Cell Death Signaling

Douglas R. Green and Fabien Llambi

Department of Immunology, St. Jude Children's Research Hospital, Memphis, Tennessee 38105

Correspondence: douglas.green@stjude.org

SUMMARY

In multicellular organisms, cell death is a critical and active process that maintains tissue homeostasis and eliminates potentially harmful cells. There are three major types of morphologically distinct cell death: apoptosis (type I cell death), autophagic cell death (type II), and necrosis (type III). All three can be executed through distinct, and sometimes overlapping, signaling pathways that are engaged in response to specific stimuli. Apoptosis is triggered when cell-surface death receptors such as Fas are bound by their ligands (the extrinsic pathway) or when Bcl2-family proapoptotic proteins cause the permeabilization of the mitochondrial outer membrane (the intrinsic pathway). Both pathways converge on the activation of the caspase protease family, which is ultimately responsible for the dismantling of the cell. Autophagy defines a catabolic process in which parts of the cytosol and specific organelles are engulfed by a double-membrane structure, known as the autophagosome, and eventually degraded. Autophagy is mostly a survival mechanism; nevertheless, there are a few examples of autophagic cell death in which components of the autophagic signaling pathway actively promote cell death. Necrotic cell death is characterized by the rapid loss of plasma membrane integrity. This form of cell death can result from active signaling pathways, the best characterized of which is dependent on the activity of the protein kinase RIP3.

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Outline

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1 INTRODUCTION

Although cell death can happen as a result of overwhelming damage, most cell deaths in animals occur in an active manner, as a consequence of specific signaling events. In general, there are three types of cell death, defined in large part by the appearance of the dying cell: apoptosis (also known as type I cell death), autophagic cell death (type II), and necrosis (type III) (Galluzzi et al. 2007).

Apoptosis is characterized by cell shrinkage, membrane blebbing, and condensation of the chromatin (pyknosis) (Kerr et al. 1972). It can be further defined as cell death accompanied by the activation of caspase proteases (Galluzzi et al. 2012). Two major signaling pathways trigger apoptotic cell death: the mitochondrial (the intrinsic) pathway and the death receptor (the extrinsic) pathway. The latter involves a classical ligand–cell-surface-receptor interaction. For example, cytotoxic lymphocytes can kill infected or transformed cells by expressing ligands for death receptors (DRs), a subset of the tumor necrosis factor (TNF) receptor (TNFR) family. These ligands induce apoptotic cell death of the targeted cells provided they express such DRs. DR-induced cell death in general is critical for immune system function and homeostasis. In contrast, the mitochondrial apoptotic pathway is usually initiated in a cell-autonomous manner. Most cellular stresses, such as DNA damage (induced by genotoxic agents or defects in DNA repair) or endoplasmic reticulum (ER) stress (induced by the accumulation of unfolded proteins), actively engage apoptosis when cells are damaged beyond repair.

Conversely, the lack of a signal, such as those activated by growth factors (e.g., cytokines and neurotrophic factors), can lead to cell death. This mechanism is critical for the development of the nervous system in vertebrates and it is estimated that half of the neurons generated die during this process (Buss et al. 2006). This cell death is due, in part, to the failure of some neuronal precursors to properly migrate or innervate their targets and the consequent lack of neurotrophic factor stimulation. Similarly, during an immune response, cytokine deprivation (together with the DR pathway) is responsible for the acute contraction of the lymphocyte population after clearance of the pathogen. Another example of "loss-of-signal"-induced cell death is the particular form of apoptosis called anoikis, which occurs when epithelial or endothelial cells detach from the extracellular matrix (ECM). In this scenario, unligated ECM receptors of the integrin family cease to induce prosurvival signaling pathways, eventually leading to apoptosis. This mechanism prevents cells shedding from their original location from colonizing elsewhere (a characteristic of metastatic cancer cells). Finally, apoptosis can be induced by oncogenes (e.g., Myc) as a safeguard mechanism against cancer development. This process is controlled in part by a p53-dependent apoptotic pathway, which is activated in response to aberrant mitogenic signals resulting from oncogene overexpression or mutation. As a consequence, evasion of apoptotic cell death is often a requisite to sustain oncogene transformation (see Sever and Brugge 2014).

Autophagic cell death is characterized by the appearance of large intracellular vesicles and engagement of the autophagy machinery. Note that although autophagy (i.e., the membrane engulfment and catabolic degradation of parts of the cytoplasm) is a well-defined process, its function as an active cell death mechanism remains highly controversial. Autophagy is mainly a survival process engaged in response to a metabolic crisis (e.g., low ATP levels and nutrient and amino acid deprivation) or to remove damaged organelles (e.g., mitochondria with low membrane potential) and protein aggregates. As a stress response, autophagy accompanies rather than promotes cell death in most scenarios and merely represents a failed survival attempt (Shen et al. 2012). Nevertheless, there are specific examples in which the autophagy machinery is absolutely required for cell death. During *Drosophila* metamorphosis, obsolete larval tissues such as the midgut and salivary glands regress through massive autophagic cell death, a process triggered by the steroid hormone ecdysone. In this particular case, a deficiency in genes of the autophagic signaling pathway alters the cell death program (Berry and Baehrecke 2007; Denton et al. 2009). Autophagic cell death has also been reported in response to deregulated H-Ras activity and could therefore represent a safeguard mechanism against oncogenic transformation (Elgendy et al. 2011).

Necrosis is characterized by cell swelling and plasma membrane rupture, and a loss of organellar structure without chromatin condensation. Although necrosis can occur as a consequence of irreparable cell damage, at least one pathway of active necrosis exists. This form of cell death, sometimes called necroptosis, is engaged by several signaling pathways that all converge on the activation of receptor-interacting protein kinase 3 (RIP3). RIP3 is activated upon recruitment to macromolecular complexes downstream from various cell-surface receptors: DRs, Toll-like receptors (TLRs), and the T-cell receptor (TCR). Additionally, DNA damage can directly induce the formation of a RIP3-activation platform, independently of cell-surface receptor ligation. Finally, RIP3-dependent necrosis is also triggered by the cytosolic DNA sensor, DNA-dependent activator of interferon (DAI) regulatory factors, following virus infection and the presence, in the cytosol, of double-stranded viral DNA.

Here we are concerned with signaling leading to cell death in vertebrates, and focus on processes that are at least

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partially understood at the molecular level. We do not discuss the physiology and pathology of cell death in detail or processes involved in the clearance of dying cells. Readers will find a more complete overview of these topics and other aspects of cell death elsewhere (Green 2011b).

2 TYPE I CELL DEATH: APOPTOSIS

Apoptotic cell death is predominantly initiated either by the DR or mitochondrial pathway, although additional pathways exist. DRs—for example, Fas (also known as CD95), Trail receptor (TRAIL-R), or TNFR1—induce apoptosis by directly recruiting a caspase-activation platform upon binding to their respective ligand. The mitochondrial pathway of apoptosis, on the other hand, is triggered upon loss of integrity of the mitochondrial outer membrane, which allows the release of proapoptotic factors (e.g., cytochrome *c*) from the mitochondria into the cytosol. This process is controlled by the Bcl2 protein family. Once in the cytosol, cytochrome *c* induces the assembly of a caspase-activation complex: the apoptosome. Both pathways culminate in the activation of caspase proteases and the cleavage of intracellular proteins, ultimately leading to the dismantling of the cell. Below, we explore these processes in detail, starting with the end point: caspase activation.

2.1 Caspase Activation, Function, and Regulation

Apoptosis involves the activation of caspases, which orchestrate all of the morphological changes that characterize this form of cell death. Caspases are cysteine proteases with specificity for aspartic acid residues in their substrates. Although at least 17 different caspases exist in mammals, our focus is on only a subset of these for which the activation is at least partially understood and roles in cell death have been established.

The executioner caspases (caspase-3, caspase-6, and caspase-7) effect the destruction and are produced as inactive dimers that lack protein-interaction domains (Fig. 1). Activation is due to proteolytic cleavage between what will be the large and small subunits of the mature enzyme (Salvesen and Riedl 2008). Upon cleavage, the new ends fold into the dimer interface and promote conformational changes to create two active sites in the mature protease.

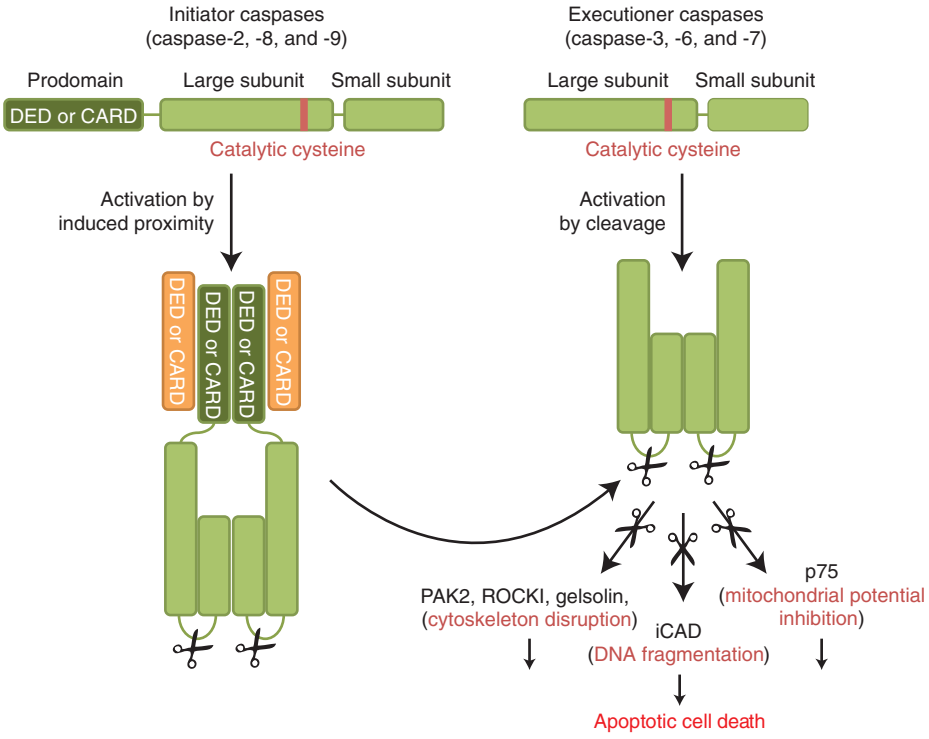

Figure 1. The caspase protein family. Initiator caspases (caspase-2, caspase-8, and caspase-9) are the apical caspases of the apoptotic-signaling cascade. Initiator caspases are produced as inactive zymogens composed of a prodomain (containing a CARD or a death effector domain [DED]) and a large and small subunit. They are recruited through their prodomains into large activation platforms and activated by dimerization. In contrast, executioner caspases (caspase-3, caspase-6, and caspase-7) are activated by cleavage of the zymogen between the large and small subunits and are therefore dependent on initiator caspases for their activation. Catalytically active caspases are composed of a heterotetramer of two small and two large subunits.

