‘In parallel’ interconnectivity of the dorsal longitudinal anastomotic vessels requires both VEGF signaling and circulatory flow

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Summary
Blood vessels deliver oxygen, nutrients, hormones and immunity factors throughout the body. To perform these vital functions, vascular cords branch, lumenate and interconnect. Yet, little is known about the cellular, molecular and physiological mechanisms that control how circulatory networks form and interconnect. Specifically, how circulatory networks merge by interconnecting ‘in parallel’ along their boundaries remains unexplored. To examine this process we studied the formation and functional maturation of the plexus that forms between the dorsal longitudinal anastomotic vessels (DLAVs) in the zebrafish. We find that the migration and proliferation of endothelial cells within the DLAVs and their segmental (Se) vessel precursors drives DLAV plexus formation. Remarkably, the presence of Se vessels containing only endothelial cells of the arterial lineage is sufficient for DLAV plexus morphogenesis, suggesting that endothelial cells from the venous lineage make a dispensable or null contribution to this process. The discovery of a circuit that integrates the inputs of circulatory flow and vascular endothelial growth factor (VEGF) signaling to modulate aortic arch angiogenesis, together with the expression of components of this circuit in the trunk vasculature, prompted us to investigate the role of these inputs and their relationship during DLAV plexus formation. We find that circulatory flow and VEGF signaling make additive contributions to DLAV plexus morphogenesis, rather than acting as essential inputs with equivalent contributions as they do during aortic arch angiogenesis. Our observations underscore the existence of context-dependent differences in the integration of physiological stimuli and signaling cascades during vascular development.

Key words: Angiogenesis, Circulatory flow, Endothelium, VEGF, Vascular development, Zebrafish

Introduction
Blood and lymphatic circulatory networks enable survival and homeostasis (Adams and Alitalo, 2007; Xu and Cleaver, 2011). Accordingly, formation of too many, too few, and/or ectopic vascular interconnections, like those between arteries and veins or blood and lymphatic vessels, is deleterious (Brouillard and Vikkula, 2007; Schulte-Merker et al., 2011). Blood vessel networks that are insufficiently interconnected provide poor circulatory flow, leading to ischemia, cognitive and/or motor deficits, and death (Vikkula, 2007; Schulte-Merker et al., 2011). Conversely, the reconnection of vascular networks disrupted by trauma is essential for wound healing, the incorporation of tissue grafts and transplanted organs, and recovery from both coronary artery disease and limb ischemia (Laschke et al., 2009).

Vascular networks arise by diverse mechanisms. For example, the vasculogenic coalescence of endothelial cell precursors forms the primary vascular plexi of the yolk sac and embryo (Patan, 2000), radial waves of sprouting angiogenesis drive the expansion of the primary retinal vascular plexus (Fruttiger, 2007) and bilateral segmental (Se) vessels interconnect ipsilaterally ‘in series’ to form the paired dorsal longitudinal anastomotic vessels (DLAVs) of the trunk that flank the roof of the neural tube (Isogai et al., 2003; Walls et al., 2008). However, little is known about how circulatory networks merge by interconnecting ‘in parallel’ along their boundaries to yield larger circuits.

To learn about this process we exploited the advantages of the zebrafish embryo as a model system for the study of vascular development (McKinney and Weinstein, 2008) to determine how the paired DLAVs interconnect with each other across the midline (see Isogai et al., 2001). The zebrafish DLAVs derive from chevron-shaped primary segmental vessel sprouts [primary Se; nomenclature according to Isogai et al. (Isogai et al., 2003)] that arise bilaterally at 21 hours post-fertilization (hpf) from the trunk arterial arch (aorta) near the somite boundaries. As the primary Se bifurcate along the anteroposterior axis, near the dorsal side of the neural tube, they anastomose with their ipsilateral neighbors forming two continuous and independent DLAVs that diverge from each other anterogradely (Isogai et al., 2001; Childs et al., 2002; Isogai et al., 2003; Blum et al., 2008; Herwig et al., 2011). Shortly after (~36 hpf), secondary segmental vessel sprouts [secondary Se; nomenclature according to Isogai et al. (Isogai et al., 2003)] start arising bilaterally from the axial vein. Some of
these sprouts will contribute to the lymphatic vasculature. Others connect to an adjacent ipsilateral primary Se, thus transforming half of the segmental vasculature into veins (SeVs) after regression of the aortic roots of the primary Se. Others connect to an adjacent ipsilateral primary Se, thus transforming half of the segmental vasculature into veins (SeVs) after regression of the latter’s aortic root. Henceforth, primary Se connected to the aorta are called Se arteries (SeAs). These two kinds of Se vessels generally occupy non-stereotypical, alternating positions along the trunk and can be distinguished according to the directionality of the flow they carry and via transgenic reporter expression (Isogai et al., 2003; Hogan et al., 2009a; Bussmann et al., 2010).

