First publ. in : Nature Reviews : Molecular Cell Biology ; 2 (2001), 8. - S. 589-598
DOI : 10.1038/35085008

FOUR DEATHS AND A FUNERAL: FROM CASPASES TO ALTERNATIVE MECHANISMS

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A single family of proteases, the caspases, has long been considered the pivotal executioner of all programmed cell death. However, recent findings of evolutionarily conserved, caspase-independent controlled death mechanisms have opened new perspectives on the biology of cell demise, with particular implications for neurobiology, cancer research and immunological processes.

CASPASES
Family of cysteine proteases that can be divided into inflammatory caspases, and pro-apoptotic caspases, which can be further grouped into initiator and effector caspases.

PHAGOCYTOSIS
Uptake of dying cells by macrophages or neighbouring cells. Recognition of 'eat-me' signals by specific receptors on the phagocytosing cell.

DEATH RECEPTORS
A family of cell-surface receptors that can mediate cell death upon ligand-induced trimerization.

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Programmed cell death (PCD) is essential for the development and maintenance of multicellular organisms. Many eukaryotic cells that die and are removed in a programmed way undergo an astonishingly stereotypical series of biochemical and morphological changes, the most defining features of which are the activation of CASPASES, chromatin condensation and the display of PHAGOCYTOSIS markers on the cell surface[1–3]. The underlying death process has been called apoptosis to delineate it clearly from other death programmes (BOX 1).

The unexpected ability of certain cells to survive the activation of pro-apoptotic caspases[4–9] demonstrates a remarkable plasticity of the cellular death programme, and does not support the idea that caspases alone are sufficient for the induction of mammalian PCD. Furthermore, recent evidence indicates a diversification of the apoptosis programme in higher eukaryotes with respect to the necessity and role of caspases. Namely, apoptosis-like cell death can occur without the activation of effector caspases[9–18], and signals emanating from the established key factors of apoptosis — including DEATH RECEPTORS and caspases themselves — may result in a non-apoptotic death[19–21] (BOX 1).

Remarkably, modifications in the mode of death do not necessarily affect the efficient removal of dying cells[22,23]. So, some of the alternative caspase-independent death pathways might have evolved to fulfil the same purpose as that proposed for classical apoptosis — that is, to guarantee a safe and non-inflammatory removal of corpses. We present here a differentiated view on PCD, based not on the activation of caspases, but rather on the morphology and fate of dying cells (BOX 1). The discussion of medically relevant fields, such as neurology and oncology, also takes into account the implications of the death mode for the surrounding tissue and the potential of caspase-independent PCD signalling mechanisms as therapeutic targets.

Evolution of cell death principles

The driving evolutionary pressures for the development of several cell-death programmes have been increasing in parallel with the increased complexity and life span of the organisms[24]. But when in evolution did the caspase-independent death mechanisms arise? Caspase-coding sequences are absent from the known genomes of many non-animal species[24]. Nevertheless, such organisms — including plants and several single-celled eukaryotes — undergo PCD under conditions of stress[25,26]. For instance, in yeast, this apoptosis-like death is associated with DNA fragmentation, ZEIOSIS, PHOSPHATIDYLSERINE EXPOSURE and chromatin condensation[25], and can be selectively triggered or blocked by Bax-like or CED-9-related genes, respectively. Furthermore, programmed PARAPTOSIS-like death is well characterized in caspase-deficient slime moulds[27].

The introduction of the caspases, and especially of the mitochondrial CED-9/Bcl-2-related death switches[24,26], might represent a decisive refinement of the old caspase-independent death programmes. The relative importance of different death mechanisms seems to

Box 1 **Four patterns of death: from apoptosis to necrosis**

Apoptosis is defined by stereotypic morphological changes, especially evident in the nucleus where the chromatin condenses to compact and apparently simple geometric (globular, crescent-shaped) figures[1]. Other typical features include phosphatidylserine exposure, cytoplasmic shrinkage, zeiosis and the formation of apoptotic bodies (with nuclear fragments) (BOX 2). In its most classic form, apoptosis is observed almost exclusively when caspases, in particular caspase-3, are activated. When death can be blocked by inhibition of any signal or activity (for example, caspases) within the target cell, then the simplest condition for programmed cell death (PCD) is met. Apoptotic morphology results from one of the most elaborate forms of PCD, and it may be viewed as a far end of a continuum of death modes, with varying contributions of the cellular machinery.

Apoptosis-like PCD is used here to describe forms of PCD with chromatin condensation that is less compact/complete than in apoptosis (geometrically more complex and lumpier shapes), and with the display of phagocytosis-recognition molecules before lysis of the plasma membrane. Any degree and combination of other apoptotic features can be found. Most published forms of 'caspase-independent apoptosis' fall into this class. Notably, some classic 'caspase-dependent apoptosis' models, such as tumour-necrosis-factor-induced death of MCF-7 cells, also have this morphology. For comparative examples see BOX 2.

Necrosis-like PCD is used here to define PCD in the absence of chromatin condensation, or at best with chromatin clustering to speckles[19,20,33,34,104]. Varying degrees of other apoptosis-like features — including externalization of phosphatidylserine — might occur before the lysis[33,34]. Necrotic PCD usually involves specialized caspase-independent signalling pathways. However, caspase-8 might be activated[21] and caspase-1-driven necrosis has also been observed[112]. A subgroup of necrotic PCD models are often classified as 'aborted apoptosis'; that is, a standard apoptosis programme is initiated, then blocked at the level of caspase activation and finally terminated by alternative, caspase-independent routes[30].

Accidental necrosis/cell lysis is the conceptual counterpart to PCD, as it is prevented only by removal of the stimulus. It occurs after exposure to high concentrations of detergents, oxidants, ionophores or high intensities of pathologic insult[30]. Necrosis is often associated with cellular OEDEMA (organelle swelling) and devoid of zeiosis **(see movies 3 and 4 online)**. The necrotic tissue morphology is, in large part, due to post-mortem events (occurring after lysis of the plasma membrane)[30].

OEDEMA
Water accumulation and swelling within a tissue, cell or organelle.

ZEIOSIS
Dynamic plasma membrane blebbing of a dying cell, analogous to the bubbling of fermenting yeast.

PHOSPHATIDYLSERINE EXPOSURE
Translocation of phosphatidylserine, which is confined to the inner leaflet of the plasma membrane in healthy cells, to the outside of the plasma membrane where it is recognized by a specific receptor on macrophages.

