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News and Commentary

Macroautophagy *versus* mitochondrial autophagy: a question of fate?

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Autophagy is a catabolic process that allows recycling of cytoplasmic components (including organelles) into basic components, offering a bioenergetically efficient alternative to *de novo* synthesis. In yeast, autophagy functions primarily as an adaptive mechanism allowing survival in the response to changes in the availability of nutrients in the environment. Over the past decade, genetic screens performed in yeast have elucidated many of the molecular components involved in this adaptive response.[1–3] More recently, the roles of homologous genes are being explored in multicellular organisms and in cells derived from these organisms.[4] These studies have demonstrated that while autophagy is often induced as part of an adaptive response, induction of autophagy may also lead to cell death. During *Drosophila* larval development, for example, starvation-induced autophagy occurs in the larval fat body and is required for maintaining circulating nutrient levels and survival of the larvae under unfavorable nutritional conditions;[5] by contrast, programmed autophagy results in cell death and is the primary means of removing certain larval organs during metamorphosis.[6] On a cellular level, autophagy is observed prior to apoptosis in growth factor-deprived neuronal cultures.[7,8] In cells lacking critical proapoptotic proteins, the adaptive process of autophagy aimed at maintaining bioenergetic homeostasis can be unmasked in response to growth-factor withdrawal.[9] In contrast, cell death in response to a variety of chemicals has been suggested to result from autophagic destruction of the cell and to be suppressed by inhibition of autophagy.[10] The factors that determine whether induction of autophagy contributes to cell survival or cell death are not well-elucidated; however, two key issues may be the rate of autophagic degradation and the specificity of the engulfed components. Although autophagy is generally considered a nonspecific process, there are instances where the mitochondria (and other organelles) appear to be specifically targeted. Here, we focus on the signals and pathways involved in regulating the induction of autophagy and mitochondrial autophagy in yeast and how these processes are relevant to survival and death of mammalian cells.

In general terms, 'autophagy' refers to any intracellular process that involves the degradation of cytosolic components by the lysosome. There are at least three distinct autophagic pathways: macroautophagy, microautophagy and chaperone-mediated autophagy. Macroautophagy is a multistep process by which portions of cytoplasm and/or organelles are sequestered in a double or multimembrane structure ('autophagosome') and delivered to the lysosome for degradation (Figure 1). Upon fusion of the autophagosome with the lysosome, it becomes an autophagolysosome or autophagic vacuole. Although macroautophagy is generally considered a nonspecific process there are instances in which organelles, such as mitochondria and peroxisomes, appear to be preferentially sequestered. These processes have been termed mitophagy, and pexophagy, respectively (Figure 1). In yeast, the cytoplasm to vacuole targeting (cvt) pathway functions to deliver specific proteases to the vacuole and may be considered a selective form of autophagy (Figure 1). Microautophagy is similar to macroautophagy, but involves sequestration of cytosolic components directly by the lysosomal membrane. Chaperone-mediated autophagy involves translocation of targeted proteins directly into the lysosome via a chaperone, and has only been described in mammalian cells. For simplicity, as the focus of this review is macroautophagy, the terms autophagy and macroautophagy will be used interchangeably.

Macroautophagy in yeast is a multistep process that is regulated by the Tor signaling pathway

The molecular pathways regulating autophagy have been best characterized in yeast, in which macroautophagy is induced in response to starvation. In yeast cells, nutrient deprivation results in a suppression of protein synthesis to limit the expenditure of energy and cellular resources, and a simultaneous increase in the regeneration of amino acids and other small metabolites through autophagy. This starvation response is coordinated by the Tor kinases, Tor1p and Tor2p.[11] Under nutrient-rich conditions, the Tor kinases are maintained in an active state, while under starvation conditions the Tor kinases become inactive. Tor2 inhibits autophagy, at least in part, by regulating the interaction between Atg13p and Atg1p.[12] Under vegetative conditions, Atg13 is hyperphosphorylated in a Tor-dependent manner, and exhibits a reduced affinity for Atg1p. Under starvation conditions, Atg13p becomes hypophosphorylated and is able to form a complex with Atg1p, which alters Atg1p kinase activity and is critical for the induction of autophagy[13] (Figure 2). In addition to induction of autophagy by Atg13p

