Supporting Information

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SI Materials and Methods

Materials and Reagents. MEM, FBS, HBSS, AlexaFluor546 NHS ester, and 4,4-difluoro-4-bora-3a,4a-diaza-*s*-indacene-dodecanoic acid (BODIPY-C12) were from Invitrogen. Methyl-β-cyclodextrin (MβCD), water-soluble cholesterol (cholesterol loaded onto MβCD), hydroxypropyl-β-cyclodextrin, β-sitosterol, filipin, paraformaldehyde (PFA), BSA, compound S58-035, 1-naphthol, and other chemicals were from Sigma Chemicals. U18666A (3-β-[(2-diethyl-amino) ethoxy] androst-5-en-17-one) was from Cayman Chemicals. Anti-BMP [bis(monoacylglycerol)phosphate], clone 6C4, antibody was from Echelon Biosciences. TLC plates and Typhoon Trio Imager were from GE Healthcare.

Software. MetaExpress and MetaMorph were from Molecular Devices (MDS Analytical Technologies). MATLAB was from The MathWorks.

Statistical Analysis. *P* values were computed using unpaired, two-tailed, Student's *t* test.

Cell Lines. Human fibroblast cell lines GM03123 (NPC1), GM18455 (NPC2), and GM05659 (apparently normal) were obtained from Coriell Institute. Fibroblasts were grown in MEM with 2.2 g/L sodium bicarbonate, supplemented with 10% (vol/vol) FBS (growth medium).

Antibody Staining. Anti-BMP staining was performed mostly as described previously (1). Cells were fixed with 1% (wt/vol) paraformaldehyde for 10 min; permeabilized with 0.05% (wt/vol) saponin; blocked with 2% (wt/vol) BSA; labeled with primary antibody against BMP (1:150) (2–4) for 1 to 2 h; washed three times with PBS; and labeled with goat anti-mouse Alexa488-IgG (Invitrogen) for 2 h, followed by three washes with PBS. Filipin labeling (100 μg/mL) was done concurrently with the secondary antibody incubation.

Automated Fluorescence Microscopy and Image Analysis. Filipin staining and automated image analysis [lysosomal storage organelle (LSO) filipin assay] were done as described previously (5). Images were acquired using an ImageXpress^{MICRO} imaging system from Molecular Devices (MDS Analytical Technologies) equipped with a 300-W xenon arc lamp. A CoolSnapHQ camera (1,392 × 1,040 pixels) from Roper Scientific was used to acquire images. Filipin images were acquired using 377/50-nm excitation and 447/60-nm emission filters with a 415-nm dichroic filter using a 10× Plan Fluor 0.3 numerical aperture (NA) objective from Nikon. AlexaFluor488 immunofluorescence images of BMP were acquired using 482/35-nm excitation and 536/40-nm emission bandpass filters with a 513-nm dichroic filter using a 20× Plan Fluor 0.5 NA objective from Nikon. Images were acquired at either four (filipin) or nine (AlexaFluor488) sites per well using 2 × 2 pixel binning. Each site was individually focused using a high-speed laser autofocus comprising a 690-nm diode laser and a dedicated 8-bit CMOS camera. 696 × 520 pixel images were acquired at 12 intensity bits per pixel.

Images were background corrected as described previously (6), and either the filipin or the BMP LSO ratio was quantified as integrated thresholded fluorescence power of filipin or anti-BMP fluorescence within the LSOs, normalized to the total cell area as defined by a low threshold using filipin fluorescence.

Wide-Field Fluorescence Microscopy. Filipin and AlexaFluor546 images were acquired mostly as described previously (1), using a 63× 1.4 NA oil immersion objective with 2 × 2 pixel binning using a Leica DMIRB microscope.

Free Cholesterol Measurement by Gas Chromatography/Mass Spectrometry (GC/MS). Lipids were extracted from cells with hexane/2-propanol (3:2) and separated on a Varian Factor Four capillary column (VF-1ms 30 m × 0.25 mm ID DF 0.25) using a Varian 4000 GC/MS/MS system. Injector temperature was 270 °C. The following temperature gradient was used: initial temperature was 115 °C, then it was raised to 260 °C at 9 °C/min and held for 2.89 min, then raised to 269 °C at 3 °C/min and again to 290 °C at 9 °C/min and held for 4.67 min. Helium gas flow rate was 5 mL/min. Electron ionization detection was used with current set at 10 μAmp. Total ionic current was used for detection (50–1,000 *m/z*). β-sitosterol was used as an internal standard for quantification. Protein concentration after solubilization with 0.5 M NaOH was determined with modified Lowry reagent (Bio-Rad) against BSA standards.

