Supporting Information

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Materials and Methods

Antibodies, Expression Plasmids, and Reagents. Affinity-purified rabbit antibodies against human BIG1 and BIG2 (1) and rabbit anti-KANK1 and mouse anti-KANK1 antibodies (2) have been described. Mouse monoclonal antibodies against HA were purchased from Covance Research Products, against α-tubulin and γ-tubulin from Sigma-Aldrich, against GM130 and BiP from BD Bioscience; rabbit polyclonal antibodies against KIF21A were from Upstate Biotechnology, and against KANK1 from Bethyl Laboratories. Horseradish peroxidase-conjugated sheep anti-rabbit, anti-mouse Ig (IgG), and TnT T7 Coupled Wheat Germ Extract System was purchased from Promega, Alexa Fluor 594- or 488-conjugated anti-rabbit and anti-mouse antibodies from Invitrogen, and EDTA-free protease-inhibitor mixture from Sigma-Aldrich. Plasmids encoding HA-KIF21A and HA-BIG1 fragments have been described (3). Plasmids encoding full length human KANK1 or KANK-myc were from Origene Technologies. For synthesis of proteins in vitro (wheat germ extract system), BIG1 fragments were subcloned into pGAD T7 vector (Clontech).

Cell Culture, Transfection, and Preparation for Immunoprecipitation or Western Blotting. HeLa cells (American Type Culture Collection) were grown in DMEM (Invitrogen) with 10% FBS (Invitrogen) in a humidified 5% CO_2 atmosphere at 37 °C. PolyJet DNA in vitro transfection reagent (SignaGen Laboratories) was used for transient transfection 24 h before cell analysis according to the manufacturer's protocol. For depletion of selected proteins, ON-TARGETplus siRNAs and related reagents were designed and sold by Dharmacon RNA Technologies. BIG2 siRNA was SMARTpool siRNA mixture of four different siRNA duplexes. Sense sequences of other specific siRNAs are shown in Table S1. Negative control was Dharmacon siCONTROL nontargeting siRNA. HeLa cells (~50% confluent) were transfected with 100 nM or indicated concentration of siRNA using DharmaFECT1 reagent according to the manufacturer's instructions and harvested 48 h later. To quantify levels of depleted or overexpressed proteins, cells were scraped and broken (vortex) in radioimmunoprecipitation assay (RIPA) 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) followed by repeated (about five times) passage through a 26-gauge needle before analysis of samples (20 μg of protein) by Western blotting.

To cross-link protein complexes for immunoprecipitation analyses, cells were treated with a thiol-cleavable cross-linker, 2 mM dithiobis(succinimidylpropionate) (Pierce), according to the manufacturer's instructions, for 30 min before scraping in ice-cold Nonidet P-40 buffer (50 mM Tris·HCl, pH 7.4/150 mM NaCl/1% Nonidet P-40 and EDTA-free protease inhibitor mixture). After 30 min on ice and centrifugation (13,000 × *g*, 15 min, 4 °C), samples (1 mL, 1 mg of protein) of supernatant (cell extract) were incubated (4 °C, mixing overnight) with 5 μg of specific antibodies or normal rabbit IgG and 50 μL of protein A agarose (Millipore). Beads were washed five times in lysis buffer before elution of bound proteins (95 °C, 10 min) in gel-loading buffer for separation in 4–12% NuPAGE gel (Invitrogen) and transfer to polyvinylidene difluoride membranes (Millipore), which were incubated overnight (4 °C) in PBS containing 0.1% Tween 20 and 5% nonfat dry milk. Membranes were incubated (1 h, room temperature) with primary or secondary (coupled to horseradish peroxidase) antibodies in the same solution. Reaction with Super Signal Chemiluminescent substrate (Pierce) was used for densitometric quantification of specific proteins.

Wound-Healing Assay and Analysis of Cell Migration. Cells (5×10^5 per well in 3 mL of growth medium) were seeded in six-well plates. After 24 h, confluent cells were scratched with a fine pipette tip, rinsed with PBS, and time-lapse microscopy (TE2000E2, Nikon) was initiated using 10×/0.4NA objective lens, equipped with a temperature and CO_2 controller. Images were recorded at 20-min intervals for 16 h using a CCD video camera (CoolSNAP HQ2 CCD camera, Photometrics) operated by MetaMorph 7.5 image analysis software (Molecular Devices). Quantification of cell migration was performed as described by Santy and Casanova (4). Wound area covered by the monolayer was traced and measured using MetaMorph 7.5 software. Motility characteristics, including migration path, distance, rate, and directional persistence, were obtained from time-lapse movies. To track migration paths of individual cells at the wound edge, the centers of nuclei were manually traced in each frame by MetaMorph software to generate x, y coordinates that were used to compute motility parameters. Migration paths were recorded as graphs (Excel program, Microsoft). Velocity of cell migration is the ratio of total length of migration path to duration (6 h) of migration. Migration distance is length of the cell track from 0 to 6 h. Directional persistence is the ratio of direct distance from 0 to 6 h to total length of the path in that period.

