

### **3,4-Difluorobenzocurcumin inhibits Vegfc-Vegfr3-Erk signalling to block developmental lymphangiogenesis in zebrafish**

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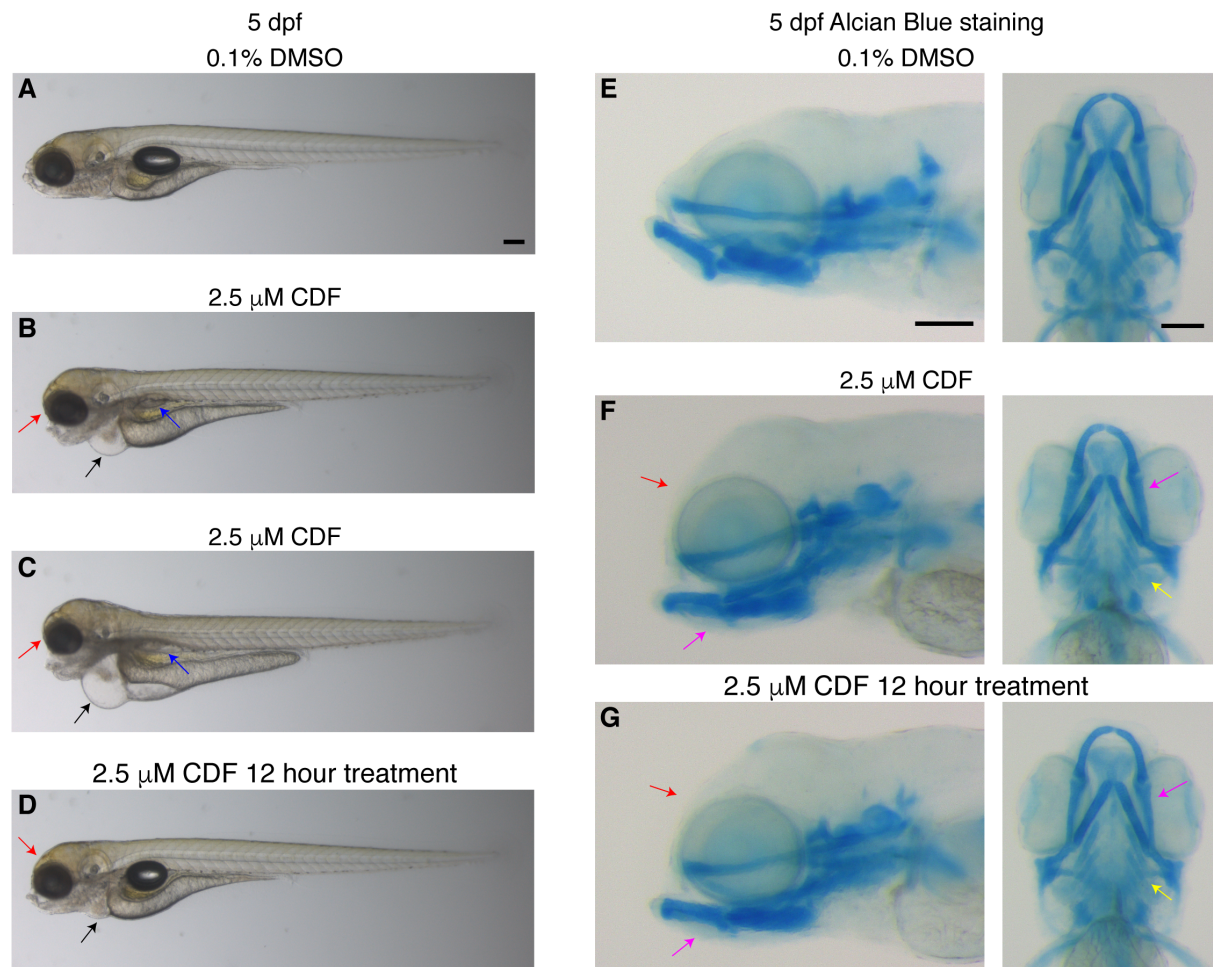
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## Supplementary figures

