Inactivation of NPC1L1 Causes Multiple Lipid Transport Defects and Protects against Diet-induced Hypercholesterolemia*

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NPC1L1, a recently identified relative of Niemann-Pick C1, was characterized to determine its subcellular location and potential function(s). NPC1L1 was highly expressed in HepG2 cells and localized in a subcellular vesicular compartment rich in the small GTPase Rab5. mRNA expression profiling revealed significant differences between mouse and man with highest expression found in human liver and significant expression in the small intestine. In contrast, liver expression in mouse was extremely low with mouse small intestine exhibiting the highest NPC1L1 expression. A mouse knock-out model of NPC1L1 was generated and revealed that mice lacking a functional NPC1L1 have multiple lipid transport defects. Surprisingly, lack of NPC1L1 exerts a protective effect against diet-induced hyperlipidemia. Further characterization of cell lines generated from wild-type and knock-out mice revealed that in contrast to wild-type cells, NPC1L1 cells exhibit aberrant plasma membrane uptake and subsequent transport of various lipids, including cholesterol and sphingolipids. Furthermore, lack of NPC1L1 activity causes a deregulation of caveolin transport and localization, suggesting that the observed lipid transport defects may be the indirect result of an inability of NPC1L1 null cells to properly target and/or regulate caveolin expression.

Niemann Pick C1-like 1 protein (NPC1L1)1 was previously identified based on its high degree of similarity, 42% amino acid identity and 51% similarity to the polytopic, late endosome-resident protein, NPC1. Both possess a putative sterol-sensing domain, suggesting roles in sterol/lipid transport (1), and they also have an amino-terminal “NPC1 domain” (2). Based on this homology and preliminary data, we hypothesized that NPC1L1 has a lipid permease function similar to that of NPC1 (3). In contrast, however, the two proteins have variant targeting signals and thus are predicted to function similarly but at different intracellular locations.

To gain a further understanding of the function of this family of proteins, we have carried out cell and molecular studies to determine the location and tissue expression of NPC1L1. In addition, since no known disorders map at 7p13, the chromosomal location of human NPC1L1 (1), we have generated a mouse knock-out of NPC1L1. Our results are in contrast to a published report suggesting that NPC1L1 resides at the plasma membrane (4) and indicate that this protein is predominantly intracellular and colocalizes with the small GTPase Rab5. In addition, the expression profile of human NPC1L1 shows this protein to be highly enriched in liver. Further, inactivation of NPC1L1 leads to multiple lipid transport defects including cholesterol and sphingolipids, suggesting that NPC1L1 plays a critical role in lipid homeostasis and transport in support of our original hypothesis.

MATERIALS AND METHODS

Tissue Culture, Transfection, and Immunofluorescence Studies—All cells, including COS7, HepG2, and Caco-2 cells, were obtained from ATCC (Manassas, VA). Cells were maintained at 37 °C in a humidified environment with 5% CO2 in Dulbecco’s modified Eagle’s medium containing 2 mM glutamine, 10% fetal bovine serum, and 10 μg/ml gentamicin. HepG2 cells and Caco-2 cells were grown on glass coverslips for 2 days before staining. All other cells were grown overnight before transfection using Lipofectamine and Plus reagents (Invitrogen), according to the manufacturer’s instructions. Unless indicated otherwise, cells were fixed using methanol at 4 °C for 6 min and then processed for indirect immunofluorescence staining using the appropriate anti-IgG secondary antibodies that were tagged with either Alexa 488 or Alexa 594 (Molecular Probes).

For immunofluorescence with transferrin-Alexa 568, the cells were incubated with 50 μg/ml transferrin for 25 min followed by a 15-min chase period. They were subsequently washed, fixed with methanol, and stained as above. Cells were photographed using a Nikon Eclipse microscope equipped with a CCD camera. Images were deconvoluted using the MetaMorph Software package (Universal Imaging).

Antibodies—Monoclonal antibodies for the Golgi markers GS15, GS28, GM130, Vti1b, GS27, and p230 and those for Rab8, calnexin, and EEA1 were purchased from Pharmingen. Goat polyclonal antibodies for ABCD3, Rab11, and calnexin and rabbit polyclonal for Rab5A were obtained from Santa Cruz Biotechnology. Transferrin-Alexa 568 and Golgin 97 were from Molecular Probes. The Alexa Fluor 594 labeling kit (Molecular Probes) was used to directly label the affinity-purified NPC1L1 rabbit polyclonal antibody with Alexa 594, enabling it to be used for colocalization of NPC1L1 with other rabbit polyclonal antibody markers.

