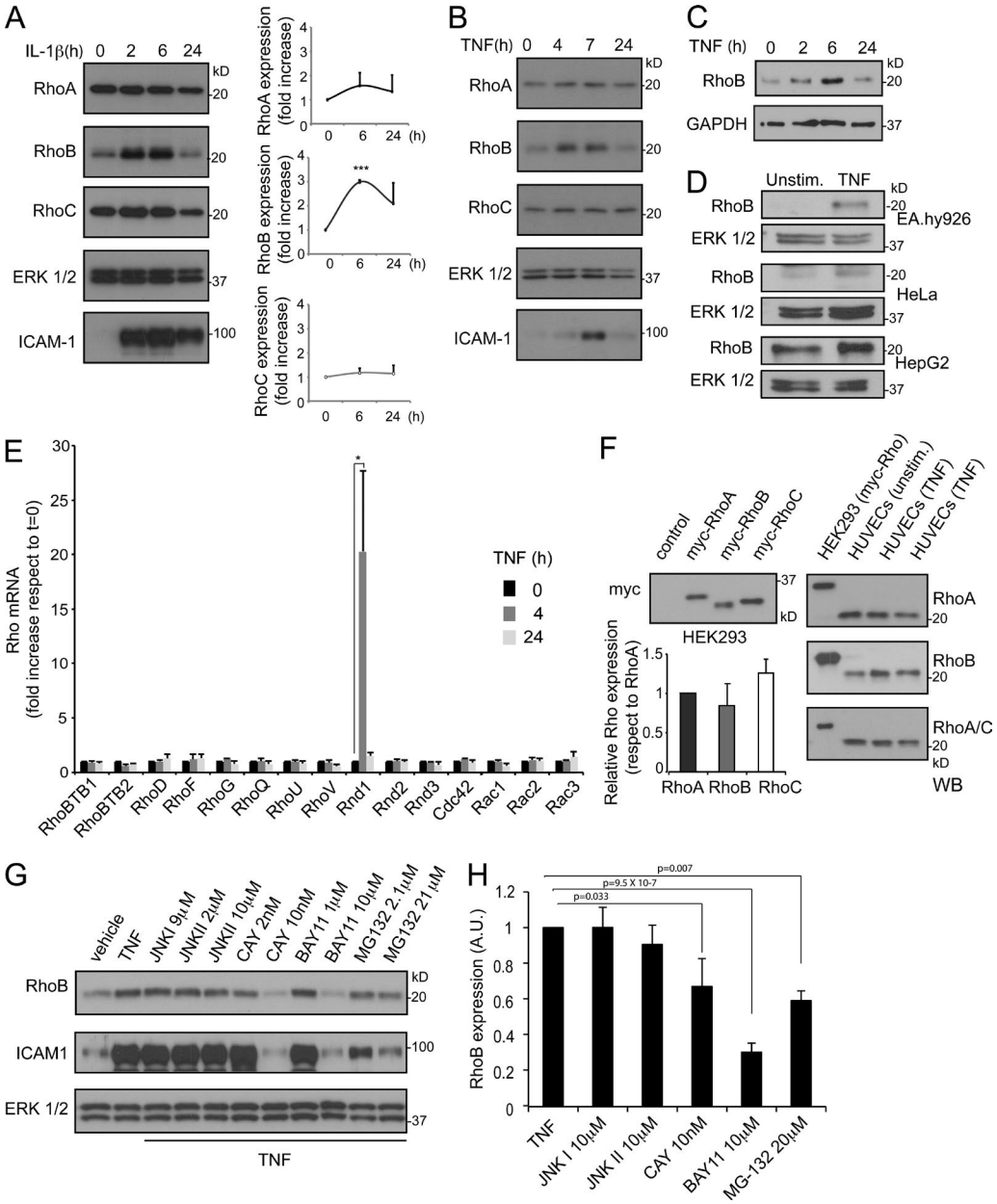


Figure S1. **Changes in RhoB protein expression in response to inflammatory cytokines in primary human microvascular endothelial cells and endothelial and epithelial cell lines.** (A) HUVECs were starved for 12 h and stimulated at different times with 10 ng/ml IL-1β, lysed, and immunoblotted for the indicated antibodies. Right graphs quantify the expression changes of RhoA, RhoB, and RhoC in response to IL-1β, and the mean + SEM of three different experiments are shown. ***, $P < 0.001$. (B) HDMVECs were starved for 12 h and stimulated at different times with TNF, lysed, and immunoblotted for the indicated antibodies. (C) Human bone marrow endothelial cells (HBMECs) were starved for 12 h, stimulated at the indicated times with 10 ng/ml TNF and lysed. Western blot (WB) was performed for the indicated proteins. (D) EA.hy926, HeLa, and HepG2 cells were left unstimulated or stimulated with TNF for 7 h and lysed, and RhoB was detected by Western blot. (E) HUVECs were stimulated with TNF at different times, their RNA was isolated and the indicated transcripts were analyzed by qPCR. Results were normalized to mRNA levels of housekeeping genes (β-actin and GADPH). Graphs represent the mean + SEM from three different experiments. *, $P < 0.05$. (F) Relative expression levels of RhoA, RhoB, and RhoC in HUVECs. Three different lysates of HEK293 cells containing similar levels of exogenous RhoA, RhoB, or RhoC tagged with myc were generated as internal standards (left). One lysate from unstimulated HUVECs and two lysates from TNF-stimulated HUVECs were blotted with antibodies against RhoA, RhoB, or RhoC, together with the HEK293 lysate containing the corresponding exogenous myc-Rho (right). The expression level of each endogenous Rho protein in TNF-stimulated HUVECs was then normalized to the intensity signal from their corresponding standard lysate, so the relative abundance of RhoB and RhoC could be quantified and represented with respect to RhoA protein expression (bottom left graph). Graph represents the mean + SEM from six different lysates of TNF-stimulated HUVECs. (G) HUVECs were stimulated with TNF for 7 h when indicated in the presence or absence of the indicated inhibitors. Cells were lysed and Western blot was performed for the indicated proteins. (H) The quantification of RhoB expression after the indicated treatments represents the mean + SEM of three different experiments.

