

## SIGNALING PATHWAYS THAT REGULATE LIFE AND CELL DEATH

### Evolution of Apoptosis in the Context of Self-Defense

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**Abstract:** Programmed Cell Death is essential for the life cycle of many organisms. Cell death in multicellular organisms can occur as a consequence of massive damage (necrosis) or in a controlled form, through engagement of diverse biochemical programs. The best well known form of programmed cell death is apoptosis. Apoptosis occurs in animals as a consequence of a variety of stimuli including stress and social signals and it plays essential roles in morphogenesis and immune defense. The machinery of apoptosis is well conserved among animals and it is composed of caspases (the proteases which execute cell death), adapter proteins (caspase activators), Bcl-2 family proteins and Inhibitor of Apoptosis Proteins (IAPs). We will describe in this review the main apoptotic pathways in animals: the extrinsic (death receptor-mediated), the intrinsic/mitochondrial and the Granzyme B pathway. Other forms of non-apoptotic Programmed Cell Death which occur in animals will also be discussed. We will summarize the current knowledge about apoptotic-like and other forms of cell death in other organisms such as plants and protists. Additionally, we will discuss the hypothesis that apoptosis originated as part of a host defense mechanism. We will explore the similarities between the protein complexes which mediate apoptosis (apoptosomes) and complexes involved in immunity: inflammasomes. Additional functions of apoptotic proteins related to immune function will be summarized, in an effort to explore the evolutionary origins of cell death.

## INTRODUCTION: PROGRAMMED CELL DEATH

Cell death regulated by a genetic program, or Programmed Cell Death (PCD), is a fundamental mechanism of homeostasis of tissues. For this reason, it is a critical process for many multicellular organisms, which have developed different programs of programmed cell death such as apoptosis. Programmed cell death, however, does not only occur in multicellular organisms: it has also been shown to occur in yeast, protozoans, bacteria and unicellular algae. This suggests that a program designed for self killing of individual cells is in the long term beneficial for the colony or 'group of individuals'.

Programmed Cell Death (PCD) has been studied thoroughly in animals. In response to stress due to starvation or damage, animal cells engage a biochemical pathway to destruct themselves. Moreover, upon detection of a virally infected cell, cytotoxic cells kill other cells from the same organism by inducing their suicide. Cell death is not only employed to remove damaged or infected cells, but it is also important for sculpting tissues. During embryonic development, animals produce many cells which are no longer needed in the adult animal and which therefore undergo PCD to eliminate themselves. Classical examples of this are the removal of the tadpole of frogs during metamorphosis or the disappearance of interdigital tissues during mammalian development to sculpt fingers. In the adult animal, unwanted cells are eliminated through PCD when they are no longer needed. For instance, during an immune response there is a rapid production of lymphocytes which respond to a specific pathogen. These lymphocytes have to be eliminated after the pathogen has been cleared. For these reasons, these cells are 'programmed' to eliminate themselves after a few days of life.

Programmed cell death can occur through different biochemical programs. Apoptosis is the best known of these programs and it is the major form of cell death in animals. This process occurs through a coordinated dismantling of a cell in a matter of hours. Cells shrink, detach and end up forming small pieces or 'apoptotic bodies', which are immediately cleared by phagocytes and are thus removed from the body in a silent way. During apoptosis, cells maintain integrity of their plasma membrane, which helps avoid inflammation. In contrast, cell death by 'necrosis', which occurs in situations of uncontrolled tissue damage (for instance after traumatic injury due to heat shock or radiation) is a 'passive' form of cell death which triggers inflammation.

Apoptosis requires a number of genes that are well conserved across animal evolution. In the past few years, multiple genetic and cell biology experiments have shown that apoptosis is executed through similar biochemical pathways in vertebrates, *Drosophila* and *C. elegans*. These biochemical pathways lead to activation of proteins termed 'caspases'. What distinguishes apoptosis from other forms of cell death is a number of morphological and biochemical features which are consequence of the activity of caspases.

## APOPTOSIS IS EXECUTED THROUGH ACTIVATION OF CASPASE PROTEASES

Caspases are cysteine proteases (they have a cysteine in the active site) which achieve apoptotic cell death through the cleavage of several substrates. A few hundred caspase substrates have been described in human cells. Roughly, we can say that during apoptosis, caspases cleave two different subsets of proteins. The first group comprises proteins that are necessary for maintenance of cellular structures (cytoskeletal components, organelle

proteins, etc.) and proteins whose cleavage induces activation of proteins that destroy the cells, such as nucleases. Cleavage of these substrates produces the morphological changes associated with apoptosis. The second group of apoptotic caspase substrates comprises proteins that are involved in what we could define as “life support” functions (transcription and translation, metabolism, growth promoting signaling molecules etc.).<sup>1</sup> By cleaving these substrates, caspases ensure the termination of the life of the cell.

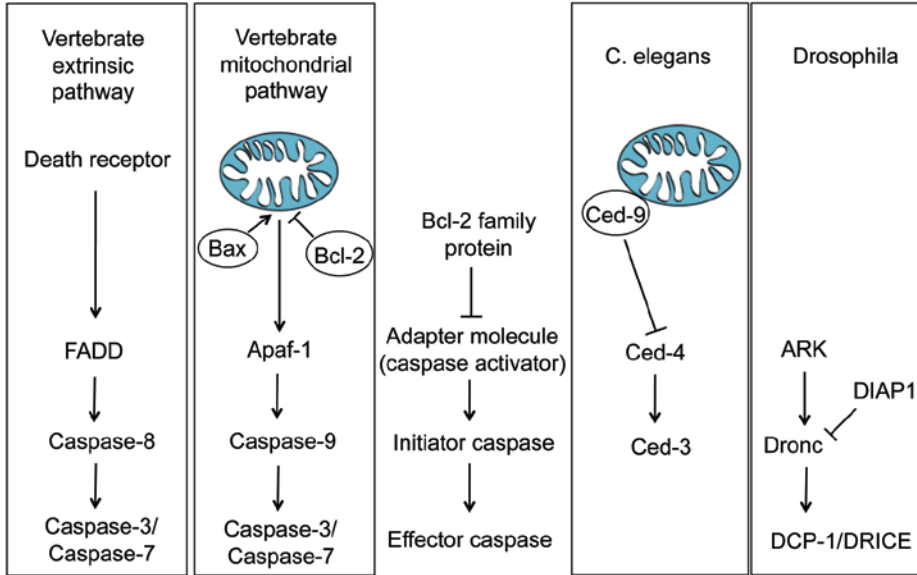
Caspases are normally present in the cytosol. Since killer proteases are obviously dangerous, they are kept in check by a number of safety mechanisms. The most important is that they are inactive until a ‘deadly’ stimulus activates them by promoting their oligomerization and subsequent cleavage. Cleavage of the caspase precursors, which we call the ‘procaspases’, allows the formation of the mature proteases. When activated, caspases can cleave and activate other caspase molecules and this leads to an irreversible proteolytic cascade that ends up killing the cell. It should be noted that not all caspases are involved in apoptosis; there is a group of caspases which includes caspase-1 which play roles in inflammation but not in cell death, as will be discussed in more detail later. Caspases of this group are called ‘inflammatory caspases’ as opposed to the rest of caspases, which are apoptotic caspases. We can classify apoptotic caspases in two groups based on their sequence homology and their role in the proteolytic cascade: initiator (apical) and executioner (effector) caspases. Initiator caspases (caspase-8 and -9 in mammals) have a long domain in the N-terminus of the protein: the pro-domain. Pro-domains are responsible for interactions between initiator caspases and the molecules which activate them. These domains are mainly of two types: CARD (Caspase Recruitment Domain), present in caspase-9 and DED (Death Effector Domain), present in caspase-8. These domains are protein interaction modules composed of six alpha-helical bundles. Upon an apoptotic stimulus, homotypic CARD-CARD or DED-DED interactions bring two molecules of initiator caspases in close proximity to each other and this event leads to their activation and subsequent inter-molecule cleavage.

