CHOLESTEROL ACCUMULATION SEQUESTERS RAB9 AND DISRUPTS LATE ENDOSONME FUNCTION IN NPC1-DEFICIENT CELLS*
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Niemann-Pick type C disease is an autosomal recessive disorder that leads to massive accumulation of cholesterol and glycosphingolipids in late endosomes and lysosomes. To understand how cholesterol accumulation influences late endosome function, we investigated the effect of elevated cholesterol on Rab9-dependent export of mannose 6-phosphate receptors from this compartment. Endogenous Rab9 levels were elevated 1.8-fold in Niemann-Pick type C cells relative to wild type cells, and its half-life increased 1.6-fold, suggesting that Rab9 accumulation is caused by impaired protein turnover. Reduced Rab9 degradation was accompanied by stabilization on endosome membranes, as shown by a reduction in Rab9's capacity for guanine nucleotide dissociation inhibitor-mediated extraction from Niemann-Pick type C membranes. Cholesterol appeared to stabilize Rab9 directly, as liposomes loaded with prenylated Rab9 showed decreased extractability with increasing cholesterol content. Rab9 is likely sequestered in an inactive form on Niemann-Pick type C membranes, as cation-dependent mannose 6-phosphate receptors were missorted to the lysosome for degradation, a process that was reversed by overexpression of GFP-tagged Rab9. In addition to using primary fibroblasts isolated from Niemann-Pick type C patients, RNA interference was utilized to recapitulate the disease phenotype in cultured cells, greatly facilitating the analysis of cholesterol accumulation and late endosome function. We conclude that cholesterol contributes directly to the sequestration of Rab9 on Niemann-Pick type C cell membranes which in turn, disrupts mannose 6-phosphate receptor trafficking.

Niemann-Pick type C (NPC) is an autosomal recessive, neurodegenerative disorder. A hallmark of NPC is the massive accumulation of cholesterol and glycosphingolipids within late endosomes and lysosomes (reviewed in refs 1-3). In normal cells, endocytosed low density lipoproteins are delivered to endosomes, where they are hydrolyzed and free cholesterol is released. This cholesterol is transported rapidly out of endosomes to the plasma membrane and ER (4,5). In NPC cells, the cholesterol does not exit the endocytic pathway and it accumulates within lysosomes.

Approximately 95% of NPC patients harbor mutations in the NPC1 gene that encodes a large, late endosomal protein with 13 transmembrane domains (6-8). Though NPC1 binds cholesterol weakly (9), the precise function of NPC1 is unknown; it may be involved in cholesterol export from late endosomes (10). The remainder of NPC patients carry mutations in the NPC2 gene that encodes a small, soluble protein present in the lumen of late endosomes and lysosomes (11). Unlike NPC1, NPC2 binds cholesterol with high affinity (12), but like NPC1, its precise role is unclear.

Late endosomes act as sorting stations to deliver endocytosed molecules to lysosomes for degradation, while at the same time, retrieving other classes of proteins and lipids for transport back to non-degradative compartments. Mannose 6-phosphate receptors (MPRs) represent recycling, late endosomal cargo proteins. MPRs carry newly synthesized lysosomal enzymes from the trans-Golgi Network (TGN) to endosomes, and then return to the TGN for another round of transport (13,14). Two distinct MPRs have been identified: the dimeric, ~46kDa cation-dependent (CD-) MPR and the ~300kDa cation-independent (CI-) MPR. Transport of MPRs from late endosomes to the trans Golgi is coordinated by the Rab9 GTPase (15,16) and requires the Rab9 effector and cargo adaptor, TIP47 (17,18), a Rab9 effector named p40 (19), the putative Golgi tether and Rab9 effector GCC185 (20), NSF, alpha SNAP, and a protein named mapmodulin (21).
Previous work showed that the motility of cholesterol-laden late endosomes is greatly reduced (22,23); they also accumulate CI-MPRs (24) implying that late endosome export is compromised. Another possible link between late endosome sorting and NPC comes from the observation that overexpression of GFP-tagged Rab9 in NPC fibroblasts relieves the accumulation of cholesterol and glycosphingolipids (25,26). The mechanism by which Rab9 might achieve this is currently unknown, but given the role of Rab9 in MPR export from late endosomes, the data suggest that this pathway might also be important for lipid and cholesterol export.

We have explored the consequences of cholesterol and glycosphingolipid accumulation in NPC mutant cells for late endosomal sorting and Rab9 function. We show here that increased cholesterol stabilizes Rab9 on late endosome membranes, and disrupts late endosomal export of MPRs in NPC1-deficient cells.

EXPERIMENTAL PROCEDURES

Recombinant Proteins, Antibodies and expression plasmids—Prenylated Rab9 and Rab5 were purified from Baculovirus infected insect cell membranes and complexed to bovine serum albumin (BSA) as described (27). Guanine nucleotide dissociation inhibitor (GDI) was purified from bovine brain (28). Antibodies were described earlier (29), or were mouse anti-Rab5 from BD Biosciences (San Jose, CA) and rabbit anti-GFP from Molecular Probes (Eugene, OR). Construction of mammalian expression vectors encoding GFP-tagged Rab9 and Rab7 has been described (30).

Cell culture—HeLa cells from ATCC (Manassas, VA) were cultured at 37°C and 5% CO₂ in Dulbecco’s modified Eagle’s media (DMEM) supplemented with 7.5% fetal calf serum, penicillin and streptomycin. Wild-type human fibroblasts (AG10803) and human Niemann-Pick type C fibroblasts (GM03123) from Coriell Cell Repositories (Camden, NJ) were grown in DMEM supplemented with 10% fetal calf serum, penicillin and streptomycin. HEK293 cells, from ATCC, were stably transfected with myc-tagged CD-MPR as described (20) and were cultured in DMEM with 7.5% fetal calf serum and 250 µg/ml G418. For RNA interference (RNAi), HeLa cells were transfected at 50% confluency with duplex RNA (Dharmacon Research, Lafayette, CO) using Oligofectamine (Invitrogen, Carlsbad, CA) according to the manufacturer. HEK293 cells were transfected, with RNA or DNA, at a confluency of 90% using Lipofectamine 2000 (Invitrogen) according to the manufacturer. For RNAi, NPC1 was targeted with siRNA against the sequence CCAGGTCTTTAAGTTACAA. TIP47 siRNA was reported previously (29). siRNA directed against GFP was used as a negative control (31). Unless stated, all RNAi depletions were carried out for 72 h. Specific silencing of targeted genes was confirmed by at least three independent experiments. For NPC1, good commercial antibodies were unavailable, thus efficiency of depletion was estimated by immunofluorescence staining for cholesterol using BC-theta (see below). Cholesterol accumulation was observed 48 h post transfection; experiments were carried out after 72 h depletion as the cholesterol accumulation was greater. About 70% of HeLa cells showed a large accumulation of cholesterol upon siRNA treatment.

