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Review

Caspase function in programmed cell death

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The first proapoptotic caspase, CED-3, was cloned from *Caenorhabditis elegans* in 1993 and shown to be essential for the developmental death of all somatic cells. Following the discovery of CED-3, caspases have been cloned from several vertebrate and invertebrate species. As reviewed in other articles in this issue of *Cell Death and Differentiation*, many caspases function in nonapoptotic pathways. However, as is clear from the worm studies, the evolutionarily conserved role of caspases is to execute programmed cell death. In this article, I will specifically focus on caspases that function primarily in cell death execution. In particular, the physiological function of caspases in apoptosis is discussed using examples from the worm, fly and mammals.
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Caspases are cysteinyl aspartate proteinases (cysteine proteases that cleave their substrates following an Asp residue). The first known member of the caspase family was caspase-1, initially known as interleukin-1β-converting enzyme (ICE), an enzyme required for the maturation of IL1β.[1,2] In 1993, the *Caenorhabditis elegans* cell death gene *ced-3* was cloned and its product was found to be similar to ICE and a developmentally regulated protein Nedd2 (now called caspase-2).[3–6] In subsequent years, a number of caspases have been cloned from various mammalian and non-mammalian species. Eleven caspases have been described in human, 10 in mouse, four in chicken, four in zebrafish, seven in *Drosophila melanogaster* and four in *C. elegans*.[7] As discussed in other review articles in this issue of *Cell Death and Differentiation*, many of the cloned caspases, including caspase-1, do not have a role in apoptosis execution, whereas some caspases have dual functionality having roles both in apoptotic and nonapoptotic signalling. In this article, I focus primarily on caspases that either have a clearly defined function in apoptosis or have an implied function in cell death pathways. Given that there is much in common in the apoptotic function of caspases from various model organisms, I have discussed here work from *C. elegans*, *D. melanogaster* and mammals.

Two Types of Apoptotic Caspases

Almost all healthy cells contain several caspases as inactive precursors, often called caspase zymogens. Although caspase zymogens contain a small amount of catalytic activity, they are kept in check by a variety of regulatory molecules. Upon receiving an apoptotic signal, the caspase zymogens undergo proteolytic processing to generate two subunits that comprise the active enzyme.[8,9] The cleavage of the zymogen is not always an obligatory requirement for caspase activation, but all activated caspases can be detected as cleaved fragments in apoptotic cells.[8,9] The structural studies predict that the mature caspase is a heterotetramer, composed of two heterodimers derived from two precursor molecules (see review by Bao and Shi in this issue). In addition to the regions that give rise to the two subunits, procaspases contain N-terminal prodomains of varying lengths. Based on the length of the prodomain, caspases can be divided into two groups: those that have a relatively long prodomain, and those containing a short prodomain.[8,9] The long prodomains in many caspases consist of specific protein–protein interaction domains that play a crucial role in caspase activation.[8,9] These domains mediate recruitment of the procaspase molecules to specific death signalling complexes, leading to their autocatalytic activation, by mechanism(s), the precise details of which are still a matter of some debate, but often termed 'proximity-induced' activation.[8,9] The caspases that get activated via recruitment to signalling complexes are known as the initiator caspases, as they provide a link between cell signalling and apoptotic execution (Figure 1). The main initiator caspases are caspase-2, -8, -9 and -10 in mammals and *Drosophila* Nedd2-like caspase (DRONC) in *Drosophila*. These caspases either contain a caspase recruitment domain (CARD), as in caspase-2, -9 and DRONC, or a pair of death effector domains (DEDs), as in caspase-8 and -10 (Figure 2). These domains bind similar motifs in adaptor proteins (Table 1). The caspases lacking a long prodomain lack the ability to self-activate and require cleavage by activated initiator caspases.[8,9] Given that most of the cellular substrates are cleaved by these downstream caspases (see

