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Supplemental Information

VE-Cadherin Is Required for Lymphatic Valve Formation and Maintenance

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SUPPLEMENTAL INFORMATION

Figure S1

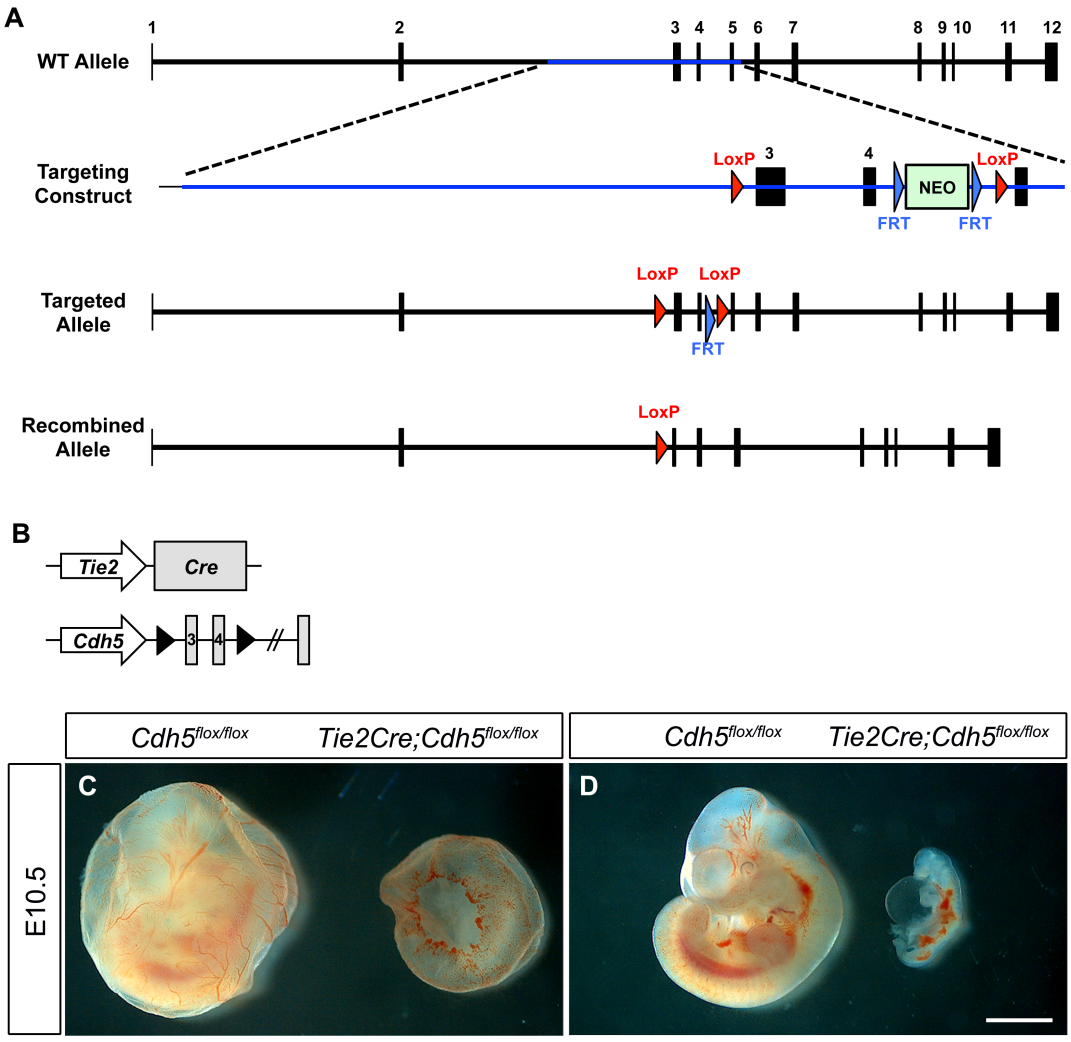

Figure S1. Generation of VE-cadherin^{flox} mice and pan-endothelial deletion (related to Figure 1). (**A**) To enable the conditional deletion of VE-cadherin from the vasculature, homologous recombination was used to insert loxP sites flanking exons 3 and 4 of the *Cdh5* gene. Distal to exon 4, a Frt-flanked neo cassette was inserted for positive selection and was later removed by Flp recombinase. Embryonic stem cells were selected and microinjected to yield three founders. Southern blot and PCR were used to

