Lutter et al., http://www.jcb.org/cgi/content/full/jcb.201110132/DC1

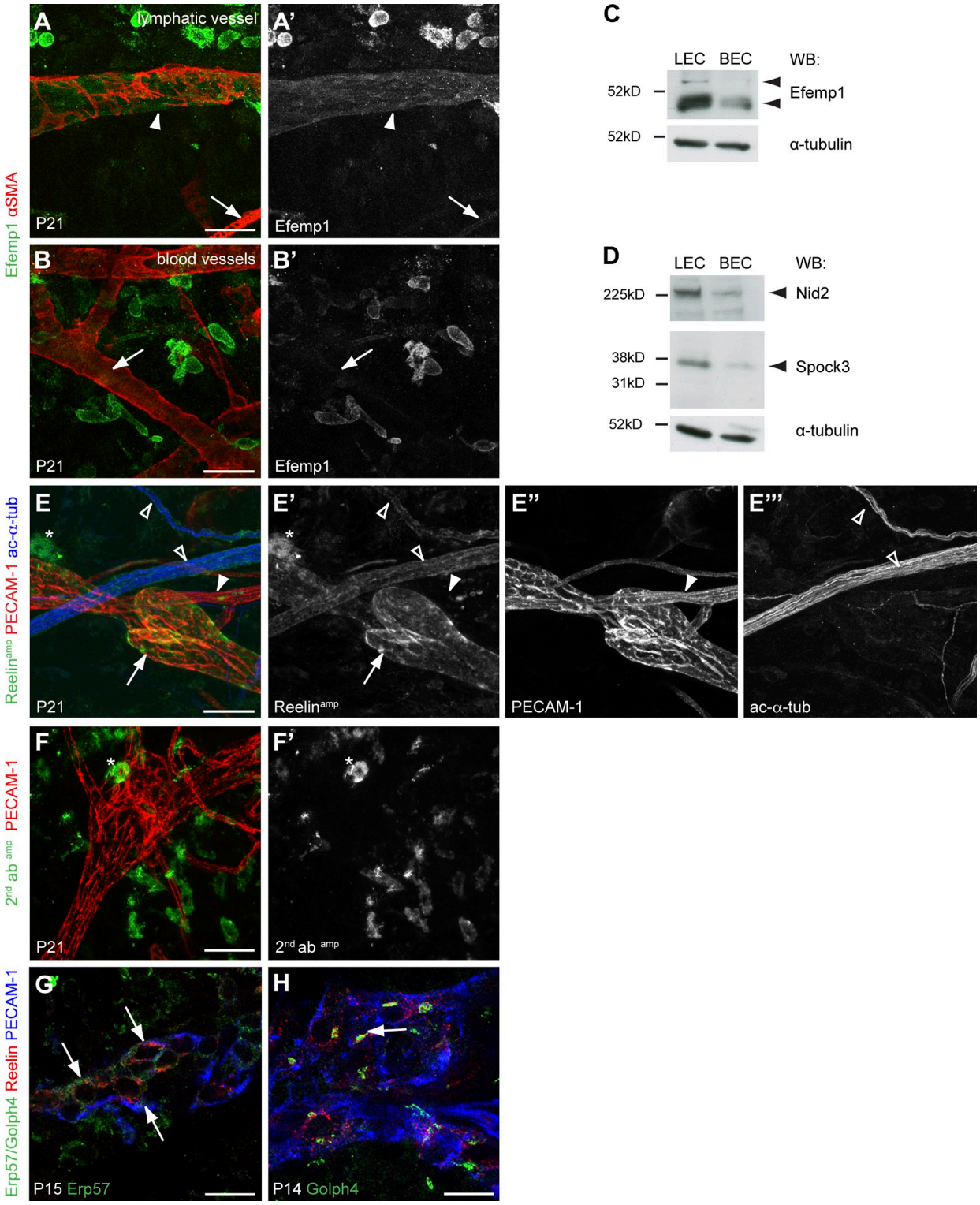

Figure S1. **Identification of extracellular matrix–associated proteins preferentially expressed in lymphatic vessels.** (A–B′) Immunofluorescence staining of P21 ear skin using the indicated antibodies. The arrowheads indicates Efemp1$^+$ collecting lymphatic vessels (A), and the arrows points to Efemp1$^-$ blood vessels (A and B). (C and D) Western Blot analysis of LEC and BEC lysates using antibodies against Efemp1 (C) and Nid2 and Spock3 (D), with α-tubulin as a loading control. (E–E‴) Immunofluorescence staining of P21 ear skin using antibodies against Reelin (green; signal amplified using TSA procedure), PECAM-1 (red), and acetylated α-tubulin (blue). Single-channel images are shown in E′–E‴. Arrows and arrowheads indicate lymphatic and blood vessels, respectively. Open arrowheads show Reelin-positive nerves, and the asterisk indicates unspecific signal in scattered cells. (F and F′) Immunofluorescence staining of P21 ear skin with secondary antibodies against Reelin used in E (green; signal amplified using TSA procedure), and antibodies against PECAM-1 (red). Nonspecific signal (green) was detected in scattered cells (asterisks). (G and H) Immunofluorescence staining of P15 (G) and P14 (H) ear skin using antibodies against Erp57 (green; G, to stain endoplasmic reticulum), Golph4 (green; H, to stain Golgi), Reelin (red), and PECAM-1 (blue). The images represent single confocal sections. Arrows indicate areas of colocalization. Bars: (A–F′) 50 μm; (G and H) 31.75 μm.

