mafba is a downstream transcriptional effector of Vegfc signaling essential for embryonic lymphangiogenesis in zebrafish

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The lymphatic vasculature plays roles in tissue fluid balance, immune cell trafficking, fatty acid absorption, cancer metastasis, and cardiovascular disease. Lymphatic vessels form by lymphangiogenesis, the sprouting of new lymphatics from pre-existing vessels, in both development and disease contexts. The apical signaling pathway in lymphangiogenesis is the VEGFC/VEGFR3 pathway, yet how signaling controls cellular transcriptional output remains unknown. We used a forward genetic screen in zebrafish to identify the transcription factor mafb as essential for lymphatic vessel development. We found that mafb is required for the migration of lymphatic precursors after their initial sprouting from the posterior cardinal vein. mafb expression is enriched in sprouts emerging from veins, and we show that mafb functions cell-autonomously during lymphatic vessel development. Mechanistically, Vegfc signaling increases mafb expression to control downstream transcription, and this regulatory relationship is dependent on the activity of SoxF transcription factors, which are essential for mafb expression in venous endothelium. Here we identify an indispensable Vegfc–SoxF–Mafba pathway in lymphatic development.

[Keywords: lymphatic; vascular; Mafb; Vegfc; Sox18; Sox7; zebrafish]

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Lymphangiogenesis is the formation of new lymphatic vessels from pre-existing vessels. Lymphangiogenesis plays integral roles in cancer metastasis, lymphedema, and cardiovascular disease (Lim et al. 2013; Martel et al. 2013; Stacker et al. 2014). Common molecular pathways control lymphangiogenesis in development and disease, and much of our current understanding has come from the study of embryogenesis. In the embryo, the lymphatic vasculature derives chiefly from pre-existing veins through a process involving cellular transdifferentiation, migration, and proliferation and vessel morphogenesis (Oliver and Srinivasan 2010; Koltowska et al. 2013).

Lymphangiogenesis is dependent on VEGFR3 signaling in all known contexts, including during development. Knockout mice for Vegfr3 are embryonic-lethal due to cardiovascular failure (Dumont et al. 1998), and heterozygous mutation of Vegfr3 in the Chy mouse model leads to lymphedema and lymphatic vascular defects (Karkkainen et al. 2001). Furthermore, the transgenic overexpression of a soluble inhibitory [ligand trap] form of VEGF3 disrupts tissue lymphangiogenesis (Makinen et al. 2001). Mouse mutants for Vegfc fail to form the earliest lymphatic sprouts from embryonic veins, and, indicative of the instructive role for VEGFC, overexpression of this ligand promotes ectopic tissue lymphangiogenesis (Jeltsch et al. 1997; Karkkainen et al. 2004; Hagerling et al. 2013).

In humans, the VEGFC/VEGFR3 pathway also controls lymphatic vessel development, and patients with mutations in either VEGFR3 or VEGFC develop primary lymphedema in familial Milroy’s disease or Milroy-like lymphedema, respectively (Irthum et al. 2000; Karkkainen et al. 2000; Gordon et al. 2013). In humans, the VEGFC/VEGFR3 pathway also controls lymphatic vessel development, and patients with mutations in either VEGFR3 or VEGFC develop primary lymphedema in familial Milroy’s disease or Milroy-like lymphedema, respectively (Irthum et al. 2000; Karkkainen et al. 2000; Gordon et al. 2013). Finally, several coreceptors and modulators of VEGFC/VEGFR3 signaling play crucial roles in lymphangiogenesis (for review, see

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Schulte-Merker et al. 2011; Koltowska et al. 2013; Zheng et al. 2014), underscoring how central the pathway is in lymphatic development.

