Tbx1 regulates Vegfr3 and is required for lymphatic vessel development

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Lymphatic dysfunction causes several human diseases, and tumor lymphangiogenesis is implicated in cancer spreading. TBX1 is the major gene for DiGeorge syndrome, which is associated with multiple congenital anomalies. Mutation of Tbx1 in mice recapitulates the human disease phenotype. In this study, we use molecular, cellular, and genetic approaches to show, unexpectedly, that Tbx1 plays a critical role in lymphatic vessel development and regulates the expression of Vegfr3, a gene that is essential for lymphangiogenesis. Tbx1 activates Vegfr3 transcription in endothelial cells (ECs) by binding to an enhancer element in the Vegfr3 gene. Conditional deletion of Tbx1 in ECs causes widespread lymphangiogenesis defects in mouse embryos and perinatal death. Using the mesentery as a model tissue, we show that Tbx1 is not required for lymphatic EC differentiation; rather, it is required for the growth and maintenance of lymphatic vessels. Our findings reveal a novel pathway for the development of the lymphatic vessel network.

Introduction

The lymphatic vascular system is involved in many pathological processes, including lymphedema and the metastatic spread of cancer. Despite the importance of the lymphatic system for human health, our knowledge of lymphatic vessel development lags considerably behind that of blood vascular development, and it is only in recent years that some of the molecules that regulate lymphangiogenesis have been identified (for review see Tammela et al., 2005). The data presented in this study establish the transcription factor TBX1 as a new player in lymphangiogenesis.

TBX1 encodes a T box transcription factor involved in the 22q11 deletion syndrome (22q11DS), also known as DiGeorge syndrome. 22q11DS patients have a complex phenotype that includes cardiovascular defects, craniofacial dysmorphism, hypocalcemia, immune deficiency, behavioral disorders, and psychiatric diseases. Most of the physical abnormalities are caused by TBX1 haploinsufficiency (Yagi et al., 2003), and we have shown that TBX1 may contribute to the psychiatric disorders (Paylor et al., 2006). Lymphatic defects have not been reported in 22q11DS patients, with a single exception (Mansir et al., 1999). However, the recurrent infections associated with the disease may be contributed by subtle lymphatic abnormalities in addition to the known T cell immune deficiency.

In the mouse, mutation of Tbx1 recapitulates the common physical abnormalities seen in patients (Jerome and Papaioannou, 2001; Lindsay et al., 2001; Merscher et al., 2001). During mouse development, Tbx1 is widely expressed in the developing pharynx, ear, and the endothelial lining of some blood vessels (Vitelli et al., 2002; Paylor et al., 2006). Intriguingly, a
A similar pathology has been reported in several mouse mutants that have mutations in genes involved in lymphatic vessel development (see Tammela et al., 2005), suggesting that Tbx1 may be required in ECs for lymphangiogenesis. In view of the dramatic phenotype seen in Tie2-Cre; Tbx1flox/lacZ mutants, we first evaluated lymphangiogenesis in the gastrointestinal (GI) tract of preterm embryos. For this, we performed immunostaining on isolated GI tracts of E18.5 embryos using antibodies against three EC-specific genes, CD31 (Pecam-1), Vegfr3, and Lyve1. Anti–Pecam-1 identifies ECs of arteries, veins, and lymphatic vessels, whereas anti-Vegfr3 and anti-Lyve1 are specific for lymphatic ECs (LECs) at this embryonic stage. Anti–Pecam-1 revealed an overall reduction in the number of vessels in the mesentery of both Tie2-Cre; Tbx1flox/lacZ mutants (Fig. 1, B and B'), and Tbx1lacz/lacz (null) mutants (not depicted). We used anti-Vegfr3 to confirm that the missing vessels were indeed lymphatic vessels (Fig. S1). Similar results were obtained with anti-Lyve1 (Fig. S1), thereby confirming that the lack of staining with anti-Vegfr3 was caused by the absence of the lymphatic vessels. Thus, EC-specific loss of Tbx1 causes a developmental failure of the entire GI lymphatic vasculature.