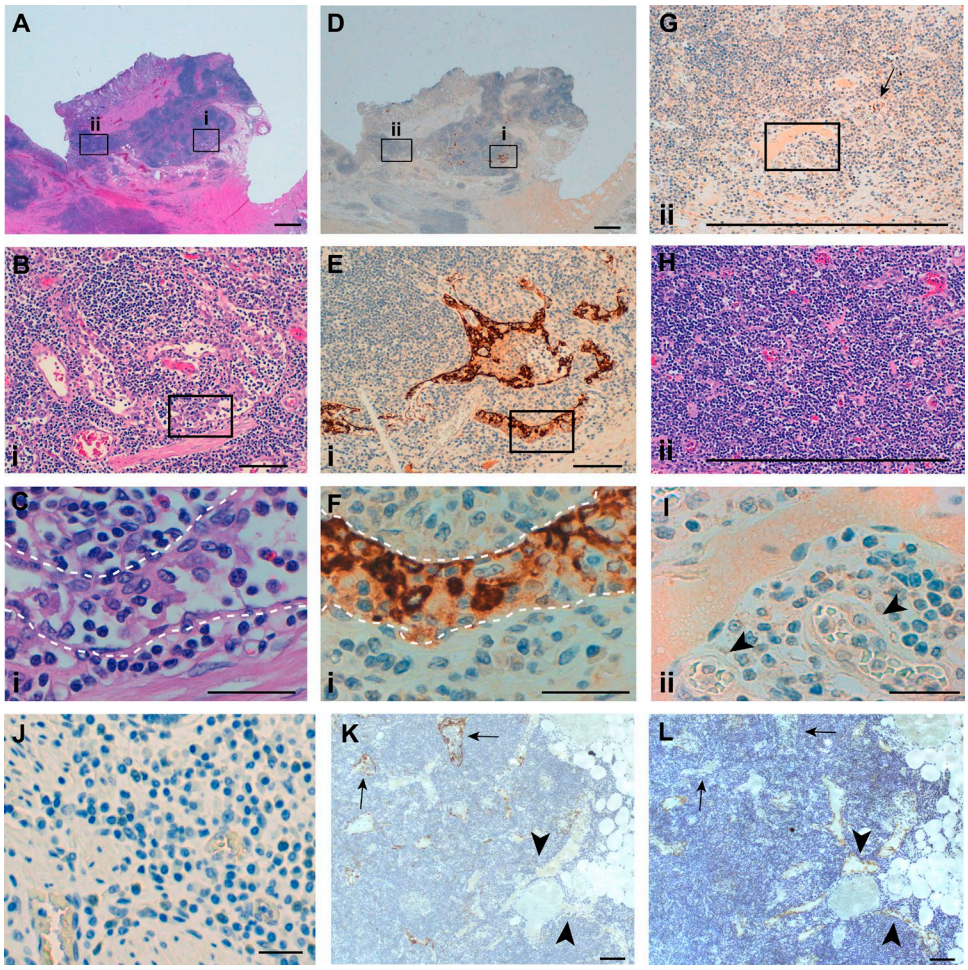


Figure S2. **RhoB is expressed in fissures from intestinal vessels of patients with Crohn's disease and in a colon adenopathy.** (A–C) Hematoxylin and eosin staining of a fissure in a small intestine from a patient with Crohn's disease. (B) Enlargement of the squared area (i) in A. (C) Enlargement of the squared area in B. (D–F) Immunodetection of RhoB in a consecutive paraffin section of the same tissue area. RhoB staining corresponds to capillaries or postcapillary venules of larger size. (E), enlargement of the squared area (i) in D. (F) Enlargement of the squared area in E. (C and F) A small vessel marked with a discontinuous white line shows capillary hyperplasia, with RhoB-positive endothelial cells emitting protrusions into the vessel lumen. RhoB is also expressed in blood cells. (G) Enlargement of the squared area (ii) in D. The arrow points to a small vessel positive for RhoB. (H) Enlargement of the squared area (ii) in A. (I) Enlargement of the squared area in G. (J) Isotype-specific control antibody (cytokeratin 7) showing no staining in small vessels. (K and L) Consecutive paraffin sections of a ganglion from a colon adenopathy stained for RhoB (K), and the lymphatic vessel marker podoplanin (L). Note that RhoB is preferentially expressed in vessels negative for podoplanin (arrows), whereas RhoB expression in podoplanin-positive vessels (arrowheads) is weaker or negative. Bars: (A and D) 1 mm; (B, E, G, and H) 100 µm; (C, F, I, and J) 20 µm; (K and L) 100 µm.

