Supplemental Data

Supplemental Figure Legends

**Figure S1.** Multiple independent siRNAs directed to EB1, p150<sup>Glued</sup> and Clasp1 block EC lumen and tube formation in 3D matrices. (a-c) ECs were transfected with multiple siRNA for EB1, p150<sup>Glued</sup> and Clasp1. The data shows the mean of EC lumen area per field ± S.D (n=3). (d) A siRNA to APC blocks EC lumen formation while one directed to CLIP-170 did not. Statistical significance **P < 0.01, ***P < 0.0005. (e) Western blots indicate siRNA-mediated knockdown of APC and CLIP-170.

**Figure S2.** Vascular guidance tunnels are generated during EC tube formation and post-translationally modified tubulins are strongly expressed by ECs during lumen formation and tube assembly. Cultures were established for 24 or 72 hr and then were stained for collagen type I and the indicated tubulin post-translational modifications or total α-tubulin. The stained cultures were imaged by confocal microscopy. Arrowheads indicate the borders of vascular guidance tunnels which represent matrix-free spaces created by ECs during EC tubulogenesis. L indicates luminal spaces. Bar equals 20 μm.

**Figure S3.** The microtubule stabilizing protein, Tau, controls EC lumen and tube formation and affects levels of acetylated and detyrosinated tubulin during this process. (a) siRNA suppression of Tau leads to blockade of EC lumen formation. ECs were treated with control or Tau siRNAs and EC lumen assays were performed and quantitated and cell lysates were obtained. Western blots were probed with the indicated antibodies. (b) Increased expression of Tau stimulates EC lumen formation. ECs were treated with control GFP or Tau recombinant adenoviruses and EC lumen assays were performed and quantitated and cell lysates were obtained. (c) siRNA suppression of tubulin tyrosine ligase (TTL) leads to increased EC lumen formation. ECs were treated with control or TTL siRNAs and EC lumen assays were performed and quantitated and cell lysates were obtained.
**Figure S4.** Colchicine-induced EC tube collapse in 3D matrices leads to rapid decreases in acetylated tubulin and loss of basally distributed F-actin that is coincident with the loss of EC lumens and apical-basal polarization. EC cultures were established for 48 hr in 3D collagen matrices and colchicine was added at 20 µM or was not added. (a) Cultures were fixed after 60 min and were stained and imaged by confocal microscopy. Arrowheads indicate increased F-actin staining which is no longer polarized compared to control. Bar equals 20 µm. Cell lysates were prepared at the indicated times (b) and Western blots were performed showing marked decreases in acetylated tubulin and with detectable decreases in detyrosinated tubulin and γ-tubulin, but not α-tubulin or actin.

**Figure S5.** Asymmetric localization of acetylated tubulin in a subapical domain while F-actin is basally localized during EC lumen formation in 3D collagen matrices. ECs were induced to express GFP within the cytoplasm using a GFP adenovirus and were placed in 3D collagen matrices for 24 hr. Fixed cultures were stained for acetylated tubulin, F-actin, and total α-tubulin, and then imaged by confocal microscopy. L indicates luminal spaces. Bar equals 20 µm.

**Figure S6.** Serial confocal microscopic sections reveal subapical localization of acetylated tubulin and basal localization of F-actin during EC lumen formation in 3D collagen matrices. ECs were seeded in collagen matrices and after 24 hr, were fixed and stained for acetylated tubulin and F-actin. L indicates luminal spaces. Bar equals 25 µm.

**Supplemental Videos**

**Video 1.** Real-time video of ECs treated with a control siRNA that are forming lumens and tubes in 3D collagen matrices (over a 24 hr period). Frames were collected every 10 minutes and the video is shown at 7 frames per second.
**Video 2.** Real-time video of ECs treated with a EB1 siRNA shows reduced lumen and tube formation compared to control siRNA-treated ECs in 3D collagen matrices (over a 24 hr period). Frames were collected every 10 minutes and the video is shown at 7 frames per second.

**Video 3.** Real-time video of ECs treated with a p150Glued siRNA shows reduced lumen and tube formation compared to control siRNA-treated ECs (over a 24 hr period). Frames were collected every 10 minutes and the video is shown at 7 frames per second.

**Video 4.** Real-time video of ECs treated with a Clasp1 siRNA shows reduced lumen and tube formation compared to control siRNA-treated ECs (over a 24 hr period). Frames were collected every 10 minutes and the video is shown at 7 frames per second.

