Artery-vein specification in the zebrafish trunk is pre-patterned by heterogeneous Notch activity and balanced by flow-mediated fine tuning

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ABSTRACT

How developing vascular networks acquire the right balance of arteries, veins and lymphatic vessels to efficiently supply and drain tissues is poorly understood. In zebrafish embryos, the robust and regular 50:50 global balance of intersegmental veins and arteries that form along the trunk, prompts the intriguing question how the organism keeps “count”. Previous studies suggest that the ultimate fate of an intersegmental vessel (ISV) is determined by the identity of the approaching secondary sprout emerging from the posterior cardinal vein (PCV). Here, we show that the formation of a balanced trunk vasculature involves an early heterogeneity in endothelial cell (EC) behavior and Notch signaling activity in the seemingly identical primary ISVs that is independent of secondary sprouting and flow. We show that Notch signaling mediates the local patterning of ISVs, and an adaptive flow-mediated mechanism subsequently fine-tunes the global balance of arteries and veins along the trunk. We propose that this dual mechanism provides the adaptability required to establish a balanced network of arteries, veins and lymphatic vessels.
INTRODUCTION

Efficient supply of oxygen and nutrient to tissues and organs is dependent on the formation of a hierarchically branched blood vessel network, comprised of feeding arteries, capillaries and draining veins. During zebrafish development, the first axial artery and vein assemble from progenitor cells guided by local cues in the tissue (Kohli et al. 2013). However, the subsequent expansion of vascular networks sees arteries and veins arise through the sprouting and remodeling from the primitive vascular plexus (Isogai et al. 2003). When and where to form an artery or vein is a complex biological problem, as endothelial cells (ECs) adopt distinct gene expression repertoires associated with specific morphogenic behaviors in arteries and veins (Torres-Vazquez, Kamei, and Weinstein 2003). Organ-specific signatures additionally contribute to EC heterogeneity. The complexity of these differentiation processes and the multitude of chemical and physical morphogenic cues applied to the network appear to provide a daunting task for patterning (Nolan et al. 2013). Observations of stereotyped branching patterns of arteries and veins suggested that localized guidance cues drive artery formation (Carmeliet and Tessier-Lavigne 2005). At the same time, blood flow is a critical determinant of artery-vein formation in the chick and mouse yolk sac, and appears essential for all aspects of vascular remodeling and plasticity (le Noble et al. 2004; Lucitti et al. 2007). Rerouting flow is able to shape new arteries, and the plasticity reported after infarct illustrates that even rerouting flow in the established network triggers plastic responses (le Noble et al. 2004). The fact that many genes are differentially regulated by shear stress in ECs following exposure to blood flow suggests that genetic regulation and flow-dependent mechanisms are not necessarily exclusive, but integrated (Lehoux and Tedgui 2003; Wragg et al. 2014). Yet how this is achieved and coordinates the correct number, branching pattern and spacing of arteries and veins remains largely unknown.

In the zebrafish trunk vasculature, the question takes on an additional dimension, as it first arises as an all-arterial network and is subsequently remodeled into a balanced network of arteries and veins. Although the order of arteries and veins along the trunk is not fixed, every embryo forms a balanced number of arteries and veins (Bussmann et al. 2010). How exactly this remodeling is organized to result in this balance is currently unknown. The intersegmental vessels (ISVs) initially arise as arterial vessels but then remodel into veins, or alternatively remain arterial and guide lymphatic structures (Geudens et al. 2010; Isogai et al. 2010).
This process has been described to follow local cues in the tissue, and gene regulatory mechanisms are thought to drive fate decisions into artery, vein or lymphatic structures. The main axial vessels, the dorsal aorta (DA) and posterior cardinal vein (PCV), are formed through the coalescence of angioblasts in a process termed vasculogenesis. Around 23 hours post-fertilization (hpf) ECs sprout from the DA to form the primary ISVs, which are consequently all arterial by origin. These primary ISVs fuse at the dorsal side of the trunk to form the dorsal longitudinal anastomotic vessel (DLAV). Around 30-32 hpf, a second wave of sprouting occurs, this time originating from the PCV. These secondary sprouts either form a stable connection to a primary ISV, remodeling it into a venous ISV following its disconnection from the DA, or sprout to the level of the horizontal myoseptum to contribute to lymphatic structures (Kuchler et al. 2006; Isogai et al. 2003; Geudens et al. 2010; Yaniv et al. 2006). This process leads to the formation of a balanced network of arteries and veins that efficiently delivers blood throughout the trunk. What drives the outcome of secondary sprouting – vein formation or lymphatic contribution – is still poorly understood. Several publications have suggested that secondary sprouts forming either venous ISVs or lymphatic structures are genetically different when emerging from the PCV (Hogan, Bos, et al. 2009; Nicenboim et al. 2015; Koltowska et al. 2015; Geudens et al. 2010; Yaniv et al. 2006). This concept proposes that the ultimate fate of an ISV is determined by the nature of the approaching secondary sprout, i.e. a secondary sprout with a lymphatic fate restriction will lead to arterial maintenance, whereas a secondary sprout lacking lymphangioblast identity will remodel the ISV into a vein. Current concepts favor the idea that secondary sprouts are fate-restricted by their level of expression of the lymphatic determinant Prox1; cells expressing low levels of Prox1 will connect to the primary ISV and form a vein, whereas sprouts expressing high levels continue to the level of the horizontal myoseptum and give rise to the lymphatic precursor structures (Nicenboim et al. 2015; Koltowska et al. 2015). However, how such a deterministic program would establish the arterio-venous balance observed throughout the whole organism remains unclear.

Here, using high-resolution live imaging, advanced cell tracking and computational analytics, we made a series of discoveries showing that the formation of a balanced trunk vasculature involves an unexpected early heterogeneity in EC behavior in the seemingly identical primary ISVs and an adaptive flow-mediated mechanism that fine-tunes the balance
of arteries and veins along the trunk. Even before connection of the secondary sprouts and in absence of blood flow, the ECs constituting the primary ISVs show distinct behaviour that is predictive for later arteriovenous patterning, showing that the primary ISVs rather than the secondary sprouts, are pre-programmed to become either an artery or a vein. In addition, and subsequently to this pre-patterning, a flow-mediated mechanism provides flexibility and adaptation to the system to fine-tune the balance of arteries and veins.

RESULTS

The trunk vasculature exhibits a global balance of arteries and veins and local patterns favoring alternating vessel identities.

The zebrafish trunk vasculature consists of a balanced network of arteries and veins (Figure 1A) (Bussmann et al. 2010). To explore the nature of this global artery-vein balance, we analyzed the sequence of arteries and veins on both sides of a 10-somite segment in 6 days post fertilization (dpf) wild-type embryos, in which arteries and veins are already functionally specified. Performing a neighborhood analysis to determine the conditional probabilities of forming an artery or a vein given the status of the neighboring vessels uncovered a strong, albeit imperfect ipsilateral patterning of alternating vessel fates (Figure 1B, Figure S1A-B). Contra-lateral patterning between opposing vessels in the zebrafish trunk is less pronounced, providing only for a weak prediction of vessel identity (Figure 1C). Taken together, an ISV surrounded by arterial ISVs (aISV) has a high probability of being a venous ISV (vISV).

Secondary sprouts emerging from the PCV commonly establish a connection with primary ISVs regardless of their future identity.

Previous studies identified an early patterning event that occurs in the PCV, establishing either venous or lymphatic cell fate in cells forming the secondary sprouts (Koltowska et al. 2015; Nicenboim et al. 2015). The resulting fate restriction is thought to determine whether the secondary sprout will connect to the primary ISV to form a vein, or will not connect and instead form lymphatic structures. Surprisingly, we found regular interactions between the primary ISV and the secondary sprout irrespective of the final outcome of the patterning event (Figure 1D; Supplementary Movie S1). In most segments, secondary sprouts fuse with the primary ISV, forming three-way connections between the DA, ISV and PCV. Ultimately
however, the connection to the DA either regresses, turning the ISV into a vein, or remains stable, thus preserving arterial ISV identity. In the case of the latter, the secondary sprout disconnects again from the ISV and contributes to lymphatic formation (Figure 1D). Occasional occurrence of these three-way connections has previously been reported, albeit in only 3% of the ISVs (Isogai et al. 2003). Interestingly, our quantification reveals that at least 77.5% of future aISVs (40 aISVs, n=43 embryo) are transiently connected to secondary sprouts, forming a lumenized and perfused shunt (Supplementary Figure S1C, Supplementary Movie S2).

**Notch mediates local patterning of the trunk vasculature.**

Because of the known role of Notch in regulating artery-vein specification (Lawson et al. 2001; Lawson and Weinstein 2002; Zhong et al. 2001; Zhong et al. 2000), and the increased formation of vISVs upon Notch inhibition (Geudens et al. 2010; Hogan, Herpers, et al. 2009), we asked whether Notch activity cell-autonomously influences ISV specification. We used Tol2 transgenesis to mosaically overexpress the intracellular domain of Notch1a (NICD) together with a mCherry reporter in single ECs of embryos expressing the vascular reporter Tg[fli1a:EGFP]y1 (Supplementary Figure S1D). At 6 dpf, mCherry-positive NICD-overexpressing cells were found almost exclusively in the arterial compartment of the trunk vasculature (DA and aISVs) (Figure 1F-G), indicating that Notch signaling might play a cell-autonomous role in ISV patterning. Overexpressing the intracellular domain of the parologue Notch1b (N1bICD) and a dominant active version of Suppressor of Hairless (Su(H)), the transcription factor mediating canonical Notch signaling, confirmed this idea (supplementary figure S1E). Live imaging revealed that the ISV containing NICD-overexpressing cells remained arterial, while still forming transient lumenized three-way connections (Supplementary Figure S1F, Supplementary Movie S3). Interestingly, when studying all vessels in these embryos, we observed a strong bias towards the formation of veins in ISVs containing only WT cells (Figure 1G). Moreover, when analyzing individual embryos, we observed that the more ISVs contained NICD-overexpressing cells, the more wild-type ISVs turned into veins (Figure 1H). As a result, embryos with mosaic overexpression of NICD maintained the global artery-vein balance (Figure 1G). Neighborhood analysis in these embryos showed a complete disruption of ipsilateral patterning, rendering all local patterns equally probable (Figure 1I). Taken together, these results demonstrate a cell autonomous role for high Notch activity in locally instructing
artery formation, and suggest the existence of a compensation mechanism that maintains the global balance between arteries and veins, independently of the local patterning.

