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Supplemental Information

Cholesterol-Dependent Degradation of Squalene Monooxygenase, a Control Point in Cholesterol Synthesis beyond HMG-CoA Reductase

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Supplemental Experimental Procedures

Materials

Chemicals and reagents used are listed below with the supplier. Dulbecco’s Modified Eagle’s Medium/Ham’s Nutrient Mixture F-12 (DMEM/F12), Dulbecco’s Modified Eagle’s Medium (DMEM), DMEM low glucose, DMEM high glucose (L-methionine, and L-cysteine free), Fetal Bovine Serum (FBS), Newborn Calf Serum (NCS), penicillin-streptomycin, TRIzol Reagent, SuperScript III First Strand cDNA Synthesis Kit, Lipofectamine LTX, Opti-MEM I medium, Dynabeads, Amplex Red Cholesterol Assay kit, and anti-V5 antibody were purchased from Invitrogen (Carlsbad, CA). [1-14C]-acetic acid sodium salt (specific radioactivity: 56 mCi/mmol) and Glutathione Sepharose 4B beads were purchased from GE Healthcare (Chalfont St. Giles, UK). [2-14C]-mevalonolactone (mevalonate) (specific radioactivity: 40-60 mCi/mmol) and [35S]-Protein Labeling Mix (EXPRE35S35S Protein Labeling Mix, specific radioactivity: >1000 Ci/mmol) were purchased from Perkin Elmer (Waltham, MA). Anti-SQLE (SM) antibody was purchased from Protein Tech Group (Chicago, IL). HA.11 monoclonal antibody was purchased from Covance (Princeton, NJ). Peroxidase-conjugated AffiniPure Donkey Anti-Mouse IgG and Peroxidase-conjugated AffiniPure Donkey Anti-Rabbit IgG were
obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). Lipoprotein-Deficient Serum (LPDS) was prepared from NCS as described previously (Goldstein et al., 1983). LDL (d=1.019-1.063 g/ml) was isolated by standard ultracentrifugation techniques from the plasma of healthy male volunteers (Brown et al., 1996). N-Acetyl-Leu-Leu-Norleu-al (ALLN), anti-α-tubulin antibody, butylated hydroxytoluene, chloroquine, compactin (also called mevastatin), cycloheximide, desmosterol, Dulbecco’s phosphate buffered saline (PBS), lactacystin, methyl-β-cyclodextrin, mevalonate, Z-Leu-Leu-Leu-al (MG132), primers, protease inhibitor cocktail, N-ethylemaleimide, sodium oleate, sodium dodecyl sulfate (SDS), IGEPAL CA-630, sodium deoxycholate, Zaragozic acid A trisodium salt (squalene synthase inhibitor, SSi), methionine, and cysteine were obtained from Sigma (St. Louis, MO). The squalene epoxidase inhibitor, GR144000X (squalene monooxygenase inhibitor, SMi) was kindly donated by GlaxoSmithKline (Middlesex, UK). iProof High-Fidelity DNA Polymerase and Precision Plus Kaleidoscope protein marker were from Bio-Rad Laboratories (Hercules, CA). SYBR Green SensiMix dT was from Quantace (Norwood, MA). 24(S),24-Epoxycholesterol (24,25EC) was obtained from Enzo Life Sciences (Farmingdale, NY). Cholesterol, lanosterol, lathosterol, 7-dehydrocholesterol (7DHC), 24,25-dihydrolanosterol (24,25DHL), 7α-hydroxycholesterol (7αHC), 7β-hydroxycholesterol (7βHC), 7-ketocholesterol (7KC), 19-hydroxycholesterol (19HC), 25-hydroxycholesterol (25HC), and 27-hydroxycholesterol (27HC) were obtained from Steraloids (Newport, RI). If not otherwise mentioned, oxysterols were delivered in ethanol. Sterols and oxysterols complexed with methyl-β-cyclodextrin were prepared as described (Brown et al., 2002). Sterol/CD complexes were diluted without addition of further cyclodextrin, so a constant molar ratio of ~0.1 sterol to methyl-β-cyclodextrin was used. All solvents used for thin layer chromatography (TLC) were analytical reagent grade from Ajax Finechem (Taren Point, NSW, Australia). Chinese Hamster Ovary-7 (CHO-7), SRD-1, SRD-13A, and HEK293 cells were
generous gifts of Drs. Michael S. Brown and Joseph L. Goldstein (UT Southwestern Medical Center, Dallas, TX). SRD-15 cells were generously donated by Dr Russell DeBose-Boyd (UT Southwestern Medical Center, Dallas, TX). HepG2 cells and primary human fibroblasts were kind gifts from the Centre for Vascular Research (UNSW, Sydney, NSW, Australia). BE(2)C cells were generously donated by Dr Louise Lutze-Mann (UNSW, Sydney, NSW, Australia). The HA-tagged ubiquitin plasmid, pMT123, encoding 8 tandem HA-ubiquitins (Treier et al., 1994), was a gift from Dr Dirk Bohmann (University of Rochester Medical Center, Rochester, NY).

