Materials and Methods

Cell culture - Primary human endothelial cells from various tissues of origin were used in these studies (Clonetics, Lonza, Walkersville, MD, USA). Each cell type tested was from a single donor. These included human aortic endothelial cells (HAEC), human coronary artery endothelial cells, human umbilical vein endothelial cells, and human microvascular endothelial cells derived from dermis or retina (ACBRI, Kirkland, WA, USA). All were grown in endothelial growth media (EGM2, Clonetics, Lonza) under standard cell culture conditions (37°C and 5% CO_2), and used for experiments before passage 8. After validation of the phenotype in three different donors of HAEC (Supplemental Fig. S3), one was chosen for further analysis. Experiments were performed in 6-well plates (Greiner Bio-One, Monroe, NC, USA) and HAEC were seeded at 50,000 cells per well in 2mL EGM2 to optimize ligand stimulation but minimize confluence-mediated contact inhibition over the course of 24h of growth.

Ligand stimulation - Recombinant human DLL4 (R&D Systems, Minneapolis, MN, USA, M.W. 67kDa) was reconstituted in sterile PBS to 50µg/mL, and coated to 6-well plates at 1.25µg (~20 picomols)[1] in 975µL sterile PBS per well for 24-48h at 4°C. Equivalent volumes of vehicle PBS were added to control wells during coating incubations. Prior to seeding cells, PBS from wells containing DLL4 or vehicle PBS was aspirated. Recombinant human BMP9 (R&D Systems, M.W. 13kDa) was reconstituted in sterile-filtered solutions of PBS containing 0.01% BSA and 4µM HCl for a stock concentration of 10µg/mL (800pmol/mL). A dose response curve of HAEC proliferation to increasing amounts of BMP9 was performed (Supplemental Fig. S1). Based on the physiological levels of circulating BMP9[2] and our titration, we used a final concentration of 5ng/mL (0.4pmol/mL) BMP9 for experiments, compared to the same volume of vehicle control. For immunofluorescence studies and the mass spectrometry experiment, the vehicle used to reconstitute BMP9 was sterile PBS, such that the DLL4, BMP9 and combined ligand stimulation conditions shared one vehicle control (PBS).

BrdU (5-bromo-2'deoxyuridine) assays - The proliferation rate of HAEC was calculated by measuring the incorporation of BrdU during the DNA synthesis phase of the cell cycle. BrdU (Sigma) was reconstituted to a stock concentration of 10mM in PBS, sterile filtered, and added to the media atop the cells for a 20µM final concentration. HAEC were pulsed with BrdU for the final 6h of their 24h ligand stimulation before being fixed in 4% buffered formalin phosphate (Affymetrix, Santa Clara, CA, USA). Staining for BrdU was performed by first permeabilizing the cells in 1% Triton-X100 for 30 minutes, followed by 30 minutes of DNA denaturation in 2M hydrochloric acid, and then incubating 2.5h with 1µg/mL Alexa-488-conjugated primary anti-BrdU antibodies (Cat. # B35130, Invitrogen, Carlsbad, CA, USA) in a solution of 5% bovine serum albumin (BSA, Sigma Aldrich Chemical Co.) which also contained 2.5µg/mL 4',6-diamidino-2-phenylindole (DAPI, Calbiochem). Two color epifluorescent images of cells (N=10-15/well) were used to calculate the percentage of proliferating cells by dividing the number of BrdU-positive nuclei by DAPI-positive nuclei in each image, using ImageJ software (U.S. National Institutes of Health, Bethesda, MD, USA, http://imagej.nih.gov/ij/).

Quantitative RT-PCR (qPCR) - Analysis of relative change in gene transcript was performed by qPCR assay. Total RNA was extracted from cells following manufacturer's protocols, using miRCURY RNA Isolation Kits (Exiqon, Vedbaek, Denmark), and cDNA synthesis was performed following manufacturer's protocols, using qScript cDNA Synthesis Kits (Quanta Biosciences, Gaithersburg, MD, USA). qPCR was performed using iQ SYBR-Green Supermix protocols in MyIQ and iQ5 RT-PCR