The so-called ‘executioner’ or ‘effector caspases’ (caspase-3 and -7 in mammals) are the ones that cleave the substrates required for death of the cell. These proteins are inactive until they are cleaved by ‘initiator’ caspases. Since activation of executioner caspases require previous activation of initiator caspases, the first steps leading to initiator caspase activation are highly regulated events which determine the onset of apoptosis.

In vertebrates there are several biochemical pathways that can activate caspases. Notably, these pathways are quite similar to apoptotic pathways in other species such as *C. elegans* and *D. melanogaster* (Fig. 1). As a summary, a stimulus (coming either from within the cell or from the outside) triggers the formation of a multi-protein complex which recruits and activates an initiator caspase. Activation of these caspases occurs when several molecules are recruited to these ‘deadly complexes’ by oligomerization with the so-called ‘adapter molecules’ or caspase activators. Initiator caspases then cleave and activate effector caspases and this triggers death of the cell.

### **CONSERVED APOPTOTIC REGULATORS IN VERTEBRATES, FLIES AND NEMATODES: CASPASES, IAPS, ADAPTER MOLECULES AND BCL-2 FAMILY PROTEINS**

Stress and developmental or social cues induce apoptosis through a biochemical pathway which is very similar in mammals, flies and nematodes. The caspase activation

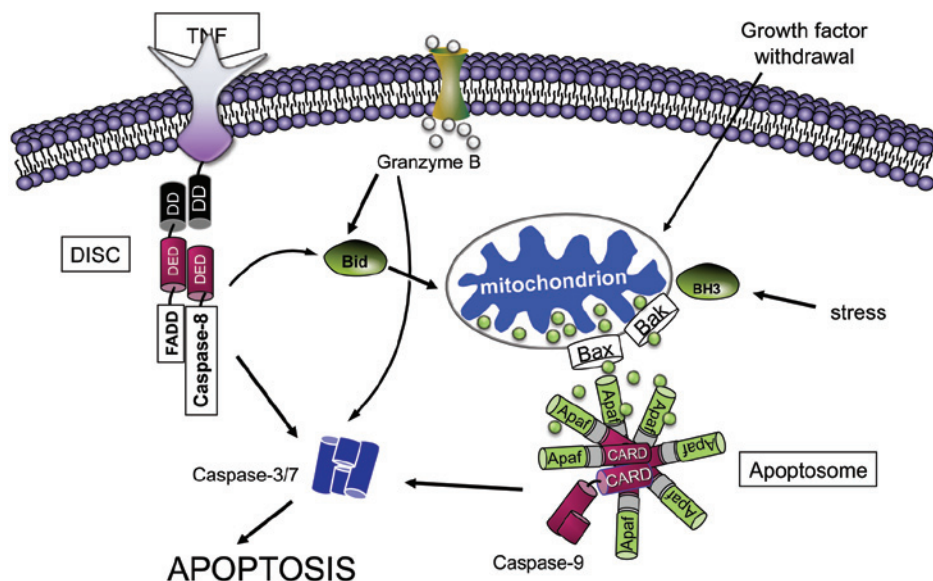


**Figure 1.** Apoptosis in animal models. The apoptotic machinery in insects (*D. melanogaster*), nematodes (*C. elegans*) and vertebrates is very similar. Adapter proteins such as Apaf-1 or FADD recruit activator caspases, which cleave and activate effector caspases. In vertebrates and *C. elegans*, Bcl-2 proteins control the pathway either directly by inhibiting the Apaf-1-like molecule (in *C. elegans*) or indirectly by controlling mitochondrial permeabilization, which in vertebrates is required for formation of the Apaf-1/caspase-9 complex (apoptosome).

cascade begins when several molecules of initiator caspases are activated through oligomerization in a complex termed ‘apoptosome’. In mammalian cells, the apoptosome is nucleated by the adapter molecule Apaf-1, which recruits the initiator caspase-9 through interactions between the CARD domain present both in Apaf-1 and caspase-9 (Fig. 2). Caspase-9 bound to the apoptosome can cleave and activate effector caspases-3 and -7, which are responsible for the death of the cell.

In the nematode *C. elegans*, caspases are also activated through the formation of apoptosomes around Apaf-1-like molecules. In fact, the homolog of Apaf-1 in nematodes, Ced-4, was discovered before Apaf-1. *C. elegans* has been an invaluable tool to identify the proteins which participate in apoptosis. The reason is that adult animals all have exactly the same number of cells. During development, a number of cells that are no longer needed in the adult animal die by apoptosis. This way, it is easy to identify mutants with ‘extra’ cells (mutated in genes essential for apoptosis) or with an excess in cell death (possibly due to a mutation in a gene which inhibits apoptosis). This system led to the identification of the essential apoptotic genes. Caspases, adapter molecules (Apaf-1-like) and Bcl-2 family proteins were first identified as key components of the apoptotic cell death machinery in the nematode and their homologs are also key for apoptosis in mammals.

The apoptotic pathway in *C. elegans* is relatively simple (Fig. 1). It comprises three proteins: a protein located in the mitochondrial membrane, Ced-9 (homolog of human Bcl-2 family proteins), a caspase activator, Ced-4 (Apaf-1 homolog) and a caspase, Ced-3.<sup>2</sup> Ced-9 holds the caspase activator Ced-4 inactive. At a point during development of the



**Figure 2.** Two main pathways of apoptosis in vertebrates: the extrinsic and the mitochondrial pathway. In the extrinsic pathway (left), a death ligand such as TNF, TRAIL or Fas Ligand activates a death receptor, which recruits FADD through its Death Domain (DD). FADD recruits Caspase-8 through Death Effector Domain (DED) interactions. Caspase-8 can directly cleave and activate effector caspases such as -3 and -7, or cleave Bid to induce Bax/Bak activation. In the mitochondrial pathway, a BH3-only protein (labeled “BH3”) such as Bid, Puma, Noxa etc. activates Bax and/or Bak which permeabilize mitochondria triggering cytochrome c release (depicted as small spheres). Cytochrome c triggers the oligomerization of Apaf-1 and formation of the apoptosome, where Caspase-9 is activated. Caspase-9 cleaves and activates effector caspases.

animal, an inhibitor of Ced-9 is synthesized in some cells and this promotes the release of Ced-4. Ced-4 then forms an apoptosome that recruits and activates the caspase Ced-3 through CARD-CARD interactions and this triggers death of the cell. This pathway is simpler than in mammals, since no initiator caspases are needed: the caspase Ced-3 functions as both activator and effector caspase and executes death upon activation.

The apoptotic process has been also characterized in detail in the fly *Drosophila melanogaster*. Caspase activation pathways are also quite similar, with caspases being activated through recruitment to an apoptosome, which is nucleated by a protein very similar to Ced-4 and Apaf-1: ARK. In *Drosophila*, however, activation of the apoptosome is triggered in a different form: during development, several proteins are synthesized that inactivate a caspase inhibitor: DIAP1. DIAP1 is holding the initiator caspase-9 homolog, DRONC, inactive. Degradation of DIAP1 is sufficient to trigger apoptosome formation and apoptosis. DIAP1 belongs to a family of proteins whose homologs have anti-apoptotic functions in mammals. These proteins, named IAPs (Inhibitor of Apoptosis Protein) bind and inactivate caspases directly or promote their ubiquitination and proteasomal degradation.

