Chapter 2
Vascular Development in the Zebrafish

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Abbreviations

DA	Dorsal aorta
DLAV	Dorsal longitudinal anastomotic vessel
HLT	Hypotrichosis-lymphedema-telangiectasia
HMG	High-mobility group
HPF	Hours post-fertilization
ISV	Intersegmental vessels
LDA	Lateral dorsal aorta
LPM	Lateral plate mesoderm
MO	Morpholino
NICD	Notch intracellular domain
PCV	Posterior cardinal vein
PHBC	Primordial hindbrain channel
TAD	Transactivation domain

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2.1 Zebrafish Vasculogenesis and Arteriovenous Specification

The first organ system that becomes functional in most vertebrates is the cardiovascular system. Given the transparency of the early zebrafish embryo, the development of this particular organ system has been traditionally a subject of interest. Large offspring numbers and external development of zebrafish embryos makes them very well suited for developmental research, aided by the fact that circulation within the zebrafish embryo commences very quickly; the first signs of heart beat can be appreciated at approximately 22 h post-fertilization (hpf), while the first erythrocytes move through the embryonic body only a few hours later [89]. The appeal of the zebrafish embryo for *in vivo* observation [43], the availability of transgenic lines that mark precursor cells of the vascular endothelium and that allow distinguishing between arterial and venous cells within the same embryo, the possibility of interfering with this process experimentally, and the generation of mutants that affect the process [73] have resulted in significant insight into various aspects of vascular development [84]. In this chapter, we will mainly focus on vasculogenesis and the early events of arteriovenous specification. The ensuing processes of arterial and venous differentiation, and the behavior of endothelial cells that contribute to later aspects of angiogenesis and lymphangiogenesis have recently been reviewed [65, 77] (see also Chap. 5) and will only be touched on here where necessary.

It was previously thought that the differentiation between arteries and veins is mainly established by the difference in hemodynamic forces such as blood pressure [22]; however, in recent years more and more evidence has emerged that molecular differences between arterial and venous precursor cells regulate the formation of the arteriovenous system irrespective of hemodynamic forces [81]. Specifically, recent studies have shown that molecular differences between arterial and venous cells have already been established in the early stages when endothelial precursors begin to arise [88].

Formation of the vascular system starts with vasculogenesis, which is defined as the *de novo* formation of vessels from individual mesenchymal cells. In the zebrafish trunk, this process results in the formation of the dorsal aorta (DA) and the posterior cardinal vein (PCV). How do these vessels arise during early development? It has been apparent for some time that there is a set of bilaterally aligned cells in the posterior lateral plate mesoderm (LPM) which constitute a precursor pool for these axial vessels. At this point in time, these cells stain positive for a variety of endothelial markers, and are commonly referred to as angioblasts, a term used by Sabin as early as 1917 when studying equivalent processes in the chicken embryo. Angioblasts migrate over the endodermal layer to the embryonic midline, where they form a vascular cord [60, 61, 63]. The migration of these angioblasts and their importance for DA and PCV formation is undisputed, but where these cells are initially localized in the LPM and whether they are actually already specified at the onset of migration is far less clear. In addition, the exact events upon reaching the embryonic midline still need to be resolved. Both issues will be discussed herein.

Independent reports showed two distinct waves of angioblast migration during vascular development in the zebrafish trunk. The first wave of migration starts at approximately 14 hpf, and the second wave starts at approximately 16 hpf. This has been confirmed by a number of groups, most recently by Kohli et al. [17, 34, 39]. Before the actual onset of migration, and as early as the 4-somite stage (12 hpf), a subset of medial angioblasts can be found to express the marker *etv2/etsrp*, a transcription factor that has key roles during vasculogenesis. Three hours later, a second and more laterally positioned line of *etv2/etsrp*-expressing cells becomes apparent [58]. The heterochrony of *etv2/etsrp* expression among these two angioblast populations is further substantiated when looking at other marker genes; *kdrl* (formerly known as *flk1* [6]) highlights medial angioblasts at the 10-somite stage but gets expressed in lateral angioblasts only a few hours later. Similarly, the widely used pan-endothelial marker *fli1a* [76] is first expressed in medial angioblasts, before then becoming expressed in the lateral angioblast population [39]. The arterial marker *gridlock/hey2* can only be found to be expressed in the medial population, not in the lateral population [87], suggesting that the medial angioblast population constitutes a pool of arterial precursor cells, while the lateral population contributes to the PCV.

