SUPPLEMENTARY MATERIALS AND METHODS

Plasmids

The Tol2 vector system was kindly provided by K. Kawakami (National Institute of Genetics, Japan) (Kawakami et al., 2004; Urasaki et al., 2006). Tol2_amp and pCS2_GAL4FF_KanR vector for BAC recombineering were kindly provided by S. Schulte-Merker (University of Münster, Germany).

To construct the plasmid encoding EGFP-Notch2 intracellular domain (GFP-N2ICD) and mCherry-N2ICD, a cDNA encoding zebrafish *notch2* (NM_001115094) was amplified by PCR using cDNA library derived from zebrafish embryos as a template and cloned into pEGFP-C1 vector or pmCherry-C1 vector (Clontech), respectively. Primers to amplify N2ICD are as follows; 5'-ATTGTCGACACCATGgtgctgggtgttttggctgc-3' and 5'-ATTAAGCTTCACCATGgttggcatgttgattgc-3'. Lowercase; homology arm to *notch2*. Uppercase; restriction enzyme sites, start codon. pCSegfpN5ICD (#22467) (Villefranc et al., 2007) was obtained from Addgene, which is referred to as GFP-N3ICD in this text, and mRNA was in vitro transcribed with SP6 RNA polymerase from NotI-linearised pCSegfpN5ICD vector using the mMESSAGE mMACHINE kit (Ambion, Austin, TX). A cDNA encoding EGFP was subcloned into the pTol2-fli1a vector to construct the pTol2-fli1a:GFP plasmid (Wakayama et al., 2015). Then, the cDNA encoding H2B was inserted into the pTol2-fli1a-GFP vector to generate the pTol2-fli1a:H2B-GFP plasmid.

BAC recombineering

BAC recombineering was done as previously described (Ando et al., 2016). Briefly, pRedET plasmid (GeneBridge) was introduced into E. coli containing CH211-39M8 BAC clone encoding *notch2* gene (BacPAC resources) by electroporation (1800 V, 25 μF, 200 Ω) to increase the efficiency of homologous recombination. To amplify and insert GAL4FF at C-

terminus of *notch2* in CH211-39M8 BAC clone, pCS2_GAL4FF_KanR plasmid vector and following PCR primers were used: 5'-ccgcgcagccaacacagtcccagcagtcccagcgcagcagtgtgtacgcaATGAAGCTACTGTCTTCTATC-3' and 5'-gatggtttgctgctgaagagagagaatcaatacactctcaaaggtctgagTCAGAAGAACTCGTCAAGAAGGCG-3'. (lowercase; homology arm to BAC vector. uppercase; primer binding site to the template plasmid)

Transgenic zebrafish lines

Tol2 transposase mRNA was in vitro transcribed with SP6 RNA polymerase from NotI-linearised pCS-TP vector using the mMESSAGE mMACHINE kit (Ambion). To generate the *TgBAC(notch2:Notch2-GAL4FF)*^{cbz1} or *Tg(fli1a:H2B-GFP)*^{ncv69}, the corresponding BAC DNA or plasmid as described above was co-injected with Tol2 transposase mRNA into one-cell stage embryos of *Tg(UAS:GFP)* or wild type (AB) strain, respectively.

Injections of morpholino oligonucleotides

Control morpholino oligonucleotide (MO) (Gene Tools, LLC, Philomath, OR), 3-6 ng of *alk1* MO, 5-10 ng of *notch2* MO, 2-10 ng of *notch3* MO, 10 ng *dll4* MO, and 10 ng *notch1b* MO was injected into embryos at one-cell stage. These MOs have been previously reported to be specific and effective. The sequence for the already-validated MOs used in this study are: splicing-*alk1* MO, 5'-ATCGGTTTCACTCACCAACACACTC-3' (Corti et al., 2011), ATG-*notch2* MO, 5'-TTCGAATGTGAAAGTCTTACCTGCA-3' (Kim et al., 2014), ATG-*notch3* MO, 5'-ATATCCAAAGGCTGTAATTCCCCAT-3' (Wang et al., 2014), splicing-*dll4* MO, 5'-TAGGGTTTAGTCTTACCTTGGTCAC-3' (Leslie et al., 2007), splicing-*notch1b* MO, 5'-AATCTCAAACTGACCTCAAACCGAC-3' (Milan et al., 2006).

Whole-mount *in situ* hybridization

The antisense *deltaC* and *ephrinB2a* RNA probes labeled with digoxigenin (DIG) were prepared by using an RNA labeling kit (Roche). Briefly, fixed embryos were hybridized with antisense RNA probes at 65ºC overnight in hybridization buffer (5 x SSC, 50% formamide, 5 mM EDTA, 0.1% Tween 20, 50 µg/ml heparin, and 1 mg/ml torula RNA). Embryos washed after hybridization were preincubation with blocking buffer (0.1 M maleic acid [pH 7.5], 5% sheep serum, and 2% blocking reagent [Roche]) at room temperature for 2 h and subsequently incubated with anti-DIG antibody conjugated with alkaline phosphatase (Roche) in blocking buffer at room temperature for 4 h. After embryos were washed, colorimetric reaction was carried out using BM purple (Roche) as the substrate. To stop reaction, embryos were washed by PBS-T and fixed by 4% PFA. The following primers were used for amplification of RNA probes; *deltaC-S* (5'-GAGCACCTCAAACACCAGTG-3'), *deltaC-AS* (5'-CACCTCCTCCACCCATAAGG-3') and *ephrinB2a-S* (5'-CGGGATCCATGGGCGACTCTTTGTG -3'), *ephrinB2a-AS* (5'-GCTCTAGATCACACCTTGTAATAGATG -3').

Table S1. qPCR primers.

