

Review

Does Autophagy Contribute to Cell Death?

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ABSTRACT

Autophagy (specifically macroautophagy) is an evolutionarily conserved catabolic process where the cytoplasmic contents of a cell are sequestered within double membrane vacuoles, called autophagosomes, and subsequently delivered to the lysosome for degradation. Autophagy can function as a survival mechanism in starving cells. At the same time, extensive autophagy is commonly observed in dying cells, leading to its classification as an alternative form of programmed cell death. The functional contribution of autophagy to cell death has been a subject of great controversy. However, several recent loss-of-function studies of autophagy (*atg*) genes have begun to address the roles of autophagy in both cell death and survival. Here, we review the emerging evidence in favor of and against autophagic cell death, discuss the possible roles that autophagic degradation might play in dying cells, and identify salient issues for future investigation.

INTRODUCTION

Programmed cell death plays a critical role during tissue development and homeostasis of all animals; it is responsible for sculpting tissues and organs, controlling cell numbers and eliminating abnormal damaged cells. Aberrations in cell death contribute to the pathology of numerous disorders, including neurodegeneration, immune cell dysfunction, and cancer. Apoptosis is the most common form of programmed cell death, and a tremendous amount of information has accumulated with regard to molecular mechanisms governing this process.¹ Nevertheless, in recent years, alternative cell death programs have been receiving increased attention; in particular, autophagy has been proposed as an important nonapoptotic cell death mechanism.²⁻⁴

Autophagic death has historically been classified as type 2 programmed cell death based on morphological grounds.^{5,6} However, the existence of autophagic cell death as a bona fide death process is a matter of intense debate. This is because autophagy is well recognized as a survival mechanism during conditions of nutrient limitation; through the bulk degradation of cytoplasmic material, autophagy is utilized to generate both nutrients and energy in these starving cells (reviewed in refs. 10–12).⁷⁻⁹ Under these circumstances, autophagy is critical for maintaining cell viability. Accordingly, the presence of autophagy in dying cells has been proposed to function as an adaptive stress response to prolong cell viability, rather than herald the onset of autophagic type 2 cell death.¹³

Nevertheless, interest in autophagic cell death persists, primarily because autophagic degradation is a fundamental catabolic process that potentially could be coopted for the destruction of a cell by literally “eating itself” to death. The degradation and recycling of proteins, organelles, and other cytoplasmic components is vital for the maintenance of both cell and tissue homeostasis. Two major pathways for protein degradation have been described—the ubiquitin-proteasome system, for the degradation of short-lived proteins; and autophagy, which involves the large-scale delivery of proteins and organelles to the lysosome for destruction.¹² Importantly, multiple routes for autophagic degradation actually exist in cells, including: (1) macroautophagy (often simply called autophagy) in which cytoplasmic contents and organelles are encompassed in double or multimembrane autophagosomes, and subsequently delivered to the lysosome;¹⁴ (2) microautophagy, where cytoplasm is directly engulfed by lysosomal membrane;¹⁵ and (3) chaperone-mediated autophagy, where proteins with a specific signal sequence are transported to the lysosomal lumen by a receptor-mediated process.¹⁶ Of these routes, only macroautophagy has been associated with type 2 cell death; this process will be the exclusive focus of this review and henceforth be referred to as autophagy.

Table 1 Summary of recent studies investigating the role of ATG function on programmed cell death

Death stimulus	Experimental Model	Atg Loss-of-Function	Effect on Programmed Cell Death and Other Major Conclusions	Reference
Z-VAD; Caspase-8 knockdown	L929 fibroblast cells	Atg7 Atg6/Beclin1	Reduced autophagic death; Increased cell viability	Yu et al. ⁷¹
Interferon- γ	HeLa carcinoma cells	Atg5	Decreased cell death and vacuole formation; Death requires FADD and caspase	Pyo et al. ⁷⁷
Etoposide; staurosporine	<i>bax</i> ^{-/-} <i>bak</i> ^{-/-} fibroblasts	Atg5 Atg6/Beclin 1	Reduced autophagic death; Increased cell viability	Shimizu et al. ⁷⁹
Nutrient depletion+DIF	Dictyostelium discooidium	Atg1	Continued cell death without autophagy or vacuolar morphology	Kosta et al. ⁹³
Serum withdrawal UV treatment	<i>Beclin 1</i> ^{-/-} ES cells	Atg6/Beclin 1	No change in apoptotic cell death versus control ES cells.	Yue et al. ⁸⁷
Nutrient depletion	HeLa carcinoma cells	Atg5 Atg6/Becn1 Atg10 Atg12	Increased apoptosis; Autophagy is cytoprotective	Boya et al. ⁹⁷
IL-3 Withdrawal	<i>bax</i> ^{-/-} <i>bak</i> ^{-/-} hematopoietic cells	Atg5 Atg7	Increased cell death due to bioenergetic crisis; Autophagy is cytoprotective	Lum et al. ¹⁰³

Because of the growing interest in the role of autophagy during cell death, the focus of this article will be to overview the current evidence both in favor of and against autophagy as a regulator of programmed cell death, and to discuss the potential functions of autophagic degradation in dying cells. The reader should consult a plethora of excellent reviews to better understand the processes, molecules, and signaling pathways regulating autophagy (examples include^{10-12,17}). Of special note, the genes and proteins constituting the core machinery of the autophagic process have been identified through genetic screens in yeast; many of these genes, now called *ATGs* (for *AuTophagy Genes*), are conserved in higher organisms.¹⁸ These landmark genetic studies have also facilitated the investigation of how the core autophagic machinery influences a variety of key physiological and pathological processes in higher organisms, including programmed cell death. Accordingly, several loss-of-function studies of *atg* orthologues that more directly address the role of autophagy during cell death have been published in recent months; these reports are summarized in Table 1 and will be further discussed throughout this article.

