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Autophagy and Cell Death

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Autophagy is a physiological and evolutionarily conserved phenomenon maintaining homeostatic functions like protein degradation and organelle turnover. It is rapidly upregulated under conditions leading to cellular stress, such as nutrient or growth factor deprivation, providing an alternative source of intracellular building blocks and substrates for energy generation to enable continuous cell survival. Yet accumulating data provide evidence that the autophagic machinery can be also recruited to kill cells under certain conditions generating a caspase-independent form of programed cell death (PCD), named autophagic cell death. Due to increasing interest in nonapoptotic PCD forms and the development of mammalian genetic tools to study autophagy, autophagic cell death has achieved major prominence, and is recognized now as a legitimate alternative death pathway to apoptosis. This chapter aims at summarizing the recent data in the field of autophagy signaling and autophagic cell death. © 2007, Elsevier Inc.

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I. Introduction

Programed cell death (PCD) is an evolutionarily conserved phenomenon observed especially in multicellular organisms, which is crucial for several vital functions, including developmental morphogenesis, tissue homeostasis, and defense against pathogens. In their seminal article published in 1972, Kerr, Wyllie, and Currie described two major types of cell death: apoptosis, the genetically controlled PCD, and necrosis, the nonprogramed and accidental type of cell death (Kerr *et al*., 1972). In the following three decades, the term ''apoptosis'' was used as a general term to describe PCD and an impressive amount of information has accumulated regarding the molecular mechanisms governing this phenomenon. Because the apoptosis versus necrosis concept of cell death was so dominant, early observations about the existence of alternative, nonapoptotic PCD types were ignored by the majority of the scientific community (Schweichel and Merker, 1973). In recent years, however, increasing interest in alternative PCD types emerged once different tools to study these other genetically controlled systems at the molecular level became available. One type, autophagic cell death, recently received considerable momentum in light of the identification of the mammalian orthologues of the yeast autophagic genes. As a consequence, autophagic cell death is currently recognized as one of the major alternative or complementary cell death pathways to apoptosis in several experimental systems.

In this chapter, we will describe the morphological and molecular basis of autophagy and autophagic cell death, document what is known so far about proteins and pathways regulating this phenomenon and finally discuss its crosstalk with apoptosis. A crucial issue in the field is how to reconcile the catabolic and survival-related role of autophagy with its cell death-inducing properties. We will also discuss this important issue in light of recent observations.

II. Description of Programed Cell Death Morphologies

Revisiting the earlier work of Schweichel and Merker (Schweichel and Merker, 1973), in a review article from 1990, Clarke described three major cell death morphologies of cell death during embryonic development or after toxin treatment (Clarke, 1990). Clarke classified classical apoptosis as Type I cell death. This type of cell death is characterized morphologically by cell shrinkage, chromatin condensation, nucleosomal DNA degradation and finally, fragmentation of the cell into so-called apoptotic bodies. Caspases are the key mediators of this type of cell death and most of the

observed morphological changes can be attributed to the cleavage of key cellular target proteins by these cysteine proteases. The final destination of the apoptotic bodies is the lysosomes of phagocytes or neighboring cells after heterophagocytosis.

Autophagic cell death was classified as the Type II cell death by Clarke. The most prominent morphological change observed in this type of cell death is the appearance of double- or multiple-membrane enclosed vesicles in the cytoplasm that engulf portions of cytoplasm and/or organelles such as mitochondria and endoplasmic reticulum. These vesicles fuse with lysosomes and deliver their cargo for degradation by lysosomal enzymes of the same cell. This process is termed as autophagy (self-eating in Greek), corresponds to the previous definition of macroautophagy, referred to as the term autophagy in this text. While the initial observation that certain dying cells display morphological hallmarks of autophagy is beyond dispute, the question of whether autophagy is causative for cell death became a major issue for intensive research. One working hypothesis suggested that under specific circumstances that depend on the character of the stimulus, its amplitude, and duration, extensive autophagy may cause cell death. According to this possibility, the cell cannibalizes itself from inside, and the ultimate cause(s) of cellular demise should be identified as will be detailed in this chapter. Of note, nuclear changes, such as chromatin condensation, appear later in autophagic cell death than they do in apoptosis, and there is no DNA fragmentation or formation of apoptotic bodies. Clearance of the remnants of the dead cells by phagocytosis may occur later and more sporadically than that seen in apoptosis.

Type III cell death, defined by Clarke as nonlysosomal vesiculate degradation, which represent a less well-studied type of cell death, is out of the scope of this chapter, therefore it will not be discussed further. Cells simultaneously harboring the morphological characteristics of more than one of the types of cell death described above have also been observed in certain tissues and under certain conditions. This suggests the existence of additional mixed types of PCD that proceed with concomitant activation of several death mechanisms in the same cell (Clarke, 1990).

III. Autophagic Cell Death

A. Autophagy May Kill: Molecular Evidence

Morphological analysis of cells and tissues revealed an increased autophagic activity during developmental cell death in several organisms. The list includes cell death during insect metamorphosis, limb bud morphogenesis in birds, and palatal closure in mammals (Bursch, 2001; Clarke, 1990;

Schweichel and Merker, 1973). Certain toxins also caused cell death with ultrastructural characteristics of autophagic cell death (Schweichel and Merker, 1973). Although a causal relationship between the autophagic activity and cell death could not be measured and the observation stayed at the correlative level, these studies opened the way to subsequent studies dissecting the role of autophagy in cell death.

The discovery that some chemical compounds like 3-MA, wortmannin, and LY294002 inhibited autophagy, was a turning point in the analysis of autophagic cell death (Blommaart *et al*., 1997; Seglen and Gordon, 1982). Several independent groups using different cell types and death stimuli reported the existence of a caspase-independent cell death which proceeded with the accumulation of autophagic vesicles and increased lysosomal activity, and which was attenuated by inhibitors of autophagy. Antiestrogen-induced death of the breast cancer line MCF-7 (Bursch *et al*., 1996), TNF-α-induced death of leukemia cells (Jia *et al*., 1997), oncogenic Ras-induced death of gastric or glioma cells (Chi *et al*., 1999) or growth factor withdrawal induced death of neuronal cells (Xue *et al*., 1999) may be cited as examples of these studies. Yet the most commonly used autophagy inhibitor, 3-MA, used at the dose range that optimally inhibits autophagy, also inhibited JNK and p38 activation (Xue *et al*., 1999), attenuated mitochondrial permeability transition pore opening (Xue *et al*., 2002) and increased lysosomal pH (Caro *et al*., 1988). Due to these multiple pleiotropic effects, the results obtained from these studies were evaluated with caution by the scientific community and the role of autophagy observed in dying cells remained a debated issue (reviewed by Gozuacik and Kimchi, 2004; Levine and Yuan, 2005). More convincing indications for the relevance of this phenomenon to death regulation was provided by studies demonstrating autophagic cell death induction by well-established death-promoting proteins like BNIP3 (Vande Velde *et al*., 2000), death-associated protein kinase (DAPk) and DRP-1/DAPk2 (Inbal *et al*., 2002), and from studies documenting the suppression of autophagy by death protective proteins like Bcl-2 (Cardenas-Aguayo Mdel *et al*., 2003; Saeki *et al*., 2000; Vande Velde *et al*., 2000; Xue *et al*., 2001; Yanagisawa *et al*., 2003).

