Supporting Information

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SI Results

FC Loading Effects on SMPD2 and Plasma Membrane Lipid Rafts. The protein levels of SMPD2, the neutral sphingomyelinase that breaks SM into ceramide and phosphocholine, were compared in unloaded and FC-loaded cells and no significant differences were observed (Fig. S1*A*), suggesting that alterations in SM degradation were not responsible for the increased levels of SM in FC-loaded cells. Because cholesterol and SM have high affinity toward each other and both molecules are enriched in specialized lipid-raft microdomains of plasma membrane (PM), we tested whether FC-induced SM accumulation led to the increased formation of lipid rafts at the PM. Using a previously described flow-cytometry–based cholera toxin B (CT-B) binding assay for the lipid-raft constituent ganglioside M1, FC-loaded cells were found to have significantly more lipid rafts compared with unloaded cells (Fig. S1*B*). These results were confirmed by fluorescent microscopy with the FC-loaded cells showing higher cell surface binding of CT-B–Alex-647 compared with unloaded cells (Fig. S1*C*).

Lysosomal Inhibitor Impairs ORMDL1 Degradation upon FC Loading. To determine the mechanistic pathway/s for FC-mediated degradation of ORMDL1, we tested whether FC-induced degradation of ORMDL1 could be blocked by treatment with either proteasomal or lysosomal inhibitors. Neither MG132 (reversible) nor lactacystin (irreversible) proteasome inhibitors prevented the FC-mediated induction of ORMDL1 turnover in RAW264.7 cells (Fig. S3 *D* and *E*). These data ruled out the role of the proteasomal degradation pathway in FC-induced ORMDL1 degradation.

SI Materials and Methods

Reagents. Myriocin, cycloheximide, cholesterol-CD, ACATi (Sandoz 58-035), MG132, lactacystin, chloroquine, sphingomyelin, sphingomyelinase, *N*-ethyl-*N*-(2-hydroxy-3-sulfopropyl)-3,5-dimethoxyaniline sodium salt (DAOS), aminoptyrine, choline oxidase, peroxidase, palmitoyl-CoA, L-serine, sucrose-monolaurate, and pyridoxal 5′-phosphate hydrate were obtained from Sigma-Aldrich. Antibodies against ORMDL1, GAPDH, SPTLC1, SPTLC2, LC3, and Atg7 were obtained from Thermo Scientific, Abcam, Novas Biological, and Santa Cruz Biotechnology. ORMDL1–tGFP and ORMDL3–tGFP constructs and tGFP antibody were bought from Origene. Radiochemicals were obtained from Perkin-Elmer.

SM Measurement Assay. SM was hydrolyzed by bacterial SMase and converted to *N*-acylsphingosine and phosphorylcholine. Alkaline phosphatase was used to convert phosphorylcholine to choline, which was then oxidized by choline oxidase and further acted upon peroxidase in the presence of DAOS, H_2O_2, and 4-aminoantipyrine to generate a blue-colored dye. The SM standard solution was prepared by dissolving 5 mg of SM in 10 mL of 2% Triton X-100 in ethanol. Cellular lipid fractions were resuspended in 200 μL of Reagent 1 (2 units SMase, 4.86 units alkaline phosphatase, 2 mM DAOS, and 0.05% Triton X-100, 0.05 M Tris·HCl, 0.66 mM calcium chloride, pH 8). The cell suspension was then gently mixed by pipetting up and down several times and transferred to a 96-well plate for a 20-min incubation at 37 °C. A total of 100 μL of Reagent 2 (0.07 units choline oxidase, 3 units peroxidase, 0.72 mM 4-aminoantipyrine, 0.05 M Tris·HCl, 1 mM calcium chloride, pH 8) was added and incubated at 37 °C for 30 min. Absorption was measured at 600 nm using a plate spectrophotometer. The amount of SM was calculated by comparing it to the standard curve of SM and was plotted per milligram of cell protein determined using the BCA assay (Pierce). For FC measurements, total cell cholesterol was extracted from the cells using 1 mL hexane:isopropanol (3:2, v:v). The extracts were transferred to microfuge tubes and dried. Then the protein from the same wells was dissolved by addition of 1 mL of 0.2 M NaOH. The plate was incubated at 37 °C for 3 h then rocked for 5 min at room temperature. Protein concentration in cell lysate was determined using the BCA assay. Cholesterol standards and samples were dissolved in isopropanol:Nonidet P-40 (9:1, v:v). In a black 96-well plate, 10 mL of a 100 units/mL catalase solution was distributed in each well and 40 μL of each sample was mixed followed by a 15-min incubation at 37 °C to eliminate any peroxides present in reagents or samples. Next, 150 μL of Reagent A (0.1 M potassium phosphate buffer, pH 7.4, 0.25 M NaCl, 5 mM cholic acid, 0.1% Triton X-100, 0.3 units/mL cholesterol oxidase, 1.3 units/mL HRP, and 0.4 mM ADHP) was added and mixed in each well. The plate was incubated at 37 °C for an additional 15 min and fluorescence was measured at an excitation wavelength of 530 nm and an emission wavelength of 580 nm.

