DeepCAGE Transcriptomics Reveal an Important Role of the Transcription Factor MAFB in the Lymphatic Endothelium

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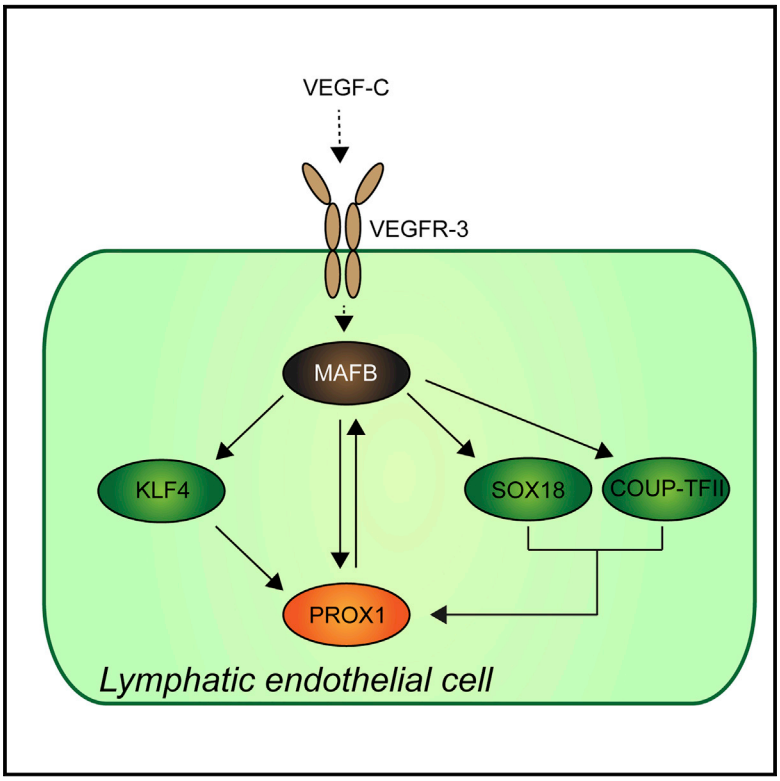
Graphical Abstract



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In Brief

VEGF-C signaling through VEGFR-3 is one of the most important pathways for induction of lymphangiogenesis. Using CAGE RNA sequencing, Dieterich et al. characterize gene expression changes in response to VEGFR-3 activation and identify MAFB as an important transcription factor in lymphatic endothelial cells.

Highlights

- CAGE RNA sequencing identifies transcription factors activated by VEGFR-3
- MAFB is a lymphatic transcription factor specifically activated by VEGFR-3
- MAFB controls the expression of other transcription factors and lymphatic marker genes
- MAFB-deficient mice show abnormal lymphatic patterning in the skin

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DeepCAGE Transcriptomics Reveal an Important Role of the Transcription Factor MAFB in the Lymphatic Endothelium

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SUMMARY

VEGF-C/VEGFR-3 signaling plays a central role in lymphatic development, regulating the budding of lymphatic progenitor cells from embryonic veins and maintaining the expression of PROX1 during later developmental stages. However, how VEGFR-3 activation translates into target gene expression is still not completely understood. We used cap analysis of gene expression (CAGE) RNA sequencing to characterize the transcriptional changes invoked by VEGF-C in LECs and to identify the transcription factors (TFs) involved. We found that MAFB, a TF involved in differentiation of various cell types, is rapidly induced and activated by VEGF-C. MAFB induced expression of PROX1 as well as other TFs and markers of differentiated LECs, indicating a role in the maintenance of the mature LEC phenotype. Correspondingly, E14.5 *Mafb*$^{-/-}$ embryos showed impaired lymphatic patterning in the skin. This suggests that MAFB is an important TF involved in lymphangiogenesis.

INTRODUCTION

The development of the lymphatic system in mice begins around embryonic day (E) 9.5, when a subpopulation of venous endothelial cells starts expressing the transcription factor (TF) prospero homeobox 1 (PROX1) and forms patches of lymphatic endothelial cell (LEC) progenitors (reviewed in Koltowska et al., 2013 and Yang and Oliver, 2014). Around E10.5–E11.5, LEC progenitor cells bud off the veins and form primary lymphatic structures (François et al., 2012; Hägerling et al., 2013; Yang et al., 2012). During this stage, migrating cells upregulate podoplanin (PDPN), a marker of differentiated LECs that is absent from the PROX1+ LEC progenitors in the veins (François et al., 2012; Yang et al., 2012). Continuous sprouting and migration of LECs during the following days give rise to a primitive lymphatic plexus that undergoes further maturation to form initial and collecting lymphatic vessels. At E14.5, the subcutaneous lymphatic network is forming, with lymphatic vessels growing dorsolaterally from the sides toward the dorsal midline. Finally, formation of lymphatic valves in collecting vessels begins around E16.0 and indicates complete differentiation of these vessels (Sabine et al., 2012). Of note, a recent report has also suggested a non-venous origin of lymphatic vessels in the lumbar region of the back skin (Martinez-Corral et al., 2015).

Several TFs play a crucial role in the coordination of lymphatic development. PROX1 is a key TF for LEC differentiation. *Prox1*$^{-/-}$ embryos fail to develop any lymphatic vessels because LEC fate specification in the embryonic veins is impaired (Wigle and Oliver, 1999). Even in *Prox1* heterozygous mice, the number of LEC progenitors is reduced severely (Harvey et al., 2005; Srinivasan and Oliver, 2011), indicating that the expression level of PROX1 needs tight regulation for normal lymphangiogenesis. Two TFs, sex-determining region Y box 18 (SOX18) and nuclear receptor subfamily 2, group F, member 2 (NR2F2, also called COUP-TFII) have been shown to be required for the initial induction of PROX1 expression (François et al., 2008; Srinivasan et al., 2010). Furthermore, COUP-TFII is needed for maintenance of PROX1 expression during LEC specification but not in fully differentiated LECs (Johnson

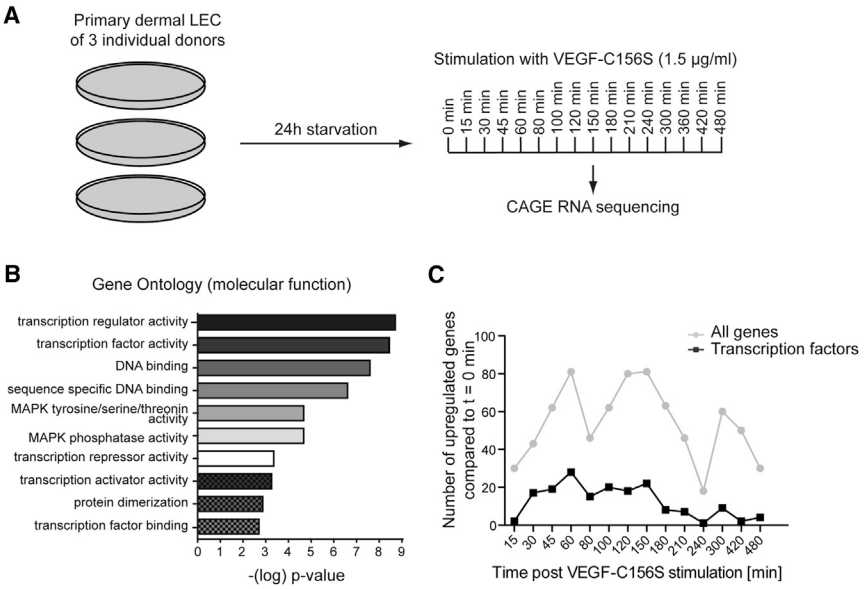

Figure 1. RNA Sequencing of Primary Human LECs

(A) Diagram of the CAGE RNA sequencing approach. Primary human LECs from three individual donors were stimulated with VEGF-C156S, and RNA was extracted and subjected to sequencing at 16 different time points over a period of 0–480 min.

(B) Diagram of the ten most significantly enriched GO terms (molecular function) associated with all upregulated genes.

(C) Graph representing the number of upregulated TFs in comparison with all upregulated genes for each time point.

