Supplemental Materials

Molecular Biology of the Cell

Taha et al.

Supplemental Material

Legends to Supplemental Figures

Supplemental Figure S1 (related to Figure 1). The VE-cadherin-mCherry fusion protein is a functional component of cell junctions**.** Western blot analysis of native HUVEC and HUVEC expressing mCherry or VE-cadherin-mCherry. The membrane was probed by respective antibodies as indicated. The secondary antibodies against rabbit-anti-mCherry and mouse-anti-β- and -γ-catenin are labeled in red (A1), and the secondary antibody to goat-anti-VE-cadherin is labeled in green (A2). (A3) Merged image of B1 and B2. (B) IP using mCherry antibody from soluble Triton X-100 (1%) extracts of HUVEC expressing VE-cadherin-mCherry followed by Western blot analysis. The membrane was probed with anti-mCherry, anti-VE-cadherin, and anti-β- and -γ-catenin antibodies, as indicated. (C, D) HUVEC and VE-cadherin null endothelioma cells were either left unmodified (C1, D1), or transduced with VE-cadherin-mCherry (C2-6 and D2-5) followed by immunolabeling with anti-VE-cadherin, anti-β- and -γ-catenin or anti-p120 antibodies, as indicated. Nuclei were visualized by DAPI staining (blue).

Supplemental Figure S2 (related to Figure 1 and 2). (A) VE-cadherin-mCherry fusion protein expressed in HUVEC is functionally incorporated into cell junctions. (A1) Live-cell imaging of HUVEC expressing VE-cadherin-mCherry under control conditions or (A2) during treatment with 3 mM EGTA for 60 min (dotted lines indicate cell borders), followed by (A3) re-calcification (see Video 1). Arrows (white and blue) point to VE-cadherin-mCherry. (B) HUVEC plating assay. (B1 and B2) side and top view of medium droplet spotted on glass-based bottom chamber. (B3) Scheme of decreasing cell density as the distance gets further away from the center of the droplet. (B4) DIC images of HUVEC from the same culture. (C) VE-cadherin-mCherry localizes at cell junctions, based on density.

Labeling of VE-cadherin by antibody of naive, subconfluent (C1), and confluent (C3) HUVEC cultures. VE-cadherin-mCherry expressed HUVEC display the same pattern as unmodified culture; interrupted patterning in subconfluent cells (C2) and a continuous line in confluent (C4) cells. White boxes indicate cropped and enlarged areas, as indicated. (D and E) Quantification of (D1) antibody-labelled and junction-localized VE-cadherin in HUVEC cultures. (D2) Segmentation based on junction localized anti-VE-cadherin staining was performed (Seebach et al., submitted), followed by relative intensity measurements of the region of interest (ROI) at cell junctions. (D3 and D4) ROI was defined by a 4-pixel extension to each side of the segmentation line (yellow arrows).

Supplemental Figure S3 (related to Figure 2). LifeAct-EGFP and EGFP-p20 fusion proteins are functionally competent when expressed in HUVEC. (A) Western blot analysis of non-transduced HUVEC or HUVEC expressing either EGFP alone or LifeAct-EGFP. The membrane was dissected and probed with anti-EGFP, anti- α-tubulin as internal control and anti-VE-cadherin as indicated. Protein ladder (red). (B) HUVEC expressing LifeAct-EGFP (B1) were fixed and subsequently labeled with phalloidin rhodamine (B2, red). DAPI stains the nucleus (blue). (C) Western blot analysis of non-transduced HUVEC or HUVEC expressing either EGFP or EGFP-p20. The membrane was probed with anti-EGFP (C1, green channel or anti-ARP 3 (C2, red channel) antibodies. (C3) Merged image. (D) Western blot analysis of anti-EGFP IP of HUVEC expressing EGFP-p20. The membrane was probed with anti-EGFP or anti-ARP3 antibodies. (E) Time-lapse recording (h:min:sec) of HUVEC expressing both EGFP-p20 and mCherry-β-actin (related to Video 5). (E1-3) Overview at t = 0 min; box indicates cropped and merged images as shown below. Arrows indicate lamellipodia. (F) HUVEC expressing EGFP alone (F1-4) or EGFP-p20 (F4-16) were double immunolabeled with anti-EGFP (green) and either anti-ARP3 (red) or anti-ARP2 (red) antibodies or phalloidin rhodamine (red) respectively as indicated. Nuclei are labelled by

DAPI (blue). Boxes indicate cropped and enlarged areas shown in panels on the right. Arrows indicate lammellipodia.

Supplemental Figure S4 (related to Figure 3). Density-dependent distribution of the ARP2/3 complex at endothelial cell junctions**.**

(A-G) HUVEC cultures were immunolabeled with anti-ARP3 (green) and either anti-p21 (red), anti-VE-cadherin (red), or anti-γ-catenin (red) antibodies in densities as indicated. DAPI stains the nucleus (blue). (A) Sparse cultures (3 x 10^4 cells/cm^2) display lamellipodia at free cell borders (arrows), while (B) subconfluent cultures (6 x 10^4 cells/cm^2) exhibit large junction-associated lamellipodia (arrows). (C) Confluent cultures (9-12 x 10^4 cells/cm^2) exhibit only small locally restricted junction-associated lamellipodia. (A1, B2 and C3) Fluorescence line plots done from a line as indicated by the red lines of the cropped areas. (A2, B3 and C3) Co-localization plots of with anti-ARP2 and anti-ARP3 antibodies. (D-G) Subconfluent and confluent HUVEC cultures labeled as indicated. Boxes indicate cropped areas. JAIL (arrows) preferentially appear at spaces between VE-cadherin/catenin complex (arrowheads) as indicated. Shown are representative images from 10 independent experiments that yielded similar results.

