Ezetimibe-sensitive cholesterol uptake by NPC1L1 protein does not require endocytosis

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ABSTRACT Human NPC1L1 protein mediates cholesterol absorption in the intestine and liver and is the target of the drug ezetimibe, which is used to treat hypercholesterolemia. Previous studies concluded that NPC1L1-GFP protein trafficking is regulated by cholesterol binding and that ezetimibe blocks NPC1L1-GFP function by inhibiting its endocytosis. We used cell surface biotinylation to monitor NPC1L1-GFP endocytosis and show that ezetimibe does not alter the rate of NPC1L1-GFP endocytosis in cultured rat hepatocytes grown under normal growth conditions. As expected, NPC1L1-GFP endocytosis depends in part on C-terminal, cytoplasmically oriented sequences, but endocytosis does not require cholesterol binding to NPC1L1’s N-terminal domain. In addition, two small-molecule inhibitors of general (and NPC1L1-GFP) endocytosis failed to inhibit the ezetimibe-sensitive uptake of [3H]cholesterol from taurocholate micelles. These experiments demonstrate that cholesterol uptake by NPC1L1 does not require endocytosis; moreover, ezetimibe interferes with NPC1L1’s cholesterol adsorption activity without blocking NPC1L1 internalization in RH7777 cells.

INTRODUCTION
NPC1L1 is responsible for ~70% of cholesterol absorption at the surface of intestinal epithelial cells (Altmann et al., 2004; Weinglass et al., 2008a; Jia et al., 2011). It is the target of the drug ezetimibe (Zetia), a drug discovered based on its ability to lower plasma low-density lipoprotein cholesterol levels (Rosenblum et al., 1998; van Heek et al., 2000; Davis et al., 2001). Although in mice, NPC1L1 is expressed uniquely in the intestine, humans also express NPC1L1 in the liver (Yu et al., 2006; Temel et al., 2007). In the liver, NPC1L1 is believed to mediate biliary readsorption of cholesterol, a process that is also inhibited by ezetimibe administration (Temel et al., 2007). The precise mechanism by which NPC1L1 protein transfers cholesterol across the membrane is unknown.

The predominant splice form of NPC1L1 yields a protein of 1332 amino acids with 13 transmembrane domains and three relatively large extracellular domains (Wang et al., 2009), analogous to the related NPC1 protein. The first, N-terminal, extracellular domain binds cholesterol in both NPC1 (Infante et al., 2008) and NPC1L1 (Kwon et al., 2011; Zhang et al., 2011). NPC2 protein is believed to transfer cholesterol to the NPC1L1-related protein NPC1 at the limiting membrane of late endosomes and lysosomes. No such partner is known for NPC1L1: bile salts likely solubilize cholesterol in mixed micelles for NPC1L1 binding (Yamanashi et al., 2007; Haikal et al., 2008). The second extracellular domain of NPC1L1 contributes significantly to the binding site for ezetimibe (Weinglass et al., 2008a).

At steady state in normal growth medium, NPC1L1-GFP is localized primarily to perinuclear recycling endosomes in rat hepatocyte cell lines, colocalizing with transferrin receptor and Rab11 GTPase (Yu et al., 2006; Ge et al., 2008). Yu et al. (2006) were the first to show that NPC1L1-GFP cycles between the endocytic recycling compartment and the cell surface. Nevertheless, most NPC1L1 resides in recycling endosomes at steady state. McArdle RH7777/CRL-1601 hepatoma cells (Yu et al., 2006; Ge et al., 2008; Li et al., 2014) and CaCo-2 (Yamanashi et al., 2007) and MDCKII cell lines (Weinglass et al., 2008a, and references therein) have been used to study NPC1L1-dependent cholesterol uptake in cell culture.

