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Growth Factor Regulation of Autophagy and Cell Survival in the Absence of Apoptosis

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Following growth factor withdrawal, Bax$^{-/-}$ Bak$^{-/-}$ cells activate autophagy, undergo progressive atrophy, and ultimately succumb to cell death.

1

Cells from Bax$^{-/-}$ Bak$^{-/-}$ animals fail to undergo apoptosis in response to serum deprivation, loss of attachment, and growth factor withdrawal. Thus, Bax and Bak are essential and redundant regulators of apoptosis and extracellular signals.

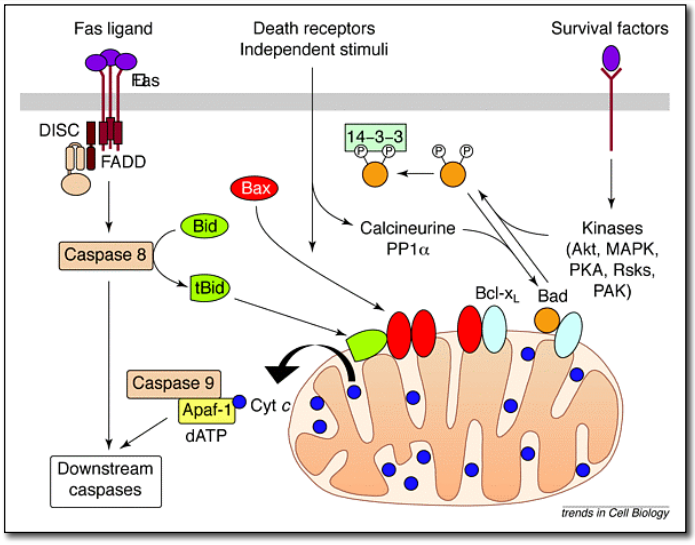

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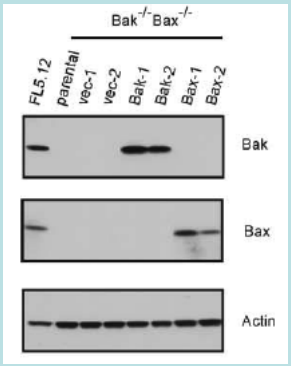
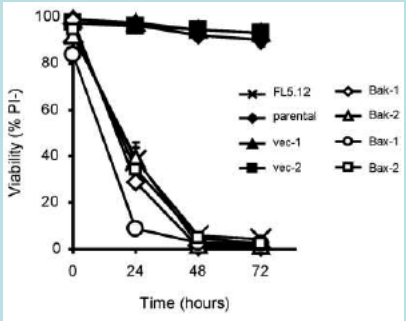
Figure 1. Bax -/- Bak -/- Cells Undergo Atrophy and Maintain Prolonged Survival Following Withdrawal of Growth Factor



(A) Two independent clones of Bax -/- Bak -/- IL-3-dependent cells (parental) stably transfected with either Bax, Bak, or empty vector (vec) were generated, and expression levels were assessed by Western blot. The IL-3-dependent Bax / Bak / cell line FL5.12 is shown for comparison.



(B) Kinetics of cell death in Bax- or Bak-reconstituted cells following IL-3 withdrawal. Viability was measured by propidium iodide exclusion. Data are averages of three experiments standard deviation (SD).

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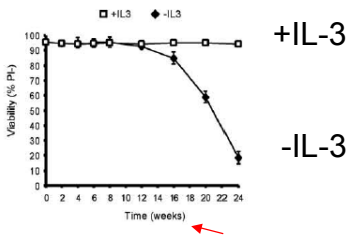
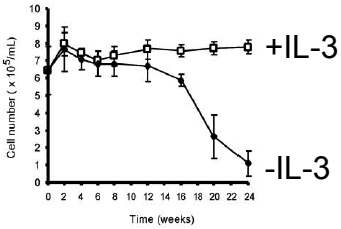
(C) **Cell viability** of Bax -/- Bak -/- cells in the presence or absence of IL-3. Cells were washed and cultured in the presence (open squares) or absence (closed diamonds) of IL-3. At the indicated time points, cells were collected and viability was assessed. Cells grown in the presence of IL-3 were passaged every 2–3 days to restore a cell concentration of 7.5 x10^5 cells/ml. The medium in IL-3-deprived cultures was replaced with an identical volume of fresh complete medium without IL-3 every 10 days. Data are averages of three independent experiments SD.



+IL-3

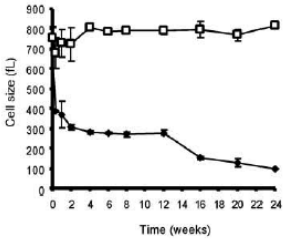
-IL-3



+IL-3

-IL-3

(D) **Cell numbers** of cultures that were grown in the presence or absence of IL-3 and were cultured as in (C). Data are averages of three independent experiments SD.



+IL-3

-IL-3

(E) **Cell size** of cultures that were grown in the presence or absence of IL-3 and were cultured as in (C). Data are averages of three independent experiments SD.

4

An additional consequence of growth factor limitation is a rapid decline in the surface expression of nutrient transporters including the major glucose transporter GLUT1, the LDL receptor, amino acid transporters and receptors for iron uptake

This decrease in nutrient transporter expression has been proposed to perturb mitochondrial physiology resulting in the induction of apoptotic cell death.