**Flow mediates the global artery-vein balance of the trunk vasculature.**

Since a balanced artery-vein network is required for optimal blood flow distribution in the fish trunk, we speculated that flow and flow sensing play an important role in the patterning and/or compensation of vessel specification. To test this hypothesis, we slowed down the heart rate by treating embryos with tricaine (tricaine mesylate, MS-222), a muscle relaxant commonly used as an anesthetic in fish. Treatment of the embryos with twice the dose normally used for anesthesia, after the onset of secondary sprouting, from 31 to 52 hpf, significantly reduced heart rate and blood flow speed (Supplementary Figure 2). Flow reduction in wild-type embryos disrupted the global balance of arteries and veins (61.2% ± 0.06 aISV) (Figure 2A) whilst retaining ipsilateral patterning (Figure 2B). Accordingly, blood flow could be a critical determinant in the compensation mechanism that establishes the overall balance in the number of arteries and veins along the trunk. Indeed, treating embryos harboring mosaic overexpression of NICD in the vasculature with a similar dose of tricaine resulted in a reduction in the number of WT vessels becoming vISVs, thus abolishing the compensation effect (Figure 2C).

**Differential polarity and directional movement of endothelial cells predict arterial or venous fate**

Given the prevalence of three-way connections as an intermediate step in remodeling, the question whether a given ISV remains arterial or becomes a vein is determined by which of the branches of the three-way connection is stabilized or lost. This is reminiscent of the pruning process in capillary networks where directional migration of cells drives regression of poorly perfused vessel segments (Chen et al. 2012; Franco et al. 2015). Previous studies of developmental vessel pruning in mouse retina and zebrafish brain established that ECs exposed to physiologically high levels of blood flow, above a certain threshold, align in the direction of the flow and polarize against it (Franco et al. 2015; Tzima et al. 2005). As a
consequence, adjacent vessel segments (branches) that experience different levels of flow show opposite endothelial polarization and movement. ECs polarize and migrate away from the branch experiencing low or sub-threshold flow, into the branch under high flow. The divergent polarity of cells in the low-flow branch causes cells to disconnect. Therefore, we analyzed the polarity of ECs during the remodeling process in Tg[fli1a:GFP]y1;Tg[fli1a:B4GalT-mCherry]bns9 embryos, labeling ECs in green, and endothelial Golgi apparatus in red. We analyzed polarity at three different phases: I) before secondary sprout connection, II) when the three-way connection is present and III) after resolution (Figure 3A, Supplementary Figure S4A). We found that most ECs polarize against the flow, leading to ventral polarity in aISVs (60% (105/175) of ECs) and dorsal polarity in vISVs (52% (71/137) of ECs) after remodeling (phase III) (Figure 3B-E, Supplementary Movies S4-S5). Surprisingly, ECs in future aISVs and vISVs already showed differential polarity before secondary sprout connection (Figure 3E - phase I). Moreover, we tracked the nucleus of ECs in all three phases in double transgenic Tg[-0.8flt1:RFP]hu5333;Tg[fli1a:EGFP]y1 embryos that allow clear distinction of arteries (labeled in green and red) and veins (labeled only green). We observed that cells in the primary ISV moved dorsally in future vISVs, whereas cells in future aISVs remained largely at the same position or moved slightly ventrally (Figure 3F). Again, this difference was already present in phase I, illustrating that ECs within the primary ISVs forming future arteries or veins behave differently early on, before any interactions with the secondary sprouts from the PCV take place.

Since vessel regression events are characterized by a progressive conversion from multicellular to unicellular arrangements (Franco et al. 2015; Lenard et al. 2015), we analyzed the remodeling process by imaging cellular junctions at the base of the primary ISV in Tg[fli1a:pecam1-EGFP]ncv27;Tg[-0.8flt1:RFP]hu5333 (junctions in green, arterial structures in red) embryos during remodeling. For most future vISVs, at the moment of connection to the secondary sprout, the base of the ISV was made of a single EC connecting the vessel to the DA. In contrast, the majority of future aISVs had a multicellular arrangement at the ISV base (Figure 3G-H, Supplementary Movie S6). Combined, these findings uncover a heterogeneity in primary ISVs, showing differential behavior of ECs that is predictive of their specification, prior to the connection with secondary sprouts (Figure 3N). These findings also suggest the possibility that the process of disconnecting from the DA to form a vISV may be initiated independently of the approaching secondary sprout.
Primary ISV pre-patterning occurs independently of secondary sprouts

To test this hypothesis, we inhibited the formation of secondary sprouts by inactivating ccbe1, a critical mediator of Vegfc processing (Hogan, Bos, et al. 2009). Despite the absence of secondary sprouts in ccbe1 morpholino treated embryos, a subset of ISVs (on average 38.2% ± 4.8) showed a dynamic behaviour consistent with regression of the DA connection (Figure 3I-K, Supplementary Movie S7). Regressing behaviour was evident by the presence of only a thin membrane connection, lumen collapse and reconnection, and even full detachment of the ISV from the DA in 36.6% (± 11.32) of regressing ISVs (Supplementary Figure S3A-B). The regressing ECs exhibited dorsal polarity and movement, a behaviour consistent with venous specification (Supplementary Figure S4C-D). Interestingly, co-injection with a dll4 morpholino, which leads to the formation of an increased number of veins in the trunk (Leslie et al. 2007), resulted in a dramatic increase of ISV regression (MO-dll4 + MO-ccbe1: 70.9% ± 21.54) (Figure 3K-L, supplementary Figure S3C, Supplementary Movie S8). Together these results suggest that the autonomous regression behaviour observed in the absence of secondary sprouts is associated with a venous specification that is established early on in primary ISVs.

The majority of primary ISVs are not perfused before secondary sprouts connection.

To assess if flow could play a role in the specification of primary ISV prior to Phase II, we set to investigate the perfusion status of these vessels. At the end of phase I / inception of phase II, 25% (3/12) of future vISVs and 11% (1/9) of future aISVs were lumenized throughout (from the DA to the DLAV) (N=3, 7 zebrafish embryos). However, none appeared to exhibit full ventral to dorsal lumenization prior to the connection to the secondary sprout. As lumenization itself is not sufficient to assess perfusion (as defined by the transit of blood through the ISV), we investigated perfusion both with erythrocytes and serum. To assess perfusion with serum, we injected Tg[fli1a:EGFP]y1 embryos with fluorescent beads in the general circulation (Qtracker™ 705 quantum dots), after the initiation of general blood circulation (Figure 4A-B)(Martin et al. 2013). We found that 27.3% (3/11) of future aISVs and 30.4% (7/23) of future vISVs showed perfusion with the beads (as characterized by the presence of a continuous detection of the fluorescent beads throughout the primary ISV, from...
the dorsal aorta to a putative outlet in an adjacent ISV) at the end of phase I/inception of phase II (connection of the primary ISV to the secondary sprout). When analyzed one hour earlier, in phase I, only 18.2% (2/11) of aISVs and 26.1% (6/23) of vISVs showed serum perfusion (Figure 4C) (N=4, 13 embryos). In Tg[gata1a:dsRed]^{td2};Tg[fli1a:EGFP]^{y1} embryos, we were unable to detect any red-labeled erythrocytes transiting through the future aISV (n=7) or vISVs (n=11) prior to, or at the inception of, Phase II (Figure 4D) (n=7 embryos).

Overall, our analysis suggests that the blood perfusion status of the primary ISV prior to the connection to the secondary sprout is unlikely to significantly influence the specification of primary ISVs in phase I. In addition, these results support the idea that polarization of ECs during phase I (see Figure 3) precedes the polarization against the direction of flow.

**Notch signalling mediates early primary ISV specification**

Given the strong influence of Notch activity on primary ISV specification, we analyzed the effect of manipulating Notch signaling on EC behavior within the ISV. Tracking of cell movement in dll4 morphants showed that the primary ISV cells migrated dorsally like wild-type venous cells, while in ISVs overexpressing NICD, ECs instead migrated ventrally like arterial cells. Both behaviors are visible in all phases of remodeling (Figure 5A). Analysis of cell polarity during remodeling in dll4 morphant embryos showed that the majority of cells are polarized dorsally, as in wild-type vISVs, but that an increased number of cells appear unpolarized (Supplementary Figure S4E). Similarly, in flow chamber experiments under shear stress conditions mimicking physiological flow, we found that chemical inhibition of Notch signaling using the gamma-secretase inhibitor DAPT prevented HUVECs from efficiently orienting parallel to the flow and from polarizing against the flow (Supplementary Figure S4F).

Thus, our combined *in vivo* and *in vitro* results indicate that Notch signaling influences EC polarity and movement in ways that are predictive of vessel specification into future arteries. However, such a mechanism of artery-vein specification would imply that endogenous Notch activity should be heterogenous early on in primary ISVs.

