# Endothelial cell specification -

# Crossroads in vascular development

Dorien Hermkens

The research described in this thesis was performed at the Hubrecht Institute of the Royal Netherlands of Arts and Sciences (KNAW), within the framework of the graduate school of Cancer, Stem cells & Developmental biology (CS&D) in Utrecht. The research was performed in close collaboration with the department of Experimental Cardiology of the Erasmus University Medical Centre, within the framework of the Cardiovascular Research School Erasmus MC Rotterdam (COEUR), The Netherlands.

Cover: View of vein interior in fractal form

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# Endothelial cell specification -

# Crossroads in vascular development

Endotheelcel specificatie -

Knooppunten in de vasculaire ontwikkeling

(met een samenvatting in het Nederlands)

## Proefschrift

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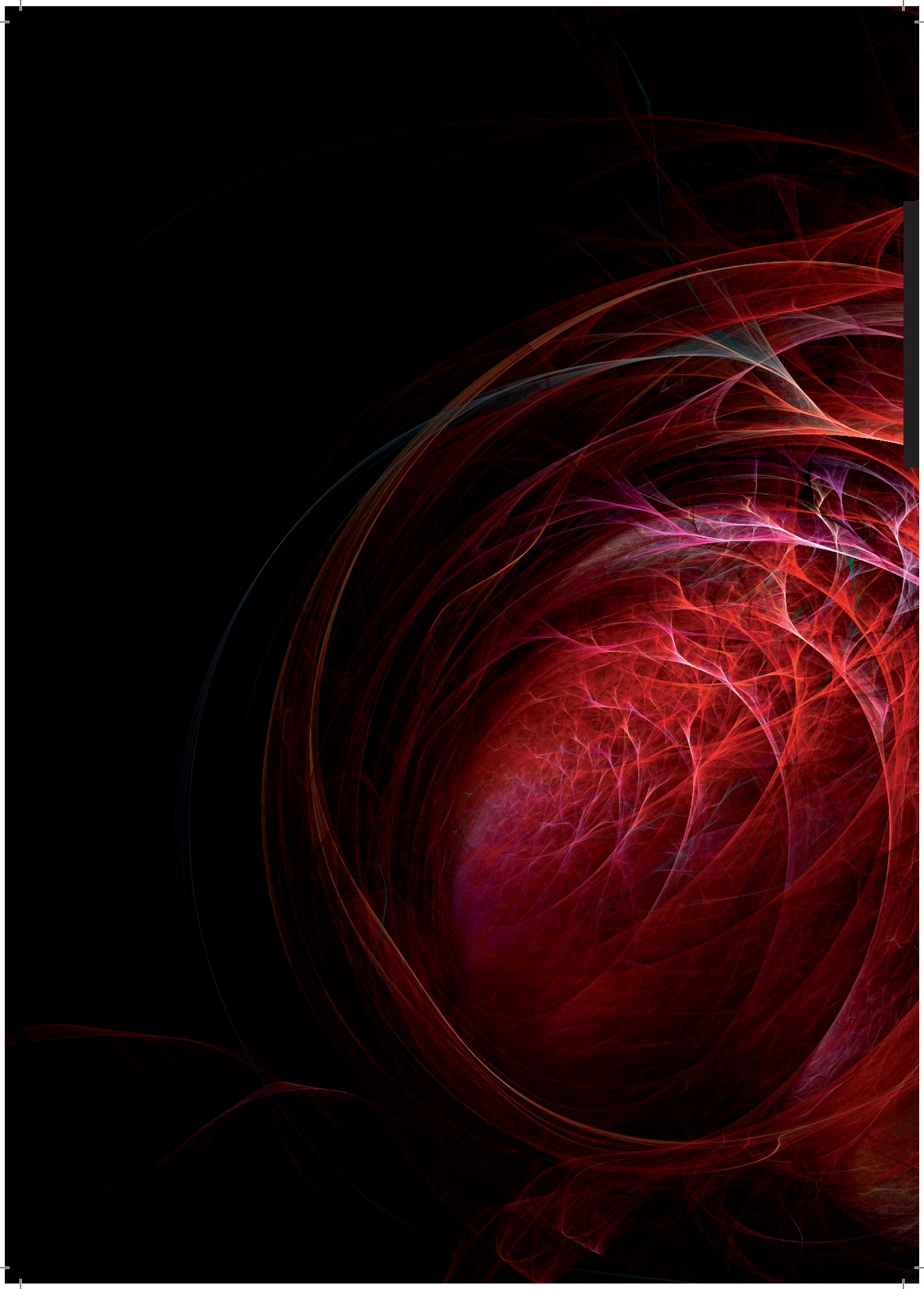

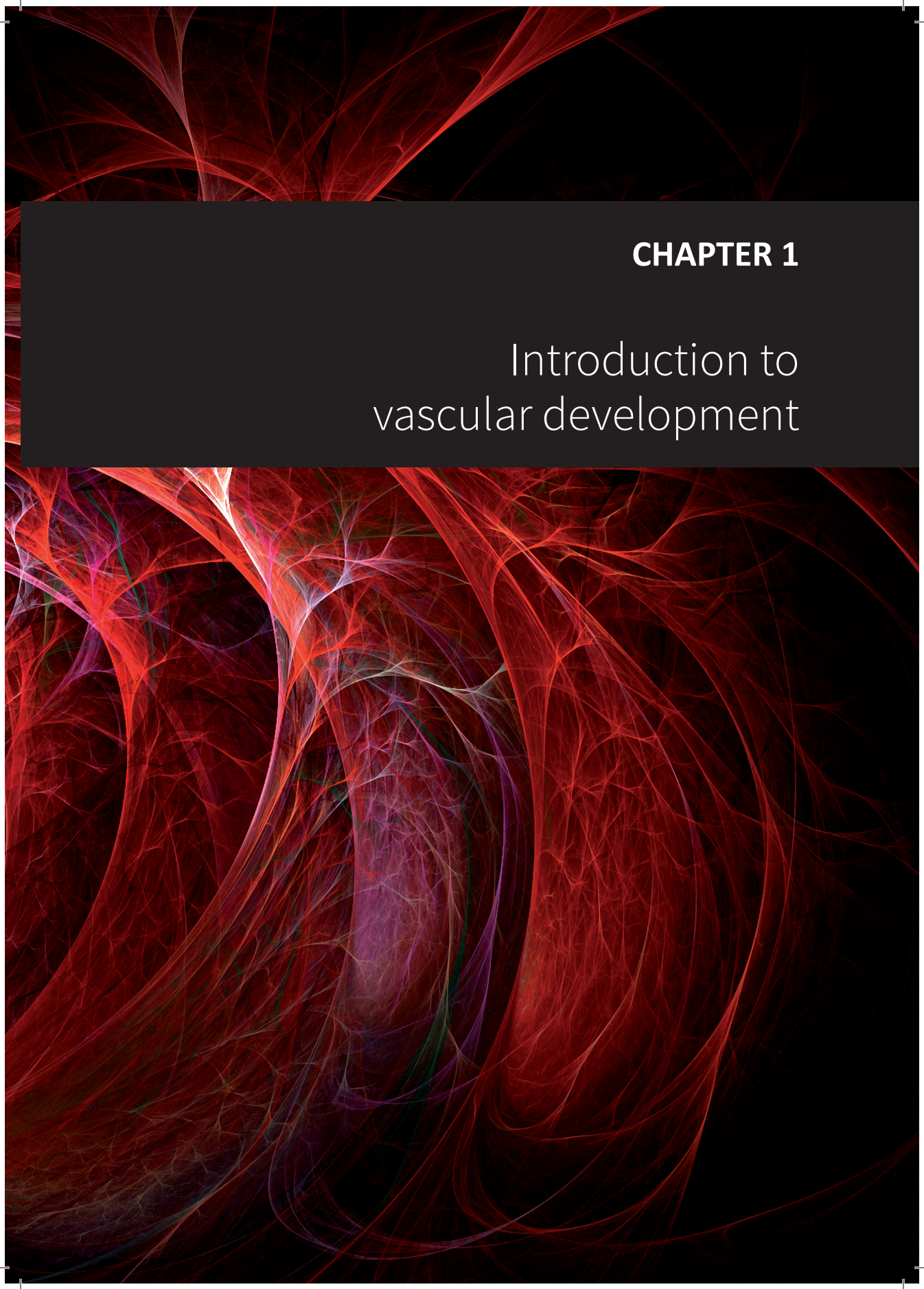

# CHAPTER 1

# Introduction to vascular development

**The vasculature is formed through vasculogenesis, a process in which mesodermal-derived angioblasts migrate and aggregate to form the very first vessels, and through angiogenesis, a process in which endothelial cells from pre-existing vessels sprout to form new vessels. To form a functional vasculature, endothelial cells subsequently differentiate into arterial, venous and lymphatic cells. The arteries transport blood from the heart towards all perfused tissue, the veins return the blood from the peripheral tissues back to the heart. Lymphatic vessels have a somewhat different function in that they collect interstitial fluid and control fluid homeostasis. Disturbances in the development of the vasculature will consequently result in severe malformations. The zebrafish (*Danio rerio*) model system is highly suitable for studying the very early defects in vascular development by virtue of the fast development, the transparency of its embryos and its ability to rely on passive oxygen diffusion during the first week of life. Since the embryonic development of the vasculature is highly comparable with the regulation of the vasculature in later stages, studying early vascular development will continue to provide significant insights into the malformation of the vasculature in cardiovascular diseases or tumor formation.**

The cardiovascular system was already acknowledged in the 16$^{th}$ century B.C. The Ebers Papyrus, an ancient Egyptian medical document, contains the first recognition of the circulatory system. Here it was suggested that air enters via the mouth and then flows directly to the heart, from where it moves through the whole body making use of vessels (Saba et al., 2006). This was the first step towards the recognition of the circulatory system where the heart and the vessels are interconnected and are responsible of delivering air (oxygen) throughout the body. However, it was not until the seventeenth century that William Harvey first described the functionality of the arteries and veins in circulating blood through the body (Harvey, 1950). This appreciation of the existence of blood running through different kinds of vessels was a great step forward in understanding the importance and complexity of the circulation system. To this day, studying the cardiovascular system is crucial to better understand its functioning and dys-functioning, shedding more light on the pathology of cardiovascular diseases.

## PATHOLOGIES OF THE VASCULAR SYSTEM

Cardiovascular diseases are currently the number one cause of death globally. Under the umbrella term 'cardiovascular diseases' all problems with the heart or/and blood vessels are included, among the best known being myocardial infarction, stroke and atherosclerosis. A myocardial infarction, more commonly referred to as a heart attack, occurs when the heart does not get enough blood due to a blockage in one of the vessels supplying the heart with oxygen. A stroke is a disturbance of blood supply to the brain, either by physical blockage of cerebral vessels or by hemorrhages. These blockages in vessels can have several causes, the best-known being an atherosclerotic plaque. Atherosclerosis is a thickening (and resulting loss of elasticity) of an arterial wall by chronic vascular inflammation and

lipid accumulation. An atherosclerotic plaque can rupture and eventually cause thrombus formation and complete blockage of the vessel resulting in local ischemia (Jackson, 2011; Libby et al., 2011). The risk to get a cardiovascular disease depends on many factors. Diet, lifestyle and age are often mentioned as possible risk factors, but also genetic predisposition plays a crucial role; many genes have been linked to cardiovascular diseases.



Whereas in cardiovascular diseases the circulation is disturbed in specific locations, in cancer the blood supply and also vascular growth is often locally enhanced. Malignant tumors contain a complex network of vessels, allowing the tumor to grow (Folkman, 1992; Holash et al., 1999). Chemotherapy is often focused on this aspect of tumor growth. Well-known examples are Sunitinib and Imatinib, which inhibit certain receptor tyrosine kinases, like vascular growth factor receptor and platelet-derived growth factor, thereby causing inhibition of angiogenesis and endothelial cell proliferation (Ferrara, 2004; Faivre et al., 2007; Iqbal, 2014). Furthermore, metastasis in cancer is mainly supported by the spreading of tumor cells through blood- and lymphatic vessels.

The molecular mechanisms involved in these processes are similar to those occurring during embryonic vascular development, and therefore studying vascular development will continue to provide important insights into (how to interfere with) these pathological conditions.

## VASCULAR DEVELOPMENT

To understand the pathology of cardiovascular diseases it is crucial to understand the composition and development of the cardiovascular system in detail. Here we will discuss the basic principles of the vascular- and circulatory system, which will be addressed further in this thesis.

### The vascular system

The cardiovascular system is one of the earliest organ systems to develop in all vertebrates (Fishman and Chien, 1997). The vasculature gives rise to a network of different sized tubes and consists of arteries, veins and lymphatic vessels. The heart pumps oxygen-rich blood with high pressure towards the arteries, after which the arteries branch into multiple arterioles and capillaries. With very few exceptions, capillaries supply every cell and organ of the body with oxygen, nutrients, hormones and multiple other molecules. At the same time the blood circulation in the capillaries makes it possible to collect, and eventually discard, waste products, such as carbon dioxide and unwanted metabolites. After the exchange, the blood is collected into a network of venules and veins, returning the blood towards the heart. In mammals, the heart pumps the oxygen-low blood towards the lungs, where the blood is replenished with oxygen, ready to supply other tissues. This continuous

exchange process is necessary for the maintenance and renewal of all organs and cells. Furthermore, the constant circulation enables a variety of cells, such as immune cells and platelets to be transported to the required places, hereby playing an important role in - among others - wound healing and maintaining homeostasis of physiological pH levels and body-temperature.

The vasculature consists of a monolayer of endothelial cells (ECs) that (in most cases) bind tightly to each other lining a lumen, often surrounded by supporting cells such as smooth muscle cells or pericytes (Allt and Lawrenson, 2001; Armulik et al., 2005; Bergers and Song, 2005). To form these vessels and to create this highly complex vasculature system, two main processes are involved. The differentiation and migration of endothelial progenitor cells (also called angioblasts) and the clustering of these cells to line up as a vessel is called vasculogenesis (Risau and Flamme, 1995; Adams and Alitalo, 2007). From these preexisting vessels, ECs can migrate and sprout to form new vessels in a process referred to as angiogenesis (Risau, 1997). Vasculogenesis and angiogenesis are the key processes that build the entirety of the vascular complex. ECs differentiate further into arterial and venous ECs, making up the important distinction between these two aspects of the vasculature (Fig. 1). The arterial network consists of (besides ECs) a thick muscle layer to cope with the high pressure of the blood flow from the heart. The venous vasculature transports relatively low pressured blood, and therefor only needs a thin layer of muscle cells surrounding the endothelial cells (Armulik et al., 2005). Furthermore, venous vessels often have valves to direct the blood flow towards the heart and to prevent back flow.

An aspect of the vasculature that serves completely different functions is the lymphatic system. Here too, endothelial cells line luminized vessels, but in the case of the lymphatic system, these vessels are blind-ending and do not transport blood. In terms of their embryonic development, a subset of venous cells will differentiate into lymphatic (precursor) cells, which then branch out and form a network of lymphatic vessels (Petrova et al., 2002; Wigle et al., 2002) (Fig. 1). The lymphatic vasculature collects interstitial fluid and drains its contents back into the venous system. The lymphatic system is also important in the transportation of immune cells and several waste products. These waste products can be transported to the blood and to, e.g., the liver or kidneys where they are metabolized and excreted (Alitalo et al., 2005).

The molecular regulation of these processes has gained much attention during the last decade and several key players have been identified. The molecular regulation of vasculogenesis as well as the migration of ECs during early development and the specification of EC into arterial and venous ECS will be discussed further in chapter 2.

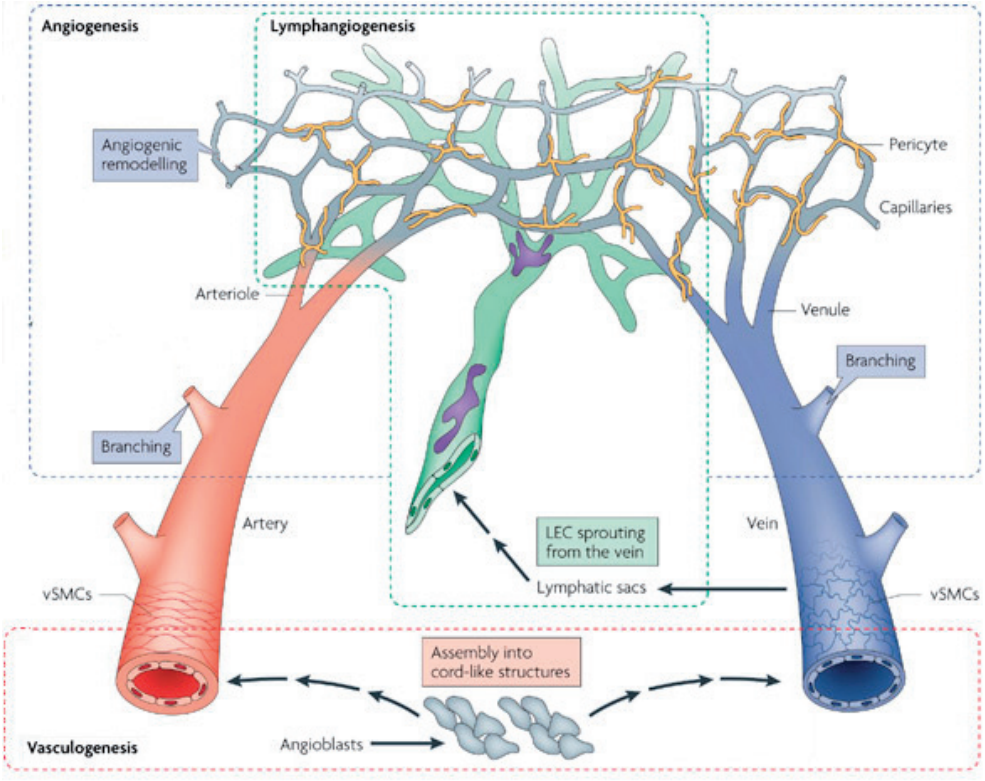


**Fig.1 Assembly of the vascular network.** Initially, angioblasts migrate and assemble to form cord-like structures in a process called vasculogenesis (lower part of the panel). Subsequently, sprouting and branching of new vessels from the pre-existing vessels occurs and is referred to as angiogenesis (upper part of panel). Endothelial cells further differentiate into arterial and venous cells. In a functional vascular network, blood is guided from the arteries to the arterioles which branch into capillaries. The blood from the capillaries will assemble into venules and gets transported to the veins. Venous cells can differentiate into lymphatic endothelial cells and form the lymphatic system. Pericytes (yellow), smooth muscle cells (purple) and vascular smooth muscle cells (vSMCs) support the endothelial cells. *Adapted from Adams and Alitalo, 2007.*

## The circulatory system

The blood contains several cell-types generated in a process called hematopoiesis. At least a subset of blood cells and ECs derives from the same precursor cells, the mesoderm-derived hemangioblasts, as suggested by (1) overlap in gene expression patterns in EC precursors and blood cell precursors, (2) their close spatial and temporal association during migration, and (3) direct lineage tracing experiments (Choi et al., 1998; Vogeli et al., 2006). In mammalian development, mesodermal cells from the primitive streak will migrate to the lateral plate mesoderm (LPM) in the extra-embryonic yolk sac and differentiate into endothelial and hematopoietic progenitors while forming blood islands. In these extra-embryonic yolk sac blood islands the first wave of hematopoiesis takes places (Detrich et

al., 1995). Also in the zebrafish, like other vertebrates, mesodermal cells can differentiate into angioblasts and blood cell precursors. These first steps of primitive hematopoiesis and vasculogenesis initiate in the zebrafish embryo at the anterior and posterior LPM, and manifest themselves in the form of two bilateral mesodermal cell populations. From the 10-12 somite stage, erythroid and EC precursor cells from the LPM start to migrate over the endoderm to the midline, where the EC precursor cells from the LPM coalesce to from the axial vessels. Erythroid precursors coalesce into the Intermediate Cell mass (ICM), which is located in the trunk of the embryo, ventral to the notochord. In this position, the first wave of hematopoiesis in zebrafish occurs (Fouquet et al., 1997; Jin et al., 2005).

During the primitive wave the erythroid progenitors will differentiate into primitive erythrocytes and primitive macrophages. During the second wave of hematopoiesis, hematopoietic stem cells (HSC) will arise. These stem cells will retain their pluripotent state and are able to form all aspects of the mature hematopoietic lineages during lifetime, such as definitive erythrocytes. This second wave takes place in the aorta-gonad-mesonephros (AGM) in mammals, and in the ventral wall of the aorta in zebrafish. Later on these aspects of mature hematopoiesis are taken over by the liver and subsequently by the bone marrow and thymus in mammals, and by the kidneys in zebrafish (Davidson and Zon, 2004).

Gata-1, a zinc finger transcription factor, is expressed in primitive erythrocytes even before the formation of blood islands in mice (Yokomizo et al., 2007) and from the 5-somite stage in zebrafish onwards, which makes Gata-1 a suitable marker for studying erythropoiesis. Gata1-positive primitive erythrocytes are the only circulating erythroid cells in zebrafish for the first 4dpf. After 6dpf the number of these cells starts to decline and definitive erythrocytes will populate the circulation (Detrich et al., 1995; Weinstein et al., 1996).

## ZEBRAFISH AS A MODEL SYSTEM

The molecular mechanisms regulating the vasculature are highly similar between vertebrates (Isogai et al., 2001). To study the complex regulation of the circulatory system, the vertebrate zebrafish (*Danio rerio*) is a highly attractive model. Zebrafish are relative easy to maintain and they produce high offspring numbers: one mating can produce hundreds of eggs. Zebrafish embryos develop externally and are transparent during early development, making them suitable for research in diverse fields by real-time observations of organ formation or developmental processes. Zebrafish are convenient for embryonic manipulations and for use in genetic screens, making it possible to explore the genetic mechanisms involved in various diseases. Furthermore, transgenic embryos can be raised which, for example, express a fluorophore under the control of an endothelial-specific promoter. The use of these transgenic reporter lines in combination with live imaging has enabled detailed studies of the developing vasculature (Lawson and Weinstein, 2002; Beis and Stainier, 2006; McKinney and Weinstein, 2008). Importantly, zebrafish embryos obtain

oxygen via passive diffusion from the water during early development, making them a good model to study vasculature defects from an early time point in development onward as effectively organ systems are not dependent on blood flow. Zebrafish embryos without a complete vascular system (*cloche* mutants) (Liao et al., 1997) or without circulation (*silent heart/tnnt2* mutants) (Sehnert et al., 2002) are able to survive up to a week. In mammalian model systems the lack of normal vasculature and blood circulation often results in death in utero, whereas these defects can be studied in detail during development in zebrafish embryos.

## Zebrafish development

Zebrafish development proceeds completely via an extra-uterine mode, and starts after fertilization with multiple cell divisions, reaching the 4-cell stage within one hour. Cell divisions proceed quickly, and cells undergo epiboly movements around 4 hours post fertilization (hpf). From 10 hpf onwards, somites start to form, and around 14 hpf a premature embryo can be recognized. Around 20 hpf the tail of the embryo becomes clearly visible, and at 24 hpf the embryo is elongated and the heart starts to beat. After 2 days the embryo will hatch out of the chorion and start to swim around, entirely relying on nutritional support from the yolk. The larval stage will start from 5dpf onwards, when the swim bladder is formed and the fish are able to use external food sources. Depending on food availability and other factors the fish will become adult in 2-3 months (Kimmel et al., 1995).

## The zebrafish vasculature

Heart beating and blood circulation in a zebrafish embryo starts at around 24 hpf and 27hpf, respectively. The first vessels that develop are the dorsal aorta (DA) and the posterior cardinal vein (PCV), which align in a parallel manner along the length of the trunk (Fouquet et al., 1997). The aorta is subdivided in the ventral aorta with the aortic arches, the lateral dorsal aorta (LDA) in the head region, and the dorsal aorta in the trunk. The aortic arches will provide the blood with oxygen from the gills. The blood from the trunk is returned via the PCV towards the common cardinal vein (CCV) and subsequently to the heart. The blood circulation enters the head region via the ventral aorta and the primitive internal carotid artery, which will branch in multiple arties. The posterior hindbrain channel, the anterior cardinal vein and the primary head sinus (PHS) will bring the blood back to the CCV and the heart (Isogai et al., 2001) (Fig. 2).

After the formation of the main vessels, angiogenic sprouting will further increase the complexity of the vasculature. From 24 somite stage onwards, intersegmental vessels (ISV) sprout from the dorsal aorta and migrate along the somite boundaries to the dorsal side, where they T-branch and form the dorsal longitudinal anastomotic vessel (DLAV) (Isogai et al., 2001)[7]. These sprouts consist of stalk cells that support the dorsal most filopodia-rich

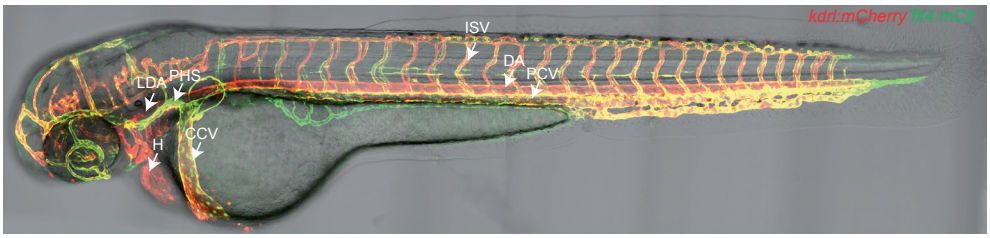


**Fig.2 Transgenic zebrafish embryo with primitive vasculature.** *Kdrl:mCherry;Flt4:mCitrine* embryo at 2 dpf depicting the arterial vessels in red and the venous vessels in yellow. H = heart, LDA = lateral dorsal aorta, PHS = primary head sinus, CCV = common cardinal vein, ISV = intersegmental vessel, DA = dorsal aorta, PCV = posterior cardinal vein.

tip cell (Childs et al., 2002). This primary sprouting in the trunk is followed by secondary sprouting around 36 hpf when cells from the PCV start to grow dorsally. This secondary sprouting behaves in a distinct manner compared to primary sprouting. In secondary sprouting, every other sprout will connect to the arterial ISVs, thereby remodeling them into venous ISVs. The other sprouts will migrate along the remaining aISVs and differentiate into parachordal lymphangioblast (PLs) (Isogai et al., 2003; Bussmann et al., 2010). The PLs will first migrate to the horizontal myoseptum and subsequently migrate to the dorsal and ventral side to form the lymphatic network, consisting of the thoracic duct (the main lymphatic structure at this stage, positioned in between the DA and PCV), and the dorsal longitudinal lymphatic vessel, running along the DLAV (Yaniv et al., 2006; Hogan et al., 2009; Bussmann et al., 2010) (Fig. 3). The guidance of these sprouts needs to be tightly regulated, however the involvement of specific extrinsic and intrinsic signaling pathways is currently under debate (van Impel et al., 2014) and will be discussed further in chapter 4.

## EXPERIMENTAL TECHNIQUES IN THE ZEBRAFISH

As mentioned before, zebrafish are experimentally very accessible and can be used for genetic studies to get more insight into vertebrate genetics, ultimately leading to a better understanding of human diseases. Currently the four most common techniques in genetic zebrafish studies are mutagenesis screening, morpholino knock downs, genome engineering via TALEN/CRISPR, and transgenesis. These techniques will be referred to throughout this thesis.

### Large-scale genetic screens

One of the first large-scale genetic studies performed in zebrafish was based on forward mutagenesis-based screening. Using this genetic approach, the spermatogonial DNA of male fish is mutagenized by ethylnitrosourea (ENU)-treatment (Grunwald and Streisinger, 1992;

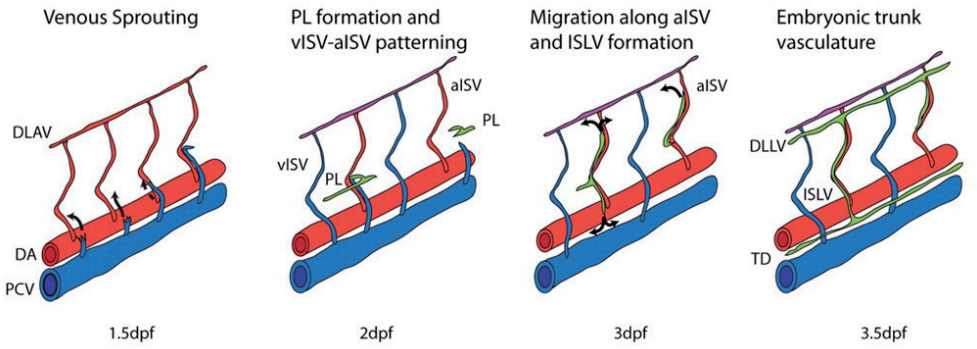


**Fig.3 Angiogenesis and lymphangiogenesis in the zebrafish trunk.** After the formation of the primary ISVs by sprouting from the dorsal aorta (DA), secondary sprouts will arise around 1.5 dpf. These sprouts from the posterior cardinal vein (PCV) will migrate dorsally, where half of the venous sprouts will connect to an arterial ISV (aISV), remodeling it into a venous ISV (vISV). The other sprouts will not connect with the aISVs but differentiate into parachordal lymphangioblast (PLs) and migrate to the horizontal myoseptum. Around 3 dpf these PLs will migrate dorsally and ventrally, forming the intersegmental lymphatic vessels (ISLVs), the thoracic duct (TD) running between the DA and the PCV, and the dorsal longitudinal lymphatic vessel (DLLV), running along the dorsal longitudinal anastomotic vessel (DLAV). *Adapted from Bussmann et al., 2010.*

Solnica-Krezel et al., 1994). These males are crossed to untreated wild-type females, resulting in offspring with heterozygous point-mutations in their DNA. After further propagating these mutagenized fish, they can be screened (Mullins et al., 1994) by in-crossing the F2 offspring and screening for the phenotype of interest to identify heterozygous carriers (Driever et al., 1996; Haffter et al., 1996). The mutation causing the phenotype can then be mapped to a specific gene/chromosome. These screens have been tremendously successful, generating thousands of mutants. Based on the same ENU mutagenesis principle, ways have been devised to achieve reverse genetic approaches. Initially (Wienholds et al., 2002), Targeted Induced Local Lesions in Genomes (TILLING) was designed to allow large-scale reverse genetic screening. The near complete sequence of the genome of zebrafish made it possible to target and sequence specific regions of interest within mutagenized genomes. Following ENU mutagenesis, possible mutations in a gene of choice are analyzed by high-throughput sequencing. Further outcrossing the fish with the mutation of interest and subsequent analysis of the phenotype makes it possible to study the effect of loss of function genes in zebrafish (Fig. 4A). Genetic screening is quite labor and time intensive because several generations of fish need to be generated and sequenced, however, the off-target effects are minimal and specific mutations can be analyzed (Wienholds et al., 2002; Wienholds et al., 2003). Recently, Kettleborough et al. published a genome-wide analysis of protein-coding gene functions by a combination of mutagenesis and high-throughput sequencing and identified more than 1000 mutant alleles along with their phenotypes (Kettleborough et al., 2013).

## The use of morpholinos for transient knock-downs

Morpholinos are small synthetic anti-sense oligomers that are designed to target a specific region of interest (Summerton, 1999; Nasevicius and Ekker, 2000). Morpholino oligomers are around 25 base pairs long and consist of morpholine rings instead of deoxyribose rings, and are connected through phosphorodiamidate groups instead of phosphates. Morpholinos are injected at the one-cell stage and persist up to a maximum of 5 days in the embryo (Corey and Abrams, 2001). The targeting strategy when using morpholinos is often divided into 2 groups: morpholinos that block translation, and morpholinos that bind a splice-site and interfere with proper splicing. In the first instance, the morpholinos are designed to bind to the ATG site or the 5'untranslated region of the gene of interest. These morpholinos inhibit the binding of the ribosomal initiation complex, blocking translation (Summerton, 1999). The second class of morpholinos binds at splice-sites of pre-mRNA, inhibiting the binding of proteins necessary for normal splice-events (Draper et al., 2001) (Fig. 4B). While morpholinos are relative easy to use and while the effect can be studied directly, morpholinos often cause severe toxic effects. It is also challenging to address the effectiveness of the morpholinos and distinguish between non-specific and targeted effects (Eisen and Smith, 2008; Schulte-Merker and Stainier, 2014).

## The TALEN/CRISPR genome engineering methodologies

Recent approaches in genetic zebrafish studies are making use of transcription activator-like effector nucleases (TALENs) and the clustered regularly interspaced short palindromic repeats (CRISPR) associated (Cas) system. TALENs consist of two Fok1 nuclease domains, each fused to a TAL effector DNA binding domain that bind to the region of interest. The DNA binding domains consist of amino acid repeat units, each repeat unit recognizing a single nucleotide of DNA, making strong sequence-specific binding possible (Boch et al., 2009; Moscou and Bogdanove, 2009). Binding of both TAL effector DNA binding domains to their specific recognition sites within the genome will cause heterodimerization of the nuclease domains. The dimerized nuclease domain then creates double-stranded DNA breaks in the region of interest. Non-homologous end-joining (NHEJ) will repair this break, in the process inserting or deleting a few base pairs. This in-precise repair mechanism can cause a frame-shift leading to a premature STOP-codon and thus a loss-of-function allele (Bedell et al., 2012; Moore et al., 2012; Hwang et al., 2014) (Fig. 4C).

The CRISPR system relies on a similar principle as the TALEN system, however when using CRISPR short palindromic repeats (sgRNA) flank the specific region of interest, recruiting Cas9 for target specific cleavage (Auer and Del Bene, 2014). Generating CRISPRs and TALENs are less labor and time intensive than genetic screens and current evidence suggests off-target effects to be low. Recently, Schulte-Merker & Stainier (2014) reported on the advantages of TALENs/CRISPRs in comparison to the morpholino approach.