Intracellular transport of NPC1L1 has been reported to be cholesterol regulated (Yu et al., 2006; Ge et al., 2008). When NPC1L1-expressing cells are treated with 1.5% cyclodextrin (plus compactin and mevalonate to block de novo cholesterol biosynthesis), GFP-NPC1L1 accumulates at the cell surface. This relocation is
RESULTS AND DISCUSSION

We used a quantitative assay to monitor the trafficking of NPC1L1-GFP protein in stably expressing RH7777/CRL-1601 cells that have been used in previous studies of NPC1L1–green fluorescent protein (GFP) trafficking (Yu et al., 2006; Ge et al., 2008; Li et al., 2104). The presence of the C-terminal GFP moiety did not alter the fraction of NPC1L1 that acquires complex oligosaccharides, consistent with proper folding and transport through the Golgi complex (Yu et al., 2006). Although we cannot rule out the possibility that the presence of the GFP may influence the efficiency of endocytosis, it has not interfered with the ability of the tagged protein to enhance cholesterol uptake in a manner that is sensitive to ezetimibe inhibition (Yu et al., 2006; Ge et al., 2008; this study).

Schmid and coworkers pioneered the use of membrane-impermeable reducing agents as sensitive tools with which to monitor endocytosis of disulfide-linked biotin molecules (Schmid and Carter, 1990; Schmid and Smythe, 1991). After labeling the surface with a disulfide-linked biotin or biotinylated ligand, cells are warmed for various times and endocytosis protects internalized constituents from subsequent reduction upon addition of membrane-impermeant β-mercaptopethanol sulfonate.

To measure NPC1L1-GFP endocytosis, we transferred RH7777 hepatoma cells stably expressing human NPC1L1-GFP to ice and incubated them with a thiol-cleavable, biotinylation reagent to label all cell surface proteins. Cells were then warmed for various times to permit endocytosis, then chilled and treated with β-mercaptopethanol sulfonate to remove biotin from remaining, cell surface–localized molecules. Endocytosed NPC1L1-GFP, still biotinylated, was collected on NeutrAvidin agarose and detected by immunoblot analysis. Total NPC1L1-GFP, as well as the NPC1L1-GFP that was not biotinylated and flowed through the streptavidin agarose column, was monitored in parallel by immunoblot.

As shown in Figure 1A, NPC1L1-GFP was biotinylated at the surface of RH7777 hepatoma cells, as detected by immunoblot of streptavidin agarose–eluted proteins. On incubation of cells for 20 min at 37°C, ~45% of total cell surface NPC1L1 was endocytosed, as determined by its protection from reducing agent action (Figure 1A). Protection from reducing agent required 37°C incubation to permit internalization, as NPC1L1 was no longer detected on the surface of cells held at 0°C (see later discussion) or in which endocytosis was inhibited (see later discussion). The abundant, control cytosolic protein GDP dissociation inhibitor (GDI) (Figure 1B) was easily detected in input samples of whole-cell lysates (7.5% analyzed) but not after binding to NeutrAvidin resin (40% analyzed). This shows that biotinylation was specific for surface localized proteins. Capture of biotinylated proteins on NeutrAvidin resin was essentially 100% as determined by monitoring the capture of bulk, unidentified, biotinylated cell surface proteins (Figure 1C). These results indicate that this assay monitors cell surface–localized NPC1L1-GFP molecules.

To verify that cell surface biotinylation is a reliable means to monitor NPC1L1 trafficking, we compared the efficiency of biotin labeling with direct antibody labeling. Antibodies to native NPC1L1 extracellular domain were not available; thus we introduced a 3xMyc
at 37°C. Thus all subsequent endocytosis graphs will be presented in this manner, with an example of actual gel data included.

**Ezetimibe does not alter NPC1L1 endocytosis rate in RH7777 cells**

Previous reports evaluated the effect of ezetimibe by monitoring the internalization of NPC1L1 in cells pretreated with cyclodextrin (Ge et al., 2008; Li et al., 2014). Under those conditions, internalization of NPC1L1 protein is cholesterol dependent. In contrast, when the rate of NPC1L1 internalization was measured in normal medium, it was completely unchanged after addition of 30 μM ezetimibe, a concentration that is far above the $K_D$ for drug binding (Figure 4, A and B). It is important to note that the preparations of ezetimibe used in these experiments were fully capable of blocking NPC1L1-dependent uptake of cholesterol from bile salts (see later discussion). These data show that in RH7777 cells, NPC1L1 internalization from the cell surface relies at least in part on cytoplasmic domain sequences (as expected) but is insensitive to ezetimibe addition.