Cholesterol Esterification. Cells were plated in six-well plates, pulsed with either 3 mM MβCD or HPβCD, and then allowed to recover for 16 h in growth medium. They were then incubated with fresh medium with 3 μM BODIPY-C12 with or without compound S58-035 [acyl CoA:cholesterol acyl transferase (ACAT) inhibitor] for 6 h. Lipids were extracted twice with hexane/2-propanol (3:2). Lipids were resolved on normal-phase TLC plates using the following system: hexane/2-propanol/diethyl ether/glacial acetic acid (80:10:10:1) and imaged using a Typhoon Trio imager using 488 nm excitation/520 nm emission. Spots corresponding to BODIPY-cholesteryl ester and free BODYPY-C12 were quantified in MetaMorph and background corrected. Protein concentration after solubilization with 0.5 M NaOH was determined with modified Lowry reagent (Bio-Rad) against BSA standards.

Synthesis of MβCD-Dextran. Unless otherwise stated, chemicals were purchased from Aldrich, TCI, or Alfa Aesar and were used without any further purification. Aminodextran was purchased from Invitrogen. When necessary, solvents and reagents were dried before use, using standard protocols. All nonaqueous reactions were carried out in oven-dried glassware under an atmosphere of argon. 6^{A}-*O*-*p*-toluenesulfonyl-β-cyclodextrin (7) and mono(6^{A}-*O*-*p*-toluenesulfonyl) permethyl-β-cyclodextrin (8) were prepared according to previously published procedures.

Boc-NH-(PEG)$_3$-CONH-Aminodextran. Pyridine was added to a suspension of aminodextran (70,000 MW, 500 mg) in dimethylformamide (DMF) (10 mL) (400 μL, 5 mmol) and the reaction was stirred at ambient temperature for 10 min. At that point, Boc-NH-(PEG)$_3$-COOH (510 mg, 1.4 mmol) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (540 mg, 2.8 mmol) were added sequentially. The resulting mixture was vigorously stirred at ambient temperature for 18 h and then purified by extensive dialysis against water (Spectra/Por tubing, molecular weight cutoff 12–14 kDa) to remove unreacted small molecules. Concentration by lyophilization (×2) furnished the title compound as a white powder (600 mg).

NH$_2$-(PEG)$_3$-CONH-Aminodextran. Boc-NH-(PEG)$_3$-CONH-aminodextran (600 mg) was added to a flask containing trifluoroacetic acid (15 mL) at 0 °C and stirred for 30 min then gradually warmed to ambient temperature and stirred for additional 45 min. The reaction was then diluted with water and extensively dialyzed against water. The pure title compound was obtained as a white powder (600 mg) after lyophilization (×3).

MβCD-NH-(PEG)$_3$-CONH-Aminodextran. Mono(6^A-*O*-*p*-toluenesulfonyl) permethyl-β-cyclodextrin (560 mg, 0.4 mmol) was added to a solution of NH_2-(PEG)$_3$-CONH-aminodextran (600 mg) in formamide (45 mL). The resulting solution was heated to 100 °C and stirred for 48 h, at which point it was diluted with water and extensively dialyzed against water. After lyophilization (×2), the title compound was obtained as a white powder (530 mg).

AlexaFluor546-MβCD-NH-(PEG)$_3$-CONH-Aminodextran. MβCD-NH-(PEG)$_3$-CONH-aminodextran (530 mg) was added to a solution of PBS, and the mixture was stirred at ambient temperature for 1 h. At that point, AlexaFluor546 NHS ester (400 μg, 0.37 μmol) was added and the reaction continued to stir for additional 48 h. After extensive dialysis against water and lyophilization (×3), the title compound was obtained as a pink, cotton-like solid (427 mg).

Estimation of MβCD Substitution on Aminodextran. Dextran derivitization with MβCD was estimated according to a method described previously (9). We used a SpectraMax M2 fluorometer (MDS Analytical Technologies) to detect changes in 1-naphthol fluorescence as the result of MβCD binding.