The MLN64 rabbit polyclonal antibodies were generated against the carboxyl terminus region of MLN64, spanning amino acids 239–444. The antibodies were purified by affinity chromatography using Affi-Gel resin (Bio-Rad) coupled to the MLN64 polypeptide. The anti-Rab9 polyclonal antibodies were generated and purified in a similar manner.

Generation of Anti-NPC1L1 Polyclonal Antibodies—Histidine-tagged fragments of human NPC1L1 (accession number AY513526) amino acids 416–635 and amino acids 1276–1332 were expressed in E. coli.

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‡‡ The abbreviations used are: NPC1L1, Niemann Pick C1-like 1 protein; YFP, yellow fluorescent protein; CFP, cyan fluorescent protein; m, monomeric; SRE, sterol-response element; MOPS, 4-morpholinepropane sulfonic acid; WT, wild type; L1, NPC1L1 knock-out; LDL, low density lipoprotein; HDL, high density lipoprotein; NBD, nucleotide-binding domains; bis-tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxy- methyl)propane-1,3-diol; ES, embryonic stem; LacCer, lactosylceramide; ABC, ATP-binding cassette.
richia coli and purified using nickel affinity chromatography. These proteins were used to immunize two rabbits each, and polyclonal antiserum, MS42 and MS44, respectively, were collected and purified using a Protein A-Sepharose column (Amersham Biosciences). In addition, a peptide antibody against the sequence GCSRFMYHKPLKNSQDYTE (aa 1047–1064), Ben4052, was generated by introducing the above peptide to an Affi-Gel resin according to the manufacturer's recommendations (Pierce).

**Vector Construction—**Monomeric (m) YFP and CFP were generated using eYFP and eCFP plasmids (Clontech) as templates. The L221K and Q69M mutations for mYFP and the L221K mutation in mCFP were created using the megaprimer PCR mutagenesis method (5) and verified by sequencing. To generate mYFP and mCFP fusions with NPC1L1, the stop codon of the human NPC1L1 sequence (GenBank™ accession number AY151256) (1) was removed by PCR amplification, and the resulting cdNA was verified by sequencing and fused to mYFP and mCFP cdNAs. To introduce a FLAG tag into the mYFP fusion, an adapter encoding the FLAG tag amino acid sequence DYKDDDDK was ligated in-frame into the NPC1L1 at the unique BsmI restriction site. The sterol-response element (SRE) luciferase vector, SRE-generate the mYFP-Caveolin-1 fusion. The sterol-response element (SRE) luciferase vector, SRE-Renilla, was generated by introducing a dual SRE element from SRE-GFP (green fluorescent protein) (6) into the phRG synthetic Renilla luciferase vector (Promega).

**Real-time PCR—**Human multiple tissue cdNA panels that had been normalized to four different control genes (β-actin, α-tubulin, G3PDH, phospho-basement membranes) and the manufacturer's instructions. The full-length form of NPC1L1. Real-time PCR amplification was achieved using the Lightcycler 2 (Roche Applied Sciences). Data analysis was carried out using the accompanying software (version 4.0). The primers used for amplifying mouse NPC1L1 were: 5'-GCTTCTTCGAGATGATACACTCCC-3' (forward) and 5'-GCGGATCCTTATATTTCTTTCTGCAAGTTGAT-3' (reverse). The primers used for human NPC1L1 were designed (7). Briefly, cells were washed twice with phosphate-buffered saline, and plasma membrane proteins were biotinylated for 15 min on ice using 1 mg/ml N-hydroxysuccinimide (NHS)-biotin (Pierce). Cells were washed as above to remove unreacted biotin. Half of the dishes were then transfected with 200 μg/ml pCMV-β-gal for 30 min. Cells were washed three times with growth medium containing fetal calf serum to inactivate the trypsin and subsequently lysed in the presence of protease inhibitors as above. Samples were processed for immunoblotting as above and probed either with the Ben4052 anti-NPC1L1 antibody or with streptavidin-HRP (Vector laboratories) to detect biotinylated proteins.

**Western Blot Analysis of NPC1L1—**HepG2 and Caco-2 cells were seeded at 50 × 10^6 cells/well overnight in 4 °C. Remaining samples were incubated at 4 °C for 1 h with 2 mM EDTA, 1% IGEPAL, 0.01% SDS; CD32, 50 mM Tris, pH 7.4, 120 mM NaCl, 25 mM KCl, 0.2% Triton X-100) containing protease inhibitor cocktail, and electrophoresed in a solution of 10% acetic acid, 20% methanol for 10 min and immersed in a solution of 10% acetic acid, 20% methanol for 10 min and immersed in NuPAGE transfer buffer and NPC1L1 was detected prior to electrophoresis as above. The samples were blotted onto nitrocellulose using NuPAGE transfer buffer, and NPC1L1 was detected using the Ben4052 antibody (1 μg/ml) and Lumilight Plus reagent detection (Roche Applied Science).

