

Review

Cell death by autophagy: facts and apparent artefacts

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Autophagy (the process of self-digestion by a cell through the action of enzymes originating within the lysosome of the same cell) is a catabolic process that is generally used by the cell as a mechanism for quality control and survival under nutrient stress conditions. As autophagy is often induced under conditions of stress that could also lead to cell death, there has been a propagation of the idea that autophagy can act as a cell death mechanism. Although there is growing evidence of cell death by autophagy, this type of cell death, often called autophagic cell death, remains poorly defined and somewhat controversial. Merely the presence of autophagic markers in a cell undergoing death does not necessarily equate to autophagic cell death. Nevertheless, studies involving genetic manipulation of autophagy in physiological settings provide evidence for a direct role of autophagy in specific scenarios. This article endeavours to summarise these physiological studies where autophagy has a clear role in mediating the death process and discusses the potential significance of cell death by autophagy.

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Facts

- In many *in vitro* and *in vivo* systems cell death is often accompanied by features of autophagy.
- Autophagy does not have a universal role in the execution of programmed cell death; rather it is required in a context-specific manner.
- Known examples of physiological cell death involving autophagy are more commonly associated with development, especially in insects.

Open Questions

- How widespread is autophagic cell death in the animal kingdom?
- How do cells die by autophagy and does this require components of the apoptotic machinery?
- Are upstream signals that lead to cell death by autophagy different from cell death by other means (such as apoptosis and programmed necrosis)?
- Is autophagic cell death relevant to human pathologies and can it be targeted therapeutically for treatment of disease?
- What is the evolutionary significance of autophagic cell death?

Programmed cell death (PCD) is a fundamental biological process that is highly evolutionarily conserved. In animal development PCD is required for removal of unnecessary or

excess cells during tissue pattern formation and to maintain tissue homeostasis. PCD also functions to remove abnormal or damaged cells such as those subjected to genotoxic damage or infected with pathogens. Until a few years ago cell deaths were classified largely on the basis of morphology, as apoptosis or necrosis.¹ However, it now appears from animal models and biochemical studies that multiple additional modalities contribute to PCD during development and in the adult. Hence more accurate definitions of cell death pathways based on molecular characteristics, rather than the classical morphological descriptions, include extrinsic apoptosis, caspase-dependent or caspase-independent intrinsic apoptosis, regulated (programmed) necrosis, mitotic catastrophe and autophagic cell death.²

Despite the presence of multiple apparent death modalities, it is important to emphasise that the majority of the described physiological cell death in metazoans is mediated by caspase-dependent apoptotic mechanisms. The two main caspase-dependent apoptotic pathways in mammals are the extrinsic and intrinsic pathways. A key step in the initiation of both of these apoptotic pathways is caspase activation, which involves oligomerisation and/or proteolytic cleavage into two subunits that constitute the active enzyme.³ The extrinsic pathway involves ligand-mediated activation of death receptors of the tumor necrosis factor family. This leads to the recruitment of caspase-8 through the adaptor protein FADD to form the death-inducing signalling complex resulting in caspase-8 activation and cell death.^{2,3} The intrinsic

This article is dedicated to Jürg Tschopp.

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Abbreviations: PCD, programmed cell death; Atg, autophagy-related gene; TOR, target of rapamycin; PE, phosphatidylethanolamine; GFP-LC3, green fluorescent protein fused to LC3; PI3K, class-I phosphoinositide-3-kinase

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caspase-dependent pathway is characterized by disruption of mitochondria in response to various intracellular stresses. Mitochondrial outer membrane permeabilisation caused by accumulation of pro-apoptotic members of the Bcl-2 protein family Bak and Bax results in the release of proteins, including cytochrome-*c*. Release of cytochrome-*c* facilitates the formation of the apoptosome with Apaf-1 and dATP, which recruits caspase-9 and triggers its activation.³ In many cases, the active initiator caspases are required for processing and activation of effector caspases that cleave a wide range of cellular proteins resulting in cell death.

By contrast, the precise molecular mechanisms regulating autophagic cell death, the focus of this review, remain unclear. Originally identified as a survival mechanism after stress induced by starvation, macroautophagy (hereafter referred to as autophagy) has an important role in many biological processes, including cell survival, cell metabolism, development, aging and immunity.^{4,5} This conserved catabolic process involves engulfment of cytoplasmic material by a double membrane vesicle, the autophagosome, for eventual degradation by the lysosome.⁴ Although the presence of autophagy in dying cells is well documented, the precise role of autophagy in cell death is still unclear in many circumstances and is the subject of some controversy.⁶

The highly regulated dynamic process of autophagy can be divided into several stages: induction, autophagosome nucleation, expansion and completion, followed by lysosome fusion, degradation and recycling (Figure 1).⁴ Induction of autophagy is initiated by the activation of the autophagy-related gene-1 (Atg1) complex, comprising Atg1, Atg13 and Atg17, as well as accessory proteins.⁷ After this, vesicle nucleation requires activation of the class-III phosphatidylinositol-3-kinase (Vps34) and Beclin-1/Atg6, as well as several other factors to recruit proteins and lipids for autophagosome

