

Supplementary Fig. 1. Zebrafish facial lymphatic development

Confocal images of the developing facial lymphatics in *lyve1:egfp;kdrl:rfp* or *lyve1:dsred;kdrl:egfp* embryos at (A) 2 dpf (B) 3 dpf and (C) 5 dpf with *lyve1* in green and *kdrl* in magenta with diagrams highlighting in red arteries that only express *kdrl* (AA), in orange veins that express both *kdrl* and *lyve1* (PHS, CCV, and PCeV) and in green developing lymphatic vessels that only express *lyve1* (FLS, LFL, MFL, OLV and LAA) at each stage. (A-C) Confocal images showing *kdrl*-expressing vessels. (A'-C') Confocal images showing the *lyve1*-expressing vessels. (A''-C'') Confocal images of *kdrl* and *lyve1* expression. CCV – common cardinal vein, PHS – primary head sinus, AA – branchial arteries, PCeV – posterior cerebral vein, FLS – facial lymphatic sprout, LFL – lateral facial lymphatic, OLV – otolithic lymphatic vessel, MFL – medial facial lymphatic and LAA – lymphatic branchial arches. Scale bars: 50 µm.

Supplementary Fig. 2. *flt4*, *ccbe1* and *vegfc* are required for secondary sprouting from the PCV

(A-D) Confocal images of 1.5 dpf *lyve1:egfp* embryos treated with (A) control MO (B) *flt4* MO (C) *ccbe1* MO (D) *vegfc* MO-1. Secondary sprouts from the PCV (arrowheads) are only present in embryos injected with control MO. (E) Quantitation of secondary sprout formation at 1.5 dpf in embryos injected with morpholinos. *** p<0.001, by a Mann-Whitney test to control; error bars indicate s.d. Scale bar: 50 µm.

Supplementary Fig. 3. Microangiography and lymphangiography

(A) Confocal images of 2 dpf *lyve1:egfp* embryos injected with fluorescent beads into the bloodstream to highlight blood flow. Vessels that express *lyve1* (green) and contain blood flow (magenta) are veins (PHS, CCV and PCeV) while blood-containing vessels that do not express *lyve1* are arteries (LDA). n=3 for control and *flt4* morphants (B) Confocal images of 5 dpf *lyve1:dsred* embryos injected with fluorescein dextran to highlight functional lymphatics (lymphangiography). Data is representative of n=3 for wild type and n=4 for *vegfc^{hu5055}*. The *vegfc^{hu5055}* mutant has a reduction in functional lymphatics at 5 dpf. CCV – common cardinal vein, PHS – primary head sinus, PCeV – posterior cerebral vein, LDA – lateral dorsal aorta, FLS – facial lymphatic sprout, LFL – lateral facial lymphatic, OLV – otolithic lymphatic vessel, MFL – medial facial lymphatic. Scale bars: 50 μ m.

Supplementary Fig. 4. Validation of *vegfc* morpholinos

RT-PCR using RNA isolated from 1 dpf embryos injected with either control MO, 0.5 pmoles of *vegfc* MO-1 or 0.5 pmoles of *vegfc* MO-2, with primers against *vegfc* (top panel) or *eflα* (bottom panel). The *vegfc* MO-1 causes a complete loss of the *vegfc* transcripts while *vegfc* MO-2 causes a partial loss. The 1190bp band is full-length *vegfc* while the 1071bp band is a shorter splice variant of *vegfc*.

Supplementary Fig. 5. *flt4*, *ccbe1* and *vegfc* are required for development of the intestinal lymphatic network

(A-D) Confocal images of the right intestinal lymphatics at 6 dpf in *lyve1:dsred;kdr1:egfp* embryos treated with (A) control MO (B) *flt4* MO (C) *ccbe1* MO (D) *vegfc* MO-1 with

lyve1 in green and *kdrl* in magenta. (**A'-D'**) Confocal images of the *lyve1:dsred* vessels only. The right intestinal lymphatics are marked with arrows. *flt4*, *ccbe1* and *vegfc* are all required for the formation of the intestinal lymphatic network. Scale bar: 50 μ m.

Supplementary Fig. 6. *cxcr4a* and *cxcl12a* are not required for development of the facial or intestinal lymphatics

(**A-I**) Representative images of whole mount *in situ* hybridisation showing the expression of (A-C) *cxcr4a* (D-F) *cxcl12a* and (G-I) *cxcl12b* in (A,C,D,F,G) 2 dpf and (B,E,H,I) 3 dpf embryos showing that *cxcr4a*, *cxcl12a* and *cxcl12b* are expressed in the head. (**J-O**) Confocal images of the facial lymphatics in *lyve1* embryos at (J-L) 3 dpf (M-O) 5 dpf (**P-R**) trunk lymphatics at 5 dpf (**S-U**) intestinal lymphatics at 6 dpf in *lyve1:dsred;kdrl:egfp* embryos injected with (J,M,P,S) control MO (K,N,Q,T) *cxcr4a* MO or (L,O,R,U) *cxcl12a* MO. (**S'-U'**) Confocal images of the *lyve1:dsred* vessels only. *lyve1* in green and *kdrl* in magenta. The LFL (white arrowheads) and right intestinal lymphatics (white arrows) form normally in *cxcr4a* or *cxcl12a* morphant embryos but the development of the thoracic duct (red asterisks) is impaired. The PHS is marked with a white asterisk. (**V**) Quantitation of the length of the lateral facial lymphatic (LFL) vessel in 3 dpf embryos. (**W**) Quantitation of the percentage thoracic duct formation in 5 dpf embryos. ** p<0.01, by a Mann-Whitney test to control; error bars indicate s.d. Scale bars: 50 μ m.

