SUPPLEMENTAL MATERIALS

Fig 1s

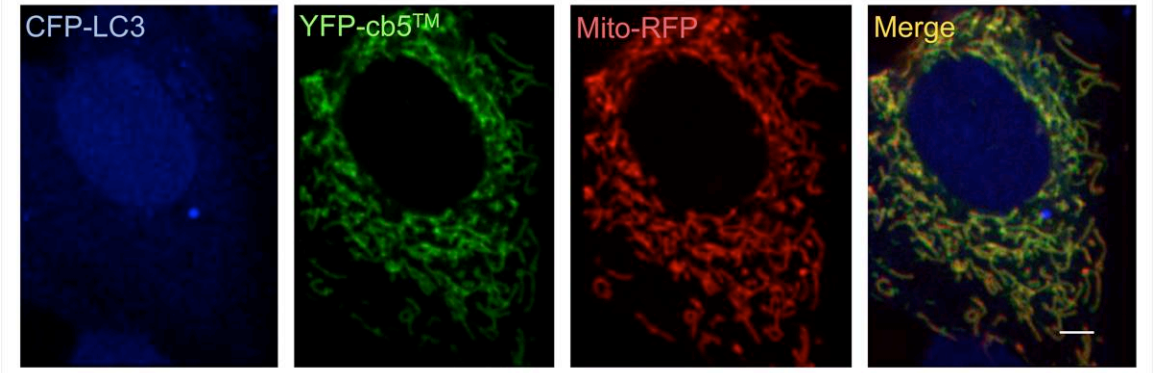


Figure 1s: **Targeting of mitochondrial targeted YFP-cb5^{TM} in unstarved NRK58B cells.** Cells were transfected with YFP-cb5^{TM} and Mito-RFP and imaged 16 hours later. The YFP-cb5^{TM} signal overlaps completely with the mitochondrial signal of Mito-RFP. No signal in the endoplasmic reticulum is observed. (Scale bar: 5μm.)

