Chapter 30
Autophagy and Cell Death

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Abstract Autophagy is an evolutionarily conserved biological phenomenon related to protein degradation and organelle turnover. Three types of autophagy have been defined: macroautophagy, microautophagy, and chaperone-mediated autophagy, which differ the way of in the delivery of substrates to the lysosome. In macroautophagy, substrates are wrapped in a double membrane structure, called the autophagosome. The formation of the autophagosome and its fusion with the lysosome are genetically controlled by a series of autophagy molecules and are activated in response to a number of environmental cues. Much has yet to be learned about the signaling pathway and the molecular mechanisms about this process. Autophagy is important to multiple cellular functions, particularly for nutrient and energy balance, and the turnover of cellular substances. The relationship of autophagy with cell death is complicated and may be context-dependent. Whereas the nature of autophagic death has yet to be carefully defined, it seems that autophagy may, in fact, be a key regulator of both apoptosis and necrosis. In this context, the roles of macroautophagy in both prosurvival and prodeath have been identified. Understanding the circumstance in which autophagy affects cell functions and therefore cell viability is critical for the future intervention of this process to control cancer, tissue injury, and other disease processes.

Keywords Autophagy · Macroautophagy · Cell death · Apoptosis · Necrosis · Atg molecules · Bcl-2 family proteins · Starvation · Metabolic stress · Mitophagy · ER stress · Hypoxia

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Introduction

Two major protein degradation systems are present in eukaryotic cells: the proteasome and the lysosome. They differ in their functional significance and the type of substrates they take in for degradation. In the lysosome system, the degradation of extracellular materials is mediated by endocytosis (heterophagy), whereas the degradation of intracellular components is mediated by three types of autophagy: macroautophagy, microautophagy, and chaperone-mediated autophagy (CMA) **(1–7)**, which differ in how the cytoplasmic materials are delivered to the lysosome. In macroautophagy, the content is sequestered in a double-membrane structure called the *autophagosome*, which subsequently fuses with the lysosome. In microautophagy, the content is directly taken up by the lysosomes through membrane invagination, whereas in CMA, the content binds to Hsc70 and its co-chaperones. The complex then binds to LAMP2a on the surface of the lysosome. The substrate protein is then transported into the lysosome **(6)**. This chapter discusses macroautophagy (hereafter referred to as autophagy) and its relationship with cell death in mammalian cells.

The term *autophagy* comes from Greek, meaning "self-eating." Autophagy as a biological phenomenon was first systemically described by de Duve and Wattiaux 40 years ago **(1)**, although it seems that the phenomenon, as a process of bulk segregation of cellular constituents, was reported as early as in 1957 in mammalian cells **(8)**. Unlike the ubiquitin proteasome system, autophagy is responsible for the degradation of long-lived proteins and is the only system that can degrade organelles, such as mitochondria **(4**, **9)**. Although autophagy has long been recognized, progression of the study was slow due to a lack of understanding of its molecular mechanisms. The breakthrough came in the 1990 s when the phenomenon was studied in yeast. The powerful yeast genetics allows the identification of multiple genes required for autophagy **(10**, **11)**. These works have since revolutionized the field and brought the research of autophagy into the molecular era.

Basic Autophagy Machinery

Autophagy is evolutionarily conserved and operates in plants, yeast, *C. elegans*, *Drosophila*, and mammals. A large portion of the molecular machinery of autophagy is conserved in these organisms **(11)**. Currently, 31 autophagy-related genes (ATG) have been identified since the first gene, Atg1, was discovered from a genetic screening in yeast **(12**, **13)**. Detailed discussions of these genes, particularly those of yeast, can be found in several recent reviews **(14**, **15)**. A brief summary of the mammalian system is given below.

The core autophagy machinery seems to be built around two ubiquitin-like conjugation systems **(3)** (Fig. 30.1). In one system, the ubiquitin-like protein