Supplementary Fig. 7. Expression of *flt4* and *ccbe1*

Representative images of whole mount *in situ* hybridisation showing the expression of (**A-E**) *flt4*, and (**F-J**) *ccbe1* in (A,F,) 1.5 dpf, (B-D,G-I) 2 dpf and (E,J) 3 dpf embryos.

(C,H) The dorsal aspect of the head region at 2 dpf and (D,I,) lateral aspect of the trunk region at 2 dpf.

Supplementary Fig. 8. *vegfd* is not essential for development of the trunk or intestinal lymphatic networks

(A) RT-PCR using RNA isolated from 1 dpf embryos injected with either control MO or 1 pmole of *vegfd* MO using primers against *vegfd* (top panel) or *eflα* (bottom panel). The *vegfd* MO causes aberrant splicing of the *vegfd* mRNA, resulting in deletion of 65 bp. (B-E) Confocal images of the trunk lymphatics in *lyve1:egfp* embryos at (B,C) 1.5 dpf (D,E) 5 dpf and (F,G) intestinal lymphatics at 6 dpf in *lyve1:dsred;kdr1:egfp* embryos injected with (B,D,F) control MO or (C,E,G) *vegfd* MO. (F',G') Confocal images of the *lyve1:dsred* vessels only. *lyve1* in green and *kdr1* in magenta. *vegfd* morphants display normal secondary sprouting from the PCV (white arrowheads), normal thoracic duct formation (red asterisks), and normal intestinal lymphatic formation (white arrows). Scale bars: 50 μm.

Supplementary Movie 1: Facial lymphatic sprouting in a *lyve1:egfp* embryo treated with control MO

Confocal time-lapse imaging of the facial lymphatic sprout in a *lyve1:egfp* embryo from 1.5 dpf to 1.9 dpf (9:03h), injected with control MO. Time-lapse image z-stacks were collected 10 min apart; movie was made at 7 frames per second. Red arrow marks the FLS, the green arrowhead marks the formation of the pectoral vein.

Supplementary Movie 2: Facial lymphatic sprouting in a *lyve1:egfp* embryo treated with *flt4* MO

Confocal time-lapse imaging of the facial lymphatic sprout in a *lyve1:egfp* embryo from 1.5 dpf to 1.9 dpf (9:03h), injected with *flt4* MO. Time-lapse image z-stacks were collected 10 min apart; movie was made at 7 frames per second. The green arrowhead marks the formation of the pectoral vein.

Supplementary Movie 3: Facial lymphatic sprouting in a *lyve1:egfp* embryo treated with *vegfc* MO-1

Confocal time-lapse imaging of the facial lymphatic sprout in a *lyve1:egfp* embryo from 1.5 dpf to 1.9 dpf (9:03h), injected with *vegfc* MO-1. Time-lapse image z-stacks were collected 10 min apart; movie was made at 7 frames per second. Red arrow marks the FLS, the green arrowhead marks the formation of the pectoral vein.

Supplementary Movie 4: Facial lymphatic sprouting in a *lyve1:egfp* embryo treated with *vegfc* MO-1 and control MO

Confocal time-lapse imaging of the facial lymphatic sprout in a *lyve1:egfp* embryo from 1.5 dpf to 1.9 dpf (9:03h), injected with *vegfc* MO-1 and control MO. Time-lapse image z-stacks were collected 10 min apart; movie was made at 7 frames per second. Red arrow marks the FLS, the green arrowhead marks the formation of the pectoral vein.

Supplementary Movie 5: Facial lymphatic sprouting in a *lyve1:egfp* embryo treated with *vegfc* MO-1 and *vegfd* MO

Confocal time-lapse imaging of the facial lymphatic sprout in a *lyve1:egfp* embryo from 1.5 dpf to 1.9 dpf (9:03h), injected with *vegfc* MO-1 and *vegfd* MO. Time-lapse image z-stacks were collected 10 min apart; movie was made at 7 frames per second. The green arrowhead marks the formation of the pectoral vein.

