Supplemental Materials

Endothelial Epsin deficiency decreases tumor growth by enhancing VEGF signaling

Satish Pasula[1#], Xiaofeng Cai[1#], Yunzhou Dong[1#], Mirko Messa[3], John McManus[1], Baojun Chang[1], Xiaolei Liu[1], Hua Zhu[1], Robert Silasi Mansat[1], Seon-Joo Yoon[1], Scott Hahn[1], Jacob Keeling[1], Debra Saunders[1], Genevieve Ko[3], John Knight[1], Gail Newton[5], Francis Luscinskas[5], Xiaohong Sun[1], Rheal Towner[1], Florea Lupu[1], Lijun Xia[1,2], Ottavio Cremona[6,7,8], Pietro De Camilli[3], Wang Min[4]*, Hong Chen[1,2]*

[1]Cardiovascular Biology Program, Oklahoma Medical Research Foundation, [2]Department of Biochemistry and Molecular Biology, University of Oklahoma Health Science Center, Oklahoma City, OK 73104.

[3]Howard Hughes Medical Institute, [3]Departments of Cell Biology and [4]Pathology, [3]Program in Cellular Neuroscience, Neurodegeneration and Repair, [4]Interdepartmental Program in Vascular Biology and Therapeutics, Yale University School of Medicine, New Haven, CT 06536.

[5]Center for Excellence in Vascular Biology, Department of Pathology, Brigham and Women's Hospital, Harvard University, Boston, MA 02115. [6]IFOM, the FIRC Institute of Molecular Oncology Foundation, [7]Università Vita—Salute San Raffaele and [8]Istituto Nazionale di Neuroscienze, Milano, Italy.

[#]These authors contributed equally to this work.

*To whom correspondence should be addressed.

E-mail: hong-chen@omrf.org.

Conflict of interest: The authors have declared that no conflict of interest exists.

Supplemental Methods

Generation of conditional Epn1$^{fl/fl}$ mice and EC-specific DKO mice. We recently reported a strategy for generation of an epsins 1 and 2 global double knockout (DKO) mouse model (1). We used a similar strategy with modifications to create a conditional knockout of epsin 1 (*Epn1$^{fl/fl}$* mice). *Epn1$^{fl/fl}$* mice were mated with *Epn2$^{-/-}$* to generate *Epn1$^{fl/fl}$/Epn2$^{-/-}$* mice, which are on C57BL/6J congenic background. Tamoxifen inducible endothelial cell-specific DKO mice (EC-iDKO) were obtained by crossing *Epn1$^{fl/fl}$/Epn2$^{-/-}$* mice with *VEcad-ER^{T2} Cre* deleter mice on C57BL/6J background, which inactivates the epsin 1 gene specifically in endothelial cells upon tamoxifen administration. To induce postnatal deletion of endothelial epsin 1, we administered 4-hydroxytamoxifen (150 µg per 30g of body weight) by IP injection into ten-week-old WT or *Epn 1$^{fl/fl}$/VEcad-ER^{T2} Cre/Epn2$^{-/-}$* mice with or without NICD transgene. Injections were performed once per day for 5-7 consecutive days, followed by a 5-7 day resting period to obtain WT, EC-iDKO or EC-iDKO:NICD mice. EC-DKO mice were generated by crossing *Epn1$^{fl/fl}$/ Epn2$^{-/-}$* mice with *VEcad-Cre* deleter mice on C57BL/6J background. C57BL/6J WT mice bred with Cre transgenic deleter mice or other transgenic mice were used as WT controls throughout in the study.

Antibodies and reagents. Polyclonal rabbit antibodies for epsins 1 and 2 were obtained as previously described (2, 3); anti-VE-cadherin, anti-CD31 and PE-conjugated anti-CD31 from BD; anti-ZO-1 from Invitrogen; anti-EGFR from Rockland; Rabbit anti-VEGFR2, VEGFR1, VEGFR3, PDGFR-β, TGF-βR1, NICD, anti-phospho-VEGFR2 (pY1175), anti-PLCγ, anti-phospho-PLCγ, anti-ERK, and anti-phospho-ERK from Cell Signaling Technology; Rabbit anti-VEGF-A and anti-phospho-VEGFR2 (pY1054/1059) from Millipore. Anti-EEA1, goat anti-epsin 1, mouse anti-VEGFR2, and goat anti-transferrin receptor were obtained from Santa Cruz. Anti-LAMP1 were from BD Bioscience; anti-CD63 from Chemicon; anti-clathrin heavy chain from Affinity BioReagents VEGF-A, FGF and PDGF were from R&D systems. Alexa Fluor 594

labeled human transferrin from Invitrogen and bovine and human holo-transferrin were from Sigma Aldrich. 4-hydroxytamoxifen and human fibronectin were from Sigma. γ-secretase inhibitor, VEGFR2 inhibitor (SU1498), VEGFR2 kinase Inhibitor I (Cat# 676480) were obtained from Calbiochem. Matrigel was from BD. DSS was from MP Chemicals, Inc. AOM was obtained from Sigma-Aldrich.

Plasmids and transfection. Notch and NICD expression plasmids are kind gifts from Dr. Michael Potente, from Goethe University in Frankfurt, Germany. MECs were transfected with NICD and full length Notch constructs using an Amaxa Nucleofector device (Lonza) according to the manufacture's protocol. Mammalian expression plasmids for epsin 1, VEGFR2 and their mutants were described previously (4, 5).

Cell culture. HUVECs and BAECs were purchased from Lonza and cultured according to the manufacture's protocol. Cells were used between passage 2 and 5. The murine GL261 cell line was kindly provided by Dr. Safrany, from the Frederic Joliot-Curie National Research Institute for Radiobiology and Radiohygiene, Budapest, Hungary. (6). GL261 cells were cultured at 37°C with 5% CO_2 in high glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (7, 8). Before implantation, the cells were briefly trypsinized, centrifuged and resuspended in DMEM or EMEM. Agarose (Sigma) was added to the final suspension to obtain a 1% ultra-low temperature gelling agarose cell suspension (7), which was kept in a sterile vial at 37 ºC until implantation. Primary mouse endothelial cell (MECs) isolation from brain or lungs was performed as described previously (4). MECs isolated from WT, WT*/VEcad-ER*^{T2} or *Epn1*$^{fl/fl}$*/Epn2*$^{-/-}$*/VEcad-ER*^{T2} *Cre* mice were treated with 5 μM of 4-hydroxytamoxifen dissolved in ethanol for two days at 37 ^{0}C followed by incubation for additional two days without 4-hydroxytamoxifen. Deletion of epsin 1 was confirmed by both western blot and immunohistochemistry using epsin 1 antibodies. We

observed no difference between MECs isolated from WT or WT/*VEcad-ER^{T2} mice.* Freshly isolated primary MECs were used for all experiments without any further passages.

RNA interference. HUVECs were transfected by Oligofectamine or RNAiMAX according to the manufacture's instructions with siRNA duplexes of scrambled or human epsin 1 (UGCUCUUCUCGGCUCAAACUAAGGG) and epsin 2 (AAAUCCAACAGCGUAGUCUGCUGUG), designed using Ambion® Silencer® Select Pre-designed siRNAs (Invitrogen). At 48 - 72 h post transfection, cells were processed for biochemical, wound and network formation assays.

Immunoprecipitation and western blot analyses. For VEGF, FGF and PDGF signaling assays, MECs that had been starved 16 h in serum free medium were treated with 50 ng/ml of VEGF-A, 25 ng/ml FGF and 25 ng/ml PDGF for 0, 5 or 15 m at 37 ^{0}C and processed for western blotting directly. For sequential immunoprecipitation (IP), transfected 293T cells were lysed with RIPA Buffer (1%Triton X-100/0.1%SDS/0.5% sodium deoxycholic acid/5 mM tetrasodium pyrophosphate/50 mM sodium fluoride/5 mM EDTA/150 mM NaCl/25 mM Tris, pH 7.5/5 mM Na_3VO_4/ 5 mM N-ethylmaleimide and protease inhibitor cocktail). Cell lysates were precleared with mouse IgG and protein G beads for 2 h at 4 ^{0}C followed by incubation with anti-Flag for 4 h at 4 ^{0}C. Precipitated proteins were eluted from beads using 2% SDS in 50 mM Tris, pH 7.5 and diluted 1:20 with RIPA Buffer followed by anti-VEGFR2 immunoprecipitation and western blotting. For immunoprecipitation using BAECs cells, 90% confluent BAECs were starved for 24 h at 37 ^{0}C with DMEM. Cells were stimulated with 50 ng/ml of VEGF-A for 0, 2, 5, 15, 30 min and harvested using RIPA buffer. Cell lysates were precleared with goat IgG and protein G sepharose beads at 4 ^{0}C for 2 h followed by incubation with goat anti-epsin 1 as described above. For negative controls, goat IgG was added instead of goat anti-epsin 1 and immunoprecipitation was carried out using cell lysate prepared from cells exposed to 50 ng/ml

of VEGF-A for 2 or 5 min. Quantifications for western blots were performed using NIH ImageJ software.