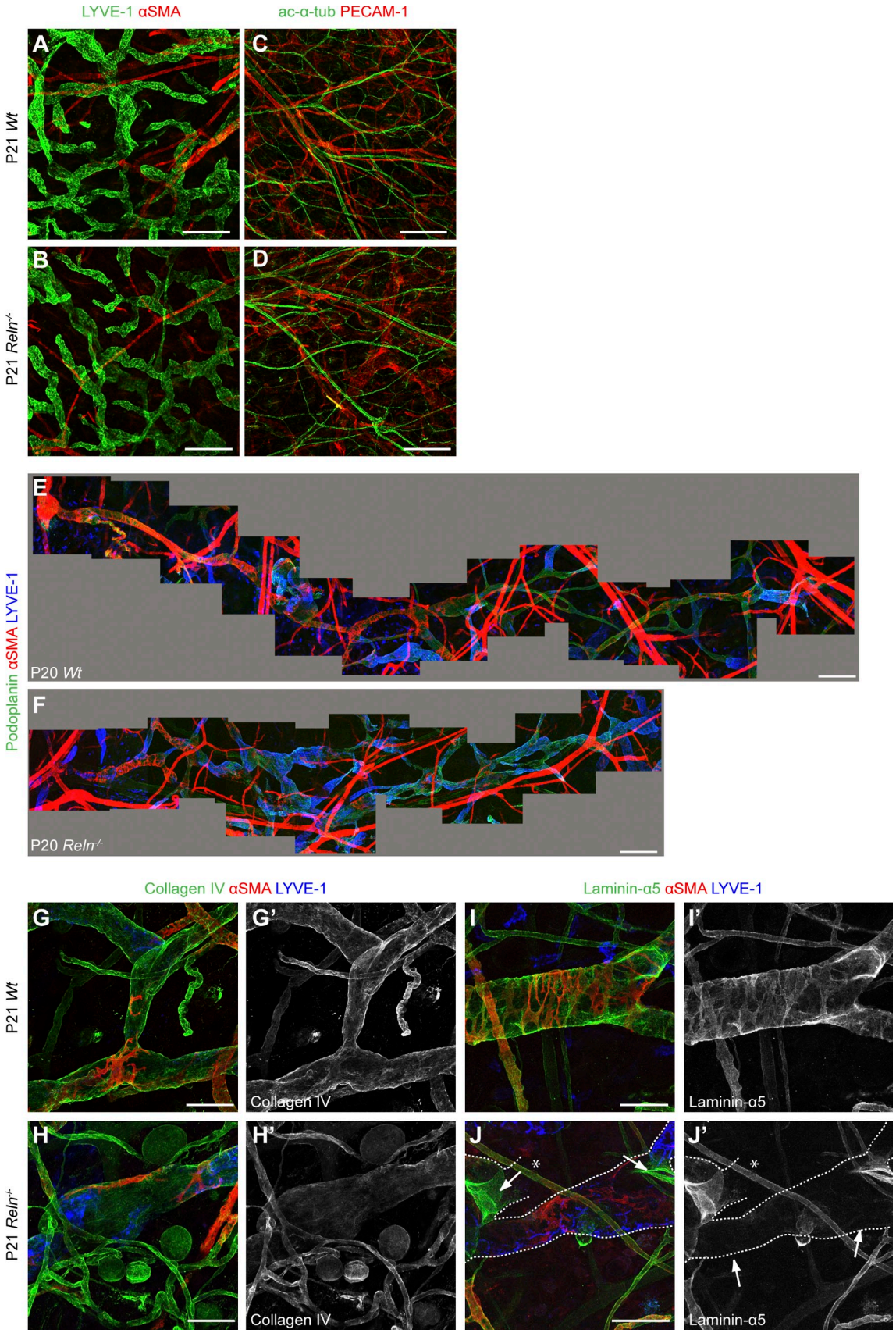

Figure S2. **Characterization of *Reeler* phenotype in the skin.** (A–D) Immunofluorescence staining of P21 wild-type (A and C) and *Reln*$^{-/-}$ (B and D) ear skin using antibodies against LYVE-1 (green; A and B) and α-SMA (red; A and B), or acetylated α-tubulin (green; C and D, to detect neurites) and PECAM-1 (red; C and D, to detect blood vessels). (E and F) Immunofluorescence staining of whole vessels shown in Fig. 4 (A and B) using antibodies against Podoplanin (green), α-SMA (red), and LYVE-1 (blue), but without removing the overlapping α-SMA–positive blood vasculature. (G–J′) Immunofluorescence staining of P21 wild-type (G, G′, I, and I′) and *Reln*$^{-/-}$ (H, H′, J, and J′) ear skin, incubated with antibodies against Collagen IV (green; G–H′), Laminin-α5 (green; I–J′), α-SMA (red), and LYVE-1 (blue). Arrows indicated Laminin-α5–positive luminal valves, which develop normally in *Reln*$^{-/-}$ mice. The broken line in J and J′ outlines the collecting vessel, and the asterisks in J and J′ indicate a vessel that is only partially visible because a full z stack was not included. Bars: (A–F) 200 μm; (G–J′) 50 μm.

