Materials and Methods

Materials. All cell culture media, reagents, buffers, restriction enzymes, modifying enzymes, synthetic oligomers, Platinum Quantitative PCR SuperMix-UDG (Cat No 11730-017), Ultramax-DH5α competent Escherichia coli (E. coli), and NuPAGE Novex, were purchased from Invitrogen Life Sciences (Carlsbad, CA) unless otherwise noted. All solvents, chemicals, bovine serum albumin (cat # 6003) and cholesterol (cat # C3045) were purchased from Sigma-Aldrich Co. (St. Louis, MO). The digoxigenin (DIG) RNA Labeling Kit (cat # 1175025) and DIG Nucleic Acid Detection Kit (cat # 1175041) were purchased from Roche Molecular Biochemicals (Germany). Streptavidin-biotin-peroxidase immunostaining was carried out using Histostain-SP kit (cat # 95-6143) from Zymed Laboratories (Burlingame, CA). ^14C-cholesterol, ([4-14C]-cholesterol, 45-60 mCi/ mmol) (cat # NEC018) and ^14C-triglyceride (carboxyl-^14C -triolen, 100 mCi/ mmol) (cat # NEC 674) were purchased from New England Nuclear (Boston, MA). Tritiated sitostanol ( [5,6-^3H] 50 Ci/ mmol) (cat # ART361) was purchased from American Radiolabeled Chemicals Inc. (St. Louis, MO). Additional items purchased: Tri-Reagent from Molecular Research Center, Inc. (Cincinnati, OH); ECL Plus (cat # RPN2132) from Amersham Biosciences (England), Coomassie
Plus Protein assay (cat # 1856210) from Pierce, (Rockford, IL), and O.C.T. from Miles Scientific (Elkhart, IN),

**Animals.** All animals were housed, treated, and cared for according to NIH guidelines for the humane treatment of laboratory animals and the Animal Welfare Act in a program accredited by the American Association for Accreditation of Laboratory Animal Care under a protocol approved by the Schering-Plough Research Institute’s Animal Care and Use Committee. Animals were kept under controlled conditions of humidity, light, and temperature. Male Sprague-Dawley rats (~250-300 g) (Charles River Laboratories, MA) were utilized for tissue isolation procedures. Mouse strain C57Bl6 were used in Taqman analysis for NPC1L1 tissue distribution. NPC1L1 (-/-) mice were generated by Deltagen Inc. (Palo Alto, CA) using a NPC1L1 targeting vector consisting of a LacZ-Neo encoding cassette flanked by ~1.9 kb of 5’- and 3.2 kb of 3’- mouse genomic DNA sequence resulting in the deleted sequence from base 790 to 998 of the mouse NPC1L1 sequence (Accession # AK078947). NPC1L1 (-/-) mice were generated using 129/OlaHsd ES cells injected into C57BL/6 blastocysts to generate chimeric mice. The colony was established and maintained at Schering-Plough Research Institute.

**Antibodies**

Rabbit polyclonal antisera used for immunoblotting and western analysis were generated against KLH coupled rat NPC1L1 specific peptides as follows:
A0715 (AA #1007-1025), EQFHKYLPWFLNDPPNIRC-(KLH); A0868 (AA #511-525), GQTSLVDWKDHFLYCY-(KLH) and A1801 (AA #525-541), (KLH)-CANAPLTFKDGTALALS (ResGen Inc., Huntsville, AL). Anti-tubulin mouse monoclonal ascites fluid B-5-1-2 (cat # T5168) was purchased from Sigma-Aldrich (St Louis, MO)

Recombinant DNA and sequencing protocols.

The *E. coli* strain DH5α was used as host strain for the propagation and maintenance of plasmid DNA. Plasmid pCR2.1 was used for cloning of polymerase chain reaction (PCR) products. All recombinant DNA experiments were conducted using standard procedures (1). Sequencing was performed using Big Dye Terminator RR Mix on the ABI 373XL automated sequencer (Perkin-Elmer, Applied Biosystems Division; Foster City, CA).

Gene expression profile in humans

Targeted gene expression profile for human NPC1L1 was conducted using the Affymetrix HG-U95 by Gene Logic Inc. (Gaithersburg, MD). A proprietary biorepository of 2045 normal human samples representing 36 common tissues were profiled. Messenger RNA levels are determined using “spike-in” standard curve generation and normalized using the Affymetrix MAS 4.0 or MAS 5.0 algorithms. Expression levels are reported as the median of the range of expression value for all samples defining the tissue set. The tissue
(small intestine) with the most abundant signal was set to a normalized value of 100 arbitrary units and lesser values for other tissues reported as a fraction.

