Supplementary Materials and Methods for: Kdo2-Lipid A, a TLR4 specific agonist, induces de novo sphingolipid biosynthesis in RAW264.7 macrophages, which is essential for induction of autophagy by Kacee Sims, Christopher A. Haynes, Samuel Kelly, Jeremy C. Allegood, Elaine Wang, Amin Momin, Martina Leipelt, Donna Reicher, Christopher K. Glass, M. Cameron Sullards & Alfred H. Merrill, Jr.

Methods

Lipid MAPS Consortium Standard Cell Culture Protocol- Warm the growth medium for 15 – 20 minutes in a 37°C water bath. After warming, place a volume of the warmed fresh medium into new vessels. (See below for plate size, cell density, and medium volume recommendations). To begin subculturing, aspirate the old medium from the cells and rinse them once with DPBS. Add fresh medium (~10 ml to 100 mm dish) to the cells and gently scrape the cells until all are dislodged. Pipette the cell suspension, with a 10 ml pipette, up and down five times, without introducing bubbles. Count the cells using a hemacytometer and dispense the cells into the new vessels growing the cells to no more than 80% confluence, (~2-3 days) before passing or harvesting. Recommended cell seeding and media volume for specific plate formats to obtain approximately 80% confluence in 2-3 days: 150 mm plates: 5 x 10^6/20 ml medium, 100 mm plates: 2 x 10^6/10 ml medium, 60 mm plates: 5 x 10^5/5 ml medium, 6-well plates: 3 x 10^5/well/3 ml medium, 12-well plates: 7.5 x 10^4/well/2 ml medium, 24-well plates: 5 x 10^4/well/1 ml medium, 96-well plates: 2 x 10^4/well/100 µl medium

Stable Isotope Labeling of Sphingolipids- Uniformly labeled ^{13}C-palmitate was mixed with fatty acid-free bovine serum albumin (BSA) at a 1:1 molar ratio to make a 1 mM solution[1] which was diluted to 0.1 mM with complete cell culture medium and filter sterilized just before use. The [U-^{13}C]-palmitate/BSA was added to cells at the same time as the KLA or vehicle, incubated for up to 24 h, then the cells were extracted for lipid analysis as described previously (1,2). To quantitate the three categories of SLs labeled with [U-^{13}C]-palmitate (i.e., in the sphingoid base backbone alone—which we designate "Base-labeled"—in the fatty acid only—"Fatty acid-labeled"—and both the sphingoid base and fatty acid—"Dual-labeled"), the additional multiple reaction monitoring (MRM) pairs were added: the [M + 16] precursor ions and [M + 16] product ions for Base-labeled; [M + 16] precursor ions and [M + 0] product ions for "Fatty acid-labeled"; and [M + 32] precursor ions and [M + 16] product ions for "Dual-labeled." The MRM extracted ion chromatogram peak areas of all four SL isotopomers were integrated (Analyst 1.4), converted to picomoles using the peak areas of the internal standards, and normalized to the micrograms of DNA in the sample, except where otherwise noted.

Generation of RAW264.7 cells stably expressing GFP-LC3- LC3A cDNA was obtained from Origene. To generate N-terminal GFP-tagged LC3, the LC3A gene was amplified using the following gene-specific primers: 5'-ATGCCCTCAGACCGGCCTTT-3' and 5'-GAAGCCGAAGGTTTCCTGGGAGG-3'. The PCR fragment corresponding to the coding sequence of LC3A was then cloned into pEGFP-C2/*SmaI* (Clonetech, Mountain View, CA) resulting pEGFP-C2-LC3A. Sequencing was used to confirm the fidelity of the construct.