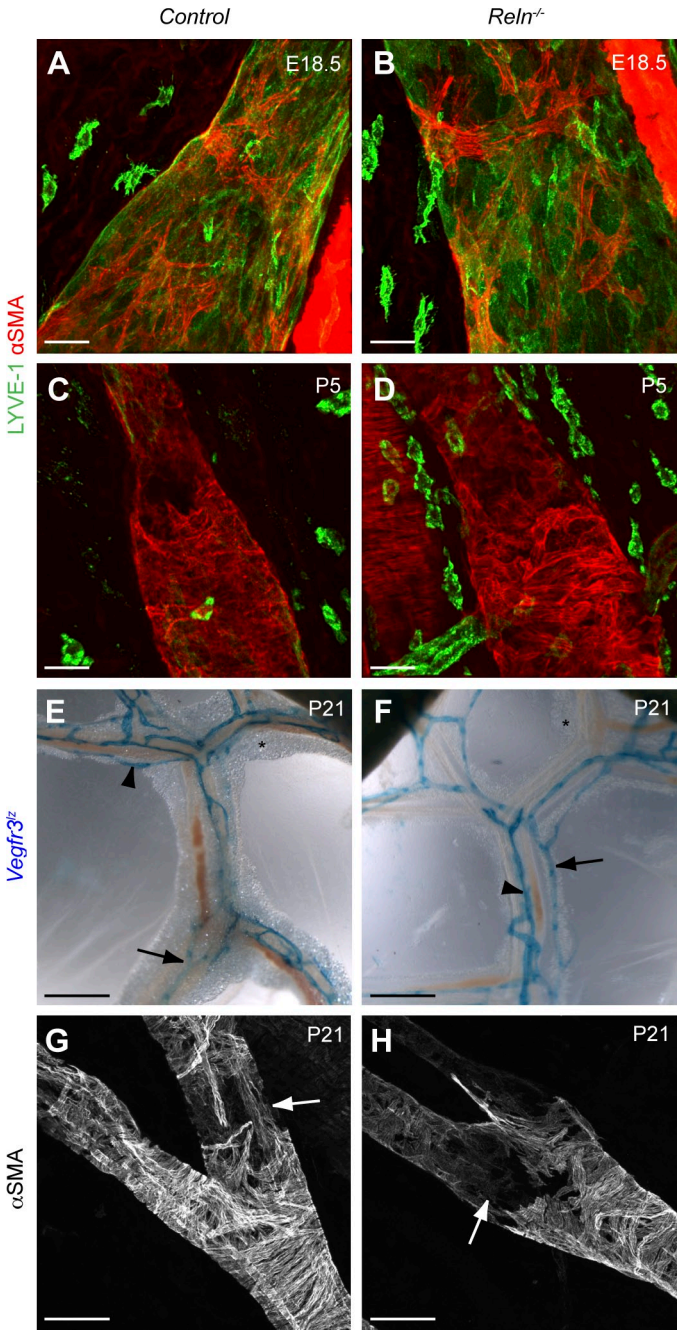

Figure S3. **Characterization of the *Reeler* phenotype in the mesentery.** (A–D) Immunofluorescence staining of wild-type (A and C) and *Reln*$^{-/-}$ (B and D) mesenteric vessels at embryonic day 18.5 (E18.5) and P5 using antibodies against LYVE-1 (green) and α-SMA (red). (E and F) X-Gal staining of mesenteric lymphatic vessels for *Vegfr3*^{lz} reporter activity in control and *Reln*$^{-/-}$mice. Arrows point to collecting vessels that contain luminal valves, and arrowheads indicate strongly X-Gal–positive capillaries. (G and H) Immunofluorescence staining of wild-type and *Reln*$^{-/-}$ mesenteric vessels at P21 using antibodies against α-SMA. Arrows indicate valve areas that have sparse SMC coverage. Bars: (A–D) 40 μm; (E and F) 1 mm; (G and H) 100 μm.

