Ando et al., http://www.jcb.org/cgi/content/full/jcb.201301115/DC1

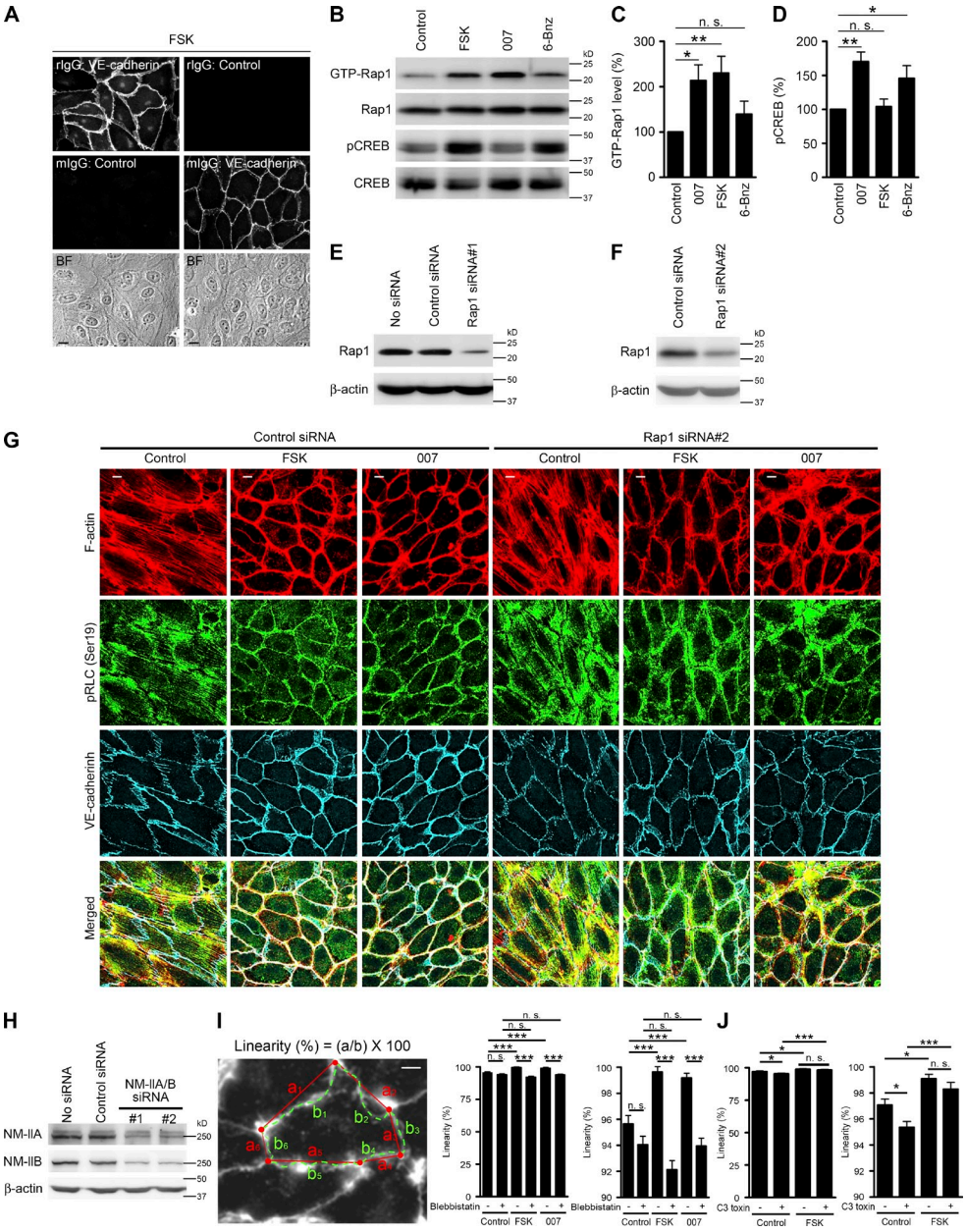

Figure S1. **Rap1 induces phosphorylation of RLC and formation of CAB at cell–cell contacts.** (A) Monolayer-cultured HUVECs plated on collagen-coated dish were starved in 0.5% BSA-containing medium 199 for 4 h, and stimulated with FSK for 20 min. Then, the cells were immunostained either with rabbit anti–VE-cadherin antibody (rIgG: VE-cadherin) and mouse control IgG (mIgG: Control; left) or with rabbit control IgG (rIgG: Control) and mouse anti–VE-cadherin antibody (mIgG: VE-cadherin; right). Rabbit and mouse antibodies are visualized with Alexa Fluor 488–labeled anti–rabbit (top) and Alexa Fluor 546–labeled anti–mouse (middle) secondary antibodies, respectively. The bright field (BF) images are shown on the bottom. Note that no fluorescence signal was observed in the staining with mouse and rabbit control IgG, confirming the specificity of immunofluorescence staining. (B) Levels of the GTP-bound form of Rap1 (GTP-Rap1), total Rap1 (Rap1), phosphorylated CREB (pCREB), and total CREB (CREB) in the HUVECs stimulated with vehicle (Control), FSK, 007, or 6-Bnz. (C and D) Levels of GTP-Rap1 (C) and pCREB (D) observed in B were quantified. Values are expressed as a percentage relative to those observed in the cells stimulated with vehicle (Control), and shown as mean ± SEM (error bars) from 10 independent experiments. (E) Lysates from the HUVECs transfected without (No siRNA) or with control siRNA or Rap1 siRNA #1 for 3 d were subjected to Western blot analyses with anti-Rap1 and anti–β-actin antibodies. (F) HUVECs transfected with control siRNA or a mixture of siRNAs targeting against Rap1A and Rap1B (Rap1 siRNA #2) were cultured for 3 d, and subjected to Western blot analysis with anti-Rap1 and anti–β-actin antibodies. (G) HUVECs were transfected with control siRNA or Rap1 siRNA #2, cultured for 2 d, and replated on a collagen-coated glass-base dish. The next day, the cells were starved in 0.5% BSA-containing medium 199 for 4 h, stimulated with vehicle (Control), FSK, or 007 for 20 min, stained with rhodamine-phalloidin (F-actin), and immunostained with anti-pRLC at Ser19 (pRLC (Ser19)) and anti–VE-cadherin antibodies. The merged images are shown at the bottom. (H) Knockdown