Supplementary Materials and Methods

Plasmids

A cDNA fragment encoding β-catenin binding domain of human TCF4E (amino acid 1-314) was amplified by PCR using pCMXGAL4/hTCF4E vector, a gift from A. Kikuchi (Osaka University, Japan), as a template, and subcloned into pCMV-BD vector (Stratagene) to construct pCMV-Gal4db-TΔC plasmid that encodes the β-catenin binding domain of TCF4 fused to the DNA binding domain of Gal4 (Gal4db-TΔC). The pCMV-Gal4db-TΔC (D16A) plasmid encoding Gal4db-TΔC mutant in which Asp-16 of TCF4E was replaced with Ala was generated using QuickChange Site-directed mutagenesis kit (Stratagene).

The Tol2 vector system was kindly provided by K. Kawakami (National Institute of Genetics, Japan) (Kawakami et al., 2004; Urasaki et al., 2006). The pTol2-fli1 vector was constructed by removing a cDNA fragment containing GFP and Gateway cassette from the pTolfli1epEGFPDest plasmid, a gift from N. Lawson (University of Massachusetts Medical School, USA) (Lawson and Weinstein, 2002). To generate the pTolflk1 plasmid, the *fli1* enhancer/promoter was removed from the pTolfli1 vector, and replaced with the *flk1* promoter, a gift from D.Y. Stainier (Max Planck Institute). The cDNA encoding Gal4db-TΔC followed by 2A peptide and mCherry was subcloned into pTol2-fli1 vector to generate the pTol2-fli1:Gal4db-TΔC-2A-mC plasmid. The pTol1 vector was constructed by modifying pDon122 vector (COSMO BIO CO., LTD). To generate the pTol1-UAS and pTol2-UAS plasmids, the upstream activating sequence (UAS) derived from pBluescript II-UAS:GFP vector, a gift from M. Hibi (Nagoya University, Japan), was inserted into pTol1 and pTol2 vectors, respectively.

cDNA fragments encoding zebrafish *tcf3*, *dkk1*, *noggin3*, *bmp2b*, *aggf1*, *nr2f2*, *flt4*, *fli1a*, *axin1* and *wnt3a* were amplified from cDNAs library derived from zebrafish embryos by PCR and cloned into pCR4 Blunt TOPO vector (Invitrogen). The pTol1-UAS:TΔN-GFP plasmid was constructed by inserting a cDNA fragment that encodes Tcf3 lacking β-catenin binding domain (amino acid 55-561) followed by GFP (TΔN-GFP) into pTol1-UAS vector. An oligonucleotide encoding nuclear localization signal (NLS) derived from SV40 (PKKKRKV) was inserted into pEGFP-C1 and pmCherry-C1 vectors (Clontech, Takara Bio Inc.) to generate the plasmids expressing NLS-tagged GFP (NLS-GFP) and NLS-tagged mCherry (NLS-mC), respectively. The NLS-GFP cDNA was subcloned into the pTol2-UAS vector to construct the pTol2-UAS:NLS-GFP plasmid. An oligonucleotide encoding the myristoylation (Myr) signal derived from Lyn kinase was subcloned into pmCherry-N1 vector (Clontech, Takara Bio Inc.) to construct the plasmid encoding Myr signal-tagged mCherry (Myr-mC). The pTol2-fli1:Myr-mC plasmid was constructed by inserting Myr-mC cDNA into the pTol2-fli1 vector. An oligonucleotide encoding NLS derived from SV40 was inserted into the EcoRI/XhoI sites of a pcDNA3-td-EosFP vector (Molecular Biotechnology) to generate the plasmid encoding NLS-tagged tandem dimer Eos fluorescence protein (NLS-Eos). To construct pTolflk1-NLS-Eos vector, the NLS-Eos cDNA was subcloned into the pTolflk1 vector.

The DNA sequence of zebrafish heat shock protein 70 promoter was derived from pHSP70/4 EGFP plasmid (provided by J. Kuwada, University of Michigan, USA) (Halloran et al., 2000), and subcloned into the pTol2, namely the pTol2-hsp70l plasmid. The pTol2-hsp70l:dkk1-FLAG and the pTol2-hsp70l:wnt3a-FLAG plasmids were generated by inserting a cDNA fragment encoding C-terminally FLAG-tagged dkk1 and

that encoding C-terminally FLAG-tagged wnt3a into the pTol2-hsp70l plasmid, respectively.

The DNA sequence encoding zebrafish cardiac myosin light chain 2 (cmlc2) promoter was cloned by PCR using zebrafish genomic DNA as a template, and inserted into the pTol2 vector to construct the pTol2-cmlc2. The pTol2-cmlc2:NLS-mC plasmid was constructed by inserting the NLS-mC cDNA into the pTol2-cmlc2. Then, the chicken β-globin insulator (HS4) derived from pJC13-1 vector, a gift from G. Felsenfeld (National Institute of Health, USA) (Chung et al., 1993), and the zebrafish heat shock protein 70 promoter were sequentially subcloned into pTol2-cmlc2:NLS-mC to construct the pTol2-cmlc2:NLS-mC-HS4-hsp70l plasmid. The cDNA fragments expressing FLAG-tagged noggin3 and FLAG-tagged bmp2b, in which FLAG tag was inserted immediately after their signal sequence, were subcloned into the pTol2-cmlc2:NLS-mC-HS4-hsp70l vector to generate the pTol2-cmlc2:NLS-mC-HS4-hsp70l:noggin3-FLAG (hsp70l:noggin3-FLAG) and the pTol2-cmlc2:NLS-mC-HS4-hsp70l:FLAG-bmp2b-FLAG (hsp70l:bmp2b-FLAG) plasmids, respectively.

A cDNA fragment encoding human AGGF1 was amplified by PCR from human heart cDNAs, and subcloed into pEGFP-N1 vector to generate pEGFP-N1-AGGF1. The pmCherry-N1-Aggf1 was constructed by inserting the zebrafish aggf1 cDNA into pmCherry-N1 vector. The aggf1-mCherry cDNA was subcloned into pCS2+ vector, namely pCS2-Aggf1-mCherry.

The expression vectors encoding wild type and constitutive active β-catenin have already been reported (Zhang et al., 2011). To construct pCMV-Gal4-β-catenin that encodes β-catenin N-terminally fused to DNA binding domain of Gal4, the β-catenin

cDNA was subcloned into pCMV-BD vector. The pCS2+Gal4FF vector that expresses an engineered transcriptional activator consisting of the DNA-binding domain of Gal4 fused to two transcription activation modules from VP16 (Gal4-VP16) was kindly provided by M. Hibi (Nagoya University, Japan). pFR-Luc plasmid was purchased from Stratagene. pRL-SV40, pRL-TK, TOPflash and FOPflash vectors were obtained from Addgene.

To construct the pTol2-E1b-UAS-E1b vector, the UAS flanked by E1b minimal promoters on both sides was inserted into the pTol2 vector. A cDNA encoding NLS-GFP together with polyA signal and that encoding FLAG-tagged axin1 with polyA signal were sequentially subcloned into the pTol2-E1b-UAS-E1b vector to construct the pTol2-UAS:NLS-GFP,Axin plasmid (UAS:NLS-GFP,Axin), which drives expression of both NLS-GFP and FLAG-tagged Axin simultaneously in a Gal4-dependent manner.