The N-terminal extracellular domain of NPC1L1 protein binds cholesterol (Zhang et al., 2011), and mutations in this domain have
be reported to both block cholesterol binding (Zhang et al., 2011) and influence NPC1L1 transport (Ge et al., 2008). To investigate the importance of cholesterol binding directly to NPC1L1 for its endocytosis, we tested an NPC1L1 mutant protein (L216A) that was shown previously to display impaired cholesterol binding (Zhang et al., 2011). A purified, soluble version of NPC1L1’s N-terminal domain (residues 22–284) carrying this mutation (from insect cells) showed a significant decrease in its capacity to bind [3H]cholesterol in vitro compared with the wild-type NPC1L1 protein (from 293F cells; Figure 5A). Thus, although our proteins were obtained from different cell lines, these findings are entirely consistent with previous reports (Zhang et al., 2011).

As shown in Figure 5B, despite impaired cholesterol binding via N-terminal domain sequences, full-length NPC1L1-GFP L216A protein was endocytosed at a rate that was indistinguishable from that of wild type NPC1L1-GFP. To verify further the importance of N-terminal domain cholesterol binding on NPC1L1 endocytosis, we obtained a construct that lacks the entire N-terminal, cholesterol-binding domain (Zhang et al., 2011) and monitored its uptake in RH7777 hepatocytes. Figure 5C shows that the complete absence of an N-terminal cholesterol-binding site had little, if any, effect on the initial rate of NPC1L1 internalization. These data show that under normal culture conditions, endocytosis of NPC1L1 protein is independent of cholesterol binding to its N-terminal, cholesterol-binding domain.

NPC1L1 function does not require endocytosis

NPC1L1 picks up cholesterol from bile salts in the intestine. To explore further the mechanism of ezetimibe action, we used an established assay (Reboul et al., 2011) to monitor ezetimibe-sensitive cholesterol uptake from bile salt micelles, using cultured RH7777 hepatoma cells. Although some labs have monitored NPC1L1 function using cyclodextrin-cholesterol complexes as substrate donor, we were not able to detect NPC1L1-dependent cholesterol uptake after 20 min using that donor substrate in our cell culture system (unpublished data); this may be because others used longer incubation times of 1–2 h. Nevertheless, we succeeded in monitoring NPC1L1-dependent cholesterol uptake when cholesterol was provided to cells in bile salt micelles (Figure 6). Briefly, cultures were incubated with [3H]cholesterol taurocholate micelles; NPC1L1-mediated uptake was determined by comparing cholesterol uptake with that detected for the parental hepatoma cell line, which does not express NPC1L1 protein. Although prolonged incubation of cultured cells with bile salts can lead to issues of toxicity, this was mitigated by monitoring cholesterol uptake and endocytosis within a 20-min time frame. Presumably, the extensive glycocalyx of the intact intestine protects cells from toxicity, and well-polarized monolayers of CaCo2 cells may also be less sensitive over longer incubations (Yamanashi et al., 2007). In CaCo2 cells, cholesterol uptake from bile salt micelles continues linearly for several hours (Yamanashi et al., 2007).
We first tested Dyngo-4a, a drug that inhibits endocytosis by blocking the GTPase activity of Dynamin1 (Robertson et al., 2014) and may have additional targets (Park et al., 2013). Figure 7, A and B, shows that Dyngo-4a blocked NPC1L1 endocytosis by >90%, as monitored by cell surface biotinylation. Nevertheless, Dyngo-4a had no effect on NPC1L1-dependent (or -independent) cholesterol uptake (Figure 7C).

