**Pkd1 Regulates Lymphatic Vascular Morphogenesis during Development**

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**SUMMARY**

Lymphatic vessels arise during development through sprouting of precursor cells from veins, which is regulated by known signaling and transcriptional mechanisms. The ongoing elaboration of vessels to form a network is less well understood. This involves cell polarization, coordinated migration, adhesion, mixing, regression, and shape rearrangements. We identified a zebrafish mutant, *lymphatic and cardiac defects 1* (*lyc1*), with reduced lymphatic vessel development. A mutation in *polycystic kidney disease 1a* was responsible for the phenotype. *PKD1* is the most frequently mutated gene in autosomal dominant polycystic kidney disease (ADPKD). Initial lymphatic precursor sprouting is normal in *lyc1* mutants, but ongoing migration fails. Loss of *Pkd1* in mice has no effect on precursor sprouting but leads to failed morphogenesis of the subcutaneous lymphatic network. Individual lymphatic endothelial cells display defective polarity, elongation, and adherens junctions. This work identifies a highly selective and unexpected role for *Pkd1* in lymphatic vessel morphogenesis during development.

**INTRODUCTION**

The lymphatic vasculature forms in the embryo as a result of specification of lymphatic endothelial cell (LEC) fate, followed by coordinated sprouting, morphogenesis, and network elaboration. LEC fate is specified through key transcription factors, which act in embryonic veins (François et al., 2008; Srinivasan et al., 2010; Wigle and Oliver, 1999). LEC precursors subsequently sprout from veins and migrate through the embryo (reviewed in Koltowska et al., 2013). This process is under the control of VEGFC/VEGFR3 signaling (Karkkainen et al., 2004) and its modulators (reviewed in Koltowska et al., 2013). In mouse, lymphatic precursors form lymph sacs in the anterior of the embryo (François et al., 2012; Yang et al., 2012), which likely remodel into major lymphatic vessels (Hägerling et al., 2013). Superficial LECs (sLECs) migrate dorsally as loosely attached individual cells to form the subcutaneous lymphatic network (Hägerling et al., 2013). Although several guidance molecules, cellular interactions, and extrinsic forces pattern embryonic lymphangiogenesis (reviewed in Koltowska et al., 2013), much remains to be understood about the cellular mechanisms that regulate LEC polarization, adhesion, outgrowth, remodeling, and morphogenesis.

In zebrafish, there are strong parallels with mammals in the processes that regulate lymphatic vascular development (Hogan et al., 2009b; Küchler et al., 2006; Yaniv et al., 2006). We have used forward genetic screens to identify zebrafish mutants that lack lymphatic vessels. Here, one zebrafish mutant uncovers a surprising role for the ADPKD gene *Pkd1* in lymphatic vascular development. We show that this function of *Pkd1* is conserved and cell autonomous in endothelial knockout mice. Our findings suggest a uniquely staged role for PKD1 in the regulation of lymphatic vascular morphogenesis.

**RESULTS**

**lyc1** Mutants Fail to Form a Lymphatic Vasculature

We identified a zebrafish mutant dubbed *lymphatic and cardiac defects 1* (*lyc1*). *lyc1* mutants exhibited a reduction or loss of the main axial lymphatic vessel, the thoracic duct (TD) at 4 days postfertilization (dpf) as well as mild cardiac edema, while retaining blood circulation (Figures 1A–1D and 1I). By 5 dpf, mutant blood flow was reduced and cardiac edema increased in severity (Figure S1; data not shown). To determine the origins of the phenotype, we examined gene expression for arteriovenous genes, lymphangiogenesis regulators (including chemokines and receptors), and flow-induced pathways at 32 hr postfertilization (hpf), during the initiation of lymphatic development. These markers were unchanged in *lyc1* embryos (Figure S1). In the zebrafish, precursor
Figure 1. lyc1 Mutants Display Reduced Lymphatic Development

(A and B) Overall morphology of wild-type siblings (A) and lyc1 mutants (B) at 4 dpf.

(C and D) The vasculature Tg(fli1a:EGFPy1; flt1:tomato)^WT^ of (C) wild-type (WT) (arrowheads indicate thoracic duct) and (D) lyc1 mutants at 4 dpf (asterisks indicate absence of thoracic duct).

(E and G) The vasculature Tg(fli1a:EGFP^WT^; flt1:tomato)^WT^ in wild-type sibling (E) and mutant embryos (G) at 56 hpf (arrows indicate lymphatic precursors known as parachordal lymphangioblasts, PLs).

(F and H) flt1:tomato^WT^ expression marks the arterial ECs, a loss of signal (brackets) indicating venous intersegmental vessels (vISVs).

(I–K) Quantification of (I) thoracic duct extent across ten somites (WT n = 40, lyc1 n = 17), (J) parachordal lymphangioblasts (WT n = 78, lyc1 n = 17), and (K) venous sprouts (WT n = 40, lyc1 n = 15). DA, dorsal aorta; PCV, posterior cardinal vein.

Error bars indicate SEM. See also Figures S1 and S2.
LECs emerge from the posterior cardinal vein (PCV) during secondary angiogenesis and migrate dorsally to the horizontal myoseptum to form parachordal lymphangioblasts (PLs). Concomitantly, venous sprouts form intersegmental veins (vISVs). Strikingly, the numbers of vISVs and PLs were normal in lyc1 mutants (Figures 1E–1H, 1J, and 1K). This phenotype differs from described mutants for vegfc, vegfr3, or ccbe1 (Hogan et al., 2009a, 2009b; Le Guen et al., 2014; Villefranc et al., 2013), which lack all venous sprouting. Time-lapse imaging showed that the lymphatic defect resulted from a block in the migration of PLs out of the horizontal myoseptum (Movies S1 and S2). Quantitative analysis of cell behavior spanning this period of altered migration revealed that mutant precursor LECs remain mobile but show altered exploratory behavior and filopodial extension dynamics, consistent with impaired directional migration (Movies S3 and S4; Figure S2).

**A Loss-of-Function Mutation in p kd1a Is Responsible for the lyc1 Phenotype**

Meiotic mapping (see the Experimental Procedures) was used to identify a region of chromosome 1 containing the lyc1 locus. The critical interval (Figure 2A) contained two genes, tuberous sclerosis 2 (tsc2) and polycystic kidney disease 1a (pkd1a). In the zebrafish genome, pkd1 (encoding Polycystin1) is present as duplicate genes, with pkd1a coding for a conserved 4281 amino acid protein. Sequencing revealed a mutation in pkd1a, introducing a premature stop codon (R3607X) (Figure 2B). This mutation was predicted to result in the failed translation of six of the 11 transmembrane domains and essential C-terminal cytoplasmic tail of the protein.

In humans, PKD1 and PKD2 (encoding POLYCYSTIN2) are the most commonly mutated genes in ADPKD (for review, see Chapin and Caplan, 2010; Zhou, 2009). PKD1 haploinsufficiency and loss of function have also been frequently associated with cardiovascular complications (reviewed in Rossetti and Harris, 2013). In mammals, POLYCYSTIN1 protein localizes to primary cilia, apical membranes, adherens, and desmosomal junctions. It can act as a mechanosensory signaling protein, transducing extracellular signals through its cytoplasmic C-terminal domain (reviewed in Zhou, 2009). POLYCYSTIN1 binds to POLYCYSTIN2 (a calcium pump) at the membrane to regulate Ca2+ influx and signaling but also binds to E-cadherin, β-catenin, and components/effectors of the planar cell polarity pathway (Castelli et al., 2013; Lal et al., 2008; Roitbak et al., 2004).

Previous studies depleting Polycystin1 (a and b) in zebrafish found that MO-pkd1a/b embryos exhibit a specific body curvature phenotype (Mangos et al., 2010). We injected MO-pkd1b into our pkd1a mutant embryos and robustly induced this phenotype.
Figure 3. Pkd1 Cell-Autonomously Regulates Subcutaneous Lymphatic Vascular Development in Mice

(A–C) Morphology of WT, Pkd1KO, and Pkd1DECKO embryos at 14.5 dpc (arrowhead indicates edema).