The genes encoding proteins of these four groups of apoptosis-related proteins (Bcl-2 homologs, Apaf-1 homologs, IAPs and caspases) exist in all animals studied.<sup>3,4</sup> This suggests that the apoptotic process existed in the precursor of animals (Table 1). To summarize,

**Table 1.** Summary of presence of apoptotic proteins in animals

	Ecdysozoa		Cnidaria	Deuterostomia		
Phyla	Arthropoda (insects and others)	Nematoda (round-worms and others)	(jelly-fish, Hydra)	Echinodermata (starfish, sea urchins)	Chordata: Vertebrata (fish, mammals and others)	Chordata: Urochordata (tunicates)
Caspases	7 in <i>Drosophila</i>	+	+	+	+	+
Death receptors/ligands	TNFR-like without DD	-	?	Several genes	Several proteins	?
Bcl-2-like proteins	+	+	+	+	+	+
Apaf-1	+	+	+	+	+	not detected
NOD-like proteins, non-Apaf	+	+	?	+	+	?
IAPs or BIR-containing proteins	Several IAP genes	BIR-containing proteins	Several genes	Several genes	Several IAP genes	BIR-containing proteins

Caspases are the proteases responsible for cell death and they exist in all animals studied. Death receptors and their ligands activate the extrinsic apoptotic pathway in vertebrates and they are involved in immune responses in insects and mammals. Bcl-2 family proteins regulate apoptosis in vertebrates and *C.elegans*, but their role in other organisms is unclear. IAPs (Inhibitors of apoptosis proteins) inhibit apoptosis in *Drosophila* and mammals. BIR-containing proteins are homologs of IAPs which contain only the BIR domains present in IAPs, but not other domains. These proteins do not necessarily play roles in apoptosis.

we can conclude that apoptotic pathways in animals from three different animal phyla are similar, but they differ in the form in which initiator caspases are activated. In *Drosophila* and *C.elegans*, apoptosomes are held constitutively inactive due to the presence of an inhibitor and they are activated upon the synthesis of a molecule that neutralizes this inhibitor. In *Drosophila*, this inhibitor is a caspase inhibitor (DIAP1). In *C.elegans*, the inhibitor is a Bcl-2 family protein, Ced-9. On the contrary, in mammals, the apoptosome is formed upon the presence of an activator. This activator is cytochrome c, which is a molecule which sits in the mitochondria in healthy cells and during apoptosis it is released to the cytosol where it activates the apoptosome (Fig. 2).

### CELL SUICIDE: MITOCHONDRIA AND BCL-2 FAMILY PROTEINS REGULATE “SELF-INDUCED” CELL DEATH IN MAMMALS

In vertebrates, including Zebrafish and mammals, cell death is controlled by a group of proteins that belong to the family of the oncogene Bcl-2.<sup>5</sup> As discussed above, in *C.elegans*, a Bcl-2 family protein (Ced-9) controls apoptosis in a direct manner by holding

the apoptosome-forming protein Ced-4 inactive. In vertebrates the situation is different. Bcl-2 proteins control caspase activation indirectly, through controlling mitochondrial permeabilization. Mitochondria are organelles which provide energy and metabolites to the cell. But these organelles also participate in apoptosis in several organisms, because they contain proteins which can activate caspases when the outer membrane is permeabilized. As discussed above, apoptosis is initiated upon the formation of the apoptosome, a cytosolic complex formed by multimers of Apaf-1, similar to the complex which initiates apoptosis in flies and nematodes.<sup>6</sup> This complex is formed by seven molecules of Apaf-1 which recruit and activate Caspase-9 (Fig. 2). The stimulus which triggers the formation of the mammalian apoptosome is the binding of cytochrome c to Apaf-1. The discovery of the requirement of cytochrome c for apoptosis was surprising, since this is a protein of the mitochondrial respiratory chain. Cytochrome c is normally playing a role in cell metabolism, inside the mitochondria. If so, how does cytochrome c locate to the cytosol during apoptosis? A number of models have been proposed, including the formation of putative channels that would provoke mitochondrial swelling, leading to rupture of the outer mitochondrial membrane. However, the current model implies that two proteins of the Bcl-2 family, Bax and Bak, integrate in the mitochondria during apoptosis and form pores that allow the passage of intermembrane space proteins, including cytochrome c.<sup>7</sup> Bax and Bak are required and perhaps sufficient to form pores in the outer mitochondrial membrane. These proteins, when activated, can form pores in liposomes.<sup>8</sup>

Bax and Bak are part of the Bcl-2 family of proteins. This family is divided in three groups. The first group is comprised of homologs of the mammalian protein Bcl-2 which have an antiapoptotic function, including Bcl-2 itself. Members of this family such as Mcl-1 and Bcl-xL, like Bcl-2, are overexpressed in many human tumors and protect tumor cells from apoptosis. The proapoptotic proteins Bax and Bak are part of the second subset of Bcl-2 family proteins: these proteins are called 'multidomain' proapoptotic Bcl-2 proteins because they share a number of domains with the antiapoptotic Bcl-2 homologs. In fact, the tridimensional structure of the anti-apoptotic and the pro-apoptotic multidomain Bcl-2 proteins is remarkably similar, although they have opposite functions. Bax and Bak form pores in the mitochondrial membranes, Bcl-2, Bcl-xL and Mcl-1 inhibit the formation of these pores and the release of cytochrome c.

The third group of Bcl-2 family proteins comprises pro-apoptotic proteins such as Bid, Bim, Bad, Puma and Noxa. These proteins are less similar to Bcl-2 than Bax and Bak: they only share the BH3 domain, which is a short motive of around 25 amino acids. For these reason, these proteins are called 'BH3-only' proteins. The BH3 domain is required for these proteins to bind to the multidomain Bcl-2 proteins and exert their proapoptotic function. BH3 proteins can directly bind and activate Bax and Bak, while they also inactivate antiapoptotic Bcl-2 proteins. Apoptosis is initiated when a sufficient number of BH3-only molecules inactivates the antiapoptotic proteins and activates the proapoptotic ones.<sup>9</sup> Some BH3-only proteins are specifically induced after some types of stress. For instance, Puma is induced in a p53-dependent manner after genotoxic stress and Bim is induced after endoplasmic reticulum stress. Other BH3-only proteins are constantly present in some cell types but they are kept inactive through posttranslational mechanisms such as phosphorylation, and certain stresses activate them through removal of these modifications. This way, BH3-only proteins function as 'stress sensors' that determine whether a cell will die.

Social signals such as growth factor limitation or loss of cell-to-cell contact also induce apoptosis through activation of BH3-only proteins and induction of the mitochondrial

pathway. As discussed above, in multicellular organisms, cell death contributes to maintenance of tissue homeostasis. When cells lack proliferation factors due, for instance, to tissue overgrowth, they stop proliferating and in many cases, they die by apoptosis. This is critical for the maintenance of cell numbers in tissues with high cell turnover, particularly the immune system. Some immune cells are “addicted” to cytokines and when they are deprived of cytokines they stop growing and undergo apoptosis mediated by the activation of BH3-only proteins.<sup>10</sup> Indeed, mutations of several components of the mitochondrial pathway produce a number of phenotypes related with hyperproliferation of immune cells in mice and mutations in BH3-only proteins are associated with human immune diseases.<sup>11</sup>

### IS THERE A ROLE OF THE MITOCHONDRIA IN APOPTOSIS OF INVERTEBRATES?

Apoptotic pathways are fairly similar in nematodes, flies and vertebrates. Caspases are required for apoptosis in all animals studied and so are their activators (Apaf-1-like molecules). In mammals, the mitochondrial pathway is of great relevance, because it is activated in response to multiple stimuli. Mitochondria (and cytochrome c) are required for apoptosome-mediated caspase activation in response to a diversity of stimuli, including developmental cues, growth factor withdrawal, heat shock, nutrient deprivation, endoplasmic reticulum stress and DNA damage. As discussed above, during apoptosis mitochondria release cytochrome c upon formation of pores composed of Bax and/or Bak. Cytochrome c then binds Apaf-1 and the subsequent conformational change of Apaf-1 attracts Caspase-9, leading to the formation of the apoptosome.