To analyze Notch activity in wild-type embryos during phase II we used a Notch activity reporter line based on the Epstein-Barr virus tp1 enhancer containing 12 concatamers of...
Su(H)/Rbpj binding sites and a minimal promoter (Parsons et al. 2009). To avoid misinterpretation due to time delays in protein degradation, we turned to a photoconvertible version of the Notch activity reporter Tg[tp1-MmHbb:kaede]um15 (Clements et al. 2011) and crossed it to Tg[kdr-l:ras-Cherry]s916 to visualize blood vessels. The photoconvertible fluorescent protein Kaede was converted from Kaede green into Kaede red protein by UV exposure at 29-30hpf, a time-point before the start of secondary sprouting. This allowed us to analyze the generation of newly formed Kaede green protein, indicating still active Notch signaling. The embryos were imaged at 52hpf and again at 6dpf to determine the Notch activity status during remodeling and the final artery-vein sequence, respectively. This experiment clearly revealed an early heterogeneity in Notch signaling in the primary ISVs, before and during connection of the secondary sprouts, with multiple ECs within the ISV being labelled in a majority of cases (2.4 ±1.1 cells/ISV; n=31 ISVs) (Figure 5B). Active Notch signaling during phase II of vascular remodeling correlated significantly with arterial specification (87.3% ±16.4 aISV; n=20 embryos), whereas the absence of Notch activity during phase II correlated with venous specification (69.2% ±18.3 vISV; n=20 embryos) (Figure 5D). Of note, we also observed that a portion of ISVs expressed higher levels of Kaede green indicating stronger Notch activity than others, correlating even more strongly with arterial development (94.7% ±13.3 aISV; n=18 embryos) (Figure 5B-D). These high Notch activity ISVs were often found in alternating positions with lower Notch activity ISVs (Figure 5B-C, red arrows), corresponding with the observed local patterning favoring alternating vessel identities (Figure 1B). Indeed, neighborhood analysis determining the conditional probability of finding a Notch activated vessel (i.e. tp1 positive) given the Notch activity status of its neighbors showed that tp1 positive vessels are more frequently flanked by tp1 negative vessels (Figure 5G). This effect was even more pronounced for the high Notch activity ISVs (not shown).

Given the effect of flow inhibition on the global artery-vein balance, we investigated the effect of flow inhibition on Notch activity in embryos with a shifted artery-vein balance. After conversion of the Kaede protein, embryos were treated with 2x tricaine from 30-52 hpf (during phase II). Again, the embryos were imaged at 52hpf and 6dpf. The overall percentage of ISVs with active Notch signaling did not change after flow inhibition (40.5% ±14.2 vs 33.8% ±16.7, n=20 control vs 12 tricaine treated embryos, P>0.99) (Figure 5E). Also, the arterial specification of ISVs with active Notch signaling was not affected (87.3% ±16.4 vs 89.0% ±14.2, n=20 control
vs 12 tricaine treated embryos, $P=0.99$) (Figure 5F). However, we found that ISVs negative for the tp1-reporter formed significantly more arteries in flow inhibited embryos than in untreated control embryos (30.8% ±18.3 vs 48.3% ±17.9, n=20 control vs 12 tricaine treated embryos, $P=0.026$) (Figure 5F). Finally, and crucially, we show that in the majority of tp1-positive future aISVs (86.4%, n=22) the Notch pathway is activated before connection of the secondary sprout (Figure 5H, Supplementary Movie S10).

Taken together, these results indicate that Notch signaling is an important determinant for arterial specification, whereas blood flow is required to fine-tune the global artery-vein balance.

**DISCUSSION**

Initial formation of the stereotyped primary vascular network in the zebrafish trunk has been extensively studied (Lawson and Weinstein, 2002; Isogai et al., 2003; Jin et al., 2005; Blum et al., 2008; Herbert et al., 2009). However, the mechanisms through which the trunk vasculature remodels into a balanced network of arteries and veins, and especially how primary ISV either remain arterial or remodel into veins, is less well understood.

An important research focus has been the heterogeneous nature of secondary vascular sprouts emerging from the PCV. Recent work has shown that $prox1$ expression levels in the nascent secondary sprouts can be correlated with their future specification as lymphatic endothelial cells (LECs) or venous ECs (Nicenboim et al. 2015; Koltowska et al. 2015). Accordingly, lymphatic precursors arise from secondary sprouts expressing $prox1a$, while the majority of Prox1a negative sprouts connect to the primary ISVs and contribute to the formation of vISVs (Nicenboim et al. 2015; Koltowska et al. 2015). Although never directly tested, this concept would seem to imply that the question of whether any given primary ISV will remain arterial or will remodel into a vein is determined by the Prox1a-mediated fate restriction of the secondary sprout it encounters.

However, our work demonstrates that the future arterial or venous fate of the ISV can be largely predicted by endothelial heterogeneity in the primary ISVs. Furthermore, this
heterogeneity in signaling activity, EC polarity and directional EC movement appears pre-specified prior to the formation of any connection with the approaching secondary sprout. At first glance, these results are therefore incompatible with the idea that vascular remodeling events in the zebrafish trunk are driven by the lymphatic-vein specification of secondary sprouts under the governance of Prox1a activity. However, a more detailed examination of the remodeling process, the different early specification events, the intermediate steps in remodeling, and continuous tracking of cells during the process, leads to a picture that may partially reconcile both models.

Whilst our present results clearly identify Notch-mediated specification of primary ISV arterio-venous fate through directional movements and junctional configurations that appear to stabilize ISV connections to the DA, and thereby retain arterial identity, they do not necessarily mean that Prox1a levels cannot determine lympho-venous EC fate decisions. Our study identifies that most secondary sprouts engage in a connection with the primary ISV irrespective of any pre-specification of either the primary ISV or the secondary sprout. This would mean that the ultimate fate of ECs to become arterial, venous, or lymphatic is not determined by a decision governing with what they initially connect, but instead by a decision that governs from what they will ultimately disconnect. In the case of an arterial ISV, the critical disconnection will need to occur between ISV and secondary sprout. A venous ISV will instead need to disconnect from the DA. Similarly, lymphatic EC will need to disconnect from the primary ISV in order to form the parachordal lymphangioblasts. The formation of these parachordal lymphangioblasts has generally been seen to occur wherever the ISV remains arterial, linking the acquisition of lymphatic fate in the secondary sprout to the acquisition of arterial fate in the ISV. Closer observations however identify additional and alternative behavior: Even where secondary sprouts have remodeled an ISV into a vein, lymphangioblasts can occasionally be seen to emerge and disconnect from this vein at a later time point (See Supplementary Movies S1 and S5). This phenomenon has already been described by Isogai and colleagues in their landmark study (Isogai et al. 2003). Thus, a secondary sprout can give rise to both venous and lymphatic structures, suggesting that the original idea of prox1a promoting lymphatic fate may hold true even if the cells intermittently help to form a vein. Such a model would also fit with the observed overabundance of Prox1a high secondary sprouts (Koltowska et al. 2015). Accordingly, 65% of secondary sprouts show high Prox1a
expression, demonstrating that some will need to form veins despite of their Prox1a expression.

The question how the overall balance of arteries and veins is established, or “how the embryo keeps count” is therefore not answered by early fate determination events in the secondary sprout. Neither is it determined by the number of Notch high primary ISV, or the number of vessels that show features of pre-specified arteries. Our quantification points to roughly 60% of vessels showing ventral polarity, and ventral movement, as well as a multicellular junction arrangement at their base. Consequently, this pre-specification would predict that embryos form around 60% arteries and only 40% veins. Intriguingly, blocking flow leads to numbers very close to this pre-specification, with an overabundance of arteries formed, indicating that flow-mediated compensation induces re-specification of some arteries into veins even in wild-type embryos. A similar 60/40 ratio was observed in artery/vein-specific primary ISVs EC behaviour when blocking secondary sprouting, together suggesting that flow acts on the three-way connection to drive compensation. The extent of this compensation effect appears scalable for larger deviations from the optimal 50/50 ratio as shown by the mosaic Notch activation experiments, indicating there is no fixed subpopulation of adaptable ISVs. When looking at intrinsic Notch activity, the ISVs containing highest reporter activity showed almost no deviation from arterial fate no matter whether flow is inhibited or not. The Notch low vessels however appear to shift to more arteries in the absence of flow (Figure 5F). Therefore, it is tempting to speculate that ECs showing lower levels of Notch are more plastic and amenable to flow-mediated adaptation.

The question of how the local pattern favoring alternating arteries and veins is achieved, at least in the ipsilateral analysis, is equally intriguing. Although it would be conceivable that the three-way connection alone coupled to flow-induced polarity can work to favor alternating fates, and thus balance flow distribution, the data suggest something else is at play. Embryos lacking flow maintain local patterning favoring alternating fate, even if the global balance is skewed. Mosaic overexpression of NICD however complete disrupted local patterning, suggesting that Notch levels are also part of the local patterning. This idea is supported by the fact that even the early Notch reporter heterogeneity assessed in the Tg[tp1-MmHbb:kaede]um15 line shows local patterning. How this is patterning of high and low Notch
activity in the early ISV is achieved remains to be studied in future work. Further studies will also need to uncover how flow overwrites the early ISV specification. Previous work suggests that it could do so by directly modulating Notch levels (Watson et al. 2013), although the possibility of a distinct specification pathway cannot be excluded.

Recently, work by Weijts and colleagues (Weijts et al. 2018) has suggested that the artery-vein balance in the zebrafish trunk can be primarily explained by a flow mediated activation of Notch activity preventing primary ISVs from turning into vISVs. Although we agree on the fact that Notch and flow play a role in artery-vein balance, our models differ significantly, most notably by decoupling the role of Notch and flow during primary ISV specification. While we also show that flow plays a role in the global artery-vein balance in the trunk vasculature (Figure 2), our work strongly suggests that primary ISVs are pre-specified in a Notch-dependent process prior to, and in the absence of, connection to the secondary sprouts originating from the PCV (Figure 3 and 5). In addition, we show that this pre-specification occurs before the majority of primary ISVs are perfused, suggesting that this process is flow-independent (Figure 4). Finally, we found that inhibition of blood flow with tricaine result in an increased number of arteries (Figure 2), and not veins, like presented in that study. Overall, these results are hard to reconcile with our own.

