Supporting Information

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SI Materials and Methods

Cell Culture. Cell culture reagents were obtained from Invitrogen. The HeLa epithelial cell line was cultured in DMEM, 10% (vol/vol) heat-inactivated FBS, 100 U/mL penicillin G, 100 μg/mL streptomycin, 2 mM L-glutamine, and 15 to 20 mM Hepes. NIH 3T3 cells were cultured in the same medium but with 10% heat-inactivated bovine calf serum instead of FBS. T cells were maintained in RPMI-1640, 10% (vol/vol) heat-inactivated FCS, 100 U/mL penicillin G, 100 μg/mL streptomycin, 2 mM L-glutamine, 20 mM Hepes, 1 mM sodium pyruvate, 100 μM nonessential amino acids, 55 μM β-mercaptoethanol, and essential amino acids.

Constructs and Transfection. Arl13b-EGFP was a kind gift from Dr. K. Kontani (University of Tokyo, Tokyo, Japan) (7). The construct pcDNA3.1(+)-Arl13b was obtained by PCR using human Arl13b cDNA (Open Biosystems) as a template. The primers used were 5′-CGCGGATCCGCGCCGCCACCATGTTCAGTCTGATGGCCAGTTGCT-3′ (forward) and 5′-CCGCTCGAGCGGTTATGAGATCACATCATGAGCATC-3′ (reverse). After digestion with BamHI and XhoI, the PCR product was cloned into pcDNA3.1(+) (Invitrogen). Arl13b-FLAG was generated from pcDNA3.1(+)-Arl13b using the Quickchange II Site-Directed Mutagenesis Kit (Agilent Technologies), using forward primer 5′-ATGATGTGATCTCAGATTATAAAGATGATGATGATAAATAACCGCTCGAGTC-3′ and complementary reverse primer. The mutant Arl13b 1–193 was generated by PCR using human Arl13b cDNA (Open Biosystems) as a template. The primers used were 5′-CGCAAGCTTGCCACCATGTTCAGTCTGATGGCCAGTTGCT-3′ (forward) and 5′-CCGGTCGACCGGGTCTCTTGCAATAACATGTAGC-3′ (reverse). After digestion with HindIII and SalI, the PCR product was cloned into Arl13b-EGFP that had been previously digested with the same enzymes to remove Arl13b.

To rescue the effect of Arl13b knockdown, Arl13b WT was cloned into KpnI and BamHI sites of pcDNA3.1(+) (Invitrogen) with a blasticydin resistance gene (cloned between EcoRI and XhoI sites). The primers used were 5′-CGCGGTACCGCGCCGCCATGTTCAGTCTGATGGCCAGTTGCT-3′ (forward) and 5′-CCGGGATCCCGGTTATGAGATCACATCATGAGCATCA-3′ (reverse).

Plasmid transfections were performed using Fugene 6 (Roche) or Lipofectamine 2000 (Invitrogen) in accordance with the manufacturer's instructions. An Arl13b mutant with silent mutations on the final base of each of the codons of the target sequence was generated from this construct as explained above, with forward primer 5′-GACCCAGAACCAACGAATCCTTTCCAACCCATCGCCTCCGTCATCATTGAGAATGAAGGAAAACTTGA-3′ and complementary reverse primer. All constructs were verified by sequencing.

The EGFP-Rab22a Q64L construct was a kind gift from Dr. J. Donaldson (National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD) (3). HA-Arf6-Q67L and T27N have been described previously (27).

Recycling Assay and Flow Cytometry Analysis. The recycling assay and flow cytometry analysis were performed essentially as described previously (2).

Cell Transduction. HeLa:CD1a stable transfectants were plated at 2×10^5 cells per well onto six-well plates and infected with shRNA-containing lentiviruses on the next day. For this, 6 μg/mL polybrene (Sigma-Aldrich) was added to the cells before virus was added. After 24 h, 2 μg/mL puromycin (Sigma-Aldrich) was added, and the cells were incubated for at least 1 wk before being assayed. shRNA target sequences were as follows: E3: CCTGTCAGAAAGGTGACTCTT; E4: CCAGCCAATAGCATCTGTAAT; Mission (negative control sequence, Sigma-Aldrich): CAACAAGATGAAGAGCACCAA.