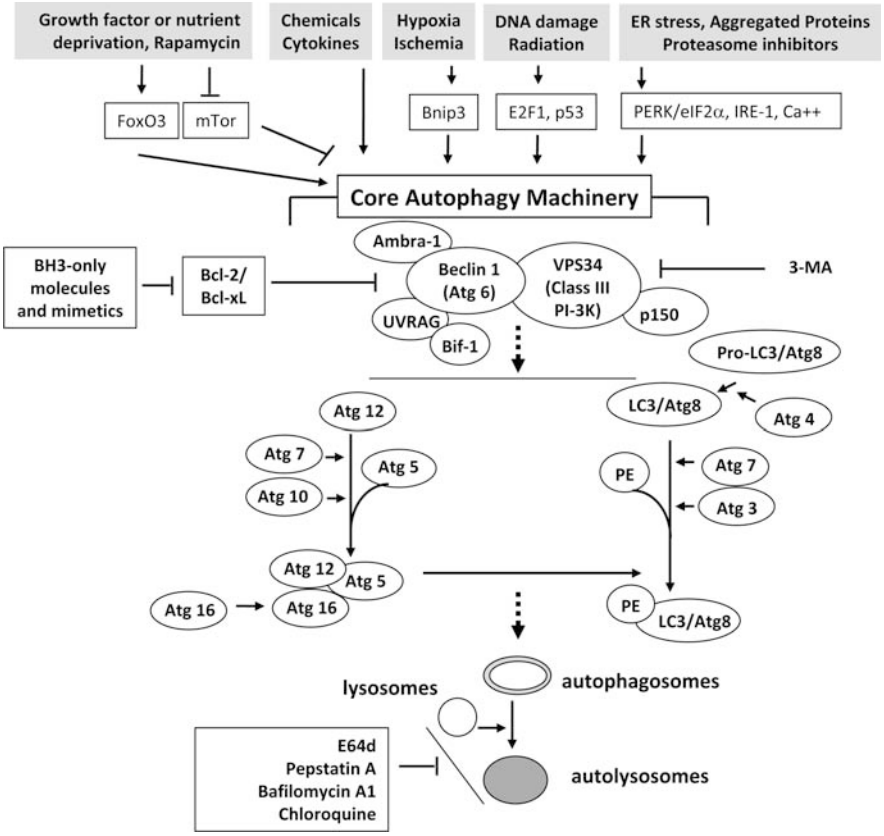

Fig. 30.1 Activation of macroautophagy in mammalian cells. Macroautophagy in mammalian cells could be activated by multiple signals. Some of them are listed at the top of the diagram. The signaling to the core autophagy machinery is only partially revealed, and remains largely unknown to many of the stimuli. The effects of mTOR, Bnip3, calcium, and the unfolded protein response components are among the better-understood mechanisms. The Beclin-1/Atg6 complex, composed of Beclin-1, VPS34 (the class III PI-3 kinase), and its regulatory partner, p150/VPS15, Ambra-1, UVRAG, and its binding partner, Bif-1, seems to act upstream by responding to the stimulation. The activity of this complex can be regulated by a number of other factors, including the Bcl-2 family proteins, and some pharmacological agents that suppress VPS34 enzymatic activity. It is not entirely clear how the Beclin-1 complex positively regulates the two conjugation systems: the Atg12-Atg5-Atg16 and the PE-LC3/Atg8 complexes, which form in the presence of Atg7, an E1-like enzyme, and Atg10 or Atg3, E2-like enzymes. These two complexes are required for the formation of an autophagosome (AV), which is a double-membrane structure. During the formation of AV, the cytosolic materials to be degraded are enclosed within the structure. Autophagosomes are then fused with the lysosomes to form autolysosomes, which degrade the enclosed materials. Both the fusion process and the lysosome activity can be blocked by multiple chemicals as indicated, which can thus block autophagy at a later stage

Atg12 was first activated by Atg7, a ubiquitin-activating enzyme (E1)-like protein, and then transferred by Atg10, a ubiquitin carrier protein (E2)-like protein, to Atg5 through a covalent bond. The Atg5-Atg12 complex interacts with Atg16 to form a multimer complex, which is localized to membranes of early autophagosomes. It seems that the assembly of this system is independent of autophagy activation. Thus, the complex appears to provide the necessary platform for autophagy activation.

In another system, the microtubule-associated protein 1 light chain 3 (LC3), or GATE-16 or GABARAP, all mammalian homologues of the yeast Atg8, is first cleaved by Atg4 to expose the conserved Gly^{120} at its C-terminus. LC3 is then conjugated to phosphatidylethanolamine (PE), also via Atg7, and Atg3, another ubiquitin carrier protein (E2)-like protein **(3**, **16)**. The unconjugated form of LC3 (called LC3-I) is in the cytosol, while the conjugated form (called LC3-II) targets to the autophagosomal membrane **(16)** with the assist of the Atg5-Atg12-Atg16 complex **(17)**. This association of LC3-PE to the autophagosomes is considered important for the membrane extension of the autophagosome and the eventual enclosure of the membrane to form the vacuoles. The Atg5-Atg12-Atg16 complex is recycled, while the LC3 complex stays on the membrane until it is degraded by the lysosome. LC3 is thus widely used as a marker for monitoring the autophagy process.

