eGFP-Jam signals at intercellular  
1053 contacts of aortic cells, using the Tubeskinner plugin, according to the protocol described in

1054 Lancino et al 2018. Briefly, the aorta was rotated to face the lumen, a circular ROI fitting the  
1055 interior of the aorta was initialized by the experimenter on the first plane, and for each plane  
1056 sequentially, the algorithm will more accurately fit the ROI to the aortic perimeter and collect  
1057 the fluorescence intensity all along its longitudinal axis. The collected intensity was then  
1058 projected on a 2D-plane, where the Y-axis of the 2D-cartography corresponds to the perimeter  
1059 of the aorta, and the X-axis corresponds to the X-axis of the original z-stack.

1060 Before further processing, a metadata table recapitulating the dates and conditions of  
1061 experiments for each embryo was generated, and a random label was assigned to rename  
1062 each acquisition. This allowed for the later part of the analysis, that involves semi-automated  
1063 segmentation and classification by the user, to be blind and avoid potential bias.

1064 Each 2D-cartography was then manually pre-segmented using the ROI Polygon tool to draw  
1065 the contour of each aortic cell, using the original 3D z-stack as reference. Due to technical  
1066 limitation (decreased fluorescence signal collection on the farthest lateral side of the aorta),  
1067 only about 2/3 of the 2D-cartographies could be segmented (comprising the floor, the right  
1068 lateral side of the aorta and the roof). The manual segmentation was then improved using the  
1069 “Active Contour” plugin, that improved the fit of the cellular contour to the fluorescence signal  
1070 and slightly dilated the cell contours until the ROIs formed a joint paving. All morphometric  
1071 descriptors were extracted for each cell. The number of neighbours was automatically  
1072 generated for each cell by counting the number of touching adjacent ROIs.

1073 Additionally, cells were manually classified into cell types according to morphometric  
1074 parameters using as reference the original 3D z-stack and in particular the nls-mKate2 nuclear  
1075 signal. EHT-undergoing cells were positioned on the floor of the aorta, have reduce antero-  
1076 posterior axis length, luminal membrane inward bending for EHT pol+ cells and luminal  
1077 membrane outward bending for EHT pol- cells, and a thickened nucleus. Hemogenic cells  
1078 whose criteria were established in Lancino et al 2018 (elongated in the antero-posterior axis,  
1079 with lateral membranes not trespassing the equatorial plane of the aortic wall), are positioned  
1080 on the floor of the aorta, systematically have a reduced width perpendicular to the blood flow

1081 and, importantly, a thickened nucleus that protrudes out of the aortic plane. Finally, endothelial  
1082 cells have a flattened nucleus positioned high on the lateral side or on the roof of the aorta.

1083

#### 1084 **Measurement of the aortic perimeter**

1085 An accurate measurement of the aortic perimeter was obtained using the z-stack generated  
1086 for the aortic cells' morphometric analysis. Briefly, the z-stack was rotated to face the lumen of  
1087 the aorta and a ROI fitting the aortic perimeter was drawn by the user and optimized by the  
1088 "Cell Contour" plugin. The length of the perimeter was then extracted from the ROI statistics.

1089

#### 1090 **Fluorescence Recovery After Photobleaching (FRAP) measurements and analysis**

1091 Measurement of junctional turn-over was performed on 48-55 hpf *Tg(kdrl:eGFP-JAM3b;*  
1092 *kdrl:nls-mKate2)* embryos using a Nikon Ti2e spinning disk microscope equipped with a  
1093 sCMOS camera (Photometrics, Prime 95B, pixel size 11 $\mu$ m, 1200 $\times$ 1200 pixels, QE 95%) and  
1094 a 40x water objective (Numerical Aperture 1.15, Working Distance 0.6mm, xy pixel size of  
1095 0.27 $\mu$ m). Embryos were mounted as previously described.

#### 1096 *Samples*

1097 For ArhGEF11 splicing morpholino phenotypic analysis, 10 different embryos (injected with the  
1098 splicing morpholino) obtained from 3 separate experiments and 30 control embryos (non-  
1099 injected siblings or control morpholino injected siblings) obtained from 6 separate experiments  
1100 were analyzed.

1101 For EHT analysis, 16 embryos over 4 separate experiments were analyzed.

1102 For ArhGEF11 CRISPR/Cas9 Cter deletion phenotypic analysis, 8 mutant embryos and 12  
1103 wild type siblings over 3 separate experiments were analyzed.

1104 Overall, the exact number of interfaces analyzed is written on each figure panel.

#### 1105 *Acquisitions*

1106 For each acquisition, a wide two channels z-stack of the aorta was generated to capture the  
1107 whole aortic volume and generate a 2D-cartography of the aortic environment (Icy, plugin

1108 Tubeskinner). This 2D-cartography of the cellular contours and the z-stack with the cellular  
1109 contours and nuclei were used jointly to identify un-ambiguously cellular and junctional types  
1110 and define one or multiple single point regions of interest (ROIs) for subsequent bleaching and  
1111 fluorescence recovery measurement.

1112 The sequence of acquisitions before/after bleach focused only on the eGFP-Jam3b signal to  
1113 speed up the acquisition and improve the temporal resolution. The 5 steps of the acquisition  
1114 can be recapitulated as follows and we obtained/performed: (1) a single z-stack of 20 $\mu$ m  
1115 centered on the plane of focus, (2) a 30 sec time-lapse acquisition on the plane of focus to  
1116 measure average pre-bleach intensity with an image every second, (3) a high intensity  
1117 stimulation of the ROIs (25% 300ms 488 FRAP laser exposure, leading to the bleaching of the  
1118 Jam3b-eGFP signal), (4) a 3 min time-lapse acquisition on the plane of focus to measure fast  
1119 post-bleach recovery with an image every second, and (5) a 7 min time lapse acquisition of  
1120 8 $\mu$ m z-stack centered around focus plane with a 20 sec time interval to measure slow post-  
1121 bleach recovery. Each step was save separately as .nd2 files.

#### 1122 *Signal quantification and recovery analysis*

1123 File were converted from .nd2 to .tiff using FIJI batch converter, scale factor 1.00, bilinear  
1124 interpolation, no average when downsizing, images read using bioformat.

1125 To collect fluorescence intensity, ROIs for each stimulation point was manually drawn using  
1126 Fiji and named with an individual label using Fiji ROI manager. A metadata table was manually  
1127 curated with all information for each ROI (junction type, condition, date of experiment, embryo  
1128 number). An additional ROI was created to collect background intensity for subsequent  
1129 corrections. Acquisitions with clear embryo movement or drifting were either completely  
1130 removed from the dataset (~5% of the acquisitions) or only kept for early recovery analysis  
1131 (see below) if the drift only occurred in the last step (fifth) of the acquisition.

1132 Fluorescence intensity for each ROI was collected and saved automatically as a .csv file for  
1133 each acquisition using a homemade Fiji macro.

1134 All further analysis were performed using R for automatization.

1135 Briefly, for each ROI: the fluorescent signal was normalized to the background signal ( $I_{\text{background}}$   
1136  $\text{normalized} = I_{\text{roi}} - I_{\text{background}}$ ), then normalized to mean intensity pre-bleach  
1137 ( $I_{\text{normalized}} = I_{\text{background normalized ROI post-bleach}} / I_{\text{background normalized ROI pre-bleach}}$ ), and finally scaled  
1138 ( $I_{\text{full scaled normalized}} = (I_{\text{normalized}} - \min(I_{\text{normalized}})) / (1 - \min(I_{\text{normalized}}))$ ).

1139 A single exponential equation was used to fit the data using the `nls:multistart` package for  
1140 parameter optimization:

$$1141 F(t) = A \cdot (1 - e^{-t \cdot \tau})$$

1142 With  $A$  the maximum amplitude of the curve,  $t$  the time, and  $\tau$  a constant defining the growth  
1143 rate of the curve.

1144 The fitted curve allowed to extract quantitative information describing the recovery of each type  
1145 of junction in different conditions: the mobile fraction of the bleached pool (corresponding to  $A$ ,  
1146 the amplitude of recovery) and the time of half recovery, related to the speed of recovery, that  
1147 can be extracted from  $\tau$ :

$$1148 T_{\text{half-recovery}} = \ln(0.5) / (-\tau)$$

1149 A more precise description of the recovery speed was extracted from the observation of the  
1150 early time period (until 30s post bleach), where the fluorescence intensity increases uniformly.

1151 A first-degree polynomial equation was fitted to the data (using `lm()` function from the `stats`  
1152 package), and the coefficient corresponding to the slope of the fitted curve was used as a  
1153 metric for speed of fluorescence recovery.

1154

## 1155 **Statistical analysis**

1156 Statistical tests used are described for each figure panel in the legend. Since our samples are  
1157 usually small, the test used is an unpaired two samples non-parametrical Wilcoxon mean  
1158 comparison test, without a priori knowledge on sample normal distribution or homoscedasticity.

1159

## 1160 **Data and software availability**

1161 All graphics and plots were generated using RStudio and packages including: ggplot2  
1162 (Wickham 2016), readr (Wickham, Hester, and Bryan 2022), dplyr (Wickham et al. 2023),  
1163 stringr (Wickham 2022), ggstatsplot (Patil 2022), ggpubr (Kassambara 2020), wesanderson  
1164 (Ram and Wickham 2018), cowplot(Wilke 2020), ggsci (Xiao 2018), ggbeeswarm (Clarke and  
1165 Sherrill-Mix 2017), viridis (Garnier 2021), DT (Xie, Cheng, and Tan 2022), SciViews (Grosjean  
1166 2019), nls.multstart (Padfield and Matheson 2020), matrixStats (Bengtsson 2022).

1167

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1179

## 1180 **Authors contributions**

1181 LT designed, performed and analyzed experiments, and wrote the Method section and the  
1182 Figure legends. SM designed and conducted experiments. CV conducted experiments. AS  
1183 conceived the project, designed, performed and analyzed experiments, wrote the manuscript  
1184 with input from LT, and obtained funding.

1185

## 1186 **Declaration of interests**

1187 The authors declare no competing interests.

## 1188 **Figure legends**

1189

1190 **Figure 1: Tracing the evolution of the luminal membrane using the polarity marker**

1191 **Podocalyxin points at the biological significance of apicobasal polarity establishment**

1192 **in EHT cell emergence complexity. A**, cartoons depicting the early and late steps of EHT

1193 cells emerging from the aortic floor (steps 1 and 2, as previously described in the zebrafish

1194 embryo, see (Kissa and Herbomel 2010; Lancino et al. 2018)) and with hypothetical evolution

1195 of the luminal membrane (in green) before (3 and 4) and after the release (2', the cell detaches

1196 from the endothelial layer via junction downregulation leading to exposure of the luminal

1197 membrane with the extracellular milieu; 3', the luminal membrane is consumed via endocytic

1198 recycling (E) and/or lysosomal degradation (Lys) prior to detachment; 4', the luminal

1199 membrane in 4 is released inside the cell (twisted arrow) before detachment. Grey area=

1200 nucleus. **B**, PodocalyxinL2 (Podxl2) construct designed to establish transgenic fish lines.

1201 Cartoons representing full length (top drawing) and deleted Podxl2 (amino-acid sequence 341-

1202 587) in which the mucin domain (serine/threonine-rich O-glycosylation domain) is replaced by

1203 either eGFP or mKate2. The tetracysteine-containing globular domain (subjected to N-

1204 glycosylation) was kept as favoring apical membrane retention. TMD, transmembrane domain;

1205 DTHL, C-terminal peptidic motif involved in partnership with PDZ domain containing proteins.

1206 **C-D**, EHT performing cells visualized using *Tg(Kdrl:Gal4;UAS:RFP;4xNR:eGFP-Podxl2)*

1207 embryos and time-lapse sequences initiated at 55 hpf obtained with spinning disk confocal

1208 microscopy (imaging was performed at the boundary between the most downstream region of

1209 the AGM and the beginning of the caudal hematopoietic tissue). Top grey panels show the

1210 green, eGFP channels for eGFP-Podxl2 only. Bottom panels show the merge between green

1211 and red (soluble RFP, in magenta) channels. Bars: 8 $\mu$ m. **C**, single plane images of 2 EHT pol+

1212 cells extracted from a time-lapse sequence at t = 0 and t = 45 min, with the right cell (eht cell

1213 2) more advanced in the emergence process than the left one (eht cell1). Note the enrichment

1214 of eGFP-Podxl2 at the luminal membrane (surrounding the cavity labelled with an asterisk) in  
1215 comparison to the basal membrane (white arrow). Note also the evolution of the luminal  
1216 membranes with time, with the aortic and eht cell 1 lumens still connecting at  $t = 0$  (green  
1217 arrow), the apparent fragmentation of the cytosolic vacuole (2 asterisks for eht cell 1 at  $t = 45$   
1218 min) and the compaction of Podxl2-containing membranes for eht cell 2 at  $t = 45$  min. More  
1219 details on the evolution of the connection between the aortic/eht cell lumens are shown in  
1220 **Figure 1 – figure supplement 1A. D**, single plane images of 2 EHT pol- cells extracted from  
1221 a time-lapse sequence at  $t = 30$  min and  $t = 210$  min (see **Figure 1 – video 3** for the full-time  
1222 lapse sequence), with the right cell (eht cell 2) slightly more advanced in the emergence than  
1223 the left one (eht cell 1, with the latest attachment point between the emerging cell and the  
1224 aortic floor (pink arrow)). Note, in comparison with the cells in panel C, the ovoid shapes of  
1225 cells, the absence of enrichment of eGFP-Podxl2 at luminal membranes (green arrows) and  
1226 the accumulation of eGFP-Podxl2 at basal membrane rounded protrusions (white arrows).

1227  
1228 **Figure 2: Immature HE is not polarized and controls membrane delivery of intra-**  
1229 **cytosolic vesicular pools. A**, *Tg(Kdrl:Gal4;UAS:RFP;4xNR:eGFP-Podxl2)* embryo imaged  
1230 using spinning disk confocal microscopy. Black and white images show eGFP-Podxl2 only.  
1231 Images were obtained from a time-lapse sequence (initiated at 35 hpf) lasting for 435 min (7.25  
1232 hrs), with intervals of 15 min between each z-stack. Example of an HE cell with equal  
1233 partitioning of eGFP-podxl2 between luminal and abluminal membranes (at  $t = 0$  min), with  
1234 eGFP-podxl2 containing intra-cytosolic vesicles (one labelled with a green asterisk) and  
1235 undergoing mitosis at  $t = 30$  min (HE cell 1' and HE cell 1'' are daughter cells). Note the  
1236 inheritance of the largest micropinocytic-like vacuole by HE cell 1' and its maintenance through  
1237 time until EHT emergence initiation at  $t = 180$  min (green asterisk in 1.5x magnified areas at  $t$   
1238 = 60 and 90 min). At  $t = 360$  min (green box) both fluorescence channels are shown; bottom  
1239 panel: green (eGFP-Podxl2), magenta (soluble RFP). The magenta arrow points at the basal

1240 side of the EHT pol + cell that does not contain any detectable eGFP-Podxl2; on the contrary,  
1241 eGFP-Podxl2 is enriched at the luminal/apical membrane (note that exocytosis of the large  
1242 vacuolar structure may have contributed to increase the surface of the apical/luminal  
1243 membrane (the green asterisk labels the lumen of the EHT pol + cell). The green arrow points  
1244 at the abluminal membrane of the EHT cell derived from HE cell 1” and that contains eGFP-  
1245 Podxl2 (with no evidence of a significant expansion of a luminal/apical membrane); this  
1246 indicates that this cell is more likely to be an EHT pol- cell that does not sort the vesicular cargo  
1247 to the luminal/apical membrane. Bar = 10 $\mu$ m. **B**, model summarizing the evolution of HE cells  
1248 that involves the tuning of apicobasal polarity thus leading to cells competent for giving birth to  
1249 either EHT pol+ or EHT pol- cells (including the oriented release of large vesicular  
1250 macropinocytic-like vacuoles preferentially toward the luminal membrane of future EHT pol +  
1251 cells). The polarity status of the HE is proposed to evolve throughout the entire time window  
1252 of the EHT, leading to asynchrony in its ability to give birth to EHT cells (emergence of EHT  
1253 pol+ and EHT pol- cells are both observed until 72 hpf, see main text).

1254

1255 **Figure 3: Molecular variants and deletion mutants to investigate the control of**  
1256 **apicobasal polarity during the 30-32 and 48-50 hpf time-windows. A**, Expression of  
1257 Pard3ab and bb isoforms in the zebrafish embryo at 35 hpf using whole mount in situ  
1258 hybridization (WISH). Expression in the dorsal aorta, in the trunk region (delimited areas), is  
1259 marked by arrowheads as well as in the venous plexus of the caudal hematopoietic tissue, in  
1260 the tail. ey = elongated yolk. **B**, Pard3ab and bb zebrafish isoforms and natural variants to  
1261 investigate the regulation of apicobasal polarity during the 30-32 hpf and 48-50 hpf time  
1262 windows. The cartoons show the 3 sequential PDZ domains (PDZ1-3), the CR1 and CR3  
1263 conserved regions involved in oligomerization and atypical protein kinase C (aPKC) binding,  
1264 respectively. For each isoform, the variants that we have cloned either contain or exclude (+/-  
1265 exon) the peptides encoded by specific exons (see **Materials and Methods**); of relevance for  
1266 our purpose (investigating the expression of potential interfering isoforms), we focused on

1267 isoforms deleted from exons encoding for part of the PDZ domains (covering the amino-  
1268 terminus of Pard3ab PDZ2 and the carboxy-terminus of Pard3bb PDZ3). Note that deletion of  
1269 exon 10 of Pard3ab triggers a frame shift in the ORF leading to a complete deletion of PDZ2  
1270 and to a premature stop codon upstream of PDZ3. In this case, this should lead to the synthesis  
1271 of a deleted proteins with major loss of function or, alternatively, the degradation of the mRNA  
1272 via alternative splicing activated non-sense mediated decay (NMD, as exemplified in the EMT  
1273 process, see (Pradella et al. 2017)). The position of the primers used to amplify cDNA  
1274 fragments is depicted (fw (forward), rev (reverse), magenta: primers for WISH probes; see  
1275 **Materials and Methods**). Note that, in this study and for the PDZ1 domain of Pard3ab, we did  
1276 not address the expression levels of the isoforms containing or not the insert encoding for the  
1277 YVFR peptide. **C, left:** cartoons representing the zebrafish full-length runx1a amino-acid  
1278 sequence and the dt-runx1 mutant deleted from the trans-activation domain and of the C-  
1279 terminus (note that the construct encodes for a C-terminal fusion with eGFP that is released  
1280 upon expression via a cleavable T2A peptide (introduced between the 2xHA tag and the N-  
1281 terminus of eGFP, to prevent from potential steric hindrance). nls, nuclear localization signal.  
1282 **Right:** anti-HA tag immunofluorescence obtained after z-projection of the dorsal aorta of a 50  
1283 hpf *Tg(dt-runx1)* embryo. Note the localization of the 2xHA-tagged dt-runx1 protein in nuclei  
1284 (some of them are pointed by red arrowheads) and of eGFP in nuclei and the cytosol of aortic  
1285 cells. Bar: 25 $\mu$ m.

1286  
1287 **Figure 4: Pard3 isoforms and variants are differentially regulated during the 30-32 and**  
1288 **48-50 hpf time-windows.** qRT-PCR analysis of gene products of interest in trunks isolated  
1289 from 30-32 hpf and 48-50 hpf embryos. Trunks were collected from eGFP positive or eGFP  
1290 negative incrosses of heterozygous *Tg(dt-runx1)* fishes (dt-runx1 incross and sibling controls,  
1291 respectively) and from *Tg(kdrl:Gal4;UAS:RFP)* embryos injected or not at the single cell stage  
1292 with the *sih* morpholino (morpholino *sih*). Graphs show the measured mean fold changes  
1293 relative to the expression of *ef1a* and to the expression in control embryos. Statistical tests:

1294 two sided unpaired two samples Wilcoxon test, with p-values of significant differences. **A**, gene  
1295 expression levels of (**left**) the 3 isoforms of Pard3 proteins (Pard3ab, bb, b and ba, see  
1296 **Materials and Methods** for accession numbers and primers) and (**right**) the hematopoietic  
1297 marker *cmyb* and the polarity protein *Scrib* (for the cellular basolateral domain  
1298 establishment/maintenance). **B**, Pard3ab and Pard3bb gene expression levels for *dt-runx1*  
1299 mutants (red boxes) and for the *sih* morpholino (yellow boxes), all relative to control conditions.  
1300 **C**, Pard3ab +/- PDZ2 (top 2 panels) and Pard3bb +/- PDZ3 (bottom 2 panels) gene splicing  
1301 variants expression levels for *dt-runx1* mutants (left panels, red boxes) or the *sih* morpholino  
1302 (right panels, yellow boxes), all relative to control conditions.

1303

1304 **Figure 5: eGFP-Jam3b localization is reinforced at antero-posterior sites of the**  
1305 **endothelial/EHT interface and at tri-cellular junctions.** **A**, cartoons representing  
1306 homodimers of full-length JAMs with the C-terminal cytosolic part interacting with Pard3 (JAMs  
1307 interact with the first PDZ domain of Pard3) as well as the constructs generated in this study  
1308 and containing eGFP inserted between the Immunoglobulin-like (Ig) domains and the trans-  
1309 membrane region (TMD). The constructs were obtained for zebrafish JAM2a and JAM3b. **B**,  
1310 **C**, 52 hpf *Tg(kdrl:eGFP-Jam3b; kdrl:nls-mKate2)* embryos were imaged in the trunk region  
1311 (AGM) using spinning disc confocal microscopy. The panels are either maximum z-projections  
1312 (top two) or single plane z-sections (bottom two, focusing on the aortic floor) of aortic  
1313 segments, with either the merged *nls-mkate2* and eGFP-Jam3b fluorescence signals  
1314 (magenta and green) or the eGFP-Jam3b signal only (black and white images). Bottom of the  
1315 Figure: 2D-cartographies obtained after deploying aortic cylinders and showing the eGFP-  
1316 Jam3b signals only. **B**, example of an EHT pol+ cell (cell 1, white arrows point at reinforcement  
1317 of signal at antero-posterior junctions). On the 2D cartography, cell 1 (red) is contacting  
1318 endothelial cells 2 and 3; note the reinforcement of eGFP-Jam3b signals along antero-  
1319 posterior membrane interfaces perpendicular to blood flow (red arrows) as well as at the two  
1320 tri-cellular junctions visible between cells 1, 2 and 3 (black arrows). **C**, example of two EHT

1321 pol- cells (cells 1 and 2, white arrows point at reinforcement of signal at antero-posterior  
1322 junctions). On the 2D cartography, cells 1 and 2 (red) are contacting endothelial cells 3, 4, 6  
1323 and 3, 6 respectively; note the reinforcement of eGFP-Jam3b signals along antero-posterior  
1324 membrane interfaces perpendicular to blood flow (red arrows) and endothelial cell 6 that has  
1325 intercalated between endothelial cell 7 and EHT pol- cell 2 (blue arrow). In right margins,  
1326 magenta and green arrowheads designate the aortic floor and roof, respectively. Bars = 10 $\mu$ m.

