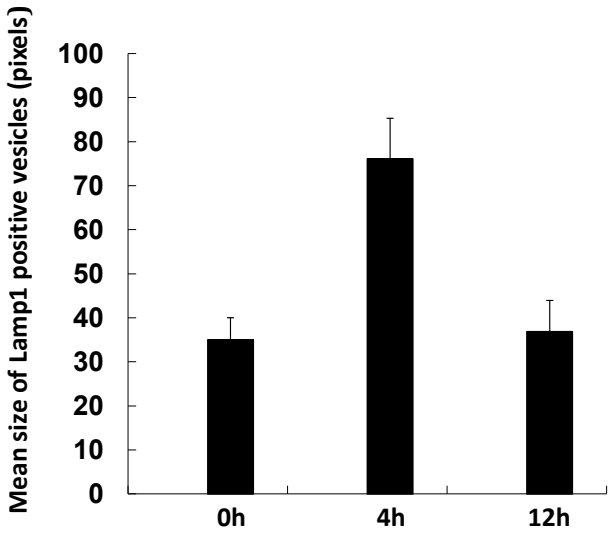
Supplemental Data

Supplemental Figure Legends

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.

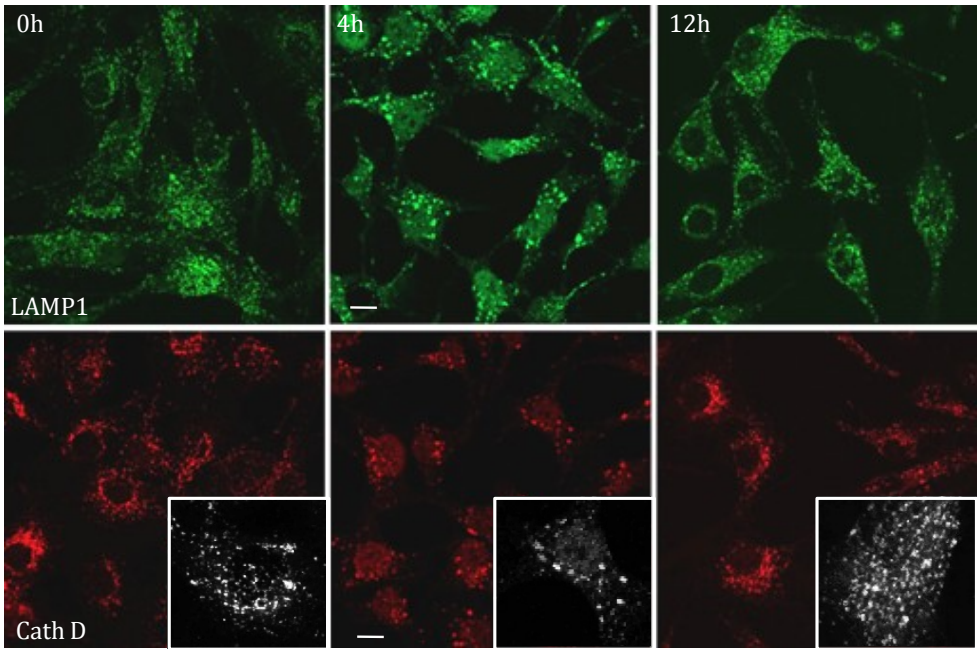
S2



S2. 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

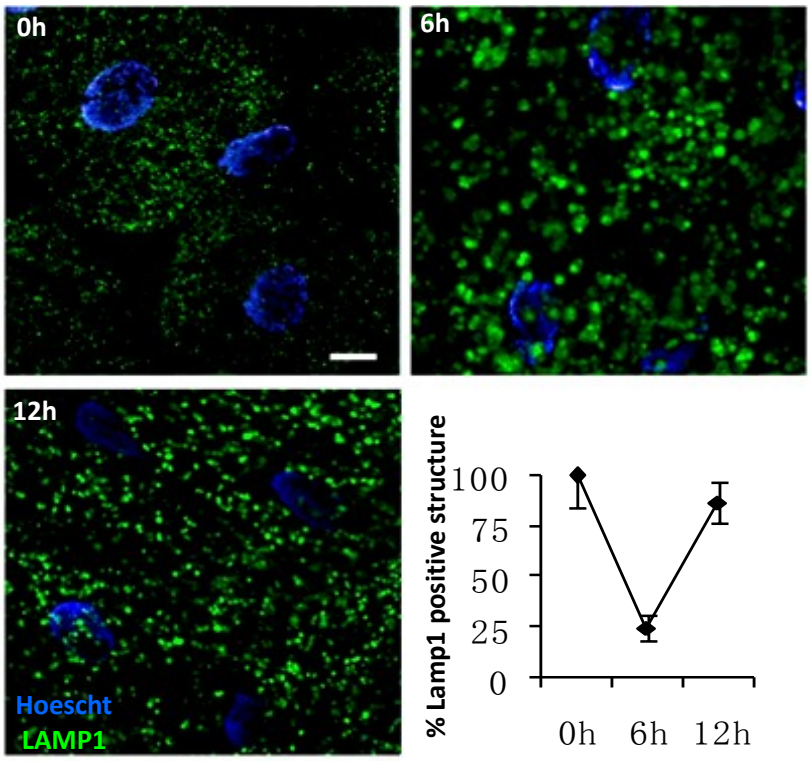
Quantified by Image Pro-Plus. Error bar show s.e.m (n=3).

S3



S3. NRK cells were starved for 0, 6, or 12 hours as indicated and stained for Lamp1 (top) and cathepsin D (bottom).

