A local VE-cadherin and Trio-based signaling complex stabilizes endothelial junctions through Rac1


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Supplemental Figures
**Figure S1. Trio controls endothelial barrier function.** (A) TagRFP-shTrio constructs were expressed in ECs. Western blot shows efficient Trio knockdown in TagRFP-expressing ECs. (B) ECs, transiently transfected with VE-cadherin-GFP and treated with shCTRL or shTrio, are analyzed for FAJ length. Red line represents FAJ length and white line represents linear junction. When VE-cadherin staining showed zigzag pattern, it was quantified as FAJ. Graph on the right shows quantification of ratio between FAJ length and total junction length. Experiment is carried out three times independently from each other and 25 cells per experiment are analyzed. Data are mean±SEM. (C) Permeability was measured by culturing HUVECs on FN-coated Transwell filters and fluorescently-labeled Dextran was allowed to diffuse through the monolayer for four hours. Both dextran 3K and 10K showed increased permeability across Trio-deficient ECs. This experiment is carried out three times in duplicate. Data are mean±SD. *p<0.05. (D) ECs were cultured on FN-coated ECIS arrays and treated with ITX3 (100µM) or DMSO. Resistance was determined after 10h treatment and showed decreased resistance. Experiment is carried out three times independently from each other. Data are mean±SEM. *p<0.01. (E) Western blot analysis on the left shows efficient overexpression of GFP, GFP-TrioN and GFP-TrioC in endothelial cells. Blots on the right show expression of GFP-TrioN or GFP only in Trio-deficient endothelial cells. Actin is shown as loading control. (F) Intercellular gaps are quantified based on DIC images. shCTRL or shTrio-HUVECs were transfected with VE-Cadherin-GFP and 1U/mL Thrombin was administered for time periods (minutes) indicated in left lower corner. Stills show the gap size, illustrated with the white line. Bar: 10 μm. (G) ECs were cultured on FN-coated glass covers, transfected as indicated, stained. The constitutively active mutant of RhoG (Q61L), GFP-CAAX or GFP do not co-localize with VE-cadherin (red), whereas constitutively active Rac1 (Q61L) does co-localize with VE-cadherin. Bar: 20 μm. (H) Western blot confirms efficient VE-cadherin knock down. For the experiment, 1:50 dilution was used. The same blot was re-probed for β-catenin . Actin is used as loading control. (I) Trio-GEF1 activity is not required for its interaction with the VE-cadherin complex. DMSO- or ITX3-treated HUVECs were lysed and subjected to an IP for Trio. (J) ECs were cultured on FN-coated glass covers, transfected as indicated, stained. The N-terminal GEF1 domain of Trio (GEF1) as well as the C-terminal GEF domain of Trio (GEF2) do not show clear co-localization with VE-cadherin (red). Bar: 20 μm.
Figure S2. Trio interacts with VE-cadherin. (A) GST-spectrin-repeats 1-4 and 5-8 and GST alone were incubated with VE #2 or scrambled peptide as indicated. Western blot analysis shows that spectrin-repeats 5-8 bound to the VE and not to the scrambled peptide. (B) Western blot shows the input of the GST proteins as indicated for pull down experiments in Figure 4G and S2A. (C) Trio-deficient (shTrio) or control (shCTRL) ECs were treated with 4mM EGTA as indicated to chelate calcium and calcium (1mM CaCl₂) was added back for 30-60 min. Cells were stained as indicated. Bar: 20 μm. (D) ECs were grown to confluence and stimulated with 50ng/mL VEGF for 30 min. Cells were lysed and subjected to Trio IP. Association of VE-cadherin and β-catenin to Trio was determined by Western blotting. Quantification of three independent experiments is shown in lower panel. Data are mean±SEM. *p<0.05. (E) ECs were transduced with control or Trio shRNA, grown to confluency and lysed. Expression levels of several junction markers were determined by Western Blotting. Quantification of three independent experiments is shown in lower panel. (F) ECs were transduced with control or Trio shRNA, grown to confluency, lysed and an immunoprecipitation (IP) for VE-cadherin was performed. Blot shows that binding of catenins to VE-cadherin is not altered in Trio-depleted cells. Quantification of three independent experiments is shown in lower panel.