1327

1328 **Figure 6: Junctional recycling at tri-cellular contacts is differentially controlled between**  
1329 **the two EHT types.** 48-55 hpf *Tg(kdrl:eGFP-Jam3b; kdrl:nls-mKate2)* embryos were imaged  
1330 using spinning disc confocal microscopy and illuminated for Fluorescence Recovery After  
1331 Photobleaching (FRAP) in the trunk region (AGM, Aorta Gonad Mesonephros). **A, B**, panels  
1332 are either maximum z-projections (top left) or single plane z-sections (bottom left and top right,  
1333 focusing on the aortic floor) of aortic segments, with either the merged nls-mkate2 and eGFP-  
1334 Jam3b fluorescence signals (magenta and green) or the eGFP-Jam3b signal only (black and  
1335 white images). White arrows point at reinforcement of signal at antero-posterior junctional  
1336 pools of an EHT pol+ cell (**A**) or of an EHT pol- cell (**B**), both marked by asterisks. Bottom right:  
1337 2D-cartographies obtained after deploying aortic cylinders and showing the eGFP-Jam3b  
1338 signals only. Black arrows point at antero-posterior junctional pools, in particular at tri-  
1339 junctional regions that exhibit increase in signal density (well visible in **A**, black arrows). 2 and  
1340 3 endothelial cells are contacting the EHT pol+ cell (**A**) and the EHT pol- cell (**B**), respectively.  
1341 In right margins, magenta and green arrowheads designate the aortic floor and roof,  
1342 respectively. Bars = 20 $\mu$ m. **C - G**, FRAP analyses. EGFP-Jam3b junctional pools  
1343 corresponding to the brightest spots inside junctional regions of interest were bleached for  
1344 FRAP measurements (these high intensity pools were localized at the level of bi- and tri-  
1345 junctions for endothelial cells (EC) and in tri-junctional regions for EHT pol+ and EHT pol- cells;  
1346 all these junctional pools were systematically visualized by deploying each aortic segment  
1347 before bleaching as shown in the 2D-cartographies in A and B as well as in **Figure 6 – figure**

1348 **supplement 1**, see also **Materials and Methods**). FRAP analysis concerned 3 types of  
1349 junctional interfaces: between endothelial cells (EC – EC, black and grey), EHT pol- and  
1350 endothelial cells (pol- – EC, brown), EHT pol+ and endothelial cells (pol+ – EC, blue). **C, D**,  
1351 evolution of mean fluorescence intensity for each type of junctional interface over time (10  
1352 min), after photobleaching (t = 0s). **E**, median maximum amplitude of recovery of all  
1353 determinations and for each type of junctional interface (maximum of simple exponential fitted  
1354 curves). **F, G**, early fluorescence recovery. Early evolution (over the first 30s) of the mean  
1355 fluorescence intensity for each type of junctional interface over time after photobleaching (t =  
1356 0s). **F**, the fitted lines correspond to linear regressions of mean fluorescence intensities. **G**,  
1357 median values of fluorescence recovery slopes (linear regressions) of all determinations and  
1358 for each type of junctional interface. Statistical tests: two sided unpaired two samples Wilcoxon  
1359 test.

1360

1361 **Figure 7: Interfering with ArhGEF11/PDZ-RhoGEF function leads to the accumulation of**  
1362 **hemogenic cells and impairs EHT progression. A-C**, numeration and morphometric  
1363 analyses of aorta and cell types for *Tg(kdrl:eGFP-Jam3b; kdrl:nls-mKate2)* ArhGEF11 exon  
1364 38 splicing morpholino-injected and control embryos (**A**), or for (*Kdrl:eGFP-Jam3b;*  
1365 *kdrl:ArhGEF11<sup>CRISPR-Cterdel+/+</sup>*) homozygous ArhGEF11 C-ter deletion mutants and control  
1366 siblings (**B**). 48-55 hpf embryos were imaged using spinning disk confocal microscopy. **Aa**,  
1367 **Bb**, 2D-cartographies obtained after deploying aortic cylinders and showing the eGFP-Jam3b  
1368 signals only with cell contours delineated either in blue (endothelial cells), yellow (hemogenic  
1369 cells, see **Materials and Methods** for their morphological definition), red (morphologically  
1370 characterized EHT cells, for controls), and small cells delineated by cyan boxes  
1371 (morphologically uncharacterized EHT cells and putative post-mitotic cells remaining as pairs,  
1372 included in the numeration as hemogenic cells). Cellular contours have been semi-  
1373 automatically segmented along the cellular interfaces labelled with eGFP-Jam3b (see

1374 **Materials and Methods**). Scale bars: 10 $\mu$ m. **Aa'**, **Bb'**, **left**: numeration of endothelial,  
1375 hemogenic and EHT-undergoing cells according to the position of their geometrical center  
1376 (either on the aortic floor, or on the roof, or on the lateral side), for each condition; **right**: number  
1377 of endothelial, hemogenic and EHT-undergoing cells in each condition calculated from the  
1378 segmentation of 3 x 2D-projections per embryo and covering the entire aortic regions in the  
1379 trunk. **Aa''**, **Bb''**, **left**: length of hemogenic cells (in the longest axis) in function of their  
1380 orientation ( $^{\circ}$ ) relative to the blood flow axis (0 – 180 $^{\circ}$ ); **right**: distribution of the orientation of  
1381 hemogenic cells relative to the blood flow axis, displayed as a mean distribution of cells per  
1382 embryo. **Aa'''**, **Bb'''**, hemogenic cell elongation factors in arbitrary Units (scale factor given by  
1383 the ratio between the first- and the second-best fitting ellipse diameters, the minimum value  
1384 being 1 for a non-elongated object) represented as boxplot distribution of all segmented cells  
1385 (**left**) or as the distribution of cell elongation factor per embryo (**right**), for controls and for  
1386 interfering conditions as indicated. **Aa''''**, **Bb''''**, hemogenic cell area represented as boxplot  
1387 distribution of all segmented cells (**left**) or as the distribution of cell area per embryo (**right**),  
1388 for controls and for interfering conditions as indicated. **C**, aortic perimeter (in  $\mu$ m) for controls  
1389 and mutant conditions as indicated. Statistical tests: two sided unpaired two samples Wilcoxon  
1390 test. For the ArhGEF11 exon 38 splicing morpholino condition, analysis was performed on 2  
1391 x control (non-injected embryos) and 3 x embryos injected at the one-cell stage; for the  
1392 CRISPR mutant condition, analysis was performed on 2 x wild-type siblings for control and 2  
1393 x homozygous mutant embryos whose DNA mutation was confirmed by sequencing. 3  
1394 consecutive aortic segments per embryo were analyzed to cover the whole length of the dorsal  
1395 aorta, in the trunk region (covering a distance of 990 $\mu$ m per embryo).

1396

1397 **Figure 8: Interfering with the function of ArhGEF11/PDZ-RhoGEF suggests an activity**  
1398 **at the interface between endothelial and hemogenic cells that relies on restricting the**  
1399 **mobility of junctional pools at tri-cellular junctions, with a prominent role of the +exon**

1400 **38 variant during endothelial cell intercalation. A**, 2D-cartography of an aortic segment of  
1401 a *Tg(kdrl:eGFP-Jam3b; kdrl:nls-mkate2)* embryo injected at the one cell stage with the  
1402 ArhGEF11 exon 38 splicing morpholino. All the arrows pointing at reinforced junctional  
1403 contacts between cells were bleached and imaged for FRAP analysis (the bleached areas  
1404 correspond to the spots of highest intensities in the regions of interest, as visualized at the  
1405 fluorescent confocal microscope, see **Materials and Methods**). Black and Magenta arrows  
1406 point at he-he-ec and he-ec-ec tri-cellular junctions, respectively (he: hemogenic cell; ec:  
1407 endothelial cell). Green arrows point at he-ec bi-cellular junctions. ec: endothelial cell, he:  
1408 hemogenic cell. Bar = 20 $\mu$ m. **B, C**, FRAP analysis of bleached eGFP-Jam3b localized in  
1409 regions of interest in controls (grey) and ArhGEF11 exon 38 splicing morpholino injected  
1410 embryos (blue) or homozygous ArhGEF11<sup>CRISPR-Cterdel+/+</sup> mutants (green). Statistical tests: two  
1411 sided unpaired two samples Wilcoxon test. Bb, Cc, evolution, after photobleaching (at t=0s),  
1412 of the mean fluorescence intensity per condition at HE-EC, HE-EC-EC and HE-HE-EC bi- and  
1413 tri-junctions over time (10 min). Bb', Cc', median values for maximum amplitude of recovery  
1414 (maximum of simple exponential fitted curves, see **Materials and Methods**). Bb'', Cc'', early  
1415 evolution, after photobleaching (at t=0s), of the mean fluorescence intensity per condition over  
1416 time (for the first 30 seconds). The fitted lines correspond to the linear regression of the mean  
1417 fluorescence intensities. Bb''', Cc''', median fluorescence recovery slopes (linear regression).  
1418 **D**, Model (2D deployment of the aortic wall) representing the endothelial/hemogenic dynamic  
1419 interplay and the proposed function of ArhGEF11 and of its +exon 38 peptide encoding variant  
1420 at junctional and membrane interfaces. This interplay involves 2 essential dynamic events  
1421 requiring junctional remodeling: **1** (left cartoon, magenta arrows), the mobility of he-ec-ec tri-  
1422 junctional contacts accompanying the movement of endothelial cells (ex: for ec1 and ec2)  
1423 along lateral sides of hemogenic cells which is required to decrease the number of adjoining  
1424 cells (see Lancino et al. 2018). This takes place contemporarily to - or in alternance with -, the  
1425 contraction of HE and EHT cells as they are progressing throughout the emergence and  
1426 reducing contacting membrane surfaces along the longitudinal axis (the reduction in contacting

1427 membrane surfaces also involves membrane retrieval, hypothetically via endocytosis). Our  
1428 data obtained with the CRISPR deletion mutants (a slight tendency to increase, on average,  
1429 the turnover and mobile pool of these he-ec-ec junctions) suggest that ArhGEF11, in the wild  
1430 type condition, should be slowing down the recycling of junctional components at tri-junctions,  
1431 which hypothetically should contribute to increase adhesion strength. This may be required  
1432 also to stabilize the junction-cytoskeleton interface involved in controlling the  
1433 contraction/shrinkage of HE cells along the longitudinal axis, a hypothesis that is compatible  
1434 with the mutant phenotype observed in this study, i.e the increase in frequency of more  
1435 elongated HE cells and the decrease in HE cell progression throughout EHT (see **Figure 7**); **2**  
1436 (right cartoon, cyan arrow and bottom cartoons a-c), the intercalation of an endothelial cell to  
1437 isolate 2 adjacent hemogenic cells or 2 daughter cells after mitosis (not depicted). This is  
1438 mandatory for EHT progression and completion which requires adjoining endothelial cells to  
1439 protrude membrane extensions that will anastomose to seal the aortic floor (see Lancino et al.  
1440 2018). The accumulation of adjoining cells of rather small length and apparently impaired in  
1441 EHT progression that we describe in **Figure 7** upon MO interference (that may also indicate  
1442 impairment in abscission completion) suggests that the ArhGEF11 +exon 38 peptide encoding  
1443 variant is more specifically involved in controlling the remodeling of the he/he interface that  
1444 leads to endothelial cell intercalation (bottom cartoons, the remodeling of he-he-ec junctions is  
1445 leading to he-ec-ec junctions and takes place between b and c). The increase in the recycling  
1446 parameters that we measure in this interfering condition (mobile pool and early speed of  
1447 recovery) indicates that the activity of ArhGEF11, and in particular of its +exon 38 peptide  
1448 encoding variant, negatively controls junctional recycling. Hypothetically, the junctional  
1449 adhesion strengthening triggered by reducing the recycling of junctional components at the  
1450 he/he interface may be required during the early phase and progression of intercalation to  
1451 support increase in membrane tension and environmental constraints (intercalation contributes  
1452 to reducing the length/surface of the he/he membrane interface preceding junctional

1453 remodeling (cartoons b and c); this reducing membrane interface is in addition submitted to  
1454 the shear stress imposed by blood flow).  
1455

1456 **Legends to supplement figures**

1457

1458 **Figure 1 – figure supplement 1. A**, 2 EHT pol+ cells visualized using a  
1459 *Tg(Kdrl:Gal4;UAS:RFP;4xNR:eGFP-Podxl2)* embryo and time-lapse sequence (initiated at 55  
1460 hpf) obtained with spinning disk confocal microscopy. The images are extracted from the same  
1461 time lapse sequence than the one used for **Figure 1C**. Green channel (eGFP-Podxl2) only is  
1462 shown. Green arrows point at the evolution of the connection between the aortic/eht cell  
1463 lumens at t = 0, 10, 30 min. Asterisks label the lumen delimited by the luminal/apical  
1464 membranes. Bar = 8µm. **B**, Model summarizing and interpreting the temporal evolution of the  
1465 luminal/apical membrane (in green, the asterisks mark the lumen of the apparent vacuole-like  
1466 intracellular membrane structures) after the release of an EHT pol+ cell from the aortic floor.  
1467 **Step 1**, the pseudo-vacuole filled with fluid and delimited by eGFP-Podxl2 as visualized at t =  
1468 65 min in **A** (and also at 45 min in **Figure 1C**, 2 asterisks) is consumed partly via budding (after  
1469 sorting of eGFP-Podxl2); these budding profiles can be seen on the left image (pink  
1470 arrowheads) corresponding, for eht cell 1, to the plane 16 of the time point t = 80 min of the  
1471 time lapse sequence (see the Z-stack in **Figure 1 – video 2**). **Step 2**, after sorting and budding,  
1472 the cell remains with pseudo-endocytic Podxl2 containing membranes and the remaining  
1473 vacuolar structures filled up with fluid regress, putatively by chasing water as illustrated in **step**  
1474 **3. Step 4**, the eht cell remains with pseudo-endocytic Podxl2 containing membranes that label  
1475 newly born precursors of HSPCs. Note that since the cell remains in contact with the aortic  
1476 floor while the pseudo-vacuole is regressing, the vacuole-like intracellular membrane proximal  
1477 to aortic cells may never undergo fission (see **Figure 1A**, steps 4-4') but gets consumed via  
1478 budding and flattening upon water chase (steps 1-3 that are similar to **Figure 1A**, steps 3-3').  
1479  
1480 **Figure 1 – video 1. EHT pol+ cells at early timing.** Z-stack (37 planes) corresponding to the  
1481 time point t = 0 min whose plane 21 is shown **Figure 1C**.

1482

1483 **Figure 1 – video 2. EHT pol+ cells at late timing.** Z-stack (37 planes) corresponding to the  
1484 time point  $t = 80$  min whose planes 16 (for eht cell 1) and 22 (for eht cell 2) are shown **Figure**  
1485 **1 – figure supplement 1B.**

1486

1487 **Figure 1 – video 3. Emergence of EHT pol- cells.** Time-lapse sequence using a  
1488 *Tg(Kdrl:Gal4;UAS:RFP;4xNR:eGFP-Podxl2)* embryo, obtained with spinning disk confocal  
1489 microscopy (38 timing points, each acquired with 10 min intervals, starting at 55 hpf) and from  
1490 which the images of the 2 emerging EHT pol- cells shown Figure 1D were extracted.

1491

1492 **Figure 2 – figure supplement 1. HE cells are not polarized at 30 hpf.**  
1493 *Tg(Kdrl:Gal4;UAS:RFP;4xNR:eGFP-Podxl2)* 30 hpf embryo imaged using spinning disk  
1494 confocal microscopy. **Top 2 panels:** green (eGFP-Podxl2) and red (soluble RFP, in magenta)  
1495 channels. White arrows point at 2 individual EHT cells. Note that HE cell 1 protrudes long  
1496 filopodia, some of which inside the aortic lumen (live imaging shows that they are moving along  
1497 the blood flow, data not shown). **Bottom 2 panels:** 1.625X magnification of EHT cell 1 and 2  
1498 in top panels. Green channel (eGFP-Podxl2) only. Green arrows point at very large intracellular  
1499 vesicles, the largest reaching approximately  $30\mu\text{m}$ . Asterisks mark the cytoplasm. Bar:  $100\mu\text{m}$ .

1500

1501 **Figure 2 – figure supplement 2. Evolution of non-polarized HE cells throughout**  
1502 **emergence.** *Tg(Kdrl:Gal4;UAS:RFP;4xNR:eGFP-Podxl2)* embryo imaged using spinning disk  
1503 confocal microscopy. Images were obtained from discontinuous time-lapse sequences  
1504 covering a period of 13 hours (from 35 – to – 48 hpf). Successive phases of the evolution of  
1505 HE cells are visible, from a non-polarized status (with the accumulation of Podxl2-containing  
1506 intra-cytosolic vesicles as well as cell division) to post-emergence EHT cells remaining  
1507 beneath the aortic floor. The top panel is a z-projection of 69 consecutive z-sections  
1508 interspaced by  $0.3\mu\text{m}$ ; green (eGFP-Podxl2) and red (soluble RFP, in magenta) channels are

1509 shown; 4 individual HE cells (1 – 4, green arrows)) are marked, with cells 1 and 4 more  
1510 advanced in the process of emergence. All the other panels are z-sections focused on the  
1511 aortic floor, allowing visualizing the progression of the EHT, in particular at 35 hpf ( $t = 0$ ), a  
1512 timing point at which the separation between the luminal and basal membranes are equally  
1513 labelled with eGFP-Podxl2, attesting for the absence of apicobasal polarity (see cells 1 and 4,  
1514 see also cell 1 at  $t = 2.5$  hours (hrs) and cells 3' and 3'' at  $t = 5$  hrs). Note, at  $t = 2.5$  and 5.0  
1515 hrs, the presence of intracytoplasmic eGFP-Podxl2 labelled vesicles inside HE cells,  
1516 suggesting vesicular transport (white arrowheads, to be compared with images **Figure 2 –**  
1517 **figure supplement 1**, at 30 hpf). At 48 hpf (bottom panel), HE cells have emerged and remain,  
1518 for some of them, in close contact with the aortic floor (after having performed mitosis (notably  
1519 for HE cell 2 (the division is visualized at 5hrs) and for HE cell 3, each one giving rise to cells  
1520 2' - 2'' and 3' - 3'' respectively, all containing residual eGFP-Podxl2 containing membranes as  
1521 EHT signature). Bar =  $80\mu\text{m}$ .

1522

1523 **Figure 3 – figure supplement 1. Phenotypic analysis of dt-runx1 expressing mutants:**  
1524 **expansion of the thymus. A**, maximum z-projections of confocal spinning disk sections of the  
1525 thymus region of 5 dpf *Tg(kdrl:gal4;UAS:RFP)* (left, control) and increased  
1526 *Tg(kdrl:Gal4;UAS:RFP;4xNR:dt-runx1-eGFP)* (right, dt-runx1) larva. The area for each thymus  
1527 is delimited (white lines); note the systematic expansion of thymic cells on the right sides of  
1528 each image for dt-runx1 mutants in comparison to controls. Bar:  $20\mu\text{m}$ . **B**, Area of projected  
1529 thymus for the 2 conditions illustrated in A. Statistical tests: two sided unpaired two samples  
1530 Wilcoxon test, with p-values of significant differences only.

1531

1532

1533 **Figure 3 – figure supplement 2. Phenotypic analysis of dt-runx1 expressing mutants:**  
1534 **evidence for apicobasal polarity of hemogenic cells.** 30-32 hpf embryos obtained from

1535 *outcrossed Tg(Kdrl:Gal4;UAS:RFP;4xNR:mKate2-Podxl2) X Tg(kdrl:Gal4;UAS:RFP;4xNR:dt-*  
1536 *runx1-eGFP)* fishes and imaged in the trunk (AGM) region using spinning disk confocal  
1537 microscopy. Images are depicting typical HE cells (in boxes) from hemogenic regions, with  
1538 apical and basal membranes clearly separated from each other owing to reduction of their  
1539 surface area (in comparison to flat aortic cells) and elongation in the antero-posterior axis.  
1540 Luminal membranes are enriched with (magenta arrows) or more or less devoid of (green  
1541 arrows) *mkate2-Podxl2*. Green: cytosolic eGFP released from the cleavage of *dt-runx1-eGFP*.  
1542 Note that because of mosaicism, he cell 2 does not express *mkate2-Podxl2*. Bars:100 $\mu$ m.

1543

1544 **Figure 3 – figure supplement 3. Phenotypic analysis of *dt-runx1* expressing mutants:**  
1545 **slowing down of the emergence process and accumulation of EHT cells.**

1546 *Tg(kdrl:Gal4;UAS:RFP;4xNR:dt-runx1-eGFP)* embryos imaged using spinning disk confocal  
1547 microscopy and analyzed in the AGM/trunk region. **A**, z-projections of the dorsal aorta obtained  
1548 from 52-55 hpf embryos. Top panel: fluorescence from the red channel is shown for the  
1549 *Tg(Kdrl:Gal4;UAS:RFP)* control. Bottom panels: fluorescence in the green channel only is  
1550 shown for the mutants (eGFP, released from the *dt-runx1-eGFP* cleavage). The black asterisks  
1551 point at emerged cells that are in close contact with the aortic floor. Green arrows: EHT pol+  
1552 cells on aortic floor; magenta arrows: EHT pol+ cells in the lateral aortic wall; white arrows:  
1553 uncharacterized emerging cells; he: hemogenic cells. **B**, evolution of an EHT cell extracted  
1554 from a 7 hrs time-lapse sequence (0 to 420 min) and showing significant changes in its  
1555 morphology throughout emergence. Note the sub-luminal and cytosolic localization of pools of  
1556 *Podxl2* (notably at t = 30 min, magenta arrows) suggesting enhanced trafficking of the protein  
1557 and relative instability of apical polarity features, consistently with apparent fluctuation of the  
1558 luminal membrane surface contacting the aortic lumen (green arrows, in particular at t = 240  
1559 min). At t = 420 min, the cell has emerged and *Podxl2* containing membranes remain in close  
1560 contact with the membrane contacting the aortic floor. Bars:20 $\mu$ m.

1561

1562 **Figure 5 – figure supplement 1. Model depicting the evolution of junctional interfaces**  
1563 **and of the differential mobility of antero-posterior junctional complexes for EHT pol+**  
1564 **and EHT pol- cell emergence types. Top panel:** EHT pol+ cell whose emergence depends  
1565 on the constriction of circumferential actomyosin (orange, see Lancino et al. 2018) and the  
1566 reduction of the membrane interface contacting endothelial neighbors shrinking along the  
1567 longitudinal axis (horizontal double arrows) and in the 2D plane (endothelial neighbors are not  
1568 depicted and are embedded in the blue X-Y plane). Presumably, the reduction of membrane  
1569 interfaces relies on consumption via endocytosis. Green ovals: junctional complexes  
1570 reinforced at antero-posterior poles. Green line: apical/luminal membrane with typical inward  
1571 bending. **Bottom panel:** EHT pol- cell whose emergence depends on the dynamics of  
1572 adhesion pools that move synchronously in 3D (X, Y, Z), both at antero-posterior poles (blue  
1573 ovals) and at lateral sides of the emerging cell contacting endothelial neighbors (adjoining  
1574 endothelial cells are not depicted and are embedded in the blue X-Y plane). Presumably, this  
1575 type of emergence in which endothelial cells crawl over the EHT cell (curved arrows) involves,  
1576 for the latter, a partial retrograde endocytic recycling of junctional complexes (opposite to the  
1577 direction of emergence). Note that nuclei are not drawn at scale.

