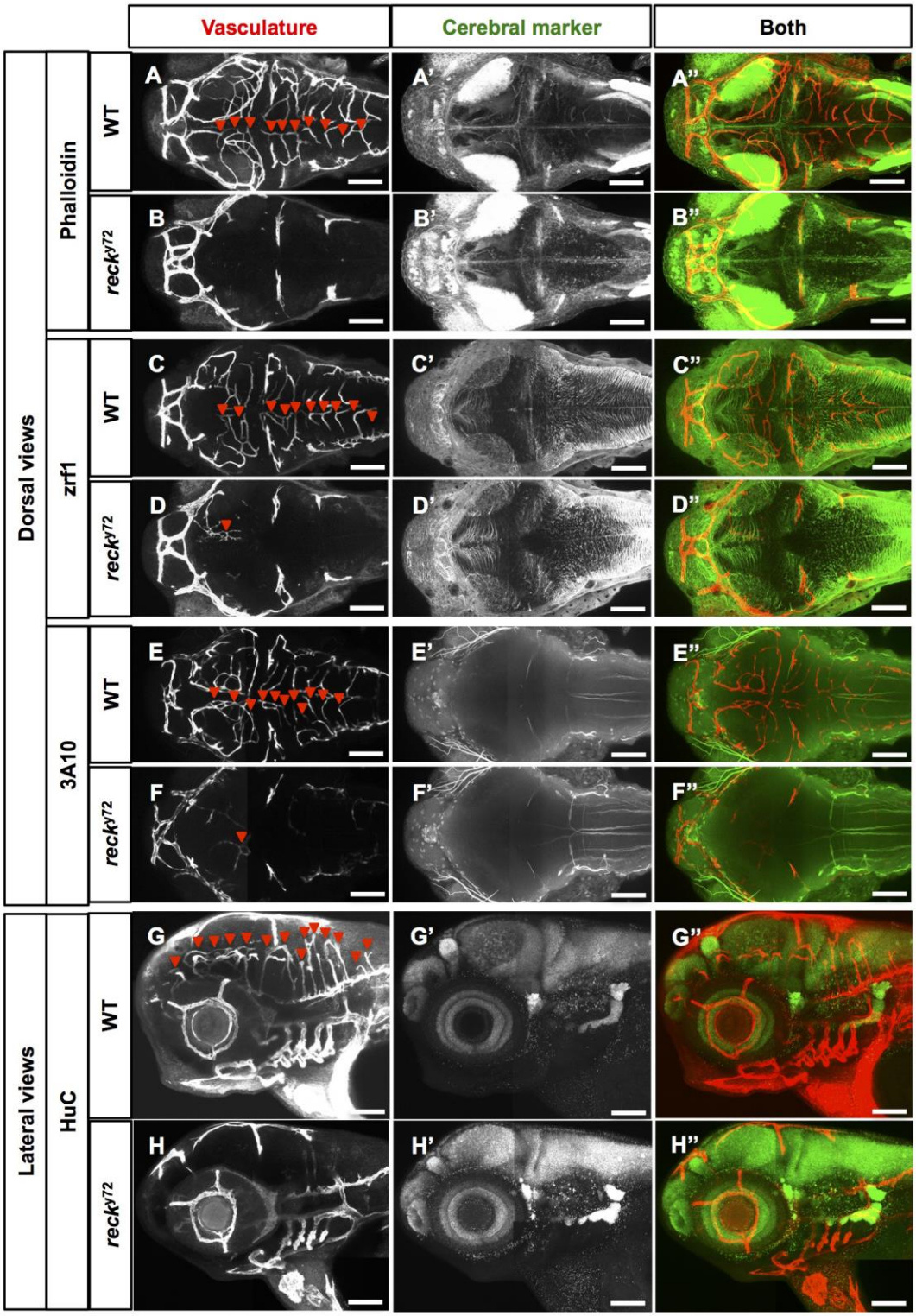


Fig. S1. Normal gross cerebral organization in *reck*^{y72}. (A-H'') Confocal views; 72 hpf embryo heads. Anterior, left. (A-F"') Dorsal views; left side, bottom. (G-H") Lateral views; dorsal, up. Scale Bar: 100 µm. Genotypes, vasculature and cerebral markers as indicated. Vasculature: *Tg(kdrl:RFP)*^{s896} or *Tg(kdrl:eGFP)*^{1a116}. Cerebral markers: phalloidin staining (labels F-actin at the interface of actin subunits), zrf1 (radial glia), 3A10 (labels a subset of hindbrain spinal cord projecting neurons, including Mauthner neurons) and HuC (pan-neuronal marker) immunofluorescence; see (Brand et al., 1996; Kim et al., 1996; Lowery et al., 2009)(Ulrich et al., 2011).

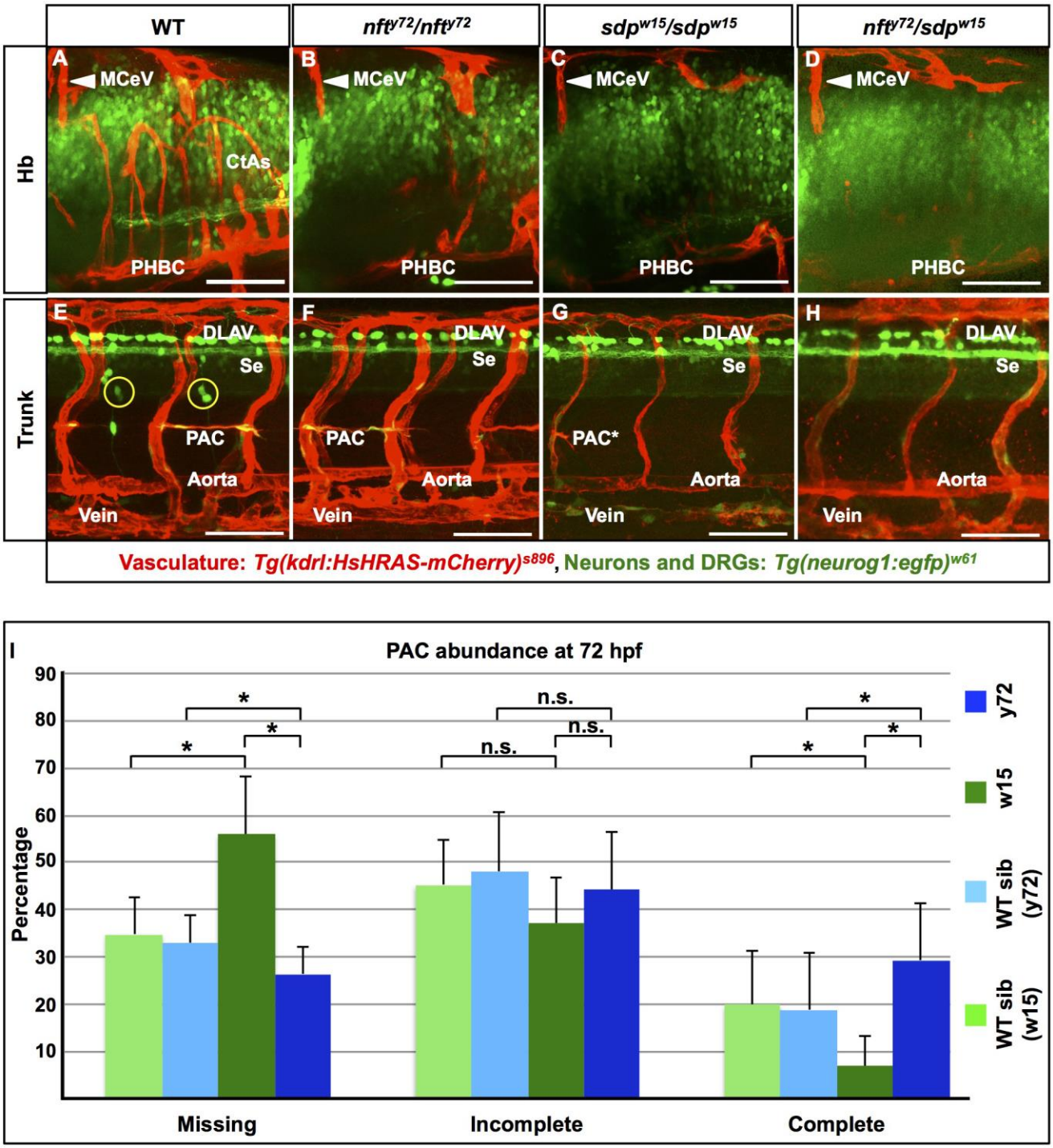


Fig. S2. *nft*^{y72}/*sdp*^{w15} trans-heterozygotes and both *nft*^{y72} and *sdp*^{w15} homozygotes have identical CtA and DRG deficits. (A-H) Confocal lateral images of the Hb (A-D) and trunk (E-H) of 72 hpf embryos. Anterior, left; dorsal, up. Vasculature (white labels), red (*Tg(kdrl:RFP)*^{s896}); DRG neurons, green (in yellow circles, *Tg(neurog1:eGFP)*^{w61}). Scale bars: 100 µm. (I) Quantification of the abundance of missing, incomplete and complete Parachordal Chains (PAC) at 72 hpf in *nft*^{y72} (*reck*^{y72}) and *sdp*^{w15} (*Df(Chr24:reck)*^{w15}) mutants and their phenotypically-WT siblings. A complete PAC spans the full distance between an ipsilateral Se vessel pair. PAC percentages were thus calculated in reference to the total number of Se vessel pairs scored. Asterisks, significant differences ($p < 0.05$) between genotypes (Student's *t*-test); n.s., no significant. n=10 embryos per genotype or phenotype.

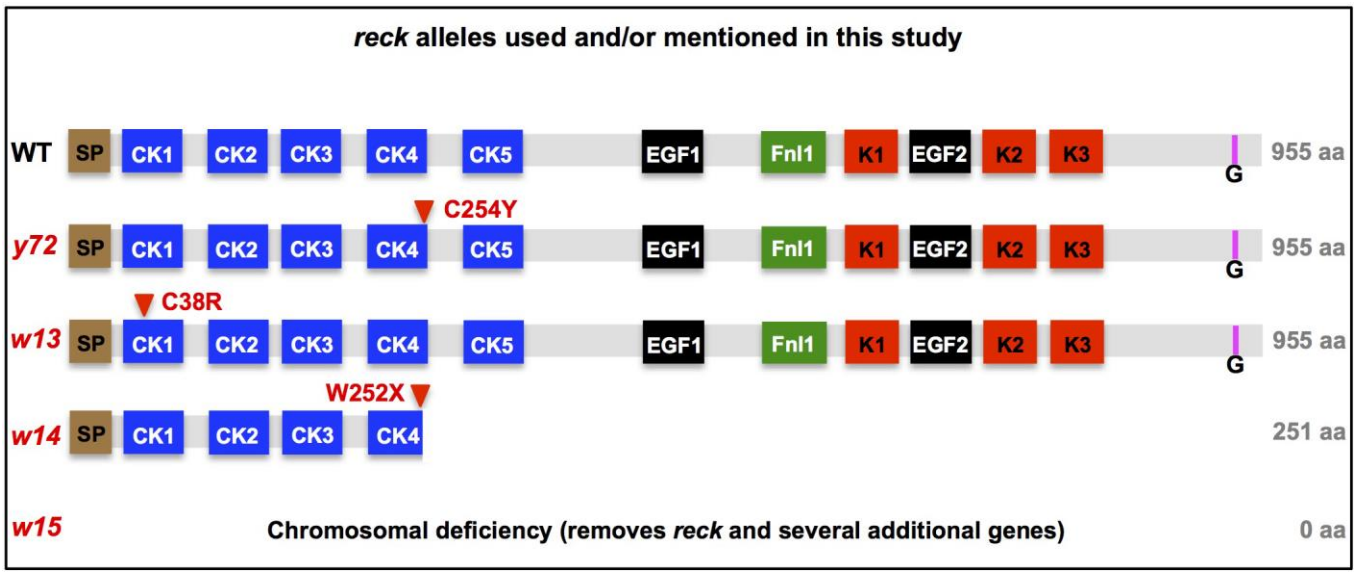


Fig. S3. Domains and sizes of Reck proteins (based on conceptual translation) encoded by the mutant alleles used and/or mentioned in this study. See Table S1.

