Supplemental Material

Talin-dependent integrin activation regulates VE-cadherin localization and endothelial cell barrier function.

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Online Detailed Methods

Cell and Tissue Culture

Human umbilical vein endothelial cells (HUVECs) were purchased from Lonza (C-2519A) and cultured in endothelial cell growth medium (Lonza). Cells used for experiments were not cultured beyond passage 8. For mouse lung endothelial cell cultures, lungs were harvested and pooled from 2 adult mice and enzymatically dissociated with collagenase I (Worthington Chemical) for 50 minutes at 37°C. Endothelial cell enrichment and culture was performed as previously described.[1, 2] Briefly, sheep anti-rat Dynal Beads (Fisher) coated with CD31 antibody (BD Pharm-MEC 13.3) were incubated with the lung single-cell suspension. Magnetically sorted cells were plated in flasks coated with 5μg/mL human fibronectin (Sigma). Upon reaching confluency, ECs were were magnetically sorted a second time using sheep anti-rat Dynal beads coated with CD102 antibody (Life Technologies). Cre-mediated deletion of *Tln1* was induced by adding 500nM 4-hyrdoxy-tamoxifen (Cayman) to the culture media.

Immunostaining, Western blot and Antibodies

For detection of talin1/2 protein levels by Western blotting, cells were lysed in RIPA buffer (150mM NaCl, 50mM Tris pH 7.4, 0.1% SDS, 1% Triton X-100, 1% Sodium deoxycholate, 1mM PMSF, 1mM NaVO4, 1mM NaF, 1mM EDTA, complete protease inhibitor (Roche) and samples were clarified by centrifugation at 13,000g for 10 min at 4°C. Protein samples were boiled for 5 minutes in Laemmli buffer containing 10mM DTT and separated on 6% Tris-glyine gels (Invitrogen). Immunoblotting was done using a goat anti-mouse Talin1/2 (Santa Cruz, sc-7534, 1:1000) antibody. Primary antibody was detected using donkey anti-goat IR800 (Thermo, 1:10000) with an Odyssey CLx imager (LI-COR). For retinal preparations, retinas were fixed and whole-mounted as described below and immunofluorescence was performed using activation-sensitive integrin β1 9EG7 antibody (BD Biosciences, 550531, 1:100), hamster anti-β1 integrin HMβ1-1 (Biolegend,102206, 1:100), rat anti-mouse VE-cadherin (BD Biosciences, 550548, 1:50), and Rabbit anti-mouse ZO-1 (ThermoFisher, 61-7300, 1:200). Immunofluorescence on cryosections of 4% PFA-fixed, 10μm frozen sections was performed using rabbit anti-collagen IV (Thermo, NC0530614, 1:500), rabbit anti-mouse Laminin (Sigma, L9393, 1:400). Fixed-cell immunofluorescence was performed as described below with antibodies rabbit anti-mouse VE-cadherin (Enzo- ALX-210-232-C100, 1:300), mouse anti-VE-cadherin (Enzo, ALX-803-305-C100, 1:200), goat anti-mouse talin1/2 (Santa Cruz, sc-7534, 1:300), mouse anti-p120 catenin (BD Labs, 610133, 1:200), Vinculin (Sigma- V9131, 1:500), β1 Integrin TS2/16 (Biolegend, 303002, 1:100), Phalloidin- Alexa568 (Life Technologies, A12380, 1:500), Secondary antibodies used were goat anti-mouse Alexa488 and Alexa568 (Life Tech, 1:400), goat

anti-rabbit Alexa-488/568/647 (Life Tech, 1:400), donkey anti-goat Alexa-488/647 (Life Tech, 1:400). For antibody internalization assays rabbit anti-mouse VE-cadherin (Enzo, ALX-210-232-C100, 1:300) and mouse anti-VE-cadherin (Enzo, ALX-803-305-C100, 1:200) were utilized.

Flow Cytometry

For flow cytometry assessment of surface β1 integrin expression, whole lungs were enzymatically dissociated in Collagenase I (Worthington) as described below, antibody staining was performed for 15 minutes on ice with mouse anti-CD31-PE (Biolegend, 102408, 1:100) and mouse anti-CD45-APC (Biolegend, 103112, 1:100). For flow cytometry assessment of surface integrin subunit expression in cultured ECs, staining was performed as mentioned above using anti-α1-PE (Biolegend, 328303, 1:50), anti-α2-PE (Biolegend, 359307, 1:50), anti-α3-PE (Biolegend, 343803, 1:50), anti-α4-PE (Biolegend, 304303, 1:50), anti-α5-PE (Biolegend, 328009, 1:50), anti-α6-PE (Biolegend, 313611, 1:50), anti-αv-PE (Biolegend, 327909, 1:50), anti-β1-PE (Biolegend, 303003, 1:50), or anti-β3-PE (Biolegend, 336405, 1:50). Mouse IgG1-PE (Biolegend, 400111, 1:50), mouse IgG2a-PE (Biolegend, 400211, 1:50) or mouse-IgG2b-PE (Biolegend, 401207, 1:50) were used as isotype controls.

shRNA Transduction and Plasmid Transfection

Lentivirus was generated using the pLKO.1 backbone expressing either human Talin1 or scramble shRNAs sequences (Talin1 #1: 5'- GCCTCAGATAATCTGGTGAAA, Talin1

#2: 5'- TCCGAATGACCAAGGGTATTA, scramble: 5'-CGAGGGCGACTTAACCTTAGG).

HUVECs were infected with virus overnight, puromycin selected 24 hours after infection and 48 hours after infection trypsinized and replated onto glass coverslips coated with human fibronectin or gelatin or seeded into ECIS electrode arrays as described below. HUVECs were transfected with DNA plasmids encoding GFP fused to the N-terminus of either full-length mouse talin or talin head head domain (amino acids 1-435) using an Amaxa Nucleofector IIb (Lonza) and nucleofector kit for HUVECs (Lonza) per manufacturer's instruction. Transfection was done 48 hours after lentiviral transduction of shScramble or shTln1 shRNA and cells were fixed and analyzed by confocal microscopy 24 hours after transfection.

Cell and Tissue Immunofluorescence

For *in vitro* immunofluorescence, HUVECs were transduced with either shScramble or shTln1 shRNA and replated as described above on fibronectin (5μg/mL, Sigma) or gelatin (0.1%, Sigma) coated coverslips. Cells were fixed for 10 minutes with 4% paraformaldehyde and washed with PBS+ (Ca^{2+}/Mg^{2+}). Cells were then permeabilized with 0.1% Triton X-100/PBS+ for 10 minutes and washed with PBS+. Primary and secondary antibody incubations were performed in 0.1% BSA/PBS+ for 30 minutes at 37°C. Cells were mounted using Vectashield with DAPI (Vector Labs) and sealed with nail polish. Where noted, antibodies used for β1 integrin activation (9EG7, Biolegend), function-blocking (P5D2, Abcam) and non-functional (K-20, Santa Cruz) were added

during cell seeding and incubated for 24 hours or after seeding for blocking experiments.