**Generation of NPC1L1 Knock-out Mice—**The genomic databases for bacterial artificial chromosomes containing the mouse genomic sequence were searched, and one clone that contained the mouse NPC1L1 promoter and entire coding region was identified (RP23-6412P2 from a bacterial artificial chromosome containing the mouse NPC1L1 gene). The gene was disrupted at the unique n-dodecyl-β-maltoside (w/v), 0.12% cholesteryl hemisuccinate ester (w/v), 20 mM HEPES, pH 7.4, 1 mg/100 μg, 5 mM MgCl2, 10% glycerol (v/v), and protease inhibitor mixture (Sigma). Total protein was measured using the Bradford assay (Bio-Rad), and cell extracts containing 40 μg of protein were heated at 70 °C for 10 min in NuPAGE sample buffer prior to electrophoresis as above. The samples were blotted onto nitrocellulose using NuPAGE transfer buffer, and NPC1L1 was detected using the Ben4052 antibody (1 μg/ml) and Lumilight Plus reagent detection (Roche Applied Science).

**Animal Care—**All mice were housed in the Mount Sinai animal care facility according to protocols approved by the Institutional Animal Care and Use Committee. For colony maintenance, the mice were given a regular chow diet (LabDiet rodent diet 20, PMI Nutritional International, Richmond, IN) and water ad libitum. For studying the effects of an atherogenic diet, the Pagien high cholesterol, high fat diet (8) was administered (Research Diets, catalog number D12336) and contained 12.5% cholesterol, 5% sodium cholic acid, and a fat content of 35 kcal%. The matched low fat diet (catalog number D12337) contained 0.3% cholesterol, no cholic acid, and a fat content of 10 kcal%.

**Generation of SV40-immortalized Cell Lines—**Wild-type and NPC1L1 knock-out mice that were 3–6 days old were euthanized in a sterile environment, and liver tissue was removed and minced into 3–4-mm pieces. These were washed in phosphate-buffered saline, transferred to 1 ml of ice-cold 0.25% trypsin/100 mg of tissue, and incubated at 4 °C for 16 h. Cells were dispersed by pipetting and then kept in culture until they began to proliferate. Cells were transfected with the pT7TNeo plasmid as described previously (9). Clones of SV40-transformed cells were picked, and expression of the SV40 antigen was confirmed by immunofluorescence analysis using an anti-SV40 T antigen monoclonal antibody (BD Biosciences).
SRE Dual-Luciferase Reporter and LDL Cholesterol Esterification Assays—The mouse wild-type (WT) and NPC1L1 knock-out (L1) cell lines were grown in 12-well dishes, and each was cotransfected with 1 μg of SRE-Renilla plasmid and 50 ng of pGL3-control luciferase vector (Promega), using 2 μl of Lipofectamine and 5 μl of Plus reagent. At 5 h after transfection, the medium was replaced, and the cells were incubated overnight in the presence or absence of 2 mg/ml U18666A, medium containing LDL (10% fetal calf serum), LDL-deficient medium, or medium containing 10 μg of cholesterol complexed with cyclodextrin. They were then washed with phosphate-buffered saline and processed for analysis using the Dual-Luciferase assay kit as recommended (Promega) to measure SRE promoter induction of Renilla expression. The Renilla values were normalized to the control luciferase activity measurements to compensate for variations in cell state and transfection efficiency between wells. All measurements were performed in triplicate. LDL cholesterol esterification was carried out essentially as described previously (9).

Cholesterol Uptake Assays—Cholesterol uptake was carried out as follows. [3H]Cholesterol was solubilized using cyclodextrin essentially as described (10). Briefly, a mixture containing 110 μl of [3H]cholesterol (52.9mCi/mmol, PerkinElmer Life Sciences), 1 mg of cholesterol, and methyl-β-cyclodextrin solution (methyl-β-cyclodextrin/cholesterol 8:1 mol/mol) was sonicated in a bath sonicator for 15 min prior to an overnight incubation at 37 °C. Confluent cells were incubated with 1 ml of Dulbecco’s modified Eagle’s medium containing 10 μl of solubilized cholesterol at 37 °C for 0–40 min.

NBD Cholesterol and NBD LacCer Uptake—The fluorescent sphingolipid NBD lactosylceramide (LacCer) was obtained complexed to bovine serum albumin (Molecular Probes) and incubated with subconfluent cultures in serum-free media for 5–10 min. The fluorescent probe was removed, and fresh medium containing serum was added. Cells were processed and imaged as above.