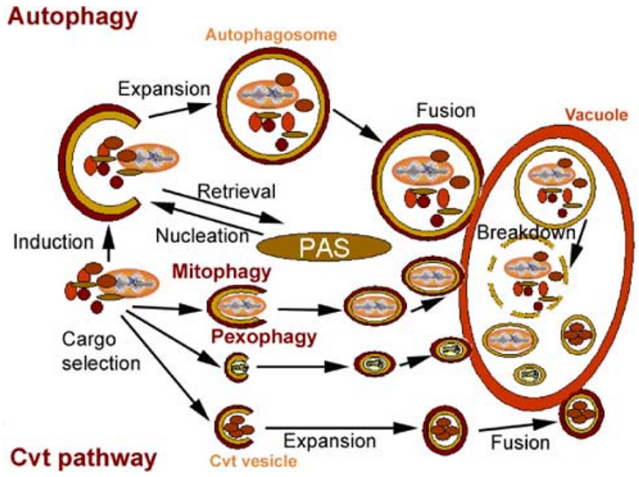

Figure 1 Lysosome-centric view of a cell demonstrating the steps involved in macroautophagy, mitophagy, pexophagy and the Cvt pathway. The steps involved in autophagy include the induction of autophagy, nucleation (at the PAS) and formation of the isolation membrane, expansion and completion of the autophagosome, fusion of the outer vesicle membrane with the lysosome and finally, breakdown of the inner autophagosome membrane and degradation of engulfed contents. Some of the differences between cvt and autophagy include the size of the vacuole and specificity (cargo selection) of the engulfed contents

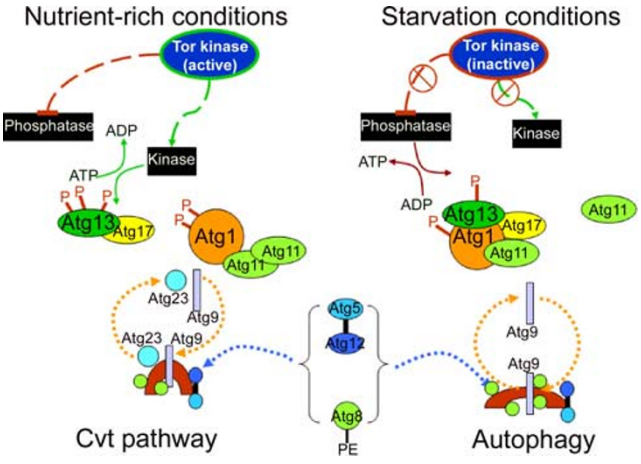

Figure 2 Role of Tor kinase and Atg1 in cvt and autophagy pathways. The activity of the Tor kinases are dependent on the availability of nutrients in the environment. Atg13 is directly or indirectly (black boxes) regulated by Tor. Regulation of Atg1 activity through its interaction with Atg13 mediates the switch between the cvt pathway and autophagy. Some of the other events required for formation of autophagosomes and cvt vesicles include the covalent attachment of Atg12 to Atg5, a process that requires the function of Atg7 and Atg10 (not shown); linkage of pairs of Atg5-Atg12 conjugates by interaction with Atg16 dimers (not shown), which is either required for the formation or completion of the sequestering vesicle; and conjugation of Atg8 to phosphatidylethanolamine (PE), which promotes membrane interaction and sequestration inside the vesicle along with the cargo. Atg11 and Atg23 are required primarily for the cvt pathway

and Atg1p, additional steps in autophagy include vesicle nucleation, vesicle expansion, membrane retrieval, vesicle completion and docking/fusion of the completed vesicle with the vacuole (Figure 1, reviewed in Klionsky[14]). Vesicle nucleation involves assembly of proteins at the preautophagosomal structure (PAS) and is dependent on the yeast Class III PI3-kinase, Vps34, as well as Vps15, Atg14 and Atg6 (homologue of mammalian Beclin1).[15,16] Vesicle expansion and completion involve conjugation of Atg5 and Atg12 and conjugation of Atg8 to phosphatidylethanolamine (Figure 2) (reviewed in Ohsumi and Mizushima[17]). Atg8 and Atg12 are both activated for conjugation by Atg7, an E1-like enzyme of the ubiquitin system.[18,19] Atg9 is the only currently characterized transmembrane protein involved in the formation of double-membrane vesicles, and is thought to mark membranes to be donated to the PAS.[20] Atg1 regulates the shuttling of Atg9 between the PAS and a peripheral pool and in this way is thought to control retrieval of Atg9 and the associated membrane[20] (Figure 2).