S4



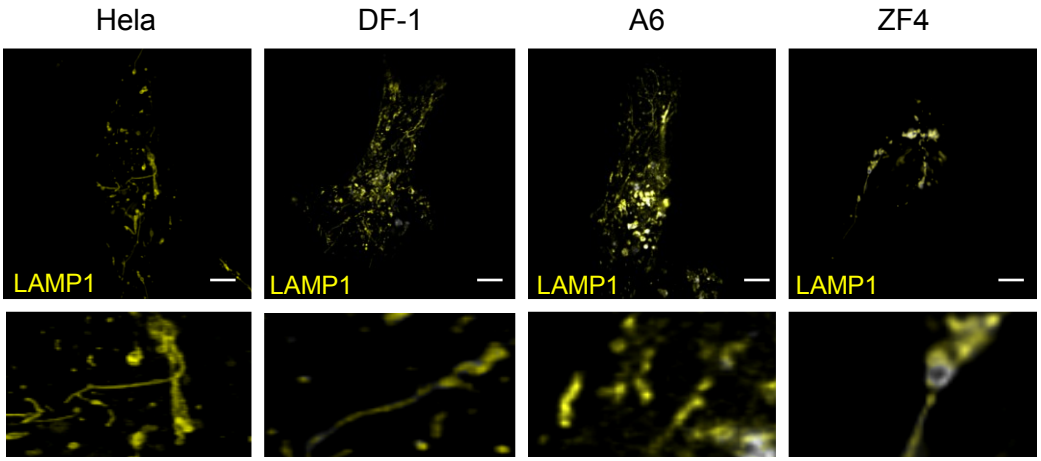
S4. *Drosophila* larvae expressing *tub*-LAMP1-GFP were starved for 0, 6, and 12 hours, and lysosomes in fat cells were imaged by fluorescent microscopy. Green: LAMP1. Blue, Höechst. Scale bar, 20 um. *Drosophila* lysosomes were quantified using Zeiss Automeasure software. Lysosomes were quantified in 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. Error bar show s.e.m (n=3).

S5

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.					

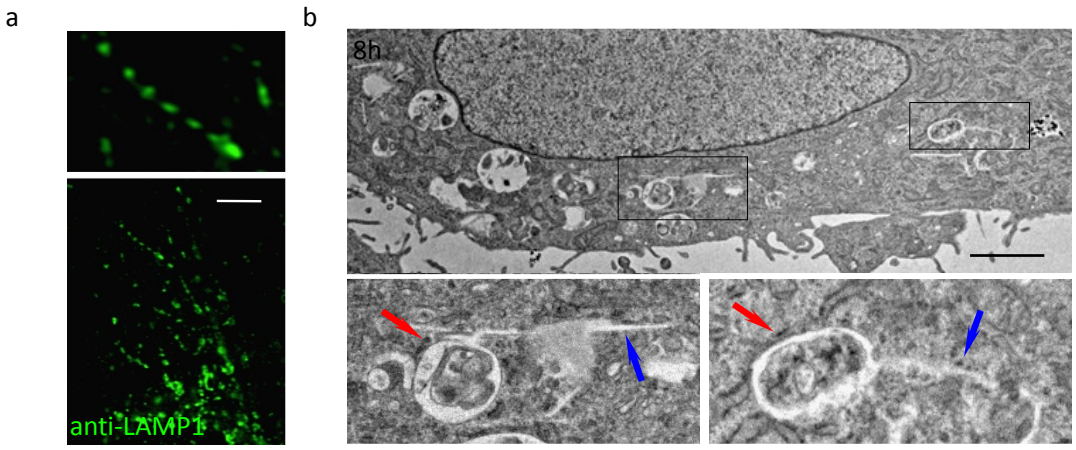
b



S5. Lysosome reformation occurs in cell lines from diverse tissues and animal species.

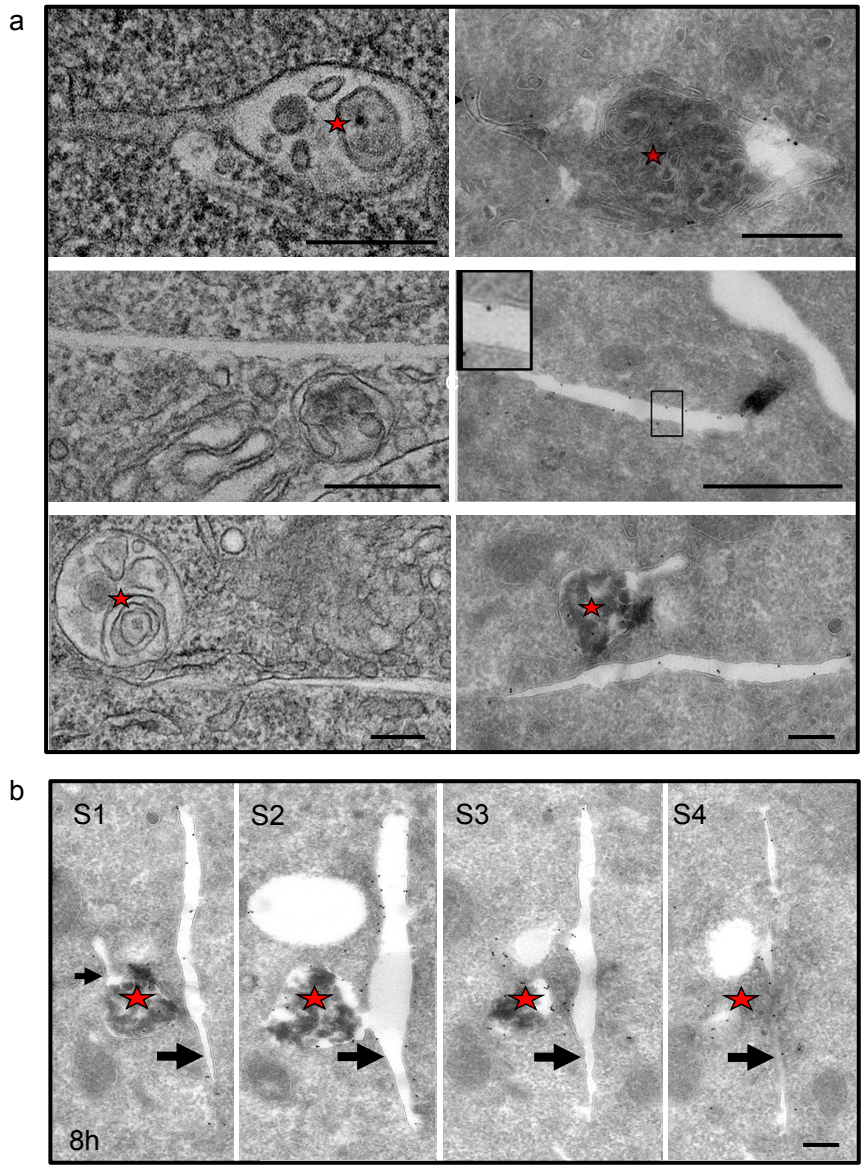
Cells were starved for 0, 4, 8, or 12 hours, and the number and size of lysosomes were monitored by lysotracker. Reformation tubules were visualized in cells expressing Lamp1-YFP. (a) Table showing what cell types were monitored, and which ones had tubules. (b) Images of reformation tubules from representative cell lines of diverse origins.