Several other key autophagy genes are important to the initiation and regulation of autophagy. Beclin-1, a mammalian homologue of Atg6, is particularly important. Beclin-1 forms a complex with VPS34, p150/VPS15. VPS34 is a class III PI-3-kinase that is required for autophagy and can be suppressed by 3-methyladenine (3-MA) and also Wortmannin. In yeast, Atg6 also binds to Atg14, which has not been identified in mammalian cells. However, several prosurvival Bcl-2 family proteins **(18)** and Ambra-1**(19)** are found in mammalian cells that can also interact with Beclin-1. However, Bcl-2 suppresses, but Ambra-1 promotes, Beclin-1/VPS34 activity, and therefore autophagy. While the exact mechanism is not clear, the recent finding that Beclin-1 contains a BH3-only domain **(18**, **20**, **21)** suggests that Bcl-2 may sequester Beclin-1 from its interaction with VPS34. Finally, while UVRAG has not been found to be instrumental in yeast autophagy, it seems important for mammalian autophagy due to its interaction with Beclin-1 **(22)**. Another recently discovered molecule, Bif-1, promotes autophagy by interacting with UVRAG **(23)**. Thus, there are extensive molecular interactions at the early stage of the formation of autophagosomes (Fig. 30.1).

Activation of Autophagy

A key regulatory of autophagy in yeast and mammalian cells is the TOR complex **(5**, **24)**. A functional TOR activity would suppress autophagy. Because the TOR pathway is central to the signaling of growth and energy metabolism,

autophagy is intimately coupled with growth and energy control (Fig. 30.1). It can be affected by the upstream signaling of the TOR pathway. For example, growth factors, insulin, and the class I PI3-kinase and Akt will all suppress autophagy by activating Tor **(5)**. On the other hand, suppressing TOR function with rapamycin could induce autophagy.

At the moment, it is not clear how TOR suppression could cause autophagy. In yeast, this seems to be coupled with the activity of the Atg1-Atg13 complex. Although the mammalian cells express two Atg1 homologues, ULK1 and ULK2, they do not seem to possess the Atg13 homologue. Furthermore, genetic deletion of ULK1, unlike that of Atg5 or Atg7, does not result in global defects in autophagy, but specifically affects mitochondria autophagy during erythrocyte maturation **(25)**. It is possible, however, that the Beclin-1 complex would be among the first to be activated by the signals from the TOR suppression.

Several other signaling pathways have been defined in mammalian cells, some of which are likely mTOR-independent (Fig. 30.1). One such pathway can be characterized as calcium-calpain-Gsα–mediated **(26)**. In a number of cases, the induction of autophagy in mammalian cells seems to be related to the transcriptional activation of a number of molecules that are either involved in the core process of autophagy or involved in yet-to-be defined manners. Several transcription factors can be involved. In muscle cells, activation of the FoxO3 transcription by the suppression of the IGF-1-PI-3 K-Akt pathway could in turn enhance the transcription of several autophagy genes, including LC3 and Bnip3 **(27, 28)**. Bnip3 could be also transcriptionally activated by HIF-1, which is induced during hypoxia **(29, 30)**. Bnip3 is responsible for the autophagy induced by hypoxia and ischemia. Furthermore, E2F1 can be responsible for the upregulation of Atg1, Atg5, LC3, and DRAM, which can be important for DNA damage-induced autophagy **(31)**. DRAM is a lysosomal membrane protein participating in autophagy that had previously been identified as a p53 transcriptional target **(32)**. Whereas nucleic p53 could promote autophagy via upregulating DRAM, a recent study has also indicated that cytoplasmic p53 can suppress autophagy by unknown mechanisms **(33)**.

Finally, ER stress caused by chemicals and misfolded protein aggregates can induce autophagy, which helps to relieve ER stress **(34–39)**. In this case, it seems that the unfolded protein response (UPR) pathways, PERK/eIF2alpha and IRE-1/JNK, may be responsible for the autophagy induction. The PERK/eIF2alpha pathway could promote Atg12 expression, which may provide a mechanism of autophagy induction in this case **(35)**. ER stress could elevate the intracellular calcium level, which in turn can activate calmodulin-dependent kinase kinase beta to promote the activity of AMPK, leading to mTOR suppression and autophagy **(40)**. Alternatively, calcium can induce the phosphorylation of PCKθ, which seems to specifically participate in ER-stress-induced, but not in amino acid deprivation-induced, autophagy **(41)**. In this case, the activation of PKCθ is independent of either mTOR or the UPR.

