Supporting Information

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SI Materials and Methods

Animals, Materials, Primary Antibodies, and Plasmids. Animal protocols were approved by the institutional animal care and use committee of the Shanghai Institutes for Biological Sciences (Chinese Academy of Sciences). Experiments were performed at age matched 8-wk-old imprinting control region or C57/B6 strain male mice (five mice for each experimental group) housed in a specific pathogen-free animal facility in plastic cages in a temperature-controlled room (22 °C) with a 12-h light, 12-h dark cycle. The mice were fed ad libitum a cereal-based rodent chow diet.

As for materials, we obtained horseradish peroxidase-conjugated donkey anti-mouse and anti-rabbit IgG from Jackson ImmunoResearch Laboratories; filipin, FITC, or peroxidase-labeled Cholera Toxin, subunit B (CTxB) (to stain GM1) from Sigma; Alexa fluor® 555 donkey anti-mouse IgG and iron-loaded transferrin-rhodamine conjugate from Invitrogen; sulfosuccinimidyl 6-(biotinamido) hexanoate and NeutrAvidin-agarose from Pierce; proaerolysin (to stain GPI-anchored protein) from Protox Biotech; methyl-β-cyclodextrin (CDX) from Cyclodextrin Technologies Development, Inc.; cholesterol from Steraloids, Inc.; and other reagents from previously described sources (1). Lipoprotein-deficient serum (LPDS) ($d > 1.215$ g/mL) was prepared from newborn calf serum by ultracentrifugation (2).

Antibodies against EGFP, Niemann-Pick C1-like 1 (NPC1L1), transferrin receptor, clathrin heavy chain (CHC), and AP-2 complex subunit (μ2) are described as before (3). Mouse monoclonal anti-flotillin-1 and anti-flotillin-2 and anti-GM130 antibodies are purchased from BD Transduction Laboritories™. Mouse monoclonal anti-FLAG and rabbit polyclonal anti-calnexin antibodies are purchased from Sigma, respectively. Rabbit polyclonal anti-cathepsin D, mouse monoclonal anti-prohibitin, rabbit polyclonal anti-PMP70, and mouse polyclonal anti-C-AMP Response Element Binding antibodies, are purchased from Santa Cruz, Calbiochem, and ZYMED Laboratories, respectively.

The coding regions of human flotillin-1, flotillin-2, stomatin, or caveolin-1 were amplified from human liver cDNA by standard PCR and cloned into vectors with indicated tags described in the text.

RNA Interference. Duplexes of siRNA were synthesized by Genepharma. The siRNA targeting rat CHC was described before (3). The sequences of siRNAs targeting rat flotillin-1 were 5′-ggatctctctcaacacact-3′, 5′-ggagatctataaggacaga-3′, and 5′-ggccaagcaggagaaggta-3′; rat flotillin-2 were 5′-ccatcaaggatgtctatga-3′; human flotillin-1 were 5′-gagaagtcccaactaatta-3′and 5′-gagattacgaactgaagaa-3′; human flotillin-2 were 5′-ccatcaaggacgtgtatga-3′ and 5′-ccaagattgctgactctaa-3′; human NPC1L1 was 5′-gctacaaggtatgactctct-3′. The siRNA against vesicular stomatitis virus G was described before and used as a control (4). Transfection of siRNAs was carried out as previously described (4).

Cell Culture. CRL-1601 (McArdle RH7777 rat hepatoma cell), CRL1601–NPC1L1-EGFP, CRL1601–NPC1L1-myc, and L02 (a human liver cell line) cells were grown in monolayer at 37 °C in 5% CO2. The cells were maintained in medium A (DMEM containing 100 units/mL penicillin and 100 μg/mL streptomycin sulfate) supplemented with 10% FBS. For CRL1601–NPC1L1-EGFP and CRL1601–NPC1L1-myc cells, 200 μg/mL G418 was supplemented. Cholesterol-depleting medium is medium A supplemented with 5% LPDS, 10 μM compactin, 50 μM mevalonate, and 1.5% CDX. Cholesterol-replenishing medium contains medium A supplemented with 5% LPDS, 10 μM compactin, 50 μM mevalonate, and 15 μg/mL cholesterol/CDX. The cholesterol/CDX inclusion complexes were prepared as described before (5).

Cell Surface Biotinylation Assay. Surface biotinylation assay was performed as described previously (3). In brief, the cells were washed twice with ice-cold PBS and incubated with 1 mg/mL sulfosuccinimidyl 6-(biotinamido) hexanoate in PBS for 40 min at 4 °C. Then the cells were washed twice with buffer A [20 mM Tris • HCl (pH 8.0) and 150 mM NaCl] with incubation for 15 min in the second wash. The cells were collected by scraping and lysed in buffer B [10 mM Tris • HCl (pH 8.0), 150 mM NaCl, and 1% NP-40]. The cell lysates were incubated with NeutrAvidin-agarose and rotated overnight at 4 °C. The agarose beads were then washed three times with buffer B. Biotinylated proteins were eluted with SDS-PAGE sample buffer and analyzed by SDS-PAGE followed by immunoblot.