Biotinylation of cell surface VEGFR2. MECs were starved overnight before being treated with 50 ng/ml of VEGF-A for 0, 5 or 20 min at 37 ^{0}C to allow internalization of cell surface VEGFR2. At the end point of treatment, cells were incubated with 1 mM EZ-Link Sulfo-NHS-LC-Biotin on ice for 30 min, washed with 50 mM glycine followed by cell lysis with RIPA buffer and processed for streptavidin bead pull down. Cell surface biotinylated VEGFR2 was visualized by western blotting using anti-VEGFR2 antibodies and quantified by NIH ImageJ software.

Internalization of biotinylated VEGFR2. MECs were starved overnight and incubated with 1 mM cleavable EZ-Link Sulfo-NHS-S-S-Biotin dissolved in cold PBS at 4 ^{0}C for 30 min. Cells were washed with cold PBS/50 mM glycine to stop biotinylation. Cells were then changed to warm media with 50 ng/ml VEGF-A and incubated at 37 ^{0}C for 0, 5 15 or 20 min. Remaining surface biotin attached to uninternalized plasma membrane proteins was then removed by incubating with cleavage buffer (23mM NaH_2PO_4/27mM Na_2HPO_4/75mM NaCl/1% BSA/10 mM EDTA, pH 8.0/50 mM DTT) for 15 min for 2 times at 4 ^{0}C with gentle shaking. Cells were lysed in RIPA buffer and processed for streptavidin bead pull down. 30% of the pull down from lysates prepared from cells that were not treated with cleavage buffer was loaded for western blotting. Endocytosed VEGFR2 or VEGFR1 was visualized by western blotting using anti-VEGFR2 or anti-VEGFR1 antibodies and quantified by NIH ImageJ softwate.

Internalization of biotinylated VEGFR2 and transferrin receptor. MECs were starved overnight and incubated with 1 mM cleavable EZ-Link Sulfo-NHS-S-S-Biotin dissolved in cold PBS at 4 ^{0}C for 30 min. Cells were washed with cold PBS/50 mM glycine to stop biotinylation. Cells were then changed to warm media with 50 ng/ml VEGF-A and 10 µg/ml holo-bovine transferrin and

incubated at 37 ^{0}C for 0 and 5 min. Remaining surface biotin attached to uninternalized plasma membrane proteins was then removed by incubating with cleavage buffer (23mM NaH_2PO_4/27mM Na_2HPO_4/75mM NaCl/1% BSA/10 mM EDTA, pH 8.0/50 mM DTT) for 15 min for 2 times at 4 ^{0}C with gentle shaking. Cells were lysed in RIPA buffer and processed for streptavidin bead pull down. 30% of the pull down from lysates prepared from cells that were not treated with cleavage buffer was loaded for western blotting. Endocytosed VEGFR2 or transferrin receptor was visualized by western blotting using anti-VEGFR2 or anti-transferrin receptor antibodies.

Internalization of VEGFR2 bound to biotinylated VEGF/streptavidin-Alexa 488. VEGF-A was labeled with Biotin (EZ-Link® Micro Sulfo-NHS-LC Biotinylation Kit) according to the manufacture's instructions. HUVECs or MECs were plated on coverslips pre-coated with 0.2% gelatin and grown to 75% confluency. Cells were serum starved overnight and incubated with 100 ng/ml of biotinylated VEGF-A prebound to Streptavidin Alexa Fluor 488 for 30 min at 4 ^{0}C. Cells were then shifted to 37 ^{0}C for 2, 10, and 20 min to allow internalization of VEGFR2. At the end of 2, 10, and 20 min, WT MECs but not DKO MECs were acid washed (0.15 M NaCl, 0.5M acetic acid [pH4.5]) for 5 min at 4 ^{0}C to remove cell surface bound biotinylated VEGF-A/Streptavidin Alexa Fluor 488 and allow visualization of internalized VEGFR2, and fixed with 1% formaldehyde in PBS for colocalization analysis. Cells were permeabilized, incubated with primary goat anti-epsin 1, goat anti-EEA1, mouse anti-CD63 or LAMP1 antibodies followed by incubation with fluorescent secondary antibodies. Cells were then washed and mounted, and photomicrographs were obtained using an Olympus IX81 Spinning Disc Confocal Microscope with an Olympus plan Apo Chromat 60x objective and Hamamatsu Orca-R^2 Monochrome Digital Camera C1D600.

Simultaneously internalization of transferrin and VEGFR2. WT or DKO MECs were incubated with 100 ng/ml of biotinylated VEGF pre-conjugated to Streptavidin Alexa Fluor 488 and 10 µg/ml human transferrin Alexa Fluor 594 conjugate at 4 ^{0}C for 30 min, shifted to 37 ^{0}C for 0 to 10 min and processed for immunofluorescence. WT MECs at 10 min incubation were further subjected to acid wash to remove cell surface labeling for selective visualization of internalized proteins.

FACS analysis of cell surface expression of VEGFR2. Antibody for VEGFR2 for FACS was purchased from Santa Cruz for HUVECs or from R&D systems for primary mouse MECs. The expression of epsins in HUVECs was downregulated by siRNAs treatment for 48 h and cells were starved overnight. Endothelial cells were treated with VEGF (50 ng/ml) as indicated followed by shifting to 4 ^{0}C for detachment with EDTA. Cells were stained with primary antibody for 30 min on ice, followed by fluorescent secondary antibody staining for another 30 min on ice. Isotype antibody serves as isotype control. Flow cytometry was performed on a FACSCalibur (BD Biosciences). Data were analyzed with BD CellQuest Pro software.

Confocal imaging of cells. Immunofluorescence was performed as described with modifications (1, 9). WT or DKO MECs were starved for 16 h in serum free medium, stimulated with 50 ng/ml of VEGF-A at 37 ^{0}C for 0 to 20 min. Cells were fixed, processed for immunostaining with anti-VE-cadherin and goat anti-epsin 1 or anti-ZO-1 and goat anti-epsin 1 antibodies for 2 h at RT, then incubated with fluorescent secondary antibodies for 1 h at RT. Cells were washed and mounted, and photomicrographs were obtained using an Olympus IX81 Spinning Disc Confocal Microscope with an Olympus plan Apo Chromat 60x objective and Hamamatsu Orca-R^2 Monochrome Digital Camera. Colocalization of two proteins were quantified in 30 cells at 63x magnification.

Tumor implantation. To assess tumor growth, we implanted Lewis Lung Carcinoma cells (LLC cells, ATCC, $1x10^6$ cells/tumor) or Melanoma cells ($0.25 x10^6$ cells/tumor) in twelve-week-old WT, EC-DKO, EC-iDKO or EC-iDKO:NICD mice, all on C57BL/6J background. We estimated the time of tumor appearance and monitored the tumor growth in four groups of mice by measuring tumor size with digital calipers. We recognized tumors more than 2 mm in diameter as positive and calculated tumor volume based on the formula 0.5326 (length [mm] × width $[mm]^2$). For VEGFR2 kinase inhibitor experiments, twelve-week-old WT or EC-iDKO mice were injected with LLC cells as above, followed by intravenous injection of 100 µl of either DMSO or 0.4mM kinase inhibitor every other day for six times. Tumors were harvested and processed for western blotting, immunofluorescence or EM analysis.

Glioma tumor model. WT and EC-iDKO male mice were implanted with $2 x 10^4$ GL261 cells (6) in a volume of 4 µL, at 1 mm anterior and 2 mm lateral to the bregma, at a depth of 1.5 mm, using an injection rate of 0.6 µL/min. For the GL261 mouse glioma cell implantations we did not use cells that had undergone more than 20 passages. Animals were imaged at the OMRF Advanced Magnetic Resonance Center, using a 30cm-horizontal bore 7 Tesla magnet (Bruker BioSpin MRI Gmbh, Ettlingen, Germany) at day 24 after cell implantation for the orthotopic GL261 glioma model. Each animal was anesthetized (2% isoflurane at 0.7 L/min oxygen) and immobilized in a MR probe head first and in a prone position. A head surface coil was used to receive the induced MR signal. A quadrature volume coil was used for transmission of all RF pulses (Bruker BioSpin MRI Gmbh, Ettlingen, Germany). After checking the correct positioning of the brain at the isocenter, morphological T_2-weighted images of the whole cerebrum were acquired using a multi-slice RARE (rapid acquisition with relaxation enhancement) sequence. Mouse brain morphological images were acquired with a slice thickness of 0.5 mm and a FOV of 2 × 2 cm2, for an approximate in-plane resolution of 80 µm. Tumor volumes were determined from the T_2-weighted images, as described previously (10).

