

Chapter 2

Transcriptional Control of Lymphatic Endothelial Cell Type Specification

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Abstract The lymphatic vasculature is the “sewer system” of our body as it plays an important role in transporting tissue fluids and extravasated plasma proteins back to the blood circulation and absorbs lipids from the intestinal tract. Malfunction of the lymphatic vasculature can result in lymphedema and obesity. The lymphatic system is also important for the immune response and is one of the main routes for the spreading of metastatic tumor cells. The development of the mammalian lymphatic vasculature is a stepwise process that requires the specification of lymphatic endothelial cell (LEC) progenitors in the embryonic veins, and the subsequent budding of those LEC progenitors from the embryonic veins to give rise to the primitive lymph sacs from which the entire lymphatic vasculature will eventually be derived. This process was first proposed by Florence Sabin over a century ago and was recently confirmed by several studies using lineage tracing and gene manipulation. Over the last decade, significant advances have been made in understanding the transcriptional control of lymphatic endothelial cell type differentiation. Here we summarize our current knowledge about the key transcription factors that are necessary to regulate several aspects of lymphatic endothelial specification and differentiation.

2.1 Overview of the Stepwise Process Leading to the Formation of the Lymphatic Network

Mammals have two interdependent circulatory systems—the blood vasculature and the lymphatic vasculature. Although detailed descriptions of the blood vascular system were available as early as the sixth century BC, those of the lymphatic vasculature were in the seventeenth century AD by Asellius. In contrast to the

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function of the blood vasculature in transporting blood throughout the body, the lymphatic vasculature is essential for maintaining interstitial fluid homeostasis. The main physiological functions of the lymphatic vasculature include draining and returning fluid from the extracellular tissue spaces back to the blood circulation, absorbing lipids from the intestinal tract and tissues, and transporting immune cells to lymphoid organs.

During the formation of the blood vascular network, the Notch signaling pathway is required to promote arterial cell fate differentiation (De Val and Black 2009; Kokubo et al. 2005; Lawson et al. 2001). On the other hand, the orphan nuclear receptor *COUP-TFII* promotes venous fate differentiation by inhibiting Notch signaling and other arterial specification genes (You et al. 2005).

Studies on the lymphatic network in the past decade have brought important advances in the understanding of the molecular events that lead to the development of the lymphatic vasculature. At least in the mammalian embryo, the formation of the lymphatic vasculature is a stepwise process that is closely associated with the venous vasculature. The pioneering work by Florence Sabin at the beginning of the last century proposed that the lymphatic vasculature arises from the embryonic veins (Sabin 1902). Detailed lineage tracing analysis performed in mouse embryos almost a century later confirmed Sabin's prediction that lymphatic endothelial cells (LECs) have a venous origin (Srinivasan et al. 2007). Therefore, a key prerequisite for the genesis of the lymphatic network is the prior formation of the blood vasculature.

In the mouse embryo, the process leading to the formation of the entire lymphatic vascular network starts inside the cardinal vein (CV) and intersomitic vessels at around embryonic day 9.5 (E9.5) (Srinivasan et al. 2007; Wigle and Oliver 1999; Yang et al. 2012; Hagerling et al. 2013) (Fig. 2.1a). As discussed in detail later, at this stage a subpopulation of venous ECs become specified into the lymphatic lineage by acquiring a very specific molecular footprint. Concomitant with their stepwise loss of venous fate, these ECs will gain lymphatic fate and as such they should be considered LEC progenitors. The formation of the entire lymphatic vascular network is mediated by intermediate structures called lymph sacs. Lymph sacs are formed when most of the LEC progenitors bud off from the veins and migrate into the surrounding mesenchyme. Electron microscopy and immunostaining studies have revealed that adhesion junctions between the venous endothelial cells (VECs) and LEC progenitors are important for maintaining vein integrity during the budding process (Yang et al. 2012; Yao et al. 2012) (Fig. 2.2). As part of the stepwise differentiation process, as they bud off, LEC progenitors start expressing additional genes (e.g., podoplanin) and differentiate into more mature LECs outside the veins (Yang et al. 2012) (Fig. 2.1a, b). Importantly, at the level of the junction of the jugular and subclavian veins, a small fraction of LEC progenitors will remain inside the CV and contribute to the formation of the lymphovenous valves, which are the main valves responsible for the unidirectional return of lymph fluid into the blood circulation (Srinivasan and Oliver 2011) (Fig. 2.1b, d).

