Supplementary Materials for

Lysosomal Cholesterol Activates mTORC1 via an SLC38A9-Niemann Pick C1 Signaling Complex

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This PDF file includes:

Figure S1: Castellano BM, Thelen AM et al

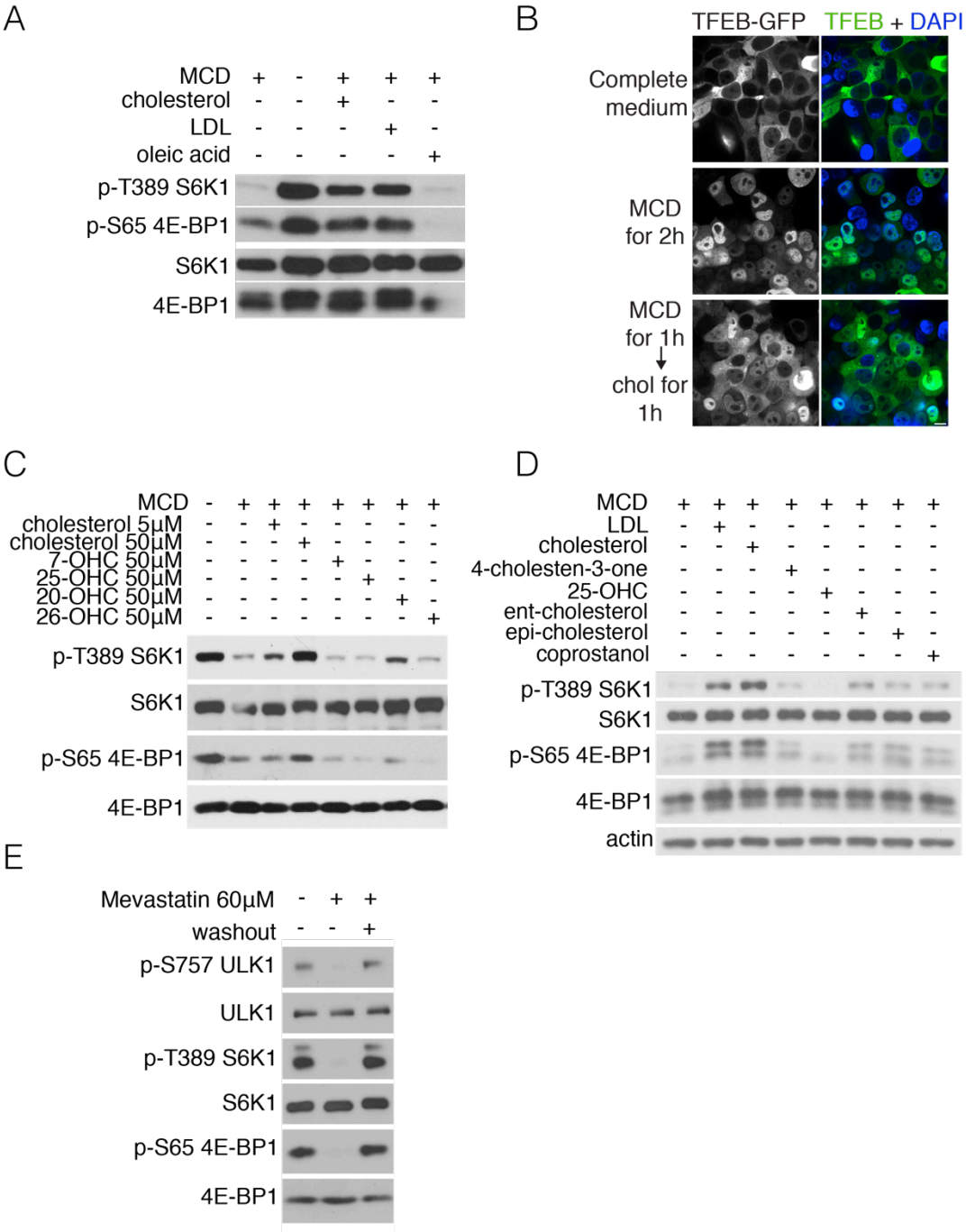

Fig. S1. Specificity of mTORC1 regulation by cellular cholesterol

(**A**) CHO cells were sterol-depleted with 1% MCD for 2h and, where indicated, restimulated for 1h with 50μM cholesterol in 1:1 complex with MCD, 5μg/ml LDL or 5μg/ml oleic acid. Cell lysates were analyzed for phosphorylation status of S6K and 4EBP1. (**B**) Regulation of TFEB nuclear localization by cholesterol levels. HEK-293T cells stably expressing TFEB-GFP were sterol-depleted with 0.75% MCD for 2h and, where indicated, restimulated for 1h with 50μM cholesterol in 1:1 complex with MCD. Scale bar, 10μm. (**C**) HEK-293T cells were sterol-depleted for 2h and, where indicated, restimulated for 1h with the indicated concentrations of cholesterol or oxysterols. Cell lysates were analyzed for phosphorylation status of S6K1 and 4E-BP1. (**D**) HEK-293T cells were sterol-depleted for 2h and, where indicated, restimulated for 1h with the indicated concentrations of sterol-related ligands. Cell lysates were analyzed for phosphorylation status of S6K1 and 4E-BP1. (**E**) Regulation of mTORC1 signaling by statins. HEK-293T cells were incubated with mevastatin for 3h followed by 1h washout and immunoblotting for the indicated proteins and phospho-proteins.

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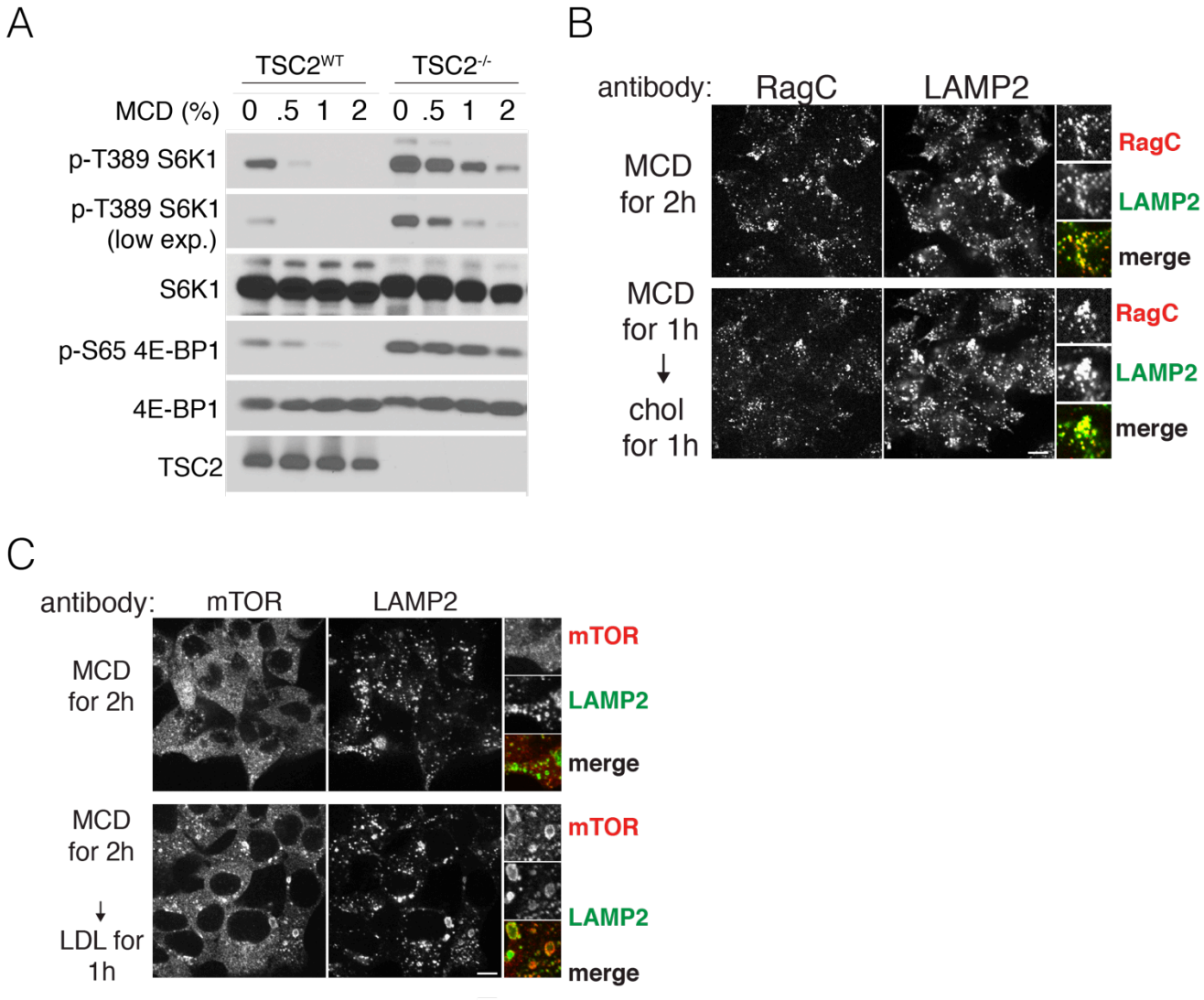

Fig. S2. Cholesterol regulates mTORC1 via a pathway distinct from growth factor signaling
(**A**) Cholesterol dependence of mTORC1 signaling in $TSC2^{WT}$ and $TSC2^{-/-}$ MEFs. Cells of the indicated genotype were incubated for 3h with increasing concentrations of MCD, followed by immunoblotting for the indicated proteins. (**B**) Cholesterol levels do not affect localization of RagC to LAMP2-positive lysosomes. HEK-293T cells were sterol-depleted for 2h and, where indicated, restimulated with LDL for 1h, followed by immunofluorescence for the indicated endogenous proteins. Scale bar, 10μm. For quantification, see Fig. 1I. (**C**) LDL regulates mTORC1 recruitment to LAMP2-positive lysosomes. HEK-293T cells were sterol-depleted for 2h and, where indicated, restimulated with LDL for 1h, followed by immunofluorescence for the indicated endogenous proteins. Scale bar, 10μm.

