© 2014. Published by The Company of Biologists Ltd | Development (2014) 141, 1228-1238 doi:10.1242/dev.105031

RESEARCH ARTICLE

Divergence of zebrafish and mouse lymphatic cell fate specification pathways

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ABSTRACT

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 pre-eminent role of Prox1 within the lymphatic vasculature is conserved in other vertebrate classes has remained unresolved, mainly owing to the lack of availability 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.

KEY WORDS: Lymphangiogenesis, Prox1, Coup-TFII, Sox18, Nr2f2

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 (Nr2f2 – Mouse Genome Informatics) (Srinivasan et al., 2010) and Sox18 (François et al., 2008) leads to the polarized expression of *Prox1* in a subset of endothelial cells (ECs) within the cardinal vein at embryonic day (E) 9.5. Shortly thereafter, Prox1-positive ECs leave the cardinal vein in a dorsal direction, mediated by Vegfc- and Flt4-driven processes of polarized sprouting and migration, resulting in the formation of the first lymphatic structures in the embryo (Karkkainen et al., 2004; Hägerling 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 murine 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 the dorsal aorta (DA) and the posterior cardinal vein (PCV). Then, in a wave of angiogenic sprouting from the DA (primary or arterial sprouting), a set of ~30 arterial intersegmental vessels (ISVs) is formed on each side of the embryo. Shortly thereafter, at about 36 hours post-fertilization (hpf), another group of ~30 sprouts emerges from each side of 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 [at 2.5 days post-fertilization (dpf)] 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 of the 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). Although expression of *prox1a* within lymphatic structures has been reported (Yaniv et al.,

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Received 22 October 2013; Accepted 20 December 2013

Development (2014) doi:10.1242/dev.105031

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 (Küchler et al., 2006). Thus, although 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 Nr2f2 (referred to as Coup-TFII in the following) 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.

RESULTS

To obtain a more precise and sensitive readout for lymphatic structures during development, we created a bacterial artificial chromosome (BAC) reporter line for *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 (supplementary material Fig. S1A,B) and 36 hpf (supplementary material Fig. S1C-F), respectively. In contrast to *Flt4* expression in mice (Hägerling 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 such as *fli1a:eGFP*, the TD is the only lymphatic structure that is readily distinguishable from blood vessels. By comparison, the *flt4* reporter line also reliably highlights more delicate lymphatic structures such as ISLVs or the DLLV in the trunk (supplementary material 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* ortholog in zebrafish and would therefore be expressed specifically in LECs, we generated different *prox1a* BAC reporter lines that were analyzed in combination with the aforementioned *flt4:mCit* line. As 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 colocalized with *flt4:mCit* expression at 5 dpf (Fig. 1B-D). In contrast to previously published lymphatic markers such as *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; supplementary material Fig. 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, mark LECs at earlier stages, possibly even within the venous endothelium of the PCV at a time point 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 and 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. 1M,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 (supplementary material 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 (supplementary material 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 (supplementary material Fig. S2G).

Because *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, because 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.