T-Cell Assay and ELISA. HeLa:CD1a stable transfectant cells transduced with lentiviruses encoding Arl13b-targeting shRNA E4, shRNA control (Mission; Sigma-Aldrich), or empty vector and selected in puromycin were irradiated at 5,000 rad, washed with T-cell medium, and plated onto 96-well flat-bottomed plates at 50,000 cells per well. *Mycobacterium tuberculosis* H37Ra extract in acetone (Difco) was evaporated in a glass tube under nitrogen and then sonicated for 2 min in complete medium. Washed T cells (25,000 DDM-reactive) were added in a total volume of 200 μL to each well, and the mixture was incubated for 22 h at 37 °C. Secreted IFN-γ was measured by ELISA essentially as described previously (2).

RNA Extraction and Quantitative RT-PCR. RNA from cells lysed using QIAshredder columns (Qiagen) was extracted with the RNeasy Mini Kit (Qiagen) in accordance with the manufacturer's instructions. cDNA was synthesized with the QuantiTect Reverse-Transcription Kit (Qiagen) in accordance with the manufacturer's instructions. Quantitative PCR was performed using Brilliant SYBR Green QPCR Master Mix (Agilent Technologies) on an Mx3000P QPCR System (Agilent Technologies). The primers used were 5′-GAATCCAAGGAGAATACCCTG-3′ (forward) and 5′-CCAACACCAATATAGGCTTTCC-3′ (reverse) for Arl13b and 5′-CATTTCCTGGTATGACAACGA-3′ (forward) and 5′-GTCTACATGGCAACTGTGAG-3′ (reverse) for GAPDH.

Immunofluorescence Staining. The stainings for internalized CD1a, CD1b, and Tf to label the recycling pathway were done essentially as described previously (2). When dextran was used, cells were incubated with Alexa Fluor 546-conjugated dextran (0.5 mg/mL; Invitrogen) and immediately fixed. Where cytochalasin D (Sigma-Aldrich) was used, cells were incubated with the drug for 45 min at the indicated concentration. Staining with primary Ab [50 ng/mL anti-EEA1 mAb clone 14/EEA1 (BD Biosciences), 1 μg/mL anti-EEA1 polyclonal Ab (Calbiochem), 0.5–0.67 μg/mL anti–α-tubulin clone B512 (Sigma-Aldrich), and 0.1 μg/mL polyclonal anti-HA tag (Abcam)], secondary Ab [2–4 μg/mL F(ab′)$_2$ goat anti-mouse Alexa Fluor 546-conjugated (Invitrogen) or 1.33–4 μg/mL F(ab′)$_2$ goat anti-rabbit Alexa Fluor 488-conjugated (Invitrogen)], 1.67 μg/mL Cy3-conjugated anti-FLAG (Sigma-Aldrich), or 0.2–0.8 U/mL Alexa Fluor 546- or 568-conjugated phalloidin were performed as described previously (2) and analyzed with a Nikon C-1 confocal microscope with EZ C1 software, a Leica TCS SP5 confocal microscope using Leica Application Suite software, or a Zeiss LSM710 confocal microscope with Zeiss Zen 2010 software. Images were processed with Adobe Photoshop CS version 8.0, with the levels or brightness of each channel adjusted up to a maximum threshold defined by the absence of signal in the negative controls.

Immunoprecipitation. HeLa and NIH 3T3 cells were lysed in cold lysis buffer [50 mM Tris·HCl (pH 7.4), 0.1% Triton X-100, 150 mM NaCl, 1 mM EDTA, 0.1% SDS) in the presence of protease and phosphatase inhibitors for 30 min on ice, followed by centrifugation at 12,000 × *g* for 15 min at 4 °C. Cell lysates (900 μg) were precleared for 1 h with protein G-Sepharose beads (Sigma-

Aldrich) and then incubated overnight at 4 °C with anti–β-actin (clone AC-15, 1 μg/mL; Sigma-Aldrich) or affinity-purified rabbit polyclonal anti-Arl13b (2 μg/mL; see below). Protein G-Sepharose beads were then added and mixed for 2 h at 4 °C. Beads were recovered by centrifugation, washed with lysis buffer with high salt concentration (500 mM NaCl) and then with lysis buffer, and finally resuspended in Laemmli buffer.

