Supporting Information

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SI Materials and Methods

Antibodies and Expression Plasmids. Affinity-purified rabbit antibodies against human brefeldin A-inhibited guanine nucleotide-exchange proteins (BIG)1 and BIG2 were described (1). Rabbit polyclonal antibodies against nonmuscle myosin II heavy chain proteins (NMHC) IIA (PRB-440P) were purchased from Covance Research Products Inc., against HA (A190-108A) and myosin phosphatase-targeting subunit 1 (MYPT1) (A300-889A) from Bethyl Laboratories, against c-myc from Sigma-Aldrich (C3956), against GFP from Clontech (632460), against regulatory light chain (RLC) (3672) and phospho-RLC (T18/S19) (3674) from Cell Signaling. Rabbit polyclonal antibodies against NMHC IIB and IIC were kindly provided by Robert S. Adelstein and Mary Ann Conti (National Heart, Lung, and Blood Institute, National Institutes of Health). Mouse monoclonal antibodies against HA were purchased from Covance Research Products Inc. (MMS-101P), against c-myc from Invitrogen (P/N 46-0603), against myosin light chain kinase (MLCK) from Sigma-Aldrich (M7905), against protein phosphatase type 1(PP1) (sc-7482), PP1cδ (sc-365678), and rho-associated protein kinase 1 (ROCK1) (sc-17794) from Santa Cruz Biotechnology, against ROCK2 from BD Bioscience (610623), and against GAPDH from Millipore (MAB374). Goat polyclonal antibodies against PP1cα (sc-6104) and PP1cγ (sc-6108) were purchased from Santa Cruz Biotechnology.

Plasmids encoding HA-BIG1 fragments were previously described (2). Plasmids encoding full-length human NMHC IIA (3), C-terminal GFP-tagged RLC mutants (T18A/S19A and RLC-T18D/S19D) (4), and myc-MYPT1 (5) were from Addgene. Plasmids pCMV6-XL4-BIG2, pCMV6-Entry-PP1cδ, and pCMV6-Entry-RLC were from Origene Technologies. For synthesis of proteins in vitro in wheat germ extract, BIG1 and BIG2 fragments were subcloned into pGADT7 vector (Clontech). Plasmid pCMV6-XL6-myosin IIA was a generous gift from Zissis C. Chroneos (The Pennsylvania State University, University Park, PA).

Cell Culture and Transfection. HeLa cells (American Type Culture Collection) were grown in DMEM (Invitrogen) with 10% (vol/vol) FBS (Invitrogen) in an atmosphere of 5% CO_2 at 37 °C. HilyMax DNA transfection reagent (Dojindo Molecular Technologies) was used, according to the manufacturer's protocol, for transient transfection 24 h before cell analyses. ON-TARGETplus siRNAs and related reagents were designed to deplete selected proteins (Dharmacon RNA Technologies). Sense sequences of BIG1- or BIG2-specific siRNAs were GUCCAAAUGUCCUCGCAUA and CCAAAAGAUUGACCGAUUA, respectively. Negative control was Dharmacon siCONTROL nontargeting siRNA. HeLa cells, 24 h after plating (*ca.* 50% confluent), were transfected with 100 nM siRNA using DharmaFECT1 reagent according to the manufacturer's instructions and harvested 72 h later. To quantify endogenous or overexpressed proteins, cells were broken in radioimmunoprecipitation assay buffer [50 mM Tris·HCl (pH 7.4), 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, and EDTA-free protease inhibitor mixture] or Nonidet P-40 buffer [20 mM Tris·HCl (pH 7.4), 50 mM NaCl, 0.1% Nonidet P-40, and EDTA-free protease inhibitor mixture] before analysis of samples (5 μg protein) by Western blotting.

Immunoprecipitation and Western Blotting. To cross-link interacting proteins before IP, cells were treated (30 min, room temperature) with 2 mM dithiobis (succinimidylpropionate) (Pierce), a thiol-cleavable cross-linker dissolved in dry DMSO, which was quenched with 20 mM Tris buffer (pH 7.5); cells were mechanically broken (passage through a 28-gauge needle) in ice-cold Nonidet P-40 buffer. Samples of proteins (100 μg) were incubated (4 °C with mixing, overnight) with 2 μg of specific antibodies or control IgG and 30 μL of Dynabeads Protein G (4 °C with mixing, 6 h) (Invitrogen). After immunoprecipitation (IP), proteins were eluted from beads, separated in 4–12% NuPAGE gel (Invitrogen), and transferred to nitrocellulose membranes (Invitrogen), which were blocked with 5% nonfat milk (Bio-Rad), reacted with primary and secondary (coupled to horseradish peroxidase) antibodies, and exposed in an LAS-4000 system (FUJIFILM) after development using Super Signal Chemiluminescent substrate (Pierce). Proteins were quantified using a BCA Protein Assay Kit (Pierce).

