

1 **Proper migration of lymphatic endothelial cells requires survival and guidance cues**  
2 **from arterial mural cells**

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23 **Keywords:** Lymphatics, Lymphangiogenesis, mural cells, Pdgfr $\beta$ , cell migration, Vegfc,  
24 Cxcl12b

25

26 Word count: 26879

27 **ABSTRACT**

28 The migration of lymphatic endothelial cells (LECs) is key for the development of the complex  
29 and vast lymphatic vascular network that pervades most of the tissues in an organism. In  
30 zebrafish, arterial intersegmental vessels together with chemokines have been shown to  
31 promote lymphatic cell migration from the horizontal myoseptum (HM). Here we found that  
32 LECs departure from HM coincides with the emergence of mural cells around the  
33 intersegmental arteries, raising the possibility that arterial mural cells promote LEC migration.  
34 Our live imaging and cell ablation experiments revealed that LECs migrate slower and fail to  
35 establish the lymphatic vascular network in the absence of arterial mural cells. We determined  
36 that mural cells are a source for the C-X-C motif chemokine 12 (Cxcl12a and Cxcl12b) and  
37 vascular endothelial growth factor C (Vegfc). We showed that ERK, a downstream component  
38 of Vegfc-Vegfr3 signaling cascade, is activated in migrating LECs and that both chemokine and  
39 growth factor signalling is required for the robust migration. Furthermore, Vegfc-Vegfr3 has a  
40 pro-survival role in LECs during the migration. Together, the identification of mural cells a  
41 source for signals that guide LEC migration and survival will be important in the future design  
42 for rebuilding lymphatic vessels in the disease contexts.

43

44 **INTRODUCTION**

45

46 The lymphatic vessel network spans across the whole body to balance the tissue fluid  
47 homeostasis, coordinate the immune responses, and enable dietary fat absorption in the  
48 intestine. The robustness of the formation and reproducibility of the vascular tree is dependent  
49 on molecular dynamics and tissue-tissue interaction required for the precision and fine-tuning  
50 of lymphatic endothelial cell (LEC) migration. Although multiple previous studies have  
51 uncovered important signals and cells guiding lymphatic vessel formation (Bussmann et al.,  
52 2010; Cha et al., 2012; Jafree et al., 2021), the recent technological developments and new  
53 transgenic lines (Ando et al., 2016; Wang et al., 2020) have opened up opportunities to identify  
54 further regulators of LEC migration.

55

56 In zebrafish trunk, lymphatic vessel specification is marked by the expression of transcription  
57 factor Prox1 in ECs around 32 hours post fertilization (hpf) in response to Vegfc-Vegfr3  
58 signalling (Koltowska et al., 2015a). Around 34 hpf, venous-derived Prox1 positive cells sprout  
59 from the posterior cardinal vein (PCV) and migrate to the horizontal myoseptum (HM),  
60 establishing parachordal lymphatic endothelial cells (PL) (Hogan and Schulte-Merker, 2017).  
61 After about 10 hours the PLs move out from HM and migrate dorsally or ventrally and by 5  
62 days post fertilization (dpf) give rise to the main trunk lymphatic vessels, including dorsal  
63 longitude lymphatic vessel (DLLV), intersegmental lymphatic vessel (ISLV) and thoracic duct

64 (TD), by 5 days post fertilization (dpf) (Kuchler et al., 2006; Yaniv et al., 2006). During this later  
65 migration, vast majority of the LECs are associated with the arterial intersegmental vessels  
66 (aISV) (Bussmann *et al.*, 2010). In mutant embryos lacking aISV  
67 (*plcy*<sup>l26480</sup> and *kdr*<sup>hu5088</sup> mutants), PLs remain in HM, and subsequent the lymphatic network  
68 formation is compromised (Bussmann *et al.*, 2010). On molecular level, Cxcl12b secreted from  
69 arterial ECs (aECs) in ISV guides this LECs migration via Cxcr4 receptor expressed in LECs  
70 (Cha *et al.*, 2012). Yet, if other tissues or cells cooperate with arterial ECs to support this  
71 migration remains unknown.

72

73 Simultaneous with LEC development described above, vascular mural cells (MC) are formed  
74 *de novo* along aISVs and beneath the dorsal aorta. aISVs play a critical role in this process,  
75 and MC-emergence is completely abolished in the absence of arterial ECs (Ando *et al.*, 2016).  
76 The spatio-temporal similarity of MC and lymphatic vessel development around aISV raises  
77 the question about possible interaction between MCs and LECs in this region.

78

79 VEGFC-mediated signalling through the VEGFR3 receptor is essential for multiple steps of  
80 lymphatic vessels formation, including LEC proliferation, differentiation, and migration. *In vitro*  
81 cell culture experiments have demonstrated that VEGFC-VEGFR3 and  $\beta$ 1 integrin to promote  
82 LEC migration (Makinen *et al.*, 2001; Wang *et al.*, 2001). Studies using *vegfc* reporter  
83 zebrafish line has uncovered multiple sources of *vegfc*, including the fibroblasts and neurons,  
84 which contributes to the initial sprouting and migration of lymphatic vessel into the HM (early  
85 migration) (Wang *et al.*, 2020). The requirement of VEGFC-VEGFR3 and its source(s) in the  
86 LEC migration out of the HM (late migration) remains to be defined. Mechanistically,  
87 transcription factor Mafba, which regulates LEC migration but not proliferation, has been  
88 shown to act downstream of VEGFC-VEGFR3 signalling (Dieterich *et al.*, 2015; Koltowska *et*  
89 *al.*, 2015b). A genome-wide analysis further indicted the presence of a transcriptional network  
90 controlling LEC migration, through the induction of chemokine receptors that promote  
91 chemotaxis in migrating LECs (Williams *et al.*, 2017). Although the migratory regulators have  
92 been identified, the upstream cellular source of the signals initiating the migration is unknown.

93

94 Here, we took advantage of the transgenic zebrafish reporters which allow to visualize MC  
95 and LEC simultaneously at high spatio-temporal resolution *in vivo*, and to investigate their  
96 communication during lymphangiogenesis. We found that MCs emergence precedes LEC  
97 migration along aISV and that LECs interact with MCs residing at the aISVs. Moreover, in the  
98 absence of MCs, the LECs migration was inhibited, and lymphatic vessel formation was  
99 compromised. We further determined that MCs produce lymphangiogenic factors including

100 *vegfc*, *cxcl12a*, and *cxcl12b*. Thus, this study uncovers a close interaction between MC-LEC  
101 of functional importance for lymphatic vessel formation in the zebrafish trunk.

102

## 103 **RESULTS AND DISCUSSION**

104

### 105 **MCs and LECs interact during LEC migration**

106

107 To address a potential interaction between MCs and LECs around arteries, we examined their  
108 distribution around aISVs, using the reporter lines  
109 *Tg(lyve1b:DsRed);Tg(flt1:YFP);Tg(pdgfrb:GFP)* and found the spatial proximity, with MCs  
110 being sandwiched between the aISV and the migrating LEC at 4 dpf (**Figure 1A**). Time lapse  
111 imaging showed that LECs migrated out from HM immediately after the emergence of *pdgfrb*<sup>+</sup>  
112 MCs (**Figure 1B and C, Supplemental Figure 1A**). The number of MCs was not changed  
113 before and after the LEC migration along aISV (**Figure 1D**) and in approximately 90% of the  
114 cases, LEC migrated towards and interacted with the MC residing on aISV (n=24 **Figure 1E-**  
115 **F**). When LEC migrated out from HM, we noticed that LEC dynamically extended and  
116 regressed protrusions as if they sensed the environment and searched for the nearby located  
117 MC (**Figure 1E, Supplemental Figure 1A**). To understand the biological significance of this  
118 interaction, we quantified the velocity of LEC migration along aISV and found that the LECs in  
119 contact with the MCs migrated two times faster than LECs migrating along aISV without MCs  
120 (**Figure 1G**). These observations suggest that MCs might provide directional cues to promote  
121 robust LEC migration.

122

### 123 **MCs promote lymphatic vessel formation**

124

125 We next asked if *pdgfrb* positive MCs are necessary for lymphatic vessels formation. PDGFR $\beta$   
126 is known to be essential for MC development, especially their proliferation and migration (Ando  
127 *et al.*, 2016; Gaengel *et al.*, 2009). The *pdgfrb*<sup>um148</sup> mutant zebrafish (Kok *et al.*, 2015) showed  
128 30% reduction of MC-number around aISVs (**Figure 1H-I**). Coincidentally, trunk lymphatic  
129 vasculature formation in *pdgfrb*<sup>um148</sup> mutant zebrafish revealed slight reduction in the network  
130 formation. The rendering of the area of *lyve:DsRed* labelled lymphatic vessels, as a measure  
131 of lymphatic vessels density, revealed on average an area of 100 mm<sup>2</sup> in the sibling vs. 70  
132 mm<sup>2</sup> in the *pdgfrb*<sup>um148</sup> mutants (**Figure 1H and 1J**). Treatment with a PDGFR $\beta$  inhibitor,  
133 AG1296 from 48 hpf, led to a greater reduction in MC-coverage and LECs number  
134 (**Supplementary Figure 1B**). Together, although it cannot be excluded that AG1296 inhibitor  
135 directly affected lymphatic vessels development, these observations suggest a requirement of  
136 *pdgfrb*<sup>+</sup> MCs for lymphatic development.

137 As both mutant or AG1296-treated larvae retained a substantial proportion of their MCs, we  
138 decided to eliminate *pdgfrb*<sup>+</sup> MCs utilizing MC-selective Nitroreductases (NTR) and  
139 metorodinazole (Mtz) system (NTR-system) (Curado et al., 2008),  
140 *TgBAC(pdgfrb:Gal4FF);Tg(14xUAS:3xFLAG-NTR, NLS-mCherry)* to confirm the involvement  
141 of MCs for lymphatic vessel formation. In this transgenic line, Mtz is converted to its cytotoxic  
142 form by NTR expressed in *pdgfrb*<sup>+</sup> MCs leading to selective MC-death in the trunk  
143 (**Supplementary Figure 2A**). When ablating MCs just prior to LEC migration out from HM by  
144 utilizing this MC-selective NTR-MTZ ablation system, LEC migration along aISV and  
145 subsequent TD formation were severely compromised (**Figure 2 A-C**). To further determine if  
146 MCs are necessary for LEC migration, we ablated MCs locally by multi-photon laser after LEC  
147 just migrated out of HM (**Figure 2D**). As a control we targeted the tissue adjacent to the MCs  
148 in the same embryo (**Figure 2E**). We imaged the same embryos one day later and observed  
149 that 40% of the LECs in the MC-ablated group fail to migrate to form DLLV (**Figure 2E-H**).  
150 The time-lapse imaging over 5 hours post ablation (pa) confirmed that LEC migration was  
151 dramatically inhibited in the MC-ablated group compared to the control non-MC ablated group  
152 (**Figure 2E-H**).  
153 Together, these data show the MC-LEC interaction at high spatio-temporal resolution during  
154 lymphatic development and demonstrate an important role of arterial associated MCs for the  
155 robust LEC migration.

