Supporting Information (SI):

Last step in the path of LDL-cholesterol from the lysosome to plasma membrane to ER is governed by phosphatidylserine

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Dataset S1 (Excel File)
## Supporting Information

### Materials

| REAGENTS | SOURCE | IDENTIFIER |
|-----------|--------|------------|
| **Antibodies** | | |
| Mouse monoclonal anti-human LDLR, Phycoerythrin-conjugated (PE-anti-LDLR) | BD Pharmingen | 565653, RRID: AB_2739325 |
| Mouse monoclonal anti-DDDDK (Flag) | MBL International | M185-3LL, RRID:AB_11126775 |
| Rabbit monoclonal anti-NPC1 | Abcam | ab134113, RRID: AB_2734695 |
| Horse anti-mouse conjugated to HRP | Cell Signaling Technology | 7076, RRID: |
| Goat anti-rabbit conjugated to HRP | Cell Signaling Technology | 7074, RRID: |
| Rabbit polyclonal anti-H3K9me3 | Invitrogen | 49-1008, RRID: AB_2533859 |
| Rabbit monoclonal anti-SREBP-2 (22D5) | This paper | |
| **Bacterial and Virus Strains** | | |
| One Shot BL21(DE3) pLysS E. coli | Thermo Fisher | C606003 |
| **Chemicals, Peptides, and Recombinant Proteins** | | |
| [1-14C]Oleic acid (50 mCi/mmol) | Perkin Elmer | NEC31705 |
| [125]I]Nal | Perkin Elmer | NEZ033A005MC |
| 1:1 Mixture of Ham's F-12 medium and DMEM | Corning | 10–090-CV |
| 1-Palmitoyl-2-oleoyl-glycero-3-phosphocholine (PC) | Avanti Polar Lipids | 850457C-25mg |
| 1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (PE) | Avanti Polar Lipids | 850757C-25mg |
| 1-Palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine (sodium salt) (PS) | Avanti Polar Lipids | 840034C-25mg |
| 25-Hydroxycholesterol | Steraloids | C6510-000 |
| 5X Loading Dye (250 mM Tris HCl, pH 6.8, 10% (w/v) SDS, 50% (v/v) Glycerol, 10 mM DTT, 0.05% (w/v) Bromophenol Blue) | This Paper | N/A |
| Acetic acid | Fisher Scientific | A38C |
| Acetonitrile | Sigma-Aldrich | 34998 |
| AF488 N-Hydroxy succinimidyl ester | Thermo Fisher | A-20000 |
| AF647 N-Hydroxy succinimidyl ester | Thermo Fisher | A-20006 |
| Agencourt AMPure XP beads | Beckman Coulter | A63880 |
| Ammonium acetate | Sigma-Aldrich | 431311 |
| BbsI-HF restriction enzyme | New England BioLabs | R3539L |
| Benzonase nuclease | Sigma-Aldrich | E1014-25KU |
| Blasticidin | Thermo Fisher | R21001 |
| Bolt 4–12% Bis-Tris Plus gradient gels | Thermo Fisher | NW04125BOX |
| Cholesteryl [1,2,6,7-3H(N)]oleate (60 ci/mmol) | American Radiolabeled Chemicals | ART1204 |
| Cholesterol methyl-β-cyclodextrin (cholesterol-MCD) | This Paper | (2) |
| Chloroquine diphosphate | Sigma-Aldrich | C6628 |
| CO2 Independent Medium | Gibco | 18045-088 |
| Compacting (CPN) | This Paper | (3) |
| Dichloromethane | Sigma-Aldrich | 34856 |
| Chemical Name                                      | Manufacturer           | Catalog Number |
|---------------------------------------------------|------------------------|----------------|
| Dimethyl Sulfoxide (DMSO)                         | Sigma-Aldrich          | D8418          |
| DMEM-high glucose (4500 mg/l)                     | Sigma-Aldrich          | D6429          |
| DMEM-low glucose (1000 mg/l)                      | Sigma-Aldrich          | D6046          |
| EDTA, sodium, pH 8.0                              | Thermo Fisher          | 15575020       |
| Esp3I (BsmBI) restriction enzyme                  | Thermo Fisher          | ER0452         |
| Ethanol                                           | Pharmco                | 111000200CSPP  |
| Ethyl Ether                                       | Fisher Scientific      | E138           |
| Fetal Calf Serum (FCS)                            | Sigma                  | F2442-500ML    |
| Filipin III from *Streptomyces filipinensis*      | Sigma-Aldrich          | F4767-1ML      |
| Formaldehyde                                      | Macron Fine            | 5016-02 Chemicals |
| FuGENE HD                                         | Promega                | E2311          |
| Hank's Balanced Salt Solution (HBSS)              | Corning                | 21-022-CV      |
| Heptane                                           | Fisher Scientific      | H350           |
| Herculase II                                      | Agilent                | 600679         |
| Hexane                                            | Fisher Scientific      | H292           |
| Hexane for LC-MS/MS                               | Sigma-Aldrich          | 293253         |
| Human and newborn calf lipoprotein-deficient serum (LPDS) (d<1.215 g/ml) | This Paper (4) | |
| Human LDL (d1.019–1.063 g/ml)                     | This Paper (4)         | |
| Imidazole                                         | USB Corp.              | 17525          |
| Isopropanol                                       | Fisher Scientific      | A416           |
| Lenti-X Concentrator                              | Takara                 | 631232         |
| L-Glutamine                                       | Corning                | 25-005-CI      |
| L-Glutamine-free DMEM                             | Sigma-Aldrich          | D5546          |
| Methanol                                          | Fisher Scientific      | A412-4         |
| Methanol for LC-MS/MS                             | Sigma-Aldrich          | 34860          |
| Mevalonate (MEV)                                  | This Paper (3)         | |
| Mowiol 4-88                                       | Calbiochem             | 475904         |
| N-Acetyl-Leu-Leu-norLeucinal (ALLN)               | Calbiochem             | 208719         |
| Ni-NTA Agarose                                    | Qiagen                 | 30230          |
| NEBuilder HiFi DNA Assembly Cloning Kit           | New England BioLabs    | E2621L         |
| OptiMEM                                           | Gibco                  | 11058-21       |
| Penicillin-Streptomycin Solution                   | Corning                | 30-002 Cl      |
| Phosphate Buffered Saline (PBS)                   | Corning                | 21-031-CV      |
| Phenylmethylsulfonyl Fluoride (PMSF)               | Thermo Fisher          | 36978          |
| Polybrene                                         | Sigma                  | TR-1003-G      |
| Puromycin                                         | Sigma                  | P8833          |
| QuikChange II XL Site-Directed Mutagenesis Kit    | Agilent                | 200522         |
| Sodium Dodecyl Sulfate (SDS)                      | Sigma-Aldrich          | 71736          |
| SuperSignal West Pico Chemiluminescent Substrate  | Thermo Fisher          | 34580          |
| T4 DNA Ligase                                     | New England Biolabs    | M0202S         |
| Trichloroacetic acid                              | Sigma-Aldrich          | T6399-500G     |
| Water for LC-MS/MS                                | Sigma-Aldrich          | 270733         |

