SUPPLEMENTAL MATERIAL

Supplemental Methods

Cell Culture and Adenovirus-Mediated Rap1 Knockdown hLECs

Human neonatal dermal lymphatic cells (HMVEC-dLyNeo-Der Lym Endo Cells, referred to as hLECs) were obtained commercially (Lonza, CC-2812). Cells of 6 passages or less were maintained in EGM-2MV media (Lonza) and cultured in a 37 °C incubator under 5% CO_2. 293A cells (Invitrogen, R705-07) were cultured in DMEM (high glucose) with 10% FBS, 0.1 mM non-essential amino acid supplements, and 2 mM L-glutamine (Gibco).

Adenovirus containing engineered microRNA constructs designed to specifically target *Rap1A* (shRap1A), *Rap1B* (shRap1B) or a negative control microRNA which is predicted not to target any known genes (shCtrl) were generated in 293A cells using the BLOCK-iT™ Pol II miR RNAi Expression system (ThermoFisher) as previously described. In this system, co-cistronically expressed GFP serves as a marker for LECs containing shRap1a and shRap1b, or shCtrl adenovirus.

RNA Extraction and Real-time PCR

Total hLEC RNA was isolated using the RNeasy Mini Kit (Qiagen) and reverse transcribed using the iScript cDNA Synthesis Kit (Bio-Rad) according to the manufacturer's protocol. Real-time quantification of human *RAP1A*, *RAP1B* and *GAPDH* mRNA transcript levels was performed with iQ SYBR Green Supermix (Bio-Rad) in a 20 µl reaction in an iCycler instrument (Bio-Rad) according to the manufacturer's instructions. *RAP1A*, *RAP1B* and *GAPDH* cRNA was amplified using human single-tube assays (ThermoFisher): Hs01092205_g1 and Hs04275955_g1 and 4310884E. Amplification conditions were 95°C for 3 min; 95°C for 10 s; 60°C for 45 s for a total of 45 cycles.

Immunofluorescence

hLECs were split into 24-well plate with acid-washed cover slips at $1x10^5$ cells/ well and allowed to grow until a monolayer was formed.

For Rap1 knockdown, hLECs were infected with either shCtrl or a combination of shRap1a and shRap1b adenovirus overnight (shRap1A/B) with medium change the next day[1]. Experiments were performed 72 hours after infection. After treatments, hLECs were rinsed in HBSS, fixed with 4% PFA for 20 min and rinsed 3 x 5 min with PBS, and then permeabilized in 0.2% Triton X-100 in PBS for 2 min, followed with 3 x 5 min washes in PBS. hLECs were blocked using 3% BSA in PBS for 1 hour at room temperature. The following primary antibodies were used: anti-VE-Cadherin (C-19, Santa Cruz) at 1:200 overnight at 4°C, and anti-pMLC (3671, Cell Signaling Technology) at 1:500. Secondary antibodies were: AffiniPure Donkey Anti-Goat IgG Cy3 (1:200, Jackson ImmunoResearch), and AffiniPure Donkey Anti-Rabbit IgG Cy5 (1:200; Jackson ImmunoResearch) for 2 hours at room temperature, followed by 3 x 5 min washes in PBS. F-actin was detected using TRITC-conjugated phalloidin (Sigma-Aldrich). The coverslips were then mounted on slides using ProLong Gold Antifade Mountant (Thermo Scientific).

Mice

Rap1a$^{fl/fl}$; *Rap1b*$^{fl/fl}$,[2] *Lyve1*$^{Cre+/+}$,[3] and *Prox1*Cre^{ERT2} mice[4] have been described previously. For the developmental studies, we bred *Rap1a*$^{fl/fl}$; *Rap1b*$^{fl/fl}$ to either *Lyve1*$^{Cre+/+}$ mice or *Prox1*^{CreERT2} mice to generate heterzygote *Rap1a*$^{fl/+}$; *Rap1b*$^{fl/+}$; *Lyve1*$^{Cre+/-}$ mice or *Rap1a*$^{fl/+}$; *Rap1b*$^{fl/+}$; *Prox1*^{CreERT2} (mixed C57/B6 and 129/SvEv bacground), which were then bred to *Rap1a*$^{fl/fl}$; *Rap1b*$^{fl/fl}$ mice to produce *Rap1a*$^{fl/fl}$; *Rap1b*$^{fl/fl}$; *Lyve1*$^{Cre+/-}$ or *Rap1a*$^{fl/fl}$; *Rap1b*$^{fl/fl}$; *Prox1*^{CreERT2} mice with both *Rap1a* and *Rap1b* deleted specifically in lymphatics. Pregnant *Rap1a*$^{fl/fl}$; *Rap1b*$^{fl/fl}$ mice crossing to *Rap1a*$^{fl/fl}$; *Rap1b*$^{fl/fl}$; *Prox1*^{CreERT2} males were injected with 5 mg/ 40g Tamoxifen from

