Supplementary Material:

Materials and Methods

Plasmid construction and site directed mutagenesis of HsAtg4A – An ORF encoding HsAtg4A (Genbank accession no. AB066214) was used to construct pQE-30-HsAtg4A as detailed previously (Scherz-Shouval et al., 2003). Three mutant genes were constructed based on the WT construct: HsAtg4A^{C77A}, in which cysteine 77 was replaced by alanine, and HsAtg4A^{C81S} and HsAtg4A^{C92S} in which the designated cysteines were replaced by serines. Mutagenesis was carried out by the ExTaq DNA polymerase (Takara). HsAtg4A as well as the various mutants were also cloned into the mammalian expression vector pcDNA4/TO/myc-His. An ORF encoding HsAtg4B, human GATE-16 or LC3 was inserted into the pEGFP-C1 vector (Clontech Laboratories) to create a fusion protein of HsAtg4B, GATE-16 or LC3 with GFP at its N-terminus. HsAtg4B^{C78S} mutagenesis was carried out by the ExTaq DNA polymerase. Cloning of GATE-16 into a pQE30 expression vector containing a haemagglutinin (HA) tag was carried out as described previously (Scherz-Shouval et al., 2003).

Antibodies and reagents - Minimal essential medium (α–MEM), Earle's balanced salt solution (EBSS), valine-free α–MEM medium and fetal calf serum (FCS) were obtained from Biological Industries (Beit Haemek Laboratories, Israel). Where indicated, cells were treated with 100 nM wortmannin, 10 mM 3-methyladenine, 100 nM Bafilomycin A1, 10 mM N-acetyl-cysteine or 1000 u/ml bovine brain catalase. Extracellularly-added catalase affects intracellular levels of H_2O_2 since it serves as a "sink". H_2O_2 can diffuse freely through the cell membrane and, therefore, once it is decomposed in the extracellular medium, intracellular levels of H_2O_2 are affected as well (Preston et al.,

2001; Sakurai and Cederbaum, 1998; Xu et al., 2003). Wortmannin, 3-methyladenine, N-acetyl-cysteine, catalase and bafilomycin A1 were provided by Sigma-Aldrich Co. L-[U-^{14}C]-Valine and [^{35}S]-Metionine were obtained from Amersham Pharmacia Biotech. 2',7'-dichlorofluorescin diacetate (DCF-DA), dihydroethidium (DHE) and MitoTracker-Red were purchased from molecular probes. The following antibodies were used: mouse monoclonal anti-γ-tubulin, anti-His (Sigma) and anti-GFP (BAbCO), rabbit polyclonal anti-Beclin1 (Sigma) and anti-hVps34 (Abgent), rhodamine-conjugated goat anti-mouse IgG (Jackson Immuno Research Laboratories), and horseradish peroxidase (HRP)-coupled goat antibody against mouse IgG (Bio-Rad). Anti-HsAtg4A antibody was produced by immunization of a rabbit with recombinant His_6-HsAtg4A. Affinity purification of this antibody was carried out as detailed below. Anti-LC3 antibody was produced by immunization of a rabbit with a peptide corresponding to the 14 amino acids of the N-terminus of LC3 with an additional cysteine (PSEKTFKQRRTFEQC).

Affinity purification of anti-HsAtg4A antibodies and cleavage-inhibition reaction - Recombinant His_6-HsAtg4A (0.5-1 mg) was run on SDS-PAGE, transferred onto nitrocellulose blot, and incubated with 1 ml of anti-HsAtg4A serum for 2 h at 4°C. After 3 washes with PBS, acidic elution (0.1 M Glycine pH 2.5) followed by basic elution (0.1 M triethylamine pH 11.5) were performed. The eluted fractions were united, dialyzed 1000 fold in PBS, tested in a Western blot of rat brain cytosol and found to recognize specifically a band corresponding to the expected size of HsAtg4A. To confirm that the cleavage defect presented in Figure 4C results from regulation of Atg4, we utilized the affinity-purified anti-HsAtg4A antibody (which recognizes specifically Atg4A and not other Atg4 homologues, data not shown). After confirming that this antibody inhibits the

cleavage activity of recombinant HsAtg4A, whereas a control antibody does not (Supplementary Figure 4B, left panel), we added the antibody to a lysate prepared from CHO cells grown in control medium. As shown in Supplementary Figure 4B (right panel), addition of anti-HsAtg4A antibody to the lysate resulted in inhibition of the cleavage of GATE-16,