S6



S6. 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.

S7



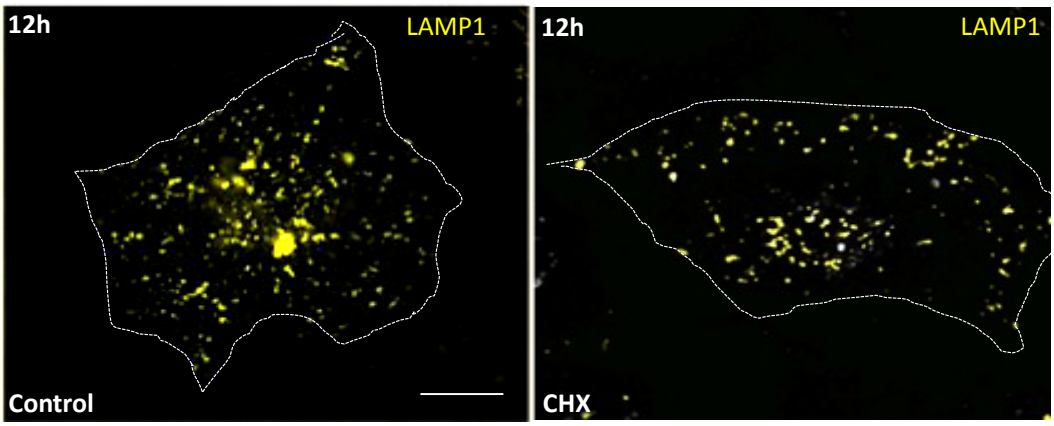
S7. a) Representative transmission electron micrographs (TEM) and immuno-transmission electron micrographs (I-TEM) of NRK cells starved 8 hours with tubules ,

Scale bar, 200 nm. left panel, TEM, right panel, I-TEM b) Immuno- transmission electron micrographs (I-TEM) of NRK cells starved 8 hours with tubules, NRK cells were starved for 8 hours and were consecutively sectioned, then analyzed by I-TEM using Lamp1 antibody. Scale bar, 2 um, red star, autolysosome, arrow indicate the membrane limited nature of tubules.

S8. 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.

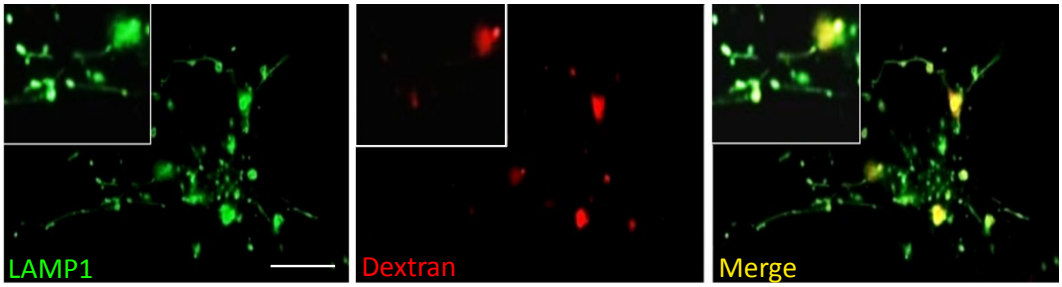
S9. Movie of tubules breaking away from autolysosomes and condensing into Lamp1-positive vesicles. NRK cells transfected with Lamp1 (photoactivatable) PAGFP/Lamp1-RFP (3:1) were starved for 6 hours. LAMP1-PAGFP was photo-activated by a 405 laser, and Lamp1-GFP structures were followed by time-lapse photography. Time (minutes) is indicated in the top left of each panel.