Plasma Lipid Assays—For plasma lipid assays, mice were given the high and low cholesterol diets for 14 weeks and then fasted for 16 h. They were euthanized using a lethal dose of the anesthetic Avertin, and total body blood was withdrawn from the inferior vena cava. Four male and four female mice were used for each diet. Plasma levels of glucose, total cholesterol, HDL cholesterol, and triglycerides were determined by the clinical chemistry laboratory at the Mount Sinai School of Medicine.
NPC1L1 mRNA Is Highest in Human Liver—To further our functional studies of NPC1L1, we evaluated the tissue distribution of NPC1L1 mRNA in both human and mouse tissues. In human tissues, NPC1L1 is predominantly expressed in liver with detectable levels in lung, heart, brain, pancreas, and kidney, ranging in expression from about 0.5 to 3% of liver expression (Fig. 1A). Since it has been reported that mouse NPC1L1 is predominantly expressed in the small intestine (4), analyses using a human panel of digestive tract tissues were also carried out. Human NPC1L1 is expressed in the small intestine at 1–4% of the levels expressed in liver (Fig. 1B), suggesting that there are significant differences between the expression of human and mouse NPC1L1. Interestingly, the expression of human NPC1L1 mirrors that of the ABC phytosterol transporter ABCG5 (Fig. 1C). In contrast, mouse NPC1L1 was not highly expressed in liver but was expressed at high levels in the small intestine and was detectable in lung, heart, brain, testis, skin, and liver with significant expression also detected in muscle and the stomach (Fig. 1D). Similar to the human tissues, expression of the ABCG5 transporter in mouse tissues was highest in liver and small intestine (Fig. 1E).

Further support for the expression of NPC1L1 in human tissues was provided by analyzing the expression of this gene in the colorectal adenocarcinoma cell line Caco-2, which displays characteristics of enterocytes, and in the liver-derived hepatocellular carcinoma cell line, HepG2. In agreement with the results of the expression profile, human NPC1L1 is expressed at much higher levels in the liver-derived HepG2 cells than in Caco-2 cells (Fig. 1F). Growth of HepG2 cells in the presence of LDL cholesterol causes a slight increase in the expression of this gene (Fig. 1F).

NPC1L1 Localizes to Internal Membranes and Colocalizes with Rab5—Since the mRNA profiling results above indicated that HepG2 and Caco-2 cells express detectable levels of NPC1L1 mRNA, we initially carried out Western blotting analyses using cellular extracts from these cells. In complete agreement with the mRNA analysis results, HepG2 cells express higher levels of NPC1L1 protein than Caco-2 cells (Fig. 2A, +peptide). Thereafter, since HepG2 cells express significant amounts of NPC1L1 protein, we carried out immunofluorescence analysis of endogenous NPC1L1 in these cells. In complete agreement with the mRNA analysis results, HepG2 cells express higher levels of NPC1L1 protein than Caco-2 cells (Fig. 2A, –peptide), and this signal can be completely blocked in the presence of the immunogen peptide (Fig. 2A, +peptide). Therefore, since HepG2 cells express significant amounts of NPC1L1 protein, we carried out immunofluorescence analysis of endogenous NPC1L1 protein in these cells. Staining with the Bsn4052 antibody revealed a perinuclear, vesicular localization, and this staining was abolished in the presence of the competing peptide immunogen (Fig. 2B and C). Expression of FLAG-tagged NPC1L1 in COS7 cells (Fig. 2D) and NPC1L1 fused to mYFP in Caco-2 cells (Fig. 2E) provided further confirmation of the subcellular location of NPC1L1. As can be clearly seen in the HepG2 endogenous staining and in the FLAG-tagged NPC1L1 in COS7 cells, the NPC1L1 protein...
decorates large vesicular structures to give them a beaded appearance (Fig. 2, B and D). Similar results were obtained using antibodies MS42 and MS44 (data not shown).

To determine the identity of the NPC1L1 subcellular compartment, co-localization studies with various subcellular organelle markers were carried out. Markers for endoplasmic reticulum (calnexin), Golgi (GS15, GS28, GM130, Vti1b, GS27, p230, golgin 97), endosomes (EEA1, Tsf; transferrin, clathrin, Rab5, Rab8, Rab9, Rab11, Min64), and peroxisomes (ABCD3). Strong co-localization was seen with the Bsn4052 antibody and Rab5A and with the MS44 NPC1L1 antibody and Rab5 (Rab5A/MS44). The Bsn4052 antibody was detected with an anti-IgG-Alexa 594 (red) antibody except in panels Tsf and Rab11, where it was labeled with anti-IgG-fluorescein isothiocyanate (green). B, HepG2 cells stained with the Bsn4052 antibody and Rab5A. The addition of the immunogen peptide (+ peptide) eliminates the co-localization signal.

