Supplemental Experimental Procedure

Antibodies and Reagents

Mouse monoclonal antibody to γ-adaptin was from Thermo Scientific (Rockford, Il). Mouse monoclonal antibody to δ-adaptin was from the Developmental Studies Hybridoma bank at the University of Iowa [S1]. Rabbit polyclonal antibody to Rab4 was from Sigma-Aldrich (St Louis, MI). Rabbit polyclonal antibodies to Arf1, Arf3 and Arl1 were gifts from Rick Kahn (Emory University, Atlanta). Rabbit polyclonal antibody to BIG1 and GBF1 was a gift from Paul Melancon (University of Alberta, Edmonton, AB, Canada). Rabbit polyclonal antibody to BIG2 was from Martha Vaughan, (NIH, USA). Mouse monoclonal antibodies to GGA3 and EEA1 were from BD Biosciences (Mississauga, ON, Canada). Mouse monoclonal antibody to HA (16B12) was from Covance (Princeton, NJ). Rabbit polyclonal antibody to EEA1 was from AbCam (Cambridge, MA). Sheep polyclonal antibody to TGN46 was from Serotec (Oxford, UK). Transferrin conjugated to Alexa Flour 488 or 546 was from Molecular Probes (Eugene, OR).

Plasmids

GFP-Rab4a and GFP-Rab5 were gifts from Marino Zerial (Max Planck Institute of Molecular Cell Biology, Dresden, Germany). mCherry-Rab4a was prepared by subcloning from GFP-Rab4a into the XhoI and Hind III sites of mCherry-C3. GFP and mCherry-N1 tagged Arf1, Arf3, Arf4 and Arf5 were gifts from Paul Melancon (University of Alberta, Edmonton, AB, Canada). HA tagged BIG1 and BIG2 were gifts from Kazuhisa Nakayama (Kyoto University, Kyoto, Japan). Arl1 plasmid was a gift from Rick Kahn (Emory University, Atlanta); Arl1 was subsequently subcloned into EGFP-N1 into the BglII and EcoRI sites of EGFP-N1.

Transferrin uptake into endosomes

Cos7 cells were incubated with Alexa 488-labeled transferrin (25µg/ml) at 0^0C on ice for 60 minutes. After washing to remove unbound ligand, cells were transferred to a 37^0C water bath for 5 minutes to allow internalization. Cells were then fixed with 4% paraformaldehyde and processed for immunofluorescence microscopy.

Transfections

Cos 7 cells were transfected using nucleofection reagent from Lonza. Briefly, 1 X 10^6 cells were mixed with 1-5 µg of the indicated expression vector and pulsed using the W-001 program of an Amaxa nucleofector system. Cells were imaged 48 hours after transfection.

Knockdowns

For Arf depletion, Cos7 cells were transfected with siRNA previously used by Volipicelli-Daley et al [S2], targeting Arf1 (ACCGUGGAGUACAAGAACA) or Arf3 (UGUGGAGACAGUGGAGUAU), either singly or as a pair using RNAi Max transfection reagent (Invitrogen). Cells were subjected to two rounds of transfection on two consecutive days (final concentration of siRNA in the medium was 20 nM) and analyzed 48hrs after the last transfection. Given the close sequence homology between Arf1 and Arf3, we checked for specificity of knockdown by immunoblotting for Arf1 in Arf3 depleted cells and Arf3 in Arf1 depleted cells. No off-target effects were observed (Fig. S2). Lentiviral vectors targeting Rab4a, Rab4b and Arl1 were purchased from Sigma Aldrich, (St Louis, MI) and lentiviral particles were prepared as per the manufacturer's protocol. Empty pLKO vector was used as a control. Cos7 cells were transduced with virus containing the shRNA for 24 hours, selected using 2 µg/ml of

puromycin and analyzed 6 days later. The following sequences were used for knockdown, for Rab4-Seq1 (Rab4a-CGTGAAGTTACCTTCTTAGAA and Rab4b-ACTGGCAAATCATGTCTCCTT), Rab4-Seq2 (Rab4a-GCATGGAATCTGATGTATGAT and Rab4b-ACTGGCAAATCATGTCTCCTT), for Arl1-seq1 (GCCATACTGGAGATGTTACTA) and Arl1-seq2 (CAAACACAGATGCAGTCATTT).

Western blotting

Cos7 cells were lysed in RIPA buffer (50mM Tris pH 7.4, 150mM NaCl, 1% NP40, 0.5% deoxycholate, 10% Glycerol, 0.1% SDS, 2mM EDTA). Protein lysates were spun at 13,000 rpm for 5 minutes and cleared lysates were mixed with SDS-PAGE sample buffer. Protein samples were resolved on a 13 % SDS gel, then transferred to PVDF membrane. Detection and quantification was carried out using a LI-COR Odyssey infrared scanning system using fluorescently labelled secondary antibodies.

Brefeldin A Treatment

Cells were treated with 5µg/ml of BFA for 10 minutes, fixed and immediately processed for immunofluorescence studies. Alternatively, cells were treated with BFA and imaged lived for the next 10 minutes.

Immunofluorescence microscopy

Cells were plated on fibronectin (1:200 dilution in PBS from a 0.1% stock solution) coated coverslips. The next day cells were fixed with 4 % paraformaldehyde for 10 minutes and blocked in PBS containing 0.1 % BSA (Bovine Serum Albumin) and 0.1 % Triton X (PBST) for 30 minutes. Incubation with both primary and secondary antibodies was for 60 minutes at room

temperature in blocking buffer. Coverslips were mounted on slides with Polong Gold Antifade reagent (Invitrogen).

Live Cell Imaging

24 hours after transfection, cells were plated on fibronectin coated Matek dishes (P35G-1.5-14c) and imaged 24 hours later (48 hours after transfection). For imaging, phenol free DMEM containing 10mM HEPES pH 7.4 with 10% FBS or without FBS was used accordingly. Cells were imaged using a 60X or 100X objective fitted to a Nikon TE 2000 microscope equipped with Yokogawa CSU 10 spinning disc and 512X512 Hamamatsu 9100c-13 EM-BT camera. Time lapse movies were captured at a typical frame rate of 1-3 seconds.

Supplemental References

S1. Peden, A.A., Oorschot, V., Hesser, B.A., Austin, C.D., Scheller, R.H., Klumperman, J. (2004). Localization of the AP-3 adaptor complex defines a novel endosomal exit site for lysosomal membrane proteins. J. Cell Biol. 164, 1065–1076.

S2. Volpicelli-Daley, L.A., Li, Y., Zhang, C., Kahn, R.A. (2005). Isoform-selective effects of the depletion of ADP- ribosylation factors 1–5 on membrane traffic. Mol. Biol. Cell. 16, 4495–4508.