Supplementary Table 1. Primer Sequences

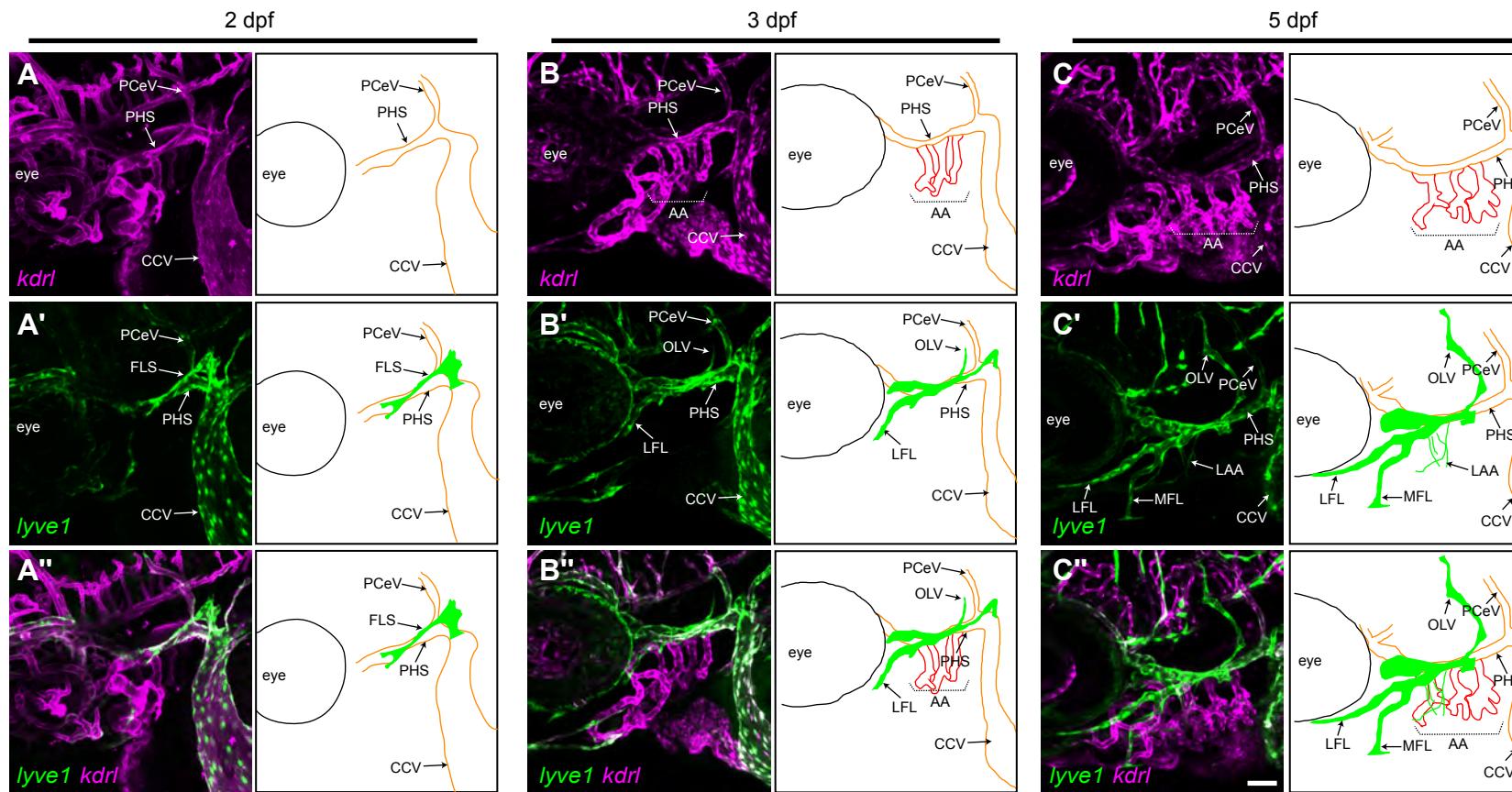
Name	Sequence	Use
<i>vegfc</i> -F <i>vegfc</i> -R	ATG CAC TTA TTT GGA TTT TC TTA GTC CAG TCT TCC CCA GT	RT-PCR expression analysis
<i>vegfc</i> -F <i>vegfc</i> -R	AAG GGC CCT AAC AGA ATG TC TTT GAA TGA AGG GTG TCA GG	qPCR (Fig 3)
<i>vegfc</i> -F EcoRI <i>vegfc</i> -R XhoI	gcg cga att cac cAT GCA CTT ATT TGG ATT TTC gcg cct cga gTT AGT CCA GTC TTC CCC AGT	Cloning cDNA for expression of mRNA, antisense probe and xenografts
<i>vegfd</i> -F <i>vegfd</i> -R	ATG AAG AAA CAG AAA TGT GCT GGA C TCA CGT ATA GTG TAG TCT GTG TG	RT-PCR expression analysis
<i>vegfd</i> -F <i>vegfd</i> -R	GCT GGA CTT CAC ATG TTG CT CTCAGTTCCCTGCTCCCCACTT	qPCR (Fig 3)
<i>vegfd</i> -F EcoRI <i>vegfd</i> -R XhoI	gcg cga att cac cAT GAA GAA ACA GAA ATG TGC gcg cct cga gTC ACG TAT AGT GTA GTC TGT GTG	Cloning cDNA for expression of mRNA, antisense probe and xenografts
<i>eflα</i> -F <i>eflα</i> -R	TGC CTT CGT CCC AAT TTC AG TAC CCT CCT TGC GCT CAA TC	qPCR (Fig 3)
<i>eflα</i> -F <i>eflα</i> -R	ATC TAC AAA TGC GGT GGA AT ATA CCA GCC TCA AAC TCA CC	Zebrafish RT-PCR control
<i>β-actin</i> -F <i>β-actin</i> -R	CAT TGC CGA CAG GAT GCA CTC AGG AGG AGC AAT GAT CTT GA	Human RT-PCR control