The discovery of genes which are part of the basic machinery of autophagy, initially made in yeast by several independent groups, opened a new era in autophagy studies and led to a better understanding of molecular events underlying this biological phenomenon (Kim and Klionsky, 2000; Ohsumi, 2001). Orthologues of the yeast genes were identified soon thereafter in several organisms including *Dictyostelium*, *C. elegans*, *Drosophila*, mouse, and human (Klionsky *et al*., 2003). Further studies confirmed that the autophagic function of these orthologues in higher organisms was conserved. These major advances opened the way to study the role of autophagy in cell death, using genetic approaches like knockout of autophagic genes

and RNAi or antisense-mediated knockdown strategies. It was shown that the RNAi-mediated knockdown of autophagic proteins like Atg5, Atg7, and Beclin-1 (see below) attenuated cell death developing under certain conditions. This included death of L929 mouse fibroblastic cells, U937 monocytic cells and macrophages in the presence of the pan-caspase inhibitor zVAD (Xu *et al*., 2006; Yu *et al*., 2004), etoposide and staurosporine-induced death of Bax/Bak knockout fibroblasts (Shimizu *et al*., 2004) and cell death-induced by overexpression of a short mitochondrial form of the p19ARF tumor suppressor (smARF) (Reef *et al*., 2006). The finding that the inhibition of autophagic activity by knocking down these autophagic genes could under certain conditions attenuate the death responses of cells supported very significantly the existence of a nonapoptotic, caspase-independent cell death type proceeding with accumulation of autophagic vesicles, and depending on autophagy proteins. In light of these new molecular strategies to inhibit autophagy, the earlier studies that utilized less specific autophagic inhibitors such as 3-MA and wortmannin, which nevertheless conferred a protection from cell death comparable to the RNAi knockdown studies, should be revisited.

B. How Does Autophagy Kill?

In principle, autophagic activity above a certain threshold which destroys a major portion of the cytosol and organelles could lead to an irreversible type of cellular atrophy and cause a total collapse of cellular functions. Indeed, it was observed that during extensive autophagy, the total area of autophagic vacuoles and dense bodies may be roughly equal to, or greater than, that of cytosol and organelles outside the vacuoles (Clarke, 1990; Lum *et al*., 2005). It is therefore plausible to think that such a degree of cellular destruction could lead to cellular demise. In line with this hypothesis, it was shown that autophagy induction by growth factor deprivation in neurons, or by staurosporine treatment of HeLa or CHO cell lines exposed to caspase inhibitors, was accompanied by the destruction of the majority of the mitochondria and by irreversible commitment to death (Xue *et al*., 2001). In IL-13-dependent bone marrow cells derived from the Bax/Bak knockout mouse, cytokine deprivation led to progressive atrophy by autophagy and finally, cell death ensued (Lum *et al*., 2005). Nevertheless, in this cellular setting, damage to the cell was reversible up until a certain threshold, and the cell death observed in these studies was relatively slow, indicating that other mechanisms contributing to cellular demise may exist in autophagic cell death.

A second possible mechanism of killing by autophagy may include the selective degradation of vital proteins in the cell. Although macroautophagy,

the relevant type of autophagy discussed in this chapter, is generally considered a nonselective phenomenon, there is evidence indicating a selective destruction of certain vital cellular components which could contribute to death induction by autophagy. Selective autophagic elimination of depolarized mitochondria, endoplasmic reticulum, and peroxisomes was observed by several independent groups (Elmore *et al*., 2001; Hamasaki *et al*., 2005; Iwata *et al*., 2006; Kissova *et al*., 2004). Likewise, long-lived proteins, mutant protein aggregates, and certain intracellular bacteria and viruses were specifically recognized and selectively eliminated by autophagy, indicating that selectivity may be a general property of autophagy under certain circumstances (Reggiori and Klionsky, 2005; Webb *et al*., 2003; Yu *et al*., 2005). Therefore, an alternative death-induction mechanism by autophagy may consist of selective elimination of vital organelles and/or proteins involved in cell survival and homeostasis, although the mechanisms regulating this selectivity still remain obscure.

Evidence in support of the contribution of selective protein elimination to autophagic cell death in mammalian cells comes from a recent study of zVAD-induced autophagic cell death observed in certain cell types. Lenardo and coworkers elegantly showed that catalase, a key enzyme of the cellular antioxidant defense mechanism, was selectively eliminated during autophagic cell death (Yu *et al*., 2006). Oxidative stress induced by the degradation of catalase was directly responsible for cellular demise, since inhibitors of autophagy, RNAi-mediated knockdown of autophagic genes, or various ROS inhibitors, prevented cell death. Thus, catalase may be an example of the selective protein target(s) of autophagy contributing to the irreversible cellular damage that leads to cell death. Discovery of additional selective autophagy targets and mechanisms controlling their degradation may further clarify how a catabolic and prosurvival mechanism can be used for cellular suicide.

IV. Autophagy and Autophagic Cell Death Regulatory Mechanisms

Although initial morphology-based studies were performed in mammalian cells and tissues, genes regulating autophagy were discovered by several independent groups in yeast (Harding *et al*., 1995; Thumm *et al*., 1994; Tsukada and Ohsumi, 1993). These studies resulted in the cloning of a partially overlapping set of APG, AUT, and CVT genes by independent groups. The confusing nomenclature has been simplified by consensus to a unified nomenclature of ATG genes (*a*u*t*ophagy-related genes) (Klionsky *et al*., 2003). The list to date includes 27 proteins that are involved in various stages of the autophagic

process, including vesicle enucleation and their subsequent expansion, autophagic vesicle fusion to late endosome/lysosome, and cargo degradation. Although we are still far from having a complete picture, upstream pathways regulating autophagy started to be uncovered and some of the genes which mediate the formation of the autophagosomes were organized along molecular pathways. In this section, we will describe these pathways and discuss their relevance to autophagic cell death.