verify correct targeting. (**B**) To further validate the new *Cdh5*^{flox} allele, which targets different exons from a previously reported strain (Frye et al., 2015), we crossed it to the pan-endothelial Tie2-Cre strain to completely delete both alleles of VE-cadherin from all endothelial cells. (**C**) Representative brightfield images of two E10.5 embryos within their yolk sacs. Note the blood islands in the VE-cadherin knockout embryos compared to the blood vessels in the control yolk sac. (**D**) Representative brightfield images of the same two embryos in **C** after removal of the yolk sacs. Note that the VE-cadherin-deficient embryo exhibits stunted growth, blood pooling in malformed vessels, and a dilated pericardial sac as reported previously for global VE-cadherin null embryos (Carmeliet et al., 1999). Scale bar is 1 mm in **C-D**.

Figure S2

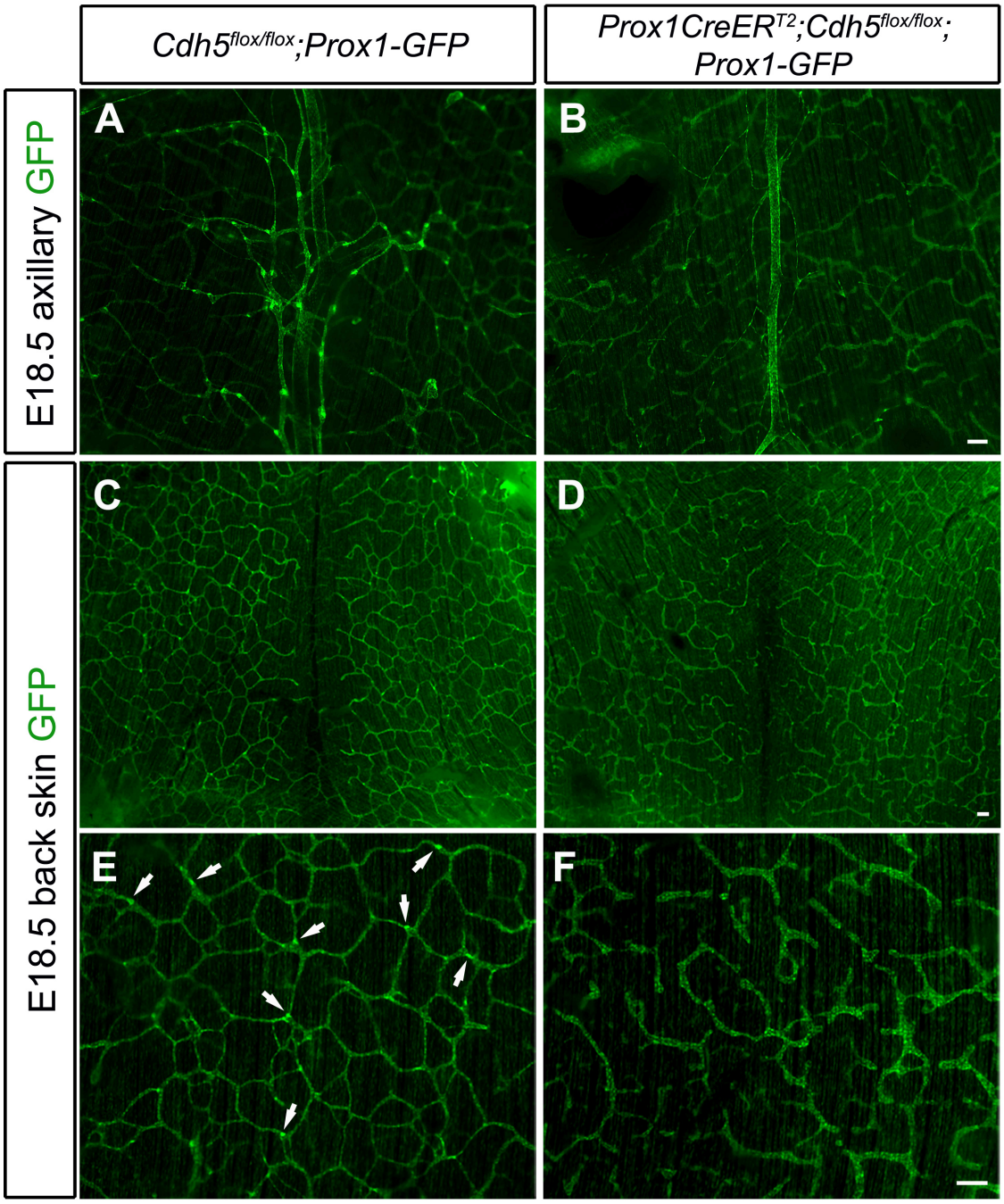

Figure S2. VE-cadherin deletion results in loss of lymphatic valves and lymphatic defects in embryonic back skin and axillary regions (related to Figure 1). (**A** and **B**) The axillary lymphatic vasculature of E18.5 control embryos expressing the Prox1-GFP