Confocal Immunofluorescence Microscopy. For γ-tubulin staining, cells were fixed and permeabilized in methanol (5 min, −20 °C) then incubated (30 min) in blocking buffer (0.1% saponin/0.2% BSA in PBS). In other experiments, cells were fixed (4% paraformaldehyde in PBS, 15 min, 25 °C), permeabilized (0.01% Triton X-100/0.05% SDS in PBS, 5 min). After blocking buffer, all cells were incubated with primary antibodies in blocking buffer for 1 h followed by extensive washing with PBS, incubation with Alexa Fluor-conjugated secondary antibodies in blocking buffer (1 h), and mounting in VECTASHIELD Mounting Medium with DAPI (Vector Laboratories).

F-actin was stained with Alexa Fluor 594-conjugated phalloidin (Invitrogen). For wound-healing, 1×10^5 cells per well in Lab-Tek 4-well chamber slides (Nunc) were grown for 24 h before wounding, fixed 6h later, and prepared for inspection/imaging using LSM 510 META laser confocal microscope (Carl Zeiss) with 40×/1.3 NA oil objective lens and 488- or 543-nm lasers. Pinholes were set to scan ~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).

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* 97:2567–2572.
2. Sarkar S, et al. (2002) A novel ankyrin repeat-containing gene (Kank) located at 9p24 is a growth suppressor of renal cell carcinoma. *J Biol Chem* 277:36585–36591.
3. 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: 18788–18793.
4. Santy LC, Casanova JE (2001) Activation of ARF6 by ARNO stimulates epithelial cell migration through downstream activation of both Rac1 and phospholipase D. *J Cell Biol* 154:599–610.

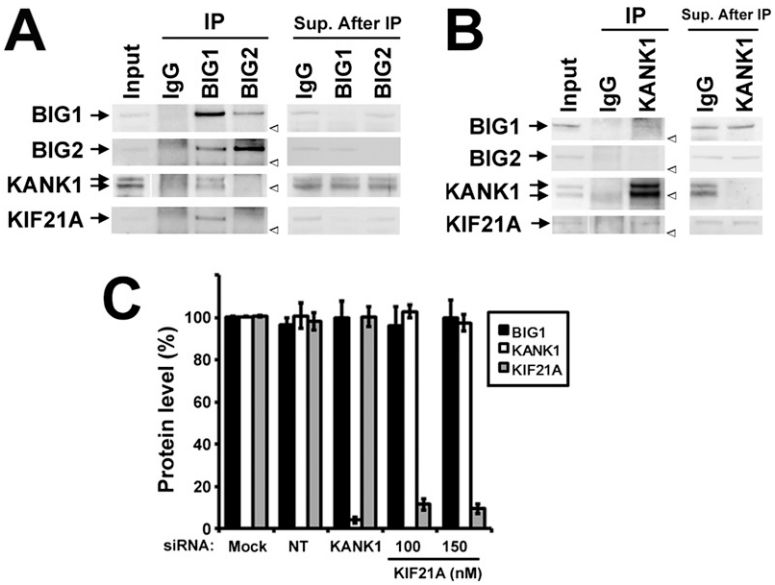

Fig. S1. Co-IP of KANK1 and BIG1. (*A* and *B*) Enlargements of gels in Fig. 1 *A* and *B*. Samples (25%) of proteins precipitated from extracts of HeLa cells (1 mg) with antibodies against BIG1, BIG2, or KANK1, or control IgG, and 2.5% of input or supernatant proteins after IP were separated by SDS/PAGE before reaction with indicated antibodies. (*C*) Amounts of each protein (input) used for IP in Fig. 1*D* expressed relative to that of the same protein in control (Mock) in the same experiment = 100. Data are reported as means ± SEM from three experiments.