A. Class III Phosphatidylinositol 3-Kinase, Beclin-1, and Autophagy

Phosphatidylinositol 3-phosphate (PI3-P) generated by the Class III PI3-kinase is the major lipid signal controlling autophagic vesicle formation (Petiot *et al*., 2000). Indeed, the widely used chemical inhibitors of autophagy, 3-MA, wortmannin and LY294002, target the Class III PI3-kinase through competition for ATP binding in the active site of its kinase domain, with variable efficiency and specificity (Petiot *et al*., 2000; Seglen and Gordon, 1982). Consequently, treating cells with the Class III product, PI3-P, accelerates autophagy, while Class I PI3-kinase products inhibit it (Petiot *et al*., 2000).

Studies performed mainly in yeast and mammalian cells showed that a protein complex consisting of Atg6 (Vps30 or Beclin-1 in mammalians) and myristylated serine kinase Vps15/p150 regulates the activity of the autophagy-related Class III PI3-kinase Vps34 (Stack *et al*., 1995). In line with this, expression of Vps15/p150 or Beclin-1 stimulated autophagy in mammalian cell lines (Liang *et al*., 1999; Petiot *et al*., 2000). Furthermore, Class III PI3-kinase inhibitors and knockdown of Beclin-1 attenuated death in several different cell types (Reef *et al*., 2006; Shimizu *et al*., 2004; Xu *et al*., 2006; Yu *et al*., 2004). Atg14 is also part of the complex in the yeast. Unlike the other members of the complex, the mammalian homologue of Atg14 has yet to be discovered. In the yeast, Atg14 localizes to yeast-specific autophagy organizing structures called preautophagosomal structures (PAS) and serves as an adaptor for the Atg6/Vps34 complex (Kim *et al*., 2002). Mammalian Beclin-1 localizes to the trans-Golgi network and the endoplasmic reticulum, suggesting that multiple foci in these compartments may be the sites of PI3-P formation and autophagic vesicle nucleation in mammals (Kihara *et al*., 2001; Liang *et al*., 1998; Pattingre *et al*., 2005). In general, PI3-P mediates docking of FYVE (for conserved in *F*ab1, *Y*OTB, *V*ac1, and *E*EA1) or PX (*P*ho*x* homology) domain-containing proteins to nucleation sites (Gillooly *et al*., 2001; Wishart *et al*., 2001). The identity of the PI3-P-binding proteins that regulate autophagy pathways have yet to be discovered.

Beclin-1, the mammalian orthologue of Atg6, was cloned as a Bcl-2 interacting protein (Liang *et al*., 1998). In fact, Bcl-2 blocked the Beclin-1

interaction with Vps34, decreased Class III PI3-kinase activity in cells and downregulated starvation-induced autophagy, both in cell culture and in mouse cardiac muscle (Pattingre *et al.*, 2005). In line with this, immunoprecipitation of Beclin-1 from starved cells brought down less Bcl-2 compared to controls grown in nutrient rich conditions, indicating that the Bcl-2/Beclin-1 interaction may be regulated by autophagy-inducing signals. Strikingly, Beclin-1 mutants which can no longer bind Bcl-2 caused excessive autophagy and cell death, highly suggesting that the protective effect of Bcl-2 on autophagic cell death may be related to its ability to sequester Beclin-1 and thus inhibit Vps34 PI3-kinase activity (Canu *et al.*, 2005; Cardenas-Aguayo Mdel *et al.*, 2003; Saeki *et al.*, 2000; Vande Velde *et al.*, 2000).

B. Two Ubiquitin-Like Pathways Involved in Autophagic Vesicle Formation

Two ubiquitin-like pathways play a central role in autophagic vesicle formation. The first system involves Atg12, which is covalently conjugated to Atg5 through the sequential action of the E1 ligase-like protein Atg7 and E2-like protein Atg10 (Mizushima *et al.*, 1998; Shintani *et al.*, 1999; Tanida *et al.*, 1999). Atg12 is conjugated to Atg5 just after its synthesis, and this event seems not to be regulated by autophagy-inducing signals. The Atg12/Atg5 complex then binds to Atg16 which, through its homo-oligomerization capacity, leads to the formation and stabilization of larger protein complexes (350 kDa in yeast and 800 kDa in mammals) (Mizushima *et al.*, 1999, 2003). Atg12 conjugation to Atg5 is necessary for the second ubiquitin-like pathway to proceed and autophagic vesicles to form (Mizushima *et al.*, 2001; Suzuki *et al.*, 2001).

The second ubiquitin-like pathway involves the conjugation of Atg8 protein (mammalian MAP LC3 protein) to a lipid molecule, phosphatidylethanolamine (PE) (Ichimura *et al.*, 2000) or, at least *in vitro*, phosphatidylserine (Sou *et al.*, 2006). In this manner, several Atg8 proteins recruit lipids molecules to expand the autophagic membranes. As soon as Atg8 is translated, a protease called Atg4 cleaves off a portion of its C-terminus, exposing a critical C-terminal glycine residue (Kirisako *et al.*, 2000). Following the ubiquitination-like reaction mediated by Atg7 and Atg3, the amino group of the lipid molecule is conjugated via an amide bond to this glycine residue (Ichimura *et al.*, 2000). Lipid conjugation leads to the conversion of the soluble 18 kDa form of Atg8 (mammalian LC3-I) to an autophagic vesicle-associated, faster migrating 16 kDa form on SDS-PAGE gels (mammalian LC3-II). Immunostaining of Atg8/LC3 indicates a change from a diffuse cytoplasmic or nuclear distribution to punctate dots which reflect autophagic

vesicles (Kamada *et al.*, 2000; Kirisako *et al.*, 1999). Analysis of LC3 by SDS-PAGE and immunolocalization is now, in addition to the more labor-intensive transmission electron microscopy, commonly used to detect and quantify autophagic activity in cells and whole animals (Mizushima, 2004).

C. The Tor Pathway

Discovered as the target of the drug rapamycin, the Tor serine/threonine (Ser/Thr) kinase (also known as RAFT1, FRAP or SEPT) is the central player of a complex signaling network regulating several cellular events, including protein synthesis, cell growth and proliferation, as well as autophagy (Fingar and Blenis, 2004). The Tor pathway is modulated by growth factor, nutrient and energy availability, osmotic stress and DNA damage, and its activation suppresses autophagy.

In yeast under nutrient rich conditions, Tor phosphorylates autophagy-related protein Atg13, leading to it dissociation from a protein complex containing Atg1 kinase. Atg13 dissociation attenuates Atg1 kinase activity, which correlates with autophagy induction (Kamada *et al.*, 2000; Scott *et al.*, 2000). Under nutrient limitation, Tor activity is blocked, Atg13 is rapidly dephosphorylated, and it tightly associates with Atg1 kinase. This promotes activation of Atg1, leading to autophagy induction (Abeliovich *et al.*, 2003; Kamada *et al.*, 2000). Additionally, the Tor complex regulates the function of the three yeast orthologues of the Ser/Thr phosphatase PP2Ac (PPH21/22 proteins), through phosphorylation of their regulatory subunit TAP42 (Di Como and Arndt, 1996). The relevance of these pathways to mammalian autophagy and autophagic cell death is yet to be discovered.

