SUPPLEMENTAL MATERIAL

Pkd1 regulates lymphatic vascular morphogenesis during development

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SUPPLEMENTAL 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 *= $P \leq 0.05$, **= $P \leq 0.01$, ***= $P \leq 0.001$ and ****= $P \leq 0.0001$. 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 (Thisse et al., 1993, Habeck et al., 2002), with NBT/BCIP staining solution (Roche). Expression analysis and plasmid probes for *flt4*, *dab2*, *couptfII*, *ephrinb2a*, *vegfc* probes has been previously described in (Thompson et al., 1998, Hogan et al., 2009, Aranguren et al., 2011, Song et al., 2004, Lawson et al., 2001). Antibodies and primers used in this study are reported below.

Primers and antibody

Primers and antibodies used in this study are presented in Tables 1-5

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 RNeasy 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 containg 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, sh*PKD1*_a (TTGTAGACACAGAACTCCTCG) (Sigma, TRCN0000062320) and sh*PKD1*_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 were: 18s-Fw (AGGAATTCCCAGTAAGTGCG), 18s-Rev (GCCTCACTAAACCATCCAA), PKD1-Fw (CTTCCGGTGGACCATCAACG), and PKD1-Rev (GCCGCGCTCTGATAAATGAC). 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 embyos 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 LEGENDS

Supplemental Movie S1

Brightfield visualization of blood flow in the trunk of a WT embryo at 4 dpf. Lateral view is shown and blood flow can be seen in the major axial vessels. Related to Figure 1.

Supplemental Movie S2

Brightfield visualization of blood flow in the trunk of a *lyc1* embryo at 4 dpf. Lateral view is shown and blood flow can be seen in the major axial vessels. Related to Figure 1.

Supplemental Movie S3

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. Related to Figure 1.

Supplemental Movie S4

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. Related to Figure 1.

Supplemental Movie S5

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. Related to Figure 1.

Supplemental Movie S6

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. Related to Figure 1.

Supplemental Movie 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*$^{tm9(CAG\text{-}tdTomato)Hze}$*/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. Related to Figure S7.

Supplemental Figure S1: *lyc1* mutants present with a heart defect as early as 3 dpf. 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.

Supplemental Figure S2: Expression of known regulators of vascular development is unchanged in *lyc1* mutants. Related to Figure 1.

(A) *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. **(B)** 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). **(C)** 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 S3: 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/*MO-pkd1b* (5ng MO) (n=3 embryos, n=4 leading fronts) and (**B**) phenotypically mutant *lyc1*/ *MO-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 in WT/*MO-pkd1b* (5ng MO) (n=3 embryos, n=4 leading front) and phenotypically mutant *lyc1*/ *MO-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/*MO-pkd1b* (n=3) and phenotypically mutant *lyc1/MO-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/*MO-pkd1b* (n=3) and phenotypically mutant *lyc1/MO-pkd1b* embryos (n=4) (5ng MO) between 56-64 hpf.

Supplemental Figure S4: 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; kdrl:Cherry*). 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. 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.

Supplemental Figure S5: 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 when an ECM collagen is reduced, 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 S6: Endothelial knockout of *Pkd1* does not affect blood vascular development and lymphatic morphological defects are observed in *Pkd1*^{KO} embryos from 11.5 dpc. Related to Figure 3.

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

(**C**) Representative morphology of subcutaneous blood vascular network in *WT* (n=7), *Pkd1*^{KO} (n=7), *Pkd1*$^{i\Delta ECKO}$ (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 *WT* (n=5) *and Pkd1*^{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 *Pkd1*$^{i\Delta ECKO}$ (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 *WT* (n=6) and *Pkd1*$^{i\Delta ECKO}$ (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**) *Pkd1*^{KO} (n=5) bisected embryos with PROX1 at 10.5 dpc stained with ENDOMUCIN and PROX1. Scale bar: 50 μm

(I,J) Morphology of representative sLECs in (**I**) WT (n=5) and (**J**) *Pkd1*^{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 *Pkd1*^{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 $Pkd1^{KO}$ (n=5) (n=39, n=66 measurements respectively) at 11.5dpc.

Supplemental Figure S7: 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 *Pkd1*$^{i\Delta ECKO}$ with primers F4/R4 (Piontek 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)26Sor*$^{tm9(CAG-tdTomato)Hze}$*/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)26Sor*$^{tm9(CAG\text{-}tdTomato)Hze}$*/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 S8: *Tie2:Cre* mediated *Pkd1* knockout does not lead to lymphatic vascular defects in subcutaneous vessels. Related to Figure 3.

A-B) Subcutaneous lymphatic vasculature in WT and *Pkd1*^{ECKO} (*Tie2:Cre+; Pkd1*$^{f/f}$) mutants at 14.5 dpc.

(C) Quantification of branch points/area (2000*1500 µm area on both sides of the midline) in WT (n=4 embryos) and *Pkd1*^{ECKO} (*Tie2:Cre+; Pkd1*$^{f/f}$) (n=4 embryos) embryos at 14.5 dpc.

(D) Quantification of the average width of lymphatic vessels (µm) across the whole skin in WT (n=4 embryos) and *Pkd1*^{ECKO} (*Tie2:Cre+; Pkd1*$^{f/f}$) (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.