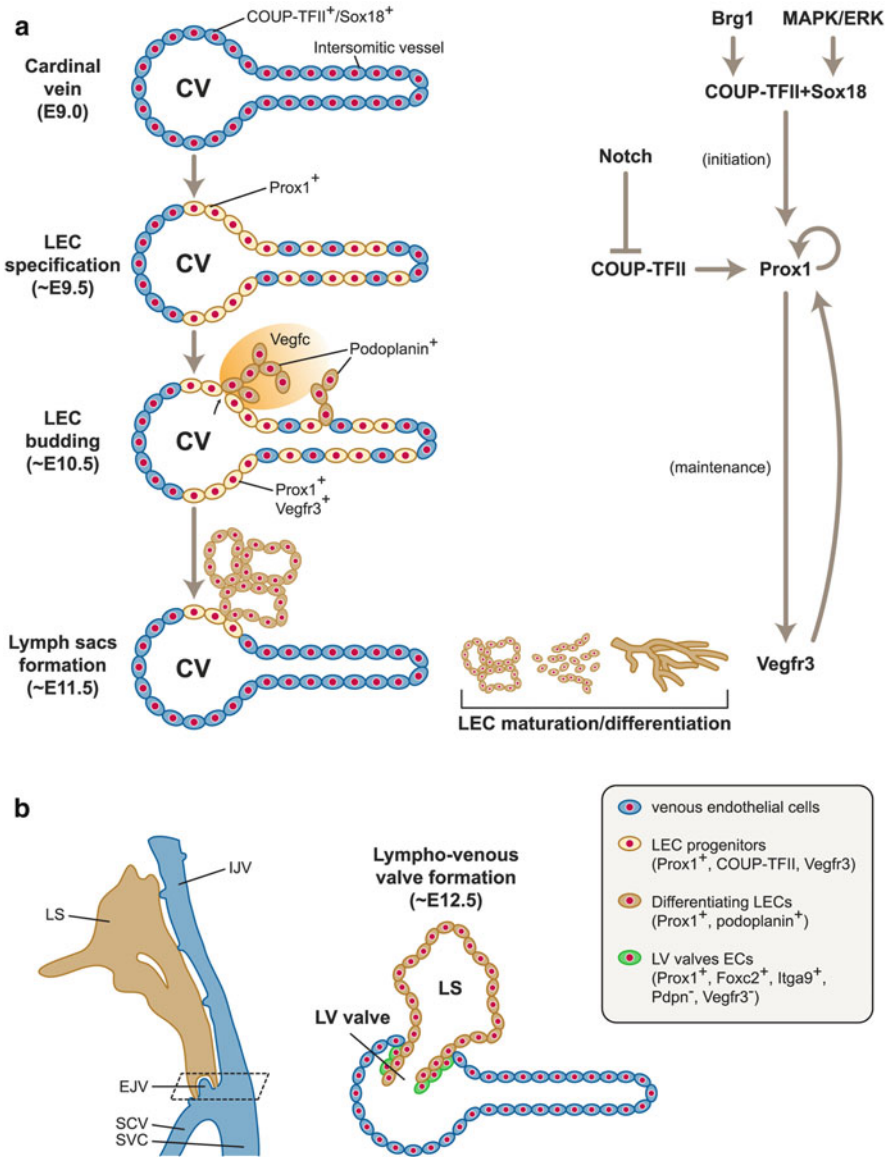


Fig. 2.1 Schematic representation of the stepwise process leading to the formation of the mammalian lymphatic vasculature. **(a)** The cardinal vein (CV) is the source from where LECs are going to be specified. Early during development, the CV expresses the transcription factors *COUP-TFII* and *Sox18*. Together, the activity of these factors will be necessary to induce the expression of *Prox1* in a subpopulation of venous ECs. The initiation of *Prox1* expression at around E9.5 is an indication that LEC specification started and the venous *Prox1*-expressing ECs should be considered as LEC progenitors. Approximately a day later (E10.5), most of those progenitors will start to bud off from the CV. This process is guided by the graded expression of *Vegfc* in the surrounding mesenchyme. LECs will bud off from the CV and intersomitic vessels as a chain of interconnected cells. *Vegfr3* expression in LECs is maintained by *Prox1*. As soon as LECs bud from the vein they start to express podoplanin, an indication that lymphatic maturation

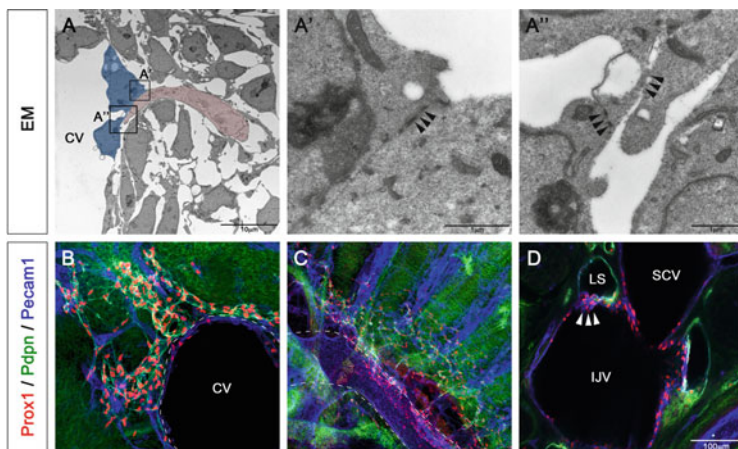


Fig. 2.2 *Prox1*-expressing LEC progenitors bud from the CV (a, a', a''). EM analysis of E10.5 *Prox1*^{+/LacZ} embryos showing that *Prox1*-expressing cells (pink) exit the CV via an active budding mechanism. The budding cell is attached to the venous endothelial cells (blue) by adhesion junctions (arrowheads in (a') and (a'')). Panels (a', a'') are high-power magnifications of the black boxed area in (a). (b) Podoplanin expression is only detected once *Prox1*-expressing LEC progenitors fully exit the CV (dashed line) at around E11.5. (c) *Prox1*-expressing LEC progenitors that bud off from the CV (dashed line) and ISVs migrate as an interconnected group of cells dorsally and longitudinally into the surrounding mesenchymal tissue at approximately E11.0 in the anterior region of the embryo. (d) A small subpopulation of *Prox1*-expressing venous ECs remain in the veins and form the lymphovenous valves at the junction of the jugular and subclavian veins at E15.5 (arrowheads in (d)). These valve cells are negative for podoplanin. CV cardinal vein, IJV internal jugular vein, SCV subclavian vein. Scale bars: 10 μm (a), 1 μm (a', a''), 100 μm (b-d)