S10



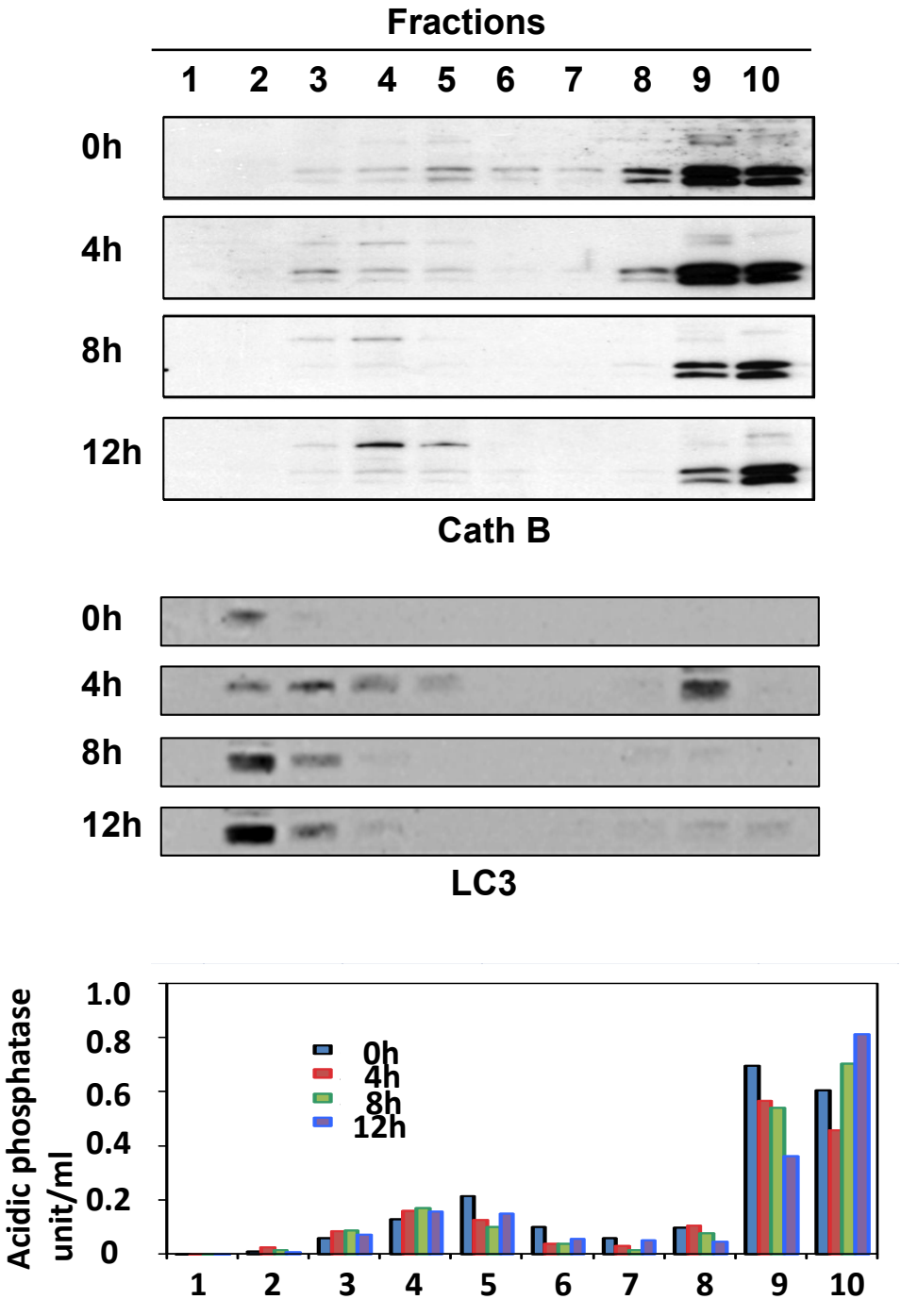
S10. Formation of proto-lysosomes does not need new Lamp1 synthesis. NRK cells were transfected with Lamp1-YFP, and 24 hours after transfection, cells were starved for 12 hours with or without presence of 10ug/ml CHX. Scale bar, 5 um

S11



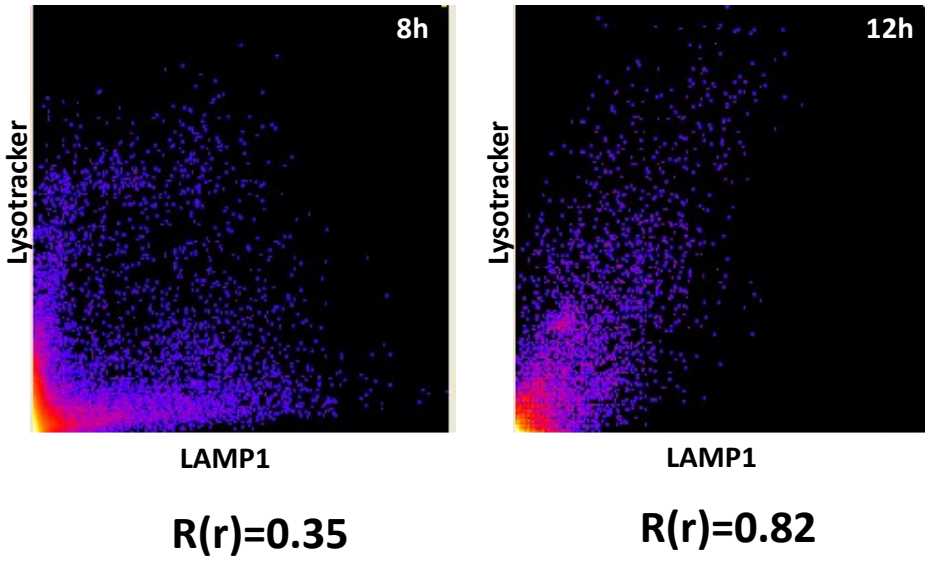
S11. NRK cell were transfected with Lamp1-YFP, 12 hours after transfection, cells were loaded with 10ug/ul dextran-TR and then starving for 8 hours. green: Lamp1, red, dextran, Scale bar, 5um.

S12

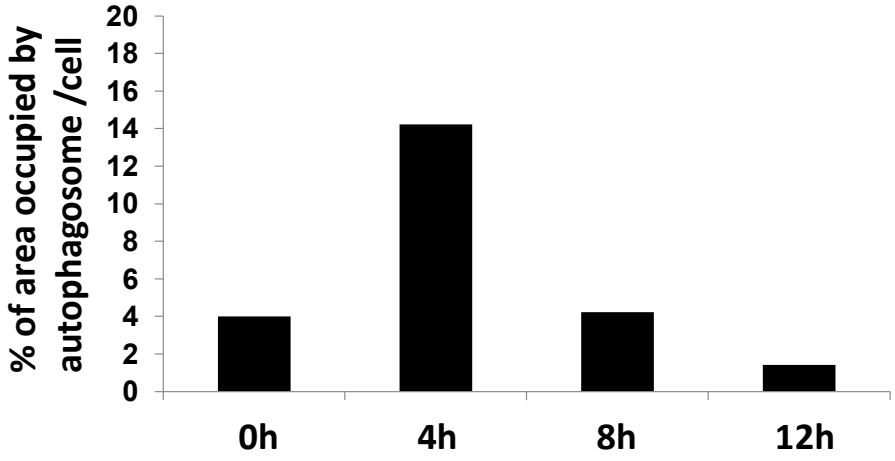

Fractions
1 2 3 4 5 6 7 8 9 10
0h
4h
8h
12h
Cath B
0h
4h
8h
12h
LC3
Acidic phosphatase unit/ml
1.0
0.8
0.6
0.4
0.2
0
0h
4h
8h
12h
1 2 3 4 5 6 7 8 9 10

S12. NRK cells were starved for 0, 4, 8, or 12 hours and then subjected to gradient ultracentrifugation. Fractions were collected, protein extracts were made and Western blots were probed with anti- Cathepsin B, LC3. Fraction 1 is the bottom of the gradient and fraction 5 is the top of the gradient. The acidic phasphotase activityof each fraction were measured by acidic phasphotase kit from Sigma.