**Figure S3.** *Trio promotes linear F-actin-rich cell-cell junctions and Rac1 activation.* (A) ECs were cultured on FN-coated glass covers, transfected with GFP-TrioN and stained as indicated. Region of interest (ROI) shows increase in F-actin at cell-TrioN and stained as indicated. Region of interest (ROI) shows increase in F-actin at cell-cell junctions. Bar: 20 μm. (B) VE-cadherin-ectodomain-Fc- or Fc-coated magnetic dyna-beads were added to an EC monolayer to induce VE-cadherin ligation. ECs were lysed and adhered beads were isolated using a magnetic holder. Binding of endogenous VE-cadherin and β-catenin to the beads was determined by Western blotting. (C) VE-cadherin ligation does not increase RhoG activation, as analyzed using a GST-ELMO pull-down assay. (D) RhoA activation was analyzed using a G-LISA kit and showed a significant decrease in RhoA activation upon VE-cadherin ligation. Experiment is carried out three times. Data are mean±SEM. *p<0.05. (E) HUVEC were transduced with control or Trio shRNA #1 and incubated with VE-cadherin-ectodomain-Fc-coated magnetic beads to induce VE-cadherin ligation. Right panels show quantification, including Western blot control for Trio knockdown. Data are mean±SEM. *p<0.05. (F) HEK293 cells were transfected with GFP, GFP-TrioN-wt or GFP-TrioN-1406A/D1407A and Rac1 and RhoG activity was measured as described in the Method section. The catalytic-dead mutant N1406A/D1407A of TrioN showed no increase in Rac1 activation. Upper panel shows GTPase activity after pull down and panels below show total cell lysates and protein loading. Experiment is carried out three times independently from each other. (G) HUVEC were cultured to confluency and RhoG was silenced using shRNA as indicated. GFP-TrioN was overexpressed and localized to cell-cell junctions. Cells were fixed, permeabilized and stained for VE-cadherin in red, F-actin in blue. Merge shows co-localization between GFP-Trio, VE-cadherin and F-actin. RhoG depletion did not affect TrioN-induced linearization of cell-cell junctions. Western blot analysis showed efficient RhoG knock down (shRhoG) in HUVECs. Actin is shown as loading control. Previous work from our group showed that this shRNA did not affect Rac1 levels (Van Rijssel et al., MBoC 2012).
**Figure S4. Quantification of DORA Rac1 sensor.** (A) Schematic drawing of the Rac1 single-chain DORA sensor. Rac1-wt and the Pak-binding domain (PBD) are both located at the outside of the sensor. Cerulean3 and Venus are used to generate FRET when Rac1 and PBD are in close proximity. Both parts are linked through the ribosome linker protein L9, ensuring low off-rate FRET. (B) Stills from Movie 5 (left) showing spontaneous protrusions of Rac1 DORA sensor transfected ECs with spatial and temporal Rac1 activation at protrusions. Heat map (LUT) on the right shows FRET ratio. Warm colors indicate FRET. (C) HeLa cells were transfected with the Rac1 sensor and stimulated with EGF (concentration; arrow/asterisk) for indicated time points. Rac1 activity, as result of FRET, was shown in warm colors according to the heat map and showed transient activation upon EGF treatment. Right graph shows normalized intensity of Cerulean (blue) and Venus (yellow) and red line indicates transient increased FRET (YFP/CFP) ratio upon EGF (asterisk). Second most right graph shows average of three independent experiments of FRET ratio upon EGF treatment. (D) Western blot analysis of Rac1 activity upon EGF reflects FRET data presented under C. (E) ECs transfected with DORA Rac1 sensor were stimulated with thrombin (1U/mL; arrow/asterisk). Rapid decrease in FRET signals was detected, followed by a local increase in FRET signal during the recovery phase. Warm colors indicate increased FRET. Graph on the right shows FRET ratio. (F) Quantitative imaging of active and wt Rac1 biosensor by fluorescence lifetime imaging (FLIM). Reduced FLIM of the constitutively active Rac1 (Q61L) sensor mutant compared to the Rac1-wt sensor was measured, indicating efficient FRET. (G) Dominant negative DORA Rac1-PAK-mutant sensor shows no increase in FRET, indicating low background sensor signals. Also the graphs on the right show no change in fluorescent intensity for Cerulean, Venus or FRET ratio. (H) Trio-deficient ECs (marked by TagRFP-shTrio) show high FRET signals, reflecting Rac1 activity at the front of a protrusion (white arrow). However, upon initial cell-cell contact, marked by VE-cad-647 (red arrow), no increase in FRET is measured (green arrow). LUT: heat map on right shows warm colors as high FRET.
