Supplemental Expanded Methods:

Tissue Preparation, Immunohistochemistry and Microscopy

Tissues or cells, plated on glass coverslips, were fixed in 4% paraformaldehyde in PBS overnight. For immunofluorescent staining, tissues were then cryoprotected with 30% sucrose in PBS overnight, embedded in OCT (Tissue-Tek) and cryosectioned at 6-8μm. Sections or cells were rehydrated in PBS, permeablized with 0.4% Triton-X100 in PBS, blocked with 4% BSA in PBS and incubated in primary antibody overnight. After washing, fluorescently labeled secondary antibodies (Jackson ImmunoResearch, West Grove, PA) were incubated for 2 hours at room temperature, sections were washed and mounted for imaging. EGFP was imaged immediately after permeablization. Images were acquired on a Nikon E800 microscope with a Hammamatsu ORCA-ER CCD camera using Metamorph Software (Molecluar Devices) and processed in Photoshop8.0 (Adobe). For hematoxylin and eosin staining, fixed tissues were put through an ethanol gradient, embedded in paraffin and sectioned at 10μm. Sections were deparaffinized and stained with Mayer's hematoxylin and eosin following standard protocols.

Quantitative RT-PCR

Lymphatic vessels were isolated from the hind limbs of adult SvEv129/6 mice. Briefly, the hind footpads of anesthetized mice were injected with 3% Chicago sky blue in Tyrode's Buffer. After incorporation, mice were euthanized and lymphatic vessels labeled with blue dye were excised under a dissecting microscope. RNA was isolated using the RNeasy Micro kit (Qiagen, Valencia, CA) with additional DNAse treatment (Promega). RNA from embryos and cultured cells was isolated using the RNeasy Mini kit. cDNA was then generated using the iScript Select cDNA synthesis kit (Bio-Rad, Hurcules, CA).

Quantitative PCR was performed on the Stratagene Mx-3000p machine (La Jolla, CA) using ABgene ROX master mix (Rochester, NY) and rodent GAPDH control reagents (Applied Biosystems, Foster City, CA). Relative levels

of gene expression were determined using the comparative quantitation ($\Delta\Delta$CT) method with MxPro software (Stratagene). Sequences for the mouse *AM*, *calcrl* and *RAMP2* primers and probes have been published (33-35) and all other primer and probe sequences are provided in Supplemental Figure 6 online. All assays were repeated at least twice, and all samples were run in triplicate. Results were analyzed using the two-tailed Student's t-test assuming unequal variance.

Interstitial fluid analysis

To collect interstitial fluid from homozygous null embryos, *RAMP2*$^{-/-}$ E14.5 embryos were dissected in PBS. A 30.5 gauge needle was inserted under the edematous skin and fluid was slowly drained. Rat lymph was collected directly from the thoracic duct of an adult Sprague-Dawley rat and mouse serum was collected by tail bleed from adult SvEv129/6 mice. Samples were centrifuged at 30,000x g and supernatants were loaded onto 8-12% gradient polyacrlymide tris-glycine gels. Gels were stained with coomassie blue 250 (Sigma.)

Quantification of Lymph Sac Area

Hematoxylin and eosin stained transverse sections of the jugular region of *AM, calcrl and RAMP2* null embryos were imaged on a Leica MZ16 dissecting microscope. Sections containing jugular lymph sacs that were matched for the same anteroposterior level were blindly measured using ImageJ software (NIH, USA). In order to normalize for section variability, the area of the lymph sac (defined as the area within the perimeter) was divided by the area of the adjacent jugular vein.

Transmission Electron Microscopy

Embryos were collected and fixed in 2% formaldehyde/2.5% glutaraldehyde in 0.15M sodium phosphate buffer, pH 7.4, overnight at 4˚C. Following several washes in sodium phosphate buffer, the samples were post-fixed for 1 hour in potassium ferrocyanide-reduced osmium, dehydrated through a graded series of

ethanol and embedded in PolyBed 812 epoxy resin (Polysciences, Warrington, PA). Using a diamond knife, 1 μm cross-sections were cut, stained with 1% toluidine blue and examined by light microscopy to isolate areas containing jugular lymph sacs. Ultrathin sections were cut with a diamond knife (70-80 nm thickness), mounted on 200 mesh copper grids and stained with 4% aqueous uranyl acetate for 15 minutes followed by Reynolds' lead citrate for 8 minutes. The sections were observed using a LEO EM-910 transmission electron microscope (LEO Electron Microscopy, Inc., Thornwood, NY), accelerating voltage of 80 kV, and micrographs were taken using a Gatan Orius SC 1000 CCD Camera (Gatan, Inc., Pleasanton, CA).