Detection System thermal cyclers (Bio-Rad, Hercules, CA, USA). Cycling conditions were 95°C for 10 minutes, followed by 40 cycles of 95°C for 30 seconds, 58°C for 30 seconds, and 72°C for 30 seconds. The comparative C_T ($2^{-\Delta\Delta C(T)}$) method was used to calculate fold changes, utilizing Cyclophilin-A (*PPIA*) as a normalizing gene. Melting curve analysis was performed for validation of primers, over cycling temperatures of 55°C to 95°C, ramping in increments of 0.5°C, with 10 second dwell times. Primer sequences are listed below:

Gene	Forward primer (5' to 3')	Reverse primer (5' to 3')
HEY1	GCGCGTCAAAGTAACCTTTC	TCGGCTCTAGGTTCCATGTC
HEY2	TGGGGAGCGAGAACAATTAC	TCAAAAGCAGTTGGCACAAG
CDKN1B ($P27^{kip1)}$	ACGTTTGACGTCTTCTGAGG	GTGGACCACGAAGAGTTAAC
THBS1 (TSP1)	AGACTCCGCATCGCAAAGG	TCACCACGTTGTTGTCAAGGG
PPIA (Cyclophilin-A)	CTCGAATAAGTTTGACTTGTGTTT	CTAGGCATGGGAGGGAACA

siRNA mediated gene knockdown - Transfection of small interfering RNA constructs into HAEC was performed in 6-well plates 12h-16h before ligand stimulation, to permit transfection of the constructs, down-regulation of mRNA targets, and turnover of existing proteins. Following the transfection period, cells were trypsinized and transferred to new 6-well plates coated and/or dedicated for 24h ligand stimulation. Transfection of 100pmols per well of interfering RNA constructs was performed following manufacturer's protocol, using GeneSilencer Transfection Reagent Kits (GenLantis, San Diego, CA, USA). Knockdown constructs were purchased and prepared according to manufacturer's instructions: Notch1 (Cat. #GS4851), Notch4 (Cat. #GS4855), CBF1 (Cat. #GS3516), TSP1 (Cat. #GS7057), and NT control (Cat. #1027281) were purchased from Qiagen (Hilden, Germany); ALK1 (Cat. #SR300060), SMAD1 (Cat. #SR302765), SMAD5 (Cat. #SR302769), SMAD9 (Cat. #SR302772), and $P27^{KIP1}$ (Cat. #SR300741) were purchased from Origene (Rockville, MD, USA). Cell lysates were collected after ligand stimulation and assayed by western blots to verify that knockdown efficiencies were carried through to the end of the experiment.

Western blotting - Western blot gel loading schema (L to R) corresponded to lysates from HAEC from wells with: PBS coating (vehicle control #1), DLL4, vehicle for BMP9 (vehicle control #2), BMP9, and DLL4+BMP9, flanked by kaleidoscope molecular weight markers (Bio-Rad). Whole cell lysates were collected in 2x sample buffer and heated to 95°C for 10 minutes. Conditioned media were collected and concentrated in Ultracel-3 3kDa centrifugal filter units (Amicon, EMD Millipore, Billerica, MA, USA), concentrations were normalized using a Dc protein concentration assay kit (Bio-Rad), and normalized volumes were mixed with 6X sample buffer (to 2X) and heated to 95°C for 10 minutes. Tissue proteins were obtained by mincing 50mg tissue in 500 μL RIPA buffer (20mM Tris-HCL pH 7.5, 150mM NaCl, 1mM Na_2EDTA, 1mM EGTA, 1% NP-40, 1% Na deoxycholate, 2.5mM Na pyrophosphate, 1mM β-glycerophosphate) containing protease and phosphatase inhibitor cocktails (Sigma-Aldrich, St. Louis, MO, USA) with a scalpel on ice, followed by 5s probe sonication (constant duty cycle, 2.5 output control, Branson Sonifier 250, Branson Ultrasonics, Danbury, CT, USA), then centrifuged 15 minutes at 8000xg at 4°C. Supernatants were collected, quantified and prepared for immunoblot as described. Western blot (immunoblot) assays were performed per standard protocols on 8%, 10% or 12%, 7-lane SDS polyacrylamide gels, and transferred for 1h at 18V and 0.4A using the Transblot Turbo System and midi PVDF transfer kits (Bio-Rad). Membranes were blocked in 5% milk/TBST overnight at 4°C, and incubated minimum 48h with primary antibodies in 5% bovine serum albumin (BSA) in PBS with 0.2% sodium azide. All primary antibodies were diluted 1:1000, except GAPDH, β-actin and β-tubulin,