Fluorescence Quantification. For quantification of relative intracellular NPC1L1, flotillin-1 and flotillin-2, or cholesterol signal, the detailed procedure was described previously (3). In brief, the selected cell was magnified and encircled by multiangle AOI (area of interest) and the indicated fluorescence signal was quantified by Image-Pro Plus 5.02 as total. Then another multiangle AOI was drawn immediately adjacent to the plasma membrane within the same cell and the indicated fluorescence signal was quantified as internal signal. The relative intracellular localization was calculated with the formula: internal-background/total-background.

Overlap coefficient was also calculated using Image-Pro Plus 5.02. In brief, all images were taken with equal acquisition parameters and single cell in each merged image was selected and background was subtracted. The overlap coefficient (R) between green and red signals was calculated automatically by the "colocalization" tool in the software. About 50–100 cells from each experimental group were quantified in each experiment and the data are representative of three experiments.

Transfection, Immunoprecipitation, and Immunoblot Analysis. Transfection of cells with Fugene HD (Roche) was performed according to the manufacture's manual. Conditions of drug incubations were described in figure legends. Triplicate dishes of cells for each treatment were harvested and lysed in immunoprecipitation (IP) buffer (PBS, 5 mM EDTA, 5 mM EGTA, 1% digitonin) plus protease inhibitors. Whole-cell lysate was incubated with anti-EGFP antibody coupled agarose and rotated at 4 °C for 2 h. The agarose beads were then washed five times with IP buffer. Proteins binding to the beads were eluted by elution buffer (0.2 M glycine/HAc, pH 2.9) and proteins were precipitated by acetone at −70 °C overnight. The precipitates were incubated in SDS-PAGE loading buffer at 37 °C for 30 min. Immunoblot (IB) analysis was carried out as previously described (6).

Fluorescence Recovery After Photobleaching (FRAP) Analysis. The cells were grown on 35-mm glass bottom microwell dishes (Mat Tek) and cotransfected with indicated plasmids. After transfection (48 h), the cells were depleted of cholesterol by incubating in the cholesterol-depleting medium. For microscopy, the cells were maintained in phenol-free medium F-15 (GIBCO) plus 5% LPDS and 10 μM compactin. The confocal microscope used in the FRAP experiments was a Leica True Confocal Scanner (TCS) SP5 equipped with a temp-control 37 °C stage. FRAP was performed with "FRAP wizard" program in Leica TCS SP5 system. Bleaching of outlined regions of interest was performed at 37 °C

with high-intensity illumination (100% transmittance); recovery was observed at low-intensity illumination (5–10% transmittance) to minimize undesired photobleach. Leica confocal software was used to measure pixel intensity in the regions of interest. The recovery values are the percentage of prebleach values. More than 20 cells from each experimental group were quantified in each experiment and the data are representative of three experiments.

Plasma-Endocytic Membrane Purification. The experiments were performed as described previously (7). In brief, the cells was homogenized in buffer C containing 20 mM Hepes (pH 7.5), 250 mM sucrose, 15 mM KCl, 1 mM EDTA, and 2 mM $MgCl_2$ and protease inhibitors. Then nucleus and debris were removed by spin down at $1,000 \times g$ for 5 min. Membranes were collected by high speed ($100,000 \times g$) centrifugation for 30 min. The membranes were resuspended in buffer C and layered on buffer D containing 20 mM Hepes (pH 7.5), 1.12 M sucrose, 15 mM KCl, 1 mM EDTA, and 2 mM $MgCl_2$ and protease inhibitors. Centrifugation ($100,000 \times g$) was performed for 2 h and the opaque layer (plasma-endocytic membrane) between the boundary of buffers C and D was collected. Then the purity of the plasma-endocytic membrane was checked by IB with different markers followed by sucrose gradient analysis of indicated proteins and lipids.

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2. Goldstein J-L, Basu S-K, Brown M-S (1983) Receptor-mediated endocytosis of low-density lipoprotein in cultured cells. *Methods Enzymol* 98:241–260.
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4. Song B-L, Sever N, Bose-Boyd R-A (2005) Gp78, a membrane-anchored ubiquitin ligase, associates with Insig-1 and couples sterol-regulated ubiquitination to degradation of HMG CoA reductase. *Mol Cell* 19:829–840.
5. Brown A-J, Sun L, Feramisco J-D, Brown M-S, Goldstein J-L (2002) Cholesterol addition to ER membranes alters conformation of SCAP, the SREBP escort protein that regulates cholesterol metabolism. *Mol Cell* 10:237–245.
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7. Yuan T, Hong S, Yao Y, Liao, K (2007) Glut-4 is translocated to both caveolae and non-caveolar lipid rafts, but is partially internalized through caveolae in insulin-stimulated adipocytes. *Cell Res* 17:772–782.

