Divergence of lymphatic cell fate specification pathways between zebrafish and mice

running title: Prox1a in lymphatic development

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keywords: lymphangiogenesis, prox1, coup-TFII, sox18, nr2f2

SUMMARY

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

INTRODUCTION

Lymphatic vessels play pivotal roles in tissue fluid homeostasis, immune cell trafficking and the uptake of dietary fats in the small intestine. A failure in lymphatic vessel development (lymphangiogenesis) or in lymphatic function is causative for several inherited or acquired pathological conditions that lead to tissue swelling by accumulation of extravasated fluids (Tammela and Alitalo, 2010; Schulte-Merker et al., 2011). In mice, combined activity of the transcription factors Coup-TFII (Srinivasan et al., 2010) and Sox18 (Francois et al., 2008) leads to the polarized expression of *Prox1* in a subset of endothelial cells (ECs) within the cardinal vein at E9.5. Shortly thereafter, Prox1 positive ECs leave the cardinal vein in a dorsal direction, mediated by Vegfc/Flt4 driven processes of polarized sprouting and migration, resulting in the formation of the first lymphatic structures in the embryo (Karkkainen et al., 2004; Hagerling et al., 2013). *Prox1* expression in lymphatic precursor cells is essential for the initiation of a lymphatic gene expression program, and *Prox1* knockout mice lack all lymphatic structures (Wigle et al., 2002). Forced expression of *Prox1* is sufficient to confer lymphatic identity to blood ECs, demonstrating the pivotal role of the gene for lymphatic specification (Hong et al., 2002; Petrova et al., 2002). Continuous expression of *Prox1* in lymphatic ECs is also indispensable for the maintenance of lymphatic cell fate during later stages of development (Johnson et al., 2008), thus firmly establishing Prox1 as the central determining factor of lymphatic identity.

In the zebrafish trunk, the process of vasculogenesis establishes an initial primitive circulatory loop consisting of dorsal aorta (DA) and posterior cardinal vein (PCV). Then, in a wave of angiogenic sprouting from the DA (primary or arterial sprouting), a set of about 30 arterial intersegmental vessels (ISVs) is formed on each side of the embryo. Shortly thereafter, at about 36 hpf, another group of roughly two times 30 sprouts emerge bilaterally from the PCV. These venous (or secondary) sprouts also migrate dorsally, and about half of them make a stable connection to pre-existing arterial ISVs, thereby remodeling them into intersegmental veins. Venous sprouts that fail to connect to arteries migrate further

dorsally towards the midline of the embryo where they populate the region of the horizontal myoseptum as parachordal lymphangioblasts (PLs) (Hogan et al., 2009a), which constitute a pool of lymphatic precursors in the embryonic trunk. These PLs will subsequently migrate away from the horizontal myoseptum (2.5 dpf) by using arterial ISVs as migration routes to populate the different regions of the trunk, eventually giving rise to the thoracic duct (TD; situated between the DA and PCV), a number of intersegmental lymphatic vessels (ISLVs) in close proximity to arterial ISVs, and the dorsal longitudinal lymphatic vessel (DLLV) (Bussmann et al., 2010).

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

Using a novel transgenic reporter line, we show here that *prox1a* exhibits a dynamic expression pattern in different endothelial compartments during early vascular development. In contrast to the situation in mice, we found that expression of this reporter gene only becomes a specific and reliable marker for lymphatic ECs at later stages of lymphangiogenesis, arguing against a lymphatic specification function during the onset of venous sprouting. In line with this, using a novel targeted allele of *prox1a* in combination with the previously described *prox1b* mutation, we show that lymphangiogenesis can proceed in the complete absence of Prox1 in zebrafish. In addition, we show that the functionally related transcription factors Coup-TFII and Sox18 are also dispensable during lymphangiogenesis. These results indicate that the Sox18/Coup-TFII/Prox1 lymphatic specification code is not conserved in fish, suggesting an alternative mode of lymphatic commitment in this vertebrate class.

MATERIALS AND METHODS

Zebrafish husbandry

Strains were maintained under standard husbandry conditions. Animal experiments have been performed according to the rules of the Animal Experimentation Committee (DEC) of the KNAW. The following published transgenic lines have been employed in this study: *Tg(flt1^{enh}:tdTomato)* (Bussmann et al., 2010),

Tg(fli1a:eGFP)^{y1} (Lawson and Weinstein, 2002), *Tg(kdrl:HRAS-mCherry)*^{s916} (*kdrl:mCherry-Caax* hereafter) (Hogan et al., 2009a).

Transgenesis

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

Genome editing by zinc-finger nuclease and TALENs

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

Genotyping

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

Immunohistochemistry and in situ hybridisation (ISH)

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

Microscopy

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

RESULTS

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

prox1a is expressed within different endothelial cell populations during development

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

Next, we investigated whether zebrafish *prox1a* expression would, like its murine counterpart, already mark LECs at earlier stages, possibly even within the venous endothelium of the PCV at a point in time prior to venous (secondary) sprouting. At 32 hpf, shortly before the emergence of the first sprouts from the PCV, *prox1a* could indeed be detected in a subset of venous cells (Fig. 1I, J) and subsequently in sprouting venous ECs (Fig. 1K). To determine whether *prox1a* might exclusively mark secondary sprouts that will give rise to lymphangioblasts, expression was analyzed at 44 hpf, a stage when PL cells have

extended to the horizontal myoseptum while venous destined sprouts have already established a connection to the respective ISV. We found *prox1a* reporter expression only in a proportion of PLs (Fig. 1L; 17/78 PLs), indicating that not all lymphatic precursor cells are positive for *prox1a* at this stage. Equally important, the transgene also transiently marked individual venous ISVs (in 12/21 imaged regions) (Fig. 1L, O, R), suggesting no clear correlation between *prox1a* expression and the fate of secondary sprouts.

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

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

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

Lymphatic development is not blocked in *prox1a* mutant embryos

To assess the role of Prox1a in lymphangiogenesis, we generated a loss-of-function allele for *prox1a* using zinc-finger nucleases (Fig. S3A). The *prox1a*^{i278} allele harbors a 10bp deletion in the first coding exon, resulting in a frame-shift after amino acid 149, and a premature STOP after additional 4 amino acids (Fig. 2A). Homozygous mutants are devoid of full-length Prox1a protein as assessed by immunostaining of slow muscle fibres at 30 hpf (Fig. 2B, C) The overall appearance of homozygous *prox1a*^{i278} mutants was normal until between days 4 and 5, at which stage mutant larvae started to develop severe edema around the gut and the eye (Fig. 2D, E). At 5.5–6 dpf most mutants had impaired blood circulation and showed signs of tissue necrosis. Importantly, timing as well as severity of edema was different from *ccbe1*, *vegfc* and *flt4* mutant scenarios: While completely lacking lymphatic structures, mutants of each of those genes only develop mild edema starting from 5-6 dpf. The later onset of edema in those lymphatic mutants presumably results from the lymphatic system beginning to perform its drainage function at early day 5 (Karpanen and Schulte-Merker, 2011); thus atypical tissue fluid accumulations can only arise from this time point. It follows that the edema observed in *prox1a* mutants does not reflect a sign of impaired lymphatic drainage function.

In *Prox1*-null mutant mice, the failure of LEC progenitors to leave the cardinal vein results in the absence of lymphatic structures (Yang et al., 2012) and even heterozygous embryos die shortly after birth with

dysfunctional lymphatics and a lack of lymphovenous valves (Harvey et al., 2005; Srinivasan and Oliver, 2011). If *prox1a* was equally important in specifying lymphatic cell fate in zebrafish, homozygous mutants should be deficient in lymphatic structures. However, when analyzing the appearance of PLs at the horizontal myoseptum at 2 dpf, we found that *prox1a*^{i278} mutants show only a marginal reduction in the number of PLs (Fig. 2F-H). Since it is possible that this initially mild defect results in more dramatic effects at later stages, we evaluated whether the formation of the TD was impaired. At 5 dpf, no defects in heterozygous embryos and only minor defects in a proportion of homozygous mutants were evident (Fig. 2I-K). On average, homozygous mutants lacked the TD only in 1.4 of the first 10 somites, a mild phenotype that could also reflect a secondary defect caused by the early edema formation and overall impaired appearance of mutants at 5dpf (Fig. 2L). Hence, expression of *prox1a* in the venous and lymphatic endothelium as revealed by the reporter gene described above is not essential for the specification of lymphatic structures. To confirm that the loss of *prox1a* did not affect secondary sprouts that are committed to a venous fate, the ratio of arteries and veins as well as the total number of ISVs was quantified. Overall, no defects on the blood vasculature could be observed and the arterio-venous ratio was also unaffected in mutants (Fig. 2I-K, M, N). We also found that expression of the *prox1a* reporter line was present in homozygous *prox1a*^{i278} mutants, indicating that, in contrast to the situation in mice (Srinivasan et al., 2010), Prox1a protein is not required for maintaining its own lymphatic expression (Fig. S4A-D). Furthermore, considering that the *prox1a* reporter represents a specific lymphatic marker at 5 dpf, the unaltered expression of both, *flt4:mCit* and *prox1a:KalTA4,UAS:tagRFP* in the TD of homozygous *prox1a* mutants argues for correct lymphatic specification of LECs even in the absence of functional Prox1a protein. Given the specific zygotic expression pattern of *prox1a* in different subsets of ECs, our finding that Prox1a protein was undetectable in slow muscle fibres of *prox1a*^{i278} mutants prior to secondary sprouting as well as the fact that maternally expressed *prox1a* transcripts have not been identified (Harvey et al., 2013), it seems unlikely that the lack of zygotic phenotype can be attributed to maternal rescue. We therefore conclude that *prox1a* function is not essential for any aspect of lympho-venous sprouting and that the gene is dispensable for lymphatic cell fate determination in zebrafish.

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

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

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

The principal factor that promotes venous cell identity in many vertebrate embryos is the transcription factor COUP-TFII (NR2F2), which suppresses expression of arterial genes in venous ECs (You et al., 2005). Since lymphatics are venous-derived, loss of endothelial Coup-TFII expression in mice not only causes venous specification defects but also results in a lack of LECs (Srinivasan et al., 2007). Coup-TFII has been reported to be required for the initiation of *Prox1* expression within the cardinal vein by direct binding to its promoter region (Srinivasan et al., 2010) and subsequently also for the maintenance of *Prox1* expression in future LECs during early lymphangiogenesis. The latter function is thought to be dependent on a direct physical interaction between Coup-TFII and Prox1, and heterodimers have been reported to act as co-regulators of several lymphatic lineage specific genes such as *FLT4* in cultured LECs (Lee et al., 2009). More recently, vascular defects including a reduction in PL number and aberrant TD formation has been reported to result from MO-knockdown of zebrafish *coup-TFII* (Aranguren et al., 2011). We therefore wondered whether lymphatic lineage specification in fish might be entirely regulated by *coup-TFII* instead of *prox1* genes. To explore this possibility, we employed TALEN constructs targeting the first exon of *coup-TFII* and generated a one base pair insertion allele *nr2f2*^{hu10330} (*coup-TFII*^{hu10330} hereafter). Sequencing of cDNA from *coup-TFII*^{hu10330} mutant embryos did not reveal alternative transcripts lacking the 1bp insertion (data not shown). This suggests that the allele represents a loss-of-function situation since the insertion results in a frame-shift and premature stop codon 5' to both the nuclear receptor-DNA binding domain and the ligand-binding domain (Fig. 4A).
Homozygous *coup-TFII*^{hu10330} mutants were viable beyond 6 dpf (Fig. 4B, C) and developed a normal blood vasculature without any sign of arterio-venous identity defects in the main trunk vessels. In addition, when scoring TD formation at 5 dpf, we observed only negligible defects and the majority of mutants were indistinguishable from siblings (Fig. 4D-G). Since also the ratio of intersegmental arteries and veins was unaffected in *coup-TFII* mutant embryos (Fig. 4F, H) these results demonstrate that zygotically expressed *coup-TFII* is not essential for venous specification and lymphatic development.