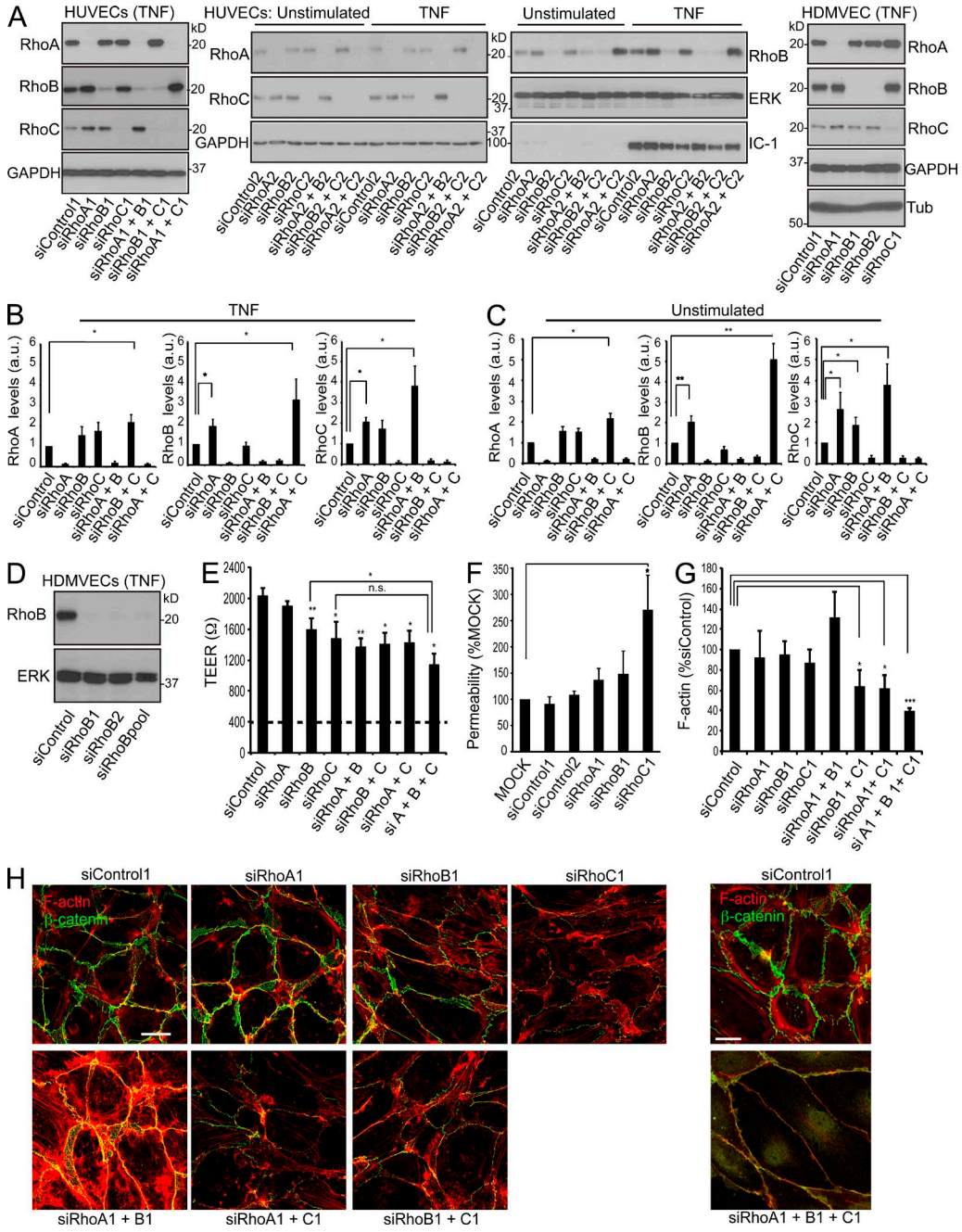


Figure S3. **Effect of siRNA oligonucleotides on RhoA subfamily member expression; additive and compensatory roles of RhoA, RhoB, and RhoC; and major role of RhoC in regulating constitutive permeability in unstimulated HUVECs.** (A) TNF-stimulated and unstimulated HUVECs and HDMVECs were transfected in parallel with control siRNA (siControl) or with siRNA targeting RhoA, RhoB, or RhoC (siRho). Rho expression was analyzed by immunoblotting 72 h after transfection. GAPDH and ERK immunoblots are shown as loading controls. (B and C) Quantification of three experiments performed with set 1 of siRNA oligonucleotides and three experiments performed with set 2. Pooled data are shown. Similar results were obtained when siRNA sets were quantified separately. *, $P < 0.05$; **, $P < 0.005$. Graph represents the mean + SEM. (D) Efficiency of individual siRNA targeting RhoB, compared with that of a pool of four siRNA oligonucleotides, which contain a patented modification pattern to reduce off-target effects (siRhoB pool). (E) Absolute TEER of HUVECs transfected with the indicated combination of siRNA oligonucleotides for 72 h. Quantification of a minimum of three experiments performed with set 1 siRNA oligonucleotides and three experiments performed with set 2. Pooled data are shown. Similar results were obtained when siRNA sets were quantified separately. Discontinuous line marks the basal resistance values in empty electrodes. *, $P < 0.05$; **, $P < 0.005$. Graph represents the mean + SEM. (F) Permeability to dextran-FITC in HUVECs previously transfected with the indicated siRNAs and plated on transwells for 48 h. Graph represents the mean + SEM from three different experiments. *, $P < 0.05$. (G and H) siRNA-transfected HUVECs were fixed and stained for F-actin and β-catenin. Quantification of the intensity of F-actin staining normalized to cells transfected with siControl1. *, $P < 0.05$; ***, $P = 2.19 \times 10^{-6}$. Graph represents the mean + SEM F-actin detected with phalloidin-TRITC. Bar, 20 μm.