The mammalian Tor orthologue, mTor, exists in the cell as part of two distinct multiprotein complexes (Fingar and Blenis, 2004): raptor/mTor and rictor/mTor complexes. The complex containing the raptor protein regulates cell survival and autophagy through p70S6K and 4E-BP1 phosphorylation. p70S6K phosphorylates a wide spectrum of proteins involved in transcription, protein synthesis and RNA splicing, including the 40S ribosomal protein S6, eukaryotic elongation factor 2 kinase (eEF-2 kinase), and transcription and splicing related proteins. mTor mediated activation of the 4E-BP1 also contributes to the prosurvival role of Tor, since it leads to upregulation of cap-dependent translation. Furthermore, p70S6K phosphorylates and inactivates the proapoptotic BH3-only protein Bad (Harada *et al.*, 2001). Another p70S6K target, eEF-2 kinase, inhibits protein synthesis through phosphorylation and inactivation of elongation factor 2. Its phosphorylation by p70S6K relieves this inhibition by inactivation of the kinase (Wang *et al.*, 2001).

Binding of the small GTPase Rheb is a critical step in the activation of the mTor complex. Rheb is under strict control through regulation of its GTPase activating proteins TSC1 (hamartin) and TSC2 (tuberin) (Fingar and Blenis, 2004). Phosphorylation of TSC2 by an intracellular energy status sensor and an AMP-activated kinase, AMPK, leads to the stabilization of the TSC1/2 complex, increases its GTPase activity toward Rheb, inactivating it and thereby inhibiting the raptor/mTor complex and activating autophagy (Inoki *et al*., 2003). On the other hand, survival-related kinases Akt/PKB, ERK (Ma *et al*., 2005), and RSK1 (Roux *et al*., 2004) activate the mTor pathway through phosphorylation and inhibition of TSC2. Hypoxia also inhibits mTor activity through hypoxia inducible factor (HIF) transcriptional targets REDD1 (Brugarolas *et al*., 2004), REDD2 (Corradetti *et al*., 2005) and in a TSC1/2-dependent manner. Thus, TSC1/2-dependent control of Rheb is a convergence point of several metabolic, prosurvival, and stress-related signals regulating mTor activity.

AMPK and TSC1/2 may also connect DNA damage-induced stress to mTor inhibition and to autophagy activation, since this pathway was necessary for etoposide-induced and p53-dependent autophagy activation (Feng *et al*., 2005). In line with this, the DNA damaging agent etoposide was capable of inducing autophagic cell death in Bax/Bak knockout cells (Shimizu *et al*., 2004). The second Tor complex, consisting of rictor and mTor, is not inhibited by rapamycin, and therefore its relevance to autophagy pathways is not clear.

D. Class I Phosphatidylinositol 3-Kinase and Autophagy

Class I PI3-kinase antagonizes apoptosis and autophagy through activation of the PDK1 and Akt/PKB pathway. Following growth factor binding to cell surface receptors, Class I PI3-kinase is activated to generate PI3,4-diphosphate and PI3,4,5-triphosphate. These lipid products recruit PDK1 and Akt/PKB to cell membranes through their Pleckstrin homology (PH) domains, where PDK1 activates Akt/PKB by phosphorylation. Akt/PKB promotes cell survival through phosphorylation of several substrates. Phoshorylation of the cell death-related Bcl-2 family member Bad leads to its inactivation through sequestration to the cytoplasm by 14-3-3 proteins. Transcriptional induction of death-related genes is also blocked following the nuclear exclusion of forkhead transcription factors as a result of their phosphorylation by Akt/PKB. In another survival promoting mechanism, Mdm2 phosphorylation by Akt/PKB enhances p53 ubiquitination and degradation (Zhou *et al*., 2001). Akt/PKB has also been shown to activate the antiautophagic mTor pathway either by directly phosphorylating and inhibiting TSC2 (Inoki *et al*., 2002) or by decreasing AMP/ATP ratios and hence

suppressing AMPK activity (Hahn-Windgassen *et al.*, 2005). The tumor suppressor PTEN, which inactivates the Akt/PKB pathway by dephosphorylation of Class I PI3,4,5-triphosphate, upregulated autophagy in HT-29 colon cancer cells (Arico *et al.*, 2001). Indeed, PTEN overexpression was shown to counteract Akt/PKB-mediated survival signals and caused cell death in various cell types (Li *et al.*, 1998; Wang *et al.*, 2000).

Interestingly, the antiautophagic nature of Class I PI3-kinase is not universal. Class I PI3-kinases and growth hormones that activate this pathway can actually induce autophagic cell death in glucose-deprived muscle derived H9c2 and C2C12 cell lines, but not in PC12 or HepG2 carcinoma cell lines. In other cell types such as HT-29 colon cancer cell lines, treating cells with Class I PI3-kinase products, expression of activated Akt/PKB kinase or IL-13 treatment inhibited autophagy, while Akt/PKB inhibitors stimulated autophagy (Petiot *et al.*, 2000; Takeuchi *et al.*, 2005). Therefore, cell type and/or tissue-specific signaling connections may modulate the outcome of the stimulation of this pathway (Aki *et al.*, 2003).

E. Protein Transcription/Translation-Related Pathways

Phosphorylation of the eukaryotic initiation factor 2α?(eIF2α) on serine-51 by a conserved family of protein kinases represents a central and evolutionarily conserved mechanism of stress-induced translation regulation and autophagy. In yeast during amino acid starvation, eIF2α phophorylation by the yeast eIF2α kinase GCN2 inhibits global protein synthesis and favors translation of GCN4, a stress-related transcription factor, by an alternative mechanism (Dever *et al.*, 1992). In addition to the transcriptional activation of several stress-related genes, including those involved in amino acid biosynthesis and transport, GCN4 also activates the transcription of autophagy-regulating genes ATG1, ATG13, and ATG14 (Natarajan *et al.*, 2001). Indeed, GCN2 and GCN4, and eIF2α phosphorylation were essential for starvation-induced autophagy. In addition, GCN4 was also necessary for rapamycin-induced autophagy (Talloczy *et al.*, 2002).