The formation of the simple and evolutionarily conserved anatomy of the DLAVs (Isogai et al., 2003; Levine et al., 2003; Doherty et al., 2007; Walls et al., 2008) and the eventual establishment of the plexus that interconnects them are vital. The DLAVs communicate in series with cephalic vessels (Isogai et al., 2003; Levine et al., 2003; Doherty et al., 2007; Walls et al., 2008), are thought to be precursors for the perineural vascular plexus that later surrounds and invades the spinal cord (Walls et al., 2008; Bautch and James, 2009), and are likely to guide the migration of the neighboring dorsal lymphatic vasculature (see Hogan et al., 2009a; Hogan et al., 2009b; Bussmann et al., 2010). Thus, studying how the DLAV plexus arises and remodels should also illuminate the etiology of spinal cord vascular malformations, a major cause of debilitating myelopathies (Caragine et al., 2002).

**Results**

**Development and anatomy of the DLAV plexus**

To visualize the process of DLAV plexus morphogenesis we imaged the mid-trunk region (which approximately spans somites 8–13) of zebrafish embryos carrying the endothelial-specific...
reporter $Tgf(fli:EGFP)^y_1$ (Lawson and Weinstein, 2002). In this trunk region DLAV assembly is complete by $\sim 32$ hpf (Fig. 1A–D). Shortly after, at around 36–38 hpf, interconnections between the two DLAVs begin to form as angiogenic sprouts launch from the dorsal surface of the DLAVs and project in a dorsomedial fashion (Fig. 1E,F). These DLAV-derived sprouts connect either with their closest contra-lateral neighbors or to the opposite DLAV to form a plexus. For simplicity, from this stage onwards we collectively refer to the paired DLAVs and their interconnections as the DLAV plexus. The DLAV plexus progressively displays a ladder-like appearance in dorsal views, such that by 48 hpf this structure has many ‘rungs’ along its length (Fig. 1G–I). Almost simultaneously, these rungs branch at the midline in an anteroposterior fashion, anastomosing in series (supplementary material Movie 1), to yield by 56 hpf a third longitudinal vessel that runs along the midline at a more dorsal position than the DLAVs (Fig. 1J–L; supplementary material Movie 2). At this stage the DLAV plexus displays a complex and non-stereotypical braid-like organization in which the longitudinal vessels display different diameters along their length. By 60 hpf two or three longitudinal vessels are found along the DLAV plexus (Fig. 1M–M′′).

To determine the spatiotemporal dynamics of initial DLAV plexus formation we scored the position of DLAV–DLAV interconnections along the trunk and tail between 36 and 48 hpf (Fig. 1N). We found that the DLAV plexus forms first at the mid and posterior trunk regions, spreading both rostrally and caudally while becoming denser. Notably, the plexus is absent from the region corresponding approximately to the first eight somites (Fig. 1N,O–P), thus indicating that the anterior limit for DLAV plexus morphogenesis falls within the transition zone between anterior and posterior trunk somitogenesis (Holley, 2006). However, at 72 hpf a single vessel interconnects the DLAVs at the level of the putative hindbrain-spinal cord interface (Ma et al., 2010), namely between the third and fourth somites (Fig. 1P).

Next, we used endothelial PECAM-1 immunostaining to visualize the vasculature in mouse embryos to determine if and how DLAV plexus formation occurs. Our observations reveal that, at a minimum, the initial steps of this process are evolutionarily conserved between zebrafish and mammals. However, at 72 hpf a single vessel interconnects the DLAVs at the level of the putative hindbrain-spinal cord interface (Ma et al., 2010), namely between the third and fourth somites (Fig. 1P).

To explore the cellular basis of DLAV plexus formation and remodeling we first quantified endothelial cell abundance within DLAV plexus morphogenesis. Our observations reveal that, at a minimum, the initial steps of this process are evolutionarily conserved between zebrafish and mammals. However, at 72 hpf a single vessel interconnects the DLAVs at the level of the putative hindbrain-spinal cord interface (Ma et al., 2010), namely between the third and fourth somites (Fig. 1P).