Functional Roles of Autophagy in Mammalian Cells

It is now clear that autophagy is activated under and regulated by many physiological and pathological conditions and, in turn, affects these processes **(4**, **6**, **7**, **42)**. Thus, autophagy is inevitably associated with the pathogenesis of many human diseases **(6**, **43)**. It is required for normal development and participates in the clearance of apoptotic cells during embryogenesis **(42)**. In adults, autophagy seems to be involved in the extension of life span and in protecting cells from stress response, such as starvation. The autophagic degradation of cellular constituents can efficiently recycle essential nutrients to sustain basic biological processes. Thus, autophagy is important for the regulation of energy and nutrient metabolism **(5**, **24)**. Autophagy is commonly known to be activated by amino acid starvation (in mammalian cells) or nitrogen deprivation (in yeast cells). Under these conditions, autophagy is activated to degrade proteins and recycle amino acids to meet the cell's energy requirement **(5**, **10**, **24**, **44)**. This function of autophagy in meeting the energy and nutritional needs of cells was evolutionarily conserved. Genetic deletion of Atg5 in mice led to perinatal death due to the lack of sufficient nutrients in the cardiac and diaphragm muscles for their vital functions **(45)**.

In addition, the degradation of mitochondria, peroxisomes, endoplasmic reticulum (ER), or ribosomes by autophagy is most likely associated with cellular homeostasis as well as changing metabolic needs **(7**, **13**, **46)**. The ability of autophagy to degrade misfolded proteins is an important beneficial function in the pathogenesis of conformational diseases **(6**, **37)**. Autophagy is also employed as a defense mechanism to clear up intracellular microbes, misfolded proteins, and damaged organelles **(7)**.

The role of autophagy in cancer development and cancer therapy has been an area of intense study in recent years **(47**, **48)**. In the first study aiming to understand the function of Beclin-1/Atg6, a mouse stain deficient in this gene was constructed. While Beclin-1-null mice were embryonic lethal, Beclin-1 heterozygous mice were normal at the beginning, but developed multiple tumors later on, suggesting that Beclin-1 is a haplo-insufficiency tumor suppressor **(49)**. The loss of heterozygosity of Beclin-1 is frequently seen in breast and ovarian cancers **(50)**. While the mechanism of how Beclin-1 serves as a tumor suppressor is not known, it is possible that its proautophagy function is important in maintaining cellular homeostasis by removing damaged organelles, such as mitochondria **(51)**. Damaged mitochondria may become a major source of intracellular free radicals that could cause genomic instability and tumors. As a consequence, autophagy may suppression tumorigenesis by preventing genomic instability **(48**, **52)**.

Autophagy in the Regulation of Cell Death

The relationship between autophagy and cell death has been hotly debated in recent years. There are ample observations indicating that the two processes are intimately connected. Evidence for the role of autophagy in promoting cell

survival or in promoting cell death is compelling in both cases **(53, 54)**. In addition, it seems that the regulation of autophagy, apoptosis, and necrosis can be coupled so that one type of mechanism may activate or inactivate the other.

Programmed cell death was initially classified into several categories, primarily based on the ultrastructural morphology of the dying cells **(55)**. While one category of PCD demonstrates features of apoptosis, another category shows the accumulation of autophagosomes **(56)**. The latter was classified as autophagic death. However, considerable controversy exists regarding whether the autophagy process actually promotes cell death or instead is a reactive process that may actually provide protection **(53–56)**. These concerns were not just for the developmental biology process, but also for many pathological processes where autophagy is induced significantly in response to cytotoxic or metabolic stress.

With the understanding of the molecular machinery of autophagy, it is now easier to address these issues. Thus, by inhibiting the key autophagy genes through genetic deletion, RNAi-mediated knockdown, or pharmacological interventions, one may determine whether cell death is suppressed, enhanced, or not changed at all. In this way, the influence of autophagy on cell death could be determined. Indeed, depending on the circumstances, both pro- and anti-death functions of autophagy could thus be identified.

Autophagy Promotes Cell Survival

There are numerous conditions in which autophagy clearly plays a prosurvival role. In neonatal mice, autophagy is required for the endogenous generation of nutrients in such energy-dependent organs as the heart and diaphragm as the newborn adapts to taking in nutrients from an exogenous source, i.e., milk **(45, 57)**. Autophagy deficiency due to the deletion of key autophagy genes, such as Atg5, can thus lead to the premature death of newborn mice. A similar dependency of survival on autophagy during starvation has been demonstrated in *Drosophila*, which occurs primarily in the nutrient-sensing organ, the fat body **(58, 59)**. At the cellular level, the importance of autophagy in survival during nutrient or growth factor deprivation can also be shown in mammalian cells **(44, 60)** and yeast cells **(61)**.