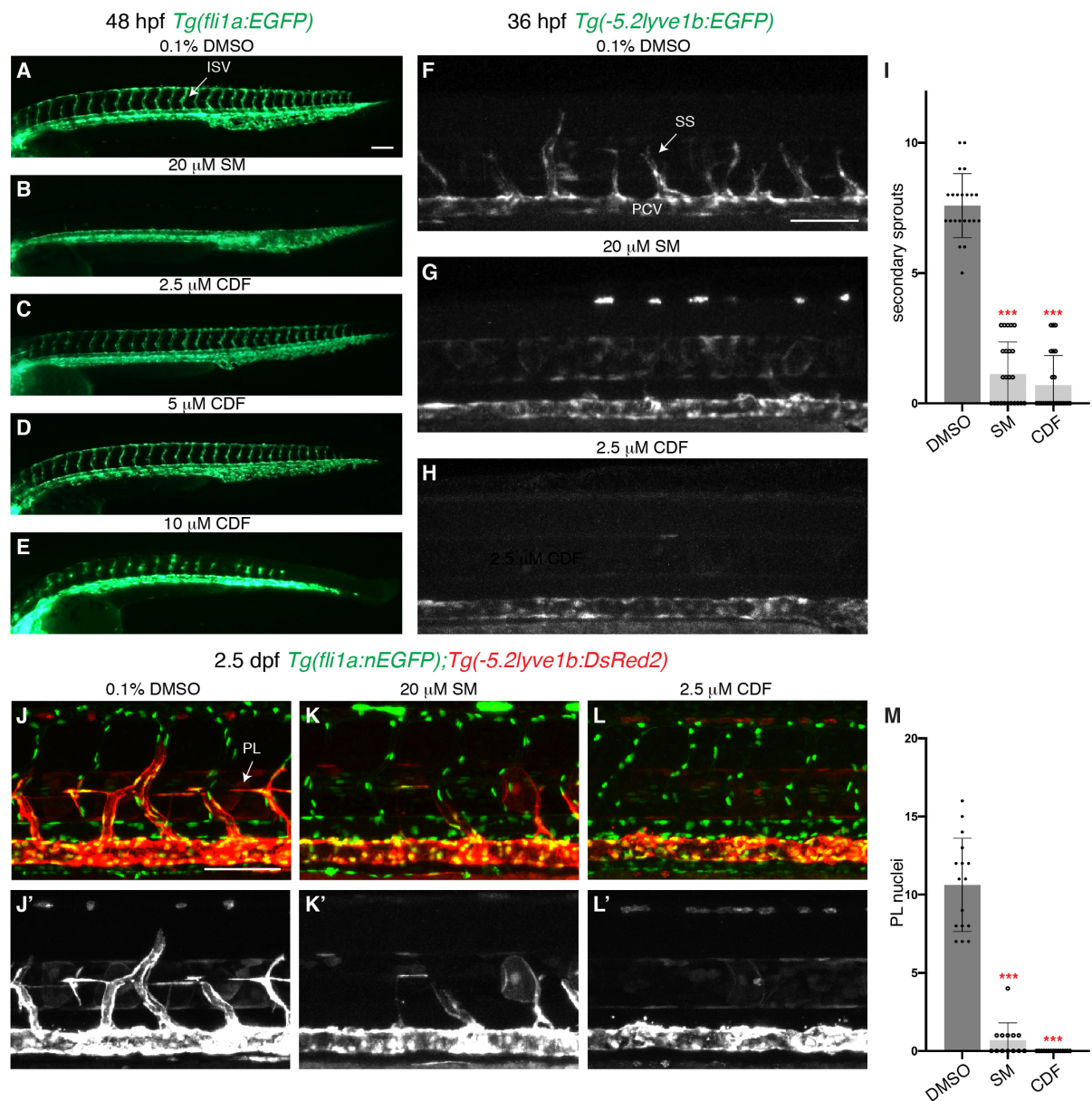


**Figure S1: Brief treatment of 3,4-Difluorobenzocurcumin causes less morphological phenotypes than longer treatment.**

**(A-D)** Lateral trans-light images of 5 dpf larvae either continuously treated with 0.1% DMSO (A: 40/40), 2.5  $\mu$ M 3,4-Difluorobenzocurcumin (CDF, B: 15/40, C: 25/40), or treated for 12 hours with 2.5  $\mu$ M CDF (D: 34/40). **(E-G)** Lateral (left) and ventral (right) trans-light images of 5 dpf larvae either continuously treated with 0.1% DMSO (E: 45/45), 2.5  $\mu$ M CDF (F: 41/45), or treated for 12 hours with 2.5  $\mu$ M CDF (G: 37/45) stained with Alcian Blue. Purple arrows show the major jaw elements that form normally and along which facial lymphatics normally develop. Other arrows show morphological phenotypes (impaired anterior cranial protrusion (red), pericardial oedema (black), lack of swim bladder inflation (blue), delayed pharyngeal