Atg1 plays a critical role in yeast in switching between the cvt pathway and autophagy

Under vegetative conditions, core components of the autophagic machinery are involved in the cvt pathway, which is a mechanism for targeting specific cytosolic proteins, such as the vacuolar protease aminopeptidase I (ApeI) and α-mannosidase, to the yeast vacuole.[21,22] The process is similar to autophagy in that it involves sequestration in double membrane vesicles (cvt vesicles) that fuse with the yeast vacuole; but differs from autophagy in the specificity of the engulfed material and the size of the vesicles [23,24](Figure 1). Although there are proteins that are specific to one pathway or the other, many components of the cvt and autophagy pathways are shared. Atg1p is one of the proteins that are required for both processes, but is unique in that through modulation of its kinase activity and its association with Atg13p, Atg1p functions as a molecular switch between the cvt and autophagy pathways (Figure 2). Although the mechanism by which Atg1p regulates the switch are not fully elucidated, there is circumferential evidence to suggest that Atg1 may be involved in regulating both the size and composition of the vesicles during cvt and autophagy. First of all, Atg1 interacts with Atg11, a protein that is required for cvt (but not autophagy) and is involved in the specific packaging of cargo through its interaction with Atg19 and Atg8.[25,26] It is possible that the interaction of Atg13p and Atg1p diminishes the role of Atg11 by excluding it from the complex or inhibiting the formation of Atg11 homo-dimers or -oligomers. Atg13 and Atg1 also form a complex with Atg17, which is involved in regulating the size of the vesicles during autophagy.[27,28] Another way that Atg1p differentially regulates the cvt and autophagy pathways is through the recycling of Atg9p and/or Atg23p (a peripheral membrane protein) depending on the kinase activity of Atg1p[20]. Similar to Atg11, Atg23 is essential for cvt.[29] The regulation of Atg1 by Tor and some of the differences between the complexes formed during autophagy and cvt are depicted in Figure 2. Although there is no direct mammalian correlate of the cvt pathway, the observation that the mammalian and *Caenorhabdhitis elegans* Atg1 homologues may be involved in the trafficking of neuronal receptors during axonal growth suggests that this

primitive pathway may have been adapted for cell-type-specific functions in multicellular organisms.[30–32]

Autophagy is essential for degradation of peroxisomes in yeast

In addition to degradation of bulk cytoplasm for recycling of metabolic components during starvation, autophagy is involved in degradation of organelles that become unnecessary during a shift in environmental conditions. In methylotropic yeast, such as *H. polymorpha*, peroxisomes proliferate when methanol is used as the sole source of carbon and energy. When these methanol-grown cells are transferred into media supplemented with glucose or ethanol, the peroxisomes disappear rapidly by a process called macropexophagy, which is similar to macroautophagy (reviewed in Leao and Keil[33]). During macropexoaphagy, peroxisomes are individually and specifically sequestered in a multimembrane vesicle and delivered to the vacuole for degradation. In *S. cerevisiae*, growth on a media containing fatty acids, such as oleate, stimulates peroxisome biogenesis. When the cells are then shifted into a media in which the peroxisomes become redundant for growth, the organelles are specifically degraded.[34] A number of genes involved in autophagy and/or the cvt pathway, including Atg1, Atg11 and Atg17, have been implicated in regulation of pexophagy in *H. polymorpha* and in *Saccharomyces cerevisiae*, suggesting that there is overlap between the three pathways.[28,33–35] In addition to activation of the autophagy machinery, a key determinant of pexophagy appears to be localized on the peroxisome membrane. Pex14, which is conserved from yeast to humans, has been shown to be a prime target for macropexophagy in *H. polymorpha*.[36] As a component of the receptor docking site at the peroxisomal membrane, in small import-competent peroxisomes active pex14 is involved in peroxisome biogenesis and in peroxisomal matrix protein import; while in large mature peroxisomes, pex14 is inactive and is well-positioned to be targeted by the autophagic machinery under appropriate conditions.

