Polydom Is an Extracellular Matrix Protein Involved in Lymphatic Vessel Remodeling

Nanami Morooka, Sugiko Futaki, Ryoko Sato-Nishituchi, Masafumi Nishino, Yuta Totani, Chisei Shimono, Itsuko Nakano, Hiroyuki Nakajima, Naoki Mochizuki, Kiyotoshi Sekiguchi

Rationale: Lymphatic vasculature constitutes a second vascular system essential for immune surveillance and tissue fluid homeostasis. Maturation of the hierarchical vascular structure, with a highly branched network of capillaries and ducts, is crucial for its function. Environmental cues mediate the remodeling process, but the mechanism that underlies this process is largely unknown.

Objective: Polydom (also called Svep1) is an extracellular matrix protein identified as a high-affinity ligand for integrin α9β1. However, its physiological function is unclear. Here, we investigated the role of Polydom in lymphatic development.

Methods and Results: We generated Polydom-deficient mice. Polydom−/− mice showed severe edema and died immediately after birth because of respiratory failure. We found that although a primitive lymphatic plexus was formed, it failed to undergo remodeling in Polydom−/− embryos, including sprouting of new capillaries and formation of collecting lymphatic vessels. Impaired lymphatic development was also observed after knockdown/knockout of polydom in zebrafish. Polydom was deposited around lymphatic vessels, but secreted from surrounding mesenchymal cells. Expression of Foxc2 (forkhead box protein c2), a transcription factor involved in lymphatic remodeling, was decreased in Polydom−/− mice. Polydom bound to the lymphangiogenic factor Ang-2 (angiopoietin-2), which was found to upregulate Foxc2 expression in cultured lymphatic endothelial cells. Expressions of Tie1/Tie2 receptors for angiopoietins were also decreased in Polydom−/− mice.

Conclusions: Polydom affects remodeling of lymphatic vessels in both mouse and zebrafish. Polydom deposited around lymphatic vessels seems to ensure Foxc2 upregulation in lymphatic endothelial cells, possibly via the Ang-2 and Tie1/Tie2 receptor system. (Circ Res. 2017;120:1276-1288. DOI: 10.1161/CIRCRESAHA.116.308825.)

Key Words: embryonic development ■ extracellular matrix ■ lymphatic capillary ■ Polydom/Svep1 ■ vascular remodeling

The main function of the lymphatic vasculature is to collect the protein-rich tissue fluid extravasated from blood vessels and return it to the blood circulation.1 Lymphatic vessels are required for lipid absorption, as well as immune cell trafficking and surveillance. Defects in lymphatic function lead to lymph accumulation in tissues, known as lymphedema.2,3 Differentiation of lymphatic endothelial cells (LECs) in mice starts around E9.0 in a subset of endothelial cells of the cardinal vein via upregulation of Proxl, a master regulator of LECs.4 Prox1-positive LECs bud and migrate from the vein in response to vascular endothelial growth factor (VEGF)-C5 to form the first lymphatic structures, designated the jugular lymph sac or primordial thoracic duct (TD) at E12.5,6,7 Lymphatic vessels further sprout from these structures to form the lymphatic network throughout the body.

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From the Laboratory of Extracellular Matrix Biochemistry, Institute for Protein Research, Osaka University, Suita, Osaka, Japan (N. Morooka, S.F., R.S.-N., M.N., Y.T., C.S., I.N., K.S.); Laboratory of Anatomy and Cell Biology, Osaka Medical College, Takatsuki, Osaka, Japan (S.F.); Department of Cell Biology (M.N., H.N., M. Mochizuki) and AMED-CREST (N. Mochizuki), National Cerebral and Cardiovascular Center Research Institute, Suita, Osaka, Japan.

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Correspondence to Kiyotoshi Sekiguchi, Institute for Protein Research, Osaka University, 3-2 Yamadaoka, Suita, Osaka 565-0871, Japan. E-mail sekiguchi@protein.osaka-u.ac.jp

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Lymphatic vessels regulate tissue fluid homeostasis, immune cell trafficking, and absorption of dietary fats. Development of the primitive lymphatic plexus into a hierarchical vascular tree, comprising lymphatic capillaries and collecting vessels, is essential to its function. Both extracellular and intracellular factors (such as angiopoietin-2 and Foxc2, and cell-surface receptors Tie1 and ephrinB2) are crucial to this development; however, mechanisms that underlie lymphatic remodeling are not well understood. This study shows that extracellular matrix protein Polydom/Svep1 is essential for lymphatic development, including sprouting of new capillaries and formation of collecting vessels. Polydom/Svep1 accumulates on lymphatic endothelial cells but is secreted from mesenchymal cells. Polydom/Svep1 upregulates transcription factor Foxc2 in lymphatic endothelial cells, possibly through its known receptor, integrin α9β1. Understanding the functions of this newly identified extracellular protein will help elucidate the mechanisms by which extracellular factors regulate lymphatic vessel development.

What Is Known?

• Polydom/Svep1 is an extracellular matrix protein and a high-affinity ligand for integrin α9β1, a cell-adhesion receptor that facilitates lymphangiogenesis.
• In embryonic mice, the primitive lymphatic plexus develops into a hierarchical vascular network of lymphatic capillaries and collecting vessels.
• Loss-of-function studies have shown that Ang-2 (angiopoietin-2), Tie1, ephrinB2, and transcription factor Foxc2 (forkhead box protein c2) play a crucial role in these lymphatic development processes.

What New Information Does This Article Contribute?

• Mesenchymal cell-derived Polydom/Svep1 is essential for lymphatic system development, including sprouting of new capillaries and formation of collecting vessels.
• Polydom/Svep1 upregulates Foxc2 expression, possibly through the pathway involving angiopoietin-2 and Tie1/Tie2 receptors rather than through its receptor integrin α9β1.

Nonstandard Abbreviations and Acronyms

<table>
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<th>Abbreviation</th>
<th>Explanation</th>
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<tr>
<td>Ang</td>
<td>angiopoietin</td>
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<tr>
<td>dpf</td>
<td>days postfertilization</td>
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<tr>
<td>Foxc2</td>
<td>forkhead box protein c2</td>
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<tr>
<td>ISLV</td>
<td>intersegmental lymphatic vessel</td>
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<tr>
<td>Itga9</td>
<td>integrin α9</td>
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<tr>
<td>LEC</td>
<td>lymphatic endothelial cell</td>
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<td>MO</td>
<td>morpholino</td>
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<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
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<td>PDGFR</td>
<td>platelet-derived growth factor receptor</td>
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<td>PL</td>
<td>parachordal lymphangioblast</td>
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<td>RT-PCR</td>
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<td>TD</td>
<td>thoracic duct</td>
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<td>VEGF</td>
<td>vascular endothelial growth factor</td>
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These mutant mice also display defective sprouting of lymphatic capillaries with ectopic smooth muscle cell coverage. The lymphatic maturation defects during late gestation and the postnatal period lead to lymphatic dysfunction and impaired postnatal survival. However, the detailed mechanisms underlying lymphatic maturation remain unknown, especially with regard to the interactions with environmental factors. Here we show that the extracellular matrix protein Polydom/Svep1 (hereafter referred to as Polydom) is required for lymphatic remodeling.

Polydom is a large extracellular matrix protein of >300 kDa expressed in cultured bone marrow stromal cells.15–17 Recently, we identified Polydom as a high-affinity ligand for integrin α9β1,18 a cell adhesion receptor involved in lymphangiogenesis. Integrin α9 (Itga9) is predominantly expressed in lymphatic valves. Itga9 deficiency in mice leads to defects in formation of luminal valves in collecting lymphatic vessels and early postnatal death from chylothorax between 6 and 12 days of age.19,20 The ligand for integrin α9β1 that functions in lymphangiogenesis remains to be defined because gene knockout of known integrin α9β1 ligands like osteopontin and tenascin-C do not cause any defects in lymphatic vessels.21,22

In this study, we addressed the physiological function of Polydom by generating Polydom-deficient mice and zebrafish. Polydom−/− mice showed severe edema from the mid-gestation stage and died immediately after birth. In Polydom−/− embryos, the primitive lymphatic plexus was developed, but the subsequent remodeling was impaired. Impaired lymphatic development was also observed in zebrafish after knockdown/knockout of polydom. We explored the mechanism underlying the phenotype of Polydom−/− mice by focusing on a panel of transcription factors and growth factors involved in lymphangiogenesis.