efficiency of two sets of siRNA mixtures for NM-IIA and NM-IIB (#1 and #2) was analyzed similar to as in D. (I) The linearity of cell–cell junctions observed in Fig. 1 E was quantified. The direct distance (a, red) and junction length (b, green) between vertices were measured. Junction linearity was defined by the ratio of the direct distance between vertices (a) to the junction length (b), and expressed as a percentage [(a/b) × 100]. Values are shown as mean ± SEM from three independent experiments ($n \geq 58$). (J) Linearity of cell–cell junctions observed in Fig. 2 C was quantified similar to as in I. Values are shown as mean ± SEM (error bars) from three independent experiments ($n \geq 64$). For I and J, the full-scale (left) and zoomed-in versions (right) of the graphs are shown. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, significant difference between two groups. n.s., no significance between two groups. Bars, 10 µm.

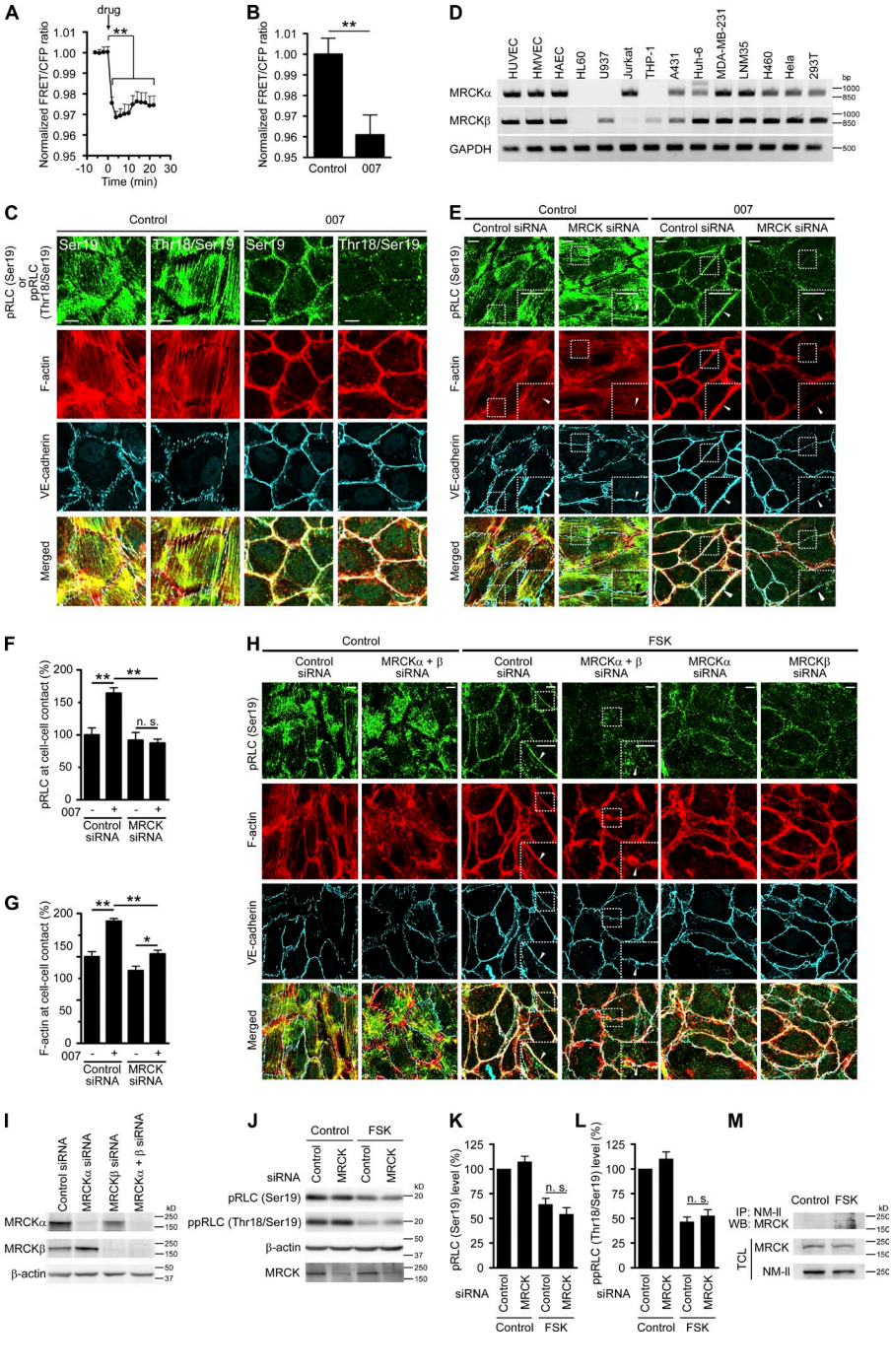

Figure S2. **MRCK is required for Rap1-induced phosphorylation of RLC and formation of CAB at cell–cell junctions.** (A) HUVECs expressing Raichu-RhoA, a FRET-based RhoA activity-monitoring probe, were stimulated with vehicle or FSK and subjected to time-lapse FRET imaging as described in the legend of Fig. 5 E. The normalized FRET/CFP ratio was calculated by dividing the FRET/CFP ratio in the cells stimulated with FSK by those in the vehicle-treated cells at the corresponding time points. The drugs were added to the culture media at time 0 as indicated by the arrow. Data are expressed as fold increase relative to that at time −6 min, and are shown as mean ± SEM (error bars) of 10 independent experiments. (B) Raichu-RhoA–expressing HUVECs were stimulated with vehicle (Control) or 007, and subjected to time-lapse FRET imaging similar to Fig. 5 E. The fold increase in FRET/CFP ratios ∼15 min after the stimulation was calculated similar to as in Fig. 5 F and shown as mean ± SEM (error bars; $n \geq 89$). (C) HUVECs were stimulated with vehicle (Control) or 007, and immunostained with either anti-pRLC at Ser19 (pRLC (Ser19)) or anti-pRLC at Thr18/Ser19 (ppRLC (Thr18/Ser19)) antibody, and anti-VE-cadherin antibody, then stained with rhodamine-phalloidin (F-actin). pRLC (Ser19) or ppRLC (Thr18/Ser19) images, F-actin