SUPPLEMENTAL MATERIALS

Endothelial Nitric Oxide Synthase Regulates Lymphatic Valve Specification By Controlling β - catenin Signaling During Embryogenesis in Mice

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Supplemental Figure 1

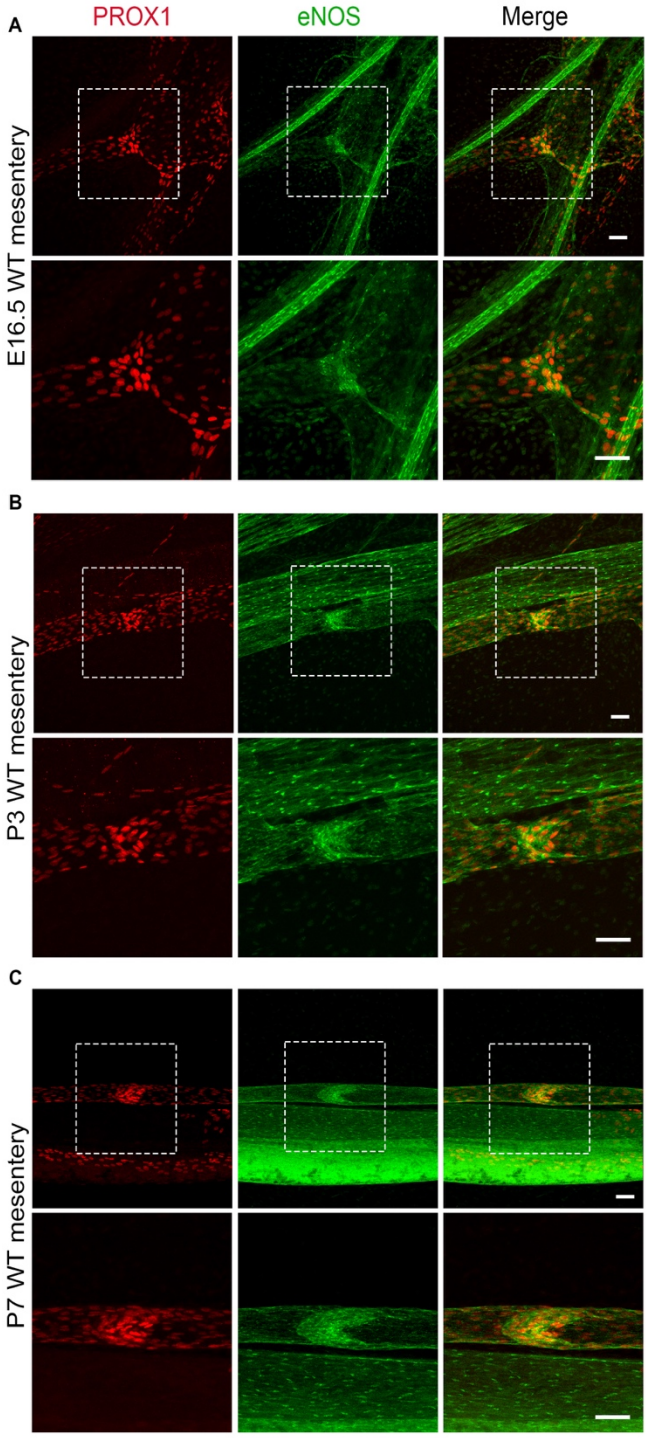

Figure S1: eNOS is highly expressed in developing and mature lymphatic valves.
(A-C) Whole-mount immunostaining of PROX1 (red) and eNOS (green) in WT mesenteries at E16.5, P3 and P7. The bottom images are high-magnification images of the white boxed areas. Scale bars are 50μm in A-C.

