NPC1L1 (Niemann–Pick C1-like 1) mediates sterol-specific unidirectional transport of non-esterified cholesterol in McArdle-RH7777 hepatoma cells

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Recent evidence suggests that NPC1L1 (Niemann–Pick C1-like 1) is critical for intestinal sterol absorption in mice, yet mechanisms by which NPC1L1 regulates cellular sterol transport are lacking. In the study we used a McArdle-RH7777 rat hepatoma cell line stably expressing NPC1L1 to examine the sterol-specificity and directionality of NPC1L1-mediated sterol transport. As previously described, cholesterol-depletion-driven recycling of NPC1L1 to the cell surface facilitates cellular uptake of non-esterified (free) cholesterol. However, it has no impact on the uptake of esterified cholesterol, indicating free sterol specificity. Interestingly, the endocytic recycling of NPC1L1 was also without effect on β -sitosterol uptake, indicating that NPC1L1 can differentiate between free sterols of animal and plant origin in hepatoma cells. Furthermore, NPC1L1-driven free cholesterol transport was unidirectional, since cellular cholesterol efflux

to apolipoprotein A-I, high-density lipoprotein or serum was unaffected by NPC1L1 expression or localization. Additionally, NPC1L1 facilitates mass non-esterified-cholesterol uptake only when it is located on the cell surface and not when it resides intracellularly. Finally, NPC1L1-dependent cholesterol uptake required adequate intracellular K^+ , yet did not rely on intracellular Ca^{2+} , the cytoskeleton or signalling downstream of protein kinase A, protein kinase C or pertussis-toxin-sensitive G-protein-coupled receptors. Collectively, these findings support the notion that NPC1L1 can selectively recognize non-esterified cholesterol and promote its unidirectional transport into hepatoma cells.

Key words: cholesterol efflux, ezetimibe, Niemann–Pick C1-like 1 (NPC1L1), sitosterol, sterol uptake.

INTRODUCTION

Niemann–Pick C1-like 1 (NPC1L1) was recently identified as a protein sharing high sequence identity (42 %) with, and similarity (51 %) to, the late-endosome-resident protein NPC1 (Niemann–Pick C1) [1]. Like NPC1, NPC1L1 was found to contain a putative SSD (sterol-sensing domain) and an N-terminal ‘NPC1 domain’ [1], indicating that this protein may play a role in cellular sterol homeostasis. In subsequent studies NPC1L1 was identified as a major component of the cholesterol-uptake pathway by the enterocyte, evidenced by the fact that mice lacking NPC1L1 have dramatically reduced intestinal cholesterol absorption and are resistant to diet-induced hypercholesterolaemia [2–4]. In further support of NPC1L1’s role in cellular cholesterol homeostasis, our group has recently reported that cholesterol-depletion-driven translocation of NPC1L1 to the cell surface facilitates FC [free (non-esterified) cholesterol] uptake [5]. Furthermore, the potent cholesterol absorption inhibitor ezetimibe can block NPC1L1-dependent cholesterol uptake in McArdle-RH7777 hepatoma cells [5], and ezetimibe binding to intestinal brush-border membranes is defective in NPC1L1 knockout mice [6]. Collectively, these findings support a crucial role for NPC1L1 in cellular sterol homeostasis, yet defined mechanisms by which NPC1L1 affects sterol transport are lacking.

Acquisition of cholesterol in essentially all cells occurs primarily through *de novo* synthesis [7–9]. However, several proteins located at the plasma membrane have been documented to facilitate sterol movement from extracellular compartments

to supplement the *de-novo*-synthesized pools within the cell. The first protein described to facilitate cellular cholesterol uptake is the LDLR (low-density-lipoprotein receptor). The LDLR recognizes one of two ligands, ApoB100 (apolipoprotein B100) or ApoE, resident on LDLs (low-density lipoproteins) and remnants of VLDLs (very-low-density lipoproteins) or chylomicrons [10,11]. Once the ligand interacts with its cognate receptor, the lipoprotein cargo is clustered into clathrin-coated pits. Then the holoparticle is internalized via receptor-mediated endocytosis and the protein and lipid components are subsequently delivered into an endosomal/lysosomal compartment [12]. Thereafter, acidification causes release of the LDLR from its lipoprotein cargo, and resident CEs (cholesterol esters) are hydrolysed locally by an acidic cholesteryl esterase to liberate FC to metabolically active pools [13–15]. Collectively, LDLR-mediated endocytosis delivers both FC and EC (esterified cholesterol) to an endosomal compartment, which can subsequently be used to meet the metabolic needs of the cell.

Another protein that mediates cellular cholesterol uptake is the SR-BI (scavenger receptor class B type I), yet the mechanism by which it facilitates cholesterol uptake is significantly different from that described for the LDLR. SR-BI recognizes lipoproteins containing apoAI, and has therefore been termed the ‘HDL (high-density lipoprotein) receptor’ [16,17]. In a process that has not been clearly delineated, SR-BI binds HDL particles and selectively transports EC into the cell, while the protein component of the lipoprotein is left in the extracellular space or plasma to either be re-used or cleared by the kidney [18–21]. In contrast with LDLR-mediated EC delivery, EC liberated from

Abbreviations used: ABC, ATP-binding cassette; Apo, apolipoprotein; BAPTA-AM, 1,2-bis-(2-aminophenoxy)ethane-*N,N,N',N'*-tetra-acetic acid tetrakis(acetoxymethyl ester); BCA, bicinchoninic acid; CE, cholesteryl ester; CETP, cholesterol ester transfer protein; COE, cholesterol oleoyl ether; DMEM, Dulbecco’s modified Eagle’s medium; EC, esterified cholesterol; EGFP, enhanced green fluorescent protein; ERC, endocytic recycling compartment; FC, free (non-esterified) cholesterol; GPCR, G-protein-coupled receptor; H-89, *N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide; HDL, high-density lipoprotein; LDL, low-density lipoprotein; LDLR, low-density-lipoprotein receptor; NPC1, Niemann–Pick C1; NPC1L1, Niemann–Pick C1-like 1; M β CD, methyl- β -cyclodextrin; PKA, protein kinase A; PKC, protein kinase C; SR-BI, scavenger receptor class B type I; SSD, sterol-sensing domain; TC, total cholesterol.

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SR-BI-dependent uptake is hydrolysed by a neutral cholesteryl esterase and enters directly into metabolically active pools, bypassing the acidification step needed in the LDLR-mediated endocytosis [22,23]. In addition to facilitating selective CE uptake into cells, SR-BI can act bidirectionally to promote cellular cholesterol efflux out of cells [24]. Collectively, SR-BI facilitates the selective uptake of CE from HDL, yet can also act to promote cholesterol efflux.

In addition to these well-defined (LDLR and SR-BI) protein-mediated pathways for cellular cholesterol uptake, we have recently demonstrated that NPC1L1 can also act to facilitate cholesterol movement into cells when properly targeted to the plasma membrane [5]. Our previous work demonstrated that NPC1L1 trafficked from a transferrin-positive endocytic recycling compartment to an 'apical-like' subdomain of the plasma membrane when cells were depleted of cholesterol. This cholesterol-depletion-driven translocation of NPC1L1 to the cell surface resulted in augmented cellular cholesterol uptake [5]. However, the sterol specificity and the possibility that transport could be bidirectional, like that of SR-BI, was not addressed in our original work. Therefore the purpose of the present study was to define the specificity and directionality of sterol transport by NPC1L1. We also sought to establish a relationship between the subcellular localization of NPC1L1 and mass cholesterol movement into cells. Additionally, the present study examines the potential involvement of signalling cascades and the cytoskeleton in NPC1L1-dependent cholesterol uptake. Collectively, the results from the present study establish that NPC1L1 mediates the selective unidirectional cellular transport of FC, yet not non-esterified β -sitosterol, which occurs in a K^+ -sensitive manner in hepatoma cells.