Characterization of the zebrafish lymphatic vasculature has demonstrated the highly conserved function of the VEGFC/VEGFR3 signaling pathway in vertebrates [Kuchler et al. 2006; Yaniv et al. 2006]. Forward genetic screens for mutants lacking lymphatics in zebrafish discovered a function for Cceb1 [Hogan et al. 2009a], which was subsequently shown to play a conserved role in mice and humans [Alders et al. 2009; Connell et al. 2010; Bos et al. 2011]. CCBE1 regulates the processing and activation of immature VEGFC to its mature, functional form necessary for lymphangiogenesis [Jeltsch et al. 2014; Le Guen et al. 2014]. In addition, zebrafish mutants have been described in vegfc and vegfr3 themselves, and we now know that zebrafish Vegfc signaling controls all secondary angiogenesis, including the formation of lymphatic precursors [PLs] from the posterior cardinal vein [PCV] [Hogan et al. 2009a,b; Villefranc et al. 2013; Le Guen et al. 2014]. The coordinated activity of both Vegfc and Vegfr3 further controls lymphangiogenesis of the facial lymphatic network [Okuda et al. 2012; Astin et al. 2014].

Although the apical signaling pathway that governs lymphatic sprouting is well established, we still have a very limited understanding of how signaling controls downstream pathways and regulates endothelial cell (EC) transcription. Several transcription factors are known to control the initial specification and maintenance of lymphatic EC [LEC] fate in mice, including PROX1, COUP-TFI, and SOX18 [Wigle and Oliver 1999; Francois et al. 2008; Srinivasan et al. 2010; Srinivasan and Oliver 2011]. Other transcription factors controlling later differentiation and specialization of lymphatic vessels include FOXC2 and GATA2 [Petrova et al. 2004; Norrmen et al. 2009; Kazenwadel et al. 2012; Lim et al. 2012]. Interestingly, emerging data have suggested that both the maintenance of PROX1 and the induction of SOX18 activity can be driven by VEGF signaling mechanisms [Deng et al. 2013; Duong et al. 2014; Srinivasan et al. 2014]. While this integration of cell-extrinsic signaling and cell-intrinsic transcriptional information appears to play an important role in lymphatic development, the spatiotemporal control of signaling and the complexity of EC transcriptional mechanisms remain far from fully understood.

The initial genetic screens performed for zebrafish lymphatic development used a pan-endothelial marker [Tg(fl1a:EGFP)y1] and scored the presence of the thoracic duct as a proxy for systemic lymphangiogenesis. We took advantage of the recently described Tg(-5.2lyve1b:dsRed) strain, which labels the comprehensive network of developing lymphatic vessels and embryonic veins (Okuda et al. 2012), to perform a phenotypically sensitized forward genetic screen and integrated whole-genome sequence mapping for rapid gene discovery. Here, we report the first mutant characterized from this screen, a mafb mutant. We found that mafb is an essential Vegfc-regulated transcription factor controlling lymphangiogenesis and identified a role for SoxF transcription factors in inducing mafb expression.

**Results**

**uqabgh mutants fail to form lymphatic vasculature**

We designed and performed an ENU mutagenesis screen in zebrafish. Briefly, we mutagenized the Tg(-5.2lyve1b:dsRed) strain and performed a classical F3 embryonic screen in this background. We sequenced the genomes of the transgenic mutagenized founders and our in-house WIK strain, which we used to generate mapping crosses with a predefined genomic variation. In total, we used 838 mutagenized F1 genomes (419 F2 families) with, on average, four F2 in-crosses scored per family (or 573 genomes screened). We identified 34 mutants that gave lymphatic deficiency/venous sprouting defects at either of two time points: 3 dpf and 5 dpf.

The **uqabgh mutant** showed a highly selective reduction, up to a complete loss, of lymphatic vessels in the embryonic trunk and face but formed a grossly normal blood vasculature (Fig. 1A; Supplemental Fig. 1B). Overall, the body plan was normal except for an otic vesicle defect and edema by 7 dpf [Figs. 1B, 2D, Supplemental Fig. 1A]. Quantification of the number of LECs in the trunk and face using a nuclear marker of ECs coupled with Tg(-5.2lyve1b:dsRed) demonstrated a significant reduction in mutants [Fig. 1C–E]. In the developing facial lymphatics, reductions were observed in medial facial lymphatics and branchial arch lymphatics (Fig. 1D,E; Supplemental Fig. 1C). The number of LECs in the otolithic lymphatic vessel was increased but was presumed to reflect altered, local tissue patterning. Earlier in development, examination of parachordal PL cell number showed no difference in mutants [Fig. 1F,G]. We analyzed the expression of prox1a in the Tg(prox1a:Kalt4-4xUAS:uncTagRFP) transgenic line that has been previously reported [Dunworth et al. 2014; van Impel et al. 2014] crossed onto a Tg(10xUAS:Venus) reporter strain. Interestingly, we saw no change in prox1a expression in sprouts leaving the PCV, suggesting normal establishment of cell identity in mutants [Fig. 1H, I]. Overall, these observations indicate that the **uqabgh mutants initiate lymphatic fate and sprouting secondary angiogenesis, but ongoing formation of a comprehensive lymphatic network fails.**