But when did this connection between mitochondria and apoptosis arise? A role for cytochrome c has not yet been found in animals other than mammals. Our other two main animal models to study apoptosis, *C. elegans* and *D. melanogaster*, do not seem to require neither homologs of Bax/Bak proteins acting on mitochondria, nor mitochondrial permeabilization or the release of cytochrome c. In *C. elegans*, the Bcl-2 homolog Ced-9, which is a mitochondrial protein, controls apoptosis by keeping the Apaf-1 homolog Ced-4 inactive and not by releasing cytochrome c. Indeed, cytochrome c could not possibly activate Ced-4 in the same manner in which cytochrome c activates Apaf-1, because Ced-4 does not contain the domain which is responsible for Apaf-1 binding to cytochrome c: the WD40 domain. It is intriguing, though, that *C. elegans* Ced-9, which controls apoptosis, is a mitochondrial protein, like mammalian Bcl-2 proteins. This suggests an ancestral link between mitochondrial permeabilization and apoptosis that may have not been maintained in nematodes.<sup>12</sup>

*Drosophila* apoptosome does not require cytochrome c either. The Apaf-1 homolog, ARK, does contain the WD repeats which are responsible for the interaction of Apaf-1 with cytochrome c. It is possible that during evolution of insects, an ancestral role for cytochrome c was lost in favor of a more direct way to control apoptosis: the synthesis of DIAP inhibitors. We definitively need to expand our knowledge of how apoptosis proceeds in other metazoan phyla in order to understand how the mitochondria-caspase connection arose. It is possible that it appeared early during evolution and that it was lost in nematodes and insects, which are relatively close groups in evolutionary terms. These organisms would have simplified the apoptotic pathways by eliminating the mitochondrial control, reducing this way the number of molecules implicated in induction of apoptosis.

This hypothesis is supported by the fact that cnidarians and equinoderms contain numerous Apaf-1 homologs, some of which can potentially interact with cytochrome c through their WD repeats.<sup>3</sup> However, the alternative possibility, that mitochondrial control of apoptosis appeared during evolution of vertebrates, cannot be discarded at this point.

### CELL DEATH BY SUICIDE INDUCTION: THE DEATH RECEPTOR (EXTRINSIC) APOPTOTIC PATHWAY

In vertebrates, the mitochondrial pathway is responsible for induction of apoptosis upon stress, or upon loss of survival signals such as cytokines or growth factors. As discussed, this pathway is regulated by Bcl-2 family proteins and it is mediated by cytochrome c release from mitochondria, apoptosome formation and activation of caspase-9, which is the apical or initiator caspase in this pathway. While the mitochondrial pathway is of vital importance for tissue homeostasis and stress responses, there is a second apoptotic pathway with important roles in immune response and tumorigenesis: the extrinsic pathway.

The extrinsic or death receptor-mediated pathway is induced upon activation of receptors related to Tumor Necrosis Factor (TNF) receptor. TNF- $\alpha$  is a cytokine which participates in immune responses through the activation of NF-kappaB. But under some circumstances, the outcome of stimulating a cell with TNF is death of the cell instead of NF-kappaB activation. TNF- $\alpha$  and its receptors belong to a family of proteins (the TNF superfamily) which comprises a few dozen proteins with roles in inflammation and immunity. A subset of the TNF-related proteins can induce cell death and are named death ligands. These proteins are TNF- $\alpha$ , Fas ligand (CD95L) and TRAIL (TNF-related apoptosis-inducing ligand). Death ligands are secreted proteins which behave as cytokines that regulate inflammation and other immune-related processes. They can also be expressed in a membrane-bound form by lymphocytes and Natural Killer cells, which use them to kill infected or antigenic tumor cells through the induction of apoptosis in their targets.

The extrinsic pathway is very similar in mammals and fish.<sup>13</sup> This form of apoptosis requires a few molecules that couple the signal from the ligand to caspase activation (Fig. 2). Death ligands induce oligomerization and conformational change of their receptors, the so-called 'death receptors'. These receptors contain in the intracellular portion a 'Death Domain' (DD), which is evolutionarily related to the CARD domain present in caspase-9 and other caspases. Upon oligomerization, the receptor recruits through a homotypic interaction an adapter molecule that also contains a Death Domain. In most models, this molecule is FADD. The other portion of FADD comprises a Death Effector Domain (DED). This domain, which is also structurally and evolutionarily related to CARD domains, is present in the initiator caspase-8. When FADD aggregates in death receptor complexes, caspase-8 is recruited to the complex through homotypic DED-DED interactions, in a manner that resembles CARD-CARD interactions to form the apoptosome. The complex that contains the death receptor, FADD and caspase-8 is called the Death Receptor Signaling Complex (DISC).

The activation of caspase-8 at the DISC is not sufficient per se to kill the cell. This protease, upon activation, cleaves and activates effector caspases such as caspase-3 and this leads to death of the cell. In some cell lines, however, activation of caspase-8 is not sufficient to activate caspase-3 directly. In these cells, caspase-8 needs an amplification signal to induce effector caspase activation: it cleaves the BH3-only protein Bid. Bid then acts on Bax and/or Bak on the mitochondrial membrane to trigger the release of

cytochrome c and the formation of the apoptosome. For this reason, in some cell types Bcl-2 or Bcl-xL overexpression blocks death receptor-induced cell death.<sup>14</sup>

This apoptotic pathway seems to only be present in vertebrates. No death receptors have been found in *C. elegans*. In *Drosophila*, the TNF-receptor homolog, Wengen, can induce cell death, but as we will discuss later, this form of cell death is not classical apoptosis and it does not require caspase-8.<sup>15</sup>

## CELL DEATH BY MURDER: THE GRANZYME PATHWAY

Cytotoxic lymphocytes (CTL) and Natural Killer (NK) cells owe their names to their ability to induce death of their target cells. In order to avoid propagation of a virus, cytotoxic cells attack and kill the infected cells and they use several effector mechanisms in order to do so. One mechanism is the induction of apoptosis in the target cell through the extrinsic pathway. CTL and NK cells can express Fas Ligand in their surface. This death ligand, as discussed above, can activate the death receptor Fas in the target cell and thus induce its suicide through caspase-8 activation. Moreover, cytotoxic cells deliver the content of toxic granules towards the target cell. These granules contain, among other components, TNF, which in a manner similar to Fas Ligand can interact with its receptor, leading to activation of the TNF-receptor mediated extrinsic pathway and subsequent caspase-8-mediated death. Additionally, granules contain perforin, a protein that forms transmembrane channels and facilitates intracellular delivery of the protease Granzyme B, another component of cytotoxic granules.

Granzyme B can induce apoptosis through directly activating caspases.<sup>16</sup> This protease is able to activate caspases through its proteolysis, in a similar manner than initiator caspases activate other caspases. Several caspases have shown to be activated after treatment with a combination of Granzyme B and perforin, including caspase-8 and the effector caspases -3 and -7. Cleavage and activation of caspase-3 can lead directly to 'classical' apoptosis. However, Granzyme B can also use a mitochondrial pathway to induce apoptosis, by cleaving the BH3-only protein Bid (Fig. 2). Bid cleavage activates the Bax/Bak mediated mitochondrial apoptotic pathway, with subsequent activation of the apoptosome and the caspase-9 mediated pathway. Granzyme B-mediated killing can be inhibited by overexpression of Bcl-2 in human cells, indicating that the mitochondrial pathway is more relevant than direct activation of effector caspases.

