Supplement

Methods

Lipid and lipoprotein assays. The SM, total cholesterol, total phospholipids, and triglycerides in plasma were assayed by enzymatic methods.[24, 25] Lipoprotein profiles were obtained by fast protein liquid chromatography (FPLC), using a Sepharose 6B column.[25]

Apolipoprotein measurement. Plasma apoE, apoB, and apoA-I levels were determined as previously described.[26] Briefly, 0.2 µl plasma were separated by 4-15% SDS gel electrophoresis, and immunoblotted with polyclonal antibodies against apoE (Abcam), apoB (US Biological), and apoA-I (Biodesign).

Lipid analyses by LC/MS/MS. The liver and plasma sphingomyelin, phosphatidylcholine, ceramides, sphingosine-1-phosphate were measured by LC/MS/MS as described previously.[22]

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 mouse primers used for SMS1 RT-PCR were: Forward 5'-GTGCTCAGACCGGAAGAAAG-3', and reverse 5'-

ACTAGCTTCTCCGCGTGTTG-3'. The human primers used for SMS2 were: Forward 5'-ATAATGCAACAAGGCTTGGAA-3' and reverse 5'-GAAGGTAGCCATCCGATTACTG-3'. 18S rRNA was used as an internal control. The forward and reverse primer sequences for 18S rRNA were: 5'-AGTCCCTGCCCTTTGTACACA-3' and 5'-GATCCGAGGGCCTCACTAAAC-3', respectively, and the probe sequence was 5'-CGCCCGTCGCTACTACCGATTGGT-3'.

SMS activity assay. The liver SMS activity were determined as previously described.[22]

Lysenin treatment and cell mortality measurement. Hepatocytes were washed twice in PBS and incubated with lysenin, 50 ng/ml for 1 hour. Cell viability was measured using the WST-1 cell proliferation reagent according to the manufacturer's instructions (Roche).

Ceramidase Assay. The activity of ceramidase was measured using C6-NBD-Cer as a substrate. The reaction mixture contained 550pmol of C6-NBD-Cer and 100μg protein of liver homogenate in 20μl of 25mM Tris-HCl buffer, pH 7.5, containing 1% sodium cholate. Following incubation at 37°C for 30 min, the reaction was terminated by the addition of 100μl of chloroform/methanol (2/1, v/v), dried in a SpeedVac concentrator, and redissolved in 30μl of chloroform/methanol (2/1, v/v), and an appropriate amount of sample was applied to a TLC plate, which was developed with chloroform, methanol, 25% ammonia (90/20/0.5, v/v/v). NBD-hexanoic acid released by the action of the enzyme and the remaining C6-NBD-Cer were separated by TLC, then analyzed, and quantified with an image-Pro plus 4.5.

Ceramide synthase Assay. A microsomal fraction was isolated from mouse liver. The assay mixture for ceramide synthase contained 1μM ^{3}H-sphingosine, 25mM potassium, phosphate buffer(PH 7.4), 0.5mM dithiothreitol, 200μM palmitoyl-CoA and 0.2mg of microsomal protein in a total volume of 0.1ml. The reaction was incubated at 37°C for 15 min. The reaction was stopped by extraction of lipids using 2ml of chloroform/methanol (1/2, v/v). The organic phase was dried under nitrogen and applied to TLC plate. Radiolabeled ceramide was resolved by using a solvent: chloroform/methanol/1N ammonium hydroxide(40/10/1, v/v/v). The radioactive spots were scraped from the plates and counted. The solvent system can separate both sphingosine and ceramide.

Sphingosine kinase assay. The reaction solution had 100μg protein of liver homogenate, 45μl assay buffer (50mM K_3PO_4 , 0.267% fatty acid free bovine serum albumin, 3mM $MgCl_2$, 40mM NaF, 2mM semicarbazide, 1mM DTT, 50 mM phosphoenolpyruvate, 10 mM ATP) containing 0.01mM sphingosine and 16uCi/ml [3-^{3}H] sphingosine. The reaction was performed at 37°C for 30 min and stopped by extraction of lipids using 2 volumes of chloroform/methanol/HCl (100/200/1, v/v/v). The organic phase was evaporated in a SpeedVac centrifuge and applied to TLC plate. Radiolabeled sphingosine-1-phosphate was resolved by using a solvent: 1-butanol/acetic acid/water(3:1:1, v/v/v). Radioactive spots were scraped from the plate and counted. The solvent system can separate both sphingosine and sphingosine-1-phosphate.