Cleavage of caspase-6 is mediated by caspase-3 and caspase-7 (Slee et al. 1999), whereas activation of the latter two caspases is generally the function of "initiator" caspases. It is these initiator caspases, and their activation, that define the apoptotic signaling pathways (see below).

Following activation, the executioner caspases, particularly caspase-3 and caspase-7, can process at least 1000 proteins (Crawford and Wells 2011). The cleavage of these caspase substrates can result in either a gain or a loss of function of these proteins and eventually leads to the cellular changes associated with apoptosis. Notably, caspase proteolysis inactivates components of essential physiological processes. For example, caspase cleavage of the p75 subunit of complex I of the electron transport chain disrupts the mitochondrial transmembrane potential, electron transport, and ATP production during apoptosis (Ricci et al. 2004). Conversely, caspase cleavage can also activate specific pathways. This is the case for caspase-activated nuclease (CAD), a DNAse that cuts chromatin between nucleosomes when its inhibitor, iCAD, is cleaved by caspase-3 (Enari et al. 1998; Sakahira et al. 1998). Additionally, caspases can hijack signaling pathways through constitutive activation of some of their components. For example, the characteristic morphology of apoptotic cells is caused by caspase-mediated activation of several actin cytoskeleton modulators: gelsolin, p21-activated kinase 2 (PAK2), and Rho-associated kinase I (ROCKI). Gelsolin, a calcium-regulated actin-severing protein, becomes constitutively active following caspase processing (Kothakota et al. 1997). PAK2 and ROCK I are serine–threonine kinase effectors of the Rho GTPase family (Rac1, Cdc42, and Rho) that regulate actin polymerization and actin–myosin contractility. Upon caspase cleavage, their kinase activity becomes independent of the GTPases, thus inducing an aberrant reorganization of the actin cytoskeleton and the characteristic membrane blebbing observed in apoptotic cells (Rudel and Bokoch 1997; Coleman et al. 2001; Sebbagh et al. 2001) (for more details on cytoskeleton-modulating proteins, see Devreotes and Horwitz 2014).

Unlike the executioner caspases, initiator caspases (Fig. 1) exist as inactive monomers in cells and are not activated by cleavage. Instead, adaptor molecules that assemble into caspase-activation platforms recruit these initiator caspases, forcing monomers into close proximity and causing conformational changes that result in the formation of active sites. In some (but not all) cases, subsequent autocleavage of the caspase is necessary to stabilize the mature, active enzyme (Pop et al. 2007; Oberst et al. 2010). Note that although cleavage of an executioner caspase is indicative of its activation, that of initiator caspases is not necessarily an indication of activation (McStay et al. 2008).

The interactions between caspase-activation platforms and caspases involve "death fold" domains of the proteins (Kersse et al. 2011). Such death fold elements are present in adaptor proteins and caspases: the caspase-recruitment domain (CARD), and the death effector domain (DED). Other death folds, the death domain (DD) and the pyrin (PYR) domain, are involved in the assembly of some caspase-activation platforms but not present on caspases (Kersse et al. 2011). Death fold domains typically mediate protein–protein interaction through homotypic interaction. They do not share sequence similarity but have similar structures composed of six amphipathic α helices.

2.2 Caspase-9 Activation: The Mitochondrial Pathway of Apoptosis

The mitochondrial pathway of apoptosis, also called the intrinsic pathway, is the most common mechanism of apoptosis in vertebrates. It is activated in response to a variety of cellular stresses, including DNA damage, growth factor deprivation, ER stress, and developmental cues. In this pathway, the executioner caspases are cleaved and activated by caspase-9, which is itself activated by a caspase-activation platform called the apoptosome (Fig. 2) (Bratton et al. 2001; Bratton and Salvesen 2010).

Apoptotic protease-activating factor 1 (APAF1) constitutes the scaffold around which the apoptosome is assembled. During intrinsic apoptosis, cytochrome *c* is released from mitochondria into the cytosol (see below) and binds to Apaf1 (Zou et al. 1997). This interaction triggers hydrolysis of the Apaf1 cofactor dATP to dADP (Kim et al. 2005). The subsequent exchange of dADP with exogenous dATP allows the oligomerization of seven APAF1–dATP–cytochrome-*c* units into an active apoptosome. At the center of the apoptosome, exposed CARDs on APAF1 bind to the CARD of caspase-9, thus bringing the inactive caspase-9 monomers into close proximity for activation and autoprocessing (Yu et al. 2005; Yuan et al. 2010). Owing to its higher affinity for the apoptosome, full-length caspase-9 displaces the processed form, creating a continuous cycle of caspase-9 recruitment, activation, processing, and release (Malladi et al. 2009). Because caspase-9 only sustains catalytic activity in this bound state (Rodriguez and Lazebnik 1999; Stennicke et al. 1999; Bratton et al. 2001), the apoptosome functions as a molecular timer in which its lifetime is directly proportional to the amount of unprocessed caspase-9 present (Malladi et al. 2009).

In healthy cells, cytochrome *c* is found only in the mitochondrial intermembrane space. For it to interact with APAF1, mitochondrial outer membrane permeabilization (MOMP) triggered by apoptotic stimuli must occur (Tait and Green 2010). MOMP induces the release of all soluble

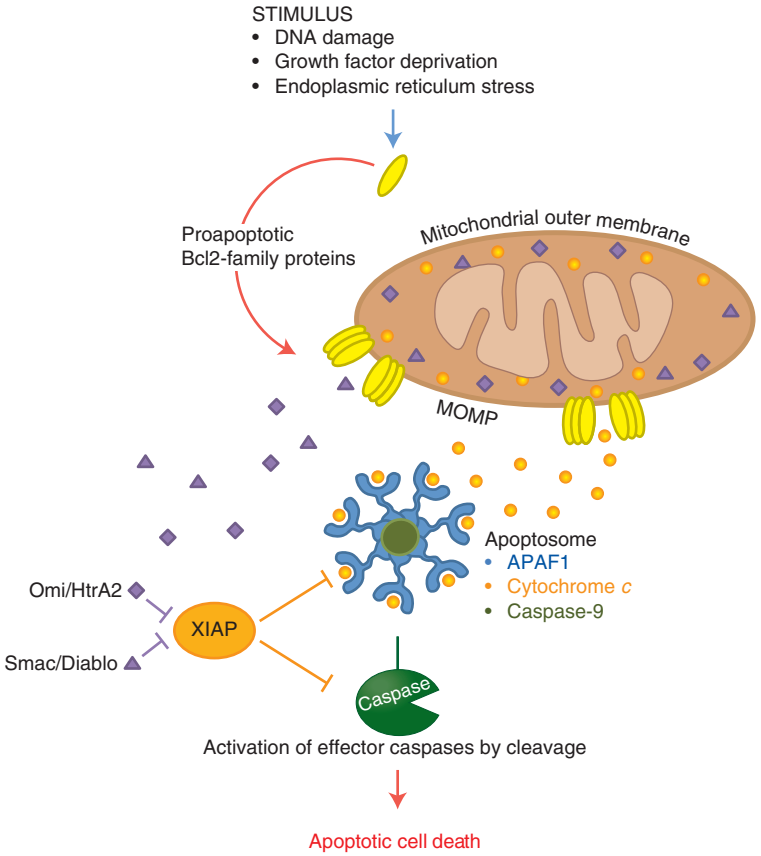

Figure 2. The mitochondrial apoptotic pathway. In response to various cellular stresses, proapoptotic members of the Bcl2 family induce mitochondrial outer membrane permeabilization (MOMP), allowing the release into the cytosol of proapoptotic factors that are normally sequestered in the intermembrane space of the mitochondria (including cytochrome *c*, Smac, and Omi). In the cytosol, cytochrome *c* binds to APAF1 and triggers its oligomerization. Caspase-9 is then recruited and activated by this platform, known as the apoptosome. Catalytically active caspase-9 cleaves and activates the executioner caspases-3 and -7. Upon release in the cytosol, Smac and Omi bind to and inhibit the caspase inhibitor X-linked inhibitor of apoptosis (XIAP). In doing so, they relieve XIAP inhibition of caspase-9, caspase-3, and caspase-7, and potentiate overall caspase activation by the apoptosome.

Cold Spring Harbor Perspectives in Biology

proteins of the mitochondrial intermembrane space into the cytosol. In addition to cytochrome *c*, two other proapoptotic proteins are released during that process: Smac (also known as Diablo) and Omi (also known as HtrA2). Smac and Omi potentiate the apoptosome activity by antagonizing the caspase inhibitor X-linked inhibitor of apoptosis (XIAP) (Fig. 2). In the absence of Smac and Omi, XIAP binds to, and inhibits the catalytic activity of, the initiator caspase-9 and the executioner caspases-3 and -7. XIAP further dampens intrinsic apoptosis induction through direct ubiquitylation and proteasomal degradation of active caspases (Eckelman et al. 2006).

MOMP is a tightly regulated event controlled by members of the Bcl2 family. These share one or more Bcl2 homology (BH) regions defined by sequence, structure, and function (Fig. 3A). There are three broad classes of Bcl2 proteins: the proapoptotic effector proteins (Bax and Bak), which are necessary and sufficient for MOMP; the antiapoptotic Bcl2 proteins (e.g., Bcl2, Bcl-xL, and Mcl1), which block MOMP; and the BH3-only proteins (e.g., Bid, Bim, Bad, and Noxa), which activate the proapoptotic effectors and/or neutralize the antiapoptotic Bcl2 proteins. Bcl2 proteins also control several other cellular processes, including mitochondrial fusion, autophagy, and calcium efflux from the ER (Chipuk et al. 2010).