**Media Recipes**

Media formulations are described below.

| Medium* |配方 |
|---------|-----|
| A | DMEM/F12 supplemented with 5% LPDS |
| B | Medium A containing 5 µM compactin, and 50 µM mevalonate |
| C | DMEM/F12 supplemented with 5% NCS |
| D | DMEM low glucose supplemented with 10% FBS |
| E | DMEM low glucose supplemented with 5% LPDS |
| F | Medium E containing 5 µM compactin, and 50 µM mevalonate |
| G | DMEM high glucose supplemented with 10% FBS |
| H | DMEM high glucose supplemented with 5% LPDS |
| I | Medium H containing 5 µM compactin, and 50 µM mevalonate |

*All media containing penicillin (100 U/ml), streptomycin (100 µg/ml), and L-glutamine (2 mM)*
Cell Culture

All cells were maintained in monolayer at 37°C in 5% CO₂. Cells were seeded at the following densities: 1×10⁵ cells/well in triplicate in 12-well plates for quantitative real-time PCR; 2×10⁶ cells/6 cm dish for immunoprecipitation; 4×10⁵ cells/10 cm dish for cell fractionation and glutathione sepharose pulldown; 2×10⁵ cells/well in 6-well plates for all other experiments. CHO-7, SRD-1, and SRD-15 cells were maintained in medium A, HepG2 cells in medium D, and BE(2)C, HEK293, and fibroblasts in medium G. SRD-13A cells were maintained in medium C supplemented with 5 μg/ml cholesterol, 1 mM mevalonate, and 20 μM sodium oleate. Unless otherwise stated, cells were statin pretreated in media containing 5 μM statin (compactin) and 50 μM mevalonate overnight (CHO-7, SRD-1, SRD-13A, and SRD-15 cells in medium B, HepG2 cells in medium F, and BE(2)C, HEK293, and fibroblasts in medium I). Cells were then treated with test agents (added in ethanol or dimethylsulfoxide to refreshed media) as indicated in the figure legends. Within an experiment, the final concentration of solvents was kept constant between conditions and did not exceed 0.28 % (v/v).
**Primer Sequences**

Primers are described in the following two tables.

*Primer sequences for quantitative real-time PCR analysis*

| Gene   | Direction | Primer Sequence (5’-3’) | Reference                  |
|--------|-----------|-------------------------|----------------------------|
| *SQLE* (hamster) | Forward   | TCTGATACACGGCTACATAG    | Present study              |
|        | Reverse   | ACTTGCCATGGTGGAAGCAAC   | (Du et al., 2006)          |
| *HMGCR* (hamster) | Forward   | CTGGTGATGGGAGCTTGCTGTG  | (Wong et al., 2006)        |
|        | Reverse   | AATCACAAGCAGGAAAGAC     |                            |
| *Pbgd* (mouse)   | Forward   | AGATTCTTGATACTGCACCTC   |                            |
|        | Reverse   | TGAAAGACAACAGCATCACA    |                            |
**Primer sequences used for cloning and site-directed mutagenesis**