Immunoblot Analysis. Protein concentration in cell lysates was determined using the DC Protein Assay Kit (Bio-Rad), and equal amounts (50 μg) of each sample were boiled at 95 °C for 5 min, loaded on a 12% SDS-polyacrilamide gel, transferred onto nitrocellulose membranes in transfer buffer (25 mM Tris; 192 mM glycine; 0.5% SDS; 10% methanol) for 90 min at 500 mA, and processed for immunoblot analysis. Membranes were blocked with blocking buffer (5% skim milk and 0.1% Tween-20 in PBS) and the Ab incubated in the same buffer. Affinity-purified rabbit polyclonal Ab anti-Arl13b (see below) was used at 2 μg/mL, anti–α-tubulin (Sigma-Aldrich) was used at 0.2 μg/mL, anti–β-actin (Sigma-Aldrich) was used at 0.4 μg/mL, and HRP-conjugated secondary Ab (Santa Cruz Biotechnology) was used at 0.4 μg/mL. Blots were developed with ECL (GE Healthcare) in accordance with the manufacturer's instructions. and a Molecular Imager Chemidoc XRS System (Bio-Rad) was used to visualize the presence of chemiluminescence. Band intensities were quantified using ImageJ version 1.38x.

Anti-Arl13b Polyclonal Ab. Rabbit anti-Arl13b polyclonal Ab was raised by YenZym Antibodies against peptide residues 273–286 from the human protein (CREKKNQKMEKDSDG-amide). The serum was affinity-purified using the same peptide.

Materials and Methods for SI Figures. ***Antibodies.*** Anti-Sorting nexin 1 (0.83 μg/mL; BD Biosciences), anti-EEA1 polyclonal Ab (1 μg/mL; Calbiochem), anti-acetylated α-tubulin (0.2 μg/mL; Sigma-Aldrich), Cy3-conjugated anti-FLAG (1.67 μg/mL; Sigma-Aldrich), polyclonal anti-HA tag (0.1 μg/mL; Abcam) and anti-TfR mAb (5E9 ascites; 1:8,000 dilution) were used.

Drug Treatments. Where cytochalasin D (Sigma-Aldrich) was used, cells were incubated with the drug for 45 min at the indicated concentrations. Nocodazole (10 μg/mL) was used for 2 h.

Constructs. Mouse Rab11a coding sequence with part of the 3′ UTR sequence was cloned by RT-PCR using total RNA isolated from AtT20 cell line. Rab11a was amplified by PCR using primers 5′-TGAGGAATTCATGGGCACCCGCGACGACGAGTA-3′ (forward) and 5′-AATAGTCGACCATGCTGGTTGCTGAATATGGTG-3′ (reverse), and then cloned into pcDNA ENTR BP V5 mCherry-C2 using EcoRI and SalI. pcDNA ENTR BP V5 mCherry-C2 was constructed on a backbone of pcDNA6.2/GW/EmGFP (Invitrogen), an expression mammalian vector with Gateway technology (Invitrogen), by swapping EmGFP by V5 and polylinker using DraI. mCherry was amplified from pCMV myc-mCherry with primers 5′-GATCAGATCTATGGTGAGCAAGGGCGAGGAGGATAAC-3′ (forward) and 5′-AGCTCTCGAGTCCTTGTACAGCTCGTCCATGCGCCG-3′ (reverse), and then cloned using BglII and XhoI.