Loss of *sox18* does not interfere with lymphatic development

Another transcription factor that has been implicated in lymphatic development in mice and humans is Sox18 (Irrthum et al., 2003). Mice lacking functional Sox18 develop edema in certain genetic backgrounds (Francois et al., 2008) because of a failure in LEC development. *Sox18* is expressed in a subset of cardinal vein cells that subsequently initiate *Prox1* expression. In vitro experiments further showed that Sox18 can activate *Prox1* expression by direct binding to its promoter region, placing *Sox18* upstream of *Prox1* during lymphatic specification (Francois et al., 2008). More recent morpholino knockdown studies as well as over-expression experiments employing a dominant-negative mouse Sox18 variant in zebrafish suggested a specific requirement for *sox18* during sprouting of lymphatic-fated secondary sprouts from the PCV. In contrast to mouse however, zebrafish *sox18* is expressed in both DA and PCV in a non-polarized fashion during venous sprouting (Cermenati et al., 2013) which is counterintuitive in the context of lymphatic specification.
We generated a mutant with a 1bp insertion 5' to the HMG-box encoding sequence (Fig. 4I). This allele is predicted to encode a truncated protein that neither contains the essential HMG nor the trans-activation domains [reviewed in (Downes and Koopman, 2001)], and since no alternative transcripts could be detected in mutants (data not shown) we consider it to represent a loss-of-function allele. Loss of Sox18

function did not lead to any obvious differences in the formation of arterial and venous ISVs, which is in line with previous morpholino knockdown data (Cermenati et al., 2008; Herpers et al., 2008; Pendeville et al., 2008; Cermenati et al., 2013). At 5 dpf the overall appearance of homozygous *sox18*^{hu10320} mutants was indistinguishable from siblings (Fig. 4J, K) and when scored for the presence of the TD, no lymphatic or blood vascular phenotypes were detectable (Fig. 4L-O). Accordingly, the quantification of PLs at the horizontal myoseptum at 48 hpf did not reflect any significant differences (Fig. 4N, P), demonstrating that Sox18 is dispensable for venous sprouting and lymphatic cell fate determination.

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

It has been reported that overexpression of Prox1 in blood ECs in vitro is sufficient to shift their gene expression profile towards a LEC phenotype (Hong et al., 2002; Petrova et al., 2002). Similar results were also obtained from mouse models, where the forced expression of *Prox1* in blood ECs led to a LEC-like gene expression pattern, severe edema formation, and embryonic lethality at E13.5 (Kim et al., 2010). Interestingly, the ability of *Prox1* to transform blood into lymphatic ECs seems to be restricted to the venous endothelium in vivo (Kim et al., 2013), indicating that the additional expression of only one transcription factor is sufficient to differentiate a venous ECs into a LEC. Although we showed here that mutations in the zebrafish orthologues of *Prox1* and two other central genes that govern and maintain lymphatic commitment in mammals did not block lymphatic specification, we wondered whether *prox1a* expression might nevertheless be sufficient to force blood ECs into a lymphatic cell fate. Therefore, we aimed to mis-express *prox1a* in blood ECs and injected an *UAS:prox1a* construct into a *flt4:Gal4FF* driver-line (Fig. 5A) which drives expression from a 3.8kb *flt4* promoter fragment from Medaka (Deguchi et al., 2012). Upon transient injection of the UAS-construct, overexpression of *prox1a* transcripts was verified by whole-mount ISH. As expected, embryos injected with the UAS-construct expressed *prox1a* in a mosaic pattern in venous and arterial ECs at considerably higher levels than the endogenous endothelial expression in uninjected siblings at 32 hpf (Fig. 5B-E). We checked at 38 hpf (data not shown) and 48 hpf whether this *prox1a* mis-expression would have an influence on the behavior of positive ECs (marked by the co-expression of mRFP) during venous sprouting. However, neither the overall number of secondary sprouts nor the positioning of RFP–positive cells within the vasculature indicated a differential cell behavior upon *prox1a* over-expression (Fig. 5F, H). Furthermore and in contrast to the mouse experiments, continuous mis-expression of *prox1a* did also not result in any vascular or lymphatic defects at later stages of lymphangiogenesis. In fact, at 5 dpf no differences in vascular morphology or in the expression pattern of the *flt4:Gal4FF; UAS:GFP* marker could be appreciated and cells expressing the construct ended up in all endothelial compartments (Fig. 5G, I, data not shown). Together these results indicate that zebrafish *prox1a* is not only dispensable for lymphatic specification, but is also unable to commit ECs to a lymphatic fate.

DISCUSSION

Specification of the lymphatic lineage in mice is intimately linked to the restricted expression of the transcription factor Prox1 in a subpopulation of cardinal vein cells, and Prox1 function is essential for future LECs to migrate out of the cardinal vein (Yang et al., 2012). In the absence of mutants for Prox1

orthologues in other vertebrate species, a stringent assessment of conserved Prox1 function during lymphangiogenesis has been hampered. Nevertheless, expression studies in, e.g. Xenopus (Ny et al., 2005) and zebrafish (Yaniv et al., 2006) suggest that there is evolutionary conservation.

Previous morpholino-knockdown strategies for zebrafish *prox1a* did not provide a fully conclusive picture and we had previously pointed out that different morpholinos directed against *prox1a* result in developmental anomalies which we interpreted as being unspecific (Kuchler et al., 2006). Hence, we generated a *prox1a* loss-of-function model to conclusively address the role of *prox1*-like genes for lymphangiogenesis in fish. Our results demonstrate that *prox1a* expression is not essential and also not sufficient for specification of LECs. Even the combined loss of *prox1a* and *prox1b* does not inhibit lymphatic development.

In combination with our finding that *coup-TFII* and *sox18* are also not essential for lymphangiogenesis to occur, this suggests that lymphatic specification must be achieved in a different way and that the Coup-TFII/Sox18/Prox1 function has been evolving only in higher vertebrates or has been eliminated in the zebrafish/teleost lineage. How can one explain the strict requirement for Prox1 in mice, while in fish the Coup-TFII/Sox18/Prox1 code seems dispensable?

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

In fish, however, the timing of events is different: Angiogenesis begins with a first wave of VegfA/Vegfr2 governed sprouting from the DA, giving rise to a set of intersegmental arteries (Fig. 6B1). Subsequently, a second set of sprouts arises exclusively from the PCV (Fig. 6B2). The emergence of these venous sprouts that eventually will give rise to both intersegmental veins and lymphatic precursors, depends on the Vegfc/Flt4 pathway and on Ccbe1, indicating that in zebrafish the mechanisms that drive the initial migration of both subpopulations is identical. This might also explain why genes, which at some point specifically mark LECs in mice (like *Flt4* or *Lyve1*), are expressed within the whole venous endothelium in zebrafish. It is only slightly later, when secondary sprouts reach the level of the arterial ISVs that both populations display a differential behavior, suggesting that only from this point in time lymphatic and venous fates are separating (Fig. 6B3, B4). It is therefore plausible that it is not an intrinsic mechanism but rather external cues, possibly provided by the ISV themselves, that control which sprout will connect to an ISV and which will migrate further to the horizontal myoseptum. In fact, the only known signaling pathway so far that has been shown to affect the cell fate decision made by secondary sprouts is Dll4/Notch. Knockdown of *dll4* leads to a dramatically enhanced number of venous ISVs and only a small number of PL cells, suggesting that the fate of secondary sprouts is strongly shifted towards venous identity (Geudens et al., 2010). Whether this requirement for Notch signaling is artery-intrinsic (*dll4* and *notch1b* are both expressed in arterial ECs) or reflects a signaling interaction between ISV and secondary sprouts requires further analysis, but in either case arterial ISVs directly or indirectly influence the fate decisions made by venous sprouts.

In summary, the scheme depicted in Fig. 6 offers an explanation as to why the Coup-TFII/Sox18/Prox1 signaling axis is required in mice, but dispensable in zebrafish: in mice (and probably other vertebrate classes) Prox1 function specifies LEC fate within a subset of venous ECs within the cardinal vein. In

zebrafish (and likely all teleosts), this specification step is not required within the endothelial epithelium of the cardinal vein. Our findings therefore reveal an astonishing difference in the earliest step of lymphangiogenesis between vertebrates. In the absence of a full understanding of the exact point in time when LECs become specified in zebrafish, it remains difficult to appreciate fully the degree of similarity between mice and other vertebrates at the level of genetic control and cellular behavior. However, other steps of lymphangiogenesis, such as the migration of future LECs away from the major vein, are conserved in vertebrates, as evidenced by the conserved functions of *vegfc* (Karkkainen et al., 2004; Ny et al., 2008; Villefranc et al., 2013), *flt4* (Jeltsch et al., 1997; Ny et al., 2008; Hogan et al., 2009b) and *ccbe1* (Hogan et al., 2009a; Bos et al., 2011).

Acknowledgements

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

Funding

AvI was supported by a MC-IEF award and MGR by a VENI grant from NWO. ZZ and PWI were supported by the Agency for Science Technology and Research (A*STAR). JF and EAO were supported by MRC grant U117581329.

Author contributions

AvI generated the *flt4* transgenic lines, analyzed the *prox1a/b* mutants and the *prox1a* reporter line and wrote the manuscript. ZZ, DMAH, GR generated and analyzed new mutant alleles, in part using TALEN constructs generated by JPM. JCF generated the *prox1a* reporter line. HD, EAO, PWI and SSM conceived and supervised the study and edited the manuscript.

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Fig. 1. *prox1a* expression marks different endothelial compartments during vascular development.

In all pictures (except for E), *flt4:mCit* expression is shown in green while *prox1a:KalTA4,UAS:tagRFP* expression is highlighted in red.
(A) In full z-projections, *prox1a* expression at 5dpf is apparent in various tissues, including liver (white arrowhead), lense (arrow) and myotome (blue arrowhead). (A') Same z-projection displaying only the *flt4:mCit* expression in venous and lymphatic ECs.
(B) Partial z-projection of the same embryo (comprising only optical sections without myotome signal) reveals additional expression in the spinal cord (blue arrowhead) and in the lymphatic vasculature of the head (arrow) and the trunk (white arrowhead).

(C, D) Higher magnification of trunk lymphatics at 5 dpf exhibiting combinatorial *prox1a* (red) and *flt4* (green) reporter expression restricted to the TD (arrow) and ISLVs (arrowhead).

(E) Depth color-coded z-projection (projecting each slice in a different color according to its position within the stack; see color bar) of indicated region in B (only *prox1a* channel) reveals the presence of two separate *prox1a*-positive vessels above the swim bladder (arrows point to lymphatics on the right, arrowheads to lymphatics on the left body side), which merge near the 6th ISVs with the TD in the trunk region (yellow arrow).

(F) Dorsal view on the *prox1a*-positive bilateral anterior TD (arrowheads), which connects to the trunk TD at the indicated location (yellow arrow).

(G, H) Full (G) and partial z-projection (H) of the area (compare to box in A) where the anterior TD (arrowhead in G) and the facial lymphatic network (arrow in G) fuse and drain into the common cardinal vein (arrow in H) in a 5 dpf embryo. [JLV-jugular lymphatic vessel, TD-thoracic duct, PCV-posterior cardinal vein, ACV-anterior cardinal vein, CCV-common cardinal vein].
(I, J) At 32 hpf, *prox1a* positive cells (red, arrows) are located within the PCV (green). Note that in this lateral view, *prox1a* positive cells are located in both the dorsal and ventral aspect of the PCV.