Vascular endothelial growth factor C (*Vegfc*) signaling is indispensable for the budding process. A newly identified player in this pathway, *Ccbe1*, is also required for LEC budding (Bos et al. 2011; Hogan et al. 2009). In the absence of either molecule, LEC progenitors fail to bud off from the embryonic veins (Karkkainen et al. 2004; Hagerling et al. 2013; Bos et al. 2011). Recent studies have shown that as LECs bud off from the veins, they remain interconnected to each other during migration (Yang et al. 2012; Hagerling et al. 2013) (Fig. 2.2c). These interconnected LECs form strings that gradually organize into the lumenized structures called lymph sacs. Earlier studies argued that by sprouting and remodeling, the lymph sacs and the early lymphatic plexus give rise to the mature

Fig. 2.1 (continued) and differentiation is progressing. As LECs bud, they will start to form the different lymph sacs (starts at around E11.5), intermediate structures from which upon maturation and differentiation, the entire lymphatic network will be formed. (b) Although most *Prox1*-expressing LEC progenitors will move out from the veins, a small fraction located at the junction of the internal (IJV) or external jugular veins (EJV) with the subclavian vein (SCV) will remain and upon their intercalation with lymph sac (LS) LECs, they will help to form the lymphovenous valves

lymphatic vasculature, including both superficial lymphatics and the thoracic duct (TD) (Sabin 1902; van der Putte 1975; Oliver 2004). However, digital three-dimensional reconstructions using ultramicroscopy suggest a different model. According to this new model, simultaneous with the formation of primitive lymph sacs, LECs accumulate at the first lateral branch of the intersomitic vessels to form a peripheral longitudinal lymphatic vessel that will give rise to the superficial lymphatic plexus while the primitive lymph sacs will later develop into the TD (Hagerling et al. 2013). After the primitive lymphatic vessels are formed, they will further differentiate into larger collecting vessels and smaller capillaries, which are the two predominant types of vessels of the lymphatic vasculature. Lymphatic capillaries absorb interstitial fluid and carry this lymph toward the larger collecting lymphatic vessels. In the collecting lymphatics, valves form to prevent the backflow of the lymph and separate the lymphatic vessels into functional units called lymphangions (Sacchi et al. 1997; Casley-Smith 1980).

2.2 Transcriptional Control of Lymphatic Specification and Differentiation

2.2.1 *Sox18 and COUP-TFII Initiate Lymphatic Endothelial Cell Transcriptional Profiling*

So far, only a few transcription factors critical during LEC specification and differentiation have been identified, and how these genes are regulated and how they interact with other critical signaling pathways (e.g., *Vegfc-Vegfr3*, MAPK/ERK, and Notch signaling) remain to be fully elucidated.

Many transcription factors play an important role in regulating blood endothelial cell (BEC) fate differentiation. Given the common origin and close relationship of venous and lymphatic endothelial cells, it is not surprising that at least some venous endothelial transcription factors are also required for the development of the lymphatic vasculature. For instance, *Sox18* encodes an SRY-type HMG box transcription factor and is a member of the SOX gene family. Mutations in *SOX18* in humans are associated with hypotrichosis–lymphedema–telangiectasia (Irrthum et al. 2003). *Sox18* is expressed in the vascular endothelium and hair follicles in mouse embryos (Pennisi et al. 2000b). Point mutations in *Sox18* result in cardiovascular and hair follicle defects in mice; as a consequence, these mice are known as *ragged* mice (Carter and Phillips 1954; Slee 1957a, b). On the other hand, *Sox18* knockout mice display a milder coat defect and no obvious cardiovascular defects in a mixed genetic background (Pennisi et al. 2000a).

Some evidence supports that other Sox transcription factors have redundant functions. For example, *Sox18* and *Sox7* can compensate for the loss of each other in arteriovenous specification in zebrafish (Herpers et al. 2008; Cermenati et al. 2008; Pendeveille et al. 2008). In the mouse, the expression of *Sox18* in

Table 2.1 Key players in the lymphatic endothelial transcriptional network

Gene	Binding sites in enhancers or promoters	Binding site validation	Interaction protein	References
<i>Sox18</i>	Ets1; Egr1	N/A	N/A	Deng et al. (2013)
<i>COUP-TFII</i>	Brg1	ChIP	Prox1	Davis et al. (2013), Lee et al. (2009), Yamazaki et al. (2009)
<i>Prox1</i>	SOXF (Sox18); COUP-TFII; miR-181; miR-31	EMSA; ChIP; Tg; TA	COUP-TFII; Ets2; NF-κB	Francois et al. (2008), Srinivasan et al. (2010)
<i>Vegfr3</i>	Prox1; Ets2; Tbx1	ChIP; TA	N/A	Yoshimatsu et al. (2011), Chen et al. (2010)
<i>Nrp2</i>	Sp1 (COUP-TFII)	ChIP; TA	N/A	Lin et al. (2010)
<i>Foxc2</i>	N/A	N/A	NFATc1	Norrmén et al. (2009)
<i>NFATc1</i>	N/A	N/A	Foxc2	Norrmén et al. (2009)
<i>Gata2</i>	ETS; E box (Tal1)	EMSA; Tg	N/A	Khandekar et al. (2007)