This notion was further confirmed by *in vivo* observations that tracked individual cells and addressed the question as to whether angioblasts, irrespective of their position within the LPM, can contribute to both DA or PCV, or whether early positioning within either the medial or lateral angioblast population is largely, or even entirely, predictive for cells to become part of the DA or PCV. The zebrafish embryo is very suitable for this type of analysis as one can mark individual cells *in vivo* and follow the fate of labeled cells over time. Of course, *in vivo* imaging tracks cell movements, while in situ hybridizations (such as the ones discussed above) provide static representations of gene expression, which makes the direct comparison between these different modes of acquiring data difficult. That notwithstanding, lineage tracing has provided a number of important insights. When labeling a single cell at the margin of a gastrula stage embryo (6 hpf), Vogeli et al. [80] reported that a few of these cells contribute exclusively to the endothelial and the hematopoietic lineage, providing direct demonstration of the existence of hemangioblasts. However, only part of the hematopoietic and the endothelial lineage arise from these bi-potential cells, indicating that there are other cellular sources for the vasculature [80]. Another study labeled cells of the LPM at the 7- to 12-somite stage, and only observed contribution to either the arterial or venous lineage (consistent with *gridlock/hey2* expression) [87]. Hence, based on both marker expression studies and *in vivo* tracing experiments, it appears that there are lineage-restricted angioblasts in the zebrafish LPM during the early stages of somitogenesis that have been specified to become either arteries or veins (Fig. 2.1a).

How about the movement of these angioblasts from the posterior LPM to the midline? As mentioned above, it has been reported a number of times that first the medial, then the lateral, angioblast population migrates to the midline. Both migration waves occur in an anterior to posterior manner. The use of a transgenic line that expresses the photo-convertible fluorophore *kaede* from the *etv2* promoter has enabled elegant studies which demonstrated that the medial angioblasts give rise to