Methods

Targeted Disruption of the Polydom Gene in Mice Causes Severe Edema

We generated Polydom-deficient mice to clarify the physiological roles of Polydom. The targeting vector was designed to excise exon 2 of the Polydom gene, thereby resulting in aberrant termination of Polydom protein translation (Figure 1A). The targeted Polydom gene was confirmed by Southern blotting (Figure 1B) and genomic polymerase chain reaction (PCR; Figure 1C). Reverse transcription PCR analysis showed that exon 2 was deleted, but the following exons were transcribed in the targeted allele (Figure 1D). Immunohistochemical staining with an anti-Polydom antibody failed to detect any Polydom protein in Polydom−/− mice (Figure 1E), confirming that excision of exon 2 resulted in loss of the functional protein.

Polydom−/− embryos were born at a nearly Mendelian frequency at E18.5, although no homozygotes survived to day...
7 after birth (Figure 1F). Further examination revealed that Polydom–/– mice died immediately after birth: Polydom–/– embryos obtained by Caesarian section at E18.5 were alive but cyanotic and died within 30 minutes after birth (Figure 1G). Polydom–/– pups exhibited severe edema, with excessive fluid accumulation in the thoracic and abdominal cavities and...
subcutaneous space (Figure 1H). Hydrostatic and histological examinations of the lungs of Polydom−/− pups revealed that the excessive accumulation of pleural fluid rendered the alveolar airspace unable to inflate, thereby, resulting in respiratory failure (Online Figure I). These findings point to a role of Polydom in fluid homeostasis.

**Polydom-Deficient Mice Exhibit Aberrant Lymphatic Vessel Formation and Dysfunction of Fluid Drainage**

The edema observed in Polydom−/− embryos suggested a dysfunction of the cardiovascular or lymphatic system. Because vascular development was not significantly impaired in Polydom−/− embryos (Online Figure II), we explored any defects in lymphatic development that could cause severe edema. No apparent defects were observed for Prox1-positive primordial TD formation at E12.5 (Online Figure III), suggesting that the specification of LECs and the formation of the first lymphatic structures were not affected by disruption of Polydom. The primitive lymphatic plexus was formed in the Polydom−/− embryos at E15.5, but the vascular patterning was impaired: VEGF receptor (VEGFR)-3-positive lymphatic vessels in the skin of mutant mice were heterogeneous in size and had many bumps, while the skin of wild-type littermates showed a uniformly sized lymphatic plexus (Figure 2A, 2B, and 2I). At E18.5, the lymphatic vessels gave rise to secondary sprouts that invaded into the upper dermal layers in wild-type mice (Figure 2E and 2I). In Polydom−/− mice, the initiation of sprouting appeared to occur, but the LECs failed to elongate and yielded rounded bumps, suggesting that the migration of LECs was impaired in Polydom−/− mice (Figure 2F and 2J).

In the mesentery, loss of Polydom resulted in a failure of collecting lymphatic vessel formation. In wild-type mice, the primitive lymphatic plexus was remodeled to form a single lymphatic trunk with luminal valves that were detected as laminin α5-positive structures (Figure 2C, 2G, and 2K). However, the lymphatic vessels in Polydom−/− mice remained highly branched and did not develop luminal valves (Figure 2D, 2H, and 2K). These phenotypes in Polydom−/− embryos were more severe than those in Itga9−/− embryos, which can develop collecting lymphatic vessels, but fail to form luminal valves.19,20 Although Polydom is a ligand for integrin α9β1, these findings raise the possibility that the failure of lymphatic vessel remodeling in Polydom−/− mice may not be explained by impaired Polydom–integrin α9β1 interactions.

Aberrant remodeling of lymphatic capillaries was also observed in the intestine and heart of Polydom−/− embryos. In the intestine, lymphatic vessels are first organized into the lymphatic plexus in the intestinal wall and then sprout into villi to form lacteals.21 Similar to the findings for the skin, the lymphatic plexus developed in the intestinal wall, but the lacteals could not migrate into the villi in Polydom−/− mice (Figure 2L through 2N). Furthermore, extracardiac LECs were found to migrate into the ventricular surface in Polydom−/− mice, but the vessels failed to extend toward the apex of the heart (Figure 2O and 2P). Thus, Polydom-deficient mice have multiple defects in lymphatic vessel remodeling.

To determine whether the morphological defects observed in the lymphatic vessels lead to impaired lymphatic drainage, we assessed the lymphatic function by intradermal injection of Indian ink. Ink uptake was not observed in Polydom−/− mice, indicating failure of the lymphatic flow (Figure 2Q and 2R; Online Figure IV). Consistent with the impaired lymphatic drainage, immunofluorescence staining of VE-cadherin demonstrated that the LECs in Polydom−/− embryos had a cuboidal shape and did not align longitudinally, while the LECs in wild-type embryos elongated in the direction of the lymphatic flow (Figure 2S through 2U).24 These findings indicated that Polydom is involved in the remodeling of lymphatic vessels, including the sprouting of new capillaries and formation of collecting lymphatic vessels.

**Role of Polydom in Thoracic Duct Formation in Zebrafish**

Zebrafish possess a well-defined lymphatic vascular system that shares morphological, molecular, and functional characteristics with the lymphatic vessels in mammals.25 Because Polydom is highly conserved in vertebrates,27 we examined whether polydom is also involved in the trunk lymphatic network in zebrafish. We performed knockdown of polydom in zebrafish using a splice-blocking morpholino (polydom MO). The knockdown efficiency of MO was assessed by quantitative reverse transcription PCR (Online Figure VA). MO-mediated knockdown of zebrafish polydom led to loss of formation of the TD between the dorsal aorta and the posterior cardinal vein at 4 days postfertilization (dpf; Figure 3A and 3B). The lymphatic progenitors in zebrafish initially emerge from the posterior cardinal vein at 1.5 dpf as lymphovenous sprouts and grow to form a string of parachordal lymphangioblasts (PLs) at the horizontal myoseptum.26,27 The PL formation in polydom-knockdown embryos was comparable to that in control embryos at 2.5 dpf (Online Figure VB and VC). By 3 dpf, the PLs in control fish started to migrate along arterial intersegmental vessels either dorsally or ventrally, thereby, leading to formation of the intersegmental lymphatic vessels (ISLVs), dorsal lymphatic lines, and the TD (Online Figure VG and VE and Online Movie I). In contrast, PLs did not migrate away from the midline and failed to form ISLVs in polydom morphants (Online Figure VG and VE and Online Movie II), suggesting that impaired ISLV formation results in loss of the TD.

To visualize polydom-expressing cells, we generated embryos expressing mCherry under control of the polydom promoter. Polydom-expressing cells were detected along the PLs, ISLVs, and TD region where the knockdown phenotypes became evident (Online Figure VF).

To rule out possible off-target effects of the polydom MO,28 we used TALEN (transcription activator-like effector nuclease)-mediated gene editing and generated a mutant polydomrev106 allele with an 11-bp deletion in the first exon of polydom. This allele resulted in a frameshift after amino acid 17 and a premature stop codon after an additional 75 amino acids (Online Figure VG through VI). Homozygous mutants lacked the TD at 6 dpf in accordance with the MO-induced phenotypes (Figure 3C and 3E). Most mutants developed severe edema around the gut and the eye at 6 dpf (Figure 3D and 3F; Online Figure VJ), presumably resulting from dysfunction of the lymphatic system.25,29 These phenotypes of the mutant fish were consistent with those of Polydom−/− mice, corroborating the involvement of polydom in the lymphatic vessel formation in zebrafish.
Figure 2. Aberrant lymphatic vessel formation and dysfunction of fluid drainage in Polydom-deficient mice. A–H. Whole-mount immunofluorescence staining for vascular endothelial growth factor receptor (VEGFR)-3 (green) and laminin α5 (red) in E15.5 and E18.5 Polydom−/− or wild-type (+/+) embryos. VEGFR-3-positive lymphatic vessels showed a meshwork that was uniform in lumen size in the wild-type skin, but uneven in lumen size in the Polydom−/− skin (arrowheads in B and F). Lymphatic sprouts at E18.5 (arrows in E) were only detected in the wild-type skin. The mesenteric lymphatic vessels at E15.5 in Polydom−/− mice were comparable to those (Continued)
Polydom Is Produced by Mesenchymal Cells and Deposited Around Lymphatic Vessels

To investigate whether Polydom interacts with lymphatic vessels, we performed immunofluorescence staining of mouse embryos with an anti-Polydom antibody. In E12.5 embryos, Polydom was expressed around the primordial TD (Online Figure VI). Whole-mount immunofluorescence staining of the skin and mesentery revealed that Polydom was deposited in a fibrillar pattern around the primitive lymphatic plexus at E15.5 and the collecting lymphatic vessels at E18.5 (Figure 4A through 4H; Online Figure VII). In addition, Polydom was strongly detected at lymphatic valves (Figure 4E through 4H).

