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Correction notice

Termination of autophagy and reformation of lysosomes regulated by mTOR

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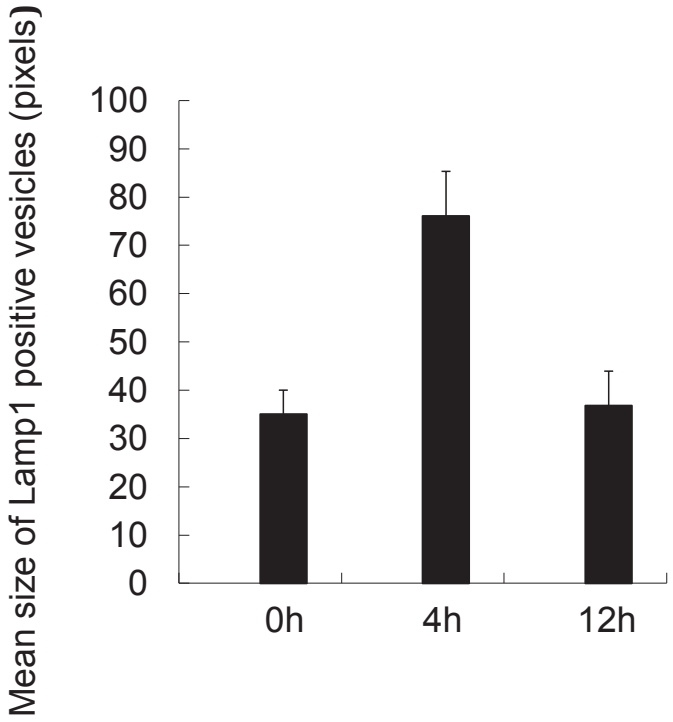
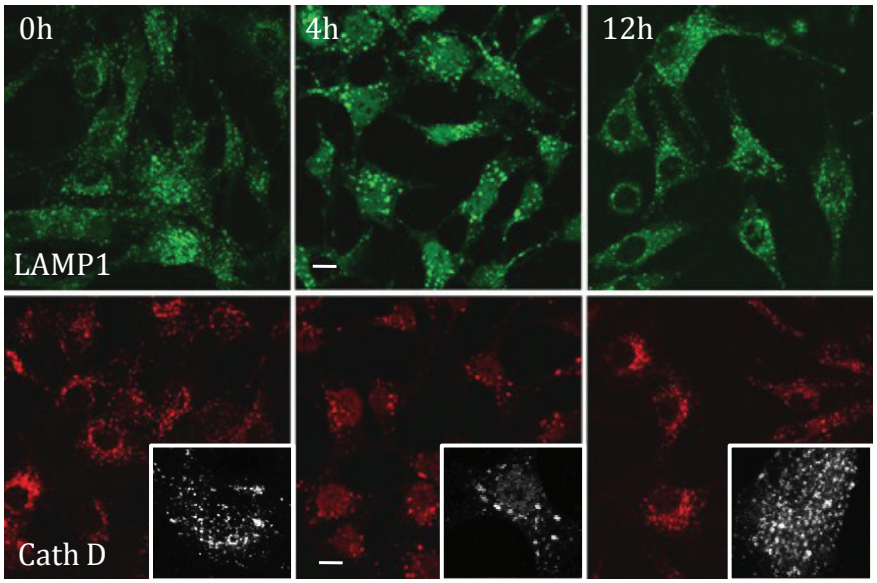
In the version of the Supplementary Information originally posted online, there were some discrepancies between the file numbering and the in-text file citations. These have now been corrected and experimental methods have also been added; see Supplementary Information Table of Contents for details.

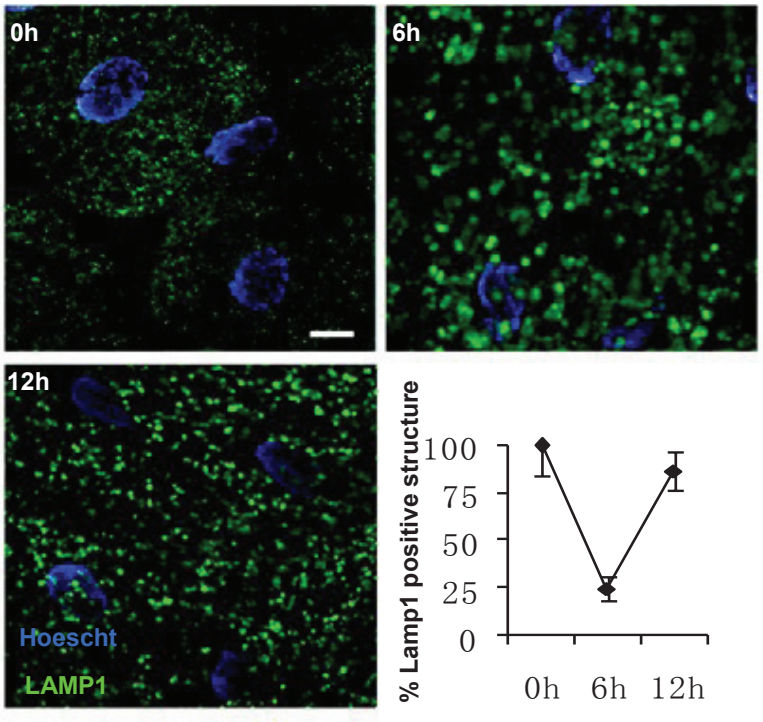
SUPPLEMENTARY INFORMATION



S1. NRK cells were starved for 0, 4, and 12 hours (h) and cells were stained with anti-Lamp1 antibody to quantify lysosomes. The mean size of Lamp1 positive vesicles were Quantified by Image Pro-Plus.



S2. NRK cells were starved for 0, 4, or 12 hours (h) as indicated and stained for Lamp1 (top) and cathepsin D (bottom). Boxes display enlargements of individual cells.