See also Figure S1 and Table S1.

et al., 2008; Lin et al., 2010; Srinivasan et al., 2010). Another important molecular cue for lymphatic development is signal transduction through the vascular endothelial growth factor C (VEGF-C)/VEGF receptor 3 (VEGFR-3) axis. During embryonic development, VEGFR-3, encoded by the *Flt4* gene, is expressed by venous endothelial cells but is upregulated in LEC precursors (Kukk et al., 1996). Its ligand VEGF-C is expressed in the mesenchyme, and VEGF-C/VEGFR-3 signaling is crucial for budding and guidance of primitive LECs (Karkkainen et al., 2004). In addition, VEGF-C/VEGFR-3, together with the VEGF-C co-receptor neuropilin 2 (NRP2), is needed for lymphatic vessel sprouting and lymphangiogenesis during later stages of development and for postnatal lymphangiogenesis (Rutkowski et al., 2013; Xu et al., 2010; Yuan et al., 2002). A recent report has demonstrated that a positive feedback loop between PROX1 and VEGFR-3 expression is also required for the maintenance of the LEC phenotype during later stages of development (Srinivasan et al., 2014). In this study, the authors showed that PROX1 is a direct regulator of VEGFR-3 expression and that VEGFR-3 is needed to maintain PROX1 expression, although the precise molecular pathway linking VEGFR-3 and PROX1 induction remains unknown.

Binding of VEGF-C to VEGFR-3 is well known to result in rapid phosphorylation of several tyrosine residues in the cytoplasmic tails of VEGFR-3, activating downstream signaling cascades, including the phosphatidylinositol 3-kinase (PI3K) and Raf-MEK-ERK pathways (reviewed in Koch et al., 2011). However, despite the importance of this signaling axis for the development and maintenance of the lymphatic system, it is still not understood how these signals are translated into a transcriptional response in LECs and which TFs are involved. Here we characterized the immediate transcriptional changes downstream of VEGFR-3 activation in LECs in detail using cap analysis of gene expression (CAGE) RNA sequencing (Kanamori-Katayama et al., 2011; Shiraki et al., 2003) in conjunction with the FANTOM5 project (http://fantom.gsc.riken.jp/5/). Of note, we found upregulation of multiple TFs, including the well known regulators of PROX1 expression, SOX18 and kruppel-like factor 4 (KLF4) (François et al., 2008; Park et al., 2014). Importantly, we identified MAFB as a lymphatic TF activated by VEGF-C and suggest that MAFB regulates lymphangiogenesis by upregulation of PROX1.

The work presented here is part of the FANTOM5 project (Arner et al., 2015; Forrest et al., 2014). Data downloads, genomic tools, and co-published manuscripts have been summarized at http://fantom.gsc.riken.jp/5/.

RESULTS

Activation of VEGFR-3 Induces Expression of Multiple Transcription Factors

To investigate acute transcriptional changes in response to VEGFR-3 stimulation, we treated primary LECs derived from three individual donors with the VEGFR-3-specific ligand VEGF-C156S (Joukov et al., 1998), isolated RNA at 16 different time points from 0–480 min, and subjected it to CAGE RNA sequencing (Figure 1A). In total, we found 241 genes upregulated at at least one of the time points (Table S1). Gene ontology (GO) analysis showed that a large number of those genes were either TFs or otherwise related to transcriptional regulation. In fact, 7 of the 10 most significantly enriched GO molecular function terms were related to the regulation of gene expression (Figure 1B). Induction of TFs was particularly strong during the early time points after stimulation, from 15–210 min (Figure 1C), indicating that a wave of immediately induced TFs controls downstream effects of VEGFR-3 stimulation in LECs.

VEGFR-3 Stimulation Specifically Induces Expression of MAFB, KLF4, and SOX18 in LECs

In total, we found 50 TFs among the VEGF-C156S-induced genes (Figure 1C; Table S1). Using qPCR, we could validate upregulation of 17 of these TFs during the early phase of the stimulation (expression peak between 30 and 80 min after stimulation) (Figure 2B; Figures S1A–S1P). Many of these 17 TFs, such as *EGR*s, *FOS*, *JUN*, etc., are considered "immediate early genes" (IEGs) that are well known to be upregulated in various

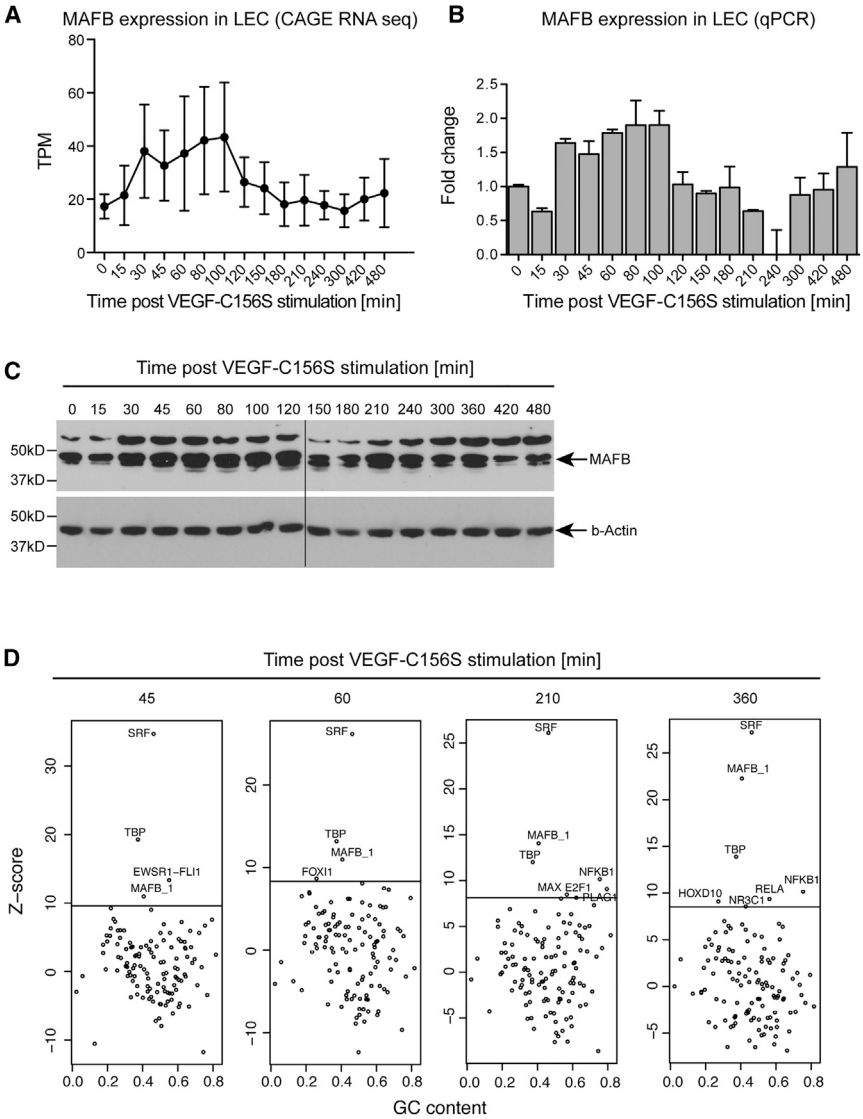

Figure 2. MAFB Is Upregulated and Active in VEGF-C156S-Stimulated LECs

(A) Normalized upregulation of *MAFB* transcripts (tags per million [TPM]) according to CAGE RNA sequencing (RNA-seq). Data points represent the mean ± SD of LECs from three individual donors.

(B) Validation of *MAFB* upregulation by qPCR (one representative donor). Error bars represent mean + SD.

(C) Western blot showing transient upregulation of MAFB protein.

(D) Activity plots showing significant enrichment of predicted MAFB_1 binding sites in promoters of differentially expressed (*Z*-score higher than the mean + 1.5 × SD, indicated by a horizontal line) at the time points of 45, 60, 210, and 360 min. Each plot provides the Z-score of the TF binding profiles on the y axis, whereas the x axis represents the percent guanine and cytosine (%GC) content of the profiles.