Autophagy is also important for cellular survival under other stressful conditions. In mammalian cells, autophagy could be activated in response to metabolic stress, ischemia, or hypoxia **(48, 62)**. The suppression of autophagy can result in increased cell death. Cytotoxic agents, including many chemotherapeutic agents, such as proteasome inhibitors **(39)**, ER stressors **(34–36, 39)**, DNA-damaging agents **(31, 32, 63)**, and histone deacetylase inhibitor **(64)**, can all activate autophagy, likely in response to the damage caused by these agents. Under pathological conditions, such as the accumulation of misfolded proteins,

autophagy is required for the cellular clearance of these proteins and survival **(37)**. In *C. elegans*, limited food, high temperature, a highly dense population, and mutation in the insulin-like growth receptor (*daf-2*) could all cause development arrest in the form of dauer diapause, which is specialized for survival under these adverse conditions. Autophagy is required for dauer entry and therefore for the survival of the worm **(65)**.

Autophagy promotes cellular survival through its basic function of degrading intracellular components. In the nutrient/growth factor depletion/deficiency condition, autophagic degradation recycles the cellular proteins and glycogen to provide amino acids and glucose for ATP generation **(44)**. In cells under DNA damage or metabolic stress, autophagy may play an important role in removing damaged organelles, such as mitochondria, to reduce the cellular ROS level and maintain genomic stability **(48, 62)**. The clearance of misfolded proteins resulting from ER stress, proteasome inhibition, or genetic mutation is another important function of autophagy in maintaining cellular viability **(37)**.

The mechanisms of autophagy induction under these different conditions are not all well defined. Growth factor deprivation seems to be linked to the downregulation of the Akt signaling, which leads, on one hand, to the suppression of the Tor signaling and, on the other hand, to the activation of FoxO3, a transcription factor that can cause the upregulation of several autophagy genes (discussed earlier). Both events can lead to autophagy activation.

Autophagy induced by the deprivation of nitrogen in yeast is also critically related to the suppression of Tor signaling **(61)**. However, amino acid deprivation in the mammalian cells does not seem to be completely dependent on the mTOR pathway **(5)**. The PERK/eIF-2α signaling, part of the UPR initiated at the ER, can also contribute **(66)**. This pathway, together with another UPR pathway orchestrated by IRE-1, is also involved in autophagy initiated by the misfolded proteins and ER stress **(35, 36, 39)**. However, in response to proteasome inhibitors, which also cause ER stress and the accumulation of misfolded proteins, only the IRE-1 pathway is required **(39)**. ER stress can lead to calcium release, which in turn can activate calmodulin kinase kinase beta and AMPK to suppress mTOR **(40)**. Interestingly, in these cases, cytoplasmic p53 can suppress the induction of autophagy, thereby ensuring its rapid degradation following the induction **(67)**.

Autophagy-mediated prosurvival function could suppress either apoptosis or necrosis. In many apoptosis-competent cells, autophagy could co-exist with apoptosis, and the suppression of autophagy increases apoptosis **(35, 36, 39, 44, 60, 63, 64)**. However, in apoptosis-incompetent cells, caused by the deletion of key proapoptosis genes, such as Bax and Bak, or the overexpression of anti-apoptosis genes, such as Bcl-2 or Bcl-xL, or the use of caspase inhibitors, the inhibition of autophagy often leads to necrosis **(48, 68, 69)**. Many agents could induce both apoptosis and necrosis, although apoptosis can be a dominant type of death, and autophagy can suppress both types. The latter suggests that autophagy acts at the upstream level, where the death stimulation is derived,

so it can mitigate the cause for both apoptosis and necrosis. This thinking is consistent with the idea that the clearance function of autophagy is responsible for its prosurvival function by removing the "damaged" cellular content.

Autophagy Can Participate in Cell Death

Although early studies largely employed morphological criteria to define autophagic death, which is subject to verification, more recent works are based on molecular evidence to substantiate the role of autophagy in promoting cell death.

A typical example for autophagy to participate in developmentally regulated programmed cell death is the degradation of the salivary glands during *Drosophila*'s pupal stage, which is triggered by the steroid hormone ecdysone. Cell death is accompanied with both autophagy and apoptosis features, and determining whether or not autophagy promotes cell death had previously been confusing. A recent study using autophagy gene mutants now indicates that cell death can be significantly inhibited, suggesting that autophagy can promote cell death **(70)**. Furthermore, autophagy is activated by growth arrest and can induce cell death in the absence of caspase activation. In this case, it seems that autophagy and apoptosis independently contribute to the death. Indeed, a combined inhibition of both pathways increased the suppression of the gland degradation **(70)**.