In conclusion, our work identifies transient artery-vein connections as intermediate structures that resolve through a combination of pre-specified and flow-induced directional migration of ECs. We propose that vascular remodeling, based on a dual mechanism of molecular specification and flow-mediated patterning control, provides the necessary plasticity to allow formation of an overall balanced and efficiently perfused vascular network in the zebrafish trunk.
MATERIALS AND METHODS

Zebrasfish husbandry and transgenic lines

Zebrafish (Danio rerio) were raised and staged as previously described (Kimmel et al. 1995). The following transgenic lines were used: Tg[fli1a:EGFP] y1 (Lawson and Weinstein 2002) (labels all endothelial cells), Tg[fli1a:nEGFP] y7 (labels all endothelial cell nuclei) (Roman et al. 2002), Tg[fli1a:dsRedEX] um13 (Covassin et al. 2009) (label all endothelial cells), Tg[gata1a:dsRed] d2 (Traver et al. 2003) (label all erythrocytes), Tg[-0.8flt1:RFP] hu5333 (Bussmann et al. 2010) (strongly labels arterial endothelial cells), Tg[fli1a:B4GalT1-mCherry] bns9 (Kwon et al. 2016) (label the Golgi apparatus of endothelial cells), Tg[fli1a:pecam1-EGFP] ncv27 (Ando et al. 2016) (label the endothelial cell junctions), Tg[tp1-MmHbb:kaede] um15 (Clements et al. 2011) (label cells with an active Notch signalling pathway), Tg[kdr-l:ras-Cherry] s916 (Hogan, Bos, et al. 2009) (label endothelial cell membrane). For growing and breeding of transgenic lines we comply with regulations of the ethical commission animal science of KU Leuven and MDC Berlin.

Vessel patterning analysis

Figures 1A-C, 1G-J, 2A-C, S1A-B, S2A-B show analyses of the regularity of local and global vessel arrangements of aISVs and vISVs in the zebrafish trunk. To perform those analyses, 6dpf zebrafish embryos were screened under a fluorescent stereomicroscope (Leica M205 FA) to identify the sequence of arterial and venous ISVs on both flanks of a 10 somites segment in the trunk, starting after then junction of DA and PCV. The identity of arteries and veins was determined by their connection to respectively the DA or the PCV, and by direction of blood flow. This manual characterization yielded for each vessel its ISV type, its collateral neighbor’s ISV type, as well as the left ISV neighbor type for all but the leftmost vessels, and the right ISV neighbor type for all but the rightmost vessels. In mosaic NICD overexpression experiments, in addition to the vessel type it was also registered if a given vessel contained a NICD overexpressing cell (used in Figures 1H-J). This dataset was then analyzed to obtain the global and local distribution patterns of aISVs and vISVs for different experimental conditions using a custom-made Python script.

To obtain the global vessel ratio, the number of aISVs and vISVs of all ISVs was compared in Figures 1A,G,H and Figure 2A.

To study the local ISV patterning, the relative frequency of aISVs given the type of the neighboring vessels was obtained: Figures 1C, 1J and 2C show the frequency of aISVs given the type of the collateral neighbor (aISV or vISV); Figures 1B, 1I and 2B show the frequency of
aISVs given the vessels’ corresponding number of ipsilateral aISV neighbors (0, 1 or 2 aISVs); Supplementary Figure 2A shows the frequency of aISVs for the given number of aISVs in the 2-neighborhood of the vessel (0, 1, 2, 3 or 4 aISVs); and Supplementary Figure 1B shows the frequency of aISVs given the type of the right neighbor (aISV or vISV). Figure 5G similarly shows the frequency of tp1 positive and negative ISVs given the Notch activity status (tp1 signal) of the ipsilateral neighbors.

Mosaic overexpression using Tol2 transgenesis

Transgenic zebrafish embryos Tg[fli1a:EGFP]y1 were injected at one-cell stage with 100 pg of Tol2 mRNA (Kwan et al. 2007) and 15 pg of plasmid DNA pTol2-N1alCD-basfli-mCherry (De Bock et al. 2013), 25pg of plasmid DNA pTol2-N1bICD-basfli-mCherry or 40pg of plasmid DNA pTol2-Su(H)VP16-basfli-mCherry. The pTol2-N1bICD-basfli-mCherry and pTol2-Su(H)VP16-basfli-mCherry constructs were generated by Multisite Gateway cloning (Life Technologies). Embryos were raised at 28°C and screened for transient expression at ~30 hpf.

Quantification of arterial and venous ISV distribution was performed in 6-day old embryos by scoring their percentile presence in 10 consecutive somite segments in the trunk after the junction of DA and PCV (i.e. somites 5-15).

Live imaging

Embryos were anaesthetized in 0.014% tricaine (MS-222, Sigma), mounted in a 35 mm glass bottom petri dish (0.17 mm, MatTek) using 0.6-1% low melting point agarose (Sigma) containing 0.014% tricaine, and bathed in Danieu’s buffer containing 0.007%(0.5X)– 0.014% (1X) tricaine and 0.003% PTU (as indicated). Time-lapse imaging was performed using a Leica TCS SP8 upright microscope with a Leica HCX IRAPO L x25/0.95 water-dipping objective and heating chamber, or on an upright 3i spinning-disc confocal using a Zeiss Plan-Apochromat, 20x, 40x or 63x/1.0 NA water-dipping objective. Image processing was performed using Fiji software (Schindelin et al. 2012).

Tricaine treatment

To slow down heart rate during the secondary sprouting and ISV remodeling process embryos were treated with 0.028% (2X) tricaine (MS-222, Sigma) between 31 and 52 hpf, after which the compound was washed away again.
Cell polarity analysis

To analyze polarity of ECs during vascular remodeling, time-lapse movies were made of transgenic Tg[fli1a:EGFP]^{y1};Tg[fli1a:B4GalT1-mCherry]^{bns9} embryos during vascular remodeling in the trunk (~32 hpf to ~54 hpf). Polarity arrows from the center of the nucleus to the center of the Golgi apparatus were drawn manually using Fiji software. For every primary ISV cell the polarity was scored per time point: dorsal polarity, ventral polarity or unpolarized, depending on the relative position of Golgi and nucleus, i.e. respectively, Golgi dorsal, ventral or parallel to the nucleus in respect to the local angle of the ISV. Per developmental phase all scores were added over the different timepoints, as indicated in the figure legends.

Cell movement analysis

To analyze the upward movement of ECs within ISVs of Tg[fli1a:GFP]^{y1} or Tg[fli1a:nEGFP]^{y7}; Tg[fli1a:dsRedEX]^{um13} embryos in different stages of development, we use a mix of manual segmentation of developmental timelapse and computational analysis in Python (Figures 3F & 3K). Confocal stacks were registered using StackReg (ImageJ plugin - http://bigwww.epfl.ch/thevenaz/stackreg/). The cells’ distance to the dorsal aorta was tracked through time in a 2D maximum projection manually in Fiji, and combined with the information about the later fate (aISV or vISV) of the vessel containing the cell, and the current phase of development of the ISV (cf. Figure 3A). To obtain the upward speed of the cells in the individual phases of the vessel development, the initial distance of the cell’s nucleus to the aorta was compared to the final position in the given phase (in [μm]) divided by the duration of the cell’s trajectory in the phase (in [min]). Note that in this definition a positive upward speed corresponds to an increasing distance from the dorsal aorta (i.e. movement towards the DLAV), while a negative upward speed corresponds to an average movement towards the dorsal aorta.

Perfusion assay

The Qtracker™ 705 quantum dots solution was injected in the duct of Cuviers of zebrafish embryos anesthetized with 0.014% tricaine and mountain laterally in 0.0014% low-melting agarose after establishment of blood flow (28 to 30 hpf). The quantum dots were excited with a 561 nm laser and their emission detected between 665.5 and 735.5 nm.
**Morpholino knockdown**

Morpholinos against *ccbe1* and *dll4* were used as previously described (Hogan, Bos, et al. 2009; Leslie et al. 2007).

**Notch activity reporter assay**

For analysis of Notch activity during phase II of vascular remodeling, the Kaede\textsuperscript{green} protein in embryos of the Notch activity reporter *Tg[tp1-MmHbb:kaede]\textsuperscript{um15} Tg[kdr-l:ras-Cherry]\textsuperscript{s916}* was converted to Kaede\textsuperscript{red} at 29hpf by illumination with a 405nm laser. These embryos were imaged at 52hpf to assess Notch activity during phase II, i.e. newly formed Kaede\textsuperscript{green} protein. At 6dpf the same embryos were imaged again to analyze the artery-vein sequence.

For the tricaine experiment, embryos were treated with 0.028\% (2x) tricaine from 29-52hpf after conversion of the Kaede protein.

**In vitro flow chamber experiments**

HUVECs (Promocell, primary cells from pooled donors; characterized by flow cytometry with the following markers CD31+, vWF+, Dil-Ac-LDL uptake+, SMA- and tested for mycoplasma contamination) were seeded and grown to confluency on a slide in EBM2 medium (Promocell) coated with gelatin. Unidirectional laminar shear stress was applied to confluent HUVECs using a parallel plate chamber system (Ramkhelawon et al. 2009) for 24 hours and cells were treated with 5\(\mu\)M DAPT or a similar amount of DMSO in controls for the duration of the experiment. Local shear stress was calculated using Poiseuille law and averaged 20 dyne/cm\(^2\). Cells were fixed in 100\% methanol for 10min at -20°C and stained for DAPI, VE-cadherin (Santa Cruz, sc-6458, dilution 1/100) and GM130 (BD Biosciences, 610822, dilution 1/400) (Franco et al. 2016). Matlab was used to analyze cell orientation (direction of the main axis of the nucleus) and cell polarity (angle between center of the nucleus and center of the Golgi).