Retinal mounts and immunofluorescence were performed as previously described[3]. Briefly, retinas were dissected out of mice at specified times after tamoxifen treatment, fixed in 4% PFA and whole-mounted following antibody staining. Tissue was mounted using Fluoromount (Life Technologies) and imaging was performed on an Olympus FV1000 inverted confocal microscope. For intestinal whole-mount imaging, mice were euthanized at the specified time after tamoxifen injection and a small piece of the small intestine was splayed onto a silicon plate with micro dissecting pins and fixed in 4% PFA at 4°C for 2 hours before being mounted on glass coverslips in Fluoromount. Total and Active β1 integrin levels in P7 Tln1 CTRL and Tln1 EC-KO retinal vessels were visualized using hamster anti-β1 integrin HMβ1-1 (Biolegend, 102206, 1:100) or β1 9EG7 antibody (BD Biosciences, 550531, 1:100) respectively from 3 mice per group. 5-6 images per retina were acquired with endothelium specifically visualized with either CD31 (BD Biosciences, 550274, 1:100) or FITC-Lectin (Vector Labs, FL1101-5, 1:25). Mean fluorescence of either total or active β1 integrin levels were measured only in CD31+ or FITC-Lectin+ areas of the acquired images to exclude any non-endothelial integrin signal using FIJI software's threshold masking (Li Threshold) and selection.

For analysis of frozen tissue sections, mice were perfused through the heart with 4% PFA, further fixed overnight in 4% PFA at 4°C and incubated in 30% sucrose/PBS at 4°C overnight. Organs were washed with PBS, embedded in O.C.T Compound (TissueTek), snap-frozen in liquid nitrogen and 10μm cryosections were cut at 50μm intervals. Tissue was permeablized and blocked with PBS containing 0.3%

Triton-X and 1% BSA. Primary antibody incubation was performed overnight at 4 degrees in perm/block buffer. Secondary antibody incubation was performed in PBS with 0.1% BSA at 37 degrees for 2 hours. Sections were mounted using Vectashield with DAPI (Vector Labs). Data collected from frozen sections and animal tissue was done so by annotating microscope slides with the animal ID number and without the genotype of each animal. Only after all pertinent data was collected and organized was the user unblinded to the genotypes of the mice in order to analyze the groups being compared in each experiment. For quantitative fluorescence analysis through FIJI, datasets were thresholded using FIJI software's triangle or default algorithm where appropriate to ensure unbiased quantitation of datasets.

As a negative control for immunostaining experiments, a secondary antibody only condition was included in at least one biological replicate of each assay presented. Negative control data was collected alongside experimental groups to ensure specificity of the antibody signals detected.

Junctional Width Quantitation, Line Intensity Profiles, Internalization Quantitation and Cell Contraction Analysis

HUVEC junctions were visualized by immunostaining for VE-cadherin (Enzo). Junctional width was measured using FIJI software using the line tool to select 3 unique parts of a cell-cell edge. The average of the 3 measurements is reported as the junctional width of a single cell-cell border. Cell junctions between 40-50 cells from 5-6 fields of view were measured and the data shown is from 3 independent experiments.

Line intensity profiling was done using FIJI to plot pixel intensity (Grey Values) of 20μm regions of cell-cell contacts. Profiles of VE-cadherin and vinculin staining were separately collected and overlayed to generate co-localization line-intensity profiles.

Antibody internalization assays were performed as previously described[4, 5] and analyzed using FIJI software. Mean fluorescence intensity (MFI) was measured by calculating the area of fluorescence signal of the internalized antibody per field of view from 4-6 fields of view for the two conditions analyzed. MFI was divided across the number of cells counted per field view as determined by DAPI staining and MFI/cell was compared across the groups.

Cell contraction was measured in talin-deficient and control HUVECs by staining for pMLC2 (Cell Signaling, 3674s, 1:100). Staining was performed as described above. 4-6 images (~20 cells per image) were collected per group and unbiasedly analyzed using FIJI software's threshold algorithm, triangle. pMLC2+ area was reported as a representation of pMLC2 activity and statistically analyzed as indicated in the figure legend. Actin stress fiber formation in talin-deficient and control HUVECs/HDMVECs was analyzed by immunofluorescence staining using FITC-Phalloidin (Sigma, P5282, 1:300). Staining was performed in conditions as described above and quantitated by counting the number of actin stress fiber positive cells (>3 perinuclear stress fibers/cell) from 4-6 fields of view (~15-20 cells/field) per condition tested. Where indicated, the number of perinuclear actin stress fibers/cell was also analyzed in HUVECs treated with either shTln1#1, shTln1#2 or shScramble shRNA by assessing the number of perinuclear fibers in each cell from 4-6 fields of view. Statistical analysis was performed as indicated in each figure legend.

Electrical Cell-Substrate Impedance Sensing

Barrier function of endothelial monolayers was assessed using an ECIS-Z-Theta (Applied Biophysics) instrument. Electrode arrays (8W1E; iBidi) were pre-treated with 10mM L-cysteine for 10 minutes at room temperature and coated with 0.1% gelatin and 5µg/ml human fibronectin for 1 hour at 37°C. 1.0 x 10^5 cells were seeded into each well 24 hours prior to starting time-point for multiple-frequency measurements. Cells were cultured at 37°C in 5% CO_2 for 24 hours and experimental groups were averaged from 2-3 wells of a standard 8-well set-up. Monolayer resistance values reported were measured at 24 hours after seeding and normalized to basal resistance in a cell-free well. All resistance values were recorded at a frequency of 4000 Hz. For β1-integrin activating/blocking antibody analyses at the 24 hour point, ECIS measurements were paused, media was changed to 400µL of fresh EGM-2 media containing antibody and wells brought to 37°C by equilibrating in the incubator for 15 minutes prior to restarting data collection. Data points reported are 3 hours after antibody addition. For antibody functional assays, cells were treated with β1 integrin K20 (Santa Cruz, sc-18887, 5μg/mL), β1 integrin 9EG7 (BD Biosciences, 550531, 5μg/mL), and β1 integrin P5D2 (R and D Systems, MAB17781, 5μg/mL).

***In vivo* Permeability**

To measure vascular leak, we performed an Evans blue dye (EBD) assay with slight modifications[6]. (Sigma, St Louis, MO, USA, 1%) was injected into the retroorbital plexus of mice. Blood was taken 5 and 120 minuntes post-injection. Two hours following EBD injection, mice were euthanized and organs (kidney, liver, brain and intestine) were

excised. Organs were then weighed and incubated in formamide for 48 hours at 56°C to extract EBD from the tissue. The extravasation of dye was measured spectrophotometrically at 620nm and 740nm. Values were corrected for hemoglobin with the following formula: OD620-(1.426×OD740+0.03). Concentrations were calculated by using a standard curve of known concentrations of EBD and normalized by tissue weight.