SPT Activity Assay. Microsomal fractions were prepared from monolayers of RAW264.7 cells by sonication followed by a low-speed spin. RAW264.7 cells were treated with 50 μg/mL cholesterol-CD or with 100 μg/mL AcLDL ± 2 μg/mL ACATi for 16 h at 37 °C. Cells were washed twice with 3 mL of PBS and suspended in 500 μL of 50 mM Hepes (pH 8) and 1 mM EDTA. The cell suspension was sonicated for 15 s at 50% power and 50% pulsation and centrifuged at 2,500 × *g* for 2 min. The supernatant was isolated and protein concentration in cell extract was determined by micro BSA protein assay (Pierce). A total of 190 μL of total cell lysate (~200–400 μg total protein) was mixed with 10 μL of the corresponding 20× assay mix (10 mM l-serine, 0.4 mM pyridoxal 5′-phosphate, 1 mM palmitoyl-CoA, 20 μCi/mL [^{14}C]L-serine), and vortexed, and kept on ice. For negative control, 2 μL of 1 mg/mL myriocin solution was added. The mixture was incubated at 37 °C for 60 min for the SPT reaction. Then 0.5 mL of methanol/KOH:$CHCl_3$ (4:1) was added and mixed intensively. A total of 0.5 mL $CHCl_3$, 0.5 mL alkaline water (2 mM NH_4OH), and 100 μL of 2 M NH_4OH was added sequentially and mixed intensively, followed by centrifugation at 12,000 × *g* for 1 min at room temperature. The upper phase was removed with gentle aspiration. The lower phase was washed twice with 900 μL of alkaline water. Then 400 μL (2/3 volume) of the lower phase was transferred to a polyethylene scintillation vial. $CHCl_3$ was evaporated under a stream of nitrogen. A total of 5 mL of Ecolite liquid scintillation mixture was added and radioactivity was determined using a Beckman LS6500 scintillation counter.

Lipid-Raft Quantification. RAW264.7 cells were loaded with 50 μg/mL cholesterol-CD at 37 °C for 16 h. These cells were then washed twice with PBS and gently scraped in 1.5 mL PBS. The Alexa-647–CT-B (1 μL of 1.5 mg/mL stock in PBS was added to 1.5 mL cells) was added at a final concentration of 1 μg/mL to cells in 5 mL polystyrene round-bottom tubes (12 × 75 mm). The samples were subjected to flow analysis with a BD Biosciences LSRII cytometer using laser [Alexa-647 (red) Ex: 639, Em: 650–670, filter 660/20] and data were analyzed by FlowJo software. Images of Alexa-647–CT-B–labeled cells were captured using epifluorescent microscopy.