| Name            | Primer Sequence (5'-3')                                                |
|-----------------|------------------------------------------------------------------------|
| topoSQEF        | ATGTGGGACTTTTCTGGGCATTGC                                               |
| topoSQER        | ATGAACCATACTATCTCTTCTTGAG                                             |
| pcTOPO TK F     | GCTTGGGTTAGGGCGTTTTGCCTGTCTTGGCATGGCAGCTTCA                           |
|                 | TCCCCGTGAC                                                             |
| pcTOPO TK R     | GCTTGGGTTAGGGCGTTTTGCCTGTCTTGGCATGGCAGCTTCA                           |
|                 | GCTTTTAGAGC                                                           |
| SM Ntrm KO F    | CCAGTGTGGGTGGAATTGCCCTTATGGAACCTATCACATTTCCAGA                        |
|                 | AACCAATATTTCAGA                                                       |
|                 | AACAAGC                                                               |
| pcV155 R        | TCTTCATGCAATTGTCGGTC                                                  |
| pcDNA3 MCS C F  | AAGGGCAATTCTGCAGATATCCAGACAGATGG                                      |
| pcTOPO EV R     | CTGCAGAATTGCCTTAAGGGCAATTCCACCACACTGGAC                               |
| GAr oligo F     | GCTGGGAGCAGGCGGTGGAAGGCTGAGTGGCAGGTGACAGGTGAGGCTGAGGCGGTGAGGCGGTGAGGCA |
| GAr oligo R     | ACGCAGGGCGGTGACGGAGGCA                                               |
| SM N GAr F      | CCAGTGTGGGTGGAATTGCCCTTATGGAAGGACAGGCGGTGACAGGCGGTGAGGCGGTGAGGCGGTGAG |
| SM N GAr R      | GAAAGTGGCAATGCCCAGAAAAGTCCAACCTGCTCCACCTCCACCTGCTCCACCTCCACCTGCTCCACCTGCA |
| pcC GAr F       | CGTACCAGGTCTCATCATACCACCATCATCACATGCTGGAGCAGGCGGTGAGGCGGTGAGGCGGTGAGGCA |
| Sequence       | Gene   | Description | Sequence       | Gene   | Description |
|---------------|--------|-------------|---------------|--------|-------------|
| GGAGC         | pcGAr  | R           | GAGGCTGATCAGCGGGGTTAACTCAACCTGCTCCACCTCCA GCAC | pcGFP F | GCCGCTCGAGTCTAGAGGGCCCGGCTCAGAAATGAGTGAGC AAGGGCGAGGAG |
|               | pcGFP R|             | CGAGACCCAGGAGGAGGGTTAGGGATAGGGTACCCATCTGTCAGCC | Cd GFP  | GCCGGCAGCGGGCAGCGCGTGCAGAAAGGGCGAGGAGC |
|               | SM d476| GFP R       | GCCGCCGCTGCCGGCTTTCTGGCCTCCTCCTGGCCTC          | C GST   | GCCGGCAGCGGGCCTCCCTCCTTAACCTAGTTATGGAATTT |
|               | M1     | F           | GCCGGCAGCGGGCCTCCCCTATACTAGGTTATTGGGAAATTTTT | C GST   | GTTGGGATAGGGCTTACCCTACGATGCGGCGGCGGCTCG |
|               | V5     | F           | GCCGGCAGCGGGCCTCCCCTATACTAGGTTATTGGGAAATTTTT | C V5    | GTTGGGATAGGGCTTACCCTACGATGCGGCGGCGGCTCG |
|               | N Ub   | F           | GTGGTGGAATTGCCCTTATGGGCTACCCCTATGGGATG      | SM N Ub | CCCAGAAAAGTCCACATACCACCTCTGAGACGGAGGAGGACC |
|               | M1     | F           | ATGTGGGAATTTTCTGGGCATTGC                   | SM M1   | ATGTGGGAATTTTCTGGGCATTGC |
|               | pcDNA3 | n R         | AAGGGCAATTCCACCACACTGG                    | pcDNA3  | AAGGGCAATTCCACCACACTGG |
|               | 2K15R  | F           | CTATTTTTATAGGAGGTTCCGGGGACTTC            | SM 2K15R| CTATTTTTATAGGAGGTTCCGGGGACTTC |
|               | K82R   | R           | GGGGATCTGGGCCAGAAGAAG                      | SM K82R | GGGGATCTGGGCCAGAAGAAG |
|               | K90R   | F           | CAGAAAATAGGGAGCAGGTCCTC                  | SM K90R | CAGAAAATAGGGAGCAGGTCTC |
|               | K100R  | R           | TATTGGTTCCCTCTTCTGCC                    | SM K100R| TATTGGTTCCCTCTTCTGCC |
|               | d476 K0| GFP R       | GCCGCCGCTGCCGGCGCTCCTCTGGCCTC           | SM d476 K0GFP R | GCCGCCGCTGCCGGCGCTCCTCTGGCCTC |
|               | K157R  | R           | CTGTCAGGGCTCCCTTAAAGTCTCTC              | SM K157R| CTGTCAGGGCTCCCTTAAAGTCTCTC |
|               | K268R  | F           | GGGAGTTCAGTACAAGGATAGGGAGACTGGGAGATATCAAGG | SM K268R| GGGAGTTCAGTACAAGGATAGGGAGACTGGGAGATATCAAGG |
| SM K293R R | GGAGACCAGGCTCCTCTGAACTTGG |
|------------|---------------------------|
| SM K318R F | CTTTCTTATGAAGAATGCACCACAGTTAGGGGAAATCATGC TGAAC |
| SM 2K400R R | GAAGAACACCTCCTCCTCCTCACTGATGAAGG |
| SM K429R R | CAGTTTTCTCCATAGCCTTTATATCTTTTAAAAGC |
| SM K436R F | GAAAACTGCTAAGGGGTATCCCTGACC |
| SM K496R R | CATTGCCACCAAGCCTGAAATAAAGAAAAAC |
| SM K536R F | GTATTTTTGCTTTAGGTACAGAACCTTGG |
| SM K570R R | GAACCATATACCTCATTCTGAG |
| SM L5A F | CCTTATGTTGGACTTTTGCCGGCATTGCC |
| SM SFS F | GCATTGCCACCTCCACCCAGCCTTTAGCAAGAAGTTCCGGGACTTCAT |
| SM Y44S F | CTCGCTGGGCTGCTGCTCTCCAGCCGCTGCGCCACC |