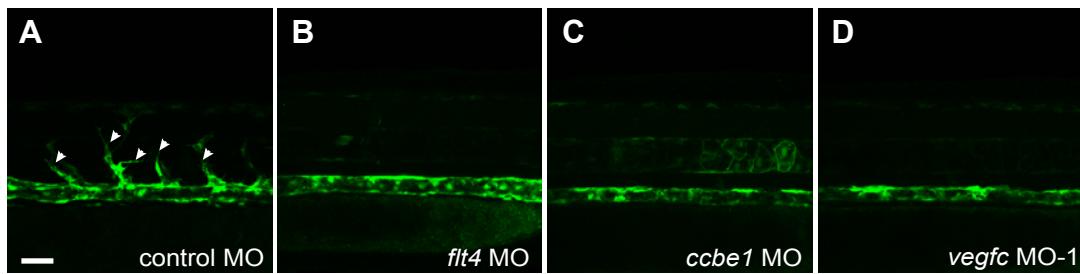
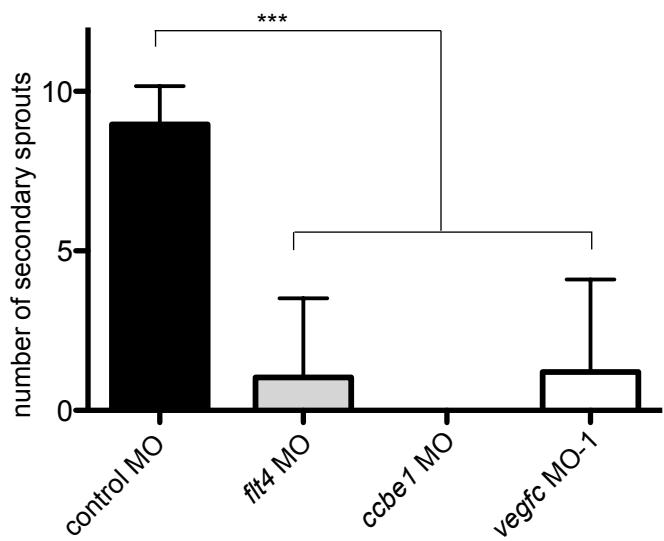
Supplementary Table 2. Morpholino Sequences

Name	Sequence	Target	Dose (pmole)	Reference
<i>vegfc</i> -MO1	ATGCTCCTGCTGAGACACAGACAAG	intron1/exon2	0.5	[1]
<i>vegfc</i> -MO2	ACTTGACTCACCGTCTGCTGATG	exon3/intron3	0.5	[1]
<i>flt4</i> MO	CTCTTCATTCCAGGTTCAAGTCC	translation	0.5	[2]
<i>ccbe1</i> MO	CGGGTAGATCATTTCAGACACTCTG	translation	1.0	[3]
<i>cxcr4a</i> MO	AGACGATGTGTCCGTAATAAGCCAT	translation	0.5	[4]
<i>cxcl12a</i> MO	GTGCAGATACTCACATGACTTGGAA	exon2/intron2	0.5	[4]
<i>vegfd</i> MO	CAAATGAATCCGATACTGACCTGTT	exon4/intron4	1.0	This study
Control MO	CCTCTTACCTCAGTTACAATTATA		0.5	

1. Flores MV, Hall CJ, Crosier KE, Crosier PS (2010) Visualization of embryonic lymphangiogenesis advances the use of the zebrafish model for research in cancer and lymphatic pathologies. *Dev Dyn* 239: 2128-2135.
2. Hogan BM, Herpers R, Witte M, Helotera H, Alitalo K, et al. (2009) Vegfc/Flt4 signalling is suppressed by Dll4 in developing zebrafish intersegmental arteries. *Development* 136: 4001-4009.
3. Hogan BM, Bos FL, Bussmann J, Witte M, Chi NC, et al. (2009) Ccbe1 is required for embryonic lymphangiogenesis and venous sprouting. *Nat Genet* 41: 396-398.
4. Cha YR, Fujita M, Butler M, Isogai S, Kochhan E, et al. (2012) Chemokine signaling directs trunk lymphatic network formation along the preexisting blood vasculature. *Dev Cell* 22: 824-836.

