Figures and figure supplements

Heart of glass anchors Rasip1 at endothelial cell-cell junctions to support vascular integrity

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Figure 1. Rap1 effector Rasip1 interacts with the transmembrane protein Heart-of-Glass 1. (A) Schematic representation of the Heart-of-Glass 1 cytoplasmic tail (aa1274-1381) peptide coupled to NeutrAvidin beads. The C-terminal YF motif is indicated in red. (B) Mass spectrometry analysis was performed to identify novel HEG1 interactors. HEG1 wild-type or ΔYF cytoplasmic tail coupled to NeutrAvidin beads was used as bait using lysates from Human Umbilical Vein Endothelial Cells (HUVEC) or HeLa cells. HEG1 wild-type as well as ΔYF bound to Rasip1 from HUVEC lysate but not from HeLa lysate. In contrast, KRIT1, CCM2, and ITGB1BP1 (ICAP1) bound to wild-type HEG1 but not ΔYF in both HUVEC and HeLa. Color and size of dots indicate spectral count and relative abundance respectively (Antonio Vizcaíno et al., 2015). False-Discovery-Rate (FDR) was less than 1%. (C) Western blot analysis shows that wild-type (WT) HEG1 cytoplasmic tail and ΔYF bound to Rasip1 from HUVEC lysates, whereas KRIT1 only bound to wild-type HEG1 cytoplasmic tail. mtlb cytoplasmic tail was used as a control. Affinity Matrix was visualized by Ponceau staining. Data are representative of at least 3 independent experiments. (D) Top section: Schematic representation of HEG1 cytoplasmic tail fused to mCherry fluorescent protein and a mitochondrial targeting sequence. Bottom section: HUVECs, transfected with mito-mCherry-HEG1, were incubated with DeepRed-conjugated Mitotracker (500 nM; 30 minutes) and analyzed by Spinning Disk Confocal Microscopy (SDCM). Mito-mCherry-HEG1 intracellular distribution colocalized with mitochondria as visualized by Mitotracker. Higher magnification images of the boxed area are included. Scale bars, 10 μm. (E) HUVECs, transfected with mito-mCherry-HEG1 wild-type (WT) or mito-mCherry alone, were analyzed by SDCM for endogenous Rasip1 localization. A fraction of Rasip1 was targeted to mito-mCherry-HEG1 positive structures but not to mito-mCherry. Representative images of 3 independent experiments are shown. Scale bars, 10 μm. (F) HEK293T cells were transfected with GFP-tagged full-length Rasip1, FLAG-tagged full-length murine HEG1/FLAG empty vector, or both. Immunoprecipitation was done by using anti-FLAG G1 resin and bound proteins were separated by SDS-PAGE. Western blot analysis shows that wild-type GFP-tagged Rasip1 was co-immunoprecipitated with full-length FLAG-tagged murine HEG1. See also Figure 1—figure supplement 1.

