< Supporting Information >

Discovery of autophagy modulators through the construction of high-content screening platform via monitoring of lipid droplets

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I. General information

Reagents and materials

Chemicals for autophagy regulation were purchased from Sigma-Aldrich. Fluorescent dyes such as SF44 and Hoechst 33342 were obtained by reported procedure[1] or purchased from Invitrogen. Micro BCA™ Protein Assay Kit was purchased from PIERCE and was used for the measurement of protein concentration of cell lysate. Cell culture reagents including fatal bovine serum, culture media, and antibiotic-antimycotic solution were purchased from GIBCO, Invitrogen. The culturing dish or screening plates were purchased from CORNING. All antibodies for western blot analysis were purchased from Abcam and Cell Signalling. Developing for western blot analysis was performed by Amersham ECL Prime Western Blotting Detection System from GE Healthcare Life Science.

Instruments and programs

Fluorescence microscopy studies were carried with Olympus Inverted Microscope Model IX71, equipped for epi-illumination using a halogen bulb (Philips No. 7724). Emission signal of each experiment was observed at the indicated spectral setting: green channel, using a 450–480 band pass exciter filter, a 500 nm center wavelength chromatic beam splitter, a 515 nm-long pass barrier filter (Olympus filter set U-MWB2). Emission signal of each experiments were detected with 12.5M pixel recording digital color camera (Olympus, DP71). Quantification of fluorescence images was analyzed by Image-Pro Plus® 6.2 program and all graphs were figured by GraphPad Prism 5. The quantified data are the mean measurements of 40–50 cells from at least three independent experiments and these measurements were normalized by DMSO as a control.

High-contents screening was performed by InCell Analyzer 2000 (GE Healthcare). Images of randomly selected 4 different spots per individual well in a 96-well plate were automatically captured. Images were taken by auto-focusing mode and 20× scale. Fluorescence imaging was also performed with SF44 for lipid dropet (LD) and Hoechst for nuclei at the indicated filter setting; Excitation filter: 430/24× and Emission filter 605/64 nm for LD; Excitation filter: 350/50× and Emission filter: 455/50 nm for nuclei. Data were analyzed by InCell Analyzer 1000 workstation 3.6 program according to the manufacturer's protocol. Fluorescence intensity of LD was interpreted as a cellular organelle using granularity module and the area of individual cell was recognized by nuclei staining using collar segmentation.

Chemiluminescent signal was monitored by ChemiDoc™ MP imaging system (Bio-Rad) and quantified by ImageLab 4.0.1 program.

[1] *J. Am. Chem. Soc.* **2011,** *133,* 6642–6649.