MATERIALS AND METHODS

Materials

All cell cultureware and TLC plates (Whatman no. 4410221) were purchased from Fisher Scientific. [9,10- $^3H(n)$]Oleic acid (NET-289) and β -[22,23- 3H]sitosterol (custom-synthesized) were purchased from PerkinElmer Life Sciences. [4- ^{14}C]Cholesterol (CFA128) and [3H]COE {[1 α ,2 α (n- 3H)cholesteryl oleyl ether; TRK888} were purchased from Amersham Biosciences. The BCA (bicinchoninic acid) colorimetric assay system was purchased from Pierce (no. 23225). The enzymatic total-cholesterol assay system was purchased from Boehringer-Mannheim (no. 236691). The enzymatic assay for FC was purchased from Wako chemical (no. 274-47109). Pertussis toxin, H-89 {N-[2-(p-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide}, BAPTA-AM [1,2-bis-(2-aminophenoxy)ethane-N,N,N',N'-tetra-acetic acid tetrakis(acetoxymethyl ester)], calphostin C and monensin were all purchased from Calbiochem. Nocodazole, cytochalasin D, M β CD (methyl- β -cyclodextrin) and all other chemicals and reagents were purchased from Sigma-Aldrich, unless otherwise stated.

Cell lines and tissue culture

McArdle-RH7777 rat hepatoma cells stably expressing EGFP (enhanced green fluorescent protein) alone (EGFP cells) or those expressing an NPC1L1-EGFP fusion protein (L1-EGFP cells) were used in the present study. These latter cells have one copy of EGFP appended to the C-terminus of NPC1L1, and the extent of overexpression has been previously described [5]. For normal growth, cells were maintained in Medium A [DMEM (Dulbecco's modified Eagle's medium) containing 4.5 g/litre

glucose, 100 units/ml penicillin, 100 μ g/ml streptomycin sulfate and 10% (v/v) FBS (fetal bovine serum)] in a humidified 5% CO₂ incubator at 37°C. For cholesterol depletion, cells were changed to Medium B [DMEM containing 4.5 g/litre glucose, 100 units/ml penicillin, 100 μ g/ml streptomycin sulfate and 5% (v/v) calf lipoprotein-deficient serum] with 2% (w/v) M β CD.

Cholesterol loading

Cholesterol was delivered to cells using cholesterol-M β CD complexes (Sigma no. C4951), which contain $\approx$ 50 mg of cholesterol/g of solid (molar ratio cholesterol/M β CD, 1:6). All treatment concentrations involving cholesterol/M β CD were based on cholesterol weight. Cells were incubated with 10 μ g/ml cholesterol/M β CD acutely for 2 or 4 h in Medium A.

K⁺ depletion

Stable cells were rinsed with K^+ -free buffer (140 mM NaCl, 20 mM Hepes, 1 mM CaCl₂, 1 mM MgCl₂ and 1 mg/ml D-glucose, pH 7.4) and subsequently incubated for 5 min with a hypotonic shock buffer (K^+ -free buffer diluted 1:1 with deionized water). Thereafter, cells were washed twice with K^+ -free buffer and maintained in K^+ -free buffer for cholesterol-uptake assay. For subsequent cholesterol depletion of K^+ -depleted cells, 2% (w/v) M β CD was added directly to K^+ -free buffer, and cells were incubated with this for 1 h.

Isolation and labelling of human lipoprotein

Human HDL [d (density) 1.063–1.225 g/ml] and LDL [d 1.019–1.063 g/ml] were isolated from the blood of healthy volunteers, with their informed consent, by differential ultracentrifugation as described previously [25], and were dialysed against PBS (0.01 M; pH 7.4) before labelling. The total cholesterol and protein mass of the lipoproteins was measured; the mass ratios (cholesterol/protein) were 0.25:1 for HDL and 1.2:1 for LDL. The purity of all lipoprotein preparations was confirmed by determining protein and cholesterol profiles following FPLC separation on a Pharmacia Superose-6 column. For [4- ^{14}C]cholesterol labelling of HDL and LDL, 80 μ g/ml PMSF was added to the lipoproteins to inhibit residual lecithin:cholesterol acyltransferase activity, and 100 units/ml penicillin and 100 μ g/ml streptomycin were added to prevent bacterial growth. Thereafter, [4- ^{14}C]cholesterol (0.2 μ Ci added/100 μ g of protein) was dried under a nitrogen stream and resuspended in 100 μ l of ethanol. The ethanolic [4- ^{14}C]cholesterol solution was added dropwise to lipoprotein samples with gentle mixing and incubated for 24 h at 37°C. To ensure no significant esterification had occurred, an aliquot of the labelled lipoproteins was extracted using the method of Bligh and Dyer [26] and free and esterified [4- ^{14}C]cholesterol radioactivity was determined after TLC separation. The specific radioactivity of [4- ^{14}C]cholesterol-labelled LDL was 4150 d.p.m./ μ g of LDL proteins, and that of [4- ^{14}C]cholesterol-labelled HDL was 4110 d.p.m./ μ g of HDL proteins. Esterification of [4- ^{14}C]cholesterol in both HDL and LDL samples was less than 0.01%. For [3H]COE labelling of HDL and LDL, 100 units/ml penicillin and 100 μ g/ml streptomycin were added to dialysed lipoprotein preparations to prevent bacterial growth. The lipoproteins were mixed 1:1 (v/v) with a CETP (cholesterol ester transfer protein) source [d = 1.225 g/ml bottom from African-green-monkey (*Cercopithecus aethiops*) plasma dialysed against 0.01 M PBS, pH 7.4]. Thereafter, [3H]COE (0.5 μ Ci/100 μ g of HDL or LDL protein) was dried under a nitrogen stream and resuspended in 25 μ l of acetone. The acetone/[3H]COE mixture was

added dropwise to a lipoprotein/CETP source mixture with gentle mixing. Residual acetone was evaporated under a nitrogen stream and lipoproteins were incubated overnight at 37 °C in a shaking water bath. After labelling, [³H]COE-labelled lipoproteins were separated on a Sepharose CL4B [4 % (w/v) agarose] column to remove [³H]COE that was not incorporated into lipoproteins. The final preparation of [³H]COE-labelled HDL had a specific radioactivity of 1.2 d.p.m./ng of HDL protein, and that of [³H]COE-labelled LDL had a specific radioactivity of 3.3 d.p.m./ng of LDL protein.

Lipoprotein-mediated [¹⁴C]cholesterol or [³H]COE uptake assay

EGFP or L1-EGFP cells were seeded in 35-mm-diameter dishes at a density of 2×10^4 cells/cm² and allowed to proliferate for ≈ 36 h. In a subset of dishes, the culture medium was then changed to Medium B with 2 % M β CD for 1 h to cholesterol-deplete cells. Following cholesterol depletion, all plates were washed once with PBS and then supplemented with 1 ml of assay medium (serum-free DMEM). Immediately thereafter, 100 μ g (HDL or LDL protein) of [¹⁴C]cholesterol-labelled lipoproteins or [³H]COE-labelled lipoproteins was added per plate, and cultures were incubated at 37 °C in a humidified 5 % CO₂ incubator for the indicated times. Following pulse-labelling, monolayers were gently washed three times with PBS and subsequently lysed in 0.1 % SDS/PBS. The total cellular lysate was subjected to liquid-scintillation counting to determine cellular cholesterol uptake. To control for unincorporated cell-associated [¹⁴C]cholesterol or [³H]COE, a set of cultures was briefly exposed to labelled lipoproteins (< 30 s) and immediately washed and harvested; the resulting radioactivity was subtracted from all experimental results. For normalization, the protein concentration of parallel cultures was determined using the BCA colorimetric assay.

