## MATERIAL 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). pCS2_EGFP_KanR or pCS2_mCherry_KanR was generated by replacing Gal4FF in pCS2_Gal4FF_KanR vector with a PCR-amplified EGFP or mCherry cDNA.

A cDNA encoding nuclear localization signal (NLS)-tagged mCherry was amplified by PCR using pTolflk1-NLS-mCherry vector as a template (Fukuhara et al., 2014) and subcloned into the pTol2-E1b-UAS-E1b vector to construct the pTol2-NLS-mCherry-E1b-UAS-E1b plasmid. To generate the pTol2-UAS:NLS-mCherry, mVenus-geminin plasmid, a cDNA encoding mVenus fused to the N-terminal 100 aa of zebrafish geminin (mVenus-geminin) was amplified by PCR using pTolflk1-mVenus-zGem (1/100) plasmid as a template (Fukuhara et al., 2014) and subsequently inserted into the pTol2-NLS-mCherry-E1b-UAS-E1b vector.

To construct the plasmid encoding Pecam1-EGFP, a cDNA encoding full length of zebrafish *pecam1* (NM_001113799) was amplified by PCR using cDNA library derived from zebrafish embryos as a template and cloned into pEGFP-C1 vector (Clontech, Takara Bio Inc., Shiga, Japan). The pecam1-EGFP cDNA was subcloned into pTol2-fli1a vector (Fukuhara et al., 2014) to generate pTol2-fli1a-pecam1-EGFP plasmid.

To construct the plasmid encoding pTol2-5xUAS-loxP-mCherry-loxP-mVenus, two oligonucleotides encoding loxP sequence (ataacttcgtatagcatacattatacgaagttat) were

inserted into the pcDNA3.1 vector (Invitrogen, Carlsbad, CA). Then, a cDNA encoding mCherry and that expressing mVenus were PCR-amplified and sequentially inserted between two loxP sites and downstream of the second loxP site to generate the pcDNA3.1-loxP-mCherry-loxP-mVenus, respectively. Finally, the loxP-mCherry-loxP-mVenus cDNA was subcloned into the pTol2-5xUAS vector, a gift from K. Kawakami (National Institute of Genetics, Japan) (Asakawa et al., 2008; Distel et al., 2009), to generate the pTol2-5xUAS-loxP-mCherry-loxP-mVenus.

For construction of pTol2-IF-NLS-mCherry-HS4-fli-iRFP plasmid, PCR-amplified cDNA encoding NLS-tagged mCherry was subcloned into pTol2 vector to construct pTol2-NLS-mCherry. Then, a cDNA encoding the chicken β-globin insulator (HS4) was amplified by PCR using pJC13-1 vector, a gift from G. Felsenfeld (National Institute of Health, USA) (Yusufzai and Felsenfeld, 2004), as a template and subcloned into the pTol2-NLS-mCherry vector to generate pTol2-NLS-mCherry-HS4. To construct pTol2-NLS-mCherry-HS4-fli1a, a cDNA encoding fli1a promoter was PCR-amplified using pTol2-fli1a vector (Fukuhara et al., 2014) as a template and inserted downstream of HS4 insulator in the pTol2-NLS-mCherry-HS4 vector. Subsequently, a cDNA encoding -4.5 kb intestinal fatty acid binding protein (IF) promoter region (Her et al., 2004) was amplified by PCR using genomic DNA derived from zebrafish embryos as a template and 5'- aaagagactgaaagggcagaagcacagg-3' and 5'- gatgatgacagactgttgtgtgatc-3' primers. This DNA was subcloned into the upstream of NLS-mCherry in the pTol2-NLS-mCherry-HS4-fli1a vector to generate pTol2-IF-NLS-mCherry-HS4-fli1a. Finally, a pTol2-IF-NLS-mCherry-HS4-fli-iRFP plasmid was constructed by inserting a PCR-amplified cDNA encoding iRFP670, a gift from A. Sakakibara (Chubu University, Japan), into the downstream of fli1a promoter in the pTol2-IF-NLS-mCherry-HS4-fli1a

vector.

A cDNA encoding Cre recombinase (Kogata et al., 2006) followed by 2A peptide and mCherry was subcloned into pTol2-fli1a vector to generate pTol2-Cre-2A-mCherry. Then, a cDNA encoding -2.1 kb ntla promoter region was amplified by PCR using genomic DNA derived from zebrafish embryos as a template and 5'-TAACGCGTATACAATTCCTTTGTGCTGTTGCAACAC-3' and 5'-ATGCTAGCATTTCCGATCAAATAAAGCTTGAGAT-3' primers, and subcloned into the pTol2-Cre-2A-mCherry vector to generate pTol2-ntla-Cre-2A-mCherry.

**BAC recombineering**

pRedET plasmid (GeneBridge, Heidelberg, Germany) was introduced into E. coli containing either CH1073-606I16 BAC clone encoding *pdgfrb* gene (BacPAC resources, Oakland, CA) or CH1073-307D13 BAC clone encoding *tagln* gene by electroporation (1800 V, 25 μF, 200 Ω) to increase the efficiency of homologous recombination. Next, two Tol2 long terminal repeats in opposing directions flanking an ampicillin resistance cassette was amplified by PCR using Tol2_amp as a template and were inserted into the BAC vector backbone. Then, the cDNA encoding either EGFP, mCherry or Gal4FF together with a kanamycin resistance cassette (EGFP_KanR, mCherry_KanR or Gal4FF_KanR) was amplified by PCR using pCS2_EGFP_KanR, pCS2_mCherry_KanR or pCS2_Gal4FF_KanR plasmid as a template, respectively, and inserted at the start ATG of the *pdgfrb* or *tagln* gene. Primers to amplify the EGFP_KanR, mCherry_KanR or Gal4FF_KanR PCR product are as follows:

EGFP_KanR for CH1073-606I16,

5'-tgttgttttctctccgtctgcagtgttgaatgtgtcctgctctagaagaaACCATGGTGAGCAAGGGCGAG

GAG-3' and

5'-ttgtgatagcagtgaataggaagtggatgcggctgatggtcgaactcttTCAGAAGAACTCGTCAAGA AGGCG-3'; To amplify the mCherry_KanR for CH1073-606I16, the same primer pair for EGFP_KanR for CH1073-606I16 was used; Gal4FF_KanR for CH1073-606I16, 5'-tgttgttttctctccgtctgcagtgttgaatgtgtcctgctctagaagaaACCATGAAGCTACTGTCTTCTA TCGAAC-3' and

5'-ttgtgatagcagtgaataggaagtggatgcggctgatggtcgaactcttTCAGAAGAACTCGTCAAGA AGGCG-3'; EGFP_KanR for CH1073-307D13,

5'-atcacaggtgtttctctgtggacgctctgctgtttctccatcagggagcgACCATGGTGAGCAAGGGCGA GGAG-3' and

5'-atcttatcctgcacctgccggctcagcccataagacggccccttgtttgcTCAGAAGAACTCGTCAAGA AGGCG-3' (lowercase; homology arm to BAC vector. uppercase; primer binding site to the template plasmid).

**Transgenic and mutant 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, Austin, TX). To generate the *TgBAC(pdgfrb:EGFP)*$^{ncv22}$, *TgBAC(pdgfrb:mCherry)*$^{ncv23}$, *TgBAC(pdgfrb:Gal4FF)*$^{ncv24}$ and *TgBAC(tagln:EGFP)*$^{ncv25}$ zebrafish lines, the corresponding BAC DNA was co-injected with Tol2 transposase mRNA into one-cell stage embryos of wild type strain, AB. To establish the *Tg(UAS:NLS-mCherry,mVenus-geminin)*$^{ncv26}$, *Tg(fli1a:pecam1-EGFP)*$^{ncv27}$, *Tg(UAS:loxP-mCherry-loxP-mVenus)*$^{ncv28}$ and *Tg(IF:NLS-mCherry,fli1a:iRFP)*$^{ncv29}$ zebrafish lines, pTol2-UAS:NLS-mCherry,mVenus-geminin, pTol2-fli1a-pecam1-EGFP,

pTol2-5UAS-loxP-mCherry-loxP-mVenus and pTol2-IF-NLS-mCherry-HS4-fli1a-iRFP plasmids were injected along with Tol2 transposase mRNA into one-cell stage embryos, respectively. *Tg(UAS:GFP)* and *Tg(UAS:RFP)* fish lines were kindly provided by K. Kawakami (National Institute of Genetics, Japan) (Asakawa et al., 2008). *Tg(tbx6:Cre,myl7:EGFP)*$^{sq6}$ fish was kindly provided by T. J. Carney (Agency for Science, Technology and Research, Singapore) (Lee et al., 2013). *Tg(sox17:DsRed)*$^{s903}$ fish was obtained from the Zebrafish International Resource Center (University of Oregon, OR, USA). *Tg(fli1a:Myr-mCherry)*$^{ncv1}$, *Tg(fli1a:Myr-EGFP)*$^{ncv2}$ (Fukuhara et al., 2014), *Tg(flt1:mCherry)*$^{ncv30}$ (Kwon et al., 2013) and *Tg(sox10:Cre)*$^{ba74}$ (Rodrigues et al., 2012) fish lines were previously described. Throughout the text, all Tg lines used in this study are simply described without their line numbers. For example, *TgBAC(pdgfrb:EGFP)*$^{ncv22}$ is abbreviated to *TgBAC(pdgfrb:EGFP)*.