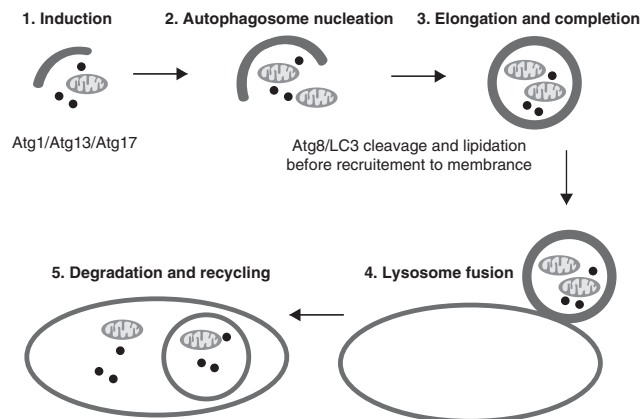


Figure 1 Various steps involved in macroautophagy. Autophagy is the process of engulfment of cytoplasmic material, including organelles and protein aggregates, into a double membrane vesicle, the autophagosome. Induction of autophagy is initiated by activation of the Atg1 complex (Atg1/Atg13/Atg17 and other components). Autophagosome nucleation requires class-III phosphatidylinositol-3-kinase (Vps34) and Beclin-1/Atg6 to recruit proteins and lipids required for autophagosome formation. Elongation and completion are mediated by two-ubiquitin-like systems, which result in lipidated LC3 binding to the autophagosome membrane. The completed autophagosome then fuses with the lysosome, where the autophagosome contents are degraded

formation. Vesicle elongation and completion are mediated by two-ubiquitin-like systems; Atg7 (E1-like) and Atg3 (E2-like) regulate the lipid modification (phosphatidylethanolamine, PE) of LC3 (the mammalian orthologue of Atg8), which requires initial cleavage of LC3 by Atg4 protease; Atg7 and Atg10 (E2-like) regulate the conjugation of Atg12 to Atg5, followed by transfer to Atg16. The Atg12/Atg5/Atg16 complex mediates LC3-PE binding to the autophagosome membrane.^{8–10} The completed autophagosome is transported to the lysosome where the membranes fuse, resulting in breakdown of the autophagosome contents by the lysosome.^{4,7}

Regulation of autophagy induction has predominantly come from studies of the role of autophagy as a survival response during growth factor withdrawal or starvation. In this situation, induction of autophagy requires inactivation/inhibition of the target of rapamycin (TOR) kinase and activation of the canonical autophagy pathway involving multiple *Atg* genes. In the presence of growth factors and nutrients, active insulin receptor/class-I phosphoinositide-3-kinase (INR/PI3K) signalling pathways activate TOR kinase, resulting in growth and inhibition of the Atg1 complex.¹¹ In response to low nutrients, TOR is no longer activated, enabling initiation of autophagy.^{7,12} The interplay between growth signalling and induction of autophagy in cell death is beginning to be revealed,¹³ and it will be important to expand on this to understand how these signals are integrated.

Autophagic Cell Death

Observations that in many instances dying cells have increased autophagic markers and morphological features of autophagy originally led to the proposal of autophagic cell death.¹⁴ Given that autophagy is a pathway activated after cellular stress, as caused by starvation and increased metabolism (as in cancer cells), it is not surprising that cells undergoing death after stress also show features of autophagy. For example, cultured cells deprived of survival factors (such as serum) will undergo apoptosis, but as they go through the starvation process, they also activate their autophagic machinery for survival. This conundrum has led to some confusion in the literature where signs of autophagy in dying cells have been interpreted to mean that cells are in fact using autophagy as a death mechanism. While some claims of cell death by autophagy were possibly artefactual, there is accumulating evidence of a complex interplay between the apoptotic and autophagic machinery, and examples where autophagy is required for PCD to occur are beginning to come to light.^{14,15} As discussed below, such examples, although relatively few, show an expanding direct role of autophagy in physiological cell death in multiple organisms. That said, one must keep in mind that the majority of PCD is not mediated by autophagy in the physiological systems employed so far, even when cell death is accompanied by features of autophagy.

It is important then to discriminate between whether autophagy is determining if a cell dies or not, or whether autophagy is altering the dynamics of death. Caution needs to be taken in assigning this mode of cell death and requires establishing a functional role of the autophagy pathway in cell death. Shen and Codogno¹⁶ have proposed that the following

three criteria should be met to define cell death by autophagy: (i) cell death occurs independent of apoptosis; (ii) there is an increase in autophagic flux, not simply an increase in autophagy markers; and (iii) that suppression of autophagy using genetic means or chemical inhibitors is able to prevent cell death. To fulfil such criteria it is important to definitively assess whether autophagy is arrested or occurs to completion. Consequently several approaches are needed to determine whether there is an increase in autophagic flux where the pathway occurs to completion rather than measure autophagy by steady-state methods that are less able to detect arrest.^{17,18}

One of the important criteria in assigning autophagic cell death is the ability to block cell death by inhibiting autophagy. Several autophagy inhibitors are currently used and they each have their own drawbacks. In a recent cell-based screen to determine the cellular response to a collection of cytotoxic compounds, several agents were found to induce autophagy as determined by GFP-LC3 puncta.¹⁹ In response to these agents, inhibition of autophagy by knockdown of *Atg7* was not able to attenuate cell death. This indicates that among the collection of 59 cytotoxic agents that were capable of inducing autophagy in this *in vitro* model, none induced autophagic cell death. The authors concluded that the inability to detect a compound that can induce cell death by autophagy implies that in human cells, autophagy is rarely, if ever, a mechanism of cell death. While this may be true to some extent, these *in vitro* experiments do not take into account any context-dependent, *in vivo* (as in tumours) and developmental role of autophagy in cell death.