Results

Table I. Plasma lipid measurement in SMS2 KO, SMS2LTg, and WT mice

Mice	PL(mg/dl)	Chol(mg/dl)	TG(mg/dl)
Chow			
WT	112±16	85±19	52±8
SMS2 KO	123±29	99±10	48±5
WT	128±24	115±12	57±16
SMS2LTg	139±11	110±11	53±14
High-fat			
WT	164±22	139±10	69±6
SMS2 KO	165±25	152±26	65±6
WT	179±18	176±15	73±9
SMS2LTg	182±32	170±13	77±16

Value: mean±SD; n=4-5. PL, choline-containing phospholipids; Chol, total cholesterol; TG, triglyceride. *P<0.01.

Table II. Liver lipid measurement in SMS2 KO, SMS2LTg, and WT mice.

Mice	SM (μg/mg liver)	PC (μg/mg liver)	Cer (ng/mg liver)	Sph (ng/mg liver)	S-1-P (ng/mg liver)	DHS-1-P (ng/mg liver)
WT	0.93±0.11	18±2	82±10	1.6±0.3	0.16±0.01	0.15±0.02
SMS2 KO	0.70±0.05*	19±3	92±9	2.2±0.5	0.11±0.02*	0.13±0.03
WT	0.61±0.03	19±2	87±7	1.2±0.2	0.08±0.02	0.14±0.03
SMS2LTg	0.74±0.09*	20±1	70±9*	1.1±0.1	0.12±0.01*	0.13±0.01

Value: mean±SD; n=4-5. SM, sphingomyelin; PC, phosphatidylcholine; Cer, ceramide; Sph, sphingosine; S-1-P, sphingosine-1-phosphate; DHS-1-P, dihydroxyl-sphingosine-1-phosphate. *P<0.05.

Supplement Figure legends

Supplement Fig.I. Strategy used to disrupt the mouse SMS2 gene. A, The bottom line represents the map of the endogenous mouse SMS2 gene and its flanking sequence. The top line shows the predicted organization of the locus after homologous recombination. A pair of PCR primers indicated was used to confirm the integrity of site-specific integration. B, Tail tip DNA was extracted. Genomic PCR was performed. Wild type mouse (+/+) DNA shows a 760bp band; heterozygous knockout mouse (+/-) DNA shows 760bp and 970bp bands; and homozygous knockout mouse (-/-) DNA shows a 970bp band. NE, neomycin-resistant gene; WT, wild type; KO, knockout.

Supplement Fig.II. Strategy used to establish liver-specific SMS2 transgenic mice. A 2.5-kilobase cDNA fragment of human SMS2 was cloned into the *Sal*I and *Xho*I sites of the pLIV-7 plasmid (29), kindly provided by Dr. John M. Taylor (Gladstone Institute of Cardiovascular Disease, University of California, San Francisco). A linearized fragment of the construct containing the promoter, first exon, first intron, and part of the second exon of the human apoE gene, the human SMS2 cDNA, and the polyadenylation sequence, and hepatic control region of the apoE/C-I gene locus were used to generate transgenic mice by standard procedures.

Supplement Fig. III. Liver ceramidase activity measurement. The ceramidase activity was measured as described in Supplement "Methods".

Supplement Fig. IV. Liver ceramide synthase activity measurement. The ceramide synthase activity was measured as described in Supplement "Methods".

Supplement Fig.V. Liver sphingosine kinase activity measurement. The sphingosine kinase activity was measured as described in Supplement "Methods".