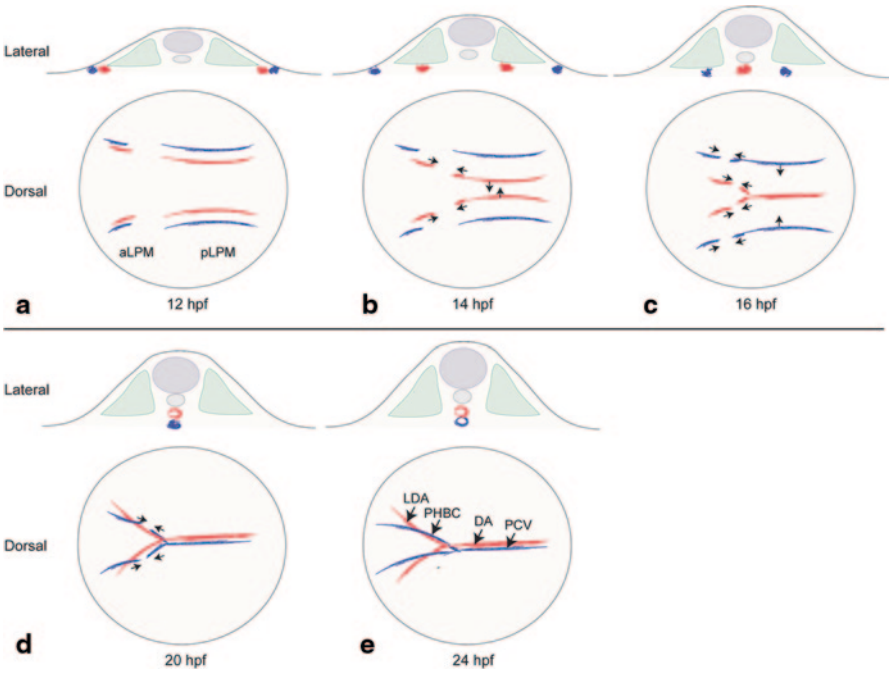

Fig. 2.1 Arterial and venous precursor cell migration. **a** At 12 hpf, angioblasts are located within the anterior and posterior lateral plate mesoderm (*LPM*) in two bilateral stripes. Presumably, even at this early stage, the medial angioblast population consists of arterial precursor cells (*red*), while the lateral population consists of venous precursor cells (*blue*). **b** At approximately 14 hpf, the precursor cells located at more medial positions within the posterior LPM start to migrate to the midline. At the same time, some arterial cells of the posterior LPM start to migrate anteriorly, whereas arterial cells from the anterior LPM start to migrate posteriorly. **c** At 16 hpf, arterial precursor cells will form the first axial vessel, the dorsal aorta. Cells within the more lateral located stripes of the posterior LPM start to migrate to the midline. In addition, some of these cells will migrate anteriorly, whereas in the anterior LPM some venous progenitor cells start to migrate posteriorly. **d** At 20 hpf, the venous precursor cells have migrated to the midline and form the posterior cardinal vein (PCV). The migration of arterial cells in the anterior region results in formation of the lateral dorsal aortae. **e** At 24 hpf, venous precursor cells in the anterior region have migrated to form the Primordial hindbrain channel (*PHBC*). *Green* indicates somites, *purple* indicates notochord and hypochord, *yellow* indicates embryonic tissue

the DA, and that lateral angioblasts constitute the PCV [39] (Fig. 2.1). Furthermore, these studies support a model where angioblasts move directly to a more dorsal location and form the DA, while lateral angioblasts migrate directly to a more ventral position and form the PCV. This is in contrast to a study by Herbert et al. [26], who suggested a ventral sprouting mechanism, during which angioblasts from the DA contribute directly to the PCV [26]. Whether this discrepancy is possible due to imaging in different regions of the embryo needs to be resolved.

As a consequence of early specification events and a highly orchestrated (both in time and space) array of cell movements, two cords of angioblasts/endothelial cells align along the embryonic axis. These cells establish cell–cell junctional complexes

among each other, express markers of apical and basal polarity, and eventually form a luminized DA and PCV, even before the onset of circulation (reviewed by Schuermann et al. [65]).

2.2 Molecular Cues During Vasculogenesis

There is a plethora of genes and factors that have been connected to the genetic control of vasculogenesis and angiogenesis, but only a few for which zebrafish mutants are available and for which we fully understand the mechanistic implications. One of the first cardiovascular mutants described presents with a near-complete failure to specify blood and endothelial lineages, and these *cloche* mutants have been very instructive to understand many aspects of early vascular development [72]. It has been suggested that mutations in the *lycat* gene, encoding an acyl transferase, are causative for the phenotype [86]. The *cloche* mutant phenotype can be rescued via forced expression of the ETS1-related protein, placing this key transcription factor downstream of cloche [74]. As mentioned above, the ETS-domain transcription factor Etv2/Etsrp/ER71 is one of the earliest markers specifically expressed in angioblasts, and Etsrp is required for the expression of *vegfr2/kdrl* in early development. In *etsrp* zebrafish morphants, angioblasts are unable to differentiate, migrate, or form functional axial vessels. Overexpression of *etsrp* causes the induction of vascular endothelial markers in several cell types. Etsrp is thus a key regulator in the induction of vascular endothelial fate in early development [58, 74].

