HHS Public Access

Author manuscript

Nat Rev Mol Cell Biol. Author manuscript; available in PMC 2016 June 01.

Published in final edited form as:

Nat Rev Mol Cell Biol. 2015 June ; 16(6): 329–344. doi:10.1038/nrm3999.

Live to die another way: modes of programmed cell death and the signals emanating from dying cells

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Preface

All life ends in death, but perhaps one of life's grander ironies is that it also depends on death. Cell-intrinsic suicide pathways, termed programmed cell death (PCD), are crucial for animal development, tissue homeostasis and pathogenesis. Originally, PCD was virtually synonymous with apoptosis, but recently, alternative PCD mechanisms have been reported. Here, we provide an overview of several distinct PCD mechanisms, namely apoptosis, autophagy and necroptosis. In addition, we discuss the complex signals emanating from dying cells, which can either fuel regeneration or instruct additional killing. Further advances in understanding the physiological role of multiple cell death mechanisms and associated signals will be important to selectively manipulate PCD for therapeutic purposes.

"Life and death are one thread, the same line viewed from different sides."

Lao Tzu (ancient Chinese philosopher)

Introduction

The concept of naturally occurring cell death dates back to 1842, when Karl Vogt showed that the notochord of the midwife toad is removed by cell death during metamorphosis enabling the formation of vertebrae[1]. However, for many years it was assumed that cell death is no more than an inevitable passive finale of life. This notion was challenged by the observation in the mid 1960s that developmental cell death relies on the expression of endogenous genes, thus giving rise to the term programmed cell death (PCD)[2, 3]. Another important contribution came from electron microscopy studies carried out by Kerr, Wylie and Currie in the 1970s that defined the ultra-structural hallmarks of different types of cell death[4]. Although multiple types of cell death had already been described in the 19th century,

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Competing interests statement

The authors declare no competing interests.

electron microscopy enabled a clear distinction to be made between these different types of death. Cells that die during normal development and tissue homeostasis have characteristic morphological hallmarks, including cytoplasmic shrinkage, nuclear condensation, and the retention of membrane and organelle integrity — a process termed "apoptosis"[4]. By contrast, as a result of overwhelming trauma, dying cells swell and rupture, in a mode of cell death named "necrosis".

Historically, *Caenorhabditis elegans*, *Drosophila melanogaster* and *Mus musculus* have been instrumental in developing our understanding of PCD and its role during animal development (Box 1). The pioneering work in *C. elegans* from Horvitz and colleagues defined the core apoptotic pathway and revealed the conserved role of caspases in the execution of apoptosis[5, 6] (Figure 1A). Since then, additional cell death mechanisms have been reported, indicating that apoptosis is not the only mode of PCD. Here, we provide an overview of several major PCD mechanisms and critically discuss the biological significance of these pathways *in vivo.* Additional details summarizing cell-based and biochemical studies for individual forms of PCD can be found in several excellent recent reviews[7–15]. Another rapidly expanding area of research that we cover is signaling by apoptotic cells. Traditionally, it was thought that dying cells have limited signaling capacity being rapidly cleared by phagocytes. However, it is now clear that apoptotic cells release a multitude of signals that profoundly affect their cellular environment. These signals include mitogens to promote proliferation and tissue repair, and death factors to stimulate coordinated cell killing. This extraordinary complexity in the regulation and execution of cell death poses significant experimental challenges, but also presents exciting new opportunities for clinical translation.

Box 1

Programmed cell death in model organisms

The *Caenorhabditis elegans*, *Drosophila melanogaster* and *Mus musculus* model systems have shaped our understanding of how cells undergo programmed cell death (PCD). *C. elegans* provides unique opportunities for experimentation due to its defined and invariant cell lineage. In ontogeny of the hermaphrodite worm, 131 of 1090 somatic cells are eliminated by PCD, generating adults with 959 cells[172]. In loss-of-function mutants for the pro-apoptotic genes *egl-1*, *ced-3* and *ced-4*, cell death is blocked resulting in survival of these 131 cells; however, the lifespan and behavior of the worm are not affected.

D. melanogaster is considerably more complex, and cell fate and number are not pre-determined but depend on extracellular signals and environmental factors. Therefore, *D. melanogaster* offers unique opportunities for studying PCD in the context of developmental plasticity and tissue homeostasis. The most prominent form of developmental PCD in the fly is apoptosis, and inhibition of this process causes severe developmental defects, malformations and organismal lethality[40–42, 173]. However, inhibition of apoptosis does not affect the elimination of specific cells such as nurse cells, indicating that apoptosis is not the only PCD mechanism in flies[174].

Consistent with increased organismal complexity, the apoptotic machinery in vertebrates is even more intricate, and is involved in regulating crucial events throughout the organism's life span. Therefore, it was surprising that mice deleted for key components of the apoptotic machinery only have minor developmental defects and can reach adulthood[11]. The simplest explanation for the lack of overt phenotypes may be functional redundancy between apoptotic proteins[22]. However, another possibility is that cells are eliminated by alternative PCD mechanisms when apoptosis is blocked[11]. Nevertheless, the inhibition of apoptosis in many situations causes embryonic lethality, developmental abnormalities and various pathologies (Table 1).

These developmental studies have been complemented by different models to explain why cells need to die during development: sculpting; deleting structures; supplying nutrients; regulating cell number; and eliminating abnormal cells[8, 175].

Type I cell death: apoptosis

Caspases: the cellular executioners

Apoptosis is the most prominent and best-studied mode of PCD during development[9, 16]. This conserved process, which can be triggered both intrinsically (for example, by DNA damage) or extrinsically (for example, by growth factor withdrawal, steroid hormones, ligation of death receptors), culminates in the activation of caspases, a class of cysteine proteases that are expressed as inactive zymogens in virtually all cells (Figure 1)[17, 18]. Interestingly, whereas *C. elegans* is equipped with four caspases, flies and mice contain multiple caspases (7 and 13, respectively), suggesting that higher organismal complexity is matched with a larger number of caspases. Although many of the caspases are instrumental in the execution of apoptosis, these proteins also have non-apoptotic functions in various processes, including immunity, cellular remodeling, learning, memory and differentiation[8, 9, 19].

Traditionally, caspases have been subdivided into initiator caspases **[G]** (Dredd and Dronc in *Drosophila*; caspase-1, -2, -4, -5, -8, -9, -10, -11, and -12 in mammals) and executioner caspases **[G]** (Ice and Dcp-1 in *Drosophila;* caspase-3, -6, -7, and -14 in mammals). Some members of the caspase family can be compensated for whereas others have non-redundant essential functions *in vivo* (Table 1) [10, 11, 13]. Initiator caspases have long N-terminal prodomains enabling the formation of protein platforms that regulate caspase activation. An example is the interaction of the caspase-9 prodomain with apoptotic peptidase-activating factor 1 (APAF1) and cytochrome c, which produces the apoptosome **[G]** that is thought to initiate apoptosis[20]. Alternatively, it has been suggested that this platform is responsible for signal amplification rather than initiating the apoptotic casacade, which could explain how executioner caspases can be activated in mice (and flies) mutant for APAF1 (*Drosophila* Ark) and caspase-9 (Dronc)[21, 22, 23]. In addition, this provides a potential mechanism for how executioner Caspase -3 and Caspase-7 can regulate upstream events, such as mitochondrial outer membrane permeabilization (MOMP) **[G]** and the release of cytochrome c[24, 25]. According to this model, at least in some scenarios, executioner caspases may actually function upstream of initiator caspases.

Apoptosome formation

A decisive point that determines whether the cell commits to apoptosis is MOMP, which results in the release of cytochrome c from the mitochondria. MOMP is regulated by members of the B-cell lymphoma-2 (BCL-2) family, which contains both anti-apoptotic and pro-apoptotic members (Figure 1)[10]. The interplay between BCL-2 proteins regulates whether a cell undergoes apoptosis. The assembly of BAX–BAK oligomers within the mitochondrial outer membrane promotes MOMP and the release of intermembrane proteins, such as cytochrome c, to the cytosol. This in turn promotes apoptosome formation and the activation of executioner caspases, thus propagating a proteolytic cascade that leads to the cleavage of large numbers of crucial cellular target proteins, including inactivation of a DNase inhibitor, which causes nuclear DNA fragmentation [17, 22, 26].

The activation of caspases can also occur as a result of death receptor ligation, which results in binding of the initiator caspase-8 to its adaptor FADD (Fas-associated via death domain) and assembly of the death-inducing signaling complex (DISC) **[G]**, as discussed in the necroptosis section below.