Movies

**Movie 1. Related to figure 1.** Time-lapse recording of HUVECs expressing VE-cadherin-GFP and Tag-RFP-shCTRL (left) or Tag-RFP-shTrio (right). Movie shows EC monolayer dynamics for 30 minutes. Images were taken every 30 seconds. Box represents ROI shown in Figure 1A.
Movie 2. Related to figure 2. Time-lapse recording of thrombin-stimulated HUVECs expressing VE-cadherin-GFP and RFP-Lifeact. Movie shows retracting endothelial cells upon thrombin (1U/mL) stimulation, followed by recovery phase and re-assembly of cell-cell junctions. Timeframe of the movie is 1.5 hours and images were taken every 16 seconds.
Movie 3. Related to figure 2. Time-lapse recordings showing linear stable cell-cell junctions in GFP-Trio-N (N-terminus) expressing cells after thrombin stimulation (arrowheads), whereas a major part of cell-cell junctions of non-transfected cells are disrupted (arrows). VE-cadherin is visualized using an Alexa-647-conjugated VE-cadherin antibody. Timeframe of the movie is 75 minutes and images were taken every 45 seconds.
**Movie 4. Related to figure 2.** Time-lapse recording of thrombin-stimulated shCTRL- (left) or shTrio- (right) treated HUVECs expressing VE-cadherin-GFP. Movie shows disruption of VE-cadherin-based cell-cell junctions (white and red arrows), followed by junction recovery. In control conditions, recovery results in linear junction morphology (yellow arrows). In shTrio ECs, junctions first recover (arrowheads) but disassemble rapidly after (red arrows). Timeframe of the movie is 150 minutes and images were taken every 30 seconds.
**Movie 5. Related to figure 7.** Time-lapse movie of a Rac1 DORA-biosensor-expressing endothelial cell making spontaneous membrane protrusions (left) or stimulated with thrombin (1U/mL) (right). Note the high FRET ratio and the rapid on and off state of the sensor, comparable with the formation and contraction of the membrane protrusions. See for LUT, figure S4B,E. Timeframe of the left movie is 20 minutes and images were taken every 5 seconds. Timeframe of the right movie is 45 minutes and images were taken every 30 seconds.
**Movie 6. Related to figure 7.** Time-lapse movie of a Rac1 DORA-biosensor-expressing endothelial cell making cell-cell contact with a non-expressing cell. Movie panel on the left shows DIC with time indication and white arrow shows initial cell-cell contact. FRET signal of Venus/Cerulean3 at cell-cell junction regions is indicated by arrowheads, α-catenin-mCherry (α-Cat) upon junction formation is shown by white arrows and the merge of Rac1 activity (FRET signal) with α-catenin-mCherry is depicted in the right frame. At the right, the color bar represents FRET ratio with warm colors as the highest signal. Arrowheads indicate local FRET signals upon the formation of cell-cell contact. Timeframe of the movie is 44 minutes and images were taken every 8 seconds.

**Movie 7. Related to figure 7.** Time-lapse movie of Trio-deficient endothelial cells expressing the Rac1-biosensor and VE-cadherin-ALEXA-647 in an attempt to re-assemble cell-cell contact. Movie panel on the left shows DIC with time indication, and white arrows indicating dis- and re-assembly of cell-cell junctions; second panel shows FRET signal of Venus/Cerulean3, and arrowheads show FRET for cell-cell junction selected region; third panel shows VE-cadherin-ALEXA-647 as cell-cell junction marker in white and fourth panel shows the merge of Rac1 activity (FRET signal) in red with VE-cadherin in green. At the right, the color bar represents FRET ratio with warm colors as the highest signal. Timeframe of the movie is 60 minutes and images were taken every 16 seconds.
Movie 8. Related to figure 7. Time-lapse movie of Trio-deficient endothelial cells expressing the Rac1-biosensor and VE-cadherin-ALEXA-647. Movie panel on the left shows DIC with time indication; second panel shows FRET signal of Venus/Cerulean3, third panel shows VE-cadherin-ALEXA-647 as cell-cell junction marker in white and fourth panel shows the merge of Rac1 activity (FRET signal) with VE-cadherin. At the right, the color bar represents FRET ratio with warm colors as the highest signal. White arrows show local Rac1 activity upon the induction of spontaneous protrusions in the absence of VE-cadherin. Green and red arrow show the formation of cell-cell contact, marked with VE-cadherin, and an absence of FRET signal. Timeframe of the movie is 90 minutes and images were taken every 20 seconds.