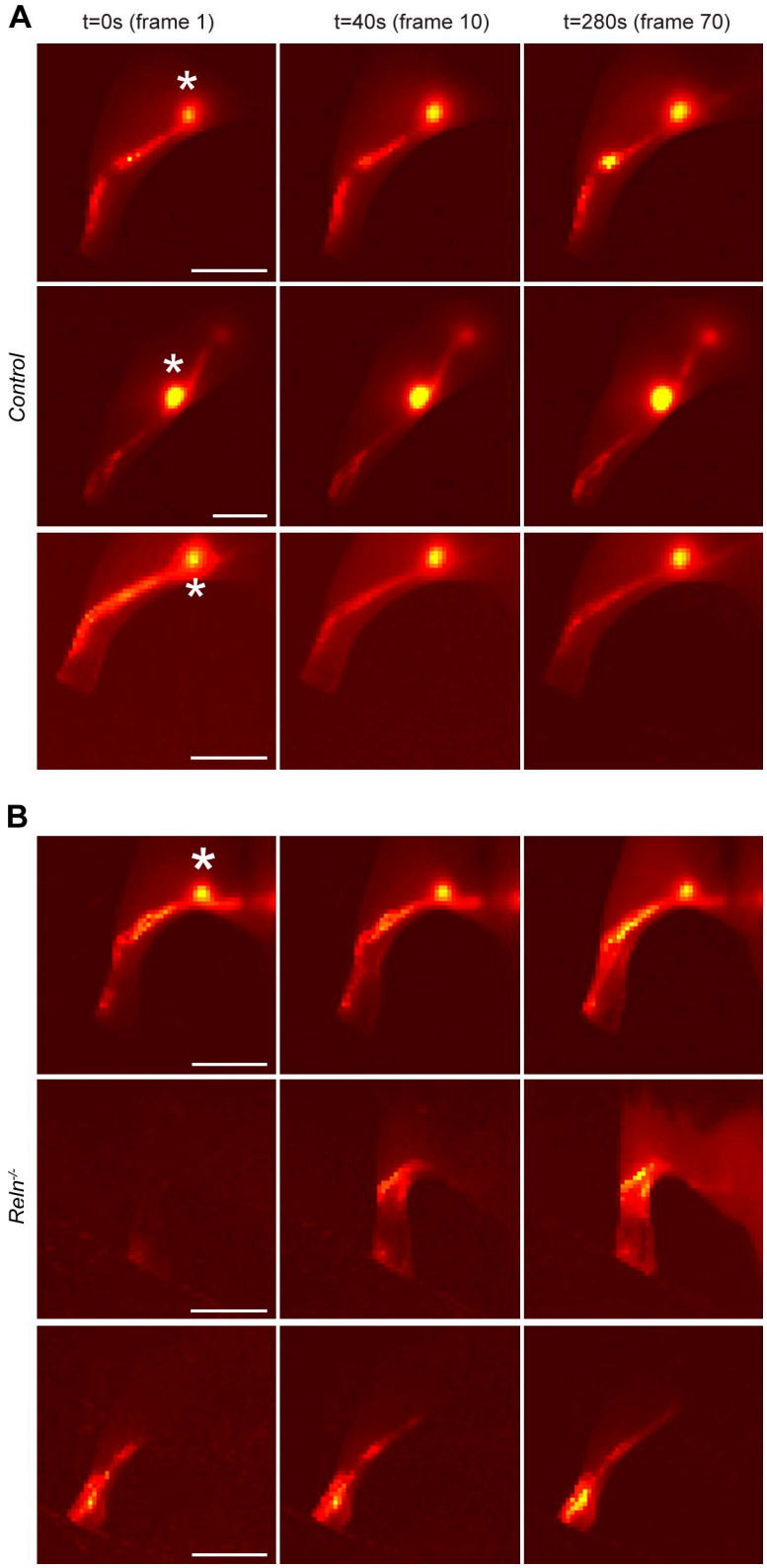

Figure S4. **Real-time imaging of lymphatic function in 3-wk-old control and *Reln*-deficient mice.** Still images of three control (A) and three *Reln*$^{-/-}$ (B) hind limbs after ICG dye injections at three time points (excluding a 6–8-s delay before recording was started). In control but not in *Reln*$^{-/-}$ mice, except in one mutant mouse (top row in B), the dye reached the inguinal lymph node (asterisks). Bars, 0.5 cm.