1578  
1579 **Figure 5 – figure supplement 2. JAM2a and JAM3b expression and localization in**  
1580 **diverse embryonic tissues.** Localization of transiently expressed eGFP-Jam2a (**A-D**) and  
1581 eGFP-Jam3b (**E-G**) in 52 hpf embryos, using spinning disk confocal microscopy. Plasmid  
1582 constructs were expressed under the control of the heat shock *Hsp70* promoter. For both  
1583 constructs, expression was induced approximately 6 hrs before imaging, by 1 hour balneation  
1584 in 39°C embryo medium. All images are maximum z-projections. **A, E**, ependymal cells. White  
1585 and red arrows point at reinforcement of eGFP-Jam2a and eGFP-Jam3b at apical intercellular  
1586 junctions and at basolateral membranes, respectively. **B**, pronephric tubule cells. White arrows  
1587 point at the reinforcement of the eGFP-Jam2a signal at apical sides of membranes of polarized

1588 cells constituting pronephric tubules. The red arrow points at baso-lateral localization of eGFP-  
1589 Jam2a. **C, F**, skin epithelial cells. White arrows point at the localization of eGFP-Jam2a and  
1590 eGFP-Jam3b at lateral junctional interfaces between two neighboring cells. Red arrows point  
1591 at tri-cellular junctions at which the density of eGFP-Jam2a and eGFP-Jam3b is reinforced. **D**,  
1592 skin epithelial cells. White arrows point at membrane protrusions. **G**, Striated muscle cells. Red  
1593 arrows point at T-tubules (invagination of the sarcolemmal membranes); white arrows point at  
1594 the plasma membranes of myofibrils. Bars: 20 $\mu$ m.

1595

1596 **Figure 6 – figure supplement 1. Examples of junctional contacts targeted by FRAP in**  
1597 **the aortic landscape.** After performing z-stack acquisitions in trunk regions followed by 2D-  
1598 deployment of aortic segments (**A-C**), 4 different *Tg(kdrl:eGFP-Jam3b; kdrl:nls-mKate2)* 48-  
1599 55 hpf embryos were illuminated for FRAP. Panels (**A**) and (**B**) are the entire segments from  
1600 which cropped images of EHT pol+ and EHT pol- cells (black asterisks) and surroundings were  
1601 extracted to build the panels A and B of **Figure 6**. Panels (**C**) and (**D**) are from 2 other embryos.  
1602 The 2D-cartographies allow visualizing the junctions that were selected for FRAP, with the  
1603 black arrows pointing at junctional interfaces between EHT and endothelial cells (all are in the  
1604 area of tri-junctions) and the green arrows pointing at bi-junctions (Bj) or tri-junctions between  
1605 endothelial cells (green asterisks). Bar = 20 $\mu$ m.

1606

1607 **Figure 7 – figure supplement 1. Searching for PDZ-domain containing RhoGEFs**  
1608 **potentially involved in the EHT.** **A**, cartoons representing the domains composing the 9 PDZ  
1609 domain-containing RhoGEFs that were investigated in this study (all these RhoGEFs are  
1610 encoded by different genes in the zebrafish; protein respective length is not drawn at scale).  
1611 **B**, results obtained from 3 independent qRT-PCR experiments and performed on material  
1612 extracted from trunk regions of 35 and 48 hpf embryos obtained from incrossed  
1613 *Tg(kdrl:Gal4;UAS:RFP;4xNR:dt-runx1-eGFP)* adult fishes. Statistical tests: two sided unpaired  
1614 two samples Wilcoxon test, with p-values of significant differences. GEF: guanine nucleotide-

1615 exchange factor; PDZ: postsynaptic density protein of 95 kDa, Discs large and Zona  
1616 occludens-1; PH: Plekstrin homology; RGS: regulator of G-protein signaling; DH: Dbl (diffuse  
1617 B-cell lymphoma) homology; RBD: Ras-binding domain; DEP: Dishevelled Egl-10 and  
1618 Plekstrin; InsP4-PTPase: PtdIns(1,3,4,5)P4 phosphatidylinositol phosphatase; TIAM: T-cell-  
1619 lymphoma invasion and metastasis; LARG: leukemia-associated Rho guanine-nucleotide  
1620 exchange factor; PReX: PtdIns(3,4,5)P3-dependant Rac exchanger-1 and 2.

1621

1622 **Figure 7 – figure supplement 2.** Whole mount in situ hybridizations (WISH) performed on 30-  
1623 32 hpf and 48-50 hpf embryos, with probes specific for all 9 PDZ-domain containing RhoGEFs  
1624 that were investigated in this study. Note that all RhoGEFs are detected in the dorsal aorta of  
1625 the trunk region (white arrowheads).

1626

1627 **Figure 7 – figure supplement 3. A N-terminal fragment of ArhGEF11/PDZ-RhoGEF**  
1628 **localizes at junctional membranes with enrichment at antero-posterior sites of EHT**  
1629 **cells. A**, domains of ArhGEF11, including its actin binding site (L/IxxFE) and the position of  
1630 exon 38 encoded peptide. The bottom cartoon represents a protein deleted from its DH-PH  
1631 domains and C-terminus that are replaced by eGFP (PDZ-PRD-RGS-eGFP) to follow the  
1632 localization of the truncated fusion protein in expressing cells. **B**, localization of the ArhGEF11  
1633 PDZ-PRD-RGS-eGFP fusion protein after transient expression in the aorta (expression was  
1634 obtained after injection of the plasmid, at the one cell stage, in the *Tg(Kdrl:Gal4;UAS:RFP)* fish  
1635 line). Confocal images extracted from a time-lapse sequence (timing points  $t = 0$  and  $t = 77$   
1636 min) and showing either the eGFP channel only (left), or the merge between the green and the  
1637 red channels (RFP labels the cytoplasm). Note the localization of PDZ-PRD-RGS-eGFP at  
1638 membrane interfaces at early time point of the emergence and at the rim of an emerging cell  
1639 (white arrowheads, second cell from the right) and its enrichment at antero-posterior sites of  
1640 an EHT  $pol^+$  cell proceeding throughout emergence (right cell, at  $t = 0$  and  $t = 77$  min and  
1641 bottom cartoons, green arrows point at localization/concentration of PDZ-PRD-RGS-eGFP ).

1642

1643 **Figure 7 – figure supplement 4. MO and CRISPR approaches to investigate the function**  
1644 **of ArhGEF11/PDZ-RhoGEF in the EHT. A**, splicing MO interfering with integration of exon  
1645 **38. Top panel:** cartoon representing exons/introns (not drawn at scale) composing the 3-  
1646 prime region of the gene encoding for ArhGEF11, with the position of the splicing MO. **Middle**  
1647 **panel:** agarose gel showing the 2 alternative mRNAs encoding for ArhGEF11 in control  
1648 animals (left track, control) and after injection of the MO at the one cell stage (right track, +MO  
1649 at 2 and 5ng). **Bottom panel:** ArhGEF11 DNA sequences obtained after RT-PCR, cloning and  
1650 sequencing for 1 control and 2 +MO clones (+MO1, +MO3). **B**, morphologies of control and  
1651 +MO injected 48 hpf embryos. Note the absence of malformations. **C, top panel:** ArhGEF11  
1652 wild type and CRISPR-mediated 7bp deletion in the 3-prime region of exon 38 leading to a  
1653 frame-shift in the ORF and a downstream premature stop codon; **middle panel:** CRISPR  
1654 del/C-ter nucleotide/aa sequence and wild type nucleotide/aa sequence covering the extreme  
1655 C-ter of the full-length protein; **bottom panel:** CLUSTAL 2.1 multiple sequence alignment of  
1656 mouse and zebrafish C-termini highlighting the sequences of spliced variants of potentially  
1657 similar activity in the regulation of ArhGEF11 activity on RhoA. Accession numbers:  
1658 NP\_001003912.1 and NP\_001027010.1 for the mouse and Danio rerio sequence,  
1659 respectively. **D**, morphologies of control and homozygous ArhGEF11<sup>CRISPR-Cterdel/+</sup> 48 hpf  
1660 embryos ((*Kdrl:eGFP-Jam3b*; *kdrl:ArhGEF11<sup>CRISPR-Cterdel/+</sup>*) embryos). Note the edema in the  
1661 cardiac region (35 hpf embryos also exhibited retardation in blood circulation).

1662

1663 **Figure 7 – figure supplement 5. Supplementary data on the ArhGEF11/PDZ-RhoGEF**  
1664 **exon 38 splicing morpholino phenotype.** *Tg(kdrl:eGFP-Jam2a; kdrl:nls-mKate2)* control (**A**,  
1665 **C, E**) or MO-injected (**B, D, F**) embryos were imaged using spinning disk confocal microscopy  
1666 (48-55 hpf time-window). Aorta segments (330  $\mu$ m each) were imaged in the trunk region  
1667 (AGM). The 2D-cartographies with delimited cellular contours in panels **A** and **B** are presented

1668 **Figure 7A** with the corresponding un-modified 2D-cartographies shown on top for unmasking  
1669 of contours. Panels **A** and **C** illustrate 2 different segments from the same embryo. Panels **B**,  
1670 **D**, **F** illustrate segments from 3 different embryos. (a-f) Maximum z-projections of merged nls-  
1671 mkate2 and eGFP-Jam2a signals for control (a, c, e) and for ArhGEF11 exon 38 splicing  
1672 morpholino (b, d, f) conditions. For panels (a) and (b), maximum z-projections of the eGFP-  
1673 Jam2a signal only are also shown. (a'-f') Single z-plane images of merged nls-mkate2 and  
1674 eGFP-Jam2a signals for control (a', c', e') and for ArhGEF11 exon 38 splicing morpholino (b',  
1675 d', f') conditions. For **e'**, the image is a composition of 2 different z-planes from the same field  
1676 (the boundaries are marked with white ticks). In right margins, magenta and green arrowheads  
1677 designate the aortic floor and roof, respectively. (a''-f'') 2D-cartographies (bottom, with  
1678 delineated cellular contours) obtained from eGFP-Jam2a signals for control (a'', c'', e'') and for  
1679 the ArhGEF11 exon 38 splicing morpholino (b'', d'', f'') conditions, respectively. Cell contours  
1680 are delineated either in blue (endothelial cells), yellow (hemogenic cells), red (morphologically  
1681 characterized EHT cells, red arrows), and small cells delineated by cyan boxes  
1682 (morphologically uncharacterized EHT cells and putative post-mitotic cells remaining as pairs).  
1683 Cellular contours have been semi-automatically segmented along the cellular interfaces  
1684 labelled with eGFP-Jam3b (see **Materials and Methods**). White and black arrows designate  
1685 hemogenic cells with their nucleus visible on the z-section. Analyses were performed on 2 x  
1686 control non-injected embryos and 3 x embryos injected at the one-cell stage with the  
1687 ArhGEF11 exon 38 splicing MO. Bars = 20µm.

1688

1689 **Figure 7 – figure supplement 6. Supplementary data on the ArhGEF11/PDZ-RhoGEF**  
1690 **CRISPR C-ter deletion phenotype.** (*Kdrl:eGFP-Jam3b; kdrl:ArhGEF11<sup>CRISPR-Cterdel+/+</sup>*)  
1691 homozygous ArhGEF11 C-ter deletion mutants (CRISPR ArhGEF11: panels **B**, **D**, **F**) and  
1692 control siblings (Control: panels **A**, **C**, **E**) were imaged using spinning disk confocal microscopy  
1693 (48-55hpf time-window). Note that the genetic background of the CRISPR fish line is

1694 (Kdrl:GAL4; UAS:RFP) thus allowing the red cytosolic staining of aortic and hemogenic cells.  
1695 Aorta segments (330  $\mu\text{m}$  each) were imaged in the trunk region. The 2D-cartographies with  
1696 delimited cellular contours in panels **A** and **B** are presented **Figure 7B** with the corresponding  
1697 un-modified 2D-cartographies shown on top for unmasking of contours. Panels **A**, **C** and **D**, **F**  
1698 illustrate 2 different segments from the same embryo. (a-f) Maximum z-projections of merged  
1699 nls-mkate2 and eGFP-Jam3b signals for control (a, c, e) and for the CRISPR mutant (b, d, f)  
1700 conditions. For panels (a) and (b), maximum z-projections of the eGFP-Jam2a signal only are  
1701 also shown. (a'-f') Single z-plane images of merged nls-mkate2 and eGFP-Jam3b signals for  
1702 control (a', c', e') and for ArhGEF11 exon 38 splicing morpholino (b', d', f') conditions. For b'  
1703 and d', the image is a composition of 2 different z-planes from the same field (the boundaries  
1704 are marked with white ticks). In right margins, magenta and green arrowheads designate the  
1705 aortic floor and roof, respectively. (a''-f'') 2D-cartographies (bottom, with delineated cellular  
1706 contours) obtained from eGFP-Jam3b signals for control (a'', c'', e'') and for the CRIPR mutant  
1707 (b'', d'', f'') conditions, respectively.  
1708 Cell contours are delineated either in blue (endothelial cells), yellow (hemogenic cells), red  
1709 (morphologically characterized EHT cells, red arrows), and small cells delineated by cyan  
1710 boxes (morphologically uncharacterized EHT cells). Cellular contours have been semi-  
1711 automatically segmented along the cellular interfaces labelled with eGFP-Jam3b (see  
1712 **Materials and Methods**). White and black arrows designate hemogenic cells with their  
1713 nucleus visible on the z-section. Analyses were performed on 2 x wild-type siblings for controls  
1714 and 2 x mutant embryos whose mutation was confirmed by DNA sequencing. Bars = 20 $\mu\text{m}$ .  
1715

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2124 Endothelium.” *Blood* 136 (7): 845–56. <https://doi.org/10.1182/blood.2020004801>.
- 2125  
2126  
2127

2128 **Supplementary files**

2129

2130 **Supplementary file 1: Materials and Methods - Supplementary Table**

2131

2132

2133 **Figure 1 – video 1. EHT pol+ cells at early timing.** Z-stack (37 planes) corresponding to the  
2134 time point t = 0 min whose plane 21 is shown **Figure 1C**.

2135

2136 **Figure 1 – video 2. EHT pol+ cells at late timing.** Z-stack (37 planes) corresponding to the  
2137 time point t = 80 min whose planes 16 (for eht cell 1) and 22 (for eht cell 2) are shown **Figure**  
2138 **1 – figure supplement 1B**.

2139

2140 **Figure 1 – video 3. Emergence of EHT pol- cells.** Time-lapse sequence using a  
2141 *Tg(Kdrl:Gal4;UAS:RFP;4xNR:eGFP-Podxl2)* embryo, obtained with spinning disk confocal  
2142 microscopy (38 timing points, each acquired with 10 min intervals, starting at 55 hpf) and from  
2143 which the images of the 2 emerging EHT pol- cells shown Figure 1D were extracted.

2144

# Figure 1

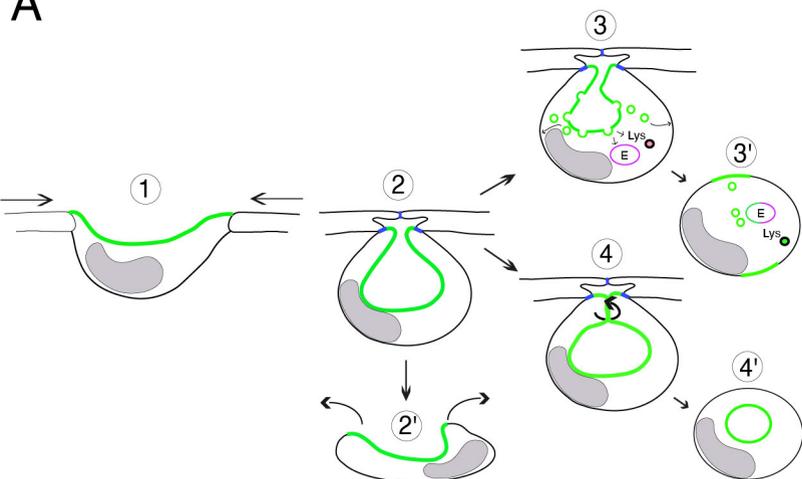
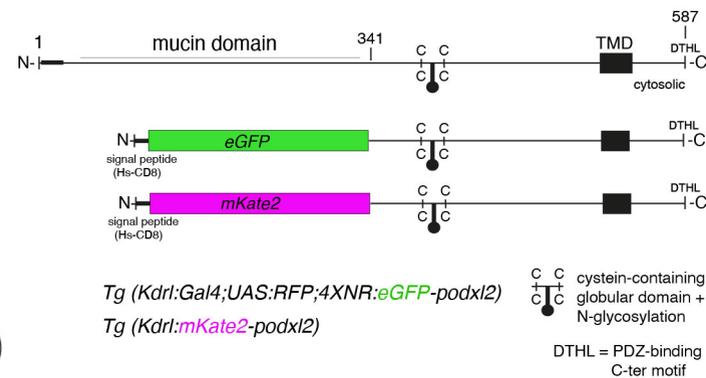
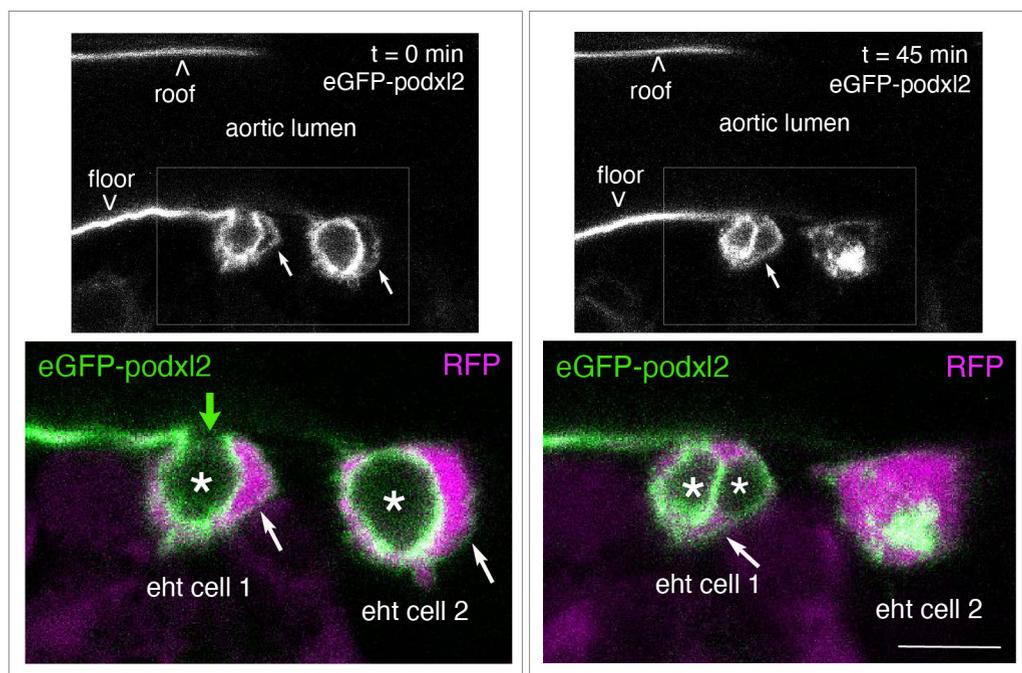
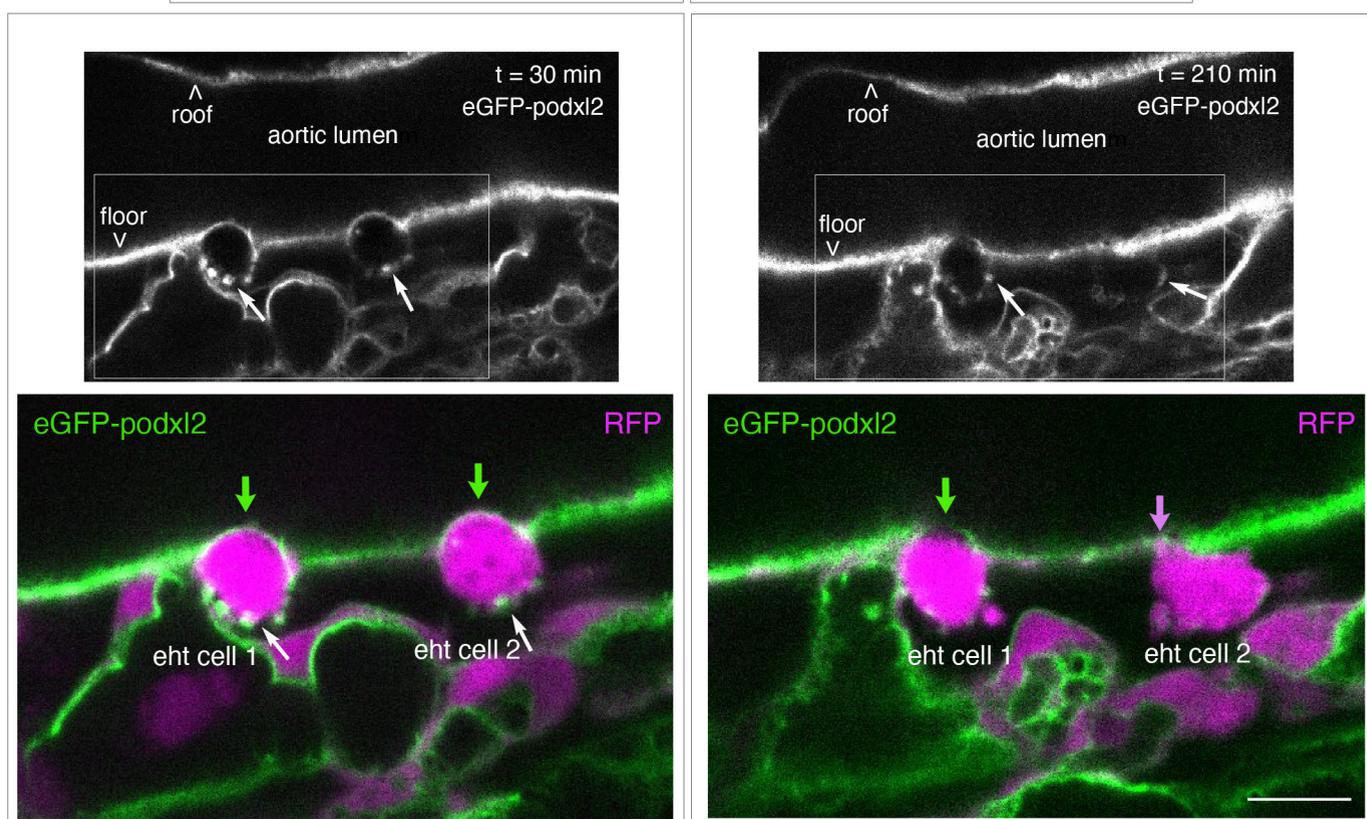
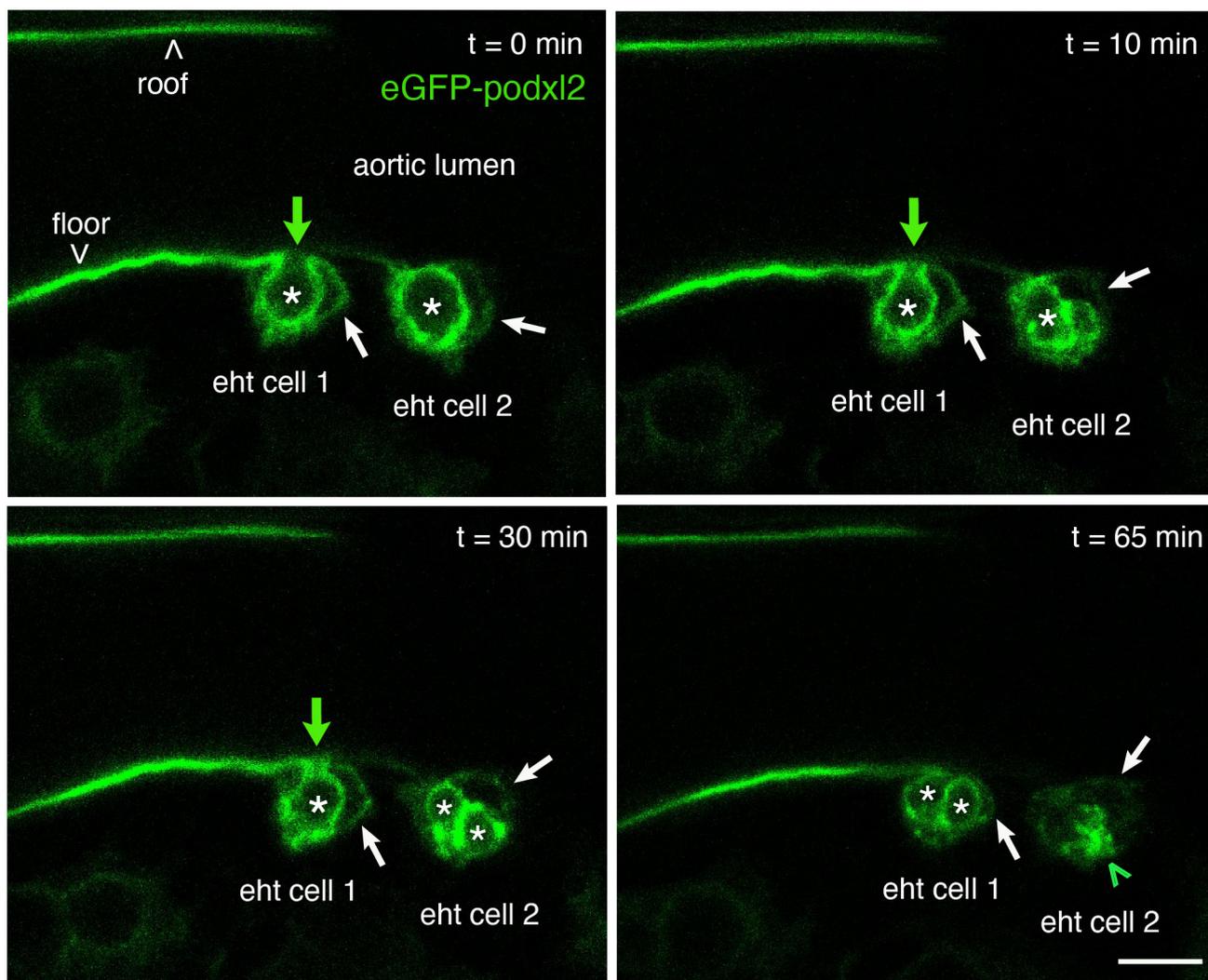
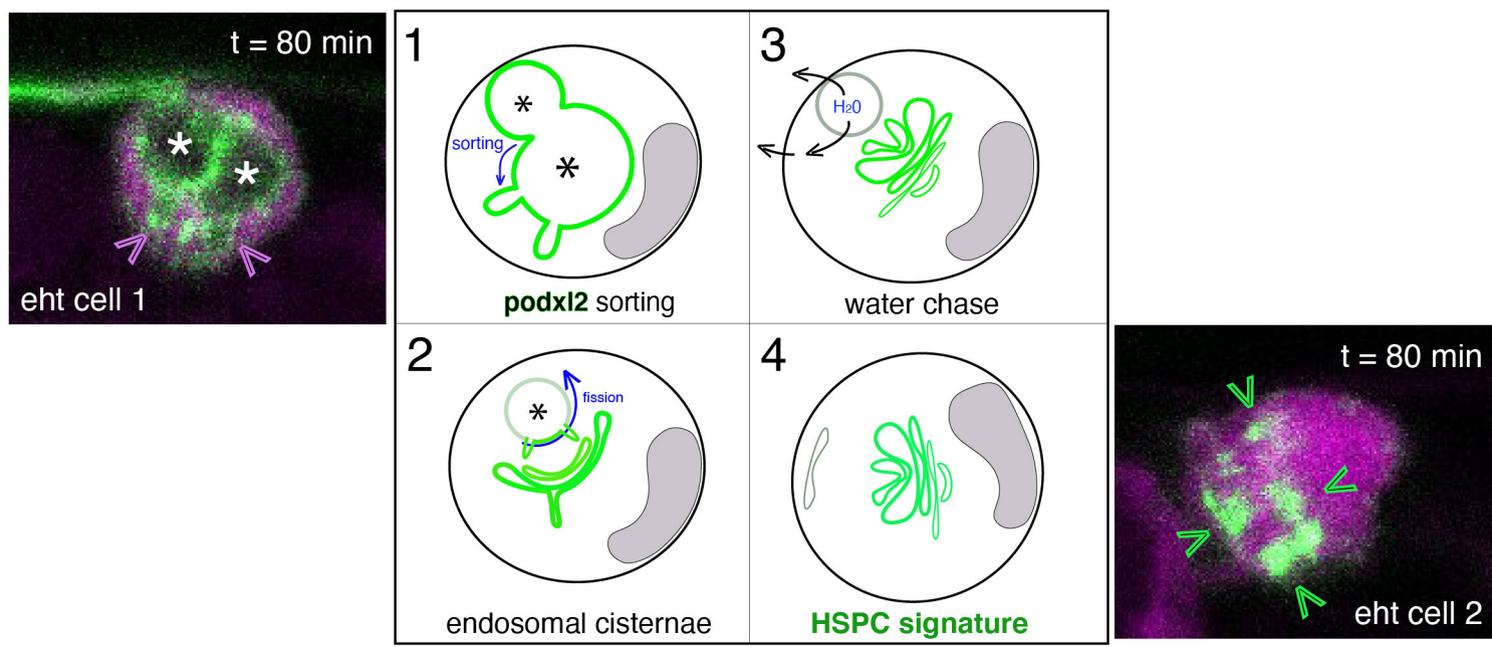
**A**