156

### 157 **Chemokines are expressed in *pdgfrb*<sup>+</sup> mural cells during LEC migration**

158

159 The importance of MCs for LEC migration might represent a direct interaction between the  
160 two cells or an indirect effect mediated via aISV ECs, which were previously shown to be  
161 necessary for LEC guidance (Bussmann et al., 2010). The absence of MCs does not affect  
162 arterial identity at early stages in zebrafish (Ando et al., 2019), arguing that the importance of  
163 MC in LEC development is not simply to regulate aEC presence or abundance. However, in  
164 order to directly test aEC function is critical for MC-dependent LEC migration in aISV, we also  
165 ablated ECs in aISV after the emergence of MCs using the multi-photon laser system. We  
166 observed that even in the absence of aEC, but remaining presence of MCs, LEC migration  
167 progressed (**Figure 3A**), suggesting that signals from MCs are sufficient to promote LEC  
168 migration. Thus, the previously reported strong inhibition of LEC development in aISV-  
169 depleted mutants (Bussmann et al., 2010) might include the effects of MC-loss as aISVs ECs  
170 are essential for MC-formation (Ando et al., 2016). However, as the aISV rapidly regrow  
171 following laser ablation system (**Figure 3A-C**), the long-term effects cannot be assessed, and  
172 it remains to be determined to what extent the molecular signals from MC act in synergy with  
173 aECs to promote LEC migration.

174 Based on the above observations, we hypothesise that MC may also secrete chemoattractant  
175 molecules to guide the LECs (**Figure 3A**). It has been shown that the LEC migration is driven  
176 by the chemokines Cxcl12a and Cxcl12b (Cha *et al.*, 2012), which are expressed in ECs. To  
177 test if Cxcl12a and Cxcl12b are also expressed in MCs we collected cells by fluorescent  
178 activated cell sorting (FACS) from *Tg(flt1:YFP); TgBAC(abcc9:Gal4FF); Tg(UAS:GFP);*  
179 *Tg(lyve1b:DsRED); Tg(kdrl:mCherry)* larvae at 3 dpf and analysed candidate genes  
180 expression in aECs (double positive for green (YFP) and red (mCherry) fluorescence), MCs  
181 (single positive for green (GFP) fluorescence), and venous ECs and LECs (single positive for  
182 red (DsRED/mCherry) fluorescence) (**Figure 3D, Supplementary Figure 3A**). Although  
183 *abcc9* reporter is highly selective for ISV-MCs in the trunk, posterior notochord is also labelled  
184 (Ando *et al.*, 2019). Therefore, to avoid the possible contamination of other cell types than  
185 *abcc9* positive MCs, we used micro-dissection to isolate the trunk region containing *abcc9*-  
186 positive ISV-MCs for FACS. We confirmed the identity of sorted MCs by expression of *abcc9*  
187 and *pdgfrb* (**Supplementary Figure 3B**). In line with published data, that *cxcr4a* and *cxcr4b*  
188 are expressed by LECs and *cxcl12b* by aECs (Cha *et al.*, 2012). In addition, we found that  
189 *cxcl12a* and *cxcl12b* are expressed in the *abcc9*-positive MC population (**Figure 3E**). To  
190 further understand if signalling by these ligands is essential for LEC migration, we have treated  
191 embryos with a Cxcr4 inhibitor, AMD3100 (**Figure 3F, Supplementary Figure 3C**), and found  
192 LECs migrated less and their migration velocity is decreased (**Figure 3G-J**). This coincided  
193 with an increased number of filopodia formation on LEC (**Figure 3K**), indicating loss of  
194 guidance (Meyen *et al.*, 2015). Together our data show that MCs produce the LEC chemo-  
195 attractants driving LEC migration.

196

### 197 **MC and arterial derived *vegfc* promotes LEC migration and survival**

198

199 Vegfc-Vegfr3 initiates a signalling cascade driving lymphangiogenesis in zebrafish and is  
200 required for sprouting and LEC migration towards the HM (Hogan *et al.*, 2009a; Yaniv *et al.*,  
201 2006). As Vegfc has been shown to be expressed in vascular smooth muscle cells (VSMCs)  
202 in mice (Antila *et al.*, 2017), we hypothesised that it might be expressed also in zebrafish MC.  
203 Firstly, we checked *vegfc* expression in FAC-sorted cells by RT-qPCR and found that *vegfc*  
204 expression in aISV-MCs as well as aECs (**Figure 4A**). We also found that phospho(p)-ERK  
205 staining of LEC, which is known to be activated downstream of Vegfc-Vegfr3 or Cxcl12-Cxcr4  
206 (Spinosa *et al.*, 2019; Xing *et al.*, 2017), during LEC migration along aISV (**Figure 4B**). To  
207 assess if ERK activation is required for the LEC migration from HM we have used the MEK  
208 inhibitor, SL327 (**Supplementary Figure 4A**). We treated embryos at 51 hpf just prior to their  
209 migration and assessed the phenotypes by time-lapse imaging (**Figure 4C-D**). We traced the  
210 migration distance and observed a reduced number of migrating cells and increased number

211 of cells that stalled or regressed their migration in SL327-treated embryos (n=5) (**Figure 4E**).  
212 In addition, we found a dramatic decrease of LEC cell division from 25% controls to 1.5 %  
213 percent in SL327-treated embryos (**Figure 4G**), which is in agreement with the known  
214 necessary role of *Vegfc-Vegfr3* in cell proliferation (Cao et al., 1998). SL327-treatment induced  
215 cell death in 7 out of 12 cells, which was further confirmed by TUNEL staining (**Figure 4 H-I**),  
216 suggesting that during this lymphatic developmental window ERK activation acts as a LEC  
217 pro-survival factor. These effects were not observed in AMD3100 treated embryos, indicating  
218 that ERK activation may be induced mainly via *Vegfc-Vegfr3* signalling rather than *Cxcl12-*  
219 *Cxcr4* signalling. Together, these results indicate that in addition to LEC specification and  
220 sprouting from the PCV (Karkkainen et al., 2004; Koltowska *et al.*, 2015a), *Vegfc-Vegfr3*  
221 signalling also instructs LEC in the subsequent migratory events to establish the lymphatic  
222 vessel network in the trunk.

223

224 In summary, we demonstrated MC-LEC interaction at high spatio-temporal resolution during  
225 lymphatic development. We further uncovered an important role for artery-associated MCs in  
226 guidance of LECs, which is mediated by their secretion of chemoattractants including *Cxcl12*  
227 and *Vegfc*. Since other sources of *Cxcl12* and *Vegfc* have already been demonstrated in the  
228 zebrafish embryonic trunk (Cha *et al.*, 2012; Wang *et al.*, 2020), we propose that colonisation  
229 of the aISV by MC may provide a signalling threshold needed for LECs to depart from the HM  
230 and begin migrating along the aISVs. Our study underscores the importance of spatial and  
231 temporal control of the guidance cues and mitogens in order to promote and refine the  
232 migratory path and survival of LECs. Our finding of MC as molecular source for  
233 lymphangiogenic factors should have relevance to future designs aiming at re-establishing  
234 lymphatic vessels in disease contexts.

235

236

## 237 **Material and methods**

238

### 239 **Zebrafish**

240 Zebrafish were maintained in Genome Engineering Zebrafish National Facility, Uppsala  
241 University using standard husbandry conditions (Alestrom et al., 2020). Animal experiments  
242 were carried out under ethical approval from the Swedish Board of Agriculture (5.2.18-  
243 7558/14). Previously published transgenic lines used were *Tg(fli1a:nEGFP)<sup>y7</sup>*, *Tg(-*  
244 *5.2lyve1b:DsRed2)<sup>nz101</sup>* (Okuda et al., 2012), *Tg(1xUAS:GFP)* (Asakawa et al., 2008),  
245 *TgBAC(pdgfrb:Gal4FF)*, (Ando et al., 2016) *TgBAC(pdgfrb:GFP)* (Ando et al., 2016),  
246 *TgBAC(abcc9:GAL4FF)* (Ando et al., 2019), *Tg(flt1:YFP)<sup>hu4881</sup>* (Hogan et al., 2009b),  
247 *Tg(fli1a:GFP)<sup>y1</sup>* (Nathan and Weinstein, 2002), *Tg(-7kdr1:DsRed2)<sup>pd27</sup>* (Kikuchi et al., 2011),

248 *pdgfrb*<sup>um148</sup> (Kok et al., 2015), *Tg(kdr1:TagBFP)*<sup>mu293Tg</sup> (Matsuoka et al., 2016), *Tg(fli1a:Myr-*  
249 *GFP)*<sup>ncv2Tg</sup> (Fukuhara et al., 2014), *Tg(dab2:GFP)*<sup>ncv67Tg</sup> (Shin et al., 2019).  
250 *Tg(lyve1:mCherry)*<sup>ncv87Tg</sup> and *Tg(14xUAS:3xFLAG-NTR,NLS-mCherry)*<sup>ncv514Tg</sup> were generated  
251 in this study.

252

### 253 **Genotyping**

254 For *pdgfrb*<sup>um148</sup> the following primers were used for PCR

255 *pdgfrb* Forward                               5'- ATGCGCTAAAGGTGAATTGG- 3'

256 *pdgfrb* Reverse                               5'- GCGTCTGCCATAGTTGAACA- 3'

257 The PCR product was digested with Mbo1 restriction enzyme at 37 °C for 1 h. The digested  
258 product was run on 2% agarose gel. The wild type fragment is cut resulting in two bands of  
259 200 bp and 300 bp long; *pdgfrb*<sup>um148</sup> mutant is not cut and the band is 500 bp long;  
260 heterozygous *pdgfrb*<sup>um148</sup> is a combination of three fragments with bands sizes of 200 bp, 300  
261 bp and 500 bp.

262

### 263 **Immunohistochemistry**

264 Immunohistochemistry was performed according to a previously published protocol (Le Guen  
265 et al., 2014; Shin et al., 2016) with the following modifications. After acetone treatment  
266 embryos were treated with Proteinase K at 10 mg/ml diluted in PBST for 35 mins. Antibodies  
267 used were chicken  $\alpha$ -GFP (1:400, ab13970 Abcam), rabbit  $\alpha$ -DsRed (1:400, Living colors,  
268 632496 Takara Bio), rabbit  $\alpha$ -Phospho-p44/42 MAPK (1:250, #4370 Cell Signaling  
269 Technology), and  $\alpha$ -rabbit IgG-HRP (1:1000, #7074 Cell Signaling Technology). TUNEL  
270 staining was performed with *In Situ* Cell Death Detection Kit, Fluorescein (Merck,  
271 11684795910) with the instruction provided by manufacture.