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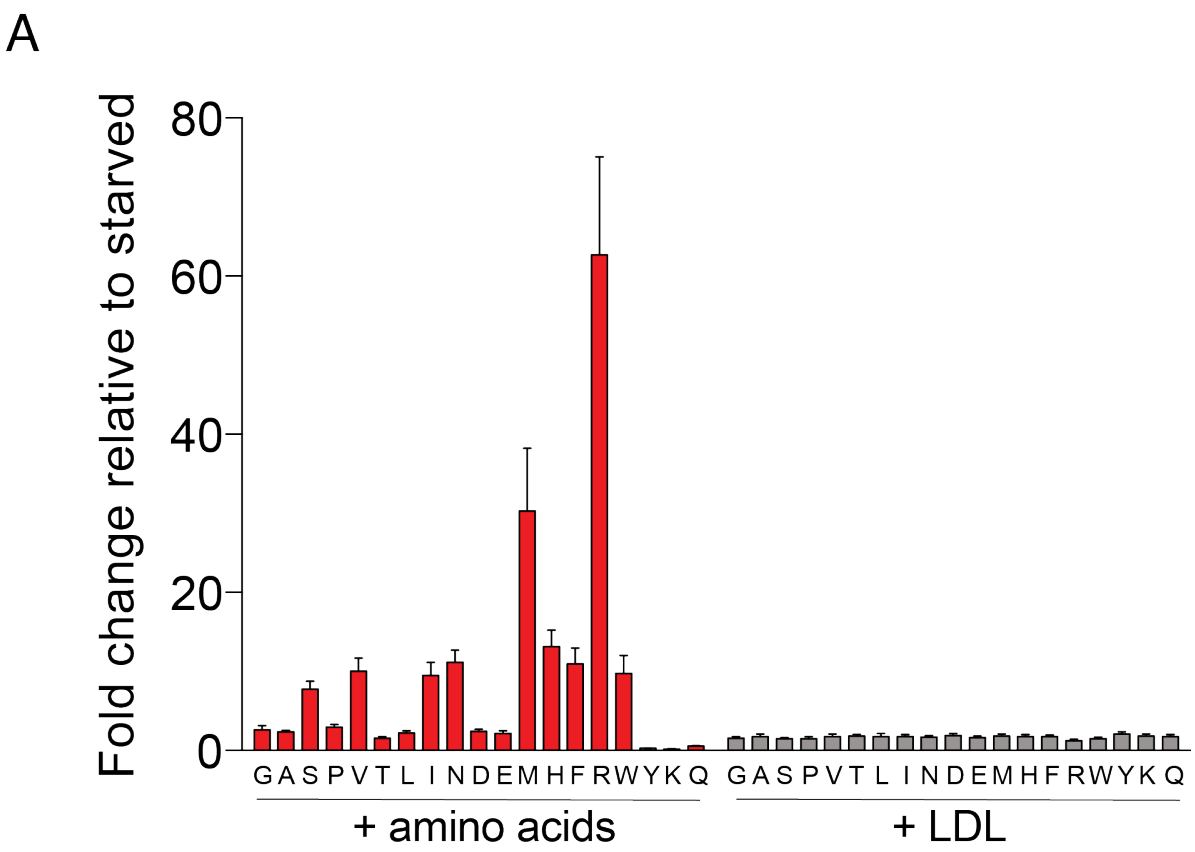

Fig. S3. Cholesterol starvation/refeeding does not alter intracellular amino acid levels HEK-293T cells were subjected to starvation and refeeding with amino acids (red bars) or by MCD-mediated sterol depletion followed by refeeding with LDL (grey bars). Following cell lysis, amino acid levels were measured using single reaction monitoring (SRM)-based LC-MS/MS using previously established methods **(*1*)**. Metabolites were quantified by integrating the area under the curve for individual SRMs and normalized to internal standard levels and external standard curves. Shown are fold changes relative to starved. Each bar represents the mean + SD from N=5 independent samples per condition.

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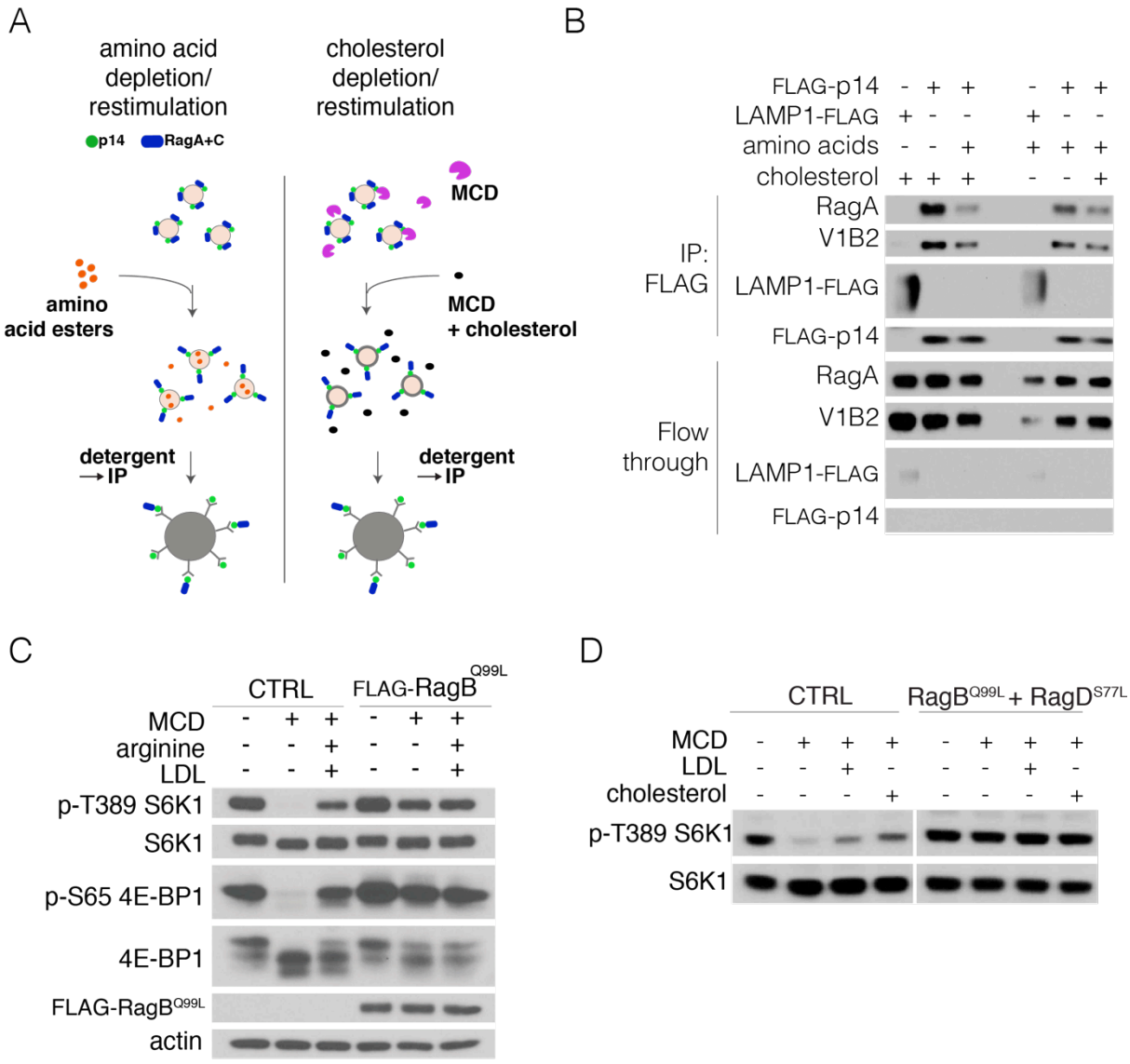

Fig. S4. Cholesterol regulates mTORC1 upstream of the Rag GTPases

(**A**) Organelle-based *in vitro* assay. A light organelle preparation stably expressing FLAG-tagged proteins is stimulated with amino acid esters for 15 min (*left*) or sterol-depleted with 0.1% MCD for 1h followed by restimulation with MCD:cholesterol for 15 min (*right*). Interaction with endogenous proteins is determined by immunoblotting following detergent solubilization and FLAG immunoprecipitation. (**B**) Regulation of the interaction between Ragulator and endogenous Rag GTPases and v-ATPase by amino acids and cholesterol *in vitro*. Organelle preps stably expressing FLAG-p14 or LAMP1-mRFP-FLAG^{X2} (LRF, negative control) were subjected to starvation/restimulation with amino acids or cholesterol. Interaction with RagA and V1B2 was determined by immunoblotting. (**C**) HEK-293T cells stably expressing the constitutively active FLAG-RagB^{Q99L} mutant, along with control HEK-293T cells, were sterol- and arginine-starved for 2h and, where indicated, restimulated with LDL for 1h. Cell lysates were immunoblotted for the indicated proteins and phospho-proteins. (**D**) CHO cells stably expressing the constitutively active GFP-RagB^{Q99L} and RagD^{S77L} mutants, along with control CHO cells, were sterol-depleted for 2h and, where indicated, restimulated for 1h. Cell lysates were immunoblotted for the indicated proteins and phospho-proteins.

