Supplemental materials

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Plasmids

VASP point mutants were generated by site-directed mutagenesis using the QuikChange Multi kit (Stratagene). Human VASP in the prokaryotic expression vector pQE-30 (QIAGEN) and primers (see Table S1) were used to exchange S157, S239, and T278 with alanines (S157A, S239A, T278A) or acidic amino acids (S157D, S239D, T278E) and P177, P178, and P179 with alanines (P177A, P178A, P179A). 5′ His_6- or VSV-epitope (YTDIEMNRLGK)-tagged VASP and C-terminally truncated VASP mutants (VSV-EVH1-PRR and VSV-EVH1, coding for VASP amino acids 1–195 and 1–114, respectively) were subcloned into pcDNA3 vector (Invitrogen). SPCN (IRAKp961A1733Q), claudin-5 (IRATp970A0452D, GenBank/EMBL/DDBJ accession no. BC032363), and EVL (IRATp970C0752D) cDNAs were obtained from RZPD; VAV1 cDNA was a gift from Dr. A. Obergfell (University of Würzburg, Würzburg, Germany). DNA fragments encoding amino acids 970–1025 of SPCN (SPCN_SH3; GenBank/EMBL/DDBJ accession no. AAH53521), and 595–659 (N-VAV1_SH3) and 785–835 (C-VAV1_SH3) of VAV1 (GenBank/EMBL/DDBJ accession no. NM_005428) were cloned into pGEX-4T3 (GE Healthcare) to generate GST fusion proteins. C-terminally VSV-tagged claudin-5 (Cl-VSV) in pcDNA3 was generated by PCR using a reverse primer that replaced the stop codon with VSV tag. For the generation of the chimeric protein claudin-5-VSV-SPCN_SH3 (Cl-VSV-SH3), the native stop codon of claudin-5 cDNA was deleted and the construct was cloned into pcDNA3 vector. Then the 5′ VSV-tagged SPCN SH3 domain (VSV-SH3) was inserted in-frame at the 3′ end.

Protein purification, coupling, and phosphorylation

His_6-tagged wild-type and mutant VASP (VASP-AAA, -DDE, -DAA, -ADA, -AAE, -P177A, -P178A, and -P179A) was expressed and purified as described (Bachmann et al., 1999). GST or GST fusion proteins (SPCN_SH3, N-VAV1_SH3, and C-VAV1_SH3) were *Escherichia coli* expressed, affinity purified on glutathione sepharose beads (GE Healthcare), and then covalently coupled to Affi-Gel 15 (Bio-Rad Laboratories). His_6-tagged VASP was phosphorylated in vitro with porcine PKA catalytic subunit (1 unit for 1 μg VASP; 5 min, 30°C). In Western blotting, our phosphorylation status–specific antibodies (anti-pS157, anti-pS239, and anti-pT278; see below) and polyclonal anti-VASP antibody (M4) revealed that PKA-treated VASP was completely S157 phosphorylated, whereas S239 phosphorylation was minor and T278 phosphorylation was not detectable. Profilin was purified from human platelets (Reinhard et al., 1995) and used for competition experiments as described (Lambrechts et al., 2000).

Antibodies and fluorescent phalloidin

Antibodies used were: anti-VASP (M4, Immunoglobe, for immunofluorescence [IF], Western Blot [WB], immunoprecipitation [IP]), anti-VASP (IE273, Immunoglobe, IF, WB, IP), anti-pS157-VASP (5C6, Nanotools identical with 3111, Cell Signaling Technology IF, WB; note: as detailed by the supplier, anti-pS157-VASP antibodies unspecifically stain cell nuclei), anti-pS239-VASP (16C2, Nanotools, WB), anti-pT278-VASP ([Blume et al., 2007], WB), anti-SPCN (2122, Cell Signaling Technology, IF, WB, IP), anti-SPCN (MAB1622, Chemicon, WB, IP), anti-ZO-1 (BD Transduction Laboratories, IF), anti-GST (Dr. K. Bundschu, University of Würzburg, WB), anti-His_6 (#70796, Novagen, WB), anti-His_6 (H1029, Sigma-Aldrich, WB, IP), anti-VSV (Clone P5D4, Sigma-Aldrich, IF, WB), and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH, mAB 374, Chemicon, WB). Unrelated anti-plasma kallikrein antibodies served as negative controls (Renne et al., 2005). Horseradish peroxidase–conjugated secondary antibodies were from Dianova (WB), fluorescence-conjugated antibodies (Alexa-fluor 488, 594, or 647, IF) from Invitrogen. TRITC-phalloidin (P1951, Sigma-Aldrich, IF) was used to visualize F-actin.