In mammalians, the eIF2α kinase family consists of four proteins that connect different stress conditions to cellular responses, including autophagy. GCN2 is activated during amino acid starvation by uncharged tRNAs. PKR is activated during viral infection by double stranded RNA. PERK is activated during endoplasmic reticulum stress by unfolded proteins, and HRI is activated by low heme levels. In addition to its role in starvation-induced autophagy, as in the yeast, mammalian eIF2α phosphorylation was also found to be necessary for virus-induced autophagy in primary murine embryonic fibroblasts (Talloczy *et al.*, 2002). The eIF2α kinase PKR was also shown

to be essential for virus-induced autophagy, and it was able to complement the yeast GCN2 to induce autophagy in response to starvation (Talloczy *et al.*, 2002). Therefore, in mammalian cells, stress responses triggered by eIF2α phosphorylation regulate autophagy activation by viral infection or amino acid starvation. Whether this pathway plays a role in the autophagic cell death observed following additional cellular stresses still needs to be studied.

Other molecular events controlling global protein synthesis include 4E-BP1 phosphorylation and eEF-2 kinase activation. 4E-BP1 (also known as PHAS-I) is a repressor of the mRNA cap-binding factor eIF4E. Growth factor stimulation under nutrient rich conditions leads to 4E-BP1 phosphorylation by mTor and other kinases, and induces its dissociation from eIF4E. eIF4E is then free to start a cascade of events leading to the correct positioning of the initiation complex and the 40S ribosomal subunit at the $5'$ end of mRNA, leading to cap-dependent translation. mTor inhibition was shown to block 4E-BP1 phopshorylation and thus cap-dependent translation (Beretta *et al.*, 1996).

eEF-2 kinase, another protein involved in translation regulation, is a target of the mTor downstream kinase p70S6K (Wang *et al.*, 2001). eEF-2 kinase was shown to block the elongation phase of translation by phosphorylation of eEF-2. A study showed that eEF-2 kinase was crucial for nutrient deprivation-triggered autophagy (Wu *et al.*, 2006). Like some other autophagy-related kinases [e.g., DAPk or DRP-1, see below], eEF-2 kinase is also regulated by calcium/calmodulin (Nairn *et al.*, 1985). Therefore, eEF-2 kinase may also connect calcium-dependent signals to autophagy induction through translation regulation.

As observed in yeast, inhibition of mammalian cap-dependent protein translation through the above-mentioned mechanisms could lead to upregulation of certain proteins involved in the transcriptional regulation of autophagy-related genes. Upregulation of these autophagy-related proteins could stimulate or support the progress of autophagy and even autophagic cell death. In fact, in several mammalian experimental systems, accumulation of autophagy regulating proteins was observed by several independent groups (Daido *et al.*, 2004; Kanzawa *et al.*, 2003; Scarlatti *et al.*, 2004; Shimizu *et al.*, 2004). Furthermore, overexpression of key autophagy-regulating proteins like Beclin-1 and Atg5, induced cell death with autophagic morphology, indicating the importance of transcriptional and/or translational regulation of autophagic cell death (Pattingre *et al.*, 2005; Pyo *et al.*, 2005). Supporting the concept of transcriptional control of autophagy, in *Drosophila*, a transcriptional program involving the steroid hormone ecdysone regulates autophagic cell death-mediated destruction of the salivary gland (Baehrecke, 2003). On the other hand, the protein translation inhibitor cycloheximide did not block the initial stages of autophagy in mammalian liver cells (Lawrence and Brown, 1993). Therefore, further studies are necessary to fully appreciate

the contribution of these transcriptional and/or translational mechanisms to autophagy and autophagic cell death.

F. G Proteins

In HT-29 colon cancer cells, amino acids and the Ras/Raf1/ERK1/2 pathway were shown to modulate autophagy through regulation of ER- and Golgi-localized heterotrimeric G proteins (Houri *et al*., 1993; Ogier-Denis *et al*., 1995; Pattingre *et al*., 2003a). Amino acid starvation was shown to relieve Raf1 from an inhibitory dephosphorylation and enhance phosphorylation of ERK1/2 by Raf1. Activated ERK1/2 in turn caused direct activation of the GAIP protein (Ogier-Denis *et al*., 2000; Pattingre *et al*., 2003a). GAIP is a GTPase activating protein for the Gαi3 class of G proteins. Activation of GTP hydrolysis by Gαi3 and stabilization of its GDP-bound form by AGS3 protein resulted in the stimulation of autophagy (Ogier-Denis *et al*., 1995, 1997; Pattingre *et al*., 2003b). To date, the mechanism of autophagy induction by the heterotrimeric G protein complex is unknown. Considering the Golgi/ER localization of the complex, hypothesized that Gαi3 could contribute to the control the flux of membrane from these compartments to autophagy organizing centers (Meijer and Codogno, 2004).

Akt/PKB can also modulate this G protein-dependent pathway through direct phosphorylation and inactivation of Raf1. Consequently, simultaneous stimulation of the Akt/PKB and Ras/Raf1/ERK1/2 pathways by EGF treatment failed to induce autophagy (Pattingre *et al*., 2003a). On the other hand, PTEN, which is a negative regulator of Akt, stimulated autophagy in the same system (Arico *et al*., 2001). Thus, a complex network which consists of at least three modulators, namely amino acid availability, Ras/Raf1/ERK1/2 signaling, and the Akt/PKB pathway, regulate autophagy activation in this system. In line with this, overexpression of Ras was able to activate autophagy and autophagic cell death in several systems (Chi *et al*., 1999; Pattingre *et al*., 2003a).

G. DAPk Family

A functional genetic screen for mediators of interferon-γ-induced cell death led to the isolation of several new death-associated proteins (Deiss *et al*., 1995). One of the genes isolated by this approach, DAPk (DAPk or DAPk1), and its homologues DRP-1/DAPk2 and ZIPk/DAPk3, were later shown to be involved in cell death-induced by other stimuli as well, including activation of Fas receptors, TNF-α, TGF-β, detachment from extracellular

matrix and oncogenes (Cohen and Kimchi, 2001; Inbal *et al*., 2002; Jang *et al*., 2002; Kawai *et al*., 2003; Raveh *et al*., 2001; Wang *et al*., 2002). While the DAPk family of Ser/Thr kinases has been shown to modulate the apoptotic death in some experimental settings, their overexpression in HEK 293 or HeLa cells induced autophagic Type II cell death (Gozuacik and Kimchi, 2004; Inbal *et al*., 2002; Shani *et al*., 2004).