**FACS and RT-PCR**

Cranial parts of 60 *TgBAC(pdgfrb:EGFP);Tg(fli:Myr-mCherry)* larvae at 7 dpf were cut out and dissociated in 2 ml of protease solution (1% trypsin, 1 mM EDTA, pH 8.0 in PBS) containing 2.7 mg/ml collagenase P (Roche, Mannheim, Germany) for 1 h with gently pipetting every 30 min. The reaction was terminated by addition of 200 µl of stop solution (30% fetal bovine serum, 6 mM $CaCl_2$ in PBS). The dissociated cells were pelleted by centrifugation at 3,000 rpm for 5 min at 4 °C and washed with 1 ml ice-cold suspension media (1% fetal bovine serum, 0.8 mM $CaCl_2$, 50 U/ml penicillin, 0.05 mg/ml streptomycin in phenol red free DMEM [GIBCO, Grand Island, NY]). After the centrifugation, the cells were resuspended in 1 ml of ice-cold suspension media and sorted using a FACS Aria III sorter (BD Bioscience, San Jose, CA). The sorted cells

were then grouped into EGFP-negative/mCherry negative (double negative), EGFP-positive/mCherry negative (pdgfrb:EGFP (+), fli1a:Myr-mCherry (-)) and EGFP-negative/mCherry-positive (pdgfrb:EGFP (-), fli1a:Myr-mCherry (+)) cell populations. Total RNAs were purified from the sorted cells by using NucleoSpin RNA XS kit (Macherey-Nagel, Düren, Germany) and reverse transcribed by random hexamer primers using Superscript III (Invitrogen) according to the manufacturers' instructions. PCR amplification was carried out using KOD-FX (TOYOBO, Osaka, Japan) and the following primer sets: *pdgfrb*, 5'- CGTTCCCAGGAGCCTTTTCT-3' and 5'- TTGGGATCAGGGATGGGGAT-3'; *cspg4*, 5'-AAGTGGCAAGATGAGAGCCC-3' and 5'- ATGCTCCATTGGTGGTCTGG-3'; *acta2*, 5'- CCCAGCACTGTCAGGTGATT-3' and 5'- AAGCCTGCCTTACACAGTCC-3'; *tie1*, 5'- CATGGAGATCGCTGTCGTAA-3' and 5'- TGCATTTGCCTTTGTTCTTG-3'; *tie2*, 5'- AGCACACTCTCCTCACAGCA-3' and 5'- TTCGCCACAAAGTTCTCTCC-3'; *ef1a1l1* (*ef1a*), 5'- TCACCCTGGGAGTGAAACAGC-3' and 5'- ACTTGCAGGCGATGTGAGCAG-3'.

**Immunohistochemistry**

Approximately 1 mpf *TgBAC(pdgfrb:EGFP);Tg(fli1a:Myr-mCherry)* and *TgBAC(tagln:EGFP);Tg(fli1a:Myr-mCherry)* zebrafish were anesthetized and fixed with 4% paraformaldehyde at 4 °C overnight. After washing with PBS, their skins and scales were removed using scissors and forceps. The pleura region as shown in Fig. 1H and the caudal region as depicted in Fig. S1H were embedded in 4% agarose and cut transversely with VT1200S vibratome (Leica Microsystems, Wetzlar, Germany) into 200 µm sections. The tissue sections were washed with PBS-Tween 20 (PBST)

containing 0.1% TritonX-100 three times every for 5 min at 4 °C with shaking and treated with blocking reagent (5% sheep serum, 1% BSA and 0.1% TritonX-100 in PBST) for 1 h at R.T. Then, the samples were immunostained with anti-α-SMA antibody (sc-53142) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at 4°C overnight. Protein reacting with primary antibody was visualized with Alexa Fluor 633-labeled anti-mouse IgG (Molecular Probes, Invitrogen, Eugene, OR). Fluorescence images were obtained using a FluoView FV1000 or FV1200 confocal upright microscope (Olympus) equipped with water-immersion 20x (XLUMPlanFL, 1.0 NA) lens.

**Injections of morpholino oligonucleotides**

Control morpholino oligonucleotide (MO) (Gene Tools, LLC, Philomath, OR), 2 ng of *tnnt2a* MO, 4 ng of *foxd3* MO, 4 ng of *tfap2a* MO, 5 ng of *tbx6* MO or 5 ng of *hand2* MO was injected into embryos at one-cell stage. The sequence for the already-validated MOs used in this study are: *tnnt2a* MO, 5'-CATGTTTGCTCTGATCTGACACGCA-3' (Sehnert et al., 2002); *foxd3* MO, 5'- TGCTGCTGGAGCAACCCAAGGTAAG-3' (Whitesell et al., 2014); *tfap2a* MO, 5'- CCTCCATTCTTAGATTTGGCCCTAT-3' (Whitesell et al., 2014); *tbx6* MO, 5'- CATTTCCACACCCAGCATGTCTCGG-3' (Kawamura et al., 2005); *hand2* MO, 5'- CCTCCAACTAAACTCATGGCGACAG-3' (Reichenbach et al., 2008).

## Supplemental Figures

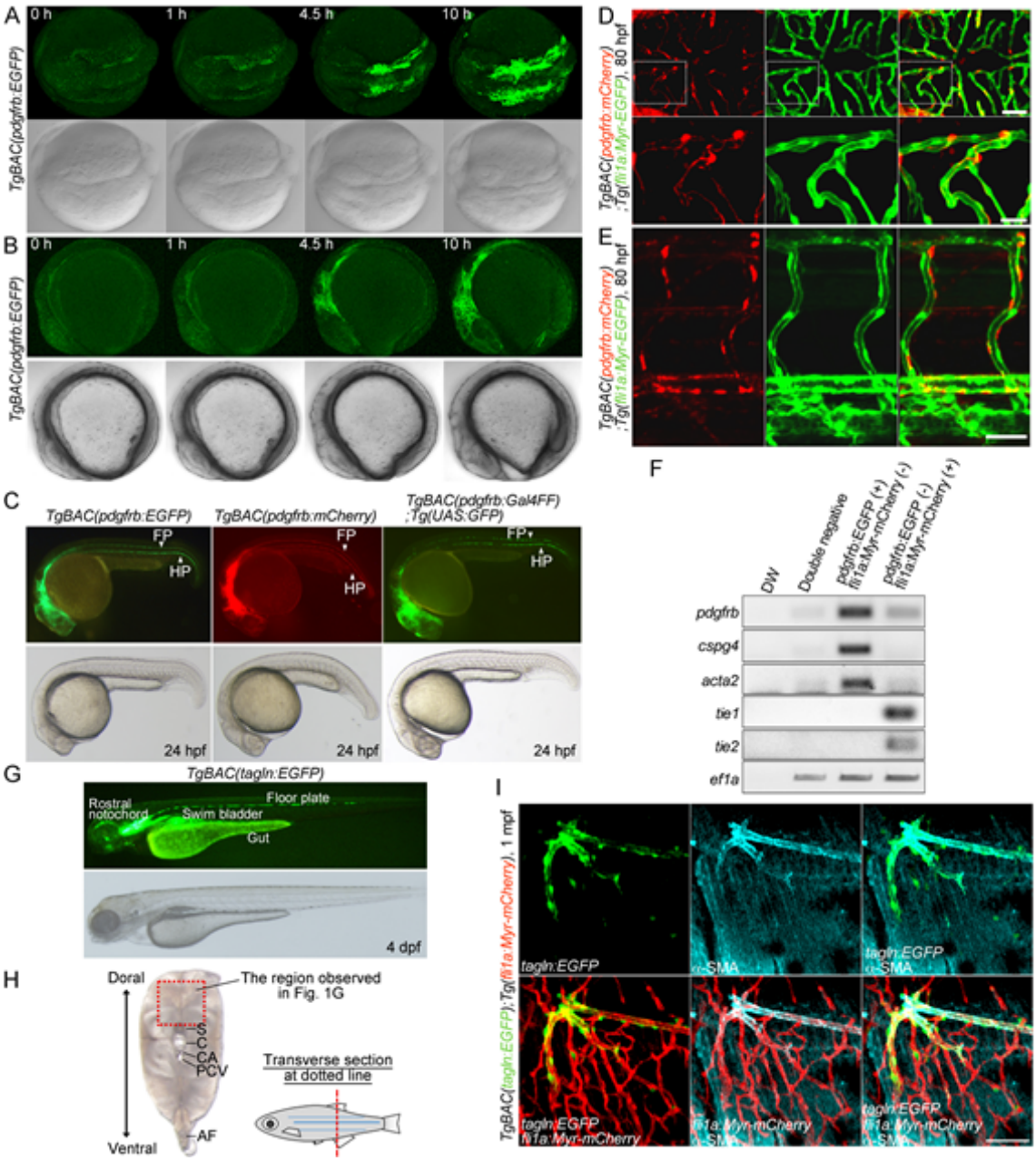



**Figure S1. Characterization of *TgBAC(pdgfrb:EGFP)* and *TgBAC(tagln:EGFP)* zebrafish lines.** (**A**, **B**) Time-lapse confocal images of the *TgBAC(pdgfrb:EGFP)* embryos from 8 somite stage. Dorsal (**A**) and lateral (**B**) view images at 8 somite stage (leftmost column) and their subsequent time-lapse images with the elapsed time (h) at the top. Upper, *pdgfrb:EGFP*; lower, bright field image. (**C**) Lateral views of *TgBAC(pdgfrb:EGFP)* (left), *TgBAC(pdgfrb:mCherry)* (center) and *TgBAC(pdgfrb:Gal4FF);Tg(UAS:GFP)* (right) zebrafish embryos at 24 hpf. Upper, *pdgfrb:EGFP* (left), *pdgfrb:mCherry* (center) and *pdgfrb:Gal4FF;UAS:GFP* (right);