TYPES OF PROGRAMMED CELL DEATH

Cell death is traditionally classified into two groups: necrosis, a passive, uncontrolled way to die, and programmed cell death, a highly regulated process with defined cellular pathways. In turn, programmed cell death has been historically categorized into three types, which are largely defined on the basis of morphological features; they include apoptotic (type 1), autophagic (type 2), and nonlysosomal (type 3) cell death.^{5,6} Apoptosis (type 1) is by far the most common of these morphologies. Autophagic (type 2) cell death, which will be discussed in detail in the ensuing sections, is also frequently observed both in vitro and in vivo; consequently, it has often been proposed to be an “alternative” mechanism of programmed cell death. Finally, nonlysosomal (type 3) cell death, which resembles necrosis, is notable for organelle swelling and the formation of empty cytoplasmic spaces. Compared to the type 1 and type 2 morphologies, type 3 cell death is rarely observed in physiological situations.⁶ The morphological features and detection methods for type 1 and type 2 cell death are discussed in greater detail below.

Type 1 cell death. The characteristic morphological features of apoptosis include cell shrinkage, condensation of the nuclear chromatin, nuclear fragmentation, and membrane blebbing.¹⁹ The clearance of dying apoptotic cells is mediated by the engulfment by neighboring phagocytic cells, which is also called heterophagy.^{20,21} Over the last decade, extraordinary progress has been made in elucidating the molecular pathways governing apoptosis. During apoptotic cell death, the death signals elicited by various stimuli ultimately converge upon two fundamental processes—(1) mitochondrial membrane depolarization and the release of death-inducing factors (e.g., cytochrome c); and (2) the activation of caspases.^{1,22,23} Caspases are cysteine proteases that dismantle the cell during apoptosis; they exist in catalytically inactive forms, called procaspases, which are proteolytically cleaved to form active enzymes. During apoptosis, certain caspases, such as caspase-8 and caspase-9, serve as upstream initiators, whereas others, like caspase 3, serve as downstream executioners. Activation of the initiator caspases by death signals triggers a rapidly amplified proteolytic cascade, ultimately resulting in the activation of executioner caspases, and the degradation of a wide spectrum of substrates as the cell dies.²⁴ The absence of caspase activation is usually viewed as a requisite in order to categorize a death process as being “alternative”.^{3,25}

Importantly, a variety of biochemical and in situ detection methods exist to detect these cardinal events associated with apoptosis in cells and tissues, which obviates the need for ultrastructural studies. Although this list is not comprehensive, a few examples include the biochemical or in situ detection of caspase activation and cytochrome c release, the detection of nuclear fragmentation by Fluorescence Activated Cell Sorting (FACS) for a sub-G₁ cell population, and the detection of DNA fragmentation by terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end-labeling (TUNEL).²⁶ These methods can be used to quantify apoptosis in various experimental situations.

Type 2 cell death. Autophagic cell death is notable for the presence of autophagic vacuoles in the dying cell.^{5,6} It has been hypothesized that the level of autophagic degradation during type 2 cell death is far more extensive than the rate of autophagy observed during the turnover of organelles in normal cells; hence, this self-degradation ultimately leads to a cell's demise. Although the heterophagic clearance

of dead cells may occur, it is not a primary morphological feature of type 2 cell death.²⁷

Unlike apoptosis, there are limited tools for the detection or quantification of autophagy in biological situations, including programmed cell death.²⁸ The gold standard has been the demonstration of the autophagic vacuoles by electron microscopy; however, this method requires considerable skill. Autophagy has also been assayed by staining with the fluorescent compound monodansylcadaverine (MDC) or by the measuring the degradation rate of radioactively labeled long-lived proteins, but neither of these assays are specific for protein degradation by autophagic pathways.²⁸⁻³⁰ More recently, monitoring the intracellular location of Atg proteins, using Green Fluorescent Protein (GFP)-tagged molecules, has emerged as an effective and reliable method to monitor autophagy.^{28,31,32} During autophagy, Atg8 is modified with the lipid phosphatidylethanolamine (PE) through an ubiquitin-like conjugation process, upon which it specifically relocates to early autophagosomes in yeast.^{33,34} Similar changes have been observed in mammalian cells and tissues during autophagic degradation, including the relocation of the Atg8 orthologue, MAP-LC3, to autophagosomes, as well as changes in MAP-LC3 electrophoretic mobility upon PE lipid conjugation.^{31,32} These new molecular markers for detecting and quantifying autophagic activity should assist in more effectively investigating the role and regulation of autophagy during programmed cell death.