An alternative explanation is that the decline in surface expression of nutrient transporters simply reflects a secondary response to the decreased metabolic demand on the cell following the cessation of growth and the withdrawal from the cell cycle.

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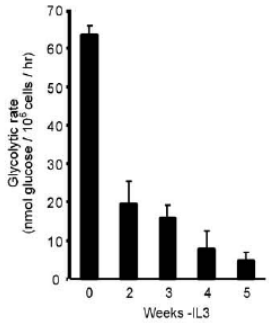
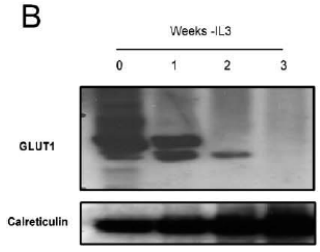
Figure 2. Metabolic Effects of IL-3 Withdrawal on Bax-/- Bak -/- Cells

(A) Glycolytic rate of cells grown in the absence of IL-3 as measured by the conversion of 5-H^3-glucose to ^3H2O at the indicated time points. The data presented at week 0 represent values of control cells growing in IL-3 throughout the time course of the experiment. Data are averages of three experiments SD.



time-dependent loss of GLUT1, the major glucose transporter expressed on these cells



(B) Western blot analysis of GLUT1 expression in cells cultured in the absence of IL-3. The GLUT1 expression at week 0 is representative of GLUT1 expression of cells grown in IL-3.

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Figure 2. Metabolic Effects of IL-3 Withdrawal on Bax-/- Bak -/- Cells

Coincident with the decline in glycolysis, there was a decline in mitochondrial membrane potential

(C) Mitochondrial membrane potential as measured by TMRE staining in cells grown without IL-3 (solid histogram) at the indicated time point. Baseline TMRE was determined by using cells treated with the uncoupler CCCP (dotted histogram). The numbers in the top right corner indicate the averge mean fluorescence intensity of three independent experiments. The week 0 time point indicates the mean fluorescence intensity of cells growing in IL-3 and is representative of the values obtained for such cells over the time course of the experiment.

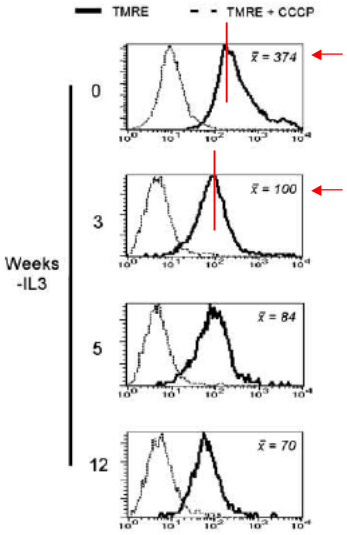

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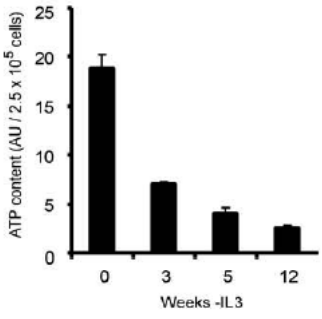
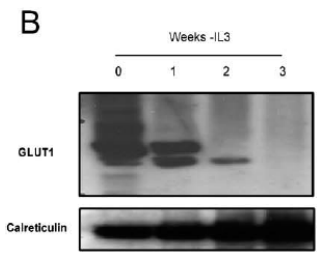
Figure 2. Metabolic Effects of IL-3 Withdrawal on Bax-/- Bak -/- Cells

(D) ATP levels in cells grown without IL-3 and expressed as arbitrary units (AU). ATP levels for IL-3-grown cells did not decline significantly over the time course of the experiment (data not shown). Data are averages of three independent experiments SD.



Cellular ATP levels also fell, but the decline in glucose transporter expression was greater than that expected based on the ATP decline, suggesting that cells were utilizing alternative substrates to maintain their bioenergetics.



8

The continued decline in cell size of the G0/G1 arrested cells following growth factor withdrawal suggested the possibility that cells were utilizing **macroautophagy to catabolize intracellular substrates to maintain their survival**.

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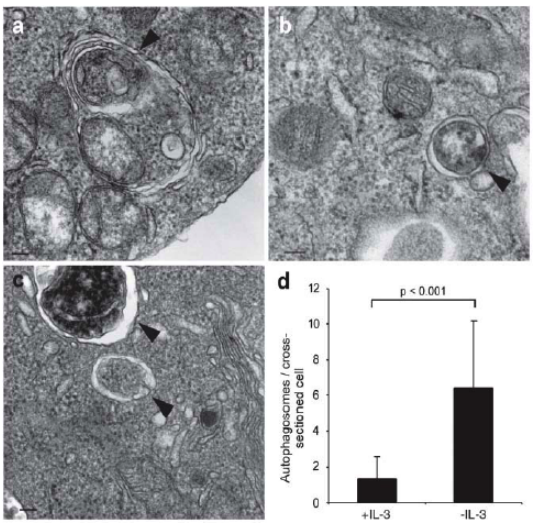
Figure 3. Growth Factor Withdrawal Induced Autophagosome Formation Is Required for Survival

(A) Electron microscopy of cells grown in the absence of IL-3 for 48 hr (a–c) showing the presence of autophagosomes. Arrowheads depict representative autophagosomes quantitated in (d). Scale bar, 100 nm. (d) Quantitation of the number of autophagosomes per cross-sectioned cell cultured in the presence or absence of IL-3 for 48 hr. Error bar represents SD. Statistical significance determined by Student's t test.