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Keywords: DRONC; DRICE; Apaf-1; cytochrome *c*; apoptosome; caspase activation; caspase function
Abbreviations: PCD, programmed cell death; DIAP1, *Drosophila* inhibitor of apoptosis protein 1; DED, death effector domain; CARD, caspase recruitment domain; DRONC, *Drosophila* Nedd2-like caspase; MEFs, mouse embryonic fibroblasts; IAP, inhibitor of apoptosis protein; RNAi, RNA interference; KO, (gene) knockout; DKO, double knockout; BH, Bcl-2 homology (domain); CNS, central nervous system; RHG, REAPER/HID/GRIM (domain); EcR, ecdysone receptor; Usp, ultraspiracle
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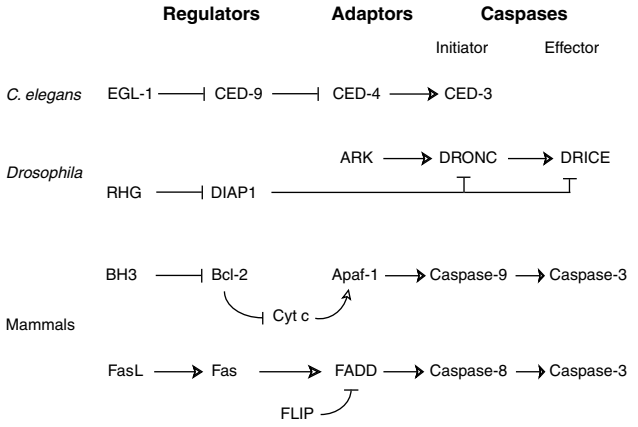

Figure 1 Evolutionary conservation of the caspase activation pathways. Various regulators that initiate caspase activation or keep caspase activation in check, adaptors that mediate activation of initiator caspases, the initiator and effectors caspases, in *Caenorhabditis elegans*, *Drosophila melanogaster* and mammals are shown

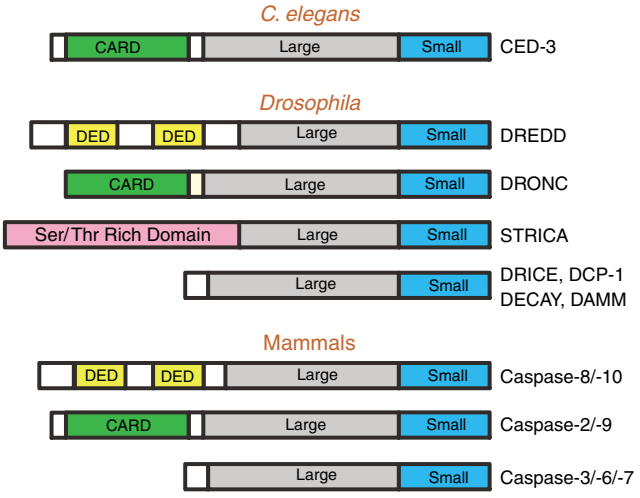

Figure 2 The initiator and effector caspases in *C. elegans*, *Drosophila* and mammals. Only caspases with potential or demonstrated role in apoptosis are shown. The small and large subunits, and the protein–protein interaction motifs (such as CARD and DED) are shown. Note that the long prodomain in STRICA does not contain any protein–protein interaction motif(s). The relative lengths of various domains and subunits are not drawn to size and only serve as a guide here to demonstrate the various types of apoptotic caspases found in *C. elegans*, *Drosophila* and mammals

review by Timmer and Salvesen in this issue), these caspases are often called the effector caspases. The key effector caspases in mammals include caspases-3, -6 and -7, and in *Drosophila* DRICE.

CED-3, the Death Caspase in *C. elegans*

Although there are four caspase-like proteins in *C. elegans*, CED-3 is the only one that has been shown to be required for apoptosis.[10,11] It is a CARD-containing caspase that acts both as initiator and effector caspase. EGL-1, a Bcl-2 homology (domain) (BH)3-only protein, CED-3 and CED-4, the adaptor

Table 1 Various adaptors for initiator caspases and the domains that mediate caspase/adaptor interactions

Caspase	Adaptor	Domain mediating interaction
CED-3	CED-4	CARD
DRONC	ARK	CARD
DREDD	dFADD	DED
Caspase-2	RAIDD	CARD
Caspase-8	FADD	DED
Caspase-9	Apaf-1	CARD
Caspase-10	FADD	DED

