

RESEARCH ARTICLE

Sox7 controls arterial specification in conjunction with *hey2* and *efnb2* function

Dorien M. A. Hermkens^{1,2}, Andreas van Impel¹, Akihiro Urasaki¹, Jeroen Bussmann¹, Henricus J. Duckers² and Stefan Schulte-Merker^{1,3,4,*}**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 Vikkula, 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 sprouting 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 PLC γ /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). 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 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 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 human hypotrichosis-lymphedema-telangiectasia (HLT) syndrome, in which individuals have severe lymphedema, vascular leakages

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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 *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; supplementary material Fig. S1). Sequencing of cDNA from *sox7^{hu5626}* 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 *kdr1: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

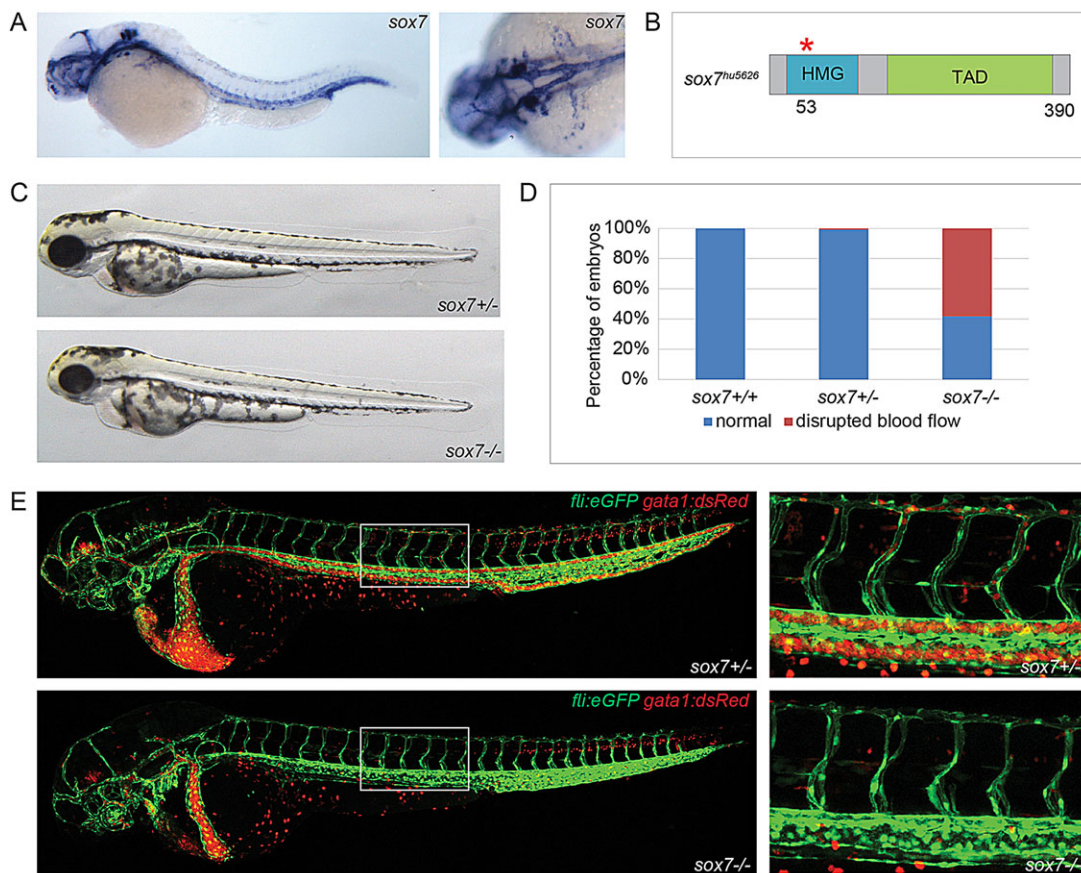


Fig. 1. Disrupted blood circulation in *sox7* zebrafish mutants. (A) *sox7* *in situ* hybridization of 3 dpf wild-type embryos (left panel, lateral view; right panel, dorsal view) showing *sox7* expression in all main vessels. (B) Schematic diagram of the *sox7^{hu5626}* allele with a premature stop-codon 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) On average, 59% of *sox7^{hu5626}* 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) *kdr1:eGFP;gata1:dsRed*-positive *sox7^{hu5626}* 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.