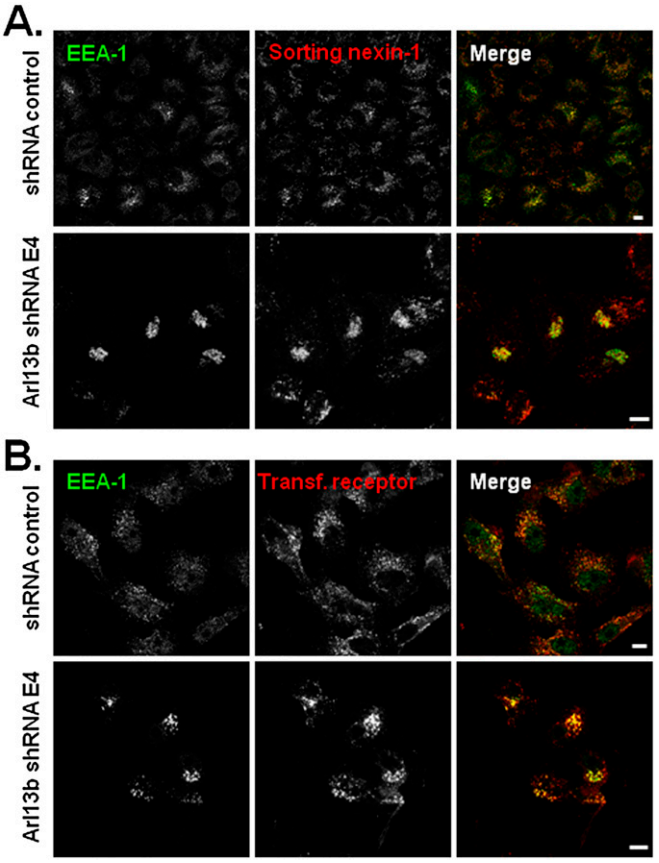

Fig. S1. Arl13b silencing leads to the clustering of early endosomes. HeLa cells were transfected with Arl13b-targeting shRNA (*Lower*) or control (*Upper*) and selected in puromycin. Cells were fixed, permeabilized, and costained for EEA1 (green) and Sorting nexin-1 (red) (*A*) or for EEA1 (green) and transferrin receptor (red) (*B*). (Scale bars: 10 μm.)

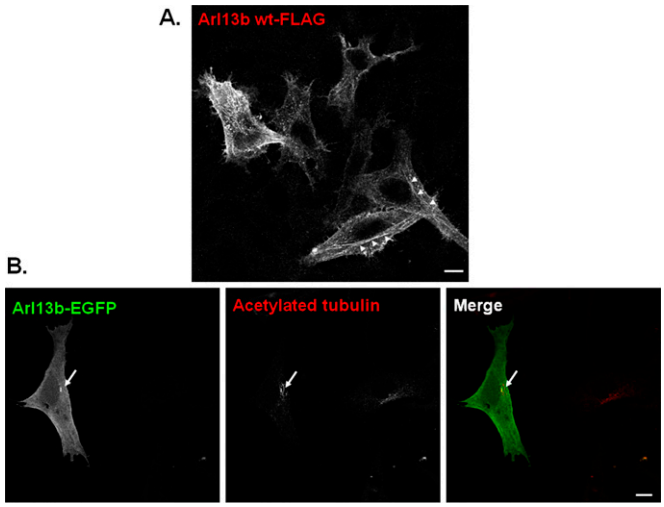

Fig. S2. Arl13b localizes to tubular structures of HeLa cells and primary cilia of NIH 3T3 cells. (*A*) HeLa cells were transfected with Arl13b-FLAG, fixed, permeabilized, and stained with Cy3-conjugated anti-FLAG Ab. Arrowheads denote Arl13b-labeled tubular structures. (*B*) NIH 3T3 cells were transiently transfected with Arl13b-EGFP, fixed, permeabilized, and stained with anti-acetylated α-tubulin (a primary cilium marker) mAb, followed by Alexa Fluor 546-conjugated anti-mouse secondary Ab (red). Arrow indicates a primary cilium. (Scale bars: 10 μm).