Transgenic zebrafish lines

Tol1 and Tol2 transposase mRNAs were *in vitro* transcribed with SP6 RNA polymerase from NotI-linearised pCS-TP vector using the mMESSAGE mMACHINE kit (Ambion). To generate the *Tg(fli1:Gal4db-TΔC-2A-mC)*, *Tg(fli1:Myr-mC)*, *Tg(UAS:TΔN-GFP)*, *Tg(UAS:NLS-GFP)*, *Tg(flk1:NLS-Eos)* and *Tg(hsp70l:dkk1-FLAG)* zebrafish lines, the corresponding Tol1- or Tol2-based plasmid DNAs (25 ng) were microinjected along with Tol1 or Tol2 transposase RNA (25 ng) into one-cell stage embryos of wild type strain, AB. To establish the *Tg(fli1:Gal4db-TΔC-2A-mC)*, *Tg(fli1:Myr-mC)* and *Tg(flk1:NLS-Eos)* fish lines, the embryos showing transient expression of mCherry or Eos fluorescence in the vasculature were selected, raised to adulthood, and crossed with wild type AB to identify germline transmitting founder fishes. To develop the

Tg(UAS:TΔN-GFP) and *Tg(UAS:NLS-GFP)* Tg lines, the fish carrying the corresponding genes were first screened by genomic PCR, and confirmed by crossing with *Tg(fli1:Gal4FF)* zebrafish to check the transgene expression. To generate the *Tg(hsp70l:dkk1-FLAG)* fish line, all embryos were raised to adulthood, and subjected to genomic PCR screening. Embryos obtained from crossing the genomic PCR-positive fish with wild type AB were heat-shocked to check the transgene expression. The *Tg(flt1:mC)* zebrafish line was generated as previously reported (Bussmann et al., 2010).

Tg(fli1:GFP) fish were provided by N. Lawson (University Massachusetts Medical School, USA) (Lawson and Weinstein, 2002). *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(fli1:Gal4FF)* fish line was a gift from M. Affolter (University of Basel, Switzerland) (Totong et al., 2011; Zygmunt et al., 2011). *Tg(hsp70l:dkk1-GFP)* fish were obtained from the Zebrafish International Resource Center (University of Oregon, OR, USA).

Mounting of zebrafish embryos

Pigmentation of embryos was inhibited by 1-phenyl-2-thiourea (PTU) (Sigma-Aldrich). Embryos were dechorionated, anesthetised in 0.016% tricaine (Sigma-Aldrich) in E3 embryo medium, and mounted in 1% low-melting agarose dissolved in E3 medium poured on a 35-mm-diameter glass-base dish (Asahi Techno Glass). The mounted embryos were submerged in E3 medium supplemented with 0.016% tricaine and 0.2 mM PTU.

Quantitative analyses of fluorescence, CV defects and ectopic vessel formation

To quantify the β-catenin transcriptional activity in CVP in the Figures 1D, 3B, 3D, 5D, 5F, S3G and S6E, 3D-renderd confocal stack fluorescence images of GFP and mCherry in the caudal regions of *Tg(fli1:Gal4db-TΔC-2A-mC);(UAS:GFP);(fli1:Myr-mC)* fish embryos at 36 hpf were acquired using confocal microscopy. The mCherry fluorescence-marked CVP on the 3D-images was manually cropped using Volocity software. Then, the volume of the cropped areas and the total GFP fluorescence intensity within the areas were calculated. The β-catenin transcriptional activity in the CVP was determined by dividing the total GFP fluorescence intensity by the volume of CVP.

To quantify the defective CV formation in the Figures 2D, 4D, 6I, S6B and S8F, the CV of *Tg(fli1:Myr-mC)* or *Tg(fli1:GFP)* embryos at 48 hpf were analyzed and classified into two groups: mild group and severe group. In the Figures 2D, 4D and S6B, the embryos with mild phenotype exhibited no blood circulation in the CV, whereas those with severe phenotype lacked CV. In the Figures 6I and S8F, the embryos with the mild phenotype lacked the CV, but developed the CVP, whereas those with the severe phenotypes exhibited lack of the CV and defective CVP. Frequency of mild and severe phenotypes is expressed as percentage relative to the total number of analyzed embryos.

To quantify formation of ectopic venous vessels in Bmp2b-overexpressing embryos in Figure 4E, the *Tg(fli1:GFP)* embryos were analyzed at 48 hpf. The areas covered by ectopic venous vessels within 11 somites in the caudal regions were measured using Volocity software.

RNA-seq analyses

Using total RNA purified from β-catenin (+) ECs and β-catenin (-) ECs, reverse transcription and cDNA library preparation were performed with SMARTer Ultra Low RNA Kit (Clontech). cDNA was fragmented with a Covaris S220 instrument (Covaris). Subsequently, the sample was end-repaired, dA-tailed, adaptor ligated, and then subjected to PCR by using the NEBNext® DNA Library Preparation and NEBNext Multiplex oligos For Illumina. The sample was sequenced by using MiSeq (Illumina) to generate pair-end 150-bp reads.

Raw reads were mapped to Zebrafish genome (DanRer7). Expression levels were measured by calculating RPKM (reads per kilobase per million sequenced reads), and normalised TMM (Trimmed Mean of M-values) method by using Avadis NGS software (Strand Life Sciences).

TUNEL assay

EC apoptosis was analyzed by TUNEL assay. *Tg(UAS:NLS-GFP);(fli1:Gal4FF)* or *Tg(UAS:TΔN-GFP);(fli1:Gal4FF)* embryos at 32 hpf were dechorinated, fixed with 4% paraformaldehyde (PFA) at 4 °C overnight, and dehydrated with methanol (MeOH) at -20 °C. After gradual hydration, the embryos were permeabilised with 5 μg/ml proteinase K, refixed with 4% PFA, and washed three times with PBS-T. Then, apoptotic cells were stained by using In Situ Cell Death Detection Kit, TMR red (Roche). To quantify the EC apoptosis, we counted TUNEL-positive cells among the GFP-positive ECs.

Heat shock and chemical treatment

Tg(hsp70l:dkk1-GFP) and *Tg(hsp70l:dkk1-FLAG)* embryos were heat shocked at either 12 or 24 hpf for 1 h at 39 °C. *Tg(hsp70l:dkk1-GFP)* embryos were identified by the expression of GFP. *Tg(hsp70l:dkk1-FLAG)* embryos were identified based on the appearance of defective caudal fin formation as described in Figure S3, and confirmed by genomic PCR after the analyses. To ubiquitously express wnt3a-FLAG, the *Tg(fli1:Gal4db-TΔC-2A-mC);(UAS:GFP);(fli1:Myr-mC)* embryos were injected with 25 ng pTol2-hsp70l:wnt3a-FLAG plasmid along with Tol2 transposase RNA (25 ng), and heat-shocked at 22 hpf for 1 h at 39 °C. To ubiquitously express noggin3-FLAG and bmp2b-FLAG, the *Tg(fli1:Gal4db-TΔC-2A-mC);(UAS:GFP);(fli1:Myr-mC)* embryos were injected with 25 ng pTol2-cmlc2:NLS-mC-HS4-hsp70l:noggin3-FLAG (hsp70l:noggin3-FLAG) and 25 ng pTol2-cmlc2:NLS-mC-HS4-hsp70l:bmp2b-FLAG (hsp70l:bmp2b-FLAG) plasmids along with Tol2 transposase RNA (25 ng), and heat-shocked at 22 hpf for 2 h and at 24 hpf for 30 min at 39 °C, respectively. The embryos carrying the corresponding genes were selected by the expression of mCherry in cardiac myocytes.

Tg(fli1:Gal4db-TΔC-2A-mC);(UAS:GFP);(fli1:Myr-mC) embryos were dechorinated, and incubated from 24 to 33 or 48 hpf and from 15 to 36 hpf in the E3 medium containing 1 or 3 μM BIO, a glycogen synthase kinase 3 inhibitor (6-bromoindirubin-3'-oxime, EMD Biosciences) and 10 μM IWR-1, an axin-stabilising compound (Calbiochem), respectively. As a control, the embryos were also incubated in the E3 solution containing DMSO. *Tg(flk1:NLS-Eos)* embryos were incubated in the E3 medium containing either DMSO or 1.5 μM Ki 8457, an inhibitor for Vegf-A receptor (Tocris).