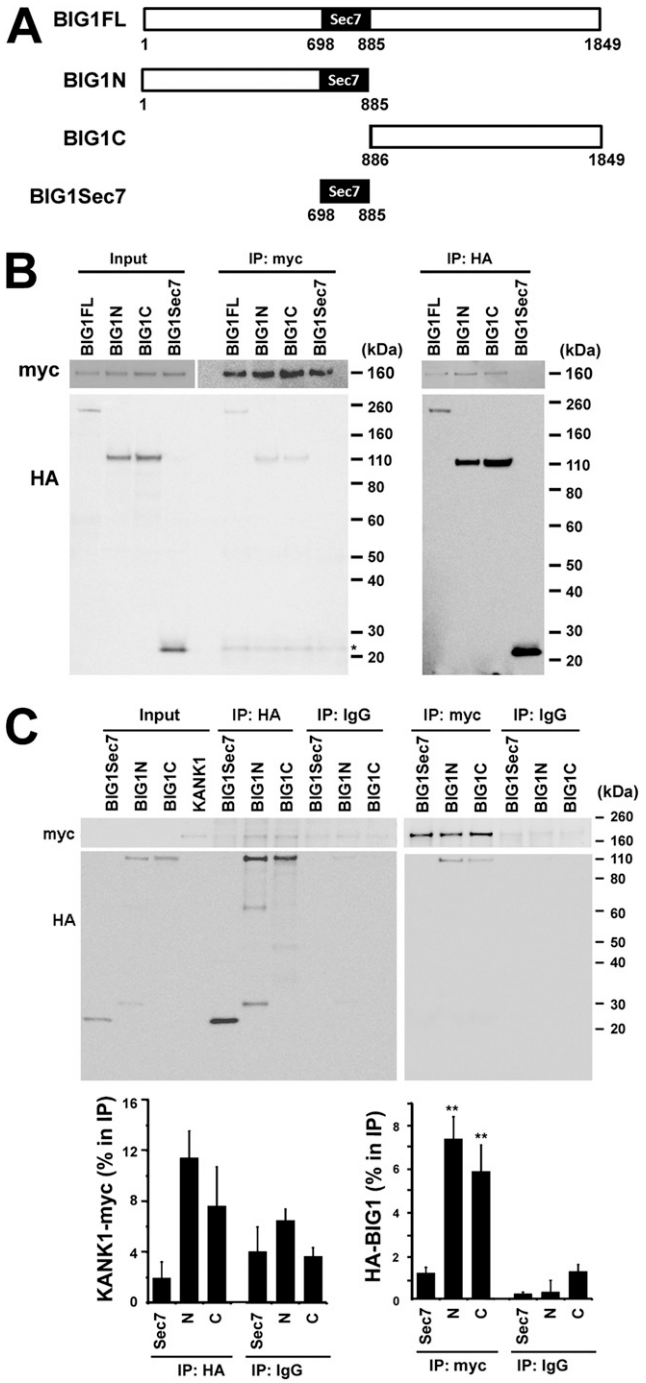

Fig. S2. Immunoprecipitation of overexpressed BIG1 and KANK1. (*A*) BIG1, full-length or indicated fragment with N-terminal HA tag. (*B*) HeLa cells co-transfected with constructs encoding full-length KANK1-myc and HA-BIG1 or its fragment were harvested 24 h later by scraping in ice-cold Nonidet P-40 buffer. After 30 min on ice and centrifugation (13,000 × *g*, 15 min, 4 °C), samples (0.5 mL, 100 μg of protein) of extracts were incubated (4 °C, mixing, overnight) with 2 μg of antibodies against HA or myc and 30 μL of protein A agarose (Millipore). Beads were washed three times in Nonidet P-40 buffer and once in RIPA buffer before elution of bound proteins. Samples (50%) of agarose-bound proteins were separated before Western blotting with mouse monoclonal antibodies against myc (KANK1) or HA (BIG1). Input (10 μg) was 10% of total proteins used for IP. Asterisk indicates light chains of IgG. (*C*) HA-BIG1N, HA-BIG1C, HA-BIG1Sec7, and KANK1-myc were synthesized by in vitro wheat germ transcription/translation according to the manufacturer's instruction. Samples of HA-BIG1 and KANK1-myc proteins were mixed at 4 °C for 1 h, then with 3 μg of antibodies against HA or myc for 1 h before addition of 40 μL of protein G beads (final 1 mL in PBS) and mixing overnight. Beads were washed five times with ice-cold Nonidet P-40 buffer before eluting bound proteins by boiling in 30 μL of sample buffer and separation by SDS/PAGE. Total eluted proteins or sample (10%) of input proteins were subjected to Western blotting, densitometric quantification, and calculation of percentage of each input protein in IP. Data are means ± SEM of values from three experiments. $^{**}P < 0.005$ (*t* test), versus IgG control.