1. Pipalia NH, Hao M, Mukherjee S, Maxfield FR (2007) Sterol, protein and lipid trafficking in Chinese hamster ovary cells with Niemann-Pick type C1 defect. *Traffic* 8:130–141.
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5. Rosenbaum AI, et al. (2009) Chemical screen to reduce sterol accumulation in Niemann-Pick C disease cells identifies novel lysosomal acid lipase inhibitors. *Biochim Biophys Acta* 1791:1155–1165.
6. Pipalia NH, Huang A, Ralph H, Rujoi M, Maxfield FR (2006) Automated microscopy screening for compounds that partially revert cholesterol accumulation in Niemann-Pick C cells. *J Lipid Res* 47:284–301.
7. Brady B, Lynam N, O'Sullivan T, Ahern C, Darcy R (2000) 6^A-O-p-toluenesulfonyl-beta-cyclodextrin. *Org Synth* 77:220–224.
8. Faiz JA, Spencer N, Pikramenou Z (2005) Acetylenic cyclodextrins for multireceptor architectures: Cups with sticky ends for the formation of extension wires and junctions. *Org Biomol Chem* 3:4239–4245.
9. Grosse PY, Pinguet F, Joulia JM, Astre C, Bressolle F (1997) High-performance liquid chromatographic assay for methyl-beta-cyclodextrin in plasma and cell lysate. *J Chromatogr B Biomed Sci Appl* 694:219–226.

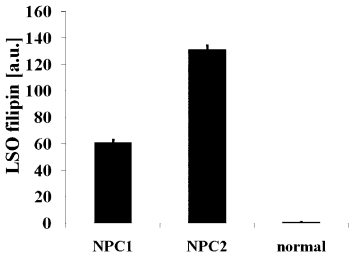

Fig. S1. Comparison of cholesterol accumulation as measured by the LSO assay in NPC1-defective, NPC2-defective, and apparently normal human fibroblasts. Cells were plated in 384-well assay plates at a density to produce ≈80–90% confluency after 4 days. At the end of the experiment, cells were fixed with 1% PFA and stained with 50 μg/mL filipin. Images were acquired using an ImageXpress^{MICRO} imaging system with a 10× objective. Quantification of filipin labeling of the LSOs was done as described previously [Pipalia NH, Huang A, Ralph H, Rujoi M, Maxfield FR (2006) Automated microscopy screening for compounds that partially revert cholesterol accumulation in Niemann-Pick C cells. *J Lipid Res* 47:284–301]. Data represent averages ± SEM of one representative experiment ($11 \leq n \leq 16$, where n is total number of wells per condition used for quantification).