II. Monitoring of autophagy regulation with SF44

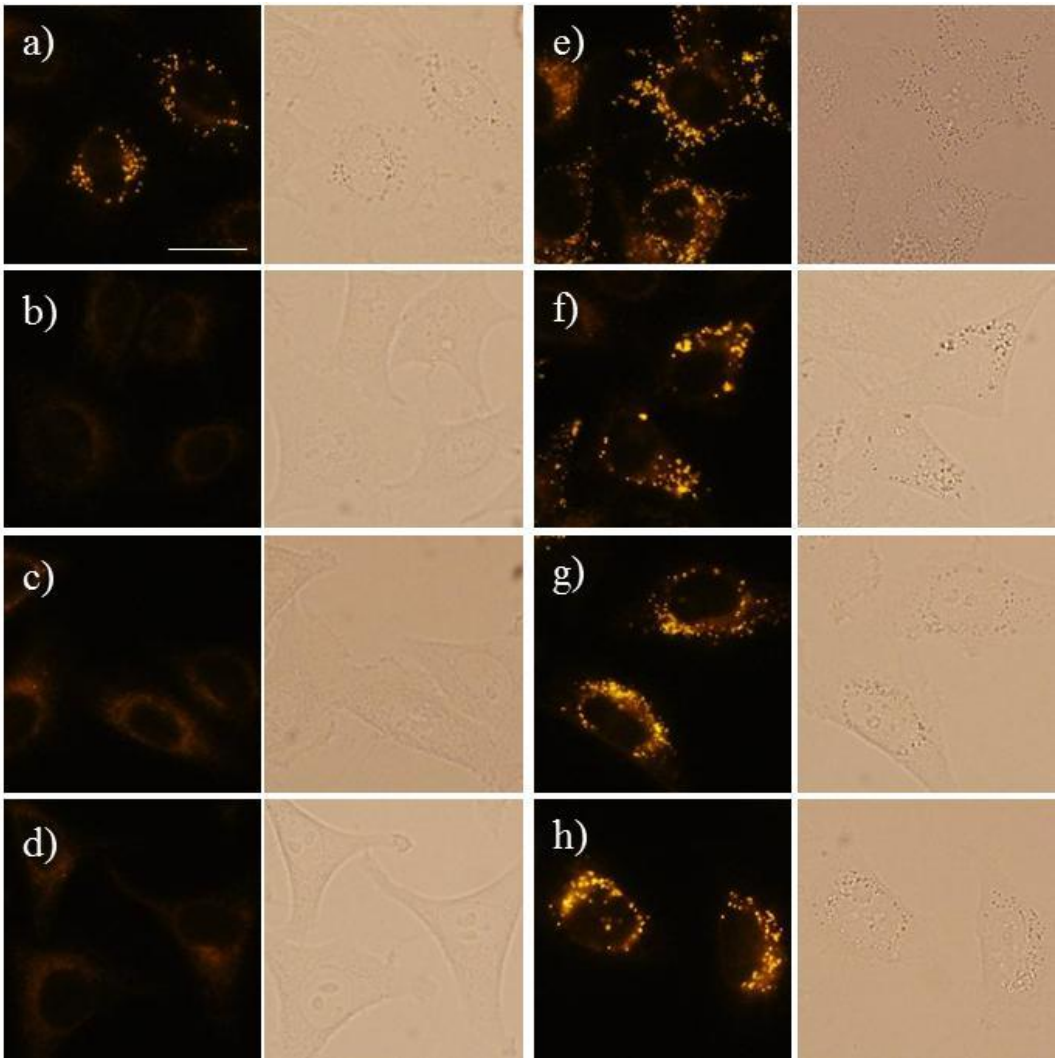

Fig. S1 Fluorescence (left) and bright field (right) images of autophagic regulation in HeLa cells under various conditions [corresponding to Fig. 2a–h]. Cellular LDs were visualized with SF44. Cells were incubated with (a) DMSO as a control; (b) serum-free media, (c) rapamycin, and (d) tamoxifen to activate autophagy; (e) 3-methyladenine, (f) wortmannin, (g) bafilomycin A1, and (h) nocodazole to inhibit autophagy. The scale bar represents 20 μm.

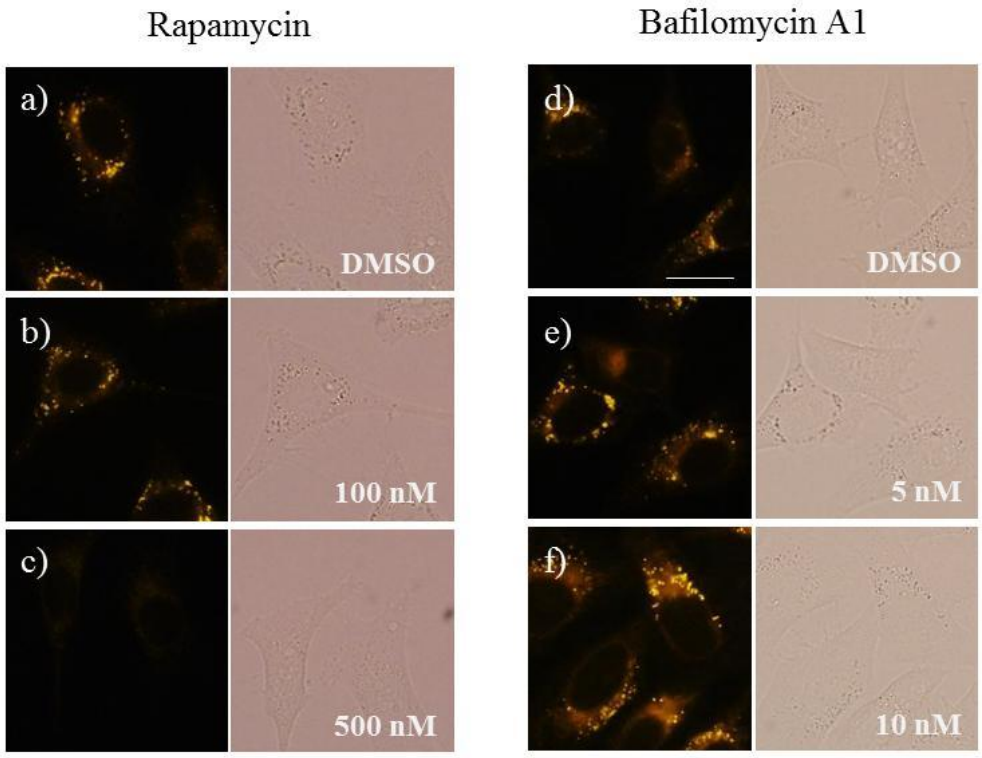

Fig. S2 Fluorescence (left) and bright field (right) images of autophagy regulation in HeLa cell with rapamycin and bafilomycin A1 [corresponding to Fig. 2i, j]. Cellular LDs were visualized with SF44. Cells were incubated with rapamycin in (a–c) and bafilomycin A1 in (d–f) with different concentrations indicated in each figure. The scale bar represents 20 μm.