**uqabgh is a mafb mutant**

We used a whole-genome sequence-based approach to map the genomic location of the affected gene (Supplemental Material; data not shown). We identified a region of homozygosity on chromosome 23, and, within this linked region, a candidate mutation was identified in the mafb gene [Fig. 2A; Supplemental Fig. 2A]. A C/T nonsense mutation was identified [encoding Q155*] and predicted to truncate Mafba prior to the critical basic region leucine zipper [BRLZ] domain and thus is a predicted loss-of-function allele [Fig. 2B,C]. The zebrafish neural segmentation mutant valentino [Moens et al. 1996] is a mafb mutant and displays the same otic vesicle phenotype as **uqabgh** [Fig. 2D], suggesting a causative mafb mutation. MAFB is a bZIP transcription factor that can act as an activator or repressor. MAFB homologs regulate
cellular differentiation in various developmental contexts, controlling posterior hindbrain/otic fate decisions (Moens et al. 1996; Moens and Prince 2002), podocyte development (Sadl et al. 2002; Moriguchi et al. 2006), pancreatic β-cell differentiation (Artner et al. 2007), and hematopoietic lineage decisions (Sieweke et al. 1996, 1997; Kelly et al. 2000; Bakri et al. 2005). However, MafB has no previously described role in either lymphangiogenesis or vascular development.

To confirm that the mutation in mafba causes the lymphatic vascular developmental phenotype, we used CRISPR genome editing to generate germline mosaic zebrafish transmitting mutations in mafba (Supplemental Fig. 2B). Mosaic founders were then crossed to carriers for the uq4bh mutation, and the progeny were analyzed for phenotypes. Compound heterozygous embryos [confirmed by genotyping] displayed the described valentino otic phenotype, and this was coincident with a loss of lymphatic vascular development (Fig. 2D,E). This complementation test confirmed the mutant as mafba\textasciitilde uq4bh (Fig. 2E; Supplemental Fig. 2C,D).

mafba is expressed in the PCV and enriched in secondary sprouts

To determine the cell type in which mafba is active, we examined gene expression by in situ hybridization (ISH). mafba expression was observed in neurons, rhombomeres, and the pancreas (data not shown) as well as the PCV. We observed weak PCV expression at 24 h post-fertilization (hpf), which was increased by 36 h post-fertilization (hpf), preceding and concomitant with secondary angiogenesis (Fig. 3A; Supplemental Fig. 3A). At 48 hpf, when secondary sprouts have formed, we observed enriched expression of mafba dorsally in ECs sprouting or sprouted from the PCV (Fig. 3A). As ISH is insensitive at later developmental stages, we examined mafba expression in FACS (fluorescent-activated cell sorting)-sorted populations of arterial ECs (AECs), venous ECs (VECs), and LECs that we isolated from 60 hpf, 3 dpf, and 5 dpf embryos (Fig. 3B,C; Coxam et al. 2014, 2015). Indicative of lineage-restricted expression in the vasculature, we found that mafba was highly enriched in VECs compared with AECs at early time.
points [comparable with known VEC markers] and was enriched in LECs compared with VECs by 5 dpf [comparable with known venous and lymphatic markers lyve1b and prox1a]. We examined expression of other Maf family genes and the duplicate Mafb homolog mafb, which showed detectable EC expression, suggesting that it could play a compensatory role [Supplemental Fig. 4B,C]; however, given the phenotype of mafb mutants, any compensation must be partial.

