Supplemental Material

Selective Targeting of a Novel Epsin-VEGFR2 Interaction Promotes VEGF-mediated Angiogenesis

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Online-Only Supplement of Materials and Methods

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Antibodies and reagents: Unless specified otherwise, common laboratory chemicals and reagents were from either Sigma-Aldrich or Fisher Scientific. Media and additives for cell culture were from Gibco, Mediatech or Biowest. Mouse anti-GAPDH, anti-c-Cbl, anti-Transferrin Receptor (CD71), EEA 1 and goat anti-epsin 1 antibodies were from Santa Cruz (sc-166545, sc-1651, sc-32272, sc-6145 and sc-8673). Rabbit anti-epsin 1 and 2 antibodies were previously described[1, 2]. Rabbit anti-epsin 3 antibody was from Abcam (ab 185577). Rat anti-CD31 antibody was from BD Pharmingen (550274). Rabbit anti-VEGFR2, rabbit anti-phospho-VEGFR2 (pY1175), rabbit anti-AKT, rabbit anti-phospho-Akt (pS473), mouse anti-ERK (p44/42 MAPK), and mouse anti-phospho-ERK (p-p44/42 MAPK; pT202/pY204) antibodies were from Cell Signaling Technology (2479, 2478, 9272s, 9271s, 4695s, 9106s). Mouse anti-HA antibody was from Covance (MMS-101-P). Mouse anti-ubiquitin antibody was from Cytoskeleton (AUB01). Mouse anti-tubulin antibody (12G10) was from Developmental Studies Hybridoma Bank. Rabbit anti-epsin 1 & 2 were obtained as previously described[2-4] Biotinylated isolectin B4 was from Vector Laboratories (B-1205). Secondary antibodies and fluorescently labeled streptavidin were from Invitrogen and Jackson ImmunoResearch Laboratories. VEGF-A was from R&D systems (293-VE/CF). EdU (A10044) and Alexa Fluor 594 Azide (C10339) were from Invitrogen. Matrigel was from BD Biosciences (354230). Peptides were purchased from China Peptides. Peptide sequences are as listed: AP-Scrambled Peptide – RQIKIWFQNRRMKWKKQLSQEKAESMELALEGEL; AP-UIM Peptide – RQIKIWFQNRRMKWKKSGEEELQLQLALAMSKEE; AP-UIM^{E3,4,5A} Peptide – RQIKIWFQNRRMKWKKSGAAALQLQLALAMSKEE.

Conditional endothelial cell-specific epsin deficient mouse generation (EC-iDKO): Epn2$^{-/-}$ mice were obtained as described[5]. Conditional Epn1$^{fl/fl}$ mice were obtained as described (**Online-Only Figure IA**)[1]. Epn1$^{fl/fl}$ mice were mated with Epn2$^{-/-}$ mice to generate Epn1$^{fl/fl}$; Epn2$^{-/-}$ mice. Tamoxifen-inducible endothelial cell-specific DKO mice (EC-iDKO) were obtained by crossing Epn1$^{fl/fl}$; Epn2$^{-/-}$ mice with iCDH5-ER^{T2} Cre deleter mice on C57BL/6J background (**Online-Only Figure IB**)[1, 2, 6]. To induce early postnatal deletion, pups were administered 50 μg of 4-hydroxytamoxifen by intraperitoneal injection once daily on postnatal day (P) 2, P3, P4[2, 6, 7]. All mice were bred on C57BL/6J background[1, 2].

Structural modeling: *(1) Molecular Dynamics Simulation:* The three-dimensional (3D) structures of epsin UIM was predicted by PEP-FOLD (http://bioserv.rpbs.univ-paris-diderot.fr/PEP-FOLD)[8, 9]. In this method, models were clustered by the sOPEP energy value (the coarse grained energy), and then ranked based on their cluster scores. Among the top 5 ranks, the first model of epsin UIM with best score was selected for docking experiment (**Online-Only Figure IVA**). *(2) Molecular Docking Procedure:* Docking experiments were performed using ClusPro program[10, 11]. The 3D crystal structures of ubiquitin (PDB ID: 1UBQ), VEGFR2 kinase domain (PDB ID: 3U6J) and epsin 1 ENTH domain (PDB ID: 1H0A) were obtained from PDB Data Bank. The apo-open form of crystal structure of VEGFR2 kinase domain was obtained by removing pyrazolone, the co-crystallized VEGFR2 kinase inhibitor, from its binding site. Simulated models of epsin UIM or established ubiquitin structure were then docked into the VEGFR2 kinase domain independently to generate predicted binding models. Models with the highest scores and good topologies were selected as candidate structures for the proposed models of interaction: epsin UIM:VEGFR2 KD or ubiquitin:VEGFR2 KD. Epsin 1 ENTH was further docked into ubiquitin:VEGFR2 KD to generate the model of Epsin:Ubiquitin:VEGFR2 KD super complex.