reporter contains many valves (GFP^{hi} cell clusters) near branch points that are completely absent in the lymphatic-specific VE-cadherin knockout embryos. (**C** and **D**) Low magnification fluorescence images of control and lymphatic-specific VE-cadherin knockout back skin expressing the Prox1-GFP reporter. The midline runs vertically through each image. Note that the lymphatic plexus of the VE-cadherin deficient embryos appears broken and the migration of the lymphatic vessels to the midline appears faster than in controls. (**E** and **F**) High magnification fluorescence images of areas in **C** and **D**. Note that the developing lymphatic plexus in the VE-cadherin deficient embryonic back skin contains dilated vessels that are broken such that lymphatic islands have formed compared to the control lymphatic vasculature. Scale bars are 200 μm.

Figure S3

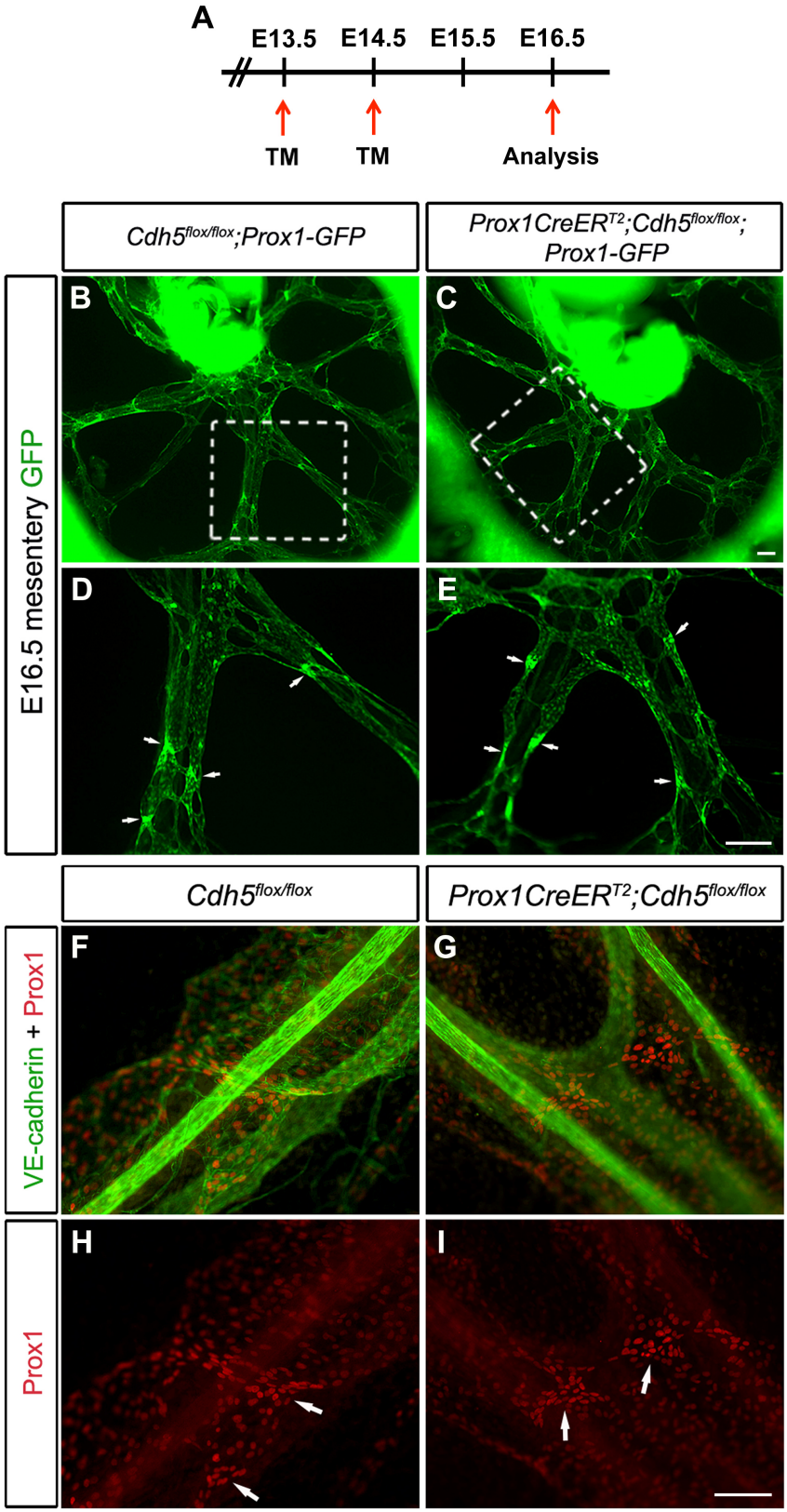
A
E13.5
E14.5
E15.5
E16.5
TM
TM
Analysis
Cdh5flox/flox;Prox1-GFP
Prox1CreERT2;Cdh5flox/flox;
Prox1-GFP
E16.5 mesentery GFP
B
C
D
E
Cdh5flox/flox
Prox1CreERT2;Cdh5flox/flox
VE-cadherin + Prox1
F
G
Prox1
H
I