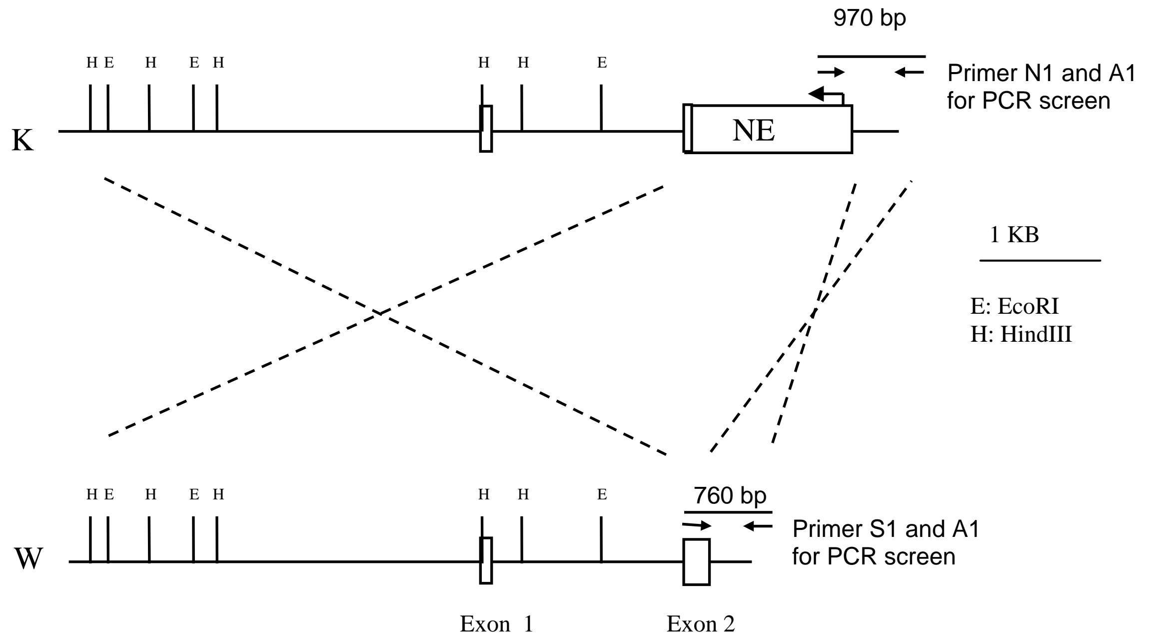
Supplement Fig.VI. Plasma apolipoprotein analyses. Plasma apoE, apoB, and apoA-I levels were determined as previously described. Briefly, 0.2µl plasma was separated by

4-15% SDS gel electrophoresis and immunoblotted with polyclonal antibodies against apoE (Abcam), apoB (US Biological), and apoA-I (Biodesign).). Values are mean ± S.D., N=5, *P<0.01.

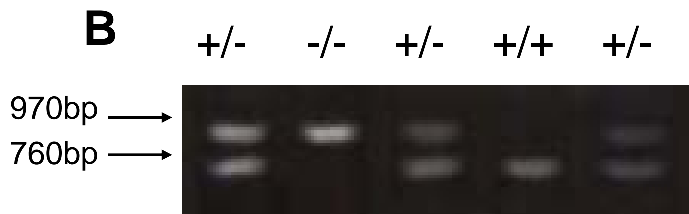
Supplement Fig.VII. Macrophage cholesterol mass efflux was evaluated in WT, SMS2 KO and SMS2Tg mice. WT macrophages (10^5 cells/well) were grown in DMEM with 10% FBS. Cell cholesterol-loading was done by growing the cells overnight in DMEM with 10% FBS containing 50μg/ml acetylated LDL. Cells were washed with PBS and the medium replaced with DMEM containing 10% plasma from WT and SMS2LTg mice (Panel A) or WT and SMS2 KO mice (Panel B). We measured cholesterol in the medium after 24-hours incubation. The increment of cholesterol in the medium stands for the cholesterol effluxed from the macrophages during the experimental period. Values are mean ± S.D., N= 5, P<0.05.

Supplement Fig. I. Liu et al.