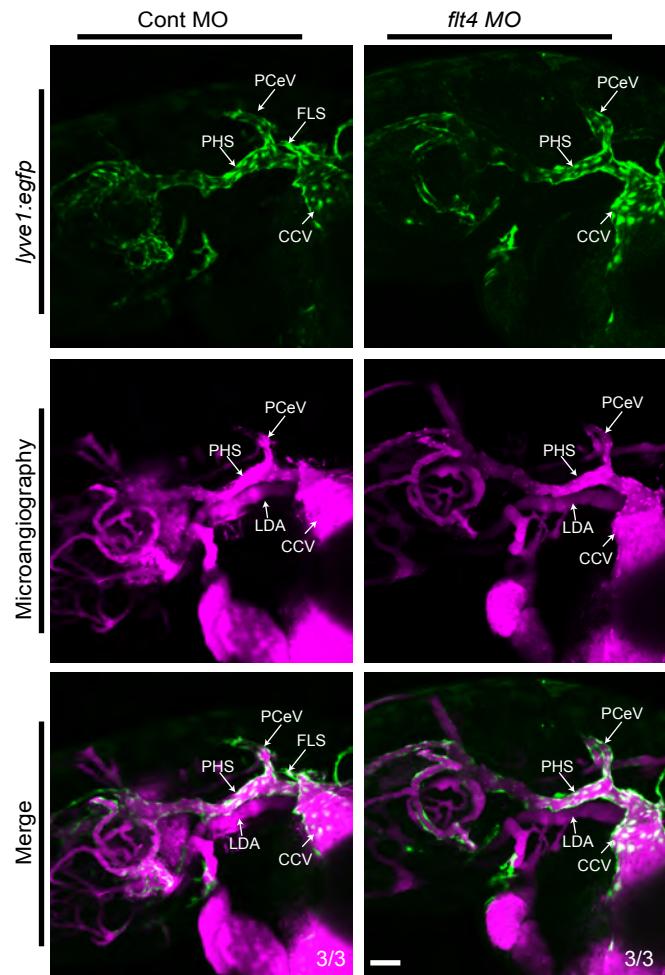
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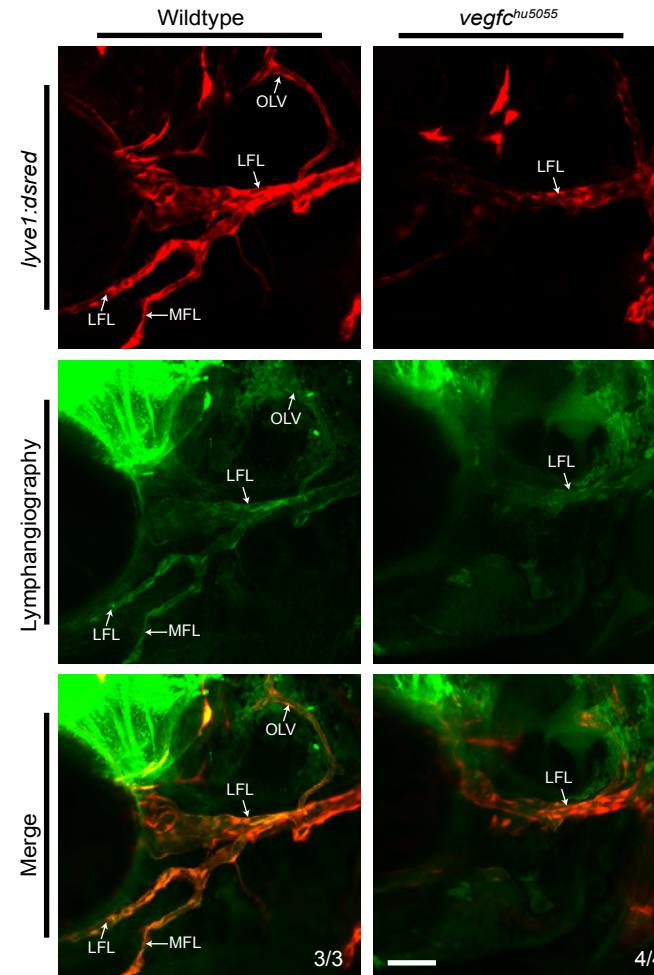
**E**