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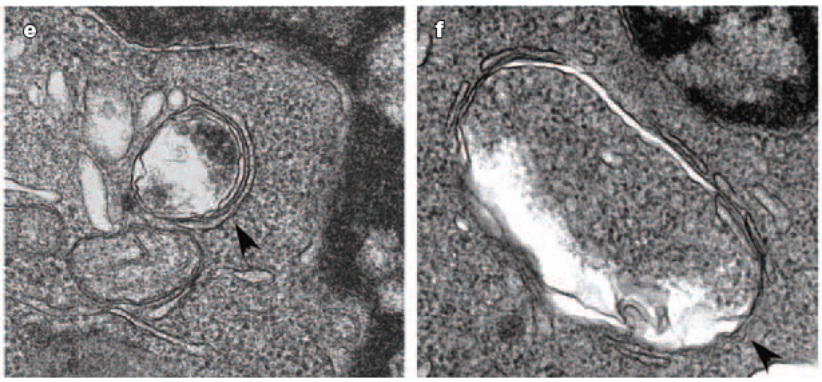
Higher-power magnification photomicrographs of IL3-dependent cells deprived of IL3 show autophagosomes that contain intracellular contents. Autophagosomes are indicated by arrowheads.



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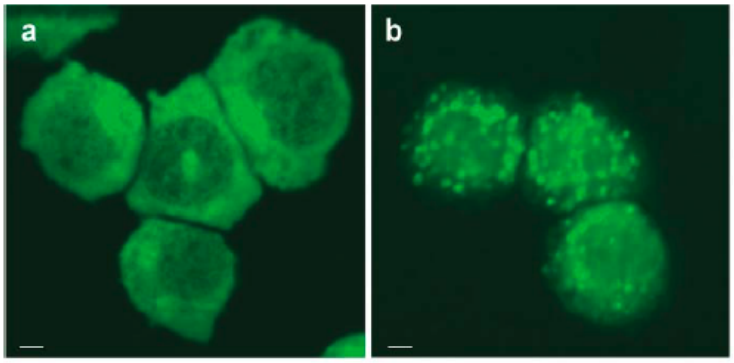
Immunofluoresence with anti-LC3* antibody on cells grown in the presence (a) or absence (b) of IL-3 for 48 hr.



(*) antibody specific for the mammalian homolog of the yeast Atg8 protein, microtubule-associated protein-1 light chain-3 (LC3).

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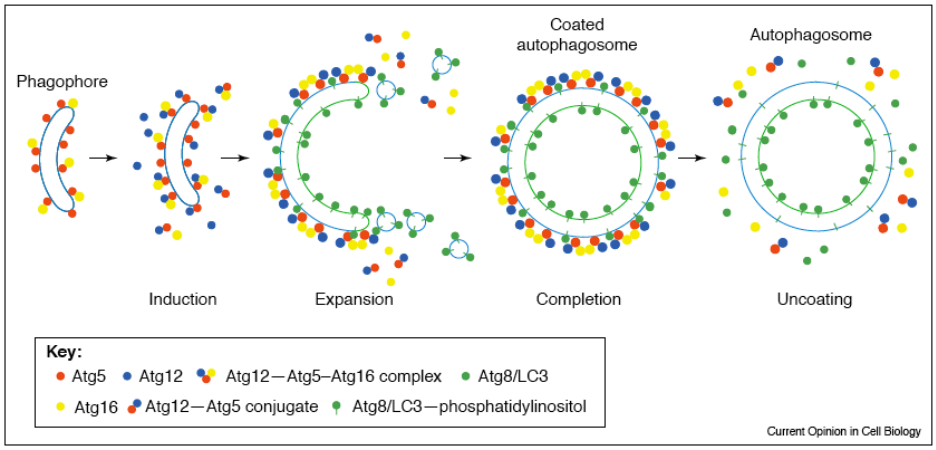
Atg: autophagocytosis associated molecules in yeast



Current Opinion in Cell Biology 2005, **17**:415–422

What hapen if autophagy is blocked?

How to block autophagy?

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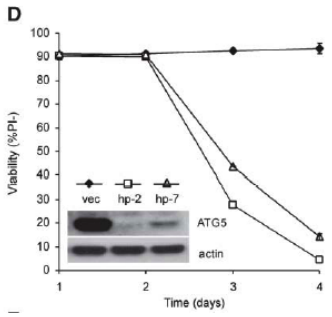
Inhibition of Autophagy Leads to Cell Death

absence of IL-3 for 48 hr

(D) Time course of cell viability following IL-3 withdrawal in cells with inactivation of ATG5. Data are averages of three experiments SD.
Western blot analysis of ATG5 protein expression in cells transfected with vector control, hp-2, or hp-7 shRNA is shown as a representative experiment. Actin was used as loading control.



(E) Time course of cell viability following IL-3 withdrawal in cells transfected with FITC tagged-siRNA for ATG7 (Yu et al., 2004) or a control siRNA. Cells which had incorporated the siRNA for ATG7 or control were purified by FACS sorting based on FITC-positive cells, and viability was assessed at the indicated time points. Data are averages of three experiments SD.