(K) A sprouting venous EC (arrow) at 38 hpf expressing both *flt4* and *prox1a* transgenes.

(L+O) At 48 hpf only a proportion of PLs are *prox1a:KalTA4*-positive (white arrow) while the majority shows no signs of reporter expression (yellow arrow). In addition, venous ISVs positive for the *prox1a* reporter are evident (white arrowheads). (L) Overlay of a full z-projection of the *flt4:mCit* signal (green, to outline the complete vasculature) and identical partial z-projections of the *prox1a* (red, see also O) and *flt4* reporter (blue, to highlight the part of the vasculature that is included in the partial z-projection of the *prox1a* signal). Note that *prox1a* positive ECs will appear white in the overlay.

(M, N) The *prox1a* reporter line also labels individual arterial sprouts (arrowheads) at 27 hpf.

(P, Q) Single z-plain of the trunk region in a 25 hpf embryo showing DA cells positive for *prox1a* reporter expression (arrowhead). Note ventral domain expression of the DA.

(R) Intersegmental vein showing both *prox1a* and *flt4* reporter expression at 48 hpf.

(S, T) At 3.5 dpf, all LECs display expression of the *prox1a* reporter while expression in other endothelial domains has disappeared.

(U) Expression of *prox1a* in the head region at 3.5 dpf, highlighting the facial lymphatic network (arrow) including the drainage point with the common cardinal vein (arrowhead).

Fig. 2. Lymphangiogenesis in *prox1a* mutant embryos.

(A) Schematic representation of the homeodomain (HD) containing Prox1a protein, indicating the predicted effect of the 10bp deletion in the *prox1a*^{i278} allele, leading to a frame-shift (red aminoacids) and a truncated protein after 153 aminoacids.

(B, C) Prox1a immunostaining of slow muscle fibres in sibling (B) and homozygous mutant *prox1a*^{i278} embryos (C) demonstrates a complete loss of wild-type Prox1a protein (green) at 30 hpf (slow myosin heavy chain-1 is shown in red).

(D, E) Bright-field pictures of 5 dpf sibling (D) and homozygous *prox1a*^{i278} mutant (E) embryos. Note the strong edema formation around the eye and gut area (arrowheads) which can be even more pronounced in other *prox1a* mutants at this stage.

(F, G) In both, heterozygous siblings (F) and homozygous *prox1a*^{i278} mutants (G), PLs appear at the level of the horizontal myoseptum at 2 dpf (arrows).

(H) Average PL numbers per embryo are mildly reduced in *prox1a* mutants at 2dpf (student's t-test p=0.025). Error-bars indicate standard deviations of wild-type (green), heterozygous (orange) and mutant (red) groups in embryos from a *prox1a*$^{+/-}$ incross.

(I-K) *flt4:mCit*; *flt1*^{enh}*:tdTom* double transgenic embryos highlighting arterial ISVs in red and venous and lymphatic structures in green. Compared to heterozygous siblings (I), most homozygous *prox1a*^{i278} mutants do not display TD defects at 5 dpf (J), while others display a mild reduction (K) in some areas of the trunk (arrows point at TD; asterisks mark the lack of TD). Note the overall unaffected ratio of venous and arterial ISVs in mutants (J, K).

(L) Average number of segments positive for TD cells, scored in the first 10 segments above the yolk extension at 5 dpf. Error-bars indicate the s.d. for the respective genotypic class from a *prox1a*$^{+/-}$ incross. The p-value for the difference between wild-type and mutant population is p=2.3E-08 (student's t-test).

(M) The average percentage of intersegmental veins does not differ between genotypic classes in an incross of *prox1a*^{i278} carriers.

(N) Overview table indicating the average percentage of arteries and veins per embryo, the average total number of ISVs and the average total number of PLs in the progeny of a *prox1a*^{i278} carrier incross.

Fig. 3. Lymphatic specification is not blocked in *prox1a*^{i278};*prox1b*^{SA0035} double mutants.

(A-C) In all genotype combinations of a *prox1a*^{i278};*prox1b*^{SA0035} double heterozygous incross, including double heterozygotes (A), homozygous *prox1b* mutants (B) as well as homozygous double mutants (C), PLs are apparent at the horizontal myoseptum at 2 dpf (arrows).

(D-F) As in double heterozygous embryos (D), also the loss of both copies of *prox1b* (E) does not lead to lymphatic defects on the level of TD formation. In *prox1a*^{i278};*prox1b*^{SA0035} double homozygous embryos (F) mild TD defects are occasionally visible (arrows indicate the TD while asterisks highlight segments without TD).

(G) Average number of TD-positive segments within the first 10 segments above the yolk extension for the indicated genotypic classes of a *prox1a*$^{+/-}$;*prox1b*$^{+/-}$ incross. The moderate reduction in TD length is statistically significant in the *prox1a* single mutants (student's t-test p=0,01) and the *prox1a;prox1b* double mutant embryos (p.=0.002) when compared to wild-type siblings.

(H) Overview about the average TD length (TD) in 10 scored segments, the corresponding standard deviations and the number of scored embryos (n) in a *prox1a*$^{+/-}$;*prox1b*$^{+/-}$ incross.

Fig. 4. Specification of the lymphatic lineage is independent of *coup-TFII* and *sox18*.

(A) Schematic representation of the Coup-TFII protein indicating the position of the first affected amino acid in the 1bp insertion allele *nr2f2*^{hu10330} (*coupTFII* ^{hu10330} hereafter) which leads to a premature stop codon after additional 4 amino acids N-terminal to the nuclear receptor- DNA binding and ligand binding domain.

(B, C) Bright-field images of wild-type (B) and homozygous mutant *coup-TFII*^{hu10330} (C) embryos, with no signs of edema or morphological abnormalities at 6 dpf.

(D, E) *flt4:mCit;flt1*^{enh}*:tdTom* positive embryos from a *coup-TFII*^{hu10330} incross. Compared to heterozygous siblings (D), *coup-TFII* mutants (E) show only marginal TD defects (asterisk) in a small proportion of embryos at 5 dpf. Note the overall normal vascular morphology with proper PCV, DA and ISVs present in homozygous mutants.

(F) Overview about the average TD length in the first ten segments, the proportion of venous ISVs and the respective standard deviations and the corresponding number of scored embryos from *coup-TFII*^{hu10330} incrosses.

(G) The average length of the TD in the first 10 segments above the yolk extension is not significantly affected in *coup-TFII*^{hu10330} mutants.

(H) The proportion of venous ISVs is unaltered in homozygous *coup-TFII*^{hu10330} mutants at 5 dpf.

(I) Schematic overview of the Sox18 protein, indicating the first affected amino acid in the 1bp insertion allele *sox18*^{hu10320} preceding the HMG-box. The resulting frame-shift leads to a premature stop codon after additional 50 amino acids.

(J, K) Bright-field pictures of heterozygous (J) and homozygous mutant *sox18*^{hu10320} embryos at 5 dpf. Note the overall wild-type appearance of mutant embryos (K).

(L, M) *sox18*^{hu10320} heterozygous (L) and homozygous mutant (M) embryos expressing *fli1a:eGFP* in all ECs. Even in a *sox18* loss-of-function situation, embryos do not show defects in the formation of the TD (arrowheads).

(N) Overview table summarizing the average TD length in the first 10 segments, the average total number of PLs per embryo and the respective standard deviations and number (n) of scored embryos from a *sox18*^{hu10320} incross.

(O) The average length of the TD in wild-type (green), heterozygous (yellow) and homozygous mutant (red) *sox18*^{hu10320} embryos does not differ significantly.

(P) The average number of PLs present in *sox18*^{hu10320} mutants is not significantly affected at 54 hpf.

Fig. 5. Forced expression of *prox1a* does not commit venous ECs to a lymphatic phenotype.

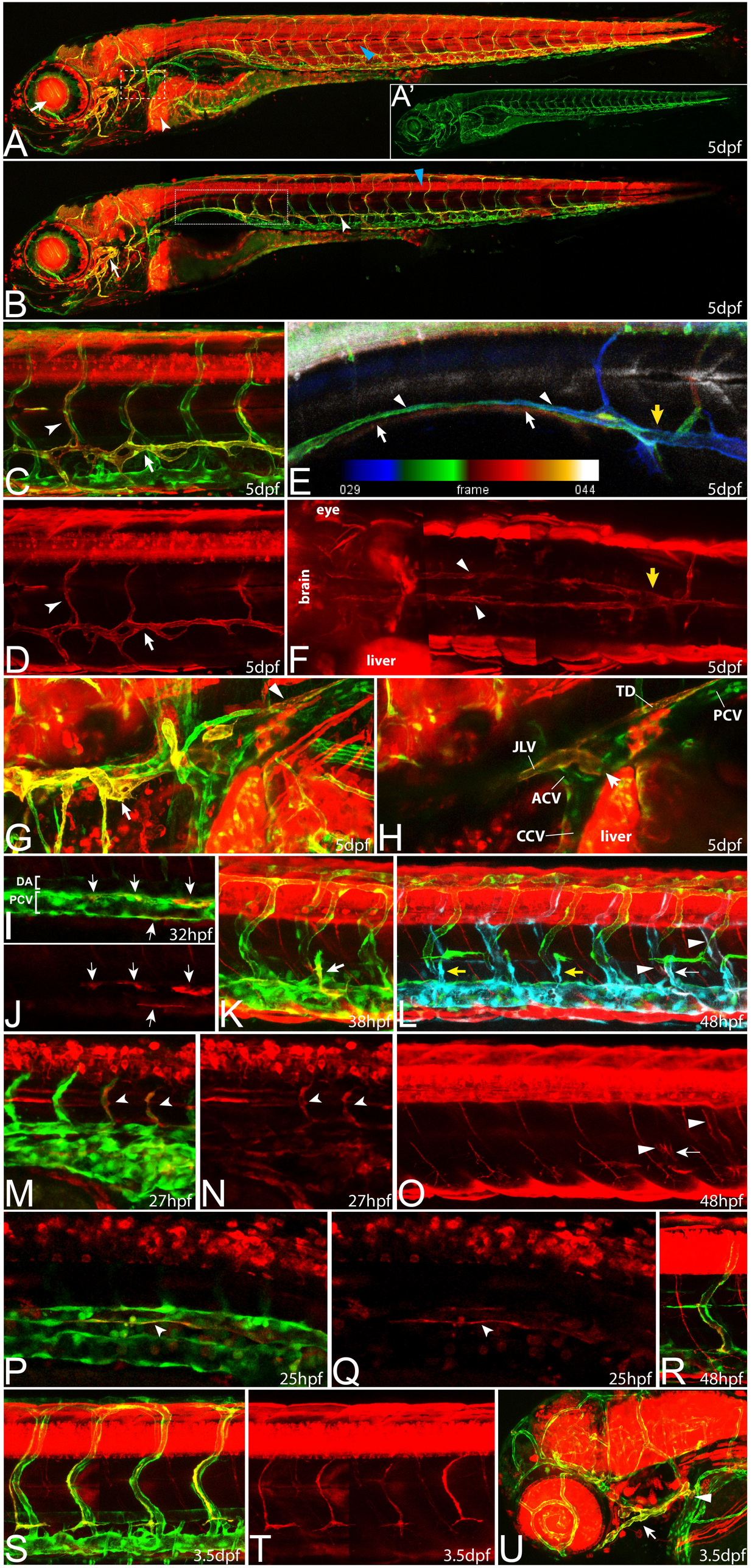
(A) Schematic representation of the *prox1a* overexpression construct and the *flt4:Gal4FF* line which drives Gal4FF expression under the control of a Medaka 3.8kb *flt4* promoter fragment.