and VE-cadherin images, and the merged images are shown. (D) Expression of MRCKα, MRCKβ, and GAPDH mRNA in the ECs (HUVEC, human microvascular EC [HMVEC], and human arterial EC [HAEC]) and in the non-ECs (HL60, U937, Jurkat, THP-1, A431, Huh-6, MDA-MB-231, LNM35, H460, HeLa, and 293T) was analyzed by RT-PCR analysis. (E) HUVECs transfected with control or MRCK siRNA for 2 d were stimulated with vehicle (Control) or 007, and stained similar to as in Fig. 3 C. (F and G) Quantitative relative expression values of pRLC at Ser19 (F) and F-actin (G) at cell–cell contacts compared to those in the control siRNA-transfected cells stimulated with vehicle observed in E are shown as mean ± SEM (error bars; $n \geq 40$). Similar results were obtained in three independent experiments. (H) HUVECs were transfected with control siRNA, MRCKα siRNA, MRCKβ siRNA, or a mixture of MRCKα and MRCKβ siRNAs (MRCK α + β siRNA) as indicated at the top, and stimulated with vehicle (Control) or FSK. The cells were then stained similar to as in E. (I) HUVECs were transfected similar to as in H, and subjected to Western blot analysis with anti-MRCKα, anti-MRCKβ, and anti–β-actin antibodies. (J) HUVECs transfected with control or MRCK siRNA were stimulated with vehicle (Control) or FSK similar to Fig. 3 C, and subjected to Western blot analysis with anti-pRLC at Ser19 (pRLC (Ser19)), anti-pRLC at Thr18/Ser19 (ppRLC (Thr18/Ser19)), anti–β-actin, and MRCK antibodies. (K and L) Levels of pRLC at Ser19 (K) and pRLC at Thr18/Ser19 (L) observed in J were quantified. Values are expressed as a percentage relative to those in the control siRNA-transfected cells stimulated with vehicle (Control), and shown as mean ± SEM (error bars) from eight independent experiments. (M) Cellular lysates derived from confluent HUVECs stimulated with vehicle (Control) or FSK for 10 min were immunoprecipitated with anti–NM-II antibody. Immunoprecipitates (IP: NM-II) and aliquots of total cell lysates (TCL) were subjected to Western blot analyses with anti-MRCK and anti–NM-II antibodies as indicated on the left. In E and H, the boxed areas are enlarged in the insets. Arrowheads indicate cell–cell junctions. *, $P < 0.05$; **, $P < 0.01$, significant difference between two groups. n.s., no significance between two groups. Bars, 10 µm.