Inhibitor of apoptosis proteins (IAPs)

Another important layer of PCD regulation involves the inhibition of caspases by the IAP family (Figure 1)[8]. IAPs were originally discovered in insect viruses and contain at least one baculovirus inhibitory repeat (BIR) domain **[G]**[27, 28]. A large number of cellular IAPs have been described in insects and vertebrates and were grouped into a “family”, but it is important to recognize that these are functionally diverse proteins, many of which have no direct role in apoptosis. Compelling evidence for a crucial role of some IAPs in the regulation of apoptosis came from the analyses of loss-of-function and gain-of-function mutations of *Drosophila IAP1* (*Diap1*)[29–31]. Inactivation of *Diap1* causes large-scale caspase activation and apoptosis, whereas gain-of-function mutations inhibit cell death, indicating that DIAP1 has an essential role in caspase regulation *in vivo*. IAPs can bind and inhibit caspases *in vitro* through their BIR domain[28]. Several IAPs also have a RING (really interesting new gene) motif, enabling them to function as E3 ubiquitin ligases and promote ubiquitylation of key cell death proteins[28, 32, 33]. In addition, some IAPs contain a ubiquitin-associated (UBA) domain, facilitating interaction with ubiquitylated proteins, and a caspase-recruitment domain (CARD) that mediates homophilic interaction with caspases[32, 33]. DIAP1 has two important functions by virtue of its RING domain: first, it ubiquitylates caspases thus suppressing apoptosis; second, it auto-ubiquitylates and targets itself for degradation once the cell has committed to apoptosis[30, 34–36]. Interestingly, DIAP1-mediated ubiquitylation involves both degradative ubiquitylation and non-degradative poly-ubiquitylation that can regulate caspase activity [37, 38].

The mammalian orthologue of DIAP1 is the X-linked inhibitor of apoptosis (XIAP) protein, which also functions as an E3 ligase and is considered to be the most potent caspase inhibitor *in vitro*[39]. XIAP-deficient mice are viable and for many years were thought to have only “mild” phenotypes[32]. However it has recently become clear that XIAP has a crucial role in stem cell apoptosis (see below).

IAP antagonists

For a cell to commit to apoptosis, IAPs must be inactivated by endogenous antagonists (Figure 1). A family of natural IAP antagonists was originally discovered by screening for cell death-defective mutants in *Drosophila*[40]. This revealed the essential role of three closely linked genes — *reaper*, *hid* and *grim* (which encode the RHG proteins) — for the induction of apoptosis [40–42]. RHG proteins have a common N-terminal IAP-binding motif (IBM) that is structurally conserved between flies and mammals and is essential for IAP binding, IAP inactivation and cell killing[43].

Deletion of RHG genes blocks apoptosis, and their ectopic expression is sufficient for the killing of many different cell types[40–42, 44]. In addition, DIAP1 mutations that reduce RHG protein binding protect against apoptosis, indicating that DIAP1 is a direct and functionally relevant target for the pro-apoptotic activity of RHG proteins (Figure 1B). Furthermore, RHG proteins physically interact with each other to form heteromeric complexes at the mitochondrial outer membrane that are crucial for cell killing *in vivo*[45]. Significantly, RHG genes are transcriptionally regulated by embryonic patterning pathways and by many different pro-apoptotic signals[14]. This indicates that the convergence of different death-inducing signals occurs, at least in part, via a transcriptional mechanism. Finally, the pro-apoptotic activity of the Hid protein is blocked upon phosphorylation by the MAP kinase ROLLED, and Hid is targeted for cell survival by the epidermal growth factor receptor–Ras signaling pathway[46, 47]. Collectively, these studies established the important physiological role of IAPs and their antagonists and revealed that caspases are controlled by both activating and inhibitory pathways[48, 49].

Mammals also have IAP-binding proteins, most notably SMAC (also known as DIABLO) and HTRA2 (also known as Omi and PARK13)[50–52]. In addition, a large number of mitochondrial proteins that can interact with IAPs through their N-terminal IBM have been identified but remain to be functionally characterized[53]. SMAC and HTRA2 are localized to the mitochondrial intermembrane and translocate to the cytoplasm upon MOMP (Figure 1C). It is thought that upon cytoplasmic release, these proteins promote cell death by binding IAPs, which promotes caspase activation. However, rigorous genetic evidence to support this model *in vivo* is still lacking. *HtrA2*$^{-/-}$ animals and cells do not have reduced cell death rates and actually lose a population of neurons in the striatum, resulting in a lethal postnatal neurodegenerative disorder with features of Parkinson disease. This phenotype appears to be due to loss of the protease function of HTRA2 and not its IAP-binding IBM[54].

Similarly, *Smac*$^{-/-}$ mice have no overt phenotype, and *Smac*$^{-/-}$ mouse embryonic fibroblasts (MEFs), lymphocytes and hepatocytes respond normally to apoptotic stimuli[55]. Although a redundancy between SMAC and HTRA2 has been proposed, *Smac*$^{-/-}$*HtrA2*$^{-/-}$ mice have a similar phenotype to their parental single-knockout strains[54]. An alternative explanation for the physical association of SMAC and HTRA2 with XIAP is that binding functions to promote the ubiquitylation and proteasome-mediated degradation of SMAC and HTRA2 upon release into the cytoplasm (a “cleanup” mechanism)[53]. Small-molecule IAP antagonists based on the conserved IBM of RHG proteins and SMAC (often referred to as “SMAC-mimetics”) have been developed and are currently under clinical investigation as

pro-apoptotic cancer therapeutics[56]. Initially designed to inhibit XIAP, these compounds target primarily cIAP1 and cIAP2 and sensitize cancer cells to tumour necrosis factor (TNF)-related apoptosis[57, 58].

Another mammalian IAP antagonist is ARTS, which is encoded by the *SEPT4* gene. ARTS does not contain an IBM but similarly to the RHG proteins, it is localized to the mitochondrial outer membrane[59, 60]. ARTS functions upstream of MOMP and mitochondrial protein release[25]. *Sept4*$^{-/-}$ mice are defective in caspase-mediated elimination of bulk cytoplasm during spermiogenesis, have increased numbers of hematopoietic stem and progenitor cells and have increased susceptibility towards developing malignancies[61, 62]. XIAP is the direct biochemical and physiological target of ARTS, and both proteins are crucial for the regulation of cell death in hair follicle stem cells (HFSCs) **[G]**[60, 63]. Loss of ARTS protects HFSCs against apoptosis, increases HFSC number, and leads to marked improvement in wound healing[63]. Furthermore, *Sept4*$^{-/-}$ mice have improved regeneration of hair follicles from the wound bed and significantly smaller scars[63]. Conversely, inactivation of XIAP abrogates these phenotypes and impairs wound healing, revealing an *in vivo* role for XIAP and the importance of apoptosis in regulating HFSC expansion[63].

In summary, opposing regulatory pathways simultaneously regulate caspase activity during apoptosis, through either caspase activation (by APAF1) or caspase inhibition (by IAPs), which are coordinately subjected to many layers of upstream regulation. The cell is killed by the apoptotic machinery only after multiple checkpoints have been cleared to enable effector caspase activity that is sufficiently widespread and at a sufficiently high level to cause extensive proteolysis of crucial cellular proteins. Finally, even at a late stage in apoptosis cells can sometimes recover, a phenomenon termed "anastasis" (Greek for “rising to life”)[64]. This could be a mechanism to preserve cells that are difficult to replace.

Non-apoptotic programmed cell death

Although apoptosis is the most prominent form of PCD during development, emerging evidence indicates that it is not the only mechanism[11]. In *C. elegans*, virtually all PCD occurs by apoptosis, except for the linker cell **[G]**, which dies in a caspase-independent manner[5, 65]. Dying linker cells have unique morphological features, which are also seen in certain neurons that die during spinal cord development of vertebrates, suggesting that this PCD mechanism is conserved throughout evolution (Box 2). In addition, as detailed in the following sections, autophagy and necroptosis have been intensively studied in the context of PCD.

Box 2

Linker cell death

The linker cell in *Caenorhabditis elegans* migrates along a predetermined path and completes its migration when positioned between the gonad and cloacal tube, which functions as the sperm’s exit channel. Death of the linker cell prior to completion of its migration results in severe defects in gonadal elongation and impaired male fertility[65].

The microRNA let-7 and its downstream target, LIN-29, are crucial components regulating linker cell death. In let-7 and LIN-29 mutant animals, the linker cell survives and the connection between gonadal and cloacal tubes fails to form, resulting in sperm accumulation[65]. However, LIN-29 is also expressed in other cells that are not doomed to die, indicating that LIN-29 is necessary but not sufficient for linker cell death. Recently, it was elegantly shown that linker cell death also requires SEK-1, TIR-1 and the polyglutatamine-repeat protein pqn-41[176]. However, elucidation of the downstream targets responsible for the demolition phase of the linker cell, as well as of other key death-inducing genes that control linker cell death, is ongoing. Of particular interest, the *Drosophila* and mouse TIR-1 orthologues (Ect4 and SARM1, respectively) have a role in Wallerian degeneration. Upon injury, loss of Ect4 or SARM1 suppresses axonal degeneration, which indicates that axons can actively promote their own demise and that TIR-1, Ect4 and SARM1 are implicated in non-apoptotic mechanisms of programmed cell death[177].

