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Death without caspases, caspases without death

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Apoptosis is a conserved cell-death process displaying characteristic morphological and molecular changes including activation of caspase proteases. Recent work challenges the accepted roles of these proteases. New investigations in mice and the nematode *Caenorhabditis elegans* suggest that there could be caspase-independent pathways leading to cell death. In addition, another type of cell death displaying autophagic features might depend on caspases. Recent studies also indicate that caspase activation does not always lead to cell death and, instead, might be important for cell differentiation. Here, we review recent evidence for both the expanded roles of caspases and the existence of caspase-independent cell-death processes. We suggest that cellular context plays an important role in defining the consequences of caspase activation.

PROGRAMMED CELL DEATH (PCD) (see Glossary) is important for sculpting tissues and destroying harmful cells such as autoreactive immune cells and tumor cells. Excess PCD can be harmful and contributes to various degenerative pathologies [1], whereas lack of PCD can contribute to the development of proliferative disorders such as cancer [2]. Historically, morphological criteria have been used to identify two types of PCD – APOPTOSIS and autophagic cell death. Here, we define apoptosis as a type of PCD that is dependent on the activity of CASPASE proteases (see below) and is accompanied by highly conserved morphological changes including chromatin compaction, membrane blebbing and cell shrinkage (Figure 1). We define autophagic death by the presence of autophagic vesicles within dying cells (see below). Cells can also die in a nonprogrammed way in response to injury by a morphologically distinct yet ill-defined process termed NECROSIS.

Apoptosis has been the most investigated form of PCD, and many proteins involved in this process have been identified. Regulators of apoptosis directly or indirectly control the activities of caspases – cysteine proteases that are key executors of apoptosis, cleaving their substrates following specific aspartate residues. Caspases are synthesized as proproteins, possessing weak proteolytic activity [3,4]. When cleaved at internal aspartate residues, procaspases are converted into mature enzymes with increased activity (Box 1). Three major pathways regulate caspase activity (Figure 2). Certain protein complexes on the cell surface that contain receptors for extracellular ligands can directly activate caspases. Mitochondrial proteins, including members of the BCL-2 family, control caspase activity by regulating CASPASE ACTIVATORS such as the *Caenorhabditis elegans* protein CED-4 or its mammalian homolog Apaf-1 [5]; caspase activators act as scaffolds, thereby allowing cross-activation of adjacent caspase zymogens [6]. Finally, members of the IAP family inhibit apoptosis by binding to and inactivating mature caspases (Figure 2); for detailed reviews of these upstream regulators see [7,8]. The relationship between caspase activation and the morphological changes that accompany apoptosis remains ill defined.

Here, we review evidence suggesting that caspases are key executioners of apoptosis and discuss recent studies indicating that caspases also promote autophagic cell death. In addition, we survey the literature to address two questions: are there caspase-independent forms of PCD,

Glossary

Adaptor Proteins: Proteins such as mammalian FADD that bind to both membrane-bound death receptor proteins, such as Fas, and long prodomain caspases such as Caspase-8.
Apoptosis: A type of programmed cell death (PCD) requiring caspase activity and in which the dying cell shows highly conserved morphological changes, including chromatin compaction, membrane blebbing and cell shrinkage.
Autophagy: A process by which a cell uses its lysosomes to degrade vesicles containing cytoplasm and organelles. Autophagy has been seen in some dying cells such as the salivary-gland cells of *Drosophila* larva; however, it is not clear whether autophagy is a mechanism used to promote cell death.
Bcl-2 (B Cell Lymphoma 2): A conserved anti-apoptotic protein that can be negatively regulated by pro-apoptotic BH3-domain-only proteins.
BH3 (Bcl-2 Homology 3) Domain Only Proteins: Pro-apoptotic proteins found in *C. elegans* and mammals. Are thought to regulate apoptosis by negatively regulating Bcl-2-related proteins.
Caspase: A cysteine protease that cleaves substrates after aspartate residues. Caspase activity is required for apoptosis.
Caspase Activator: Caspases can be activated by other caspases or by proteins such as CED-4 or Apaf-1 that act as scaffolds for caspase binding.
Cavitation: The process by which the pro-amniotic cavity is created when PCD occurs in the ectoderm layer of a mouse embryo.
IAP (Inhibitor Of Apoptosis): An anti-apoptotic protein found in *Drosophila* and mammals. IAP can inhibit apoptosis by binding to and inhibiting caspases.
Necrosis: A type of cell death that is not programmed during development or homeostasis but results from physical trauma. Cells undergoing necrosis have a distinctive morphology and require a different set of molecules than those required by cells undergoing apoptosis.
Phagocytosis: The process through which a dying cell is engulfed by a healthy cell.
Pcd: The natural death of cells during development and/or homeostasis. Apoptosis is a type of PCD involving caspase activation and distinct morphological changes.
RNA Interference: A technique in which the introduction of double-stranded RNA corresponding to a gene of interest results in decreased expression of that gene product.