Non-lipoprotein mediated cholesterol uptake assay

EGFP or L1-EGFP cells were seeded in 35-mm-diameter dishes at a density of 2×10^4 cells/cm² and allowed to proliferate for ≈ 36 h. For experiments where pharmacological inhibitors were used, all inhibitors were present for 1 h prior to M β CD treatment, during M β CD treatment and during the pulse-labelling period. In a subset of dishes, the culture medium was then changed to Medium B with 2 % M β CD for 1 h to cholesterol-deplete cells. Following cholesterol depletion, all plates were washed once with PBS and then supplemented with 1 ml of assay medium (serum-free DMEM). Immediately thereafter, 0.1 μ Ci of [4-¹⁴C]cholesterol was added per plate in an ethanolic BSA suspension, and cultures were incubated at 37 °C for 90 min. The preparation of the ethanolic BSA/[¹⁴C]cholesterol suspension and cell-harvesting procedure have been previously described [5].

Sitosterol uptake assay

EGFP or L1-EGFP cells were seeded in 35-mm-diameter dishes at a density of 2×10^4 cells/cm² and allowed to proliferate for ≈ 36 h. In a subset of dishes, the culture medium was then changed to Medium B with M β CD for 1 h to cholesterol-deplete cells. Following cholesterol depletion, all plates were washed once with PBS and then supplemented with 1 ml of assay medium (serum-free DMEM). Immediately thereafter, 0.1 μ Ci of β -[22,23-³H]sitosterol was added per plate for the indicated times, and cultures were incubated at 37 °C in a humidified 5 % CO₂ incubator. Prior to administration, the original β -[22,23-³H]sitosterol suspension in toluene was dried under a N₂ stream and resuspended in 100 % ethanol. The ethanolic β -[22,23-³H]sitosterol was diluted

20-fold into PBS supplemented with 1.5 % (w/v) fatty-acid-free BSA, which, when diluted into the assay medium, resulted in a final concentration of 0.0375 % BSA and 0.1 % ethanol. Following pulse labelling, monolayers were gently washed three times with PBS and subsequently lysed in 0.1 % SDS/PBS. The total cellular lysate was subjected to liquid-scintillation counting to determine cellular β -[22,23-³H]sitosterol uptake. To control for unincorporated cell-associated β -[22,23-³H]sitosterol, a set of cultures was briefly exposed to β -[22,23-³H]sitosterol (< 30 s) and immediately washed and harvested; the resulting radioactivity was subtracted from all experimental results. For normalization, the protein concentration of parallel cultures was determined using the BCA colorimetric assay.

Dual sterol-uptake assay

To determine the simultaneous kinetics of [4-¹⁴C]cholesterol and β -[22,23-³H]sitosterol uptake, each substrate was provided at the same specific radioactivity over a short time course (5 min–2 h). Briefly, EGFP or L1-EGFP cells were seeded in 35-mm-diameter dishes at a density of 2×10^4 cells/cm² and allowed to proliferate for ≈ 36 h. In a subset of dishes, the culture medium was then changed to Medium B with 2 % M β CD for 1 h to cholesterol-deplete cells. Following cholesterol depletion, all plates were washed once with PBS and then supplemented with 1 ml of assay medium (serum-free DMEM). Immediately thereafter, 0.1 μ Ci of β -[22,23-³H]sitosterol and 0.1 μ Ci of [4-¹⁴C]cholesterol was added per plate for the indicated times and cultures were incubated at 37 °C in a humidified 5 % CO₂ incubator. Prior to administration, the original β -[22,23-³H]sitosterol and [4-¹⁴C]cholesterol/toluene suspension were dried under a N₂ stream and resuspended in 100 % ethanol. The ethanolic β -[22,23-³H]sitosterol was diluted 20-fold into PBS supplemented with 1.5 % fatty-acid-free BSA, which, when diluted into the assay medium, resulted in a final concentration of 0.0375 % BSA and 0.1 % ethanol. Following pulse labelling, monolayers were gently washed three times with PBS and subsequently lysed in 0.1 % SDS/PBS. The total cellular lysate was subjected to liquid-scintillation counting to determine cellular β -[22,23-³H]sitosterol and [4-¹⁴C]cholesterol uptake. To control for unincorporated cell-associated β -[22,23-³H]sitosterol and [4-¹⁴C]cholesterol, a set of cultures was briefly exposed to the substrates (< 30 s), and immediately washed and harvested; the resulting radioactivity was subtracted from all experimental results. For normalization, protein concentration of parallel cultures was determined using the BCA colorimetric assay.

Fatty-acid-uptake assay

EGFP or L1-EGFP cells were seeded in 35-mm-diameter dishes at a density of 2×10^4 cells/cm² and allowed to proliferate for ≈ 36 h. The culture medium was then changed to medium B with 2 % M β CD for 1 h to cholesterol-deplete cells. Following cholesterol depletion, all plates were washed once with PBS and then supplemented with 1 ml of assay medium (serum-free DMEM). Immediately thereafter, 0.5 μ Ci of [9,10-³H(n)]oleic acid was added per plate for the indicated times and cultures were incubated at 37 °C. Prior to incubation, [9,10-³H(n)]oleic acid was complexed to fatty-acid-free (> 98 %) BSA at a 4:1 molar ratio using a 1 mM BSA stock. Uptake was terminated by the addition of an ice-cold stop buffer (PBS supplemented with 2 % fatty-acid-free BSA). Monolayers were carefully washed an additional two times with stop buffer to remove background radioactivity and lysed in 0.1 % SDS/PBS. The total cellular lysate was subjected to liquid-scintillation counting to determine cellular uptake.

Protein concentration of parallel cultures was determined using the BCA assay. To control for unincorporated cell-associated [9,10-³H(n)]oleic acid, a set of cultures was briefly exposed to [9,10-³H(n)]oleic acid (<30 s) and immediately washed and harvested; the resulting radioactivity was subtracted from all experimental results. For normalization, protein concentration of parallel cultures was determined using the BCA colorimetric assay.

Cholesterol-efflux assay

EGFP or L1-EGFP cells were seeded in 12-well plates at a density of 2×10^4 cells/cm² and allowed to proliferate for ≈ 36 h. Then cultures were supplemented with labelling medium (DMEM, 10 % FBS, 100 units/ml penicillin, 100 μ g/ml streptomycin and 0.2 μ Ci [4-¹⁴C]cholesterol) to label cellular cholesterol pools. Cultures were incubated in the labelling medium for 48 h to allow for equilibration. Thereafter, the culture medium was then changed to Medium B with 2 % M β CD for 1 h to cholesterol-deplete a subset of cells. Following the cholesterol-depletion period, all plates were gently washed three times with PBS and then supplemented with 0.5 ml of efflux medium (serum-free DMEM and 0.1 % fatty-acid-free BSA) in the absence or presence of cholesterol acceptors (100 μ g/ml HDL, 20 μ g/ml ApoA-I or 5 % FBS) for 4 h at 37 °C. To determine cholesterol efflux, the conditioned media were collected and centrifuged for 5 min at 16000 g to pellet cellular debris. An aliquot was removed and the radioactivity present in the incubation medium was determined by liquid-scintillation counting. The percentage of radiolabelled cholesterol released (% efflux) was calculated as:

$$\text{Efflux (\%)} = (\text{d.p.m. in medium after 4 h}) / (\text{d.p.m. in cells at zero time}) \times 100$$

To control for background efflux, a set of cultures was briefly (<30 s) exposed to efflux medium $\pm$ efflux acceptor, and medium was immediately removed and counted for radioactivity. The resulting radioactivity was subtracted from all experimental results. Cellular protein content was also determined for all cultures using the BCA assay.

Cellular lipid mass determinations

EGFP or L1-EGFP cells were seeded in 150-mm-diameter dishes, and allowed to grow to ~ 70 % confluence. The culture medium was then changed to Medium B with 2 % M β CD for 1 h to cholesterol-deplete cells. Thereafter, monolayers were washed twice with PBS and total cellular lipid extracts were prepared [26]. Thereafter, detergent-solubilized lipid was used to determine triacylglycerol, FC and total cholesterol using the commercial enzymatic assays as previously described [5]. EC was calculated as the difference between total cholesterol and FC. For normalization, the protein concentration of parallel cultures was determined using the BCA assay.