AOM/DSS-induced mouse colorectal tumorogenesis model. Twelve-week-old WT, EC-iDKO mice were given a single intraperitoneal injection of AOM (10 mg/kg body weight; Sigma-Aldrich) (11). 1 week after injection, mice were fed with 1.75% DSS (40 kD; MP Biomedicals, Inc.) in drinking water for 7 days and then received no further treatment. Mice were killed at 16, 17, 18, 19 and 20 weeks after the 7-day DSS treatment. The colon and rectum were cut open longitudinally and examined for tumors. The number and size of tumors were recorded.

Prostate tumor model. We crossed EC-iDKO and WT with Transgenic Adenocarcinoma of Mouse Prostate (TRAMP) mice on C57BL/6J background (12) to generate TRAMP-EC-iDKO or TRAMP-WT mice. Ten week-old, sex and genetic background matched TRAMP-EC-iDKO or TRAMP-WT mice were IP injected with 4-hydroxytamoxifen (150 μg per 30g of body weight) for five to seven consecutive days to induce deletion of endothelial epsin 1. Mortality of TRAMP mice was recorded and survival rate was plotted for TRAMP mice with and without epsins 1 and 2. Mice were also dissected at weeks 20, 24, 28, 32 and 36 and checked for prostate tumors.

Immunohistochemistry and immunofluorescence of tissue samples. Immunohistochemistry and immunofluorescence were performed as described with modifications (1, 9). Diameter of tumor vessels from WT and epsin mutant mice were measured using the scale bar tool in the Slidebook 5.0 software on Olympus Spinning Disc Confocal microscope. At least 60 vessels for each genotype were randomly selected for measurements from 20 micrographs at 40x magnification. Perfused vessels were scored from 60 randomly selected vessels from 20 micrographs at 40x magnification. Area containing leaked FITC-dextran or covered by α-SMA was quantified using the mask tool in the Slidebook 5.0 software on Olympus Spinning Disc Confocal microscope. TUNEL staining of frozen sections of tumor samples was performed using the In Situ Cell Death Detection kit (Roche) according to the manufacture's instructions.

RT-PCR. Total RNA was extracted from WT or EC-iDKO tumors or MECs with the Trizol Reagent (Invitrogen). One µg total RNA was treated with 1 unit RNase-free DNase I (Invitrogen) to eliminate genomic DNA. The first strand cDNA was synthesized by using the SuperScript III First-Strand Synthesis SuperMix (Invitrogen). An aliquot of 1 µl of the product was subjected to PCR using gene-specific primer pairs: Hes1 (5'~ACACCGGACAAACCAAAGAC~3', 5'~GTCACCTCGTTCATGCACTC~3'); Hey2 (5'~GACAACTACCTCTCAGATTATGGC~3', 5'~CGGGAGCATGGGAAAAGC~3'); VEGFR2 (5'~AGTCTACGCCAACCCTCC~3', 5'~CATTCTTTACAAGCATACGG~3'); Beta-Actin (5'~GACGGCCAGGTCATCACTAT~3', 5'~ACATCTGCTGGAAGGTGGAC~3'); VEGF-A (5'~AAGGAGAGCAGAAGTCCCATGA~3', 5'~CACAGGACGGCTTGAAGATGT~3).

Evans blue permeability assay. Evans Blue Dye (100 µl of a 1% solution in 0.9% NaCl; Sigma-Aldrich) was injected into the retro-orbital plexus of anesthetized WT and mutant mice followed by an intradermal injection of 10 µl VEGF (200 ng/µl) or PBS into the ears. 30 minutes after the injection, mice were sacrificed and perfused with PBS through the left ventricle to clear the dye from the vascular volume. Ears were removed, dried at 60°C overnight, and weighed before Evans blue extraction using 1 ml formamide at 55°C for 16 h. Evans blue content was quantified by reading at 630 nm in a spectrophotometer.

BrdU labeling of mouse endothelial cells.

WT and DKO MEC were grown in a 48-well plate until they reached 50% percent confluency. Cells were starved overnight and stimulated with growth factors or growth factors plus inhibitors for 6 h. BrdU labeling and detection kit (Roche) was then used to label the proliferating cells. Briefly, cells were incubated with BrdU labeling medium (1:500 diluted in medium) for 3 h. Cells were washed three times with wash buffer and fixed with ethanol for 20 min at -20^{0}C followed by washing the cells three times with wash buffer and incubated with 6M HCl/0.1% Triton for 30

minutes at room temperature. This was followed by six washes with PBS/0.1% Triton. Cells were blocked with PBS/0.3% Triton/3% BSA/3% donkey serum for 30 min at room temperature followed by incubation with anti-BrdU working solution for 30 min at 37^0C. After three washes with wash buffer, cells were again incubated with donkey anti-mouse Ig-fluorescein for 30 min at 37^0C. After washing the cells three times with wash buffer they were stained with DAPI and visualized using Olympus Fluorescent microscope. Percentage of proliferating cells was calculated based on the ratio of BrdU-positive cells vs DAPI-positive cells.

Wound and network/tube formation assays.

Monolayer EC wound assay. Monolayer EC wound assays were performed as described (5). Quantification of wound distance at 12 h was performed using NIH ImageJ software.

EC network or tube formation. EC network/tube formation in Matrigel was performed as described (5). Quantification of tube formation (capillary-like networks) at 16 h was performed using NIH ImageJ software.

Ear neovascularization in mice.

Adenovirus encoding $VEGF_{164}$ ($1x10^9$ pfu) (Ad-VEGF) or β-galactosidase (Ad-LacZ) ($1x10^9$ pfu) was intradermally injected into the mice (WT and EC-iDKO) right and left ear skin, respectively. Ear vasculature was visualized by whole-mount staining of the ear samples with PE-conjugated anti-CD31.

Transmission electron microscopy. Tumor tissues were fixed with 3% PFA and 2% glutaraldehyde in 0.1 M cacodylate buffer, pH7.4, post-fixed in 1% osmium tetroxide and mordanted in 1% tannic acid, both in cacodylate buffer. Postfixed tissues were dehydrated, treated with propylene oxide and embedded in epoxy resin (EMS Inc., Hatfield, PA). Ultra-thin sections (80 nm), counterstained with 1 % lead citrate and 0.5 % uranyl acetate, were examined

on a Hitachi H7650 electron microscope (Hitachi High Technologies America, Inc.). Morphometry of EM was done based on at least 30 to 40 micrographs taken from random fields in each sample.

Statistical analysis. Data were shown as ± SEM. Data were analyzed by the two-tailed student's *t* test or ANOVA, where appropriate. The Wilcoxon signed-rank test was used to compare data that did not satisfy the student's *t* test or ANOVA. *P* value ≤ *0.05* was considered significant.

Supplemental References

1. Chen, H., Ko, G., Zatti, A., Di Giacomo, G., Liu, L., Raiteri, E., Perucco, E., Collesi, C., Min, W., Zeiss, C., et al. 2009. Embryonic arrest at midgestation and disruption of Notch signaling produced by the absence of both epsin 1 and epsin 2 in mice. *Proc Natl Acad Sci U S A* 106:13838-13843.
2. Rosenthal, J.A., Chen, H., Slepnev, V.I., Pellegrini, L., Salcini, A.E., Di Fiore, P.P., and De Camilli, P. 1999. The epsins define a family of proteins that interact with components of the clathrin coat and contain a new protein module. *J Biol Chem* 274:33959-33965.
3. Chen, H., Fre, S., Slepnev, V.I., Capua, M.R., Takei, K., Butler, M.H., Di Fiore, P.P., and De Camilli, P. 1998. Epsin is an EH-domain-binding protein implicated in clathrin-mediated endocytosis. *Nature* 394:793-797
4. Chen, H., and De Camilli, P. 2005. The association of epsin with ubiquitinated cargo along the endocytic pathway is negatively regulated by its interaction with clathrin. *Proc Natl Acad Sci U S A* 102:2766-2771.
5. Zhang, H., He, Y., Dai, S., Xu, Z., Luo, Y., Wan, T., Luo, D., Jones, D., Tang, S., Chen, H., et al. 2008. AIP1 functions as an endogenous inhibitor of VEGFR2-mediated signaling and inflammatory angiogenesis in mice. *J Clin Invest* 118:3904-3916.
6. Szatmari, T., Lumniczky, K., Desaknai, S., Trajcevski, S., Hidvegi, E.J., Hamada, H., and Safrany, G. 2006. Detailed characterization of the mouse glioma 261 tumor model for experimental glioblastoma therapy. *Cancer science* 97:546-553.
7. von Kienlin, M., Ziegler, A., Le Fur, Y., Rubin, C., Decorps, M., and Remy, C. 2000. 2D-spatial/2D-spectral spectroscopic imaging of intracerebral gliomas in rat brain. *Magnetic resonance in medicine : official journal of the Society of Magnetic Resonance in Medicine / Society of Magnetic Resonance in Medicine* 43:211-219.