Cell culture, transfection and luciferase reporter assay

HEK 293 and HEK 293T cells and human umbilical vein ECs were cultured and transfected as described previously (Fukuhara et al., 2008). To investigate whether β-catenin stimulates transcriptional activity of Gal4db-TΔC, HEK 293 cells were transfected with either pCMV-Gal4db-TΔC or pCMV-Gal4db-TΔC (D16A) vector together with pFR-Luc and pRL-TK that encode firefly and renilla luciferase under the control of five tandem repeats of Gal4 binding sites and herpes simplex virus thymidine kinase promoter, respectively. The transfection mixture also included either the expression plasmid encoding wild type β-catenin or the empty vector. The total amount of plasmid DNA was adjusted with empty vector. The cells were lysed using passive lysis buffer (Promega) 16 h after the transfection. To examine the effect of BIO, the cells were stimulated with 5 μM BIO for 16 h. The luciferase activities in cell extracts were determined by using a Dual-Luciferase assay system (Promega). To normalize the levels of the experimental reporter activity, firefly luciferase activity are divided by renilla luciferase activity for each sample.

To examine the effect of Aggf1 on β-catenin transcriptional activity, the cells were transfected with either TOPflash or FOPflash plasmid together with pRL-SV40 vector. The transfection mixtures also included the expression plasmids as described in the figure legends. The luciferase activities in the cells were measured 24 h after the transfection. To normalize the levels of the experimental reporter activity, firefly luciferase activity were divided by renilla luciferase activity for each sample. The levels of β-catenin/Tcf-dependent transcription were indicated by the value of TOPflash luciferase activity divided by that of FOPflash activity. The cells were also transfected

with the expression plasmid encoding either Gal4-β-catenin or Gal4-VP16 together with pEGFP-N1-Aggf1 or the empty plasmid, and lysed 24 h after the transfection.

Injections of morpholino oligonucleotide (MO) and mRNA

For morpholino oligonucleotide (MO)-mediated gene knockdown, embryos were injected at the one-cell or two-cell stage with control MO (Gene Tools), 2.5 ng of *aggf1* splicing MO (*aggf1* MO1), 10 ng of *aggf1* translation blocking MO (*aggf1* MO2), 4 ng of *nr2f2* splicing MO (*nr2f2* E1I1 MO), 2 ng of *nr2f2* translation blocking MO (*nr2f2* ATG MO) (Gene Tools). To suppress the cell death induced by MO off-targeting, *p53* MO was co-injected 1.5-fold (w/w) to the other MO used, as previously reported (Robu et al., 2007). The sequences for the already-validated MOs used in this study are: *aggf1* MO1, 5'-GCCCTGCTCACCTGCTGTCGGAGAT-3' (Chen et al., 2013); *aggf1* MO2, 5′-CGCATCAATAGGGAGCAACCGCGAT-3′ (Chen et al., 2013); *nr2f2* E1I1 MO, 5′-ACAAAAATCCGAATACCTTCCCGTC-3′ (Aranguren et al., 2011); *nr2f2* ATG MO, 5′-AGCCTCTCCACACTACCATTGCCAT-3′ (Aranguren et al., 2011).

aggf1-mCherry mRNA was *in vitro* transcribed with SP6 RNA polymerase from pCS2-Aggf1-mCherry vector using the mMESSAGE mMACHINE kit (Ambion). To ubiquitously express Aggf1-mCherry, 200 pg of *aggf1-mcherry* mRNA was injected into one cell stage of *Tg(fli1:Gal4db-TΔC-2A-mC);(UAS:GFP);(fli1:Myr-mC)* embryo.

Genotyping

For the genotyping of the zebrafish Tg lines, genomic PCR were performed using the following primer sets: for *Tg(UAS:TΔN-GFP)* and *Tg(UAS:NLS-GFP)*, 5'-GAAGCAGCACGACTTCTTCAAGTCC-3' and

5’-CCTTGATGCCGTTCTTCTGCTTGTC-3’; for *Tg(hsp70l:dkk1-FLAG)*,

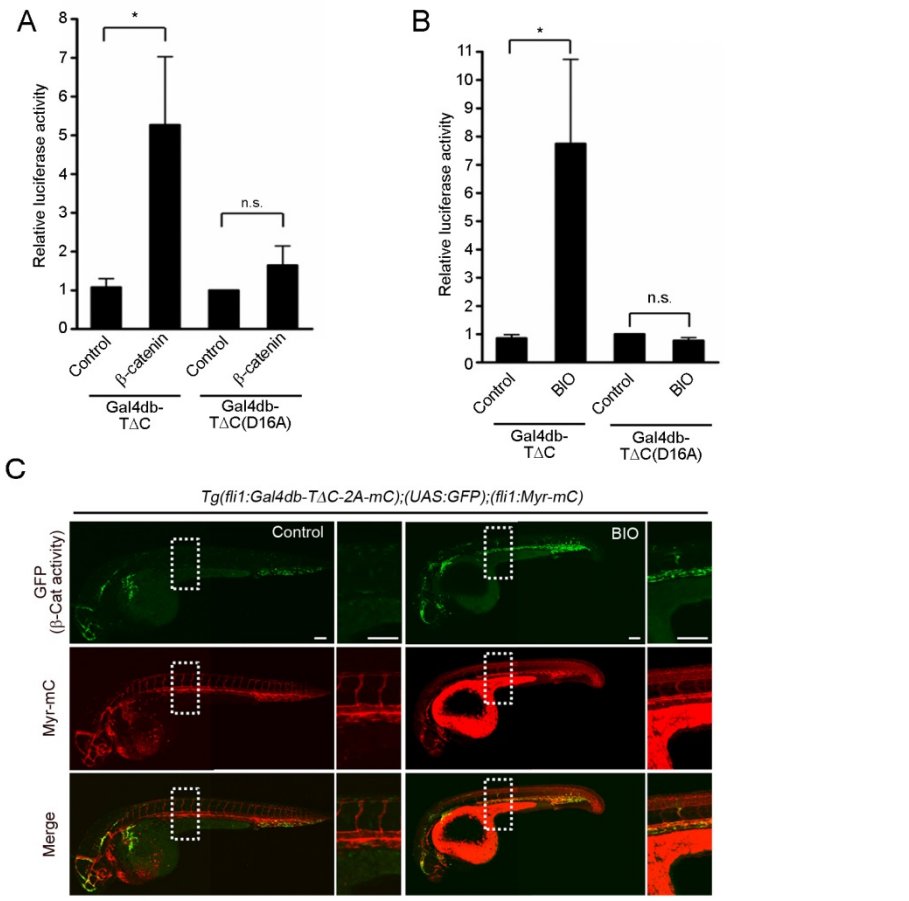
5’-ATAATGAAACAATTGCACCGATAA-3’ and

5’-GAAGTTCAGACTGTCCAAAAGTGA-3’.

Real-time reverse transcription PCR

Real-time reverse transcription PCR was performed as previously described (Fukuhara et al., 2008).

