Life in the balance – a mechanistic view of the crosstalk between autophagy and apoptosis

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Journal of Cell Science 125, 5259–5268

doi: 10.1242/jcs.115865

Summary

Cellular stress triggers a fascinating decision-making process in cells; they can either attempt to survive until the stress is resolved through the activation of cytoprotective pathways, such as autophagy, or can commit suicide by apoptosis in order to prevent further damage to surrounding healthy cells. Although autophagy and apoptosis constitute distinct cellular processes with often opposing outcomes, their signalling pathways are extensively interconnected through various mechanisms of crosstalk. The physiological relevance of the autophagy–apoptosis crosstalk is not well understood, but it is presumed to facilitate a controlled and well-balanced cellular response to a given stress signal. In this Commentary, we explore the various mechanisms by which autophagy and apoptosis regulate each other, and define general paradigms of crosstalk on the basis of mechanistic features. One paradigm relates to physical and functional interactions between pairs of specific apoptotic and autophagic proteins. In a second mechanistic paradigm, the apoptosis or autophagy processes (as opposed to individual proteins) regulate each other through induced caspase and autolysosomal activity, respectively. In a third paradigm unique to autophagy, caspases are recruited and activated on autophagosomal membranes. These mechanistic paradigms are discernible experimentally, and can therefore be used as a practical guide for the interpretation of experimental data.

Key words: Apoptosis, Autophagy, Cancer, Cell death, Crosstalk

Introduction

The evolutionary transition to multicellularity has dictated the emergence of a highly controlled genetic program of cellular suicide, known as ‘apoptosis’. Apoptosis constitutes the main route of programmed cell death, and the molecular players involved in its regulation and execution have been well characterised (see Box 1 for more details). Apoptosis enables the precise elimination of superfluous cells during normal development, and of malfunctioning cells in the adult organism. Although various forms of programmed cell death have been described in unicellular organisms, their molecular makeup differs in many aspects from that of apoptosis in multicellular eukaryotes (Vercammen et al., 2007; Nedelcu et al., 2011; Bender et al., 2012). In addition, from simple, near-linear death cascades found in organisms such as the nematode *Caenorhabditis elegans*, apoptosis has evolved to form a highly elaborate, interwoven network of proteins in higher eukaryotes, which relentlessly surveys almost every aspect of cellular function. It is, therefore, not surprising that the apoptotic network interacts with and receives inputs from other cellular stress-response pathways, such as autophagy.

Autophagy (‘self-eating’) is a catabolic process that involves the engulfment of proteins and entire organelles within double-membrane vesicles, the autophagosomes. The autophagosomes are subsequently delivered to lysosomes, wherein their content is degraded and recycled back into the cytosol in the form of basic biomolecules (Levine and Kroemer, 2008). From an evolutionary perspective, autophagy precedes apoptosis, and is universally found in eukaryotes, from yeast to mammals (Meijer et al., 2007). Cells utilise autophagy to recycle basic biomolecules during nutrient deprivation, to scavenge damaged organelles and harmful proteins, and to eliminate intracellular pathogens. As such, the autophagic process plays a crucial role in maintaining cellular homeostasis, and also contributes to cell survival during times of stress (Levine and Kroemer, 2008). At the molecular level, autophagy is controlled by a group of autophagy-related (Atg) genes that operate concertedly during autophagosome biogenesis (Yang and Klionsky, 2010). Most of the molecular components of autophagy have been identified by genetic studies in yeast. Owing to a high degree of conservation, many homologues of the yeast autophagy genes were subsequently identified in higher eukaryotes (see Box 2 for more details).

Notably, autophagy is often observed in dying cells. In many settings, this represents an attempt of the cell to mitigate the stress before resorting to the irreversible and final solution of apoptosis. In other settings, activation of autophagy might reflect a crosstalk between the two processes, as will be discussed here in detail. Indeed, autophagy is induced in response to many stresses that ultimately lead to apoptosis, including organelle dysfunction, metabolic stress and pathogen infection. If the stress is resolved, cells typically restore autophagy to baseline levels and return to their initial state. However, if the stress persists and autophagy can no longer support cell survival, cells might respond by activating apoptosis in order to ensure their controlled and efficient elimination, without triggering local inflammation. Therefore, it is conceivable that the formation of regulatory ties between autophagy and apoptosis (and vice versa) conferred an evolutionary advantage to cells, as it enabled a more controlled and precise response to a given stress signal. Thus, although autophagy and apoptosis undoubtedly represent distinct cellular processes with fundamentally different biochemical and

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Box 1. Basic molecular mechanisms of apoptosis

Two main pathways that control the activation of apoptosis have been characterised – the extrinsic (death receptor) pathway and the intrinsic pathway.

Extrinsic apoptosis is initiated following the activation of death receptors at the plasma membrane by their cognate extracellular death ligands, such as FASL, TNF-α or TRAIL (Barnhart et al., 2003; Dempsey et al., 2003; Thorburn, 2004). Binding of the death ligand induces receptor trimerization and subsequent activation of initiator caspase-8 by an intracellular multi-protein complex, known as DISC (Barnhart et al., 2003). Activated caspase-8, in turn, activates the downstream effector caspases, caspase-3 and caspase-7, which ultimately lead to cell death by cleavage of a broad spectrum of cellular targets.

The intrinsic pathway is triggered by a wide range of cellular stress signals, including DNA damage, ER stress, oxidative stress and growth factor withdrawal (Tait and Green, 2010). A key step in the initiation of intrinsic apoptosis is mitochondrial outer membrane permeabilization (MOMP), which enables the release of pro-apoptotic factors, such as cytochrome c, from the mitochondrial intermembrane space (Tait and Green, 2010). Release of cytochrome c to the cytosol drives the formation of a protein complex known as the 'apoptosome', which induces the activation of initiator caspase-9 (Riedl and Salvesen, 2007; Tait and Green, 2010). Similar to the role of caspase-8, caspase-9 proteolytically activates the effector caspases, resulting in cell death. IAP family proteins can suppress cell death by binding to caspases (Salvesen and Duckett, 2002).