(Fig. 1D,E). 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

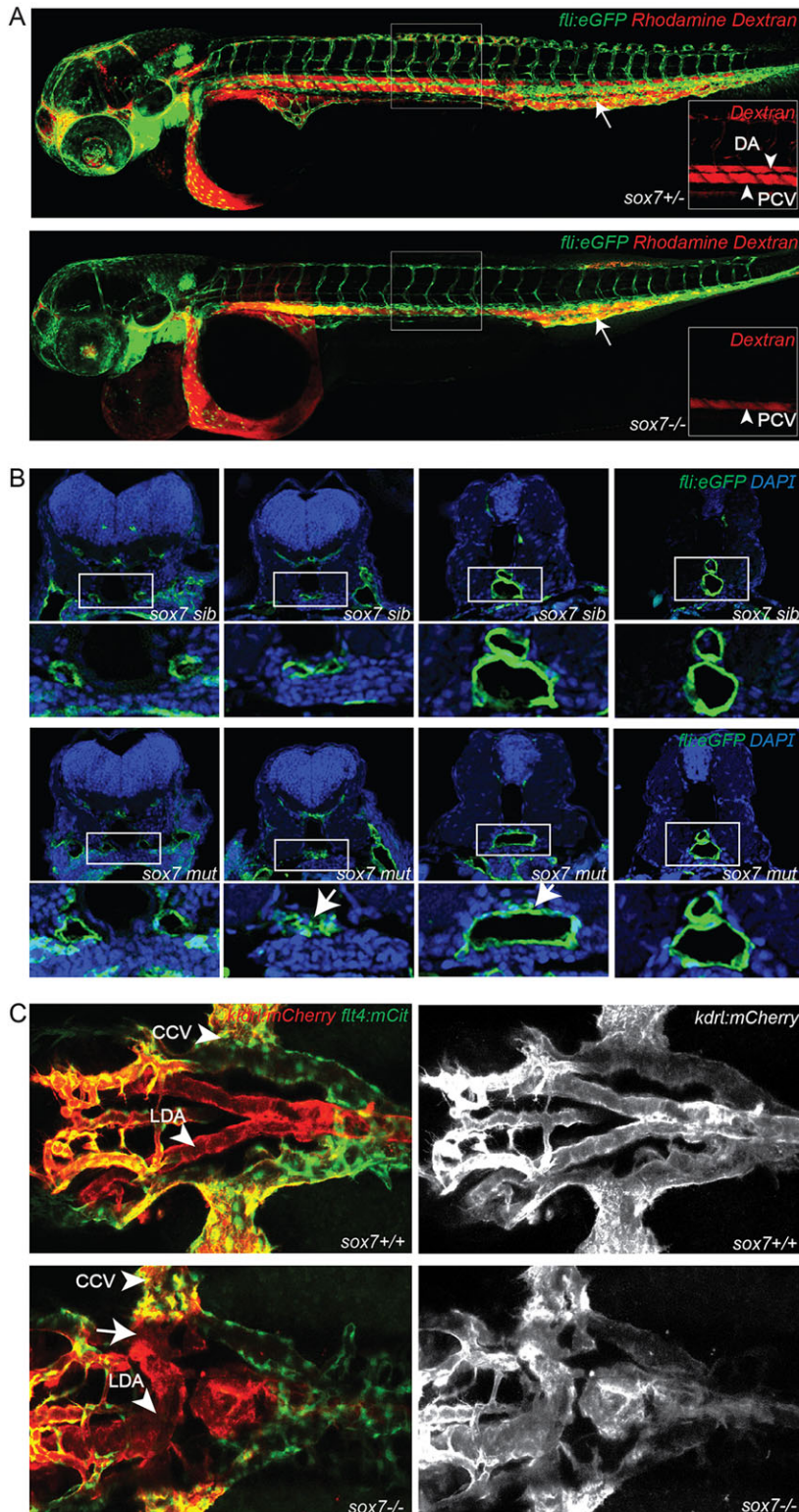


Fig. 2. *sox7* mutants show an altered morphology of the LDA while displaying normal DA-PCV segregation in the trunk. (A) Rhodamine-dextran angiograms (injected into the PCV at the position of the arrow) of *sox7*^{hu5626} *fil1a:eGFP* siblings (upper panel) and mutants (lower panel) at 2 dpf. Insets depict the rhodamine-dextran channel of the boxed area with higher magnification highlighting the lack of dye uptake in the DA of *sox7*^{hu5626} mutants. (B) Transverse sections of *fil1a:eGFP*-positive *sox7*^{hu5626} sibling and mutant embryos at 2 dpf, stained with anti-GFP (green) and DAPI (blue). In *sox7*^{hu5626} 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*^{hu5626} *kdr1:mCherry*; *fil1a:mCherry* sibling and mutant embryos at 2.5 dpf. Right panel *kdr1:mCherry* channel only. Shunt formation (arrow) occurs in *sox7*^{hu5626} 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 the CCV/PHS, 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 (Siekman et al., 2009). In *sox7* mutants, we found that ECs from the developing LDA and the CCV (called Duct of Cuvier at this stage) formed 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 even 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 *vegfr3*, to be upregulated in arterial cells at the location of ectopic arterio-venous connections in most *sox7* mutants at 20 hpf (Fig. 4A,D). Vegfr3/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*, *dab2*, *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 *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 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