In mammalian cells, autophagy can contribute to cell death under several stressful conditions. Thus, autophagy can be induced in response to certain chemotherapeutic drugs **(71**, **72)**, radiation **(73)**, hypoxia **(74)**, ischemia in the brain **(75)**, cytokines such as INF-γ **(76)**, and ligands such as HIV-1-encoded envelope glycoproteins **(77)**. In these cases, the deletion or RNAi-mediated knockdown of key autophagy genes can significantly reduce cell death, while overexpressing these genes can promote it.

How autophagy promotes cell death is not entirely clear. Although it may be tempting to assume that excessive self-digestion could lead to the depletion of key molecules or organelles essential to the process, the mechanism of killing may be as diverse as the stress signals that induce autophagy in the first place. The autophagic machinery may be interfaced with the apoptotic machinery or the necrotic mechanism to promote apoptosis or necrosis. For example, it was found that in INF-γ-induced autophagic death in HeLa cells, Atg5 can bind to FADD and activate caspase-8 and downstream caspases as if there were a death receptor engagement **(76)**. In another case, when Atg5 was overexpressed, it could be cut by calpains. The 24-Kda cleaved Atg5 N-terminal fragment (aa 1-193) is then translocated to the mitochondria, where it binds to Bcl-xL and inactivates it, resulting in cytochrome c release and cell death **(71)**. In these cases, autophagy seems to link to the classical apoptosis pathway; therefore, the death is actually mediated by the apoptosis machinery.

However, in other cases, autophagy-induced cell death is activated by the loss of the ability to mount an effective apoptosis. Thus, in apoptosis-deficient fibroblasts lacking both Bax and Bak, treatment with etopside **(72)** or prolonged

ER stress **(78)** caused autophagy. Similarly, radiation-induced cell death would switch from apoptosis to autophagic death if caspase activity could be blocked **(73)**. The molecular mechanism is not clear in this type of autophagy-mediated nonapoptotic death, although the role of JNK has been implicated **(72)**. It is likely that cell death in these circumstances is necrotic **(78)**. Similarly, ROS-induced autophagic death can also be necrotic **(79)**. Autophagy-promoted necrosis has also been reported in *C. elegans* **(80)**.

Interestingly, a number of BH3-only Bcl-2 family proteins or their binding partners, when overexpressed, can directly induce cell death with contributions from the autophagic process. Bnip3 is a BH3-only Bcl-2 family protein that has been shown to induce autophagic death in a number of cases. A more detailed discussion of this molecule is given in the following section, since autophagy induced by this molecule can also be prosurvival in other cases **(81)**. Other BH3-only molecules that can induce autophagic death include Apolipoprotein L1 (ApoL1) **(82)** and Bik **(83)**. Finally, hSpin1, a human homologue of the *Drosophila* spinster (spin) gene, can induce nonapoptotic death, which can be inhibited by Bcl-xL **(84)**. The latter effect may not be related to apoptosis regulation but to a direct physical interaction. This type of nonapoptotic autophagy-mediated death is necrotic **(84)**.

One potential mechanism by which BH3-only molecules can induce autophagy is through competitive binding to Bcl-2 or Bcl-xL to disrupt the interaction of the latter with Beclin-1 (Fig. 30.1). Beclin-1 is an important autophagy molecule and interacts with multiple other molecules, such as the class III PI-3 kinase VPS34, UVRAG, and Ambra-1, to promote autophagy **(85)**. However, Beclin-1 also possesses a conserved BH3 domain of the Bcl-2 family proteins and, in fact, can interact with multiple antideath Bcl-2 family members, such as Bcl-2 and Bcl-xL **(85)**, which suppress the function of Beclin-1 as a proautophagy molecule. However, this interaction can be disrupted by the phosphorylation of Bcl-2 or by other BH3-only molecules, such as Bad, or BH3 mimetics, such as ABT737 (see Chapter 2) **(85)**. As a result, Beclin-1 can be desuppressed and can in turn promote autophagy. Indeed, in a human leukemia cell line, HL60, simply knocking down Bcl-2 can cause autophagy, which seems to contribute to the accompanying cell death **(86)**. In the case of Bik-induced autophagic death, the loss of Bcl-2 is a prerequisite **(83)**. Likewise, ApoL1-induced autophagy depends on its BH3 domain, which may thus be involved in the competitive binding with Bcl-2 to release Beclin-1.