### Endothelial cell behaviors associated with DLAV plexus assembly

To explore the cellular basis of DLAV plexus formation and remodeling we first quantified endothelial cell abundance within the two separate DLAVs or the DLAV plexus during development. To do this we co-visualized endothelial cell nuclei and blood vessel anatomy in $Tgf(flk1:EGFP-NLS); Tgf(flk1:ras-mCherry)^{s896}$ embryos (see Blum et al., 2008; Chi et al., 2008). Specifically, we scored endothelial cell abundance at 34, 48 and 72 hpf in two areas: the anterior region where the DLAVs remain separate due to lack of plexus formation (somites 3–5) and the DLAV plexus-forming mid-trunk region (somites 8–12). This analysis revealed that endothelial cell abundance increases significantly over time in both regions and that endothelial cell abundance is similar between both regions at each stage analyzed (Fig. 2A–C). These observations indicate that the lack of DLAV plexus formation in the anterior trunk is due to a key difference in the way endothelial cells distribute, rather than to an anterior deficit in endothelial cell abundance.

We next asked whether endothelial cell migration and/or proliferation contribute to the observed increase in DLAV plexus endothelial cell abundance in the mid-trunk. To do this we performed cell transplantation experiments between $Tgf(flk1:EGFP-NLS)$ donors and $Tgf(flk1:ras-mCherry)^{s896}$ hosts and periodically imaged the chimeras to track changes in both the position and number of donor endothelial cells (Zygmunt et al., 2011). Consistent with prior reports describing complex, non-stereotypical cell rearrangements during DLAV formation (Blum et al., 2008) we found that donor endothelial cells found within the mid-trunk primary Se of the host at 32 hpf could later be found within the DLAV plexus at 72 hpf (Fig. 2D–F′). Hence, endothelial cells found within the primary Se after the DLAVs have already formed contribute to DLAV plexus morphogenesis by migrating dorsally. We also found that donor endothelial cells located within the DLAVs of the host at 32 hpf had migrated...
and proliferated by 72 hpf (supplementary material Fig. S2). Accordingly, time-lapse imaging of Tg(fli1::NLS-mCherry) embryos (32–44 hpf) confirms the occurrence of both endothelial cell proliferation and migration during DLAV plexus morphogenesis (supplementary material Movie 3).

The DLAV plexus communicates with SeAs and SeVs arranged in a generally alternating and non-stereotypical pattern (see Isogai et al., 2003; Hogan et al., 2009a; Bussmann et al., 2010). While SeAs are made exclusively of angiogenic cells of an arterial lineage (the aorta), SeVs contain angiogenic cells from both the axial artery and vein (Isogai et al., 2003; Hogan et al., 2009b; Bussmann et al., 2010). Thus, to investigate if endothelial cells from the venous lineage found within Se vessels are required for DLAV plexus formation, we analyzed wild-type (WT) embryos injected with a morpholino (MO) that induces a functional knockdown of ccbe1 (collagen and calcium-binding EGF domain-1). ccbe1 morphants lack venous secondary Se sprouts and thus cannot establish SeVs (Hogan et al., 2009a). As expected, comparison of WT embryos and ccbe1 morphants carrying the endothelial-specific reporter Tg(fli:EGFP)y1 revealed a lack of Se venous flow in ccbe1 morphants at both 48 and 72 hpf (supplementary material Fig. S3). However, we observed venous flow in the first, third and fourth Se vessels of ccbe1 morphants, consistent with the notion that these vessels are genetically hardwired to become veins (data not shown) (see Isogai et al., 2003; Hogan et al., 2009a). ccbe1 morphants do not exhibit obvious abnormalities in DLAV plexus morphogenesis despite abnormal DLAV plexus hemodynamics, including unidirectional anterograde circulatory flow and reduced venous outflow (data not shown). Collectively, these observations indicate that endothelial cells from the venous lineage make null or dispensable contributions to DLAV plexus morphogenesis in zebrafish (see Fig. 4H for a graphical summary of our findings) and that this process is not significantly impaired by a partial but significant reduction in its venous outflow.