Results and discussion

Loss of Tbx1 in ECs causes perinatal lethality and lymphatic abnormalities

We deleted Tbx1 specifically in ECs using an EC-specific Cre driver, Tie2-Cre (Kisanuki et al., 2001). We crossed Tie2-Cre; Tbx1lacz/lacz mice with Tbx1flox/flox mice and genotyped 114 offspring at weaning. We did not recover any Tie2-Cre; Tbx1flox/lacZ pups (i.e., null for Tbx1 in ECs and heterozygous in all other tissues). In contrast, this genotype was recovered at the normal Mendelian ratio (10/39) on embryonic day (E) 18.5. We established that Tie2-Cre; Tbx1flox/lacZ mutants died between postnatal day (P) 2 and 4, when they showed growth failure, dehydration, and abdominal chylos ascites (Fig. 1 A'), which are features that were not present in controls (Fig. 1 A).

Figure 1. Lymphatic abnormalities in EC-specific Tbx1-null mutants. (A) In control embryos, chyle is visible in mesenteric lymphatic vessels (arrowheads). (A') On P4, Tie2-Cre; Tbx1flox/lacZ mutants show growth retardation, abdominal distention, and accumulation of chyle in the intestinal wall (arrows) and membranous mesentery (arrowheads). (B and B') Immunohistochemistry on isolated intestines at E18.5. Anti–Pecam-1 shows that Tie2Cre; Tbx1flox/lacZ mutants (B') have fewer mesenteric vessels than controls (B). (C and C') Anti–Vegfr3 reveals that Tie2Cre/+; Tbx1flox/lacZ mutants lack the mesenteric lymphatic vessels. (C) Control mesentery is shown. L, lymphatic vessel; A, artery; V, vein; I, intestine. Bars, 1 mm.
We next examined lymphatic vessels in other tissues, including heart, diaphragm, and skin in Tbx1lacZ/+ and Tbx1lacZ/lacZ embryos at E18.5 (Fig. S2). In the heart, anti-Vegfr3 immunostaining revealed a severe reduction in the number of lymphatic vessels in Tbx1lacZ/lacZ embryos, with only a few vessels adjacent to the aorta being identifiable (Fig. S2, A–B’). Anti–Pecam-1 immunostaining showed similar staining patterns in both genotypes (Fig. S2, C and C’), although in Tbx1lacZ/lacZ embryos, some vessels were dilated, and there was a reduced vessel density. In the diaphragm, an extensive network of lymphatic vessels was observed in control embryos (Fig. S2, D and E), whereas in Tbx1lacZ/lacZ embryos, this network was severely reduced (Fig. S2, D’ and E’). Similar results were obtained with anti-Lyve1 (Fig. S2, F and F’). In the skin, Tbx1lacZ/lacZ embryos had fewer subcutaneous lymphatic vessels than control embryos (Fig. S2, G’ and H’ and G and H, respectively). Consistent with this observation, Tbx1lacZ/lacZ embryos showed subcutaneous edema at E14.5 (Fig. S2, I and I’).

Tbx1 is required early in mesenteric lymphangiogenesis

Because of the severity of the mesenteric phenotype, we focused our attention on the function of Tbx1 in this tissue. In the mouse, the first mesenteric lymphatic vessels form around E13 (van der Putte, 1975; Kim et al., 2007). To monitor Tbx1 expression, we used a lacZ reporter allele (Lindsay et al., 2001) and an anti-Tbx1 antibody. At E13.5, we identified β-galactosidase (β-gal) activity in the proximal mesentery, where it colocalized with anti-Vegfr3 immunostaining (Fig. 2, A and B), indicating that Tbx1 is expressed in the LECs of early mesenteric lymphatic vessels. Note that Tbx1 expression was not LEC specific at this or later embryonic stages, as β-gal activity was also identifiable in some ECs of the mesenteric artery and vein. Robust β-gal activity in lymphatic vessels continued through E16.5 (Fig. 2, C, D, and F). Anti-Tbx1 immunostaining showed a similar pattern of expression (Fig. 2 E). We determined the time requirement for Tbx1 in mesenteric lymphangiogenesis using transgenic mice that carry a tamoxifen (TM)-inducible Cre recombinase under the control of a ubiquitous promoter (TgCAGG-CreERTM; Hayashi and McMahon, 2002). TgCAGG-CreERTM, Tbx1flox/+ male mice were bred with Tbx1flox/flox females. Pregnant females were injected with TM between E11.5 and E14.5 to effect Cre-induced excision of the floxed Tbx1 exon 5, and thereby gene inactivation between E12.5 and E15.5 (see Materials and methods). Mice with the genotype TgCAGG-CreERTM, Tbx1flox/lacZ constituted the test group, whereas embryos with the genotype TgCAGG-CreERTM, Tbx1flox/+ constituted the control group. Embryos were collected at E18.5, and intestines were immunostained with anti-Vegfr3. In control embryos exposed to TM at E11.5, the mesenteric lymphatics...
Tbx1 is required for lymphatic vessel maintenance