Sup Fig 3

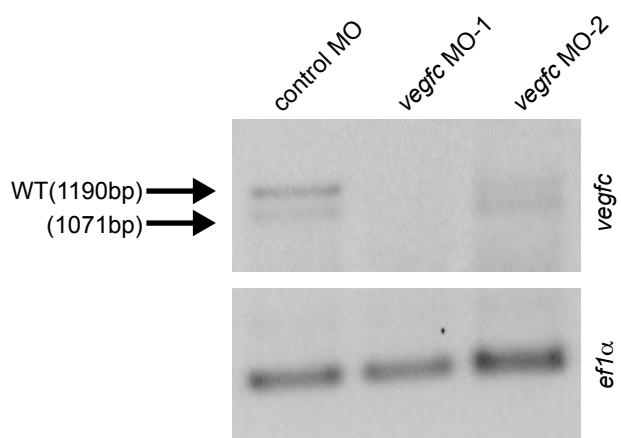
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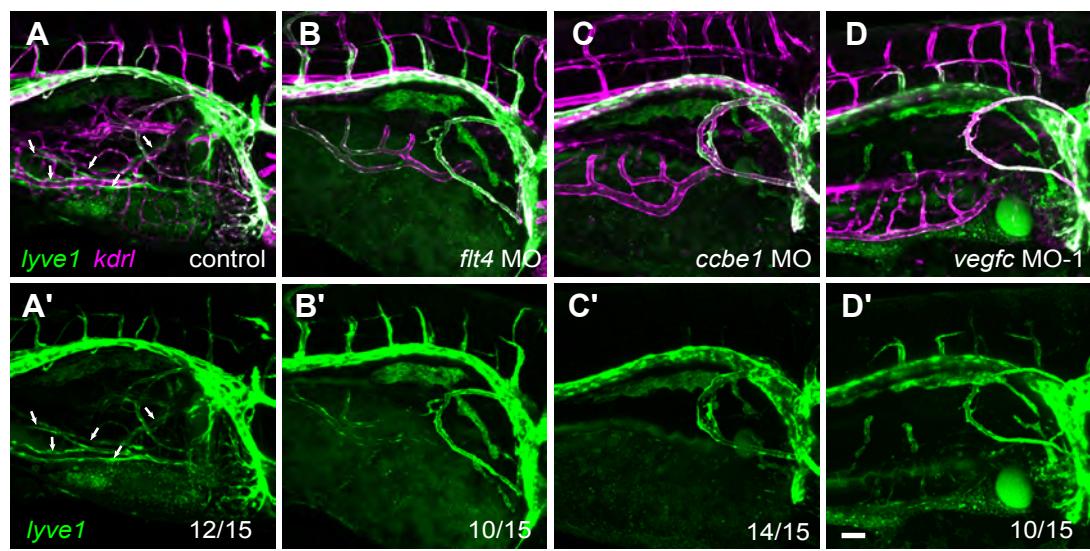
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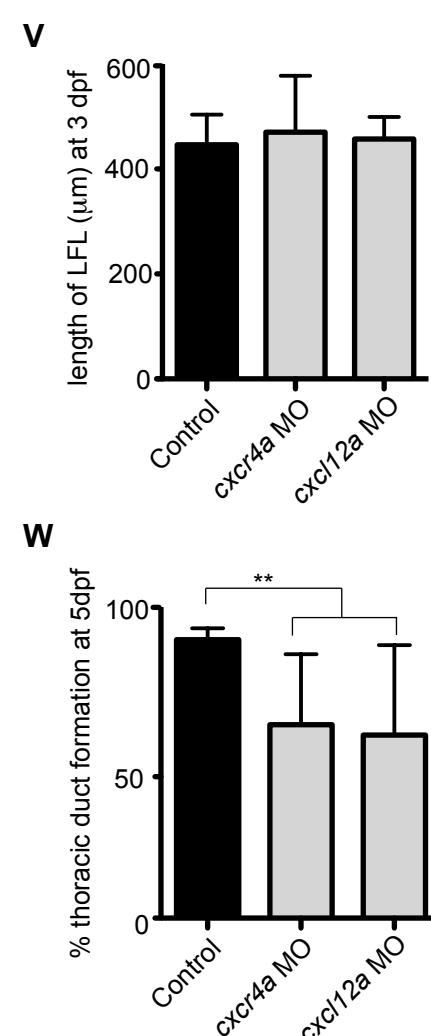
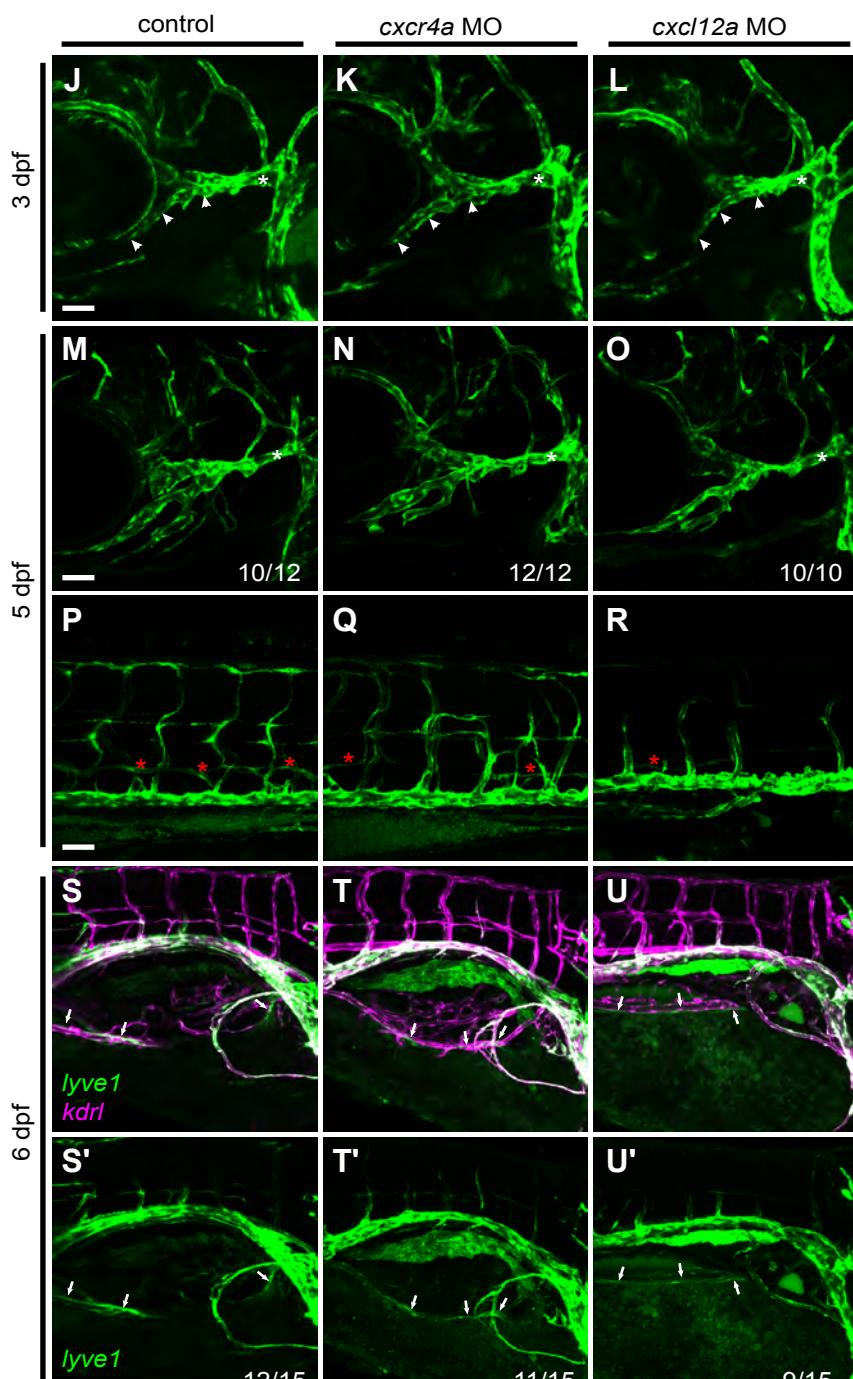
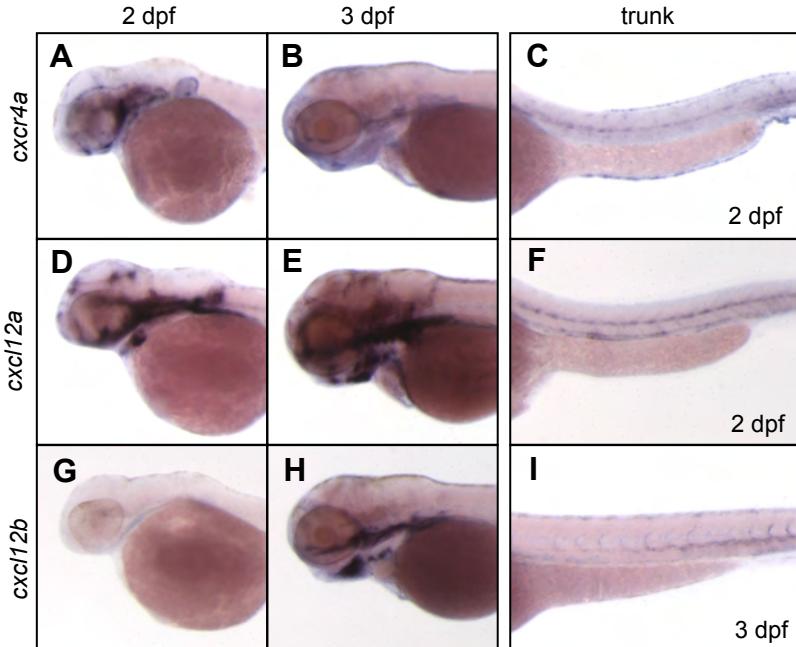
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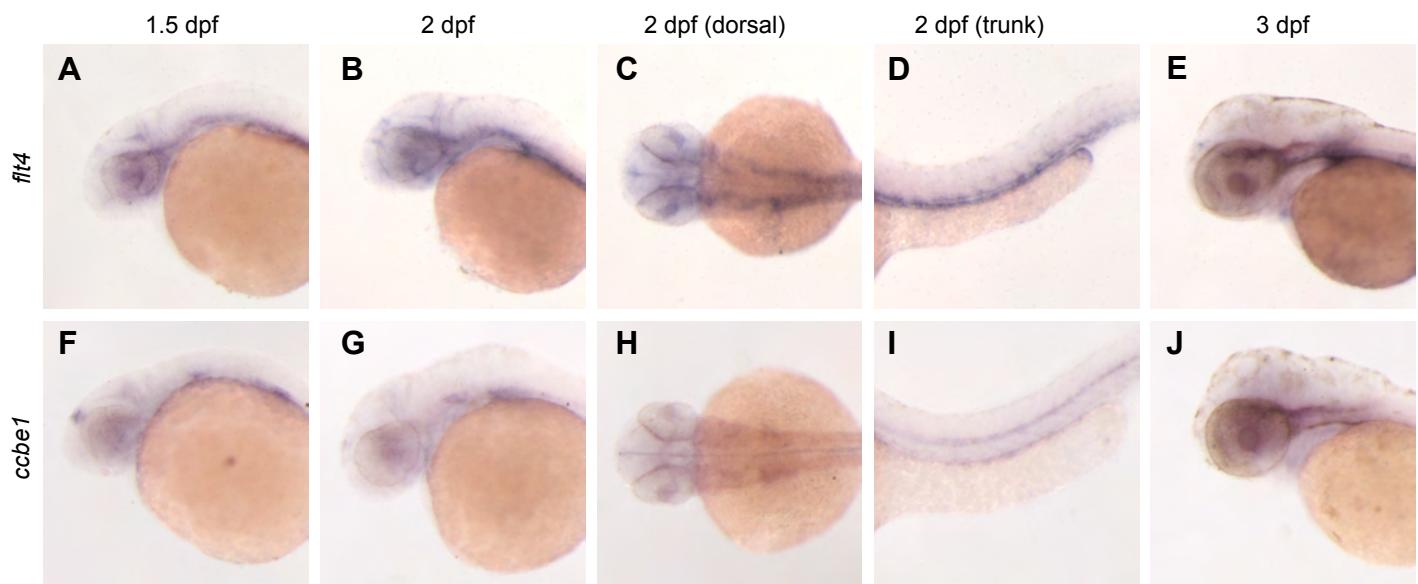


