SUPPLEMENTAL INFORMATION

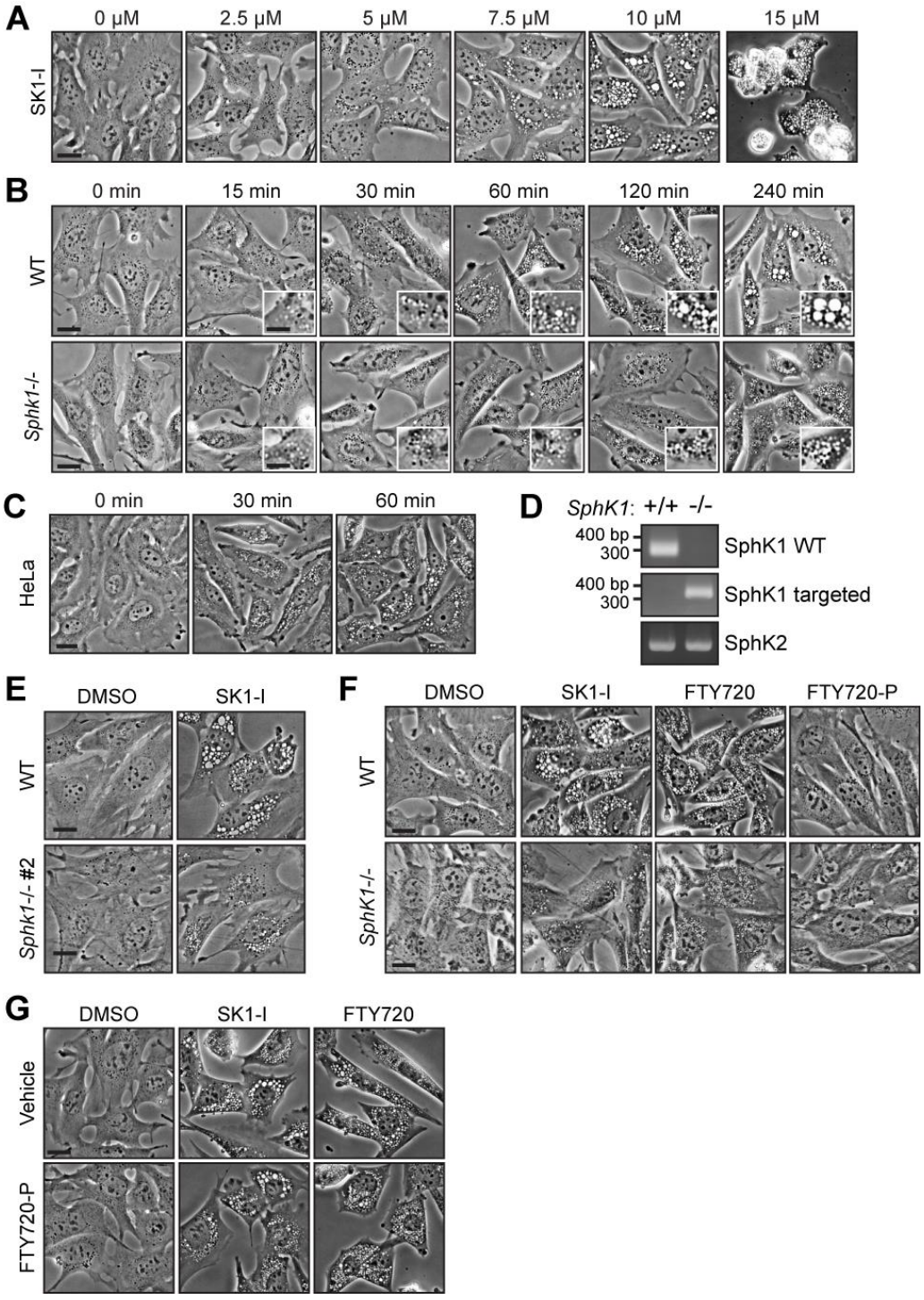

Figure S1, Related to Figure 1. (**A**) Phase contrast images of WT MEFs subjected to a dose-response of SK1-I for 4 h. (**B**) Phase contrast images of WT or *SphK1*-/- MEFs treated with 10 µM SK1-I for the indicated durations. (**C**) Phase contrast images of HeLa cells treated with 10 µM SK1-I for the indicated durations. (**D**) PCR genotyping of established WT and *SphK1*-/- MEFs. (**E**) Phase contrast images of WT MEFs or an additional *SphK1*-/- clone (*SphK1*-/- #2) treated with 10 µM SK1-I for 2 h. (**F**) Phase contrast images of WT or *SphK1*-/- MEFs treated with 10 µM SK1-I, FTY720 or FTY720-phosphate (FTY720-P) for 4 h. (**G**) Phase contrast images of WT MEFs pre-treated with 100 nM FTY720-P for 30 min prior to the addition of 10 µM SK1-I or FTY720 for an additional 4 h. Scale bars represent: 20 µm; 10 µm in enlarged panels of (**B**).