Bax and Bak are directly responsible for the loss of mitochondrial outer membrane integrity (Fig. 3B). Upon activation, they form large oligomers that insert into the mitochondrial outer membrane, disrupting it (Eskes et al. 2000; Korsmeyer et al. 2000; Dewson et al. 2008, 2009). The precise nature of the disruption remains unclear, but it allows the near simultaneous release of all intermembrane space proteins (Goldstein et al. 2000; Munoz-Pinedo et al. 2006).

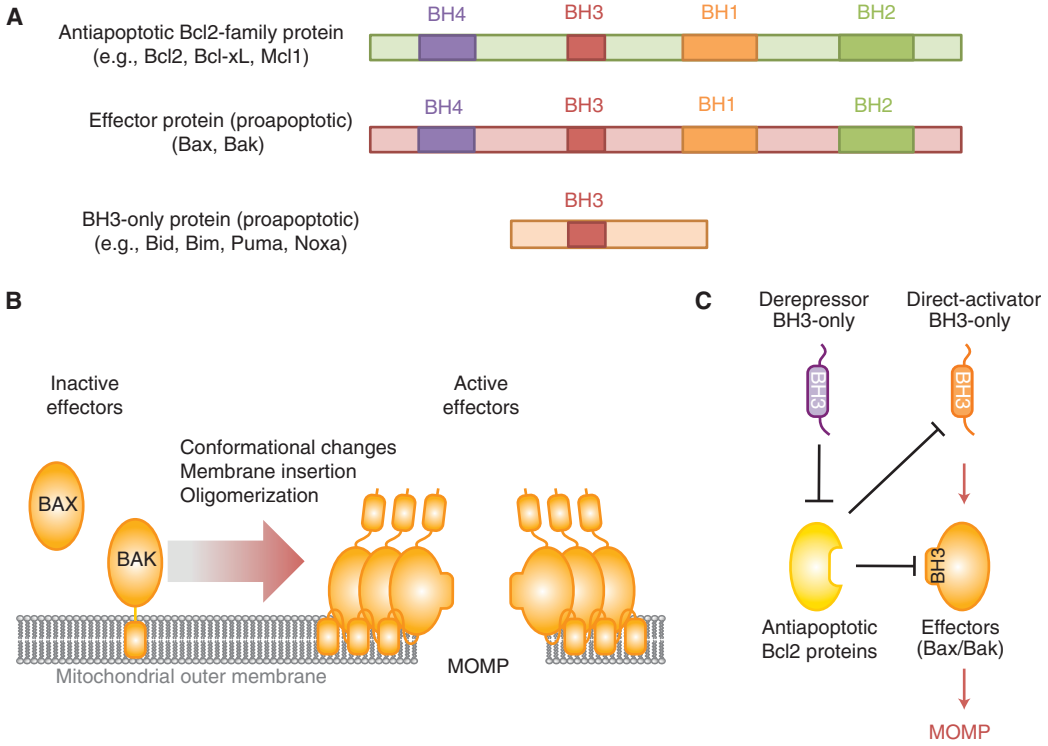

Figure 3. Regulation of mitochondrial outer membrane integrity by the Bcl2 protein family. (*A*) Members of the Bcl2 protein family are characterized by the presence of one or more Bcl2 homology (BH) region. The antiapoptotic Bcl2 proteins (e.g., Bcl2, Bcl-xL, and Mcl1) and the proapoptotic effectors (e.g., Bax and Bak) share four BH regions and a similar globular structure. BH3-only proteins (e.g., Bid, Bim, Bad, and Noxa) are characterized by a single BH region (BH3). (*B*) The proapoptotic effectors reside in cells in inactive forms tethered to the outer mitochondrial membrane (Bak) or soluble in the cytosol (Bax). Upon activation, Bax and Bak oligomerize and further insert into the membrane, causing mitochondrial outer membrane permeabilization (MOMP) and thus apoptosis. (*C*) Bax/Bak activation is triggered following the transient binding of a subset of direct activator BH3-only proteins (e.g., Bid and Bim). Antiapoptotic Bcl2 proteins inhibit MOMP by sequestering the direct activator proteins and/or the effectors. Another group of BH3-only proteins, called sensitizers or derepressors (e.g., Bad and Noxa) promote MOMP by antagonizing antiapoptotic Bcl2 proteins, thereby releasing both direct activator BH3-only proteins and Bax/Bak.

Bax and Bak act redundantly in MOMP and at least one of them is required to permeabilize mitochondria. In living cells these proteins are generally inactive, but become activated in response to upstream events. At least two of the BH3-only proteins (Bim and active Bid) activate Bax and Bak through transient interaction (Fig. 3C), although other conditions (such as heat, changes in pH, and changes in the lipid milieu) may activate the effectors independently of BH3-only proteins (Wei et al. 2000; Kuwana et al. 2002, 2005; Letai et al. 2002).

MOMP is antagonized by the antiapoptotic Bcl2 proteins, which bind to and inhibit both Bax/Bak and the BH3-only proteins by interacting with their BH3 domains (Llambi et al. 2011) (Fig. 3C). Antiapoptotic Bcl2 proteins are also regulated at both the transcriptional and posttranslational levels. In particular, Mcl1 degradation by the ubiquitin-proteasome system participates in apoptosis induction following several cellular stresses. Upon DNA damage, the BH3-domain-containing protein Mcl1 ubiquitin ligase E3 (MULE) directly binds to Mcl1 and catalyzes its ubiquitylation and degradation (Warr et al. 2005). During antitubulin chemotherapeutic-induced mitotic arrest, Mcl1 is phosphorylated by stress-activated and mitotic kinases such as the MAP kinase (MAPK) Jun amino-terminal kinase (JNK), casein kinase II (CKII), and p38 MAPK. These phosphorylation events unveil a degron on Mcl1 that recruits the SCF-Fbw7 ubiquitin ligase complex, thus targeting Mcl1 for ubiquitylation and degradation (Wertz et al. 2011). Similarly, Mcl1 is degraded following growth factor withdrawal (e.g., IL3) and the subsequent loss of phosphoinositide 3-kinase (PI3K)-Akt signaling. This process relieves glycogen synthase kinase 3 (GSK3) from Akt inhibition. GSK3 then phosphorylates Mcl1, allowing its ubiquitylation by the E3 ligase β-transducin-repeat-containing protein (β-TrCP) and its subsequent degradation (Maurer et al. 2006; Ding et al. 2007). Conversely, cancer cells

can increase Mcl1 stability and overall resistance to cellular stress by expressing USP9X, a deubiquitylase that removes polyubiquitin chains from Mcl1 (Schwickart et al. 2010).

Proteins of the Bcl2 family integrate pro- and antiapoptotic signals in healthy and stressed cells and therefore constitute one of the main signaling nodes in the life or death decision. Within this family, BH3-only proteins constitute the main upstream sensors of the mitochondrial apoptotic pathway. A wide variety of signaling pathways converge on the BH3-only family of proteins and regulate their expression level and activity both transcriptionally and posttranscriptionally (see Table 1). For example, Bid is activated upon cleavage by caspase-8 following DR ligation (Li et al. 1998, Luo et al. 1998). In doing so, Bid coordinates the cross-regulation between the extrinsic and intrinsic apoptotic pathways. Additionally, proteolytic activation of Bid can be achieved by granzyme B during the cytotoxic lymphocyte killing process (Heibein et al. 2000; Sutton et al. 2000). The apoptotic response to genotoxic stress is performed, in part, by Puma and Noxa (Jeffers et al. 2003; Villunger et al. 2003). Both are direct transcriptional targets of the tumor suppressor p53 (Oda et al. 2000; Nakano and Vousden 2001; Yu et al. 2003), although other stimuli regulate their expression levels (see Table 1). Similarly, Bim is transcriptionally up-regulated by the forkhead transcription factor FOXO3A upon cytokine deprivation (Dijkers et al. 2000; Gilley et al. 2003; Urbich et al. 2005). Bim is also a major apoptotic factor of the ER stress pathway engaged in response to accumulation of unfolded proteins (Puthalakath et al. 2007). Finally, Bad activity is negatively regulated through phosphorylation by several kinases, such as Akt (Datta et al. 1997; del Peso et al. 1997), which induces its sequestration by 14-3-3 proteins. Upon growth factor deprivation and loss of Akt signaling, Bad is released and antagonizes antiapoptotic Bcl2 proteins (Zha et al. 1996; Datta et al. 1997).

MOMP often condemns a cell to death even if caspase activation is blocked or disrupted (Tait and Green 2010). This is probably the consequence of a catastrophic loss of mitochondrial function that results in bioenergetic failure (Lartigue et al. 2009). Under these conditions, some cells can recover from MOMP, however, and survive (Tait et al. 2010). Therefore, the regulation of caspase activation downstream from MOMP may be important in some settings. For example following cytochrome *c* binding to APAF1, the failure to replace hydrolyzed dADP by exogenous dATP triggers nonfunctional aggregation and irreversible inactivation of APAF1 (Kim et al. 2005). Nucleotide exchange, and therefore functional apoptosome assembly, is facilitated by a complex composed of heat shock protein 70 (Hsp70), cellular apoptosis susceptibility (CAS) protein, and putative HLA-DR-associated protein I (PHAPI) (Kim et al. 2008). Accordingly, cellular levels of these proteins modulate caspase-activation efficiency during intrinsic apoptosis. tRNA levels also regulate apoptosome activity by binding to cytochrome *c*, blocking its interaction with APAF1 (Mei et al. 2010). Finally, intrinsic apoptosis can be perturbed downstream from MOMP through phosphorylation and inhibition of caspase-9 (see below) (Allan and Clarke 2009).

2.3 Caspase-8 Activation: The Death Receptor Pathway

Caspase-8 is activated predominantly by the DR pathway of vertebrate apoptosis. DRs are a subset of the TNFR superfamily and include TNFR1, Fas, and TRAIL-R1/2 (Dickens et al. 2012). They contain a DD in their intracellular regions. Through a series of homotypic interactions, these initiate the assembly of large macromolecular complexes that recruit and activate caspase-8 for apoptotic signaling, as well as other signaling molecules that control processes such as inflammation and cell adhesion (Newton and Dixit 2012).