The list includes validated binding sites and interaction proteins for lymphatic endothelial transcription factors. Relevant references for each promoter/enhancer and interaction protein are listed. *EMSA* gel shift assay, *ChIP* chromatin immunoprecipitation, *Tg* transgenic embryos, *TA* trans-activation assays in cell culture, *N/A* not applicable

vascular endothelial cells starts as early as E7.5 in the allantois and yolk-sac blood islands (Pennisi et al. 2000b). *Sox18* expression in ECs in the CV is detected at around E9.0, but gets downregulated in LECs at around E14.5 and is not maintained in the adult lymphatic vasculature (Francois et al. 2008). *Sox18* is indispensable for generating venous LEC progenitors via its activation of the critical transcription factor *Prox1* (prospero homeobox 1, *Drosophila prospero*-related vertebrate gene) in VECs (Francois et al. 2008). However, this lack of LEC progenitors in *Sox18* mutant mice has only been observed in a pure C57BL/6 (B6) background; *Sox7* and *Sox17* functionally substitute for *Sox18* in a mixed background (Hosking et al. 2009). A 4 kb *Prox1* promoter region that is sufficient to recapitulate *Prox1* expression in vivo contains two conserved *SoxF* binding sites that in vitro can be bound directly by *Sox18* (Francois et al. 2008) (Table 2.1). These results show that *Sox18* is a direct upstream activator of *Prox1* in ECs in the CV, and as such, also an important early player in the acquisition of LEC progenitor fate. Previously, the MAPK/ERK pathway was shown to regulate lymphatic vessel growth by modulating *Vegfr3* expression in mice (Ichise et al. 2010). A recent report revealed that MAPK/ERK signaling might be responsible for activating *Sox18* expression in the CV (Deng et al. 2013). Normally, induction of *Prox1* expression by *Sox18* occurs only in the embryonic veins (i.e., not in the arteries). This specificity could be because arteries fail to express certain specific factors required to activate *Prox1* in combination with *Sox18* (Francois et al. 2008) or because they express certain genes (repressors) that inhibit *Prox1* activation. The finding that gain of function of

the MAPK/ERK signaling component RAF1 in ECs induces an abnormal expression level of *Sox18* in the veins and dorsal aorta, which in turn activates *Prox1* expression in these vessels (Deng et al. 2013), argues that aberrantly activated MAPK/ERK signaling either inhibits arterial-specific repressor(s) or turns on the expression of venous *Sox18* coactivators in the arteries.

As mentioned above, in addition to *Sox18*, other coactivators are necessary to induce *Prox1* expression in the veins and initiate the specification of LEC progenitors. The chicken ovalbumin upstream promoter-transcription factor II (*COUP-TFII*) is a venous cofactor that can bind to the *Prox1* promoter and activate its expression (Srinivasan et al. 2010). *COUP-TFII* is a member of the steroid/thyroid hormone receptor superfamily and is highly expressed in the mesenchymal tissue as well as in the blood vascular endothelium during development (Pereira et al. 1995). Within the vasculature, its expression is restricted to the veins, where its activity is required to promote and maintain venous identity by inhibiting Notch activity in VECs, thus blocking the arterial transcriptional program (You et al. 2005). A recent study reported that *COUP-TFII* activity in the veins can be regulated epigenetically by the chromatin-remodeling enzyme gene *Brg1*, a member of the SWI/SNF protein family (Davis et al. 2013). *Brg1* remodels the chromatin structure of the *COUP-TFII* promoter region by direct binding, thereby preventing the access of the transcriptional machinery to that region (Davis et al. 2013). Interestingly, *COUP-TFII* is not only essential for venous cell fate differentiation but is also involved in the specification of LEC progenitors during lymphatic vasculature development. Deletion of *COUP-TFII* expression from LEC progenitors causes a drastic reduction in the number of LECs (Srinivasan et al. 2007). Moreover, *COUP-TFII* directly binds conserved binding sites located approximately 9.5 kb upstream of the *Prox1* open reading frame (ORF) (Srinivasan et al. 2010) (Table 2.1). These results argue that *COUP-TFII* is another direct in vivo regulator of *Prox1* expression during the early phases of LEC specification in the CV (Fig. 2.1). The maintenance of *Prox1* expression in LECs also requires *COUP-TFII* (see below). In addition, conditional inactivation of *COUP-TFII* at different embryonic stages revealed that its activity is not only essential for the specification of LEC progenitors but also for the sprouting of dermal lymphatic capillaries prenatally (Lin et al. 2010). In the absence of *COUP-TFII*, the lymphatic capillaries failed to form filopodial extensions projecting from the vessels. This function of *COUP-TFII* is via direct transcriptional regulation of *Nrp2*, a coreceptor for *Vegfc* in LECs (Lin et al. 2010; Xu et al. 2010) (Table 2.1). However, although *COUP-TFII* is expressed in LECs throughout life, its activity is not required to maintain quiescent lymphatic vessels in the adult, as normal lymphatic function remains intact when *COUP-TFII* is inactivated in adult mice (Lin et al. 2010). Thus, *Sox18* and *COUP-TFII* are two transcription factors crucial to initiate *Prox1*-mediated LEC progenitor specification in the veins. Although both *Sox18* and *COUP-TFII* bind to the *Prox1* promoter to induce its expression, either one is sufficient to activate *Prox1* expression by itself. However, how these two transcription factors interact with each other in this process remains uncertain.