PARAPTOSIS
A form of programmed cell death without prominent chromatin condensation and mainly characterized by cytoplasmic vacuolization.

have been optimized subsequently in various ways. One form of extreme specialization is exemplified by somatic cell death in the nematode *Caenorhabditis elegans*, which sits on a branch of the phylogenetic tree that separated early from the branch leading to mammalian development. Accordingly, the requirements for PCD in *C. elegans* are adapted to its specific needs and have diverged widely from those of mammals[24]. As the environmental pressure to provide a flexible death response is very low in this short-lived organism, it has been enough to optimize only one stereotyped caspase-dependent apoptosis programme. In contrast to mammals, control by mitochondrial proteins might not be required, and most degradative enzymes are supplied by the phagocytosing cell rather than by the dying cell itself[2,3]. Apoptosis in *C. elegans* is often cell autonomous — that is, it is neither signalled nor controlled from outside and the entire system of death receptors seems to be absent. In accordance with this minimalist programme, somatic PCD is not essential for the survival or the development of *C. elegans*[28]. Rudimentary remainders of alternative apoptotic programmes are, however, still found in the MALE LINKER CELL, in which PCD that might be independent of CED-3 is triggered from outside[28].

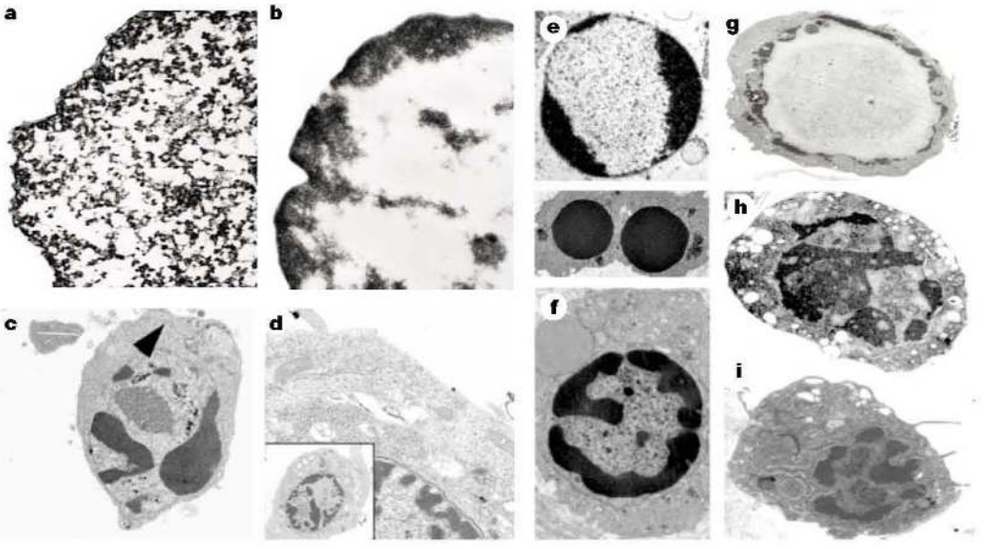
The mammalian system of death programmes could represent an opposite form of evolutionary direction in which, apart from the many caspases, other cysteine proteases and mitochondrial factors have taken additional roles during development and life[3,29]. The essential nature of some factors (knockout lethality[18,29]) combined with the redundancy of others (difficulty with interpretation of knockouts[29]) has made the study of their specific role in PCD technically challenging. In addition, it has remained unclear which mechanisms are essential for commitment to death and which ones only determine the phenotypic outcome[30].

PCD can take many forms

If one keeps to the strict morphological criteria of apoptosis — including the geometrical shape of chromatin after its condensation (BOX 2) — caspases seem to be indispensable for apoptosis (BOX 1). However, there are many forms of 'apoptosis-like PCD', in which the chromatin condenses into less geometric shapes and phagocytosis markers on the plasma membrane are displayed before cell lysis. An array of well-characterized cell death models that occur in the absence of caspase activation falls into this category[9–17,31] (BOX 2). Furthermore, an analysis of cell membrane dynamics in different death models has revealed that an important hallmark of apoptosis, zeiosis, can occur independently of caspase activation[9,32] **(see movies 1 and 2 online)**. PCD can also occur in the absence of chromatin condensation[20,33,34]. Such necrosis-like PCD is the result of active cellular processes that can be intercepted by, for instance, oxygen-radical scavengers[20,35], inhibition of poly(ADP) ribose polymerase (PARP)[36] or mutations in intracellular signalling molecules[34]. Further caspase-independent modes of PCD include autophagy — characterized by the formation of large, lysosome-derived cytosolic vacuoles[17,37,38] — and DARK CELL DEATH in specialized cells such as chondrocytes[39] or neurons[40].

Contrary to earlier expectations, the inhibition of caspase activation does not necessarily protect against cell death stimuli. Instead it might reveal, or even enhance, underlying caspase-independent death programmes. These programmes might resemble apoptosis-like PCD[9,41–43], autophagy[44] or even necrosis[19,20,32,45–48]. In many experimental apoptosis models, including those triggered by death receptors[20,34,42,46,48], cancer drugs[49], growth-factor deprivation[44], STAUROSPORINE[41], anti-CD2[41], oncogenes[32], COLCHICINE[43] or the expression of Bax-related proteins[32,45], the existence of back-up death pathways has been uncovered after inhibition of caspase activity by pharmaceutical PAN-CASPASE INHIBITORS. However, several lines of evidence also support the relevance of such 'second-line' mechanisms for normal physiology and pathology. For example, caspase pathways can be inactivated not only by pharmacological inhibitors, but also by other factors such as mutations[47], energy depletion[19], nitrative/oxidative stress[21], other proteases that are activated simultaneously[50,51], members of

Box 2 **Nuclear alterations in different forms of programmed cell death**



The use of chromatin condensation as a criterion to distinguish apoptosis from apoptosis-like PCD has been inconsistent in the scientific literature, and the potential for overlapping definitions and errors is large. The following examples of classical apoptosis (c,e) and apoptosis-like PCD (b,d,f,g–i) might provide a general guideline. Examples of control chromatin (a), and caspase-independent chromatin margination triggered directly by microinjection of AIF (b) (reproduced with permission from REF. 78 © (1999) Macmillan Magazines Ltd). Caspase-dependent strong chromatin compaction (c) versus caspase-independent, AIF-driven lumpy chromatin condensation (d) in PCD of mouse embryonic stem cells (reproduced with permission from REF. 18 © (2001) Macmillan Magazines Ltd). (e) Caspase-dependent chromatin compaction to crescent shaped masses at the nuclear periphery and chromatin fragmentation to two compact spheres (f) or caspase-independent lumpy chromatin condensation without nuclear fragmentation in colchicine-induced neuronal cell death (reproduced with permission from REF. 43 © (2001) The John Hopkins University Press). Incomplete, lumpy chromatin condensation (compare with b,d) in caspase-independent apoptosis-like PCD triggered by Hsp70 depletion[16] (g) or the active form of vitamin D[15] (i), and in caspase-dependent TNF-induced apoptosis-like PCD (h) in caspase-3 deficient MCF-7 cells. (L. Bastholm, F. Elling, I. Stenfeldt Mathiasen and J. Nylandsted are acknowledged for providing the unpublished panels g–i.)