Western Blotting. RAW264.7 cells were grown in six-well plates and loaded with 50 μg/mL cholesterol-CD or with 100 μg/mL AcLDL with or without 2 μg/mL ACATi for 16 h. The PBS-washed cell pellet was lysed in 200 μL of Nonidet P-40 lysis buffer. After discarding the nuclear pellet, the protein concentration was determined using the BCA protein assay (Pierce). A total of 10 μg of cell protein samples were run on Novex 4–20% Tris-glycine gels (Invitrogen) and transferred onto polyvinylidene fluoride membranes (Invitrogen). Blots were incubated sequentially with 1:1,000 rabbit polyclonal antibody raised against SMPD2 (LS bioSciences), or 1:5,000 rabbit polyclonal antibody raised against ORMDL1 (Thermo Scientific), or 1:1,000 rabbit polyclonal antibody raised against SPTLC1 (H-300, Santa Cruz), or 1:1,000 mouse polyclonal antibody raised against SPTLC2 (A01, Abnova), and 1:10,000 horseradish peroxidase-conjugated goat anti-rabbit or goat anti-mouse secondary antibody (Bio-Rad). The signal was detected with an enhanced chemiluminescent substrate (Pierce) and direct chemiluminescent quantification was performed using a Bio-Rad Chemi Doc system. For LC3 Western blot, RAW264.7 cells were incubated with or without 50 μg/mL cholesterol-CD for 16 h, indicated wells received 30 μM chloroquine during the last 2 h of incubation. Blots were sequentially incubated with 1:1,000 rabbit polyclonal antibody raised against LC3B (Novus Biologicals) and 1:10,000 horseradish peroxidase-conjugated goat anti-rabbit as described above. GAPDH was used as a loading control.

Coimmunoprecipitation. RAW264.7 cells were washed with solution I (1 M sorbitol, 10 mM $MgCl_2$, 30 mM DTT, 100 μg/mL phenylmethylsulfonyl fluoride, 50 mM K_2HPO_4), resuspended in solution II (1 M sorbitol, 10 mM $MgCl_2$, 30 mM DTT, 100 μg/mL phenylmethylsulfonyl fluoride, 50 mM K_2HPO_4, and 25 mM sodium succinate, pH 5.5), and incubated at 30 °C for 30 min. Cells were collected by centrifuging at 4,500 × *g* for 20 min at 4 °C and lysed using Nonidet P-40 lysis buffer, followed by the addition of 2 mM EDTA, 200 μM sodium vanadate, and 50 mM sodium fluoride. Lysis was accomplished by incubating cell suspensions for 30 min on ice. Protein extracts were clarified by centrifuging lysates at 14,000 rpm for 5 min. For immunoprecipitation, extracts were incubated with mouse polyclonal antibodies (raised against either SPTLC2 or ubiquitin) or guinea pig monoclonal antibody (raised against p62) for 4 h at 4 °C. Prewashed protein A agarose beads were added to these samples and incubated for 2 h. Beads were washed and immunoprecipitated proteins along with input proteins (10% of IP proteins) were then loaded on a Novex 4–20% Tris-glycine gel and analyzed by Western blotting with rabbit polyclonal antibody raised against ORMDL1.

Indirect Immunofluorescence/Fluorescent Microscopy. RAW264.7 cells were grown in four-chamber slides in indicated growth media. Cells were washed with 1× PBS, fixed with paraformaldehyde, and then incubated with permeabilization solution (1× PBS/0.2% Triton X-100) for 10 min at room temperature (RT). Cells were washed with 1× PBS three times and blocked with normal goat serum (1× PBS/1% BSA/5% normal goat serum) at 37 °C for 30 min. The cells were probed with p62 antibody and Alexa-647–labeled secondary antibody. For LC3 staining, RAW264.7 cells were transfected with LC3–GFP (Addgene) using lipofectamine LTX according to manufacturer protocol (Life Technologies). Cells were treated with or without 50 μg/mL cholesterol-CD for 16 h or starved for amino acids for 4 h as a positive control of autophagy activation. For endogenous ORMDL1, p62, and calreticulin staining, RAW264.7 cells were treated with or without 50 μg/mL cholesterol-CD or 100 μg/mL AcLDL + ACATi for 16 h. The formaldehyde-fixed cells were incubated with rabbit-ORMDL1 and mouse-calreticulin or guineas pig-p62 antibodies and probed with Alexa-488 anti-rabbit, Alexa-647 anti-mouse, or anti-guinea pig secondary antibodies. All images were captured using an Olympus IX51 inverted epifluorescent microscope, Olympus LUCPlanFl ×40/0.6 lens, with a Q-Image EXi aqua camera and Olympus cellSens Dimension version 1.7 software. Postimaging processing was performed in Adobe Photoshop CS2.

