Materials and Methods

Animals. All animal studies were approved by the Institutional Animal Care and Use Committee of Yale University. Littermates of WT ($AIP1^{lox/lox}$) and global AIP1-KO ($AIP1^{lox/lox}$:β-actin-Cre), littermates of WT ($AIP1^{lox/lox}$) and the AIP1-ecKO ($AIP1^{lox/lox}$:Tie2-Cre), and littermates of WT ($AIP1^{lox/lox}$) and the AIP1-nKO ($AIP1^{lox/lox}$:Nestin-Cre) were used for experiments.

Assessment of developmental retinal angiogenesis. To compare superficial retinal vasculature development, AIP1-KO and WT pups were sacrificed and retinal whole-mounts were stained with isolectin B4 to visualize endothelial cells as described previously[1-4]. The area of the retina covered by vascular bed was outlined using NIH ImageJ software, and determined as the percentage of the total retinal area for comparison. In addition, cross sections from eyes harvested on P7, P9 and P12 were stained with isolectin to visualize the vessel formation in the deep retina.

Cornea lymphangiogenesis assay. Recombinant human VEGF-C (*Cys156Ser*) was formulated into Hydron pellet at 250 ng/ml and implanted into the cornea of WT and AIP1-KO mice as we previously described[5]. Hemangiogenesis was assessed by stereomicroscopy before mice were sacrificed on day 14. The corneal tissue was dissected and flattened before fixation with 3% paraformaldehyde (PFA). The tissues were digested with proteinase K (20g/mL), followed by whole mount staining overnight at 4°C.

Fluorescein staining of whole-mount retinas. Eyes from neonatal mice were collected on P4-P17 and fixed in 4% paraformaldehyde for 4 hours at 4°C. For dissection, the cornea, lens, sclera and hyaloid vessels were removed. Retinas were permeabilized in 0.5%Triton/PBS (5% normal donkey serum) overnight at 4°C, followed by incubation with fluorescein labeled isolectin B4 (Invitrogen) diluted 1:50 in 0.1% Triton/PBS (1% normal donkey serum) overnight at 4°C. Retinas were then washed 5 times with PBS and mounted by making four incisions in fluorescent mounting medium. Pictures were taken with the same exposure and gain using an Olympus confocal microscope (La Jolla, CA). Vascular areas were outlined using NIH Image J software and quantified as the percentage of total area of retina analyzed [3, 4].

Immunofluorescence analysis. Paraffin-embedded samples were used for immunostaining analysis. Eye sections were deparaffinized, rehydrated through a graded alcohol series, and heated in 10 mM sodium citrate for antigen retrieval. Paraffin sections were preincubated with 5% normal donkey serum/PBS for 1 h, and then incubated with respective antibodies or isolectin B4 overnight at 4°C. Bound antibodies were detected with fluorescence-conjugated second antibody (Vector Labs) and mounted in medium with DAPI.

Gene expression in the tissues. Total RNAs were isolated from tissues by using the RNeasy kit with DNase I digestion (Qiagen, Valencia, CA). Reverse transcription (RT) was done by standard procedure (Super Script first-strand synthesis system; Qiagen) using 1 μg total RNA. Quantitative real-time polymerase chain reaction (PCR) was performed by using iQ SYBR Green Supermix on iCycler real-time detection system (Bio-Rad Laboratories, Inc., Hercules, CA). RT-PCR with specific primers were performed as previously described [6-8].

Protein extraction and western blot analysis. Freshly dissected unfixed tissue was homogenized in lysis buffer. The lysates were centrifuged at 13000*g* for 10 minutes at 4°C. Supernatants were collected and determined with a Bradford Protein Assay kit (Bio-Rad, Hercules, CA). The cell lysates were subjected to SDS-PAGE followed by immunoblotting (Immobilon P; Millipore, Milford, MA) with specific antibodies followed by detection using an enhanced chemiluminescence kit (Amersham Life Science, Arlington Heights, IL).

BrdU incorporation assay in vivo. For the 5-bromo-2'-deoxyuridine (BrdU) incorporation assay, 100 μg BrdU (Sigma) per gram of body weight dissolved in sterile PBS was injected intraperitoneally into the pups. 2 h after BrdU injection, retinas were harvested and stained with anti-BrdU (Abcam) according to the published protocol [9].