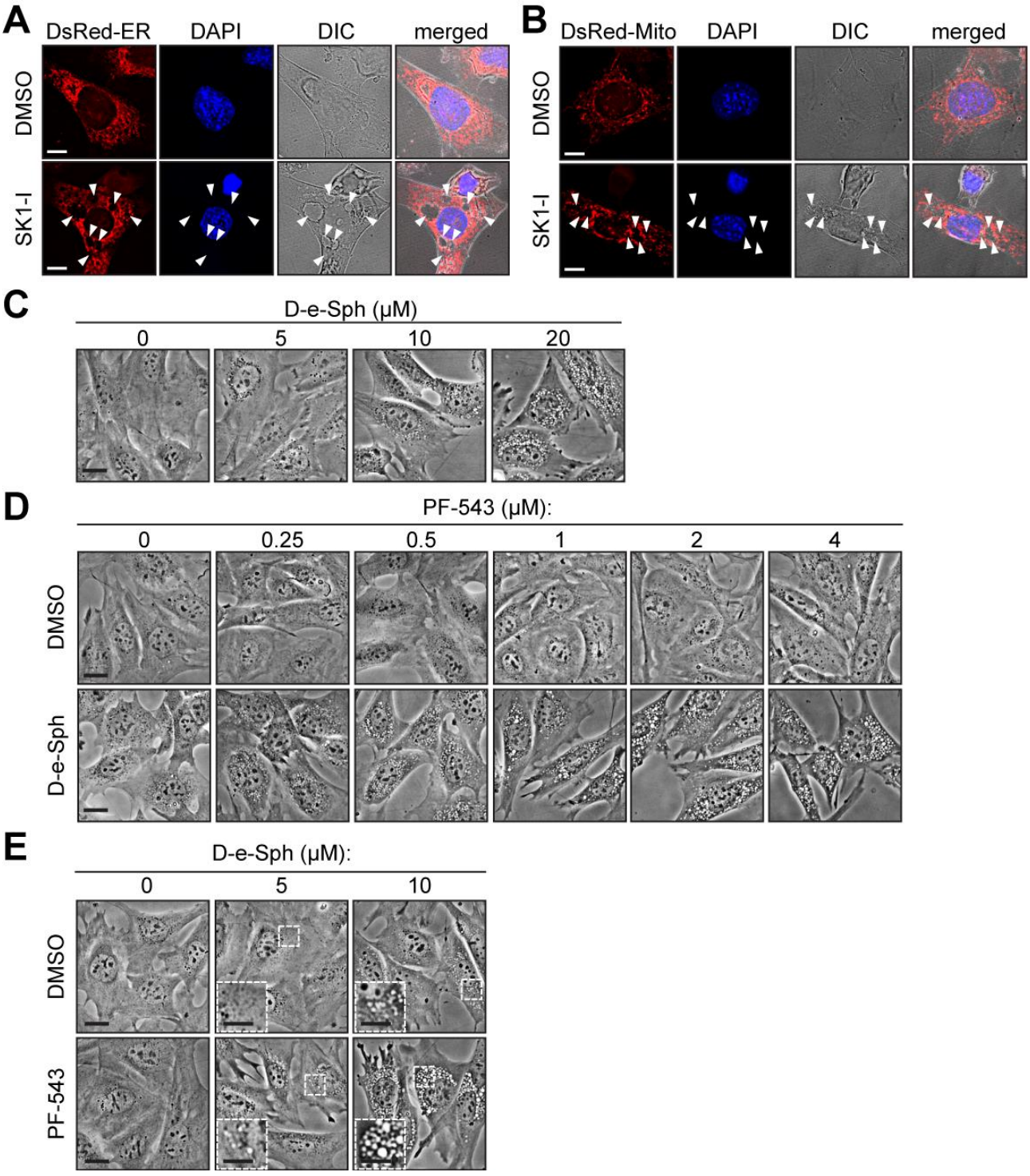

Figure S2, Related to Figures 1 & 2. (**A-B**), Fluorescence images of WT MEFs stably expressing (**A**) DsRed-ER or (**B**) DsRed-Mito and treated with 10 μM SK1-I for 6 h followed by fixation and nuclei staining with DAPI. Arrowheads indicate vacuoles. (**C**) Phase contrast images of WT MEFs subjected to a dose-response of Sph for 4 h. (**D**) Phase contrast images of WT MEFs pre-treated with various concentrations of PF-543 for 30 min prior to 10 μM Sph for an additional 2 h. (**E**) Phase contrast images of WT MEFs pre-treated with 2 μM PF-543 for 30 min prior to the addition of 5 μM or 10 μM Sph for 