Finally, necrosis, commonly considered a passive form of cell death, has been proposed to be regulated by defined cellular pathways in certain contexts.^{2,35} Like autophagy, this process is postulated to be an alternative cell death mechanism. However, the distinct and interconnected roles of autophagy and necrosis as alternative death processes both require further investigation. In addition, other alternative forms of cell death have been described based on studies of derived cell lines, but the physiological relevance of these morphologies remains unclear.²⁵

THE CASE IN FAVOR OF AUTOPHAGIC CELL DEATH

The presence of autophagic vacuoles in many types of dying cells suggests that autophagy might play a causal role in the regulation of type 2 cell death. Treatment of MCF-7 mammary cancer cells with the selective estrogen antagonist, 4-hydroxytamoxifen triggers cells to die over a period of several days, and these cells possess massive amounts of autophagic vacuoles.³⁶ MCF-7 cells lack critical apoptosis regulators, such as the caspase executioner, caspase-3, broaching the possibility that autophagy can compensate for defects in apoptosis.³⁷ Type 2 cell death has also been described in a variety of cancer cells upon treatment with chemotherapeutic agents *in vitro*, which may have important clinical implications for the treatment of cancers with apoptotic defects.³⁸⁻⁴²

Autophagic vacuoles are also associated with degenerating neurons in Parkinson's and Alzheimer's patients, and with neuronal cell lines expressing toxic proteins.⁴³⁻⁴⁸ Although these autophagic structures could be used to remove accumulating protein aggregates, it is also possible that autophagy plays a role in the death of cells during neurodegeneration.^{49,50} Type 2 cell death has been most commonly observed in developing animals. Autophagic vacuoles have been observed in dying cells from animals of diverse taxa including insects, amphibians and mammals.⁶ In mammals, cell death with a type 2 morphology is observed throughout development, including the regression of the corpus luteum,⁵¹ the involution of mammary

and prostate glands,⁵²⁻⁵⁴ and the regression of Mullerian duct structures during male genital development.⁵⁵ Therefore, type 2 cell is observed during normal development, and in association with disorders such as neurodegeneration, all of which provide correlative evidence that autophagy plays a functional role in the death of cells.

Combined activation of autophagy and caspases during programmed cell death. The larval salivary glands of the fruit fly *Drosophila melanogaster* are destroyed by type 2 autophagic cell death, and have served as a particularly useful model for studying the relationship between autophagy and cell death under physiological conditions.⁵⁶ An increase in the steroid hormone, 20-hydroxyecdysone (ecdysone), triggers complex morphological and biochemical changes that culminate in removal of salivary glands six hours after this rise in hormone. These changes occur two to four hours after the rise in ecdysone, and include DNA fragmentation, changes in structural protein localization, and dynamic changes in cell structure including the formation of autophagic vacuoles.⁵⁷⁻⁵⁹ A two-step transcription regulatory hierarchy mediates these changes. Ecdysone is bound by a heterodimeric receptor complex composed of the nuclear receptor proteins Usp and EcR; this receptor complex binds to DNA and activates transcription of the early genes *BR-C*, *E74A* and *E93*. Mutations in any of these genes prevents the death of salivary glands, and also prevents transcription of many secondary response late genes including caspases and caspase regulators, as well as *Atg* genes.⁶⁰⁻⁶³

Caspase function is required for type 2 autophagic death of salivary glands, and this raises the issue of what role autophagy might play in these dying cells. Expression of the caspase inhibitor p35 prevents destruction of salivary glands,⁵⁷ and salivary glands of animals with mutations in the caspase *Dronc* also fail to die.⁶⁴ Inhibition of caspase activity does not completely block vacuolar changes following the rise in ecdysone that triggers cell death, raising the possibility that autophagy might play an active role in cell removal of salivary glands.⁵⁸ This hypothesis is supported by data indicating that caspases are activated at the same time as *Atg* gene induction, and prior to the formation of autophagic vacuoles in these cells.⁵⁹ Although it is possible that autophagy is being utilized to maintain cell viability after the activation of caspases, it seems counter-productive that the same steroid signal that promotes the transcription of cell death regulators would simultaneously promote *Atg* gene transcription for survival; this does not appear to be an efficient use of energy and resources. It appears more likely that autophagy participates in the death or degradation of salivary glands in *Drosophila*.

The concurrent activation of autophagic and apoptotic pathways has also been observed in mammalian cells. The deprivation of nerve growth factor (NGF) from cultured sympathetic neurons elicits cell death that involves both caspases and autophagy; based on pharmacological inhibition studies, autophagic degradation has been proposed to be triggered during the early stages of cell death and function upstream of principal apoptotic events, including cytochrome c release and caspase activation.⁶⁵ In addition, serum deprivation of PC12 cells induces cell death that exhibits both apoptotic and autophagic features.⁶⁶ Finally, the characteristic features of autophagy and apoptosis have also been observed during lumen formation of mammary epithelial acini grown in three-dimensional culture. When cultured on reconstituted basement membrane, MCF-10A cells, a cell line derived from normal mammary epithelium, form acini—spherical structures in which a layer of polarized epithelial cells surround a hollow lumen and resemble glandular epithelium *in vivo*.⁶⁷ Interestingly, lumen formation involves the selective death of centrally located cells, and both autophagic vacuoles and processed