Cell culture: HEK 293T cells (CRL-11268) and MCF-7 cells (HTB-22) were purchased from ATCC and cultured at 37 °C with 5% CO_2 in DMEM (Mediatech; 10-013-CM) supplemented with 10% fetal bovine serum (FBS; Biowest; 501520H1). Human umbilical vein endothelial cells (HUVEC) were purchased from Lonza, cultured according to the manufacturer's protocol and used between passages 2 and 5. Primary mouse endothelial cells (MEC) were isolated from mouse lungs or brains and cultured as described previously[12]. In short, for MECs isolation from the lungs, 4-6 mouse neonatal pups (7–14 days old) were used. Initially a 25-µl intramuscular injection of heparin (1,000 USP U/ml) was injected to each mouse. After 10 minutes, the mice were anesthetized, followed by exposure of the thoracic cavity. To flush blood cells from lungs, 5 ml of ice cold DMEM was then injected via the right ventricle. 1ml of collagenase Type 1(1mg/ml; Gibco Laboratories) was then quickly instilled through the trachea into the lungs, and the trachea was then tied off. The lungs (without the heart) were subsequently removed and minced finely. Then incubated with 2 mg/ml collagenase Type I (Gibco Laboratories), 5 mg/ml Dispase II (neutral protease, Grade II) (Roche) in DMEM (Sigma-Aldrich) in a 50-ml tube for 30 min in a 37°C water bath. Every 5–8 min during this incubation, the tube was gently agitated for a few seconds. After the 30-min incubation, the resulting tissue/cell suspension was filtered through a 40-µm strainer. The filter was washed with 5 ml of 1× PBS. The filtered cell suspension (~35 ml) was centrifuged for 15 min at 1000 rpm. The cell pellet was washed once with complete DMEM, resuspended in warm DMEM supplemented with 20% FBS, 1% Antibiotic Antimycotic Solution (Corning) and 1 ng/ml bFGF (Roche), and plated into a gelatin-coated T-75 tissue culture flask. The following day, the cells from each lung were removed by trypsin and pooled together into one suspension for the sorting. The lung cell suspension was then subjected to magnetic beads sorting using anti-CD31 antibody. The sorted cells were resuspended in culture media described above and used for experiments. For MECs isolation from the brains, meninges from 14 days old mice were carefully removed from the forebrain and gray matter was minced and then digested with 2 mg/ml collagenase Type I (Gibco Laboratories), 5 mg/ml Dispase II (neutral protease, Grade II) (Roche) in DMEM (Sigma-Aldrich) containing 50 mg/ml gentamicin and 2 mM glutamine in 37°C water bath for 2 hours. The cell pellet was separated by centrifugation in 20% BSA–DMEM (1,000 × g, 20 minutes). The microvessels obtained in the pellet were further digested with 2mg/ml collagenase-dispase (Roche) in DMEM for 1.5 hours at 37°C, pelleted, washed with DMEM, and plated in DMEM supplemented with 20% FBS and 1 ng/ml bFGF (Roche). Freshly isolated primary MECs were used for all experiments without any further passages. We saw no difference in results between MECs isolated from lungs and brains.

MECs isolated from WT or $Flk1^{fl/fl}$; iCDH5-ER^{T2} Cre (VEGFR2 $^{fl/fl}$/iCDH5-ER^{T2} Cre) mice were treated with 5 µM of 4 hydroxytamoxifen (Sigma) dissolved in ethanol for two days at 37^0C followed by incubation for additional two days without 4-hydroxytamoxifen. Deletion of VEGFR2 was confirmed by western blot using VEGFR2 antibody. Freshly isolated primary MECs were used for all experiments without any further passages.

Plasmids and transfection: Mammalian expression plasmids for epsin 1 and VEGFR2 were described previously[12, 13]. Mutagenesis was performed using Quick Change II Site-Directed Mutagenesis Kit (Agilent Technologies; 200524). Mutagenesis primers and/or expression plasmids are available upon request. HEK 293T cells were transfected with indicated plasmids

using Lipofectamine 2000 (Invitrogen; 11668-019) according to the manufacture's instruction. MECs or HUVECs were transfected using an Amaxa Nucleofector device (Lonza) according to the manufacture's protocol. At least 1-2 folds increase observed in native vs mutant epsin expression after transfection.

RNA interference: HUVECs were transfected by Oligofectamine or RNAiMAX according to the manufacturer's instructions (Invitrogen; 12252-011, 13778-150) with siRNA duplexes. Human c-Cbl siRNA and respective scrambled control siRNA were obtained based on what described in previously published paper[14]. Human VEGFR2 siRNA (Sense: GGGAAUACCCUUCUUCGAATT; Anti-Sense: UUCGAAGAAGGGUAUUCCCTT), and respective scrambled control siRNA were purchased from Invitrogen. Cells were processed for biochemical immunoprecipitation or immunofluorescence assays 48-72 hrs after transfection as previously described[1].

qRT-PCR: Total RNA from HUVECs were extracted by using commercially available kits (Qiagen, Valencia, CA, USA) followed by DNase I treatment. The extracted RNA was then reverse-transcribed into cDNA by using Oligo (dT) 20 Primers (Invitrogen-18418-020) according to the manufacturer's protocol. Quantitative real-time PCR was performed using the StepOnePlus Real-Time PCR detection system (Applied Biosystems, Foster City, CA, USA) and Platinum SYBR Green qPCR super Mix (Invitrogen). The amplification cycles consisted of initial heating at 95 °C for 10 min, followed by 40 cycles each consisting of 15 s of heating at 95 °C, 1 minute of annealing at 60 °C, and 45 s of extension at 72 °C. The average threshold cycles (Ct) of samples were used to compare the relative abundance of the mRNA. The Ct of β-actin RNA was used to normalize all samples. Each sample was run in triplicates for analysis. Primer sequences for PCR were described as follows: epsin 1 (antisense primer, 5'-GTGAGGTCGGCAATCTCTGA-3', and the sense primer, 5'-AGATCAAGGTTCGAGAGGCCA-3'), epsin 2 (antisense primer, 5'-GGGACAGATTGTACGGTGGC-3', and the sense primer, 5'-TCTCTCCCACAGCAGACTACG-3') and epsin 3 (antisense primer, 5'-GGAGTTTGTGGTGGGGAGAG', and the sense primer, 5'-ACATCCCAGGTTTTAGGCCG-3') and β-actin (antisense primer, 5'-AGCACTGTGTTGGCGTACAG-3', and the sense primer, 5'-AGAGCTACGAGCTGCCTGAC-3').

Immunoprecipitation and Western blot analyses: Transfected HEK 293T cells, MECs or HUVECs were serum-starved for 16 hrs in DMEM, then stimulated with 50 ng/mL VEGF-A as specified. Cells were then 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 pre-cleared with appropriate species IgG and protein G Sepharose beads for 2 hrs with rotation at 4 °C, followed by incubation with indicated antibody for 4 hrs with rotation at 4 °C. Precipitated proteins were eluted from beads using 2X Laemmli buffer, resolved by SDS-PAGE (7.5% acrylamide), electroblotted to polyvinylidene difluoride membrane and blocked with 3% skim milk (weight per volume) according to standard procedures. Standard Western blotting procedures using specified antibodies, enhanced chemiluminescence and autoradiography were used to detect immunoreactive proteins[1, 15]. Band intensities were quantified using NIH Image J software.