See also Figure S2 and Table S2.

cell types by a wide variety of stimuli. To identify TFs that are specifically induced by VEGFR-3 stimulation in LECs, we compared our data to the results of published gene set enrichment analyses (GSEAs) on other stimulation experiments (VEGF-A-treated human umbilical vein endothelial cells (HUVECs) and epidermal growth factor [EGF]-treated MCF7, MCF10A, and HeLa cells; Abe and Sato, 2001; Amit et al., 2007; Nagashima et al., 2007). As expected, the majority of TFs were upregulated in one or several of the comparison datasets, meaning that they represent IEGs. Upregulation of only three TFs, *MAFB* (V-maf musculoaponeurotic fibrosarcoma oncogene homolog B), *KLF4*, and *SOX18*, was induced selectively in VEGF-C156S-stimulated LECs.

MAFB regulates the differentiation of multiple cell types, including monocytes/macrophages, kidney podocytes, and pancreatic α and β cells (Friedman, 2007; Hang and Stein, 2011; Yang and Cvekl, 2007). In contrast to KLF4 and SOX18, a role of MAFB in LECs has not been described before. In our dataset, *MAFB* was induced rapidly in LECs and reached maximal expression during the first 2 hr after VEGFR-3 stimulation (Figures 2A and 2B). This induction was reflected by an increase in MAFB protein, which slowly accumulated during the first 3–4 hr after stimulation and dropped back toward the baseline at later time points (Figure 2C).

MAFB Actively Regulates Gene Expression in Response to VEGFR-3 Stimulation

Next we aimed to investigate whether MAFB is involved in the regulation of target genes in LECs in response to VEGFR-3 stimulation. Using the CAGE RNA sequencing data, we identified the transcriptional start sites (TSSs) of all upregulated genes in our dataset, extracted the core promoter sequence around them (−1,500 to +500 base pairs [bp]), and searched for MAFB binding sites using oPOSSUM-3 (Kwon et al., 2012). MAFB is known to bind MAFB response elements (MAREs) with the consensus sequence TGCTGAC, and to ''degenerated'' MAREs with the consensus sequence TGCNNNN (Eychène et al., 2008). Accordingly, analyses of protein binding microarray data have identified two distinct types of TF binding profiles for MAFB (Badis et al., 2009). These TF binding profiles are denoted as ''MAFB_1'' and ''MAFB_2'' in the publically available TF binding site database JASPAR (Mathelier et al., 2014; Figure S2A). Using oPOSSUM-3, we found a significant enrichment of MAREs in the core promoters of upregulated genes after 45, 60, 210, and 360 min (Figure 2D; Table S2). Similarly, we also found a significant enrichment for the number of genes with at least one MAFB_1 or MAFB_2 element in the core promoter among all upregulated genes after 15, 210, and 360 min (Figure S2B). This is a strong indication that MAFB is indeed active after VEGFR-3

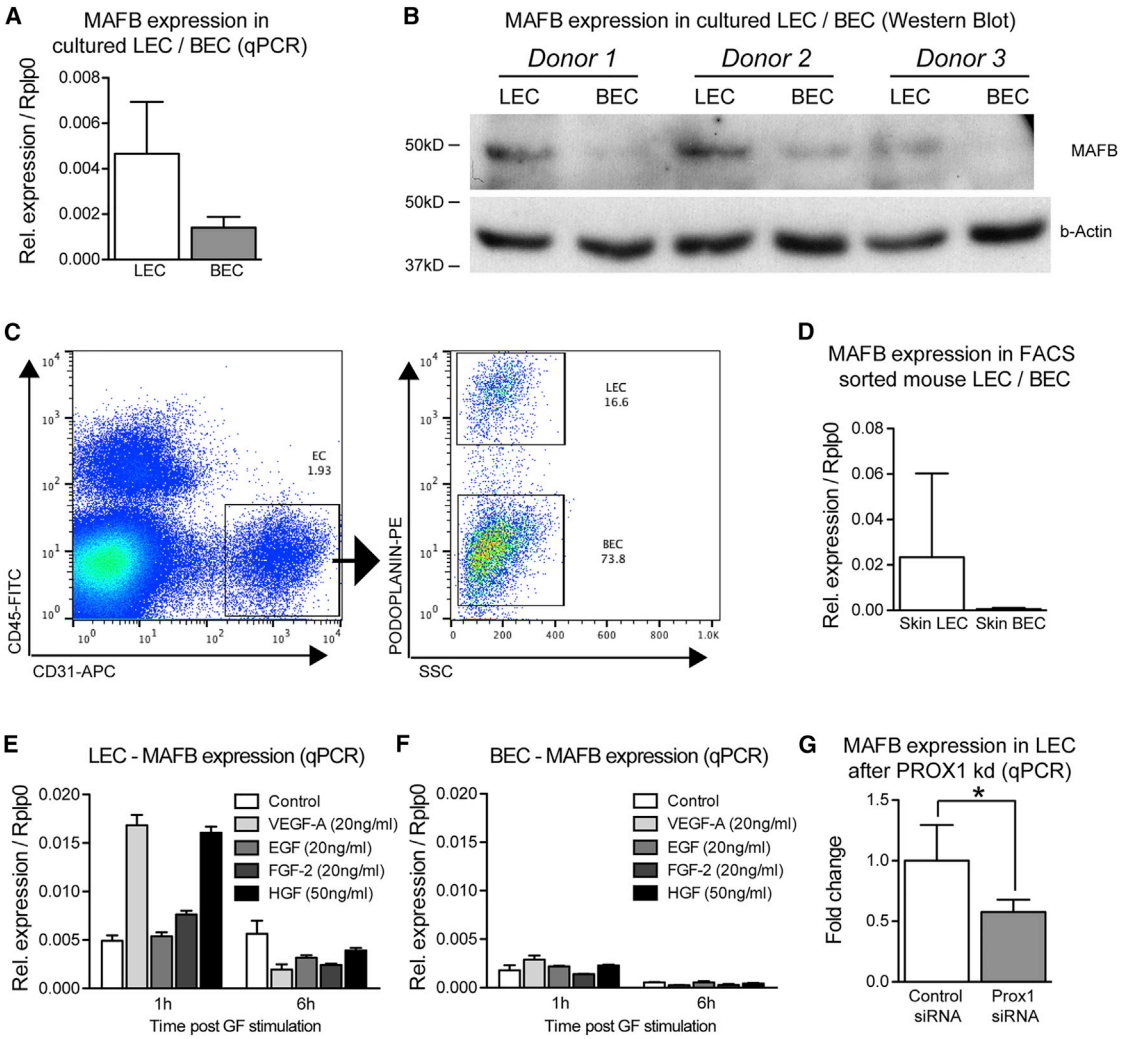

Figure 3. MAFB Is Selectively Expressed in LECs Compared with BECs

(A) qPCR showing relative expression of *MAFB* in cultured human LECs and BECs of the same donor. Error bars represent mean + SD of three independent experiments.

(B) Western Blot for MAFB protein in cultured human LECs and BECs of three individual donors.

(C) Gating strategy used for FACS of LECs and BECs from mouse back skin (plots pre-gated for live, single cells). $CD45^{-}CD31^{+}Podoplanin^{+}$ cells were considered as LECs and $CD45^{-}CD31^{+}Podoplanin^{-}$ cells as BECs.

(D) qPCR showing *MAFB* expression in LECs and BECs sorted by FACS from mouse back skin. Error bars represent mean + SD (n = 5–6).

(E and F) Representative qPCR of *MAFB* expression in LECs (E) and BECs (F) treated with VEGF-A, EGF, FGF-2, and HGF at the indicated doses for 1 or 6 hr. One representative of two independent experiments is shown. See also Figure S3. Error bars represent mean + SD.

(G) qPCR of *MAFB* expression in LECs after siRNA knockdown of *PROX1*. Error bars represent mean + SD of four independent experiments (*$p < 0.05$).

stimulation and induces further target genes, mediating at least a part of the downstream effects.