caspase-3 are present in the dying cells that occupy the developing acinar lumen. Preventing apoptosis by the overexpression of either Bcl-2 or Bcl-x_L delays the clearance of cells, but does not completely inhibit lumen formation in this system; notably, the central cells in Bcl-2-expressing acini also exhibit extensive autophagy.⁶⁸ On the other hand, inhibiting downstream signaling by the Tumor Necrosis Factor Related Apoptosis Inducing Ligand (TRAIL) reduces the formation of vacuoles in the central cells, but does not prevent luminal apoptosis. However, the combined expression of Bcl-x_L and inhibition of TRAIL signaling results in the stronger defects in lumen formation, intimating that both caspase activation and autophagy contribute to proper clearance during lumen formation.^{68,69} Although these results may support the notion that autophagy functions as an alternative cell death pathway upon apoptotic inhibition, they do not exclude the possibility that autophagy could function as a stress response mechanism in dying cells occupying the lumen. Loss-of-function studies of *atg* orthologues during acini development are needed to clearly establish the role of autophagy during cell death or clearance in the lumen. Nevertheless, these studies in mammalian neuronal and epithelial cells, similar to data from studies of *Drosophila* larval salivary gland destruction, suggest that cell death could involve two mechanisms that either function in parallel or through interconnected regulatory pathways.

Death processes that require *atg* genes. Recent studies also indicate that autophagy is required for the caspase-independent death of cells. Murine L929 fibrosarcoma cells die when caspase function, most notably caspase-8, is inhibited, and these cells possess large numbers of autophagic vacuoles.^{70,71} The autophagic death of L929 cells depends on the function of the receptor interacting protein (RIP), which has been described in previous studies of necrosis-like cell death.⁷² L929 autophagic cell death also requires components of the Jun N-terminal kinase (JNK) pathway including MKK7 and c-Jun.⁷¹ Significantly, the induction of this type 2 autophagic cell death and formation of autophagic vacuoles requires the function of the autophagy proteins Atg7 and Atg6 (Beclin1). Importantly, caspase-8 also plays a significant role in apoptosis of L929 cells when they are exposed to death inducing stimuli, such as tumor necrosis factor alpha (TNF- α).⁷¹ Activation of its receptor by TNF triggers recruitment of the adaptor proteins FADD, TRADD, and RIP, all of which function in various aspects of downstream signaling. Importantly, FADD (Fas-Associated Death Domain-containing protein) recruits pro-caspase-8 to this complex, thereby activating it and inducing apoptosis.^{73,74} RIP is a caspase-8 substrate; hence through RIP cleavage, FADD and caspase-8 may regulate an important switch that promotes apoptosis and inhibits autophagic cell death.^{75,76}

Autophagy also appears to be required for death induced by the cytokine interferon-gamma (IFN- γ). In HeLa carcinoma cells, the antisense downregulation of Atg5 suppresses both autophagic vacuole formation and cell death mediated by IFN- γ . Conversely, overexpression of Atg5 induces both cell death and autophagy. Interestingly, Atg5 interacts with FADD both in vitro and in vivo; and downregulation of FADD inhibits both IFN- γ and Atg5-mediated cell death without affecting vacuole formation. Hence, the death-promoting activity of Atg5 requires FADD as a downstream mediator. Notably, a pan-caspase inhibitor (zVAD-fmk) blocks IFN- γ -induced cell death, but not vacuole formation.⁷⁷ Overall, these results favor the hypothesis in which autophagy ensues during the early stages of cell death, and subsequently triggers the activation of caspases. As delineated above, a similar paradigm has been proposed for sympathetic neuron cell death during NGF withdrawal.⁶⁵

The Bcl-2 family proteins function as important regulators of apoptosis. Mouse embryonic fibroblasts which lack the multidomain Bcl-2 family members Bax and Bak, two proapoptotic proteins critical for mitochondrial membrane permeability, are resistant to apoptosis induced by numerous agents.⁷⁸ A recent study indicates, however, that cells doubly deficient for Bax and Bak are capable of dying with a nonapoptotic morphology in response to apoptosis-inducing agents. These dying cells possess autophagic vacuoles and elevated levels of Atg proteins. Furthermore, this autophagic cell death requires the function of two autophagy genes, *atg5* and *atg6/beclin1*.⁷⁹ Similar to the results observed in L929 cells, these studies of Bax/Bak double-knockout cells indicate that caspase activity and autophagic degradation may serve complementary roles that regulate distinct forms of cell death. Because viruses commonly possess genes products that inhibit caspase activation, autophagic death could serve as an important “failsafe” mechanism to elicit non-apoptotic cell death in infected cells, and thus, prevent further pathogenic effects. Further investigation is needed to determine if autophagy can regulate a caspase-independent cell death in vivo.

EVIDENCE AGAINST AUTOPHAGY AS A CONTRIBUTOR TO PROGRAMMED CELL DEATH

Although increased autophagy has been observed during programmed cell death in a variety of situations in vitro and in vivo, many of these studies have not demonstrated a causal role for autophagy as an initiator of cell death. As discussed above, dissecting the true role of autophagy during programmed cell death is often complicated by the fact that dying cells that exhibit characteristic type 2 morphological features cell also demonstrate caspase activation and mitochondrial membrane permeability, two cardinal features of classical apoptosis (type 1 cell death). From a kinetic standpoint, one may speculate that caspase-mediated proteolysis can destroy a cell more rapidly than self-degradation by autophagy; if so, it is likely that apoptotic processes represent the predominant death mechanism when both processes take place simultaneously, even if extensive autophagic degradation exists. Nevertheless, the functional role of autophagy during cell death in many situations remains unclear and requires further study.