MALE LINKER CELL
The linker cell is a male-specific cell at the tip of the developing gonad. It 'guides' growth of the male gonad from the midbody region towards the tail. When the gonad has reached the tail late in larval development, the linker cell is 'murdered' by one of two neighbouring cells.

DARK CELL DEATH
Slow neuronal death observed, for example, during Huntington's disease. Characterized by strong cytoplasmic condensation, chromatin clumping, ruffling of the cell membrane, but no blebbing of the nucleus or plasma membrane.

PARP
Poly(ADP)-ribose polymerase. A nuclear enzyme activated by DNA damage and reducing cellular ATP levels when overactivated.

STAUROSPORINE
Plant-derived cytotoxin known to trigger mitochondria-dependent apoptosis in most cell types. Model apoptosis inducer.

the INHIBITOR OF APOPTOSIS PROTEIN (IAP) family[3,52] or an array of viral proteins that can silence caspases[3].

Upon caspase inhibition, the alternative death pathways also surface *in vivo*. They are involved in processes such as the negative selection of lymphocytes[53,54], embryonic removal of interdigital webs[47], tumour necrosis factor (TNF)-mediated liver injury[55] and the death of chondrocytes controlling the longitudinal growth of bones[39]. These examples might be just the tip of the iceberg for the complexity of death signalling *in vivo*. And the overlapping death pathways initiated by a single stimulus seem to be the rule rather than the exception[18,34]. The examination of potential crossovers of death pathways that lead eventually to different phenotypic outcomes might allow us to understand which events really determine commitment to death and which ones are involved in upstream signalling or downstream execution.

The funeral: removal of corpses

The classic caspase-dependent programme is optimized to ensure that signals for phagocytosis are displayed well before cellular constituents might be released[3,56]. Does this also apply to caspase-independent programmes? A dominant uptake signal in mammalian cells is the translocation of phosphatidylserine to the outer leaflet of the plasma membrane (for more details, see the article by Peter Henson and colleagues in this issue). This 'eat-me' indicator is uncoupled from caspase activation in many model systems[9,12,23,25,33], and non-apoptotically-dying eukaryotic cells can be efficiently phagocytosed[23]. Mechanisms that can lead to the translocation of phosphatidylserine and phagocytosis in cells undergoing caspase-independent death include disturbances of cellular calcium homeostasis and the activation of protein kinase C[23,43]. Non-caspase cysteine proteases might also be involved in these alternative signal pathways. For instance, cathepsin B activity is required for the translocation of phosphatidylserine in some tumour cells challenged with TNF[9]. During the apoptosis-like death of platelets, phagocytosis signals are selectively blocked by calpain inhibitors[57]. Finally, genetic analysis in *C. elegans* has shown that the same phagocytosis recognition molecules are involved in removing corpses that have been produced by caspase-dependent apoptosis and caspase-independent necrosis[22].

Signalling in caspase-independent PCD

Several molecular mediators of classic caspase-mediated apoptosis pathways were characterized during the past decade[2,3,58], whereas the description of most alternative death routines remained limited to the phenomenological level. But recent mechanistic findings have opened a new era for this field. Like classic apoptosis, alternative death programmes can be mediated by proteases (TABLE 1) and switched on by death receptors (FIG. 1) or mitochondrial alterations (FIG. 2). The alternative signalling pathways regulated by these factors are discussed in more detail below.

Non-caspase proteases. Caspase-mediated cleavage of specific substrates explains several of the characteristic

Table 1 | **Death by more than a thousand cuts**

Protease activation	Protease	Antiprotease	Antiprotease inactivation	Comments
Cysteine proteases				
Several proteolytic steps; autoproteolysis/ proteolysis by other proteases (caspases, granzyme B, cathepsins).	Caspase	IAP	Proteolysis of IAP proteins (for example, caspases); displacement (DIABLO).	At least 14 isoenzymes in different cellular organelles; cleavage at defined motifs (Asp at cleavage site P1).
Autoproteolysis (pH dependent); other lysosomal proteases.	Cys-cathepsins	Cystatins	Proteolysis (for example, cathepsin D). Some cystatins are cytosolic (A,B) or extracellular (C), indicating many roles of cathepsins in these compartments.	Large protease family usually confined to lysosomes. Potential for change of substrate specificity and localization (cytosol, extracellular).
Many proteolytic steps; autoproteolysis (Ca^{2+} dependent); membrane translocation.	Calpains	Calpastatin	Proteolysis (for example, caspases, calpains).	Small family of proteases with important role in cytosolic/nuclear proteolytic signalling.
Other proteases				
Proteolysis, for example, by caspases; serine proteases (SerProt) are no longer inhibited upon cleavage of LEI.	SerProt	Serpins (for example, LEI)	Proteolysis (for example, elastase) or oxidation; protease inhibitor LEI is transformed into L-DNase II.	AP24 and other non-characterized serine proteases act as essential execution proteases together with, or independent of, caspases.
This specific class of serine proteases is activated by proteolysis (dipeptidylpeptidase I).	Granzymes	Serpins	Proteolysis	Many proteolytic roles in the the activation of caspases or Bid; direct induction of PCD and cleavage of nuclear lamins; found in the granules of T cells.
Ubiquitylation of substrates (ATP dependent).	Proteasome			Continuous turnover of short-lived proteins. Different proteolytic activities. Regulation of steady state of factors relevant for PCD.
Proteolysis Ceramide p53-dependent transcription.	Cathepsin D			Aspartyl protease; might translocate from lysosomes to cytosol or extracellular space and trigger apoptosis.