Fig 2s

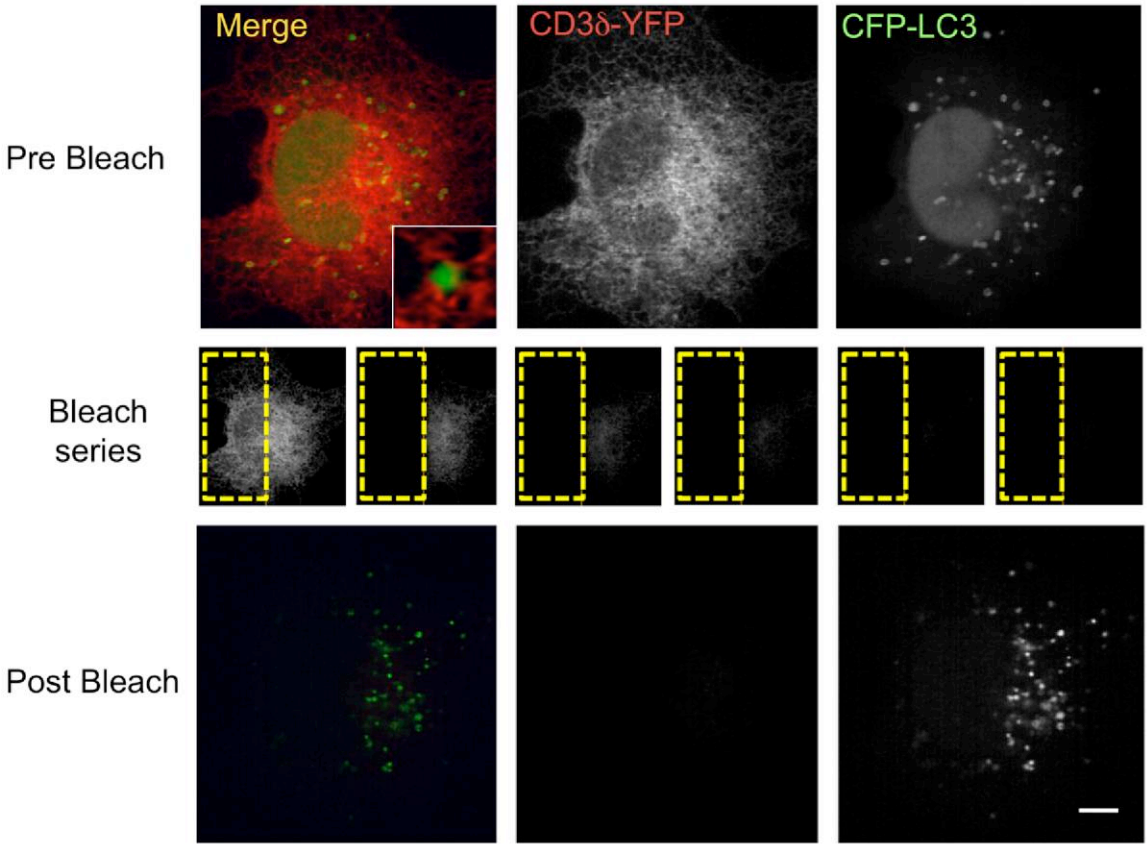


Figure 2s: **Repetative photobleaching to determine if CD3δ-YFP is captured by autophagosomes.** NRK58B cells were transfected with CD3δ-YFP and starved 16 hours later. High resolution imaging at sites of low ER network density show lack of overlap with the ER marker and CFP-LC3 (inset, upper left). To further evaluate potential tranfer of the ER marker to autophagosomes, the ER network was bleached. Because the ER network is continuous, repetative photobleaching part of the ER (yellow box above) depletes signal throughout the entire ER network. No signal was retained in autophagosomes following photobleaching, indicating no ER marker was present on autophagosomes. If the ER marker was present on membranes of mature autophagosomes separated from the ER network, they would retain signal if they were outside the bleached region. (Scale bar: 5μm.)

Fig 3s

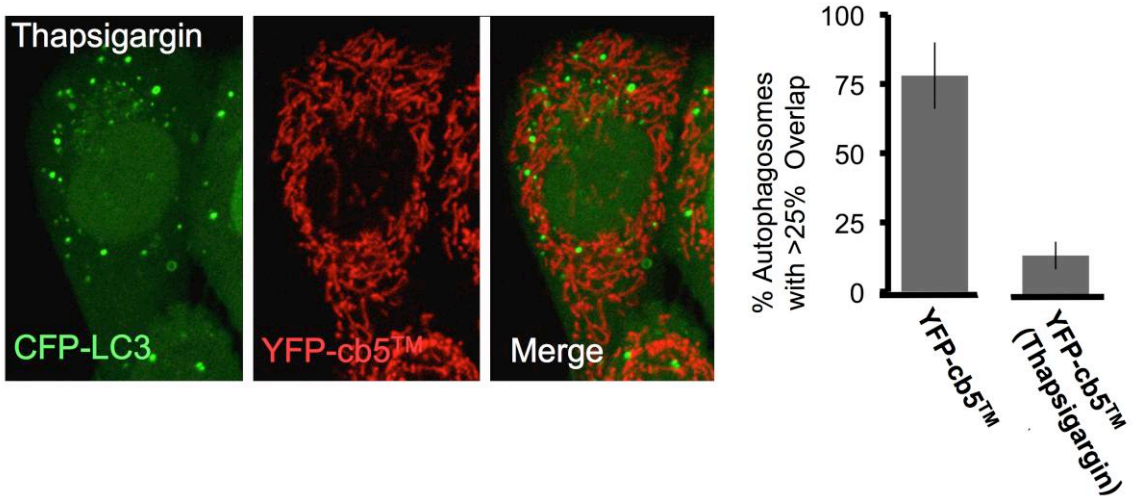


Figure 3s: **ER stress induced autophagosomes do not utilize outer membrane**. A four hour treatment of NRK58B cells with 25nM thapsigargin induced autophagosomes that did not acquire the YFP-cb5^{TM} marker.

Figure 4s:

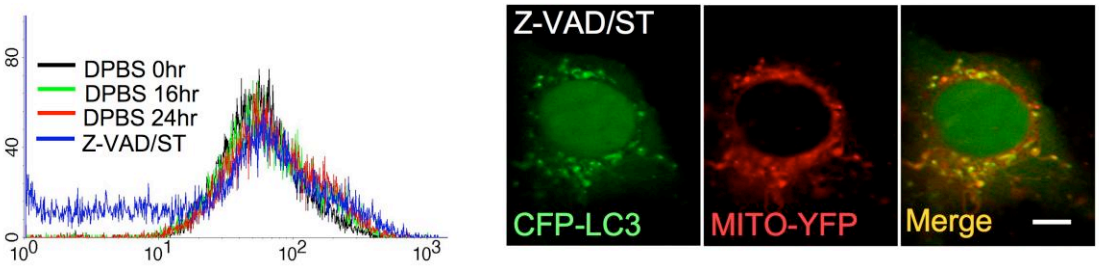


Figure 4s: **Assessment of whether mitophagy might underlie cb5 delivery.** Cells were either starved or treated with ZVAD/Staurosporine and stained with a potential insensitive mitochondrial marker. Subsequent flow cytometry revealed loss of mitochondrial mass in response to ZVAD/Staurosporine treatment (left hand panel; left hand