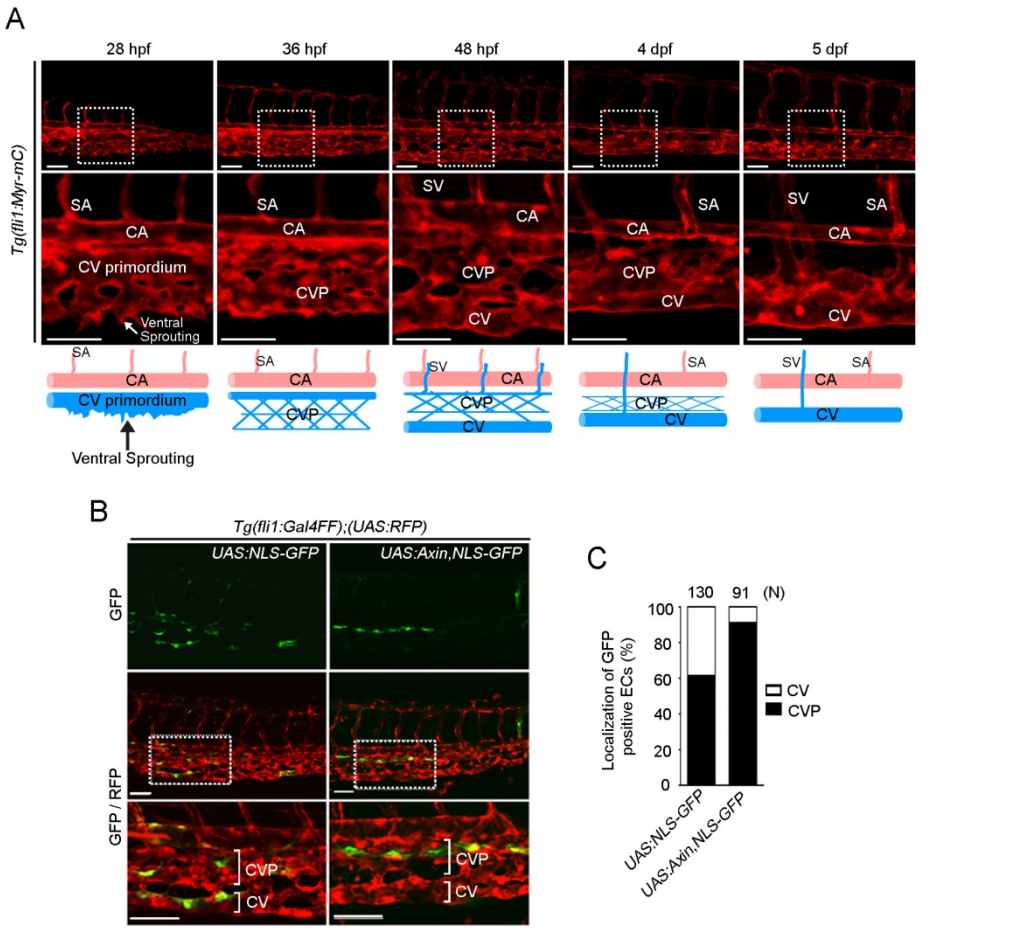
Kashiwada_Supplementary Figure S1



Supplementary Figure S1. Reporter gene expression driven by Gal4db-TΔC reflects transcriptional activity of β-catenin. (A) Relative luciferase activity in HEK 293T cells transfected with UAS-luciferase reporter and the plasmid encoding either Gal4db-TΔC or its mutant (Gal4db-TΔC(D16A)) that lacks the ability to bind β-catenin together with the empty vector (Control) or that encoding β-catenin. Data are expressed relative to that observed in the empty vector-transfected cells that express Gal4db-TΔC (D16A), and shown as mean ± s.e.m. of four independent experiments. (B) Relative luciferase activity in 5 μM BIO-stimulated HEK 293T cells transfected with UAS-luciferase reporter and the plasmid encoding either Gal4db-TΔC or Gal4db-TΔC (D16A). The cells were stimulated with vehicle (Control) or BIO for 16 h. Data are expressed relative to that observed in the vehicle-treated cells that express Gal4db-TΔC (D16A), and shown as mean ± s.e.m. of three independent experiments. (C) 3D-rendered confocal stack fluorescence images of the

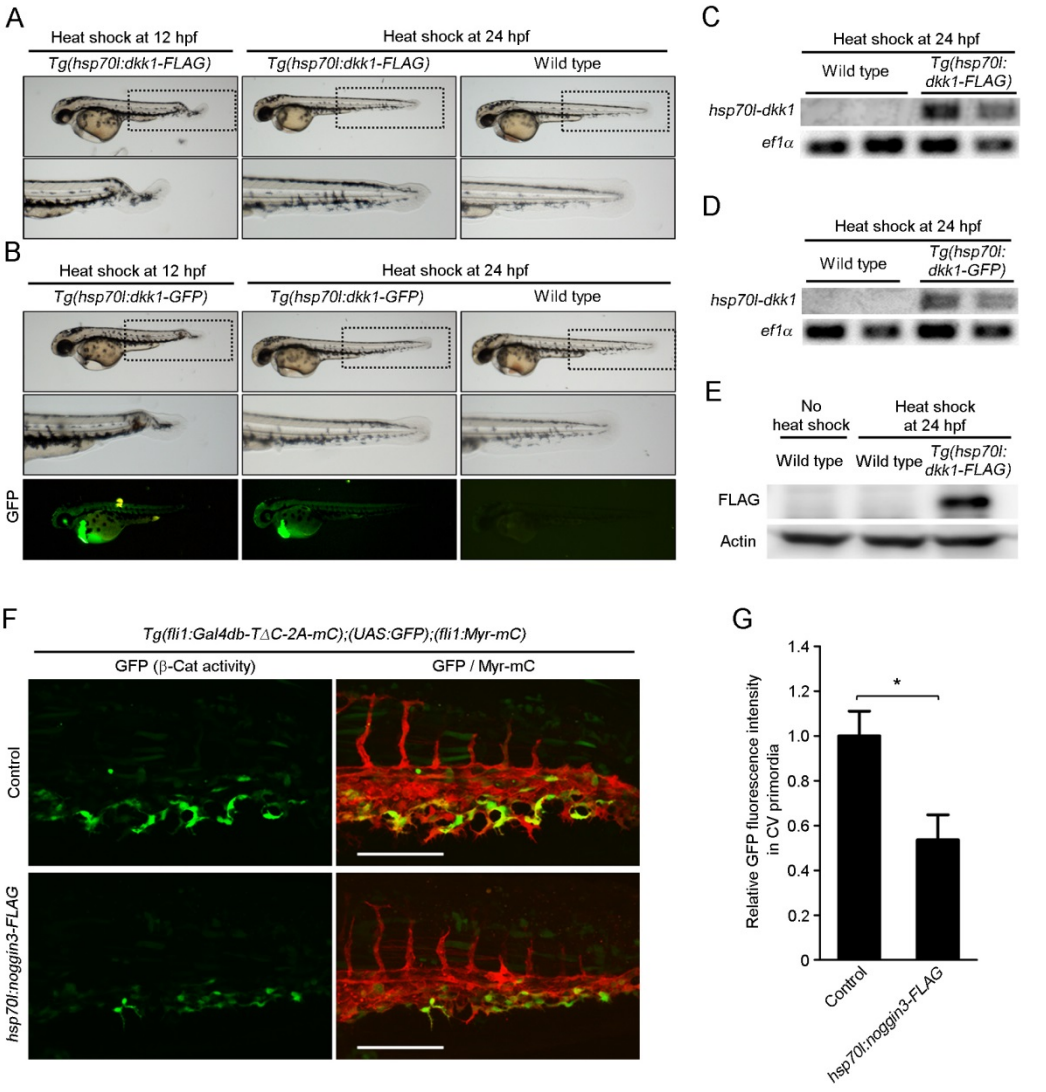
Tg(fli1:Gal4db-TΔC-2A-mC);(UAS:GFP);(fli1:Myr-mC) embryos treated with vehicle (Control) or 3 μM BIO, a glycogen synthase kinase 3 inhibitor, from 24 to 33 hours post-fertilization (hpf). Top, GFP images (β-Cat activity); middle, mCherry images (Myr-mC); bottom, the merged images (Merge). The images are composites of two images, since it was not possible to capture the whole animal at sufficiently high resolution in a single field of view. The boxed areas are enlarged on the right side of the original images. *p<0.05. n.s., no significance.