While cell death by autophagy, to our current knowledge, is relatively uncommon, studies using specific model organisms and systems have provided convincing data to support a direct role of autophagy in cell death. Here we discuss some of these physiologically relevant scenarios from *Arabidopsis*, *Dictyostelium*, *Drosophila* and mammalian cells (Table 1). These studies have also revealed that many context-dependent cell death pathways exist that rely on both autophagy and apoptosis, and highlight the complexity of developmentally regulated PCD. In this regard, it may be important to distinguish cell death that requires autophagy as well as caspase activation (Figure 2). Another consideration is the complex requirement of autophagy in tumour cells, which again appears to be context-dependent having distinct roles in cell growth, survival and death (Figure 2).

Table 1 Examples from various organisms where loss of the autophagic machinery suppresses physiological cell death

Organism	Tissue/cells	Autophagy inhibition
<i>Arabidopsis</i>	Tracheary elements	<i>Atg5</i> mutant
<i>Dictyostelium</i>	Fruiting body formation	<i>Atg1</i> mutants
<i>Drosophila</i>	Removal of larval salivary glands	<i>Atg</i> mutants
<i>Drosophila</i>	Removal of larval midgut	<i>Atg2</i> , <i>Atg18</i> mutants and <i>Atg1</i> , <i>Atg18</i> knockdown
Mammals	Ras-expressing HOSE cells	<i>Atg5</i> and <i>Atg7</i> knockdown
Mammals	HCN after insulin withdrawal	<i>Atg7</i> knockdown

Autophagic Cell Death in Plants

The mechanism of autophagy is conserved in yeast, plants and metazoans, and involves the action of the canonical *Atg* genes as described above. The process of autophagic/vacuolar cell death in plants resembles metazoan autophagy, and examples of autophagic/vacuolar cell deaths during plant development and under stress conditions are beginning to be revealed.²⁰ In particular, autophagic cell death appears to have a critical role in the differentiation of the plant vascular system, which is important for transport of water and nutrients. Vascular development involves differentiation of xylem cells, including formation of tracheary elements by secondary cell wall thickening and cell death. The developmental PCD of tracheary elements in *Arabidopsis* has been shown to require autophagy.^{21,22} Differentiation of *Arabidopsis* tracheary elements in *Atg5* mutant cells is disrupted, owing to an inability to activate autophagy and a failure to undergo cell death, supporting a critical role of autophagy in developmental cell death in plants.^{21,22}

Autophagic Cell Death in *Dictyostelium discoideum*

The amoeba *D. discoideum* exists in a vegetative life state in soil where it feeds on bacteria and when food is limited it differentiates by aggregation to form a fruiting body. This process requires cell death to carry the spore-forming cells to the stalk, enabling sporulation and dispersal. The genome of *Dictyostelium* does not encode caspases or Bcl-2-family members, therefore the developmental PCD does not involve caspase-mediated apoptotic cell death.²³ However, *Atg* genes are present in *Dictyostelium* and analysis of *Atg1* loss-of-function mutation reveals distinct roles in survival during starvation and in cell death.²⁴ Autophagy is induced by starvation and cAMP, but this alone does not result in cell death. A second signal, the differentiation factor DIF-1, is required to induce PCD of the starved cells, and DIF-1 alone is unable to induce death of non-starved cells. After starvation, *Atg1* mutants show decreased autophagy with mitochondrial alterations, indicating that *Atg1* is required for a survival role.

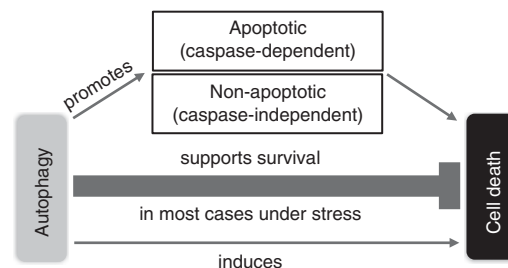


Figure 2 Autophagy in cell survival and cell death. In most circumstances autophagy acts as a survival mechanism, for example, by lowering cell metabolism and supplying nutrients under starvation conditions, or by maintaining quality control in rapidly growing cells (such as oncogene-transformed cells). Autophagy also has specific and context-dependent roles in cell death. It can promote both caspase-dependent apoptotic cell death (e.g., during *Drosophila* larval salivary gland degradation) and non-apoptotic cell death when caspases are inhibited (e.g., in certain tumour cells). In addition, autophagy has a more direct role in mediating cell death in specific contexts (e.g., in the degradation of *Drosophila* larval midgut). The exact mechanism(s) regulating autophagic cell death remains to be determined

When the starved *Atg1* mutant cells are exposed to DIF-1, they no longer die, indicating that *Atg1* is essential for cell death to occur. This study suggests a model whereby autophagic cell death requires an initial signal, such as starvation, and that a second signal, like DIF-1, is required to induce cell death.²⁴ As both of these signals require the function of *Atg1*, this raises the issue of how *Atg1* function is converted from a survival signal to a death signal. This critical question remains to be addressed.