Table S1. **Primers for the generation of VASP point mutants by site-directed mutagenesis**

Point mutation	Primer Sequence
S157A	5′-CG GAG CAC ATA GAG CGC CGG GTC GCC AAT GCA GGA GGC CCA CC-3′
S157D	5′-CG GAG CAC ATA GAG CGC CGG GTC GAC AAT GCA GGA GGC CCA CC-3′
S239A	5′-GGA GCC AAA CTC AGG AAA GTC GCC AAG CAG GAG GAG GCC TCA GGG-3′
S239D	5′-GGA GCC AAA CTC AGG AAA GTC GAC AAG CAG GAG GAG GCC TCA GGG-3′
T278A	5′-G CTG GCC CGG AGA AGG AAA GCC GCG CAA GTT GGG GAG AAA ACC-3′
T278E	5′-G CTG GCC CGG AGA AGG AAA GCC GAG CAA GTT GGG GAG AAA ACC-3′
P177A	5′-CA CCA CCA GGA CCT GCC CCT CCT CCA GGT C-3′
P178A	5′-CA CCA GGA CCT CCC GCT CCT CCA GGT CCC C-3′
P179A	5′-CA GGA CCT CCC CCT GCT CCA GGT CCC CCC C-3′

Immunofluorescence and phase-contrast microscopy

IF studies were performed as described (Blume et al., 2007) with minor modifications. In brief, ECV304 cells, either untransfected or transfected with Cl-VSV or Cl-VSV-SH3, were grown in chamberslides (Nunc), fixed in ice-cold 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, and blocked with 10% goat serum. After incubation with primary antibodies (8 h, 4°C), cells were incubated with fluorescence-conjugated secondary antibodies and/or fluorescence-conjugated phalloidin for 1.5 h and mounted onto glass slides with Mowiol (Hoechst). Stained sections were investigated using a microscope (Eclipse E600; Nikon) equipped with a C1 confocal scanning head and a 100-fold oil immersion objective. 14 × 0.3-μm sections were collected as Z-stacks. One image from the center of each stack is shown. Images were acquired and prepared for presentation using the EZ-C1 software (Nikon, version 3.00). Colocalization was quantified using ImageJ 1.37 software and colocalization plug-in. Phase-contrast images of living ECV304 cells were captured using a microscope (Axiovert 25; Carl Zeiss, Inc.) equipped with a 20-fold objective and a camera (EOS 500; Canon).

Peptide and alanine substitution scans

For peptide scans, 15-mer peptides with 14 amino acid overlap, spanning the VASP protein sequence from E114 to P224 (including the entire PRR), were synthesized in an array of 10 × 10 spots on a cellulose membrane (Frank and Overwin, 1996). For alanine substitution scans, the 15-mer VASP peptide P171-P185 or mutant peptides, in which each residue was successively substituted for alanine, were prepared accordingly. For peptide and alanine substitution scans, membranes were rinsed in methanol, washed with Tris-buffered saline (TBS), blocked for 2 h in 2% milk/TBS at room temperature, and incubated overnight with GST fusion proteins (1.6 μg/ml) in 2% milk/TBS at 4°C. After washing, bound protein was probed with an anti-GST antibody in 2% milk/TBS and detected with horseradish peroxidase–conjugated secondary antibody and chemiluminescence substrate (ECL Plus, GE Healthcare).