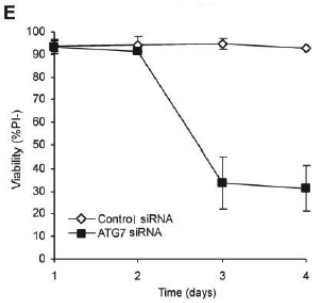
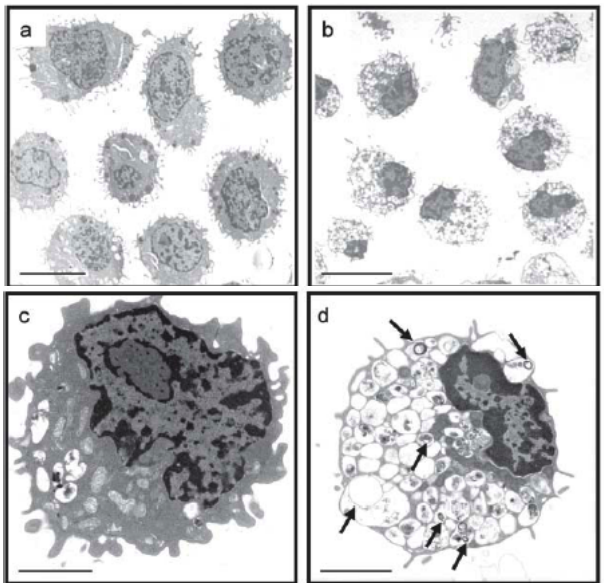


Figure 4. Persistent Autophagy in Long-Term (6 weeks) Growth Factor-Withdrawn Cells

IL-3



-IL-3

(A) Electron microscopy of cells grown in the presence (a) or absence (b) of IL-3 for 6 weeks. Scale bar, 8.5 m. Magnification image of a cell grown in the presence (c) or absence (d) of IL-3 showing autophagosomes (arrows). Scale bar, 2.3 um.

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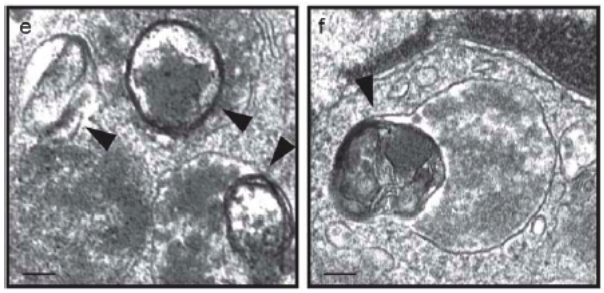
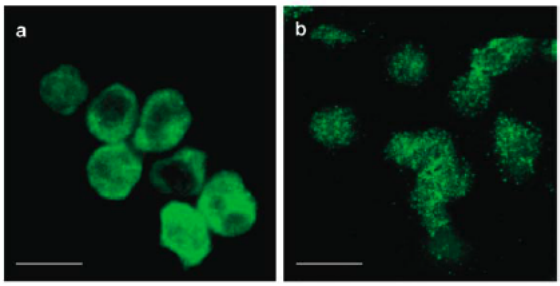
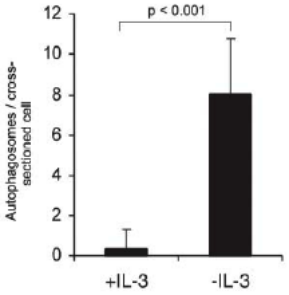
Higher magnification of cells grown in the absence of IL-3 (e and f). Arrowheads depict autophagosomes in cells containing recognizable cellular material (e) or a late autophagosome fusing with a lysosome (f). Arrowheads depict representative autophagosomes quantitated in (B).

Long term deprivation (6 weeks)







While macroautophagy in yeast and plant cells is required to promote cell survival in the absence of nutrients, the macroautophagy observed following IL-3 deprivation occurred in the presence of abundant extracellular nutrients.

The IL-3-deprived cells were maintained in complete RPMI medium supplemented with 10% serum, and the medium was replaced every 10 days. The medium removed from these cultures was not nutrient deficient since it supported proliferative expansion of the parental $Bax^{-/-}$ $Bak^{-/-}$ cells when supplemented with IL-3 (data not shown). Therefore, macroautophagy in $Bax^{-/-}$ $Bak^{-/-}$ cells was induced by growth factor withdrawal and not by a lack of nutrients in the extracellular environment.

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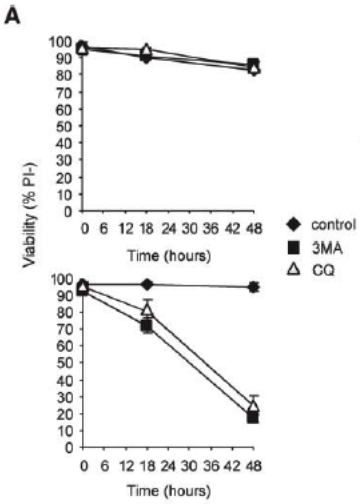
Figure 5. Cell Death Following Inhibition of Autophagy

(A) Viability of cells grown in the presence (top panel) or absence (bottom panel) of IL-3 for 6 weeks treated with 5 mM 3-MA (closed squares) or 10 M CQ (open triangles). PBS was used as a vehicle control (closed diamonds).



Existing shRNA transfection methods proved ineffective in cells that had undergone prolonged growth factor withdrawal, therefore we used two independent and widely used inhibitors of macroautophagy, 3-methyladenine (3-MA) and chloroquine (CQ) to block autophagy.