that mediates CED-3 activation, are all essential for all developmentally programmed cell death (PCD) in the worm and loss of function mutants of *egl-1*, *ced-4* and *ced-3* result in the survival of all 131 somatic cells that are destined to die.[10,12] The Bcl-2-like protein CED-9 by contrast prevents cell death by blocking the activation of CED-3 (Figure 1). Recent structural and biochemical data demonstrate that CED-3 is activated by a tetrameric CED-4 complex (apoptosome) that is prevented from assembling by interaction with CED-9 until EGL-1 sequesters CED-9 away.[13] In healthy cells, CED-4 is a dimer directly bound to CED-9, and presumably this interaction prevents CED-4 activation by tetramerization.[13] The elevated levels of EGL-1, primarily regulated by transcription in response to developmental cues, allow sequestration of CED-9 and release of CED-4. Following the release from CED-9, two CED-4 dimers form the active tetramer that recruits and facilitates CED-3 activation.[12,13] Like mammalian APAF-1 and *Drosophila* ARK (see below), CED-4 is a member of the P-loop ATPase family that comprises a CARD followed by a nucleotide-binding/oligomerisation domain, and a second conserved helical domain.[12,13] The CARDs in the CED-4 apoptosome directly bind the CARD of CED-3 facilitating proximity-induced autoactivation of CED-3.[12,13]

Cell Death Caspases in *Drosophila*

There are seven caspases in *Drosophila* named DCP-1, DREDD/DCP-2, DRICE, DRONC, DECAY, DAMM and STRICA/DREAM.[14–20] Similar to mammalian caspases, *Drosophila* caspases can be divided into initiator and effector caspases based on their prodomains.[21] The caspases DREDD, DRONC and STRICA contain long amino-terminal prodomains, whereas DCP-1, DRICE, DECAY and DAMM have short prodomains (Figure 2). DREDD contains two DEDs in its prodomain region, whereas DRONC is the only CARD-containing caspase in flies.[15,17] STRICA, the third fly caspase with a long amino-terminal region contains a Ser/Thr-rich prodomain that lacks any CARD or DED-like structures.[20] The significance and function of this unusual prodomain in STRICA is not currently known, and similar prodomains have not been reported in mammals. DCP-1 and DRICE are highly homologous to each other, whereas DECAY is the next closest relative of DRICE and DCP-1.[21] All these three caspases share a high degree of homology with the mammalian caspase-3.[21] Despite the presence of two DEDs in its prodomain, DREDD shows more overall sequence

homology to the CARD-containing mammalian caspase-2, than to the DED-containing caspase-8.[21] The primary amino-acid sequence of DRONC is also most similar to caspase-2, rather than its predicted functional homologue caspase-9.[21] DAMM and STRICA are distantly related to other fly caspases, and despite the difference in the length of their prodomains, share highly related caspase domains.[19–21]

Functions of Fly Caspases

DREDD. An initial report suggested that DREDD functions in cell death.[15] This conclusion was based on expression analysis and overexpression studies that showed that the mRNA for *dredd* accumulates in preapoptotic cells, and that signalling by RPR, HID and GRIM results in processing of DREDD to its active form in transfected *Drosophila* S2 cells.[15] Additional evidence for a function of DREDD in apoptosis came from the observation that heterozygosity at the *dredd* locus suppresses cell death induced by the ectopic expression of *rpr*, *grim* and *hid* in transgenic models, indicating that the concentration of DREDD may be a rate-limiting step in apoptosis.[15]

However, subsequent genetic data from the analysis of *dredd* mutants suggest that the primary function of DREDD is in innate immune response by regulating the activation of RELISH, a member of the NF-κB family.[22] Flies mutated at the *dredd* locus fail to induce the synthesis of antimicrobial peptides and are highly susceptible to infection by Gram-negative bacteria.[22] Recent data indicate that DREDD and its adaptor dFADD[23] are required for the activation of IKK and JNK, components of the *Drosophila* antibacterial immunity pathway.[24] dFADD and DREDD have also been shown to participate in spermatid individualization in *Drosophila*;[25] however, their exact function in that process remains unknown.

DRONC. DRONC was first cloned as a caspase with most homology to mammalian caspase-2 (Nedd2).[17] As it is the only CARD-containing caspase in fly, it can be regarded as the true CED-3/caspase-9 orthologue. Indeed, a number of recent studies establish DRONC as the main (and perhaps only) initiator caspase in *Drosophila*.[26–32] Earlier studies had shown that the heterozygosity at the *dronc* locus or the expression of a catalytically inactive DRONC mutant suppress the ablated eye phenotype caused by the overexpression of *rpr*, *hid* and *grim*, consistent with the idea that DRONC is a downstream effector in the pathways mediated by these DIAP1 antagonists (see below).[33–35] More recent studies using *dronc*-null animals have confirmed these findings and also shown that to suppress HID-induced apoptosis, both copies of *dronc* need to be inactivated.[26–30] Loss of DRONC function by RNAi results in almost complete loss of apoptosis in embryos, indicating that DRONC is required for most PCD during embryogenesis.[34] Similarly, in the germline *dronc* mutant embryos, most PCD is absent and these embryos fail to hatch.[28] Both *in vitro* and *in vivo* studies show that DRONC is essential for most PCD in larval tissues and apoptosis induced by cytotoxic agents, including X- and γ-irradiation.[26–35]