Figure S3. Embryonic deletion of VE-cadherin does not affect valve initiation/specification at E16.5 (related to Figure 1). (**A**) Schematic of the tamoxifen schedule used for early embryonic deletion of VE-cadherin. TM, tamoxifen. (**B**-**E**) Direct fluorescence imaging of GFP (green) of freshly dissected mesenteries from E16.5 control and *VE-cadherin*$^{LEC\text{-}KO}$ embryos at low (**B**, **C**) and high (**D**, **E**) magnification. White arrows indicate Prox1^{hi} cell clusters where valves are forming. (**G**-**J**) Whole mount immunostaining for VE-cadherin (green) and PROX1 (red) in control and *VE-cadherin*$^{LEC\text{-}KO}$ embryonic mesenteries lacking the Prox1-GFP reporter. White arrows indicate PROX1^{hi} cell clusters where valves are forming. Scale bars are 200 μm in **B**-**E** and 100 μm in **F**-**I**.

Figure S4

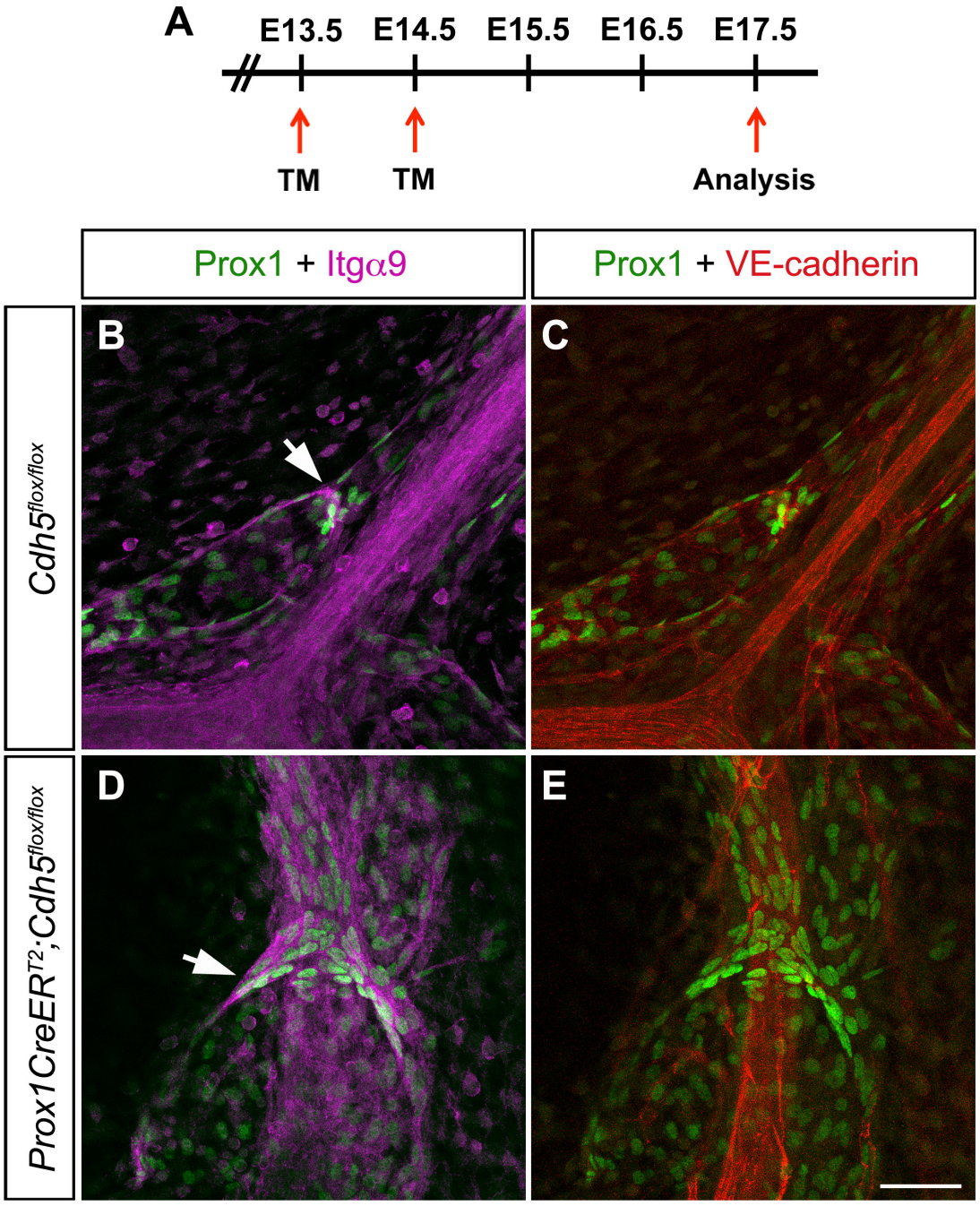

Figure S4. Embryonic deletion of VE-cadherin does not affect valve condensation or elongation at E17.5 (related to Figure 1). (**A**) Schematic of the tamoxifen schedule

used for early embryonic deletion of VE-cadherin. TM, tamoxifen. (**B**-**E**) Confocal imaging of whole mount immunostaining of E17.5 mesentery for PROX1 (green), VE-cadherin (red), and Integrin-α9 (violet) from controls (**B**,**C**) and VE-cadherin^{LEC-KO} (**D**,**E**) embryos. White arrows indicate valves at the elongation stage of valve formation that express similar levels of Integrin-α9. Scale bar is 50 μm.