After densitometric quantification, amounts of indicated proteins in each experiment were expressed relative to that in the control (usually nonspecific siRNA) for the same cells =100%. Data are reported as means ± SEM of values from three experiments. In most experiments, samples analyzed were 5% of input (before IP) and 50% of IP, or less if a large percentage of IP was expected.

Immunofluorescence and Image Quantification. Cells grown on collagen I (50 μg/mL; BD Bioscience)-coated slides (Nunc Brand Product) were fixed and prepared for confocal microscopy as previously described (6). F-actin was reacted with Alexa Fluor 594-conjugated phalloidin (Invitrogen). Images were acquired by an LSM 510 META laser confocal microscope (Carl Zeiss) using 40×/1.3 N.A. oil objective lens, set to scan *ca.* 1-μm layers, at a resolution of 512 × 512 pixels. Projection view and optical sections were processed digitally using CLSM5 Zeiss Browse Image software. Figures were assembled and labeled using Adobe Photoshop (Adobe Systems). Phalloidin staining and quantification of F-actin fluorescence were performed as previously described (7). Fluorescence intensity within an outlined perimeter of each cell was measured in pixels by ImageJ software (http://rsb.info.nih.gov/ij/).

In Vitro Protein Synthesis in Wheat Germ Extract System. Reactions using the TNT Coupled Wheat Germ Extract System (Promega), performed according to the manufacturer's instructions, contained 25 μL of wheat germ extract, 2 μL of reaction buffer, 1 μL RNA polymerase (SP6 or T7), 1 μL of 1 mM amino acid mixture minus methionine, 1 μL of 1 mM amino acid mixture minus leucine, 1 μg DNA template, and nuclease-free water to a total volume of 50 μL. After incubation at 30 °C for 2 h, 20 μL protein products were analyzed by IP and Western blotting. All findings were replicated at least three times.

Migration Assays. Details are in ref. 7. Briefly, Transwell membranes (6.5-mm diameter, 8-μm pore; Corning Life Sciences) were coated (overnight, 4 °C) on the bottom with fibronectin (10 μg/mL in DMEM; Millipore) and washed in PBS to remove excess ligand. The lower chamber contained 600 μL of 10% FBS in DMEM. Serum-deprived (overnight) cells (10^5 cells) in 0.1 mL DMEM with 1% BSA in upper chambers were incubated (4 h, 37 °C) before fixation (4% formaldehyde, 15 min, room temperature) of cells on filters, and staining with 1% crystal violet (3 h, RT). Cells in images of five fields per filter-bottom surfaces captured using a phase-contrast microscope equipped with a digital camera were counted by Image-Pro Plus software.

Statistics. Data are presented as means ± SEM of values from three experiments. One-way ANOVA was used to compare the mean values of multiple groups. Considered statistical significance was $P < 0.05$.

1. Yamaji R, et al. (2000) Identification and localization of two brefeldin A-inhibited guanine nucleotide-exchange proteins for ADP-ribosylation factors in a macromolecular complex. *Proc Natl Acad Sci USA* 97(6):2567–2572.
2. Shen X, et al. (2008) Interaction of brefeldin A-inhibited guanine nucleotide-exchange protein (BIG) 1 and kinesin motor protein KIF21A. *Proc Natl Acad Sci USA* 105(48):18788–18793.
3. Wei Q, Adelstein RS (2000) Conditional expression of a truncated fragment of nonmuscle myosin II-A alters cell shape but not cytokinesis in HeLa cells. *Mol Biol Cell* 11(10):3617–3627.
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5. Eto M, Kirkbride JA, Brautigan DL (2005) Assembly of MYPT1 with protein phosphatase-1 in fibroblasts redirects localization and reorganizes the actin cytoskeleton. *Cell Motil Cytoskeleton* 62(2):100–109.
6. Li CC, et al. (2011) Effects of brefeldin A-inhibited guanine nucleotide-exchange (BIG) 1 and KANK1 proteins on cell polarity and directed migration during wound healing. *Proc Natl Acad Sci USA* 108(48):19228–19233.
7. Li CC, et al. (2007) ARL4D recruits cytohesin-2/ARNO to modulate actin remodeling. *Mol Biol Cell* 18(11):4420–4437.