When Fas or a TRAIL-R is bound by its ligand, clustering of the receptors recruits a DD-containing adaptor molecule, FADD, through DD-DD interactions (Fig. 4A). This exposes another death fold domain in FADD, its DED. The complex represents a caspase-activation platform called a death-inducing signaling complex (DISC), as the FADD DEDs bind to DEDs in the prodomain of caspase-8. This brings caspase-8 monomers into close proximity, triggering their protease activity. Caspase-8 then undergoes autocatalytic cleavage both between the large and small subunits and between the large subunit and the prodomain. The first cleavage stabilizes the active dimer (and is required for homodimer activity in this pathway); the second cleavage releases it from the DISC (Dickens et al. 2012).

In some cells (called type I cells), active caspase-8 then promotes apoptosis by cleaving and activating caspase-3 and caspase-7. However, in many cell types (type II cells), the active executioner caspases are inhibited by XIAP, and thus apoptosis is blocked (Jost et al. 2009). In these cases, another caspase-8 substrate comes into play, the BH3-only protein Bid (see above). Caspase-8-mediated cleavage activates Bid, which in turn activates Bax and Bak to promote MOMP. The IAP antagonists Smac and Omi are then released, and these neutralize XIAP to allow apoptosis to proceed.

The signaling pathways engaged downstream from TNFR1 are more complex and potentially lead to three different outcomes: survival, apoptosis or necrosis (Fig. 4B). Upon TNFR1 ligation, a different DD-containing adaptor, TNFR-associated death domain protein (TRADD), is first recruited. TRADD does not directly bind or activate cas-

Table 1. Signaling to BH3-only proteins

BH3-only protein	Stimulus/input	Type of regulation	Signaling pathway	Output	References
Bad	Growth factors and cytokines	Phosphorylation	Akt	Cell survival	Datta et al. 1997, 2002; del Peso et al. 1997
		Phosphorylation	p70 S6K	Cell survival	Harada et al. 2001
		Phosphorylation	PKA	Cell survival	Harada et al. 1999
		Phosphorylation	PIM kinases	Cell survival	Fox et al. 2003; Yan et al. 2003
		Phosphorylation	PAK	Cell survival	Schürmann et al. 2000; Cotteret et al. 2003
		Sequestration	14-3-3	Cell survival	Zha et al. 1996; Datta et al. 2000
	TNF	Phosphorylation	IKK	Cell survival	Yan et al. 2013
	Neuronal activity deprivation	Phosphorylation	Cdc2	Apoptosis	Konishi et al. 2002
	Growth factor or cytokine deprivation	Phosphorylation	JNK	Apoptosis	Donovan et al. 2002
		Dephosphorylation	PP1, PP2A, and PP2C	Apoptosis	Ayllón et al. 2000; Chiang et al. 2001; Klumpp et al. 2003
	Calcium	Dephosphorylation	Calcineurin	Apoptosis	Wang et al. 1999
Bid	Cytotoxic T cell	Cleavage	Granzyme B	Apoptosis	Heibein et al. 2000; Sutton et al. 2000
	Fas/TNF/TRAIL	Cleavage	Caspase-8	Apoptosis	Li et al. 1998; Luo et al. 1998
	Heat shock or ER stress	Cleavage	Caspase-2	Apoptosis	Bonzon et al. 2006; Upton et al. 2008
	Ischemia or cisplatin	Cleavage	Calpain	Apoptosis	Chen et al. 2001; Mandic et al. 2002
	Lysosome permeabilization	Cleavage	Cathepsin	Apoptosis	Stoka et al. 2001; Reiners et al. 2002
Bim	Growth factors or cytokines	Phosphorylation	ERK1/2 and RSK1/2	Cell survival	Ley et al. 2003; Hubner et al. 2008; Dehan et al. 2009
		Ubiquitylation	βTrCP	Cell survival	Akiyama et al. 2003; Ley et al. 2003; Dehan et al. 2009
		mRNA stability	Hsc70	Cell survival	Matsui et al. 2007
	?	mRNA stability	miR-17-92	Cell survival	Xiao et al. 2008
	ER stress	Transcription	CHOP-C/EBPa	Apoptosis	Puthalakath et al. 2007
		Dephosphorylation	PP2A	Apoptosis	Puthalakath et al. 2007
	Glucocorticoid	Transcription	GR	Apoptosis	Erlacher et al. 2005; Ploner et al. 2008
	Growth factor deprivation or DNA damage	Phosphorylation	JNK	Apoptosis	Lei and Davis 2003; Putcha et al. 2003
	Growth factor or cytokine deprivation	Transcription	FOXO3a	Apoptosis	Dijkers et al. 2000; Gilley et al. 2003
Noxa	DNA damage	Transcription	p53	Apoptosis	Oda et al. 2000; Villunger et al. 2003; Naik et al. 2007
	DNA damage	Transcription	p63	Apoptosis	Kerr et al. 2012
	DNA damage	Deubiquitylation	UCH-L1	Apoptosis	Brinkmann et al. 2013
	Glucose deprivation	Transcription	?	Apoptosis	Alves et al. 2006
	Hypoxia	Transcription	HIF1α	Apoptosis	Kim et al. 2004
	Proteasome inhibition	Transcription/ protein stabilization	Myc, others	Apoptosis	Fernandez et al. 2005; Qin et al. 2005; Nikiforov et al. 2007
Puma	Cytokine deprivation	Transcription	FOXO3a	Apoptosis	Jeffers et al. 2003; Villunger et al. 2003; You et al. 2006; Ekoff et al. 2007
	DNA damage	Transcription	p53	Apoptosis	Nakano and Vousden 2001; Yu et al. 2001; Jeffers et al. 2003; Villunger et al. 2003
	DNA damage or ER stress	Transcription	p63	Apoptosis	Pyati et al. 2011; Kerr et al. 2012
	Glucocorticoid	Transcription	GR	Apoptosis	Villunger et al. 2003; Erlacher et al. 2005
BIK	DNA damage	Transcription	Smad	Proapoptotic	Spender 2009
	TGFβ	Transcription	E2F1	Proapoptotic	Real 2006
		Ubiquitylation?		Proapoptotic	Nikrad 2005; Zhu 2005
	Proteasome inhibition	Cleavage	RHBDD1	Antiapoptotic	Wang et al. 2008a
		Phosphorylation	Casein kinase	Proapoptotic	Verma 2001; Li 2003
		Transcription	Smad	Proapoptotic	Ramjaun 2007

Continued

Table 1. *Continued*

BH3-only protein	Stimulus/input	Type of regulation	Signaling pathway	Output	References
BMF	TGFβ	Sequestration	DLC2	Antiapoptotic	Puthalakath 2001
		Transcription	HIFα	Proapoptotic	Bruick 2000; Guo 2001
BNIP3	Hypoxia	Transcription	E2F1	Proapoptotic	Yurkova 2008
		Transcription inhibition	NF-κB	Antiapoptotic	Shaw 2006, 2008
		Transcription	Jun	Proapoptotic	Ma 2007
HRK	Potassium deprivation	Transcription	E2F1	Proapoptotic	Hershko 2004
		Transcription inhibition	DREAM	Antiapoptotic	Sanz 2001
	Cytokines	Transcription	HIFα	Proapoptotic	Sowter 2001
NIX	Hypoxia				

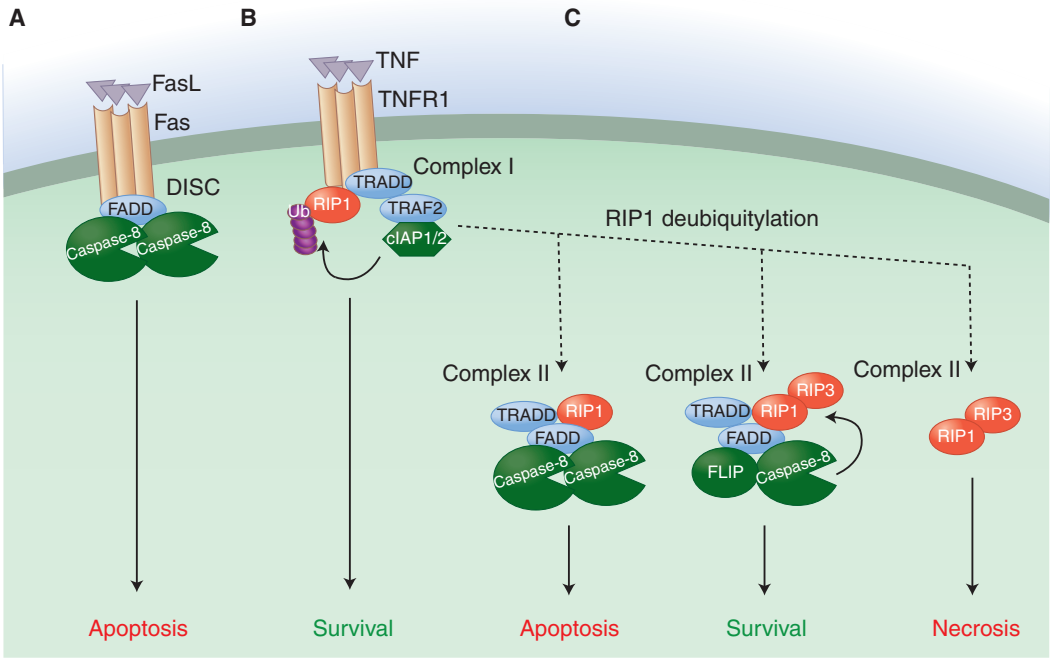

Figure 4. Death receptor signaling pathway. The death receptor signaling pathway is triggered by ligation of a death receptor (TNFR1, Fas, or TRAIL-R1/2) and potentially leads to three different outcomes: survival, apoptosis, or necrosis. (*A*) Upon ligation (by FasL and TRAIL, respectively), Fas/CD95 and TRAIL-Rs assemble a caspase-activation platform called the DISC. This platform recruits and activates caspase-8 via the adaptor protein FADD and thus engages the extrinsic apoptotic pathway. (*B*) Upon ligation to TNF, TNFR1 recruits the adaptor protein TRADD and the kinase RIP1, which in turn mobilizes additional partners, such as the ubiquitin ligases TRAF2 and cIAP1/2. These catalyze the nondegradative ubiquitylation of RIP1 as well as other components of the protein complex, resulting in the stabilization of a prosurvival and proinflammatory signaling platform called complex I. (*C*) The removal of ubiquitin chains of RIP1 by inhibition of cIAP1/2 or through the action of deubiquitylases destabilizes complex I, thus allowing the release of TRADD and RIP1. TRADD and RIP1 then promote the formation of a series of cytosolic complexes (complex II) that can initiate either apoptotic or necrotic cell death. In the proapoptotic configuration of the complex, TRADD and RIP1 recruit FADD and caspase-8, resulting in caspase activation and apoptosis. When FADD or caspase-8 are absent, or when caspase activity is blocked, RIP1 binds to and activates RIP3, thus triggering necrotic cell death. Expression of FLICE-like inhibitory protein (FLIP) inhibits the activity of both complexes. FLIP forms a heterodimer with caspase-8 that cannot induce apoptosis but still displays catalytic activity, and this activity antagonizes the activation of RIP3. Therefore, complexes containing FLIP–caspase-8 heterodimers simultaneously block apoptosis and RIP-dependent necrosis.