Figure S5

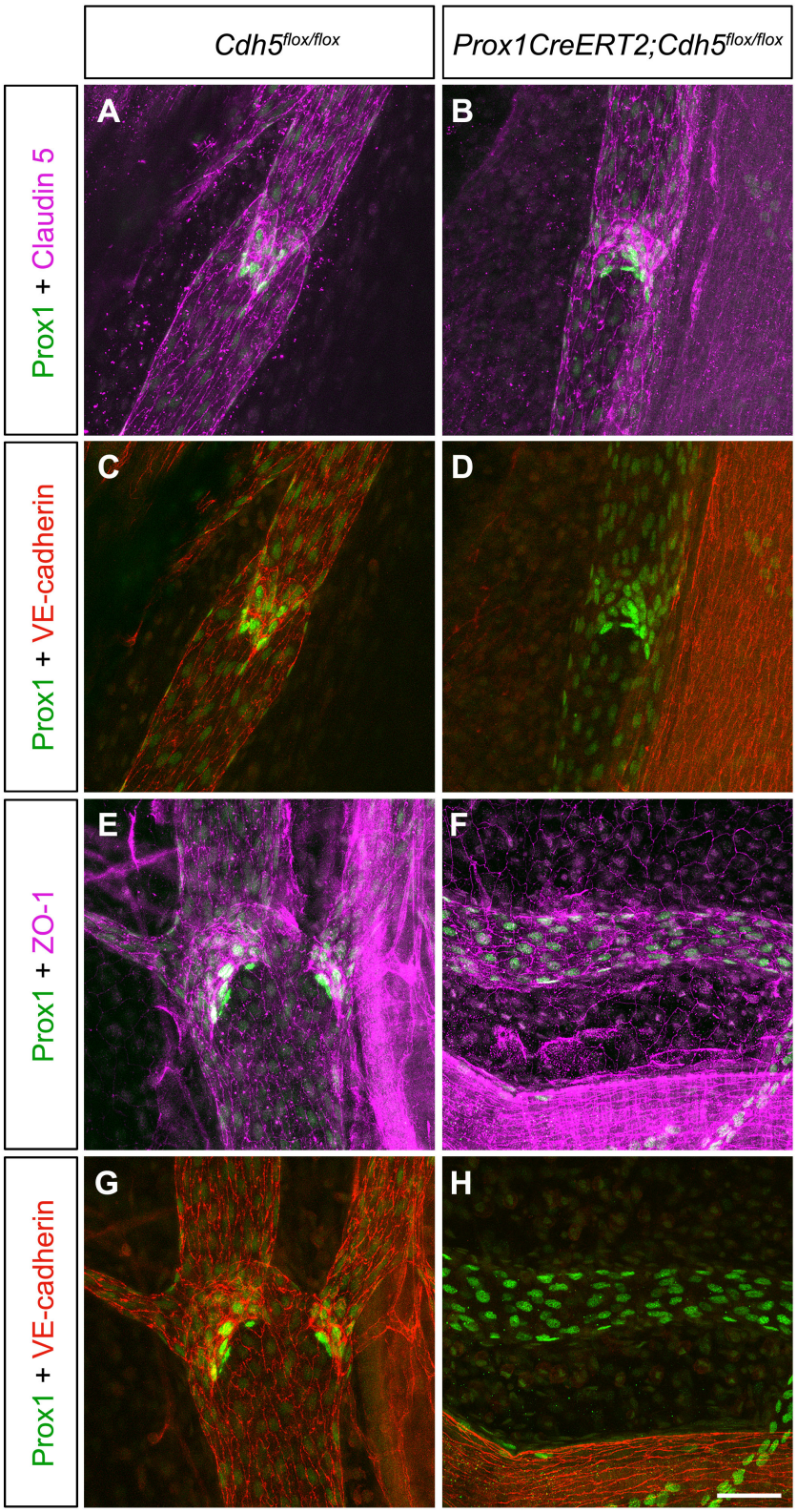
Cdh5flox/flox
Prox1CreERT2;Cdh5flox/flox
Prox1 + Claudin 5
Prox1 + VE-cadherin
Prox1 + ZO-1
Prox1 + VE-cadherin
A
B
C
D
E
F
G
H

Figure S5. Postnatal deletion of VE-cadherin does not alter tight junction protein expression (related to Figure 3). (**A**-**D**) Whole mount immunostaining for PROX1 (green), VE-cadherin (red), and Claudin-5 (violet) of P8 mesentery from control or VE-cadherin^{LEC-KO} mice imaged with confocal microscopy. Both control and VE-cadherin^{LEC-KO} lymphatic vessels express Claudin-5 (**A**-**B**) and ZO-1 (**E**-**F**). Scale bar is 50 μm.