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

Supplemental Figure S9: 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 (sh*Pkd1*_a and sh*Pkd1*_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 (sh*Pkd1*_a and sh*Pkd1*_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 S10: No evidence for a contribution of primary cilia to lymphangiogenesis. Related to Discussion section.

(**A-B**) The vasculature Tg(*lyve1:DsRed2*) in (**A**) wild-type sibling and (**B**) *ift88*^{tz288} 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**) *ift88*^{tz288} mutants at 5 dpf.

(**E-H**) The vasculature Tg(*lyve1:DsRed2; flt1:YFP*) of (**E,G**) WT and (**F**,**H**) *ift88*^{tz288} mutants at 5 dpf.

(**I-J**) Quantification of (**I**) secondary sprouts in WT (n=12) and *ift88*^{tz288} (n=10) at 56 hpf and (**J**) thoracic duct extent in WT (n=16) and ift88^{tz288} (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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Table 1: Antibodies used:

NAME	Reference	Company	Dilution
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**
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**

Table 2: primer used during mapping

Mapping marker 1		
location Zv9 54.65Mb	AI92-6F	**5'**-GACTACACCGGTTGTGTCTG-**3'**
	AI92-6R	**5'**-GTCCCTTCAGATCGGTCAC-**3'**
Mapping marker 2		
location Zv9 57.73Mb	CAB52-2F	**5'**-ACCACACAGACAAACTCTGG-**3'**
	CAB52-2R	**5'**-CGTCACATGTTGATCACAGAC-**3'**
Mapping marker 3		
location Zv9 54.77Mb	CAB19-2F	**5'**-CAGAGGCTTCTCCTATCACAC-**3'**
	CAB19-2R	**5'**-TGACAACCATGCTTTGAGTC-**3'**
Mapping marker 4		
location Zv9 54.92Mb	Z8678-F	**5**'-TGAACAAATGGTCGCATGTT-**3'**
	Z8678-R	**5'**-ACTTTTCCATCTGCTGCTCC-**3'**

Table 3: primers used to genotype *lyc1* mutation

lyc1 mutation site	Pkd1stopcodon-1F	**5'**-TTGCTATTCCTGTGTGTTCTG-**3'**
	Pkd1stopcodon-1R	**5'**-ACATCAACCGTCTGACATC-**3'**
lyc1 mutation site (RFLP)	Hinp1I stopcodon-R	**5'**-CGCACTCGGGGGATCCGCTGGACCACGC-**3'**

Table 4: Primers used to genotype Pkd1 mice models

Cre coding sequence	CRE1F	**5'**-CGAACGCACTGATTTCGACC-**3'**
	CRE1R	**5'**-AACCAGCGTTTTCGTTCTGC-**3'**
Cre coding sequence (alternative)	CRE5NF	**5'**-GTTTCACTGGTTATGCGGCG-**3'**
	CRE5NFR	**5'**-GGTGCTAACCAGCGTTTTCG-**3'**
LacZ coding sequence	LZF	**5'**-GGCGGCTTCGTCTGGGACTG-**3'**
	LZR	**5'**-CAGGCGGCAGTAAGGCGGTC-**3'**

Table 5: primers used to generate riboprobes

pkd1a	*pkd1* cDNA template1F	**5'**-ACGTGTGTGTGTCTCTGGAC-**3'**
	pkd1 cDNA template 1R	**5'**GGATCCATTAACCCTCACTAAAGGGAACTGTACTCTGGGTATTGTGTGC-**3'**
cxcl12a	*cxcl12a* cDNA template F	**5'**-GCGC**GAATTC**AAAAAGCCCAACAGCAGCAGG-**3'**
	cxcl12a cDNA template R	**5'**-GCGC**CTCGAG**ACACGGAGCAAACAGGACTCC-**3'**
cxcl12b	*cxcl12b* cDNA template F	**5'**-GCGC**GAATTC**TATTGCCCAGCAATGTTCGC-**3'**
	cxcl12b cDNA template R	**5'**-GCGC**CTCGAG**TGTGACCAGAGGGCTAGTGT-**3'**
cxcr4a	*cxcr4a* cDNA template F	**5'**-GCGC**GAATTC**TGCTCACTCTGCCATTCTGG-**3'**
	cxcr4a cDNA template F	**5'**-GCGC**CTCGAG**ACAGCAGTGAAAGTACGCGA-**3'**
cxcr4b	*cxcr4b* cDNA template F	**5'**-GCGC**GAATTC**TCGCAGACCTCCTGTTTGTC-**3'**
	cxcr4b cDNA template F	**5'**-GCGC**CTCGAG**GCAGTGGAAATATGCCAGCG-**3'**
col9a2	col9a2 cDNA template F	**5'**-GCGC**GAATTC**TTTCAGTGTCCAACCAACTG-**3'**
	col9a2 cDNA template R	**5'**-GCGC**CTCGAG**GATCCTTGCATTCCCATC-**3'**
col12a1	*col12a1* template F	**5'**-GCGC**GAATTC**TCTGTATGCTGATGGAGAGG-**3'**
	col12a1 template R	**5'**-GCGC**CTCGAG**GCATCATACTGAGCGTAAACC-**3'**