RAW264.7 cells stably expressing green fluorescent protein (GFP)-tagged LC3 were generated by transfection with pEFGP-C2-LC3A. Transfection was performed in 100-mm dishes (3.0 x 10^6 cells/dish) with 6 µg plasmid DNA/plate with GeneJuice® (Novagen; EMD Biosciences, San Diego, CA) according to the manufacturer's protocol. Following 48 h of

[1] Solutions containing BSA were tested for endotoxin contamination because we noted that fatty acid-free BSA from some suppliers was contaminated or became so during incubation at 37°C for the preparation of the fatty acid-BSA complex.

exposure, cells were washed with PBS and selected in media containing 300 µg/mL of G418 (Sigma-Aldrich) for 14 days. The media was changed every other day. After two weeks, selected G418-resistant colonies were cloned by two rounds of limiting dilution in a 96 well plate order to establish fluorescent colonies originating from a single cell. In order to confirm localization and expression of the LC3, GFP-LC3-transfected RAW264.7 cells were cultured on 12 mm cover slips (VWR; West Chester, PA). Following exposure to Kdo_2-Lipid A (Avanti Polar Lipids; Alabaster, AL) for 24 h, cells were fixed in 4% formaldehyde and analyzed by fluorescence confocal microscopy.

Immunohistochemical Analysis of the ER and Golgi- For visualization of the ER and Golgi, fixed cells were permeabilized with 0.2% Triton X-100 for 5 min, blocked in 3% BSA, 2% serum in PBS for 2 h, and subjected to indirect immunofluorescence staining. Cells were incubated overnight at 4 °C with anti-BiP (1:500) for visualization of the ER or anti-GM130 (1:500) for visualization of the Golgi diluted in 0.1% BSA in PBS. The cells were washed three times with PBS and incubated with the Alexa Fluor-conjugated secondary antibody (1:200) diluted in 0.1% BSA in PBS for 3 h at room temperature prior to nucleic acid staining.

Immunohistochemical Analysis of Ceramide Localization- Cer staining was performed through the adaptation of (26). Briefly, for surface Cer staining, the non-specific binding sites of fixed cells were saturated for 2 h at room temperature with 3% BSA, 2% serum in PBS followed by overnight incubation at 4°C with anti-ceramide (1:50) diluted in 0.1% BSA. In order to visualize intracellular ceramide, the staining protocol was continued with a second fixation with 4% formaldehyde for 20 min at room temperature. The cells were then washed twice with PBS and permeabilized with 0.2% Triton X-100 for 5 min. Non-specific intracellular binding sites were blocked with 3% BSA, 2% serum in PBS for 45 min followed by overnight incubation at 4°C with anti-Cer Ab (1:50) diluted in 0.1% BSA. The cells were then washed three times and incubated with the Alexa Fluor-conjugated secondary antibody (1:200) diluted in 0.1% BSA for 3 h at room temperature prior to nucleic acid staining.

Immunohistochemical Analysis of SPT and 3KSR Localization- Serine palmitoyltransferase (SPT) staining was performed as previously described (35). For 3-ketosphinganine reductase (3KSR) staining, fixed cells were permeabilized with 0.2% Triton X-100 for 5 min, blocked in 10% FBS in PBS for 1 h, and subjected to indirect immunofluorescence staining. Cells were incubated overnight at 4 °C with anti-HA-tag (1:800) diluted in 10% FBS in PBS. The cells were washed three times with 10% FBS and incubated with the Alexa Fluor-conjugated secondary antibody (1:200) diluted in 10% FBS for 2 h at room temperature.

Measurement of Cell Diameter and Area- RAW264.7 cells were cultured on glass coverslips (VWR, Inc. West Chester, PA) in a 24-well plate. Following treatment, cells were fixed with 4% formaldehyde in PBS at room temperature for 15 min. Fixed cells were rinsed in PBS and mounted in Fluoromount G (Southern Biotechnology Associates, Inc., Birmingham, AL) prior to observation under a Zeiss LSM 510 inverted laser scanning confocal microscope (Heidelberg, Germany). Bright field images were collected with the resident Zeiss confocal microscope software and cellular diameter measured using ImageJ version 1.41a (National Institute of Health). The surface area was calculated using the measured diameter.

