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# Supplemental Data

# RhoBTB3: A Rho GTPase-Family ATPase Required for Endosome to Golgi Transport

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## Supplemental Experimental Procedures

*Plasmid generation*-Human cDNA clone KIAA0878 (clone hk07362) was obtained from Kazusa DNA Research Institute (Kisarazu, Chiba Japan). RhoBTB3 was cloned into the NdeI/XhoI sites of pET14b (Novagen, Madison, WI) into EcoRI/XbaI sites of pCDNA 3.1(+) (Invitrogen, Carlsbad, CA) and into EcoRI/XhoI sites of pGEX4T-1 (Amersham Biosciences, Piscataway, NJ). A GST-tagged, 191 C-terminal amino acid fragment of RhoBTB3 was generated by PCR using clone KIAA0878 as a template followed by digestion and ligation into the EcoRI/XhoI site of pGEX4T-1. RhoBTB3 truncations were generated by PCR and subcloned into EcoRI/XbaI sites of pCDNA3.1 (+). RhoBTB3-D532E, N138D and ΔCAAX were generated using a Quikchange II mutagenesis kit (Stratagene, La Jolla, CA).

*Antibodies and proteins*-Recombinant Rab9, Rab5, GST-Rab9, and GST-Rab5 were purified as described (Aivazian et al, 2006). pGST, pGST-RhoBTB3 (420-611), pGST-RhoBTB3 were transformed into Rosetta cells (Invitrogen). Cultures were grown to an $OD_{600}$ of 0.2, transferred to room temperature, and induced with 200mM IPTG at $OD_{600}$ =0.5. For protein purification, cells were lysed by French press in a buffer containing 40mM Hepes pH7.4, 250mM KCl, 1mM DTT, 5mM $MgCl_2$ and protease inhibitor cocktail (Roche). Lysates were centrifuged at 40,000 rpm (Ti45 rotor, Beckman). Supernatants were diluted 10-fold in lysis buffer with no KCl and passed through a 25ml Q-Sepharose Fast Flow column (GE Healthcare). Proteins were eluted with a 0-500mM NaCl gradient. A pool was then passed over a Glutathione agarose column and eluted with 20mM glutathione. Proteins were dialyzed into 40mM Hepes pH7.4, 200mM KCl, 40% glycerol and 1mM DTT. Rabbit anti-His-RhoBTB3 antibodies were prepared using 100µg antigen per immunization (Josman Laboratories, Napa Valley, CA). Mouse anti-Rab9, rabbit anti CI-MPR, mouse anti-EEA1 and anti-myc have been described (Reddy et al., 2006). Chicken anti-myc (Bethyl, Manassas, VA), sheep anti-TGN46, Alexa Fluor 488 or 647 goat anti-mouse and Alexa Fluor 594 goat anti-rabbit (Invitrogen, Carlsbad, CA) were purchased. The Rho domain of RhoBTB3 (amino acids 1-220) was cloned into pGEX4T-1 (GE Biosciences) and transformed into Rosetta BL21 DE3 cells. Saturated, overnight starter culture was added to 2L LB Broth supplemented with carbenicillin and chloramphenicol. Cultures were grown to $OD_{600}$~0.6 at 37°C and then moved to 30°C and induced with 0.5mM IPTG for 3 hours. Cells were resuspended in lysis Buffer (50mM Tris pH 8.0, 400mM NaCl, 2mM $MgCl_2$, 0.5mM EDTA, 1mM DTT, and 1mM PMSF), lysed with two passes at 15,000 psi in an C5-Emulsiflex (Avisten, Inc). Homogenate was spun 40,000 X g for 30 minutes; the supernatant was incubated with Glutathione-Sepharose (GE biosciences) equilibrated in lysis buffer for 90 minutes at 4°C. GST-RhoBTB3 Rho domain was eluted with 50mM Tris pH 8.0, 400mM NaCl, 2mM $MgCl_2$, 0.5mM EDTA, and 20mM Glutathione. The

eluate was dialyzed overnight against 50mM Tris pH 8.0, 400mM NaCl, 2mM $MgCl_2$, 0.5mM EDTA then snap frozen and stored at -80°C.