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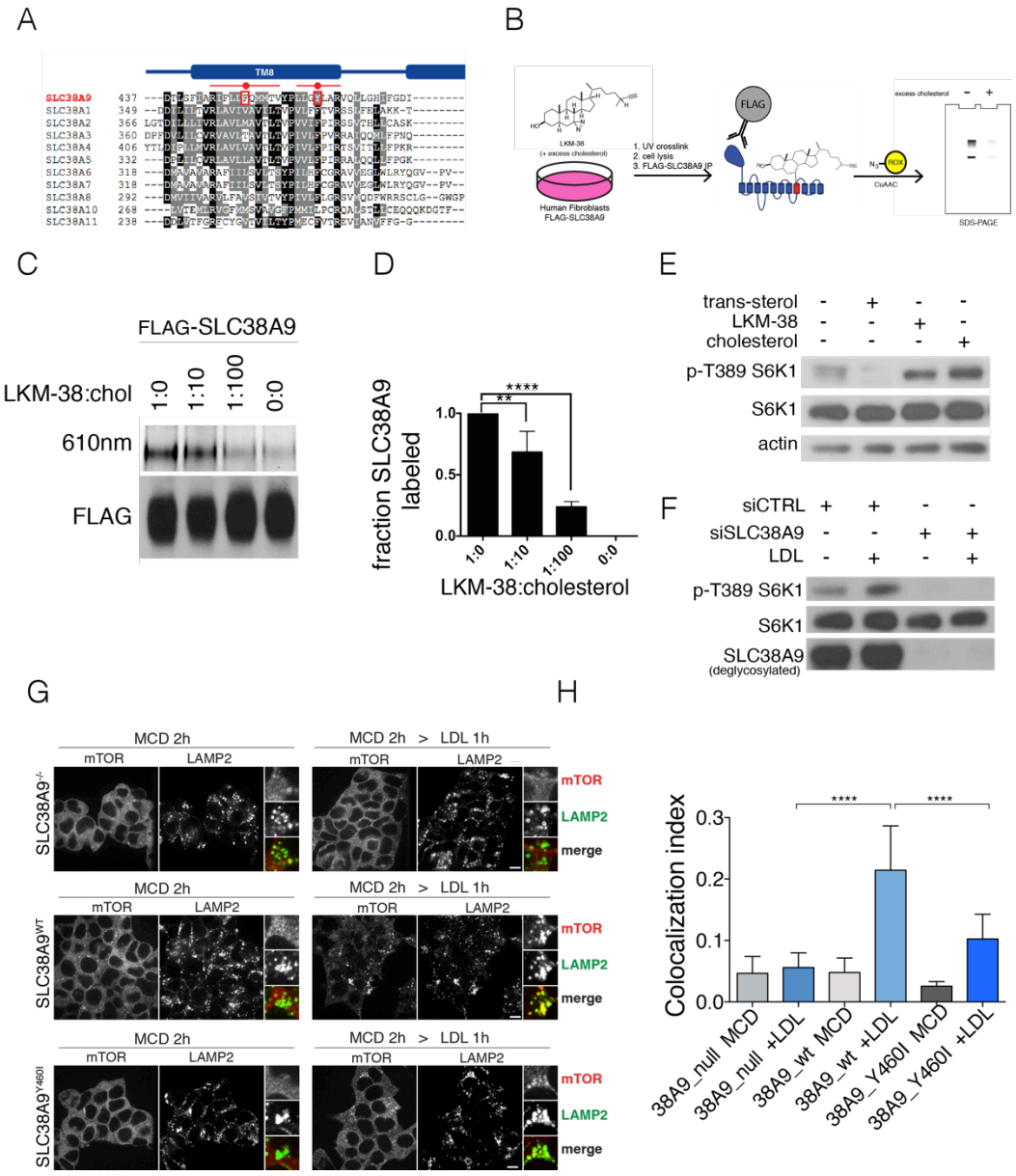

Fig. S5. SLC38A9 interacts with cholesterol and is required for mTORC1 activation in response to cholesterol

(A) Alignment of transmembrane helix 8 (TM8) of SLC38A9 with other members of the SLC38 transporter family. The red boxes highlight essential Phe 449 and Tyr 460 within the CARC and CRAC domains, respectively. **(B)** Schematic of the cholesterol-photoactivation assay. Human fibroblast cells stably expressing FLAG-SLC38A9 are treated with the photoactivatable cholesterol analogue LKM-38, followed by UV-crosslinking, FLAG immunoprecipitation and clicking to rhodamine-X azide (ROX). (**C**) Cross-linking of the photoactivatable-cholesterol analogue LKM-38 to FLAG-SLC38A9 as described in (B), in the presence of increasing amounts of competing native cholesterol. The FLAG blot shows the immunoprecipitated FLAG-SLC38A9 in each condition (**D**) Quantification of 3 independent sterol cross-linking experiments as described in (C). Shown are mean + SD. N=3 samples/condition. ANOVA: $p < 0.0001$ followed by Tukey's t-test: $^{**}p < 0.01$, $^{****}p < 0.0001$. (**E**) HEK-293T cells were sterol-depleted for 2h and, where indicated, restimulated for 1h with identical concentrations of the indicated sterol-related ligands. Cell lysates were analyzed for phosphorylation status of S6K1. (**F**) HEK-293T cells treated with a non-targeting siRNA or with a siRNA pool against SLC38A9 were sterol-depleted for 2h and, where indicated, restimulated with LDL for 1h. Cell lysates were immunoblotted for the indicated proteins and phospho-proteins. (**G**) SLC38A9-deleted HEK-293T cells re-expressing wild-type or Y460I-mutated FLAG-SLC38A9 were subjected to the indicated treatments, followed by immunofluorescence for endogenous mTOR and LAMP2. Scale bar, 10µm. (**H**) Quantification of mTOR-LAMP2 co-localization from the experiment in (E). Shown are mean + SD. N=15 cells/condition. ANOVA: $p < 0.0001$ followed by Tukey's t-test: $^{****}p < 0.0001$.

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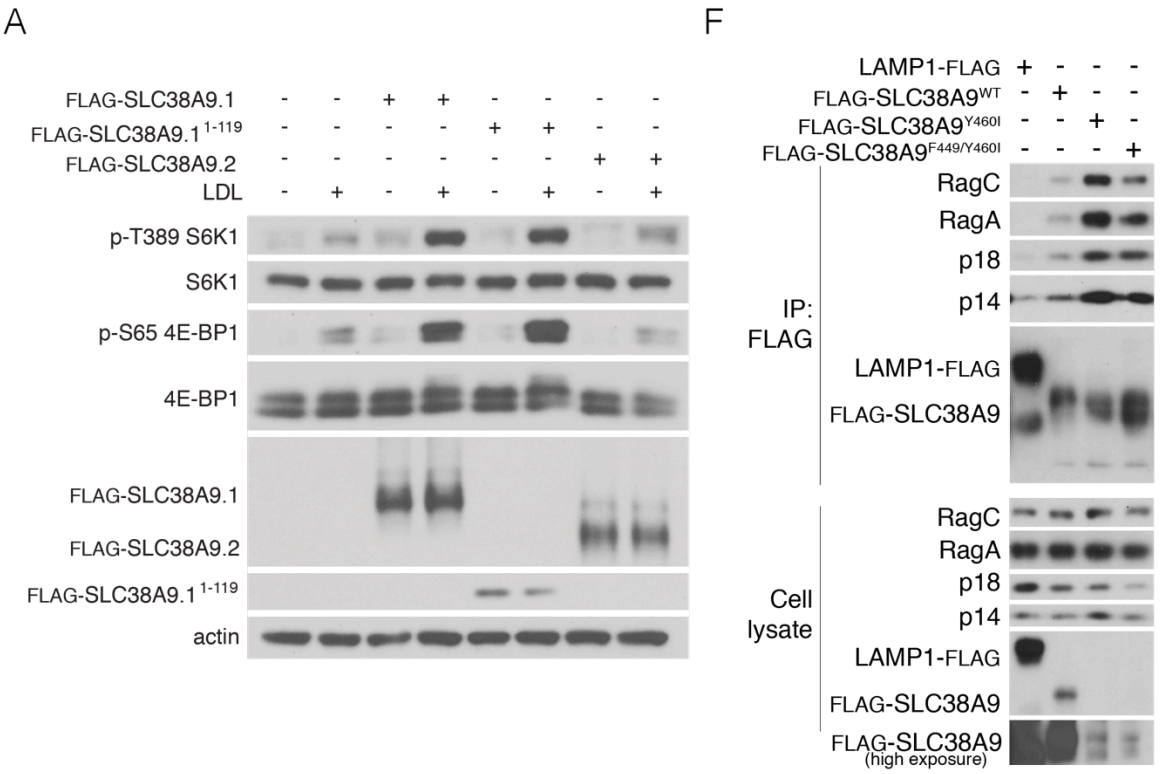

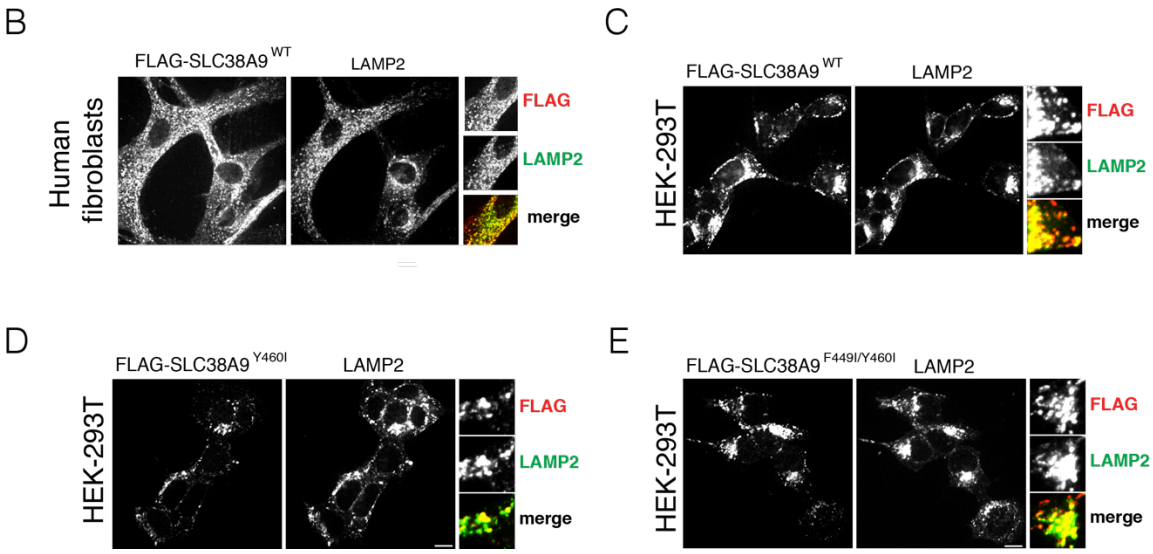

Fig. S6. SLC38A9 participates in mTORC1 regulation by cholesterol

(**A**) Overexpression of SLC38A9.1 (full length) and its 1-119 aa N-terminal fragment, but not SLC38A9.2 (which lacks the N-terminal domain) enhances mTORC1 response to cholesterol. HEK-293T cells overexpressing the indicated FLAG-tagged SLC38A9 constructs were subjected to cholesterol depletion, followed by restimulation with LDL where indicated. Cell lysates were analyzed for phosphorylation status of S6K (T389) and 4E-BP1 (S65) and for total protein levels. (**B-E**) The CARC/CRAC mutants of SLC38A9 are correctly targeted to the lysosome. Human fibroblasts and HEK-293T cells stably expressing the indicated FLAG-tagged SLC38A9 constructs were fixed and subjected to double immunofluorescence for FLAG and endogenous LAMP2. Scale bar, 10μm. (**F**) Enhanced interaction of the SLC38A9 CARC-CRAC mutants with the mTORC1 scaffolding complex. SLC38A9-deleted HEK-293T cells stably expressing the indicated FLAG-SLC38A9 constructs were subjected to FLAG immunoprecipitation, followed by immunoblotting for the indicated proteins.