**Critical Commercial Assays**

| Assay Name                                         | Manufacturer           | Catalog Number |
|----------------------------------------------------|------------------------|----------------|
| Qubit dsDNA BR Assay Kit                           | Invitrogen             | Q32853         |
| KAPA Library Quantification Kit for Illumina platforms (KK4835) | KAPA Biosystems (Roche) | 07960204001 |
| Bioanalyzer High Sensitivity DNA Analysis          | Agilent                | 5067-4626      |
| Pierce BCA Protein Assay Kit                       | Thermo Fisher          | 23224          |
### Cell Lines

| Cell Line       | Source          | Catalog Number |
|-----------------|-----------------|----------------|
| SV589           | (5)             | N/A            |
| SV589j          | This Paper      | N/A            |
| Lenti-X 293T    | Clontech        | 632180         |
| CHO-K1          | ATCC            | CCL-61         |

### Oligonucleotides

See *SI Appendix, Table S1* Integrated DNA Technologies

### Recombinant DNA

| Construct                     | Source           | Catalog Number |
|-------------------------------|------------------|----------------|
| pSpCas9(BB)-2A-GFP (PX458)    | Addgene          | #48138, Gift from Feng Zhang |
| pSpCas9(BB)-2A-Puro V2.0 (PX459) | Addgene        | #62988, Gift from Feng Zhang |
| lentiCRISPRv2                 | Addgene          | #52961, Gift from Feng Zhang |
| pLenti CMV Blast              | Addgene          | #17486, Gift from Eric Campeau and Paul Kaufman |
| Human PTDSS1 cDNA             | Horizon          | MHS6278-202828427 |
| psPAX2                        | Addgene          | #12260, Gift from Didier Trono |
| pMD2.G                        | Addgene          | #12259, Gift from Didier Trono |
| pCMV-NPC1-Flag-TEV-Streptactin| Addgene          | # 73179, Gift from David Root and John Doench |
| CRISPR Knockout Pooled Brunello libraries | Addgene | N/A |
| Perfringolysin O* (Y181A)    |                  | Gift from Arun Radhakrishnan |

### Software and Algorithms

| Software and Algorithm         | Source           | Catalog Number |
|--------------------------------|------------------|----------------|
| Flowjo v10                     | Becton Dickinson | N/A            |
| Image Studio Lite v5.2         | LI-COR           | N/A            |
| Galaxy                         | (7)              | N/A            |
| MAGeCK                         | (8)              | N/A            |
| Openlab Imaging Software       | Improvision      | N/A            |

### Other

| Other                          | Source           | Catalog Number |
|--------------------------------|------------------|----------------|
| 0.2 µm Nitrocellulose Filters  | BioRad           | 1704271        |
| 100-mm Cell Culture Dish       | Corning          | 430167         |
| 12-mm Glass coverslips         | Fisher Scientific| 1254581        |
| 12-well Cell Culture Plate (22.1-mm) | Corning     | 3513           |
| 6-well Cell Culture Plate (34.8-mm) | Corning     | 3516           |
| 96-well Cell Culture Plate (6.4-mm) | Corning     | 3595           |
| BD Acuri C6 Plus Flow Cytometer | Becton Dickinson| N/A            |
| BD FACSAria1 Cell Sorter       | Becton Dickinson | N/A            |
| Extruder Set                   | Avanti Polar Lipids | 610023        |
| Glass tubes for LC-MS/MS       | Fischer Scientific| 1496226 F     |
| Illumina NextSeq500            | Illumina        | N/A            |
| IODO-GEN precoated tubes       | Thermo Fisher    | 28601          |
| MoFlo Cell Sorter              | Beckman Coulter | N/A            |
| NanoDrop UV-Vis Spectrophotometer | Thermo Fisher | N/A            |
| Pasteur pipettes               | VWR              | 14673-010      |
Methods

Cell Culture and Plating Conditions. Medium A is Dulbecco’s modified Eagle’s medium (DMEM)-low glucose (1000 mg/l). Medium B is DMEM-high glucose (4500 mg/l). Medium C is a 1:1 mixture of Ham’s F-12 medium and DMEM. Medium D is medium A devoid of L-glutamine. All media are supplemented with 100 U/ml of penicillin and 100 μg/ml of streptomycin sulfate. Buffer A is Hank’s Balanced Salt Solution (HBSS) containing 2% FCS and 2 mM sodium EDTA, pH 8.0.