**Construction of Expression Plasmids**

PCRs were performed using iProof polymerase, with verification by sequencing.

pCMV-SM-V5 contains the protein coding sequence of human squalene monooxygenase (identical to NM_003129.3 gi.62865634, 927-2651, NP_003120) with a C-terminal V5 epitope and His tag. It was produced by TA cloning into pcDNA3.1-V5-His TOPO vector (Invitrogen) using the primers topoSQEF and topoSQER for PCR. pTK-SM-V5 is identical to pCMV-SM-V5, but with expression driven by the thymidine kinase promoter. It was prepared through
amplification of the promoter and splicing region from pTK-beta (Invitrogen) with primers pcTOPO TK F, pcTOPO TK R, and site-directed mutagenesis (SDM) of pCMV-SM-V5 using the PCR product to replace the cytomegalovirus enhancer/promoter.

SMΔ(W2-K100)-V5 consists of an initiating methionine followed by amino-acids 101-574 of human SM, a multiple cloning site and V5-His tag. Corresponding deletions, pTK-SMΔ(W2-K100)-V5 and pCMV-SMΔ(W2-K100)-V5 were prepared from the respective vectors above with PCR and SDM (Sanchis et al., 2008) with SM Ntrm KO F and pcV155 R. Empty vectors were generated with pcDNA3 MCS C F and pcTOPO EV R.

pTK-SM-V5-GAr contains a 30 amino-acid repeat from Epstein-Barr virus nuclear antigen-1 after the V5-His tag. pTK-GAr-SM-V5-GAr contains an additional copy of the repeat after the initiating methionine. These were constructed using PCR of the repeat and SDM of pTK-SM-V5. The oligos GAr oligo F and GAr oligo R were annealed and extended, then amplified to generate mutagenic megaprimers targeting the N- or C-termini using primers SM N GAr F and SM N GAr R or pcC GAr F and pcC GAr R, respectively.

pTK-SM-N100-GFP-V5 encodes the first 100 amino-acids of human followed by a linker ‘AGSGA’, the enhanced green fluorescent protein and the V5-His tag. It was prepared through SDM of pTK-SM-V5 with a GFP megaprimer derived from pEGFP-N1 (Clontech) with the primers pcGFP F and pcGFP R, followed by a deletion using iPCR/PIPE (Klock et al., 2008) recombination cloning/SDM with the primers Cd GFP F and SM d476 GFP R. pTK-SM-N100-GST-V5 instead includes a glutathione S-transferase (GST) fusion, amplified from pGEX-4T-1 (GE Healthcare) with insert primers C GST F and C GST R, combined with the TK vector product from primers C V5 F and SM d476 GFP R.
Wild-type or mutant ubiquitin was fused to the N-terminus of SM-N100-GFP-V5 with amplification of the insert with SM N Ub F and SM N Ub R from pRK5-HA-Ubiquitin-WT or -K48R (Lim et al., 2005) (Ted Dawson, Johns Hopkins University School of Medicine, Addgene Plasmids 17608 and 17604 respectively), and the vector with SM M1 F and pcDNA3 MCS n R, yielding pTK-Ub-WT-SM-N100-GFP-V5 or pTK-Ub-K48R-SM-N100-GFP-V5, respectively.

