Sox7 controls arterial specification in conjunction with hey2 and efnb2 function

Dorien M. A. Hermkens1,2, Andreas van Impel1, Akihiro Urasaki1, Jeroen Bussmann1, Henricus J. Duckers2 and Stefan Schulte-Merker1,3,4,*

ABSTRACT
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 in 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 crucial in arterial specification in conjunction with hey2 and efnb2 function, with mutants in all three genes displaying shunt formation and an arterial block.

KEY WORDS: Vascular development, Arterial-venous specification, Sox7, Zebrafish

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 Viikula, 2007). The vascular system consists of endothelial cells (ECs) that become specified into arterial, venous and lymphatic cells, eventually forming a functional vascular system. 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. It was recently reported that medial angioblasts start migrating around 14 hpf (10-somite stage) and the lateral angioblasts initiate migration to the midline around 16 hpf (15-somite stage). The medially located angioblasts will form the arterial cells of the first arterial vessel, the dorsal aorta (DA), and the laterally localized angioblasts will give rise exclusively to the venous cells of the first venous vessel: the posterior cardinal vein (PCV) (Zhong et al., 2000; Kohli et al., 2013). Vascular endothelial growth factor (Vegf) receptors and their ligands play crucial roles during arterial-venous specification. Vegf receptor 3/Flt4 is initially expressed in all ECs and becomes restricted to venous and lymphatic ECs later in development (Kaipainen et al., 1995; Hogan et al., 2009b; van Impel et al., 2014). Inactivation of Vegfr3 in mice has been shown to result in decreased angiogenic spraying and vascular network formation (Tammela et al., 2008). Activation of Vegf receptor 2, also expressed in ECs, by Vegfa and Sonic Hedgehog (Shh) signaling induces the PLCγ2/Mek/Erk pathway and subsequently the Notch signaling pathway (Lawson et al., 2002). 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). Ephb2 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 by Francois et al., 2010). The sox gene family encodes transcription 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 contain a transactivation domain (TAD), which activates transcription of target genes (Hosking et al., 1995). One subgroup of the Sox family that is of particular interest for vascular development is the SoxF group, consisting of SOX7, SOX17 and SOX18. In vitro studies revealed that Sox7 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 human hypotrichosis-lymphedema-telangiectasia (HLT) syndrome, in which individuals have severe lymphedema, vascular leakages...
and disrupted hair follicle development (Irrthum et al., 2003). The phenotype of mice with a truncated Sox18 protein (mutation in the ragged opossum allele) resembles this syndrome, resulting in severe edema, blood vessel disruption and early lethality (Pennisi et al., 2000; James et al., 2003). On some genetic backgrounds in mice, Sox18 is required for the differentiation of ECs in lymphatic cells by initiating expression of Prox1 (François 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 DA and the PCV. sox7/sox18 double morphants display an increase in 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); 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 therefore generated a zebrafish sox7 mutant. Here, we demonstrate a highly specific arterial-venous shunt phenotype in sox7 mutants and connect Sox7 function to Hey2/Notch signaling and to Efnb2 function.

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 sox7hu5626 allele comprises a guanine-to-adenine mutation leading to a predicted premature stop-codon (after amino acid 53) within the HMG domain (Fig. 1B; supplementary material Fig. S1). Sequencing of cDNA from sox7hu5626 mutant embryos did not reveal alternative transcripts lacking the nonsense mutation (supplementary material Fig. S2), suggesting that the sox7 allele represents a loss-of-function situation. 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 kdr:GFP;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. 1. Disrupted blood circulation in sox7 zebrafish mutants. (A) sox7 in situ hybridization of 3 dpf wild-type embryos (left panel, lateral view; right panel, dorsal view) showing sox7 expression in all main vessels. (B) Schematic diagram of the sox7hu5626 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 sox7hu5626 heterozygous sibling and homozygous mutant at 2 dpf. (D) On average, 59% of sox7hu5626 mutants display disturbed blood flow at 2.5 dpf. Percentages of pooled embryos from four independent experiments (total of 275 embryos). Percentages can vary substantially between different backgrounds. (E) kdr:GFP;gata1:dsRed-positive sox7hu5626 mutants lack functional blood circulation in the trunk, whereas heterozygous siblings have normal circulation at 2.5 dpf. Right panels: higher magnifications of boxed area.
Further analysis in different transgenic backgrounds showed that although this phenotype could always be detected in a fraction of homozygous sox7 mutants, the actual penetrance of the blood circulatory defect is largely dependent on the genetic background.