BCL2 family proteins serve as important regulators of intrinsic apoptosis by controlling MOMP (Youle and Strasser, 2008). The BCL2 family is comprised of pro- and anti-apoptotic members that share one or more BCL2-homology (BH) domains. Anti-apoptotic proteins (e.g. BCL2 and MCL1) contain four BH domains (BH1–4), and promote cell survival by antagonizing the activity of the pro-apoptotic BCL2 family members. The pro-apoptotic members can be divided into two subfamilies, according to their BH domain composition: multi-domain pro-apoptotic proteins (e.g. BAX and BAK), which contain three BH domains, and 'BH3-only' proteins (e.g. BAD, BID, BIK and PUMA), which contain only the BH3 domain (Youle and Strasser, 2008; Shamas-Din et al., 2011).

morphological features, the protein networks that control their regulation and execution are highly interconnected.

In light of these observations, extensive efforts have been made over the last decade to elucidate the various points of interface between autophagy and apoptosis at the molecular level. By systematically analysing the wide spectrum of publications, we find that several common features emerge. Accordingly, we propose that the crosstalk between autophagy and apoptosis occurs at several levels that can be classified into distinct paradigms on the basis of mechanistic features (Fig. 1).

The regulation of apoptosis by autophagy can be subdivided into three main mechanistic paradigms (Fig. 1A): (1) regulation of apoptosis by specific autophagy proteins (paradigm A1) – specific autophagy proteins can directly regulate apoptosis in a manner that is not necessarily related to their canonical role in autophagy signalling. That is, the acquired apoptotic function of these specific proteins does not necessarily require the activation of the entire process of autophagy; (2) activation of caspases on autophagosomal membranes (paradigm A2) – the autophagosomal membrane can serve as a platform for caspase activation. This paradigm requires the canonical function of autophagy proteins that are involved in autophagosome formation, but not of those proteins that are involved in later steps of autolysosomal fusion and degradation; (3) regulation of apoptosis by autophagic degradation (paradigm A3) – in this paradigm, apoptosis is regulated by the autophagy process as a whole, and is therefore dependent on the function of proteins that are involved both in the early (autophagosome formation) and late (lysosomal fusion and cargo degradation) stages of autophagy. A key characteristic of this paradigm is the dependence on an active autophagic flux, which requires lysosomal activity.

Following a similar logic, the regulation of autophagy by apoptosis can be subdivided into two main mechanistic paradigms (Fig. 1B): (1) direct regulation of autophagy by specific apoptotic proteins (paradigm B1); (2) regulation of autophagy by activated caspases, which necessitates the activation of the apoptotic process as a whole (paradigm B2).

Examples of each of these paradigms will be discussed in detail below (see also Fig. 2).

Regulation of apoptosis by autophagy

Paradigm A1: regulation of apoptosis by specific autophagy proteins

The following examples relate to specific autophagy proteins that have evolved to regulate the apoptotic pathway by directly interacting with components of the apoptotic machinery (Fig. 2). As might be expected, only inhibition or genetic ablation of those specific autophagy genes is likely to affect apoptosis, because in most cases neither autophagosome formation nor autolysosomal function are required for their apoptotic function (Fig. 1A).

The ATG5–ATG12 conjugation system

The process of autophagosome formation requires the covalent conjugation of the autophagy proteins ATG12 and ATG5 in a ubiquitylation-like process that involves the E1-like enzyme, ATG7, and the E2-like enzyme ATG10 (see Box 2 for more details). ATG5 and ATG12 are, therefore, integral parts of the autophagic 'core machinery' and are indispensible for the induction of autophagy. In addition to their role in autophagy, ATG5 and ATG12 were found to have key functions in the initiation of apoptosis in response to diverse stress signals. Surprisingly, in the realm of apoptosis, it is the non-conjugated forms of ATG12 and ATG5 that contribute to induction of apoptosis, implying that their apoptotic role may be independent of their canonical role in autophagy.

In apoptotic cells, ATG5 is cleaved by calpains. The resulting N-terminal fragment of ATG5 translocates to the mitochondrion by an unknown mechanism, where it mediates the release of cytochrome c by interacting with the pro-survival BCL2 family member B-cell lymphoma-extra large ($BclX_L$) (Yousefi et al., 2006). Notably, the domains in ATG5 that are involved in the interaction with $BclX_L$ have not yet been identified, and it is unclear whether ATG5 binding results in the inactivation of the anti-apoptotic function of $BclX_L$.

Recent work from our group identified a pro-apoptotic function for ATG12, the conjugation partner of ATG5 (Rubinstein et al., 2011). In an unbiased siRNA screen, we found that ATG12 is required for caspase activation in response to a variety of apoptotic stresses. Consistent with the characteristics of paradigm A1 of crosstalk, the knockdown of several other essential autophagy genes did not result in a substantial effect on apoptosis, supporting a specific role for

Box 2. Basic molecular mechanisms of autophagy

Several key protein complexes are involved in the induction of autophagy. Suppression of the mTOR kinase, a sensor of growth factors and nutrient availability, releases its inhibitory effects on the Unc-51-like kinase (ULK1) complex, which is necessary for induction of autophagy (Yang and Klionsky, 2010). Autophagosomal membrane nucleation requires the class III PI(3)-kinase VPS34 and its associated proteins, including Beclin 1. Two essential ubiquitin-like conjugation systems (described in detail below) control the elongation of the autophagosomal membrane (Tanida et al., 2004), whereas targeting and fusion of the autophagosome to the lysosome employ canonical trafficking mechanisms of the endocytosis pathway (Yang and Klionsky, 2010).