Characterization of circulatory flow during DLAV plexus morphogenesis

During embryogenesis, conserved genetic pathways shape the stereotypical anatomy of the early vessels in a circulation-independent manner. Yet, later, mechanical inputs and chemical factors provided by circulatory flow modulate vascular development (reviewed by Jones, 2011). Hence, as a first step to define the potential role of circulatory flow in DLAV morphogenesis we used confocal microangiography (Weinstein et al., 1995) to visualize lumens with active circulation in 32–72 hpf Tg(fli:EGFP)y1 embryos in which the blood plasma was fluorescently labeled with quantum dots (Qdots) (Rieger et al., 2005) (Fig. 3).

We found that at 32 hpf dorsal aorta of the trunk and axial vein display bright angiographic signals and carry robust erythrocyte circulation (data not shown) (Bussmann et al., 2010). In contrast, the DLAVs and the primary Se are angiographically negative (Fig. 3A,B). This indicates that at this stage these structures have yet to complete tubulogenesis, i.e. form a continuous lumen (see Xu and Cleaver, 2011), consistent with prior observations based on the visualization of the luminal marker Podocalyxin-2 (Herwig et al., 2011). In contrast, at the start of DLAV plexus morphogenesis (38 hpf) the DLAVs/developing DLAV plexus and some of the primary Se carry circulatory flow (Fig. 3C,D). Later, at 43 hpf most of the Se vasculature supports plasma flow (Fig. 3C,D') and thus by 48 and 72 hpf the entire DLAV plexus and Se vasculature display strongly angiographically positive signals, indicating

![Fig. 3. Maturation of DLAV and DLAV plexus circulatory function during development. (A–J') Mid trunk DLAV and DLAV plexus vasculature (details). Endothelium is shown in green [Tg(fli:EGFP)y1]. Microangiograms of circulatory plasma flow is shown in red (Qdot-605). Anterior, left; dorsal, up (lateral views). Scale bars: 30 μm.](image-url)
that tubulogenesis is complete across the entire network (Fig. 3G–J). Hence, our microangiographic findings reveal that DLAV perfusion precedes plexus formation.

Next, to determine the directionality of circulatory flow through the DLAV plexus and Se vasculature we visualized erythrocyte movement. To do this we used Tg(fli:EGFP)y1; Tg(gata-1:DsRed)sd2 embryos, in which the endothelium and erythrocytes are labeled with fluorescent proteins of different colors (Lawson and Weinstein, 2002; Traver et al., 2003). We found that before 43 hpf erythrocytes do not circulate through the DLAV plexus and flow only through a small fraction of the Se vasculature, likely because they have yet to complete tubulogenesis and/or their luminal diameter is yet too small (data not shown). However, erythrocytes travel through both the DLAV plexus and the majority of SeAs and SeVs at 48 and 72 hpf, consistent with our angiographic findings. Notably, the DLAV plexus enables non-stereotypical patterns of erythrocyte flow in both anterior and posterior directions and from SeAs to both ipsilateral and contralateral SeVs, even those located at a different anteroposterior level (supplementary material Movies 4, 5, and data not shown). Hence, the DLAV plexus effectively dorsally merges the two Se networks.

Circulatory flow and VEGF signaling make additive contributions to DLAV plexus morphogenesis

Circulatory flow is a physiological factor that modulates signaling cascade outputs to control diverse aspects of cardiovascular development, including angiogenesis, hematopoiesis and cardiac maturation (Jones, 2011). For example, crosstalk between circulatory flow and VEGF signaling shapes aortic arch remodeling (Yashiro et al., 2007; Nicoli et al., 2010).

Our microangiographic findings suggest that DLAV plexus morphogenesis might require circulatory flow. In addition, VEGF ligands are expressed near (vegfaa and vegfab) or in (vegfc) the
trunk vasculature (Lawson et al., 2002; Covassin et al., 2006; Hogan et al., 2009b; Gore et al., 2011). Moreover, components of the circuit that integrates hemodynamic flow and VEGF signaling during aortic arch angiogenesis, such as kif2a, mir-126a and the VEGF receptors kdr and flt4 are expressed in the trunk vasculature. Finally, kif2a expression in the trunk vasculature is circulation dependent. Taken together, these observations suggest that circulatory flow and VEGF signaling might be functionally related in the context of DLAV plexus morphogenesis, as they are during aortic arch angiogenesis (Lawson et al., 2001; Wienholds et al., 2005; Covassin et al., 2006; Parmar et al., 2006; Choi et al., 2007; Siekmann and Lawson, 2007; Hogan et al., 2009b; North et al., 2009; He et al., 2011; Wang et al., 2011).