Figure 2. Continued in wild-type mice, but failed to form the lymphatic trunk and laminin α5-positive lymphatic valves (arrow in G) at E18.5 (quantified in K). E15.5 skin: wild-type (+/+), n=9 embryos; homozygous (−/−), n=9 embryos. E18.5 skin: wild-type (+/+), n=8 embryos; homozygous (−/−), n=6 embryos. E15.5 mesentery: wild-type (+/+), n=8 embryos; homozygous (−/−), n=12 embryos. E18.5 mesentery: wild-type (+/+), n=14 embryos; homozygous (−/−), n=12 embryos. I and J, Quantification of bumps and branch points in E15.5 skin (upper) and second sprouts and bumps in E18.5 skin (lower). Data are means±SEM (n=5 embryos each for E15.5; n=4 embryos each for E18.5). The differences between wild-type (+/+) and homozygous (−/−) were statistically significant (P<0.001, except for the number of branch points in E15.5 skin. L and M, Immunofluorescence staining for LYVE-1 (red) and PECAM-1 (green) in E18.5 hearts. LYVE-1-positive coronary lymphatic vessels and PECAM-1-positive coronary vessels were stained with Hoechst 33342 (blue). Arrowheads in M indicate dilated lymphatic vessels. Central lacteals were absent from the villi in Polydom−/− mice, whereas lacteals extended to the tip of villus in wild-type mice (arrows in L). Wild-type (+/+), n=3 embryos; homozygous (−/−), n=5 embryos. N, Quantification of sprouts into villi and dilated lymphatic vessels in the intestine. Data are means±SEM. Wild-type (+/+), n=5 panels from 2 embryos; homozygous (−/−), n=6 panels from 3 embryos. The number of villi with lymphatic sprouts as well as that of nondilated lymphatic vessels around intestinal wall was significantly reduced in homozygous embryos (P<0.001). O and P, Whole-mount immunofluorescence staining for LYVE-1 (red) and PECAM-1 (green) in E18.5 hearts. LYVE-1-positive coronary lymphatic vessels failed to extend toward the apex of the heart in all Polydom−/− mice examined (arrowheads). Wild-type (+/+), n=3 embryos; homozygous (−/−), n=6 embryos. Q and R, Lymphangiography by injection of Indian ink into the hindlimb footpads of E18.5 embryos. Wild-type (+/+), n=3 embryos; homozygous (−/−), n=4 embryos. None of the Polydom−/− embryos examined exhibited uptake in the ventral skin. S, and T, Whole-mount immunofluorescence staining for Prox1 (red) and VE-cadherin (green) in the E18.5 mesentery. Wild-type (+/+, n=5 embryos; homozygous (−/−), n=4 embryos. U, Quantification of the cell alignment in E18.5 mesenteric lymphatic vessels. The rotation angle of Prox1-positive cells relative to the longitudinal axis of the vessel was analyzed. Data are means±SEM. Wild-type (+/+), n=11 panels in 3 embryos; homozygous (−/−), n=13 panels in 3 embryos. *P<0.001. Bars, 100 μm. NS indicates not significant.

Figure 3. Impaired thoracic duct (TD) formation in zebrafish after knockdown/knockout of polydom. A, Lateral views of 4 days postfertilization (dpf) Tg(fli1:GFP) embryos uninjected (control) or injected with the polydom morpholino (MO). The TD (closed arrowheads in control embryos) was absent from the polydom morphants (open arrowheads). Bars, 50 μm. B, Percentages of TD formation at 4 dpf. Data are means±SEM (control, n=75 embryos; polydom MO, n=70 embryos). N=4. Embryos with TD formation at >80%, 10% to 79%, and 0% to 9% in the 4 segments above the distal intestine were counted as normal, incomplete, and loss, respectively. Impaired TD formation was statistically significant in embryos injected polydom MO (P<0.001). C, Lateral views of wild-type and polydom−/− Tg(fli1:GFP) embryos at 6 dpf. Closed arrowheads in the wild-type embryos indicate the TD, which was absent in the polydom−/− embryos (open arrowheads). Bars, 50 μm. D, Overall morphology of the wild-type and polydom−/− Tg(fli1:GFP) embryos at 6 dpf. The arrow indicates edema around the gut area. E, Percentages of TD formation at 6 dpf. Data are means±SEM (wild-type [+/+], n=32 embryos; heterozygous [+/ncv106], n=83 embryos; homozygous [ncv106/ncv106], n=29 embryos). N=3. Impaired TD formation was statistically significant in polydom−/− and polydom+/− embryos (P<0.001 against polydom−/− and polydom+/− embryos). F, Percentages of edema incidence at 6 dpf. Data are means±SEM (wild-type [+/+], n=32 embryos; heterozygous [+/ncv106], n=83 embryos; homozygous [ncv106/ncv106], n=29 embryos). N=3. Edema incidence was statistically significant in polydom−/− embryos (P<0.01 and 0.05 against polydom−/− and polydom+/− embryos, respectively).
These results indicate that Polydom directly associates with lymphatic vessels throughout embryonic development.

To examine whether Polydom is produced and deposited by LECs, we visualized the Polydom-expressing cells using the mutant mouse strain B6N(Cg)-Svep1tm1b(EUCOMM)Hmgu/J, which carries a lacZ reporter downstream of the first exon of Polydom. Contrary to our expectation, expression of the lacZ reporter was not detected on lymphatic vessels, but in cells scattered in the dermis (Figure 4I), suggesting that Polydom is expressed in mesenchymal cells, but not in LECs. To corroborate this observation, we serially fractionated cells from the skin of E15.5 embryos into blood cells, LECs, blood vascular endothelial cells, and flow-through cells (referred to as others) and compared the expression levels of Polydom mRNA in the fractionated cells. Polydom expression was predominantly detected in the flow-through cells, but not in LECs, blood vascular endothelial cells, or blood cells (Figure 4J, upper panel). Furthermore, fractionation of cells that expressed platelet-derived growth factor receptor (PDGF)-α, a cell surface marker for mesenchymal stem cells, revealed that Polydom was strongly expressed in PDGF-α-expressing cells (Figure 4J, lower panel). These results were consistent with the localized lacZ activity in the mesenchymal cells of the Polydom-lacZ reporter mice. We also examined conditional knockout mice in which Polydom expression was disrupted in endothelial cells by expression of Cre recombinase under control of a Tie2 promoter. The mice were viable and fertile with no symptoms of edema (N. Morooka, data not shown), confirming that LECs are not the major source of Polydom.

**Expression of Transcriptional Factor Foxc2 Is Reduced in Polydom−/− Mice**

To explore the mechanism by which Polydom depletion causes lymphatic defects, we compared the expression of lymphatic...
Figure 5. Expression of lymphatic endothelial markers. Whole-mount immunofluorescence staining for Prox1 (A–D), Foxc2 (forkhead box protein c2; E–H), LYVE-1 (lymphatic vessel endothelial hyaluronan receptor 1; I–L), and Podoplanin (M–P) in Polydom−/− or wild-type (+/+) embryos at E16.5 and E18.5. Foxc2 expression was reduced in Polydom−/− mesenteric lymphatic vessels from E16.5 to E18.5 (arrowheads in F and H). Asterisks indicate arterial expression of Foxc2. Mesenteric lymphatic vessels failed to downregulate Prox1 and LYVE-1 expression at E18.5 in the absence of Polydom (D and L). Prox1 staining at E16.5: wild-type (+/+), n=4 embryos; homozygous (−/−), n=4 embryos. Prox1 staining at E18.5: wild-type (+/+), n=5 embryos; homozygous (−/−), n=7 embryos. Foxc2 staining at E16.5: wild-type (+/+), n=3 embryos; homozygous (−/−), n=3 embryos. Foxc2 staining at E18.5: wild-type (+/+), n=5 embryos; homozygous (−/−), n=10 embryos. LYVE-1 and Podoplanin staining at E16.5: wild-type (+/+), n=10 embryos; homozygous (−/−), n=5 embryos. Bars, 100 μm. Q. Numbers of Prox1+ cells and percentage of Foxc2+ cells within Prox1+ cells from whole-mount staining of the mesentery. Data are means±SEM (n=10 panels each for E16.5; n=13 panels each for E18.5). R. Quantitative reverse transcription polymerase chain reaction (RT-PCR) analysis for Foxc2 expression in LECs isolated from the Polydom−/− or wild-type (+/+).
markers in mesenteric lymphatic vessels between wild-type and Polydom−/− mice. Prox1, a master regulator of lymphatic development, was expressed uniformly in wild-type LECs at E16.5 (Figure 5A). The expression of Prox1 was then downregulated at E18.5, except in the valve region, at a time when the primitive lymphatic plexus was remodeled into collecting vessels (Figure 5C). In Polydom−/− mice, the Prox1 expression remained high in LECs at E18.5 (Figure 5B, 5D, and 5Q). Foxc2, which controls lymphatic vascular maturation, was widely expressed at E16.5 in wild-type mice and thereafter restricted to the valve region (Figure 5E and 5G). However, the expression of Foxc2 was significantly low in Polydom−/− mice at both E16.5 and E18.5, compared with the levels in wild-type mice (Figure 5F and 5H).