(B-E) Whole mount *in-situ* hybridization against *prox1a* in uninjected *flt4:Gal4FF* siblings (B, C) and embryos injected with the *prox1a* mis-expression construct at 32 hpf (D, E). Note the domain of forced *prox1a* expression within the axial vessels in injected embryos (arrows in E), which does not reflect endogenous *prox1a* expression (C). Arrowheads in B, D highlight endogenous *prox1a* expression in the lateral line primordium, the arrowhead in C points at a signal in the corpuscles of Stannius.

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

Fig. 6. Chronology of angiogenic and lymphangiogenic events in mice and zebrafish.

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


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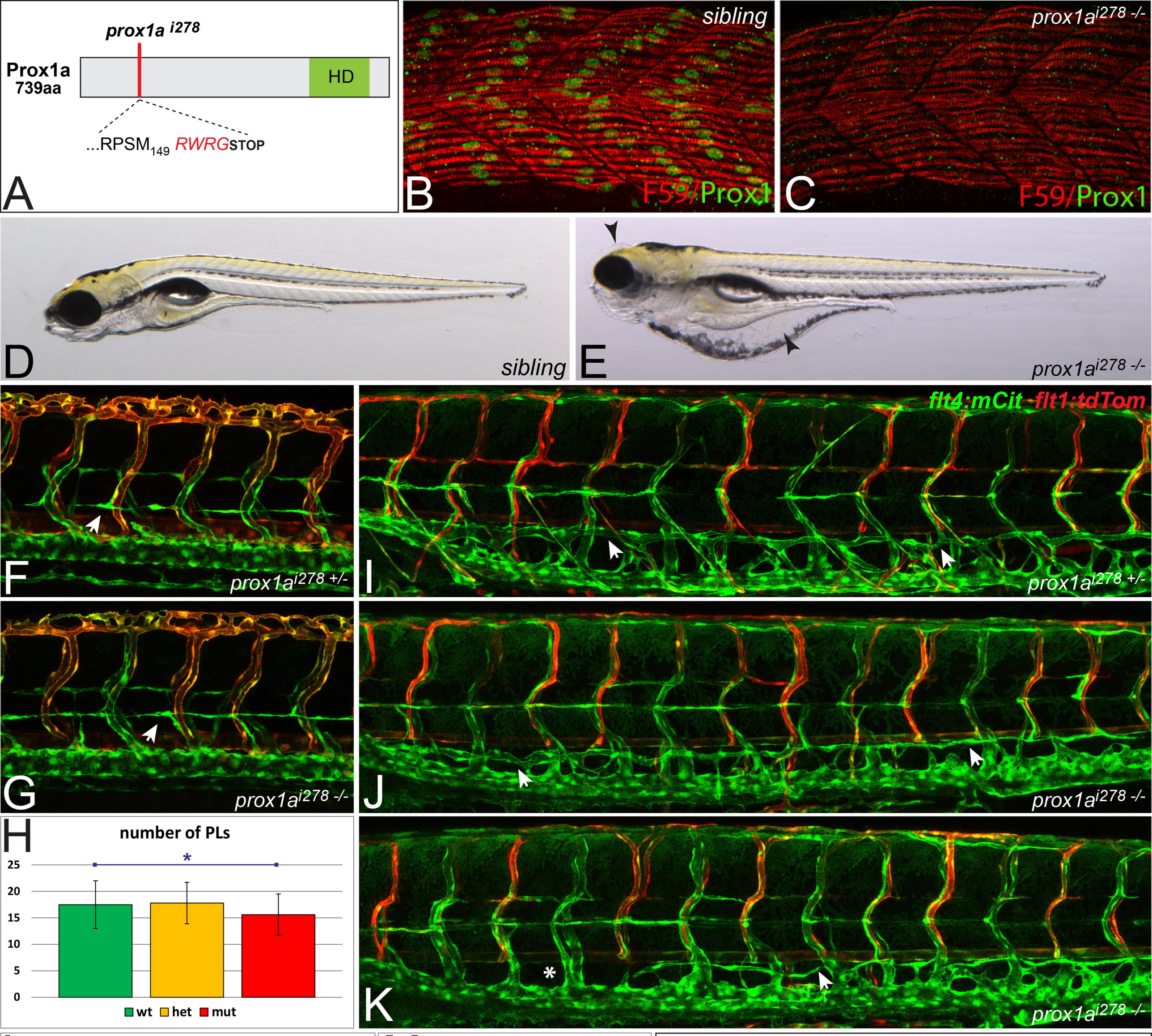
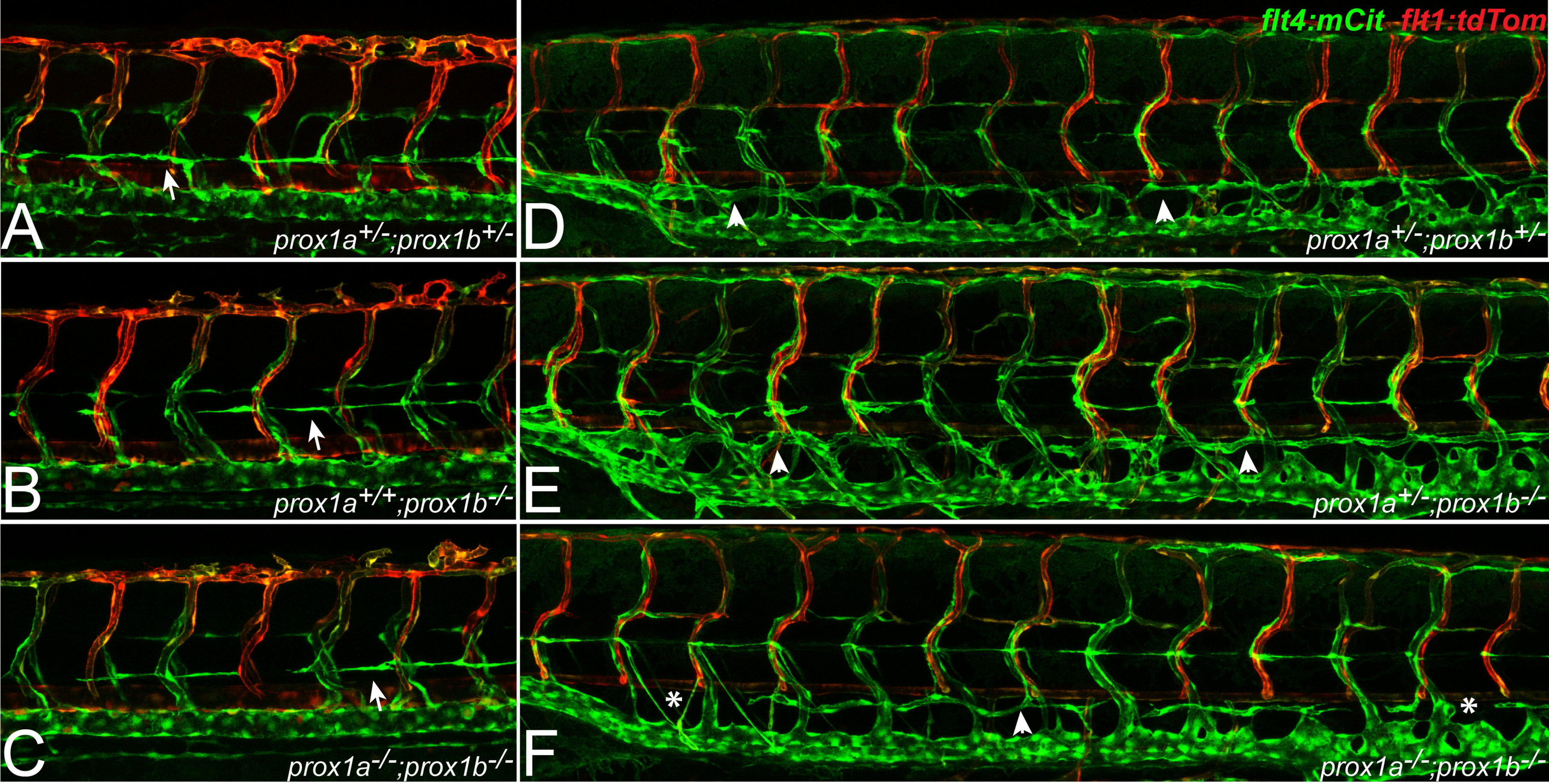
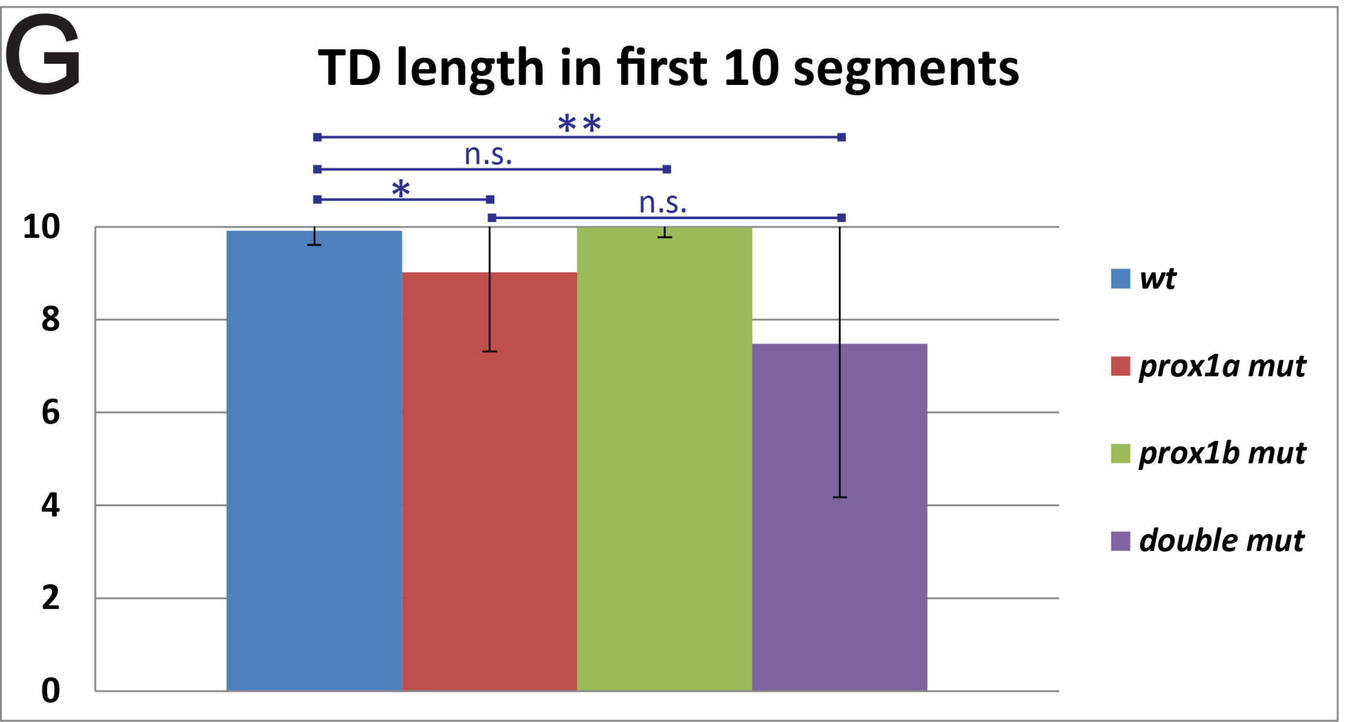
U
3.5dpf



	% arteries	% veins	total ISVs	total PLs	TD+
wt	50.22	49.78	46.4	17.5	9.9
het	50.89	49.11	46.2	17.8	9.9
mut	50.95	49.22	46.1	**15.6**	**8.6**
n			79	219	347