Although physical interactions and a complex cascade-like signaling connection exist among the members of the DAPk family (Inbal *et al*., 2000; Shani *et al*., 2004), accumulating data indicate that individual members may have specific functions in different autophagy pathways. A dominant negative DRP-1/DAPk2, but not DAPk was able to attenuate tamoxifen-induced autophagy and amino acid starvation-induced in MCF-7 breast carcinoma cells (Inbal *et al*., 2002). Knocking down DAPk on the other hand attenuated the interferon-γ-induced autophagic cell death of HeLa cells (Inbal *et al*., 2002). In contrast, DAPk knockout primary fibroblasts and hepatocytes showed similar level of autophagy activation in response to amino acid starvation and inhibition of mTor with rapamycin, indicating that DAPk is not involved in these phenomena (D. Gozuacik and A. Kimchi, unpublished observations). Moreover, while DAPk associates with the actin cytoskeleton (Cohen *et al*., 1997), overexpressed DRP-1 was highly concentrated in the lumen of autophagic vesicles (Inbal *et al*., 2002), raising the possibility that DRP-1 may have a direct role in autophagic vesicle formation, possibly through phosphorylation of the components of autophagic vesicle formation machinery. Thus, the DAPk family of proteins seems to be stress-activated kinases linking different cellular stresses like interferon-γ exposure, starvation, or growth factor deprivation to autophagy pathways and to autophagic Type II cell death.

H. Sphingolipid Pathways

One of the classes of lipid found in eukaryotic membranes is sphingolipids. The biological function of these molecules goes far beyond the structural role that had been initially attributed to them. The sphingolipid metabolites ceramide, sphingosine, and sphingosine 1-phosphate (S1P) are now recognized as lipid messengers that regulate cell growth, survival, and death. Recent work also provides evidence that ceramide and S1P are involved in autophagy regulation.

Ceramide may be derived from the hydrolysis of sphingomyelin by sphingomyelinase enzymes (SMase) or it may be synthesized *de novo* through the action of serine palmitoyl transferase and ceramide synthase. Studies in cancer cell lines have implied that ceramide may act as an autophagic cell death signaling molecule, and provided evidence that classical autophagic

cell death-inducers like tamoxifen, used ceramide as a second messenger (Daido *et al*., 2004; Scarlatti *et al*., 2004). Ceramide can induce autophagy activation through several mechanisms: Ceramide treatment blocked the antiautophagic Akt/PKB and mTor pathways, upregulated expression of key autophagy regulators Beclin-1, LC3, and the pro-death Bcl-2 family member BNIP3, and led to mitochondrial membrane potential loss. Of note, the autophagic cell death kinase DAPk was also upregulated and activated on ceramide treatment, and it was shown to be play a central role in ceramide-induced cell death (Pelled *et al*., 2002; Shohat *et al*., 2001).

S1P and one of the enzymes responsible for its production, sphingosine kinase 1 (SK1), also induced autophagy in MCF-7 cells (Lavieu *et al*., 2006). S1P is generated on phosphorylation by sphingosine kinases of sphingosine (which itself is produced by deacylation of ceramide by ceramidases). Unlike ceramide, SK1 and S1P seem to be involved in survival-related autophagy under nutrient limiting conditions, rather than autophagic cell death, since blockage of autophagy induced by SK1 and S1P killed the cells by apoptosis rather than rescuing them from death. In line with this, nutrient starvation in mammalian or yeast cells stimulated endogenous SK1 activity (Lanterman and Saba, 1998; Lavieu *et al*., 2006) and starvation-induced autophagy was blocked by a SK1 inhibitor or a dominant negative SK1 expression (Lavieu *et al*., 2006). At the molecular level, there are differences between ceramide and S1P-induced autophagy. In contrast with ceramide, SK1 did not lead to the inactivation of the Akt/PKB prosurvival pathway, but while blocked the mTor pathway by an independent mechanism. Also, upregulation of Beclin-1 expression by SK1 occurred to a lesser extent compared to that observed after ceramide treatment (Lavieu *et al*., 2006).

All these studies implicate the sphingolipids ceramide and S1P and their upstream modulators in autophagy signaling. The potential outcome of the opposing effects of these sphingolipids will be discussed in detail in the following sections.

I. Role of Mitochondria and the Bcl-2 Family Proteins in Autophagy

Mitochondria are one of the most prominent targets of autophagy, and several studies in yeast and mammalian cells addressed the role of mitochondrial destruction by autophagy in cell survival and death. In yeast, Uth1p, an outer mitochondrial membrane protein, was necessary for mitochondrial autophagy (Kissova *et al*., 2004), and inactivation of its gene conferred resistance to Bax- or rapamycin-induced cell death (Camougrand *et al*., 2003). Under nonstarvation conditions, yeast mutations causing mitochondrial dysfunction, loss of mitochondrial membrane potential, and defects in mitochondrial biogenesis were shown to trigger mitochondrial autophagy

and cell death (Priault *et al.*, 2005). Interestingly, despite the extensive elimination of mitochondria, the site of aerobic ATP production, intracellular ATP levels actually increased during autophagy in wild-type cells, probably due to the shutdown of energy-consuming processes such as protein translation. This observation may indicate that deficiencies of mitochondrial functions other than respiration and energy supply (e.g., lipid, heme, amino acid, and nucleotide synthesis) may be the critical contributors to cell death induction by mitochondrial dysfunction (Priault *et al.*, 2005).

Similar observations concerning the role of mitochondrial integrity as a signal for the activation of autophagy were also made in mammalian cells. Mutations in mitochondrial DNA or drug-induced loss of mitochondrial membrane potential led to selective elimination of these organelles by autophagy (Elmore *et al.*, 2001; Gu *et al.*, 2004). According to Lemasters and colleagues, elimination of damaged mitochondria by autophagy could in fact block cell death by limiting the release of proapoptotic mitochondrial factors to the cytoplasm (Lemasters *et al.*, 1998). Therefore, mitochondrial autophagy could act as a mechanism to protect the cells from cell death in this setting. Work by others, however, challenged this hypothesis. Amino acid starvation, which induces mitochondrial autophagy, caused death of primary hepatocytes, and the autophagy inhibitor, 3-MA, prevented cell death (Schwarze and Seglen, 1985). Furthermore, preferential destruction of mitochondria by autophagy, accompanied by a decrease in cell size, was critical to commit cells to death in the presence of caspase inhibitors (Xue *et al.*, 2001).