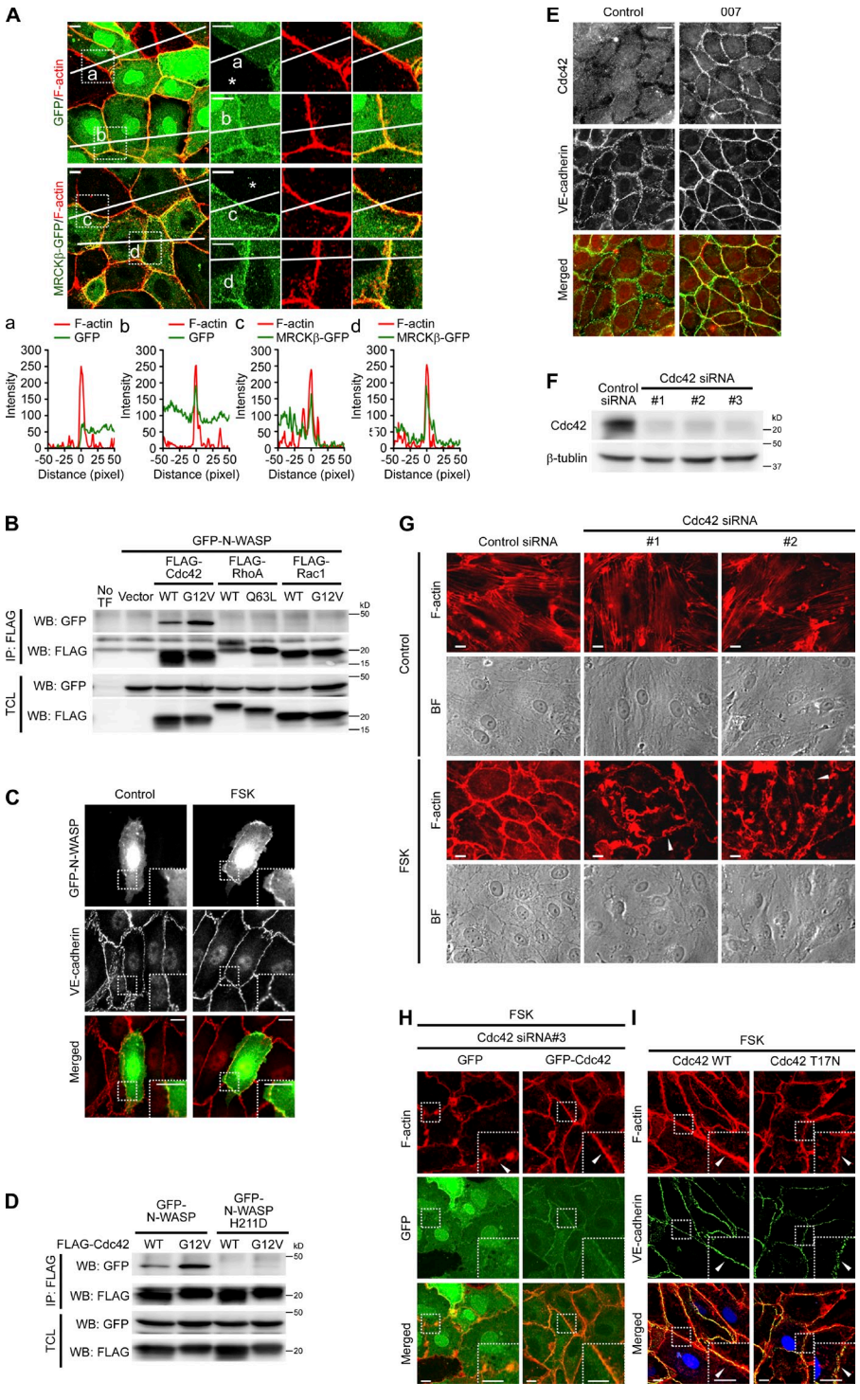

Figure S3. **FSK induces CAB formation by inducing accumulation of active Cdc42 at cell–cell junctions.** (A) Localization of GFP and MRCKβ-GFP at cell–cell junctions in the images of Fig. 3 F was quantified by line-scan analysis of fluorescence intensity. Lines are randomly drawn on the merged images of GFP (green) and F-actin (red) fluorescence in the FSK-stimulated HUVECs that express either GFP (top) or MRCKβ-GFP (bottom). The boxed areas (a–d) that include the point on the line across the cell–cell junctions are enlarged on the right. Asterisks indicate the cells that do not express GFP or MRCKβ-GFP. The fluorescence intensity profile of GFP (green line) and F-actin (red line) along the line in each boxed area are shown at the bottom. The x axes show distance from the cell–cell contacts. The increase in GFP intensity is observed at the cell–cell contact between the two GFP-positive cells (b) but not at the cell–cell contact between the GFP-positive cell and GFP-negative cell (a). Indeed, the intensity at the cell–cell contact of the two GFP-positive cells (~200) is two times higher than that of the cytoplasm (~100), which suggests that high intensity at the cell–cell contacts between the two GFP-positive cells is ascribed to the overlap of plasma membrane of GFP-positive cells. In clear contrast, high GFP signal was clearly observed not only at cell–cell contacts between the two MRCKβ-GFP-positive cells (d) but also at those between the MRCKβ-GFP–positive cell and the MRCKβ-GFP–negative cell (c), indicating that MRCKβ-GFP localizes at cell–cell contacts in FSK-stimulated cells. (B) 293T cells were transfected with the plasmid encoding the GFP-tagged CRIB domain of N-WASP (GFP-N-WASP) together with the vector expressing either FLAG-tagged wild type or constitutive active mutant of Cdc42 (Cdc42 WT and Cdc42 G12V), RhoA (RhoA WT and RhoA Q63L), and Rac1 (Rac1 WT and Rac1 G12V) as indicated at the top. Cell lysates were immunoprecipitated with anti-FLAG antibody. Immunoprecipitates (IP: FLAG) and aliquots of total cell lysates (TCL) were subjected to Western blot analyses with anti-GFP (WB: GFP) and anti-FLAG (WB: FLAG) antibodies