Analysis of Autophagy in CHO-LYB and CHO-LYB-LCB1 Cells- CHO-LYB and CHO-LYB-LCB1 cells were obtained from Ken Hanada and verified to have the phenotype of being defective in *de novo* SL biosynthesis due to mutation of SPT1 (CHO-LYB) (3). This pathway is restored in the CHO-LYB-LCB1 cell line by stable transfection with wild-type SPT1 (3). In order to monitor autophagy, CHO-LYB and CHO-LYB-LCB1 cells were transfected with GFP-

LC3. Transient transfection was performed using GeneJuice® transfection reagent (Novagen; EMB Biosciences, San Diego, CA) according to manufacturer's protocol. 24 h after transfection, cells were treated with vehicle control (EtOH) or fenretinide/4HPR (10 μM). Fenretinide/4HPR is an established inducer of autophagy in a variety of cell lines (4-6). Following 24 h treatment, cells were fixed with 4% formaldehyde in PBS at room temperature for 15 minutes. Fixed cells were rinsed in PBS and mounted in Fluoromount G (Southern Biotechnology Associates, Inc., Birmingham, AL) prior to observation under a Zeiss LSM 510 inverted laser scanning confocal microscope (Heidelberg, Germany). Autophagic cells were identified as cells with five or more GFP-LC3 punctate autophagosomes for each condition.

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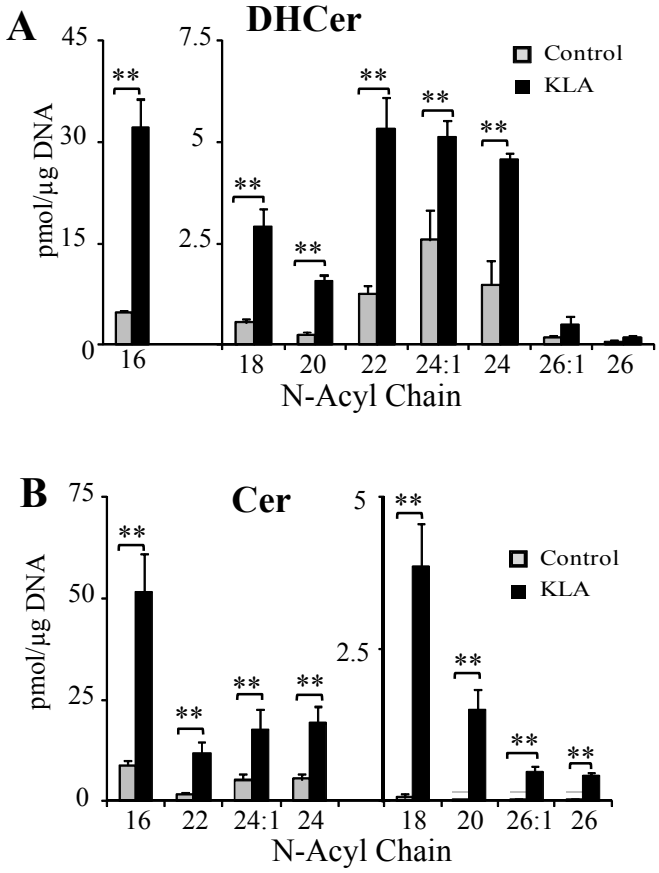
Supplementary Figures for: Kdo2-Lipid A, a TLR4 specific agonist, induces de novo sphingolipid biosynthesis in RAW264.7 macrophages, which is essential for induction of autophagy by Kacee Sims, Christopher A. Haynes, Samuel Kelly, Jeremy C. Allegood, Elaine Wang, Amin Momin, Martina Leipelt, Donna Reichart Christopher K. Glass, M. Cameron Sullards & Alfred H. Merrill, Jr.



Supplementary Figure S1. Dihydroceramides and Ceramides of RAW264.7 cells incubated PBS (Control) or KLA for 24 h. RAW264.7 cells were incubated with vehicle control (PBS) or KLA (100 ng/mL) for 24 h then the sphingolipids were analyzed by LC ESI-MS/MS. The data represent the mean + SEM (n = 9); *p < 0.05; **p < 0.001.