III. Construction of high-content screening platform

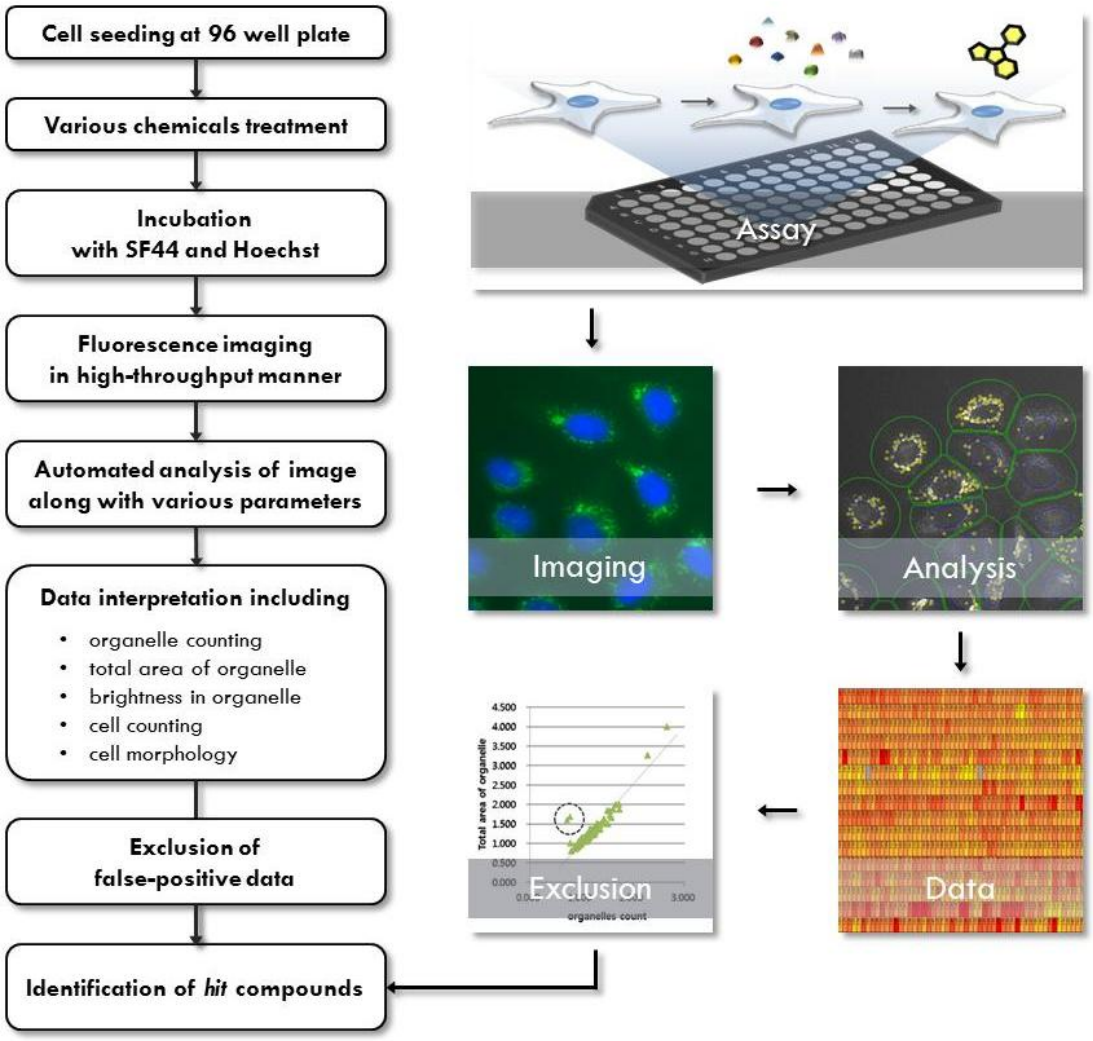

Fig. S3 Systematic flow chart and diagram of image-based screening process for the identification of *hit* compounds using automated imaging instrumentation and image analysis.