**Blood flow and heart rate measurements**

Embryos were anaesthetized in 0.014\% tricaine (MS-222, Sigma), mounted in a 35 mm glass bottom petri dish (0.17 mm, MatTek) using 1\% low melting point agarose (Sigma) containing 0.007\% (0.5X), 0.014\% (1X) or 0.028\% (2X) tricaine, and bathed in Danieau’s buffer containing 0.007\% (0.5X), 0.014\% (1X) or 0.028\% (2X) tricaine respectively and 0,003\% PTU for
1h before being imaged on an upright 3i spinning-disc confocal using a Zeiss Plan-Apochromat 20x/1.0 NA water-dipping objective with a frame interval of 10ms. Kymographs were generated using the MultipleKymograph plugin in ImageJ to quantify heart rate over a 8 second period, synced to the beginning of a heartbeat (line width: 1).

To estimate instantaneous blood flow speed, we cropped images of the dorsal aorta and measured average frame-to-frame translation of red blood cells using the Kuglin-Hines algorithm (Kuglin and Hines, 1975) for image phase-correlation. In brief, the phase correlation map between two adjacent frames was calculated by multiplying the Fast Fourier transform (FFT) of frame\textsubscript{i} and a conjugate FFT of frame\textsubscript{i+1}. The inverse FFT of the phase correlation gives a correlation map with a peak offset from the center by the relative shift between the frames. The position of the peak was determined by finding the local maximum in a Gaussian filtered correlation map. The velocity data was smoothed with a moving average filter with a span of 5 frames. Analysis was performed in Matlab (Mathworks, Inc.).

**Statistical Analysis**

No statistical method was used to predetermine sample size.

Data represent mean ± SEM of representative experiments (except when indicated otherwise). Statistical tests were conducted using Prism (GraphPad) software. Adequate tests were chosen according to the data to fulfill test assumptions. Sample sizes, number of repeat experiments, performed tests and p-values are indicated per experiment in Supplemental Table 1. The angle repartitions of the flow chamber experiments were analyzed using Kuiper two-sample test, a circular analogue of the Kolmogorov-Smirnov test. A p-value < 0.05 was considered statistically significant.

Zebrafish embryos were selected on the following pre-established criteria: normal morphology, beating heart, presence of circulating red blood cells. The experiments were not randomized. For every experiment treated and control embryos were derived from the same egg lay. The investigators were not blinded to allocation during experiments and outcome assessment.
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Author Contributions

I.G., B.C., S.A., V.G., A.-C.V. and H.G. designed the experiments. I.G., B.C., S.A., V.G., K.M., A.R. and A.-C.V. performed the experiments and analyzed the data. I.G., B.C., S.A. and H.G. wrote the manuscript.

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REFERENCES


Figure 1 – Notch mediates the local patterning of the trunk vasculature.

A) Quantification of the ratio of arterial and venous ISVs in a 10-somites region of the trunk of 6 dpf WT embryos (N=3 experiments, 74 embryos, 1480 ISVs).

B) Ipsilateral neighborhood analysis of vessel identity with 2 neighbors in 6 dpf WT embryos (N=3 experiments, 74 embryos, 1184 ISVs).

C) Contralateral neighborhood analysis of vessel identity in 6 dpf WT embryos (N=3 experiments, 74 embryos, 1480 ISVs).

D) Stills from time-lapse movie (Supplementary Movie S1) of a Tg[fli1a:EGFP]y1 / Tg[-0.8flt1:RFP]hus333 embryo showing ISV remodelling into a venous (left) and an arterial (right) intersegmental vessel (vlISV and alISV). The double transgenic labelling with GFP expression in all ECs and RFP expression in arterial ECs, facilitates distinction between arterial (yellow) and venous (green) structures. In both cases, a lumenized connection is formed between the secondary sprout and the primary ISV (arrowhead). In the case of the formation of an alISV, the connection is lost again and the secondary sprouts forms lymphatic precursors at the horizontal myoseptum (parachordal lymphangioblasts, PL). In case of vlISV remodelling, the secondary sprout connection is stabilized and the connection between primary ISV and DA regresses.

E) Tg[fli1a:EGFP]y1 embryos mosaically expressing a pT2Fli1ep-zN1aICD-basfli-mCherry construct at 50 hpf. Lymphangiogenic sprouts i.e. sprouts delivering lymphatic precursors at the horizontal myoseptum (arrowheads), can be observed at the position of NICD overexpressing (NICDOE) ISVs (asterisks).

F) Tg[fli1a:EGFP]y1 embryos mosaically expressing a pT2Fli1ep-zN1aICD-basfli-mCherry construct at 6dpf. NICDOE mCherry-positive cells were found almost exclusively in the arterial compartment of the vasculature.
G) Quantification of the ratio of arterial and venous ISVs in a 10-somite trunk region of 6dpf control embryos (N=3 experiments, 74 embryos) and mosaic NICD\textsuperscript{OE} embryos (N=3 experiments, 51 embryos). In mosaic embryos the arterio-venous distribution was quantified overall and separately in NICD\textsuperscript{OE} and wild-type ISVs. Wild-type ISVs compensate for the forced arterialization of NICD\textsuperscript{OE} ISVs by increased formation of venous connection.

H) Quantification of the percentile presence of arterial and venous ISVs in a 10 somites region of the trunk of 6dpf NICD\textsuperscript{OE} embryos. Mosaic NICD\textsuperscript{OE} embryos represented in panel G were grouped based on their relative number of NICD\textsuperscript{OE} ISVs (<20, 20-30 or >30%; n=13, 26 and 12 embryos, respectively).

I) Ipsilateral neighborhood analysis of vessel identity with 2 neighbors in 6 dpf NICD\textsuperscript{OE} embryos (N=3 experiments, 40 embryos, 592 ISVs) compared to WT embryos (N=3 experiments, 74 embryos, 1184 ISVs).

DA, dorsal aorta; PCV, posterior cardinal vein; ISV, intersegmental vessel; PL, parachordal lymphangioblasts; WT, wild-type; NICD\textsuperscript{OE}, NICD-overexpressing. Scale bars, 50\(\mu\)m
Figure 2 – Flow mediates the global artery-vein balance of the trunk vasculature.

A) Quantification of the ratio of arterial and venous ISVs in a 10-somites region in the trunk of 6dpf wild-type embryos either untreated (control; N=3 experiments, 74 embryos) or treated with 2x tricaine to slow down the heart rate (N=3 experiments, 65 embryos). (*, P<0.0001, two-tailed unpaired t-test)

B) Ipsilateral neighborhood analysis of vessel identity with 2 neighbors in 6dpf wild-type embryos either untreated (control; N=3 experiments, 74 embryos, 1184 ISVs) or treated with tricaine to slow down the heart rate (N=3 experiments, 65 embryos, 950 ISVs).

C) Quantification of the ratio of arterial and venous ISVs in a 10-somites trunk region of 6dpf mosaic NICD\textsuperscript{OE} embryos either untreated (-) or treated (+) with 2x tricaine to slow down
the heart rate (respectively: N=3 experiments, 51 embryos; N=2 experiments, 20 embryos). In mosaic embryos the arterio-venous distribution was quantified overall and separately in mosaic NICD$^{OE}$ and wild-type ISVs (WT). Blood flow reduction eliminates the compensating venous remodeling in wild-type (WT) ISVs in mosaic NICD$^{OE}$ embryos, resulting in and overall shift towards more arterial ISVs.
Figure 3 – primary ISVs are specified into aISVs and vISVs prior to connection by secondary sprouts originating from the PCV.
A) Schematic representation of the three phases of primary ISV remodeling; phase I: before secondary sprout connection to the primary ISV, phase II: When a lumenized connection is formed between the secondary sprout and the primary ISV, phase III: when the three-way connection resolves into aISV or vISV.

B-D) Stills from time-lapse movies (see Supplementary Movie S4-S5) of ECs polarity in aISVs and vISVs of Tg[fli1a:GFP]y1;Tg[fli1a:B4GalT-mCherry]bns9 embryos during the 3 different phases: I) before secondary sprout connection, II) during three-way connection and III) after resolution. Arrows indicate the angle from the centre of the nucleus to the centre of the Golgi complex: green arrows indicate dorsal polarity, blue arrows indicate ventral polarity, yellow arrows indicate unpolarised ECs.

E) Quantification of EC polarity in aISVs (n=7 aISVs, 16 cells) and vISVs (n=8 vISVs, 17 cells) of Tg[fli1a:GFP]y1;Tg[fli1a:B4GalT-mCherry]bns9 embryos during the 3 different phases: I) 2.5 hours before secondary sprout connection, II) during three-way connection and III) 2.5 hours after resolution.

F) Quantification of ECs upward speed (in microns/day) in aISVs (n= 12 aISVs, 67 cells) and vISVs (n=13 vISVs, 103 cells) during the 3 different phases: I) 1 hour before secondary sprout connection, II) during three-way connection and III) 1 hour after resolution.

G) Stills from time-lapse movie (Supplementary Movie S6) of a Tg[fli1a:pecam1-EGFP]cv27;Tg[-0.8flt1:RFP]hus333 embryo showing ISV remodelling into an arterial and a venous intersegmental vessels (aISV and vISV) at 29 and 45 hpf.

H) Quantification of the cellular structure at the base of the primary ISV at the inception of Phase II. Detection or absence of GFP expression is used to characterise the nature of the connection (unicellular or multicellular) (n=34 embryos, 12 aISVs, 40 vISVs).
I) Still from time lapse movie (see supplementary movie S7) of a Tg[fli1a:pecam1-EGFP]\textsuperscript{vcv27} ; Tg[-0.8flt1:RFP]\textsuperscript{huh5333} 5ng MO-ccbe1 embryo showing ISV regression in the absence of secondary sprouting.

J) Quantification of percentage of primary ISV exhibiting a regression behaviour (full disconnection from the DA, thin membrane connection to the DA, lumen collapse and reconnection, and cell death at the base of the primary ISV – see Supplementary Figure 3) (N=4 experiments, 37 morphants, 241 morphant vessels).