S13

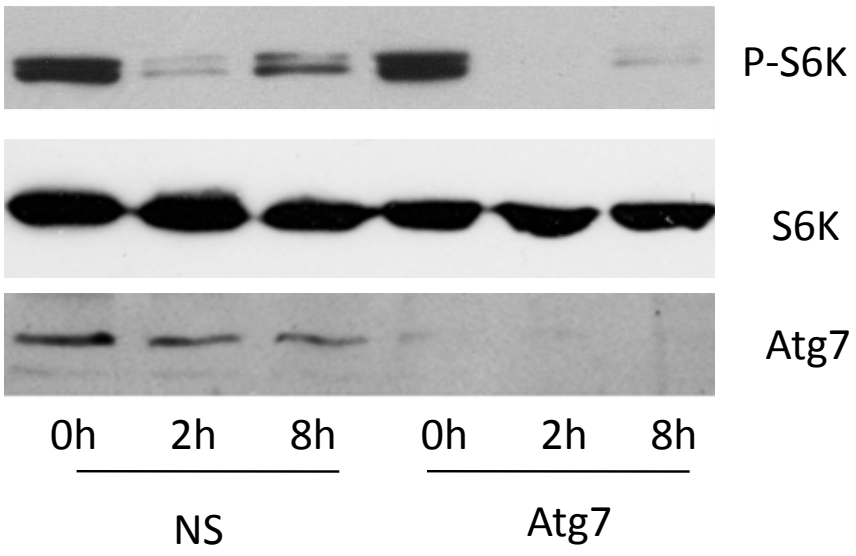


S13. The co-localization of LAMP1 and Lysotracker. LAMP1-GFP-expressing NRK cells were starved for 8 and 12 hours and stained with Lysotracker. Co-localization of LAMP1 and Lysotracker was analyzed using IMRIS software from Bitplane.



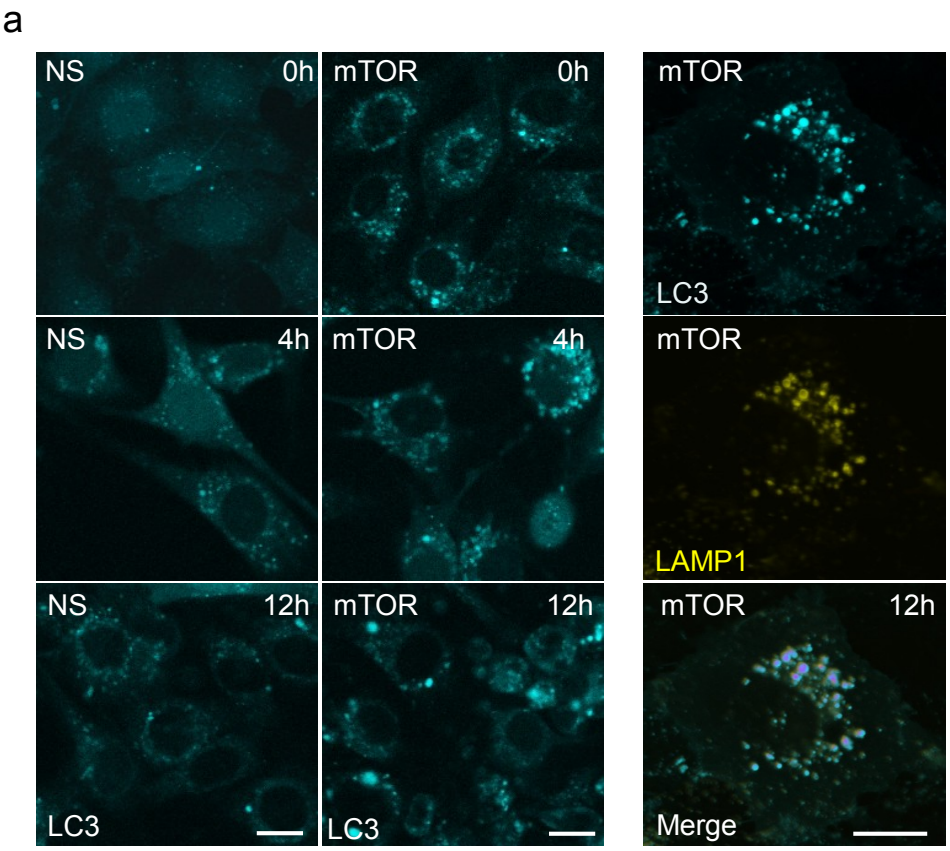
S14. Morphometric analysis of the area occupied by autophagosomes per cell for Fig. 3a.