We asked whether Tbx1-deficient lymphatic vessels form, and if so, what their fate is. To address this, we used two previously characterized Tbx1-null alleles, Tbx1<sup>CES</sup> (Xu et al., 2004) and Tbx1<sup>Cre</sup> (Huynh et al., 2007), in conjunction with the Cre reporter R26R (Soriano, 1999). X-gal staining of isolated intestines at E14.5 shows extensive contribution of Tbx1-traced cells to the mesenteric vessels of control (Tbx1<sup>Cre</sup>−; R26R) embryos (Fig. 3 A) and null mutant (Tbx1<sup>CES</sup>; R26R) embryos (Fig. 3 A'). Double staining (X-gal and anti-Lyve1) of Tbx1<sup>Cre</sup>−; R26R embryos at E14.5 (Fig. 3 B) and E15.5 (Fig. 3 C) revealed that the mesenteric lymphatic endothelium is largely composed of Tbx1-traced cells (Fig. 3, black arrowheads). In Tbx1<sup>CES</sup>; R26R embryos (Fig. 3, B' and C'), Tbx1-traced cells contributed to clusters of vessels (Fig. 3, B' and C', red arrowheads) that were unlike the characteristic network of lymphatic vessels seen in controls. Nevertheless, some of these abnormal vessels were Lyve1<sup>+</sup>, indicating their lymphatic nature. In the distal mesentery, β-gal<sup>+</sup> vessels were Lyve1 negative (Fig. 3, D and D') and are likely tributaries of the mesenteric artery and vein (note that the punctate brown signals in mutant and control sections are Lyve1<sup>+</sup> macrophages). Thus, Tbx1 is not essential for mesenteric lymphatic vessel formation. We determined the fate of the abnormal lymphatic vessels at later embryonic stages. At E16.5, only a few β-gal<sup>+</sup> vessels were identifiable in the proximal mesentery of Tbx1<sup>CES</sup>; R26R embryos (Fig. 3 E'), some of which were also Lyve1<sup>+</sup> (Fig. 3 E', red arrowhead), whereas in the distal mesentery, β-gal<sup>+</sup> vessels were Lyve1 negative, as seen at E15.5 (not depicted). At E18.5, β-gal<sup>+</sup> vessels were Lyve1 negative and had the anatomy of veins and arteries (unpublished data). Similar results were obtained at the aforementioned embryonic stages with anti-Vegfr3 (unpublished data). Thus, on a Tbx1-null background, the mesenteric lymphatic vessels form, but they are abnormal, they fail to extend into the distal mesentery, and between E15.5 and E16.5, they are almost completely lost. To determine whether this loss is caused by cell death, we performed anti–caspase 3 immunostaining on isolated GI tracts of Tbx1<sup>lacz</sup> and Tbx1<sup>lacz</sup> embryos at E14.5 and E16.5. Results showed a significant increase in the number of apoptotic cells adjacent to the mesenteric artery of Tbx1<sup>lacz</sup> embryos (P < 0.01) at E16.5 (Fig. S3, F–G) but not at E14.5 (Fig. S3, E, E', and G).