Immunofluorescence Microscopy—A freeze-thaw protocol was used to permeabilize cells for Rab localization. Cells, grown and transfected on glass coverslips, were washed once in cold PBS and then once with cold glutamate lysis buffer (25 mM Hepes, pH 7.4, 25 mM KCl, 2.5 mM Mg-acetate, 5 mM EGTA, 150 mM K-glutamate). Coverslips, drained of excess buffer, were snap frozen by immersion in liquid nitrogen and then thawed at room temperature for 60 seconds before placing on ice. Cells were washed twice in glutamate lysis buffer then fixed at room temperature with 3.7% formaldehyde in 200 mM Hepes, pH 7.4 for 30 min, with one change of fixative after 10 min. After fixing, cells were washed twice in glutamate lysis buffer then fixed at room temperature with 3.7% formaldehyde in 200 mM Hepes, pH 7.4, followed by two washes and 15 min in PBS+1% BSA. For the staining of other proteins, Triton X-100 permeabilization was used (29). Cells were incubated with primary antibody (in BSA/PBS) for 30 min followed by washes, and 30 min in secondary antibody diluted 1: 1000 in BSA/PBS. Cholesterol was visualized by incubating coverslips in 0.05 mg/ml filipin (Sigma-Aldrich, St. Louis, MO) for 1 h prior to antibody staining. Alternatively, following permeabilization, cholesterol was visualized by incubating coverslips with 10 µg/ml of biotinylated streptavidin (Invitrogen, Carlsbad, CA) as described (32). BC0 was kindly supplied by Dr. Yoshiko Ohno-
Iwashita, Tokyo Metropolitan Institute of Gerontology. After washes and coverslip mounting, cells were imaged and processed as described (29).

**Immunoblot analysis**—Immunoblot analysis and quantification of indicated proteins was carried out as described (29).

**Half-life determination**—Pulse chase labeling of cells with Tran[^35S]-label to determine protein half-life has been described (29).

**Crude membrane fractionation**—Wild type and NPC fibroblasts, grown in 100 mm plates (or transfected HeLa cells) were washed three times with PBS and once with 10 mM Hepes, pH 7.4, and then swollen for 15 min at 4°C in 10 mM Hepes, pH 7.4, supplemented with protease inhibitors. Cells were harvested by scraping in homogenization buffer (20 mM Hepes, pH 7.4, 250 mM sucrose, 1 mM EDTA, 1 mM DTT plus protease inhibitors), and were homogenized with 5 passes through a 22g needle. A post nuclear supernatant (PNS) was obtained by centrifuging the homogenate at 3000 rpm, 4°C for 5 min. The PNS was further centrifuged at 98,000 rpm in TLA 100.2 rotor (Beckman-Coulter, Fullerton, CA) for 15 min at 4°C; the supernatant (cytosolic fraction) was removed and the pellet (membrane fraction) was resuspended in homogenization buffer. Protein concentration was determined by Bio-Rad protein assay (Bio-Rad, Hercules, CA) using bovine serum albumin as standard and Rab9 levels were quantified by SDS-PAGE and immunoblot.

**Salt-stripping of NPC membranes**—NPC crude membranes were mixed in 20 mM Hepes, pH 7.4, 250 mM sucrose, 0.5 mg/ml BSA containing either 1 M KCl, 1.5M KCl, 2 M KCl or 2 M urea on ice for 2 min. Membranes were then washed twice by centrifugation at 98,000 rpm, 4°C; the supernatant (cytosolic fraction) was removed and the pellet (membrane fraction) was resuspended in homogenization buffer. Protein concentration was determined by Bio-Rad protein assay (Bio-Rad, Hercules, CA) using bovine serum albumin as standard and Rab9 levels were quantified by SDS-PAGE and immunoblot.

**GDI-mediated Rab9 membrane extraction**—Crude membranes, in amounts that were normalized to contain equal amounts of Rab, were incubated in buffer containing 20 mM Hepes, pH 7.4, 100 mM KCl, 1 mM MgCl₂, 1 mM GDP, 0.5 mg/ml BSA and protease inhibitors. GDI, in the indicated amounts, was added to each sample for 10 min at 37°C. Samples were placed on ice and either spun down at 98,000 rpm for 10 min in a TLA 100.1 rotor (Beckman-Coulter) and the supernatant removed (to obtain the extracted Rab) or not centrifuged (to obtain the total, input Rab, used in the reaction). The quantity of Rab in each sample was then analyzed by SDS-PAGE and immunoblot.

**Crude endosome isolation**—HeLa cells were grown to 90% confluency in 150 mm plates. Where indicated, cells were incubated with 3 µM U18666A (BIOMOL International, Plymouth Meeting, PA) for 20 h prior to harvesting. Cells were washed three times with PBS and once with 10 mM Hepes, pH 7.4, and then swollen for 15 min at 4°C in 10 mM Hepes, pH 7.4, supplemented with protease inhibitors. Cells were harvested by scraping in 20 mM Hepes, pH 7.4, 250 mM sucrose, 1 mM EDTA, 1 mM DTT supplemented with protease inhibitors, and were then dounce-homogenized with 10-15 strokes. The homogenate was adjusted to 1.5 M sucrose, loaded at the bottom of a non-linear sucrose gradient (1.5 M sucrose, 1.2 M sucrose and 0.25 M sucrose) and centrifuged at 25,000 rpm in an SW28 rotor (Beckman-Coulter) for 2h at 4°C. Crude endosomes were isolated from the 1.2-0.25M sucrose interface. Membranes were concentrated by pelleting at 98,000 rpm for 10 min at 4°C and resuspended in 20 mM Hepes, pH 7.4, 100 mM KCl, 250 mM sucrose, 1 mM EDTA, 1 mM DTT and protease inhibitors, and snap frozen in liquid nitrogen. Samples were analyzed by immunoblotting to determine the levels of Cl-MPR, TIP47 and Rab9.

**Liposome preparation**—Liposomes were prepared according to (www.avantilipids.com/pdf/MorrisseyLabProtocolForPrepSuvBySonication.pdf).

Soybean polar lipid extract (3µmol, Avanti Polar Lipids, Alabaster, AL) with or without 3 µmol cholesterol (Sigma-Aldrich) were dissolved in 200 µl benzene:methanol (19:1). Lipids were then dried under a stream of nitrogen, followed by 2 h in a speed-vac. Dried lipids were then hydrated in 3 ml HBS (20 mM HEPES, pH 7.4, 100 mM NaCl) for 1 h with vigorous vortexing every 5 min. Following resuspension, lipids were subject to six cycles of freeze/thaw in liquid nitrogen and then sonicated in a water bath until the lipid suspension changed from a chalky to opalescent appearance indicative of liposomes. The liposome preparations (2 mM lipid) were stored under nitrogen at 4°C until needed.