Overexpression studies showed that vascular endothelial growth factor A (*vegf-a*) and Sonic Hedgehog (Shh) are involved in the localization of the medial and lateral angioblasts to the midline, with high levels of *vegf-a* or *shh* resulting in a random distribution of medial and lateral angioblasts at the midline [39]. Similarly, *vegf-a* morphants showed a single vessel consisting of lateral and medial angioblasts [39], suggesting that Vegf and Shh are critical factors for arteriovenous specification at the time point of angioblast positioning at the midline. In zebrafish, *shh* is expressed in the notochord and floorplate [16], whereas *vegf-a* is expressed in the somites [49]. It is hypothesized that the medially located angioblasts receive a higher concentration of Shh and Vegf signaling than the angioblasts localized at the lateral positions [39]. This difference in concentration could induce the distinct pathways for arterial or venous specification. This model is intriguingly simple but important questions remain. Are these morphogens (Shh, Vegf) sufficient to both serve as chemoattractants for cell migration and for specifying arterial and venous cell fates? Or is there a stochastic initiation of some angioblasts in the LPM to migrate medially in a first wave, and these ‘front runners’ inhibit arterial fates in the trailing cells of the second wave? A thorough fate mapping of migration events might clarify some of these issues; if all angioblasts are equally naïve before the onset of migration, then fate mapping should reveal that the cells located most medially in the LPM (and which should therefore perceive the highest levels of Vegf and Shh) should invariably end up in the aorta. However, should more medially located cells be overtaken by more distally located cells, this would argue that the cells might not be naïve.

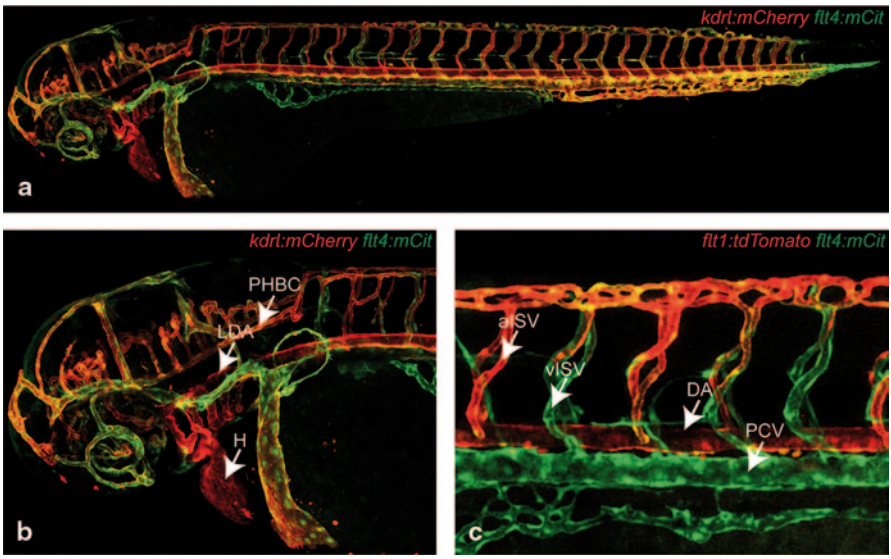

Fig. 2.2 Transgenic zebrafish vasculature. **a** Overview of *kdrl:mCherry;flt4:mCit* zebrafish embryo at 2 days post-fertilization (dpf), with arteries depicted in *red* and veins depicted in *yellow*. **b** Enlarged head region of (a), including the heart (*H*), lateral dorsal aorta (*LDA*), and the primordial hindbrain channel (PHBC). **c** Trunk region of *flt1:tdTomato;flt4:mCit* embryo at 3 dpf, with arteries depicted in *red* and veins depicted in *green*, including the arterial intersegmental vessel (*aISV*), venous intersegmental vessel (*vISV*), dorsal aorta (*DA*), and posterior cardinal vein (*PCV*)

While the axial vasculature is defective in *vegf*-deficient embryos and *shh* mutants, the anterior vasculature is unaffected, suggesting a different mechanism of vessel formation in the anterior LPM of the zebrafish. Time-lapse analysis showed that the anterior part of the DA, the lateral DA (LDA) develops in a different manner. At 14 hpf, a subset of angioblasts localized at the anterior LPM migrate posteriorly, while a subset of angioblasts from the posterior LPM start to migrate anteriorly. These cell populations migrate towards each other, eventually connecting and forming the LDA. This process of angioblast migration is suggested to be mediated by the chemokine Cxcr4 [70]. The expression of *cxcr4* is restricted to the angioblasts that will form the anterior-most LDA. Knockdown of *cxcr4* resulted in inappropriate fusion of anterior and posterior angioblasts, and consequently formed a disrupted LDA [70]. At approximately 17 hpf, the same process of migration happens for venous angioblasts that form the largest anterior vein in the head, the primordial hindbrain channel (PHBC) [7, 70] (Fig. 2.1 and 2.2a, b).