Full length His-RhoBTB3 in pET14b was transformed into Arctic Express RIL E. coli cells (Agilent). Cells were grown to saturation overnight at 37°C in carbenicillin and gentamicin. A sample (50ml) was diluted to 2L, transferred to 30°C for 3 hours, then moved to 12°C for 16 hours with 0.2mM IPTG. Cells pellets were resuspended in 50mM HEPES pH7.4, 400mM NaCl, 2mM $MgCl_2$, 1mM DTT, 1mM PMSF, 10mM imidazole and lysed with two passes at 15,000psi in an C5-Emulsiflex (Avisten, Inc). The homogenate was spun at 40,000 X g for 30 minutes; the supernatant was incubated with Ni-Sepharose (GE Biosciences) for 60 minutes at 4°C. His RhoBTB3 was eluted with 300mM imidazole and immediately applied onto an AKTA Sephadex 75 Prep grade column (GE Bioscience) equilibrated in 50mM HEPES pH7.4, 400mM NaCl, 2mM $MgCl_2$, 1mM DTT.

*HPLC*--Purified RhoBTB3 Rho domain was injected onto a reverse phase HPLC column (Varian) as described (Tucker et al., 1986). Peak fractions were collected and subjected to mass spectrometry (Waters Micromass ZQ single Quadrople, Waters Corp., Milford, MA).

*Kinetic Measurements*--Purified GST-RhoBTB3 Rho domain (8nmol) was incubated in 50mM Tris pH 7.4, 150mM NaCl, 10mM EDTA, 0.1% BSA (0.5ml) for 10 minutes at room temperature to remove bound ADP. EDTA and ADP was removed on a 10ml PD-10 column (GE Bioscience) equilibrated in exchange buffer to separate ADP from RhoBTB3. GST-RhoBTB3 Rho domain apoprotein was then added to [$\gamma$-$^{32}$P]-ATP and 20mM $MgCl_2$ in 420µl at 37°C. At 0, 2, 5, and 10 min, 50µl of sample was added to 750µl, 5% activated charcoal in 50mM phosphate pH 3.0. Samples were immediately vortexed and stored on ice until all reactions were complete. Samples were centrifuged; released radioactive phosphate in supernatants was determined by scintillation counting.

*siRNA depletion of RhoBTB3 and gene rescue*--Human RhoBTB3 was targeted using either of two duplexes: 5'-CATTACGAGTCATTGTTAA- 3' (NT:977-995) or 5'-CAGACAAAATGAAGAGTTA- 3' (NT: 405-423) for 72 hours. HeLa cell transfections were performed with Oligofectamine (Invitrogen, Carlsbad, CA). In rescue experiments, the siRNA was directly labeled with AlexaFluor 488-hydrazine according to Odom et al., 1980. Eight silent point mutations were introduced to make myc-RhoBTB3 constructs containing 977-CTCTTAGCGTTATCGTCAA-995 protected from siRNA mediated destruction. After 24 hours with siRNA, cells were transfected with rescue plasmid using Fugene 6 (Roche Diagnostics). After 72 hours of siRNA transfection and 48 hours of plasmid, cells were fixed and permeablized as described (Ganley, *et al* 2008).

*Binding and secretion assays*--Binding of RhoBTB3 C-terminus to Rab GTPases was as described (Reddy et al, 2006). Rab protein (2.6µM) was incubated with 3µM GTP$\gamma$S or 3µM GDP, 5µCi of [$^{35}$S]GTP$\gamma$S (MP Biomedicals, Irvine, CA) or 5µCi [$^{3}$H]GDP (GE Healthcare), and free nucleotide was removed on PD10 columns (GE Healthcare). Filter binding assays were performed to determine the fraction active Rab proteins. Nucleotide-loaded Rabs (600nM) were incubated with GST-RhoBTB3 C-terminus for 1 hr at 20°C. Complexes then were collected on glutathione resin and washed three times. Beads were counted in a scintillation

counter to determine the fraction of Rab bound to GST-RhoBTB3 C-terminus. For full length RhoBTB3, recombinant His-RhoBTB3 wild-type or D532E mutant (100nM) was incubated with 3μM Rab9-$^{35}$S-GTPγS for 1 hour at 20°C with 100mM ATPγS, in 20mM HEPES pH7.4, 200mM KCl, 5mM $MgCl_2$. Complexes were collected with Ni-NTA agarose, washed extensively in the binding buffer and eluted with 25mM EDTA in the same buffer. Quantitation by scintillation counting. In vitro transport and lysosomal enzyme secretion assays were as described (Reddy et al, 2006).