Cold Spring Harbor Perspectives in Biology

pase-8 but instead acts as a membrane-bond scaffold for the recruitment of additional signaling molecules, including a kinase, receptor-interacting protein 1 (RIP1), and the ubiquitin ligases TRAF2 and cIAP1/2. Components of this complex, including RIP1, are modified by a complex series of nondegradative ubiquitylation events performed by both the cIAPs and the linear ubiquitin assembly complex (LUBAC). The assembly and ubiquitylation of this signaling platform (referred to as complex I) culminates in the activation of the NF-κB signaling pathway and an inflammatory response rather than cell death (Newton and Dixit 2012).

TNFR1 ligation leads to cell death when ubiquitylation of RIP1 is compromised either by inhibition of ubiquitin ligases contained in complex I (e.g., cIAP1/2) or through direct de-ubiquitylation of RIP1 by enzymes such as CYLD (and probably other signaling events). RIP1 and TRADD are then released from TNFR1 to form a series of dynamic cytosolic signaling platforms (collectively referred to as complex II) that have the potential to cause both apoptotic and necrotic cell death (Fig. 4C). Cytosolic TRADD recruits FADD (via DD-DD interaction), which in turn can bind to and activate caspase-8 similarly to the DISC (Fig. 4C, left panel) (Christofferson and Yuan 2010; Declercq et al. 2009). In this configuration, complex II formation leads to apoptotic cell death. In many cases, however, apoptosis does not proceed upon TNFR1 ligation, at least in part because the activation of NF-κB induces the expression of the caspase-8-like protein FLICE-like inhibitory protein (FLIP) (Micheau et al. 2001). FLIP has a domain structure similar to that of caspase-8, but it lacks a catalytic cysteine. It preferentially binds to FADD (via DED-DED interactions) and a monomer of caspase-8. The caspase-8–FLIP heterodimer is catalytically active (Micheau et al. 2002; Pop et al. 2011), but does not promote apoptosis. Why this is remains obscure, but may relate to more rapid degradation of the complex (Geserick et al. 2009). Consequently, the assembly of complex II under conditions of active NF-κB signaling, and therefore FLIP expression, leads to a survival outcome (Fig. 4C, middle panel). Finally, complex II formation can engage a necrotic form of cell death (see section below). As we will see below, the activity of the caspase-8–FLIP heterodimer is also important for preventing this necrotic cell death signaling engaged by ligated TNFR1.

2.4 Caspase -1 and Caspase-5/-11 Activation: The Inflammasome Pathway

Caspase-1 and human caspase-5 (caspase-11 in rodents) have large prodomains containing CARDs. These are engaged by caspase-activation platforms called inflammasomes. Generally these platforms form in response to infectious agents (e.g., viruses, bacteria, and fungi) and inert substances that induce inflammation (e.g., uric acid crystals, calcium phosphate crystals, alum, asbestos) (Franchi et al. 2012).

The platforms that engage caspase-1 are established by activated NOD-like receptors (NLRs), which bear either a CARD or, more often, a PYR domain (Fig. 5). In most cases, the NLR engages the adaptor molecule ASC via PYR–PYR interactions. ASC also contains a CARD that binds to and activates caspase-1 (Martinon et al. 2002; Faustin et al. 2007; Franchi et al. 2009).

Another caspase-1 inflammasome involves the sensor AIM2, which binds to cytosolic DNA (e.g., from viruses) and also engages ASC (Fig. 5) (Hornung et al. 2009). Caspase-1 cleaves and promotes the secretion of two inflammatory cytokines, interleukin (IL) 1β and IL18. It can also cleave and activate Bid, as well as caspase-3 and caspase-7 to promote apoptosis. This form of caspase-1-mediated cell death is sometimes called pyroptosis (Brennan and Cookson 2000; Cookson and Brennan 2001; Fink and Cookson 2006; Bergsbaken and Cookson 2007).

Little is known about the activation platform for caspase -5/-11, except that it does not involve ASC or the NLRs that function in caspase-1 activation (Kayagaki et al. 2011). Nevertheless, caspase-11 activation can result in apoptosis (Kayagaki et al. 2011). Caspase-11 can probably also bind to and activate caspase-1 (Green 2011a). In addition, caspase-11, but not caspase-1, is involved in the lethal effects of bacterial lipolysaccharides in mice in vivo (endotoxemia) (Kayagaki et al. 2011), which may also involve one of the executioner caspases, caspase-7 (Lamkanfi et al. 2009).

2.5 Caspase-2 Activation: The PIDDosome Pathway

The functions of caspase-2 in mammalian apoptosis remain somewhat obscure (Krumschnabel et al. 2009). Caspase-2 is activated in response to heat shock, microtubule disruption and DNA damage (Bouchier-Hayes et al. 2009), and it has been implicated in apoptosis in oocytes (Nutt et al. 2005) and degenerating neurons (Troy et al. 1997, 2000, 2001).

The activation platform for caspase-2 includes the adaptor molecule RAIDD (Duan and Dixit 1997; Hofmann et al. 1997; Chou et al. 1998), which bears a CARD that binds to the CARD in the prodomain of the caspase. Like caspase-8, caspase-2 is activated by induced proximity, following which intrachain autocleavage stabilizes the mature enzyme (Baliga et al. 2004).

In addition to a CARD, RAIDD also has a DD. This binds to the DD-containing protein PIDD (Lin et al. 2000; Telliez et al. 2000) to form what has been called the PIDDosome (Tinel and Tschopp 2004). PIDD contains two intein regions that promote its autocleavage. The first cleavage produces PIDD-C, a molecule that functions in

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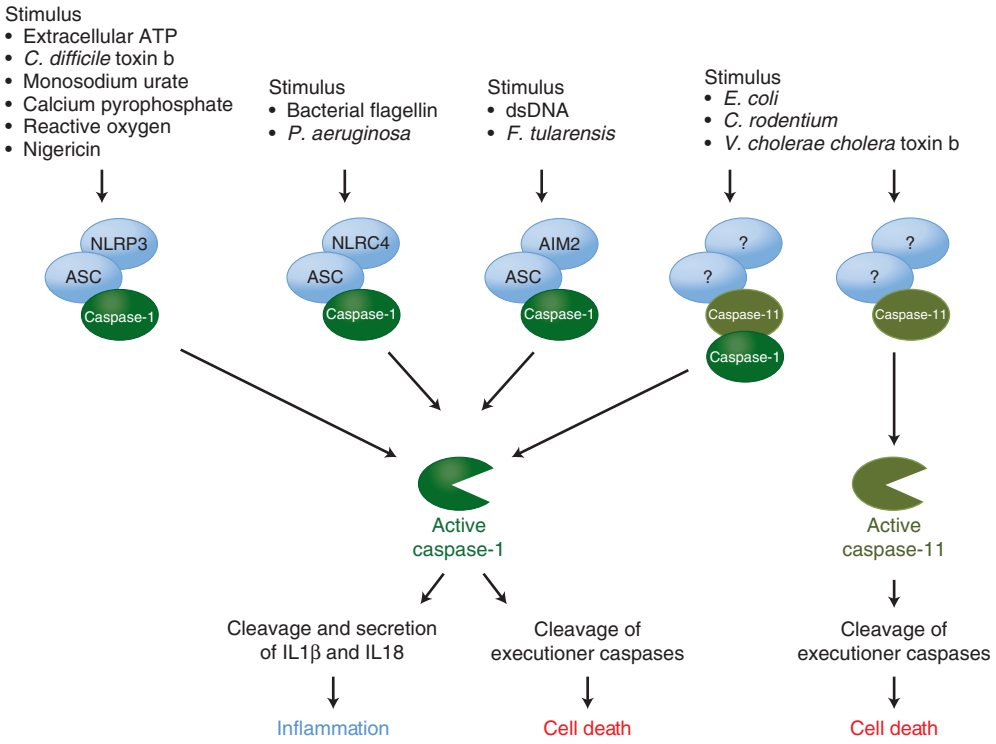


Figure 5. The inflammasomes. The inflammasomes are caspase-activation platforms that assemble in response to infectious agents (e.g., viruses, bacteria, and fungi) and inert substances that induce inflammation (e.g., uric acid crystals, calcium phosphate crystals, alum, asbestos). They recruit and activate the inflammatory caspase-1 and -11. The caspase-1 activation platforms are supported by the NOD-like receptors (NLRs; e.g., NLRP3 and NLRC4) and AIM. Upon activation by inflammatory agents, these proteins recruit the adaptor molecule ASC and caspase-1. The subsequent activation of caspase-1 induces cleavage and secretion of two inflammatory cytokines, interleukin (IL) 1β and IL18.

NF-κB activation (Janssens et al. 2005; Tinel et al. 2007). The second generates PIDD-CC, which binds to RAIDD (Tinel and Tschopp 2004; Tinel et al. 2007). However, caspase-2 activation occurs in the absence of PIDD (Manzl et al. 2009; Ribe et al. 2012), and it is not clear when or if PIDD is required (Manzl et al. 2012). The interaction of caspase-2 with RAIDD is regulated by phosphorylation of the caspase (see below).

2.6 Regulation of Caspase Activation by Kinases

Much of the regulation of apoptosis inevitably occurs upstream of caspase-activation platforms. But other signaling events can modify caspases such that, even when a suitable activation platform forms, their activation is inhibited. XIAP and FLIP are two examples. Below we consider some others.