(D–F) Lymph sacs (LS) in WT, Pkd1KO, and Pkd1DECKO embryos stained with endomucin, LYVE1, and PROX1. JV, jugular vein. Scale bar represents 100 μm.

(G–I) Hematoxylin and eosin staining in WT, Pkd1KO, Pkd1DECKO embryos at 14.5 dpc. Lymph sacs (LS) indicated.

(J and K) Subcutaneous lymphatics in Sox18:GFP-Cre-ERT2, Cg-Gt(Rosa)26Sor(CAG-tdTOMATO)Hze/J costained with NRP2 and PROX1 (n = 663/1,138 scored LECs were tdTOMATO positive (58.2%), from n = 2 embryos, 13.5 dpc).

(L–N) Subcutaneous lymphatic vasculature in WT, Pkd1KO, and Pkd1DECKO mutants at 14.5 dpc. Dashed line indicated the dorsal midline of the embryo. Scale bar represents 400 μm.

(O–Q) Representative subcutaneous lymphatic sprout in WT, Pkd1KO, and Pkd1DECKO mutants at 14.5 dpc.

(R and S) Quantification of branchpoints/area (2,000 × 1,500 μm area on both sides of the midline) in (R) WT (n = 7 embryos) and Pkd1KO (n = 7 embryos) and (S) WT (n = 8 embryos) and Pkd1DECKO (n = 6 embryos) embryos at 14.5 dpc.

(legend continued on next page)
phenotype, confirming that the lyc1 mutation is a loss-of-function allele (Figures 2C and S3). Pkd1 and Pkd2 can modulate extracellular matrix (ECM) formation (Mangos et al., 2010). Importantly, even the most phenotypically penetrant pkd1a mutants for lymphangiogenesis do not display the body curvature associated with altered ECM. We examined several markers and knockdown scenarios but found no evidence for increased ECM or a role of altered matrix in the lyc1 lymphatic phenotype (Figure S4).

**pdk1a Is Expressed in Migrating LECs and Loss of Function in the ADPKD Complex Mimics lyc1 Defects**

We found that pdk1a expression was ubiquitous in the 24 hpf embryo but was enriched in the trunk during secondary angiogenesis at 32 hpf (Figure 2D). We saw no evidence for nonsense-mediated decay in mutants using in situ hybridization at 32 hpf (n = 130 embryos from a carrier incross analyzed; data not shown). As in situ hybridization has proved insensitive in LECs in older zebrafish (post 3 dpf), we isolated LECs using fluorescence-activated cell sorting (FACS). Taking advantage of a new transgenic line Tg(flyve1:DsRed2)ota1tot (Okuda et al., 2012) labeling embryonic veins and lymphatic vessels, crossed onto the Tg(kdrl:egfp)fo43 line (restricted to blood vessels; Jin et al., 2005), we isolated LECs and venous ECs (VECs). We performed quantitative PCR (qPCR) for known markers, validating the specificity of cell populations (Figures 2E and S3). Consistent with the timing of the lyc1 phenotype, pkd1a and pkd2 were expressed in VECs and LECs, with pkd1a in both populations at 3 dpf but reduced in LECs at 5 dpf. pkd1b was expressed at low, almost undetectable levels at all stages (Figures 2F and S3).

In endothelial cells, Polycystin1 can regulate calcium signaling through Polycystin2 activity (Chapin and Caplan, 2010; Naull et al., 2003). To investigate this potential mechanism, we knocked down Pkd2. Embryos depleted for Pkd2 exhibited a phenotype similar to that of lyc1/MO-pkd1b embryos and reduced TD extent (Figures 2G–2I and S3). We next treated embryos with previously validated Ca2+ signaling antagonist and agonists (North et al., 2009). These treatments generated activity throughout the vasculature (Figure S6). We also used an inducible tdTomato reporter to quantify activity in subcutaneous lymphatic vessels (Figure S6). Hence, previous work would not have uncovered function in these vessels. We generated Tie2:Cre-mediated knockout embryos for Pkd1 and found no subcutaneous lymphatic phenotype (Figure S5). Therefore, we utilized Sox18:GFP-CreERT2(GCE) as an additional endothelial CRE strain (Kartopawiro et al., 2014). We validated the use of Sox18:GCE on a Rosa26rt-LacZ background, which demonstrated CRE activity throughout the vasculature (Figure S6). We also used an inducible tdTomato reporter to quantify activity in subcutaneous lymphatics by co-staining with LEC markers NRP2 and PROX1. We found that induced Sox18:GCE was active in 58% of sprouting subcutaneous LECs at 13.5 dpc and frequently in clonal regions spanning whole vessels (Figures 3J, 3K, and S6H–S6Q; Movie S5).

We generated induced Pkd1 endothelial cell knockout (iADcko) embryos using this line. Pkd1iADcko embryos displayed either mild or no subcutaneous edema at 14.5 dpc (Figure 3C), with lymph sacs present but not containing blood (Figures 3I and 3F). In the subcutaneous lymphatic vasculature, Pkd1iADcko embryos displayed similar dramatic effects to germ-line KO animals, if marginally milder on quantification (Figures
KO embryos. Although we saw defects in Pkd1KO embryos, these were at the dorsal midline associated with edema and considered secondary to altered tissue architecture (Figure S5). In contrast, Pkd1TKO embryos displayed normal blood vasculature, including normal vessel width and branching (Figure S5). Interestingly, Pkd1TKO embryos did not show reduced LEC migration toward the midline (Figure 3N). This would be expected for mutants in known pathways such as VEGF/VEGFR3.

**PKD1 Regulates Sprouting and Cell-Cell Junctions In Vitro in Human LECs**

Next, we examined the sprouting of human LECs in vitro in response to VEGF using a spheroid outgrowth assay. Small interfering RNA (siRNA)-mediated knockdown of PKD1 in LECs resulted in a reduced number of cells within individual spheroid sprouts, with extensions exhibiting reduced length and abnormal morphology (Figures 4A–4H; Figure S7). The efficacy of knockdown with the siRNA mix was validated by qPCR, and the specificity was verified with an independent small hairpin RNA (shRNA) knockdown (Figure S7). We examined the phenotype of LECs in cultured monolayers and observed a rapid change in morphology following PKD1 knockdown (Figures 4I–4P). Stress fibers were disorganized in these cells (Figures 4I and 4M), and analysis of cell junctions revealed reduced VE-cadherin and β-catenin and disorganized junctions following knockdown (Figures 4J, 4K, 4N, and 4O). ZO-1 localization at tight junctions was relatively unaffected in these assays, despite altered cell morphology, suggesting a level of selectivity to adherens junctions (Figures 4L and 4P). The levels of VE-cadherin were not altered by western blot although β-catenin showed a mild reduction (Figure S7), probably indicative of destabilized junctional complexes.

**Pkd1 Regulates Polarity and Cell-Cell Junctions during Lymphatic Vessel Morphogenesis in Mice**

Pkd1 has been implicated in the regulation of polarity in epithelial cells and shown to regulate cellular convergent extension and polarity during kidney tubule morphogenesis through planar cell polarity (PCP) signaling (Castelli et al., 2013). Pkd1 binds to PAR3 and aPKC as well as E-cadherin and β-catenin therefore being associated with both polarity and junctional components (Castelli et al., 2013; Lal et al., 2008; Roitbak et al., 2004). Recently, the PCP pathway has been shown to regulate junctional rearrangements in developing LECs, at least during valve morphogenesis (Tatin et al., 2013).

We examined cell polarity in sprouting embryonic lymphatic vessels. The Golgi apparatus orient toward the migration front relative to the nucleus in many cell types including LECs (Figures 5A and 5C), serving as an ideal readout for polarity. We quantified Golgi orientation in Pkd1KO embryos and found it to be significantly randomized in 14.5 dpc lymphatic vessels compared with siblings (Figures 5A–5D and 5G). Furthermore, this loss of polarity was associated with increased nucleus sphericity in mutant vessels, a previously described proxy for polarity and migratory behavior (Hägerling et al., 2013) (Figure 5H).