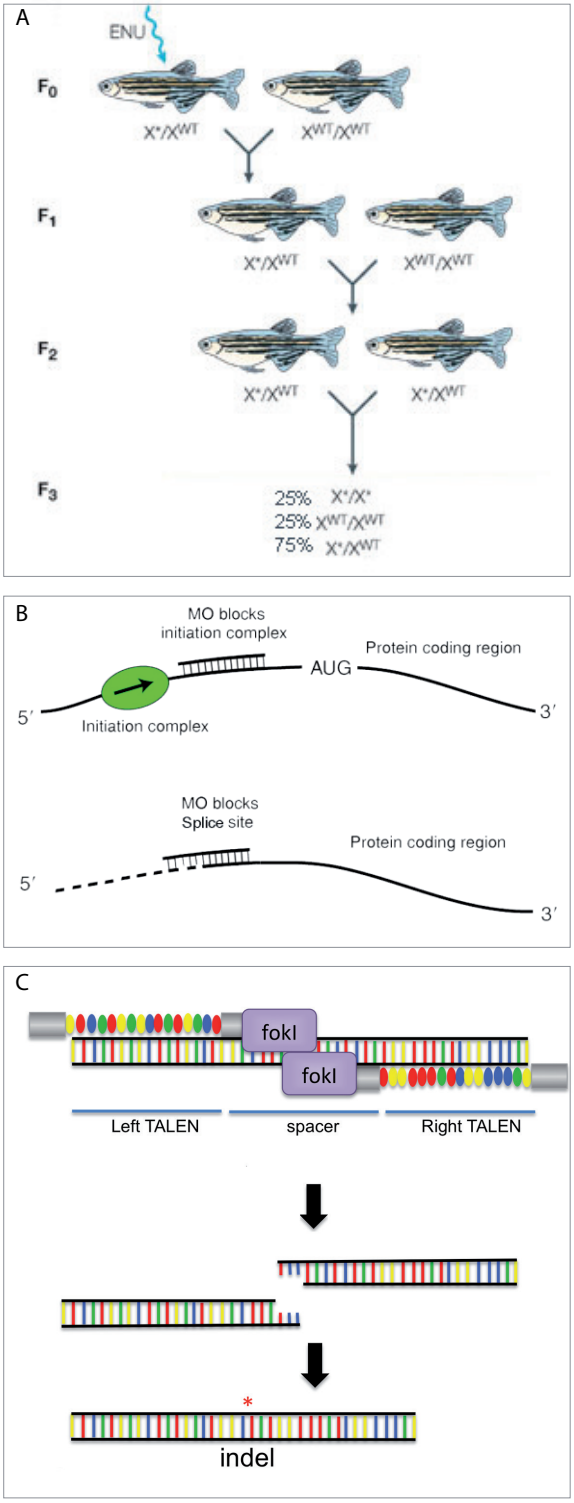


**Fig.4 ENU-based screening, morpholino knockdown and TALEN genome engineering in zebrafish.** (A) In ENU-based screening spermatogonia are mutagenized with ENU and these males are crossed to untreated females. The F1 offspring contains heterozygous mutations and can be further propagated and used to analyze specific mutations and their phenotypes. *Adapted from (Langenau and Zon, 2005).* (B) Morpholinos bind to specific (pre-) mRNA and inhibit translation or splicing by blocking the binding of the initiation complex (upper panel) or the splice-site (lower panel) respectively. *Adapted from (Eisen and Smith, 2008).* (C) TAL effector DNA binding domains (Left and Right TALEN) fused to Fok1 nuclease domains can bind specifically to the region of interest. The dimerized activated nuclease domain induces double-stranded breaks which results in non-homologous end-joining with base pair insertions and/or deletions (indels). *Adapted from (Moore et al., 2012).*

### The use of transgenic zebrafish to visualize (vascular) development

Besides their usefulness for loss-of-function studies, zebrafish are also highly suitable as transgenic models. Using this approach, a gene or promoter of interest is linked to a fluorescent protein (e.g. eGFP, mCitrine, dsRed, mCherry), which makes it possible to visualize the expression of the gene of interest (Koster and Fraser, 2001). Bacterial artificial chromosomes (BAC) are also used in which a reporter gene is inserted into a pre-defined target locus. BACs are cloning vectors that consist of large DNA inserts, including all or a majority of regulatory elements. Injecting the DNA construct into the one cell-stage can result in a stable transgenic line when the transgene is integrated in the germline DNA (Yang et al., 2006; Suster et al., 2009; Bussmann and Schulte-Merker, 2011). Furthermore, it is possible to insert a tissue-specific promoter in the vector, restricting the expression to the tissue of interest. Transgenic zebrafish lines that are commonly used in studying the vasculature (and also used in this thesis) are *fli1a:eGFP* labelling all ECs (Lawson and Weinstein, 2002), *kdrl:eGFP* labelling arterial and venous ECs (Jin et al., 2005)*, Flt1:tdTomato* labelling only arterial ECs (Bussmann et al., 2010), *Flt4:mCitrine* labelling venous cells (Bussmann and Schulte-Merker, 2011), and *Gata1:dsRed* labelling all primitive erythrocytes (Traver et al., 2003). Also double transgenic embryos can be studied, for example when analyzing both blood and the vascular system, labelling the primitive erythrocytes and the ECs independently. Furthermore, impressive new insights on the *in vivo* behavior of specific EC types were obtained using real-time imaging of transgenic zebrafish embryos.

## OUTLINE OF THIS THESIS

As introduced in the previous chapter, this thesis will focus on endothelial specification in vascular development. In **chapter 2** we report on endothelial cell migration during vasculogenesis in zebrafish. We will discuss in detail the arterial-venous specification of endothelial cells and the molecular mechanisms involved in the process. In **chapter 3** we will analyze the specific function of an important player in arterial-venous specification, the transcription factor Sox7, by generating a *sox7* zebrafish mutant. We report on the analysis of *hey2* and *efnb2* zebrafish mutants and link *sox7* genetically to Hey2 and Efnb2 function. Furthermore we place Sox7 upstream of Notch signaling in arterial specification and discuss its cooperation with other SoxF family members. In **chapter 4** we will focus on lymphatic endothelial specification. We analyze the function of Prox1 in zebrafish, making use of a *prox1* transgenic reporter line and *prox1a/b* zebrafish mutants. Furthermore we generated *sox18* and *coupTF2* mutants and discuss the regulation of *prox1*, *sox18* and *coupTF2* in lymphatic specification in zebrafish and compare this with lymphatic specification events in mice. In **chapter 5** we conclude this thesis with a summarizing discussion about current work and new insights in endothelial cell specification during vascular development.

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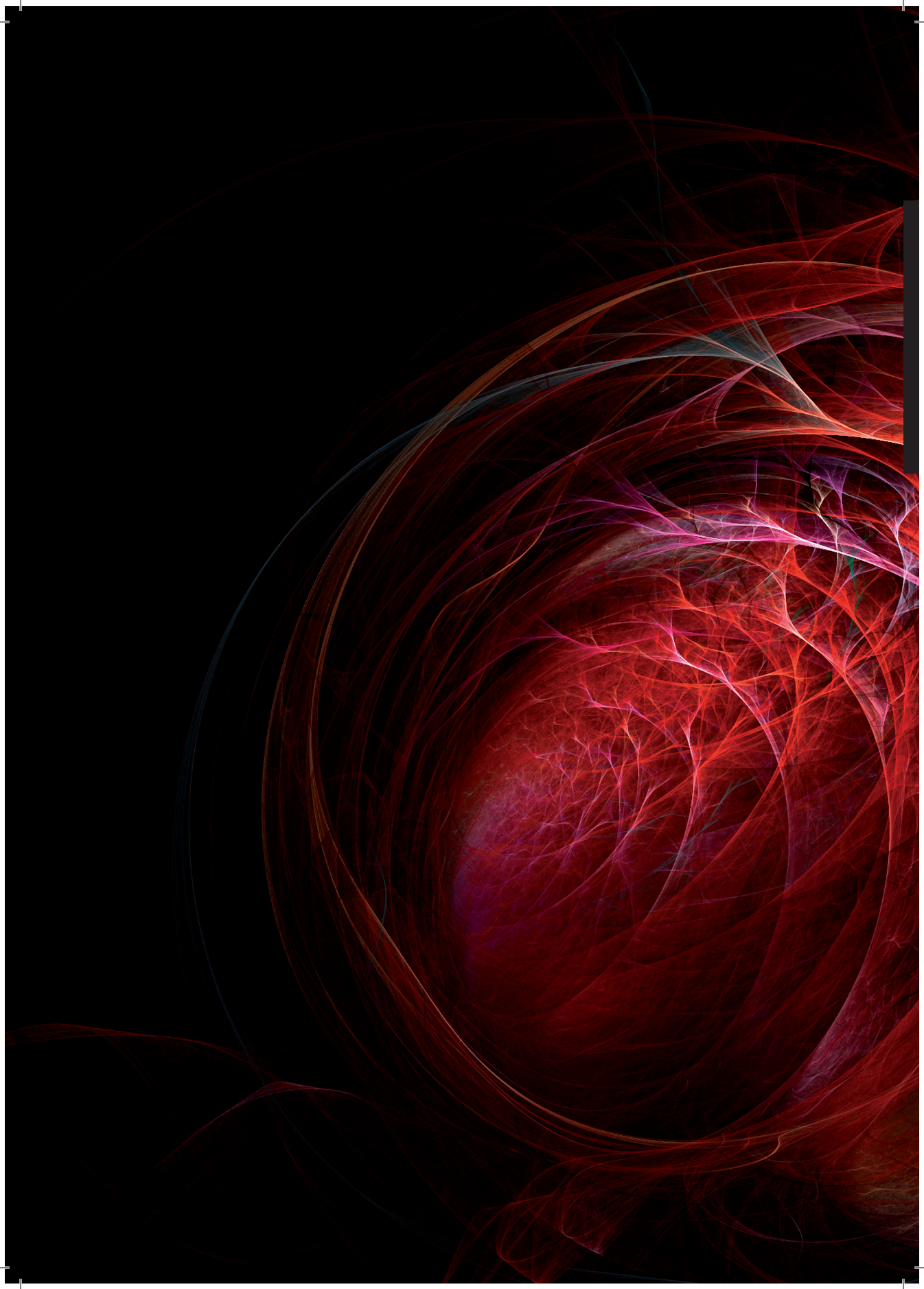

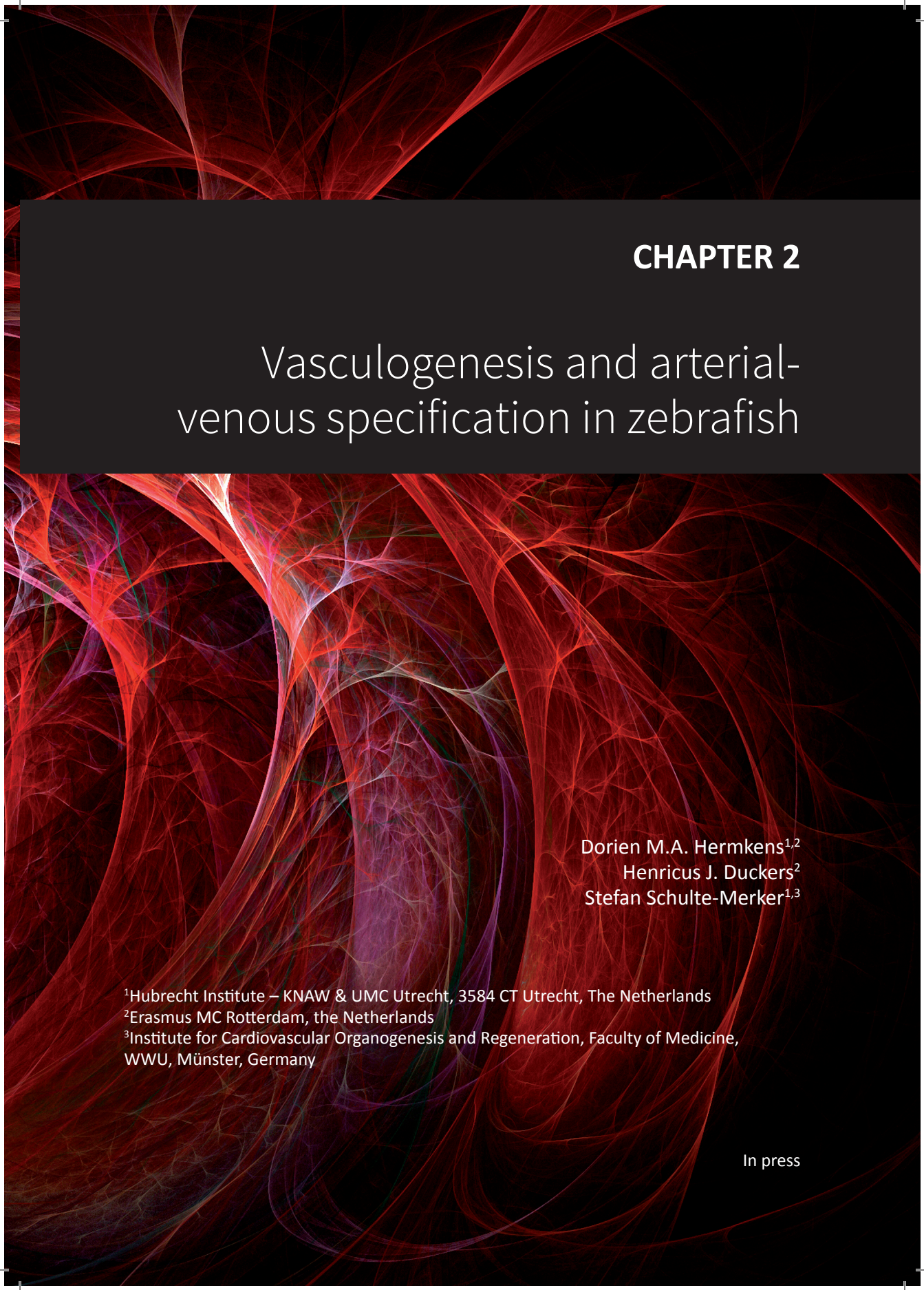

# CHAPTER 2

# Vasculogenesis and arterial-venous specification in zebrafish

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In press

**One of the earliest processes during vertebrate development is the formation of the cardiovascular system. This highly complex system of differently sized tubes permits circulation of blood while enabling the exchange of oxygen and nutrients in all perfused tissues. Recent studies in zebrafish have provided additional insight into the initial establishment of the vasculature, and particularly the process of vasculogenesis. During vasculogenesis, angioblasts exhibit a complex migratory behavior, before they differentiate into endothelial cells and form a primitive vascular network. In addition to migrating to specific locations, endothelial precursor cells also need to be specified to take on arterial and venous fates, respectively. In particular the interdependence between the spatial and temporal localization of early angioblasts and their arterial-venous specification has gained some interest, and is one focus of this review. Furthermore, we are discussing the complex network of genetic interactions that play a role in arterial-venous specification.**

## ZEBRAFISH VASCULOGENESIS AND ARTERIO-VENOUS 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, aided by the fact that circulation within the zebrafish embryo commences very quickly: the first signs of heart beat can be appreciated around 22 hours post fertilization (hpf), while the first erythrocytes move through the embryonic body only a few hours later (Zon, 1995). The appeal of the zebrafish embryo for in vivo observation, the availability of transgenic lines that mark precursor cells of the vascular endothelium, the possibility to interfere with this process experimentally, and the generation of mutants that affect the process, have resulted in obtaining significant insight into various aspects of vascular development (Driever et al., 1996; Lawson and Weinstein, 2002). In this review, we will mainly focus on vasculogenesis and the early events of arterio-venous 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 been recently reviewed (Schuermann et al., 2014; van Impel and Schulte-Merker, 2014) and will be only touched upon here where necessary.

Previously it was thought that the differentiation between arteries and veins is mainly established by the difference in hemodynamic forces such as blood pressure (Gonzalez-Crussi, 1971), however, in recent years more and more evidence has emerged that molecular differences between arterial and venous precursor cells regulate the formation of the arterio-venous system irrespective of hemodynamic forces (Wang et al., 1998). Specifically, recent studies showed that molecular differences between arterial and venous cells are already established in early stages when endothelial precursors just begin to arise (Zhong et al., 2001).

The 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 F. 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 (Poole and Coffin, 1989; Risau and Flamme, 1995; Sabin, 2002). 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 actually are already specified at the onset of migration is far less clear. Also, the exact events upon reaching the embryonic midline still need to be resolved. We will discuss both issues in the following.

Independent reports showed two distinct waves of angioblast migration during vascular development in the zebrafish trunk. The first wave of migration starts around 14 hpf, the second wave around 16 hpf. This has been confirmed by a number of groups, most recently by Kohli et al. (Fouquet et al., 1997; Jin et al., 2005; Kohli et al., 2013) . 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 *etv2*/*etsrp*, a transcription factor that has key roles during vasculogenesis. Three hours later, a second and more lateral positioned line of *etv2*/*etsrp* expressing cells becomes apparent (Pham et al., 2007). The heterochrony of *etv2*/*etsrp* expression among these two angioblast populations is further substantiated when looking at other marker genes: *kdrl* (formerly called *flk1* (Bussmann et al., 2008)) 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* (Thompson et al., 1998) is expressed first in medial angioblasts, before then becoming expressed in the lateral angioblast population (Kohli et al., 2013). The arterial marker *gridlock*/*hey2* can only be found to be expressed in the medial population, not in the lateral one (Zhong et al., 2000), 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 which tracked individual cells and addressed the question whether angioblasts, irrespective of their position within the LPM, can contribute to both DA or PCV, or whether the 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 the 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 labelled 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. (2006) reported that a few of these cells contribute exclusively to the endothelial and the haematopoietic lineage, providing direct demonstration of the existence of haemangioblasts. However, only a part of the haematopoietic and the endothelial lineage arise from these bi-potential cells, indicating that there are other cellular sources for the vasculature. Another study labeled cells of the LPM at the 7-12 somite stage, and observed only contribution to either the arterial or venous lineage (consistent with *gridlock/hey2* expression) (Zhong et al., 2000). Hence, based both on marker expression studies and *in vivo* tracing experiments, it appears that there are lineage-restricted angioblasts in the zebrafish LPM during early stages of somitogenesis which have been specified to become either arteries or veins (Fig. 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 fashion. The use of a transgenic line which expresses the photo-convertible fluorophore *kaede* from the *etv2* promoter, has enabled elegant studies which demonstrated that the medial angioblasts give rise to the dorsal aorta, and that lateral angioblasts constitute the PCV (Kohli et al., 2013). 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 (Fig. 1). This is in contrast to a study by Herbert et al. (2009), who suggested a ventral sprouting mechanism, during which angioblasts from the DA contribute directly to the PCV. 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 in Schuermann et al., 2014).

## 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 there are only 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* mutant has been very instructive to understand many aspects of early vascular development (Stainier et al., 1995). Mutations in the *lycat* gene,

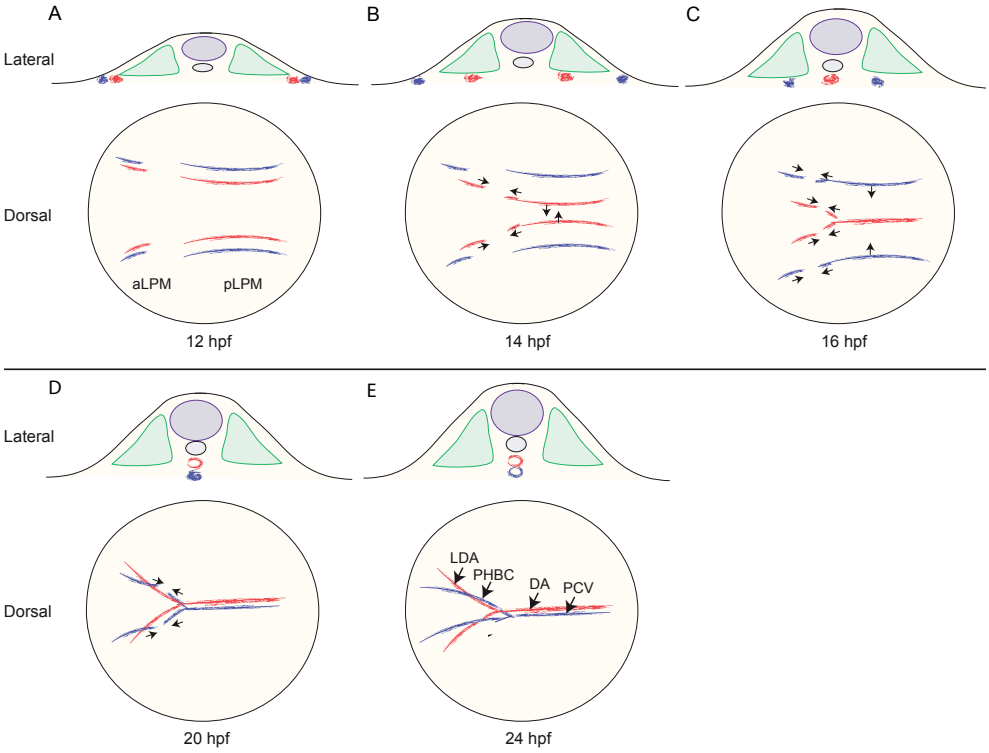


**Fig.1 Arterial and venous precursor cell migration.** (A) At 12 hpf angioblasts are located within the anterior and posterior lateral plate mesoderm (LPM) in 2 bilateral stripes. Presumably, even at this early stage the medial angioblast population consists of arterial precursor cells (red), while the lateral population consists of venous precursor cells (blue). (B) Around 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 LMP start to migrate posteriorly. (C) At 16 hpf, arterial precursor cells will form the first axial vessel, the dorsal aorta (DA). Cells within the more lateral located stripes of the posterior LMP start to migrate to the midline. Also 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 PCV. The migration of arterial cells in the anterior region results in formation of the lateral dorsal aortae (LDA). (E) At 24 hpf venous precursor cells in the anterior region have migrated to form the posterior hindbrain channel (PHBC). Green = somites, purple = notochord and hypochord, yellow = embryonic tissue.

encoding an acyl transferase, have been suggested to be causative for the phenotype (Xiong et al., 2008). The *cloche* mutant phenotype can be rescued via forced expression of the Ets1-related protein, placing this key transcription factor downstream of Cloche (Sumanas and Lin, 2006). As mentioned above, the ETS domain transcription factor Etv2/Etsrp/ER71 is one of the earliest makers 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, to migrate or to form functional axial vessels. Over-expression 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 (Sumanas and Lin, 2006; Pham et al., 2007).

Overexpression studies showed that vascular endothelial growth factor A (VegfA) and Sonic Hedgehog (Shh) are involved in the localization of the medial and lateral angioblasts to the midline, with high levels of VegfA or Shh resulting in a random distribution of medial and lateral angioblasts at the midline (Kohli et al., 2013). Similarly, *vegf-A* morphants showed a single vessel consistent of lateral and medial angioblasts (Kim et al., 2013), suggesting that Vegf and Shh are critical factors for arterio-venous specification at the time point of angioblast positioning at the midline. In zebrafish, *shh* is expressed in the notochord and floorplate (Ekker et al., 1995), whereas *vegf-A* is expressed in the somites (Liang et al., 1998). It is hypothesized that the medial located angioblasts receive a higher concentration of Shh and Vegf than the angioblasts localized at the lateral positions (Kohli et al., 2013). 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 chemo-attractants 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 be able to 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 highest levels of Vegf and Shh) should invariably end up in the aorta. Should, however, more medially cells be overtaken by more distally located cells, this would argue that the cells might not be naïve.

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 dorsal aorta (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 cells migrate towards each other, eventually connect and form the LDA. This process of angioblast migration is suggested to be mediated by the chemokine Cxcr4 (Siekmann et al., 2009). The expression of *cxcr4* is restricted to the angioblasts that will form the anterior most LDA. Knock down of *cxcr4* resulted in inappropriate fusion of anterior and posterior angioblasts and consequently form a disrupted LDA (Siekmann et al., 2009). Around 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) (Siekmann et al., 2009; Bussmann et al., 2011) (Fig. 1 and Fig. 2A,B).

After the initiation of vasculogenesis, angiogenesis will remodel the vascular network further. 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 (Isogai et al., 2003). 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 (Hogan et al., 2009a, reviewed in van Impel and Schulte-Merker, 2014) (Fig. 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 that and contribute to the pool of lymphatic precursor cells. One might presume that this process might involve Notch and Delta, but up to now no members of the Notch/Delta signaling pathway have been detected to be expressed in the PCV or in venous sprouts.

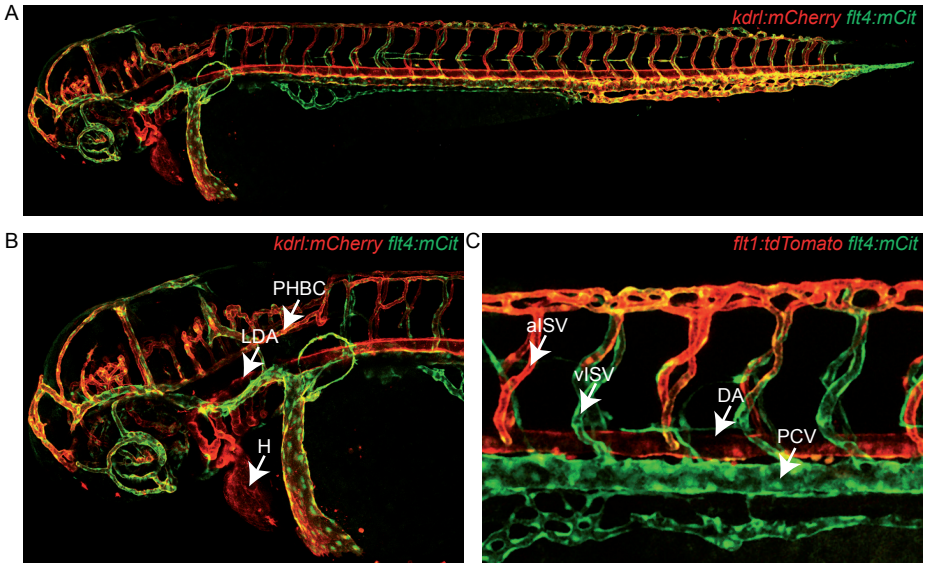


**Fig.2 Transgenic zebrafish vasculature.** (A) Overview of *kdrl:mCherry;flt4:mCit* zebrafish embryo at 2 dpf. Arteries in red, veins in yellow. (B) Enlarged head region of (A) with the heart (H), lateral dorsal aorta (LDA) and the posterior hind brain sinus (PHBC). (C) trunk region of *flt1:tdTomato;flt4:mCit* embryo at 3 dpf with arteries in red and veins in green, including the arterial intersegmental vessel (aISV), venous intersegmental vessel (vISV), dorsal aorta (DA) and posterior cardinal vein (PCV).

## MOLECULAR REGULATION OF ARTERIAL-VENOUS SPECIFICATION

### EphrinB2/Eph receptor B4

One of the most widely referred-to markers for arteriovenous specification are EphrinB2 (Efnb2) and Eph receptor B4 (Ephb4), based on the finding that Efnb2 and Ephb4 receptor are differentially expressed in arteries versus veins, respectively (Wang et al., 1998). The Ephb4 receptor belongs to the receptor tyrosine kinase family and is the only Ephrin receptor that specifically binds to Efnb2, which is a membrane-bound ligand of the Ephrin ligand family (Gerety et al., 1999). The Ephrin ligands and the Eph receptors are both transmembrane proteins, and signaling requires cell-to-cell contact and can be bidirectional (Bruckner et al., 1997). Eph receptors and their ligands are often, but not always, localized in adjacent cell population (Gale et al., 1996; Adams et al., 1999). '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 SH2 (Src-homology-2) domain containing adaptor protein and SH3 binding partners (reviewed in Kullander and Klein, 2002). The exact contribution of forward and/or reverse signaling in arterio-venous specification is still unclear.

In zebrafish, *efnb2* expression is restricted to the arterial ECs. Expression of *efnb2* is initiated around the 20-somite stage, when the angioblasts have migrated to the midline, but circulation has not commenced yet. *ephb4* receptor mRNA is also expressed in the vasculature but the expression is restricted to the venous ECs (Wang et al., 1998). 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 arterio-venous 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 (Lawson et al., 2001). 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 wildtype host embryos. In hosts that received *efnb2a* MO donor cells, the donor cells ectopically localized to the vein. In hosts embryos that contain *ephb4* MO donor cells, fewer cells contribute to the vein compared to their controls, again suggesting a role for Efnb2 and Ephb4 in arteriovenous specification (Herbert et al., 2009). 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 (Gerety et al., 1999) .

## Vegf and Shh

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) (Terman et al., 1991; de Vries et al., 1992; Bussmann et al., 2008). The expression pattern of these receptors has been examined in detail (Bussmann et al., 2008; Lohela et al., 2009; Shibuya, 2013), 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 only morpholino-based data exist (Lee et al., 2002; Martyn and Schulte-Merker, 2003) 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 knock down of *vegf-Aa* was reported to result in deficiency of intersegmental vessel sprouting, with no major deficiencies in the DA or PCV (Nasevicius et al., 2000). Analyzing the dependence of vasculogenesis on Vegf-A is confounded, however, by the duplication of zebrafish *vegf-A* genes (Bahary et al., 2007), and by maternal expression of the respective mRNAs. Stable mutant lines, or maternal zygotic mutants have not been generated, and this precludes a final assessment on the role of Vegf-A during early stages of vasculogenesis; current evidence based on double knock downs suggests diverged functions of Vegf-Aa and Vegf-Ab, but no major effects on vasculogenesis (Bahary et al., 2007). Mutants for *vegfr4/kdrl* (Habeck et al., 2002) and *vegfr3/Flt4* (Hogan et al., 2009b), however, 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 later stages of vascular development, during arterial and venous intersegmental vessel sprouting, but their role during earlier stages of vasculogenesis remains somewhat enigmatic: more than one Vegf receptor might be required to be mutated to appreciate a phenotype.

A key regulator in the early steps of angioblast migration and in later events of arterial and venous specification has been suggested in Sonic Hedgehog (Shh), a member of the Hedgehog family which can act as a ligand for the transmembrane receptors Patched and Smoothened (Lawson et al., 2002). *smoothened* mutants, which are devoid of Shh signaling, show comparatively normal angioblast migration (Wilkinson et al., 2012), and the receptors for Shh, the duplicated *patched* genes, appear not to be expressed within the posterior LPM (Lewis et al., 1999). Shh regulates expression of *semaphorin 3a1*, which has been shown to have an effect on angioblast migration (Shoji et al., 2003). Also, Shh signaling from the midline is essential for normal *vegf-A* expression in the medial aspects of the somites (Lawson et al., 2002). While more work needs to be done to clearly carve out the role for Shh and Vegf-A during the very first events of vasculogenesis, the requirement for both genes in later steps of arterio-venous 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 (Gering and Patient, 2005). Interestingly, Shh has been demonstrated to positively influence the expression of *calcitonin receptor-like receptor alpha (calcrla)* (Nicoli et al., 2008), which ultimately results in *vegf-A* expression upstream of Notch. This is particularly significant in light of a recent finding (Wilkinson et al., 2012), where Hedgehog signaling was found to induce somatic *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 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. The requirement at later stages, however, is supported by a number of observations, and a picture has emerged where Vegf signaling in presumptive arteries induces PLC-γ1. Zebrafish mutant for *PlC-γ1* show a marked defect in formation of arteries and strongly reduced expression of *efnb2* (Lawson et al., 2003). *PLC-γ1* mutants cannot be rescued with *vegf-A* overexpression, suggesting PLC-γ1 downstream of Vegf receptor function in arterial signaling (Lawson et al., 2001; Lawson et al., 2003)(Lawson, Vogel et al. 2002).

## Notch and Hey2

The zebrafish Notch family members consist of four Notch receptors (notch1a, notch1b, notch2 and notch3) and several Notch ligands (DeltaA-D, Dll4, Jagged1a, Jagged1b and Jagged2) 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) (Kao et al., 1998; Nakagawa et al., 2000). The Notch signaling pathway has long been recognized as a key driver of arterial identity, and in recent years it has been extensively evaluated in this respect and also in its involvement during tip cell/stalk cell formation (Phng and Gerhardt, 2009). The latter aspect has been reviewed in detail elsewhere (Wilkinson and van Eeden, 2014), and we will here focus only on the arterio-venous specification role of Notch-Delta signaling.

The Notch-Delta signaling pathway appears to be restricted to the arterial endothelium in zebrafish (Villa et al., 2001), 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 (Lawson et al., 2001). 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 (Zhong et al., 2000). Gain-of-function of Notch family members causes a reduction of venous fate (Lawson et al., 2001). 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 (Lawson et al., 2001; Villa et al., 2001)



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, where after *gridlock* expression continues to be restricted to arteries (Zhong et al., 2000). Loss of Gridlock function in early zebrafish development results in defective proliferation of angioblasts at the level of the LPM (Chun et al., 2011). Later on, loss of *gridlock* results in a circulatory short-cut through a disrupted dorsal aorta, with concomitant increase of the venous maker *ephb4* and a decrease in the arterial marker *efnb2* (Zhong et al., 2000). More specifically, the point of fusion of the LDA to the DA is affected, which represents a remarkably specific and locally restricted phenotype (Weinstein et al., 1995; Zhong et al., 2001). Furthermore, overexpression of *gridlock* causes suppression of venous markers (Zhong et al., 2001). 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 (Nakagawa et al., 2000; Zhong et al., 2001). 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 the Gridlock phenotype. These results show that Gridlock functions downstream of the Hh-Vegf-Notch signaling pathway in arterial specification (Peterson et al., 2004; Rowlinson and Gering, 2010). As in other cases, the defect observable in mutants appears to be more pronounced in arterio-venous specification during angiogenesis, and less during vasculogenesis.