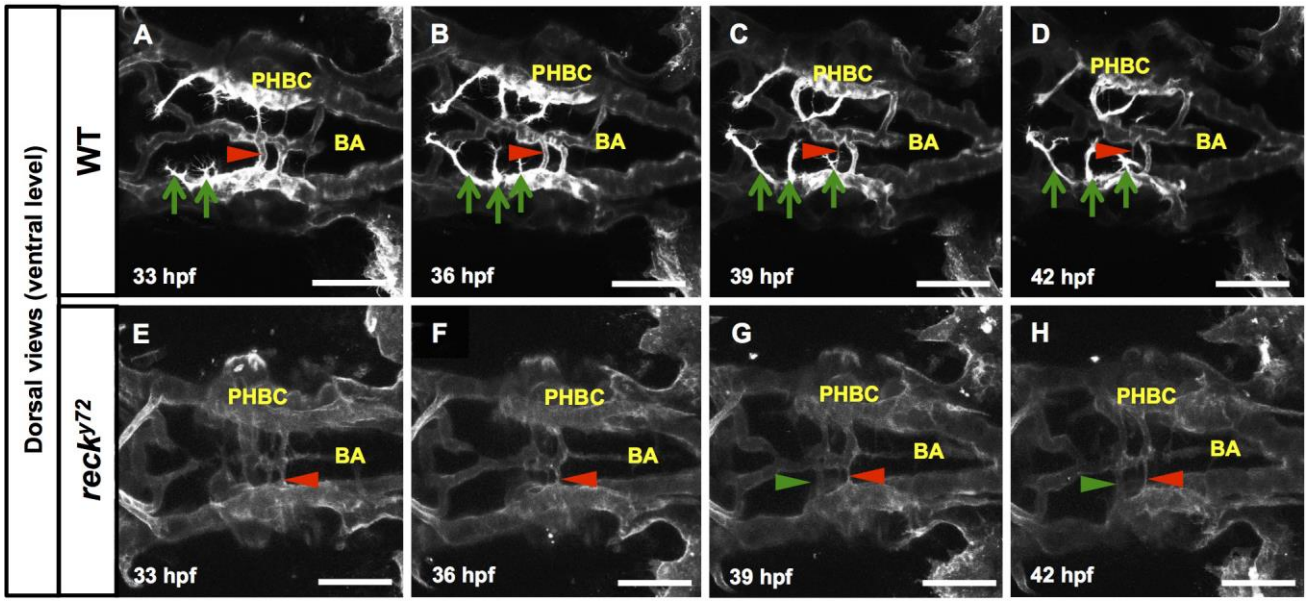


Fig. S4. In *reck*^{y72} the PHBCs are deficient in CtA sprout formation and display abnormal avc dynamics. Time-lapse stills (dorsal views, ventral-level) of Hb vascular development (visualized with *Tg(kdrl:GFP)*^{la116}) in WT (A-D) and *reck*^{y72} (E-H) from 33 to 42 hpf. Anterior, left; right side, up. Green arrows, developing CtA sprouts on the embryo's left side. Green arrows, *de novo* avc formation. Red arrowheads, regressing avc. In this WT (A-D) three filopodia-rich CtA sprouts launch from the dorsal side of the left PHBC, one PHBC-BA avc thins and regresses but no new avc develop. In this *reck*^{y72} (E-H) filopodial activity is absent from the dorsal face of the PHBCs and, accordingly, no CtA-like, dorsally projecting sprouts emerge. On the mutant's left side a PHBC-BA avc regresses and another forms. There is no evidence that the mutant's supernumerary avc are mistargeted CtAs unable to invade the brain. Scale bars: 100 µm. See Movies S2A-S2B.

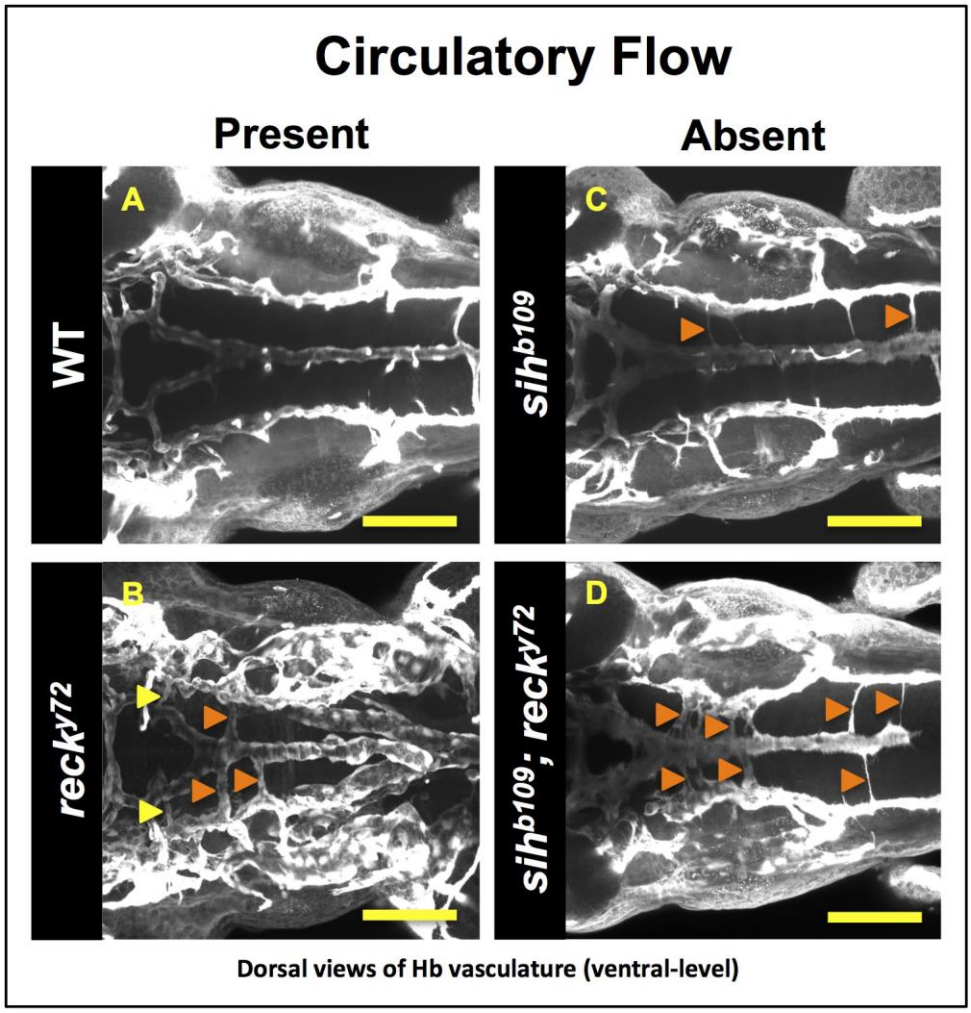


Fig. S5. *reck* limits avc abundance even in the absence of circulatory flow. (A-D) Confocal images (dorsal view) of the 48 hpf peri-neural vasculature. Genotypes as indicated. Arrowheads, avc (PCS-connected, yellow; BA-connected, orange). Scale bars: 100 µm. See Table S2.

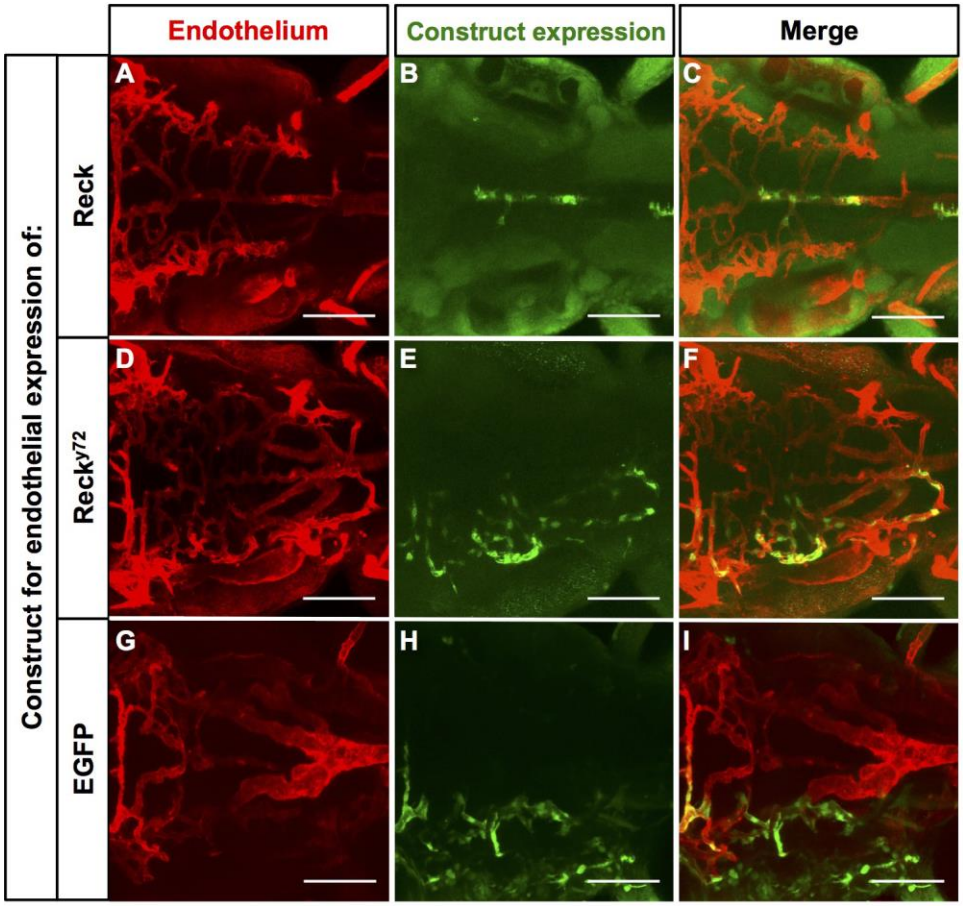


Fig. S6. Examples of the distribution of clones with exogenous endothelial expression of Reck, Reck^{y72} or EGFP in the 72 hpf Hb vasculature of *reck*^{y72} mutants. (A-I) Dorsal views (ventral-level) of the Hb extra-cerebral vasculature (red, *Tg(kdrl:RFP)*^{s896}) of *reck*^{y72} injected with constructs driving endothelial expression of exogenous Reck, Reck^{y72} (both HA-tagged, see Fig. 2L) or EGFP proteins (green). Anterior, left. Right side, up. Scale Bars: 100 µm. See also Figure 5 and Graph S1.