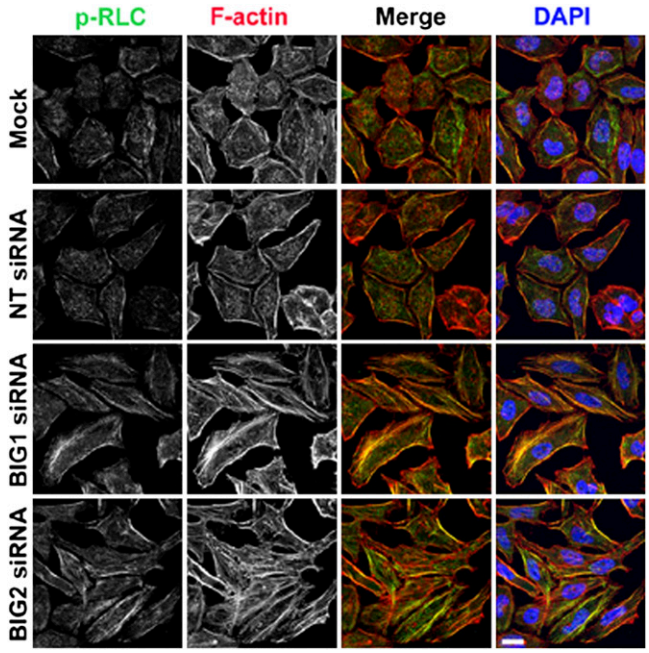

Fig. S1. Effects of BIG1 or BIG2 depletion on intracellular distribution of p-RLC and F-actin. After incubation for 72 h with vehicle alone (Mock) or control (NT) or specific BIG1 or BIG2 siRNA, cells were fixed and reacted with indicated antibodies. (Scale bars, 10 μm.)

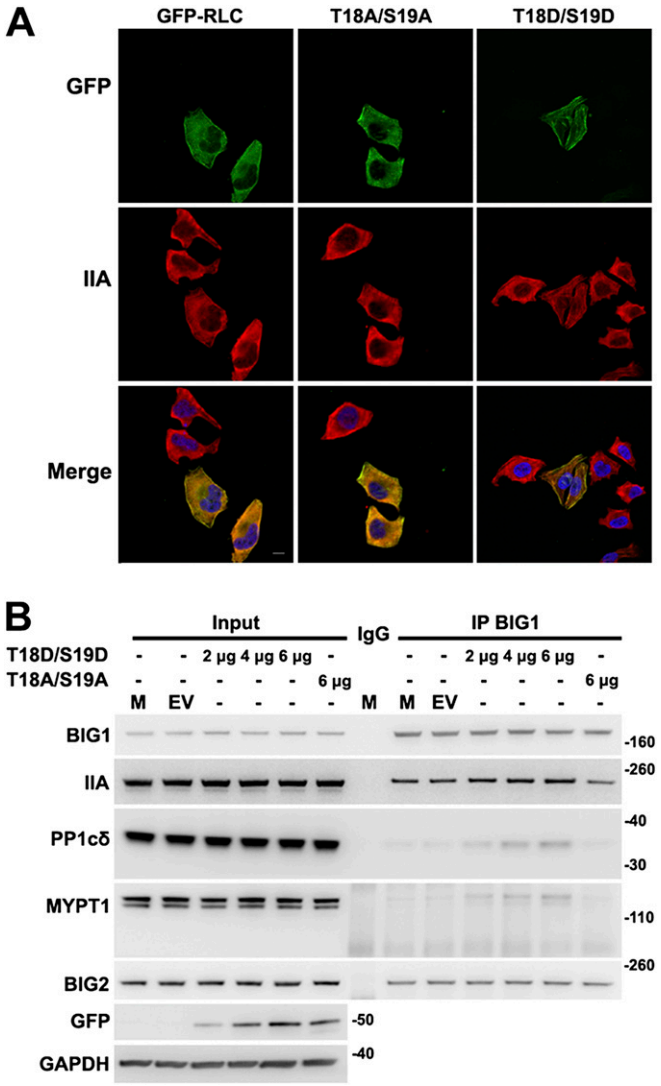

Fig. S2. Effects of overexpression of RLC mutant constructs: intracellular distribution and effects on co-IP of NMHC IIA, PP1cδ, MYPT1, and BIG2 with BIG1. (*A*) HeLa cells expressing GFP-RLC, GFP-RLC-T18A/S19A, or GFP-RLC-T18D/S19D (green) were fixed and immunostained for NMHC IIA (red). (Scale bar, 10 μm.) (*B*) Cells were incubated with vehicle (M), empty vector (EV), or 2, 4, or 6 μg of GFP-RLC-T18D/S19D or 6 μg of GFP-RLC-T18A/S19A for 24 h before BIG1 IP for analysis of collected proteins.