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How to characterize cell death by genomic DNA analysis?

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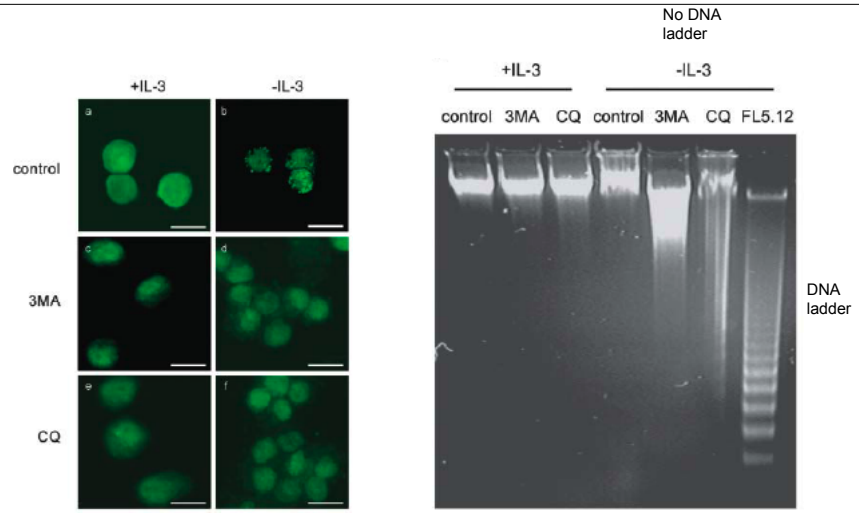

(B) Immunofluorescence staining of LC3 in cells grown in the presence (a) or absence (b) of IL-3 for 6 weeks. Cells grown in the presence or absence of IL-3 were treated for 18 hr with 5 mM 3-MA (c and d) or 10 M CQ (e and f) followed by LC3 staining. PBS was used as a vehicle control.

(C) DNA fragmentation assay was performed on Bax / Bak / cells grown in the presence or absence of IL-3 for 6 weeks and treated for 36 hr with 5 mM 3-MA, 10 M CQ, or PBS as a vehicle control. IL-3-dependent Bax -/- Bak -/- FL5.12 cells grown in the absence of IL-3 for 36 hr were used as a positive control for DNA laddering.

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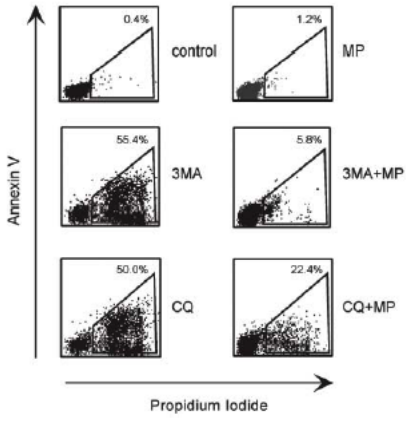
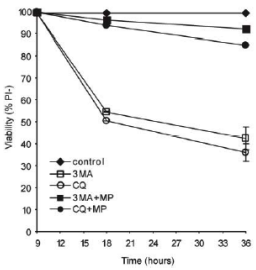
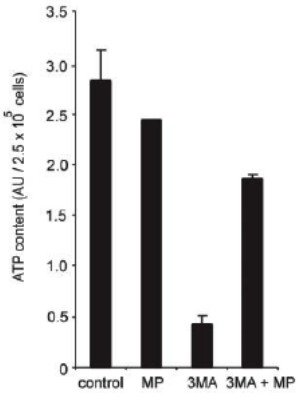
Cell Death Following Inhibition of Autophagy Is Reversed by Methylpyruvate



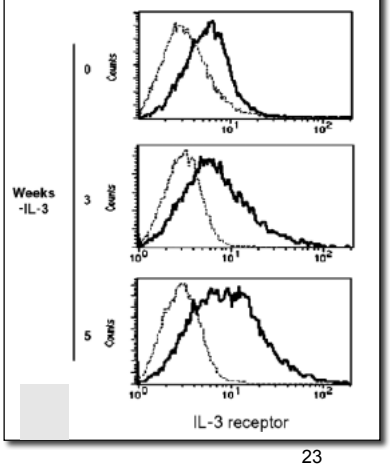




A cell-permeable form of pyruvate, methylpyruvate (MP), was added to the cultures at the time 3-MA or CQ treatment. Once internalized, this substrate can be oxidized in the tricarboxylic acid cycle to produce NADH to fuel electron transport and ATP production.

Cells cultured in the absence of IL-3 had higher levels of surface IL-3 receptor than cells grown in the presence of IL-3.

Cell surface staining of IL-3 receptor chain. Dotted histogram represents isotype control and solid histogram represents IL-3 receptor expression.



Despite the loss of cell surface nutrient transporters, the absence of an observable Golgi/ER, and a profound decline in total protein content, the cells cultured in the absence of IL-3 had higher levels of surface IL-3 receptor than cells grown in the presence of IL-3.

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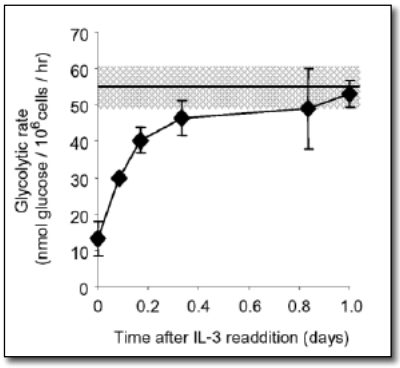
IL-3 Restimulates Glycolysis and Growth/Proliferation in Growth Factor-Deprived Cells

Glycolytic rate of cells following readdition of IL-3.