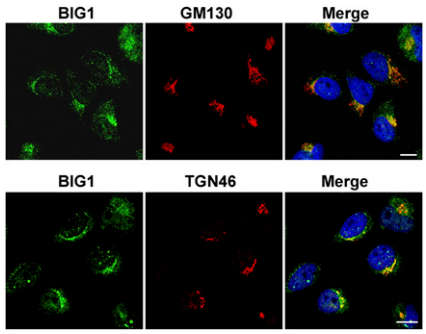

Fig. S3. Endogenous BIG1 and Golgi membrane markers GM130 and TGN46 in HeLa cells. Cells were reacted with rabbit anti-BIG1 and mouse Golgi marker antibodies before confocal immunofluorescence microscopy. (Scale bar, 10 μm.)

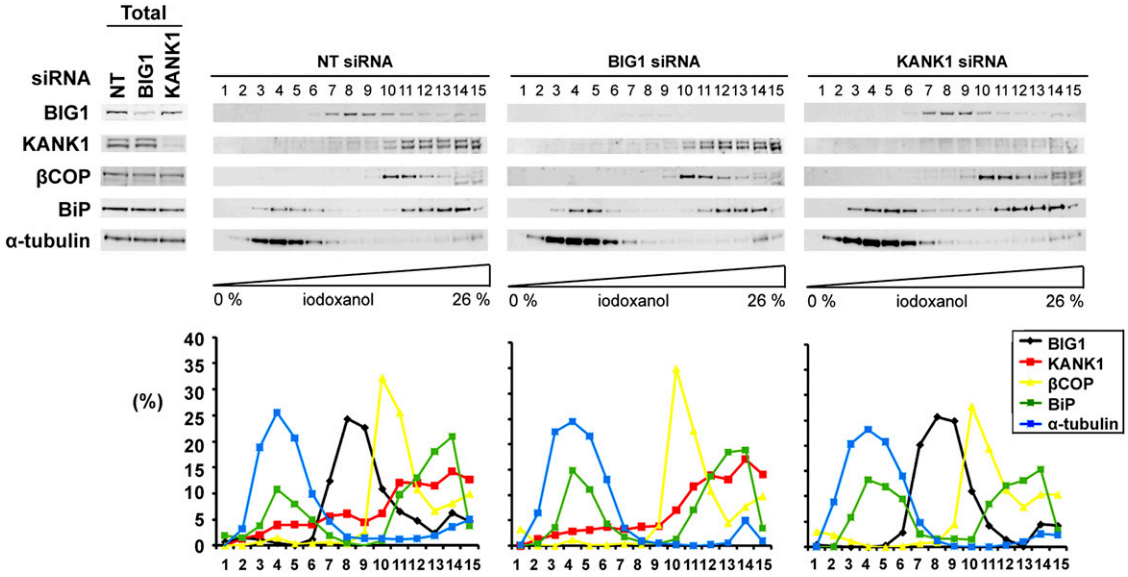

Fig. S4. Fractionation of BIG1- or KANK1-depleted HeLa cells by gradient centrifugation. Cells transfected 48 h earlier with nontargeted (NT), BIG1, or KANK1 siRNA were scraped in homogenization buffer (0.25 M sucrose/1 mM EDTA/20 mM Hepes-KOH, pH 7.4, and EDTA-free protease inhibitors) and passed ~25 times through a 26-gauge needle to ~90% complete breakage, followed by centrifugation (800 × *g*, 10 min, 4 °C). Samples (0.6 mL, ~1.5 mg protein) of postnuclear supernatant (PNS) were applied to gradients of 2%, 6%, 10%, 14%, 18%, 22%, and 26% iodixanol (0.6 mL each, total volume 4.2 mL) according to the manufacturer's protocol (Axis Shield; protocol S36). After centrifugation (100,000 x *g*, 4 °C, 16 h), 15 fractions (collected from the top) were analyzed by Western blotting. Amounts of proteins, quantified by densitometry, are reported as percentage in each fraction of total protein recovered. Data are from one experiment representative of three.