IV. Analysis of a pilot screening results with various parameters

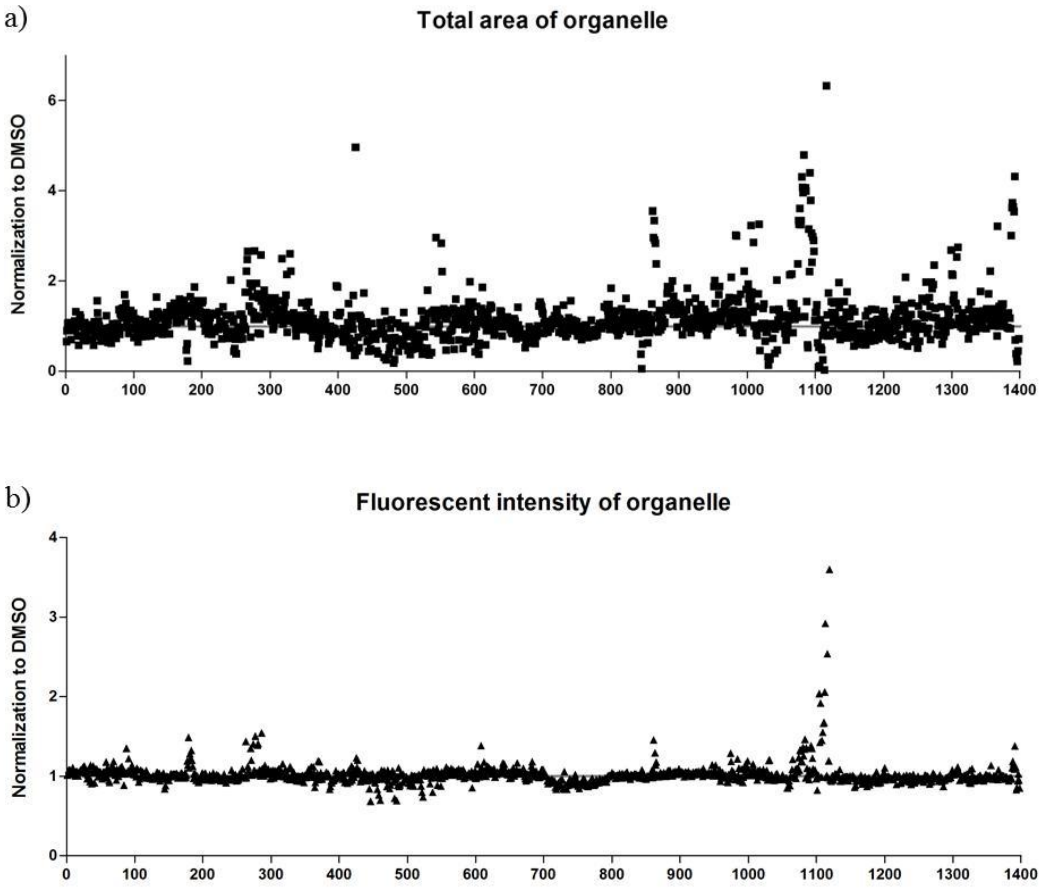

Fig. S4 Screening outcomes [corresponding to Fig. 3a]. Image data were analyzed by (a) total area of organelles per cell and (b) fluorescent intensity of organelle.

V. Protocol for exclusion of false positives from screening results

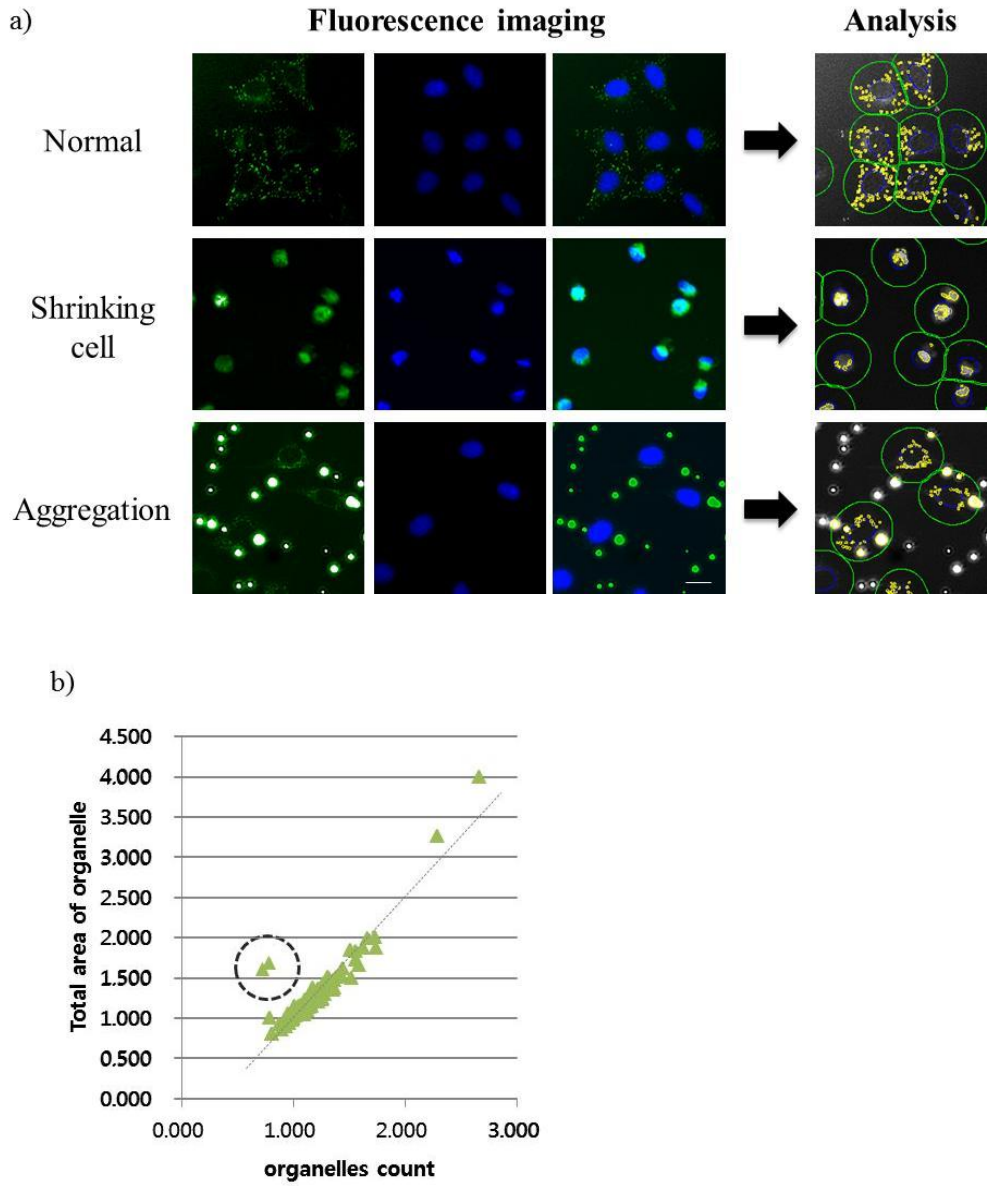

Fig. S5 Systematic exclusion of false positives from imaging data. (a) Representative images of false positives according to the automatic image analysis. Representative fluorescence images (in left panel) of normal cells, shrinking cells associated with cell death, and aggregation of SF44 upon treatment with various compounds. LD staining with SF44 (left), nuclei staining with Hoechst (middle), and their merged image (right) were displayed in a pseudo color. Monocromatic images after automatic image analysis (in right panel) under the identical conditions. Blue, green, and yellow circle indicated the recognition of nuclei, cell area, and LD, respectively. The scale bar represents 20 μm. (b) Two-dimensional fitting between organelle counting and total organelle area. The deviated points from linearity (dotted circle) were excluded as false positive via automatic analysis.