Hence, to evaluate if circulatory flow is required for DLAV plexus morphogenesis we first analyzed silent heart mutants (sihb109). These embryos lack heart contractility due to the inactivation of the tnt2a/cardiak tropomin-2a gene, yet form continuous DLAVs on schedule (Sehnert et al., 2002; Isogai et al., 2003). We found that DLAV plexus formation is impaired, but not absent, in both 48 hpf sihb109 mutants and tnt2a morphants (supplementary material Fig. S4, and data not shown), indicating that circulation promotes DLAV plexus morphogenesis. We next abrogated circulatory flow on demand by stopping the heartbeat with the action potential blocker tricaine (Muntean et al., 2010) or the myosin ATPase inhibitor BDM/2,3-butanedione 2-monoxime (Forster et al., 2004), as in Nicoli et al. (Nicoli et al., 2010). Surprisingly, we found that post-DLAV formation (32–48 hpf) circulatory flow abrogation with either tricaine or BDM prevented, rather than impaired, DLAV plexus morphogenesis in WT embryos. Moreover, DLAV plexus morphogenesis was absent in 48 hpf sihb109 mutants treated with tricaine during the same period. In addition, while both 48 hpf sihb109 mutants and tnt2a morphants display prominent cerebral Central Arteries, these brain vessels fail to form in both WT and sihb109 mutants treated with either tricaine or BDM (data not shown) (see Bussmann et al., 2011; Corti et al., 2011). Hence, tricaine and BDM treatments induce circulation-independent effects that impair vascular development.

We sought to define whether VEGF signaling also modulates DLAV plexus morphogenesis. We began by analyzing VEGF receptor-2 ortholog kinase insert domain receptor-like mutants (kdrP17), which have impaired primary Se sprouting/growth and circulatory deficits but can form continuous DLAVs (Covassin et al., 2006; Bussmann et al., 2008; Covassin et al., 2009; Bussmann et al., 2010). We found that 48 and 72 kdrP17 fail to form contra-lateral interconnections between the DLAVs (supplementary material Fig. S5A,B). To abrogate VEGF receptor (VEGFR) activation on demand we employed a dose of the highly specific tyrosine kinase inhibitor SU5416 (to abrogate VEGFR activation), injected with tnt2a MO to prevent circulation [sih phenocopy (Sehnert et al., 2002)] and the combination of the latter two treatments (both). To do this we measured endothelial cell abundance within the whole trunk vasculature (axial vasculogenic and angiogenic vessels), the angiogenic vasculature (Se vessels and DLAV plexus) and the DLAV plexus (idem).

Overall, we found that lack of circulatory flow induces greater defects on DLAV plexus morphogenesis than VEGFR inactivation. In addition, the combination of these two conditions exacerbates the morphological defects induced by either treatment (Fig. 4A–G). In particular, the lack of both circulatory flow and VEGFR signaling has a synergistic effect on endothelial cell abundance within the whole trunk vasculature (Fig. 4E), but additive effects within both the angiogenic vasculature (Fig. 4F) and the DLAV plexus (Fig. 4G).

Next, we aimed to determine whether lack of circulatory flow and/or VEGFR inactivation affects DLAV plexus morphogenesis by impairing its development or inducing its regression (supplementary material Fig. S6). To do this we measured endothelial cell abundance in both control and tnt2a morphant embryos at 43 hpf (supplementary material Fig. S6A,D,G) and compared these values with those exhibited by 56 hpf animals under the four conditions mentioned before (supplementary material Fig. S6B,C,E,F,H,I). We found that endothelial cell abundance in 56 hpf animals lacking both circulatory flow and VEGFR signaling is significantly smaller than that of both 43 hpf control and tnt2a morphant embryos. Thus, our results support the notion that the DLAV plexus regresses when both circulatory flow and VEGFR signaling are absent.

**Discussion**

Our analysis of DLAV plexus formation in zebrafish and mouse embryos provides the first description of the cellular, molecular and physiological mechanisms by which circulatory networks merge by interconnecting in parallel along their boundaries.