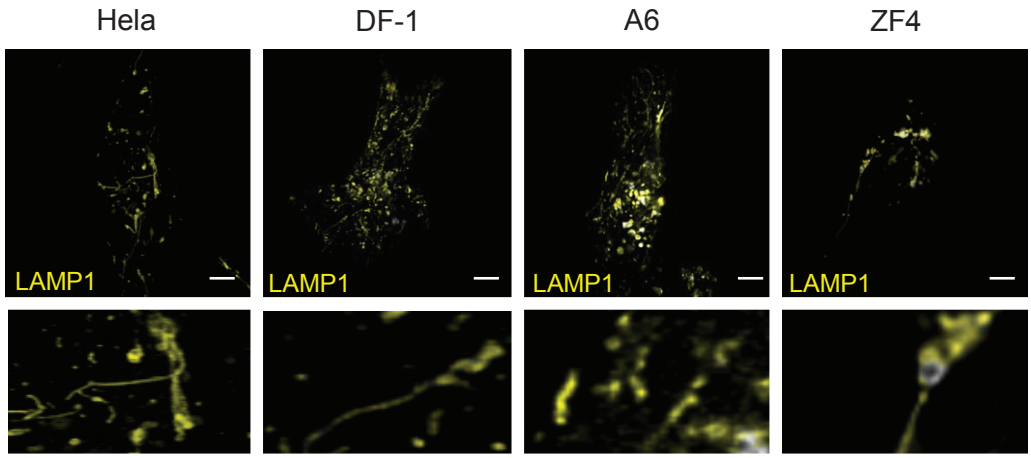


S3. *Drosophila* larvae expressing *tub*-LAMP1-GFP were starved for 0, 6, and 12 hours (h), and lysosomes in fat cells were imaged by fluorescence microscopy. Green: LAMP1. Blue, Höechst. Scale bar, 20 um. *Drosophila* lysosomes were quantified using Zeiss Automeasure software. Lysosomes were quantified in at least 7 different larvae per time-point, in fat cells from one fat body per larva and 2 fields per fat body. Each field contained 3-4 cells. For a and b, the number of lysosomes per time point was normalized to the average number of lysosomes in non-starved (0 h) cells or larvae (designated 100%). Error bars indicate the standard deviation.

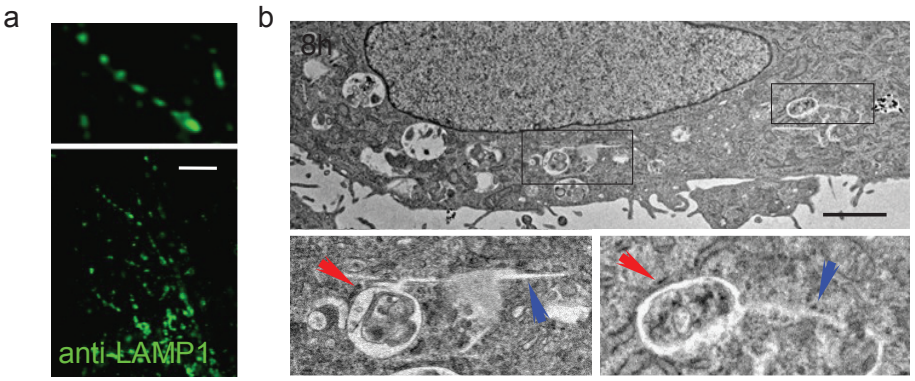
a

	Hela	C166	MRC-5	SH-SY5Y	MEF
Organism	Homo sapiens	Mus musculus	Homo sapiens	Homo sapiens	Mus musculus
Organ	cervix	yolk sac	lung	brain	embryo
Cell Type	epithelial	endothelial	fibroblast	epithelial	fibroblast
Lysosome recovery	Yes	Yes	Yes	No	Yes
Tubules	Yes	Yes	Yes	No	Yes
	OP9	vero	HuH7*	DF-1	ZF4
Organism	Mus musculus	Cercopithecus aethiops	Homo sapiens	Gallus gallus	Danio rerio
Organ	bone marrow	kidney	liver	embryo	embryo
Cell Type	fibroblast	epithelial	epithelial-	fibroblast	fibroblast
Lysosome recovery	Yes	No	Yes	Yes	Yes
Tubules	Yes	No	No	Yes	Yes
	A6	Hep G2	OMK*	A549	H4-II-E-C3
Organism	Xenopus laevis	Homo sapiens	Aotus trivirgatus	Homo sapiens	Rattus norvegicus
Organ	kidney	liver	kidney	Lung	liver
Cell Type	epithelial	epithelial	epithelial	Epithelial	epithelial
Lysosome recovery	Yes	Yes	Yes	Yes	Yes
Tubules	Yes	Yes	Yes	Yes	Yes
* kinetic of autophagy is slower in these cell lines.					