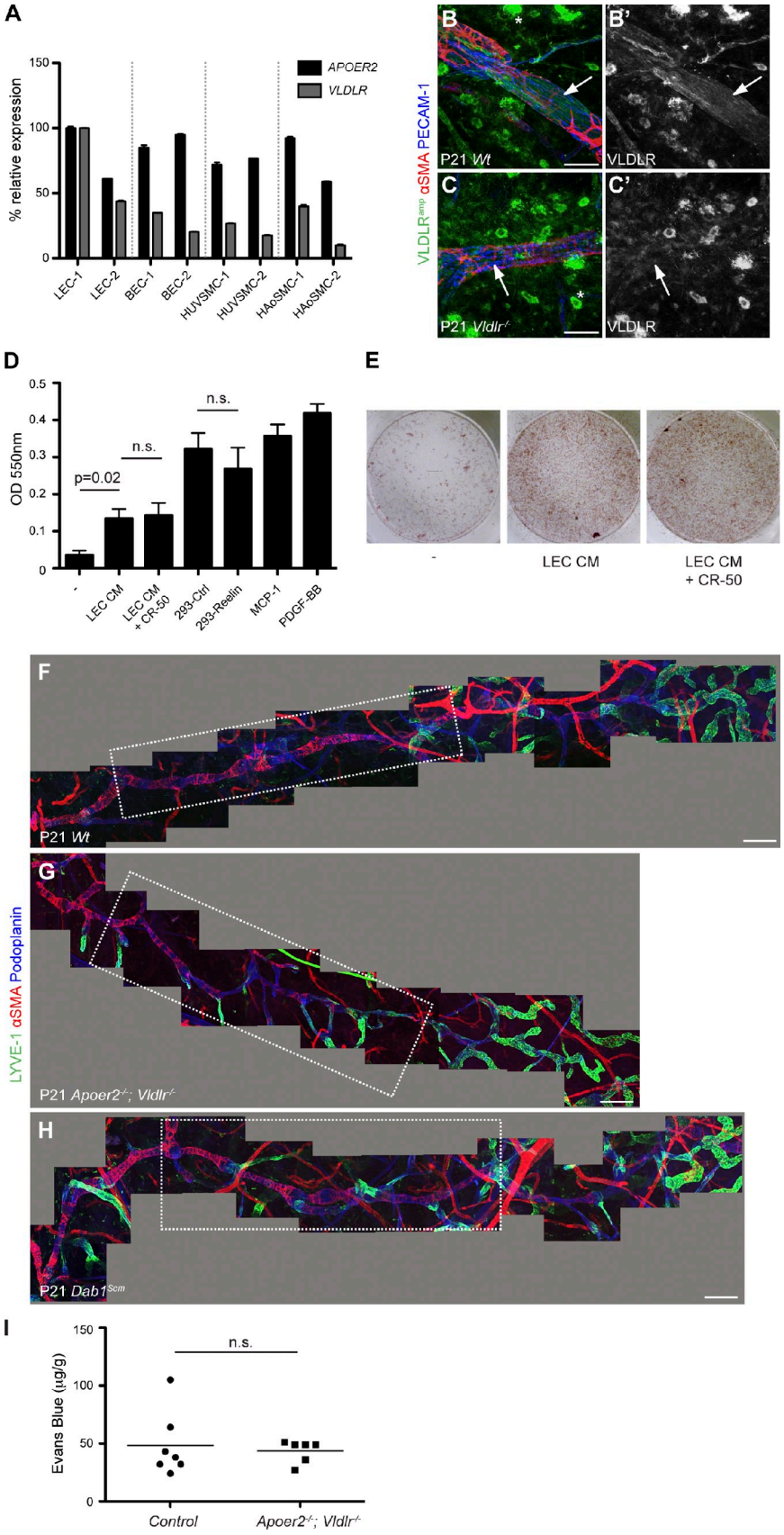

Figure S5. **Reelin signaling pathway components in lymphatic development.** (A) qPCR data showing expression of *APOER2* and *VLDLR* in BEC, HUVSMC, and HAoSMC, relative to LEC. Two independent samples were analyzed. Data represent mean ± SEM (error bars). (B–C′) Immunofluorescence staining of P21 ear skin in wild-type (B and B′) and *Vldlr*$^{-/-}$ (C and C′) mice, using antibodies against VLDLR (green; signal amplified using TSA procedure), α-SMA (red), and PECAM-1 (blue). Absence of signal in C and C′ (arrows) demonstrates specificity of staining in B and B′ (arrows), and the asterisk indicates nonspecific staining in scattered cells. (D) Migration of HUVSMC toward LEC-conditioned medium (CM) +/− function-blocking antibody against Reelin (CR-50), and toward conditioned medium from untransfected or full-length Reelin-expressing 293 cells in a transwell filter migration assay. 10 ng/ml MCP-1 and 5 ng/ml PDGF-BB were used as positive controls. OD values determined by crystal violet staining of the migrated cells are mean ± SEM from three experiments (error bars). n.s., not significant. (E) Representative images of HUVSMCs on transwell filters stained with crystal violet after migration. (F–H) Original images of vessels from *Wt* (F), *Apoer2*$^{-/-}$;*Vldlr*$^{-/-}$ (G), and *Dab1*^{Scm} (H) mice shown in Fig. 6, without removing overlapping α-SMA–positive blood vasculature. White boxes indicate areas of vessels magnified in Fig. 6. (I) Quantification of Evans blue dye content in inguinal lymph nodes of control (*Apoer*$^{+/+}$;*Vldlr*$^{-/-}$ and *Apoer*$^{+/-}$;*Vldlr*$^{-/-}$) and *Apoer2*$^{-/-}$;*Vldlr*$^{-/-}$ mice after 5 min of injection into the hind limb footpad. Values from individual lymph nodes and mean values are plotted ($n \geq 6$). Bars: (B–C′) 50 µm; (F–H) 200 µm.