**mafba acts cell-autonomously during zebrafish lymphangiogenesis**

mafba expression is suggestive of a cell-autonomous function; hence, we performed cellular transplantation experiments to formally test autonomy. We transplanted wild-type Tg(fli1a:EGFP) cells into Tg(-5.2lyve1b:DsRed) recipients from a mafb heterozygous in-cross and scored for the presence of grafted [EGFP-positive] cells in arteries [AECs], veins [VECs], and lymphatics [LECs] at 5 dpf. We found that wild-type cells could as readily contribute AECs, VECs, and LECs in mutant embryos (n = 13 vascular grafts) as they could in sibling controls (n = 11 vascular grafts) [Fig. 4A,B]. We traced all grafted cells with dextran and noted the positions of non-ECs, which were in variable locations and not consistent in rescued mutant embryos. Together, these analyses suggest that mafb is sufficient in ECs to direct lymphangiogenesis in mutant embryos.

Reciprocally, we transplanted mutant cells from Tg(kdrl:EGFP);Tg(-5.2lyve1b:DsRed) double-transgenic embryos into unlabeled wild-type hosts. In this experimental setting, AECs will express only EGFP, VECs will express EGFP plus dsRED, and LECs will express only dsRED in successful vascular grafts. Transplanted mutant cells contributed to AECs (n = 12/12 vascular grafts) and VECs [n = 3/12 vascular grafts] but not Tg(lyve1:DsRed)-expressing LECs [Fig. 4C,D]. Interestingly, in one transplanted embryo (n = 1/12), mutant cells formed a section of vasculature that appeared to be lymphatic based on
morphology, but this vessel expressed Tg(kdrl:EGFP), which is normally restricted to blood vessels. Hence, this grafted vessel was considered to have a differentiation defect [Supplemental Fig. 3B]. Taken together, reciprocal cellular mosaic experiments demonstrate that mafba acts autonomously in ECs during lymphatic vessel development.

**A set of mafba-dependent endothelial genes are responsive to Vegfc signaling**

We next isolated Tg(kdrl:EGFP)-expressing ECs from sibling and mafba\textsuperscript{muqta} mutant embryos at 48 hpf by embryo dissociation and FACS for EGFP [Supplemental Fig. 4A]. We performed RNA sequencing [RNA-seq] in triplicate and generated a concordant data set identifying 23 down-regulated and 68 up-regulated genes (Fig. 5A; Supplemental Table 1). These dysregulated genes were generally lowly expressed, probably indicative of low numbers of cells derived from secondary sprouts (which express mafba) in the larger pool of Tg(kdrl:EGFP)-expressing ECs. To confirm that these genes were dysregulated, we used quantitative PCR (qPCR) for 15 down-regulated and 47 up-regulated genes and confirmed that the majority was misexpressed, as indicated in the RNA-seq data [Fig. 5B].

To understand how selective to secondary angiogenesis these genes are, we sorted ECs using FACS from 30-hpf MO-vegfc mutants, we time-lapse-imaged PLs in the horizontal myoseptum from 48 hpf onward in Tg(-5.2lyve1:DsRed);Tg(kdrl:EGFP) double-transgenic embryos. While wild-type PLs actively migrated out of the myoseptum and elongated along their arterial substrates [Bussmann et al. 2010; Cha et al. 2012], mutant PLs commonly failed to migrate from the myoseptum and elongated along their arterial substrates. We observed vastly increased ECs, mafba-dependent genes are also dependent on normal Vegfc signaling.