as indicated on the left. (C) HUVECs expressing GFP-N-WASP were stimulated with vehicle (Control) or FSK, and stained with anti–VE-cadherin antibody. GFP (GFP-N-WASP) and VE-cadherin images and the merged images (GFP-N-WASP, green; VE-cadherin, red) are shown. (D) 293T cells were transfected with the plasmid encoding either GFP-N-WASP or its mutant (GFP-N-WASP H211D), in which His-211 of N-WASP was replaced with Asp, together with the vector expressing either FLAG-tagged wild type or a constitutive active mutant of Cdc42 (Cdc42 WT and Cdc42 G12V). Immunoprecipitation and subsequent Western blot analyses were performed similar to as in B. (E) HUVECs were stimulated with vehicle (Control) or FSK and immunostained with anti-Cdc42 and anti–VE-cadherin antibodies. Images of Cdc42 and VE-cadherin and the merged images (Cdc42, red; VE-cadherin, green) are shown. (F) HUVECs were transfected with control siRNA or Cdc42 siRNA (#1, #2, or #3) for 3 d, and subjected to Western blot analysis with anti-Cdc42 and anti–β-actin antibodies. (G) HUVECs transfected with control siRNA or Cdc42 siRNA (#1 or #2) were stimulated with vehicle (Control) or FSK, and stained with rhodamine-phalloidin (F-actin). F-actin and bright field (BF) images are shown. (H) HUVECs expressing either GFP or siRNA-insensitive GFP-Cdc42 were transfected with control siRNA or Cdc42 siRNA #3. The next day, the cells were replated at confluent density on collagen-coated glass base dish, starved in 0.5% BSA-containing medium 199, and stimulated with FSK. The cells were then stained with rhodamine-phalloidin (F-actin). F-actin (red) and GFP (green) images and the merged images are shown. (I) HUVECs were transfected with the plasmid encoding either Cdc42 WT-IRES-Express red fused to nuclear localization signal (ERed-NLS) or Cdc42 T17N-IRES-ERed-NLS, stimulated with FSK, stained with rhodamine-phalloidin (F-actin), and immunostained with anti–VE-cadherin antibody. F-actin and VE-cadherin images and the merged images (F-actin, red; VE-cadherin, green; ERed-NLS, blue) are shown. In C, H, and I, the boxed areas are enlarged in the bottom right corner of each image. Arrowheads indicate cell–cell junctions. Bars, 10 μm.