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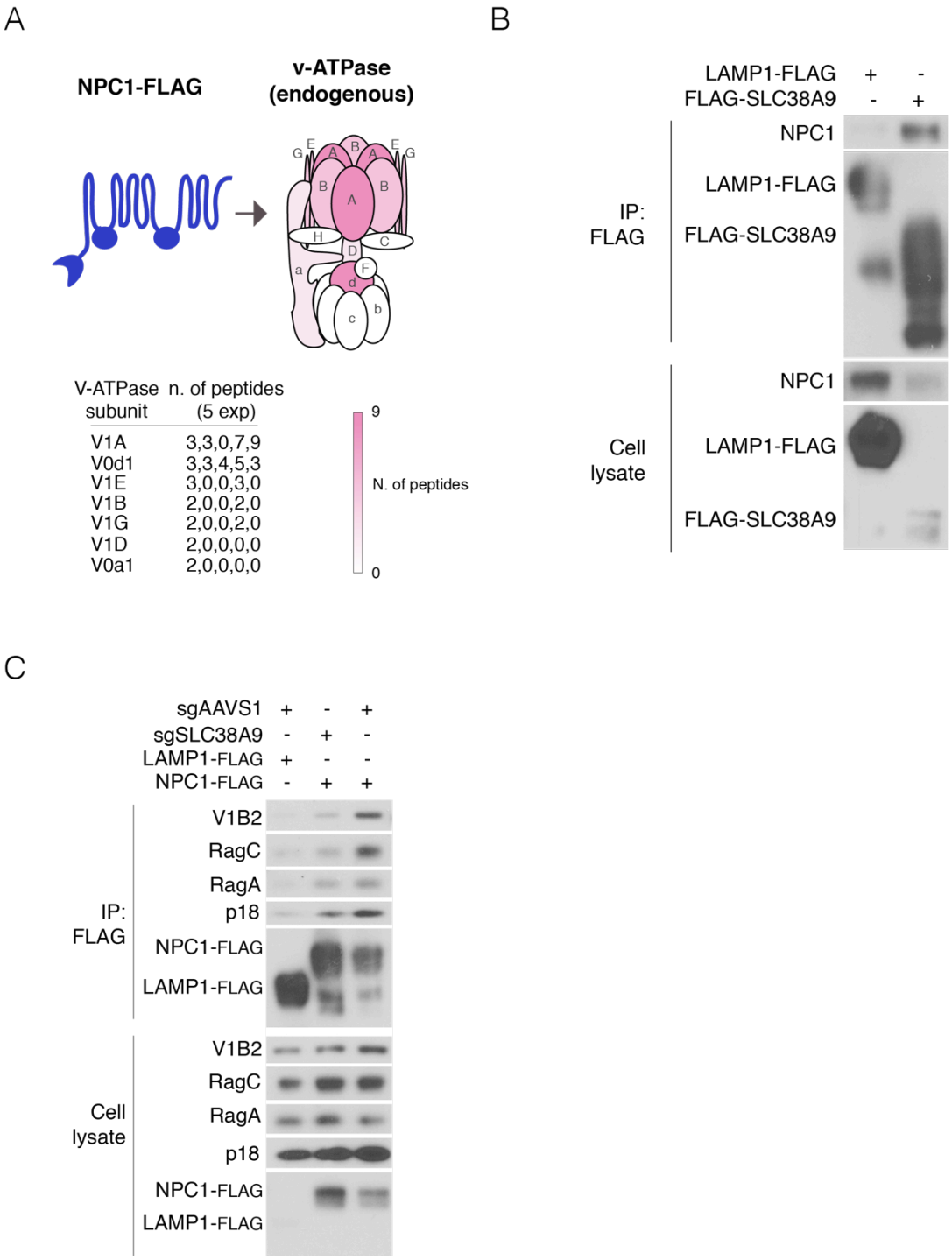

Fig. S7. NPC1 binds to the mTORC1 scaffolding complex via SLC38A9

(A) Cartoon summarizing mass spectrometry analyses of immunoprecipitates from HEK-293T cells expressing FLAG-NPC1. v-ATPase subunits are color-coded according to their peptide representation. Peptide counts for v-ATPase subunits from 5 independent experiments are shown in the table below. **(B)** HEK-293T cells stably expressing LAMP1-FLAG or NPC1-FLAG were lysed in the presence of Triton X-100 and subjected to FLAG immunoprecipitation followed by immunoblotting for the indicated proteins. **(C)** SLC38A9 is required for the interaction of NPC1 with the mTORC1 scaffolding complex. Control or SLC38A9-deleted HEK-293T cells stably expressing NPC1-FLAG were lysed and subjected to FLAG IP followed by immunoblotting for the indicated proteins. LAMP1-FLAG expressing HEK-293T cells were used as negative control.

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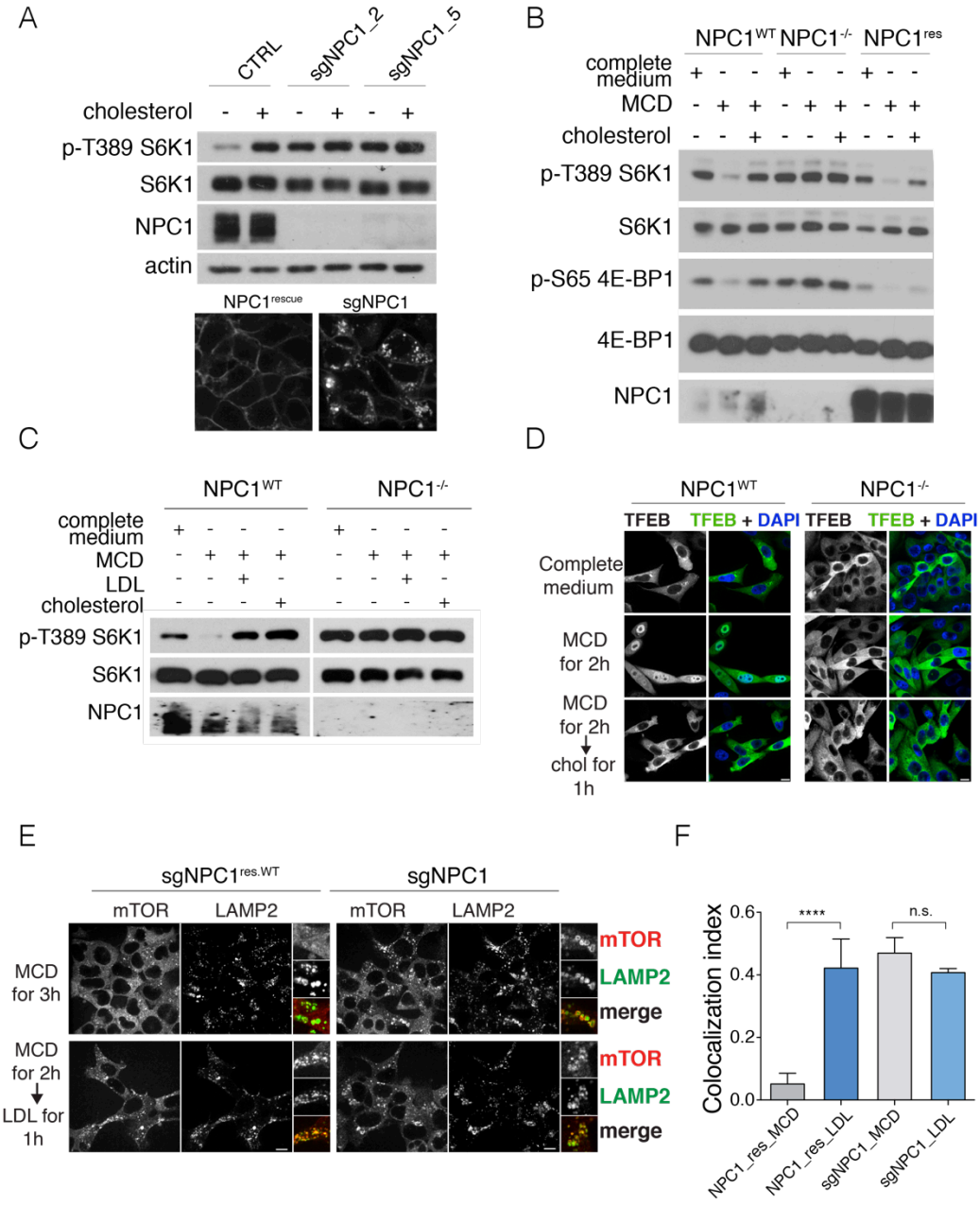

Fig. S8. Loss of NPC1 renders mTORC1 insensitive to sterol starvation

(**A**) (*top*) Control HEK-293T cells, or HEK-293T cells deleted for the NPC1 gene via CRISPR/Cas9 (clones 2 and 5) were sterol-depleted for 2h, or depleted and restimulated with MCD:cholesterol for 1h. Cell lysates were immunoblotted for the indicated proteins and phospho-proteins. (*bottom*) Filipin staining reveals lysosomal cholesterol accumulation in NPC1-deleted but not in control HEK-293T cells. (**B**) WT, NPC1$^{-/-}$ and NPC1 rescue CHO cells were sterol-depleted for 2h and, where indicated, restimulated with MCD: cholesterol for 1h. Cell lysates were immunoblotted for the indicated proteins and phospho-proteins. (**C**) WT and NPC1$^{-/-}$ MEFs were sterol-depleted for 2h or depleted and restimulated with MCD:cholesterol or 25μg/ml LDL for 1h. Cell lysates were immunoblotted for the indicated proteins and phospho-proteins. (**D**) Nuclear translocation of TFEB in response to cholesterol levels requires NPC1. Control and NPC1$^{-/-}$ CHO cells stably expressing TFEB-GFP were sterol-depleted for 2h or depleted and restimulated with MCD: cholesterol for 1h. Scale bar, 10μm. (**E**) Lysosomal localization of mTORC1 is insensitive to sterol depletion in the absence of NPC1. NPC1-deleted or NPC1-FLAG rescued HEK-293T cells were sterol-depleted for 2h or depleted and restimulated with 25μg/ml LDL, and subjected to double immunofluorescence for mTOR and LAMP1. Scale bar, 10μm. (**F**) Quantification of mTOR-LAMP2 co-localization from the experiment in (E). Shown are mean + SD. N=15 cells/condition. ANOVA: $p < 0.0001$ followed by Tukey's t-test: ****$p < 0.0001$.