**mafba expression is up-regulated by Vegfc**

We investigated mafba expression in MO-vegfc and MO-vegfr3 embryos at 30 hpf and saw no change in mafba expression [Supplemental Fig. 4D]. At 48 hpf, when mafba normally becomes enriched in the dorsal PCV, this enrichment was lost in MO-vegfc and MO-vegfr3 embryos, and overall EC mafba levels (normalized) were reduced by qPCR [Fig. 5D,E]. Venous sprouts fail to form in MO-vegfc and MO-vegfr3 embryos, and so the loss of expression may be a consequence of failed morphogenesis. However, the observation is consistent with selective enrichment in cells responding to Vegfc signaling, so we next examined mafba expression in vegfc-induced embryos. We observed vastly increased mafba expression in the vasculature of vegfc-induced embryos by ISH and confirmed this increase by qPCR (normalized) using FACS-sorted ECs from vegfc-induced embryos [Fig. 5F,G]. We did not find any evidence for regulation of vegfc, vegfr3, or other Vegf family downstream from mafba [Supplemental Fig. 4E–G].

mafba regulates LEC migration from the horizontal myoseptum but is dispensable for vegfc-induced proliferation

To determine the earliest cellular defect in trunk lymphangiogenesis in mafba mutants, we time-lapse-imaged PLs in the horizontal myoseptum from 48 hpf onward in Tg(-5.2lyve1:DsRed);Tg(kdrl:EGFP) double-transgenic embryos. While wild-type PLs actively migrated out of the myoseptum and elongated along their arterial substrates [Bussmann et al. 2010; Cha et al. 2012], mutant PLs commonly failed to migrate from the myoseptum [Fig. 6A; Supplemental Movies 1, 2]. Some PLs displayed distinctly broad and rounded morphology during
migration, which was not observed in wild-type cells [Fig. 6A]. Interestingly, we did not find any evidence for dysregulation of chemokine signaling components that control this migratory event [Cha et al. 2012] or ephrinb2a/ephrB4, which have been implicated in EC migration [Supplemental Fig. 5A–C]. We next examined mafba mutants in the vegfc-induced transgenic overexpression background and found that vegfc overexpression induced the proliferation of VECs at the same level in mafba mutants as in wild-type transgenic embryos (Fig. 6B,C). Together, these data show that mafba controls the ongoing migration of PLs after initial secondary angiogenesis but is not required for vegfc transgene-induced venous proliferation.

Vegfc up-regulates mafba expression in a SoxF transcription factor-dependent manner

We and others have previously shown that VEGF signaling can control the activity of SOX18 in human ECs, mice, and zebrafish [Deng et al. 2013; Duong et al. 2014]. To determine whether SoxF family transcription factors play a role in mafba induction, we examined mafba expression in sox7/sox18 double morpholino (dMO)-injected embryos. We found a strong reduction to complete absence of mafba expression in the PCV at 30 and 48 hpf [Fig. 7A], an unexpected observation because sox7/sox18 dMO embryos display expanded expression of classical VEC markers [Herpers et al. 2008; Pendeville et al. 2008]. Given that SOXF transcription factors can respond to VEGFs and that we observed that mafba expression is also Vegfc-responsive, we next examined whether the up-regulation of mafba by Vegfc is dependent on SoxF transcription factors. We found that knockdown of sox7 alone did not have an impact on the induction of mafba by Vegfc [Fig. 7B,C]. sox18 knockdown strongly reduced the intensity of induction, and the dMO knockdown further reduced the induction of mafba expression [Fig. 7B,C]. Supporting this regulatory relationship in vitro, transfection of human umbilical vein ECs with SOX18 but not SOX7 induced MABP expression [Supplemental Fig. 6A,B]. Furthermore, expression of sox18 and sox7 was normal in mafba mutant embryos, suggesting that these genes are upstream of but not downstream from mafba [Supplemental Fig. 6D,E].

Given that the transgenic overexpression of Vegfc induces proliferation of VECs, we asked whether the reduction of mafba VEC expression in vegfc-induced/MO-sox7-injected embryos was concurrent with a reduction in VEC proliferation. Importantly, Vegfc overexpression in sox7/sox18 double-knockdown embryos still induced robust VEC proliferation [Fig. 7D]. This observation is strikingly in line with the fact that mafba also played no role in vegfc-induced VEC proliferation in this transgenic background. Finally, we examined sox7 and sox18 expression by qPCR on embryonic ECs FACS-sorted [using Tg(kdrl:EGFP)] from MO-vegfc and vegfc-induced embryos at 48 hpf. We found that both were mildly reduced in loss-of-function scenarios and increased in gain-of-function
scenarios, where normalized expression of endothelial egfp was not changed (Fig. 7E; Supplemental Fig. 6C).