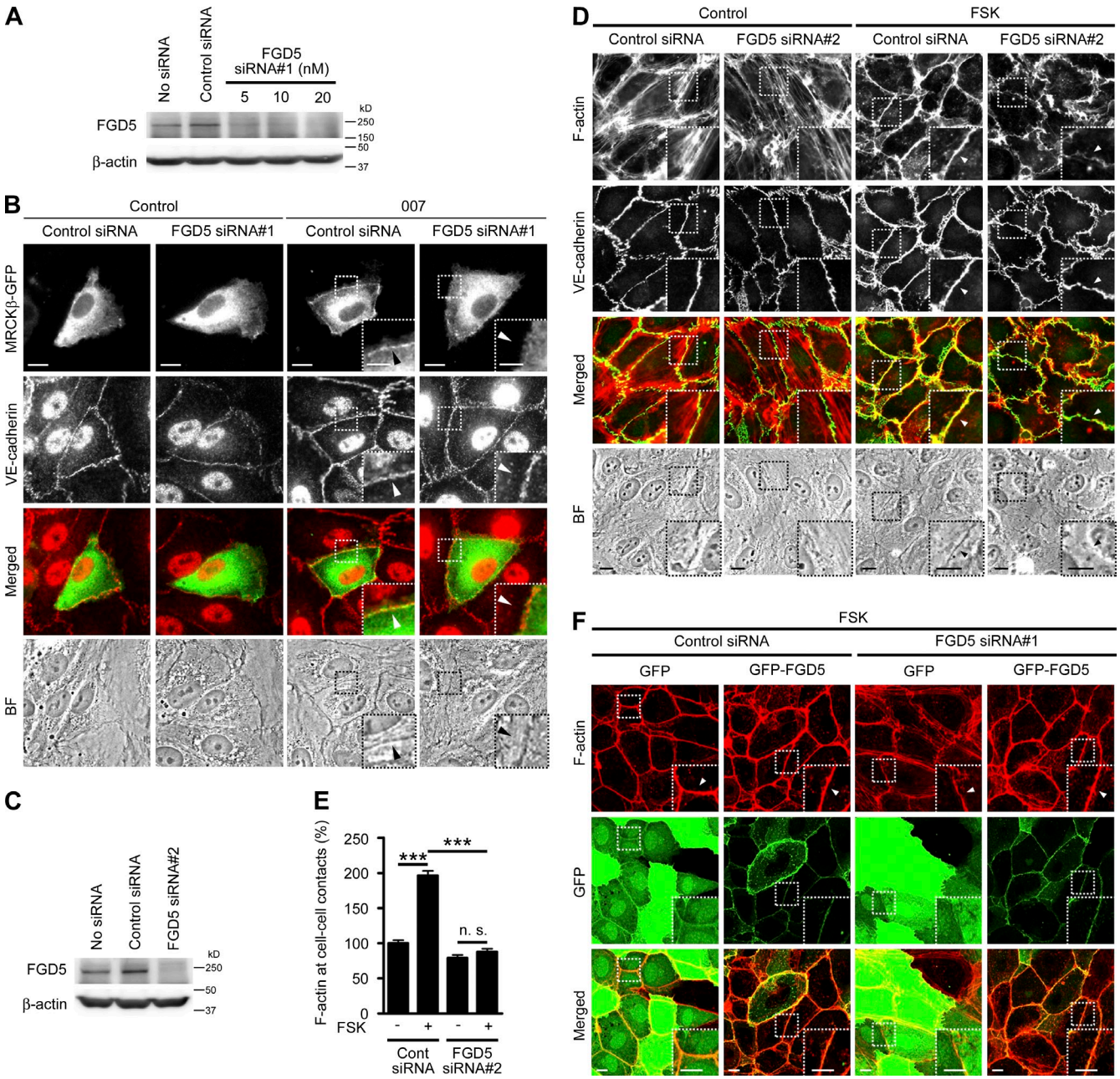

Figure S4. **Rap1 induces CAB formation though FGD5.** (A) HUVECs were transfected without (No siRNA) or with control siRNA or FGD5 siRNA #1 for 3 d, and subjected to Western blot analysis with anti-FGD5 and anti–β-actin antibodies. (B) HUVECs transfected with the plasmid encoding MRCKβ-GFP together with control siRNA or FGD5 siRNA #1 were stimulated with 007, and immunostained with anti–VE-cadherin antibody. GFP (MRCKβ-GFP) and VE-cadherin images, the merged images (MRCKβ-GFP, green; VE-cadherin, red), and bright field (BF) images are shown. (C) HUVECs transfected with FGD5 siRNA #2 were subjected to Western blot analysis similar to as in A. (D) HUVECs transfected with control siRNA or FGD5 siRNA #2 were stimulated with vehicle (Control) or FSK similar to Fig. S1 A, and stained with rhodamine-phalloidin (F-actin) and immunostained with anti–VE-cadherin antibody. F-actin and VE-cadherin images, the merged images (F-actin, red; VE-cadherin, green), and BF images are shown. (E) F-actin that accumulated at cell–cell contacts, as observed in D, was quantified. Values are expressed as a percentage relative to that in control siRNA-transfected cells stimulated with vehicle, and are shown as mean ± SEM (error bars; $n \geq 40$). Similar results were obtained in three independent experiments. ***, $P < 0.001$, significant difference between two groups. n.s., no significance between two groups. (F) HUVECs expressing either GFP or siRNA-insensitive GFP-FGD5 were transfected with control siRNA or FGD5 siRNA #1, stimulated with FSK, and stained with rhodamine-phalloidin (F-actin). F-actin and GFP images and the merged images (F-actin, red; GFP, green) are shown. In B, D, and F, the boxed areas are enlarged in the insets. Arrowheads indicate cell–cell junctions. Bars: (B, main panels, D, and F) 10 µm; (B, insets) 5 µm.