### SoxF family members

*SRY-related high mobility group box (sox)* genes form a family of transcription factors involved in diverse developmental processes, including vascular development (reviewed in (Chew and Gallo, 2009)). The Sox proteins contain two main domains, the high mobility group (HMG) domain which can bind target DNA motifs, and the transactivation domain, which mediates the transcriptional response (Bowles et al., 2000). The *sox* family is divided in several subfamilies, with the *sox-F* family members comprised of the *sox7, sox17* and *sox18* genes. Sox17 is involved in hematopoietic stem cell regulation and formation of endoderm (Alexander and Stainier, 1999; Kanai-Azuma et al., 2002; Kim et al., 2007; Chung et al., 2011), but has recently also been shown to play a role in arterial specification in the

mouse (Corada et al., 2013). Sox7 and Sox18 have long been recognized as being involved in vascular development (Cermenati et al., 2008; Herpers et al., 2008; Pendeville et al., 2008; Wat et al., 2012). The human syndrome Hypotrichosis-lymphedema-telangiectasia is linked to mutations in *SOX18*, and patients present with disrupted blood and lymphatic vessels (Irrthum et al., 2003). Mice with truncated SOX18 protein ('Ragged' mice) resemble HLT and display defects in blood and lymphatic vasculature development (Pennisi et al., 2000; James et al., 2003; Downes et al., 2009). In mice, *Sox18* starts to be expressed at ~E9.0 in a subpopulation of venous endothelial cells which induces *Prox1* expression in these cells. Subsequently, these *Prox1*-expressing cells migrate away from the veins under the influence of VEGF-C and become lymphatic endothelial cells (LECs) which will later form the lymphatic vascular network (Francois et al., 2008; Srinivasan et al., 2010). In contrast, zebrafish *prox1* and *sox18* are dispensable for vascular development, revealed by *prox1* and *sox18* mutants not having vascular problems (van Impel et al., 2014)

In zebrafish, Sox18 and Sox7 appear to play redundant roles in vascular development. *sox7* and *sox18* are expressed in the early pre-migratory angioblasts at the LPM, then in the migrating angioblast population, and later in the specified vasculature. Double morpholino knock-down showed defective blood circulation, and the DA and PCV are fused together in the trunk of the embryo, which results in arterio-venous shunt formation at a relatively late stage of development. Venous markers are up-regulated in the DA, whereas the arterial markers are down-regulated, suggesting a role for Sox7/Sox18 in arterio-venous specification (Cermenati et al., 2008; Herpers et al., 2008; Pendeville et al., 2008). The exact mechanism of Sox7 and Sox18 function in arterio-venous regulation is not known yet, however, recent evidence showed that the enhancer for the Notch ligand Dll4 contains a binding site for SoxF factors. Both SoxF and Rbpj transcription factors can bind and regulate Dll4 enhancer activity, suggesting an important role for SoxF family members in regulating Dll4 activity and subsequent arterio-venous specification (Sacilotto et al., 2013).

### Foxc and Ets

Forkhead (fox) transcription factors are helix-turn-helix proteins. Foxc transcription factors are expressed in the vasculature and *Foxc*-null mice die during embryonic development with severe vascular defects, including arteriovenous malformations and loss of arterial markers (Kume et al., 2001; Seo et al., 2006). Similarly, in zebrafish combined knockdown of *foxc1a* and *foxc1b* results in severe disruption of the vascular system (De Val et al., 2008). In vitro studies showed that Vegf signaling can induce the transcriptional activity of Foxc proteins (Seo et al., 2006). Overexpression of *foxc* genes induces expression of arterial markers, such as Notch1 and Dll4. Foxc can bind and activate the *dll4* promoter, suggesting that Foxc acts upstream of Notch signaling in arteriovenous specification (Seo et al., 2006). Furthermore, Foxc2 can interact with the Su(H)/NICD complex to induce *hey2* promoter activity (Hayashi and Kume, 2008). Foxc, together with the Ets factor Etsrp, bind to a FOX:ETS motif inducing

enhancer activation. This FOX:ETS domain is present in many endothelial specific enhancers suggesting the importance of the Foxc and Ets transcription factors in vascular development (De Val et al., 2008).



## SUMMARY

Within the last few years, we have witnessed a considerable number of studies that have significantly advanced our understanding of vasculogenesis and angiogenesis. Careful lineage analysis and meticulous comparison of expression data of various marker genes have shed new light on the nature of early angioblasts (Fig. 3). The later events of arterio-venous specification and differentiation have in turn benefited from genetic interference studies – here, it will be necessary to repeat some of the work with stable mutant lines rather than relying on morpholino data. This notwithstanding, we will continue to gain more insight into the early events of cardiovascular development from additional work in zebrafish embryos, which are so well suited for the combined application of genetics and in vivo imaging.

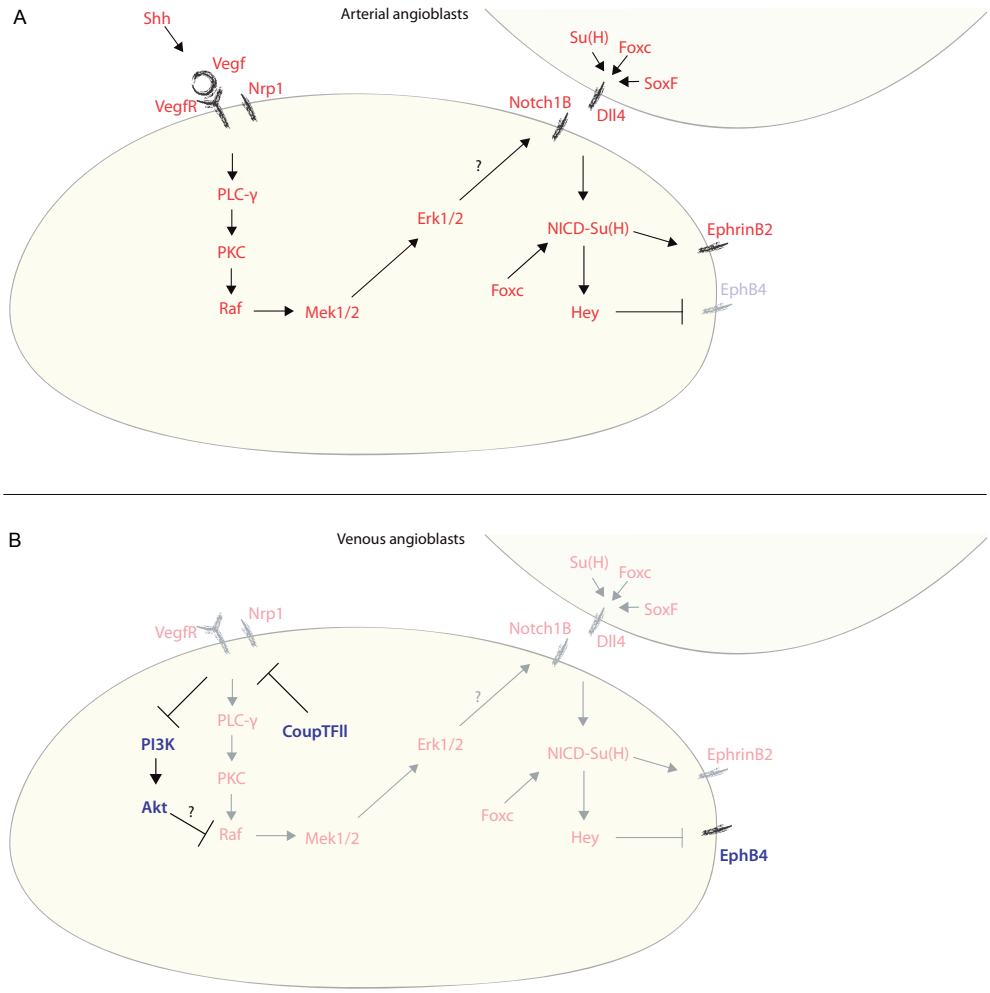


**Fig.3 Model of the molecular pathways in arterial and venous fated angioblasts.** Arterial specific genes are depicted in red, venous specific genes in blue. (A) Shh induces Vegf/Nrp1 signaling which activates PLC-γ, and subsequently the Mek/ERK pathway in arterial cells. Su(H), Foxc and SoxF can bind to the Dll4 enhancer activating Notch signaling. The Notch-intracellular domain (NICD) will be proteolytic cleaved upon activation and translocated to the nucleus, where it binds, together with Foxc, to Su(H). This complex can activate Hey2 signaling and induces the expression of EphrinB2 on arterial membranes. Hey2 is suggested to function as a transcriptional suppressor for Ephb4. (B) In venous fated angioblasts, these pathways are inhibited by the PI3K/Akt pathway, which counteracts the PLCγ/Mek/Erk pathway, and (in mice) by COUPTF2, which is suggested to suppress the Nrp1/VegfR pathway. In venous cells, Ephb4 expression on the membrane is no longer inhibited. Question marks indicate interactions that are unknown.

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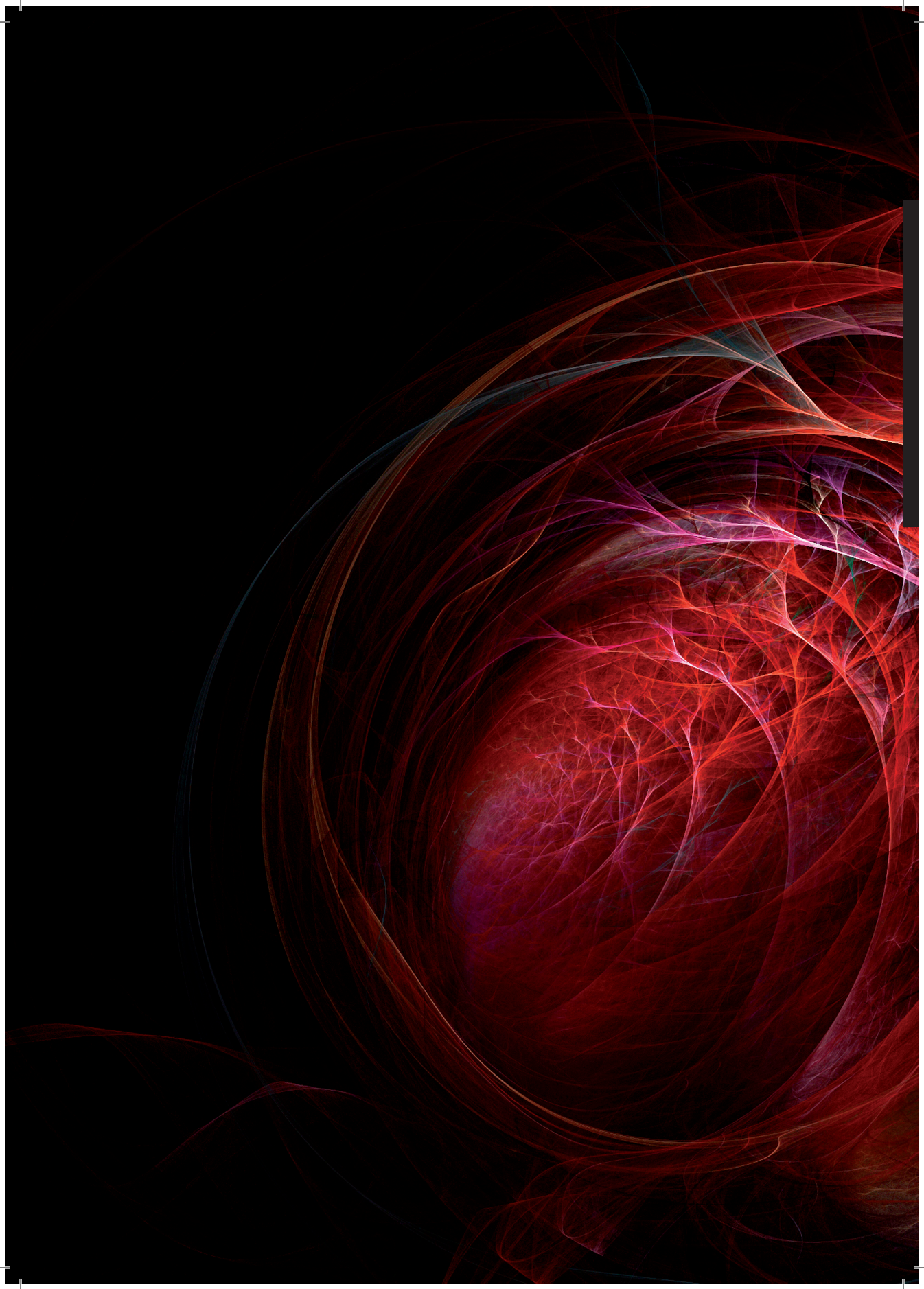

# CHAPTER 3

# Sox7 controls arterial specification in conjunction with Hey2 and EfnB2 function

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Development invited revision

**SoxF family members have been linked to arterio-venous specification events and human pathological conditions, but in contrast to Sox17 and Sox18, a detailed *in vivo* analysis of a Sox7 mutant model is still lacking. In this study we generated zebrafish *sox7* mutants to understand the role of Sox7 during vascular development. By *in vivo* imaging of transgenic zebrafish lines we show that *sox7* mutants display a short circulatory loop around the heart as a result of aberrant connections between the lateral dorsal aorta (LDA) and either the venous primary head sinus (PHS) or the common cardinal vein (CCV). *in situ* hybridization and live observations in *flt4:mCitrine* transgenic embryos revealed increased expression levels of *flt4* in arterial endothelial cells at the exact location of the aberrant vascular connections *sox7* mutants. An identical circulatory short loop could also be observed in newly generated mutants for *hey2* and *efnb2*. By genetically modulating levels of *sox7*, *hey2* and *efnb2* we demonstrate a genetic interaction of *sox7* with *hey2* and *efnb2*. The specific, spatially confined effect of loss of Sox7 function can be rescued by overexpressing the Notch-intracellular domain (NICD) in arterial cells of *sox7* mutants, placing Sox7 upstream of Notch in this aspect of arterial development. Hence, *sox7* levels are critical in arterial specification in conjunction with *hey2* and *efnb2* function, with mutants in all three genes displaying shunt formation and an arterial block.**

## INTRODUCTION

One of the first organs that develop in the vertebrate body is the vascular system. Abnormalities in vascular development can cause endothelial malformations ranging from severe birth defects to mild lesions (Brouillard and Vikkula, 2007). The vascular system consists of endothelial cells (ECs) which become specified into arterial, venous and lymphatic cells, eventually forming a functional vascular system. Arterial-venous specification starts early in development: in zebrafish the first arterial and venous vessels are formed around the 20-somite stage. Vascular endothelial growth factor (Vegf) receptors and their ligands play crucial roles during arterial-venous specification. Activation of the Vegf receptor by Vegf and Sonic Hedgehog (Shh) induces the PLC-gamma/Mek/Erk pathway and subsequently the Notch signaling pathway. Upon activation of the Notch receptor by binding to one of its ligands (Delta-like, Jagged), the Notch intracellular domain (NICD) is released from the plasma membrane via proteolytic processing. The NICD translocates to the nucleus where it can bind to Suppressor of Hairless (Su(H)). This complex can mediate transcription of *Hairy/Enhancer of Split (Hes)* and *Hes*-related genes *(Hey/HRT/HERP)* and the expression of *ephrinB2 (efnb2)* on the arterial membrane (Lawson et al., 2001; Zhong et al., 2001). Efnb2 is a member of the Ephrin family and is a largely arterial-specific transmembrane protein that functions as a ligand for the venous receptor tyrosine kinase Eph receptor B4 (Ephb4). Signaling requires cell-to-cell contact and can be bidirectional. The reciprocal signaling between Efnb2 and Ephb4 is crucial in arterial-venous specification (Wang et al., 1998; Gerety et al., 1999).

Previous work has suggested a role for the *SRY-related HMG box (sox)* gene family in various aspects of vascular development (reviewed in Francois et al., 2010). The *sox* gene family encodes transcriptions factors and consists of 10 subgroups (SoxA-J). All members of the *sox* family contain a high mobility group box (HMG) domain which facilitates DNA binding in the minor groove and mediates DNA bending (Giese et al., 1992), and a transactivation domain (TAD), which activates transcription of target genes (Hosking et al., 1995). One subgroup of the Sox family which is of particular interest for vascular development is the SoxF group, consisting of SOX7, SOX17 and SOX18. *In vitro* studies revealed that SoxF transcription factors can bind the arterial specific enhancer of the notch ligand *dll4* (Sacilotto et al., 2013). Furthermore, Sox17 has recently been shown to play a key role in endoderm formation, hematopoietic stem cell regulation and the acquisition of arterial identity by functioning upstream of Notch signaling (Hudson et al., 1997; Kanai-Azuma et al., 2002; He et al., 2011; Corada et al., 2013). Mutations in *SOX18* are linked to the Hypotrichosis-lymphedema-telangiectasia (HLT) syndrome in humans in which patients have severe lymphedema, vascular leakages and disrupted hair follicle development (Irrthum et al., 2003). Mice with a truncated Sox18 protein (mutation in the *ragged opossum* allele) resemble this syndrome, resulting in severe edema, blood vessel disruption and early lethality (Pennisi et al., 2000; James et al., 2003). On certain genetic backgrounds in mice, Sox18 is required for the differentiation of ECs in lymphatic cells by initiating expression of Prox1 (Francois et al., 2008). Recent work in zebrafish, however, has shown that *sox18* is dispensable for lymphatic specification in the fish (van Impel et al., 2014). A simultaneous knockdown of *sox7* and *sox18* transcripts in zebrafish results in disruption of arterial-venous segregation at 48 hpf, followed by shunt formation between the dorsal aorta (DA) and the posterior cardinal vein (PCV). *sox7/sox18* double morphants display an increase of venous markers and a decrease of arterial markers in the DA, corroborating the involvement of Sox7/Sox18 in arterial-venous specification (Cermenati et al., 2008; Herpers et al., 2008; Pendeville et al., 2008). Previous reports in zebrafish could not identify a specific vascular function for Sox7, though these studies were limited by the use of morpholinos (Cermenati et al., 2008; Herpers et al., 2008; Pendeville et al., 2008). Mice lacking *Sox7* die at embryonic day 10.5 due to cardiovascular failure (Wat et al., 2012), and this early lethality, together with delayed development, pericardial edema and failure of yolk sac remodeling, precludes analysis of underlying cellular mechanisms. To understand the specific role of Sox7 during vascular development, we generated a zebrafish *sox7* mutant. We here demonstrate a highly specific arterial-venous shunt phenotype in *sox7* mutants and connect Sox7 function to Hey2/Notch signaling and to Efnb2 function.

## METHODS

### Zebrafish

Zebrafish were maintained under standard husbandry conditions according to the rules of the Animal Experimentation Committee (DEC) of the KNAW. Transgenic lines used are *Tg(fli1a:eGFP)*$^{y1}$ (Lawson and Weinstein, 2002), *Tg(kdrl:eGFP)*$^{s843}$ (Jin et al., 2005), *Tg(gata1:dsRed)*$^{sd2}$ (Traver et al., 2003), *Tg(kdrl:HRAS-mCherry)*$^{s916}$ *(Hogan et al., 2009a)*, *Tg(flt4:mCitrine)*$^{hu7135}$ (van Impel et al., 2014), *Tg(Sox17:eGFP) (Mizoguchi et al., 2008)* and *Tg(UAS:myc-NICD) (Scheer and Campos-Ortega, 1999).* The *Tg(dll4:Gal4FF*$^{hu10049}$) line was generated from BAC CH211-19M2 following standard recombineering procedures (Bussmann and Schulte-Merker, 2011). The *sox18*$^{hu10320}$ allele has been previously described (van Impel et al., 2014).

### Genotyping, morpholinos and TALEN constructs

Genotyping of *sox7*, *efnb2a* and *efnb2b* was performed by KASPAR with primers listed in table S1. *hey2* MO used: 5'-CGCGCAGGTACAGACACCAAAAACT-3'. *hey2* TALEN binding sites: TAL1: 5'-TGTCGTTGTAGGTGA GCCA-3' and TAL2: 5'-TATTCCCAGCCAAAGCAATGG-3' (primers mentioned in table S1).

### Histology and *in situ* hybridization

ISH and immunohistochemistry (IH) were performed as described previously (Schulte-Merker, 2002). For IH the embryos were stained with Rabbit anti-GFP 1:1000 and anti-Rabbit Alexa 488 1:1000 (Invitrogen), after which the embryos were paraffin-embedded and sectioned (7µm). For ISH we used previously described *hey2* (Zhong et al., 2000), *sox18* (Herpers et al., 2008), *efnb2* (Chan et al., 2001), *notch3* (Lawson et al., 2001) and *flt4* (Bussmann et al., 2007) probes.

### Microscopy

Confocal imaging was performed on live embryos embedded in 0.5% low melting point agarose (Invitrogen) with a Leica TCS SPE. Bright field pictures are taken with the Zeiss Axioplan microscope. Images were processed using Adobe Photoshop CS5.1 and Fiji (http://fiji.sc/Fiji).

## RESULTS

### Blood circulation is perturbed in *sox7* mutant embryos

Previous observations in zebrafish demonstrated that *sox7* is expressed in ECs of the major vessels in the head and trunk, such as the PCV and the (lateral) DA (Cermenati et al., 2008; Herpers et al., 2008; Pendeville et al., 2008) (Fig. 1A). To assess the specific function of Sox7, we generated *sox7* mutants by targeting induced local lesions in genomes (TILLING) (Wienholds et al., 2002). The *sox7*$^{hu5626}$ allele comprises a guanine-to-adenine mutation leading to a predicted premature stop-codon (after amino acid 53) within the HMG domain (Fig. 1B, S1). The overall appearance of these mutants is normal during early development (Fig. 1C), however we noticed severe edema formation in mutant embryos from 72 hpf onwards leading to lethality by day 5. Analysis of homozygous *sox7* mutants in a *kdrl:eGFP;gata1:dsRed* transgenic background revealed that the majority (59%, n = 275 embryos) lack blood circulation in the trunk from the start of circulation onwards (Fig. 1D,E).

To further characterize the blood circulation defect in *sox7* mutants, we performed micro-angiographies. In *sox7* mutants, the Rhodamine Dextran dye distribution can be detected from the injection site within the PCV towards the beating heart, however, we never observed distribution from the heart into the axial DA. This indicates complete blockage of blood flow at the anterior part of the DA (Fig. 2A). Cross-sections of 48 hpf *sox7* mutants revealed a disrupted morphology of ECs and a defective lumen formation at the position where in sibling embryos both lateral dorsal aortae (LDA) fuse to form the dorsal aorta (DA) (Fig. 2B,S2). Further analysis of *sox7* mutants at 2.5 dpf in *kdrl:mCherry;flt4:mCitrine* transgenic embryos, in which we could distinguish arterial and venous ECs, revealed a direct connection between the LDA and the venous primary head sinus (PHS) or the common cardinal vein (CCV) (Fig. 2C).

In wild type embryos the blood is guided from the bulbus arteriosus (BA) and ventral aorta (VA) into either the LDA and subsequently to the DA in the trunk, or from the VA to the primitive internal carotid artery towards the head region. The blood from the trunk is returned towards the heart by the posterior cardinal vein (PCV) and the CCV and from the head via the posterior hindbrain channel, the anterior cardinal vein and the primary head sinus (PHS) to the CCV and the heart. The shunt in *sox7* mutants causes the blood to circulate from the BA and VA into the LDA from where it directly returns via the PHS and CCV to the heart without entering the trunk region. Consistently, ectopic connections between the LDA and the PHS and between the LDA and CCV are evident in *sox7* mutants (Fig. 3A,B).

Previously reported *sox7/sox18* double morphants (Fig. 3D), have multiple ectopic connections between the DA and PCV (Cermenati et al., 2008; Herpers et al., 2008; Pendeville et al., 2008). To exclude toxic effects of combined morpholino knockdown, we generated *sox7;sox18* double mutants and found multiple shunts between the DA and PCV in double mutant embryos. In contrast, no ectopic connections between the DA and PCV

were observed in *sox7* single mutants (Fig. 3C), pointing to a highly specific non-redundant local requirement of normal Sox7 levels in the LDA.

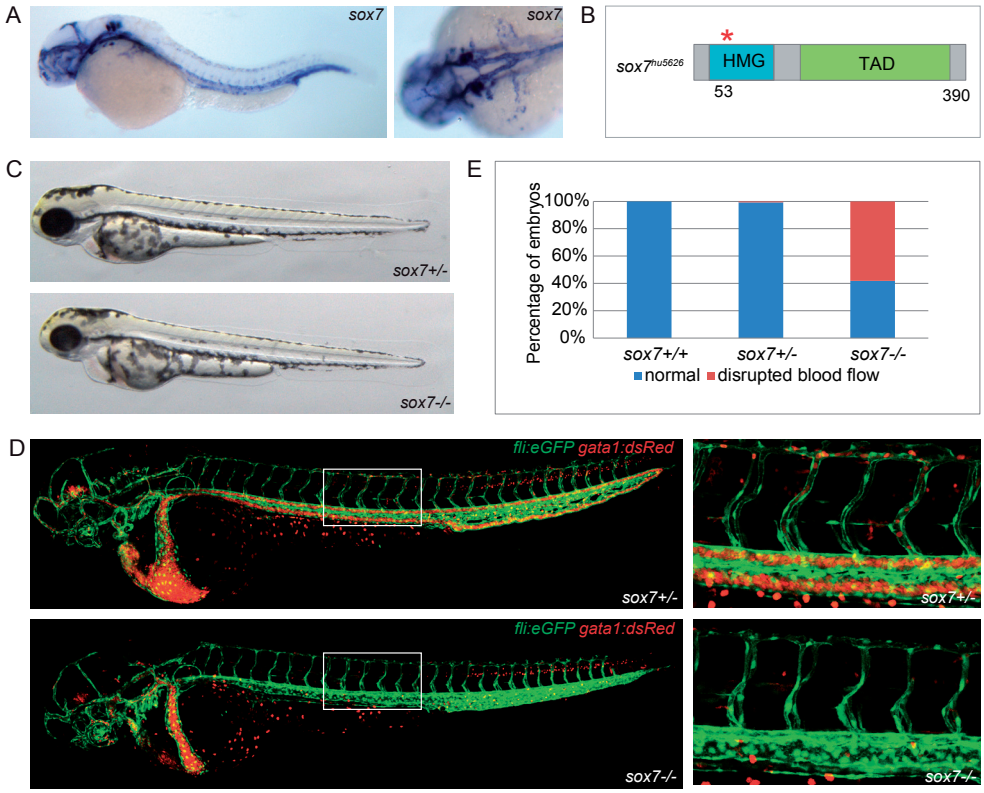


**Fig.1 Disrupted blood circulation in *sox7* zebrafish mutants.** (A) *sox7* ISH of 3 dpf wild-type embryos, left panel lateral view, right panel dorsal view. Note *sox7* expression in all main vessels. (B) Schematic diagram of *sox7*$^{hu5626}$ allele with a premature stop-codon after amino acid 53 (red asterisk). HMG = high mobility group box, TAD = trans-activating domain. (C) Overall normal appearance of *sox7*$^{hu5626}$ heterozygous sibling and homozygous mutant at 2 dpf. (D) *kdrl:GFP;gata1:dsRed*-positive *sox7*$^{hu5626}$ mutants depict a short circulatory loop around the heart, whereas heterozygous siblings have normal circulation. Right panel: higher magnification of boxed area. (E) On average, 59% of *sox7*$^{hu5626}$ mutants display disturbed blood flow at 2.5 dpf. Percentages of pooled embryos from 4 independent experiments (275 embryos). Note that percentages can vary substantially between different backgrounds.

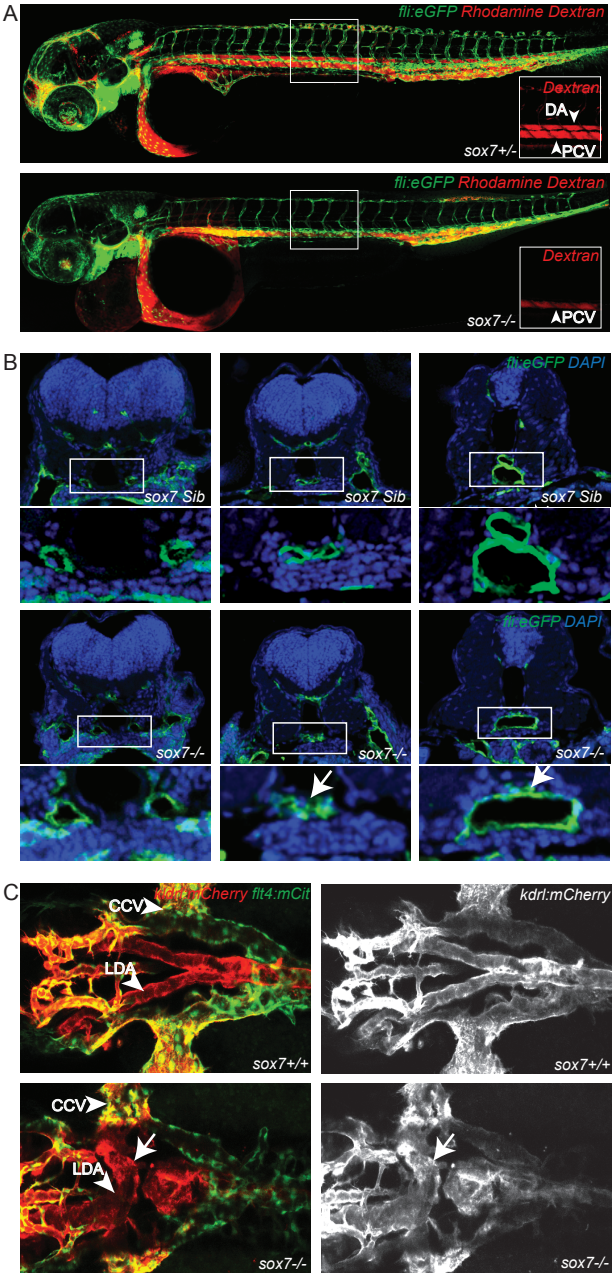


**Fig.2 *sox7* mutants show an altered morphology of the LDA while displaying normal DA-PCV segregation in the trunk.** (A) Rhodamine-Dextran angiograms (injected into the PCV) of *sox7*$^{hu5626}$ *Fli1:eGFP* siblings (upper panel) and mutants (lower panel). Insert lower right corner depicts Rhodamine-Dextran channel only of boxed area with higher magnification. Note lack of dye uptake in DA of *sox7*$^{hu5626}$ mutants. (B) Transverse sections of *fli1a:eGFP*-positive *sox7*$^{hu5626}$ sibling and mutant embryos, stained with anti-GFP (green) and Dapi (blue). Aorta morphology is disturbed at the position of LDA/DA fusion in *sox7*$^{hu5626}$ mutants (arrows). For positions of sections see Fig.S2. (C) Dorsal view of *sox7*$^{hu5626}$ *kdrl:mCherry;flt4:mCitrine (kdrl:mCherry;fl4:mCit)* sibling and mutant embryos at 2.5 dpf. Right panel *kdrl:mCherry* channel only. Note shunt formation in *sox7*$^{hu5626}$ mutants at the position of LDA fusion (arrow). (L)DA = (lateral) dorsal aorta, PCV = posterior cardinal vein, CCV = common cardinal vein.

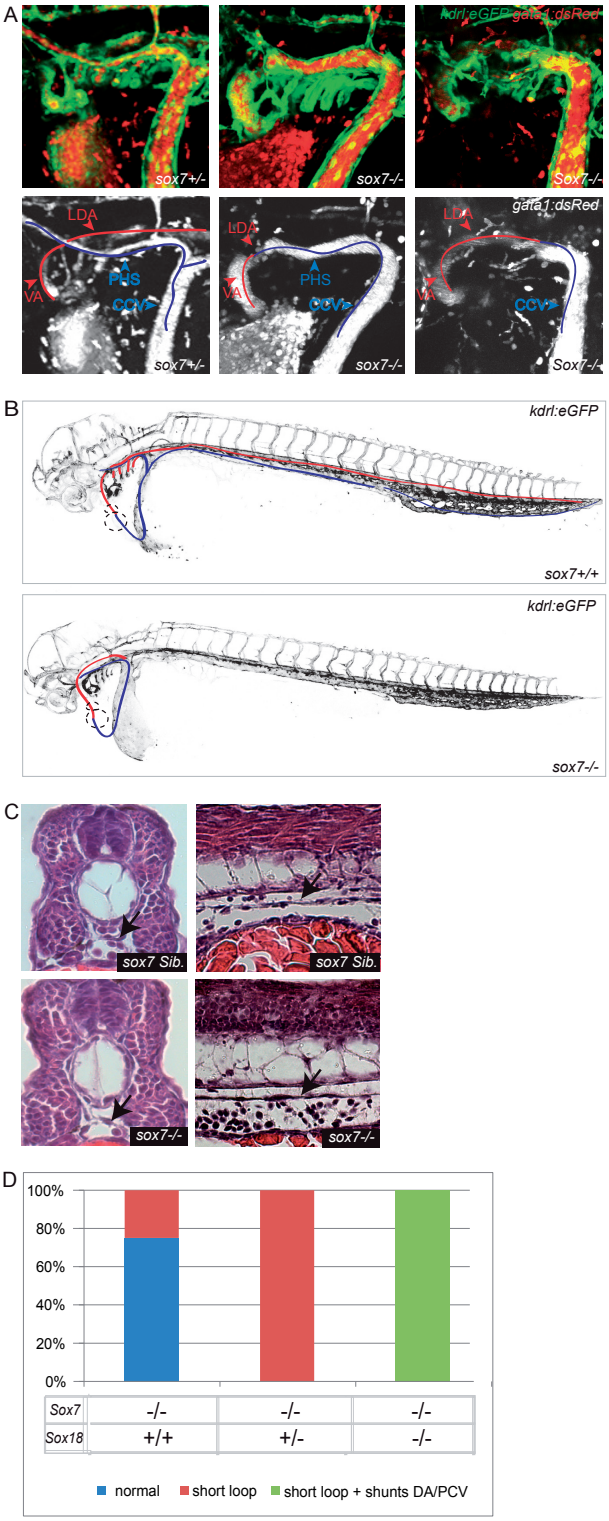


**Fig.3 *sox7* mutants develop a circulatory short-loop phenotype.** (A) *sox7*$^{hu5626}$ *kdrl:eGFP;gata1:dsRed* mutants at 2.5 dpf with shunt formation resulting in a circulatory short-loop (middle and right panel) in contrast to normal circulation in *sox7*$^{hu5626}$ siblings (left panel). Middle panel depicts ectopic connection between LDA and PHS, right panel between LDA and CCV. Lower panel only *gata1:dsRed* with detailed schematic representation of blood flow. CCV = common cardinal vein, LDA = lateral dorsal aorta, PHS = primary head sinus, VA = ventral aorta. (B) Schematic representation of normal blood flow in wild-type embryos compared to the short-circuit in *sox7*$^{hu5626}$ mutants. Dashed circles highlights the position of the heart. (A+B) Red and blue lines depict blood flow in arteries and veins, respectively. (C) Hematoxylin and eosin stained transverse (left panel) and sagittal (right panel) cross-sections of trunks of 2 dpf *sox7*$^{hu5626}$ siblings (top) and homozygous mutants (bottom). Note normal DA-PCV segregation in both siblings and mutants (arrows). (D) Quantification of the phenotypes in *sox7*$^{hu5626}$*;sox18*$^{hu10320}$ loss-of-function embryos. Note all *sox7*$^{hu5626}$*;sox18*$^{hu10320}$ double homozygous mutants have a short-loop of circulation and shunts between DA and PCV, whereas *sox7-/-sox18+/+* and *sox7-/-sox18+/-* embryos have no shunts between DA and PCV. The remaining, non-shown genotypic combinations of *sox7;sox18* embryos have all 100% wild-type phenotype. Bars show percentages of embryos of a representative experiment (n = 77 embryos).