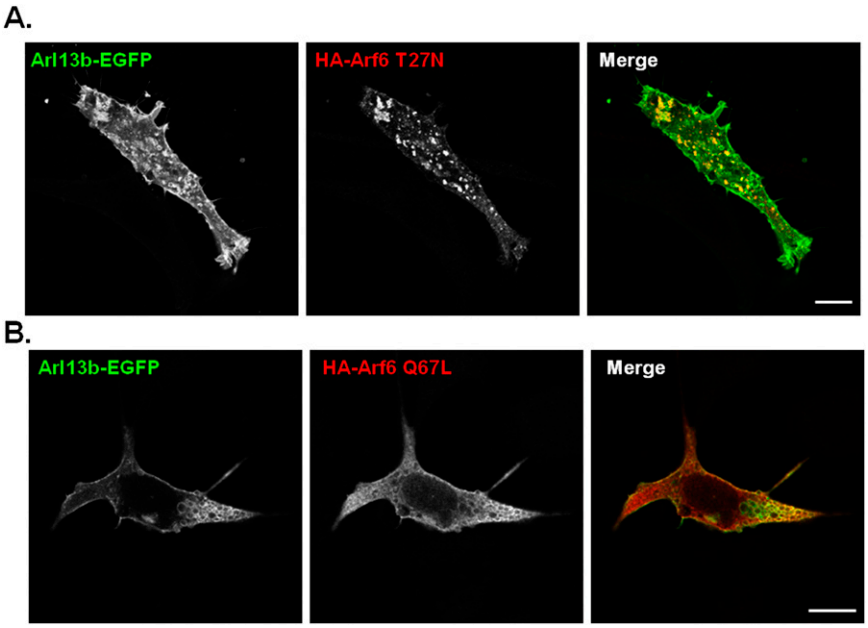

Fig. S3. Arl13b-EGFP colocalizes with Arf6 in NIH 3T3 cells. NIH 3T3 cells were transiently cotransfected with Arl13b-EGFP and HA-Arf6 T27N (*A*) or HA-Arf6 Q67L (*B*), fixed, permeabilized, and stained with anti-HA tag polyclonal Ab followed by Alexa Fluor 546-conjugated anti-rabbit secondary Ab (red). (Scale bars: 10 μm.)