which were diluted 1:2000 (Table M2). Secondary HRP-conjugated antibodies were diluted 1:2000 in 5% milk/TBST, and used to incubate membranes 2h at room temperature. Chemiluminescence was performed using Luminata Forte HRP substrate (Millipore), and images were captured digitally using a Fujifilm Luminescent LAS-4000 Imager (G.E. Life Sciences, Piscataway, NJ, USA). Image analysis was performed using Fuji Colony software, v.1.1 (Fujifilm, Tokyo, Japan). Band densitometry quantification was performed using ImageJ (NIH), measuring the band size and grayscale intensity of experimental and vehicle lanes, compared to normalizing protein band size and grayscale intensity in their respective lanes, and the results used to determine the ratio of experimental to vehicle for % change. The % change values are presented in graphs, tables or as text directly on the blot image, with vehicles controls set at 100%. Antibodies used were as follows:

Antibody target	Host	Company	Catalog number
β-actin	Mouse	Sigma	A2228
β-tubulin	Mouse	Sigma	T4026
Notch1	Rabbit	Cell Signaling	3608
Cleaved Notch1(V1744)	Rabbit	Cell Signaling	4147
Notch2	Rabbit	Cell Signaling	4530
Cleaved Notch2 (D1733/A1734)	Rabbit	Millipore	07-1234
Notch4	Mouse	Cell Signaling	2423
Cleaved Notch4 (V1432)	Rabbit	Genetex	GTX86910
CBF1	Rabbit	Cell Signaling	5313
ALK1	Rabbit	Sigma	HPA007041
DLL4	Rabbit	Abcam	AB7280
Total SMAD1/5/9	Rabbit	Genetex	GTX121939
pSMAD1/5/9 (pS463/465) IHC/WB	Rabbit	www.mmcri.org/antibody	VLI 31-5
pSMAD1/5/9 (pS463/465) IF	Rabbit	www.mmcri.org/antibody	VLI D108
P14	Mouse	Cell Signaling	2407
P15	Rabbit	Cell Signaling	4822
P16	Rabbit	Cell Signaling	4824
P18	Mouse	Cell Signaling	2896
P19	Rabbit	Genetex	GTX102414
P21	Mouse	Cell Signaling	2946
$P27^{kip1}$	Rabbit	Cell Signaling	2552
$pP27^{kip1}$ (S10)	Rabbit	Abcam	AB62364
$pP27^{kip1}$ (T157)	Rabbit	Abcam	AB85047
P53	Rabbit	Cell Signaling	9282
pP53	Rabbit	Cell Signaling	9284
Cyclin D1	Mouse	Cell Signaling	2926
Cyclin D3	Mouse	Cell Signaling	2936
Cyclin E1	Mouse	Cell Signaling	4129
Cyclin E2	Rabbit	Cell Signaling	4132
CDK2	Rabbit	Cell Signaling	2546
CDK4	Mouse	Cell Signaling	2906

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Notch2	Rabbit	Cell Signaling	4530
Cleaved Notch2 (D1733/A1734)	Rabbit	Millipore	07-1234
Notch4	Mouse	Cell Signaling	2423
Cleaved Notch4 (V1432)	Rabbit	Genetex	GTX86910
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pSMAD1/5/9 (pS463/465) IF	Rabbit	www.mmcri.org/antibody	VLI D108
P14	Mouse	Cell Signaling	2407
P15	Rabbit	Cell Signaling	4822
P16	Rabbit	Cell Signaling	4824
P18	Mouse	Cell Signaling	2896
P19	Rabbit	Genetex	GTX102414
P21	Mouse	Cell Signaling	2946
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$pP27^{kip1}$ (S10)	Rabbit	Abcam	AB62364
$pP27^{kip1}$ (T157)	Rabbit	Abcam	AB85047
P53	Rabbit	Cell Signaling	9282
pP53	Rabbit	Cell Signaling	9284
CDK6	Mouse	Cell Signaling	3136
Ki-67	Rabbit	Abcam	AB15580
SKP2	Rabbit	Cell Signaling	4313
thrombospondin1	Rabbit	Abcam	AB85762
fibronectin	Mouse	Sigma	F7387
COL IV	Goat	Chemicon	AB769
β-catenin	Rabbit	Biolegend	600101
PECAM	Mouse	Cell Signaling	3528
CD36	Rabbit	Genetex	GTX100642
CD47	Rabbit	Genetex	GTX63166