Discussion

Taken together, the observations above demonstrate that the transcription factor Mafba is essential for embryonic lymphangiogenesis during zebrafish development. In mice, MAFB has established roles in directing hindbrain segmentation and podocyte, pancreatic β-cell, and hematopoietic lineage differentiation but no known function in vascular lineages (Sieweke et al. 1996, 1997; Moens et al. 1998; Kelly et al. 2000; Sadl et al. 2002; Bakri et al. 2005; Moriguchi et al. 2006; Artner et al. 2007). Our findings of a cell-autonomous role in lymphatic vessel development, given the previously described functions in cell fate and differentiation, suggest a likely role in endothelial lineage decisions or differentiation. A function in ongoing LEC differentiation could explain the migration defect observed if critical machinery was not switched on in PLs after they initially sprout from the PCV. To fully understand how Mafba elicits such a specific phenotype in LECs, it is clear that the characterization of downstream genes and pathways is now needed.

Vegfc is the major driver of developmental lymphangiogenesis in vertebrates (Karkkainen et al. 2004). In zebrafish, Vegfc induces VEC proliferation in the PCV (Helker et al. 2013; Le Guen et al. 2014) and the sprouting of venous precursors and PLs from the PCV during secondary angiogenesis (Hogan et al. 2009a; Villefranc et al. 2013). Our observations of mafba expression and the expression of a subset of mafba-dependent genes in Vegfc loss-of-function and gain-of-function embryos indicate that mafba is responsive to Vegfc signaling during development. We showed previously that mutations in genes within the Vegfc pathway completely block the phenotypes caused by overexpression of Vegfc (Le Guen et al. 2014), yet here we observed that loss of mafba has no impact on transgene-induced proliferation while being crucial for lymphatic development. This suggests that Vegfc signaling has multiple downstream outcomes in ECs.
and that different cellular responses are controlled by different effectors. In line with this observation, the cellular defect in mafb<sup>auq4bh</sup> mutants occurs later than in vegfc, vegfr3, or ccbe1 mutants (Hogan et al. 2009a; Villefran et al. 2013; Le Guen et al. 2014), with mafb controlling LEC migration from the horizontal myoseptum rather than sprouting of LEC precursors from the PCV. It will be intriguing to discover whether MAFB proteins play specialized roles regulating a discrete subset of functional genes rather than broad roles in LEC identity/transcription and determine how this compares with other transcription factors, such as PROX1, which is maintained by VEGFC signaling in mice (Srinivasan et al. 2014).

We further investigated potential mechanisms by which Vegfc might regulate the levels of mafb in zebrafish. In mice, MafB can act together with Ets1, and there is evidence that Vegfc can modulate Ets-mediated transcription (Sieweke et al. 1996; Yoshimatsu et al. 2011); however, we examined morpholino knockdown models and gene expression levels and found no evidence that Mafb acts coordinately with Ets factors in lymphatic development (Supplemental Fig. 6F–J). In mice, the transcription factor Sox18 controls lymphangiogenesis in a partially redundant manner with other SOXF transcription factors in different mouse strains (Francois et al. 2008; Hosking et al. 2009). The transcription factors Sox18 and Sox7 function redundantly during zebrafish blood vascular development, with dMO-injected embryos displaying early arterial–venous defects that are recapitulated in genetic mutants and do not allow for controlled analysis of later lymphangiogenesis (Cermenati et al. 2008; Herpers et al. 2008; Pendeville et al. 2008; Hermkens et al. 2015). Interestingly, recent work has shown that SOXF transcription factors can be up-regulated and nuclear-localized in response to VEGF/VEGFC–ERK signaling to control downstream gene expression and vascular lineage decisions (Deng et al. 2013; Duong et al. 2014). We investigated whether SOXF transcription factors could link Vegfc signaling to mafb expression. We found that Sox18 and Sox7 are necessary for normal mafb expression and further showed that they indeed mediate the observed induction of mafb by transgenic overexpression of Vegfc. Taken together, these observations led us to a working model of how this pathway functions to control lymphatic development (Fig. 7F).