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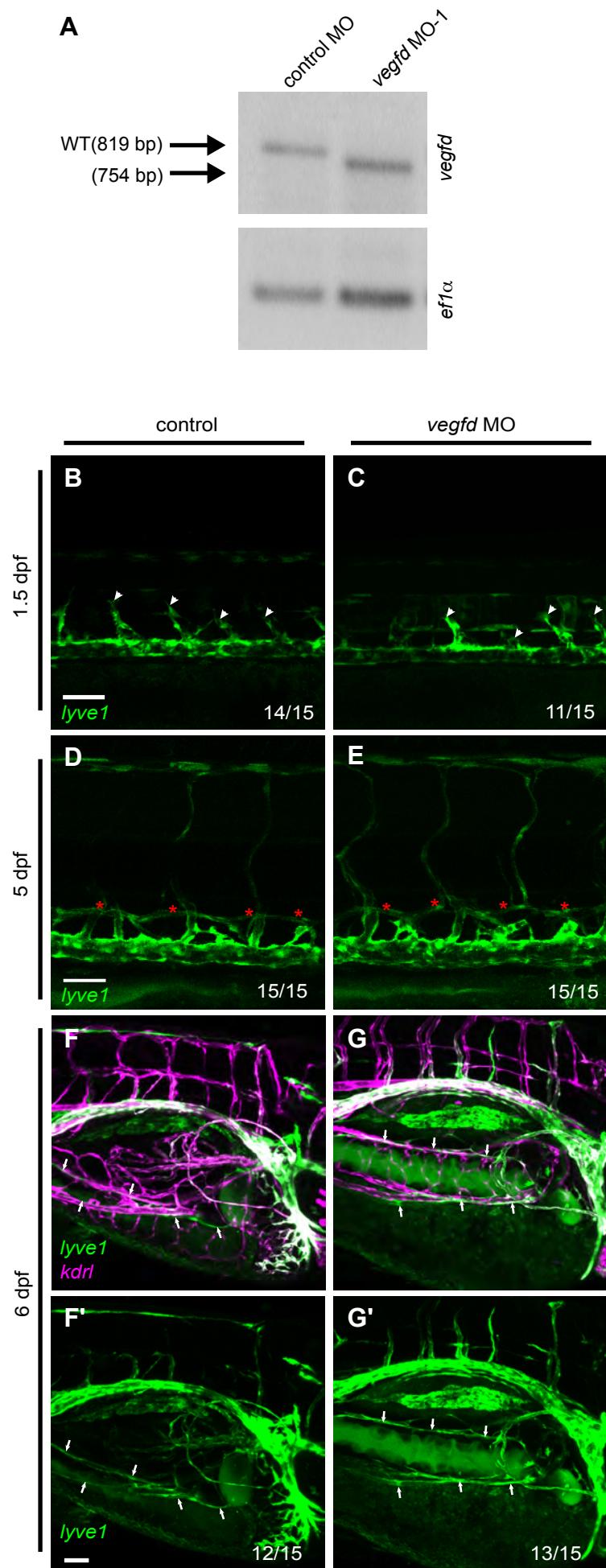