Information taken from REFS 4,9,64,54,114. IAP, inhibitor of apoptosis; PCD, programmed cell death.

features of apoptosis: for example, cleavage of the inhibitor of caspase-activated DNase (ICAD) leads to chromatin changes; cleavage of lamins results in nuclear shrinkage; cleavage of cytoskeletal proteins leads to cytosolic reorganization; and cleavage of p21-activated kinase-2 or Rho-activated serine/threonine kinase leads to blebbing[2,59]. So what brings about the apoptotic features observed in cells that die in a caspase-independent manner?

The first guess is, naturally, other proteases (for instance, cathepsins, calpains, serine proteases and the proteasome complex; TABLE 1). Indeed, data based on activity measurements, protease inhibitors and/or genetic deletion support their roles as essential cofactors either upstream or downstream of caspases in several cell death models[4,9,10,60–69]. Furthermore, many non-caspase proteases can cleave at least some of the classic caspase substrates, indicating that they might also mimic the cellular effects of caspases[62,64,65,68]. Accordingly, evidence is emerging for the ability of other proteases to induce apoptosis-like PCD in the absence of caspase activation. Examples include the roles of cathepsins D and B in camptothecin-induced death of liver cancer cells[10]; of cathepsin B in fibrosarcoma cells treated with TNF[9]; of the proteasome in colchicine-treated neurons[43]; and of calpains in vitamin-D-treated breast-cancer cells (M.J., unpublished observation). But more work is needed to define the role of the individual proteases in the complex process of PCD. Genetic approaches need to be combined with meticulous pharmacological titration of inhibitors[9], as it turns out that pan-caspase inhibitors, as well as many active-site inhibitors of other proteases, are highly unspecific at the concentrations widely used to test their role in PCD[9,62,64,70].

Death receptors as triggers. The best-studied members of the death-receptor family are TNF receptor-1 (TNFR1) and Fas (also known as CD95 or Apo-1). Whereas it has long been known that TNF-induced death can take the shape of either apoptosis or necrosis[71], the ability of Fas to induce necrosis-like PCD has been described only recently[19–21,34,72]. Interestingly, in activated primary T lymphocytes, this caspase-independent necrosis-like PCD seems, at least in some cases, to be the dominant mode of death[34]. This might explain why inhibition of caspase activity in mouse T lymphocytes *in vivo* does not induce the lymphadenopathy and/or autoimmune disease usually manifested in mice with inactivating mutations in Fas or Fas ligand[53].

The demonstration that caspase-8 is recruited to ligand-activated receptors through a receptor-associated

COLCHICINE
A microtubule-depolymerizing poison. Leads to loss of neurites in neurons and to apoptosis in most cell types.

PAN-CASPASE INHIBITORS
Cell-permeable irreversible inhibitors of all caspases examined so far that block or retard caspase-dependent processes. Widely used examples include z-Val-Ala-Asp-fluoromethylkethone (z-VAD-fmk) and Boc-Asp-fluoromethylketone.

INHIBITOR OF APOPTOSIS PROTEIN
(IAP). A class of proteins (IAP, XIAP, NAIP) containing a BIR domain that can act as an intracellular caspase inhibitor.

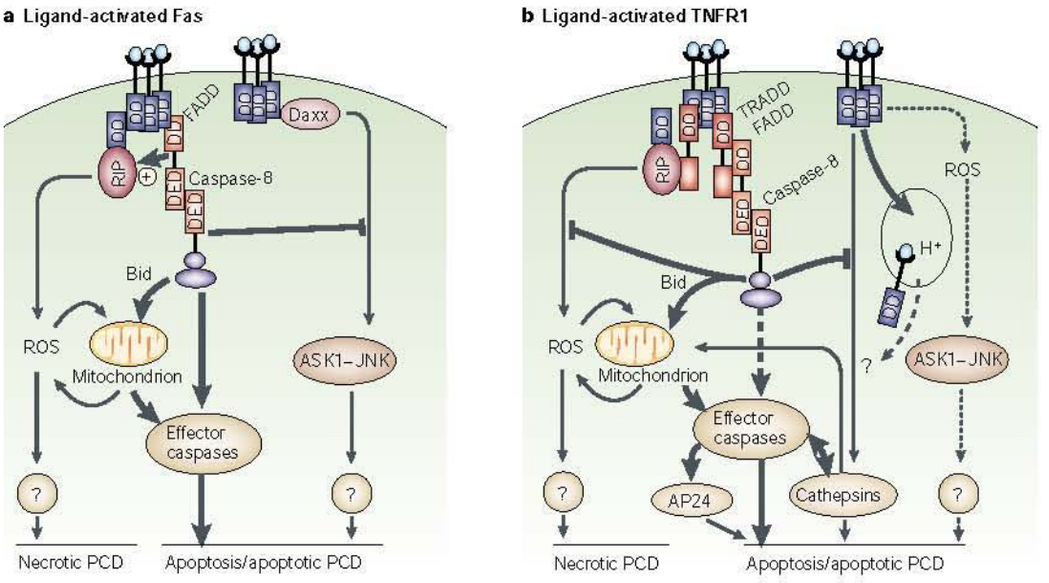


Figure 1 | **Multiple death pathways triggered by death receptors.** Death-receptor signalling is initiated by ligand-induced receptor trimerization. **a** | Receptor death domains (DD) of Fas then recruit FADD, RIP and/or Daxx to the receptor complex. Caspase-8 is activated after recruitment to FADD through interactions between the death effector domains (DED) in the two proteins, and triggers effector caspases either directly or through a Bid-mediated mitochondrial pathway[3] (thick arrows). RIP initiates a caspase-independent (thin arrows) necrotic pathway mediated by the formation of reactive oxygen species (ROS)[34]. Daxx activates the apoptosis-stimulating kinase 1/Jun amino-terminal kinase (ASK1–JNK) pathway, leading to caspase-independent apoptosis[73,74,77]. **b** | Tumour necrosis factor receptor-1 (TNFR1) signalling differs from that of Fas in the following steps. First, binding of FADD and RIP to the receptor complex requires the adaptor protein TRADD[3]. Second, binding of Daxx to TNFR1 has not been shown and the ASK1–JNK pathway is activated by ROS[73,75] (dotted line, caspase involvement unclear). Third, the RIP-mediated necrotic pathway is inhibited[34] by caspase-8. Fourth, TNFR1 can initiate a caspase-independent direct cathepsin-B-mediated pathway[9]. Fifth, cathepsin B can enhance the mitochondrial death pathway[61]. Last, the final execution of the death — that is, phosphatidylserine exposure, chromatin condensation and loss of viability — is brought about by effector caspases, the serine protease AP24 or cathepsin B in a cell-type-specific manner[3,4,9,61]. PCD, programmed cell death.