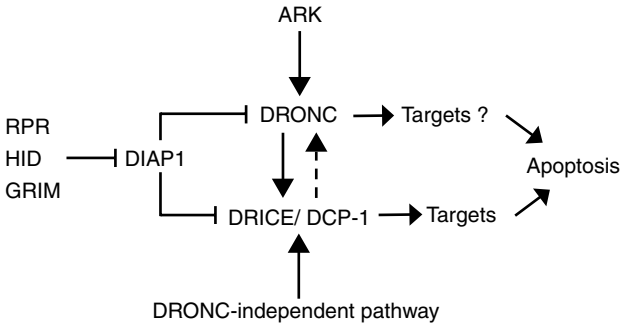

Figure 3 Pathways of caspase activation in *Drosophila*. The canonical caspase activation pathway in *Drosophila* involves removal of DIAP1 from DRONC via the RPR, HID and GRIM proteins that bind and facilitate autoubiquitination of DIAP1. Removal of DIAP1 allows DRONC to be activated in an ARK-dependent manner. Activated DRONC activates downstream effector caspases such as DRICE and DCP-1. In some cases, such as in larval midguts, effector caspases can also be activated by an unknown mechanism that is independent of DRONC

DRONC ablation also blocks apoptosis induced by the loss of DIAP1, suggesting that DRONC mediates cell death by signals that lead to DIAP1 degradation.[36]

dronc is upregulated by the steroid hormone ecdysone, which mediates the histolysis of larval tissues during metamorphosis, and loss of *dronc* delays the removal of larval salivary glands.[17,26,29] Furthermore, in cultured cells, *dronc* ablation by RNA interference (RNAi) inhibits cell death induced by ecdysone.[37,38] However, surprisingly, midgut removal and downstream caspase activation in larval midguts occur normally in *dronc* mutant animals,[26] suggesting that alternative ways of activating effector caspases exists in some cases (Figure 3). Gene expression and promoter analysis indicate that the *dronc* gene is regulated by the heterodimeric nuclear receptor EcR/Usp (ecdysone receptor/ultraspiracle) directly in response to ecdysone and also via the ecdysone-induced transcription factor BR-C.[37,38] CARMER, the fly homologue of the mammalian CARM1 histone methyl transferase, is also recruited to the *dronc* promoter via the EcR/Usp complex and positively regulates ecdysone-induced *dronc* expression.[39] *dronc* promoter is spatially and temporally regulated during *Drosophila* development.[40] Therefore, the levels of DRONC may determine the cell survival/death of specific cell populations during development. Although precisely how *dronc* expression is regulated is not understood, complex regulation by various transcription factors and chromatin modification/remodelling are likely to contribute to this process.[41]

Although DRONC is clearly a caspase which efficiently cleaves synthetic substrates such as VDVAD, and downstream caspases DRICE and DCP-1, following an Asp residue, recombinant DRONC expressed in *Escherichia coli* can autoprocess following a Glu residue in the P_1 position.[35] This unusual substrate cleavage activity of DRONC may be attributed to an atypical sequence of the catalytic site.[17] The known targets of DRONC include effector caspases DRICE and DCP-1 and removal of DRONC inhibits the processing of these caspases, suggesting that DRONC is required for downstream caspase activation.[31,32,42] DRONC cleaves and activates DRICE and DCP-1 at a TETD sequence.[31,35] In addition to its essential function in cell death, DRONC has also

been implicated in several nonapoptotic functions,[25,30,32] and it appears that low levels of spatially regulated activation is required for its nonapoptotic roles, whereas high level activation leads to apoptosis.[43]