272

### 273 **Image acquisition**

274 Embryos were anesthetized and mounted in 1% low-melting agarose on a 35-mm-diameter  
275 glass-base dish (627870 or 627861 Greiner). Confocal images were obtained using a Leica  
276 TCS SP8 confocal microscope (Leica Microsystems) equipped with water-immersion 25X  
277 (Fluotar VISR, 0.95 NA) objective, water-immersion 40X (HC PL APO CS2, 1.1 NA) objective  
278 and glycerol-immersion 63X (HC PL APO CS2, 1.3 NA) objective or FluoView  
279 FV1000/FV1200/FV3000 confocal upright microscope (Olympus) equipped with a water-  
280 immersion 20x (XLUMPlanFL, 1.0 NA) lens. The 473 nm (for GFP), 559 nm (for mCherry),  
281 and 633 nm (for Qdot 655) laser lines in FluoView FV1000/FV1200/FV3000 confocal  
282 microscope and the 488 nm (for GFP) and 587 nm (for mCherry) in Leica TCS SP8 confocal  
283 microscope were employed, and 488 nm and 651 nm on the Zeiss NLO710, respectively.



284

## 285 **Image analysis**

286 Image quantification was performed using z-stacks in ImageJ 2.0.0 (Schindelin et al., 2012),  
287 Olympus Fluoview (FV10-ASW, FV31S-SW), or IMARISx64 9.5.1 software (Bitplane). Total  
288 LEC number was counted manually using the overlay of DsRed and GFP channels over five  
289 somites in the trunk. Lymphatic vessel area was calculated by rendering the surface using  
290 DsRed channel, the non- lymphatic structures were manually removed. Measurements of  
291 surface area were exported directly from Imaris (Bitplane).

292

## 293 **Cell tracking**

294 To quantify the migrating distance the centre of PL nuclei in figure 3G and figure 4D was  
295 manually tracked until either cell dying or disappearing from the view in Imaris (Bitplane). The  
296 individual cell track was generated by “spot” and “cell track” function and then manually edited  
297 if needed. The data was exported to GraphPad for plotting and statistical analysis.

298

## 299 **Distance measurement**

300 The migrating distance in all figures were measured in three-dimension using spot function in  
301 Imaris (Bitplane), see cell tracking. In figure 3B-C, the sprouting front of PL just migrating out  
302 of HM was chosen as start point (T0) and the migrating front at the end of the time lapse video  
303 was chosen as end point (T1). A direct line was used to connect the dots and the length of  
304 line segment is measured. In figure 2H, the perpendicular distance between point T1 and HM  
305 was also measured in addition to the measurement above.

306

## 307 **Chemical treatment**

308 To inhibit Cxcr4 signalling, the embryos were treated in 20  $\mu$ M antagonist ADM3100 (Merk)  
309 from 51 hpf to 72 hpf. To block phosphorylation and activation of ERK1/2, embryos were  
310 treated in 10  $\mu$ M SL327 (EMD Millipore) from 51 hpf to 72 hpf. Embryos were anesthetized  
311 and mounted in 1% low-melting agarose in a 2-well slide with separate chambers which allows  
312 . The prepared chemical solution (3 ml) was added on top of the agarose layer in one chamber  
313 and the E3 water or DMSO (3 ml) to the other.

314

## 315 **Fluorescence Activated Cell Sorting (FACS) and qPCR analysis**

316 Embryos of *Tg(flt1:YFP);Tg(-5.2lyve1b:DsRed2)* and *Tg(flt1:YFP);Tg(-5.2lyve1b:DsRed2)*  
317 were collected at 3 dpf and screened as described in figure 3D, dissociation was performed  
318 as previously described (Kartopawiro et al., 2014). The dissociated cells were sorted using a  
319 FACS Aria III (BD Biosciences) into 300 $\mu$ l TRIzol™ LS Reagent (Thermo Fisher). Total RNA  
320 was extracted using the Quick-RNA Microprep kit (Cambridge Bioscience) following the

321 manufacturer's instructions. RNA quality and concentration were determined using 2100  
 322 Bioanalyser Instrument (Agilent) together with Bioanalyser High Sensitivity RNA Analysis Kit  
 323 (Agilent). 1 ng of RNA template was subjected to cDNA synthesis using SuperScript™ VILO™  
 324 cDNA Synthesis Kit (Thermo Fisher). qPCR analysis was performed using the primers in Table  
 325 S1 on CFX384 Touch Real-Time PCR Detection System (BioRad). Data were analysed using  
 326 the CFX Maestro Software (BioRad). The geometric average of *rpl13* and *β-actin* expression  
 327 was used as reference to calculate relative gene expression of target genes with the ddCT  
 328 method. Primer sequences listed below in Table 1.  
 329

Target gene	Forward primer Sequence 5'-3'	Forward primer Sequence 5'-3'	Reference
<i>β-actin</i>	CGAGCTGTCTTCCCATCCA	TCACCAACGTAGCTGTCTTT	Designed for this study
<i>rpl13</i>	CATCTCTGTTGACTCACGTC G	CATCTTGAGCTCCTCCTCAGTA C	Designed for this study
<i>cxcr4a</i>	CATGACAGACAAGTACCGT CT	TGCTGTACAAGTTTACCGTGTA	qPrimerDB
<i>cxcr4b</i>	TGCTAACATTCTGATAAGA CC	GTACTTTTATTGCCAGACCTAAA GG	qPrimerDB
<i>cxcl12a</i>	GCAAGTGCTTTGACACAAAA AG	TTTGTGGCAAAGTAACCCTG	qPrimerDB
<i>cxcl12b</i>	GATCGTGATAGCTTTGTGAA CC	AATGTTAACAATGCTTGGCCTC	qPrimerDB
<i>vegfc</i>	TCTTAAAAGGGAGACGGTTT CA	TACATTTCTTCTCTTGGGGTC	qPrimerDB
<i>abcc9</i>	AAAGTTATGGAAGTTTGCCG AG	AAAGTTATGGAAGTTTGCCGAG	qPrimerDB
<i>pdgfrb</i>	CGTTCCAGGAGCCTTTTCT	TTGGGATCAGGGATGGGGAT	(Ando <i>et al.</i> , 2019)

330

### 331 Ablation experiments

332 Ablation with multi-photon microscopy

333 For mural cells ablation, embryos of *Tg(-5.2lyve1b:DsRed2);Tg(pdgfrb:GFP)* were laterally  
 334 mounted in 1% low melting-point agarose at 57 hpf. An aSV with migrating LEC was chosen  
 335 randomly, a GFP-positive MC were identified using 488-nm laser. MC locating ahead of  
 336 migrating route were ablated using a two-photon laser at 790 nm (Mai Tai, Spectr-Physics

337 Millenia PRO). Control ablations were performed as above but the adjacent area to the pdgfrb+  
338 cell targeted with the two-photon laser. For aISV ablation, *Tg(-*  
339 *5.2lyve1b:DsRed2);Tg(flt:YFP);Tg(pdgfrb:GFP)* embryos were prepared as described above  
340 and an aISV, with LEC migrating along, was ablated using the two-photon laser targeting the  
341 nuclei of aEC. Larvae were imaged before and after ablation with a Zeiss LSM 710 FCS  
342 confocal microscope, which was followed by either time-lapse imaging for around 5 h or follow-  
343 up z-stack imaging at 3 dpf.

344

#### 345 Ablation with NTR-Mtz system

346 Embryos from *Tg(Pdgfrb:Gal4FF,UAS:NTR-mCherry);Tg(fli1a:GFP)* were collected and  
347 screened as described in figure 2A. Embryos both positive and negative for *mCherry* were  
348 treated with medium mixture of E3 water and either 5mM MTZ or DMSO from 48 hpf, and  
349 replaced daily with fresh medium mixture. The region above the yolk extension was imaged  
350 at 120 hpf. The formation of thoracic duct was analyzed by scoring the extend of the thoracic  
351 duct formation.

352

#### 353 **Statistical analysis**

354 Statistical analysis was performed using Prism software (GraphPad). Gaussian distribution of  
355 samples was tested with either D'Agostino–Pearson test or Shapiro-Wilk normality test.  
356 Student's t test was used for comparison of two means. One-way ANOVA with post hoc test  
357 was used for multiple comparison as stated in corresponding figure legend.

358

#### 359 **Acknowledgment**

360 This work was supported by Wallenberg Academy Fellowship (2017.0144), Ragnar Söderbergs  
361 Fellowship (M13/17), Vetenskapsrådet (VR-MH-2016-01437) and Jeansson's Foundation. The  
362 SciLifeLab Zebrafish facility in Uppsala hosted zebrafish. FACS was performed at BioVis at Uppsala  
363 University. We are grateful to A. Chiba, H. Nakajima, S. Yuge, T. Babazono, W. Koeda, K. Hiratomi, M.  
364 Sone, E. Nakamura, K. Kato, and H. Ichimiya for technical assistance.

365

366

#### 367 **Author contribution**

368 D.P, K.A. and K.K, conceptualised the project, performed, analysed experiments and co-wrote the  
369 manuscript. R.S. and M.G. performed and analysed experiments. N. M, C. B., and S.F unpublished  
370 reagents and resources contribution.

371

372

#### 373 **Figure Legends**

374 **Figure 1. *pdgfrb*<sup>high</sup> mural cells emerge around aISVs prior to LEC migration and provide**  
375 **guidance.**

376 (A) Confocal stack image of trunk aISV in 4 dpf *Tg(-5.2lyve1b:DsRed);Tg(flt1:YFP);*  
377 *TgBAC(pdgfrb:GFP)* of lymphatic endothelial cells (gray, LEC), arterial intersegmental vessels  
378 (magenta, aISV) and MCs (green, MC). Scale bar; 10  $\mu$ m.

379 (B) Confocal stack images from time-lapse images in the trunk of 2 dpf *Tg(lyve1b:*  
380 *mCherry);Tg(kdrl:TagBFP);TgBAC(pdgfrb:GFP)* embryo. Boxed regions are enlarged in the  
381 bottom.

382 Arrowheads indicate *pdgfrb*<sup>+</sup> MCs (green) around aISV (magenta) prior to (bottom left) and  
383 during (bottom middle and right) LEC (gray) migration. Scale bars; 50  $\mu$ m or 30  $\mu$ m (enlarged  
384 image).