**B**

**C**

**D**


FIGURE 1 - figure supplement 1

A

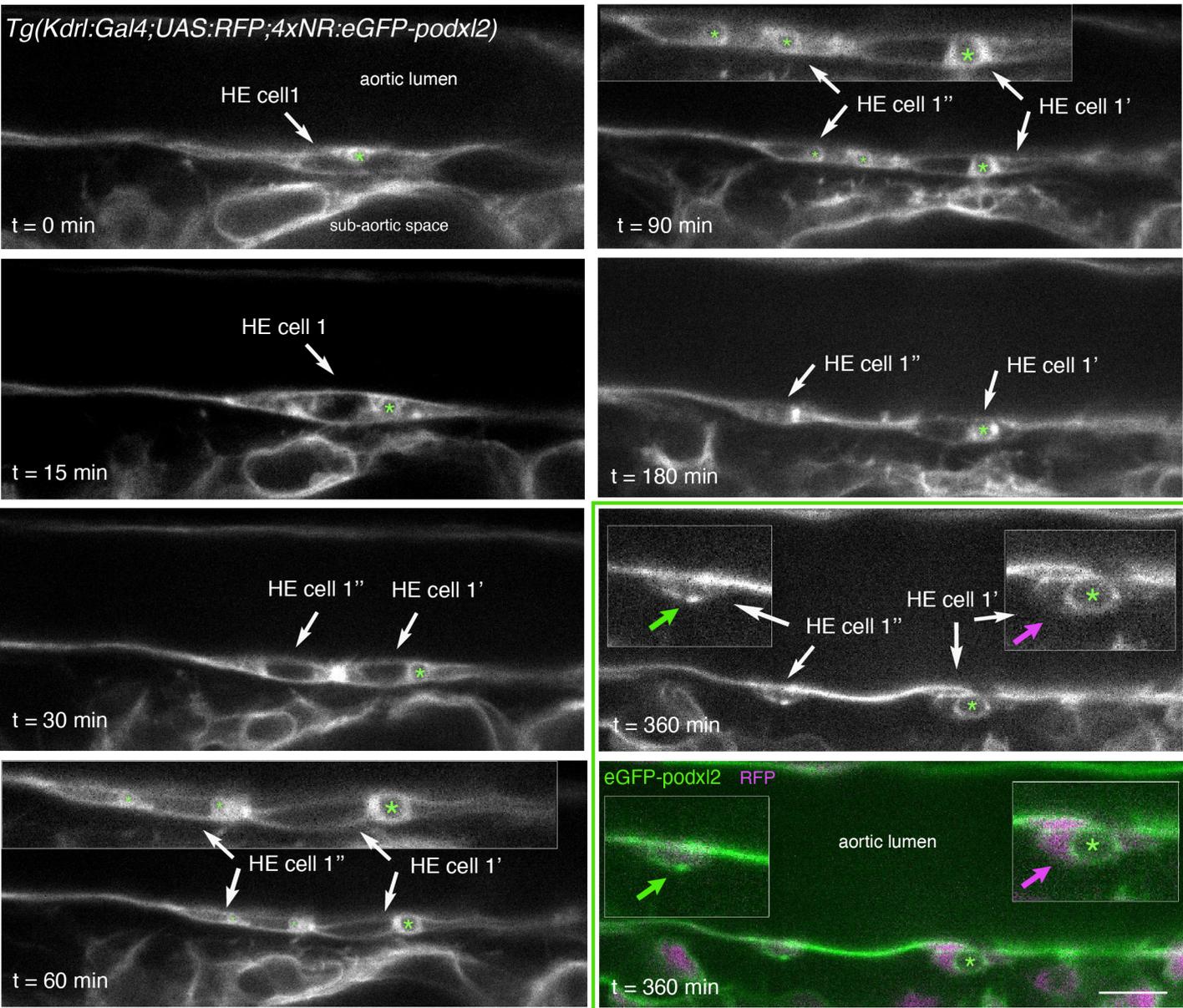


B



# Figure 2

## A



## B

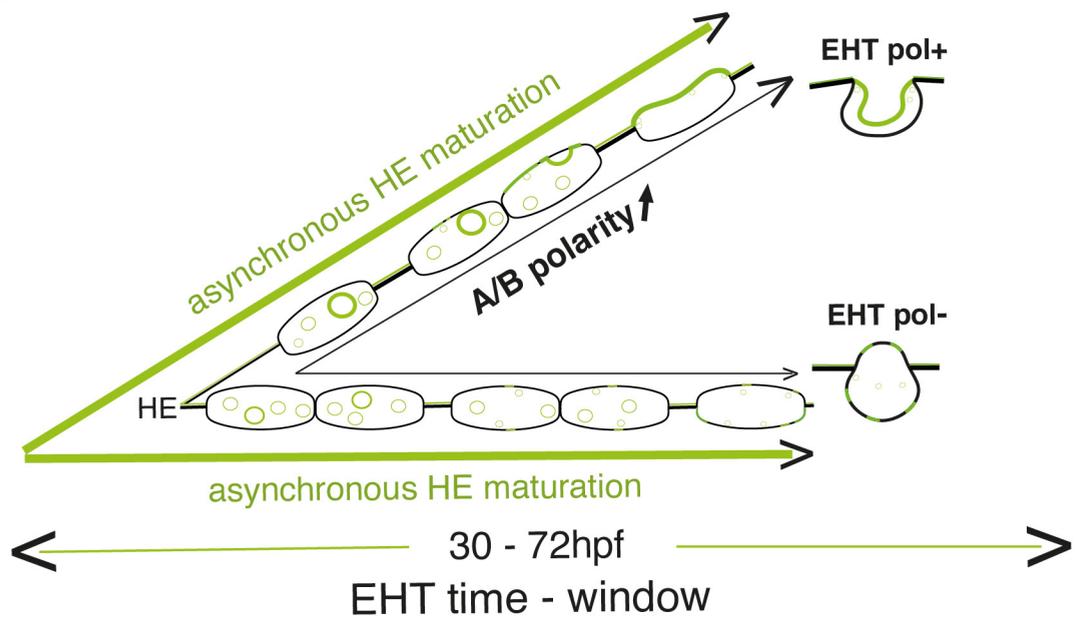


Figure 2  
- figure supplement 1

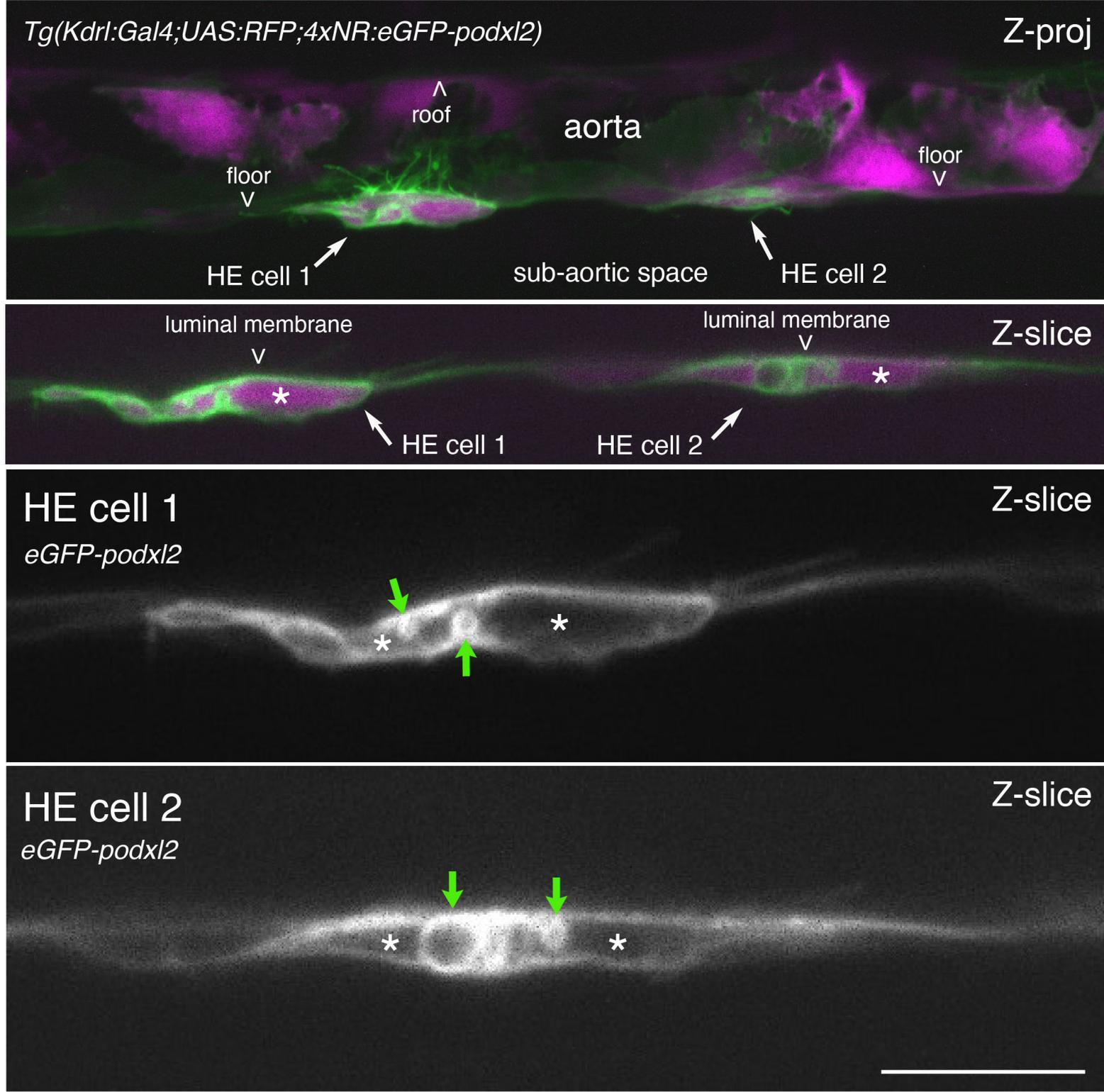
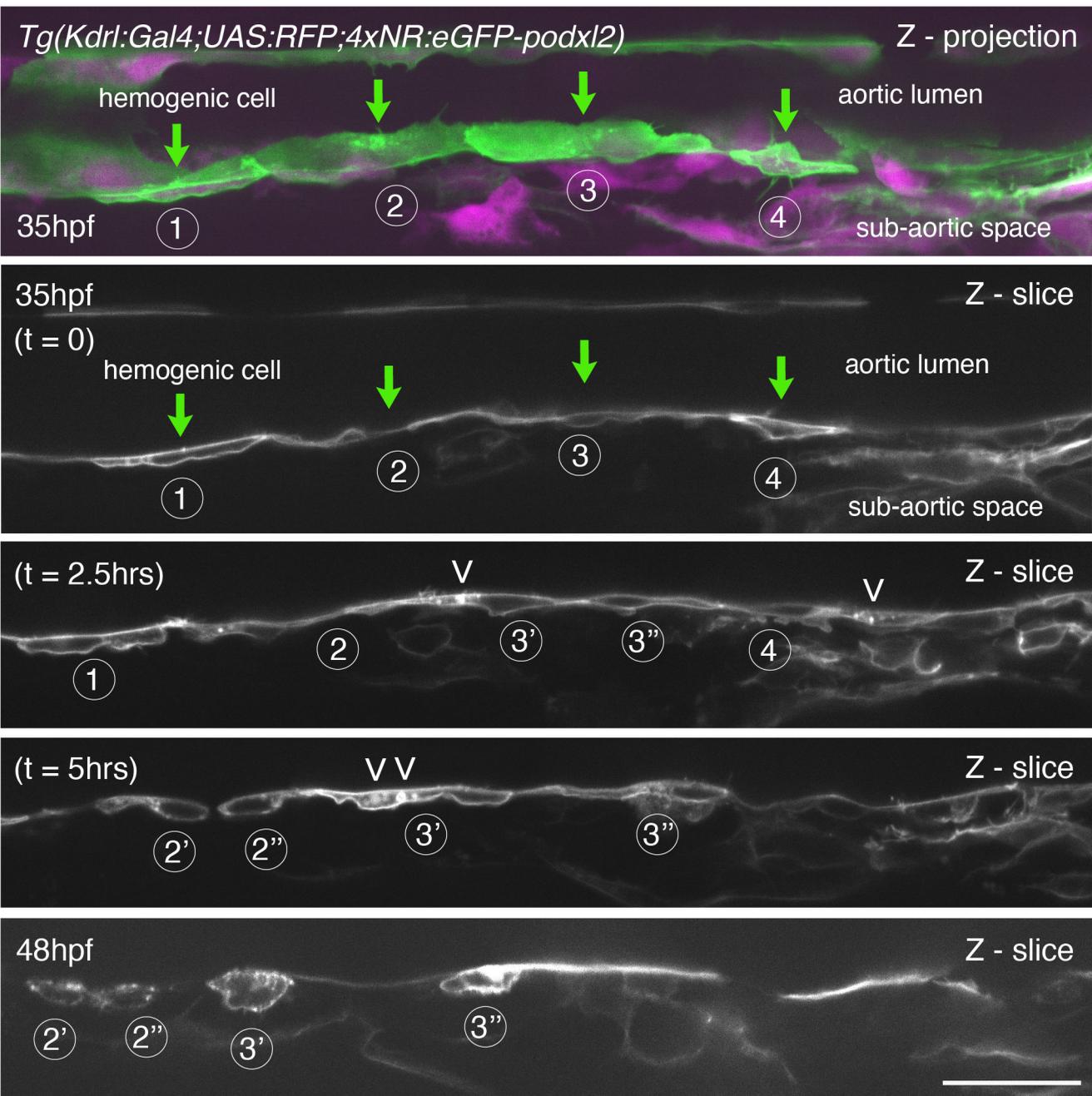
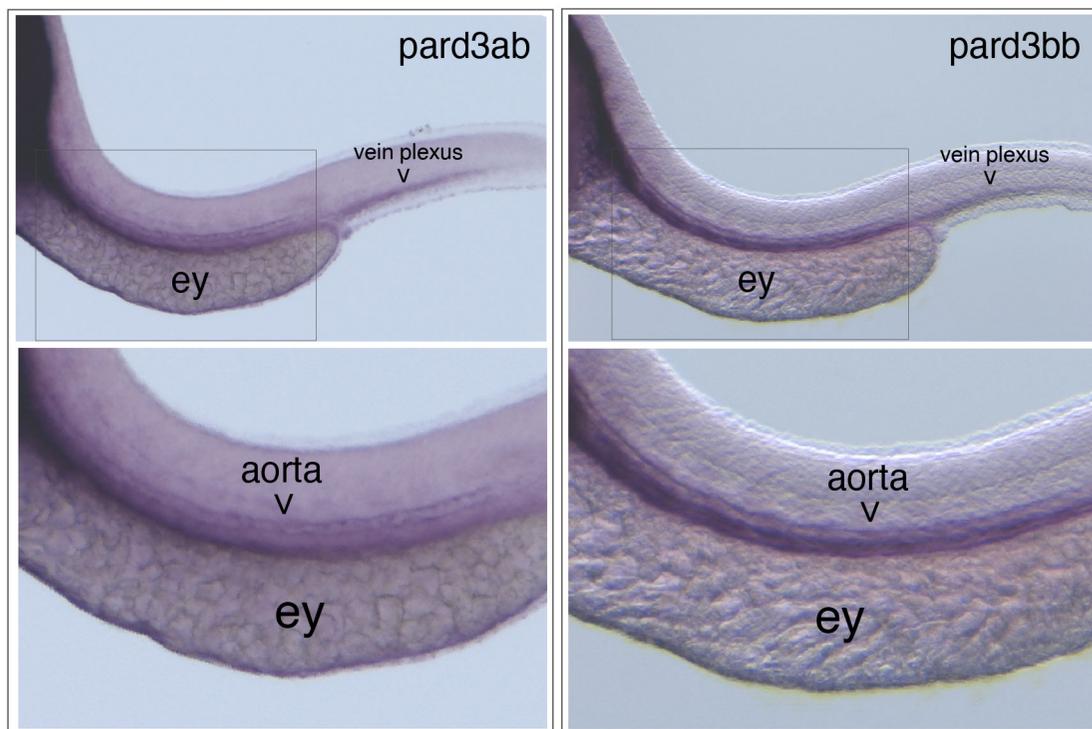