cartilage development (yellow)) associated with treatment with CDF. Brief treatment of CDF causes less morphological phenotypes.

Scale bars: 100  $\mu\text{m}$ .



**Figure S2: 3,4-Difluorobenzocurcumin treatment inhibits lymphatic and venous sprouting.**

**(A-E)** Lateral fluorescent images of 48 hpf *Tg(fli1a:EGFP)* embryos treated with either 0.1% DMSO (A), 20  $\mu$ M sunitinib malate (SM, B), or 3,4-Difluorobenzocurcumin (CDF, C-E) at indicated concentrations. CDF at 5 and 2.5  $\mu$ M do not inhibit primary angiogenesis.

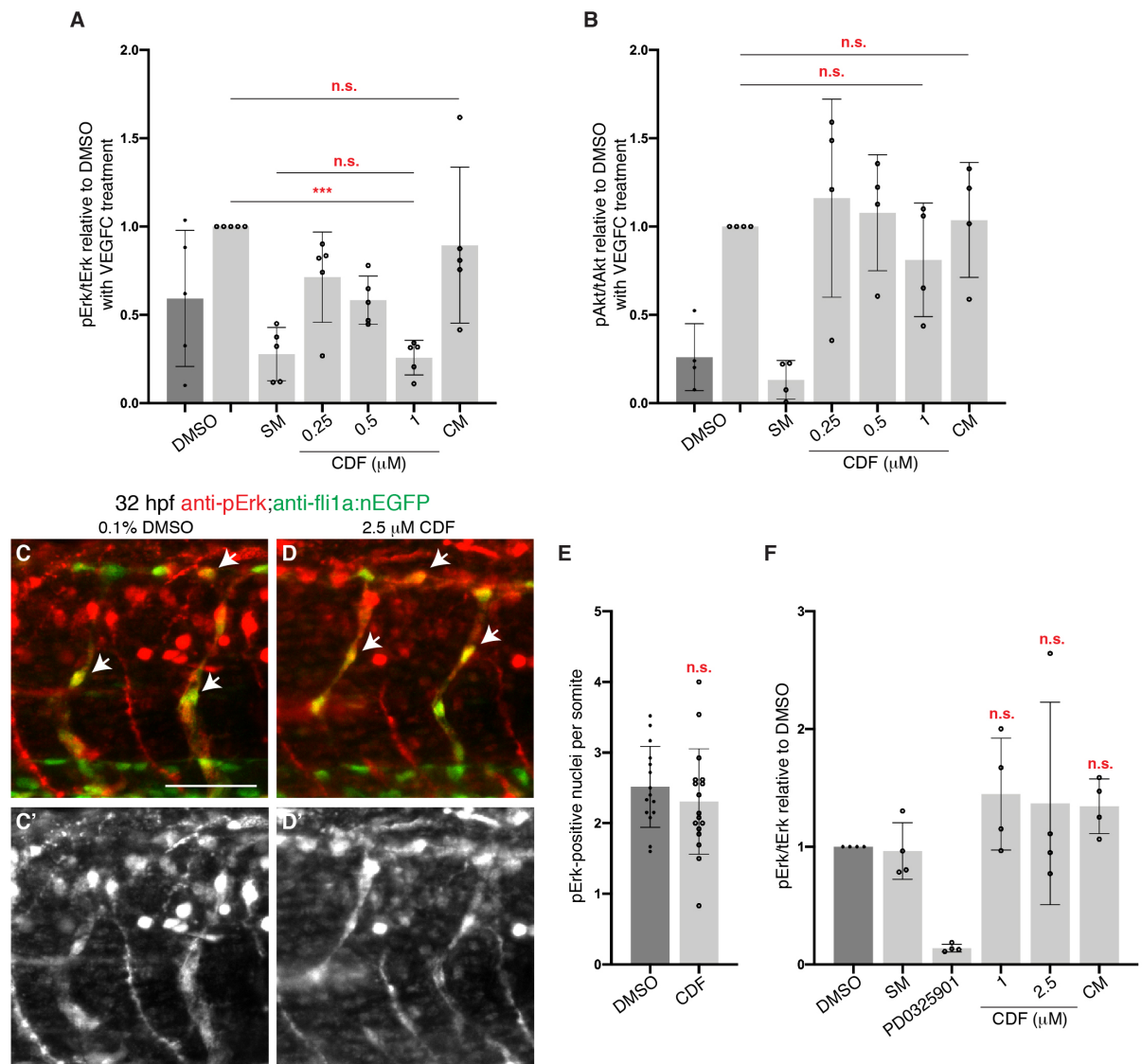
**(F-H)** Lateral confocal images of 36 hpf *Tg(-5.2lyve1b:EGFP)* embryos treated with either 0.1% DMSO (F), 20  $\mu$ M SM (G), or 2.5  $\mu$ M CDF (H). CDF at 2.5  $\mu$ M blocks secondary sprouting.