Autophagy is involved in degradation of mitochondria in yeast

While the role of autophagy in specific degradation of peroxisomes has been well documented, its role in the degradation of mitochondria has only recently emerged. In yeast, the mitochondria exist in autophagosomes following induction of autophagy by nitrogen starvation,[37] but until recently, it was unclear whether their presence in autophagosomes was reflective of a specific or a nonspecific process. The identification of a mitochondrial protein, Uth1p, which is required for mitochondrial autophagy (mitophagy) but is not required for macroautophagy, provides the first indication that the mitochondria can be specifically targeted for degradation by autophagy.[38] Both macroautophagy and mitophagy are induced in wild-type yeast strains in response to nitrogen starvation or rapamycin treatment, and the latter is more pronounced when cells are grown under aerobic conditions on a nonfermentable carbon source and mitochondrial oxidative phosphorylation is required for ATP production. ΔUth1 yeast strains exhibit the normal autophagic response to nitrogen starvation or rapamycin treatment, but are defective in mitophagy, a phenotype that allows them to survive rapamycin treatment on media containing a nonfermentable carbon source.[38] Yeast strains with deletions in autophagy genes, such as *Atg5* and *Atg7*, also exhibit defects in mitophagy,[38] confirming that the autophagic machinery is involved in mitochondrial degradation. Further evidence that the signal for mitophagy can originate from the mitochondria itself comes from the observation that yeast mutants unable to maintain an electrical potential across the inner mitochondrial membrane exhibit an increase in mitophagy compared to wild-type cells.[39] There is preferential degradation of the damaged mitochondria, which is inhibited by mutations in Atg5.[39] An independent study has confirmed that several genes involved in macroautophagy, including Atg1, play a role in the normal turnover of mitochondria, and that defects in autophagy lead to compromised mitochondrial function and accumulation of mutations in nuclear DNA (Shengkan Jin, personal communication). Together, these data suggest that autophagy is involved in mitochondrial degradation during both vegetative (with degradation of individual damaged mitochondria) and starvation conditions (with bulk degradation of mitochondria). The difference in the rate of mitochondrial degradation appears to be a function of changes in the autophagy/cvt machinery and signals originating from the mitochondria. Since changes in the phosphorylation status and activity of Atg1 and Atg13 regulate the switch between the cvt and autophagy pathways, and may be involved in regulating the size of the vacuoles and specificity of the engulfed material, the role of Atg1 kinase activity in mitochondrial clearance during stress will be important to determine. Additional insight into mitophagy may be gained by a detailed comparison of factors involved in mitophagy with those regulating macropexophagy.

Autophagy is involved in mitochondrial degradation in mammalian cells

A requirement for multicellular organisms is that growth occurs within the constraints of tissue and organismal homoestasis – therefore, unlike in unicellular organisms (such as yeast) where growth is primarily dependent on nutrient availability, proliferation of mammalian cells is also dependent on extrinsic growth factors. Insulin and glucagon are among the factors that regulate the metabolism of fat and glucose in the liver and changes in the circulating concentrations of these hormones signal fed or starvation states and elicit counter-regulatory responses that maintain normoglycemia.[40] Morphologic studies have demonstrated that starvation and glucagon can induce an autophagic response in hepatocytes that includes degradation of the mitochondria, presumably aimed at increasing the intracellular availability of amino acids.[41] Genetic evidence that autophagy is involved in organelle degradation comes from a recent study characterizing the phenotype of an Atg7 conditional knockout mouse model.[42] Atg7 deletion in hepatocytes results in a dramatic decrease in autophagosome-like structures in fed and fasted mutant mice livers compared to controls. The few

autophagosomes that are visualized tend to be smaller than those observed in fasted control liver and do not contain large cytoplasmic organelles. Unlike control animals, in which fasting is associated with a significant decrease in the activity of the mitochondrial enzyme succinate dehydrogenase (SDH) and in the amount of the mitochondrial protein, cytochrome *c* (indicative of mitochondrial degradation), fasting is not associated with any change in SDH activity or cytochrome *c* levels in the Atg7$^{-/-}$ livers. The defect in autophagy is associated with an accumulation of peroxisomes and deformed mitochondria in Atg7-deficient hepatocytes. These results suggest that autophagy is involved in turnover of organelles in mammalian cells. However, the specific autophagy genes (other than Atg7) involved in the pathway remain to be elucidated.