Quantification of Foxc2+ cells within the Prox1+ cell population showed that ≈40% of Prox1+ cells were Foxc2+ in wild-type mice but <5% were Foxc2+ in Polydom−/− mice (Figure 5Q). LYVE-1 expression was high at E16.5 and then decreased at E18.5 in wild-type mice, while it remained high in Polydom−/− lymphatic vessels at E18.5 (Figure 5I through 5L). No significant difference was detected in Podoplanin expression between wild-type and Polydom−/− mice (Figure 5M through 5P). These results indicated that the expression of Foxc2, but not other lymphatic markers, is reduced in Polydom−/− mice. To corroborate this observation, we examined the expression level of Foxc2 transcripts in LECs isolated from Polydom−/− mouse skin by reverse transcription PCR (Figure 5R). The results showed that Foxc2 expression was reduced by ≈60% at E15.5 and ≈80% at E18.5 in skin LECs from Polydom−/− embryos, confirming the involvement of Polydom in the transcriptional regulation of Foxc2 expression. As Foxc2−/− mice show similar defects in lymphatic remodeling, these results raise the possibility that the phenotype of Polydom−/− mice primarily stems from the reduced expression of Foxc2.

**Ang-2 Binds to Polydom and Potentiates Foxc2 Expression in LECs**

To explore the mechanism by which Polydom regulates Foxc2 expression in LECs, we investigated the interactions of Polydom with a panel of growth factors involved in lymphatic development, including VEGF-C and Ang-2, by solid-phase binding assays. Recombinant integrin α9β1 and type I collagen were used as positive and negative controls, respectively, in the assays. Polydom did not show any significant binding to VEGF-C, but was capable of binding to Ang-1 and Ang-2 (Figure 6A). When human dermal LECs were cultured in the presence of Ang-1 or Ang-2, only Ang-2 enhanced the expression of Foxc2 in human dermal LECs (Figure 6B and 6C), suggesting that Polydom exerts its effect on Foxc2 expression through interaction with Ang-2.

Ang-2 has been shown to act as an agonist/antagonist for Tie receptors in a context-dependent manner. To investigate whether Polydom affects the expressions of Tie1 and Tie2 receptors, we compared expression of Tie1 and Tie2 transcripts in dermal LECs from wild-type and Polydom−/− mice by quantitative reverse transcription PCR. Transcripts for both Tie1 and Tie2 were significantly reduced in Polydom−/− mice (Figure 7A). Consistent with the results, immunofluorescence of Tie1 was reduced in Polydom−/− lymphatic vessels (Figure 7B and 7C). Tie2 immunofluorescence was also decreased (although to a lesser extent) in Polydom−/− mice. These results suggest that signaling events downstream of Tie receptors are compromised in Polydom−/− mice because of reduced Tie1/Tie2 expression.

**Discussion**

The lymphatic vasculature develops through a series of events, including formation of lymph sacs, sprouting of lymphatic vessels, and remodeling and maturation of the primitive lymphatic plexus. These events are regulated by factors secreted by either mesodermal/mesenchymal cells or LECs, including VEGF-C, Cebe1 (collagen- and calcium-binding EGF domain–containing protein 1), fibronectin EIIIA (extra type-III repeat A), EMILIN1 (elastin microfibril interface located protein 1).35 Here, we provide evidence that Polydom, an extracellular matrix protein that acts as a high-affinity ligand for integrin α9β1, is involved in lymphatic development, particularly in the remodeling and maturation process. Polydom-deficient mice developed severe edema and died immediately after birth because of respiratory failure. The primitive lymphatic plexus failed to remodel into collecting lymphatic vessels with luminal valves in Polydom−/− mice.
Polydom was also involved in the TD formation in zebrafish, indicating an evolutionarily conserved role of Polydom in lymphangiogenesis.

Our results showed that the expression of Foxc2 was significantly reduced in Polydom−/− mice. The involvement of Foxc2 in lymphatic remodeling has been demonstrated by Petrova et al.8,9 Mouse embryos deficient in Foxc2 expression exhibit irregular lymphatic vasculature patterning and a failure to form the collecting trunk, both of which are phenotypic features reminiscent to those of Polydom−/− embryos. In lymphatic vascular development, upregulation of Foxc2 precedes the morphological changes and is followed by downregulation of Prox1 and LYVE-1 expression. The lymphatic vessels in Polydom−/− embryos failed to downregulate Prox1 and LYVE-1 expression, consistent with the phenotypes of Foxc2-deficient mice.9 Furthermore, Polydom−/− mice showed increased recruitment of smooth muscle cells to dermal lymphatic capillaries, as well as overexpression of Ang2 in LECs (N. Morooka, unpublished observations), similar to the case for Foxc2−/− mice.8,9 These phenotypic similarities between Polydom−/− and Foxc2−/− mice suggest that the downregulation of Foxc2 is responsible for the lymphatic defects in Polydom−/− mice.

Because Polydom is a high-affinity ligand for integrin α9β1,18 the interaction with integrin α9β1 may lead to the upregulation of Foxc2 expression. The involvement of integrin α9β1 in lymphatic development, particularly in lymphatic valve formation, has been documented.20 However, the phenotype of Itga9−/− mice is less severe than that of Polydom−/− mice, given that Itga9−/− mice survive for 6 to 12 days after birth,19 while Polydom−/− neonates die within 30 minutes. The tortuous structure of the lymphatic plexus in the skin and the remodeling defects observed in the mesenteric vessels in Polydom−/− mice were not found in Itga9−/− mice.20,36 These discrepancies between the phenotypes of Itga9−/− and Polydom−/− mice argue against the possibility that integrin α9β1 is the putative Polydom receptor responsible for the sprouting of new capillaries and formation of collecting lymphatic vessels. This conclusion is further supported by the fact that zebrafish polydom lacks the EDDMMEVPY sequence within the 21st CCP (complement control protein) module, which is the sequence recognized by integrin α9β1,18 thus, precluding the role of integrin α9β1 as a polydom receptor in zebrafish. Furthermore, Karpanen et al37 have demonstrated that zebrafish Itga9 mutants do not show a lymphatic phenotype. It should be noted, however, that Polydom may contribute to lymphatic valve formation in mice as an integrin α9β1 ligand because depletion of Polydom after E15.5 did not compromise the formation of collecting lymphatic vessels, but did result in failure of luminal valve formation (Online Figure VIII).

Accumulating evidence indicates that extracellular matrix proteins bind to growth factors and, thereby, regulate their distribution, activation, and presentation to cognate receptors on cells.38,39 Although VEGF-C/VEGFR-3 signaling is a well-known pathway involved in lymphangiogenesis, our

Figure 7. Expression of Tie receptors is lower in Polydom-deficient mice. A, Quantitative reverse transcriptase polymerase chain reaction (RT-PCR) analysis of Tie1 and Tie2 expression in lymphatic endothelial cells (LECs) isolated from the Polydom−/− or wild-type (+/+) skin at E18.5. Actb was used as a control. Data represents means±SEM of 3 independent experiments. *P<0.05. B, Whole-mount immunofluorescence staining for Tie1 and Tie2 with LYVE-1 (lymphatic vessel endothelial hyaluronan receptor 1) in Polydom−/− or wild-type (+/+) embryos at E16.5. Expression of Tie1 and Tie2 was significantly less in Polydom−/− dermal lymphatic vessels (arrowheads in B; quantified in C). Tie1 staining: wild-type (+/+), n=3 embryos; homozygous (−/−), n=5 embryos. Tie2 staining: wild-type (+/+), n=3 embryos; homozygous (−/−), n=6 embryos. Bars, 50 μm. C, Quantification of immunofluorescence signal intensity for Tie1 and Tie2 in Polydom−/− or wild-type (+/+) embryos at E16.5. Data are means±SEM. Tie1: wild-type (+/+), n=4 panels; homozygous (−/−), n=5 panels. Tie2: wild-type (+/+), n=3 panels; homozygous (−/−), n=3 panels. *P<0.05; **P<0.001.
solid-phase binding assays failed to demonstrate any significant interaction between Polydom and VEGF-C. In line with our finding, Karpanen et al. showed that the overexpression of VEGF-C in polydom mutant fish did not show any difference in VEGF-C signaling compared with that in sibling control. We tried to express recombinant CCBE1 in human 293 cells, but the yield of the recombinant protein was low, and we could not obtain enough amount of CCBE1 for solid-phase assays. Karpanen et al. demonstrated that double svec/ccbe1 heterozygous zebrafish embryos did not show TD defects. These results lead us to conclude that Polydom does not contribute to the vegfc/vegfr3 pathway that is essential for early-stage lymphangiogenesis.