Target	Primers (5'-3')		Detection
pdgfrb (Fig. S3C)	*pdgfrb-S*	CGTTCCCAGGAGCCTTTTCT	SYBR Green
	pdgfrb-AS	TTGGGATCAGGGATGGGGAT	
cspg4 (Fig. S3C)	*cspg4-S*	AAGTGGCAAGATGAGAGCCC	SYBR Green
	cspg4-AS	ATGCTCCATTGGTGGTCTGG	
dll4 (Fig. S3C)	*dll4-S*	GGACAAATGCACCAGTATGC	SYBR Green
	dll4-AS	GTTTGCGCAGTCGTTAATGT	
notch1b (Fig. S3C)	*notch1b-S*	ATGCATCTTTTCTTCGTGAA	SYBR Green
	notch1b-AS	TCTCCCGTCTGCAGTTGGTT	
notch2 (Fig. S3C)	*notch2-S*	GCCTGGACTGCTCTGAAAAC	SYBR Green
	notch2-AS	CATTGACAGGGTTTGTGTCG	
18S ribosomal RNA (Fig. 3C)	*18S-S*	AATCGCTCCACCAACTAAGAAC	SYBR Green
	18S-AS	AGTATGGTTGCAAAGCTGAAAC	
notch2 (Fig. S3D,F)	Dr03436779 (Thermo fisher scientific)		FAM
notch3 (Fig. S3D,G)	Dr03432970 (Thermo fisher scientific)		FAM
dlc (Fig. S5A)	Dr03073935 (Thermo fisher scientific)		FAM
dll4 (Fig. S5A)	Dr03428646 (Thermo fisher scientific)		FAM
actb1 (Fig. S3D,F,5A)	Dr03432610 (Thermo fisher scientific)		VIC

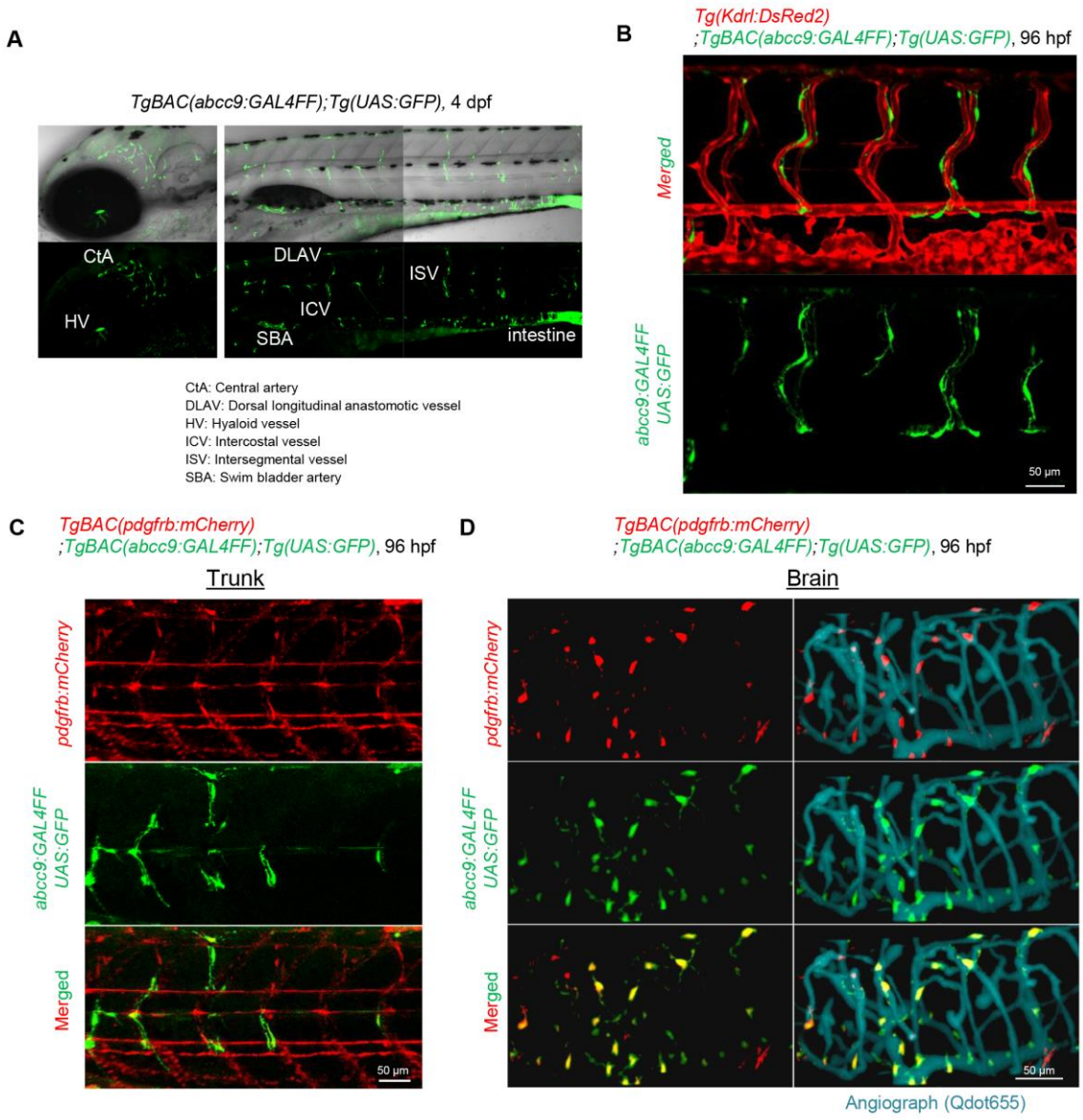


Figure S1. *TgBAC(abcc9:GAL4FF)* zebrafish line. (**A**) Lateral view of the *TgBAC(abcc9:GAL4FF);Tg(UAS:GFP)* larva at 4 dpf. Upper, the merged image of bright field image and *UAS:*GFP; lower, *UAS:*GFP. (**B-D**) Lateral view of confocal stack image of trunk vasculature of *TgBAC(abcc9:GAL4FF);Tg(UAS:GFP);Tg(kdrl:DsRed2)* larva (**B**), trunk (**C**) or brain vasculature (**D**) of *TgBAC(abcc9:GAL4FF);Tg(UAS:GFP);Tg(pdgfrb:mCherry)* larva at 96 hpf. Lateral view, anterior to the left. Note that *abcc9* reporter positive cells were hardly detected beneath the DA (**B**, **C**). In **D**, vascular structure was visualized by injecting Qdot655 into the circulation (aqua).