2 h. Scale bars represent: 10 μm in (**A**), (**B**), & enlarged panels of (**E**); 20 μm in (**C**)-(**E**).

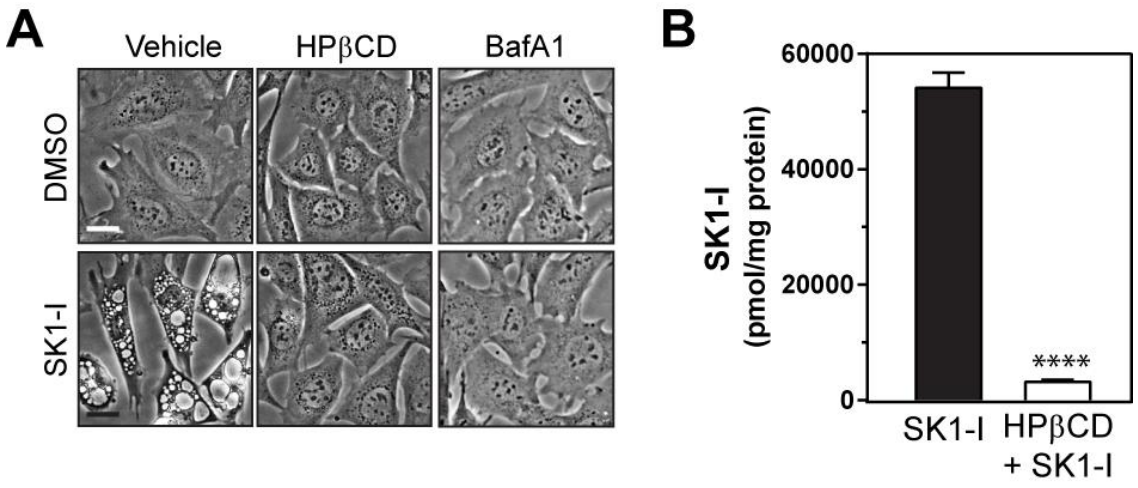

Figure S3, Related to Figure 3. (**A**), Phase contrast images of WT MEFs pre-treated with 2% HPβCD or 100 nM bafilomycin A1 (BafA1) for 20 min prior to DMSO or 10 μM SK1-I treatment for an additional 1 h. Scale bars represent 20 μm. (**B**), LC-MS/MS quantification of SK1-I uptake using total cell lysates from WT MEFs pre-treated with vehicle or 2% HPβCD for 20 min followed by 10 μM SK1i for an additional 4 h. Data represent the mean ± SD of triplicate samples. Student's t-test. ****, $p<0.0001$.