VI. Dose-dependent analysis of hit compounds

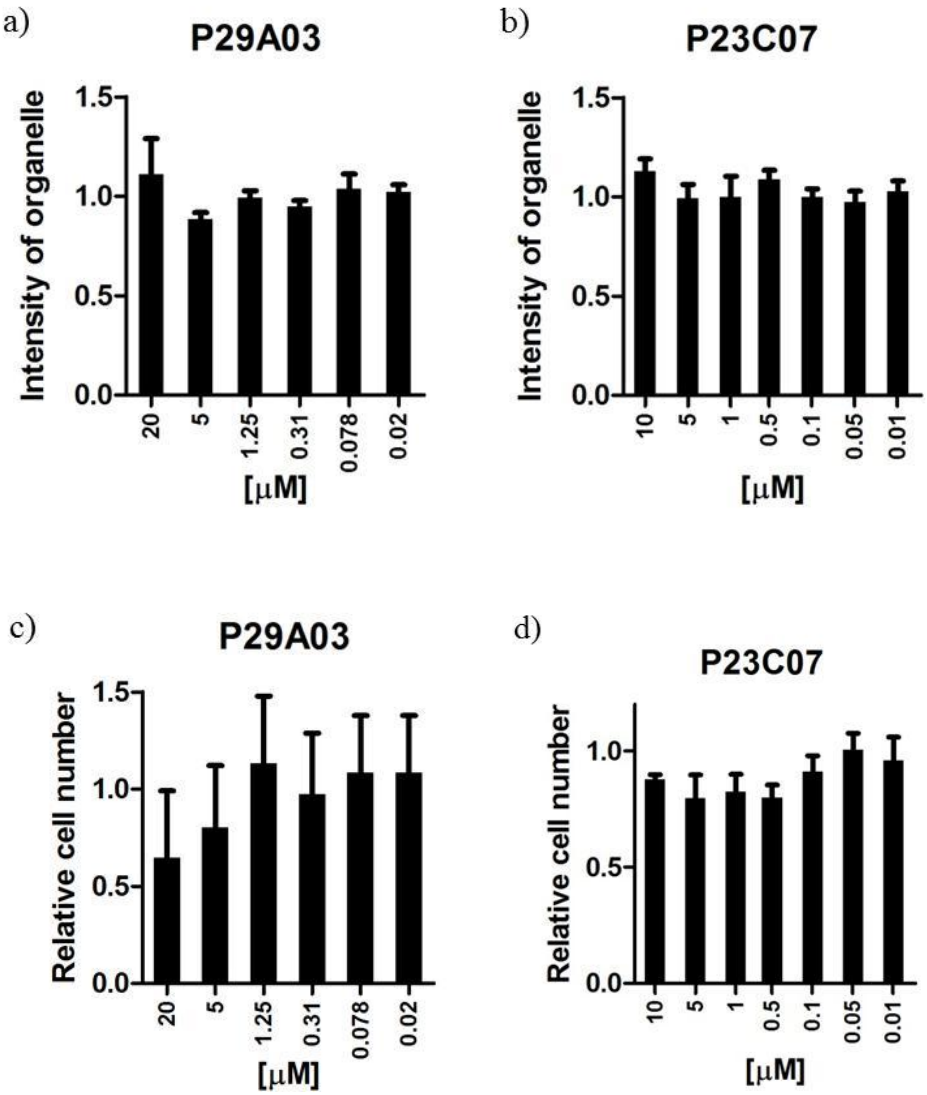

Fig. S6 Dose-dependent results analyzed by 2 different parameters (along with 2 other parameters displayed in Fig. 3d, e) upon treatment with P29A03 and P23C07 in HeLa cells. (a, b) Analysis results on the basis of organelle intentisy for (a) P29A03 and (b) P23C07. (c, d) Analysis results on the basis of relative cell numbers for (c) P29A03 and (d) P23C07. Each data were normalized with DMSO as a control. The graphs described means with standard deviation (SD) at least 4 independent imaging experiments with over hundreds of cells.