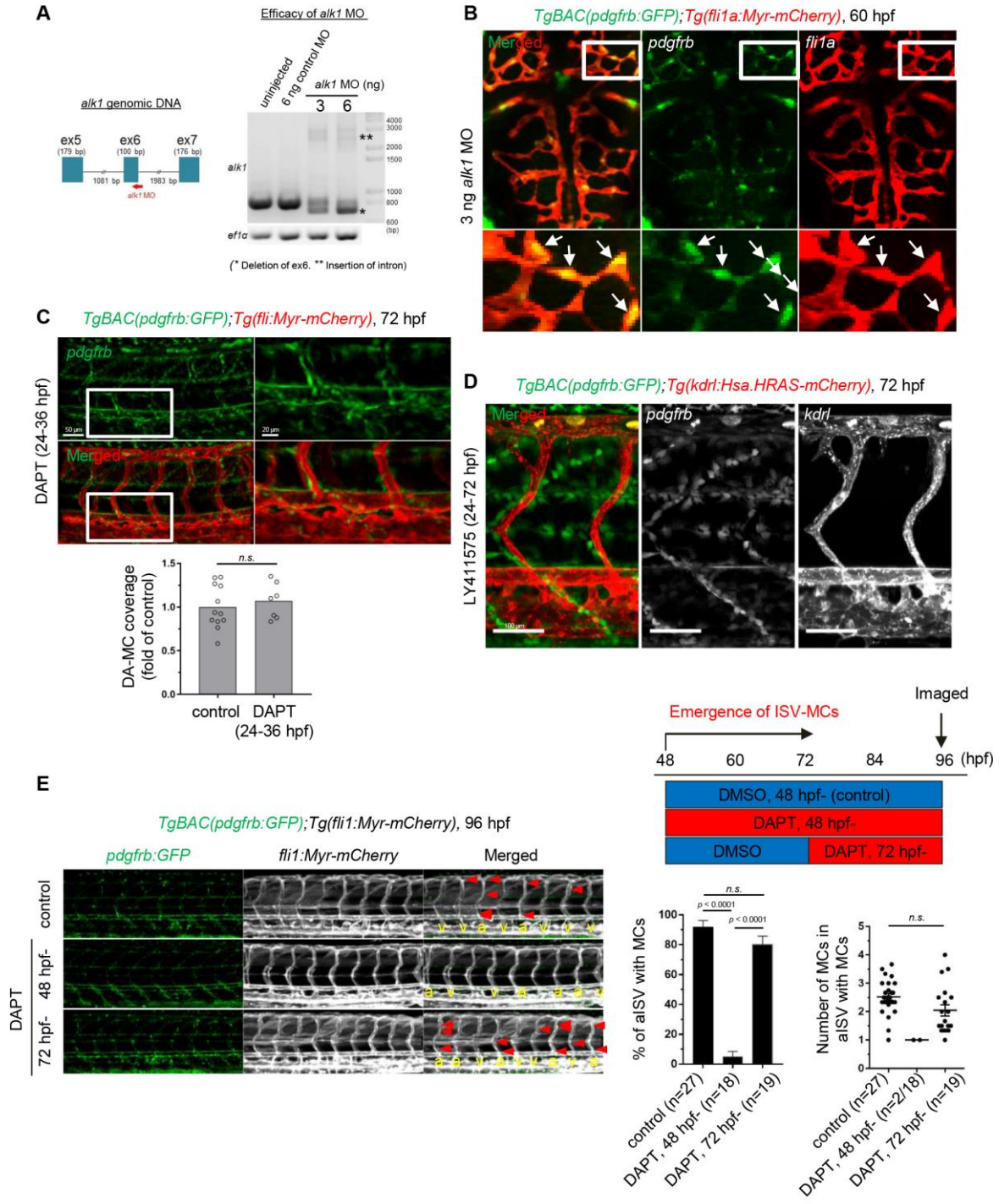


Figure S2. *alk1* MO efficacy and effects of DAPT on MC emergence. (**A**) The schematic model of *alk1* MO target site (left). *alk1* MO targets the border between exon 6 and intron 7, resulted in the skipping of exon 6 (*) or the failure of removal of intron 7 (**) as shown in the right PCR result. (**B**) Confocal stack images of the central arteries of 60 hpf *TgBAC(pdgfrb:GFP);Tg(fli1a:Myr-mCherry)* embryos injected with 3 ng *alk1* MO. Boxed regions are enlarged to the bottom. Note that *pdgfrb*^{high} MCs were not detected around central arteries but *fli1a:*Myr-mCherry-positive ECs became positive for *pdgfrb:*GFP (arrows). (**C**) Representative confocal stack images of the trunk vasculature of 72 hpf *TgBAC(pdgfrb:GFP);Tg(fli1a:Myr-mCherry)* larva treated with 100 μM DAPT from 24-36

hpf. Boxed regions are enlarged to the right. The quantitative analysis of DA-MC coverage is shown below the images. Note that DAPT treatment before the DA-MC emergence period (36 hpf-) did not affect the DA-MC emergence. (**D**) Representative confocal stack images of the trunk vasculature of 72 hpf *TgBAC(pdgfrb:GFP);Tg(fli1a:Myr-mCherry)* larva treated with 5 μM LY411575 from 24-72 hpf. (**E**) Confocal stack images of ISVs in the 96 hpf *TgBAC(pdgfrb:GFP);Tg(fli1a:Myr-mCherry)* embryos treated with DMSO (control), 100 μM DAPT from 48 hpf to 96 hpf, or DMSO from 48 hpf to 72 hpf and subsequent DAPT from 72 hpf to 96 hpf, as shown in the schematic model on the right top. Arrowheads indicate MCs in ISVs. a, aISV. v, vISV, The percentage of aISV covered by *pdgfrb*^{high} MCs and the number of MCs in one aISV with MCs, as observed in the right images, are shown on the right. In embryos treated with DAPT from 48 hpf, only two out of 18 embryos showed the aISV with *pdgfrb*^{high} MC.