After the initiation of vasculogenesis, angiogenesis will further remodel the vascular network. Angiogenesis is the formation of blood vessels from pre-existing blood vessels by sprouting and remodeling of endothelial cells. In the zebrafish trunk, endothelial cells start to sprout dorsally from the DA at the somite boundaries and form the intersegmental vessels (ISVs). This primary sprouting results in ISVs

that connect at the dorsal site to form the dorsal longitudinal anastomotic vessel (DLAV). After establishing this arterial network, secondary sprouting will occur in which endothelial cells sprout dorsally from the PCV [32]. These sprouts can connect to the ISVs and remodel the arterial ISV into a venous ISV. Statistically, only every second sprout becomes a venous ISV. The other endothelial sprouts will migrate further to the horizontal myoseptum and constitute a population of parachordal lymphangioblasts, which then migrate either dorsally or ventrally and start to form the lymphatic vasculature [29] (reviewed by van Impel and Schulte-Merker [77, 78]) (Fig. 2.2c).

One of the prevalent questions concerning the above angiogenic processes is how venous sprouts make the decision on whether to connect to an intersegmental artery and to remodel it into a vein in the process, or whether not to do this and contribute to the pool of lymphatic precursor cells. One might presume that this process might involve Notch and Delta but, until now, no members of the Notch/Delta signaling pathway have been detected to be expressed in the PCV or in venous sprouts.

2.3 Molecular Regulation of Arterial-Venous Specification

2.3.1 EphrinB2/Eph Receptor B4

Two of the most widely referred to markers for arteriovenous specification are Ephrin-B2 (Efnb2) and Eph receptor B4 (EphB4), based on the finding that Efnb2 and the EphB4 receptor are differentially expressed in arteries versus veins, respectively [81]. The EphB4 receptor belongs to the receptor tyrosine kinase family and is the only Ephrin receptor that specifically binds to Efnb2, a membrane-bound ligand of the Ephrin ligand family [20]. The Ephrin ligands and Eph receptors are both transmembrane proteins, and signaling requires cell-to-cell contact, which can be bidirectional [5]. Eph receptors and their ligands are often, but not always, localized in the adjacent cell population [1, 19]. ‘Forward’ signaling starts with the binding of an Ephrin ligand to a receptor dimer. This leads to trans-phosphorylation of the intracellular domain of the receptor, and results in a conformational change that can activate the kinase domain. ‘Reverse’ signaling occurs when the conserved tyrosine residues of the cytoplasmic domain of the Ephrin ligand are phosphorylated upon contact with the Eph receptor ectodomain, or by an Eph receptor-independent mechanism. This causes the recruitment of an SH2 (Src-homology-2) domain-containing adaptor protein and SH3 binding partners (reviewed by Kullander and Klein [41]). The exact contribution of forward and/or reverse signaling in arteriovenous specification is still unclear.

In zebrafish, *efnb2* expression is restricted to the arterial endothelial cells (ECs). Expression of *efnb2* is initiated at approximately the 20-somite stage, when the

angioblasts have migrated to the midline but circulation has not yet commenced. *ephb4* receptor messenger RNA (mRNA) is also expressed in the vasculature but the expression is restricted to the venous ECs [87]. These clear expression patterns in arterial versus venous ECs make *EphB4* and *Efnb2* suitable markers for arterial and venous differentiation and, accordingly, their expression changes upon altered arteriovenous specification. For example, inhibition of Notch signaling results in a decrease of arterial fates, which can be appreciated by reduced expression of *efnb2* in the DA [44]. Herbert et al. suggested that Efnb2a limits the ventral migration of arterial angioblasts, whereas Ephb4a promotes it, based on results obtained upon transplanting *efnb2a or ephB4a* morpholino (MO) donor cells into wild-type host embryos. In hosts that received *efnb2a* MO donor cells, the donor cells ectopically localized to the vein. In host embryos that contain *ephb4* MO donor cells, fewer cells contribute to the vein compared with their controls, again suggesting a role for Efnb2 and EphB4 in arteriovenous specification [26]. This is largely consistent with other systems, and mice mutants for *Efnb2* have been shown to present the same phenotype as mutants for *EphB4*, characterized by defective morphogenesis of the vasculature. Mutant vasculature suffers from a lack of distinct boundaries between the arteries and the veins, again stressing the importance of Efnb2 and EphB4 in arteriovenous specification [20].