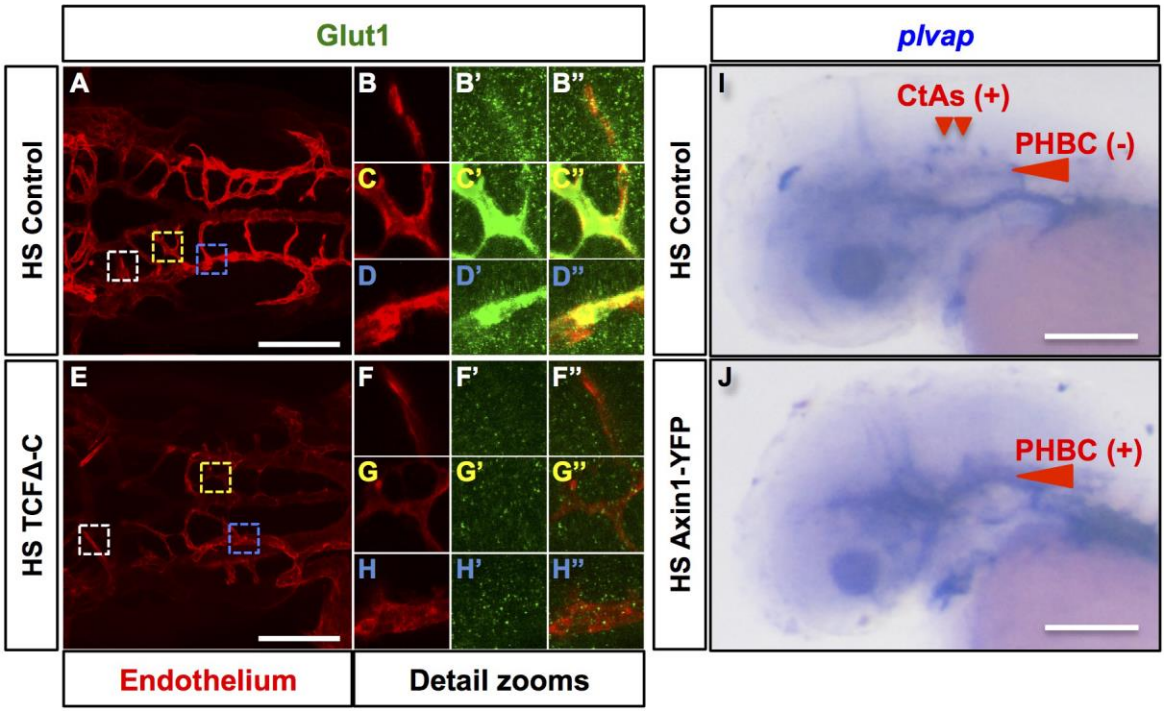


Fig. S7. Forced inhibition of canonical Wnt signaling impairs CtA formation and leads to aberrant cerebrovascular expression of the Wnt-responsive markers of barriergenic differentiation Glut1 and *plvap*. (A-H") Confocal dorsal images of the 48 hpf Hb vasculatures of heat-shocked (HS) embryos without (Control) or with TCFΔC (*Tg(hsp70l:Xla.TCFΔC-EGFP*) over-expression. Heat-shock: 40ºC for 30 minutes at 30 hpf. Anterior, left. Right side, up. Endothelium, red (*Tg(kdrl:RFP)*^{s896}); Glut1 immunofluorescence, green. Colored dashed boxes (A, E) demarcate a region of the following vessels: white, MtA (zooms: B-B", F-F"); yellow, CtAs (zooms: C-C", G-G"); blue, PHBCs (zooms: D-D", H-H"). Merged images of zooms: B", C", D", F", G", H". Glut1 decorates the MtA, CtAs and PHBCs of the Control (n=2 embryos). In contrast, in the TCFΔC over-expressing embryo CtA abundance is reduced (n=10 embryos) and cerebrovascular Glut1 decoration is missing (n=2 embryos). (I-J) Transmitted-light lateral images of the 48 hpf heads of heat-shocked (HS) embryos without (Control) or with Axin1-YFP (*Tg(hsp70l:Mmu.Axin1-YFP)*^{w35}) over-expression subjected to whole mount RNA *in situ* hybridization with *plvap* riboprobes. Heat-shock: 39ºC for 1 hour at 24 hpf. (I) Control. *plvap* is expressed in the dorsal aspect of CtAs but not in the PHBCs (n=9 embryos). (J) Axin1-YFP over-expressing embryo. *plvap* is ectopically expressed in the PHBCs (n=7 embryos). Anterior left. Dorsal up. Scale bar: 100 μm.

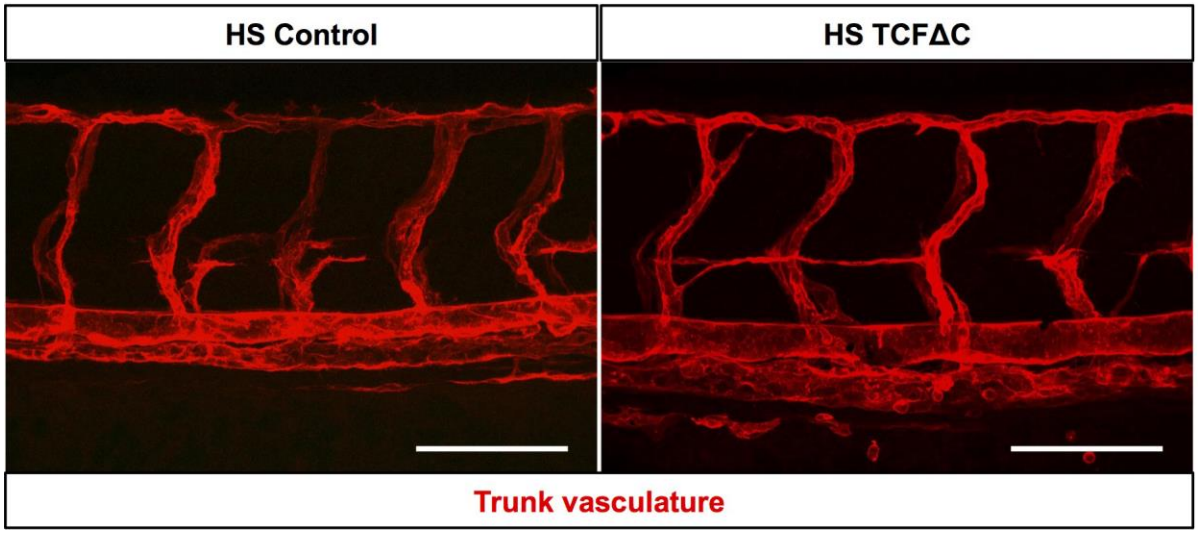


Fig. S8. Forced inhibition of canonical Wnt signaling at 30 hpf has no impact on the patterning of the trunk vasculature. Confocal lateral images of the 48 hpf trunk vasculatures (red; *Tg(kdrl:RFP)*^{s896}) of heat-shocked (HS) embryos without (Control) or with TCFΔC (*Tg(hsp70l:Xla.TCFΔC-EGFP)* over-expression. Heat-shock: 40ºC for 30 minutes at 30 hpf. Anterior, left; dorsal, up. Scale bars: 100 µm.

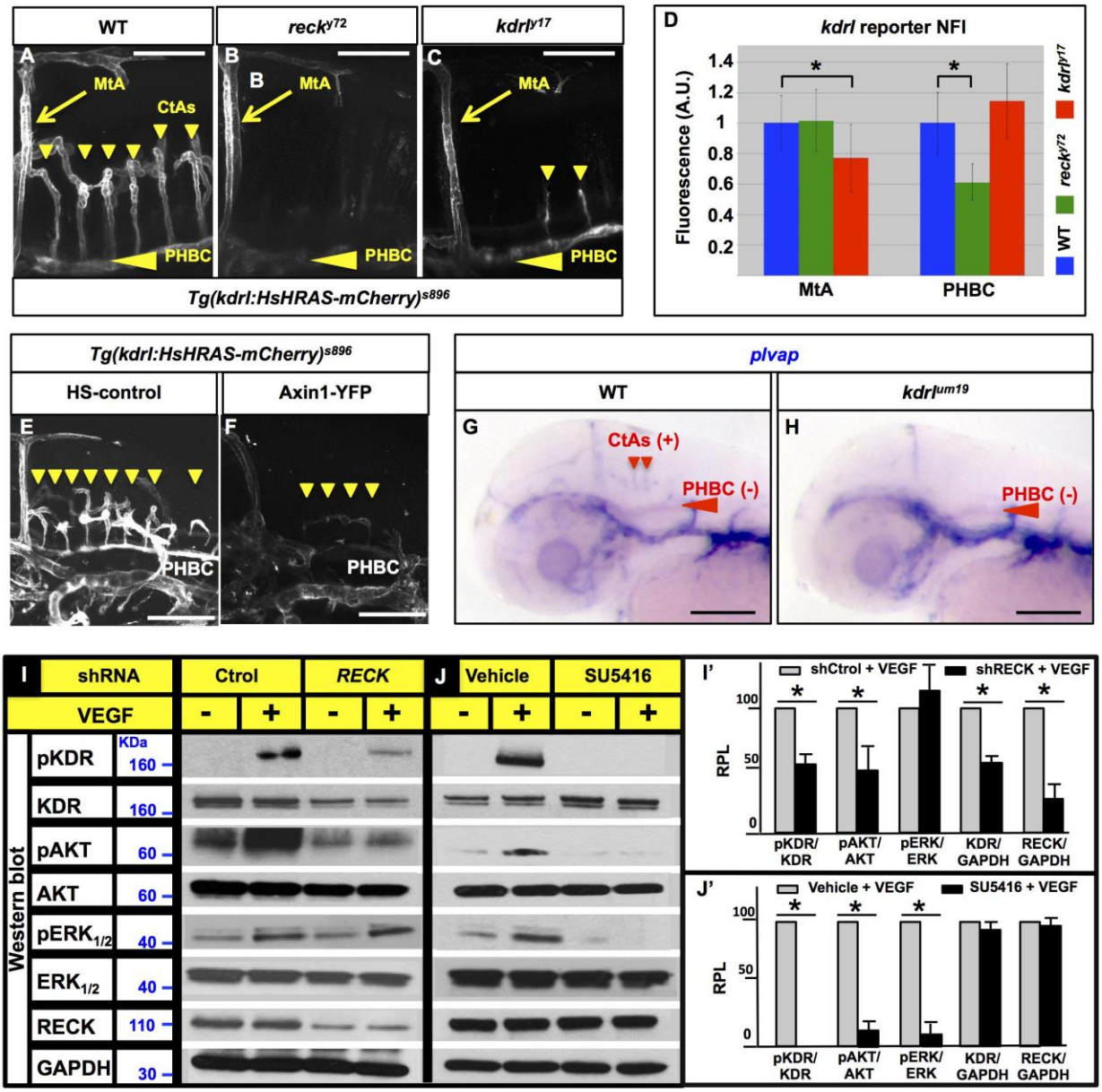
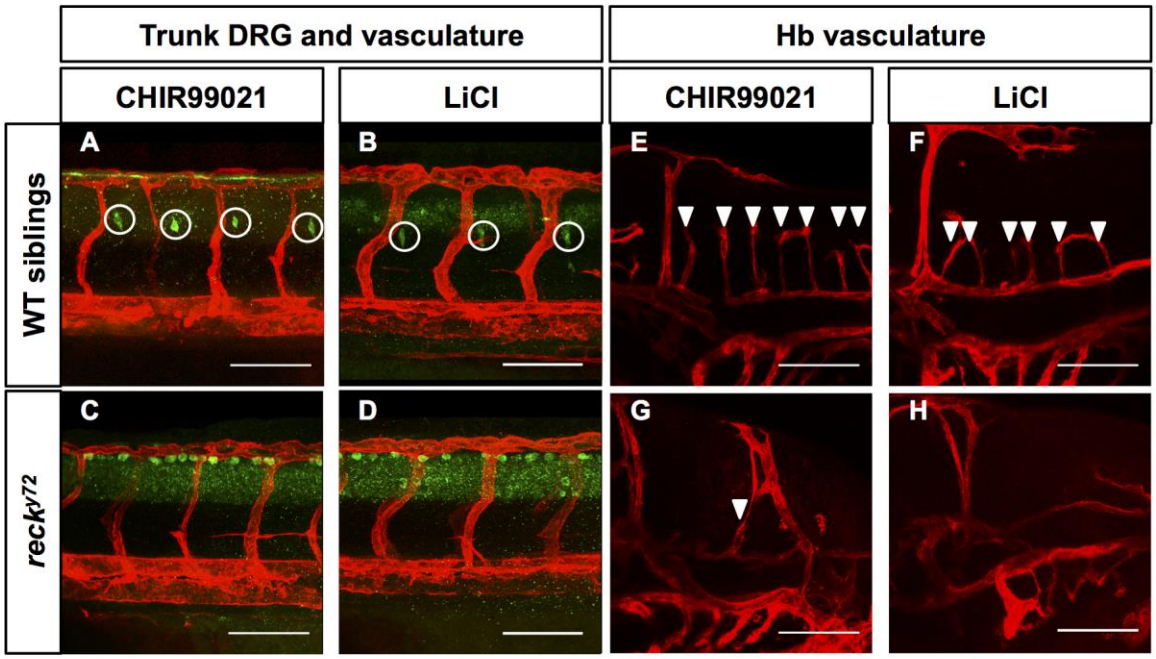