Factors that increase mitochondrial permeability promote degradation of mitochondria by autophagy

In yeast, the characterization of the *Uth1* gene and the observation that loss of the mitochondrial membrane potential can promote autophagy provide evidence that mitochondrial proteins may provide signals targeting the organelle for degradation by autophagy.[38,39] In mammalian cells, although specific molecular targets have not been identified, confocal imaging studies have demonstrated that mitochondrial depolarization precedes mitochondrial autophagy in cultured hepatocytes (in response to serum starvation and glucagon treatment) and that inhibition of the mitochondrial permeability transition prevents mitochondrial autophagy.[41] These results suggest that factors increasing mitochondrial permeability may also promote mitophagy in mammalian cells. Since apoptosis is associated with an increase in mitochondrial permeability, it is not entirely surprising that mitophagy is observed in cells that subsequently die by apoptosis in several mammalian cell systems.[7,8,43] Sympathetic neurons die within a few days after nerve growth factor (NGF) withdrawal and the cell death is characterized by an early increase in autophagosomes containing the mitochondria and other cytoplasmic components, followed by degradation of organelles within the autophagosome.[7] The classical features of apoptosis appear at later time points. Inhibition of autophagy using 3-methyladenine (3-MA, an inhibitor of Class III PI3 kinase) also blocks apoptosis with overlapping dose–response curves, suggesting that there may be a common signaling pathway that activates both autophagy and apoptosis.[7] Furthermore, treatment of NGF-deprived neurons with the pan-caspase inhibitor, Boc-Asp-(OMe)-fluoromethylketone (BAF), prevents apoptosis, but the mitochondria selectively disappear by autophagy within three days and the cells die shortly thereafter.[8] Similarly, in Hela cells treated with BAF for the duration of the culture, a 9-h incubation with staurosporin results in complete mitochondrial loss via autophagy within 3 days after removal of staurosporine.[8] These results demonstrate that apoptotic stimuli target mitochondria for degradation by autophagy – a feature of apoptosis that may not be readily appreciated due to the rapid cell death that follows initiation of the caspase cascade.

Inhibition of apoptosis upstream of the mitochondria prevents mitochondrial autophagy

In contrast to inhibiting apoptosis using caspase inhibitors, inhibition of NGF-withdrawal-induced apoptosis upstream of the mitochondria with Bcl-2 prevents mitochondrial autophagy and prolongs survival.[8] The effect of Bcl-2 may be a reflection of its ability to prevent mitochondrial depolarization and permeability by inhibiting the functions of Bax and Bak. In mammalian cells, Bax and Bak are proapoptotic members of the Bcl-2 family of proteins, which are potent regulators of apoptosis that can influence the permeability of the outer mitochondrial membrane. The mechanism by which Bax promotes mitochondrial membrane permeability remains controversial (reviewed in Sharpe *et al.*[44]). In yeast, overexpression of Bax induces cell death and even though yeast do not undergo apoptosis and the yeast genome does not contain any obvious homologues of mammalian cell death executors (caspases, Bcl-2 family members, apoptosis-inducing factor, etc.), these unicellular organisms may contain an alternative cell death machinery that is similar enough to the mammalian counterpart to be activated by Bax.[45] The cell death induced by Bax in yeast is dependent on mitochondria and occurs more rapidly under conditions in which mitochondrial proliferation is stimulated (i.e. on nonfermentable carbon source). Interestingly, the *Uth1* gene that is required for mitophagy was also identified in a screen for mutants resistant to Bax-induced cell death in yeast, suggesting that one of the mechanisms by which Bax may exert its effects is by promoting mitophagy.[46] Other mechanisms by which Bcl-2 may be influencing mitochondrial autophagy include inhibiting the activation of BNIP3 or mammalian homologues of the *Drosophila Spin* gene. BNIP3 is a member of the Bcl-2 family that forms heterodimers with Bcl-2 and Bcl-xL. It is loosely associated with the mitochondrial membrane in normal cells, but integrates into mitochondrial membranes during induction of cell death.[47] Overexpression of BNIP3 results in a caspase-independent cell death with features of necrosis, including opening of the permeability transition pore, and evidence of mitochondrial autophagy.[48] Hspin is a transmembrane protein that is localized primarily in the mitochondria. Overexpression of HSpin (the human Spin homologue) also induces a caspase-independent cell death that involves mitochondrial autophagy and is inhibited by Bcl-2 and Bcl-xL.[49] In addition to regulating mitochondrial signals, Bcl-2 and Bcl-xL may also influence mitochondrial autophagy through their direct interaction with Beclin1, the mammalian homologue of Atg6. Atg6/Beclin1 is part of the Class III PI3-kinase complex that promotes nucleation of autophagy at the preautosomal structure in yeast, and at the trans-golgi network in mammalian cells.[15,16] In murine embryonic fibroblasts (MEFs), Beclin1 expression is upregulated in response to DNA damaging agents and contributes to autophagic cell death in response to these toxic agents.[10] It is interesting to note that Beclin1 expression was not increased in response to amino-acid starvation, which also induces autophagic cell death in MEFs.[10] Beclin1$^{-/-}$ mice die during embryogenesis, and although Beclin1$^{+/-}$

heterozygotes are viable, they exhibit an increased incidence of tumors.[50,51]