**Rab loading of liposomes and GDI extraction**—Rabs were loaded onto liposomes using the following ratios: approximately 10 pmol of prenylated Rab, complexed to BSA, was incubated with 100 µl of liposomes containing 1 mM MgCl₂ and 1 mM GDP for 20 min at 37°C. GDI extractions were carried out by incubating 50 µl of Rab-loaded liposomes in buffer A (20 mM
HEPES, pH 7.4, 100 mM NaCl, 1 mM MgCl$_2$, 1 mM GDP, 0.5 mg/ml BSA) with the indicated amount of GDI for 10 min at 37°C. Following this, samples were placed on ice and the volume made up to 400 µl in buffer A with Nycodenz (Sigma-Aldrich) to a final concentration of 47% (w/v). This was then overlayed with 250 µl of 30% Nycodenz followed by 100 µl buffer A. Samples were then centrifuged at 95,000 rpm for 1 h at 4°C in TLA 100.2 rotor (Beckman-Coulter). 150 µl fractions were taken from the top of the sample and analyzed for Rab content by dot-blot. Liposomes were present in the top fraction, while solubilized Rab remained in the bottom two fractions. Initial loading efficiency of Rabs on liposomes was approximately 20%, calculated by comparing the quantity of prenyl Rab present on liposomes after isolation by flotation on a Nycodenz gradient, with the initial quantity of prenyl Rab/BSA complex employed. Rab rescue of CD-MPR degradation—CD-MPR expressing HEK293 cells, grown in 100 mm plates, were transfected with NPC siRNA or control transfected as indicated. 24 h post-transfection cells were split into multiple 35 mm plates and 48 h post transfection cells were re-transfected in duplicate with the indicated GFP-Rab construct. Following 72 h of RNAi depletion, cells were harvested and proteins analyzed by SDS-PAGE and immunoblot.

RESULTS

Rab9 is not essential for cholesterol trafficking

Previous reports concluded that Rab9 overexpression could rescue cholesterol and lipid accumulation in NPC fibroblasts (25,26). Rescue may have been achieved by stimulating Rab9-mediated, vesicular transport from late endosomes to the TGN. If so, it was possible that excess cholesterol used this route in Rab9 overexpressing cells for removal from late endosomes.

To test whether Rab9 is normally required for cholesterol export from late endosomes, we used RNA interference to deplete Rab9 from HeLa cells and looked at cholesterol levels and localization using filipin staining (Fig. 1). If the Rab9 pathway represents a significant route for cholesterol egress from late endosomes, Rab9 depletion would be expected to cause an accumulation of cholesterol in late endosomes. As shown in Figure 1, filipin staining in control cells was essentially the same as that seen in cells that had lost more than 90% of their Rab9 protein content. Examination of >100 cells indicated no significant change in filipin staining in Rab9 depleted cells compared with control cells. This demonstrates that Rab9 is not essential for cholesterol export from late endosomes in normal cells. These findings do not preclude cholesterol export via a Rab9 pathway in NPC cells overexpressing Rab9.

Recapitulation of the NPC1 disease phenotype using siRNA

We used small interfering RNA (siRNA) to disrupt NPC1 protein in cultured cells. This approach has the potential to reveal the earliest consequences of the loss of NPC1 protein function, separate from compensatory changes that may occur in cells lacking NPC1 function over many generations.

As shown in Figure 2, siRNA depletion of NPC1 was readily evidenced by a large increase in perinuclear, cholesterol accumulation detected by BC0 staining (Fig. 2, middle row). The siRNA transfection efficiency was about 70%, consistent with the fraction of cells that displayed this phenotype. The accumulation observed was highly reminiscent of that seen in fibroblasts isolated from patients carrying NPC1 mutations (33,34). After 72 h of NPC1 siRNA treatment, the bulk of the accumulated cholesterol was not detected in early endosomes (marked by co-staining with anti-Rab5 antibody (Fig. 2, left column)), nor in the majority of late endosomes (marked by co-staining with Rab7 or Rab9 antibody (Fig. 2, center columns)). Cholesterol did accumulate in a compartment that co-stained for the lysosomal protein, LAMP1 (Fig. 2, right column), suggesting that cholesterol first accumulates in a lysosome-related organelle, as shown previously for NPC fibroblasts (33-35). It should also be noted that the overall localization of early and late endosomes appeared unchanged in cells that accumulated cholesterol (Fig. 2, compare cells within each panel), whereas LAMP1-positive compartments were somewhat more dispersed when they contained excess cholesterol.

This technique provides a powerful approach to study NPC disease, as any cultured cell line amenable to siRNA transfection can now be used as a model for the loss of NPC1 function. This approach alleviates the challenges associated with primary cell culture, as well as secondary effects of drugs (e.g. U18666A) used to trigger cholesterol accumulation. In addition, siRNA transfection permits analysis of the initial stages of cholesterol accumulation, in the
absence of long term adaptive changes that primary cells may have made during their chronic exposure to high levels of accumulated cholesterol.

**Rab9-positive endosomes also acquire excess cholesterol**

Cholesterol derived from the hydrolysis of LDL is rapidly transported out of late endosomes (36,37). Thus, in normal cells at steady state, very little cholesterol is detected in late endosomes and lysosomes (36-38). However, in NPC cells, cholesterol accumulates primarily in terminal, lysosome-related organelles that are distinct from late endosomes in that they are LAMP1-positive and Rab7- and Rab9-negative (Figs. 2 and 3). Because rapid cholesterol export from late endosomes is blocked in NPC, an increase in late endosome cholesterol is also predicted. Indeed, the cholesterol content of Rab9-positive late endosomes does appear to be increased in NPC fibroblasts (Fig. 3).

Filipin staining for cholesterol in wild type human fibroblasts revealed peripheral punctae (green; Fig. 3, left panels) that did not overlap with Rab9 staining (red; Fig. 3, left panels). A high proportion of the filipin-labeled structures were positive for Rab5, suggesting that they are early endosomal in nature (data not shown). In NPC fibroblasts, a large amount of the accumulated cholesterol (green; Fig. 3, right panels) was distinct from Rab9-positive structures (red; Fig. 3, right panels). Nevertheless, there were an increased number of Rab9 structures that were positive for cholesterol staining (arrows in Fig. 3, right panels). Given the tendency of filipin to photobleach at a rapid rate, it was not possible to determine whether cholesterol concentration was increased in the majority of Rab9-positive late endosomes or in only a subset of them. Similar increases in Rab9-cholesterol colocalization were observed in BsC-1 cells treated with the hydrophobic amine U18666A to mimic the NPC cholesterol accumulation phenotype (data not shown). Thus, in NPC cells, most cholesterol accumulates in lysosomes (Fig. 2); in addition, cholesterol can accumulate in late endosomes (Fig. 3), and this pool is likely a precursor of the cholesterol present in LAMP-1 positive lysosomes.