Table S1. **Validation of differential gene expression of known EC markers in Affymetrix GeneChip analysis between lymphatic vessels, arteries, and veins**

EC type markers	Gene symbol	Lymphatic vs. vein	Lymphatic vs. artery	Artery vs. vein
Lymphatic	*Prox1*	**5.1**	**17.8**	−3.5
	Pdpn	**2.3**	**2.8**	−1.3
	Lyve1	**2.6**	**3.6**	−1.4
	Flt4 (Vegfr3)	**4.6**	**3.8**	1.2
	Stab1	**1.7**	**2.0**	−1.2
Blood vessel	*Vegfc*	**−3.8**	**−5.4**	1.4
	Figf (Vegfd)	**−3.3**	**−1.8**	−1.9
	Flt1 (Vegfr1)	**−2.7**	**−1.9**	−1.4
	Pdgfrb	**−2.0**	**−1.6**	−1.2
	Vwf	**−7.6**	**−4.3**	−1.8
Arterial	*Efnb2**	1.7	−1.1	**1.9**
	Hey1	1.2	−1.3	**1.6**
	Dll4	1.3	−1.4	**1.9**
Venous	*Ephb4**	−1.1	1.7	**−1.9**
	*Nrp2**	1.5	3.5	**−2.4**

The fold change in expression of each gene in lymphatic vessels compared to veins, lymphatic vessels compared to arteries, and arteries compared to veins. A number >0 indicates up-regulation and <0 indicates down-regulation. Known lymphatic, blood vessel, and arterial and venous markers are up- or down-regulated in the expected pattern. Asterisks indicates arterial/venous genes with known expression also in the lymphatics. Bolt text indicates genes that are up-regulated in lymphatic vessels compared to arteries or veins ("Lymphatic"), in arteries or veins compared to lymphatic vessels ("Blood vessels"), or in arteries compared to veins or vice versa ("Arterial/"Venous"). Fold changes represent the mean of two independent samples.