Dying linker cells exhibit non-apoptotic characteristics, such as nuclear envelope crenellation, lack of chromatin condensation, organelle swelling, and increased cytoplasmic membrane-bound structures[65, 176]. These morphological features resemble cell death during mammalian development, as in the case of chick ciliary ganglion cells and chick spinal cord motor neurons, suggesting that linker cell death is a morphologically conserved form of cell death. Furthermore, nuclear envelope crenellation is implicated in several polyglutatamine expansion diseases, raising the possibility that neurodegeneration is promoted by inappropriately activating a linker cell death like machinery. Future work will shed light on the evolutionary conservation of this intriguing cell death program and its role in development and disease.

Type II cell death: autophagy

Macroautophagy (hereafter referred to as autophagy) is a catabolic process involving the degradation of cytoplasmic components, protein aggregates and organelles by forming autophagosomes, which are degraded by fusion to lysosomes[66]. This process has been extensively studied in the yeast *Saccharomyces cerevisiae* in response to starvation, where it functions to protect cells from death by recycling cell contents. Autophagy depends on a large group of evolutionarily conserved *ATG* (autophagy-related) genes[66]. In accordance with the protective, pro-survival function of autophagy, silencing and deletion of *ATG* genes resulted in accelerated cell death[12, 67]. However, in certain scenarios (and dependent on the organism) it has been suggested that autophagy can lead or contribute to cell death.

Historically, type II cell death was termed autophagic cell death **[G]**, which perhaps misled people to perceive that cell death is achieved by the autophagic machinery[12]. However, this name simply indicates that cell death is accompanied by large numbers of autophagosomes, thus raising the question of whether autophagy is an unsuccessful attempt at survival rather than a *bona fide* cell death mechanism.

In *Drosophila*, dying cells of the salivary glands contain autophagic vacuoles and express ATG genes[7, 68]. Moreover, deletion of *ATG* genes attenuates both autophagy and PCD[69].

Interestingly, the engulfment receptor *Draper* is required for autophagy during the death of salivary gland cells[70]. Furthermore, compelling evidence indicates that caspase activity is necessary for the degradation of these cells, indicating the involvement of an apoptotic mechanism. First, anti-apoptotic *Diap1* is repressed and pro-apoptotic genes (*rpr* and *hid*) are activated prior to the death of salivary gland cells[71]. Second, dying cells are positive for apoptotic markers[66] (such as acridine orange and TUNEL). Third, deletion of *Ark* (the *Drosophila* ortholog of *Apaf1*) or caspase inhibition rescues these cells from death[68, 72–76]. Thus, autophagy alone is not sufficient for salivary gland cell death.

Autophagic cell death is also associated with the destruction of *Drosophila* midgut cells. Death of these cells has been characterized by both caspase activation and autophagy; however, recent experiments show that mutations in caspases or their regulators do not rescue these cells from destruction[77]. By contrast, the inhibition of autophagy genes (*Atg1*, *Atg2* and *Atg18*) delays midgut degradation without a decrease in caspase activity, which indicates that the death of midgut cells involves a caspase-independent form of PCD that requires autophagy[77]. Intriguingly, this work also suggests an unknown mechanism for the activation of caspases in these cells, as the executioner caspase Decay was activated in the absence of the initiator caspase Dronc.

In mice, it has also been suggested that autophagy functions as an alternate killing mechanism in situations where apoptosis cannot be executed. $Bax^{-/-}Bak^{-/-}$ MEFs are resistant to various inducers of apoptosis but can undergo cell death in what seems to be an autophagy-mediated manner[78, 79]. Knockdown of the mammalian *ATG* gene *Beclin 1* (*Becn1; ATG6* in yeast) or of *Atg5* reduced cell death in $Bax^{-/-}Bak^{-/-}$ MEFs in response to etoposide and staurosporin[73]. Intriguingly, autophagy was not induced when downstream apoptotic processes were inhibited in $Casp9^{-/-}$ or $Apaf1^{-/-}$ MEFs or in response to the addition of the pan caspase inhibitor zVAD-fmk, indicating that BAX and BAK might have a specific role in regulating autophagy. In this regard, it is intriguing that BECN1 has a BH3 domain, which enables the interaction with pro-survival BCL-2 family members (BCL-2, BCL-XL and MCL-1)[12, 80]. This interaction is conserved and is also seen in *C. elegans*, where BECN1 binds CED-9 (a BCL-2-like protein)[81]. The direct interaction between BCL-2, BCL-XL and BECN1 inhibits the pro-autophagy function of BECN1 and is dependent upon subcellular localization[80] (Figure 2A). The autophagy-inhibiting BCL-2–BCL-XL–BECN1 complexes localize to the endoplasmic reticulum (ER), indicating that autophagy is regulated in an organelle-specific manner[82] (Figure 2A). Interestingly, the BH3 domain of BECN1 also regulates dissociation of this complex. The death-associated protein kinase 1 (DAPK1) can phosphorylate the BH3 domain of BECN1, resulting in reduced affinity for BCL-XL and dissociation of the complex, which leads to the induction of autophagy[83]. In addition, by competitive interaction, the BH3-only BNIP3 protein can also dissociate the BCL-2–BCL-XL–BECN1 complex[84]. Finally, BECN1 has been found to be a substrate of caspase-3, and caspase-mediated cleavage of BECN1 abrogated its interaction with BCL-2 (Figure 2A)[85]. Taken together, the results indicate that the BH3 domain of BECN1 can regulate the association–dissociation kinetics of the BCL-2–BCL-XL–BECN1 complex and thus shift the balance between autophagy and apoptosis[85].

ATG5 may also have a dual role in regulating both autophagy and apoptosis. ATG5 can undergo calpain-dependent cleavage, which switches its function from a pro-survival autophagy protein to death induction. This cleavage removes the C-terminal domain of ATG5 giving rise to a pro-apoptotic product that translocates to the mitochondria where it induces the intrinsic apoptotic program (Figure 2B)[86]. Interestingly, ATG5 can also bind FADD via its C-terminal domain and stimulate caspase-dependent apoptotic cell death[87].

There is some indication that autophagy functions to enable the classic hallmarks of apoptosis, such as phosphatidylserine exposure and membrane blebbing. These processes depend on ATP, and autophagy may help in this regard by generating the metabolic substrates required for the bioenergetics needs of the dying cell. An elegant study demonstrated that ATP produced by the autophagic machinery is responsible for the generation of the find-me and eat-me signals, lysophosphatidylcholine and phosphatidylserine, respectively, in mouse embryoid bodies (Figure 2B)[88]. Embryoid bodies derived from *Becn1*$^{-/-}$ or *Atg5*$^{-/-}$ embryonic stem cells were able to undergo the initial stages of apoptosis, but were unsuccessful in producing the signals required for phagocytic clearance[88]. Additionally, autophagy might supply the ATP required for membrane blebbing during apoptosis[89].

A major unresolved question is to what extent autophagy actually mediates cell death, rather than simply occurs with it. Recently, using a systematic approach to examine the existence of autophagic cell death in mammalian cells, it was shown that of 1,400 death-inducing compounds not one eliminated cells through the induction of autophagy[90]. Silencing of key autophagy genes was not only ineffective in preventing cell death but actually accelerated it[90]. As these results are negative in nature they do not exclude the possibility of cell death mediated by autophagy, but they indicate that autophagy does not have a major role in promoting cell death.

Type III cell death: regulated necrosis

For many years necrosis was regarded as an unregulated mode of cell death caused by overwhelming trauma. However, a large number of recent studies point to the existence of multiple modes of regulated necrosis[15]. Necrosis is characterized by organelle and cellular swelling, rupture of the plasma membrane and release of the intracellular content[15]. Different modes of regulated necrosis share common morphological features[15]. Here, we focus on the best-studied form of regulated necrosis, termed necroptosis, which denotes a type of necrotic cell death that depends on RIPK1 (Receptor-interacting protein kinase 1) and/or RIPK3[13–15]. Additional terms have been suggested for necrosis induced by different stimuli, but it remains to be shown that these actually represent different execution mechanisms for programmed necrosis[15].

Necroptosis

The term “necroptosis” was coined to specify a non-apoptotic mode of cell death that is elicited by TNF receptor 1 (TNFR1) when caspases are inhibited[91]. This form of PCD can be inhibited by necrostatin-1[91] **[G]**. Although it is just one type of regulated necrosis, necroptosis has often been regarded as synonymous with regulated necrosis[91, 92].