Filipin Staining. To determine the cellular localization of free cholesterol, RAW264.7 cells treated with or without 50 μg/mL cholesterol-CD and fixed with paraformaldehyde (3%) for 1 h at RT. Cells were first washed with PBS, followed by PBS-glycine to quench excess paraformaldehyde. Cells were incubated with filipin (0.05 mg/mL in PBS/10% FBS) and images were acquired by fluorescent microscopy using a UV filter set.

LIVE/DEAD Fixable Dead Cell Staining. To quantify damaged and dead cell population, RAW macrophages were cultured in six-well plates in growth media containing either RAW264.7 cells treated with Chol-CD or 100 μg/mL AcLDL + ACATi for 16 h. As a negative control, cells were left untreated and a subset of untreated cells was heat killed as a positive control. The dead cells were quantified by using LIVE/DEAD Fixable Blue Dead Cell Stain kit for UV excitation from Molecular Probes following manufacturer protocol.

Annexin–Cy5 Labeling. To determine apoptosis status of cells, Annexin-Cy5 labeling was performed in cholesterol-loaded and control macrophages. For positive control, apoptosis was induced in RAW macrophages by treatment with staurosporine (final concentration 1 μM) for 6 h at 37 °C. To measure cell surface PS, cells were cultured in six-well plates in growth media, washed twice with PBS, and resuspended in 500 μL Annexin V binding buffer plus 1 μL of Annexin V-Cy5 (Biovision). The samples were incubated at room temperature for 5 min in the dark. Flow cytometry analysis was performed using a BD Biosciences LSRFortessa cytometer using a 639-nm excitation laser and emission at 650–670 nm; data were analyzed using FlowJo software.

Generation of Stably Transfected Human ORMDL–tGFP Cell Lines. The human ORMDL1/ORMDL3–tGFP clones were transfected into HEK293 cells. Transfected cells were examined by epifluorescence microscopy to confirm tGFP expression. tGFP positive colonies were subsequently picked and expanded in 1.5 mg/mL G418. For visualizing changes in ORMDL1 localization, HEK293 ORMDL1–tGFP cells grown in 24-well plates were treated with 50 μg/mL water-soluble cholesterol-CD for 16 h at 37 °C. The localization of ORMDL1/3 in cells was determined by confocal fluorescence microscopy.

Pulse-Chase Analysis of ORMDL1 Turnover. Raw264.7 cells were cultured in a 12-well plate and switched to L-leucine-free medium for 1 h. Then cells were pulse labeled for 4 h with 10 μCi/mL of [^{14}C]L-leucine in medium lacking L-leucine. Wells for the 0-h chase were scrapped and the PBS-washed cell pellet was lysed in 50 μL of Nonidet P-40 lysis buffer. For the remaining wells, the medium was removed, and the cells were chased for 16 h in medium supplemented with 1 mM unlabeled L-leucine in the absence or presence of 100 μg/mL AcLDL with 2 μg/mL ACATi. Following the chase, the PBS-washed cell pellet was lysed in 50 μL of Nonidet P-40 lysis buffer. After discarding the nuclear pellet, an aliquot (2 μL) was removed for trichloroacetic acid (TCA) precipitation followed by scintillation counting of the precipitate. The remainder of the supernatant (48 μL) was incubated with 5 μL rabbit polyclonal antibody raised against ORMDL1 for 4 h at 4 °C. Protein A/G Agarose beads were added to these samples and incubated for 2 h. Immunoprecipitates were extensively washed and run on a Novex 12% Tris-glycine polyacrylamide gel. The fixed gel was impregnated in 1 M sodium salicylate for fluorography. After