References

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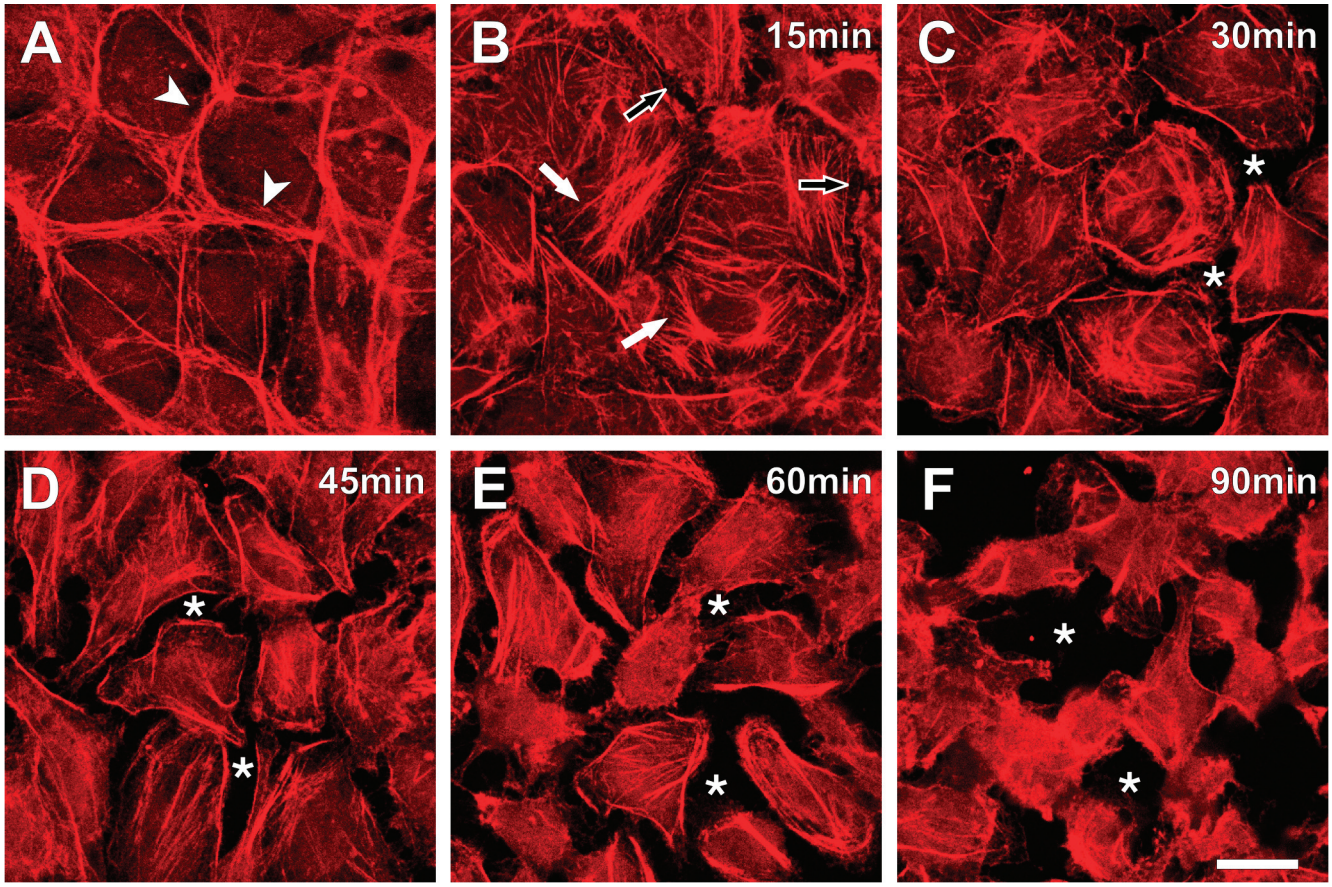


Figure S1. **Cell–cell contact disassembly and actin reorganization after calcium switch in ECV304 cells.** ECV304 cells were grown to confluence and extracellular calcium was removed by addition of 4 mM EGTA (Ca^{2+} switch) for the indicated times. Subsequently, cells were fixed and processed for confocal immunofluorescence microscopy. Actin filaments are shown in red. Confluent ECV304 cells exhibited the characteristic endothelial cobblestone morphology, intercellular junctions appeared closed, and actin fibers formed a cortical ring that lined the cytoplasmic face of cell–cell contacts (A, arrowheads). 15 min after complexation of extracellular calcium, interendothelial junctions started to open (B, filled arrows) and the actin cytoskeleton reorganized to form focal adhesion associated stress fibers (B, open arrows), while the perijunctional actin ring disappeared. 30 min after Ca^{2+} switch, interendothelial junctions were disturbed and gaps formed between adjacent cells (C, asterisks), allowing paracellular flux of macromolecules. 45, 60, and 90 min after removal of extracellular calcium, cell–cell contact disruption progressed, leading to an increasing number of large intercellular gaps (D–F; asterisks). For analyses of increased paracellular permeability of ECV304 cells at these time points after Ca^{2+} switch, see Fig. 10 A. Bar, 30 μm.