Fig. S9. *reck* and canonical Wnt signaling promote the cerebrovascular expression of the transgenic *kdrl* transcriptional reporter *Tg(kdrl:RFP)*^{s896} in zebrafish and *RECK* promotes VEGF signaling in HUVEC. (A-C, E-F) Confocal lateral images of the live fluorescence of *Tg(kdrl:RFP)*^{s896} (white) in the Hb vasculatures of WT (A), *reck*^{y72} (B), *kdrl*^{y17} (C) and heat-shocked (HS) embryos without (E) or with (F) inhibition of canonical Wnt signaling via Axin1-YFP (*Tg(hsp70l:Mmu.Axin1-YFP)*^{w35}) over-expression. Anterior, left; dorsal, up. (A-C) 58 hpf. (E-F) 48 hpf. (D) Quantification of the Normalized Fluorescence Intensity (NFI) of *Tg(kdrl:RFP)*^{s896} in the MtAs and PHBCs of WT, *reck*^{y72} and *kdrl*^{y17} at 58 hpf expressed in fluorescence Arbitrary Units (A. U.). Asterisks, significant differences (p<0.05). Student's t-test. n=10 embryos per genotype. Vertical lines, SD. (A-D) The PHBC fluorescence of *Tg(kdrl:RFP)*^{s896} is reduced in *reck*^{y72} but not in *kdrl*^{y17}, while that of the MtA is only reduced in *kdrl*^{y17}. (E-F) Both CtA abundance and *Tg(kdrl:RFP)*^{s896} cerebrovascular fluorescence are reduced in Axin1-YFP over-expressing 48 hpf embryos. Heat-shock: 39°C for 1 hour at 24 hpf. (G-H) Transmitted-light lateral images of the 48 hpf heads of phenotypically WT-siblings (G) and *kdrl*^{um19} (H) subjected to whole mount RNA *in situ* hybridization with *plvap* riboprobes. In the WT siblings *plvap* is expressed (+) in the dorsal aspect of CtAs but not (-) in the PHBCs (n=11 embryos). In *kdrl*^{um19} mutants *plvap* is also not expressed (-) in the PHBCs (n=11 embryos) and the CtAs are missing. (A-C, E-H) Small down-pointing arrowheads (yellow or red), CtAs; large left-pointing arrowheads (yellow or red), PHBCs. Scale bars: 100 µm. (I, J) Western Blots of biochemical readouts of VEGF-A signaling. Conditions, above. Detected proteins and molecular weight markers, left. (I', J') Bar graphs of Western Blot quantifications. y axis, Relative Protein Level (RPL) measured by densitometry. x axis, protein and/or phospho-isoform. n=3. Asterisks, significant differences (p<0.05); Student's t-test (vertical lines, SEM).



I	DRG abundance (%)		
Genotype	DMSO	CHIR99021	LiCl
WT	21/21 = 100% n = 8 embryos	14/14 = 100% n = 4 embryos	25/27 = 92.6% n = 8 embryos
reck^{y72}	0/19 = 0% n = 5 embryos	0/37 = 0% n = 10 embryos	0/34 = 0% n = 10 embryos

Fig. S10. Chemical activation of canonical Wnt signaling with the GSK3β inhibitors CHIR99021 and LiCl fails to rescue the DRG and CtA deficits of *reck*^{y72} mutants. (A-H) Confocal lateral images at 72 hpf. Anterior, left; dorsal, up. Scale Bars: 100 µm. Genotypes and treatments as indicated. (A-D) DRG (white circles, green HuC immunofluorescence) and vasculature (*Tg(kdrl:HsHRAS-mCherry)*^{s896}; red) in the trunk. (E-H) Hb vasculature (*Tg(kdrl:HsHRAS-mCherry)*^{s896}; red). Central Arteries (CtA; white arrowheads). (I) Quantification of DRG abundance in WT and *reck*^{y72} mutants treated with DMSO vehicle, CHIR99021 and LiCl. DRG abundance was scored by imaging a region of the anterior trunk at the level of the yolk extension spanning 3-4 Se vessels (in WT animals there is one DRG per Se vessel). Thus, the percentages were calculated from the ratio of DRG found/DRG expected.

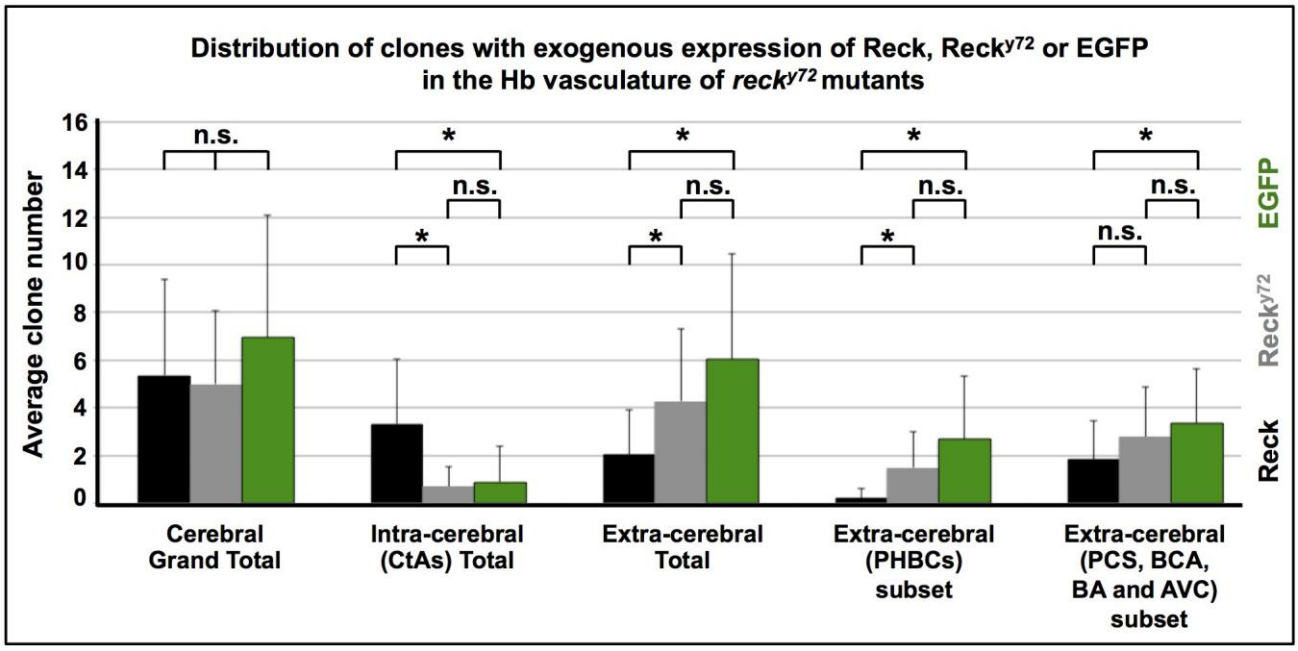
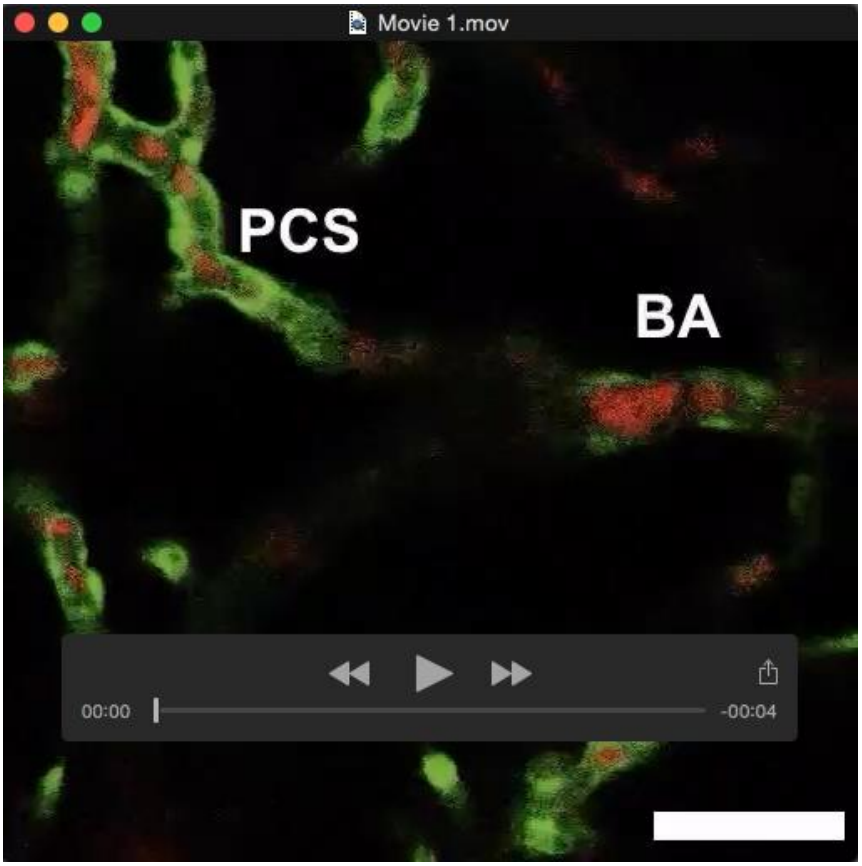
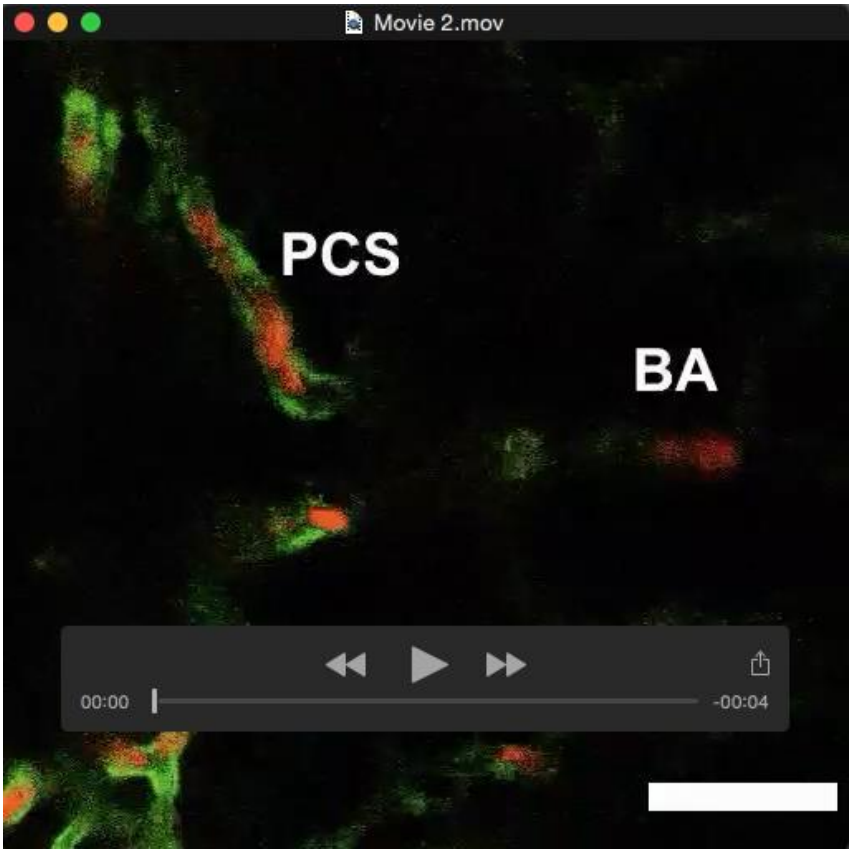