385 (C) Quantification of aISV with (n=10) or without (n=3) MCs presence when LECs left HM for  
386 time lapse videos as in (E).

387 (D) Quantification of MC number around aISV (n=13) at the start and end of the migration for  
388 time lapse videos as in (E).

389 (E) Confocal stack images from time lapse of LEC migration.  
390 *TgBAC(pdgfrb:GAL4FF);(UAS:GFP)* in green and *Tg(-5.2lyve1b:DsRed2)* in grey. Scale bar;  
391 50  $\mu$ m.

392 (F) Quantification of LEC and MC interaction during migration. Migrating following MC n=21,  
393 migrating not following MC n=3.

394 (G) Quantification of duration of LEC migration with (n=5) or without (n=3) contacting MCs.  
395 Data are presented as mean  $\pm$  SEM. \*\*p<0.005

396 (H) Confocal stack images of *Tg(pdgfrb:GAL4FF); Tg(UAS:GFP)* (green) and *Tg(-*  
397 *5.2lyve1b:DsRed2)* (grey) in the trunk of sibling (top) and *pdgfrb*<sup>UM148</sup> mutant (bottom) embryos  
398 at 5 dpf. Lymphatic structure is rendered with *lyve* channel in IMARIS (right panel). Scale bar;  
399 100  $\mu$ m.

400 (I) Quantification of number of *pdgfrb*<sup>+</sup> mural cells around ISVs in siblings (n=20) and  
401 *pdgfrb*<sup>UM148</sup> mutant (n=10). Data are presented as mean  $\pm$  SEM, unpaired two-tailed Student's  
402 t-test was used. \*\*\*p<0.0001.

403 (J) Quantification of surface area of lymphatic vasculature in siblings (n=20) and *pdgfrb*<sup>UM148</sup>  
404 mutant (n=10). Data are presented as mean  $\pm$  SEM, unpaired two-tailed Student's t-test was  
405 used. \*p<0.05.

406

407 **Figure 2. Mural cells are required for robust formation of lymphatic vascular bed.**

408 (A) Work flow of cell ablation by NTR-MTZ system. *Tg(pdgfrb:Gal4FF);Tg(14xUAS:3xFLAG-*  
409 *NTR,NLS-mCherry)* (red) and *Tg(fli1a:GFP)* (grey) were imaged at 120 hpf after treatment of  
410 DMSO or 5mM MTZ from 48 hpf, and the formation of thoracic duct (TD) was analyzed.  
411 (B) Confocal stack images of the trunk in 5 dpf embryos treated as described in (A). Arrows  
412 indicates TD forming beneath dorsal aorta. Asterisks indicates the absence of TD. Scale bar;  
413 100  $\mu\text{m}$ .  
414 (C) Quantification of (B). Embryos were scored as fully, partially and hardly formed based on  
415 the TD development. Data were presented as ratio to total number of embryos analyzed.  
416 (D) Work flow of cell ablation by multi-photon microscopy. Mural cells (MC, green) labelled by  
417 *TgBAC(pdgfrb:GAL4FF);(UAS:GFP)* and PL by *-5.2lyve1b:DsRed2* (grey) embryo. The MC  
418 on intersegmental vessels in proximity to PL were ablated at 57 hpf. For analysis, ablation was  
419 either followed by time-lapse imaging or confocal imaging at 3 dpf.  
420 (E) Confocal stack images of before and after ablation (top two row) and follow-up at 3 dpf  
421 (bottom two row). Control ablation (dashed box) at the adjacent region of GFP<sup>+</sup> MCs and GFP+  
422 MC on aISV ablation (solid grey box) was performed in the same embryos. Arrowheads  
423 indicate ablated GFP-positive cells and development of targeted LEC at 3 dpf. Scale bar; 100  
424  $\mu\text{m}$ . Middle and right panel, zoom-in images cropped in z-stacked.  
425 (F) Live imaging of the lymphatic endothelial cells (LEC) migration in the context of control  
426 (top images) and GFP+ MC on aISV (bottom images) ablation, confocal stack images from  
427 time lapse at selected timepoints from 0 hours post ablation (hpa) to 4.96 (hpa). Scale bar; 50  
428  $\mu\text{m}$ .  
429 (G) Quantification of ablated LEC forming DLLV at 3 dpf. DLLV forming (n=3), not forming  
430 (n=2).  
431 (H) Quantification of migrating distance from time-lapse videos corresponding in (D). Distance  
432 was calculated as both T0-T1 and the perpendicular distance between the T1 and HM for  
433 embryos with (ablated, n=4) or without (control, n=3) ablation. T0, the sprouting front of LEC  
434 at the start of video; T1, sprouting front of LEC at the end of video. Data are presented as  
435 mean  $\pm$  SEM, unpaired two-tailed Student's t-test was used on two types of measurements  
436 respectively. \*p<0.05.

437

### 438 **Figure 3. Chemokines are expressed in ISV-mural cells during LEC migration**

439 (A) Confocal stack images from time lapse post multi-photon laser ablation in 2 dpf  
440 *Tg(flt1:YFP)* (magenta); *TgBAC(pdgfrb:GFP)* (green) and *Tg(-5.2lyve1b:DsRed2)* (grey).  
441 Arrowheads indicate remained GFP<sup>+</sup> mural cells without aISV. White arrows indicate the  
442 ablated site of aISV. Scale bar; 50  $\mu\text{m}$ .

443 (B-C) Quantification of LEC (n=4) and aISV (n=4) migration distance post two-photon laser  
444 ablation.

445 (D) Illustration of FACS sorting and qPCR analysis on 3 dpf embryos.

446 (E) qRT-qPCR of *cxcr4a*, *cxcr4b*, *cxcl12a* and *cxcl12b* in FACS sorted population cells at 3  
447 dpf. Graph represents gene expression relative to geometric average of *rpl13* and  *$\beta$ -actin* from  
448 three biological repeats (mean  $\pm$  SEM). Kruskal-Wallis test with Dunn's post hoc test was  
449 used. No significance (ns),  $p \geq 0.9999$ .

450 (F) Work flow of Cxcr4 inhibitor treatment. Embryos of *Tg(fli1:GFP);Tg(lyve1b:mCherry)* was  
451 grown in PTU from 24 hpf, then changed to 20  $\mu$ M ADM3100 or E3 water at 51 hpf. Time-  
452 lapse imaging was started then in a two-well slide that allows spontaneous imaging for both  
453 groups.

454 (G) Confocal stack images from time lapse imaging indicated in (F). Scale bar; 50  $\mu$ m.

455 (H) Quantification of dorso-ventral migration showing individual tracks for sprouting front of  
456 PLs in E3 water (n=10) and AMD3100 (n=25) treated embryos as described in (G).

457 (I) Quantification of dorso-ventral migration showing average (mean) of tracks in DMSO and  
458 AMD3100 treated group. Data are presented as mean  $\pm$  SEM, unpaired two-tailed Student's  
459 t-test was used. \*\* $p < 0.005$ .

460 (J) Quantification of velocity of dorso-ventral migration from time lapse video described in (3F).  
461 Nuclei of PLs in E3 water (n=10) and AMD3100 (n=25) treated embryos were tracked and the  
462 distance between begin and end position of nuclei was measured then divided by duration.  
463 Data are presented as mean  $\pm$  SEM. unpaired two-tailed Student's t-test was used.

464 (K) Quantification of frequency of filopodia formation from time lapse video described in (3F).  
465 (left) Number of protrusions other than the sprouting front was counted and divided by the  
466 corresponding duration in E3 (n=12) and AMD3100 (n=20) treated embryos. Data are  
467 presented as mean  $\pm$  SEM. unpaired two-tailed Student's t-test was used. (right) Number of  
468 protrusions other than the sprouting front was counted and divided by the corresponding  
469 duration in E3 (n=12) and AMD3100 (n=20) treated embryos. Data are presented as mean  $\pm$   
470 SEM. unpaired two-tailed Student's t-test was used.

471

#### 472 **Figure 4. Mural cell and arterial derived *vegfc* promotes LEC migration**

473 (A) qRT-qPCR of *vegfc* expression in trunk ECs and MCs cells at 3 dpf as described in (3E).  
474 Graph represents gene expression relative to geometric average of *rpl13* and  *$\beta$ -actin* from  
475 three biological repeats (mean  $\pm$  SEM). Kruskal-Wallis test with Dunn's post hoc test was  
476 used. \* $p < 0.05$

477 (B) Endogenous pErk (cyan, right) in migrating LEC in trunk of *Tg(-5.2lyve1b:venus)* embryos  
478 ( $\alpha$ -GFP, grey, middle) at 3 dpf. Scale bar 100  $\mu$ m; 50  $\mu$ m in enlarged images.

479 (C) Work flow of *vegfc* inhibitor treatment. Embryos of *Tg(fli1:GFP);Tg(lyve1b:mCherry)* was  
480 grown in PTU from 24 hpf, then changed to 10  $\mu$ M SL327 or DMSO at 51 hpf. Time-lapse  
481 imaging was started at 57 hpf.

482 (D) Confocal stack images from time lapse of 57 hpf *Tg(fli1a:nEGFP)<sup>y7</sup>* (green) and *Tg(-*  
483 *5.2lyve1b:DsRed2)* (grey) treated with DMSO or 10  $\mu$ M SL327 from 51 hpf. Grey arrowheads  
484 indicate cell death. Scale bar in 50  $\mu$ m.

485 (E) Quantification of dorso-ventral migration showing individual cell tracks for nuclei of PLs  
486 (top panel) in DMSO (n=11) and SL327 (n=12) treated embryos as described in (4C). Red  
487 cross indicates cell death at the end of tracking. Bottom panel, average (mean) of tracks in  
488 DMSO and SL327 treated group. Data are presented as mean  $\pm$  SEM, unpaired two-tailed  
489 Student's t-test was used. \*\*\*\*p<0.0001

490 (F) Quantification of cell proliferation in DMSO (n=14) and SL327 treated (n=17) embryos as  
491 described in (4C). Nuclei marker in green was used to count the cell division event.

492 (G) Quantification of total LEC number at beginning (T0) and end (T1) of the time lapse in  
493 DMSO (n=10) and SL327 (n=9) treated embryos as described in (C), data are presented as  
494 mean  $\pm$  SEM. T0 DMSO vs T1 SL327 p<0.0001, T0 SL327 vs T1 SL327, T1 DMSO vs T1  
495 SL327 p<0.0001. Other comparisons were ns, p $\geq$ 0.9999. One-way ANOVA with Tukey's post  
496 hoc test for statistical analysis. \*\*\*\*p<0.0001

497 (H) Confocal stack image of embryos treated with DMSO or SL327 as described in (4C).  
498 Embryos were fixed at 3 dpf and used for TUNEL (cyan) and  $\alpha$ -DsRed staining (grey). Zoom-  
499 in single z stack images of TUNEL staining in merged and TUNEL channel showing the  
500 colocalization of the signal. Scale bar; 100  $\mu$ m, 30  $\mu$ m (enlarged images).