Removal of Midgut and Salivary Glands during *Drosophila* Metamorphosis

Significant contributions to the study of autophagy have come from yeast and mammalian cultured cells. In addition, *Drosophila* has proved to be an excellent model to study the *in vivo* role and mechanisms regulating autophagy during development.⁸ The major developmental transitions through the *Drosophila* life cycle, including molting and metamorphosis, are coordinated by change in the titre of the steroid hormone ecdysone. Binding of ecdysone to its heterodimeric receptor (EcR/Usp) results in the spatial and temporal regulation of proliferation, differentiation and PCD.²⁵ During the transition from larvae to adult, ecdysone acts to regulate the removal of obsolete larval tissues, including the salivary glands and midgut.^{25,26} This results in the transcriptional upregulation of several cell death genes, including caspases (*dronc* and *drice*), Apaf-1 like adaptor (*ark*), IAP antagonist (*reaper*), as well as multiple *atg* genes in these tissues.^{25,27–32} Initial studies examining removal of the salivary gland observed features of both apoptosis, such as induction of pro-apoptotic genes, increased caspase activity and DNA cleavage, as well as autophagy with cytoplasmic vacuoles.^{26,33–36} The role of apoptosis in the removal of salivary glands was revealed in *dronc* and *ark* mutants where the salivary glands persisted for longer without DNA cleavage.^{37,38} However, removal of salivary glands is only partially blocked by genetic ablation or inhibition of the canonical caspase activation machinery, suggesting that other processes are required for their complete removal. Indeed, when autophagy is blocked by loss-of-function mutations in *atg* genes, degradation of salivary glands is delayed.¹³ Furthermore, when both caspase and autophagy inhibition are combined, a severe delay in removal is observed showing greater inhibition of degradation than either pathway alone.¹³ These genetic studies suggest a model whereby developmental PCD of the salivary glands requires both apoptosis and autophagy acting in parallel pathways.

This concept is further supported by studies involving induction of autophagy by overexpression of a key autophagy gene, *atg1*, which was found to be sufficient to promote salivary gland cell death independent of caspase activation.¹³ Interestingly, *Atg1* overexpression in the *Drosophila* larval fat body results in caspase-dependent cell death.³⁹ The *Atg1*-induced cell death also required other downstream *atg* genes, indicating that the cell death is not due to non-specific effects of overexpressing *Atg1*. Although these studies support an important role for autophagy in cell death, high level of autophagy induction may result in non-physiological consequences.

It is not clear what distinguishes the role of autophagy in cell death from survival, but it is reasonable to suggest that autophagy is different in surviving and dying cells. The *Drosophila* cell corpse engulfment receptor gene *draper*, the homologue of the *Caenorhabditis elegans* cell death gene *ced-1*, as well as other components of the engulfment pathway are specifically required for activation of autophagy and cell death of salivary glands.⁴⁰ A dramatic reduction in autophagy and persistence of salivary glands is seen in *draper* mutants, implying that induction of autophagy requires *Draper*. The requirement for *Draper* is cell-autonomous, as clonal analysis of *draper* mutants showed a reduction in autophagy only in salivary gland cells that are depleted of *draper*. Furthermore, *Draper* is not required for starvation-induced autophagy, which is necessary for fat body survival.⁴⁰ Thus, the role of *draper* in autophagy and cell death is context-dependent, specifically it is required for removal of salivary glands but not for starvation-induced autophagy required for survival in the fat body.⁴¹

Removal of the larval midgut during larval–pupal transition is also dependent on autophagy; however this is distinct from salivary gland removal. Despite upregulation of many components of the apoptotic machinery and the presence of high levels of caspase activity in dying midguts, inhibition of caspases by expression of the caspase inhibitor p35 or genetic ablation/mutation of the canonical apoptotic machinery does not affect midgut removal (Figure 3).⁴² The dying midguts accumulate markers of autophagy (e.g. GFP-*Atg8a* puncta), and inhibition of autophagy by loss-of-function mutations in *atg2* and *atg18* or knockdown by RNA interference of *atg1* or *atg18* severely delays their removal.⁴² However, in contrast to salivary gland histolysis, combined inhibition of caspase activity and autophagy shows no more delay in midgut removal compared with inhibition of autophagy alone.^{42,43} Given that caspases are activated during midgut cell death, but their removal/inhibition does not suppress PCD, it appears that there is a complex interaction between the apoptotic and autophagic machinery in this tissue.⁴³ Autophagy induction by overexpression of *Atg1* in the larval midgut is sufficient to induce autophagy and premature degradation (Denton *et al.*, unpublished data). This implies that autophagy is required for midgut PCD and is not dependent on caspase activity. Nevertheless, while the midgut clearly requires autophagy for removal, loss of autophagy only delays PCD, does not abolish it. Therefore, much still remains to be understood about the role of autophagy in PCD during metamorphosis.⁴³