Table S2. **Genes associated with extracellular matrix structure, function, assembly, or degradation that were differentially expressed in Affymetrix GeneChip analysis between lymphatic vessels, veins, and arteries**

Gene name	Gene symbol	Lymphatic vs. vein	Lymphatic vs. artery	Artery vs. vein
EGF-like protein[a]	*Egfl6*	**7.5**	**10.9**	−1.4
Reelin[a]	*Reln*	**5.0**	**1.7**	2.9
Fibulin-3[a]	*Efemp1*	**4.0**	**5.4**	−1.4
Prolargin[a]	*Prelp*	**3.4**	**3.3**	1.0
Osteonectin[a]	*Spock3*	**3.2**	**4.5**	−1.4
Collagen, Type VI, α6[a]	*E330026B02Rik*	**2.9**	**3.5**	−1.2
TGFbeta1 binding protein[a]	*Ltbp1*	**2.5**	**1.0**	2.4
Galectin-3 binding protein[a]	*Lgals3bp*	**2.4**	**2.1**	1.2
Thrombospondin, type 1, domain containing 4[a]	*Thsd4*	**2.2**	**−1.1**	2.4
ADAM metallopeptidase with thrombospondin type 1 motif, 19[a]	*Adamts19*	**2.1**	**2.6**	−1.2
Nidogen 2[a]	*Nid2*	**2.1**	**1.9**	1.1
Extracellular matrix protein FRAS1[a]	*Fras1*	**1.9**	**3.1**	−1.7
Cartilage oligomeric matrix protein[a]	*Comp*	**1.7**	**2.7**	−1.6
Matrix metalloproteinase 19[a]	*Mmp19*	**1.5**	**2.1**	−1.4
Connective tissue growth factor[b]	*Ctgf**	−2.2	**−4.4**	2.0
F-spondin[b]	*Spon1**	−2.1	**−3.6**	1.7
Collagen, type VIII, α1[b]	*Col8a1*	1.4	**−3.5**	5.1
Tropoelastin[b]	*Eln**	−2.4	**−3.2**	1.4
ADAM metallopeptidase with thrombospondin type 1 motif, 8[b]	*Adamts8*	−1.6	**−2.7**	1.2
Mamcan[b]	*Mamdc2*	−2.3	**−2.7**	1.2
Emilin-3[b]	*Mmrn2*	−1.8	**−2.5**	1.4
Kazal-type serine protease inhibitor domain 1[b]	*Kazald1*	−1.2	**−2.4**	2.0
Aggrecan[b]	*Acan*	1.0	**−2.2**	2.3
Melanoma inhibitory activity protein[b]	*Mia1**	−3.0	**−2.2**	−1.4
Chondroadherin[c]	*Chad*	**−5.0**	−1.1	−4.5
ADAM metallopeptidase with thrombospondin type 1 motif, 18[c]	*Adamts18*	**−3.1**	1.1	−3.3
Tenascin C[c]	*Tnc*	**−2.9**	−1.9	−1.5
EGF-like, fibronectin type III and laminin G domains[c]	*Egflam*	**−2.1**	−1.7	−1.3
ADAM metallopeptidase with thrombospondins type 1 motif, 2[c]	*Adamts2*	**−2.1**	−2.0	−1.1

Genes associated with extracellular matrix structure, function, assembly, or degradation that are up-regulated ≥2-fold in lymphatic vessels compared to veins or arteries, and veins or arteries compared to lymphatic vessels in an Affymetrix GeneChip array. Asterisks indicate genes that are up-regulated ≥2-fold in both arteries and veins compared to lymphatic vessels. Fold changes represent the mean of two independent samples. Bold text indicates genes that are up-regulated in lymphatic vessels compared to arteries or veins (first set), in arteries compared to lymphatic vessels (second set), or in veins compared to lymphatic vessels (last set).
[a]Up-regulated in lymphatic vessels compared to arteries or veins.
[b]Red up-regulated in arteries compared to lymphatic vessels.
[c]Up-regulated in veins compared to lymphatic vessels.

Table S3 is available for download as a Microsoft Excel file.