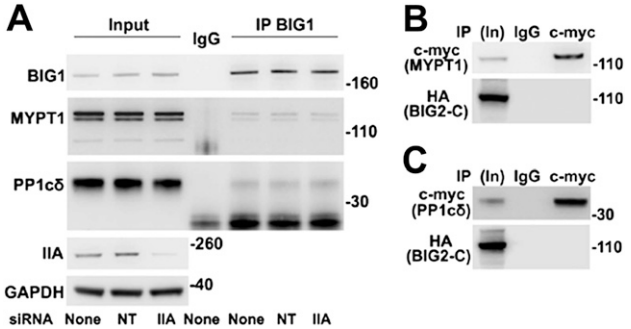

Fig. S3. Effects of depletion of NM IIA on co-IP of MYPT1 or PP1cδ with BIG1, and interactions of BIG2-C with MYPT1 and PP1cδ. (*A*) Cells were incubated for 72 h without (None) or with NMHC IIA siRNA before IP of endogenous proteins with antibodies against BIG1 and analysis of collected proteins (50%) by Western blotting with indicated antibodies. (*B* and *C*) In vitro-synthesized BIG2-C and c-myc-MYPT1 or c-myc-PP1cδ were mixed before IP with control IgG or c-myc antibodies, yielding no evidence of their interaction with BIG2-C.

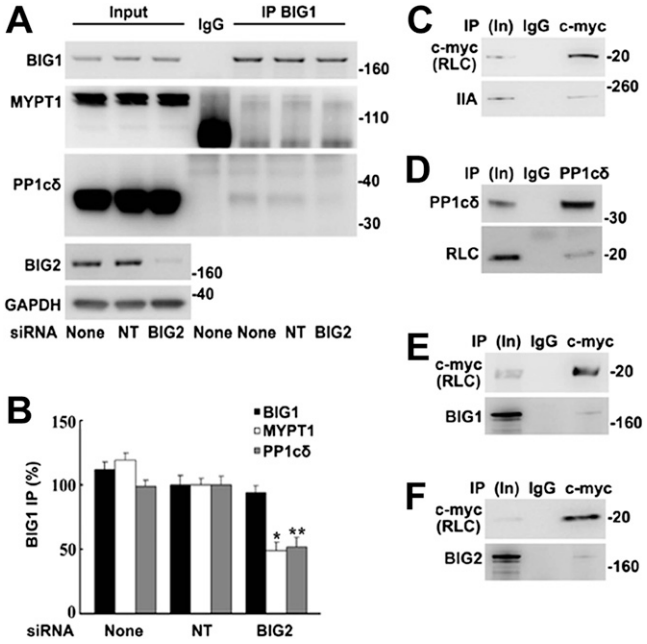

Fig. S4. Effects of BIG2 depletion on co-IP of MYPT1 or PP1cδ with BIG1, and interactions of RLC with NMHC IIA, PP1cδ, BIG1, and BIG2. (*A* and *B*) Cells were incubated without (None) or with BIG2 siRNA for 72 h before IP of BIG1 and quantification of BIG1, MYPT1, and PP1cδ among collected proteins. Amounts of BIG1, MYPT1, and PP1cδ from BIG1 IP were expressed relative to that of the same protein in NT cells (=100%) in each experiment. Data are means ± SEM of values from three experiments. *$P < 0.005$ vs. NT, **$P < 0.01$ vs. NT. (*C* and *D*) In vitro-synthesized RLC and NMHC IIA or PP1cδ were mixed before IP and analysis of collected proteins by Western blotting with indicated antibodies. (*E*) HA-BIG1 full-length and c-myc-RLC were synthesized and mixed as indicated. Proteins (50%) from IP with antibodies against c-myc were analyzed by Western blotting with antibodies against c-myc (RLC) and BIG1. (*F*) Full-length BIG2 and c-myc-RLC were synthesized and mixed. Samples taken before (In, 5%) or from IP (50%) with antibodies against c-myc were analyzed by Western blotting with indicated antibodies.