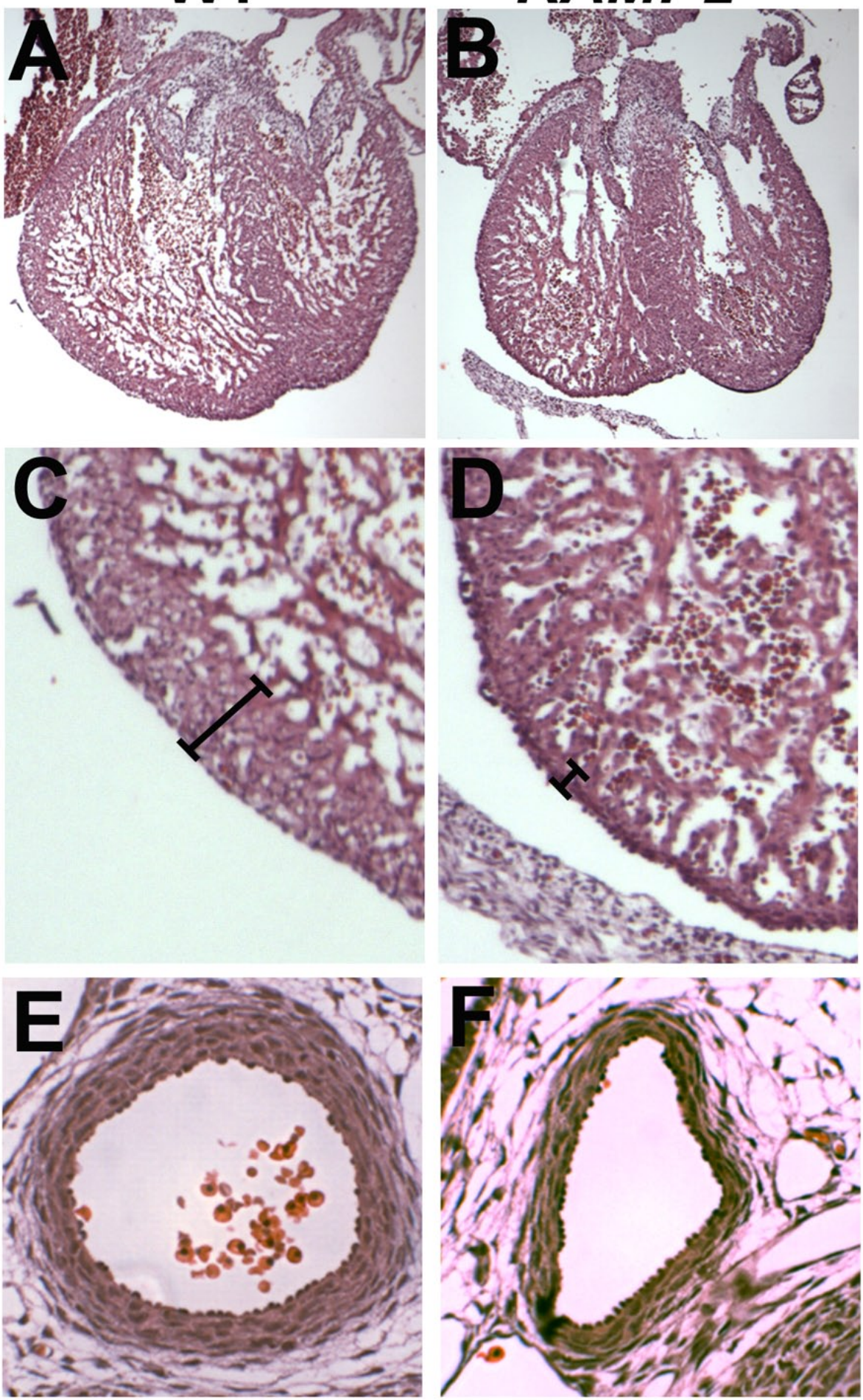
In Cell Western

$8x10^4$ cells/ml were cultured under normal growth conditions and after 24 hours media was changed to basic medium containing 0.5% FBS for 24 hours. Cells were then treated with 10nm AM for indicated times. At the end of incubation, the medium was removed and the cells were fixed, permeabilized, blocked, and treated with the appropriate antibodies per manufacturer's instructions (LI-COR). The primary antibodies, anti-phosph-ERK1/2 and anti-ERK1/2, were used at dilutions 1:100 and 1:25, respectively, and detected with goat-anti-mouse IR680 and goat-anti-rabbit IR800 each at 1:5000 dilutions. The plates were scanned with the Odyssey infrared scanner, data was obtained using scanner software and total area under the curve was determined by Gaussian best-fit analysis.

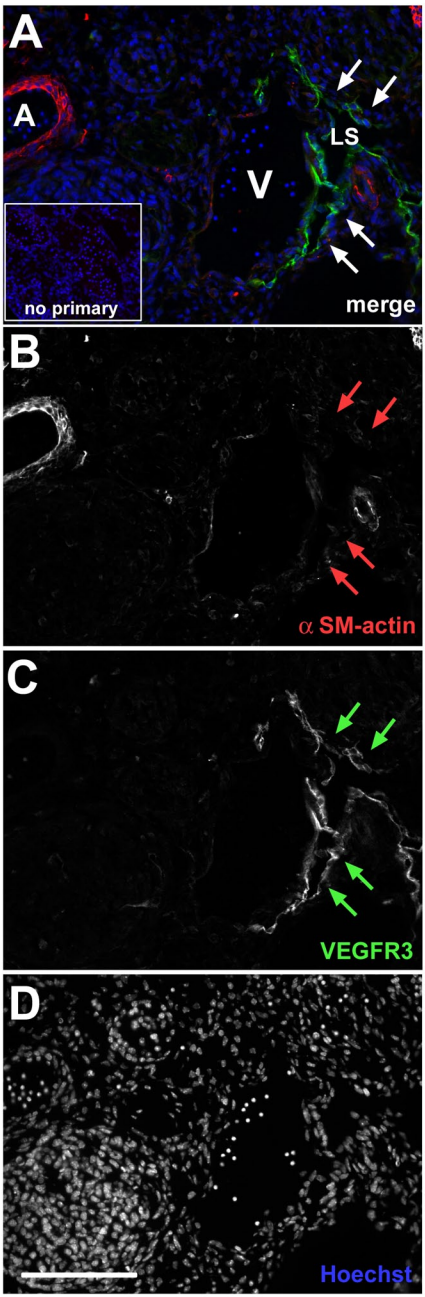
Western Blotting

LECs were treated with 10nm AM for indicated time points, washed three times with PBS, lysed in 100μl of sample buffer, boiled for 10 min, loaded onto a 10% polyacrylimide gel and resolved by SDS-PAGE. Protein was then transferred to a polyvinylidene diflouride (PVDF) membrane which was subsequently blocked in 5% non-fat dry milk overnight at 4 degrees. The primary antibodies were incubated overnight at 4 degrees at 1 mg/ml and detected with secondary