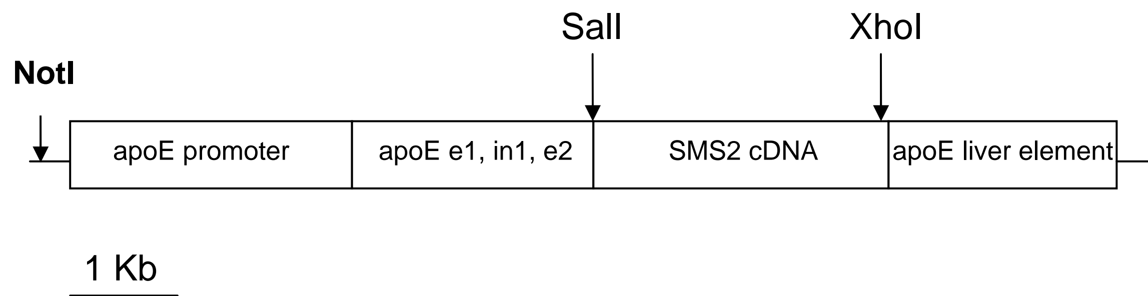
A



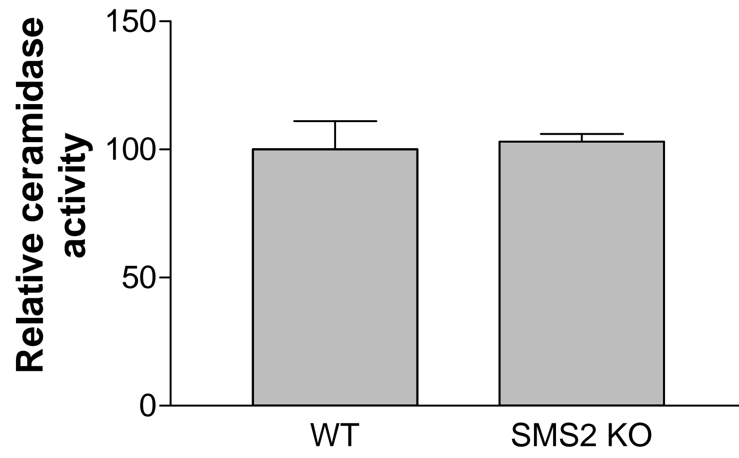
B



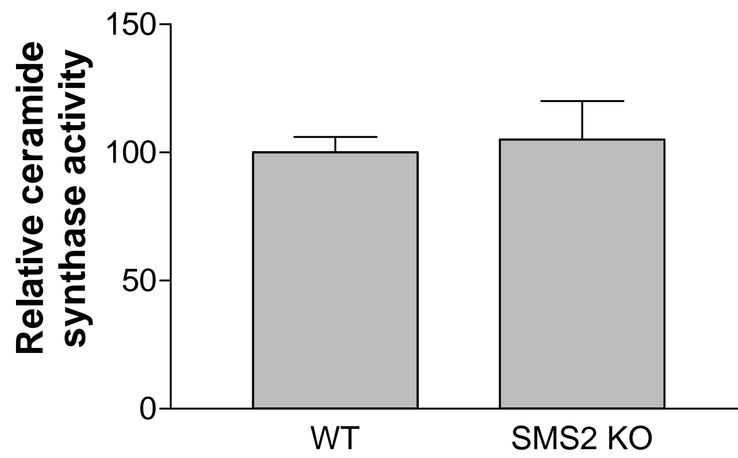
Supplement Fig. II. Liu et al.



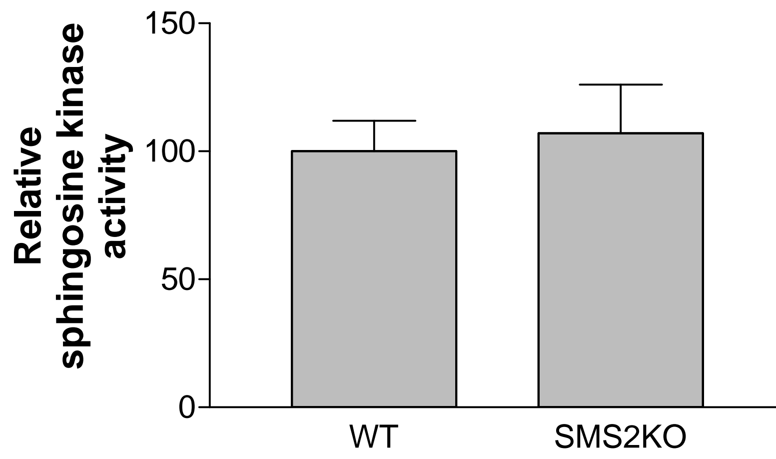
Supplement Fig. III. Liu et al.