that, the gel was dried and exposed to X-ray film at −80 °C. The exposed film was used to cut out the dried gel band corresponding to the immunoprecipitated ORMDL1, and the protein was extracted in 30% hydrogen peroxide followed by scintillation counting. The fraction of [^{14}C] in ORMDL1 compared with the total TCA precipitate was calculated.

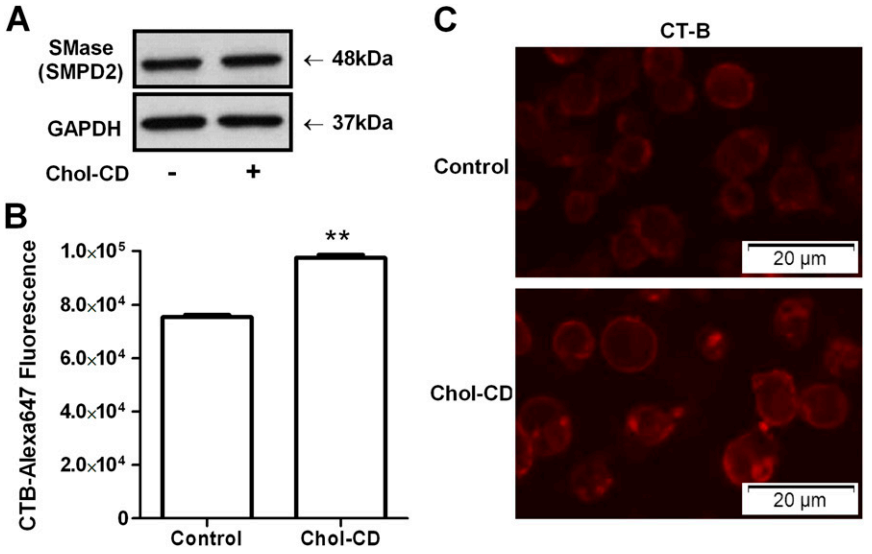

Fig. S1. Cholesterol loading does not alter SMPD2 levels, but increases cellular lipid rafts. (*A*) Western blot analyses of neutral SMase (SMPD2) expression in RAW264.7 cells unloaded or loaded with 50 μg/mL cholesterol-CD for 16 h. GAPDH levels were determined by Western blotting and used as a loading control. (*B*) Lipid rafts evaluated in RAW264.7 cells unloaded or loaded with 50 μg/mL cholesterol-CD for 16 h by incubation with Alexa-647 conjugated to cholera toxin subunit B (CT-B) and flow cytometry ($n = 3$, mean ± SD, $P < 0.005$, by t test). (*C*) Lipid rafts in RAW264.7 cells demonstrated by fluorescence microscopy using Alexa-647–labeled CT-B.