Figure 2 - figure supplement 2

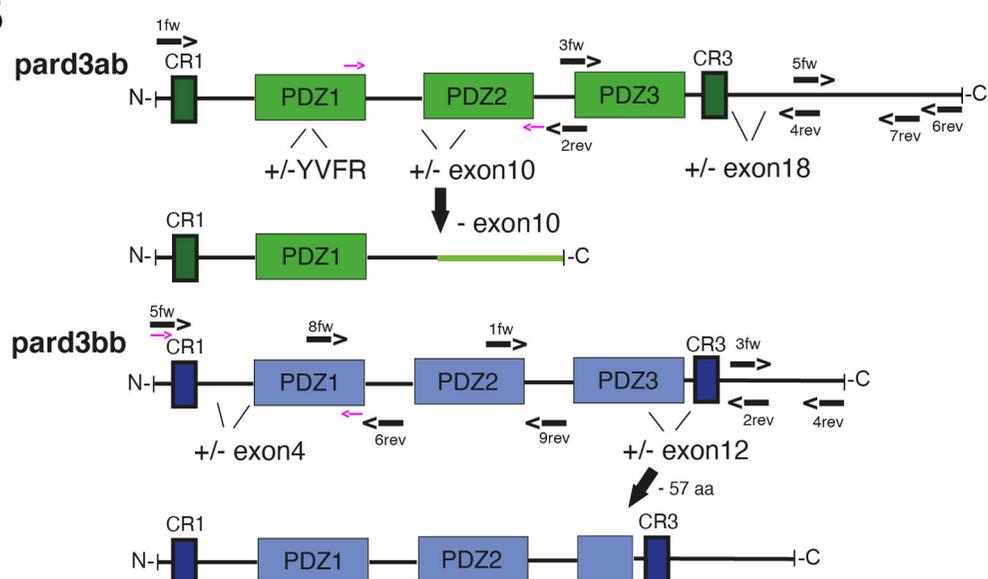


## Figure 3

A

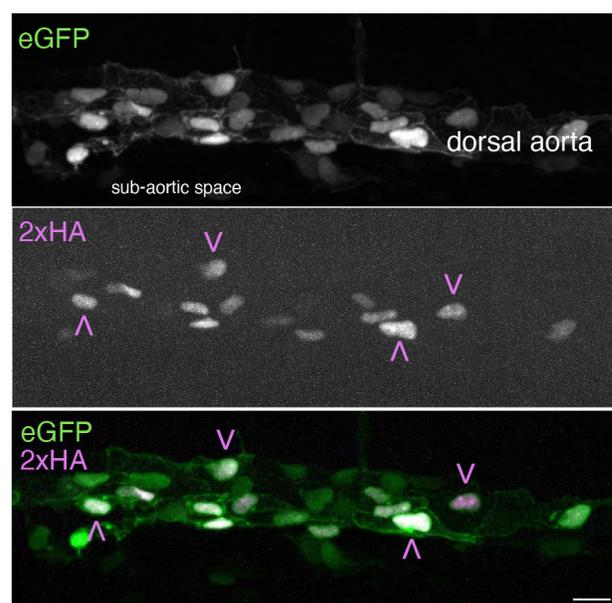
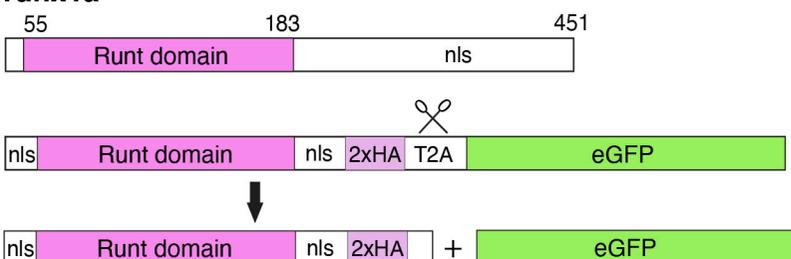


B



C

runx1a



Bar = 25 μm

Figure 3 - figure supplement 1

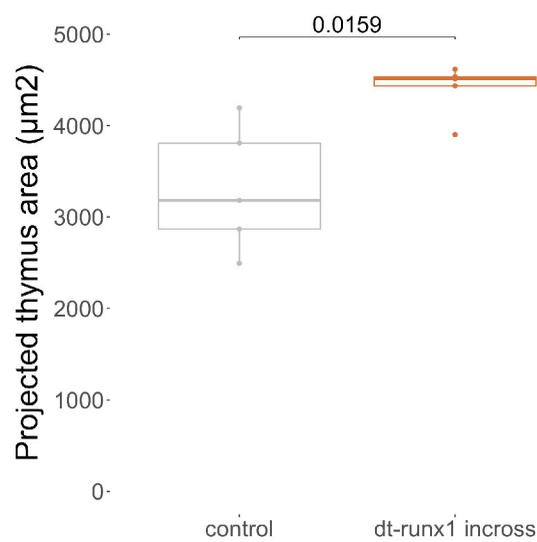
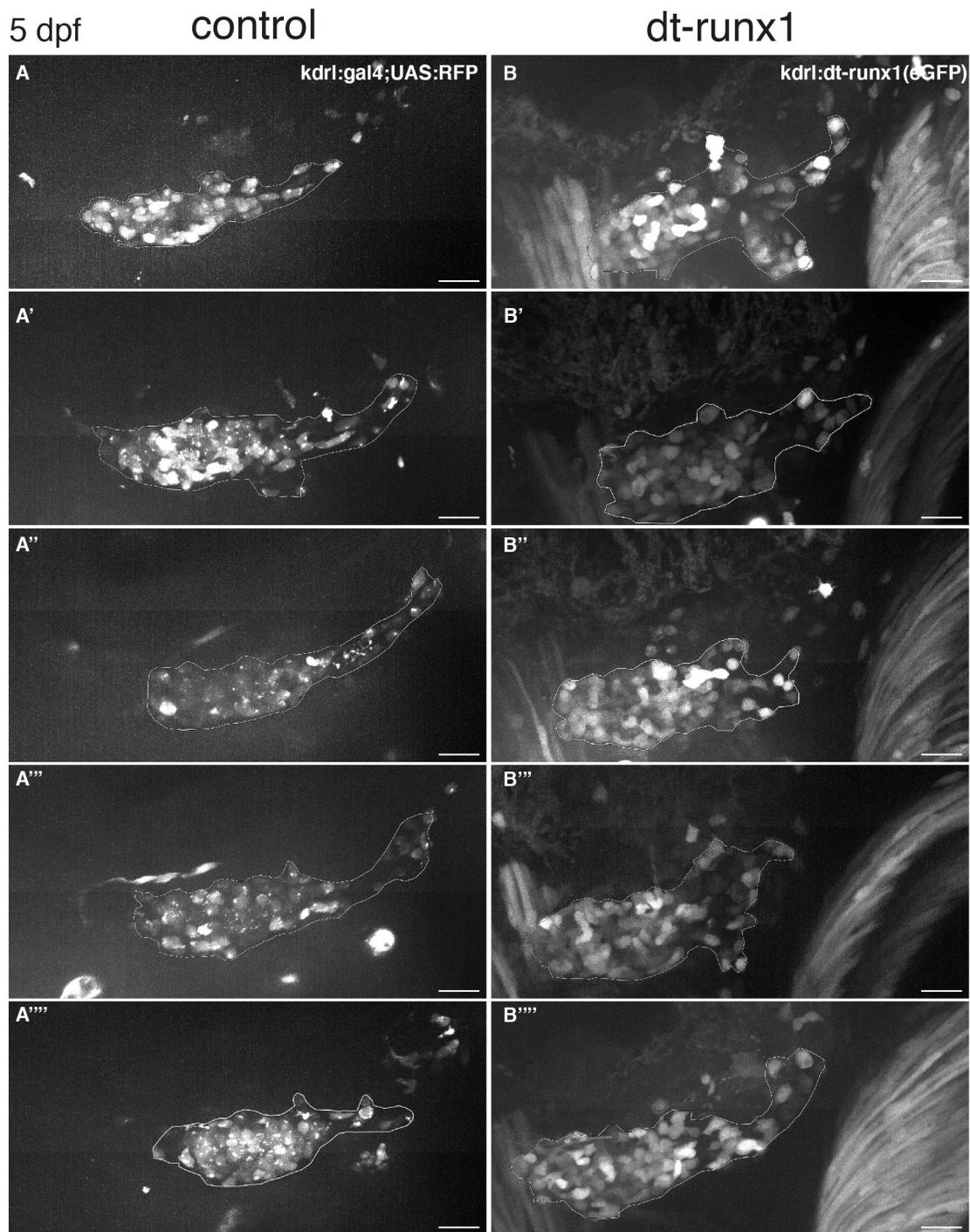


Figure 3 - figure supplement 2

mKate2-podxl2

eGFP

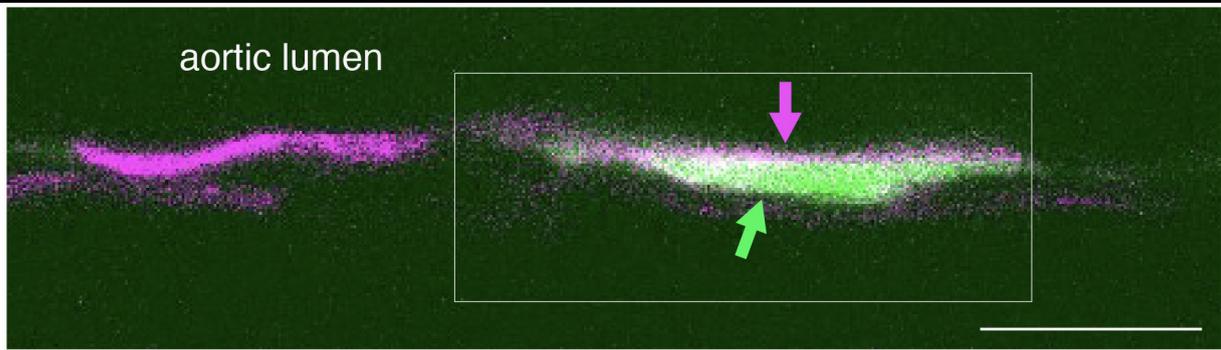
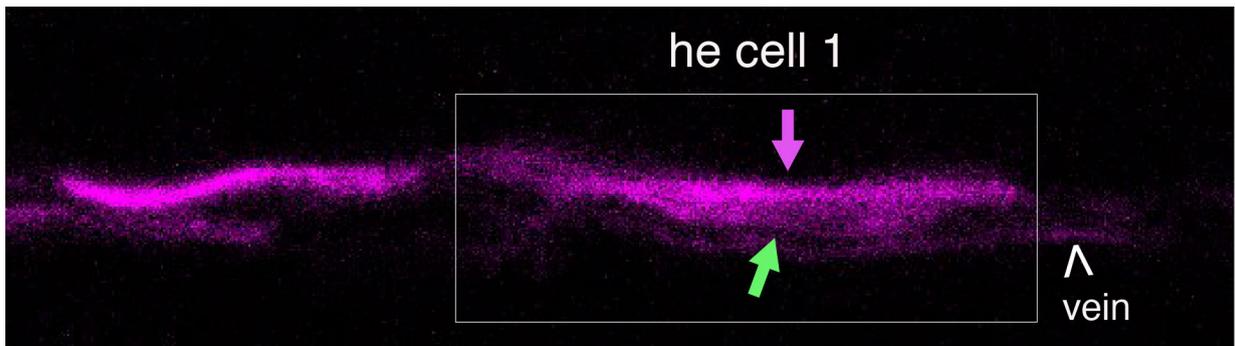
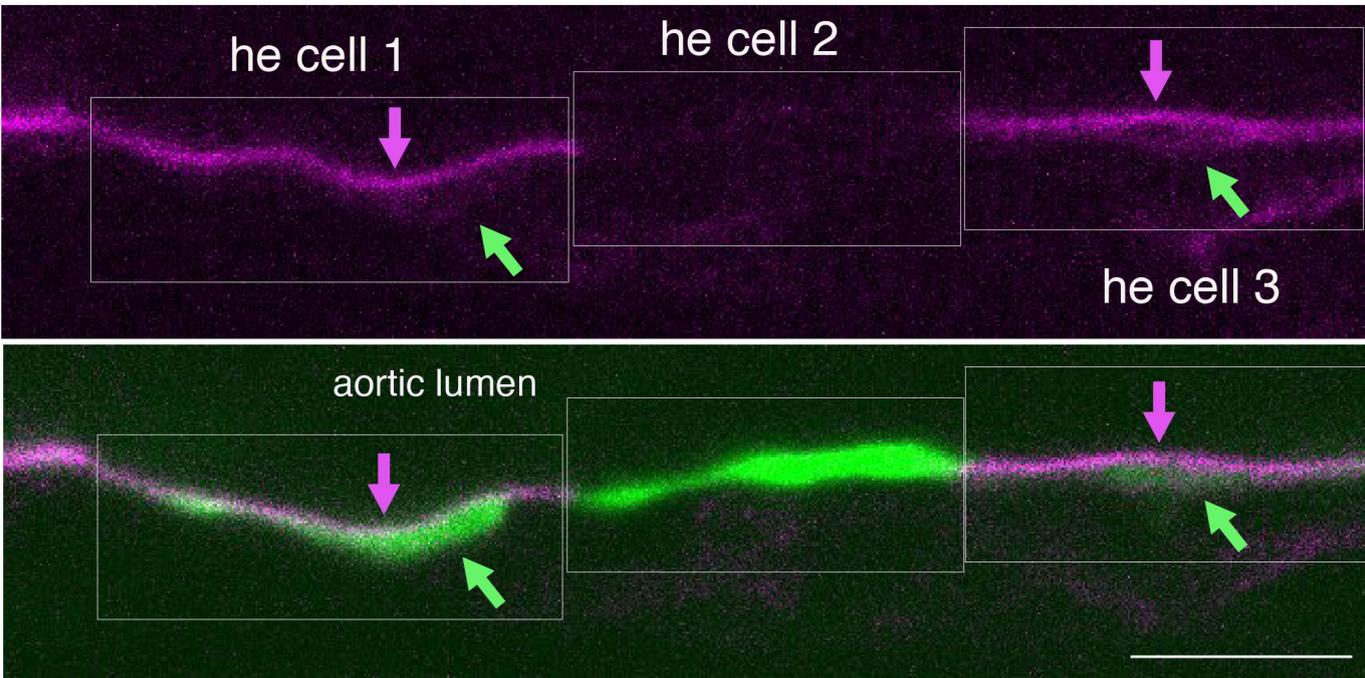
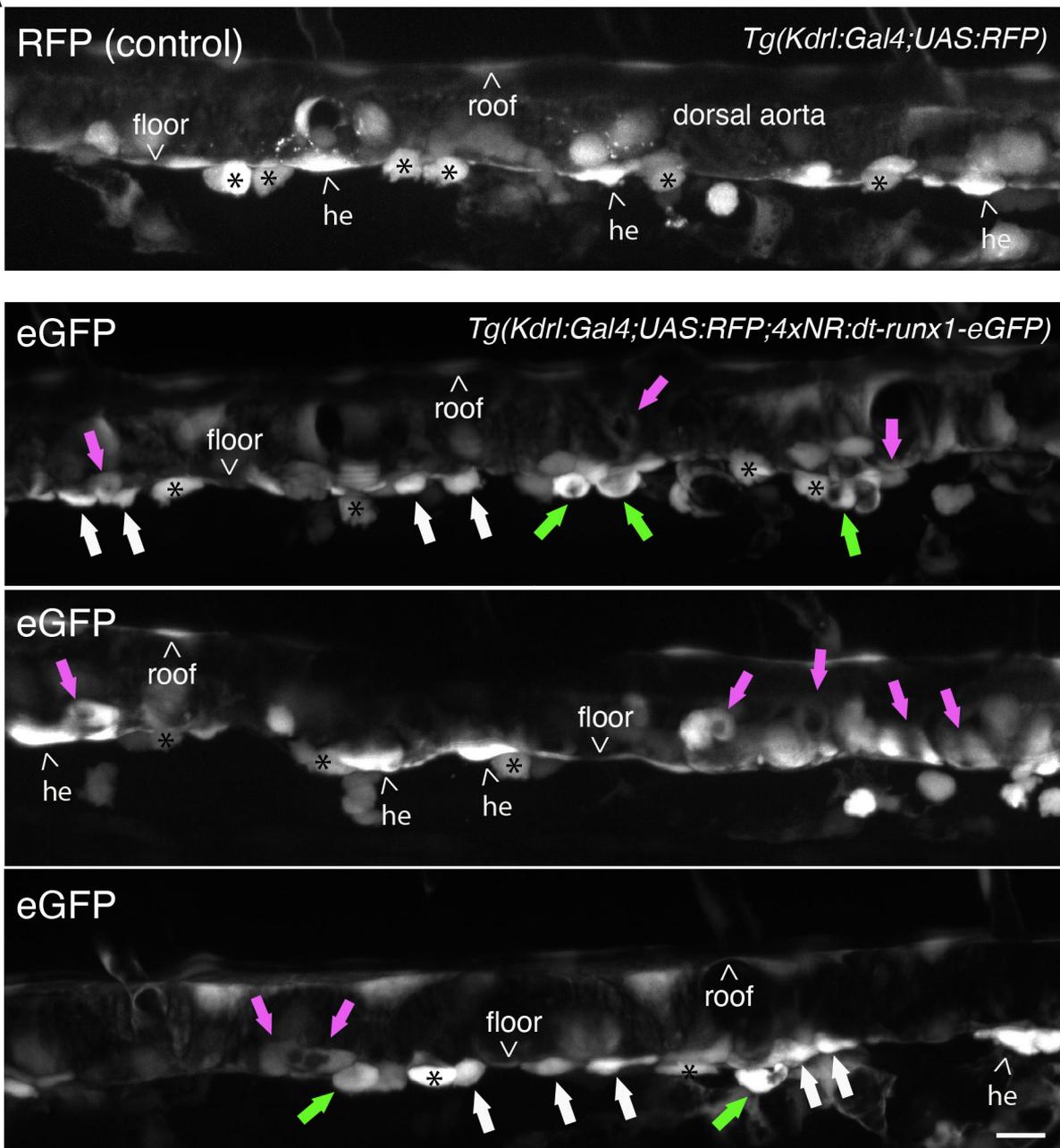