K) Quantification of percentage of primary ISV exhibiting a regression behaviour in ccbe1 morphants compared to the percentage of veins in control clutch mates (n=37 morphants, n=29 WT controls).

L) Stills from time-lapse movie (Supplementary Movie S8) of a Tg[fli1a:EGFP]\textsuperscript{y1};Tg[-0.8flt1:RFP]\textsuperscript{huh5333} 5ng MO-ccbe1 / 10ng MO-dll4 embryo showing ISV regression in the absence of secondary sprouting.

M) Quantification of percentage of primary ISV exhibiting a regression behaviour (full disconnection from the DA, thin membrane connection to the DA, lumen collapse and reconnection, and cell death at the base of the primary ISV – see Supplementary Figure 2) (N=7 experiments, 62 morphants, 531 morphant vessels).

N) Quantification of percentage of primary ISV exhibiting a regression behaviour in MO-ccbe1(5ng)/MO-dll4 (10ng) double morphants compared to the percentage of veins in control clutch mates (n=62 morphants, n=17 WT controls).

O) Schematic representation of ISV specification prior to and at the inception of the three-way connection, quantifiable through EC polarity, upward movement speed and cellular structure at the connection to the dorsal aorta.

A, aISV; V, vISV. Scale bars, 50\textmu m
**Figure 4** - The majority of primary ISVs are not perfused prior to connection to the secondary sprouts originated from the PCV.

A) Stills from time-lapse movie of the trunk region a Tg[fli1a:EGFP]y1 embryos showing perfusion with Qtracker™ 705 quantum dots between 33 and 34:20 hpf. Scale bar, 50 μm.

B) Stills from time-lapse movie of a Tg[fli1a:EGFP]y1 embryos at time of connection a primary ISV to a secondary sprout (transition from Phase I to Phase II) injected with Qtracker™ 705 quantum dots 705 fluorescent beads.

C) Quantification of primary ISV perfusion in Phase I and at time of connection to the secondary sprout (Phase I / Phase II transition). Phase I perfusion is quantified 1h before connection to the secondary sprout. Perfusion is defined by the continuous labelling of the lumen area ISV with the quantum dots and visible presence of a probable inlet and outlet for flow. (n=13 embryos, 23 vISVs, 11 aISVs).
D) Stills from time-lapse movie of a Tg[fli1a:EGFP], Tg[gata1a:dsRed]$^{4d2}$ embryo (labeling ECs in green and blood cells in red) at time of connection a primary ISV to a secondary sprout (transition from Phase I to Phase II). (representative of n=7 embryos, 7aISVs, 11 vISVs)
Figure 5 - Notch signalling mediates early primary ISV specification

A) Quantification of ECs upward speed (in microns/day) in ISVs of WT (n=12 aISV (67 cells), 13 vISV (103 cells)), NICD\textsuperscript{OE} (n=30 aISV, 29 NICD\textsuperscript{OE} cells) and MO-dll4 (n=9 vISV, 85 cells) embryos (32 to 54 hpf) at 3 different time points: I) 2.5 hours before secondary sprout
connection, II) during three-way connection and III) 2.5 hours after resolution of the three-way connection.

B-C) Notch activity reporter $Tg(tp1-MmHbb:kaede)_{um15}^{um15};Tg(kdr-l:ras-Cherry)_{s916}^{s916}$ imaged at 52 hpf (B) and at 6 dpf (C) in the same embryo, after conversion of the Kaede photoconvertible fluorescent protein at 29 hpf (Notch activity reporter shown in green, all ECs labelled in red). Red arrows point to ISVs expressing high levels of the Kaede$^{green}$ protein, both at 52hpf and at 6dpf.

D) Quantification of the ratio of arteries and veins, determined at 6 dpf, correlated to the Notch activity status at 52hpf, after conversion of the Kaede photoconvertible fluorescent protein at 29 hpf (tp1 positive, negative or high (as indicated by red arrowheads in panel B)) (n=20 embryos). (*, P<0.0001, two-ways ANOVA)

E) Quantification of the percentage of tp1 positive (tp1+) and tp1 negative (tp1-) ISVs in untreated (n=20 embryos) versus tricaine 2x treated (tric 2x) embryos (n=12 embryos) at 52hpf, after conversion of the Kaede photoconvertible fluorescent protein at 29hpf. Flow inhibition between 29 and 52 hpf does not affect tp1 promoter activity during this period.

F) Quantification of the ratio of arteries and veins, determined at 6 dpf, correlated to the Notch activity status at 52hpf (tp1 positive or tp1 negative), in untreated (n=20 embryos) versus tricaine 2x treated embryos (n=12 embryos). Flow inhibition does not affect the balance of arteries and veins formed from tp1 positive ISVs, but tp1 negative ISVs form significantly more arteries after tricaine treatment (*, P=0.027, two-ways ANOVA)

G) Ipsilateral neighborhood analysis of Notch activity status of vessels with 2 neighbors in 52 hpf embryos after conversion of the photoconvertible Kaede protein at 29 hpf. Graph shows the frequency of finding a tp1 positive or negative ISV, given the Notch activity status of the neighboring ISVs (tp1 positive (+) or tp1 negative (-)) (n= 19 embryos, 199 ISVs) (*, P<0.0001, one-way ANOVA)
H) Quantification of the ratio of arterial ISVs with activated Notch signaling first detectable (as indicated by tp1 signal) before connection of the secondary sprout (i.e. during phase I) or after connection of the secondary sprout (i.e. during phase II) (n=22 ISVs).

NS, not significant. Scale bars, 100 μm
Figure 6

Alternative mechanism of artery-vein specification in the zebrafish trunk.
Figure S1

A) Ipsilateral neighborhood analysis of vessel identity with 4 neighbors in 6 dpf WT embryos (N=3 experiments, 74 embryos, 888 ISVs).

B) Ipsilateral neighborhood analysis of vessel identity with 1 neighbor in 6 dpf WT embryos (N=3 experiments, 74 embryos, 1332 ISVs).

C) Stills from time-lapse movie (Supplementary Movie S2) in Tg[fli1a:GFP];Tg[cd34:DsRed]td2 labeling ECs in green and blood cells in red showing formation of a transient perfused three-way connection (C'-C") as circulating blood cells can be observed in the DA-ISV-secondary sprout-PCV shunt. In panel C" it is clear that the ISV-PCV connection is disconnected again and the secondary sprout takes part in lymphatic development, whereas strong dorsal flow is established in the arterial ISV.

D) Tg[tp1-MmHbb:kaede]um15 embryo mosaically expressing a pT2Fli1ep-zN1aICD-basfli-mCherry construct (NICD^{OE}) at 52 hpf. Endogenous Notch activity was blocked by treatment with 25µM DAPT from 24 till 52 hpf in order to observe Notch activation by NICD overexpression.

E) Quantification of the ratio of arterial and venous ISVs containing cells overexpressing Su(H)VP16, a constitutively active variant of the Su(H) transcription factor (Su(H)VP16^{OE}) (N=3 experiments, 29 embryos) or mosaically overexpressing Notch-1bICD (N1bICD^{OE}), the intracellular domain of Notch1b, the parologue of Notch1a (N=2 experiments, 43 embryos). (*P=0.0001)

F) Stills from time-lapse movie (Supplementary Movie S3) in Tg[fli1a:GFP]^1 embryos mosaically overexpressing a pTol2-zN1aICD-basfli-mCherry construct showing formation of a transient perfused three-way connection between a wild-type secondary sprout and a NICD overexpressing (NICD^{OE}) primary ISV, indicating that Notch activation does not prevent interaction between primary ISVs and secondary sprouts.

Scale bars, 10µm
Figure S2

A) Quantification of mean blood flow speed (in µm/s) in the dorsal aorta of Tg[gata1a:dsRed]sd2 embryos at 50 hpf, after 1h incubation at 28.5°C in fish media containing 0.007% (0.5X), 0.014% (1X) or 0.028% (2x) tricaine. (n=16 0.5X tricaine treated embryos, n=18 1X tricaine treated embryos, n=17 2x tricaine treated embryos).

B) Quantification of average blood flow speed (in µm/s) in the dorsal aorta of Tg[gata1a:dsRed]sd,Tg[fli1α:GFP]y1 WT, MO-ccbe1 (5ng) and MO-dll4 (10ng) embryos at 50 hpf, in 0.014% tricaine. (n=19 WT, n=22 MO-ccbe1, n=7 MO-dll4). Only embryos exhibiting no secondary sprouts were used for quantification.

C) Representation of a kymograph displaying red blood cell movement in the dorsal aorta of Tg[gata1a:dsRed]sd2 embryos.

D) Quantification of heart rate (heartbeats/minute) in the dorsal aorta of Tg[gata1a:dsRed]sd2 embryos at 50 hpf, after 1h incubation at 28.5°C in fish media containing 0.007% (0.5X), 0.014% (1X) or 0.028% (2x) tricaine. (n=18 0.5X tricaine treated embryos, n=19 1X tricaine treated embryos, n=19 2x tricaine treated embryos).

E) Quantification of heart rate (heartbeats/minute) in the dorsal aorta of Tg[gata1a:dsRed]sd,Tg[fli1α:GFP]y1 WT, MO-ccbe1 (5ng) and MO-dll4 (10ng) embryos at 50 hpf, in 0.014% tricaine. (n=20 WT, n=22 MO-ccbe1, n=9 MO-dll4). Only embryos exhibiting no secondary sprouts were used for quantification.
Figure S3

A) Example of two different type of regression behaviour, imaged in Tg[fli1a:pecam1-EGFP]ncv27; Tg[-0.8flt1:RFP]hu5333 5ng MO-ccbe1 embryos: Full disconnection and thin membrane connection left.

B) Quantification of the nature of ISV regression behaviour in MO-ccbe1 embryos (N=4 experiments, 37 embryos).