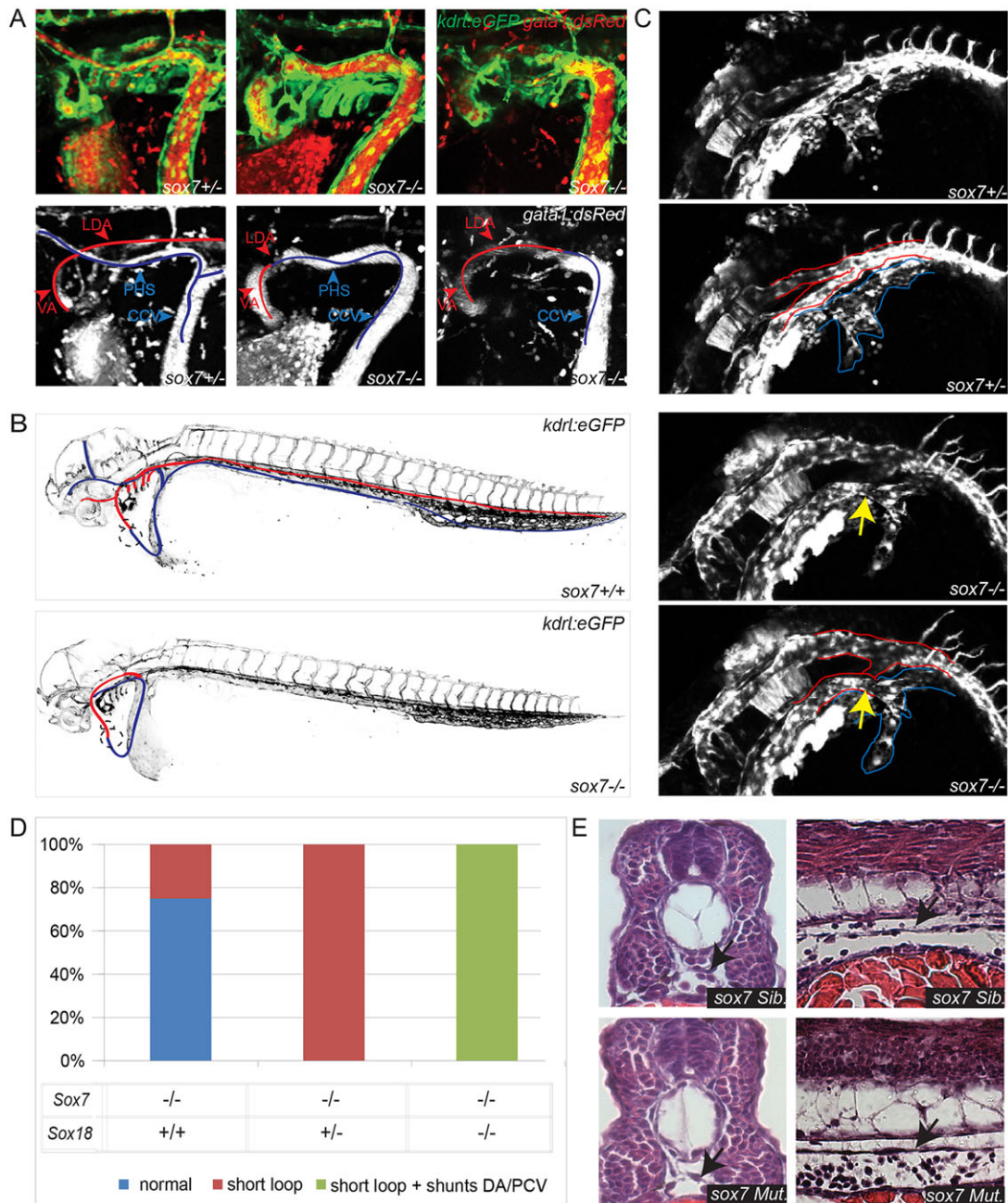


Fig. 3. *sox7* mutants develop a circulatory short-loop phenotype. (A) *sox7^{hu5626} kdr:eGFP;gata1:dsRed* mutants at 2.5 dpf with shunt formation resulting in a circulatory short-loop (middle and right panel) in contrast to normal circulation in *sox7^{hu5626}* siblings (left panel). Middle panel depicts ectopic connection between LDA and PHS, right panel between LDA and CCV. Lower panel depicts 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 with the short-circuit in *sox7^{hu5626}* mutants at 2.5 dpf. Dashed circles highlight the position of the heart. (A,B) Lines depict blood flow in arteries (red) and veins (blue). (C) Stills of time-lapse movies of *kdr:eGFP*-positive *sox7* mutant and sibling at the time point of ectopic connection formation (see supplementary material Movies 3 and 4). ECs of the LDA (outlined by red lines) connect to ECs of the CCV (outlined by blue lines) in the *sox7* mutant at 26 hpf (yellow arrow). (D) Quantification of the phenotypes in 3 dpf *sox7^{hu5626};sox18^{hu10320}* loss-of-function embryos. 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 all have 100% wild-type phenotype. Bars show percentages of embryos of a representative experiment (total of 77 embryos). (E) 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) showing normal DA-PCV segregation in both siblings and mutants (arrows).