lower, bright field images. FP, floor plate; HP, hypochord. (**D**, **E**) Dorsal view of CtAs (**D**) and lateral view of trunk vessels (**E**) in the *TgBAC(pdgfrb:mCherry);Tg(fli1a:Myr-EGFP)* larvae at 80 hpf. Anterior to the left. Left; *pdgfrb:mCherry*, center; *fli1a:Myr-EGFP*, right; the merged images of *pdgfrb:mCherry* (red) and *fli1a:Myr-EGFP* (green). The boxed areas in **D** are enlarged at the bottom. (**F**) Expression of MC markers (*pdgfrb*, *cspg4*, and *acta2*), EC markers (*tie1* and *tie2*) and *ef1a* in the EGFF and mCherry double-negative cells (Double negative), EGFP-positive and mCherry-negative cells (pdgfrb:EGFP (+), fli1a:Myr-mCherry (-)) and EGFP-negative and mCherry-positive cells (pdgfrb:EGFP (-), fli1a:Myr-mCherry (+)) isolated from the *TgBAC(pdgfrb:EGFP);Tg(fli:Myr-mCherry)* larvae by FACS at 7 dpf was analyzed by RT-PCR analyses. (**G**) Lateral view of the *TgBAC(tagln:EGFP)* zebrafish larva at 4 dpf. Upper, *tagln:EGFP*; lower, bright field image. (**H**) Transverse sectional image of caudal region of adult zebrafish. The boxed area indicates the region where the images shown in Figure **1G** were taken. S, spinal cord; C, caudal vertebra; CA, caudal artery; PCV, posterior cardinal vein; AF, anal fin. (**I**) Confocal images of blood vessels in the intercostal muscle of the 1 mpf *TgBAC(tagln:EGFP);Tg(fli1a:Myr-mCherry)* juvenile. The pleural tissue was immunostained with anti-α-SMA antibody to visualize VSMC, as in Fig. **1H**. *tagln:EGFP* (green), α-SMA (blue) and *fli1a:Myr-mCherry* (red) images are shown as indicated at the bottom left corner of each image. Scale bars, 20 μm (enlarged image in **D**), 50 μm (**D**, **E**, **I**).

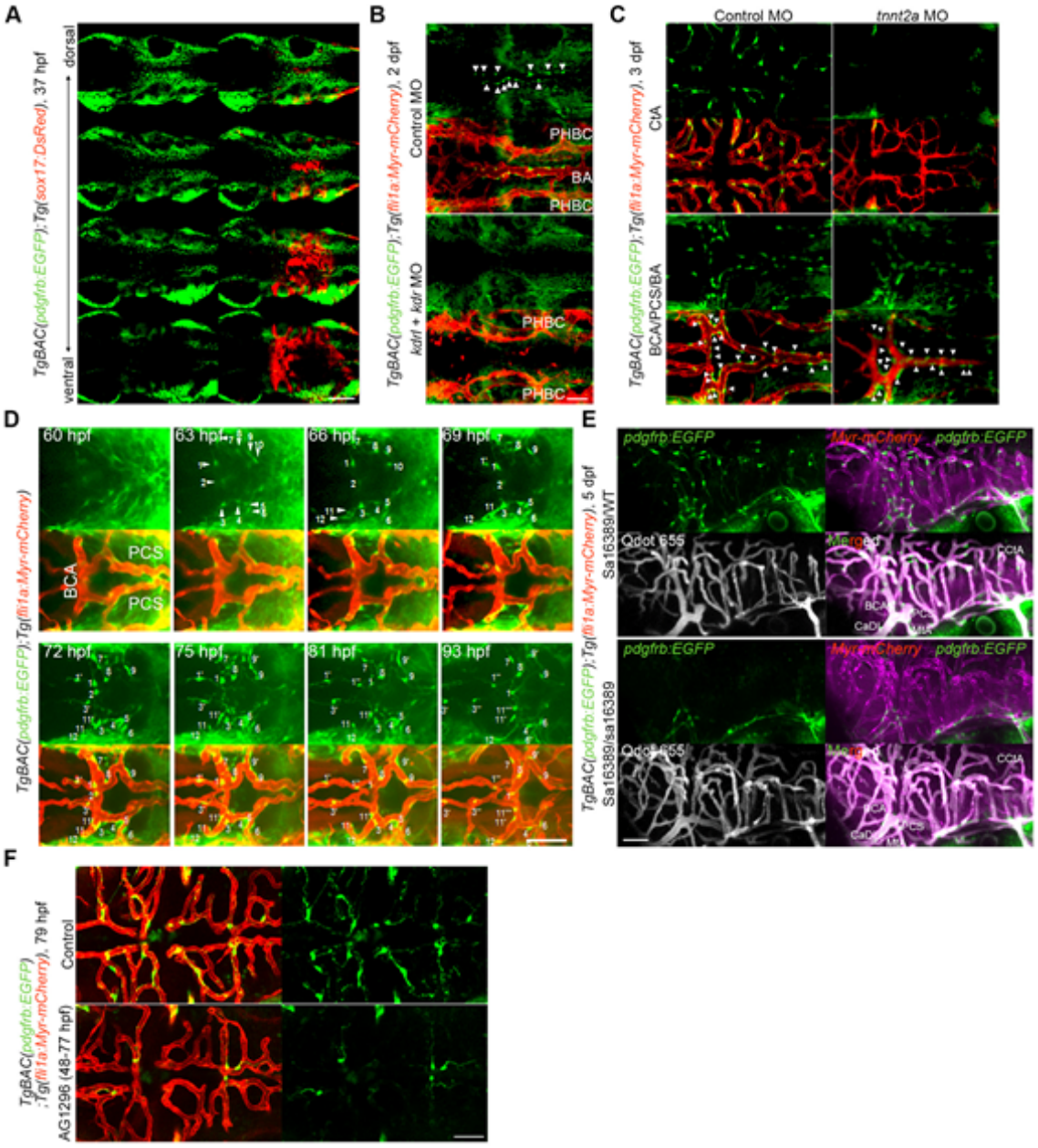



**Figure S2. MC coverage of cranial vessels.** (**A**) Confocal images of cranial region in the *TgBAC(pdgfrb:EGFP);Tg(sox17:DsRed)* embryo at 37 hpf. Dorsal view, anterior to the left. Multiple single z-plane images are shown (from the top (dorsal) to the bottom (ventral)). Left, *pdgfrb:EGFP*; right, the merged images of *pdgfrb:EGFP* (green) and *sox17:DsRed* (red). Note that EGFP-positive cells were not identical to DsRed-positive endodermal cells. (**B**) Confocal images of hindbrain vasculature in the 2 dpf *TgBAC(pdgfrb:EGFP);Tg(fli1a:Myr-mCherry)* embryos injected with control MO (upper two panels) or both *kdrl* and *kdr* MOs (lower two panels). Upper, *pdgfrb:EGFP*; lower, the merged images of *pdgfrb:EGFP* (green) and *fli1a:Myr-mCherry* (red).

Arrowheads indicate the MCs covering the BA. Note that *kdrl*/*kdr*-double morphant exhibited not only defective formation of BA but also lack of MCs in the midline of the cerebral base. (**C**) Confocal images of CtA (upper tow panels) and the vessels in the cerebral base (BCA/PCS/BA, lower two panels) in the 3 dpf *TgBAC(pdgfrb:EGFP);Tg(fli1a:Myr-mCherry)* larvae injected with control MO (left column) or *tnnt2a* MO (right column). Upper, *pdgfrb:EGFP*; lower, the merged images of *pdgfrb:EGFP* (green) and *fli1a:Myr-mCherry* (red). Arrowheads indicate the MCs covering the BCA, PCS and BA. Note that MC coverage was observed in the vessels in the cerebral base but not in the CtA of *tnn2a* morphants. (**D**) Time-lapse confocal images of the cranial vasculature in the *TgBAC(pdgfrb:EGFP);Tg(fli1a:Myr-mCherry)* embryo (60-93 hpf). Dorsal view, anterior to the left. Upper, *pdgfrb:EGFP*; lower, the merged images of *pdgfrb:EGFP* (green) and *fli1a:Myr-mCherry* (red). Numbers indicate individual EGFP-positive cells emerging around the PCS and BCA. EGFP-positive cells designated with prime marks indicate progeny cells. (**E**) Confocal images of cranial vessels of *pdgfrb* heterozygous (upper two panels, sa16389/WT) and homozygous (lower two panels, sa16389/sa16389) larvae in the *TgBAC(pdgfrb:EGFP);Tg(fli1a:Myr-mCherry)* background at 5 dpf. Lateral view, anterior to the left. To assess blood flow and blood vessel lumenization, Qdot 655 fluorescent probe was injected into the common cardinal vein just before imaging. Upper left, *pdgfrb:EGFP*; lower left, Qdot 655; upper right, the merged images of *pdgfrb:EGFP* (green) and *fli1a:Myr-mCherry* (magenta); lower right, the merged images of *pdgfrb:EGFP* (green), *fli1a:Myr-mCherry* (magenta) and Qdot 655 (white). (**F**) Confocal images of CtA in the *TgBAC(pdgfrb:EGFP);Tg(fli1a:Myr-mCherry)* larva treated with vehicle (upper) or 20 μM AG1296 (lower) during 48-77 hpf. Dorsal view, anterior to the left. Left, the merged images of *pdgfrb:EGFP* (green) and *fli1a:Myr-mCherry* (red); right, *pdgfrb:EGFP*. BA, basilar artery; PHBC, primordial hindbrain channel; CCtA, cerebellar central artery; BCA, basal communicating artery; PCS, posterior communicating segment; CaDI, caudal division of the internal carotid artery; MtA, metencephalic artery. Scale bars, 50 μm.