MAFB Is Selectively Expressed and Inducible in LECs Compared with BECs

Having established the specific induction and activity of *MAFB* in VEGF-C156S-stimulated LECs, we next investigated the expression pattern of *MAFB* in the endothelium in greater detail. In line with previous reports (Hirakawa et al., 2003; Petrova et al., 2002), we found that *MAFB* expression was detectable in both cultured human LECs and blood vascular endothelial cells (BECs) and was increased markedly in LECs both at the mRNA and the protein level (Figures 3A and 3B). Similarly, we found that *MAFB* expression was higher in LECs than in BECs isolated from mouse back skin by fluorescence-activated cell sorting (FACS) (Figures 3C and 3D).

We next tested the effect of other growth factors that have been implied in lymphangiogenesis on the *MAFB* expression level in LECs and BECs. *MAFB* expression in LECs was induced by VEGF-A and HGF after 1 hr and returned to baseline levels after 6 hr (Figure 3E). The effect of VEGF-A was comparable with that of VEGF-C and VEGF-C156S (Figure S3A). In contrast,

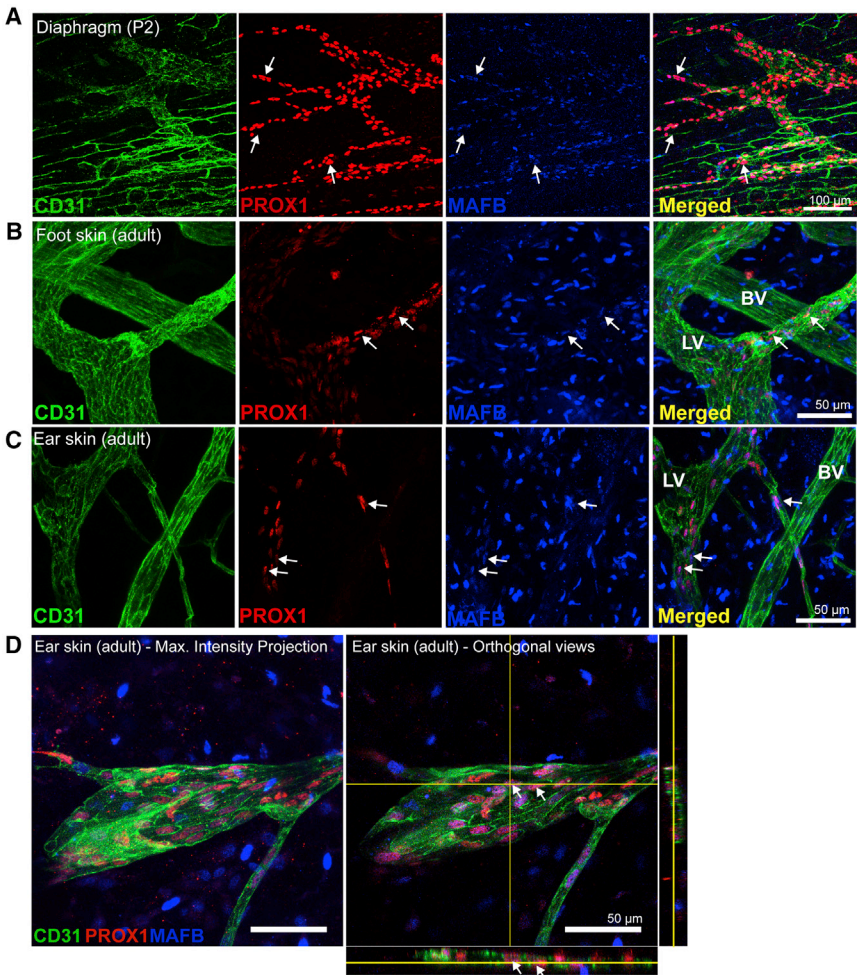

Figure 4. MAFB Is Present in Lymphatic Vessels in the Mouse

Maximum-intensity projections of confocal images of mouse (C57BL/6 wild-type) tissue whole mounts stained for CD31 (green), PROX1 (red), and MAFB (blue).

(A) Postnatal diaphragm (P2).

(B) Foot skin (adult).

(C) Ear skin (adult). Arrows indicate colocalization of MAFB and PROX1. LV, lymphatic vessel; BV, blood vessel.

(D) Maximum intensity projection and 3D reconstruction with orthogonal views of a lymphatic vessel in the ear skin. Colocalization of PROX1 and MAFB in the z direction is indicated by arrows.

See also Figure S4.

no effect on *MAFB* expression was seen in BECs (Figure 3F; Figure S3B). This is in line with the GSEA that indicated that *MAFB* is not induced in VEGF-A-treated HUVECs (Abe and Sato, 2001).

PROX1 is considered a fate-determining transcription factor for LECs, regulating the expression of many genes involved in LEC differentiation (Koltowska et al., 2013; Yang and Oliver, 2014). To investigate whether PROX1 might be responsible for the preferential expression of *MAFB* in LECs, we used small interfering RNA (siRNA) to deplete *PROX1* expression. Knockdown of PROX1 in cultured LECs with two individual siRNAs was associated with decreased *MAFB* expression (Figure 3G; Figure S3C), indicating that *MAFB* is a PROX1 target gene.

MAFB Is Expressed in Lymphatic Vessels In Vivo

To further investigate the expression of MAFB in lymphatic endothelium in vivo, we stained tissue whole mounts with a MAFB-specific antibody in combination with antibodies to the lymphatic marker PROX1 and the pan-vascular marker CD31. MAFB staining was detected in the developing lymphatic vessels in the diaphragm (P2) as well as in the foot and ear skin of adult mice (Figures 4A–4C). Lymphatic MAFB staining was nuclear and completely overlapped with PROX1 staining (Figure 4D), confirming that MAFB is indeed expressed by lymphatic endothelial cells in vivo. MAFB staining was not detected in CD31+ PROX1− blood vessels (Figures 4A–4C). Strong MAFB expression was also found in non-vascular single cells in all tissues analyzed. Co-staining for CD68, CD206, and LYVE-1 revealed that virtually all of these cells were macrophages (Figures S4A and S4B), in agreement with previous studies (Sieweke et al., 1996).

MAFB Regulates the Expression of Several Key Transcription Factors and Differentiation Markers in LECs

To investigate whether MAFB expression might affect the LEC phenotype, we overexpressed MAFB in cultured LECs using adenovirus (Ad-MAFB). Cells infected with a GFP virus (Ad-GFP) served as a control. We achieved high MAFB expression both at the RNA and protein level for at least 72 hr after infection (Figures S5A and S5B). CAGE RNA sequencing of LECs 24 hr after adenovirus infection showed that MAFB overexpression resulted in significant (log_2 fold change [log_2FC] > 1, false discovery rate [FDR] < 5%) upregulation of a large set of target genes (Table S3). Among the upregulated genes were *LYVE1* and *PDPN*, two major markers of lymphatic differentiation. We therefore investigated the expression of additional key LEC markers at several time points (24, 48, and 72 hr) after adenovirus infection by qPCR. We found a strong upregulation of *PROX1* mRNA 72 hr after MAFB overexpression (Figure 5A). In line with this, the protein level of PROX1 was also higher than in control cells at this time point. There was a steady decrease of PROX1 expression in control LECs over time that was not seen in MAFB-overexpressing LECs (Figures 5B and 5C). We also found upregulation of *KLF4* after 48 and 72 hr and of *NR2F2* (COUP-TFII) after 72 hr (Figures 5D and 5E). Surprisingly, expression of *SOX18* was initially decreased but later increased, which may be due to MAFB acting as transcriptional activator or repressor, depending on the context (Figure 5F). Additionally, we found a strong induction