It is well established that modulation of growth-signalling pathways required for induction of autophagy as a survival response acts by regulating TOR kinase activity. Do these same pathways have a role in the induction of autophagy in cell death? Indeed, induction of cell death in salivary glands follows downregulation of growth signalling. Maintenance of growth signalling, by expression of activated Ras or positive regulators of the class-I PI3K pathway, inhibited autophagy and prevented salivary gland removal.¹³ Moreover, the Hippo/Warts pathway of growth arrest is also required for salivary gland cell death, as loss-of-function *warts* mutants show defective growth arrest, which can be rescued by expression of *Atg1* and inhibition of PI3K signalling.⁴⁴ Despite the distinct

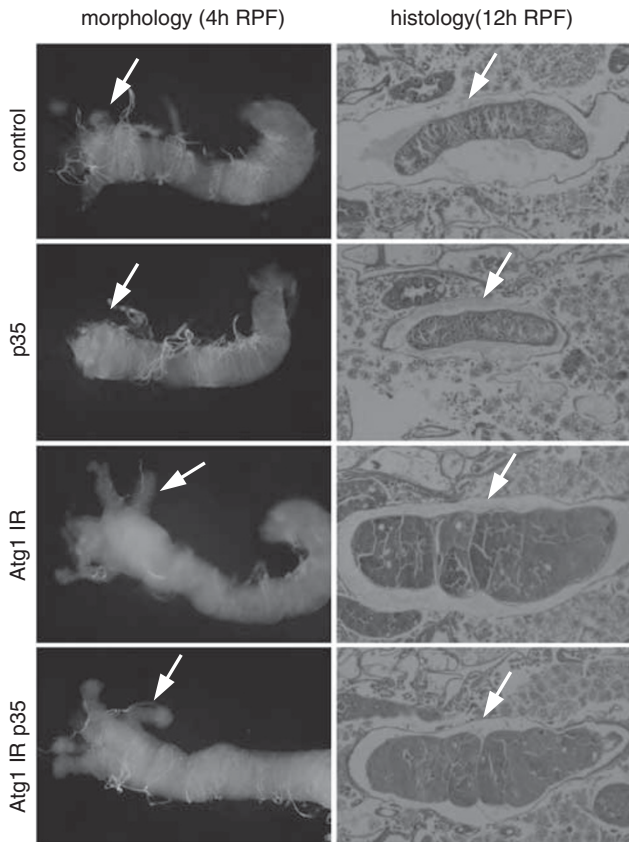


Figure 3 Blocking autophagy delays removal of the *Drosophila* larval midgut. An example where autophagy seems to have a direct role in PCD is the removal of the *Drosophila* larval midguts during larval–pupal transition.⁴² Morphology of dying midguts at 4 h relative to puparium formation (RPF) (left) and histological analysis of paraffin sections at 12 h RPF. A significant delay in midgut histolysis when autophagy is inhibited by knocking down the *Atg1* gene (*Atg1 IR*) as seen by the presence of less contracted gastric caeca (left) and a less condensed midgut (right) compared with a control, as indicated by arrows. Inhibition of caspases by expression of baculovirus p35 has no effect on midgut degradation. Combined knockdown of *Atg1* and p35 expression shows a delay in midgut histolysis, as seen by the presence of gastric caeca (arrows) that is similar to *Atg1* knockdown alone

roles of autophagy in cell survival and cell death, activation of autophagy appears to occur in response to similar signals. However, the mechanism regulating integration of signals between the growth and cell death pathways remain unknown.

Developmental and Starvation-Induced Autophagy during *Drosophila* Oogenesis

Studies involving *Drosophila* salivary gland and midgut PCD suggest that there is a context-dependent complex relationship between the apoptotic and autophagy pathways. While autophagy alone (in the complete absence of caspases) appears sufficient for midgut PCD, autophagy and caspase activation can occur in parallel pathways and both are required for removal of the salivary glands. There also seems to be a relationship between caspases and autophagy in dying cells during *Drosophila* oogenesis. There are two

nutrient-sensitive checkpoint stages, the germarium and mid-stage egg chambers, which undergo starvation-induced cell death to remove defective egg chambers.^{45,46} In addition, normal maturation of egg chambers requires developmental PCD of germline nurse cells and somatic follicle cells late in oogenesis. The starvation-induced cell death that occurs during oogenesis induces autophagy when nutrients are low, as seen by accumulation of GFP-LC3 puncta. This requires key elements of the autophagy pathway, and *atg7* mutants fail to induce autophagy under starvation conditions.^{45,46} These checkpoints also require the caspase Dcp-1 and the inhibitor of apoptosis protein dBruce for autophagy.⁴⁵ In response to starvation Dcp-1 mutants show reduced GFP-LC3 puncta, and overexpression of Dcp-1 was sufficient to induce autophagy under nutrient-rich conditions. Furthermore, *dBruce* mutants resembled Dcp-1 overexpression with degenerating egg chambers and increased autophagy under nutrient-rich conditions, suggesting a role of dBruce in inhibition of autophagy. Although this study provides evidence that starvation-induced degeneration during oogenesis requires autophagy and components of the apoptotic machinery for a coordinated response, the underlying mechanism of how autophagy contributes to cell death remains unclear.