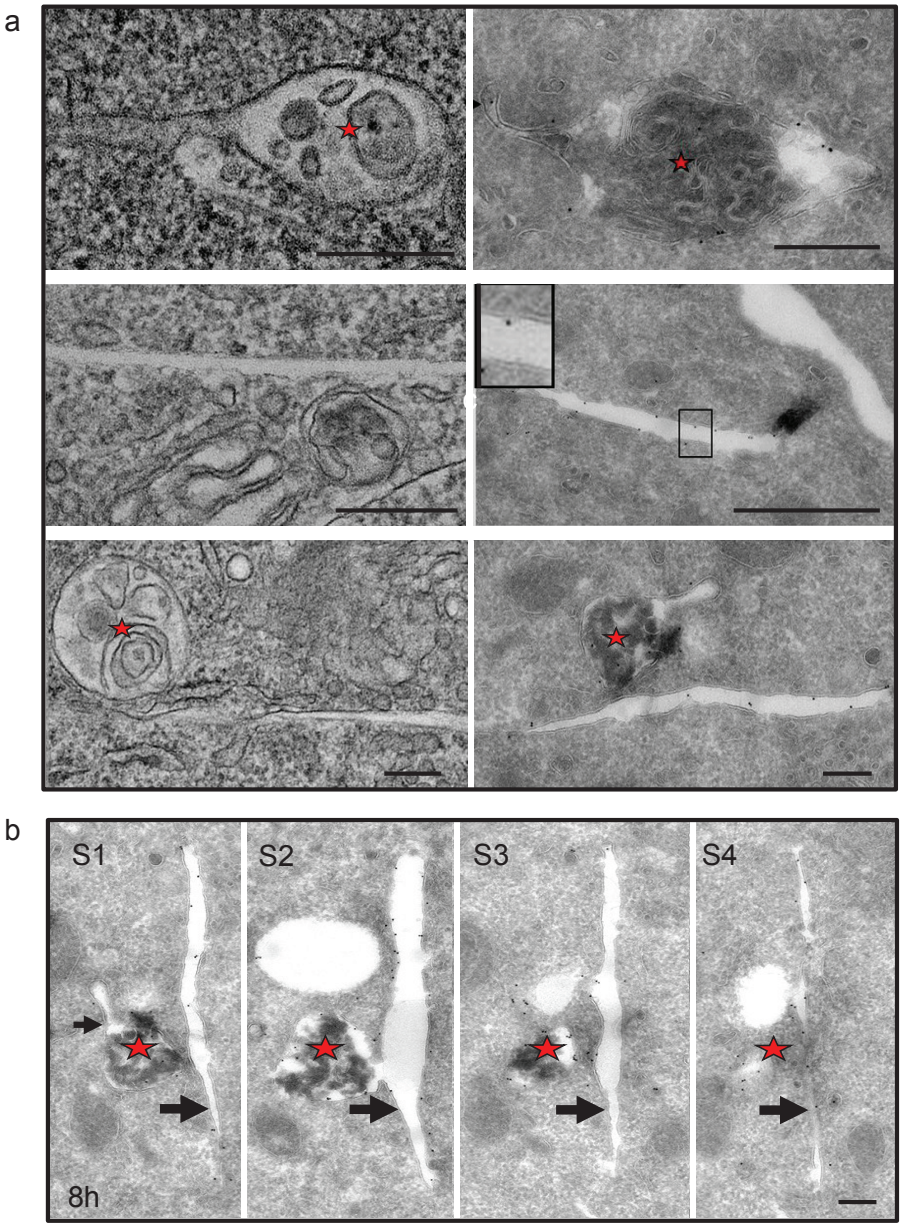
b



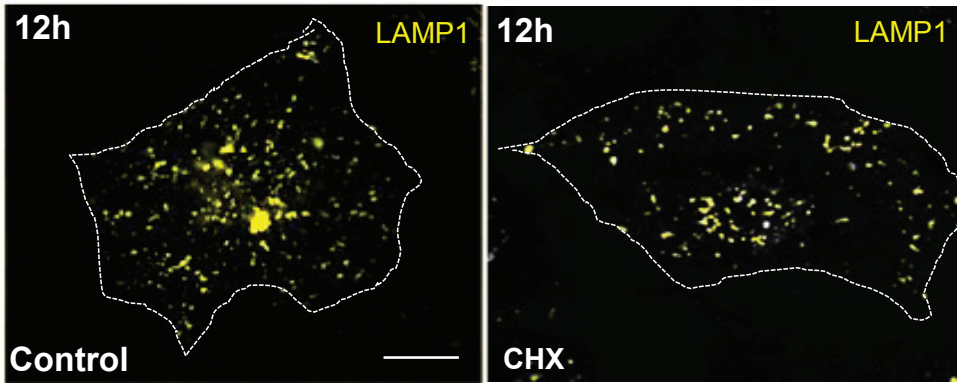
S4. Lysosome reformation occurs in cell lines from diverse tissues and animal species. Cells were starved for 0, 4, 8, or 12 hours, and the number and size of lysosomes were monitored by lysotracker. Reformation tubules were visualized in cells expressing Lamp1-YFP. Note we did not observe clear recovery of lysosomes in Vero or SH-SY5Y cells. (a) Table showing the lysosome recovery and tubule formation in various cell types from different organs and species. (b) Images of reformation tubules from representative cell lines of diverse origins as described in (a).



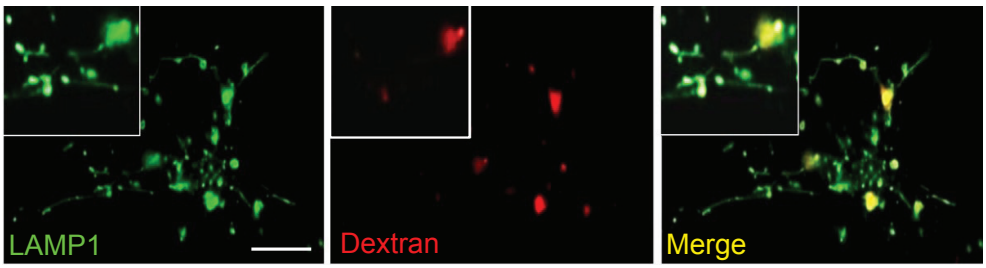
S5. NRK cells were starved for 8 hours and then stained with anti-Lamp1 antibody. Upper panel shows enlargement of a tubule beginning to vesiculate (a). Representative transmission electron micrographs (TEM) of NRK cells starved 8 hours with tubules (b). Lower panels show enlargements of boxed regions in the upper panels illustrating tubules apparently connected to autolysosomes. Red arrowhead, autolysosome; blue arrowhead, tubules.



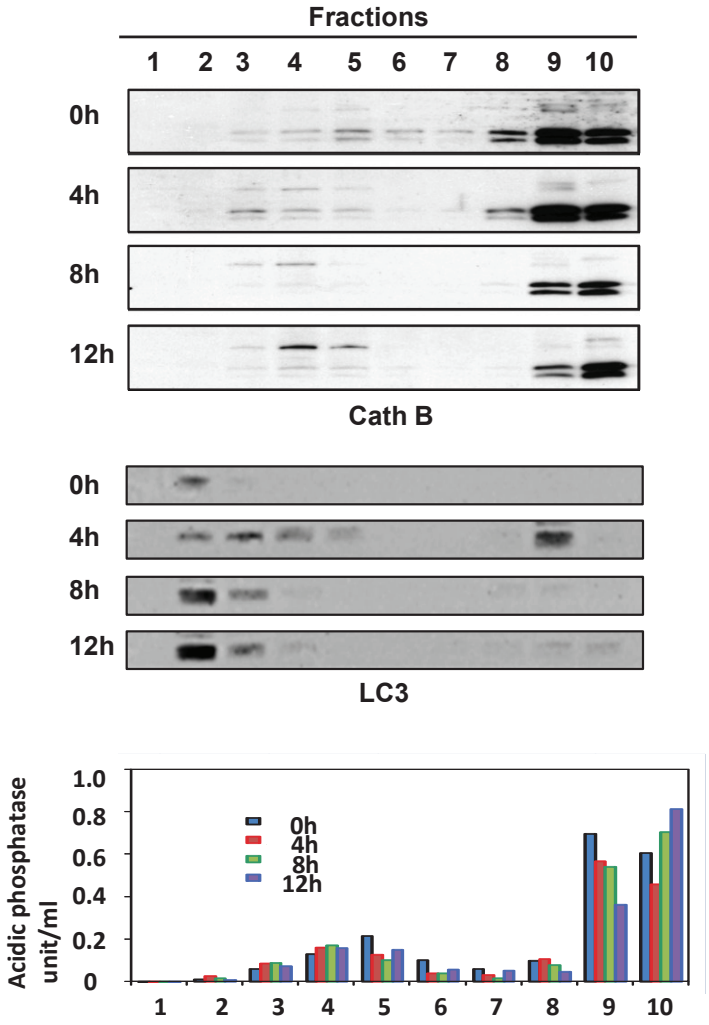
S6. Membrane limited tubules in starved cells. (a) Representative transmission electron micrographs (TEM)(left) and immuno-TEM (I-TEM)(right) of NRK cells starved 8 hours displaying tubules, Scale bar, 200 nm. Box indicated enlargement showing immunogold marking of LAMP-1 on tubule membrane. (b) Reoriented I-TEM from lower panel of (a) including further serial sections (S1 – S4) analyzed by I-TEM using Lamp1 antibody marked by immuno-gold. Scale bar, 2 um, red star, autolysosome, arrow indicates the membrane limited nature of tubules.