**Video 5.** A video stack of serial sections of EC tubes and vascular guidance tunnels in 3D collagen matrices. EC cultures in 3D collagen matrices (24 hr) were stained for acetylated tubulin (green), collagen type I (red), and nuclei (blue) and confocal images were obtained. Confocal z-stacks were then utilized to generate the video which demonstrates EC-lined tubes which are present within vascular guidance tunnel spaces (negative staining areas).

**Video 6.** A video stack of serial sections of EC tubes in 3D collagen matrices showing acetylated tubulin present in a subapical domain while F-actin is present basally. EC cultures in 3D collagen matrices (24 hr) were stained for acetylated tubulin (green), F-actin (red), and nuclei (blue) and confocal images were obtained. Confocal z-stacks were then utilized to generate the video which demonstrates EC-lined tubes that show acetylated tubulin present in a polarized subapical region and F-actin distributed basally.
**Video 7.** A 3D reconstruction of EC tubes in 3D collagen showing acetylated tubulin present in a subapical domain while F-actin is present basally. EC cultures in 3D collagen matrices (24 hr) were stained for acetylated tubulin (green), F-actin (red), and nuclei (blue) and confocal images were obtained. Confocal z-stacks were then utilized using Imaris software to construct a 3D image using these labels and assemble a video which rotates the image to provide different views of the EC lumen structure.

### Supplemental Methods

**Reagents**

Rat tail collagen type I was prepared as described. Antibody to alpha-tubulin (Sigma), Delta 2 tubulin (Millipore), Detyrosinated tubulin (Millipore), Tyrosinated tubulin (Sigma), Polyglutamyalted tubulin (Sigma), Acetylated tubulin (Sigma), Gamma tubulin (Sigma or Epitomics), Tubulin tyrosine ligase (Sigma), CLIP170 (ProSci incorporated), p150^Glued^ (BD Biosciences), EB1 (Sigma or BD Biosciences), CLASP1 (Epitomics), collagen type I (Sigma), Adenomatous Polyposis Coli (Epitomics), HDAC6 (Sigma), β-Actin (Sigma), ERK ½ (Cell signaling), PAK2(cell signaling), PAK4 (cell signaling), Phospho-PAK2 (cell signaling), Phospho-PAK4 (cell signaling), Phospho-Src (Cell signaling), Phospho-ERK (Cell signaling), Alexa fluor® 633 phalloidin (Molecular Probes), CD31/PECAM-1 (Epitomics), Tau (Sigma and Epitomics), HDAC6 (Epitomics), Sirt2 (Epitomics) were used for western blot or immunofluorescence analysis. Colchicine was from Calbiochem (La Jolla, CA). Other reagents were from Sigma-Aldrich.

**Transfection of ECs with siRNA**

A luciferase controls were purchased from Dharmacon RNA Technologies. Single siRNA duplexes were obtained from Invitrogen and the transfections were performed as described previously. The siRNA sequences are as follows: 

- **EB1 si#1**, 5' - CAUGACAUUGCUGCCUUG AUCAAUG and 5' - CAUUGAUCCAGGCCAGCAUGUCAUG-3';
- **EB1 si#2**, 5' - CAGGUG AGUUGAUGCAGCAGGUCAA-3' and 5' - UUGACCUCUGCAUAACUCAGCUG-3';
- **EB1 si#3**, 5' - UCCAUAAACUGACAAUACGCAGCCC-3' and 5' - UUGACCUCUGCAUAACUCAGCUG-3';
- **EB1 si#4**, 5' - UUGACCUCUGCAUAACUCAGCUG-3' and 5' - UCAGUUGUUGGA-3'.
UGUACUCGUGUUCUAGCUUAGCUUG-3'; p150Glued si#1, 5’-CCGGGAUCUGGAA
ACUUAUGCAGU-3’ and 5’-UUCAACUCCUUAAACUGUCUCUC-3’; p150Glued
si#2, 5’-ACUGCAUGAAGUUCCAGACAGC-3’ and 5’-GAGAGACAGUUAAUA
AGGAGUUGAA-3; p150Glued si#3, 5’-GCGCUGCAUCCAGUAAUCACUC-3’ and
5’-UGUACUCGUGUUCUAGCUUAGCUUG-3’; p150Glued si#4, 5’-UCUCUCAGUGCA
UGUGGAGUUGUA-3’ and UACACAUCACACUCUCACUCAGACA-3’; CLASP1 si#1,
5’-CACUAAGCAAGUGUGCCACAUAU-3’ and 5’-AUAUGUGGCCACAAUCUUGCU
UAGUG-3’; CLASP1 si#2, 5’-CAGAUUGUGCUAAUCUCUCUCGUUUG-3’ and CAAACA
GAGAGAUAUGACACAAUCUG-3’; SIRT2, 5’-UAGCUGAUCUAAGAUGGCCUCUG-
3’ and 5’-CAGAGGCCAUCUUUGAGUACACUA-3’; HDAC6, 5’-GCUGCAGCGUGA
GAGUCCAACUUU-3’ and 5’-AAAGUUGGACUCACCGUGCAGC-3’; TTL, 5’-U
GUAGUAAGAUCACUUCUGGGG-3’ and 5’-CCCCAAAGUGAUACUUCACUA-
ACA-3’; Tau, 5’-GCUCUAAUGGGAACACUCACAAUA-3’ and 5’-UUAUGAGUGGAUG
UGCCUAAGAGGC-3’; APC, 5’-AAUGCUUUCACUCUCACUUCAAUA-3’ and 5’-
AAAGUUGAUGACAGUUGUUCUC-3’.