E8.5 to E10.5 to induce deletion of *Rap1a* and *Rap1b* in the *Rap1a/b*$^{fl/fl}$; *Prox1*^{CreERT2} emrbyos. For timed pregnancies, the morning on which a vaginal plug was detected was considered E0.5. For inducible deletion of both *Rap1a* and *Rap1b*, we bred *Rap1a*$^{fl/fl}$; *Rap1b*$^{fl/fl}$ to *Prox1*Cre^{ERT2} mice to generate *Rap1a*$^{fl/fl}$; *Rap1b*$^{fl/fl}$; *Lyve1*$^{Cre+/-}$ mice, which were then bred to *Rap1a*$^{fl/fl}$; *Rap1b*$^{fl/fl}$ mice to obtain *Rap1a*$^{fl/fl}$; *Rap1b*$^{fl/fl}$; *Prox1*Cre^{ERT2} mice (mixed C57/B6 and 129/SvEv bacground). To induce Cre-*loxP* recombination, *Rap1b*$^{fl/fl}$; *Prox1*Cre^{ERT2} mice 3-4 months of age were IP injected with 5 mg/ 40g Tamoxifen for 5 consecutive days. These mice were anesthetized using 0.2–0.4 ml/10 g body weight of Avertin (2,2,2,-Tribromoethanol, 20 mg/ml, Sigma). Both *Rap1a/b* KO mouse lines are on a C57/BL6 and 129/SvEv mixed background. Littermates were used when compare *Rap1a/b*KO to WT mice. We did not observed difference between sexes in each experiment. All animal studies were approved by the Institutional Animal Care and Use Committee of the University of North Carolina at Chapel Hill (UNC-Chapel Hill). Animal housing, care, and husbandry were overseen by the UNC Division of Comparative Medicine Animal Resources, which is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC).

Histology

Mouse embryos were dissected at E13.5, fixed in 4% PFA overnight and embedded in paraffin for sectioning (UNC Histology Research Core Facility). 5 µm sections were used for immunofluorescence and Haemotoxylin and Eosin (H&E) staining (UNC Histology Research Core Facility). Paraffin sections were rehydrated with a series of citrizol/ethanol solutions: citrizol, 2 x 100%, then 90%, 70%, 50% ethanol and water. Antigen retrieval was performed by submerging slides in 10 mM sodium citrate buffer and microwaving for 12 min at 750 W. Slides were kept in the hot buffer for an additional 20 min before being washed 1 x 5 min in PBS. Slides were incubated in 0.1% Triton X-100 in PBS for 2 x 15 min, and blocked in 5% donkey serum for 2 hours. Anti-VE-cadherin (1:200, C-19, Santa Cruz) and anti-podoplanin (1:200, Syrian Hamster; Developmental Studies Hybridoma Bank (DSHB)) primary antibodies were incubated overnight at 4°C. Coverslips were washed 3 x 5 min in PBS, and incubated in AffiniPure Donkey Anti-Goat IgG Cy3 (1:200, Jackson ImmunoResearch) and AffiniPure Rabbit Anti-Syrian Hamster IgG 647 (1:200, Jackson ImmunoResearch) for 2 hours at room temperature, then rinsed 3 x 5 min in PBS. The slides were mounted with ProLong Gold Antifade Mountant (Thermo Scientific).