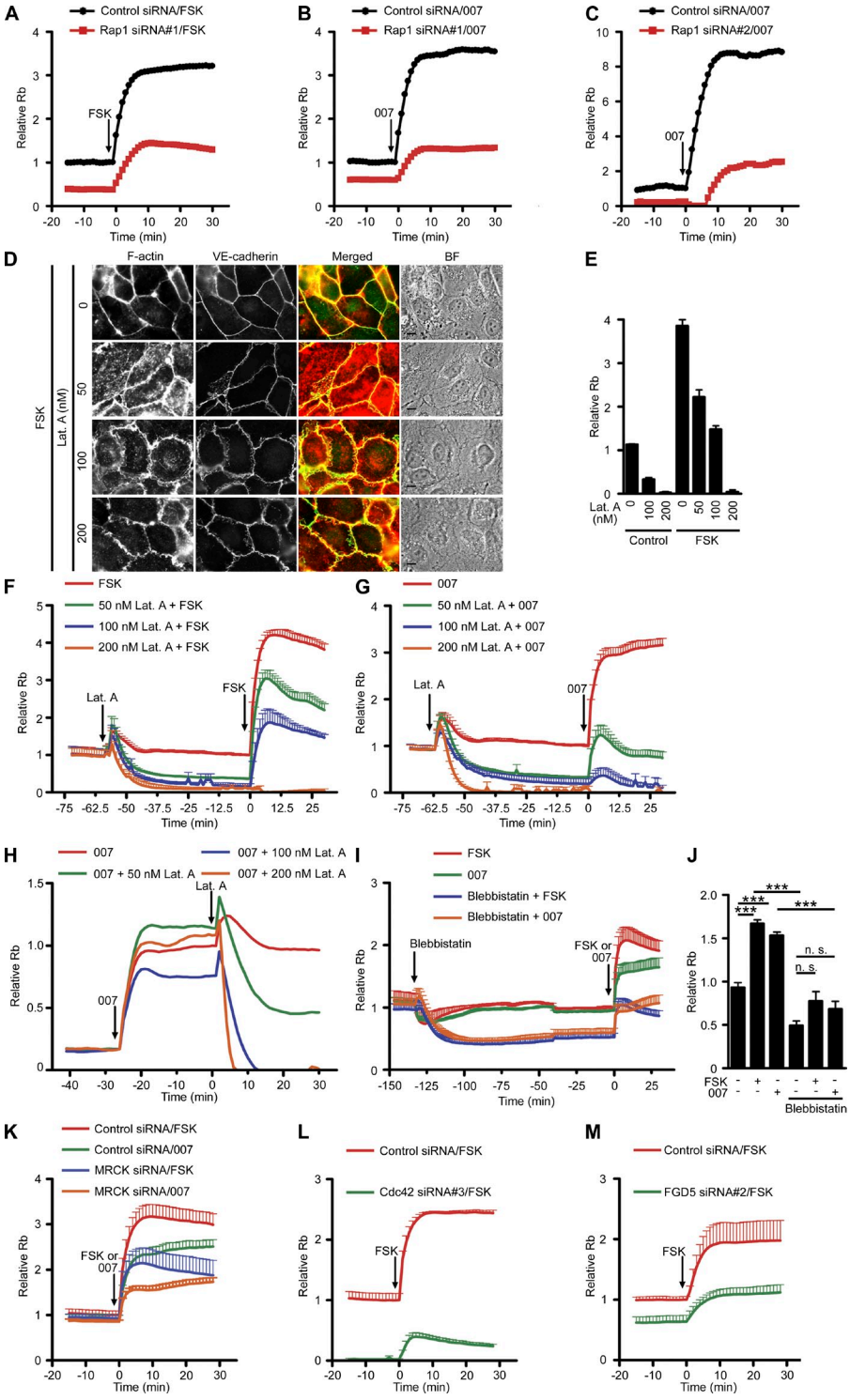

Figure S5. **Rap1 potentiates EC barrier function through FGD5–Cdc42–MRCK–NM-II–mediated reorganization of actin cytoskeleton.** (A–C) The Rb values in monolayer-cultured HUVECs were measured using the ESIC system. HUVECs transfected with control siRNA or either Rap1 siRNA #1 (A and B) or Rap1 siRNA#2 (C) were stimulated with FSK (A) or 007 (B and C). The drugs were added to the culture media at time 0. Lines in graphs show the representative Rb values versus time (min). Data are expressed as fold increase in Rb values relative to that obtained from the control siRNA-transfected cells at time 0. (D) HUVECs were treated with the indicated concentrations of latrunculin A (Lat. A) for 45 min, stimulated with FSK, stained with rhodamine-phalloidin (F-actin), and immunostained with anti–VE-cadherin antibody. F-actin and VE-cadherin images, the merged images (F-actin, red; VE-cadherin, green), and bright field images (BF) are shown. Bars, 10 µm. (E–G) HUVECs were pretreated with the indicated concentrations of latrunculin A (Lat. A), stimulated with vehicle (Control), FSK, or 007, and subjected to ECIS analysis. In E, relative Rb values at 30 min after the stimulation are shown. Data are expressed as fold increase in Rb values obtained from latrunculin A–untreated cells stimulated with vehicle, and shown as mean ± SEM (error bars; $n = 3$). In F and G, lines in graphs show the mean ± SEM (error bars) of relative Rb values ($n \geq 3$) versus time (min). The stimulants were added to the culture media at time 0. Data are expressed as fold increase in Rb values relative to those obtained from the latrunculin A–untreated cells at time 0. (H) 