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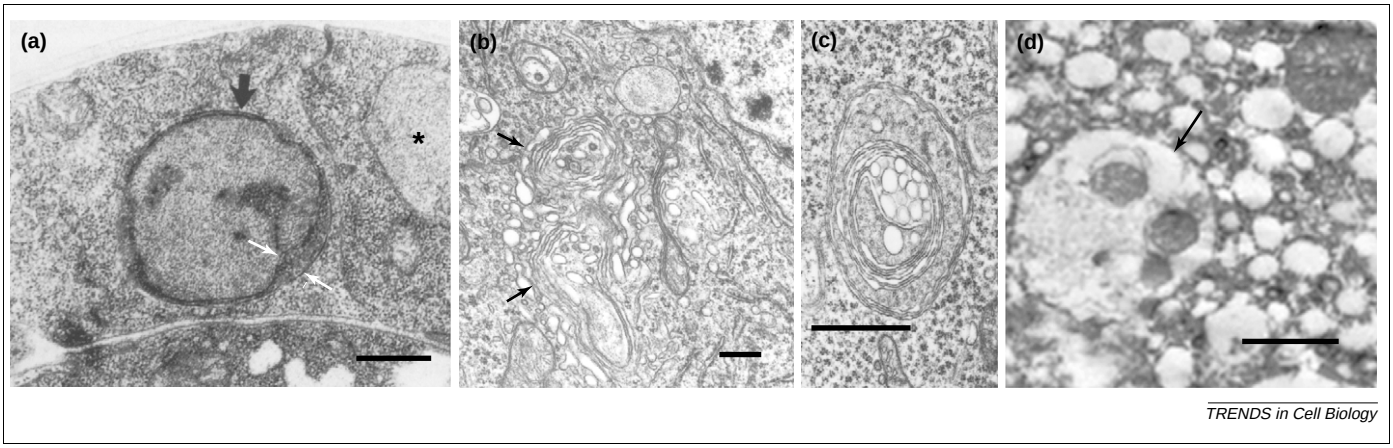

Figure 1. Images of dying cells obtained by electron microscopy. **(a)** An apoptotic cell in *C. elegans* (black arrow) engulfed by a neighboring cell. Note the shrunken darkly stained cytoplasm (flanked by two white arrows) and compare the nucleus with the healthy nucleus at the right (asterisk). **(b)** Autophagic vesicles in a dying Purkinje cell of a lurcher mouse (arrows). **(c)** Higher-magnification image of an autophagic vesicle in a dying Purkinje cell of a lurcher mouse. **(d)** Autophagic vacuole (arrow) in a dying *Drosophila* salivary gland cell. Images adapted, with permission, from [10,29,32]. Scale bars represent 1 μm.

and do caspases function in processes other than cell death?

The caspase connection to apoptosis

Caspases were first revealed as executors of apoptosis from studies in the nematode *C. elegans*. In animals carrying a mutation in the *ced-3* gene, which encodes a caspase, virtually all cells normally fated to die by apoptosis survived [9]. Survival of these extra cells did not affect the viability, life span or gross development of the mutant animals [9]. Epistasis studies between mutations in *ced-3* and mutations in other genes encoding cell-death regulators suggested that *ced-3* was the most downstream regulator of apoptosis known in *C. elegans* [10].

Genetic studies in other organisms also suggest that caspases are at the center of the apoptotic program. Mutations in several mammalian caspases disrupt apoptosis (Table 1). However, in contrast to *ced-3* mutants, in which virtually all apoptosis is prevented, mammalian caspase mutants often display tissue-specific defects in apoptosis, probably because mammals possess an expanded repertoire of caspases compared with *C. elegans* (Table 1). For example, mice homozygous for knockout alleles of either *caspase-9* [11,12] or *caspase-3* [13] died shortly after birth and had excess brain tissue, which appeared to be a consequence of defective apoptosis [11,12]. Cell death in other major organs was, however, less prominently affected. In human beings, two different alterations in *caspase-10* are associated with type II autoimmune lymphoproliferative syndrome – a condition characterized by defective apoptosis of lymphocytes [14]. The altered Caspase-10 proteins exhibited decreased apoptotic and enzymatic activities, suggesting that the alterations might cause disease. One of the alterations was present in a heterozygous state in 6% of a Danish control group [15], and thus might be a naturally occurring

Box 1. General principles of caspase activation

Caspases are cysteine proteases that cleave substrates after specific aspartate residues. The specificity of target sites seems to be determined by a four-amino-acid recognition motif, as well as by other aspects of the three-dimensional structure of the target protein. Caspases are synthesized as proenzymes that are activated through cleavage at internal aspartate residues by other caspases (Fig. I); however, caspases might also have weak catalytic activity in their unprocessed form. Proteins such as *C. elegans* CED-4 or its mammalian homolog Apaf-1 can bind to procaspases and can also multimerize. Multimerization might support cross-activation of adjacent caspase zymogens. Activated caspases consist of dimers of a large and a small subunit that, together, form the active site of the enzyme. Structures obtained by X-ray crystallography suggest that these heterodimers themselves dimerize to form an enzyme with two active sites. Procaspases are often divided into two classes; those with long N-terminal domains are termed initiator caspases, and those with short N-terminal domains are called executor caspases. Long prodomains can bind to activator molecules, such as Apaf-1, or adaptor molecules associated with membrane receptors, such as Fas. It is thought that long prodomain caspases activate short prodomain caspases; however, this assertion is only supported by a limited number of experiments.

