Materials and Methods

Zebrafish lines and maintenance
Zebrafish were raised and maintained according to established techniques. The following strains were used: AB (from the Wilson lab, UCL, London, UK), tg(fli1a:EGFP)\textsuperscript{y1} (from the Lawson lab, University of Massachusetts Medical School, MA), tg(fli1\textsuperscript{enh}:RFP) line.

MO/RNA microinjections
Antisense morpholinos (MOs; Gene Tools, Philomath, OR) used in this study were already described: sox18-MO1, sox18-MO2, sox7-MO1, sox7-MO2, \textsuperscript{4} vegfc-MO, \textsuperscript{5} flt4-MO. MOs, diluted in Danieau buffer, were injected at 1- to 2-cell stage. Escalating doses of each MO were tested for phenotypic effects; as control for unspecific effects, each experiment was performed in parallel with a std-MO (standard control oligo) with no target in zebrafish embryos. We usually injected 1 pmol/embryo of sox18-MO2, 0.5 pmol/embryo of vegfc-MO, and 0.8 pmol/embryo of sox18-MO1, sox7-MO1 and sox7-MO2. For combined knockdown experiments, we injected sox18-MO2 and vegfc-MO at 0.5 and 0.06 pmol/embryo or 0.25 and 0.03 pmol/embryo, respectively; sox18-MO2 and flt4-MO at 0.5 and 0.06 pmol/embryo, respectively. For sox18 RNA, a SmaI-XbaI fragment from pBSKS\textsuperscript{+}\textsuperscript{sox18}, containing zebrafish sox18 cDNA, was subcloned into pCS2\textsuperscript{+}; the resulting plasmid was digested with NotI and transcribed with Sp6 RNA Polymerase (Roche). Rescue experiments were performed with the coinjection, into 1-cell stage embryos, of 1 pmol/embryo sox18-MO2 and 25 or 50pg/embryo sox18 RNA diluted in Danieau buffer. For vegfc RNA injections, plasmid pCS2+vegfc was digested with NotI and transcribed with Sp6 RNA polymerase. As an internal control, we wanted to check if vegfc RNA could rescue the severe lymphatic phenotype in vegfc morphants. Given the full complementarity of vegfc-MO to vegfc RNA, all injections with vegfc RNA were carried out using two independent needles: the same 1-cell stage embryos were injected first with 1 nl of vegfc RNA (50 pg) and then with 1 nl of vegfc-MO or sox18-MO2 (0.5 or 1 pmol, respectively).
Specificity of sox18/sox7-MOs

All sox18/sox7-MOs used in this study had been previously described⁴. Specificity of the splicing morpholinos (sox18-MO2, sox7-MO2) had been addressed by checking through RT-PCR that each splicing morpholino was altering processing of its target pre-mRNA, without affecting the splicing of the other sox transcripts (as shown in Figure S4 of the above mentioned paper). The efficacy of the translation blocking MOs (targeting the AUG region or the 5’UTR) could not be directly tested due to lack of specific antibodies. However, we could show that three independent sets of MOs (sox18-MO1+sox7-MO1, sox18-MO2+sox7-MO2, sox18-MO4+sox7-MO4) when coinjected at low doses, but not when injected separately, were producing the same circulatory phenotype in the trunk region. Taken together, these observations strongly support the notion that all sox18/sox7-MOs presented in Cermenati et al., 2008⁴ are specific for their targets.

The sequences of the sox18/sox7-MOs used in this study are reported below. For splicing MOs, sequences complementary to the intron sequence of the pre-mRNA target are shown in lowercase letters:

\[
\begin{align*}
\text{sox18-MO1} & \quad 5’-\text{TATTCATTCAGCAAGACCAACAG-3’}, \\
\text{sox18-MO2} & \quad 5’-\text{gtgagtgctttacCCAGCATTTTAC-3’}, \\
\text{sox7-MO1} & \quad 5’-\text{ACGCACTTATCAAGCCGCATGTG-3’}, \\
\text{sox7-MO2} & \quad 5’-\text{gttaaatctttacCAAGCATCTTGC-3’}.
\end{align*}
\]

Phenotypic analysis

The analysis of TD formation is commonly used to study lymphatic development in zebrafish. We analyzed TD formation by scoring its length along 10 consecutive trunk segments, up to the anus, in \text{tg(fli1a:EGFP)y1} larvae at 5 dpf, as previously described⁵,⁹. Due to the variability of the lymphatic phenotype, the analyzed larvae were distributed into five phenotypic classes of increasing severity: fully formed TD (normal TD), TD present in 7-9 segments (70-90% TD), 4-6 segments (40-60% TD), 1-3 segments (10-30% TD) and absent TD. At 1.5 to 2.5 dpf, we scored 10 segments in the same region also to analyze the number of sprouts from the vein, PLs and a/v ISVs. All larvae analyzed in this study were circulating to avoid secondary defects that would interfere with TD phenotypes.
Production of tg(hsp70l:Sox18RaOp mCherry) transgenic line
The inducible construct consisting of an in frame fusion of the Sox18RaOp cDNA with mCherry, under the control of the hsp70l promoter, was generated with Gateway system (see below for detailed description). The construct is sketched in Figure 3A. The pDestTol2CG-Ragged plasmid was injected with Tol2 transposase RNA (25ng/µl) to generate tg(hsp70l:Sox18RaOp mCherry) lines. Founders, crossed with tg(fli1a:EGFP)y1 or tg(fli1a:EGFP)y1;tg(fli1enh::RFP), were heat shocked at 37-38°C for 1 hour and embryos sorted for mCherry expression 3-4 h post-heat shock to confirm induction of construct expression. PLs and a/vISVs were counted at 54-56 hpf and the presence of TD was scored at 5 dpf.