Supplemental Figure 2

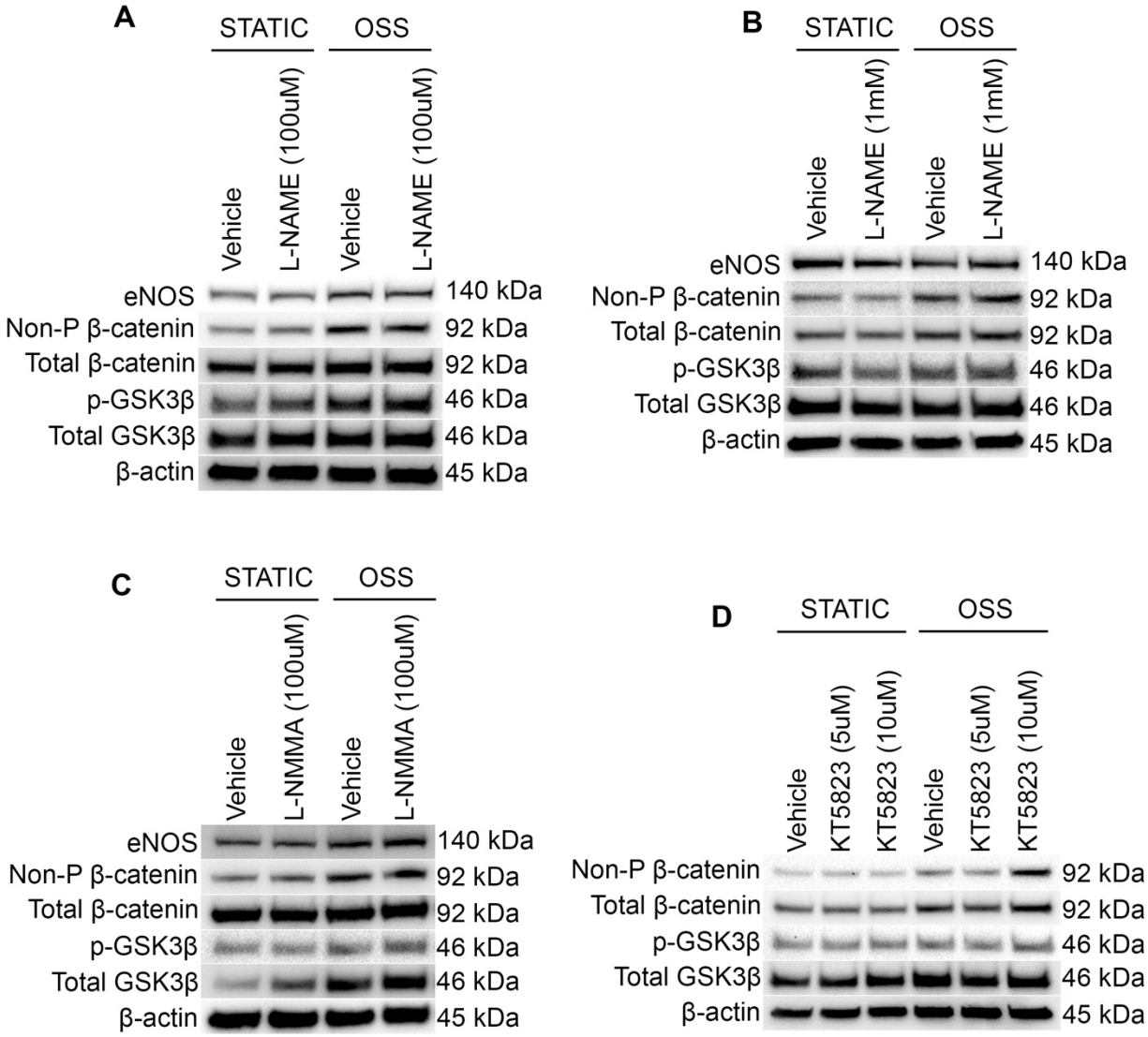

Figure S2: eNOS regulates β-catenin signaling independent of NO production.
(A-D) Western blot for the indicated proteins using lysates from hdLECs exposed to static or OSS conditions and treated with vehicle or (A) 100μM L-NAME, (B) 1mM L-NAME, (C) 100μM L-NMMA, and (D) 5μM or 10μM KT5823.