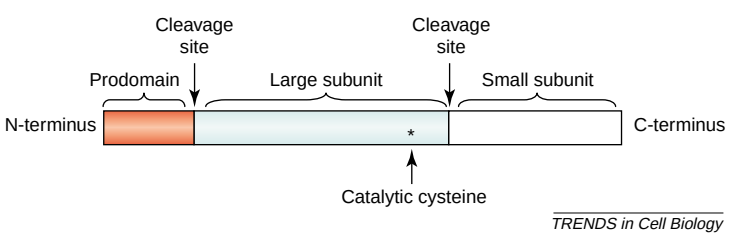

Figure I.

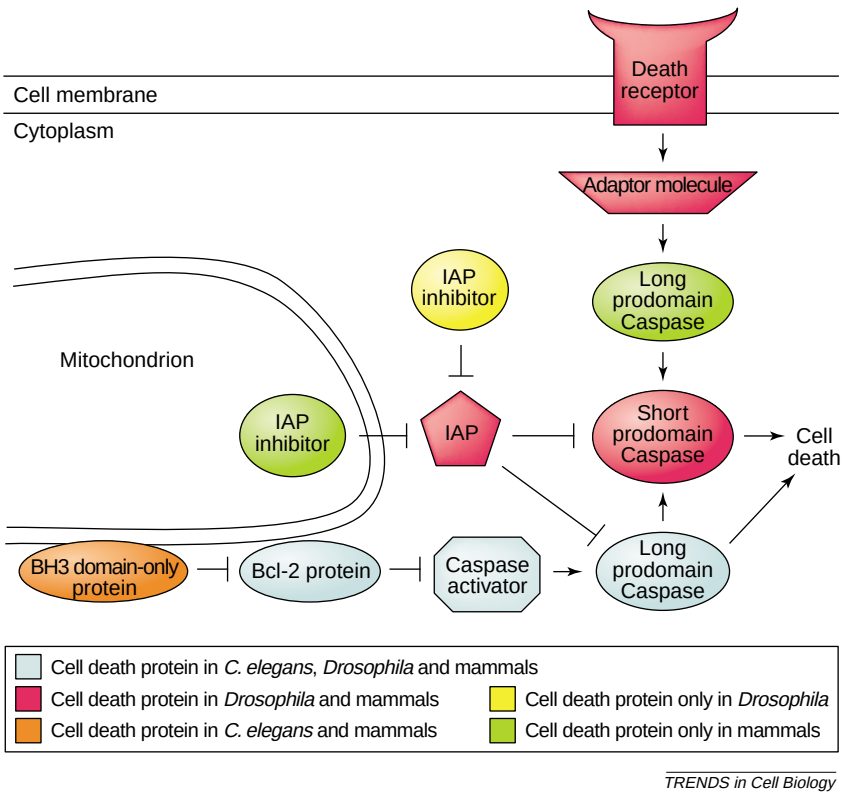

Figure 2. Pathways that regulate caspases. This figure summarizes three major pathways leading to caspase activation as gleaned from studies in mammals, *Drosophila* and *C. elegans*. The evidence used to draw this figure comprises both genetic epistasis studies and biochemical experiments. Membrane receptor complexes, such as Fas or TNF receptor complexes, can activate caspases directly following receptor aggregation. Mitochondrial proteins, including members of the Bcl-2 family, control caspase activity by regulating caspase activators such as the *C. elegans* protein CED-4 or its mammalian homolog Apaf-1. CED-4 and Apaf-1 promote caspase activation by acting as scaffolds, thereby allowing cross-activation of adjacent caspase zymogens [6]. IAP (inhibitor of apoptosis) proteins inhibit apoptosis by binding to and inactivating mature caspases.

polymorphism. The affected individual, however, was homozygous for the *caspase-10* allele. In *Drosophila*, mutants exist in only two of the seven genes encoding caspases – *dcp-1* [16] and *dredd* [17] – and neither has an overt cell death phenotype. However, depletion of the *Drosophila* caspase DRONC by RNA INTERFERENCE (RNAi) [18] or use of a dominant-negative form of the protein [19] are consistent with a role for this protein in mediating cell death.

In addition to genetic analyses, inhibitor studies also suggest important roles for caspases in apoptosis. Chemical caspase inhibitors, such as zVAD.fmk [20], or virally derived caspase inhibitors, such as baculovirus p35 protein [21], prevent apoptosis (Box 2). Immunohistochemical data using antibodies specific for the activated form of caspases are also consistent with caspase involvement in PCD. For example, antibodies specific to activated *caspase-3* bound to those regions in the mouse embryo where developmental cell death occurred naturally [22].

Box 2. Using chemical caspase inhibitors to study cell death

Chemical inhibitors of caspases are often used to study these enzymes in mammalian systems. Typically, these inhibitors are modified peptide pseudosubstrates composed of the cleavage sequence of the caspase target. These peptides are often coupled with a fluoro- or a chloromethylketone group that irreversibly inhibits the active site of the caspase to which it binds. Using chemical inhibitors has important advantages. These molecules can often rapidly manipulate caspase activity in cells. Furthermore, broad-spectrum caspase inhibitors might be the only useful tools for blocking the activity of the entire class of proteins simultaneously. Because mammals have multiple caspases, a genetic approach for removing all of them is not feasible.

Although useful for studying caspase function, there are two main issues to consider when analyzing data obtained from experiments using a chemical inhibitor. First, how specific is the inhibitor and could it affect other proteins unintentionally? For example, recent experiments using the broad-spectrum caspase inhibitor zVAD.fmk suggest that, at standard experimental concentrations, it might also inhibit the cathepsin B protease [81]. Second, how efficient is the inhibitor? Does it effectively reach its target? One problematic issue with some mammalian caspase inhibitors is their poor membrane permeability. Thus, the absence of an effect in experiments using chemical inhibitors might not truly reflect the absence of caspase function.