**(I)** Quantification of secondary sprout (SS) number across 10 somites in 36 hpf *Tg(-5.2lyve1b:EGFP)* embryos treated with either 0.1% DMSO (n=22 embryos), 20  $\mu$ M SM (n=24 embryos), or 2.5  $\mu$ M CDF (n=24 embryos).

**(J-L')** Lateral confocal images of 2.5 dpf *Tg(fli1a:nEGFP);Tg(-5.2lyve1b:DsRed2)* embryos treated with either 0.1% DMSO (J,J'), 20  $\mu$ M SM (K,K') or 2.5  $\mu$ M CDF (L,L'). Images J'-L' represent the *Tg(-5.2lyve1b:DsRed2)* expression of images J-L. CDF at 2.5  $\mu$ M inhibits parachordal LEC (PL) formation.

**(M)** Quantification of PL nuclei across 10 somites in 2.5 dpf *Tg(fli1a:nEGFP);Tg(-5.2lyve1b:DsRed2)* embryos treated with either 0.1% DMSO (n=16 embryos), 10  $\mu$ M SM (n=13 embryos), or 2.5  $\mu$ M CDF (n=15 embryos).

ISV: intersegmental vessel, PCV: posterior cardinal vein. Statistical test: Kruskal-Wallis test was conducted for graphs I and M.  $p \leq 0.001$  (\*\*\*). Scale bars: 100  $\mu$ m.



**Figure S3: 3,4-Difluorobenzocurcumin treatment reduces protein level of pERK in human dermal lymphatic microvascular endothelial cells but does not reduce the whole-organism protein level of pErk in zebrafish.**

(**A,B**) Densitometry analysis of pERK (**A**) and pAKT (**B**) protein levels from western blot analysis in **Figure 3A** ( $n=5$  for pERK analysis and  $n=4$  for pAKT analysis). CDF dose-dependently blocks VEGFC-induced ERK phosphorylation in human dermal lymphatic microvascular endothelial cells. pERK and pAKT protein levels were normalized to total ERK (tERK) and tAKT protein levels.