To explore the expression of the other SoxF member, Sox17, in the context of LDA development, we made use of the *sox17:eGFP* transgenic line (Mizoguchi et al., 2008). While *sox17* is expressed in the arterial cells of the dorsal aorta and ISVs, we could not detect *sox17:eGFP* expression in the LDA of *sox7* siblings and mutants. Furthermore, arterial *sox17:eGFP* levels in *sox7* siblings and mutants are indistinguishable at 1 and 2 dpf (Fig. S3 and data not shown). Hence, while we cannot exclude an involvement of Sox17 in LDA formation, the expression analysis does not support an involvement, while *sox7* and *sox18* mRNAs can readily be detected in ECs of the LDA.



### *fms-related tyrosine kinase 4 (flt4)* expression is altered in arterial cells of *sox7* mutants

To assess the involvement of *sox7* in vascular development, we performed whole mount *in situ* hybridization (ISH) for arterial and venous specific genes. This revealed *flt4,* also called *vegfr3,* expression to be up-regulated in arterial cells at the location of ectopic arterio-venous connections in most *sox7* mutants at 20 hpf (Fig. 4 A,D). Vegfr3/Flt4 is a transmembrane tyrosine kinase receptor for the ligands Vegf-C and Vegf-D and becomes quickly restricted to only venous and lymphatic endothelial cells (Kaipainen et al., 1995; Joukov et al., 1996; Achen et al., 1998; Hogan et al., 2009b; van Impel et al., 2014). We confirmed our observation of induced *flt4* expression with a transgenic reporter line and found *flt4:mCitrine* expression to be specifically increased in the LDA of *sox7* mutants at 26 hpf, while there is no detectable expression in the LDA of wild-type embryos at this time point (Fig. 4B). Expression of *flt4* was also increased in the DA and ISVs in 58% of *sox7* mutants at 26 hpf (Fig. 4C,E). While the ectopic expression of this venous marker would suggest differential expression of other arterial and/or venous markers, we did not detect this (for *notch1b, notch3, efnB2, hey2, dll4, foxc1, sox18*, *dab2 and ephb4*) in *sox7* mutants at several time points (20-30 hpf) (Fig. 4A,C; and data not shown).

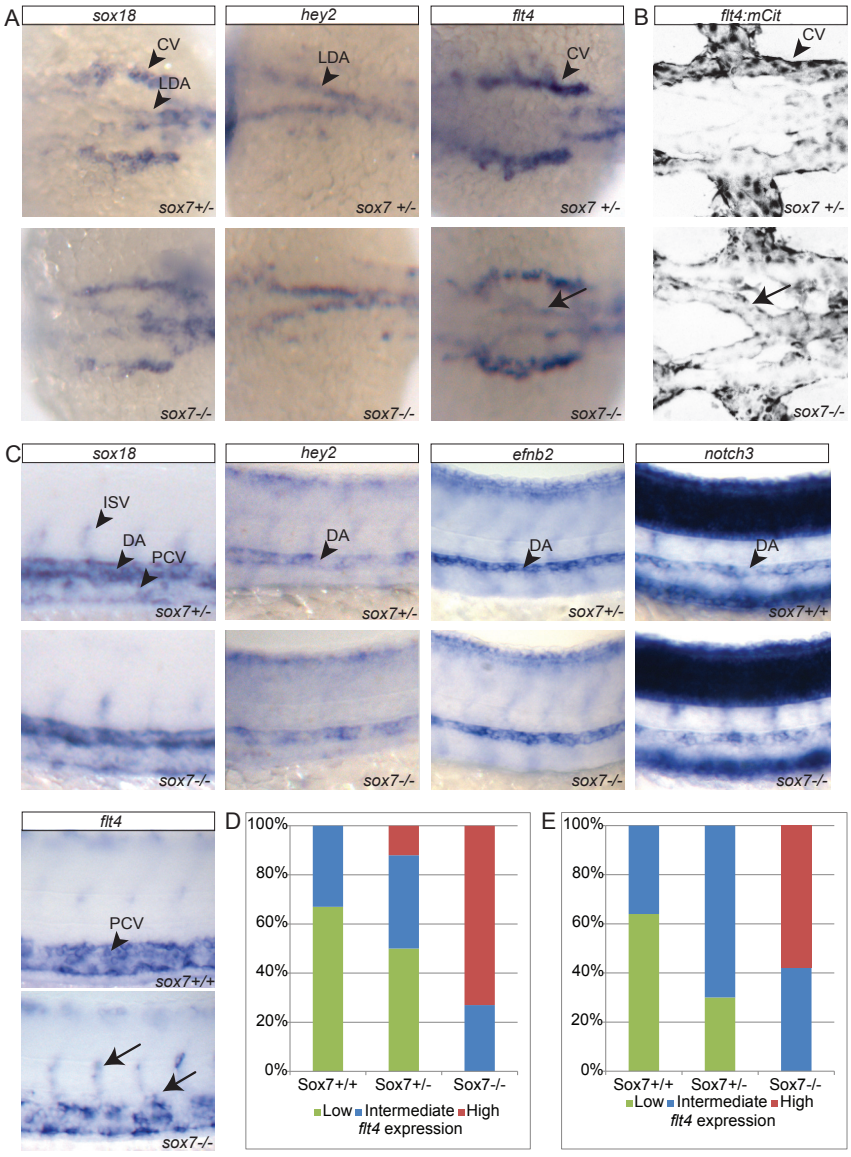


**Fig.4 *flt4* expression is altered in arterial cells of *sox7* mutants.** (A-C) *sox7*$^{hu5626}$ siblings upper panel, mutants lower panel. (A) ISH for *sox18, hey2* and *flt4* in *sox7*$^{hu5626}$ heterozygous and mutant embryos at 20 hpf (dorsal view). Note elevated *flt4* expression levels in the LDA of *sox7*$^{hu5626}$ mutants (arrow). (B) Dorsal view of *flt4:mCitrine (flt4:mCit)*-positive *sox7*$^{hu5626}$ heterozygous and homozygous mutant at 26 hpf. Note elevated *mCitrine* expression in the LDA of *sox7*$^{hu5626}$ mutant (arrow) compared to *sox7*$^{hu5626}$ sibling. (C) Lateral view of trunk region of *sox7*$^{hu5626}$ siblings and mutants with ISH for *sox18, hey2, efnB2, notch3* and *flt4* at 24-27 hpf. *sox7*$^{hu5626}$ mutants display higher *flt4* expression in DA and ISVs (arrows) compared to siblings. (D) Quantification of 20-24 hpf embryos based on their *flt4* expression levels (ISH) in the LDA. 73% of *sox7*$^{hu5626}$ mutants have high expression of *flt4* in LDA region at 20 hpf (n = 30 embryos). (E) Quantification of 24-27 hpf embryos based on their *flt4* expression levels (ISH) in the trunk. 58% of *sox7*$^{hu5626}$ mutants have elevated *flt4* expression, which was never observed in siblings (n = 57 embryos). (L) DA = (lateral) dorsal aorta, (P)CV = (posterior) cardinal vein, ISV = intersegmental vessel.

### *efnb2* and *hey2* genetically interact with *sox7*

The spatially confined defect in *sox7* mutants is a rather unique phenotype, however, we serendipitously observed identical defects in double mutants for *efnb2a* and *efnb2b*. Both *efnb2* mutants were generated by TILLING, and contain point-mutations leading to predicted premature stop-codons (after amino acid 86 in *efnb2a*$^{hu3393}$ and amino acid 78 in *efnb2b*$^{hu2971}$) (Fig. 5A). We found that *efnb2a* as well as *efnb2b* single mutants do not develop any obvious vascular defects, however simultaneous loss of both genes resulted in circulatory short-circuits in 42% of double mutants (Fig. 5B, C). We investigated a possible connection between *sox7* and *efnb2* genes by generating triple mutants. Importantly, increasing the number of loss-of-function alleles was accompanied by an increase in the amount of embryos displaying the short-loop phenotype. Furthermore, while all *efnb2a* mutants had normal circulation, and while only 17% of *sox7* mutants exhibited the short-loop phenotype in this genetic background, 78% of *sox7;ephb2a* double mutants developed a circulatory short-loop, revealing a strong genetic interaction between *ephb2a* and *sox7* (Fig. 5D).

*Hairy/enhancer-of-split related with YRPW motif 2* (*hey2*) is a *hes*-related gene that is expressed in angioblasts from early development onwards. Previous studies showed that mutations in *hey2* (*gridlock* mutants) display ectopic arterial to venous connections (Weinstein et al., 1995). *hey2* is a downstream target of the Notch signalling pathway and participates in arterial cell-fate specification (Nakagawa et al., 2000). Injections of a *hey2*-morpholino (MO) recapitulated the *sox7* loss-of-function phenotype in our hands with lack of blood flow in the trunk and a circulatory short-loop near the heart (Fig. 6A). To investigate a possible connection between *sox7* and *hey2* we titrated down the injected amounts of *hey2* MO so that only minor vascular defects in wild-type embryos are evident upon injection (8% with 0.3ng). When injected into the offspring of a *sox7*$^{+/-}$ incross, we observed an increase in the number of embryos displaying the short-loop (37% with 0.3ng *hey2* MO, 5% in un-injected s*ox7*$^{+/-}$ in-cross) (Fig. 6A). To confirm this observation we generated *hey2* TALEN constructs targeting exon 2 upstream of the two important domains, the basis helix-loop-helix (bHLH) DNA binding domain and the Orange domain (confers specificity among the *hairy/enhancer-of-split* family*)* (Fig. 6B). Transient *hey2* Talen mRNA injections also resulted in the same specific circulatory defects in a subset of embryos, again mimicking the *sox7* mutant phenotype (data not shown). When transiently injected into a *sox7*$^{+/-}$ in-cross the *hey2* Talen mRNA resulted in a moderate increase of the short-circuit penetrance (Fig. 6C), thereby confirming the MO data and providing independent support for the notion that both genes function together in arterial specification.

### Sox7 functions upstream of Notch-ICD in arterial specification

Recently, *in vitro* studies reported that SoxF transcription factors can bind the arterial specific enhancer of the notch ligand *dll4* (Sacilotto et al., 2013). Since Hey2 and Efnb2a/b have been

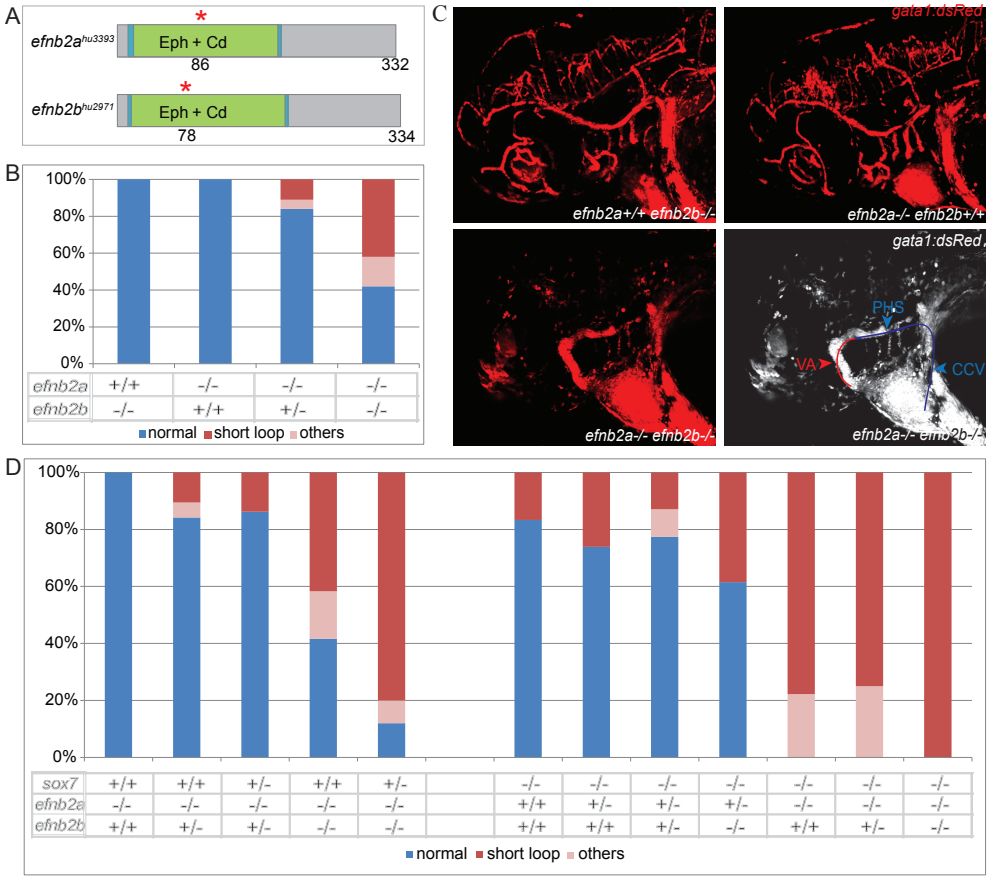


**Fig.5 *efnb2a;efnb2b* double mutants develop a circulatory short-circuit phenotype and *sox7* genetically interacts with *efnb2a*.** (A) Schematic diagram of *efnb2a*$^{hu3393}$ and *efnb2b*$^{hu2971}$ alleles with premature stop codons (red asterisks) at amino acid 86 and 78, respectively. Eph = Ephrin domain (green), Cd = Cupredoxin domain (blue). (B) Quantification of *efnb2a*$^{hu3393}$ and *efnb2b*$^{hu2971}$ single and double mutant phenotypes revealed circulatory short-looping in 42% of double homozygous mutants and in 11% of *efnb2a*$^{hu3393-/-}$;*efnb2b*$^{hu2971+/-}$ embryos. Bars represent combined results of 5 independent experiments (n = 128 embryos). (C) Blood circulation in *gata1:dsRed*-positive *efnb2a*$^{hu3393}$;*efnb2b*$^{hu2971}$ single and double mutant embryos. Note normal circulation in single mutants (upper panel) and short-loop in *efnb2a*$^{hu3393}$;*efnb2*$^{hu2971}$ double mutants (lower panel). Schematic representation of blood flow in double mutants (lower right panel). Lines depict blood flow in arteries (red) and veins (blue). VA = ventral aorta, PHS = primary head sinus, CCV = common cardinal vein. (D) Combinations of *sox7*$^{hu5626}$, *efnb2a*$^{hu3393}$ and *efnb2b*$^{hu2971}$ loss-of-function alleles result in increasingly severe effects on the circulatory system. Note that all *sox7*$^{hu5626}$;*efnb2a*$^{hu3393}$ double homozygous mutant embryos exhibit a disrupted circulatory system, whereas single mutants display no phenotype (*efnb2a*$^{hu3393}$), or are only partially penetrant (*sox7*$^{hu5626}$). Bars represent combined results of 5 independent experiments (n = 507 embryos). (B,D) The genotype combinations not shown all displayed 100% wild-type phenotype. Embryos were analyzed at 2.5 dpf. Phenotypes classified as 'others' are edema, total lack of circulation and/or shunts in trunk region.

suggested to act downstream of Notch signaling (Lawson et al., 2001) and since we show here that *sox7* genetically interacts with both factors, we wondered whether the arterial defects in *sox7* mutants are a consequence of altered Notch signaling levels. We increased Notch signaling levels in arterial ECs by expressing the Notch1 intracellular domain (NICD) under the control of a newly established, arterial-specific *dll4* BAC-transgenic line (Fig. S4). We found that *dll4:Gal4FF;UAS:NICD* expression in *sox7* mutants partially rescued the short-loop phenotype compared to *sox7* mutants lacking the *dll4:GalFF;UAS:NICD* construct (3% versus 27% within the same genetic background; n = 386 embryos Fig. 6D). This demonstrates that elevated levels of arterial Notch1 signaling are sufficient to suppress the vascular defects in *sox7* mutants providing direct *in vivo* evidence that Sox7 acts upstream of Notch1 signaling in arterial specification.

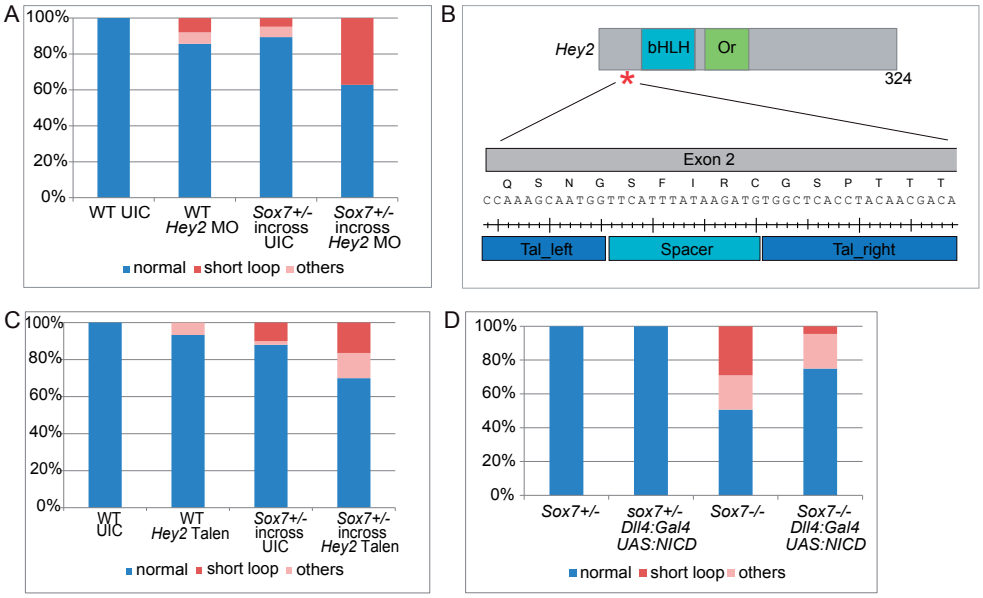


**Fig.6 *sox7* synergizes with *hey2* and functions upstream of Notch-ICD in LDA development.** (A) The partially penetrant short-loop phenotype of both, *sox7*$^{hu5626}$ mutants and *hey2* morphants (0.3ng), are exacerbated when the *hey2* MO is injected into a *sox7*$^{hu5626+/-}$ in-cross (n = 35). Bars show percentage of embryos of a representative experiment. (B) Schematic diagram of Hey2 indicating the TALEN target site in exon 2, which is upstream of the coding region of the basic helix-hoop-helix (bHLH) DNA binding domain and the orange (Or) domain. (C) Transient injections of 10pg *hey2* TALEN mRNA into wild-type and *sox7*$^{hu5626}$ heterozygous in-cross. Note the moderate enhancement of short-loop phenotype penetrance in *sox7*$^{hu5626}$ in-cross with *hey2* TALEN compared to un-injected *sox7*$^{hu5626}$ in-cross and *hey2* TALEN injections into wild-type embryos. (D) Arterial specific UAS:NICD over-expression using the *dll4:Gal4* driver line significantly (student's t-test p<0.01) rescues the short-loop circulatory phenotype in *sox7* mutants: short-loop phenotype in 27% of mutants without NICD overexpression (n = 70 embryos), and in 3% with NICD overexpression (n = 45 embryos). Bars represent pooled embryos of 3 independent experiments (n = 300). Embryos in A,C,D are 2.5 dpf. Phenotypes classified as 'others' are edema, total lack of circulation and/or shunts in trunk region.

## DISCUSSION

Here we report for the first time a detailed analysis of a genetic *sox7* loss-of-function model. *sox7* zebrafish mutants display highly specific, locally restricted defects within the vasculature. Since *sox7* is expressed in all ECs, this suggests redundant roles for SoxF family members, in which Sox17 and/or Sox18 can partially compensate for the loss of *sox7* in most vascular beds. The notion of redundancy is further supported by *sox18* zebrafish mutants not showing abnormalities during early vascular development (van Impel et al., 2014), while combined *sox7* and *sox18* loss-of-function situations display rather comprehensive defects, with multiple shunts between the DA and PCV in the trunk (Cermenati et al., 2008; Herpers et al., 2008; Pendeville et al., 2008) (and this study, Fig. 3D). Furthermore no ectopic connections between the DA and PCV were detected in *sox7*$^{-/-}$;*sox18*$^{+/-}$ embryos, whereas the short loop of circulation is present, suggesting that one copy of *sox18* (possibly in conjunction with *sox17* function) is able to establish normal DA-PCV segregation in the trunk of the zebrafish. Why these compensatory mechanisms do not prevail in the LDA remains enigmatic.

In our analysis of arterio-venous markers, only *flt4* levels appeared to be changed to a detectable level, while differential expression of other arterial and/or venous markers in *sox7* mutants could not be detected. This could theoretically be explained by very specific and direct signaling between *sox7* and *flt4.* It seems more likely, however, that the process in question is modulated by a delicate balance of genetic interactions, where very subtle changes in expression level (too subtle to detect by ISH) can lead to severe disturbance of AV specification. This can also explain the lack of differential expression of arterial markers such as the Notch target genes in *sox7* mutants, since we demonstrate here that Sox7 can act upstream of Notch. Furthermore, the changes in expression can be highly time-restricted, leading to differential expression in only a short time interval. Although our analysis was performed in 30 minutes intervals (between 20-30 hpf), it is still possible that differential expression (possibly also only on the protein level) in even a shorter time-interval can lead to severe A-V disturbances.

Flt4 expression in the trunk becomes restricted to venous cells around 24 hpf in zebrafish (Hogan et al., 2009b). Hence, it is possible that the ectopic *flt4* expression in arterial cells of *sox7* mutants results from a lack of proper down-regulation of *flt4* specifically in these arterial cells. Previous work in mice and cultured ECs demonstrated that Efnb2 can mediate the internalization and signaling of Flt4 (Wang et al., 2010). Since we showed that Sox7 can function in Efnb2 signaling and that *flt4* expression is up-regulated in *sox7* mutants, it is likely that in *sox7* mutants the internalization and signaling of Flt4 is compromised possibly via Efnb2 signaling. The precise regulation between Flt4 and Efnb2 in arterial development remains to be elucidated.

Furthermore, Flt4 and its ligands Vegf-C and Vegf-D have been shown to be involved in endothelial cell sprouting and migration of lymphatic endothelial cells (Karkkainen et

al., 2004; Hogan et al., 2009b; Villefranc et al., 2013). v*egf-d* is suggested to genetically interact with *sox18* (Duong et al., 2014) and *vegf-d* is expressed near the CCV and the PHS at 1.5dpf (Astin et al., 2014). *Vegf-c* expression is initiated at the 18 somite stage (18 hpf) in the hypochord, which is located closely to the notochord and the DA (Covassin et al., 2006). At 24 hpf *vegf-c* becomes expressed in the DA and the LDA, whereas *flt4* becomes progressively restricted to venous cells (Covassin et al., 2006). Furthermore, *vegf-c mutants* and morphants lack the venous posterior hindbrain channel (closely located to the LDA) (Covassin et al., 2006; Hogan et al., 2009b; Villefranc et al., 2013), suggesting an interplay between Vegf-C and Flt4 in these vessels. It is a possibility that the elevated *flt4* levels in the arterial cells of the *sox7* mutants results in a disrupted balance between Flt4 and Vegf-C/D signaling in ECs of the LDA and the ECs of the surrounding venous vessels, thereby misguiding the ECs and forming ectopic shunts.

The very specific circulatory defects in *sox7*, *hey2* and *efnb2* mutants, together with the genetic interaction study presented here (Fig. 3) strongly suggests a synergistic relationship between *sox7* and both *hey2* and *efnb2*. Whether these proteins function in one pathway or in parallel pathways that synergize to function in arterial development remains unclear at present. We suggest a model where Sox7 (and possibly in a partially redundant manner Sox17 and Sox18) acts upstream of the Notch-signaling pathway while controlling Flt4 expression and functioning in conjunction with Hey2 and Efnb2 in arterio-venous specification.

The locally restricted effect of the *sox7* mutation indicates a level of redundancy in terms of the activity of SoxF family members to a degree that, e.g., leads to AV defects in the trunk only upon severe reduction of both Sox7 and Sox18 (Herpers et al., 2008, this study). All endothelial cells appear to be able to compensate for the loss of Sox18 function (van Impel 2004), and most endothelial cells can compensate for loss of Sox7 function (this study) – the only exception being the endothelial cells of the LDA, which critically depend on Sox7 function. It is unclear at present why the LDA represents a vascular bed that displays this specific requirement, but the occurrence of the same locally restricted phenotype in 3 different mutant scenarios indeed points towards unique features in the genetic control of endothelial cell behavior within the LDA.

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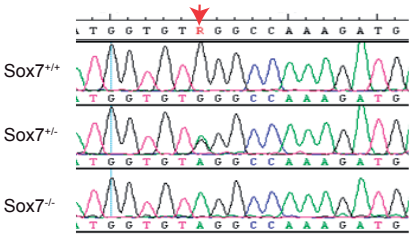
## SUPPLEMENTARY MATERIAL

### Supplementary Figures

S1




S2

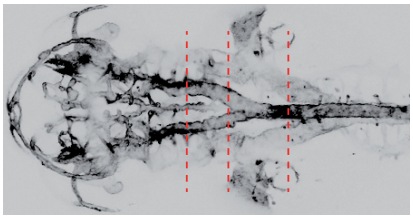


**Fig.S1 Sequence of the *sox7*$^{hu5626}$ allele.** Chromatogram of part of the *sox7* sequence comprising the site of the nonsense mutation (GTG>GTA) in a heterozygous (middle panel) and homozygous mutant *sox7*$^{huXYZ}$ embryo (bottom panel).

**Fig.S2 Indication of the relative positions of cross-sections shown in Fig.2B.** Dorsal view of the head region of a *kdrl:eGFP* wild-type embryo highlighting (dotted red lines) the relative positions of cross-sections in Fig.2B. First cross-section at location of bilateral aorta, second cross-section at location of bilateral aorta fusion in a single aorta, third section at the position of the single aorta.

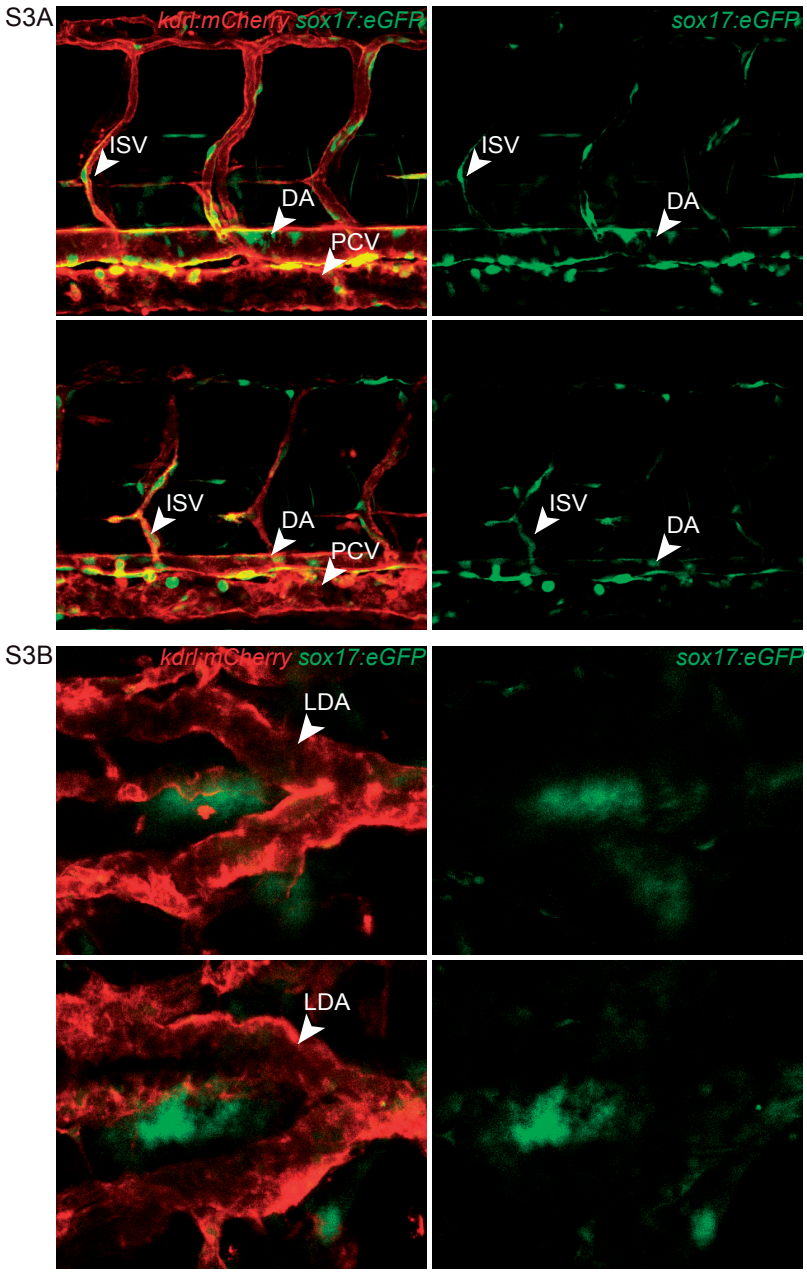



**Fig.S3 Endothelial *sox17:eGFP* expression is indistinguishable between *sox7* mutants and siblings, and is expressed in the DA and ISVs but not in LDA cells.** (S3A) Lateral view of trunk region of *kdrl:mCherry;sox17:eGFP*-positive *sox7* mutants and siblings at 2 dpf. *sox17:eGFP* is expressed in the DA and ISVs, but not in the PCV and is indistinguishable between *sox7* mutants and siblings. (S3B) Dorsal view of the head region of *kdrl:mCherry;sox17:eGFP*-positive *sox7* siblings and mutants at 2 dpf. LDA cells do not express detectable levels of the transgenic reporter line in both *sox7* siblings and mutants. Pictures in S3A and B are taken with same laser intensity and settings. (L)DA = (lateral) dorsal aorta, PCV = posterior cardinal vein, ISV = intersegmental vessel