Tbx1 regulates transcription of growth factors and angiogenic markers in ECs

We next used quantitative real-time PCR (qRT-PCR) to explore the transcriptional response of 82 genes involved in angiogenesis and lymphangiogenesis (RT2Profiler PCR Array; QIAGEN) to Tbx1 expression in cultured human umbilical vein ECs.

developed normally (Fig. S3 A). In contrast, TgCAGG-CreERT<sup>TM</sup>, Tbx1<sup>flox/lacZ</sup> mutants exposed to TM at E11.5 (Fig. S3 B) and E12.5 (not depicted) had no identifiable mesenteric lymphatics at E18.5, which is similar to Tie2-Cre; Tbx1<sup>flox/lacZ</sup> mutants. Induction at E13.5 resulted in near-complete absence of lymphatic vessels, although in two out of seven TgCAGG-CreERT<sup>TM</sup>, Tbx1<sup>flox/lacZ</sup> mutants analyzed, a few Vegfr3<sup>+</sup> vessels were seen (Fig. S3 C, arrows). Induction at E14.5 resulted in apparently normal lymphatic vessel development at E18.5 (Fig. S3 D). Thus, Tbx1 is required for lymphangiogenesis until E14. In the mouse, this is ~24 h after the onset of mesenteric lymphangiogenesis (van der Putte, 1975). As shown in Fig. 2 (A and B), Tbx1 is expressed in mesenteric LECs at this critical stage.

Figure 3. Fate mapping of Tbx1-expressing cells in the mesentery. [A and A'] X-gal staining of isolated intestines at E14.5 shows extensive contribution of Tbx1-traced cells to the mesenteric vessels of control (A; Tbx1<sup>Cre</sup>−; R26R) and mutant (Tbx1<sup>CES</sup>; R26R) embryos (A). Black lines indicate the angle of sections in B and B'. (B and B') In the proximal mesentery of E14.5 embryos, X-gal staining and anti-Lyve1 colocalize in mesenteric LECs in control (B) and mutant (B') embryos. Note the abnormal anatomy of the mutant lymphatic vessels (B', red arrowheads). (C and C') At E15.5, in the proximal mesentery, highly abnormal lymphatic vessels in mutant embryos (C', red arrowheads) are both X-gal<sup>+</sup> and Lyve1<sup>+</sup>. (D and D') In the distal mesentery, lymphatic vessels were present in control embryos (D, arrowhead) but not mutant embryos (D'). Insets show the boxed area at higher magnification. (E and E') At E16.5, very few X-gal<sup>+</sup>/Lyve1<sup>+</sup> vessels are identifiable (E', red arrowhead). Note that the vasa vasorum (E', black arrows) is X-gal<sup>+</sup>/Lyve1<sup>+</sup> negative. A, mesenteric artery; V, mesenteric vein; L, lymphatic vessel; P, proximal; D, distal. Black arrowheads indicate lymphatic vessels. Bars, 100 μm.
Among the markers of lymphangiogenesis, PROX1 and LYVE1 did not change significantly in response to TBX1 transfection. However, VEGFR3, PDPN, and VEGFD increased significantly, as did IGF1 (Fig. 4 A). We validated data obtained by qRT-PCR and immunocytofluorescence with VEGFD and IGF1 antibodies on TBX1-transfected HUVECs (Fig. 4, B–E). Results showed that ~7% of the control cell population expressed VEGFD or IGF1 protein, and this percent increased twofold after TBX1 transfection (Fig. 4 F). This suggests that at least part of the increased level of mRNA encoding those proteins is caused by an increase in the number of cells expressing those markers. We evaluated cell proliferation by immunocytofluorescence with anti–phosphohistone H3. In HUVECs transfected with 3 µg human TBX1 expression vector, cell proliferation increased >1.8-fold above that of control cultures (Fig. 4 G). We used Western blotting to confirm that transfection of HUVECs with the TBX1-expressing vector resulted in increased expression of TBX1 protein (Fig. 4 H).

**Tbx1 regulates VEGFR3 in ECs**

Of the three most highly up-regulated genes, IGF1, VEGFD, and PDPN, only Pdpn loss of function in mice is associated with lymphatic defects, namely lymphatic vessel dilatation and impaired lymphatic transport (Schacht et al., 2003), which is unlike the lymphatic phenotype of Tbx1-null mutants. In addition, anti-VEGFD immunostaining in Tbx1-null mutants was normal. Specifically, it was expressed in LECs lining the jugular lymph sacs and in subcutaneous lymphatic vessels (unpublished data), both of which are present in Tbx1<sup>−/−</sup> mutants, although they are abnormal and reduced in number.