The members of the Bcl-2 family are critical regulators of the mitochondrial-based intrinsic apoptotic pathway (Gross *et al.*, 1999). Accumulating data indicate that Bcl-2 family members may also be important for the regulation of autophagy and autophagic cell death. Section IV.A discussed the role that Bcl-2 plays in regulating autophagy through direct interaction with key autophagy proteins, such as Beclin-1 in the ER. Mitochondrial-localized Bcl-2 family members also contribute to the regulation of autophagy. First, overexpression of the antideath members, Bcl-2 or Bcl-XL, protected cells from autophagic cell death (Cardenas-Aguayo Mdel *et al.*, 2003; Saeki *et al.*, 2000; Vande Velde *et al.*, 2000; Xue *et al.*, 2001; Yanagisawa *et al.*, 2003), while overexpression of death-inducing Bcl-2 family members containing BH3 domains, such as BNIP3 or Bax, caused autophagic cell death (Camougrand *et al.*, 2003; Vande Velde *et al.*, 2000). Autophagic cell death induced by BNIP3 overexpression was dependent on mitochondrial permeability transition pore opening and membrane potential loss, and inhibitors of pore opening blocked cell death (Vande Velde *et al.*, 2000). Nevertheless, cytochrome *c* release and AIF nuclear translocation was not observed on BNIP3 overexpression, indicating that autophagic cell death does not depend on these factors. Additionally, upregulation of BNIP3 expression and BNIP3 activation

by dimerization accompanied ceramide and arsenic trioxide induced autophagic cell death (Daido *et al*., 2004; Kanzawa *et al*., 2005). In another study, a dominant negative BNIP3 was able to block ischemia/reperfusion-induced cell death of cardiac myocytes, but inhibition of BNIP3-induced autophagy in this system accelerated cell death with apoptotic characteristics (Hamacher-Brady *et al*., 2006). Overexpression of another pro-death Bcl-2 family member, Bax, in yeast, caused autophagic cell death (Camougrand *et al*., 2003). In this system, Bax led to the accumulation of reactive oxygen species, lipid oxidation and plasma membrane changes. Cell death was dependent on the Uht1 protein. Cytochrome *c* release was observed in this system but was not essential for cell death (Priault *et al*., 1999). Overexpression of HSpin1, a transmembrane protein containing a BH3 (Bcl-2 homology)-like domain that localizes to mitochondria, also caused a nonapoptotic cell death with characteristics reminiscent of autophagic cell death (Yanagisawa *et al*., 2003).

All these data indicate that, as in the case of apoptosis, prosurvival Bcl-2 members seem to inhibit autophagy and block autophagic cell death, while, prodeath Bcl-2 members have the opposite effects.

J. RIP Protein-Related Pathways and Autophagic Cell Death

Recent studies revealed the importance of death pathways involving the death-receptor associated kinase, RIP protein, in autophagy signaling. In fibroblast L929 cells, U937 monocytes, RAW 264.7 macrophages, and primary mouse peritoneal macrophages, the pancaspase inhibitor zVAD-induced cell death with autophagic characteristics and this event was blocked by RNAi-mediated knockdown of autophagy-related proteins Beclin-1 and Atg7 (Yu *et al*., 2004). zVAD's effects were attributed to the lack of proteolytic cleavage of RIP by caspase-8, which triggered a novel autophagic cell death pathway, involving MKK7, JNK, and c-Jun. Of note, *de novo* protein synthesis was required for autophagic cell death in this system, since the protein synthesis inhibitor cycloheximide prevented zVAD-induced cell death. In a second study, the same group further expanded these results by showing that zVAD-activated and RIP-dependent cell death in L929 cells resulted in selective degradation of the antioxidant enzyme catalase through autophagy, which killed the cells by causing the accumulation of ROS (Yu *et al*., 2006).

An independent group studied zVAD-induced death in RAW 264.7 macrophages and primary mouse peritoneal macrophages, in the presence of lipopolysaccharides (LPS) (Xu *et al*., 2006). Under these conditions, the cells died by autophagic cell death which could be inhibited by the chemical inhibitors of autophagy or RNAi-mediated knockdown of Beclin-1. In line

with the observations by Yue *et al.*, Xue and colleagues observed that autophagic cell death depended on the accumulation of ROS. Interestingly, PARP was also involved in cell death and was activated downstream of ROS production. Autophagic cell death activated by the combination of zVAD and LPS was attenuated in Toll-Like Receptor 4 adaptor (TRIF) knockout cells. Therefore, the authors proposed that the combination of LPS-stimulated Toll Receptor/TRIF and zVAD mediated stabilization of RIP contributed to the activation of a RIP-dependent autophagic death pathway in this system.

V. Autophagy–Apoptosis Crosstalks

Despite the fact that apoptosis and autophagy proceed through independent mechanisms, several lines of evidence point out the existence of crosstalk between the two pathways. This concept stems from several independent observations. One basic observation is that the cellular response to the same stimuli may manifest itself predominantly by autophagic or apoptotic characteristics depending on cellular context or experimental setting. Furthermore, in some cases, apoptotic and autophagic morphologies may coexist in the same cells, and the cell death may show mixed characteristics even at the molecular level. On the other hand, apoptosis and autophagy may depend on each other in some systems, so that blockage of one may affect the progress of the other. In other scenarios, one mechanism may counteract the other, or they may manifest themselves in a mutually exclusive manner. Consequently, inhibition of one PCD type may lead to enhancement or inhibition of the other type. This section will summarize the emerging data regarding the molecular basis of the connection between apoptosis and autophagic cell death.

In some systems, the very same signals or molecules that trigger apoptosis were shown to stimulate autophagy. Etoposide (Shimizu *et al.*, 2004), staurosporine (Shimizu *et al.*, 2004), interferon-γ (Inbal *et al.*, 2002), TRAIL (Mills *et al.*, 2004; Thorburn *et al.*, 2005), FADD (Thorburn *et al.*, 2005), ceramide (Scarlatti *et al.*, 2004), NGF withdrawal (Xue *et al.*, 1999), Ras (Kanzawa *et al.*, 2003; Pattingre *et al.*, 2003a), and DAPk (Inbal *et al.*, 2002) were all shown to induce cell death with apoptotic characteristics in some systems, while in others they were capable of killing the cells by autophagic cell death. According to one possibility, the same upstream signaling pathways may impinge on both apoptotic and autophagic directions, and the cellular context, for example tissue specificity or different experimental settings, may determine whether one pathway may predominate over the other during cell death induction. For example, ectopically

expressed DAPk leads to apoptotic cell death in fibroblasts, and to autophagic cell death in 293T or HeLa cells (Inbal *et al.*, 2002; Jang *et al.*, 2002; Raveh *et al.*, 2001). In another example it has been reported that while genotoxic stress activates in wild-type fibroblasts the canonical p53 pathway which leads to caspase dependent apoptosis, in the Bax/Bak-deficient fibroblasts autophagic cell death dominated (Shimizu *et al.*, 2004). In line with this, blockage of apoptosis by the inhibition of caspases was able to switch cell death from apoptosis to autophagic cell death in several systems. On the other hand, knockdown of key autophagy genes in some systems accelerates nutrient deprivation-induced apoptotic cell death (Boya *et al.*, 2005).