2.2.2 *Prox1* Regulates LEC Progenitor Specification and Differentiation

It is widely accepted that LEC specification begins with *Prox1* expression in VECs at around E9.5 (Oliver 2004) (Fig. 2.2). *Prox1* (prospero homeobox 1, vertebrate gene related to *Drosophila prospero*) (Oliver et al. 1993) was the first lymphatic endothelial transcription factor to be identified (Wigle and Oliver 1999). Although *Prox1* is expressed in many other tissue types such as the central nervous system, lens, heart, liver, and pancreas (Oliver et al. 1993), its expression in the vascular system is restricted to lymphatic ECs. *Prox1* begins to be expressed in a subpopulation of ECs on the anterior CV (LEC progenitors) at around E9.5 and its expression is maintained in LECs through life (Wigle et al. 2002; Wigle and Oliver 1999). Functional inactivation of *Prox1* in mice showed that in the absence of *Prox1* expression, the embryo was devoid of the entire lymphatic vasculature and died at around E14.5 (Wigle and Oliver 1999). It was later shown that conditional deletion of *Prox1* at any developmental or postnatal stage leads to the loss of lymphatic-specific gene expression and the concomitant upregulation of BEC-specific genes in LECs (Johnson et al. 2008). These findings show that *Prox1* activity is required for the formation of LEC progenitors and for the maintenance of LEC identity at all developmental stages, including adulthood. Furthermore, it was recently shown that *Prox1* activity is also necessary for LECs to bud from the embryonic veins (Yang et al. 2012). Finally, several in vitro gain-of-function studies indicated that ectopic expression of *Prox1* in BECs is able to initiate a mature lymphatic-specific gene expression (e.g., *Vegfr3* and *podoplanin*) while suppressing BEC-specific genes (Hong et al. 2002; Petrova et al. 2002). Taken together, these studies indicate that *Prox1* is a key transcriptional regulator not only during the specification of LEC progenitors but also during LEC differentiation.

As *Prox1* is a central regulator of lymphatic endothelial transcription, its expression is tightly regulated. Several lines of evidence suggest that *Prox1* dosage is crucial for its function. In most genetic backgrounds, haploinsufficiency of *Prox1* causes perinatal death and pups exhibit characteristics of lymphatic dysfunction (e.g., chylothorax and chylous ascites) (Harvey et al. 2005). In other backgrounds, a small proportion of *Prox1* heterozygous animals survive to adulthood; however, their lymphatic vessels are mispatterned and leaky, and mice develop adult-onset obesity (Harvey et al. 2005). All *Prox1* heterozygote embryos display edema and occasionally develop blood-filled lymphatics (Srinivasan and Oliver 2011; Harvey et al. 2005). In these embryos, the number of LEC progenitors is significantly decreased and lymphovenous valves are absent (Srinivasan and Oliver 2011). Detailed analyses of *Prox1* null embryos in which the ORF of *Prox1* was replaced with either *LacZ* or *GFP* reporter gene constructs revealed that *Prox1* is essential for maintaining its own expression in LEC progenitors; this autoregulation is crucial for LEC identity (Wigle et al. 2002; Srinivasan et al. 2010). At the molecular level, in vitro studies suggest a physical interaction between *COUP-TFII* and *Prox1*

in LECs (Lee et al. 2009; Yamazaki et al. 2009). Similarly, a recent study demonstrated that *COUP-TFII* homodimers induce VEC fate by repressing the Notch target genes *HEY1/2*, whereas *COUP-TFII/Prox1* heterodimers induce or are permissive for the expression of a subgroup of LEC-specific genes (Aranguren et al. 2013). Comprehensive phenotype analyses showed that in *COUP-TFII/Prox1* double heterozygotes embryos, the loss of *COUP-TFII* aggravates the lymphatic defects of *Prox1* heterozygotes (Srinivasan et al. 2010). Compared with *Prox1* heterozygote embryos, the number of LECs and the expression level of *Prox1* are further reduced in *COUP-TFII/Prox1* double heterozygotes (Srinivasan et al. 2010). This result argues that the activity of *COUP-TFII* is required to maintain *Prox1* expression in LECs and that the amount of *COUP-TFII/Prox1* protein complex is important to regulate *Prox1* expression in a dosage-dependent manner (Srinivasan et al. 2010; Srinivasan and Oliver 2011). In addition, embryos with a mutated *Prox1* nuclear hormone receptor-binding site in which the interaction between *Prox1* and *COUP-TFII* is abolished displayed similar LEC specification defects (Srinivasan et al. 2010). Taken together, these results support that the *COUP-TFII/Prox1* interaction is required to maintain *Prox1* expression in LEC progenitors and, therefore, LEC identity during the LEC specification stage. Once LEC progenitors are specified and start to differentiate as they bud off from the CV, *COUP-TFII* activity is no longer required to maintain *Prox1* expression (Srinivasan et al. 2010).