Figure S9: Castellano BM, Thelen AM et al

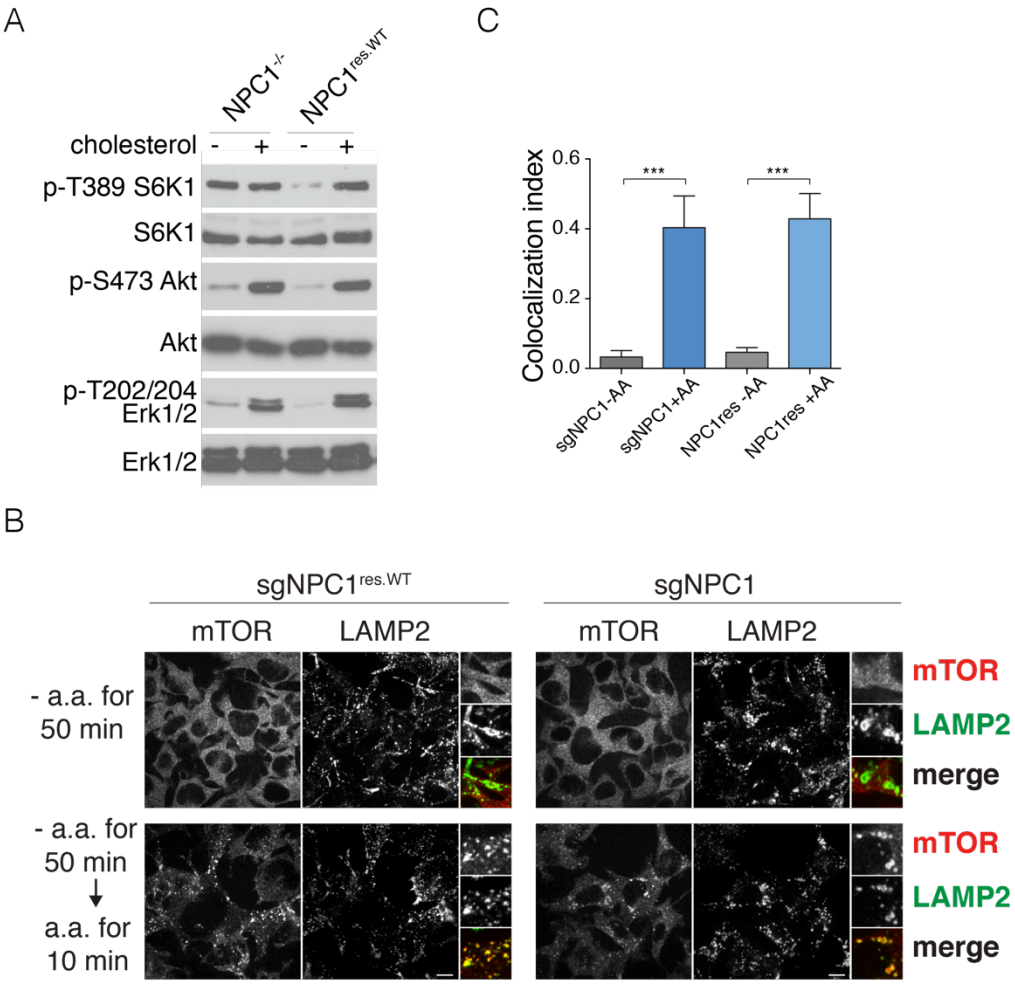

Fig. S9. NPC1 specifically functions in mTORC1 regulation by cholesterol but not amino acids
(A) NPC1 is required for cholesterol sensing by mTORC1 but not by Akt or MAPK. NPC1-null and NPC1-FLAG rescued CHO cells were sterol-depleted for 2h in 1% MCD or depleted and restimulated with MCD: cholesterol for 1h. Cell lysates were immunoblotted for the indicated proteins and phospho-proteins. (**B**) NPC1-deleted or NPC1-FLAG rescued HEK-293T cells were starved for amino acids for 50 minutes, or starved and restimulated with amino acids for 10 minutes, followed by double immunofluorescence for endogenous mTOR and LAMP2. Scale bar, 10μm. (**C**) Quantification of mTOR-LAMP2 co-localization from the experiment in (B). Shown are mean + SD. N=15 cells/condition. ANOVA: $p < 0.0001$ followed by Tukey's t-test: ****$p < 0.0001$.

MATERIALS & METHODS

Materials

Reagents were obtained from the following sources: antibodies to phospho-T389 S6K1 (9234S), S6K1 (2708S), phospho-S65 4EBP (9451S), 4EBP (9644S and 9452S), phospho-S757 ULK (6888S), ULK (8054S), TSC2 (4308S), RagA (4357S), RagC (3360S), LAMTOR1 (8975S), LAMTOR2 (8145S), mTOR (2983S and 2972S), raptor (2280S), phospho-S473 Akt (4060S), Akt (4691), phospho-T202/204 ERK1/2 (9101S), ERK1/2 (4695S) and the FLAG (2368S) epitope from Cell Signaling Technology; antibodies to NPC1 (ab36983), ATP6V1B2 (ab73404) from Abcam; antibody to LAMP2 (sc-18822) from Santa Cruz Biotechnology; antibody to SLC38A9 (HPA043785) from Sigma Aldrich; HRP-labeled anti-rabbit (PI-1000) secondary antibodies from Vector Laboratories. RPMI, FLAG M2 affinity gel, ATP, amino acids, amino acid esters, cholesterol, filipin and methyl-beta-cyclodextrin (MCD) from Sigma-Aldrich; Pierce Protease Inhibitor Tablets from Fischer Scientific; DMEM from Life Technologies; PNGaseF from New England Biolabs; Cholesterol oxidase from MilliPore; trans-sterol refers to PhotoClick Cholesterol from Avanti Polar Lipids, Inc.; Lipoprotein, low density from Alfa Aesar; oxysterols (5-CHOLESTEN-3β, 25-DIOL; 5-CHOLESTEN-3β, 7β-DIOL; 5-, 25R-CHOLESTEN-3β, 26-DIOL; 5-CHOLESTEN-3β, 20α-DIOL) and mevastatin from Steraloids. siRNA smartpools against SLC38A9 were purchased from Dharmacon.

A rabbit polyclonal antibody against NPC1 was a generous gift from Linton Traub (Univ. of Pittsburgh).

Cholesterol starvation/stimulation in cells

HEK-293T, CHO or MEF cells in culture dishes were rinsed once with serum-free media and incubated in DMEM containing 0.5-1.0% methyl-beta cyclodextrin (MCD) supplemented with 0.5% lipid-depleted serum (LDS) for 2 hours. Cells were then transferred to DMEM supplemented with 0.5% LDS and 0.1% MCD (starved condition), or to DMEM + 0.5% LDS containing 20μg/ml cholesterol pre-complexed with 0.1% MCD (resulting in MCD:cholesterol at 1:1 molar ratio, 50μM), or to DMEM + 0.5% LDS + 0.1% MCD supplemented with 25-50μg/ml low-density lipoprotein (LDL) and incubated for 1-2 hours.

MCD:cholesterol complexes were prepared by diluting a 20mg/ml cholesterol stock solution (in EtOH) 1000-fold into a 15-ml falcon tube containing DMEM + 0.1% MCD + 0.5% LDS, resulting in 50μM final concentration of both cholesterol and MCD. The tube was vortexed and incubated in a 37C water bath for 2h.

Lipid-depleted serum was prepared as described (*2*).

Cell lysis and immunoprecipitation

HEK-293T cells stably expressing FLAG-tagged proteins were rinsed once with ice-cold PBS and lysed in ice-cold lysis buffer (150mM NaCl, 20 mM HEPES [pH 7.4], 2 mM EDTA, 0.3% CHAPS or 1% Triton X-100, and one tablet of EDTA-free protease inhibitors per 50 ml). Cell lysates were cleared by centrifugation at 13,000 rpm for 10 minutes in a microfuge. For immunoprecipitations, 30 µl of a 50% slurry of anti-FLAG affinity gel (Sigma) were added to each lysate and incubated with rotation for 2-3 hours at 4°C. Immunoprecipitates were washed three times with lysis buffer. Immunoprecipitated proteins were denatured by the addition of 50 µl of sample urea buffer and heating to 37°C for 15 minutes, resolved by 4%–12% SDS-PAGE, and analyzed by immunoblotting.

A similar protocol was employed when preparing samples for mass spectrometry.

Protein mass spectrometry

Immunoprecipitates from HEK-293T cells stably expressing FLAG-NPC1 were prepared as described above. Proteins were eluted off the anti-FLAG affinity beads with a custom-synthesized 3xFLAG peptide or recovered by boiling with sample buffer, resolved on 4-12% NuPage gels (Invitrogen), and stained with simply blue stain (Invitrogen). Each gel lane was sliced into 4 pieces and the proteins in each gel slice digested overnight with trypsin. The resulting digests were analyzed by mass spectrometry as described(*3*).

Lysosome immunoprecipitation for cholesterol measurement

Confluent HEK-293T cells stably expressing LAMP1-mRFP-FLAG^{X2} (LRF), plated at 2 x 15cm dishes per condition in quadruplicate, were sterol-depleted for 2h in 0.5% MCD, or sterol-depleted and restimulated with 50µM cholesterol (in complex with MCD at 1:1 stoichiometry) for 2h. Following these incubations, cells were rinsed once in cold PBS, then scraped, spun down and resuspended in 750ul of fractionation buffer: 140mM KCl, 5mM MgCl2, 50mM Sucrose, 20mM HEPES, pH 7.4, supplemented with protease inhibitors. Cells were mechanically broken by spraying 4-5 times through a 23G needle attached to a 1ml syringe, then spun down at 2000g for 10min, yielding a post nuclear supernatant (PNS). PNS aliquots were equalized based on total protein concentration and subjected to overnight immunoprecipitation with 100µl of a 50% slurry of anti-FLAG affinity gel. The next day, beads were washed 4 times in fractionation buffer. After washing, beads were loaded on a spin column and lipids were eluted from the beads by adding hexane:ethyl acetate (1:1).

Lysosomal cholesterol measurement using mass spectrometry

The samples were extracted by modified Bligh-Dyer method in the presence of internal standard d7-cholesterol (20 µg per sample). The samples were further derivatized to improve the mass spectrometric detection sensitivity of cholesterol. Measurement of cholesterol was performed with a Shimadzu 10A HPLC system and a

Shimadzu SIL-20AC HT auto-sampler coupled to a Thermo Scientific TSQ Quantum Ultra triple quadrupole mass spectrometer operated in SRM mode under ESI(+). Data processing was conducted with Xcalibur (Thermo). A quality control (QC) sample was prepared by pooling the aliquots of the study samples and was used to monitor the instrument stability. The QC was injected eight times in the beginning to stabilize the instrument, and was injected between every four study samples. The data was accepted if the coefficient variance (CV) of cholesterol in QC sample was < 15%. The quantification of cholesterol was calculated as the peak area ratio of cholesterol to d7-cholesterol multiplied by the quantity of d7-cholesterol (20 μg).