SV589 cells (5) and SV589j cells, a clonal line of SV40-immortalized human fibroblasts derived from SV589 cells and selected by LDLR flow cytometry, were grown in medium A containing 5% FCS. Lenti-X 293T cells (human) were maintained in medium B supplemented with 5% FCS and 2 mM L-glutamine. CHO-K1 cells (Chinese hamster) were maintained in medium C supplemented with 5% FCS. Stock cultures of all cell lines were maintained in monolayer culture at 37 °C in either a 5% (SV589, SV589j, and Lenti-X 293-T cells) or 8.8% (CHO-K1 cells) CO2 incubator. All cell lines were routinely monitored for mycoplasma contamination.

For all experiments except CRISPR-Cas9 screening, cells were set up on day 0 in 6-well plates at a density of 7.5 to 30 x 10^4 cells/well in 1-2 ml of the indicated medium. Imaging experiments included 12-mm coverslips in wells. As described in the figure legends, cells were set for experiments on day 0 in medium containing 5 or 10% FCS. On day 2, the cells were switched to cholesterol depletion medium consisting of either medium A or C containing either medium A or C containing either 5% human or calf lipoprotein-deficient serum (LPDS), and compactin plus mevalonate (CPN/MEV). The concentrations of CPN/MEV were as follows: 30 μM/200 μM for SV589 cells and their derived KO cell lines and 50 μM/50 μM for CHO-K1 cells and their derived KO cell lines.

Lipoproteins and Stock Solutions. Human and newborn calf lipoprotein-deficient serum (LPDS) (d<1.215 g/ml) and human LDL (d1.019–1.063 g/ml) were prepared by ultracentrifugation as previously described (4). The concentration of LDL is expressed in terms of its protein content, i.e., μg protein/ml. Iodination of purified LDL (4 mg protein/reaction) was performed using IODO-GEN precoated tubes (2). More than 90% of the ^125I-radioactivity in ^125I-LDL was precipitable after incubation with 10% (w/v) trichloroacetic acid. Stock solutions of sodium mevalonate (MEV) and sodium compactin (CPN) (3) were prepared as described previously. Stock solutions of cholesterol methyl-β-cyclodextrin (cholesterol-MCD) were prepared as previously described (2). Stock solutions of filipin (1 mg/ml) were prepared fresh each day in DMSO. Stock solutions of 25-hydroxycholesterol (1 mg/ml) were prepared in ethanol and stored in multiple aliquots.
at 4 °C. Phycoerythrin-conjugated anti-LDLR (PE-anti-LDLR) is a mouse monoclonal antibody that binds to the human LDLR (9).

**Plasmids.** The sequences of all nucleic acids used in this study are provided in *SI Appendix*, Table S1. For creation of clonal knockout (KO) cell lines, oligonucleotides encoding the specified sgRNA sequence with adaptor overhangs were annealed and cloned into the indicated plasmid. After plasmid digestion with BbsI-HF, the sgRNA was inserted into one of the following plasmids: 1) PX458, a plasmid encoding Cas9, sgRNA scaffold, and EGFP; or 2) PX459, a plasmid encoding Cas9, sgRNA scaffold, and a puromycin-resistance cassette. Cloning was performed using NEBuilder HiFi DNA Assembly Cloning Kit in the case of PX458 or with T4 DNA Ligase in the case of PX459 as previously described (10). For creation of heterogeneous KO cell populations, oligonucleotides encoding the specified sgRNA sequence with adaptor overhangs were annealed and cloned using T4 DNA ligase. The sgRNA was inserted into Esp3I-digested lentiCRISPRv2, a lentiviral system encoding Cas9, sgRNA scaffold, and a puromycin-resistance cassette. A nontargeting sgRNA (11) was used as a negative control. For the lentiviral expression of *PTDSS1*, the cDNA for human *PTDSS1* was cloned into pLenti CMV Blast with three C-terminal Flag tags in tandem using the NEBuilder HiFi DNA Assembly Cloning Kit. Flag tags were generated from pCMV-NPC1-Flag-TEV-StrepTactin (6). The point mutations E212A and C189A in *PTDSS1* (12) were introduced into the wild-type cDNA by site-directed mutagenesis using QuikChange II LX Kit.

**Genome-wide CRISPR-Cas9 Screening.** The methodology for the genome-wide CRISPR-Cas9 KO screening involving a fluorescent reporter was based on previous work described by Golden, et al. (13). We used the human CRISPR KO Pooled Brunello Library (containing 76,441 sgRNAs in lentiCRISPRv2 described by Doench, et al. (14). The library was amplified in bacteria, and the purified plasmids were used to prepare lentivirus in Lenti-X 293T cells. Genome-wide screens were performed in SV589j cells. Two independent screens were carried out, the results of which were used for the MAGeCK analysis as described below.