Sup Fig 6





Sup Fig 8

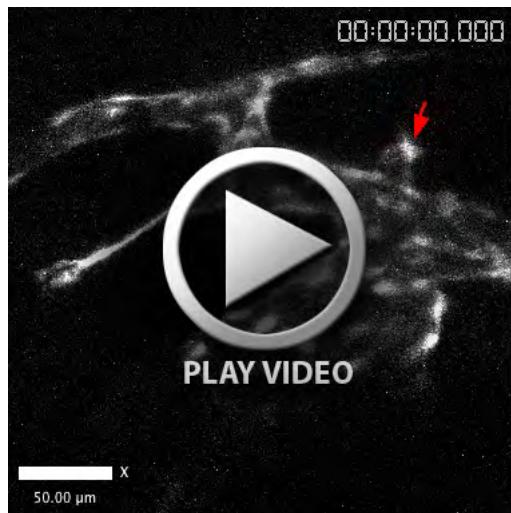




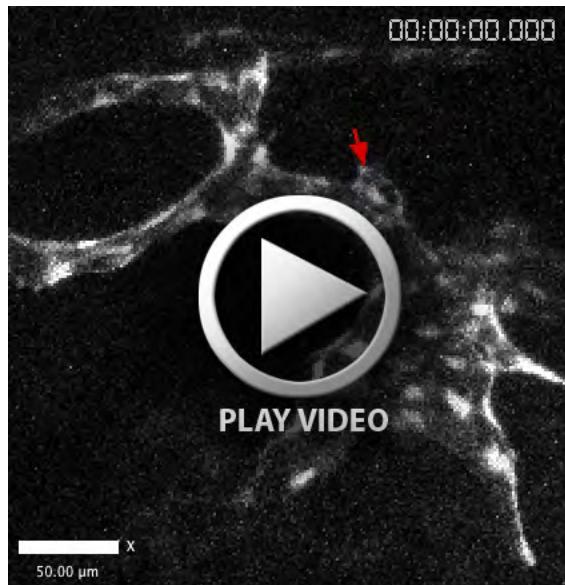
Movie 1.



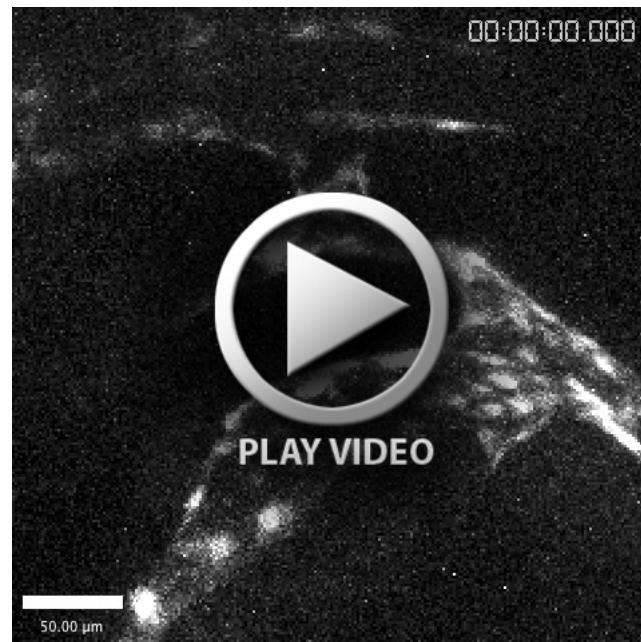
Movie 2.



Movie 3.



Movie 4.



Movie 5.