DEATH DOMAIN protein FADD led to the first molecular models of death-receptor-induced apoptosis[3] (FIG. 1). Later, Fas was also suggested to induce an alternative apoptosis programme, mediated by recruitment of the nuclear Daxx protein to the receptor, and the subsequent activation of apoptosis-stimulating kinase 1 (ASK1) and Jun amino-terminal kinases (JNK1/2)[73,74]. However, subsequent data from studies using primary fibroblasts derived from mice deficient for *Ask1* (REF. 75) or *Jnk1/2* (REF. 76) cast doubt on the relevance of this kinase pathway. This controversy might be resolved by a report indicating that, in many cells, the Daxx–ASK1–JNK pathway, which is not inhibited by pan-caspase inhibitors, might be either overruled by a more rapid induction of the caspase pathway or blocked by expression of the small heat-shock protein Hsp27 (REF. 77). ASK1 has also been found to be essential for TNF-triggered apoptosis of primary fibroblasts, but its activation by TNF seems to require REACTIVE OXYGEN SPECIES (ROS)[75] instead of Daxx[73]. It remains to be studied whether the TNFR1–ROS–ASK1 pathway is caspase independent. As noted above, TNF can induce a cathepsin-B-mediated apoptosis-like programme, even in the absence of caspase activity[9,42].

The molecular mechanisms underlying the necrosis-like PCD that can be induced by death receptors have also been worked out recently. The necrotic programmes induced by death receptors are dependent on the kinase activity of the receptor-interacting protein (RIP)[34] and the formation of ROS[20,34,48]. Whereas the function of FADD as a general and essential caspase-8 recruiter in death-receptor-induced apoptosis is well established[3], its role in the necrotic pathway is more complex. Although FADD is indispensable for Fas-induced necrosis-like PCD[34], it blocks TNFR1-induced necrosis, probably[34,46] by activating caspase-8. So, the concentration of FADD might be one of the switches between the apoptotic and necrotic pathways triggered by TNF.

The picture described above indicates a complexity of death-receptor-induced apoptotic and necrotic signalling networks that far exceeds that of the simple linear pathway originally indicated by the discovery of the receptor-triggered caspase cascade (FIG. 1).

Mitochondria as triggers. Many models of PCD involve some form of mitochondrial control, and it is useful to consider the signalling phases upstream and downstream of these organelles separately (FIG. 2). The pro-apoptotic Bcl-2-related proteins — such as Bax, Bak, Bid and Bim — have a dominant role at the mitochondrial stage of PCD signalling[2,3]. These proteins translocate to the mitochondria, or change their conformation and interaction partners on the mitochondria, in response to various death stimuli. The regulatory coun-

DEATH DOMAIN
A conserved sequence motif first identified in the intracellular parts of death receptors. Later recognized as the key motif for association of the receptors with cytosolic death-domain-containing proteins (FADD, TRADD, RIP) and the induction of cell death.

REACTIVE OXYGEN SPECIES (ROS). Collective term comprising intracellularly formed classic oxygen radicals and peroxides.

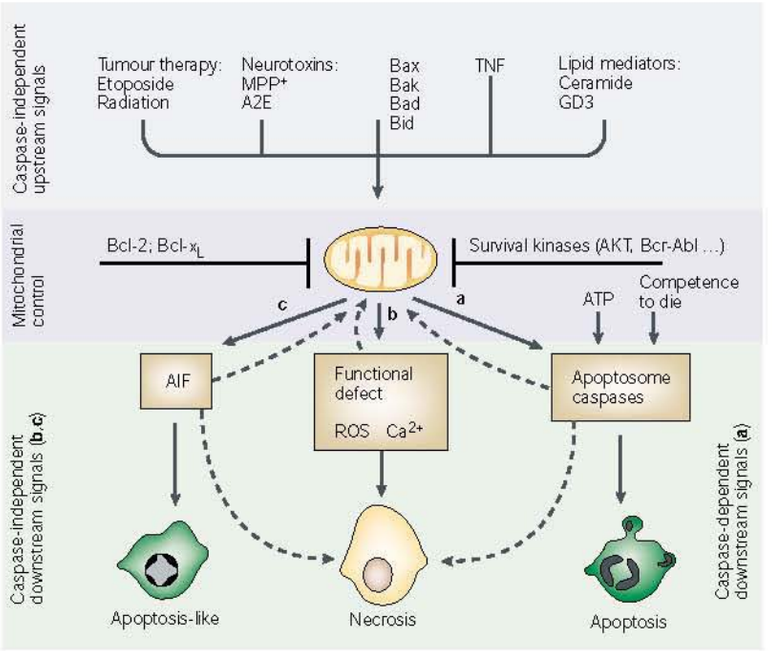


Figure 2 | **Mitochondrial roles in caspase-independent PCD.** Death triggers upstream of mitochondria usually do not require caspases. They include various chemotherapeutics, neurotoxins related to 1-methyl-4-phenylpyridinium (MPP) or the retinal cytotoxic pigment A2E, and lipid mediators such as ceramide or the disialoganglioside GD3[30,49,79]. Most signals can be blocked by anti-apoptotic Bcl-2 family members or survival kinases that act on this level[3]. Three fundamentally different and initially independent signals emanate from mitochondria. **a** | Cytochrome *c*, which leads to caspase activation in the apoptosome and thus triggers classical apoptosis[2,3]. **b** | Reactive oxygen species (ROS) and Ca^{2+}, which induce necrotic PCD[30,58]. **c** | The apoptosis inhibitory factor (AIF), which is released from mitochondria and triggers apoptotic-like death associated with chromatin condensation and margination, but not advanced chromatin compaction and nuclear fragmentation[78]. All three processes might feed back to mitochondria, affecting their function and structure, and therefore trigger one another[3,58,78]. Lack of essential cofactors for processes **a** and **c** will convert them to necrosis[30]. TNF, tumour necrosis factor.

terparts at this level include the anti-apoptotic members of the same family (for example, Bcl-2 and Bcl-x_L). Eventually, the ratio of death and survival signals sensed by the Bcl-2-family proteins determines whether the cell will live or die[2,3,30].