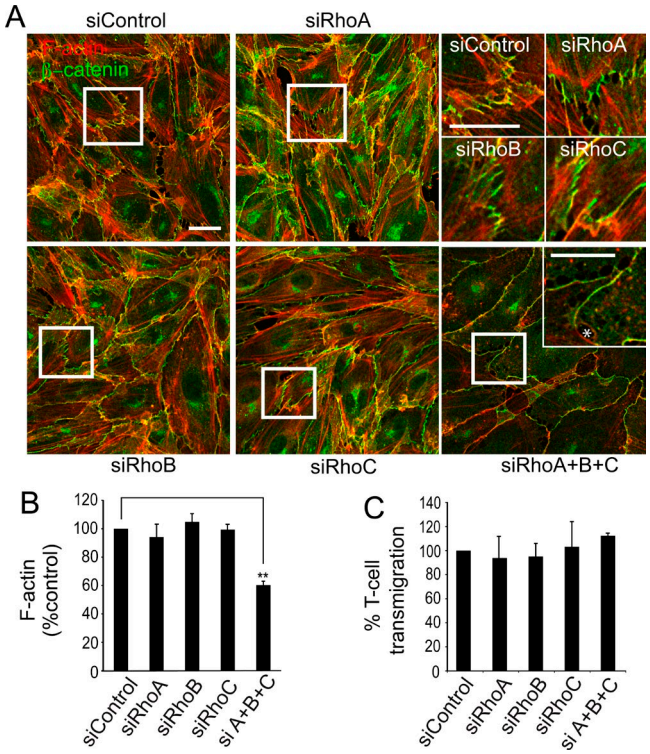


Figure S4. **Effects of single and triple knockdown of RhoA, RhoB, and RhoC on F-actin levels and T cell transmigration in TNF-stimulated HUVECs.** (A) Changes in F-actin staining intensity and adherens junction morphology in siRNA transfected HUVECs stimulated with TNF for 7 h. Bar, 20 µm. Top right enlargements corresponded to the squared areas in images from single Rho-depleted cells. (B) Intensity of F-actin staining per cell was quantified in three different experiments. Graphs shows the mean + SEM. **, $P = 4 \times 10^{-4}$. (C) HUVECs were transfected with the indicated siRNA oligonucleotides. 24 h after transfection, cells were seeded on 3-µm-pore transwells for 48 h to confluence and stimulated with TNF for 7 h. Calcein-labeled T-lymphoblasts were added to the top of the transwells. After 4 h, calcein in bottom wells was measured in a fluorescent microplate reader to detect transmigrated T cells. Graph represents the mean + SEM of three different experiments. Statistical tests between siControl and siRho cells yielded $P > 0.05$.