On day 0, SV589j cells were transduced with lentivirus containing the Brunello CRISPR library in medium A supplemented with 10% FCS and 1 µg/ml polybrene at a multiplicity of infection of 0.2 or 0.32 (15). On day 1, cells received fresh medium A with 10% FCS devoid of lentivirus. On day 2, cells were switched and maintained in medium A with 10% FCS and 0.8 µg/ml puromycin for ~10 days, after which the cells were plated in medium A with 10% FCS devoid of puromycin. One day later, the cells were depleted of cholesterol by incubation for 16 h in medium A containing 5% LPDS, 30 µM CPN and 200 µM MEV. Cells were then switched to the above medium supplemented with 50 µg protein/ml LDL. After 24 h, the cells were harvested for FACS sorting as described below. An aliquot of 40 x 10^6 cells was kept unsorted and unstained as a control; the remaining ~2 x 10^8 cells were sorted on a MoFlo cell sorter. The brightest 0.5% of cells were collected by FACS, pelleted, and frozen at -80 °C.

sgRNA coverage was maintained throughout each screen at >500 cells per sgRNA. This was achieved for each screen as follows: 76,441 sgRNAs were infected into 300 x 10^6 SV589j cells that were then selected in puromycin for 10 days, after which at
least 40 x 10^6 cells were subjected to FACS sorting (UT Southwestern Flow Cytometry Core). We selected 200 x 10^3 cells that showed the highest amount of fluorescence (0.5% of total cells). Genomic DNA was extracted from the top 0.5% and from unsorted cells using a genomic DNA (gDNA) isolation method (13). The gDNA underwent two sequential rounds of PCR to generate amplicon libraries using the Herculase II enzyme. Library DNA was then purified using Agencourt AMPure XP beads. DNA concentration of the libraries was quantified with the Qubit dsDNA BR Assay Kit and by qPCR with the KAPA Library Quantification Kit for Illumina platforms. Amplicon library size and integrity were assessed via the Bioanalyzer High Sensitivity DNA Analysis Kit. Following these quality checks, library amplicons were sequenced on an Illumina NextSeq500 with 75 bp single-end reads (McDermott Center, UT Southwestern). 30-70 x 10^6 reads were obtained per sample.

The CRISPR-Cas9 screening data was analyzed as follows: The Brunello library sgRNA reference files were obtained from Addgene. Sequences were extracted from demultiplexed FASTQ files using Galaxy (7) and normalized read counts were calculated as described previously (16). MAGeCK (Model-based Analysis of Genome-wide CRISPR-Cas9 Knockout) was used to identify statistically significant hits from the screening data (8).

Cloned Cells Lacking NPC1 or PTDSS1. Cloned SV589 cells lacking NPC1 were generated as follows: On day 0, the cells were plated at a density of 7 x 10^5 cells per 100-mm dish and then transfected with 10 µg PX459 plasmid encoding the two sgRNAs targeting exon 4 of the NPC1 gene delivered in 1 ml OptiMEM and 40 µl Fugene HD. After 48 h, the cells were switched to medium A containing 5% FCS and 1 µg/ml puromycin and then selected for ~2 weeks at the time when single colonies appeared. Colonies were harvested using cloning cylinders, and those colonies lacking NPC1 protein expression as assessed by immunoblot were cloned by serial dilution.

Cloned CHO-K1 cells lacking NPC1 were generated as follows: On day 0, CHO-K1 cells were plated at a density of 7 x 10^5 cells per 100-mm dish and then transfected as above with 10 µg PX459 plasmid encoding the two sgRNAs targeting exon 2 of NPC1. After 48 h, cells were switched to medium C containing 5% FCS and 25 µg/ml puromycin and those colonies lacking NPC1 protein were selected and cloned as above. Targeting of exon 2 of NPC1 (amino acids Q19 to Q60) (SI Appendix, Table S1) results in a frameshift mutation followed by a premature stop codon corresponding to amino acid 38, as verified by DNA sequencing of the surrounding genomic DNA.

Cloned CHO-K1 cells lacking PTDSS1 were generated as follows: cells were plated and transfected as above with 20 µg PX458 plasmid encoding the two sgRNAs targeting intronic regions flanking exon 4 of PTDSS1 (SI Appendix, Table S1 and Fig. S3). After 48 h, cells from 5 dishes were harvested in 3 ml buffer A and subjected to FACS sorting on a BD FACS Aria1 machine. The brightest 2% of GFP-positive cells were collected, subcloned in 96-well plates by serial dilution, and screened for PTDSS1 knockout (KO) by PCR and Sanger DNA sequencing of the surrounding genomic DNA. Deletion of exon 4 of PTDSS1 (amino acids G106 to M146) results in a frameshift
mutation followed by a premature stop codon corresponding to amino acid 124, as verified by DNA sequencing of the surrounding genomic DNA.

Cloned CHO-K1 cells lacking PTDSS2 were generated as follows: cells were plated and transfected as above with 20 µg PX458 plasmid encoding the two sgRNAs targeting intronic regions flanking exon 2 of PTDSS2 (SI Appendix, Table S1). After 48 h, the cells underwent FACS sorting, subcloning, and screening as above for PTDSS1−/− cells. Deletion of exon 2 of PTDSS2 (amino acids W39 to R73) is predicted to result in a premature stop codon corresponding to amino acid 39. This deletion was confirmed by sequencing this region of genomic DNA and mRNA using primers flanking exon 2. The sequence revealed a complete deletion of exon 2 with no residual normal mRNA sequence.