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**Figure 1—figure supplement 1.** Mass spectrometry identifies Rasip1 as a novel HEG1 interactor. **(A)** Sequence of the HEG1 cytoplasmic tail. Sequence necessary for Rasip1 interaction (TDVYYSPTS) is underlined. **(B)** Total and unique (in parentheses) peptide counts identified after Mass Spectrometry analysis using wild-type (WT) or ΔYF HEG1 cytoplasmic tail as bait from Human Umbilical Vein Endothelial Cells (HUVEC) or HeLa lysate. Numbers from replicate experiments are shown. **(C)** Western blot analysis shows that wild-type (WT) HEG1 cytoplasmic tail, 1364X (missing the last 18 amino acids), and ΔYF bound to Rasip1 from HUVEC lysates, whereas KRIT1 only bound to wild-type HEG1 cytoplasmic tail. Moreover, HEG1 C19 bound to KRIT1 but not to Rasip1. αlollb cytoplasmic tail was used as a control. Affinity Matrix was visualized by Ponceau staining. All lanes were from the same gel. Data are representative of at least 3 independent experiments. DOI: http://dx.doi.org/10.7554/eLife.11394.004
Figure 2. Rasip1 localization and binding to HEG1 is independent of KRIT1. (A) KRIT1 expression in HEK293T was reduced by lentiviral expression of shKRIT1. Western blot analysis shows that wild-type HEG1 cytoplasmic tail binds to GFP-tagged Rasip1 from HEK293T lysates independent of KRIT1 protein expression. Affinity Matrix was visualized by Ponceau staining. Data are representative of 3 independent experiments. (B) KRIT1 Immunoprecipitation (IP) was performed to visualize KRIT1 protein expression in Human Umbilical Vein Endothelial Cells (HUVEC). Western blot analysis shows that KRIT protein expression is reduced in shKRIT1-expressing cells. GAPDH was used as a loading control. (C) HUVECs, infected with control shRNA (shCtrl) or KRIT1-specific shRNA (shKRIT1) and subsequently transfected with mito-mCherry-HEG1, were analyzed by Spinning Disk Confocal Microscopy (SDCM) for endogenous Rasip1 localization. Independent of KRIT1 protein levels, a fraction of wild-type Rasip1 was targeted to mito-mCherry-HEG1 positive structures. See Panel B for Western blot analysis of KRIT1 protein expression. Higher magnification images of the boxed area are included. Representative images of 3 independent experiments are shown. Scale bars, 10 μm.

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Rasip1 junctional localization is dependent on HEG1. (A) Rasip1 intracellular distribution was analyzed by Spinning Disk Confocal Microscopy (SDCM) in Human Umbilical Vein Endothelial Cells (HUVEC) infected with lentiviral particles containing control shRNA (shCtrl) or HEG1-specific shRNA (shHEG1#1). Cells were treated with DMEM (5% FBS, 4 mM EGTA) to remove Calcium and disrupt adherens junctions. Subsequently, cells were incubated with DMEM containing 8-pCPT-2-O-Me-cAMP-AM (‘007’, 100 μM) and Calcium (2 mM) for 20 minutes to mimic junction formation/stabilization. Under these conditions, endogenous Rasip1 localized to cell-cell contacts in control cells (shCtrl). In contrast, shRNA-mediated depletion of HEG1 (shHEG1#1) prevented Rasip1 junctional localization. Higher magnification images of the boxed area are included. Representative images of 3 independent experiments are shown. Scale bars, 10 μm. (B) Bar diagram shows percentage of cells with Rasip1 junctional localization. Mean values ± SEM are shown. One-way analysis of variance (ANOVA) with Bonferroni’s test was used to determine statistical significance.

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to compare each condition versus untreated control (shCtrl) cells. Data are from 3 independent experiments. (C) Efficiency of HEG1 mRNA depletion in HUVEC was measured by Q-PCR for cells infected with lentiviral particles containing control shRNA or two different shRNAs specific for HEG1 (shHEG1#1 and shHEG1#2) or transfected with control (siCtrl) or HEG1-specific siRNA (siHEG1). Mean values ± SEM are shown from 3 independent measurements. (D) Rasip1 intracellular distribution was analyzed by Spinning Disk Confocal Microscopy (SDCM) in HUVEC infected with lentiviral particles containing control shRNA (shCtrl) or KRIT1-specific shRNA (shKRIT1). Cells were treated with DMEM (5% FBS, 4 mM EGTA) to remove Calcium and disrupt adherens junctions. Subsequently, cells were incubated with DMEM containing 8-pCPT-2-O-Me-cAMP-AM (‘007’, 100 μM) and Calcium (2 mM) for 20 minutes to mimic junction formation/stabilization. Under these conditions, endogenous Rasip1 localization to cell-cell contacts was observed in both control cells (shCtrl) and in KRIT1-depleted cells (shKRIT1). See Figure 2 Panel B for Western blot analysis of KRIT1 protein expression. Higher magnification images of the boxed area are included. Representative images of 3 independent experiments are shown. Scale bars, 10 μm. See also Figure 3—figure supplements 1–3.