501 (I) Corresponding quantification of TUNEL signal from (I). In SL327 treated group (n=45) LEC  
502 showed a positive TUNEL staining rate of 15.56% while it was 0% in DMSO treated group  
503 (n=45).

504 (J) A model of LEC migration in zebrafish trunk. Signals promoting cell survival and migration  
505 expressed in MCs include *vegfc*, *cxcl12a* and *cxcl12bat* at 3 dpf (top). In absence of the arterial  
506 mural cells, LECs fail to establish a robust lymphatic network at 5 dpf (bottom panel).

507

508 **Supplementary Figure 1, related to Figure 1; *pdgfrb*<sup>high</sup> mural cells emerge around**  
509 **aISVs prior to LEC migration and provide guidance.**

510 (A) Confocal stack images from time-lapse images in the trunk of  
511 *Tg(dab2:GALFF);Tg(UAS:GFP)* in grey and *Tg(pdgfrb:mCherry)* in green. White Arrow heads,  
512 MCs appear around aISV. Arrow, sprouting front of migrating LEC. Scale bar; 50  $\mu$ m.

513 (B) Confocal stack images of 5 dpf *Tg(fli1a:nEGFP);(-5.2lyve1b:DsRed2)* treated with 20  $\mu$ M  
514 PDGFR inhibitor AG1296 (n=15) or DMSO (n=15) from 48 hpf. Data are presented as mean  
515  $\pm$  SEM, unpaired two-tailed Student's t-test was used. \*\*\*p<0.0005.

516

517 **Supplementary Figure 2, related to Figure 2; Mural cells are required for robust**  
518 **formation of the lymphatic vascular bed.**

519 (A) Representative confocal stack images of 5 dpf  
520 *TgBAC(pdgfrb:Gal4FF);Tg(14xUAS:3xFlag-NTR, NLS-mCherry);Tg(fli1:GFP)* with high and  
521 low NTR expression treated with 5 mM MTZ or vehicle for 16 h. NTR expression was highly  
522 selective on MCs but not on *pdgfrb*-low population. MCs were ablated after 16 h of 5 mM MTZ  
523 treatment. Arrows indicate floorplate and hyperchord were not ablated by the treatment  
524 despite the expression of NTR. Scale bar; 100  $\mu$ m

525 (B) Confocal stack images from *Tg(fli1a:Myr-GFP)* in control and ablated embryos described  
526 in Figure 2A post injection of Qtracker 705 vascular labels (shown in grey) into common  
527 cardinal vein. Arrows indicate lymphatic vessels labeled by leaked dye when injected or during  
528 the circulation in the control but not in the MC-ablated larva. Scale bar; 100  $\mu$ m

529

530 **Supplementary Figure 3, related to Figure 3; Chemokines are expressed in ISV-mural**  
531 **cells during LEC migration.**

532 (A) Gating strategy for FACS sort of *Tg(flt1:YFP);Tg(-5.2lyve1b:DsRed2)* and *Tg(abcc9:GFP);*  
533 *Tg(kdrl:DsRed2)* as described in Figure 3D. Sorting was performed on all singlet, alive cells  
534 according to their expression of DsRed (red, 561 nm) and GFP/ YFP (green, 488 nm).

535 (B) RT-qPCR of *abcc9* and *pdgfrb* expression in trunk ECs and MCs cells at 3 dpf as described  
536 in (3E). Graph represents gene expression relative to gematric average of *rpl13* and  $\beta$ -*actin*  
537 from three biological repeats (mean  $\pm$  SEM). Kruskal-Wallis test with Dunn's post hoc test was  
538 used. No significance (ns),  $p \geq 0.9999$ .

539 (C) Confocal stack images of embryos as described in Figure 3F treated with 20  $\mu$ M ADM3100  
540 or E3 water from 51 hpf to 120 hpf. Scale bar; 100  $\mu$ m.

541

542 **Supplementary Figure 4, related to Figure 4; Mural cell and arterial derived *vegfc***  
543 **promotes lymphatic endothelial cell migration and survival**

544 (A) Confocal stack images of embryos as described in Figure 4C treated with 4  $\mu$ M SL327 or  
545 DMSO from 51 hpf to 120 hpf. Scale bar; 100  $\mu$ m.

546

547 **Supplementary Movies S1 related to Figure 1.**



548 Confocal time lapse imaging in trunk in 2 dpf *Tg(lyve1b: mCherry)*, *Tg(kdrl:TagBFP)*,  
549 *TgBAC(pdgfrb:GFP)* embryos corresponding in Figure 1B

550

551 **Supplementary Movies S2 related to Figure 1.**

552 Confocal time lapse imaging in trunk in 2 dpf *TgBAC(pdgfrb:GAL4FF);(UAS:GFP)*, *Tg(-*  
553 *5.2lyve1b:DsRed2)* embryos corresponding in Figure 1E

554

555 **Supplementary Movies S3-4 related to Figure 1.**

556 Representative confocal time lapse imaging of LEC migrating with or without contacting MCs  
557 in trunk in 2 dpf embryos corresponding Figure 1G.

558

559 **Supplementary Movies S5-6 related to Figure 2.**

560 Representative confocal time lapse imaging of multi-photon ablation of MCs in  
561 *TgBAC(pdgfrb:GAL4FF);(UAS:GFP)*, *Tg(-5.2lyve1b:DsRed2)*, corresponding Figure 2F.

562

563 **Supplementary Movies S7 related to Figure 3.**

564 Representative confocal time lapse imaging of multi-photon ablation of aISV in *Tg(flt1:YFP)*;  
565 *TgBAC(pdgfrb:GFP)*; *Tg(-5.2lyve1b:DsRed2)*, corresponding Figure 3A.

566

567 **Supplementary Movies S8-11 related to Figure 3.**

568 Representative confocal time lapse imaging of *Tg(fli1:GFP)*; *Tg(lyve1b:mCherry)* treated with  
569 20  $\mu$ M ADM3100 or E3 water at from 51 hpf, corresponding Figure 3G.

570

571 **Supplementary Movies S12-15 related to Figure 3.**

572 Representative confocal time lapse imaging of *Tg(fli1a:nEGFP)<sup>zy</sup>*; *Tg(-5.2lyve1b:DsRed2)*  
573 treated with 10  $\mu$ M SL327 or DMSO from 51 hpf. Grey arrowheads indicate cell death.