Supplemental Figure 3

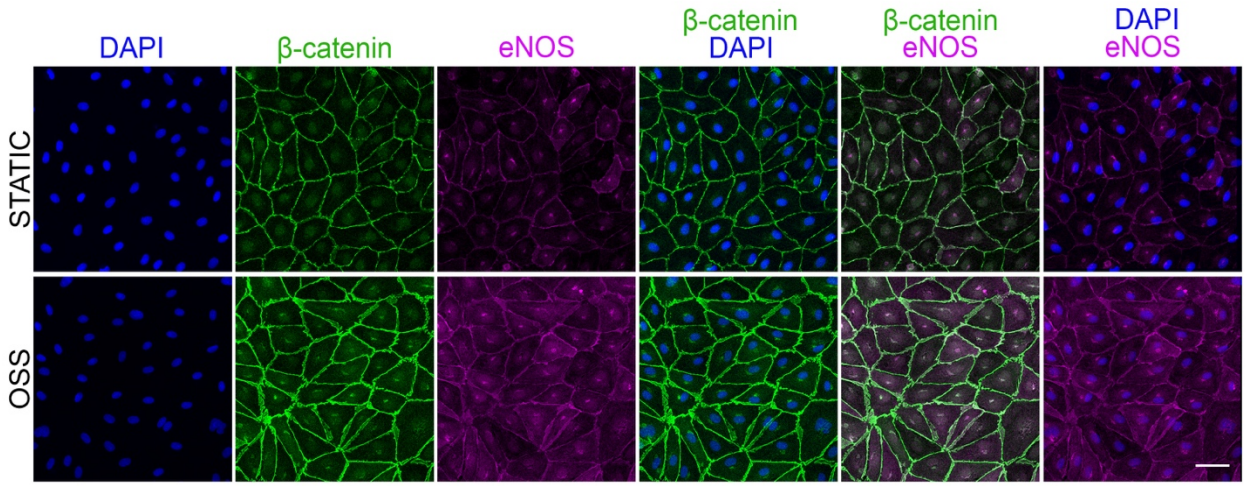

Figure S3: eNOS and β-catenin colocalize at the cell membrane and golgi. Immunostaining of DAPI (blue), β-catenin (green) and eNOS (magenta) in hdLECs cultured under static or OSS conditions for 48 hours were imaged with confocal microscopy. Scale bar is 50µm.