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Figure 3—figure supplement 1. Rasip1 junctional localization is dependent on HEG1. (A and B) Rasip1 intracellular distribution was analyzed by Spinning Disk Confocal Microscopy (SDCM) in Human Umbilical Vein Endothelial Cells (HUVEC) infected with lentiviral particles containing control shRNA (shCtrl) or HEG1-specific shRNA (shHEG1#2) (A), or HUVEC transfected with control siRNA (siCtrl) or HEG1-specific siRNA (siHEG1) (B). Cells were treated with DMEM (5% FBS, 4 mM EGTA) to remove Calcium and disrupt adherens junctions. Subsequently, cells were incubated with DMEM containing 8-pCPT-2-O-Me-cAMP-AM (’007, 100 μM) and Calcium (2 mM) for 20 minutes to mimic junction formation/stabilization. Under these conditions, endogenous Rasip1 localized to cell-cell contacts in control cells (shCtrl or siCtrl). In contrast, depletion of HEG1 (shHEG1#2 or siHEG1) prevented Rasip1 junctional localization. Higher magnification images of the boxed area are included. Representative images of 3 independent experiments are shown. Scale bars, 10 μm.

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ROCK inhibition does not restore Rasip1 junctional localization in HEG1 depleted cells. (A) Rasip1 intracellular distribution was analyzed by Spinning Disk Confocal Microscopy (SDCM) in Human Umbilical Vein Endothelial Cells (HUVEC) infected with lentiviral particles containing control shRNA (shCtrl) or HEG1-specific shRNA (shHEG1#1). Cells were incubated with ROCK inhibitor (H-1152, 3 μM) for 30 minutes prior to the Calcium Switch assay. Next, cells were treated with DMEM (5% FBS, 4 mM EGTA) to remove Calcium and disrupt adherens junctions. Subsequently, cells were incubated with DMEM containing 8-pCPT-2-O-Me-cAMP-AM (‘007’, 100 μM) and Calcium (2 mM) for 20 minutes to mimic junction formation/stabilization. Under these conditions, endogenous Rasip1 localized to cell-cell junctions in control cells (shCtrl). Even in ROCK-inhibited cells, depletion of HEG1 prevented Rasip1 junctional localization similar to cells that were not treated with ROCK (see Figure 3 and Figure 3—figure supplement 1 and 2). Higher magnification images of the boxed area are included. Representative images of 3 independent experiments are shown. Scale bars, 10 μm. (B) MLC phosphorylation was analyzed by SDCM in HUVEC treated with ROCK inhibitor H-1152 (3 μM) for 30 minutes. Inhibition of ROCK reduced levels of phosphorylated MLC compared to untreated cells confirming treatment efficiency. Representative images are shown. Scale bars, 10 μm.

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Knock-down of HEG1 does not affect Rasip1-Rap1 or Rasip1-Radil-ARHGAP29 complex formation. (A and B) U2OS cells were infected as indicated. 48 Hours post-infection, cells were transfected as indicated. Immunoprecipitation was done by using anti-FLAG G1 resin and bound proteins were separated by SDS-PAGE. Western blot analysis shows that mCherry-HA-Radil (A) or YFP-ARHGAP29 (B) co-immunoprecipitated with full-length FLAG-tagged Rasip1 in control cells (shCtrl) and silencing HEG1 expression had no effect (shHEG1#1). (C) Efficiency of HEG1 mRNA depletion in U2OS cells was measured by Q-PCR for cells infected with lentiviral particles containing control shRNA (shCtrl) or an shRNA specific for HEG1 (shHEG1#1). Mean values ± SEM are shown from 3 independent measurements. (D) U2OS cells were infected as indicated and subsequently, 48 hours post-infection, cells were transfected as indicated. Immunoprecipitation was done by using anti-FLAG G1 resin and bound proteins were separated by SDS-PAGE. Western blot analysis shows that full-length GFP-tagged Rasip1 co-immunoprecipitated with FLAG-tagged Rap1 V12 (constitutively active) in control cells (shCtrl) and silencing HEG1 expression (shHEG1#1) or addition of a purified HEG1 cytoplasmic tail peptide (5 μM) had no effect. Relative binding compared to control cells expressing Rap1-V12 and Rasip1 is shown.