Kashiwada_Supplementary Figure S2



Supplementary Figure S2. The development of the caudal vein (CV). (A) Confocal images of caudal regions of *Tg(fli1:Myr-mC)* embryos at the developmental stages indicated at the top. The boxed areas are enlarged beneath the original images. Schematic representations of the caudal vessel structures are shown at the bottom. (B) Confocal images of the caudal regions of the 48 hpf *Tg(fli1:Gal4FF);(UAS:RFP)* embryos injected with *UAS:NLS-GFP* Tol2 plasmid or *UAS:Axin,NLS-GFP* Tol2 plasmid, which drives the expression of Axin and NLS-GFP simultaneously. GFP images and the merged images of GFP (green) and RFP (red) are shown in the top and middle rows, respectively. The boxed areas in the merged images are enlarged in the bottom row. (C) Percentages of GFP-expressing cells in the CV and CVP as observed in B were quantified. The number of analyzed embryos is indicated on the top. CA, caudal artery; CV, caudal vein; CVP, caudal vein plexus; SA, segmental artery; SV, segmental vein.

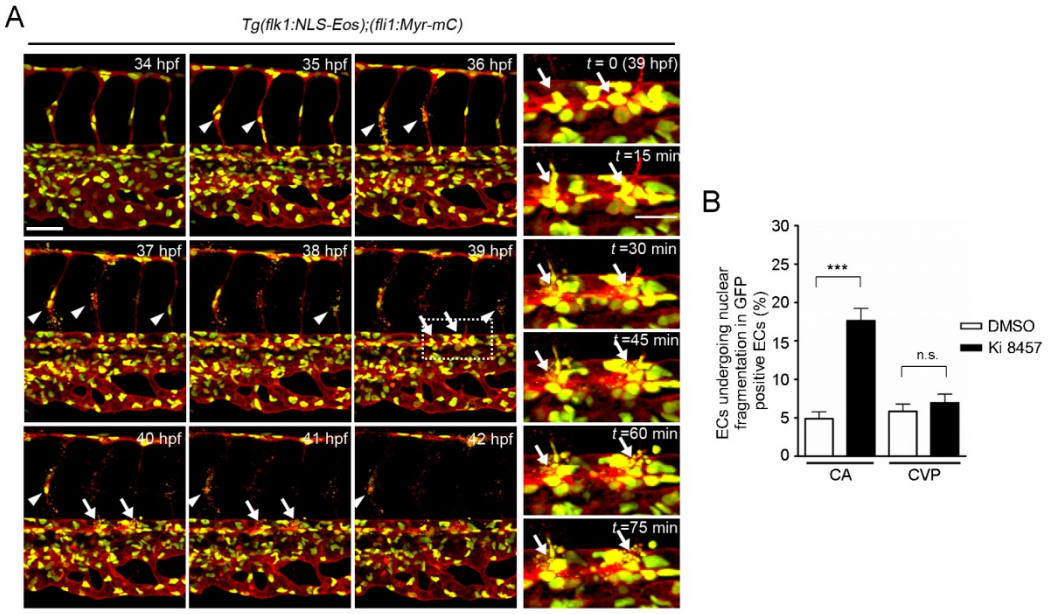
Kashiwada_Supplementary Figure S3



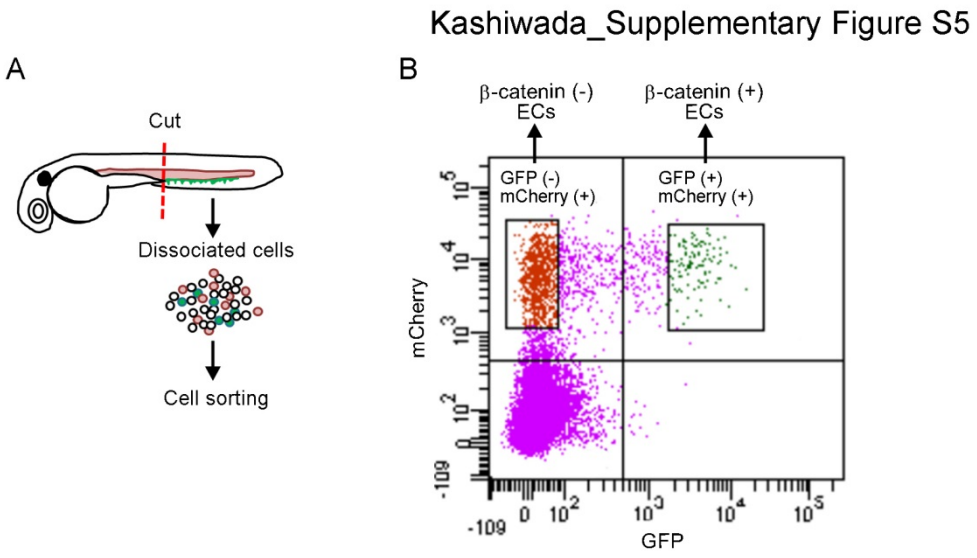
Supplementary Figure S3. Wnt signaling is suppressed by heat shock promoter-driven expression of dkk1. (A) Lateral view of wild type sibling and *Tg(hsp70l:dkk1-FLAG)* embryos heat-shocked at either 12 or 24 hpf as indicated at the top. The boxed areas are enlarged beneath the original images. (B) Lateral view of wild type sibling and *Tg(hsp70l:dkk1-GFP)* embryos heat-shocked at either 12 or 24 hpf as indicated at the top. The boxed areas are enlarged beneath the original images. GFP fluorescence images are shown at the bottom. (C, D) Genomic PCR analysis of wild type sibling, *Tg(hsp70l:dkk1-FLAG)* (C) and *Tg(hsp70l:dkk1-GFP)* (D) zebrafish

embryos were performed using primer sets to amplify the DNA fragment that contains *heat shock promoter* and *dkk1* and to amplify the *ef1α* gene. (E) Western blot analysis of lysates from heat-shocked wild type and *Tg(hsp70l:dkk1-Flag)* embryos with anti-FLAG and anti-βactin (Actin) antibodies. (F) Confocal stack fluorescence images of the 28 hpf *Tg(fli1:Gal4db-TΔC-2A-mC);(UAS:GFP);(fli1:Myr-mC)* embryos injected without (Control) or with *hsp70l:noggin3-FLAG* plasmid and heat-shocked at 24 hpf for 1h. GFP images (β-Cat activity); right, the merged (GFP/Myr-mC) of GFP (green) and mCherry (red) images. Scale bars, 100 μm. (G) Fluorescence intensities of GFP in the CV primordia and the ECs that sprouted from the CV primordia, as observed in F, were expressed as relative values to that observed in the control embryos. Data are shown as means ± s.e.m. (Control n=8, *hsp70l:noggin3-FLAG* n=5).