Substitution point mutations were prepared using SDM with combinations of the primers SM 2K15R F, SM K82R R, K90R F, SM K100R R, SM d476 K0 GFP R, SM K157R R, SM K268R F, SM K293R R, SM K318R F, SM 2K400R R, SM K429R R, SM K436R F, SM K496R R, SM K536R F, SM K570R R, and alternatively SM L5A F, SM SFS F (Y12,14S) or SM Y44S F.

Metabolic Labeling of Squalene and Cholesterol: Lipid Extraction and Thin Layer Chromatography

Cells were washed once with PBS, lysed in 500 µl 0.1 M NaOH, and rinsed with 1.25 ml H₂O. Protein concentrations were measured by the Bicinchoninic Acid method (Pierce, Rockford, IL). Lysates were saponified with 500 µl 20% KOH (w/v) in methanol, butylated hydroxytoluene (1 µl, 20 mM), and EDTA (20 µl, 20 mM) at 70ºC for 1 hr. After cooling, the lipids were extracted with 2 ml hexane and evaporated to dryness. Extracts were re-dissolved in 60 µl hexane and aliquots corresponding to equivalent amounts of protein separated on Silica Gel 60 F₂₅₄ plates (Merck, Whitehouse Station, NJ) with a mobile phase of hexane: diethyl ether: glacial acetic acid (60:40:1, v/v/v). Bands corresponding to cholesterol and squalene (with relative Rₜ values of ~0.4 and ~0.9, respectively) were visualized using the FLA-5100 phosphorimager (Fujifilm,
Tokyo, Japan). The relative intensities of bands were quantified using Sciencelab ImageGauge 4.0 Software (Fujifilm).

**Immunoprecipitation or GST Pull-Down of Ectopic SM**

Following transfection, statin pretreatment and treatment as indicated in the respective figure legends, CHO-7 cells were washed once and lysed in modified RIPA buffer (1.0% IGEPAL CA-630, 0.1% SDS, 0.5% sodium deoxycholate, 1 mM sodium orthovanadate, 150 mM NaCl, 1 mM Na EDTA, 20 mM Tris-HCl, pH 7.4) supplemented with protease inhibitor cocktail. For the ubiquitination assays, N-ethylmaleimide (10 mM) and ALLN (25 μg/ml) were also added to the RIPA buffer during lysis. Protein concentrations were measured by the Bicinchoninic Acid method (Pierce, Rockford, IL). After protein standardization, lysates were immunoprecipitated with monoclonal anti-V5-conjugated Dynabeads (Invitrogen), according to the manufacturer’s instructions. Pull-down of the N100-GST fusion protein was achieved with an overnight incubation with glutathione sepharose beads at 4°C. Following 4 washes with RIPA buffer, beads were resuspended in 50 μl of ‘loading buffer’ (2 vol RIPA: 2 vol 10% SDS: 1 vol 5x Laemmli buffer). Samples were subjected to 7.5% or 10% SDS-PAGE followed by immunoblot analysis with anti-V5 (for SM) and anti-HA (for ubiquitin) antibodies. For the [35S] metabolic labeling experiments, the gels were visualized using the FLA-5100 phosphorimager (Fujifilm, Tokyo, Japan). The relative intensities of bands were quantified using Sciencelab ImageGauge 4.0 Software (Fujifilm).
Cell Fractionation

Fractions were prepared according to (Feramisco et al., 2004), with minor modifications. CHO-7 cells in 10 cm dishes were grown in medium A (without antibiotic) and transfected with 5 µg of DNA. Cells were harvested after 24 hr by scraping into ice-cold PBS, washed, resuspended in Buffer A (10 mM HEPES-KOH pH 7.4, 10 mM KCl, 1.5 mM MgCl$_2$, 100 mM NaCl, 5 mM Na EDTA, 5 mM Na EGTA, and 250 mM sucrose), and passed through an 18 G needle 50 times. The lysate of equalized protein content was centrifuged at 1,000 x g for 5 min, 4°C, and the post-nuclear supernatant centrifuged at 100,000 x g for 30 min, 4°C, with resuspension of the resulting membrane pellet in an equal volume of the same buffer. The 1,000 x g pellet was resuspended in Buffer B (20 mM Hepes-KOH (pH 7.6), 25% (v/v) glycerol, 0.42 M NaCl, 1.5 mM MgCl$_2$, 5 mM sodium EDTA, and 5 mM sodium EGTA), rotated at 4°C for 1 hr, and similarly centrifuged at 100,000 x g for 30 min at 4°C, with the supernatant yielding the nuclear fraction. Fractions of equal volume were analyzed with SDS-PAGE and immunoblotting.
Figure S1