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Figure 4. Rasip1 binds to HEG1 upstream of the KRIT1-binding site. (A) Schematic representation of different HEG1 cytoplasmic tail peptides used to map the binding region for Rasip1. (B) HEK293T cells were transfected with GFP-tagged full-length Rasip1. Western blot analysis shows that HEG1 wild-type (WT), 1334X, C54, and 1318-1339 bound to GFP-Rasip1. In contrast, HEG1 1328X, C49, and Δ1327-1335 failed to bind to GFP-Rasip1. Endogenous KRIT1 binding was only observed for HEG1 WT, C54, C49, and Δ1327-1335 which all contain the C-terminal YF motif. Affinity Matrix was visualized by Ponceau staining. (C) Top section: Bar diagram shows binding of GFP-Rasip1 to HEG1 cytoplasmic tail peptides relative to wild-type HEG1. Mean values ± SEM are shown from at least 3 independent experiments. Bottom section: HEG1 1327-1335 (TDVYYSpts) is necessary for Rasip1 binding. (D) HUVECs, transfected with mito-mCherry-HEG1 or mito-mCherry-HEG1(Δ1327-1335), were analyzed by Spinning Disk Confocal Microscopy (SDCM) for endogenous Rasip1 localization. A fraction of Rasip1 was targeted to mito-mCherry-HEG1 positive structures but not to mito-mCherry-HEG1(Δ1327-1335). Scale bars, 10 μm. (E) HEK293T cells were transfected with GFP-tagged full-length Rasip1, FLAG-tagged murine HEG1 full-length, Δ1283-1291 (corresponding to aa 1327–1335 in human HEG1), empty vector, or both. Immunoprecipitation was done by using anti-FLAG G1 resin and bound Figure 4 continued on next page
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proteins were separated by SDS-PAGE. Western blot analysis shows that GFP-tagged Rasip1 was co-immunoprecipitated with full-length mHEG1 but not mHEG1(D1283–1291).

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Figure 5. Rasip1 central domain interacts with HEG1 cytoplasmic tail. (A) Schematic representation of Rasip1 constructs. (B) HEK293T cells were transfected with FLAG-tagged Rasip1 1-265, 266-550, or 551-963. Western blot analysis shows that the HEG1 cytoplasmic tail peptide preferentially bound to FLAG-Rasip1 266-550, which contains an FHA domain. αIIb cytoplasmic tail was used as a control. Affinity Matrix was visualized by Ponceau staining. Data are representative of at least 3 independent experiments. (C) HEK293T cells were transfected with FLAG-tagged wild-type Rasip1 (WT) or Rasip1(D334-539), which lacks the FHA domain. Western blot analysis shows that, in contrast to Rasip1 wild-type, the HEG1 cytoplasmic tail did not interact with Rasip1(D334-539). Affinity Matrix was visualized by Ponceau staining. Data are representative of at least 3 independent experiments. (D) Wild-type (WT) HEG1 cytoplasmic tail peptide, ΔYF, and HEG1 1318-1339, but not αIIb cytoplasmic tail, directly bound to recombinant MBP-Rasip1 266-550 fusion protein. Coomassie blue-stained SDS-PAGE gel is representative of 3 independent experiments. All lanes were from the same gel. (E) FLAG-Rasip1 intracellular distribution was analyzed by Spinning Disk Confocal Microscopy (SDCM) in Human Umbilical Vein Endothelial Cells (HUVEC) expressing FLAG-Rasip1 or Rasip1(Δ334-539) expressed by lentiviral infection. Cells were treated with DMEM (5% FBS, 4 mM EGTA) to remove Calcium and disrupt adherens junctions. Subsequently, cells were incubated with DMEM containing 8-pCPT-2-O-Me-cAMP-AM (‘007’, Figure 5 continued on next page)
100 μM) and Calcium (2 mM) for 20 minutes to mimic junction formation/stabilization. Under these conditions, Rasip1 WT localized to cell-cell contacts in. In contrast, Rasip1Δ334-539 failed to localize to junctions albeit Rasip1-positive vesicular structures could be found in the vicinity of cell-cell contacts (indicated by the arrows). Higher magnification images of the boxed area are included. Representative images of 3 independent experiments are shown. Scale bars, 10 μm.