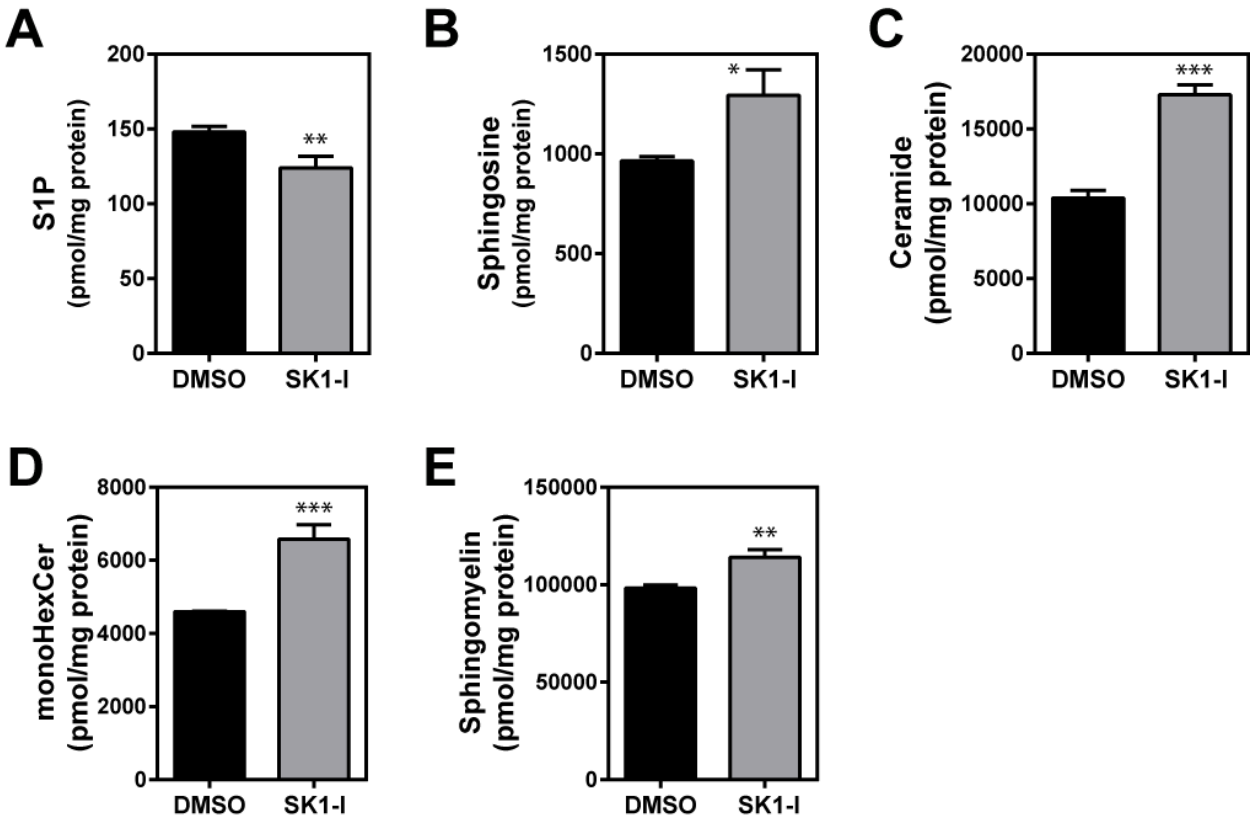

Figure S4, Related to Figure 4. LC-MS/MS quantification of cellular sphingolipids from total cell lysates after treatment of WT MEFs with DMSO or 10 µM SK1-I for 4 h. Sphingolipids are presented as mean pmol per mg protein ±SD of triplicate samples. (**A**), Sphingosine-1-phosphate. (**B**), Sphingosine. (**C**), Ceramide. (**D**), Monohexosylceramide. (**E**), Sphingomyelin. Student's t-test, ***, $p<0.001$; **, $p<0.01$; *, $p<0.05$.