2.3.2 Vascular Endothelial Growth Factor and Sonic Hedgehog

In zebrafish, as in all other non-eutherian vertebrates, four VEGF receptors are present, namely *vegfr1 (flt1), vegfr2 (flk1/kdr), vegfr3 (flt4), and vegfr4 (kdrl)* [6, 14, 75]. The expression pattern of these receptors has been examined in detail [6, 50, 68], and transgenic reporter lines exist for most of them. Neuropilins, non-tyrosine kinase transmembrane molecules, have been shown to be needed for VEGF signaling in other systems, but in zebrafish, morpholino-based data [47, 51] are not entirely consistent with recently provided mutant data [40], and generating mutant lines for the duplicated *nrp1a/b and nrp2a/b* genes is required to shed light on a requirement for these co-receptors.

Morpholino-mediated knockdown of *vegf-Aa* was reported to result in deficiency of ISV sprouting, with no major deficiencies in the DA or PCV [53]. However, analyzing the dependence of vasculogenesis on Vegf-A is confounded by the duplication of zebrafish *vegf-A* genes [3], and by maternal expression of the respective mRNAs. Stable mutant lines or maternal zygotic mutants have not been generated, precluding a final assessment on the role of Vegf-A during the early stages of vasculogenesis; current evidence based on double knockdowns suggests diverged functions of Vegf-Aa and Vegf-Ab, but no major effects on vasculogenesis [3]. Moreoever, mutants for *vegfr4/kdrl* [23] and *vegfr3/flt4* [30] have been reported and clearly demonstrate that zygotic expression of these receptors is not essential for vasculogenesis to occur; mutants in either gene have an apparently normal DA and PCV. These genes have distinct functions at the later stages of vascular

development, during arterial and venous ISV sprouting, but their role during earlier stages of vasculogenesis remains somewhat enigmatic; more than one VEGF receptor might be required to be mutated in order for a phenotype to be appreciated.

A key regulator in the early steps of angioblast migration, and in later events of arterial and venous specification, has been suggested in Shh, a member of the Hedgehog family which can act as a ligand for the transmembrane receptors Patched and Smoothened [45]. *Smoothened* mutants, which are devoid of Shh signaling, show comparatively normal angioblast migration [85], and the receptors for Shh, the duplicated *patched* genes, appear not to be expressed within the posterior LPM [48]. Shh regulates expression of *semaphorin 3a1*, which has been shown to have an effect on angioblast migration [69]. In addition, Shh signaling from the midline is essential for normal *vegf-A* expression in the medial aspects of the somites [45]. While more work needs to be carried out to clearly carve out the role for Shh and Vegf-A during the very first events of vasculogenesis, the requirement for both genes in the later steps of arteriovenous specification is better understood. *Shh* mutants show strongly reduced arterial marker gene expression, but this phenotype can be partially overcome by forced expression of *vegf-A* mRNA. Furthermore, overexpression of *shh* results in ectopic expression of arterial markers in venous ECs, again suggesting a specific role of Shh in arterial specification [21]. Interestingly, Shh has been demonstrated to positively influence the expression of *calcitonin receptor-like receptor*-a (*calcrla*) [54], which ultimately results in *vegf-A* expression upstream of Notch. This is particularly significant in light of a recent finding by Wilkinson et al. [85], where Hedgehog signaling was found to induce somitic *vegf-A* expression independent of Calcrla, while Hedgehog can also signal through Calcrla to induce arterial differentiation in angioblasts independent of Vegf-A function. Hence, at least during the later stages, there is room for both signaling pathways in parallel, which in turn might help to guide our thinking about a possibly redundant function for Shh and Vegf-A during the first steps of vasculogenesis. Indeed, it has been suggested that neither pathway is absolutely required for angioblast migration, and that one pathway can compensate for (partial) loss of the other. However, the requirement at the later stages is supported by a number of observations, and a picture has emerged where VEGF signaling in presumptive arteries induces Plc-γ1. Zebrafish mutants for *plc-γ1* show a marked defect in the formation of arteries and strongly reduced expression of *efnb2* [46]. *plc-γ1* mutants cannot be rescued with *vegf-A* overexpression, suggesting Plc-γ1 to act downstream of VEGF receptor function in arterial signaling [44, 46].