These issues make it difficult to decide whether a cell-death process is caspase-independent. Caspase inhibitors are frequently added to cultured cells that are challenged by pro-apoptotic stimuli. When the inhibitors do not block cell death, caspase-independent cell death is often pronounced. However, it is possible that the inhibitors did not access caspases efficiently, and thus they might still be active. Alternatively, caspases might be inactivated, but the cause of death could be non-specific cellular damage by the inhibitor. Thus, for convincing conclusions on caspase-dependent and/or -independent cell death, inhibitor studies should be accompanied by other types of studies.

Table 1. Known mutations in caspase genes of *C. elegans, Drosophila,* mice and human beings, and their resulting effects on cell death and other processes

Organism	Caspase	Mutations available? (mutant phenotype)	Cell death phenotype	Other roles
C. elegans	*ced-3*	Yes [9,36]. (Grossly normal development.)	Mutant alleles block almost all of the 131 somatic cell deaths [9,36]	None identified
	csp-1	No		
	csp-2	No		
	csp-3	No		
Drosophila	*dcp-1*	Yes [16]. (Larval lethality and melanotic tumors.)	None identified [16]	Possibly oogenesis [73,74]
	dredd	Yes [17]. (Grossly normal development.)	None identified	Innate immune-system function [17]
	dronc	No	Impaired embryonic cell death [18,75]	None identified
	drice	No		Might be involved in spermatid differentiation [53]
	strica/ dream	No		
	damm	No		
	decay	No		
Mouse	*caspase-1*	Yes – knockout [62,63]. (Animals develop normally.)	Defects in death-receptor-mediated apoptosis [63]	Defects in production of IL-1α and IL-1β [62,63]
	caspase-2	Yes – knockout [76]. (Animals have excess oocytes.)	Oocytes resistant to cell death [76]	None identified
	caspase-3	Yes – knockout [13]. (Perinatal lethality. Animals have excess brain tissue.)	Defects in brain apoptosis [13,77]	Skeletal muscle differentiation [78]
	caspase-6	Yes – knockout [79]. (Animals develop normally.)	Not determined	Not determined
	caspase-7	Yes – knockout [79]. (Embryonic lethality.)	Not determined	Not determined
	caspase-8	Yes – knockout [59]. (Embryonic lethality, impaired heart-muscle development and decreased pool of hematopoietic precursors.)	Defects in Fas and TNF-initiated cell death [59]	T-cell function [66]
	caspase-9	Yes – knockout [11,12]. (Perinatal lethality. Animals have excess brain tissue.)	Defects in brain apoptosis [11,12]. Defects in cell death in response to UV or γ-irradiation [12]	None identified
	caspase-11	Yes – knockout [64]. (Animals develop normally.)	Defects in oligodendrocyte-mediated cell death [67]	Defects in production of cytokines IL-1α and IL-1β [64]
	caspase-12	Yes – knockout [80]. (Animals develop normally.)	Fibroblasts are defective in cell death in response to endoplasmic reticulum stress stimuli [80]	None identified
Human	*caspase-8*	Yes – familial mutation [65].	Defects in death-receptor-mediated apoptosis [65]	Defects in activation of T, B and NK cells [65]
	caspase-10	Yes – familial mutation [14]. (Patients have autoimmune lymphoproliferative syndrome type II.)	Defects in death-receptor-mediated apoptosis [14]	None identified
	caspase-14	No		Terminal differentiation of keratinocytes [60,61]

Caspases and autophagic cell death: apoptosis in disguise?

AUTOPHAGY is a process enabling cells to dispose of cytoplasm and organelles by fusion of vesicles containing these cellular components with the lysosome (Figure 1). Autophagy has been extensively characterized in the yeast *Saccharomyces cerevisiae*, where it is triggered in response to starvation [23]. Because starved yeast can resume growth when nutritional conditions improve, the main role of autophagy in this organism is probably to protect the cell from starvation by recycling its content. In *C. elegans*, autophagy might also serve a protective role. Loss-of-function mutations in *C. elegans* homologs of human and yeast autophagy genes suggest a role for autophagy in the proper maintenance of a developmentally arrested larval state termed dauer (German for enduring), which is adapted to harsh conditions including starvation [24].

Autophagy, however, might be employed for the steroid-hormone-regulated PCD of the larval salivary glands as *Drosophila* develops from a larva to an adult. Dying cells of the salivary glands contain autophagic vacuoles (Figure 1) and express genes associated with autophagy [25,26]; they are therefore morphologically distinct from apoptotic cells. Thus, it was surprising when a series of recent publications suggested that caspases control cell death in the salivary glands of *Drosophila*. A genome-wide study of transcriptional changes during PCD of the salivary glands showed increased expression of the caspases *drice* and *dream* [25], and other studies showed increased transcription of the caspase *dronc* [26,27]. Furthermore, expression of the caspase inhibitor p35, or a dominant-negative form of the caspase *dronc*, in the cells of the salivary glands prevented their destruction [28]. How distinct is cell death in the salivary glands from apoptosis? Interestingly, dying cells of the salivary glands do possess features associated with apoptosis, including permeability to the vital dye acridine orange, phosphatidylserine exposure on the outer leaflet of the plasma membrane and DNA fragmentation [28].