VII. Conversion of LC3 I to II after treatment with hit compounds monitored via western blot analysis

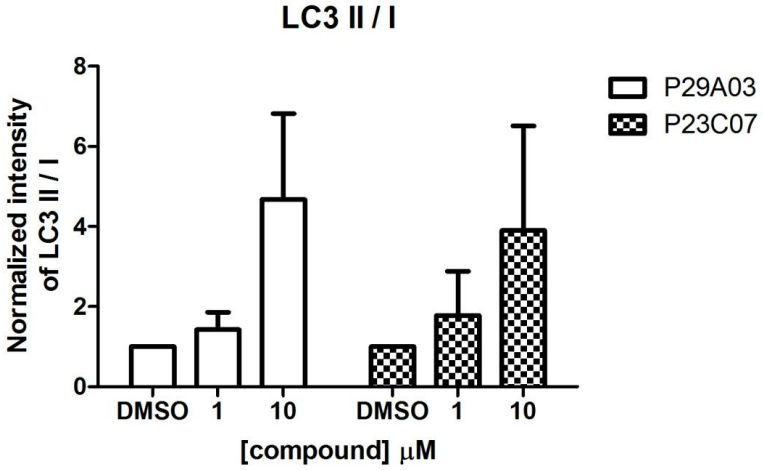

Fig. S7 Densitometry results of LC3 II/I ratio upon treatment with P29A03 and P23C07 via western blot analysis. All experimental condition was identical to that in Fig. 4. The graph described means and SD of at least 3 independent experiments.

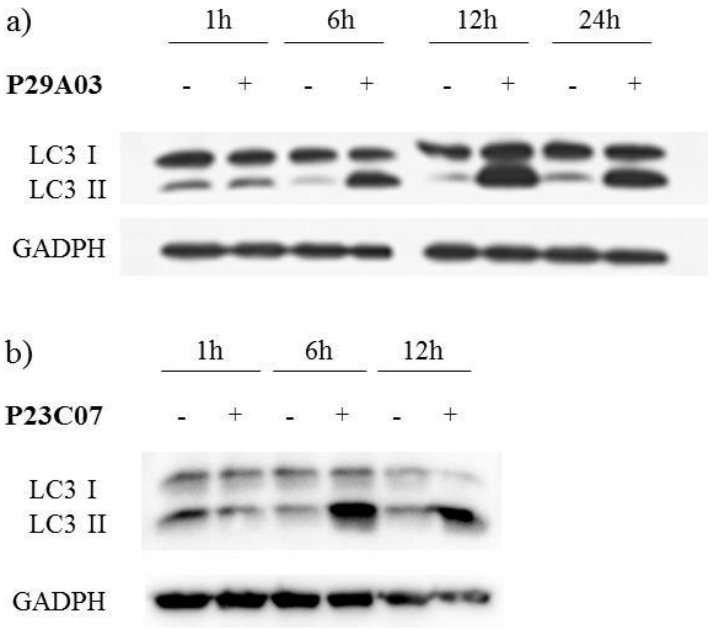

Fig. S8 Monitoring of LC3 II level via western blot analysis on the basis of time-dependent treatment with P29A03 and P23C07. HeLa cells were treated with 10 μM of (a) P29A03 and (b) P23C07 during the indicated time frame.