574

575

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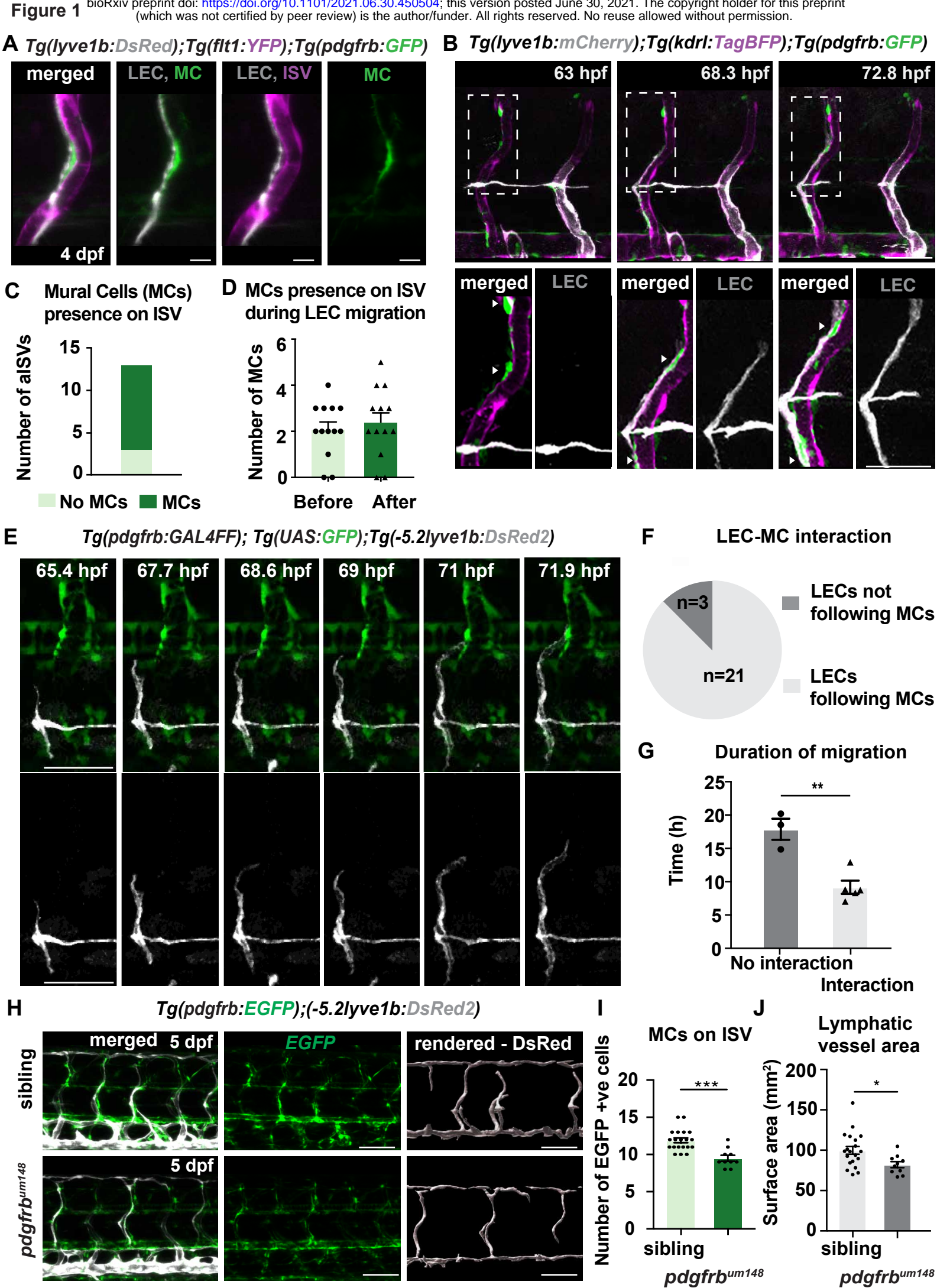
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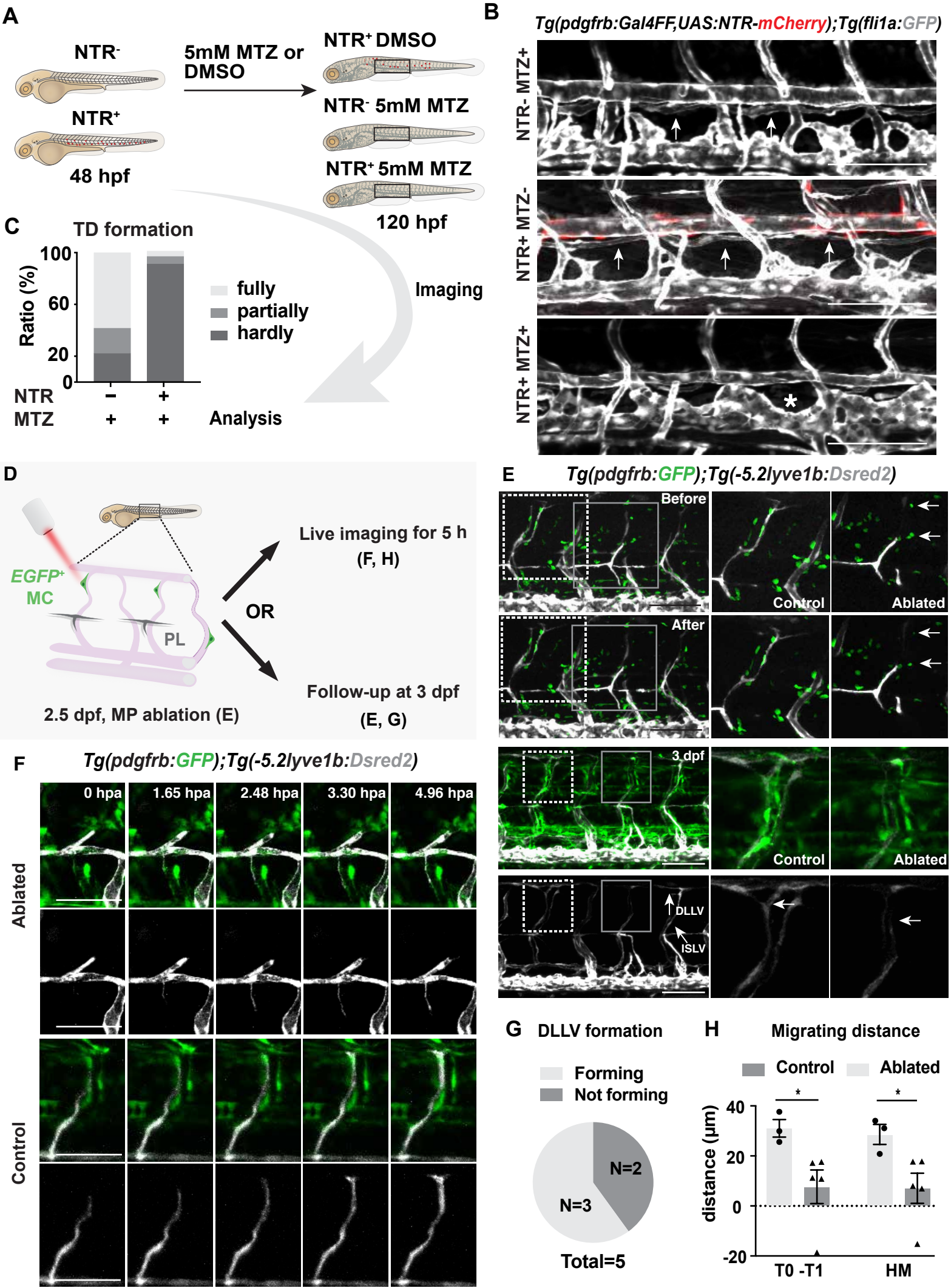
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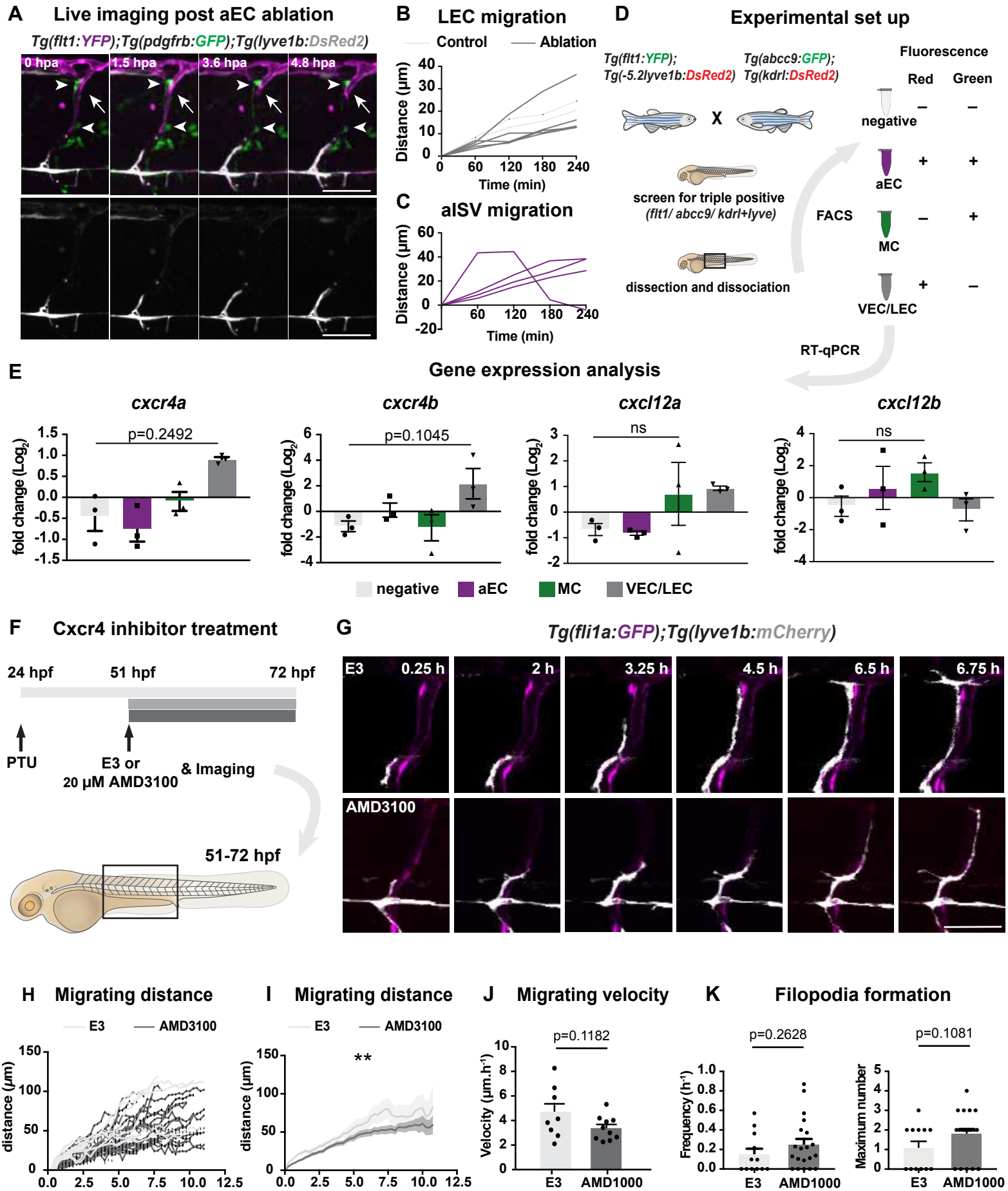
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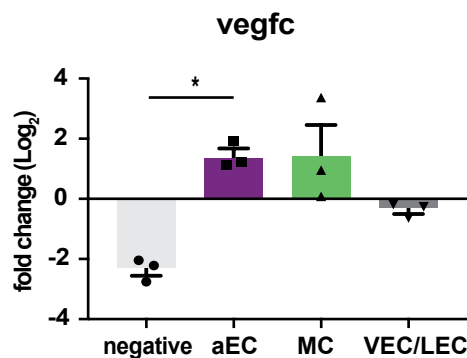




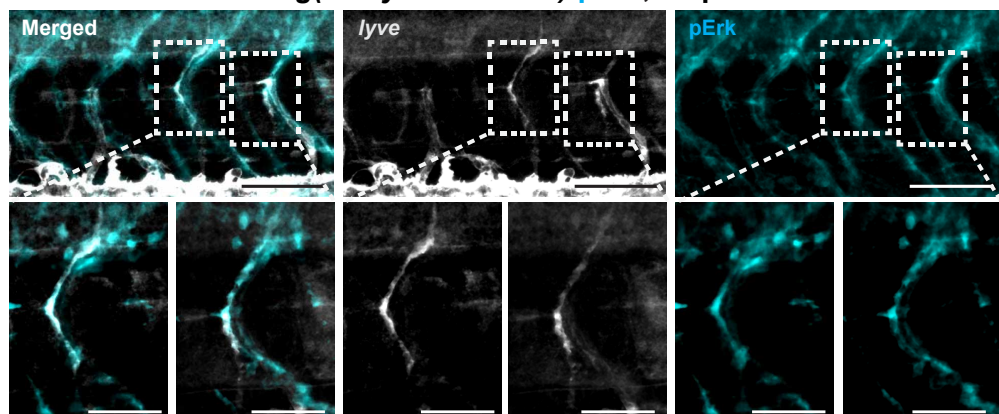
**Figure 3**



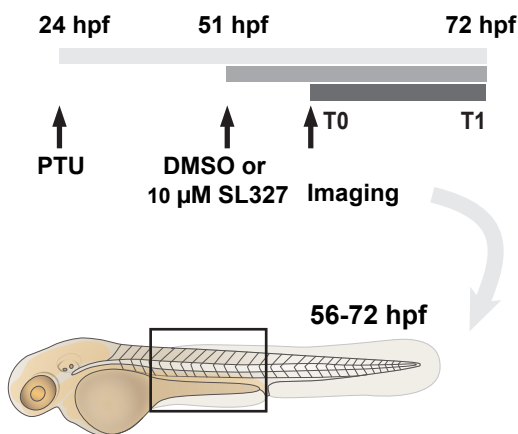
### A Gene expression analysis



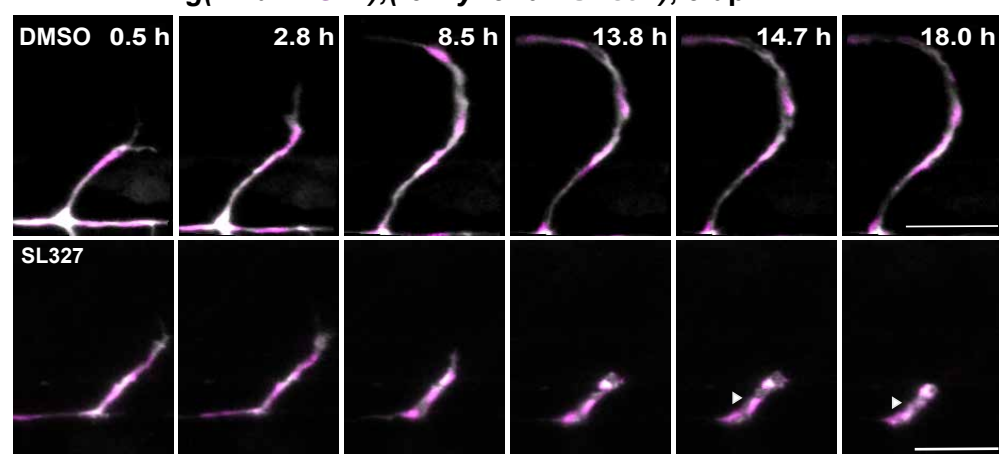
### B *Tg(-5.2lyve1b:venus)* pErk, 3 dpf