Figure S6

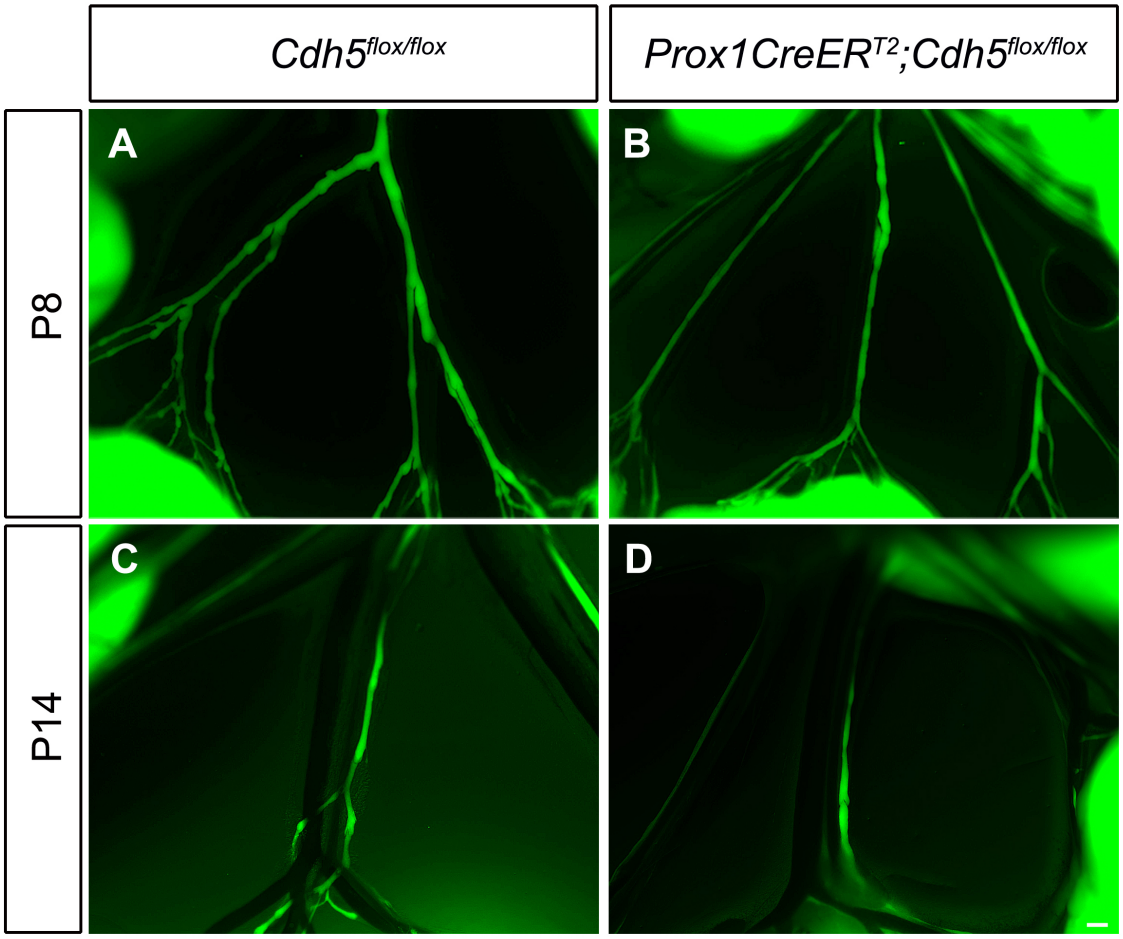

Figure S6. Lymph flow is not impaired upon postnatal deletion of VE-cadherin (related to Figure 3). (**A** and **B**) Postnatal day 8 (P8) pups were fed BODIPY FL C16, a fluorescent lipid that is absorbed by intestinal lymphatic vessels. Fluorescence images of the mesenteric lymphatic vessels of VE-cadherin$^{LEC\text{-}KO}$ pups demonstrate that lymph flow is not impaired after the deletion of VE-cadherin and subsequent valve regression. (**C** and **D**) Fluorescent images of the mesenteric lymphatic vessels of postnatal day 14 (P14) pups indicate that lymph flow is not impaired after lymphatic-specific deletion of VE-cadherin. Scale bar is 200 μm.