Dying gland cells also expressed the apoptotic activators *rpr*, *hid* and *grm* [29]. Furthermore, a time-course study showed that autophagic vesicle accumulation preceded the appearance of apoptotic changes, and the latter correlated with increased caspase activity [30]. The observation that, despite the presence of autophagic vacuoles, the expression of p35 or dominant-negative *dronc* prevented cell death in the salivary glands, suggests that caspases are required to kill these cells and autophagy alone is not sufficient to promote the demise of the salivary glands. Mutations in autophagy genes in *Drosophila* have not been described, preventing a direct test of this conclusion. Thus, although cell death in the salivary glands has been dubbed autophagic, and is distinct from apoptosis, it might harbor apoptotic characteristics at its core.

Why might cells of the salivary glands possess autophagic vesicles? During development, apoptotic cells often die in isolation and their remnants are engulfed by neighboring cells or professional phagocytes. However, cells of the larval salivary glands undergo massive synchronous destruction and might be difficult to clear efficiently. Autophagy might decrease the volume of the salivary glands and hence aid the removal of cell remnants by phagocytes.

In mammals, the relationship between autophagy and apoptosis has also been examined. Beclin, a homolog of the yeast autophagy protein Apg6 (or Vps30), can induce autophagy in human cells [31]. Intriguingly, Beclin interacted with the key apoptotic regulator Bcl-2 in two-hybrid studies, suggesting a link between autophagy and apoptosis. However, the functional consequences of this interaction have not been investigated. Further evidence for links among Beclin, autophagy and cell death has recently come from analysis of Purkinje cells in lurcher mice [32]. Lurcher mice suffer from degeneration of the cerebellar cortex because of a mutation causing constitutive activation of the GluRδ2 glutamate receptor in Purkinje cells [33]. Two-hybrid studies in yeast revealed that GluRδ2 can interact with nPIST, which in turn could bind to Beclin [32]. The death of Purkinje cells in lurcher mice had been defined as apoptotic, because electron microscopy (EM) studies revealed that in postnatal day 12 animals dying Purkinje cells possessed an apoptotic morphology [34]. However, recent EM studies of lurcher mice on postnatal day 10 revealed dying Purkinje cells with autophagic vesicles (Figure 1) [32]. It is unknown whether the autophagic cells on day 10 are the same as those undergoing apoptosis on day 12. However, the temporal order of autophagy followed by apoptosis, as seen in the salivary-gland cells of *Drosophila*, suggests that autophagy might set the stage for apoptosis to kill the Purkinje cells of lurcher mice.

Apoptosis following autophagy has also been reported in an EM study monitoring the death of sympathetic neurons from the superior cervical ganglion after the withdrawal of growth factors *in vitro* [35]. Treatment of cells with the autophagic inhibitor 3-methyladenine delayed apoptosis, suggesting that, in this system, autophagy might facilitate apoptosis [35].

In summary, to date there is no genetic evidence that autophagy alone is necessary or sufficient to cause PCD. Furthermore, cells that die an autophagic death often also have apoptotic features. Is autophagic death, therefore, apoptosis in disguise? The answer awaits a better understanding of the role of autophagy during cell death.

Evidence for caspase-independent PCD

Early cell-death studies in *C. elegans* hinted that PCD could occur in the absence of caspases. Characterization of *C. elegans ced-3* mutants using light microscopy revealed that the migratory leader cell of the male gonad, the linker cell, underwent PCD even in the absence of *ced-3* [9]. Subsequent findings were consistent with the existence of *ced-3*-independent PCD; these included reports that, in the anterior pharynx, some cells died even in animals harboring a deletion of the entire protease-encoding domain of *ced-3* [36]. The *C. elegans* genome contains three other caspase-related genes – *csp-1*, *csp-2*, and *csp-3* [37]. Investigation of these caspases by RNAi in either wild-type animals or animals homozygous for partial loss-of-function alleles of *ced-3* has failed to reveal a role for the caspase-related genes in PCD (S. Shaham, unpublished). Thus, *ced-3* might be the only caspase associated with PCD in *C. elegans*. One hint to how a *ced-3*-independent pathway might promote PCD came from genetic studies. In a *ced-4* loss-of-function mutant of *C. elegans*, PCD is blocked, suggesting that CED-4, a caspase activator (Figure 2, Box 1), is required for PCD [9]. Epistasis experiments placed *ced-4* activity upstream of *ced-3* [10]. When CED-4 was overexpressed in the ALM neurons of *C. elegans*, it could kill these cells [10]. Surprisingly, killing was reduced but not completely blocked in a *ced-3* mutant background [10]. This result suggests that *ced-4* can kill cells independently of *ced-3* (Figure 3); however, it is not clear whether the *ced-3* alleles used in these experiments completely eliminated *ced-3* activity or whether overexpressed CED-4 exhibited a novel toxicity.