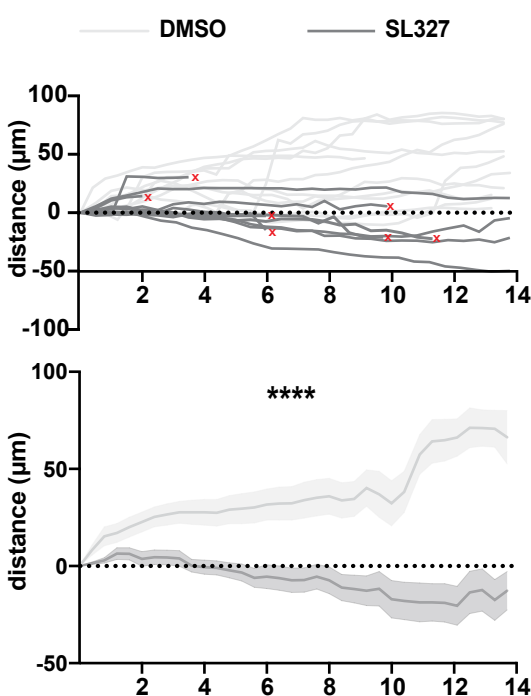
### C MEK inhibitor treatment



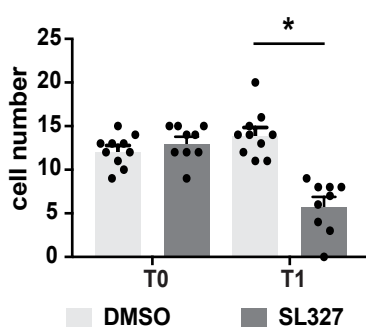
### D *Tg(fli1a:nEGFP);(-5.2lyve1b:DsRed2)*, 3 dpf



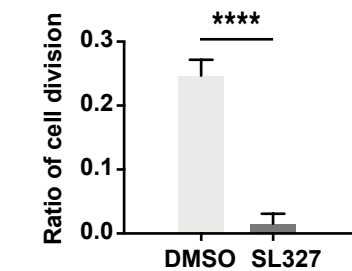
### E Migrating distance



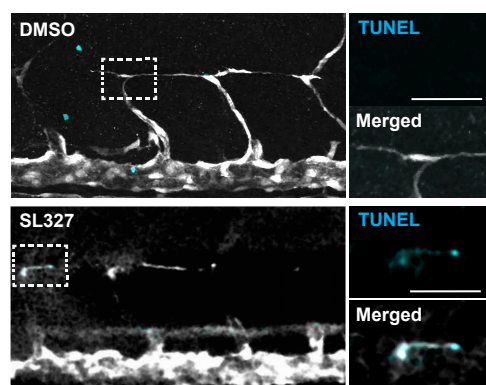
### F LEC number



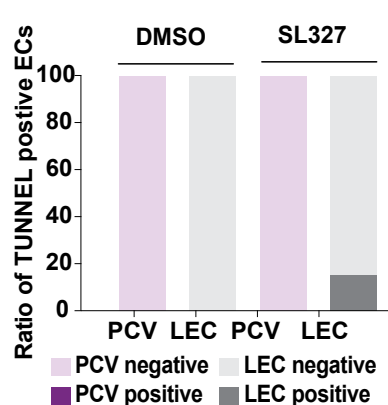
### G Proliferation



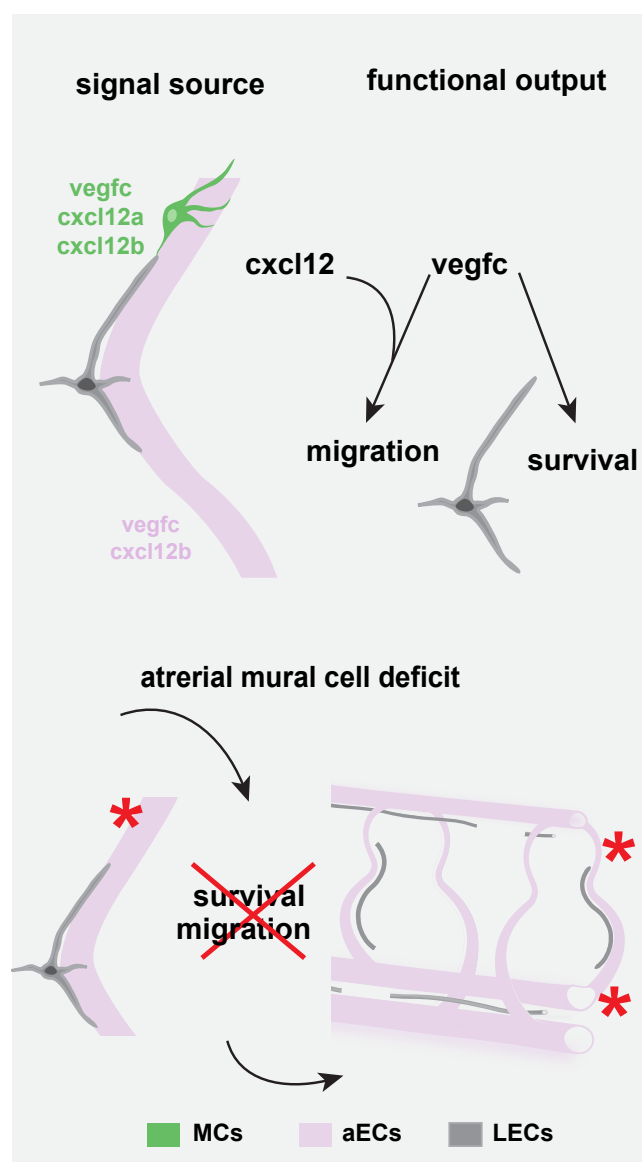
### H *Tg(-5.2lyve1b:DsRed2)* TUNEL



### I LEC death

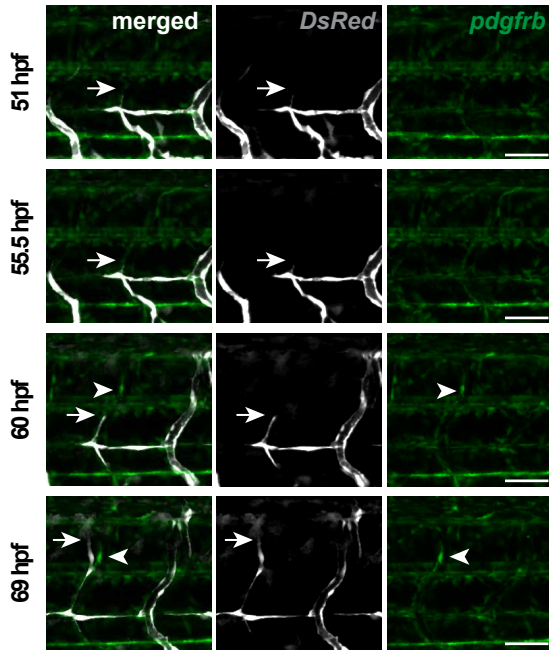


### J LEC migration

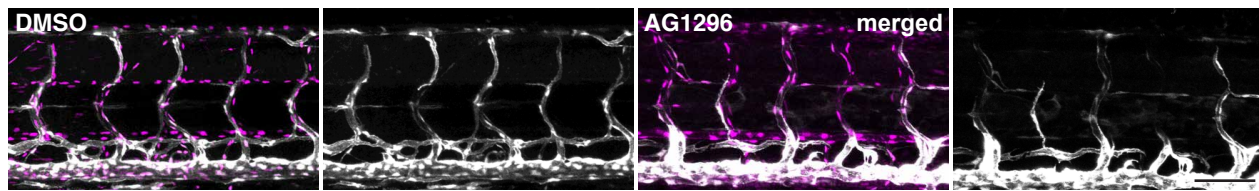




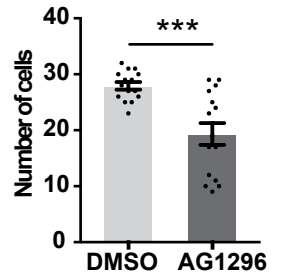
**A** *Tg(dab2:GALFF);Tg(UAS:GFP);Tg(pdgfrb:mCherry)*