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

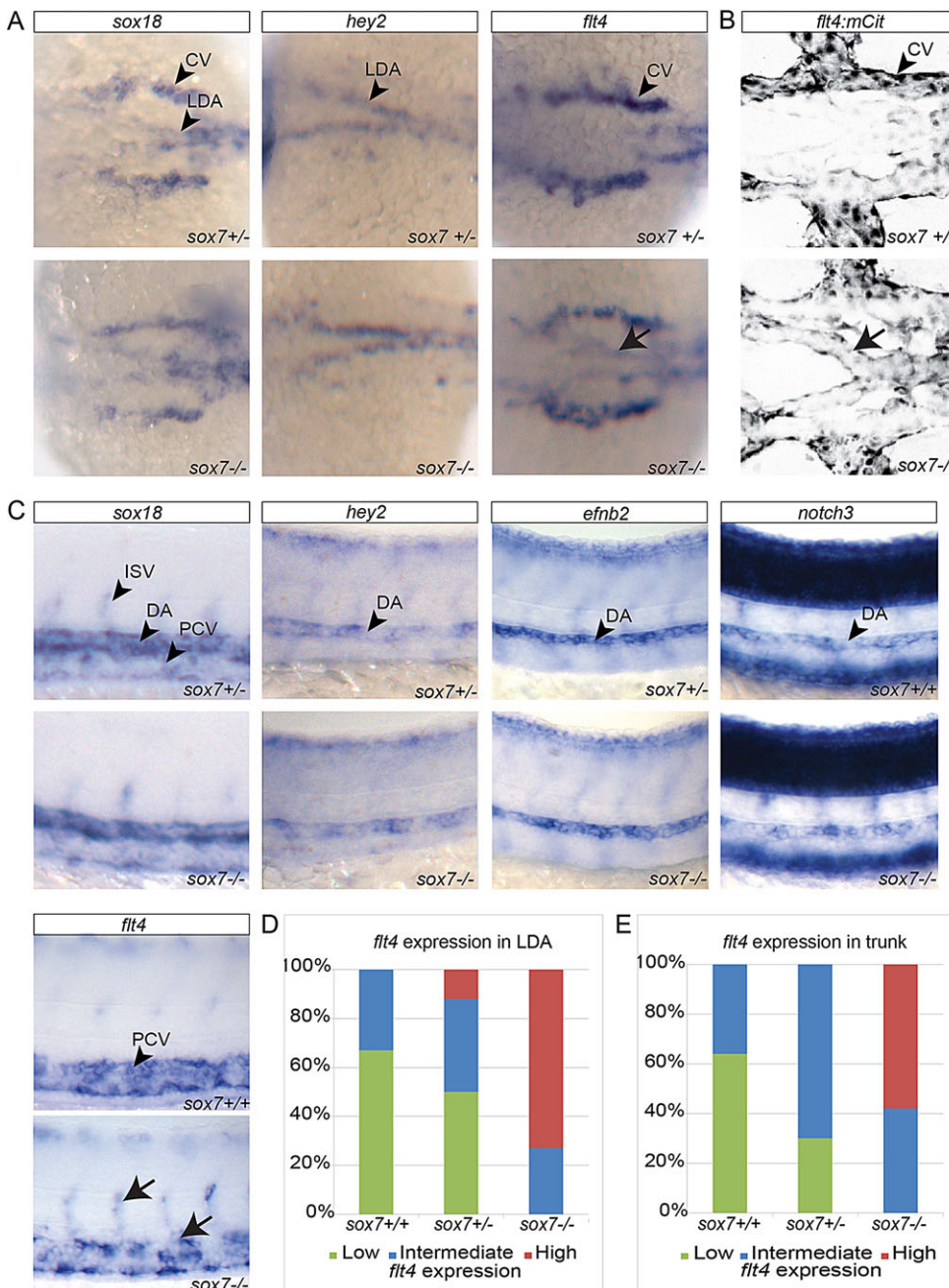


Fig. 4. *flt4* expression is altered in arterial cells of *sox7* mutants. (A-C) *sox7*^{hu5626} siblings (upper panel) and mutants (lower panel). (A) *In situ* hybridization for *sox18*, *hey2* and *flt4* in *sox7*^{hu5626} heterozygous and mutant embryos at 20 hpf (dorsal view). *sox7*^{hu5626} mutants display elevated *flt4* expression levels in the LDA (arrow). (B) Dorsal view of *flt4:mCitrine* (*flt4:mCit*)-positive *sox7*^{hu5626} heterozygous and homozygous mutant at 26 hpf showing elevated *mCitrine* expression in the LDA of *sox7*^{hu5626} mutant (arrow) compared with *sox7*^{hu5626} siblings. (C) Lateral view of the trunk region of *sox7*^{hu5626} siblings and mutants with *in situ* hybridization for *sox18*, *hey2*, *efnb2*, *notch3* and *flt4* at 24-27 hpf. *sox7*^{hu5626} mutants display higher *flt4* expression in DA and ISVs (arrows) compared with siblings. (D) Quantification of 20-24 hpf embryos based on their *flt4* expression levels (*in situ* hybridization) in the LDA. Seventy-three percent of *sox7*^{hu5626} mutants have high expression of *flt4* in LDA region at 20 hpf (total of 30 embryos). (E) Quantification of 24-27 hpf embryos based on their *flt4* expression levels (*in situ* hybridization) in the trunk. Fifty-eight percent of *sox7*^{hu5626} mutants have elevated *flt4* expression, which was never observed in siblings (total of 57 embryos). (L)DA, (lateral) dorsal aorta; (P)CV, (posterior) cardinal vein; ISV, intersegmental vessel.