Vascular leak in the intestinal villi was also assessed by retro-orbital injections of 200 μL of FITC-Lectin (2.5 μg/mL, Vector Labs, FL1101-5) into adult 8-12 week old Tln1 CTRL and EC-KO mice 15 days following tamoxifen injections. Lectin was allowed to circulate for 30 minutes and mouse tissue was perfusion fixed with 4% PFA. Following several PBS washes, a piece of the small intestine proximal to the stomach was splayed and mounted onto a silicon plate to reduce tissue elasticity overnight. A piece of the flattened tissue was mounted onto a slide where 4-6 whole villi per mouse were imaged. Max-intensity z-stack projections were quantitated using FIJI. Intravascular levels of FITC-Lectin mean fluorescence were measured by first creating a mask on all tdTomato positive areas. Extravascular areas were assessed by excluding the tdTomato+ areas followed by quantitation of FITC-Lectin fluorescence.

Quantitative real-time PCR

Total RNA was isolated from CD31+,TdTomato+ and CD31+, TdTomato- cells with a miRNeasy Mini Kit (Qiagen) according to the manufacturer's protocol and reverse transcribed to cDNA using oligodT primers and SuperScript IV reverse transcriptase (Life Technologies) according to the manufacturer's protocol. RNA concentrations were

quantified at 260/280 using the Nanodrop 2000 (Thermofisher) and 50 ng of RNA was used for first strand cDNA synthesis. A non-reverse transcribed sample control was used in which the reaction lacked reverse transcriptase. Quantitative PCR was performed using Fast SYBR Green Master Mix (Applied Biosystems) with primers specific for *Tln1* (5'-GGCTGGGAAAGCTTTGGAC, 5'-CATCTCATTGAGCCGCTGG) and β-actin (*Actb*) (5'-GGGAAATCGTGCGTGACATCAAAG, 5'-CATACCCAAGAAGGAAGGCTGGAA) using a CFX96 Touch real-time PCR detection system (Bio-Rad Laboratories). qPCR reactions were carried out in 20μL volumes containing relevant primers at 250nM and 10ng of cDNA template in duplicates. Data was analyzed and presented using the $2^{-\Delta Ct}$ method[7] for relative gene expression analysis.

References

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Online Figure Legends

Online Figure I. Inducible endothelial cell-specific deletion of talin in *Tln1*$^{f/f}$;PDGFβ-iCreERT2+ mice causes defects in the integrity of intestinal capillaries.

A. Adult, 8-12 week old, *Tln1*$^{f/f}$;PDGFβ-iCreERT2$^{+/-}$, *Tln1*$^{f/wt}$;PDGβ-iCreERT2$^{+/-}$ and *Tln1*$^{f/f}$;PDGFβ-iCreERT2$^{-/-}$ mice were treated with tamoxifen once a day for three consecutive days and survival was monitored. *Tln1*$^{f/f}$;PDGFβ-iCreERT2$^{-/-}$ mice all survived for more than 42 days and for clarity are not depicted. **B.** Intestinal vascular hemorrhage was observed 16 days after tamoxifen treatment in *Tln1*$^{f/f}$;PDGFβ-iCreERT2+ mice (i-ii). Bleeding was observed in the intestinal wall of small intestines that were cut longitudinally and everted (iii) with bleeds prominent in the villi (iv). **C.** Hematoxylin/eosin staining of organs isolated from *Tln1*$^{f/f}$;PDGFβ-iCreERT2$^{+/-}$ and *Tln1*$^{f/f}$;PDGFβ-iCreERT2$^{-/-}$ mice 16 days after tamoxifen treatment. Scale bar=25μm. **D.** Flow cytometry of peripheral blood of mice with the indicated genotypes 7 days after tamoxifen treatment. Platelets were analyzed by gating on region R6 and staining with a FITC-CD41 antibody. Leukocytes were analyzed by gating on region R7 and staining with a FITC-CD45 antibody.

Online Figure II. Cdh5-creERT2 is efficiently activated by tamoxifen in the endothelium of several organs.

Talin1 EC-KO-tdTom and Tln1 CTRL-tdTom were treated with tamoxifen and after 16days organs were fixed and frozen sections were prepared and analyzed by confocal microscopy. **A**. TdTomato expression in sections of heart, kidney, and liver. (n=3; scale=100 μm) **B**. TdTomato and either collagen IV or laminin immunofluorescence were examined in liver sections. (n=2; scale=100 μm).

Online Figure III. Talin1 is deleted in intestinal ECs.

A. Tln1 EC-KO-tdTom mice were injected with a low dose of tamoxifen (250μg, once) and 10 days later small intestine from 3 mice were pooled, enzymatically dissociated and live, CD45- cells were FACS-sorted to isolate CD31+ cells that were either TdTomato positive or TdTomato negative. **B.** Reverse transcription of RNA isolated from the sorted populations was analyzed by real time PCR with primers specific for talin1 and β-actin transcripts and talin1 expression was normalized to β-actin.

Online Figure IV. Deletion of talin1 does not alter endothelial cell integrin surface expression.

A. Flow cytometry analysis of surface β1 integrin expression of lung endothelial cells of Tln1 EC-KO and Tln1 CTRL mice. Single cell suspensions were prepared from enzymatically-dissociated lungs. β1 integrin expression was quantified on EC populations defined as CD31+,CD45-. (n=2). **B.** Flow cytometry analysis of surface

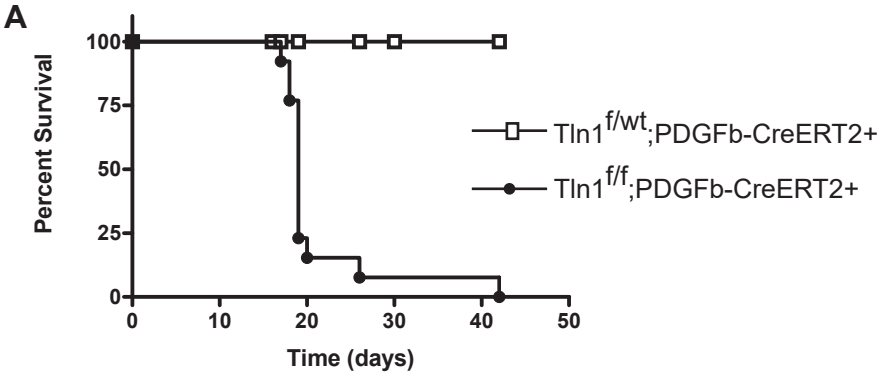
integrin subunit expression (α1, α2, α3, α4, α5, α6, αv, β1, β3) is comparable in shScramble and shTln1 HUVECs (n=2).