DRICE. DRICE is the most abundant and widely expressed effector caspase in *Drosophila*.[16,21] It is also most similar to mammalian caspase-3 and exhibits similar substrate specificity, with an optimal activity on caspase-3 substrate DEVD.[16,21] In most cases, DRICE is activated by DRONC and RNAi and genetic studies have shown that loss of *dronc* function severely impairs DRICE activation.[27,28,42] However, DRONC-independent effector caspase activation (presumably DRICE) has also been noted in larval midguts.[27] A systematic *in vivo* RNAi study demonstrated that DRONC, DRICE, STRICA and DECAY are rate limiting for apoptosis.[36] This work also suggested that HID-mediated apoptosis requires STRICA, DECAY, DRONC and DRICE, whereas apoptosis induced by DIAP1 removal is primarily dependent on DRONC and DRICE.[36] Three laboratories have recently published studies of independently generated *drice* mutants.[30,44,45] The analyses of these mutants suggest that *drice* acts genetically downstream of RPR, HID, GRIM and DIAP1 and is required for most cell deaths that occur during development. Additionally, *drice*-null cells are resistant to stress and irradiation-mediated cell death. However, around 10% of animals survive in the absence of DRICE and in some tissues cell death can occur normally. Studies with a *drice/dcp-1* double mutant suggest that some cells require DRICE for apoptosis, whereas in other DRICE and DCP-1 play a somewhat redundant function.[30,45]

Like *dronc*, *drice* expression is also regulated by ecdysone in salivary glands during metamorphosis.[46] RNAi studies in an ecdysone-responsive *Drosophila* cell line and in salivary glands suggest that *drice* is necessary for efficient apoptosis induced by ecdysone.[36,46] However, in *drice* mutants, salivary glands seem to undergo normal cell death,[44] suggesting possible functional redundancy/compensation *in vivo*. In l(2)mbn cells, *drice* expression appears to be specifically controlled by the ecdysone-regulated transcription factor BR-C.[46] DRICE also seems to contribute to the nonapoptotic process of spermatid differentiation.[45] The known targets of DRICE include lamin DmO, DIAP1, DRONC and baculovirus p35.[21,47] In mammals, caspase-3 cleaves inhibitor of caspase activated DNase (ICAD), resulting in the release and activation of CAD, which mediates DNA fragmentation.[48] Similarly, DRICE mediates cleavage of *Drosophila* ICAD (dICAD).[49] However, unlike in mammals, caspase-mediated CAD cleavage is also necessary for dCAD activation.[49]

DCP-1. DCP-1 was the first cloned *Drosophila* caspase.[14] Initial studies reported that *dcp-1*-null mutations are larval lethal, with larvae carrying melanotic tumors.[14] The mutants also had other apparent developmental abnormalities and female flies carrying the *dcp-1*$^{-}$ germline clones were sterile owing to a defect in the transfer of nurse cell cytoplasmic contents to developing oocytes.[50] Later studies revealed that these phenotypes were owing to the loss of function of an outlying gene *pita*, rather than *dcp-1*.[51] The specific *dcp-1*-null animals are viable, develop normally and do not show any apparent phenotype, suggesting that this caspase is not essential for any developmental cell death and is functionally redundant.[30,44,45] Interestingly, the study of *drice/dcp-1* double mutant reveals that some cells strictly require DRICE for apoptosis, whereas other cells require either DRICE or DCP-1.[45] For example, the embryos and larval eye discs from *drice/dcp-1* double mutants show significantly reduced cell death when compared to *drice* mutants.[45] Recently, Kondo *et al*.[30] also reported that *drice/dcp-1* double mutants essentially phenocopy *dronc* mutants, suggesting that both these effector caspases act downstream of DRONC and have some overlapping function. *In vitro* RNAi data suggest that specific ablation of DCP-1 from l(2)mbn cells significantly reduces the rate of ecdysone-induced apoptosis.[46] Thus, although it seems that most cell deaths can occur in the absence of DCP-1, this caspase may yet contribute to the efficiency of cell death execution, at least in some cell types. Given its close similarity with DRICE, DCP-1 substrate specificity mimics that of DRICE cleavage specificity and both these caspases are likely to have similar substrates.[21]

DECAY, DAMM and STRICA. DECAY is also related to DRICE, DCP-1 and mammalian caspase-3 and has the ability to cleave DEVD *in vitro*.[18] Ectopic expression of DECAY has a weak apoptotic effect in mammalian cells,[18] but its function in *Drosophila* cell death pathways is somewhat less clear at present. Recently, the Miura laboratory generated a *decay* mutant and found that *decay* mutants are viable and fertile and do not show any obvious abnormalities.[30] Furthermore, the authors state that the developmental PCD occurred normally in *decay* mutants, suggesting that DECAY function is redundant, at least during development.