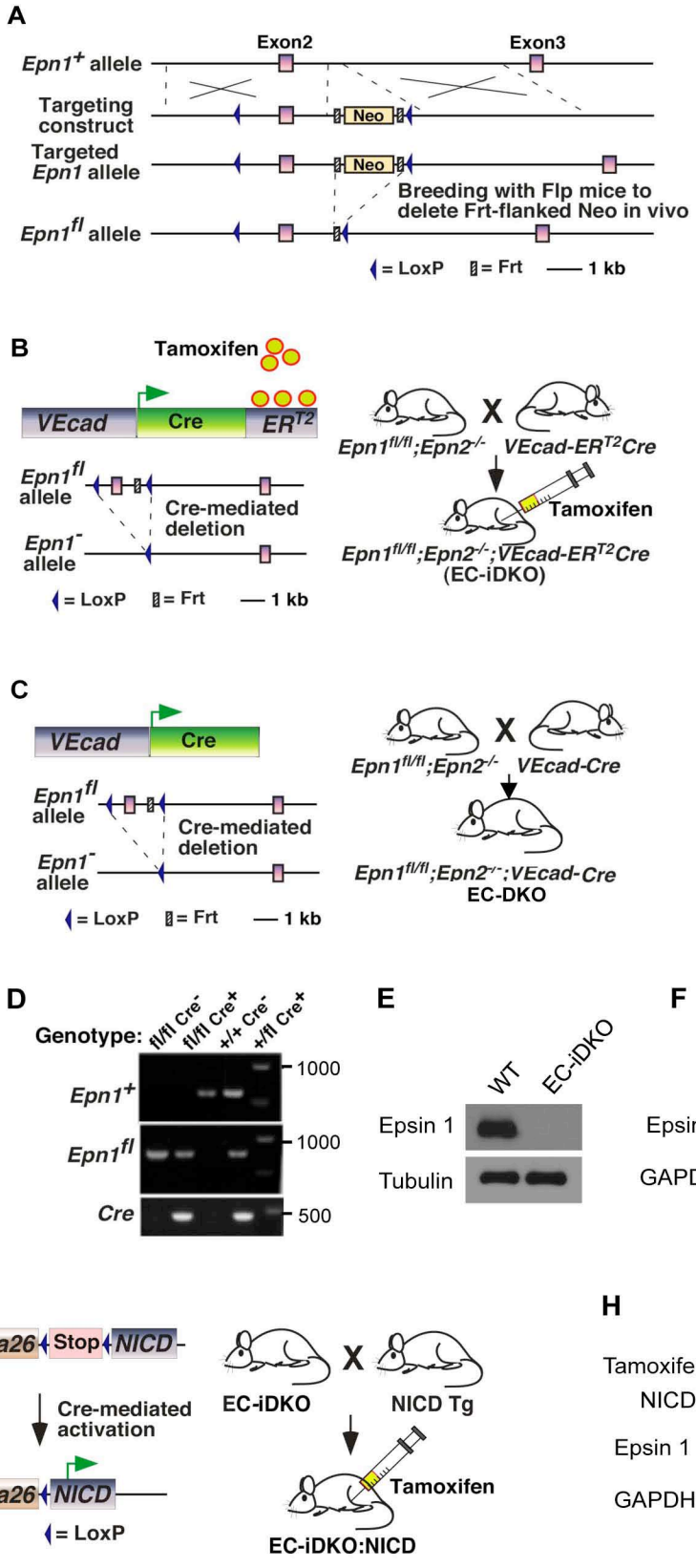
8. Rubenstein, M., Shaw, M., Mirochnik, Y., Slobodskoy, L., Glick, R., Lichtor, T., Chou, P., and Guinan, P. 1999. In vivo establishment of T98G human glioblastoma. *Methods and findings in experimental and clinical pharmacology* 21:391-393.

9. Chen, H., Fre, S., Slepnev, V.I., Capua, M.R., Takei, K., Butler, M.H., Di Fiore, P.P., and De Camilli, P. 1998. Epsin is an EH-domain-binding protein implicated in clathrin-mediated endocytosis. *Nature* 394:793-797.

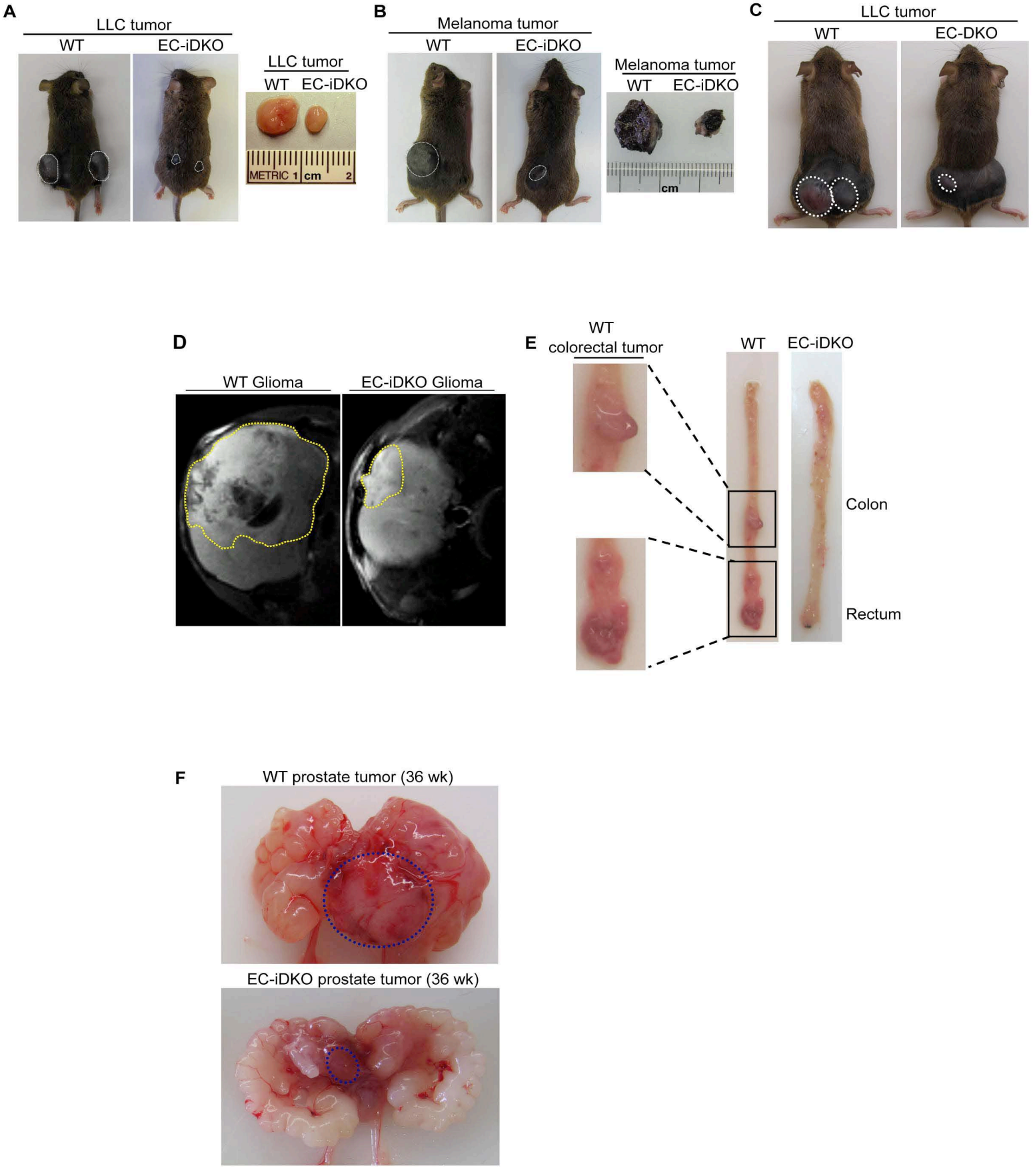
10. Doblas, S., He, T., Saunders, D., Hoyle, J., Smith, N., Pye, Q., Lerner, M., Jensen, R.L., and Towner, R.A. 2012. In vivo characterization of several rodent glioma models by (1) H MRS. *NMR in biomedicine* 25:685-694.

11. An, G., Wei, B., Xia, B., McDaniel, J.M., Ju, T., Cummings, R.D., Braun, J., and Xia, L. 2007. Increased susceptibility to colitis and colorectal tumors in mice lacking core 3-derived O-glycans. *The Journal of experimental medicine* 204:1417-1429.