Several lines of evidence indicate that Ang-2 is a lymphangiogenic factor involved in remodeling and maturation of the lymphatic vasculature. Ang-2 deficiency leads to failure of lymphatic remodeling from a primitive plexus into collecting vessels, as well as failure of intraluminal valve formation, being phenotypes reminiscent of Foxc2 mice and Polydom mice. Our results showed that Polydom bound to Ang-1 and Ang-2, but only Ang-2 could enhance Foxc2 expression in human dermal LECs, raising the possibility that Polydom exerts its effect on Foxc2 expression in LECs by potentiating Ang-2 (Online Figure IX). Consistent with this possibility, inhibition of Ang-2 in mouse embryos by treatment with an Ang-2-blocking antibody led to downregulation of Foxc2 in lymphatic vessels.

Ang regulates vascular development through interaction with Tie receptors. Several lines of evidence indicate that Tie1 is involved in lymphatic development: Tie1 deficiency in mice leads to edema and remodeling defects in lymphatic vessels. Conditional deletion of Tie1 with Nfat1Cre leads to the defects in lymphatic valve formation and collecting vessel remodeling and is associated with lower Foxc2 expression. Our results show that Tie1 expression is significantly reduced in Polydom mice, which implies that the reduced Tie1 expression downregulates signaling events downstream of the Ang–Tie system, thereby, resulting in reduced Foxc2 expression (Online Figure IX). This supposition is supported by the common phenotypic features—including severe edema and defects in lymphatic remodeling—seen in mice deficient in expression of Polydom, Ang-2, Tie1, or Foxc2, although how Polydom regulates Tie1 expression is not yet clear. Ang-2 was recently reported to induce Tie1 expression. If Polydom is an activator of Tie1 expression, it remains to be elucidated how Polydom facilitates the remodeling of lymphatic vessels, as typical observed in the mesenteric collecting trunk in Polydom mice. Consistent with this scenario, new sprouts from the primitive lymphatic plexus were significantly reduced in the Polydom mice and skin and intestinal tube. The elongation of lymphatic capillaries was also impaired in the heart of Polydom mice. Furthermore, polydom knockout in zebrafish caused defects in TD formation that were ascribed to impaired ISLV formation caused by PL migration defects. These results obtained for Polydom-deficient mice and polydom mutant zebrafish lend support for the possibility that Polydom is involved in the migration of LECs through the upregulation of Foxc2, thereby, facilitating the remodeling of the primitive lymphatic plexus into a hierarchical vascular tree, although the detailed mechanisms by which Polydom regulates LEC migration in lymphatic remodeling remain to be investigated.

Despite the localized deposition of Polydom on lymphatic vessels, our results showed that Polydom was predominantly expressed in mesenchymal cells, but not in LECs. Imaging of the β-galactosidase activity in Polydom-lacZ reporter mice demonstrated that Polydom-expressing cells were scattered in the dermis, residing beneath lymphatic vessels. Furthermore, polydom-expressing cells in TgBAC(polydom:McCherry) zebrafish were detected along the PLs and ISLVs, as well as in the region between the dorsal aorta and the posterior cardinal vein where the TD is formed. These results indicate that the mesenchymal cells residing near the primitive lymphatic plexus express and secrete Polydom, which is deposited on LECs to facilitate the remodeling of lymphatic vessels. In support of this possibility, significant Polydom expression was detected in cells that express PDGFR-α.

There is accumulating evidence that extracellular factors secreted by mesenchymal cells play essential roles in lymphatic vascular development. VEGF-C and Ccbe1, which are both secreted by mesodermal/mesenchymal cells, have been shown to act in the initial stage of lymphatic development, that is, sprouting of lymphangioblasts from the venous endothelium. However, the roles of mesenchymal factors in lymphatic remodeling and maturation have been poorly understood. Our results showed, for the first time, that Polydom is a mesenchymal factor involved in lymphatic remodeling. Although it remains to be elucidated how Polydom facilitates the remodeling and maturation of the lymphatic vasculature, further investigations into the mechanisms by which Polydom acts on the primitive lymphatic plexus to upregulate Foxc2...
expression should provide insights into the interplay between LECs and the surrounding mesenchymal cells that ensures the development of the lymphatic vasculature and maintenance of the fluid homeostasis in the body.

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Disclosures
None.

References
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References


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Nanami Morooka, Sugiko Futaki, Ryoko Sato-Nishiuchi, Masafumi Nishino, Yuta Totani, Chisei Shimono, Itsuko Nakano, Hiroyuki Nakajima, Naoki Mochizuki and Kiyotoshi Sekiguchi

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Mouse Strains

To generate Polydom<sup>−/−</sup> mice, we constructed a targeting vector containing a single loxP site upstream of exon 2 followed by a PGK promoter-driven neomycin-resistance (PGK-neo) cassette flanked by two FRT sequences at the 5’ and 3’ ends, respectively, and another loxP site in the downstream of these sequences (Figure 1A). The construct was flanked by 4.4-kb upstream and 4.6-kb downstream Polydom genomic sequences. The targeting vector was introduced into C57BL/6N-background mouse embryonic stem cells to induce homologous recombination. Mice derived from the recombinant embryonic stem cell clone containing the neo allele were crossed with a transgenic line, B6.Cg-Tg(CAG-cre)CZ-MO20sb (RIKEN BRC),<sup>1</sup> to excise exon 2 and the PGK-neo cassette. Excision of exon 2 was verified by Southern blotting. Genomic DNA was isolated from the embryonic liver using DNAzol<sup>®</sup> Reagent (Invitrogen) and digested with BamHI and XhoI for detection with a 5′ probe and SpeI for detection with a 3′ probe. Antisense probes labeled with digoxigenin (DIG) were prepared using a PCR DIG Probe Synthesis Kit (Roche) with the following primers: 5′-probe-F, 5′-TGGTTGTACTGGGTAGCTGA-3′; 5′-probe-R, 5′-CGTCTGGCATTITTTCTCTTG-3′; 3′-probe-F, 5′-TCTCGCCAGAATGTGTTCACG-3′; 3′-probe-R, 5′-TAAGGTTAGCTAGGAAACC-3′. Genotyping was performed by PCR using tail DNA with the following three primers: 5′-TGACACTGGAGCTCCTGTGCCTTTG-3’ (F1); 5′-GATCCATGAGATGAATTGAGGTGT-3′ (F2); and 5′-GATCCATGAGATGAGATGAATTGAGGTGT-3′ (R1); 5′-GATCCATGAGATGAGATGAATTGAGGTGT-3′ (Cre-F); 5′-GATCCATGAGATGAGATGAATTGAGGTGT-3′ (Cre-R). The wild-type allele gave a 243-bp band, while the flox allele and the mutant allele gave a 360-bp and a 559-bp band, respectively.

A Polydom-lacZ knock-in mouse line, B6N(Cg)-Svep1<sup>tm1b(EUCOMM)Hmgu/J</sup>, was obtained from The Jackson Laboratory.<sup>2</sup> To generate mice containing the flox allele, mice containing the neo allele were crossed with a transgenic line, C57BL/6-Tg(CAG-flpe)36Ito/ItoRbrc (RIKEN BRC),<sup>3</sup> to excise the PGK-neo cassette. For endothelial cell-specific Polydom deletion, mice containing the flox allele were bred with another mouse strain, B6.Cg-Tg(Tek-cre)1Ywa/J, in which Cre recombinase was uniformly expressed in endothelial cells under control of a Tie2 promoter (The Jackson Laboratory).<sup>4</sup> For inducible Polydom deletion, mice containing the flox allele (Polydom<sup>fl/fl</sup>) were crossed with a CAGGCre-ER<sup>TM</sup> (hemizygous allele) transgenic line, B6.Cg-Tg(CAG-cre/Esr1<sup>−</sup>)<sup>5Amc/J</sup> (The Jackson Laboratory).<sup>5</sup> Tamoxifen (TM) in sunflower oil was administered via intraperitoneal injection (5 mg/40 g) to females at E15.5.<sup>6,7</sup>

All mouse experiments were approved by the Experimental Animal Committee of the Institute for Protein Research, Osaka University, and were performed in compliance with the institutional guidelines.