To determine the earliest defect, we performed detailed phenotypic analysis at 10.5 and 11.5 dpc. At 10.5 dpc, analysis of PROX1 expression indicated that cell migration from the cardinal vein and nuclear morphology was normal in mutants (Figures 5I, S6G, and S6H). However, at 11.5 dpc, although the blood vasculature was grossly normal (Figure 5S), mutant LECs at the sprouting vessel front displayed increased nucleus sphericity (decreased elipticity) compared with wild-type (Figure 5J). We assessed Golgi orientation at these stages, but the direction of individual cell migration events was not regular, and the midline cannot be used as a direction of migration until later in development (data not shown). These early leading vessels also exhibited increased width and numbers of nuclei relative to vessel length similar to later Pkd1KO vessels (Figures S5I–S5J).

Finally, we investigated cell shape and the morphology of junctions within lymphatic vessels. At 14.5 dpc, VE-cadherin highlighted cell shape and showed that mutant cells failed to elongate along the plane of migration toward the midline compared with wild-type vessels (Figures 5K, 5L, 5Q, 5P, and 5S). At the level of individual junctional morphology, both VE-cadherin and β-catenin expression identified junctions that displayed immature morphology with irregular intracellular protrusions (arrowheads in Figures 5M, 5N, 5Q, and 5R). These phenotypes were only seen in phenotypically mutant vessels and not morphologically wild-type mutant vessels (data not shown; phenotypic variability shown in Figure 3). Quantification of the number of cells displaying immature junctions showed a significant phenotype from as early as 12.5 dpc (Figures 5T–5V).

**DISCUSSION**

Our results, along with those of Outeda et al. (2014) published in this issue of *Cell Reports* demonstrate the surprising finding that Pkd1 is a regulator of lymphatic vessel development. In zebrafish, at the cellular level, Pkd1 regulates LEC migration out of the horizontal myoseptum but not initial sprouting from veins that is regulated by ccbe1/vegf/vegfr3 (Hogan et al., 2009a, 2009b; Le Guen et al., 2014; Villefranc et al., 2013). *Pkd1a* is expressed in lymphatic precursor cells when they are actively migrating, consistent with the earliest cellular defects in the mutant.

It was important, given the highly studied nature of Pkd1, to ask if this function was conserved in mammals. In knockout mice, early specification and initial sprouting of LECs occurs normally. However, defects are seen in the morphology of migrating LECs at 11.5 dpc with morphological defects in the subcutaneous lymphatic network prominent by 14.5 dpc. This uniquely timed requirement is distinct from phenotypes in known pathways, suggesting that Pkd1 may act by an uncharacterized mechanism in LECs. Interestingly, the lymph sacs were blood filled in full knockout but not in endothelial knockout mice, which displayed only mild edema. This may be due to the staging of tamoxifen treatment to knockout *Pkd1* function from 9.5 or 11.5 dpc, when lymph sacs are already establishing (Hägerling et al., 2013). The observation that the lymphatic phenotype was reproduced by deletion with Sox18:GCE, active in LECs, but not *Tie2:Cre*, which we observed acts in BECs, suggests that Pkd1 functions in the LECs themselves during vessel morphogenesis.
Given the diverse functions of the protein, several hypotheses could explain the observed migration and morphogenesis defects. PKD1 has been previously reported to function at the primary cilium in endothelial cells (Nauli et al., 2008). However, we found lymphatic vessels developed normally in a ciliogenesis mutant (ift88; Huang and Schier, 2009), we saw no evidence for altered ciliogenesis in lyc1 mutants, and overexpression of a Pkd1a-YFP fusion protein, driven by the pkd1a promoter (BAC clone), did not lead to cilium enrichment (Figure S8). Hence, we find no supportive evidence that Pkd1 in zebrafish lymphatic development functions at the cilium. Because Pkd1 can also localize to adherens junctions, desmosomal junctions, and intracellular organelles and has a number of binding partners, it has the potential to act at diverse locations.

Figure 4. PKD1 Regulates Sprouting and Cell-Cell Junctions in LECs In Vitro
(A–F) Morphology of human LEC spheroids treated with control and PKD1 siRNA (50 nM) in BSA or VEGFC-supplemented conditions, stained with F-ACTIN (green) and DAPI (blue). Scale bar represents 100 μm in (A), (B), (D), and (E) and 30 μm in (C) and (F).
(G and H) Quantification of number of sprouts (G) and number of nuclei per 100 μm of sprouts (H) in spheroids treated with control or PKD1 siRNA in BSA or VEGFC-supplemented conditions.
(I–P) Morphology of human LECs treated with control or PKD1 siRNA (50 nM) VEGFC-supplemented conditions, stained with DAPI (blue) and F-ACTIN (green) (I and M), β-catenin (pink) (J and N), VE-cadherin (red) (K and O), or ZO-1 (L and P).
Error bars indicate SEM. See also Figure S7.
Figure 5. Pkd1 Regulates Polarity and Cell-Cell Junctions in Mouse Embryonic Lymphatic Vessels

(A and B) Subcutaneous lymphatic vessels in skin of WT and Pkd1KO embryos at 14.5 dpc, stained with endomucin, NRP2, PROX1, and GOLPH4 (Golgi apparatus), non-LEC GOLPH4 staining subtracted. Scale bar represents 20 μm.

(C and D) PROX1, GOLPH4 staining in WT and Pkd1KO lymphatic vessels. Arrowhead indicates Golgi; N, nucleus.

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The earliest consequences of loss of function are changes in cell morphology during morphogenesis, including altered polarity and adhesion. Cell polarity and adhesion are intimately associated and must be carefully regulated to control tissue morphogenesis. It is hard to determine which defect is primarily regulated by Pkd1. However, parallels can be drawn with recent findings in kidney tubule development where Pkd1 regulates cellular convergent extension during tube formation through the PCP pathway (Castelli et al., 2013). Although it will take further work to delineate the pathways modulated by Pkd1 in LECs, the finding of a crucial role in lymphatic vascular development is unexpected and serves as a unique entry point to understand lymphatic vascular morphogenesis.

EXPERIMENTAL PROCEDURES

Zebrafish Strains, Mapping, and Genotyping
Animal use conformed to guidelines of the animal ethics committee at the University of Queensland. Zebrafish were maintained and screened performed as previously described (Hogan et al., 2009a). Genotyping was performed as previously described (Hogan et al., 2009b). Primers are given in Supplemental Experimental Procedures. The lyc1 mutant allele is formally designated pkd1a*;Forbes13. The Tg(fli1a:YFP)Pkd1m232Tg, Tg(kdr:egfp)Pkd1m232, Tg(fli1a:EGFP)Pkd1m232, Tg(−6.5kdr:mcherry)Pkd1m232, Tg(−0.8fli1:tdTomato)Pkd1m232Tg, and Tg(lyve1;DsRed2)m1091 lines were previously described (Busmann et al., 2010; Hogan et al., 2009b; Jin et al., 2005; Krueger et al., 2011; Lawson and Weinstein, 2002; Okuda et al., 2012).

Mouse Strains
We generated Sox18:GFP-Cre-ErT2(GCE), B6.129S4-Pkd1tm2Ggg/J (Pkd1f/f) (C57BL/6 background) mice by crossing Sox18:GFP-Cre-ErT2 (C57BL/6 background) mice to Tie2:Cre mice and breeding resulting carriers. We generated Tie2:Crl, Rosa26rLacZ (C57BL/6 background) mice by crossing Pkd1f/f mice to both Rosa26rLacZ and Sox18:GFP-Cre-ErT2 mice and breeding resulting carriers. We generated Tie2:Crl, Rosa26rLacZ (C57BL/6 background) mice by crossing Tie2:Crl mice to Rosa26rLacZ and breeding resulting carriers. We generated Sox18:GFP-Cre-ErT2(GCE), Cg-Gt(Rosa26So(Prox1CAG-tdTomato)Pkd1F0.8flt1:tdTomato)s916 by crossing Sox18:GFP-Cre-ErT2 mice to Cg-Gt(Rosa26So(Prox1CAG-tdTomato)Pkd1F0.8flt1:tdTomato)s916 homozygous mice. We generated Pkd1f/f embryos by crossing Pkd1f/f mice to B6.C-Tg(MV-cre)Tg1/J and incising resulting progeny in subsequent generations. Genotyping primers are described in Supplemental Experimental Procedures.