When analyzing the blood flow by making use of bright-field movies, we could observe a very specific short loop of circulation in the sox7 mutants with no circulation in the trunk. This short loop of circulation was never observed in sox7 siblings (supplementary material Movies 1 and 2).

To characterize the blood circulation defect in sox7 mutants in more detail, 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 (Fig. 2A). This indicates complete blockage of blood flow at the LDA.

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 at the position of the arrow) of sox7

fli1a:eGFP siblings (upper panel) and mutants (lower panel) at 2 dpf. Insets depict the rhodamine-dextran channel of the boxed area with higher magnification highlighting the lack of dye uptake in the DA of sox7 mutants. (B) Transverse sections of fli1a:eGFP-positive sox7 siblings and mutant embryos at 2 dpf, stained with anti-GFP (green) and DAPI (blue). In sox7 mutants, the aortic morphology is disturbed at the position of LDA/DA fusion (arrows) while being unaffected more anteriorly and posteriorly to this position. For relative positions of sections, see supplementary material Fig. S3. (C) Dorsal view of sox7

kdrl:mCherry;flt4:mCitrine sibling and mutant embryos at 2.5 dpf. Right panel kdrl:mCherry channel only. Shunt formation (arrow) occurs in sox7 mutants at the position of LDA fusion. (L) DA, (lateral) dorsal aorta; PCV, posterior cardinal vein; CCV, common cardinal vein.
anterior part of the DA. Cross-sections of 48 hpf *sox7* mutants revealed a locally restricted, disrupted morphology of ECs and defective lumen formation at the position where, in sibling embryos, both lateral dorsal aortae (LDA) fuse to form the dorsal aorta (DA). Both anterior and posterior to this position, the structure of the aorta in mutant embryos appeared to be unaffected (Fig. 2B; supplementary material Fig. S3). Subsequent 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), while no other abnormalities in the development of the head vasculature could be detected (data not shown).

To investigate the temporal relationship between the blockage of circulation at the fusion site of both LDAs and the shunt formation between LDA and CCV, we performed time-lapse imaging of *kdrl:eGFP*-positive *sox7* mutants and siblings starting at the 20-somite stage. In wild-type embryos, the LDA is formed by migration of an anterior and a posterior subpopulation of ECs that fuse and give rise to a continuous LDA on both sides of the embryo (Siekmann et al., 2009). In *sox7* mutants, we found that ECs from the developing LDA and the CCV (called Duct of Cuvier at this stage) form an ectopic connection already during the formation of both vessels and prior to the onset of circulation (Fig. 3C; supplementary material Movies 3 and 4). This finding is consistent with the observation that embryos displaying the short-loop phenotype do not ever transiently establish blood flow in the trunk during early stages of development. In conclusion, these results indicate that the shunt formation in *sox7* mutants is not a secondary phenotype caused by the blockage of blood circulation at the fusion site of the two LDAs, but is a specific effect caused by the lack of functional Sox7.

It was previously reported that *sox7/sox18* double morphants 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 the combined morpholino knockdown, we generated *sox7;sox18* double mutants and also found multiple shunts between the DA and PCV in our genetic mutants, thereby fully recapitulating the morphant phenotype (Fig. 3D). However, no ectopic connections between the DA and PCV were observed in *sox7* or *sox18* single mutants (Fig. 3E) (van Impel et al., 2014), reconfirming the strong redundancy between both SoxF transcription factors in the trunk vasculature (Cermenati et al., 2008; Herpers et al., 2008; Pendeville et al., 2008). In addition, we noticed that the penetrance of the *sox7* short-loop phenotype was enhanced by the additional loss of one copy of *sox18*, whereas homozygous *sox18* mutants lacking one copy of *sox7* did not establish the circulatory short loop at all (Fig. 3D; data not shown). These results suggest a compensatory role for Sox18 in the absence of Sox7 in the LDA; however, they also emphasize the highly specific local requirement of normal Sox7 levels in this specific part of the aorta.