Statistical analysis

Unless otherwise indicated, results are expressed as means $\pm$ S.E.M. All results were analysed using one-way analysis of variance followed by Student's *t* tests for each pair for multiple comparisons. Differences were considered significant at $P < 0.05$. All analyses were performed using JMP version 5.0.12 (SAS Institute, Cary, NC, U.S.A.) software.

RESULTS

NPC1L1 facilitates the uptake of FC, but not EC

To determine whether NPC1L1 can differentiate between FC and EC, we delivered these distinct substrates via common HDL and LDL lipoprotein donors in order to make head-to-head comparisons. Using lipoprotein vehicles was necessary, since we have previously determined that delivery via NPC1L1's probable physiological donor (bile acid-containing micelles) was toxic to these cells (results not shown). As shown in Figure 1, when [¹⁴C]cholesterol was delivered via HDL, stable EGFP and L1-EGFP cell lines had similar rates of cholesterol uptake over time when cells were kept in normal culture conditions (Medium A). However, when the uptake assay was performed in cells pretreated with 2 % M β CD in Medium B for 1 h, HDL delivery of [¹⁴C]cholesterol was substantially augmented in L1-EGFP cells when compared with EGFP cells under the same conditions. For HDL delivery of [¹⁴C]cholesterol, the stimulation of cholesterol uptake due to M β CD treatment was 3.65-fold in EGFP cells versus 6.26-fold in L1-EGFP after 2 h of substrate incubation (Figure 1). We attribute this M β CD-induced augmentation of FC uptake to be due to the previous described co-ordinate translocation of NPC1L1 to the cell surface [5].

To further support this result, when [¹⁴C]FC was delivered via LDL, M β CD-stimulated cholesterol uptake was augmented in L1-EGFP when compared with EGFP cells (1.78- versus 2.20-fold respectively) after 2 h of substrate incubation (Figure 1). Interestingly, also in cells cultured under normal conditions (Medium A), L1-EGFP cells have augmented LDL-[¹⁴C]cholesterol uptake over time when compared with EGFP cells (Figure 1). These data suggest that, even in situations where NPC1L1 resides intracellularly (Medium A), LDL-mediated FC uptake can be slightly enhanced by NPC1L1 overexpression. For both HDL and LDL delivery of [¹⁴C]cholesterol, less than 1 % of the cholesterol was esterified over the 4 h incubation period (results not shown). Collectively these results strongly support the notion that, when NPC1L1 resides on the plasma membrane (i.e. after M β CD treatment), FC uptake is augmented regardless of the vehicle and, to a lesser extent, NPC1L1 expression may play a role in promoting LDL-mediated cholesterol uptake even when it resides intracellularly.

To address the question of whether NPC1L1 promotes EC uptake, we utilized the same lipoprotein vehicles (HDL and LDL) to deliver the non-hydrolysable CE analogue [³H]COE to test its potential as a possible substrate for NPC1L1. As seen in Figure 1, regardless of the vehicle (LDL or HDL), EGFP cells and L1-EGFP cells had similar rates of uptake of [³H]COE. This was true both with and without M β CD pretreatment. There was a slight, yet significant, increase in [³H]COE uptake seen in L1-EGFP cells pretreated with M β CD after 1 h of substrate incubation, but this effect was not seen at other time points, leading us to believe that NPC1L1 does not drive [³H]COE accumulation over an extended time period. These data clearly demonstrate that, although NPC1L1 can augment FC uptake when located at the plasma membrane, it cannot actively transport EC under the same conditions. Importantly, these data separate NPC1L1 as the only documented outside-in transporter with FC specificity in McA-RH7777 hepatoma cells.

NPC1L1 expression or localization does not affect oleic acid or β -sitosterol uptake

It has been demonstrated previously that NPC1, a protein highly similar to NPC1L1, can facilitate the transport of long-chain fatty acids into cells [27]. Therefore we examined whether a similar

Figure 1 Cholesterol depletion enhances NPC1L1-dependent selective FC uptake

EGFP control or L1-EGFP stable cells lines were either kept in Medium A or cholesterol-depleted in Medium B containing 2% M β CD for 1 h. Thereafter, cells were provided with 100 $\mu\text{g}/\text{ml}$ of either human HDL or LDL labelled with [^{14}C]FC or [^3H]COE for a period spanning 0–4 h as described in the Materials and methods section. Cell-associated radioactivity is expressed as d.p.m./mg of cellular protein. Data represent means $\pm$ SEM of one of two independent experiments ($n = 4$). The asterisk (*) represents significant differences between EGFP versus L1-EGFP cells within each M β CD treatment group, $P < 0.05$.

transport function could be attributed to NPC1L1. To define the function of NPC1L1 in long-chain-fatty-acid transport, a [^3H]oleic acid uptake assay was performed as described in the Materials and methods section. As seen in Figure 2(A), M β CD treatment of EGFP or L1-EGFP cells was without a significant effect on [^3H]oleic acid uptake. Furthermore, EGFP and L1-EGFP cells accumulated identical levels of [^3H]oleic acid over a period of 30 min, regardless of M β CD treatment. At 60 min a small, yet significant, increase in [^3H]oleic acid uptake was seen in the L1-EGFP cells compared with EGFP cells. However, we do not interpret this slight increase to be biologically important. Collectively, these data do not support a role for NPC1L1 in long-chain-fatty-acid transport.

Previous reports have proposed that NPC1L1 can transport both animal (cholesterol) and plant (β -sitosterol) sterols in CaCo-2 cells [28]. Furthermore, mice lacking NPC1L1 have reduced intestinal absorption of both cholesterol and β -sitosterol [4]. In order to examine whether these findings could be substantiated in our hepatoma-cell model, we performed a plant-sterol-uptake assay utilizing β -[^3H]sitosterol as a potential substrate for NPC1L1. As seen in Figure 2(B), when cells were grown under normal conditions (Medium A), β -[^3H]sitosterol uptake was identical for EGFP and L1-EGFP cells. Interestingly, M β CD treatment of the cells did result in substantial augmentation of β -[^3H]sitosterol uptake when compared with cells not treated with

M β CD, but the fold increase was identical for EGFP and L1-EGFP cells (Figure 2B).

To examine further whether NPC1L1 can differentiate between FC and β -sitosterol in McArdle-RH7777 hepatoma cells, we examined the simultaneous uptake of both substrates under conditions where NPC1L1 was resident either intracellularly ($-$ M β CD; Figure 2C) or on the plasma membrane ($+$ M β CD; Figure 2D). Interestingly, the kinetics of uptake were significantly faster for β -[^3H]sitosterol when compared with [^{14}C]cholesterol in the absence of M β CD pretreatment in both hepatoma cell lines (Figure 2C). However, only the uptake of [^{14}C]cholesterol was dependent on NPC1L1 expression and more specifically its localization on the plasma membrane. In support of this, when NPC1L1 resided intracellularly ($-$ M β CD), EGFP and L1-EGFP cells had similar rates of β -[^3H]sitosterol uptake that were faster than [^{14}C]cholesterol uptake over time (Figure 2C). However, when the dual-sterol-uptake assay was performed in cells pretreated with 2% M β CD in Medium B for 1 h, [^{14}C]cholesterol uptake was substantially augmented by the presence of NPC1L1 at the cell surface (Figure 2D). In contrast, when both cell lines were subjected to M β CD pretreatment β -[^3H]sitosterol uptake was identical in EGFP and L1-EGFP cells and essentially matched the rate of NPC1L1-assisted [^{14}C]cholesterol uptake (Figure 2D). Collectively, these data support the notion that NPC1L1 can indeed differentiate between plant and animal sterols in cultured

Figure 2 Expression of NPC1L1-EGFP does not alter oleic acid or β -sitosterol uptake