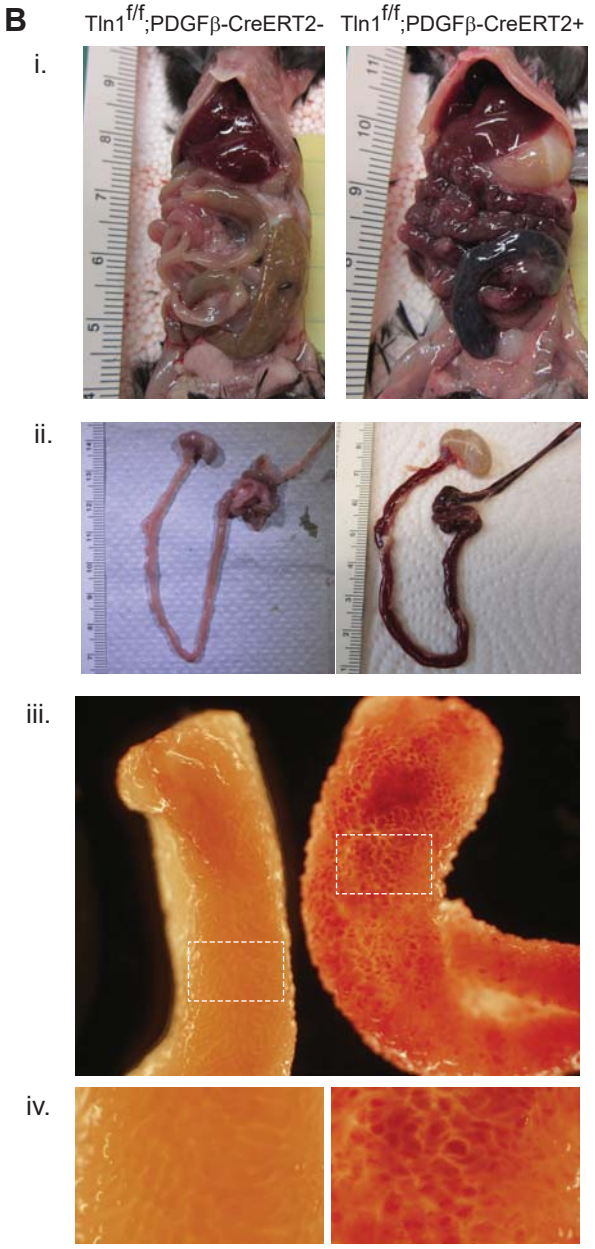
Online Figure V. Loss of talin1 in cultured ECs and retinal vasculature alters ZO-1 junctional organization

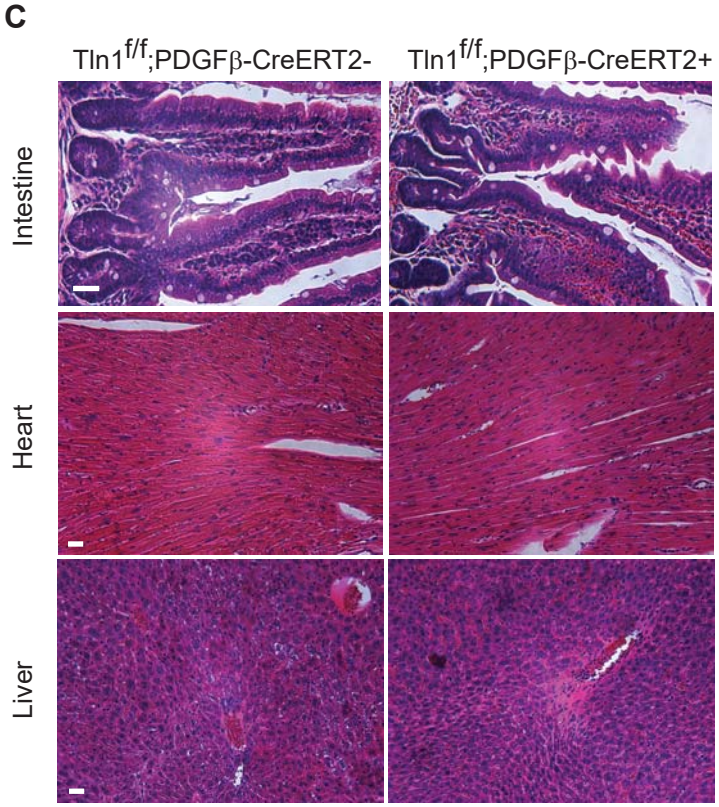
A. Immunofluorescence staining of ZO-1 in shTln1 HUVECs shows junctional widening and discontinuity of tight junctions relative to shScramble cells (n=2; scale=25 μm). **B.** P7 retinal mounts from Tln1 CTRL and Tln1 EC-KO mice stained with FITC-Lectin and ZO-1. White arrows identify junctional disorganization in retinal capillaries consistent with changes observed in VE-cadherin stained retinal capillaries (n=3; scale=25 μm).

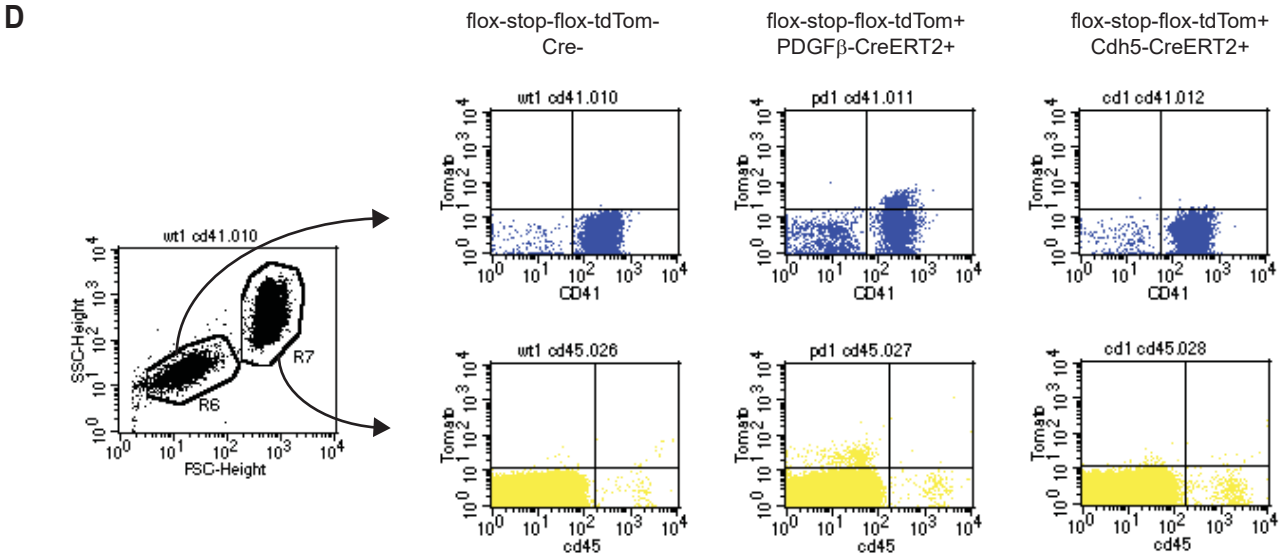
Online Figure VI. Tln1 deletion in venous and dermal microvascular ECs alters cell-cell junction organization and promotes cytoskeletal contraction