*Microscopy*-- In Supp. Fig. 2, cells were treated with or without 4μM nocodazole for 1 hr prior to fixation and processing for immunofluorescence (Warren et al, 1984). Cells were imaged using an Axiophot 2 fluorescence microscope (Carl Zeiss) fitted with a Plan-Apochromat 63X/1.40 oil objective with a Plan-Apochromat 63X/1.4 oil objective and a CCD camera (AxioCamHRc) controlled by Axiovision 4.2 software (Carl Zeiss). For Figure 3, images were acquired using a deconvolution microscope (Spectris, Applied Precision, Issaquah, WA) with an inverted epifluorescence microscope (IX70; Olympus American, Melville, NY), a PlanApo 60X 1.4 numerical aperture oil immersion objective (Olympus America), a CCD camera (CoolSNAP HQ) and acquisition and deconvolution software (Delta Vision, Applied Precision). The quantitative analysis of fluorescence intensity was done using ImageJ (NIH, Bethesda, MD). Images were processed using Adobe Photoshop (Adobe systems, Mountain View, CA). Quantitation of Golgi size used Matlab (Matworks). Cells and the Golgi complex (GCC185 or Golgin-245) were identified by a threshold intensity; the pixel area occupied by fluorescence was determined on a per cell basis. For Figure 5, images were acquired with an Eclipse 80i (Nikon, Inc) with a 60X/NA 1.40 objective lens and CCD camera (CoolSnapHQ; Photometrics, Inc.).