EGFP control or L1-EGFP stable cell lines were either kept in Medium A or cholesterol depleted in Medium B containing 2% M β CD for 1 h. Thereafter, cells were provided with [^3H]oleic acid (A), β -[^3H]sitosterol alone (B), or β -[^3H]sitosterol and [^{14}C]cholesterol together (C and D) as described in the Materials and methods section, and cell-associated radioactivity was determined after a short time course. (C) Represents dual sterol (β -[^3H]sitosterol and [^{14}C]cholesterol) uptake in cells maintained in Medium A, which exhibit perinuclear/ERC localization of NPC1L1. (D) Represents dual sterol (β -[^3H]sitosterol and [^{14}C]cholesterol) uptake in cells after 1 h treatment with 2% M β CD in Medium B to induce membrane localization of NPC1L1-EGFP. Data are expressed as d.p.m./mg of cellular protein and represent means $\pm$ S.E.M. for one of two independent experiments ($n = 4$ for A and B; $n = 3$ for C and D). * Represents significant differences between EGFP versus L1-EGFP cells within each M β CD treatment group, $P < 0.05$.

hepatoma cells, and preferentially promote the transport of FC, but not β -sitosterol. Furthermore, β -sitosterol uptake in McArdle-RH7777 hepatoma cells seems to occur in an NPC1L1-independent manner, yet can be dramatically enhanced with M β CD pretreatment, indicating that a novel facilitated mechanism of transport may exist that is independent of NPC1L1.

NPC1L1-dependent FC transport is not bi-directional

Previous studies have clearly demonstrated that SR-BI, another well-characterized cholesterol-transport protein, can transport cholesterol both into and out of cells and is therefore a bidirectional cholesterol transporter [16–24]. In order to examine whether NPC1L1 could promote the movement of cholesterol out of cells, we performed a cholesterol-efflux assay as described in the Materials and methods section using ApoAI, HDL and FBS as cholesterol acceptors. As seen in Figure 3(A), when cells were grown under normal conditions (Medium A), the efflux of cholesterol in the absence or presence of acceptors (apoAI, HDL and FBS) was identical for EGFP and L1-EGFP cells. Furthermore, when cells were treated with 2% M β CD for 1 h to promote NPC1L1 concentration to the cell surface (Figure 3B), cholesterol efflux was also identical for EGFP and L1-EGFP cells. Cellular protein content was also determined for all cultures and did not

differ across all treatment variations. These data demonstrate that NPC1L1-dependent cholesterol transport is unidirectional, and the previously reported augmentation of FC mass seen in L1-EGFP cells [5] is primarily due to enhanced uptake, and not defective efflux, of cholesterol.

NPC1L1 does not alter mass cholesterol loading when it resides intracellularly, yet promotes mass cholesterol accumulation when translocated to the plasma membrane

To determine whether the expression or localization of NPC1L1 can facilitate the mass movement of FC into cells, we examined the extent of cholesterol loading under conditions where NPC1L1 was resident either intracellularly (Figure 4) or on the plasma membrane (Figure 5). Figure 4 represents cholesterol loading under conditions where NPC1L1 resides intracellularly. We have previously described that, under these growth conditions (Medium A), most of NPC1L1-EGFP fusion proteins reside in the perinuclear ERC (endocytic recycling compartment) [5]. As previously reported [5], when grown under normal conditions (Medium A) L1-EGFP cells have 35% more TC (total cholesterol) and 27% more FC when compared with EGFP cells (Figures 4A and 4B). In addition, there was a trend towards increased EC in L1-EGFP cells, but this difference was not significant

Figure 3 Expression of NPC1L1-EGFP does not alter cellular cholesterol efflux

EGFP control or L1-EGFP stable cells lines were labelled with [14 C]cholesterol for 48 h. Thereafter, cells were either (A) kept in medium A to preserve perinuclear/ERC localization of NPC1L1 or (B) cholesterol-depleted with 2% M β CD in Medium B for 1 h to induce membrane localization of NPC1L1-EGFP. Thereafter, cells were washed and the efflux of cellular [14 C]cholesterol in the absence (no acceptor) or presence of ApoA-I (20 μ g/ml), HDL (100 μ g/ml), or FBS (5%) cholesterol acceptors was measured after 4 h incubation as described in the Materials and methods section. Results are expressed as percentage efflux and represent means $\pm$ S.E.M. for one of three independent experiments ($n = 4$). Values not sharing a common superscript differ significantly ($P < 0.05$).

(Figure 4C). When these cells were loaded with cholesterol-M β CD complexes, both EGFP and L1-EGFP cells exhibited mass loading of total cholesterol, FC and EC. Importantly, when NPC1L1-EGFP proteins resided intracellularly, cholesterol loading occurred to a similar extent in both cell lines. In this case the fold increases for EGFP and L1-EGFP cells respectively were 3.7 versus 3.5 for TC, 3.7 versus 3.6 for FC and 3.9 versus 2.8 for EC (Figures 4A–4C). Collectively these results suggest that, when cells are maintained under normal growth conditions and NPC1L1 resides intracellularly, the steady-state TC and FC masses are slightly augmented when compared with EGFP control cells. However, acute mass cholesterol loading is not augmented by NPC1L1 overexpression.

To test whether NPC1L1 could facilitate mass cholesterol transport when it resides on the plasma membrane, cells were pretreated with 2% M β CD in Medium B to facilitate the translocation of NPC1L1, as previously described [5]. Then the cells were cholesterol-loaded with M β CD-cholesterol complexes to see if loading could be augmented by membrane-localized NPC1L1. As seen in Figure 5, after 1 h of 2% M β CD pretreatment, both EGFP and L1-EGFP cells were depleted of TC, FC and EC mass by ≈ 50 –60%. Interestingly, when the EGFP control cells

Figure 4 NPC1L1 does not augment cholesterol loading when it resides intracellularly

EGFP control or L1-EGFP stable cells lines were grown in 150-mm-diameter dishes and maintained in Medium A to preserve perinuclear/ERC localization of NPC1L1. For cholesterol loading (Chol. Load), cells were grown in Medium A supplemented with 10 μ g/ml cholesterol/M β CD for 4 h. All cells were then washed twice with PBS, total cellular lipids were extracted, and TC (A), FC (B) and EC (C) mass was measured by using the enzymatic assays described in the Materials and methods section. Results are expressed as μ g of lipid/mg of cellular protein and represent means $\pm$ S.E.M. for one of two independent experiments ($n = 4$). Values not sharing a common superscript differ significantly ($P < 0.05$).

were subsequently cholesterol-loaded with M β CD-cholesterol complexes, TC, FC and EC mass increased back to levels seen prior to cholesterol depletion. However, when the L1-EGFP cells were subsequently cholesterol-loaded with M β CD-cholesterol complexes, TC and FC mass increased to levels significantly higher than those seen prior to cholesterol depletion. In further support of this, following cholesterol depletion, the increase in FC mass due to cholesterol loading was 1.54-fold in EGFP cells versus 2.59-fold in L1-EGFP cells (Figure 5B). However, NPC1L1 expression or translocation did not affect EC mass under these same conditions (Figure 5C), indicating that NPC1L1-delivered cholesterol is not adequately esterified during this 2 h period. Collectively these results suggest that, following cholesterol depletion, NPC1L1 can acutely facilitate the mass accumulation of TC and FC to levels above those seen under normal growth conditions. We attribute augmentation of FC loading, following M β CD pretreatment, to the previously described coordinate translocation of NPC1L1 to the cell surface [5].

Figure 5 Cholesterol-depletion-driven membrane localization of NPC1L1 facilitates mass cholesterol uptake

EGFP control or L1-EGFP stable cells lines were grown in 150-mm-diameter dishes and maintained in Medium A to preserve perinuclear ERC localization of NPC1L1. A subset of dishes was changed to Medium B containing 2% MβCD for 1 h (1h MβCD) to induce membrane localization of NPC1L1-EGFP. Thereafter, the dishes either received serum-free medium with or without 10 μg/ml cholesterol/MβCD for 2 h (2h Chol. Load) to replete cellular cholesterol. All cells were then washed twice with PBS, total cellular lipids were extracted and total cholesterol (TC) (A), FC (B) and EC (C) masses were measured using enzymatic assays as described in the Materials and methods section. Results are expressed as μg of lipid/mg of cellular protein and represent means ± S.E.M. of one of two independent experiments (*n* = 4). Values not sharing a common superscript differ significantly (*P* < 0.05).