Supplemental Figure S5 (related to Figure 4 and 5). (A) Western blot analyses of HUVEC expressing (A1) VE-cad-ΔC164-mCherry or control cells probed with both anti-mCherry (green) and anti-VE-cadherin-carboxy-terminal domain (red). (A2) Western blot analysis of HUVEC expressing either the mCherry-(V)CA domain or mCherry. (B) HUVEC were treated with an inhibitor of ARP2/3, CK-548, or for control purposes, the inactive inhibitor, CK-312, as indicted. This was followed by immunolabeling of VE-cadherin (green) and actin filaments with phalloidin-TRITC (red). CK-548-treated cultures exhibit interendothelial gaps (stars). White arrows indicate cell junctions. Shown is one out of 8 independent experiments that yielded similar results. (C) Time-lapse recording of HUVEC expressing both VE-cad-

mCherry and EGFP-p20 that were treated with the ARP2/3 inhibitor CK-548, followed by washout as indicated. Overview (upper panel). Time- lapse recordings showing cropped and enlarged area (white box) with no treatment, upon addition of 60 µM CK-548, and after washing, as indicated. White dots indicate cell borders. Intercellular gaps are marked by yellow arrows. Arrowheads indicate reappearance of JAIL. Shown is one of 3 independent experiments that yielded similar results.

Legends to supplemental Videos 1-13

Videos were produced with the following settings:

Video Codec: h.264
Picture Size: 1280*720 (HD)
Video bitrate: 2000Kbps
Audio Codec: AAC
Audio bitrate: 128 Kbps

Videos are illustrated and verbally annotated

Video 1, Related to Figure 1 and Supplemented Figure S2.

VE-cadherin-mCherry fusion protein expressed in HUVEC localizes at cell junctions and characteristically responds to a calcium shift.

Video 2, Related to Figure 1.

VE-cadherin-mCherry displays cell density-dependent dynamics.

Video 3, Related to Figure 1.

Overexpression of VE-cadherin reduces cell motility

Video 4, Related to Figure 2.

Actin dynamics in HUVEC cultures

Video5, Related to Figure 2 and Supplemented Figure S3

EGFP-p20 and mCherry-β-actin co-localize dynamically at lamellipodia in HUVEC.

Video6, Related to Figure 2.

The ARP2/3 complex controls actin-driven junction-associated intermittent lamellipodia (JAIL) in a cell density-dependent manner.

Video 7, Related to Figure 3.

ARP2/3-controlled **JAIL** preferentially develop at interruptions between and close to VE-cadherin-mCherry clusters.

Video 8, Related to Figure 4.

ARP2/3-controlled **JAIL** facilitate the formation of VE-cadherin adhesion sites and thus contribute to VE-cadherin dynamics.

Video 9, Related to Figure 4.

The carboxy-terminal deletion mutant VEcad-ΔC164mCherry of VE-cadherin upregulates ARP2/3-controlled JAIL formation.

Video 10, Related to Figure 5.

Inhibition of the ARP2/3 complex by expression of the mCherry-labeled VCA domain of N-WASP in HUVEC decreases JAIL formation.

Video 11, Related to Supplemental Figure S5.

Application of the ARP2/3 complex inhibitor CK-548 causes a loss of VE-cadherin-mediated cell adhesion.

Video 12, Related to Figure 5.

Application of the ARP2/3 complex inhibitor CK-666 causes a loss of VE-cadherin-mediated cell adhesion.

Supplemental Material and Methods.

Table S1: Primers

Amplicon	**Forward primer (5`- 3`)**	**Reverse primer (5`- 3`)**	**Provider**
VE-cadherin full length	TAGCTAGCCACCATGCAGAGGCTC ATGATGCT	CGGGGATCCCGATACAGCAGCT CCTCCCGGG	**Invitrogen**
VEcad-ΔC164	TAGCTAGCCACCATGCAGAGGCTC ATGATGCT	CGGGGATCCACAGGAAGATGA GCAGGGTGA	**Invitrogen**
(V)CA domain of hN-WASP	TATCCGGATGCTCTGGACGAGATG CACT	TAGAATTCTCAGTCTTCCCACT CATCATAC	**GATC Biotec**
mCherry	CGGCTAGCCACCATGGTGAGCAAG GGC	CGGGATCCCTTGTACAGCTCGT CCATG	**Invitrogen**