Necroptosis can be induced by the ligation of various death receptors, including FAS (CD95), TNFR1 and TNFR2, different TLRs (Toll-like receptors) and intracellular sensors such as DAI and PKR (protein kinase R)[93]. The TNFR signaling pathway best exemplifies how the necroptotic program can be induced. Upon TNF binding, TNFR1 recruits RIPK1, TRADD (TNFR-associated death domain), cIAP1 and cIAP2, and TRAF2 (TNFR-associated factor 2) and TRAF5, which together form a complex known as complex I (Figure 3A)[94]. cIAPs mediate Lys63-linked ubiquitylation of RIPK1, enabling the docking of TAK1, TAB2 and TAB3, which results in the activation of the IKK complex. In turn this complex targets IκBα for degradation, liberating NF-κB (nuclear factor kappa B). NF-κB translocates to the nucleus, where it drives transcription of pro-survival genes as well as genes encoding negative feedback proteins, such as IκBα and SEF[95–98]. Two such pro-survival NF-κB target genes are those encoding A20 and FLIP (Flice-Like-inhibitory-protein). A20 polyubiquitylates RIPK1 with Lys48-linked chains, thus marking it for proteasomal degradation (Figure 3A), whereas the deubiquitylating enzyme cylindromatosis (CYLD) eliminates Lys63-linked ubiquitin chains from RIPK1, leading to the dissociation of RIPK1 from complex I[98, 99]. This deubiquitylation event changes the function of RIPK1 from promoting survival to promoting death via formation of the death-inducing signaling complex (DISC; also known as complex IIa) comprised of RIPK1, RIPK3, TRADD, FADD, caspase-8 and FLIP[15, 94]. The DISC has a dual role: it facilitates the cleavage and degradation of CYLD, RIPK1 and RIPK3 to promote survival and it enables the homo-dimerization and catalytic activation of caspase-8 to stimulate apoptosis (Figure 3A). In mice, deletion of the complex IIa components caspase-8 and FLIP results in embryonic lethality[100, 101, 102]. However, these mice are rescued when RIPK3 is removed (Table 1), suggesting that a key function of complex IIa is to regulate necroptosis (see below)[103–105]. Furthermore, deletion of *Ripk1* results in postnatal lethality that can be prevented by the ablation of *Ripk3* together with either *Caspase-8* or *Fadd* (Table 1)[106, 107].

When Caspase-8 is inactivated, RIPK1 associates with RIPK3, resulting in autophophorylation, transphosphorylation and formation of the necrosome (Figure 3A)[108, 109, 110]. Accordingly, targeting the kinase domain of RIPK1 with necrostatin 1 inhibits the interaction between RIPK1 and RIPK3 but leaves the pro-survival NF-κB pathway downstream of RIPK1 unaffected[91, 111, 112]. The phosphorylation of RIPK3 (on Ser227 in humans or Ser232 in mice) enables the recruitment of MLKL (mixed lineage kinase domain like) to initiate necroptosis[113, 114]. MLKL is phosphorylated by RIPK3, leading to the formation of oligomers that translocate to the plasma membrane, bind phosphatidylinositol phosphates and form membrane-disrupting pores[115, 116]. The perturbation of membrane integrity seems to induce both Na^+ and Ca^{2+} influx, leading to a rise in osmotic pressure and membrane rupture (Figure 3A)[117, 116, 118].

Ripk3$^{-/-}$ or *Mlkl*$^{-/-}$ mice do not have developmental or homeostatic defects[106, 119]. However, as many downstream signaling components of necroptosis remain elusive, it is too early to conclude whether necroptosis has a role in developmental or homeostatic processes. By contrast, there is evidence that necroptosis functions in response to bacterial and viral infection[104]. When *Ripk3*$^{-/-}$ mice are challenged with vaccinia virus they have increased viral titers and succumb to infection more rapidly than wild-type mice[109]. This is a result of

impaired virus-induced tissue necrosis, inflammation, and control of viral replication. Hence, necroptosis can have two distinct functions. On the one hand it functions as a back-up mechanism to eliminate infected cells when apoptosis is inhibited, and on the other hand it promotes the release of DAMPs (damage associated molecular patterns) **[G]** to induce the immune response. Importantly, viruses encode specific inhibitors, such as viral inhibitor of caspase activation (v-ICA), viral FLIPs (v-FLIPs) and viral inhibitor of RIPK1 (v-IRA)[120, 121], that can affect necroptosis. Interestingly, some viruses (such as murine cytomegalovirus) can block both apoptosis and necroptosis as they encode both a caspase-8 inhibitor and a RIPK3 inhibitor[122, 123].

The induction of a pro-inflammatory response upon necroptosis has been observed in various organs, such as the skin and intestine. Epidermal deletion of *Fadd* and *caspase-8* results in skin inflammation, which can be reversed by *Ripk3* deletion[124, 125]. Similarly, mice in which the intestinal epithelial cells were deleted for *caspase-8* have ileitis as a result of increased necroptosis within the stem cell crypts[126]. These data, together with the ability of caspase-8 to cleave RIPK1 and RIPK3, identify caspase-8 as a key regulator of inflammation situated at the crossroads of apoptosis and necroptosis.

Programmed cell death through the ripoptosome

In addition to the function of cIAPs as positive regulators of canonical NF-κB signaling, they also have a key role in regulating the non-canonical NF-κB pathway[57, 127, 128]. In resting cells, non-canonical NF-κB signaling is repressed by a complex of cIAP1, cIAP2, TRAF2 and TRAF3[129]. This E3 ligase complex binds to NIK (NF-κB inducing kinase), conjugates Lys48-linked ubiquitin chains and targets NIK for proteasomal degradation. Upon ligation of a subset of TNFR-superfamily members (CD40L, TWEAK and BAFF), the TRAF2–TRAF3–cIAP complex is recruited to the receptor, which changes the substrate specificity of the complex from NIK to any of the components of the complex in a manner that depends on the cellular context and the receptor type. As a result, NIK levels are increased, resulting in phosphorylation of IKKα and the NF-κB precursor p100 (Figure 3B). Sequentially, homodimers of IKKα further phosphorylate p100 to promote its proteolytic processing to p52, which can bind to other NF-κB subunits and translocate to the nucleus. In accordance, when cIAPs are depleted (due to chemotherapeutic drugs, cytokine stimulation or SMAC mimetics) the canonical NF-κB pathway is inhibited whereas the non-canonical NF-κB pathway is activated. Under these conditions, TNF is produced and another death-inducing platform is assembled termed complex IIb or the ripoptosome[130, 131]. The ripoptosome forms in the cytoplasm independently of TNF, CD95L, TRAIL, death receptors and mitochondrial pathways. This large (~2-MDa) complex is comprised of RIPK1, FADD and caspase-8 and is negatively regulated by cIAP1, cIAP2 and XIAP. Similar to the DISC, the ripoptosome can stimulate caspase-8-mediated apoptosis, as well as necroptosis in a $FLIP_L$- and caspase-8-dependent manner (Figure 3B).

Signaling from dying cells

Apoptotic cells are rarely visible *in situ* due to efficient clearance mechanisms. When clearance is impaired, uncleared corpses become necrotic and induce inflammation and

autoimmunity[132]. In order to ensure proper cell clearance, apoptotic cells secrete “find me” signals that instruct phagocyte attraction including: lysophosphatidylcholine, sphingosine-1-phosphate, and the nucleotides ATP and UTP[132, 133,]. Additionally, these cells secrete “eat me” signals of which phosphatidylserine is the best known. In viable cells, phosphatidylserine is exclusively retained on the inner leaflet of the lipid bilayer, but is exposed on the extracellular layer during apoptosis[133]. Recent work has elegantly shown that in response to apoptotic stimuli, caspase 3 cleaves XKR8, which leads to phosphatidylserine exposure[134]. Interestingly, CED-8 (the only *C. elegans* Xk-family homologue) also promotes phosphatidylserine exposure and the engulfment of cell corpses in a caspase-mediated manner[135]. Although the exposure of phosphatidylserine during apoptosis is conserved in evolution, the exact mechanism by which it facilitates cell clearance remains controversial[132]. As apoptotic cells are rapidly cleared, it was long thought that they had limited signaling capacity besides secreting “find me” and “eat me” signals. However, recent advances have revealed that apoptotic cells are a rich source of signals that profoundly affect their cellular environment, including mitogens that instruct proliferation and regeneration, and death factors that seed additional apoptosis.

Apoptosis-induced proliferation

Regeneration is a process of regrowth or repair that enables organisms to cope with overwhelming trauma[136]. Research carried out in a diversity of model systems has now revealed that apoptotic cells can secrete mitogens to directly stimulate cell proliferation. Apoptosis-induced proliferation can aid regeneration and explain how developing tissues can compensate for massive cell loss in response to stress or injury.