Construct for tg(hsp70l:Sox18RaOp mCherry) transgenic line
The RaOp mutation was introduced into a mouse Sox18 cDNA using site-directed mutagenesis (Quickchange Lightning Site-Directed Mutagenesis Kit, Stratagene) with the primers Sox18ragmut f (5’-GAGCCTGGCGAGGCTCCTTCTTCCCA-3’) and Sox18ragmut r (5’-TGGAAGAAGGAGCCTCGCCAGGCTC-3’). This cDNA sequence was amplified using the primers attB1kozacsox18F (5’-GGGGACAAGTTTGTACAAAAAAGCAGGCTGACCATGCAGAGATCGCCGCCCGGC-3’) and sox18ragged-nostop-attB2R (5’-GGGGACCACTTTGTACAAGAAAGCAGGCTGACCATGCAGAGATCGCCGCCCGGC-3’) to remove the stop codon and clone into a Gateway entry vector. The amplified insert was cloned into DONR211 donor using Gateway technology to generate a pME-Sox18RaOp middle entry vector.

p5E-hsp70l 5’entry clone, p3E mCherry-pA 3’ entry clone, pME-Ragged middle entry vector and pDestTol2CG were combined to generate pDestTol2CG-Ragged where Sox18RaOp is fused with mCherry and under the control of the hsp70l promoter.

In situ hybridization and imaging
Whole-mount in situ hybridizations (ISHs) were carried out essentially as described. For ISHs on AB embryos, we synthesized probes as described in the following papers: sox18 and sox7, cdh5 and vegfc. ephB4 was kindly provided by R. Patient.
For ISHs on \( fli1\alpha:EGFP \)\(^\ddagger\) embryos, to avoid background problems as reported on the dedicated web page at zfin.org and recently published \(^{13}\), we generated probes using the following primers and templates:

<table>
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<tr>
<th>probe</th>
<th>primer sequence</th>
<th>template</th>
<th>Ref</th>
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| sox18 | 5′-GGAGCCAGGAGTTACAAACAC-3′  
5′-CTAATACGACTCTATAGGGTGTCCATATGTCACCAGACTTC-3′ | Image clone 6790334 | 4 |
| sox7 | 5′-CCGCGTTGATAAGATGACG-3′  
5′-CTAATACGACTCTATAGGGTGTCCATATGTCACCAGCTC-3′ | Image clone 7045912 | 4 |
| flt4 | 5′-CAAGTGACCCAGTGATGATA-3′  
5′-CTAATACGACTCTATAGGGTGTCCATATGTCACCAGGTGAG-3′ | pBSflt4 | 14 |
| dab2 | 5′-GCTCTGCTGACTGCCTCCT-3′  
5′-CTAATACGACTCTATAGGGTGTCCATATGTCACCAGCTC-3′ | pBK-CMVdab2 | 15 |
| lyve1 | 5′-AAGGTTTGGTGGCATGTTCC-3′  
5′-CTAATACGACTCTATAGGGTGTCCATATGTCACCAGCTC-3′ | Image clone 679881 | 16 |

Images were taken with a Leica MZFLIII epifluorescence stereomicroscope equipped with a DFC 480-R2 digital camera and the LAS imaging software (Leica, Wetzlar, Germany). Confocal microscopy was performed on a Leica TCS SP2 AOBS microscope, equipped with an argon laser, or a Zeiss 510 microscope. Images were processed using the Adobe Photoshop software (Adobe, San Jose, CA) or Imaris software packages.

**Histological sections**

For histological analysis after ISH, embryos were re-fixed in 4% PFA, dehydrated, wax embedded, sectioned (8 µm) with a microtome (Leitz 1516) and stained with eosin. Images were taken with a Leica microscope equipped with a Leica 480 digital camera and the LAS software (Leica, Germany).

**Statistical analysis**

Statistical analyses were performed with Student’s \( t \)-test or one-way ANOVA followed by Dunnett’s Multiple Comparison post-test, when needed, using GraphPad PRISM version 5.0 (GraphPad, San Diego, CA). In the graphs, * and ** mark statistically significant data with a p value <0.05 and <0.01, respectively. Statistically highly significant data, with a p value <0.001, are marked by ***.
Quantitative RT-PCR analysis

Quantitative Real Time reverse-transcriptase (qRT) polymerase chain reaction (PCR) analysis was performed on RNA extracted at 32.5 hpf from pools of around 30 Cherry+ embryos of the tg(hsp70l;Sox18RaOp mCherry) line heat-shocked at 29 hpf or the same number of Cherry- non heat-shocked controls. Total RNA was isolated with the RNAeasy Minikit (Qiagen) and reverse-transcribed with the Superscript III kit (Invitrogen). Quantitative PCR was performed in technical triplicates using SYBR Green PCR mastemix (Applied Biosystem) according to manufacturer’s instructions. Gene expression was normalized to hprt1; relative fold-changes were calculated by the comparative Ct or ΔΔCt method, where Ct stands for threshold cycle.

Primers were as follows: vegfc-qF 5’-ACCCTACCTACCGGATCATG-3’, vegfc-qR 5’-TCAAACAACGTCTTGCTGATG-3’; cdh5-qF 5’-AAGCCCAATGGTGACCTAAT-3’, cdh5-qR 5’-ATGGTAACACCGGTAGTGCC-3’; hprt1-qF1 5’-ATCATGGACCGAACTGAACGC-3’, hprt1-qR1 5’-AGCGATCACTGTGCGATTA-3’.
References for Materials and Methods