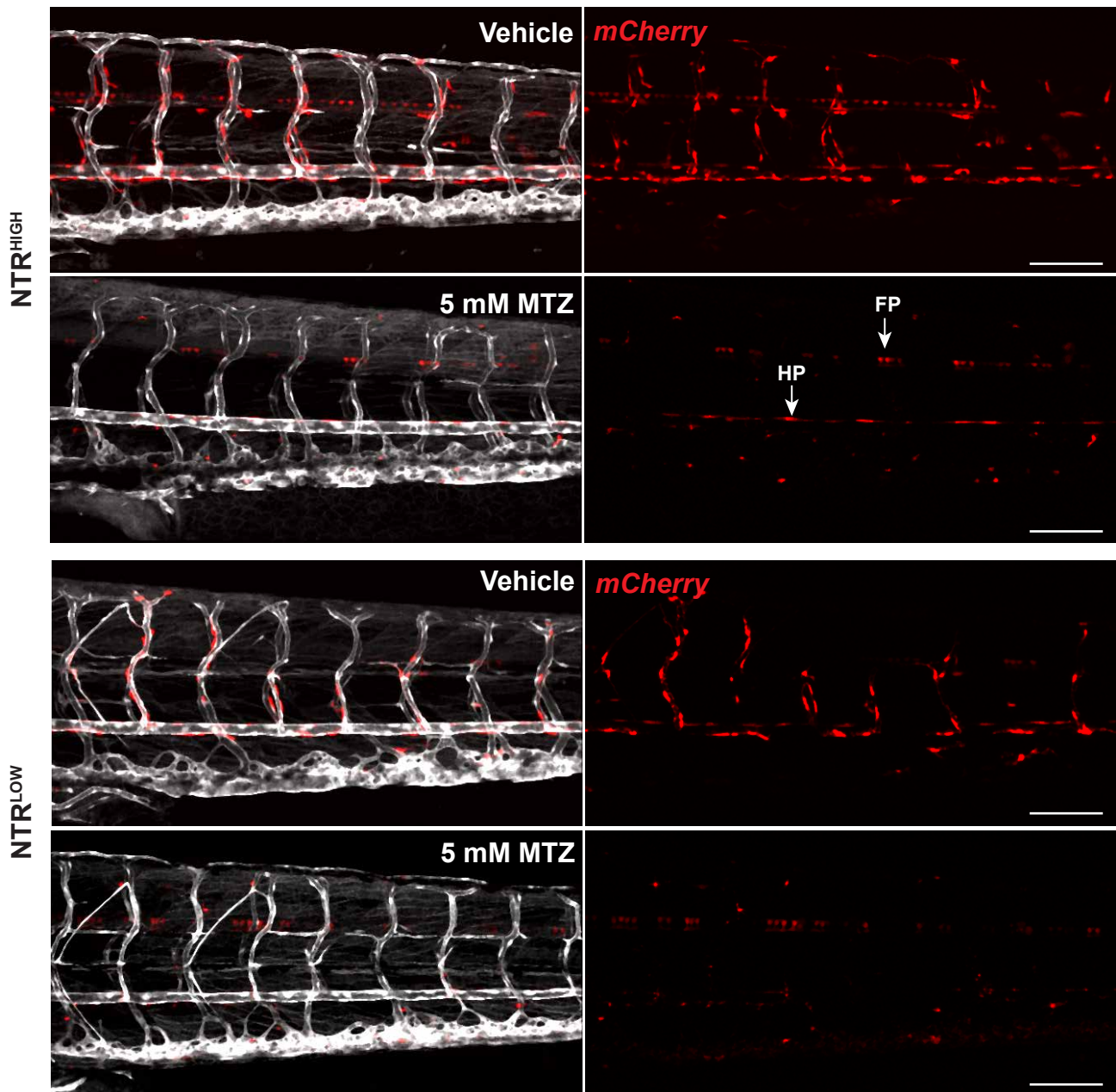
**B** *Tg(fli1a:nEGFP)<sup>y7</sup>;(-5.2lyve1b:DsRed2)*, 5 dpf



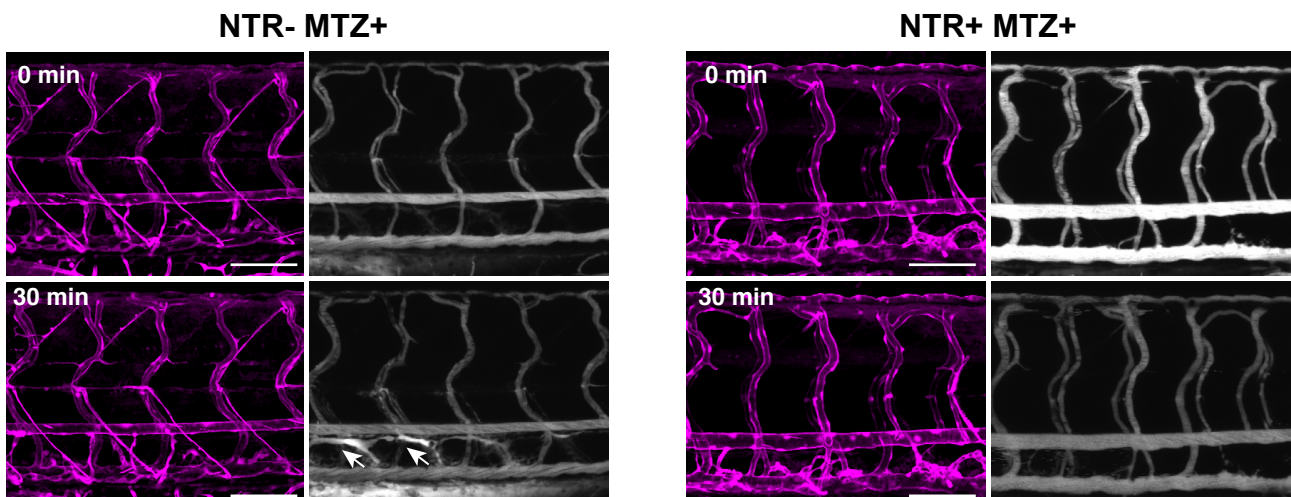
**C** Total LEC number



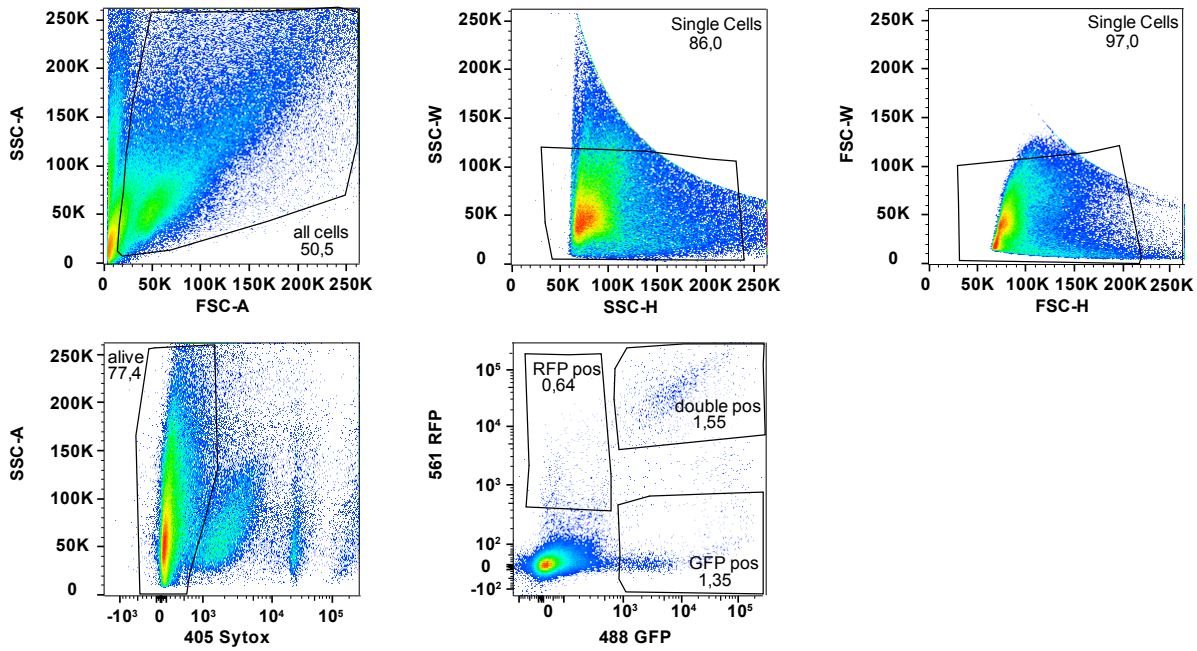
**A** *TgBAC(pdgfrb:Gal4FF);Tg(14xUAS:3xFlag-NTR, NLS-mCherry);Tg(fli1:GFP)*, 5 dpf



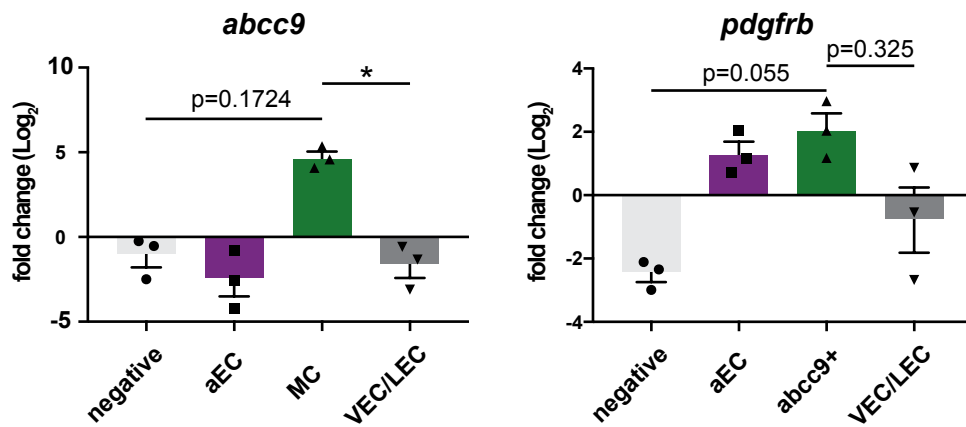
**B** *Tg(fli1a:Myr-GFP) Qtracker 705*



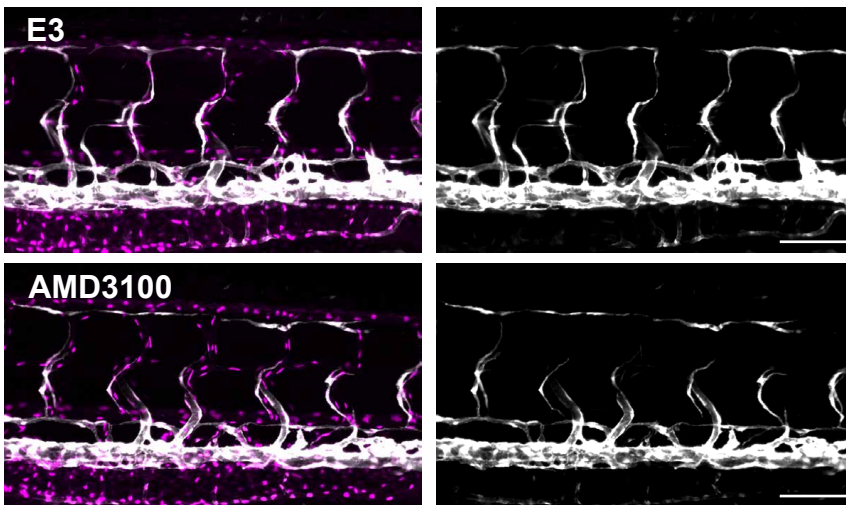
**A** **Gating for cell sorting**



**B** **qPCR analysis on FACS sorted cells**



**C** ***Tg(fli1a:nEGFP);(-5.2lyve1b:DsRed2)*, 5 dpf**



**A** *Tg(fli1a:nEGFP);(-5.2lyve1b:DsRed2)*, 5 dpf