***In vitro* cholesterol starvation and restimulation**

Confluent HEK-293T cells stably expressing FLAG-tagged proteins, plated in 2x15cm dishes, were rinsed once in cold PBS, then scraped, spun down and resuspended in 750μl of fractionation buffer: 140mM KCl, 2mM EGTA, 50mM Sucrose, 25mM HEPES, pH 7.4, supplemented with protease inhibitors. Cells were mechanically broken by spraying 4-5 times through a 23G needle attached to a 1ml syringe, then diluted with 500μl of fractionation buffer and spun down at 1973g for 10 min at 4°C, yielding a post nuclear supernatant (PNS). The PNS was supplemented with 2.5mM ATP, 2.5mM $MgCl_2$, and amino acid esters, diluted from a 100X stock. The PNS was divided into 200 μL aliquots and spiked with 1% MCD to a final concentration of 0.1%. PNS aliquots were shaken at 700 RPM for 45 min at 37°C then spun down at maximum speed for 10 min at 4°C. The supernatant was discarded and the light organelle pellets were washed and resuspended in 150μL fractionation buffer then spun down at maximum speed for 10 min at 4°C. The supernatant was discarded and the light organelle pellets were resuspended in 150μL supplemented fractionation buffer and spiked with 1% MCD -/+ cholesterol to a final concentration of 0.1% MCD and 20μg/ml cholesterol in the restimulation condition. Aliquots were shaken at 700 RPM for 15 min at 37°C followed by the addition of 900 μL of 0.3% CHAPS lysis buffer. Samples were rotated in at 4°C for 10 min then spun down at maximum speed for 10 min at 4°C. The pellet was discarded and 30 μl of a 50% slurry of anti-FLAG affinity gel (Sigma) were added to each lysate and incubated with rotation for 2-3 hours at 4°C. Immunoprecipitates were washed three times with lysis buffer. Immunoprecipitated proteins were denatured by the addition of 50 μl of sample urea buffer and heating to 37°C for 15 minutes, resolved by 4%–12% SDS-PAGE, and analyzed by immunoblotting.

***In vitro* cholesterol starvation with cholesterol oxidase**

Confluent HEK-293T cells stably expressing FLAG-tagged proteins, plated in 2x15cm dishes, were rinsed once in cold PBS, then scraped, spun down and resuspended in 750μl of fractionation buffer: 140mM KCl, 2mM EGTA, 50mM Sucrose, 25mM HEPES, 2.5mM $MgCl_2$, 0.1% MCD, pH 7.4, supplemented with protease inhibitors. Cells were mechanically broken by spraying 3-4 times through a 23G needle attached to a 1ml

syringe, then spun down at 1973g for 10 min at 4°C, yielding a post nuclear supernatant (PNS). The PNS was supplemented with 2.5mM ATP and amino acid esters, diluted from a 100X stock, then divided into 100 µl aliquots. For cholesterol competition, the PNS was incubated with increasing amounts of cholesterol (1x equal to 20 µg/mL) for 15 minutes before addition of cholesterol oxidase. The PNS was spiked with cholesterol oxidase (0.2-2 U/mL) for 5 minutes while shaking at 1100 RPM at 37°C followed by the addition of 900 µl of 0.3% CHAPS lysis buffer**.** Samples were rotated at 4°C for 10 min, then centrifuged at maximum speed for 10 min at 4°C. The pellet was discarded and 30 µl of a 50% slurry of anti-FLAG affinity gel (Sigma) were added to each lysate and incubated with rotation for 2-3 hours at 4°C. Immunoprecipitates were washed three times with lysis buffer. Immunoprecipitated proteins were denatured by the addition of 50 µl of sample urea buffer and heating to 37°C for 15 minutes, resolved by 4%–12% SDS-PAGE, and analyzed by immunoblotting.

siRNA experiments

siRNA-based experiments were modified from (*4*). HEK-293T cells were plated in a 6-well plate at 250,000 cells/well. 16 hours later, cells were transfected using Dharmafect 1 (Dharmacon) with 250 nM of pooled siRNAs (Dharmacon) targeting SLC38A9 or a non-targeting pool. 48 hours post-transfection, cells were transfected again as described. 24 hours following the second transfection, cells were replated in new 6-well plates at 1,000,000 cells/well for signaling assays . The following siRNAs were used:

Non-targeting: ON-TARGETplus Non-targeting Pool (D-001810-10-05)

SLC38A9: SMARTpool: ON-TARGETplus SLC38A9 (L-007337-02-0005)

NPC1 Knockout using Cas9 nucleofection in HEK293T cells.

Knockout of NPC1 was modified from published protocols (*5*). Briefly, sgRNAs targeted to the first exon of NPC1 were designed using online software (http://crispr.mit.edu). sgRNAs were generated by HiScribe (NEB E2050S) T7 *in vitro* transcription using PCR-generated DNA as a template. 100 pmol of Cas9-2NLS (Macrolab) was diluted to a final volume of 5 µL with Cas9 buffer (20 mM HEPES (pH 7.5), 150 mM KCl, 1 mM MgCl2, 10% glycerol and 1 mM TCEP) and mixed slowly into 5 µL of Cas9 buffer containing 120 pmol of L2 sgRNA. The resulting mixture was incubated for 10 min at RT to allow RNP formation. 2×10^{-5} HEK293t cells were harvested, washed once in PBS, and resuspended in 20 µL of SF nucleofection buffer (Lonza, Basel, Switzerland). 10 µL of RNP mixture, 100 pmol of donor DNA, and cell suspension were combined in a Lonza 4d strip nucleocuvette. Reaction mixtures were electroporated using setting DS150, incubated in the nucleocuvette at RT for 10 min, and transferred to culture dishes containing pre-warmed media. Single clones were plated and colonies were screened for NPC1 deletion via filipin staining (Sigma-Aldrich) and high throughput microscopy. Hits were validated by western blotting.

Immunofluorescence assays

HEK-293T, CHO or MEF cells were plated on fibronectin-coated glass coverslips in 6-well plates (35mm diameter/well), at 300,000-500,000 cells/well. 12-16 hours later, cells were subjected to sterol or amino acid depletion/restimulation and fixed in 4% paraformaldehyde (in PBS) for 15 min at RT. The coverslips were rinsed twice with PBS and cells were permeabilized with 0.1% (w/v) Saponin in PBS for 10 min. After rinsing twice with PBS, the slides were incubated with primary antibody in 5% normal donkey serum for 1 hr at room temperature, rinsed four times with PBS, and incubated with fluorophore-conjugated secondary antibodies produced in goat or donkey (Life Technologies, diluted 1:1000 in 5% normal donkey serum) for 45 min at room temperature in the dark, washed four times with PBS. Coverslips were mounted on glass slides using Vectashield (Vector Laboratories) and imaged on a spinning disk confocal system (Andor Revolution on a Nikon Eclipse Ti microscope).

Intracellular amino acid measurements

Metabolites from cell pellets were extracted in 40:40:20 acetonitrile:methanol:water with the inclusion of isotopic d3N15-serine as an internal standard. Samples were vortexed, sonicated, and centrifuged at 10,000 g, and an aliquot of the supernatants were injected onto an 6460 Agilent QQQ-LC-MS/MS.

Protein labeling with cholesterol alkyne probes

On Day 0, human skin fibroblasts expressing FLAG tagged SLC38A9 were plated at $5x10^5$ in 10cm plates. On Day 1, media was changed to 10% lipid-depleted serum (LDS). On Day 3, cells were treated with 2.5μM 7-Azi-27-yne (LKM-38) probe complexed with MCD in the presence or absence of cholesterol-complexed MCD, diluted in DMEM for 1 hour at 37C. Media was aspirated and replaced with cold PBS. Cells were crosslinked for 2.5 minutes on ice in a UV Stratalinker 7000, harvested by scraping, and lysed in 50mM Tris, 150mM NaCl, 1% NP-40 for 1.5 hours with rotation. Protein concentration was determined by BCA assay. Lysate was incubated with anti-FLAG M2 agarose beads (Sigma) for 2hrs at 4C to achieve SLC38A9-FLAG pull down. Beads were washed, then suspended in lysis buffer. Click reagents were added to final concentrations of 1mM $CuSO_4$, 100μM Tris(3-hydroxypropyltriazolylmethyl)amine (THPTA), 100μM ROX azide, and 5mM ascorbic acid. The click reaction proceeded at 37C for 0.5 hours, shaking at 1200 rpm. Beads were washed and then suspended in Laemmli sample buffer before analysis by SDS-PAGE. Fluorescent images were collected at 610nm using a Typhoon Trio imager.

Peptide-cholesterol binding assays

The following synthetic peptides, encompassing the CARC-CRAC region of SLC38A9, were purchased from Elim Peptides:

CARC-CRAC^{WT}: KKKRIFLLFQMMTVWPLLGYLARVKKK

CARC-CRAC^{Y460I}: KKKRIFLLFQMMTVWPLLGILARVKKK

CARC-CRAC$^{F449I/Y460I}$: KKKRIFLLIQMMTVWPLLGILARVKKK

A 'scrambled' peptide was also included in the assay:

WT-Scrambled: KKKARVRIFLYGFLLQLLMPVWTMKKK

The N-term and C-term Lys residues increase the solubility of the hydrophobic CARC-CRAC transmembrane segment. Replacing Pro^{456} located between the CARC and the CRAC with Trp conferred intrinsic fluorescence upon 280nm excitation with maximum emission at 350nm. As previously reported, the conformation of the peptide affects the 350nm emission intensity (*6*). The fluorescent titration assay was performed as previously described (*6*). Stock solutions of 2.5mM peptide in DMSO were diluted to final 2.5μM concentration in assay buffer containing 140mM NaCl, 20mM Tris (pH 8.5), supplemented with 2mM TCEP and 2% (v/v) EtOH. Stock solutions of cholesterol or cholesterol-related ligands in EtOH were diluted to final concentrations ranging between 0.5 and 50 μM in 500 μl volumes of peptide solution, which were incubated overnight at 25C in mild agitation. Fluorescent measurements were carried out in triplicates on a Cary Eclipse fluorescence spectrophotometer (Agilent Technologies) using a 280nm excitation wavelength and collecting the 350nm emission. All measured fluorescence values fell within the linear range of the instrument.