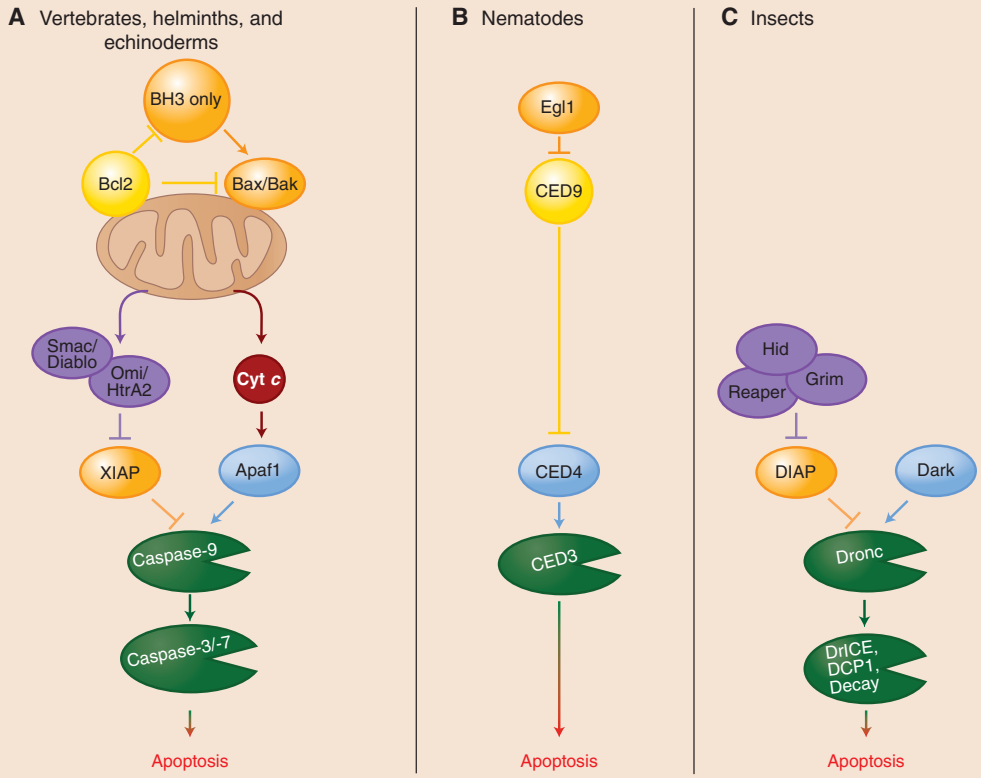
Regulation of caspase-2 activation is particularly well described. When NADPH is plentiful in the cell (e.g., owing to the activity of the pentose phosphate pathway) calcium/calmodulin-dependent protein kinase II (CaMKII) phosphorylates its CARD (Nutt et al. 2005). This allows the binding of 14-3-3ζ, which prevents association with the PIDDosome (Nutt et al. 2009). If NADPH levels decrease, protein phosphatase 1 (PP1) dephosphorylates caspase-2, permitting its activation by the PIDDosome (Nutt et al. 2009). SIRT1-mediated acetylation of 14-3-3ζ inhibits its binding to phospho-caspase-2, reinforcing this signal (Andersen et al. 2011). Intriguingly, the *Drosophila* initiator caspase Dronc (see Box 1) is also regulated by NADPH and phosphorylation by CaMKII (Yang et al. 2010; Rhind and Russel 2012).

The cell-cycle regulator cyclin-dependent kinase 1 (CDK1) bound to cyclin B1 also phosphorylates caspase-2 (Andersen et al. 2009). In this case, the phosphorylation is in the region between what will be the large and small subunits of the active caspases, and appears to directly inhibit the generation of the mature, stable enzyme.

Caspase-9 is phosphorylated on a threonine residue in the region between the prodomain and the large subunit by several kinases such as the ERK2 MAPK and CDK1 (Allan and Clarke 2009). Inhibition of caspase-9 activity by ERK2 occurs downstream from Ras in response to prosurvival stimuli such as growth factor stimulation (Allan et al.

BOX 1 CASPASE ACTIVATION IN INVERTEBRATE ORGANISMS

Homologs of mammalian components of the mitochondrial pathway (e.g., caspases, APAF1 and Bcl2 proteins) are found throughout the animal kingdom, including invertebrates. However, in neither of the two invertebrates studied extensively—*Drosophila* and *Caenorhabditis elegans*—has a role for the mitochondrial pathway involving MOMP and cytochrome-*c*-mediated activation of the apoptosome been unambiguously shown. Recent studies indicate that mitochondrial pathway may exist in helminths (Lee et al. 2011; Bender et al. 2012), and cytochrome *c* (Cyt *c*) triggers caspase activation in cytosolic extracts of a helminth and several echinoderms (Bender et al. 2012). Therefore, it is possible that the insect and nematode pathways outlined below are derived from an ancestral mitochondrial pathway resembling that of mammals (see the figure below, part A).



In *C. elegans*, the single caspase involved in apoptosis, CED3, contains a CARD and is activated by a platform composed of the APAF1 ortholog CED4 (Yuan et al. 1993; Chinnaiyan et al. 1997a). Monomeric CED4 is held inactive in living cells by the Bcl2 ortholog CED9 (Chinnaiyan et al. 1997b; Seshagiri and Miller 1997; Spector et al. 1997; Wu et al. 1997a,b). (Recall that this is not the case in mammals, in which Bcl2 proteins do not sequester APAF1, but instead restrict access to cytochrome *c* [Newmeyer et al. 2000].) In cells that are triggered to undergo apoptosis, the BH3-only protein Egl1 binds to CED9, releasing CED4, which then assembles in the caspase-activation platform for CED3 (Conradt and Horvitz 1998; Yan et al. 2004). The control of expression of Egl1 is the major way in which apoptosis in this organism is regulated (see the figure above, part B).

In *Drosophila*, there are several initiator and executioner caspases. The initiator caspase Dronc contains a CARD and is activated on a platform composed of the APAF1 ortholog, Dark (Kanuka et al. 1999; Rodriguez et al. 1999; Zhou et al. 1999). The Dark apoptosome appears to be constitutively active (Yu et al. 2006) but the activity of Dronc is inhibited by an IAP, DIAP1 (Rodriguez et al. 2002). The expression of one of several DIAP1 inhibitors, Reaper, Hid, Grim, or Sickle (Goyal et al. 2000; Lisi et al. 2000; Yoo et al. 2002), leads to displacement of DIAP1, allowing Dronc to cleave and thereby activate the executioner caspases DCP1, drICE, and Decay. Therefore, the expression of the DIAP1 inhibitors appears to directly control apoptosis in flies (see the figure above, part C). The roles of MOMP and cytochrome *c* release in *Drosophila* apoptosis are controversial. Although MOMP occurs, it appears to be predominantly caspase dependent and therefore is probably an effect rather than a cause of caspase activation.

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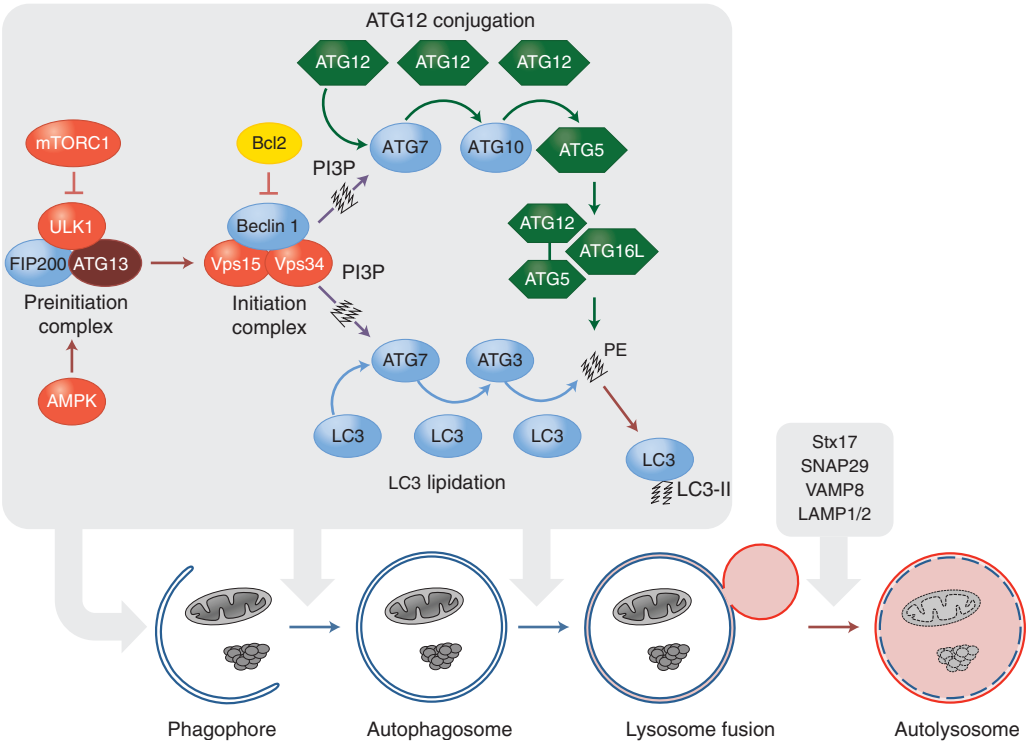

Figure 6. The autophagic signaling pathway. Under metabolic stress, AMPK activation and/or mTORC1 inhibition lead to the activation of the preinitiation complex (ULK1, FIP200, and ATG13). The latter activates the initiation complex (beclin 1, VPS15, and VPS34) that generates PI3P and recruits ATG7 to the phagophore. ATG7 functions similarly to an E1-ubiquitin ligase and initiates two conjugation pathways necessary for membrane elongation and closure of the autophagosome. In the ATG5-ATG12 conjugation pathway, ATG12 is sequentially transferred to ATG7, ATG10, and ATG12. The ATG5-12 conjugate recruits ATG16L and forms a complex necessary to stabilize the phagophore and to complete the second conjugation pathway. In the LC3-PE pathway, LC3 is cleaved by ATG4 and sequentially conjugated to ATG7 and ATG3. The ATG5-ATG12-ATG16L complex carries out the final step by transferring LC3 to PE to form an LC3-PE conjugate (also called LC3-II). LC3-II associates with the autophagosomal membrane and is crucial for the targeting of autophagosomes to lysosomes, as well as for the selective autophagy of organelles and protein aggregates.