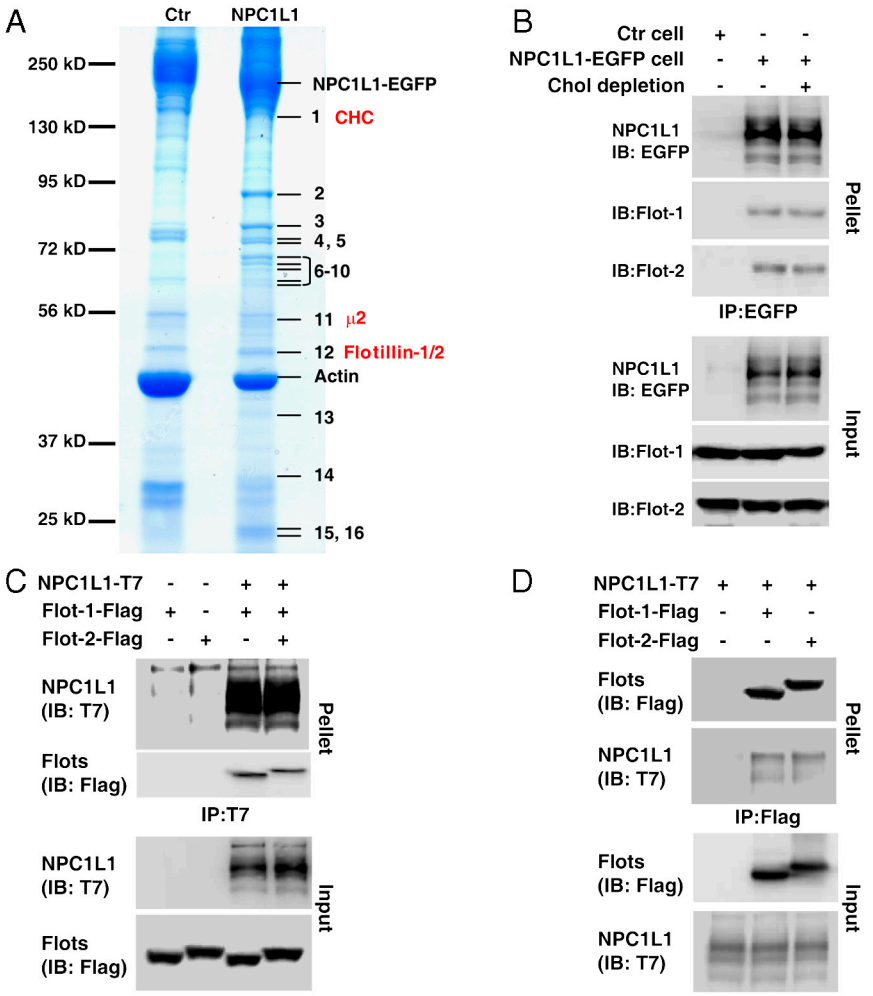


Fig. S1. NPC1L1 associates with flotillins. (*A*) Purification of the NPC1L1 protein complex. Immunoprecipitation with anti-EGFP-coupled agarose from CRL1601–NPC1L1-EGFP or control CRL1601 cells was performed as described in *SI Materials and Methods*. The eluted proteins were subjected to SDS-PAGE and stained by Colloidal blue. Protein bands (labeled 1–16) distinguished from control lane were identified by tandem mass spectrometry analysis. (*B*) The NPC1L1-flotillins complex forms constitutively during cholesterol depletion. CRL1601–NPC1L1-EGFP cells were either untreated or depleted of cholesterol for 60 min. Then coimmunoprecipitation (co-IP) was performed with anti-EGFP-coupled agarose to pull down NPC1L1-EGFP. IB showed coprecipitation of endogenous flotillins with NPC1L1-EGFP. (*C* and *D*) Reciprocal co-IP between NPC1L1 and flotillins. (*C*) NPC1L1-T7 and flotillins-FLAG were transiently coexpressed in CRL1601 cells. After transfection (48 h), co-IP was performed with anti-T7 beads to pull down NPC1L1-T7. IB showed coprecipitation of flotillins-FLAG with NPC1L1-T7. (*D*) NPC1L1-T7 and flotillins-FLAG were coexpressed in CRL1601 cells. After transfection (48 h), co-IP was performed with anti-FLAG beads to pull down flotillins-FLAG. IB showed coprecipitation of NPC1L1-T7 with flotillins-FLAG. Ctr, control; μ2, AP2 complex subunit μ2; Flot, flotillin.