Mouse stomach lysate was prepared using a previously described method[16].

His-tagged VEGFR2 pull-down of Ubiquitin: Recombinant human VEGFR2 kinase domain (amino acids 789-1356) fused with six his-tag repeats was purchased from Life Technologies™. Mono-ubiquitin and poly-ubiquitin (linear tetra-Ub) were purchased from BostonBiochem ®. To

pull down mono-ubiquitin or poly-ubiquitin by his-tagged VEGFR2, Ni-NTA beads were incubated with equal amounts of VEGFR2 for half an hour at 4^0C, then mixed with a predetermined amount of mono-ubiquitin or poly-ubiquitin (mono-ubiquitin is around 80 fold higher than poly-ubiquitin). The mixtures were further incubated at 4^0C with rotation overnight. After centrifugation, the supernatant were removed. The beads were washed three times with RIPA buffer, then denatured in 2X Laemmli buffer and processed for Western blotting.

His-tagged di-ubiquitin pull-down of VEGFR2: Di-ubiquitin construct was kindly provided by Dr. Hao Wu, Department of Cellular and Molecular Medicine at Boston Children's Hospital. The overexpression and purification of di-ubiquitin were described previously[17]. The purified di-ubiquitin was first incubated with Ni-NTA beads, and then equal volume of beads was added to each cell lysate sample (wild type or mutant VEGFR2 expressing HEK293T cells) of equal total protein. The mixtures were incubated at 4^0C with rotation overnight. After centrifugation, the supernatant were removed. The beads were washed three times with RIPA buffer, denatured in 2X Laemmli buffer, and processed for Western blotting.

Immunofluorescence staining of VEGFR2: HUVECs were transfected initially with VEGFR2-targeted siRNA, and then with wild type or mutant VEGFR2 constructs at 48 hr as described above. Twenty-four hours later, cells were serum starved overnight and incubated with 200 ng/ml of VEGF-A for 0 or 10 min at 37 °C. Cells were fixed with 4% formaldehyde in PBS, permeabilized, incubated with primary rabbit anti-VEGFR2 and goat polyclonal EEA 1 antibodies overnight, then incubation with respective donkey anti-rabbit ALEXA 488 and donkey anti goat ALEXA 594 conjugated secondary antibody for visualization. Cells were then thoroughly washed, cover slips mounted, and immunofluorescence images obtained using an Olympus IX81 Spinning Disc Confocal Microscope with an Olympus plan Apo Chromat 60x objective and Hamamatsu Orca-R2 Monochrome Digital Camera C1D600.

Biotinylation assay of cell surface VEGFR2 and Transferrin Receptor: Surface VEGFR2 and Transferrin Receptor levels were determined using a previously described surface biotinylation assay[1]. Briefly, HUVECs were transfected initially with VEGFR2-targeted siRNA, and then with wild type or mutant VEGFR2 constructs at 48 hr as described above. Twenty-four hours later, cells were serum starved overnight and incubated with 50 ng/ml of VEGF-A for 0 or 10 min at 37 °C to allow internalization of cell surface VEGFR2. At the end point, cells were placed on a rocking incubator at 4 °C with 1 mM EZ-Link Sulfo-NHS-LC-Biotin for 30 min, washed with 50 mM glycine to quench the biotinylation reaction, and lysed with RIPA buffer. Cleared lysates were processed for streptavidin bead pull down. Cell surface biotinylated VEGFR2 and Transferrin Receptor were visualized and quantified by Western blotting as described above.

***In vitro* EdU staining:** MECs were grown on 0.2% gelatin-coated coverslips until they reached 50% confluence. Cells were incubated with UIM or $UIM^{E3,4,5A}$ peptides at 12.5 μM for 16 hrs, then serum starved for 4-6 hrs in DMEM. Subsequently, EdU was added to culture medium at 10 μM and cultured for an additional 16 hrs[18]. After labeling, cells were washed with PBS, fixed in 4% paraformaldehyde and permeabilized in 0.1% Triton X-100. EdU incorporation was detected by incubating fixed cells with 100 μM fluorescent azide diluted in 100 mM Tris, 0.5 mM $CuSO_4$ and 50 mM ascorbic acid. Cells were counterstained with 0.2 μg/mL DAPI then processed for imaging as described above.

***In vitro* angiogenesis assays:** *(1) Monolayer endothelial cell scratch "wound" assay*: Monolayer endothelial cell scratch "wound" assays were performed as described[2, 12]. Briefly,

endothelial cells were cultured as described above with either UIM or UIM^{E3,4,5A} peptide (12.5 μM) for 16 hrs. Cells were subsequently starved in DMEM for 8 hrs, then subjected to "wound injury" with a plastic pipette tip. Cells were treated with fresh medium supplemented with or without 50 ng/mL VEGF-A and either UIM or UIM^{E3,4,5A} peptide (12.5 μM), and further cultured for 12 hrs. Quantification of wound distance at 12 hrs was performed using NIH Image J software. *(2) Endothelial cell network formation*: Endothelial cell network formation in Matrigel was performed as described[2, 12]. First, 0.3 mL of Matrigel was added to each well of a 24-well plate and incubated at 37°C for 1 hr. Endothelial cells were treated with UIM or UIM^{E3,4,5A} peptide (12.5 μM) for 16 hrs, then starved as described and plated on Matrigel (4 x 10^4 cells per well). After 30 min, fresh medium supplemented with or without 50 ng/mL VEGF-A and either UIM or UIM^{E3,4,5A} peptide (12.5 μM) was added and cells were incubated for an additional 16 hrs. Quantification of network formation at 16 hrs was performed using NIH ImageJ software.