Whole-Mount Immunofluorescence of Embryonic Dermal Lymphatics

Embryos were dissected and fixed in 4% PFA overnight at 4°C. The next day, embryos were rinsed 3 x 20 min with PBS. Dorsal skin was dissected carefully and placed in blocking solution (5% BSA, 3% donkey serum, and 1% Triton X-100 in PBS) for 2 hours at room temperature. Skins were then incubated with anti-podoplanin (1:200 in blocking solution) overnight at 4 °C. Skins were rinsed 4 x 20 min in PBST (1%Triton X-100 in PBS) and incubated in AffiniPure Rabbit Anti-Syrian Hamster IgG 647 (1:200 in blocking solution) for 2 hours at room temperature. Skins were rinsed 4x 30 min in washing buffer and Hoechst (10 mg/ml, 1:5000) was added to label nuclei at the last washing step. Skin pieces were mounted with the underside face up on glass slides using ProLong Gold Antifade Mountant. Fluorescent images were captured using an I83 Olympus inverted fluorescence microscope with Hamamatsu camera.

Whole-Mount Immunofluorescence and Quantification of Ear Lymphatics

Mouse ears were dissected, split into dorsal and ventral sides and fixed in 4% PFA overnight at 4 °C. The next day, both sides of the ears were rinsed 3 x 20 min with washing buffer (0.3% Triton X-100 in PBS) and then incubated in blocking solution (0.3% Triton X-100, 0.3% BSA, and 5% Donkey serum) for 2 hours at room temperature. Ears were then incubated in anti-podoplanin (1:200 in blocking solution) and anti-VE-cadherin (1:200 in blocking solution)

overnight at 4 °C. Ears were rinsed 4 x 20 min in washing buffer and incubated in AffiniPure Donkey Anti-Goat IgG Cy3 (1:200 in blocking solution) and AffiniPure Rabbit Anti-Syrian Hamster IgG 647 (1:200 in blocking solution) for 2 hours at room temperature. Ears were rinsed 4x 30 min in washing buffer and Hoechst (10 mg/ml, 1:5000, Invitrogen) was added to label nuclei at the last washing step. Ears were then rinsed 2 x 30 min in washing buffer and mounted fur side down on glass slides using ProLong Gold Antifade Mountant. Slides were analyzed and captured using both the I83 Olympus inverted fluorescence microscope with Hamamatsu camera, and a Zeiss 880 confocal laser scanning microscope (Hooker Imaging Core at UNC-Chapel Hill). We measured the thickness of lymphatic capillary vessels at its thickest area by 5 times, and for each animal 15-20 lymphatic vessels were measured to obtain the mean value.

Quantification

Quantification of hLEC Junction Formation

The number of GFP-positive hLECs with at least one gap or VE-cadherin staining discontinuity was counted with treatment condition masked to prevent experimenter bias. Results were expressed as percentage of knockdown cells having gaps, comparing shCtrl and shRap1A/B hLECs. 20-30 total GFP-positive cells per replicate, 3 replicates for each condition from 3 independent experiments were used for statistical analysis.

Quantification of RTCA Assay

Area under the curve (AUC) of each cell index (or normalized cell index) trace was calculated and used for statistical analysis of both shCtrl and shRap1A/B LECs. Sample number is indicated in the figure legend. For histamine experiments, cell index was normalized to the time point at which histamine was first introduced. For AM only or PKI plus AM studies, cell index was normalized to the time point at which AM was added.

Quantification of Junction Linearity

Linear “zipper-like” junctions were counted for both shCtrl and shRap1A/B hLECs in a blinded manner to prevent experimenter bias. The percentage of linear junctions to total junctions in each field was calculated for each image. 5-13 fields per replicate. Sample size were indicated in the figure leguend. Green lines on enlarged images in Figure 2 represent examples of junctions which were scored as linear.

Quantification of Phalloidin

We have quantified the phalloidin data using ImageJ software and line scan analysis (i.e. drawing a line across the cells and counting intensity peaks shown in plot profile. For each image, 4-6 cells per image and 9-13 images were analyzed. An intensity threshold was set at 800 and the peaks in the plot graph were counted for each group. The results were presented as peaks per micrometer.