Furthermore, until recently, a functional link between autophagy and cell death has usually been based on experiments using 3-methyladenine (3-MA), a pharmacological compound that inhibits the early sequestration events in autophagy.⁸⁰ 3-MA has been shown to prevent the formation of autophagic vacuoles as well as the eventual death of cells in various systems, such as the tamoxifen-induced death of MCF-7 mammary carcinoma cells,^{36,81} neuronal cell death upon withdrawal from NGF,⁶⁵ and the death of T-lymphoblastic leukemia cells upon treatment with TNF.⁸² However, conclusions drawn from such experiments have important caveats. First, 3-MA is a general inhibitor of phosphatidylinositol 3-kinases, enzymes that are involved in a diverse array of cellular processes in addition to autophagy.⁸³ Secondly, 3-MA is not a pharmacologically potent compound, and is commonly utilized at millimolar concentrations; at these concentrations, additional effects have been reported, including the inhibition of Jun N-terminal kinase (JNK) and p38 kinase, both of which are involved in the regulation of stress-induced cell death.^{84,85}

Clearly, more specific loss-of-function approaches to interrogate the role of various *atg* genes in cell death have been long overdue. As discussed in the previous section, several studies have identified the

requirement for *atg* orthologues in certain cell death processes. In contrast, others have revealed the opposite—that autophagy is not required for cell death or alternatively, that it actually promotes the survival of cells in response to death stimuli.

Two groups recently generated mice deficient for *beclin 1*, the mammalian orthologue of the yeast Atg6 protein.^{86,87} Complete loss of *beclin 1* is embryonic lethal and is notable for widespread cell death throughout the embryo, measured by staining with the vital dye, acridine orange. Furthermore, embryonic stem (ES) cell lines genetically null for *beclin 1* displayed no differences in classical apoptosis in response to ultraviolet radiation or to serum withdrawal, when compared to control wild type cells. Hence, *beclin 1* does not play a critical role in certain forms of cell death.⁸⁷ Importantly, the heterozygous loss of *beclin 1* in mice results in the spontaneous formation of both hematopoietic and epithelial tumors; in these tumors, the wild type allele was not deleted, corroborating previous work that *beclin 1* can act as a haploinsufficient tumor suppressor.⁸⁶⁻⁸⁸ Although further proof is needed, some speculate that retention of the wild-type allele in *beclin 1*^{+/-} tumors takes place because minimal levels of autophagy are required for tumor initiation or maintenance; if so, this would argue against autophagy serving as an alternative cell death mechanism that limits tumor progression.^{2,89}

In the social amoeba *Dictyostelium discoideum*, programmed cell death is caspase independent and notable for an autophagic vacuolar morphology, reminiscent of a type 2 process.⁹⁰⁻⁹² This cell death can be induced in *Dictyostelium* through the combined effects of starvation and the production of a morphogen called Differentiation Inducing Factor (DIF). However, studies in a mutant strain unable to produce DIF demonstrate that starvation alone can continue to induce autophagy without triggering cell death. Furthermore, upon inactivation of the *Dictyostelium atg1* autophagy gene in cells via homologous recombination, both autophagy and vacuolization are suppressed, yet cell death still proceeds with a striking nonvacuolar and centrally-condensed morphology. Hence, developmental cell death in *Dictyostelium* does not require autophagic vacuolization.⁹³

Autophagy is cytoprotective during nutrient depletion in mammalian cells. The critical role of autophagy in maintaining cell viability is well-documented in yeast; through the bulk degradation of cytoplasmic material, autophagy is utilized to generate both nutrients and energy when external nutritional sources are lacking.⁷⁻¹¹ A protective role for autophagy has also been proposed in mammalian cells through the turnover and elimination of excess or damaged organelles like peroxisomes and mitochondria. Depolarized mitochondria are rapidly eliminated by autophagy in primary hepatocytes, corroborating that autophagy may be protective against apoptosis by sequestering death promoting molecules.^{94,95}

Moreover, recent work in cultured mammalian cells has indicated that the inhibition of autophagy during nutrient depletion can actually sensitize cells to cell death by apoptosis. HeLa cells treated with lysosomotropic agents, such as hydroxychloroquine, exhibit the typical morphological features associated with type 2 cell death including the accumulation of early autophagic vacuoles, however, follow-up work has delineated that autophagic degradation is actually inhibited in these cells due to the lack of fusion between autophagosomes and lysosomes.^{96,97} Furthermore, cells exhibiting this morphology can recover from this stress, indicating that this vacuolization is not necessarily lethal; instead, a subset of these cells reach a point of no return and activate classical apoptotic programs, such as caspase activation and mitochondrial membrane permeabilization.⁹⁷ Moreover, when autophagy is inhibited, either pharmacologically or

by siRNA-mediated silencing of several autophagy genes (including *atg5*, *atg6*, *atg10* and *atg12*), cells exhibit increased sensitivity to cell death upon nutrient depletion; this death is reduced by caspase inhibition, and by the overexpression of Bcl-2. Cell death due to the combined effects of nutrient depletion and autophagy inhibition is also reduced in mouse fibroblasts doubly deficient for Bax and Bak. These results in mammalian cells substantiate a cytoprotective role for autophagy during nutrient depletion that prevents the activation of classical apoptotic pathways.⁹⁷ Similarly, the siRNA-mediated knockdown of Lysosome Associated Membrane Protein 2 (LAMP-2) can sensitize cells to cell death induced by nutrient depletion. The loss of LAMP-2, which is required for the fusion of lysosomes with autophagosomes, leads to an accumulation of autophagic vacuoles in vivo.^{98,99} Accordingly, the combined depletion of nutrients and LAMP-2 in cells in vitro leads to the acquisition of a morphology associated with type 2 cell death, which subsequently is followed by the hallmarks of apoptosis, including mitochondrial depolarization, release of cytochrome *c*, activation of caspase-3 and nuclear chromatin condensation. This example illustrates a shift from autophagic to apoptotic cell death morphology.¹⁰⁰