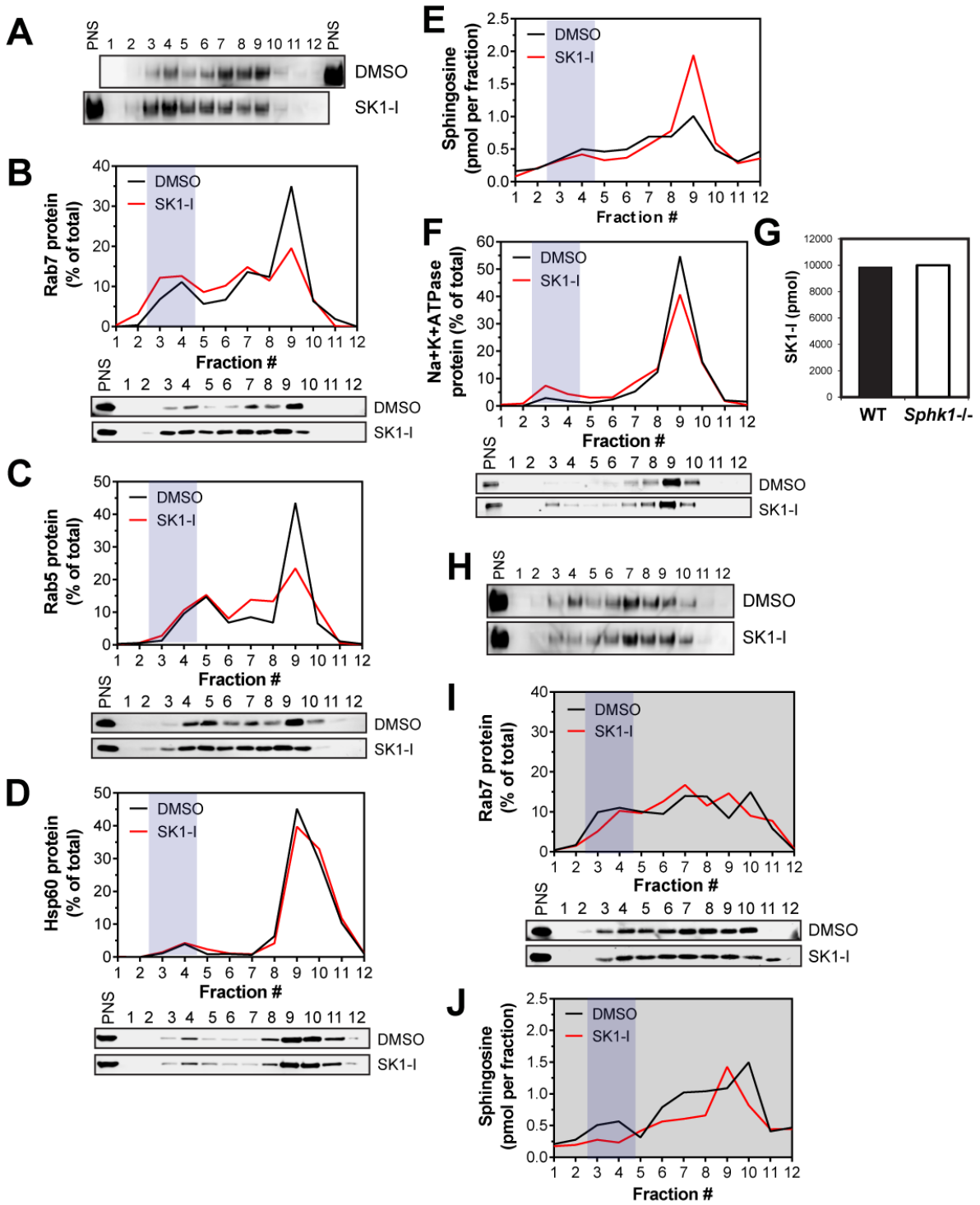

Figure S5, Related to Figure 4. WT (**A**-**G**) or *SphK1*-/- (**G**-**J**) MEFs were treated with DMSO or 10 µM SK1-I for 4 h followed by subcellular fractionation as in **Figure 4**. Protein expression was quantified by western blot and is presented as percent enrichment in each fraction. Sphingolipids were quantified by LC-MS/MS and are presented as pmol lipid per fraction. (**A**) Immunoblot of Lamp1 in subcellular fractions of WT cells. (**B**-**D** and **F**), Immunoblot and quantification of (**B**) Rab7, (**C**) Rab5, (**D**) Hsp60, and (**F**) Na+K+ ATPase in subcellular fractions of WT cells. (**E**) Sphingosine quantification in subcellular fractions of WT cells. (**G**) Sum of SK1-I in subcellular fractions of WT or *SphK1*-/- MEFs treated for 4 h. (**H**) Immunoblot of Lamp1 in subcellular fractions of *SphK1*-/- cells. (**I**), Immunoblot and quantification of Rab7 in subcellular fractions of *SphK1*-/- cells. (**J**) Sphingosine quantification in subcellular fractions of *SphK1*-/- cells. PNS, post-nuclear supernatant.