**Lentiviral-generated Knockout of NPC1, PTDSS1, and IDOL in SV589j Cells.** To generate lentivirus, 3.2 x 10⁶ Lenti-X 293T cells were plated in 100-mm dishes in 10 ml medium B supplemented with 5% FCS and 2 mM L-glutamine. After 24 h the cells were transfected in 10 ml medium B with 5 µg lentiCRISPRv2, 3 µg psPAX2, and 2 µg pMD2.G using 30 µL FuGENE HD in 0.9 ml Opti-MEM. On days 2 and 3 after transfection, the medium was collected, filtered through a 0.45-µm SFCA sterile filter, and concentrated 50X using a Lenti-X Concentrator. Pellets of lentivirus were resuspended in PBS, snap-frozen in liquid nitrogen, and stored at -80 °C.

On day 0, 6 x 10⁴ SV589j cells/well were seeded in 12-well plates and transduced with 1 ml medium A containing 10% FCS, 1 µg/ml polybrene, and a total of 80 µl of two lentiviruses, each encoding one different sgRNA directed against NPC1, PTDSS1, or IDOL (also called MYLIP). On day 1, cells received fresh medium A with 10% FCS devoid of lentivirus. On day 2, cells received medium A containing 10% FCS and 0.8 µg/mL puromycin. Cells were maintained in puromycin and replated at a lower density every two to three days over an 8-10 day period, after which the cells were used for experiments.

**Lentiviral-mediated Transduction of PTDSS1 cDNA.** Lentivirus was prepared and transduced into CHO-K1 cells as above with lentivirus (3 µl in case of WT and E212A mutant of PTDSS1 and 10 µl in case of C189A mutant of PTDSS1). On day 1, cells received fresh medium C with 10% FCS devoid of lentivirus. On day 2, cells received medium C containing 10% FCS and 15 µg/ml blasticidin. The medium was refreshed every 48 h during the 8-10 days of blasticidin selection, after which the cells were used for experiments.

**Flow Cytometry.** Cells were released from monolayers by incubation with EDTA-containing buffer A, after which ~1 x 10⁶ cells were suspended in buffer A and centrifuged for 5 min at 1000 rpm. Each cell pellet was resuspended in 0.1 ml buffer A containing either 2 µg/ml PE-anti-LDLR or 10 µg/ml AF488-conjugated PFO* (see below). After incubation for 15 min either at room temperature (anti-LDLR) or at 4 °C (PFO*), cells were washed once with buffer A and then resuspended in buffer A to a concentration of ~1 x 10⁶ cells/ml in a U-bottom 96-well plate, subjected to flow cytometry analysis on a BD Acuri C6 Plus flow cytometer, and analyzed using Flowjo v10 software. Dead cells and
cell doublets were excluded by gating so that >20,000 cells were available for analysis in each condition (SI Appendix, Fig. S1).

**Purification and Labeling of PFO**. The Y181A mutant of Perfringolysin O, hereafter referred to as PFO* (2) was produced and labeled as follows: A plasmid encoding His-tagged PFO* was expressed in One Shot BL21(DE3) pLysS E. coli (Thermo Fisher). The cell pellet was lysed by sonication in PBS with 1:1000 phenylmethylsulfonyl fluoride (PMSF). The protein was bound to Ni-NTA Agarose resin (Qiagen), washed three times with 50 mM imidazole, pH 8.0 in PBS, eluted with 250 mM imidazole, and subjected to size exclusion chromatography on Superdex 200. Fractions containing PFO* were concentrated to 2 mg/ml and incubated for 1 h with AF488 or AF647 N-hydroxy succinimidyl ester at a 5:1 dye to protein molar ratio. The labeled protein was purified by size exclusion chromatography in PBS containing 1 mM EDTA, and analyzed for labeling efficiency on a NanoDrop UV-Vis Spectrophotometer.

**Preparation of Unilamellar Liposomes**. Liposomes were prepared by drying the various phospholipids (in chloroform) in glass tubes under nitrogen gas, followed by desiccation at room temperature overnight. Lipids were resuspended to a concentration of 5 mM in PBS, snap-frozen in liquid nitrogen, and thawed at room temperature five times. Unilamellar liposomes were then prepared by extruding the lipid solutions through a 1.0-micron polycarbonate membrane 11 times, stored at 4 °C up to three weeks, and freshly extruded for each experiment. The acyl chain composition of the various phospholipids was as follows: 16:0 at the sn-1 position and 18:1 at the sn-2 position.

**Cholesteryl Ester Synthesis Assay**. The rate of incorporation of [1-14C]oleate into cholesteryl [14C]oleate was measured as described (4, 17). The details of cell plating, transfection, and pulse labeling with [14C]oleate are described in figure legends. After a 4-h incubation with [14C]oleate, the cells were washed, and the lipids were extracted from the dish in hexane:isopropanol (3:2, vol:vol). Cholesteryl [3H]oleate was added as an internal standard to estimate recovery. The lipids were separated on a silica gel G thin-layer chromatogram developed in heptane:ethyl ether:acetic acid, 90:30:1, and quantified by scintillation counting (4). Protein concentration was determined using the Pierce Bicinchoninic Acid (BCA) Assay. The amount of cholesteryl [14C]oleate formed is expressed as nmol per h per mg cell protein.