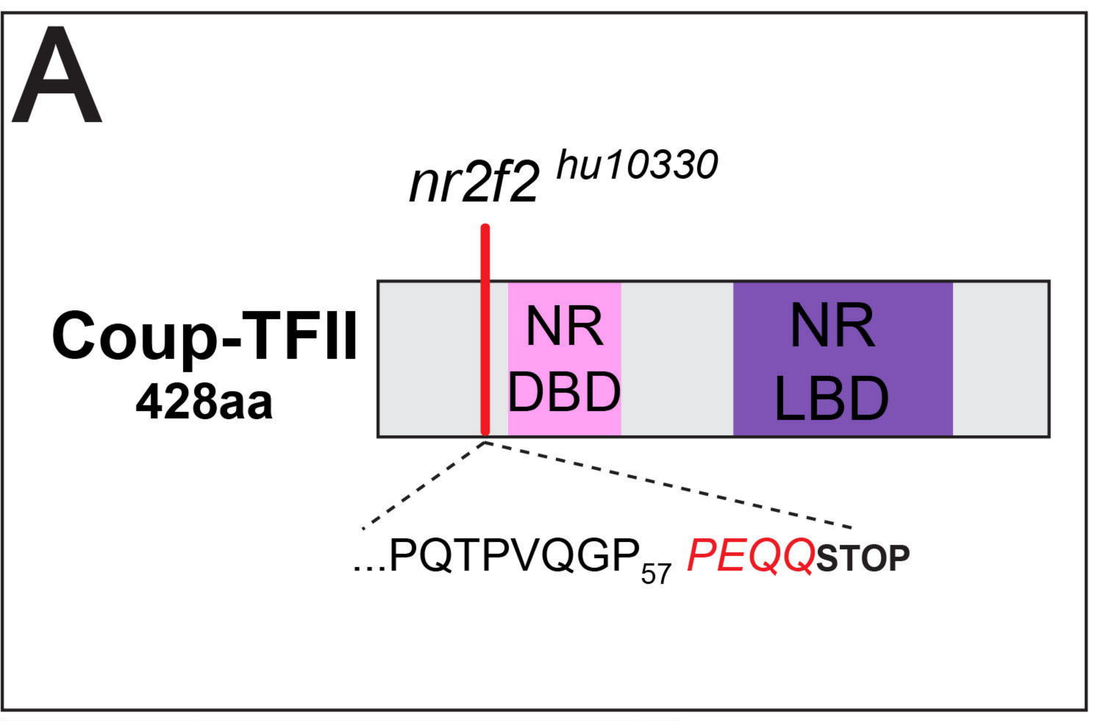
N

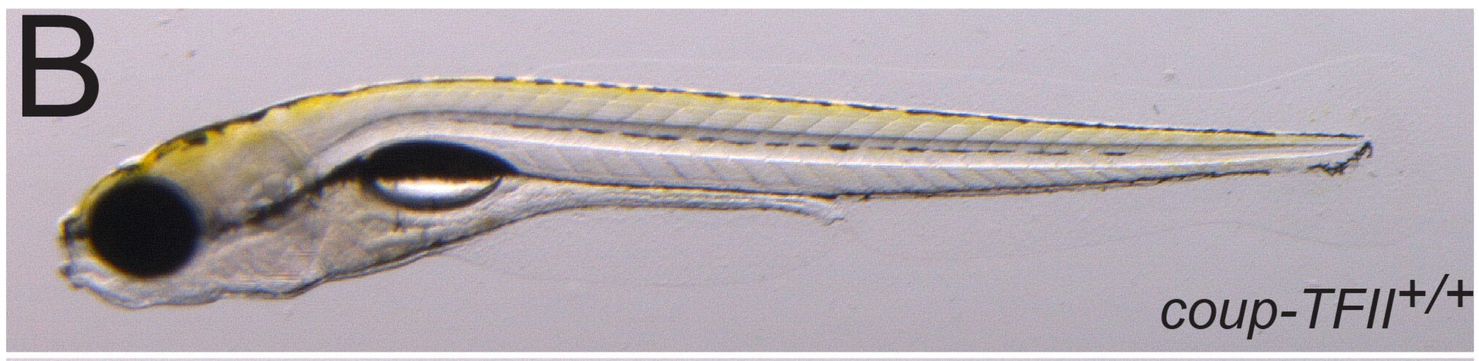


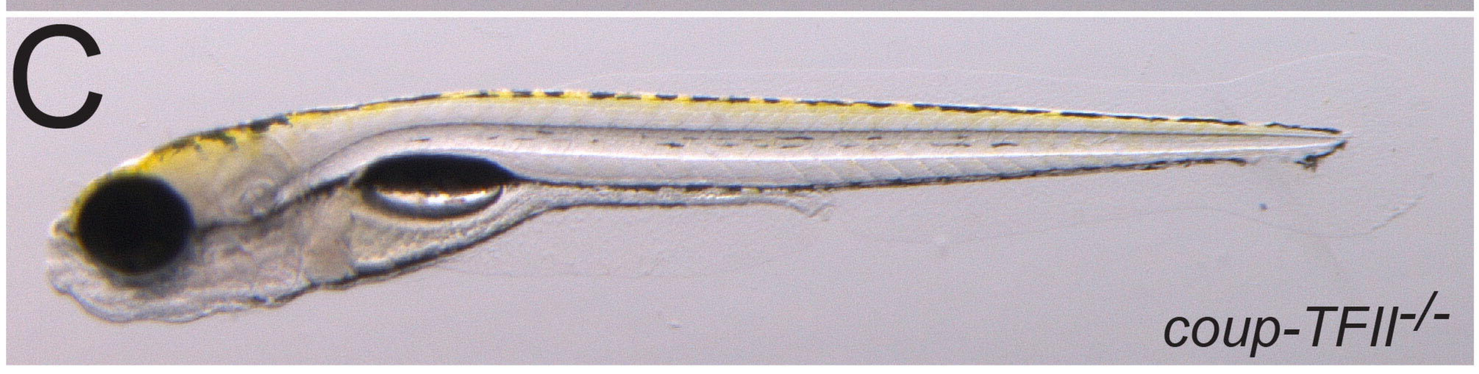


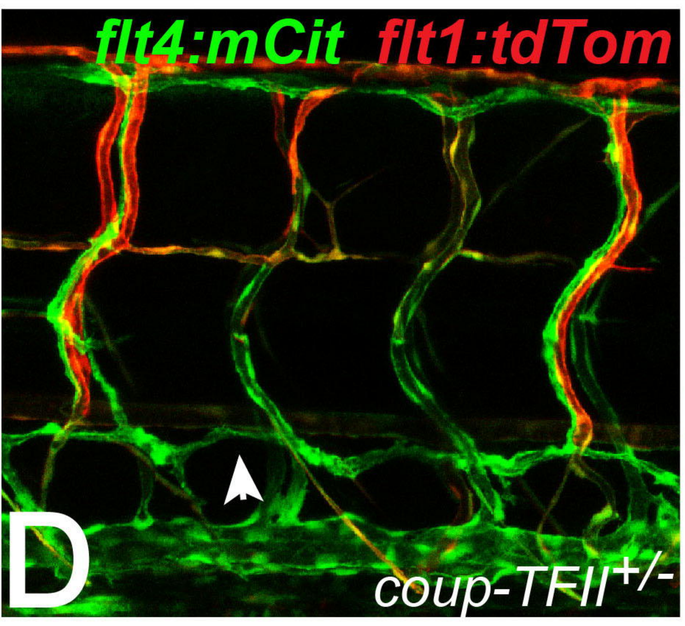
H

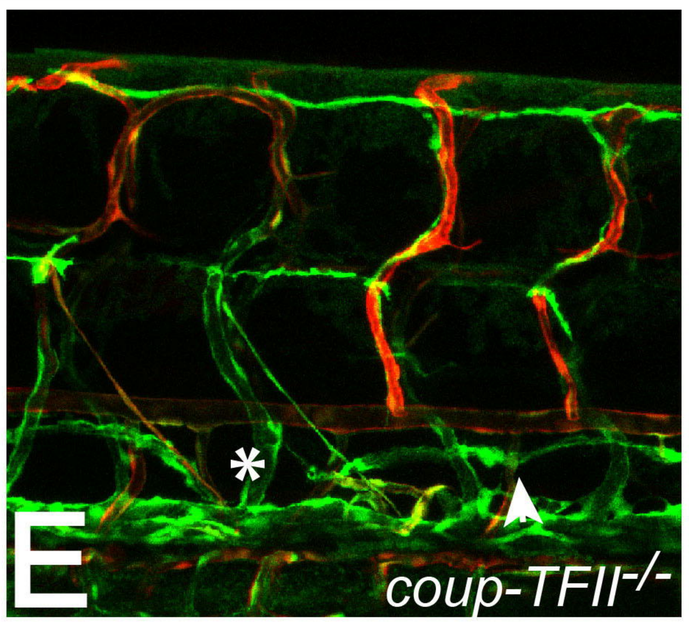
prox1a	*prox1b*	TD	s.d.	n
wt	*wt*	9.9	0.3	34
wt	*het*	10.0	0.3	60
wt	*mut*	10.0	0.2	41
het	*wt*	9.8	0.7	75
het	*het*	9.7	1.3	129
het	*mut*	9.7	1.3	56
mut	*wt*	9.0	1.7	27
mut	*het*	8.5	2.4	54
mut	*mut*	7.5	3.3	22