Development (2014) doi:10.1242/dev.105031

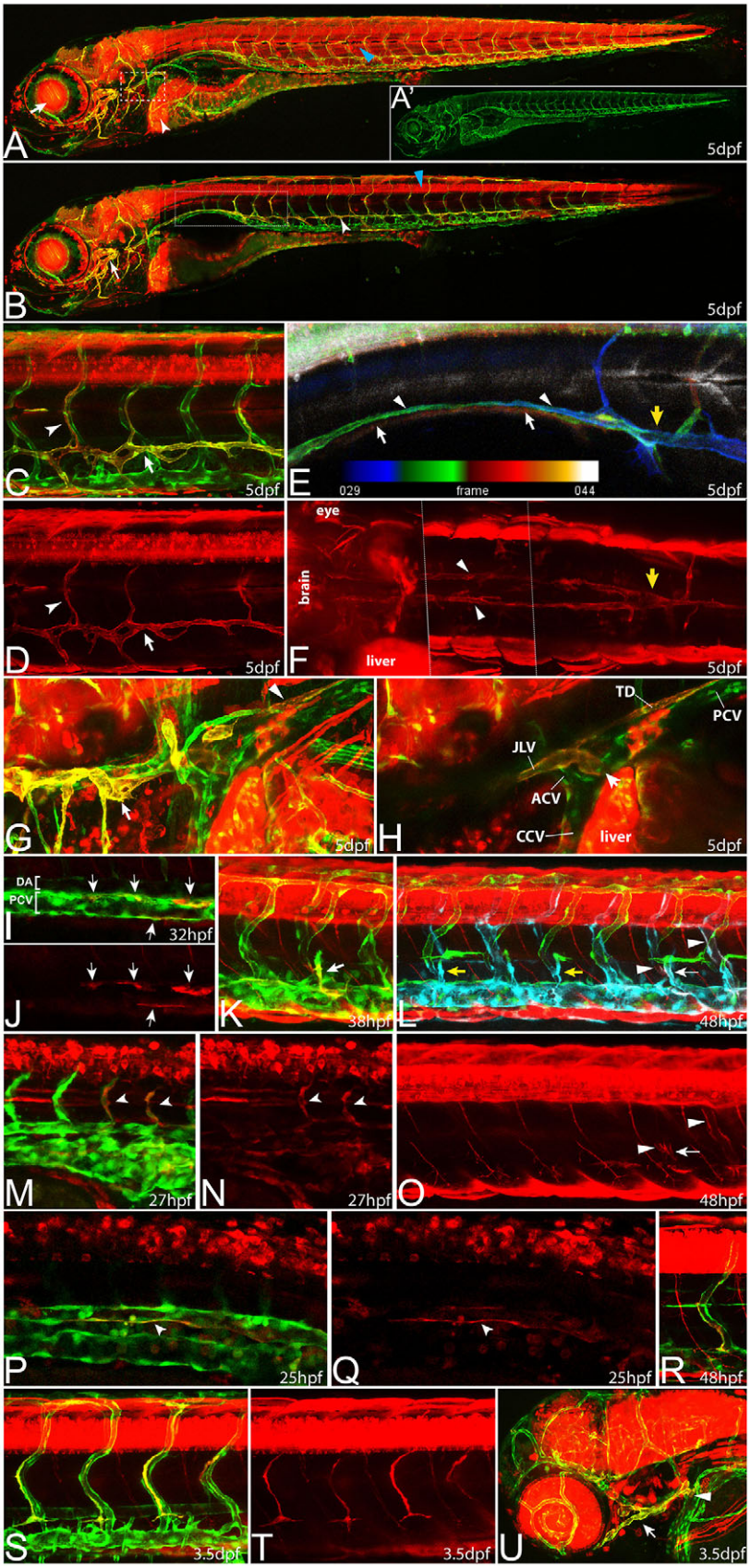


Fig. 1. *prox1a* expression marks different endothelial compartments during vascular development. In all pictures (except for E), *flt4:mCit* expression is shown in green and *prox1a:KalTA4,UAS:tagRFP* expression is highlighted in red. (A) In full *z*-projections, *prox1a* expression at 5 dpf is apparent in various tissues, including liver (white arrowhead), lens (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 in A and B are composed of several overlapping *z*-projections because the embryo was too large to fit in a single field of view. (C,D) Higher magnification of trunk lymphatics at 5 dpf exhibiting combinatorial *prox1a* (red) and *flt4* (green) reporter expression restricted to the TD (arrows) and ISLVs (arrowheads). (E) Depth color-coded *z*-projection (projecting each slice in a different color according to its position within the stack; see color bar) of boxed region in B (only *prox1a* channel) reveals the presence of two separate *prox1a*-positive vessels above the swim bladder (white arrows point to lymphatics on the right, arrowheads to lymphatics on the left body side), which merge near the sixth ISVs with the TD in the trunk region (yellow arrow). (F) Dorsal view of 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 a set of partial *z*-projections (see dotted lines) owing to interference of other *prox1a*-positive structures. (G,H) Full (G) and partial (H) *z*-projections of the area (compare with 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. ACV, anterior cardinal vein; CCV, common cardinal vein; JLV, jugular lymphatic vessel; PCV, posterior cardinal vein; TD, thoracic duct. (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 arrows) whereas the majority shows no signs of reporter expression (yellow arrows). 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*-plane of the trunk region in a 25 hpf embryo showing DA cells positive for *prox1a* reporter expression (arrowheads). 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 but 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).

Taken together, expression of *prox1a* becomes a reliable and very useful maker for LECs only at later stages of lymphangiogenesis. Therefore, 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.

Development (2014) doi:10.1242/dev.105031

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 (supplementary material Fig. S3A). The *prox1a*^{i278} allele harbors a 10 bp deletion in the first coding exon, resulting in a frame-shift after amino acid 149, and a premature stop codon after additional four amino acids (Fig. 2A). Homozygous mutants are devoid of full-length Prox1a protein as assessed by immunostaining of slow muscle fibers 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