**Immunostaining analysis**

For 2D staining, ECs were fixed for 10 min in cold methanol at -20 °C and fixed in 4 %
paraformaldehyde for 10 min at RT and then permeabilized in 0.15 % Triton X-100 for 5
min. We used indicated antibodies and stained cells mounted in slow anti-fade medium
(Molecular probe). For 3D staining, immunostaining was performed as described41.
Collagen gels were fixed in 2% paraformaldehyde for 1 hour and solubilized with
detergent. Blocking reagents for the secondary antibody were added for 1 hour and
cultures incubated with primary antibodies overnight at 4°C. Cultures were washed with
washing buffer and incubated with secondary antibodies. The stained gels were
examined by immunofluorescence microscopy. For Chorioallantoic membrane (CAM)
staining, the Japanese quail (*Coturnix coturnix japonica*) were purchased from Ozark
Egg Company and incubated at 37 °C. Chorioallantoic membrane (CAM) tissues were
harvested at embryonic day 5 for immunostaining analysis. CAM tissues were fixed in
2% paraformaldehyde for further analysis.
Generation of adenoviruses

EB1 was amplified from human cDNA clone (Addgene) using the primers: EB1 sense (5'-AGGGTACCATGGCAGTGAACGTATACTCAACGTCA-3_), EB1 antisense (5'-AGTTCTAGATTACTTGTACAGCTCCATGCGCCGAG-3_), EB1 K220A sense (5'-GAAAGAGAGGGATTTTCTACTTCCGGAGCGCTACGAACATTG-3_) and EB1 K220A antisense (5'-CAATGTTCGGTAGCGCTCCGAAGTAGAAATCCCTCTCTTTTC-3_), EB1 delta C sense (5'-AGGGTACCATGGCAGTGAACGTATACTCAACGTCA-3_), EB1 delta C antisense (5'-AGTCTAGATTCCCCCTCGTTCTCCTGGCCAAATCAA-3_)(Sigma-aldrich). Human Tau gene (addgene) was amplified by following primer set: sense (5'-AGGGTACCCGACCATGGGCTGAGCCCCGCCAGAGTTC-3_) and antisense (5'-AGTCTAGATCACAAACCCTGCTTGGCCAGGGAGGCGA-3_). HDAC6 and SIRT2 virus were purchased from Vector biolabs. Details of our adenoviral production protocol were carried out as previously described38.

Microscopy and data analysis

Image acquisition of EC lumen and tube-formation assays were done using an inverted microscope (Eclipse TE2000-E; Nikon CKX41) as previously described41. A LEICA DMI 6000B microscope captured images with an Orca 1394 camera (Hamamatsu) under transmitted light (bright field) with a 40X dry objective. Live imaging was performed in a temperature-controlled chamber set to 37°C with continuous flow of 5% CO2. Image analysis was done using MetaMorph software (Molecular Devices) by tracing EC-lumen and tube area.
Figure S3
Figure S4

(a) Control vs. Colchicine treatment

(b) Western blot analysis showing the effect of Colchicine treatment on acetylated tubulin, detyrosinated tubulin, γ-tubulin, α-tubulin, and actin.
Figure S5

GFP  Acetylated tubulin  Dapi  Merged

GFP  Actin  Dapi  Merged

GFP  α-tubulin  Dapi  Merged
Figure S6

F-Actin
Acetylated Tubulin
Dapi

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