**Cholesterol directly stabilizes prenylated Rab9 on the membrane**

As cholesterol levels were increased in the Rab9-containing late endosomes of NPC cells, we were interested to investigate how elevated cholesterol influenced the properties of endogenous Rab9 protein and its known binding partners. As shown in Fig. 4, the steady state level of Rab9 was increased almost two-fold in NPC fibroblasts compared to wild type fibroblasts; this was in contrast to the early endosome-localized Rab5, which only marginally increased (Fig. 4A and 4B). Steady state levels of the Rab9 effectors, GCC185 and TIP47 remained unchanged; there was a modest, 1.4-fold increase in the effector, p40 (Fig. 4A and B). In addition, CI-MPR levels also increased 1.75-fold in NPC cells (Fig. 4A and B). The Golgi marker, p115, did not change in NPC compared to wild type cells and served as a useful loading control (Fig. 4A and B).

It is interesting to note that immortalization of NPC1 cells by ectopic expression of telomerase reverse transcriptase led to increased levels of Rab9 and p40, suggesting that these proteins may be co-regulated (25). The increased expression of Rab9 observed here could represent an attempt by the cell to relieve cholesterol accumulation by stimulating late endosome to TGN transport. Alternatively, Rab9 could be physically stabilized in NPC cells; the increased levels could be due to a reduction in Rab9 degradation.

To distinguish between these two possibilities we examined the turnover of Rab9 protein in NPC cells compared to wild type cells (Fig. 4C). The half-life of Rab9 increased 60% from 44 hours in wild type cells to 70 hours in NPC cells, implying protein stabilization rather than gene activation. Similarly, Rab9 half-life was increased slightly (from 36 hours to 46 hours) in HeLa cells forced to accumulate cholesterol by U18666A treatment (Fig. 4C). The slight difference between drug-treated and NPC cells might reflect the different extents of cholesterol accumulation in these cell types.

The increase in Rab9 half-life in NPC cells was not due to a general decrease in protein degradation, as CI-MPR half-life was reduced 10% (from 41 hours to 36 hours; data not shown). In contrast to Rab9, while CI-MPR levels also increased in NPC cells (Fig. 4A & B), the rate of degradation was not slowed. This suggests that the rate of CI-MPR synthesis is increased. Induction of CI-MPR expression has been observed previously in cells where MPR recycling is disrupted, for example by depletion of Rab9 (29) or overexpression of a dominant negative Rab9 mutant (16). This increased rate of MPR production allows cells to maintain lysosomal enzyme delivery in the absence of MPR.
recycling. Hence, the increase in CI-MPR synthesis observed here is consistent with the possibility that MPR recycling is disrupted in NPC cells; moreover, the elevated Rab9 protein may be present in a functionally inactive form (see below).

By what mechanism is Rab9 stabilized in NPC cells? Rab proteins are doubly prenylated at their C-termini and cycle between membrane surfaces and the cytosol. Membrane-associated, GDP-bearing Rab proteins are extracted from membranes by guanine nucleotide dissociation inhibitor (GDI). This protein has the capacity to deliver Rab proteins to the appropriate membrane compartment for further membrane trafficking events (39,40). Given the increase in membrane cholesterol, we tested whether Rab9 might be stabilized on membranes in part, because it resisted extraction by GDI. This has already been demonstrated for Rab7 (22) and Rab4 but not Rab5 (41).

Using crude membrane fractions prepared from primary human fibroblasts (and normalized for Rab content), we showed that GDI could extract over 90% of Rab5 or Rab9 from wild type membranes, whereas only 35% of Rab9 or Rab5 was extracted from NPC membranes, under the same conditions (Fig. 5A and B). Rab extraction was absolutely dependent upon GDI, as no was Rab solubilized in its absence (Fig 5A). The resistance of Rab9 to GDI extraction from NPC endosomes is likely due to the increase in late endosome cholesterol, because Rab9 in membranes from HeLa cells treated with U18666A were also more resistant to GDI extraction (see below, Fig. 5C). Rab extraction was absolutely dependent upon GDI, as no was Rab solubilized in its absence (Fig 5A). The resistance of Rab9 to GDI extraction from NPC endosomes is likely due to the increase in late endosome cholesterol, because Rab9 in membranes from HeLa cells treated with U18666A were also more resistant to GDI extraction (see below, Fig. 5C). We cannot explain why we obtain a different result for Rab5 than Choudhury et al. (41) except that the assays used to measure Rab extraction differ. In our case, extraction is measured directly, after reaction in solution, using untagged, purified bovine brain GDI.

These experiments showed that prenylated Rab9 is resistant to extraction from endosome membranes containing high cholesterol. One possible explanation is that the two Rab9-associated geranylgeranyl groups interact more tightly with cholesterol-rich endosome bilayers. Another possibility is that the effect is indirect: perhaps the Rab is stabilized by a binding partner that is itself stabilized in endosomes by increased cholesterol. Choudhury et al. (41) reported that the inhibition of Rab4 extraction by GDI from NPC endosomes could be relieved by salt washing of membranes to disrupt interactions between Rab4 and other proteins. This suggested that the stabilization of Rabs by cholesterol may be indirect. To test this hypothesis for Rab9 we washed membranes under a variety of conditions to remove peripherally-associated proteins (Fig. 5C). As a positive control, we analyzed the membranes for loss of Golgi-associated p115. As shown in Fig. 5C, KCl-washed membranes contained 70% less p115 protein and urea-washed membranes contained 85% less p115 when normalized to the amount of Rab9 present in the membrane preparation. Rab9 was used as a standard because it did not change upon salt washing. In contrast to the previous report for Rab4 (41), salt washing of NPC membranes failed to relieve the block in Rab9 extraction by GDI; rather, such treatments further inhibited extraction, perhaps by triggering membrane aggregation (Fig. 5D and E).

Salt washing endosome membranes left a significant amount (>60%) of the peripheral protein and Rab9 effector, TIP47 (Fig. 5C). As TIP47 depletion reduces Rab9 stability (29), we investigated the influence of TIP47 on Rab9 membrane extraction in NPC cells. As shown above, the steady state level of TIP47 did not change in NPC cells compared with wild type cells (Fig. 4A and B). However, since only 10-20% of total TIP47 protein is membrane-associated (17), we tested whether levels of membrane-associated TIP47 changed in cells that had accumulated cholesterol.