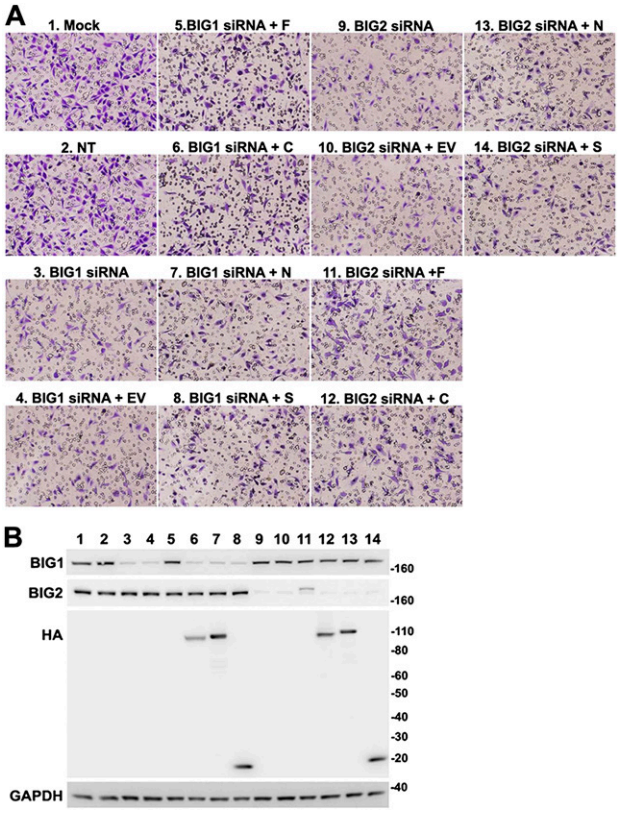

Fig. S5. Effects of BIG1-C or BIG2-C overexpression on migration of BIG1- or BIG2-depleted HeLa cells. Cells were treated as described in Fig. 6*A* before assay of Transwell migration, followed by fixation and staining of cells on the filters. (*A*) Microscopic images of cells on filters. (*B*) Western blots of HA-tagged fragments plus endogenous BIG1, BIG2, and GAPDH.

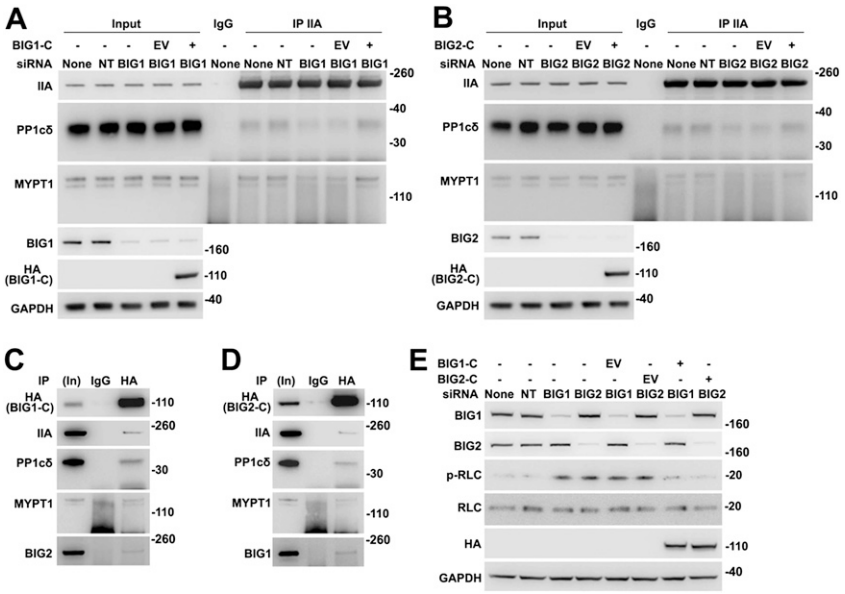

Fig. S6. Overexpression of BIG1-C or BIG2-C reversed effects of BIG1 or BIG2 depletion on co-IP of endogenous PP1cδ and MYPT1 with NMHC IIA and p-RLC. (*A* and *B*) After 72 h incubation with vehicle alone (None) or indicated siRNA, cells were incubated 24 h with empty vector (EV), or BIG1-C or BIG2-C constructs before IP of NMHC IIA. Western blot analysis of collected proteins (50%) showed increased co-IP of PP1cδ and MYPT1 after each C-fragment overexpression. (*C* and *D*) Western blots of proteins (50%) from IP with antibodies against HA of lysates of cells incubated for 72 h with BIG1 (*C*) or BIG2 (*D*) siRNA followed by 24 h with plasmid for expression, respectively, of HA-BIG1-C or BIG2-C. (*E*) After incubation with vehicle alone (None), or nontarget (NT), or specific BIG1 or BIG2 siRNA for 72 h, cells were incubated with empty vector (EV), or BIG1-C or BIG2-C plasmids for 24 h before analysis of proteins by Western blotting.