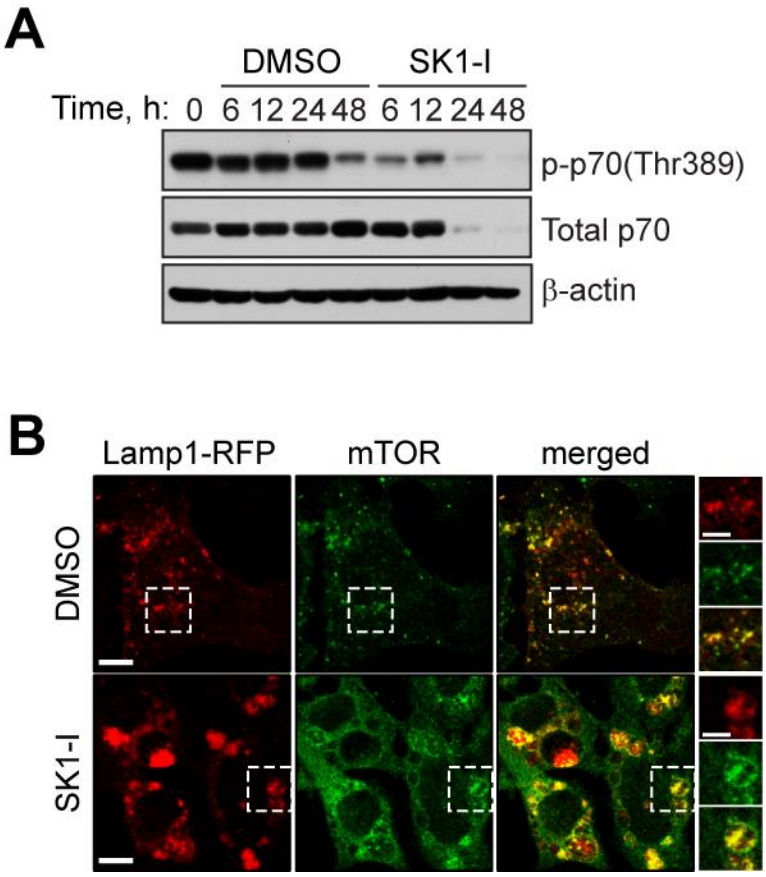

Figure S6, Related to Figure 5. (**A**) Immunoblot of WT MEFs treated with DMSO or 10 μM SK1-I for the indicated durations and probed for phospho-p70 S6 kinase (Thr389) [p-p70(Thr389)], total p70 S6 kinase (Total p70) or β-actin. (**B**) Confocal images of WT MEFs stably expressing Lamp1-RFP and treated with 10 μM SK1-I for 4 prior to immunostaining using anti-mTOR. Scale bars represent 20 μm.

Movie S1, Related to Figure 1. WT MEFs were treated with 7.5 μM SK1-I for 1 h prior to live cell time-lapse imaging at one minute intervals for an additional 4 h. Scale bar: 10 μm.

Movie S2, Related to Figure 1. WT MEFs were treated with 7.5 μM SK1-I for 30 minutes prior to live cell time-lapse imaging at 30 minute intervals for an additional 21.5 h. Scale bar: 20 μm.

Movie S3. Related to Figure 1. *SphK1*-/- MEFs were treated with 7.5 μM SK1-I for 1 h prior to live cell time-lapse imaging at one minute intervals for an additional 4 h. Scale bar: 10 μm.

Movie S4. Related to Figure 1. *SphK1*-/- MEFs were treated with 7.5 μM SK1-I for 30 minutes prior to live cell time-lapse imaging at 30 minute intervals for an additional 21.5 h. Scale bar: 20 μm.

Movie S5. Related to Figure 1. EGFP-Sphk1 was transiently expressed in *SphK1*-/- MEFs and cells were treated with 7.5 μM SK1-I for 4 h prior to live cell time-lapse imaging of GFP fluorescence. Scale bar: 10 μm.

Movie S6. Related to Figure 6. WT MEFs stably expressing Lamp1-RFP were treated with 10 μM SK1-I for 24 h prior to live-cell fluorescence time-lapse imaging. Scale bar: 10 μm.

Supplemental Experimental Procedures

Reagents. SK1-I was obtained from Enzo Life Sciences (BML-EI411) and dissolved in DMSO (note: aliquotting in glass vials at -20°C is suggested as SK1-I appears to degrade after prolonged storage in plastic tubes). D-erythro-sphingosine (d18:1; Avanti Polar Lipids) was dissolved in chloroform:methanol (90:10) and stored at -20°C. For treatment, solvent was evaporated, and D-erythro-sphingosine was resuspended in DMSO. The following reagents were purchased from the indicated sources: PF-543 (EMD Millipore); N,N-dimethyl-Sphingosine (Tocris Bioscience); FTY720, FTY720-phosphate, fumonisin B1 and E64d (Cayman Chemical). All other chemicals were purchased from Sigma.