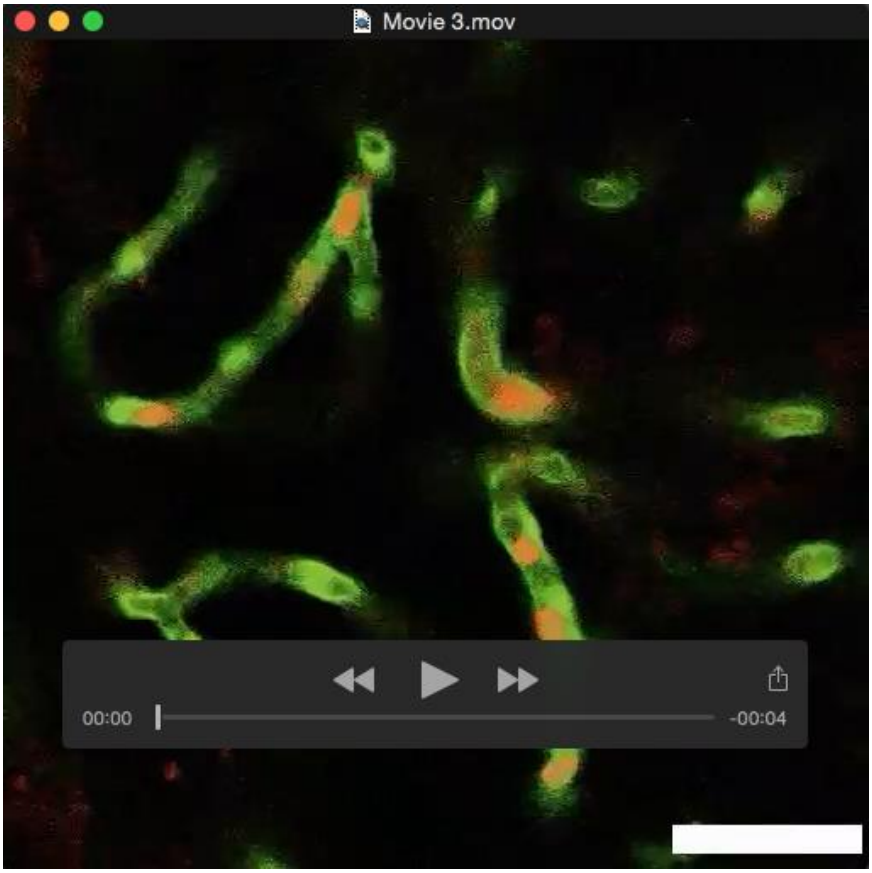
Fig. S11. Similar abundance but differential distribution of clones with exogenous endothelial expression of Reck, Reck^{y72} or EGFP in the 72 hpf Hb vasculature of *reck^{y72}* mutants. Reck-expressing clones are over-represented in the intra-cerebral vessels but under-represented in extra-cerebral vessels (particularly in the PHBCs). Conversely, Reck^{y72}- and EGFP-expressing clones are under-represented in the intra-cerebral vessels but over-represented in the extra-cerebral vessels. Together, these observations indicate that mosaic endothelial expression of Reck is sufficient to enable PHBC cells to emigrate to form CtAs, consistent with the results of our cell transplantation experiments; see Fig. 4G. Asterisks, significant differences (p<0.05); n.s., not significant. Student's t-test (vertical lines, S. E. M.). *reck^{y72}* mutants scored: Reck (n=28), Reck^{y72} (n=14), EGFP (n=19). Both Reck and Reck^{y72} are HA-tagged. See also Figs. 2L, 5.



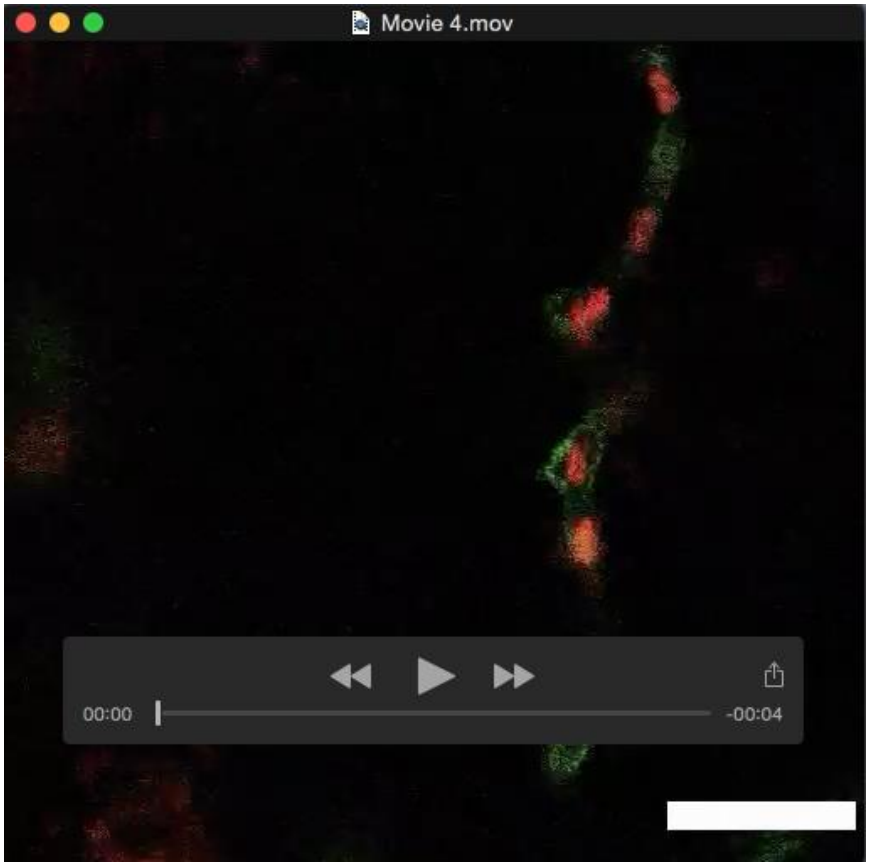
Movie 1. Dorsal view of blood flow through the anterior extra-cerebral Hb vasculature (WT, 2 dpf). Representative confocal time-lapse movie (10 frames/s); single plane. Anterior, left. Left side, bottom. Endothelium, green (*Tg(kdrl:EGFP)*^{la116}); erythrocytes, red (*Tg(gata1a:DsRed)*^{sd2}). Blood flows through the PCS and BA. Scale bar: 85 µm.



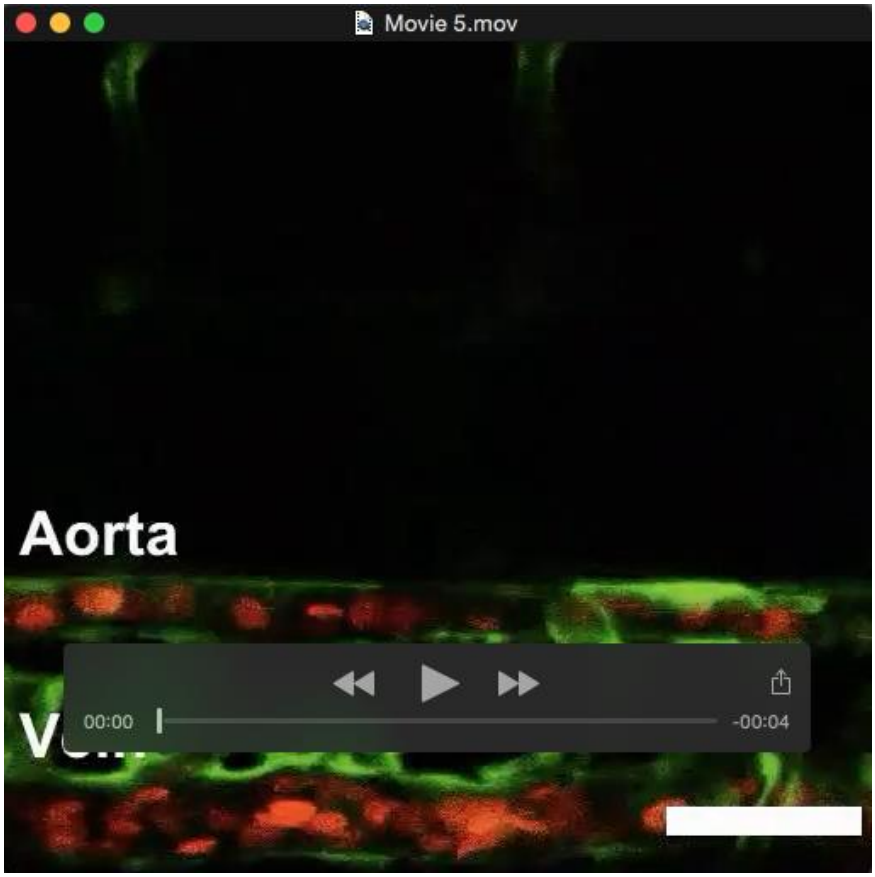
Movie 2. Dorsal view of blood flow through the anterior extra-cerebral Hb vasculature (*reck*^{y72}, 2 dpf). Representative confocal time-lapse movie (10 frames/s); single plane. Anterior, left. Left side, bottom. Endothelium, green (*Tg(kdrl:EGFP)*^{la116}); erythrocytes, red (*Tg(gata1a:DsRed)*^{sd2}). Blood flows through the PCS and BA. Scale bar: 85 µm.



Movie 3. Dorsal view of blood flow through the anterior intra-cerebral Hb vasculature (WT, 2 dpf). Representative confocal time-lapse movie (10 frames/s); single plane. Anterior, left. Left side, bottom. Endothelium, green (*Tg(kdrl:EGFP)*^{la116}); erythrocytes, red (*Tg(gata1a:DsRed)*^{sd2}). Blood flows through CtAs. Scale bar: 85 µm.

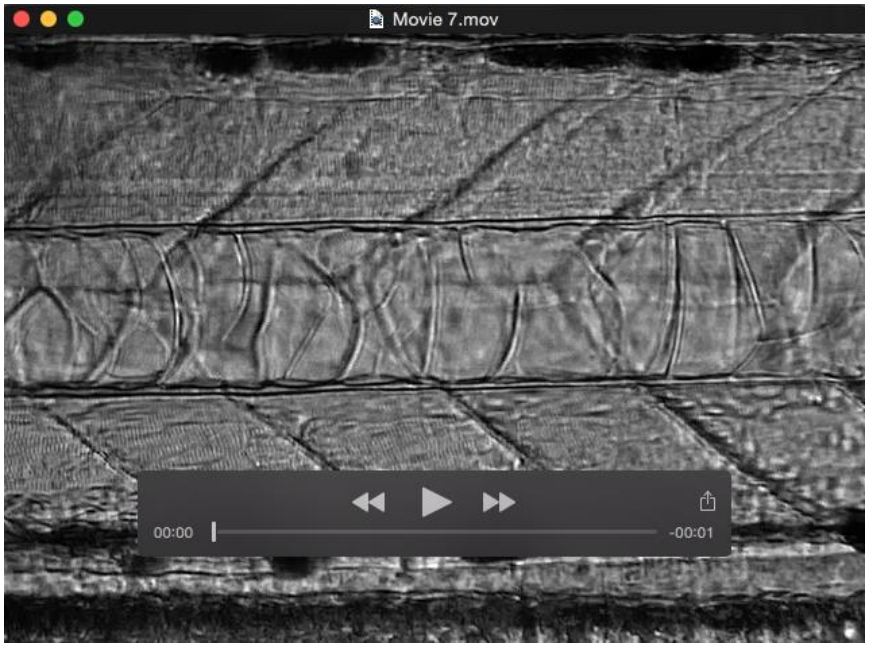


Movie 4. Dorsal view of blood flow through the anterior intra-cerebral Hb vasculature (*reck*^{y72}, 2 dpf). Representative confocal time-lapse movie (10 frames/s); single plane. Anterior, left. Left side, bottom. Endothelium, green (*Tg(kdrl:EGFP)*^{la116}); erythrocytes, red (*Tg(gata1a:DsRed)*^{sd2}). Blood flows through the two CtAs of this mutant. Scale bar: 85 µm.