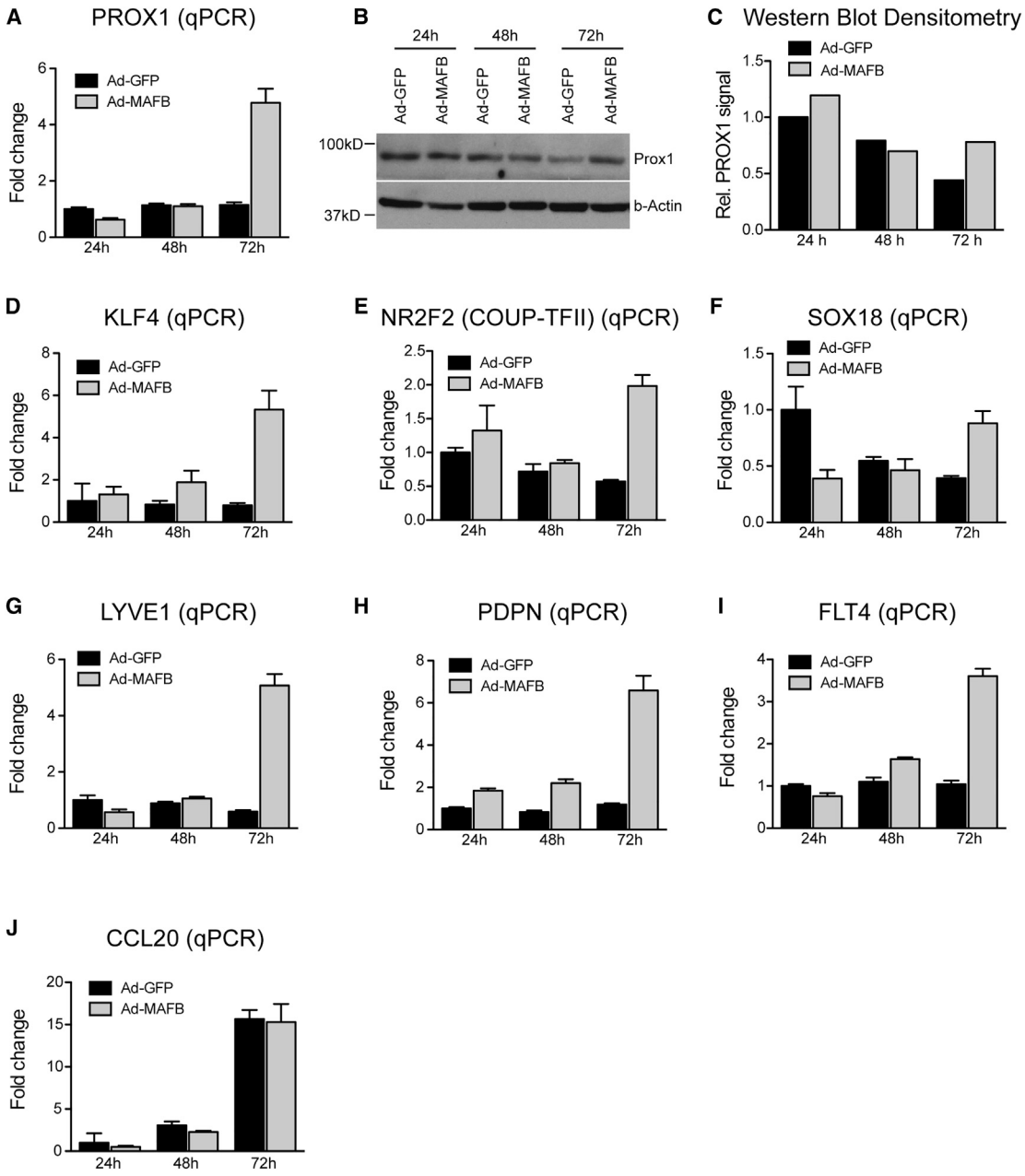

Figure 5. Overexpression of MAFB Upregulates Key Transcription Factors and Differentiation Markers in LECs

(A) Upregulation of *PROX1* mRNA in MAFB-overexpressing cells (gray bars) compared with control cells (black bars), determined by qPCR. One representative of three independent experiments is shown. Error bars represent mean + SD.

(B and C) Western Blot (B) and densitometric measurement of signal intensity (C) to determine PROX1 protein in MAFB-overexpressing LECs.

(D–J) qPCR data showing expression of *KLF4* (D), *NR2F2* (COUP-TFII) (E), *SOX18* (F), *LYVE1* (G), *PDPN* (H), *FLT4* (VEGFR-3) (I), and CCL20 (J) in LECs overexpressing MAFB compared with control cells. One representative of three independent experiments is shown. Error bars represent mean + SD.

See also Figure S5 and Table S3.

of *LYVE1, PDPN*, and *FLT4* (VEGFR-3) at one or more time points after infection (Figures 5G–5I). An unrelated control gene, CCL20, was not affected by MAFB overexpression (Figure 5J). Taken together, these data suggest that MAFB contributes to the regulation of the LEC phenotype by modulating the expression of multiple key TFs and marker genes of differentiated LECs.

MAFB Binds to Regulatory Elements in the Genes of Key LEC TFs

The effect of MAFB on the expression of LEC-associated TFs could have been direct through binding of MAFB to regulatory elements of the corresponding genes or indirect via induction of intermediate mediator genes. We next searched for potential

A *chr1:214169008 - 214169287 (PROX1 1st intron)*

..GCAGTGAATCCAACTTTGTAATAATTTGTCATTAAAAGGACATTATGAAAATGTATAAATATTCTTATA
GTTACATTAAGATATATCAACAGATATCATCTTCACCTATGATTTTACAAGTAAAAAATGCATAGCTAAG
CTAAATAAGCAGACTTATAAAATGACTATTGTGCATTTATTTCAATGCTAAACTGACCATTTATGTTTGA
AAGATGCTGCTGCTAAGGGTGTTCTCCTTCCCATTTTACATATGACAAAAATATTGTAAAATTCAAGAA..

chr9:110242741 - 110243060 (KLF4 promoter region)

..GGCAGGAGAATGGCTTGAACCCAGGTGGCGGAGGGTTGCAGTGAGCAGAGATTGCGCCACAGCACTGCA
GCCTGGGCGACAGAGCAACGTCTCAAAAAAAAAAAAAAAAAAAAAAAGACCAAGTGGAGAAAGACATTTGG
GCGAATGATTGCAACATGCTGACTCATCTAGGGTGTCGAGAAGACCGAAGCAATTTAAAAAAGTATATTA
TATCAAGGGCACCGTGGAAAACAATTAAAAAGATGGGCTCCAGATGCACATGACTCCAGAGAAGCAGTTT
GACTAATATTTCAACCCCATGCACCCGTTCCTCCCCAGCTA..

chr15:96817050 - 96817329 (NR2F2AS1 2nd intron)

..TCATGACACACTCCTGAGTCACTTTCCTAGACCCACTGATCTGGTTAGGACCCACCCACGCCGAACCAC
AGCTCCCTGTTAGAGCACGTTGCTGTCATCGCTCATTAATGGTCTTATCTCCTGCACTCAGCAGTGAGCT
CCACAGAACAGGAAGAGCATGCATCTTATTCACCTAATGCCTAATGCCTGATACAGTGCAGGGCACTTTT
AGGTGCTTAGTCATATTGGTGTGTTTCATTTGTGTTATGGTATGCAGCTACGTGTTGTAATTGTTGATTT
G..

chr20:62680411 - 62680730 (SOX18 2nd exon)

..CCCCCGCACCCCGCGGCCCGGGACCCCTGCCCCCCCCGCCCCACCCCGGCCCGAGCCCCCCCCGCCCTC
TCCCCCTTCTCTGCCGCCCTCCCGCCGCTCACCCAGCATCTTGCTGAGCACCGCGTTGTGCAGGTCCGGG
TTCTGCTGAGCCAGCCGCTTGCGCTCGTCCTTTGCCCACACCATGAAGGCGTTCATGGGCCGCCGGATGC
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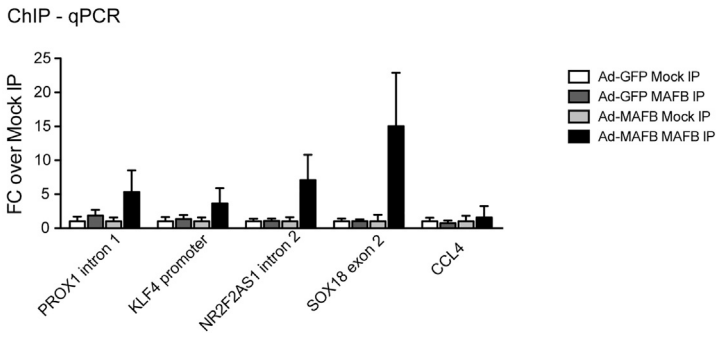

Figure 6. MAFB Binds to Regulatory Elements in the PROX1, KLF4, NR2F2AS1, and SOX18 Genes

(A) Sequences of ChIP-validated MAFF/MAFK binding areas according to ENCODE. Boxes indicate the most likely MAFB binding site as determined by JASPAR.