2003). CDK1–cyclin-B1 controls caspase-9 phosophorylation and activity during mitosis. This process is thought to dampen the threshold for intrinsic apoptosis signals during the cell cycle, especially upon prolonged mitotic arrest (Allan and Clarke 2007). The mechanism by which this phosphorylation event inhibits caspase-9 activity remains obscure because it does not affect binding to APAF1.

3 TYPE II CELL DEATH AND AUTOPHAGY

3.1 The Autophagy Pathway

Macroautophagy, referred to here simply as autophagy, is a cellular process that is highly conserved in eukaryotes.[1] It is a catabolic process engaged under metabolic stress (such as nutrient starvation and bioenergetics failure), to ensure availability of critical metabolic intermediates. It is also important for the removal of damaged organelles (including mitochondria), protein aggregates, and infecting organisms (Levine and Kroemer 2008; Kroemer et al. 2010).

Autophagy, in general, is a survival process. Its presence during so-called autophagic (type II) cell death *usually* represents a failed attempt to overcome lethal stress, and disruption of this process promotes rather than inhibits cell death in many cases (but see below). This form of cell death is therefore often referred to as caspase-independent cell death accompanied by autophagy (Kroemer and Levine 2008; Shen et al. 2012).

The autophagy pathway culminates in the formation of a double membrane structure, the autophagosome. This envelops intracellular material and ultimately fuses with lysosomes allowing degradation of the enveloped material (Fig. 6). Autophagosome formation and maturation is reg-

[1]Two other processes, microautophagy and chaperone-mediated autophagy, also exist, but are not further discussed here.

ulated by the sequential function of multiple autophagy-related (ATG) proteins. The process starts with the activation of a preinitiation complex composed of the kinase Unc-51-like kinase 1 (ULK1), FIP200, and ATG13. The activity of this kinase complex is directly regulated by two major metabolic checkpoints: mammalian target of rapamycin complex 1 (mTORC1) and AMP-activated protein kinase (AMPK) (see Hardie 2012; Laplante and Sabatini 2012). In healthy cells, mTORC1 inhibits the preinitiation complex through phosphorylation of ULK1 and ATG13. Upon metabolic stress (e.g., amino acid deprivation), mTORC1-mediated inhibition of ULK1 and ATG13 is abolished (Ganley et al. 2009; Hosokawa et al. 2009; Jung et al. 2009). In contrast, AMPK activates the preinitiation complex (Egan et al. 2011; Kim et al. 2011). When ATP synthesis is unable to meet the demands of ATP consumption in a cell, AMP and ADP accumulate and activate AMPK. Active AMPK promotes autophagy indirectly through suppression of mTORC1 activity and directly by phosphorylating and activating ULK1. Therefore, conditions under which mTORC1 is inhibited and/or AMPK is activated engage the activity of the preinitiation complex.

The preinitiation complex recruits and activates an initiation complex composed of beclin 1, a class III PI3K (Vps34), and the protein kinase Vps15, which results in the generation of the lipid phosphatidylinositol 3-phosphate (PI3P) (He and Levine 2010). The activity of the initiation complex is negatively regulated by several independent signaling pathways. Akt directly phosphorylates beclin 1, thus allowing it to bind to 14-3-3 (Wang et al. 2012). Beclin-1–14-3-3 complexes are sequestered by vimentin, a component of intermediate filaments. This Akt-mediated cytoskeletal sequestration dampens the lipid kinase activity of the initiation complex. Therefore, growth factors and the PI3K-AKT pathway can inhibit autophagy both directly through beclin 1 and indirectly through the mTOR pathway. Beclin 1 is a scaffold protein that recruits several additional partners (in addition to Vps34 and Vps15) that regulate the lipid kinases activity of the preinitiation complex (for a more comprehensive review of beclin-1-interacting proteins, see Kroemer et al. 2010). For example, beclin-1 binds to autophagy/beclin-1 regulator 1 (AMBRA1), a protein that tethers the beclin-1–Vps34 complex to microtubules through the dynein motor complex (Di Bartolomeo et al. 2010; Fimia et al. 2012). Upon autophagy induction, AMBRA1 is phosphorylated by ULK1 and released from the cytoskeleton to allow autophagosome formation.

Interestingly, members of the Bcl2 family can regulate the initiation complex. Beclin 1 contains a bona fide BH3 domain, which allows its sequestration by antiapoptotic proteins such as Bcl2 and Bcl-xL (Pattingre et al. 2005; Maiuri et al. 2007b; Oberstein et al. 2007). This dampens the activity of the initiation complex. Under stress conditions (e.g., starvation or hypoxia), the BH3-only proteins Bad and Bnip3 release beclin 1 to permit its participation in the autophagic process (Maiuri et al. 2007a,b; Bellot et al. 2009). Alternatively, dissociation of beclin 1 from Bcl2 and Bcl-xL can be achieved through phosphorylation of the beclin–1 BH3 domain by the death-associated protein kinase (DAPK) (Zalckvar et al. 2009a,b). Finally, kinases of the JNK family disrupt this complex by directly phosphorylating Bcl2, thus decreasing its affinity for beclin 1 (Wei et al. 2008). This process is essential for regulation of exercise-induced autophagy and glucose metabolism in muscles (He et al. 2012).

The elongation and ultimate closure of the autophagosome is regulated by two distinct but complementary ubiquitin-like protein conjugation systems: the ATG5-12 and LC3-PE conjugation pathways (Fig. 6). Both pathways are initiated by a single E1-ligase-like activating enzyme, ATG7, recruited to the phagophore through PI3P generated by the initiation complex. First, a small protein, ATG12, is covalently attached to the active site cysteine residue of ATG7 by a thioester linkage. ATG12 is then transferred via thioester exchange to an E2-like conjugating enzyme, ATG10, which further transfers ATG12 to ATG5. Finally, the ATG5-ATG12 conjugate noncovalently recruits ATG16L to form a large multimeric complex necessary to stabilize the forming phagophore and to complete the second conjugation pathway (Hanada et al. 2007). The LC3-PE conjugation pathway is initiated following cleavage of LC3 by the cysteine protease ATG4. Cleaved LC3 binds to ATG7 and is subsequently transferred to the E2 enzyme ATG3. The ATG5-ATG12-ATG16L complex then acts as an E3 ligase, conjugating LC3 to phosphatidylethanolamine (PE) to form an LC3-PE conjugate (also referred to as LC3-II) (Hanada et al. 2007).

This lipidated, membrane-associated form, LC3-II, is crucial for the targeting of autophagosomes to lysosomes. Once completed, the autophagosome fuses with lysosomes to form an autolysosome, thus degrading the engulfed material. This fusion event is achieved when membranes from both organelles are brought into close proximity through interaction between the autophagosomal protein syntaxin 17 (Stx17), SNAP29, and VAMP8 on the lysosome (Itakura et al. 2012). Additionally, two lysosomal proteins, LAMP1 and LAMP2, are required for the fusion process, and in their absence the autophagosome fails to fuse to lysosomes (Tanaka et al. 2000; Eskelinen et al. 2002, 2004).

During starvation (e.g., amino acid or serum deprivation), large portions of the bulk cytosol are randomly engulfed and degraded by autophagy. Additionally, autophagy can be a selective process and remove specific sub-

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cellular structures (e.g., protein aggregates and damaged or excess organelles) in the absence of metabolic stress. During selective autophagy, substrate specificity is conferred by adaptor proteins that tether the targeted structure to the nascent phagophore. Aggregates formed by unfolded proteins are ubiquitylated and linked to the phagophore by proteins such as p62 and neighbor of breast cancer 1 (NBR1) that bind both LC3 and ubiquitin (Shaid et al. 2013). Similarly, autophagic removal of mitochondria (a process called mitophagy) is controlled by mitochondrion-anchored LC3-binding proteins such as NIP-like protein X (NIX, also called Bnip3L) and FUN14-domain-containing 1 (FUNDC1). NIX, a Bcl2-family-related protein, promotes removal of the entire mitochondrial pool during terminal erythrocyte differentiation (Schweers et al. 2007; Sandoval et al. 2008), and FUNDC1 participates in hypoxia-induced mitochondrial clearance (Liu et al. 2012). Additionally, damaged mitochondria that display low membrane potential are also removed by mitophagy following ubiquitylation of mitochondrial proteins by the ubiquitin ligase parkin (Narendra and Youle 2011), although the exact mechanism by which the phagophore is recruited in that scenario is not clear.

It is relatively easy to see how autophagy is engaged under conditions of cellular stress and can ameliorate the stress to protect the cells. Two questions persist: what kills the cells in type II cell death, and *can* autophagy itself ever be a mechanism that promotes cell death? Currently, we do not have a satisfactory answer to the first question but we are closer to answering the second.

3.2 Can Autophagy Kill Cells?

Autophagy often accompanies type II cell death without participating in it. There are cases, however, in which it appears to actively promote cell death. This is best exemplified during *Drosophila* metamorphosis, in which autophagy-dependent cell death drives the involution of the salivary gland (Berry and Baehrecke 2007) and of the midgut (Denton et al. 2009). This physiological form of cell death is triggered by the steroid hormone ecdysone and occurs (in the salivary gland) following growth arrest and inhibition of the PI3K signaling pathway. In both cases, mutation of any of several essential *ATG* genes reduces these developmental cell deaths. Reciprocally, forced expression of the ULK1 ortholog ATG1 is sufficient to drive this form of cell death.

Autophagy-dependent cell death also occurs in mammalian cells transformed with a constitutively active H-Ras (Elgendy et al. 2011). In this setting, Ras-dependent cell death is inhibited if ATG5, ATG7, or beclin 1 are silenced. Intriguingly, this form of autophagic cell death appears to be regulated by Bcl2 proteins. In particular, displacement of beclin 1 from Mcl1 is thought to be triggered by the proapoptotic BH3-only protein Noxa. Again, how autophagy components promote the death of the cell remains to be elucidated.

Untransformed breast epithelial cells that lose attachment to a substrate can engulf each other, resulting in the death of the engulfed cell by entosis (Overholtzer et al. 2007). The engulfed cell undergoes apoptosis, which can be inhibited by expression of Bcl2, but this does not rescue the cell. However, if the engulfing cell lacks components of the autophagy pathway, the engulfed cell can survive (Krajcovic et al. 2011). Recent studies have shown that components of the autophagy pathway can be recruited to phagosomes that have engulfed organisms or cells, facilitating the degradation of the phagocytosed cargo (Sanjuan et al. 2007; Martinez et al. 2011). The preinitiation complex does not appear to be required. In the case of entosis for example, FIP200 is dispensable (Krajcovic et al. 2011). Thus, in these cases, autophagy may act as a "murder weapon" allowing one cell to efficiently kill another.