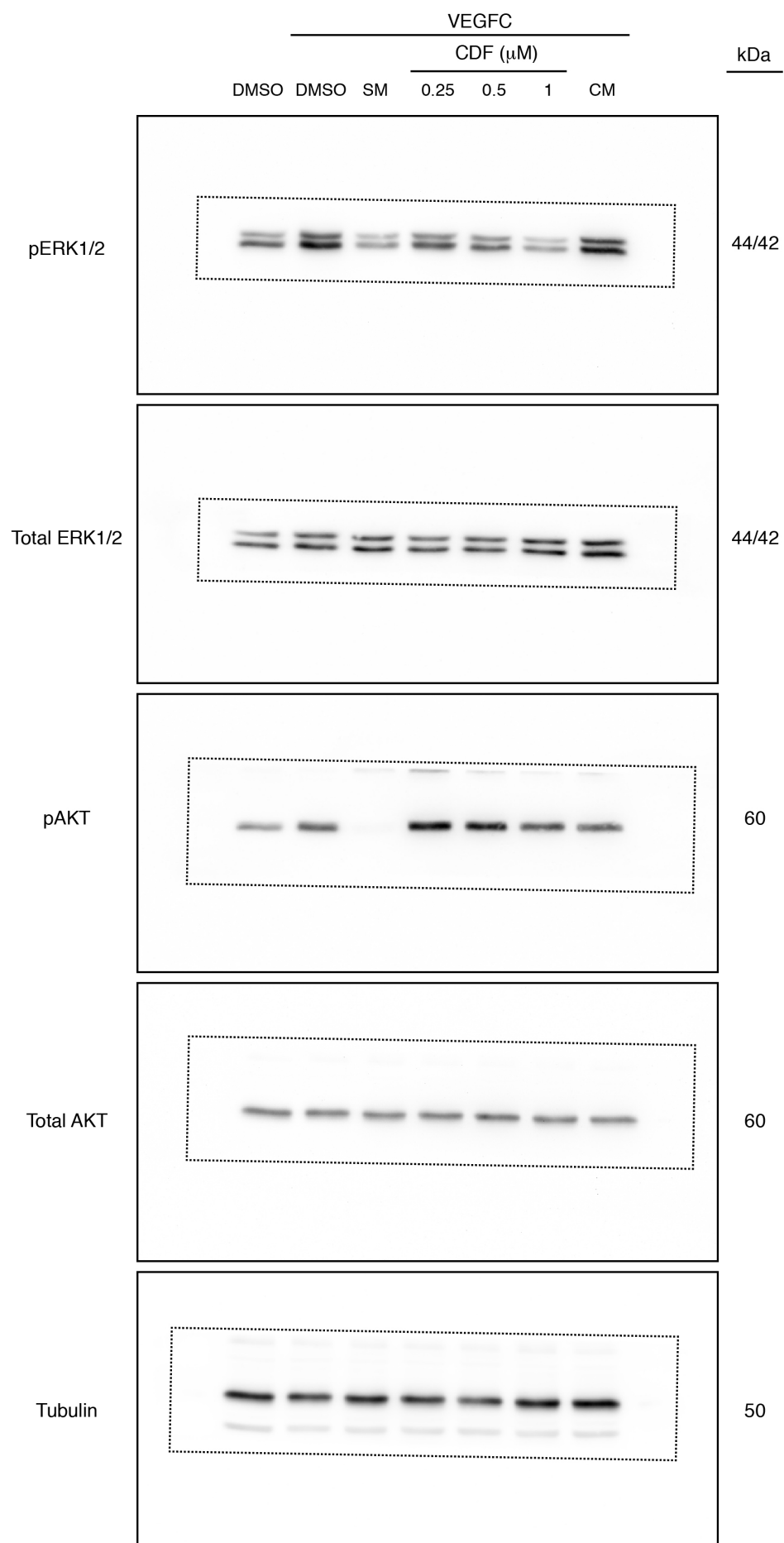
(**C-D'**) Lateral confocal images of 32 hpf *Tg(fli1a:nEGFP)* embryos treated with either 0.1% DMSO (**C,C'**) or 2.5  $\mu$ M 3,4-Difluorobenzocurcumin (CDF, **D,D'**) immunostained with anti-pErk (red) and anti-GFP (green) antibodies. CDF at 2.5  $\mu$ M

does not inhibit Erk phosphorylation in arterial endothelial cells. Images C' and D' represent the anti-pErk staining of images C and D. White arrows indicate pERK-positive arterial intersegmental vessel (aISV) or dorsal longitudinal anastomotic vessel (DLAV) endothelial cells.

**(E)** Quantification of pErk and *fli1a:EGFP*-positive nuclei in aISVs and the DLAV of 32 hpf *Tg(fli1a:nEGFP)* embryos treated with either 0.1% DMSO (n=19 embryos) or 2.5  $\mu$ M CDF (n=21 embryos).