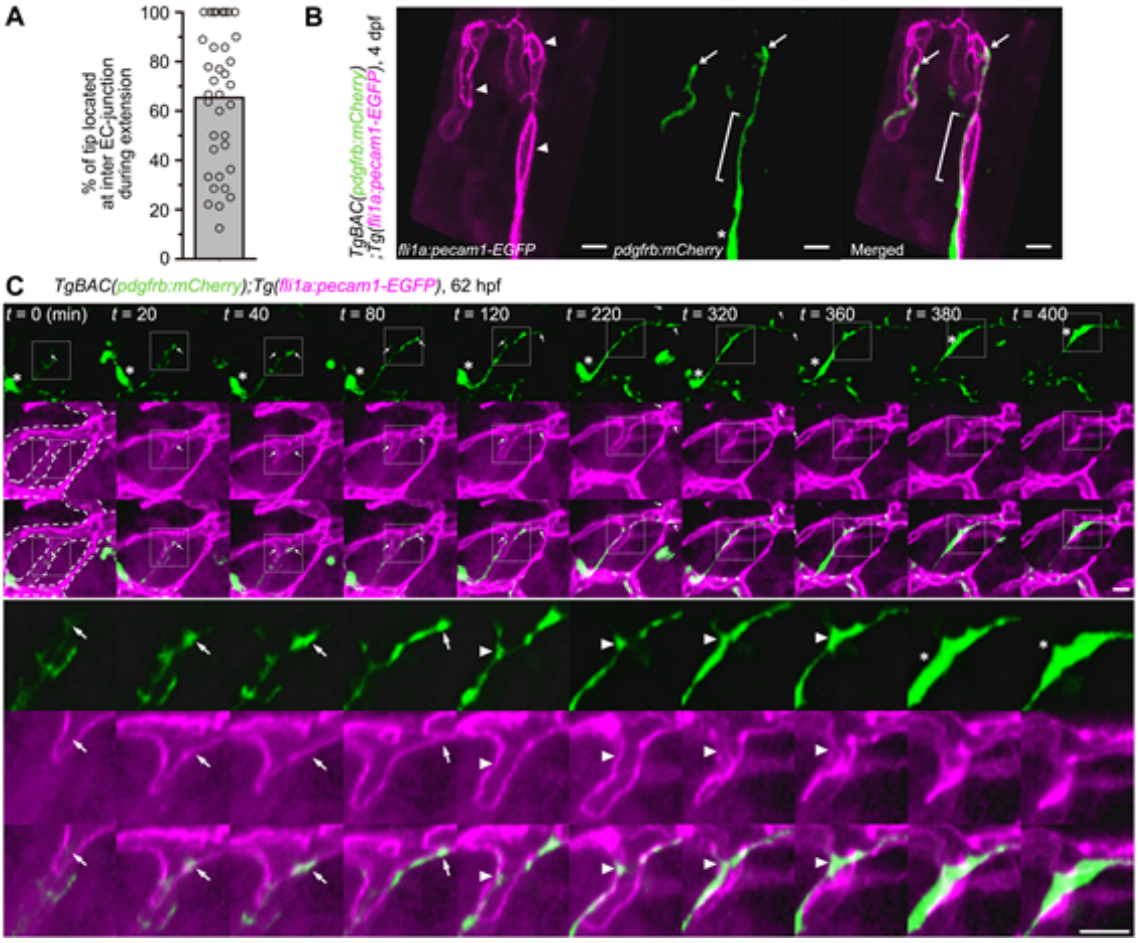



**Figure S3. Extension of MC processes and MC migration along the inter-EC junctions.** (**A**) Frequency of tip location of MC processes at the inter-EC junctions during their extension. By time-lapse imaging the *TgBAC(pdgfrb:mCherry);Tg(fli1a:pecam1-EGFP)* larvae as observed in Fig. **3A**, tip location of the MC process was examined every 15 or 20 min for more than 1 h. Data are expressed as a percentage of number of time points at which the tip of MC process located at the inter-EC junction among the total time points. Bar and circles indicate the average and the values of each process, respectively (n=36). (**B**) Confocal images of the MCs and inter-EC junctions in the CtA of the 4 dpf *TgBAC(pdgfrb:mCherry);Tg(fli1a:pecam1-EGFP)* larva. Left, *fli1a:pecam1-EGFP*; center, *pdgfrb:mCherry*; right, the merged image of *fli1a:pecam1-EGFP* (magenta) and *pdgfrb:mCherry* (green). Arrowheads indicate the inter-EC junctions visualized by Pecam1-EGFP, while arrows show the tip of MC processes. Brackets indicate the MC process aligned along the inter-EC junction. Asterisk indicates cell body of MC. The cell body of the MC shown in the left side of the image is out of frame. Scale bar, 10 µm. (**C**) Time-lapse confocal images of MC migration along the CtA in the hindbrain of the

*TgBAC(pdgfrb:mCherry);Tg(fli1a:pecam1-EGFP)* larva. 3D-rendered confocal images and its subsequent time-lapse images with the elapsed time (min) at the top are shown, as in Fig. **3A**. The boxed areas are enlarged beneath the original images. Dotted-lines in second and third rows of the leftmost column outline the vessel morphology. Arrows indicate the tip of MC process, while asterisks show the cell body. Note that the tip of MC process moved forward along the inter-EC junction and that the MC-body (asterisks) was relocated to the preceding process which aligned along the inter-EC junctions (arrowheads). Scale bars, 10 μm.

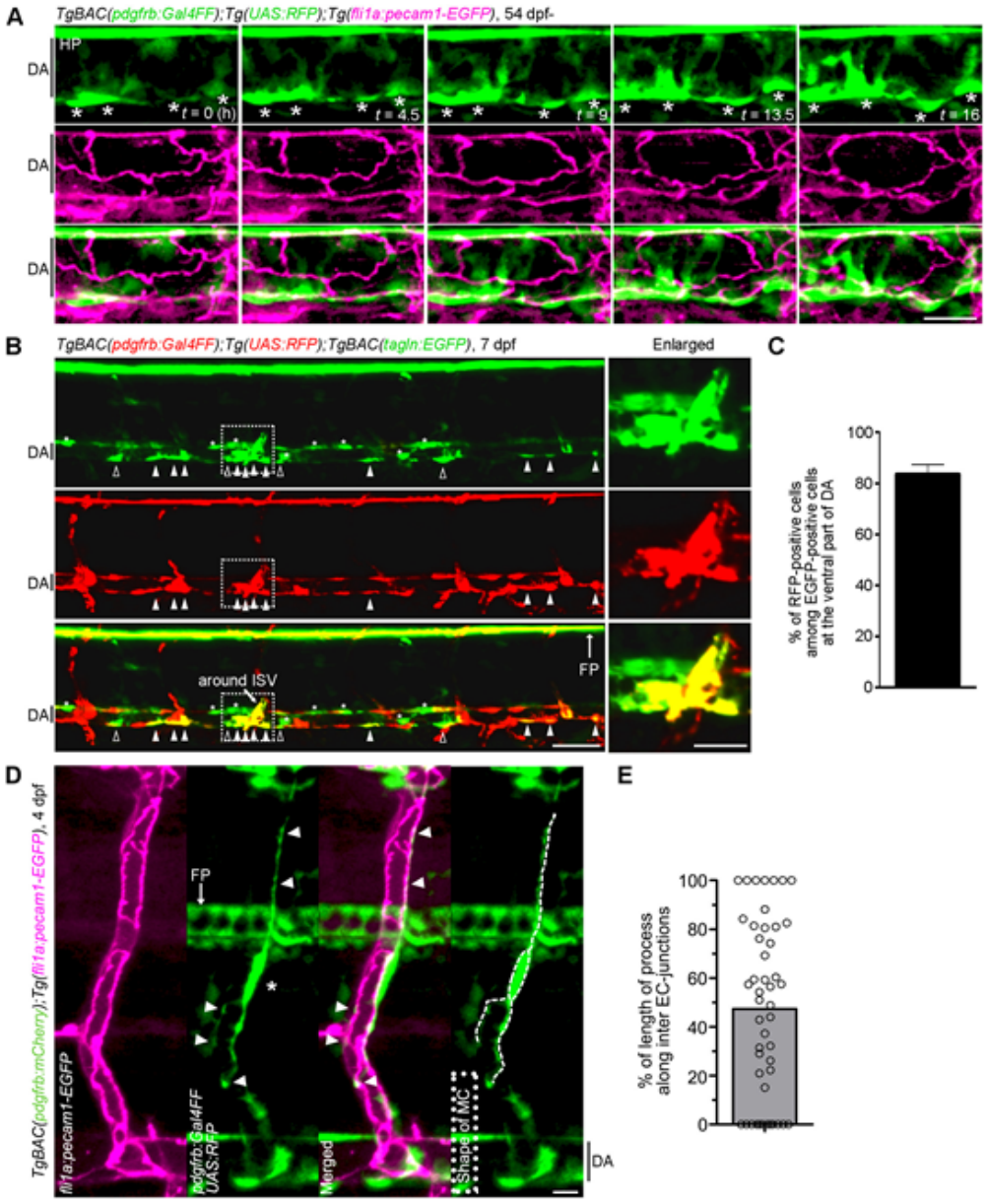



**Figure S4. MC coverage of trunk axial vessels.** (**A**) Time-lapse confocal images of MC coverage of DA. Confocal images of DA in the *TgBAC(pdgfrb:Gal4FF);Tg(UAS:RFP);Tg(fli1a:pecam1-EGFP)* embryo at 54 hpf (leftmost column) and subsequent time-lapse images with the elapsed time (h) at the bottom right. Top, *pdgfr:Gal4FF;UAS:RFP* (green); middle, *fli1a:pecam1-EGFP* (magenta); bottom, the merged images of *pdgfr:Gal4FF;UAS:RFP* (green) and *fli1a:pecam1-EGFP* (magenta). Asterisks indicate the cell body of MCs. Note that MCs located at the ventral side of DA extended multiple processes dorsally irrespective of inter EC-junctions. (**B**) Confocal images of trunk vasculature in the *TgBAC(pdgfrb:Gal4FF);Tg(UAS:RFP);TgBAC(tagln:EGFP)* larva at 7 dpf. Top,