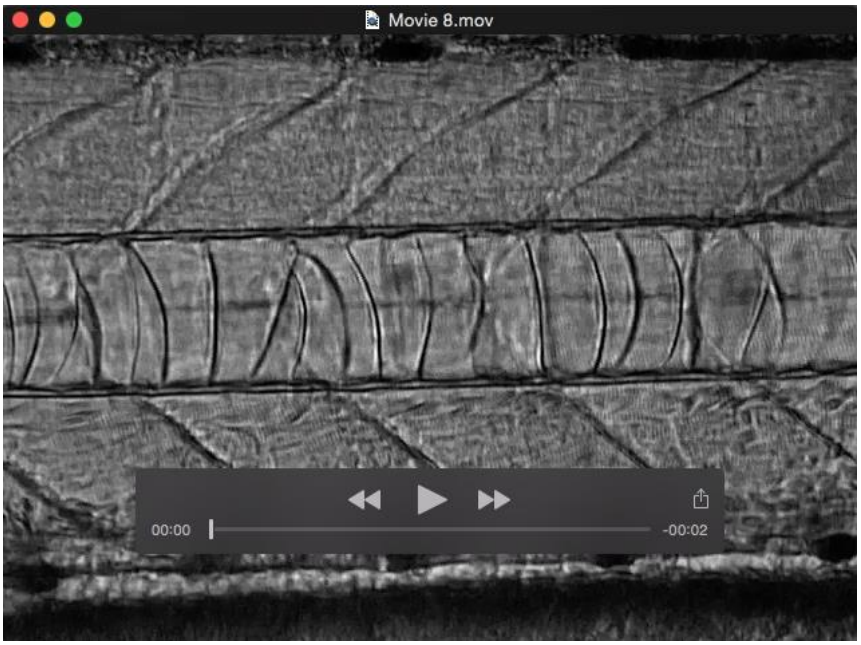


Movie 5. Lateral view of blood flow through the trunk vasculature (WT, 2 dpf). Representative confocal time-lapse movie (10 frames/s); single plane. Anterior, left. Dorsal, up. Endothelium, green (*Tg(kdrl:EGFP)*^{la116}); erythrocytes, red (*Tg(gata1a:DsRed)*^{sd2}). Blood flows through axial (Aorta and Vein; bottom) and Se vessels (unmarked; top). Scale bar: 85 µm.

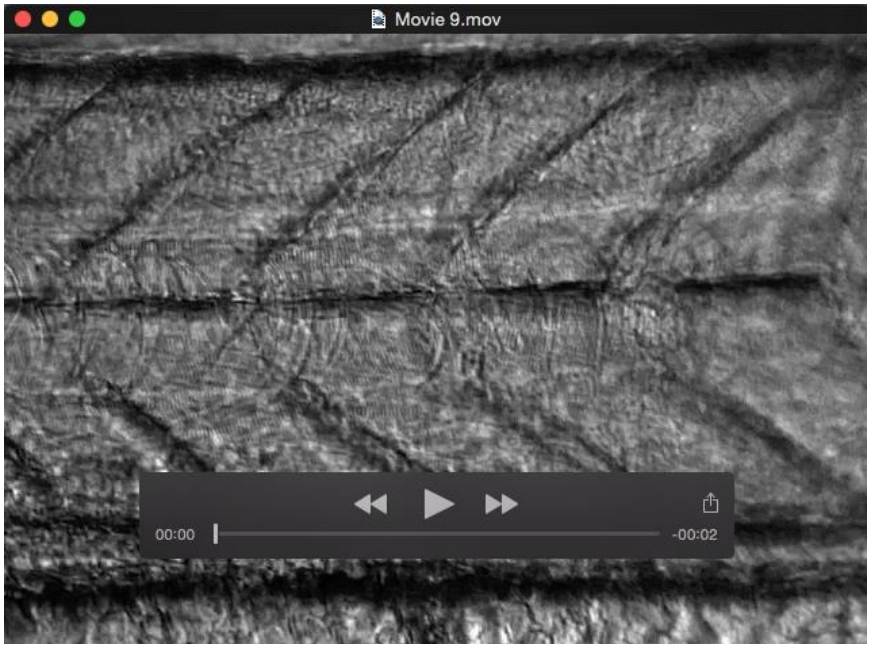
Movie 6. Lateral view of blood flow through the trunk vasculature (*reck*^{y72}, 2 dpf). Representative confocal time-lapse movie (10 frames/s); single plane. Anterior, left. Dorsal, up. Endothelium, green (*Tg(kdrl:EGFP)*^{la116}); erythrocytes, red (*Tg(gata1a:DsRed)*^{sd2}). Blood flows through axial (Aorta and Vein; bottom) and Se vessels (unmarked; top right quadrant). Scale bar: 85 µm.



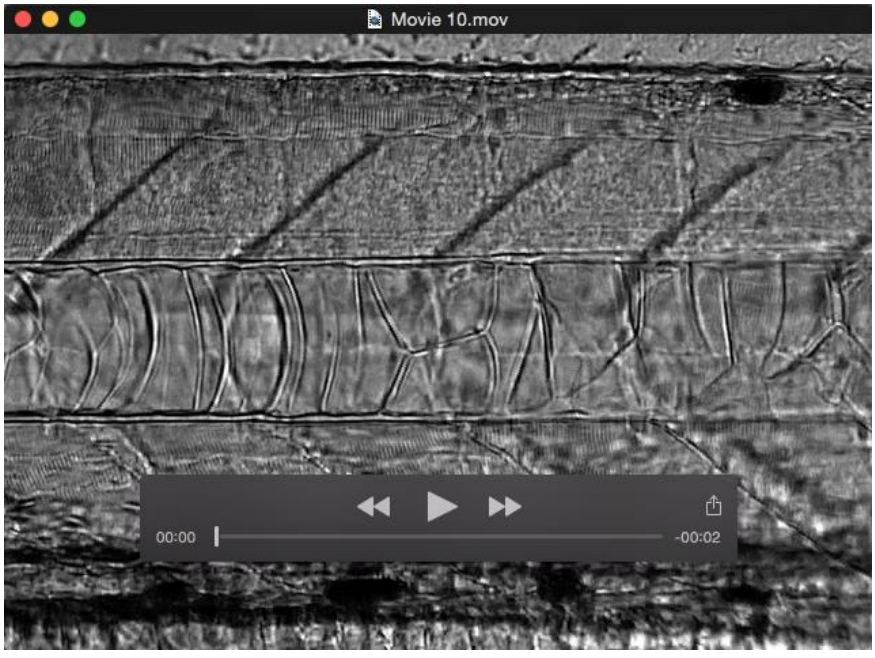
Movie 7. Lateral view of blood flow through the trunk vasculature (*reck*^{y72}, 3 dpf). Representative brightfield time-lapse movie (30 frames/s); single plane. Anterior, left. Dorsal, up. Blood flows through axial (Aorta and Vein) and Se vessels. Image size: W 740 x H 540 µm.



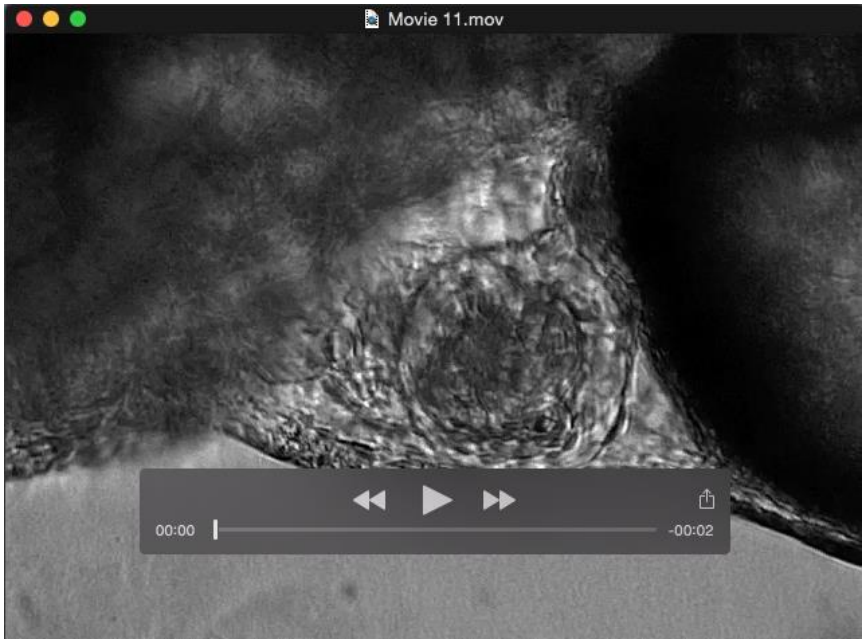
Movie 8. Lateral view of blood flow through the trunk vasculature of a WT sibling (from *reck*^{y72} in-cross; 3 dpf). Representative brightfield time-lapse movie (30 frames/s); single plane. Anterior, left. Dorsal, up. Blood flows through axial (Aorta and Vein) and Se vessels. Image size: W 740 x H 540 µm.



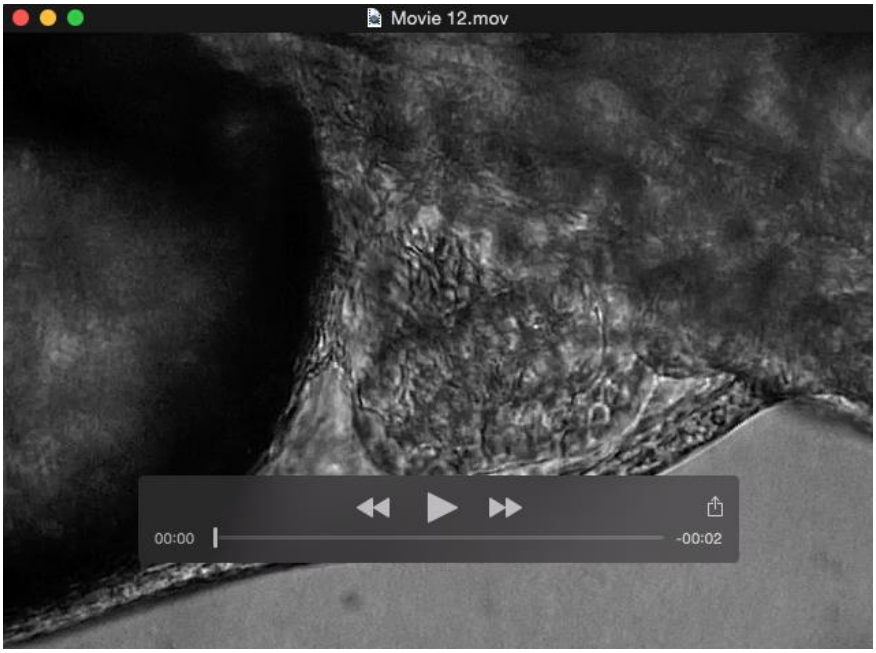
Movie 9. Lateral view of blood flow through the trunk vasculature (*Df(Chr24:reck)*^{w15}; 3 dpf). Representative brightfield time-lapse movie (30 frames/s); single plane. Anterior, left. Dorsal, up. Blood flow is absent. Image Size: W 740 x H 540 µm.