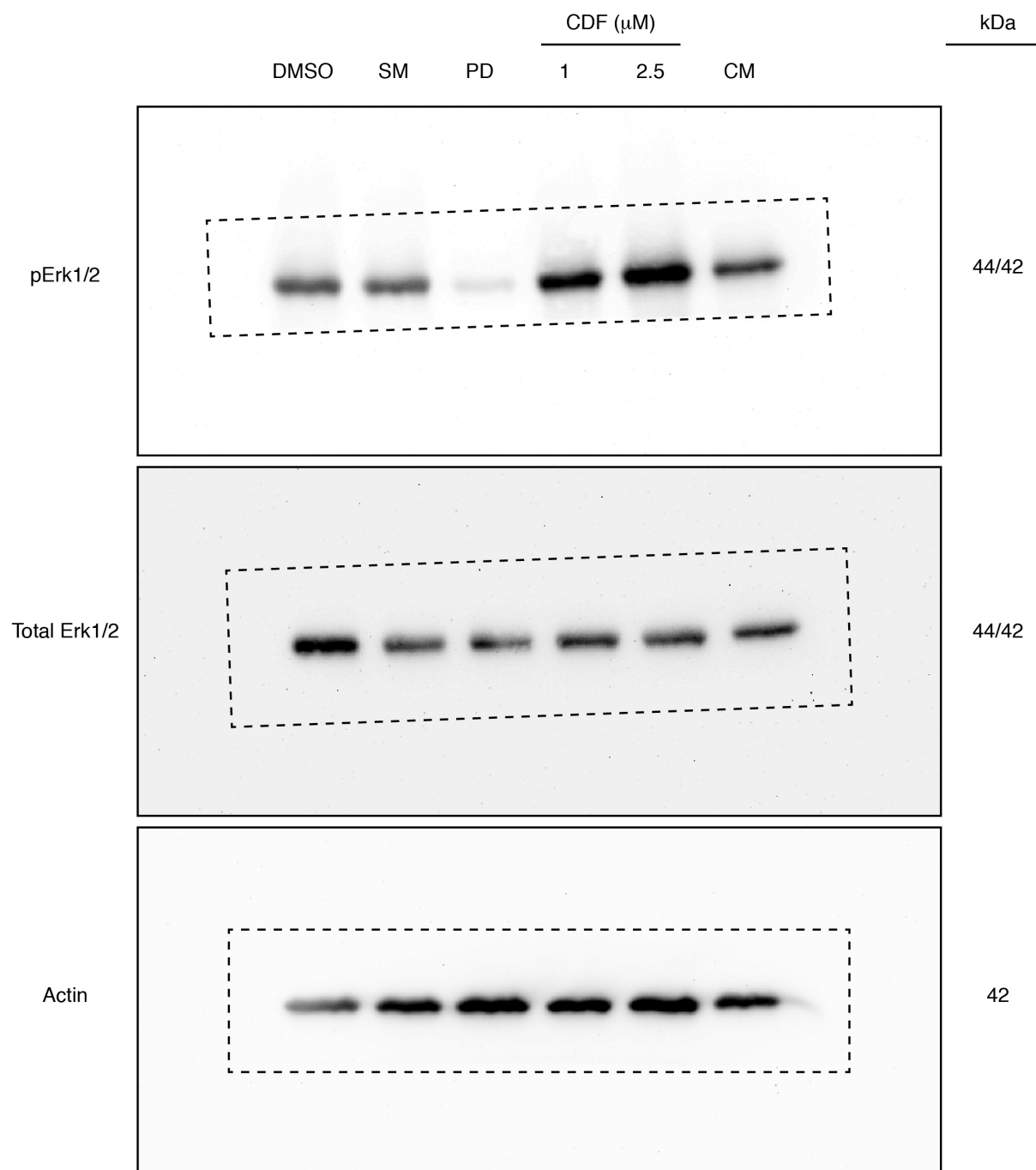
**(F)** Densitometry analysis of pErk levels from western blot analysis in **Figure 3E** (n=4). pErk protein levels were normalized to total Erk (tErk) protein levels. CDF at 2.5  $\mu$ M does not block whole-organism Erk phosphorylation.

Statistical test: Mann-Whitney test was conducted for graph E. Kruskal-Wallis test was conducted for graphs A, B, and F.  $p \leq 0.001$  (\*\*\*) and n.s. indicates not significant. Scale bar: 50  $\mu$ m.