**Tissue localization of rat NPC1L1 mRNA by *in situ* hybridization.**

DNA fragments corresponding to rNPC1L1 nucleotides 3318 to 3672 (Genbank #AY437867) were generated by PCR. The fragments were sub-cloned into plasmid pCR 2.1 in both orientations. Sense and anti-sense digoxigenin–UTP cRNA probes were generated from the T7 promoter on linearized plasmid using the DIG RNA Labeling Kit (Roche Molecular Biochemicals). Tissues were stored at –70 \(^\circ\)C until sectioned. Serial cryosections (10 µm) of fresh frozen rat jejunum were prepared and mounted on glass slides. Tissues were fixed in a solution of 4% formaldehyde in PBS for 20 min and washed thoroughly in PBS. Tissue sections were partially digested with proteinase K (5 µg/ ml) for 15 min at room temperature and then treated with 0.2 N HCl. After acetylation with 0.25% acetic anhydride in triethanolamine buffer (pH 8.0), sections were hybridized with the probes overnight at 55 \(^\circ\)C. Post hybridization washes were performed with 2X SSC at 50 \(^\circ\)C and included a 30 min incubation with 10 µg/ ml RNase at 37 \(^\circ\)C. Digoxigenin labeling was detected with the DIG Nucleic Acid Detection Kit (Roche Molecular Biochemicals). A positive signal is characterized by the deposition of a brown-red reaction product at the site of hybridization.
RT-PCR

Analysis of gene specific message expression was assessed by RT-PCR. Reverse transcription of mRNA was performed using SuperScript First Strand Synthesis System (Invitrogen) and PCR was performed using ThermalAce DNA polymerase (Invitrogen). Briefly, 600 ng of total RNA from each tissue sample was used with 0.5µg Oligo-(dT)12-18 primers in a 20 µL reaction system. The 2 µL of 10X RT buffer, 1 µL of 10 mM dNTP mix, 4 µL of MgCl, 2 µL of 0.1 M DTT and 1 µL of RNase inhibitor were also mixed in the system. After 5 min pre-incubation at 42 °C, 50 unit of Superscript II reverse transcriptase was added and the mixture was incubated at 42 °C for 75 min. The RT reaction was ended by heating the mixture at 70 °C for 15 min, chilled and stored at -30 °C until use.

Mouse NPC1L1 (Genbank #AY437866)
forward 5’-TCTGTGGAGTTGCCTGGTCCCACATTA
reverse 5’-CATAGTCCTGTTGTTGCATCAG

Real-Time Quantitative PCR Analysis

Real-time quantitative PCR analysis was performed using Platinum Quantitative PCR SuperMix –UDG (Invitrogen) on an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) Tissue expression of NPC1L1 in the rat was quantified and normalized against a control reaction for rat 18S RNA. Two independent reverse transcriptase reactions were each analyzed twice. Each analysis performed in triplicate included both 18S RNA
and the gene of interest primer/probe pairs as multiplexed samples. Briefly, total RNA (600 µg) from each tissue sample was used with 0.5 µg oligo-(dT) 12-18 primers and 2.5 µM rat 18S RNA specific primer 5’-GAGCTGGAATTACCGCGGCT in a 20 µl reverse transcriptase reaction to generate first strand cDNA (2). PCR reactions were run in 96-well format with 25 µl reaction mixture in each well containing: Platinum SuperMix (12.5 µl), 6-carboxyrhodamine-NHS-ester (ROX™) Reference Dye (0.5 µl), 50 mM magnesium chloride (2 µl), cDNA from RT reaction (0.2 µl) Multiplex reactions contained 18S primers at 200 nM each and 6-carboxyfluorescein (6FAM™)/carboxytetramethylrhodamine (TAMRA™) labeled probe at 100 nM and gene specific primers at 100 nM each and VIC™/TAMRA™ labeled probe at 50 nM. Reactions were run with a standard 2-step cycling program, 95 °C for 15 sec and 60 °C for 1 min, for 40 cycles. Each tissue was run in triplicate and the results were shown by Mean with SE.