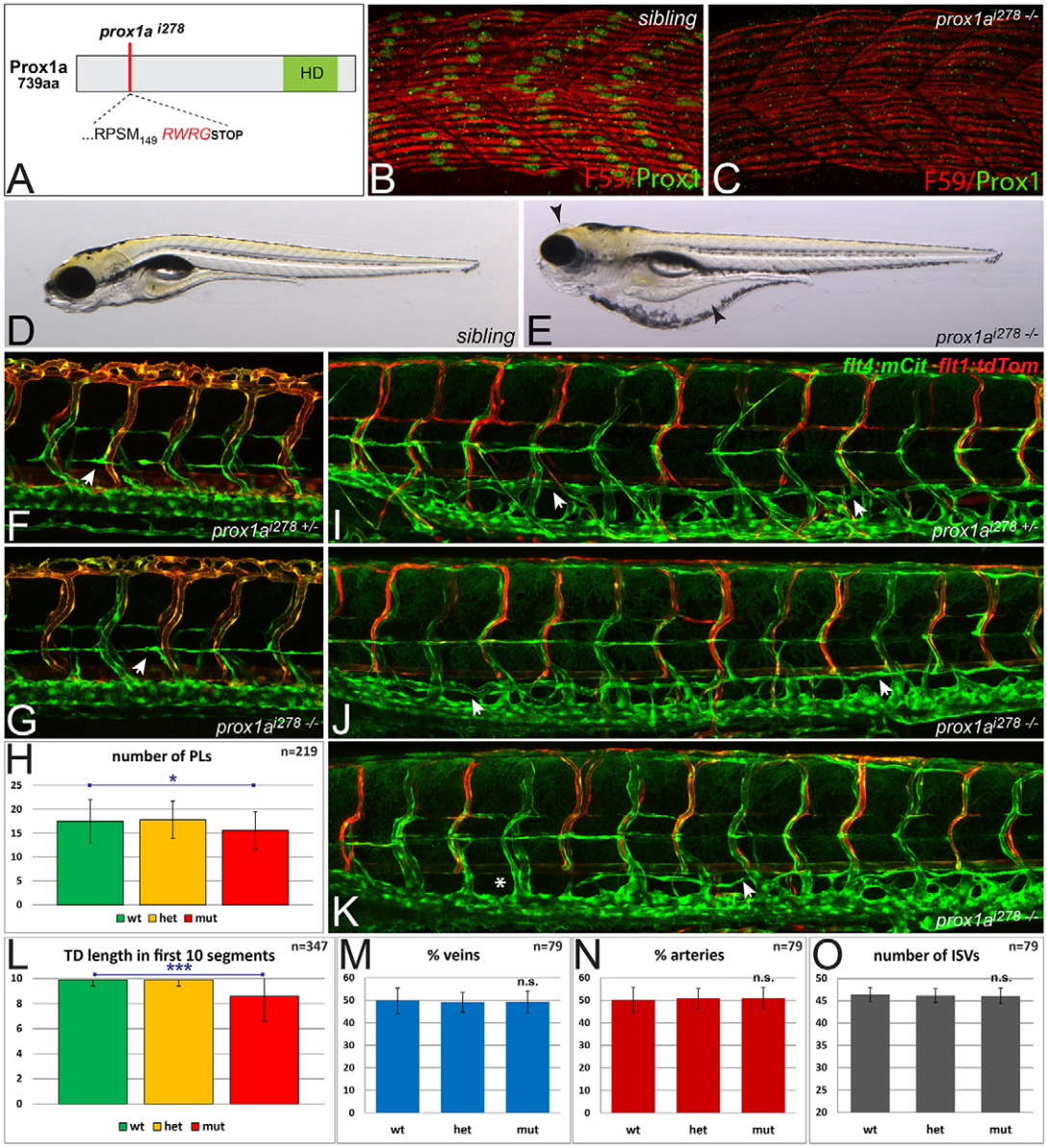


Fig. 2. Lymphangiogenesis in *prox1a* mutant embryos. (A) Schematic of the homeodomain (HD) containing Prox1a protein, indicating the predicted effect of the 10 bp deletion in the *prox1a*^{i278} allele, leading to a frame-shift (red amino acids) and a truncated protein after 153 amino acids. (B,C) Prox1a immunostaining of slow muscle fibers in sibling (B) and homozygous mutant *prox1a*^{i278} (C) embryos demonstrates a complete loss of wild-type Prox1a protein (green) at 30 hpf (slow myosin heavy chain-1 is shown in red). (D,E) Brightfield 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 2 dpf (Student's *t*-test, **P*=0.025). Error bars indicate s.d. 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 with heterozygous siblings (I), most homozygous *prox1a*^{i278} mutants do not display TD defects at 5 dpf (J), whereas 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 ten segments above the yolk extension at 5 dpf. Error bars indicate the s.d. for the respective genotypic class from a *prox1a*$^{+/-}$ incross. ****P*=2.3E–08 (Student's *t*-test, comparison of wild-type and mutant population). (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 *prox1*^{ai278} mutants, the average number of ISVs is not altered. Error bars represent s.d. n.s., not statistically significant.

Development (2014) doi:10.1242/dev.105031

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: although 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 indicate 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). As 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 ten somites, a mild phenotype that could also reflect a secondary defect caused by the early edema formation and overall impaired appearance of mutants at 5 dpf (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 were 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 (supplementary material 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 ECs, our finding that Prox1a protein was undetectable in slow muscle fibers 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

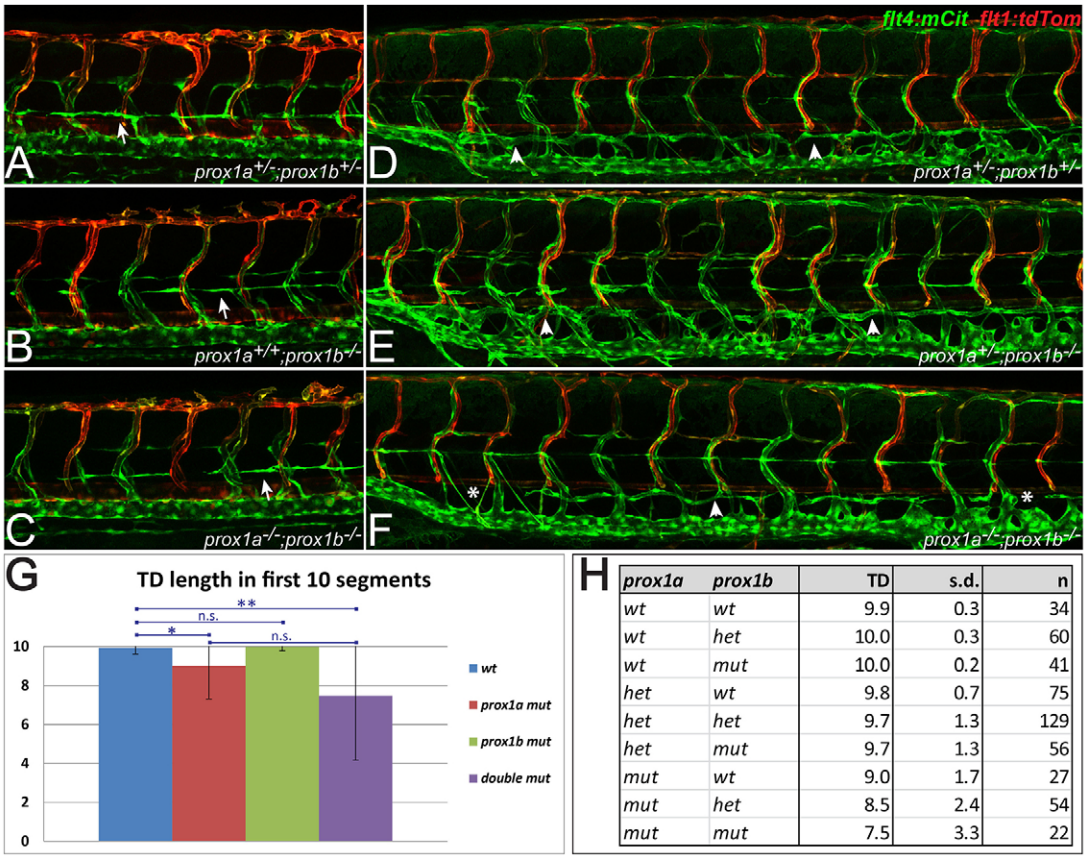
Although neither of the two *prox1* orthologs appears to be essential for zebrafish lymphatic development (Tao et al., 2011) (this study), it remains possible that they function redundantly. We therefore generated double mutants for *prox1a*^{i278} and the previously characterized loss-of-function allele *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 ten 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 (supplementary material 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 develop a wild-type lymphatic vasculature and do not show arterio-venous defects

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). As 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 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 morpholino 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 1 bp insertion allele *nr2f2*^{hu10330} (*coup-TFII*^{hu10330} hereafter). Sequencing of cDNA from *coup-TFII*^{hu10330} mutant embryos did not reveal alternative transcripts lacking the 1 bp insertion (data not shown). This suggests that the allele represents a loss-of-function situation because 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). As the ratio of intersegmental arteries and veins was also 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.

Development (2014) doi:10.1242/dev.105031



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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) and homozygous double mutants (C), PLs are apparent at the horizontal myoseptum at 2 dpf (arrows). (D-F) As in double heterozygous embryos (D), 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 and asterisks highlight segments without TD). (G) Average number of TD-positive segments within the first ten 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 with wild-type siblings. Error bars represent s.d. (H) Overview about the average TD length (TD) in ten scored segments, the corresponding s.d. and the number of scored embryos (*n*) in a *prox1a*$^{+/-}$;*prox1b*$^{+/-}$ incross. n.s., not statistically significant.