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

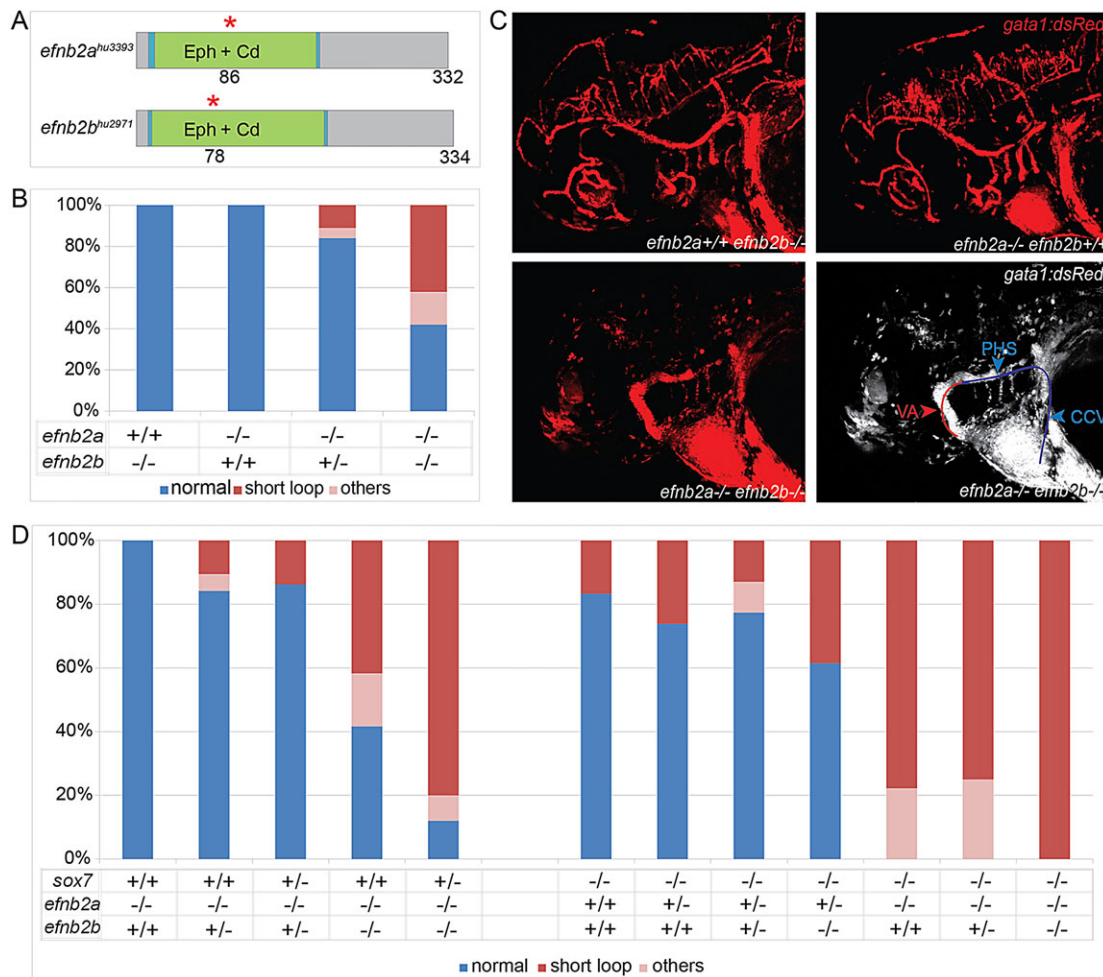


Fig. 5. *efnb2a*;*efnb2b* double mutants develop a circulatory short-circuit phenotype and *sox7* genetically interacts with *efnb2a*. (A) Schematic diagram of *efnb2a*^{hu3393} and *efnb2b*^{hu2971} alleles with premature stop codons (red asterisks) at amino acids 86 and 78, respectively. Eph, ephrin domain (green), Cd, cupredoxin domain (blue). (B) Quantification of *efnb2a*^{hu3393} and *efnb2b*^{hu2971} single and double mutant phenotypes revealed circulatory short-looping in 11% of *efnb2a*^{hu3393};*efnb2b*^{hu2971} embryos and a significant increase of short-looping in 42% of double homozygous mutants (Student's *t*-test, *P*=0.003 compared with *efnb2a*^{hu3393};*efnb2b*^{hu2971}). 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 *efnb2a*^{hu3393};*efnb2b*^{hu2971} single and double mutant embryos at 2.5 dpf. Note normal circulation in single mutants (upper panel) and short-loop phenotype in *efnb2a*^{hu3393};*efnb2b*^{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. All *sox7*^{hu5626};*efnb2a*^{hu3393} double homozygous mutant 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 *sox7*^{hu5626} single mutants or the *efnb2a*^{hu3393} single 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.

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* 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

