Materials and Methods

Mice and diet. SMSLTg and SMS KO mice were created in our laboratory. The SMS2LTg founder animals, with C57BL/6J/CBA mixed background, were backcrossed with C57BL/6J mice five generations. The original SMS2 KO mice, with 129 mouse genetic background, were backcrossed with C57BL/6 mice six generations. All mice used were littermates, or with the same genetic background. The groups of mice (male or female at age 10 weeks) were fed either rodent chow, or a high-fat and high-cholesterol (Research Diets, Inc.) diet for 8 weeks. All procedures and protocols involving the use of animals were approved by the SUNY Downstate Medical Center Animal Care and Use Committee.

Liver lipid measurements. Total liver lipids were extracted according to a modified method from Folch et al. Briefly, snap-frozen liver tissues (~100 mg) were homogenized in 5 ml of 1 N NaOH and extracted twice with 5–10 ml of a chloroform/methanol (2:1, v/v) solution. The organic layer was dried under nitrogen gas and resolubilized in 1 ml of chloroform containing 2% Triton X-100. This extract was dried again and resuspended in 1 ml of water to achieve a final concentration of 2% Triton X-100, and then measured triglyceride concentration using kit from Thermo, measured free fatty acids, total cholesterol, and total phospholipid concentration using kits from Wako.

Histological analysis. Liver tissue samples were either embedded in Tissue-Tek optimal cutting temperature compound (Sakura Finetek) and sectioned (7 μm) for neutral lipid staining using Oil Red O and a hematoxylin counterstain or fixed in 10% formalin,

embedded in paraffin, and sectioned (5 μm) for hematoxylin and eosin staining. Sections were photographed at either ×100 (hematoxylin and eosin) or ×200 (Oil Red O) magnification.

Lipid analyses by LC MS/MS. Ceramides comprised of a D-erythro-sphingosine backbone and a fatty acid amide were determined by a 2D LC-ESI MS/MS method. Lipid extracts from cells were injected onto a normal-phase column, where the polar lipids were retained, while the ceramide fractions were trapped on a reversed-phase column. Ceramides were eluted, separated, and detected using a triple quadruple mass spectrometer equipped with positive ion electrospray ionization (ESI) and selected reaction monitoring. Levels of PC and SM were measured via a flow injection ESI-MS/MS method. Protonated molecular ions of PC/SM species are selected by precursor ion scans of m/z 184 and the ion intensities across the flow injection profile were summed together, and after isotope correction, the quantities of each PC and SM species are then calculated relative to PC and SM internal standards.

mRNA analyses. RNA was isolated from cells using TriZol (Invitrogen). The mouse primers used for SMS2 RT-PCR were: Forward 5'-GGTTCCCACAGAAACCAAGA-3', and reverse 5'- GATGCCTGTTTTCCACCACT-3'. For liver SMS2 transgene, human SMS2 mRNA was determined by real-time PCR using Taqman® Gene Expression Assay (Applied Biosystems, assay ID Hs00380453_m1). The human primers used for SMS2 were: Forward 5'- TCCAGTGTGCTCCAAAGCTC-3' and reverse 5'-CACTCAGCAGCCAGCAGAT-3'. 18S rRNA was used as internal control. The forward

and reverse primer sequences for 18S rRNA are: 5'-AGTCCCTGCCCTTTGTACACA-3' and 5'-GATCCGAGGGCCTCACTAAAC-3', respectively.

SMS activity assay. Cells were homogenized in a buffer containing 50 mM Tris-HCl, 1 mM EDTA, 5% sucrose, and a cocktail of protease inhibitors (Sigma). The homogenate was centrifuged at 5000 rpm for 10 minutes and the supernatant was mixed in assay buffer containing 50 mM Tris-HCl (pH 7.4), 25 mM KCl, C_6-NBD-ceramide (3.3 μg/ml), and phosphotidylcholine (100 μg/ml). The mixture was incubated at 37^{o}C for 2 hours. Lipids were extracted in chloroform: methanol (2:1, v/v), dried under N_2 gas, and separated by thin layer chromatography (TLC) Chloroform:MeOH:20% NH_4OH (14:6:1, v/v). Band intensity was quantified by Image–Pro Plus 4.5 (Media Cybernetics Inc.).

Plasma membrane isolation. Mouse liver (0.5 g) was homogenized and plasma membrane was isolated using a kit (Biovision). The purity of the preparation was checked by Western blot, using the cytoplasmic, total membrane, and plasma membrane fractions, with respective antibodies as we did previously.

Lipid raft isolation. Liver lipid rafts were isolated, using reported protocols. Briefly, 0.1 g mouse liver was homogenized in 1.5 ml of 0.5 M Na_2CO_3 (pH 11), containing protease inhibitors. The homogenates were centrifuged (1,300 g, 5 minutes) to pellet cellular debris and nuclei. One milliliter of the postnuclear supernatant was then adjusted to 45% sucrose by addition of 1 ml of 90% sucrose, 50 mM Hepes (pH 6.5), and 150 mM NaCl. A discontinuous sucrose gradient was formed by overlaying this solution with 6 ml of

35% sucrose and 3.5 ml of 5% sucrose, both in the same buffer, containing 0.25 M Na_2CO_3, and this was centrifuged at 38,000 rpm for 18 hours in a SW41 rotor (Beckman). Fractions from top to bottom (1 ml each) were collected. Lyn kinase and caveolin-1 were used to locate the raft fractions.

Free fatty acid uptake. Mouse hepatocytes were isolated from the liver according our reported procedure. Liver fatty acid uptake was assayed according to reported method with modification. Briefly, viable hepatocytes were identified by trypan blue exclusion and counted, and incubated for 3 h in DMEM containing 10% fetal bovine serum (FBS), 1% penicillin–streptomycin mix and 1% L-glutamine. The cells were washed once with PBS, and then incubated in 1 ml of assay buffer (40 μM sodium oleate, 10 μM fatty acid-free bovine serum albumin, 5 μCi/ml [14C]oleate in phosphate-buffered saline (PBS) with 1 mM $MgCl_2$ and 1.2 mM $CaCl_2$) for 2 and 5 min at 37 °C. Assays were stopped by the addition of 1 ml of ice-cold stop solution (PBS containing 1 mM $MgCl_2$, 1.2 mM $CaCl_2$, 0.1% fatty acid-free bovine serum albumin, and 500 μM phloretin). The cells were washed three times with the above stop solution, and then lipids were extracted using Hexanes/Isopropanol (3/2, v/v). Radioactivity was analyzed by scintillation counting.

Exogenous ceramide, sphingomyelin, and phosphatidylcholine supplementation. Sphingomyelin (Sigma) and phosphatidylcholine (Sigma) were dissolved in absolute ethanol. Ceramde was dissolved in ethanol/dodecan (99.8/0.2, v/v) to make a 2-mM stock. Huh7 cells were incubated with exogenous 0, 10, and 30 μM sphingomyelin, ceramide or phosphatidylcholine for 24 h.

Statistical analysis. Each experiment was conducted at least 3 times. Unless otherwise indicated, data are expressed as mean ± SD. Differences between 2 groups were analyzed by unpaired 2-tailed Student's *t* test and among multiple groups by ANOVA followed by Student-Newman-Keuls test. A *P* value of less than 0.05 was considered significant.