Autophagy can promote cell survival in mammalian cells

It is not clear whether the mitophagy induced in response to toxic stimuli is involved in delaying cell death (by eliminating the source of and release of mitochondrial derived proapoptotic factors and reactive oxygen species) or in hastening cell death (by eliminating a major source of ATP production). The difficulty in addressing this question arises from the lack of inhibitors specifically targeting mitochondrial autophagy: currently, most inhibitors of mitochondrial autophagy in mammalian cells are also inhibitors of the general autophagic machinery. This inability to separately inhibit macroautophagy and mitophagy may also contribute to some of the conflicting results regarding the role of autophagy in promoting cell death or cell survival. We have observed that interleukin-3 (IL-3) withdrawal in an IL-3-dependent hematopoietic cell line results in cell death by apoptosis, apparently triggered by downregulation of nutrient transporters (including Glut1, the glucose transporter) and decreased glucose utilization, mimicking growth under glucose-deficient conditions.[52] By contrast, IL-3 withdrawal in a similarly derived IL-3 dependent cell line selected from $Bax^{-/-}Bak^{-/-}$ double knockout mice does not result in cell death.[9] Instead, the induction of autophagy allows prolonged survival of cells with relative preservation of mitochondrial function and provides an alternative source of energy. Consistent with the prosurvival role of autophagy in this context, inhibition of autophagy using shRNA against Atg5, siRNA against Atg7 or drugs (such as 3-MA and hydroxychloroquine) promotes cell death.[9] That the death induced after blocking autophagy is a result of a bioenergetic catastrophe is suggested by the observation that cells are rescued by methypyruvate (presumably functioning as an oxidizable substrate for at least a few functional mitochondria).[9] In Hela cells deprived of serum and amino acids, which alone does not trigger cell death, inhibition of macroautophagy by siRNA knockdown of Beclin, Atg5, Atg7 or Atg12 triggers apoptosis that can be delayed by overexpression of BCL-2 or addition of caspase inhibitors.[53] These results support the idea that macroautophagy is an adaptive process that provides a mechanism for recycling cytosolic components and contributes to bioenergetic homeostasis in cells with relatively preserved mitochondrial function. It is possible that autophagic signals which allow preservation of mitochondrial function may be involved in an adaptive process that leads to cell survival, while signals that promote autophagy and significant mitochondrial degradation are those that result in cell death.

While the central role of mitochondria in cell death is well recognized, the endoplasmic reticulum (ER) membrane is also emerging as an important source of proapoptotic signals. For example, Bax and Bak can localize to the ER membrane and promote release of Ca^{2+} in response to an apoptotic stimulus.[54,55] Moreover, it appears that under certain conditions the ER plays a more important role in inducing apoptosis than the mitochondria.[56] Recent studies have demonstrated that in yeast, starvation triggers the delivery of ER to the vacuole, in addition to the mitochondria and cytosolic proteins, by autophagy.[57] Therefore, it will be interesting to explore the possibility that damaged portions of ER membrane may be removed by autophagy and to determine the role of ER autophagy (similar to mitochondrial autophagy) in apoptosis.

It is important to recognize, however, that the relationship between autophagy and apoptosis may extend beyond the ER and mitochondria and that additional factors may link the two pathways. For example, inhibition of caspase activity in murine L929 fibroblast cells results in an autophagic cell death with morphologically preserved mitochondria and ER.[58] Similarly, Bax/Bak-deficient or Bcl-xL-overexpressing MEFs can be induced to undergo an autophagic cell death in response to DNA-damaging agents.[10] As the signals that are responsible for regulating the specificity and rate of autophagy are identified, it will become easier to dissect the role of autophagy in organelle degradation, cell survival and cell death and the mechanism by which defects in this process lead to cancer and other diseases.

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