While the initial stages of DLAV plexus morphogenesis are evolutionarily conserved, this process advances in a different way in these two species. This is surprising for two reasons. First, the sprouting of Se vessels (the DLAV precursors) progresses in a rostrocaudal direction in zebrafish, *Xenopus* and mouse, likely
reflecting the evolutionarily conserved spatiotemporal dynamics of somitogenesis and somite maturation. Second, differences in somitic specification, formation and differentiation between the anterior trunk, posterior trunk and tail are thought to be evolutionarily conserved (Isozag et al., 2001; Levine et al., 2003; Bedell et al., 2005; Holley, 2007; Walls et al., 2008). Regardless, given that somitic signals modulate the organization of the spinal cord along its anteroposterior axis (see Lewis and Eisen, 2004; Holley, 2006) it is likely that DLAV plexus formation is modulated by somitic and/or spinal cord cues. Indeed, the results of our experiments and gene expression analyses (not shown) suggest that the cues that modulate DLAV plexus morphogenesis include VEGF ligands expressed in both tissues.

Zebrafish DLAV plexus formation involves classical angiogenic behaviors, such as endothelial cell proliferation and migration. In particular, our findings indicate that endothelial cells from the arterial lineage make up the bulk, if not the entirety, of the zebrafish DLAV plexus. However, whether this is the case also in mammals remains unexplored. We note that zebrafish DLAV plexus formation appears to rely on two uncommon morphogenetic mechanisms of vascular assembly. The first is the formation of a midline vessel from two lateral precursors. This resembles the way in which the midline basilar artery of the hindbrain forms from the two laterally positioned primordial hindbrain channels in zebrafish (Bussmann et al., 2011; Corti et al., 2011; Fujita et al., 2011; Ulrich et al., 2011). The second is ‘vessel fusion’, a unique process in which vessels coalesce by behaving like highly viscous liquid drops. This is the morphogenetic mechanism employed by anemones to transform their bilateral aortae into a single axial artery (Draike and Little, 1999; Reese et al., 2004; Fleming et al., 2010; Garrock et al., 2010). Both the existence of regions within the DLAV plexus with two, rather than three longitudinal vessels (after the establishment of the third midline vessel) and our time-lapse imaging data (not shown) suggest that DLAV plexus remodeling involves vessel fusion events.

The interplay between circulatory flow and VEGF signaling is important for aortic arch angiogenesis and remodeling (Yashiro et al., 2007; Nicoli et al., 2010). For example, in the zebrafish, blood flow activates the expression of the mechano-sensitive transcription factor klk2a, which induces the expression of mir-126a. This microRNA inactivates downstream VEGF signaling inhibitors to enable angiogenesis of the fifth aortic arch. In this context, abrogation of either circulatory flow or VEGF signaling blocks angiogenesis (Nicoli et al., 2010). In contrast, we found that during DLAV plexus morphogenesis the effects of circulatory flow and VEGFR signaling are generally additive, consistent with two models. The first and most likely possibility (see Lawson et al., 2001; Wienholds et al., 2005; Covassin et al., 2006; Parmar et al., 2006; Choi et al., 2007; Siekmann and Lawson, 2007; Hogan et al., 2009b; North et al., 2009; He et al., 2011; Wang et al., 2011) is that circulatory flow promotes, but is not absolutely required, for VEGF signaling during DLAV plexus morphogenesis (Fig. 4I). This would explain why the DLAV plexus angiogenesis deficit of animals lacking flow is enhanced upon chemical inhibition of VEGFR signaling. The second alternative is that circulatory flow and VEGF signaling act in parallel pathways during DLAV morphogenesis (see Martienssen and Irish, 1999). Overall, our findings indicate that the angiogenic contributions of circulatory flow and VEGF signaling to DLAV plexus and aortic arch morphogenesis are quantitatively and qualitatively distinct (see Nicoli et al., 2010), highlighting the context-dependent nature of the circuits used by endothelial cells to integrate the inputs of physiological stimuli and signaling cascades.

### Materials and Methods

#### Zebrafish husbandry and embryo collection

Animals were kept and handled using standard laboratory conditions (Kimmel et al., 1995) under New York University IACUC guidelines. Embryo collection, incubation, staging and blockage of melanin synthesis to preserve transparency performed as described previously (Zygmont et al., 2011).