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 (François 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 (François et al., 2008). More recent morpholino knockdown studies as well as overexpression 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 the 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 1 bp insertion 5′ to the HMG-box encoding sequence (Fig. 4H). This allele is predicted to encode a truncated protein that contains neither the essential HMG nor the trans-activation domains (reviewed by Downes and Koopman, 2001), and as 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 reveal 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 an 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 an LEC-like gene expression pattern, severe edema formation, and embryonic lethality

Development (2014) doi:10.1242/dev.105031

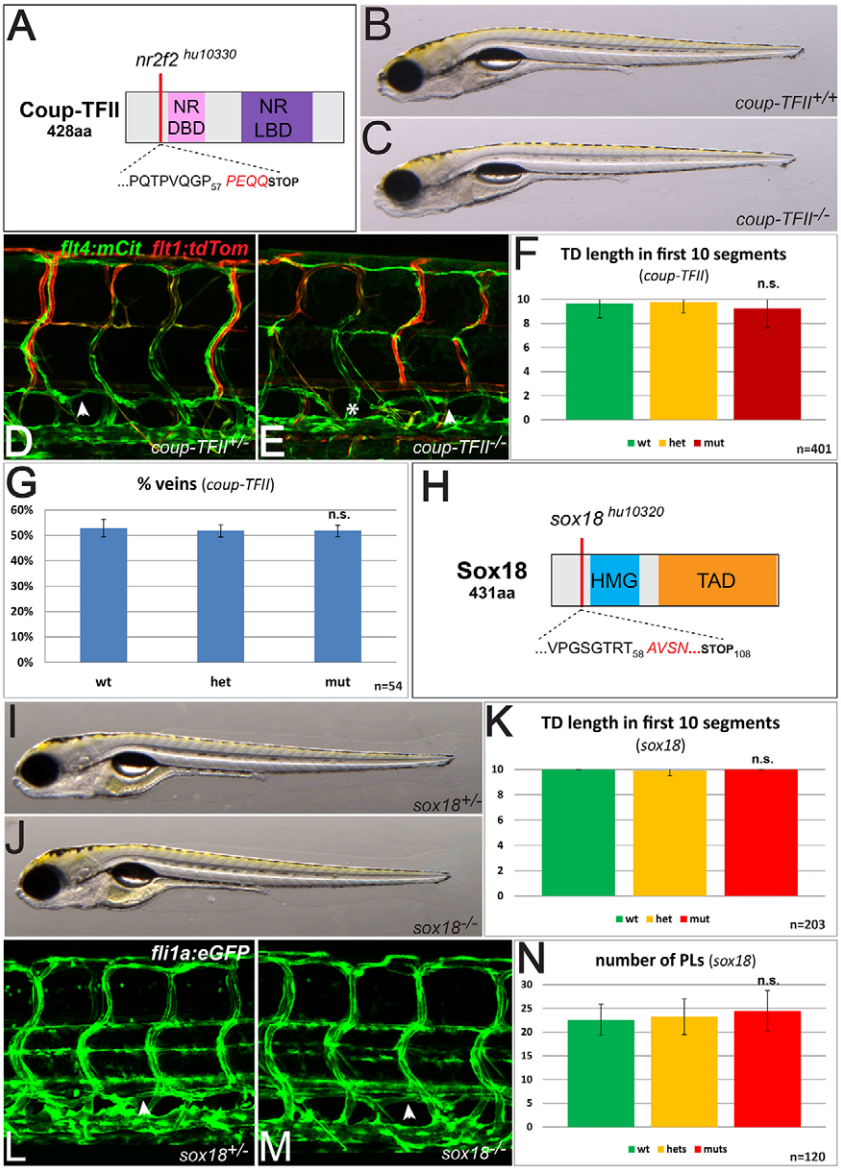

Fig. 4. Specification of the lymphatic lineage is independent of *coup-TFII* and *sox18*. (A) Schematic of the Coup-TFII protein indicating the position of the first affected amino acid in the 1 bp insertion allele *nr2f2*^{hu10330} (*coup-TFII*^{hu10330} hereafter) which leads to a premature stop codon after an additional four amino acids N-terminal to the nuclear receptor-DNA binding and ligand-binding domain. (B,C) Brightfield 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 with 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. Arrowheads indicate the presence of TD. (F) The average length of the TD in the first ten 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 1-bp 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 images of heterozygous (I) and homozygous (J) 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 (M) mutant embryos expressing *fli1a:eGFP* in all ECs. 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. Error bars represent s.d. n.s., not statistically significant.

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 EC into an LEC. Although we showed here that mutations in the zebrafish orthologs 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 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 3.8 kb *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 *in situ* hybridization (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* overexpression (Fig. 5F,G,J,K). 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. 5H,I,L,M; 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

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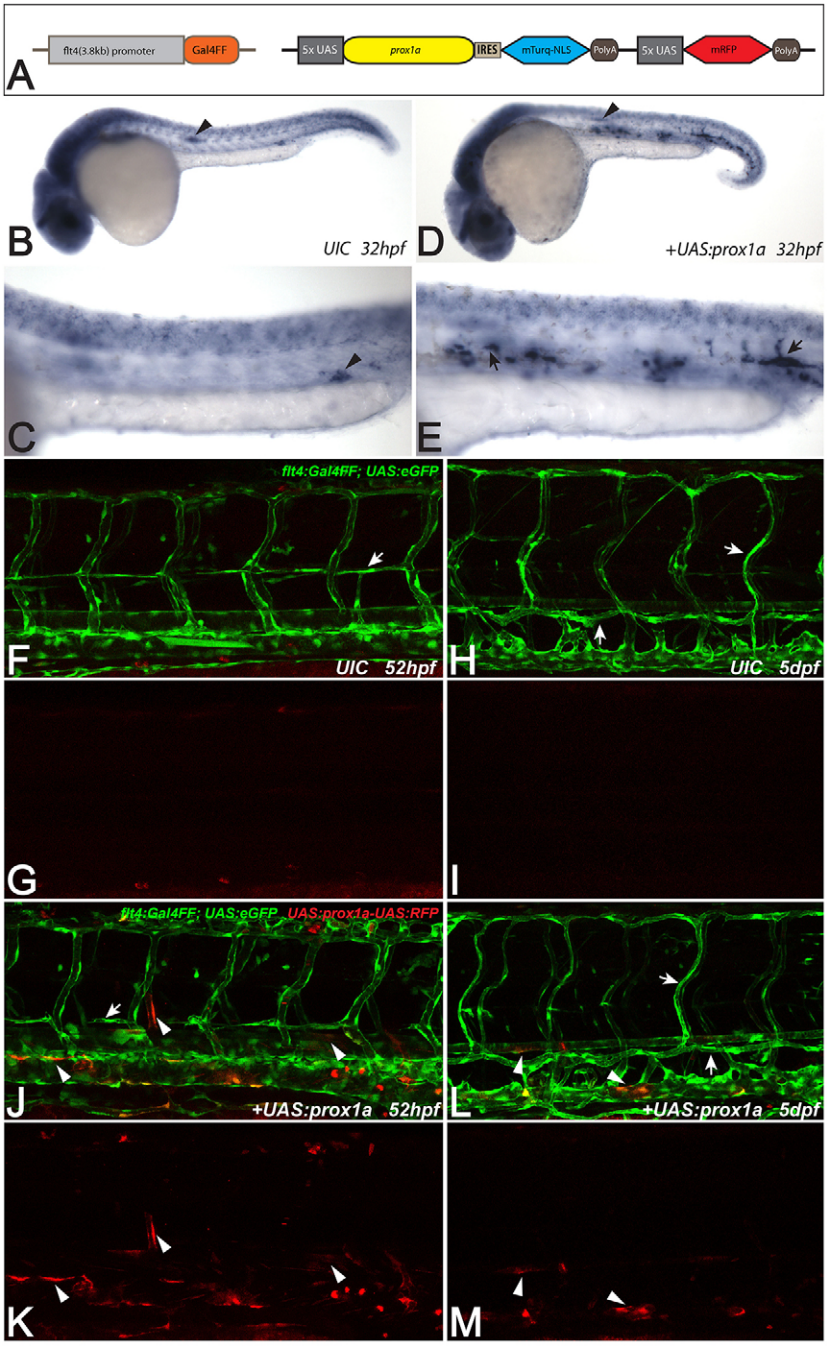