The question of how cells acquire LEC identity and differentiate has led to the characterization of a number of transcriptional regulators of LEC fate and differentiation (Wigle and Oliver 1999; Francois et al. 2008; Srinivasan et al. 2010; Srinivasan and Oliver 2011). How these transcription factors combine and integrate as a functional regulatory network and how they are controlled by extrinsic signals to modulate precise spatiotemporally controlled gene expression remain to be elucidated. The
addition of Mafb as a crucial regulator of lymphangiogenesis provides a new direction and increases the complexity of LEC gene regulation. Importantly, many transcriptional regulators of LEC development play central roles in lymphatic vascular diseases (Fang et al. 2000; Finegold et al. 2001; Irrthum et al. 2003; Ostergaard et al. 2011).

In addition to gaining a deeper mechanistic understanding of LEC fate acquisition and differentiation, it will be interesting to determine whether MAFB transcription factors contribute to vascular pathologies in the future.

Materials and methods

Zebrafish

Animal work followed the guidelines of the animal ethics committee at the University of Queensland. The forward genetic screen was based on previous studies, with mutagenesis as described previously (de Bruijn et al. 2009). The genomic sequencing pipeline was based on previous studies (Leshchiner et al. 2012) and will be described in full elsewhere. Published zebrafish lines were Tg(fli1a:nEGFP)y7 (Lawson and Weinstein 2002), Tg(-5.2lyve1b:DsRed)nz101 (Okuda et al. 2012), TgBAC(prox1a:KalTA4-4xUAS-ADV.E1b:TagRFP)nim5 (Dunworth et al. 2014; van Impel et al. 2014), Tg(flt1:YFP)hu4624 (Hogan et al. 2009a), and Tg(kdrl:EGFP)s84 3 (Jin et al. 2005).

Transgenesis, morpholinos, genotyping, and genome editing

10xUAS:vegfc plasmid DNA was generated using the full-length zebrafish vegfc cDNA cloned into the Gateway pME vector (pDON-221) using Gateway technology (Hartley et al. 2000). To generate the Tg(10xUAS:vegfc)w2bh strain, 20 ng/μL plasmid DNA and 25 ng/μL tol2 transposase mRNA were injected in one-cell stage embryos, and F1 founders were identified by

Figure 7. mafba is downstream from Vegfc and SoxF transcription factors. (A) Expression of mafba at 30 hpf in control uninjected (n=38/41) and MO-sox18/sox7 dMO-injected (n=24/25) embryos [left] and at 48 hpf in control uninjected (n=30/33) and MO-sox18/sox7 dMO-injected (n=18/18) embryos [right]. Arrows indicate PCV, and an asterisk indicates reduced expression. Bars, 100 μm. (B) mafba expression at 48 hpf in control and vegfc-induced embryos as well as vegfc-induced embryos injected with MO-sox7, MO-sox18, and MO-sox18/sox7 dMO. Arrows indicate PCV expression, and asterisks indicate reduced/absent expression. Bars, 100 μm. (C) Quantification of mafba expression in embryos from E, with embryos scored as high, medium, and low mafba expressers. Control, 88%, n=37/42, high, MO-sox7, 89%, n=39/44, high, MO-sox18, 71%, n=25/35, medium; dMO, 71%, n=34/48, low. (D) Quantification of VEC number in vegfc-induced control and MO-sox18/sox7 dMO-injected embryos. Mean ± SEM. Control, n=12, control vegfc-induced, n=34, dMO, n=11, dMO vegfc-induced, n=25; t-test. (ns) No significant difference. (E) qPCR for sox18 expression in FACS-sorted zebrafish ECs from MO-vegfc, vegfc-induced, and control embryos. egfp served as a control for EC expression levels. Expression is relative to the geometric average of kdrl, cdh5, and lyve1b expression. Mean ± SEM. (F) Working model of Mafba function in lymphangiogenesis: Vegfc up-regulates mafba expression in secondary sprouts and in a Sox18-dependent (redundancy with Sox7) manner. Mafba is essential for PL migration. Vegfc controls venous proliferation independently of Mafba or Sox18/7.
PCR. mafb full-length cDNA was cloned into the pCS2+ vector and used as a template for riboprobe synthesis. MO-vegf3, MO-vegf, MO-sox7, and MO-sox18 were described previously (Herpers et al. 2008; Le Guen et al. 2014). Two morpholinos against etsl (Pham et al. 2007) were used as described previously, and two morpholinos against eef1a2 were designed and injected (5 ng per embryo). CRISPR genome editing for mafb was performed as described in Gagnon et al. (2014) to generate the mafb