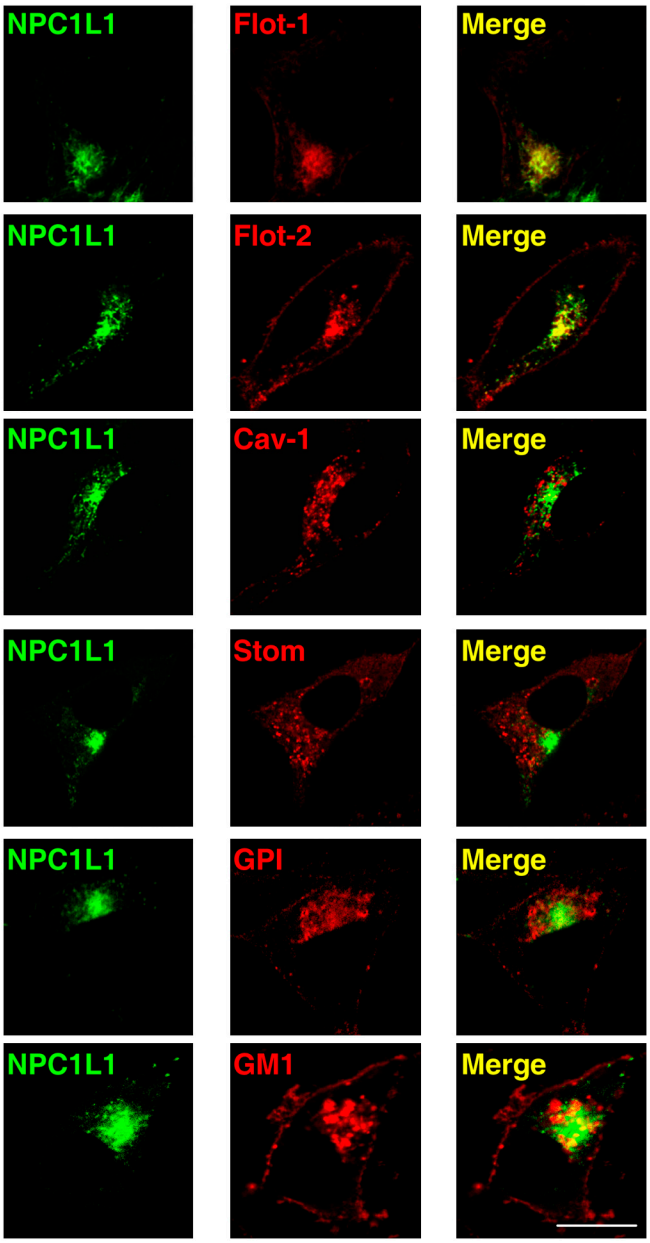


Fig. S2. NPC1L1 colocalizes with flotillin-1 and flotillin-2. NPC1L1-EGFP and red fluorescent protein tagged (row 1–3) or FLAG-tagged (row 4) membrane microdomain scaffold proteins indicated were coexpressed in CRL1601 cells, and were detected by fluorescence. The cells shown in rows 5 and 6 were transfected with NPC1L1-EGFP alone followed by staining with indicated endogenous membrane microdomain makers. Flot, flotillin; Stom, stomatin; Cav-1, caveolin-1; GPI, GPI-anchored protein; GM1, ganglioside GM1. (Scale bar: 10 μm.)

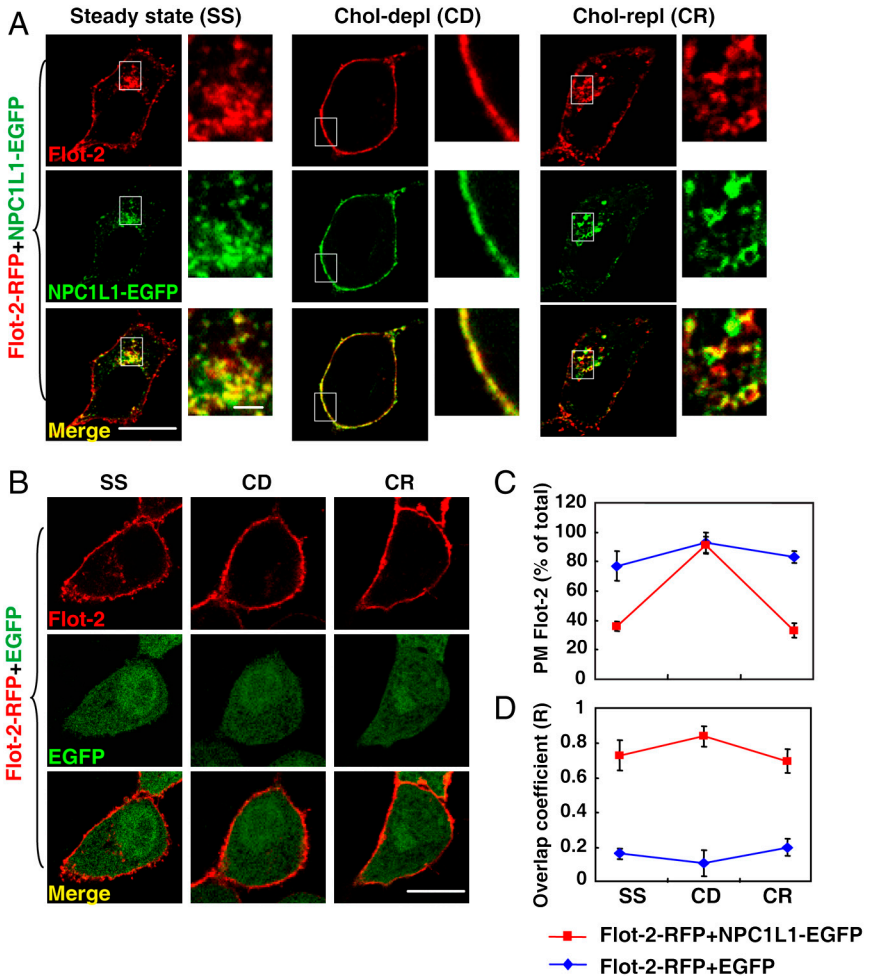


Fig. S3. NPC1L1 promotes the endocytic recycling of flotillin-2 (Flot-2). (*A* and *B*) Flotillin-2-red fluorescent protein (RFP) was coexpressed with NPC1L1-EGFP (*A*) or EGFP (*B*) in CRL1601 cells. After transfection (48 h), the cells were depleted of cholesterol for 60 min and then replenished with cholesterol for 60 min as described in Fig. 1*A*. At different time points, i.e., steady state (SS), cholesterol depletion (CD), and cholesterol replenishment (CR), the cells were fixed and imaged. [Scale bar: 10 μm or 1 μm (magnified image).] (*C* and *D*) The plasma membrane (PM) localized flotillin-2 (*C*) or the overlap (*D*) coefficient between NPC1L1-EGFP and flotillin-2-RFP shown in *A* and *B* was quantified. Error bars represent standard deviations ($n \geq 50$).