2.3.3 Notch and Hey2

The zebrafish Notch family members consist of four Notch receptors (Notch1a, 1b, 2, and 3) and several Notch ligands (DeltaA-D, Dll4, Jagged1a, 1b, and 2) in

zebrafish, which are all membrane-bound proteins. Upon binding of these ligands to the Notch transmembrane receptor, a series of proteolytic cleavages release the Notch intercellular domain (NICD) of the receptor into the cytoplasm, after which it translocates to the nucleus. The NICD can then bind to Suppressor of Hairless [Su(H)], which in turn can cause activation of several transcription factors, such as the basic helix-loop-helix (bHLH) proteins, Hairy/Enhancer of Split (Hes) and Hes-related proteins (Hey/HRT/HERP). The promoter regions of *HRT* genes have a binding site for Su(H) [36, 52]. The Notch signaling pathway has long been recognized as a key driver of arterial identity, and in recent years has been extensively evaluated in this respect, and also in its involvement during tip cell/stalk cell formation [59]. The latter aspect has been reviewed in detail elsewhere [84], therefore we will focus only on the arteriovenous specification role of Notch–Delta signaling.

The Notch–Delta signaling pathway appears to be restricted to the arterial endothelium in zebrafish [79], and both loss-of-function as well as gain-of-function studies of Notch family members revealed a disrupted vasculature, with loss of Notch signaling, such as in *mindbomb* mutant embryos, resulting in decreased arterial marker expression and arterial-venous shunts [44]. Similarly, mutants in *hey2/grl*, a factor required downstream of Notch signaling, display altered arterial gene expression and develop a distinct shunt phenotype at the level of the cranial vasculature [87]. Gain-of-function of Notch family members causes a reduction of venous fate [44]. There is a tight link between Vegf signaling function and Notch–Delta activity; Vegf-A can induce the expression of *notch*, and Notch can rescue the arterial specification defect in *vegf-A* knockdown studies, suggesting that Notch acts downstream of Vegf in arterial specification [44, 79]

One of the Notch target proteins is the hairy/enhancer-of-split-related bHLH family member Hey2. The zebrafish ortholog of the mammalian *Hey2* gene is *gridlock*. Gridlock functions as a transcriptional repressor, and is already expressed in early development, in the angioblasts that are localized within the medial aspect of the LPM, whereafter *gridlock* expression continues to be restricted to arteries [87]. The loss of Gridlock function in early zebrafish development results in defective proliferation of angioblasts at the level of the LPM [10]. Later on, loss of *gridlock* results in a circulatory shortcut through a disrupted DA, with concomitant increase of the venous maker EphB4 and a decrease in the arterial marker *efnb2* [87]. More specifically, the point of fusion of the LDA to the DA is affected, which represents a remarkably specific and locally restricted phenotype [83, 88] which has recently been shown to be mimicked by the *sox7* and *efnb2a/b* mutants [27]. Furthermore, overexpression of *gridlock* causes suppression of venous markers [88]. Both *in vitro* and *in vivo* experiments showed that induction of Notch–ICD induces *gridlock* expression, again suggesting that Gridlock is acting downstream of Notch [52, 88]. Furthermore, inhibiting Hh or VEGF signaling results in loss of *gridlock* expression in the angioblasts that will form the DA, while stimulating *vegf* expression in *gridlock* morphants rescues

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