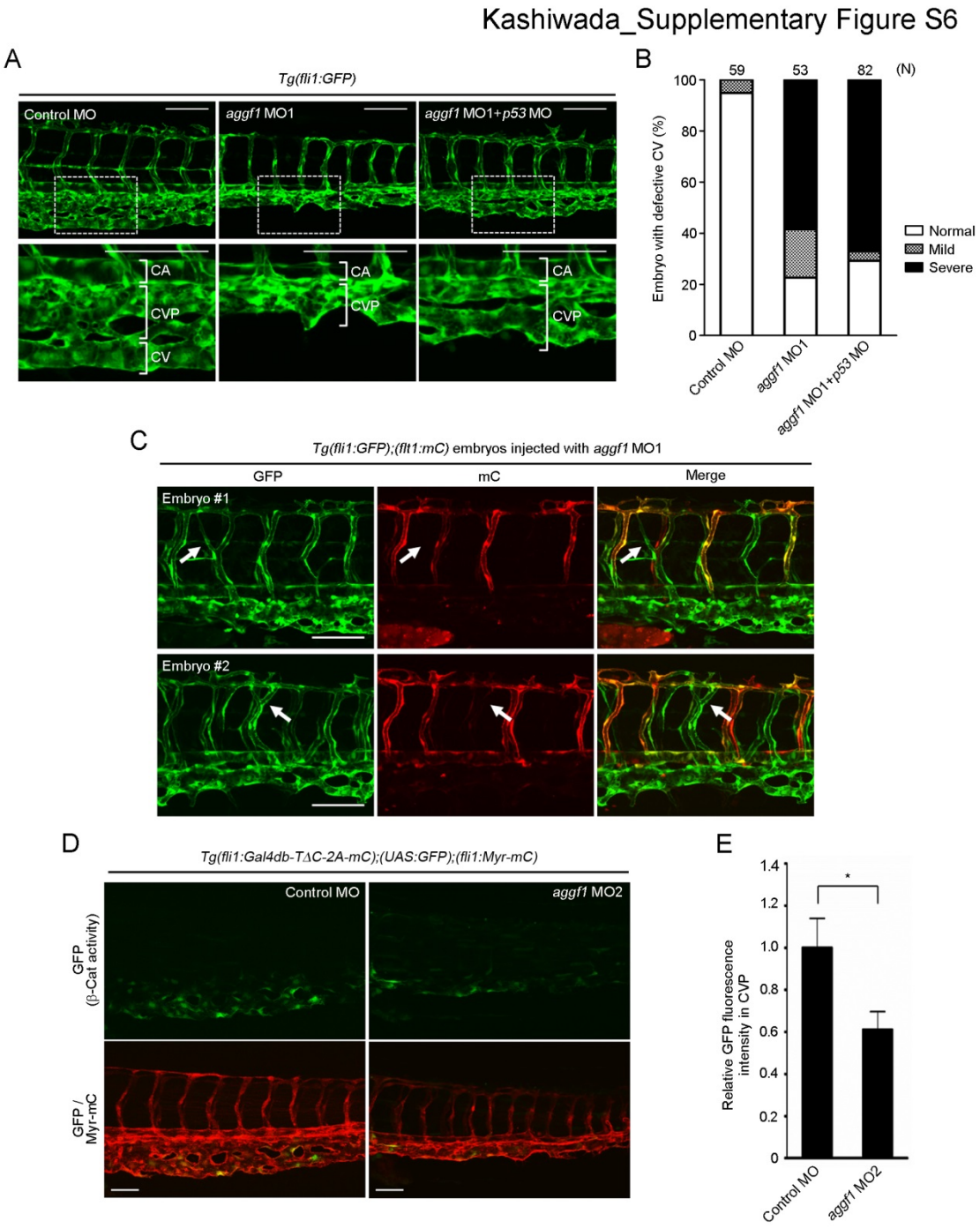
Kashiwada_Supplementary Figure S4



Supplementary Figure S4. Vegf-A promotes EC survival in the caudal artery (CA). (A) *Tg(flk1:NLS-Eos);(fli1:Myr-mC)* embryos were treated with 1.5 μM Ki 8457, an inhibitor for Vegf-A receptor, from 30 hpf, and subjected to time-lapse confocal imaging from 34 hpf to 42 hpf. The merged images of Eos (green) and mCherry (red) at every hour starting from 34 to 42 hpf are shown. The enlarged image of boxed area in the image at 39 hpf and its subsequent time-lapse images are shown at the right column. The elapsed time (min) is indicated at the top of each image. Arrowheads and arrows indicate the ECs undergoing nuclear fragmentation in the ISV and CA, respectively. Scale bars, 50 μm. (B) Percentage of DMSO-treated or Ki 8457-treated NLS-Eos-expressing ECs that undergo nuclear fragmentation in the CA and CVP between 34-42 hpf. Data are expressed as a percentage relative to the total number of NLS-Eos-expressing ECs, and shown as mean ± s.e.m. (DMSO n=6, Ki 8457 n=6). ***p<0.01. n.s., no significance.

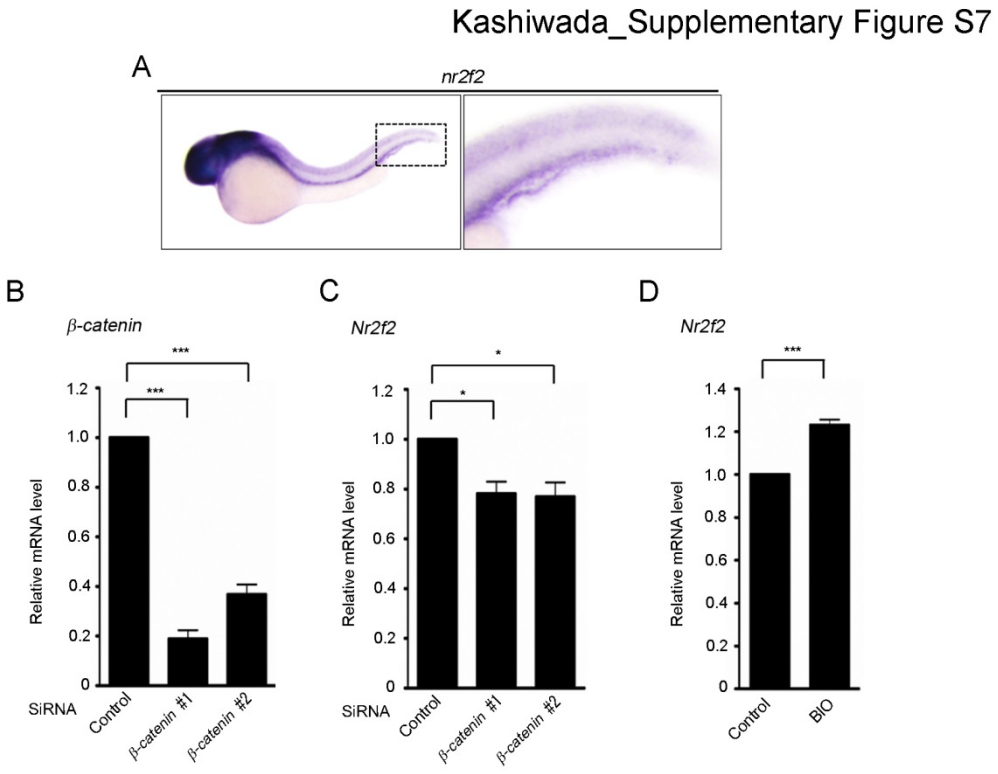


Supplementary Figure S5. Isolation of β-catenin (+) ECs from the caudal regions of EC-specific β-catenin reporter zebrafish. (A) Schematic representation of the purification of the β-catenin (+) and β-catenin (-) ECs for RNA-seq analyses. (B) To identify the genes that are upregulated and downregulated in β-catenin (+) ECs, GFP-positive and mCherry-positive ECs (β-catenin (+) ECs) and GFP-negative and mCherry-positive ECs (β-catenin (-) ECs) were isolated from the caudal parts of *Tg(fli1:Gal4db-TΔC-2A-mC);(UAS:GFP);(fli1:Myr-mC)* embryos using flow cytometry.