Two ubiquitin-like systems facilitate the covalent conjugation of the ubiquitin-like (UBL) proteins ATG12 and LC3 to ATG5 and the lipid phosphatidylethanolamine (PE), respectively (Kuma et al., 2002; Tanida et al., 2004; Geng and Klionsky, 2008). Both conjugation systems require the function of an E1-like enzyme, ATG7, which initiates the conjugation process by activating a glycine residue in the C-termini of ATG12 and LC3 (Tanida et al., 2004). Whereas ATG12 is translated with a C-terminal glycine, nascent LC3 requires pre-processing by the protease ATG4 in order to expose its C-terminal glycine.

Specific E2-like enzymes, ATG3 and ATG10, subsequently catalyse the conjugation of LC3 to PE and of ATG12 to ATG5, respectively (Geng and Klionsky, 2008). Interestingly, The ATG12–ATG5 conjugation system lacks apparent E3-like proteins, and was suggested to function as the E3-like enzyme for the LC3–PE conjugation system. The ATG12–ATG5 stable conjugate proceeds to form an active multimeric complex together with ATG16, which then localises to sites of autophagosome assembly (Kuma et al., 2002).

ATG12 that does not require autophagosome formation or lysosomal activity. Subsequent interaction assays revealed that non-conjugated ATG12 is able to bind to and inhibit BCL2 and MCL1, two anti-apoptotic members of the BCL2 family. A BH3-like domain within ATG12 was found to be required for the interaction with BCL2 and MCL1, and for the pro-apoptotic function of ATG12 (Rubinstein et al., 2011).

In addition to the role of non-conjugated ATG12 in apoptosis, Radoshevich and colleagues previously found that a stable conjugate comprised of ATG12 and ATG3 is involved in the regulation of mitochondrial homeostasis and cell death (Radoshevich et al., 2010). They showed that cells expressing a non-conjugatable mutant of ATG3 display increased mitochondrial mass and are less sensitive to mitochondrial apoptosis. Surprisingly, the ATG12–ATG3 conjugate does not appear to be involved in the regulation of autophagy.

The molecular mechanisms underlying the apoptotic function of the non-conjugated forms of ATG5 and ATG12 might be similar, as both proteins were shown to interact with anti-apoptotic members of the BCL2 family (Yousefi et al., 2006; Rubinstein et al., 2011). Notably, although the apoptotic function of ATG5 has been shown to be 'switched on' by calpain cleavage, the signals that activate the pro-apoptotic function of ATG12 are yet to be identified.

The Beclin 1–VPS34 complex

The class III phosphatidylinositol 3-kinase (PIK3C3)–Beclin 1 complex serves as a central regulator of autophagy downstream of mammalian target of rapamycin (mTOR). Beclin 1 induces the lipid kinase activity of PIK3C3 (also known as VPS34), which is necessary for the recruitment of autophagy proteins to sites of autophagosome formation. As will be discussed below in further detail, Beclin 1 was identified several years ago as a new member of the BH3-only family of proteins (Maiuri et al., 2007; Oberstein et al., 2007), and binding of BCL2 to the BH3 domain of Beclin 1 was found to inhibit autophagy (Pattingre et al., 2005).

As members of the BH3-only protein family have been well characterised as pro-apoptotic proteins, it was expected that binding of Beclin 1 to BCL2 would induce apoptosis by neutralizing the anti-apoptotic function of BCL2. However, enforced expression of Beclin 1 failed to induce or enhance apoptosis (Maiuri et al., 2007). Moreover, BCL2 was shown to retain its full anti-apoptotic activity when it is bound to Beclin 1 (Ciechomska et al., 2009b). The reason for the inability of Beclin 1 to induce apoptosis is not fully understood, but might lie in the atypical amino acid composition of its BH3 domain, which mediates a relatively weak interaction with BCL2 compared with other BH3-only proteins (as reviewed by Feng et al., 2007; Boya and Kroemer, 2009; Ciechomska et al., 2009a). Thus, the functional outcome of the interaction between Beclin 1 and BCL2 is unidirectional, and results in the inhibition of autophagy

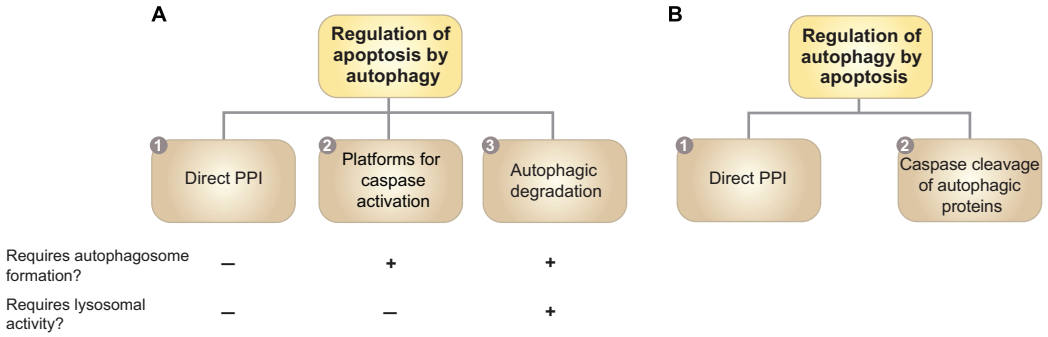

Fig. 1. Summary of the mechanistic paradigms as the basis of autophagy–apoptosis crosstalk. (**A**) The three paradigms by which autophagy regulates apoptosis: (1) regulation of apoptosis by specific autophagy proteins; (2) activation of caspases on autophagosomal membranes depends on autophagosome formation, but not on lysosomal activity; (3) regulation of apoptosis by autophagic degradation requires both autophagosome formation and lysosomal activity. (**B**) The two mechanistic paradigms by which apoptosis regulates autophagy: (1) direct regulation of autophagy by specific apoptotic proteins; (2) regulation of autophagy by activated caspases, which necessitates the activation of the apoptotic process as a whole. PPI, protein–protein interaction.