Imaging and Analysis
For confocal and spinning disk imaging, embryos were mounted as previously described (Hogan et al., 2009b). Imaging was performed on a LSM Zeiss 510 NLO, META, or Zeiss 710 FCS confocal microscope with a 10×, 20×, and 40× dry objective and 63× oil objective. Images were analyzed with the Zen software, Biplane IMARIS, Photoshop, and ImageJ.

Morpholino Oligomers
Morpholino oligomers against pkd1a (morpholino oligomer [MO] ex8), pkd1b (MO ex45), and pkd2 (MO ATG) were described in Mangos et al. (2010) and were injected at 5, 7.5, or 10 ng/embryo as described (Hogan et al., 2009).

Quantitative Real-Time PCR
Procedures were performed in order to comply with MIQE guidelines (Bustin et al., 2009) and are given in full in Supplemental Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, eight figures, one table, and five movies and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2014.03.063.

AUTHOR CONTRIBUTIONS
B.C. performed experiments, analyzed data, and cowrote the paper; A.S., N.I.B., K.A.S., C.P.-T., S.B., J.W.A., E.F., and M.J. performed experiments and analyzed data; P.S.C., R.G.P., N.L.H., T.V.P., and S.S.-M. designed experiments, analyzed data, and edited the paper; M.F. designed experiments, performed experiments, analyzed data, and edited the paper; B.M.H. designed experiments, performed experiments, and cowrote the paper.

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Supplemental Information

Pkd1 Regulates Lymphatic Vascular Morphogenesis during Development

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SUPPLEMENTAL EXPERIMENTAL PROCEDURES. Related to main experimental procedures.

Additional animal procedures
For the induction of Cre-mediated recombination in embryos, 1.5 mg tamoxifen suspension in sunflower oil were injected intra-peritoneally into pregnant females at 9.5, 10.5 and 11.5 or 11.5, 12.5, 13.5 dpc in two separate regimes.

GOLPH4 processing - subtraction of background Golgi staining
The nucleus-Golgi angle relative to the dorsal midline was measured by first using the NRP2 expressing tissues as a mask to remove non-endothelial GOLPH4 staining during processing. The angle was subsequently measured between the perpendicular to the midline and the nucleus-Golgi orientation vectors in endothelial cells. Sphericity was measured in nuclei located within 150 µm of the leading edge, on both side of the midline in 14.5 dpc embryos. At earlier stages (10.5 and 11.5dpc), polarity and nuclear sphericity were assessed in cells at the lymphatic vascular migratory front only.

Statistical analysis
We used a Mann-Whitney rank sum t-test using Prism (GraphPad software), for all figures except in Figure 2 (E), Figure 4 (G-H) and Supplementary Figure 3 and 9 where a two-tailed unpaired Student’s t test was used. P-values are represented in the Figures as *= P ≤ 0.05, **= P ≤ 0.01, ***= P ≤ 0.001 and ****= P ≤ 0.0001. Standard error of the mean is represented in error bars.

Whole-mount in situ hybridization and immunochemistry
Primers used to amplify templates for riboprobe production are presented below. All probe template cDNAs were amplified from stage mixed WT cDNA by PCR and all PCR products, except for pkd1a, were subsequently cloned into pCS2+ plasmid (Turner and Weintraub, 1994). In situ hybridization was performed essentially as described in (Habeck et al., 2002; Thisse et al., 1993), with NBT/BCIP staining solution (Roche). Expression analysis and plasmid probes for flt4, dab2, couptfII, ephrinb2a, vegfc probes has been previously described in (Aranguren et al., 2011; Hogan et al., 2009; Lawson et al.,
2001; Song et al., 2004; Thompson et al., 1998). Antibodies and primers used in this study are reported below.

**Primers and antibody**

The antibodies used in this study are: ENDOMUCIN (sc-53941, Santa Cruz Biotechnology, 1/200), NRP2 (AF567, R&D systems, 1/200), PROX1 (AF2727, R&D systems, 1/200), PROX1 (11-002, Angobio Co, 1/200), GOLPH-4 (ab28049, Abcam, 1/200), LYVE1 (Ab14917, Abcam, 1/200), LYVE1 (Ab14917, Abcam, 1/200), β-CATENIN (C2206, Sigma, 1/200), VE-CADHERIN (sc-6458, Santa Cruz Biotechnology, 1/200), Alexa fluor 647 (A21247, Invitrogen, 1/200), Alexa fluor 546 (A11010, Invitrogen, 1/200), Alexa Fluor 488 (A11055, Invitrogen, 1/200), Acetylated-tubulin (T7451, Sigma, 1/500). Primers used in this study are presented in Table 1.

**Quantitative real time PCR analysis**

*Cell isolation, RNA extraction and cDNA synthesis:* Zebrafish at 3 dpf and 5 dpf were deyolked by pipetting with 200 ul pipette tip and rinsed in Calcium free ringers solution. Embryos were dissociated via treatment with 0.25% trypsin in PBS for early time points or a 1/35 dilution of liberase Tm (Roche) PBS at 28°C with repeated pipetting. For the venous/lymphatic comparisons RNA was extracted from FACS sorted samples from 3 and 5 dpf *Tg(kdrl:GFP/lyve1:DsRed2)* zebrafish, with the DsRed/GFP positive cells corresponding to the venous cell population and the DsRed positive and GFP negative population corresponding to the lymphatic cell population. FACS analysis was performed at the Queensland Brain Institute (University of Queensland) using a Cytopeia Influx Cell Sorter (Cytopeia, Seattle USA). RNA extraction and genomic DNA removal was performed using a QIAGEN RNAs easy micro kit (Qiagen Inc., Chatsworth, CA, USA) as per manufacturers recommendations. RNA was quantified using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and the integrity confirmed using an Agilent bioanalyser. RNA was amplified using the Agilent low input Quick Amp Labelling kit. For cDNA synthesis, residual genomic DNA was removed using the genomic DNA wipeout buffer included in the Quantitect reverse transcription kit (Qiagen Inc., Chatsworth, CA, USA). 30-50 ng of amplified mRNA was reverse transcribed into cDNA for 30 min at 42°C using a Quantitect reverse transcription kit (Qiagen Inc., Chatsworth, CA, USA) as per manufacturer's
recommendations. Specificity of the qPCR reactions was assessed in the absence of reverse transcriptase enzyme by including a no-transcript control (NTC).

**Quantitative PCR**: qPCR was performed using an Applied Biosystems Viia 7 384 well qPCR machine, Applied Biosystems). Each qPCR reaction mixture contained 7.5 µl 2 x ABI SYBR green master mix (Applied Biosystems), 5ul cDNA (80-fold dilution), and 500 nM each primer to a final volume of 15 µl. Amplification was performed in duplicate in 384 well plates (Applied Biosystems) with the following thermal cycling conditions: initial UDG treatment 50°C for 10 minutes, followed by 40 cycles of 15 s at 95°C, 60 s at 60°C. Control reactions included a no template control (NTC) and a no reverse transcriptase control (-RT). Dissociation analysis of the PCR products was performed by running a gradient from 60 to 95°C to confirm the presence of a single PCR product. The efficiency of PCR amplification was determined using LinReg PCR (Ruijter et al., 2009). The stability of several reference genes was analysed including *hprt1*, *ef1a*, *rps29*, *rpl13* and β -actin. Reference gene stability was determined using GeNorm (Vandesompele et al., 2002). The geometric average of rps29, rpl13 and ef1a was used for normalisation of gene expression, except in Figure 2F where rpl13 was used, these genes being validated as the most stable across the sample population.