*tagln:EGFP*; meddle, *pdgfr:Gal4FF;UAS:RFP*; lower, the merged image of *tagln:EGFP* (green) and *pdgfr:Gal4FF;UAS:RFP* (red). The boxed areas are enlarged to the right. Open and closed triangles indicate the EGFP-positive and RFP-positive cells and the EGFP-positive and RFP-negative cells at the ventral side of the DA, respectively. Asterisks indicate the EGFP-positive and RFP-negative cells located in the dorsal and lateral side of the DA. (**C**) Percentage of RFP-positive cells among the EGFP-positive cells located at the ventral side of the DA, as observed in **B**. Data is mean ± s.e.m. ($n$ = 15). (**D**) Confocal images of an ISV in the *TgBAC(pdgfrb:mCherry);Tg(fli1a:pecam1-EGFP)* larva at 4 dpf. Lateral view, anterior to the left. Leftmost panel, *fli1a:pecam1-EGFP* (magenta); second panel from the left, *pdgfr:Gal4FF;UAS:RFP* (green); third panel from the left, the merged image of *fli1a:pecam1-EGFP* image (magenta) and *pdgfr:Gal4FF;UAS:RFP* (green); rightmost panel, *pdgfr:Gal4FF;UAS:RFP* image in which MC shape is outlined by the dotted line. Arrowheads indicate the MC processes that aligned along with Pecam1-EGFP-labeled inter-EC junctions. Asterisk shows the MC body. (**E**) Alignment of MC processes along the inter-EC junctions as observed in **D** is expressed as a percentage of the total length (n = 47). Bar and circles indicate the average and the values of individual process, respectively. In this figure, all images are shown in lateral view with anterior to the left. Scale bars; 20 μm (**D**, enlarged image in **B**) or 50 μm (**A**, **B**). DA, dorsal aorta; HP, hypochord; FP, floor plate.

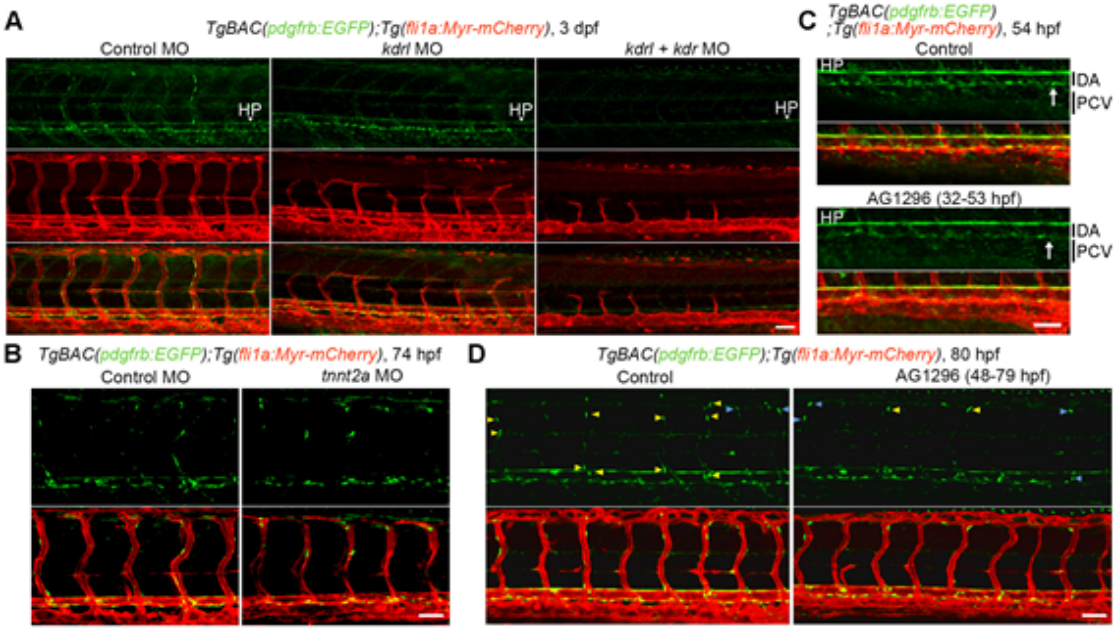



**Figure S5. Developmental mechanism of MCs in the trunk vasculature.** (**A**) Confocal images of trunk vasculature in the 3 dpf *TgBAC(pdgfrb:EGFP);Tg(fli1a:Myr-mCherry)* larvae injected with control MO (left), *kdrl* MO (center) or both *kdrl* and *kdr* MOs (right). Top, *pdgfrb:EGFP*; middle, *fli1a:Myr-mCherry* (red); bottom, the merged images of *pdgfrb:EGFP* (green) and *fli1a:Myr-mCherry* (red). (**B**) Confocal images of trunk vasculature in the 3 dpf *TgBAC(pdgfrb:EGFP);Tg(fli1a:Myr-mCherry)* embryo injected with control MO (left) or *tnnt2a* MO (right). Upper, *pdgfrb:EGFP*; lower, the merged images of *pdgfrb:EGFP* (green) and *fli1a:Myr-mCherry* (red). (**C**, **D**) Confocal images of trunk vasculature in the 54 hpf (**C**) or 80 hpf (**D**) *TgBAC(pdgfrb:EGFP);Tg(fli1a:Myr-mCherry)* larvae treated with vehicle or 20 μM AG1296 during 32-53 hpf (**C**) or 48-79 hpf (**D**). Upper, *pdgfrb:EGFP*; lower, the merged images of *pdgfrb:EGFP* (green) and *fli1a:Myr-mCherry* (red). Arrows in **C** indicate the EGFP-positive cells beneath the DA. Yellow and blue arrowheads in **D** indicate the EGFP-positive cells covering aISVs and those covering vISVs, respectively. In this figure, all images are shown in lateral view with anterior to the left. Scale bars, 20 μm (enlarged image in **A**) or 50 μm (**A**-**D**). DA, dorsal aorta; PCV, posterior cardinal vein; HP, hypochord.

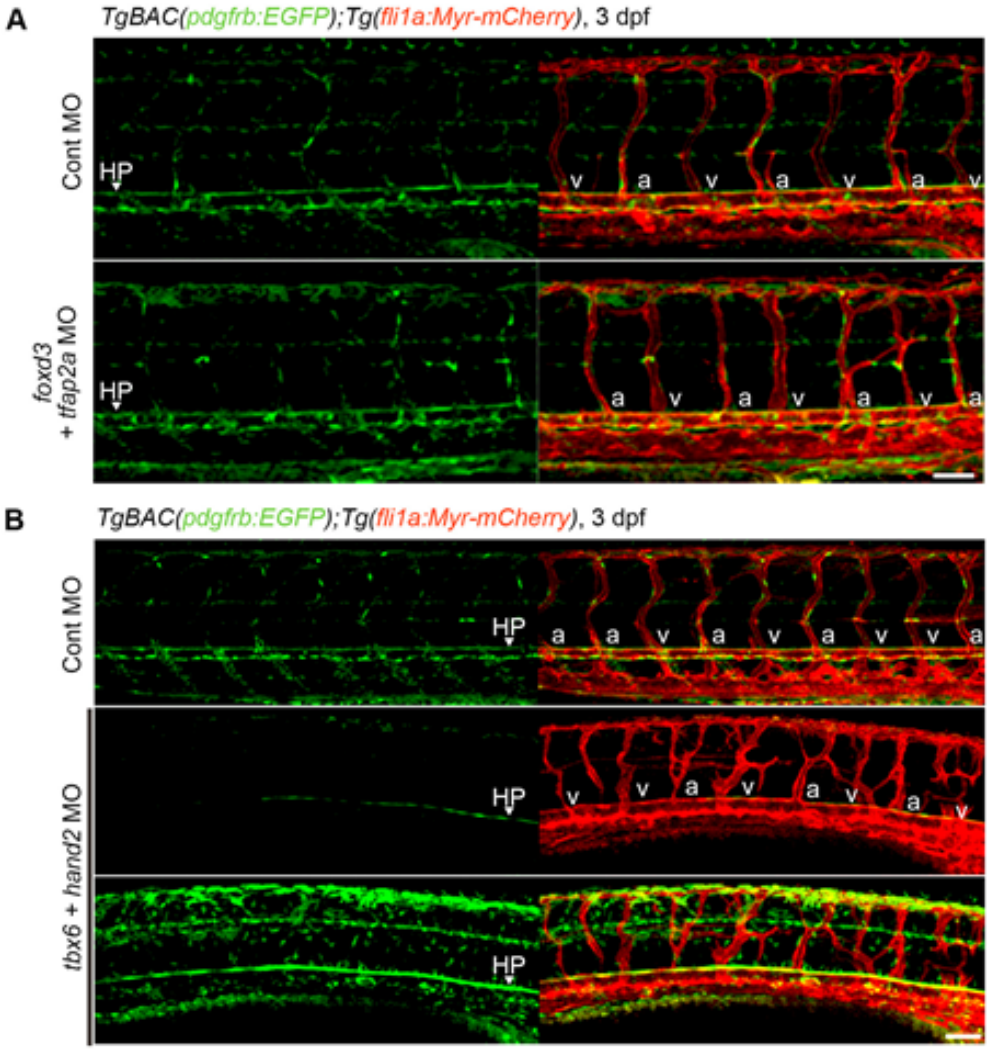
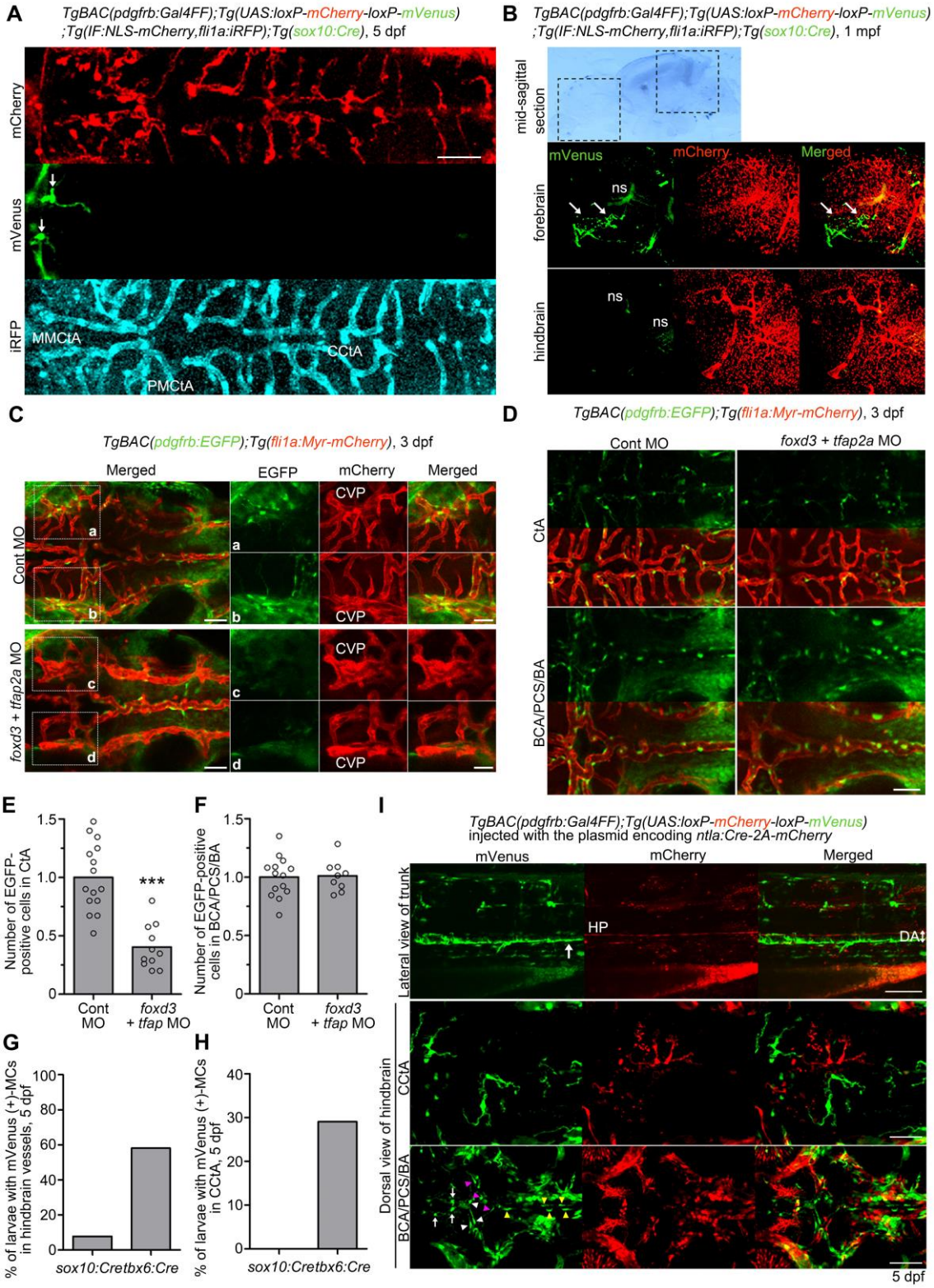