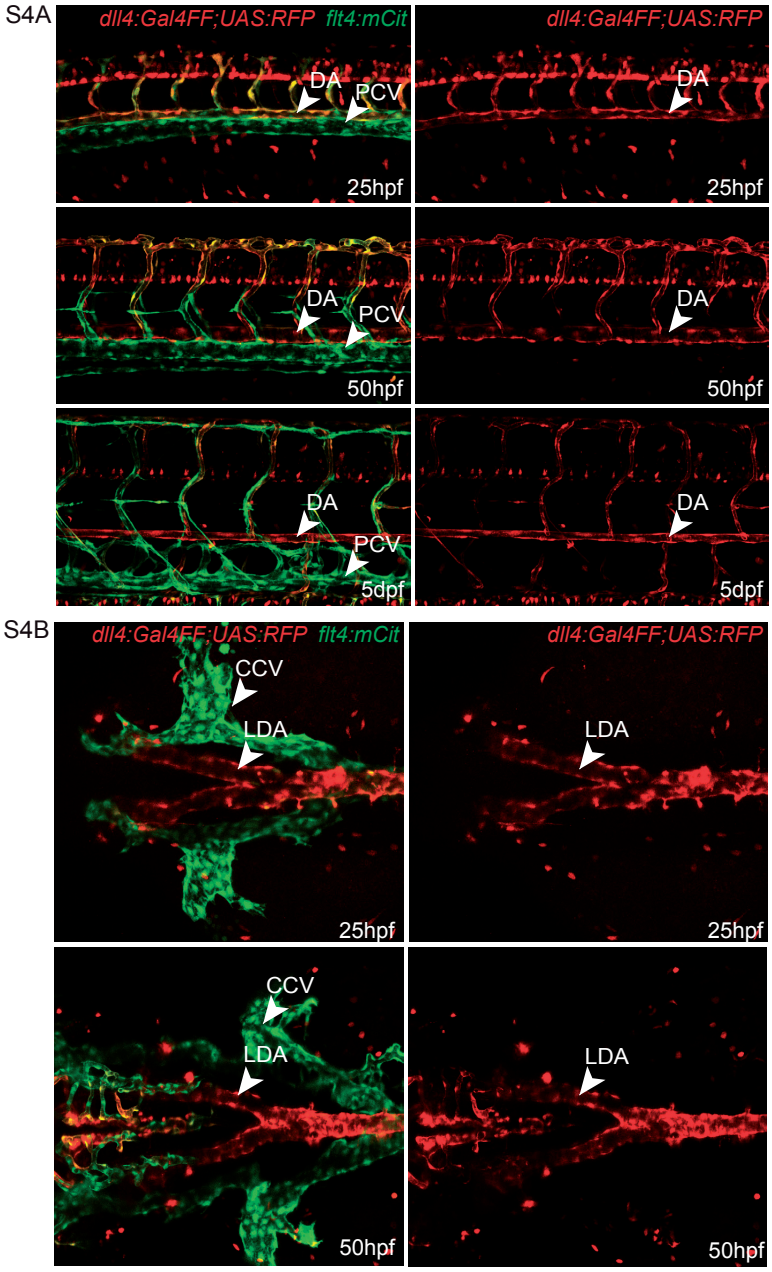
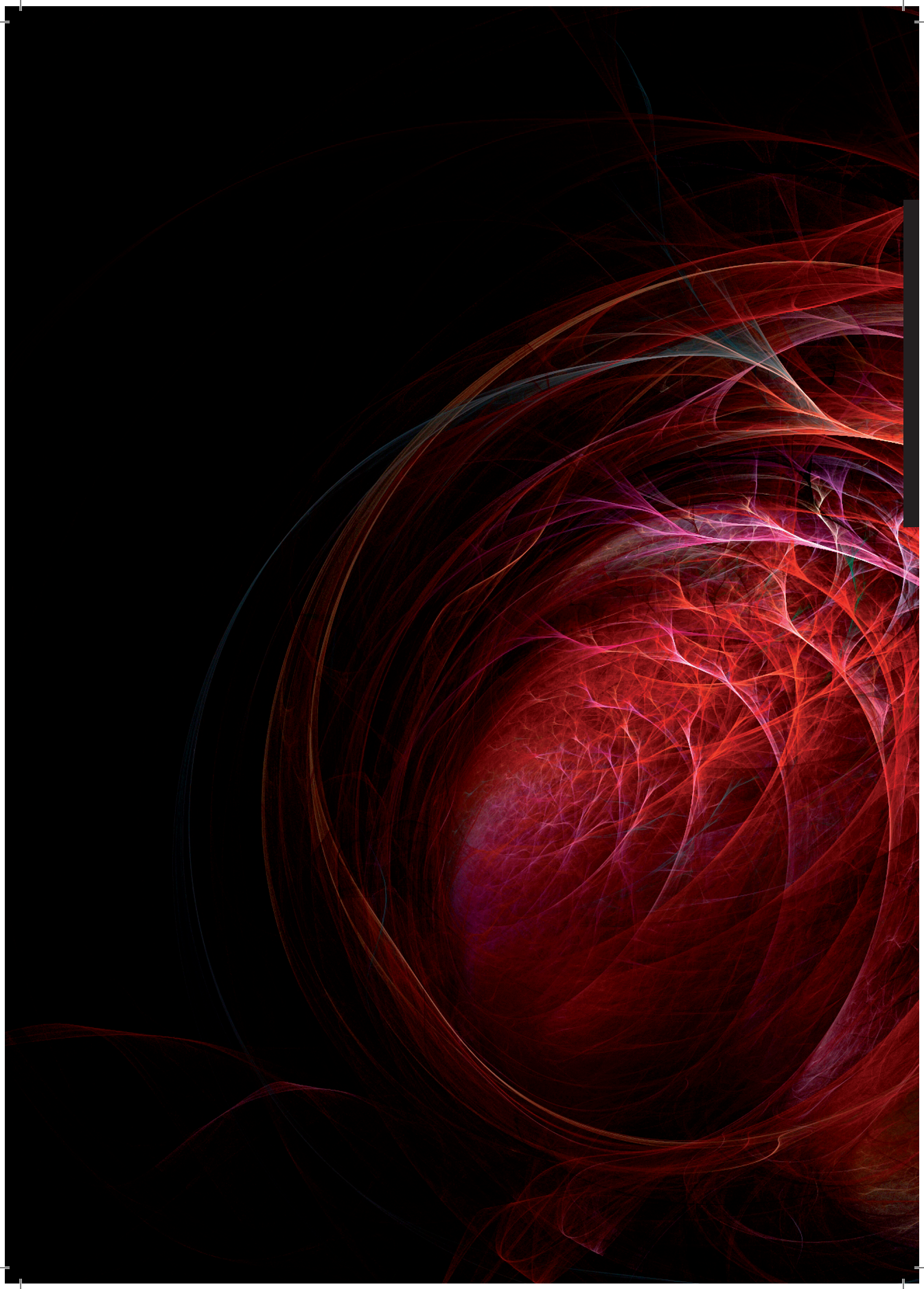



**Fig.S4 *dll4:Gal4FF;UAS:RFP* is expressed specifically in arterial endothelial cells.** (S4A) Lateral view of the trunk region of *dll4:Gal4FF;UAS:RFP;flt4:mCitrine*-positive embryos at 25 hpf, 50 hpf and 5 dpf. (S4B) Dorsal view of the head region of *dll4:Gal4FF;UAS:RFP;flt4:mCitrine*-positive embryos at 25 hpf and 50 hpf. Right panels only *dll4:gal4FF;UAS:RFP* channel. Note *dll4:gal4FF;UAS:RFP* expression in the arteries (DA and LDA) but not within venous ECs (PCV and CCV). (L)DA = lateral dorsal aorta, PCV = posterior cardinal vein, CCV = common cardinal vein.

## Supplementary Table

| Primer name | Sequence |
|---|---|
| Sox7_wt | GAA GGT GAC CAA GTT CAT GCT GAA CGC CTT CAT GGT GTG |
| Sox7_mut | GAA GGT CGG AGT CAA CGG ATT GAA CGC CTT CAT GGT GTA |
| Sox7_common | CGA GTC TCT TGC GCT CAT CTT |
| Efnb2a_wt | GAA GGT GAC CAA GTT CAT GCT CTC TGG AAC AGC TAA AGT CCTGT |
| Efnb2a_mut | GAA GGT CGG AGT CAA CGG ATT CTC TGG AAC AGC TAA AGT CCTGA |
| Efnb2a_common | AGT AGA GGC GTG TCT GCT TTT |
| Efnb2b_wt | GAA GGT GAC CAA GTT CAT GCT CGA GTT GTT CTT TGG GAA CAA GA |
| Efnb2b_mut | GAA GGT CGG AGT CAA CGG ATT CGA GTT GTT CTT TGG GAA CAA GT |
| Efnb2b_common | CTC CAC TGA GCA GAC GAA CAT |
| Hey2_ex2_Fw | TGTGAATGTGACGGATGTGA |
| Hey2_ex2_Rv | TCCGGTCCCTTCTTCTTTTT |



# CHAPTER 4

# Divergence of lymphatic cell fate specification pathways between zebrafish and mice

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**In mammals, the homeodomain transcription factor Prox1 acts as the central regulator of lymphatic cell fate. Its restricted expression in a subset of cardinal vein cells leads to a switch towards lymphatic specification and hence represents a prerequisite for the initiation of lymphangiogenesis. Murine *Prox1*-null embryos lack lymphatic structures, and sustained expression of Prox1 is indispensable for the maintenance of lymphatic cell fate even at adult stages, highlighting the unique importance of this gene for the lymphatic lineage. Whether this preeminent role of Prox1 within the lymphatic vasculature is conserved in other vertebrate classes has remained unresolved, mainly due to the unavailability of loss of function mutants. Here, we re-examine the role of Prox1a in zebrafish lymphangiogenesis: first, using a transgenic reporter line, we show that *prox1a* is initially expressed in different endothelial compartments, becoming restricted to lymphatic endothelial cells only at later stages. Second, using targeted mutagenesis, we show that Prox1a is dispensable for lymphatic specification and subsequent lymphangiogenesis in zebrafish. In line with this result, we found that the functionally related transcription factors Coup-TFII and Sox18 are also dispensable for lymphangiogenesis. Together, these findings suggest that lymphatic commitment in zebrafish and mice is controlled in fundamentally different ways.**

## INTRODUCTION

Lymphatic vessels play pivotal roles in tissue fluid homeostasis, immune cell trafficking and the uptake of dietary fats in the small intestine. A failure in lymphatic vessel development (lymphangiogenesis) or in lymphatic function is causative for several inherited or acquired pathological conditions that lead to tissue swelling by accumulation of extravasated fluids (Tammela and Alitalo, 2010; Schulte-Merker et al., 2011).

In mice, combined activity of the transcription factors Coup-TFII (Srinivasan et al., 2010) and Sox18 (Francois et al., 2008) leads to the polarized expression of *Prox1* in a subset of endothelial cells (ECs) within the cardinal vein at E9.5. Shortly thereafter, Prox1 positive ECs leave the cardinal vein in a dorsal direction, mediated by Vegfc/Flt4 driven processes of polarized sprouting and migration, resulting in the formation of the first lymphatic structures in the embryo (Karkkainen et al., 2004; Hagerling et al., 2013). *Prox1* expression in lymphatic precursor cells is essential for the initiation of a lymphatic gene expression program, and *Prox1* knockout mice lack all lymphatic structures (Wigle et al., 2002). Forced expression of *Prox1* is sufficient to confer lymphatic identity to blood ECs, demonstrating the pivotal role of the gene for lymphatic specification (Hong et al., 2002; Petrova et al., 2002). Continuous expression of *Prox1* in lymphatic ECs is also indispensable for the maintenance of lymphatic cell fate during later stages of development (Johnson et al., 2008), thus firmly establishing Prox1 as the central determining factor of lymphatic identity.

In the zebrafish trunk, the process of vasculogenesis establishes an initial primitive circulatory loop consisting of dorsal aorta (DA) and posterior cardinal vein (PCV). Then, in

a wave of angiogenic sprouting from the DA (primary or arterial sprouting), a set of about 30 arterial intersegmental vessels (ISVs) is formed on each side of the embryo. Shortly thereafter, at about 36 hpf, another group of roughly two times 30 sprouts emerge bilaterally from the PCV. These venous (or secondary) sprouts also migrate dorsally, and about half of them make a stable connection to pre-existing arterial ISVs, thereby remodeling them into intersegmental veins. Venous sprouts that fail to connect to arteries migrate further dorsally towards the midline of the embryo where they populate the region of the horizontal myoseptum as parachordal lymphangioblasts (PLs) (Hogan et al., 2009a), which constitute a pool of lymphatic precursors in the embryonic trunk. These PLs will subsequently migrate away from the horizontal myoseptum (2.5 dpf) by using arterial ISVs as migration routes to populate the different regions of the trunk, eventually giving rise to the thoracic duct (TD; situated between the DA and PCV), a number of intersegmental lymphatic vessels (ISLVs) in close proximity to arterial ISVs, and the dorsal longitudinal lymphatic vessel (DLLV) (Bussmann et al., 2010).



Previous work has indicated a strong conservation in genes controlling lymphangiogenesis between zebrafish and mammals. In all organisms examined, mutations in the transmembrane receptor Flt4, its secreted ligand Vegfc, or the more recently discovered gene *ccbe1* lead to a block of lymphangiogenesis already at the level of sprouting from the venous endothelium (Schulte-Merker et al., 2011; Koltowska et al., 2013). Although several publications have suggested that Prox1 function in lymphatic specification might be conserved both in amphibians (Ny et al., 2005) and fish, the evidence in the case of the latter has remained open to interpretation, complicated by the existence of duplicated *prox1* genes in zebrafish (Del Giacco et al., 2010; Tao et al., 2011). While expression of *prox1a* within lymphatic structures has been reported (Yaniv et al., 2006), it remains unclear whether this expression consistently marks all lymphatic structures during different stages of lymphangiogenesis. No mutant allele of *prox1a* has previously been described and its morpholino-mediated knockdown results in severely malformed embryos, making a conclusive assessment of its requirement for lymphatic development impossible (Kuchler et al., 2006). Thus, while mutations in *prox1b* do not interfere with normal lymphatic development (Tao et al., 2011), the possibility remains that *prox1a* could indeed be required during lymphatic specification in fish.

Using a novel transgenic reporter line, we show here that *prox1a* exhibits a dynamic expression pattern in different endothelial compartments during early vascular development. In contrast to the situation in mice, we found that expression of this reporter gene only becomes a specific and reliable marker for lymphatic ECs at later stages of lymphangiogenesis, arguing against a lymphatic specification function during the onset of venous sprouting. In line with this, using a novel targeted allele of *prox1a* in combination with the previously described *prox1b* mutation, we show that lymphangiogenesis can proceed in the complete absence of Prox1 in zebrafish. In addition, we show that the functionally related transcription factors Coup-TFII and Sox18 are also dispensable during lymphangiogenesis. These results indicate

that the Sox18/Coup-TFII/Prox1 lymphatic specification code is not conserved in fish, suggesting an alternative mode of lymphatic commitment in this vertebrate class.

## MATERIALS AND METHODS

### Zebrafish husbandry

Strains were maintained under standard husbandry conditions. Animal experiments have been performed according to the rules of the Animal Experimentation Committee (DEC) of the KNAW. The following published transgenic lines have been employed in this study: *Tg(flt1$^{enh}$:tdTomato)* (Bussmann et al., 2010), *Tg(fli1a:eGFP)$^{y1}$* (Lawson and Weinstein, 2002), *Tg(kdrl:HRAS-mCherry)$^{s916}$* (*kdrl:mCherry-Caax* hereafter) (Hogan et al., 2009a).

### Transgenesis

The *Tg*(*flt4$^{BAC}$:mCitrine*)$^{hu7135}$ line was generated from BAC DKEY-58G10 following standard methods (Bussmann and Schulte-Merker, 2011). The generation of the BAC transgenic line *Tg(prox1a:KalTA4-4xUAS-E1b:uncTagRFP)$^{nim5}$* will be described in detail elsewhere. For the generation of the *flt4* promotor construct *Tg(flt4:Gal4FF)$^{hu9236}$* a previously reported 3.8kb promoter fragment (Deguchi et al., 2012) was amplified from Medaka genomic DNA (Table S1) and cloned into the miniTol2 vector 5' of the Gal4FF coding sequence. In case of the *UAS:prox1a* mis-expression construct, the *prox1a* cDNA was placed behind a 5xUAS cassette in the pT2A vector followed by an IRES sequence and a mTurquoise-NLS cassette including PolyA sequences. Since mTurq-NLS expression behind the IRES turned out to be very low, an additional 5xUAS:mRFP cassette was inserted into the plasmid to identify Gal4FF-positive cells harboring the *prox1a* overexpression construct after injection. BAC DNA (100pg/embryo) or plasmids (25pg/embryo) were co-injected with Tol2 transposase mRNA (25pg/embryo) into one-cell-stage embryos and the progeny was screened for germline transmission.

### Genome editing by zinc-finger nuclease and TALENs

For the generation of *prox1a* mutants, plasmids encoding zinc-finger nucleases targeting the locus were obtained from ToolGen (Korea). The zinc-finger target sites in the first exon of *prox1a* were: 5'-TGAGATGGAGAG-3' and 5'-GGTCATGGAGGG-3' (Fig. S3). TALEN mediated genome editing for the generation of *coup-TFII* and *sox18* mutants was performed as described (Cermak et al., 2011; Bedell et al., 2012). The TALEN binding sites in *sox18* exon1 were: TAL1 5'-TGCCTGGGTCTGGAAC-3'; TAL2 5'-TGGCCTCCGCTGCTGTT-3'. For *coup-TFII*, the TALEN recognition sites were TAL1 5'-TCCGACCCCTCAGACACCCGT-3' and TAL2 5'-AACAATAACAACACACAGTCA-3'.

## Genotyping

The *prox1a*$^{i278}$, *sox18*$^{hu10320}$ as well as the *coup-TFII* allele *nr2f2*$^{hu10330}$ were genotyped by KASPAR using primers indicated in Table S1. KASPAR genotyping of the *prox1b*$^{SA0035}$ allele has been performed as described (Tao et al., 2011).

## Immunohistochemistry and in situ hybridisation (ISH)

Antibody staining using the following antibodies was performed as described (Elworthy et al., 2008): mAb F59 (anti-slow myosin heavy chain-1, DSHB) at 1:100, rabbit anti-Prox1 (Chemicon, USA) at 1:5000, goat anti-rabbit Alexa488 and goat anti-mouse Alexa546 (Invitrogen) at 1:1000. ISH was carried out as described before (Schulte-Merker, 2002).



## Microscopy

Confocal imaging was performed on living embryos embedded laterally (unless otherwise stated) in 0.5–1% low melting point agarose (Invitrogen) on Leica SPE and SP8 microscopes. Bright-field pictures were taken on an Olympus SZX16 Stereomicroscope. Images were processed using Adobe Photoshop CS5.1 and Fiji (http://fiji.sc/Fiji). Stitching of composite pictures was done with Leica LAS AF software or Adobe Illustrator CS5.1.

## RESULTS

To obtain a more precise and sensitive readout for lymphatic structures during development, we created a BAC reporter line for *vegfr3/flt4* (*Tg(flt4$^{BAC}$:mCitrine)*, *flt4:mCit* hereafter). Similar to previous observations in mice (Kaipainen et al., 1995), we found that *flt4* is initially expressed in all blood ECs but becomes progressively restricted to venous and lymphatic endothelial cells (LECs) after 26 hpf (Fig. S1A, B) and 36 hpf (Fig. S1C-F) respectively. In contrast to *Flt4* expression in mice (Hagerling et al., 2013), *flt4:mCit* expression is not lost in venous structures, making it a lymphatic-enriched, but not lymphatic-specific reporter at 5 dpf. In pan-endothelial transgenic lines like *fli1a:eGFP*, the TD is the only lymphatic structure that is readily distinguishable from blood vessels. In comparison, the *flt4* reporter line also reliably highlights more delicate lymphatic structures like ISLVs or the DLLV in the trunk (Fig. S1G, H) as well as the facial lymphatic system (Fig. 1A-B). Combined with the arterial-specific *flt1$^{enh}$:tdTom* reporter (Bussmann et al., 2010), all lymphatic, venous and arterial structures in the embryo can be easily distinguished (see below).

### *prox1a* is expressed within different endothelial cell populations during development

To assess whether *prox1a* might represent the functional *Prox1* orthologue in zebrafish and would therefore be expressed specifically in LECs, we generated different *prox1a* BAC reporter lines that were analyzed in combination with the afore mentioned *flt4:mCit* line. Since simple fluorophore expression under the control of the *prox1a*$^{BAC}$ promoter resulted in extremely weak signals (not shown), we took advantage of the enhancing effect of an optimized Gal4 variant [KalTA4 (Distel et al., 2009)] in a *prox1a$^{BAC}$:KalTA4, UAS:tagRFP* expression cassette. Consistent with previously published data (Glasgow and Tomarev, 1998; Thisse and Thisse, 2005; Pistocchi et al., 2009), the transgene marked a wide range of tissues comprising the lens, retina, liver, neuromasts and myotome (Fig. 1A). To identify expression domains masked by the myotomal expression in full z-projections, partial z-projections of the trunk were analyzed, revealing expression in all lymphatic structures within the head and trunk, where the signal co-localized with *flt4:mCit* expression at 5 dpf (Fig. 1B, C, D). In contrast to previously published lymphatic markers like *stabilin1:YFP* or *lyve1:DsRed2* (Hogan et al., 2009a; Okuda et al., 2012) or the *flt4:mCit* line, at 5 dpf the *prox1a* reporter exhibits no additional expression domains in other endothelial compartments, such as the PCV, and hence reflects the first truly LEC-specific marker at this stage of development in zebrafish. Taking advantage of this feature we found that the anterior part of the TD, which connects the head lymphatics with the TD in the trunk, comprises a bilateral structure above the developing swim bladder. The TD splits up near the sixth intersegmental vessel pair and runs in close proximity to the correspondingly bilateral PCVs (Fig. 1E, F, S2A), finally connecting to the facial lymphatic network (Okuda et al., 2012) and ultimately the common cardinal veins (Fig. 1G, H).

Next, we investigated whether zebrafish *prox1a* expression would, like its murine counterpart, already mark LECs at earlier stages, possibly even within the venous endothelium of the PCV at a point in time prior to venous (secondary) sprouting. At 32 hpf, shortly before the emergence of the first sprouts from the PCV, *prox1a* could indeed be detected in a subset of venous cells (Fig. 1I, J) and subsequently in sprouting venous ECs (Fig. 1K). To determine whether *prox1a* might exclusively mark secondary sprouts that will give rise to lymphangioblasts, expression was analyzed at 44 hpf, a stage when PL cells have extended to the horizontal myoseptum while venous destined sprouts have already established a connection to the respective ISV. We found *prox1a* reporter expression only in a proportion of PLs (Fig. 1L; 17/78 PLs), indicating that not all lymphatic precursor cells are positive for *prox1a* at this stage. Equally important, the transgene also transiently marked individual venous ISVs (in 12/21 imaged regions) (Fig. 1L, O, R), suggesting no clear correlation between *prox1a* expression and the fate of secondary sprouts.



Earlier during development, *prox1a* reporter activity could also be detected in ECs of developing arterial ISVs (Fig. 1 M, N) and in particular of the DA (Fig. 1P, Q), as well as in undefined cells within the axial vessels before the onset of circulation (Fig. S2B-E). In addition, transgene expression highlighted a subpopulation of ECs in the caudal vein region already at 26 hpf, an expression domain that could be detected up to day 3 (Fig. S2D-G). Probably as a consequence of this early and relatively wide expression within the caudal vein, *prox1a* positive venous ISVs and PL sprouts were more frequently detected in this area at 48 hpf (Fig. S2F).

Since *prox1a* is initially expressed within different endothelial subpopulations, we wanted to establish the earliest stage when *prox1a* expression would reliably and exclusively mark the lymphatic part of the vasculature. We found that, at least at 3.5 dpf, when LECs leave the horizontal myoseptum region and form the different lymphatic structures within the trunk, all LECs were highlighted by expression of the reporter, which was also the case in the head region (Fig. 1S-U). However, since the strong myotomal signal did not allow a conclusive assessment of *prox1a* expression in lymphangioblasts at the level of the horizontal myoseptum, it cannot be excluded that the gene was already expressed in all LECs while still aligning with the horizontal myoseptum.

Taken together, expression of *prox1a* becomes a reliable and very useful maker for LECs only at later stages of lymphangiogenesis. In consequence, the expression data presented here would be in line with a *prox1a* function in specifying PLs at the level of secondary sprouts but not at the level of the vein, as is the case in mice.

### Lymphatic development is not blocked in *prox1a* mutant embryos

To assess the role of Prox1a in lymphangiogenesis, we generated a loss-of-function allele for *prox1a* using zinc-finger nucleases (Fig. S3A). The *prox1a*$^{i278}$ allele harbors a 10bp deletion

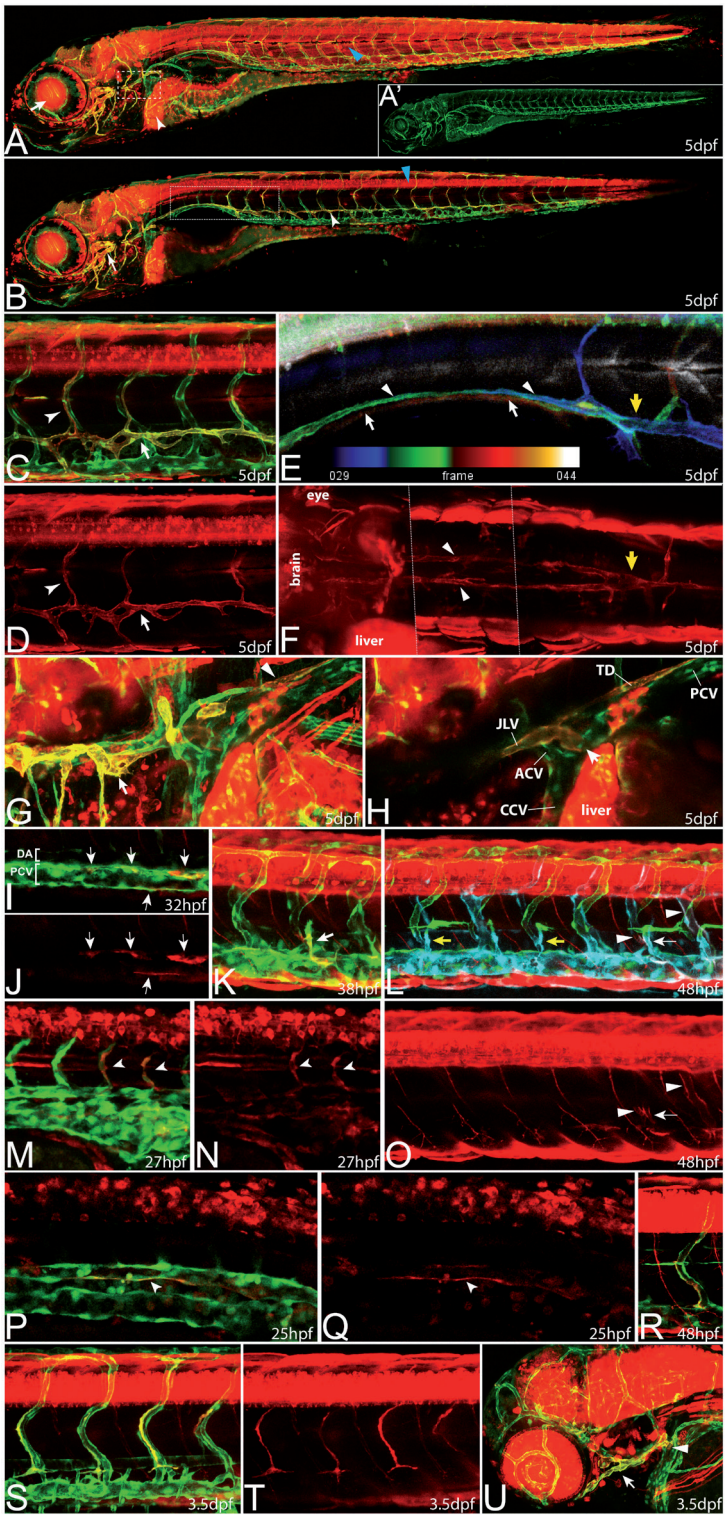

A
A'
5dpf
B
5dpf
C
5dpf
E
029
frame
044
5dpf
D
5dpf
eye
brain
liver
F
5dpf
G
5dpf
TD
PCV
JLV
ACV
CCV
liver
H
5dpf
DA
PCV
I
32hpf
J
K
38hpf
L
48hpf
M
27hpf
N
27hpf
O
48hpf
P
25hpf
Q
25hpf
R
48hpf
S
3.5dpf
T
3.5dpf
U
3.5dpf

**Fig.1 *prox1a* expression marks different endothelial compartments during vascular development.** In all pictures (except for E), *flt4:mCit* expression is shown in green while *prox1a:KalTA4,UAS:tagRFP* expression is highlighted in red. (A) In full z-projections, *prox1a* expression at 5dpf is apparent in various tissues, including liver (white arrowhead), lense (arrow) and myotome (blue arrowhead). (A') Same z-projection displaying only the *flt4:mCit* expression in venous and lymphatic ECs. (B) Partial z-projection of the same embryo (comprising only optical sections without myotome signal) reveals additional expression in the spinal cord (blue arrowhead) and in the lymphatic vasculature of the head (arrow) and the trunk (white arrowhead). Images A-B are composed of several overlapping z-projections. (C, D) Higher magnification of trunk lymphatics at 5 dpf exhibiting combinatorial *prox1a* (red) and *flt4* (green) reporter expression restricted to the TD (arrow) and ISLVs (arrowhead). (E) Depth color-coded z-projection (projecting each slice in a different color according to its position within the stack; see color bar) of indicated region in (B) (only *prox1a* channel) reveals the presence of two separate *prox1a*-positive vessels above the swim bladder (arrows point to lymphatics on the right, arrowheads to lymphatics on the left body side), which merge near the 6$^{th}$ ISVs with the TD in the trunk region (yellow arrow). (F) Dorsal view on the *prox1a*-positive bilateral anterior TD (arrowheads), which connects to the trunk TD at the indicated location (yellow arrow). The image has been assembled from individual overlapping partial z-projections (see dotted lines). (G, H) Full (G) and partial z-projection (H) of the area (compare to box in A) where the anterior TD (arrowhead in G) and the facial lymphatic network (arrow in G) fuse and drain into the common cardinal vein (arrow in H) in a 5 dpf embryo. [JLV-jugular lymphatic vessel, TD-thoracic duct, PCV-posterior cardinal vein, ACV-anterior cardinal vein, CCV-common cardinal vein]. (I, J) At 32 hpf, *prox1a* positive cells (red, arrows) are located within the PCV (green). Note that in this lateral view, *prox1a* positive cells are located in both the dorsal and ventral aspect of the PCV. (K) A sprouting venous EC (arrow) at 38 hpf expressing both *flt4* and *prox1a* transgenes. (L+O) At 48 hpf only a proportion of PLs are *prox1a:KalTA4*-positive (white arrow) while the majority shows no signs of reporter expression (yellow arrow). In addition, venous ISVs positive for the *prox1a* reporter are evident (white arrowheads). (L) Overlay of a full z-projection of the *flt4:mCit* signal (green, to outline the complete vasculature) and identical partial z-projections of the *prox1a* (red, see also O) and *flt4* reporter (blue, to highlight the part of the vasculature that is included in the partial z-projection of the *prox1a* signal). Note that *prox1a* positive ECs will appear white in the overlay. (M, N) The *prox1a* reporter line also labels individual arterial sprouts (arrowheads) at 27 hpf. (P, Q) Single z-plain of the trunk region in a 25 hpf embryo showing DA cells positive for *prox1a* reporter expression (arrowhead). Note ventral domain expression of the DA. (R) Intersegmental vein showing both *prox1a* and *flt4* reporter expression at 48 hpf. (S, T) At 3.5 dpf, all LECs display expression of the *prox1a* reporter while expression in other endothelial domains has disappeared. (U) Expression of *prox1a* in the head region at 3.5 dpf, highlighting the facial lymphatic network (arrow) including the drainage point with the common cardinal vein (arrowhead).

in the first coding exon, resulting in a frame-shift after amino acid 149, and a premature STOP after additional 4 amino acids (Fig. 2A). Homozygous mutants are devoid of full-length Prox1a protein as assessed by immunostaining of slow muscle fibres at 30 hpf (Fig. 2B, C) The overall appearance of homozygous *prox1a*$^{i278}$ mutants was normal until between days 4 and 5, at which stage mutant larvae started to develop severe edema around the gut and the eye (Fig. 2D, E). At 5.5–6 dpf most mutants had impaired blood circulation and showed signs of tissue necrosis. Importantly, timing as well as severity of edema was different from *ccbe1*, *vegfc* and *flt4* mutant scenarios: While completely lacking lymphatic structures, mutants of each of those genes only develop mild edema starting from 5-6 dpf. The later onset of edema in those lymphatic mutants presumably results from the lymphatic system beginning to perform its drainage function at early day 5 (Karpanen and Schulte-Merker, 2011); thus atypical tissue fluid accumulations can only arise from this time point. It follows that the edema observed in *prox1a* mutants does not reflect a sign of impaired lymphatic drainage function.