Three death pathways are triggered downstream of mitochondrial changes (FIG. 2). The caspase pathway leading to classical apoptosis[3,58] is initiated by the release of cytochrome *c* from the mitochondrial intermembrane space. Together with other essential factors (such as ATP), it triggers assembly of the APOPTOSOME complex, which forms the template for efficient caspase processing. As a further safeguard mechanism, caspase-inhibitory factors (IAPs) have to be removed by additional proteins (DIABLO/Smac) that are released from mitochondria before the execution caspases can become fully active and produce the typically apoptotic morphology[2,3].

The second mitochondrial death pathway leads to necrotic PCD, without necessarily activating caspases. A prominent example is TNF-induced necrosis-like PCD, mediated by mitochondria-derived ROS[35]. Intracellular control of this pathway is indicated by its susceptibility to attenuation by antioxidants[30,35].

The third distinct pathway from mitochondria is the release of the apoptosis-inducing factor (AIF) from the intermembrane space[78]. Recent genetic evidence indicates that this factor controls PCD during early development — that is, all the hallmarks of early morphogenetic death, including cytochrome *c* release, are prevented by deletion of AIF[18]. AIF induces caspase-independent formation of large (50 kb) chromatin fragments, whereas oligonucleosomal DNA fragments are generated only when caspase-activated DNase (CAD) is activated[3,78]. This biochemical difference is reflected by slight morphological differences in the shape of the condensed chromatin (FIG. 2, BOX 2).

Often, more than one of these three pathways seem to be activated simultaneously[6,58,78,79]. The cell fate (and death mechanism) is then determined by the relative speed of each process in a given model system and by the antagonists of the individual pathways that are differentially expressed in different cell types. However, the observation that AIF, cytochrome *c* and ROS/Ca^{2+} are released together in a given model does not necessarily imply that all pathways are concurrently triggered by mitochondria. Rather, AIF, caspases and ROS can feed back on mitochondria, causing enough structural and functional damage to trigger the release of other death factors, independent of the upstream signals[3,30,58,78].

Defects in any step of the cytochrome *c* or AIF pathways will result in a switch from apoptosis to cell death with a necrotic morphology[19,32,80]. This cell death would still fulfil the criteria of PCD, as it is blocked by the anti-apoptotic oncogenes Bcl-2 or Bcr-Abl[49,80], or by the deletion of pro-apoptotic Bax[81]. Also, caspase inhibition changes the mode of death, but not its extent, once the signal has arrived at mitochondria[19,30,32,46,49,80–82]. So it seems that in many models of cell death the master controllers of PCD operate at the mitochondrial level, whereas the decision on the shape of death is taken on the level of caspase activation[30].

There are, however, certain cases in which expression of Bcl-2 is not protective and in which mitochondria might not have a regulatory role[16,17,38,83,84]. Although alternative control mechanisms are not well characterized, emerging candidates include different chaperone systems, such as heat-shock proteins[6,16,77] or ORP150 (REF. 85). Organelles that have not received much attention recently, such as the endoplasmic reticulum[58] and lysosomes[65], might also have an essential role in the control of death.

The mechanism of cell death is not merely of academic interest — its importance stretches beyond the individual cell affected, as it might affect tissue reorganization and regeneration in the nervous system as well as immunological reactions in tumours. Characterization of the new death pathways throws light on diseases associated with dysregulated cell death, with possible implications for the classification and therapy of cancers and neurodegenerative disease.

Complex control of tumour cell death

Paradoxically, the cell proliferation induced by enhanced activity of ONCOPROTEINS (such as Myc, E1A, E2F and

APOPTOSOME
Multiprotein complex containing cytochrome *c*, Apaf-1 and procaspase-9. Catalyses ATP-dependent auto-activation of caspases by induced proximity. Key regulatory step of developmental apoptosis.

AIF
Apoptosis-inducing factor, a flavoprotein normally located in the mitochondrial intermembrane space. It is released during apoptosis and is involved in nuclear changes and death induction.

ONCOPROTEINS
Oncoproteins are encoded by oncogenes and their increased activity or expression promotes tumorigenesis either by inducing proliferation or by inhibiting cell death.

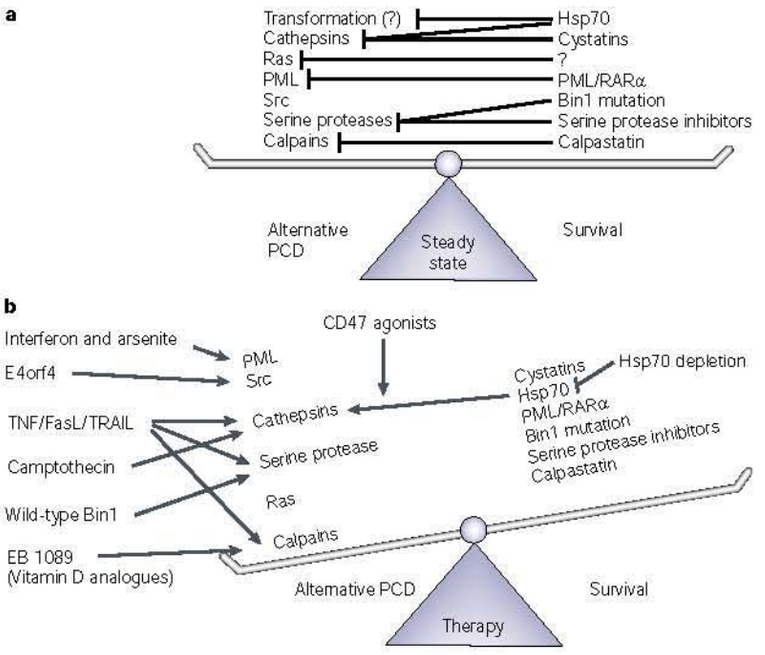


Figure 3 | **Alternative death pathways as regulators of tumour cell survival and as putative targets for cancer therapy. a** | Transformation is associated with upregulation of proteins that sensitize cells to caspase-independent programmed cell death (PCD)[38,88,90]. As a counterweight, death-promoting proteins are inactivated or expression of survival proteins is enhanced[17,52,65,92]. Analogous changes in proteins that regulate caspase-dependent apoptosis have also been shown in cancer[52,86]. **b** | Strategies of cancer therapy aimed at facilitating alternative death pathways[9–11,15–17,33,88,92]. Hsp, heat-shock protein; PML, promyelocytic leukaemia; TNF, tumour necrosis factor; TRAIL, TNF-related apoptosis-inducing ligand; RARα, retinoic acid receptor-α.