Quantification of XPerT assay

Low magnification images (20x objective) taken from multiple random fields for each condition were analyzed in ImageJ by thresholding and using the “area fraction measurement” tool to obtain the number of pixels representing the areas of fluorescent-streptavidin binding (i.e. monolayer gaps); and this was expressed as percent gap area of the total image field. 5 images per coverslip and 3 triplicate coverslips were used for each experiment. We normalized fluorescence gap area by plotting the ratio between AM- and media-treated for both shCtrl and

shRap1A/B monolayers. For the histamine study, the ratio of fluorescence gap area between histamine-treated and histamine plus AM-treated hLECs was calculated for both shCtrl and shRap1A/B. Both of these studies were plotted as gap area change comparing shCtrl with shRap1A/B. For the PKI study, we determined whether PKA inhibition would change the gap area difference caused by *Rap1A/B* deletion upon AM treatment. We calculated the ratio of fluorescence gap area in shRap1A/B to the gap area in shCtrl hLECs under AM-treatment vs. PKI plus AM-treatment conditions. 3 independent experiments were used.

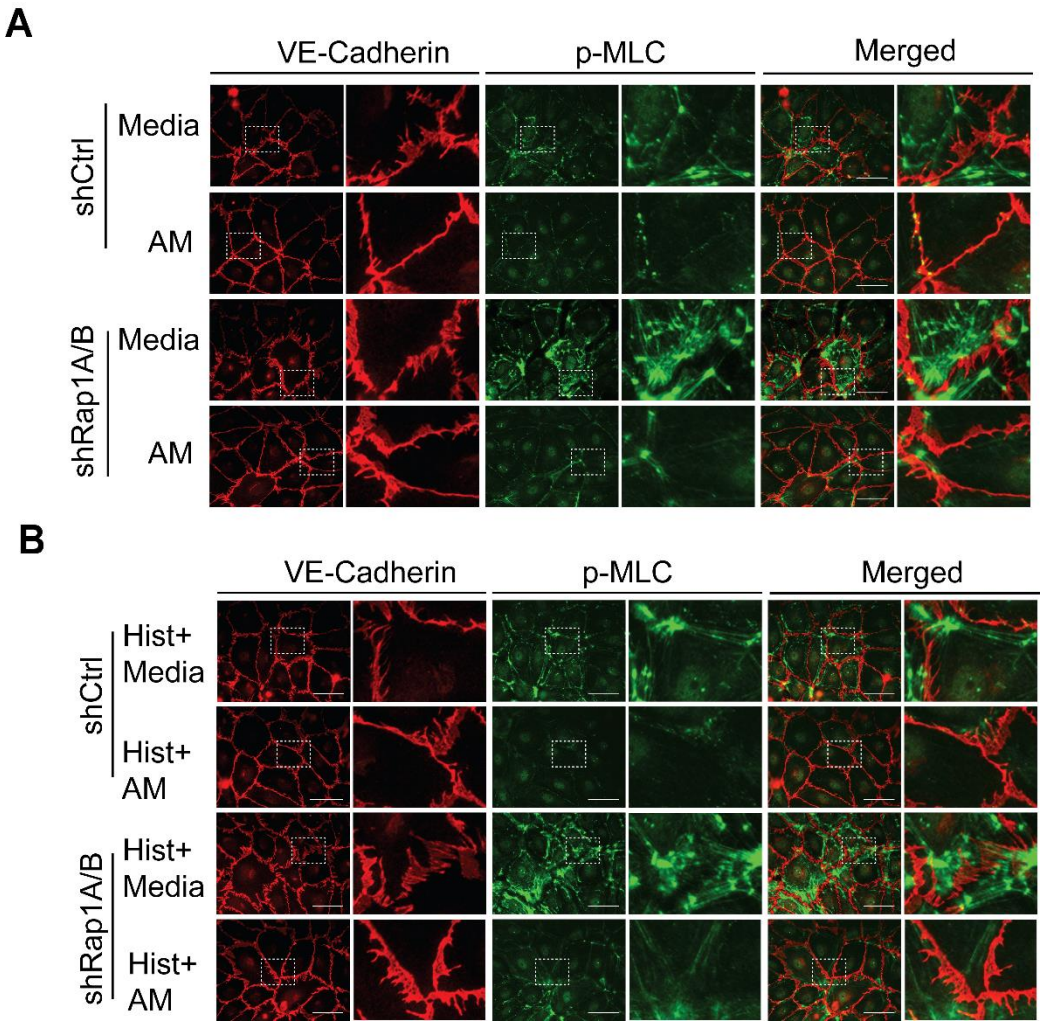
Quantification of RhoA activity

RhoA activity was quantified using the RhoA G-LISA Activation Assay Kit (Cytoskeleton. Inc, BK124). In this assay, active GTP-bound RhoA is captured by affinity interaction with a Rho-family effector protein that is immobilized onto the wells of a 96-well plate. Bound GTP-RhoA is detected by a specific RhoA antibody provided by the kit. We used lysates containing the same amount of total protein, and equal total RhoA levels were also confirmed by using the Total Rho ELISA assay kit (Cytoskeleton. Inc, BK150). We normalized the readings from the active RhoA assay to those from total RhoA assay to get the relative RhoA activity.

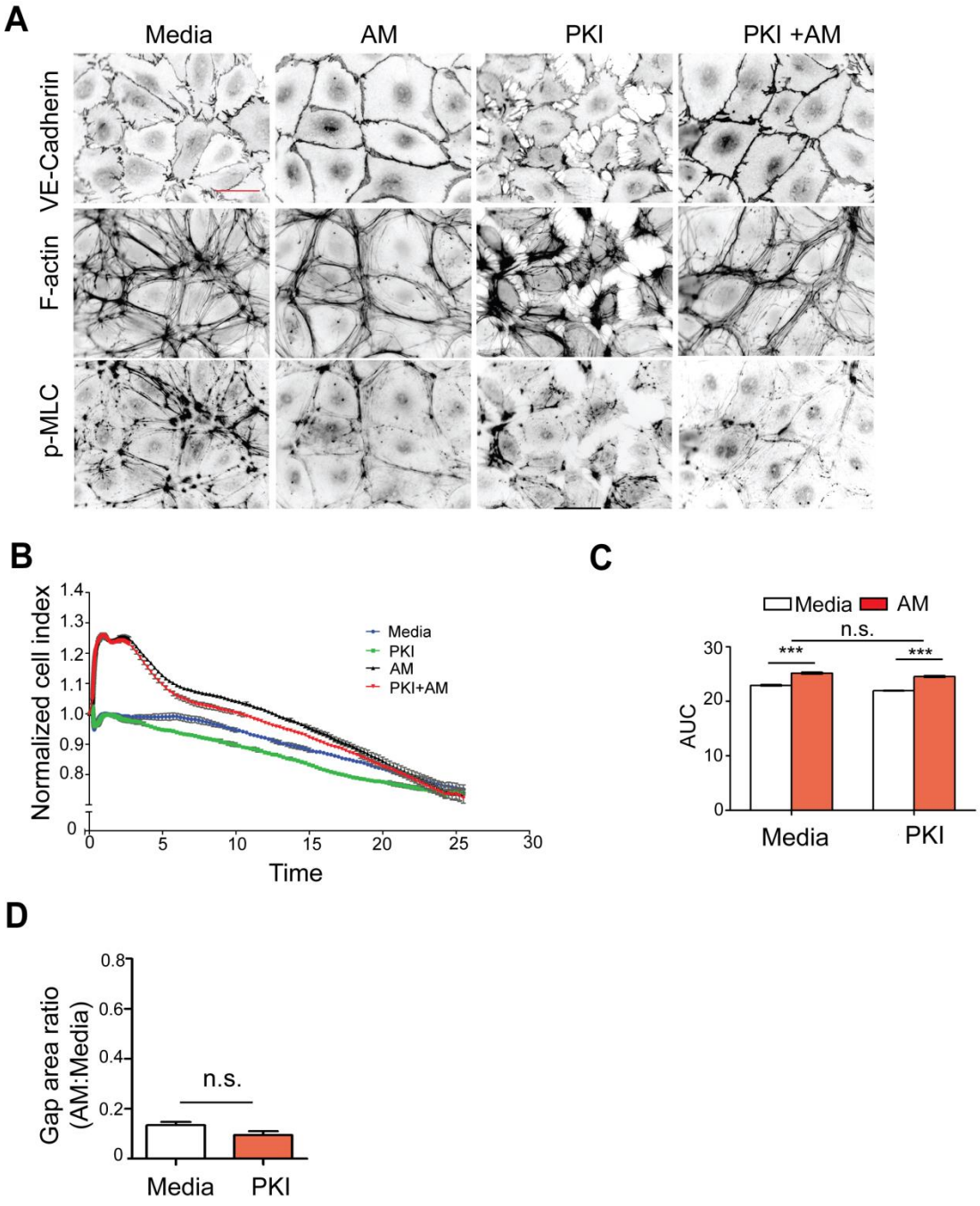
Supplemental Table 1 Embryonic Lethality of LEC-*Rap1a/b* KO