Factors That May Affect Whether Autophagy Presents a Prosurvival or Prodeath Effect

The relationship between autophagy and cell death can be quite complicated and may be affected by many factors. Autophagy likely evolves as a physiological process but can be diverted to a prodeath pathway under pathological

conditions. A very unique example of autophagy in promoting cellular injury has recently been reported in Atg5-deficient mice **(87)**. Autophagy has been found to be required for the activation of trypsinogen to trypsin under normal conditions. The enzyme is harmful to tissue if its activation and release are not properly controlled, which can be a cause of pancreatitis. Notably, because of autophagy's role in promoting enzyme maturation, it participates both in normal pancreatic functions, essentially of a "prosurvival" nature, and in pancreatitis in pathological conditions, essentially of a "prodeath" nature.

The level of autophagy could determine the outcome. In *C. elegans*, it has been suggested that only the physiological level of autophagy during starvation is prosurvival and that excessive autophagy could be prodeath **(88)**. Excessive autophagy can cause cellular atrophy and the deficiency of vital cellular components.

The presence of compensatory mechanisms, such as chaperone-mediated autophagy (CMA), may also determine whether the inhibition of macroautophagy renders cells to be more sensitive or more resistant to certain stressful signals. Thus, murine fibroblasts prepared from Atg5-deficient embryos were more sensitive to death receptor-initiated death, but were more resistant to menadione- and UV radiation-induced death due to increased CMA **(89)**.

In a more general way, whether autophagy can be prosurvival or prodeath can also be dependent on both the agents used for stimulation and the status of the cells. Thus, autophagy induced by ER stress **(38)** or proteasome inhibitors (Ding and Yin, unpublished observation) is protective in tumor cells but indifferent or detrimental in nontransformed cells. In contrast, ROS-induced autophagy is prodeath in cancer cells but may not be so in nontransformed cells **(79)**.

Cell death and autophagy can be mediated by the same molecule, but through separate mechanisms. Thus, while autophagy and cell death may seem to be coupled by the same molecule, their relationship can be more complicated than a simple interpretation. This point may be best illustrated in the case of Bnip3, a BH3-only Bcl-2 family protein originally identified as an E1B19K and Bcl-2 interacting molecule **(90)** (see Chapter 2). Bnip3 is usually localized at the mitochondrial outer membrane. Unlike other BH3-only molecules, its transmembrane (TM) domain, but not its BH3-domain, is required for its activity and membrane targeting **(91, 92)**. The expression of Bnip3 is often low in normal conditions, but it can be rapidly induced in adverse conditions, in particular, in hypoxia or ischemia **(29, 30, 93, 94)**. Bnip3 can be transcriptionally activated by HIF-1 **(29, 30)** or FoxO3 **(27)** but repressed by NF-κB **(95)** or Rb/E2F **(96)**.

The increased expression of Bnip3 is often accompanied by apoptotic or necrotic cell death, and autophagy, in cell lines subjected to hypoxia **(29, 74, 96)**, or treated with ceramide or arsenic trioxide **(97, 98)**, or in cardiac myocytes subjected to ischemia-reperfusion injury **(99, 100)**. While both cell death and autophagy can be attributed to Bnip3, in most studies, direct evidence that autophagy contributes to cell death is limited, e.g., with tumor cells under hypoxia **(74)**. In contrast, in a cardiac myocyte line under simulated ischemia-reperfusion

conditions **(99)**, or in murine embryonic fibroblasts subjected to hypoxia **(101)**, autophagy was found to be important for cell survival. In both cases, it was found that this protective effect is due to the Bnip3-promoted autophagic removal of damaged mitochondria (mitophagy) **(99**, **101)**, which is necessary to prevent increased levels of reactive oxygen species **(101)**. Interestingly, a close homologue of Bnip3, Bnip3L/Nix, can also be induced by hypoxia and has been shown to be required for mitophagy during erythrocyte maturation **(102**, **103)**.

Could the different roles of Bnip3-mediated autophagy be related to different cell types under different treatments or be related to other factors? The mechanism by which Bnip3 induces autophagy has not been completely elucidated, although one study indicated that Bnip3 could bind to and inactivate Rheb, therefore inactivating mTOR during hypoxia **(104)**. As mTOR is a major factor of autophagy suppression, the negative effect of Bnip 3 on mTOR can be expected to induce autophagy. Bnip3 is thus considered to be required for both hypoxia-induced autophagy and hypoxia-induced mTOR repression. Alternatively, Bnip3 may compete with Bcl-2 for binding to Beclin-1 and thus can cause the derepression of Beclin-1, which in turn promotes autophagy **(101)** (Fig. 30.1).