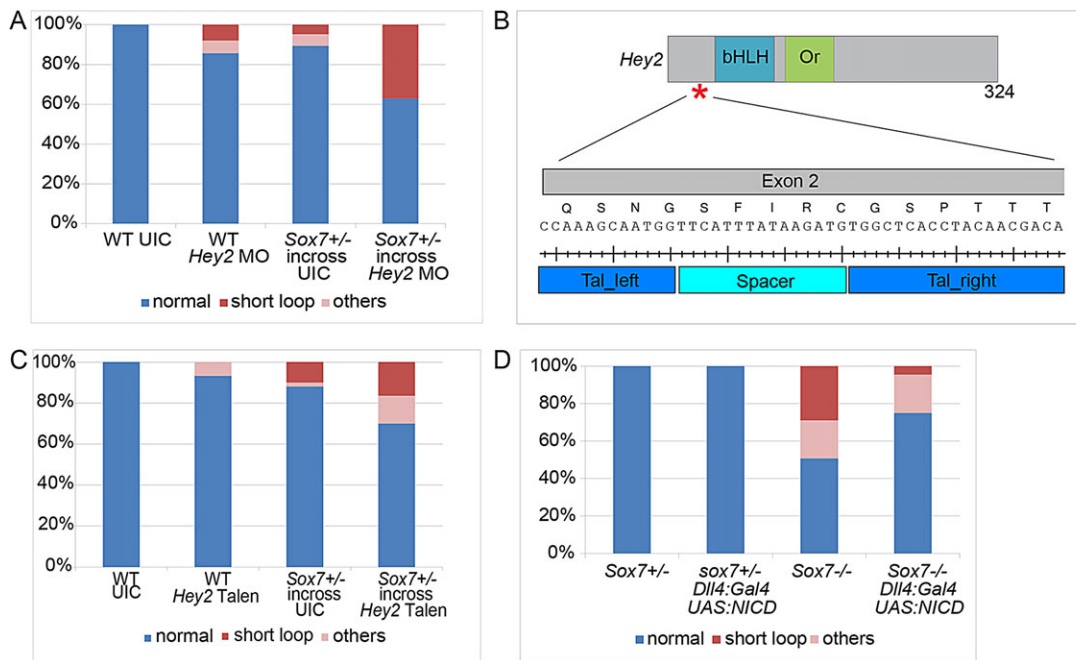


Fig. 6. *sox7* synergizes with *hey2* and functions upstream of Notch ICD in LDA development. (A) The partially penetrant short-loop phenotype of both *sox7*^{hu5626} mutants ($n=104$) and *hey2* morphants (0.3 ng, $n=63$) is exacerbated when the *hey2* MO is injected into a *sox7*^{hu5626/+} in-cross ($n=35$). 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 *sox7*^{hu5626} heterozygous in-cross. There is a moderate enhancement of penetrance of the short-loop phenotype in *sox7*^{hu5626} in-cross injected with *hey2* TALEN ($n=140$ embryos) compared with un-injected *sox7*^{hu5626} in-cross ($n=100$ embryos) and *hey2* TALEN injections into wild-type embryos ($n=90$ embryos). (D) Arterial specific *UAS:NICD* overexpression using the *dll4: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.

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 *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. However, we showed that the additional loss of one *sox18* allele enhances the penetrance of the short-loop phenotype in *sox7* mutants, suggesting a partially overlapping function of both transcription factors also in this specific part of the arterial compartment. However, why these compensatory mechanisms do not fully prevail in the LDA remains enigmatic and suggests a unique regulation within the LDA cells. Although the DA is formed by migration of medial angioblasts originating from the posterior lateral plate mesoderm towards the midline, the LDA is formed by two distinct populations of medial angioblasts from the anterior and posterior lateral plate mesoderm that migrate towards each other and eventually connect (Siekman et al., 2009). In addition, the initiation of LDA formation is slightly later (around 22-somite stage) compared with the formation of the DA (around 20-somite stage) (Siekman et al., 2009), indicating that the developmental program controlling LDA formation differs from the one in other arterial ECs.

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 observed. 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 *in situ* hybridization) are sufficient for severely disturbing arterio-venous patterning. This would also explain why we did not find differential expression of arterial markers such as the Notch target genes in *sox7* mutants, even though 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 *in situ* hybridization analysis was performed at 30 min intervals (between 20 and 30 hpf), it is still possible that differential expression (possibly also only at the protein level) in even a shorter time-interval can lead to severe disturbances in AV patterning.

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 used were *Tg(fli1a:eGFP)^{y1}* (Lawson and Weinstein, 2002), *Tg(kdrl:eGFP)^{s843}* (Jin et al., 2005), *Tg(gatal: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 recombining 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 supplementary material Table S1. The *hey2* MO used was 5'-CGCGCAGGTACAGACACCAAAAACT-3' (GeneTools) (Zhong et al., 2001); 0.3 ng of *hey2* MO was used for suboptimal effect. *hey2* TALEN binding sites are: TAL1, 5'-TGTCGTTGTAGGTGAGCCA-3'; TAL2, 5'-TATTCCCAGCCAAAGCAATGG-3'; primers are 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 Axioplan 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

Supplementary material available online at <http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.117275/-/DC1>

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