Retina angiogenesis: C57BL/6J WT and EC-iDKO mice were administered UIM or UIM^{E3,4,5A} peptide on postnatal day 2 (P2), P3, P4 and P5 via intraperitoneal injection (20 μg/g body weight). Pups were euthanized at P6, then the retinas were harvested from the dissected eyeball and processed for immunofluorescence whole-mount staining using biotinylated isolectin B4. Briefly, retinas were fixed in 4% paraformaldehyde for 1 hour then blocked in phosphate buffered saline (PBS) solution containing 5% donkey serum and 0.3% Triton X-100 for 1 hr and incubated at 4 °C for 16 hrs with biotinylated isolectin B4 diluted in blocking buffer (20 μg/mL). Retinas were subsequently washed with PBS and incubated for 1 hr at room temperature with fluorescently labeled streptavidin (Alexa Fluor) (Invitrogen). Samples were washed; retinas were mounted onto Superfrost/Plus microscope slides (Fisher Scientific). Flat-mounted retinas were imaged at 5X magnification with AxioObserver.Z1 microscope (Zeiss) with a digital camera (AxioCam MRm; Zeiss). Individual images were merged to create one entire retinal image using the automated merge function (mosaiX; Zeiss) in the software (AxioVision 4.6.3.0; Zeiss). Vascular area was quantified using NIH Image J software. Quantification was determined by calculating isolectin B4-positive stained retinal area relative to total retinal area examined. Data is presented as relative vascular area.

***In vivo* Matrigel angiogenesis assay:** Matrigel plug assay was performed as described previously[2, 19]. Briefly, twelve-week-old C57BL/6J WT mice were subcutaneously injected with 400 μL of unpolymerized growth factor-reduced phenol-free Matrigel supplemented with either PBS or VEGF-A (50 ng/mL) and specified peptides (100 μg/mL) while under anesthesia. Mice were euthanized after 7 days and the plugs removed. Plugs were photographed, formalin-fixed, cryopreserved and processed for immunostaining with anti-CD31 antibody as described previously.

***In vivo* wound healing angiogenesis assay:** In vivo Wound Healing Assay was performed as described previously[2]. Under anesthesia, mice were wounded using a 4-mm-diameter full-thickness dorsal skin punch biopsy. Wounds were then photographed daily for 7 days after injury, and wound area was measured using NIH ImageJ software. On Day 7, the wounds were biopsied to include the margin of normal surrounding skin, fixed in cold 4% paraformaldehyde overnight and cryopreserved in Tissue-Tek OCT (Sakura). Cryosections were processed for immunofluorescence staining using anti-CD31 antibodies as described previously.

Animal study approval: All animal studies were performed in compliance with institutional guidelines and were approved by Institutional Animal Care and Use Committee (IACUC), Oklahoma Medical Research Foundation, Oklahoma City, OK and Boston Children's Hospital, Boston, MA.

Statistical analysis: Data was shown as mean + S.E.M. Data was 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. A *P* value of less than 0.05 was considered significant.

References:

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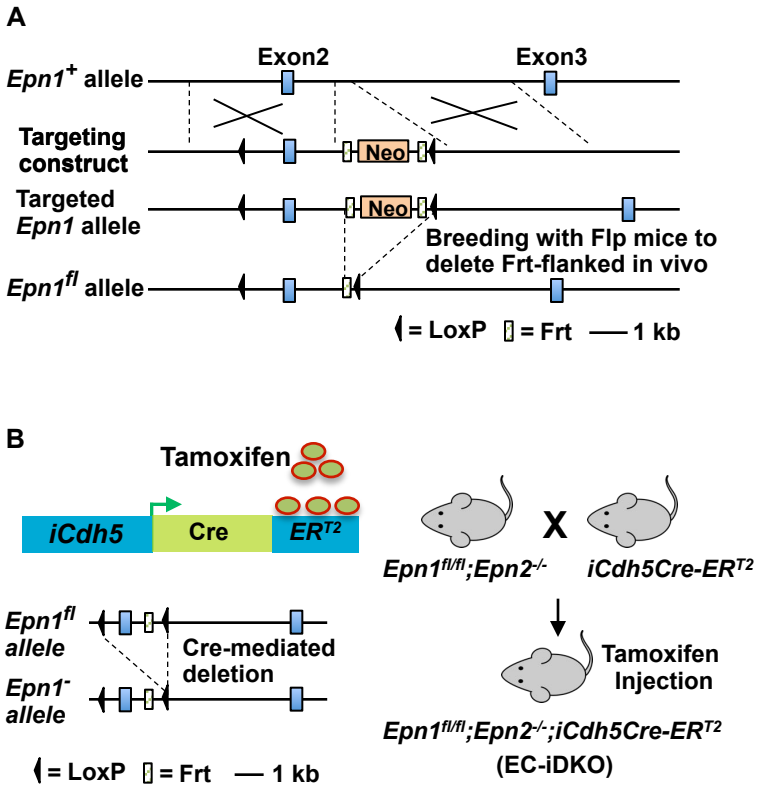
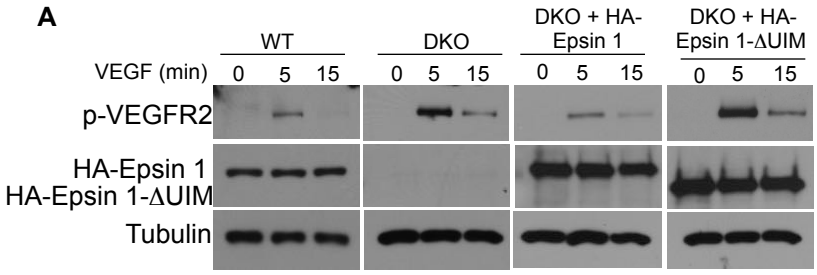
Online Figures:



Online Figure I. **Generation of EC-iDKO 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 epsins 1 and 2 double knockout (EC-iDKO) mice by crossing *Epn1*$^{fl/fl}$*; Epn2*$^{-/-}$ mice with *iCDH5 Cre deleter* mice.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WT
DKO
DKO + HA-Epsin 1
DKO + HA-Epsin 1-ΔUIM
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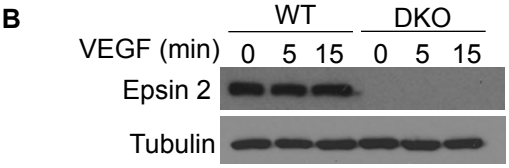
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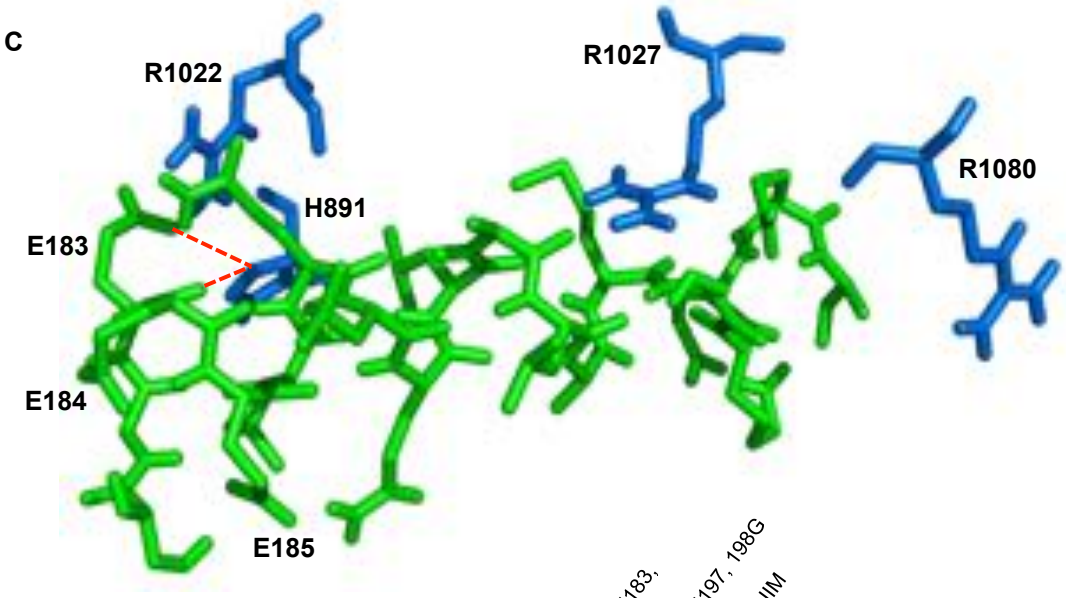
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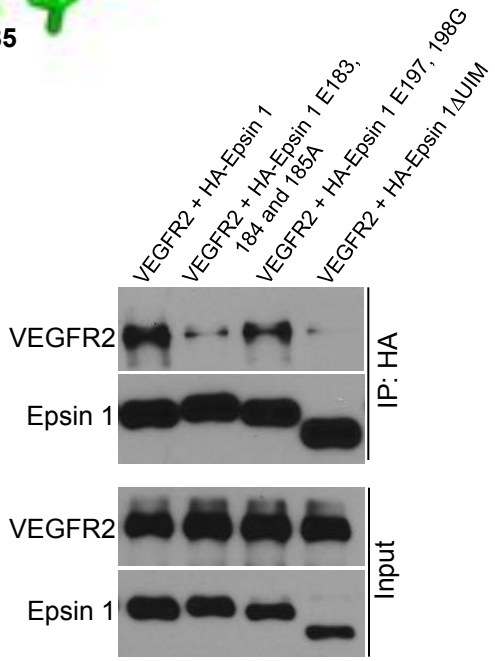
D
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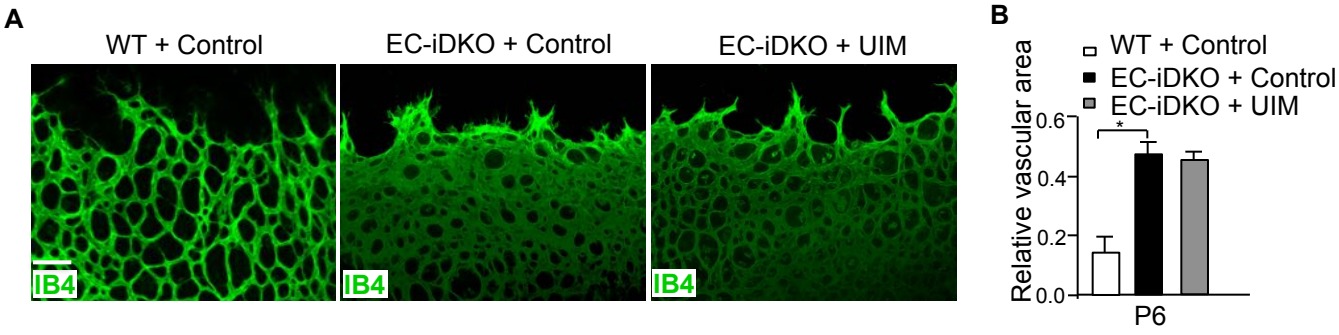
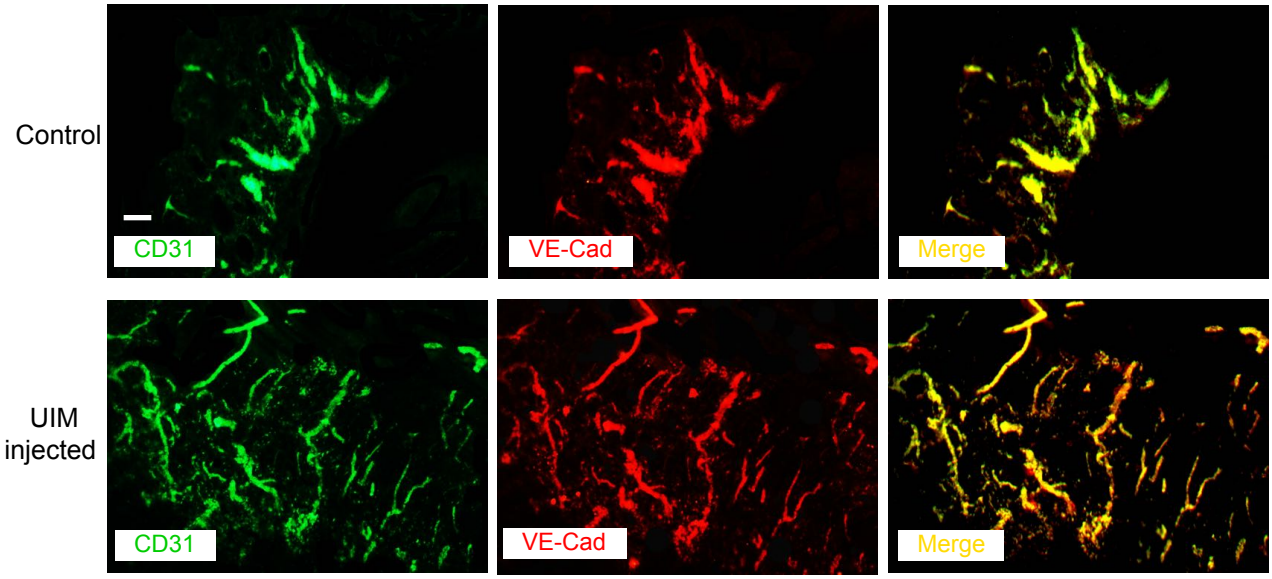
Epsin 1
IP: HA
VEGFR2
Epsin 1
Input