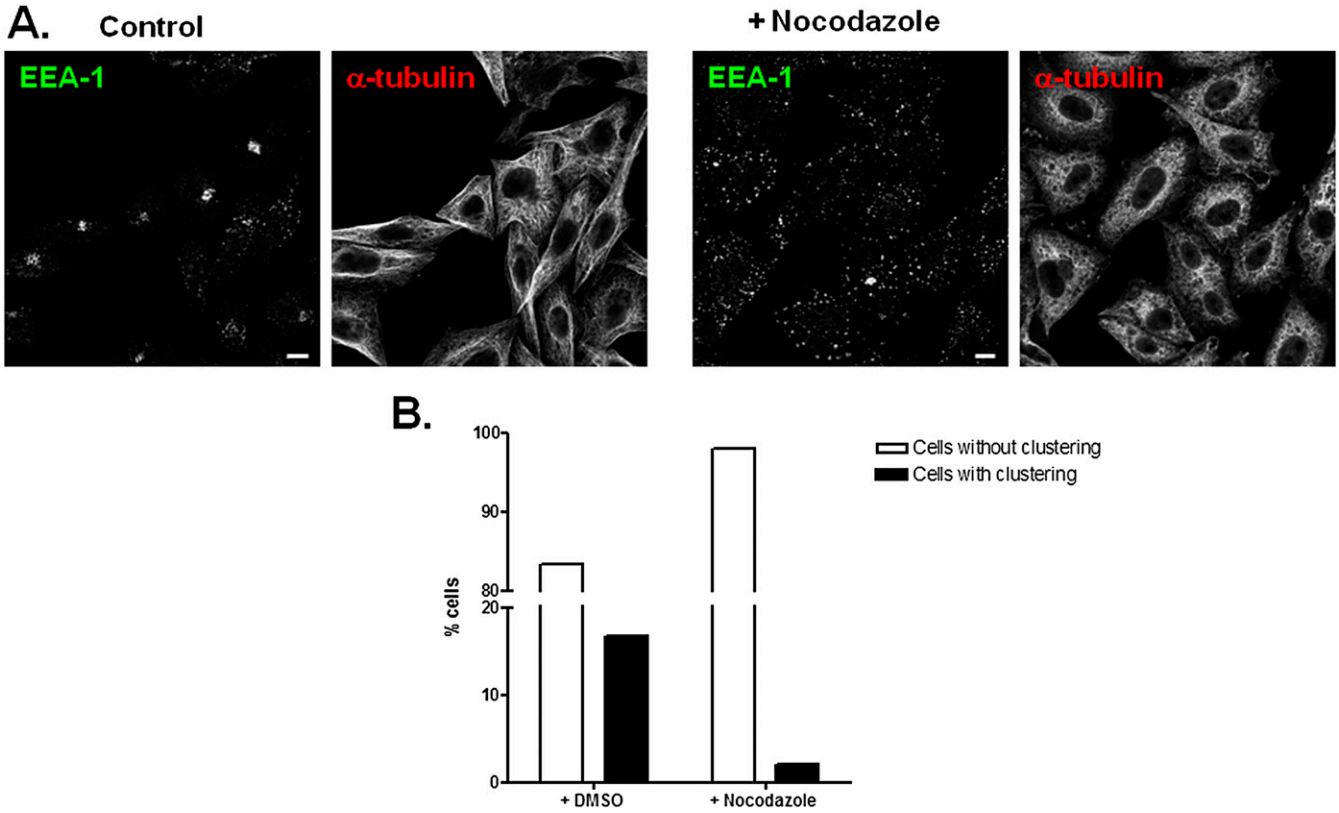

Fig. S4. Arl13b silencing-induced early endosome clustering is microtubule-dependent. (*A*) HeLa cells were transfected with Arl13b-targeting shRNA and selected in puromycin. Cells were then incubated with 10 μg/mL nocodazole in DMSO or DMSO only for 2 h and then fixed, permeabilized, and stained for EEA1 with a rabbit polyclonal Ab (green) and α-tubulin (red). (*B*) Cells with and without clustered EEA1-positive early endosomes were counted ($n = 100$). (Scale bars: 10 μm.)

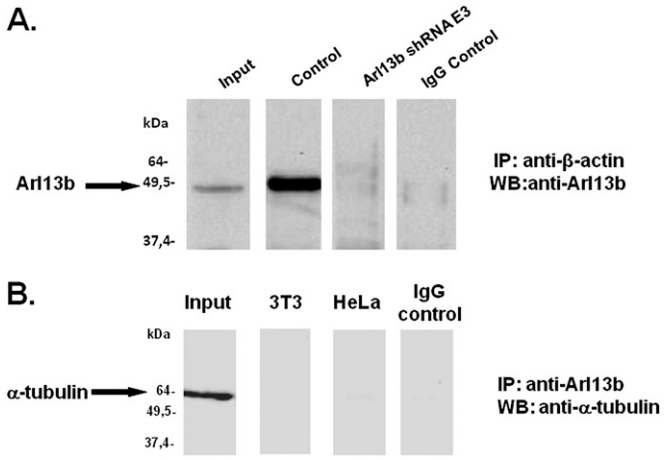

Fig. S5. Arl13b interacts with actin specifically, but not with α-tubulin. (*A*) HeLa cells were transduced with Arl13b-targeting shRNA (E3) or control (empty vector) and selected in puromycin. Total cell lysates were immunoprecipitated with mouse monoclonal anti–β-actin Ab, separated by SDS/PAGE, and immunoblotted with anti-Arl13b Ab, followed by HRP-conjugated anti-rabbit secondary Ab. Mouse IgG was used as a negative control, and 40 μg of nontransduced cell lysate was analyzed as input. (*B*) HeLa and NIH 3T3 cell lysates were immunoprecipitated with rabbit polyclonal anti-Arl13b Ab, separated by SDS/PAGE, and immunoblotted with mouse monoclonal anti–α-tubulin Ab, followed by HRP-conjugated anti-mouse secondary Ab. Rabbit IgG was used as a negative control, and 40 μg of nontransduced cell lysate was analyzed as input.