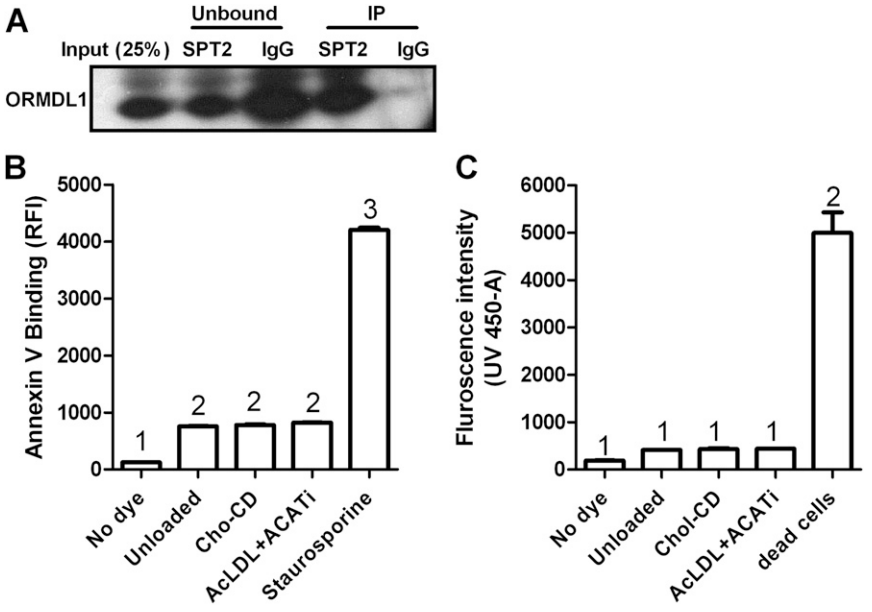

Fig. S2. (*A*) Pellet and supernatant fraction showing extent of ORMDL1 association with SPT2: RAW264.7 cell lysates were immunoprecipitated with anti-SPT2, pellet (IP), and supernatant (unbound) fractions were resolved by SDS/PAGE and immunoblotted with anti-ORMDL1 antibody, demonstrating that ORMDL1 and SPT2 are in a protein complex. (*B*) Cells were unloaded or incubated with 50 μg/mL cholesterol-CD or 100 μg/mL AcLDL + 2 μg/mL ACATi for 16 h. Staurosporine treatment was used to induce apoptosis as a positive control. The relative number of apoptotic cells was evaluated by cell surface PS as measured using Annexin V binding in a flow-cytometry assay. (*C*) Flow-cytometry analysis of dead cells using the Molecular Probes Dead Cell ethidium homodimer-1 dye. Cells were incubated with 50 μg/mL cholesterol-CD or 100 μg/mL AcLDL + 2 μg/mL ACATi for 16 h. Heat-treated cells were used as a positive control. (*B* and *C*) Values are the mean ± SD of the median fluorescence from three independent wells; different numbers above the bars show $P < 0.01$ by ANOVA posttest.