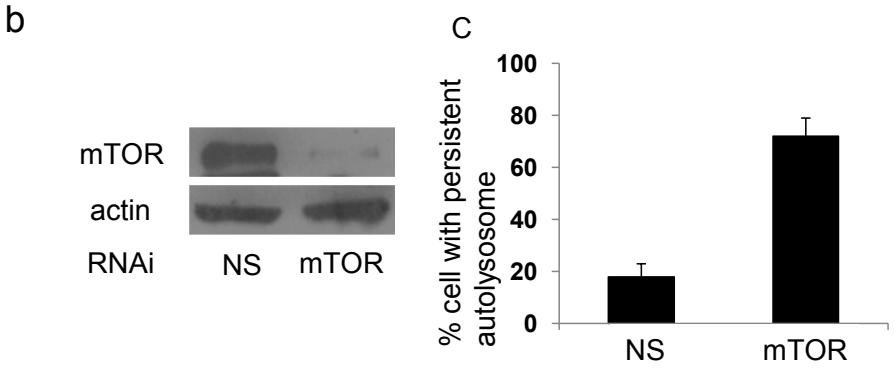
S15



S15. NRK cells were transfected with non-specific RNAi (NS) or RNAi against *atg7*. 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 Atg5, as indicated.

S16

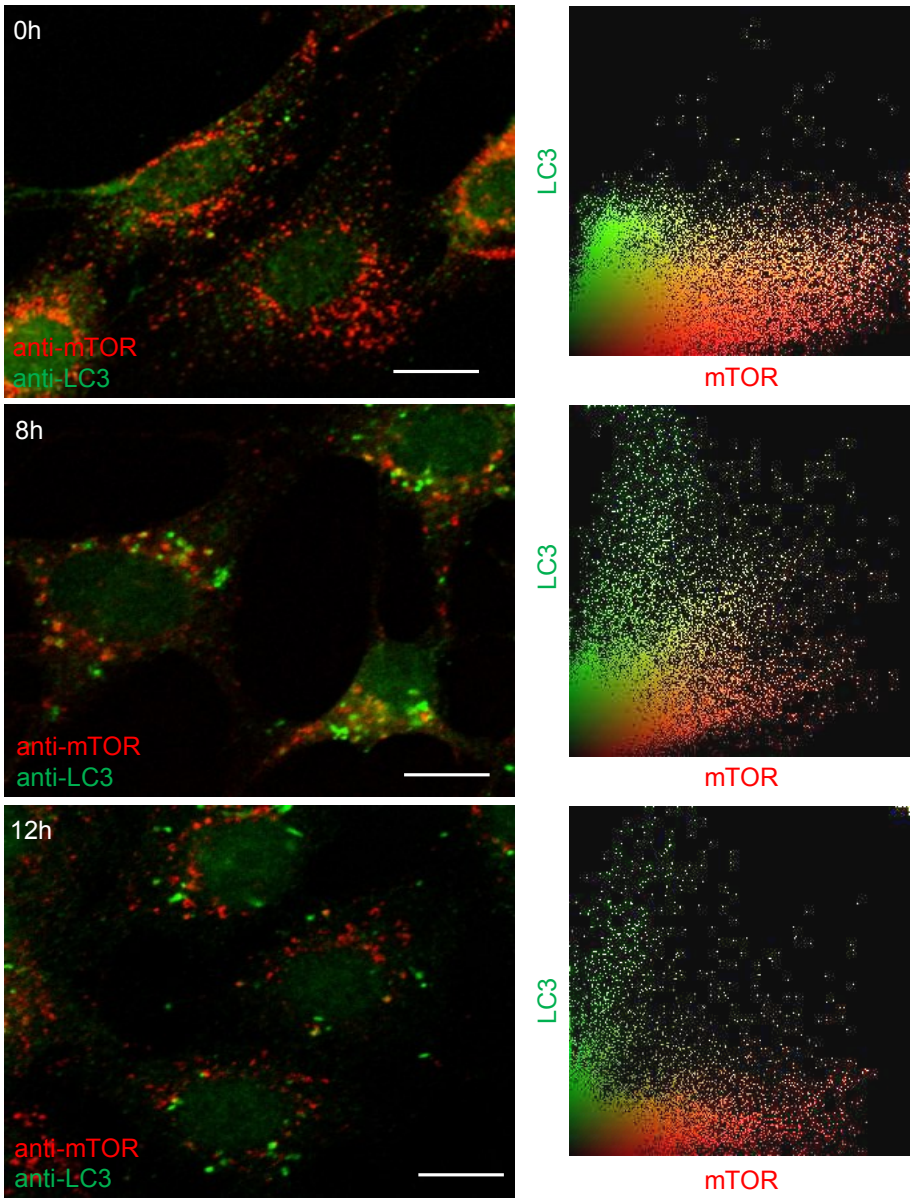




6. Knockdown of mTOR inhibits ALR and leads to accumulation of persistent, large

autolysosomes. a) NRK-LC3-CFP cells were transfected with non-specific RNAi (NS) or RNAi against *mTOR*. 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 and imaged by confocal microscopy for Lamp1 (green) and LC3 (blue). Scale bar, 5 uM. b) Cell lysates were prepared from an aliquot of the cells transfected above, subjected to gel electrophoresis, and blotted for the mTOR protein (upper blot) or actin (lower blot) as a loading control. C) Quantification of cell with persistent autolysosome 12 hours after starvation. 50 cells were counted. Error bar show s.e.m (n=3).