Therefore, we focused our attention on VEGFR3, which is essential for lymphangiogenesis (Karkkainen et al., 2000, 2001) and is regulated by TBX1 in a dose-dependent manner (Fig. 4 A). Because HUVECs express endogenous TBX1, we knocked it down using siRNA to test whether VEGFR3 expression responds to it. We obtained an ~80% reduction in TBX1 expression and a 40% reduction in VEGFR3 expression in multiple experiments (Fig. 4 I). Similar experiments performed on cultured human microvascular LECs (HMLECs) yielded a 60% reduction in VEGFR3 expression (Fig. 4 J). siRNA knockdown of TBX1 mRNA resulted in reduced TBX1 protein expression in both HMLECs (Fig. 4 K) and in HUVECs (not depicted).

To identify putative TBX1-responsive enhancers within the VEGFR3 gene, we searched for evolutionarily conserved DNA segments containing conserved T box-binding elements (TBE). A 1.1-kb segment was identified that encompassed intron 11-12 and contained a single TBE. We cloned this DNA fragment from the mouse genome and named it In11-12TBE. We assayed whether the In11-12TBE enhancer responds to Tbx1 in a tissue culture system. To test this, we cotransfected JEG3 cells with a Tbx1 expression vector and a luciferase reporter containing In11-12TBE and a basal promoter (Fig. 5 A). Overexpression of Tbx1 led to a fourfold increase in luciferase activity in multiple experiments (Fig. 5 B). In contrast, mutation of TBE
Figure 5. Tbx1 regulates VEGFR3 expression via a conserved TBE in the endogenous VEGFR3 gene. (A) Schematic of the In\(^{11-12TBE}\) luciferase reporter. A mutant version of the reporter construct was generated by site-directed mutagenesis of the TBE located within the putative VEGFR3 enhancer (In\(^{11-12TBE}\)). (B) Co-transfection of JEG3 cells with the luciferase reporter and increasing amounts of Tbx1 expression vector increased activity of the luciferase reporter containing the wild-type enhancer (wt-TBE) but not the mutant enhancer (mut-TBE). Control cultures were transfected with empty expression vector. (C) PCR of chromatin immunoprecipitation (ChIP) from HUVECs, which express endogenous TBX1 and VEGFR3. DNA fragments precipitated by anti-TBX1 were purified and tested for the presence of the aforementioned TBE by PCR. Results showed enrichment of DNA fragments containing the TBE in the ChIP sample (Fig. 5 C), indicating that TBX1 occupies this site in these cells.

Next, to test whether In\(^{11-12TBE}\) functions as an LEC enhancer in vivo, we generated transient transgenic mice carrying an HSP-lacZ reporter driven by In\(^{11-12TBE}\)wt-lacZ with either the wild-type or mutated TBE (see Materials and methods). We obtained 10 transgenic embryos with the wild-type transgene (In\(^{11-12TBE}\)wt-lacZ) at E15.5, of which five were β-gal+ in these embryos, no β-gal–expressing cells were seen in Vegfr3\(^+\) mesenteric LECs (Fig. 5, D–E) or in skin or heart lymphatic vessels (not depicted), indicating that the mutated transgene was not activated in these cells. These results demonstrate that In\(^{11-12TBE}\) functions as an LEC enhancer in vivo and that its activity depends on its TBE. In transgenic embryos carrying the wild-type or mutated TBE, we observed strong X-gal staining in ECs of the mesenteric artery (Fig. 5, D–E), suggesting that the endogenous Vegfr3 gene harbors a suppressor element that is absent in the transgene.