CDC25) or inactivation of tumour-suppressor proteins (retinoblastoma protein, for example) is often associated with caspase activation and accelerated apoptosis[86]. The coupling of cell division to cell death has therefore been suggested to act as a barrier that must be circumvented for cancer to occur[52,86]. Indeed, high expression of anti-apoptotic proteins (Bcl-2, Bcl-x_L, survivin, Bcr-Abl) and/or inactivation of pro-apoptotic tumour-suppressor proteins (p53, $p19^{arf}$, PTEN) controlling caspase-dependent apoptosis pathways are often seen in human tumours[52,86].

Alternative death pathways in cancer. Despite showing severe defects in classic apoptosis pathways, cancer cells have not lost the ability to commit suicide. On the contrary, spontaneous apoptosis is common in aggressive tumours, and most of them respond to some therapy[87]. One explanation might be that the different strategies used by cancer cells to escape apoptosis are not enough to counteract the lethal signals coupled to transformation. Alternatively, defects in the signalling pathways that lead to caspase activation might allow caspase-independent death pathways to take a dominant role in tumour cell death.

The alternative death pathways might be enhanced by transformation (FIG. 3). For example, oncogenic Ras can induce caspase- and Bcl-2-independent autophagic death[14], and tumour-associated Src-family kinases are involved in caspase-independent cytoplasmic execution of apoptotic programmes induced by adenovirus protein E4orf4 (REF. 88). Furthermore, a transformation-associated caspase-, p53- and Bcl-2-independent apoptosis-like death programme can be activated in tumour cell lines of different origins by depletion of a 70-kDa heat-shock protein (Hsp70)[16,89]. This death is preceded by translocation of active cysteine cathepsins from lysosomes to cytosol, and inhibitors of their activity partially protect against death (M.J., unpublished observation). Interestingly, cysteine cathepsins, as well as other non-caspase proteases, are highly expressed in aggressive tumours[90]. So expression of protease inhibitors might increase a cancer cell's chances of survival by impairing alternative death routes[9,65,91].

Alternative death pathways can also function at an initial step of tumorigenesis to limit tumour formation. Bin1, a tumour-suppressor protein that is often missing or functionally inactivated in human cancer, can activate a caspase-independent apoptosis-like death process that is blocked by a serine protease inhibitor or simian virus large T antigen, but not by overexpression of Bcl-2 or inactivation of p53 (REF. 17). Similarly, promyelocytic leukaemia (PML)/RARα oncoprotein also inhibits caspase-independent PCD induced by the PML tumour-suppressor protein[92]. Interestingly, cytoplasmic apoptotic features induced by ectopic expression of PML can even be enhanced by pan-caspase inhibitors[92]. It should, however, be noted that PML/RARα is also thought to interfere with caspase activation in some death models[93].

From theory to therapy. Although many cancer therapies induce classic apoptosis[87], potential drugs engaging other death routines are emerging (FIG. 3). For instance, the topoisomerase inhibitor camptothecin induces cathepsin D/B-mediated apoptosis-like PCD in hepatocellular carcinoma cells[10]; activation of a thrombospondin receptor (CD47) triggers programmed necrosis in B-cell chronic lymphoma cells[33]; interferons and arsenite initiate a caspase-independent death pathway, possibly mediated by PML[92]; and EB 1089, a synthetic vitamin D analogue, kills breast-cancer cells through a caspase-independent apoptosis-like PCD[15] mediated by calpains (M.J., unpublished observations). Moreover, increased tumour cell death observed *in vitro* when combining stimuli that activate different death-inducing proteases indicates that therapies activating various PCD pathways simultaneously might also be effective in the clinic[94,95].

Experimental gene-therapy approaches also point to alternative death pathways as promising targets for tumour therapy. For example, expression of Bin1 or adenovirus protein E4orf4, as well as depletion of Hsp70, result in tumour-specific induction of caspase-independent apoptosis-like PCD[11,16,17,89].

Alternative cell death in the nervous system

Caspase-driven neuronal apoptosis strictly following the classic apoptosome pathway is best documented during development of the nervous system[29], in which many superfluous cells are produced and turned over[96],

PTEN
A tumour-suppressor protein with phosphatase activity specific for inositol phospholipids. Negative regulator of protein kinase B (Akt) pathway in cells.

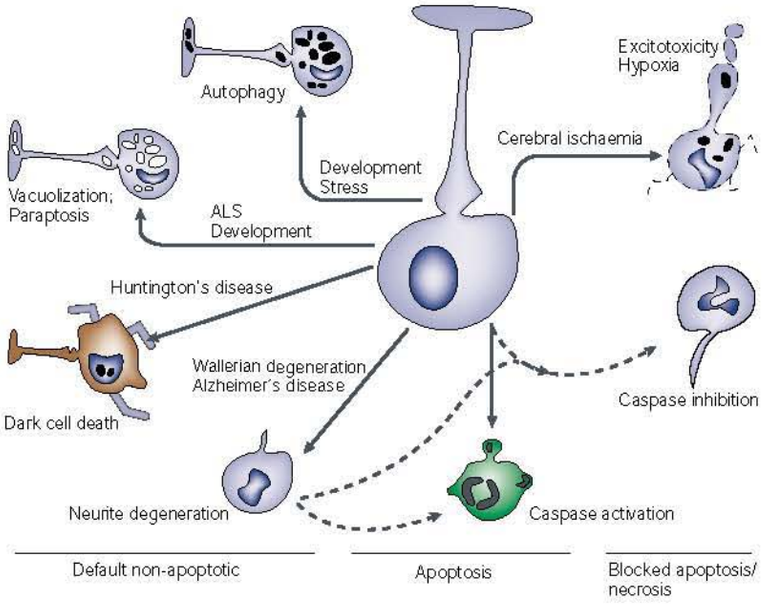


Figure 4 | **Different modes of neuronal death.** Developmental cell death occurs by caspase-dependent apoptosis[58] or morphologically and mechanistically distinct autophagy. In various human diseases or animal models of them, the dominant form of neuronal disease is, for example, dark cell death in a Huntington's disease model[40] or paraptosis in a model of amyotrophic lateral sclerosis (ALS)[104]. Selective neurite degeneration occurs independently of caspase activation in different situations, and might eventually lead either to caspase-dependent apoptosis of cell bodies or to non-apoptotic death with irregular chromatin condensation[43,83]. EXCITOTOXIC death may take many shapes and mechanisms, depending on the intensity of insult, the age of the animal and the region of the brain affected[105,106]. It often results in mixed apoptotic–necrotic features[30], including cellular swelling, blebbing, nuclear PYKNOSIS, display of phosphatidylserine and some autophagic processes, such as uptake of mitochondria into lysosomes.

and in *in vitro* cultures of cells derived from developing brain[58]. Evidence is scarce for adult neurons, and here caspase-dependent mechanisms might yield to alternative death pathways[97].