antibodies as described for the In Cell Western above. The blot was imaged with the Odyssey infrared scanner and data was obtained using scanner software.


WT
RAMP2-/-
A
B
C
D
E
F

Interstitial Fluid
Rat Lymph
Mouse Serum
220
97
66
45
30


A
A
LS
V
no primary
merge
B
α SM-actin
C
VEGFR3
D
Hoechst

Supplemental Figure 4

ES/Mouse Genotyping

	Primer Name	Primer Sequence (5' to 3')	Product size
AM	AM Forward	gtgctgacgggatcgtgctg	WT: 600
	AM Reverse	catgcagtacccgagggacct	KO: 400
	GFP Reverse	gggtcttgtagttgccgtcgt	
RAMP2	R2 Forward	tctgtctggatgctgccttgc	WT: 900
	R2 Reverse	gaagtcaggcagtcagggttg	KO: 675
	Neo Reverse	ttctatcgccttcttgacgagttc	
Calcrl	CLR Forward	gcggagcatattcaatcacaag	WT: 967
	CLR Reverse	gaaatgtgctgtatgttcaagc	KO: 800
	Neo Reverse	tggcggaccgctatcaggac	
Tie2-Cre	Cre Forward	ggcatggtgcaagttgaata	Cre: 1400
	Cre Reverse	ctggggtcttctacctttctctt	
Calcrl Flox	P1	cagattagctcagctgtatcacac	WT: 540
	P2	gcggagcatattcaatcacaag	Flox: 230
	P3	gaaatgtgctgtatgttcaagc	
	P4	ttctatcgccttcttgacgagttc	