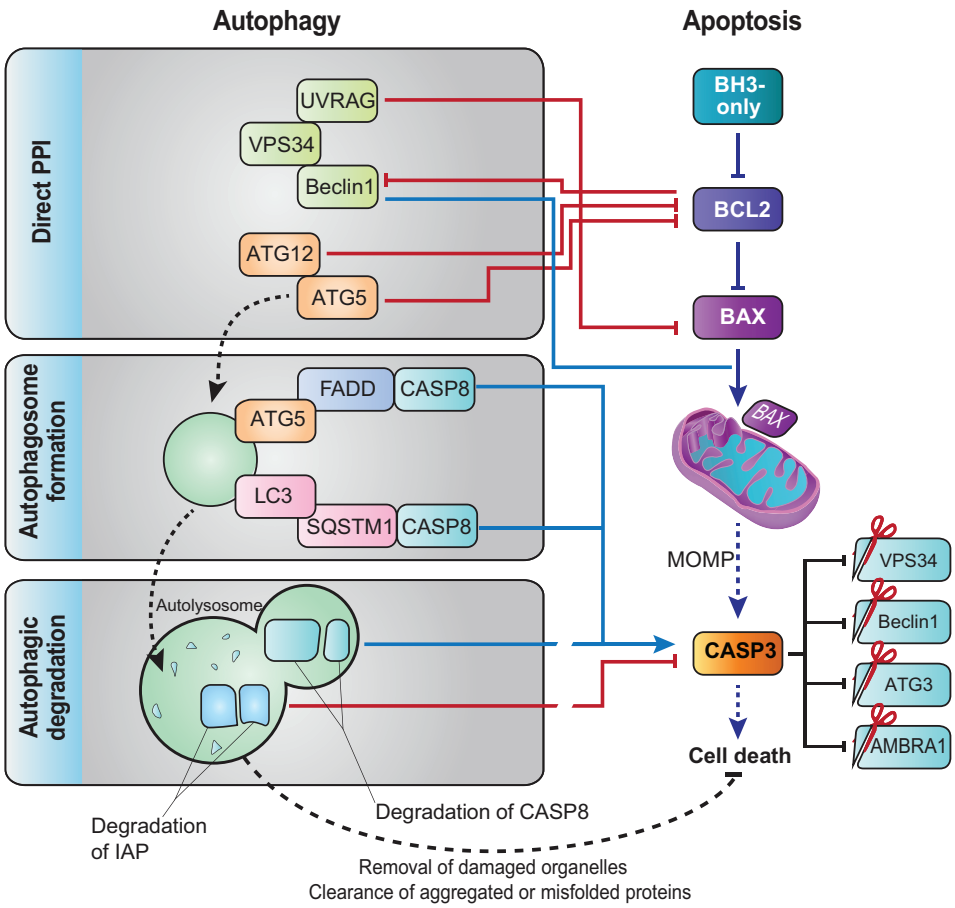

Fig. 2. Overview of the molecular mechanisms underlying the autophagy–apoptosis crosstalk. The three boxes on the left illustrate the three mechanistic paradigms of apoptosis regulation by autophagy. The central apoptotic nodes and their interactions with autophagy proteins are illustrated on the right. Stimulatory interactions are depicted in blue, whereas inhibitory interactions are depicted in red. Dotted lines indicate interactions that are interrupted. PPI, protein–protein interaction; MOMP, mitochondrial outer-membrane permeabilization.

without having a reciprocal effect on apoptosis. However, a number of groups have observed that upon induction of apoptosis, Beclin 1 is cleaved by caspases at specific sites (Cho et al., 2009; Luo and Rubinsztein, 2010; Wirawan et al., 2010). Because several other autophagy proteins are cleaved during apoptosis, it was hypothesised that cleavage of Beclin 1 serves to inhibit cytoprotective autophagy in cells that have committed to cell death (i.e. paradigm B2). However, the C-terminal cleavage product of Beclin 1 has been found to localise predominantly to mitochondria where it sensitises cells to apoptosis induced by growth factor withdrawal (Wirawan et al., 2010). In an *in vitro* assay, the authors demonstrated that the C-terminal fragment of Beclin 1 is able to induce release of cytochrome c from isolated mitochondria, providing a possible clue to its pro-apoptotic function *in vivo* (Wirawan et al., 2010).

The similarities between the Beclin 1 and ATG5 findings are striking. In both cases, activation of apoptosis leads to site-specific cleavage of these autophagic proteins, which not only serves to suppress cytoprotective autophagy (paradigm B2), but also generates mitochondria-targeted cleavage products that function as positive-feedback loops to enhance the apoptotic response (paradigm A1). These scenarios, thus, demonstrate that several paradigms of crosstalk can operate simultaneously in the cell.

The Beclin 1 interacting UV radiation resistance-associated gene (UVRAG) protein promotes autophagosome formation by inducing the activity of the Beclin 1 complex (Liang et al., 2006). In addition to its role in autophagy, UVRAG was found to function as an inhibitor of apoptosis by preventing the translocation of the pro-apoptotic protein BAX to mitochondria, a requisite step for the induction of mitochondrial apoptosis (Yin et al., 2011). The binding of UVRAG to BAX is maximal in non-apoptotic cells and substantially decreases when apoptosis is induced. Pull-down assays revealed that Beclin 1 is not found in the same complex with UVRAG and BAX, suggesting that two different UVRAG-containing complexes are involved in the regulation of autophagy and apoptosis (Yin et al., 2011). Taken together, caspase-cleaved Beclin-1 and UVRAG have opposing effects on apoptosis (Fig. 2). Future studies of the dynamics of these interactions are required to fully understand how they are coordinated *in vivo*.