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Figure 6. Deletion of the Rasip1-binding site in HEG1 prevents suppression of MLC phosphorylation. (A) Levels of activated β1 integrin (9EG7) in Rasip1- or HEG1-depleted Human Umbilical Vein Endothelial Cells (HUVEC) were measured by flow cytometry. Depletion of Rasip1 (shRasip1) decreased levels of activated β1 integrin compared to control cells (shCtrl). In contrast, depletion of HEG1 (shHEG1#1) had no effect on levels of activated β1 integrin. Levels of 9EG7 binding were corrected for total β1 integrin expression. Mean values ± SEM of three independent experiments are shown. One-way analysis of variance (ANOVA) with Bonferroni’s test was used to compare each condition with control cells (shCtrl). (B and C) Myosin light chain phosphorylation (pMLC) was analyzed by Spinning Disk Confocal Microscopy (SDCM) in HUVEC, transfected or infected as indicated. siRNA- or shRNA-mediated Figure 6 continued on next page
depletion of HEG1 (B&C, siHEG1, shHEG1#1, shHEG1#2) increased MLC phosphorylation and stress fiber formation. Similarly, lentiviral depletion of Rasip1 (shRasip1) also increased MLC phosphorylation and stress fiber formation. Representative images of 3 independent experiments are shown. Scale bars, 10 µm. (D) Myosin light chain phosphorylation (pMLC) and F-Actin were analyzed by Spinning Disk Confocal Microscopy (SDCM) in Human Umbilical Vein Endothelial Cells (HUVEC), infected as indicated. Lentiviral depletion of HEG1 (shHEG1) increased levels of pMLC and actin stress fiber formation in HUVEC compared to control cells (shCtrl) which was rescued by expression of FLAG-tagged shHEG1#1-resistant wild-type murine HEG1. In contrast, Rasip1-binding deficient murine HEG1 (Δ1283-1291) (corresponding to aa 1327-1335 in human HEG1) failed to rescue pMLC expression and the increase in actin stress fibers. Expression of rescue constructs, analyzed by flow cytometry, is shown in Figure 6—figure supplement 1. Representative images of 3 independent experiments are shown. Scale bars, 10 µm.