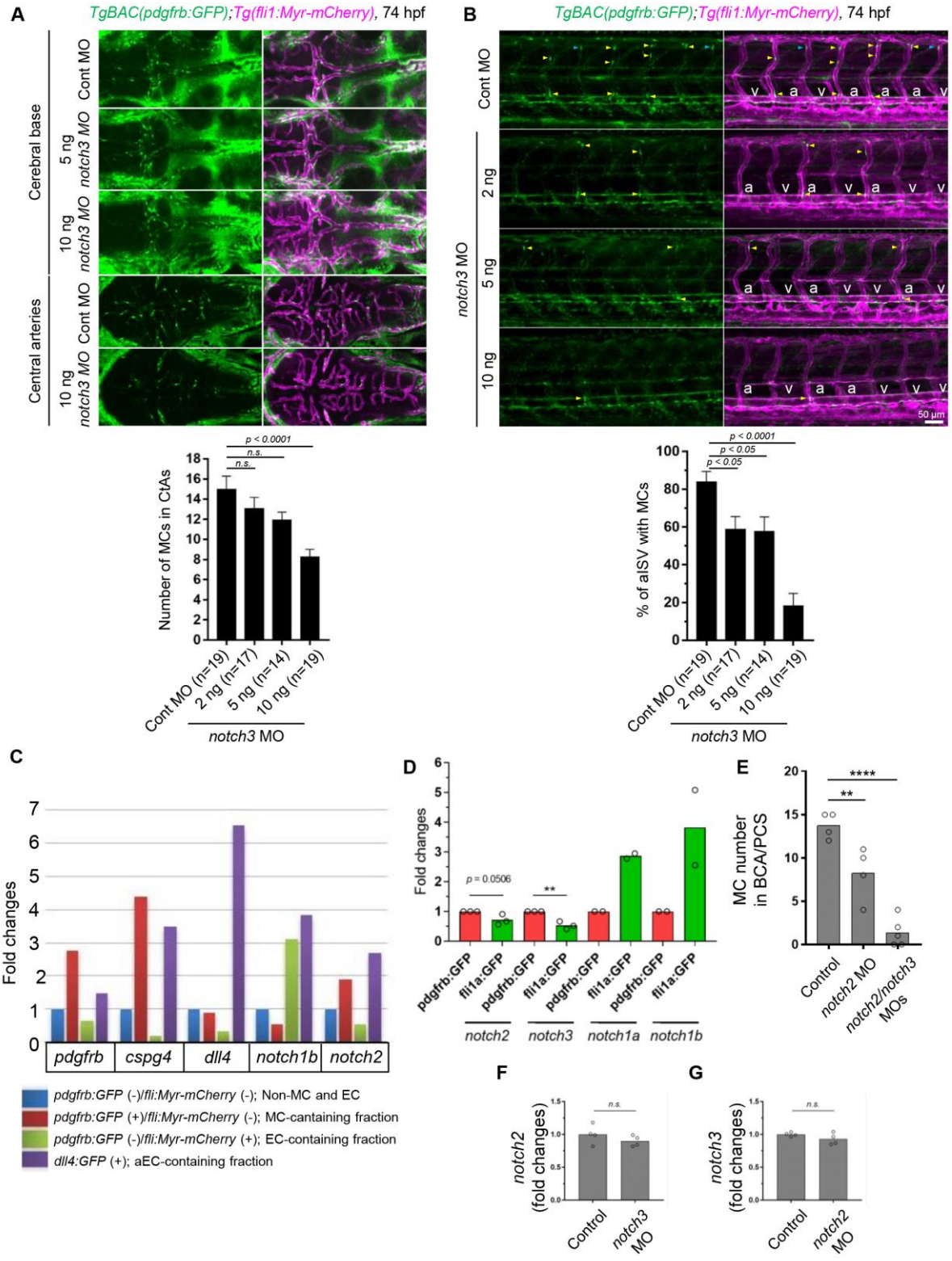


Figure S3. Effects of *notch2* or *notch3* depletion on MC emergence. (**A**) Confocal stack images of brain vessels in the 74 hpf *TgBAC(pdgfrb:GFP);Tg(fli1a:Myr-mCherry)* larvae injected with 10 ng control MO or 2-10 ng *notch3* MO. The MC number in central arteries of larvae injected with control MO or 2-10 ng *notch3* MO is shown on the bottom of confocal images. (**B**) Confocal stack images of trunk vessels in the 74 hpf

TgBAC(pdgfrb:GFP);Tg(fli1a:Myr-mCherry) larvae injected with 10 ng control MO or 2-10 ng *notch3* MO. Arrowheads indicate MCs in ISVs. a, aISV. v, vISV, The percent of aISV covered by *pdgfrb*^{high} MCs is shown on the bottom of confocal images. The percentage of aISV with MCs was significantly reduced at 10 ng *notch3* MO, however, even 10 ng *notch3* MO did not completely block the MC-emergence in aISV and beneath the DA. (**C**) qPCR analysis of *pdgfrb*, *cspg4, dll4*, *notch1b,* and *notch2* expression. The *TgBAC(pdgfrb:GFP);Tg(fli1a:Myr-mCherry)* or *Tg(dll4:GFP)* larvae at 4 dpf were subjected to FACS Aria and *pdgfrb*-GFP (-)/*fli1a*-mCherry (-), *pdgfrb*-GFP (+)/*fli1a*-mCherry (-), *pdgfrb*-GFP (-)/*fli1a*-mCherry (+), or *dll4*-GFP (+) cells were sorted. Then, after purification of mRNAs from sorted cells and subsequent cDNA synthesis, qPCR analysis was performed against targets in the figure. (**D**) *pdgfrb:*GFP (+) or *fli1a:*GFP (+) cells were sorted from 72 hpf *TgBAC(pdgfrb:GFP)* or *Tg(fli1a:GFP)* larvae and subjected to qPCR analysis of *notch2, notch3, notch1a,* and *notch1b* expression. While *notch2* and *notch3* expressions were higher in MC-containing *pdgfrb*+ population, *notch1a* and *notch1b* expression were higher in EC-containing *fli1a*+ population. (**E**) The MC number in BCA and PCS of 78 hpf larvae injected with 10 ng control MO, 10 ng *notch2* MO, or 5 ng each of *notch2* and *notch3* MOs, shown in Fig. 3A. (**F**, **G**) qPCR analysis of *notch2* or *notch3* expression in whole 72 hpf larvae injected with 5 ng *notch3* MO or 5 ng *notch2* MO, respectively. **p<0.01, and ****p<0.0001, significant difference between indicated two groups. *n.s.,* not significant between two groups.