Table S2: Antibodies

IS = immunostaining; WB = western blotting; IP: immune-precipitation

Antibody	Supplier	Concentration	Dilution	Application
Alexa fluor 488 goat-anti-mouse	Invitrogen. Eugene, Oregon USA	2 mg/ml	1:200	IS
Alexa fluor 488 Donkey anti-rabbit	Invitrogen. Eugene, Oregon USA	2 mg/ml	1:200	IS
Alexa fluor 488 Donkey anti-goat	Invitrogen. Eugene, Oregon USA	2 mg/ml	1:400	IS
Alexa fluor 568 Donkey anti-goat	Invitrogen. Eugene, Oregon USA	2 mg/ml	1:200	IS
Alexa fluor 488 Goat anti-rabbit	Invitrogen. Eugene, Oregon USA	2 mg/ml	1:200	IS
Alexa fluor 488 Donkey anti-rabbit	Invitrogen. Eugene, Oregon USA	2 mg/ml	1:200	IS
Alexa fluor 568 Goat anti-rabbit	Invitrogen. Eugene, Oregon USA	2 mg/ml	1:400	IS
Cy3 g-anti-rabbit	Jackson, Hamburg, Germany	0.66 mg/ml	1:400	IS
Cy3 g-anti-mouse	Jackson, Hamburg, Germany	0.66 mg/ml	1:400	IS
Goat anti-VE-cadherin (C-19)	Santa Cruz Biotechnology, Inc., Dallas, Texas U.S.A.	250 µg/ml	1:50 and 1:500	IS and WB
Mouse anti- α -catenin	(BD)Transduction Lab. Heidelberg, Germany	250 µg/ml	1:50 and 1:500	IS and WB
Mouse anti- α -tubulin	Sigma, Deisenhofen, Germany	3.2 mg/ml	1: 5000	WB
Mouse anti-ß-catenin	(BD)Transduction Lab. Heidelberg, Germany	250 µg/ml	1:50 and 1:500	IS and WB
Mouse anti-EGFP	Clontech Laboratories, Inc. Mountain View, CA, USA	1mg/ml	1:100 and 1:1000	IS, WB and IP
Mouse anti- γ -catenin	BD Biosciences, Heidelberg, Germany	250 µg/ml	1:50 and 1:500	IS and WB
Mouse anti-ARP 3	(BD)Transduction Lab. Heidelberg, Germany	250 µg/ml	1:500	WB
Mouse anti-P21	(BD)Transduction Lab. Heidelberg, Germany	250 µg/ml	1:50 and 1:500	IS and WB
Mouse anti-VE-cadherin	(BD)Transduction Lab. Heidelberg, Germany	250 µg/ml	1:50 and 1:500	IS and WB
Mouse anti-P120	(BD)Transduction Lab. Heidelberg, Germany	250 µg/ml	1:50 and 1:500	IS and WB
Rabbit polyclonal anti- α -catenin	A kind gift from Dietmar Vestweber, MPI-Münster, Münster, Germany.	-	1:50	IS
Rabbit anti-ARP2	Santa Cruz Biotechnology, Inc. Dallas, Texas, U.S.A.	200 µg/ml	1:50 and 1:500	IS and WB
Rabbit anti-ARP3	Millipore, Billerica, MA, USA	-	1:100 and 1:1000	IS and WB
Rabbit anti-mCherry (serum)	Dirk Lindemann, MTZ, Dresden, Germany	-	1:300 and 1:3000	IS, WB and IP
Phalloidin-TRITC Tetramethyl rhodamine Isothyocyanate	Sigma, Deisenhofen, Germany	1 mg/ml	1:500	IS
Phalloidin- Alexa fluor 488	Sigma, Deisenhofen, Germany	1 mg/ml	1:500	IS

Antibodies and reagents

Mouse IgG2a myeloma were from Calbiochem (Bad Soden, Germany) and normal rabbit serum was from Accurate Chemical and Scientific Corporation (Westbury, NY, USA); both were used as negative controls in the co-IP experiments. Near-infrared dye-coupled secondary antibodies 600 CW and 800 CW (anti-mouse, goat, and rabbit) were from LI-COR® Biosciences (Lincoln, NE, USA). ARP2/3 complex inactive inhibitor (CK-312, CK-666 and CK-689) was purchased from Calbiochem (Bad Soden, Germany) and the active inhibitor (CK-548), also called K205-1650, was purchased from ChemDiv (San Diego, CA, USA). Polyethyleneimine (PEI) transfection reagent was from Polysciences (Eppelheim, Germany). Protein-G sepharose beads were from GE-Healthcare (Uppsala, Sweden) and Protein-A sepharose CL-48 was from Amersham Biosciences (Uppsala, Sweden). All other reagents and antibodies were from Sigma (Deisenhofen, Germany).

Cell culture

Human umbilical cord vein endothelial cells (HUVEC) were isolated according to the principles outlined in the Declaration of Helsinki; this was approved by the ethics boards of the WW-University of Münster (2009-537-f-S). For examination of cells at different cell densities within the same culture, the cells were seeded in 10 mm self-manufactured glass-bottomed chambers. Briefly, 50 µl of cell suspension was spotted as a bubble in the center of a glass-bottomed chamber and allowed to settle for 30 min. Due to the bubble surface tension, cells attach at high cell density in the center and become more and sparser towards the periphery of the culture dish.

Immunofluorescence staining and antigen retrieval

Immunolabeling of HUVEC was performed after fixation with freshly prepared 2% para-formaldehyde dissolved in PBS for 10 min at RT, followed by three washes for 5 min each with 1% BSA in PBS. Cells were made permeable with 0.1% Triton X-100 in PBS for 10 min

at 4 °C. After three washes with 1% BSA in PBS, cells were incubated with the respective antibodies for 1 h at RT or overnight at 4°C, and after another washing step they were incubated with appropriate secondary antibodies. Cultures were mounted in Dako fluorescence mounting medium and observed by CLM (Carl Zeiss, Göttingen, Germany). For ARP3 and p21 staining, cells were fixed, permeabilized, and treated for 7 min at 120 °C in citrate buffer (0.378 g citric acid and 2.41 g Na-citrate-dihydrate in 1 l water, pH 6.0) for antigen retrieval. Antibody labeling was performed subsequently as described above.

Cloning and recombinant lentiviruses,

For construction of mCherry-tagged full-length VE-cadherin (VE-cad-mCherry), the cDNA of human VE-cadherin (a kind gift of Dr. Sunil K. Shaw, Women and Infants' Hospital of Brown University, Providence, RI, USA) was amplified by PCR using Hotstart Ultra Pfu polymerase; (Stratagene, La Jolla, CA, USA) and cloned into pmCherry-N1 (modified from pEGFP-N1; Clontech, Mountainview, CA, USA) using *Nhe*I and *BamHI.* The pmCherry-N1 vector containing VE-cadherin was double-digested with *NheI* and *XbaI* to yield VE-cadherin- and the fluorescent protein fragment. The fusion fragments were gel-purified and subcloned into the lentiviral vector pFUGW (Kronstein et al., 2012) using the cloning sites of *XbaI*, which has a similar overhang to that of *NheI*. See Table S1 for the primer details.