To explore the involvement of the third SoxF family member, Sox17, in the context of LDA development, we analyzed its expression pattern by employing a *sox17:eGFP* transgenic reporter line (Mizoguchi et al., 2008). Although *sox17* is expressed in arterial cells of the dorsal aorta and ISVs (supplementary material Fig. S4A), we could not detect *sox17:eGFP* expression in the LDA of *sox7* siblings or mutants (supplementary material Fig. S4B). Furthermore, the overall *sox17:eGFP* expression levels in the arterial ECs of *sox7* siblings and mutants are indistinguishable at 1 and 2 dpf (supplementary material Fig. S4A; data not shown). Therefore, although we cannot fully exclude an involvement of Sox17 in some aspect of LDA development, our analysis suggests that *sox17* is not expressed at the right stages within the LDA to affect its formation, unlike *sox7* and *sox18*, the mRNAs of which can readily be detected in ECs of this specific vascular structure.

**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 for arterial and venous specific genes. This revealed expression of *flt4*, also called *vegf3*, to be upregulated in arterial cells at the location of ectopic arteriovenous connections in most *sox7* mutants at 20 hpf (Fig. 4A,D). Vegf3/Flt4 is a transmembrane tyrosine kinase receptor for the ligands VegfC and VegfD, 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, compared with the 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). Although the ectopic expression of this venous marker would suggest differential expression of other arterial and/or venous markers, we did not detect this for a wide range of other marker genes (notch1b, notch3, efnb2, hey2, dll4, foxc1, sox18, dah2, nr2f2 and ephb4) in *sox7* mutants at several time points (20-30 hpf) (Fig. 4A,C; data not shown).

**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 *efnb2ahu3393* and amino acid 78 in *efnb2ao20779*) (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 a significant increase of circulatory short-circuits in 42% of double mutants (Fig. 5B,C). As *sox7* mutants also show only a partially penetrant phenotype, this allowed us to investigate a possible connection between *sox7* and *efnb2* genes by testing for genetic interactions and hence we generated 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* single 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. This revealed a significant increase of the short-loop phenotype when combining both mutants and suggests 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 have shown that mutations in hey2 (gridlock mutants) display ectopic arterial to venous connections (Weinstein et al., 1995). hey2 is a downstream target of
the Notch signaling 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. 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 were evident upon injection (8% with 0.3 ng) (Fig. 6A). When injected into the offspring of a sox7+/- in-cross, we observed an increase in the number of embryos displaying the short-loop (37% with 0.3 ng hey2 MO, 5% in un-injected sox7+/- in-cross) (Fig. 6A). To confirm this observation, we generated hey2 TALEN constructs targeting exon 2 upstream of the two important domains: the basic helix-loop-helix (bHLH) DNA-binding domain and the Orange domain (which 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, a phenotype that could further be reconfirmed in stable genetic hey2 mutant embryos, again mimicking the loss of sox7 (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.

Increased arterial Notch signaling suppresses the vascular defects in sox7 mutants

Recently, in vitro studies reported that SoxF transcription factors can bind the arterial-specific enhancer of the notch ligand dll4 (Sacilotto et al., 2013). As Hey2 and Efnb2a/b have been suggested to act downstream of Notch signaling (Lawson et al., 2001) and as 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 a UAS construct encoding the Notch1 intracellular domain (NICD) (Scheer and Campos-Ortega, 1999) under the control of a newly established, arterial-specific dll4 BAC-transgenic line, the expression of which did not appear to be altered in sox7 mutant embryos (supplementary material Fig. S5; data not shown). We found that dll4:Gal4FF;UAS:NICD expression in sox7 mutants significantly rescued the short-loop phenotype compared with sox7 mutants lacking the UAS:NICD construct (3% versus 27% within the same genetic background; n=300 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 for the notion that Sox7 acts upstream of Notch1 signaling in arterial specification.