Hydrogen peroxide measurements – H_2O_2 was measured using dihydroethidium (DHE, molecular probes) and 2',7'-dichlorofluorescin diacetate (DCFDA, Molecular Probes). DHE fluoresces upon DNA binding. The absorption wavelength depends on the redox state of this compound, so that interaction with ROS, which oxidizes DHE, leads to a change in fluorescence once the compound binds DNA in the nucleus or mitochondria (Vanden Hoek et al., 1997). DCFDA penetrates into cells where it is cleaved into 2',7'-dichlorofluorescin (DCF) and reacts with H_2O_2 to form fluorescent 2',7'-dichlorofluoroscein. This assay is chemically specific for hydroperoxides. Because H_2O_2 is the major peroxide in cells, it is generally accepted that DCF is proportional to H_2O_2 concentration (Cathcart et al., 1983; Vanden Hoek et al., 1997). Cells were treated with 50 μM DHE or 30 μM DCFDA for 10 min at 37°C, after which the reagent was washed out and the cells were kept in the medium at 37°C in a micro-incubator (PDMI-2, Harvard apparatus). The fluorescent signal was detected by an Olympus IX-70 confocal microscope. Images were taken 30 min after DHE washout, or 2 min after DCFDA washout (further exposures following DCFDA treatment resulted in increased cytosolic staining). Each experiment was repeated at least 3 times, representative images are shown. The DCFDA signal was also measured by a SPECTRAmax gimini fluorimeter, set to 485 excitation and 535 emission, and kept at 37°C, for 40 min. Data collected from

the fluorometric measurements was analyzed as follows: The mean of measurements obtained from replicate samples at time 0 was subtracted from the average measurement obtained at 20 min. The result of the starved cells was set to 100%, and all other treatments were normalized accordingly. The results presented for all fluorimetric measurements are the means ± s.d. of at least 3 experiments, in duplicates or triplicates.

shRNA experiments – pSuper plasmids targeted against Beclin1 (nucleotides 1339–1357 of accession number NM003766) or against hcRed (nucleotides 99–117 of accession number AF363776) as control were a kind gift from the lab of Adi Kimchi (Weizmann Institute of Science). pLKO.1-puro plasmids targeted against hVps34 or against a scrambled sequence corresponding to hVps34 were provided by Sigma.

mRNA measurements - RNA was isolated by Tri-reagent (Molecular Research Center, Inc.) from CHO or HeLa cells (for mammalian Atg8s) or *S. cerevisiae* (for ScAtg8) that were incubated in starvation medium (EBSS plus vitamins and 0.1 mM Pyruvate or SD-N, respectively) for different time periods as indicated. RNA concentration was determined by a spectrophotometer. Equal amounts of total RNA were used in a reverse-transcription reaction to produce first strand DNAs according to the manufacturer's procedure (Life Technologies). Equal volumes of the first strand DNAs were then used for quantitative-PCR with the following oligonucleotide primers, using the light cycler (Roche): GATE-16 sense GGTCAGTACATATGAAATGGATGTTCAAGGAGGACC, reverse GTATGTGAGCTCTCAGAAGCCGAAAGTGTTCTCGCC; GABARAP sense ATGAAGTTCGTGTACAAAGARGAG, reverse TCACAGACCGTAGACRCTTTCR-TC; LC3 sense ATGCCGTCSGAGAAGACCTTC, reverse TCCCGAACGTCTCCTGG-GAG, ScAtg8 sense CCGGAATTCATGAAGTCTACATTTAAGTCTGAA, reverse

CGCGGATCCCTACCTGCCAAATGTATTTTCTCC. For each gene, data obtained from non-starved cells was set to an arbitrary value of 1, and results from the corresponding starved cells were normalized accordingly.

Fractionation of cells to membrane and cytosol fractions – Cells were homogenized in a buffer containing 0.25 M Sucrose, 25 mM Tris, pH 7.5, 50 mM KCl and protease inhibitors with a Dounce homogenizer. The homogenates were centrifuged at 700 g to dispose of unbroken cells, and the supernatant was then centrifuged for 30 min at 200,000 g. The supernatant containing the cytosol was collected, and the pellet containing the membranes was resuspended in homogenization buffer and stored separately.