For construction of mCherry-tagged carboxy-terminal truncated VE-cadherin (VE-cad-Δ-C164-mCherry), the cDNA of human VE-cadherin (nucleotides 1-1860) was amplified by PCR using Hotstart Ultra Pfu polymerase (Stratagene, La Jolla, CA, USA) and cloned into pmCherry-N1 (modified from pEGFP; Clontech) using *Nhe*I and *BamHI.* The pmCherry-N1 vector containing the truncated VE-cadherin was double-digested with *NheI* and *XbaI* to yield the truncated VE-cadherin and the fluorescent protein fragment. The fusion fragments were

gel-purified and subcloned into the lentiviral vector pFUGW using the cloning sites of *XbaI*, which has a similar overhang to that of *NheI*. See Table S1 for the primer details.

The cDNA of human N-WASP was purchased from imaGenes (Berlin, Germany). A sequence was selected that contain a part from the V-domain (V) and the total sequence of the CA-domain designated as (V)CA-domain (nucleotides 1291-1519). (V)CA-domain was amplified by PCR and cloned into pmCherry-C1 (Clontech) using *BspEI* and *EcoRI*. To subclone the mCherry-(V)CA fusion protein fragments into the lentiviral vector pFUGW, pmCherry-N1 containing the (V)CA domain was double-digested with *NheI* and *EcoRI* to yield the mCherry-(V)CA fusion protein. The fusion fragment was gel-purified and subcloned into the lentiviral vector pFUGW using *XbaI* cloning sites and *EcoRI*. See Table S1 for the primer details.

The plasmid encoding the EGFP-p20 fusion protein was a kind gift of Prof. Theresia Stradal (WWU Münster-Germany). It was double-digested with *Nhe*I and *Xba*I, and the p20-EGFP fusion fragment was gel-purified and sub-cloned into the lentiviral vector pFUGW using *XbaI* cloning sites. Positive clones were verified by sequencing.

The plasmid encoding human β-actin was a kind gift from Prof Beat A. Imhof (Centre Medical Universitaire, Geneva, Switzerland). It was double-digested with *BamHI* and *EcoRI*, and the β-actin fragment was gel-purified and sub-cloned into the lentiviral vector pFUGW using *BamH1* and *EcoRI* cloning sites. The cDNA of mCherry was amplified by PCR where *NheI* and *BamHI* restriction sites were added as overhangs to the primers. See table S1 for the primer details. Subsequently, the cDNA of mCherry was double-digested with *NheI* and *BamHI*, gel purified and sub-cloned into the lentiviral vector pFUGW containing β-actin which was previously double digested with *XbaI* and *BamHI*. Positive clones were verified by sequencing. The plasmid encoding LifeAct-EGFP was kindly given by Dr. Wedlich-Söldner {Riedl, 2008 #8012}. It was double-digested with *Nhe*I and *Xba*I, and the LifeAct-EGFP

fusion fragment, gel-purified and sub-cloned into the lentiviral vector pFUGW using *XbaI* cloning sites. Positive clones were verified by sequencing.

Live-cell imaging

Live-cell imaging was performed on cells expressing the respective protein by spinning disk confocal microscope (Carl Zeiss, Göttingen, Germany) under 37°C and 5% CO2 using a culture dish perfusion system developed in our laboratory that allows application of drugs via a connected tube. Images were acquired using either Plan Apo 1.3 oil 40X or alpha Plan Apo 63X 1.46 oil objective lenses (Carl Zeiss, Göttingen, Germany) respectively. The EGFP was excited by 488 nm laser line of an Argon laser and detected by (38 HE Green filter) filter. The mCherry was excited by 543 nm laser line and detected through a 43 HE DsRed filter. The acquisition software was AxioVision-Carl Zeiss software (Carl Zeiss, Göttingen, Germany).

Production of lentivirus particles

Approximately 1.4 x 10^7 293T cells were seeded in 15-cm cell culture dishes in DMEM high glucose medium (see cell culture section). The next day, the medium was replaced 4 h before transfection by 15 ml DMEM with 10 % FCS (without antibiotics). Triple transfection of 293T cells with pFUGW carrying the gene of interest, packaging vectors (pCMV-ΔR8.74), and the envelope vector (pMD2G) carrying the VSV glycoprotein was carried out as follows:

Two solutions were prepared: Solution A contained pFUGW carrying the gene of interest (23 µg plasmid/15-cm dish), packaging vectors (pCMV-ΔR8.74; 23 µg plasmid/15-cm dish), and the envelope vector (pMD2G) carrying the VSV glycoprotein (11.5 µg plasmid/15-cm dish); the final volume of the solution was adjusted to 1.725 ml by adding DMEM-/- (without FCS or antibiotics). Solution B consisted of 124.2 µl of the transfection reagent PEI (1 mg/ml) dissolved in 1600.8 µl DMEM-/-. After 20 to 30 min of incubation at RT, solution B was added to solution A and the DNA-PEI mixture was incubated for 30 min at RT. Finally, the