The original observation that apoptotic cells can secrete mitogens to stimulate the proliferation of adjacent precursor cells came from studies in *Drosophila*[36, 137, 138] (Figure 4A). A technical problem these studies faced was the rapid clearance of dying cells. The key to overcoming this hurdle was to block the execution of apoptosis by expression of p35, which specifically inhibits executioner caspases. The resulting “undead cells” remain in a prolonged apoptotic state and stimulate tissue overgrowth by releasing mitogenic signals[139, 140] (Figure 4A). Candidates for these mitogens include Wingless (Wg, a WNT ortholog) and Decapentaplegic (Dpp, the ortholog of mammalian TGF-β and bone morphogenetic proteins), which are activated under these conditions (Figure 4A)[36, 138]. Furthermore, Wg signaling is required for cell proliferation in this system[36]. Finally, the JNK pathway also has a key role in promoting apoptosis-induced proliferation and wound healing in *Drosophila*[36, 141].

Initially, it was not clear whether JNK functions independently of the apoptotic program, is a downstream target of Dronc or is an inducer of pro-apoptotic genes[36, 142–144]. However, a recent study reconciles these differences by revealing a positive feedback loop in which JNK and p53 function both upstream and downstream of Dronc and RHG genes to amplify the apoptotic signal (Figure 4A)[145]. Interestingly, disruption of the *Drosophila* Cdc42-Par6-aPKC epithelial polarity complex stimulates JNK-dependent apoptosis and apoptosis-induced proliferation[146].

These observations were further extended in p35-independent systems, demonstrating the requirement of Wg and JNK for proper regeneration[147, 148] (Figure 4B). Significantly, in the differentiating *Drosophila* retina, apoptosis-induced proliferation involves Hedghog (Hh) as the primary mitogenic signal[149]. Hh secretion is induced in apoptotic photoreceptor neurons, dependent not only on the initiator caspase Dronc but also on the executioner caspases Ice and Dcp-1 (Figure 4C). Therefore, p35 blocks apoptosis-induced proliferation in this tissue (but not in the wing disc), which indicates that distinct mechanisms of apoptosis-induced proliferation might operate in different cell types and tissues.

Subsequently, apoptosis-induced proliferation was also observed in other organisms, including *Hydra*, *Planaria*, *Xenopus*, newts, zebrafish and mice. In the freshwater polyp *Hydra*, apoptosis is both required and sufficient to induce Wnt3 production for head regeneration after mid-gastric bisection[150]. The MAPK/CREB pathway triggers apoptosis in *Hydra* and, reminiscent of the *Drosophila* apoptosis-induced proliferation mechanism, apoptotic cells release Wnt3 in a caspase-dependent manner to promote cell proliferation and regeneration (Figure 4D)[150, 151]. Planarians and newts also have large numbers of apoptotic cells at the site of amputation, but whether these cells drive regeneration through apoptosis-induced proliferation is still unknown (Figure 4E)[152, 153–156]. In the *Xenopus* tadpole, many apoptotic cells are detected 12 hours after tail amputation and caspase activity is necessary for tail regeneration[157]. However, it remains to be seen whether Wnt, which is known to have a key role in *Xenopus* regeneration, is secreted from apoptotic cells (Figure 4F). In zebrafish, reactive oxygen species (ROS) are produced in response to adult fin amputation. ROS stimulate both caspases and JNK in a parallel manner, both of which are required for apoptosis-induced proliferation. Furthermore, the data hint that Fgf20, Sdf1 and Wnts might be secreted from dying cells in this scenario (Figure 4G)[158]. Similarly, it was recently shown in mice that interleukin-11 (IL-11), a member of the IL-6 pro-inflammatory family of cytokines, is produced by hepatocytes in a ROS-dependent manner. IL-11 is released from apoptotic hepatocytes and induces STAT3 phosphorylation and apoptosis-induced proliferation (Figure 4H)[159]. Interestingly, ROS can also result in hepatocyte necrosis that releases IL-1, which in turn stimulates and induces apoptosis-induced proliferation [160]. In addition, dying mouse MEFs can stimulate the proliferation of stem and progenitor cells in a caspase-dependent manner[161]. Mice deficient for caspase 3 and caspase 7 have impaired wound healing and liver regeneration. Interestingly, the downstream target of these caspases is prostaglandin E_2, a promoter of stem or progenitor cell proliferation and tissue regeneration (Figure 4I)[161].

Taken together, there is now a considerable body of evidence indicating that a key function of caspases in dying cells is to facilitate the release of mitogens that stimulate stem/progenitor cell proliferation and thereby mediate regeneration. Given the strong connections between stem cells, regeneration and tumor growth, apoptosis-induced proliferation may also contribute to cancer. Collectively, these findings challenge the simplistic view of apoptosis as a tumor-suppressive or -preventive mechanism. On the one hand, apoptosis suppresses tumor formation by removing damaged and unwanted cells. On the other hand, apoptosis may stimulate tumor growth through apoptosis-induced proliferation. Additional support for this idea comes from the finding that prostaglandin E_2 is released by bladder

cancer cells and stimulates the proliferation of cancer stem cells, thus leading to tumor repopulation[162]. Furthermore, deficiency for caspase 3 causes sensitivity towards radiotherapy, suggesting a cell death-induced tumor repopulation mechanism[163].

In the future, it will be interesting to examine apoptosis-induced proliferation in different human cancers, and to explore dual-agent therapies designed to kill cancer cells while simultaneously blocking mitogens that might induce proliferation.

Apoptosis-induced apoptosis

Developmental apoptosis often involves the removal of large groups of cells in a cohort[8, 16]. Examples of "communal suicide" include various morphogenic events in *Drosophila*, the elimination of the *Xenopus* tadpole tail and removal of the interdigital webbing in vertebrates. However, how this collective death is achieved remains largely undefined. New insights into this question have come from the recent finding that apoptotic cells have the capacity to instruct additional death in their neighborhood in a phenomenon termed apoptosis-induced apoptosis[164]. In the D*rosophila* wing disc, which is comprised of an anterior and a posterior compartment, induction of large-scale apoptosis in the posterior compartment surprisingly resulted in the apoptosis of cells located at a considerable distance in the anterior compartment[164].

Apoptosis-induced apoptosis in the wing disc uses Eiger (an orthologue of mammalian TNF) to instruct death from afar. Eiger is produced by dying cells of the posterior compartment and activates JNK in anterior compartment cells to initiate apoptosis (Figure 5A, B)[164]. Apoptosis-induced apoptosis also operates in vertebrates and has an important physiological function in the coordinated elimination of hair follicle cells. The hair follicle undergoes cycles of rest (telogen), growth (anagen) and degeneration (catagen; where cells located in the lower portion of the hair follicle are eliminated by apoptosis)[165]. During catagen, apoptotic cells produce TNF that is required for cell death and progression of the hair follicle cycle (Figure 5C)[164]. Similarly, in situations where DNA damage is too great to repair, TNF is expressed by apoptotic cells. In this scenario, ataxia telangiectasia mutated (ATM) activates NF-κB, which induces TNF expression, thus creating a feed-forward loop that promotes apoptosis and IL-8 secretion[166]. Interestingly, in contrast to these findings, TNF produced by dying retinal neurons in zebrafish drives glia proliferation during retinal regeneration[167].

In *Planaria*, amputation induces two successive waves of apoptosis: an immediate localized response (peaking after 1–4 hours) and a second systemic response (peaking 3 days post amputation) that can be induced in uninjured organs, presumably to ensure proper remodeling[168].

Inter-organ communication can also be seen in *Drosophila* in response to tissue damage. Wounding the cuticle results in apoptosis in the midgut, an organ distant from the wound site. ROS can mediate the induction of apoptosis in midgut cells, which activates a tissue stem cell regeneration pathway. Intriguingly blocking midgut cell death post wounding leads to fly mortality, suggesting that this mechanism dampens the dangerous systemic wound reaction[169].

The observation that apoptotic cells can facilitate additional cell death potentially has many important implications. Apoptosis-induced apoptosis may function in sculpting and deleting tissues where the complete elimination of entire cell groups is required. Additionally, this process might be an efficient means to inhibit viral spreading and might occur in pathologies associated with extensive cell death, such as ischemic stroke, liver failure and neuronal degeneration. Furthermore, apoptosis-induced apoptosis may provide an explanation for the 'bystander effect", whereby non-irradiated cells respond in the same manner as radiation-exposed cells[170, 171].

In summary, apoptotic cells have the unexpected capacity to stimulate either cell proliferation or further cell death. At this time, it is not clear how the decision between these opposing outcomes is made, and we still have a very incomplete understanding of the role and regulation of cell death *in vivo.* Further work in this area will likely yield many new surprises, and facilitate the design of rational therapies targeting cell death pathways.