BrdU incorporation assay in vitro. Cells were incubated with 10 μM BrdU for 4 h prior to fixation with 4% PFA for 30 minutes at room temperature. Once fixed, cells were acid denatured with 2 M HCl in 0.1% PBS-Tween for 30 minutes and then washed three times in PBS. Cells were then permeabilized and blocked nonspecific epitopes by 1%BSA/10% normal donkey serum in 0.1% PBS-Tween for 1 h following by primary anti-BrdU antibody and secondary antibody incubation.

Cell Culture and growth factors. HLEC (HMVEC-dLyAd) were purchased from Lonza. HLEC were cultured in EGM-2 MV media. All flasks for ECs were coated with 0.1% gelatin. 293T cells were cultured in DMEM containing 10% FBS. All cell lines were cultured at 37°C in 5% CO2. Recombinant human VEGF-A, VEGF-C, and VEGF-C (*Cys156Ser*) were purchased from R&D Systems.

Primary mouse retinal endothelial cells were isolated from P7 WT and AIP1-KO retinas by the method described previously [10]. Mouse retinal EC were confirmed by positive immunostaining for EC markers CD31 (or PECAM-1) and von Willebrand factor (vWF) with negative for astrocyte marker GFAP and pericyte marker NG2. Primary mouse lymphatic endothelial cells (LEC) were isolated from skins of 3-week old both WT and AIP1-KO mice by the method described previously [11]. Mouse LEC were further purified by streptavidin conjugated magnetic beads (Pierce) after cells were incubated with biotinylated anti-podoplanin antibody. LECs identity was confirmed with LYVE-1 and VEGFR-3 staining by flow cytometry analysis. AIP1 deletion in these cells was confirmed by western blot using anti-AIP1.

Transfection, stimulation and Western blot analysis. We used 3 different small interfering (si)RNA for human AIP1. Sense Sequence: GGAGCGCAACAGUUACCUGTT; Antisense Sequence: CAGGUAACUGUUGCGCUCCTT; 2: Sense Sequence: GGUGAAGGACUUCCUGACATT; Antisense Sequence: UGUCAGGAAGUCCUUCACCTT; 3: Sense Sequence: GGACUUGUUUUUUGUCACATT; Antisense Sequence: UGUGACAAAAAACAAGUCCTT. SiRNAs were purchased from Ambion and transfected into cells (20 nmol/L) by Oligofectamine following protocols provided by the manufacturer (Invitrogen). Cells were stimulated at different time points with recombinant human VEGF-A (50 ng/mL), VEGF-C WT (100 ng/mL), or VEGF-C (Cys156Ser, 250 ng/ml). After various treatments, cells were washed twice with PBS and lysed with SDS-PAGE sample buffer. For 293T cell transfection, 1 μg of mammalian expression plasmids for empty vector, AIP1, VEGFR-2, and VEGFR-3 were transfected in 293T cells using Lipofectamine according to the manufacturer's protocol (Invitrogen). Cells were harvested at 24-36 h post-transfection, and cell lysates were used for protein assays. For all immunoprecipitation, cells were lysed in cold lysis buffer. Lysates were then incubated with the first protein-specific antibody. Then, protein A/G PLUS-agarose (Santa Cruz Biotechnology Inc.) was added. All protein samples were boiled for 5 minutes, resolved in 10% polyacrylamide gels and transferred to polyvinylidene difluoride membrane and blocked with 5% milk diluted in PBS containing 0.05 % Tween 20 (PBST)). Membranes were then immunoblotted with the specified antibodies (1:1000 dilution in PBS containing 0.05 % Tween 20 (PBST) using horseradish peroxidase–conjugated secondary antibodies (1:2,000 dilution; GE Healthcare Life Sciences/Amersham Biosciences) and enhanced chemiluminescence detection system (GE Healthcare Life Sciences, Amersham Biosciences).

Lymphatic EC migration assay. For monolayer migration, HLEC were seeded onto gelatin-coated 12-well tissue culture plates and grown to confluence. Briefly, HLEC were transfected with control or AIP1 siRNA. On the next day, serum starved cells were subjected to “wound injury” assay with a 200 μl plastic pipette tip. Fresh media containing 3% serum in the absence or presence of VEGF-C

(Cys156Ser, 250 ng/ml) and were further cultured for 24h. The LEC migration in culture was determined by measuring wound areas in cell monolayers. Wound images were captured by a digital camera under a Zeiss Axiovert microscope (10X). Wound healing (% closed) was measured and analyzed by NIH Image 1.60 (http://rsbweb.nih.gov/nih-image/).