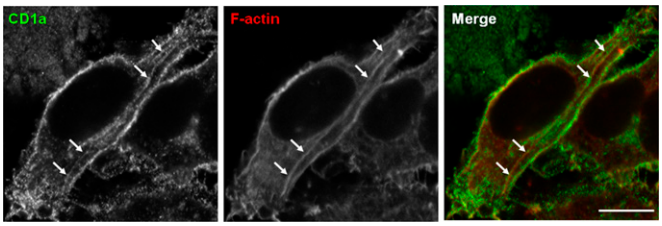

Fig. S6. CD1a-labeled tubules colocalize with actin filaments. HeLa:CD1a stable transfectant cells were fixed, permeabilized, and stained with anti-CD1a mAb (green) and Alexa Fluor 568-conjugated phalloidin, which labels filamentous actin (red). Arrows indicate actin filaments colocalizing with tubules containing CD1a. (Scale bar: 10 μm.)

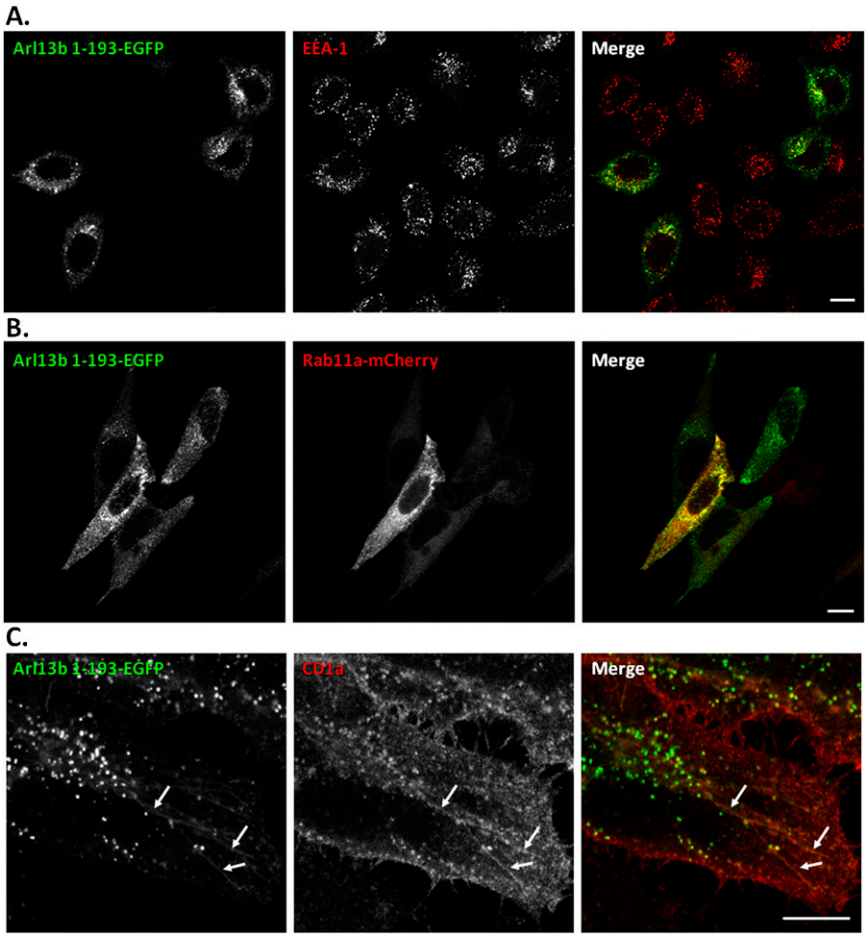

Fig. S7. Arl13b 1–193 truncation mutant partially colocalizes with early and recycling endosomes. (*A* and *C*) HeLa cells were transiently transfected with Arl13b 1–193-EGFP, fixed, permeabilized, and stained with either rabbit polyclonal anti-EEA1 (*A*) or mouse anti-CD1a mAb (*C*), followed by Alexa Fluor 546-conjugated anti-rabbit or Alexa Fluor 568-conjugated anti-mouse secondary Ab, respectively (red). Arrows indicate tubules containing CD1a and labeled by Arl13b 1–193-EGFP. (*B*) HeLa cells were transiently cotransfected with Arl13b 1–193-EGFP and mCherry-Rab11 (red) and fixed. (Scale bars: 10 μm.)

Other Supporting Information Files

Dataset S1 (XLS)