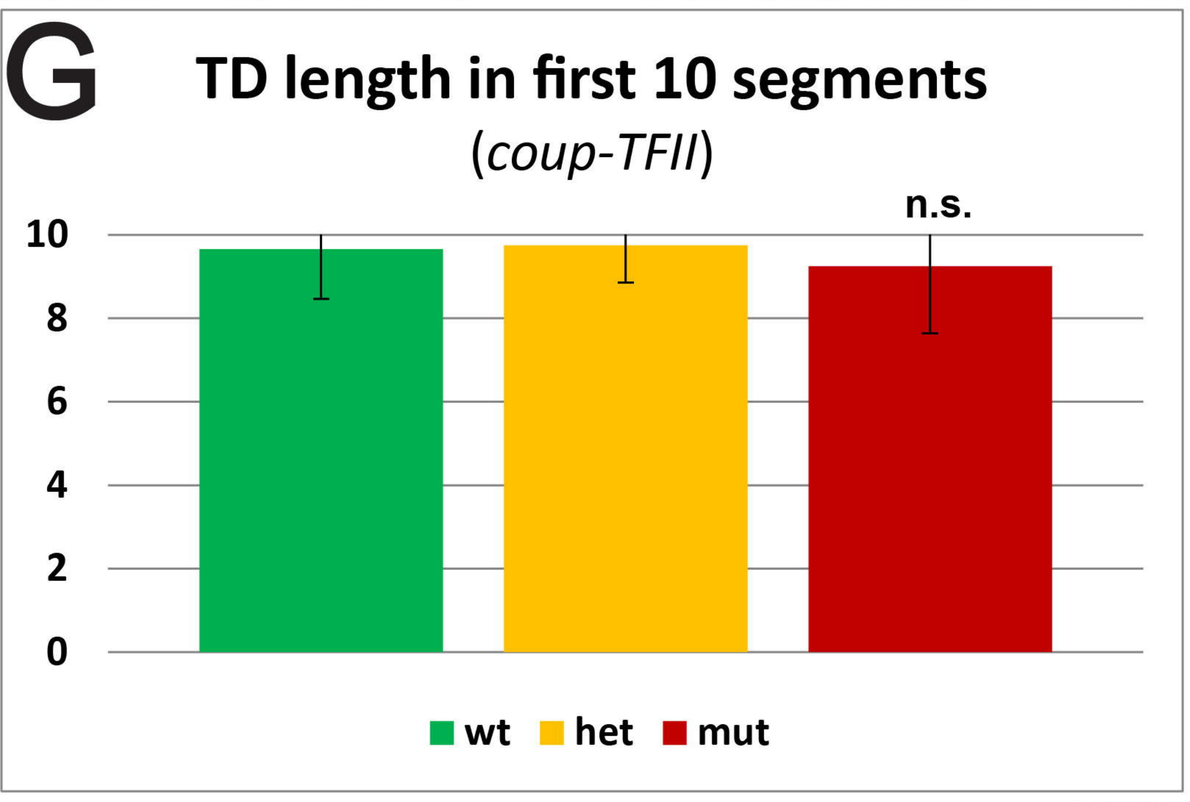


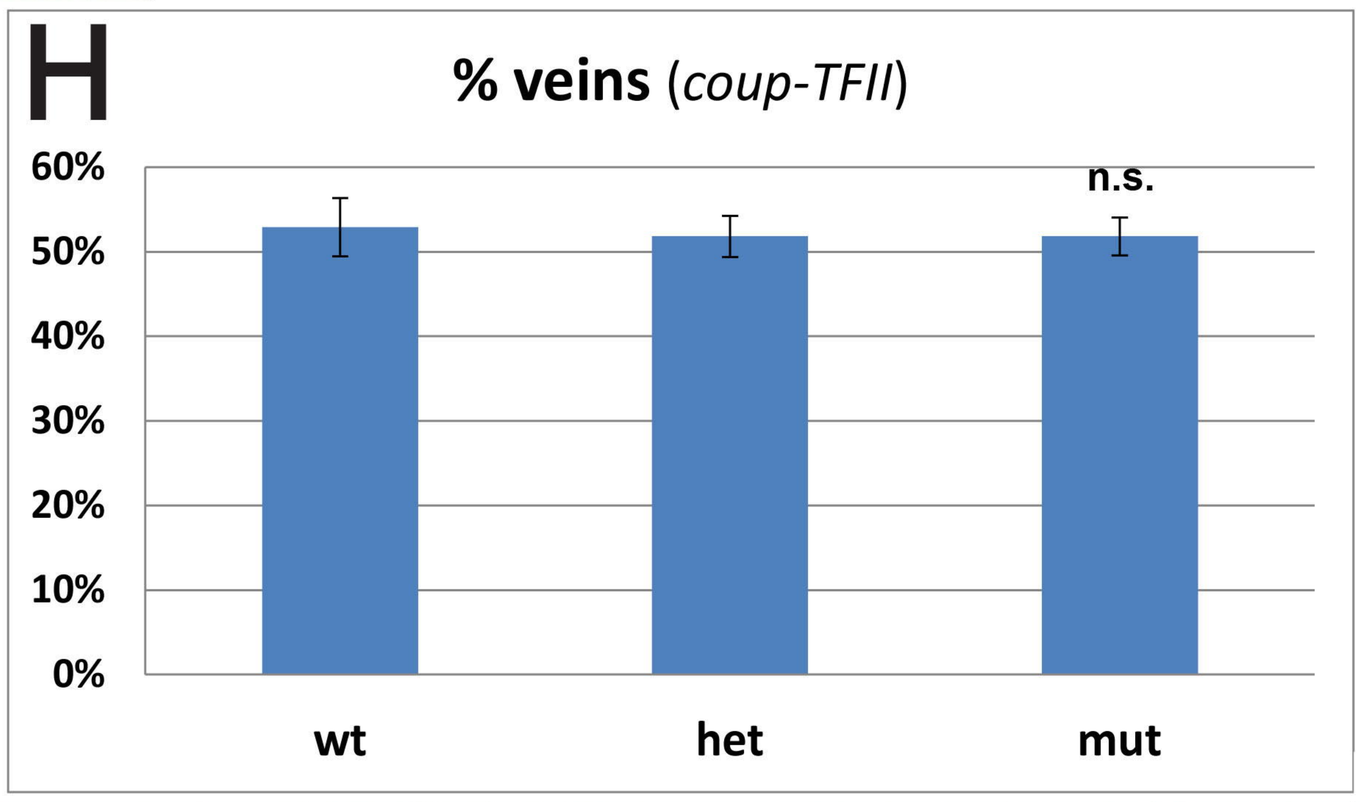


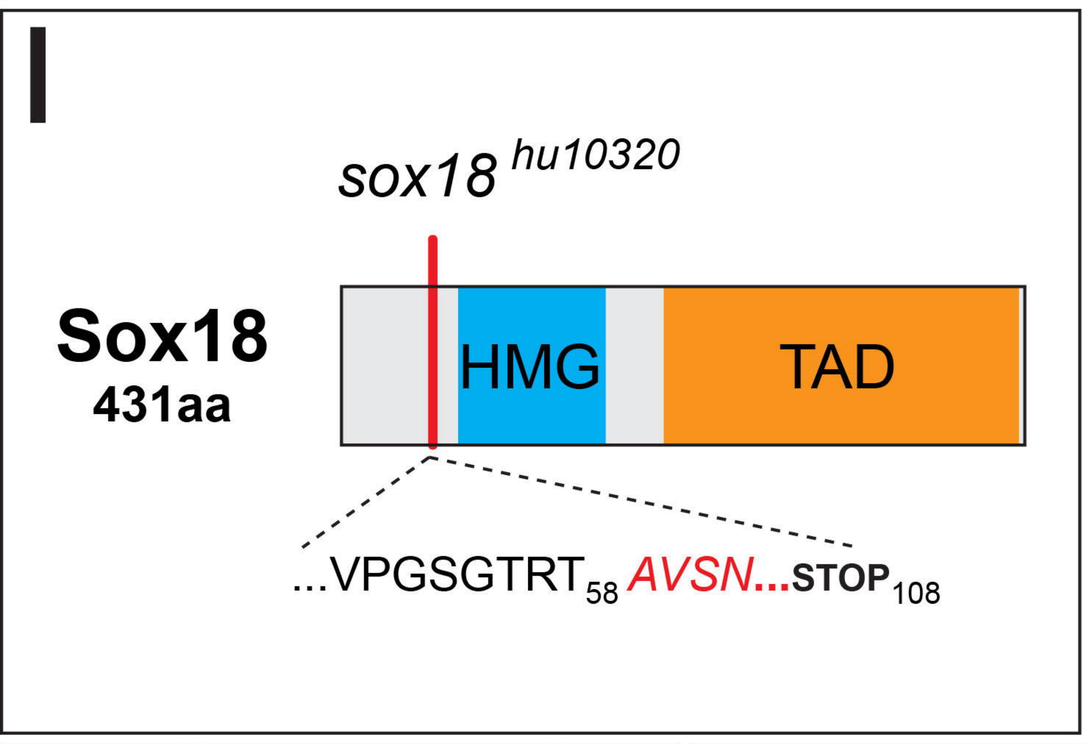


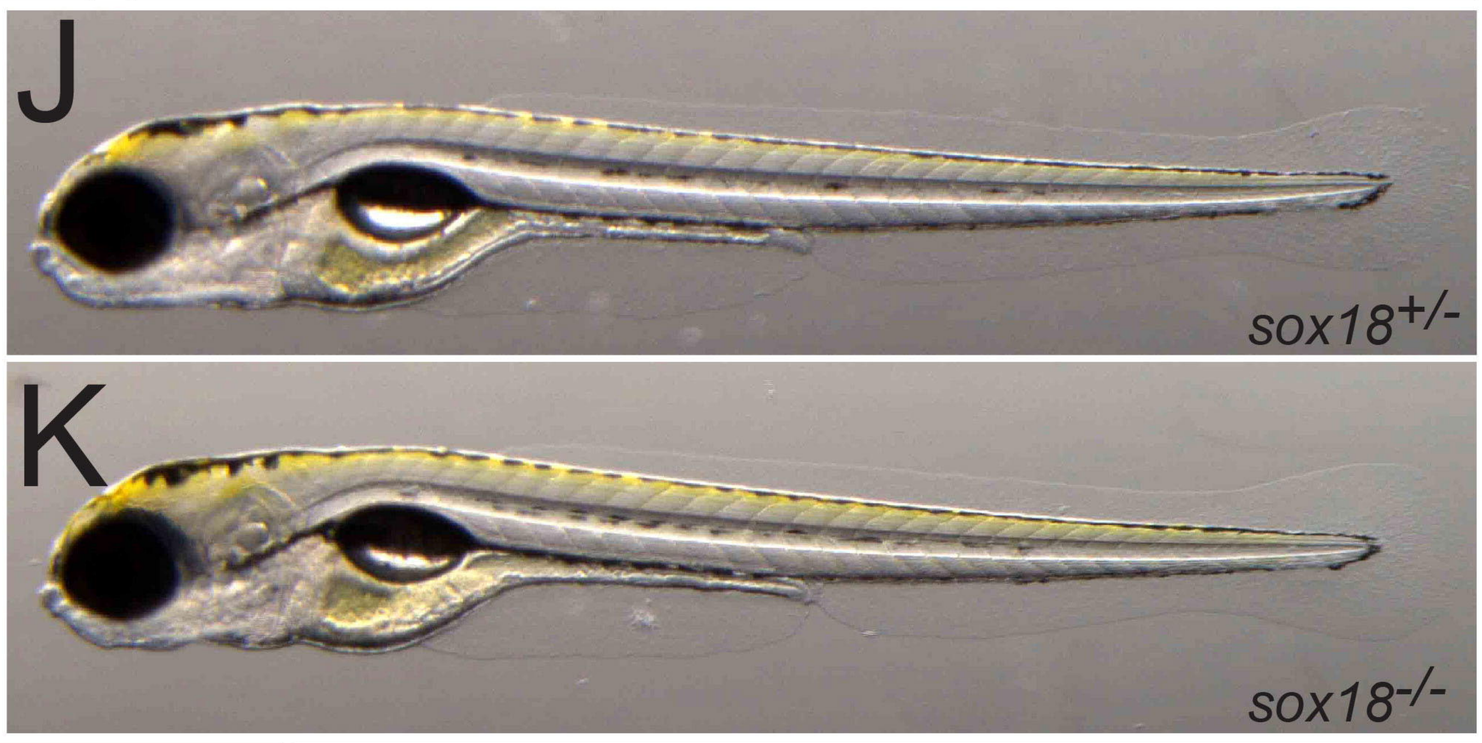
F

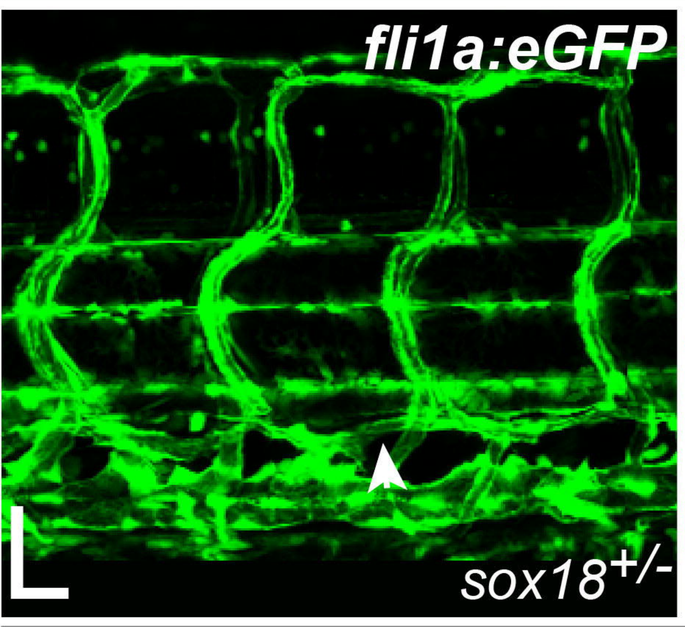
	TD+	s.d.	% veins	s.d.
wt	9.7	1.2	52.9%	3.4%
het	9.8	0.9	51.8%	2.4%
mut	9.2	1.6	51.8%	2.2%
n		401		54