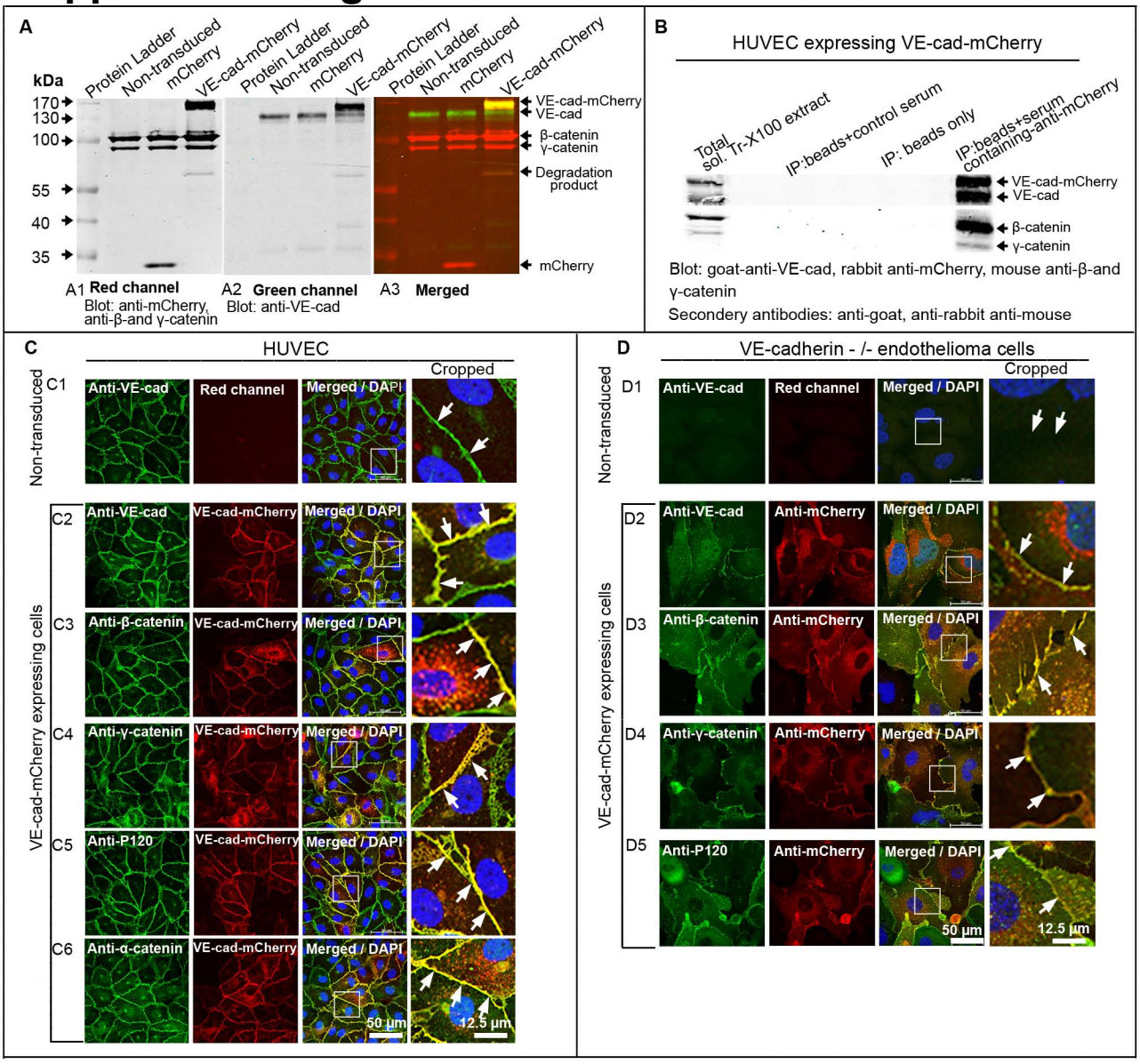
DNA-PEI mixture was added drop-wise to the Petri dish containing the 293T cells. The cells were incubated at 37°C overnight. After 14-16 h of incubation, the medium was exchanged for 20 ml fresh, pre-warmed DMEM containing antibiotics. After 24 h the medium was collected and cleared at 3000 rpm for 10 min. The supernatant was filtered through 0.45-µm filters, the virus was pelleted by ultracentrifugation (1.5 h at 25,000 rpm; 4°C), and the pellet was dissolved in 150 µl 1% BSA in PBS (sterile-filtered), aliquotted, and stored at -80° C until use.