**Fig. 3. NPC1L1 colocalizes with Rab5A in intracellular vesicles.** A, co-localization studies of NPC1L1 and various subcellular organelle markers. HepG2 cells were stained with the Bsn4052 antibody to visualize NPC1L1 and with markers for endoplasmic reticulum (calnexin), Golgi (GS15, GS28, GM130, Vti1b, GS27, p230, golgin 97), endosomes (EEA1, Tsf; transferrin, clathrin, Rab5, Rab8, Rab9, Rab11, Min64), and peroxisomes (ABCD3). Strong co-localization was seen with the Bsn4052 antibody and Rab5A and with the MS44 NPC1L1 antibody and Rab5 (Rab5A/MS44). The Bsn4052 antibody was detected with an anti-IgG-Alexa 594 (red) antibody except in panels Tsf and Rab11, where it was labeled with anti-IgG-fluorescein isothiocyanate (green). B, HepG2 cells stained with the Bsn4052 antibody and Rab5A. The addition of the immunogen peptide (+ peptide) eliminates the co-localization signal.
Since no known human disease resulting from defects in this gene is currently
apparent, we chose to further our studies of NPC1L1 function by
analyzing the role of NPC1L1 in lipid transport. Mouse fibroblasts were isolated from
mice lacking NPC1 (L1) and wild-type (WT) mice and were immortalized by
transfection with the SV40 large T antigen. To characterize the
expression and function of NPC1L1 in these cells, we constructed
expression plasmids containing the SV40 promoter and intron
up-regulating their SRE response when grown in the absence of LDL
cholesterol (L1/LDL); this response is reversed in the presence of LDL cholesterol
(L1+LDL) and in the presence of free cholesterol complexed to
cyclodextrin (L1+CD). p < 0.001. B, both WT and L1 cells are able to
erosify LDL-derived cholesterol.

**NPC1L1 Null Cells Have Multiple Lipid Transport Defects**—To further characterize the role of NPC1L1 in lipid
transport, mouse fibroblasts were isolated from NPC1L1+/− (WT) and NPC1L1−/− (L1) mice and were immortalized by
transfection with the SV40 large T antigen. To characterize the
response of these cells to changing lipid levels, we constructed
expression plasmids containing the SV40 promoter and intron
up-regulating their SRE response when grown in the absence of LDL
cholesterol (L1/LDL); this response is reversed in the presence of LDL cholesterol
(L1+LDL) and in the presence of free cholesterol complexed to
cyclodextrin (L1+CD). p < 0.001. B, both WT and L1 cells are able to
erosify LDL-derived cholesterol.

**Generation of NPC1L1 Null C57BL6 Mice**—Since no known
human disease resulting from defects in this gene is currently
apparent, we chose to further our studies of NPC1L1 function by
generating C57BL6 mice with a disrupted NPC1L1 gene. Chi-
ermic C57BL6 ES cell/BALBc mice were successfully generated and
crossed with C57BL6 females. Homozygous NPC1L1−/− mice were identified by long range PCR amplification to verify
that the neomycin/NPC1L1 gene knock-out cassette was correctly inserted by homologous recombination (Fig. 6A). Mice were routinely screened by PCR to determine their genotype
(Fig. 6B); the resulting NPC1L1−/− mice were found to breed normally and showed no obvious phenotype when compared with their wild-type NPC1L1+/+ counterparts. This was surprising considering that mice lacking NPC1 are generally ster-
ile and exhibit severe neurodegeneration that leads to death at about 2.5 months of age.

**NPC1L1 and Hypercholesterolemia**

**FIG. 5.** A, long range PCR was used to amplify the 5′ and 3′ regions (as indicated) of the NPC1L1 knock-out gene allele, producing a 9- and
5.5-kb product, respectively. Mw, 1 kb Plus DNA marker set (Invitrogen). B, multiplex PCR products from routine genotype analysis of the
NPC1L1 knock-out mice were amplified using one neomycin primer and
two NPC1L1 primers flanking the insertion to amplify the 815- and
601-bp mutant and wild-type alleles, respectively, in homozygous
knock-out (+/−), heterozygote (−/+), and wild-type (+/+). The molecular weight marker is set as in a.

**FIG. 6.** SRE response of SV40-immortalized cultured cells derived from WT and L1 mice. A, L1 cells have a hypersensitive SRE response both in the presence of LDL cholesterol (WT and L1) and when LDL cholesterol egress from endosomes is inhibited with U18666A (WT+U18 and L1+U18). Similarly, L1 cells respond by up-regulating their SRE response when grown in the absence of LDL (L1−/L1); this response is reversed in the presence of LDL cholesterol
(L1+LDL) and in the presence of free cholesterol complexed to
cyclodextrin (L1+CD). p < 0.001. B, both WT and L1 cells are able to
erosify LDL-derived cholesterol.