Fluorescence intensity values were averaged and expressed as relative increase over the 0μM ligand condition. Data were fitted to saturation binding equations using GraphPad Prism 6. Dotted lines indicate where data could not be fitted.

Synthesis of 7-Azi-27-yne

(3β,16β,25*R*)-Cholest-5-ene-3,16,26-triol (1). Known compound **1** was made as reported,[i] by reacting diosgenin (4.4g, 11.0 mmol) with 6 N HCl (800 mL) and zinc dust (90 g, 1.38 mmol) in EtOH (1 L) at reflux. Upon reaction completion, the work up was slightly modified, finding that more solvent volume was necessary to avoid incomplete phase separation of the organic and water phases. Unreacted zinc was removed by filtration of the hot reaction mixture. After cooling to room temperature, water (2 L) and then diethyl ether (~ 1 L) were added until the layers separated. The two layers were separated and the aqueous layer was extracted with diethyl ether (2 x 300 mL). The combined organic layers were washed with a saturated solution of sodium hydrogen carbonate (100 mL) and brine (100 mL) and then dried over anhydrous magnesium sulfate. Removal of the solvent and recrystallization of the crude product from ethanol/water (1:1) provided compound **1** (85%, 3.9 g, 9.3 mmol). Spectroscopic data matched the literature data.[1]

(3β,16β,25*R*)-Cholest-5-ene-3,16,26-triol, 26-(2,2-dimethylpropanoate) (2). Known compound **2** was made as reported,[1] by reacting compound **1** (1.53 g, 3.68 mmol) with pivaloyl chloride (0.59 mL, 4.78 mmol), triethylamine (1.12 mL, 8.04 mmol), and 4-dimethylaminopyridine (45 mg, 0.37 mmol) in THF (150 mL). The procedure was modified slightly by reacting the reagents at 45ºC for 16 h (instead of the originally reported

room temperature for 16 h) to yield compound **2** (73%, 1.35 g, 2.7 mmol). Spectroscopic data matched the literature data.[1]

(3β,16β,25*R*)-3-[[(1,1-Dimethylethyl)dimethylsilyl]oxy]-cholest-5-ene-16,26-diol, 26-(2,2-dimethylpropanoate) (3). Known compound **3** was made as reported,[1] by reacting compound **2** (1.19 g, 3.68 mmol) with *tert*-butylchlorodimethylsilane (0.71 g, 4.72 mmol) and DBU (0.7 mL, 4.74 mmol) in THF (25 mL) at room temperature. Compound **3** (99%,1.44 g, 2.33 mmol) had spectroscopic data that matched the literature data.[1]

(3β,25*R*)-3-[[(1,1-Dimethylethyl)dimethylsilyl]oxy]-cholest-5-ene-26-ol (4). Known compound **4** was made as reported,[1] by first reacting compound **3** (300 mg, 0.49 mmol) with MsCl (14.4 mL, 1.85 mmol) in pyridine (4.5 mL), followed by treatment with $LiAlH_4$ (83 mg, 2.19 mmol) in Et_2O (10 mL). The procedure was modified slightly by keeping the mesylation reaction for 16 h at 0ºC in a freezer overnight, instead of the reported 16 h at room temperature because decomposition was observed at room temperature. Additionally, in the $LiAlH_4$ reaction, powdered $LiAlH_4$ was found to give more consistent results than was a preformed solution of the reagent. Compound **4** (72%, 180 mg, 0.35 mmol) had spectroscopic data that matched the literature data.[1]

(3β,25*R*)-3-[[(1,1-Dimethylethyl)dimethylsilyl]oxy]-cholest-5-en-26-ol, 26-acetate (5). Known compound **5**[1] was made by reacting compound **4** (0.56 g, 1.08 mmol) with acetic anhydride (0.61 mL, 6.48 mmol) and 4-dimethylaminopyridine (6.6 mg, 0.05 mmol) in pyridine (7 mL) at room temperature for 16 h. Upon completion, the reaction was cooled to 0ºC, MeOH (0.5 mL) was added and the reaction was stirred for 5 min. Solvents were evaporated under vacuum, and the reaction was re-dissolved in CH_2Cl_2 (30 mL) and washed with water (10 mL), 1 N HCl (10 mL), water (10 mL), sat. aq. $NaHCO_3$ (10 mL) and water (10 mL). The organic layer was concentrated *in vacuo*, and the residue was purified by column chromatography on silica gel (ethyl acetate–hexanes, gradient elution), to yield compound **5** (99%, 0.59 g, 1.07 mmol). Spectroscopic data matched the literature data.[1]

(3β,25*R*)- 26-(Acetyloxy) 3-[[(1,1-dimethylethyl)dimethylsilyl]oxy]-cholest-5-en-7-one (6). Following a literature method used for the conversion of Δ^5-steroids to Δ^5-7-ketosteroids,[ii] CrO_3 (1.82 g, 18.2 mmol) was added to dry CH_2Cl_2 (15 mL) and the reaction was cooled to -20ºC. 3,5-Dimethylpyrazole (1.75 g, 18.2 mmol) was added. After stirring for 30 min at -20ºC, steroid **5** (0.68 g, 1.21 mmol) dissolved in dry CH_2Cl_2 (7.5 mL) and added dropwise. The reaction was stirred at -20ºC for 4 h, upon which time the reaction mixture was diluted with a (7:3) mixture of hexanes-ethyl acetate and filtered through a short column of silica gel layered with celite using the same solvent mixture, and the solvents were evaporated under vacuum. The crude product was purified by column chromatography on silica gel (ethyl acetate–hexanes, gradient elution) to give compound **6** as colorless crystals (64%, 0.44 g, 0.77 mmol) which was characterized spectroscopically. ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 5.63 (s, 1H), 3.94-3.89 (m, 1H), 3.83-3.78 (m, 1H), 3.57 (m, 1H), 2.41-2.31 (m, 2H), 2.19 (t, 1H, *J* = 11.6 Hz), 2.02 (s, 3H), 2.02-1.95 (m, 1H), 1.15 (s, 3H), 0.89-0.85 (m, 15H), 0.64 (s, 3H), 0.03 (s, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) δ ppm 202.3, 171.3, 165.8, 125.9, 71.4, 69.6, 54.8, 50.0, 50.0, 45.5, 43.2, 42.6, 38.8, 38.4, 36.5, 36.1, 35.7, 33.8, 32.5, 31.8, 28.7, 26.4, 25.9 (x3), 23.3, 21.3, 21.0, 18.9, 18.2, 17.4, 16.9, 12.1, -4.5, -4.6.

(3β,5α,25*R*)- 26-(Acetyloxy)-3-[[(1,1-dimethylethyl)dimethylsilyl]oxy]-cholestan-7-one (7). Following a literature method used for the hydrogenation of Δ^5-7-ketosteroids, 2 compound **6** (0.44 g, 0.77 mmol) and 10% Pd/C (150 mg) were added to a borosilicate sealable glass vessel containing EtOAc (30 mL). The reaction vessel was attached to a Parr Hydrogenator (shaker-type), and the air was evacuated and replaced with H_2 at 45 psi. The reaction was allowed to shake for 2 h, upon which time the H_2 was evacuated under vacuum for 30 min. The vessel was re-pressurized and removed from the hydrogenator, and the reaction mixture was filtered through celite (taking care to wash the Pd/C several times with EtOAc), and the eluent was concentrated *in vacuo*. As some 7-hydroxy steroid was detected on TLC, the crude reaction mixture was dissolved in CH_2Cl_2 (10 mL) and cooled to 0ºC. Pyridinium chlorochromate (0.36 g, 1.67 mmol) was added and the reaction was slowly warmed to room temperature over 2 h. Upon completion, the reaction was filtered through a short column of silica gel, and the crude product was collected and concentrated *in vacuo*. The crude product was then purified by column chromatography on silica gel (ethyl acetate–hexanes, gradient elution) to give compound **7** (93%, 0.41 g, 0.71 mmol) which was characterized spectroscopically. ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 3.94-3.90 (m, 1H), 3.85-3.74 (m, 1H), 3.53 (m, 1H), 2.33 (t, 1H, *J* = 11.6 Hz), 2.21-2.13 (m, 1H) 2.04 (s, 3H), 1.06 (s, 3H), 0.91-0.85 (m, 15H), 0.63 (s, 3H), 0.02 (s, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) δ ppm 212.4, 171.4, 71.7, 69.7, 55.6, 55.2, 50.2, 49.0, 47.3, 46.4, 42.7, 39.0, 38.6, 36.4, 36.2, 36.2, 35.7, 33.9, 32.6, 31.7, 28.6, 26.1 (x3), 25.1, 23.3, 22.0, 21.1, 18.9, 18.4, 16.9, 12.2, 12.0, -4.5 (x2).

(3β,5α,25*R*)- 26-(Acetyloxy)-3-[[(1,1-dimethylethyl)dimethylsilyl]oxy]-cholestan-7-one, cyclic 7-(1,2-ethanediyl acetal) (8). In a 2-necked flask fitted with a Dean-Stark apparatus, compound **7** (0.40 g, 0.70 mmol) and ethylene glycol (2 mL) were added to anhydrous toluene (30 mL). Pyridinium p-toluenesulfonate (0.035 g, 0.139 mmol) was then added and the reaction was stirred at 120ºC for 16 h. The reaction was then cooled to 0ºC, and brought to neutral pH with sat. aq. $NaHCO_3$. The reaction mixture was then washed with water (2 x 10 mL), concentrated in vacuo and immediately purified by column chromatography on silica gel (ethyl acetate–hexanes, gradient elution) to give compound **8** (colorless crystals, 93%, 0.40 g, 0.65 mmol) which was characterized spectroscopically. ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 4.00-3.90 (m, 5H), 3.85-3.80 (m, 1H), 3.57 (m, 1H), 2.05 (s, 3H), 1.94 (d, 1H, *J* = 12.4 Hz) 0.92-0.87 (m, 15H), 0.82 (s, 3H), 0.65 (s, 3H), 0.04 (s, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) δ ppm 174.5, 111.3, 72.0, 69.8, 64.4, 62.8, 55.3, 51.1, 49.7, 43.7, 42.3, 40.9, 39.7, 38.3, 38.0, 37.1, 36.2, 35.8, 35.3, 34.0, 32.7, 32.1, 28.9, 26.4, 26.1 (x3), 23.4, 21.8, 21.2, 19.0, 18.4, 17.0, 12.2, 11.8, -4.3, -4.4.