A. Immunofluorescence analysis of talin1-depleted HUVECs with multiple shRNAs against *Tln1* showing junctional disorganization relative to control cells (n=3; scale=25 μm). **B.** Efficient deletion of talin1 in HUVECs does not appear to consistently reduce total VE-cadherin and p120 protein expression as measured by western blot (n=3). **C.** Deletion of talin1 using two different *Tln1* shRNAs promotes cellular contraction as highlighted by qualitative changes in actin stress fiber formation (n=3; scale=25 μm). **D.** HDMVECs treated with shTln1 lentivirus increases the number of stress fiber positive cells per field of view relative to shScramble control cells (n=3; scale=25 μm; *p=0.0186 two-tailed unpaired t-test).

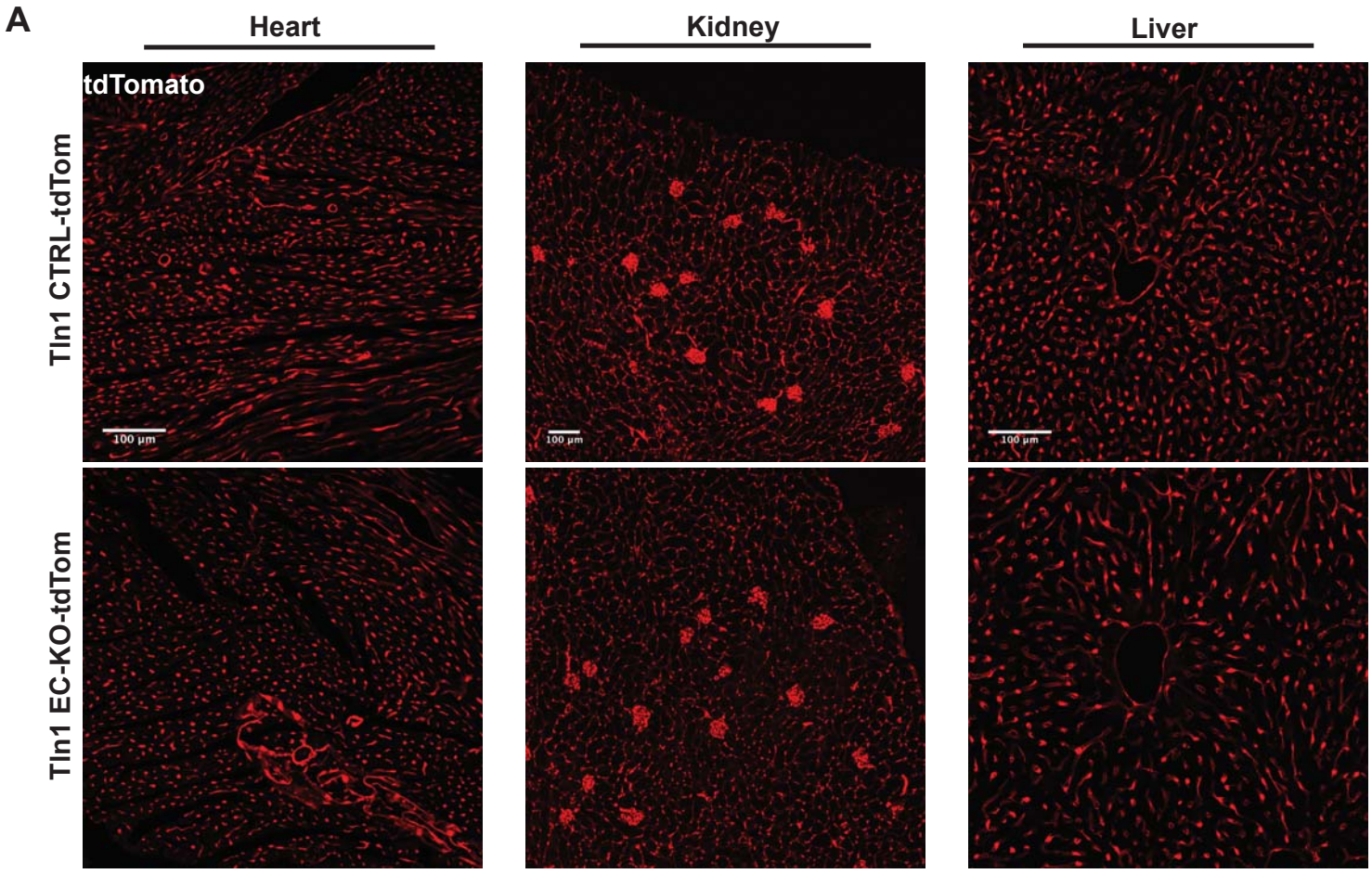


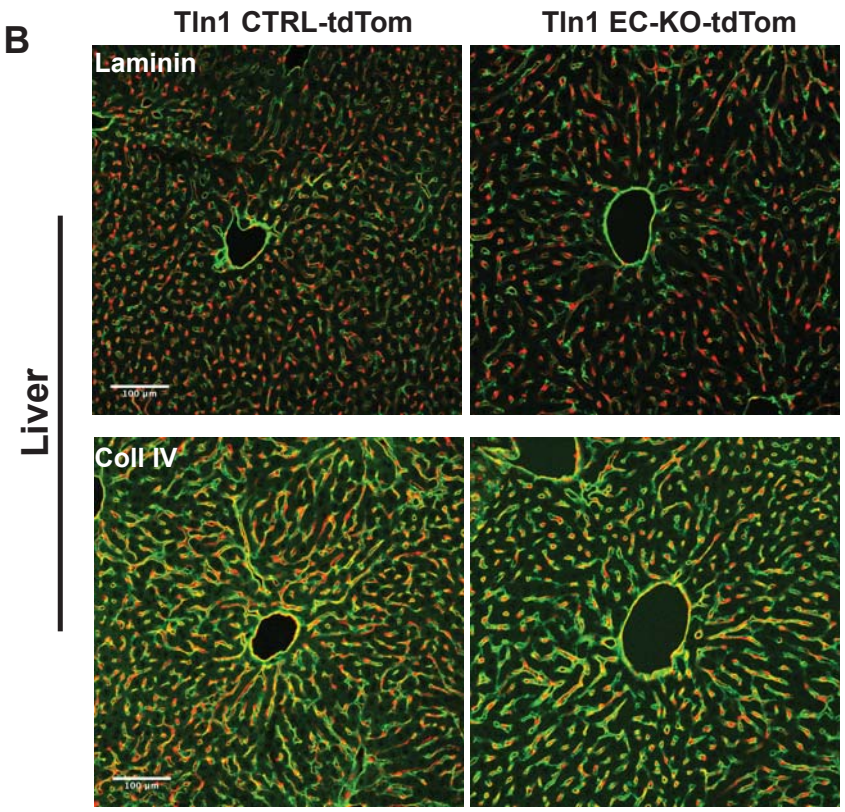






Online Figure I





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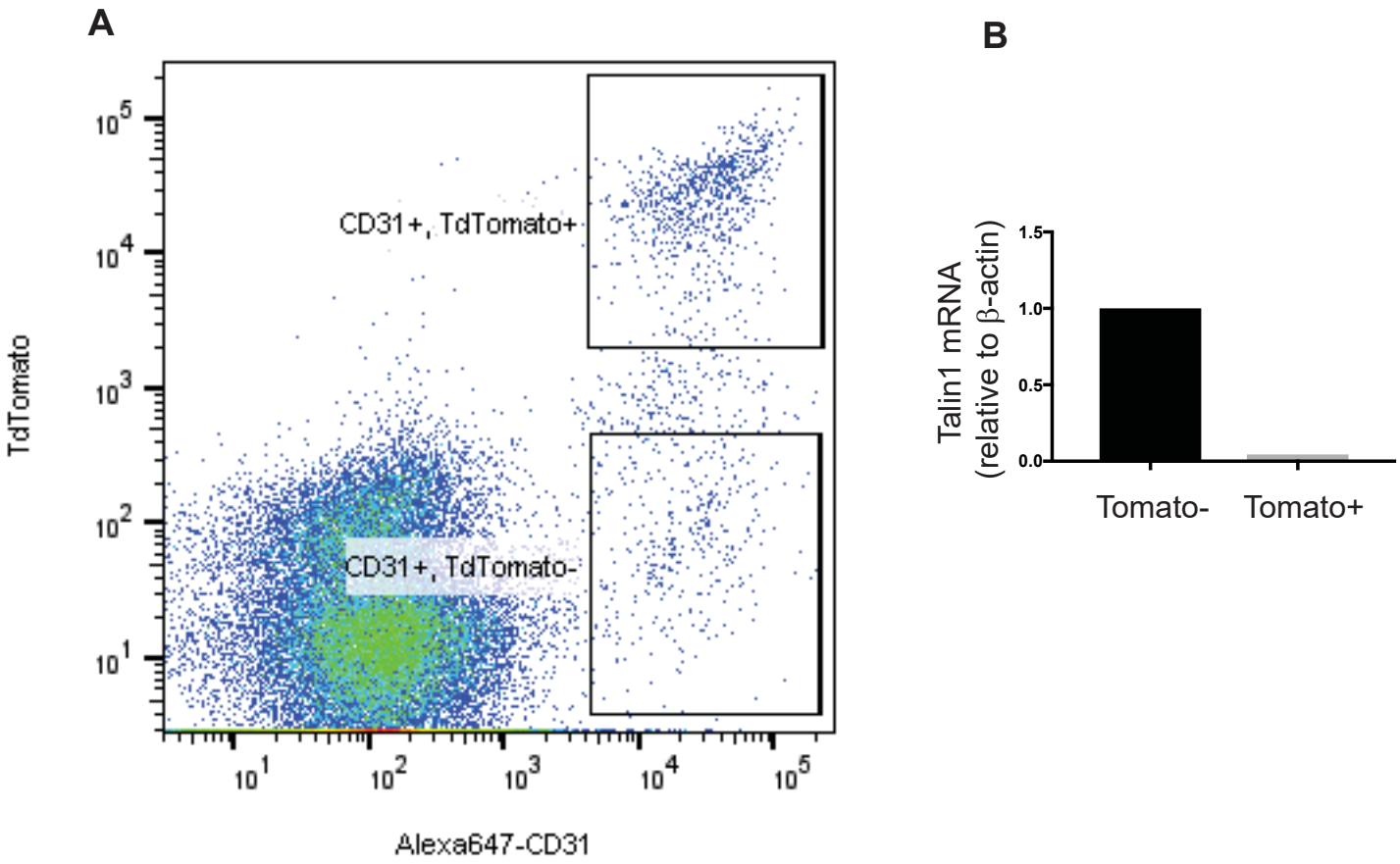

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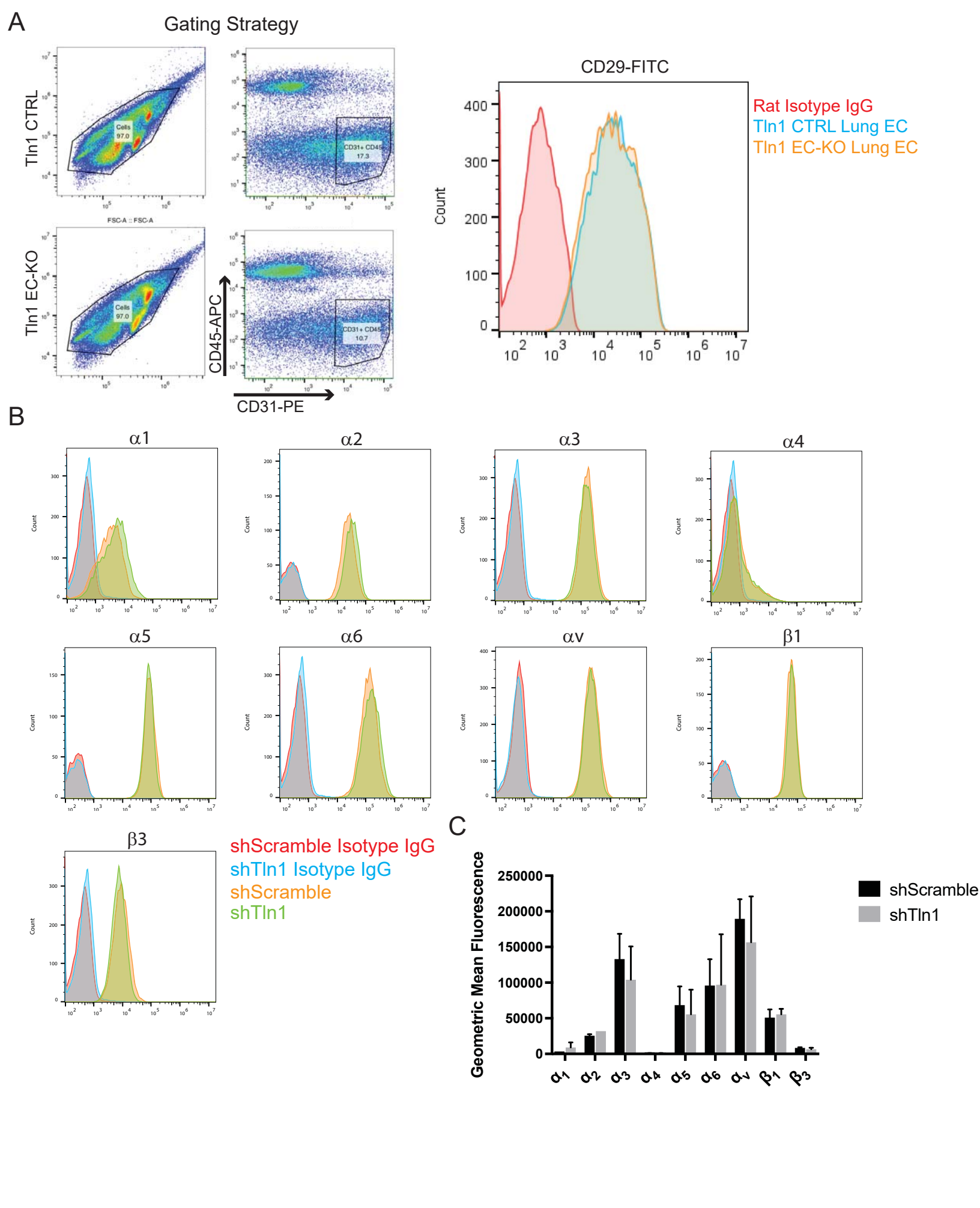