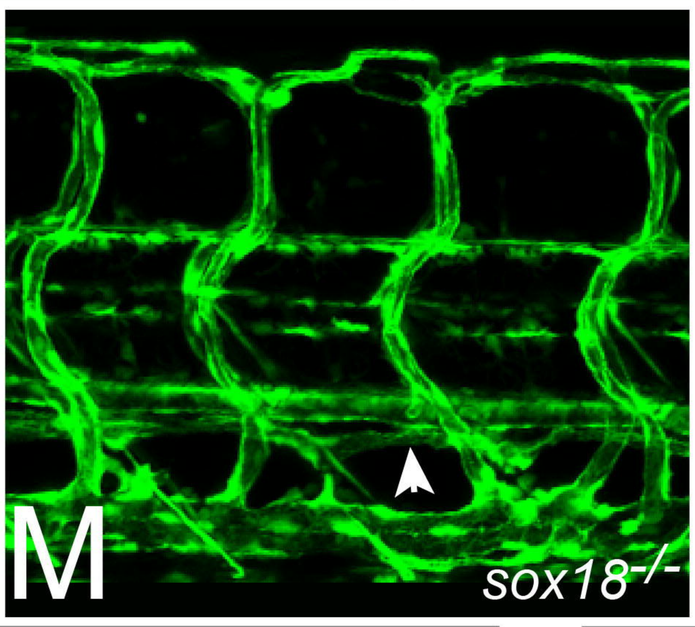






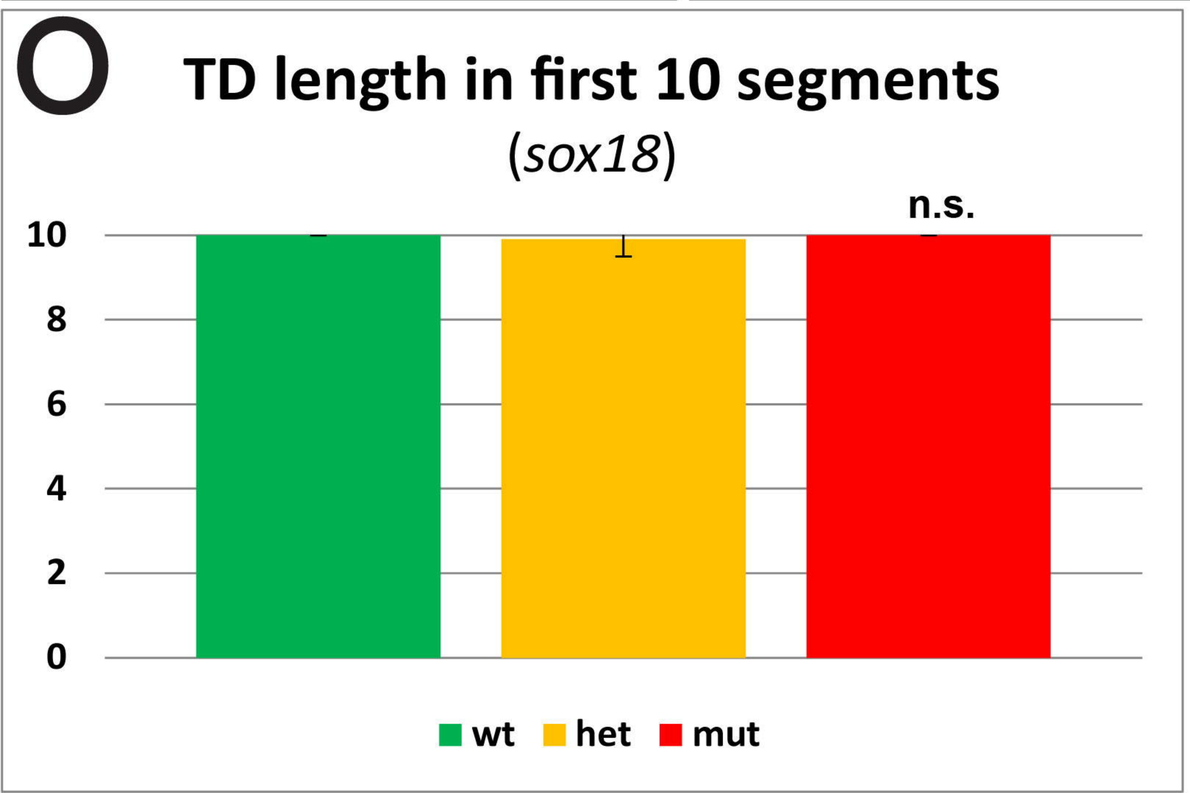


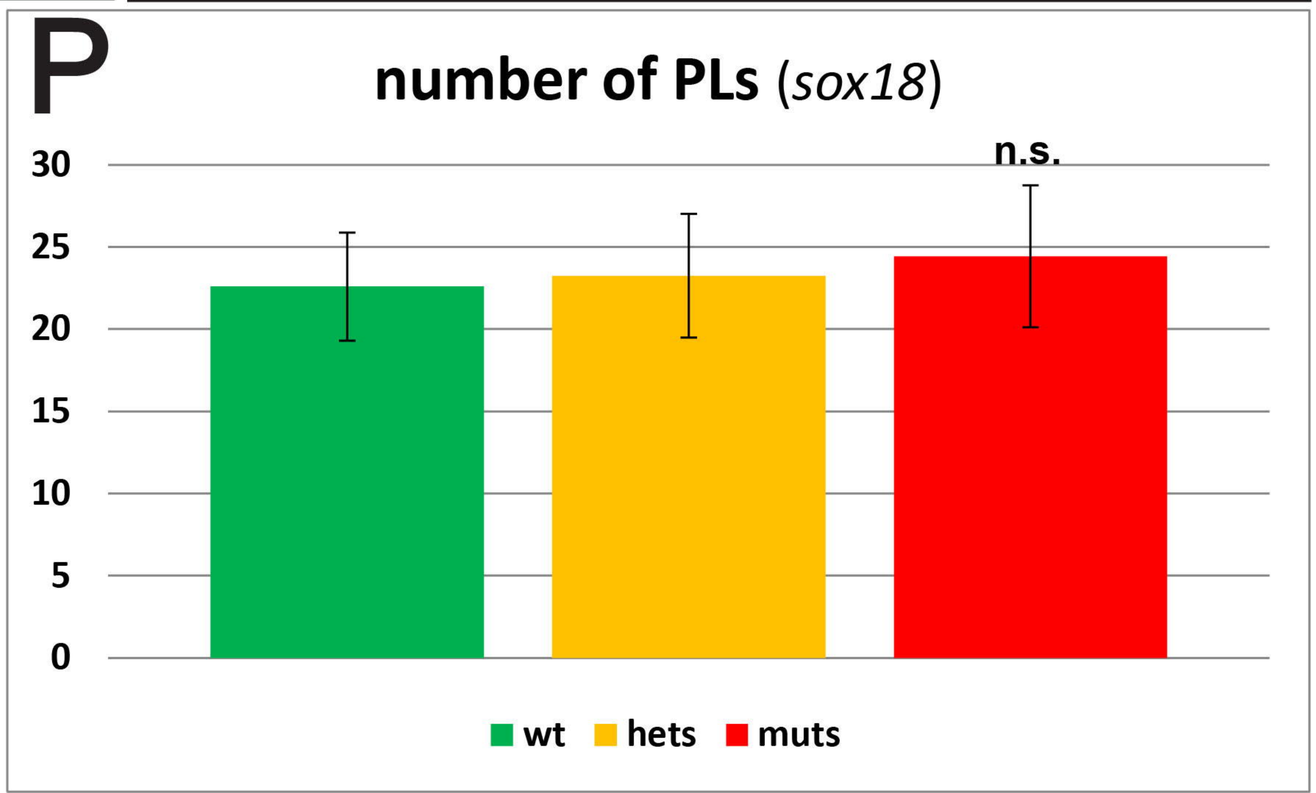


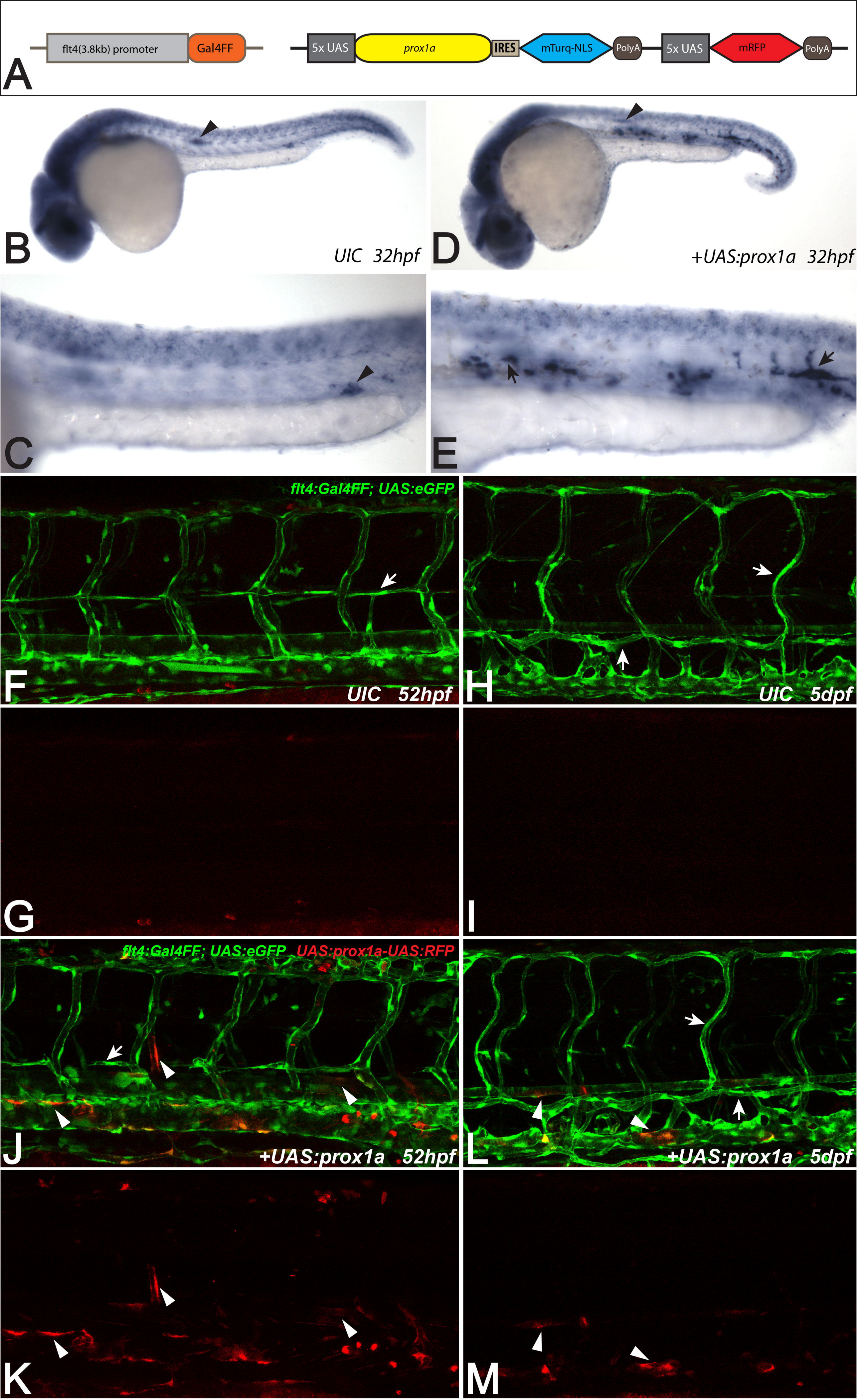


	TD+	s.d.	total PLs	s.d.
wt	10.0	0.0	22.6	3.3
het	9.9	0.4	23.2	3.8
mut	10.0	0.0	24.4	4.3
n		203		120