**FIG. 4.** NPC1L1 does not reside at the plasma membranes. A, a
plasma membrane labeling assay was carried out to confirm the intra-
cellular location of NPC1L1. The amount of NPC1L1 found at the
plasma membrane (PM) is significantly lower than the total amount of NPC1L1 (Total) that can be labeled following cell lysis (Ct; control
immunoprecipitation of NPC1L1). Mw, molecular size marker. B, pro-
tease protection assay of endogenous NPC1L1 in HepG2 cells. Plasma
membrane-resident proteins were biotinylated as described under “Ma-
terials and Methods” prior to treatment with trypsin. Cellular extracts
were immunoblotted and probed for NPC1L1 (left panel) or streptavidin
to visualize biotinylated proteins (right panel).

**FIG. 3.** A, long range PCR was used to amplify the 5′ and 3′ regions (as indicated) of the NPC1L1 knock-out gene allele, producing a 9- and
5.5-kb product, respectively. Mw, 1 kb Plus DNA marker set (Invitrogen). B, multiplex PCR products from routine genotype analysis of the
NPC1L1 knock-out mice were amplified using one neomycin primer and
two NPC1L1 primers flanking the insertion to amplify the 815- and
601-bp mutant and wild-type alleles, respectively, in homozygous
knock-out (+/−), heterozygote (−/+), and wild-type (+/+). The molecular weight marker is set as in a.
SRE response was greatly amplified when sterol transport was inhibited with U18666A. However, the 4-fold induction of SRE response in the presence of U18666A was similar between the WT and L1 cells. To determine whether the L1 cells were more sensitive to a lack of LDL cholesterol from the endosomal/lysosomal system, these cells were transfected with the SRE-luciferase construct in the presence or absence of LDL cholesterol. In the presence of LDL cholesterol, the L1 cells were able to down-regulate their SRE response (Fig. 6A, L1−LDL) when compared with their response in the absence of LDL (Fig. 6A, WT), suggesting that they do not appear to have a block in LDL cholesterol transport. Similarly, they were also able to down-regulate their SRE response in the presence of cholesterol-cyclodextrin (Fig. 6A, L1+CD), albeit not as effectively as in the presence of LDL cholesterol, which may reflect a partial inhibition of plasma membrane-derived cholesterol endocytosis (Fig. 7). Cholesterol esterification assays indicated that there was no statistically significant difference between WT and L1

Fig. 7. Lack of NPC1L1 results in impaired uptake of multiple lipids, as shown from transport studies using WT and L1 mouse cells. A, WT and L1 cells were incubated with [14C]cholesterol complexed with cyclodextrin. *, p < 0.05; **, p < 0.01. L1 cells exhibit a decrease in the uptake of cholesterol (about 30%) when compared with WT cells. *, p < 0.05; **, p < 0.005. Uptake of the fluorescent lipid analogues NBD cholesterol complexed with methyl-β-cyclodextrin (B) and NBD LacCer complexed to bovine serum albumin (C) by WT and L1 cells. Cholesterol and LacCer decorate the plasma membrane of both WT and L1 cells initially; however, at later time points, the two lipids are found predominantly in the Golgi of WT cells in contrast to L1 cells that are unable to process these lipids and retain them in punctate structures throughout the cytosol.
cells in their ability to transport and esterify LDL-derived cholesterol from their endosomal/lysosomal system (Fig. 6B). These results indicate that the higher level of SRE response seen in the L1 cells is not due to an inability to transport endosome-derived LDL cholesterol and may reflect changes in the SREBP processing machinery due to other lipid changes in these cells. This hypothesis is currently under investigation.