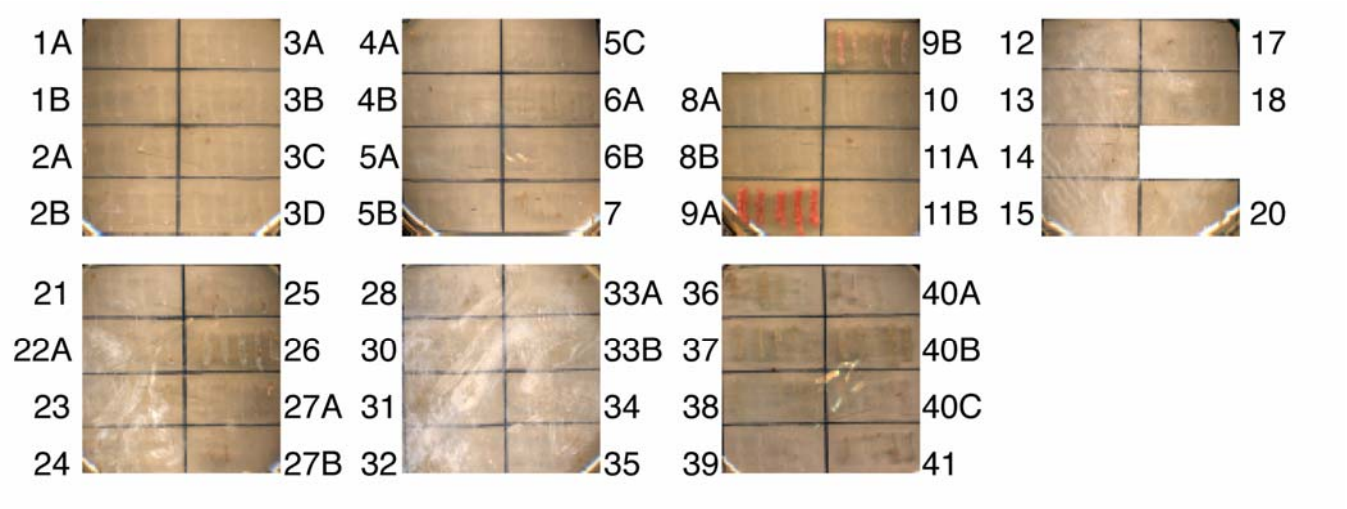


**Figure S1.**
RhoBTB3 is highly specific for Rabs 9A and 9B by yeast two hybrid screen.

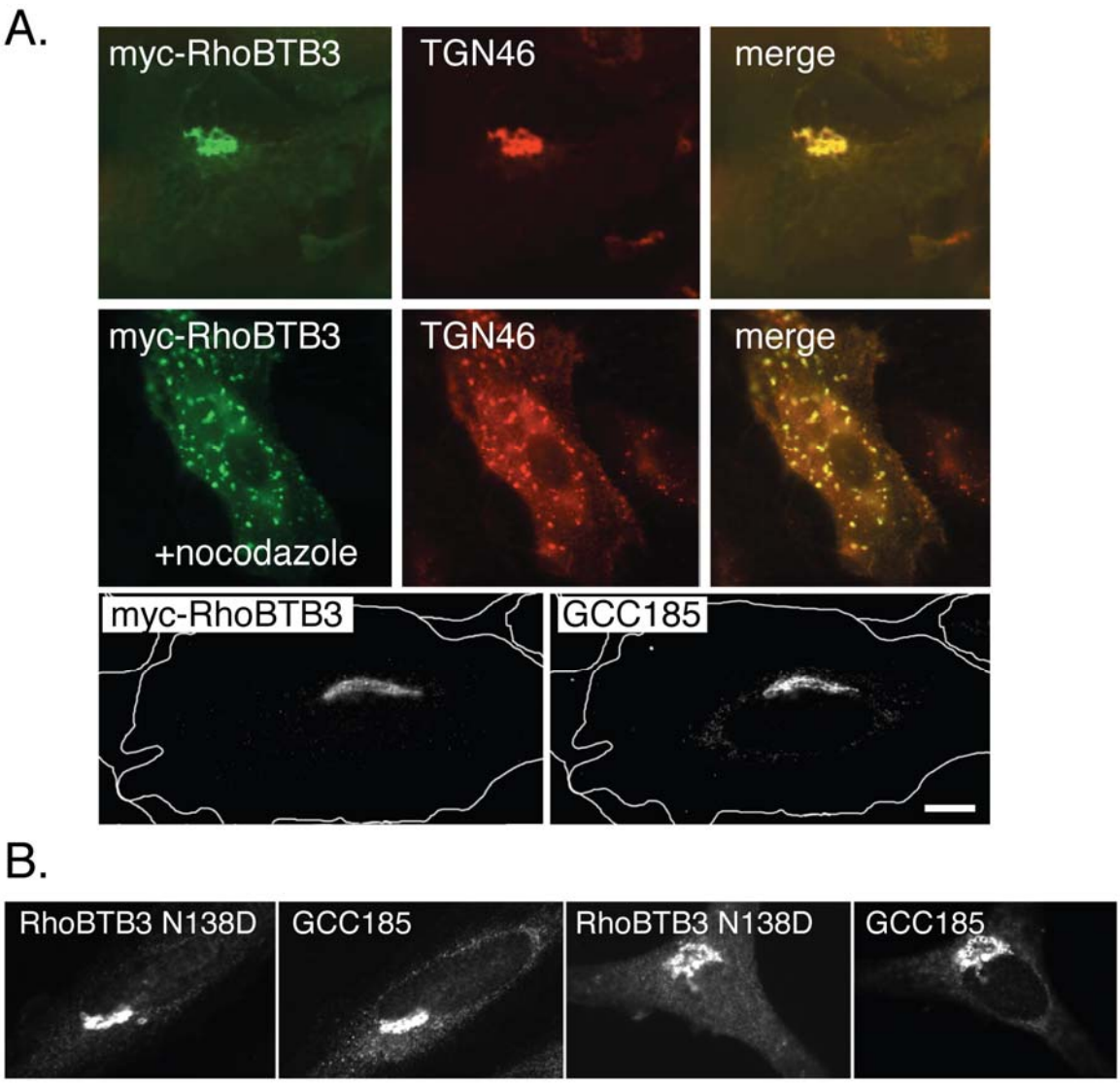


**Figure S2.**

A. RhoBTB3 is localized to the trans Golgi network. HeLa cells were transfected with a myc-RhoBTB3 plasmid for 16 hours. Top, myc-RhoBTB3 colocalizes with TGN46 in control cells and in cells treated with 4μM nocadazole for 1 hour prior to fixation (middle row). Bottom, myc-RhoBTB3 localizes to the same compartment as GCC185. Scale bar is 10μm; white line is approximate cell outline.

B. Myc-RhoBTB3 N138D colocalizes with TGN marker, GCC185**.**

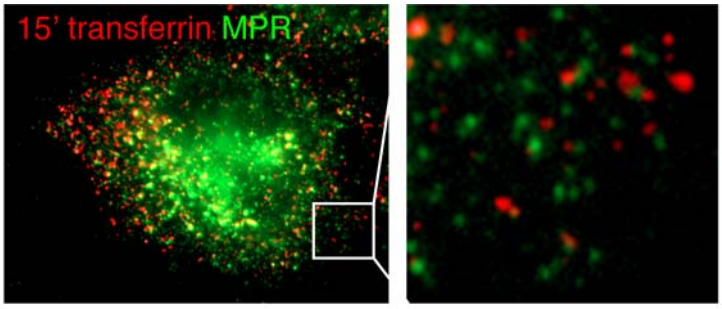


**Figure S3.**
Peripheral vesicles containing Mannose 6-phosphate receptors do no contain transferrin endocytosed for 15 minutes in RhoBTB3-depleted cells.