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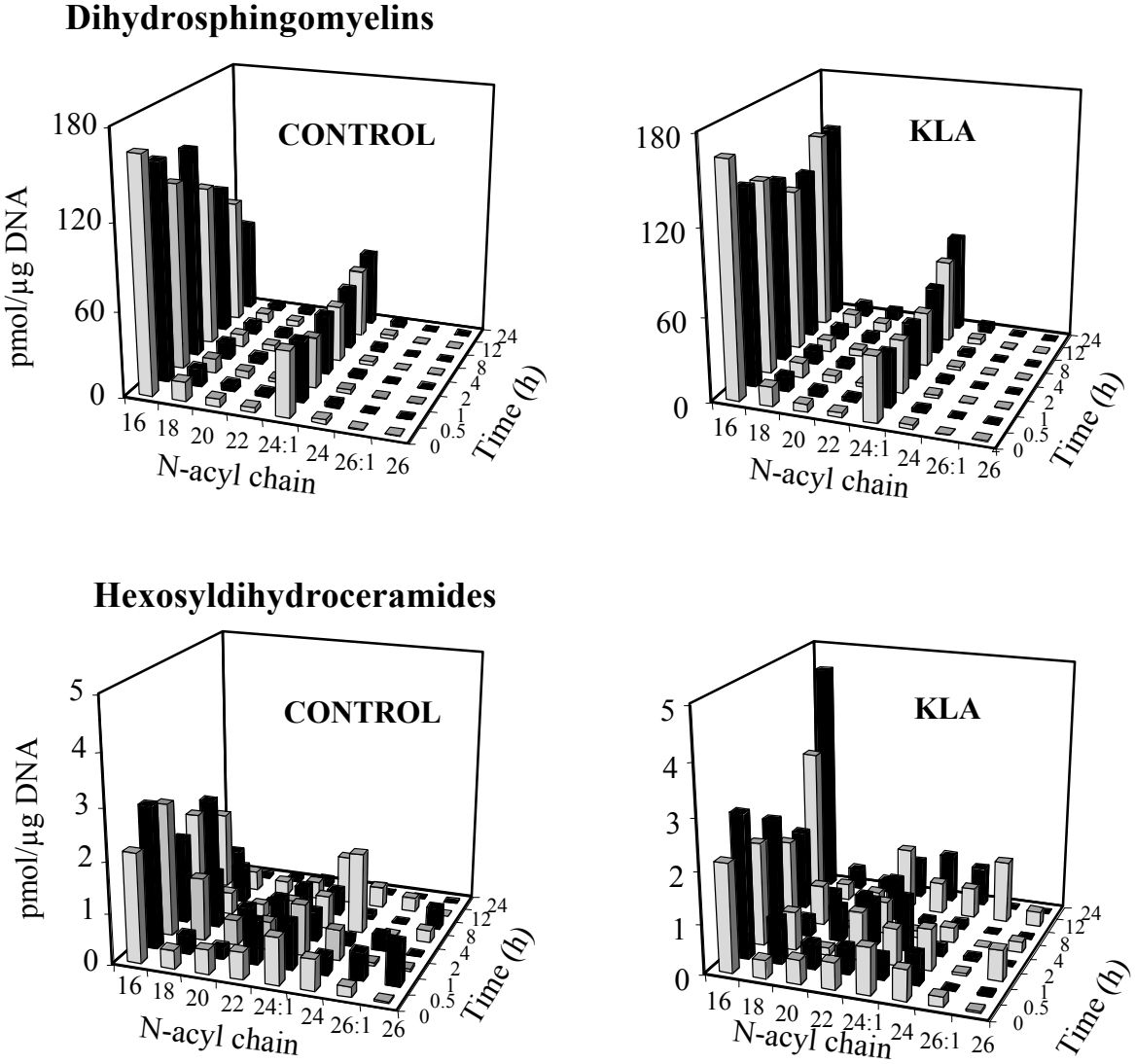

Supplementary Figure S2: KLA Increases Dihydroceramide Metabolites. RAW264.7 cells were incubated for various times with vehicle control (PBS) or KLA (100 ng/mL). Following treatment, cells were harvested at the indicated time points for lipid extraction and analysis by LC ESI-MS/MS. Data represent mean (n = 9).