The cytoprotective role of autophagy is also important during growth factor withdrawal. Growth factor withdrawal has been associated with high rates of autophagy as well as cell death; an important question is whether autophagy contributes to programmed cell death in these cells, or instead, represents an adaptive response to stress. Recent work using hematopoietic cell lines dependent on the growth factor interleukin-3 (IL-3) for cell survival has provided valuable insight into this question. Upon withdrawal of IL-3, the cell surface expression of multiple nutrient transporters is reduced; as a result, these cells cannot take up the abundant nutrients present in their extracellular milieu, and thus, rapidly undergo apoptosis.^{101,102} In cells unable to initiate apoptosis because they are doubly deficient for Bax and Bak, a progressive atrophy ensues due to the lack of nutrient uptake. Further examination reveals that these growth factor deprived cells survive for several weeks because they utilize autophagy to digest intracellular components, which provides a source of energy to maintain ATP production. If IL-3 is readded to the cultures, these cells can recover from their atrophic state and begin to exhibit normal rates of cell growth and proliferation. Importantly, the inhibition of autophagy in these growth factor deprived Bax/Bak double knockout cells, either by knockdown of *atg5* or *atg7* or by treatment with 3-MA, results in rapid cell death that is associated with compromised bioenergetics and reduced ATP levels. The results illustrate a critical role for autophagy in maintaining proper bioenergetics and promoting cell viability.¹⁰³

Notably, these studies also delineate that autophagy is self-limiting as a survival mechanism in cells unable to undergo apoptosis; the nutrient and energy supplies obtained through autophagy are ultimately depleted, upon which cells do die, albeit after several weeks.¹⁰³ Based on these results, one can construe that cells notable for extensive autophagic vacuolation are probably predisposed to die and that, under these circumstances, the cell death would likely exhibit the morphological features ascribed to type 2 cell death. Still, the major issue that remains is determining if cell death in these situations is due to the autophagic process gone awry, or whether other mechanisms are the primary initiators of death.

Autophagy and survival in vivo. The pro-survival role of autophagy in mammalian cells delineated in the above studies argues against autophagy as a mediator of cell death. Furthermore, the recent examination of *atg* orthologues in various model systems

strongly indicates that this catabolic process can promote survival in higher organisms *in vivo* by regulating a variety of developmental and systemic programs. When faced with unfavorable environmental conditions, the nematode *Caenorhabditis elegans* undergoes a reversible form of developmental arrest known as the dauer diapause. Recent work using nematodes with a mutation in *daf-2* has illustrated the importance of autophagy in this critical process; *daf-2* encodes for an insulin-like receptor, and mutant worms exhibit high rates of dauer entry.^{104,105} Dauer formation is associated with increased autophagy in hypodermal seam cells, a cell type required for multiple aspects of dauer morphogenesis. Furthermore, abnormal dauers result as a consequence of loss-of-function mutations or RNAi-mediated silencing of multiple Atg genes, including *atg1* (*unc51*), *atg6* (*bec-1*), *atg7*, *atg8* and *atg18/aut10*.¹⁰⁵ These results demonstrate that autophagy functionally contributes to an important environmental stress response program in the worm. Moreover, *bec-1* is also required for the lifespan extension phenotype associated with *daf-2* mutant nematodes, suggesting that autophagy components contribute to the enhanced survival of a multicellular organism.^{104,105}

Autophagy possesses a similar survival function during nutrient starvation in *Drosophila*. In the fly, the fat body is thought to function as a sensor of nutritional status by secreting both nutrients and trophic factors.^{106,107} During starvation, the fat body is the predominant organ to exhibit high rates of autophagy, presumably to maintain adequate nutrient levels for the entire organism.^{108,109} Accordingly, the disruption of autophagy machinery genes, either in the whole organism or specifically in the fat body, decreases survival. When combined with the loss of TOR, an essential regulator of cell growth and nutrient uptake, the deleterious effects of decreased autophagy are exacerbated.¹⁰⁸

Recent studies in mice have also revealed an essential role for autophagy for survival during the neonatal period. Mice lacking Atg5 exhibit reduced autophagy and die in their first day of life; the reduced viability of these mice is independent of their ability to nurse. Instead, mice lacking Atg5 display significantly reduced systemic levels of amino acids, as well as signs of energy depletion, such as activation of the energy sensor, AMP-activated protein kinase (AMPK). Interestingly, the highest rates of autophagy in the newborn are found in tissues that exhibit substantial increases in energy requirements (e.g., heart, diaphragm), or marked environmental changes due to the switch from amniotic fluid to air (e.g., lung alveoli, skin).¹¹⁰

Although these *in vivo* studies support the role of autophagy in promoting the survival of whole organisms, they do not formally remove the possibility that autophagic degradation may execute or promote cell death in tissue-specific contexts. As discussed in the previous section, high levels of autophagy have been observed in certain mammalian tissues *in vivo*, such as the involution of the prostate and mammary gland.⁵²⁻⁵⁴ The enormous amounts of autophagy observed in these circumstances have bolstered the case