Image analysis

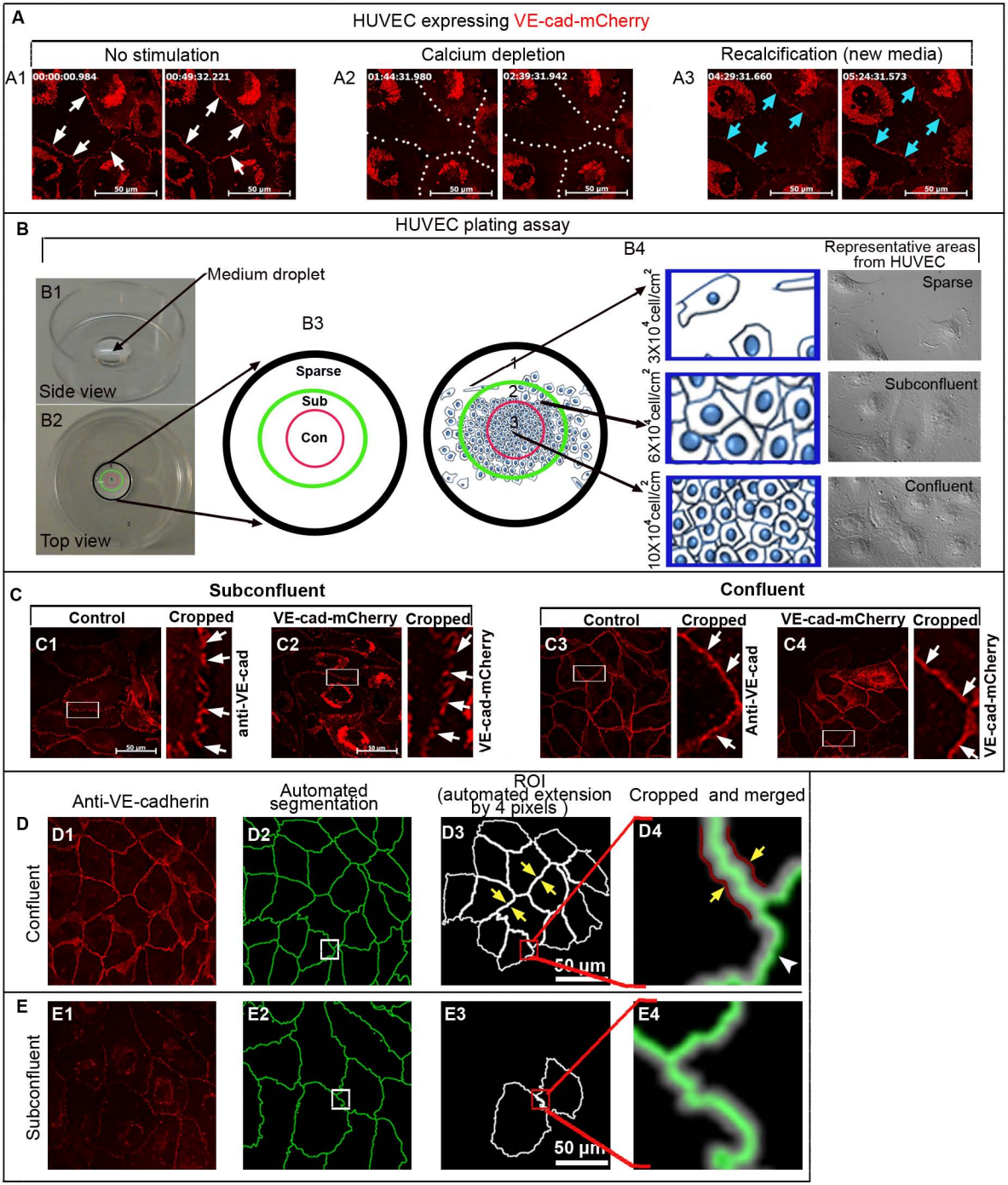
For image analysis of immunolabeled proteins at cell junctions, both labeling and image acquisition was performed under standardized conditions (related to concentrations, incubation time, temperature, laser intensity, acquisition time,). Image analysis was performed using the *Cell Border Tracker*, a newly developed algorithm that allows image segmentation and analyses of cell junction dynamics (Seebach *et al.,* submitted; see accompanying manuscript). In two particular cases manual selection of junction-associated lamellipodia was necessary as automated selection required cell junction labeling (*e.g.* VE-cadherin-mCherry) of all cells within the images to be analyzed. This requirement was not fulfilled when 1) EGFP-p20 was expressed in HUVEC alone and 2) the expression of VEcad-ΔC164mCherry was not obtained in all cells. Therefore, junction-associated lamellipodia appearing at cell junctions (EGFP-p20-labeled) were manually selected using the drawing tool from AxioVision-Carl Zeiss software. The selected junction-associated lamellipodia were manually traced on the subsequent frames until the largest area was reached. Both the surface area of the junction-associated lamellipodia and the time from the beginning until the junction-associated lamellipodia disappeared were determined. Quantification of junction-associated lamellipodia in those HUVEC expressing both EGFP-p20 and either the VEcad-ΔC164mCherry or VE-cadherin-mCherry was performed by manual selection on each frame

with an interactive junction selection tool (Seebach et al., submitted). ROI were defined and frames with selected junction-associated lamellipodia were exported to image J; a threshold was then set, and the number of junction-associated lamellipodia and area per frame were determined.