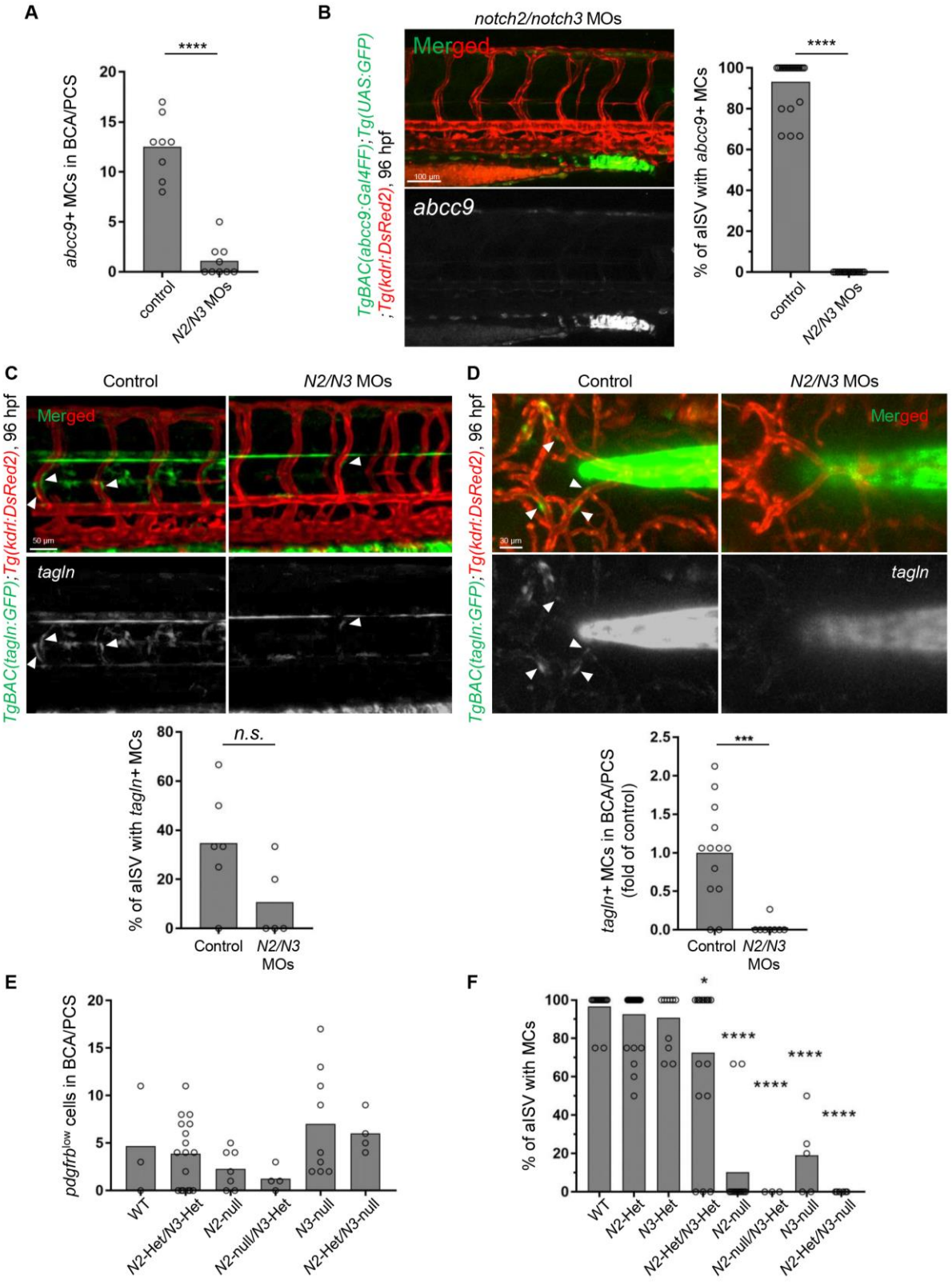


Figure S4. Effects of both *notch2* and *notch3* depletion on MC emergence. (**A**) The number of *abcc9*+ MCs in BCA and PCS in Fig. 3D. (**B**) Confocal stack images of trunk vessels in the 96 hpf *TgBAC(abcc9:GAL4FF);Tg(UAS:GFP);Tg(kdrl:DsRed2)* larva injected

with 5 ng each of *notch2* MO and *notch3* MO (N2/N3 MOs). The percent of aISV covered by *abcc9*+ MCs is shown on the right. (**C**, **D**) Confocal stack images of trunk vessels (**C**) or vessels located on cerebral base (**D**) in the 96 hpf *TgBAC(tagln:GFP);Tg(kdrl:DsRed2)* larvae injected with 5 ng each of *notch2* MO and *notch3* MO (N2/N3 MOs). The percent of aISV covered by *tagln*+ MCs (**C**) or the number of *tagln*+ MCs in BCA/PCS (**D**) are shown on the bottom of images. (**E**) The number of *pdgfrb*^{low} cells in BCA and PCS of *notch2/notch3* (N2/N3) mutants observed in Fig. 3E. No significant differences between WT and any mutants. (**F)** The percent of aISV with MCs in *notch2/notch3* (N2/N3) mutants observed in Fig. 3G. *p<0.05, ***p<0.001, and ****p<0.0001, significant difference comparing to control or WT. *n.s.,* not significant between two groups.