**Primer design**: Primers were designed using Primer blast (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) to have Tm of 60°C and to cross an exon-exon junction to avoid amplification of genomic DNA, whenever possible. Primers were used at a final concentration of 500 nM.

**Human lymphatic endothelial cells**

Human LECs were isolated and cultured as described (Norrmen et al., 2010). All experiments were performed with confluent cells.

**PKD1 knockdown in human LECs**

**siRNA transfection**

Human LECs were transfected with 50 nM of control siRNA (Qiagen, AllStars Negative Control siRNA) or *PKD1* siRNA (ThermoScientific, SmartPool containing 4 different siRNAs) using Lipofectamine RNAiMAX (Invitrogen).
**shRNA transduction**

Lentiviral particles were prepared using PLKO.1 lentivector (Sigma) either empty as a control or carrying shRNA against *PKD1*. Two different constructs were used, shPKD1_a (TTGTAGACACAGAACTCCTC) (Sigma, TRCN0000062320) and shPKD1_b (AATGTCTTGCCAAAGACGGAC) (Sigma, TRCN0000062322). shRNA sequences do not overlap with siRNAs used in the transfection experiments. Lentiviral particles were quantified using p24 Elisa kit (Gentaur) according to manufacturer's instructions. Human LECs were transduced with a multiplicity of infection of 10. Three days post-transduction, cells were selected for two days in 300 ng/ml puromycin.

**PKD1 knockdown validation**

Total RNA was isolated using Qiagen RNeasy Plus Mini Kit (Qiagen) 24h post-transfection or 3 days post-transduction. Reverse transcription was performed using Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics). Real-time qPCR analyses were performed on StepOnePlus (Applied Biosystems) using SYBR Green PCR Master Mix (Applied Biosystems). Sequences of PCR primers are included in table 1. Analysis of *PKD1* expression relative to 18s was carried out using the comparative Ct (ΔΔCt) method as described by the manufacturer.

**Lymphatic endothelial cell spheroid sprouting assay**

800 LECs were seeded in round-bottom 96-well plates as described previously (Korff et al., 1999). The spheroids were collected and embedded in fibrin gels (2.5 mg/ml fibrinogen, 0.625 U/ml thrombin and 0.15 U/ml aprotinin), treated with 100 ng/ml BSA (Sigma) or ΔNΔC-VEGFC (kindly provided by Dr. M. Jeltsch and Dr. K. Alitalo) for 48 h. The spheroids were fixed in 4% PFA for 1 h at RT. Nuclei were stained with bis-Benzimide (Sigma) and F-actin cytoskeleton with Alexa 488-conjugated phalloidin (Molecular Probes). Spheroids (8 per condition) were imaged using Zeiss LSM 510 META scanning confocal microscope. The confocal images were processed using Bitplane IMARIS Suite 6.3.1 and Photoshop softwares. The number of sprouts, the cumulated length of sprouts, the average of sprout length and the density of nuclei composing the sprouts were measured for each spheroid using ImageJ.
software. A two-tailed unpaired Student’s t test was used to analyze the statistical significance of the difference between BSA- and VEGFC-treated, or Control and PKD1-knockdown groups.

**Cell staining procedures and image acquisition**

Cells cultured on coverlips were fixed with 4% PFA, permeabilized with 0.1% Triton X-100 and blocked with 5% donkey serum. We used Alexa 488-conjugated phalloidin (Molecular Probes), rabbit anti-human β-CATENIN (Upstate), goat anti-mouse VE-CADHERIN (R&D systems) and rabbit anti-human ZO-1 (Invitrogen). Coverslips were mounted using Prolong Gold anti-fade reagent containing Dapi (Invitrogen). Cells were imaged using Zeiss LSM 510 META scanning confocal microscope. The confocal images were processed using Bitplane IMARIS Suite 6.3.1 and Photoshop softwares.

**Western blotting**

Cells were lysed in a modified RIPA buffer, containing 50 mM Tris- HCl pH7.4, 0.25 mM Na-deoxycholate, 150 mM NaCl, 2 mM EGTA, 0.1 mM Na3VO4, 10 mM NaF, 1 mM PMSF, 1% Triton-X 100 and a complete protease inhibitor mixture (Roche). Protein concentration was measured using BCA kit (Pierce) and samples were resolved by SDS-PAGE, transferred onto Immobilon-P membrane (Millipore), and blotted with antibodies against rabbit anti-human GAPDH (Sigma), mouse anti-human β-CATENIN (BD transduction) and goat anti-mouse VE-CADHERIN (R&D systems). Western blots were developed using the ECL method (SuperSignal West Femto Maximum Sensitivity Substrate; Thermo Fisher Scientific).

**Calcium drug treatment**

Zebrafish embryos were exposed to DMSO, Nifedipine and Bayk8644 (Tocris Bioscience ref 1075 and 1544 respectively) at the concentration indicated in E3 media, from 24 hpf to 4 dpf. The treatment media was changed twice a day. Nifedipine and Bayk8644 were stored according to manufacturer recommendations.
SUPPLEMENTAL MOVIE LEGENDS

Supplemental Movie S1. Related to Figure 1.
Time-lapse visualization of PL (arrow) migration between 62 hpf and 92 hpf in the trunk of a WT embryo in Tg(fli1a:EGFP; flt1:tomato). The video was acquired using a 10x objective, one frame every 20 minutes.

Supplemental Movie S2. Related to Figure 1.
Time-lapse visualization of PL (arrow) migration between 62hpf and 92hpf in the trunk of a lyc1 embryo in Tg(fli1a:EGFP; flt1:tomato). The video was acquired using a 10x objective, one frame every 20 minutes.

Supplemental Movie S3. Related to Figure 1.
Spinning disk visualization of a parachordal lymphangioblast between 56 and 64 hpf in the trunk of a control (WT/MO-pkd1b) embryo (5ng MO), which develop wildtype lymphatics. The video was acquired using a 40x dry objective, one frame every 4 minutes.

Supplemental Movie S4. Related to Figure 1.
Spinning disk visualization of a parachordal lymphangioblast between 56 and 64 hpf in the trunk of a lyc1/MO-pkd1b embryo (5ng MO). The video was acquired using a 40x dry objective, one frame every 4 minutes.

Supplemental Movie S5. Related to Figure S7.
Analysis of co-localisation of tdTOMATO with PROX1 and NRP2 during validation of the Sox18:GFP-Cre-ERT2 using the Cg-Gt(Rosa)26Sor^{tm9(CAG-tdTomato)Hze}\textsuperscript{J} reporter strain. Computational masking of the area spanned by NRP2 expression using Imaris software allows for removal of the majority of blood vascular tdTOMATO. This identifies high activity of Sox18:GCE in LECs, allowing for counting of individual PROX1/tdTOMATO co-expressing LEC nuclei (CAG-tdTOMATO localizes strongly to nuclei) and revealing large clonal patches of CRE activity in subcutaneous lymphatic vessels.
Supplemental Figure S1: Further phenotypic analysis of lyc1. Related to Figure 1

**A-B** Representative overall morphology of WT and lyc1 mutant embryonic hearts and pericardial cavities at 3 (A) and 5 dpf (B). Double-sided arrows indicate the distance between the pericardium and heart. Dashed line indicates the outline of the myocardium.

**C** In situ hybridization analysis of flt4 (n=22), dab2 (n=16), coup-TFII (n=20), ephrinb2a (n=17) and vegf-c (n=28) in lyc1 mutant embryos revealed no alterations in normal expression patterns. N values indicate the total number of embryos examined from an incross of known heterozygotes (expected 25% lyc1 mutants). Individual genotype confirmed mutant embryos are shown in the right hand panels. **D** Expression of cxcr4a was unchanged at 32 hpf in control WT/MO-pkd1b (embryos show no phenotype after pkd1b knockdown only and internally control for MO toxicity) (n=39/40) compared with phenotypically mutant (based on body curvature in the presence of MO-pkd1b) lyc1/MO-pkd1b embryos (5ng MO) (n=16/16). Expression of cxcr4b was unchanged at 32 hpf in control (WT/MO-pkd1b) (n=36/38) compared with phenotypically mutant lyc1/MO-pkd1b embryos (5ng MO) (n=7/7). Expression of cxcl12a was unchanged at 32 hpf in control (WT/MO-pkd1b) (n=50/52) compared with phenotypically mutant lyc1/MO-pkd1b embryos (5ng MO) (n=14/14). Expression of cxcl12b was unchanged at 32 hpf in control (WT/MO-pkd1b) (n=28/37) compared with phenotypically mutant lyc1/MO-pkd1b embryos (5ng MO) (n=6/7).