In summary, our data show that Tbx1 is required in ECs for development of the GI lymphatic vasculature. In mice, the loss of this population of lymphatic vessels is lethal after birth. Elsewhere, lymphatic vessels develop in both Tie2-Cre; Tbx1\(^{fl/f-lox}\) mutants and in Tbx1 germine-null mutants, although in the tissues surveyed, they were much reduced in number and abnormal.
Thus, Tbx1 is not essential for lymphangiogenesis per se; rather, it is required for the development of the lymphatic network. We have gained insights into when and how Tbx1 functions in mesenteric lymphatic development. On a Tbx1-null background, lymphatic vessels form at the appropriate time, but they are highly disorganized and fail to extend distally. Between E15.5 and E16.5, these abnormal lymphatic vessels are lost, most likely as a result of cell death. This suggests that Tbx1 has at least two roles in the mesentery. First, it is required for growth and expansion of the lymphatic network. This is consistent with our data from cultured LECs that show increased cell proliferation in response to Tbx1 transfection. Second, it is required to maintain mesenteric lymphatic vessels.

In a search for potential effectors of these functions, we found that the expression of Vegfr3 is not maintained in Tbx1flox/lacz mutants or in Tie2-Cre; Tbx1flox/lacz mutants. Through tissue culture and in vivo genetic experiments, we have demonstrated that Vegfr3 is a direct transcriptional target of Tbx1 and identified a Tbx1-responsive element in the Vegfr3 gene. Thus, we have directly linked Tbx1 to an essential lymphangiogenesis signaling pathway.

Our data support the hypothesis that, in the absence of Tbx1, lymphatic vessels are not maintained, secondary to a failure to maintain Vegfr3 expression in LECs. This hypothesis is consistent with in vitro and in vivo studies that have shown a critical role for VEGF-C/Vegfr3 signaling in the growth, migration, and survival of LECs (Mäkinen et al., 2001a,b; Veikkola et al., 2001).

Could loss of Vegfr3 in Tbx1-null ECs account for the entire lymphatic phenotype of Tbx1-null mutants? In the future, it will be interesting to see whether forced expression of Vegfr3 in ECs rescues lymphatic defects in Tbx1 mutants.

How is Tbx1 regulated? There is very little information to date. A Forkhead (Fox) transcription factor–response element has been identified upstream of the Tbx1 gene (Yamagishi et al., 2003). The enhancer responds in tissue culture to Foxc2, which has also been involved in lymphatic development (for review see Tammela et al., 2005). However, it is unknown whether Foxc2 is required for Tbx1 expression in endothelium, and, in any case, the phenotypes resulting from Tbx1 and Foxc1 or Foxc2 loss of function are different. In particular, Foxc1/2 has a critical role for VEGFR3 signaling in the growth, migration, and survival of LECs (Mäkinen et al., 2001a,b; Veikkola et al., 2001).

Materials and methods

Mouse lines

The following mouse lines were used: Tbx1flox/+ (Lindsay et al., 2001), Tbx1flox/lacZ (Xu et al., 2004), Tie2Cre (Kisounoki et al., 2001), R26R (Soriano, 1999), TgCAGG-CreERTM (Hayashi and McMahon, 2002), Tbx1flox/+ (Xu et al., 2004), and Tbx1flox/lacZ (Huyhn et al., 2007). Genotyping was performed according to the aforementioned original reports. Activation of CAGG-CreERTM was performed with a single intraperitoneal injection of 75 mg/kg body weight TM (Xu et al., 2005).

X-gal staining, immunostaining, and immunocytofluorescence

β-Gal activity was revealed on 4% PFA fixed samples by X-gal staining. Samples were photographed directly or paraffin embedded and sectioned at 10 μm. Immunostaining and immunocytofluorescence were performed using the following antibodies: rat anti–Vegfr3 (eBioscience), rat anti–PECAM-1 (BD), rabbit anti-Lyve1 (Abcam), monoclonal anti–VEGFD (R&D Systems), and rabbit anti–FGF1 (Santa Cruz Biotechnology, Inc.). Nonfluorescent antibodies were visualized after mounting slides in Cytoseal (Thermo Fisher Scientific). Fluorescent antibodies were visualized using imaging medium (Vectorshield; Vector Laboratories). Whole-mount specimens were photographed using a dissecting microscope (Stemi 2000; Carl Zeiss, Inc.) equipped with a camera (Axiocam; Carl Zeiss, Inc.) and the manufacturer’s acquisition software. Images of histological sections were acquired using a microscope (Axioskop 40; Carl Zeiss, Inc.) equipped with a camera (Axiocam; Carl Zeiss, Inc.) and the manufacturer’s acquisition software. Photoshop (Adobe) was used for trimming and labeling images.