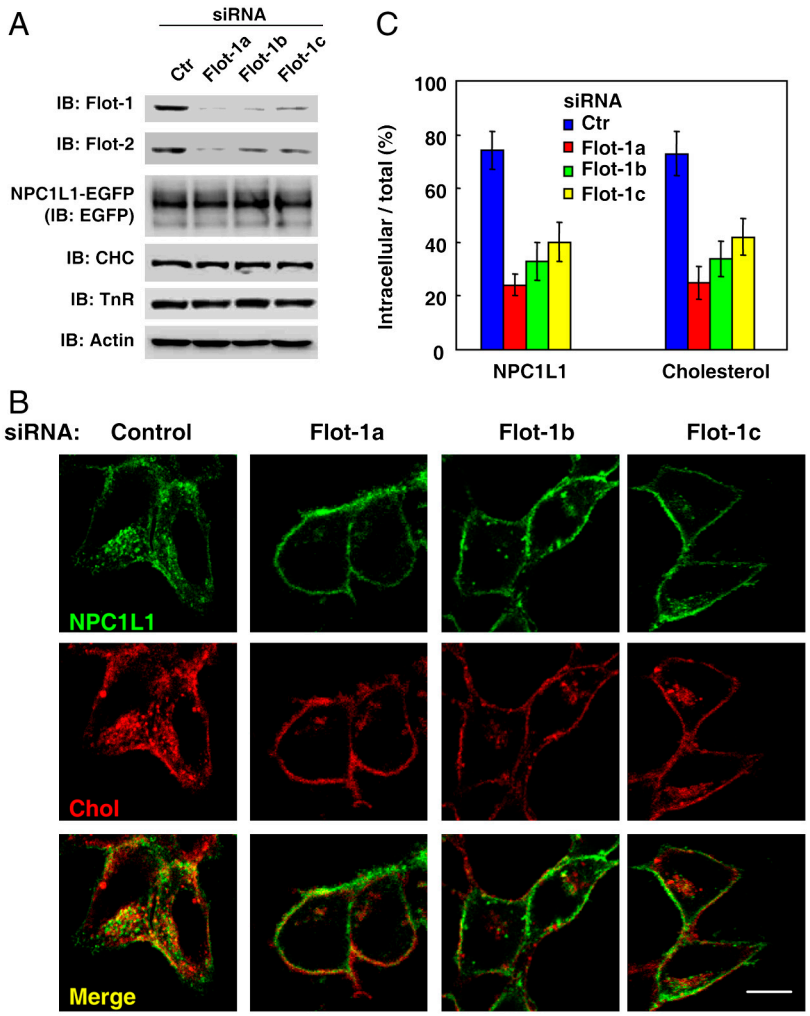


Fig. S4. Knockdown of flotillin-1 impairs NPC1L1-mediated cholesterol uptake. CRL1601–NPC1L1-EGFP cells were transfected with different siRNAs targeting flotillin-1. After transfection (96 h), the cells were either harvested for IB (*A*) or replenished with cholesterol for 60 min after cholesterol depletion followed by fixation and filipin staining (*B*). (Scale bar: 10 μm.) The intracellular localized NPC1L1 or cholesterol was quantified (*C*). Error bars represent standard deviations ($n \geq 100$). Ctr, control; Flot-1, flotillin-1; TnR, transferrin receptor.