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. As sox7 is expressed in all ECs, this suggests redundant roles for SoxF family members and a scenario where Sox18 (and possibly Sox17) can partially compensate for the loss of sox7 in most vascular beds. The notion of redundancy is further supported by sox18 mutant situations displaying rather comprehensive Fig. 5. efnb2ahu3393 and efnb2ahu2971 alleles with premature stop codons (red asterisks) at amino acids 86 and 78, respectively. Eph, ephrin domain (green), Cd, cupredoxin domain (blue). (B) Quantification of efnb2ahu3393 and efnb2ahu2971 single and double mutant phenotypes revealed circulatory short-looping in 11% of efnb2ahu3393-/-;efnb2ahu2971+/- embryos and a significant increase of short-looping in 42% of double homozygous mutants (Student’s t-test, P=0.003 compared with efnb2ahu3393-/-;efnb2ahu2971+/-). The genotype combinations not shown all displayed 100% wild-type phenotype. Bars represent combined results of five independent experiments (total of 128 embryos). (C) Blood circulation in gata1:dsRed-positive efnb2ahu3393;efnb2ahu2971 single and double mutant embryos at 2.5 dpf. Note normal circulation in single mutants (upper panel) and short-loop phenotype in efnb2ahu3393-/-;efnb2ahu2971+/- 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 sox7hu5626, efnb2ahu3393 double heterozygous embryos exhibit a significant (Student’s t-test, P=0.002) increase in the penetrance of the short-circuit phenotype compared with the only partially penetrant sox7hu5626 single mutants or the efnb2ahu3393 double mutants lacking the short-loop defect completely. The genotype combinations not shown all displayed 100% wild-type phenotype. Bars represent combined results of five independent experiments (total of 507 embryos). (B,D) Embryos were analyzed at 2.5 dpf. Phenotypes classified as ‘others’ are edema, total lack of circulation and/or shunts in trunk region.
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). In addition, no ectopic connections between the DA and PCV were detected in or synergizes with hey2 and functions upstream of Notch ICD in LDA development. (A) The partially penetrant short-loop phenotype of both sox7hu5626 mutants \(n=104\) and hey2 morphants \(0.3\ ng, n=63\) is exacerbated when the hey2 MO is injected into a sox7hu5626 in-cross \(n=35\). Bars show the percentage of embryos from 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-loop-helix (bHLH) DNA-binding domain and the orange (Or) domain. (C) Transient injections of 10 pg per embryo hey2 TALEN mRNA into wild-type and injected with \(n=140\) embryos) compared with un-injected sox7hu5626 in-cross \(n=90\) embryos. (D) Arterial specific UAS:NICD overexpression using the dil4:Gal4 driver line significantly (Student's t-test, \(P=0.004\)) rescues the short-loop circulatory phenotype in sox7 mutants: short-loop phenotype in 27% of mutants without NICD overexpression \(n=69\) embryos) and in 3% with NICD overexpression \(n=44\) embryos. Bars represent pooled embryos of three independent experiments (total of 300 embryos). (A,C,D) Embryos were analyzed at 2.5 dpf. Phenotypes classified as ‘others’ are edema, total lack of circulation and/or shunts in trunk region.

Like all other Vegf receptors, flt4 is initially expressed widely throughout all vascular beds, with its expression in the trunk becoming restricted to venous cells after 26 hpf in zebrafish (Hogan et al., 2009b). Hence, a plausible scenario is that the ectopic flt4 expression in arterial cells of sox7 mutants results from a lack of proper downregulation of flt4 specifically in these arterial cells. Flt4 and its ligands VegfC and VegfD have been shown to be involved in endothelial cell sprouting and migration (Karkkainen et al., 2004; Tammela et al., 2008; Hogan et al., 2009b; Villefranc et al., 2013). It is therefore a possibility that the elevated flt4 levels in the arterial cells of the sox7 mutants result in a disrupted balance between Flt4 signaling levels in ECs of the LDA and the surrounding venous vessels, which in conjunction with a potential mis-regulation of other arterial and venous patterning genes causes misguidance of ECs and the formation of ectopic shunts.
On the other hand, previous work in mice and cultured ECs demonstrated that Efnb2 can mediate the internalization and thereby contribute to the signaling activity of Flt4 (Wang et al., 2010). As we show here that efnb2 functions together with sox7 and both genes are essential for proper LDA development, the lack of efnb2 (and potentially indirectly also of sox7) could also result in reduced internalization of Flt4 and therefore to a decrease in its signaling potential. It is plausible that the embryo would react to such a decrease in Flt4 signaling activity by increasing the expression of the receptor. Hence, the elevated mRNA levels of flt4 in sox7 mutants could also hint towards a compensation mechanism rather than being a sign of increased Flt4 signaling levels in LDA cells. This would make the elevated flt4 mRNA levels a consequence of the sox7 defects instead of being the actual cause of the shunt formation.