Cell culture. SV40 large T-antigen immortalized mouse embryonic fibroblasts (MEFs) were kindly provided by: Dr. Noboru Mizushima (*Atg5+/+* and *Atg5−/−,* Tokyo Medical and Dental University, Tokyo, Japan) and Dr. Shengkan Jin (*Atg3+/+* and *Atg3−/−,* Robert Wood Johnson Medical School, New Brunswick, NJ). HeLa S3 cells were obtained from ATCC. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 100μgml^{-1} streptomycin, 100unitsml^{-1} penicillin, and 250ngml^{-1} amphotericin B. As glutamine undergoes spontaneous breakdown to ammonia and both regulate autophagy (Eng et al., 2010), experiments were performed using L-glutamine-free DMEM supplemented with fresh 3 mM L-glutamine immediately prior to experiments. All treatments were performed at 50-60% cell confluency.

Plasmids. The following cDNAs were subcloned into the lentiviral transfer vector backbone pCDH1-MCS1-EF1-puro (System Biosciences) using the indicated 5' and 3' restriction sites: Lamp1-RFP (Addgene 1817; 5'-EcoRI, 3'-HpaI/SwaI), DsRed2-Mito (Clontech 632421; 5'-NheI, 3'-NotI), DsRed2-ER (Clontech 632409; 5'-NheI, 3'-BglII/BamHI) and Atg5(K130R)-HA (Addgene 22957; 5'-NheI, 3'-NotI).

Murine SphK1a and catalytic mutant SphK1a^{G82D} cDNAs were a generous gift from Dr. Sarah Spiegel (Virginia Commonwealth University, Richmond, VA). SphK1a or SphK1a(G82D) cDNA was amplified by PCR (forward primer: 5'-AATACGTCGACGTGAACCAGAATGCCCTC-3', and reverse primer: 5'-CCGCCGATATCTTATGGTTCTTCTGGAGGTG-3') and subcloned into a pLJM1-EGFP vector using 5'-SalI and 3'-EcoRV restriction sites. Plasmids were expressed by nucleofection (Lonza) or lentiviral transduction. Recombinant lentiviruses were produced in HEK293T/17 cells using the third generation lentiviral packaging system (Invitrogen). Stable cell lines were generated by selection with 1 μgml^{-1} puromycin.

Antibodies for Immunostaining. Antibodies used for immunostaining were as follows: anti-Rab7 (Cell Signaling 9367; 1:150); anti-EEA1 (MBL M176-2; 1:1000); anti-Lamp-1 (Santa Cruz sc-19992; 1:100); anti-mTOR (Cell Signaling 2983; 1:150); goat anti-mouse IgG AlexaFluor488 (Invitrogen A11029; 1:2000); goat anti-rabbit IgG

AlexaFluor568 (Invitrogen A11011; 1:2000); goat anti-rat IgG AlexaFluor647 (Invitrogen A21247; 1:2000); goat anti-rabbit IgG AlexaFluor488 (Invitrogen A11008; 1:2000).

Antibodies of Immunoblotting. Antibodies used for immunoblotting were as follows: anti-LC3 (Novus Biologicals NB100-2220); anti-p62 (American Research Products 03-GP62-C); β-actin (Sigma A5441); anti-Lamp1 (Santa Cruz sc-19992); anti-Rab7 (Sigma R8779); anti-Rab5 (Cell Signaling 3547); anti-Hsp60 (BD Biosciences 611562); anti-Na+/K+ ATPase (Cell Signaling 3010); anti-α-tubulin (Sigma T5168); anti-TFEB (Bethyl Laboratories A303-673A); anti-PARP (Cell Signaling 9542); anti-phospho-p70 S6 kinase(Thr389) (Cell Signaling 9206); anti-p70 S6 kinase (Cell Signaling 9202). HRP-conjugated secondary antibodies were from GE Healthcare, and IR dye-conjugated secondary antibodies were obtained from LI-COR.

Supplemental References

Eng, C.H., Yu, K., Lucas, J., White, E., and Abraham, R.T. (2010). Ammonia Derived from Glutaminolysis Is a Diffusible Regulator of Autophagy. Sci. Signal. *3*, ra31–ra31.