Paradigm A2: autophagosomes as platforms for caspase activation

Data from several groups point to a rather unexpected mechanism by which autophagy can directly contribute to the induction of apoptosis. In this paradigm, caspases are recruited to autophagosomes, which serve as intracellular platforms for

their activation. Thus far, this mechanism of activation has been described for caspase-8, which can be activated by a death-inducing signalling complex (DISC)-like complex that assembles on autophagosomal membranes (Laussmann et al., 2011; Young et al., 2012). Autophagy-dependent activation of caspase-8 that was observed following treatment with proteasome inhibitors did not require death ligands, suggesting that autophagy links caspase-8 to apoptosis that is induced by intrinsic stress signals (Laussmann et al., 2011). Interestingly, activation of caspase-8 by autophagy has also been implicated in non-apoptotic scenarios, such as regulation of T cell proliferation following mitogenic stimulation (Bell et al., 2008). In T cells, caspase-8 reciprocally regulates autophagy, as cells that are deficient in caspase-8 or the adaptor protein Fas-associated protein with death domain (FADD) display increased autophagy (Bell et al., 2008).

Two different routes for the recruitment of caspase-8 to autophagosomes have been described in the literature (Fig. 2). According to the first route, ubiquitylated caspase-8 binds to the autophagic cargo receptor p62 (also known as SQSTM1) through the ubiquitin-binding domain (UBD) of p62, and is subsequently recruited to autophagosomes through a direct interaction between p62 and LC3. Importantly, p62 is required not only for the recruitment of caspase-8 to autophagosomes, but also for its activation, by facilitating self-oligomerization of caspase-8 (Jin et al., 2009; Young et al., 2012). In the second route, caspase-8 is recruited to autophagosomes through an interaction between the adaptor protein FADD and ATG5 (Pyo et al., 2005; Young et al., 2012). Thus, both scenarios involve an adaptor protein that mediates the recruitment of caspase-8 to autophagosomes; however, the scenarios differ in the identity of the adaptor protein (p62 versus FADD), and the autophagy protein that binds to the adaptor (LC3 versus ATG5). Although the caspase-8–p62–LC3 route implies that FADD is dispensable for the recruitment of caspase-8 to autophagosomes, it seems to be an essential component for the activation of caspase-8 in both scenarios (Young et al., 2012).

It remains to be determined what the relative contribution of each route is to caspase activation (e.g. by testing caspase-8 activation in cells expressing mutants of ATG5 and LC3 that are defective in their ability to bind FADD and p62, respectively), and whether other caspases are activated in the same way.

The paradigm of caspase activation ‘on the backs of autophagosomes’ is unique in the sense that it requires the formation of autophagosomes (or at least autophagosomal membranes), but does not require autolysosomal activity. Accordingly, Young and colleagues found that depletion of autophagy genes inhibits caspase-8 activation, whereas pharmacological inhibition of lysosomal fusion enhances caspase-8 activation, presumably owing to the decreased turnover of autophagosomes (Young et al., 2012). The opposing effects of inhibition of early events (autophagosome formation) and late events (lysosomal fusion) provide a useful way to discern between this paradigm and others.

Paradigm A3: regulation of apoptosis by autophagic degradation

The apoptotic response can be profoundly affected by the ability of the autophagic degradation process to control energy availability, organelle function and protein turnover. In this paradigm, interfering with either autophagosome formation or lysosomal activity is likely to affect the apoptotic response, because both processes are required to maintain the autophagic flux.

As mentioned above, autophagy has a central function in cell survival during nutrient starvation (Boya et al., 2005). Hence, in the absence of autophagy, the kinetics of apoptosis that is induced by starvation is often accelerated (Boya et al., 2005; Suzuki et al., 2011). The same principle can be extended to non-physiological stresses that activate cytoprotective autophagy, such as various chemotherapeutic agents. Indeed, inhibition of autophagy can sensitise tumour cells to a variety of drugs (Kanzawa et al., 2003; Kanzawa et al., 2004; Carew et al., 2007). In essence, autophagy-dependent processes that contribute to the maintenance of cellular homeostasis could indirectly limit or delay the onset of apoptosis, for example, by eliminating damaged organelles and cytotoxic protein aggregates (Komatsu et al., 2006; Ravikumar et al., 2006; Suzuki et al., 2011).

Although in the examples above, the autophagic flux prevents or delays apoptosis, in other settings it might actually facilitate certain events that are related to apoptotic cell death. For example, apoptosis-dependent cavitation of embryoid bodies requires autophagy for efficient removal of cell corpses (Qu et al., 2007). During the cavitation process, autophagy helps to maintain the levels of cellular ATP, which is required for the exposure of the ‘eat me’ signal, phosphatidylserine, and for the release of the ‘come get me’ signal, lysophosphatidylcholine. Consequently, autophagy-deficient embryoid bodies display reduced levels of ATP and fail to cavitate owing to defective phagocytic engulfment of apoptotic cell corpses. In addition, a function for autophagy in dismantling of apoptotic cells within the phagocytic cell has recently been suggested (Li et al., 2012). It is conceivable that additional energy-dependent stages of apoptosis, such as membrane blebbing, are affected by autophagic degradation in a similar manner.