In *Prox1*-null mutant mice, the failure of LEC progenitors to leave the cardinal vein results in the absence of lymphatic structures (Yang et al., 2012) and even heterozygous embryos die shortly after birth with dysfunctional lymphatics and a lack of lymphovenous valves (Harvey et al., 2005; Srinivasan and Oliver, 2011). If *prox1a* was equally important in specifying lymphatic cell fate in zebrafish, homozygous mutants should be deficient in lymphatic structures. However, when analyzing the appearance of PLs at the horizontal myoseptum at 2 dpf, we found that *prox1a*$^{i278}$ mutants show only a marginal reduction in the number of PLs (Fig. 2F-H). Since it is possible that this initially mild defect results in more dramatic effects at later stages, we evaluated whether the formation of the TD was impaired. At 5 dpf, no defects in heterozygous embryos and only minor defects in a proportion of homozygous mutants were evident (Fig. 2I-K). On average, homozygous mutants lacked the TD only in 1.4 of the first 10 somites, a mild phenotype that could also reflect a secondary defect caused by the early edema formation and overall impaired appearance of mutants at 5dpf (Fig. 2L). Hence, expression of *prox1a* in the venous and lymphatic endothelium as revealed by the reporter gene described above is not essential for the specification of lymphatic structures. To confirm that the loss of *prox1a* did not affect secondary sprouts that are committed to a venous fate, the ratio of arteries and veins as well as the total number of ISVs was quantified. Overall, no defects on the blood vasculature could be observed and the arterio-venous ratio was also unaffected in mutants (Fig. 2I-K, M-O). We also found that expression of the *prox1a* reporter line was present in homozygous *prox1a*$^{i278}$ mutants, indicating that, in contrast to the situation in mice (Srinivasan et al., 2010), Prox1a protein is not required for maintaining its own lymphatic expression (Fig. S4A-D). Furthermore, considering that the *prox1a* reporter represents a specific lymphatic marker at 5 dpf, the unaltered expression of both, *flt4:mCit* and *prox1a:KalTA4,UAS:tagRFP* in the TD of homozygous *prox1a* mutants argues for correct lymphatic specification of LECs even in the absence of functional Prox1a protein. Given the specific zygotic expression pattern of *prox1a* in different subsets of

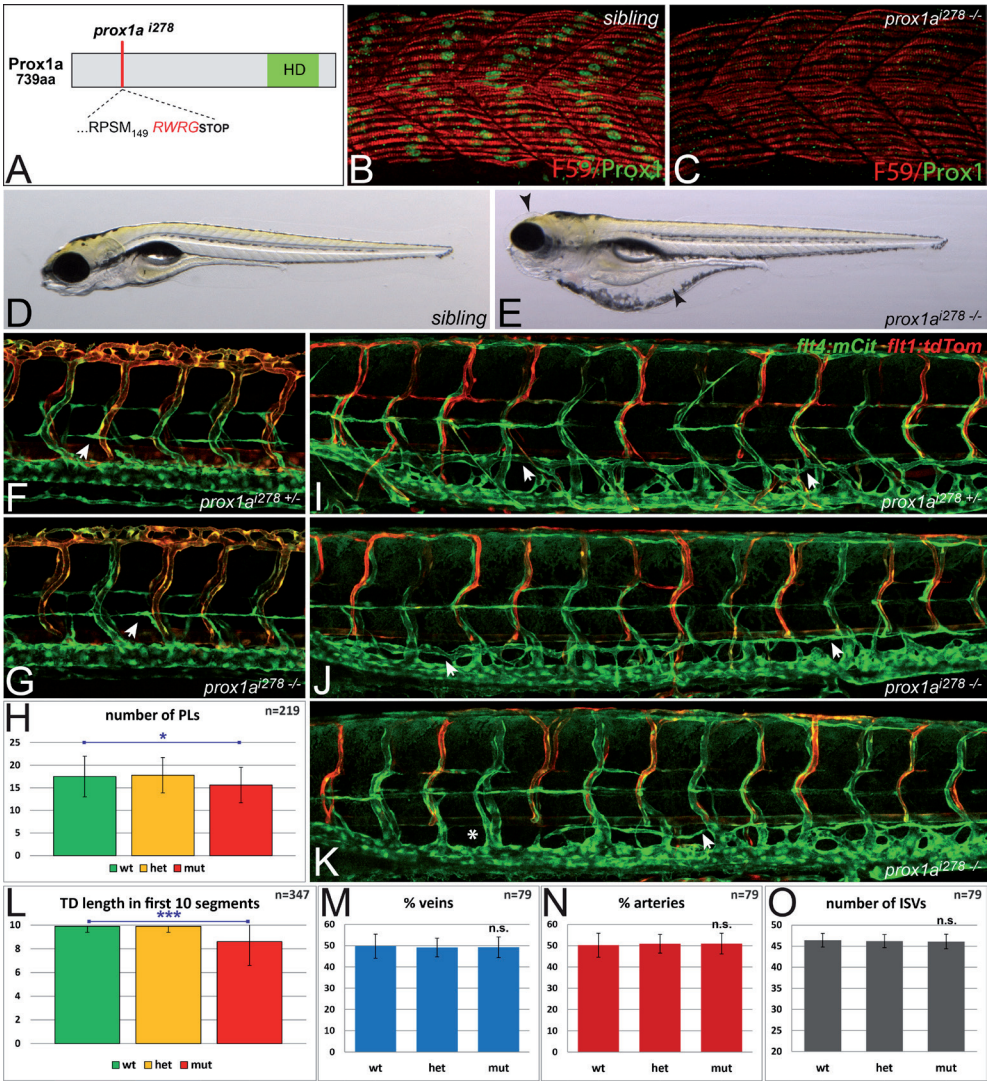



**Fig.2 Lymphangiogenesis in *prox1a* mutant embryos.** (A) Schematic representation of the homeodomain (HD) containing Prox1a protein, indicating the predicted effect of the 10bp deletion in the *prox1a*$^{i278}$ allele, leading to a frame-shift (red aminoacids) and a truncated protein after 153 aminoacids. (B, C) Prox1a immunostaining of slow muscle fibres in sibling (B) and homozygous mutant *prox1a*$^{i278}$ embryos (C) demonstrates a complete loss of wild-type Prox1a protein (green) at 30 hpf (slow myosin heavy chain-1 is shown in red). (D, E) Bright-field pictures of 5 dpf sibling (D) and homozygous *prox1a*$^{i278}$ mutant (E) embryos. Note the strong edema formation around the eye and gut area (arrowheads) which can be even more pronounced in other *prox1a* mutants at this stage. (F, G) In both, heterozygous siblings (F) and homozygous *prox1a*$^{i278}$ mutants (G), PLs appear at the level of the horizontal myoseptum at 2 dpf (arrows). (H) Average PL numbers per embryo are mildly reduced in *prox1a* mutants at 2dpf (student's t-test p=0.025). Error-bars indicate standard deviations of wild-type (green), heterozygous (orange) and mutant (red) groups in embryos from a *prox1a*$^{+/-}$ incross. (I-K) *flt4:mCit*; *flt1*$^{enh}$*:tdTom* double transgenic embryos highlighting arterial ISVs in red and venous and lymphatic structures in green. Compared to heterozygous siblings (I), most homozygous *prox1a*$^{i278}$ mutants do not display TD defects at 5 dpf (J), while others display a mild reduction

(K) in some areas of the trunk (arrows point at TD; asterisks mark the lack of TD). Note the overall unaffected ratio of venous and arterial ISVs in mutants (J, K). (L) Average number of segments positive for TD cells, scored in the first 10 segments above the yolk extension at 5 dpf. Error-bars indicate the s.d. for the respective genotypic class from a *prox1a*$^{+/-}$ incross. The p-value for the difference between wild-type and mutant population is p=2.3E-08 (student's t-test). (M, N) The average percentage of intersegmental veins (M) and arteries (N) does not differ between genotypic classes in an incross of *prox1a*$^{i278}$ carriers. (O) In *prox1a*$^{i278}$ mutants the average total number of ISVs is not altered.

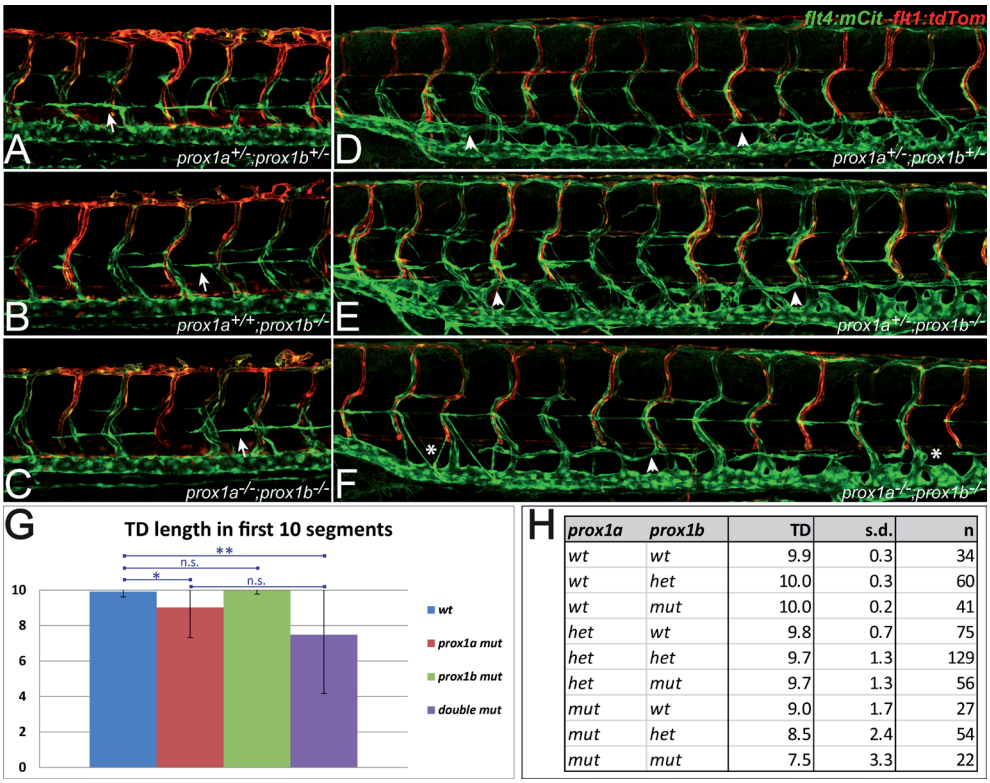
ECs, our finding that Prox1a protein was undetectable in slow muscle fibres of *prox1a*$^{i278}$ mutants prior to secondary sprouting as well as the fact that maternally expressed *prox1a* transcripts have not been identified (Harvey et al., 2013), it seems unlikely that the lack of zygotic phenotype can be attributed to maternal rescue. We therefore conclude that *prox1a* function is not essential for any aspect of lympho-venous sprouting and that the gene is dispensable for lymphatic cell fate determination in zebrafish.

## Double mutants for *prox1a* and *prox1b* only show minor lymphatic defects

Although neither of the two *prox1* orthologues appears essential for zebrafish lymphatic development , ((Tao et al., 2011), this study) it remains possible that they function redundantly. We therefore generated double mutants for *prox1a*$^{i278}$ and the previously characterized loss-of-function allele *prox1b*$^{SA0035}$ (Tao et al., 2011). In all allelic combinations including double mutant embryos, PLs were observed at the horizontal myoseptum (Fig. 3A-C), indicating that even the absence of all Prox1 function fails to block the appearance of lymphatic-fated secondary sprouts. Subsequent analysis of double mutants for the formation of TD at 5 dpf demonstrated that a moderate but significant reduction in the length of the TD within the first 10 segments was evident (Fig. 3D-H) – whether this is a specific effect of *prox1* deficiency, or whether this phenotype represents a secondary consequence of more general defects within the embryo (see Fig. 2E) is difficult to assess. The presence of PL cells, facial lymphatics (Fig. S5A-D) and TD tissue in double mutants however clearly shows that lymphatic commitment is not governed by zygotic expression of the Prox1a and Prox1b transcription factors. It follows that lymphatic specification in zebrafish must be achieved in a different way, possibly through the broadened activity of another functionally related transcription factor.

## *coup-TFII* mutants do not show arterio-venous defects and develop a wild-type lymphatic vasculature

The principal factor that promotes venous cell identity in many vertebrate embryos is the transcription factor COUP-TFII (NR2F2), which suppresses expression of arterial genes in venous ECs (You et al., 2005). Since lymphatics are venous-derived, loss of endothelial Coup-TFII expression in mice not only causes venous specification defects but also results in a lack of LECs (Srinivasan et al., 2007). Coup-TFII has been reported to be required for the




H

| prox1a | prox1b | TD | s.d. | n |
|---|---|---|---|---|
| wt | wt | 9.9 | 0.3 | 34 |
| wt | het | 10.0 | 0.3 | 60 |
| wt | mut | 10.0 | 0.2 | 41 |
| het | wt | 9.8 | 0.7 | 75 |
| het | het | 9.7 | 1.3 | 129 |
| het | mut | 9.7 | 1.3 | 56 |
| mut | wt | 9.0 | 1.7 | 27 |
| mut | het | 8.5 | 2.4 | 54 |
| mut | mut | 7.5 | 3.3 | 22 |

**Fig.3 Lymphatic specification is not blocked in *prox1a*$^{i278}$*;prox1b*$^{SA0035}$ double mutants.** (A-C) In all genotype combinations of a *prox1a*$^{i278}$*;prox1b*$^{SA0035}$ double heterozygous incross, including double heterozygotes (A), homozygous *prox1b* mutants (B) as well as homozygous double mutants (C), PLs are apparent at the horizontal myoseptum at 2 dpf (arrows). (D-F) As in double heterozygous embryos (D), also the loss of both copies of *prox1b* (E) does not lead to lymphatic defects on the level of TD formation. In *prox1a*$^{i278}$*;prox1b*$^{SA0035}$ double homozygous embryos (F) mild TD defects are occasionally visible (arrows indicate the TD while asterisks highlight segments without TD). (G) Average number of TD-positive segments within the first 10 segments above the yolk extension for the indicated genotypic classes of a *prox1a*$^{+/-}$*;prox1b*$^{+/-}$ incross. The moderate reduction in TD length is statistically significant in the *prox1a* single mutants (student's t-test p=0,01) and the *prox1a;prox1b* double mutant embryos (p.=0.002) when compared to wild-type siblings. (H) Overview about the average TD length (TD) in 10 scored segments, the corresponding standard deviations and the number of scored embryos (n) in a *prox1a*$^{+/-}$*;prox1b*$^{+/-}$ incross.

initiation of *Prox1* expression within the cardinal vein by direct binding to its promoter region (Srinivasan et al., 2010) and subsequently also for the maintenance of *Prox1* expression in future LECs during early lymphangiogenesis. The latter function is thought to be dependent on a direct physical interaction between Coup-TFII and Prox1, and heterodimers have been reported to act as co-regulators of several lymphatic lineage specific genes such as *FLT4* in cultured LECs (Lee et al., 2009). More recently, vascular defects including a reduction in PL number and aberrant TD formation has been reported to result from MO-knockdown of zebrafish *coup-TFII* (Aranguren et al., 2011). We therefore wondered whether lymphatic lineage specification in fish might be entirely regulated by *coup-TFII* instead of *prox1* genes. To explore this possibility, we employed TALEN constructs targeting the first exon of *coup-TFII* and generated a one base pair insertion allele *nr2f2*$^{hu10330}$ (*coup-TFII*$^{hu10330}$ hereafter). Sequencing of cDNA from *coup-TFII*$^{hu10330}$ mutant embryos did not reveal alternative transcripts lacking the 1bp insertion (data not shown). This suggests that the allele represents a loss-of-function situation since the insertion results in a frame-shift and premature stop codon 5' to both the nuclear receptor-DNA binding domain and the ligand-binding domain (Fig. 4A).

Homozygous *coup-TFII*$^{hu10330}$ mutants were viable beyond 6 dpf (Fig. 4B, C) and developed a normal blood vasculature without any sign of arterio-venous identity defects in the main trunk vessels. In addition, when scoring TD formation at 5 dpf, we observed only negligible defects and the majority of mutants were indistinguishable from siblings (Fig. 4D-F). Since also the ratio of intersegmental arteries and veins was unaffected in *coup-TFII* mutant embryos (Fig. 4G) these results demonstrate that zygotically expressed *coup-TFII* is not essential for venous specification and lymphatic development.

### Loss of *sox18* does not interfere with lymphatic development

Another transcription factor that has been implicated in lymphatic development in mice and humans is Sox18 (Irrthum et al., 2003). Mice lacking functional Sox18 develop edema in certain genetic backgrounds (Francois et al., 2008) because of a failure in LEC development. *Sox18* is expressed in a subset of cardinal vein cells that subsequently initiate *Prox1* expression. In vitro experiments further showed that Sox18 can activate *Prox1* expression by direct binding to its promoter region, placing *Sox18* upstream of *Prox1* during lymphatic specification (Francois et al., 2008). More recent morpholino knockdown studies as well as over-expression experiments employing a dominant-negative mouse Sox18 variant in zebrafish suggested a specific requirement for *sox18* during sprouting of lymphatic-fated secondary sprouts from the PCV. In contrast to mouse however, zebrafish *sox18* is expressed in both DA and PCV in a non-polarized fashion during venous sprouting (Cermenati et al., 2013) which is counterintuitive in the context of lymphatic specification.

We generated a mutant with a 1bp insertion 5' to the HMG-box encoding sequence (Fig. 4H). This allele is predicted to encode a truncated protein that neither contains the essential

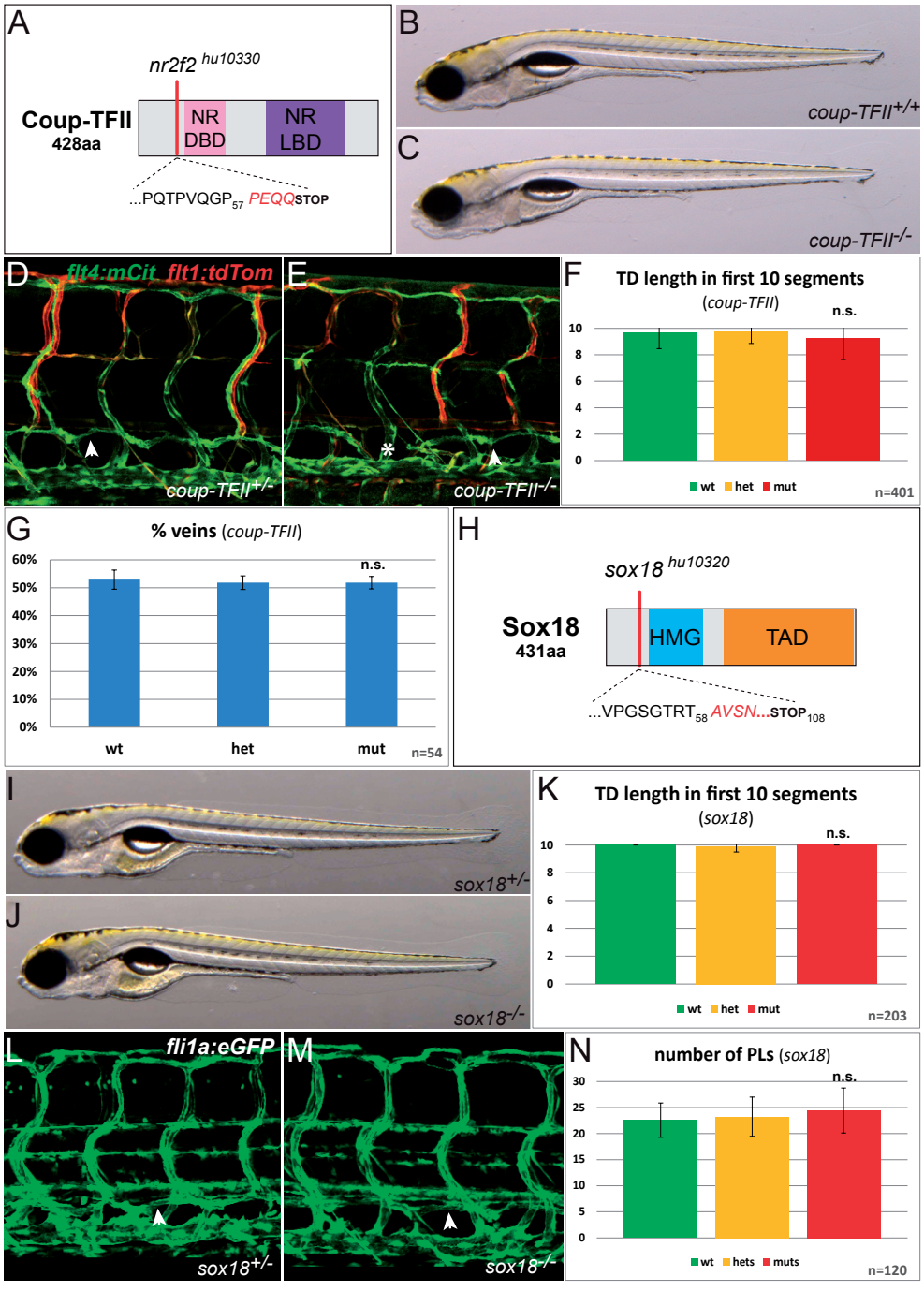



**Fig.4 Specification of the lymphatic lineage is independent of *coup-TFII* and *sox18*.** (A) Schematic representation of the Coup-TFII protein indicating the position of the first affected amino acid in the 1bp insertion allele *nr2f2*$^{hu10330}$ (*coupTFII* $^{hu10330}$ hereafter) which leads to a premature stop codon after additional 4 amino acids N-terminal to the nuclear receptor- DNA binding and ligand binding domain. (B, C) Bright-field images of wild-type (B) and

homozygous mutant *coup-TFII*$^{hu10330}$ (C) embryos, with no signs of edema or morphological abnormalities at 6 dpf. (D, E) *flt4:mCit;flt1*$^{enh}$*:tdTom* positive embryos from a *coup-TFII*$^{hu10330}$ incross. Compared to heterozygous siblings (D), *coup-TFII* mutants (E) show only marginal TD defects (asterisk) in a small proportion of embryos at 5 dpf. Note the overall normal vascular morphology with proper PCV, DA and ISVs present in homozygous mutants. (F) The average length of the TD in the first 10 segments above the yolk extension is not significantly affected in *coup-TFII*$^{hu10330}$ mutants. (G) The proportion of venous ISVs is unaltered in homozygous *coup-TFII*$^{hu10330}$ mutants at 5 dpf. (H) Schematic overview of the Sox18 protein, indicating the first affected amino acid in the 1bp insertion allele *sox18*$^{hu10320}$ preceding the HMG-box. The resulting frame-shift leads to a premature stop codon after additional 50 amino acids. (I, J) Bright-field pictures of heterozygous (I) and homozygous mutant *sox18*$^{hu10320}$ embryos at 5 dpf. Note the overall wild-type appearance of mutant embryos (J). (K) The average length of the TD in wild-type (green), heterozygous (yellow) and homozygous mutant (red) *sox18*$^{hu10320}$ embryos does not differ significantly. (L, M) *sox18*$^{hu10320}$ heterozygous (L) and homozygous mutant (M) embryos expressing *fli1a:eGFP* in all ECs. Even in a *sox18* loss-of-function situation, embryos do not show defects in the formation of the TD (arrowheads). (N) The average number of PLs present in *sox18*$^{hu10320}$ mutants is not significantly affected at 54 hpf.

HMG nor the trans-activation domains [reviewed in (Downes and Koopman, 2001)], and since no alternative transcripts could be detected in mutants (data not shown) we consider it to represent a loss-of-function allele. Loss of Sox18 function did not lead to any obvious differences in the formation of arterial and venous ISVs, which is in line with previous morpholino knockdown data (Cermenati et al., 2008; Herpers et al., 2008; Pendeville et al., 2008; Cermenati et al., 2013). At 5 dpf the overall appearance of homozygous *sox18*$^{hu10320}$ mutants was indistinguishable from siblings (Fig. 4I, J) and when scored for the presence of the TD, no lymphatic or blood vascular phenotypes were detectable (Fig. 4K-M). Accordingly, the quantification of PLs at the horizontal myoseptum at 48 hpf did not reflect any significant differences (Fig. 4N), demonstrating that Sox18 is dispensable for venous sprouting and lymphatic cell fate determination.

## Overexpression of prox1a in the venous endothelium does not affect endothelial cell behavior

It has been reported that overexpression of Prox1 in blood ECs in vitro is sufficient to shift their gene expression profile towards a LEC phenotype (Hong et al., 2002; Petrova et al., 2002). Similar results were also obtained from mouse models, where the forced expression of *Prox1* in blood ECs led to a LEC-like gene expression pattern, severe edema formation, and embryonic lethality at E13.5 (Kim et al., 2010). Interestingly, the ability of *Prox1* to transform blood into lymphatic ECs seems to be restricted to the venous endothelium in vivo (Kim et al., 2013), indicating that the additional expression of only one transcription factor is sufficient to differentiate a venous ECs into a LEC. Although we showed here that mutations in the zebrafish orthologues of *Prox1* and two other central genes that govern and maintain lymphatic commitment in mammals did not block lymphatic specification, we wondered whether *prox1a* expression might nevertheless be sufficient to force blood ECs into a

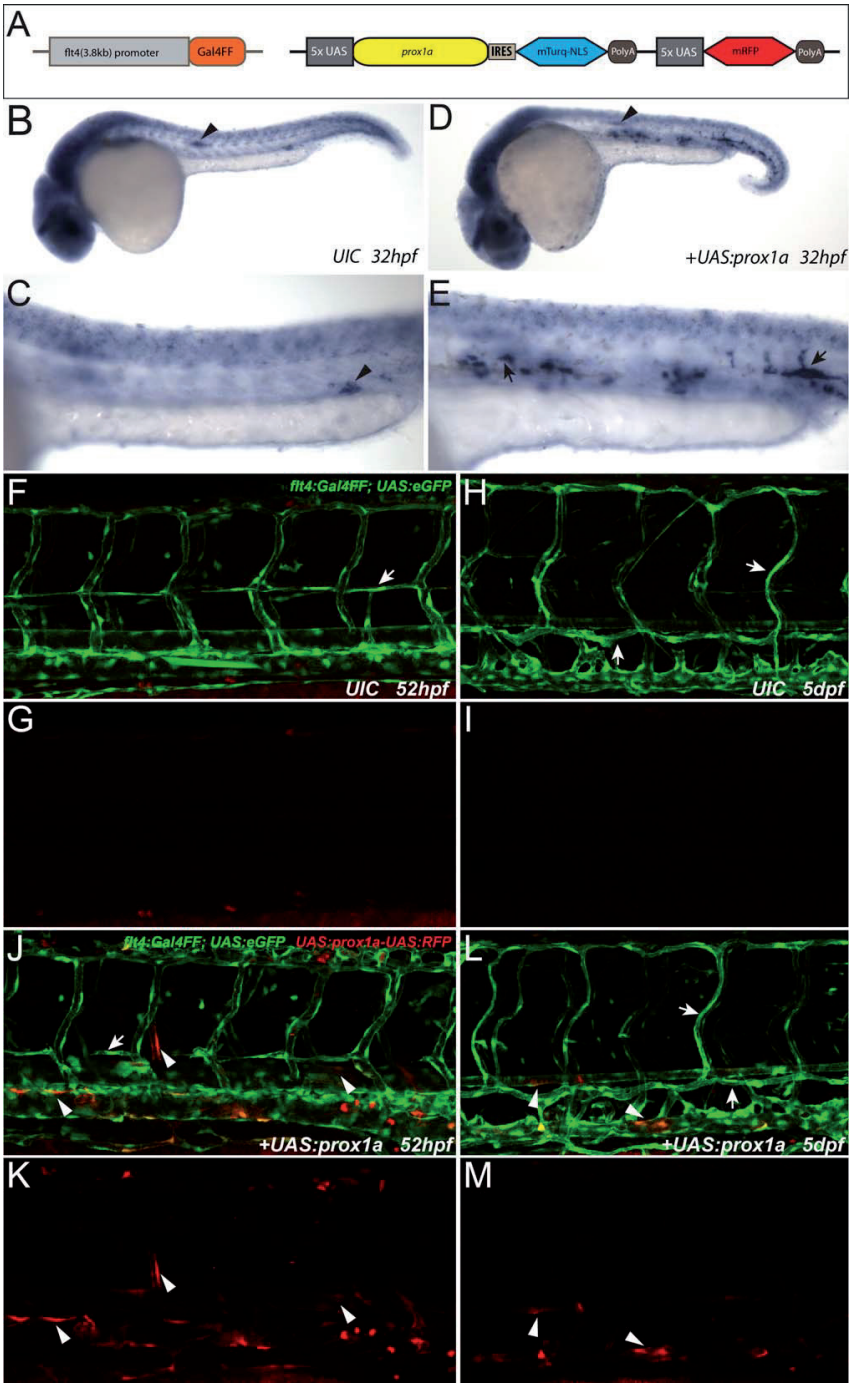



**Fig.5 Forced expression of *prox1a* does not commit venous ECs to a lymphatic phenotype.** (A) Schematic representation of the *prox1a* overexpression construct and the *flt4:Gal4FF* line which drives Gal4FF expression under the control of a Medaka 3.8kb *flt4* promoter fragment. (B-E) Whole mount *in-situ* hybridization against

*prox1a* in uninjected *flt4:Gal4FF* siblings (B, C) and embryos injected with the *prox1a* mis-expression construct at 32 hpf (D, E). Note the domain of forced *prox1a* expression within the axial vessels in injected embryos (arrows in E), which does not reflect endogenous *prox1a* expression (C). Arrowheads in B, D highlight endogenous *prox1a* expression in the lateral line primordium, the arrowhead in C points at a signal in the corpuscles of Stannius. (F-M) *flt4:Gal4FF;UAS:eGFP* wild-type siblings (F-I) and embryos injected with *UAS:prox1a_IRES_mTurq-NLS-UAS:mRFP* plasmid (J-M). At 2 dpf, forced expression of *prox1a* in ECs (marked by mRFP expression in J, K) does not lead to the emergence of ectopic secondary sprouts as compared to uninjected siblings (F, G). At 5 dpf, *UAS:prox1a-UAS:mRFP* positive ECs are still evident in arterial and venous ECs without any signs of lymphatic or venous defects (L), suggesting that *prox1a* expression is not capable of influencing the cell fate and behavior of arterial and venous ECs (red in L, M). Arrows in F, J point at PLs. In H, L, arrows highlight the TD and ISLVs and white arrowheads in J-M mark exemplary ECs harboring the *prox1a* mis-expression construct. Note that the *flt4:Gal4FF* reporter lines shows occasional expression in myotome cells as well as in a subset of neurons in the spinal cord.

lymphatic cell fate. Therefore, we aimed to mis-express *prox1a* in blood ECs and injected an *UAS:prox1a* construct into a *flt4:Gal4FF* driver-line (Fig. 5A) which drives expression from a 3.8kb *flt4* promoter fragment from Medaka (Deguchi et al., 2012). Upon transient injection of the UAS-construct, overexpression of *prox1a* transcripts was verified by whole-mount ISH. As expected, embryos injected with the UAS-construct expressed *prox1a* in a mosaic pattern in venous and arterial ECs at considerably higher levels than the endogenous endothelial expression in uninjected siblings at 32 hpf (Fig. 5B-E). We checked at 38 hpf (data not shown) and 48 hpf whether this *prox1a* mis-expression would have an influence on the behavior of positive ECs (marked by the co-expression of mRFP) during venous sprouting. However, neither the overall number of secondary sprouts nor the positioning of RFP–positive cells within the vasculature indicated a differential cell behavior upon *prox1a* over-expression (Fig. 5F, H). Furthermore and in contrast to the mouse experiments, continuous mis-expression of *prox1a* did also not result in any vascular or lymphatic defects at later stages of lymphangiogenesis. In fact, at 5 dpf no differences in vascular morphology or in the expression pattern of the *flt4:Gal4FF; UAS:GFP* marker could be appreciated and cells expressing the construct ended up in all endothelial compartments (Fig. 5G, I, data not shown). Together these results indicate that zebrafish *prox1a* is not only dispensable for lymphatic specification, but is also unable to commit ECs to a lymphatic fate.

## DISCUSSION

Specification of the lymphatic lineage in mice is intimately linked to the restricted expression of the transcription factor Prox1 in a subpopulation of cardinal vein cells, and Prox1 function is essential for future LECs to migrate out of the cardinal vein (Yang et al., 2012). In the absence of mutants for Prox1 orthologues in other vertebrate species, a stringent assessment of conserved Prox1 function during lymphangiogenesis has been hampered. Nevertheless, expression studies in, e.g. Xenopus (Ny et al., 2005) and zebrafish (Yaniv et al., 2006) suggest that there is evolutionary conservation.



Previous morpholino-knockdown strategies for zebrafish *prox1a* did not provide a fully conclusive picture and we had previously pointed out that different morpholinos directed against *prox1a* result in developmental anomalies which we interpreted as being unspecific (Kuchler et al., 2006). Hence, we generated a *prox1a* loss-of-function model to conclusively address the role of *prox1*-like genes for lymphangiogenesis in fish. Our results demonstrate that *prox1a* expression is not essential and also not sufficient for specification of LECs. Even the combined loss of *prox1a* and *prox1b* does not inhibit lymphatic development. In combination with our finding that *coup-TFII* and *sox18* are also not essential for lymphangiogenesis to occur, this suggests that lymphatic specification must be achieved in a different way and that the Coup-TFII/Sox18/Prox1 function has been evolving only in higher vertebrates or has been eliminated in the zebrafish/teleost lineage. How can one explain the strict requirement for Prox1 in mice, while in fish the Coup-TFII/Sox18/Prox1 code seems dispensable?

In mice, angiogenesis and lymphangiogenesis are two temporally separated processes. Initially, starting at E8.0, a network of intersegmental arteries and veins is formed by angiogenesis (Fig. 6A1) (Walls et al., 2008). In a second step, a subpopulation of cardinal vein cells is specified to the lymphatic lineage by Prox1 expression at E9.5 (Fig. 6A2), making only those cells responsive to the VegfC signal. These lymphatic precursors become motile then and leave the epithelial layer of the vein to form the first lymphatic structures (Fig. 6A3, A4).

In fish, however, the timing of events is different: Angiogenesis begins with a first wave of VegfA/Vegfr2 governed sprouting from the DA, giving rise to a set of intersegmental arteries (Fig. 6B1). Subsequently, a second set of sprouts arises exclusively from the PCV (Fig. 6B2). The emergence of these venous sprouts that eventually will give rise to both intersegmental veins and lymphatic precursors, depends on the Vegfc/Flt4 pathway and on Ccbe1, indicating that in zebrafish the mechanisms that drive the initial migration of both subpopulations is identical. This might also explain why genes, which at some point specifically mark LECs in mice (like *Flt4* or *Lyve1*), are expressed within the whole venous endothelium in zebrafish. It is only slightly later, when secondary sprouts reach the level of the arterial ISVs that both populations display a differential behavior, suggesting that only from this point in time lymphatic and venous fates are separating (Fig. 6B3, B4). It is therefore plausible that it is not an intrinsic mechanism but rather external cues, possibly

provided by the ISV themselves, that control which sprout will connect to an ISV and which will migrate further to the horizontal myoseptum. In fact, the only known signaling pathway so far that has been shown to affect the cell fate decision made by secondary sprouts is Dll4/Notch. Knockdown of *dll4* leads to a dramatically enhanced number of venous ISVs and only a small number of PL cells, suggesting that the fate of secondary sprouts is strongly shifted towards venous identity (Geudens et al., 2010). Whether this requirement for Notch signaling is artery-intrinsic (*dll4* and *notch1b* are both expressed in arterial ECs) or reflects a signaling interaction between ISV and secondary sprouts requires further analysis, but in either case arterial ISVs directly or indirectly influence the fate decisions made by venous sprouts.

In summary, the scheme depicted in Fig. 6 offers an explanation as to why the Coup-TFII/Sox18/Prox1 signaling axis is required in mice, but dispensable in zebrafish: in mice (and probably other vertebrate classes) Prox1 function specifies LEC fate within a subset of venous ECs within the cardinal vein. In zebrafish (and likely all teleosts), this specification step is not required within the endothelial epithelium of the cardinal vein. Our findings therefore reveal an astonishing difference in the earliest step of lymphangiogenesis between vertebrates. In the absence of a full understanding of the exact point in time when LECs become specified in zebrafish, it remains difficult to appreciate fully the degree of similarity between mice and other vertebrates at the level of genetic control and cellular behavior. However, other steps of lymphangiogenesis, such as the migration of future LECs away from the major vein, are conserved in vertebrates, as evidenced by the conserved functions of *vegfc* (Karkkainen et al., 2004; Ny et al., 2008; Villefranc et al., 2013), *flt4* (Jeltsch et al., 1997; Ny et al., 2008; Hogan et al., 2009b) and *ccbe1* (Hogan et al., 2009a; Bos et al., 2011).