Crude endosomes isolated from HeLa cells treated with U18666A showed a greater than 3.5-fold increase in membrane-associated TIP47 compared with control-treated endosomes (Fig. 6A). Because of protein mass differences, this increase is equivalent to one mole additional TIP47 per mole, stabilized Rab9 protein. NPC cells also showed higher amounts of total membrane-associated TIP47 than control cell extracts (data not shown).

To test if TIP47 interaction with Rab9 was responsible for the observed inhibition in GDI extraction, we depleted TIP47 from cells using siRNA. Figure 6B shows the effect of TIP47 depletion on Rab9 protein levels in the post nuclear supernatant (PNS) of cells treated with or without U18666A. siRNA treatment led to >90% reduction in TIP47 protein levels (Fig. 6B, top panels). We found previously that TIP47 depletion led to a four-fold increase in Rab9 turnover, balanced by a concomitant increase in Rab9 gene expression (29). Similarly, in these experiments, Rab9 levels increased only slightly upon TIP47 depletion (Fig. 6B, left 2 lanes).
Surprisingly, a combination of TIP47 depletion and U1866A treatment led to a three-fold increase in steady state Rab9 levels (Fig. 6B, bottom left). This suggests that the stabilizing effect of cholesterol might override increase in Rab9 degradation seen in cells lacking TIP47. The same results were obtained for Rab9 when total membranes from control and TIP47-depleted PNS were analyzed (Fig. 6B, right panels). Similar to the isolated endosomes (Fig. 6A), crude PNS membranes showed increased TIP47 levels when cells were forced to accumulate cholesterol in the presence of U1866A (Fig. 6B, top right panel). As Rab9 levels were increased with U18666A in the absence of TIP47, these data argue against the possibility that TIP47 binding to Rab9 is responsible for the block in GDI extraction seen upon cholesterol accumulation.

Further support for this conclusion came directly from GDI extraction assays using membranes from cells depleted of TIP47. As with membranes derived from NPC fibroblasts (Fig. 5), membranes from HeLa cells treated with U18666A showed a two-fold decrease in GDI-extractable Rab9 when compared with control treated membranes (Fig. 6C). Loss of TIP47 from these membranes via siRNA did not significantly alter the cholesterol-induced block in Rab9 extraction (Fig. 6C). Therefore TIP47 is not responsible for the increased Rab9 membrane stability upon cholesterol accumulation. As TIP47 and Rab9 interact directly (18), the increased levels of TIP47 on cholesterol-laden membranes can be most readily explained by the presence of more membrane-stabilized Rab9 with which it can interact.

We reconstituted prenylated Rab9 in artificial liposomes to investigate if cholesterol could directly stabilize Rab9 in membrane bilayers. Liposomes were prepared from purified soybean phospholipids, with or without 50% (1:1 mol:mol) cholesterol, together with purified prenylated Rab9. This represents the maximum capacity of liposomes for cholesterol. GDI extraction assays were then carried out to test the direct effect of cholesterol on Rab9 membrane stability. Following GDI extraction, reactions were loaded on the bottom of a Nycodenz gradient and subjected to density gradient centrifugation, such that the liposomes and lipidome-associated Rab proteins float to the top of the gradient; the GDI-extracted Rab proteins remain at the bottom.

Figure 7 shows dot-blot analyses of Rab9 and Rab5 proteins in liposome fractions obtained after GDI extraction (upper panels) and their quantification (lower panels). As expected, up to 80% of liposome-associated Rab proteins were extracted by GDI when liposomes were prepared in the absence of cholesterol (dashed lines). In contrast, the presence of 50% cholesterol in the membranes interfered with GDI’s extraction capability. The presence of cholesterol decreased the potency of GDI by about two-fold, in good agreement with the earlier results obtained for NPC membranes or HeLa membranes treated with U18666A. The effect was dependent on cholesterol concentration as 5 pmol of GDI extracted 75% of Rab9 from liposomes containing no cholesterol, 55% of Rab9 from liposomes with 40% cholesterol, and 40% of Rab9 from liposomes containing 50% cholesterol under these conditions (data not shown). Therefore, these data show for the first time, that cholesterol can directly stabilize a Rab in a phospholipid bilayer. Stabilization was observed with both Rab9 and Rab5, and thus seems to be a general feature of prenyl group:cholesterol interaction.

Rab9 function is blocked in NPC cells, leading to MPR missorting

The increase in CI-MPR protein levels in NPC cells (Figure 4) suggested that MPR trafficking might be inhibited. Rab9 levels were also increased (Fig. 4), but the Rab protein resisted extraction by GDI (Fig. 5) and recruited additional TIP47 protein onto endosomes (Fig. 6). To determine whether MPR export was impaired in NPC cells, we took advantage of the observation that blocks in MPR export from late endosomes usually lead to MPR missorting to lysosomes and subsequent degradation (16,29,42). To facilitate analysis, we utilized a HEK293 cell line that stably expresses a myc-tagged version of the CD-MPR. NPC1 siRNA was then used to deplete NPC1 protein from these cells.

As seen with HeLa cells depleted of NPC1 (Figs. 2, 8A left panel), HEK293 cells depleted of NPC1 protein showed an increase in cholesterol staining (Fig. 8B, left panels, asterisks). The localization of both CI-MPRs and CD-MPRs changed significantly in cells lacking NPC1. The CI-MPRs were much more dispersed (Fig. 8A, right panel). More striking was the change seen for CD-MPRs which were almost completely absent (Fig. 8B, right panel asterisk). This loss of CD-MPRs was confirmed by immunoblot analysis of NPC1-depleted cell lysates: 72h treatment with NPC1 siRNA resulted in a 70% reduction of CD-MPR levels (Fig. 8C). This reduction in CD-MPR is almost identical to the reduction seen in...
cells depleted of Rab9 (Fig. 8C). Given that disruption of Rab9 is already known to cause missorting and degradation of MPRs (16, 29), the observed loss of CD-MPR upon NPC1 depletion correlates well with a block in Rab9 function in these cells.