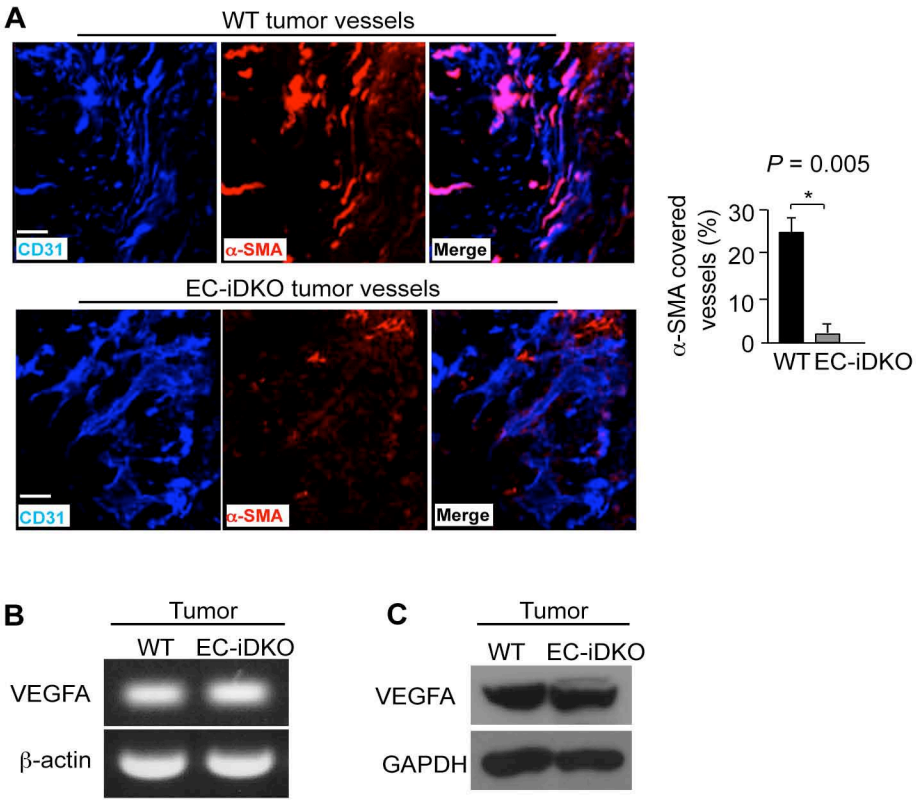
12. Greenberg, N.M., DeMayo, F., Finegold, M.J., Medina, D., Tilley, W.D., Aspinall, J.O., Cunha, G.R., Donjacour, A.A., Matusik, R.J., Rosen, J.M. 1995. Prostate cancer in a transgenic mouse. *Proc Natl Acad Sci U S A* 92(8):3439-3443.


A
Exon2
Exon3
Epn1+ allele
Targeting construct
Neo
Targeted Epn1 allele
Breeding with Flp mice to delete Frt-flanked Neo in vivo
Epn1fl allele
= LoxP
= Frt
1 kb
B
Tamoxifen
VEcad
Cre
ERT2
Epn1fl allele
Cre-mediated deletion
Epn1- allele
Epn1fl/fl;Epn2-/-
VEcad-ERT2Cre
Epn1fl/fl;Epn2-/-;VEcad-ERT2Cre
(EC-iDKO)
C
VEcad
Cre
Epn1fl allele
Cre-mediated deletion
Epn1- allele
Epn1fl/fl;Epn2-/-
VEcad-Cre
Epn1fl/fl;Epn2-/-;VEcad-Cre
EC-DKO
D
Genotype:
fl/fl Cre-
fl/fl Cre+
+/+ Cre-
+/fl Cre+
Epn1+
Epn1fl
Cre
1000
1000
500
E
WT
EC-iDKO
Epsin 1
Tubulin
F
WT
EC-DKO
Epsin 1
GAPDH
G
NICD Tg
Rosa26
Stop
NICD
Cre-mediated activation
Rosa26
NICD
= LoxP
EC-iDKO
NICD Tg
Tamoxifen
EC-iDKO:NICD
H
WT
EC-iDKO
EC-iDKO:NICD
Tamoxifen
+
+
+
NICD
Epsin 1
GAPDH

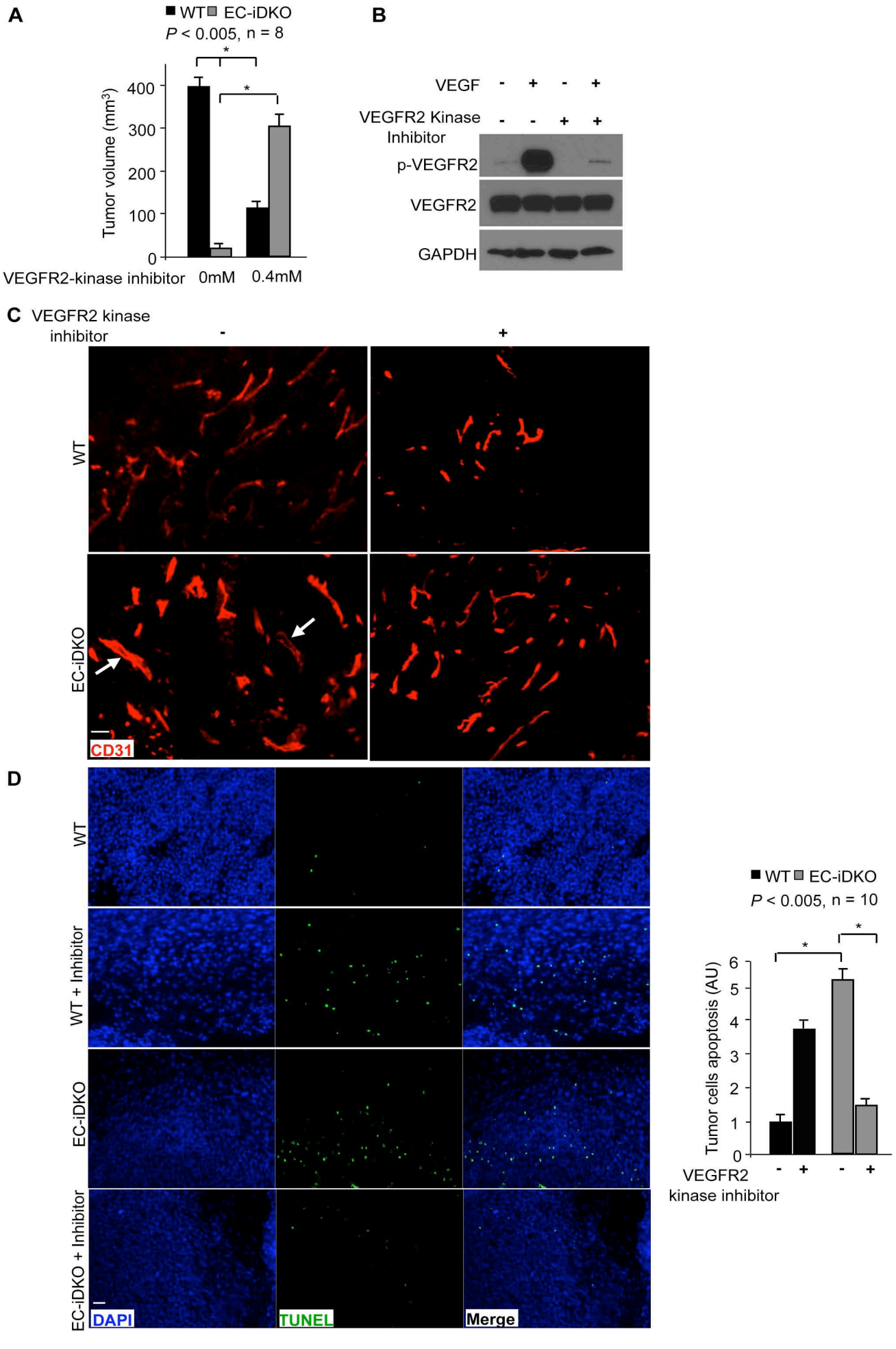
Supplemental Figure 1. Generation of conditional *Epn1flox/flox* (*Epn1*$^{fl/fl}$), EC-DKO, EC-iDKO or EC-iDKO:NICD mice. (**A**) Diagram shows homologous recombination of the floxed gene-targeting vector at the *Epn1* locus. (**B**) Strategy to generate tamoxifen inducible endothelial cell-specific DKO mice (EC-iDKO) by crossing *Epn1*$^{fl/fl}$*/Epn2*$^{-/-}$ mice with *VEcad-ER*^{T2} *Cre* deleter mice, which specifically express Cre in endothelial cells upon tamoxifen administration. (**C**) Strategy to generate endothelial cell-specific DKO mice (EC-DKO) by crossing *Epn1*$^{fl/fl}$*/Epn2*$^{-/-}$ mice with constitutive *VEcad-Cre* deleter mice, which specifically express Cre in endothelial cells. (**D**) PCR analysis of genomic DNA isolated from mice tails. Genotypes for *Epn1* or *VEcad-ER*^{T2} *Cre* are indicated. (**E**) Lysates from endothelial cells isolated from WT and EC-iDKO mice treated with tamoxifen are analyzed by western blotting for epsin 1 deletion. (**F**) Lysates from endothelial cells isolated from WT and EC-DKO mice are analyzed by western blotting for epsin 1 deletion. (**G**) Strategy to generate EC-iDKO with NICD transgene by crossing *Epn1*$^{fl/fl}$*/Epn2*$^{-/-}$*/VEcad-ER*^{T2} *Cre* mice with NICD transgenic mice (NICD Tg). Induction of *VEcad-ER*^{T2} *Cre* by tamoxifen administration specifically inactivates the epsin 1 gene and at the same time activates Notch by turning on the expression of NICD in endothelial cells. (**H**) Lysates from endothelial cells isolated from WT, EC-iDKO or EC-iDKO:NICD mice treated with tamoxifen were analyzed by western blotting for epsin 1 deletion and NICD expression.