To evaluate the extent of this potential transport defect, we next determined whether the absorption and endocytosis of lipids at the plasma membrane was also altered. To assess cholesterol influx rates, radiolabeled cholesterol was incubated with cells for 0–40 min. Both cell lines exhibited saturable uptake, but transport into the L1 cells was reduced by 30% (Fig. 7A). Next, cells were labeled as above with a fluorescent cholesterol analog and chased for various lengths of time. Initially, cholesterol decorates the plasma membrane of both WT and L1 cells in a punctate manner (Fig. 7B). However, by 180 min, in WT cells, NBD cholesterol was localized at a single intracellular site, presumably the Golgi apparatus, whereas in the L1 cells, cholesterol accumulated in multiple intracellular pools (Fig. 7B). It should be noted that transport of NBD cholesterol might not occur in an identical manner to native cholesterol in some systems (12); however, these results support the conclusions derived from the native cholesterol absorption studies shown above (Fig. 7A). Interestingly, incubation with the fluorescent sphingolipid NBD-lactosylceramide indicated that in addition to differences in the transport of cholesterol, L1 cells also have defects in sphingolipid transport. As shown in Fig. 7C, following 15 min of chase, NBD-lactosylceramide localized to the Golgi apparatus of WT cells, and this localization was complete by 40 min (Fig. 7C). However, in L1 cells, NBD-lactosylceramide was trapped in intracellular vesicular structures and did not reach the Golgi complex even after 120 min of chase (Fig. 7C). Intriguingly, this phenotype has recently been described in NPC1-defective cells (13), lending further support to the notion that NPC1 and NPC1L1 may perform similar functions.

Loss of NPC1L1 Affects Caveolin Endocytosis—The differences in lipid endocytosis between WT and L1 cells suggested that the lack of NPC1L1 activity causes a generalized lipid transport block that may involve deregulation of caveolae formation and/or internalization (14). Expression of a mYFP-caveolin construct showed that in WT cells, caveolin localizes in a perinuclear Golgi area and in ring structures located close to the plasma membrane (Fig. 8A, WT). In striking contrast, the caveolin in L1 cells appears to be trapped at the plasma membrane (Fig. 8A, L1), suggesting that lack of NPC1L1 activity causes its aberrant trafficking or mislocalization. To determine whether this increased plasma membrane localization of caveolin in the L1 cells is due to a lack of NPC1L1 activity, these cells were transfected as above with the YFP-caveolin construct and a construct encoding NPC1L1 fused to CFP. L1 cells transfected with NPC1L1 no longer exhibited the plasma membrane caveolin phenotype and appeared similar to the WT cells (Fig. 8B), further suggesting that caveolin mislocalization is affected by a lack of NPC1L1 activity. The inability of L1 cells to endocytose caveolae may partially explain their multiple lipid transport defects. To determine whether NPC1L1 is active in caveolae, we carried out co-localization studies between mYFP-caveolin and NPC1L1-mCFP. No significant co-localization between the two proteins was detected (data not shown), suggesting that the effects seen in L1 cells are not a direct effect of the lack of NPC1L1 activity in caveolae.

NPC1L1 Null Mice Are Protected from Diet-induced Hyperlipidemia—To further evaluate the role of NPC1L1 in lipid transport and/or metabolism, we placed NPC1L1+/+ and NPC1L1−/− mice on a high cholesterol diet for 14 weeks. When serum lipid levels from these mice were evaluated, no significant differences were observed between NPC1L1+/+ and NPC1L1−/− mice on normal low cholesterol diet (Fig. 9). As expected, WT mice on the high fat diet exhibited an increase in total cholesterol and LDL cholesterol and a decrease in their triglycerides, whereas HDL cholesterol levels were similar to those of animals kept on the low fat diet (Fig. 9). However, the NPC1L1−/− mice given a high fat diet showed no elevation in total and LDL cholesterol and in fact showed a significant decrease in total cholesterol (Fig. 9). These animals had a decrease in HDL levels and had similar triglyceride levels to mice kept on the low fat diet (Fig. 9). In addition, NPC1L1−/− mice on the high fat diet had a significant decrease in plasma glucose when compared with NPC1L1+/+ mice, which has a small but significant increase in plasma glucose (assayed following overnight fasting; Fig. 9).

Histochemical analysis of liver tissues from these animals showed that NPC1L1+/+ mice on the high fat diet had larger, fat-laden livers, whereas livers from the knock-out mice were normal but smaller than the WT high fat livers, indicating that these animals resisted the diet-induced fatty liver (Fig. 10A). Liver sections from NPC1L1+/+ and NPC1L1−/− mice confirmed the lipid-laden status of the NPC1L1+/+ livers and the resistance of NPC1L1−/− animals to this diet-induced lipid accumulation (Fig. 10B). Also, gall bladders from WT and NPC1L1−/− mice on the high fat diet were dramatically different with NPC1L1+/+ gall bladder tissues, showing obvious signs of lipid-induced cholestasis that were absent in the NPC1L1−/− mouse (Fig. 10C).