IL-3 was readded to cells that were cultured in the absence of IL-3 for 4 weeks and collected at the indicated time points for measurement of glycolytic rate. Solid line indicates average glycolytic rate of cells grown in the presence of IL-3 over the time course of the experiment.



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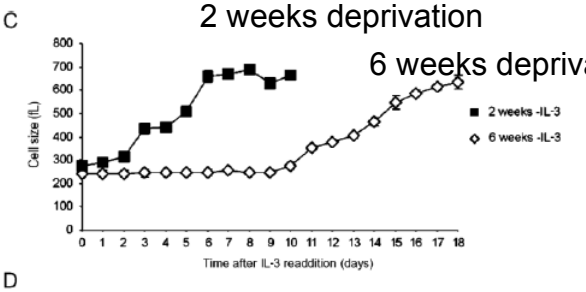
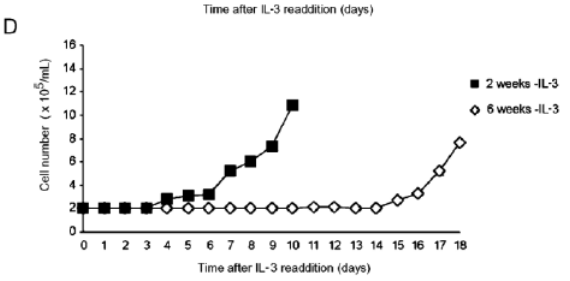
Cell size and cell number of cultures cultured without IL-3 for 2 (closed squares) or 6 (open triangles) weeks followed by readdition of IL-3.

Cell size



Cell number



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Macroautophagy Is a Conserved but Self-Limited Survival Mechanism

Based on the results, macroautophagy appears to be an evolutionarily conserved survival strategy. Macroautophagy can support growth factor-independent cell survival of hematopoietic cells for several weeks.

Thus, it appears eukaryotic cells share a common survival pathway that promotes cell-autonomous survival in the face of starvation and/or neglect. Animal cells may have evolved an apoptotic response in part to limit this form of cell-autonomous survival. Nevertheless, as previously demonstrated in unicellular organisms, macroautophagy is a self limited survival strategy and ultimately will result in cell death if not reversed.

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Cell Death and Differentiation (2009) 16, 3–11

Classification of cell death: recommendations of the Nomenclature Committee on Cell Death 2009

G Kroemer*,[1,2,3], L Galluzzi[1,2,3], P Vandenabeele[4,5], J Abrams[6], ES Alnemri[7], EH Baehrecke[8], MV Blagosklonny[9], WS El-Deiry[10], P Golstein[11,12,13], DR Green[14], M Hengartner[15], RA Knight[16], S Kumar[17], SA Lipton[18,19,20], W Malorni[21], G Nuñez[22], ME Peter[23], J Tschopp[24], J Yuan[25], M Piacentini[26,27], B Zhivotovsky[28] and G Melino[29,30]

Different types of cell death are often defined by morphological criteria, without a clear reference to precise biochemical mechanisms. The Nomenclature Committee on Cell Death (NCCD) proposes unified criteria for the definition of cell death and of its different morphologies, while formulating several caveats against the misuse of words and concepts that slow down progress in the area of cell death research. Authors, reviewers and editors of scientific periodicals are invited to abandon expressions like 'percentage apoptosis' and to replace them with more accurate descriptions of the biochemical and cellular parameters that are actually measured. Moreover, at the present stage, it should be accepted that caspase-independent mechanisms can cooperate with (or substitute for) caspases in the execution of lethal signaling pathways and that 'autophagic cell death' is a type of cell death occurring together with (but not necessarily by) autophagic vacuolization. This study details the 2009 recommendations of the NCCD on the use of cell death-related terminology including 'entosis', 'mitotic catastrophe', 'necrosis', 'necroptosis' and 'pyroptosis'.

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Table 2 Distinct modalities of cell death

Cell death mode	Morphological features	Notes
Apoptosis	Rounding-up of the cell Retraction of pseudopodes Reduction of cellular and nuclear volume (pyknosis) Nuclear fragmentation (karyorrhexis) Minor modification of cytoplasmic organelles Plasma membrane blebbing Engulfment by resident phagocytes, *in vivo*	'Apoptosis' is the original term introduced by Kerr *et al.*[14] to define a type of cell death with specific morphological features. Apoptosis is NOT a synonym of programmed cell death or caspase activation.
Autophagy	Lack of chromatin condensation Massive vacuolization of the cytoplasm Accumulation of (double-membraned) autophagic vacuoles Little or no uptake by phagocytic cells, *in vivo*	'Autophagic cell death' defines cell death occurring with autophagy, though it may misleadingly suggest a form of death occurring by autophagy as this process often promotes cell survival.[15,16]
Cornification	Elimination of cytosolic organelles Modifications of plasma membrane Accumulation of lipids in F and L granules Extrusion of lipids in the extracellular space Desquamation (loss of corneocytes) by protease activation	'Cornified envelope' formation or 'keratinization' is specific of the skin to create a barrier function. Although apoptosis can be induced by injury in the basal epidermal layer (e.g., UV irradiation), cornification is exclusive of the upper layers (granular layer and stratum corneum).[17,18]
Necrosis	Cytoplasmic swelling (oncosis) Rupture of plasma membrane Swelling of cytoplasmic organelles Moderate chromatin condensation	'Necrosis' identifies, in a negative fashion, cell death lacking the features of apoptosis or autophagy.[4] Note that necrosis can occur in a regulated fashion, involving a precise sequence of signals.