Since *COUP-TFII* is a crucial regulator in both venous and lymphatic specification (by suppressing Notch activity and triggering *Prox1* expression in the veins), it can be speculated that Notch signaling may also be involved in lymphatic vasculature development. It is well known that Notch signaling is critical for the development of the blood vasculature, including arteriovenous specification and angiogenic sprouting (Gridley 2010; Roca and Adams 2007). However, until recently the role of Notch signaling in lymphatic vasculature development remained a matter of debate, mainly because there were no conclusive data demonstrating the presence of either in vivo expression of Notch pathway components in LECs or in vivo functional studies. For example, in vivo deletion of *Rbpj* (a key mediator of Notch signaling) in ECs did not result in LEC specification defect in the embryos (Srinivasan et al. 2010). On the other hand, in vitro studies showed that ectopic expression of an activated Notch receptor in cultured LECs repressed *Prox1*, *COUP-TFII*, and the mature lymphatic marker podoplanin through Hey proteins (downstream effectors of Notch signaling) (Kang et al. 2010). Likewise, addition of soluble Jag1 or Dll4 recombinant protein (Notch ligands) into the culture medium suppressed the expression of *Prox1*, *COUP-TFII*, and podoplanin also through Hey proteins (Kang et al. 2010). These data suggest that in vitro Notch signaling inhibits LEC fate. Another study also proposed that blocking Notch promotes LEC sprouting in vitro. This Notch inhibition-induced lymphangiogenesis required *Vegfr2* and *Vegfr3* signaling (Zheng et al. 2011). Thus, these data indicate that in vitro Notch signaling is involved in both the specification and sprouting of LECs. Contradictory to these results, another study demonstrated that by treating neonatal mouse tail dermis, ears, and retinas with

blocking antibodies targeting Notch1 and Dll4, lymphatic vessel sprouting and growth were impaired (Niessen et al. 2011). Recently, some in vivo studies have supported that Notch activity plays a role during embryonic lymphatic development (Murtomaki et al. 2013). These studies showed that Notch signaling components are present in LECs during embryonic development. Also, removal of Notch1 or disturbing Notch transcription in LECs leads to an increase in the number of LEC progenitors and to larger lymph sacs (Murtomaki et al. 2013). These results argue that Notch signaling acts as a negative regulator of LEC specification by repressing *Prox1* expression. Taken together, these findings indicate that Notch signaling is a negative regulator of LEC fate decisions during lymphatic vasculature development (Fig. 2.1).

Because of the limited number of LEC progenitors on the embryonic veins and the lack of specific surface markers to sort these cells, the identification of direct in vivo target genes of *Prox1* in these cells has been challenging. Several lymphatic genes are regulated by *Prox1* expression in vitro (Mishima et al. 2007; Hong et al. 2002; Fritz-Six et al. 2008; Sabine et al. 2012; Harada et al. 2009; Shin et al. 2006). Some studies have suggested that the main receptor of *Vegfc* signaling, *Vegfr3*, is a downstream target of *Prox1*. *Vegfr3* is expressed in BECs and is essential for blood vasculature development. *Vegfr3* null mouse embryos die at around E10.0 with severe defects in remodeling of the primary vessel networks (Dumont et al. 1998). In wild-type embryos LECs start to express *Vegfr3* after E10.5. *Vegfc* is the most well-characterized *Vegfr3* ligand, and in the absence of *Vegfc* signaling, *Prox1*⁺ LEC progenitors fail to bud off from the embryonic veins (Karkkainen et al. 2004). Previous microarray data indicate that ectopic expression of *Prox1* in cultured VECs leads to a significant increase in *Vegfr3* expression (Hong et al. 2002; Petrova et al. 2002). Furthermore, during inflammation-induced lymphangiogenesis, *Prox1* transcriptionally regulates *Vegfr3* expression by binding to its promoter together with NF- κ B or Ets2 in vitro (Flister et al. 2009; Yoshimatsu et al. 2011). These in vitro data suggest that *Vegfr3* is a direct target of *Prox1* and that other coactivators such as *COUP-TFII*, NF- κ B, or Ets2 may be involved in this regulatory process (Table 2.1). Our own unpublished data recently confirmed that *Vegfr3* is a direct in vivo target of *Prox1* in a dosage-dependent manner (Table 2.1). We determined that *Prox1* maintains *Vegfr3* expression in LEC progenitors and the number of LEC progenitors. Furthermore, the expression level of *Prox1* in those cells is further reduced in *Vegfr3*^{+/-}; *Prox1*^{+/-} embryos, revealing the existence of a regulatory feedback loop between *Prox1* and *Vegfr3*. Therefore, in addition to *COUP-TFII*, *Vegfr3* also regulates *Prox1* expression during the early specification and differentiation of LEC progenitors.