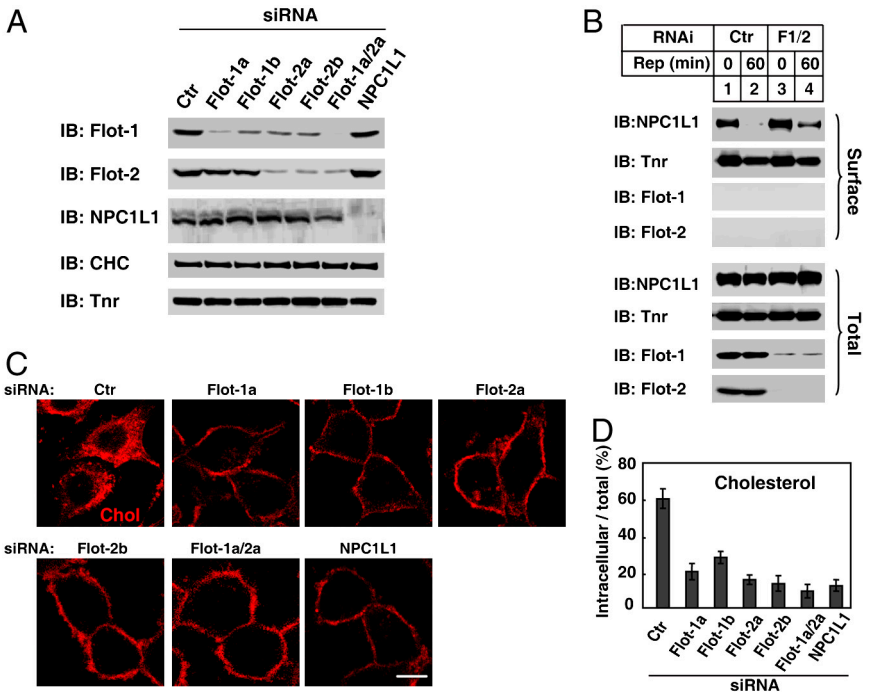


Fig. S5. Knockdown of flotillins inhibits endogenous NPC1L1-mediated cholesterol uptake in L02 cells. (*A*) L02 cells were transfected with indicated siRNAs, respectively. After transfection (96 h), the cells were harvested for IB. (*B*) The cells transfected with indicated siRNAs shown in *A* were depleted of cholesterol for 60 min (Rep 0) and then replenished with cholesterol for 60 min (Rep 60) as described in Fig. 1*A*. Surface biotinylation was performed to analyze plasma-membrane-localized NPC1L1 after cholesterol replenishment. (*C*) The cells were depleted of cholesterol for 60 min and then replenished with cholesterol for 60 min as described in Fig. 1*A*. The cells were then fixed, stained with filipin, and examined by two-photon microscopy. (Scale bar: 10 μm.) (*D*) Quantification of the internalized cholesterol shown in *C*. Error bars represent standard deviations ($n \geq 50$). Flot, flotillin; Tnr, transferrin receptor; Rep, cholesterol replenishment.

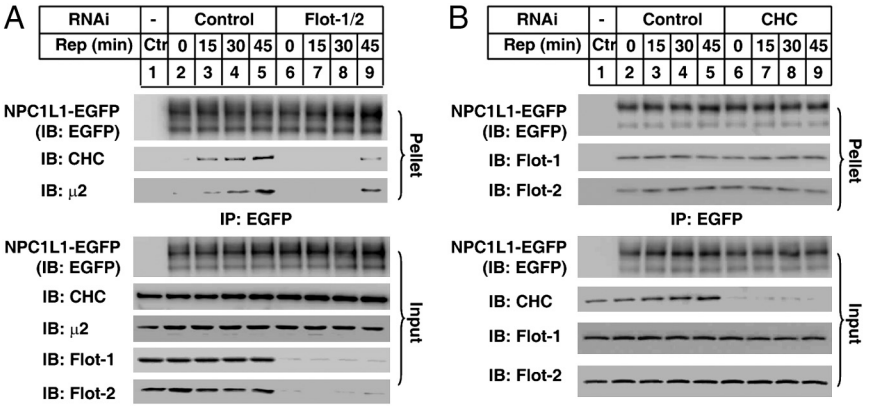


Fig. S6. Flotillins are required for recruitment of clathrin–AP2 in NPC1L1-mediated cholesterol uptake. (*A*) Coimmunoprecipitation between NPC1L1 and CHC/μ2 after flotillins knockdown. CRL1601–NPC1L1-EGFP cells transfected with flotillin-1 and flotillin-2 siRNA or control siRNA were depleted of cholesterol (Rep 0), and then replenished with cholesterol for indicated time durations (Rep 15, 30, 45). Then the cells were collected and IP was performed. The proteins were detected by IB. (*B*) Coimmunoprecipitation between NPC1L1 and flotillins after CHC knockdown. CRL1601–NPC1L1-EGFP cells were transfected with CHC siRNA or control siRNA and treated as shown in *A*. IP and IB were performed as described in *A*. Flot, flotillin; Rep, cholesterol replenishment.