**Figure S6. Investigation of the origin of the MCs in trunk vessels.** (**A**) Confocal images of trunk vasculature in the 3 dpf *TgBAC(pdgfrb:EGFP);Tg(fli1a:Myr-mCherry)* larvae injected with control MO (upper) or both *foxd3* and *tfap2a* MOs (lower). Left, *pdgfrb:EGFP*; right, the merged images of *pdgfrb:EGFP* (green) and *fli1a:Myr-mCherry* (red). (**B**) Confocal images of trunk vasculature in the 3 dpf *TgBAC(pdgfrb:EGFP);Tg(fli1a:Myr-mCherry)* larvae injected with control MO (top) or both *tbx6* and *hand2* MOs (middle and bottom). Left, *pdgfrb:EGFP*; right, the merged images of *pdgfrb:EGFP* (green) and *fli1a:Myr-mCherry* (red). In the bottom panel, GFP signal is intensified to detect the weak signal. HP, hypochord. "a" and "v" indicate arterial ISVs (aISV) and venous ISVs (vISV), respectively. Scale bars, 50 μm.



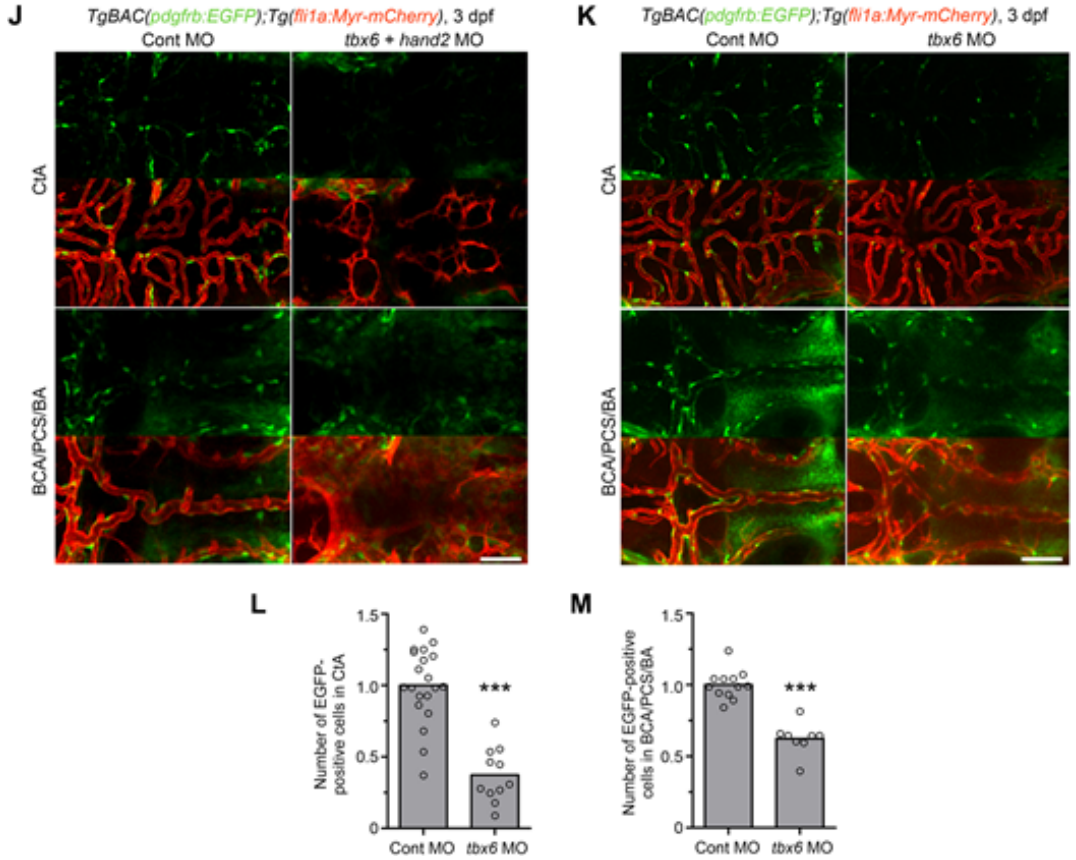



**Figure S7. Investigation of the origin of the MCs in cranial vessels.** (**A**) Confocal images of CtA in the *TgBAC(pdgfrb:Gal4FF);Tg(UAS:loxP-mCherry-loxP-mVenus);Tg(IF:NLS-mCherry,fli1a:iRFP);Tg(sox10:Cre)* larva at 5 dpf. The larvae expressing iRFP670 under the control of *fli1a* promoter was identified by *intestinal fatty acid binding protein* (*IF*) promoter-driven expression of NLS-mCherry in the intestine. Dorsal view, anterior to the left. Top, mCherry; middle, mVenus; bottom, iRFP. Arrows indicate mVenus-positive MCs. Note that the mVenus-positive MCs existed in anterior part of MMCtA, but not in the PMCtA and CCtA, at 5 dpf. (**B**) Midsagittal section of brain of the *TgBAC(pdgfrb:Gal4FF):Tg(UAS:loxP-mCherry-loxP-mVenus);Tg(IF:NLS-mCherry,fli1a:iRFP);Tg(sox10:Cre)* juvenile at 1 mpf. Confocal images of forebrain (middle panel) and hindbrain (bottom panel) indicated by the boxed areas in the bright field image (upper panel). Left, mVenus; center, mCherry; right, the merged images of mVenus (green) and mCherry (red). Arrows indicate mVenus-positive MCs. Note that the mVenus-positive MCs existed in the forebrain, but not in the hindbrain, at 1 mpf. ns indicates non-specific intrinsic fluorescence signal. (**C**) Confocal images of the vessels in the cerebral base in the 3 dpf *TgBAC(pdgfrb:EGFP);Tg(fli1:Myr-mCherry)* larvae