Zebrafish Strains

The Tg(fli1:GFP)<sup>Y1</sup> line was kindly provided by Prof. Nathan Lawson (University of Massachusetts Medical School).<sup>8</sup> The Tg(SAGFF27C;UAS:EGFP) line for specific labeling of the lymphatic vasculature was kindly provided by Prof. Koichi Kawakami (National Institute of Genetics, Japan).<sup>9,10</sup> The Tg(fli1:myr-mCherry) line was generated according to a previously described protocol.<sup>11</sup> All experiments using zebrafish were approved by the Institutional Animal Committee of the National Cerebral and Cardiovascular Center, and were performed according to the institutional guidelines.

Antibodies and Reagents

Rabbit anti-N-terminal region of mouse Polydom<sup>12</sup> and rat anti-mouse laminin α5 (M5N8-C8) were raised in our laboratory. Goat anti-mouse vascular endothelial growth factor receptor (VEGFR)-3 and lympathic vessel hyaluron receptor (LYVE)-1, rat anti-mouse LYVE-1, sheep anti-mouse Foxc2, sheep anti-human FOXC2, goat anti-human Tie1 and goat anti-mouse/rat Tie2 were purchased from R&D Systems. Rabbit anti-mouse Prox1 and LYVE-1, and hamster anti-mouse podoplanin were obtained from AngioBio. Rat anti-mouse VE-cadherin was purchased from Abcam. Cy3-conjugated mouse anti-human α-smooth muscle actin (αSMA) (clone 1A4) was obtained from Sigma. Goat anti-human Connexin 40 was purchased from Santa Cruz Biotechnology. Mouse anti-human COUP-TFII
was purchased from Perseus Proteomics. Rat anti-mouse CD45 and F4/80 were obtained from Molecular Probes. Rat anti-mouse PECAM-1 was purchased from BD Pharmingen. Rabbit anti-human SP-C was obtained from Millipore. Alexa Fluor™-conjugated secondary antibodies were purchased from Invitrogen. HRP-conjugated secondary antibody was obtained from Jackson Immune Research. EnVision+ System-HRP Labeled Polymer Anti-Rabbit was purchased from Dako. Goat anti-rat IgG Microbeads, anti-rabbit IgG Microbeads, and the LS column for the magnetic-activated cell sorting (MACS) System were purchased from Miltenyi Biotec. Collagenase type II, collagenase type IV, and deoxyribonuclease I were obtained from Worthington. Type I collagen (Cellmatrix type I-A and type I-C) was purchased from Nitta gelatin. Recombinant human Angiopoietin-1 and Angiopoietin-2 were purchased from R&D Systems.

Quantitative RT-PCR
Total RNA was isolated using an RNeasy Mini Kit (Qiagen) or a NucleoSpin RNA XS Kit (Macherey-Nagel), in accordance with the corresponding manufacturer’s instructions. For investigation of mRNA expression, single-stranded cDNA was transcribed from total RNA using a SuperScript III™ First-Strand Synthesis System for RT-PCR and random hexamers (Invitrogen). The primers used for quantitative RT-PCR are shown in Online Table I. PCR was performed with Power SYBR® Green PCR Master Mix (Applied Biosystems) and analyzed using an ABI PRISM® 7000 Sequence Detection System (Applied Biosystems) or Mastercycler ep realplex (Eppendorf).

Immunohistochemistry
Whole-mount staining of embryonic skin, mesentery, and heart was performed in accordance with the methods of Hirashima et al.14 with some modifications. Briefly, embryonic tissues were dissected and fixed with 4% paraformaldehyde (PFA) in PBS overnight at 4°C. The fixed tissues were washed three times in PBS containing 0.2% Triton X-100 (PBS-T) for 30 min at 4°C, blocked in PBS-T containing 1% bovine serum albumin (BSA) for 1 h at 4°C, and incubated with primary antibodies for 1–4 days at 4°C. After six washes in PBS-T for 30 min at 4°C, the bound antibodies were visualized with Alexa Fluor™-conjugated secondary antibodies overnight at 4°C. After another six washes in PBS-T for 30 min at 4°C, the tissues were flat-mounted on glass slides with Mount-quick (Daido Sangyo). The immunostained tissues were analyzed with an LSM5 PASCAL (Zeiss) or Fluoview FV1200 (Olympus) confocal microscope.

Whole embryonic skin was stained for β-galactosidase activity according to Yamauchi et al.15 with some modifications. Briefly, samples were fixed with 1% PFA and 0.02% Nonidet P-40 (NP-40) in 0.2 mol/L phosphate buffer (PB) for 30 min at 4°C. After washing in PB, the skin was dissected and re-fixed with 1% PFA and 0.02% NP-40 in PB for 30 min at 4°C. The fixed samples were washed in PB and incubated overnight at 37°C in staining solution (5 mmol/L potassium ferricyanide, 5 mmol/L potassium ferrocyanide, 2 mmol/L MgCl₂, 0.5% X-gal, 0.02% NP-40 in PB). The samples were rinsed twice in PB, postfixed with 4% PFA overnight at 4°C, and immunostained for VEGFR-3 using the following steps. The tissues were dehydrated with methanol, and incubated with 30% H₂O₂:DMSO:methanol (1:1:4) for >5 h at 4°C to inactivate endogenous peroxidase. After rehydration, the samples were washed three times with PB containing 0.2% Triton X-100 (PB-T), blocked with PB-T containing 1% BSA for 1 h at 4°C, and stained with primary antibodies for 3 days at 4°C. After three washes in PB-T for 30 min at 4°C, the bound antibodies were visualized with HRP-conjugated secondary antibodies and DAB buffer tablets (Merck Millipore).

For immunostaining of cryosections, mouse embryos were embedded in OCT compound (Sakura Finetek). Sections at 10-μm thickness were fixed in ice-cold acetone or 4% PFA, and incubated with 0.3% H₂O₂ to inactivate endogenous peroxidase. After washing in PBS, the sections were blocked with 3% BSA in PBS for 1 h at room temperature, and incubated with primary antibodies overnight at 4°C. The sections were washed in PBS, and the bound antibodies were visualized with EnVision+ System-HRP Labeled Polymer or Alexa Fluor-conjugated secondary antibodies. Finally, the sections were counterstained with Mayer’s hematoxylin (for DAB staining) or Hoechst 33342 (for immunofluorescence staining) and mounted with Mount-quick (Daido Sangyo) or FluorSave™ Reagent (Calbiochem).

Lymphangiography
Indian ink was intradermally injected into the footpad of the hindlimb of newborn mice delivered by Caesarean section to visualize functional lymphatic vessels, or the buccal region and hindlimb of E15.5 mice for observation of the facial lymphatic plexus and retroperitoneum collecting vessels. The lymphatic flow carrying Indian ink in the embryos was analyzed with an Olympus SZX12 stereomicroscope.

**Isolation of LECs from Dermal Cells**

LECs were isolated from dermal cells using the method of Kazenwadel et al.\(^{16}\) with some modifications. Mouse embryonic skin was dissected and digested with PBS containing 1% fetal bovine serum (FBS), 2.5 mg/ml collagenase type II and type IV, and 1 mg/ml deoxyribonuclease I for 30 min at 37°C. The digested skin was hemolyzed with distilled water and filtered through a 40-µm cell strainer. Cell fractionation was carried out using a MACS System (Miltenyi Biotec). Briefly, cells were centrifuged at 400×g for 5 min and suspended at approximately 1×10⁸ cells/ml in 1% FBS in PBS containing anti-F4/80 and anti-CD45 antibodies (1:100). The cells were incubated for 30 min at 4°C, washed with 10 ml of MACS buffer (2 mmol/L EDTA, 1% FBS in PBS), centrifuged as described above, and resuspended at 1×10⁸ cells/ml in MACS buffer. F4/80⁺/CD45⁺ cells were separated using goat anti-rat IgG Microbeads (200 µl beads/1×10⁸ cells) and a MACS LS column according to the manufacturer’s instructions. F4/80⁻/CD45⁻ cells were collected and the entire process was repeated to ensure maximal depletion of hematopoietic cells. F4/80⁻/CD45⁻ cells were centrifuged at 400×g for 5 min and resuspended at approximately 1×10⁸ cells/ml in 1% FBS in PBS with an anti-LYVE-1 antibody (1:200). LYVE-1⁺ lymphatic endothelial cells were separated using goat anti-rabbit IgG Microbeads and a MACS LS column. F4/80⁻/CD45⁻/LYVE-1⁻ cells were centrifuged at 400×g for 5 min and resuspended at approximately 1×10⁸ cells/ml in 1% FBS in PBS containing an anti-CD31 antibody (1:100). CD31⁺ blood vascular endothelial cells were purified using goat anti-rat IgG Microbeads and a MACS LS column according to the manufacturer’s instructions. The fractionated cells were subjected to RNA isolation.