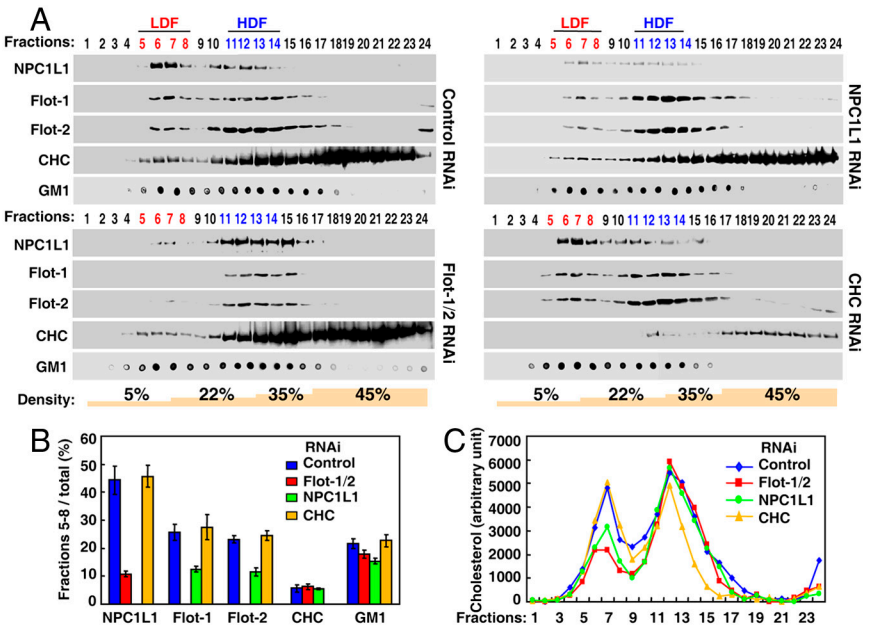


Fig. S7. Silencing of NPC1L1 or flotillins impairs the formation of cholesterol-enriched low-density membrane microdomains. (*A*–*C*) CRL1601–NPC1L1-EGFP cells were transfected with control, flotillin-1 and flotillin-2, NPC1L1, or CHC siRNA, respectively. After transfection (96 h) with the indicated siRNAs, the cells were depleted of cholesterol and then replenished with cholesterol as described in Fig. 1*A*. Subsequently, sucrose gradient fractionation experiments were conducted. Fractions were collected and subjected to IB or dot blot (*A*) or cholesterol assay (*C*). The relative content of proteins and GM1 in low-density fractions (LDF) were quantified (*B*). Error bars represent standard deviations of three experiments. HDF, high-density fractions; Flot, flotillin.

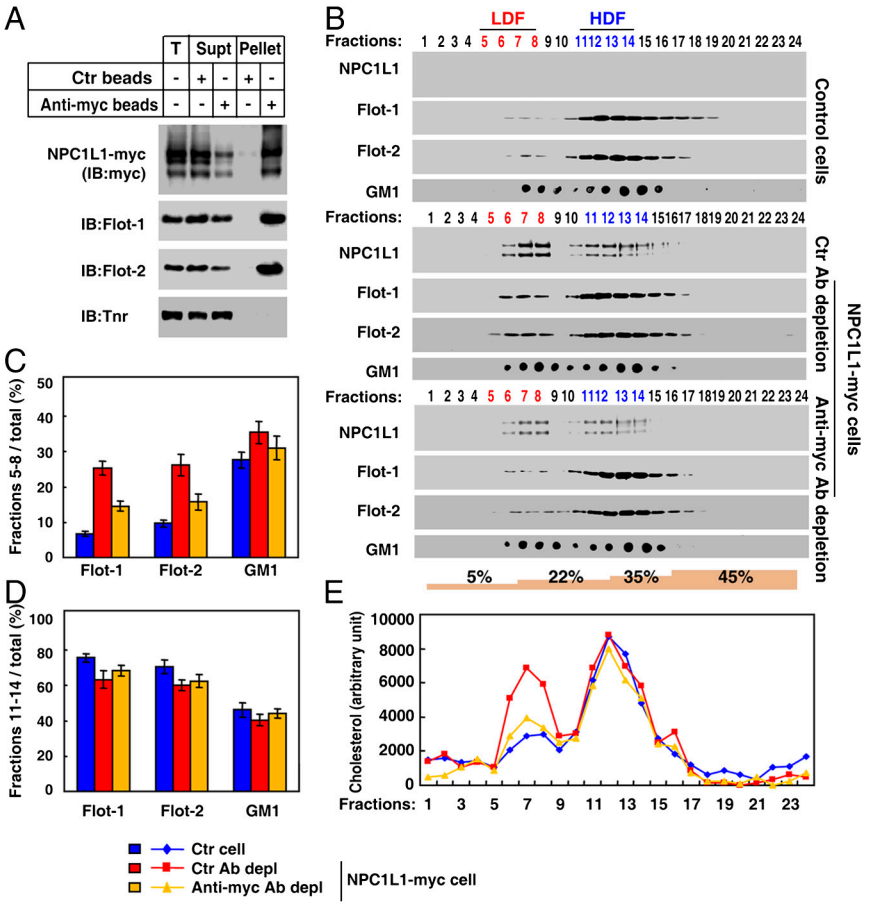
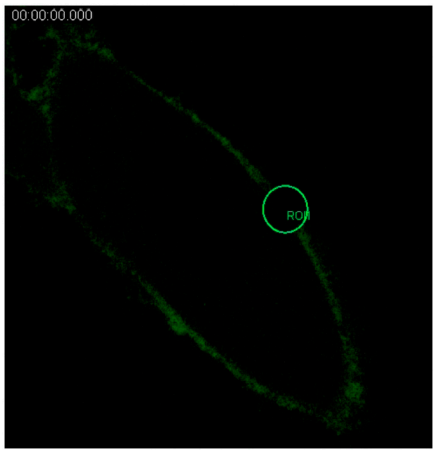
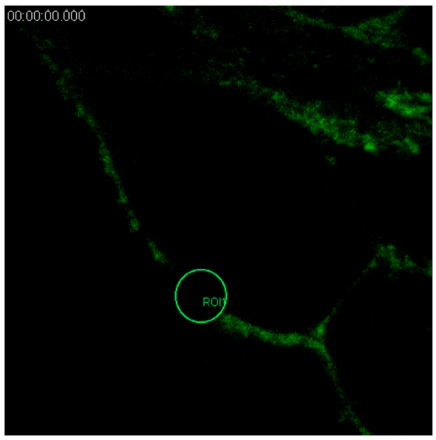