**Measurement of 125I-LDL Degradation**. The proteolytic degradation of 125I-LDL by cultured cells was measured as previously described (4). The details of cell plating and incubation are described in the figure legends. The amount of 125I-LDL degraded is expressed as μg per mg cell protein.

**Visualization of PFO** Binding by Fluorescence Microscopy. Cells were plated on 12-mm glass coverslips at a density of 0.5-1.5 × 10⁵ cells/well in a 6-well plate. Detailed incubation conditions are described in the figure legends. For imaging with AF488-conjugated PFO*, cells were cooled on ice, washed once with ice cold PBS with 2% calf LPDS and stained for 15 min at 4 °C with 10 μg/ml AF-488-conjugated PFO* diluted in CO₂ Independent Medium with 2% LPDS. The cells were then washed 3 times in PBS with 2% LPDS, fixed in methanol at -20 °C for 15 min, washed once with PBS, and
mounted in Mowiol mounting solution. For staining with filipin, the cells were fixed in 3.7% (w/v) formaldehyde in PBS for 15 min at room temperature, washed in PBS, labelled with 25 µg/ml filipin in PBS for 30 min at room temperature, washed 3 times in PBS, and mounted in Mowiol mounting solution. Fluorescence images were acquired using a 40x objective (Zeiss Plan-Neofluar 40x/1.3 DIC), an Axiovert 200M microscope (Zeiss), an Orca-285 CCD camera (Hamamatsu) and Openlab Imaging Software. Images of different conditions were captured with the same parameters.

**Measurement of Phospholipids by LC-MS/MS.** Cells were transferred to fresh glass tubes for liquid-liquid extraction by a modification of the method of Bligh and Dyer (18). Briefly, 1 ml each of dichloromethane, methanol, and water were added to a glass tube containing the sample. The mixture was vortexed and centrifuged at 2700g for 5 min, resulting in two distinct liquid phases. The lower phase was removed to a fresh glass tube with a Pasteur pipette and dried under N₂. Samples were resuspended in hexane. Lipids were analyzed by Liquid Chromatography-Mass Spectrometry (LC-MS/MS) using a SCIEX QTRAP 6500+ equipped with a Shimadzu LC-30AD HPLC system and a 150 x 2.1-mm, 5 µm Supelco Ascentis silica column. The gradient program conditions are detailed in SI Appendix, Table S2. A solution containing 95:5 (v/v) acetonitrile-water with 10 mM ammonium acetate was infused post-column at 0.03 ml/min. Column oven temperature was kept at 25 °C. Data was acquired in negative ionization mode using multiple reaction monitoring. The LC-MS data was analyzed using MultiQuant (SCIEX). The fatty acid content of each individual species of phospholipid was summed within each lipid class in order to provide the PC, PE, and PS contents.

**SREBP-2 Processing.** The details of cell plating and treatment are described in figure legends. Cells were treated with 25 µg/ml of N-acetyl-Leu-Leu-norLeucinal (ALLN) 1 h prior to harvesting for immunoblot analysis of SREBP-2 and H3K9Me3 as described below.

**Immunoblot Analysis.** Cells were lysed by addition to the dish of a solution of PBS containing 0.3% (w/v) SDS and 100 U/ml of Benzonase Nuclease. Aliquots were mixed with 5X loading dye and applied to Bolt 4-12% gradient gels. After electrophoresis, the proteins were transferred to nitrocellulose filters, which were then incubated with one of the following antibodies: 5 µg/ml rabbit anti-SREBP-2, 18 ng/ml rabbit anti-H3K9me3, 1 µg/ml mouse anti-Flag or 0.35 µg/ml rabbit anti-NPC1. Bound antibodies were visualized by chemiluminescence using Supersignal West Pico Chemiluminescent Substrate after incubation for 1 h with either 31 ng/ml of horse anti-mouse IgG or 13 ng/ml of goat anti-rabbit IgG conjugated to horseradish peroxidase. The images were scanned using an Odyssey FC Imager (Dual-Mode Imaging System; 2 min integration time) and analyzed using Image Studio lite v5.2.

**Reproducibility.** All metabolic and imaging experiments were repeated at least two times on different days. Similar results were obtained.
References

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Fig. S1

Live Cells

Singlet Cells

Top 0.5% LDLRs Sorted

Forward Scatter

Pulse Width

Forward Scatter

FITC

Side Scatter

PE-Anti-LDLR Fluorescence
Fig. S2

A

Addition

- LDL
+ LDL

Cholesteryl $^{14}$C-Oleate Formed (nmol/hr per mg protein)

Wild-type  
NPC1$^{-/-}$  
IDOL$^{-/-}$

B

Normalized Cell Count

Wild-type  
NPC1$^{-/-}$  
IDOL$^{-/-}$

PE-anti-LDLR (fluorescence)
Fig. S3

Exon 4

* 5' | | | | | | | | | | | | | 3'

sgRNA-1 sgRNA-2

AA106 AA146
Fig. S4

A  Plasma Membrane Cholesterol

B  Cholesterol Ester Synthesis

Addition

- LPDS
- FCS

Cholesteryl 14C-Oleate Formed (nmol/hr per mg)

Wild-type  PTDSS1^-/-  PTDSS2^-/-
Table S1. Nucleic Acid Sequences Used in This Study