injected with control MO (upper panels) or both *foxd3* and *tfap2a* MOs (lower panels). Dorsal view, anterior to the left. The merged images of *pdgfrb:EGFP* (green) and *fli1a:Myr-mCherry* (red) are shown at the leftmost column. Boxed areas (**a-d**) are enlarged to the right, showing *pdgfrb:EGFP* (left), *fli1a:Myr-mCherry* (center) and the merged images of *pdgfrb:EGFP* (green) and *fli1a:Myr-mCherry* (red) (right). Note that *pdgfrb:EGFP*-positive cells around CVP were dramatically reduced in the *foxd3*/*tfap2a*-double morphant larvae. (**D**) Confocal images of CtA (upper two panel) and the vessels in the cerebral base (lower two panels, BCA/PCS/BA) in the 3 dpf *TgBAC(pdgfrb:EGFP);Tg(fli1a:Myr-mCherry)* larvae injected with control MO (left column) or both *foxd3* and *tfap2a* MOs (right column). Dorsal view, anterior to the left. Upper, *pdgfrb:EGFP*; lower, the merged images of *pdgfrb:EGFP* (green) and *fli1a:Myr-mCherry* (red). (**E**, **F**) The number of EGFP-positive cells covering the CtA (**E**) or the BCA, PCS and BA (**F**), as observed in **D**. Data are expressed relative to the average of controls ($n \geq 9$). (**G**, **H**) Percentage of larvae showing mVenus-positive MCs covering the vessels in the hindbrain such as CCtA, BCA, PCS and BA (**G**) or in the CCtA (**H**) of the 5 dpf *TgBAC(pdgfrb:Gal4FF):Tg(UAS:loxP-mCherry-loxP-mVenus)* larvae crossed with *Tg(sox10:Cre)* (sox10:Cre) or *Tg(tbx6:Cre,myl7:EGFP)* (tbx6:Cre) fish line (sox10:Cre n=13, tbx6:Cre n=31). (**I**) Confocal images of trunk (upper panel; lateral view, anterior to the left) and head (middle (CCtA) and lower (BCA, PCS, and BA) panels; dorsal view, anterior to the left) regions in the 5 dpf *TgBAC(pdgfrb:Gal4FF):Tg(UAS:loxP-mCherry-loxP-mVenus)* larvae injected with the plasmid encoding *ntla:Cre-2A-mCherry*. Note that mVenus-positive MCs covered the ventral part of DA (arrow) in the trunk and the CCtA, BCA (white arrowheads), PCS (magenta arrowheads), BA (yellow arrowheads) and PMCtA (arrows) in the head. HP; hypochord. (**J**) Confocal images of the CtA (upper two panels) and the vessels in the cerebral base (BCA/PCS/BA, lower two panels) in the 3 dpf *TgBAC(pdgfrb:EGFP);Tg(fli1a:Myr-mCherry)* larvae injected with control MO (left column) or both *tbx6* and *hand2* MOs (right column). Upper, *pdgfrb:EGFP*; lower, the merged images of *pdgfrb:EGFP* (green) and *fli1a:Myr-mCherry* (red). Note that *tbx6/hand2*-double morphant exhibited defective formation of hindbrain vessels. (**K**) Confocal images of the CtA and the vessels in the cerebral base in the 3 dpf *TgBAC(pdgfrb:EGFP);Tg(fli1a:Myr-mCherry)* larvae injected with control MO (left column) or *tbx6* MO (right column) are shown, as in **J**. (**L**, **M**) The number of EGFP-positive cells covering the CtA (**L**) or the BCA, PCS and BA (**M**), as observed in **K**. Data are expressed relative to the average of controls ($n \geq 8$). Bars and circles indicate averages and each value, respectively. Scale bars, 20 μm (enlarged images in

**C**) or 50 μm (**A-D**, **I-K**). In **E**, **F**, **L** and **M**, bars and circles indicate averages and each value, respectively. In **E**, **L** and **M**, ***p<0.001, significant difference between two groups.

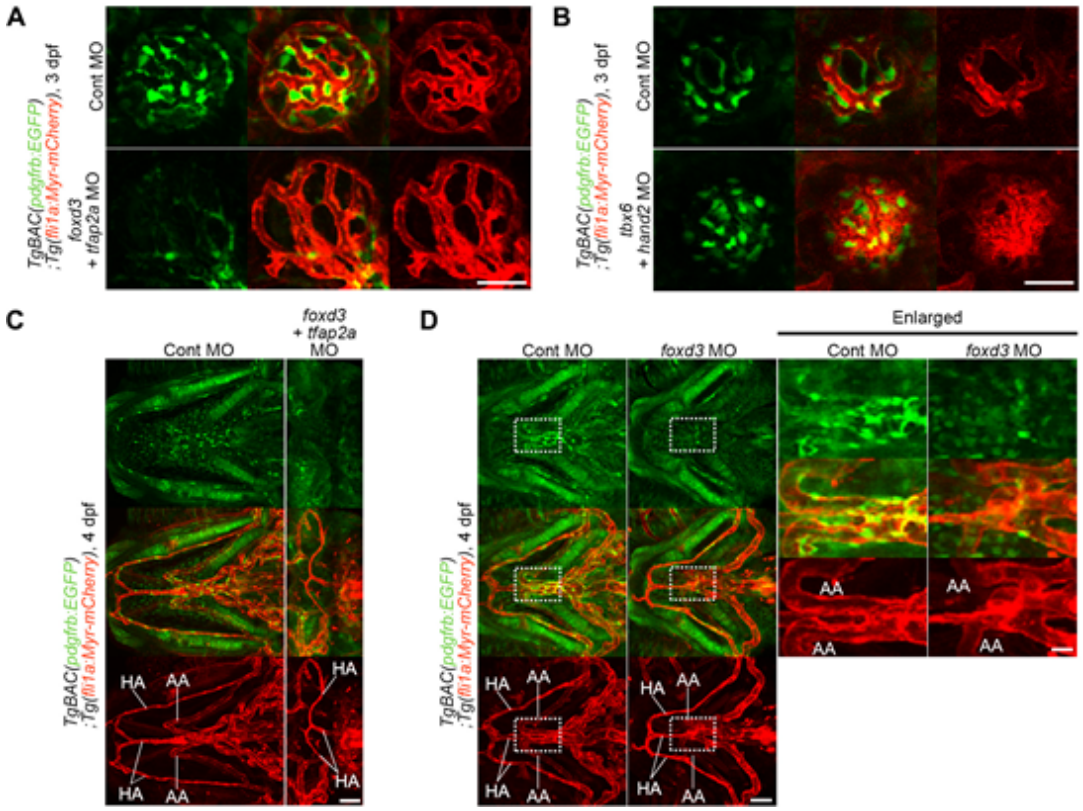
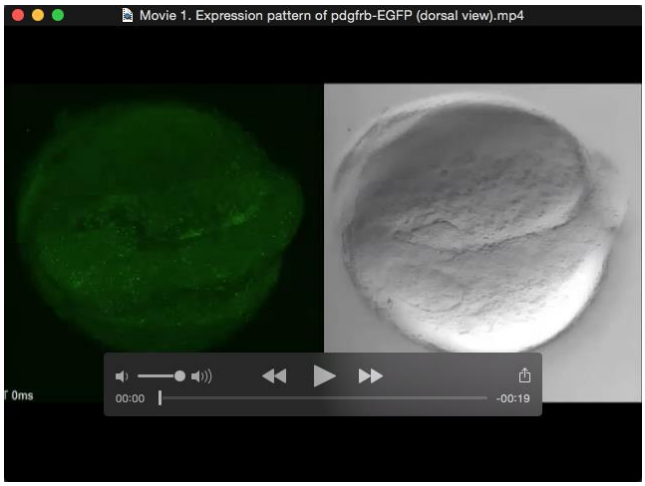
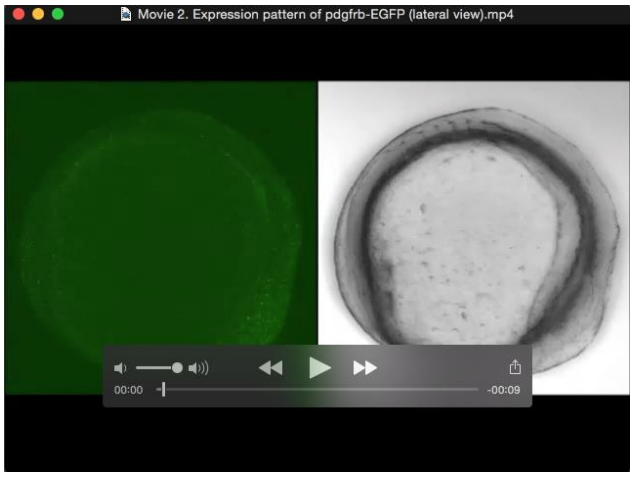


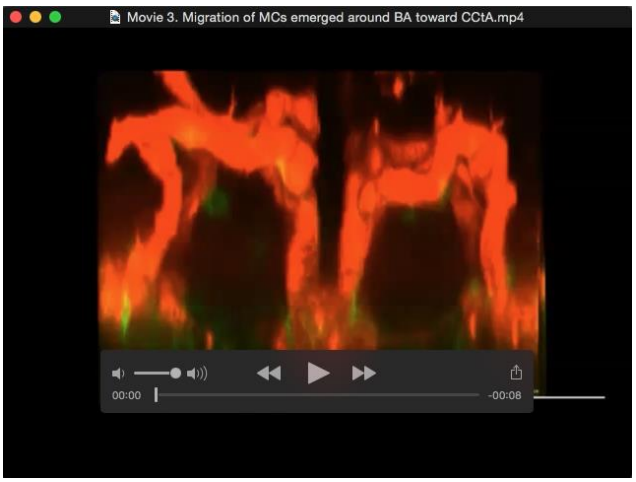

**Figure S8 Investigation of the origin of MCs in the hyaloid vessels and the vessels in the pharyngeal region.** (**A**) Confocal images of hyaloid vessels in the 3 dpf *TgBAC(pdgfrb:EGFP);Tg(fli1a:Myr-mCherry)* larvae injected with control MO (upper panels in **A** and **B**) or either both *foxd3* and *tfap2a* MOs (lower panel in **A**) or both *tbx6* and *hand2* MOs (lower panel in **B**). Lateral view, anterior to the left. Left, *pdgfrb:EGFP*; center, the merged images of *pdgfrb:EGFP* (green) and *fli1a:Myr-mCherry* (red); right, *fli1a:Myr-mCherry*. (**C**, **D**) Confocal images of vessels in the pharyngeal region in the 4 dpf *TgBAC(pdgfrb:EGFP);Tg(fli1a:Myr-mCherry)* larvae injected with control MO (left columns in **C** and **D**) or either both *foxd3* and *tfap2a* MOs (right column in **C**) or *foxd3* MO (right column in **D**). Top, *pdgfrb:EGFP*; middle, the merged images of *pdgfrb:EGFP* (green) and *fli1a:Myr-mCherry* (red); bottom, *fli1a:Myr-mCherry*. In **D**, the boxed areas are enlarged to the right. Note that *foxd3*/*tfap2a*-double morphant larva exhibited severe structural defects in the head, although it could form abnormal HA. AA, aortic arches; HA, hypobranchial artery. Scale bars, 20 μm (enlarged image in **D**) or 50 μm (**A**-**E**).