**Morpholino Injections**

For morpholino oligonucleotide (MO)-mediated gene knockdown, zebrafish embryos were injected at the one-cell or two-cell stage with 10 ng of control MO (Gene Tools) or 5–15 ng of polydom splice-blocking MO, 5’-ATTACACAGCTGCTCTTACCGCTGC-3’. The efficiency of the polydom MO was confirmed by RT-PCR.

**Zebrafish Image Acquisition, Processing, and Quantification**

Pigmentation of zebrafish embryos was inhibited by 1-phenyl-2-thiourea (Sigma). The embryos were treated with 100 µg/ml tricaine (Sigma), mounted in a drop of 1% low-melting agarose in E3 medium, and placed onto a glass-based dish. Confocal images were obtained using an FV1000 confocal microscope system (Olympus) equipped with a water immersion 20× lens (XLUMPlanFL; 1.0NA; Olympus). For confocal time-lapse imaging, images were collected every 40 min for 13 h. The video images were analyzed using ImageJ software.

**Knockout by Transcription Activator-like Effector Nuclease (TALEN)**

TALENs targeting polydom were designed using TAL Effector Nucleotide Targeter 2.0 (https://talent.cac.cornell.edu/node/add/talen-old) and assembled by the Golden Gate method.\(^{17}\) TALEN repeat variable di-residues (RVDs) were cloned into an RCIscript-GoldyTALEN vector (Addgene). The polydom TALEN mRNAs were in vitro-transcribed from SacI-linearized expression plasmids with T3 RNA polymerase using an mMessage mMachine mRNA kit (Ambion).

Embryos injected with 50 pg of polydom TALEN mRNA at the one-cell stage were raised to adulthood, and crossed with Tg(fli1:GFP) y1 to identify germline-mutated founders. Screening for founders was conducted by genomic PCR and subsequent sequencing (Online Figure VH and VI). PCR primers were designed to amplify a 400-bp region bordering the TALEN target site: 5’- AGCTCAACAGATGCCCTGAGCCCAAC-3’ and 5’- CCCGCTCTCGAAGAAGTCGTACGTT-3’. The polydom allele harboring an 11-nucleotide deletion in the first exon was identified by sequencing of the PCR products. For genotyping of polydom mutants, PCR analyses of genomic DNAs were routinely performed using the same primer set.
**Plasmid Construction**

The Tol2 vector system was kindly provided by Prof. Koichi Kawakami (National Institute of Genetics, Japan). A BAC clone harboring a genomic DNA encoding the polydom gene (CH211-79D10) was obtained from BAC PAC Resources. Subcloning was carried using a BAC Subcloning Kit, Red® ET® Recombination (Gene Bridge), following the manufacturer’s instructions. Briefly, an iTol2 cassette was introduced downstream of polydom using the following primers: 5’-gcgtaagcggggcacatttcattacctttctccgcacccgacatagatccctgctcgagccgggcccaagtg-3’ (F); 5’-gcggggcatgactattggcgcgccggatcgatccttaattaagtctactaattatgatcctctagatcagatc-3’ (R). Subsequently, the coding sequence of polydom was replaced with an mCherry cassette.

**Transgenic Zebrafish Lines**

Tol2 transposase mRNAs were in vitro-transcribed with SP6 RNA polymerase from the NotI-linearized pCS-TP vector using an mMessage mMachine Kit (Ambion). To generate the TgBAC(polydom:mCherry) zebrafish lines, the Tol2-based plasmid DNA (25 pg) was injected together with the Tol2 transposase RNA (25 pg) into one-cell stage embryos of the wild-type strain, AB. The embryos showing transient expression of mCherry were selected at 3 dpf, raised to adulthood, and crossed with AB to identify germline-transmitting founder fishes.

**cDNA Cloning and Construction of Expression Vectors**

The expression vector for recombinant Polydom with an N-terminal FLAG tag and a C-terminal His6 tag was constructed as described previously. A cDNA encoding human VEGF-C (proVEGF-C) was obtained by RT-PCR using human fetal liver total RNA (Clontech). The signal sequence of proVEGF-C was substituted with the Ig κ-chain signal sequence of the pSecTag2A mammalian expression vector (Thermo Fisher Scientific) and a His6 tag by extension PCR, with an NheI site at the 5’ end and a NotI site at the 3’ end. The PCR products were digested with NheI/NotI and inserted into the corresponding restriction sites of the pSecTag2A vector.

**Expression and Purification of Recombinant Proteins**

All recombinant proteins were produced using a FreeStyle 293 Expression System (Thermo Fisher Scientific). For purification of recombinant Polydom, conditioned media were applied to anti-FLAG M2-agarose (Sigma), and the bound proteins were eluted with 100 µg/ml FLAG peptide (Sigma). Recombinant truncated integrin α9β1 was purified as described. For purification of recombinant human proVEGF-C, conditioned media were subjected to affinity chromatography using Ni-NTA agarose (Qiagen). The bound proteins were eluted with 200 mmol/L imidazole, and dialyzed against PBS.

**Solid-phase Binding Assay**

Microtiter plates (Maxisorp; Nunc) were coated with 5 µg/ml integrin α9β1, type I collagen, proVEGF-C, Angiopoetin-1, or Angiopoetin-2 overnight at 4°C, and then blocked with 1% BSA for 1 h at room temperature. After washing with TBS containing 0.1% BSA and 0.02% Tween-20 (Buffer W) or Buffer W containing 1 mmol/L MnCl2 (for binding to integrin α9β1 only), recombinant Polydom (5 µg/ml) was allowed to bind to the microtiter plates and incubated for 3 h at room temperature. After three washes, the bound Polydom was quantified. Briefly, the wells were incubated with an anti-Polydom antibody for 1 h at room temperature, washed three times, and incubated with an HRP-conjugated anti-rabbit IgG antibody for 40 min. After three washes, the bound Polydom was quantified by measuring the absorbance at 490 nm after incubation with o-phenylenediamine.

**Cell Culture**

Human dermal lymphatic endothelial cells (HDLECs; PromoCell) were plated on type I-C collagen-coated dishes in EBM-MV2 medium supplemented with EGM-MV2 (Promocell) and grown at 37°C in a humidified atmosphere containing 5% CO2. Freshly isolated LECs were seeded on type I-C collagen-coated wells at a density of 1.2×104 cells per 0.4 ml in Lab-Tek™ II Chamber Slide System 8 wells (Nunc) and cultured for 3 days. Cells were starved with EBM-MV2 for 1 h and overlaid with 0.2 ml of EBM-MV2 containing recombinant human Angiopoietin-1 or Angiopoietin-2. After incubation at 37°C
in a humidified atmosphere containing 5% CO₂ for 12 h, the cells were fixed with 4% PFA in PBS and washed in PBS-T for 30 min. The staining procedure was the same as the protocol for embryonic section staining. Nuclear FOXC2 intensities were analyzed using ImageJ software.