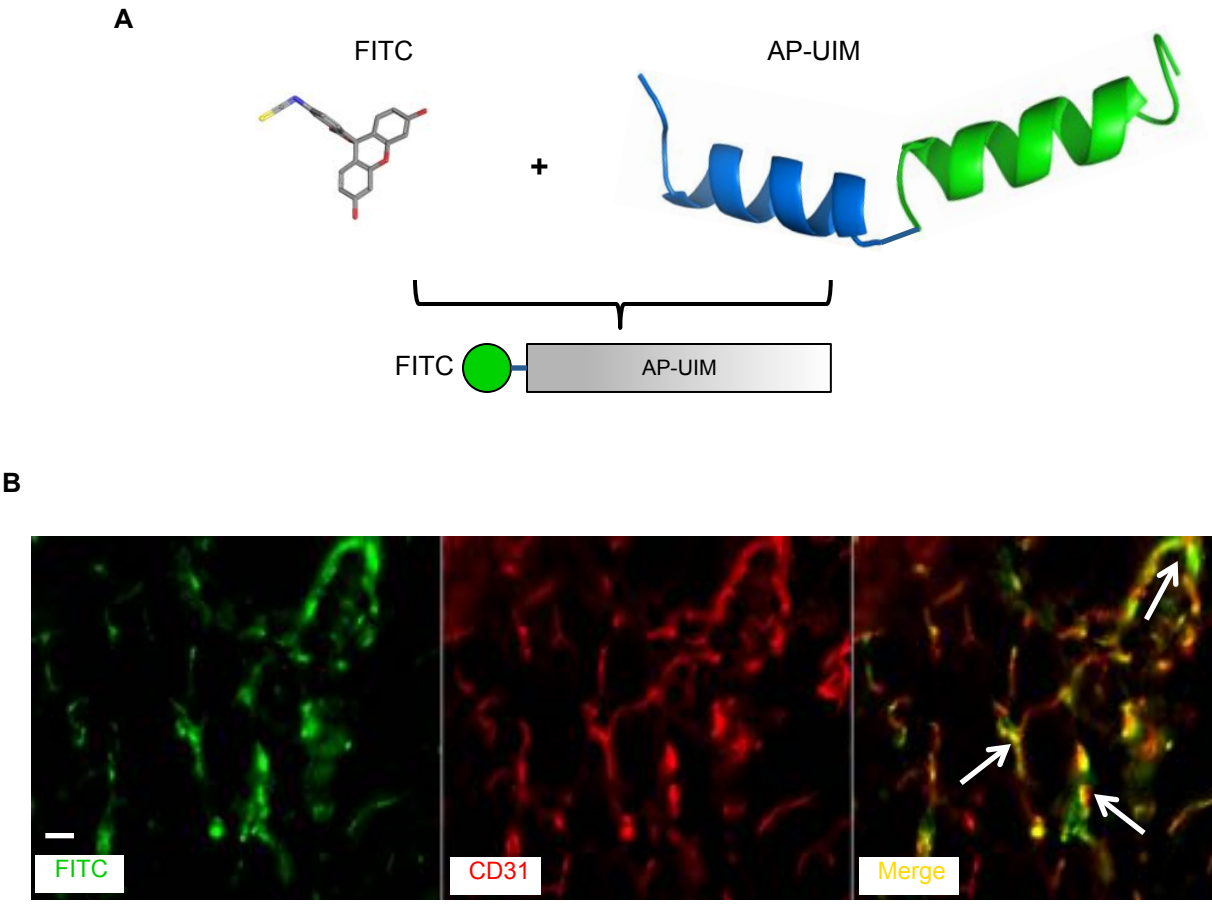
Online Figure II. Epsin binds VEGFR2 via critical interacting residues in epsin UIM and VEGFR2 Kinase domain (KD). **(A)** Western blot analysis of VEGF-induced VEGFR2 signaling in mouse endothelial cells (MEC) isolated from WT, epsins 1 and 2 knock out (DKO), and DKO cells transfected with either full-length HA-Epsin 1 or HA-Epsin 1 lacking UIM (HA-Epsin 1-ΔUIM). **(B)** Western blot analysis of epsin 2 in mouse endothelial cells (MEC) isolated from WT and epsins 1 and 2 knockout (DKO) cells. **(C)** Alternative orientation of the stick representation of epsin UIM (green) docked into the putative hairpin shaped-binding pocket of VEGFR2 KD (blue) shown in Figure 3C. VEGFR2 KD crystal structure (3U6J) was obtained from the Protein Data Bank. Stick representation for epsin UIM was predicted using PEP-FOLD. ClusPro and PyMol software were used for the docking; highest scoring model with good topologies is shown. **(D)** Western blot analysis of VEGFR2 co-immunoprecipitated by HA-specific antibodies in HEK 293T cells transfected with VEGFR2 and either HA-Epsin 1; HA-Epsin 1 E183A, E184A, E185A; HA-Epsin 1 E197G, 198G or HA-Epsin 1 ΔUIM. All representative Western blots were selected from n=3.