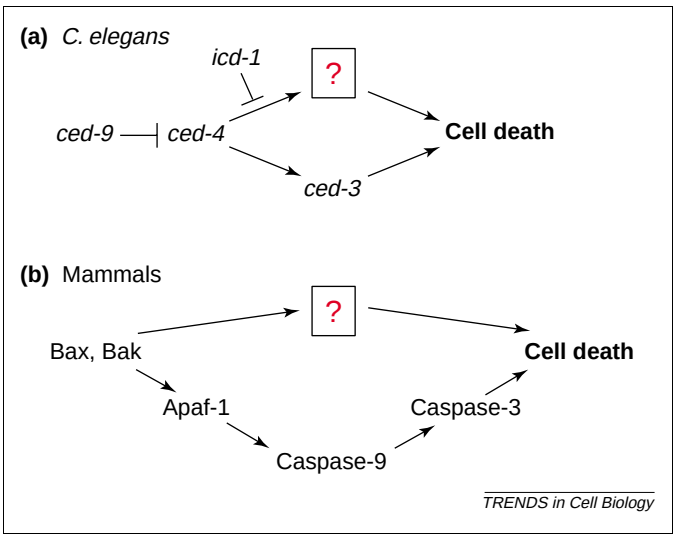

Figure 3. Possible caspase-independent cell-death pathways. In *C. elegans* and mammals, postulated caspase-independent pathways might differ. In *C. elegans* the death pathway might bifurcate downstream of CED-4, a caspase activator protein. Evidence from murine studies suggests that bifurcation might occur upstream of the CED-4 homolog Apaf-1. **(a)** The cell-death pathway in *C. elegans* is based on genetic epistasis experiments [10,36,38]. **(b)** Murine pathway is based on data from [39,40].

Consistent with the ability of CED-4 to kill ALM neurons in the absence of CED-3, Rothman and colleagues [38] recently showed that inactivation of a gene they termed *icd-1* (inhibitor of cell death) by RNAi promoted *ced-4*-dependent, but *ced-3*-independent, death of a range of cell types including neurons and male ray cells (Figure 3). ICD-1 protein is similar to both the *Drosophila* protein bicoid and the β subunit of the nascent-polypeptide-associated complex. EM analysis of a dying cell in an *icd-1*(RNAi); *ced-3* double mutant revealed morphological changes characteristic of apoptosis, including compacted chromatin and membrane blebbing [38]. Furthermore, although overexpression of proteins rarely promotes cell survival, global overexpression of ICD-1 resulted in the survival of some embryonic cells in *C. elegans* that would have normally died by apoptosis. Regardless of whether *icd-1* normally inhibits apoptosis in *C. elegans*, these studies suggest that the caspase CED-3 is not required for some types of cell death in this organism. The mechanism for these *ced-4*-dependent *ced-3*-independent cell deaths is unknown, neither is it clear whether these deaths are truly caspase independent. It would be interesting to know whether a broad-spectrum caspase inhibitor, such as baculovirus p35, could block the *icd-1*-dependent cell deaths or whether any of the other caspase homologs in *C. elegans* play a role here.

In mammals, there is also evidence for caspase-independent PCD. Genetic studies on PCD induced by BH3-domain-only proteins, such as tBID, BIM and BAD (Figure 2), showed that these proteins, which have been shown to promote caspase activation and apoptosis, can also kill cells independently of *Apaf-1* and downstream caspases. Specifically, *Apaf-1* $^{-/-}$ mouse embryonic fibroblasts (MEFs) could still die in response to the overexpression of BH3-domain-only proteins [39]. Although death was reported as apoptotic, because dying cells stained with Annexin V, a marker for external exposure of the normally intracellular membrane lipid PtdSer, such staining also might indicate non-apoptotic dying cells with membrane damage [39]. Caspase activation was not detected in the dying cells when assayed with fluorogenic substrates for caspase-2, -3, -6 or -7, nor could cell death be blocked by the pan-caspase inhibitor zVAD.fmk (Box 2) [39]. Although it is possible that another known mammalian caspase that was not assayed for is the cell-death executor in these cells, the results are intriguing and suggestive of caspase-independent cell death. A clue to possible mediators of this potentially caspase-independent death came from studies of mice lacking the *bcl-2*-related genes *bax* and *bak*. *bax* $^{-/-}$*bak* $^{-/-}$ mice died just after birth and showed a brain overgrowth defect, lymphoid-cell accumulation and retained interdigital webs, all defects associated with lack of PCD. Interestingly, MEFs derived from these mice and overexpressing BH3-domain-only proteins were almost completely resistant to death [40]. These results suggest that the PCD pathway induced by BH3-domain-only proteins bifurcates downstream of Bax and Bak (Figure 3), with one branch being caspase- and Apaf-1-dependent and the other not. It is noteworthy that this proposed caspase-independent pathway might be different from the *ced-3*-independent pathway hypothesized in *C. elegans*, because the points at which these pathways bifurcate are different (Figure 3).

Another candidate mammalian mediator of caspase-independent PCD is apoptosis-inducing factor (AIF), a conserved mitochondrial oxidoreductase. In the early mouse embryo, PCD occurs in the ectoderm to create the pro-amniotic cavity. An *in vitro* model of this event uses an aggregate of embryonic stem (ES) cells in which the inner ectodermal cells undergo cell death [41]. CAVITATION might be caspase-independent because it occurs in aggregates of ES cells from *caspase-9* knockout mice, which do not bind to antibodies directed against activated Caspase-3 [42]. Histological studies show that the cavitation of ES cells is blocked when AIF knockout cells are used [42]. A cell-death block in the ectoderm layer probably prevents cavitation, because chromatin condensation is not detected using the DNA stain DAPI, and dying cells are not seen by EM [42]. Further evidence that AIF might mediate caspase-independent cell death includes the observation that microinjection of AIF into cells induced apoptotic changes, such as chromatin compaction and PtdSer exposure, that could not be blocked by either zVAD.fmk or the overexpression of Bcl-2 [43]. AIF knockout ES cells could not be used for generating a knockout mouse to determine the role of AIF *in vivo* [42], suggesting that cavitation is perhaps an essential process.