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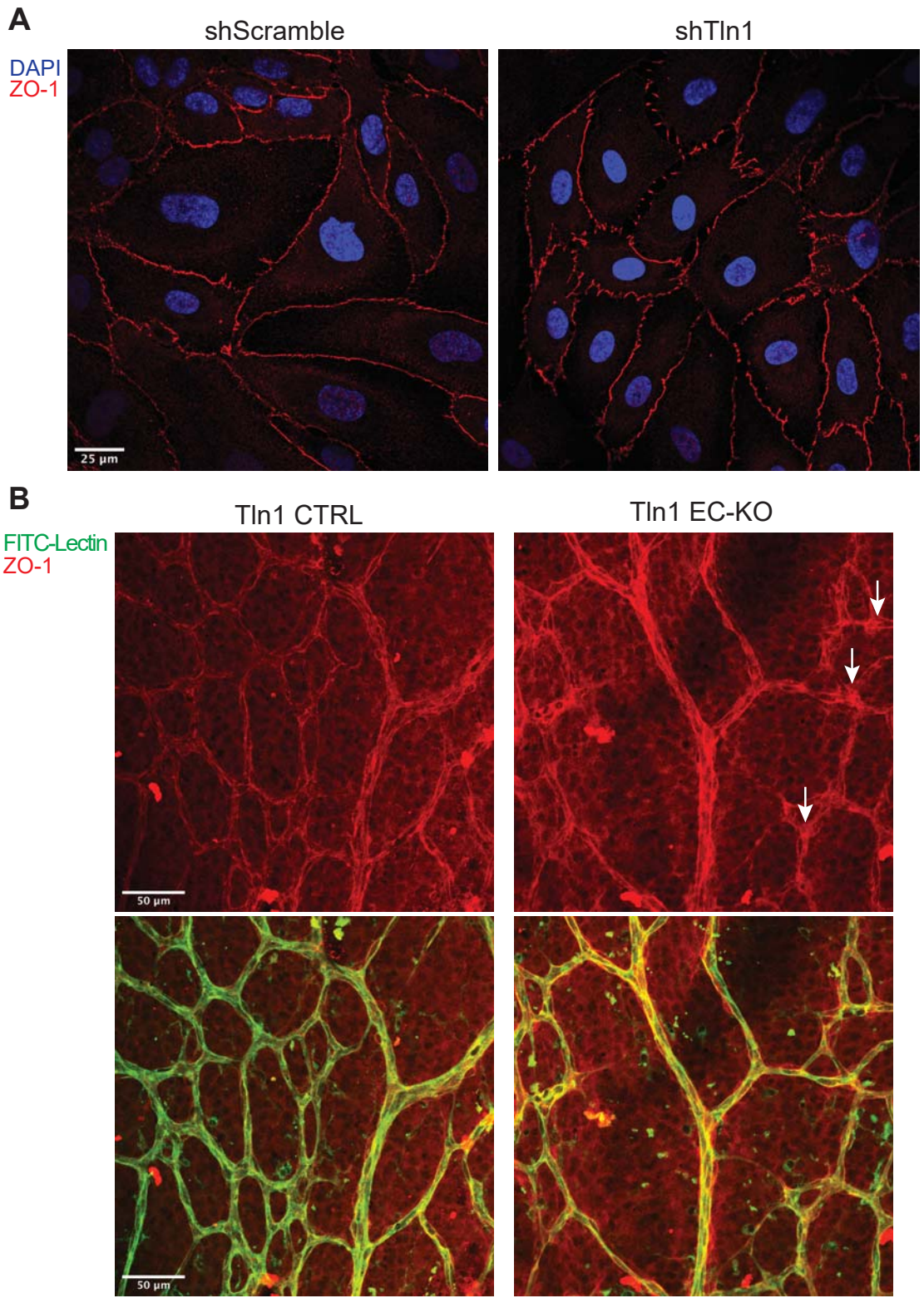

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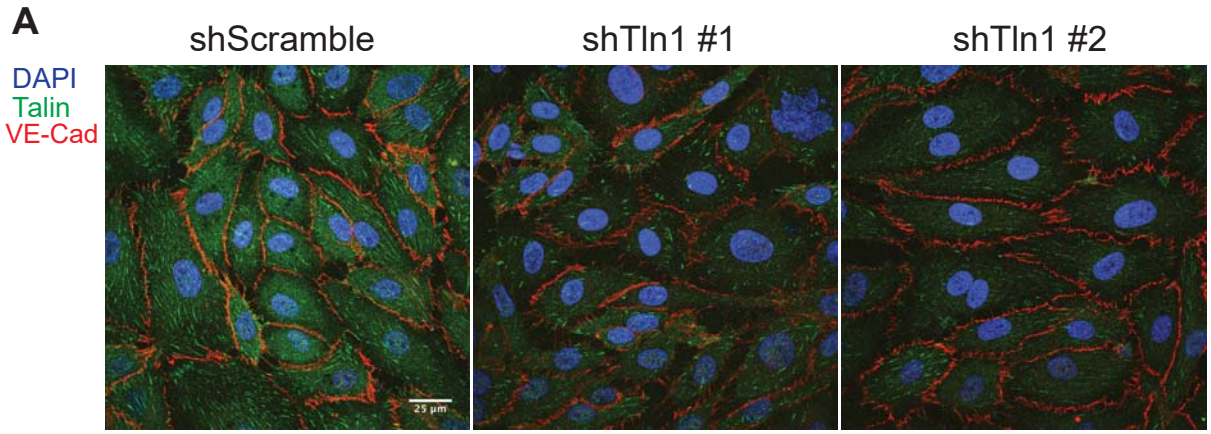