Apoptosis and autophagy may also be activated simultaneously. Gene expression profiling studies of steroid-triggered development in *Drosophila* revealed that several apoptosis-related genes are upregulated together with autophagy-related genes (Gorski *et al.*, 2003; Lee *et al.*, 2003). The concept of mixed type of cell death is also a central issue to be studied from a clinical point of view, since several disorders such as neurodegenerative diseases or myocardial infarction seem to involve cell death with mixed morphologies, although the contribution of individual pathways to cell death is still a matter of controversy in this field. In our hands, certain cellular stresses activated caspases (typical sign of apoptosis) and autophagy in the same cells, and both phenomena contributed to cellular demise, indicating cooperation between the two pathways to ensure irreversible elimination of damaged cells (D. Gozuacik and A. Kimchi, unpublished results).

Alternative scenarios also exist. In neurons, apoptosis may be preceded by autophagy, and full manifestation of apoptosis may depend on prior autophagy activation, since autophagy inhibitors like 3-MA was able to delay cytochrome *c* release and caspase activation (Xue *et al.*, 1999). Another example of autophagy-dependent apoptosis is the TNF-α-induced apoptosis of T-lymphoblastic leukemia cell lines (Jia *et al.*, 1997). In some systems, autophagy may also depend on apoptosis, since caspase activation was shown to be necessary for ecdysone-induced autophagic cell death in the *Drosophila* salivary gland (Martin and Baehrecke, 2004).

In other cellular settings, autophagy may antagonize apoptosis. For example, in sulindac sulfide (a nonsteroidal anti-inflammatory drug) treated HT-29 colon carcinoma cells, inhibition of autophagy increased the sensitivity of the cells to apoptotic signals, which manifested itself by faster cytochrome *c* release (Bauvy *et al.*, 2001). Another example of this antagonism is nutrient or growth factor deprivation-induced cell death. Treatment of deprived cells with autophagy inhibitors or blockage of autophagy by gene knockdown accelerated apoptotic cell death (Boya *et al.*, 2005).

All the scenarios presented above underline the complexity of the apoptosis/autophagy interconnection. Presumably, there must be molecular

regulators that control the switch between the two pathways. Any of the proteins that have been shown to regulate both apoptosis and autophagy are possible candidates, such as the Bcl-2 family members, DAPk, and FADD. In fact, Atg5 was shown to interact with the proapoptotic protein FADD and overexpression of Atg5-induced apoptosis through FADD (Pyo *et al.*, 2005). The list will probably continue to grow in the future.

VI. Survival Versus Death Aspects of Autophagy

Intense scientific debate revolved around the question of whether the autophagic activity observed in dying cells plays a causal role in cellular demise. The initial skepticism stemmed from the well-established catabolic role of autophagy. Even under normal growth conditions, cells use autophagy as the major pathway for the degradation and recycling of long-lived proteins, some ubiquitinated proteins and organelles like mitochondria (Komatsu *et al.*, 2005; Kuma *et al.*, 2004). Stress condition imposed on cultured cells or in the context of the entire organism, such as nutrient and/or growth factor deprivation, strongly upregulate autophagy to allow sustained viability under these unfavorable circumstances (Levine and Yuan, 2005). Examples of the prosurvival role of autophagy include yeast (Tsukada and Ohsumi, 1993) and plant (Doelling *et al.*, 2002; Hanaoka *et al.*, 2002; Liu *et al.*, 2005) autophagy under nutrient deprivation, *C. elegans* autophagy during dauer diapause (Melendez *et al.*, 2003), autophagy observed in human carcinoma cells during nutrient starvation (Boya *et al.*, 2005), autophagy in Bax/Bak knockout hematopoietic cells under growth factor deprivation (Lum *et al.*, 2005), and autophagy observed in postnatal mice during the starvation period that exists between the interruption of placental blood supply and the beginning of nursing (Kuma *et al.*, 2004). Deletion or knockdown of key autophagic genes during nutrient and growth factor deprivation led to increased cell death by apoptosis, rather than protecting from cell death, indicating that, under these circumstances, autophagy functions as a protective mechanism.

In light of this substantial body of data, how can it be that the same cellular mechanism is involved in both survival and death of cells? Although survival-related autophagy and autophagic cell death may share the same morphological features and the same basic molecular machinery of autophagic vesicle formation, several lines of evidence indicate differences at molecular level. For example, zVAD-induced autophagic cell death in L929 mouse fibroblastic cells involved the selective autophagic degradation of antioxidant protein catalase, but such elimination of catalase was not observed in starvation-induced autophagy in these same cells (Yu *et al.*, 2006). Furthermore, although both ceramide and nutrient starvation

activate autophagy via sphingolipid signaling and subsequent activation of mTor, only the former, which leads to cell death, involved inhibition of the prosurvival PKB/Akt pathway, and upregulation of Beclin-1 and BNIP3 (Daido *et al.*, 2004). The critical factor determining the final cellular outcome of death or survival may be the relative levels between the two sphingolipids SK1 and S1P, with accumulation of S1P favoring survival. This hypothesis has been termed the "S1P rheostat" (Cuvillier *et al.*, 1996).

In line with these observations, Levine and coworkers proposed a model of cell survival versus death, regulated by the balance between Beclin-1 and Bcl-2 proteins (Pattingre *et al.*, 2005). According to this model, when cellular Bcl-2 levels are high, Bcl-2 binds to and inactivates Beclin-1, thereby inhibiting autophagy. Imbalances in the Beclin-1/Bcl-2 ratios due to modest increases in Beclin-1 may lead to controlled catabolic and prosurvival autophagy activation. When Beclin-1 levels are induced above a certain threshold and no longer bound by Bcl-2, however, extensive autophagy and autophagic cell death will result. In support of this hypothesis, overexpression of Beclin-1 mutants unable to bind Bcl-2 caused autophagic cell death (Pattingre *et al.*, 2005).

All data presented above underline the importance of the cellular context created by the combinatory activation/inactivation of prosurvival or death pathways, intracellular levels of key regulatory molecules, and the regulation of selective degradation in converting autophagy from a protective mechanism to a killing machine. Therefore, although the morphological characteristics of autophagy may seem similar in both cases, survival-related autophagy and autophagic cell death mechanisms differ at the molecular level.

VII. Conclusions

The field of autophagy has greatly benefited from increasing scientific interest in the last decade, and this fact is documented by the dramatic increase in autophagy-related publications in recent years. Orthologues of the yeast autophagy genes begin to be characterized, and several model organisms lacking key autophagic genes or carrying autophagy markers are now available and under analysis. Signaling pathways regulating autophagy and autophagic cell death are beginning to be characterized. Advances in this field will not only broaden our knowledge about a fundamental process regulating cell survival and death, but it may also help to better understand certain human diseases and to characterize new drug targets, since autophagy was shown to play a role in several pathological conditions in various organisms from plants to human beings.

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