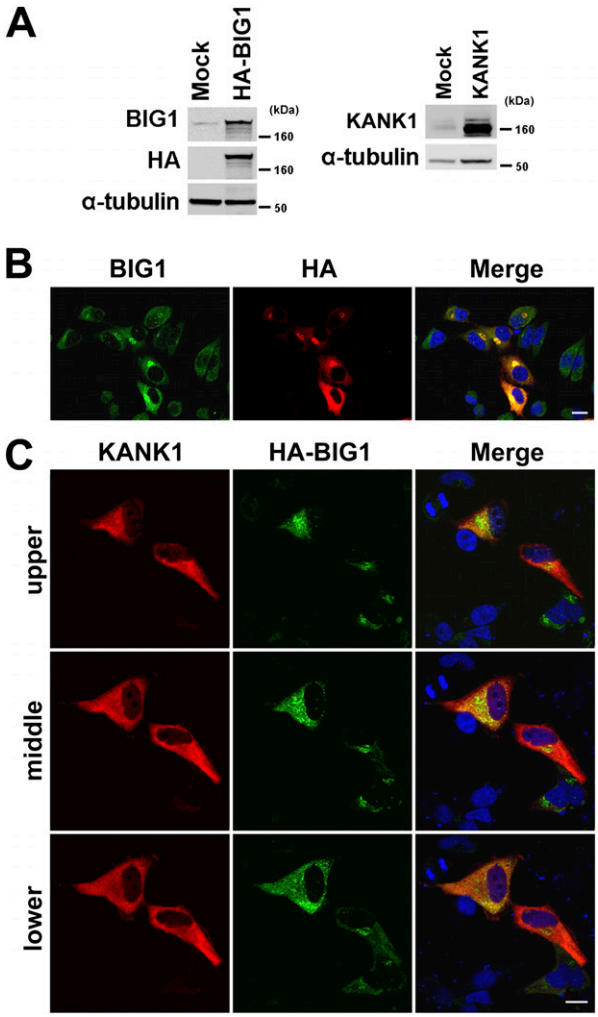

Fig. S5. Intracellular localization of overexpressed HA-BIG1 and untagged KANK1. (*A*) HeLa cells transfected with plasmids encoding HA-BIG1 and KANK1 24 h earlier were lysed in RIPA buffer, and Western blotting of proteins in samples (20 μg) of extracts reacted with antibodies against BIG1, HA, KANK1, or α-tubulin. Densitometric values for amounts of each protein were expressed relative to that of α-tubulin in the same lane to compare total amounts of BIG1 or KANK1 in cells overexpressing HA-BIG1 or untagged KANK1 with amounts of endogenous proteins in control (Mock) cells, which were ∼10.5- and 7-fold the endogenous amounts, respectively. These means, however, obscure the much wider range in amounts of overexpressed proteins seen microscopically. KANK1, presumably largely cytosolic, appears much more widely distributed than HA-BIG1. (*B*) In cells transfected with plasmids encoding HA-BIG1 and reacted with rabbit anti-BIG1 and mouse anti-HA antibodies, endogenous and overexpressed BIG1 were apparently similarly distributed. (*C*) Images from three focal planes (top to bottom, as in Fig. 2) of cells overexpressing HA-BIG1 (green) and untagged-KANK1 (red) are shown. (Scale bar, 10 μm.)