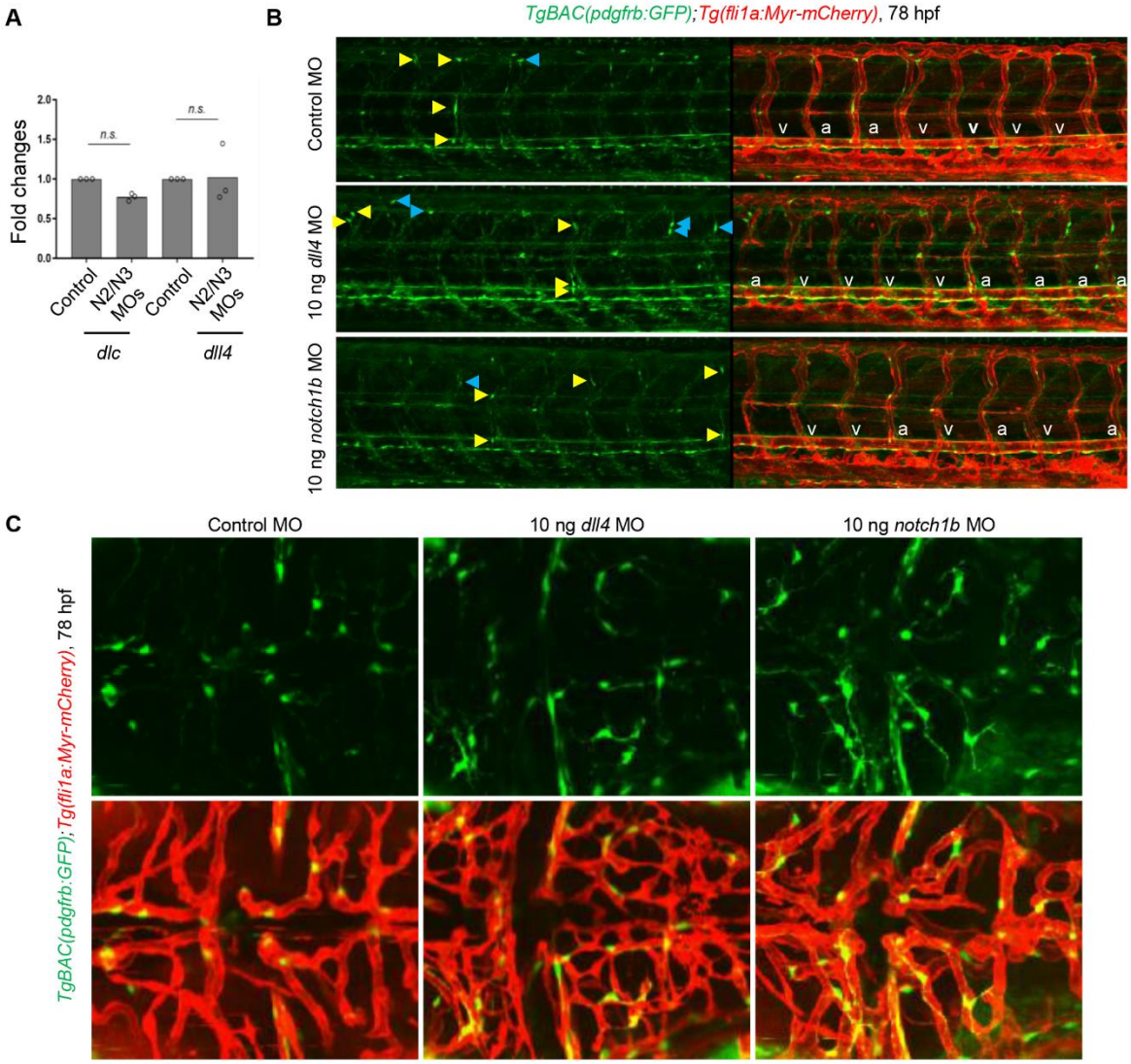


Figure S5. Effects of *notch2/3*, *dll4,* or *notch1b* depletion. (**A**) qPCR analysis of *dlc* and *dll4* expression in whole 72 hpf larvae injected with 5 ng each of *notch2* and *notch3* MOs (N2/N3 MOs). (**B**) Confocal stack images of trunk vessels in the 78 hpf *TgBAC(pdgfrb:GFP);Tg(fli1a:Myr-mCherry)* larvae injected with control MO, 10 ng *dll4*, or 10 ng *notch1b* MO. a, aISV. v, vISV. Yellow or blue arrowheads, *pdghrb*^{high} MCs in aISV or vISV, respectively. (**C**) Dorsal view of confocal stack images of brain vessels (mainly CtAs) in the 78 hpf *TgBAC(pdgfrb:GFP);Tg(fli1a:Myr-mCherry)* larvae injected with control MO, 10 ng *dll4*, or 10 ng *notch1b* MO.