## APOPTOSIS IS NOT THE ONLY WAY TO DIE: NON-APOPTOTIC FORMS OF PROGRAMMED CELL DEATH IN METAZOANS

Apoptosis is the main form of cell death in animals. However, it is not the only form of programmed cell death which has been observed in animals. Cells can die by pathways that do not involve caspase activation and the morphology of dying cells can be completely different from the classical morphology of apoptotic cells.<sup>17</sup> It should be noted that there is still a good level of disagreement regarding the definition and the physiological roles of some of these alternative forms of cell death. Traditionally, before we had been able to start the molecular characterization of apoptosis (which started in the nineties), cell death had been classified in three different types according to morphology: apoptotic (Type I), autophagic (Type II) and necrotic (Type III). This classification was

based on microscopic analysis of developing animals and has long been abandoned by researchers in the field. After the discovery of the molecular pathways of apoptosis, we could characterize apoptosis more precisely than if we regarded morphology only. We now define apoptosis as caspase-dependent cell death, which is usually accompanied by morphological changes which are consequences of caspase activation, including chromatin condensation and cell shrinkage.

After the beginning of the molecular characterization of apoptosis, for many years, other forms of cell death used to be classified by default as ‘necrosis’, or accidental, nonregulated death. However, in recent years, it has been acknowledged that non-apoptotic cell death can occur in a regulated fashion and some molecules that regulate non-apoptotic cell death have been identified. At present, we still use the term ‘necrotic cell death’ to define death which proceeds without signs of apoptosis. But this includes true “accidental”, sudden, uncontrolled cell death (for instance, after severe heat shock, ischemia or mechanical rupture) but also other forms of non-apoptotic cell death such as necroptosis, which is regulated by a number of proteins which we are beginning to identify (Table 2).

Necroptosis (or death receptor-induced necrosis) is a form of cell death induced by death ligands and mediated by the protein RIP1.<sup>18,19</sup> RIP (RIPK1) is a kinase which associates with death receptor/NF-kappaB-activating complexes. As discussed above, activation of death receptors usually kills cells through caspase-8 activation and the extrinsic pathway. However, in some cell types, ligation of death receptors—in particular, TNF-receptors—induces a form of cell death which is not mediated by caspases. Not only cells die in a caspase-independent manner, but indeed, caspase inhibition can enhance necroptotic cell death. Necroptosis is mediated by production of Reactive Oxygen Species (ROS) and by the JNK kinase. This form of cell death, although only described in mammals so far, resembles the way by which *Drosophila* TNF kills cells: in *Drosophila*, overexpression of Wengen (the TNF receptor homolog) or Eiger (its ligand) kill in an ROS and JNK-dependent manner, independently of caspase-8.<sup>15</sup> The involvement of the apoptosome and caspases in Eiger-induced cell death is unclear, since several reports show contradictory results. The resemblance between Eiger-induced cell death and necroptosis is strengthened by the fact that a similar group of proteins (such as RIP1, JNK, CYLD and TRAFs) regulate necroptosis in mammals, as well as Eiger-induced cell death in *Drosophila*.<sup>20</sup> This suggests a possible conservation of a non-apoptotic cell death pathway which role seems to be related to immune defense, since necroptosis has been shown to occur after treatment of virally infected cells with TNF and upon ligation of Toll-like receptors.

‘Autophagic cell death’ is a form of cell death observed during development of salivary glands in *Drosophila*.<sup>21</sup> By morphological criteria, this form of cell death is different from apoptosis, since it is associated with massive vacuolization due to the presence of a great number of autophagic vesicles. These vesicles are doubled membrane vesicles which engulf organelles or cytoplasm to target them to lysosomes for degradation. Autophagic cell death is induced in salivary glands or the midgut of *Drosophila* upon developmental cues. Although this form of cell death has been shown to exist in the fly, an enormous debate has been generated in the past few years regarding whether autophagic cell death exists in mammals. In general, autophagy is a cytoprotective process. In some cases, autophagic vesicles are observed in cells that undergo apoptosis and in other cases, autophagic proteins have been shown to promote caspase activation and apoptosis. This had lead to the erroneous conclusion that cell death under some circumstances is “autophagic”. To complicate the issue, ‘autophagic cell death’ has been

**Table 2.** Types of Programmed Cell Death

Type of Programmed Cell Death	Organism	Function	Key Molecules	Morphological Characteristics
Apoptosis—extrinsic pathway	Vertebrates	Immune homeostasis	Death receptors, death ligands, caspases	Chromatin condensation, cell shrinkage, phosphatidylserine exposure, DNA degradation
Apoptosis— intrinsic/ mitochondrial pathway	Vertebrates, <i>C. elegans</i>	Development, elimination of damaged cells	Bcl-2 family proteins, caspases	Same as above
Apoptosis	<i>Drosophila</i>	Development	DIAP1, caspases	Same as above
Necroptosis	Mammals	Possibly, immune defense	RIPK1, ROS, JNK	Plasma membrane rupture
Necrosis	All organisms	Probably, no function	unknown	Cytoplasmic swelling, plasma membrane rupture
Hypersensitive Response	Plants	Immune response	ROS, proteases, resistance (R) gene products	Large cytoplasmic vesicles, release of hydrolytic enzymes into the cytoplasm
Viral, aging or stress-induced cell death	Yeast	Possibly, main- tenance of fittest cells	ROS, prote- ases	Chromatin condensation, shrinkage
Autophagic cell death	<i>Dictiostelium</i>	Development	Autophagy genes	Massive vacuolization of the cytoplasm
Autophagic cell death	<i>Drosophila</i>	Organ involution during development	Autophagy (Atg) genes, caspases	Massive vacuolization of the cytoplasm (autophagic vesicles)

described in mammalian cells in culture upon treatment with caspase inhibitors and it has been shown to be mediated by JNK and RIP1; all of these are features of necroptosis. This suggests that autophagic cell death and necroptosis are the same form of cell death. The involvement of autophagy—but not caspases—in a physiologically relevant cell death process in mammals remains to be proven.

A process involving programmed cell death which we are all familiar with is death of skin cells. The skin is constantly being renewed and this renovation involves death of cells in outer layers of the skin. These cells do not die by apoptosis, but by a process called ‘cornification’ or ‘keratinization’ of the outer layers of the epidermis. Keratinization involves caspases such as caspase-14 which do not play roles in apoptosis. Besides

keratinization, there is another form of cell death which involves non-apoptotic caspases. Pyroptosis requires the activation of caspase-1, which is an inflammatory caspase that does not play a role in apoptosis. Pyroptosis, also called ‘caspase-1 dependent necrosis’, has been observed in macrophages upon infection or treatment with lipopolysaccharide. This form of death does not require effector caspases. Dying cells display mixed morphological features of apoptosis and necrosis. Because this form of death occurs upon infection and it is pro-inflammatory, it is likely that the purpose of this form of cell death is to awaken the immune system.

## CELL DEATH IN PLANTS, FUNGI AND PROTISTS

Apoptosis is not the only form of cell death in animals, which indicates that a number of alternative cell death programs exist in nature. Programmed cell death has been observed during development or in response to infection in a number of organisms (Table 2).

In plants, cell death is part of the normal cycle of the organism. Developmental cell death is observed in many plant tissues, being crucial for instance for senescence of leaves and flowers, formation of pollen and seeds and terminal differentiation of vascular elements. Moreover, plant cells undergo Programmed Cell Death in response to infection. When attacked by a pathogen, plants engage in a form of immune response which involves death of the tissue which surrounds the infected site. This phenomenon was named the ‘hypersensitive response’ because the plants which could respond and resist a pathogenic infection seemed to be “hypersensitive”, in the sense that abnormal cell death was observed in the sites of infection.<sup>22</sup> Cell death is mediated by early massive production of Reactive Oxygen Species and by proteases. The morphology of dying cells in these tissues shares some characteristics of apoptosis such as chromatin condensation and protoplast retraction (shrinkage). However, the morphology of dying plant cells is in general more similar to necrosis of mammalian cells, with massive vacuolization. Intriguingly, caspase-like protease activities have been readily detected during several forms of cell death in plants and cell death can frequently be attenuated by the use of peptidic inhibitors of mammalian caspases. This has led to the proposal that cell death in plants can occur by apoptosis. Since there are no caspases in plants, it was proposed that their distant relatives, metacaspases, participate in apoptotic-like cell death of plants and unicellular organisms, but this is still under debate. Metacaspases differ from caspases in their cleavage specificity and probably in their physiological substrates.<sup>23</sup> Moreover, it is unclear why plants would undergo apoptotic-like cell death, whose main purpose is, in opposition to other forms of cell death, to eliminate cells in a “clean” manner without rupture of the plasma membrane and without inflammation. In animals, apoptotic bodies are rapidly eliminated by phagocytosis. However, the cell wall in plant cells would preclude any possible phagocytosis of dying cells.