We also tested a second inhibitor of endocytosis, Pitstop 2, which was designed to block the binding of partner proteins to the N-terminal domain of clathrin; it is a potent inhibitor of endocytosis but may function in cells by a less specific mechanism (von Kleist et al., 2011; Willox et al., 2014). Figure 7, D and E, shows that Pitstop 2 blocked NPC1L1 endocytosis completely. Consistent with our findings with Dyngo-4a, Pitstop 2 had no effect on total cholesterol uptake or on ezetimibe-sensitive, NPC1L1-mediated cholesterol uptake (Figure 7F). Pitstop 2 showed some toxicity when cells were incubated for times >20 min. Nevertheless, cholesterol uptake proceeded normally in 20-min assays (Figure 7, E and F). These data demonstrate that NPC1L1-dependent cholesterol uptake from bile salt micelles does not require endocytosis.

In summary, the mechanism by which human NPC1L1 protein catalyzes cholesterol transport in the intestine and liver is not yet known. Others have proposed that the protein acts as an endocytic transporter of cholesterol, shuttling it to intracellular compartments, perhaps one or a few molecules at a time (Ge et al., 2008; Li et al., 2014). In this study, we showed that ezetimibe-sensitive, NPC1L1-mediated cholesterol import occurs normally in cells in which endocytosis of NPC1L1 (and other cell surface proteins) has been completely blocked. Furthermore, using a cell surface biotinylation assay to monitor NPC1L1 endocytosis quantitatively, we showed that ezetimibe does not change the rate with which NPC1L1 protein is internalized from the cell surface and delivered to intracellular compartments. These experiments suggest that NPC1L1 is capable of transferring bound cholesterol from the extracellular space to intracellular stores. Precisely how the protein accomplishes this transfer and how ezetimibe binding blocks cholesterol import will be important issues for future investigation.

**MATERIALS AND METHODS**

**Materials**

McArdle RH7777/CRL-1601 cells were obtained from the American Type Culture Collection (Manassas, VA) and cultured in DMEM containing 12% fetal bovine serum plus 2 mM l-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Stable cell lines expressing NPC1L1-GFP protein were established after transfection and G418 selection; cells were maintained in 0.75 mg/ml G418. The NPC1L1ΔN mutant lacks residues 18–260 (deleted from the signal sequence junction); NPC1L1ΔC lacks residues 1306–1332 at the end of the C-terminus. Chicken anti-GFP antibodies were from Life Technologies (Grand Island, NY); goat anti-chicken Dylight488 antibodies were from Abcam (Cambridge, MA); anti-chicken-horseradish peroxidase conjugate was from Promega (Sunnyvale, CA); and rabbit anti-bovine GDI was raised in the lab. All were used at 1:1000 for immunoblots. Goat anti-mouse Alexa Fluor 647 and mouse anti-Myc were from Invitrogen (Waltham, MA) and used at 1:500 and 10 μg/ml, respectively. Mouse anti-Myc monoclonal antibody was produced in the lab. IRDye 680RD donkey anti-chicken and IRDye 800CW streptavidin were from LI-COR (Lincoln, NE) and used at 1:10,000. EZ-link Sulfo-NHS-SS-Biotin was from Life Technologies. The [3H]cholesterol was from New England Nuclear.

**Methods**

As shown in Figure 6A, NPC1L1-expressing cells displayed about twice the amount of total cholesterol uptake from bile salt micelles compared with the control, RH7777 parental cell line, entirely comparable to previous results (Yamanashi et al., 2007; Weinglass et al., 2008b; Reboul et al., 2011). As expected, all NPC1L1-dependent cholesterol uptake was lost if cells were pretreated with 30 μM ezetimibe for 60 min with continued treatment during cholesterol uptake. This confirms that the additional cholesterol uptake seen in RH7777 cells expressing NPC1L1-GFP is mediated by NPC1L1 protein.