Although protein degradation by autophagy is considered to be largely non-selective, an interesting concept for the direct regulation of apoptosis by autophagy is the selective targeting of apoptotic proteins for autophagic degradation. In this way, autophagy could shift the balance between anti- and pro-apoptotic factors, leading to initiation or inhibition of apoptosis. Indeed, in TNF-related apoptosis-inducing ligand (TRAIL)-resistant colon carcinoma cells, active caspase-8 was found to be specifically targeted for autophagic degradation, leading to attenuation of the apoptotic response to TRAIL (Hou et al., 2010). Conversely, selective degradation of the *Drosophila* anti-apoptotic inhibitor of apoptosis (IAP) homologue, Bruce, led to increased caspase activation and DNA fragmentation during oogenesis (Nezis et al., 2010). Taken together, these first few examples support the notion that selective targeting of proteins might allow autophagy to directly regulate the apoptotic process, and in both directions (Fig. 2). Future studies should focus on identifying the specific cargo receptors that are responsible for the recognition of apoptotic proteins.

Regulation of autophagy by apoptosis

Paradigm B1: regulation of autophagy by specific apoptosis proteins

BCL2 family proteins

Components of the apoptotic machinery can directly impact autophagy through molecular interactions with autophagy proteins (Fig. 2). Perhaps the best-studied example for this type of regulation is the dual function played by BCL2 in the

inhibition of both pathways. As mentioned above, BCL2 has been identified as a direct binding partner of Beclin 1, leading to decreased autophagic activity (Pattingre et al., 2005). In resting cells, BCL2 is constitutively bound to Beclin 1, thereby allowing for only low (basal) levels of autophagy. Under autophagy-inducing conditions, BCL2 dissociates from Beclin 1, resulting in increased autophagy (Pattingre et al., 2005).

Several mechanisms that control the dissociation of BCL2 from Beclin 1 under autophagy-inducing conditions have emerged. First, the identification of Beclin 1 as a BH3-only protein suggested that other members of the BH3-only family competitively displace BCL2 from Beclin 1 (Maiuri et al., 2007). Indeed, Maiuri and colleagues have found that knockdown of the BH3-only protein BAD reduces the extent of autophagy in response to starvation, whereas its ectopic expression is sufficient to induce autophagy (Maiuri et al., 2007). Moreover, they showed that starvation leads to an increase in the interaction between $BclX_L$ and BAD, which correlates with decreased binding of $BclX_L$ to Beclin 1 and elevated levels of autophagy, supporting a competitive type of interaction.

Regulation of the BCL2–Beclin-1 interaction by BAD (Maiuri et al., 2007), as well as by other BH3-only proteins, including Noxa (Rashmi et al., 2008; Zhang et al., 2008; Chang et al., 2010; Elgendy et al., 2011), represents an additional layer of crosstalk, in which pro-apoptotic BH3-only proteins can act as positive regulators of autophagy by displacing BCL2 from Beclin 1. Interestingly, Luo and colleagues recently discovered that overexpression of the BH3-only protein BIM does not affect the interaction between BCL2 and Beclin 1. Instead, BIM functions as an inhibitor of autophagy, by anchoring Beclin 1 to microtubules (Luo et al., 2012).

Phosphorylation of Beclin 1 and BCL2 constitutes another post-translational mechanism that controls the dissociation of BCL2 from Beclin 1. Multi-site phosphorylation of BCL2 by c-Jun N-terminal protein kinase 1 (JNK1, also known as MAPK8) has been shown to substantially reduce the affinity of BCL2 for Beclin 1, leading to activation of autophagy in response to starvation (Wei et al., 2008b) or ceramide treatment (Pattingre et al., 2009). Likewise, phosphorylation of Beclin 1 within its BH3 domain by death-associated protein kinase (DAP kinase) was shown to induce autophagy by promoting its dissociation from $BclX_L$ (Zalckvar et al., 2009). Notably, both JNK1 and DAPK have been implicated in the regulation of apoptosis (reviewed by Raveh et al., 2001; Jang et al., 2002; Bialik and Kimchi, 2006).

One of the most intriguing aspects of the crosstalk between apoptosis and autophagy is the elegant way in which cells balance between the ‘economical’ solution of utilising a single protein (e.g. BCL2) to regulate two pathways, and the need to maintain a complex and individual regulation of each pathway. In the case of BCL2, this is achieved, in part, through differential binding affinities and spatial separation of apoptotic and autophagic components. For example, Wei and colleagues found that at early time points of amino acid starvation, JNK1-mediated phosphorylation of BCL2 leads to its rapid dissociation from Beclin 1 and subsequent induction of pro-survival autophagy (Wei et al., 2008b). Because pro-apoptotic proteins, such as BAX, bind BCL2 with higher affinity than Beclin 1, this low level of BCL2 phosphorylation is insufficient to displace them from BCL2. However, if the stress of starvation persists, accumulation of hyper-phosphorylated BCL2 eventually results in the dissociation of BCL2 from BAX and the activation of apoptosis (Bassik et al., 2004; Wei et al., 2008a). Thus, differential binding affinities, fine-tuned by evolution, provide a way to achieve sequential activation of autophagy and apoptosis with the use of a single protein regulator.

Spatial separation of proteins to different cellular compartments provides an additional way to achieve independent regulation of autophagy and apoptosis. Two distinct cellular pools of BCL2 at the endoplasmic reticulum (ER) and mitochondria appear to regulate autophagy and apoptosis, respectively (Pattingre et al., 2005) (Fig. 3). A localised group of ER proteins ensures the partitioning of BCL2 to the autophagy pathway at this organelle. For example, regulation of autophagy by binding of BCL2 to Beclin 1 at the ER is facilitated by the ER protein nutrient-deprivation autophagy factor-1 (NAF1), and is inhibited by the ER-localised BH3-only protein BIK (Chang et al., 2010). Moreover, JNK1 specifically phosphorylates the ER pool of BCL2 during starvation to induce autophagy (Wei et al., 2008b). Conversely, interactions between BCL2, BH3-only proteins and BAX or BAK at the mitochondrion control the activation of apoptosis (Adams and Cory, 2007; Martinou and Youle, 2011). Thus, compartmentalization of BCL2, together with organelle-specific sets of interacting proteins, enable the dynamic and independent regulation of autophagy and apoptosis (Fig. 3). Nevertheless, it has been suggested that mitochondria-localised BCL2 is also involved in inhibition of autophagy, by sequestering a mitochondrial pool of AMBRA1, a positive regulator of Beclin 1 (Strappazzon et al., 2011).