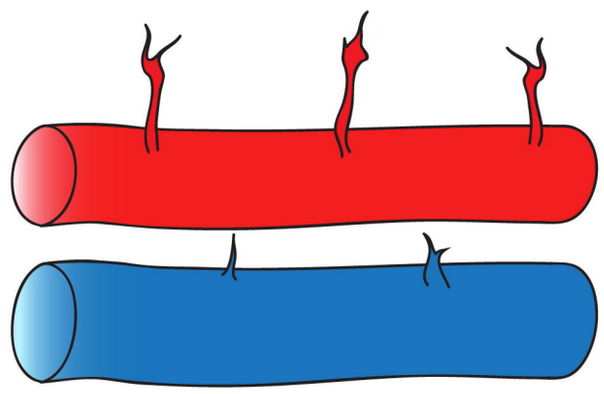
N





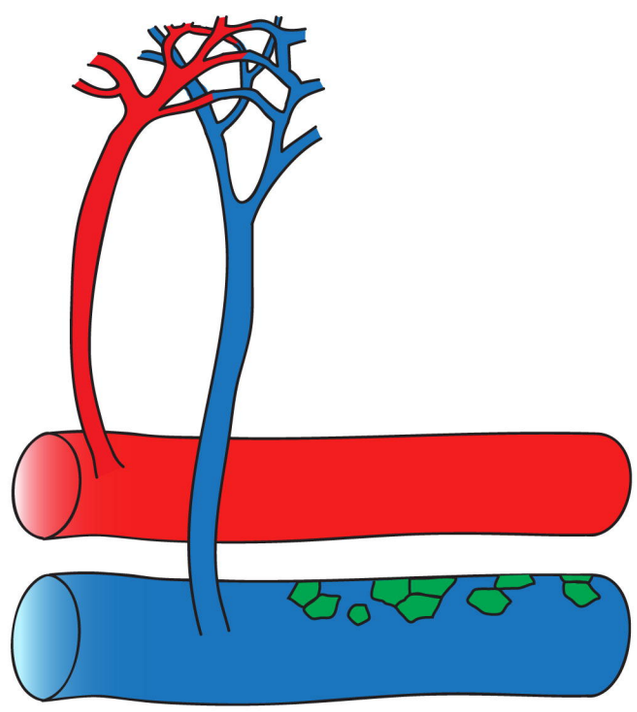

A
flt4(3.8kb) promoter
Gal4FF
5x UAS
prox1a
IRES
mTurq-NLS
PolyA
5x UAS
mRFP
PolyA
B
UIC 32hpf
D
+UAS:prox1a 32hpf
C
E
flt4:Gal4FF; UAS:eGFP
F
UIC 52hpf
H
UIC 5dpf
G
I
flt4:Gal4FF; UAS:eGFP UAS:prox1a-UAS:RFP
J
+UAS:prox1a 52hpf
L
+UAS:prox1a 5dpf
K
M

A **mouse**



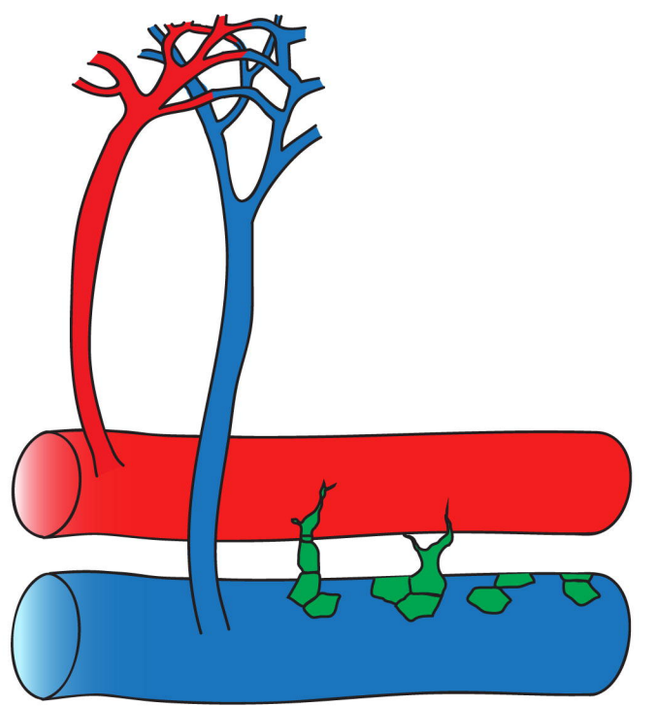
arterial and venous angiogenesis

1



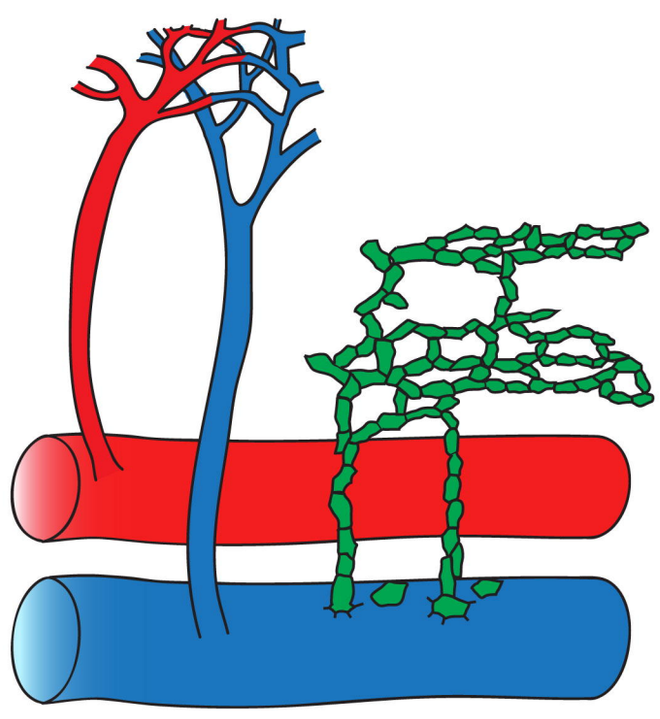
lymphatic specification

2



lymphatic sprouting

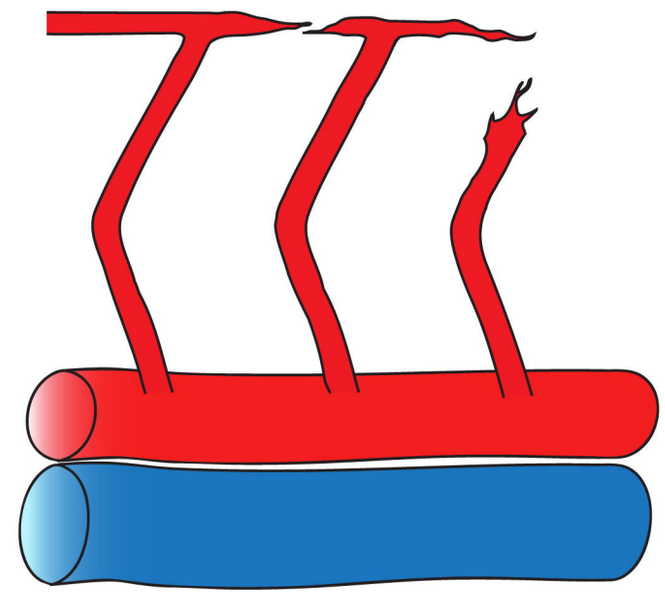
3



formation of lymphatic vasculature

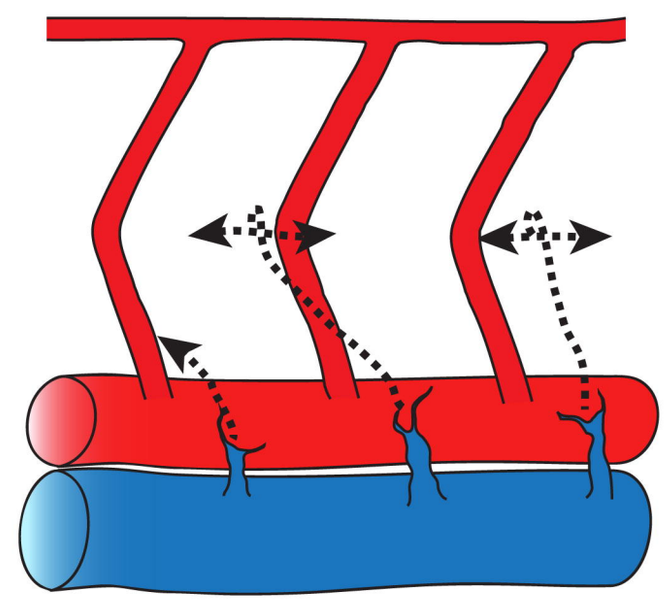
4

zebrafish **B**



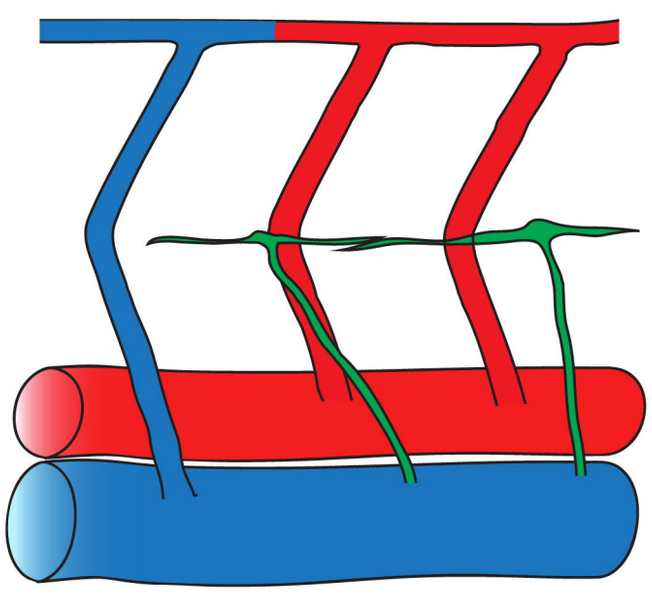
arterial sprouting

1



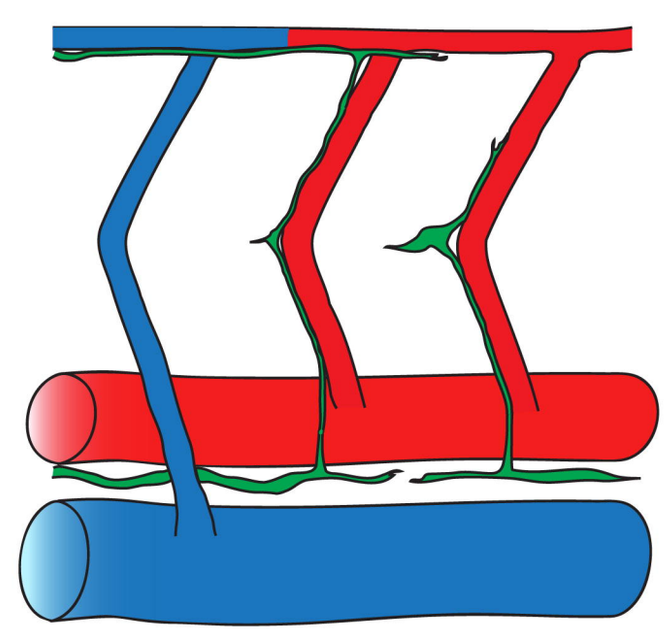
lymphovenous sprouting

2



formation of PLs and vISVs

3



formation of lymphatic vasculature

4