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Figure 6—figure supplement 1. Expression of murine HEG1 in HUVEC. Human Umbilical Vein Endothelial Cells (HUVEC) were infected with FLAG-tagged murine HEG1 wild-type or Rasip1-binding deficient murine HEG1 (D1283-1291) (corresponding to aa 1327-1335 in human HEG1). Expression of FLAG-tagged mHEG1 constructs was measured by flow cytometry using an APC-conjugated anti-FLAG antibody. Analysis shows that mHEG1 wild-type (in red) and mHEG1(D1283-1291) (in blue) are equally expressed in HUVEC. In grey, non-infected cells are shown. DOI: http://dx.doi.org/10.7554/eLife.11394.013
Figure 7. HEG1-binding deficient Rasip1 fails to rescue MLC phosphorylation and EC permeability. (A and B) Myosin light chain phosphorylation (pMLC) was analyzed by Spinning Disk Confocal Microscopy (SDCM) in Human Umbilical Vein Endothelial Cells (HUVEC), infected as indicated (A). Integrated Density was measured to quantify levels of pMLC expression (B). Lentiviral depletion of Rasip1 (shRasip1) increased levels of pMLC in HUVEC by 40% compared to control cells (shCtrl) which can be rescued by expression of FLAG-tagged shRasip1-resistant wild-type Rasip1. In contrast, HEG1-binding deficient Rasip1(Δ334-539) failed to rescue pMLC expression. Expression of rescue constructs is shown by FLAG staining. Mean values ± SEM are shown. One-way analysis of variance (ANOVA) with Bonferroni’s test was used to compare each condition versus Rasip1-depleted cells. Figure 7 continued on next page.
(shRasip1). Data are from 3 independent experiments. Scale bars, 10 μm. (C) pMLC expression was analyzed by SDCM in control HUVEC (shCtrl) or HUVEC expressing FLAG-tagged Rasip1Δ334-539 (shCtrl+Δ334-539). Expression of Rasip1Δ334-539 alone induces expression of pMLC similar to Rasip1 knock-down (Panel A). Scale bars, 10 μm. (D and E) Permeability of HUVEC, seeded on fibronectin-coated Transwell filters (pore size 0.4 μm, membrane diam. 12 mm) and infected as indicated, was measured using 70kD-FITC-Dextran (D). Western blot analysis confirmed Rasip1 knock-down and expression of FLAG-tagged rescue constructs (E). Depletion of Rasip1 (shRasip1) increased permeability by two-fold compared to control cells (shCtrl) which can be rescued by expression shRasip1-resistant wild-type Rasip1. In contrast, HEG1-binding deficient Rasip1Δ334-539 failed to rescue HUVEC permeability. Mean values ± SEM are shown. One-way analysis of variance (ANOVA) with Bonferroni’s test was used to compare each condition versus control cells (shCtrl). Data are from 3 independent experiments. See also Figure 7—figure supplement 1.

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Figure 7—figure supplement 1. Generating FLAG-Rasip1 shRNA-resistant cDNA. (A) Silent mutations have been introduced in FLAG-tagged Rasip1 to render it resistant to shRNA-mediated depletion of Rasip1 protein levels. HEK293T cells were transfected with wild-type or shRNA resistant Rasip1 and co-transfected with either control shRNA (shCtrl) or Rasip1-specific shRNA (shRasip1). Western blot analysis shows that Rasip1 shRNA-resistant protein levels are not reduced when co-transfected with shRNA targeting Rasip1. (B) shRNA-resistant Rasip1 was cloned into a lentiviral expression vector (2K7). Human Umbilical Vein Endothelial Cells (HUVEC) were infected with lentiviral particles containing control shRNA (shCtrl) or Rasip1-specific shRNA (shRasip1). Endogenous Rasip1 protein levels are reduced upon expression of shRasip1. In contrast, lentivirally expressed Rasip1 (shRNA resistant) protein levels are not affected by expression of shRasip1.