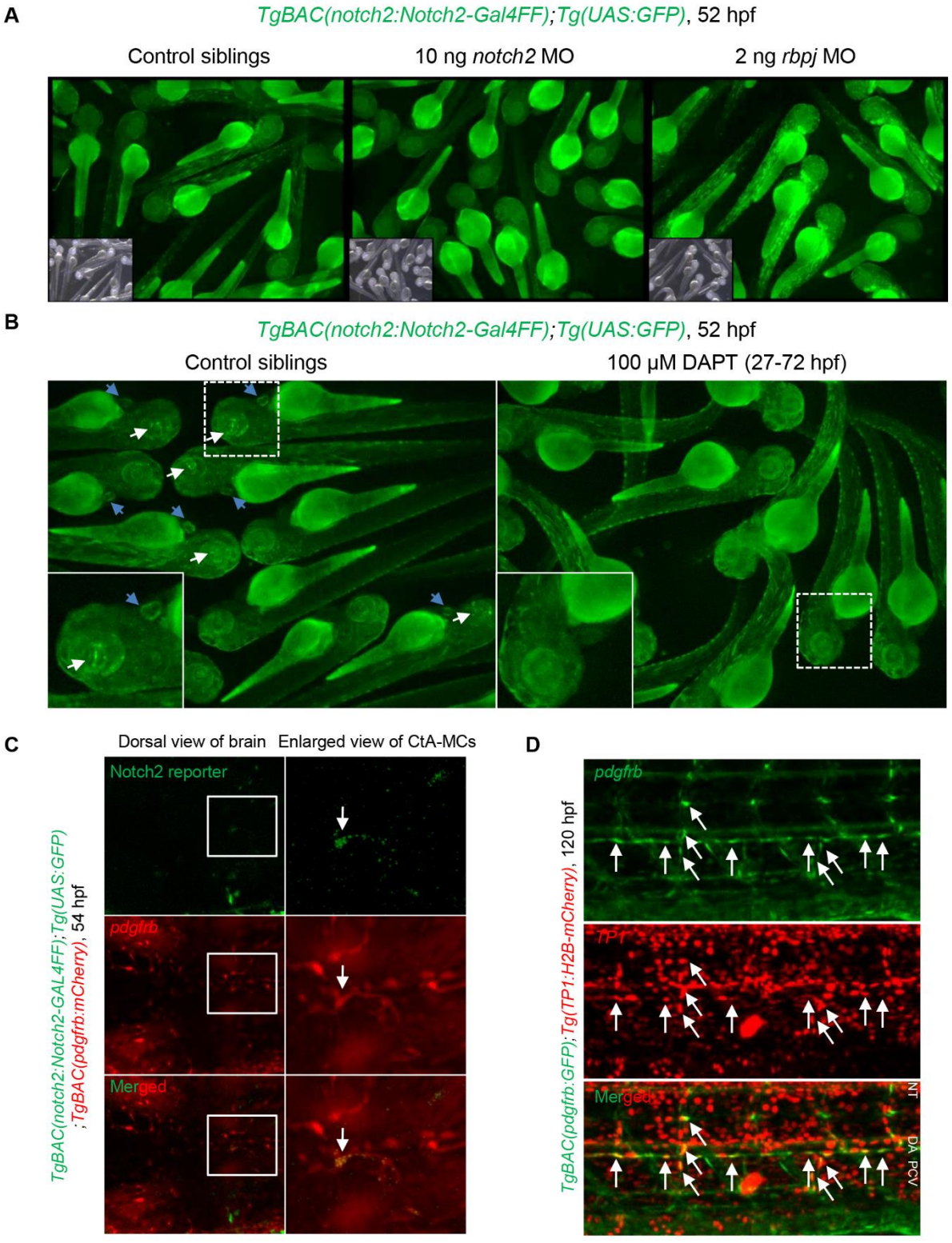


Figure S6. Visualization of Notch activity. (**A**) The 52 hpf *TgBAC(notch2:Notch2-GAL4FF);Tg(UAS:GFP)* uninjected embryos (Control siblings) or embryos injected with 10 ng *notch2* MO or 2 ng *rbpj* MO. Bright field images are inserted bottom left of each *UAS:*GFP images. Note that GFP expression was canceled by *notch2* MO whereas that was enhanced by *rbpj* MO. (**B**) The 72 hpf *TgBAC(notch2:Notch2-GAL4FF);Tg(UAS:GFP)*

larvae treated with DMSO or 100 μM DAPT from 22 to 72 hpf. Boxed regions are enlarged to bottom left of each images. GFP signals detected in the heart (blue arrows) or in the eye (white arrows) in control siblings were inhibited in DAPT treated larvae, although the reduction of GFP signal in trunk was not apparent in this DAPT treatment condition. (**C**) Confocal stacked images of 54 hpf brain (dorsal view) of *TgBAC(notch2:Notch2-GAL4FF);Tg(UAS.GFP);TgBAC(pdgfrb:mCherry)* embryos. Arrows indicate faint Notch2-activation in a CtA-MC. Embryos with Notch2-activity positive MCs in brain at 54 hpf were 7.14 % (1/14 embryos). (**D**) Confocal stacked images of trunk region of 120 hpf *TgBAC(pdgfrb:GFP);Tg(TP1:H2B-mCherry)* larva. Arrows indicate *TP1* positive MCs.

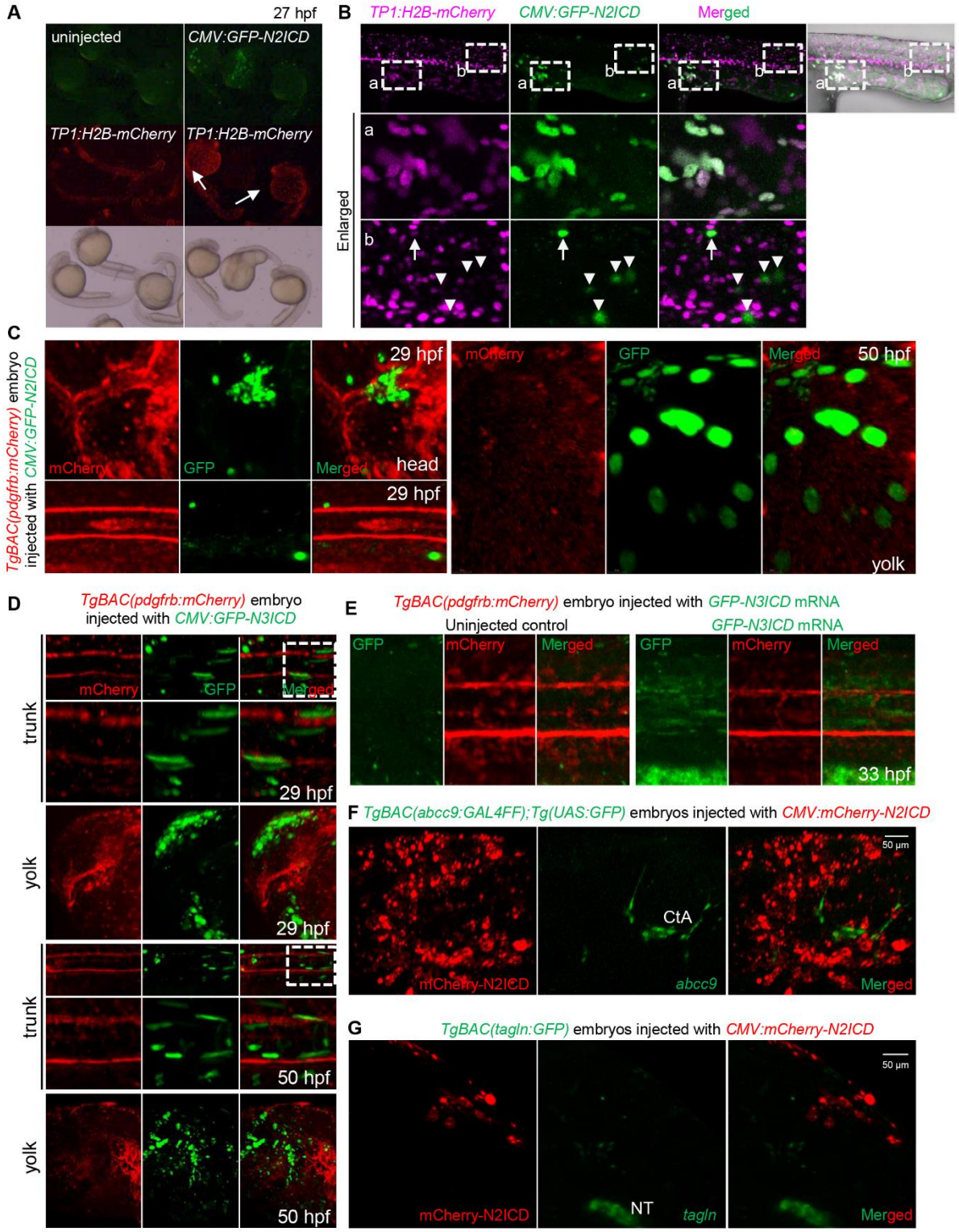


Figure S7. Effects of overexpression of N2ICD or N3ICD on MC markers expression. (**A**) The 27 hpf *Tg(TP1:H2B-mCherry)* embryos injected with a plasmid encoding *CMV:GFP-N2ICD* (right) or uninjected control (left). Overexpression of GFP-N2ICD enhanced *TP1:*H2B-mCherry expression especially around yolk sac. (**B**) Confocal stack images of the 27 hpf *Tg(TP1:H2B-mCherry)* embryos injected with a plasmid encoding *CMV:GFP-N2ICD.* Boxed regions are enlarged below. In yolk tube, all GFP-N2ICD positive