#### Zebrafish lines

We used the endothelial-specific fluorescent reporter lines Tg(fli:EGFP) y1 (cytosolic EGFP) (Lawson and Weinstein, 2002), Tg(fli1:ras-mCherry) y966 (membrane-targeted mCherry) (Chi et al., 2008), Tg(fli1:EGFP-NLS) (nuclear-targeted EGFP) (Blum et al., 2008), Tg(fli1:nslmCherry) (nuclear-targeted mCherry) (Wang et al., 2010) and the erythrocyte-specific fluorescent reporter Tg(gata1:DsRed) y1074 (Traver et al., 2003). Mutant alleles: silent heart (nih) mutants lack heartbeat due to a lesion in the cardiac troponin-12a gene (Schnert et al., 2002). The kinase dead hypomorphic allele of the duplicate canonical VEGFR2 kinase insert domain receptor-like (kdrkiD) displays reduced angiogenesis (Covassin et al., 2006; Covassin et al., 2009). Lines were used alone or in combination.

#### Fixation and immunofluorescent staining of zebrafish embryos

Fixation and immunofluorescent staining of zebrafish embryos was carried out as described previously (Zygmont et al., 2011).

#### Drug treatments of zebrafish embryos

Pools of 20 dechorionated embryos were treated in 4 ml aqueous solutions (standard embryo medium or SE medium) inside capped glass vials (Fisherbrand) with gentle rocking in a 28.5°C incubator. Treatment periods as described. Control: 0.025% DMSO/DMSO (CAS number 67-68-5; Sigma). VEGFR inhibition: 0.5 μM SU5416 (CAS number: 204005-46-9; Sigma), 0.025% DMSO. Flow inhibition: 0.66 mg/ml tricaine methanesulphonate/MS-222 (CAS number 886-86-2; Sigma-Aldrich) or BD2M, 2.3-butanedione 2-monoxime (Sigma number 57-71-6; Sigma-Aldrich). Treatments were performed as described previously (Nicoli et al., 2010; Zygmont et al., 2011).

#### Confocal imaging and image processing of zebrafish embryos

Live and fixed, immunostained embryos were mounted as described by Zygmont et al. (Zygmont et al., 2011). Confocal imaging performed with a Leica TCS SP5 microscope (Leipzig, Germany) using the water dipping 40× lens (Fisherbrand) with gentle rocking in a 28.5°C incubator. Treatment periods as described. Control: 0.025% DMSO/DMSO (CAS number 67-68-5; Sigma). VEGFR inhibition: 0.5 μM SU5416 (CAS number: 204005-46-9; Sigma), 0.025% DMSO. Flow inhibition: 0.66 mg/ml tricaine methanesulphonate/MS-222 (CAS number 886-86-2; Sigma-Aldrich) or BD2M, 2.3-butanedione 2-monoxime (Sigma number 57-71-6; Sigma-Aldrich). Treatments were performed as described previously (Nicoli et al., 2010; Zygmont et al., 2011).

#### Zebrafish confocal microangiography with quantum dots (Qdots) as contrast reagent

Tg(fli1:EGFP)y1 embryos were briefly treated with a sub-effective Tricaine dose to slow the heartbeat. Immediately 9 nl of a 1 mM Qdot-605 streptavidin conjugate solution (Q10101MP, Invitrogen) were delivered into the duct of Cuvier at 32, 38, 43, 48 or 72 hpf by injection with a glass needle; as described previously (Rieger et al., 2005). Embryos were transferred to SE medium for 5 minutes to enable full circulatory recovery, fixed, immunostained (to highlight the vasculature) and confocal imaged.

#### MO injection into zebrafish embryos

MOs (Genotools, LLC) were delivered into single-cell stage embryos as follows. 7.5 ng of the cceh1 translation blocker MO (5'-CGGTTATAGCATTCTTCAGACACTCTG-3') were delivered into Tg(fli1:EGFP)y1 embryos, as described previously. 4 ng of the tnt2a translation blocker MO (5’- CATGGTTGCTGCTGACAGCAGCAGCA-3’) were delivered into Tg(fli1:EGFP-NLS); Tg(fli1:ras-mCherry)y966 embryos, as described previously. 4 nl of the translation blocker MO (5’- CATGGTTGCTGCTGACAGCAGCAGCA-3’) were delivered into Tg(fli1:EGFP-NLS); Tg(fli1:ras-mCherry)y966 embryos, as described previously (Sehnert et al., 2002).