**sgRNAs for Human NPC1 Gene Exon 4 (see Fig. 1B)**

| Name | sgRNA Sequence (5’ to 3’) | Oligonucleotide Sequence Used for Cloning (5’ to 3’) |
|------|----------------------------|-----------------------------------------------|
| sgRNA-1 | AAAGAGTTACAATACTACGT | CACCAGGAAGTTTTGACTACGT |
| sgRNA-2 | CGCACAGAGTCAGTTTTGAA | CACCACGACAGTCAGTTTTGAA |

**sgRNAs for Human Genes from Brunello Library (see Fig. 2 and Fig.S2)**

| Name | sgRNA Sequence (5’ to 3’) | Oligonucleotide Sequence Used for Cloning (5’ to 3’) |
|------|----------------------------|-----------------------------------------------|
| Nontargeting Control (Joung et al., 2017) | CTGAAAAAGGAAGGAGTTGA | TATATCTTTGTGAAAAGGAGTTGA |
| NPC1 sgRNA-1 | AAAGAGTTACAATACTACGT | TATATCCCTGTGAAAAGGAGTTACGTTT |
| NPC1 sgRNA-2 | CAAACTTGTATCATTCAGAG | TATATCTTTGTGAAAAGGAGTTACGTTT |
| PTDSS1 sgRNA-1 | AGCACTCGGCAAATTTGAAA | TATATCTTTGTGAAAAGGAGTTATTT |
| PTDSS1 sgRNA-2 | AAATCTTCGATACGCCACAAG | TATATCTTTGTGAAAAGGAGTTATTT |
| IDOL sgRNA-1 | CATTCTGTGCGGATAGCGAAG | TATATCTTTGTGAAAAGGAGTTATTT |
| IDOL sgRNA-2 | CTGCAGTTTACGGGATAGCGAAG | TATATCTTTGTGAAAAGGAGTTATTT |
### sgRNAs for Chinese Hamster Genes (see Figs. 3-5 and Fig. S4)

| Name          | sgRNA Sequence (5' to 3')                     | Oligonucleotide Sequence Used for Cloning (5' to 3') |
|---------------|-----------------------------------------------|---------------------------------------------------|
| **NPC1** sgRNA-1 (Within Exon 2) | GGACGGAAATGACTTGCTAC | CACCCGGGACGGAAATGACTTGCTAC |
|               |                                               | AAACGTAGCAAGTCATTTCCGTC |
| **NPC1** sgRNA-2 (Within Exon 2) | ACACTCTCCATAACCAACAC | CACCCGACACTCTCCATAACCAACAC |
|               |                                               | AAACACACTCTCCATAACCAACAC |
| **PTDSS1** sgRNA-1 (Flanking Exon 4) | AAAGGGATCAGCTTAAGATG | TATATATTTTGGGAGTCTAGGCTAGGAGCTAGAATAGCAAGTTAAA |
|               |                                               | AAACGTAGCAAGTCATTTCCGTC |
| **PTDSS1** sgRNA-2 (Flanking Exon 4) | CACACATTAATTAGACCCAG | TATATATTTTGGGAGTCTAGGCTAGGAGCTAGAATAGCAAGTTAAA |
| **PTDSS2** sgRNA-1 (Flanking Exon 2) | GCTGGGTAGTACAGTATTCCT | TATATATTTTGGGAGTCTAGGCTAGGAGCTAGAATAGCAAGTTAAA |
| **PTDSS2** sgRNA-2 (Flanking Exon 2) | GCTAGTACCTGAGTACAGTATTCCT | TATATATTTTGGGAGTCTAGGCTAGGAGCTAGAATAGCAAGTTAAA |

### Oligonucleotides Used to Subclone Human PTDSS1 cDNA into pLenti CMV Blast (see Fig. 3)

| Primer Name | Oligonucleotide Sequence Used for Cloning (5' to 3') |
|-------------|---------------------------------------------------|
| A771-1      | ATAGAAGACACCGACTCTAGGCGAATTGCCTGGCTGGGAG         |
| A771-2      | CTCGCCAGCGAGCAGCGACTCTAGGCGAATTGCCTGGCTGGGAG        |
| A771-3      | CAATGGGGGAGTCTGGCTGGGAGTCTAGGCTAGGAGCTAGAATAGCAAGTTAAA |
| A771-4      | GCATCTTTTGTAGGCGTGTCTTCTTCTTCTCTAGGCGATCTAGGAGCTAGAATAGCAAGTTAAA |
| A771-5      | AGTAGAAGACGACGACTCTAGGCGAATTGCCTGGCTGGGAGTCTAGGAGCTAGAATAGCAAGTTAAA |
| A771-6      | GTTGATTGTTGCGAGCGCCCGCTACTTTGTAGGCGATCTAGGAGCTAGAATAGCAAGTTAAA |
| A771-7      | CAATGGGGGAGTCTGGCTGGGAGTCTAGGCTAGGAGCTAGAATAGCAAGTTAAA |
| A771-8      | GCTAGTACCTGAGTACAGTATTCCT | TATATATTTTGGGAGTCTAGGCTAGGAGCTAGAATAGCAAGTTAAA |