C) Quantification of the nature of ISV regression behaviour in MO-ccbe1/MO-dll4 embryos (N=4 experiments, 62 morphants).
**A**

Dorsal

Golgi apparatus

Ventral

Nucleus

**B**

**MO-ccbe1**

ISVs never disconnecting from dorsal aorta

% EC

Time before average disconnection time of fully disconnecting ISVs in the same embryos

2h30 1h

**C**

**MO-ccbe1**

ISVs fully disconnecting from dorsal aorta

% EC

Time before disconnection from DA

2h30 1h

**D**

**Controls**

isSV

% EC

Time before connection to secondary sprout

2h30 1h

**E**

**Phase I**

**Phase II**

**Phase III**

% of ECs

Ventral polarity

Dorsal polarity

Unpolarized

**F**

Cell orientation

Polarity

control

DAPT

Direction of flow

Development: doi:10.1242/dev.181024: Supplementary information
Figure S4

A) Clarification of the method used to classify to analyse polarity of ECs during vascular remodelling in Figure 3 and Supplementary Figure 4. Time-lapse movies were made of transgenic Tg[fli1a:GFP]y1; Tg[fli1a:B4GalT1-mCherry]bns9 embryos during vascular remodelling in the trunk (~32 hpf to ~54 hpf). Polarity arrows from the centre of the nucleus to the centre of the Golgi apparatus were drawn manually. For every primary ISV cell the polarity was scored per time point: dorsal polarity, ventral polarity or unpolarized, depending on the relative position of Golgi and nucleus, i.e. respectively, Golgi dorsal, ventral or parallel to the nucleus in respect to the local angle of the ISV.

B) Quantification of EC polarity in ISV never disconnecting from the dorsal aorta in Tg[fli1a:Hsa.B4GALT1-mCherry]bns9;Tg[fli1a:EGFP]y1 embryos injected with 5ng MO-ccbe1 during presumptive phase I (measured at both 1h and 2h30 prior to the average disconnecting time of ISVs in the same embryos). (n=7 morphants, 12 ISVs, 33 ECs at both 1h and 2h30 before average disconnection time).

C) Quantification of EC polarity in ISVs fully disconnecting from the dorsal aorta in Tg[fli1a:Hsa.B4GALT1-mCherry]bns9;Tg[fli1a:EGFP]y1 embryos injected with 5ng MO-ccbe1 during presumptive Phase I (measured at both 1h and 2h30 before disconnection from the dorsal aorta). (n=7 morphants, 14 ISVs, 33 and 34 ECs at 1h and 2h30 before disconnection from the dorsal aorta, respectively).

D) Quantification of EC polarity in vISVs of Tg[fli1a:Hsa.B4GALT1-mCherry]bns9; Tg[fli1a: EGFP]y1 control embryos during phase I. (n=6 fish, 10 ISVs, 28 and 29 ECs at 1h and 2h30 before connection to the secondary sprout, respectively).

E) Quantification of EC polarity in aISVs and vISVs of Tg[fli1a:EGFP]y1;Tg[fli1a:B4GalT-mCherry]bns9 MO-dll4 and WT embryos at 3 different time points: I) 2.5 hours before secondary sprout connection, II) during three-way connection and III) 2.5 hours after resolution (n=7 WT aISV, 8 WT vISV, 10 MO-dll4 vISV).
F) Flow chamber experiment. A confluent layer of HUVEC cells was exposed to high shear stress and treated with 5μM DAPT or DMSO (control). Cells were stained for DAPI (nuclei), VE-cadherin (cell boundaries) and GM130 (Golgi). Cell orientation was analyzed by plotting the direction of the main axis of the nucleus. Cell polarity was determined by defining the angle between the center of the nucleus and the center of the Golgi. The graphs represent the percentage of cells with a certain angle of direction or polarity relative to the direction of flow (10° intervals), the red line indicates the mean.
<table>
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<th>Figure</th>
<th>test</th>
<th>n value</th>
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<th>P value</th>
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<tr>
<td>Figure 1B</td>
<td>one-way ANOVA + Tukey’s multiple comparisons test</td>
<td>SEM 74 embryos, 1184 ISVs (A&amp;A: 344; A&amp;V: 526; V&amp;V: 314)</td>
<td>3 experiments</td>
<td>ANOVA: P&lt;0.0001 (F=202) A&amp;A vs A&amp;V: P&lt;0.0001 A&amp;V vs V&amp;V: P&lt;0.0001</td>
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<td>Figure 1C</td>
<td>Unpaired t-test with Welch’s correction</td>
<td>SEM 74 embryos, 1480 ISVs (A: 760; V:720)</td>
<td>3 experiments</td>
<td>A vs V: P&lt;0.0001 (2-tailed; t=4.638; df=1475)</td>
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<tr>
<td>Figure 1G</td>
<td>one-way ANOVA + Tukey’s multiple comparisons test</td>
<td>SEM WT: 74 embryos NICD OE: 51 embryos</td>
<td>WT: 3 experiments NICD OE: 3 experiments</td>
<td>ANOVA: P&lt;0.0001 (F=493) A&amp;A vs NICD OE: P&lt;0.0001 A&amp;V vs NICD OE: P&lt;0.0001 V&amp;V vs NICD OE: P&lt;0.0001</td>
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<td>Figure 1H</td>
<td>one-way ANOVA + Tukey’s multiple comparisons test</td>
<td>SEM WT: 74 embryos NICD OE: 51 embryos</td>
<td>&lt;20%; n = 13 embryos 20-30%; n = 26 embryos &gt;30%; n = 12 embryos</td>
<td>ANOVA: P&lt;0.0001 (F=12.91) &lt;20% vs 20-30%: NS (P = 0.1672) &lt;20% vs &gt;30%: P&lt;0.0001 20-30% vs &gt;30%: P&lt;0.0009</td>
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<tr>
<td>Figure 1I</td>
<td>one-way ANOVA + Sidak’s multiple comparisons test</td>
<td>SEM WT: 74 embryos, 1184 ISVs (A&amp;A: 344; A&amp;V: 526; V&amp;V: 314) NICD OE: 40 embryos, 592 ISVs (A&amp;A: 188; A&amp;V: 229; V&amp;V: 174)</td>
<td>WT: 3 experiments NICD OE: 3 experiments</td>
<td>ANOVA: P&lt;0.0001 (F=74.39) A&amp;A WT vs A&amp;V NICD OE: P&lt;0.0001 A&amp;V WT vs A&amp;V NICD OE: P&lt;0.0001 V&amp;V WT vs V&amp;V NICD OE: P&lt;0.0001 A&amp;A NICD OE vs A&amp;V NICD OE: NS (P=0.9999) A&amp;V NICD OE vs V&amp;V NICD OE: NS (P=0.0510)</td>
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<td>Suppl Figure 1A</td>
<td>one-way ANOVA + Dunnett’s multiple comparisons test</td>
<td>SEM 74 embryos, 888 ISVs (A&amp;A: 27; A&amp;V: 203; A&amp;V: 450; 1A/3V: 193; 4V: 15)</td>
<td>3 experiments</td>
<td>UIC vs Su(H)VP16 OE: P&lt;0.0001 UIC vs N1bICD: P&lt;0.0001</td>
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<td>Suppl Figure 1B</td>
<td>Unpaired t-test with Welch’s correction</td>
<td>SEM 74 embryos, 1332 ISVs (V: 642; A: 690)</td>
<td>3 experiments</td>
<td>A vs V: P=0.0001 (2-tailed; t=14.91; df=1328)</td>
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<tr>
<td>Suppl Figure 1E</td>
<td>one-way ANOVA + Dunnett’s multiple comparisons test</td>
<td>SEM control: 74 embryos, 65 ISVs</td>
<td>control: 3 experiments</td>
<td>ctrl vs tricaine: P&gt;0.0001 (2-tailed; t=6.001; df=119.4)</td>
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<td>Figure 2A</td>
<td>Unpaired t-test with Welch’s correction</td>
<td>SEM control: 74 embryos, 1184 ISVs (A&amp;A: 344; A&amp;V: 526; V&amp;V: 314) tricaine (2x): 65 embryos</td>
<td>control: 3 experiments</td>
<td>tricaine: 3 experiments</td>
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<tr>
<td>Figure 2B</td>
<td>one-way ANOVA + Sidak’s multiple comparisons test</td>
<td>SEM NICD OE untreated (-): 51 embryos NICD OE + tricaine (+): 20 embryos</td>
<td>control: 3 experiments</td>
<td>NICD OE untreated: 3 experiments NICD OE + tricaine: 2 experiments</td>
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<tr>
<td>Figure 2C</td>
<td>Unpaired t-test with Welch’s correction</td>
<td>SEM NICD OE untreated: 51 embryos</td>
<td>NICD OE untreated: 3 experiments</td>
<td>NICD OE (-) vs NICD OE (+): NS (P=0.8859) (2-tailed; t=0.1826; df=48.15)</td>
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<td>Figure 3E</td>
<td>Chi-square</td>
<td>N/A 24 ISVs, 12 aISVs, 40 eISVs</td>
<td>6 experiments</td>
<td>phase I A vs V: P=0.0075 (X²=9.782; df=2) phase II A vs V: P&lt;0.0001 (X²=152.7; df=2) phase III A vs V: P=0.0003 (X²=74.22; df=2)</td>
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<td>Figure 3F</td>
<td>Unpaired t-test with Welch’s correction</td>
<td>SEM aISV: 12 ISVs, 67 cells (I: 48; II: 54; III: 67) eISV: 13 ISVs, 103 cells (I: 49; II: 54; III: 93)</td>
<td>5 experiments</td>
<td>phase I A vs V: P=0.0302 (t=2.177; df=94.94) phase II A vs V: P=0.0169 (t=2.429; df=101.5) phase III A vs V: P=0.0213 (t=2.328; df=147.4)</td>
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<td>Figure 3H</td>
<td>Chi-square</td>
<td>N/A 34 embryos, 12 aISVs, 40 eISVs</td>
<td>5 experiments</td>
<td>A vs V: P&lt;0.0001 (X²=23.92; df=2)</td>
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<td>Figure 3J</td>
<td>SEM 37 morphants, 241 morphant vessels</td>
<td>4 experiments</td>
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<tr>
<td>Figure 3K</td>
<td>SEM 37 morphants, 29 WT controls</td>
<td>4 experiments</td>
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<tr>
<td>Figure 3M</td>
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<tr>
<td>Figure 3N</td>
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<tr>
<td>Figure 4C</td>
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<td>4 experiments</td>
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<td>Suppl Figure 2A</td>
<td>Brown-Forsythe and Welch Anova test ANOVA</td>
<td>SD 16 embryos (0.5X treatment), 18 embryos (1X treatment), 17 embryos (2X treatment)</td>
<td>2 experiments</td>
<td>0.5X vs 1X (P=0.0003), 0.5X vs 2X (P&lt;0.0001)</td>
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<td>Suppl Figure 2B</td>
<td>Brown-Forsythe and Welch Anova test ANOVA</td>
<td>SD 19 WT controls, 22 ccbe1 morphants, 7 d1a/ccbe1 morphants</td>
<td>2 experiments</td>
<td>WT vs MO-ccbe1 (P=0.9949), WT vs MO-ccbe1/MO-d1a (P=0.5496)</td>
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<tr>
<td>Suppl Figure 2D</td>
<td>One way ANOVA with Kruskall-Wallis test</td>
<td>SD</td>
<td>18 embryos (0.5X treatment), 19 embryos (1X treatment), 19 embryos (2x treatment)</td>
<td>2 experiments</td>
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<tr>
<td>Suppl Figure 2E</td>
<td>One way ANOVA with Kruskall Wallis test</td>
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<td>2 experiments</td>
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<tr>
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<td>SEM</td>
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<td>7 experiments</td>
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<tr>
<td>Suppl Figure 4B</td>
<td>N/A</td>
<td>N/A</td>
<td>7 ccbe1 morphants, 12 ISVs, 33 ECs (2h30), 33 ECs (1h)</td>
<td>3 experiments</td>
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<td>N/A</td>
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<td>3 experiments</td>
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<td>Suppl Figure 4D</td>
<td>N/A</td>
<td>N/A</td>
<td>6 WT controls, 12 ISVs, 29 ECs (2h30), 28 ECs (1h)</td>
<td>3 experiments</td>
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<td>Suppl Figure 4E</td>
<td>Chi-square</td>
<td>N/A</td>
<td>7 WT aISV (16 cells), 8 WT vISV (17 cells), 10 Dll4 KD vISV (31 cells)</td>
<td>Dll4 KD: 2 experiments</td>
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<td>Suppl Figure 4F</td>
<td>Kuiper two-sample test</td>
<td>N/A</td>
<td>5 experiments (min 1500 cells/exp)</td>
<td>5 experiments</td>
</tr>
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| Figure 5A | One-way ANOVA + Tukey's multiple comparisons test | SEM | WT: 12 aISV (I: 48; II: 54; III: 67), 13 vISV (I: 49; II: 54; III: 93) N
dICD OE: 30 aISV, 29 cells (I: 29; II: 9; III: 13) Dll4 KD: 9 vISV, 85 cells (I: 53; II: 85; III: 45) | WT: 5 experiments | ANOVA phase I: P=0.0071 (F=4.154) ANOVA phase II: P=0.0001 (F=7.221) ANOVA phase III: P=0.0003 (F=6.623) A I vs N
ICD OE I: NS (P=0.9923) A II vs NICD OE II: NS (P=0.8909) A III vs N
ICD OE III: NS (P=0.9447) V I vs Dll4 KD I: NS (P=0.0956) V II vs Dll4 KD II: NS (P=0.9999) V III vs Dll4 KD III: NS (P=0.9999) |
| Figure 5D | 2way ANOVA + Tukey's multiple comparisons test | SEM | 20 embryos | 5 experiments | tp1 pos ctrl vs tp1 pos tric2x: P=0.0269 tp1 neg ctrl vs tp1 neg tric2x: P=0.0099 |
| Figure 5E | Paired t-test | SEM | control: 20 embryos; tricaine 2x: 12 embryos control: 5 experiments tricaine 2x: 3 experiments | control vs tric2x: NS (P=0.9999) |
| Figure 5F | 2way ANOVA + Tukey's multiple comparisons test | SEM | control: 20 embryos; tricaine 2x: 12 embryos control: 5 experiments tricaine 2x: 3 experiments | tp1 pos ctrl vs tp1 pos tric2x: NS (P=0.9923) tp1 neg ctrl vs tp1 neg tric2x: P=0.0269 |
| Figure 5G | One-way ANOVA + Sidak's multiple comparisons test | SEM | WT: 19 embryos, 199 ISVs (0&0: 78; 0&1: 83; 1&1: 38) | 2 experiments | ANOVA: P=0.0001 (F=12.19) 0&0 vs 0&1: P=0.0035 0&1 vs 1&1: P=0.0001 0&1 vs 1&1: NS (P=0.1065) |
Supplementary Movies