trailing blue line) but not DPBS treatment. Fluorescence microscopy confirmed mitophagy in ZVAD/staurosporine treated cells. ZVAD/Staurosporine treated cells showed extensive Mito-YFP signal in autophagosomes (right hand panel) unlike starved cells (see Figure 4). (Scale bar: 10μm)

Figure 5s

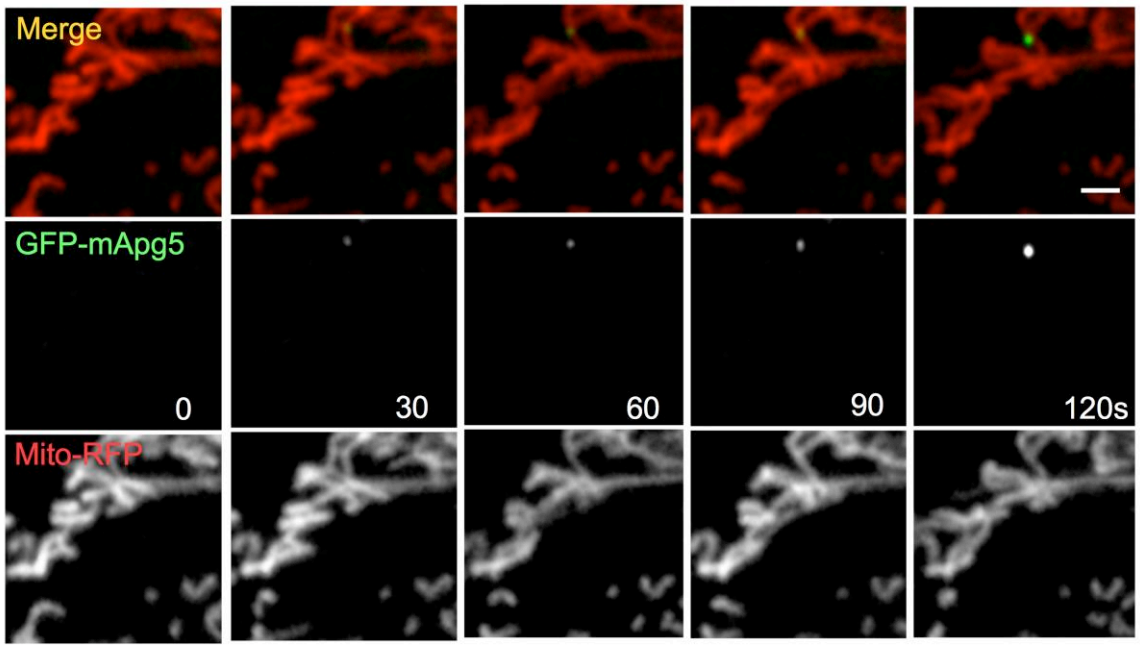
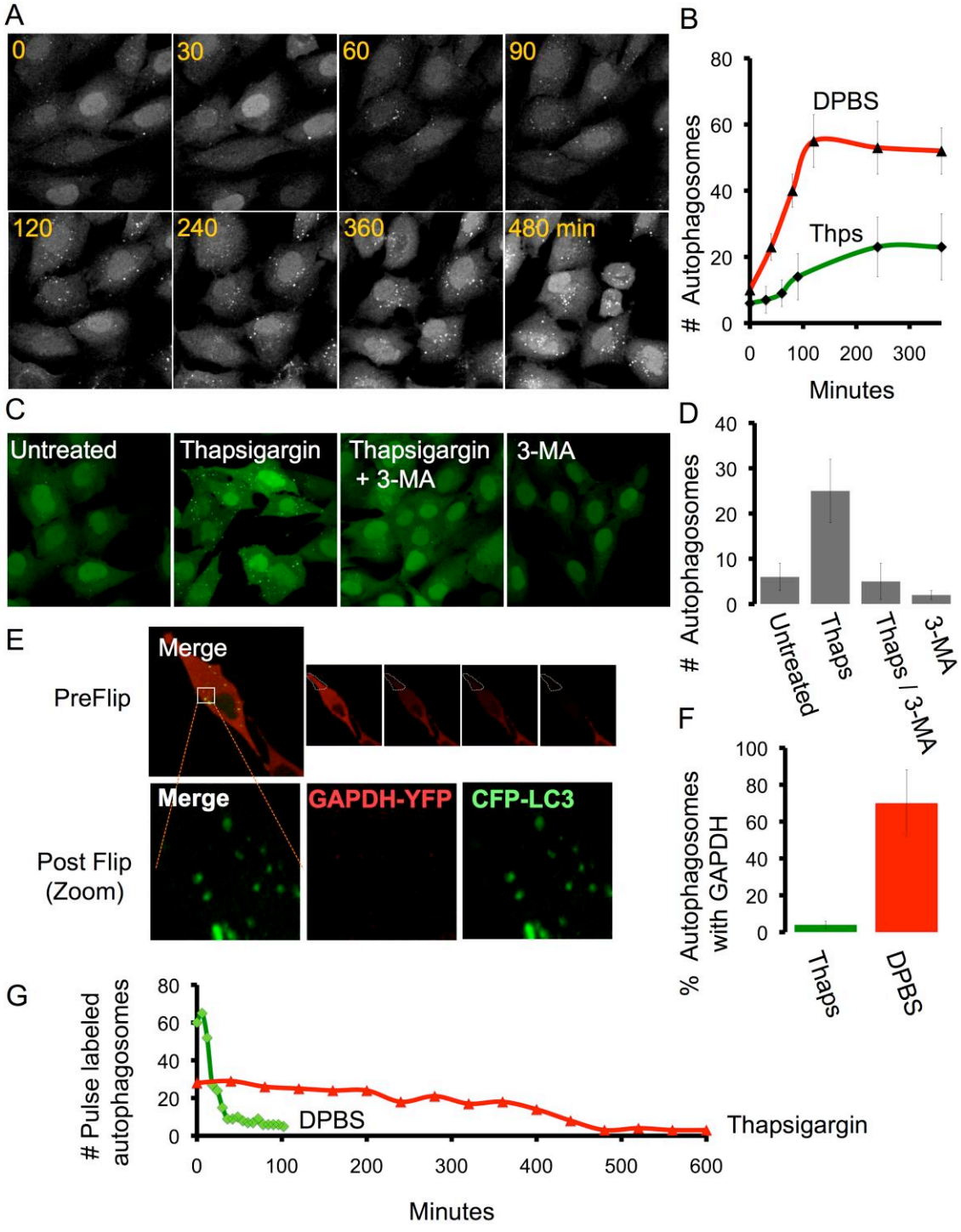