Online Figure III. Endothelial epsins are essential for proper postnatal retinal angiogenesis which is increased by the administration of UIM. **(A)** Representative images with higher magnification of whole-mount retinas isolated from WT and EC-iDKO P6 pups after intraperitoneal injection with control and UIM and immunofluorescently labeled with biotinylated-isolectin B4. **(A)** is representative of n=6. Respective quantification for **A** is shown in **B**. Scale bar in **A**: 50 µm. Error bars indicate the mean ± s.e.m. **P*<0.05.

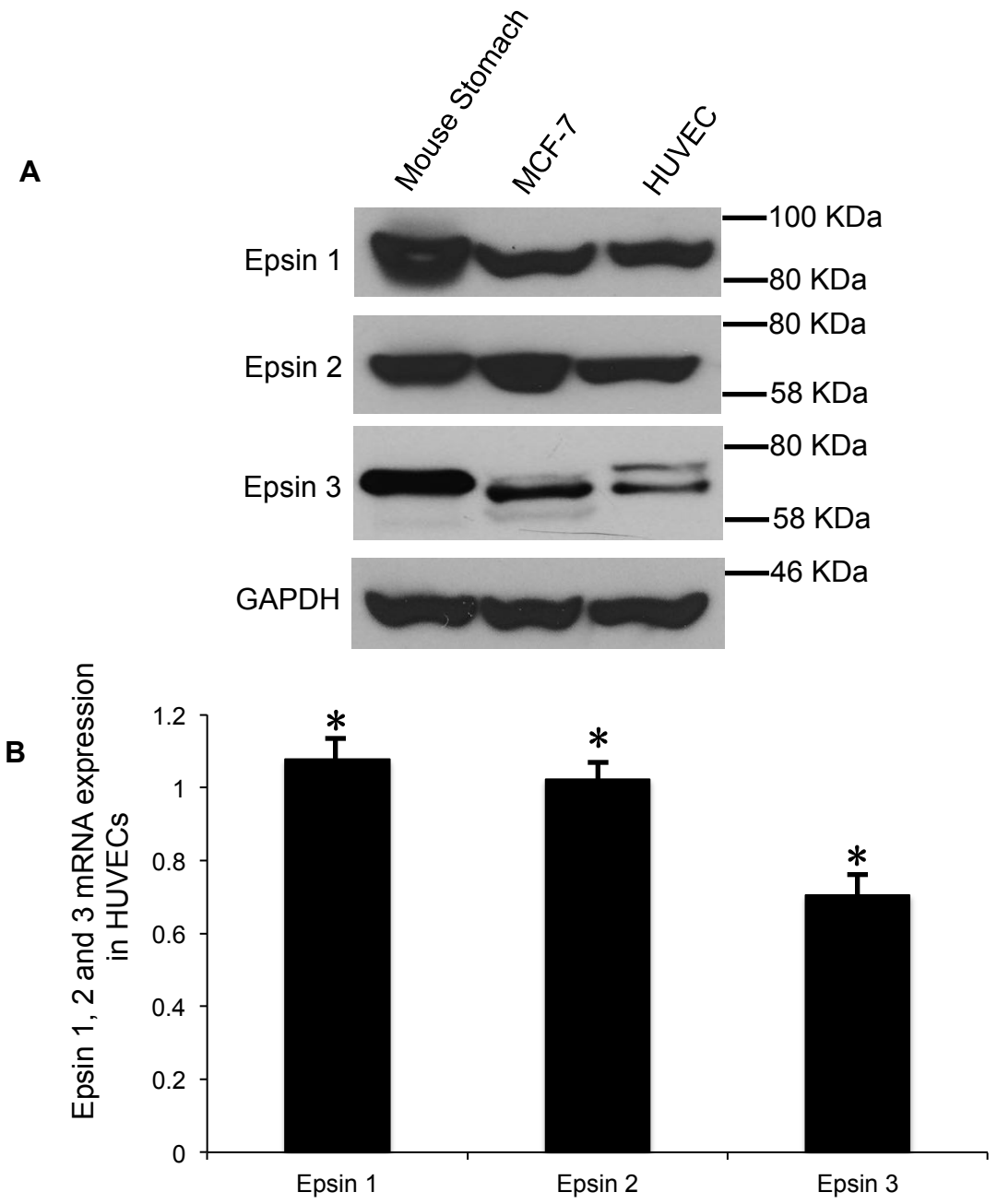


Online Figure IV. Physiological angiogenesis is increased by administration of UIM in WT mice. Representative images for the co-localization of CD31 and VE-Cadherin labeled blood vessels in cryopreserved, sectioned and immunofluorescently stained skin wound isolated at day 7 of dermal punch in WT mice receiving intratumoral injection of control or UIM peptide. Scale bar: 50 μm.