If Rab9 function is impaired in cells lacking NPC1, the loss of function might be rescued by the addition of exogenous Rab9 protein. To test this, we overexpressed GFP-tagged Rab9 in NPC1 depleted cells, and monitored the sorting of CD-MPRs. As shown in Fig 9A, overexpression of GFP-Rab9 alone resulted in a two-fold increase in CD-MPRs in the HEK293 cells (compare lanes 1 and 5; Fig. 9B). This shows that Rab9 is normally limiting and not present in sufficient amount to recycle all CD-MPRs to the Golgi complex (Fig. 9A and B). In contrast, overexpression of GFP-Rab7 did not increase CD-MPR levels but instead led to a 30% decrease (Fig. 9A compare lanes 1 & 3; Fig. 9B). This is perhaps not surprising as Rab7 functions in traffic from endosomes to lysosomes (43-46); overexpression presumably increased the rate of this transport step, and hence the rate of CD-MPR degradation. As expected, overexpression of the dominant negative Rab9 mutant, Rab9S21N, resulted in a 58% reduction in CD-MPR levels (16) (Fig. 9A and B). When the cells were depleted of NPC1, overexpression of GFP-Rab7 or CFP-Rab9S21N did not rescue CD-MPRs from degradation (Fig. 9A, B). To highlight the specific consequences of NPC-depletion in this data set, we replotted the amount of CD-MPR in GFP-Rab transfected, NPC1-depleted cells, compared to the amount of CD-MPR in cells that were only transfected with GFP-Rab-constructs (Fig. 9C). The control bar represents the amount of CD-MPR measured in cells that were mock transfected in the absence of siRNA and absence of Rab plasmid; the mock bar represents cells transfected with NPC1 siRNA but no Rab plasmid.

For the mock-, GFP-Rab7- and CFP-Rab9S21N-transfected cells, approximately 60% of the CD-MPR had been degraded. In contrast, GFP-Rab9 overexpressing cells showed only an 11% loss of CD-MPRs at steady state. Therefore, specific overexpression of wild type Rab9 GTPase rescues the cholesterol-induced degradation of CD-MPR in these HEK293 cells.

Quantitative immunoblots revealed that there was an approximate 20-fold increase in total GFP-Rab9 over endogenous Rab9 (120 pmol and 6 pmol per mg cell extract respectively), but only an 8-fold increase in membrane-associated Rab9 (data not shown). Endogenous Rab9 is almost entirely membrane-associated at steady state (15) and control experiments have shown that ~50% of expressed, GFP-Rab9 is unprenylated. It is thus likely that the 8-fold increase in membrane-associated, prenylated Rab9 is responsible for rescuing CD-MPR from degradation, as Rab9 lacking prenylation sites does not stimulate MPR transport in vitro (15). We were not able to determine the minimum amount of Rab9 overexpression required for rescue, as similar expression levels were obtained even 12 hours after transfection.

In summary, these data support a model in which Rab9 function is inhibited on cholesterol-laden late endosomes of NPC cells. They also confirm a key role for Rab9 in stabilizing CD-MPRs in living cells.

DISCUSSION

The work presented here was initiated to understand the mechanism by which Rab9 protein can alleviate the cholesterol accumulation phenotype of cells lacking functional NPC1 protein (25, 26). We show here that depletion of Rab9 protein does not yield the same phenotype as depletion of NPC1 protein. The simplest explanation for these findings is that Rab9 acts to relieve cholesterol accumulation by a pathway that is quite distinct from the normal mechanism by which NPC1 drives cholesterol efflux from late endosomal compartments. Although it has not yet been demonstrated directly, it seems likely that NPC1 protein is itself a cholesterol transporter or it acts together with another membrane associated protein to drive cholesterol efflux. In contrast, Rab9 is likely to stimulate the vesicular export of membrane vesicles from late endosome compartments. Perhaps enhancing this pathway by Rab9 expression provides a molecular bypass to enable cholesterol to be removed from late endosome compartments.

Cholesterol plays an important role in determining the physical properties of biological membranes, and also in organizing membranes into discrete microdomains essential for their normal functions (47). The activities of many integral membrane proteins are modulated by the physical properties of the membranes in which they reside (48). We show here that the function of the peripheral membrane protein Rab9 is also influenced by the composition of the lipid bilayer. Cholesterol interferes directly with the ability of the Rab protein to be
Cholesterol appears to be a general factor in stabilizing Rabs on membranes, as both Rab9 and Rab5 resisted GDI extraction, and Rab7 and Rab4 have also been shown to be stabilized on cholesterol rich membranes (22,41). From these earlier studies, it was unclear whether cholesterol was directly stabilizing the Rab or whether it influenced GDI access indirectly, through other proteins. We have shown here that cholesterol alone can stabilize a prenylated Rab in a lipid bilayer.

The lipid content of endosomal membranes is thought to consist of ~30-40% cholesterol (1). The limiting membrane of late endosomes in NPC cells is enriched in glycosphingolipids. Because of the larger head group of these lipids, the bilayer can likely accommodate higher levels of cholesterol than wild type endosome membranes (1). Thus, although it has not been measured directly, we assume that the limiting membrane of NPC cell late endosomes contains more cholesterol than endosomes present in wild type cells.

Prenyl Rab5 and prenyl Rab9 were more resistant to GDI extraction from liposomes containing cholesterol. The effect was cholesterol concentration dependent. If our assumption is correct that NPC late endosome limiting membranes contain more cholesterol than wild type membranes, our findings offer a molecular explanation for the inhibition of GDI function on Rabs bound to NPC-endosome membranes.

Cholesterol increases order in a mixed lipid bilayer, which in turn allows the hydrophobic core to pack more tightly (49). This tighter packing could increase the intermolecular interactions between the Rab prenyl groups and the lipid bilayer, and in doing so, make it physically more difficult for GDI to extract the Rab. In NPC1-deficient cells, glycosphingolipids also accumulate and might also contribute to Rab stability. We have not tested glycosphingolipids in the Rab-GDI extraction analyses presented here as their asymmetric distribution on the lumenal side of the lipid bilayer is technically very difficult to reproduce in purified liposomes. Importantly, however, cholesterol alone was sufficient to alter the GDI-extraction properties of Rab proteins. We also cannot rule out further protein-mediated stabilization of Rab proteins in living cells. Nevertheless, the stabilization of Rab9 on membranes was associated with a decrease in Rab9 degradation rate in vivo, leading to an increase in Rab9's steady state protein level.

Stabilization of Rab9 on the membrane appears to inhibit its ability to catalyze MPR export from late endosomes. A trafficking defect for MPRs in NPC cells was suggested previously by Kobayashi et al. (24) who reported a redistribution of CI-MPR to cholesterol-laden endosomes at steady state. The CI-MPR relocation that we observed upon siRNA-mediated, NPC1 depletion is consistent with their data. In addition, we have shown that CD-MPR is rapidly degraded in cells depleted of NPC1. CD-MPR was degraded more rapidly than CI-MPR, whose levels did not change significantly upon NPC1 depletion in these cells (data not shown). One possible explanation for this difference could relate to the differences in the precise localizations of the two receptor types. Klumperman et al. (50) showed by electron microscopy that within a given, late endosome, much more CI-MPR was associated with internal endosomal membranes; the CD-MPR was instead, more concentrated on the outer, limiting membrane of this compartment. It is possible that sequestration of CI-MPRs within the internal membranes in some way protects this receptor type from the more rapid degradation seen for CD-MPRs.