Immunofluorescence microscopy (IF). HLEC were grown on fibronectin-coated glass chamber slides (VWR Scientific International), fixed with 4% PFA in PBS for 15 minutes at room temperature, permeabilized with 0.1% triton-X buffer, blocked in 5% donkey serum diluted in PBS for 1 hr and stained 2 hr at room temperature or 4 °C overnight using specified antibodies, followed by Alexa Fluor 488- or 594-conjugated secondary antibodies (donkey anti-goat, donkey anti-rabbit, donkey anti-mouse or a combination for double IF diluted at 1:300 in PBS; Invitrogen Molecular Probes, Eugene, OR). Slides were observed using a Zeiss Axiovert 200 fluorescence microscope (Carl Zeiss MicroImaging; Thornwood, NY), and images were captured using Openlab3 software (Improvision, Lexington, MA). For tissue, 5 μm serial sections cut from frozen, OCT-embedded tissues were fixed in -20°C acetone for 10 minutes, dried for 15 minutes, followed by the same blocking/antibody protocol for cells as listed above.

FACS analysis of cell surface expression of VEGFR3. FACS analysis of cell surface VEGFR3 expression were performed as methods described previously for VEGFR-2[12]. Briefly, mouse LECs suspensions were stained with VEGFR3 primary antibody for 30 min on ice, followed by fluorescent 2nd antibody staining for another 30 min on ice. Isotype antibody serves as negative control. Flow cytometry was performed on a FACSCalibur (BD Biosciences). Data were analyzed with BD CellQuest Pro software.

Cell surface VEGFR3 endocytosis assay. HLEC cell surface VEGFR3 endocytosis were performed as methods described previously for VEGFR-2[12]. Briefly, HLEC were stimulated with VEGF-C for 0-30 min to allow receptors endocytosis. Cells were then incubated with 1 mM non-cleavable EZ-Link Sulfo-NHS-LC-Biotin (Pierce) on ice for 30 min. Remaining uninternalized surface biotinylated plasma membrane proteins were then removed by incubation with iced cold PBS/ 50 mM glycine for 30 min on ice. Cells were lysed and processed for streptavidin bead pull down. Endocytosed VEGFR3 was visualized by western blotting using anti-VEGFR3 antibodies and quantified by NIH Image 1.60.

Statistical Analysis. Data are represented as mean±SEM. Comparisons between two groups were by paired t-test, between more than two groups by one-way ANOVA followed by Bonferroni's post-hoc test or by two-way ANOVA using Prism 4.0 software (GraphPad). *P* values were two-tailed and values <0.05 were considered to indicate statistical significance.

Antibodies. The following antibodies were used for this study:

Antibody name	**Company**	**Cat#**	**Dilution**
BrdU	Abcam	ab6326	1:40-1:100
Prox1	AngioBio	11-002P	1:200
LYVE-1	Abcam	ab14917	1:100
GFAP	eBioscience	53-9892-82	1:50
	R&D	AF743	1:100

VEGFR3			
DLL4	R&D	AF1389	1:50
Alexa Flour 594 Isolectin GS-IB4 conjugate	Invitrogen	I21413	1:50
Alexa Flour 647 Isolectin GS-IB4 conjugate	Invitrogen	I32450	1:50
NG2	Millipore	AB5320	1:50
Nestin	Millipore	MAB353	1:100
AIP1(C-17)	Santa Cruz	sc-285	1:500
Phospho-VEGFR3 (pY1063/68)	Cell Application	CB5793	1:100
Phospho-VEGFR2 (pY1054/1059)	Cell Signaling	3817	1:1000
VEGFR2	R&D	AF644	1:500
Phospho-PLC-gamma1 (Tyr783)	Cell Signaling	2821	1:1000
PLC-gamma	Cell Signaling	2822	1:1000
Phospho-Akt (Thr308)	Cell Signaling	4056	1:1000
Phospho-Histone H3	Cell Signaling	9701	1:50
Akt	Cell Signaling	9272	1:1000
Alexa Flour 488 Donkey Anti-Rat IgG	Invitrogen	A21208	1:200
Alexa Flour 488 Donkey Anti-Goat IgG	Invitrogen	A11055	1:200
Alexa Flour 594 Donkey Anti-Goat IgG	Invitrogen	A11058	1:200
Alexa Flour 594 Donkey Anti-Rabbit IgG	Invitrogen	A21207	1:200
Alexa Flour 594 Donkey Anti-Mouse IgG	Invitrogen	A21203	1:200
AIP1/Dab2IP	Min lab		1:1000

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