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**Figure 8.** Rasip1 RA domain forms a dimer, and Rasip1 Arg182 is important for high affinity Rap1 binding. (A) Sequence alignment of human Rasip1 RA domain with human Radil. Symbols denote the degree of conservation: (*) identical, (:) conservative substitution, and (.) semi-conservative substitutions. Secondary structure elements of Radil are shown below the alignment. (B) Left: Crystal structure of Radil dimer with the dimer interface highlighted in grey. Right: Close-up view of the dimer interface. (C) Rasip1 RA domain dimer bound to two Rap1 molecules. (D) Size exclusion chromatography (SEC) analysis of Rasip1 RA domain and Rap1B alone, and in combination. (E) Binding affinity of Rasip1 RA182E with Rap1B. **Figure 8 continued on next page**
blue (PDB: 3EC8). Right: Residues 134-285 from Rasip1 were modeled using the Radil RA domain crystal structure as a template. View of Rasip1 RA domain dimer with the dimer interface highlighted: identical to Radil (magenta) and conserved (yellow). The residues highlighted are also shown in panel A with the same color code. (C) Model of the Rasip1 dimer with two RA motifs located at opposite ends suggesting it can bind two Rap1 molecules as shown. (D) Binding of the Rasip1 RA domain wild-type and R182E to Rap1 as analyzed on a Superdex-75 (10/300) gel filtration column at room temperature. Incubation of Rasip1 wild-type with Rap1 (red) resulted in complex formation with a shift towards lower volume of elution. In contrast, incubation of Rasip1(R182E) with Rap1 (black) resulted in no interaction with both proteins staying in the free state, suggesting a large reduction in affinity. Furthermore, purified Rasip1 RA domain (50 μM) alone had a large apparent molecular mass (37 kDa; green) as determined by gel filtration compared with a calculated value of 16.3 kDa, suggesting that it forms a dimer in solution. (E) Calorimetric titration of Rap1, out of the syringe into Rasip1 RA domain in the sample cell (kd = 0.77 μM). The titrations were done using monomer concentrations of Rasip1 RA domain. These data show that each Rasip1 dimer can bind two Rap1 monomers. (F) HUVECs, transfected with mito-mCherry-HEG1, were analyzed by Spinning Disk Confocal Microscopy (SDCM) for wild-type (WT) Rasip1 or Rap1-binding deficient Rasip1(R182E) localization which was visualized by FLAG staining. A fraction of wild-type Rasip1, but not Rasip1(R182E), was targeted to mito-mCherry-HEG1 positive structures. Representative images of 3 independent experiments are shown. Scale bars, 10 μm.

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Figure 9. Rap1-binding deficient Rasip1 fails to rescue MLC phosphorylation and EC permeability. (A and B) Myosin light chain phosphorylation (pMLC) was analyzed by Spinning Disk Confocal Microscopy (SDCM) in Human Umbilical Vein Endothelial Cells (HUVEC), infected as indicated (A). Integrated Density was measured to quantify levels of pMLC expression (B). Depletion of Rasip1 (shRasip1) expression in HUVEC increased levels of pMLC in HUVEC by 40% compared to control cells (shCtrl). This was rescued by expression of FLAG-tagged shRasip1-resistant wild-type Rasip1. In contrast, Rap1-binding deficient Rasip1(R182E) failed to rescue pMLC expression. Expression of rescue constructs is shown by FLAG staining. Mean values ± SEM are shown. One-way analysis of variance (ANOVA) with Bonferroni’s test was used to compare each condition versus Rasip1-depleted cells (shRasip1). Data are from 3 independent experiments. Scale bars, 10 μm. (C and D) Permeability of HUVEC, seeded on fibronectin-coated Transwell filters (pore Figure 9 continued on next page
size 0.4 µm, membrane diam. 12 mm) and infected as indicated, was measured using 70kD-FITC-Dextran (G). Western blot analysis confirmed Rasip1 knock-down and expression of FLAG-tagged rescue constructs (D). Depletion of Rasip1 (shRasip1) increased permeability by two-fold compared to control cells (shCtrl). Permeability was rescued by expression of shRasip1-resistant wild-type Rasip1. In contrast, Rap1-binding deficient Rasip1(R182E) failed to rescue HUVEC permeability. Mean values ± SEM are shown. One-way analysis of variance (ANOVA) with Bonferroni’s test was used to compare each condition versus control cells (shCtrl). Data are from 3 independent experiments.

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HEG1 supports vascular integrity by binding multiple Rap1 effectors. In endothelial cells, Rap1 activation targets Rasip1 and KRIT1 to cell-cell junctions through a direct interaction with the transmembrane receptor HEG1. Here, Rasip1 and KRIT1 regulate junction stability and vascular integrity in part by suppressing ROCK signaling.

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