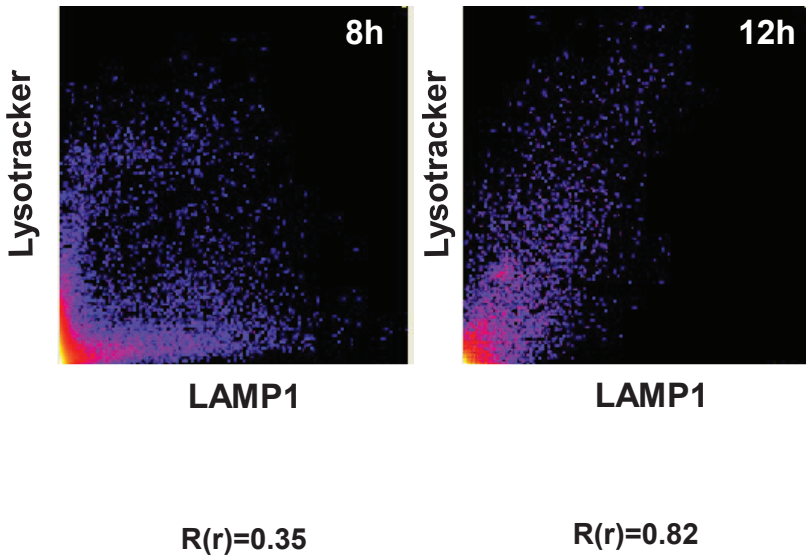
S7. Formation of proto-lysosomes does not require new Lamp1 synthesis. NRK cells were transfected with Lamp1-YFP, and 24 hours after transfection, cells were starved for 12 hours with or without presence of 10 ug/ml cycloheximide and imaged. At 12 hours of starvation, the reformation tubules will have disappeared and lysosome size and number will have recovered. Scale bar, 5 um



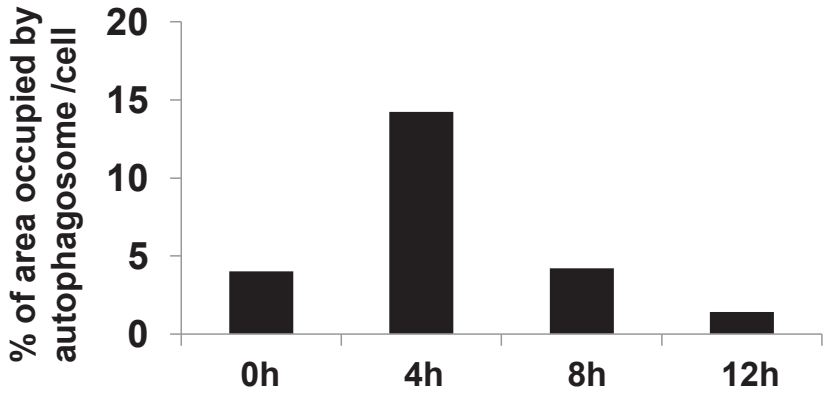
S8. NRK cell were transfected with Lamp1-YFP, 12 hours after transfection, cells were loaded with 10 ug/ul dextran-Texas red conjugate and then starved for 8 more hours and imaged. green: Lamp1, red: dextran, Scale bar, 5um.



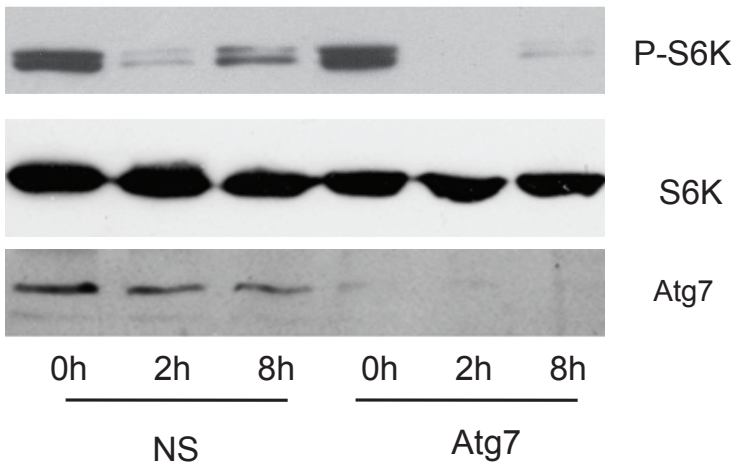
S9. NRK cells were starved for 0, 4, 8, or 12 hours and then subjected to Optiprep gradient ultracentrifugation. Fractions were collected, protein extracts were made and Western blots were probed with antibodies against cathepsin B (top) or LC3 (middle). Fraction 1 is the bottom of the gradient and fraction 10 is the top of the gradient. The lysosomal acid phosphatase activity of each fraction at each time point was measured by the Sigma kit (bottom).