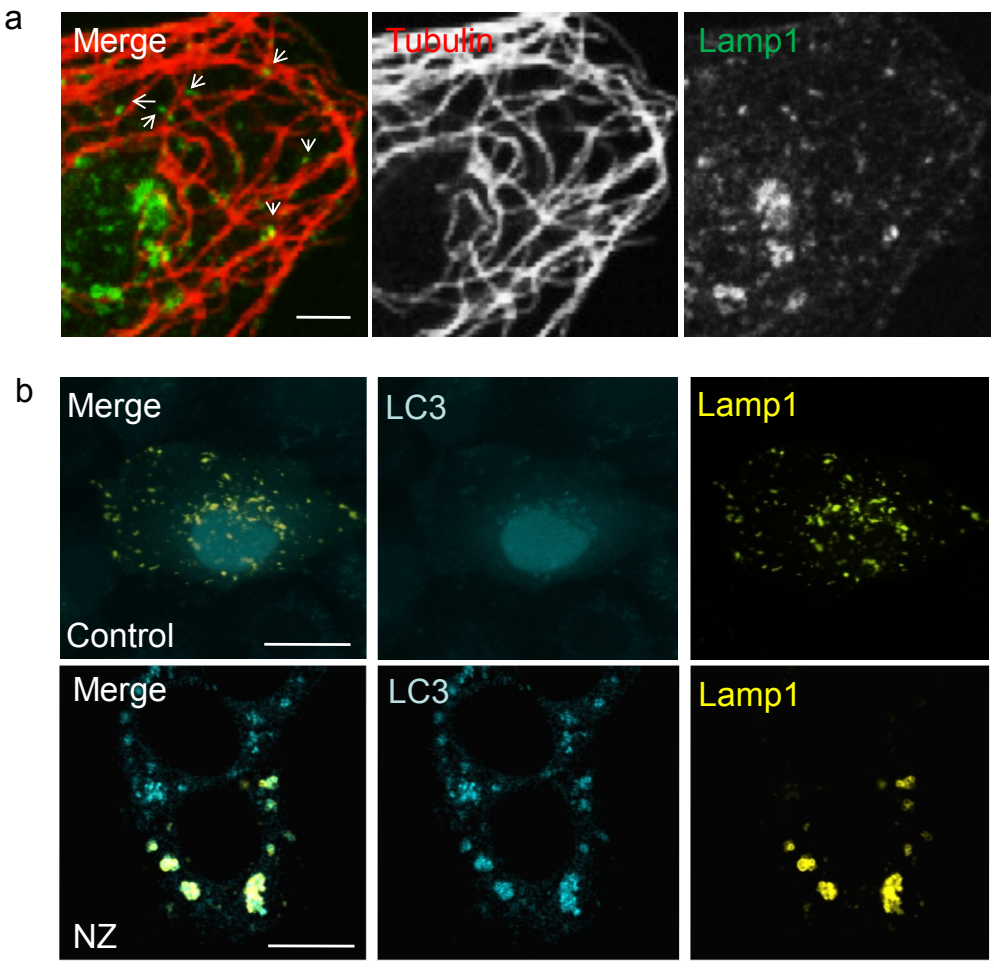
S17



S17. NRK-LC3 cells were starved for 0, 8, and 12 hours. Cells were stained with anti-

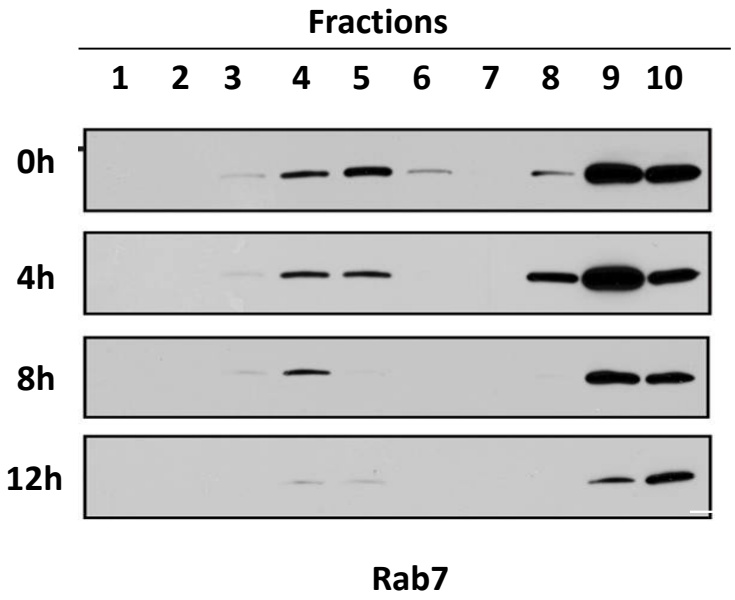
LC3(green) and anti-mTOR(red) antibody and analyzed by confocal microscopy, Scale bar, 5 um.

S18



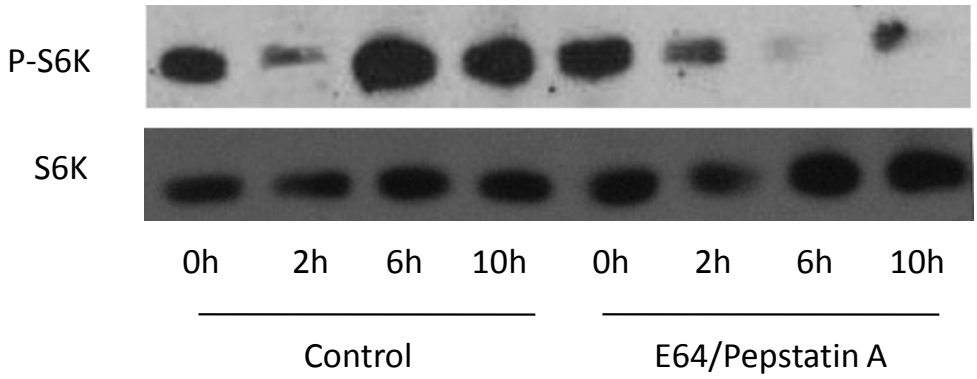
S18. (a) NRK cells were starved for 8 hours and then stained for tubulin (red) and Lamp1 (green). The arrows show protolysosomes budding at various points on the lysosome reformation tubules. Quantification showed that 97% of the proto-lysosomes were directly associated with the microtubules; (b) NRK cells were starved for 12 hours with or without the addition of nocodazole (NZ) and then stained for LC3 (blue) and Lamp 1 (yellow).