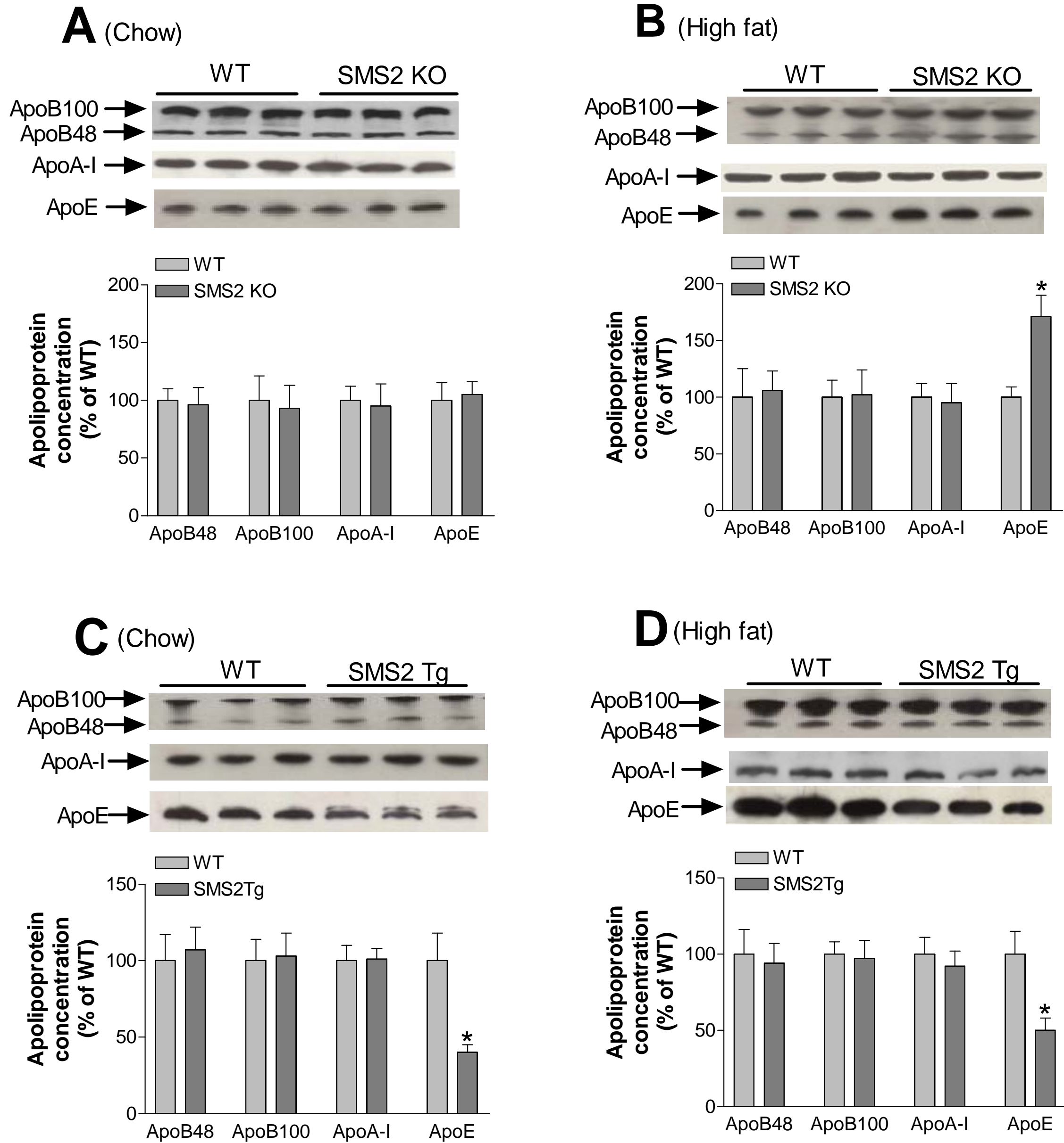
Supplement Fig. IV. Liu et al.



Supplement Fig. V. Liu et al.



Supplement Fig. VI. Liu et al.



Supplement Fig. VII. Liu et al.