#### Quantification of arterial and venous circulatory blood flow through zebrafish Se vessels

Erythrocyte flow directionality through Se vessels scored under transmitted light using an M165 FC stereomicroscope (Leica Microsystems) at 48 and 72 hpf in Tg(fli1:EGFP)y1 embryos (uninjected and cceh1 morphants). 10 consecutive left Se vessels scored per embryo at the approximate level of Se vessels 5–15. 20 embryos scored per condition and stage. Flow scoring: arterial (dorsal-bound); venous (ventral-bound); unscorable (lacking erythrocyte flow).
Endothelial cell abundance quantification in zebrafish embryos


Modulation of DLAV plexus morphogenesis

Antiangiogenic effect by SU5416 is partly attributable to inhibition of Flh-I receptor signaling. Mol. Cancer Ther. 1, 295-302.


Fig. S1. DLA plexus morphogenesis in mouse embryos. (A-J) Dorsal views of DLA plexus development in 22 (A,F), 26 (B,G), 27 (C,H), 32 (D,I) and 34 (E,J)-somite stage Swiss-Webster wild-type mouse embryos. Anterior, top. Scale bars: 250 μm. (A-E) PECAM-1 immunofluorescence. Endothelium, white. (F-J) 3D-rendered OPT data (see Materials and Methods) collected from the same embryos. Endothelium, gold. Red dashed lines denote cropped regions displayed in Fig. 1Q-U.
Fig. S2. Endothelial cell proliferation contributes to DLAV plexus morphogenesis. Lateral (A-C; dorsal, up) and dorsal (A’-C’) images of the trunk vasculature of a chimeric embryo made from a Tg(flk1:EGFP-NLS) donor and a Tg(flk1:ras-mCherry)s896 host at 32 (A-A’), 56 (B-B’), and 72 (C-C’) hpf. Anterior, left. White asterisks, endothelial cell nuclei of donor origin found within the DLAVs and/or DLAV plexus. (A-C’) Endothelium, red. Endothelial cell nuclei, green. Scale bars: 30 μm.
Fig. S3. The percentage of Se vessels carrying venous flow is dramatically reduced in ccbe1 morphants. Quantification. Arterial and venous flow through Se vessels in WT and ccbe1 morphants (ccbe1 MO) at 48 (A) and 72 (B) hpf. n=20 animals per condition and stage. ***P<0.001.
Fig. S4. Circulatory flow promotes DLAV plexus formation. (A,B) Dorsal views of the trunk vasculature of 48 hpf WT (A) and \textit{sih}^{b109} mutant (B) \textit{Tg(fli:EGFP)}^{y1} embryos. Anterior, left. White asterisks, contra-lateral DLAV interconnections. Endothelium, green. Somite boundaries, red. Scale bars: 30 \textmu m.
Fig. S5. Lack of VEGF signaling prevents the initiation of DLAV plexus formation. (A,B) Dorsal views of the trunk vasculature of 48 (A) and 72 (B) hpf kdrly17; Tg(fli:EGFP)y1 mutant embryos. Note lack of DLAV plexus formation. Anterior, left. (C-D') Lateral (C,D) and dorsal (C',D') views of the mid trunk vasculature of 48 hpf Tg(flk1:EGFP-NLS); Tg(flk1:ras-mCherry);g56 embryos treated post-DLA V formation (32 hpf onwards) with either vehicle (Ctrl; DMSO) or with VEGFR inhibitor (SU5416). (A-D') Anterior, left; dorsal, up; endothelium, green (A,B) or red (C-D'); endothelial cell nuclei, green (C-D'); somite boundaries, red (A,B) or blue (C-D'); scale bars: 30 μm. (E) Quantification of the effect of VEGFR inhibition (32-48 hpf) on DLAV and/or DLAV plexus endothelial cell abundance. n=10 animals per treatment. Error bars, s.e.m. ***P<0.001.
Fig. S6. Additive effects of VEGF signaling and circulatory flow on DLAV plexus development and maintenance. (A-I)
Quantifications of the vasculature of Tg(flk1:EGFP-NLS); Tg(flk1:ras-mCherry)^s896 embryos. 43 hpf, green bars (control and tnnt2a morphants); 56 hpf, black (control) or blue bars (experimental conditions); as in Fig. 4 (see Materials and Methods). The red dotted lines (at the level of the corresponding 43 hpf value) are included to facilitate comparisons. n=6-10 animals per treatment and stage. Error bars, s.e.m. *P<0.5; ***P<0.001.