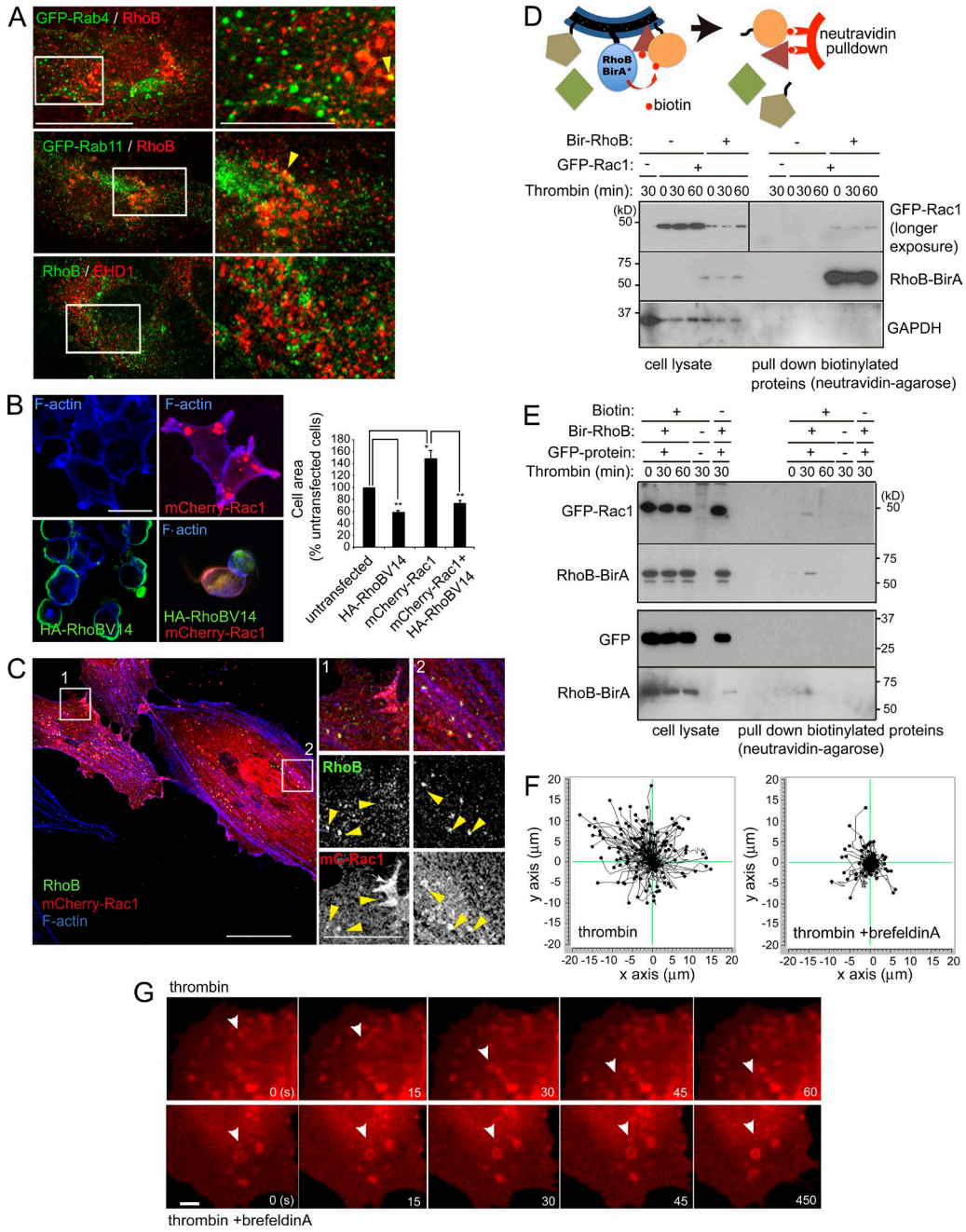
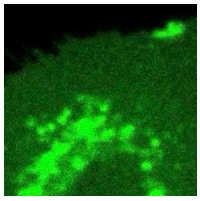
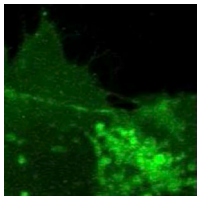