Supplemental Figure 4

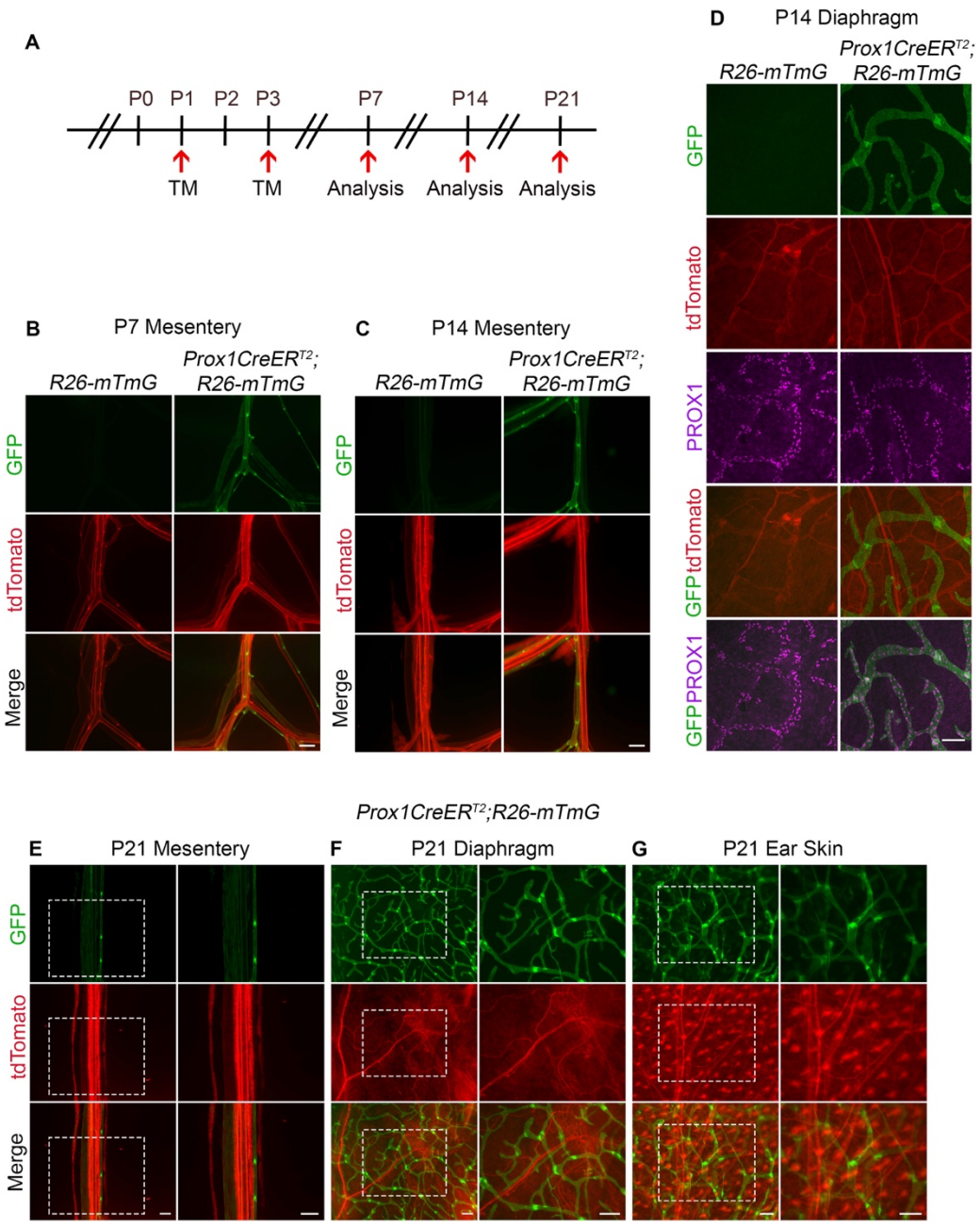

Figure S4: *Prox1CreER^{T2}* (Jscal) shows 100% recombination efficiency in a lymphatic-specific manner.

(A) Tamoxifen injection schedule to evaluate Cre-mediated recombination of reporter gene. (B,C) Fluorescence imaging of mesenteries from *R26-mTmG* littermates with and without *Prox1CreER^{T2}* (Jscal) at (B) P7 and (C) P14. (D) Whole-mount immunostaining of diaphragms from *R26-mTmG* littermates with and without *Prox1CreER^{T2}* (Jscal) at P14. (E-G) Fluorescence imaging of *Prox1CreER^{T2};R26mTmG* (Jscal) mesentery, diaphragm, and ear skin at P21. The images on the right are high-magnification images of the white boxed areas. Scale bars are 300µm in B-C and E, 100µm in D and 150µm in F-G.