A
LLC tumor
WT
EC-iDKO
LLC tumor
WT EC-iDKO
METRIC 1 cm 2
B
Melanoma tumor
WT
EC-iDKO
Melanoma tumor
WT EC-iDKO
cm
C
LLC tumor
WT
EC-DKO
D
WT Glioma
EC-iDKO Glioma
E
WT
colorectal tumor
WT
EC-iDKO
Colon
Rectum
F
WT prostate tumor (36 wk)
EC-iDKO prostate tumor (36 wk)

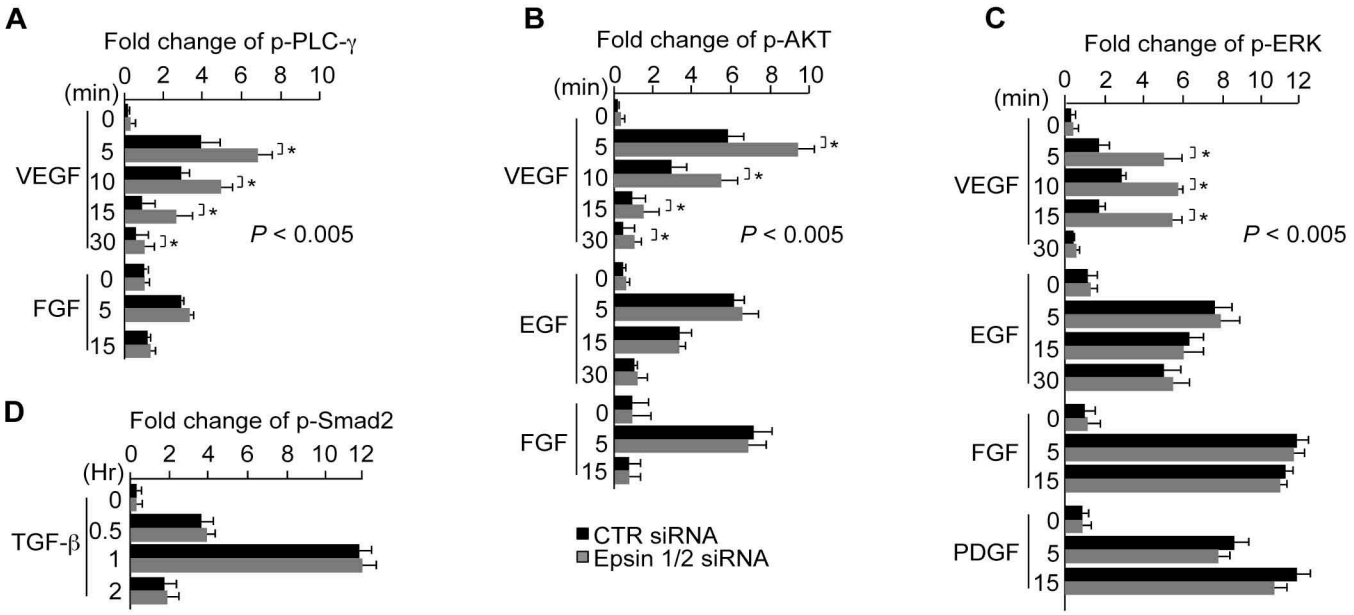
Supplemental Figure 2. Reduced tumor growth in the absence of endothelial epsins 1 and 2. (**A** and **B**) Representative WT or EC-iDKO mice bearing LLC tumors (**A**) or Melanoma tumors (**B**) at 18 days post inoculation of tumor cells. Dotted lines indicate tumors. (**C**) Representative WT or EC-DKO mice bearing LLC tumors at 18 days post inoculation of tumor cells. (**D**) Representative mouse brain *T2*-weighted images for WT and EC-iDKO gliomas, 24 days following GL261 cells injection. Images were acquired with a slice thickness of 0.5 mm and a FOV of 2 × 2 cm2, for an approximate in-plane resolution of 80 μm. Dashed yellow lines mark tumors. (**E**) Longitudinally cut colon and rectum regions of WT and EC-iDKO mice challenged with AOM and DSS. Mice were sacrificed after 18 weeks following treatment. (**F**) Male urogenital systems of TRAMP-WT and TRAMP-EC-iDKO mice show prostate tumors. Blue dotted line indicates tumor in prostates. Mice were sacrificed at 36 weeks.



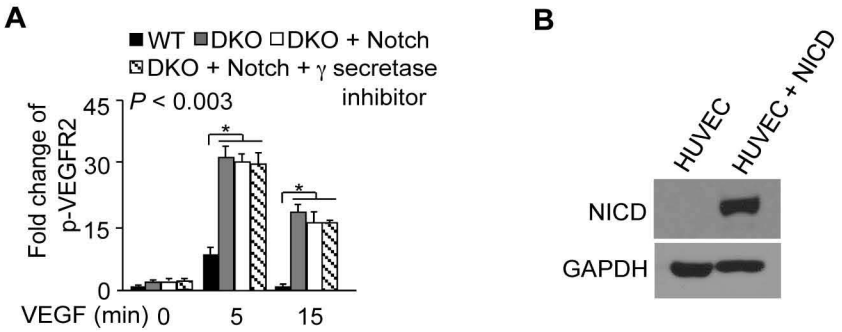
Supplemental Figure 3. Loss of endothelial epsins 1 and 2 results in reduced vascular smooth muscle cell coverage. (**A**) Reduced mural cell coverage revealed by CD31 and α-SMA immunostaining of LLC tumor vessels due to loss of endothelial epsins 1 and 2. Quantification of α-SMA coverage is shown on right. (**B**) RT-PCR showing VEGF-A expression in WT or EC-iDKO tumors. (**C**) Lysates of WT and EC-iDKO tumors were analyzed by western blotting with anti-VEGF-A antibodies. n = 10 for quantifications of (**A**). Scale bar: 50 μm (**A**).


A
WT EC-iDKO
P < 0.005, n = 8
Tumor volume (mm3)
400
300
200
100
0
VEGFR2-kinase inhibitor 0mM 0.4mM
B
VEGF - + - +
VEGFR2 Kinase Inhibitor - - + +
p-VEGFR2
VEGFR2
GAPDH
C
VEGFR2 kinase inhibitor - +
WT
EC-iDKO
CD31
D
WT
WT + Inhibitor
EC-iDKO
EC-iDKO + Inhibitor
DAPI
TUNEL
Merge
WT EC-iDKO
P < 0.005, n = 10
Tumor cells apoptosis (AU)
6
5
4
3
2
1
0
VEGFR2 kinase inhibitor - + - +