(B) qPCR showing enrichment of all four DNA areas shown in (A) in MAFB-immunoprecipitated chromatin compared with mock-immunoprecipitated chromatin from MAFB-overexpressing cells. Enrichment of the binding site in the *KLF4* gene could also be detected in control cells. A DNA stretch without MAFB bindings sites (CCL4) served as a negative control. One representative of three independent experiments is shown. Error bars represent mean + SD.

MAREs in the promoters and the coding regions of *PROX1*, *KLF4*, *NR2F2*, and *SOX18*. Recently, the Encyclopedia of DNA Elements (ENCODE) project published chromosome immunoprecipitation (ChIP) data for multiple transcription factors (ENCODE Project Consortium, 2011; Wang et al., 2012). Although MAFB itself was not investigated, the closely related MAFF and MAFK, which can bind to identical DNA sequences, are included. ChIP-validated MAFF/MAFK binding sites are present in the first intron of *PROX1*, the promoter region of *KLF4*, and the second exon of *SOX18*. No binding site was found within or in close proximity to the *NR2F2* gene. However, there is a MAFF binding site about 60 kb upstream of the TSS, in a region coding for an NR2F2 antisense RNA (*NR2F2AS1*). MAFB binding to this site might regulate expression of NR2F2 directly or indirectly through regulation of *NR2F2AS1*. We designed primers for the corresponding DNA elements (Figure 6A) and performed ChIP with a MAFB-specific antibody, followed by qPCR on LECs 72 hr after infection with Ad-MAFB (at the same time point when *PROX1* expression was induced). Ad-GFP-infected cells as well as mock immunoprecipitation (IP) with a nonspecific antibody served as controls. There was a clear ChIP signal for all four putative binding sites in MAFB-overexpressing cells, whereas a locus without a predicted MAFB binding site (*CCL4*) was not enriched (Figure 6B). We also repeatedly detected a slight enrichment of the suggested binding site in the KLF4 promoter in control cells (~1.3-to 2.2-fold over mock IP), indicating that this binding site is occupied by MAFB protein even in unstimulated, resting LECs. Although these findings do not preclude any indirect regulation of *PROX1*, *KLF4*, or *SOX18* by MAFB, they strongly suggest that MAFB regulates their expression by directly binding to *cis*-regulatory elements of the corresponding genes and that MAFB might regulate expression of *NR2F2* indirectly through *NR2F2AS1*.

MAFB Controls Lymphatic Patterning during Embryogenesis

We next investigated whether MAFB might also play a role in lymphatic vessel development in vivo. We studied lymphatic vessels in the embryonic back skin at E14.5, a time point when lateral lymphatic vessels are growing toward the dorsal midline in a highly stereotypical manner, in *Mafb*$^{-/-}$ mice and wild-type (WT) littermates. Using back skin whole mounts stained for NRP2 and PROX1 to identify lymphatic vessels (Figure 7A), we observed a slight trend toward an increased distance from the lymphatic vessel tips to the midline in *Mafb*$^{-/-}$ mice compared with WT littermates, indicating that lymphangiogenesis may be retarded (Figure 7B). There were also significant aberrations in the lymphatic patterning in the skin of *Mafb*$^{-/-}$ mice, with an increased number of vessel

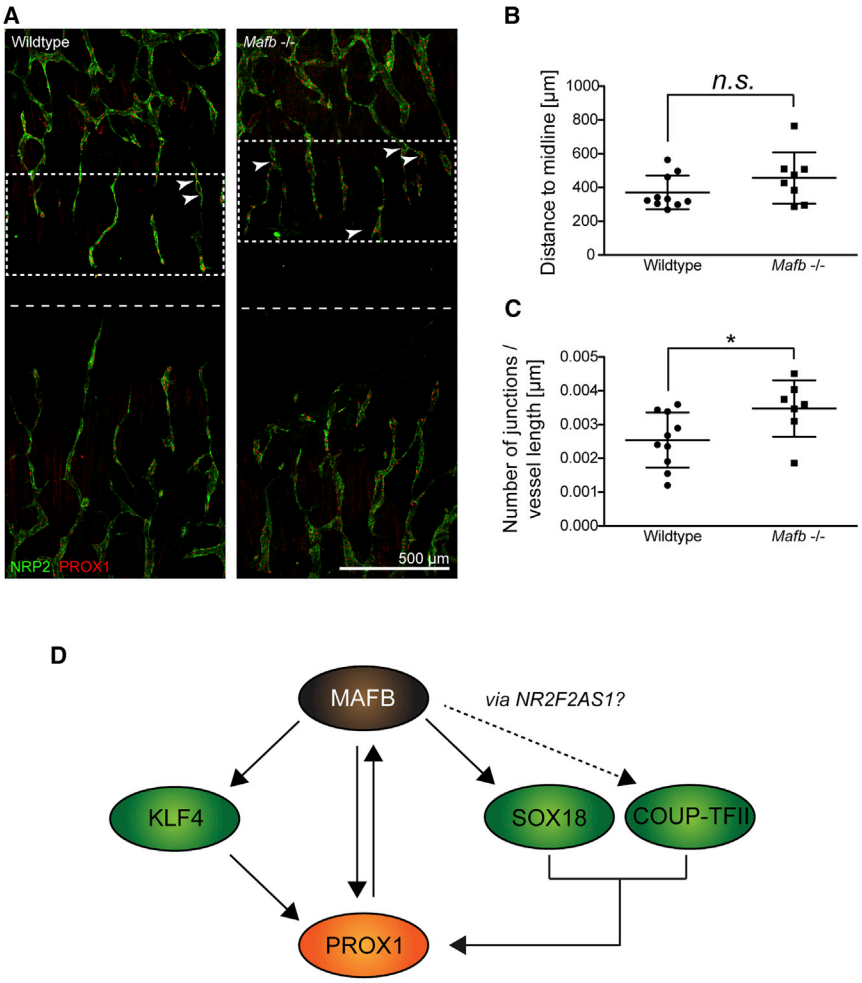

Figure 7. Lymphatic Patterning Is Impaired during Embryogenesis in *Mafb*$^{-/-}$ Mice

(A) Representative confocal images (maximum-intensity projections) of E14.5 embryonic back skin stained for NRP2 (green) and PROX1 (red). Dashed lines indicate the midline. Dashed boxes indicate the distal sprouting area, defined as a 500-µm-wide area from the lymphatic vessel tips toward the sides. Arrowheads point to vessel junctions (branch points) within the distal sprouting area.

(B) Average distance to the midline in wild-type (n = 10) and *Mafb*$^{-/-}$ (n = 7) embryos. n.s., not significant.

(C) Number of vessel junctions normalized to total vessel length in the distal sprouting area of wild-type (n = 10) and *Mafb*$^{-/-}$ (n = 7) embryos (*$p < 0.05$).

(D) Proposed model of MAFB involvement in the regulation of lymphangiogenesis. MAFB regulates PROX1 expression both directly and indirectly through KLF4, SOX18, and COUP-TFII upregulation, the latter possibly through the antisense RNA NR2F2AS1. PROX1, in turn, maintains MAFB expression in differentiated LECs.