Supplemental Figure 5

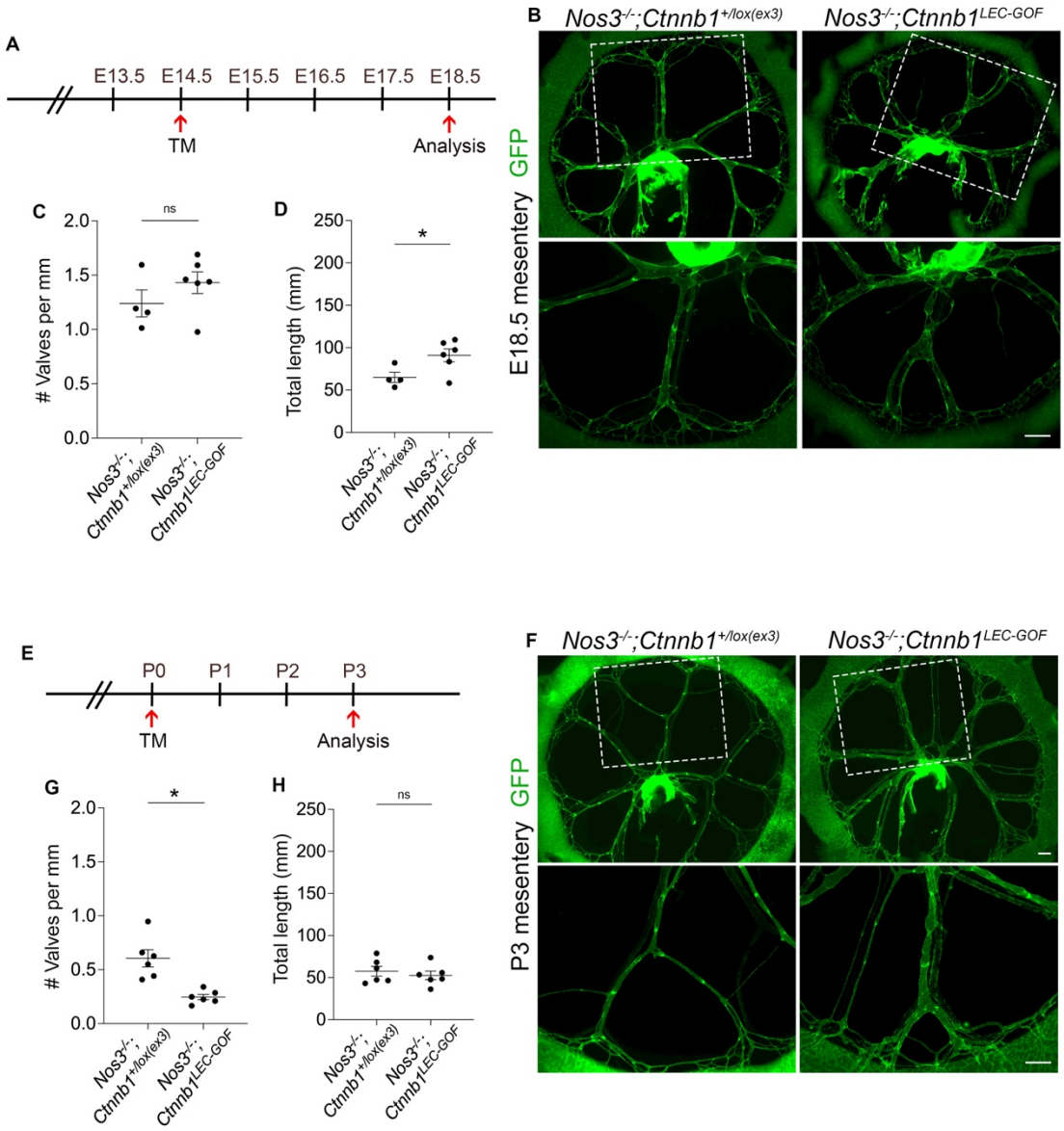

Figure S5: β-catenin gain-of-function signaling does not rescue valve loss in *Nos3*$^{-/-}$ mice. (A) Tamoxifen injection schedule for embryonic deletion of *Ctnnb1* exon 3. (B) Fluorescence imaging of E18.5 *Prox1-GFP* (green) mesenteries from *Nos3*$^{-/-}$*;Ctnnb1*$^{LEC\text{-}GOF}$ and control littermates lacking *Prox1CreER*^{T2} (Jscal). (C) Valves per millimeter from each mesentery at E18.5. (D) Total length of lymphatic vessels from each mesentery at E18.5. (E) Tamoxifen injection schedule for postnatal deletion of exon 3 of *Ctnnb1*. (F) Fluorescence imaging of *Prox1-GFP* (green) mesenteries from *Nos3*$^{-/-}$*;Ctnnb1*$^{LEC\text{-}GOF}$ and control littermates lacking *Prox1CreER*^{T2} (Jscal) at P3. (G) Valves per millimeter from each mesentery at P3. (H) Total length of lymphatic vessels from each mesentery at P3. All values are means ± SEM of n=4-6 littermates per

genotype. *P<0.05, unpaired Student's t-test. Scale bars are 500μm in B and F. Sex was not determined and data from both sexes were combined.

Major Resources Table

In order to allow validation and replication of experiments, all essential research materials listed in the Methods should be included in the Major Resources Table below. Authors are encouraged to use public repositories for protocols, data, code, and other materials and provide persistent identifiers and/or links to repositories when available. Authors may add or delete rows as needed.

Animals (in vivo studies)

Species	Vendor or Source	Background Strain	Sex	Persistent ID / URL
Mouse	Jackson Laboratories	C57Bl6/J	Male	RRID:IMSR_JAX:000664
Mouse	Jackson Laboratories	C57Bl6/J	Female	RRID:IMSR_JAX:000664