Recent data from a mouse with reduced levels of AIF expression have, however, called the role of AIF in PCD into question. The harlequin mouse has a proviral insertion that is reported to downregulate AIF mRNA and protein levels by 80% [44]. Neonatal harlequin mice are indistinguishable from wild-type animals; however, they succumb to gradual-onset neurodegeneration [44]. The levels of lipid peroxide and catalase activities in these mice suggest that they suffer from oxidative stress [44], and cultured harlequin neuronal cells were more sensitive to peroxide than wild-type cells [44]. Based on these data, AIF, which has structural similarities to peroxide scavengers, was hypothesized to protect adult neurons from oxidative stress. Thus, in the absence of AIF, oxidative stress would promote aberrant cell cycle reentry leading to neurodegeneration [44]. An analysis of cavitation in the harlequin mouse has not been reported. If AIF is required for PCD during cavitation, why do harlequin mice initially develop normally? It is possible that the ES-cell aggregate model of cavitation does not faithfully recapitulate the *in vivo* process, or that the lowered AIF expression in the harlequin mouse might provide adequate levels of AIF for cavitation to proceed *in vivo*. Alternatively, AIF levels in the harlequin mouse might only be reduced postnatally but not during embryogenesis. To reconcile the data from harlequin and AIF knockout mice, and to determine whether AIF promotes caspase-independent PCD, it would be worthwhile investigating if there are any *in vivo* or *in vitro* cavitation abnormalities in the harlequin mouse. In addition, RNAi of AIF in the ES-cell aggregate system [45] could be used for determining whether reduction of AIF to only 20% of its wild-type levels would be sufficient for cavitation to occur normally.

AIF function *in vivo* has also been studied in *C. elegans*. In this organism, inhibition of the AIF gene homolog *wah-1*

by RNAi had only very mild effects on developmental cell death, although it is unclear whether gene function was completely eliminated in the animals studied [46].

Other support for caspase-independent PCD comes from numerous *in vitro* studies demonstrating that cell death can proceed even in the presence of broad-spectrum caspase inhibitors. It is unclear, however, whether such experiments truly mimic PCD *in vivo* (Box 2).

Other deadly proteases?

We have discussed some experimental data that hint at caspase-independent PCD; however, the caspase substitute that allows cell death to proceed in these cases is unknown. Because caspases are proteases, it is possible that caspase-independent cell death might require other proteases. Indeed, there is weak, yet suggestive, evidence that might support roles for cathepsins, calpains and granzyme B in caspase-independent PCD. Cathepsins are lysosomal proteases, and mice lacking cystatin B, an inhibitor of cathepsins B, H, L and S, show cerebellar cell death [47]. This suggests that cathepsins might mediate cell death, although it is also possible that the phenotype was caused by a cathepsin-independent effect of cystatin B. Calpains are a family of calcium-dependent proteases that have also been described as possible facilitators of cell death. In breast cancer cells, vitamin-D-induced cell death appeared to be caspase-independent [48] and promoted by calpains [49]. Interestingly, cathepsin and calpain proteases have been described as key regulators of necrotic cell death in *C. elegans* [50]. Granzyme B is important for cell death mediated by cytotoxic T lymphocytes and might be an alternative to caspases. However, analysis of the role of granzyme B is complicated by the fact that it might also activate caspases [51]. Nonetheless, granzyme B can mediate some typical apoptotic changes, such as DNA fragmentation and PtdSer exposure, in the presence of caspase inhibitors [52].

Not always killers

Despite the stereotyped view that caspases are chief executioners of cell death, there are several instances where caspase activation fails to trigger cell death. Somewhat paradoxically, deadly caspases play an important role at the beginning of life itself, during sperm formation in *Drosophila*. Caspases aid the process of sperm individualization, through which spermatids become separated from syncytia and lose the bulk of their cytoplasm [53]. During individualization, a cytoskeletal membrane complex, known as the individualization complex, translocates along spermatids, disconnecting cytoplasmic 'bridges' between them and expelling spermatid cytoplasm and unnecessary organelles into a membrane bag called the cystic bulge. Immunostaining for activated drice marked the pre-individualized part of the spermatid and the cystic bulge [53]. Indeed, the cystic bulge also stained with the apoptosis-associated marker acridine orange [53]. Synthetic and viral pan-caspase inhibitors severely impaired movement of the individualization complex and prevented removal of bulk cytoplasm from the spermatids [53]. It is not known how the activated caspase facilitates the movement of the individualization complex. It is also unclear whether caspases have a direct role in exclusion of cytoplasm from the spermatid, although it is reasonable to hypothesize that caspase activity might aid the degradation of expelled cytoplasm in the cystic bulge. The morphological defects and sterility of *Drosophila* sperm treated with caspase inhibitors strikingly resemble a common abnormality in human sperm; mouse knockouts of some apoptotic genes also cause male sterility [53]. Thus, although these mammalian defects are largely uncharacterized, they might also point to a non-lethal role of caspases.