**Movie 1** Time-lapse imaging of a Tg[fltl:EGFP]$^{y1}$;Tg[-0.8flt1:RFP]$^{hu5333}$ embryo (all ECs labeled in green, arterial ECs labeled in red) showing ISV remodelling into a venous (left) and an arterial (right) intersegmental vessel from 26 to 55 hpf (frame interval: 15 minutes). In both cases, a lumenized connection is formed between the secondary sprout and the primary ISV. In the case of the formation of an aISV, the connection is lost again and the secondary sprouts forms lymphatic precursors at the horizontal myoseptum (Parachordal lymphangioblasts). In case of vISV remodelling, the secondary sprout connection is stabilized and the connection between primary ISV and DA regresses. Scale bars, 50μm
Movie 2 Time-lapse recording of a Tg[fli1a:EGFP]y1;Tg[gata1a:DsRed]sd2 embryo (with ECs labeled in green and erythrocytes in red) between 35 and 40 hpf (frame interval: 2 minutes) showing a transient perfused three-way connection in which the ISV-PCV connection dissociates again to form an arterial ISV and a lymphangiogenic sprout. Scale bars, 10μm

Movie 3 Time-lapse recording of a Tg[fli1a:EGFP]y1 embryo (labeling ECs in green) mosaically overexpressing a pT2-zN1aICD-basfli-mCherry construct (orange-red) between 31 and 43hpf (frame interval: 6 minutes) showing formation of a transient perfused three-way connection between a wild-type secondary sprout and a NICD^{OE} primary ISV. Scale bar, 15μm
Movie 4 Time-lapse imaging of a Tg[fli1a:EGFP];Tg[fli1a:B4GalT-mCherry] embryo (ECs depicted in grey, endothelial Golgi apparatus in red) showing EC polarity in an arterial ISV from 32 to 51 hpf. Arrows (green in primary ISV, blue in secondary sprout) point from the centre of the nucleus to the centre of the Golgi complex (frame interval: 15 minutes). Scale bars, 25μm
Movie 5 Time-lapse imaging of a Tg[fli1a:EGFP]y1;Tg[fli1a:B4GalT-mCherry]bns9 embryo (ECs depicted in grey, endothelial Golgi apparatus in red) showing EC polarity in a venous ISV from 33 to 55 hpf. Arrows (green in primary ISV, blue in secondary sprout) point from the centre of the nucleus to the centre of the Golgi complex (frame interval: 15 minutes). Scale bars, 25μm
Movie 6 Time-lapse imaging of Tg[fli1a:pecam1-EGFP]$^{ncv27}$;Tg[-0.8flt1:RFP]$^{hy5333}$ embryo (junctions in green, arterial structures in red) from 29 to 53 hpf showing ISV remodelling into arterial and intersegmental vessels (aISV and vISV) (frame interval: 15 minutes). Scale bars, 50μm

Movie 7 Time-lapse imaging of a Tg[fli1a:pecam1-EGFP]$^{ncv27}$;Tg[-0.8flt1:RFP]$^{hy5333}$ MO-ccbe1 embryo (junctions in green, arterial structures in red) from 30 to 54h30 hpf showing ISV regression in the absence of secondary sprouting (frame interval: 15 minutes). Scale bars, 50μm
Movie 8 Time-lapse imaging of a Tg[fli1a:EGFP]v1;Tg[-0.8flt1:RFP]hu5333 MO-ccbe1/MO-dll4 embryo (all ECs labeled in green, arterial ECs labeled in red) from 30 to 54h30 hpf showing ISV regression in the absence of secondary sprouting (frame interval: 15 minutes). Scale bars, 50μm
Movie 9 Time-lapse imaging of the Notch activity reporter *Tg[tp1-MmHbb:kaede]um15*;*Tg[kdr-l:ras-Cherry]32916* imaged from 30 hpf (right after conversion of the Kaede\textsuperscript{green} protein into Kaede\textsuperscript{red}) to 50hpf. The Notch activity reporter is shown in kaede (red: old signal after conversion/green: new signal), all ECs are labeled in red) (frame interval: 45 minutes). Scale bars, 50μm
Movie 10 Time-lapse imaging of the Notch activity reporter \( Tg[tp1-MmHbb:kaede]^{um15};Tg[kdr-l:ras-Cherry]^{916} \) in a future arterial ISV, imaged from 29 hpf (right after conversion of the Kaede\textsubscript{green} protein into Kaede\textsubscript{red}) to 48.5 hpf. The Notch activity reporter is shown in kaede (red: old signal after conversion/green: new signal), all ECs are labeled in red). The tip of the growing secondary sprout is indicated by arrows. The \( tp1 \) signal (Notch activity) builds up before connection of the secondary sprout. (frame interval: 30 minutes). Scale bars, 25\( \mu \)m