VIII. Image-based measurement of LC3 puncta after treatment of hit compounds via immunohistochemistry

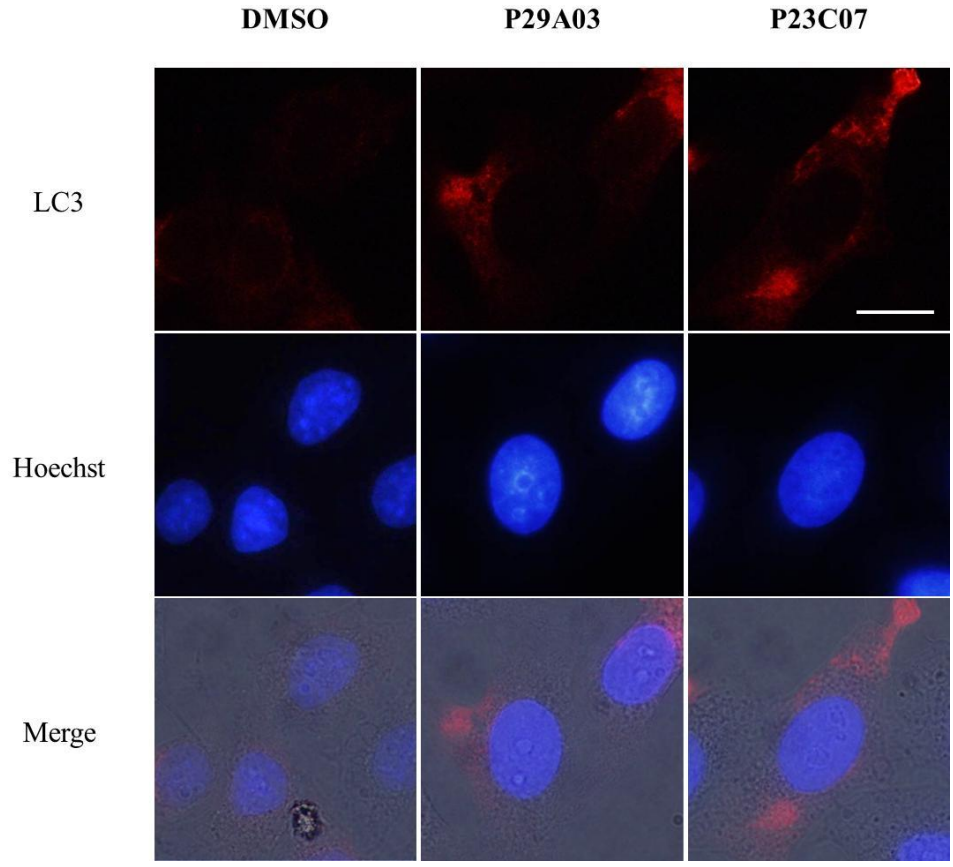

Fig. S9 Visualization of LC3 puncta upon treatment with P29A03 and P23C07 in HeLa cells via immunohistochemistry. Cells were incubated with individual compounds as 10 μM for 12 h. LC3 was visualized with anti-LC3 antibody (top), and nuclei were stained with Hoechst (middle). Both images were merged in the bottom along with bright field images. The scale bar is 20 μm.

IX. Mode-of-action studies of hit compounds

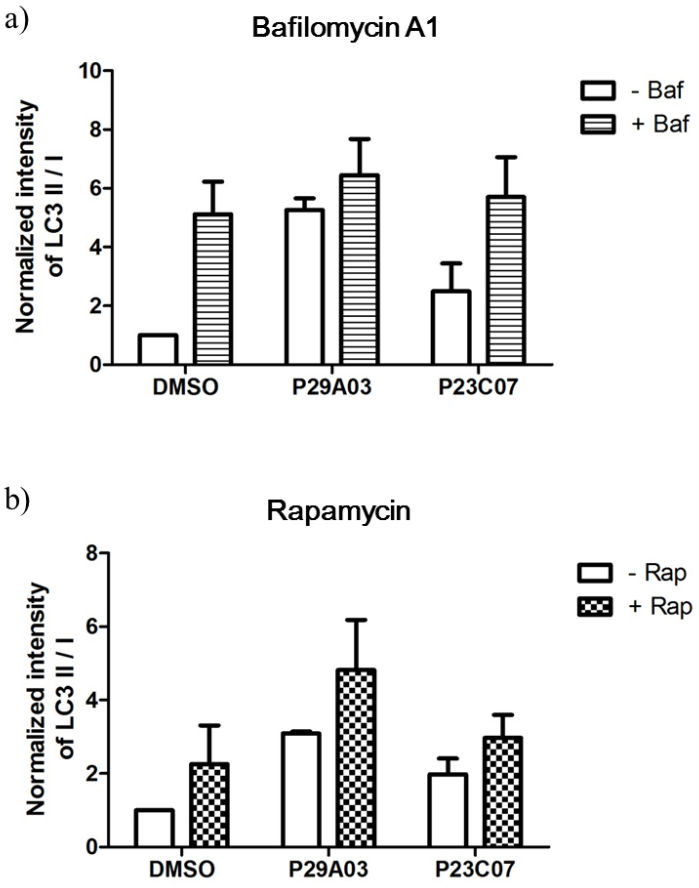

Fig. S10 Densitometry results of LC3 II/I ratio in a co-treatment assay of P29A03 and P23C07. Co-treamtent assay of both compounds was performed with (a) bafilomycin A1 and (b) rapamycin. All experimental condition was identical to that in Fig. 5a–b. Western blot results were analyzed with at least 3 independent experiments. The graph described means and SD. Baf is bafilomycin A1 and Rap is rapamycin.

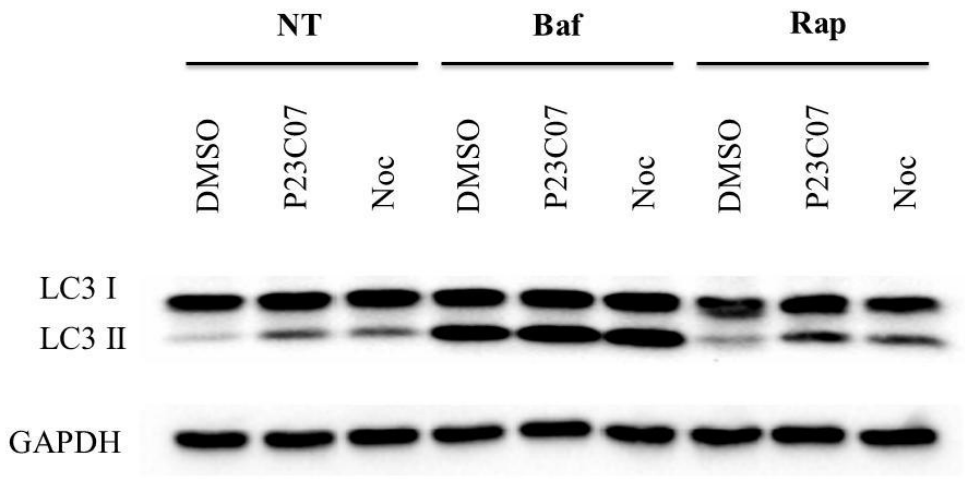

Fig. S11 Co-treatment assay of P23C07 with Baf and Rap to elucidate its microtubule-associated inhibitory effects on autophagy. HeLa cells were treated either with P23C07 (10 μM) or with nocodazole (10 μM) for 6 h in the presence of DMSO, bafilomycine A1 (10 nM), or rapamycin (100 nM), respectively. Cell lysates were subject to western blot analysis toward LC3 and GAPDH. Nocodazole was used as a positive control. Noc: nocodazole, NT: non-treated, Baf: bafilomycin A1, Rap: rapamycin.