See also Figure S6.

junctions and vessel segments, and a correspondingly decreased average segment length (Figure 7C; Figures S6D and S6E) measured in the "distal sprouting area," defined as the area spanning 500 µm from the lymphatic tips toward the sides (Figure 7A). There were no major differences in total lymphatic vessel length, vessel area, or number of PROX1+ cells (Figures S6A–S6C). No differences in the lymphatic vessels were found in the "proximal network area," defined as the area beginning 100 µm laterally from the distal sprouting area (Figures S6F–S6K). Taken together, these data show that MAFB is not absolutely required for lymphangiogenesis but involved in the fine patterning of growing lymphatic vessel tips in the embryonic back skin. *Mafb*$^{-/-}$ mice have a complex phenotype because MAFB is expressed in multiple cell types and tissues, including tissue macrophages, which might modulate lymphangiogenesis. However, we found that the number of LYVE-1+ macrophages was not changed in the back skin of *Mafb*$^{-/-}$ embryos (Figures S6L and S6M), in line with previous reports that macrophages in *Mafb*$^{-/-}$ mice only display slight phenotypic changes compared with WT mice (Aziz et al., 2006; Moriguchi et al., 2006), indicating that MAFB regulates lymphatic patterning most likely in a cell-autonomous fashion.

DISCUSSION

VEGFR-3 signaling plays a central role in lymphatic biology, both in the development of the lymphatic network during embryogenesis as well as in postembryonic lymphangiogenesis and the regulation of lymphatic function (reviewed in Koltowska et al., 2013; Yang and Oliver, 2014). Although the immediate signaling events downstream of receptor stimulation leading to activation of the MAPK and PI3K pathways have been investigated in great detail (Koch et al., 2011), little is known about how these signals are translated into a transcriptional response (Shin et al., 2008). Here, we comprehensively characterized immediate gene expression changes in LECs after VEGFR-3 stimulation using CAGE RNA sequencing in conjunction with the FANTOM5 project (http://fantom.gsc.riken.jp/5/). Because VEGF-C, the principal ligand of VEGFR-3, has been reported to bind to and activate VEGFR-2 (Joukov et al., 1997), we used VEGF-C156S, a mutated form of VEGF-C selectively binding to VEGFR-3 but not VEGFR-2 (Joukov et al., 1998), and followed changes in gene expression over a period of 8 hr by CAGE RNA sequencing. This analysis revealed that, of the genes upregulated by VEGF-C156S, a significant portion were TFs.

Many of these TFs are well known immediate-early TFs that are induced non-selectively by MAPK signaling in many cell types. However, when comparing with GSEA on other relevant stimulation experiments, we identified three TFs induced selectively in VEGF-C156S-stimulated LECs: KLF4, SOX18, and MAFB. Importantly, both SOX18 and KLF4 have been implicated previously in the regulation of PROX1 (François et al., 2008; Park

et al., 2014), the "master" TF of LECs required for differentiation and maintenance of the mature LEC phenotype (Srinivasan et al., 2014). SOX18 is necessary for the induction of PROX1 expression in venous endothelial cells (ECs) during embryonic development around E9.5, giving rise to a pool of progenitor cells (François et al., 2008). KLF4, on the other hand, has been shown to be induced transcriptionally by shear stress in endothelial cells and to bind to a regulatory element in the *PROX1* gene (Park et al., 2014). MAFB, the third specific TF identified by our analysis, has been implicated in the differentiation and function of various cell types, including macrophages, kidney podocytes, and pancreatic α and β cells (Friedman, 2007; Hamada et al., 2014; Hang and Stein, 2011; Yang and Cvekl, 2007).

Using the full power of CAGE RNA sequencing, which not only allows identification of differentially expressed genes but also of the precise TSSs used, we performed a statistical analysis of predicted transcription factor binding sites in the promoters of upregulated genes. Although not direct proof, significant enrichment of TF binding sites in the promoter regions of upregulated genes at a given time point is a strong indication that the corresponding TF is actively regulating gene expression. This analysis suggested significant activity of MAFB both during the early phase of the time course (45–60 min), concomitant with the upregulation of the *MAFB* gene, and at later phases (210 and 360 min). The significant enrichment of genes with degenerated MAREs (MAFB_2) already after 15 min of stimulation might result from activation of pre-existing MAFB protein in the LECs because post-transcriptional regulation of the transcriptional activity of MAF proteins has been described (Rocques et al., 2007; Tillmanns et al., 2007). On the other hand, because the degenerated MARE is less strictly defined as the MARE site, we cannot exclude that the significant enrichment of this site at the 15-min time point is a false positive of the analysis.

Our data suggest that MAFB, similar to SOX18 and KLF4, regulates expression of PROX1, implying that MAFB might contribute to the maintenance of the LEC phenotype and/or to lymphangiogenesis and that the regulation of PROX1 is mediated by direct binding of MAFB to a regulatory element in the first intron of the *PROX1* gene. However, indirect regulation through upregulation of KLF4, SOX18, and COUP-TFII cannot be excluded, especially because the MAFB-induced upregulation of KLF4 preceded that of PROX1. On the other hand, it is interesting to note that the reported binding site of KLF4 is also located in the first intron of *PROX1* (Park et al., 2014), suggesting that close cooperation of MAFB and KLF4 in the same *cis*-regulatory region is needed for *PROX1* induction. Of note, our observation that MAFB induces expression of KLF4 is in line with a previous report that demonstrated a similar relation between MAFB and KLF4 during epidermal development (Lopez-Pajares et al., 2015). We also found that MAFB expression depends on PROX1, indicating the existence of a positive feedback loop between these two TFs. Taken together, our findings suggest that a transcription factor network consisting of MAFB, KLF4, SOX18, COUP-TFII, and PROX1 contributes to the establishment and maintenance of the LEC phenotype during and/or after differentiation (Figure 7D). Indeed, we found impaired patterning of lymphatic vessels in the back skin of E14.5 embryos deficient of MAFB. Increased vessel branching supports a role for MAFB in the fine-tuning of lymphatic development. In addition, our data could also indicate that MAFB is involved in sensing of VEGF-C gradients, which are believed to direct lymphatic vessel growth during development. These findings are in line with the very recent observation in zebrafish that *mafba* was required for the migration of lymphatic precursors after their initial sprouting from the cardinal vein (Koltowska et al., 2015). On the other hand, the phenotype observed by us was much less severe than the one observed for *Prox1* heterozygous animals (Harvey et al., 2005; Srinivasan and Oliver, 2011), indicating that there is a significant redundancy among the function of MAFB and other TFs, such as KLF4, in regulating PROX1 expression.

Given the established interactions between SOX18 and VEGF-C signaling already at initial stages of lymphangiogenesis (Cermenati et al., 2013), MAFB deficiency may have additional effects on the generation or budding of LEC progenitors from the veins during earlier stages of embryonic development (E.9.5–E13.5). Furthermore, continuous expression of MAFB in lymphatic vessels into adulthood suggests a role in the function and/or the maintenance of the lymphatic system in the adult organism and, possibly, in the pathological lymphangiogenesis associated with inflammatory and neoplastic diseases. However, global *Mafb* knockout in mice results in lethality within 24 hr after birth because of a defect in the PreBöt-C complex in the hindbrain responsible for correct breathing (Blanchi et al., 2003; Moriguchi et al., 2006), precluding any morphological and functional studies in postnatal mice. Therefore, it would be of interest to investigate the consequences of a conditional, targeted knockout of *Mafb* only in lymphatic endothelial cells in the future.

EXPERIMENTAL PROCEDURES

Animals

C57BL/6 WT mice (Charles River Laboratories) and heterozygous *Mafb*^{tm1Jeng} mice on the C57BL/6 background (Moriguchi et al., 2006) (provided by Dr. Satoru Takahashi, Institute of Medical Sciences, University of Tsukuba, Japan) were bred in-house under pathogen-free conditions. For lymphatic vessel analysis, E14.5 *Mafb*$^{-/-}$ and WT littermates derived from heterozygous mating pairs were used. All animal experiments were approved by the Veterinäramt of Kanton Zurich.

Cells

Primary human dermal microvascular LECs and donor-matched BECs from foreskin (Hirakawa et al., 2003) were used. For the RNA sequencing screen, early-passage LECs from three individual donors were starved overnight (O/N), stimulated with VEGF-C156S (1.5 μg/ml, a gift from Prof. Kari Alitalo, Wihuri Research Center, University of Helsinki), and lysed at 16 different time points (0–480 min) using TRIzol (Life Technologies). For other stimulation experiments, LECs and BECs were starved O/N and stimulated with VEGF-A (Cell Sciences), EGF, fibroblast growth factor 2 (FGF-2) (both from PeproTech), and HGF (R&D Systems) at the indicated doses.