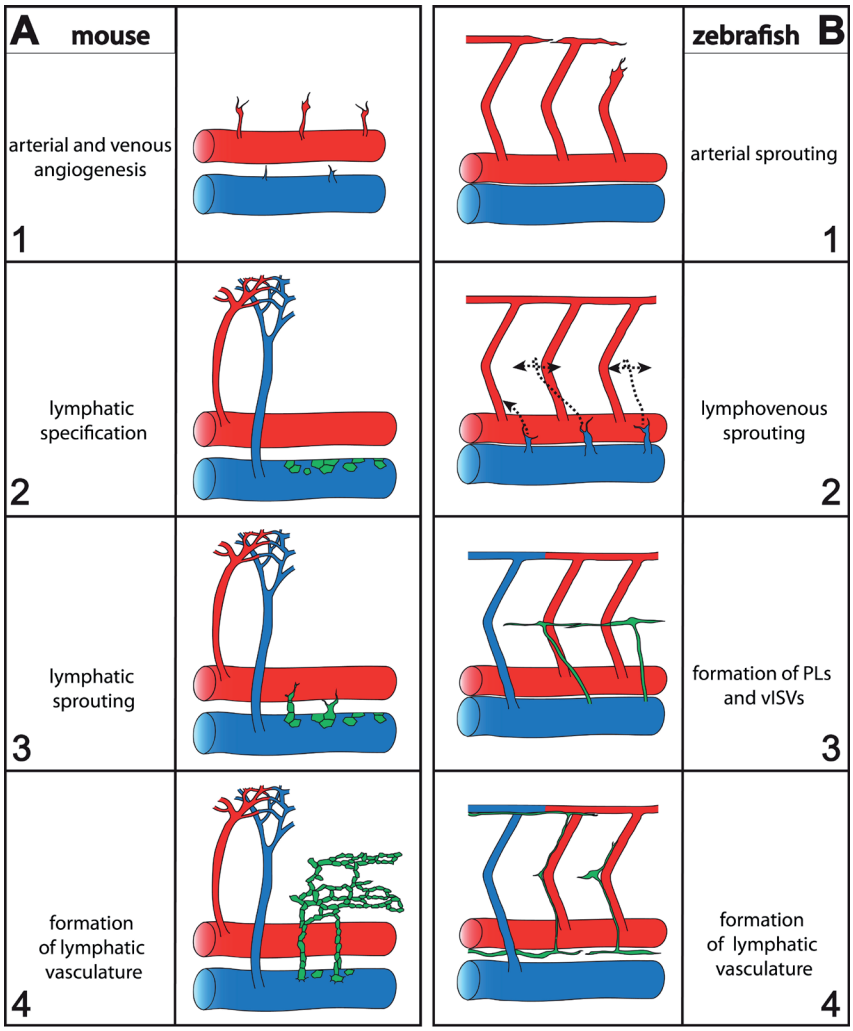



**Fig.6 Chronology of angiogenic and lymphangiogenic events in mice and zebrafish.** (A) In mice, angiogenic sprouting from the DA and cardinal vein starts at E8.0 and leads to the formation of intersegmental arteries and veins (A1). Subsequently, a subpopulation of venous ECs is specified to the lymphatic lineage by the restricted expression of *Prox1* (green cells; A2). These lymphatic precursor cells then start to leave the venous epithelium in a dorsal direction (A3), where they will eventually form transient lymphatic structures comprising the primordial TD and the dorsal peripheral longitudinal lymphatic vessel (Hagerling et al., 2013). (B) Different from the situation in mice, zebrafish angiogenic cell behavior is initially restricted to arterial ECs, which form ISVs in the trunk (B1). In a second step, venous angiogenesis and lymphangiogenesis take place simultaneously and both processes depend on the same genes (*vegfc, flt4, ccbe1*). All emerging sprouts from the PCV initially migrate dorsally but shortly thereafter they display two different cell behaviors, reflecting the first visible signs of differential cell fate amongst the sprouts: Some of them will connect to intersegmental arteries (venous fated cells) while others proceed towards the horizontal myoseptum (lymphatic fated cells), often even passing an ISV on their route (B2, dotted arrows). As a result, a set of intersegmental veins on the one hand and a pool of parachordal lymphangioblast at the horizontal myoseptum on the other hand is formed (B3). The latter ones will subsequently migrate away from this region, to form the initial lymphatic structures in the trunk (B4).

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## SUPPLEMENTARY DATA

### Supplementary figures

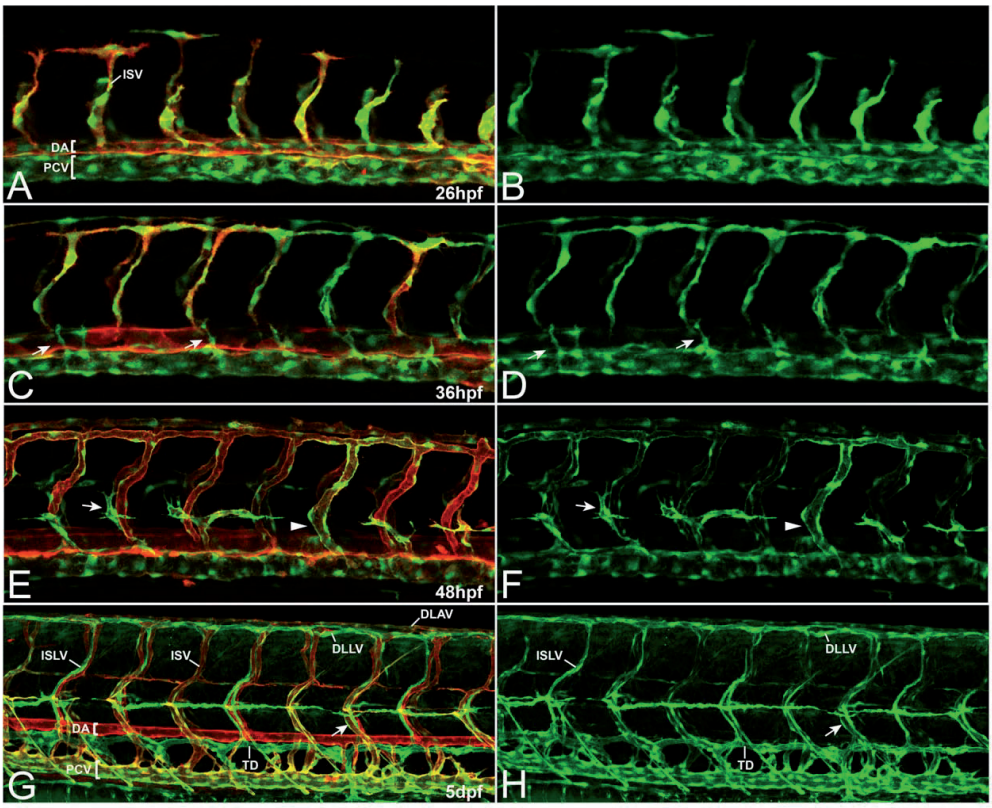



**Fig.S1 Expression pattern of *flt4*$^{BAC}$*:mCitrine* reporter line at different stages of vascular development.** (A-H) Double transgenic embryos for *flt4:mCit* (green) and *kdrl:mCherry-Caax* (red) at different stages of vascular development. (A, B) Initially, the *flt4* reporter shows expression in both, arterial and venous ECs with an enriched signal within the venous compartment (26 hpf). (C, D) From about 26 hpf onwards, arterial expression of the construct decreases, so that emerging secondary sprouts can be easily followed at around 36 hpf (arrows). (E, F) At 2 dpf, the *flt4* reporter expression is strongly confined to venous derived structures (venous ISV marked by arrowhead) and the signal gradually increases in the lymphatic lineage (see PLs highlighted by arrows). (G, H) By day 5, *flt4:mCit* expression is still evident in the PCV and venous ISVs (arrow). In addition, lymphatic structures including the TD, ISLVs as well as the DLAV are clearly highlighted throughout the trunk. DA = dorsal aorta, PCV = posterior cardinal vein, TD = thoracic duct, ISV = intersegmental vessel, ISLV = intersegmental lymphatic vessel, DLAV = dorsal longitudinal anastomotic vessel, DLLV = dorsal longitudinal lymphatic vessel.

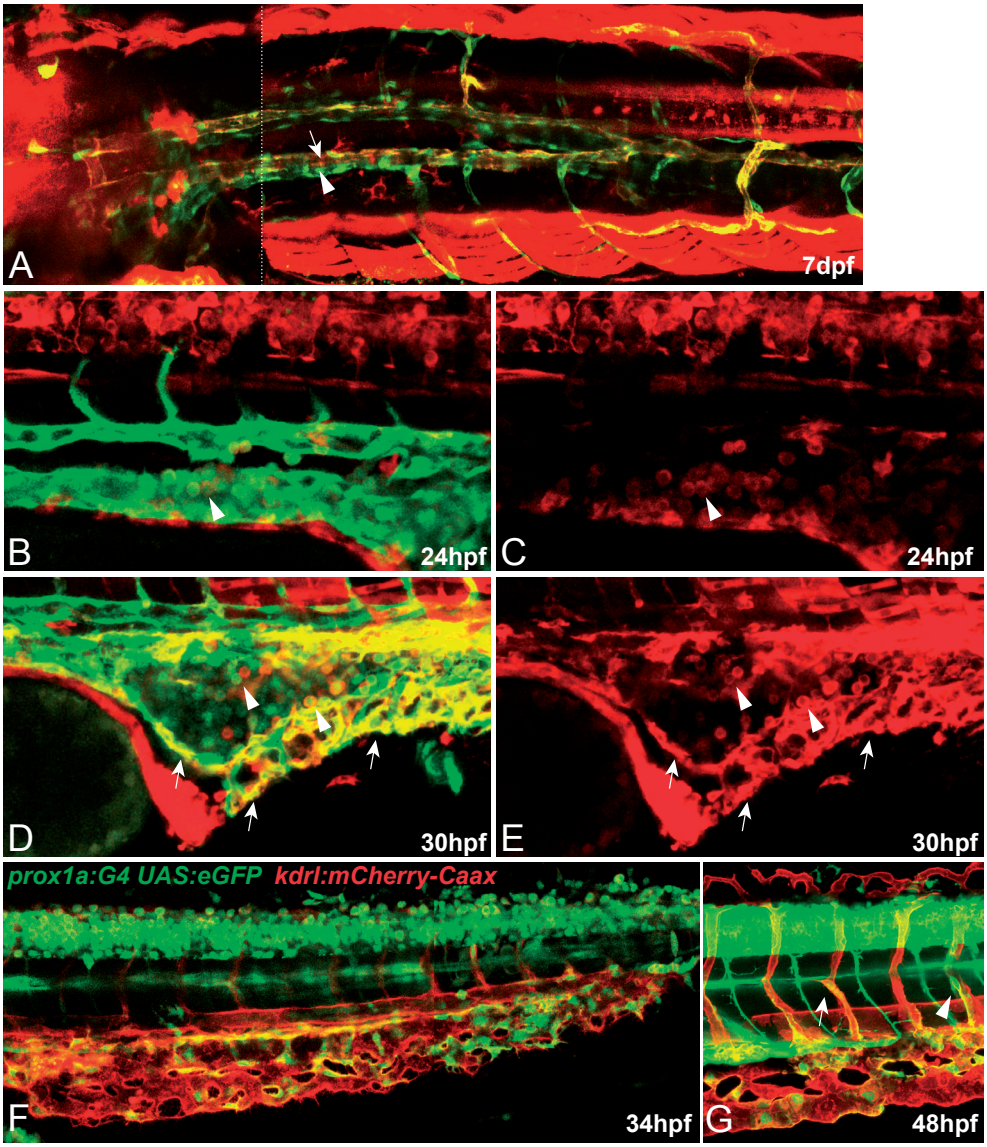



**Fig.S2 Additional expression domains of the *prox1a* reporter line.** (A-E) *Prox1a* reporter expression is shown in red, *flt4:mCit* expression is marked in green. (F, G) *kdr-l* expression domains are highlighted in red while expression of the *prox1a* reporter line is shown in green. (A) Dorsal view on the anterior part of the TD which splits up in two vessels (arrow) closely aligning to the likewise bilateral PCV (arrowhead) in a 7 dpf embryo. The image has been assembled from two overlapping partial z-projections (see dotted line). (B-E) At 24 hpf, before the onset of circulation, as yet undefined round cells within the axial vessels display expression of both markers (arrowheads in B, C). At 30 hpf, similar cells can still be seen with in the cardinal vein region (arrowhead in D, E). In addition, strong expression of the *prox1a* reporter is evident in a subpopulation of caudal vein cells (arrows in D, E). (F, G) *prox1a* is expressed within a subgroup of caudal vein cells at 34 hpf which most likely results in the higher amounts of *prox1a* positive venous (arrowhead) and lymphatic secondary sprouts (arrow) in this area at 48 hpf (G). Image F is composed of two overlapping z-projections.

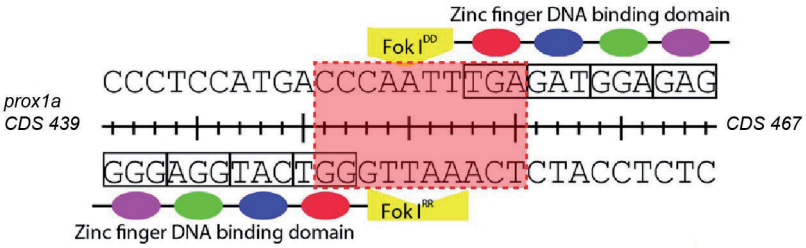



**Fig.S3 Zinc-finger nuclease mediated generation of a *prox1a* mutant allele.** Schematic representation of the zinc-finger nuclease target area in exon 1 of the *prox1a* gene. The red square indicates the deleted 10bp in the *prox1a*$^{i278}$ allele.

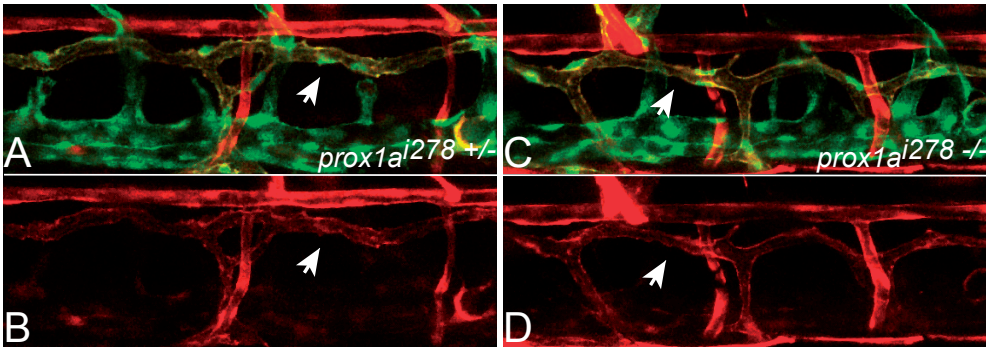



**Fig.S4 Expression of the *prox1a* reporter line in *prox1a*$^{i278}$ mutants.** (A-D) *prox1a:KalTA4,UAS:tagRFP* (TD in red), *flt1*$^{enh}$*:tdTom* (DA and arterial ISVs in red) and *flt4:mCit* positive triple transgenic embryos at 5 dpf. As in heterozygous siblings (A, B) *prox1a* reporter expression also marks the TD (see arrows) in homozygous *prox1a*$^{i278}$ mutants (C, D), suggesting that Prox1a is not required for the maintenance of its own expression.

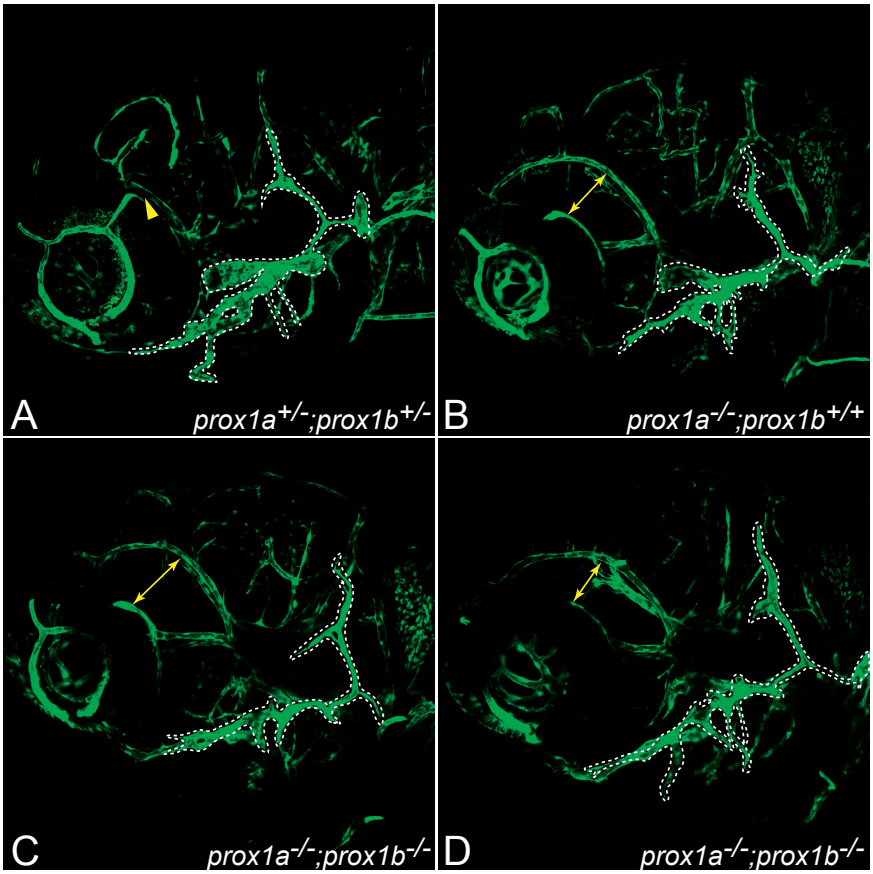
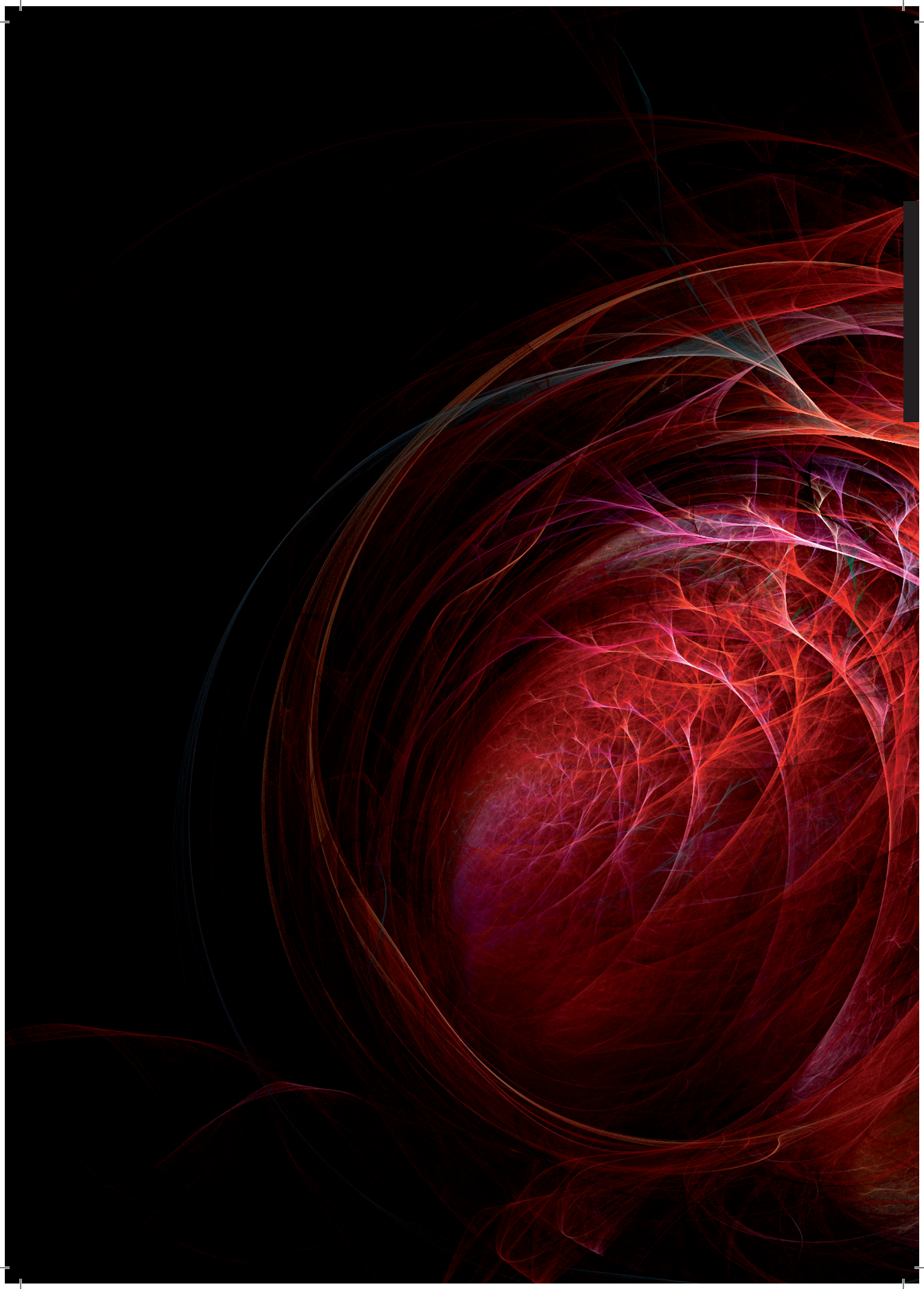
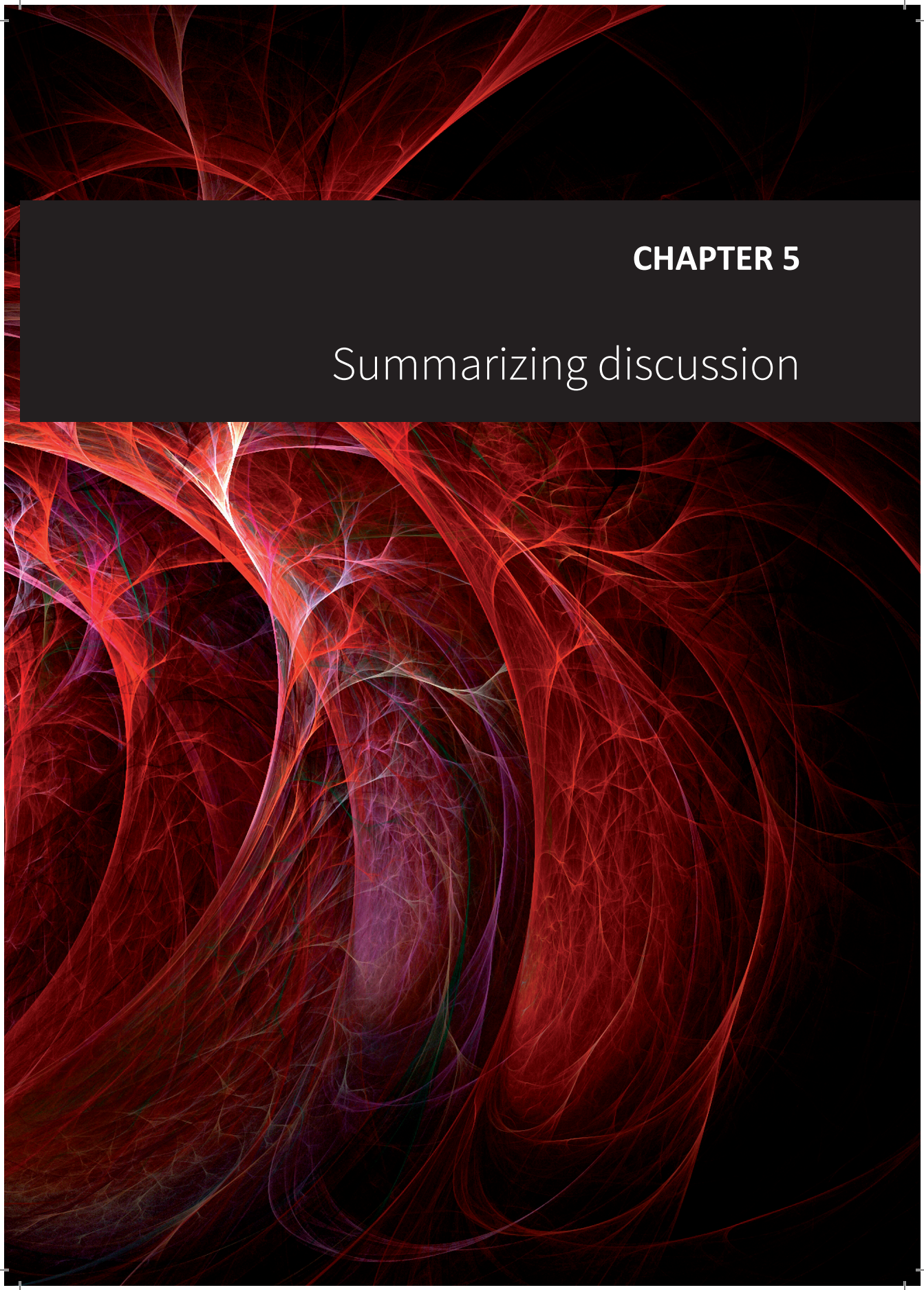



**Fig.S5 Facial lymphatics are formed in *prox1a*$^{i278}$ and *prox1a*$^{i278}$*;prox1b*$^{SA0035}$ double mutants.** (A-D) Facial lymphatic network in *flt4:mCit* expressing embryos at 5dpf. As in wild-type siblings (A), the facial lymphatics (outlined by dotted lines) also emerge in *prox1a* single (B) and *prox1a;prox1b* double mutant embryos (C-D), but seem to be less developed compared to wild-type structures at the same age. Yellow arrows indicate the distance between the dorsal ciliary vein and the primordial midbrain channel that is enlarged in *prox1a*$^{-/-}$ embryos due to massive eye edema (also see Fig. 2E).

**Supplementary Table S1: Primers used in this study**

| primer name | Sequence |
|---|---|
| flt4_3.8kb_F | 5’-CCGGAATTCCGCAGTCCGTCAATACTGAGG-3’ |
| flt4_3.8kb_R | 5’-GCGGAATTCCTCCAGATCTCCAGTCCAGAA-3’ |
| prox1a_wt | 5’-GAAGGTGACCAAGTTCATGCTACCATTCAGCAGGCCCTCCATGAC-3’ |
| prox1a_mut | 5’-GAAGGTCGGAGTCAACGGATTACCATTCAGCAGGCCCTCCATGAG-3’ |
| prox1a_common | 5’-GCCCTTAGATGCTCATCTGTTAGCCT-3’ |
| Sox18_wt | 5’-GAAGGTGACCAAGTTCATGCTCGCTGCTGTTCGAGACAC-3’ |
| Sox18_mut | 5’-GAAGGTCGGAGTCAACGGATTCGCTGCTGTTCGAGACAG-3’ |
| Sox18_common | 5’-GCACCAGTGCCTGGGTCTGGAA-3’ |
| CoupTFII_wt | 5’-GAAGGTGACCAAGTTCATGCTACACCCGTTCAAGGACCC-3’ |
| CoupTFII_mut | 5’- GAAGGTCGGAGTCAACGGATTACACCCGTTCAAGGACCC-3’ |
| CoupTFII_common | 5’-CGGGGTTGACTGTGTGTTGTT-3’ |





# CHAPTER 5

# Summarizing discussion

The differentiation of endothelial cells is an important process during vascular development, however its precise regulation remains incompletely understood. Here we shed light on several aspects of endothelial specification, thereby clarifying certain issues and opening up new paths in the understanding of endothelial differentiation during vascular development.

## ENDOTHELIAL SPECIFICATION IN ARTERIAL AND VENOUS FATES

In this thesis we give a detailed analysis of the first events in endothelial cell specification. Vascular development starts with the migration of mesodermal-derived angioblasts, which in zebrafish are localized in two bilaterally positioned populations in the lateral plate mesoderm. At the 4-somite stage a subset of angioblasts starts to express *etv2*/*etsrp,* a transcription factor that has important functions during vasculogenesis (Sumanas and Lin, 2006; Pham et al., 2007). *etv2*/*etsrp* expression starts within medially located angioblasts and appears a few hours later in a more lateral population of angioblasts (Pham et al., 2007). Also *fli1a* and *kdrl* expression, both marking endothelial cells, appears first in the medially located angioblasts and a few hours later in the laterally positioned angioblasts (Kohli et al., 2013). Whether laterally located angioblasts arise later in development or whether they only start to express these markers later still remains to be resolved: nevertheless this is a first clue to differentially specified angioblasts. Zhong et al. reported on the expression of *hey2* specifically in the medially located angioblasts at the 10 somite stage (Zhong et al., 2000). Hey2 is known to be expressed exclusively in the arterial vasculature at later stages, suggesting that the medial angioblasts will differentiate into arterial ECs. Recently, single cell tracing by Kohli et al. demonstrated that the medial angioblasts will start migrating around 14 hpf (10-somite stage) and the lateral angioblasts will start to migrate to the midline around 16 hpf (15-somite stage). Furthermore, the medially located angioblasts will form the arterial cells in the DA, and the laterally localized angioblasts will give rise exclusively to the venous cells in the PCV (Kohli et al., 2013). These expression studies and cell tracing experiments suggest that the position of the angioblasts in early development is predictive for their arterial or venous fate. In contrast, Herbert et al. demonstrated that venous cells of the PCV arise from endothelial cells in the DA migrating ventrally (Herbert et al., 2009). However, this notion is not supported by previously mentioned studies - whether this is due to technical discrepancies remains to be resolved. When and how the angioblasts commit to the arterial or venous fate remains an interesting and still largely unresolved question. Further detailed lineage-tracing studies will help to understand the migration routes of angioblasts and clarify the timeframe of arterial-venous commitment.

Overexpressing and loss-of-function studies revealed that VegfA and Shh are crucial in regulating angioblast migration. VegfA and Shh are suggested to work as morphogens, guiding the medial and lateral angioblasts towards the midline. The medially located angioblasts presumably receive higher concentrations of Vegf and Shh than the laterally located angioblasts, causing the medial angioblast to differentiate into arterial cells and the

lateral angioblast to differentiate into venous cells (Kim et al., 2013; Kohli et al., 2013). Besides the involvement of secreted factors in arterial-venous specification, also cell-autonomous regulations are known to be involved in specifying angioblasts into arterial and venous ECs. Several well-studied pathways are linked to cell autonomous regulation of arterial development, such as the VegfR-Plcγ-Mek-Erk and Dll4-Notch-Hey2 pathways (Lawson and Weinstein, 2002; Lamont and Childs, 2006; Lin et al., 2007). However, these proteins are not detected until the angioblasts have migrated to the midline (at the 20-somite stage), suggesting either a full commitment to the arterial or venous fate once the angioblasts reach the midline, or the requirement of these genes only in the actual formation of functional arterial vessels, rather than during angioblast migration. In venous cells, these pathways are inhibited resulting in the expression of venous markers, like Ephb4, on the membrane. EphB4 functions as a venous transmembrane receptor for the arterial Efnb2 ligand, and both start to be expressed around the 20 somite stage. Reciprocal signaling between *efnb2* and *ephb4* is crucial for proper arterial-venous specification (Wang et al., 1998).



It is suggested that the venous state is the default pathway that can be remodeled to the arterial fate by activation of the VegfR and Notch pathways (Thurston and Yancopoulos, 2001). However, phosphatidylinositol-3 kinase (PI3k) and Akt can be activated in venous cells which inhibit the Plcγ-Mek-Erk pathway (Hong et al., 2006). Furthermore, in the vasculature of mice lacking Coup-TFII the venous fate is shifted towards the arterial fate, suggesting a role for Coup-TFII is venous specification (You et al., 2005). The regulation of venous specification by Coup-TFII and the PI3K-Akt pathway suggests that venous specification in mice is under genetic control rather than functioning as a default pathway. In contrast, we show that zebrafish *coup-TFII* mutants have no abnormalities in venous and lymphatic vasculature development (van Impel et al., 2014), hinting at substantial differences in venous specification between different vertebrate classes. The distinct requirement of several proteins in arterial versus venous cells is based on a complex degree of interplay. To understand the specific regulation of arterial-venous development, further studies about the different (known and yet unknown) factors involved in this process is required. High-throughput screening for mutations or specific loss of function studies in vertebrate model systems as well as large-scale analysis of the differential expression on arterial and venous cells will help to clarify these regulations.

## SOXF-FAMILY MEMBERS IN ARTERIAL-VENOUS SPECIFICATION

In this thesis we report on the function of the SoxF family members during vascular development. Whereas the functions of Sox17 and Sox18 are well studied (reviewed in Francois et al., 2010), the understanding of the role of Sox7 was still inconclusive. Mice lacking Sox7 function have severe cardiovascular malformations, but a detailed study on the causative defects is precluded by early lethality (Wat et al., 2012). Previous studies in zebrafish with the use of morpholinos could not identify a vascular function of Sox7

(Cermenati et al., 2008; Herpers et al., 2008; Pendeville et al., 2008), however, this could also have been due to the use of suboptimal concentrations of the morpholino in question. In contrast, by generating *sox7* zebrafish mutants, our data revealed an important role for Sox7 in vascular development. *sox7* loss of function results in defective blood circulation. Detailed histological and live observations revealed specific ectopic connections between the arterial lateral dorsal aorta (LDA) and the venous primary head sinus or the common cardinal vein in *sox7* mutants. This ectopic connection results in a short loop of circulation close to the heart. Expression analysis of arterial and venous markers revealed elevated levels of *flt4/vegfr3* in the arterial cells of the LDA of *sox7* mutants, exactly at the location of the ectopic arterial-venous connection. We suggest a disturbance in the arterial-venous regulation in the LDA cells, resulting in defective circulation.