(A) A schematic of the cholesterol biosynthesis pathway. Abbreviations: 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR), 2,3(S)-monooxidosqualene (MOS), 2,3(S):22,23(S)-dioxidosqualene (DOS). SM also catalyzes the conversion of MOS into...
2,3(S):22,23(S)-dioxidosqualene, the precursor for the potent oxysterol 24(S),25-epoxycholesterol, which fine-tunes acute cholesterol synthesis (Wong et al., 2008). Adapted from (Gill et al., 2008).

(B) CHO-7 cells were statin pretreated as indicated and then treated in medium A with 50 μg/ml LDL, 20 μg/ml Chol/CD, or 1 μg/ml 25HC and labeled with [14C]-acetate for 4 hr. Bands corresponding to cholesterol and squalene were visualized by phosphorimaging and the image shown is representative of at least 2 separate experiments.
Figure S2.

**Figure S2.** Serum derived cholesterol promotes degradation of transfected human SM. (Related to Figure 3.)

CHO-7 cells were transfected with 1 μg of pTK-SM-V5, statin pretreated overnight, and treated in medium B or C (supplemented with 5 μM compactin and 50 μM mevalonate) as indicated for 8 hr, both containing 10 μg/ml cycloheximide. The immunoblot shown is representative of at least 2 separate experiments.
Figure S3. Effect of sterol depletion on cellular cholesterol levels and comparison of sterol specificity for selected processes. (Related to Figure 4.)

(A) CHO-7 cells were pretreated overnight (16 hr) in either medium A, medium B, medium C, or medium C with 5 µM compactin (statin) and 50 µM mevalonate. The media was then refreshed with the same formulations between conditions as for the pretreatments, but with the addition of 10

| Sterol                        | SREBP Inhibition | In vitro Binding | Ub/Deg. | HMGR | SM |
|-------------------------------|------------------|------------------|---------|------|----|
| 7α-Hydroxycholesterol         | +/-              | +/-              | -       | +    |    |
| 7β-Hydroxycholesterol         | +/-              | +/-              | -       | +    |    |
| 7-Ketocholesterol             | +/-              | +/-              | -       | +    |    |
| 19-Hydroxycholesterol         | -                | -                | +       | +    |    |
| 24(S),25-Epoxycholesterol     | +                | -                | +       | -    |    |
| 25-Hydroxycholesterol         | +                | -                | +       | -    |    |
| 27-Hydroxycholesterol         | +                | -                | +       | -    |    |
| Cholesterol                   | +                | +                | -       | -    | +  |
| Desmosterol                   | +                | +                | -       | -    | +  |
| 7-Dehydrocholesterol          | -                | nd               | nd      | +/-  | -  |
| Lathosterol                   | -                | nd               | nd      | nd   | -  |
| 24,25-Dihydrolanosterol       | -                | nd               | nd      | +    | -  |
| Lanosterol                    | -                | -                | -       | -    | -  |
µg/mL cycloheximide, and harvested after 8 hr for cholesterol mass determination similarly to Figure 4H. The data is presented as mean + SEM from 3 separate experiments.

(B) Comparison of the ability (high, +; moderate, +/-; negligible, -; not determined, nd) of the listed sterols to inhibit SREBP processing, bind to SCAP or Insig, and stimulate ubiquitination (or degradation) of HMGR or SM. Adapted from (Radhakrishnan et al., 2007), and partly derived from data from (Adams et al., 2004; Song et al., 2004; Song et al., 2005) and (Fitzky et al., 2001). The last column summarizes the results from Figures 4F and G of the current work.
Figure S4

(A and B) SRD-1 cells were statin pretreated overnight and treated in medium B containing cycloheximide (10 μg/ml) with or without Chol/CD (20 μg/ml) and with MG132 (10 μM), ALLN (25 μg/ml), lactacystin (10 μM) and chloroquine (200 μM) as indicated for 4 hr.