Yeast are unicellular fungi. At first sight, the existence of programmed cell death in unicellular organisms does not make much sense. However, yeast undergo a form of programmed cell death which resembles apoptosis. A number of stress-inducing agents such as UV radiation, acetic acid or antimicrobial peptides trigger apoptotic-like cell death which can be inhibited by inactivation of certain genes, including the yeast metacaspase Yca1. Moreover, apoptotic-like cell death has been shown to occur as a consequence of chronological aging. The “age” of the individual is determined, in budding yeast, by the number of daughter cells that a mother cell produces. In strains which do not undergo

asymmetric divisions, the concept of age is determined by the lifespan of postmitotic cells in culture media which is not replenished. It is believed that programmed cell death in this unicellular organism is beneficial for the colony because it saves limited nutrients for the healthy cells. Additionally, yeast cells infected with certain viruses secrete toxins which can induce necrotic-like or apoptotic-like features in neighboring non-infected cells. It can be hypothesized that programmed cell death contributes to limit the infection by isolating the virus, which would not be able to reproduce in neighboring dying cells. A number of apoptotic markers have been observed in dying yeast, including chromatin condensation, DNA fragmentation and phosphatidyl-serine exposure in the outer membrane, which is a feature of apoptosis which helps macrophages recognize and engulf apoptotic bodies. As in the case of plants, it is unclear why yeast would undergo an apoptotic-like form of programmed cell death, since they cannot phagocytose neighboring cells. And again, as with plants, it remains unclear whether the metacaspase present in *S. cerevisiae* is responsible for the caspase-like protease activities detected during yeast cell death.

Human unicellular eukaryotic parasites have also been shown to undergo cell death with apoptotic features.<sup>24</sup> Cell death has been extensively studied in the flagellated parasites *Trypanosoma* and *Leishmania* and it is frequently associated with production of Reactive Oxygen Species. The genes responsible for death, as well as the role in vivo of cell death still need characterization; for this reason, it might be a bit premature to assert that these organisms undergo programmed cell death. Cell death can frequently be inhibited by peptidic caspase inhibitors, but it has been shown in many cases that the metacaspases present in these organisms do not play a role in cell death in protozoans. As discussed, metacaspases have different substrate affinity than caspases and their physiological role in trypanosomes and *Leishmania* is linked to cell cycle progression.<sup>25</sup> The putative role of a programmed form of cell death in unicellular parasites is unclear. Dying parasites have been detected inside infected macrophages and in the midgut of the insect vector which transmits the parasite to humans. It has been suggested that programmed cell death would contribute to eliminate the least fit (less infective) organisms in the insect. It could also contribute to limit parasite infection intensity in the human host, in order to reduce the risk of death of the host cell, which could boost an immune response.

*Dictyostelium discoideum*, or slime mold, is one of the non-animal organisms in which programmed cell death has been studied in more detail. *Dictyostelium* belongs to a group of eukaryotes named Amoebozoa. This organism is particularly interesting from an evolutionary point of view because it transitions from a unicellular to a pluricellular stage during its life cycle. Upon starvation, *Dictyostelium* individuals aggregate and form a fruiting body: a fungus-like structure with a stalk composed of dead cells. Massive autophagy is detected in dying cells and death has been shown to depend on autophagic genes but not on caspase-like activity. *Dictyostelium* is thus, with *Drosophila* and possibly some plant tissues, another organism whose cells undergo autophagic cell death during development.<sup>26</sup>

We can conclude that there are multiple programmed cell death programs in nature. Interestingly, cell death in some non-animal organisms is accompanied by features which resemble animal apoptosis. This suggests that an apoptotic-like program may have originated in primitive unicellular eukaryotes.

## HOST DEFENSE AND THE ORIGINS OF APOPTOSIS. PATHOGEN-SENSING COMPLEXES AND APOPTOSOMES ARE STRUCTURALLY SIMILAR

Apoptosis serves three main purposes in animals. The first one is the removal of cells which are no longer necessary, such as cells which played a role during development but are not needed in the adult, or immune cells after the pathogen has been cleared. The second purpose is the elimination of damaged cells, which may not be able to perform their function properly, or are potentially dangerous to the organism (for instance, cells with DNA damage which may lead to mutations). The third main function is the elimination of infected cells. Finding out which of these functions is older in evolutionary terms may help elucidate the origins of cell death. So, which one was the most ancestral function of apoptosis? Several pieces of evidence suggest that apoptosis originated as a means to eliminate infected cells and that this mechanism could have arisen in primitive organisms such as unicellular precursors of metazoans.

In 2002, James and Green<sup>27</sup> proposed a theory to explain how a suicidal program could have originated in unicellular organisms: this would have occurred in the context of infection and self defense. The origins of a suicidal program in single-celled organisms are hard to understand, because the cells that acquired the ability to kill themselves would likely have a disadvantage and the emergence of “cheaters” with mutant genes would impair the maintenance of such a program. However, the emergence of such a process could be explained in the context of an infection. We can envision the situation as follows. In certain unicellular organisms, a program to detect a parasite engaged proteases, perhaps as a means to degrade pathogens upon infection. At certain point during evolution, these proteases acquired the ability to kill the infected cell upon detection of the pathogen. Engagement in such a self-killing program would be beneficial for the kin or group of genetically identical organisms, because this would limit the spread of the infection. And if the infected cell “cheated” or it contained a mutated version of the killer gene, it would die anyway from the infection. Therefore, in the long term, the acquisition of this suicidal program would be beneficial for the colony. Moreover, this program would be maintained in multicellular organisms, in which the machinery could be “recycled” and used for more diverse functions such as body sculpture. Let’s review some of the multiple links between the apoptotic machinery and immune responses which offer support to this hypothesis.

Perhaps the best piece of evidence in favor of an origin of apoptosis in the context of host defense is the fact that apoptosis-initiating complexes such as the apoptosome and immunity-activating complexes such as the inflammasomes are very similar to each other. Apaf-1, the nucleating component of the apoptosome, is very similar to a family of proteins that play immune roles: the NOD-like receptors. These proteins recognize intracellular pathogens and activate immune responses. Upon recognition of a pathogen or a danger signal, NOD-like receptors oligomerize and nucleate multiprotein complexes which then recruit caspase-1, which is a non-apoptotic caspase responsible for formation of the mature form of the cytokine interleukin-1. The function of inflammasomes is to promote NF-kappaB activation and/or formation of the proinflammatory cytokine interleukin-1.

NOD-like receptors are grouped in this family due to the presence of a conserved nucleotide-binding domain in the central regions of the molecule: a NOD domain, which is also present in Apaf-1. This domain is responsible for oligomerization of the molecule

and it is essential for the formation of oligomeric complexes such as the inflammasomes and apoptosomes. Apart from the NOD domain, the other portions of NOD-like receptors contain a variety of different domains which include CARDs and Death Domains, which are domains present in apoptotic proteins. The structure of some NOD-like receptors resembles enormously the structure of Apaf-1, which contains a c-terminal WD40 domain (involved in the binding to cytochrome c), a NOD domain and an n-terminal CARD domain, responsible for recruitment of caspase-9 to the apoptosome.