Fig. 5. Forced expression of *prox1a* does not commit venous ECs to a lymphatic phenotype. (A) Schematic of the *prox1a* overexpression construct and the *flt4:Gal4FF* line, which drives Gal4FF expression under the control of a Medaka 3.8 kb *flt4* promoter fragment. (B-E) Whole-mount *in situ* hybridization 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; 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 (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 line shows occasional expression in myotome cells as well as in a subset of neurons in the spinal cord. UIC, uninjected control.

for future LECs to migrate out of the cardinal vein (Yang et al., 2012). In the absence of mutants for Prox1 orthologs in other vertebrate species, a stringent assessment of conserved Prox1 function during lymphangiogenesis has been hampered. Nevertheless, expression studies in, for example, *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 (Küchler et al., 2006). Hence, we generated a *prox1a* loss-of-function model to address conclusively 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

Development (2014) doi:10.1242/dev.105031

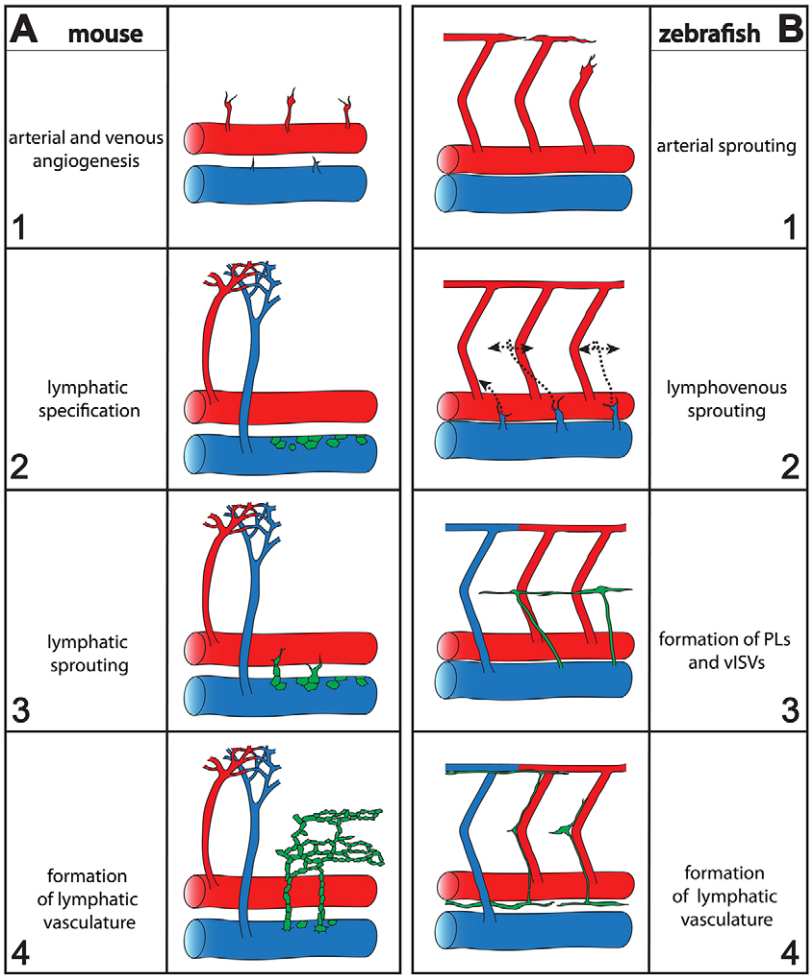

Fig. 6. Chronology of angiogenic and lymphangiogenic events in mice and zebrafish. (A) In mice, angiogenic sprouting from the DA and cardinal vein starts at E8.0 and leads to the formation of intersegmental arteries and veins (A1). Subsequently, a subpopulation of venous ECs is specified to the lymphatic 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 (Hägerling et al., 2013). (B) In contrast to 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 among the sprouts: some of them will connect to intersegmental arteries (venous fated cells) and others proceed towards the horizontal myoseptum (lymphatic fated cells), often even passing an ISV on their route (B2, dotted arrows). As a result, both a set of intersegmental veins and a pool of parachordal lymphangioblast at the horizontal myoseptum are formed (B3). The latter will subsequently migrate away from this region, to form the initial lymphatic structures in the trunk (B4).

(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. 6A3,4).

In fish, however, the timing of events is different: angiogenesis begins with a first wave of VegfA- and 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 that at some point specifically mark LECs in mice (such as *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 are lymphatic and venous fates separating (Fig. 6B3,4). It is therefore plausible that it is not an intrinsic mechanism but rather external cues, possibly provided by the ISVs 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 probably 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).

Development (2014) doi:10.1242/dev.105031

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 and the A*STAR Biological Resource Centre Institutional Animal Care and Use Committee (IACUC Project #110638). 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}* (Hogan et al., 2009a).

Transgenesis

The *Tg(flt4^{BAC}:mCitrine)^{hu7135}* line was generated from BAC DKEY-58G10 and the *Tg(prox1a^{BAC}:KalTA4-4xUAS-E1b:uncTagRFP)^{nim5}* from BAC DKEY-5J3 following standard BAC recombineering procedures (Bussmann and Schulte-Merker, 2011). For the generation of the *flt4* promoter construct *Tg(flt4:Gal4FF)^{hu9236}*, a previously reported 3.8-kb promoter fragment (Deguchi et al., 2012) was amplified from Medaka genomic DNA (supplementary material 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. Because 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 (100 pg/embryo) or plasmids (25 pg/embryo) were co-injected with Tol2 transposase mRNA (25 pg/embryo) into one-cell-stage embryos and the progeny was screened for germline transmission.