**Statistical Analysis**
The cell alignment in E18.5 mesenteric lymphatic vessels and the signal intensity of Tie1 and Tie2 in E16.5 skin were analyzed using ImageJ. All data were presented as means ± SEM. The statistical significance of differences between paired samples was determined by Student’s *t*-test. Data were considered statistically significant at *P*<0.05.
Online Figure I. Loss of Polydom does not affect the development of lung cell types, but does result in failure of lung inflation. (A) Flotation of Polydom−/− neonate lungs (right) in saline compared with those from control littermates (left). (B and C) Weights of the whole body (B) and lungs (C) for wild-type (+/+), heterozygous (+/−), and homozygous (−/−) mice. Data are means ± SEM (n=10 for wild-type pups; n=15 for heterozygous pups; n=8 for homozygous pups). *P<0.001; NS, not significant. (D and H) H&E staining of lung sections at E18.5 showing thickened walls with a smaller alveolar space in Polydom−/− mice. (E–G and I–K) Immunofluorescence staining of lung sections at E18.5 for Podoplanin (B and F) as a type I epithelial cell marker, SP-C (C and G) as a type II epithelial cell marker, and PECAM-1 (D and H) as a blood capillary marker. Nuclei are stained with Hoechst 33342 (blue). Note that the differentiation of lung epithelial cells and formation of blood capillaries are comparable between wild-type and Polydom−/− lungs. Bar, 100 µm.
Online Figure II. Vascular development is not significantly impaired in Polydom<sup>−/−</sup> embryos. (A and B) Whole-mount immunofluorescence staining for PECAM-1 in the heart of E15.5 wild-type (+/+) or Polydom<sup>−/−</sup> embryos. Bar, 100 µm. (C) Immunofluorescence staining of E16.5 heart sections for Connexin 40 (green in left panels) as an arterial marker and COUP-TFII (green in right panels) as a venous marker with PECAM-1 (red). Nuclei were stained with Hoechst 33342 (blue). Note that differentiation of arteries and veins in the heart occurs comparably between wild-type and Polydom<sup>−/−</sup> embryos. Bar, 50 µm. (D and E) Whole-mount immunofluorescence staining for αSMA in the mesentery of E17.5 wild-type (+/+) or Polydom<sup>−/−</sup> embryos. A, artery; V, vein; asterisk, pericyte recruitment on collecting lymphatic vessel. Bar, 100 µm. (F and G) Whole-mount immunofluorescence staining for PECAM-1 in the skin of E15.5 wild-type (+/+) or Polydom<sup>−/−</sup> embryos. Alignment of larger-diameter vessels in wild-type skin (closed arrowheads) was not detected in the Polydom<sup>−/−</sup> skin (open arrowheads). Bar, 100 µm.
**Online Figure III.** LEC specification and pTD formation are not impaired in *Polydom*−/− embryos. (A and B) Immunofluorescence staining of transverse sections of the cervical region of E12.5 wild-type (+/+) or *Polydom*−/− embryos for PECAM-1 (red) and Prox1 (green). Nuclei were stained with Hoechst 33342 (blue). Wild-type (+/+), *n*=3 embryos; homozygous (−/−), *n*=4 embryos. Prox1-positive pTD (*asterisk*) was formed in all *Polydom*−/− embryos examined. CV, cardinal vein; DA, dorsal aorta. Bar, 100 µm.

**Online Figure IV.** Lymphangiography of E15.5 embryos. (A) Lateral views of whole-mount embryos at E15.5. More than 90% of *Polydom*−/− embryos exhibited severe edema (50/52), whereas none of wild-type embryos showed edema (0/75). (B–F) Lymphangiography of E15.5 embryos. Indian ink was injected into the buccal region (B and C) or the hindlimb footpad (D and F). The buccal lymphatic capillaries (B and C) and retroperitoneal collecting lymphatic vessels (D and F) were filled with ink in wild-type embryos (bucca, 6/7, 86%; retroperitoneum, 2/6, 33%), but not in *Polydom*−/− embryos (bucca, 0/4, 0%; retroperitoneum, 0/4, 0%).
**A** Relative expression (normalized by **ef1a**) of polydom MO

**B** *Tg(fli1:GFP)y1* 2.5 dpf

**C** PL formation at 2.5 dpf

**D** 80 hpf 86 hpf 93 hpf

**E** ISLV formation at 3.5 dpf

**F** *Tg(fli1:GFP)y1: TgBAC(polydom:mCherry)* 4 dpf

**G** *polydom* - Exon 1

**H** TALEN mRNA

**I** RsI

**J** 6 dpf
Online Figure V. Zebrafish analysis. (A) Knockdown efficiencies of polydom transcripts determined by quantitative RT-PCR. (B) Lateral views of 2.5 dpf Tg(fli1:GFP) embryos uninjected (control) or injected with the polydom MO. Arrowheads indicate parachordal lymphangioblasts (PLs). Bar, 50 µm. (C) Percentages of PL formation at 2.5 dpf. Data are means ± SEM (control, n=112 embryos; polydom MO, n=91 embryos). N=4. Embryos with PL formation at >80%, 10–79%, and 0–9% in the four segments above the distal intestine were counted as Normal, Incomplete, and Loss, respectively. No statistically significant difference was detected in PL formation between control and polydom MO-treated embryos. (D) Time-lapse imaging of Tg(fli1:myr-mCherry);Tg(SAGFF27C;UAS:EGFP) embryos uninjected (control) or injected with the polydom MO at the indicated time points. The sprouting front of ISLVs moved along the arterial intersegmental vessels in control embryos (arrows), but not in morphants (asterisks). Bar, 50 µm. (E) Percentages of ISLV formation at 3.5 dpf. ISLVs that grew dorsally, ventrally, or on both sides, or did not grow from PLs were counted as Dorsal, Ventral, Bidirectional, and None, respectively. Data are means ± SEM (control, n=29 hemisegments; polydom MO, n=68 hemisegments). Bidirectional ISLV formation was decreased in polydom MO (8.8%) compared with control (69.0%). (F) Lateral view of a Tg(fli1:GFP);TgBAC(polydom:mCherry) embryo at 4 dpf. mCherry-positive polydom-expressing cells were localized along the PLs, ISLVs, and space between the DA and the PCV. Bar, 50 µm. (G) Schematic diagram of the TALEN target region at the polydom locus. The light blue boxes and green boxes represent the TALEN recognition sequences and the spacer sequence, respectively. Red letters indicate a BsrI site. (H) TALEN efficiency assessed by BsrI digestion of PCR-amplified cDNA. (I) Sequencing of the cDNA isolated from the germline-transmitting line. The 11-bp deletion results in a frameshift and a truncated protein after 91 amino acids. (J) Dorsal images of wild-type (+/+) and polydomncv106/ncv106 embryos at 6 dpf. Arrows indicate edema around the eye and the intestine.
Online Figure VI. Expression of Polydom in the pTD at E12.5. (A and B) Immunofluorescence staining of transverse sections of the E12.5 cervical region for PECAM-1 (red) and Prox1 (green in A) or Polydom (green in B). Nuclei were stained with Hoechst 33342 (blue). Polydom is localized around the pTD (asterisks) at E12.5. CV, cardinal vein; DA, dorsal aorta; arrowhead, perineurium. Bar, 100 µm.

Online Figure VII. Polydom is deposited around lymphatic vessels. Immunofluorescence staining for Polydom (red) and VEGFR-3 (green) in cross-sections of the wild-type skin at E18.5. Nuclei were stained with Hoechst 33342 (blue). Bar, 50 µm. Note that Polydom is deposited to surround VEGFR-3-positive lymphatic vessels. Arrows, Polydom-expressing cells; arrowheads, perineurium.
Online Figure VIII. Deletion of Polydom after E15.5 rescues collecting vessel formation, but causes a defect in valve formation. (A) Lateral views of E18.5 Polydom\textsuperscript{fl/fl} (control) and Polydom\textsuperscript{fl/fl};CreERT\textsuperscript{Tm} (fl/fl;CreERT\textsuperscript{Tm}) embryos exposed to TM at E15.5. Note that TM-induced Polydom deletion causes mild edema. (B) PCR genotyping with Polydom primers (F1, F2, and R1; left) and Cre primers (Cre-F and Cre-R; right). (C) Whole-mount immunofluorescence staining for Polydom (red), VEGFR-3 (green), and laminin α5 (white) in the mesentery of E18.5 Polydom\textsuperscript{fl/fl} (control) and Polydom\textsuperscript{fl/fl};CreERT\textsuperscript{Tm} (fl/fl;CreERT\textsuperscript{Tm}) embryos exposed to TM at E15.5. Arrows indicate laminin α5-positive lymphatic valves. Bar, 100 µm.
Online Figure IX. Schematic model of lymphatic remodeling. Polydom secreted from mesenchymal cells is deposited around lymphatic vessels in a fibrillar meshwork and upregulates Foxc2 expression through an ill-defined mechanism that involves Ang-2 and Tie1/Tie2 receptors, thereby facilitating lymphatic vascular remodeling, with sprouting of new capillaries and fusion or pruning of the vessels.
Online Table I

Oligonucleotide Primers for Quantitative RT-PCR

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Supplemental References


identified the minimal cis-sequence and a highly repetitive sequence in the subterminal region essential for transposition. Genetics. 2006;174:639-649.


Legend for Video Files

**Online Movie I.** Time-lapse movie of Tg(fli1:myr-mCherry);Tg(SAGFF27C;UAS:EGFP) embryo uninjected (control). SAGFF27C;UAS:EGFP* LECs migrate along fli1:myr-mCherry* arterial intersegmental vessel while establishing the ISLV in the trunk between 80 and 93 hpf.

**Online Movie II.** Time-lapse movie of Tg(fli1:myr-mCherry);Tg(SAGFF27C;UAS:EGFP) embryo injected with polydom MO. SAGFF27C;UAS:EGFP* LECs fail to migrate along fli1:myr-mCherry* arterial intersegmental vessel.