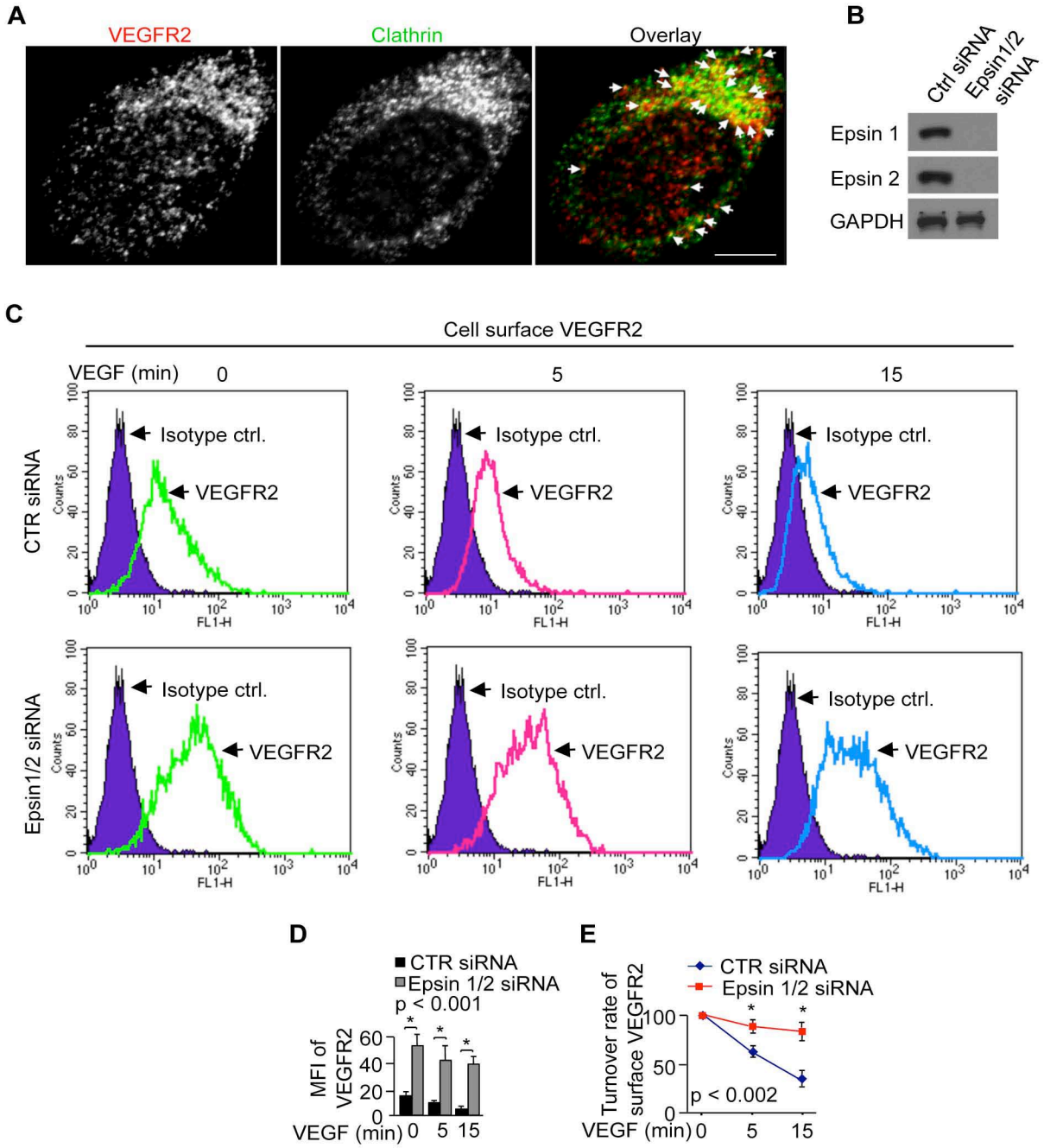
Supplemental Figure 4. VEGFR2 kinase inhibitor reduces tumor size in WT but not in EC-iDKO mice. (**A**) WT and EC-iDKO mice were injected with LLC tumor cells, followed by intravenous injection of VEGFR2 kinase inhibitor every other day with indicated concentrations. Tumor volume measured at 13 days of post inoculation of LLC is shown. (**B**) HUVECs were preincubated with either DMSO or 4 μm VEGFR2 kinase inhibitor for 3 h in DMEM and stimulated with 50 ng/ml of VEGF-A for 5 min. Cell lysates were western blotted using the indicated antibodies. (**C**) Frozen sections of LLC tumors from WT and EC-iDKO mice intravenously injected with DMSO or VEGFR2-Kinase inhibitor were immunostained with CD31. Arrows indicate some of the enlarged tumor vessels. (**D**) Frozen sections of LLC tumors of WT and EC-iDKO mice as in (**C**) were stained for apoptotic cells using TUNEL kit. Quantification of apoptotic cells is shown on right. Scale bars: 50 μm (**C** and **D**).



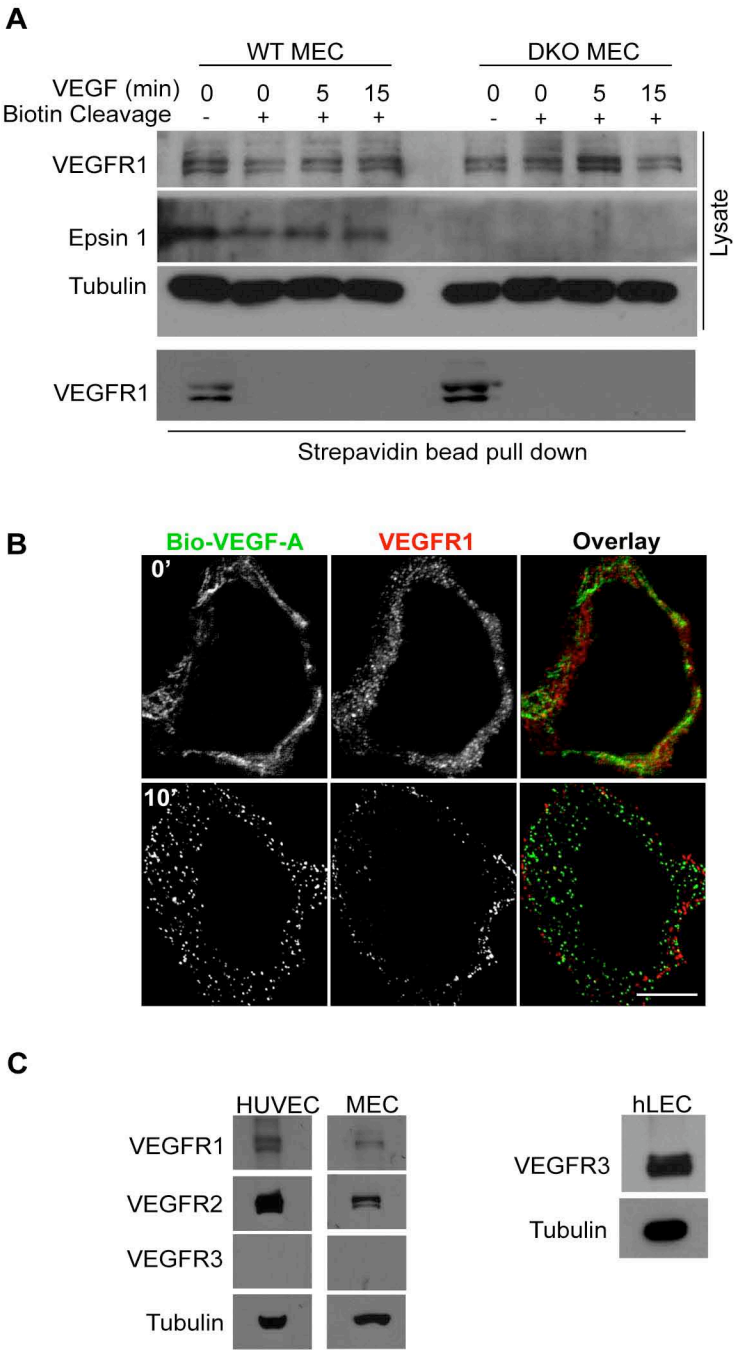
Supplemental Figure 5. Endothelial epsins 1 and 2 regulate VEGFR2 but not other angiogenic receptors signaling. (**A**-**D**) HUVECs transfected with either control or epsins 1 and 2 siRNAs were stimulated with VEGF-A (50 ng/ml) (**A**-**C**), FGF (25 ng/ml) (**A**-**C**), PDGF (25 ng/ml) (**C**), EGF (30 ng/ml) (**B** and **C**) and TGF-β (5 ng/ml) (**D**) and signaling was analyzed by western blotting with antibodies indicated as shown in figure 3. VEGF but not FGF, PDGF, EGF or TGF-β signaling is increased in the absence of epsins 1 and 2. Quantification was performed using NIH ImageJ software. n > 5 per group in all panels.