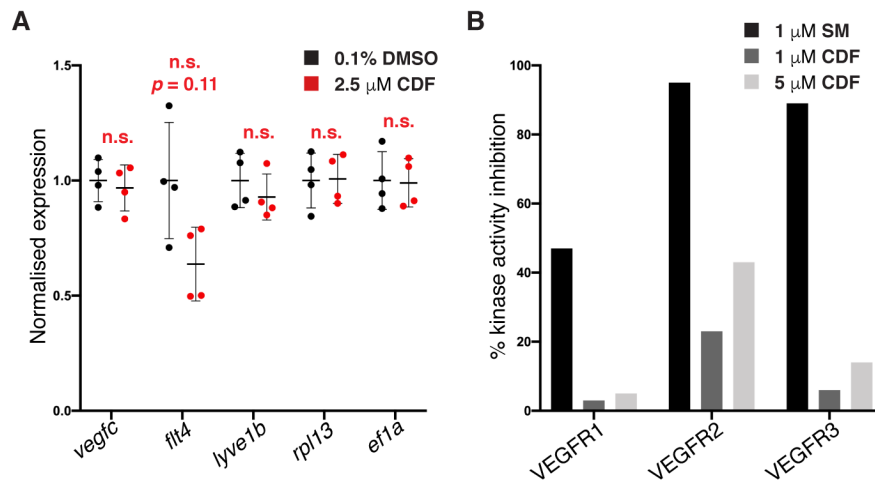
**Figure S4: Original western blot images of Figure 3A.**

Blots were pre-cropped at the dashed lines as indicated before incubation with respective primary antibodies. Images were taken using the Chemilmager™ Imaging System (Alpha Innotech, CA, USA). Original western blot images were processed using the Fiji image processing software.



**Figure S5: Original western blot images of Figure 3E.**

Blots were pre-cropped at the dashed lines as indicated before incubation with respective primary antibodies. Images were taken using the Chemilmager™ Imaging System (Alpha Innotech, CA, USA). Original western blot images were processed using the Fiji image processing software.