007-stimulated HUVECs were treated with the indicated concentrations of latrunculin A (Lat. A). Lines in the graph show the representative Rb values versus time (min). Latrunculin A was added to the culture media at time 0. Data are expressed as fold increase in Rb values relative to that obtained from the latrunculin A–untreated cells at time 0. (I) HUVECs were pretreated with or without blebbistatin, stimulated with FSK or 007, and subjected to ECIS analysis. Lines in the graph show the mean ± SEM (error bars) of relative Rb values ($n \geq 3$) versus time (min). The stimulants were added to the culture media at time 0. Data are expressed as fold increase in Rb values relative to that obtained from the blebbistatin-untreated cells stimulated with FSK at time 0. (J) Confluent HUVECs cultured for 3 d were stimulated with vehicle, FSK, or 007 in the absence or presence of blebbistatin. The relative Rb values at 30 min after the stimulation are shown. Data are expressed as fold increase in Rb values obtained from blebbistatin-untreated cells stimulated with vehicle, and shown as mean ± SEM (error bars; $n = 4$). ***, $P < 0.001$, significant difference between two groups. n.s., no significance between two groups. (K–M) HUVECs were transfected with either control siRNA or MRCK (K), Cdc42 #3 (L), or FGD5 #2 (M) siRNA, stimulated with FSK or 007, and subjected to ECIS analysis. Lines in graphs show the mean ± SEM (error bars) of relative Rb values ($n \geq 3$) versus time (min). The stimulants were added to the culture media at time 0. Data are expressed as fold increase in Rb values relative to that obtained from the control siRNA-transfected cells stimulated with FSK at time 0. In A–C, F–I, and K–M, arrows indicate the time of addition of inhibitors or stimulants.