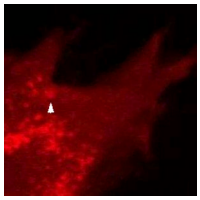
Figure S5. **Subcellular localization of RhoB, RhoB effect on Rac1-induced protrusions, BioID analysis with neutravidin pulldowns, and effect of brefeldinA on mCherry-Rac trafficking.** (A) Minor colocalization between endogenous RhoB and the indicated Rab-GFP proteins and EHD-1. (B) Active RhoB negatively regulates Rac1-induced membrane extensions. mCherry-Rac1 expression in HEK293 cells is sufficient to induce membrane extensions. RhoBV14 co expression prevents Rac1 membrane protrusions. Right graph quantifies cell area in at least 12 cells per condition from three different experiments and shows the mean + SEM; *, P = 0.011; **, P ≤ 0.003. Bars: 20 µm; (enlarged area) 10 µm. (C) Endogenous RhoB and mCherry-Rac1 partly colocalize in a vesicular compartment in confluent, TNF-pretreated HDMECs stimulated with thrombin for 2.5 h (arrowheads). mCherry-Rac1 is also localized in nascent membrane protrusions (arrow). Bars: 20 µm; (enlarged area) 10 µm. (D) BioID assay by expressing BirA-RhoB. HEK293 cells were transfected with BirA-RhoB and GFP-Rac1 when indicated. Biotinylated proteins were precipitated by pull down assays with neutravidin-agarose. Cell lysates and pull downs were immunoblotted for the indicated proteins. In the presence of BirA-RhoB, a fraction GFP-Rac1 is detected in the pull-down assay. BirA-RhoB biotinylates itself and also precipitates with neutravidin-agarose. Note in the cell lysates that BirA-RhoB reduces the GFP-Rac-1 expression. (E) Parallel BioID assay between GFP-Rac1 and BirA-RhoB (top Western blots) and GFP and BirA-RhoB (bottom Western blots) in the presence or absence of 50 µm biotin. (F and G) mCherry-Rac1 was expressed in HUVECs for at least 24 h, as in Fig. 7 I. TNF-pretreated cells were stimulated with thrombin between 60 and 100 min, and 10 µg/ml Brefeldin A was added 20 min after thrombin stimulation when indicated. Rac1 vesicular movement was recorded by time-lapse confocal microscopy at 15-s intervals during a minimum of 5 min. (F) Graphs represents the tracks of a mean of eight vesicles per cell, six cells per experiment, from three different experiments. Tracks are plotted with a common origin. (G) Arrowheads point to mCherry-Rac–positive vesicles. Bar, 20 µm.



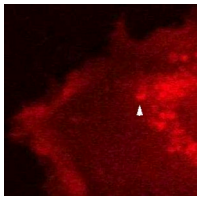
Video 1. **GFP-RhoB was expressed in HUVECs for 24 h.** TNF-pretreated cells were analyzed by time-lapse confocal microscopy at 20-s interval during a 20-min period. Video is displayed at six frames per second. Frames in Fig. 6 A.



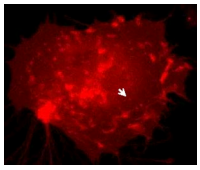
Video 2. **GFP-RhoB was expressed in HUVECs for 24 h.** TNF-pretreated cells were stimulated with thrombin for at least 60 min and then analyzed by time-lapse confocal microscopy at 20-s interval during a 20-min period. Video is displayed at six frames per second. Yellow arrowhead points to vesicles moving toward the cell periphery. Red arrows point to vesicles moving away from cell periphery. Frames in Fig. 6 A.



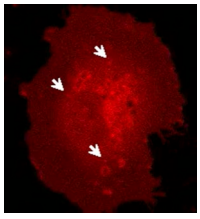
Video 3. **HUVECs were transfected with control siRNA for 72 h.** mCherry-Rac1 was expressed for the last 24 h. TNF-pretreated cells were stimulated with thrombin between 60 and 100 min and Rac1 vesicular movement was recorded by time-lapse confocal microscopy at 15-s intervals during a minimum of 5 min. Arrowhead points to a mCherry-Rac positive vesicle. Video is displayed at 6 frames per second. Frames in Fig. 7 I.



Video 4. **HUVECs were transfected with siRNA targeting Rac1 for 72 h.** mCherry-Rac1 was expressed for the last 24 h. TNF-pretreated cells were stimulated with thrombin between 60 and 100 min and Rac1 vesicular movement was recorded by time-lapse confocal microscopy at 15-s intervals during a minimum of 5 min. Arrowhead points to a mCherry-Rac positive vesicle that divides in two and merges again. Video is displayed at six frames per second. Frames in Fig. 7 I.



Video 5. **HUVECs expressing mCherry-Rac1 were pretreated with TNF for 7 h and stimulated with thrombin between 60 and 100 min.** Rac1 vesicular movement was recorded by time-lapse confocal microscopy at 15-s intervals during a minimum of 5 min. Arrowheads point to mCherry-Rac–positive vesicles. Video is displayed at 10 frames per second. Frames in Fig. S5 G.



Video 6. **HUVECs expressing mCherry-Rac1 were pretreated with TNF for 7 h and stimulated with thrombin between 60 and 100 min.** 20 min after thrombin stimulation, cells were incubated with 10 µm/ml brefeldin A. Rac1 vesicular movement was recorded by time-lapse confocal microscopy at 15-s intervals during a minimum of 5 min. Arrowheads point to mCherry-Rac–positive vesicles. Video is displayed at 10 frames per second. Frames in Fig. S5 G.