Genotypes	Embryo Number	Ratio (%)	Expected Ratio (%)
Rap1a$^{fl/fl}$; *Rap1b*$^{fl/+}$; *Lyve1*$^{Cre+/-}$ (*Rap1a* KO *Rap1b* Het)	20	11.83	12.5
Rap1a$^{fl/+}$; *Rap1b*$^{fl/fl}$; *Lyve1*$^{Cre+/-}$ (*Rap1a* Het *Rap1b* KO)	24	14.2	12.5
Rap1a$^{fl/fl}$; *Rap1b*$^{fl/fl}$; *Lyve1*$^{Cre+/-}$ (double KO)	12	7.10	12.5
Rap1a$^{fl/+}$; *Rap1b*$^{fl/+}$; *Lyve1*$^{Cre+/-}$ (*Rap1a* Het *Rap1b* Het)	17	10.06	12.5
Rap1a$^{fl/+}$; *Rap1b*$^{fl/+}$, *Rap1a*$^{fl/+}$; *Rap1b*$^{fl/fl}$, *Rap1a*$^{fl/fl}$; *Rap1b*$^{fl/+}$, *Rap1a*$^{fl/fl}$; *Rap1b*$^{fl/fl}$,	82	48.52	50
Total No. of mice	155		

Number of E13.5 embryos from *Rap1a*$^{fl/+}$; *Rap1b*$^{fl/+}$; *Lyve1*$^{Cre+/-}$ and *Rap1a*$^{fl/fl}$; *Rap1b*$^{fl/fl}$ breeding.



Online Figure I. AM decreases p-MLC2 partially independent of Rap1. p-MLC2 levels and patterns were determined in shCtrl and shRap1A/B hLECs by immunostaining with anti-pMLC antibodies (green) under basal conditions (A) and histamine treatment (B). Anti-VE-cadherin staining was used to label cell-cell junctions (red). Scale bar, 50 μm.



Online Figure II. AM rescues junctional disruption induced by inactivation of the PKA pathway. A, p-MLC2 and LEC junctions were visualized using anti-VE-cadherin and anti-p-MLC2 antibodies, F-actin was visualized with phalloidin. Scale bar, 50 μm. **B**, Representative RTCA trace of cell index normalized to time point when hLECs were treated with AM or media after 1-hour PKI or media pretreatment. Cell index was normalized to the point at which AM (10 nM) was added. **C**, Quantification of area under the curve (AUC) of media-, PKI (10 μM, 1 hour)-, media and AM (10 nM)-, PKI (10 μM, 1 hour) and AM- treated groups; n=4. **D**, Quantification of XperT assay showing gap area ratio after AM treatment in media- and PKI- treated hLECs; n=3. Data are presented as means ± SEM. n.s., not significant, and ***$p < 0.001$.

Detailed Method reference:

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3. Pham TH, Baluk P, Xu Y, Grigorova I, Bankovich AJ, Pappu R, Coughlin SR, McDonald DM, Schwab SR and Cyster JG. Lymphatic endothelial cell sphingosine kinase activity is required for lymphocyte egress and lymphatic patterning. *J Exp Med*. 2010;207:17-27.

4. Srinivasan RS, Dillard ME, Lagutin OV, Lin FJ, Tsai S, Tsai MJ, Samokhvalov IM and Oliver G. Lineage tracing demonstrates the venous origin of the mammalian lymphatic vasculature. *Genes Dev*. 2007;21:2422-32.