CAGE RNA Sequencing and Analysis

RNA was extracted from TRIzol lysates according to the manufacturer's instructions (Life Technologies). Genomic DNA was removed using g-eliminator columns (QIAGEN), and RNA quality was monitored using a bioanalyzer (Agilent). RNA samples were sequenced according to the CAGE method at the RIKEN Institute (Kanamori-Katayama et al., 2011; Shiraki et al., 2003), using the HeliScope CAGE protocol. Gene expression analysis was performed using edgeR (Robinson et al., 2010). A list of 241 upregulated genes (encompassing

50 TFs) is provided in Table S1. GO terms enrichment analysis for the corresponding genes was performed using the Database for Annotation, Visualization, and Integrated Discovery (DAVID) (http://david.abcc.ncifcrf.gov/home.jsp). For adenovirus-infected LECs, gene expression was analyzed using DESeq2 (Love et al., 2014). Differentially expressed genes are summarized in Table S3. The entire gene expression data are available at http://fantom.gsc.riken.jp/5/datafiles/phase2.0/extra (VEGF-C stimulation of LECs) and at http://fantom.gsc.riken.jp/5/suppl/Dieterich_et_al_2015/ (MAFB overexpression in LECs).

GSEA Datasets

To identify TFs that were specifically upregulated by VEGFR-3 stimulation in LECs, we compared our dataset with other stimulatory time courses from the GSEA Molecular Signatures Database (http://www.broadinstitute.org/gsea/msigdb/): VEGF-A stimulation of HUVECs (Abe and Sato, 2001), EGF stimulation of HeLa and MCF10A cells (Amit et al., 2007), and EGF stimulation of MCF7 cells (Nagashima et al., 2007).

oPOSSUM-3 Transcription Factor Activity Prediction

For prediction of TF activity, we used oPOSSUM-3, a tool looking for predicted TF binding site overrepresentation in DNA sequences (Kwon et al., 2012). Using TSSs defined by CAGE sequencing associated with refSeq transcripts, we extracted differentially expressed refSeq transcripts using edgeR ($p < 0.01$) at each time point *t* compared with time point *t-1*. Then the oPOSSUM-3 tool was applied to core promoter sequences (1, 500 bp upstream to 500 bp downstream of the TSS) of each transcript using TF binding profiles from the JASPAR database (http://www.jaspar.genereg.net) (Mathelier et al., 2014). TF binding profiles were defined as significantly overrepresented when their corresponding *Z* score or Fisher score was more than the mean + 1.5 × SD.

siRNA Knockdown of PROX1

LECs were electroporated with two different PROX1-specific siRNAs using the Nucleofactor II kit (Amaxa Biosystems), and RNA was extracted 48 hr later for qPCR analysis.

In Vitro Overexpression of MAFB

For overexpression of MAFB, we used ready-made adenoviral vectors (Sirion Biotech) with the human *MAFB* cDNA under control of the cytomegalovirus (CMV) promoter (Ad-MAFB). A CMV-GFP (Ad-GFP) vector served as a control. For transduction, LECs were infected with adenovirus at an MOI of 25. 4 hr later, the medium was replaced, and cells were harvested at the indicated time points.

Isolation of Primary Mouse Endothelial Cells

For isolation of primary mouse endothelial cells, back skin of C57BL/6 mice was excised, minced, and digested with collagenase (Sigma-Aldrich). Cells were depleted of erythrocytes using PharmLyse buffer (BD Biosciences) and stained with primary antibodies (mouse anti-mouse CD45.2-FITC [BD Biosciences], rat anti-mouse CD31-APC [BD Biosciences], and hamster anti-mouse podoplanin [Developmental Studies Hybridoma Bank, University of Iowa]), followed by secondary antibody (goat anti-hamster phycoerythrin [PE], Life Technologies). LECs and BECs were gated and sorted as described previously (Halin et al., 2007).

ChIP

ChIP of LECs 72 hr after infection with Ad-MAFB or Ad-GFP was performed essentially as described before (Nelson et al., 2006). Chromatin lysates were incubated with rabbit anti-MAFB (Sigma-Aldrich) or corresponding control rabbit immunoglobulin G (IgG) (Santa Cruz Biotechnology) (mock IP), cleared by centrifugation, and incubated with Protein G Sepharose (GE Healthcare). Pellets were collected by centrifugation and washed six times with IP buffer, and then DNA was extracted by boiling in a Chelex100 slurry (Bio-Rad). Primers were designed for selected ChIP-validated MAFF/MAFK binding sites (Wang et al., 2013) in the genes of *PROX1*, *KLF4*, *SOX18*, and *NR2F2AS1*. qPCR was performed to measure these DNA elements in specific and mock (control IgG) IPs, with a gene without known MAFB binding site (CCL4) serving as a negative control. Ct values were expressed as fold change compared with mock IP.

Tissue Whole-Mount Staining

Tissue whole mounts were fixed with paraformaldehyde (PFA), blocked in blocking solution (5% donkey serum, 0.2% BSA, 0.3% Triton X-100, and 0.05% NaN_3 in PBS), and stained with primary antibodies (in blocking solution) O/N at 4°C, followed by washing in PBS and incubation with secondary antibody (in PBS) for 2 hr at room temperature (RT).

Primary antibodies were as follows: rat anti-CD31 (BD Biosciences), rabbit anti-LYVE1 (Angiobio), rabbit anti-PROX1 (Angiobio), goat anti-PROX1 (R&D), goat anti-NRP2 (R&D), goat anti-CD206 (R&D), rat anti-CD68 (Abcam), and rabbit anti-MAFB (Sigma-Aldrich). Secondary antibodies were as follows: donkey anti-goat Al488, donkey anti-rat Al488, donkey anti-rabbit Al594, donkey anti-goat Al594, and donkey anti-rabbit Al647 (all from Life Technologies). Confocal images (z stacks) were taken with an LSM710 or LSM780 microscope (Zeiss), and maximum intensity projections, 3D reconstructions, and image analysis were made with ImageJ.

Statistics

All graphs and statistical tests were done using GraphPad Prism. If not stated differently, error bars represent mean values + SD. Student's t test was used to detect significant differences ($p < 0.05$).

More detailed methods can be found in the Supplemental Experimental Procedures.

ACCESSION NUMBERS

Gene expression data are available at http://fantom.gsc.riken.jp/5/datafiles/phase2.0/extra (VEGF-C stimulation of LECs) and at http://fantom.gsc.riken.jp/5/suppl/Dieterich_et_al_2015/ (MAFB overexpression in LECs).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures, and three tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.10.002.

AUTHOR CONTRIBUTIONS

L.C.D. and S.K. designed and performed the experiments, analyzed and interpreted the data, and wrote the paper. A.M. and W.W.W. performed and interpreted the in silico analyses. A.P., Q.M., and Y.K.H. performed the experiments. M.H. managed mouse breeding and supplied embryos and live mice. J.W.S. designed the experiments. T.L. was responsible for all data processing pipelines and developed the mapping algorithm. M.I. was responsible for CAGE data production. H.K. managed data handling. M.L. carried out the CAGE data analysis on the MAFB overexpression samples. E.A. carried out quality control on the libraries. P.C., Y.H., C.O.D., and A.R.R.F., were responsible for FANTOM5 phase2 management. M.D. designed the experiments, interpreted the data, and wrote the paper.

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WEB RESOURCES

The URLs for data presented herein are as follows:

Database for Annotation, Visualization, and Integrated Discovery (DAVID): http://david.abcc.ncifcrf.gov/home.jsp

FANTOM5 project: http://fantom.gsc.riken.jp/5/

GSEA Molecular Signatures Database (MSigDB): http://www.broadinstitute.org/gsea/msigdb/

JASPAR database: http://www.jaspaer.genereg.net

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