Cell suicide in the adult nervous system has serious implications for the whole organism, as turnover is classically limited. So a rapid caspase cascade, which is advantageous for efficient elimination of unwanted or rapidly replaceable cells, is dangerous in the developed brain and must be tightly controlled. For instance, neurons can survive cytochrome *c* release from mitochondria, provided that they do not simultaneously receive a second signal leading to a 'competence to die'[98]. Neuronally expressed apoptosis inhibitor proteins (IAP, NAIP) buffer the caspase system, and need to be inactivated before classic apoptosis can occur[2]. This buffering capacity might allow the localized activation of caspases[58] (within synapses or neurites, for example) or the sequestration of active caspases[99], without a build-up of the death cascade affecting the entire neuron. Stressed neurons might also acquire a temporary resistance, which allows them to withstand otherwise lethal insults — by excitotoxins, for example[100]. Such circumstances favour activation of slow, caspase-independent elimination routines, in which damaged organelles are digested within a stressed cell, and the chance for rescue and reversibility is maintained until the process is complete[101–103].

Although some caspase-dependent apoptosis might occur in the adult brain[58], at least part of PCD in chronic neurodegenerative disease follows alternative mechanisms and results in different morphologies[40,81,99,102,104–107] (FIG. 4). Further variation is observed in acute insults such as ischaemia or traumatic brain injury. In these cases, neurons within one brain region are exposed to different intensities of stress that trigger different death programmes. Some of the main excitotoxic processes, such as mitochondrial impairment and dissipation of cell membrane potential, differentially impair various secondary routines of PCD[30,105,106]. For instance, rapid ATP depletion or disturbance of the intracellular ion composition impair cytochrome *c*-induced caspase activation, and massive production of nitric oxide or calpain activation directly inactivate caspases[30,61]. Accordingly, cell death has mixed features of apoptosis and necrosis, and might rely on either caspases or calpains as the dominant execution proteases[68], or the activation of PARP[36] as a controller of programmed necrosis. Another group of proteases implicated as executors of ischaemic death are the cysteine cathepsins[67]. They might interact with calpains, and, notably, there is massive PCD in the brains of mice that lack the cathepsin inhibitor cystatin B[108].

The special shape of neurons (with projections up to 40,000 times longer than their cell bodies) allows degradative processes to be localized to a part of neurons and different death processes to be activated in different subsections of the cell[30,58]. For instance, synaptic damage and neurite regression can occur by Bcl-2- and caspase-independent mechanisms[43,83,109], whereas final elimination of cells might depend on caspases or proteasomal activities[43]. The role of caspases as enhancers of the final phase of cell degeneration might apply to many common diseases. The longevity of neurons, combined with their dependence on effective intracellular transport, makes them sensitive to a slow form of death, associated with the formation of intracellular polypeptide aggregates involving the amyloid-β precursor protein (APP), ataxins, presenilins, huntingtin, tau and α-synuclein[58]. As most of these proteins are caspase targets[110] and become more toxic after cleavage, caspases might contribute to the accelerated death of neurons at the end of a caspase-independent degeneration phase.

Outlook

The discovery and understanding of alternative death pathways will open new perspectives for the treatment of disease. On the one hand, the existence of caspase-independent pathways provides new options to kill tumour cells (FIG. 3), with one of these therapies (vitamin D analogues) already having advanced into phase III clinical trials. On the other hand, combination of caspase-directed and alternative therapies provides a more efficient approach to circumvent the commonly observed therapy resistance of transformed cells[94,95]. New options and targets also emerge for the prevention of death processes in neurodegenerative disease. Prominent examples that have reached the stage of clini-

PYKNOSIS
Poorly defined pathological term for nuclear condensation. Mainly used to describe forms of non-apoptotic chromatin condensation.

EXCITOTOXICITY
Neuronal death triggered by overexcitation of ion channel-gating (ionotropic) receptors (for example, ionotropic glutamate receptors). Release of endogenous excitotoxins triggers excitotoxic processes as common mechanism of neuronal loss during cerebral ischaemia.

HORIZONTAL TRANSFER
Non-germline transmission of genetic material.

cal trials target molecules such as PARP in necrosis or calpains in excitotoxicity[36,68,97].

On a more general biological level, the mode of cell death might have differential effects on the surrounding tissue[56]. The important roles of caspase-independent/alternative death for development of tumour immunity are just emerging (reviewed in REF. 23). Most recent evidence shows that the mode of cell demise controls the HORIZONTAL spread of oncogenic information[111] and infection[112]. As these processes can be favoured by caspase activation, the classic apoptosis pathways can, in fact, be detrimental to the organism. This might explain the need for extremely tight control of caspase activation by the cellular energy level[19]. The apparent paradox that death-bound ATP-depleted cells are not 'allowed' to activate caspases might then be explained by the fact that such cells would release activated caspases into the extracellular space upon premature lysis[113]. So non-apoptotic death might not only be a passive accident, but also, in some cases, be a desirable death option for long-lived organisms having to deal with tumours, infections and other non-lethal tissue insults throughout their lives.

Links

DATABASE LINKS Bax | CED-9 | Bcl-2 | CED-3 | poly(ADP) ribose polymerase | CD2 | TNF | p21-activated kinase-2 | cathepsin D | cathepsin B | TNFR1 | Fas | FADD | Daxx | ASK1 | JNK1 | Hsp27 | Bak | Bid | Bim | Bcl-x_L | DIABLO/Smac | AIF | CAD | ORP150 | Myc | E2F | CDC25 | survivin | p53 | $p19^{arf}$ | PML | RARα | CD47 | Bin1 | APP | ataxins | presenilins | huntingtin | tau | α-synuclein |

FURTHER INFORMATION Cell Death Society | Jäättelä lab

ENCYCLOPEDIA OF LIFE SCIENCES Apoptosis: molecular mechanisms | Apoptosis: morphological criteria and other assays

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Acknowledgements
We acknowledge our colleagues for stimulating discussions and the Danish Cancer Society, the German Research Council and the Danish Medical Research Council for financial support. We also apologize to those whose work could only be cited indirectly.