Genome editing by zinc-finger nuclease and transcription activator-like effector nucleases (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′ (supplementary material 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′-TGGCCTCCGCTG-CTGTT-3′. For *coup-TFII*, the TALEN recognition sites were TAL1, 5′-TCCGACCCCTCAGACACCCGT-3′ and TAL2, 5′-AACAATAACAAC-ACACAGTCA-3′.

Genotyping

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

Immunohistochemistry and *in situ* hybridization (ISH)

Antibody staining using the following antibodies was performed as described (Elworthy et al., 2008): mAb F59 [anti-slow myosin heavy chain-1, Developmental Studies Hybridoma Bank (DSHB); 1:100], rabbit anti-Prox1 (AB5475, Chemicon, USA; 1:5000), goat anti-rabbit Alexa488 and goat anti-mouse Alexa546 (Invitrogen; 1:1000). ISH was carried out as described previously (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. Brightfield images 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 performed using Leica LAS AF software or Adobe Illustrator CS5.1.

Acknowledgements

We thank M. Witte and Audrey Iyu for technical assistance; D. Schulte, S. van de Pavert and F. Kiefer for critical comments on the manuscript; and A. de Graaff and the Hubrecht Imaging Centre for supporting the imaging.

Competing interests

The authors declare no competing financial interests.

Author contributions

A.v.I. generated the *flt4* transgenic lines, analyzed the *prox1a/b* mutants and the *prox1a* reporter line and wrote the manuscript. Z.Z., D.M.A.H. and M.G.R. generated and analyzed new mutant alleles, in part using TALEN constructs generated by J.P.-M. J.C.F. generated the *prox1a* reporter line. H.D., E.A.O., P.W.I. and S.S.-M. conceived and supervised the study and edited the manuscript.

Funding

This work was supported by a Marie Curie Intra-European Fellowship award (to A.v.I.); a VENI grant from the Netherlands Organisation for Scientific Research (NWO) [863.11.022 to M.G.R.]; the Agency for Science Technology and Research (A*STAR) (Z.Z. and P.W.I.); and a Medical Research Council grant [U117581329 to J.F. and E.A.O.]. Deposited in PMC for release after 6 months.

Supplementary material

Supplementary material available online at
http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.105031/-/DC1

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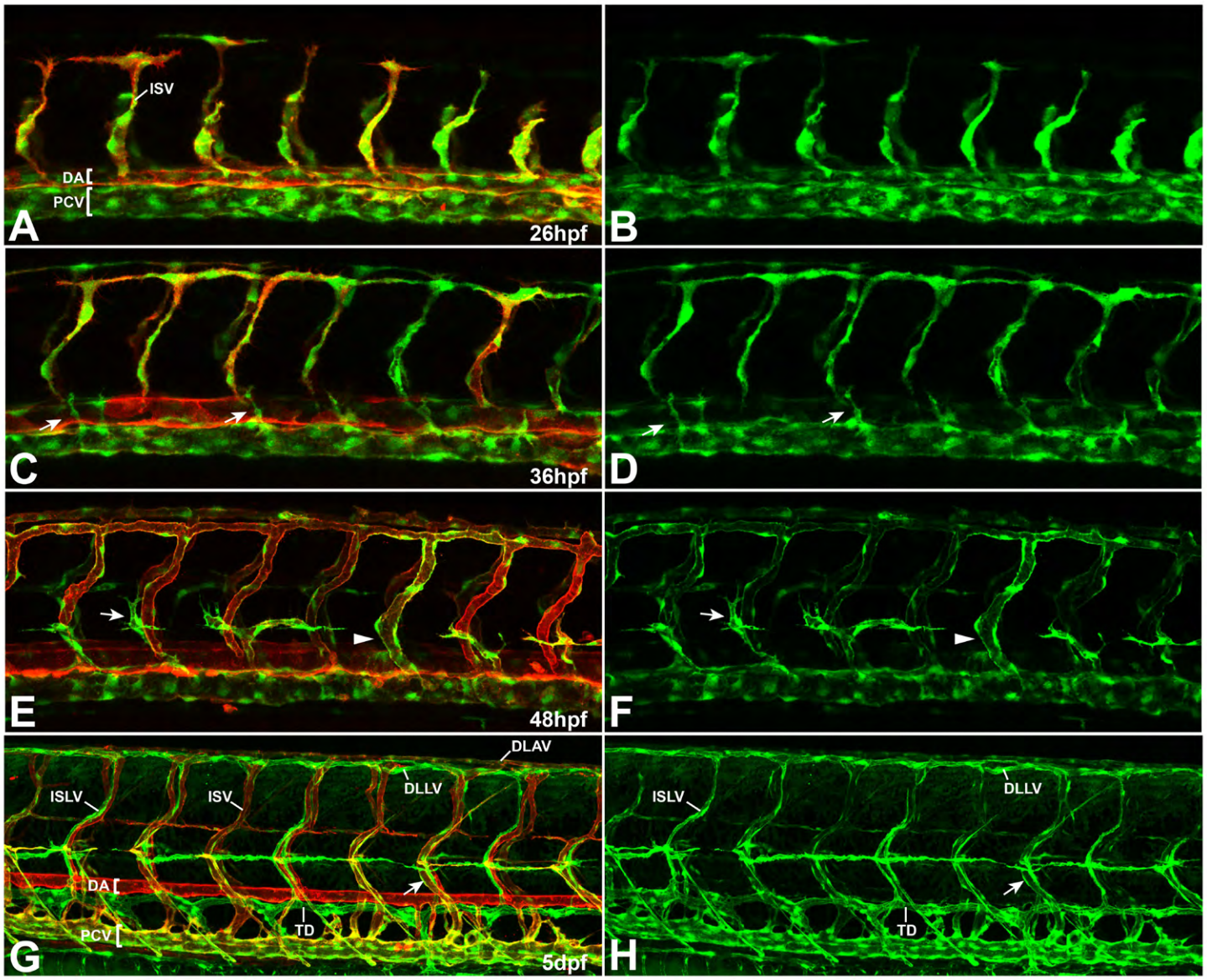

Fig. S1. Expression pattern of *flt4*^{BAC}*:mCitrine* reporter line at different stages of vascular development.