Besides transcriptional regulation, at least in vitro *Prox1* expression is also controlled by posttranscriptional regulation. It has been reported that lysine 556 is the major sumoylation site for *Prox1* and that sumoylation of *Prox1* influences its activity (Pan et al. 2009; Shan et al. 2008). In addition, in vitro data suggest that microRNAs regulate *Prox1* levels in LECs, as *Prox1* expression is negatively regulated by *miR-181* or *miR-31* in cultured LECs (Kazenwadel et al. 2010; Pedrioli et al. 2010) (Table 2.1). However, the in vivo function of the posttranscriptional

regulation of *Prox1* remains unknown. Interestingly, blood flow plays a significant role in modulating lymphatic identity *in vivo*. In this context, the expression of *Prox1* is rapidly lost when lymphatic vessels are exposed to high shear rates from blood flow, leading to the loss of lymphatic identity (Chen et al. 2012). Taken together, these results highlight that as a central player during LEC specification and differentiation, the level of *Prox1* is strictly regulated by numerous environmental and genetic factors. More transcription factors and signaling pathways that affect *Prox1* expression remain to be discovered.

2.3 Not All *Prox1*-Expressing LEC Progenitors Will Leave the CV

As discussed above, *Prox1* activity is required for the specification of LEC progenitors and for those progenitors to bud off from the CV. However, a recent study has identified a small subpopulation of *Prox1*-expressing LEC progenitors that will remain in the veins and help to form the lymphovenous valves (Srinivasan and Oliver 2011) (Fig. 2.1b, d). These cells are located at the junction of the jugular and subclavian veins and will not acquire LEC features (e.g., will not express podoplanin). Instead, they express an additional set of markers such as *Foxc2* and *Itga9* (Fig. 2.1b). Following intercalation with a subpopulation of venous ECs they will form the lymphovenous valves (Figs. 2.1 and 2.2). The formation of *Prox1*-expressing venous ECs and the derived lymphovenous valves is also dependent on *Prox1* activity, as these valves are absent in *Prox1* heterozygous mice (Srinivasan and Oliver 2011). This defect is a consequence of defective maintenance of *Prox1* expression in LEC progenitors, which is promoted by a reduction in the formation of the COUP-TFII/*Prox1* complex (Srinivasan and Oliver 2011). Together, these results support that *Prox1*-expressing venous ECs are the source of cells that will produce both LECs progenitors and lymphovenous valves. However, what makes some *Prox1*-expressing ECs remain on the vein remains to be determined.

2.4 *Foxc2* Is an Essential Regulator of Lymphatic Maturation and Valve Formation

Once the specified, mature LECs form the primitive lymph sacs and lymphatic plexus, they will differentiate further and give rise to the collecting lymphatic vessels and lymphatic capillaries. The formation of the lymphatic valves is an important step during the maturation of the primitive lymphatic plexus into collecting lymphatics. *Foxc2*, a member of the forkhead/winged-helix family of transcription factors, is one of the main players in the regulation of this critical step. In humans, point mutations in *FOXC2* have been identified as the cause of

lymphedema-distichiasis (LD) (Fang et al. 2000; Brice et al. 2002). A similar phenotype was observed in *Foxc2*^{+/-} mice (additional row of eyelashes, increased number of lymph nodes, and lymph backflow), suggesting that *Foxc2*^{+/-} is a suitable mouse model for LD (Kriederman et al. 2003). *Foxc2* is necessary for lymphatic patterning, lymphatic valve formation, and mural cell recruitment during the maturation stage (Petrova et al. 2004). Inactivation of *Foxc2* results in dilated lymphatic capillaries that become ectopically covered with smooth muscle actin-positive perivascular cells, whereas normal lymphatic capillaries lack mural cell coverage. It has been suggested that *Foxc2* and *Vegfr3* cooperate during the patterning of the lymphatic vasculature, and *Foxc2* presumably functions downstream of *Vegfr3* (Petrova et al. 2004). Although *Foxc2* is normally expressed in LECs from E9.5 to adult stages, its activity is not required for the budding and migration of LEC progenitors from the embryonic veins or the formation of lymph sacs (Dagenais et al. 2004). Studies have shown that *Foxc2* is also essential for the maturation of collecting lymphatics (Norrmén et al., 2009). Collecting lymphatics start to form around E14.5–15.5, and during their maturation markers for lymphatic capillaries such as *Prox1*, *Vegfr3*, and *Lyve1* get downregulated and valves start to form. In the absence of *Foxc2*, the expression of these markers remains high and valves do not form; as a consequence, the primary lymphatic plexus fails to mature into functional collecting lymphatics (Norrmén et al. 2009). Coimmunoprecipitation assays and genome-wide location mapping revealed that *Foxc2* physically interacts with *NFATc1*, a regulator of cardiac valve development (Chang et al. 2004; de la Pompa et al. 1998; Ranger et al. 1998), and functionally cooperates with calcineurin/*NFATc1* signaling in transcriptional regulation during the development of collecting lymphatics (Norrmén et al. 2009) (Table 2.1). Importantly, calcineurin/*NFATc1* signaling is required for normal lymphatic vascular patterning and LEC-specific gene expression during development. Blocking calcineurin/*NFATc1* signaling with the calcineurin inhibitor cyclosporine A in utero results in the loss of podoplanin and *Fgfr3* expression in LECs (Kulkarni et al. 2009). Furthermore, *Foxc2*-calcineurin/*NFATc1* signaling is not only important during collecting lymphatic vessel maturation but also indispensable for the formation and maintenance of lymphatic valves. The formation of lymphatic valves starts around E16.0, which is indicated by elevated *Prox1* and *Foxc2* expression in lymphatic valve-forming cells (Sabine et al. 2012). *Foxc2*-calcineurin/*NFATc1* signaling is activated in developing lymphatic valves, as indicated by the accumulation of nuclear *NFATc1* in lymphatic valve-forming cells. Retrograde lymph flow is observed in *Foxc2*^{-/-} embryos because of the complete absence of lymphatic valves (Petrova et al. 2004). Removal of calcineurin in ECs is also sufficient to affect the formation of a lymphatic valve territory (Sabine et al. 2012). In addition, the inactivation of calcineurin at any developmental stage results in lymphatic valve defects, indicating that *Foxc2*-calcineurin/*NFATc1* signaling is not only crucial for the initiation of valve formation but also required for the maintenance of lymphatic valves (Sabine et al. 2012). Besides calcineurin/*NFATc1* signaling, the gap junction protein *Cx37* is also essential for the assembly of the lymphatic valve territory. The clusters of lymphatic valve-forming cells were absent in *Cx37*-