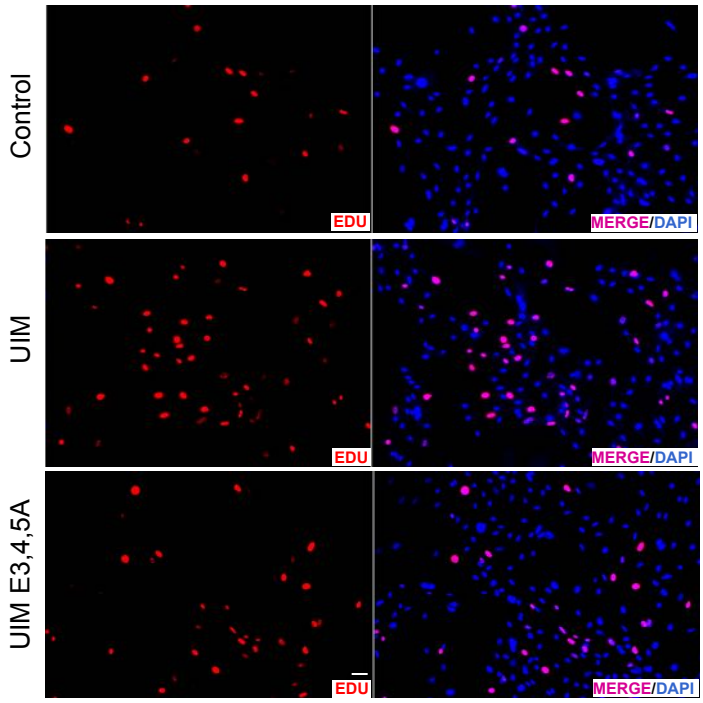


Online Figure V. FITC-UIM peptide reaches blood vessel endothelial cells *in vivo* during wound healing process. (A) Stick and schematic representations of FITC conjugated UIM peptide. **(B)** Representative images of skin wound cryosection from WT mice injected with FITC-UIM (green) that reaches blood vessels that were also immunofluorescently labeled with CD31 (red). Images were taken at 10X magnification and are representative of n=6. Scale bar in **B**: 50 μm. The arrows in the merge image of **B** pointed out the overlapped immunofluorescence signals in the blood vessels that are labeled with FITC-UIM (green) and CD31 (red) simultaneously.

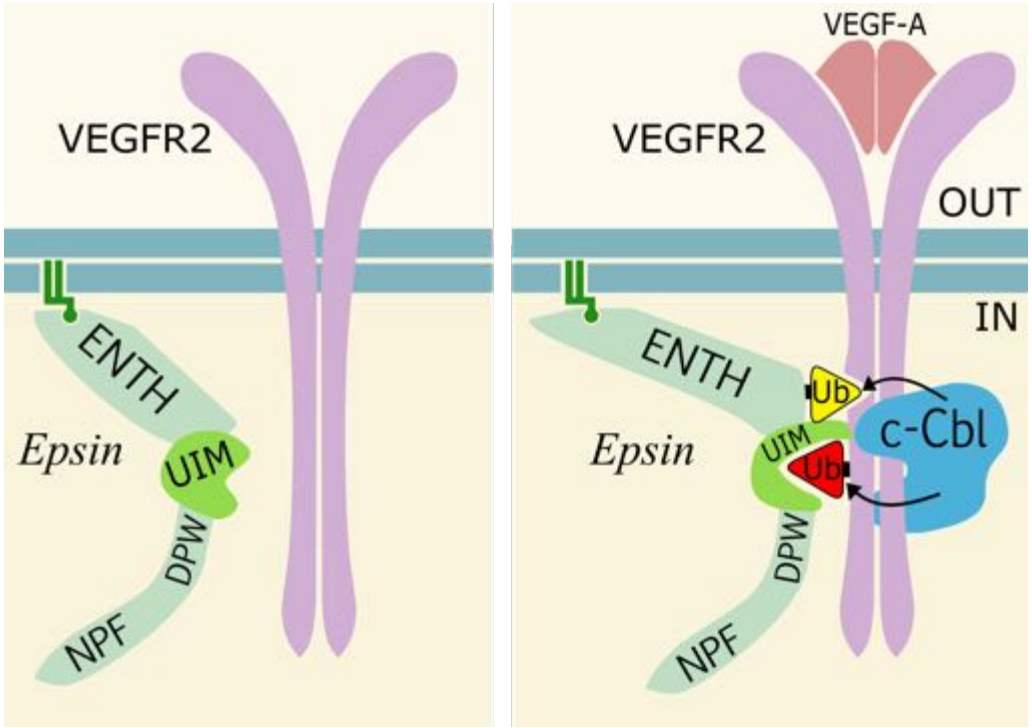
Online Figure VI. Tamoxifen-induced knockout of endogenous VEGFR2 and overexpression of exogenous VEGFR2. **(A)** Flk1$^{fl/fl}$; iCDH5 Cre (VEGFR2 $^{fl/fl}$/iCDH5-ER^{T2} Cre) mice were treated with 5 μM of 4-hydroxytamoxifen to knock down endogenous VEGFR2. **(B)** Overexpression of exogenous VEGFR2 or VEGFR2 mutants using plasmid DNA.



Online Figure VII. Epsin 3 is expressed in endothelial cells. (A) Western blot shows that epsin 3 protein is expressed in mouse stomach as reported[16], MCF-7, a human breast cancer cell line (positive control for anti-epsin 3 antibody according to the manufacture data sheet) and HUVECs. (B) Epsin 1, 2 and 3 mRNA expression in HUVECs. Total RNA was extracted from HUVECs and mRNAs were subjected to real time reverse transcriptase PCR to assess the expression of epsin 1, 2 and 3. The relative quantities of experimental mRNA were normalized to the quantity of β-actin mRNA. Data are means ± s.d. **P*<0.05.



Online Figure VIII. UIM peptide, but not UIM^{E3,4,5A} mutant peptide promotes VEGF-dependent VEGFR2 signaling and *in vitro* angiogenesis. EdU labeling analysis of MEC cell proliferation.



Online Figure IX. Model of VEGF-dependent and highly specific epsin:VEGFR2 interaction. Epsin and c-Cbl are recruited to and interact with VEGFR2 in response to VEGF stimulation. c-Cbl sequentially ubiquitinates both VEGFR2 (red ubiquitin object) and epsin (yellow ubiquitin object), thus facilitating the epsin and VEGFR2 interaction. Epsin binds VEGFR2 via its UIM low-affinity interaction with the ubiquitin moiety (red object) conjugated to VEGFR2. In turn, VEGFR2 interacts with epsin via two novel binding mechanisms: a novel epsin UIM binding cleft in VEGFR2 independent of ubiquitin, and a novel ubiquitin-interacting interface in VEGFR2 that interacts with ubiquitinated epsin (yellow ubiquitin object). Collectively, the multiple layers of binding mechanisms provide the high specificity and affinity by which epsins regulate VEGFR2, but not other angiogenic receptors.

Online Table I. Interaction residues between Epsin UIM and VEGFR2

Epsin UIM	VEGFR2	H-bonds (Å)
E183	H891	1.62
	R1027	2.65
E184	H891	1.96
	R1022	2.65
E185	R1022	1.75
E197	R1080	2.65
E198	R1080	2.69

Online Table I. Predicted interacting residues in Epsin UIM and VEGFR2, and corresponding hydrogen (H)-bond lengths between the residues.