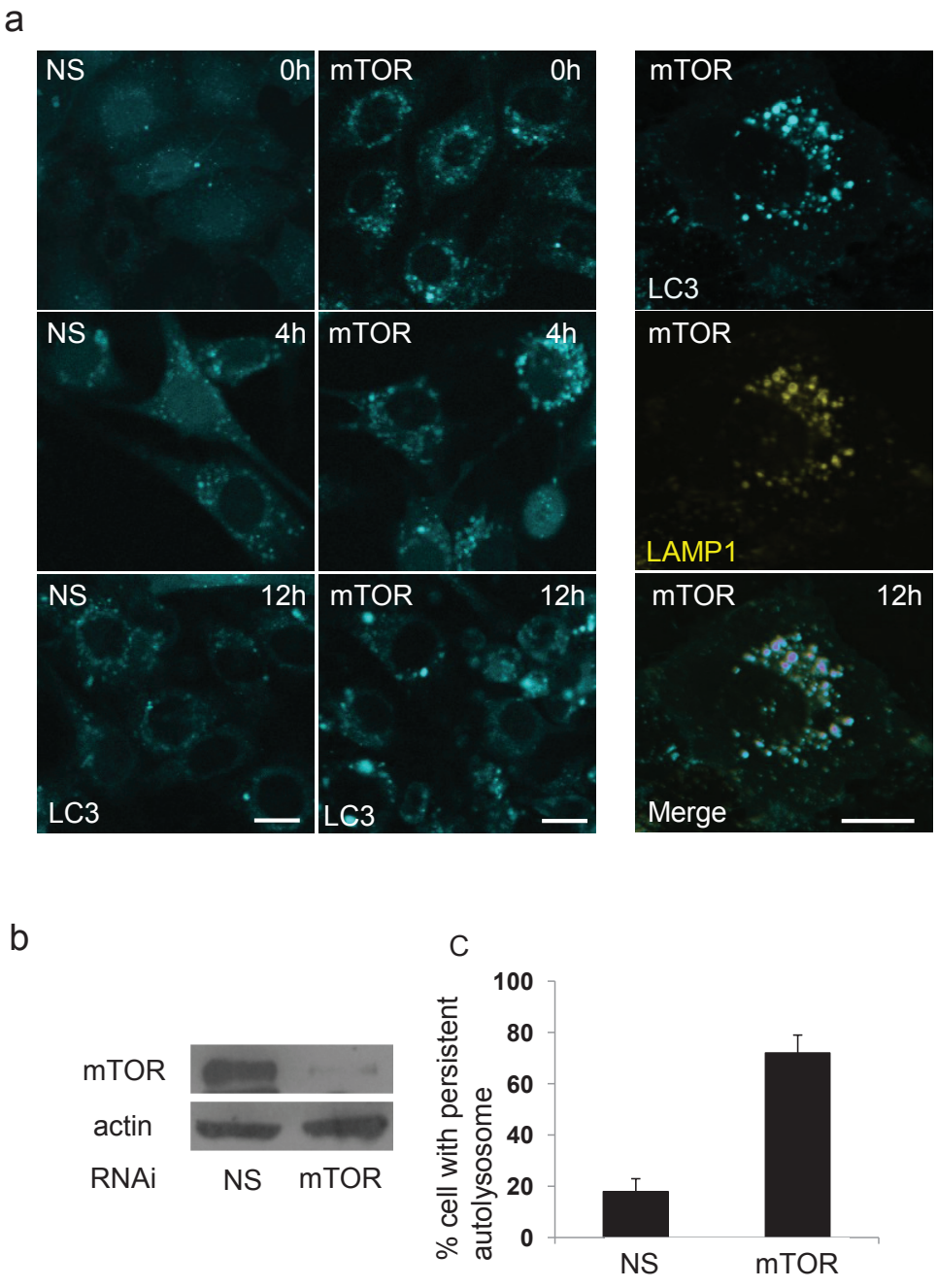
S10. The co-localization of LAMP1 and Lysotracker. LAMP1-GFP-expressing NRK cells were starved for 8 and 12 hours (h) and stained with Lysotracker. Co-localization of LAMP1 and Lysotracker was analyzed using IMARIS software from Bitplane and the Pearson's coeiffient R(r) is given.



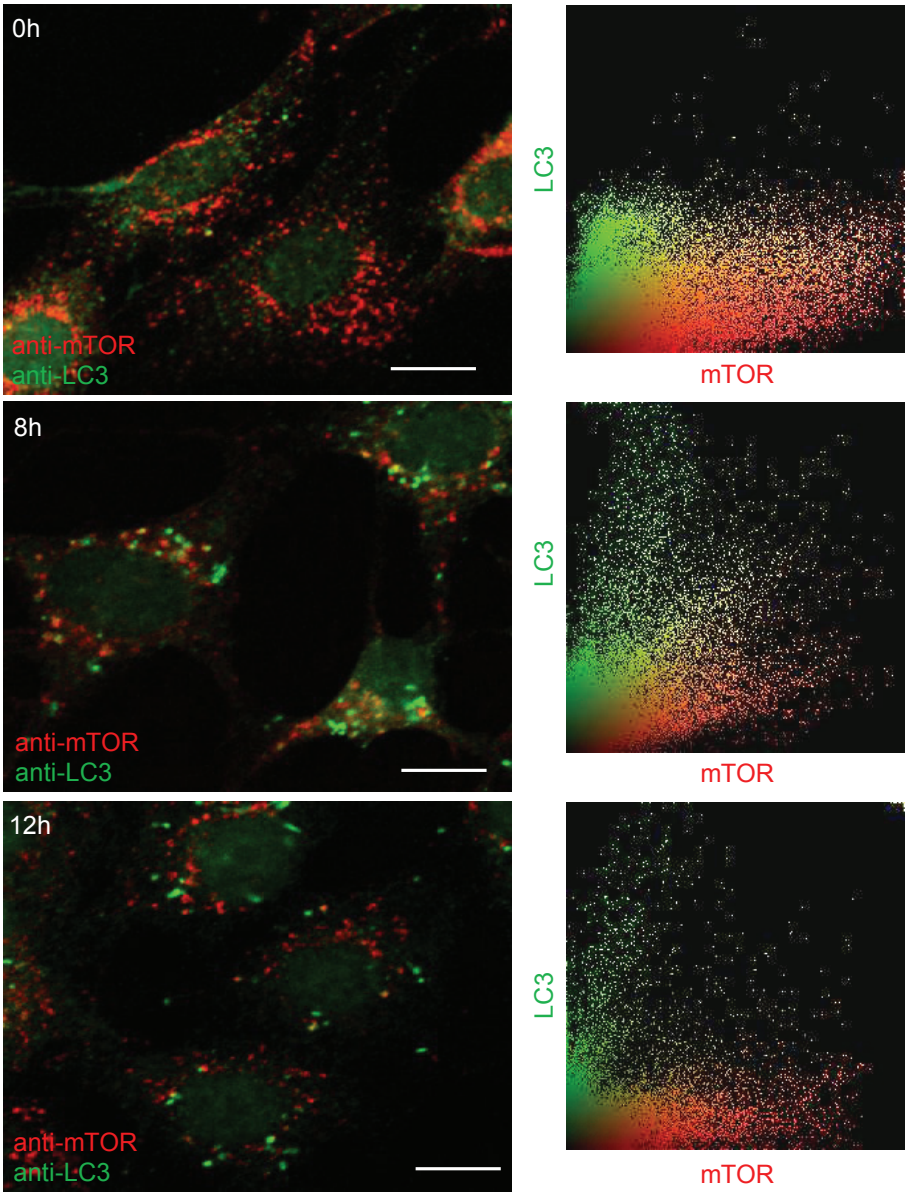
S11. Morphometric analysis of the area occupied by autophagosomes per cell for Fig. 3a. 50 cells were counted.