S19



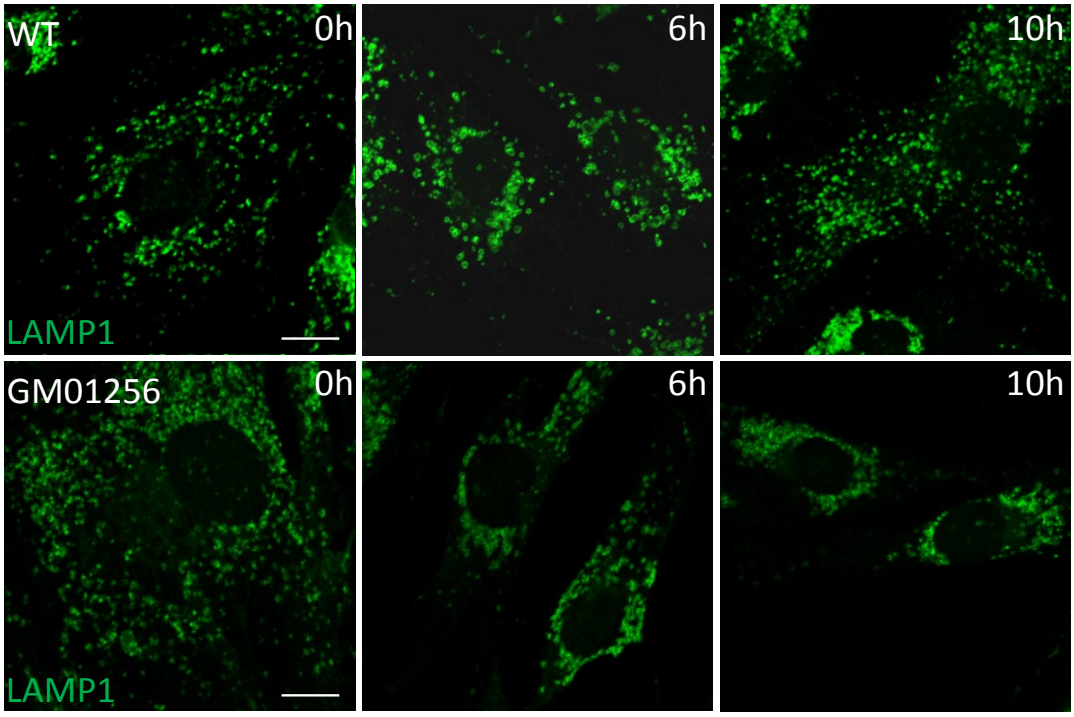
S19. NRK cells were starved for 0, 4, 8, or 12 hours and then subjected to gradient ultracentrifugation. Fractions were collected, protein extracts were made and Western blots were probed with anti- Rab7. Fraction 1 is the bottom of the gradient and fraction 5 is the top of the gradient.

S20



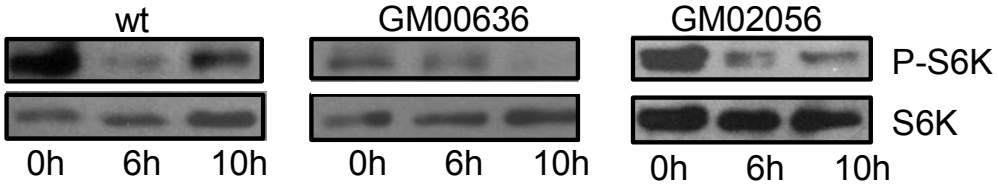
S20. NRK-LC3 cells were starved for 0, 2, 6, or 10 hours with or without the addition of E64/Pepstatin A. Cells were harvested and analyzed by Western blot using antibodies against Phospho-p70 S6 Kinase (p-S6K) and p70 S6 Kinase.

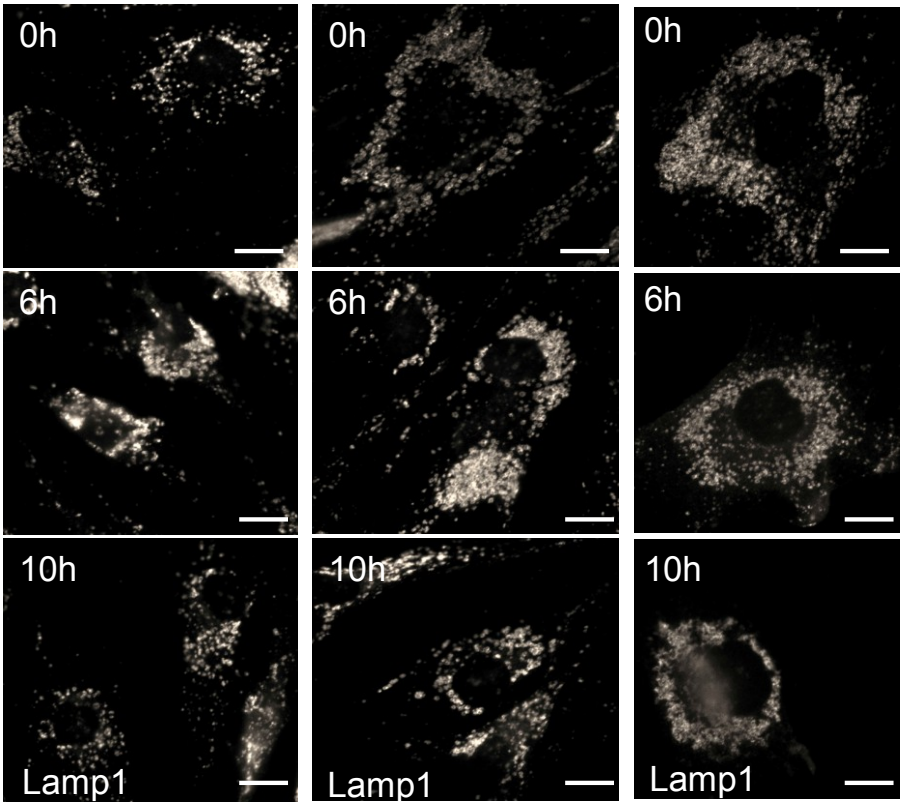
S21



S21. Original images form Figure 4g. wild type and mutant cells were starved for 0, 6, or 10 hrs 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.

S22


wt
GM00636
GM02056
P-S6K
S6K
0h
6h
10h
0h
6h
10h
0h
6h
10h


0h
0h
0h
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6h
10h
10h
10h
Lamp1
Lamp1
Lamp1

S22. wild type and mutant cells were starved for 0, 6, or 10 hrs 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.