NPC1L1-dependent cholesterol uptake requires adequate intracellular K⁺, yet does not rely on intracellular calcium, the cytoskeleton or signalling downstream of PKA (protein kinase A), PKC (protein kinase C) or pertussis-toxin-sensitive GPCRs (G-protein-coupled receptors)

Since NPC1L1-EGFP fusion proteins reside in the endocytic recycling pathways, we propose that vesicular trafficking of NPC1L1 could potentially affect its cholesterol-uptake functions,

since NPC1L1 does not facilitate cholesterol uptake when it resides intracellularly [5]. It has long been known that protein trafficking from subapical compartments to the plasma membrane is tightly controlled by a number of signalling modulators, including protein kinases and intracellular ion flux [29–31].

Therefore we utilized pharmacological inhibitors and conditions known to block different phases of vesicular trafficking to test their potential involvement in NPC1L1-dependent cholesterol uptake. To test the involvement of PKC, PKA, GPCRs and intracellular Ca²⁺ in NPC1L1-dependent FC uptake, cells were pretreated with pharmacological agents (calphostin c, H-89, pertussis toxin and BAPTA-AM respectively) known to blunt each distinct signalling capacity. As seen in Figure 6(A), pretreatment with calphostin C, H-89, pertussis toxin or BAPTA-AM were all without effect on NPC1L1-dependent FC uptake. In parallel, pretreatment with these pharmacological inhibitors had no impact on MβCD-driven translocation of NPC1L1 to the cell surface (results not shown). The efficacy of each inhibitor was confirmed by immunoblotting using phospho-specific antibodies of known downstream kinases (results not shown), indicating that these pathways are not involved in the endocytic recycling or cholesterol-transport function of NPC1L1. To test the involvement of the cytoskeleton in NPC1L1-dependent cholesterol uptake, cells were pretreated with known disruptors of microtubule (nocodazole) and actin microfilament (cytochalasin D) organization. As seen in Figure 6(B), pretreatment with nocodazole or cytochalasin D had no effect on NPC1L1-dependent cholesterol uptake. This implies that the endocytic recycling and cholesterol uptake function of NPC1L1 does not rely on the intact cytoskeleton.

Finally, we wished to test whether NPC1L1-dependent cholesterol uptake relied on clathrin-dependent internalization. Therefore, two conditions known to alter clathrin-dependent endocytic recycling of the LDLR (K⁺ depletion or monensin treatment) were utilized. As seen in Figure 6(C), K⁺ depletion in cells grown under normal growth conditions (Medium A) paradoxically increased basal [¹⁴C]cholesterol uptake by ≈ 2-fold in both EGFP and L1-EGFP cells. After cholesterol depletion, however, [¹⁴C]cholesterol uptake was diminished in K⁺-depleted L1-EGFP cells but not in K⁺-depleted EGFP cells when compared with respective vehicle-treated cells. This indicates that NPC1L1-dependent cholesterol transport requires adequate intracellular K⁺. However, monensin pretreatment, which inhibits endosomal acidification, was without effect on NPC1L1-dependent cholesterol uptake. This finding indicates that, unlike LDLR-mediated cholesterol uptake, an acidification step is not required for the endocytic recycling or transport function of NPC1L1. Collectively, these data demonstrate that NPC1L1-dependent cholesterol uptake relies on adequate intracellular K⁺ levels, but does not require PKA, PKC, pertussis toxin-sensitive GPCRs, intracellular Ca²⁺, endosomal acidification or the intact cytoskeleton.

DISCUSSION

In the present study we examined the sterol specificity and directionality of transport for NPC1L1. The major findings generated by this study include the following: (1) the endocytic recycling of NPC1L1 to the cell surface can facilitate mass uptake of FC, yet does not have an impact on CE and fatty acid uptake; (2) NPC1L1 expression and localization does not facilitate β-sitosterol uptake, indicating sterol selectivity in McA-RH7777 hepatoma cells; (3) Cholesterol uptake mediated by NPC1L1 is unidirectional; (4) NPC1L1-dependent cholesterol uptake requires adequate intracellular K⁺ levels. This is the first

Figure 6 Role of cell-signalling mediators and the cytoskeleton in NPC1L1-dependent cholesterol uptake

(A) EGFP control or L1-EGFP stable cells lines were pretreated for 1 h with protein kinase inhibitors: 200 nM calphostin C (Cal. C), 10 μ M H-89 (H-89), 100 ng/ml pertussis toxin (PTX) or 50 μ M BAPTA-AM. (B) EGFP control or L1-EGFP stable cells lines were pretreated for 1 h with 20 μ g/ml nocodazole or 10 μ g/ml cytochalasin D. (C) EGFP control or L1-EGFP stable cells lines were pretreated for 1 h with 10 μ M monensin or K⁺-depleted as described in the Materials and methods section. Thereafter, cells were either kept in Medium A (–M β CD) or cholesterol depleted by addition of 2% M β CD in Medium B (+M β CD) for 1 h in the presence of indicated inhibitors. Then cells were provided with [¹⁴C]FC in a BSA/ethanol suspension for a period spanning 90 min in the presence of the indicated inhibitors to determine cholesterol uptake. Cell-associated radioactivity is expressed as d.p.m./mg of cellular protein and results are means $\pm$ S.E.M. for one of two independent experiments ($n = 2-4$). Values not sharing a common superscript differ significantly ($P < 0.05$).

study to characterize such functions for NPC1L1 and it identifies NPC1L1 as the only known protein that selectively transports FC in an outside-in manner.

The importance of NPC1L1 in intestinal cholesterol absorption has been firmly established in mice [2]. However, the initial characterizations of NPC1L1 deficiency *in vivo* did not address whether NPC1L1 could itself actively facilitate cellular transport of sterols. Furthermore, the subcellular localization of NPC1L1

has been a matter of debate, with conflicting reports of NPC1L1 residing on the plasma membrane and in intracellular locations [3,32]. To address these issues, our group previously established a cell line overexpressing NPC1L1 fused to EGFP to better understand the intracellular itinerary and transport functions of NPC1L1. Importantly, our studies have established that NPC1L1 resides in the ERC and translocates to specialized ‘apical-like’ subdomains in a cholesterol depletion-driven manner [5]. Collectively, our previous data support a direct role for NPC1L1 in actively transporting cholesterol and the notion that the endocytic recycling of NPC1L1 appears to be critical for its ability to transport its preferred substrate across cell membranes.

The present study further supports a direct role for NPC1L1 in facilitating cellular FC uptake. Importantly, we have now shown that, when NPC1L1 resides on the plasma membrane, FC uptake is augmented from lipoproteins (HDL and LDL). However, owing to its predominant apical membrane localization in enterocytes [2] and hepatocytes [5], we do not believe that NPC1L1 would have access to plasma lipoproteins *in vivo* and therefore would have minimal impact on lipoprotein-mediated cholesterol uptake in these cell types. Instead, we believe that, in the case of lipoprotein-mediated delivery of FC in our cell system, NPC1L1 can simply remove its preferred substrate from the resident phospholipid monolayer, leaving the remaining lipoprotein cargo exclusively extracellular. This is supported by the fact that CE uptake is not augmented by NPC1L1 (Figure 1), indicating that NPC1L1 is most likely not facilitating whole-particle uptake. Instead, NPC1L1 appears to be able to selectively recognize FC, probably from membranes, and promote its intracellular retention. Whether or not this is through a ‘permease-like’ action or mediated by direct binding of FC to NPC1L1 remains to be determined. Ultimately, purification of NPC1L1 and identification of its intrinsic sterol-binding capacity may help to answer this question.