knockout mice, resulting in the absence of lymphatic valves (Kanady et al. 2011; Sabine et al. 2012). In vitro flow analyses revealed that the expression of *Cx37* and calcineurin/*NFATc1* activation in LECs is regulated by oscillatory fluid shear stress in a *Prox1*- and *Foxc2*-dependent manner and that *Cx37* depletion significantly decreases calcineurin/*NFATc1* activation (Sabine et al. 2012). In vivo, *Cx37* was almost completely absent in LECs of *Foxc2*^{-/-} embryos (Sabine et al. 2012). Taken together, these results support that *Prox1*, *Foxc2*, and shear stress coordinate the expression of *Cx37*, which in turn activates calcineurin/*NFATc1* signaling in the lymphatic valve-forming cells during lymphatic valve morphogenesis.

2.5 Additional Transcription Factors Involved in Lymphatic Development

Tbx1, a member of a conserved family of transcription factors that share a common T-box DNA-binding domain, has been recently identified as a gene whose activity is necessary during lymphatic development (Chen et al. 2010). *Tbx1* is associated with the DiGeorge syndrome; however, lymphatic defects are rarely reported in patients with this syndrome (Yagi et al. 2003; Mansir et al. 1999). During mouse development, deletion of *Tbx1* from ECs leads to embryonic edema and postnatal lethality between 2 and 4 days after birth because of abdominal chylous ascites (Chen et al. 2010). These mice have severely reduced lymphatic vessel density in the heart, diaphragm, and skin and lack the entire gastrointestinal lymphatic vasculature (Chen et al. 2010). Conditional inactivation of *Tbx1* at different developmental stages revealed that *Tbx1* activity is required until E14.5 for the formation of the mesenteric lymphatic vasculature (Chen et al. 2010). Mechanistically, chromatin immunoprecipitation analysis has shown that *Tbx1* binds to conserved T-box-binding elements in the *Vegfr3* promoter to activate its expression (Chen et al. 2010) (Table 2.1).

The Gata binding protein 2 (*Gata2*) belongs to an evolutionarily conserved family of C4 zinc finger transcription factors. *Gata2* was first demonstrated to be essential for hematopoiesis because *Gata2* knockout embryos die around E10 due to a failure in primitive hematopoiesis (Tsai et al. 1994). In addition to the hematopoietic lineage, a recent study systematically examined the expression of *Gata2* in ECs during embryonic development by using *Gata2*-GFP knock-in mice. GFP was strongly expressed in arterial and venous BECs (Khandekar et al. 2007). Interestingly, GFP expression was also observed in LECs budding from the veins and in postnatal lymphatic vessels (Khandekar et al. 2007). *Gata2* expression was also reported in lymphatic valve cells, suggesting a possible role for the gene in lymphatic valve formation (Kazenwadel et al. 2012). Importantly, conditional inactivation of *Gata2* in the endothelial lineage led to edema and hemorrhaging and ultimately embryonic demise at around E16.5. Further analysis revealed that loss of *Gata2* caused lymph sac hypoplasia and suggested defective

blood–lymphatic separation (Lim et al. 2012). More evidence for *Gata2* function in lymphatic vascular development comes from human patients in whom mutations in *Gata2* have been characterized as the cause of primary lymphedema associated with a predisposition to acute myeloid leukemia (Emberger syndrome) (Ostergaard et al. 2011). Similarly, in some patients with myelodysplastic syndrome, acute myeloid leukemia, and “MonoMAC” syndrome, mutations in *Gata2* are associated with primary lymphedema (Kazenwadel et al. 2012). Little is known about the transcriptional regulation of *Gata2*. A fragment in intron 4 of *Gata2* has been identified as an endothelium-specific enhancer of *Gata2*. Analysis of this fragment revealed that transcription factors belonging to the *Ets* family and *Scl* are activators of *Gata2* expression (Khandekar et al. 2007) (Table 2.1). In addition to the upstream regulation of *Gata2*, in vitro siRNA data suggest that *Gata2* regulates the expression of many genes required for valve formation (e.g., *Prox1*, *Foxc2*, *NFATc1*, and *Itga9*) (Kazenwadel et al. 2012). Taken together, these findings suggest that *Gata2* is another newly identified lymphatic-specific transcription factor important for early lymphatic vascular development.

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