**E** Expression of klf2a at 24 (n=10/10) and 32 hpf (n=9/10) is normal in WT embryos. Expression of klf2a at 24 hpf (n=14/14) and 32 hpf (n=19/20) in lyc1 mutant embryos.
Supplemental Figure 2

A. Movement of cell front (µm)

B. Origin to endpoint of cell front (µm)

C. Duration filipodia

D. Number of filipodia / PL
Supplemental Figure S2: Parachordal lymphangioblasts fail to directionally migrate and display altered cell dynamics in the lyc1 mutant. Related to Figure 1.

(A-B) Schematic overview of the movement of the leading front of individual parachordal lymphangioblasts (t=10.5h) from 56 hpf (scale bar: 10 µm) in (A) WT/βO-pkd1b (5ng MO) (n=3 embryos, n=4 leading fronts) and (B) phenotypically mutant lyc1/ βO-pkd1b (5ng MO) (n=5 embryos, n=5 leading fronts).

(C-D) Quantification of (C) movement of cell front and (D) origin to endpoint of cell front distance migrated by parachordal lymphangioblasts (t=10.5h) from 56 hpf in WT/βO-pkd1b (5ng MO) (n=3 embryos, n=4 leading front) and phenotypically mutant lyc1/ βO-pkd1b (5ng MO) (n=5 embryos, n=5 leading fronts) (µm).

(E) Quantification of duration of individual filipodial extensions in single parachordal lymphangioblasts at the horizontal myoseptum by time-lapse imaging (spinning disc). WT/βO-pkd1b (n=3) and phenotypically mutant lyc1/βO-pkd1b embryos (n=4) (5ng MO) were examined between 56-64 hpf (1 time unit = 4 minutes).

(F) Quantification of the number of filipodial extensions per parachordal lymphangioblasts by time-lapse imaging (spinning disc) in WT/βO-pkd1b (n=3) and phenotypically mutant lyc1/βO-pkd1b embryos (n=4) (5ng MO) between 56-64 hpf.
Supplemental Figure 3

A. Normalised expression of various genes in VEC and LEC.

B. Normalised expression of *pkd1b*.

C. Percentage of PLs/somites.

D. Percentage of TD/somites.

E. Percentage of TD/somites.

F. Normalised expression of *cacna1s*.

G. Imaging of *DMSO* and *Nifedipine* treatments.

H. Imaging of *ethanol 0.05%* and *10uM Bayk8644* treatments.

I. Percentage of PLs/somites.

J. Percentage of TD/somites.

K. Percentage of TD/somites.
Supplemental Figure S3: The lyc1 mutant lymphatic phenotype is enhanced with MO-pkd1b injection and targeting calcium signaling results in a lymphatic phenotype. Related to Figure 2.

(A) Quantitative real time PCR for kdrl, cdh5, prox1a, nfatc1, nrp2a, flt4 and lyve1 transcripts normalized expression at 3 dpf in sorted embryonic venous and lymphatic endothelial cells. Sorted cell populations display the predicted enrichment of marker genes.

(B) Quantitative real time PCR for pkd1b transcript normalized expression against ef1a and rpl13 at 3 dpf in WT and MO-pkd1a embryos at 24hpf. pkd1b is readily detectable in whole embryo cDNA but not altered by pkd1a knockdown.

(C) Quantification of parachordal lymphangioblasts in WT (n=18), lyc1 (n=9) WT/pkd1b (5ng MO) (n=21), lyc1/MO-pkd1b embryos (5ng MO) (n=23) at 56hpf.

(D-E) Quantification of thoracic duct extent in (D) WT (n=48), lyc1 (n=23), WT/MO-pkd1b (5ng MO) (n=24), lyc1/MO-pkd1b embryos (5 ng MO) (n=21), and (E) WT (n=50), MO-pkd2 embryos (7.5 ng MO)(n=136) at 4dpf.

(F) Quantitative real time PCR for cacna1s transcript normalized expression at 30 hpf in sorted embryonic venous and arterial endothelial cells. Endothelial expression of this calcium channel and Nifedipine target is confirmed.

(G-H) The vasculature of (G) DMSO 0.05% and (H) DMSO 0.05%/Nifedipine 25 µM treated embryos in Tg(fli1a:EGFP y1; kdrl:mcherry s916). The thoracic duct is markedly absent in the presence of a calcium signaling antagonist (Nifedipine).

(I) Quantification of parachordal lymphangioblasts in DMSO 0.2% (n=62) and DMSO 0.2%/Nifedipine 100µm treated embryos (n=101) at 56 hpf. PLs are unchanged in the presence of a calcium signaling antagonist (Nifedipine).

(J) Thoracic duct quantification in DMSO 0.05% (n=45) and DMSO/0.05%/Nifedipine 25 µM (n=68) at 5 dpf. Thoracic duct reduction similar to lyc1 mutants is observed.

(K) Quantification of thoracic duct extent in WT/MO-pkd1b/0.05% ethanol (5ng MO) (n=21), lyc1/MO-pkd1b/0.05% ethanol (5ng MO) (n=21), MO-pkd1b/ethanol 0.05%/Bayk8644 (5ng MO) (n=28) and lyc1/MO-pkd1b /ethanol 0.05%/Bayk8644 (5ng MO) (n=15) embryos at 4dpf. A mildly penetrant lyc1 carrier was used. Remarkably, a phenotypic interaction with the calcium agonist is observed only in the mutant animals and not in the wildtype siblings. This suggests a sensitivity of mutant cells to further fluctuations in Ca^{2+} signaling.
Number of somites spanned by col9a2 notochord expression at 48hpf

WT

lyc1/MO-pkd1b

MO-pkd2

**D**

**E**

**F**

**G**

**H**

**I**

**J**

Supplemental Figure 4
Supplemental Figure S4: The *lyc1* lymphatic vascular phenotype is independent of collagen gene expression and ECM changes. Related to Figure 2.

(A-C) Electron-microscopy imaging of the peri-notochordal region in WT, *lyc1/MO-pkd1b* (5ng MO) and *MO-pkd2* embryos (7.5 ng MO). Nt=notochord, M=muscle.

(D) Expression of *col12a1*, a vascular collagen, was unchanged at 24 and 32 hpf in control (*WT/MO-pkd1b*) (n=27, n=37 respectively) compared with phenotypically mutant *lyc1/MO-pkd1b* embryos (5ng MO) (n=37, n=12 respectively).

(E) Expression of *col9a2* was unchanged at 48 hpf in control (*WT/MO-pkd1b*) (n=10) compared with phenotypically mutant *lyc1/MO-pkd1b* embryos (5ng MO) (n=10).

(F-G) Overall morphology of (F) *MO-pkd1a/MO-pkd1b* (5ng MO each) and (G) *MO-pkd1a/MO-pkd1b/MO-col2a1a* morphants (5,5,1 ng MO respectively). Knockdown of Col2a1a rescues the gross curvature phenotype as previously described (Mangos et al., 2010).

(H). Quantification of *col9a2* expression as the anterior posterior extent of expression in the notochord, delineated by somites boundaries. No increase in *col9a2* extent was observed.

(I) Quantification of thoracic duct extent in *MO-pkd1a/MO-pkd1b* embryos (n=21) and *pkd1a/pkd1b/col2a1a* morphants (n=19). Despite rescue of the gross curvature phenotype, TD extent is not rescued.