Looking ahead

Since the original proposition of a cellular mechanism of PCD, tremendous progress has been made towards elucidating the biochemical mechanism of apoptosis. In retrospect, it is quite remarkable how the discovery of the *C. elegans* apoptotic cell death machinery paved the way for unraveling the more intricate systems seen in *Drosophila* and mammals. However, we are only now beginning to understand the *in vivo* role of key apoptotic components in mammals, which holds much promise for treating various human disorders.

The synonymy of PCD with apoptosis is now outdated as alternative cell death mechanisms have been described. One prominent process that is often associated with apoptosis is autophagy. However, additional future work is needed to clarify whether autophagy is a *bona fide* mechanism for cellular killing *in vivo*.

Another cell death process that is intensely studied is necroptosis. Perhaps the main obstacle limiting our understanding of this process is the challenge to identify necroptotic cells *in vivo*, and to distinguish whether a cell dies from necroptosis or from necrosis generated in another manner. Also, it remains to be clarified whether necroptosis drives inflammation or is driven by it.

At this time we do not understand why there are so many different ways for a cell to die, how cells are selected *in vivo* to activate any of these PCD mechanisms, and how survival and death signals are integrated to direct a specific cell fate. Finally, an exciting field of research concerns signals emanating from dying cells. Much remains to be discovered regarding the complex signals that dying cells use to instruct their neighbors, and their role under physiological conditions. Given the importance of cell death during development, homeostasis and disease, deciphering the intriguing "last words" of dying cells promises exciting new discoveries that may have considerable therapeutic value.

Acknowledgments

We thank Y. Fox for help with the illustrations and K. Fox for the endless support. This work was supported in part by NIH grant RO1GM60124 (to H.S.). H.S. is an Investigator with the Howard Hughes Medical Institute. The Deloro career advancement chair supports Y.F.

Glossary

Initiator caspases	Caspases that cleave inactive forms of executioner capases
Executioner caspases	Caspases that cleave various cellular proteins, which in many cases leads to apoptosis
Apoptosome	A protein platform that is formed during apoptosis comprised of cytochrome c that has translocated from the mitochondria to the cytoplasm and APAF1
Mitochondrial outer membrane permeabilization (MOMP)	An event regulated by the BCL-2 protein family that is considered to be the point of no return at which the cell commits to apoptosis
Death-inducing signaling complex (DISC)	A protein platform that is formed by death receptors that can drive apoptosis
Baculovirus inhibitory repeat (BIR) domain	A domain present in IAP proteins that can bind caspases as well as pro-apoptotic factors such as IAP antagonists
Hair follicle stem cells (HFSCs)	Adult stem cells normally situated in a niche called the bulge that function to replenish the hair follicle
Linker cell	A migratory cell of the *C. elegans* male gonad that dies in a non-apoptotic, caspase-independent manner
Autophagic cell death	A reported mode of PCD that is associated with the appearance of autophagosomes and depends on autophagy-related proteins
Necrostatin 1	A potent and selective inhibitor of necroptosis, originally reported as a selective allosteric inhibitor of the death domain receptor-associated adaptor kinase RIP1 in the necroptosis pathway
DAMPs (damage associated molecular patterns)	Molecules also known as alarmins that are released by stressed cells. They function as endogenous danger signals to initiate and perpetuate the inflammatory response

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Biographies

Yaron Fuchs is currently an Assistant Professor and Deloro Chair in the Technion Israel Institute of Technology. He performed a direct Ph.D. track at the Technion Israel Institute of Technology and then carried out his postdoctoral research at Rockefeller University and Howard Hughes Medical Institute (New York, USA). Upon completion, he returned to the Technion where he now heads the Laboratory of Stem Cell Biology and Regenerative Medicine. His research is focused on different modes of cell death and how they regulate diverse aspects in stem cell biology and stem cell-dependent processes.

Hermann Steller is the Strang Professor and Head of the Laboratory of Apoptosis and Cancer Biology at the Rockefeller University and an Investigator of the Howard Hughes Medical Institute. Prior to this he was Professor of Neurobiology at the Massachusetts Institute of Technology in Cambridge, Massachusetts. Hermann Steller received his Diplom (the German equivalent of an M.Sc. degree) in microbiology from the Johann Wolfgang Goethe University, Frankfurt, and his Ph.D. from the European Molecular Biology Laboratory and the University of Heidelberg. His laboratory studies the regulation of apoptosis, how defects in this process contribute to diseases, and how insights into apoptotic pathways can be exploited for the design of new therapies.

Online summary

1. During development programmed cell death performs various functions such as: sculpt and delete structures, supply nutrients, regulate cell number, and eliminate abnormal and dangerous cells.
2. Several different PCD mechanisms are used to eliminate cells and we discuss the biological significance of these pathways *in vivo.*
3. Caspase activation is coordinately subjected to many layers of upstream regulation ensuring that the cell is executed only after multiple checkpoints have been cleared.
4. Is autophagy a cell death mechanism? Here we carefully review the data supporting autophagy as a bona fide mechanism of cell destruction.
5. For many years necrosis was regarded as an unregulated mode of cell death caused by overwhelming trauma. Here, we examine a regulated form of necrosis, termed necroptosis.
6. Traditionally, it was thought that dying cells have limited effects on the cellular environment. However, it is now clear that apoptotic cells release signals that can fuel tissue regeneration.
7. Cell undergoing apoptosis have the capacity to instruct additional killing in their cellular environment, explaining how “communal suicide” can occur.

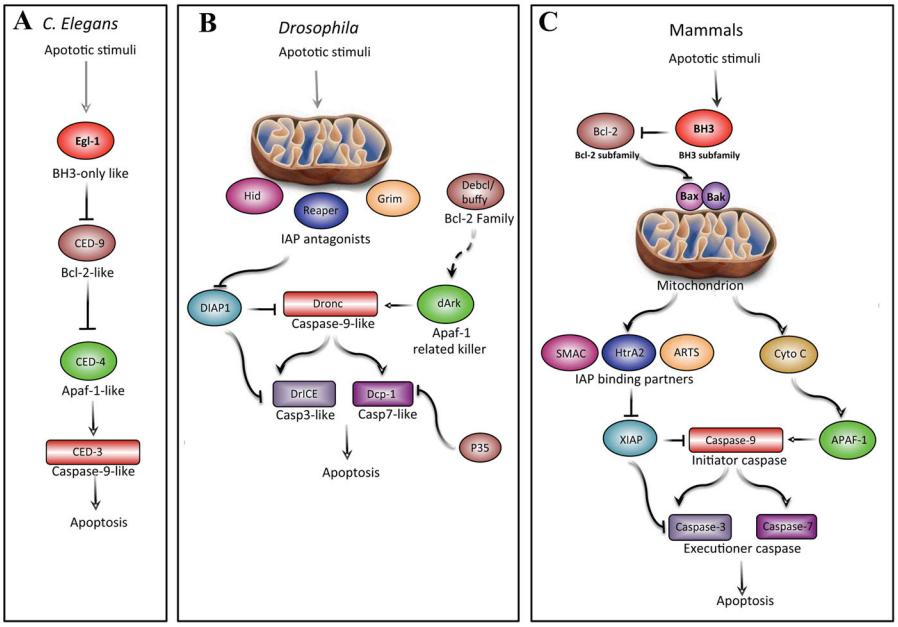

Figure 1. The core of the apoptotic machinery is conserved

Functional homologous of apoptotic proteins in *C. elegans*, *Drosophila melanogaster* and mammals uncover evolutionary conservation of the apoptotic pathway. **A**. In *C. elegans*, EGL-1 (BH3-only protein homolog) binds and inhibits CED-9 (BCL-2-family homolog), resulting in the release of CED-4 (APAF1 homolog) from the CED-9–CED-4 complex. This enables the elimination of cells by CED-3 (caspase). **B**. In *Drosophila*, the IAP antagonists Reaper, Hid and Grim (RHG) mediate the degradation of DIAP1, thus liberating Drice and Dcp-1. In addition, this enables Dronc (Caspase-9 homolog) to interact with dArk and form the apoptosome that efficiently activates executioner caspases. The p35 protein is a specific inhibitor of executioner caspases and the activation of the apoptosome might be regulated by the Bcl-2 family members Debcl and Buffy (hence depicted by a dashed line). **C**. In mammals, the crucial decision as to whether a cell commits to apoptosis is regulated by the fine interplay between the anti-apoptotic BCl-2 subfamily of proteins and the pro-apoptotic BH3-only subfamily of proteins. During apoptosis, BH3-only proteins facilitate a BAX- and BAK-dependent release of cytochrome c from the mitochondria, which binds to APAF1 and gives rise to the apoptosome. In parallel, IAP antagonists including DIABLO, HTRA2 and ARTS translocate from mitochondria and release caspases from their negative regulation by IAPs. In particular, Caspase-9 is liberated from XIAP and activated by the apoptosome, triggering executioner caspases-3 and -7. Homologous proteins are similarly depicted in the three panels.