Table S1. **Summary of RhoB expression levels detected in human tissues**

Control	Crohn´s disease (no fissure)	Crohn´s disease (fissure)
Small intestine		
–	–	+
–	–	+
–	–	+/–
–	–	–
Large intestine		
–	–	+
–	–	+/–
–	–	+/–
–		–
Liver	Hepatitis B virus infection	Allograft rejection
–	+	+
+	+	
+		
Ganglion from colon adenopathies		
Blood vessels	Lymphatic vessels	
+	–	
+	–	

Table S2. **List of qPCR primers**

mRNA	Primers
β-Actin	F: 5′-CAGGCACCAGGGCGTG-3′
	R: 5′-GTGAGGATGCCTCTCTTGCTCT-3′
Cdc42	F: 5′-TGGATACAAAACTATTTCAGCAATGC-3′
	R: 5′-GTAGGATATCAGGAGACATGTTTTACC-3′
GAPDH	F: 5′-AGCCACATCGCTCAGACAC-3′
	R: 5′-CGCCCAATACGACCAAAT-3′
Rac1	F: 5′-ACACTTGCTCTCCTATGTAGTTCTC-3′
	R: 5′-TCACTCCATTACAGTACAATGTTATGTC-3′
Rac2	F: 5′-GCAAGACCTGCCTTCTCATCA-3′
	R: 5′-GCTGTCCACCATCACATTGG-3′
Rac3	F: 5′-GTACATCCCCACCGTTTTTG-3′
	R: 5′-GGCTCACCAGAGAGAAGCAG-3′
RhoA	F: 5′-GAAGAGGCTGGACTCGGATT-3′
	R: 5′-TCACCAACAATCACCAGTTTCT-3′
RhoB	F: 5′-TATGTGGCCGACATTGAGG-3′
	R: 5′-GCGGTCGTAGTCCTCCTG-3′
RhoC	F: 5′-CCGGAGGTCTACGTCCCTAC-3′
	R: 5′-ATAGTCTTCCTGCCCTGCTG-3′
RhoD	F: 5′-GTGAATCATTTCTGCAAGAAGG-3′
	R: 5′-GCTTGTTCACCAGTGATTTG-3′
RhoF	F: 5′-GTCTATCTGGTAGTGGGTAAAC-3′
	R: 5′-AGGATACAGTGGTGCCTATC-3′
RhoG	F: 5′-TGGGAACACTGGGTATTCTCATG-3′
	R: 5′-CACCACAATAGGCAGCAACAAC-3′
RhoH	F: 5′-GAGAAGTAACATTCTGCAAATCGC-3′
	R: 5′-AGCACACGCCATTCAGCAAG-3′
RhoQ/TC10	F: 5′-TGTGAAAGAGGAGTGGGTAC-3′
	R: 5′-TAAAGTTTTGGGGTCATCTCG-3′
RhoU	F: 5′-AAATGGGTGCCGGAGATTCG-3′
	R: 5′-CCAACTCAATGAGGACTTTGACATC-3′
RhoV	F: 5′-GTCAGTCACCTCCGAGCAGTTTG-3′
	R: 5′-CCCTCCATAATGCCAAGCGTTCC-3′
RhoBTB1	F: 5′-GCAAGCAGTATTGGATTATCTC-3′
	R: 5′-TGGTCAACTCCTGAACGG-3′
RhoBTB2	F: 5′-GAGGGACCCCTGACATTTCAC-3′
	R: 5′-CATTAAACGGGACACCACTACATG-3′
Rnd1	F: 5′-CTATCCAGAGACCTATGTGCC-3′
	R: 5′-CGGACATTATCGTAGTAGGGAG-3′
Rnd2	F: 5′-TCCTGATTCTGATGCTGTGCTC-3′
	R: 5′-ATTGGGGCAGAACTCTTGAGTC-3′
Rnd3	F: 5′-GACAGTGTCCTCAAAAAGTGGAAA-3′

F, forward; R, reverse.