Genetically Modified Animals

	Species	Vendor or Source	Background Strain	Other Information	Persistent ID / URL
Parent – Male and Female	Mouse	Jackson Laboratories	C57Bl6/J	*Nos3*$^{-/-}$	RRID:IMSR_JAX:002684
Parent – Male and Female	Mouse	Choi et al. (2011)	C57Bl6/J	*Prox1-GFP*	RRID:MGI:5004059
Parent – Male and Female	Mouse	Jackson Laboratories	C57Bl6/J	*Ctnnb1*^{flox}	RRID:MGI:5909093
Parent – Male and Female	Mouse	Bazigou et al. (2011)	C57Bl6/J	*Prox1CreER*^{T2} (Tmak)	RRID:MGI:6438646
Parent – Male and Female	Mouse	Harada et al. (1999)	C57Bl6/J	*Ctnnb1*$^{ex3(loxP)}$	RRID:MGI:2673885
Parent – Male and Female	Mouse	Jackson Laboratories	C57Bl6/J	*Foxo1*^{flox}	RRID:MGI:6275124
Parent – Male and Female	Mouse	This paper	C57Bl6/J	*Prox1CreER*^{T2} (Jscal)	N/A

Antibodies

Target antigen	Vendor or Source	Catalog #	Working concentration	Persistent ID / URL
PROX1	Abcam	ab101851	1:500 (*In vivo* immunostaining)	RRID:AB_10712211
eNOS	Abcam	ab199956	1:500 (*In vivo* immunostaining) 1:1000 (Western blot) 1:100 (Co-IP western blot)	N/A
Phospho-eNOS (Ser1177)	Cell Signaling	9571	1:500 (*In vivo* immunostaining) 1:200 (Western blot)	RRID:AB_329837
AKT	Cell Signaling	4691	1:500	RRID:AB_915783

eNOS	Cell Signaling	32027	1:250 (*In vitro* immunostaining and PLA)	RRID:AB_2728756
IgG	Cell Signaling	5415	3.5µg	N/A
Phospho-AKT (Ser473)	Cell Signaling	4060	1:500	RRID:AB_2315049
β-Actin	Cell Signaling	3700	1:5000	RRID:AB_2242334
PROX1	R&D Systems	AF2727	1:500 (*In vivo* immunostaining)	RRID:AB_2170716
Alexa488-conjugated anti-GFP	Life Technologies	A21311	1:1000	RRID:AB_221477
FOXC2	R&D Systems	AF6989	1:500 (*In vivo* immunostaining)	RRID:AB_10973139
VE-cadherin	BD Pharmingen	550548	1:500	RRID:AB_2244723
FOXC2	Santa Cruz	sc515234	1:100 (Western blot)	N/A
PROX1	Proteintech	11067-2-AP	1:500 (Western blot)	RRID:AB_2268804
Non-phospho β-Catenin (Ser33/37/Thr41)	Cell Signaling	8814	1:500 (*In vivo* immunostaining and western blot)	RRID:AB_11127203
β-Catenin	Cell Signaling	2677	1:250 (*In vitro* immunostaining and PLA) 1:500 (Western blot) 3.5µg (Co-IP)	RRID:AB_1030943
Phospho-GSK3β (Ser9)	Cell Signaling	9336	1:200	RRID:AB_331405
GSK3β	Cell Signaling	12456	1:1000	RRID:AB_2636978
Alexa Fluor 488 Donkey Anti-Rabbit IgG (H+L)	Invitrogen	A-21206	1:300	RRID:AB_2535792
Alexa Fluor 488 Donkey Anti-Goat IgG (H+L)	Invitrogen	A-11055	1:300	RRID:AB_2534102
Alexa Fluor 488 Donkey Anti-Sheep IgG (H+L)	Invitrogen	A-11015	1:300	RRID:AB_2534082
Alexa Fluor 488 Donkey Anti-Rat IgG (H+L)	Invitrogen	A-21208	1:300	RRID:AB_2535794
Alexa Fluor 594 Donkey Anti-Rabbit IgG (H+L)	Invitrogen	A-21207	1:300	RRID:AB_141637

Alexa Fluor 594 Donkey Anti-Goat IgG (H+L)	Invitrogen	A-11058	1:300	RRID:AB_142540
Alexa Fluor 594 Donkey Anti-Sheep IgG (H+L)	Invitrogen	A-11016	1:300	RRID:AB_2534083
Alexa Fluor 594 Donkey Anti-Rat IgG (H+L)	Invitrogen	A-21209	1:300	RRID:AB_2535795
Alexa Fluor 647 Donkey Anti-Rabbit IgG (H+L)	Invitrogen	A-31573	1:300	RRID:AB_2536183
Alexa Fluor 647 Donkey Anti-Goat IgG (H+L)	Invitrogen	A-21447	1:300	RRID:AB_141844
Alexa Fluor 647 Donkey Anti-Rat IgG (H+L)	Jackson ImmunoResearch Laboratories	712-605-153	1:300	RRID:AB_2340694
Donkey anti-Rabbit IgG (H+L) HRP	Invitrogen	A-16035	1:2000	RRID:AB_2534709
Donkey anti-Mouse IgG (H+L) HRP	Invitrogen	A-16017	1:2000	RRID:AB_2534691