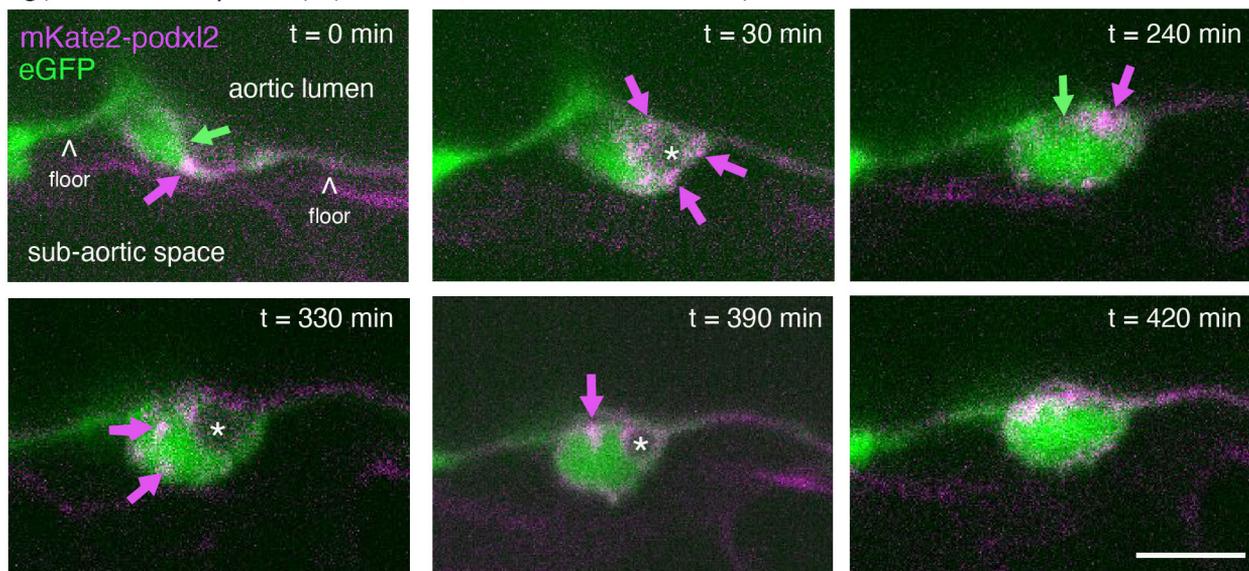
Figure 3 - figure supplement 3

A



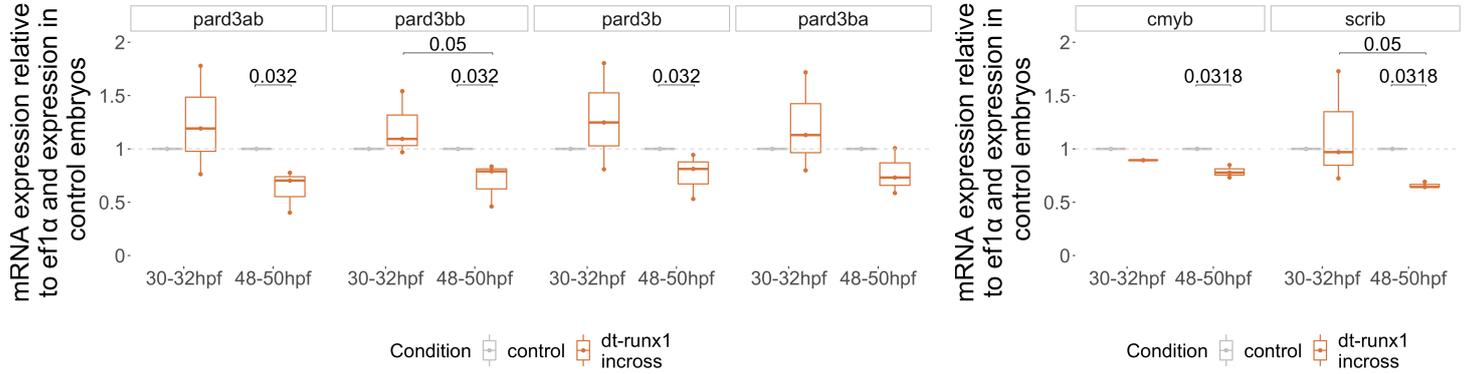
B

*Tg(kdrl:mKate2-podxl2)x(kdrl:Gal4;4xNR:dt-runx1-eGFP)*

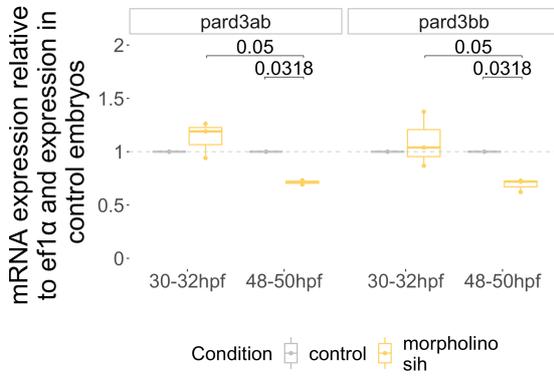


## Figure 4

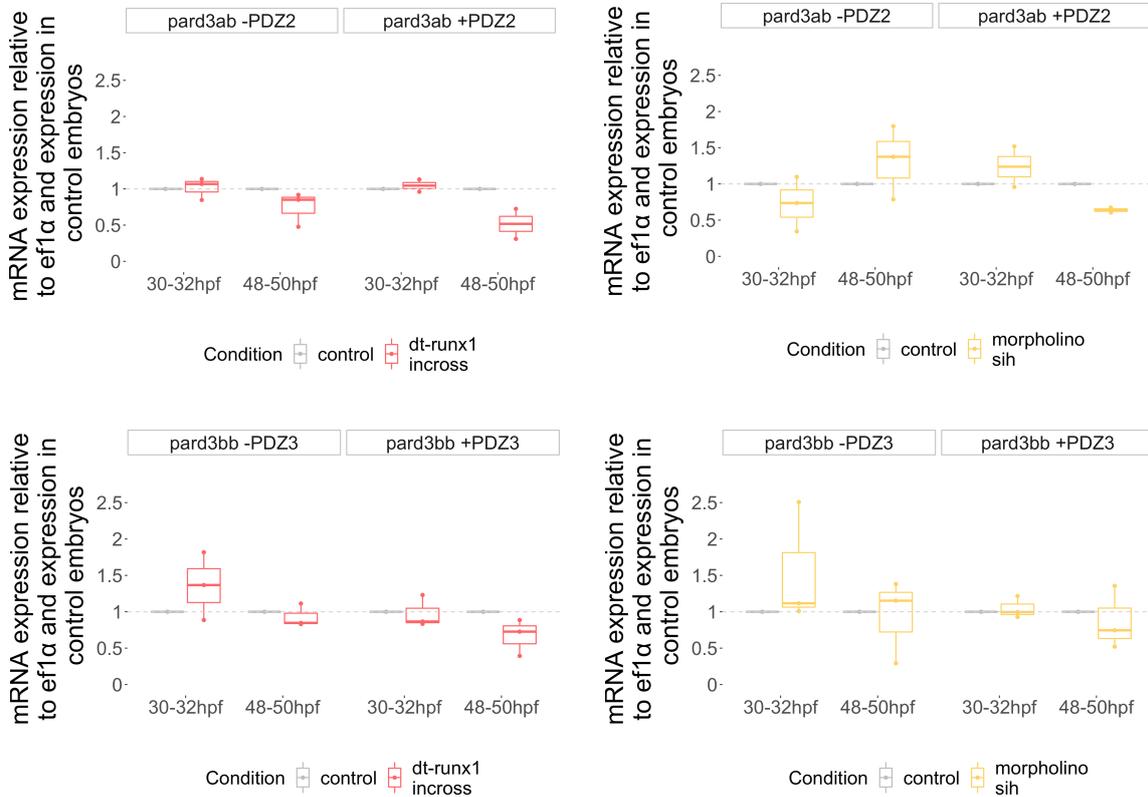
**A**



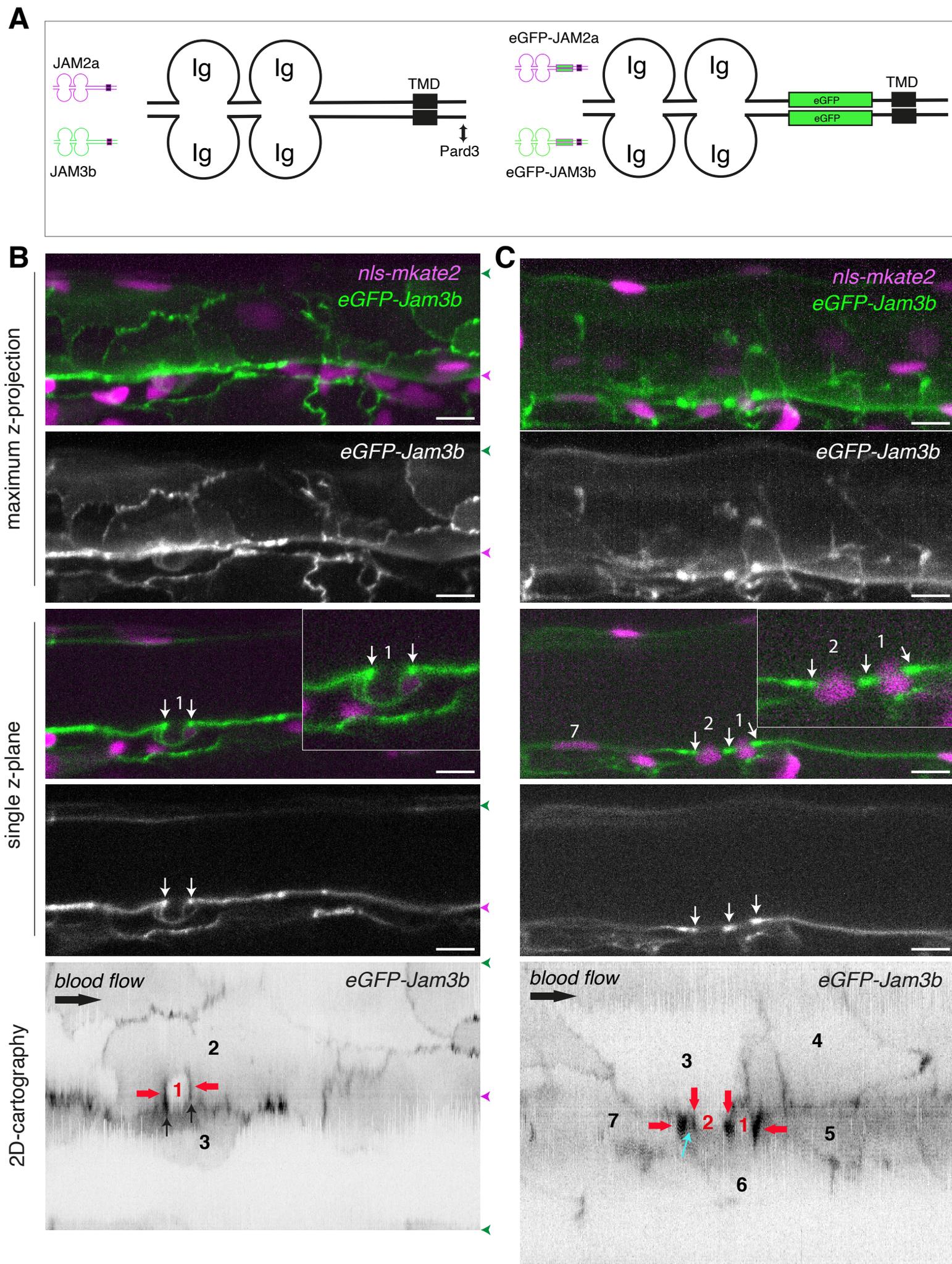
**B**



**C**



## Figure 5



## Figure 5 - figure supplement 1

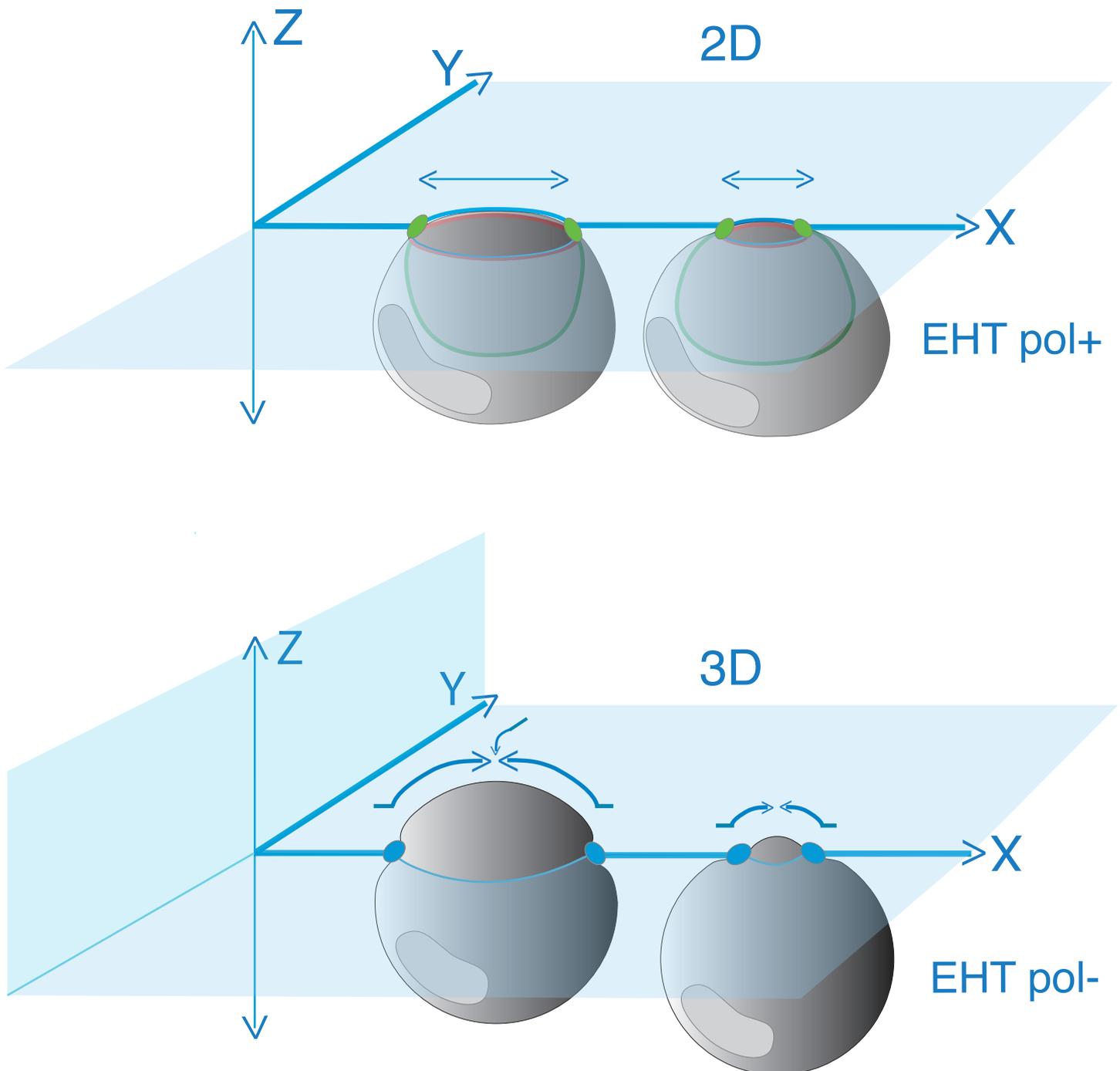


Figure 5 - figure supplement 2

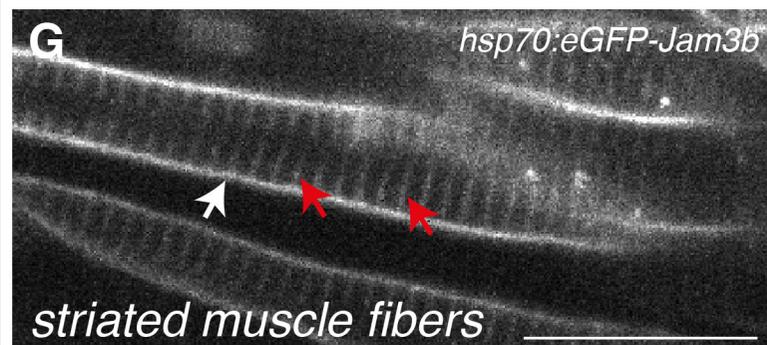
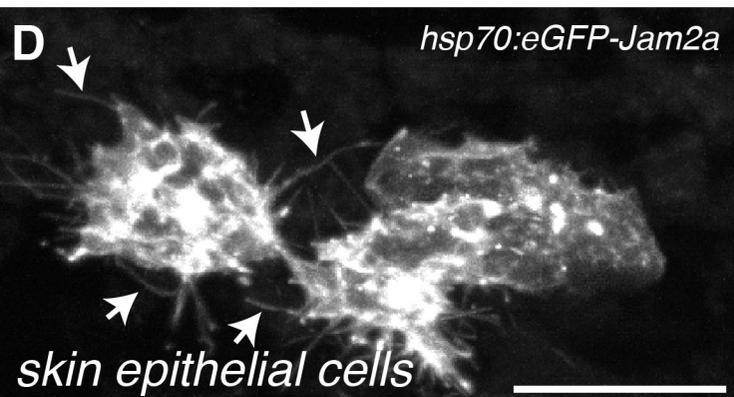
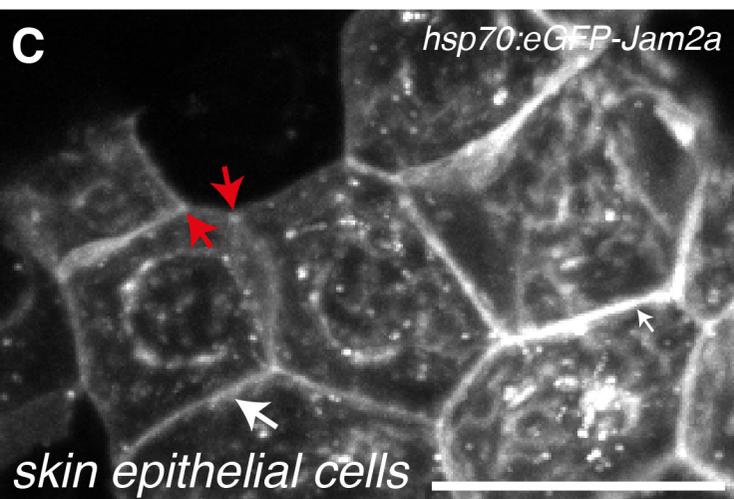
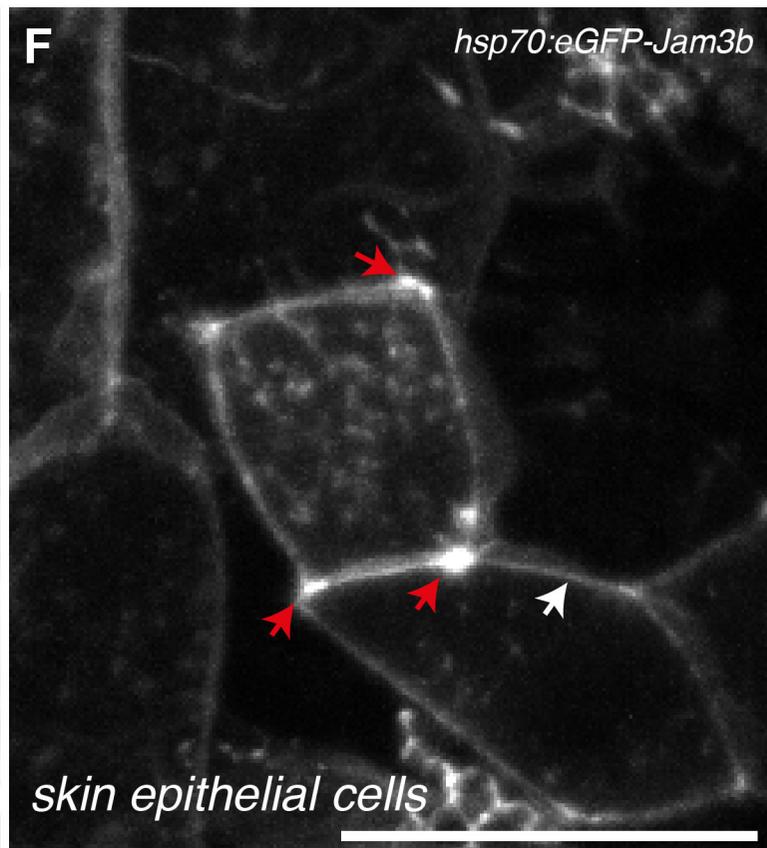
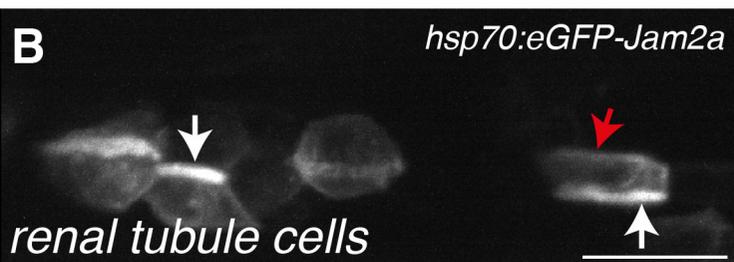
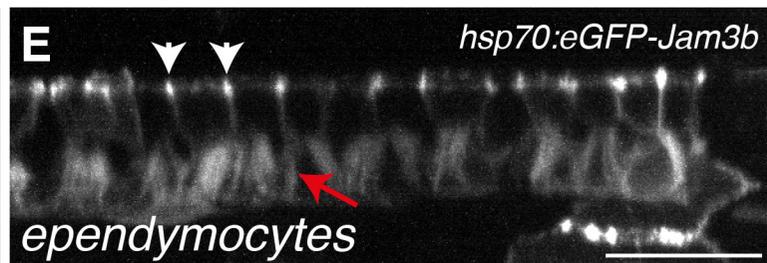
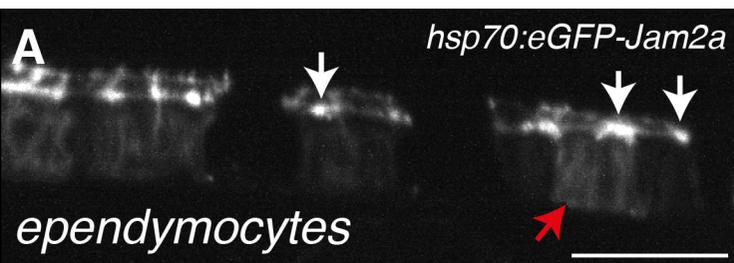


Figure 6

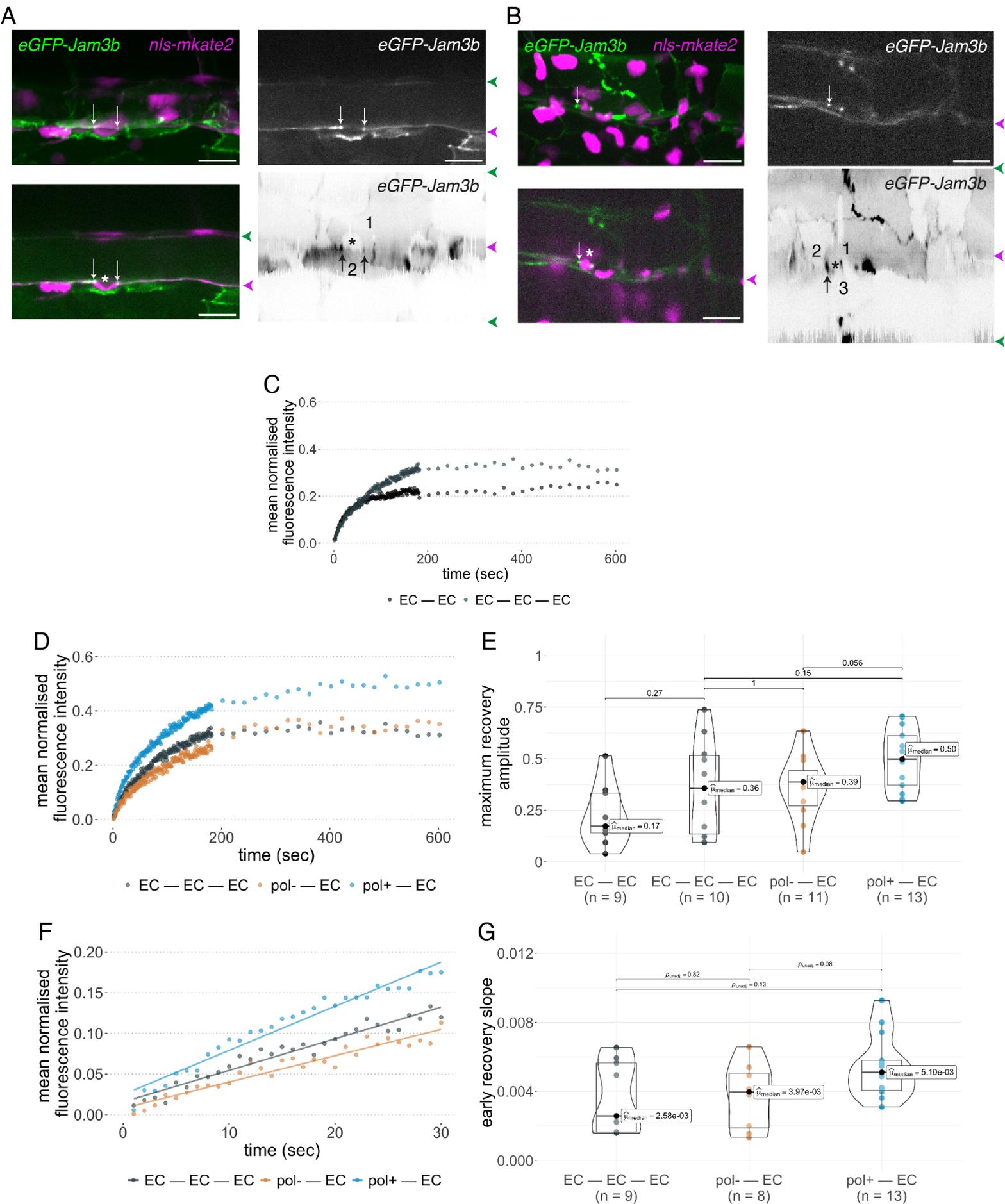
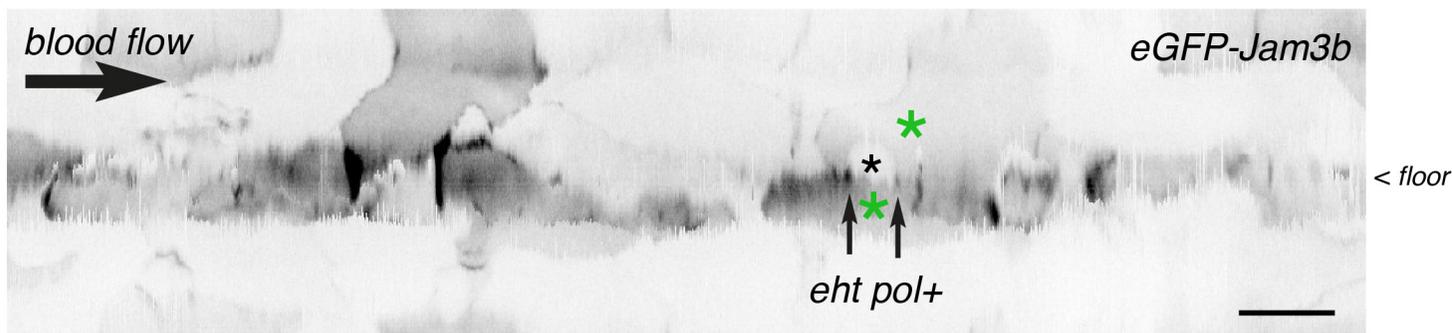
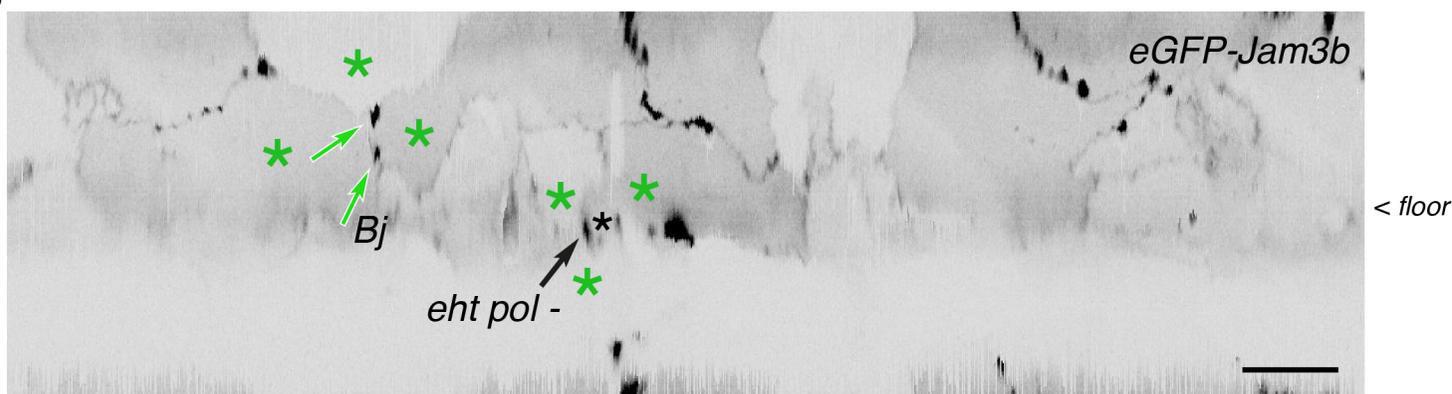