It is not completely clear whether cell death and autophagy are mechanistically coupled in the case of Bnip3. It is possible that Bnip3-induced cell death may not be related to autophagy but instead goes through a separate mechanism. Some studies suggested that cell death is related to mitochondria permeabilization by its dimerized TM domain with or without the participation of Bax and Bak **(105**, **106)**. It has been postulated that Bnip3's killing activity may be secondary to prolonged hypoxia, ischemia-reperfusion injury, and anaerobic glycolysis, which results in acidosis **(81)**. Notably, acidosis has been found to stabilize Bnip3 and to increase its association with the mitochondria and its killing ability **(107)**. Furthermore, hypoxia or ischemia does not induce cell death in cardiac myocytes in the absence of acidosis **(108)**. Thus, it is possible that Bnip3 may primarily induce autophagy, as a protective mechanism at the early stage of hypoxia/ischemia, but induce cell death at the later stage when acidosis occurs, which promotes Bnip3 interaction with the mitochondria and membrane permeabilization.

Finally, when considering the cell death's relationship with autophagy, one may need to be aware that sometimes the same phenomenon may be subjected to different interpretations, as the mechanisms of the action are not always clear, particularly at the beginning. A pan-caspase inhibitor, z-VAD, is often used to suppress caspases and, therefore, caspase-mediated apoptosis. However, this chemical could inhibit other types of proteases, notably, the lysosomal cathepsins **(69)**. The application of z-VAD to several types of cells resulted in the increased accumulation of autophagic markers and necrotic cell death that seemed to be attributable to autophagy **(109)**. However, further investigation indicated that the increased autophagosomes are not due to an increase in the induction, but to a reduction in the degradation of the autophagosome because

of the lysosomal inhibition by z-VAD **(69)**. Thus, the increased cell death is related to reduced, rather than increased, autophagy, which was confirmed by a subsequent study indicating that autophagy was prosurvival rather than prodeath following z-VAD treatment.

The Potential Clinical Significance of Modulating Autophagy to Control Cell Death

Understanding the various conditions under which autophagy may be prosurvival or prodeath can have practical benefits in controlling the disease process. This topic is particularly attractive for cancer therapy, where the goal is to eliminate tumor cells by promoting cell death. Both properties of autophagy in regulating cell death have been explored for this purpose. Thus, numerous reports have indicated that suppressing autophagy during treatment with chemotherapeutic agents such as alkylating drugs, proteasome inhibitors, and histone deactylase inhibitors **(39**, **63**, **64)** could enhance apoptosis in various types of tumor lines. Conversely, promoting autophagy by the combined use of rapamycin has been found to enhance radiation therapy **(73)**. The future challenge is to define the conditions under which autophagy plays a specific role in cell death, promotion, or inhibition. It is possible to take advantage of this dichotomic characteristic of autophagy for maximal benefits. For example, ER-stress inducers **(38)** and proteasome inhibitors (Ding and Yin, Molecular Cancer Therapeutics, inpress, 2009) can induce the prosurvival function of autophagy in the tumor cells, but they induce the prodeath function in normal cells. Thus, the suppression of autophagy *in vivo* may specifically enhance death in the tumor cells, but reduce death in the normal cells under these circumstances.

The prodeath activity of autophagy during ischemic injury in the brain **(75)** may be suppressed to reduce organ damage. Other than directly targeting the autophagy genes, it may be possible to act on specific targets if the mechanism of autophagic death is known, such as the inactivation of Bcl-xL by the cleaved Atg5 **(71)**. On the other hand, autophagic activity can be enhanced for the removal of misfolded proteins, such as the mutant huntingtin, as seen in Huntington's disease, and the alpha-1 antitrypsin Z mutant **(26**, **37**, **110)**. The prosurvival function of autophagy could thus be explored in these conformational diseases.

Conclusion

Autophagy is an evolutionarily conserved physiological process that degrades various cellular contents. Its functions are thus interfaced with cellular survival and cell death. Depending on the context, autophagy activity could contribute to either cell survival or cell death. Only in very limited cases are the mechanisms by

which autophagy modulates cell death understood. But both apoptosis and necrosis can be modulated by autophagy, and excessive digestion by autophagy can also lead to cellular atrophy and direct death. Future work should focus on understanding individual cases so that this character of autophagy can be explored for cancer therapy, control of tissue injury, and treatment of conformational diseases.

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