DISCUSSION

In these studies, we have extended our characterization of NPC1L1 function by determining its subcellular location and by generating an NPC1L1 null mouse by homologous recombination. The NPC1L1 protein co-localizes predominantly with the small GTPase Rab5, in contrast to a recent report suggesting that NPC1L1 resides at the plasma membrane (4). Knowledge of the correct NPC1L1 location is critical in providing clues as to the potential function(s) of this protein. Thus, subcellular localization studies, using endogenously expressed NPC1L1 in HepG2 cells, were carried out. The Rab5 co-localization was complete by 40 min (Fig. 7C). Intriguingly, this phenotype has recently been described in NPC1-defective cells (13), lending further support to the notion that NPC1 and NPC1L1 may perform similar functions.

Loss of NPC1L1 Affects Caveolin Endocytosis—The differences in lipid endocytosis between WT and L1 cells suggested that the lack of NPC1L1 activity causes a generalized lipid transport block that may involve deregulation of caveolae formation and/or internalization (14). Expression of a mYFP-caveolin fusion in mouse WT and L1 cells reveals differences in the location of caveolin between the two cell lines. Imaging of live cells revealed that in WT cells, caveolin forms ring structures (15, 16) below the plasma membrane (WT; inset), whereas in L1 cells, caveolin appears trapped at the cell surface (L1; inset). B, expression of NPC1L1 in L1 cells (L1+NPC1L1) corrects the caveolin phenotype. A small amount of caveolin at the plasma membrane can still be seen in the NPC1L1 cells (arrows).
zation of this protein was consistently observed in these studies using a number of different polyclonal anti-NPC1L1 antibodies. To further confirm that NPC1L1 does not reside at the plasma membrane, labeling studies were carried out to quantify the amount of NPC1L1 protein transported to the plasma membrane. The results of these labeling studies indicate that less than 5% of the protein is found at the plasma membrane, most probably representing mislocalized protein. In addition, the lack of NPC1L1 protein at the plasma membrane was confirmed by a protease protection study using endogenous expression of NPC1L1 in HepG2 cells.

The expression profile of human NPC1L1 was similar to the one that we reported previously (1), with the highest expression observed in liver. In contrast, mouse NPC1L1 showed the highest expression in the small intestine with minimal liver expression. It has been suggested that in mouse, NPC1L1 expression is confined to the small intestine, and thus, the function of this protein is to regulate cholesterol absorption at that site (4). Our results, however, suggest that NPC1L1 may have a role in other tissues such as pancreas and kidney and has lower expression in lung and heart. In contrast to the mouse expression pattern, as mentioned above, human NPC1L1 is predominantly expressed in liver, whereas the small intestine expression is only about 2–4% of that found in the liver. Thus, NPC1L1 does not function solely in the small intestine.

Generation of a mouse knock-out of NPC1L1 yielded unexpected results. These mice appeared to be phenotypically normal and did not exhibit any of the symptoms of the NPC1 null mice. By utilizing cell lines established from WT and NPC1L1 null mice, however, our studies have revealed marked defects in the transport of a number of lipids including cholesterol and sphingolipids. Interestingly, the sphingolipid transport phenotype was indistinguishable from that recently described for NPC1-deficient cells and cells from patients with sphingolipidoses (13). Both the uptake of cholesterol and sphingolipids by NPC1L1 null cells appeared to be inhibited at an endosomal step that prevented these lipids from reaching the Golgi apparatus. Intriguingly, a recent study has demonstrated that interaction of cholesterol with sphingolipids such as sphingosine affects intestinal cholesterol absorption (17). To further characterize this defect, we examined the movement of caveolin in these cells. Caveolin internalization is inhibited in the NPC1L1 null cells, suggesting that NPC1L1 may affect lipid transport by interfering with and/or regulating caveolin movement. Such inhibition of caveolin internalization has recently been shown...
to lead to increased cholesterol efflux (18). A mechanism in which efflux rather than uptake is increased by plasma membrane caveolin may explain the reduced intestinal cholesterol absorption observed in the NPC1L1 null mice.

Finally, the NPC1L1 null mice were placed on a high cholesterol diet to determine whether an absence of NPC1L1 has an effect on circulating lipid levels. Surprisingly, the knock-out mice were completely protected from the diet-induced hypercholesterolemia found in WT C57BL6 mice, as determined by their plasma lipid levels, liver, and gall bladder morphology and liver histology. These results suggest that NPC1L1 has a function in the transport of multiple lipids and their homeostasis. Inactivation of this protein clearly has a protective effect against diet-induced hypercholesterolemia in these animals.
and suggests that NPC1L1 plays a critical role in regulating lipid metabolism. However, the paradoxical observations of aberrant multiple lipid transport and caveolin mislocalization caused by a lack of NPC1L1 activity require further study. Finally, as discussed above, there are significant differences in the mRNA tissue distribution between mouse and man, and thus, any conclusion regarding the exact role of NPC1L1 in lipid metabolism and transport should be appropriately interpreted and will also require further characterization.

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