A study by Nezis *et al.*⁴⁷ identified autophagic degradation of dBruce as a trigger for developmental PCD during removal of nurse cells late in oogenesis. Inhibition of autophagy, by germline clones of *atg1* and *atg13* mutants, results in the persistence of nurse cells usually not detected at this stage in the wild type. The germline mutants of autophagy genes also have reduced caspase activity indicating that autophagy functions upstream from caspase activation. Furthermore, while dBruce is not detectable in late stages, it accumulates in the germline of autophagy mutants implying a role for autophagy in its degradation. The *dBruce atg* double mutants contain persistent nurse cell nuclei that are TUNEL-positive. This suggests that degradation of dBruce by autophagy is required for DNA fragmentation in nurse cells during oogenesis and that autophagy directly contributes to activation of cell death in this context. The precise role of autophagy during oogenesis needs to be evaluated further as analysis of chimeric egg chambers, rather than germline clones, revealed that autophagy is required in the follicle cells and not germ cells for oogenesis.⁴⁸

Removal of Extra-embryonic Tissue in *Drosophila*

Drosophila development provides yet another example of a context-dependent relationship between autophagy and apoptosis. Proper removal of the extra-embryonic amnioserosa tissue late in embryogenesis requires autophagy as a prerequisite for caspase-dependent cell death.⁴⁹ While degradation and removal of the amnioserosa requires caspase activation, inhibition of autophagy by maintenance of cell growth caused the tissue to persist beyond the normal timing of removal with a reduction in autophagy. This suggests that growth arrest and autophagy are required for elimination of this tissue. Ectopic induction of autophagy by *Atg1* expression was sufficient to induce premature amnioserosa removal and this was suppressed by co-expression of the caspase inhibitor p35. This indicates that in the amnioserosa

tissue autophagy is not an independent cell death mechanism, but rather it requires caspase activity for proper removal.

Autophagy in Tumour Cell Survival and Death

As discussed earlier, although many examples of autophagic cell death are described in the literature, it is important to distinguish whether autophagy is a cause of cell death or is induced as a secondary consequence. Many of the gene-knockout studies in mouse do not provide support for autophagy function in developmental PCD in mammals. For example, knockouts of *Atg5*, *Atg7* and *Beclin-1* do not show any deficiencies in PCD.^{50–53} This may in part be due to redundant functions of autophagy pathway components, the roles of some of which still remain to be fully understood. Further, as described below, context-specific and spatial roles of autophagy in mammalian cell death have been discovered in recent years.

There are emerging examples where some cancer cells respond to cytotoxic drugs by induction of autophagy, resulting in cell death particularly when the apoptotic pathway is non-functional.^{54–58} The role of the Bcl-2 family in apoptosis is well-characterised and double knockout *bax*^{-/-}; *bak*^{-/-} MEFs are resistant to apoptosis. However when exposed to DNA damage inducers such as etoposide, *bax*^{-/-}; *bak*^{-/-} MEFs undergo cell death that can be reduced by knockdown of components of the autophagy pathway such as *Atg5* or *Beclin-1*.⁵⁴ However, other stress stimuli do not elicit the same response. Autophagic cell death has also been observed in mouse L929 cells where cell death induced by caspase-8 inhibition can be suppressed by knockdown of *Beclin-1* and *Atg7*.⁵⁶ These studies suggest that when the apoptotic pathway is not functional, cytotoxic drugs are able to induce cell death, which appears to involve autophagy under certain circumstances.

Other examples showing requirement of autophagy for cell death again come from *in vitro* studies using cancer cells.^{57–59} Response of acute lymphoblastic leukaemia cells to the synthetic glucocorticoid dexamethasone requires induction of autophagy prior to apoptosis.⁵⁷ After dexamethasone treatment, an increase in autophagic markers, including autophagosomes detected by electron microscopy, LC3-II conversion and GFP-LC3 puncta, was seen before markers of apoptosis, including Bax activation and nuclear fragmentation. Whereas induction of autophagy in response to stress is not surprising, inhibition of autophagy by knockdown of *Beclin-1* or treatment with chemical inhibitors decreases the dexamethasone-induced cell death.⁵⁷ In an extension of these studies, dexamethasone induces autophagy as well as cell death in multiple myeloma cells.⁵⁸ Induction of autophagy has also been reported in response to oxidative stress or serum starvation, in HCT116 and HeLa human cancer cell lines, with increased p62 degradation and LC3-II accumulation.⁵⁹ This is dependent on the presence of FoxO1, a member of the forkhead family of transcription factors, but is independent of FoxO1 transcriptional activity. Furthermore, acetylated cytosolic FoxO1 interacts with *Atg7* promoting autophagy in tumour cells and in a xenograft mouse model.⁵⁹ However, it is unclear how cytosolic FoxO1 differentially prompts autophagic cell death, whereas nuclear FoxO1 is able to induce cell

death by apoptosis. Further studies are required to understand these different responses.