NPC1L1ΔC showed a decreased endocytosis rate (Figure 3, B and C). Nevertheless, cells expressing the NPC1L1ΔC protein showed wild-type levels of cholesterol adsorption when monitored after a 20 min of incubation with bile salt micelles (Figure 5B). This suggested that endocytosis might not be needed for cholesterol uptake by NPC1L1; moreover, the data demonstrate that the C-terminal sequences are not needed for cholesterol uptake from bile salt micelles. To test more directly the importance of endocytosis for NPC1L1-mediated cholesterol uptake, we also used small-molecule endocytosis inhibitors.

**FIGURE 6:** (A) Ezetimibe-sensitive cholesterol uptake in cells expressing NPC1L1 protein. Parental RH7777 cells (right) or RH7777 cells expressing human NPC1L1-GFP (left) were monitored for [3H] cholesterol uptake from sodium taurocholate micelles ± 30 μM ezetimibe for 20 min as described in Materials and Methods; 39 μg of each sample was counted. The average of two experiments carried out in quadruplicate. ****p < 0.0001 for ± ezetimibe for NPC1L1 cells (unpaired t test). (B) Cholesterol uptake in RH7777 cells expressing NPC1L1-WT or NPC1L1ΔC proteins; 19 μg of each sample was counted. A representative experiment carried out in quadruplicate. Error bars represent SEM.
American Radiolabeled Chemicals (St. Louis, MO); cholesterol, sodium taurocholate, mono-olein, oleic acid, phosphatidylcholine (2-oleoyl-1-palmitoyl-sn-glycero-3-phosphocholine), sodium 2-mercaptoethanesulfonate, iodoacetamide, paraformaldehyde, and protease inhibitor cocktail were from Sigma Aldrich (St. Louis, MO); ezetimibe and lysophosphatidylcholine (1-palmitoyl-sn-glycero-3-phosphocholine) were from Santa Cruz Biotechnology (Santa Cruz, CA); Pitstop 2 and Dyngo-4a were from Abcam; Ni-nitrilotriacetic acid (NTA) resin was from Qiagen (Valencia, CA); Lipofectamine 3000, Protein G agarose resin, and NeutrAvidin agarose were from Life Technologies (Carlsbad, CA).

Cell surface biotinylation
Endocytosis was measured as described (Weixel and Bradbury, 2002): RH7777 cells stably expressing NPC1L1-GFP (wild type or mutants) were plated on collagen-coated plates in DMEM containing 12% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. At 24 h after plating, cells were pretreated for 1 h in DMEM plus 12% FBS and 30 μM ezetimibe or 0.3% ethanol at 37°C. Medium was removed, and cells were placed on ice and washed three times with ice-cold phosphate-buffered saline (PBS). Cell proteins were surface labeled with 1 mg/ml sulfo-NHS-biotin-EZ link on ice for 30 min. Excess biotin reagent was removed by washing three times with 1% bovine serum albumin (BSA) in PBS, pH 7.4, on ice. Ice-cold (zero time point) or warm DMEM containing ezetimibe or ethanol was then placed on cells. Cells were kept on ice (zero time point) or warmed for indicated time points to permit endocytosis. After returning cells to ice, cell surface biotin on all plates was removed by treating cells with 100 mM β-mercaptoethanol sulfonate. Unreacted reducing agent was quenched with three 5-min washes in 5 mg/ml iodoacetamide in PBS, pH 7.4, on ice. Cells were lysed with buffer containing 1.25% Triton X-100, 0.25% SDS, 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 5 mM EDTA, 5 mg/ml iodoacetamide, and 1× protease inhibitor cocktail. Cleared lysate was incubated overnight with 25 μl of NeutrAvidin resin. After overnight incubation, resin was spun 700 × g for 3 min and loaded into a 1-ml syringe fitted with a frit. Resin was washed five times with 0.5 ml of cold wash buffer (0.5% Triton X-100, 0.1% SDS, 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 5 mM EDTA). Syringes were spun at 1000 rpm for 5 s to remove wash buffer; resin was then transferred to a 1.5-ml tube containing 100 μl of SDS–PAGE sample buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 100 mM dithiothreitol [DTT], 10% glycerol, and 0.02% bromophenol blue [BPP]) and heated for 20 min at 37°C. Duplicate 40-μl portions of each sample were analyzed on Bio-Rad (Hercules, CA) Mini-PROTEAN TGX 4–20% gradient gels. After transfer to polyvinylidene fluoride membrane and antibody incubation, immunoblots were visualized using a VersaDoc Imager (Bio-Rad) and analyzed using ImageJ software (National Institutes of Health, Bethesda, MD). For endocytosis experiments with Pitstop2 and Dyngo-4a, cells were grown overnight in DMEM only and then incubated for 1 h with DMEM containing 5% lipoprotein-deficient serum (LPDS; Kalen Biomedical, Montgomery Village, MD) as in cholesterol uptake assay experiments (see later discussion). Cells were pretreated for 10 min with 25 μM Pitstop 2 or 30 min 20 μM Dyngo-4a. Endocytosis was carried out in the presence of sodium taurocholate micelles to confirm...
that inhibitors were still functional under these conditions. ImmunobLOTS were visualized using the LI-COR Odyssey Imaging System and analyzed using ImageJ software. For assays with endocytosis inhibitors, all bands (± inhibitor) were normalized to the 20-min time point with no inhibitor present.