18S ribosomal RNA (Genbank # X01117)
forward 5’-GGGAGGTTAGTGA CGAAAAATAACCAT
reverse 5’-TTGCCCTCCAATGGATCCT
probe 5’-[VIC]CGAGGCCCTGTAATTGGAATGAGTCCACTT[TAMRA]
rat villin (IPI00208394)
forward 5’-AGCACCTGTCCACTGAAGATTTC
reverse 5’-TGGACGCTGAGCTTCAGTTCT
probe 5’-[VIC]CTTCTCTGCGCTGCCTCGATGGAA[TAMRA]
rat NPC1L1 (Genbank #AY437867)
forward 5’-TGATACTGTTGCTGGCGATAGG
reverse 5’-GGGTCCCACTTTGT TGACATATAGA
probe 5’-[6FAM]TATGAATATA CAGCGTTT GATTTTGCCAAGCATC[TAMRA-6FAM]
mouse NPC1L1 (Genbank #AY437866)
forward 5’-ATCCTCATCCT GGGCTTTG
reverse 5’-GCAAGGTGATCAGGAGGTTGA
probe 5’-[6FAM]CCCAGCTTATCC AGATTTTTTCTTCTTCCGC[TAMRA-6FAM]
mouse ABCG5 (Genbank # AF312713)
forward 5’-TCTCCGCGTCCAGAA ACAAC
reverse 5’-CATTGAGC ATGCCCGGTGTAT
probe 5’-[6FAM]CGCTAAAGGGCGCTGTG CAGAC[TAMRA-6FAM]
mouse ABCG8 (Genbank # NM026180)
forward 5’-CCCTCCGAT TGCTTCTTTCAG
reverse 5’-CTGAGAAATGCCCCCAGATA AAA
probe 5’-[6FAM]CCCGCTGGCTTTATAGGG TAGCT[TAMRA-6FAM]
mouse HMG-CoA synthase (Genbank # BC023851)
forward 5’-CCCTCCCTGGGAG ATAGTG
reverse 5’-CCCGTGAA TTGAAGCTGTCA
probe 5’-[6FAM]AGGCATTATTAGGT TAGTTACAGATCC[TAMRA-6FAM]

**Immunohistochemistry and Western Analysis**

Rat small intestine (jejunum) was removed, immediately embedded in O.C.T. compound (Miles Scientific) and frozen in liquid nitrogen. Tissue blocks
were stored at -70 °C until use. Cryostat microtome sections, ~6 µm, were prepared at -20 °C and mounted on glass slides, air dried at room temperature and fixed in Boulin’s fixative. Streptavidin-biotin-peroxidase immunostaining was carried out using Histostain-SP kit (Zymed Laboratories). Endogenous peroxidase activity was blocked with a 10 min incubation in 3% H₂O₂ in methanol, and nonspecific antibody binding was minimized by a 45 min incubation in 10% normal goat serum. Sections were incubated with a rabbit anti-NPC1L1 antiserum A0715 at 1:500 dilution at 4 °C overnight; they were then successively incubated with biotinylated goat anti-rabbit IgG and with streptavidin-peroxidase. Thereafter, the sections were developed in an aminoethyl carbazole (AEC)- H₂O₂ staining system and counterstained with hematoxylin. All slides were mounted and examined with a Zeiss Axioskop2 photomicroscope. A positive reaction using this protocol is characterized by the deposition of a red reaction product at the site of the antigen-antibody reaction. Nuclei appeared blue from the hematoxylin counterstain. Controls were performed simultaneously on the neighboring sections from the same tissue block. Control procedures consisted of the following: (1) substitute the primary antibody with the pre-immune serum, (2) substitute the primary antibody with the non-immune rabbit serum (data not shown), (3) substitute the primary antibody with PBS (data not shown), (4) substitute the second antibody with PBS (data not shown).

For NPC1L1 immunoblot analysis, intestinal enterocytes were prepared from NPC1L1 (-/-), (-/+), and (+/+) mice (3), and resuspended in 10 mM Tris pH
7.5, 150 mM NaCl, 5 mM EDTA and 1% Triton X-100 (TNET). Enterocytes were then lysed by incubation on ice for 60 min. followed by centrifugation at high speed in a micro-centrifuge at 4 °C for 15 min and the soluble supernatant was removed and saved. The supernatants were assayed for protein concentration using Coomassie Plus Protein assay (Pierce). The supernatants were then mixed with 4x LDS/DTT NuPage sample buffer to a final concentration of 1x and heated at 95-100 °C for 10 min. Equal quantities of protein from each sample (duplicate lanes) were then separated by SDS-PAGE using a 7% Tris-acetate NuPage Novex pre-cast gel (Invitrogen), blotted onto Invitrolon PVDF (Invitrogen) and probed with rabbit polyclonal antisera A0868 at a concentration of 1/2000 in TBS containing 5% milk (w/v) and 0.1% tween-20 (v/v). The same blot was also probed with anti-tubulin mouse monoclonal ascites fluid B-5-1-2 (Sigma-Aldrich) at a concentration of 1/20000 to blot for tubulin as a protein loading control. Immunoblots were developed using ECL Plus (Amersham).