(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-intersegmantal vessel, ISLV-intersegmental lymphatic vessel, DLAV-dorsal longitudinal anastomotic vessel, DLLV-dorsal longitudinal lymphatic vessel]

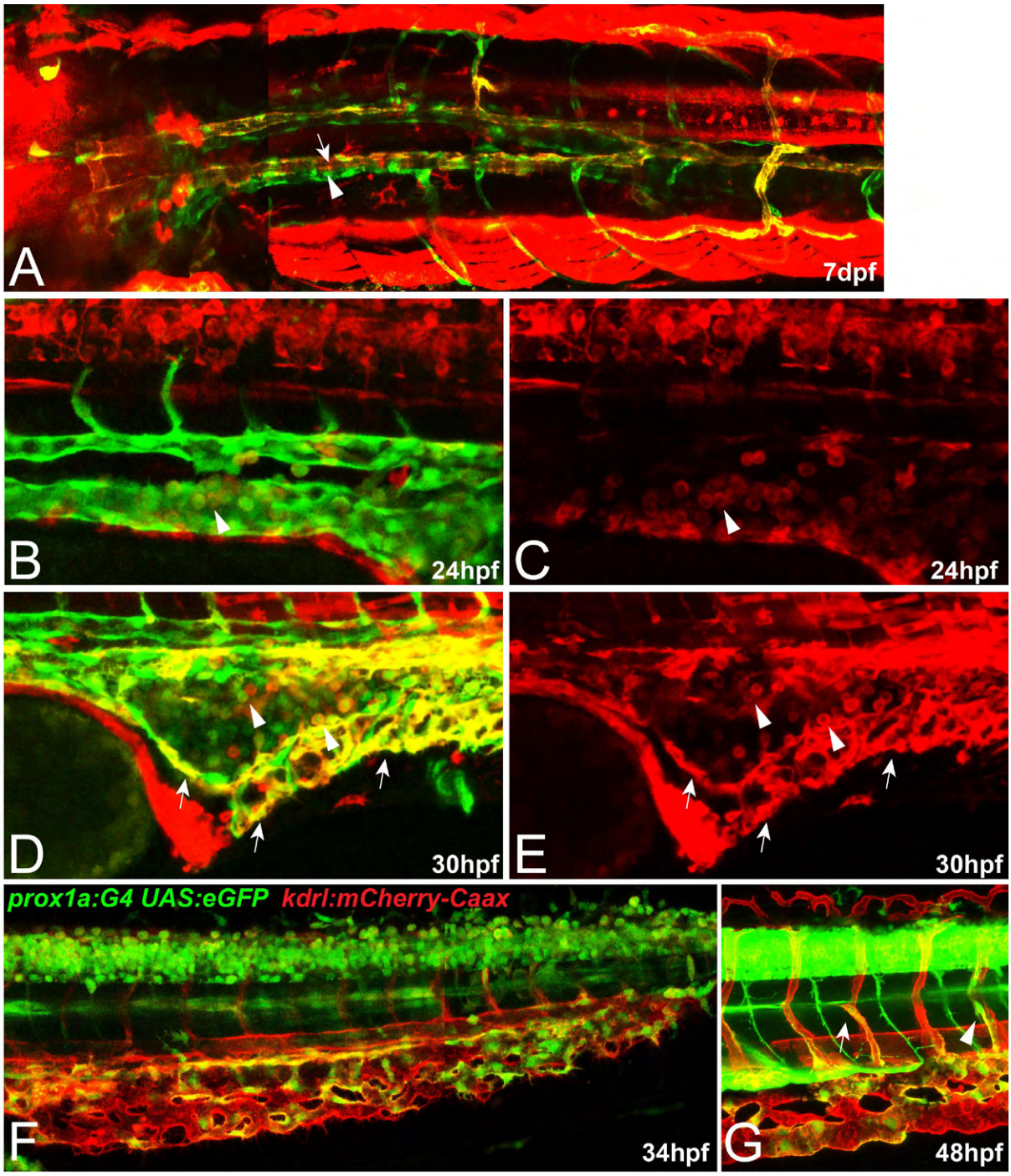

Fig. S2. Additional expression domains of the *prox1a* reporter line

(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.

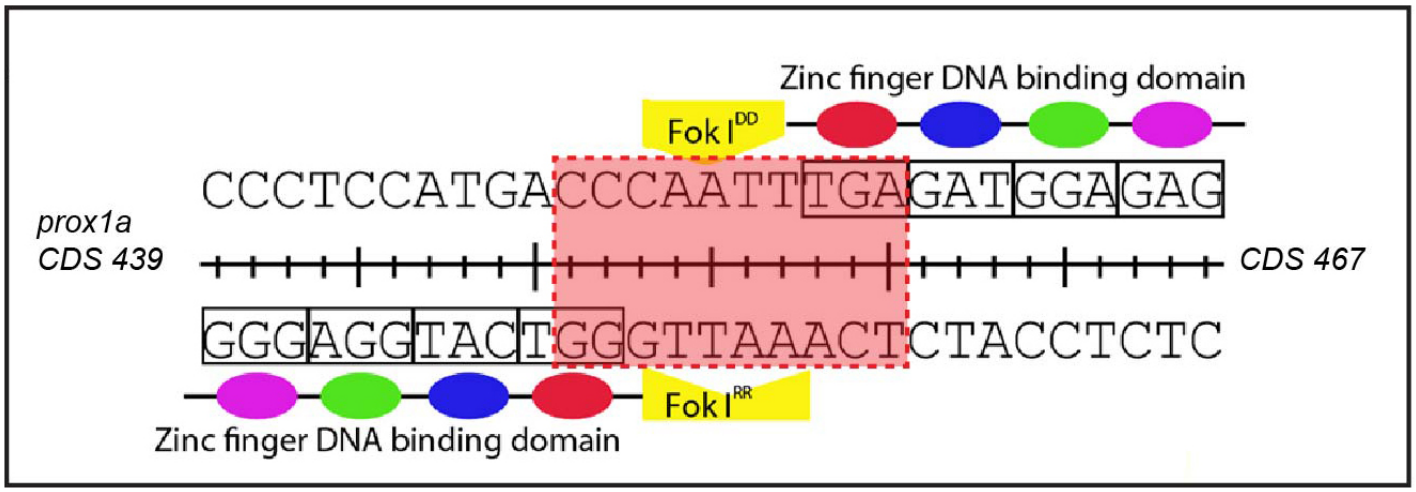

Fig. S3. Zinc-finger nuclease mediated generation of a *prox1a* mutant allele.