cells were also positive for *TP1:*H2B-mCherry (**a**). While, some N2ICD-GFP positive cells were also positive for *TP1:*H2B-mCherry (arrows) but some of them were negative for the *TP1:*H2B-mCherry (arrowheads) in the trunk (**b**). This data may suggest the differences of the sensitivity to N2ICD among the cell types. (**C**) Confocal stack images of the 29 hpf or 50 hpf *TgBAC(pdgfrb:mCherry)* embryos injected with a plasmid encoding *CMV:GFP-N2ICD.* Note that GFP-N2ICD could not induce *pdgfrb:mCherry* expression in any body parts including yolk sac even though GFP-N2ICD clearly enhanced *TP1:*H2B-mCherry expression, as shown in **A** and **B**. (**D**, **E**) Confocal stack images of the 29 hpf, 33 hpf, or 50 hpf *TgBAC(pdgfrb:mCherry)* embryos injected with a plasmid encoding *CMV:GFP-N3ICD* (**D**) or *GFP-N3ICD* mRNA (**E**). N3ICD could not induce *pdgfrb:*mCherry expression. (**F**, **G**) Confocal stack images of the 50 hpf *TgBAC(abcc9:GAL4FF);Tg(UAS:GFP)* (**F**) or *TgBAC(tagln:GFP)* embryos (**G**) injected with a plasmid encoding *CMV:mCherry-N2ICD.* N2ICD could not induce *abcc9- nor tagln*-reporter expression.

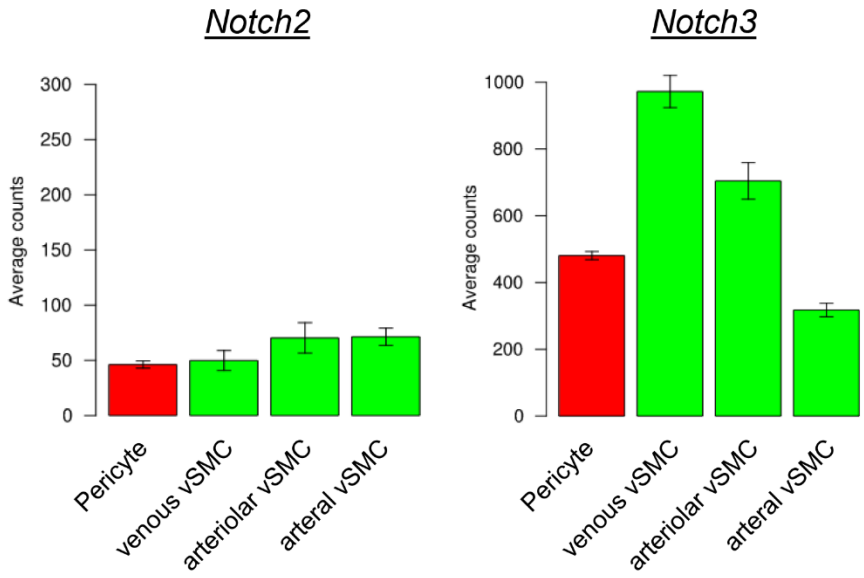
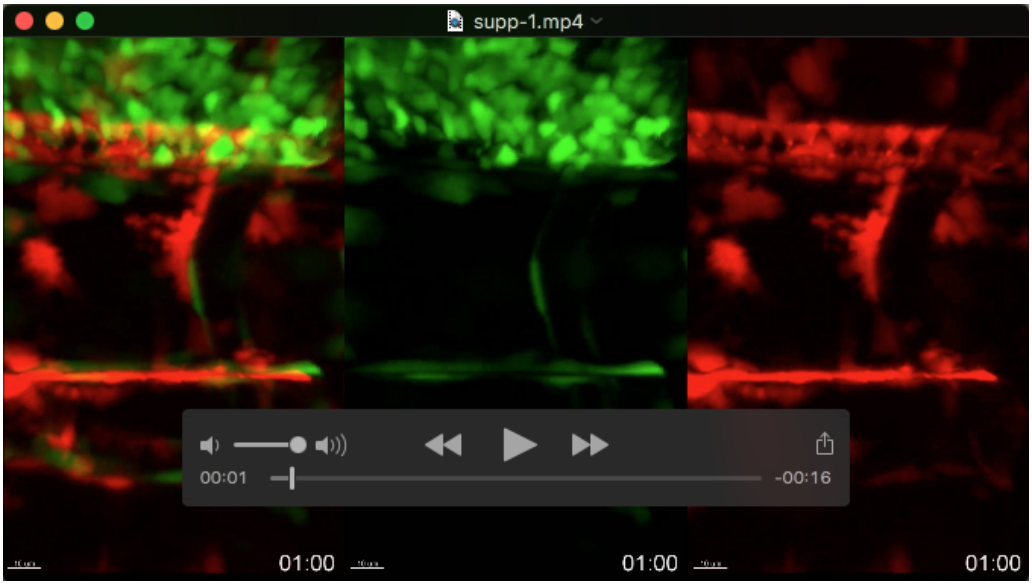


Figure S8. *Notch2* and *Notch3* mRNA expression in mouse adult brain. Average expression level of *Notch2* or *Notch3* mRNA in the adult mouse brain pericyte, venous vSMC, arteriolar vSMC, or arterial vSMC from *(http://betsholtzlab.org/VascularSingleCells/database.html)* (Vanlandewijck et al., 2018).



Movie 1. Notch activation during the specification process into *pdgfrb*^{high} MCs. Time-lapse confocal images of GFP (green) and RFP (red) fluorescence in the ISV region of *Tg(TP1:GFP);TgBAC(pdgfrb:GAL4FF);Tg(4xUAS:RFP)* from 42.6-80 hpf. Dorsal to the top, ventral to the bottom, anterior to the left. Images were obtained every 12 min and the relative time is shown on the bottom (hr:min). Arrows indicate the MCs with *TP1:*GFP and show up when *TP1:*GFP starts to be detected in *pdgfrb*+ cells, even though GFP expression in the cell above is faint at the beginning. Note that *pdgfrb*+ cells started to extend processes and migrate, or proliferate after the onset of *TP1:*GFP expression. While, *pdgfrb*+ cells without *TP1:*GFP expression reduced their fluorescence.

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