In disagreement with previous reports on CaCo-2 cells and mice [4,28], the present study demonstrates that NPC1L1 overexpression or localization did not facilitate the uptake of non-esterified β -sitosterol (Figures 2B–2D). This finding was surprising on the basis of the previously described decreases in sitosterol absorption by the intestine seen in NPC1L1-knockout mice [4] and the fact that NPC1L1 does modestly promote β -sitosterol uptake in CaCo2 cells [28]. Davis et al. [4] concluded that NPC1L1 could facilitate the intestinal transport of both cholesterol and sitosterol and thereby act as a transporter for multiple sterols of animal and plant origin. Another relevant argument against NPC1L1 mediating sterol selectivity is the fact that ezetimibe improves elevated plasma sitosterol levels present in sitosterolemia humans [33] and ABCG5/ABCG8 (ATP-binding cassette proteins G5 and G8)-knockout mice [34]. In contrast with these findings, our results (Figures 2B and 2D) indicate that NPC1L1 can indeed differentiate between animal and plant sterols in rat hepatoma cells. One possible explanation for the reported decreases in sitosterol absorption seen in NPC1L1-knockout mice is that this effect was indirect, and was instead mediated by the drastic differences in enterocytic cholesterol content and resulting sterol-regulated gene expression. For instance, Davis et al. [4] reported that mice lacking NPC1L1 showed dramatic decreases in intestinal ABCA1 mRNA expression, most likely resulting from diminished cholesterol-driven LXR (liver X receptor) signalling. Interestingly, intestinal ABCA1 expression has been recently implicated in promoting intestinal cholesterol absorption [35,36] and has been shown to mediate the basolateral transport of β -sitosterol out of CaCo-2 cells [37]. Therefore the decreases in intestinal ABCA1 seen in NPC1L1-knockout mice may partially explain the observed diminished intestinal β -sitosterol absorption.

Furthermore, the fact that ezetimibe normalizes elevated plasma sitosterol levels in sitosterolaemic humans and mice could also be a secondary effect of the potent cholesterol absorption inhibiting effects of ezetimibe and not a direct effect on NPC1L1 *per se*. However, during the preparation of this manuscript, Yamanashi and co-workers [28] were able to demonstrate that NPC1L1 could indeed facilitate β -sitosterol uptake in CaCo-2 cells, although not nearly as efficiently as cholesterol. Therefore, the lack of NPC1L1-dependent β -sitosterol uptake in our McArdle-RH7777 cell model may indicate that sterol selectivity by NPC1L1 occurs in a cell-specific manner. This raises the intriguing possibility that cell-specific cofactors may be needed to confer sterol selectivity on NPC1L1.

To fully understand the mechanism(s) by which NPC1L1 traffics through the ERC and functions to transport cholesterol, it will be critical to define the signals that control its endocytic recycling. It has long been known that endosomal trafficking is tightly controlled by a number of signalling proteins (PKA, PKC, GPCRs, Rho family GTPases and Rab GTPases) and, in some cases, relies on cytoskeletal integrity [29–31,38]. However, the involvement of the cytoskeleton and specific signalling modulators in the trafficking of NPC1L1 has not been resolved. Our findings strongly suggest that NPC1L1 trafficking and function are not influenced by PKA-, PKC-, GPCR- or Ca^{2+} -dependent signalling (Figure 6A). Furthermore, NPC1L1-dependent cholesterol uptake cannot be inhibited by nocodazole or cytochalasin D (Figure 6B), indicating that it likely traffics and functions independently of cytoskeletal integrity. Interestingly, depletion of intracellular K^+ did attenuate maximal NPC1L1-dependent cholesterol uptake (Figure 6C). Depletion of intracellular K^+ was the first method identified to specifically inhibit receptor-mediated endocytosis [39] and therefore prevent the clathrin-dependent cholesterol uptake mediated by the LDLR. The fact that NPC1L1-dependent cholesterol uptake is inhibited by K^+ depletion suggests that receptor-mediated endocytosis is in some way needed to provide the cell with NPC1L1-delivered cholesterol. However, the sequential subcellular itinerary of NPC1L1-delivered cholesterol remains to be elucidated.

In addition to previously documented proteins known to be involved in cellular cholesterol uptake (LDLR and SR-BI), NPC1L1 seems now established as another protein that functions to actively transport cholesterol across cell membranes. Unlike the LDLR and SR-BI, NPC1L1 selectively recognizes FC and facilitates only outside-in transport. Importantly, NPC1L1-dependent cholesterol uptake relies heavily on its subcellular localization [5], a mode of regulation well documented for other known solute transporters [40,41]. Therefore, further mechanistic understanding of the processes or signals that control the trafficking of NPC1L1 may provide insight into broader physiological processes, including pathways regulating intestinal cholesterol absorption and potentially re-uptake of cholesterol from canalicular bile, since the protein also localizes to the canalicular membrane of hepatocytes [5].