**Cholesterol uptake**

Uptake (20 min) was monitored using micelles as described (Reboul et al., 2011): RH7777 cells stably expressing NPC1L1-WT-GFP, or RH7777 cells as control, were plated in 12-well collagen-coated plates at 2.5 x 10^6 cells/well, four wells per condition. After allowing several hours for cells to adhere, the growth medium was removed and replaced with DMEM containing 2 mM L-glutamine. The next day, cells were incubated 1 h with DMEM containing 5% LPDS plus 2 mM L-glutamine. Ezetimibe stock was prepared in ethanol and used at a final concentration of 30 μM, final ethanol concentration 0.3%, and added during the incubation with the 5% LPDS medium. Pitstop 2 stock was prepared in dimethyl sulfoxide (DMSO) and used at a final concentration of 25 μM, final DMSO concentration 0.5%. Dynego-4a stock was prepared in DMSO and used at a final concentration of 20 μM, final DMSO concentration 0.1%. Cells were treated for 10 min with PitStop 2 or 30 min with Dynego-4a in serum-deficient DMEM containing 2 mM L-glutamine before cholesterol incubation. Drugs were also included during cholesterol uptake. Micelle lipids were first dissolved in ethanol and combined in a glass vial, and solvents were evaporated under N2. The dried lipids were incorporated into DMEM containing 24 mM sodium taurocholate by vortexing for 5 min. The solution was then diluted with DMEM containing 5% LPDS to final concentrations of 5 mM sodium taurocholate, 0.5 mM oleic acid, 0.035 mM phosphatidylcholine, 0.08 mM lysophosphatidylcholine, 0.3 mM mono-olein, 0.005 mM cholesterol, and 4 mM radiolabeled cholesterol. After 20 min at 37°C, the micelle mixture was removed and cells washed four times with ice-cold 5 mM sodium taurocholate in PBS. Cells were lysed using 100 μl of lysis buffer containing 1% Triton X-100, 50 mM Tris, pH 8, 150 mM NaCl, and 5 mM EDTA. Equal amounts of cleared lysate, measured by protein concentration, were analyzed by scintillation counting to determine cholesterol uptake.