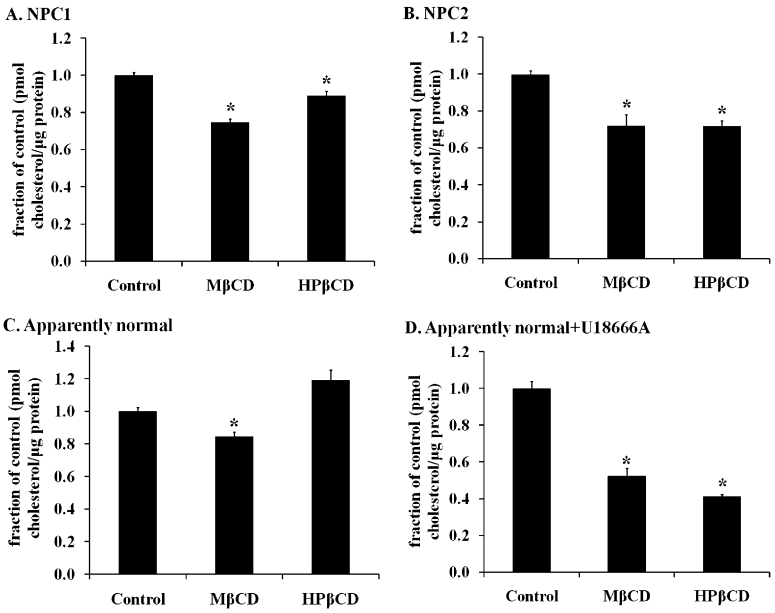

Fig. S2. GC/MS measurement of cyclodextrin effects on total free cholesterol levels in NPC1-deficient, NPC2-deficient, apparently normal, and U18666A-treated normal cells. Cells were plated in six-well plates at a density to produce ≈80–90% confluency by experiment completion. Cells were treated with 0.9 mM MβCD or HPβCD for 1 day in growth medium. At the end of the experiment, cellular lipids were extracted and analyzed by GC/MS. Data represent averages ± SEM of two independent experiments normalized to control average value for each experiment. Control average values were as follows: 0.070 ± 0.011 pmol free cholesterol per μg cell protein for NPC1 mutant cells (*A*), 0.133 ± 0.005 pmol/μg for NPC2 mutant cells (*B*), 0.044 ± 0.003 pmol/μg for normal cells (*C*), and 0.074 ± 0.006 pmol/μg for normal cells treated with 1 μM U18666A (*D*). *$P < 0.01$ vs. control ($6 \leq n \leq 9$, where n is total number of wells per condition used for quantification).

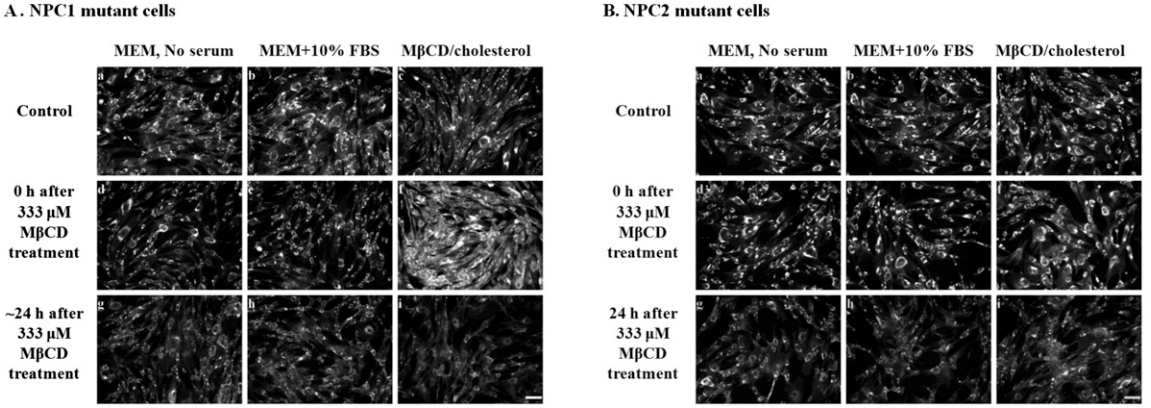

Fig. S3. Filipin images of acute cyclodextrin effects on cholesterol accumulation in NPC1- and NPC2-deficient cells. Background and shading corrected images of untreated control (*a–c*), 0 h after treatment (*d–f*), or 24 h after treatment (*g–i*) NPC1 (*A*) or NPC2 (*B*) mutant cells were obtained as in Fig. S1. Cells were treated either with 333 μM MβCD dissolved in MEM with no serum (*a*, *d*, and *g*), in MEM with 10% FBS (*b*, *e*, and *h*), or with 333 μM MβCD loaded with cholesterol (*c*, *f*, and *i*). (Scale bars, 100 μm.)

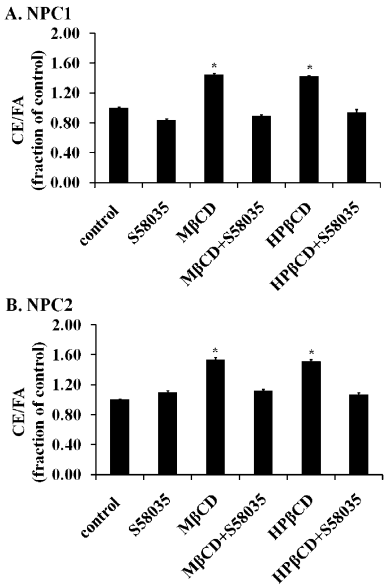

Fig. S4. Cholesterol esterification by ACAT. Cyclodextrin effects on cholesterol esterification in NPC1 (*A*) and NPC2 (*B*) mutant cells. Cells were treated for 1 h with 3 mM MβCD or HPβCD and then allowed to recover in growth medium for 16 h. Cells were then incubated with 3 μM BODIPY-C12 for 6 h. The ratio of cholesteryl-BODIPY-C12 ester (CE)/BODIPY-C12 acid (FA) after TLC separation was measured and normalized to the average control value. ACAT inhibitor S58-035 was added, where indicated, at 10 μg/mL. For NPC1-defective cells the average ratio was 0.110 ± 0.007, which corresponded to 3.73 ± 0.22 fmol CE formed per μg cell protein during 6 h. Corresponding values for NPC2-defective cells were 0.089 ± 0.003 and 2.50 ± 0.24 fmol/μg. Data represent averages ± SEM of two to three independent experiments. *$P < 0.0001$ vs. control ($6 \leq n \leq 16$, where n is total number of wells per condition used for quantification).