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REFERENCES

- Davies, J. P., Levy, B. and Ioannou, Y. A. (2000) Evidence for a Niemann–Pick C (NPC) gene family: identification and characterization of NPC1L1. *Genomics* **65**, 137–145
- Altmann, S. W., Davis, Jr., H. R., Zhu, L. J., Yao, X., Hoos, L. M., Tetzloff, G., Iyer, S. P., Maguire, M., Golovko, A., Zeng, M. et al. (2004) Niemann–Pick C1 Like 1 protein is critical for intestinal cholesterol absorption. *Science* **303**, 1201–1204
- Davies, J. P., Scott, C., Oishi, K., Liapis, A. and Ioannou, Y. A. (2005) Inactivation of NPC1L1 causes multiple lipid transport defects and protects against diet-induced hypercholesterolemia. *J. Biol. Chem.* **280**, 12710–12720
- Davis, Jr., H. R., Zhu, L. J., Hoos, L. M., Tetzloff, G., Maguire, M., Liu, J., Yao, X., Iyer, S. P., Lam, M. H., Lund, E. G. et al. (2004) Niemann–Pick C1 Like 1 (NPC1L1) is the intestinal phytosterol and cholesterol transporter and a key modulator of whole-body cholesterol homeostasis. *J. Biol. Chem.* **279**, 33586–33592
- Yu, L., Bharadwaj, S., Brown, J. M., Ma, Y., Du, W., Davis, M. A., Michaely, P., Liu, P., Willingham, M. C. and Rudel, L. L. (2006) Cholesterol-regulated translocation of NPC1L1 to the cell surface facilitates FC uptake. *J. Biol. Chem.* **281**, 6616–6624
- Garcia-Calvo, M., Lisnock, J., Bull, H. G., Hawes, B. E., Burnett, D. A., Braun, M. P., Crona, J. H., Davis, H. R., Jr., Dean, D. C., Detmers, P. A. et al. (2005) The target of ezetimibe is Niemann–Pick C1-Like 1 (NPC1L1). *Proc. Natl. Acad. Sci. U.S.A.* **102**, 8132–8137
- Dietschy, J. M., Turley, S. D. and Spady, D. K. (1993) Role of liver in the maintenance of cholesterol and low density lipoprotein homeostasis in different animal species, including humans. *J. Lipid Res.* **34**, 1637–1659
- Osono, Y., Woollett, L. A., Herz, J. and Dietschy, J. M. (1995) Role of the low density lipoprotein receptor in the flux of cholesterol through the plasma and across the tissues of the mouse. *J. Clin. Invest.* **95**, 1124–1132
- Dietschy, J. M. and Turley, S. D. (2002) Control of cholesterol turnover in the mouse. *J. Biol. Chem.* **277**, 3801–3804
- Innerarity, T. L. and Mahley, R. W. (1978) Enhanced binding by cultured human fibroblasts of apo-E-containing lipoproteins as compared with low density lipoproteins. *Biochemistry* **17**, 1440–1447
- Ishibashi, S., Herz, J., Maeda, N., Goldstein, J. L. and Brown, M. S. (1994) The two-receptor model of lipoprotein clearance: tests of the hypothesis in “knockout” mice lacking the low density lipoprotein receptor, apolipoprotein E, or both proteins. *Proc. Natl. Acad. Sci. U.S.A.* **91**, 4431–4435
- Brown, M. S. and Goldstein, J. L. (1986) A receptor-mediated pathway for cholesterol homeostasis. *Science* **232**, 34–47
- Davis, C. G., Goldstein, J. L., Sudhof, T. C., Anderson, R. G., Russell, D. W. and Brown, M. S. (1987) Acid-dependent ligand dissociation and recycling of LDL receptor mediated by growth factor homology region. *Nature* **326**, 760–765
- Rudenko, G., Henry, L., Henderson, K., Ichtchenko, K., Brown, M. S., Goldstein, J. L. and Deisenhofer, J. (2002) Structure of the LDL receptor extracellular domain at endosomal pH. *Science* **298**, 2353–2358
- Beglova, N. and Blacklow, S. C. (2005) The LDL receptor: how acid pulls the trigger. *Trends Biochem. Sci.* **30**, 309–317
- Acton, S., Rigotti, A., Landschulz, K. T., Xu, S., Hobbs, H. H. and Krieger, M. (1996) Identification of scavenger receptor SR-BI as a high density lipoprotein receptor. *Science* **271**, 518–520
- Silver, D. L. and Tall, A. R. (2001) The cellular biology of scavenger receptor class B type I. *Curr. Opin. Lipidol.* **12**, 497–504
- Silver, D. L., Wang, N., Xiao, X. and Tall, A. R. (2001) High density lipoprotein (HDL) particle uptake mediated by scavenger receptor class B type 1 results in selective sorting of HDL cholesterol from protein and polarized cholesterol secretion. *J. Biol. Chem.* **276**, 25287–25293
- Rigotti, A., Edelman, E. R., Seifert, P., Iqbal, S. N., DeMattos, R. B., Temel, R. E., Krieger, M. and Williams, D. L. (1996) Regulation by adrenocorticotrophic hormone of the *in vivo* expression of scavenger receptor class B type I (SR-BI), a high density lipoprotein receptor, in steroidogenic cells of the murine adrenal gland. *J. Biol. Chem.* **271**, 33545–33549
- Landschulz, K. T., Pathak, R. K., Rigotti, A., Krieger, M. and Hobbs, H. H. (1996) Regulation of scavenger receptor, class B, type I, a high density lipoprotein receptor, in liver and steroidogenic tissues of the rat. *J. Clin. Invest.* **98**, 984–995
- Glass, C. K., Pittman, R. C., Keller, G. A. and Steinberg, D. (1983) Tissue sites of degradation of apoprotein A-I in the rat. *J. Biol. Chem.* **258**, 7161–7167
- Shimada, A., Tamai, T., Oida, K., Takahashi, S., Suzuki, J., Nakai, T. and Miyabo, S. (1994) Increase in neutral cholesteryl ester hydrolase activity produced by extralysosomal hydrolysis of high-density lipoprotein cholesteryl esters in rat hepatoma cells (H-35). *Biochim. Biophys. Acta* **1215**, 126–132
- Connelly, M. A., Kellner-Weibel, G., Rothblat, G. H. and Williams, D. L. (2003) SR-BI-directed HDL-cholesteryl ester hydrolysis. *J. Lipid Res.* **44**, 331–341
- Ji, Y., Jian, B., Wang, N., Sun, Y., Moya, M. L., Phillips, M. C., Rothblat, G. H., Swaney, J. B. and Tall, A. R. (1997) Scavenger receptor BI promotes high density lipoprotein-mediated cellular cholesterol efflux. *J. Biol. Chem.* **272**, 20982–20985

- 25 Rudel, L. L., Greene, D. G. and Shah, R. (1977) Separation and characterization of plasma lipoproteins of rhesus monkeys (*Macaca mulatta*). *J. Lipid Res.* **18**, 734–744
- 26 Bligh, E. G. and Dyer, W. J. (1959) A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **37**, 911–917
- 27 Davies, J. P., Chen, F. W. and Ioannou, Y. A. (2000) Transmembrane molecular pump activity of Niemann–Pick C1 protein. *Science* **290**, 2295–2298
- 28 Yamanashi, Y., Takada, T. and Suzuki, H. (2007) Niemann–Pick C1-like 1 overexpression facilitates ezetimibe-sensitive cholesterol and β -sitosterol uptake in CaCo-2 cells. *J. Pharmacol. Exp. Ther.* **320**, 559–564
- 29 Hoekstra, D., Tyteca, D. and van, I. S. C. (2004) The subapical compartment: a traffic center in membrane polarity development. *J. Cell Sci.* **117**, 2183–2192
- 30 Nichols, B. J. and Lippincott-Schwartz, J. (2001) Endocytosis without clathrin coats. *Trends Cell Biol.* **11**, 406–412
- 31 Clague, M. J. and Urbe, S. (2001) The interface of receptor trafficking and signalling. *J. Cell. Sci.* **114**, 3075–3081
- 32 Iyer, S. P., Yao, X., Crona, J. H., Hoos, L. M., Tetzloff, G., Davis, Jr., H. R., Graziano, M. P. and Altmann, S. W. (2005) Characterization of the putative native and recombinant rat sterol transporter Niemann–Pick C1 Like 1 (NPC1L1) protein. *Biochim. Biophys. Acta* **1722**, 282–292
- 33 Salen, G., von Bergmann, K., Lutjohann, D., Kwiterovich, P., Kane, J., Patel, S. B., Musliner, T., Stein, P. and Musser, B. (2004) Ezetimibe effectively reduces plasma plant sterols in patients with sitosterolemia. *Circulation* **109**, 966–971
- 34 Yu, L., von Bergmann, K., Lutjohann, D., Hobbs, H. H. and Cohen, J. C. (2005) Ezetimibe normalizes metabolic defects in mice lacking ABCG5 and ABCG8. *J. Lipid Res.* **46**, 1739–1744
- 35 Brunham, L. R., Kruit, J. K., Iqbal, J., Fievet, C., Timmins, J. M., Pape, T. D., Coburn, B. A., Bissada, N., Staels, B., Groen, A. K. et al. (2006) Intestinal ABCA1 directly contributes to HDL biogenesis *in vivo*. *J. Clin. Invest.* **116**, 1052–1062
- 36 Temel, R. E., Lee, R. G., Kelley, K. L., Davis, M. A., Shah, R., Sawyer, J. K., Wilson, M. D. and Rudel, L. L. (2005) Intestinal cholesterol absorption is substantially reduced in mice deficient in both ATP-binding cassette transporter A1 (ABCA1) and Acyl-CoA/cholesterol *O*-acyltransferase 2 (ACAT2). *J. Lipid Res.* **46**, 2423–2431
- 37 Field, F. J., Born, E. and Mathur, S. N. (2004) LXR/RXR ligand activation enhances basolateral efflux of β -sitosterol in CaCo-2 cells. *J. Lipid Res.* **45**, 905–913
- 38 Kirkham, M. and Parton, R. G. (2005) Clathrin-independent endocytosis: new insights into caveolae and non-caveolar lipid raft carriers. *Biochim. Biophys. Acta* **1745**, 273–286
- 39 Larkin, J. M., Brown, M. S., Goldstein, J. L. and Anderson, R. G. (1983) Depletion of intracellular potassium arrests coated pit formation and receptor-mediated endocytosis in fibroblasts. *Cell* **33**, 273–285
- 40 Watson, R. T., Kanzaki, M. and Pessin, J. E. (2004) Regulated membrane trafficking of the insulin-responsive glucose transporter 4 in adipocytes. *Endocr. Rev.* **25**, 177–204
- 41 Valenti, G., Procino, G., Tamma, G., Carmosino, M. and Svelto, M. (2005) Aquaporin 2 trafficking. *Endocrinology* **146**, 5063–5070

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