The very specific circulatory defects in sox7, hey2 and efnb2 mutants, together with the genetic interaction study presented here strongly suggest 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 in a partially redundant manner Sox18) acts upstream of the Notch signaling pathway while controlling flt4 expression and potentially other arterio-venous patterning genes, 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 leads, for example, 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, 2014), and most endothelial cells can compensate for loss of Sox7 (this study) – the only exception being the endothelial cells of the LDA, which crucially 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 three different mutant scenarios indeed points towards unique features in the genetic control of endothelial cell behavior within the LDA.

MATERIALS AND METHODS

Zebrafish

Zebrafish were maintained under standard husbandry conditions according to the rules of the Animal Experimentation Committee (DEC) of the KNAW. Transgenic lines were used Tg(fli1a:egFP)31 (Lawson and Weinstein, 2002), Tg(kdrl:eGFP)443 (Jin et al., 2005), Tg(gata1:dsRed)482 (Traver et al., 2003), Tg(kdrl:HRAS-mCherry)229 (Hogan et al., 2009a), Tg(fli4:mcitrine)1616 (van Impel et al., 2014), Tg(sox17:eGFP) (Mizoguchi et al., 2008) and Tg(UAS:myc-NICD) (Schep and Campos-Ortega, 1999). The Tg(fli4:Gal4E/Fp)1609 line was generated from BAC CH211-19M2 following standard recombineering procedures (Bussmann and Schulte-Merker, 2011). The sox18:eGFP reporter line, J. den Hertog and A. V. I. van Impel et al., 2014, 312.

Genotyping, morpholinos and TALEN constructs

Genotyping of sox7, efnb2a and efnb2b was performed by KASPAR with primers listed in supplementary material Table S1. The hey2 MO used was 5′-CGGGAGGCTGACACACCAAAAAC-3′ (GeneTools) (Zhang et al., 2001); 0.3 ng of hey2 MO was used for suboptimal effect. hey2 TALEN binding sites are: TAL1, 5′-TGTGGGTTGGTGAGGCGAC-3′; TAL2, 5′-ATTCCCCAGCCAAAGCAATGG-3′; primers were mentioned in supplementary material Table S1. TALEn constructs were generated as described previously (Cermak et al., 2011; Bedell et al., 2012).

Histology and in situ hybridization

In situ hybridization and immunohistochemistry were performed as described previously (Schulte-Merker, 2002). For immunohistochemistry, the embryos were stained with rabbit anti-GFP 1:1000 and anti-rabbit Alexa 488 1:1000 (Invitrogen), or stained with Hematoxylin and Eosin. The embryos were paraffin-embedded and sectioned (7 µm), after which they were analyzed with a Leica TCS SPE. For in situ hybridization 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 MS222 (0.04%) and 1-phenyl-2-thiourea (PTU 0.003%) using a Leica TCS SPE. Bright-field pictures and movies were acquired on a Zeiss Axiosplan microscope and a Leica TCS SPE, respectively. Images were processed using Adobe Photoshop CS5.1 and Fiji (http://fiji.sc/Fiji).

Micro-angiography

Micro-angiography was performed by injecting rhodamine dextran (10 mg/ml) into the PCV of MS222-anaesthetized embryos, as described previously (Weinstein et al., 1995). Embryos were mounted in 0.5% low melting-point agarose and imaged with a Leica TCS SPE.

RT-PCR analysis

sox7 siblings and sox7 mutants were first selected based on the short-loop phenotype and pooled separately (n=10 embryos/pool) at 2 dpf. cDNA was synthesized from RNA with primers listed in supplementary material Table S1.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

D.M.A.H. performed the experiments, analyzed the new sox7, hey2 and efnb2 mutants, and wrote the manuscript. J.B. engineered the dll4:gal4FF BAC. A.v.i. injected and established the dll4:gal4FF:UAS:RFP transgenic line, and generated the flt4:mcitrine reporter line. A.U. analyzed the efnb2 mutants. D.M.A.H., A.v.i. and S.S.-M. designed the experiments and edited the manuscript. H.J.D. and S.S.-M. conceived and supervised the study.

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Supplementary material

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