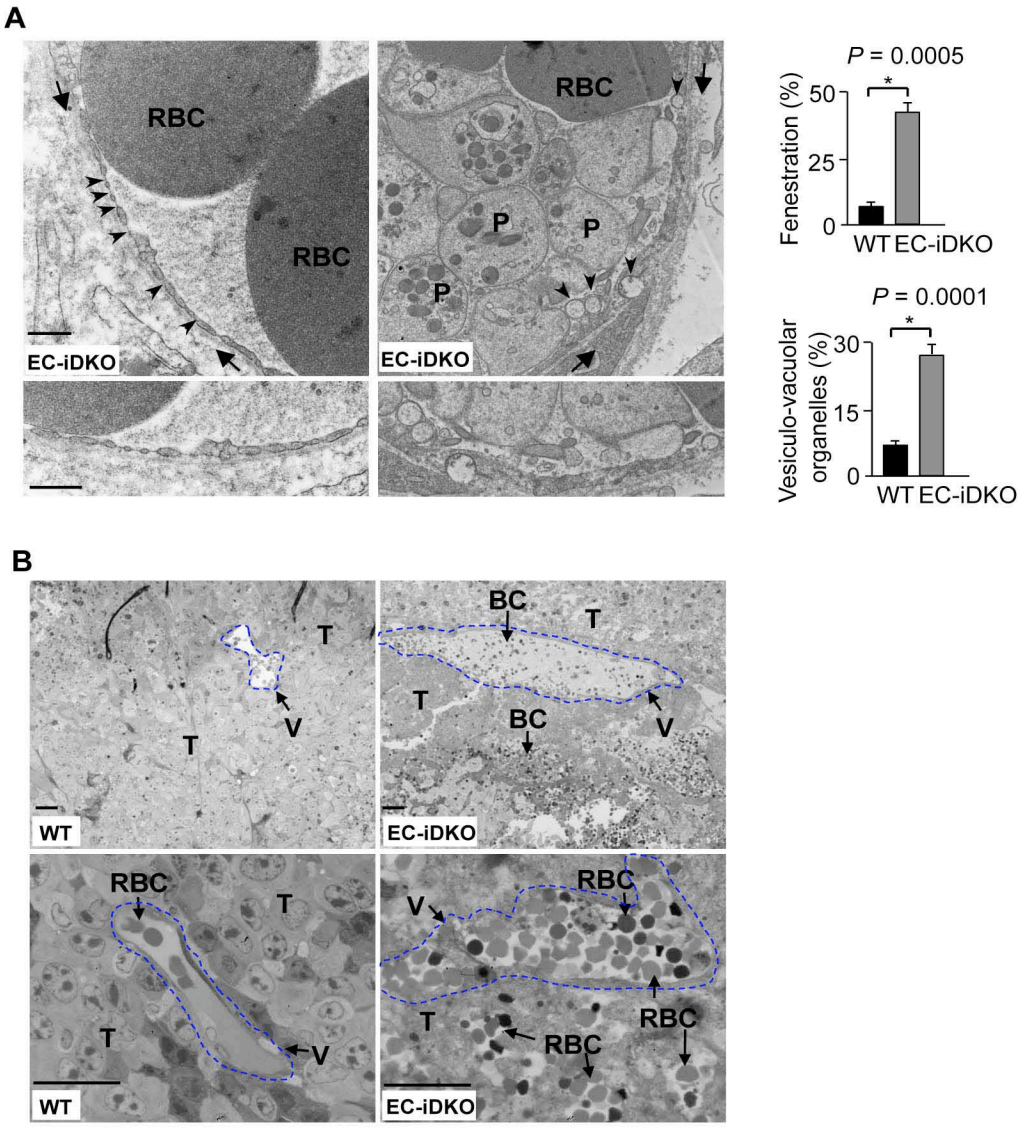
Supplemental Figure 6. Restoring Notch function fails to rescue elevated VEGFR2 signaling caused by loss of endothelial epsins 1 and 2. (**A**) DKO MECs were transfected with an empty vector or full-length Notch-myc for 24 h in the presence or absence of γ-secretase inhibitor (10 μM) followed by stimulation with VEGF-A (50 ng/ml) and analyzed using western blotting. Quantification was performed using NIH ImageJ software. (**B**) HUVECs transfected with an empty vector or a NICD plasmid were western blotted to measure NICD expression using an anti-NICD antibody. n > 5 per group in all panels.



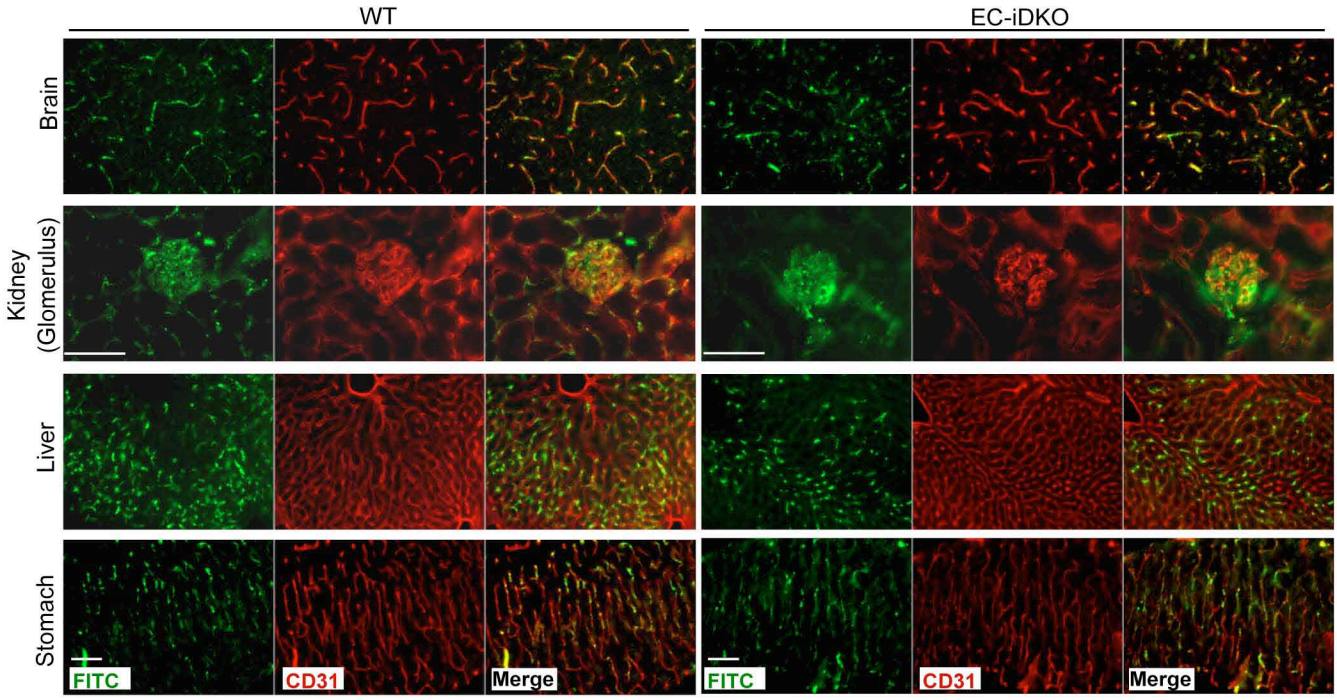
Supplemental Figure 7. VEGF induces colocalization of VEGFR2 with clathrin and accumulation of cell surface VEGFR2 in epsin-deficient endothelial cells upon VEGF stimulation. (**A**) HUVECs were incubated with 50 ng/ml of VEGF-A for 2 min and processed for immunofluorescence with VEGFR2 and clathrin antibodies. Colocalization of VEGFR2 with clathrin is seen by confocal microscopy. Arrows indicate colocalization of two proteins. (**B**) HUVECs transfected with either control or epsin 1 and epsin 2 siRNAs and cell lysates were used to western blot with indicated antibodies. (**C**-**D**) HUVECs were transfected with either control or epsin 1 and epsin 2 siRNAs and incubated with VEGF-A (50 ng/ml) for the time points as indicated and cell surface expression of VEGFR2 was measured by FACS analysis (**C**) and mean fluorescence intensity (MFI) was quantified (**D**). (**E**) Turnover rate of cell surface VEGFR2 after VEGF treatment was plotted based on the expression of cell surface VEGFR2 normalized against non-treated samples. n > 5 per group in all panels. Scale bar: 10 μm (**A**).



Supplemental Figure 8. VEGF does not induce endocytosis of VEGFR1. (**A**) WT or DKO MECs were incubated with VEGF-A (50 ng/ml) for 0, 5 and 15 min. Internalized VEGFR1 was analyzed as in figure 6***B***. Note that no internalization of VEGFR1 was observed in either WT or DKO MECs. (**B**) MECs were incubated with 100 ng/ml of biotinylated VEGF-A/Streptavidin Alexa Fluor 488 at 4 ºC for 30 min, shifted to 37 ºC for 0 to 10 min and processed for immunofluorescence using VEGFR1 antibody. (**C**) Lysates from HUVECs, MECs or human lymphatic endothelial cells (hLECs) were western blotted using antibodies against VEGFR1, VEGFR2, VEGFR3 and tubulin. No expression of VEGFR3 was detected in either HUVECs or MECs. However, high levels of VEGFR3 are detected in hLECs. Scale bar: 10 μm (**B**).



Supplemental Figure 9. Increased tumor vessel permeability due to loss of endothelial epsins 1 and 2. (**A**) Transmission EM micrographs of EC-iDKO tumor vessels showing augmented fenestration (arrowheads in left panel) and enlarged vesiculo-vacuolar organelles (arrowheads in right panel). Areas between two arrows are magnified below. Quantification of fenestration and vesiculo-vacuolar organelles is shown at right. (**B**) Light microscopy micrographs of semi-thin sections of WT or EC-iDKO tumors showing increased permeability in enlarged tumor vessels, massive leakage of blood cells and necrotic tumor areas in EC-iDKO tumor. Blue dotted line outlines tumor vessels. T: tumor cells, V: vessel, BC: blood cells, RBC: red blood cells. Scale bars: 0.5 μm (**A**), 100 μm (**B**).



Supplemental Figure 10. Loss of endothelial epsins does not produce changes in morphology and permeability of blood vessels in major organs. Frozen sections of brain, kidney (glomerulus), liver and stomach of WT and EC-iDKO mice intravenously perfused with lysine-fixable FITC-dextran were immunostained with CD31 (CD31, red, FITC-dextran, green). Scale bar: 50 μm.