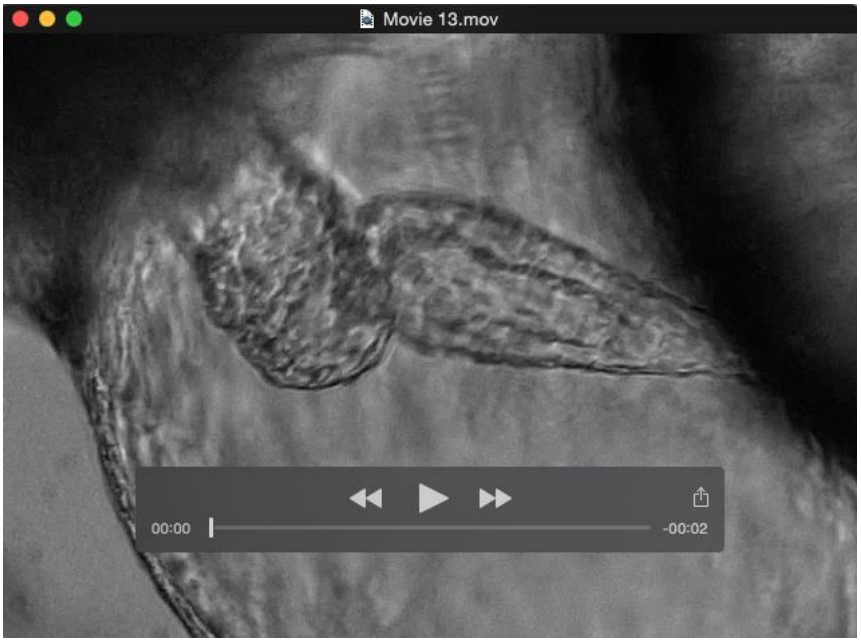
Movie 10. Lateral view of blood flow through the trunk vasculature of a WT sibling (from *Df(Chr24:reck)*^{w15} in-cross; 3 dpf). Representative brightfield time-lapse movie (30 frames/s); single plane. Anterior, left. Dorsal, up. Blood flows through axial (Aorta and Vein) and Se vessels. Image Size: W 740 x H 540 µm.



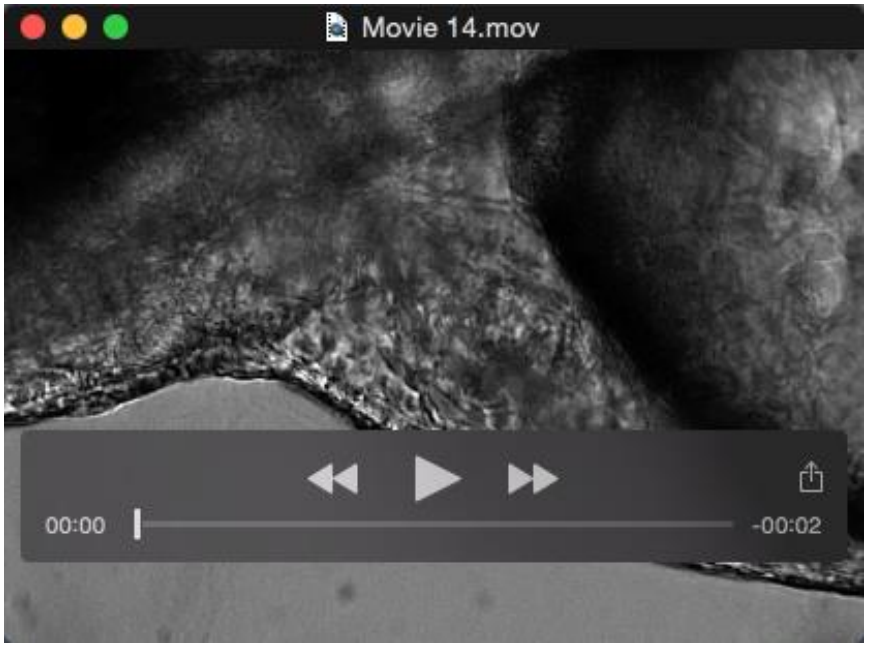
Movie 11. Cardiac contractility and circulation near the heart (*reck*^{y72}, 3 dpf). Representative brightfield time-lapse movie (30 frames/s); single plane. Anterior, left. Dorsal, up. Blood flows and cardiac contractility appears normal. Image Size: W 740 x H 540 µm.



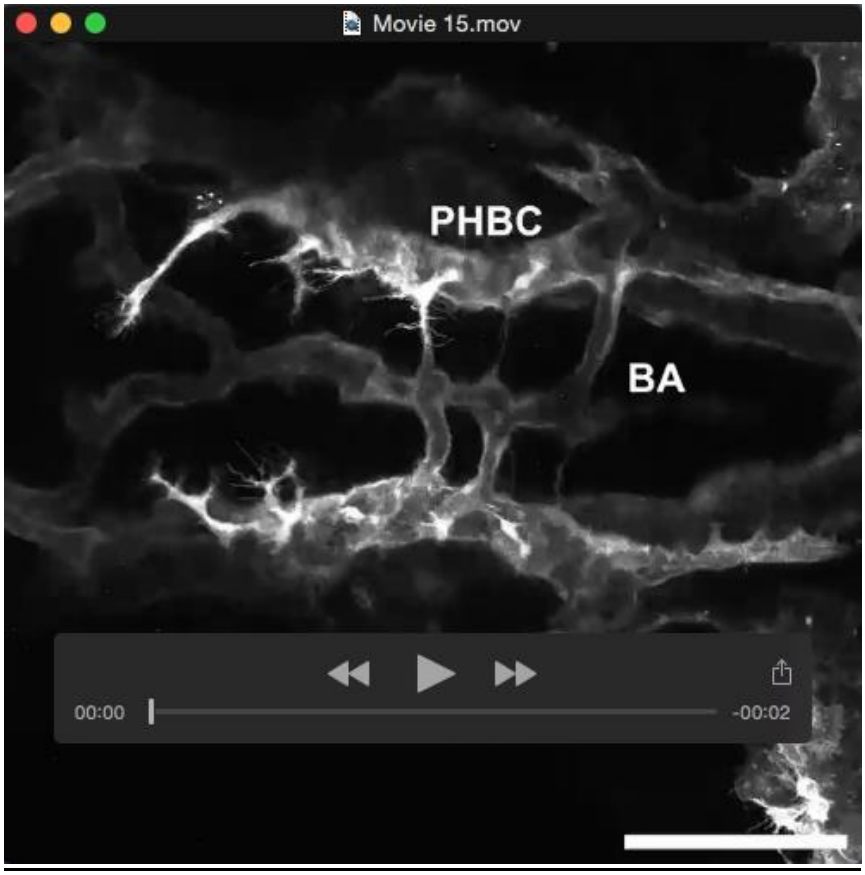
Movie 12. Cardiac contractility and circulation near the heart (WT sibling from *reck*^{y72} in-cross, 3 dpf). Representative brightfield time-lapse movie (30 frames/s); single plane. Anterior, left. Dorsal, up. Blood flows and cardiac contractility appears normal. Image Size: W 740 x H 540 µm.



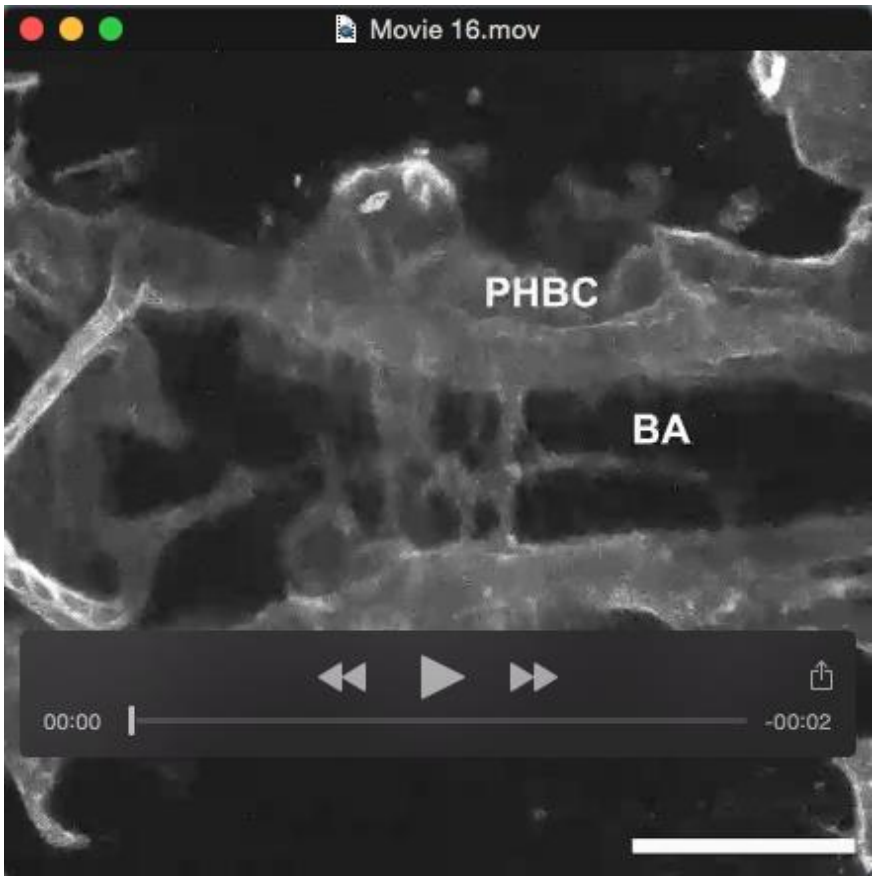
Movie 13. Cardiac contractility and circulation near the heart (*Df(Chr24:reck)*^{w15}; 3 dpf). Representative brightfield time-lapse movie (30 frames/s); single plane. Anterior, left. Dorsal, up. Note misshapen contracting heart and blood flow absence. Image Size: W 740 x H 540 µm.



Movie 14. Cardiac contractility and circulation near the heart (WT sibling from *Df(Chr24:reck)*^{w15} in-cross; 3 dpf). Representative brightfield time-lapse movie (30 frames/s); single plane. Anterior, left. Dorsal, up. Blood flows and cardiac contractility appears normal. Image Size: W 740 x H 540 µm.



Movie 15. Dorsal view (ventral level) of proper avc dynamics and CtA sprouting in the Hb vasculature (WT; from 33-43 hpf). Representative confocal time-lapse movie (hourly intervals). Anterior, left. Left side, bottom. Endothelium, white (*Tg(kdrl:RFP)*^{s896}). Scale bar: 100 μm. See Fig. S4A-D for avc and CtA sprout labeling.



Movie 16. Dorsal view (ventral level) of aberrant avc dynamics and lack of CtA sprouting in the Hb vasculature (*reck*^{y72}; from 33-45 hpf). Representative confocal time-lapse movie (hourly intervals). Anterior, left. Left side, bottom. Endothelium, white (*Tg(kdrl:RFP)*^{s896}). Scale bar: 100 µm. CtA sprouts are not observed. See Fig. S4E-H for avc labeling.