Figure 6 - figure supplement 1

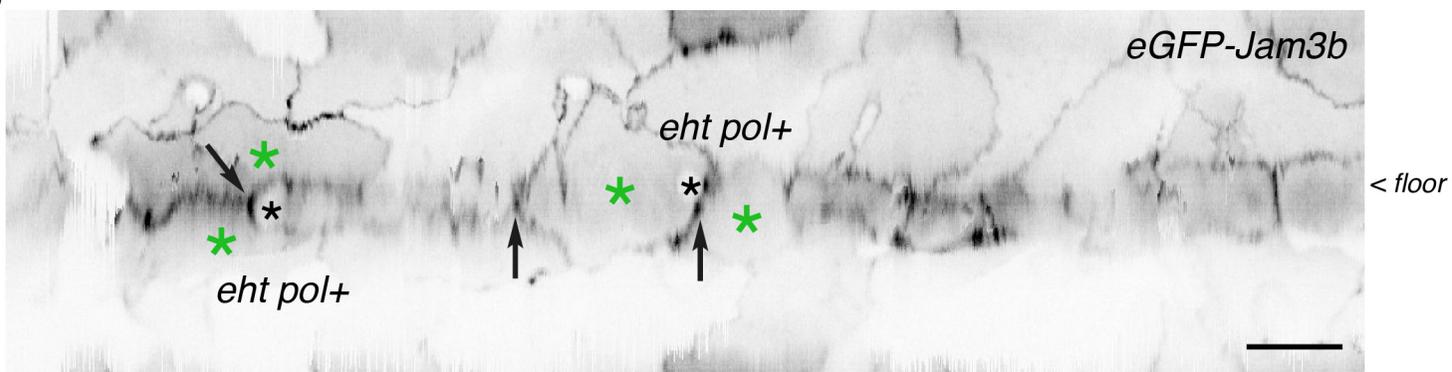
A



B



C



D

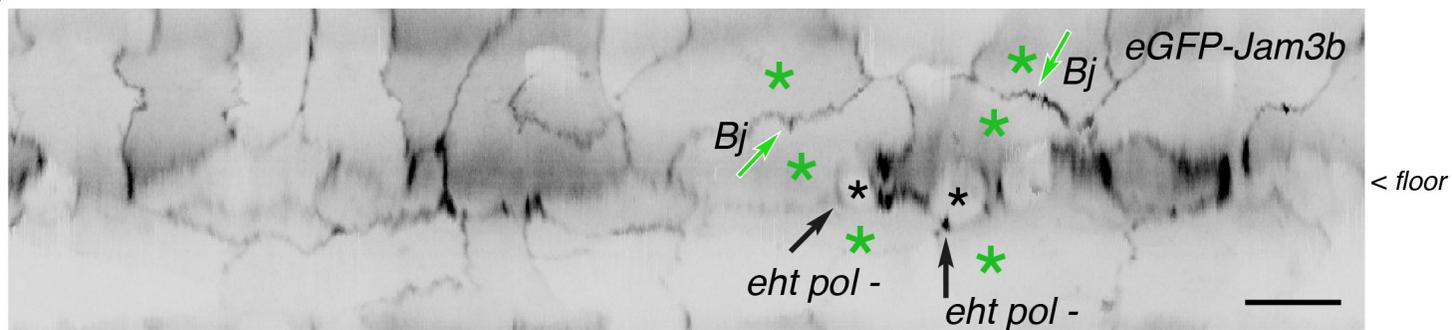
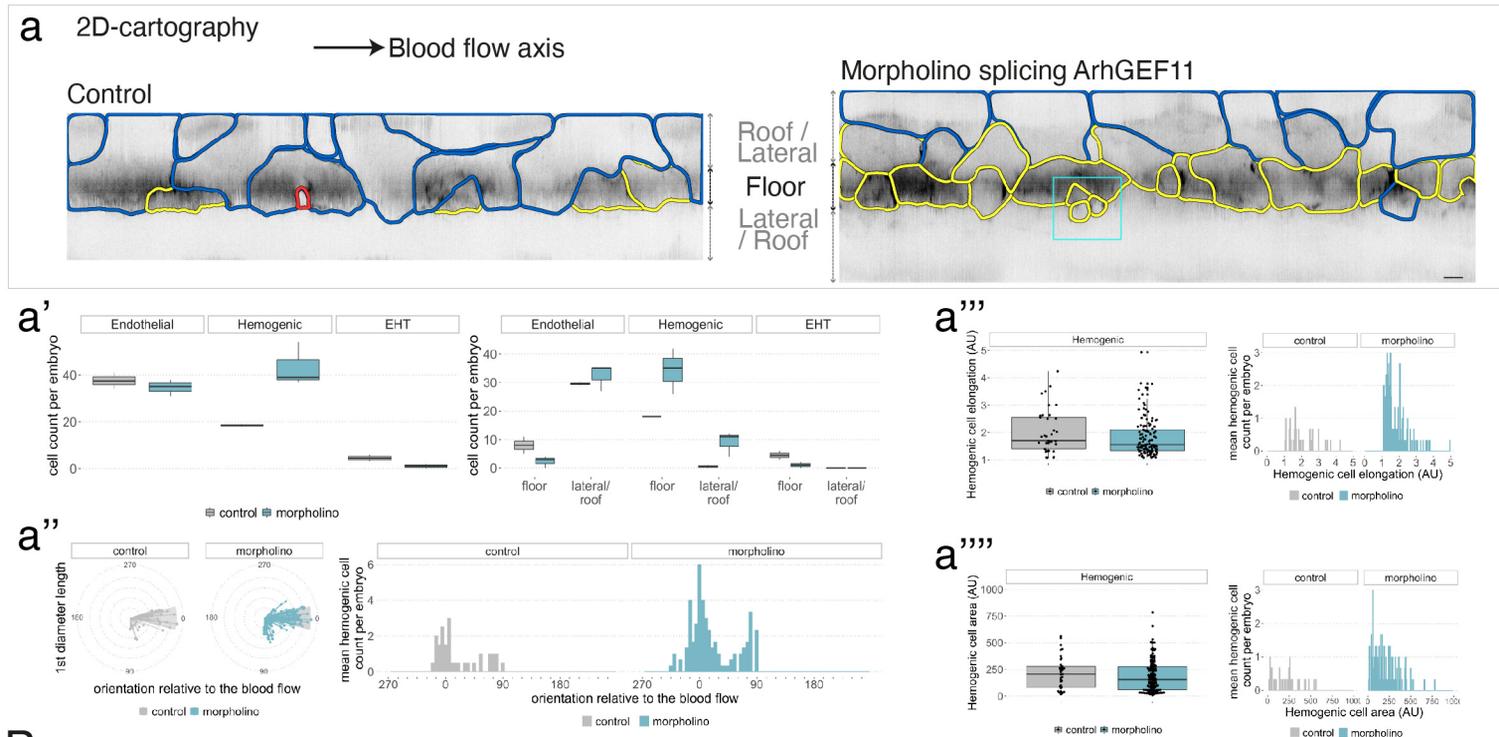
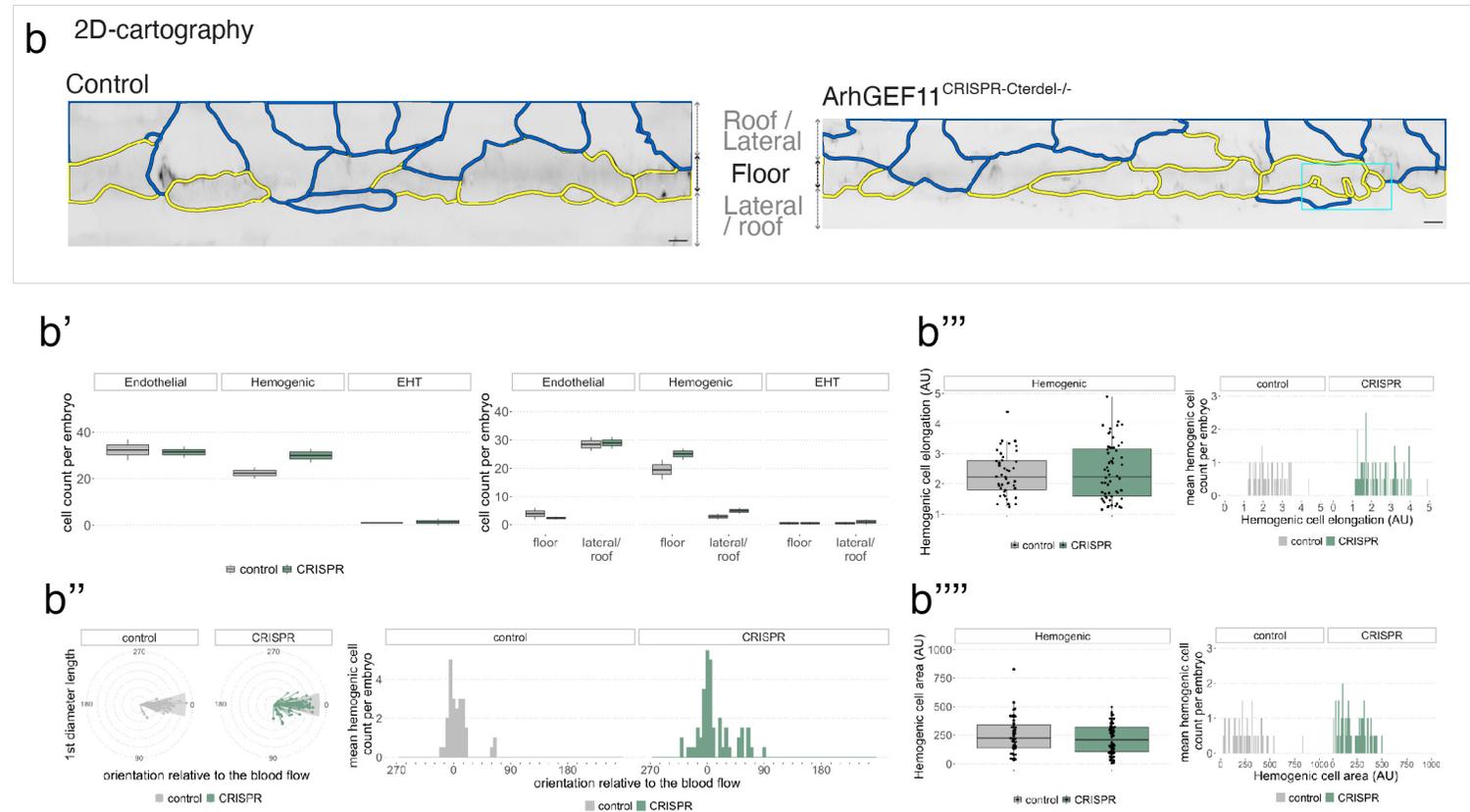


Figure 7

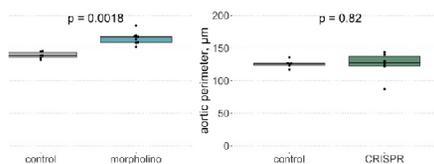
A



B

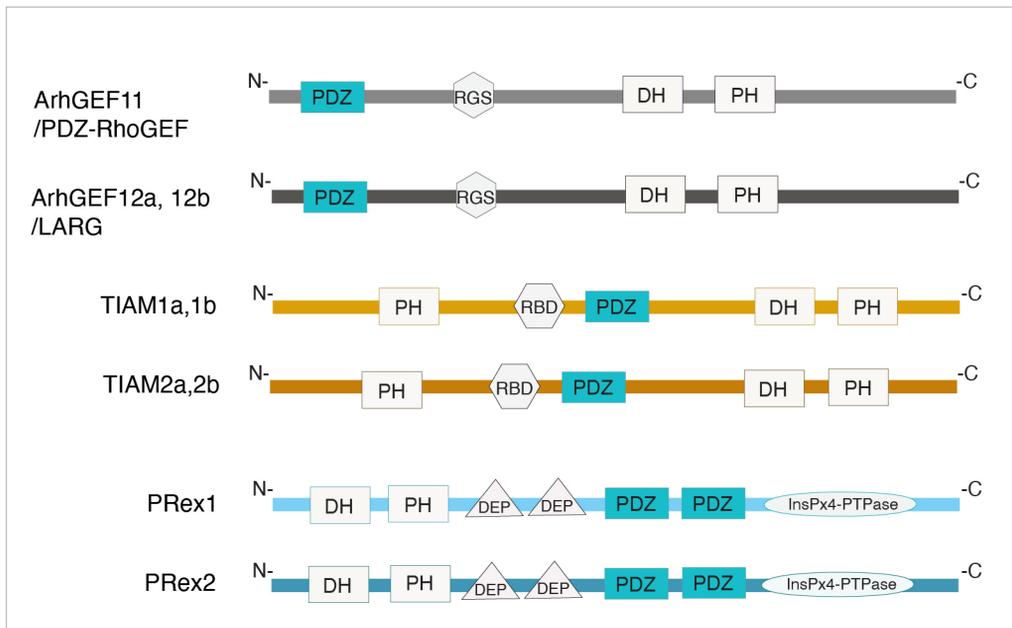


C

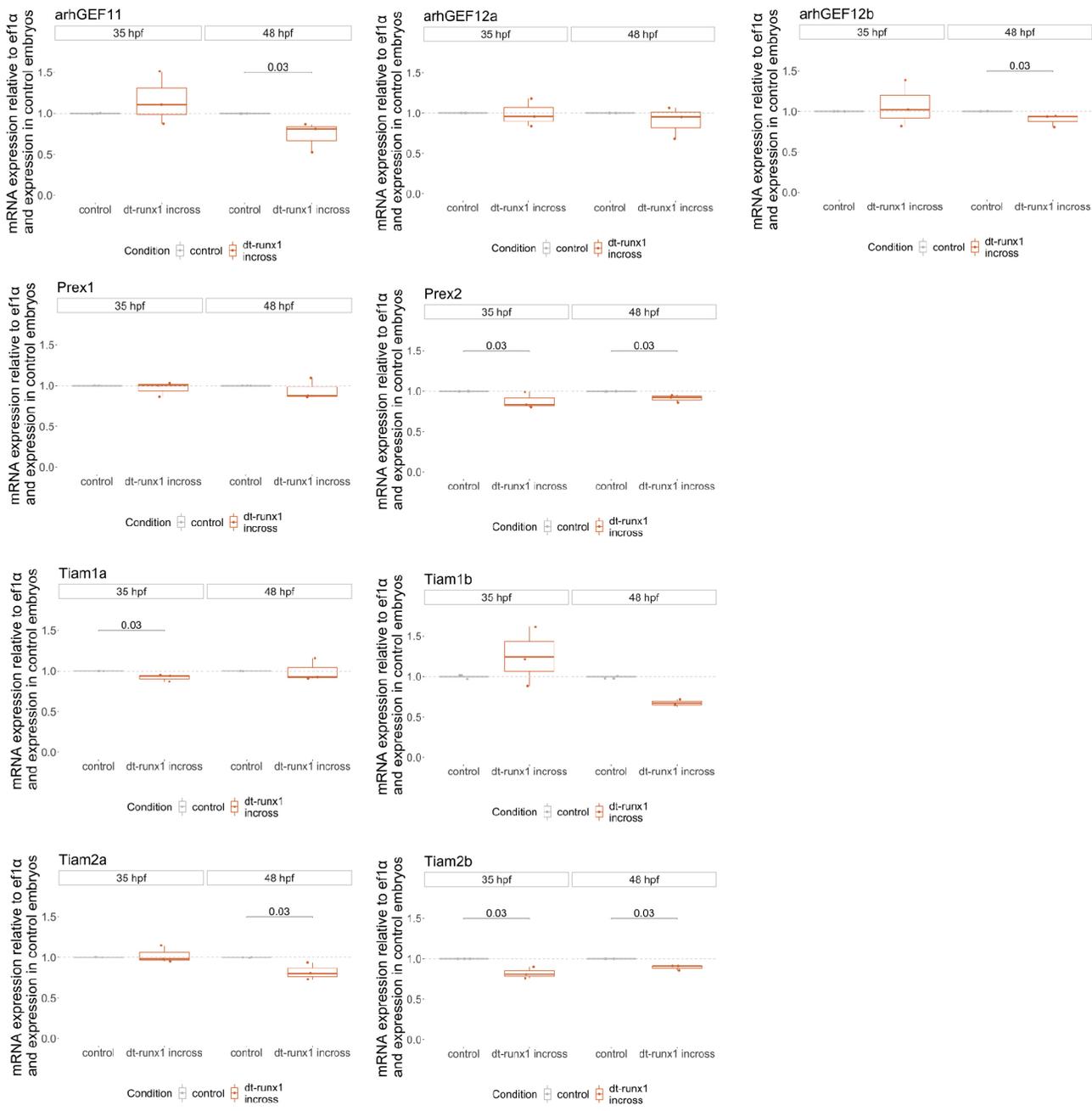


# Figure 7 - figure supplement 1

## A



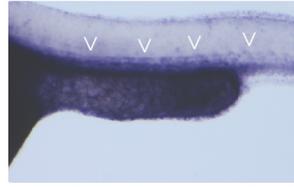
## B



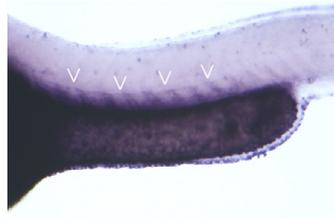
# Figure 7 - figure supplement 2

ArhGEF11/  
PDZ-RhoGEF

30-32 hpf

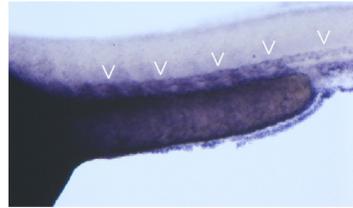
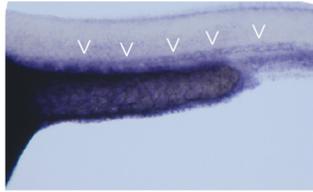


48-50 hpf



30-32 hpf

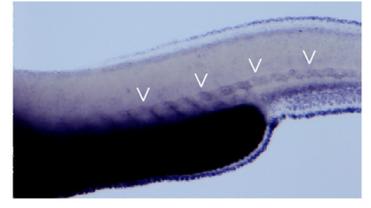
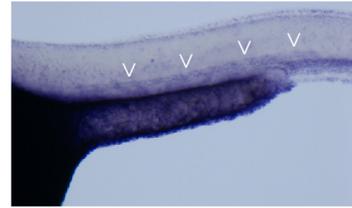
ArhGEF12a



48-50 hpf

30-32 hpf

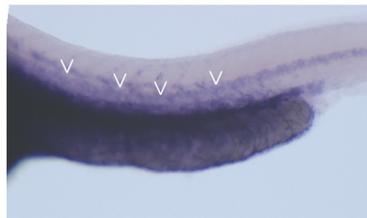
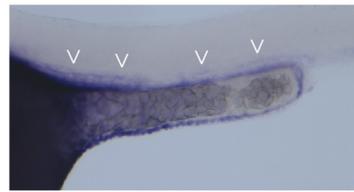
ArhGEF12b



48-50 hpf

30-32 hpf

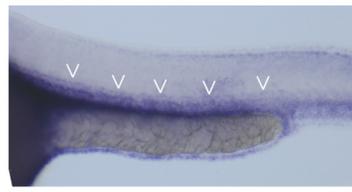
Tiam1a



48-50 hpf

30-32 hpf

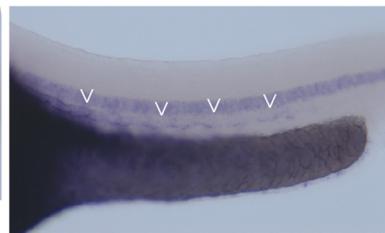
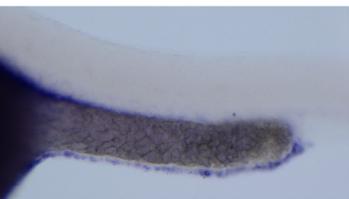
Tiam1b



48-50 hpf

30-32 hpf

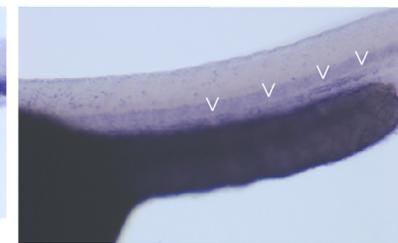
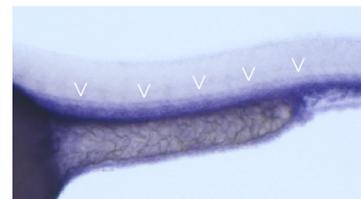
Tiam2a