Supplemental Figure-S1



Supplemental Figure-S2



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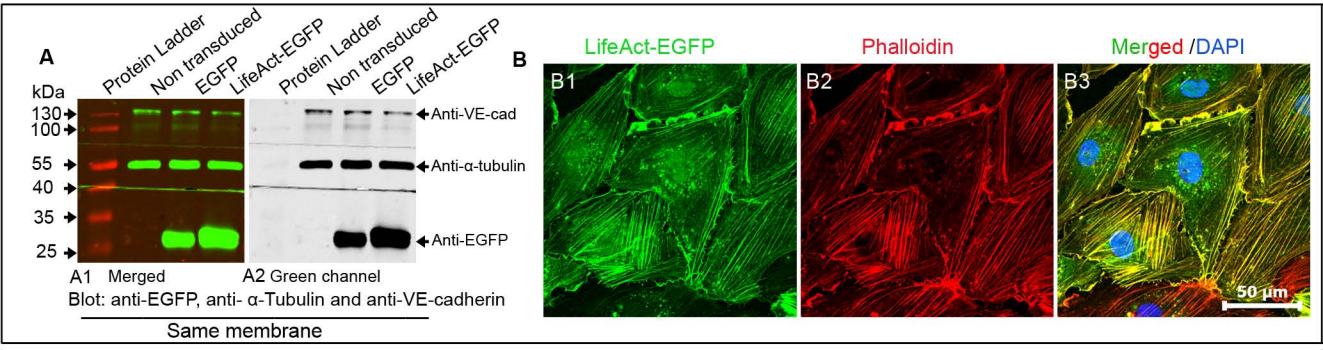
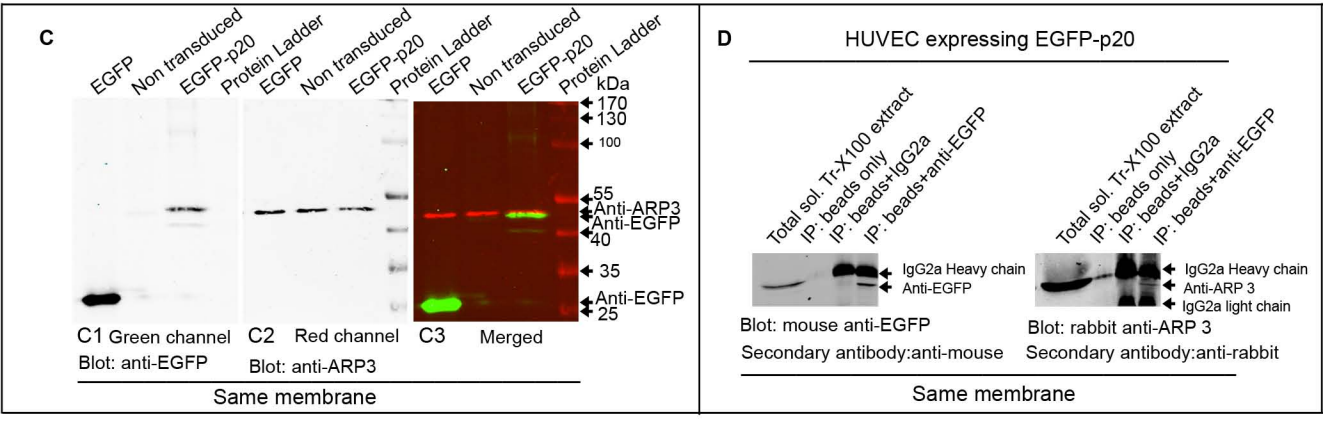