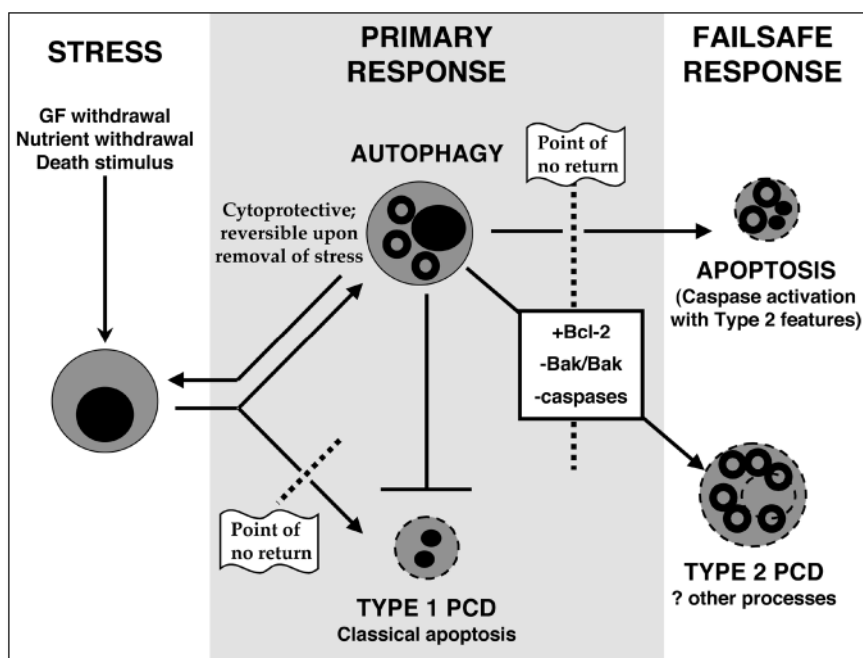


Figure 1. Potential roles of autophagy during programmed cell death. The following cartoon illustrates the possible contributions of autophagy to either the survival or death of a cell that is faced with an external stress, such as nutrient depletion, growth factor (GF) withdrawal, or a death-inducing stimulus. Primary stress response: A cell can initiate autophagy in order to maintain cell viability. This process is reversible; upon removal of the stress, a cell resumes normal cellular functions. In contrast, the cell may commit to die by apoptosis. Although these are depicted as discrete decisions, autophagy can be cytoprotective, preventing the activation of apoptotic pathways; hence, there may be interconnections between autophagy and apoptosis in this primary response. Failsafe response: If autophagy becomes excessive due to the continued presence of the stress, cell contents are depleted beyond repair, once again triggering a death response. In this case, cells can initiate apoptosis if the machinery is available; these dying cells are likely to have mixed type 1 and 2 features. When apoptosis is blocked, due to the inhibition of caspases and/or mitochondrial depolarization, the cell dies by a *bona fide* alternative mechanism, either type 2 cell death, necrosis, or another process.

for autophagy serving as either an alternative or complementary mechanism to caspases during cell death. Nonetheless, whether autophagy contributes to cell survival or cell death in these specific situations remains an important and intriguing question for further study.

POTENTIAL ROLES FOR AUTOPHAGY DURING PROGRAMMED CELL DEATH

Loss-of-function studies of Atg genes have begun to address the paradoxical functions of autophagy in both cell survival and death (summarized in Table 1). Certainly, studies of autophagy gene function in model organisms and in mammalian cells have corroborated that autophagy can serve as a survival mechanism in the primary response to various stresses.^{103,105,108-110} Furthermore, the inhibition of autophagy in starving cells can actually activate apoptosis.^{97,100} These results would support the model depicted in Figure 1, where a cell undergoes autophagy in order to keep it alive under stressful conditions. Upon removal of the initiating stress, the cell can resume normal rates of growth and proliferation. Alternatively, a cell may undergo cell death by apoptosis. In this model, autophagy does not function in cell death during the primary response to stress or death stimuli. However, this framework does not exclude the possibility that autophagy may play an active role in cell death in certain

temporal or tissue-specific contexts. Two possible roles for autophagy during cell death are discussed below, both of which require further investigation.

Autophagy as a failsafe mechanism for cell death. Autophagy can contribute to death in a caspase-independent manner, suggesting that it truly does operate as an alternative death mechanism in certain contexts.^{71,79} These alternative death pathways may function as an important failsafe response in tissue homeostasis or in diseases where apoptotic function has been compromised (Fig. 1). Such a function may also have important implications for the chemotherapeutic killing of cancer cells with apoptotic defects.^{36,38-42} Hence, loss-of-function studies of *atg* genes in response to other death stimuli are warranted in order to discover additional situations where autophagy is required in caspase-independent cell death. Studies are also needed to clarify whether a cell dies by type 2 cell death, necrosis, or another process in these situations. The use of gene-targeted mice lacking various components of the apoptotic machinery may help clarify these questions in vivo. Evidence currently indicates that alternative pathways compensate when apoptotic gene function is abolished; for example, cell death with a vacuolated or necrosis-like morphology is observed in mice lacking the apoptotic molecules, Apaf-1, caspase-3 or caspase-9.^{111,112} However, without loss-of-function studies of *atg* genes, the contribution of autophagic death in these models still remains unclear.