Figure 5s: **Live-cell imaging of GFP-LC3 labeled autophagosomes and associated mitochondrial elements.** NRK cells were transfected with the mitochondria matrix marker Mito-RFP and autophagosome marker GFP-mApg5 and subsequently starved. High-speed high resolution imaging showed autophagosome inception occurs along associated mitochondrial elements. See supplementary movie mApg5-Mito.mov.

Figure 6s


A
0
30
60
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120
240
360
480 min
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DPBS
Thps
Autophagosomes
Minutes
C
Untreated
Thapsigargin
Thapsigargin + 3-MA
3-MA
D
Autophagosomes
Untreated
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Thaps / 3-MA
3-MA
E
PreFlip
Merge
Post Flip (Zoom)
Merge
GAPDH-YFP
CFP-LC3
F
% Autophagosomes with GAPDH
Thaps
DPBS
G
Pulse labeled autophagosomes
DPBS
Thapsigargin
Minutes

Figure 6s: **Thapsigargin-induced autophagosomes are distinct from starvation-induced autophagosomes.** **(A)** Time-lapse live-cell imaging of thapsigargin treated NRK58B cells. Autophagosomes proliferated immediately following treatment. Note cytosolic and nuclear pools of CFP-LC3 were not depleted. Growth media (time 0) was replaced with media containing thapsigargin (subsequent panels). (Scale bar: 20μm) **(B)** Quantification of CFP-LC3 positive structures. Autophagosomes rapidly proliferated in response to thapsigargin treatment but reached a lower steady state number (green line) than in starvation (red line). CFP-LC3 positive structures were counted in sequential time-lapse frames. **(C)** Treatment with ClassIII PI(3) kinase inhibitor 3-methyladenine. 3-MA treatment abolished thapsigargin-induced recruitment of CFP-LC3 to membranes. Note that cytosolic and nuclear signal increased relative to untreated and 3-MA alone. (Scale bars: 20μm) **(D)** Quantification of 3-MA treatments. 3-MA robustly inhibited autophagosome formation to levels comparable to the untreated control. (Quantification was for 20 cells). **(E)** Assaying for capture of cytosolic proteins in thapsigargin-induced autophagosomes. Depletion of freely diffusing cytosolic GAPDH-YFP did not reveal punctae (i.e. cytosolic proteins within thapsigargin induced autophagosomes). Top right four panels show depletion of GAPDH-YFP signal by repetitive photobleaching within the white ROI. Bottom panels show zoom of inset outside the targeted photobleach region. Following photobleaching, no detectable GAPDH punctae remained. (Scale bar upper panel: 20μm; lower panel: 4μm) **(F)** Quantification of GAPDH-YFP captured in autophagosomes. Very few thapsigargin-induced autophagosomes showed GAPDH-YFP capture compared with a majority of starvation-induced autphagosomes. **(G)** Comparison of the lifetime of the thapsigarin-induced structures versus starvation-induced structures. As in Figure 1, PAGFP-LC3 positive autphagosomes were pulse-labeled and tracked with live cell imaging. Thapsigargin-induced autphagosomes exhibited a dramatically longer $t_{1/2}$ (~6 hours) compared to starvation-induced autophagosomes (~25minutes). Asterisk in bar graphs indicates treatment statistically different from untreated cells by Student t-test: p values < 0.001.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Plasmid Constructs

Yoshinori Ohsumi provided pEGFP-mApg5(C1) and pEGFP-LC3(C1). The BspE1/Age1 fragment of pEGFP-LC3(C1) was replaced by the BspE1/Age1 fragment from pmCFP C1 to generate pmCFP-LC3. The BspE1/BamH1 fragment of pEGFP-mApg5(C1) was moved into pmVenus C1 (cut with BspE1/BamH1) to generate pmVenus-mApg5(C1). Plasmids expressing cytosolic proteins GAPDH-YFP and GAPDH-RFP were provided by Manoj Raje. Christian Wunder and Peter Kim provided Prohibitin-YFP and YFP-cb5^{TM} respectively. YFP-cb5^{TM}(P115) was made by site directed mutagenesis of pYFP-cb5^{TM}. The plasmid was amplified using primer AAC TGG GTT ATC GCG GCG ATC TCT GCT CTG and its reverse complement. Following PCR, (methylated) template DNA was digested by the enzyme DpnI. YFP fusion to the tail anchor of mitochondrial targeted Fis1 was generated by PCR cloning using primers (5')TAC CTG TAC AAG GAG CCC CAG AAC AAC CAG GCC and (3')TAC CTC CGC GGC GAT GGC CCA CTA CGT GAA CC. The BsrG1/SacII fragment PCR amplified from Fis1 in pcDNA3.1 was cut and cloned into Venus C1. Plasmids EEA1-YFP, GPI-YFP, YFP-TGN38, GalT-YFP, CD3Delta-YFP, and YFP-HLA A2-CFP are previously published.

Fluorescence Microscopy

Single images of CFP/YFP expressing cells or fixed cells were acquired with an LSM 510 laser-scanning confocal microscope with a 63x 1.4 NA Plan-Apochromat oil objective (Carl Zeiss MicroImaging, Inc.). For live CFP/YFP imaging, the 458 nm and 514 nm laser lines of an Argon/Krypton laser (Lasos) were used to excite CFP and YFP. Line interlace scanning (wavelengths alternated between linescans) was used to minimize artifacts of motion. The pinhole was adjusted to capture non-saturated images of 0.8 μm optical slices. Emission filter passes were 470-500 nm for CFP and 535-590 nm for YFP.

For live cell imaging of induction conditions, an LSM 510 laser-scanning confocal microscope with a Zeiss 40x 1.3 NA Plan-Neofluar oil objective was used. The 458 nm

laser line was used for excitation, and 505 nm long pass filter for emission. Single plane images were captured from 1.7μm optical slices every 20 to 30 minutes for <12 hours.