X. Detailed experimental procedures

Cell culture HeLa (human cervical cancer) cells were obtained from American Type Culture Collection and cultured in RPMI 1640 supplemented with heat-inactivated 10 % (v/v) fetal bovine serum and 1 % (v/v) antibiotic-antimycotic solution. Cells were maintained in a humidified atmosphere of 5 % CO_2 incubator at 37 ℃ and cultured in 100 mm cell culture dish.

Visualization of lipid droplet in live cells using SF44 HeLa cells were seeded on a cover-glass bottom dish and incubated in 5 % CO_2 incubator at 37 ℃ overnight. Those cells were subjected to the chemical treatment or autophagy-inducing stimuli. Cells were incubated with serum-free media for 12 h, 500 nM of rapamycin for 5 h, and 20 μM of tamoxifen for 5 h to activate autophagy; 10 mM of 3-methyladenine for 24 h, 50 nM of wortmannin for 24 h, 10 nM of bafilomycin A1 for 5 h, and 10 μM of nocodazole for 5 h to inhibit autophagy. For a dose-response study, cells were treated with bafilomycine A1 for 5 h and rapamycin for 5 h with various concentrations. For LD visualization, the media were replaced with fresh media containing 5 μM of SF44. After 15 min incubation at 37 ℃, LD-staining pattern in live cells were measured using fluorescence microscopy directly without additional washing steps.

Construction of high-content screening platform and automated data analysis For the image-based screening in a high-throughput manner, HeLa cells were seeded on a 96-well plate with clear bottom and black well. Using 96 solid pin multi-blot replicators, various compounds from our in-house chemical library were transferred to individual wells of a 96 well plate with their final concentration as 10 μM. Individual screening plates contained oleic acid as a positive control, serum-free condition as a negative control, and DMSO as a vehicle. After 24 h incubation at 37 ℃, SF44 (5 μM) and Hoechst 33342 (2 μg/ml) was added to cells charged in individual wells. Serum-free condition (negative control) was needed to exchange its media with regular media before the treatment of SF44 and Hoechst. After 30-min incubation, automatic fluorescence imaging of designated plates was performed with InCell Analyzer 2000 without any washing steps.

Western blot analysis HeLa cells were seeded on 6-well plate and incubated in 5 % CO_2 incubator at 37 ℃ overnight. Cells were treated with hit compounds at various concentrations. In case of co-treatment assay, cells were treated with 10 μM of each compound in the presence of either 10 nM of bafilomycin A1 or 100 nM of rapamycin. After 6 h incubation with individual compounds, cells were washed by PBS and harvested. Cell lysates were obtained by 2 h treatment with RIPA cell lysis buffer containing protease inhibitors and phosphatase inhibitors at ice. After the centrifugation of cell lysates at 14,000 rpm and 4 ℃ for 10 min, the protein concentration in the supernatant was measured by BCA assay. The resulting proteome were analyzed by SDS-PAGE and transferred into PVDF membrane, followed by 2% BSA blocking in TBST over 1 h. The samples were subjected to immunoblotting to

detect the LC3 or p62 with specific primary antibodies, e.g. anti-LC3 (abcam), anti-p62 (cell signaling) and GAPDH (cell signaling) antibodies for overnight at 4 ℃, followed by washing with TBST for 2 h. The resulting membrane was exposed into HRP-conjugated secondary antibody for 1 h at room temperature. After 1—2 h washing with TBST, the membrane was developed by ECL prime solution and the chemiluminescent signal was measured by ChemiDoc^{TM} MP imaging system. To investigate the mode-of-action of P29A03, individual proteome samples were prepared by identical procedure after treatment of P29A03 (10 μM) for desiginated time frames. The procedure of western blot assay was identical with an above-mentioned procedure except for primary antibodies. Anti-mTOR (abcam), anti-mTOR (phosphor S2448) (abcam), anti-S6K (phospho T389) (abcam), anti-beclin-1 (cell signaling) and GAPDH (cell signaling) were used according to the manufactural recommendation.

Immunohistochemistry for LC3 puncta HeLa cells were seeded on cover glass bottom dish and incubated at 37 ℃ for overnight. After 12 h incubation of hit compounds (10 μM), cells were washed with PBS and fixed with 3.7 % paraformaldehyde in PBS containing MeOH (0.7—0.8 %) for the permeabilization for 15 min at room temperature. The resulting cells were washed with ice-cold PBS for three times, followed by the incubation with 4% BSA in PBS for 4 h at room temperature. BSA solution was decanted from glass bottom dish. Fixed cells on dish were exposed to the diluted solution (1:100) of primary antibody for LC3 (abcam) in PBS with 1% BSA, and incubated at 4 ℃ for overnight. Primary antibody was decanted and washed with PBS for three times. Then, a diluted solution (1:200) of secondary antibody, conjugated with Texas Red fluorescent dye, was added and the resulting samples were incubated at room temperature in dark for 3 h. After washing by PBS 3 times, fluorescence images were taken in PBS condition using fluorescence microscopy.