In red blood cells and lens fiber cells, caspase activation leads to a subset of apoptotic morphological changes without causing cell death. As embryonic erythroid cells differentiate into adult red blood cells, they show signs of apoptosis, including chromatin compaction, nuclear destruction and caspase activation [54]. Yet, although the cytoplasm of an apoptotic cell contracts, that of a differentiating red blood cell expands [55]. Lens fiber cells develop from epithelial cells that degrade their organelles and nuclei during differentiation, presumably to allow cellular transparency. Caspases are expressed in developing lens fiber cells [56], and zVAD.fmk can reduce nuclear destruction in an *in vitro* model of rat lens-fiber differentiation [56]. In transgenic mice that overexpressed Bcl-xL, the lens fibers did not lose their nuclei [57]. In both of these cell examples, it is unclear how caspase activity is controlled to trigger only non-lethal aspects of the apoptotic program.

There are also examples of caspase activation promoting differentiation in the absence of any morphological signs of apoptosis. For example, during infection, human monocytes differentiate to form macrophages. The differentiation process does not show morphological features of apoptosis [58]; however, antibody staining showed caspase activation at the time of the switch; and the monocyte–macrophage switch was blocked by caspase inhibitors [58]. Caspase-8 might also play a role in differentiation because *caspase-8* knockout mice exhibit defects in the development of heart muscle and also have a dramatically decreased pool of hematopoietic precursors [59]. These defects are not apparently related to abnormal cell death. The activity of human Caspase-14 was associated with the terminal differentiation of keratinocytes [60,61].

Immune functions are probably the best-characterized examples of non-lethal roles for caspases that do not involve apoptotic changes. Murine knockouts of *caspase-1* [62,63] and *caspase-11* [64] develop normally, apart from defects in the production of IL-1α and IL-1β in response to the bacterial compound lipopolysaccharide (LPS). A human family pedigree showed that *caspase-8* mutations are linked to defects in the activation of T, B and NK cells [65]. Mice studies corroborated a role for *caspase-8* in T-cell function, because a targeted *caspase-8* deletion in T cells caused defects in activation-induced expansion of peripheral T cells, T-cell activation and the ability of T cells to clear lymphocyte choriomeningitis virus [66]. Interestingly, in addition to their immune functions, *caspase-1*, *-8* and *-11* can also promote apoptosis, because cells from mice harboring knockout alleles of these caspases have

impaired death-receptor-mediated PCD [59,63,67]. The link between caspases and the immune system also extends to *Drosophila*. A fly screen to identify mutants defective in innate immunity revealed that a mutant of the caspase Dredd mounted a defective immune response when challenged with Gram-negative bacteria [17].

Caspase activity and prevention of apoptosis

How can caspases be activated without killing the cell? One possibility is the existence of subcellularly localized inhibitors that prevent local caspase activity. For example, the spermatids of *Drosophila dbruce* $^{-/-}$ males degenerate with hypercondensed nuclei, indicative of excessive nuclear caspase activity [53]. dBruce, an E2–ubiquitin-conjugating enzyme, has a baculovirus IAP repeat (BIR) domain. A similar domain in the XIAP protein can inactivate caspases by blocking their active site [68]. Thus, dBruce might protect the nuclei of differentiating fly spermatids from destruction during differentiation, despite caspase activation.

Another way that caspases might be activated without killing the cell is by blocking PHAGOCYTOSIS of the dying cell. Phagocytosis can occur early during cell death, even before a cell shows obvious signs of death [69]. In *C. elegans* some cells destined to die by apoptosis can survive if phagocytosis is defective [70,71]. Perhaps, then, red blood cells and lens fiber cells that activate caspases without cell death, survive caspase activation because they do not initiate phagocytosis.

Concluding remarks

Despite ten years of studying caspases, we are only beginning to explore the functions of these versatile enzymes, and many questions regarding their molecular functions remain. Over a hundred caspase targets have been identified, including poly ADP-ribose polymerase (PARP) and nuclear lamins [72]. However, the full range of targets is not known, nor is it clear what targets must be cleaved to cause cell death. We also do not have a thorough understanding of the biochemical and structural events that convert procaspases to mature enzymes. Recently, a complex picture has emerged of the many cellular roles of caspases, suggesting that these enzymes, thought to herald apoptotic cellular demise, might not be required for all types of PCD. Exciting new genetic data from *C. elegans* and mice suggest that some cells might die with an apoptotic morphology, but without caspase involvement. Promising leads for investigation of these potentially caspase-independent PCDs include proteins, such as AIF in mammals, and pathways downstream of CED-4 in *C. elegans* and downstream of Bak and Bax in mammals. It has also become clear that caspases can promote non-apoptotic death. New insights, particularly from studies of *Drosophila* salivary glands, suggest that caspases might also play roles in autophagic PCD. Finally, recent studies have also brought to light non-lethal roles of caspases such as findings demonstrating a need for caspase activity during *Drosophila* spermatogenesis. Taken together, these recent investigations make it clear that caspase activity can no longer be taken as the gold standard for assaying whether cells die by apoptosis. Instead, each instance of a suspected death needs to be fully explored, not only for caspase activity, but also for morphological evidence of death, and for expression of other proteins involved in death processes.

What are the molecules and mechanisms that allow PCD without caspases or caspase activity without PCD? With new experimental approaches, such as RNAi, proteomics, small-molecule inhibitors and conditional knockouts, some answers to these fascinating questions might be within reach.

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