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Science. 2004 Nov 5;306, 990-995

Autophagy in Health and Disease: A Double-Edged Sword

Takahiro Shintani and Daniel J. Klionsky*

Autophagy, the process by which cells recycle cytoplasm and dispose of excess or defective organelles, has entered the research spotlight largely owing to the discovery of the protein components that drive this process. Identifying the autophagy genes in yeast and finding orthologs in other organisms reveals the conservation of the mechanism of autophagy in eukaryotes and allows the use of molecular genetics and biology in different model systems to study this process. By mostly morphological studies, autophagy has been linked to disease processes. Whether autophagy protects from or causes disease is unclear. Here, we summarize current knowledge about the role of autophagy in disease and health.

Table 1. Possible roles of autophagy in health and disease.

Disease state	Beneficial effects of autophagy	Negative effects of autophagy
Cancer	Acts as a tumor suppressor; may be involved in type II PCD in cancer cells, could limit cell size or may remove damaged organelles that could generate free radicals and increase mutations.	May allow survival of cancer cells within the nutrient-poor environment of a tumor, could prevent cell death, and may protect against some cancer treatments.
Liver disease	Allows removal of nonfunctional endoplasmic reticulum resulting from accumulation of aggregated α_1-antitrypsin Z protein.	Increased mortality due to excessive mitochondrial autophagy.
Muscular disorder	Increased autophagy may compensate for defects in lysosome function.	Increased autophagy or defects in completing autophagy result in the accumulation of autophagosomes that may impair cell function.
Neurodegeneration	Allows the removal of protein aggregates before they become toxic.	May induce cell death in neurons that accumulate aggregated proteins.
Pathogen infection	Cellular defense against invasion by bacteria and viruses.	Subversion of the autophagic pathway allows pathogens to establish a replicative niche and supplies nutrients for growth.

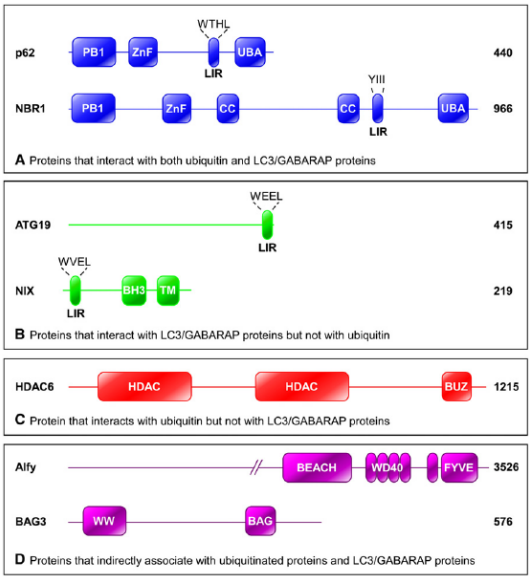
Figure 1. Proteins Involved in Selective Autophagy and Their Domain Organization.

(A) p62 and NBR1 are autophagy receptors that interact with both ubiquitin conjugated to the target and LC3/GABARAP on the autophagosome, thereby promoting autophagy of ubiquitinated targets.

(B) ATG19 is a receptor protein that interacts with both preApe1 aggregates and ATG8, thereby promoting delivery of preApe1 to the vacuole by the Cvt pathway in yeast. NIX is a mitochondrial protein that interacts with LC3/GABARAP and might participate in mitophagy.

(C) HDAC6 is a Ub-binding protein that plays a role in aggresome formation but also affects selective autophagy of ubiquitinated misfolded proteins; its precise role in autophagy is, however, not clear.

(D) ALFY is a large protein associated with both ubiquitinated proteins and autophagosomal markers; it interacts with PtdIns(3)P via the FYVE domain.



Numbers indicate length of human proteins in amino acids with the exception of ATG19, which is a yeast protein. BAG, Bcl-2-associated athanogene 1 domain; BEACH, BEACH domain; BH3, Bcl-2 homology 3 domain; BUZ, ubiquitin-binding zinc finger; CC, coiled-coil domain; FYVE, Fab1, YOTB/ZK632.12, Vac1, and EEA1 domain; HDAC, histone deacetylase domain; LIR, LC3-interacting region; PB1, Phox and Bem1p domain; TM, transmembrane domain; UBA, Ub-associated domain; WD40, WD40 repeats; WW, WW domain; ZnF, Zinc finger domain.