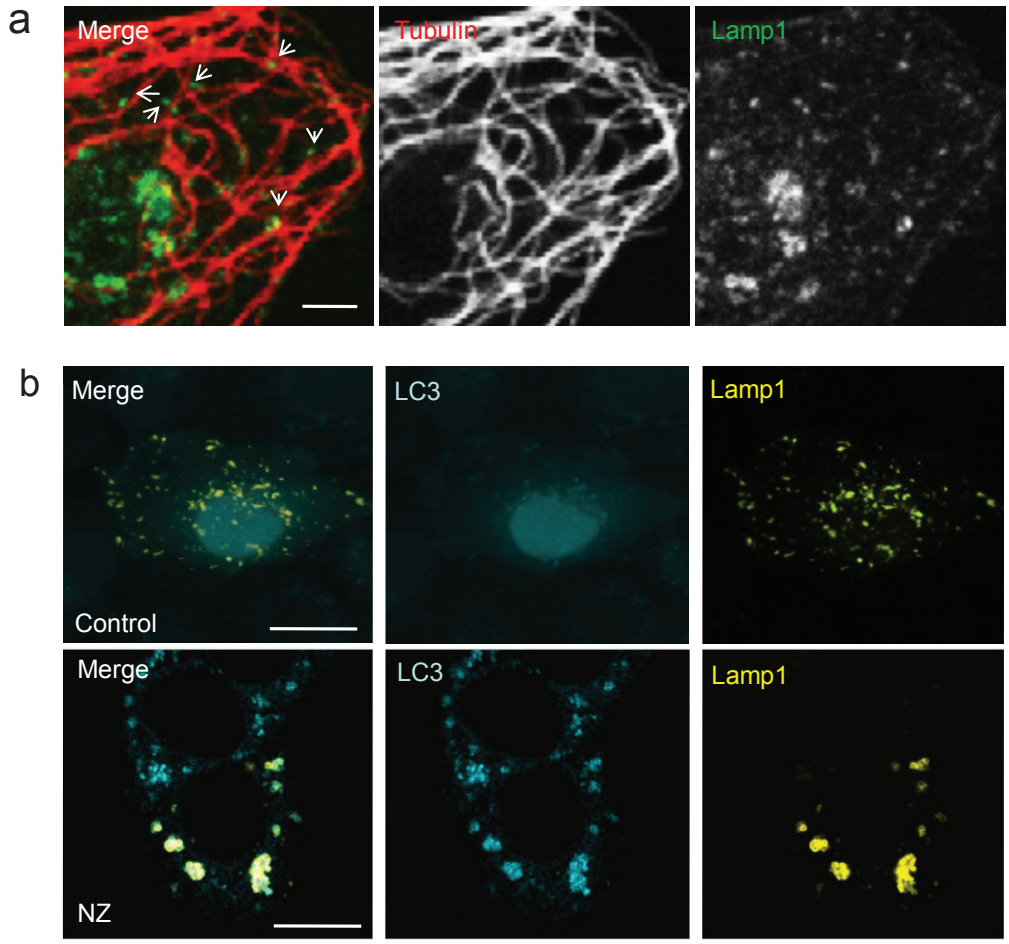
S12. NRK cells were transfected with non-specific RNAi (NS) or RNAi against *atg7*(Atg 7). 60 hours after transfection, cells were starved (NS) for 0, 2, or 8 hours, harvested and analyzed by Western blot using antibodies against Phospho-p70 S6 Kinase (p-S6K), p70 S6 Kinase, and Atg7, as indicated.



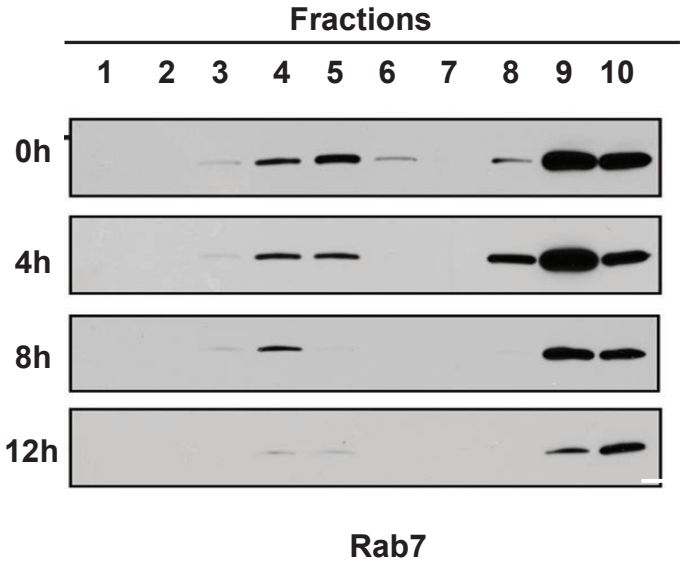
S13. Knockdown of mTOR inhibits ALR and leads to accumulation of persistent, large autolysosomes. a) NRK-LC3-CFP cells were transfected with non-specific RNAi (NS) or RNAi against *mTOR* (mTOR). After 2 days, cells were again transfected with NS- or *mTOR*-RNAi and LAMP1-YFP; 16 hrs after transfection, cells were starved for 0 or 12 hrs (h) and imaged by confocal microscopy for Lamp1 (green) and LC3 (blue). Scale bar, 5 uM. b) Cell lysates prepared from the cells in (a) were subjected to gel electrophoresis, and blotted for the mTOR protein (upper blot) or actin (lower blot). C) Quantification of cell with persistent autolysosome 12 hours after starvation. 50 cells were counted.



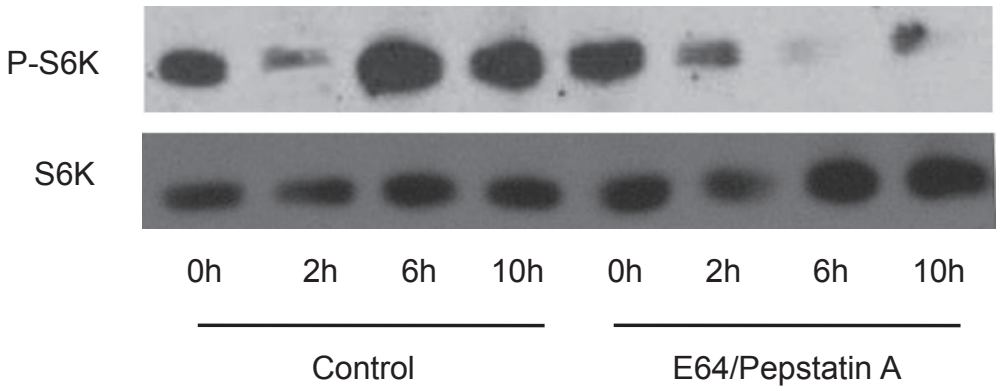
S14. NRK-LC3 cells were starved for 0, 8, and 12 hours (h). Cells were stained with anti-LC3b(green) and anti-mTOR(red) antibody and analyzed by confocal microscopy (left), Scale bar, 5 um. Co-localization of LC3 and mTOR was analyzed using IMARIS software from Bitplane and shows significant co-localization at 8 h.