For live cell imaging of autophagosome turnover, images were captured as described for induction conditions except that the 488 line was used to excite activated PAGFP and the optical slice was increased to 2.0um. For photoactivation, either a 413 nm Coherent Enterprise II laser or 405 nm diode laser was used to activate signal to <80% of maximal signal. For subsequent bleaching, the 488 nm line was repetitively targeted to a 5X5 micro region in the cell periphery.

For live cell imaging of autophagosome fusion, tracking GFP-LC3 association with RFP-labeled mitochondrial elements, and photobleaching to assay for autophagosome substrate capture and continuity, a Beta version of the Zeiss LSM DUO with a 63x 1.4 NA Plan-Apochromat oil objective (Carl Zeiss MicroImaging, Inc.) was used. The scan zoom was < 2.5 in all cases. For GFP/lysotracker and GFP/RFP tracking, 489 and 561 diode lasers and 495-555 and 575-615 bandpass filters were used. For CFP/YFP imaging, 440 and 489 diode lasers and 445-505 and 495 long pass emission filters were used. For photobleaching to identify substrates, a 561 nm laser was used. For photobleaching to evaluate continuity, 405 nm and 489 nm lasers were simultaneously used.

For live cell imaging of GFP-mApg5 association with RFP labeled mitochondrial elements, an UltraView ERS 6FO-US system with Photokinesis ERS6 module was used (Perkin Elmer). 490 nm and 561 nm diode lasers were used to excite GFP and RFP. A 63x 1.4 NA Plan-Apochromat oil objective (Carl Zeiss MicroImaging, Inc.) and Orca-ER CCD camera (Hamamatsu) were used for image acquisition.

On Zeiss systems, the autofocus macro in the Zeiss Multi-time software was used to maintain focal position over long time periods. Prior to each timepoint, a 633 nm laser line was scanned on a line of the sample. Focal plane position was detected by reflection of the laser line at the focal plane. Sample offset from this position was determined.

NBD-PS (18:1-06:0 NBD PS) formulation

(1-oleoyl-2-{6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]hexanoyl}-sn-glycero-3-phosphoserine) (Cat# 810194C , Avanti Polar Lipids, Inc) was mixed with DOPC 18:1 (Δ9-Cis) PC 1,2-di-(9Z-octadecenoyl)-sn-glycero-3-phosphocholine at a 20:80 ratio (Cat# 850375 Avanti Polar Lipids, Inc). The chloroform was removed and the NBD-PS was reconstituted to a 2.2mM stock in sterile PBS. NBD-PS was added to cells at a 0.22mM concentration for 30 minutes to label the cells.

Pharmaceutical treatments

Thapsigargin was purchased from Sigma (Cat T9033) and was used at 25nM final concentration. Autophagy was induced in <2 hours. NRK cells continued to survive and divide in the presence of 25nM thapsigargin for prolonged periods (>24 hours).

Digitonin was purchased from Calbiochem (Cat 300410) A 6% stock was made in water. When added to NRK cells at 1:1000 for cell permeabilization it readily released free GFP from the cytosol within 1 to 2 minutes. Prolonged exposure to this concentration in KHM buffer perturbed cell architecture. KHM buffer is described in (Lorenz et al., 2006)

Z-VAD-FMK and Staurosporine were purchased from Sigma (Cat# V-116 and S6942 respectively.) Z-VAD-FMK was dissolved in DMSO to make a 100mM stock solution. Cells were pretreated with Z-VAD-FMK for 30 minutes (50uM final concentration). Subsequently Staurosporine was added for 6 hours at 1uM final concentration. After 6 hours, cells were washed three times and media was replaced with media containing only Z-VAD-FMK (50uM) and incubated for 24 hours. Media was replaced again with Z-VAD-FMK containing media. Cells were incubated for an additional 24 hours and subsequently images to assess mitophagy.

Supplemental Movie Titles:

Movie 1: Transient recruitment of mApg5 (Red) precedes LC3 (Green) during autophagosome biogenesis.

Movie 2: Fusion of autophagosomes (Green) with lysosomes (Red) causes acidification of the hybrid organelle and loss of LC3 (Green).

Movie 3: Photo pulse-labeling using PAGFP-LC3 reveals rapid turnover of starvation-induced autophagosomes.

Movie 4: LC3 (Green) is recruited to structures associated with mitochondria (Red).

Movie 5: Atg5 (Green), an early autophagosomal marker, also appears on structures associated with mitochondria (Red).

[H] Supplemental Movies and Spreadsheets

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