(J) Quantification of the number of collagen fibers in the medial layer of the peri-notochordal region in WT (n=3), WT/MO-*pkd2* (7.5 ng MO) (n=3), WT/MO-*pkd1b* (5 ng MO) (n=3) and *lyc1/MO-pkd1b* embryos (5ng MO)(n=3). No change was observed in peri-notochordal collagen.
Supplemental Figure S5: Endothelial knockout of \textit{Pkd1} does not affect blood vascular development and lymphatic morphological defects are observed in \textit{Pkd1}$^{\text{KO}}$ embryos from 11.5 dpc. Related to Figure 3.

(A-B) Overall morphology of lymphovenous valves in (A) WT (n=2) and (B) \textit{Pkd1}$^{\text{KO}}$ (n=3) coronal sections stained with PROX1, LYVE1 and ENDOMUCIN based on previous studies (Srinivasan and Oliver, 2011). \textbf{LS}: Lymph sac; \textbf{CV}: Cardinal Vein. White arrow and inset indicate lymphovenous valve. Scale bar: 100 µm

(C) Representative morphology of subcutaneous blood vascular network in \textit{WT} (n=7), \textit{Pkd1}$^{\text{KO}}$ (n=7), \textit{Pkd1}$^{\Delta\text{ECKO}}$ (n=3) at 14.5 dpc, stained with ENDOMUCIN. Scale bar: 200 µm

(D) Lateral views of the representative morphology of the blood vascular network in whole mount \textit{WT} (n=5) and \textit{Pkd1}$^{\text{KO}}$ (n=5) embryos at 11.5 dpc, stained with ENDOMUCIN. Scale bar: 100 µm

(E) Quantification of the width of subcutaneous vessels (µm) across the whole skin in WT (n=6), and \textit{Pkd1}$^{\Delta\text{ECKO}}$ (n=3) embryos (n=2398, n=1200 measurements respectively. Two areas of 2000*900 µm centered on the midline were used for quantification in every embryo).

(F) Quantification of the number of branch points per area in \textit{WT} (n=6) and \textit{Pkd1}$^{\Delta\text{ECKO}}$ (n=3) embryos (3 areas of 2000*500 µm centered on the midline were used for quantification in every embryo).

(G,H) Representative lateral view of (G) WT (n=5) and (H) \textit{Pkd1}$^{\text{KO}}$ (n=5) bisected embryos with at 10.5 dpc stained with ENDOMUCIN and PROX1. Scale bar: 50 µm. CCV: common cardinal vein, iLECs: initial lymphatic endothelial cells.

(I,J) Morphology of representative sLECs in (I) WT (n=5) and (J) \textit{Pkd1}$^{\text{KO}}$ (n=5) embryos stained for NEUROPILIN2. Scale bar: 10 µm

(K) Quantification of the average width of leading sprouts (µm) in WT (n=5) and \textit{Pkd1}$^{\text{KO}}$ (n=5), (n=18, n=27 measurements respectively, across leading lymphatic vessels, averaged) at 11.5 dpc.

(L) Quantification of nuclei per µm of vessel in WT (n=5) and \textit{Pkd1}$^{\text{KO}}$ (n=5) (n=39, n=66 measurements respectively) at 11.5dpc.

(M-N) Subcutaneous lymphatic vasculature in WT and \textit{Pkd1}$^{\text{ECKO}}$ (\textit{Tie2:Cre}+; \textit{Pkd1}^{f/f}) mutants at 14.5 dpc.

(O) Quantification of branch points/area (2000*1500 µm area on both sides of the midline) in WT (n=4 embryos) and \textit{Pkd1}$^{\text{ECKO}}$ (\textit{Tie2:Cre}+; \textit{Pkd1}^{f/f}) (n=4 embryos) embryos at 14.5 dpc.
(P) Quantification of the average width of lymphatic vessels (µm) across the whole skin in WT (n=4 embryos) and Pkd1$^{ECKO}$ (Tie2:Cre+; Pkd1$^{ff}$) (n=4 embryos) embryos. The average is shown of n=800 and n=800 measurements respectively, across leading lymphatic vessels from both side of the midline at 14.5 dpc.

(Q) Quantification of nuclei/100 µm length of vessel in WT (n=4 embryos) and Pkd1$^{ECKO}$ (Tie2:Cre+; Pkd1$^{ff}$) (n=4 embryos) (n=10 representative vessels were counted per embryo at the leading edge and averaged) at 14.5 dpc.
Supplemental Figure 6

A

Tie2:Cre$^{+/+}$; LacZ$^{-/-}$

B

Tie2:Cre$^{+/-}$; LacZ$^{+/+}$

C

Tie2:Cre$^{+/-}$; LacZ$^{+/-}$

D

10.5 dpc

E

14.5 dpc

F

14.5 dpc

G

F4/R4 floxed band

F4/R4 defloxed band

Pkd1$^{+/-}$

Pkd1$^{+/+}$

H

I

J

K

NRP2

PROX1

L

M

N

O

NRP2

PROX1

P

Q

R

S

tdTOMATO

PROX1

tdTOMATO

tdTOMATO

tdTOMATO

tdTOMATO

tdTOMATO

tdTOMATO

tdTOMATO

CV

H

Pkd1$^{+/-}$

Pkd1$^{+/+}$

F4/R4 floxed band

F4/R4 defloxed band
Supplemental Figure S6: Validation of gene targeting. Related to Figure 3.

(A-C) Whole mount X-gal staining of β-gal in Tie2:Cre−/− Rosa26R−/− and Tie2:Cre+, Rosa26R+ embryos shows that the Cre activity in the subcutaneous vasculature is strong in the blood vascular endothelium but not the lymphatic vascular endothelium at 14.5 dpc.

(D-E) Whole mount X-gal staining of β-gal in Sox18-CREert2, Rosa26R embryos show CREert2 activity in 10.5 and 14.5 dpc embryos.

(F) Whole mount X-gal staining indicates CRE activity, which is selective for endothelial cells, recapitulating the Sox18 expression pattern. Note that there is no co-stain to determine vessel identity in this experiment.

(G) Genotyping of Pkd1ΔECKO with primers F4/R4 (Plontek et al., 2004) shows a band indicating a defloxing event (subsequently confirmed by sequencing). This shows that the Pkd1 locus is inactivated in a proportion of cells in the endothelial specific Sox18:GCE strain (DNA from whole tissue extract).

(H-K) Subcutaneous vessels analysed in Sox18:GFP-Cre-ERT2, Cg-Gt(ROSA)26SoRtm9(CAG-tdTomato)Hz/J co-stained with NRP2 and PROX1 to identify the lymphatic vessels at 13.5 dpc. H. Low magnification image of whole skin showing tdTOMATO expression in blood and lymphatic endothelial cells. I. PROX1 stains only the lymphatic vessels. J. Lymphatic vessels make up a proportion of total cells expressing tdTOMATO (indicated by the hashed lines). K. Image processing to remove most blood vascular tdTOMATO (by computational masking of NRP2 positive tissue using Imaris software) reveals strong activity of Sox18:GFP-Cre-ERT2 in cells of the lymphatic network. Scale bar: 200 μm

(L-O) Example of a lymphatic vessel with tdTOMATO expression as analysed by Imaris image processing to remove non-lymphatic endothelial staining (eg, panels M-O). Inset in N and O are individual co-stained nuclei (PROX1 and tdTOMATO (CAG-tdTOMATO localizes to nuclei)) allowing precise cell counting. Quantification of whole skin as described in Figure 3 and related text, using this approach revealed that n=663/1138 LECs were tdTOMATO, PROX1 and NRP2 positive (58.2%).

(P-Q) Alternative masking in Imaris using PROX1 expression as the mask identified the same co-localisation as using an NRP2 mask (inset are individual nuclei). Scale bar: 10 μm

(R-S) Sox18:GFP-Cre-ERT2, Cg-Gt(ROSA)26SoRtm9(CAG-tdTomato)Hz/J co-stained with LYVE1 and PROX1 at 11.5 dpc labels the cardinal vein from which early LECs derive. CV: Cardinal Vein, H: Heart. Scale bar: 300 μm
Supplemental Figure 7

A

Control

BSA

Control

VEGFC

shPkd1_a

BSA

VEGFC

shPkd1_b

BSA

VEGFC

G

H

Pkd1 mRNA level relative to 18s (%)

Control

shPkd1_a

shPkd1_b

Control

shPkd1

I

β-CATENIN

VE-CADHERIN

GAPDH

J

Total sprout length/spheroid (mm)

Control

siPKD1
Supplemental Figure S7: Validation of shRNA and siRNA targeting of PKD1 expression in LECs in vitro. Related to Figure 4.