### Oligonucleotides Used to Mutagenize Human PTDSS1 cDNA (see Fig. 3)

| Mutation | Oligonucleotide Sequence Used for Cloning (5' to 3') |
|----------|---------------------------------------------------|
| E212A    | CTCCTCCCCAAATTTGCGGccTGCTGGTGGGATCAAGTC         |
|          | GACTCTCCACCCGAGCAGCantGCGAAAATTTGGGAGGAGG       |
| C189A    | GATCCGTAGTTACGGTCTGcTGGACAATCATTTACCT            |
|          | AGCTGTAATCTGTGAGCCGAGCCGTACACTACGGG           |
Table S2. Gradient Program for LC-MS/MS Method

| Time (min) | Flow rate (ml/min) | Solvent A (%) | Solvent B (%) | Solvent C (%) |
|------------|--------------------|---------------|---------------|---------------|
| 0          | 0.3                | 97.5          | 2.5           | 0             |
| 3          | 0.3                | 95            | 5             | 0             |
| 9          | 0.3                | 40            | 60            | 0             |
| 9.3        | 0.3                | 80            | 0             | 20            |
| 20         | 0.3                | 60            | 0             | 40            |
| 26         | 0.3                | 56            | 0             | 44            |
| 26.5       | 0.3                | 40            | 0             | 60            |
| 27.5       | 0.3                | 40            | 0             | 60            |
| 27.6       | 1.2                | 97.5          | 2.5           | 0             |
| 32.0       | 1.2                | 97.5          | 2.5           | 0             |

Solvent A, Hexane.
Solvent B, Methyl tert-butyl ether.
Solvent C, Isopropanol:water (90:10, v/v)
LEGENDS FOR SUPPLEMENTAL FIGURES

**Fig. S1.** Gating Strategy for CRISPR-Cas9 Screen, Related to Fig. 1C. Cells were sorted by FACS and gated to restrict analysis to live and singlet cells. Cells expressing the top 0.5% LDLRs as measured by PE-anti-LDLR fluorescence (box, right panel) were collected for genomic DNA extraction. FITC, fluorescein isothiocyanate.

**Fig. S2.** Analysis of IDOL−/− Cells Compared with NPC1−/− Cells, Related to Fig. 2. (A) Cholesteryl ester synthesis in WT, NPC1−/−, and IDOL−/− SV589j cells. On day 0, the indicated lentiviral-generated knockout cells were set up in medium A with 10% FCS. On day 2, cells were depleted of cholesterol by incubation for 16 h in medium A containing 5% calf LPDS and CPN/MEV. On day 3, the cells were refed with the same medium in absence of presence of 50 µg protein/ml LDL. After incubation for 4 h, the cells were pulse-labeled for 2 h with 0.1 mM sodium [14C]oleate (6040 dpm/nmol), after which the cellular content of cholesteryl [14C]oleate was measured. Each bar represents the average of duplicate incubations with individual values shown as circles. (B) Measurement of LDLRs by flow cytometry in WT, NPC1−/−, and IDOL−/− SV589j cells incubated with LDL. On day 0, the indicated lentiviral-generated knockout cells were set up in medium A with 10% FCS. On day 1, cells were refed medium A containing 5% calf LPDS and 30 µM compactin (CPN) plus 200 µM mevalonate (MEV). After 16 h, cells were switched to the above medium with 50 µg protein/ml LDL. After 24 h, the cells were harvested by incubation with EDTA, washed, incubated with PE-anti-LDLR, and subjected to flow cytometry (SI Appendix, Methods). Same WT control histogram is shown in both panels for reference.

**Fig. S3.** Exon-Intron Structure of the Chinese Hamster PTDSS1 gene, Related to Fig. 3. sgRNAs flanking exon 4 (amino acids 106-146) were used to create the PTDSS1−/− cell lines. Asterisk (*) denotes location of premature stop codon corresponding to amino acid 124. As determined by alanine scan mutagenesis, amino acids 172-226 are crucial for the catalytic activity of hamster PTDSS1 (12).

**Fig. S4.** Analysis of PTDSS2−/− Cells Compared with PTDSS1−/− Cells. On day 0, wild-type PTDSS1−/−, and PTDSS2−/− CHO-K1 cells were set up in medium C with 5% FCS. (A) Measurement of LDL-derived cholesterol by flow cytometry in plasma membranes. On day 2, cells were switched to cholesterol depletion medium C. After incubation for 16 h, the cells received medium D containing CPN/MEV supplemented with 10% FCS. After 6 h, cells were harvested, incubated with AF647-labeled PFO*, and examined by flow cytometry. Same WT control histogram is shown in all panels for reference. (B) Cholesteryl ester synthesis. On day 2, cells were switched to cholesterol depletion medium C. After incubation for 12 h, the cells received cholesterol depletion medium D with 5% calf LPDS or 10% FCS as indicated. After incubation for 4 h, the cells were pulse-labeled for 2 h with 0.1 mM sodium [14C]oleate (9711 dpm/nmol), after which the cellular content of cholesteryl [14C]oleate was measured. Each bar represents the average of duplicate incubations with individual values shown.
Combined MAGeCK Analysis from Two CRISPR-Cas9 Screens Based on LDLR Protein Level in SV589j Cells as Quantified by FACS; Related to Fig. 1. Model-based Analysis of Genome-wide CRISPR-Cas9 Knockout (MAGeCK) of CRISPR-Cas9 screens. Positively selected genes were identified and ranked according to a Robust Rank Aggregation score based on log fold change of individual sgRNAs between sorted and unsorted cells (8). The data shown are the combined results from two independent screens, each carried out with 76,441 sgRNAs. Note that we did not perform experiments to access the validity of the gene knockouts in SI Appendix, Dataset S1 except for the genes discussed in the Results.