## Movies



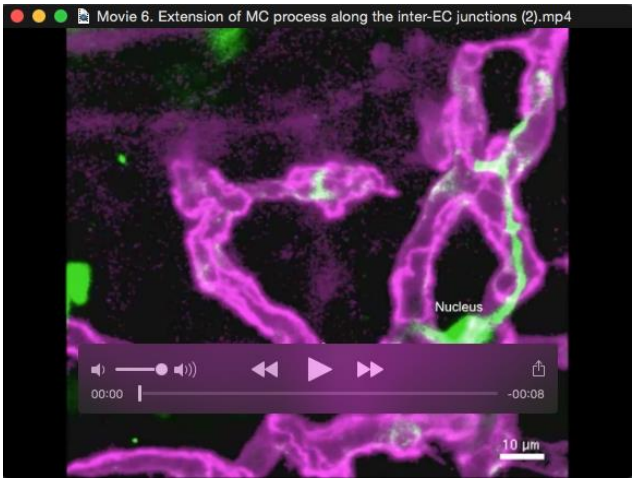

**Movie 1 and 2. Expression pattern of EGFP in the early stage of *TgBAC(pdgfrb:EGFP)* embryos.**
Time-lapse confocal imaging of EGFP fluorescence in the *TgBAC(pdgfrb:EGFP)* embryos from 8 somite stage. Images were obtained every 45 min. In movie 1, anterior to the left (dorsal view). In movie 2, anterior to the right (lateral view).



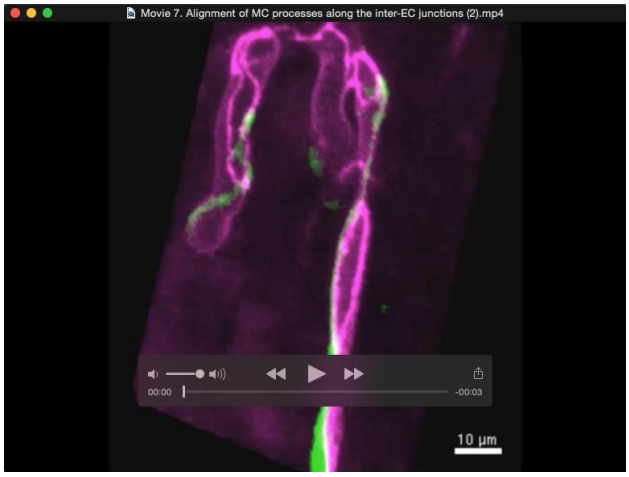

**Movie 3. Migration of MCs emerged around BA along the CCtA.**
Time-lapse confocal imaging of MC coverage of CCtAs in the *TgBAC(pdgfrb:EGFP);Tg(fli1a:Myr-mCherry)* larva (57-71 hpf). Lateral view, dorsal to the top and anterior to the front. Green, EGFP fluorescence; red, mCherry fluorescence. Note that EGFP-positive cells located around the BA dorsally migrated along the CCtA.



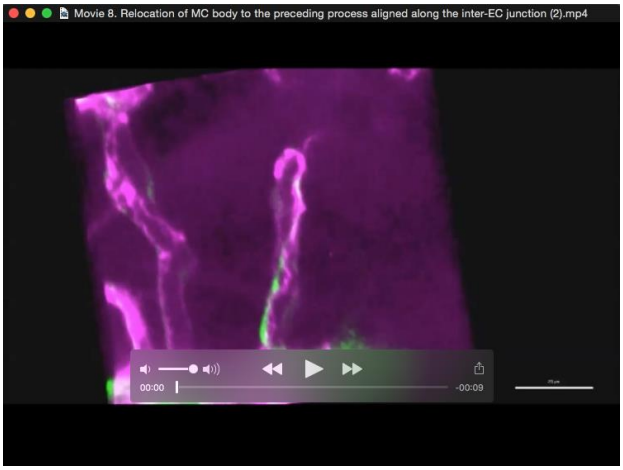
**Movie 4 and 5. Dynamics of MCs during their migration along the CtA.**
Time-lapse confocal imaging of cranial vasculature in the *TgBAC(pdgfrb:EGFP);Tg(fli1a:Myr-mCherry)* larva (57-88 hpf). Dorsal view, anterior to the left. Movie 4, merged image of EGFP (green) and mCherry (red); movie 5, EGFP image. Note that EGFP-positive cells covering the vessels located in the cerebral base such as BA, BCA, PCS and CVP migrated toward the CtAs that include CCtA, PMCtA, AMCtA and MMCtA.




**Movie 6. Extension of MC process along the inter-EC junctions.**
Time-lapse confocal imaging of CtA in the *TgBAC(pdgfrb:mCherry);Tg(fli1a:pecam1-EGFP)* larva starting from 62 hpf, as shown in Fig 3A. Images were obtained every 15 min. Green, mCherry (MC); Magenta, EGFP (Pecam1-EGFP-labeled inter-EC junctions). Arrowhead indicates the tip of MC process. Note that MC extended a process along the Pecam1-EGFP-labeled inter-EC junctions.

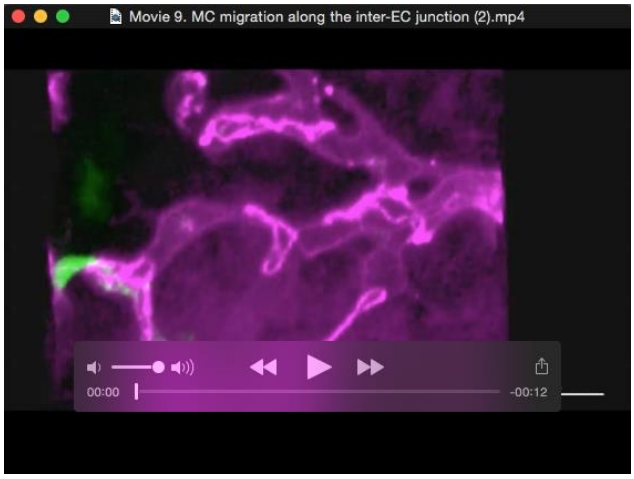



**Movie 7. Alignment of MC processes along the inter-EC junctions.**
3D-rotate image of CtA in the *TgBAC(pdgfrb:mCherry);Tg(fli1a:pecam1-EGFP)* larva at 4 dpf, as shown in Fig. **S3B**. Green, mCherry (MC); Magenta, EGFP (Pecam1-EGFP-labeled inter-EC junctions).

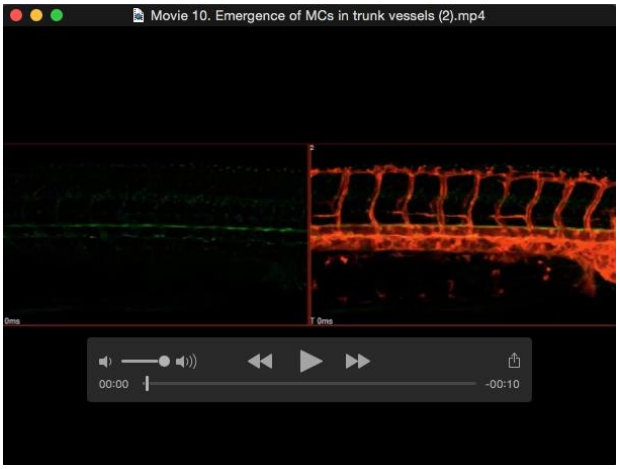



**Movie 8. Relocation of MC body to the preceding process aligned along the inter-EC junction.**

Time-lapse confocal imaging of CtA in the *TgBAC(pdgfrb:mCherry);Tg(fli1a:pecam1-EGFP)* larva starting from 62 hpf, as shown in Fig. **3C**. Images were obtained every 20 min. Green, mCherry (MC); magenta, EGFP (Pecam1-EGFP-labeled inter-EC junctions).




**Movie 9. MC migration along the inter-EC junction.**
Time-lapse confocal imaging of CtA in the *TgBAC(pdgfrb:mCherry);Tg(fli1a:pecam1-EGFP)* larva staring from 62 hpf, as shown in Fig. **S3C**. Images were obtained every 20 min. Green, mCherry (MC); magenta, EGFP (Pecam1-EGFP-labeled inter-EC junctions). The MC could extend its process across the unicellular EC tube without contacting with the inter-EC junctions. However, once the tip of the process reached to the inter-EC junction, the MC constantly extended its process along the junction and moved forward by relocating its cell body to the preceding process which aligned along the inter-EC junctions.




**Movie 10. Emergence of MCs in trunk vessels.**
Time-lapse confocal imaging of trunk vasculature in the *TgBAC(pdgfrb:EGFP);Tg(fli1a:Myr-mCherry)* embryo (46-76 hpf). Lateral view, anterior to the left. Left, EGFP image; right, merged image of EGFP (green) and mCherry (red).

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