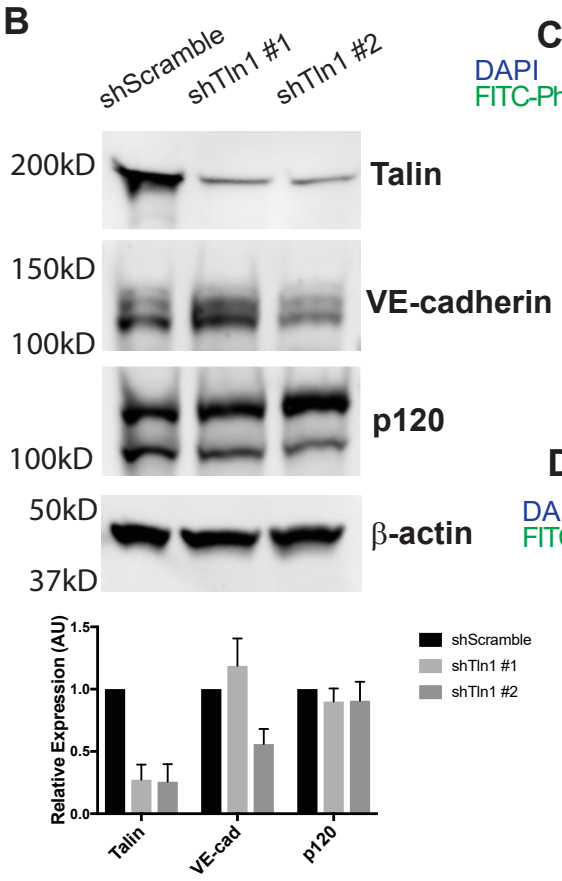

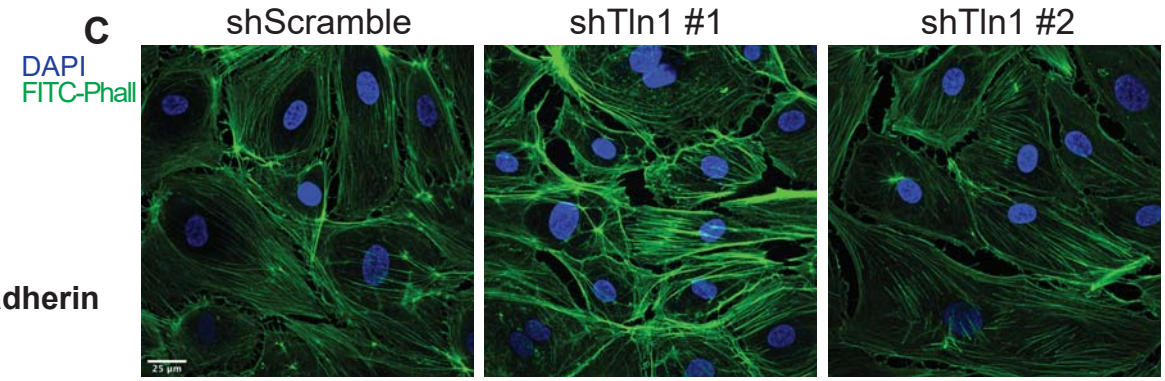

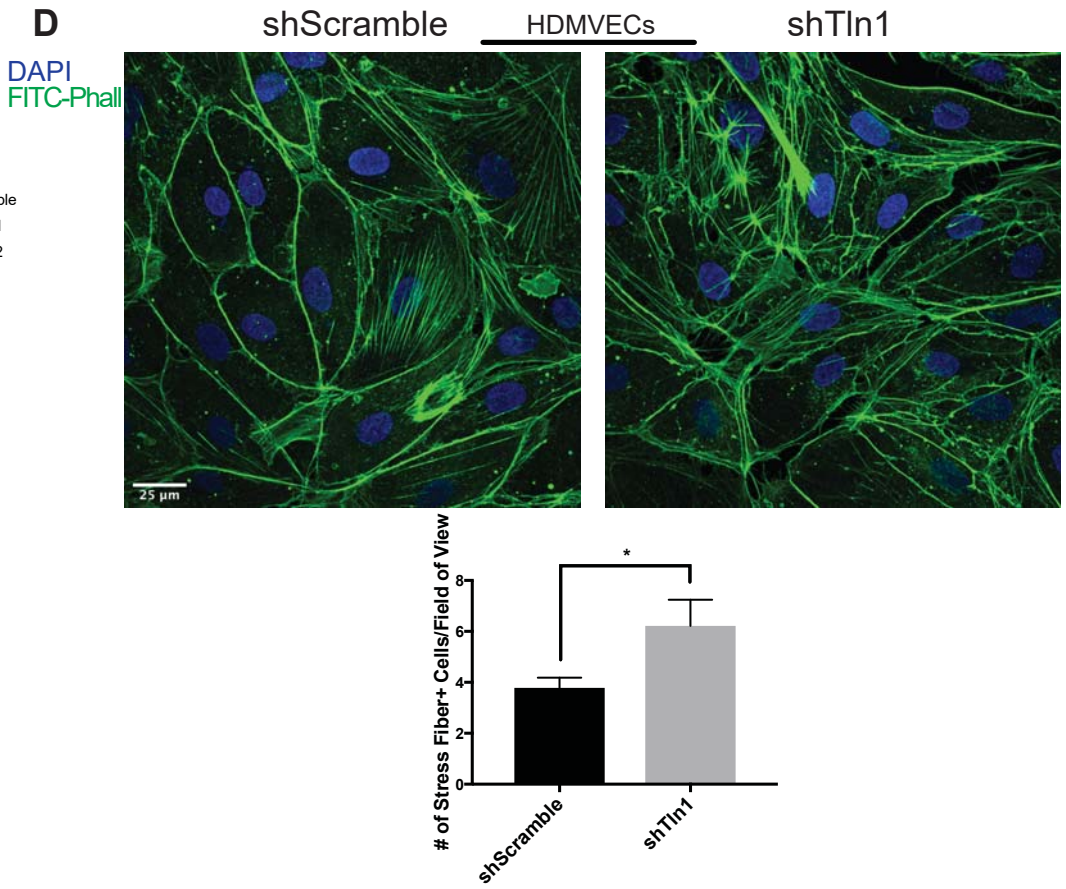

Online Figure VI