DNA/cDNA Clones

Clone Name	Sequence	Source / Repository	Persistent ID / URL
sh*NOS3*	CCGGAACAGCACAAGAGTTAT	VectorBuilder Inc.	

Cultured Cells

Name	Vendor or Source	Sex (F, M, or unknown)	Persistent ID / URL
Human neonatal dermal lymphatic endothelial cells (hdLECs)	PromoCell (C-12216)	M	https://promocell.com/product/human-dermal-lymphatic-endothelial-cells-hdlec/

Other

Description	Source / Repository	Catalog #	Persistent ID / URL
Tamoxifen	Sigma-Aldrich	T5648	https://www.sigmaaldrich.com/US/en/product/sigma/t5648
Donkey Serum	Jackson ImmunoResearch Laboratories	017-000-001	https://www.jacksonimmuno.com/catalog/products/017-000-001
Human Fibronectin	Corning	354008	https://ecatalog.corning.com/life-sciences/b2c/US/en/Surfaces/Extracellular-Matrices-ECMs/Corning%C2%AE-Fibronectin/p/354008#:~:text=Product%20Number354008&text=Corning%C2%AE%20Fibronectin%2C%20human%2C%201%20mg%2C%20is%20used%20as,a%20variety%20of%20cell%20types.
SC-79	Millipore	123871; CAS 305834-79-1	https://www.emdmillipore.com/US/en/product/Akt-Activator-II-SC79-CAS-305834-79-1-Calbiochem,EMD_BIO-123871

NG-Monomethyl-L-arginine acetate (L-NMMA)	R&D Systems	0771; CAS 53308-83-1	https://www.rndsystems.com/products/l-nmma-acetate_0771
KT5823	R&D Systems	1289; CAS 126643-37-6	https://www.rndsystems.com/products/kt-5823_1289
NG-Nitro-L-arginine methyl ester (L-NAME)	R&D Systems	0665; CAS 51298-62-5	https://www.rndsystems.com/products/l-name-hydrochloride_0665
Endothelial Cell Growth Medium MV 2 Kit	PromoCell	C-22121	https://promocell.com/product/endothelial-cell-growth-medium-mv-2/
Duolink Proximity Ligation Assay Kit	Millipore	DUO92101	https://www.sigmaaldrich.com/US/en/product/sigma/duo92101
Pierce Co-Immunoprecipitation Kit	Thermo Scientific	26149	https://www.thermofisher.com/order/catalog/product/26149

ARRIVE GUIDELINES

The ARRIVE guidelines (https://arriveguidelines.org/) are a checklist of recommendations to improve the reporting of research involving animals. Key elements of the study design should be included below to better enable readers to scrutinize the research adequately, evaluate its methodological rigor, and reproduce the methods or findings.

Study Design

Groups	Sex	Age	Number (prior to experiment)	Number (after termination)	Littermates (Yes/No)	Other description
Control Group	Not available	Embryos or postnatal pups	Whole litter	Whole litter	Yes	Wild-type or floxed controls
Treatment Group	Not available	Embryos or postnatal pups	Whole litter	Whole litter	Yes	Global knockout or LEC-specific knockout mice

Sample Size: Please explain how the sample size was decided Please provide details of any a *prior* sample size calculation, if done.

All n-numbers are stated in each figure legend. A power analysis was performed based on a pilot study to determine sample size. Using α=0.05 and a power (1-β)=0.8, a sample size of n=9 was used to detect a ≥40% difference in PROX1^{high} specification clusters from control levels, and a sample size of n=6 will be used to detect a ≥50% difference in valve number from control levels. For *in vitro* experiments, a sample size of n=3-4 independent experiments was performed.

Inclusion Criteria

All mice in a litter were used, regardless of sex.

Exclusion Criteria

None.

Randomization

None.

Blinding

None.