(A) 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.

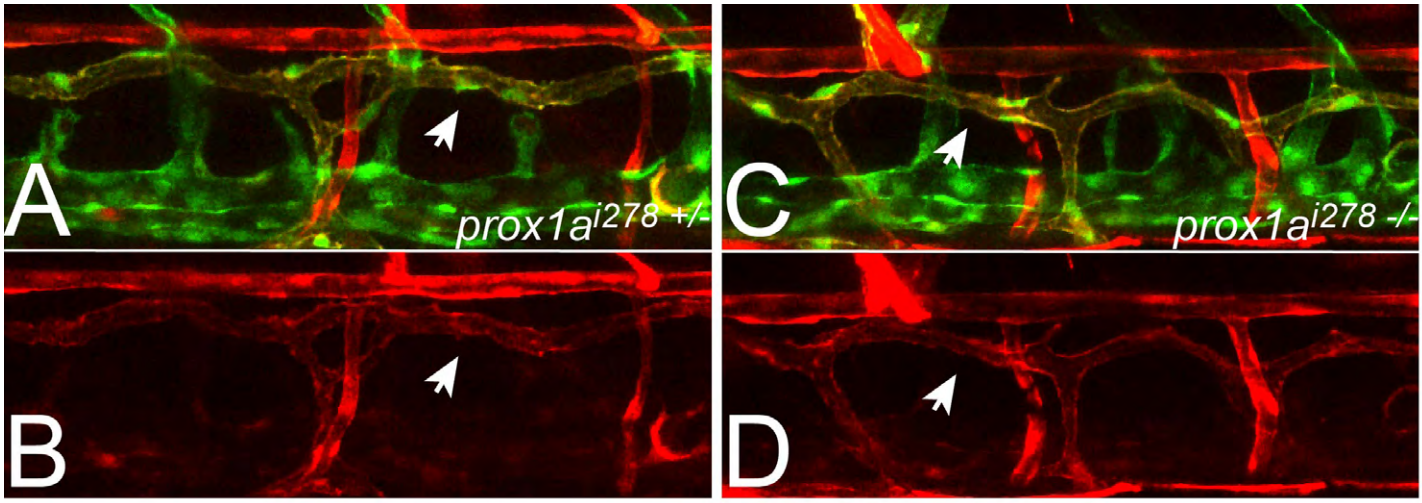

Fig. S4. Expression of the *prox1a* reporter line in *prox1a*^{i278} mutants.

(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.

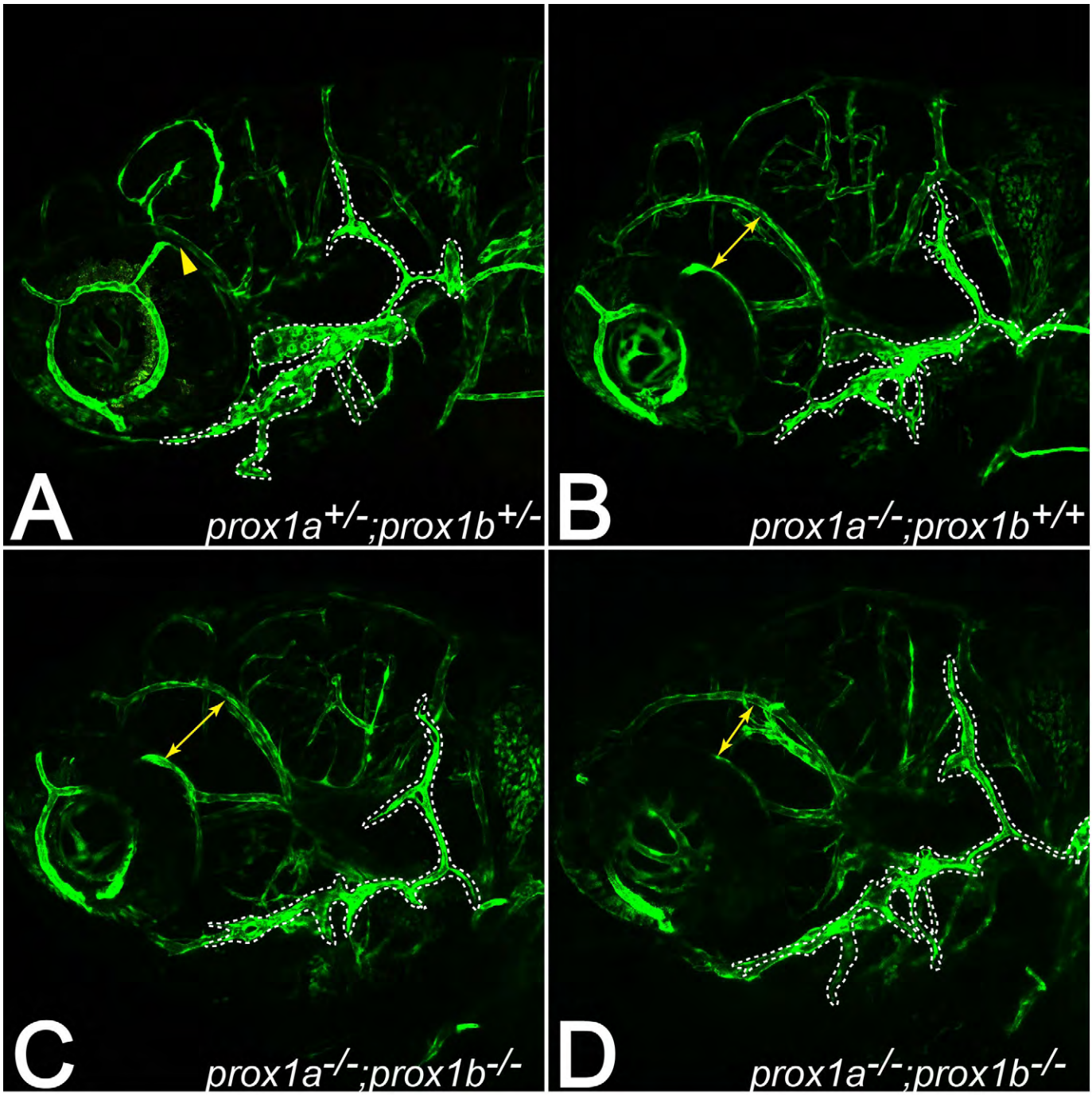

Fig. S5. Facial lymphatics are formed in *prox1a*^{i278} and *prox1a*^{i278}*;prox1b*^{SA0035} double mutants.

(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).

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'