FLIP

FLICE-like inhibitor protein (FLIP) is an anti-apoptotic protein that is implicated in suppression of death receptor-mediated apoptosis (Irmler et al., 1997). Like BCL2, cellular and viral versions of the protein exist (Irmler et al., 1997). Recently, it was found that cellular and viral FLIPs can also inhibit autophagy by

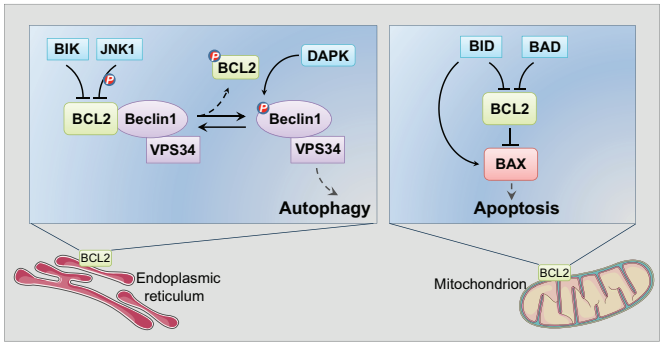

Fig. 3. Compartmentalization of BCL2 to different cellular organelles allows independent control of autophagy and apoptosis by a single protein regulator. At the ER, BCL2 inhibits autophagy by binding to Beclin 1. The binding of BCL2 to Beclin 1 is regulated by several mechanisms, including competitive binding of the BH3-only protein BIK to BCL2, phosphorylation of BCL2 by JNK1, and phosphorylation of Beclin 1 by DAPK. At the mitochondrion, BCL2 inhibits apoptosis by interacting with BAX and BAK. BH3-only proteins (e.g. BAD, NOXA, BID and others) activate apoptosis by inhibiting BCL2 and its related anti-apoptotic family members. Specific BH3-only proteins are also able to induce apoptosis by directly activating BAX and BAK (e.g. BID, BIM and PUMA).

interfering with the function of ATG3, the E2-like enzyme in the LC3 conjugation system (Lee et al., 2009). Interaction studies revealed that FLIP inhibits autophagy by competing with LC3 for binding of ATG3, attenuating the LC3 lipidation process. When autophagy is induced, the interaction between FLIP and ATG3 is substantially decreased. Notably, separate regions within the FLIP protein control its anti-autophagic and anti-apoptotic activities, consistent with the characteristics of paradigm B1 (Lee et al., 2009).

These data suggest that, similar to BCL2, FLIP can inhibit both autophagy and apoptosis. Because inhibition of apoptosis by FLIP takes place at the plasma membrane, and inhibition of autophagy presumably occurs at sites of autophagosome formation, it is possible that compartmentalization of FLIP to these two subcellular localizations provides a way to achieve independent regulation of autophagy and apoptosis by FLIP.

Paradigm B2: regulation of autophagy by activated caspases

Numerous autophagic proteins have been identified as targets for caspase-mediated cleavage, which inactivates their autophagic function, including Beclin 1, VPS34, ATG3, ATG4D and AMBRA1 (Betin and Lane, 2009; Cho et al., 2009; Luo and Rubinsztein, 2010; Norman et al., 2010; Oral et al., 2012; Pagliarini et al., 2012). A plausible explanation for this phenomenon is that inhibition of autophagy, which often precedes apoptosis, serves to prevent the simultaneous activation of contradicting pro-survival and pro-death processes in the cell, thereby enabling a smooth transition towards the 'point of no return'. In support of this concept, Pagliarini and colleagues have recently found that expression of a mutant form of the autophagy protein AMBRA1 that cannot be cleaved by caspases confers partial protection from apoptotic cell death, which also correlates with higher levels of autophagy (Pagliarini et al., 2012). As mentioned above, in some examples of caspase cleavage of autophagic proteins, there exists a twist to this function, in that the cleaved product of Beclin 1 acquires a pro-apoptotic function, thereby conferring an additional benefit for cells that are destined for demise (Wirawan et al., 2010).

Surprisingly, starvation-induced autophagy in *Drosophila* nurse cells was shown to require the caspase Dcp1 and was inhibited by Bruce (Hou et al., 2008; Kim et al., 2010). However, it is still unknown whether the autophagic function of Dcp1 requires its proteolytic activity, and if so, what its downstream autophagic substrates are.

Notably, in this paradigm, regulation of autophagy through apoptosis is dependent on the final stages of caspase activation, and would therefore be affected by genetic or pharmacological inhibitors of caspases, such as Z-VAD-FMK.

Pathological and physiological implications of crosstalk between autophagy and apoptosis

One of the current challenges in the field is to identify the physiological conditions in the context of the whole organism, under which the different paradigms of the autophagy–apoptosis crosstalk come into play. The elegant molecular studies performed in cell lines provide the basis for the discovery of the different means of crosstalk, but cannot be easily applied to *in vivo* systems. Nevertheless, some indications of a physiological and pathological relevance for autophagy–apoptosis crosstalk have emerged, albeit these examples have been less characterised at the molecular level.