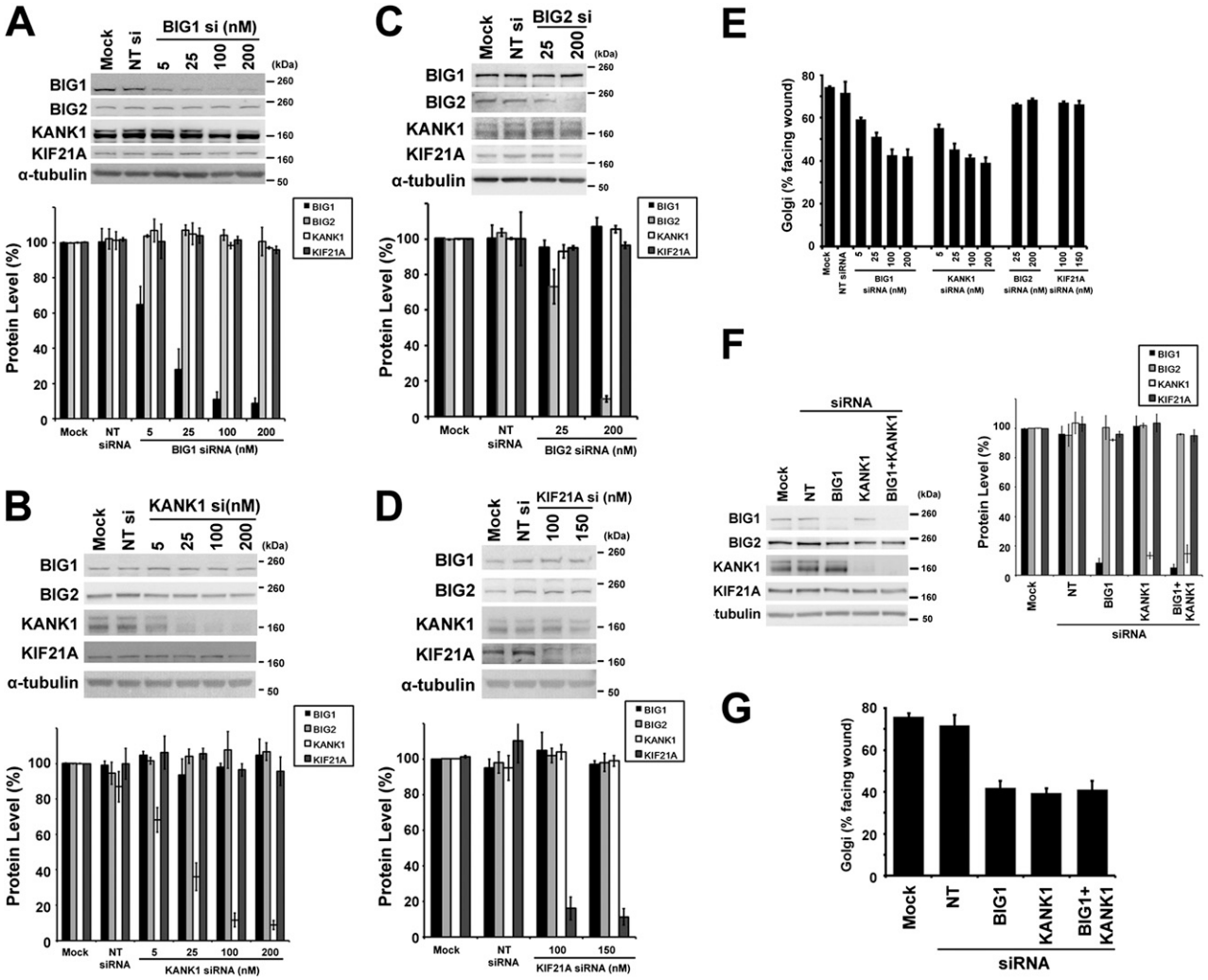

Fig. S6. Depletion of BIG1 or KANK1 interfered with cell polarization during wound healing. (*A–D*) Cells transfected with 100 nM nontargeted (NT), or indicated concentrations of specific siRNA or vehicle alone (Mock) were lysed 48 h later, and analyzed by Western blotting and densitometric quantification. Amounts of proteins were expressed relative to that of the same protein in Mock cells (=100%). Data from three experiments are reported as means ± SEM. (*E*) Confluent monolayers of HeLa cells transfected 48 h before with indicated siRNA or vehicle alone (Mock), as in Fig. 3, were wounded and fixed 6 h later, stained with DAPI and anti-GM130, and analyzed by microscopy. Percentage of wound-edge cells with Golgi in forward-facing 120° sector between nucleus and wound was recorded for at least 100 cells of each population in each experiment. Data are means ± SEM of values from three experiments. (*F*) Amounts of proteins in cells transfected with 100 nM nontargeted (NT) or specific siRNA or with vehicle alone (Mock) were analyzed by Western blotting and densitometry 48 h after transfection. Data from three experiments expressed relative to that of the same protein in Mock cells (=100%) are reported as means ± SEM. (*G*) Percentage of wound-edge cells with Golgi in forward-facing 120° sector between nucleus and wound in cells transfected with indicated siRNA shown in *E*. At least 100 cells of each population in each experiment were recorded. Data are means ± SEM of values from three experiments.

Table S1. Sense sequences of siRNAs

siRNA	Sequence (5′–3′)
BIG1 siRNA#1	GUCCAAAUGUCCUCGCAUA
BIG1 siRNA#2	GAACAGGUCUCCAACAAUU
KANK1 siRNA#1	GGUCAGUCACGGACGGAUA
KANK1 siRNA#2	GGAUAAAGGAGUUCCGGCA
KIF21A siRNA	GAGCAGAUCUACAUUCAAU