Figure S7

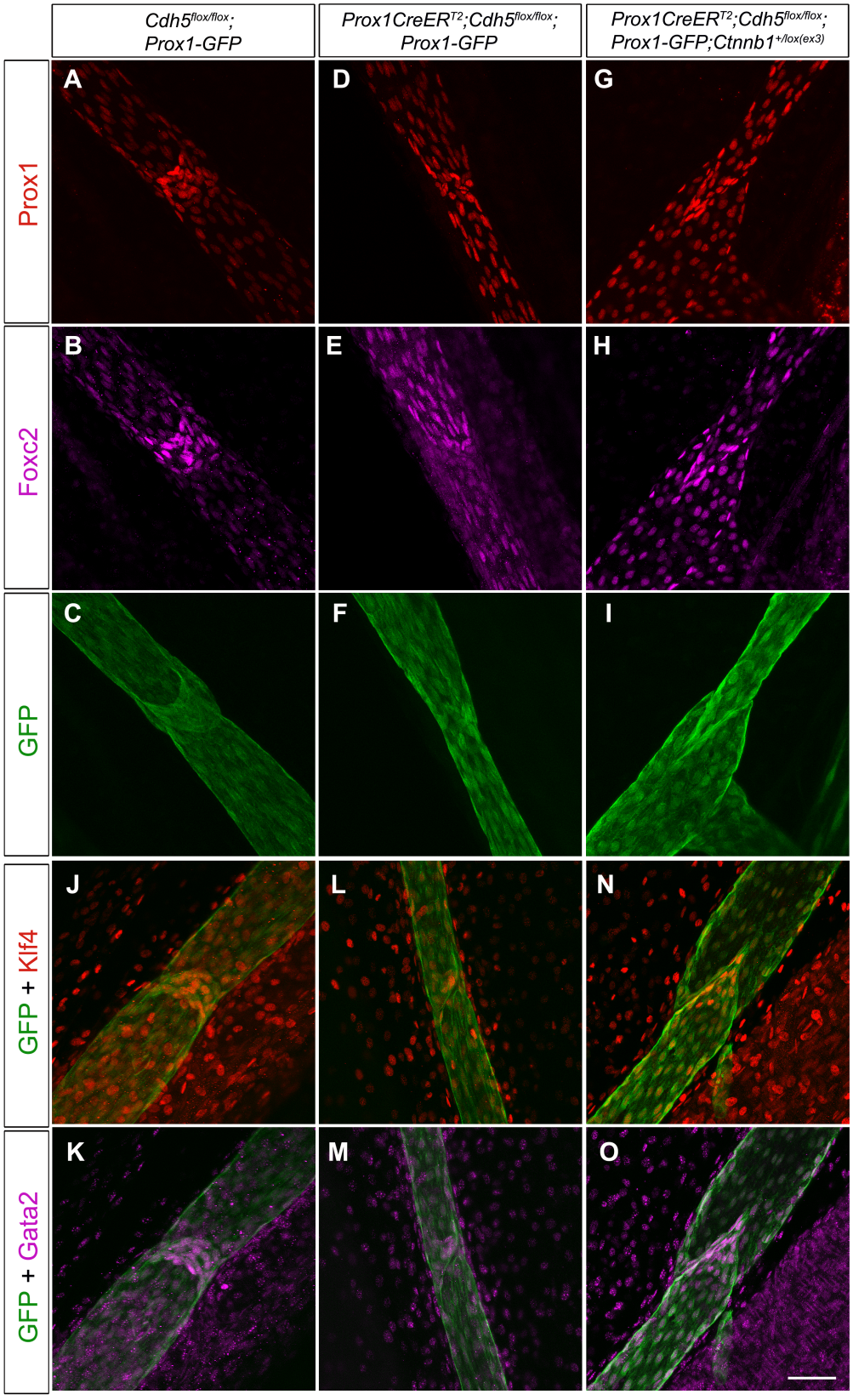
Cdh5flox/flox; Prox1-GFP
Prox1CreERT2;Cdh5flox/flox; Prox1-GFP
Prox1CreERT2;Cdh5flox/flox; Prox1-GFP;Ctnnb1+/flox(ex3)
Prox1
Foxc2
GFP
GFP + Klf4
GFP + Gata2
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O

Figure S7. Transcription factor expression *in vivo* after VE-cadherin deletion and rescue with β-catenin mutant (related to Figure 6). (**A-I**) The indicated genotypes were immunostained for Prox1 (red), GFP (green), and Foxc2 (violet). (**J-O**) The same genotypes were immunostained for Klf4 (red), GFP (green), and Gata2 (violet). Scale bar is 100 μm.

Supplemental Table 1. Primers used for qRT-PCR (related to KEY RESOURCES TABLE in STAR METHODS).

Gene	Primer Sequence
FOXC2	F: 5’ GCCTAAGGACCTGGTGAAGC R: 5’ TTGACGAAGCACTCGTTGAG
GATA2	F: 5’ CACCCCTAAGCAGAGAAGCAA R: 5’ TGGCACCACAGTTGACACACT
KLF4	F: 5’ AGGGGGTGACTGGAAGTTGT R: 5’ CCAAGCACCATCATTTAGGC
AXIN2	F: 5’ TTATGCTTTGCACTACGTCCCTCCA R: 5’ CGCAACATGGTCAACCCTCAGAC
CX37	F: 5’ CACCGTCAGCCAGATCTTAC R: 5’ ATCGTCCCCACCTCCAC
PROX1	F: 5’ GCTCCAATATGCTGAAGACC R: 5’ ATCGTTGATGGCTTGACGTG
VEGFR3	F: 5’ CGAGGAGGCCAGGTGTTTTA R: 5’ TAAAACACCTGGCCTCCTCG
GAPDH	F: 5’ GAAGGTCGGAGTCAACGGATTT R: 5’ ATGGGTGGAATCATATTGGAAC