Several studies have recently shown that expression of activated Ras increases autophagy and that this results in different outcomes, either cell growth or cell death, depending on the context. While the majority of the reports support the role of autophagy for cell survival, there are examples indicating that autophagy induced by Ras activation promotes cell death.^{60–62} In a study using human ovarian surface epithelial (HOSE) cells, induced expression of oncogenic H-Ras^{V12} was shown to cause proliferative arrest and to induce autophagy resulting in cell death.⁶⁰ Surprisingly, caspase activation is not detected in the Ras-expressing cells, whereas autophagy markers, including p62 degradation, LC3 lipidation and autophagosome formation, are evident. When key regulators of autophagy, *Atg5* or *Atg7*, are knocked down Ras-induced death is inhibited. Oncogenic H-Ras^{V12} expression stimulates the upregulation of the BH3-only protein Noxa and *Beclin-1*, and knockdown of Noxa or *Beclin-1* reduces autophagy and promotes survival. In addition, overexpression of anti-apoptotic Bcl-2-family members, Mcl-1, Bcl-2 and Bcl-xL, suppresses activated Ras-induced death. Interaction of *Beclin-1* with the anti-apoptotic proteins Bcl-2, Bcl-xL and Mcl-1 inhibits *Beclin-1* function, preventing autophagy. In the presence of Noxa, *Beclin-1* dissociates from Mcl-1, proposed to be due to the higher affinity of the Mcl-1 and Noxa interaction, resulting in *Beclin-1* promotion of autophagy. This indicates that in response to oncogenic Ras, Noxa may participate in the activation of the autophagy machinery to promote cell death by a unique caspase-independent mechanism.

In support of these findings, expression of oncogenic H-Ras or K-Ras in three different normal fibroblast cell lines, Rat2, NIH3T3 and WI38, induced cell death in a caspase-independent manner.^{61,62} Accompanying the Ras-induced cell death were *Atg5* upregulation and accumulation of autophagosomes. Treatment with several different autophagy inhibitors protected the cells from Ras-induced cell death, supporting the idea that autophagy is promoting cell death rather than survival in these cell types.

The finding that Ras-induced autophagy is required for cell death is in contrast to other reports that support a role for autophagy in promoting the growth of tumour cells.^{63–67} Expression of oncogenic Ras in immortalized baby mouse kidney (iBMK) cells results in increased autophagy, with increased LC3 puncta and LC3 lipidation under basal conditions, without affecting cell viability.⁶³ Under starvation conditions (metabolic stress), Ras-expressing iBMK cells require autophagy to survive, as knockdown of *Atg5* or *Atg7* reduces their viability. Furthermore, the ability of Ras-transduced iBMK cells to form tumours in nude mice is greatly reduced when they are deficient for *Atg5* or *Atg7*, or *p62*. Consistent with this, several human tumour cell lines with oncogenic Ras mutations showed high basal levels of autophagy. In a subset of these, inhibition of autophagy by knockdown of *Atg5* or *Atg7* greatly reduced their survival under starvation.⁶³

Similarly, autophagy is required for Ras-induced cell growth where the ability of H-Ras^{V12}-expressing MEFs to form colonies in soft agar is reduced by knockdown of *Atg7* or

Atg12.⁶⁴ In other cancer cell lines with activated Ras expression, knockdown of *Atg5* or *Atg7* prevents autophagy, reduces growth in soft agar and decreases tumour growth in nude mice.⁶⁶ This further supports the findings that autophagy is required for oncogenic Ras-mediated cell growth.

Additional support for a role of autophagy in tumour cell growth comes from a study showing that pancreatic cancer cells require autophagy for growth and survival. Several pancreatic ductal adenocarcinoma (PDAC) cell lines and primary human pancreatic tumours show increased LC3 puncta and LC3 lipidation, indicating high levels of autophagy and autophagic flux present under basal conditions.⁶⁷ Inhibition of autophagy in PDAC cells, by chemical or genetic means, results in decreases in proliferation and colony-forming ability. These findings are supported in PDAC xenografts and in a K-Ras-driven PDAC mouse model where chloroquine treatment results in a reduction in tumour size and prolonged survival. Whereas autophagy seems to be required for PDAC growth, other tumour cells, including lung or breast cancer cell lines, have no increase in basal autophagy and are not sensitive to autophagy inhibition by chloroquine. Thus this effect appears to be specific to pancreatic cancer cells.

These studies highlight the ability of oncogenic Ras to induce autophagy, which can lead to different outcomes (Figure 4). Whereas some transformed cells require autophagy for survival, others seem to require autophagy for cell death. These contradictory functions suggest that, depending on the different cellular context autophagy is required for both cell survival and cell death of cancer cells.