Previous observations and data presented here demonstrated that zebrafish embryos with loss of function for both *sox7* and *sox18* display more severe disturbances, with multiple shunts between the arterial and venous vessels and a miss-regulation of several arterial and venous markers. We also showed in an independent study that loss of *sox18* alone had no influence on vascular development. These observations imply redundant roles for *sox7*, *sox18* and possibly *sox17* in vascular development. However, in *sox7* mutants these compensatory pathways are not functional in the LDA, suggesting a unique regulation in the LDA cells. While the DA is formed by migrating medial angioblasts (from the posterior LPM) to the midline, the LDA is formed by two populations of medial angioblasts (from the anterior and posterior LPM) migrating towards each other (Siekmann et al., 2009). Also the timing of LDA formation is slightly later (around 22-somite stage) than the DA formation (around 20-somite stage) (Siekmann et al., 2009), possibly indicating that LDA cells behave differently and are under different genetic control than other arterial cells.

The miss-regulation of specifically *flt4*, but not other markers, in *sox7* mutants might be taken to suggest a strong direct connection between Sox7 and Flt4. However, an alternative and somewhat more likely scenario is that there are subtle changes in the expression level of other factors, but that these differences, while biologically meaningful, are too small to detect by current methods.

We also report on newly generated *hey2* and *efnb2* mutants, which develop similar arterial-venous specification defects as we detected in *sox7* mutants. Combining *sox7* and *hey2* loss of function alleles resulted in an increase in penetrance of the disrupted circulation and ectopic shunt formation, revealing the genetic interaction of *sox7* and *hey2*. Furthermore, combinations of *sox7* and *efnb2* loss-of-function alleles resulted in an enhancement of the specific phenotype, suggesting genetic interactions of *sox7* with *efnb2*. These results point towards an important role for Sox7 in Hey2 and Efnb2 functioning. Arterial-specific overexpression of Notch1-ICD could rescue the defect in the vasculature of *sox7* mutants, placing Sox7 upstream of Notch1 in the arterial specification pathway. Previous studies reported a role for Hey2 and Efnb2 downstream of Notch (Lawson et al., 2001), supporting

our notion that Sox7 works upstream in the arterial specification pathway, mediating Hey2 and Efnb2 function. Recently, Sacilotto et al. reported that SoxF members can bind to the arterial specific enhancer of the Notch ligand Dll4 (Sacilotto et al., 2013), supporting our *in vivo* data that Sox7 acts upstream of Notch signaling.

Besides the role of Notch signaling in specific arterial-venous specification, Notch is known to be involved in several other processes, such as the sprouting of vessels in angiogenesis. Interference of the Dll4-Notch signaling pathway by depleting Dll4 during angiogenesis results in arterial hyper-sprouting. These rather distinct mechanisms mediated by the same components suggest tight temporal and spatial control of the Notch signaling components. The requirement of these components at the right place at the right time is crucial in development. This highlights the complex and tight regulation of these and other genes and promotes future studies to broaden the focus to combinatory regulations of one specific gene as well as between several genes.



## ENDOTHELIAL SPECIFICATION IN LYMPHATIC FATE

After the arterial-venous vasculature is established within the murine embryo, a subset of venous cells will differentiate into lymphatic endothelial cells. These lymphatic endothelial cells will form the lymphatic vasculature, important in maintaining fluid homeostasis, the transport of intestinal lipid, and immune function. Studies in mice identified the expression of SOX18 and COUPTFII in a subset of venous cells, which induce the polarized expression of PROX1 (Francois et al., 2008; Srinivasan et al., 2010). These venous cells will migrate and differentiate into lymphatic cells forming the lymphatic vasculature. Mice loss- and gain-of-function studies identified *Prox1* as an essential regulator of lymphatic specification. Previous zebrafish studies using morpholinos reported a conserved role for Sox18 (Cermenati et al., 2013) and CoupTFII (Aranguren et al., 2011) in lymphangiogenesis, while the understanding of the role of Prox1 in zebrafish was still inconclusive (Kuchler et al., 2006; Tao et al., 2011), even though it had been suggested to have an evolutionary conserved and central role in lymphangiogenesis (Del Giacco et al., 2010). In contrast, by making use of loss-of-function mutants generated by TILLING and TALEN techniques, we report that in zebrafish *prox1* as well as *sox18* and *coupTFII* are dispensable for lymphatic specification. This discrepancy between phenotypes generated through the use of morpholinos versus targeted mutagenesis in zebrafish is a current debate. While morpholinos are relative easy to use and the effect can be studied directly, it can have severe toxic side-effects and these off-target effects are complicated to control. TILLING and TALEN/CRISPR approaches are therefore rapidly gaining interest in which specific mutations are induced leading to loss-of-function alleles (Schulte-Merker and Stainier, 2014).

To support the notion that *prox1* is dispensable for lymphatic specification, a zebrafish *prox1a* reporter line has been generated which revealed the details of *prox1a* expression,

with expression becoming restricted to lymphatic cells only at later stages during lymphangiogenesis. During LEC specification, zebrafish *prox1* is not exclusively expressed in lymphatic cells and not all lymphatic cells will express *prox1* until 3.5dpf. Combining this data with our loss-of-function data suggests that the lymphatic specification is differentially regulated between fish and mice. In contrast, studies on the function of *flt4* (Jeltsch et al., 1997; Hogan et al., 2009b), *vegfc* (Karkkainen et al., 2004; Villefranc et al., 2013) and *ccbe1* (Hogan et al., 2009a; Bos et al., 2011) showed that these three genes have an important and apparently fully conserved function in lymphangiogenesis, both in mice and in zebrafish. Since the detailed mechanism of lymphatic specification and the exact time point of this process are still unknown in vertebrates, it remains difficult to address any conclusions or comparisons between the species. It is possible that the CoupTFII-Sox18-Prox1 signaling axis has evolved only in higher vertebrates, or that it has been eliminated in the zebrafish. The redundant functions of SoxF genes previously discussed, suggest also in this process combinatory roles of different SoxF members. Although no redundant roles are known yet of the CoupTFII family members, it is possible that CoupTFII functions with other CoupTF family members in zebrafish lymphangiogenesis. Another alternative is that in zebrafish Prox1, Sox18 and CoupTFII compensate for each other. Combinatorial loss-of-function of these three genes (possibly with their duplicates or family members) will elucidate a possible function of these genes in zebrafish lymphangiogenesis further.

In mice, venous cells get specified in the cardinal vein; in zebrafish it is not until the secondary sprouts reach the ISV that they will differentiate into lymphatic cells or become venous ISV cells. Specific labelling of lymphatic (precursor) cells early in development will reveal the time point of lymphatic commitment, which will lead to the better understanding of the mechanisms involved and the differences between mice and zebrafish lymphangiogenesis. Secondary sprouting in zebrafish is (partially) regulated by extrinsic signals. This hypothesis is supported by Dll4/Notch signaling in arterial cells, where inhibiting Dll4 signaling in the arterial ISV will result in a shift of the secondary sprouts towards the venous fate, eventually leading to an enhanced number of venous ISVs and less lymphatic endothelial cells (Geudens et al., 2010). This suggests that arterial signaling is able to regulate the fate of the secondary sprouts, but whether this is a direct or indirect regulation remains to be resolved. Another possibility is the involvement of surrounding tissues (e.g. somites or neurons) in the regulation of the migration of the sprouts which may function as a guidance template during lymphatic cell migration. Genetic loss- and gain-of-function studies targeting these surrounding tissues will provide insights into this hypothesis. Furthermore, analyzing the specific gene expression of the venous cells at the moment of lymphatic fate commitment will yield important information on lymphatic specification.

## CLINICAL IMPLICATIONS

The molecular regulations of vascular development during embryology are highly similar to those of vascular development during later stages, and it is therefore possible to translate findings from vascular embryology to clinical settings. Disturbances in the vasculature can cause a wide array of cardiovascular diseases, whereas locally enhanced vessel formation in tumors can induce tumor growth and metastasis. Some of the genes mentioned in this thesis have been linked to clinical syndromes. Whereas in mice and zebrafish *Sox18* is suggested to be (redundantly) involved in vascular and (in mice) lymphatic development, in humans *SOX18* mutations are linked to Hypotrichosis-lymphedema-Telangiectasia (HLT). Patients with HLT have disrupted blood and lymphatic vessel (and hair follicle) development, resulting in severe lymphedema and vascular leakage (Irrthum et al., 2003). Additionally, mice lacking *Sox18* function have reduced angiogenesis in tumor development, suggesting important roles for *Sox18* in tumor progression and eventually metastasis (Young et al., 2006). Furthermore, mutations in *SOX17* are linked to primary lymphedema in humans (Ferrell et al., 2008). SOX7 is suggested to act as a tumor suppressor with a down-regulation of SOX7 expression in several cancer cell lines (Hayano et al., 2013). Decreased SOX7 expression has been linked to poor outcome of cancer patients (Li et al., 2012; Zhong et al., 2012; Stovall et al., 2013). Mutations in *COUP-TFII* are, besides previous mentioned functions, also linked to congenital heart defects in humans (Pereira et al., 1999; Al Turki et al., 2014). These observations show the importance of these genes in cardiovascular diseases and cancer. While more research is needed to explore the functions of these genes in detail, current data hint for these genes to be potential entry points for targeted therapies in cardiovascular diseases, tumor growth and metastasis. It also underscores the importance of detailed developmental research, to increase our knowledge of the regulation of the vasculature in normal and pathological development.

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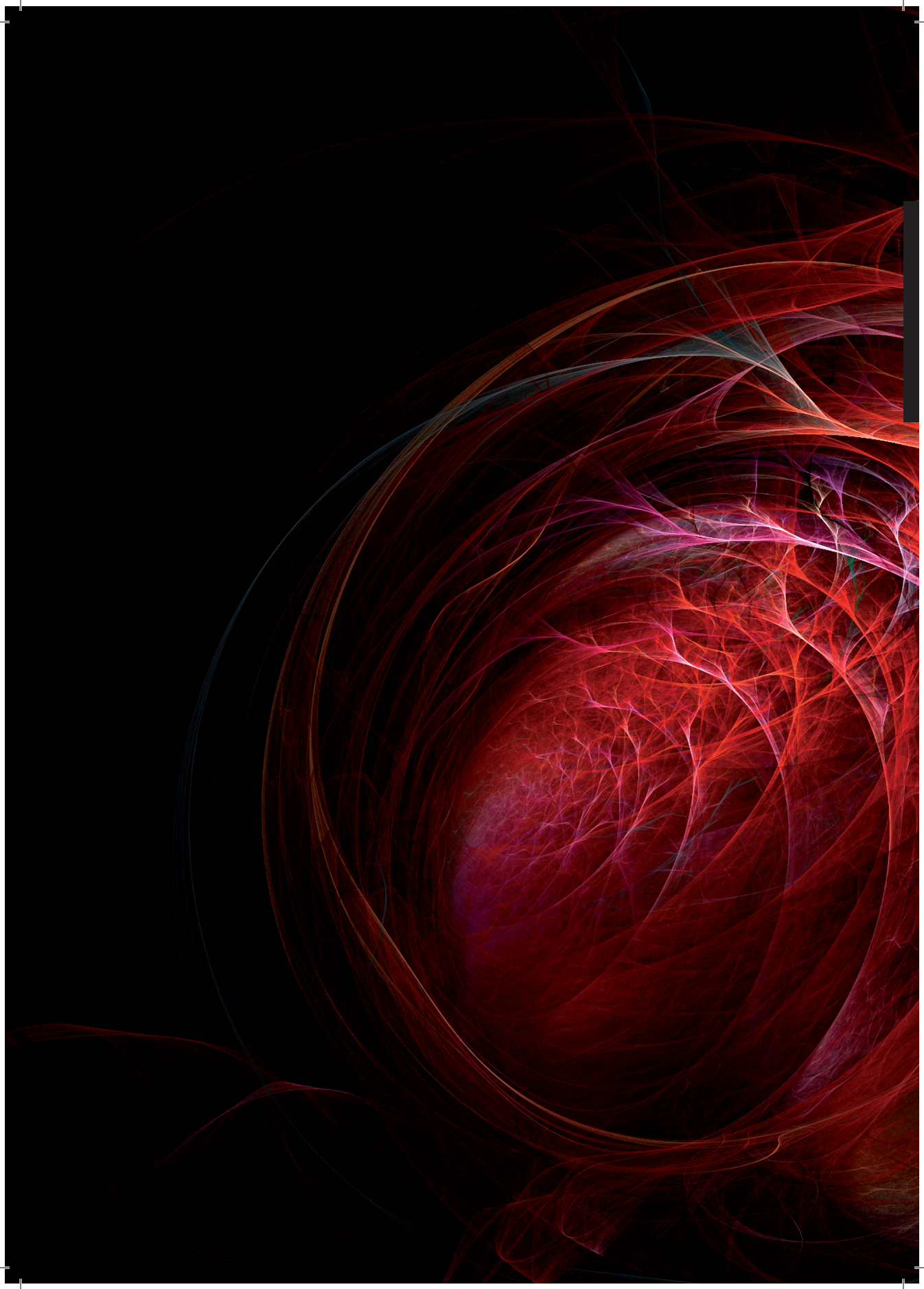
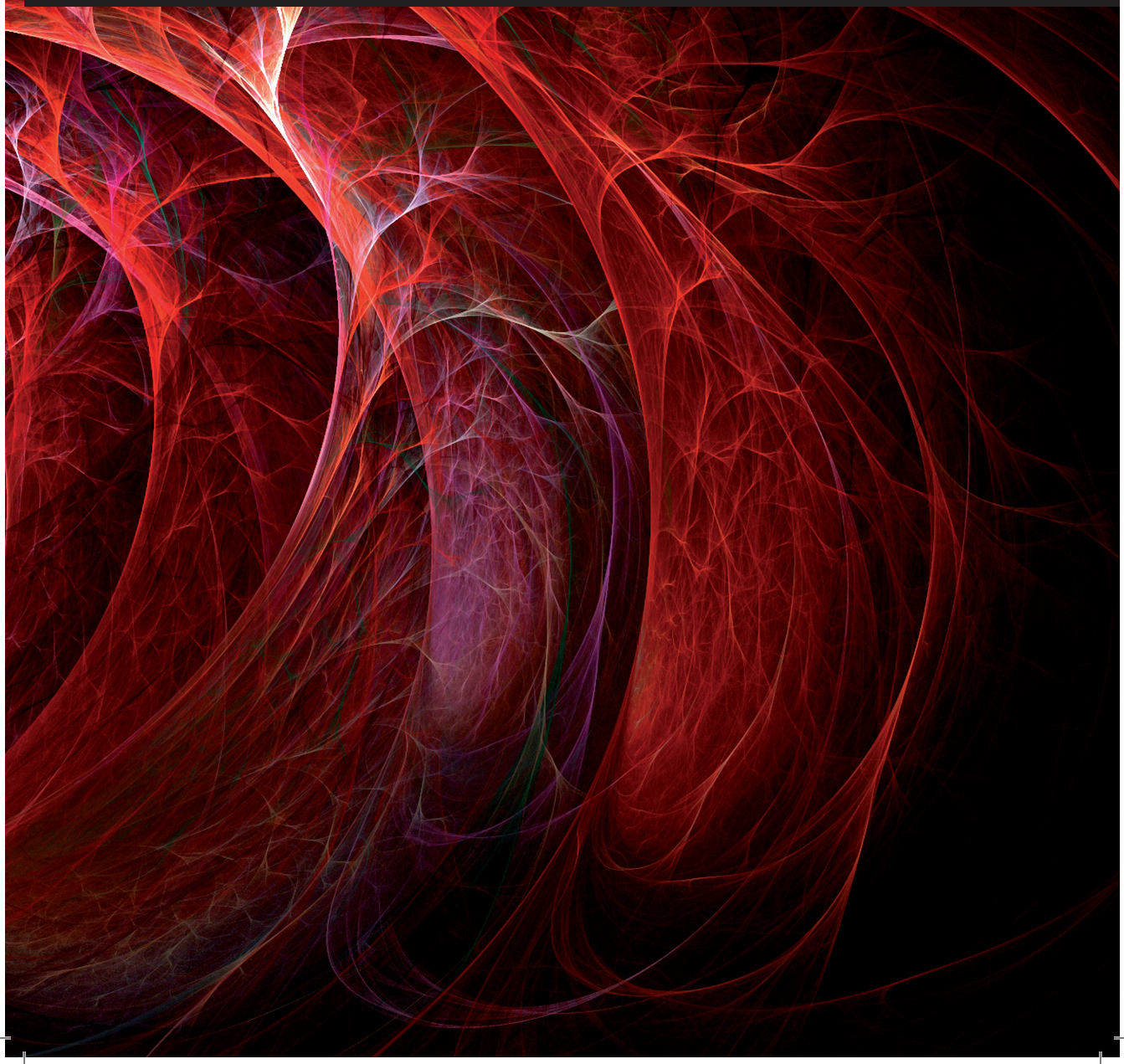


# Addendum



## NEDERLANDSE SAMENVATTING

Het hart- en vaatstelsel is het eerste functionele orgaan in een embryo en is cruciaal voor de verdere ontwikkeling. Het hart, de bloedvaten en het bloed zijn belangrijk voor het transport van voedingstoffen en afvalstoffen in het lichaam. Een verstoorde bloedcirculatie kan leiden tot hart- en vaatziekten en heeft ook een cruciale rol bij kanker. Hart- en vaatziekten en kanker zijn momenteel de belangrijkste doodsoorzaken in de westerse wereld. Met hart- en vaatziekten worden alle problemen met het hart en/of de bloedvaten bedoeld. De meest bekende en gevaarlijkste hart- en vaatziekten zijn een hartinfarct en een beroerte. Een hartinfarct ontstaat als een van de vaten die het hart voorziet van bloed niet goed functioneert, waardoor het hart niet voldoende zuurstof meer ontvangt. Een beroerte is een tekort aan bloedtoevoer naar de hersenen, doordat een bloedvat in de hersenen geblokkeerd raakt of doordat er bloedingen ontstaan. Het risico op het krijgen van hart- en vaatziekten hangt af van vele factoren. Dieet, levensstijl en leeftijd worden vaak genoemd als risicofactoren, maar ook erfelijkheid speelt een cruciale rol; vele genen zijn inmiddels al gerelateerd aan hart- en vaatziekten. Waar in hart- en vaatziekten de bloedcirculatie is geblokkeerd op specifieke plaatsen, is bij kanker de bloedtoevoer en vatengroei juist lokaal gestimuleerd. Kwaadaardige tumoren bevatten een complex netwerk van vaten die ervoor zorgen dat de tumor kan groeien. Chemotherapie is vaak gefocust op dit aspect van kanker, door de vorming van nieuwe bloedvaten in de tumoren te remmen. Het ontstaan van uitzaaiingen vindt voornamelijk plaats door de verspreiding van tumorcellen door bloed- en lymfevaten.

Om deze ziekten beter te bestrijden is het van belang de veranderingen in bloedvaten te begrijpen, maar ook de normale ontwikkeling van bloedvaten te bestuderen. De (moleculaire) processen die betrokken zijn bij hart- en vaatziekten en de groei van bloedvaten in kwaadaardige tumors zijn te vergelijken met de processen bij de embryologische ontwikkeling van de bloedvaten. Het bestuderen van de vasculaire ontwikkeling zal daarom belangrijke inzichten in de behandeling en voorkoming van hart- en vaatziekten blijven geven.

Elke cel in ons lichaam bevat erfelijk materiaal opgeslagen in het DNA. Het DNA vormt de genetische code voor het maken van vele verschillende eiwitten die belangrijke processen in de cel reguleren. Elke cel heeft een eigen set geactiveerde eiwitten die de cel specifieke eigenschappen geven. Met het onderzoek in dit proefschrift willen we de eiwitten en dus genen die betrokken zijn bij de ontwikkeling van het vatensysteem verder toelichten. Ook ontdekken we van bepaalde eiwitten een tot nu toe onbekende rol in de ontwikkeling van het vatenstelsel.

In **hoofdstuk 1** wordt het vatenstelsel in detail toegelicht. De belangrijkste bouwstenen van bloedvaten zijn de endotheelcellen. Alle bloedvaten bestaan uit een laag endotheelcellen die een lumen (holte) omringen. In de grotere vaten worden de endotheelcellen omringd door spiercellen. Endotheelcellen ontstaan vroeg in de ontwikkeling, waarbij mesodermale cellen migreren naar een specifieke regio in een embryo, het laterale plaat mesoderm. Vanuit deze locatie migreren deze endotheliale voorlopercellen naar elkaar en vormen de eerste vaten. Dit proces waarbij bloedvaten worden gevormd uit endotheliale voorlopercellen wordt vasculogenese genoemd. Na het ontstaan van een primitief vasculair systeem door vasculogenese, kunnen endotheelcellen vanuit de bestaande bloedvaten nieuwe bloedvaten vormen door middel van het vormen van vertakkingen. Dit proces wordt angiogenese genoemd. Endotheelcellen differentiëren in arteriële en veneuze endotheelcellen. Arteriële endotheelcellen vormen samen arteriën die het bloed van het hart naar alle organen en cellen in het lichaam transporteren. Veneuze endotheelcellen vormen de venen, die het bloed van de organen en weefsels terug naar het hart brengen. Naast deze circulaire bloedsomloop in arteriën en venen, bestaat er nog een specifiek vatenstelsel, het lymfatisch stelsel. Veneuze endotheelcellen kunnen zich verder differentiëren in lymfatische endotheelcellen die de lymfevaten vormen. Het lymfatische vatenstelsel neemt vloeistof op en transporteert dit naar lymfeknopen. Hier wordt de vloeistof gefilterd waarna het via de lymfevaten naar de venen wordt getransporteerd.

Om deze precieze ontwikkeling te visualiseren en de functie van een specifiek eiwit te onderzoeken, maken we gebruik van zebravissen. Dit modelsysteem heeft een vrijwel identieke bloedvatenontwikkeling als de mens. Ook het DNA van de zebravis komt sterk overeen met dat van zoogdieren. De genen voor het functioneren van het hart- en vatenstelsel en andere essentiële processen zijn bewaard gebleven tijdens de evolutie, waardoor we de zebravis goed kunnen gebruiken voor onderzoek op dit gebied. Bovendien vindt de embryonale ontwikkeling van zebravissen plaats buiten de moeder en de vele eitjes zijn transparant gedurende de vroege ontwikkeling. Dit maakt het mogelijk om de ontwikkeling van de zebravis live te bestuderen met behulp van microscopen. Omdat vrijwel het gehele DNA van de zebravis bekend is, is het tevens mogelijk om het DNA te manipuleren waarbij bijvoorbeeld het DNA zo wordt aangepast dat een stukje niet meer functioneert. Naast het aanbrengen van mutaties in het DNA op de precieze plaats van interesse, kunnen we het DNA zodanig manipuleren dat bijvoorbeeld de arteriën rood kleuren en de venen groen zodat we deze vaten separaat kunnen bestuderen.

In **hoofdstuk 2** wordt de ontwikkeling van endotheelcellen in detail besproken. We bediscussiëren de allereerste processen in de endotheelcellen en bespreken het moment van arteriële en veneuze differentiatie. De differentiatie in arteriële en veneuze endotheelcellen wordt gemedieerd door verschillende moleculaire processen. Arteriële en veneuze cellen hebben elk hun eigen complexe moleculaire regulatie. De eiwitten

VegfA en Shh werken als morfogenen waarbij ze de migratieroute (transportroute) van de endotheel voorlopercellen beïnvloeden. De fysieke locatie van deze cellen ten opzichte van de morfogenen kan mede bepalen welke migratieroute de cellen volgen. Door het volgen van een migratieroute zullen de endotheel voorlopercellen naar een specifieke plaats in het midden van het embryo migreren en daar de eerste grote arteriële of veneuze bloedvaten vormen. Ook Efnb2 en Ephb4 zijn cruciaal voor de arteriële-veneuze differentiatie. Efnb2 is een arterieel transmembraaneiwit dat functioneert als een receptor voor het veneuze transmembraanligand Efnb4. De binding van deze eiwitten en dus ook het fysieke contact van arteriële en veneuze cellen is essentieel voor de specificatie. In arteriële cellen zijn tevens de VegfR-Plcy-Mek-Erk en de Dll4-Notch-Hey signaleringsroutes geactiveerd. In veneuze cellen worden PI3K en Akt geactiveerd, deze eiwitten kunnen de Plcy-Mek-Erk signalering remmen en daarmee de arteriële identiteit onderdrukken en de veneuze identiteit aannemen. De complexe regulatie door deze en andere eiwitten zorgt dus voor de ontwikkeling van endotheelcellen in arteriële dan wel veneuze cellen, die de bouwstenen vormen voor de arteriële en veneuze bloedvaten.

In **hoofdstuk 3** wordt een belangrijke groep eiwitten in arteriële-veneuze specificatie, de soxF eiwitten, verder besproken. We belichten de tot nu toe onbekende, specifieke en cruciale functie van *sox7* in de ontwikkeling van het vatenstelsel. Zebravissen met een mutatie in hun *sox7* genoom vertonen een verstoorde bloedcirculatie. Gedetailleerde histologische en 'live' observaties lieten zien dat er ectopische connecties ontstaan tussen de specifieke vaten, namelijk de arteriële Laterale Dorsale Aorta (LDA) en de veneuze Primaire Hoofd Sinus en de Cardinale Vene, in *sox7* mutanten. Deze ectopische connecties resulteren in een verkorte circulatie-loop rond het hart. Wanneer we kijken naar andere eiwitten in deze cellen van de *sox7* mutanten, zien we dat *flt4/vegfr3*, een eiwit dat normaal alleen in veneuze cellen geactiveerd is en niet in arteriële cellen, in de arteriële cellen van de LDA in hoge mate wordt geactiveerd. Dit suggereert dat deze arteriële cellen in *sox7* mutanten een veneuze identiteit aannemen. Deze observaties suggereren een afwijking in de arteriële-veneuze regulatie in de LDA cellen in *sox7* mutanten, resulterend in een verstoorde circulatie. In dit hoofdstuk rapporteren we ook over de nieuw gegenereerde *hey2* en *efnb2* mutanten, welke dezelfde verstoorde circulatie laten zien. Ook hier is er sprake van arterieel-veneuze specificatie defecten zoals gezien in *sox7* mutanten. Door het combineren van *sox7* en *hey2* of *efnb2* mutaties konden we zien dat deze drie eiwitten in dezelfde arteriële signaleringsroute functioneren en elkaar daarbij beïnvloeden. Verder zagen we dat het specifieke vasculaire defect in *sox7* mutanten te verhelpen is met het specifiek arterieel activeren van het intracellulaire domain van Notch1. Dit wijst op een rol voor Sox7 in het beïnvloeden van Notch1 in de arteriële signaleringsroute en daarbij ook het reguleren van *hey2* en *efnb2* functie, waarmee we een stap verder komen in het begrijpen van de arteriële-veneuze specificatie.

Nadat het arteriële-veneuze vatenstelsel is ontwikkeld in embryo's, kan een deel van de veneuze cellen zich differentiëren in lymfatische cellen. Deze lymfatische cellen zullen het lymfatisch vatenstelsel vormen dat belangrijk is in homeostase, transport van o.a. lipiden en het functioneren van het immuunsysteem. In **hoofdstuk 4** gaan we dieper in op de differentiatie van lymfatische cellen en de moleculaire processen die daarbij betrokken zijn. Studies met muizen als modelsysteem hebben laten zien dat een gedeelte van de veneuze cellen Sox18 en Couptf2 activeren. De activatie van deze eiwitten induceert de activatie van Prox1, waarbij deze veneuze cellen zullen migreren en differentiëren in lymfatische cellen. In deze muizenstudies is Prox1 als een essentiële regulator voor de lymfatische ontwikkeling geïdentificeerd. Vorige zebravisstudies (met gebruik van morpholinos) suggereerden een evolutionaire rol voor *sox18* en *couptf2* in lymfe-angiogenese, tevens is de functie van *prox1* in zebravis nog onduidelijk. Door gebruik te maken van meer geavanceerde technieken, TILLING en TALEN, hebben we specifieke mutaties in zebravis *prox1*, *couptf2* en *sox18* kunnen introduceren. Deze mutanten lieten zien dat deze drie genen in zebravissen niet essentieel zijn voor lymfe-angiogenese, in tegenstelling tot de eerder genoemde studies. Door gebruik te maken van een *prox1* transgene zebravislijn, hebben we de expressie van *prox1* gedurende de vroege ontwikkeling kunnen vastleggen. In de vroege ontwikkeling komt *prox1* niet exclusief tot expressie in veneuze cellen die differentiëren in lymfatische cellen. Prox1 is ook aanwezig in arteriële cellen en in andere veneuze cellen. Prox1 blijkt pas na lymfatische specificatie exclusief in lymfatische cellen tot expressie te komen. Hiermee is aangetoond dat *prox1* niet voor de specificatie van veneuze cellen zorgt in zebravissen. Dit in tegenstelling tot de data verkregen in muizenstudies. Hieruit valt te concluderen dat de veneuze specificatie van lymfatische cellen (gedeeltelijk) anders wordt gereguleerd in zebravis en muis. Dit in tegenstelling tot de functie van *flt4*, *vegf* en *ccbe1*. De functie van deze genen is evolutionair bewaard gebleven, want zowel in muis als in zebravis zijn deze genen essentieel in lymfe-angiogenese.

In **hoofdstuk 5** bediscussiëren we de opgedane kennis over de specificatie van endotheelcellen in arteriële, veneuze en lymfatische cellen vanuit verschillende invalshoeken en plaatsen we dit in de context van de bestaande literatuur. Vervolgens eindigen we met de klinische toepassingen van de opgedane kennis. Het ontdekken van deze nieuwe functies van genen bij de vorming van vaten kan ons essentiële informatie geven over het behandelen van erfelijke hart- en vaatziekten maar ook bij het stimuleren van bloedvatengroei na bijvoorbeeld een hartinfarct. Tevens is deze informatie belangrijk bij het remmen van bloedvatengroei in tumoren en bij het begrijpen en mogelijk voorkomen van de verspreiding van tumorcellen naar andere plekken in het lichaam.

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## PUBLICATION LIST

**Vasculogenesis and arterial-venous specification in zebrafish.**
D.M.A. Hermkens, H.J. Duckers and S. Schulte-Merker. *In press*

**Sox7 interacts genetically with Hey2 and Efnb2 to control arterial specification.**
D.M.A. Hermkens, A. Urasaki, A. van Impel, J. Bussmann, H.J. Ducker, S. Schulte-Merker. *Development invited revision*

**Divergence of zebrafish and mouse lymphatic cell fate specification pathways.**
A. van Impel, D.M.A. Hermkens[*], M.G. Roukens[*], Z. Zhao[*], J.C. Fischer, J. Peterson-Maduro, H.J. Duckers, E.A. Ober, P.W. Ingham and S. Schulte-Merker. *Development. 2014 Mar;141(6):1228-38.*

**Cgnl1 controls endothelial function and tubule assembly during vascular growth.**
C. Cheng, D.M.A. Hermkens, I. Chrifi, D. Tempel, R.A. Haasdijk, M. M. Brandt, C. van Dijk, P.E. Bürgisser, E.H.M. van de Kamp, R. Herpers, C. Zhu, D.A. Mustafa, L.A.J. Blonden, M.J. Kros, S. Schulte-Merker, H.J. Duckers. *In preparation*

**9430020K01Rik (KIAA1462): a new regulator of endothelial cell proliferation in angiogenesis**
R.A. Haasdijk, D.M.A. Hermkens, D. Tempel, J.A.A. Demmers, E.H.M. van de Kamp, L.A.J. Blonden, S. Schulte-Merker, C. Cheng, H.J. Duckers. *In preparation*

**Thsd1: a new regulator of endothelial barrier function in vascular development and advanced atherosclerosis.**
R.A. Haasdijk[*], W.K. den Dekker[*], D.M.A. Hermkens[#], D. Tempel[#], R. Szulcek[#], F.L. Bos[#], I. Chrifi, M.M. Brandt, E.H.M. van de Kamp, L.A.J. Blonden, J. van Bezu, J.C. Sluimer, E.A.L. Biessen, G.P. van Nieuw Amerongen, S.Schulte-Merker, C.Cheng, H.J. Duckers. *In preparation*

**Tnfaip8l1 promotes angiogenesis by inhibition of endothelial cell apoptosis.**
D. Tempel, R.A. Haasdijk, F.L. Bos, D.M.A. Hermkens, P.E. Bürgisser, J.C. Sluimer, L. Bosman, E.A.L. Biessen, C. Cheng, S. Schulte-Merker, H.J. Duckers. *In preparation*

*,# Authors contributed equally

## CURRICULUM VITAE

Dorien Hermkens was born on the 26th of January 1986 in Herten. She graduated from the 'Bisschoppelijk College Echt' with a VWO certificate in 2004, after which she started to study Biomedical Sciences at the University of Utrecht. In 2007 she received the Bachelor's degree and subsequently enrolled in the Master's program 'Cancer, Genomics and Developmental Biology', now called 'Cancer, Stem Cells and Developmental Biology' at the University of Utrecht. She did her major research project at the department of Physiological Chemistry in the group of prof. dr. Bos under supervision of Ester Frische where she focused on the function and molecular mechanisms of Ras family proteins in processes related to cancer. After this internship she wrote her master thesis on the homing and mobilization mechanisms of bone marrow derived cells in cancer in the group of prof. dr. Voest under supervision of Jeanine Roodhart. For her minor research project between February and October 2009 she worked in the group of prof. dr. M. Milan at the department of Cell and Developmental Biology in the Institute for Research in Biomedicine, Barcelona. Under the supervision of dr. Andres Dekanty, she studied the development and growth control of the Drosophila Melanogaster wing. After successfully obtaining her master's degree, she started her PhD education in May 2010 in the group of Dr. E. Duckers at the department of Molecular Cardiology Erasmus MC Rotterdam in collaboration with the group of prof. dr. S. Schulte-Merker at the Hubrecht Institute. She focused on the specification of endothelial cells during vascular development of which the results are described in this thesis.