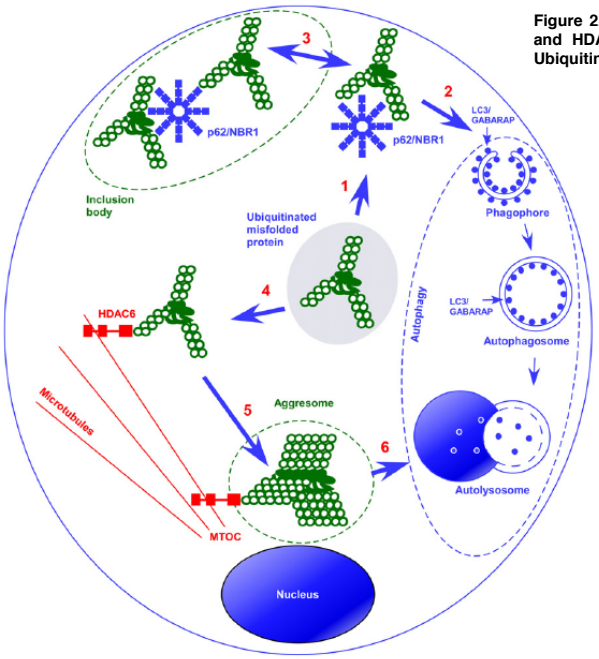


Figure 2. A Model for the Function of p62, NBR1, and HDAC6 Proteins in Selective Autophagy of Ubiquitinated Misfolded Proteins

Oligomerized misfolded proteins are ubiquitinated and recognized by the Ub-binding domain of oligomeric p62 and NBR1 proteins, drawn as spokes of a wheel (although polyUb chains are depicted, it is possible that monoubiquitination is sufficient for target recognition) (1), which target them for selective degradation by autophagy (2). Oligomeric p62 and NBR1 also mediate formation of proteinacious inclusion bodies (3). Binding of HDAC6 to ubiquitinated proteins ensures their transport along the microtubules toward the MTOC (4), where excess misfolded proteins can be organized into an aggresome (5). Inclusion bodies (3) and aggresomes (6) may allow autophagic degradation of stored misfolded proteins. Closed boxes, Ub-binding domains; empty circles, Ub; filled circles, conjugated LC3/ GABARAP proteins.

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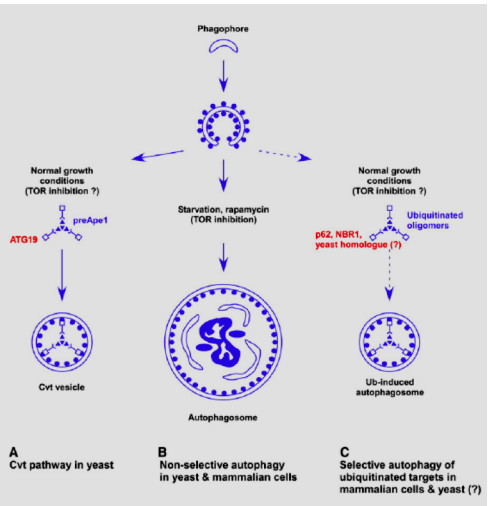
Figure 3. Comparison of the Yeast-Specific Cytosole-to-Vacuole (Cvt) Pathway with the Process of Nonselective Autophagy and the Selective Autophagy of Ubiquitinated Targets Mediated by p62 and NBR1



(A) Constitutive formation of smaller autophagosomes (Cvt vesicles) mediates delivery of cytosolic precursors of resident vacuolar hydrolyses, such as preApe1, and represents a form of selective autophagy in yeast cells. ATG19 interacts with both aggregated preApe1 and ATG8 on the autophagosome (filled circles).

(B) In contrast, larger autophagosomes are formed in yeast during starvation response, a nonselective process regulated by TOR kinase signaling.

(C) Similar to the Cvt pathway, ubiquitinated aggregates are recognized by specific receptors (like p62 and NBR1) and targeted to autophagosomes via their interaction with LC3/GABARAP proteins conjugated to the lipid membrane of the autophagosome (filled circles).

The role of TOR signaling in selective autophagy has not been clarified. The broken arrows indicate the fact that this process has not formally been in yeast cells—it, therefore, remains hypothetic.

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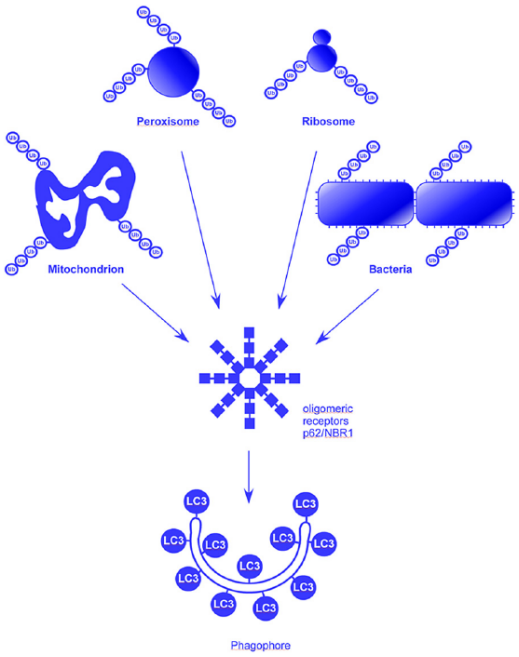

Figure 4. Proposed Role of Ubiquitination and Autophagy Receptors in Mitophagy, Pexophagy, Ribophagy and Xenophagy

Conjugation of monoUb (data not shown) or Ub chains (depicted) to the ribosomal proteins or those residing in the limiting membranes of organelles, such as mitochondria and peroxisomes, may constitute a signal that directs targeted autophagosome formation. Intracellular bacteria are also associated with Ub; however, the nature of this association is less clear. p62 and NBR1 form oligomers and bind both Ub and ATG8/LC3 on the phagophore and, thereby, might mechanistically link ubiquitination to selective autophagy.

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