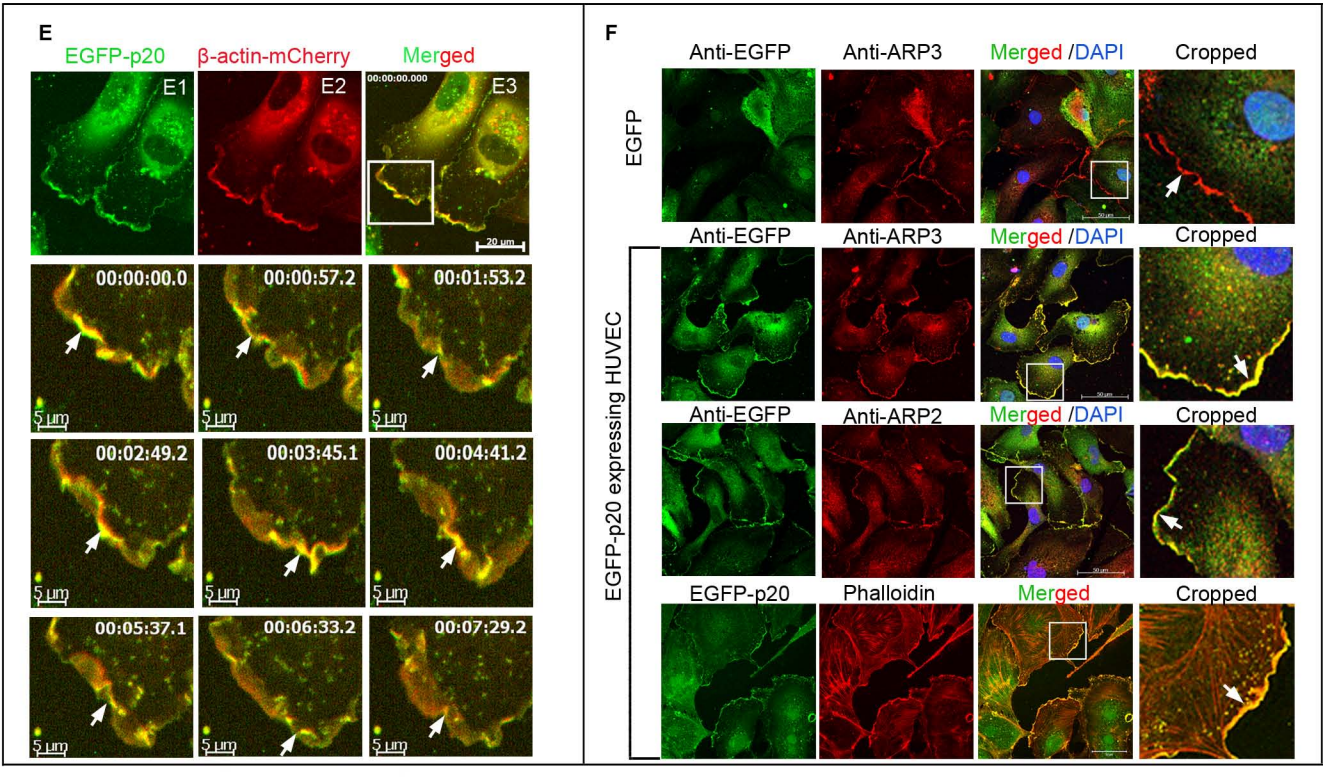
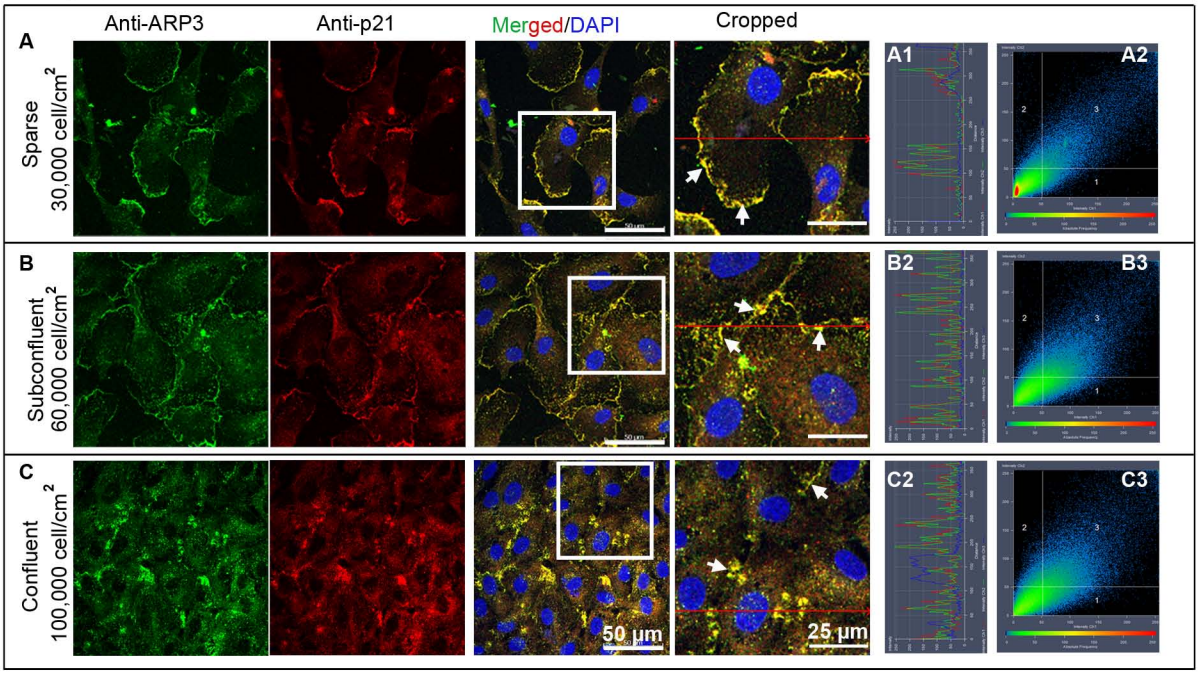
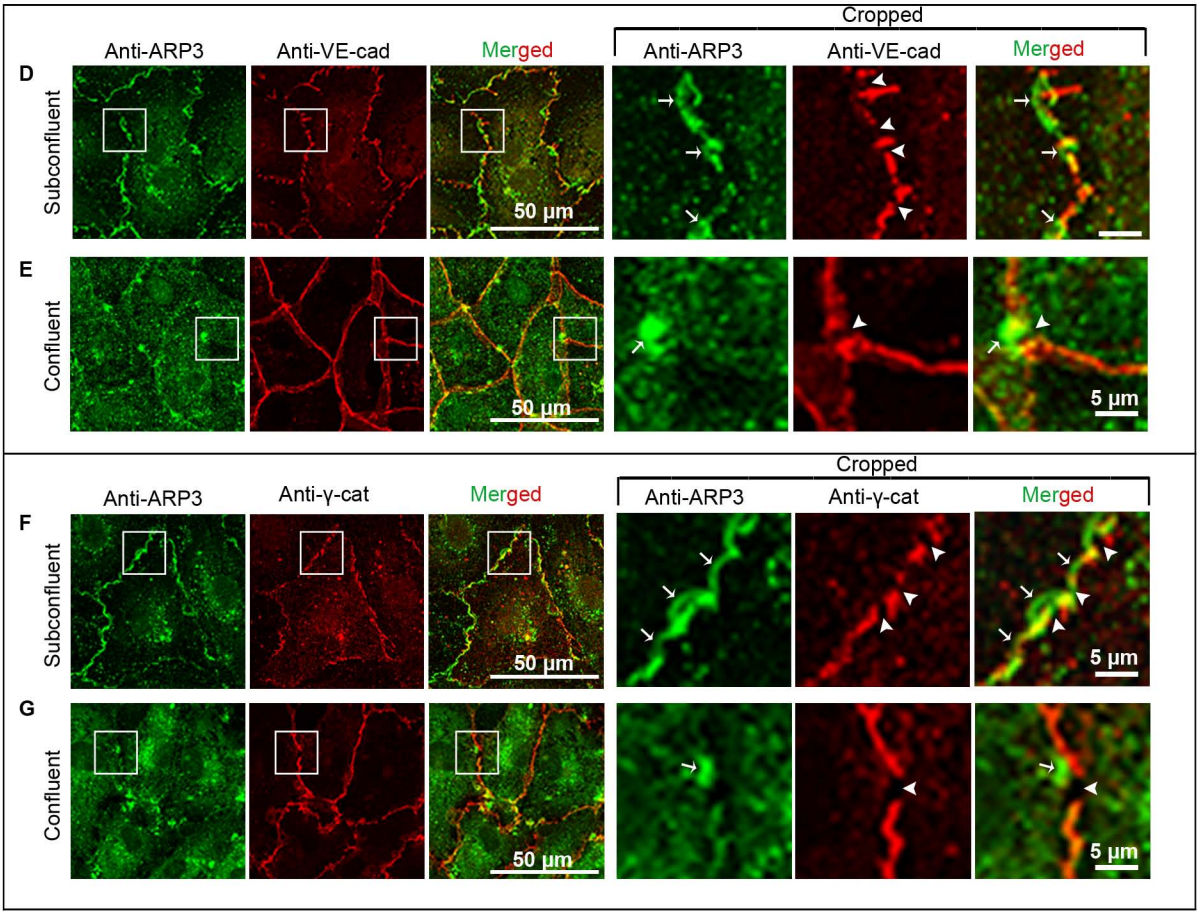






Supplemental Figure-S4





Supplemental Figure-S5