Fig. S8. Immunodepletion of NPC1L1-associated membranes reduces flotillins and cholesterol in low-density fractions (LDF). (*A*) Immunodepletion of NPC1L1-myc-associated membrane. CRL1601 (control) and CRL1601–NPC1L1-myc cells were replenished with cholesterol after cholesterol depletion as described in Fig. 1*A*. Then membrane fraction was prepared by homogenizing in hypotonic buffer. Then the membrane fraction was resuspended in the same hypotonic buffer, sonicated, and incubated with control or antimyc beads at 4 °C for 4 h. The beads were washed three times with the hypotonic buffer. Total cell lysate (T), the postimmunodepletion supernatant (Supt), and pellet were analyzed by IB. (*B–E*) Sucrose gradient ultracentrifugation analysis of the NPC1L1-depleted supernatant. The membrane from postimmunodepletion supernatant was collected and resuspended in 0.5 M Na_2CO_3 buffer (pH 11.0) and sonicated. Then sucrose gradient ultracentrifugation was performed. Indicated proteins or GM1 in each fraction were analyzed by IB or dot blot. (*C*) Quantification of indicated proteins or GM1 in LDF as relative to total. (*D*) Quantification of indicated proteins or GM1 in high-density fractions (HDF) as relative to total. (*E*) Cholesterol level in each fraction. Error bars represent standard deviations of three experiments. Ctr, control; Flot, flotillin; Ab, antibody.



Movie S1. FRAP experiments shown in Fig. 1*G* (*Upper*). FRAP was performed as described in *SI Materials and Methods*. After photo-bleach of NPC1L1-EGFP in the selected area, images of the fluorescence recovery of NPC1L1-EGFP were captured every 5 s for 195 s.

Movie S1 (AVI)



Movie S2. FRAP experiments shown in Fig. 1*G* (*Lower*). FRAP was performed as described in *SI Materials and Methods*. After photo-bleach of NPC1L1-EGFP in the selected area, images of the fluorescence recovery of NPC1L1-EGFP were captured every 5 s for 195 s.
Movie S2 (AVI)

Table S1. The list of protein names, National Center for Biotechnology Information accession numbers, and peptide sequences indicated in Fig. S1*A*

Band	Protein name	Peptide sequence
1	Cltc clathrin heavy chain (REFSEQ: NP_062172)	KADDPSSYMEVVQAANTSGNWEELVKY KAFMTADLPNELIELLEKI KAHTMTDDVTFWKW
2	Calnexin precursor (REFSEQ: NP_742005)	KAKKDDTDDEIAKY KAPVPTGEVYFADSFDRG KCESAPGCGVWQRP
3	Heat shock protein 5 (REFSEQ: NP_037215)	KDAGTIAGLNVMRI KDNHLLGTFDLTGIPPAPRG KELEEIVQPIISKL
4	Heat shock protein 9 (REFSEQ: NP_001094128)	KLFEMAYKKM KDSETGENIRQ KDAGQISGLNVLRV
5	Heat shock protein 8 (REFSEQ: NP_077327)	KCNEIISWLDKN KDAGTIAGLNVLRI KDNNLLGKF
6	Ribophorin I (REFSEQ: NP_037199)	KEDQVIQLMNTIFSKK KEETVLATVQALHTASHLSQQADLRN KFPEEEAPSTVLSQNLFTPKQ
7	Ribophorin II (REFSEQ: NP_113886)	KAVTSEIAVLQSRL KDIPAYSQDTFKV KDTYIENEKLSSGKR
8	ATPase family, AAA domain containing 3A (REFSEQ: NP_001030094)	KCSEVAQLTAGMSGRE KDKWSNFDPTGLERA
9	Heat shock protein 1 (REFSEQ: NP_071565)	KCEFQDAYVLLSEKK KDIGNIISDAMKKV KRIQEITEQLDITTSEYEKEKL
10	Coronin, actin binding protein 1C (REFSEQ: NP_001102797)	KKTTDTASVQNEAKL KLDEILKEIKS KNILDSKPAANKKS
11	AP-2 complex subunit μ-2 (REFSEQ: NP_446289)	MIGGLFIYNHKG KASENAIVWKI RSPVTNIART
12	Flotillin-1 (REFSEQ:NP_073192)	KAQLIMQAEAEAESVRM KVSAQCLSEIEMAKA RAQQVAVQEQEIARR
	Flotillin-2 (REFSEQ:NP_114018)	KNVVLQTLEGHLRS RTDKELIATVRR
13	Actin related protein 2/3 complex, subunit 2 (REFSEQ: NP_001100389)	KDSIVHQAGMLKR RASHTAPQVLFSHRE RDNTINLIHTFRD

Band	Protein name	Peptide sequence
14	EF hand domain containing 2 (REFSEQ: NP_001026818)	KAAAGELQEDSGLHVLARL KLGAPQTHLGLKS KSMIQEVDEDFDSKL
15	B-cell receptor-associated protein 31 (REFSEQ: NP_001004224)	KAENEALAMQKQ KGTAEDGGKLDVGSPEMKL KKYMEENDQLKKG
16	NEDD4 family interacting protein 1 (REFSEQ: NP_001013077)	KAEATIPLVPGRD KMPETFSNLPRT