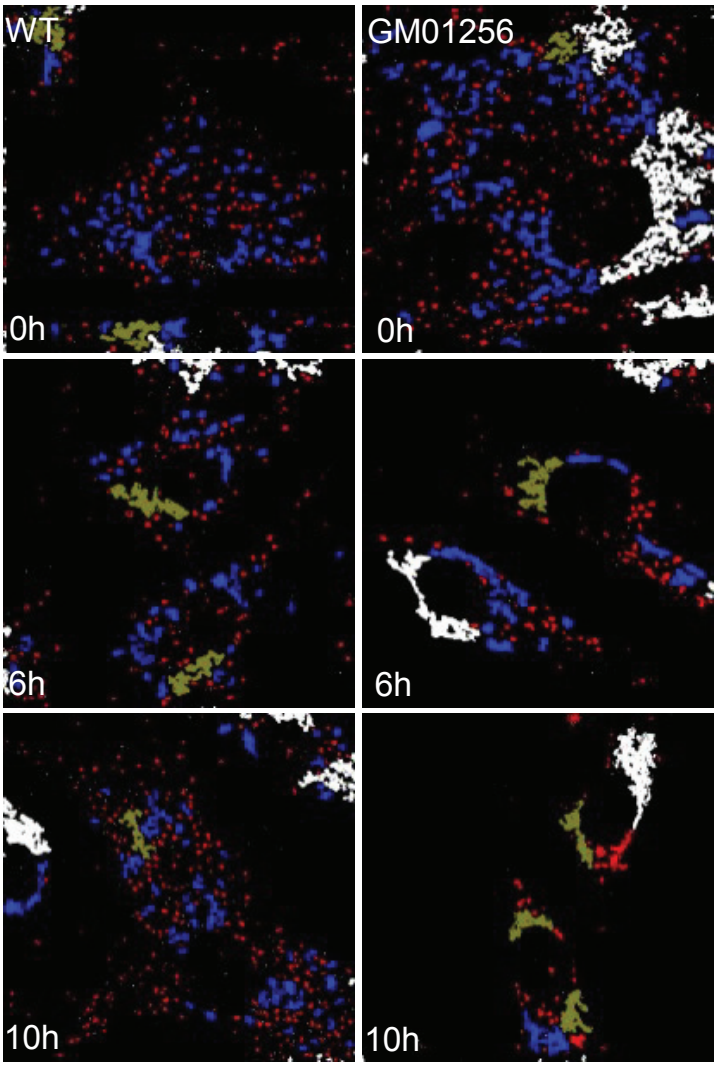
S15. (a) NRK cells were starved for 8 hours and then stained for tubulin (red) and LAMP1 (green). The arrows show protolysosomes budding at various points on the lysosome reformation tubules in close association with microtubles. Quantification showed that 97% of the proto-lysosomes were directly associated with the microtubules; (b) NRK cells were starved for 12 hours with or without the addition of nocodazole (NZ) and then stained for LC3 (blue) and LAMP1 (yellow).



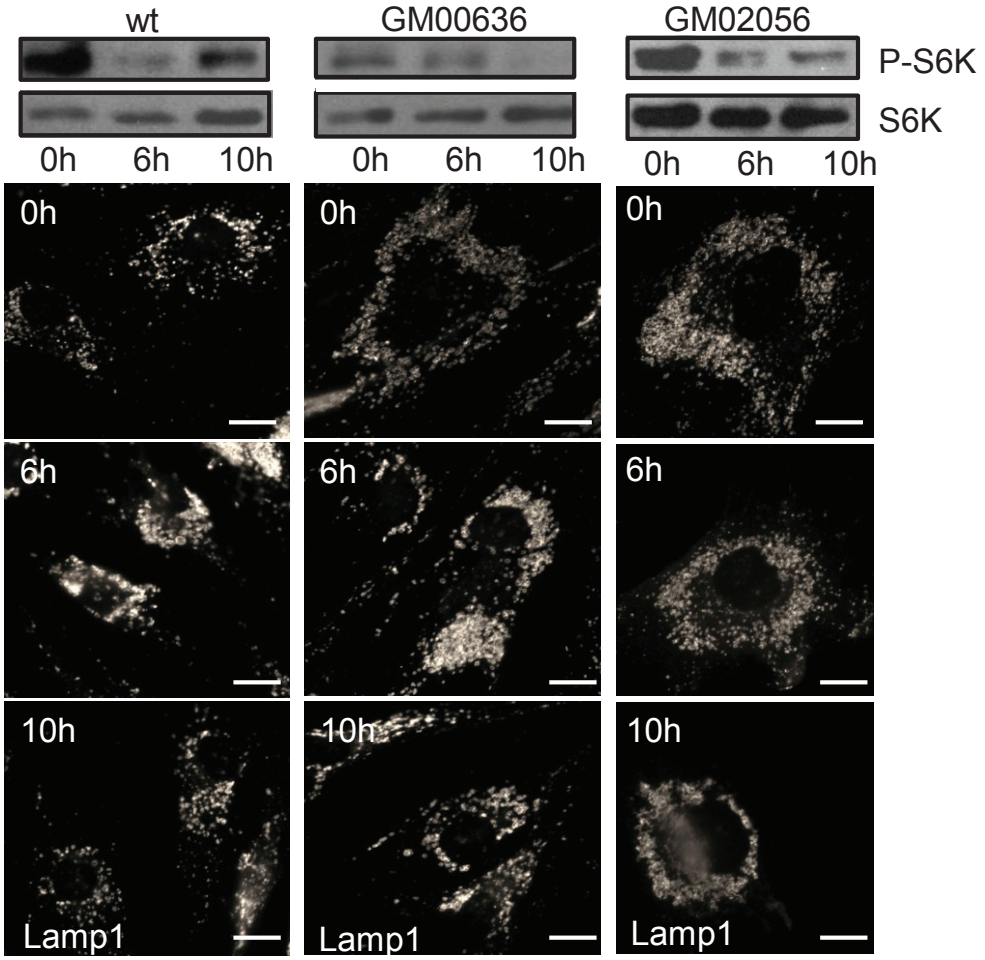
S16. Fractions from gradient ultracentrifugation as in S9 were immunoblotted with anti-Rab7.



S17. NRK-LC3 cells were starved for 0, 2, 6, or 10 hours (h) with or without the addition of E64/Pepstatin A, protein lysates were prepared and analyzed by Western blot using antibodies against Phospho-p70 S6 Kinase (p-S6K) and p70 S6 Kinase (S6K).



S18. Photomicrographs from the experiment in figure 4g. displaying wild type (WT) and LSD mutant cells (GM01256) were starved for 0, 6, or 10 hours (h) stained for Lamp1. Scale bar, 5um.



S19. Photomicrographs and protein blot analyses of wild type (wt) and mutant cells (GM00636 or GM02056) were starved for 0, 6, or 10 hours (h) and then analyzed by Western blot using antibodies against Phospho-p70 S6 Kinase (p-S6K), p70 S6 Kinase (S6K) (top panel) and stained for Lamp1 (lower panels). Scale bar, 5um.