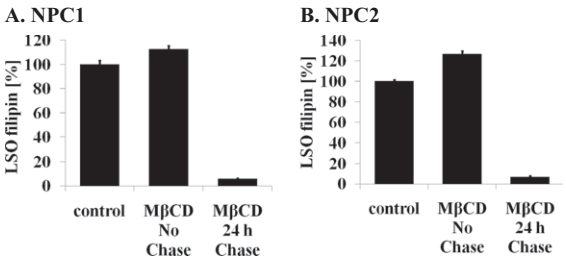

Fig. S5. LSO filipin values for the ACAT assay. LSO filipin levels were measured immediately (no chase) or after 24-h chase after a 1-h 3-mM MβCD treatment in NPC1 (*A*) and NPC2 (*B*) mutant cells. Data represent averages ± SEM of two independent experiments ($n = 8$ for treated samples, $n = 72$ for untreated control, where n is total number of wells per condition used for quantification).

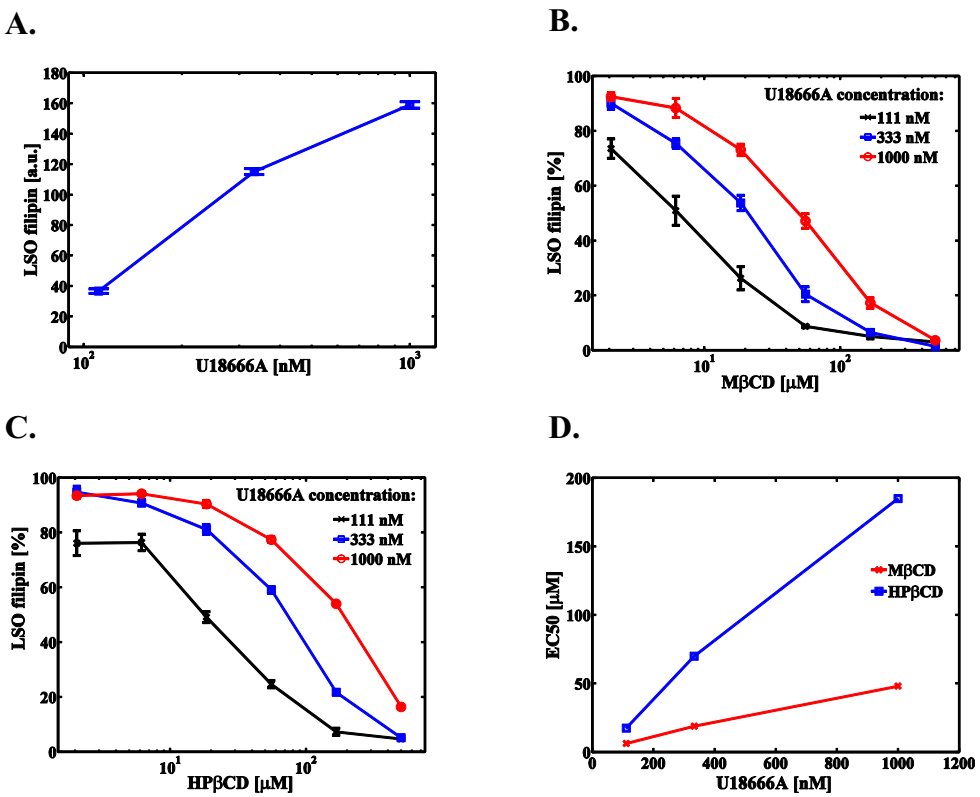

Fig. S6. Cyclodextrin effects on cholesterol accumulation in U18666A-treated apparently normal human fibroblasts. Cells were plated in 384-well assay plates at a density to produce ≈80–90% confluency by experiment completion and treated with U18666A at indicated concentrations for 24 h before addition of various concentrations of either MβCD or HPβCD for another 24 h, during which indicated U18666A levels were maintained. At the end of the experiment, cells were fixed with 1% PFA and stained with 50 μg/mL filipin. Images were acquired using an ImageXpress^{MICRO} imaging system with a 10× objective. LSO filipin quantification was done as described previously [Pipalia NH, Huang A, Ralph H, Rujoi M, Maxfield FR (2006) Automated microscopy screening for compounds that partially revert cholesterol accumulation in Niemann-Pick C cells. *J Lipid Res* 47:284–301]. (*A*) Cholesterol accumulation, as measured by the LSO filipin assay, at different concentrations of U18666A. MβCD (*B*) and HPβCD (*C*) LSO filipin dose–response curves at three different U18666A concentrations. (*D*) MβCD and HPβCD EC_{50} (from Table S2) dependence on U18666A concentration. Data represent averages ± SEM of two independent experiments (n = 12 for control and n = 6 for treated, where n is total number of wells per condition used for quantification).

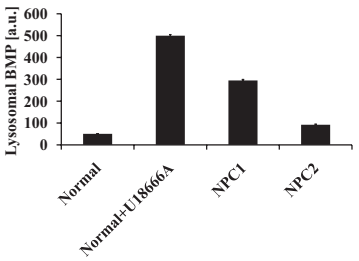