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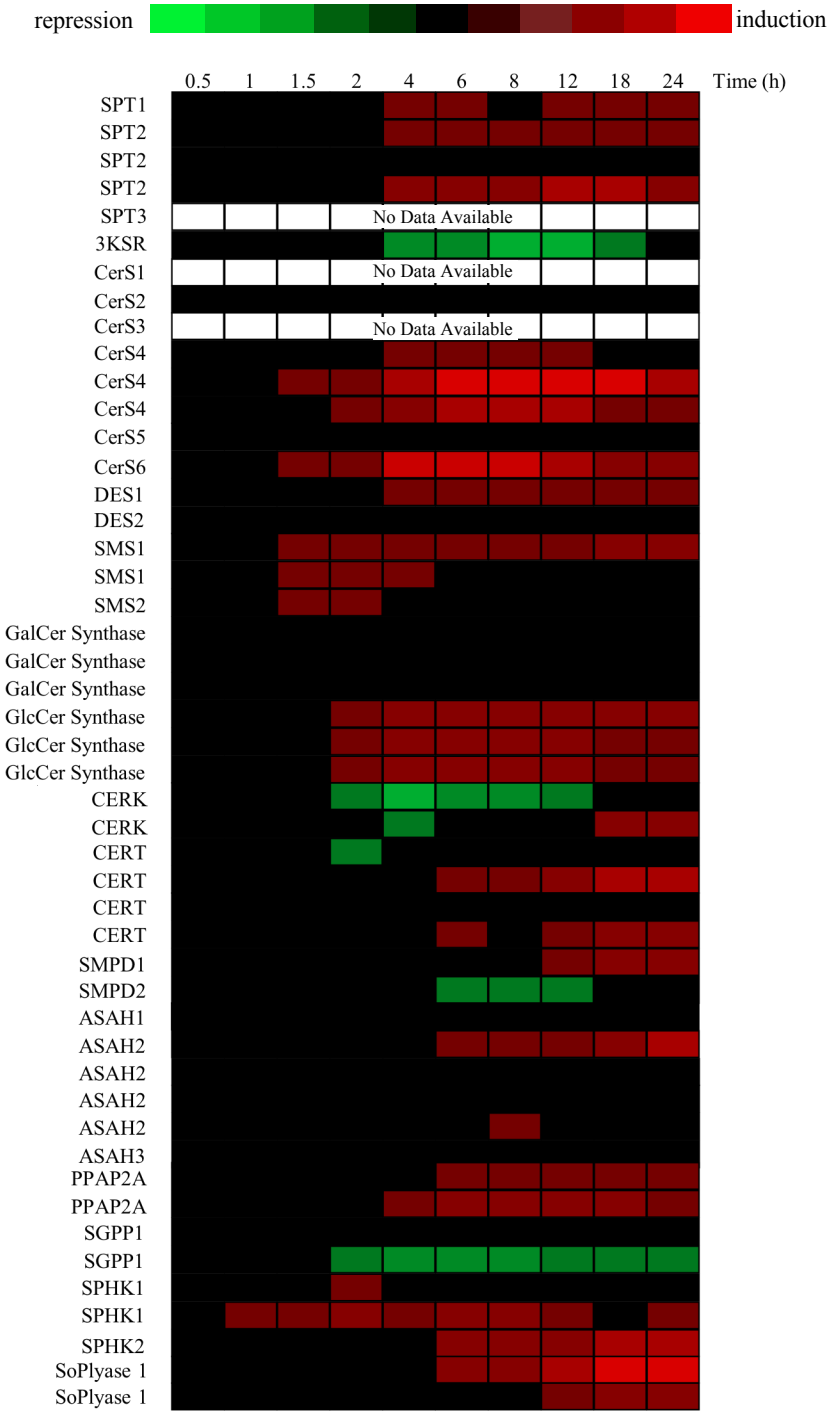