It is interesting to note that in the Chinese hamster ovary cell line LEX2 (a mutant defective in the degradation of low-density lipoprotein and the release of non-esterified, free cholesterol) MPRs accumulate in arrested, multi-vesicular endosomes; this phenotype can be reversed by addition of cholesterol to the growth medium (51). This implies that a fine balance exists between cholesterol concentration and endosome function: too little or too much can be inhibitory for MPR export.

The more diffuse localization of CI-MPRs (Fig. 8A) combined with the increased degradation of CD-MPRs (Fig. 8B) suggests that MPR recycling is disrupted in NPC cells. A likely explanation is sequestration of Rab9 in an inactive form, as introduction of exogenous Rab9 rescued the degradation phenotype (Fig. 9). In general, prenylated proteins are thought to prefer more disordered lipid domains containing lower cholesterol concentrations (52,53). The high cholesterol content of late endosomes in NPC cells may force and trap Rab9 into a more ordered domain, which in turn, could restrict the Rab9-effector interactions required to initiate vesicle budding. If that unusually ordered domain reduces the availability of Rab9 for effector interactions, increasing Rab9 concentration may increase the proportion of Rab9 that is available for
productive effector interactions. This in turn will increase the probability of vesicle budding. Although it is formally possible that overexpressed Rab9 acts in a manner distinct from endogenous Rab9 protein, we consider this unlikely because we rescue the normal, Rab9-mediated process of MPR recycling to the Golgi complex. It is equally possible that the block in MPR recycling is due to a more general effect of excess cholesterol. Excess cholesterol may interfere physically with the formation of transport vesicles, despite the presence of the protein machinery required to initiate this process. One point argue against this hypothesis: overexpression of GFP-tagged Rab9, and not GFP-Rab7 or CFP-Rab9S21N, rescued CD-MPR from degradation, and in other studies, led to removal of the accumulated cholesterol (25,26). As trapped cholesterol can apparently exit late endosomes through overexpression of Rab9 (25,26), our data suggest that the accumulation of cholesterol in NPC cells could be exacerbated by inhibition of endogenous Rab9.

Rab5 also resisted GDI extraction from membranes of NPC cells, however the function of early endosomes in these cells does not seem to be impaired (54). In wild type cells, the bulk of cholesterol normally occupies early, and not late, endocytic and recycling compartments (our unpublished observations and (38,55)). Rab5 function differs from Rab9 function in that on early endosomes, it acts to drive the fusion of entire, early endosome compartments. This homotypic process differs from the formation of transport vesicles that is triggered by Rab9 on late endosomes. Transport vesicle formation requires a highly regulated deformation of a flat membrane to form a transport vesicle, whereas wholesale endosome fusion does not. Moreover, Rab5 can undergo multiple rounds of GTP hydrolysis and rebinding, without the need for membrane extraction and membrane delivery by GDI. This may explain why Rab9 function on late endosomes is more sensitive to cholesterol accumulation than Rab5 function on early endosomes.

Excess cholesterol in NPC cells inhibited GDI-mediated Rab4 extraction, and it slowed recycling of both internalized transferrin and a fluorescent analog of lactosylceramide, to the plasma membrane (41). Thus, Rab function can be highly sensitive to changes in membrane cholesterol. Given the important roles of Rab proteins in all steps of membrane trafficking (39,40), the sensitivity of multiple Rabs to cholesterol levels helps to explain the striking array of cellular changes that accompany NPC1 deficiency (2).

In summary, cholesterol alone can influence Rab retrieval from membranes, and lead to its sequestration in an inactive form. For certain intracellular trafficking pathways, this can have serious consequences. Indeed, cholesterol accumulation interferes with late endosome export of CI-MPRs and CD-MPRs, and leads to the more rapid destruction of CD-MPRs in the lysosome.

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**FOOTNOTES**

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1The abbreviations used are: NPC, Niemann-Pick type C; MPR, mannose 6-phosphate receptor; CI-MPR, cation independent mannose 6-phosphate receptor; CD-MPR, cation dependent mannose 6-phosphate receptor; TGN, trans-Golgi Network; DMEM, Dulbecco’s modified Eagle’s Medium; HEK293, human embryonic kidney 293; RNAi, RNA interference; siRNA, small interfering RNA; PBS, phosphate buffered saline; BSA, bovine serum albumin; PNS, post nuclear supernatant; GDI, guanine nucleotide dissociation inhibitor; GFP, green fluorescent protein.

2Reddy, J.V., Burguete, A.S., Ganley, I.G. and Pfeffer, S.R. (2006) submitted for publication.

**FIGURE LEGENDS**

**Fig. 1.** Cholesterol staining is unaltered in Rab9-depleted cells. Immunofluorescence microscopy of HeLa cells transfected with control siRNA (top row) or Rab9 siRNA (bottom row) and double labeled with filipin to detect cholesterol (left column) and monoclonal anti-Rab9 antibody (right column).

**Fig. 2.** Depletion of NPC1 leads to lysosomal cholesterol accumulation. Immunofluorescence microscopy of HeLa cells transfected with NPC1 siRNA. Cells were double labeled with BC0 to detect cholesterol (middle row) and the indicated anti-Rab antibody or anti-LAMP1 antibody (top row). Transfection of siRNA is about 70% efficient, thus each panel shows cells with and without cholesterol accumulation. Lower row, images are merged to allow easy comparison.

**Fig. 3.** Rab9 positive late endosomes also accumulate cholesterol in NPC1 fibroblasts. Immunofluorescence microscopy of wild type (left) or NPC1 (right) fibroblasts double labeled with filipin to detect cholesterol (green), and monoclonal anti-Rab9 antibody (red). The boxed region in the upper panels is shown enlarged in the lower panels. Arrows indicate areas of co-localization between cholesterol and Rab9. Note that most cholesterol does not co-localize with Rab9.
**Fig 4. Rab9 levels are increased in NPC1 fibroblasts.** (A) Immunoblot analysis of indicated proteins in wild type (WT) or NPC1 fibroblast cell lysates. (B) Quantitation of immunoblots shown in A; values are mean percentages in NPC cells compared to wild type cells, +/- SD, from at least three independent experiments. (C) Wild type and NPC fibroblasts, or Hela cells treated with or without U18666A for 12 h, were labeled with 35S-methionine/cysteine and chased for various times to determine the half-life of Rab9. Shown is the mean percentage of the Rab9 half-life in NPC cells relative to wild type cells (left lane) or HeLa cells treated with U18666A relative to control treated cells (right lane). Values are from three independent experiments; error bars represent SD.