(A-C) Morphology of human LEC spheroids treated with control and two alternative PKD1 shRNA (shPkd1_a and shPkd1_b respectively) in BSA or VEGFC supplemented conditions, stained with F-ACTIN (green) and DAPI (blue). Scale bar: 100 µm.

(D-F) Quantification of number of sprouts (D), total sprout length (mm) (E) and number of nuclei per 100 µm of sprouts (F) in spheroids treated with control or PKD1 targeting shRNA (shPkd1_a and shPkd1_b respectively) in BSA or VEGFC supplemented conditions.

(G-H) Quantification of PKD1 mRNA expression level relative to 18s in human LEC spheroids treated with control and two alternative PKD1 shRNA (shPkd1_a and shPkd1_b respectively) (G) and with control and PKD1 targeting siRNA (H) in VEGFC supplemented conditions.

(I) Western-blot quantification of β-CATENIN, VE-CADHERIN and GAPDH protein levels in cultured human LECs treated with control and PKD1 targeting siRNA.

(J) Quantification of total sprout length (mm) in spheroid treated with control or PKD1 targeting siRNA in BSA or VEGFC supplemented conditions.
Supplemental Figure S8: No evidence for a contribution of primary cilia to lymphangiogenesis. Related to Discussion section.

(A-B) The vasculature Tg(lyve1:DsRed2) <sup>nzt101</sup> in (A) wild-type sibling and (B) ift88Δ<sup>if946</sup> mutant embryos at 56 hpf (arrowheads indicate parachordal lymphangioblasts and white arrows indicate venous sprouts).

(C-D) Overall morphology of (C) wild-type siblings and (D) ift88Δ<sup>if946</sup> mutants at 5 dpf. (E-H) The vasculature Tg(lyve1:DsRed2<sup>nzt101</sup>; flt1:YFP<sup>flu4624Tg</sup>) of (E,G) WT and (F,H) ift88Δ<sup>if946</sup> mutants at 5 dpf.

(I-J) Quantification of (I) secondary sprouts in WT (n=12) and ift88Δ<sup>if946</sup> (n=10) at 56 hpf and (J) thoracic duct extent in WT (n=16) and ift88Δ<sup>if946</sup> (n=18).

(K-N) Overview of primary cilia localization in the trunk of (K,M) WT (n=4) and (L,N) lyc1 mutants (n=3) embryos at 30 hpf, stained for blood vessels (Kdrl-Cherry), nuclei (DAPI) and primary cilia (Acetylated-tubulin) markers. Arrowheads indicate example of discrete primary cilia.

(O) An individual representative primary cilium in a lyc1 embryo.

(P) Transient expression of a Pkd1-YFP BAC construct in an arterial intersegmental vessel and adjacent muscle cells at 4 dpf. DA: Dorsal Aorta, PCV: Posterior Cardinal Vein, TD: Thoracic duct
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| Name                  | Use                                           | Sequence direction | Sequence                        |
|-----------------------|-----------------------------------------------|--------------------|----------------------------------|
| marker 1 - 54.65Mb    | polymorphic marker used for positional cloning | F                  | $5'$-GACTACACCAGGTGTTGTCCTG-3' |
|                       |                                               | R                  | $5'$-GTCCCTCTGAGTCGTCAC-3'      |
| marker 2 - 54.73Mb    | polymorphic marker used for positional cloning | F                  | $5'$-ACACACAGACAAACTCTGG-3'      |
|                       |                                               | R                  | $5'$-CGTCACATTGATGACACAC-3'      |
| marker 3 - 54.77Mb    | polymorphic marker used for positional cloning | F                  | $5'$-CAGAGGCTTCTCTACACAC-3'      |
|                       |                                               | R                  | $5'$-TGACAAACCCTTTGAGTC-3'      |
| marker 4 - 54.92Mb    | polymorphic marker used for positional cloning | F                  | $5'$-TGAACAAATCCTGAGTTT-3'      |
|                       |                                               | R                  | $5'$-ACTAACACCTGAGTC-3'          |
| lyc1 mutation site    | sequencing lyc1 mutation (R3607X)             | F                  | $5'$-TTGCTATTCTGTGTCTTG-3'      |
|                       |                                               | R                  | $5'$-ATCATCACGCTGTCAGAC-3'      |
| lyc1 mutation site    | Introduction of an Hinp1I restriction fragment length polymorphism by PCR for rapid genotyping of lyc1 fish. The presence of the lyc1 stop codon mutation in the pkd1a sequence removes the induced Hinp1I restriction cutting site. |                      | $5'$CGCACTCGGGGATCCCCTGGAGACCCGTAC-3' |
|                       |                                               |                      |                                  |
| pkd1a riboprobe       | riboprobe amplification with T3 polymerase Tag | F                  | $5'$-ACGTGTTGTCCTTGGAC-3'      |
|                       |                                               | R                  | $5'$-GGATCCATTAACCCTCACTAAAGGAACAGTTACCTTGCCATGGTGC-3' |
| cxxf12a cloning       | subcloning riboprobe in PCS2+ vector          | F                  | $5'$-GCCGCAATTCAAAAACACTGCCAGAC-3' |
|                       | [restriction sites in bold]                  | R                  | $5'$-GCCGCTTCAGGAGCCACCAGAGCTG-3' |
| cxxf12b cloning       | subcloning riboprobe in PCS2+ vector          | F                  | $5'$-GCCGCAATTCTATTGCCGCAAATCTG-3' |
|                       | [restriction sites in bold]                  | R                  | $5'$-GCCGCTTCAGGTGACCCAGGGCTAGT-3' |
| cxxr4a cloning        | subcloning riboprobe in PCS2+ vector          | F                  | $5'$-GCCGCAATTCTGTCCTGCAAATCTG-3' |
|                       | [restriction sites in bold]                  | R                  | $5'$-GCCGCTTCAGGAGCCACCAGAGCTG-3' |
| cxxr4b cloning        | subcloning riboprobe in PCS2+ vector          | F                  | $5'$-GCCGCAATTCTGCACTTGCAAATCTG-3' |
|                       | [restriction sites in bold]                  | R                  | $5'$-GCCGCTTCAGGAGCCACCAGAGCTG-3' |
| co9a2 cloning         | subcloning riboprobe in PCS2+ vector          | F                  | $5'$-GCCGCTTCAGGAGCCACCAGAGCTG-3' |
|                       | [restriction sites in bold]                  | R                  | $5'$-GCCGCTTCAGGAGCCACCAGAGCTG-3' |
| co12a1 cloning        | subcloning riboprobe in PCS2+ vector          | F                  | $5'$-GCCGCTTCAGGAGCCACCAGAGCTG-3' |
|                       | [restriction sites in bold]                  | R                  | $5'$-GCCGCTTCAGGAGCCACCAGAGCTG-3' |
| Cre coding sequence   | genotyping for the presence of Cre            | F                  | $5'$-CAACAGCAGTGGTTTTCGTCGAC-3' |
|                       | (alternative)                                | R                  | $5'$-GTCTCATTGATGTCGAGCC-3'      |
| LacZ coding sequence  | genotyping for the presence of LacZ           | F                  | $5'$-GGCTCAGCGAGGCTTCTGGAC-3'    |
| 18S shRNA qPCR        | quantification of 18S expression              | F                  | $5'$-AGGAATTCGCCAGTAAGTGGGTC-3' |
|                       |                                               | R                  | $5'$-GCCCTCAAAACCCTACCAAC-3'   |
| pkd1 shRNA qPCR       | quantification of PKD1 expression            | F                  | $5'$-CTTCCGGTGGCACCACATCAAC-3'   |