Supplementary Figure S3: mRNA Expression of Genes Involved in Sphingolipid Biosythesis Following KLA Stimulation RAW 264.7 cells were harvested 0.5, 1.0, 1.5, 2.0, 4.0, 6.0, 8.0, 12.0, 18.0, and 24 hours after treatment with 100ng/ml Kdo2-Lipid A. The gene symbols (*mus musculus)* used at www.lipidmaps.org for the enzymes of the sphingolipid pathway: SPT family (sptlc1 and sptlc2), 3KSR (FVT1), CerS (lass2, lass4, lass5, and lass6). DES family (Degs1 and Degs2), SMS 1 (Tmem23), SMS2 (4933405A16RIK), GalCer Synthase (ugt8a), GlcCer synthase (ugcg), CERK (CERK), CERT (Col4a3bp), SMPD1 (SMPD1), SMPD2 (SMPD2), ASAH1 (ASAH1), ASAH2 (ASAH2), ASAH3 (ASAH3), PPAP2A (PPA2A), SGPP1 (SGPP1), SPHK1 (SPHK1), SPHK2 (SPHK2), SoPlyase1 (sgp11)

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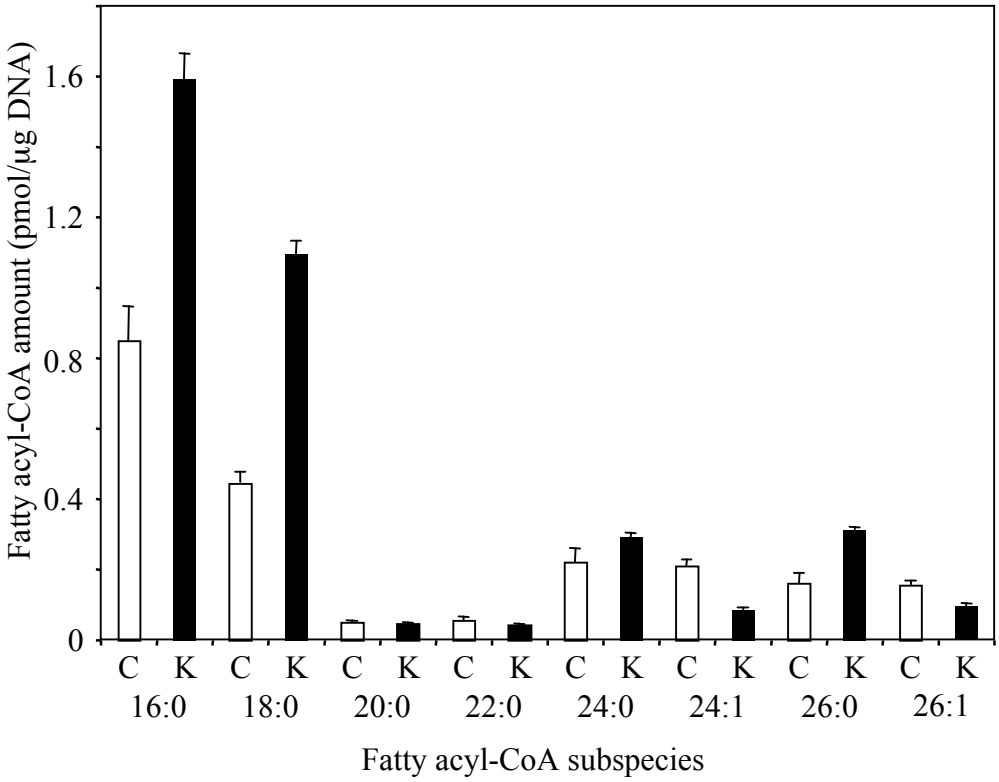

Supplementary Figure S4: Amounts of Fatty acyl-CoAs in RAW264.7 cells with and without KLA stimulation. RAW264.7 cells were incubated for 24 with vehicle control (PBS) or KLA (100 ng/mL). Following treatment, cells were harvested at the indicated time points for lipid extraction and analysis by LC ESI-MS/MS for the amounts of various chain length fatty acyl-CoAs that are utilized by CerS to make SL. Data represent mean ± SEM (n = 3).