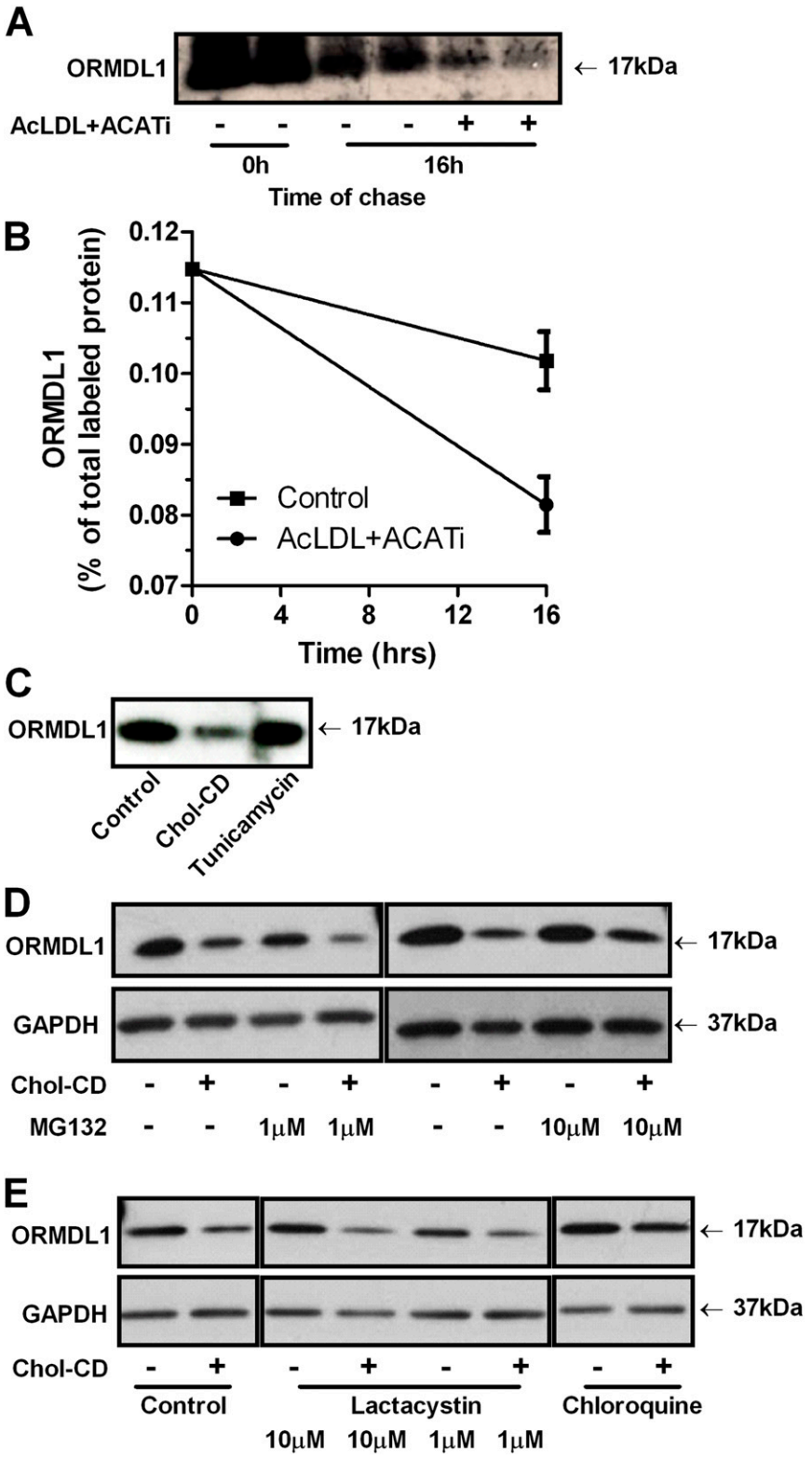

Fig. S3. ORMDL1 turnover studies. (*A*) RAW cells were pulse labeled for 4 h with 10 μCi/mL of [^{14}C]L-leucine in medium lacking L-leucine, followed by a 16-h chase in medium supplemented with 1 mM unlabeled L-leucine in the absence or presence of 100 μg/mL AcLDL with 2 μg/mL ACATi. IP was performed by using anti-ORMDL1 and fluorography shows the [^{14}C]-labeled ORMDL1. (*B*) Relative levels of [^{14}C]ORMDL1 compared with total [^{14}C]-labeled protein after labeling and after chasing in the presence or absence of 100 μg/mL AcLDL with 2 μg/mL ACATi. (*C*) ORMDL1 levels in RAW264.7 cells after the incubation with 50 μg/mL cholesterol-CD or 5 μg/mL tunicamycin. (*D*) Levels of ORMDL1 in RAW264.7 cells after a 16-h incubation with or without 50 μg/mL cholesterol-CD, in the absence or presence of MG132 (1 μM and 10 μM). (*E*) Levels of ORMDL1 in RAW264.7 cells after a 16-h incubation with or without 50 μg/mL cholesterol-CD, in the absence or presence of lactacystin (10 μM and 1 μM) or 100 μM chloroquine.