Cell manipulations

HUVECs (Lonza) were electroporated with increasing amounts of a TBX1 expression or with empty vector and an EGFP reporter to monitor transfection. Transfection of a standard amount of DNA in all samples was achieved by adjusting the quantity of empty vector in the mix. After 24 h, cells were fixed in 4% PFA for immunocytofluorescence or lysed for RNA isolation. qRT-PCR was performed using commercial primers (RT2Profiler PCR Array; PAHS-024; human angiogenesis; QIAGEN). RNA interference was performed using commercial siRNA for TBX1 (ON-TARGETplus SMARTpool; Thermo Fisher Scientific) and a control siRNA (Thermo Fisher Scientific). HUVECs or HMVECs were transfected with 40 nM siRNA and Lipofectamine 2000 (Invitrogen). TBX1 mRNA was evaluated by qRT-PCR, and TBX1 protein was evaluated by Western blotting with anti-Tbx1 (Invitrogen). Protein levels were normalized to anti–β-actin (Sigma-Aldrich).

Enhancer activity assays

For the luciferase construct, a 1.1-kb of DNA surrounding a conserved TBE in the Vegfr3 intron 11-12 was PCR amplified with primers 5′-CGAGCT-GGAAGAGGTGACC-3′ and 5′-CACCAGCTGTCACCCGT-3′. The amplified, named In11112TBE, was first cloned into a T-vector, pCRII/TOPO (Invitrogen), and after KpnI–Xhol digestion, it was subcloned into a pGL3 luciferase expression vector (Promega). Subsequently, the enhancer fragment was subjected to site-directed mutagenesis of the TBE. The native sequence 5′-TACACCCCTCA-3′ was mutated to 5′-TACGGGCTCTCA-3′ using a site-directed mutagenesis kit (QuikChange XL; Agilent Technologies). A Tbx1 expression vector was cotransfected with the enhancer reporter (wild type or mutated) into JEG3 cells, which were harvested 48 h later. JEG3 cells transfected with empty expression vector, and without the enhancer reporter, constituted the control sample. Experiments were performed in triplicate and repeated twice.

In11112TBEwt is HSP-locZ and In11112TBEmut-HSP-locZ transgenes were injected into FVB/NCr pronuclei. Embryos were harvested at E15.5, stained with Xgal, and genotyped PCR with the following primers: TT_LaczF, 5′-CTGGAAATTCCGGCATGAC-3′; TT_LaczR, 5′-CCGGATGATTAGTGTCGC-3′; TT_VR3F, 5′-GGCTTCTGCTGGGTAGACG-3′; and TT_VR3R, 5′-AGTTGCTGCACTTCACT-3′.

ChIP

ChIP experiments were performed as described previously (Breiling et al., 2004). Chromatin from cross-linked HUVECs cells was immunoprecipitated
with anti-Tbx1 (Invitrogen) or with anti-c-Myc (control; Sigma-Aldrich). DNA purified from chromatin was quantified by qRT-PCR using the following primers: TBE_ip_F, 5'-CACACCTCAGAGCTTCT-3'; TBE_ip_R, 5'-GCCCT-GCTATCATCAGAAGCAAC-3'; and TBE_ip_R1, 5'-GGGGAGAGGAGGCCATCTA-3'.

Online supplemental material

Fig. S1 shows live1 immunostaining of E18.5 Gl tracts of Tbx1 mutants. Fig. S2 shows that Tbx1 is expressed in and is required for development of lymphatic vessels of the heart, diaphragm, and skin. Fig. S3 shows that a lymphatic phenotype after time-controlled Tbx1 mutagenesis increased apoptosis in the proximal mesentry of Tbx1-null mutants. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200912037/DC1.