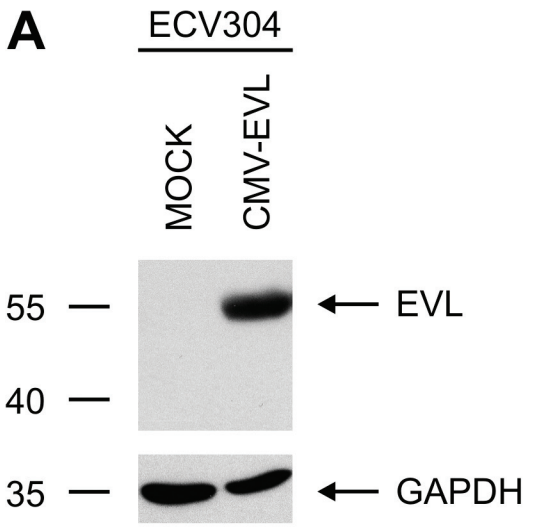
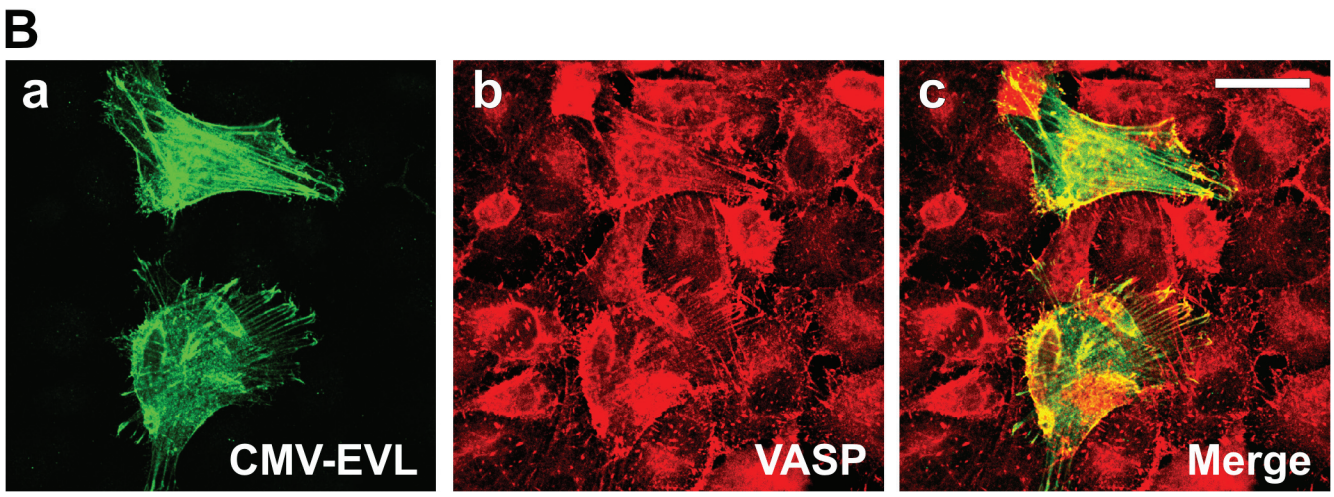


Figure S2. **ECV304 cells do not express detectable levels of endogenous EVL.** (A) ECV304 cells were transfected with a CMV-driven expression vector encoding human EVL (CMV-EVL) or the empty vector alone (MOCK, control). 24 h after transfection, cells were lysed and analyzed by Western blotting with EVL-specific antibodies. GAPDH served as loading control. (B) ECV304 cells were transfected with CMV-EVL, fixed, and processed for confocal immunofluorescence microscopy. CMV-EVL (a, green), endogenous VASP (b, red), and overlay (c) are shown. Bar, 30 μm.



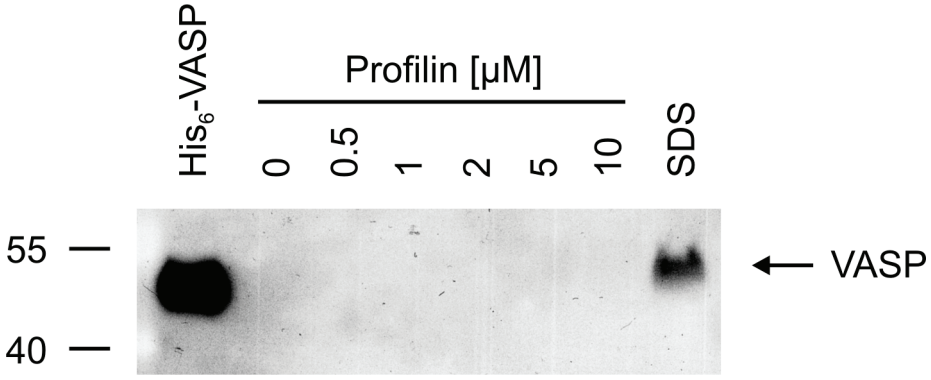


Figure S3. **Profilin does not compete with the SPCN SH3 domain for VASP binding.** Purified recombinant His_6-VASP was bound to immobilized GST-SPCN_SH3. After extensive washing, purified profilin was applied in increasing concentrations (0–10 μM). Western blotting with His_6-specific antibodies did not detect displaced VASP in the elution fractions. As positive control, SDS sample buffer was used that eluted VASP from GST-SPCN_SH3 fusion protein. Purified recombinant His_6-VASP was run on the same gel.