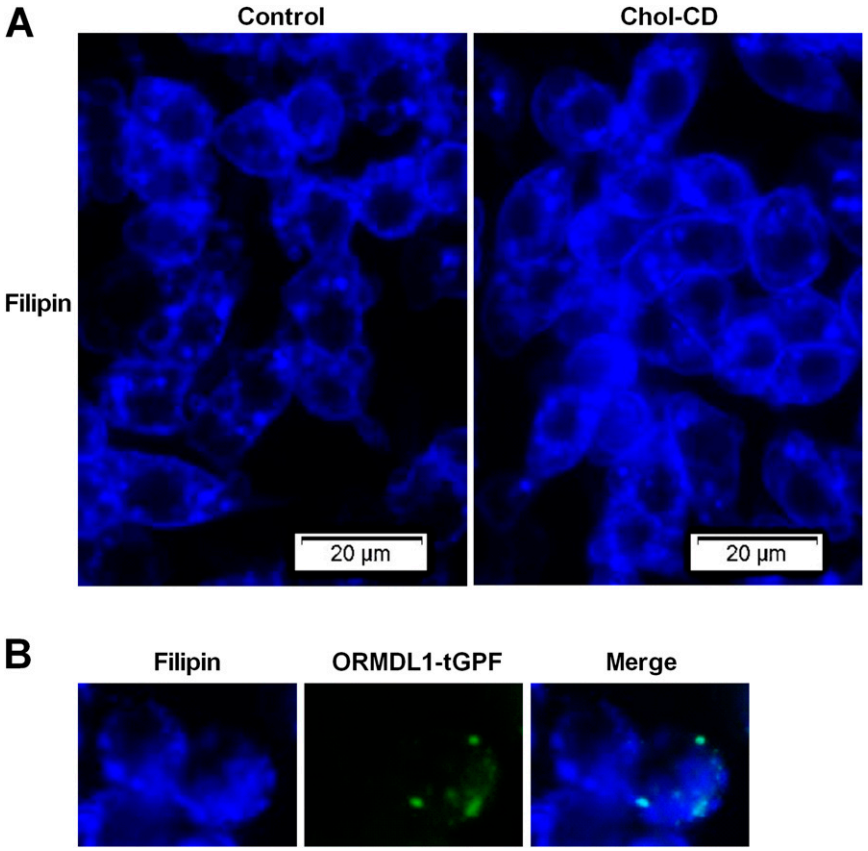

Fig. S4. Free cholesterol distribution assessed by filipin staining. (*A*) RAW cells were treated with or without 50 μg/mL cholesterol-CD and fixed with paraformaldehyde (3%) for 1 h at RT. Cells were incubated with filipin (0.05 mg/mL in PBS/10% FBS) and images were acquired by fluorescent microscopy. (*B*) RAW cells transiently transfected with ORMDL1–tGFP construct were treated with 50 μg/mL cholesterol-CD, followed by incubation with filipin (0.05 mg/mL in PBS/10% FBS) and cells were imaged by fluorescent microscopy.

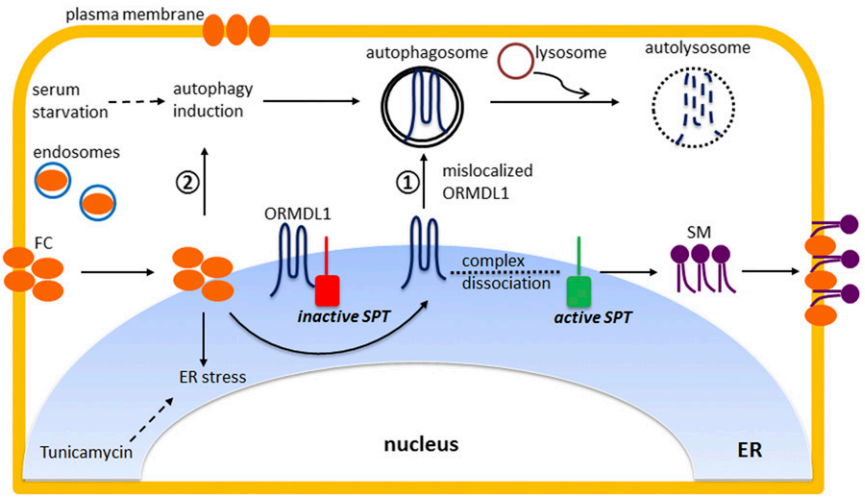

Fig. S5. Model depicting events leading to FC-mediated degradation of ORMDL1. FC loading induces (*i*) ORMDL1 exit from the ER and (*ii*) induction of autophagy. The combination of these two events results in ORMDL1 degradation via autophagy, which relieves the negative regulatory effect of ORMDLs on SPT activity and increases SM biosynthesis.