4 TYPE III CELL DEATH, NECROSIS

4.1 Types of Necrotic Cell Death

Cellular necrosis or necrotic cell death encompasses a wide variety of cell death processes with one common denominator: the loss of plasma membrane integrity followed by cytoplasmic leakage (Yuan and Kroemer 2010). Necrosis can occur simply as a consequence of such extensive damage that cell integrity is disrupted—for example, at high temperature, following freeze-thaw, or upon mechanical stress. In such cases, cell death is passive and does not require the activation of any particular signaling pathway. Note that a necrotic morphology (i.e., rupture of the plasma membrane) can also be observed at late stages of an apoptotic or autophagic cell death program, when dead cells fail to be cleared from the system by phagocytosis. This process is referred to as secondary necrosis and is independent of any other signaling event than those initially engaged (apoptotic or autophagic). However, necrotic cell death is not always an accidental or passive process and can also be the result of a directed signaling cascade.

The best-characterized form of programmed necrosis is RIP-kinase-dependent necrosis (also referred to as "necroptosis") (Degterev et al. 2005). This requires the kinase activity of RIP3 and can result in a rapid cell death with the features of necrosis (Cho et al. 2009; He et al. 2009; Zhang et al. 2009). Necroptosis can be engaged downstream from TNFR1 (Schulze-Osthoff et al. 1994; Vercammen et al. 1998; Holler et al. 2000; Matsumura et al. 2000). Following

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TNFR1 ligation, RIP3 is activated through recruitment to complex II by RIP1 (Fig. 4C). The interaction between RIP1 and RIP3 is mediated by their respective RIP homotypic interaction motifs (RHIMs). In this configuration, complex II induces necrosis rather than apoptosis and is referred to as the RIPoptosome (Feoktistova et al. 2011a; Tenev et al. 2011). The assembly and activation of the necrotic signaling platform are controlled by a series of post-translational modifications of components of this complex. RIPoptosome formation is negatively regulated by ubiquitylation of RIP1. Consequently, treatments with cIAP1/2 inhibitors or expression of the deubiquitylase CYLD promote TNFR1-induced necrosis (Wang et al. 2008b; O'Donnell et al. 2011; Moulin et al. 2012). Additionally, RIP3 activation depends on the kinase activity of RIP1 (Cho et al. 2009; He et al. 2009; Zhang et al. 2009), which can be inhibited by the drug necrostatin (Degterev et al. 2008). Accordingly, RIP-dependent necrosis is blocked by necrostatin (Degterev et al. 2005). In addition, necrosis induction by the RIPoptosome is negatively regulated by the catalytic activity of the caspase-8–FLIP heterodimers (see below).

RIP-dependent necrosis can be triggered by TLR3 and TLR4 (for more details on TLR signaling pathways, see Lim and Staudt 2013). TLR3 and TLR4 promote the assembly of the RIPoptosome by recruiting the RHIM-containing protein TIR-domain-containing adaptor-inducing interferon-β (TRIF) (Imtiyaz et al. 2006; Feoktistova et al. 2011b). TRIF recruits RIP1 and RIP3 to induce RIP-dependent necrosis (Feoktistova et al. 2011a). TRIF might, however, recruit RIP3 independently of RIP1 through direct RHIM–RHIM interaction. RIP-dependent necrosis can also be engaged during viral infection (Cho et al. 2009; Upton et al. 2010) via the RHIM-containing cytosolic DNA sensor, DNA-dependent activator of interferon regulatory factors (DAI). DAI is activated by double-stranded viral DNA and recruits RIP3 through its RHIM domain (Upton et al. 2012). Finally, DNA damage signaling can also cause RIPoptosome formation independently of any membrane receptor signaling (Tenev et al. 2011).

The mechanism by which RIP3 causes cell death remains obscure. Several mechanisms have been proposed, including the generation of reactive oxygen species (either mitochondrial or via NADPH oxidase) and elevation of metabolic processes to deplete ATP and/or NADH, but the evidence for them is not compelling (Hitomi et al. 2008). Recent studies have implicated the pseudokinase mixed lineage kinase-like (MLKL) (Sun et al. 2012; Zhao et al. 2012) as a critical player in the execution of RIP-dependent necrosis. Phosphorylation by RIP3 triggers MLKL oligomerization and translocation to the plasma membrane (Cai et al. 2014; Chen et al. 2014). The exact mechanism by which MLKL impairs the plasma membrane integrity is not known but is thought to involve a disruption of ionic homeostasis.

4.2 Control of RIPK-Dependent Necrosis by Caspases

Stimuli that activate RIP1 and the RIPoptosome (e.g., ligation of TNFR1, ligation of TLR3/4, and possibly DNA damage) engage caspase-8 and FLIP. The catalytic activity of caspase-8–FLIP heterodimers antagonizes activation of RIP3 and the ensuing necrosis (Pop et al. 2011). As a result, cells that are treated with TNF or TLR3/4 ligands do not generally undergo cell death. However, if caspase-8–FLIP activity is blocked or disrupted, this can engage necrosis in a manner that is dependent on both RIP1 and RIP3 (Cho et al. 2009; He et al. 2009; Zhang et al. 2009; Oberst et al. 2010). RIP1, RIP3, and CYLD are substrates of caspase-8 (Lin et al. 1999; Feng et al. 2007; Lu et al. 2011; O'Donnell et al. 2011). However, in the absence of FLIP, activation of caspase-8 is not sufficient to prevent RIPK-dependent necrosis, even if apoptosis downstream from caspase-8 activation is blocked (Oberst et al. 2011).

4.3 Other Necrosis Signaling Pathways

Several other forms of necrosis signaling can occur in cells, although, like RIP-dependent necrosis, none of these is fully characterized. Ischemia/reperfusion injury (I/R), for example, causes cell death when a tissue is transiently deprived of blood supply and is then restored. High levels of calcium influx and generation of reactive oxygen species (ROS) have been associated with this form of cell death (Halestrap and Pasdois 2009). These trigger cell death, in part, by causing a mitochondrial permeability transition (mPT), in which the mitochondrial inner membrane becomes permeable to small solutes (Ricchelli et al. 2011). This results in a rapid dissipation of the transmembrane potential, followed by matrix swelling and often rupture of the mitochondrial outer membrane. Cyclophilin D (CypD), a peptidyl-proline *trans*-isomerase, plays a major role in mPT. Mice lacking CypD display defective mPTs (Baines et al. 2005; Basso et al. 2005; Nakagawa et al. 2005; Schinzel et al. 2005) and display diminished tissue damage under conditions of I/R (Baines et al. 2005; Nakagawa et al. 2005; Schinzel et al. 2005). Importantly, mice lacking CypD have normal apoptosis (Baines et al. 2005; Nakagawa et al. 2005; Schinzel et al. 2005) and RIP-dependent necrosis (Ch'en et al. 2011).

Another form of active necrosis involves NADPH oxidase1 (Nox1) and can be triggered by TNF (Kim et al. 2007). Nox1 generates a ROS burst that may be important.

There may be a relationship between Nox1 and RIP-dependent necrosis (Vanden Berghe et al. 2007), but further evidence supporting this connection is needed.

In neurons, engagement of glutamate receptors can trigger a form of necrotic cell death called excitotoxicity (Wang and Qin 2010). This appears to involve a calcium influx that triggers an ROS burst and also activates calpain (Higuchi et al. 2005; Vosler et al. 2008). Excitotoxicity is associated with I/R and may occur in other pathological conditions (Szydlowska and Tymianski 2010).

5 BEYOND TYPE III—OTHER FORMS OF CELL DEATH

There are additional forms of cell death that appear to require the participation of the cell in its demise. These include mitotic catastrophe (Castedo et al. 2004), ferroptosis (Dixon et al. 2012), and others. Although outside the scope of our discussion, a useful distinction might be made between cellular suicide and cellular sabotage. Suicide mechanisms (including apoptosis, necroptosis, and perhaps autophagic cell death, as outlined above) represent signaling pathways that appear to have evolved to execute the cell. In contrast, sabotage can be thought of as the consequence of disruption of normal cellular processes that have not evolved for the purpose of cell death. Much as a train must be moving if removal of a railroad tie (a *sabot*) is to damage it, these forms of cell death occur when cellular machinery confronts a disruption that results in cellular destruction. For example, if chromosomes are cross-linked, mitosis may sufficiently damage them that the cell dies, even if mechanisms of active cell suicide cannot be engaged (mitotic catastrophe, although it is not clear that mitotic catastrophe proceeds in this way [Castedo et al. 2004]).

6 CONCLUSION

Active or programmed cell death is essential to maintain homeostasis in multicellular organisms as well as for the selective elimination of potentially harmful or infected cells. Accordingly, deregulation of the signaling pathways that trigger cell death can lead to the development of catastrophic diseases such as cancer and autoimmunity (too little cell death) as well as degenerative diseases (too much cell death). Therefore, the existence of tightly controlled and efficient means to induce cell death can be interpreted as the logical consequence of the evolution of multicellular organisms. However, the necessity for so many different death-signaling pathways might appear counterintuitive. Taken together, cell death induction could be viewed as a simple signaling process with multiple inputs/stimuli and one outcome: the death of the cell. However, the way by which a cell dies has important consequences for neighboring cells and sometimes the entire organism. For example, apoptotic and necrotic cells display divergent inflammatory properties and trigger different immune responses. Additionally, particular death programs include the release of proliferative signals that trigger compensatory proliferation in surrounding tissues. These signals might differ from one type of cell death to the next. Finally, death-signaling pathways are clearly interconnected. For example, autophagic cell death is often potentiated by caspase activation, whereas RIP-dependent necrosis is antagonized by a caspase-dependent activity. Cross talk between these pathways potentially provides numerous backup mechanisms for cell death programs and could explain why inhibition of a single program often has minor consequences for the organism. A better understanding of the impact of each type of cell death on surrounding tissues and of the interplay between these cell death programs might help to answer some of these questions.

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