Supplementary Movie S1. Movie showing that multiple lysosomes fuse with GFP-LC3-labeled autophagic vesicles. GFP-LC3-expressing NRK cells grown in chambers were induced to form autophagic vesicles by starvation. After induction, cells were labeled with the acidotropic pH-sensitive dye LysoTracker Red for five minutes. Excess dye was then washed out. Dual-channel live cell imaging revealed fusion events between individual GFP-LC3-positive autophagosomes and multiple low-pH lysosomes. Lysosome fusion causes the degradation of GFP-LC3. Arrow indicates autophagosome (green) and multiple lysosomes (red) fusing to it.

Supplementary Movie S2. Movie of tubules extending from autolysosomal membranes (arrow) and small Lamp1-positive vesicles pinching off from the tips of tubules. 8 hours after serum starvation, LAMP1-YFP-expressing NRK cells were imaged by time lapse microscopy. Time (seconds) is indicated in the top right of each panel.

Supplementary Movie S3. Movie of tubules breaking away from autolysosomes and condensing into Lamp1-positive vesicles. NRK cells transfected with Lamp1 (photoactivatable) PAGFP/Lamp1-RFP (3:1) were starved for 6 hours. LAMP1-PAGFP was photo-activated by a 405 laser, and Lamp1-GFP structures were followed by time-lapse photography. Time (minutes) is indicated in the top left of each panel.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies

Lysotracker-red, dextran-red (MW 10000), DQ-BSA-red, Hoechst and DQ-ovalbumin-green were from Invitrogen (Carlsbad, CA). Leupeptin is from Millipore (Billerica, MA). Cycloheximide and anti-LAMP1 antibody was from Sigma-Aldrich (St. Louis, MO). Anti-P-S6K, Anti-S6K, anti-S6K, anti-P-4E-BP, anti-4E-BP, anti-atg7, anti-LC3 were from Cell Signaling Technology (Danvers, MA). anti-AP-3 was from Developmental Studies Hybridoma Bank, Anti-Cath B was from Santa Cruz Biotechnology, anti-Cath D is from BD Bioscience. Western blots were performed as previously described[14].

Cell Culture and Transfection

NRK cells were obtained from American Type Culture Condition (ATCC) and cultured in DMEM (Life Technologies) medium supplemented with 10% FBS (5% CO2). Cells were transfected via Amaxa nucleofection™ using solution T and program X-001, using 200 pmol RNAi or total 2 ug DNA[15]. Cells were then cultured in growth medium for further analysis. For two rounds of transfection, cells were transfected with 200 pmol RNAi, and 72 hours after transfection, cells were transfected again with 100 pmol RNAi and up to 2 ug DNA. Starvation medium is DMEM medium without the addition of serum and glutamine. HELA, C166, MRC-5, SH-SY5Y, MEF, OP9, Vero, HuH7, DF-1, ZF4,A6, HepG2, OMK, A549, H4-II-E-C3 are from ATCC and cultured according to the repository instructions.

Constructs

The human *spinster* construct was kindly provided by Dr. D. Yamamoto, Waseda University, Japan. LAMP1 and LC3 constructs with CFP, RFP, YFP, GFP or PAGFP

tags were provided by Dr. J. Lippincott-Schwartz, NICHD, NIH. Rab7-GTP, and Rab7 were provided by Dr. J. Bonifacino, NICHD, NIH. RNAi were synthesized from Invitrogen. mTOR, CAGCAGCTGGTACATGACAAGTACTTT, atg5, GGCATTATCCAATTGGCCTACTGTT

Live Cell Imaging

Transfected cells were re-plated in Lab Tek Chambered coverglass (NUNC) the night before imaging, and cells were maintained at 37°C with 5% CO_2 in a PeCon open chamber (PeCon, Erbach, Germany). Images were acquired by a Leica sp5 confocal microscope. 3D models were constructed by collecting images by Z-stack scanning at 0.5 um intervals, and images were collapsed to construct 3D models by IMRIS (Bitplane, Zurich, Switzerland). For fly experiments, 3rd instar larvae either expressing *tub*-LAMP1-GFP (control), or trans-heterozygous *spinster* mutant larvae expressing LAMP1-GFP (*tub*-LAMP1-GFP; $spinster^{10403}$/$spinster^{K09905}$ were starved in moist petri dishes lacking food at 25°C. The fat body was dissected and stained with Hoechst, and samples were imaged immediately without fixation on a Zeiss AxiovisionZ.1 microscope with fluorescence. Lysosome number was measured using Zeiss Automeasure software. Lysosomes were quantified in at least 7 different larvae per time-point, in fat cells from one fat body per larva and 2 fields per fat body. Each field contained 3-4 cells. The number of lysosomes per timepoint was normalized to the average number of lysosomes in non-starved (0h) larvae. Error bars indicate standard deviation.

Cell fractionation

Lysosome isolation kit is from Sigma-Aldrich. Cell fractionation and lysosome isolation were performed according to manufacture's manual.

Staining

Cells were washed with Phosphate Buffered Saline (PBS), fixed in 2% paraformaldehyde for 10 min, and permeabilized in 0.2% Triton X-100 for 5 min. They were blocked with 10% FBS in PBS for 30 min, stained with 10 ug/ml of rabbit anti-LAMP1 (Sigma-Aldrich) in blocking buffer for 1 hour, and washed with PBS three times. Cells were then stained with fluorescein isothiocyanate conjugated (FITC)-anti rabbit secondary antibody (Becton-Dickinson, San Jose, CA) in PBS for 1 hour and washed with PBS three times.

Electron Microscopy

Cells were fixed in 3% glutaraldehyde in 0.1 M MOPS buffer (pH 7.0) for 8 hrs at room temperature, then 3% glutaraldehyde/1% paraformaldehyde in 0.1 M MOPS buffer (pH 7.0) for 16 hours at 4°C. They were then post-fixed in 1% osmium tetroxide for 1 hour, and embedded in Spurr's resin, sectioned, doubly stained with uranyl acetate and lead citrate, and analyzed using a Zeiss EM 10 transmission electron microscope.

Immuno-electron microscopy

NRK LAMP-1-YFP cells were starved for 8 hours and then fixed for immuno-electron microscopy by adding freshly-prepared 4% formaldehyde in 0.1 M phosphate buffer (pH 7.4) to an equal volume of culture medium. After 10 min, the fixative was replaced for

post-fixation in 4% formaldehyde at 4°C overnight. Ultrathin cryosectioning and immunogold labeling were performed as previously described[16]. The LAMP-1-YFP construct was detected by using polyclonal rabbit anti-LAMP-1/LGP120 M3 at a dilution of 1:650. This antibody is a kind gift of Dr. Ira Mellman (Genentech, San Francisco). The antibody was marked by protein A-10 nm gold particles (Cell Microscopy Center Utrecht, The Netherlands).

Additional methods and procedural details may be found in references 14-17.