**Fig 5. Rab9 and Rab5 resist extraction by GDI in NPC fibroblast membranes.** (A) Crude membranes from wild type or NPC fibroblasts, normalized to contain equal amounts of a given Rab, were treated with the indicated amounts of GDI. Extracted Rabs were analyzed by immunoblot, following removal of the membranes by high speed centrifugation, and compared to the initial membrane-bound Rab prior to GDI extraction (Input). (B) Quantitation of immunoblots shown in A; values are means +/- SD from at least three independent experiments. (C) Crude membranes from NPC fibroblasts were washed with buffers containing the indicated amounts of KCl or urea. Membranes (M) or the released wash proteins (W) were analyzed for the indicated proteins by immunoblot. (D) Crude membranes, treated as in C above, were normalized for Rab content. Membranes were then incubated with or without 4 µM GDI and the extracted Rab9 analyzed by immunoblot. (E) Quantitation of a representative immunoblot shown in D.

**Fig 6. TIP47 depletion does not relieve the cholesterol-induced block in GDI extraction.** (A, left panel) Immunoblot analysis of crude endosomes isolated from HeLa cells treated with or without U18666A for 14 h. (A, right panel) Quantitation of immunoblots shown in A; values were normalized to p115 content and are mean percentages of U18666A-treated samples compared to control-treated samples from three independent experiments. Error bars represent SD. (B) Immunoblot of HeLa cells transfected with control or TIP47 siRNA followed by 24 h of U18666A treatment. Shown are PNS (left) and crude membranes (right). (C, left) Crude membranes from C were normalized for Rab9 content, incubated with or without 4 µM GDI and the extracted Rab9 analyzed by immunoblot. (C, right) Quantitation of immunoblots shown at left: values are means from two independent experiments, each carried out in duplicate. Error bars represent SD.

**Fig 7. GDI extraction of Rabs from liposomes is impaired in the presence of cholesterol.** Liposomes prepared with or without cholesterol were loaded with prenylated Rab9 as described in Experimental Procedures. (A, top) Dot blot analysis of liposome-associated Rab9 following treatment with the indicated amounts of GDI and subsequent isolation on a Nycodenz gradient. (A, bottom) Quantitation of dot blots from three independent experiments, shown is mean percentage of liposomal-associated Rab9 following GDI-treatment compared to non-GDI treated samples. Error bars represent SD. (B, top) Same as A top but for Rab5. (B, bottom) Quantitation of dot blots shown at the top of panel B.

**Fig 8. Loss of NPC1 leads to CD-MPR missorting.** Immunofluorescence of NPC1 siRNA-treated HeLa cells (A) or NPC1 siRNA-treated HEK293 cells stably expressing myc-tagged CD-MPR (B). Cells were double labeled with BC0 to detect cholesterol (left), or anti-CI-MPR antibody (A, right panel), or anti-myc antibody to detect CD-MPR (B., right panel). Cells marked with asterisks have accumulated cholesterol. (C) Immunoblot analysis of HEK293 lysates treated with control (GFP), NPC1 or Rab9 siRNA. Top, Quantitation of immunoblots shown below: values were normalized to p115 content and are mean percentages compared to control-RNAi samples from three independent experiments. Error bars represent SD.

**Fig 9. Rab9 overexpression rescues CD-MPR degradation upon NPC1 depletion.** (A) Immunoblot analysis of HEK293 lysates from cells transfected with or without NPC1 siRNA for 48 h followed by transfection with the indicated GFP-Rab construct for an additional 24 h. (B) Quantitation of immunoblots shown in A. Values are mean percentages, normalized to p115, of CD-MPR in control (buffer only) RNAi or NPC1 RNAi transfected
cells compared to the control RNAi and mock DNA transfected cells. Error bars represent SD. (C) Data shown in A and B are replotted to highlight NPC1 depletion-specific changes. Values are mean percentages, normalized to p115, of CD-MPR in GFP-Rab transfected NPC1-depleted cells compared to GFP-Rab-transfected control-depleted cells. Error bars represent SD.
Figure 3

WT

NPC

Cholesterol  Rab9  Cholesterol  Rab9
Figure 4

A. WT NPC
CI-MPR
GCC185
p115
TIP47
p40
Rab5
Rab9

B. Protein level relative to wild type cells (%)

C. Rab9 half life relative to control (%)

NPC
HeLa +U18666A
Figure 5

A.

Input | Extracted
--- | ---
Wild type | Rab5 | Rab9
NPC1 | Rab5 | Rab9

GDI (µM) 0 2 4

B.

Rab extracted (%)

WT/Rab5
WT/Rab9
NPC/Rab5
NPC/Rab9

GDI (µM) 0 2 4

C.

M | W | M | W | M | W
p115
TIP47
Rab9

Control | 1M KCl | 1.5M KCl | 2M Urea

D.

Rab9

Control | 1M KCl | 1.5M KCl | 2M Urea

Input | Extracted
--- | ---

E.

Rab9 extracted (%)

Control | 1M KCl | 1.5M KCl | 2M Urea
NPC membrane treatment
Figure 6

A. Endosomes
- Rab9- TIP47- p115-
U1866A: - +

B. U1866A:
- + + +
siRNA:
- + + +
TIP47- Rab9-
PNS Membranes

C. Rab9 extracted (%)
control siRNA
input +U18666A
siRNA +U18666A
control siRNA
U18666A: - + - +
Figure 7

A. Liposome-associated Rab9

Phospholipid

Phospholipid + Cholesterol

0 3 6 9

Lipid

Lipid + Cholesterol

B. Liposome-associated Rab5

Phospholipid

Phospholipid + Cholesterol

0 2 4 6

Lipid

Lipid + Cholesterol

Phospholipid

Phospholipid + Cholesterol

0 3 6 9

Lipid

Lipid + Cholesterol

0 2 4 6
Figure 8

A. Cholesterol CI-MPR

B. Cholesterol CD-MPR

C. CD-MPR level (%)

siRNA: control NPC1 Rab9

p115- CD-MPR- Rab9-
Figure 9

A.

NPC1 siRNA: - - + - - + - +

p115

CD-MPR

GFP-Rab

B.

CD-MPR level relative to mock transfected control (%)

Control siRNA

NPC siRNA

Mock GFP-Rab7 GFP-Rab9 GFP-Rab9 S21N

C.

CD-MPR levels relative to control depleted cells (%)

NPC-1 depleted cells

control Mock GFP-Rab7 GFP-Rab9 GFP-Rab9 S21N
Cholesterol accumulation sequesters Rab9 and disrupts late endosome function in NPC1-deficient cells
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