Human Q-RT-PCR TaqMan

	Primer Name	Primer Sequence (5' to 3')	Product size
AM	hAM Forward	agctcaagccttgccacttc	163
	hAM Reverse	gacggaaaccagattcatcc	
	hAM Probe	ttagcagggtctgcgcttcgca	
RAMP2	hR2 Forward	gaaaaggcttggtgcgactg	153
	hR2 Reverse	gttggcaaagtggatctggt	
	hR2 Probe	agcaggccttatagcaccctgcga	
CALCRL	hCLR Forward	gcgacctgaaggaaagattg	123
	hCLR Reverse	agaattgcttgaacctctcca	
	hCLR Probe	tgcacttccagggtctttggtctcac	
LYVE1	hLyve1 Forwarc	gcaccatgtctacagaaactgaa	
	hLyve1 Reverse	gcagcaccaaagaagaggag	
	HLyve1 Probe	atgaagctgctgggtttggaggtgt	

Supplemental Figure Legends:

Supplemental Figure 1: Developmental cardiovascular defects of *RAMP2*$^{-/-}$ embryos phenocopies those of *AM*$^{-/-}$ and *calcrl*$^{-/-}$ embryos. Histological sections from E14.5 wildtype and *RAMP2*$^{-/-}$ embryos stained with H&E. The *RAMP2*$^{-/-}$ embryos had generally smaller hearts than wildtype littermates (A,B) with overtly normal artia, ventricles, valves and septum. The thickness of the compact zone of the right ventricle (RV) was significantly diminished in *RAMP2*$^{-/-}$ embryos compared to wildtype littermate controls (C,D). While the endothelial lining of aortas (Ao) from *RAMP2*$^{-/-}$ embryos appeared indistinguishable from that of wildtype littermates, there was a remarkable reduction in the extent of vascular smooth muscle coverage in *RAMP2*$^{-/-}$ embryos compared to wildtype (E,F). In summary, RAMP2$^{-/-}$ embryos displayed developmental cardiovascular defects similar to those previously reported for *AM*$^{-/-}$ and *calcrl*$^{-/-}$ mice.

Supplemental Figure 2: Interstitial fluid removed from *RAMP2*$^{-/-}$ embryos contains high molecular weight proteins. Fluid was removed from an E13.5 *RAMP2*$^{-/-}$ embryo and centrifuged to remove cellular debris. The supernatant was resolved on an SDS-PAGE gel along with lymph obtained directly from the thoracic duct of a rat, and mouse serum. Commassie staining shows that the edematous fluid contains an abundance of albumin (67kD) and numerous other high molecular weight proteins (>100kD), typical of lymph and unlike serum.

Supplemental Figure 3: Lymph sacs in developing E14.5 embryos are not lined by smooth muscle cells.

(A-D) Immunofluorescent staining of transverse sections through the jugular region of WT embryos. Newly formed lymph sacs (LS) which are lined with VEGFR3-containing endothelial cells (panel A:green, panel C) are not surrounded by smooth muscle, as indicated by lack of a SM-actin staining (panel A:red, panel B:arrows). Smooth muscle staining can be seen around the carotid artery (A) and jugular vein (V) of panels A and B. Hoechst stained nuclei are

shown in panel D and indicated in blue in panel A. Inset in panel A shows no primary antibody control.

Supplemental Figure 4: Primers and Probes used for Genotyping and Gene Expression Analysis.

Supplemental Movie 1: High resolution, signal volume rendered, 3-Dimensional movie of fluorescent immunohistochemistry on an E14.5 wildtype embryo using VEGFR3 antibody to visualize lymphatic vascular development.

Supplemental Movie 2: High resolution, signal volume rendered, 3-Dimensional movie of fluorescent immunohistochemistry on an E14.5 *RAMP2*$^{-/-}$ embryo using VEGFR3 antibody to visualize lymphatic vascular development.