(3β,5α)-3-[[(1,1-Dimethylethyl)dimethylsilyl]oxy]-7-oxo-cholestan-26-al, cyclic 7-(1,2-ethanediyl acetal) (9). Compound **8** (0.45 g, 0.72 mmol) was dissolved in THF (5 mL) and MeOH (50 mL). K_2CO_3 (1.0 g, 7.2 mmol) was added and the reaction was stirred for 16 h at room temperature. Upon completion, the reaction was carefully neutralized with 1 N HCl, water was added (50 mL) and the reaction mixture was extracted with CH_2Cl_2 (4 x 30 mL). The organic fractions were combined, dried over anhydrous Na_2SO_4, concentrated *in vacuo* and dried. The crude alcohol was dissolved in CH_2Cl_2 (25 mL) and cooled to 0ºC. $NaHCO_3$ (0.6 g, 7.2 mmol) was added followed by Dess-Martin periodinane reagent (0.61 g, 1.44 mmol). After stirring 2 h at 0ºC, another portion of Dess-Martin periodinane reagent (0.31 g, 0.72 mmol) was added and the reaction was stirred 1 h at 0ºC. Upon completion, a 3:1 mixture of sat. aq. $NaHCO_3$ (6 mL) and 10% w/w $Na_2S_2O_3$ (2 mL) were added and the reaction was stirred vigorously for 10 min. Water (30 mL) was added, and the organic layer was separated and washed again with water (10 mL). The crude product was purified by column chromatography on silica gel (ethyl acetate–hexanes, gradient elution) to afford compound **9** (85%, 0.35 g, 0.61 mmol) which was characterized spectroscopically. ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 9.59 (s, 1H), 4.01-3.88 (m, 4H), 3.55 (m, 1H), 2.33-2.28 (m, 1H), 1.92 (d, 1H, *J* = 12.4 Hz), 1.06 (dd, 3H, *J* = 7.2 Hz, *J* = 2.0 Hz), 0.88 (d, 3H, *J* = 6.8 Hz), 0.86 (s, 9H), 0.80 (s, 3H), 0.63 (s, 3H), 0.02 (s, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) δ ppm 205.5, 111.2, 72.0, 64.4, 62.7, 55.1, 51.1, 49.7, 46.5, 43.6, 42.2, 40.9, 39.7, 38.3, 37.9, 37.1, 36.1, 35.7, 35.3, 32.0, 31.1, 28.8, 26.4, 26.1 (x3), 23.5, 21.8, 19.0, 18.4, 13.5, 12.2, 11.8, -4.4 (x2).

(3β,5α)-3-[[(1,1-Dimethylethyl)dimethylsilyl]oxy]-20-(4-methyl-5-hexyn-1-yl)-pregnan-7-one, cyclic 7-(1,2-ethanediyl acetal) (10). Compound **10** was made similar to a literature protocol,[iii] by reacting compound **9** (263 mg, 0.46 mmol) with dimethyl-(1-diazo-2-oxopropyl)phosphonate (Bestmann-Ohira reagent, 0.11 mL, 0.73 mmol) and K_2CO_3 (253 mg, 1.83 mmol) in MeOH (5 mL) and THF (5 mL). The original procedure was modified slightly, by carrying out the reaction out for 4 h at 0ºC (instead of the reported 5 h at 45ºC). These modifications were necessary because, although not reported in the original published procedure, it was found that 5 h at 45ºC significantly scrambles the C25 stereocenter, which is only apparent in the alkyne peaks in the 100 MHz ^{13}C NMR spectrum. By reducing the temperature to 0ºC, the reaction still proceeded efficiently while greatly reducing C25 isomerization. The crude compound **10** was purified by column chromatography on silica gel (CH_2Cl_2–hexanes, gradient elution) to afford compound **10** (84%, 219 mg, 0.38 mmol) as a ~95:5 mix of 25(*R*) and 25(*S*) diastereomers (as detected by ^{13}C NMR), respectively, which were not separable by column chromatography. Spectroscopic data for the 25(*R*) component of compound **10**: ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 3.99-3.87 (m, 4H), 3.55 (m, 1H), 2.41-2.36 (m, 1H), 2.01 (d, 1H, *J* = 2.4 Hz, alkyne CH), 1.93 (d, 1H, *J* = 12.8 Hz), 1.15 (d, 3H, *J* = 7.2 Hz), 0.90 (d, 3H, *J* = 6.4 Hz), 0.85 (s, 9H), 0.80 (s, 3H), 0.63 (s, 3H), 0.02 (s, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) δ ppm 111.2, 89.5, 72.0, 68.2, 64.4, 62.7, 55.2, 51.1, 49.6, 43.6, 42.2, 40.9, 39.7, 38.2, 37.9, 37.4, 37.1, 35.9, 35.8, 35.3, 32.0, 28.8, 26.4, 26.1 (x3), 25.9, 23.8, 21.8, 21.1, 19.0, 18.4, 12.2, 11.8, -4.4, -4.4. Characteristic resonances for the 25(*S*) component of compound **10**: ^{13}C NMR (100 MHz, $CDCl_3$) δ ppm 89.4, 68.3.

(3β,5α)-3-[[(1,1-Dimethylethyl)dimethylsilyl]oxy]-20-(4-methyl-5-hexyn-1-yl)-pregnan-7-one (11). Compound **11** was made by dissolving compound **10** (300 mg, 0.53 mmol) in THF (30 mL) and adding 1 M tetrabutylammonium fluoride in THF (1.58 mL, 1.58 mmol) dropwise. After stirring for 16 h at room temperature, water (50 mL) was added, and the reaction was extracted with EtOAc (4 x 20 mL). The organic fractions were combined, dried over anhydrous Na_2SO_4, concentrated *in vacuo* and dried. The crude compound was then dissolved in acetone (60 mL), and p-toluenesulfonic acid (28 mg, 0.015 mmol) was added. After stirring for 5 h at room temperature, the reaction was neutralized with sat. aq. $NaHCO_3$. Solvents were evaporated under vacuum, and the reaction mixture was re-dissolved in CH_2Cl_2 (20 mL) and washed with water

(2 x 10 mL). The crude product was purified by column chromatography on silica gel (acetone–hexanes, gradient elution) to afford compound **11** (97%, 211 mg, 0.51 mmol) as a ~95:5 mixture of 25(*R*) and 25(*S*) diastereomers, respectively. Spectroscopic data for the 25(*R*) component of compound **11**: ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 3.61 (m, 1H), 2.45-2.32 (m, 3H), 2.24-2.14 (m 1H), 2.04 (d, 1H, *J* = 2.0 Hz, alkyne CH), 1.17 (d, 3H, *J* = 6.4 Hz), 1.00 (s, 3H), 0.92 (d, 3H, *J* = 6.4 Hz), 0.65 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ ppm 212.4, 89.6, 70.9, 68.2, 55.4, 55.1, 50.2, 49.0, 47.0, 46.3, 42.7, 38.9, 38.0, 37.4, 36.2, 36.2, 35.9, 35.8, 31.2, 28.6, 25.9, 25.1, 23.8, 22.0, 21.1, 19.0, 12.2, 12.0. Characteristic resonances for the 25(*S*) component of compound **11**: ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.78 (s, CH_3); ^{13}C NMR (100 MHz, $CDCl_3$) δ ppm 89.4, 68.3.

(3β,5α)-20-(4-Methyl-5-hexyn-1-yl)-spiro[pregnane-7,3'-[3*H*]diazirin]-3-ol (7-Azi-27-yne). The compound was synthesized in a similar fashion as a known procedure for the conversion of a steroid 6-ketone to a 6-diazirne.[iv] Compound **11** (0.32 g, 0.77 mmol) was dissolved in MeOH (30 mL) and the reaction was cooled to 0ºC. Ammonia gas (~10 mL) was condensed into the reaction vessel. The flask was then sealed tightly with a septum, and hydroxylamine-O-sulfonic acid (0.44 g, 3.9 mmol) was dissolved in MeOH (4 mL) and added dropwise to the reaction. After stirring for 1 h at 0ºC, the reaction was brought to room temperature and stirred for 16 h. Upon completion, the reaction was filtered through a pad of silica and concentrated *in vacuo* to give the 7-diaziridine intermediate. As the final 7-diazarine compound is photoreactive, the remainder of the synthesis was carried out under dimmed lights. The 7-diaziridine was dissolved in MeOH (25 mL) at room temperature, and Et_3N (1.0 mL) was added. I_2 crystals, (0.24 g, 0.93 mmol) were dissolved in MeOH (2.5 mL), and the solution was added dropwise over 1 min. The reaction was immediately quenched with 10% aq. $Na_2S_2O_3$ until colorless, and was then diluted with brine (25 mL) and extracted with CH_2Cl_2 (4 x 15 mL), dried over anhydrous Na_2SO_4, and concentrated *in vacuo*. The residue was purified by column chromatography on silica gel (EtOAc–hexanes, gradient elution), to yield **7-Azi-27-yne** as colorless crystals (33%, 110 mg, 0.026 mmol) containing a ~95:5 mixture of the 25(*R*) and 25(*S*) diastereomers (only detectable by ^{13}C NMR), respectively. Spectroscopic, physical and analytical data for **7-Azi-27-yne**: m.p. 103-106ºC (Et_2O:Hex); IR: ν_{max} 3308, 2969, 2933, 2855, 2112, 1573, 1449, 1377, 1031 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 3.63 (m, 1H), 2.38 (m, 1H), 2.02 (d, 1H, *J* = 2.4 Hz, alkyne CH), 1.15 (d, 3H, *J* = 7.2 Hz), 0.91 (s, 3H), 0.87 (d, 3H, *J* = 6.4 Hz), 0.79-0.65 (m, 1H), 0.58 (s, 3H), 0.31-0.22 (m, 1H), -0.02 (dd, 1H, *J* = 14.0 Hz, *J* = 3.2 Hz); ^{13}C NMR 25(*R*) component (100 MHz, $CDCl_3$) δ ppm 89.5, 71.0, 68.2, 54.6, 52.5, 50.0, 43.4, 42.6, 39.0, 37.4, 37.4, 37.3, 36.7, 36.1, 36.0, 35.8, 35.6, 32.4, 31.5, 28.1, 25.9, 25.0, 23.8, 21.2, 21.1, 19.0, 12.1, 11.6. Characteristic resonances for the 25(*S*) component of **7-Azi-27-yne**: ^{13}C NMR (100 MHz, $CDCl_3$) δ ppm 89.4, 68.3. HR-FAB MS $[M+H]^+$ calcd for 425.3532, found 425.3531.

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