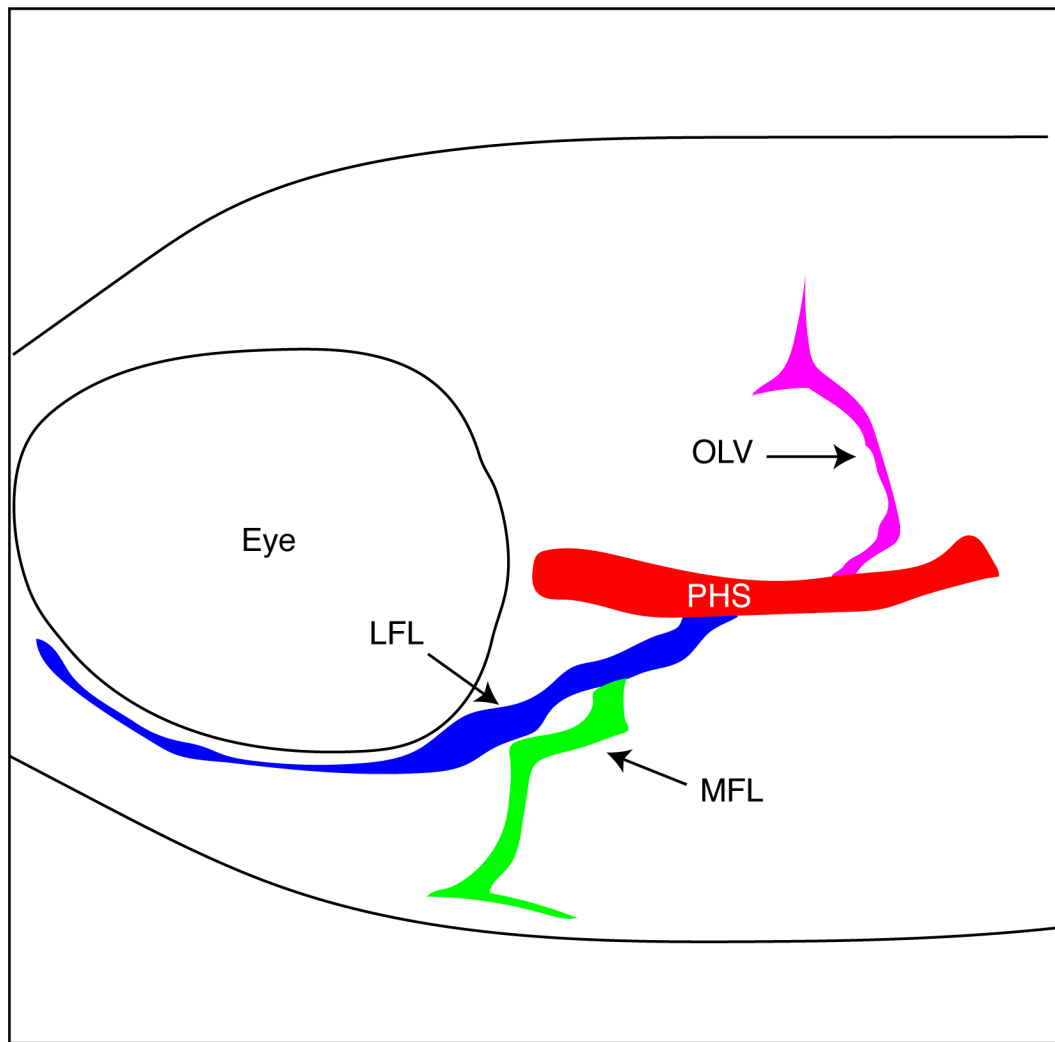


**Figure S6: 3,4-Difluorobenzocurcumin treatment does not reduce *vegfc* and *flt4* mRNA levels, and VEGFR3 kinase activity.**

(A) qPCR analysis of *vegfc*, *flt4*, *lyve1b*, *rpl13*, and *ef1a* mRNA levels in 36 hpf embryos treated with either 0.1% DMSO or 2.5  $\mu$ M 3,4-Difluorobenzocurcumin (CDF, n=4). CDF at 2.5  $\mu$ M does not significantly inhibit *vegfc* or *flt4* mRNA levels.

(B) Quantification of % kinase activity inhibition of either 1  $\mu$ M sunitinib malate (SM), 1  $\mu$ M CDF, or 5  $\mu$ M CDF against VEGFR1 (1  $\mu$ M SM=47%, 1  $\mu$ M CDF=3%, 5  $\mu$ M CDF=5%), VEGFR2 (1  $\mu$ M SM=95%, 1  $\mu$ M CDF=23%, 5  $\mu$ M CDF=43%) and VEGFR3 (1  $\mu$ M SM=89%, 1  $\mu$ M CDF=6%, 5  $\mu$ M CDF=14%) (n=2). CDF is not an inhibitor of VEGFR3 kinase activity at 1 or 5  $\mu$ M.

Statistical test: Mann-Whitney test was conducted for individual genes in graph A. n.s. indicates not significant.



**Figure S7: Schematic representation of the regions where facial lymphatic vessels were quantified.**

Schematic representation of the regions where lateral facial lymphatic (LFL, blue), medial facial lymphatic (MFL, green), or otolith lymphatic vessel (OLV, purple) were quantified in 5 dpf zebrafish larvae for this study.

PHS: primary head sinus.

### **Supplementary Table**

**Table S1: Primer sequences used in this study.**

<b>qPCR Primer name</b>	<b>Sequence</b>
<i>ef1a</i> qPCR forward	CTGGAGGCCAGCTCAAACAT
<i>ef1a</i> qPCR reverse	ATCAAGAAGAGTAGTACCGCTAGCATTAC
<i>flt4</i> qPCR forward	AAAGGGGAGACAACGACATG
<i>flt4</i> qPCR reverse	CGGCACTAACGAGAAGAGAG
<i>lyve1b</i> qPCR forward	CAGCACAGTCTGTCTAGACG
<i>lyve1b</i> qPCR reverse	ACCAAACCTGCATGTCTGTAA
<i>rpl13</i> qPCR forward	CATCTTGAGCTCCTCCTCAGTAC
<i>rpl13</i> qPCR reverse	CATCTCTGTTGACTCACGTCG
<i>vegfc</i> qPCR forward	GGCCTCAACAGAGCTTCAAC
<i>vegfc</i> qPCR reverse	TCTCTTGGGGTCCACGTTAC