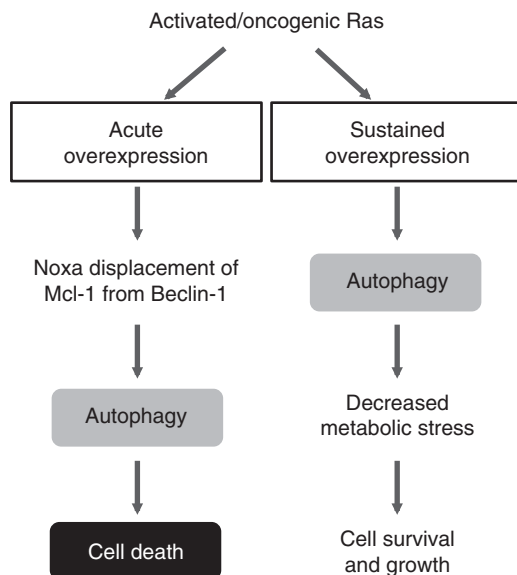


Figure 4 Autophagy induction in response to activated Ras can result in cell growth and survival or cell death. Expression of activated Ras can induce autophagy and this has different consequences depending on the context. Acute overexpression of activated Ras induces Noxa and Beclin-1, which results in Mcl-1 dissociation from Beclin-1 and induction of caspase-independent autophagic cell death. Sustained overexpression of Ras leads to metabolic stress. In this scenario autophagy promotes cell survival and growth by overcoming cellular energy deficit and/or maintaining organelle quality control in rapidly dividing transformed cells

Role of Autophagy in Neuronal Cells

The role of autophagy as a protective mechanism in several types of neurodegenerative conditions is well recognised, where it functions to remove ubiquitin aggregates, preventing neuronal degeneration.⁶⁸ Consistent with this, loss of function of *Atg5* and *Atg7* causes neuronal loss and neurodegeneration.^{69–71} By contrast, adult hippocampal neural (HCN) stem cells undergo autophagic cell death in culture in response to insulin withdrawal. HCN insulin-starved cells accumulate markers of autophagy, including increases in lipidated LC3 and Beclin-1, and knockdown of *Atg7* suppresses autophagy, reducing cell death. Conversely, when autophagy is induced after treatment with rapamycin (an inhibitor of TOR), there is an increase in cell death in the insulin-deficient HCN cells. Furthermore, even though the HCN cells have an intact apoptotic pathway, there is no evidence of caspase activation. This suggests a causative role of autophagy in HCN cell death after insulin withdrawal.^{72,73}

Concluding Remarks

Although examples of autophagic cell death have come from studies in several model organisms, the importance of this mode of PCD regulation in mammalian development is still uncertain and requires further investigation. As most of the evidence for autophagic cell death in mammals comes from cell culture studies and transformed cells, often with inactive apoptotic pathways, this produces conflicting results depending on the system and also depending on the stress stimuli. In these examples, autophagy is often inhibited by gene knock-down, and currently it cannot be ruled out that some of the canonical autophagy genes, including *Atg5*, *Atg7* and *Beclin-1*, are involved in unrelated mechanisms, in particular cell death pathways. Indeed, *Atg12* conjugation to *Atg3* has a role in mitochondrial homeostasis and cell death that does not affect starvation-induced autophagy.⁷⁴ This highlights the importance in the clinical setting for treatment of specific cancers, where it will be important to determine whether autophagy is having a protective or a cell death role.

Autophagy is normally activated after stress, which often accompanies cell death by apoptosis. However, in some circumstances cellular signalling can induce an alternative pathway that results in autophagic cell death. In these instances the mechanism of how autophagy kills cells remains unknown. For example, it remains to be determined whether autophagy causes cell death simply by proteolytic degradation of bulk of the cell mass or by limited degradation of critical cell survival factors, which then leads to activation of an alternative cell death program.

It is intriguing that certain cells and tissues require autophagy for cell death. It has been proposed that the nature and strength of the stress stimuli may have a determining role in whether autophagy promotes survival or death. However, in regard to the developmental function of autophagic cell death, specific signals are likely to have an important role. The examples of autophagic cell death from various organisms suggest there is a more fundamental, and perhaps widespread, evolutionary role of autophagy in cell death execution. In *C. elegans* defects in the apoptosis or autophagy pathway

alone are viable, whereas embryos that are deficient in both autophagy and apoptosis arrest at early developmental stages with severe morphological defects.⁷⁵ The studies summarised in this review also suggest that there is a cooperative role of the apoptotic and autophagic pathways in different developmental contexts. It is evident that there is a complex interplay between apoptosis and autophagy, yet the mechanisms remain unknown. It appears that autophagy has a fundamental role in cell removal during tissue patterning and metamorphosis, and it will be exciting to determine the mechanisms by which autophagic induction leads to cell deletion. Although autophagic cell death appears to be context-dependent, given its potential role in tumour survival and cancer therapy it will be essential to fully understand the molecular basis of this special type of PCD modality.

The studies in *Drosophila* indicate that in almost all known cases autophagic cell death coincides with developmental contexts when nutrients are limiting (such as larval–pupal–adult transition). Thus, nutrient limitation may be the main trigger for autophagy in these contexts. It is therefore tempting to speculate that under such scenarios autophagy has evolved to have an additional role in obsolete cell/tissue deletion. Perhaps the dual role of autophagy in the survival and death of transformed cells and tumours also represents this ancient adaptive function.

Conflict of Interest

The authors declare no conflict of interest.

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