Extracellular matrix proteins - Collagen IV, (COL IV, Cat. #C7521, M.W. 500kDa), derived from human placenta, was purchased from Sigma; fibronectin (FN, Cat. #1918-FN-02M, M.W. 440kDa), derived from human serum, was purchased from R&D Systems; thrombospondin-1 (TSP1, Cat. #16-20-201319, M.W. 430kDa), derived from human platelets, was purchased from Athens Research & Technology (Athens, GA, USA). COL IV and FN were reconstituted in sterile Ca^{+2}-free/Mg^{+2}-free PBS, and TSP1 was reconstituted in water. Experimental wells of 6-well plates were coated with 30pmols of reconstituted protein in 500μL PBS and allowed to dry on a flat surface at room temperature in sterile

conditions overnight, and control wells were coated with equivalent volumes of reconstitution buffers. HAEC were plated at the same density as in ligand stimulation experiments (50,000 cells/well) to minimize confluence-mediated inhibition, and BrdU was performed as described.

Immunofluorescence - Cells were prepared for immunofluorescence by fixing in 4% buffered formalin phosphate (Affymetrix) for 30-60 minutes, blocking in 5% BSA in PBS at room temperature for 30 minutes, and incubating with primary antibodies (1:250 in 5% BSA in PBS, with 0.2% sodium azide) for 24-48h at 4°C. Primary antibodies for immunofluorescence were the same as used for western blots, unless noted. After washing off primary antibodies, cells were incubated 3h with Alexa488-conjugated secondary antibodies (Invitrogen), which were diluted 1:500 in 5% BSA in PBS, which also contained 2.5µg/mL DAPI. Epifluorescence microscopy was performed on a Leica DM IRM (Leica Microsystems, Wetzlar, Germany). Images were analyzed by ImageJ (NIH). Quantification of nuclear proteins was performed by measuring average grayscale pixel intensity (0-255) within the boundaries of the nuclei.

Quantitative isotope coded affinity-tagged (ICAT) mass spectrometry – Three independent experiments were conducted per condition with $2.3x10^6$ HAEC at passage 7 (3 x 150mm dishes per condition per experiment). For the DLL4 coating of 150mm dishes, the mass of ligand coated (25.2µg or 371pmols) was scaled up proportional to surface area ($2.65pmol/cm^2$ x $140cm^2$). BMP9 stimulation was scaled up to maintain a concentration of 5ng/mL. After 24h of ligand or control stimulation, cells were collected and fractioned according to manufacturer's protocol using a QProteome Kit (Qiagen). Proteins were precipitated using 4x volume -20°C acetone and pellets were air-dried in under sterile conditions. ICAT labeling and mass spectrometry was performed as previously described[3].

Viral transduction – 2500 viral particles per cell were prepared in the serum-free medium with 34µL Genejuice reagent (EMD Millipore, Billerica, MA, USA) and incubated at room temperature for 30 minutes. Conditions in which two different virus types were mixed, half of the viral particle concentration of each was used. HAEC were washed twice with PBS and the viral cocktail was added to the cells and they were incubated under standard cell culture conditions for 8h to allow for viral transduction. After 8h, cells were gently but thoroughly washed free of virus particles and allowed to recover in normal growth medium for an additional 16 hours before further experimentation. All viral work was performed under approved Institutional Biosafety Committee (IBC) protocols, and in dedicated BSL2 facilities.

Statistical analysis - Student's two-tailed *t-tests* were used for pair-wise analyses, and comparisons were considered statistically significant at $p<0.05$. Standard deviations were derived from three or more technical replicates. Data shown represents the best representation of multiple biological replicates which showed identical trends, verifying the reproducibility of the experiments in our hands.