**Protein purification**
pFastBac NPC1L1-WT-N-terminal domain plasmid was obtained from AddGene (Cambridge, MA) and used to make virus for infection of Sf9 insect cells. At 72 h after infection, Sf9 culture was spun down and ammonium sulfate added to the supernatant to achieve 75% saturation. The resulting precipitate was resuspended in 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.4, and 150 mM NaCl and incubated with Ni-NTA resin overnight at 4°C. After elution with 20 mM HEPES, pH 7.4, 150 mM NaCl, and 400 mM imidazole, the protein was further purified using Q-Sepharose (GE Healthcare Life Sciences, Pittsburgh, PA). GFP-NPC1L1-N-terminal domain-L216A mutant protein was expressed in HEK293F cells. After 72 h the culture was spun down, and the supernatant was incubated with Ni-NTA resin overnight, eluted as described, and dialyzed into 50 mM HEPES, pH 7.4, and 150 mM NaCl.

**NPC1L1 cholesterol binding**
The assay was based on Infante et al. (2008): 80-μl solutions containing histidine-tagged NPC1L1-N-terminal domain wild-type and L216A cholesterol-binding mutant purified proteins (31.2 nM) were incubated with cholesterol (20 nM, 1:3 cold:hot) in 50 mM Tris, pH 7.4, 150 mM NaCl, 0.004% NP-40, and BSA (192 nM) overnight at 4°C. Solutions were loaded onto 1-ml syringes fitted with a frit and 20 μl of Ni-NTA resin. After 10 min, the resin was washed six times with 1 ml of wash buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 0.004% NP-40) and eluted with 1 ml of elution buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 0.004% NP-40, 400 mM imidazole). Eluted samples were mixed with BioSafeII (Research Products International, Mt. Prospect, IL), and radioactivity was determined using a scintillation counter.

**Labeling with antibody for flow cytometry**

RH7777 cells were transfected with NPC1L1-GFP containing a 3xMyc tag between residues S986 and L987 (Wang et al., 2009). At 48 h after transfection, the cells were incubated for 5 s with 0.05% trypsin and 10 mM EDTA in PBS. The trypsin was removed and immediately quenched with 10% FBS and 10 mM EDTA in PBS. Cells were spun at 1000 rpm for 5 min, washed once with PBS, and then divided into two portions. For surface labeling, one portion was gently resuspended in mouse anti-Myc supernatant and rotated for 1 h at 4°C. Both portions were fixed with 3.5% paraformaldehyde in PBS for 15 min at room temperature. For total NPC1L1 labeling, the other portion was permeabilized with 0.1% Triton X-100 and 1% BSA in PBS for 5 min at room temperature. After permeabilization, cells were resuspended in mouse anti-Myc supernatant and rotated for 1 h at 4°C. After being washed three times with PBS plus 1% BSA, portions were incubated with 1:500 goat anti-mouse Alexa Fluor 647 with 1% BSA in PBS for 1 h at room temperature. Cells were analyzed by flow cytometry, gating for single-cell GFP-positive cells, monitoring fluorescence intensity at 647 nm.

**Labeling with antibody followed by immunoblot analysis**

RH7777 cells transiently expressing NPC1L1-GFP containing a 3xMyc tag were washed three times with ice-cold PBS and then incubated on ice for 1 h with gentle rotation in 10 μg/ml mouse anti-Myc in PBS with 1% BSA. Unbound antibody was removed by washing three times with ice-cold PBS before lysis with 0.5% Triton X-100, 50 mM Tris, pH 8, 150 mM NaCl, and 5 mM EDTA. For total NPC1L1 labeling, cells were lysed, and the cleared lysate was incubated with 10 μg/ml mouse anti-Myc 1 h at 4°C. Antibody-labeled protein was incubated with protein G resin overnight at 4°C. Protein was eluted in sample buffer (50 mM Tris-HCl, pH 6.8, 4% SDS, 100 mM DTT, 10% glycerol, 0.02% BPP) at 60°C for 20 min. Samples were loaded onto a 4–20% gradient gel and immunoblotted as described.

**Other methods**

Protein was determined by bicinchoninic acid protein assay (Life Technologies) using BSA as standard; for the cholesterol uptake experiments, protein was determined using Advanced Protein Assay reagent (Cytoskeleton, Denver, CO).

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