The most extensively studied examples hinting at the physiological relevance of crosstalk relate to the field of cancer aetiology and drug treatment. Although apoptosis has an important tumour-suppressive function, the role of autophagy in cancer is more complex and largely depends on the specific stage of tumour development (Eisenberg-Lerner et al., 2009; Eisenberg-Lerner and Kimchi, 2009). Tumour cells frequently face various types of environmental stresses that are known to trigger pro-survival autophagy (e.g. hypoxia or nutrient deprivation resulting from limited blood supply), and therefore often exhibit elevated levels of autophagy (Degenhardt et al., 2006; Karantza-Wadsworth et al., 2007). Adaptation to metabolic stress by autophagy might, thus, promote tumour cell survival and prevent cells from undergoing apoptosis by mitigating the stress. Consequently, pharmacological inhibition of autophagy under these conditions, particularly in combination with other chemotherapeutic agents, might lead to a favourable outcome, compromising the ability of tumour cells to adapt to stress, thus driving apoptotic-competent cells towards cell cycle arrest or programmed cell death (Carew et al., 2007; Jin et al., 2007; Maycotte and Thorburn, 2011). Other recent work suggested that suppression of autophagy leads to inhibition of cell proliferation in non-small-cell lung carcinoma (NSCLC) and sensitises cells to cisplatin-induced apoptosis by stimulation of reactive oxygen species (ROS) formation (Kaminskyy et al., 2012). By contrast, the inhibition of autophagy in apoptosis-deficient tumours, such as those overexpressing BCL2, might have detrimental effects. In apoptosis-deficient cells, the accumulation of cellular damage as a result of defective autophagy might lead to increased genomic instability, or drive cells towards pro-inflammatory necrotic cell death, which can facilitate tumour progression and drug resistance (Degenhardt et al., 2006; Mantovani et al., 2010).

Another interesting aspect, which is currently under intense debate, is the notion that autophagy itself might lead to cell death under specific circumstances, a process known as 'autophagic cell death' (Gozuacik and Kimchi, 2007). This can be particularly relevant in apoptosis-deficient tumours as an alternative program of cell death. Considering the inhibitory effect that caspases can have on autophagy, as discussed above, tumour cells that are defective in apoptosis might exhibit excessive autophagic degradation of cellular components, leading to cell death. In this scenario, the induction of autophagic cell death in apoptosis-deficient tumours might constitute a 'failsafe' mechanism that ensures cell death by autophagy when apoptosis is defective. It remains to be tested whether pharmacological induction of autophagic cell death represents an attractive approach to tumour therapy, as several preliminary studies suggest (Kanzawa et al., 2003; Turcotte et al., 2008; Lee et al., 2009).

In light of the different crosstalk paradigms discussed above, the molecular target through which autophagy is inhibited might also be important for the treatment of cancer. For example, inhibition of autophagy in a pancreatic tumour cell line by knockdown of Beclin 1 or pharmacological inhibition of PIK3C3 was found to enhance apoptosis following treatment with chemotherapeutic agents (Ropolo et al., 2012). However, inhibition of autophagy in these cells by knockdown of ATG5 resulted in decreased levels of apoptosis, suggesting that autophagy-independent apoptotic functions of ATG5 are at play in pancreatic cancer (Ropolo et al., 2012).

There are fewer studies of other human pathologies, for which apoptosis and autophagy have been described as part of their phenotypes and disease aetiology. For example, crosstalk between autophagy and apoptosis has been documented in different forms of heart disease (Nishida et al., 2008), and after trauma and sepsis (Hsieh et al., 2009). Further studies on the exact underlying mechanisms of autophagy–apoptosis crosstalk in these diseases might help to direct the suitable mode of drug treatment.

Conclusions and perspectives

The multiple layers of connectivity between autophagy and apoptosis manifest themselves in a seamless balance between life and death in response to cellular stress. Perturbations of this delicate balance might therefore be associated with various pathologies, such as cancer and neurodegeneration.

In recent years, much emphasis has been placed in identifying direct protein–protein interactions between autophagy and apoptosis proteins (paradigms A1 and B1). In most cases, these binary protein interactions exhibit clear directionality, in that the functional outcome for a given pair of interacting proteins is unidirectional (i.e. regulating either autophagy or apoptosis, but not both). Therefore, an interesting pending question relates to the evolutionary advantage of utilising an autophagy protein to regulate apoptosis (and vice versa). Do such binary interactions reflect a 'true' crosstalk between autophagy and apoptosis, or are they simply examples of bi-functional proteins that regulate each process separately? Thus far, very little has been done to directly address this question experimentally. One compelling possibility for the utilisation of autophagy proteins to regulate apoptosis (paradigm A1), is that these specific autophagy proteins serve as rheostats that can sense the metabolic state of the cell and signal to the activation of apoptosis when the damage becomes too severe, thus, forming a switch between an early autophagic response and a late apoptotic response. Another possibility is that activation of the apoptotic function of these autophagy proteins entails deactivation of their autophagic function, resulting in activation of apoptosis and suppression of pro-survival autophagy. As discussed above, the latter hypothesis is supported by examples in which cleavage of autophagy proteins under apoptotic conditions suppresses their autophagic function, while at the same time, generates apoptosis-inducing cleavage products (Yousefi et al., 2006; Carew et al., 2007).

In addition, very little is currently known about the actual function of these binary interactions in physiological settings. A major challenge in the field is, therefore, to move forward from the identification of individual interactions towards a more global and integrative understanding of how they come together to determine the fate of a cell. Moreover, it is likely that autophagy engages in additional interplay with other forms of stress response and cell death pathways, such as programmed necrosis (Bialik et al., 2010). Studying the dynamics of several different protein interactions in animal model systems and/or in specimens derived from human diseases might shed light on their relative importance to the apoptosis–autophagy crosstalk, and the way in which they are orchestrated in physiologically relevant settings.

Acknowledgements

We apologize to those authors whose work could not be cited due to space limitations. We thank Shani Bialik and Axel Onsager Norgren for critical reading of the manuscript.

Funding

This work was supported by the Center of Excellence grant from the Flight Attendant Medical Research Institute (FAMRI) and by THE FIRST Program of the Israel Science Foundation [grant number 1477/09 to A.K.]. A.K. is the incumbent of Helena Rubinstein Chair of Cancer Research.

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