(A) Densitometric values for SM protein were normalized to the vehicle-treated control condition, which was set to 1. The data is presented as mean ± SEM from 4 separate experiments. In cholesterol-treated cells, all three proteasomal inhibitors significantly increased SM levels above control values (p < 0.05 by paired t-test).

(B) This immunoblot is representative of 3 separate experiments.
Figure S5

A

| Human  | 1 | 2 | 3 | 4 | 5 | 6 |
|--------|---|---|---|---|---|---|
| Rat    |   |   |   |   |   |   |
| Fish   |   |   |   |   |   |   |
| Sea Urchin |   |   |   |   |   |   |
| LEDGFIP |   |   |   |   |   |   |
| Yeast  |   |   |   |   |   |   |

B

| Lane | 1 | 2 | 3 | 4 | 5 | 6 |
|------|---|---|---|---|---|---|
| V5   |   |   |   |   |   |   |

| Fraction | N | M | C | N | M | C |
|-----------|---|---|---|---|---|---|
| pSM-V5    |   |   |   |   |   |   |
| WT        |   |   |   |   |   |   |
| Δ(W2-K100)|   |   |   |   |   |   |
| N100-GFP  |   |   |   |   |   |   |

| Lane | 1 | 2 | 3 |
|------|---|---|---|
| V5   |   |   |   |

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Figure S5. SM sequence conservation, membrane localization, and mutation experiments.

(Related to Figure 7.)

(A) Multiple sequence alignment of SM protein for selected species, constructed using ClustalW2. Alignment of human (Homo sapiens, NP_003120), rat (Rattus norvegicus, P52020), finch (Taeniopygia guttata, XP_002187271), zebra fish (Danio rerio, NP_001103509), lancelet (Branchiostoma floridana, XP_002594656), slime mould (Dictyostelium discoideum, XP_629022) and yeast (Saccharomyces cerevisiae, P32476).

(B) Membrane localization of truncation mutants. CHO-7 cells were transfected as indicated and harvested for cell fractionation as described in the Supplemental Experimental Procedures. SM protein was analyzed by immunoblotting. N, nuclear; M, membrane (100,000 x g pellet); C, cytosol (100,000 x g supernatant).

(C-E) Substitution mutants of SM show cholesterol-dependent regulation. CHO-7 cells were transfected with 0.5 µg (C, E) or 1 µg (D) of plasmid as indicated – SM-N100-KO-GST-V5 (KO) used in (E) contains no lysines in the first 100 hundred amino-acids, but others remain within GST and the V5 tag. Following statin pretreatment, cells were treated in medium B containing cycloheximide (10 µg/ml) with or without Chol/CD (20 µg/ml) for 8 hr. SM protein was analyzed by immunoblotting. Immunoblots shown are each representative of at least 2 separate experiments.

(F) CHO-7 cells were transfected with 0.5 µg pTK-Ub-WT-SM-N100-GFP-V5 (UbWT) or pTK-Ub-K48R-SM-N100-GFP-V5 (UbK48R), statin pretreated and treated as in (D-E) (representative of 2 separate experiments). Polyubiquitin chains recognized by the proteasome are made up of G76-K48 inter-ubiquitin linkages, so the K48R mutant causes premature chain termination, inhibiting degradation (Ward et al., 1995). Expression of TK-driven N100-GFP was abrogated when co-transfected with CMV-driven mutant ubiquitin (data not shown), possibly due to
transcriptional squelching by the CMV promoter. Hence to avoid this, ubiquitin was delivered under the control of the TK promoter by fusing it to the N-terminus of SM. This approach is possible because ubiquitin fusions are efficiently processed to liberate free ubiquitin by deubiquitinating enzymes (Treier et al., 1994). Thus, the size of the immunoblotted N100-GFP did not shift.

(G) CHO-7 cells were transfected with 1.5 μg of pTK-SM-N100-GST-V5 (N100-GST) and 0.5 μg of pMT123 (pUb-HA, HA-tagged ubiquitin). Following statin pretreatment, cells were treated in medium B with or without Chol/CD (20 μg/ml) and MG132 (10 μM) for 1 hr. N100-GST protein was pulled down with glutathione sepharose beads and immunoblotted for V5 (N100-GST) and HA-ubiquitin. Representative of 3 separate experiments.
Supplemental References

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