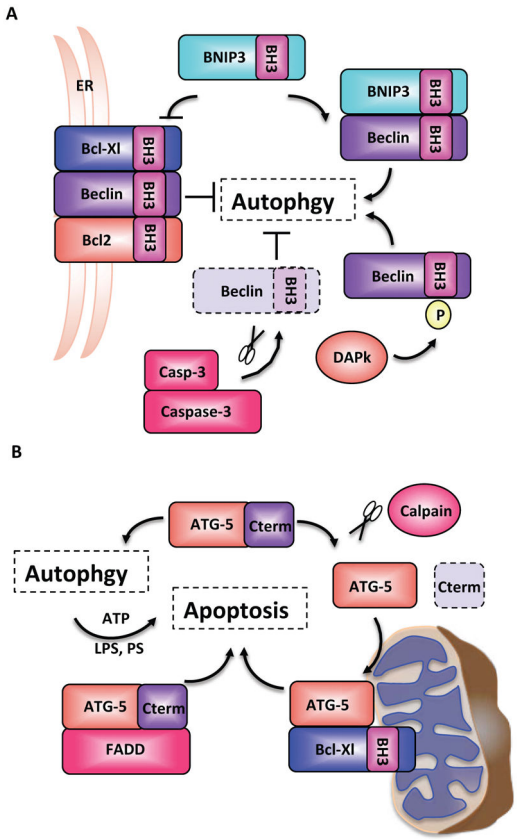

Figure 2. Crosstalk between autophagy- and apoptosis-related proteins

The BH3 domain of the autophagy-related protein Beclin 1 (BECN1) enables the formation of a BCL-2–BCL-XL–BECN1 complex that localizes to the ER and inhibits autophagy. Competitive interaction by the BH3-only protein BNIP3 and phosphorylation by the death-associated protein kinase (DAPK) regulate the dissociation of this complex and drive autophagy. In addition, BECN1 has also been found to be a substrate of caspase-3. **B**. The autophagy-related protein ATG5 can undergo calpain-dependent cleavage, which switches its function from pro-survival to pro-death. The C-terminal cleavage gives rise to a pro-apoptotic product that translocates to the mitochondria, binds BCL-XL and induces apoptosis. ATG5 can also induce apoptosis by binding to FADD, and it seems that apoptotic features such as phosphatidylserine exposure and membrane blebbing depend upon autophagy for the provision of energy in the form of ATP.

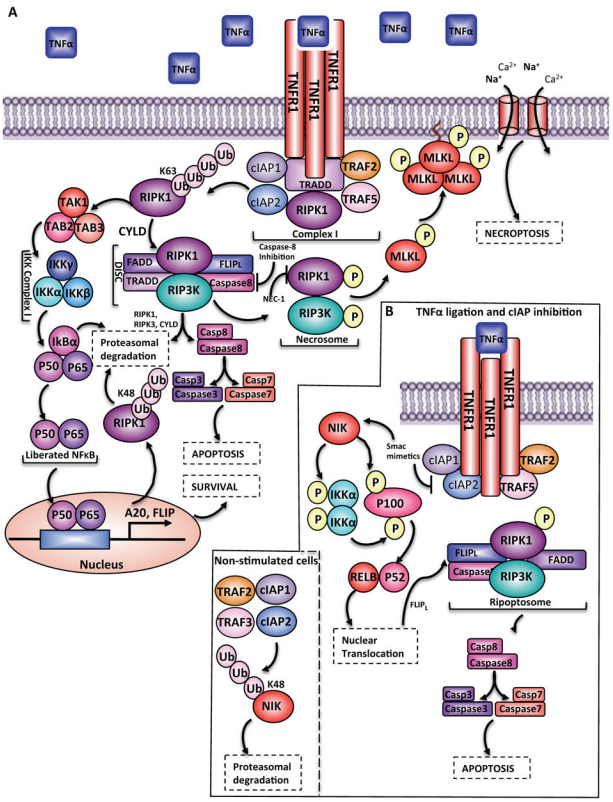

Figure 3. TNF-mediated survival, apoptosis and necroptosis

A. TNF ligation results in recruitment of RIPK1, TRADD, cIAP1 and cIAP2, and TRAF2 and TRAF5 to the TNFR1 to form complex I. cIAP1 and cIAP2 mediate Lys63-linked ubiquitylation of RIPK1, enabling docking of TAK1 (transforming-growth-factor-β-activated-kinase-1) and its binding partners TAB2 and TAB3. The signal is then perpetuated to IKK ((IκBα (inhibitor of kappa B) kinase)), which degrades IκBα, the cytoplasmic inhibitor of canonical NFκB. Once liberated from IκBα, NFκB translocates to the nucleus where it drives the transcription of pro-survival genes as well as feedback antagonists. Two pro-survival NFκB target genes are *A20* and *FLIP which promote association of* RIPK1 with cytoplasmic RIPK3, TNF-receptor-associated-death-domain (TRADD), FADD, caspase-8 and the NF-κB target FLIP to form the death-inducing signaling complex (DISC). $FLIP_L$ can hetrodimerize with Caspase-8 and facilitate the cleavage and degradation of CYLD, RIP1K and RIPK3. However, the DISC also enables homo-dimerization and catalytic activation of caspase-8, which activates caspase-3 and caspase-7 to induce apoptosis. When caspase-8 is deleted or inhibited, RIPK1 interacts with RIPK3, resulting in the formation of the necrosome, an interaction that can be inhibited by necrostatin 1 (nec-1). RIPK3 recruits and phosphorylates MLKL, leading to the formation of oligomers that translocate to the plasma membrane. Once at the plasma membrane, MLKL forms membrane-disrupting pores, regulating both Na^+ and Ca^{2+} influx, resulting in membrane rupture. **B**. cIAPs also function as negative regulators of the non-cannonical NF-kB pathway. In resting cells, a complex encompassing cIAP1, cIAP2, TRAF2 and TRAF3 targets and degrades NIK. Upon ligation of TNFR1, the TRAF2-TRAF3-cIAP1/2 complex is recruited to the receptor, changing the substrate specificity from NIK to the components of the complex. This results

in increased NIK levels, phosphorylation of IKKα and p100, proteolytic processing of p100 to p52, and translocation to the nucleus. When cIAPs are depleted the canonical NFκB pathway is inhibited whereas the non-canonical NFκB pathway is not. Under these conditions, the large (~2-MDa) ripoptosome is assembled and, similarly to complex IIa, can stimulate caspase-8-mediated apoptosis.

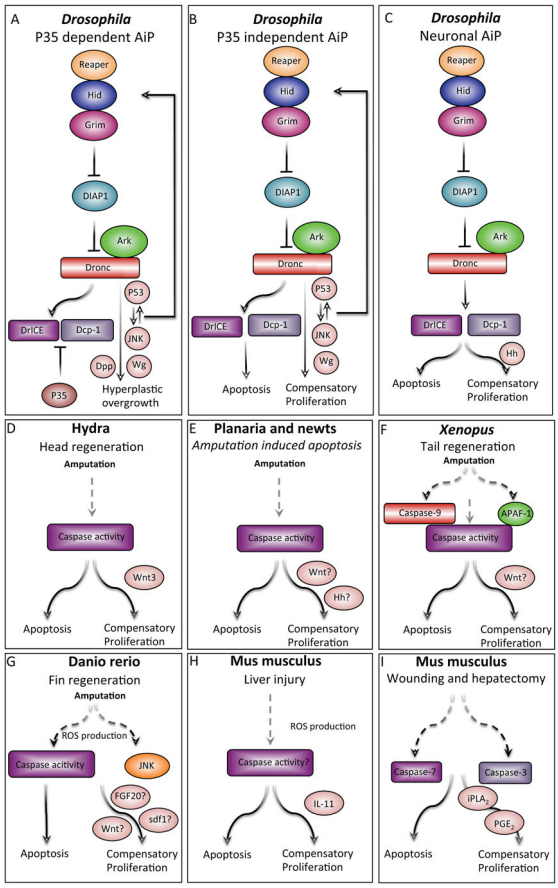

Figure 4. Apoptosis-induced proliferation

Apoptotic cells can secrete mitogens that stimulate growth and regeneration. Caspase targets and mitogenic factors are depicted in pink, and question marks indicate uncertainty. **A.** In *Drosophila*, p35 inhibits executioner caspases and retains the cells in an “undead” state. Under these conditions, p53 and JNK are activated and promote the release of Wg (Wnt ortholog) and Dpp (TGFb/BMP homolog), which promotes hyperplastic overgrowth through apoptosis-induced proliferation (AiP). **B.** In *Drosophila*, in a natural non p35-dependent system, apoptosis induces tissue regeneration by the secretion of Wg. **C**. In *Drosophila*, differentiated neurons induce apoptosis-induced compensatory proliferation via Hedghog (Hh), stimulating non-neuronal cell proliferation. In this scenario, the signal is downstream of Drice and Dcp-1 and not Dronc. **D**. In *Hydra*, head regeneration is a caspase-dependent process and apoptotic cells secrete Wnt3 to induce apoptosis-induced compensatory proliferation. **E**. In newts and *Planaria*, apoptotic cells and caspase activity are seen at the amputation site; however, it remains to be seen whether the apoptotic cells are responsible for the secretion of Wnt and Hh. **F**. In *Xenopus*, tail amputation leads to caspase activity, and inhibition of caspase-3 and caspase-9 inhibits proliferation and regeneration. It is unclear whether this form of compensatory proliferation is mediated by Wnt. **G**. In zebrafish, fin amputation results in ROS activity, which induces caspase and JNK activation. It remains to be established if FGF20, Wnt and sdf1 are secreted from apoptotic cells to regulate apoptosis-induced compensatory proliferation. **H**. In mice, liver injury results in