**Cholesterol and Triglyceride Absorption and Levels.**

Cholesterol absorption and the activity of ezetimibe were determined in the NPC1L1 (-/-), (+/-) and (+/+) age matched control mice by dual fecal isotope ratio method as previously described (4). Mice (n= 4-5/group) were fed a standard chow diet or a diet containing 0.1% sodium cholate, and treated with ezetimibe (10 mg/kg) or corn oil vehicle daily (Research Diets Inc., New Brunswick, NJ). Mice were gavaged with $^{14}$C-cholesterol (1 µCi, 0.1 mg unlabeled cholesterol) and $^{3}$H-sitostanol (2 µCi) in 0.1 ml corn oil. Feces were
collected daily for 3 days and fecal $^{14}$C-cholesterol and $^3$H-sitostanol levels were determined by combustion in a Packard Oxidizer.

Acute cholesterol and triglyceride uptake and absorption were determined in NPC1L1 (+/+) and (-/-) age-matched mice (4). Mice (n = 4-5/group) were gavaged with $^{14}$C-cholesterol, 1 µCi with 0.1 mg cold cholesterol or [carboxy-$^{14}$C]-triolein in 0.1 ml corn oil. Two hours later, blood, livers, small intestines, and intestinal saline rinses were analyzed by liquid scintillation counting.

Total plasma cholesterol and triglyceride levels were determined using the Wako Cholesterol CII enzymatic colorimetric method. (Wako Chemicals USA, Inc., Richmond, VA) and the Sigma Diagnostics GPO-Trinder reagents with Procedure No. 337, respectively. Liver free cholesterol, cholesteryl ester, and triglyceride levels were determined by HPLC essentially as described (4).
Fig. S1  Sequence alignment of predicted NPC1L1 protein sequences. Human (Genbank # AY437865), mouse (Genbank # AY437866), and rat (Genbank # AY437867) were aligned using the Cluster V algorithm (5). The putative sterol-sensing domain is boxed. Abbreviations for the amino acid residues are as follow: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Iso; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; Y, Tyr.
Fig. S2  Relative mRNA levels in small intestine and livers of chow fed mice.

Jejunum and livers were harvested from NPC1L1 (-/-) (white bars), (+/-) (hatched bars), and age-matched (+/+ ) mice (n=4/ group). Total RNA was isolated and equal amounts of RNA from individual mice were assessed for *HMG-CoA synthase*, *ABCG5* and *ABCG8* mRNA expression by real-time quantitative PCR analysis. Values are mean ±SEM.
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<th>(+/+ )</th>
<th>(+/- )</th>
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<tr>
<td><strong>Plasma (mg/dl)</strong></td>
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<td>Cholesterol</td>
<td>82 ± 4</td>
<td>81 ± 9</td>
<td>87 ± 6</td>
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<td>Triglyceride</td>
<td>110 ± 20</td>
<td>110 ± 12</td>
<td>84 ± 12</td>
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<td><strong>Liver (mg/g)</strong></td>
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<td>Cholesteryl ester</td>
<td>2.81 ± 0.29</td>
<td>2.18 ± 0.14</td>
<td>1.85 ± 0.15*</td>
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<tr>
<td>Free Cholesterol</td>
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<td>1.57 ± 0.06</td>
<td>1.52 ± 0.02</td>
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<td>Triglyceride</td>
<td>48.5 ± 8.2</td>
<td>41.6 ± 8.9</td>
<td>55.0 ± 16.3</td>
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<td><strong>Triglyceride Absorption</strong></td>
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<td>Plasma + Liver (%^{14}C dose)</td>
<td>1.7 ± 0.23</td>
<td>-</td>
<td>1.55 ± 0.16</td>
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**Table S1.** Plasma and liver lipid levels in male NPC1L1 (+/+), NPC1L1 (+/-), NPC1L1 (-/-) mice and ^{14}C triglyceride absorption in NPC1L1 (+/+ ) and (-/-) mice (n = 4-5/group, Means ± SEM, * p<0.05).

**References:**