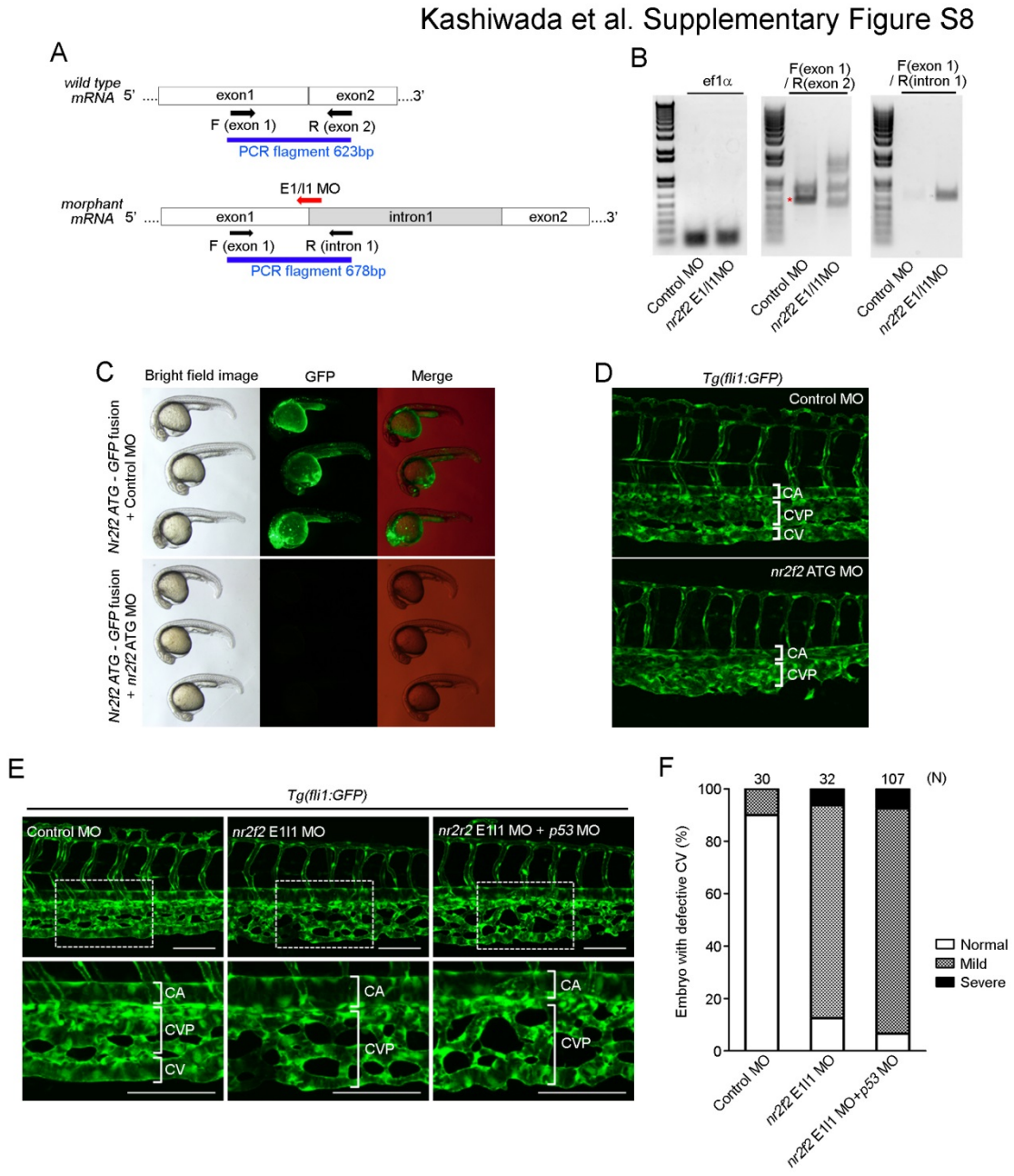


Supplementary Figure S6. Aggf1 stimulates β-catenin-dependent transcription in ECs to develop the CV. (A) Confocal stack images of the caudal regions of the 48 hpf *Tg(fli1:GFP)* embryos injected with control MO (left column), *aggf1* MO1 (middle column) or a mixture of *aggf1* MO1 and *p53* MO (right column). The boxed areas are

enlarged at the bottom. CA, caudal artery; CV, caudal vein; CVP, caudal vein plexus. (B) The CV phenotypes observed in A were quantified, as in Figure 2D. (C) Confocal images of caudal regions of 48 hpf *Tg(fli1:GFP);(flt1:mC)* embryos injected with *aggf1* MO1. Two representative embryos are shown (Embryo #1 and Embryo #2). Left, GFP images; middle, mCherry images (mC); right, the merged images (Merge). mCherry fluorescence marks the arterial ECs. Arrows indicate defective ISVs. Note that formation of venous ISVs was mainly affected by depletion of Aggf1. (D) Confocal images of caudal regions of 36 hpf *Tg(fli1:Gal4db-TΔC-2A-mC);(UAS:GFP);(fli1:Myr-mC)* embryos injected with either control MO or *aggf1* MO2. Upper, GFP images (β-Cat activity); lower, merged images of GFP (green) and mCherry (red) (GFP/Myr-mC). (E) Fluorescence intensity of GFP in the caudal vein plexus (CVP) as observed in D was quantified, and expressed as a relative value to that observed in the control MO-transfected embryos. Data are shown as mean ± s.e.m. (Control MO n=14, *aggf1* MO2 n=15). **p*<0.05. Scale bars, 100 μm (A, C) and 50 μm (D).

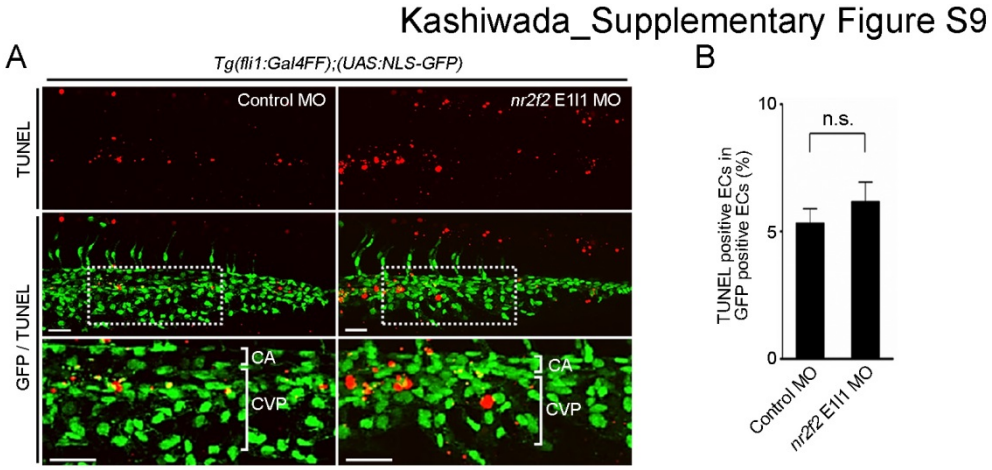


Supplementary Figure S7. β-catenin regulates expression of *NR2F2*. (A) Expression pattern of *nr2f2* mRNA in zebrafish embryos at 48 hpf, as detected by whole-mount *in situ* hybridization. The boxed area is enlarged at the right. (B, C) Total RNA was extracted from human umbilical vein ECs transfected with either control siRNA or two independent siRNAs targeting *β-catenin* (#1 and #2), and subjected to real-time RT-PCR analysis to determine the expression levels of *β-catenin* (B) and *NR2F2* (C) mRNAs. Bar graphs show relative mRNA levels of *β-catenin* and *NR2F2* normalized to that of *GAPDH*. Data are expressed relative to that in control siRNA-transfected cells, and shown as mean ± s.e.m. (n=4). (D) Total RNA was extracted from human umbilical vein ECs stimulated with vehicle (Control) or 2 μM BIO for 24 h, and subjected to real-time RT-PCR analysis to determine the expression levels of *NR2F2* mRNA, as in B. Data are expressed relative to that in control cells, and shown as mean ± s.e.m. (n=4). **p*<0.05, ***p*<0.01, ****p*<0.001.