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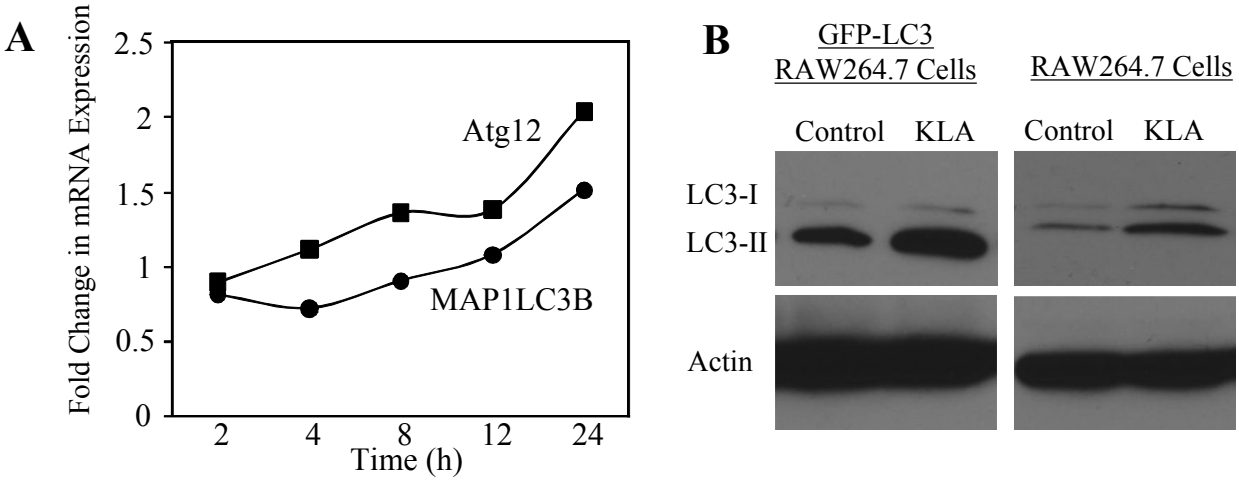

Supplementary Figure S5. Additional evidence that KLA induces autophagy in RAW264.7 cells. A, RAW264.7 cells were incubated in the absence (control) or presence of KLA (100 ng/mL), harvested at 2, 4, 8, 12, and 24 h and for each time point an Agilent array was used to determine the mRNA expression level of various genes including Map1LC3B and Atg12. B, RAW264.7 cells were incubated for 24 h with vehicle control (PBS) or KLA (100 ng/mL) prior to immunoblot analysis using LC3 or β actin antibodies.

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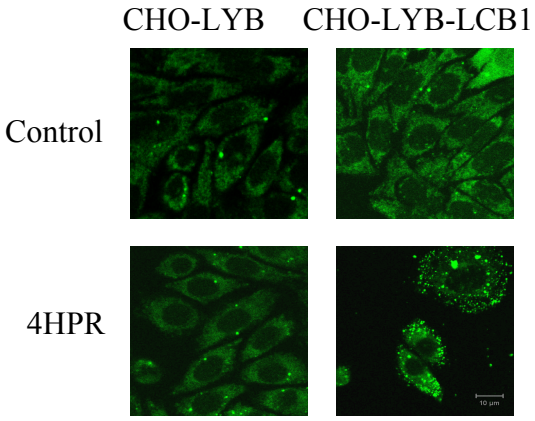

Supplementary Figure S6: Confirmation that *de novo* Sphingolipid Biosynthesis is Required for Autophagy using CHO-LYB Cells CHO-LYB and CHO-LYB-LCB1 cells transfected with GFP-LC3 were incubated with vehicle control (EtOH) or fenretinide/4HPR (10 ㅼM) for 24 h. Representative images for each condition are shown.