SUPPLEMENTARY MATERIALS AND METHODS

Zebrafish genotyping

kdrl^{y17}: We PCR-amplified a 320 bp genomic fragment using y17-F (5'-GCTTCTGTCGTTCATTCTTAA-3') and y17-R (5'-ACTAAAGATAACCTGTTACAGTTACCTCTC-3') primers. The product was digested with DdeI and run on a 3.5% agarose TBE gel. The WT allele is cut twice, yielding a visible 270 bp fragment and two smaller undetectable fragments. The mutant allele is cut only once, resulting in a visible 300 bp fragment and a smaller undetectable fragment. ***reck***^{y72}: We PCR-amplified a 489 bp fragment using y72-F (5'-CTGTCAGCTGGCCTGTAAGCGTATCC-3') and y72-R (5'-GGGGCATACAGTAGCTCCTCCCCT-3') primers. The product was purified and sequenced using the y72-R primer. ***reck***^{w14}: We PCR-amplified a 232 bp fragment using w14-F (5'-CACAGAGCAGGAGATCATGGAC-3') and w14-R (5'-TAAAGCTGCTGTTCTGGGGTAAAG-3') primers. The PCR product was digested with BfaI and ran on a 3.5% agarose TBE gel. BfaI cuts the mutant allele only, yielding 162 and 70 bp fragments. ***sih***^{b109}: This allele contains a 13 bp deletion at the promoter. The mutant allele was diagnosed by its smaller size. PCR products were obtained using the b109-F (5'-GCATAGAAGCCCTTACAACATC-3') and b109-R (5'-GCTGTCGTCTGATAACACG-3') primers and run on a 3.5% agarose TBE gel. The WT product is 123 bp, the mutant is 110 bp.

Whole-mount RNA *in situ* hybridization (WISH)

Vector templates (Torres-lab identifier, #) were used to make anti-sense riboprobes against: *cdh5/ve-cdh* (#287) (Larson et al., 2004), *tie1* (#101) (Lyons et al., 1998), *fli1a* (#100) (Thompson et al., 1998); *dll4* (#592) (Covassin et al., 2009); *kdrl* (#592) (Choi et al., 2007); *cxcr4a* (#794) (Bussmann et al., 2011); *dab2* (#899) (Bussmann et al., 2011); *mmp2* (#917) (Janssens et al., 2013), *mmp14a* (#919*)* (Coyle et al., 2008); *wnt1* (#750) (Heisenberg et al., 1999); *vegfaa* (#896), *vegfab* (#897) (Bussmann et al., 2011), *cxcl12b/sdf1b* (#886) (Fujita et al., 2011); *plvap/vsg1* (#1385) (Siekmann et al., 2009) *glut1/slc2a1* (#1390) (Zheng et al., 2010) and *reck* (#1140) (Prendergast et al., 2012).

HCR *in situ* hybridization

DNA probes (5'-3') against *reck* transcripts (Molecular Instruments): (P1-gcctcacacactgtagagtacgactcgccgttatgagcacacacctcacg, P2-ccgactgcatgacagcggccctgataatccaccgccacacgctccgagaa, P3-tggacagggaacagcgttacagccggagtcagaaccgaactcagactcca, P4-gttcatttgtgctttattccagaggatccgcagcattcctgcacacaccg, P5-ccgagttaatgagggagtcgatcttctctgcttccttactgcaggcttca). Protocol adapted from Choi et al., 2014. Briefly, embryos were fixed for 24h (4% PFA/4ºC), methanol-permeabilized, rehydrated, ProtK-digested (if >30 hpf) and incubated with probes overnight. Probes removed by washing in Wash Buffer/5xSSCT mixes. Embryos were incubated overnight with the fluorescently-tagged amplifier that specifically recognizes the probes, washed in 5xSSCT, mounted and imaged.

Cell culture

Reagents

Recombinant human VEGF165 and SU5416 were purchased from GEMINI BIO-PRODUCTS and SIGMA respectively. Antibodies: pVEGFR-2 (#2478), VEGFR-2 (#2479), pAKT (Ser473) (#4058), AKT (#9272), pERK1/2 (Ser473) (#4370), ERK1/2 (#4695), Reck (#3433), GAPDH (#4058) and HA (# 2367) from Cell Signaling. GFP and FLAG from Life Technologies and SIGMA respectively. Human Umbilical Vein Endothelial Cells (HUVEC) primary cell culture, Human Embryonic Kidney 293T (HEK293T) and COS-7 cell lines were kindly provided by Timothy Hla, Matthias Stadtfeld and Stevan R Hubbard´s laboratories respectively. Control (sc-108080) and human RECK (sc-39718-V) shRNA lentiviral particles from Santa Cruz Biotechnology Inc.

Cell culture

HUVEC cells were grown in low serum medium optimized for the culture of human endothelial cells (Vasculife EnGS VEGF-Free medium, from Lifeline Cell Technology). HEK293T and COS-7 cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM; GIBCO) supplemented with 10% fetal bovine serum (FBS). Cells grown in 10-cm diameter dishes were incubated at 37°C in a humidified atmosphere with 5% CO2.

Lentivirus infection and transfection

1 X 10^5 HUVECs/well were seeded in a 6-well plate covered with 0.1% gelatin (SIGMA). Next day, HUVECs were infected with control or human RECK shRNA lentiviral particles supplemented with 5 µg/ml polybrene (Millipore). 24 h post-infection the medium was changed and HUVECs were selected for 48 h with 10 µg/ml puromycin. Post-selection cells were starved overnight and stimulated with/without VEGF165 (10 ng/mL) for 2 min. HUVECs were used between passages 2-6. HEK 293T or COS-7 (70% confluence) cells grown in 3.5-cm diameter dishes were transfected using Lipofectamine-2000 reagent (Invitrogen) according to the manufacturer's instructions, with 2 µg of epitope-tagged

versions of the WT (^{3xFLAG}Reck or ^{2xHA}Reck) and mutant (^{3xFLAG}Reck^{y72} or ^{2xHA}Reck^{y72}) zebrafish Reck proteins. 3 h post-transfection, the OPTI-MEM medium was replaced with complete DMEM. Cells were used for western blot or immunofluorescence assays at 48 h post-transfection. Western blot assays: *To investigate whether Reck promotes VEGF-A signaling* HUVECs were cultured, infected with shRNA lentivirus particles and stimulated w/wo VEGF165 as described. In some experiments we combined VEGF-A stimulation (10 ng/ml for 2 min) and SU5416 (5 μM for 1h) mediated inhibition of VEGF-A signaling.

Measuring the abundance of WT and mutant epitope-tagged Reck proteins

COS-7 cells were transfected with CMV-driven v**ectors** (Torres-lab identifier, #) for co-expressing epitope-tagged (FLAG or HA) Reck (#1190-1) or Reck^{y72} (1334-5) with EGFP in cultured cells. For whole-cell lysates, cells on 6 well plate were placed on ice, rinsed once with phosphate-buffered saline (PBS), lysed in 0.25 ml of room temperature buffer (20 mM Tris [pH 7.5], 0.5 mM EDTA, 150 mM NaCl, 1 mM EGTA and 1% SDS) containing protease and phosphatases inhibitors (COMPLETE–EDTA Free from Roche) and loaded onto 4-12% Nu-PAGE gels. Proteins were transferred to Immobilon membranes (Millipore), detected by Western blotting and revealed with Western Lightning PLUS-ECL (PerkinElmer). Western Blot densitometry quantification performed with ImageJ software. Experiments were done at least in triplicate.

Immunofluorescence and imaging

The cell surface localization of epitope-tagged versions of WT (3xFLAG-Reck or 2xHA-Reck) and mutant (3xFLAG-Reck^{y72} or 2x-HAReck^{y72}) zebrafish Reck proteins was assayed via immunofluorescence staining of non-permeabilized HEK293T cells. 2 days post-transfection, cells were washed with PBS, fixed with fresh 4% paraformaldehyde in PBS, pH 7.4, for 20 min at room temperature, washed five times with PBS and incubated with 1% bovine serum albumin for 30 min at 37°C. FLAG and HA tags were detected with monoclonal antibodies (1/500) in 0.5% bovine serum albumin for 1 h at 37°C, washed, and followed by incubation with secondary anti-mouse antibody Alexa 546 at 1/500 in 1% bovine serum albumin for 40 min at room temperature. Images of fixed cells were acquired with an inverted Nikon Eclipse Ti-E fluorescence microscope using the 20X objective.

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Table S1. *nft^{y72}/sdp* trans-heterozygotes and both *nft^{y72}* and *sdp* homozygotes have identical CtA and DRG deficits. Percentage of embryos (different genotypes) showing indicated defects at specified ages (D2: 2 dpf; D3: 3 dpf) "*w15* sibs (+)" are embryos with CtAs and DRG from a *Df(Chr24:reck)^{w15}*/+ in-cross. A significant percentage of them, like *Df(Chr24:reck)^{w15}* homozygotes, show hemorrhage. *Df(Chr24:reck)^{w15}* homozygotes also show heart edema and circulatory deficits (see also Fig. 1, Figs. S1-3 and Movies 1-14). These defects are rare/absent in other *reck*$^{-}$ genotypes, including heterozygotes of other alleles. Thus, they are likely due to the removal of additional genes in *Df(Chr24:reck)^{w15}*. Notably, *reck* point mutants lack the global vascular defects of *reck* morphants in (Prendergast et al., 2012), which are likely due to morpholino off-target effects; see (Kok et al., 2015; Schulte-Merker and Stainier, 2014) (Stainier et al., 2015) and references therein. In contrast, the vascular phenotypes of *reck* point mutants resemble those of *reck* morphants in (Vanhollebeke et al., 2015).

***n* embryos scored at**		**Genotype**	**Percentage of embryos with**													
			Deficits in the presence of blood vessels						**Defective cardiovascular function**						**DRG deficit**	
			CtAs		**MCeV**		**Se + DLAV**		**Hemorrhage**		**Heart edema**		**Circulation**			
D2	**D3**		**D2**	**D3**	**D2**	**D3**	**D2**	**D3**	**D2**	**D3**	**D2**	**D3**	**D2**	**D3**	**D2**	**D3**
100	100	+/+	0	0	0	0	0	0	0	0	0	0	0	0	0	0
66	134	***w15* sibs (+)**	0	0	0	0	0	0	**27**	**3**	0	0	**2**	**1**	0	0
12	49	***w15 / w15***	**100**	**100**	0	0	0	0	**8**	**27**	0	**100**	**75**	**100**	**100**	**100**
19	40	***y72 / y72***	**100**	**100**	0	0	0	0	**5**	**5**	0	0	0	0	**100**	**100**
36	13	***y72 / w15***	**100**	**100**	0	0	0	0	**8**	0	**3**	0	0	0	**100**	**100**
10	10	***y72 / w14***	**100**	**100**	0	0	0	0	0	0	0	0	0	0	**100**	**100**

Table S2. *reck* limits avc abundance even without circulatory flow. Average Abundance (A) for different vessels in the indicated genotypes. n=10 embryos per genotype (50 hpf) analyzed via confocal imaging. SD, Standard deviation. Statistical significance calculated with Student's *t*-test. Pairs of genotypes with significant differences between them indicated with a plus "+" sign. Non-significant differences indicated with "NS". See also Fig. S5.

Vessels	**Genotype**								**Pairs of genotypes with significant differences (p <0.05)**					
	WT		***sih^{b109}***		***reck^{y72}***		***reck^{y72}; sih^{b109}***		**WT**			***sih^{b109}***		***reck^{y72}***
	A	SD	**A**	SD	**A**	SD	**A**	SD	***sih^{b109}***	***reck^{y72}***	***reck^{y72}; sih^{b109}***	***reck***	***reck^{y72}; sih^{b109}***	***reck^{y72}; sih^{b109}***
avc between PHBC-BA	**0.1**	0.3	**3.4**	1.7	**3.6**	1.7	**10.4**	2.1	+	+	+	**NS**	+	+
avc between PHBC-PCS	**0.2**	0.4	**0.2**	0.4	**3.2**	1.3	**1.8**	1.1	**NS**	+	+	+	+	+
avc TOTAL	**0.3**	0.5	**3.6**	1.7	**6.8**	1.8	**12.2**	2.3	+	+	+	+	+	+
CtAs	**11.4**	1.6	**11.1**	2.4	**2.0**	0.8	**0.9**	1.0	**NS**	+	+	+	+	+