***Dll4* mice –** All *in vivo* procedures were conducted in accordance with current National Institutes of Health Guidelines, and were approved by the Maine Medical Center Research Institute's Institutional Animal Care and Use Committee (IACUC). Development, characterization, and genotyping of a mouse strain with a targeted mutation in the *Dll4* gene was previously described[4]. When *Dll4* mutant embryonic stem cell chimeric mice were bred to the C57BL/6J genetic background, this mutant allele was haploinsufficient, leading to completely penetrant embryonic lethality with defects in vascular development and remodeling. However, when chimeric mice with the *Dll4* mutation were crossed to the FVB/NJ background, some *Dll4*+/- mice survived into adulthood. These mice have now been backcrossed to FVB/NJ mice for more than ten generations *Dll4*+/- mice were generated by matings

between wild type littermates and *Dll4*+/- mice, yielding approximately 29% *Dll4*+/- mice (calculated from 275 pups born).

Mouse tissue collection and analysis – *Dll4*+/- and WT littermate cohorts aged 11 weeks to 56 weeks (mean age = 25 weeks) were used in our studies. Mice were anesthetized and euthanized by exsanguination with 4% buffered formalin phosphate perfused into the left ventricle. Tissues were dissected, left in fixative overnight, and processed for paraffin embedding and sectioning (Histopathology Core Facility, Maine Medical Center Research Institute). After deparaffinization, slides were stained with hematoxylin/eosin (H&E) or immunostained to detect specific antigens, including pSmad1/5, Ki-67, and VE-Cadherin. For the lungs, the caudal apex of the lower right lobe was analyzed. For the thoracic aortas, transverse sections at the 7th posterior intercostal arteries were used for H&E and immunostaining, and *en face* staining was performed on the luminal surface of the aorta between the 8th and 12th intercostal arteries as landmarks. Slides were imaged using a Zeiss Axioskop 40 microscope (Zeiss, Oberkochen, Germany) with Canon EOS 60D mounted camera and EOS utility software (Canon, Tokyo, Japan). H&E-stained aortas were used to calculate aorta wall thickness and smooth muscle cell density (N=13 WT, 9 *Dll4*+/-). VE-Cadherin staining was used to distinguish blood vessels and measure lumen circumferences (N=13 WT, 9 *Dll4*+/-). Ki-67 was used as a proliferation marker, and Ki-67 positive endothelial cells in the lung vasculature were counted and normalized to lumen circumference (N=7/genotype). Phospho-SMAD1/5/9 staining was nuclear, and quantification was performed by measuring average grayscale pixel intensity (0-255) within the boundaries of the endothelial nuclei for each genotype (N=6/genotype).

***En face* staining and endothelial density –** Formalin-fixed thoracic aortae between the 8th and 12th posterior intercostal arteries were cut longitudinally, pinned to plastic, and stained with Hoechst nuclear dye (Hoechst 33342, 10ug/mL in water, Molecular Probes, Life Technologies, Carlsbad, CA, USA) for 30 minutes at room temperature in the dark. The tissues were washed three times with PBS-T, before being mounted on glass slides in Vectashield anti-fade mounting medium (Vector Labs, Burlingame, CA, USA) lumen-side down, and coverglass sealed with nail polish. Epifluorescence microscopy was performed as previously described. Z-stacks through the aortae were captured with a 20X objective (307,200μm^2/image, 20-25 images/field, 3 fields/animal). Images were stacked and endothelial cells were counted using ImageJ software (NIH). N=8 WT, 9 *Dll4*+/-.

Lung microfil perfusion and vascular microCT analysis – The vasculature of *Dll4*+/- and wild type mice, aged 11weeks to 56 weeks (mean age = 25 weeks), were visualized by microcomputed tomography (microCT) of tissues following vascular perfusion with Microfil (MV-122, Flow Tech, Carver, MA). Mice were anesthestized and euthanized by exsanguination with 2ml Microfil introduced into the right ventricle at a rate of 0.5ml/min using a perfusion pump. The left atrium was clipped to release blood. After perfusion, Microfil was allowed to cure at 4^{o}C, and tissues were dissected. Lungs were scanned by microCT (Scanco VivaCT-40, Scanco Medical, Basserdorf, Switzerland) at 10.5μm resolution, with a voltage of 55kVp and a current of 145μA. The resolution was set to high, which created a 2048 x 2048 pixel image matrix. The tomograms were globally thresholded based on X-ray attenuation and used to render binarized 3-D images of the lungs. Animations of 3-D lungs were generated using ImageJ software (NIH).

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