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Figure S1. **Lyve1 immunostaining of E18.5 GI tracts of Tbx1 mutants.** (A and B) In wild-type (WT) embryos, Lyve1 is expressed in mesenteric lymphatic vessels (A, arrowheads) in the wall of the intestine (A, arrow) and in the lacteals (B, arrowheads). (A' and B') In Tbx1lacZ/lacZ embryos, no lyve1+ vessels were detected, confirming data obtained with anti-Vegfr3. Note brown speckles in wild-type and mutant tissues are lyve1+ macrophages. Bars: (A and A') 1 mm; (B and B') 100 µm.
Figure S2. Tbx1 is expressed in and is required for development of lymphatic vessels of the heart, diaphragm, and skin. (A–B) Anti-Vegfr3 immunostaining of E18.5 hearts in Tbx1$$^{lacZ/+}$$ (A and B) and Tbx1$$^{lacZ/lacZ}$$ (A and B') embryos shows severely reduced immunostaining in the homozygous heart, where only a few vessels close to the aorta are identifiable (arrows). (C and C') Dorsal views of Pecam-1-immunostained E18.5 hearts in Tbx1$$^{lacZ/+}$$ (C) and Tbx1$$^{lacZ/lacZ}$$ (C') embryos show a similar staining pattern. (D–E') Anti-Vegfr3 immunostaining of E18.5 diaphragm in Tbx1$$^{lacZ/+}$$ (D and E) and Tbx1$$^{lacZ/lacZ}$$ (D' and E') embryos reveals a severe reduction of lymphatic vessels in the homozygous diaphragm (arrows). (F and F') Anti-Lyve1 immunostaining shows a similar reduction of lymphatic vessels in the homozygous diaphragm. (G–H') Double staining (X-gal and anti-Vegfr3) of the skin of Tbx1$$^{lacZ/+}$$ (G and H) and Tbx1$$^{lacZ/lacZ}$$ (G' and H') embryos at E18.5. Black arrows indicate Vegfr3$$^{+}$$ subcutaneous lymphatic vessels. In null mutants (G' and H'), the number of Vegfr3$$^{+}$$ lymphatic vessels is reduced. Red arrows show that where β-gal$$^{+}$$ vessels were present in Tbx1$$^{lacZ/lacZ}$$ mutants, anti-Vegfr3 immunostaining was reduced or absent. (I and I') Subcutaneous edema in a Tbx1$$^{lacZ/lacZ}$$ embryo at E14.5. Boxed areas in A, A; D, D'; and G, G' are shown at higher magnification in the panels below (B, B'; E, E'; and H, H'; respectively). LV, left ventricle; RV, right ventricle. Bars: (A–F, I, and I') 1 mm; (G–H') 100 µm.
Figure S3. **Lymphatic phenotype after time-controlled Tbx1 mutagenesis.** Whole-mount E18.5 GI tracts after immunostaining with anti-Vegfr3. (A) A control embryo exposed to TM at E11.5 developed normal mesenteric lymphatic vessels (arrows) and a normal network of lymphatic vessels in the intestinal wall (arrowheads). (B–D) Timed conditional null mutant embryos exposed to TM at E11.5 (B), E13.5 (C), and E14.5 (D). (B) Embryos exposed to TM at E11.5 (and E12.5; not depicted) lacked all mesenteric lymphatics at E18.5. After TM injection at E13.5, a few mesenteric lymphatic vessels were seen in some mutants (C), whereas TM injection at E14.5 resulted in normal lymphatic development (D). Arrows indicate lymphatic vessels. Bars, 1 mm. (E–G) Apoptosis in the mesentery of Tbx1-null mutants. (E–F) Anti-caspase 3 immunostaining and X-Gal staining of histological sections of the proximal mesentery of Tbx1lacZ/+ (E and F) and Tbx1lacZ/lacZ (E' and F') embryos at E14.5 (E and E') and E16.5 (F and F'). Arrows indicate caspase 3+ cells. (G) At E16.5, but not at E14.5, there were more apoptotic cells in the mesentery of Tbx1lacZ/lacZ mutants than in controls; *, P < 0.01. All caspase 3+ cells were counted in 12 fields. Values on the y axis are the mean number of apoptotic cells per field ± SEM. L, lymphatic vessel; A, artery; V, vein. Bars, 100 µm.