48-50 hpf

30-32 hpf

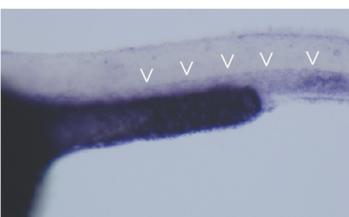
Tiam2b



48-50 hpf

30-32 hpf

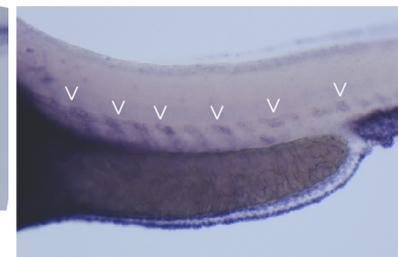
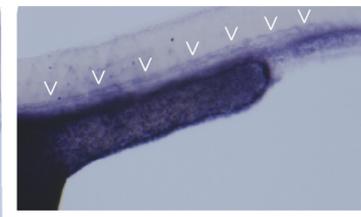
PRex1



48-50 hpf

30-32 hpf

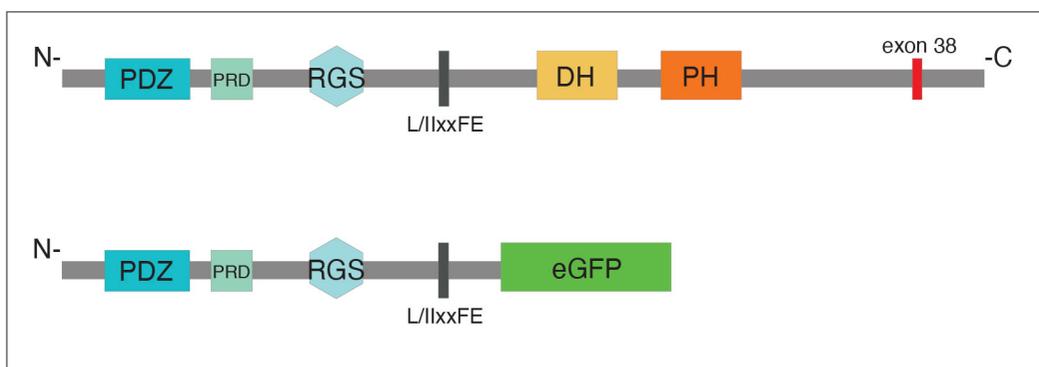
PRex2



48-50 hpf

# Figure 7 - figure supplement 3

## A



## B

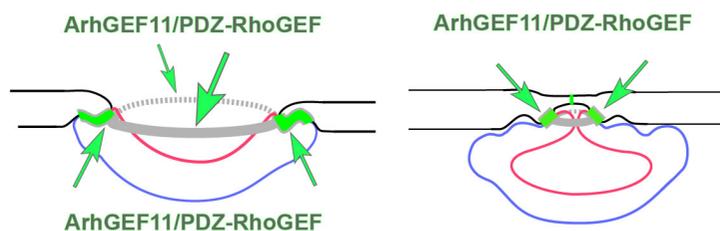
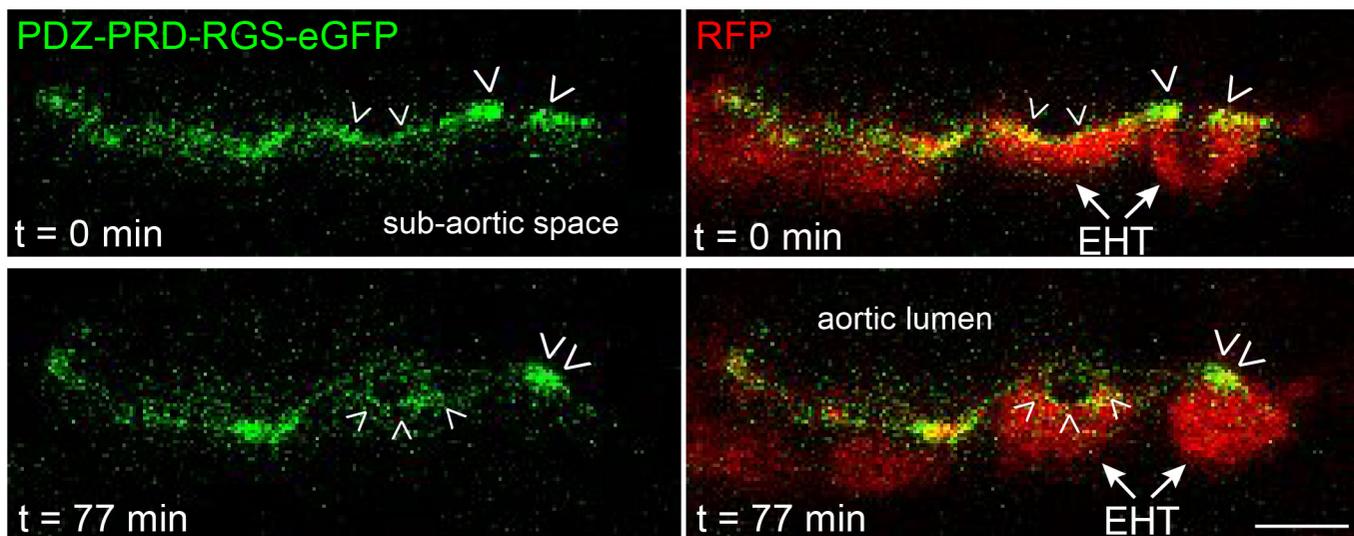
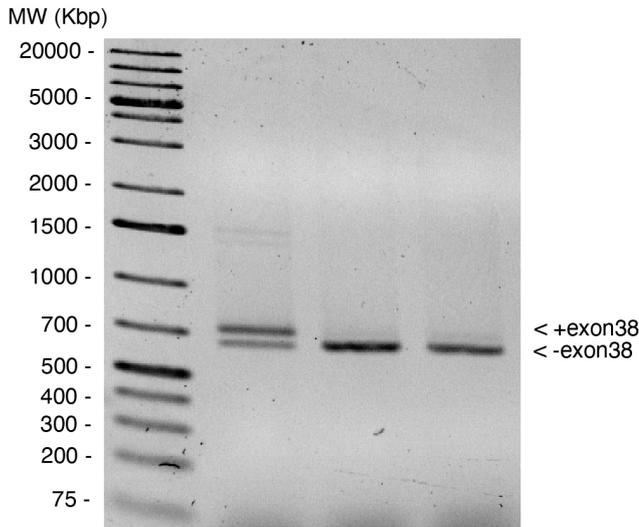
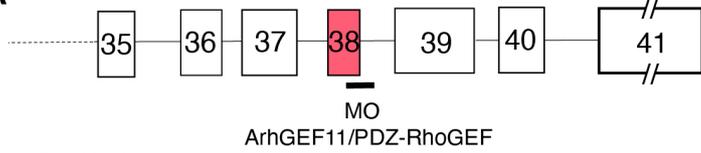


Figure 7 - figure supplement 4

A



control  
+MO1  
+MO3

control  
+MO1  
+MO3

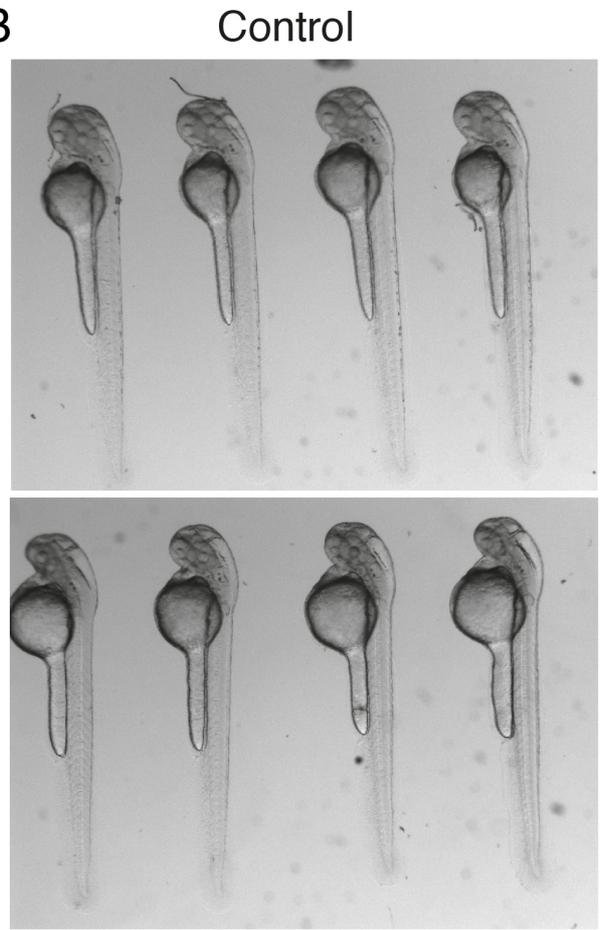
exon38

```

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+MO1        GCCTGCTGTTTCAGGTGGTGAGGAAAAG-----GAAACCTGTTCTATCTGTT
+MO3        GCCTGCTGTTTCAGGTGGTGAGGAAAAG-----GAAACCTGTTCTATCTGTT
*****

control      CACCGATGATGTCACCGCCAGCCAATCGTCTGAGGACGGAGGAAACCTGTTCTATCTGTT
+MO1        CACCGATGATGTCACCGCCAGCCAATCGTCTGAGGACGGAG-----GAAACCTGTTCTATCTGTT
+MO3        -----GAAACCTGTTCTATCTGTT
*****
    
```

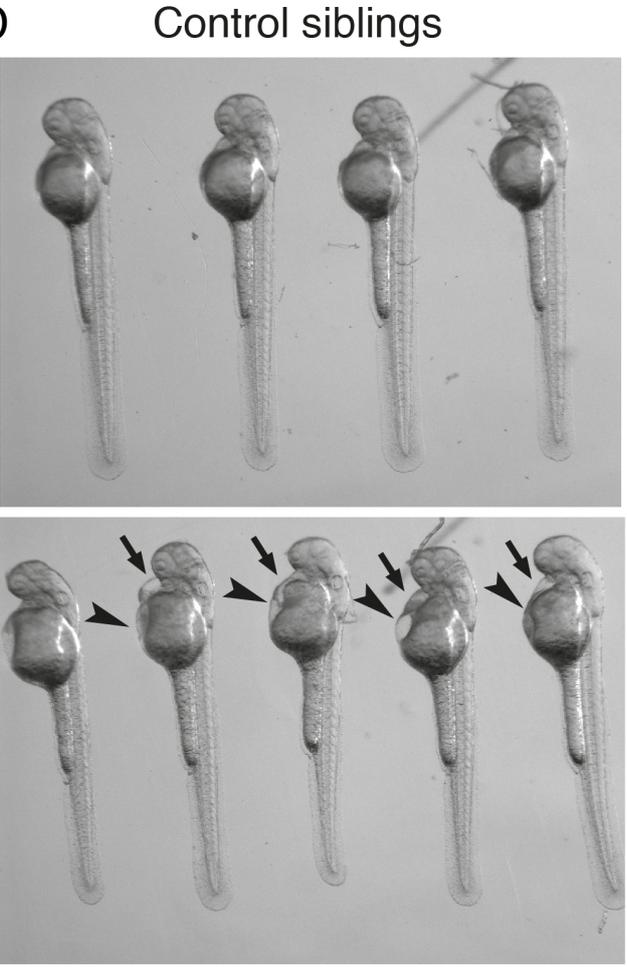
B



C

	intron 37-38	exon38
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CRISPRdelCter	CGTCGCTCTCCTCCTCCTCCTTCACACACAGCTGTGGGTGTGGGCTGTTCTCTCTCCTGATG	
Control	ACATCACCGATGATGTCACCGCCAGCCAATCGTCTGAGGACGGAGGTGGGGCTTCATTTA	
CRISPRdelCter	ACATCACCGATGATGTCACCGCCAGCCAATCGTCTGAG-----GTGGGGCTTCATTTA	
		intron 38-39
CRISPR delCter nucleotide/aa sequence		
	exon38 gtcaaccgcccagccaatcgctctgaggaacctgttctctatctggtgatgtgacggatcagg V T A S Q S S E E T C S I W -	
Wild type Cter nucleotide/aa sequence		
	exon38 gtcaaccgcccagccaatcgctctgaggaacctgttctctatctggtgatgtgacg V T A S Q S S E D G G N L F Y L V M S T gatcagggtgtctcagacagagaccacacagagctcaagatcttcaactctgtg D O V V S D E S T T D E L K D L H H S V tttctgcatctttatcaatgagagaggagcagacacgacctgtctatcgccaact F S S S F I N E E E E O D T P C L S P T gaccatcactgactcagagaccagggctcagaccagcctcagccatcagagcagc D O S L I E S Q G H R P G L S Q S Q S S gtccaactcattgatcaaaaatggtgatgagatctcaacactatggagagctgatg V Q R H V I K N V D E I F N T M E E L M aagaaactgcagcaactgagggacattgaagcggatcaccacaaactcctgaagaaacta K K L Q H L R D I E A D H H K L L K K L cgcnaaacctctctgtgacaaaatgtctagtgtgggtcccaaatctccacagc R K P L P V D K M S S D G V H K S P T A gcgcaactctctcactggaccgagcagagagatgtaaggaggcgtctcaacggaa A R L S S L D R G A G D G K E G V S T E ccagctcaacctcaaatccagtccaactggttctg P A Q P Q I O S T G F -	
NP_001003912.1	VENLRHLILWLLPGHTVKTQAAGEPEDDLTPTPSVVSIHSPWDPGSPGQAPASIDNTQ	
GEF11	VETLRQLIFRDLEDG-----WSQSDDTPTNETANERSPFTRPNESSDPAPSESS	
	**..**:* : * * : : ** .. : . : . . . . . * * : * * : * : . .	
NP_001003912.1	FPRPEGSQPEGEDVALCSLAHLPPTRNNGIWDSPELDNRNPAEEASSSEPAAGSYKVVVRKV	
ZfGEF11	QWEEEPVEAPLLEVLQPAVQVVRKAVGVGCSLPDDITDDVTASQSSSEIDGNGLFLVMST	
	* : . : * : * : : : . : . : . : . : . : . : * : * : * : * : * : . .	
NP_001003912.1	SLLPGGGVGAAKVAGSNVTPALPESGQSESELSEVGGGAQATGNCFYVSMPEPLDSSTE	
ZfGEF11	QVVS-----DETTDELKDLHHSVFSSSFINEEEQDTPCLS	
	: . . : . . : * * : * : . . : . . : . . : . .	
NP_001003912.1	PPGTPPPLSQCHSLPAWPEPPQHRGVTGGQRSSLVRDMGVIHFTIEQLTVLHLRLKDM	
ZfGEF11	PTDQSLIESQHRPGLSQSSVQR-----HVIKNVDEIFNTEELMKLQLHLRDI	
	* . . * * * : : . : * : * : . . : . . : * * : * : * : * : * : * : . .	
NP_001003912.1	ELAHRELLNSLGGESSGGTTPVGSFHTEAARWTDYLSLSP---PAKEALTSDFPQNNQEQGS	
ZfGEF11	EADHKKLLKLRKLPVDMKSSDGVHKSPTAARLSSLDRGAGDGKEGVSTEPAPQIQST	
	** : * * : * : . . . . . : * * : * * : * : * : * : * : * : * : * : . .	
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ZfGEF11	GF-----	

D



CRISPR ArhGEF11 delCter+/+

# Figure 7 - supplemental 5

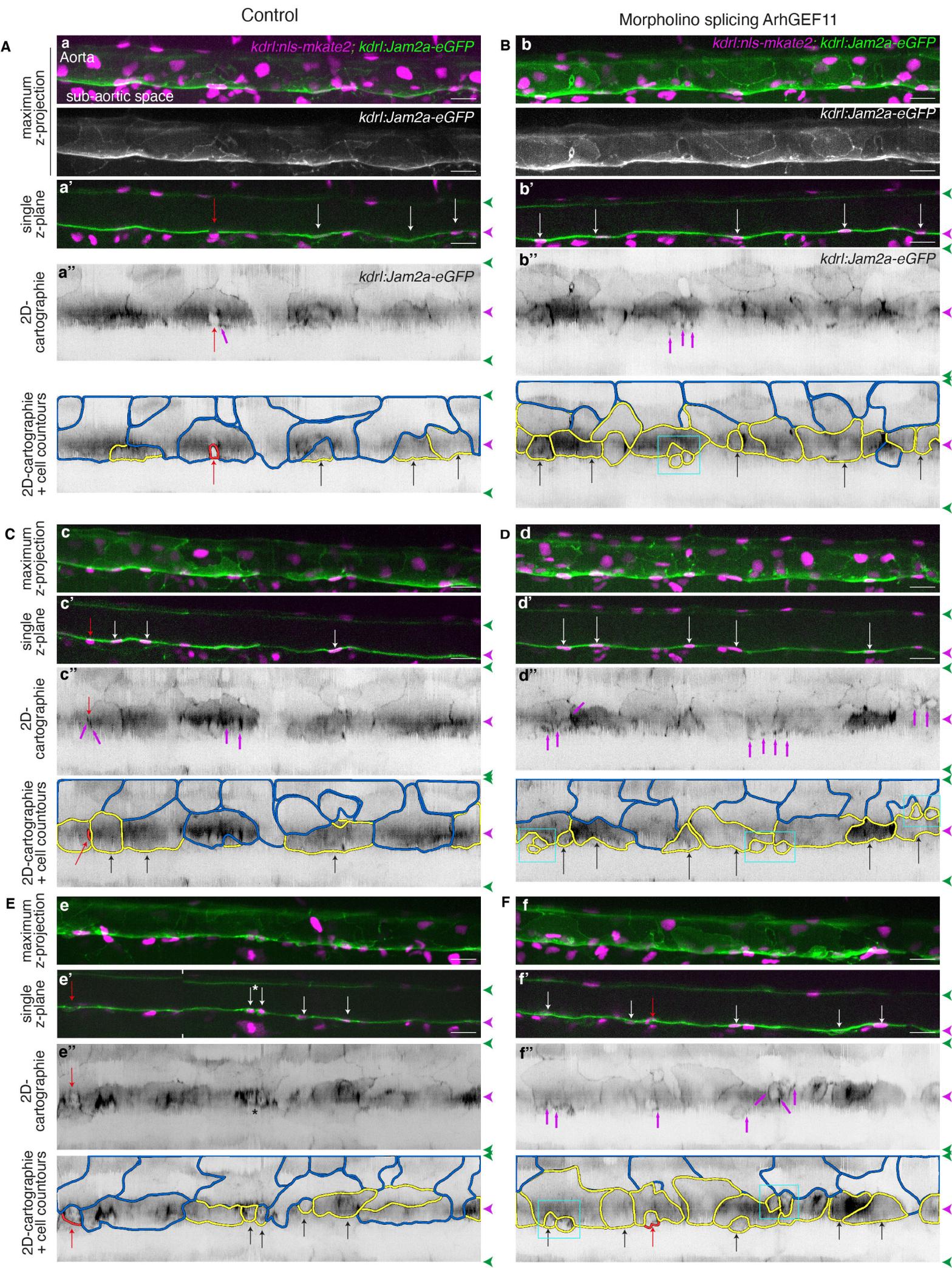


Figure 7 - supplement 6

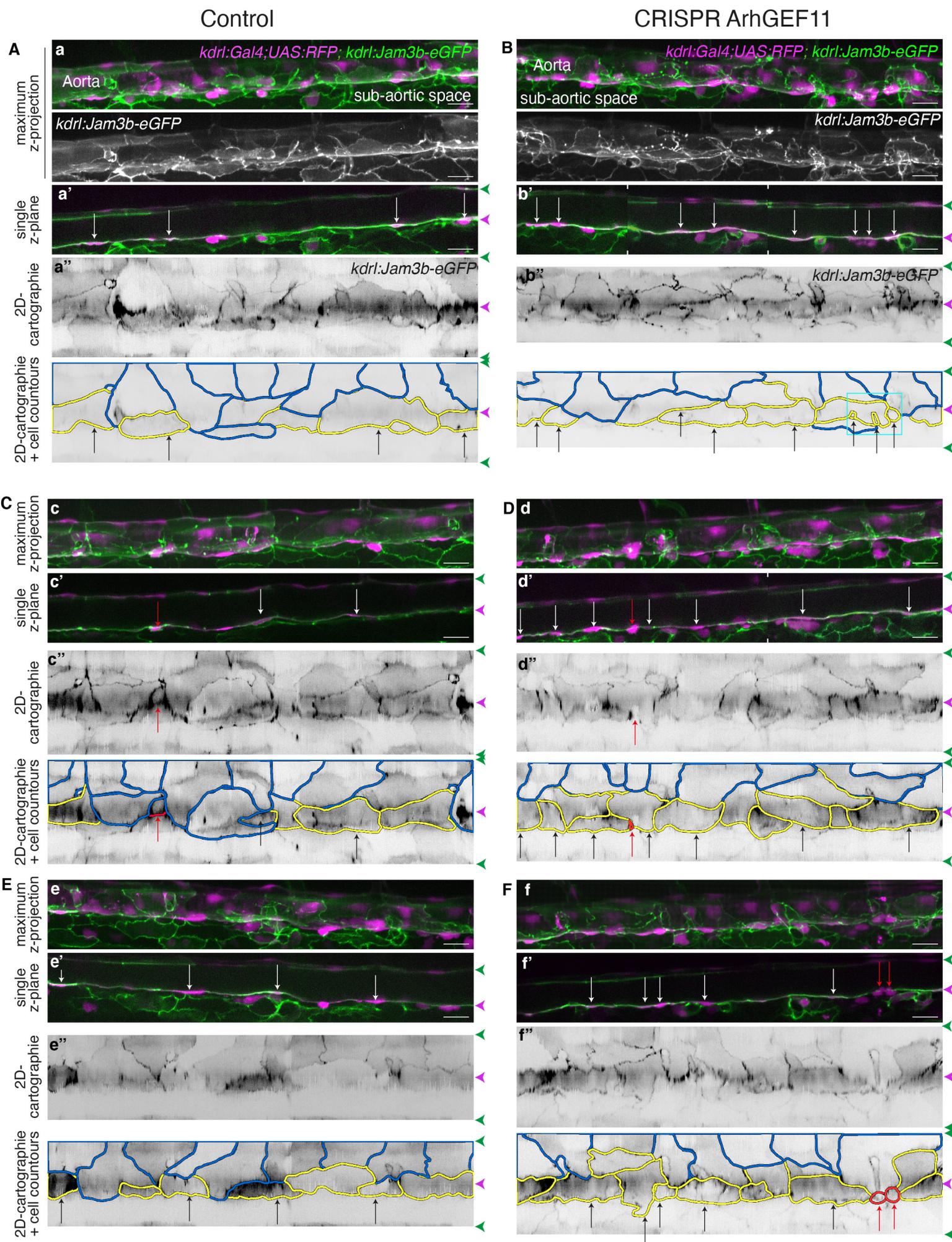


Figure 8