ROS production and secretion of IL-11 from hepatocytes, which facilitates apoptosis-induced compensatory proliferation. **G**. In mice, wound repair and liver regeneration depend on caspase-3 and caspase-7. Caspase-3 mediates the proteolysis of iPLA2, producing PGE2, which is known to stimulate stem cell proliferation, wound repair and regeneration.

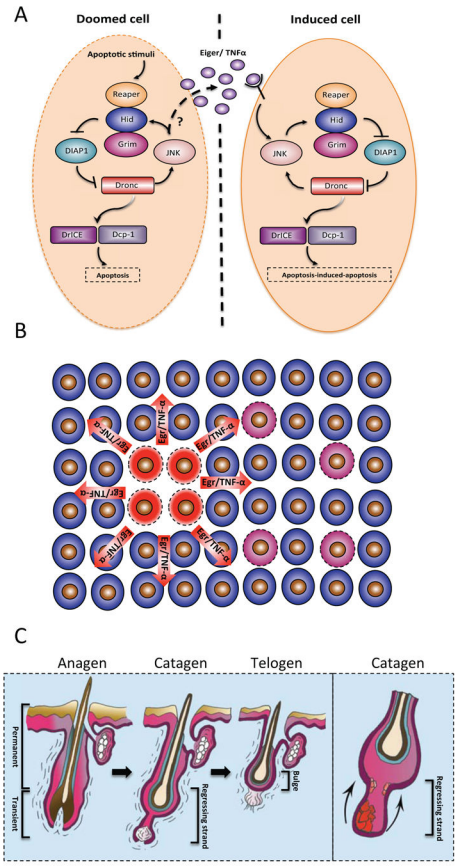

Figure 5. Apoptosis-induced apoptosis

A. Expression of the IAP antagonists Hid or Reaper in ‘doomed’ cells (left panel) can cause apoptosis in distant cells (right panel). The apoptotic signal is propagated by Eiger (a TNF homolog), which can cross compartmental borders (marked by a dashed line), to activate JNK and instruct apoptosis in distant cells. Presumably, the production of Eiger depends on JNK (depicted by a question mark). **B**. Schematic representation of apoptosis-induced apoptosis. Cells illustrated in red represent the initial apoptotic focal point. These cells emit Eiger in *Drosophila* and TNF in mice, which result in secondary apoptosis (pink cells). (**C right panel**) Schematic diagram depicting the hair follicle cycle. Hair follicles cycle between phases of growth (anagen), destruction (catagen) and rest (telogen). During catagen, apoptosis eliminates the lower two-thirds of the hair follicle. Post-catagen, the hair follicle enters the resting telogen phase, followed by a new cycle of hair growth (anagen). (**C left panel**) Apoptosis-induced apoptosis regulates the hair follicle cycle. During catagen, a primary apoptotic event (red cells), induces a wave of secondary apoptosis (pink cells) that is required for the cohort elimination of transient hair follicle cells.

Table 1

Physiological function of key cell death genes.

Key cell death genes	Functional classification	Mutant phenotype	Physiological Function from mutant and RNAi studies
C. elegans			
Egl-1	BH3 only	Animals are viable and have an additional 131 cells.	Essential for inhibiting CED-9.
ced-9	Bcl-2 like	Developmental lethality due to massive cell death.	Inhibits cell death.
ced-4	Apaf-1 like	As for *Egl-1* mutation	Essential for activating CED-3.
ced-3	Caspase-9 like	As for *Egl-1* mutation	Essential for cell death.
Drosophila			
hid	IAP antagonist	Mutants die as embryos with extensive defects in apoptosis.	Essential for most apoptosis.
Reaper, Hid and Grim	IAP antagonists	Mutants die as embryos with extensive defects in apoptosis.	Essential for most apoptosis.
Ark	APAF1 like adaptor	Pupal lethality. Defects in developmental death.	Essential for most apoptosis.
DIAP1	IAP	Embryonic lethal due to excessive apoptosis.	Inhibits Dronc, Drice and Dcp-1.
Dronc	Caspase-9 like	Pupal lethality. Defects in developmental death.	Essential for most apoptosis.
Dcp-1	Executioner caspase	Mutants are viable.	Can be compensated by Drice similar to mammalian caspases.
Ice and *Dcp-1*	Executioner caspases	Prepupal lethality. Phenotype is similar to Dronc	Essential for apoptosis.
Mammals			
Bcl-2	BCL-2 subfamily	Abnormal death of renal epithelial progenitors. Fatal kidney disease.	Inhibits apoptotic induction.
Puma, *Bid* and *Bim*	BH3 subfamily	Similar to *Bax* and *Bak* mutants.	Essential for initiation of apoptosis at the mitochondria.
Bax and *Bak*	BAX subfamily	Most mice die during embryogenesis. Neonates display partial syndatcily, imperforated vaginas and an increase in hematopoietic cells	Essential for apoptosis induction.
Smac	IAP antagonist	Mice appear normal.	Physiological function found with Caspase-3 (see below).
Htra2	IAP antagonist	Lethal postnatal neurodegenerative disease.	Phenotype does not seem to be related to apoptosis.
Sept4/Arts	IAP antagonist	Increased apoptotic resistant stem cell numbers, improved regeneration and increased malignancies.	Important for stem cell apoptosis, tumorgenesis and regeneration.
Apaf1	Capsase-9 adaptor	Similar to *Caspase-9*$^{-/-}$ mice. Some mice survive adulthood.	Essential for Caspase-9 activation.
Xiap	IAP	Mice display impaired wound healing and hair follicle stem cells exhibit increased susceptibility to apoptosis.	Important for stem cell apoptosis and skin regeneration.
Caspase-3	Executioner Caspase	Phenotype depends on genetic background. Perinatal lethal in FVB129 and no phenotype in C57BL/6 mice probably due	Essential for neuronal cell death and may have a redundant role in other tissues.

Key cell death genes	Functional classification	Mutant phenotype	Physiological Function from mutant and RNAi studies
		differences in caspase-7 expression levels.	
Caspase-7	Executioner Caspase	Mutants appear normal	No apparent apoptotic defects in mutants. Appears to compensate in the absence of caspase-3
Caspase-3 and *caspase-7*	Executioner Caspase	Perinatal lethal. Defects in cardiac development. MEFs display apoptotic resistance.	Caspase-3 and -7 are required for execution of apoptosis.
Caspase-3 and *Smac*	Executioner Caspase and IAP antagonist	Perinatal lethality. Somewhat similar to *Caspase3*$^{-/-}$ and *Caspase7*$^{-/-}$ mice.	Sensitizing deletion of caspase 3 demonstrates that SMAC modulates Caspase-7 via IAPs
Caspase-8	Initiator caspase	Embryonic lethal. Death receptor-mediated apoptosis is completely impaired	Essential for extrinsic apoptosis mediated by the TNF family and inhibition of necroptosis.
Fadd	Caspase-8 adaptor	Similar to *Caspase-8*$^{-/-}$ mice	Essential for Caspase-8 activation.
Caspase-8 and *Rip3k*	Initiator caspase and Necroptosis factor	Mice develop normally but develop lymphadenopathy by four months of age.	Caspase-8 and RIP3K together seem to be dispensable for mammalian development.
Caspase-9	Initiator caspase	Perinatal lethal, but some animals survive to adulthood. Defects in the CNS.	Required for efficient apoptosis in some cell types.

Abbreviations: MEFs- Mouse embryonic fibroblasts, CNS- central nervous system, IAP- inhibitor of apoptosis.