Autophagy may also function as a failsafe response in a cell exposed to a persistent stress. In many cases where autophagic vacuoles are observed in dying cells, there is concurrent or subsequent evidence of caspase activation.^{59,65,66,68} In such cases, the role of autophagy has justifiably been questioned; compared to caspase activation, self-degradation is an inefficient way to die. Nonetheless, because autophagy is a limited survival mechanism, it is entirely feasible that excessive autophagy could initiate a death process that utilizes both type 1 and type 2 mechanisms (Fig. 1). This death-promoting function of autophagy would presumably be triggered by the digestion of a critical threshold of cytoplasmic contents necessary for survival, by the direct activation of death-promoting molecules (e.g., FADD), or by the selective degradation of specific regulatory molecules or organelles essential for viability.^{65,77,103} Notably, recent studies indicate a specific protein, Ald6p, an acetaldehyde dehydrogenase, is preferentially targeted for autophagic degradation in yeast. Although further investigation is needed to determine the existence of other targets, this work broaches the intriguing prospect that a critical survival factor can similarly be selectively degraded by autophagy.¹¹³

Autophagy as a mechanism of cell clearance. Autophagy may also provide a cell intrinsic degradation machinery during type 2 cell death, which in the case of apoptosis is supplied via heterophagic engulfment by a secondary phagocytic cell.^{20,21} In many studies described above, both in vitro and in vivo, there is a noticeable absence of professional phagocytic activity during programmed cell death. Hence, lysosomal degradation through autophagy may be critical in situations where the phagocytosis of dying cells is overwhelmed by massive cell death, such as during insect metamorphosis. Remarkably, several molecules implicated in phagocytosis are upregulated during steroid-induced *Drosophila* salivary gland death, including *Crq*, an orthologue of the macrophage engulfment receptor, CD36. Interestingly, the *Crq* protein is found in the cytoplasm of highly autophagic cells.^{58,59} In addition, genes encoding motor proteins (*ctp*, *ck*), the dynein light chain gene *dle90F*, and members of the Rho, Rac, and Rab family of small GTPases all exhibit increases

in transcription just before salivary gland autophagic cell death.^{61,62} These molecules are known to play important roles in a variety of cytoskeletal rearrangements, including the phagocyte engulfment of apoptotic cells. Although the role of these molecules in autophagic cell death is less clear, these studies intimate that interesting parallels may exist between phagocytosis and autophagy.

In mammals, extensive autophagy is also observed during large-scale tissue remodeling. For example, the post-lactational mammary gland is notable for rapid, widespread tissue destruction, where physical barriers may be imposed on professional phagocytes due to an intact epithelial basement membrane or myoepithelial cell layer.⁵³ It is tempting to speculate that self-degradation by autophagy serves as an important cell clearance mechanism when phagocytes are absent or when widespread tissue histolysis overwhelms professional phagocytic activity. On the whole, further investigation is needed to clarify the functional role of autophagy in both cell death and cell degradation in specific tissues in vivo.

THE RELATIONSHIP BETWEEN APOPTOSIS AND AUTOPHAGY

Importantly, the current state of knowledge strongly argues against rigidly classifying apoptosis and autophagy as distinct mechanisms of programmed cell death. The terms "type 1" and "type 2" essentially describe morphological features that reflect either the death-initiating stimulus or the environmental context of the dying cell. They do not necessarily define discrete, mutually exclusive functional programs. Gene expression studies of *Drosophila* salivary gland death,^{61,62} death of sympathetic neurons during NGF withdrawal,^{65,84} and functional studies of *atg* genes all suggest that coordination between these two cellular processes exists.^{77,97,103}

Indeed, several molecules may be involved in the crosstalk between apoptosis and autophagy. Beclin 1 was identified as a Bcl-2-interacting protein, suggesting an interesting association between molecules regulating autophagy and cell death that requires further investigation.¹¹⁴ Two related Ca²⁺-calmodulin dependent kinases, Death Associated Protein Kinase (DAPK) and DAPK-related protein-1 (DRP-1), regulate membrane blebbing during apoptosis, but can also promote autophagic vacuole formation in dying cells.¹¹⁵ Ectopic expression of the human orthologue of *Drosophila* *Spinster* (*hSpin1*) in cancer cells causes a necrosis-like cell death with increased acidic compartments.¹¹⁶ *hSpin1* binds to both Bcl-2 and Bcl-x_L proteins, and expression of Bcl-x_L can inhibit *hSpin1*-induced cell death. Interestingly, *Spinster* localizes to the late endosome and lysosome, suggesting that it may function in the late stages in autophagy.^{116,117} Further studies of these molecules and pathways should provide insight into the relationships between apoptosis and autophagy during cell survival and death.

CONCLUSION

Interest in autophagy has soared in recent years, given its fundamental role in cellular homeostasis and its expanding importance in a variety of physiological and pathological contexts. Ironically, even though programmed cell death was one of the first settings to broach the biological significance of autophagy, evidence for a clear-cut and wide-ranging functional role of autophagy during programmed cell death has remained elusive. The isolation of the *ATG* genes from genetic screens in yeast has provided enormous insight into the mechanisms that regulate autophagy, and are beginning to inform studies of autophagic cell death. These studies have confirmed that

the role of autophagy in cell survival is conserved among organisms. Conversely, the role and regulation of autophagy in programmed cell death requires further investigation in both higher organisms and mammalian cell culture systems.

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