In humans we find over a dozen of NOD-containing proteins, most of which contain a Leucine Rich Repeat in the carboxyl termini, which is a ligand-recognition domain.<sup>28</sup> Apaf-1 differs from some human NOD-containing proteins because it contains the cytochrome c-binding WD40 domain instead of the Leucine Rich Repeat. This suggests an intriguing possibility. Is it possible that Apaf-1, like other NOD-containing proteins, was originally a molecule that recognized a bacterial component and triggered an immune response? This component would have been cytochrome c, which was a protein present in the –first pathogenic, later endosymbiotic- bacteria. Recent phylogenetic analyses offer more pieces of evidence that point towards an immune origin of the Apaf-1-like proteins: some Apaf-1 homologs in cnidarians contain TIR domains. These domains are found in Toll-like receptors, which are proteins involved in recognition of extracellular pathogens.<sup>3</sup> Moreover, the NOD domain in Apaf-1 is strikingly homologous to the NOD domain present in certain plant proteins which are involved in the Hypersensitive Response. These proteins, the R gene products, have Apaf-1 like NOD domains and also leucine rich repeats like mammalian NOD-like receptors. R gene products, upon pathogen detection, trigger an immune response that involves cell death, as discussed above. Given the similarity between all these proteins, it is very likely that Apaf-1 molecules were originally pathogen sensors.

As proposed by James and Green,<sup>27</sup> molecules present in the surface of the mitochondrial endosymbiont such as cytochrome c may have originally triggered an immune response in the unicellular host, which was later suppressed when the association became mutually beneficial. Then, a remnant of this immune response was maintained in animals. At a certain point the cell “learnt” to control cytochrome c release, which perhaps became a signal associated with mitochondrial damage. This would then trigger a caspase cascade that would lead to cell death instead of an immune response. Cytochrome c release then became a central point of control of what became an essential mechanism to eliminate unnecessary cells: apoptosis.

## **NON-APOPTOTIC FUNCTIONS OF APOPTOTIC PROTEINS ARE RELATED TO IMMUNITY**

As discussed above, there are four main groups of apoptotic proteins: caspases, Bcl-2 proteins, IAPs and Apaf-1- or FADD-like adapter molecules. Many of these proteins play roles in the immune response. Among the caspases, both the apoptotic caspases and the inflammatory caspases play roles in the immune system. The group of inflammatory caspases comprises caspase-1, -4, -5 and -12. These caspases are activated in inflammasomes, which are very similar to apoptosomes, as described earlier. They are activated when a pathogen or a danger signal is detected. Their proteolytic activity is linked to production of awareness signals to alert the immune system. However, these are not the only caspases with roles in immunity. Caspase-8, the apical caspase in the

apoptotic death receptor pathway, is a bona fide apoptotic caspases which also plays roles in proliferation and immunity. As discussed above, pro-apoptotic activation of caspase-8 usually occurs in response to a death ligand such as TNF. In most cells, however, the response to TNF- $\alpha$  (and to a lesser degree, to other death ligands) is not the induction of cell death but the activation of NF- $\kappa$ B, which occurs in a caspase-8-dependent manner. Caspase-8 is also required for proliferation of lymphocytes and activation of NF- $\kappa$ B after ligation of the T-cell receptor.<sup>29</sup>

In *Drosophila*, caspase-8 is not an apoptotic caspase. In flies we do not find an apoptotic pathway similar to the mammalian extrinsic pathway, in which caspase-8 is the initiator caspase. The main role of the fly caspase-8 homolog Dredd seems to be the activation of the immune response against gram-negative bacteria, which is mediated through the Immune Deficiency (IMD) signaling cascade.<sup>30</sup> IMD is a protein which is activated after infection. Similar to death receptors, IMD contains a Death Domain and it presumably forms a DISC-like complex. IMD recruits *Drosophila* FADD (dFADD) and this leads to the activation of Dredd. The IMD pathway is thus very similar to the mammalian TNF- $\alpha$  signaling pathway as it involves *Drosophila* Caspase-8 (Dredd) and the FADD homolog. Similarly to what occurs in the human non-apoptotic TNF signaling pathway, the IMD pathway promotes the activation of NF- $\kappa$ B and this requires the caspase Dredd. The IMD pathway thus presents similarities with the TNF/apoptotic extrinsic pathway but also with the inflammasome, since it is executed through interaction between caspases and adapter molecules in a complex with proteins that recognize pathogens. Thus, both caspases and the adapter protein FADD, which are apoptotic in humans, play roles in the immune response both in humans and insects.

IAPs (Inhibitors of Apoptosis Proteins) were originally identified by virtue of their homology with a protein from a baculovirus which inhibits caspases. The well studied IAP from *Drosophila* DIAP1 is a caspase inhibitor which is critical for regulation of apoptosis during embryonic development. Its downregulation is enough to activate the initiator caspase DRONC and trigger apoptosis. In mammals, however, loss of IAPs per se usually does not lead to caspase activation and cell death. The main role of IAPs is related to immunity and activation of NF- $\kappa$ B. It has recently been shown that only one of the eight IAP proteins present in humans, XIAP, is a direct caspase inhibitor. cIAP1 and cIAP2 are evolutionarily related to XIAP and they inhibit apoptosis, although they do not inhibit caspase activity. These proteins have a similar structure to XIAP but they lack aminoacid residues involved in direct caspase activation, which suggests two possible scenarios: either some of these proteins lost the capability to directly inhibit caspases, or this function was acquired late during evolution.<sup>31</sup> If this second scenario was true, what were their other, more ancient functions? In human cells, cIAPs participate in TNF receptor signaling. They are recruited to the DISC through indirect interactions with TNF receptors and they participate in TNF-mediated induction of NF- $\kappa$ B. cIAPs and paradoxically, also the sudden loss of cIAPs through chemical inhibition, activate both canonical and noncanonical NF- $\kappa$ B activation pathways.<sup>32</sup>

Activation of NF- $\kappa$ B seems to be a conserved function of IAP proteins even in invertebrates. DIAP1 is essential for apoptosis in *Drosophila*. It was recently acknowledged that another member of the IAP family, *Drosophila* IAP2, unlike DIAP1, is dispensable for development of the fly but it is necessary for the innate immune response against gram-negative bacteria. DIAP2 mediates NF- $\kappa$ B activation in the IMD pathway.<sup>33</sup> As mentioned before, the IMD pathway is activated in response to bacteria and it may involve the formation of a DISC-like complex with *Drosophila* FADD and Caspase-8 homologs.

NAIP is another human IAP protein which does not play a role in apoptosis: it participates in the formation of the inflammasome in response to intracellular bacteria.<sup>34</sup> Lastly, mammalian XIAP, which is a *bona fide* caspase inhibitor, has also been shown to participate in NF-kappaB signaling after the detection of intracellular pathogens. Thus, IAPs and caspase-8, like other caspases and NOD-containing proteins, play major roles in immune signaling.

## CONCLUSION AND PERSPECTIVES

Cell death is vital to life of multicellular organisms and it plays a role in maintenance of homeostasis of populations of unicellular organisms. It is unclear how cell death originated and it is possible that this process evolved independently in several lineages. The diversity of cell death programs described, which differ both in morphology of the dying cell and in the biochemical pathways responsible for their execution, suggests that there is not a common origin of all cell death programs. However, pathogen-recognition pathways are linked to cell death at least in animals and plants, which suggest that the origins of at least some forms of cell death are related to the ability of an organism to kill its own infected cells.

Apoptotic proteins have several alternative roles besides cell death. Some evidence suggests that these alternative roles were actually the ancestral functions of these proteins and that the currently 'apoptotic' proteins got recruited to the apoptotic machinery at different points during evolution. The exploration of these alternative roles would probably yield important information to solve the puzzle of the evolutionary origins of cell death.