Whole-mount ISH
Whole-mount ISH was performed as described previously (Kartopawiro et al. 2014) using the mafb probe (see above) generated from plasmid linearized using Clal. Probes for vegf (Ober et al. 2004), vegfr3 (Hogan et al. 2009b), cxcr4a and cxcl12b (Coxam et al. 2014), cftp6a (Durbin et al. 1998), gphb4a (Cooke et al. 1997), and sox18 and sox7 (Herpers et al. 2008) were used as previously described.

FACS and gene expression analysis
Isolation of zebrafish embryonic ECs, RNA extraction, cDNA preparation, and qPCR were performed as described previously (Coxam et al. 2014; Kartopawiro et al. 2014). For isolating cells from sibling and mafb

Immunohistochemistry
Immunohistochemistry for anti-GFP was performed according to the following protocol. Embryos were fixed in 4% PFA (paraformaldehyde) overnight and washed five times with PBST (0.1% Tween in PBS [phosphate buffered saline]). Embryos were blocked in PBSTD (PBS with 1% BSA, 1% DMSO, 0.1% Triton-100) with 10% horse serum for 3 h, anti-GFP (chicken polyclonal to GFP, 1:200; ab13970) was added, and embryos were incubated overnight. Embryos were washed five times for 30 min in PBSTD and then incubated in PBSTD and 10% horse serum with secondary antibody (goat anti-chicken IgG, 1:400; Alexa 488, Invitrogen, A11039) and DAPI (1:1000, Sigma Aldrich) overnight. Embryos were washed five times for 30 min in PBST and imaged.

Transplantation
Transplantation was performed essentially as described previously (Hogan et al. 2009a) with the following changes. Donor embryos were injected with dextran cascade blue (10,000 MW, Invitrogen) at 5 ng/mL [1 nL per embryo]. Cells from wild-type donor embryos [Tg(fli1a:nEGFP)] were transplanted into host embryos derived from mafb heterozygous in-crosses [Tg(+5.2lyve1b-DsRed)]. For reciprocal transplants, mutant cells [Tg(κdrl:EGFP), Tg(+5.2lyve1b-DsRed)] were transplanted into unlabeled wild-type hosts, and donors were genotyped. Embryos with successfully transplanted ECs were cultured until 5 dpf.

Imaging and quantification
Live and fixed embryos were mounted laterally and imaged using a Zeiss LSM 710 FCS confocal microscope. All images were processed using either ImarisX64 7.70 and/or ImageJ 1.47 [National Institutes of Health] software. The number of LEC nuclei [expressing Tg(fli1a:nEGFP)] coexpressing Tg(+5.2lyve1b-DsRed) across five somites through a Z-stack was manually counted using ImageJ 1.47 [National Institutes of Health] software (Fig. 1A(C,D–G); Supplemental Fig. 1C) The number of Prox1-positive LECs expressing Tg(proxlA:CATlTA4-4xUAS-E1b: uncdRFP) (Hogan et al. 2008; Le Guen et al. 2014) detected by a-GFP in green and costained with DAPI (to label nuclei) across five somites was manually counted using ImageJ 1.47 [National Institutes of Health] software (Fig. 1H,I). For the quantification of EC numbers shown in Figure 5, the spot tool in ImarisX64 7.70 software was used. For each sample, the Tg(fli1a:nEGFP) nuclei were selected based on fluorescence intensity, and the total number of GFP-positive nuclei (across five somites) in a full Z-stack was calculated.

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