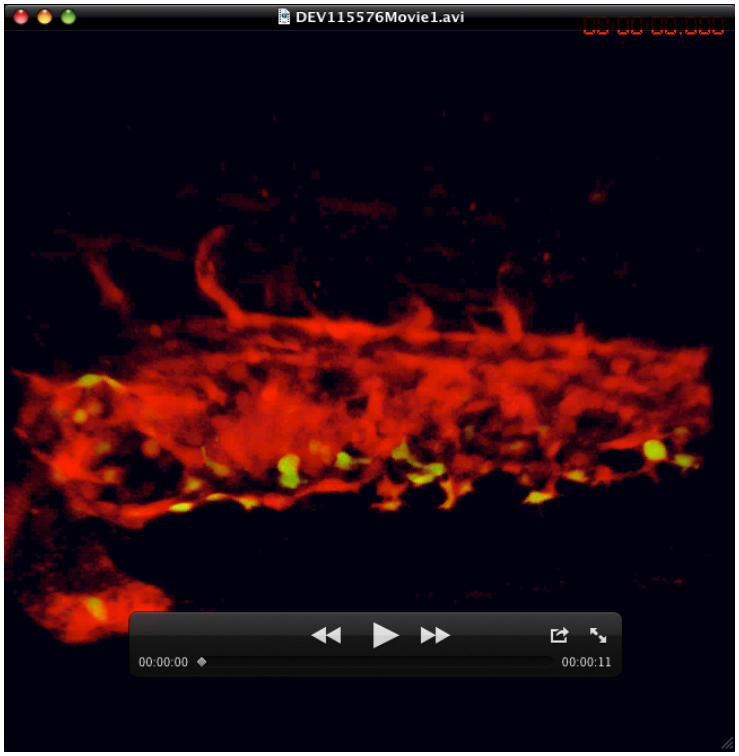


Supplementary Figure S8. Nr2f2 is required for CV formation. (A) *nr2f2* E1/I1 MO (red arrow) is a splice-blocking MO that targets the boundary between exon 1 and intron 1. Schematic diagrams of normally-spliced mRNA (upper) and aberrantly-spliced mRNA produced in *nr2f2* E1/I1 MO-injected embryos (lower). (B) RT-PCR analyses of RNAs extracted from the zebrafish embryos injected from control MO or *nr2f2* E1/I1 MO were performed using two sets of PCR primers as indicated in A (F(exon 1)/R(exon

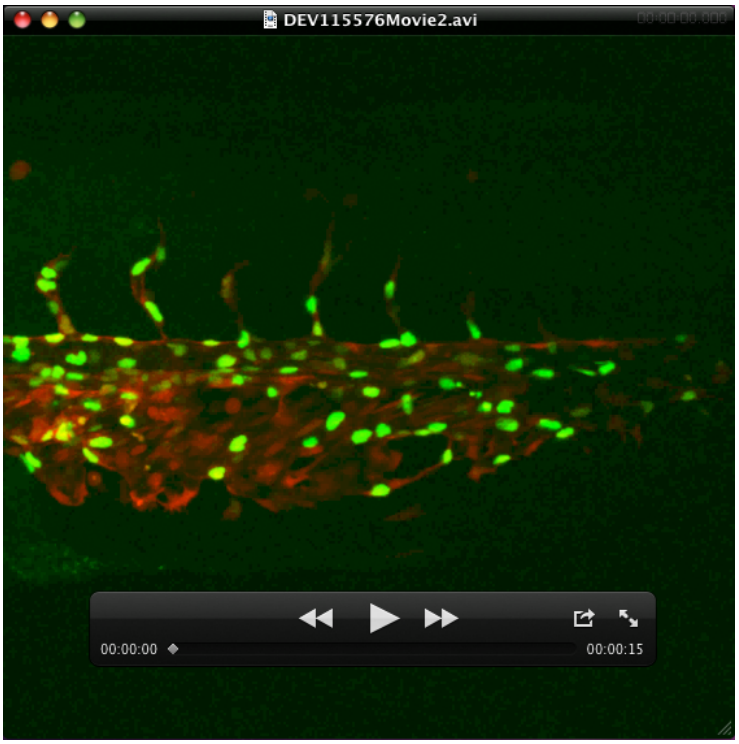
2); F(exon 1)/R(intron 1)) as well as an *ef1a* primer. PCR using a primer set of F(exon 1)/R(exon 2) amplified the 623-bp fragment (single asterisk) corresponding to normally-spliced *nr2f2* mRNA in control MO-injected embryos, but not in those injected with *nr2f2* E1I1 MO. In contrast, PCR using a primer set of F(exon 1)/R(intron 1) amplified the 678-bp fragment (double asterisks) that contains partial sequence of exon 1 and intron 1 only in *nr2f2* E1I1-injected embryos. These results suggest that *nr2f2* E1/I1 MO efficiently blocks splicing of exon 1 and 2, leading to inclusion of intron 1 and the creation of premature stop codon. (C) Bright field images (left column), GFP images (middle column) and the merged images (right column) of 24 hpf embryos injected with mRNA encoding amino acids 1-27 of Nr2f2 C-terminally tagged with GFP (Nr2f2 ATG-GFP fusion) together with control MO (upper) or *nr2f2* ATG MO (lower). Note that injection of *nr2f2* ATG MO efficiently suppressed expression of Nr2f2 ATG-GFP fusion protein. (D) Confocal stack images of the caudal regions of the 48 hpf *Tg(fli1:GFP)* embryos injected with control MO or *nr2f2* ATG MO. (E) Confocal stack images of the caudal regions of the 48 hpf *Tg(fli1:GFP)* embryos injected with control MO (left column), *nr2f2* E1I1 MO (middle column) or a mixture of *nr2f2* E1I1 MO and *p53* MO (right column). The boxed areas are enlarged at the bottom. (F) The CV phenotypes observed in E were classified and quantified, as in Figure 6H and 6I. CA, caudal artery; CV, caudal vein; CVP, caudal vein plexus. Scale bars, 100 μm.



Supplementary Figure S9. Nr2f2 is not involved in survival of venous ECs during CV formation. (A) Confocal stack fluorescence images of the 32 hpf *Tg(fli1:Gal4FF);(UAS:NLS-GFP)* embryos injected with either control MO or *nr2f2* E1I1 MO and subjected to TUNEL staining. Top, TUNEL signal; middle, the merged images (GFP/TUNEL) of GFP (green) and TUNEL (red); bottom, the enlarged images of boxed areas. Scale bars, 50 µm. (B) Percentage of TUNEL-positive ECs among the NLS-GFP-expressing ECs in the CVP of the embryos injected with control MO or *nr2f2* E1I1 MO as observed in A. n.s., no significance.



Supplementary Movie 1. β-catenin-mediated gene expression is induced in the ECs that sprout from CV primordia. Time-lapse confocal imaging of the CV formation in the *Tg(fli1:Gal4db-TΔC-2A-mC);(UAS:GFP);(fli1:Myr-mC)* embryo. Green, GFP fluorescence (β-Cat activity); red, mCherry fluorescence (ECs). The recording started at 24 hpf. Elapsed time (hr:min:sec:msec).



Supplementary Movie 2. Inhibition of β-catenin/Tcf-dependent transcription results in nuclear fragmentation of the ECs in the CVP, but not in the caudal artery. Time-lapse confocal imaging of the CV formation in the *Tg(fli1:Gal4FF);(UAS:RFP);(UAS:TΔN-GFP)* embryo. Green, GFP fluorescence (TΔN-GFP-expressing ECs); red, mCherry fluorescence (ECs). The recording started at 30 hpf. Elapsed time (hr:min:sec:msec).

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