Many questions remain to be solved. For instance, which are the most ancestral functions of caspases? Caspases do not only participate in immune function, but they also play roles in cell proliferation and differentiation.<sup>35</sup> What are the roles of metacaspases? The proteases implicated in cell death of non-animal organisms need to be identified. More intriguing is the issue of when Bcl-2 family proteins got recruited to the apoptotic machinery. Despite extensive searches, no Bcl-2 homolog has been found outside the animal kingdom. These proteins seem to have other conserved roles besides regulation of apoptosis; amongst these, the most relevant seem to be the regulation of mitochondrial dynamics and calcium homeostasis.<sup>36,37</sup> Moreover, the role of these proteins in cell death has only been proven so far in *C. elegans* and vertebrates. Much work needs to be done in animals from other animal phyla to determine the ancestral role of these proteins, which are extremely relevant in human pathologies such as cancer. Maybe one day we will be able to explain how cell suicide, the most altruistic behavior possible, arose and was maintained during evolution.

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## REFERENCES

1. Taylor RC, Cullen SP, Martin SJ. Apoptosis: controlled demolition at the cellular level. *Nat Rev Mol Cell Biol* 2008; 9:231.
2. Lettre G, Hengartner MO. Developmental apoptosis in *C. elegans*: a complex CEDnario. *Nat Rev Mol Cell Biol* 2006; 7:97.
3. Zmasek CM, Zhang Q, Ye Y et al. Surprising complexity of the ancestral apoptosis network. *Genome Biol* 2007; 8:R226.
4. Koonin EV, Aravind L. Origin and evolution of eukaryotic apoptosis: the bacterial connection. *Cell Death Differ* 2002; 9:394-404.
5. Youle RJ, Strasser A. The BCL-2 protein family: opposing activities that mediate cell death. *Nat Rev Mol Cell Biol* 2008; 9:47.
6. Li P, Nijhawan D, Budihardjo I et al. Cytochrome c and dATP-Dependent Formation of Apaf-1/Caspase-9 Complex Initiates an Apoptotic Protease Cascade. *Cell* 1997; 91:479.
7. Munoz-Pinedo C, Guio-Carrion A, Goldstein JC et al. Different mitochondrial-intermembrane space proteins are released during apoptosis in a manner that is coordinately initiated but can vary in duration. *Proc Natl Acad Sci USA* 2006; 103:11573-11578.
8. Kuwana T, Mackey MR, Perkins G et al. Bid, Bax and lipids cooperate to form supramolecular openings in the outer mitochondrial membrane. *Cell* 2002; 111:331-42.
9. Lomonosova E, Chinnadurai G. BH3-only proteins in apoptosis and beyond: an overview. *Oncogene* 2008; 27:S2.
10. Pinon JD, Labi V, Egle A et al. Bim and Bmf in tissue homeostasis and malignant disease. *Oncogene* 2009; 27:S41.
11. Strasser A. The role of BH3-only proteins in the immune system. *Nat Rev Immunol* 2005; 5:189.
12. Oberst A, Bender C, Green DR. Living with death: the evolution of the mitochondrial pathway of apoptosis in animals. *Cell Death Differ* 2008; 15:1139-46.
13. Eimon PM, Kratz E, Varfolomeev E et al. Delineation of the cell-extrinsic apoptosis pathway in the zebrafish. *Cell Death Differ* 2006; 13:1619.
14. Scaffidi C, Fulda S, Srinivasan A et al. Two CD95 (APO-1/Fas) signaling pathways. *EMBO J* 1998; 17:1675-87.
15. Igaki T, Kanda H, Yamamoto-Goto Y et al. Eiger, a TNF superfamily ligand that triggers the *Drosophila* JNK pathway. *Embo J* 2002; 21:3009-18.
16. Logue SE, Martin SJ. Caspase activation cascades in apoptosis. *Biochem Soc Trans* 2008; 036:1-9.
17. Kroemer G, Galluzzi L, Vandenabeele P et al. Classification of cell death: recommendations of the Nomenclature Committee on Cell Death 2009. *Cell Death Differ* 2008; 16:3.
18. Tait SWG, Green DR. Caspase-independent cell death: leaving the set without the final cut. *Oncogene* 2008; 27:6452.
19. Vercammen D, Beyaert R, Denecker G et al. Inhibition of caspases increases the sensitivity of L929 cells to necrosis mediated by tumor necrosis factor. *J Exp Med* 1998; 187:1477-1485.
20. Hitomi J, Christofferson DE, Ng A et al. Identification of a molecular signaling network that regulates a cellular necrotic cell death pathway. *Cell* 2008; 135:1311.
21. Martin DN, Balgley B, Dutta S et al. Proteomic analysis of steroid-triggered autophagic programmed cell death during *Drosophila* development. *Cell Death Differ* 2007; 14:916.
22. Mur LAJ, Kenton P, Lloyd AJ et al. The hypersensitive response; the centenary is upon us but how much do we know? *J Exp Bot* 2008; 59:501-520.
23. Vercammen D, Declercq W, Vandenabeele P et al. Are metacaspases caspases? *J Cell Biol* 2007; 179:375-380.
24. Deponte M. Programmed cell death in protists. *Biochimica et Biophysica Acta (BBA)—Molecular Cell Research* 2008; 1783:1396.
25. Ambit A, Fasel N, Coombs GH et al. An essential role for the *Leishmania* major metacaspase in cell cycle progression. *Cell Death Differ* 2007; 15:113.
26. Olie RA, Durrieu F, Cornillon S et al. Apparent caspase independence of programmed cell death in *Dictyostelium*. *Current Biology* 1998; 8:955.
27. James ER, Green DR. Infection and the origins of apoptosis. *Cell Death Differ* 2002; 9:355-7.
28. Petrilli V, Dostert C, Muruve DA et al. The inflammasome: a danger sensing complex triggering innate immunity. *Curr Opin Immunol* 2007; 19:615.
29. Su H, Bidere N, Zheng L et al. Requirement for Caspase-8 in NF- $\kappa$ B Activation by Antigen Receptor. *Science* 2005; 307:1465-1468.
30. Georgel P, Naitza S, Kappler C et al. *Drosophila* immune deficiency (IMD) is a death domain protein that activates antibacterial defense and can promote apoptosis. *Dev Cell* 2001; 1:503-14.
31. Eckelman BP, Salvesen GS. The human anti-apoptotic proteins cIAP1 and cIAP2 bind but do not inhibit caspases. *J Biol Chem* 2006; 281:3254-3260.

32. Vince JE, Wong WW-L, Khan N et al. IAP Antagonists Target cIAP1 to Induce TNF[alpha]-Dependent Apoptosis. *Cell* 2007; 131:682.
33. Leulier F, Lhocine N, Lemaitre B et al. The Drosophila inhibitor of apoptosis protein DIAP2 functions in innate immunity and is essential to resist gram-negative bacterial infection. *Mol Cell Biol* 2006; 26:7821-7831.
34. O'Riordan MXD, Bauler LD, Scott FL et al. Inhibitor of apoptosis proteins in eukaryotic evolution and development: a model of thematic conservation. *Dev Cell* 2008; 15:497-508.
35. Lamkanfi M, Festjens N, Declercq W et al. Caspases in cell survival, proliferation and differentiation. *Cell Death Differ* 2007; 14:44-55.
36. Autret A, Martin SJ. Emerging Role for Members of the Bcl-2 Family in Mitochondrial Morphogenesis. *Mol Cell* 2009; 36:355.
37. Hetz C, Glimcher L. The daily job of night killers: alternative roles of the BCL-2 family in organelle physiology. *Trends Cell Biol* 2008; 18(1):38-44..