Fig. S7. Comparison of BMP accumulation in NPC1-defective, NPC2-defective, normal, and U18666A-treated normal fibroblasts. BMP levels were quantified as described in the *SI Materials and Methods* using fluorescence microscopy from 20× magnification images obtained with anti-BMP antibody (clone 6C4) and AlexaFluor488-conjugated goat anti-mouse secondary antibody. Data represent averages ± SEM of one representative experiment (n = 32, where n is total number of wells per condition used for quantification).

Table S1. Apparent EC_{50} values for MβCD and HPβCD effects on LSO values of GM03123 and GM18445 cells as function of time of incubation with the compounds

Cell line	Compound	Days of treatment	EC_{50} (μM)	R^2
GM03123	MβCD	1	2.22	0.943
GM03123	MβCD	2	1.10	0.997
GM03123	MβCD	3	0.98	0.995
GM03123	MβCD	4	1.08	0.993
GM18455	MβCD	1	40.39	0.987
GM18455	MβCD	2	3.19	0.965
GM18455	MβCD	3	2.80	0.999
GM18455	MβCD	4	1.69	0.997
GM03123	HPβCD	1	3.16	0.781
GM03123	HPβCD	2	3.77	0.920
GM03123	HPβCD	3	4.18	0.966
GM03123	HPβCD	4	5.44	0.991
GM18455	HPβCD	1	272.41	0.970
GM18455	HPβCD	2	20.84	0.909
GM18455	HPβCD	3	16.94	0.998
GM18455	HPβCD	4	11.51	0.991

Apparent EC_{50}s (compound concentration at 50% reduction in LSO filipin) for MβCD and HPβCD treatment of GM03123 (NPC1) and GM18455 (NPC2) cells for 1–4 days were determined by fitting (using MATLAB, nonlinear least-squares Levenberg-Marquardt algorithm) dose–response curves (Fig. 2 in main text) to the rectangular hyperbola of the form $y = m/(x + b) + c$, and solving for $y = 50$, where y = normalized LSO filipin values (%), x = compound concentration (μM), and m, b, and c are coefficients.

Table S2. Apparent EC_{50} values for MβCD and HPβCD effects on LSO values of U186664-treated GM05659 cells as function of U18666A concentration

	Concentration U18666A (nM)		
	111	333	1,000
EC_{50} MβCD (μM)	6.2	18.9	48.1
EC_{50} HPβCD (μM)	17.4	69.9	184.9

Apparent EC_{50}s (compound concentration at 50% reduction in LSO filipin) for MβCD and HPβCD treatment of GM05659 apparently normal fibroblasts treated with 1 μM U18666A. Cells were pretreated with 1 μM U18666A for 1 day and then treated with the respective cyclodextrin for another day, in the continued presence of 1 μM U18666A compound. EC_{50}s were calculated as in Table S1. All fits had $R^2 \geq 0.97$.