The functions of DAMM and STRICA, both of which can induce cell death *in vitro* to some extent, are not fully understood, as specific mutants for these caspases are currently unavailable. Ectopic overexpression of *damm* or *strica* in the *Drosophila* eye results in a rough eye phenotype, and the expression of these caspases sensitises cells to apoptosis.[19,20] As mentioned earlier, *strica* RNAi results in delayed removal of larval salivary glands,[36] consistent with the observation that *strica* transcript is upregulated in salivary glands prior to their histolysis.[52] These observations suggest that, along with DRONC, DRICE and DCP-1, STRICA may also contribute to the efficient PCD of salivary glands during *Drosophila* metamorphosis.

Activation of Fly Caspases

The main regulator of caspase activation (and caspase activity), and thus cell surival/apoptosis in *Drosophila* is DIAP1 (*Drosophila* inhibitor of apoptosis protein 1).[53,54] Unlike in mammals, the Bcl-2 family of proteins (two in *Drosophila*[55,56]) seem to play a less important role (if any) in regulating caspase activation in flies. DIAP1 binds and inhibits both the initiator caspase DRONC and effector caspases DRICE and DCP-1 (see review by Callus and Vaux in this issue). DIAP1 consists of two BIR (Baculoviral inhibitor of apoptosis protein (IAP) repeat) domains and a RING type of

ubiquitin-ligase domain. BIR1 interacts with DRICE, whereas BIR2 binds DRONC. DIAP1 mutant embryos and cells where DIAP1 has been ablated show spontaneous caspase activation and apoptosis.[57] Thus, it seems that in healthy cells DIAP1 is bound to DRONC, preventing its interaction with ARK. Upon receiving an appropriate death signal, RPR, HID and GRIM bind DIAP1 resulting in its autoubiquitination, followed by proteasome-dependent degradation. With the DIAP1 inhibition taken away, DRONC activation can now proceed in an ARK-dependent manner. Interestingly, unlike in mammals where Apaf-1 requires binding to cytochrome *c* that is released from mitochondria for its activation and oligomerzation (see review by Bao and Shi in this issue),[58] ARK-mediated DRONC activation can occur in the absence of cytochrome *c* release from mitochondria.[59] The trigger for DRONC activation is thus likely to be the removal of the inhibitory effects of DIAP1. This could be achieved by increasing the intracellular levels of RPR, HID or GRIM, or by elevating the concentration of DRONC and ARK, the scenarios that are seen during developmental cell death in *Drosophila*.[41]

Loss-of-function mutations in *ark* block most developmentally programmed cell death, resulting in extra cells in the embryos, hyperplasia of the central nervous system and other abnormalities.[60–62] *ark* mutants essentially phenocopy *dronc* mutants and show a block in the removal of larval salivary glands, but the histolysis of midgut appears normal in *ark*-null animals.[60–62] Caspase activation is severely impaired in the absence of ARK, suggesting that ARK is required for DRONC activation.[60] Structurally, ARK is more similar to its mammalian counterpart Apaf-1 than to CED-4, in that it consists of several WD40 repeats not found in CED-4.[59] However, it does not require cytochrome *c* for the formation of an apoptosome. In response to many apoptotic insults, *Drosophila* cells fail to show cytochrome *c* release and extensive RNAi, expression and biochemical data show that cytochrome *c* is not required for caspase activation and apoptosis in *Drosophila* cells.[42,63–66] ARK can assemble into a large apoptosome complex in the presence of dATP.[67] However, cytochrome *c* is not required for the apoptosome assembly and fails to bind the complex.[67] The 3D structure of the ARK complex at 18.8 A resolution using electron cryo-microscopy showed that eight ARK molecules assemble into an apoptosome-like particle.[67] Interestingly, the ARK apoptosome is a double-ringed structure.[67] Currently, the mechanism by which DRONC is recruited and activated by this apoptosome is not understood.

The Mammalian Apoptotic Caspases

In mammals, the function and regulation of caspases in cell death is complex, often complicated by the publication of conflicting data using reagents and inhibitors of dubious specificity. The data from caspase knockout (KO) animals has also been plagued by controversies, often due to the phenotypic anomalies in different genetic backgrounds of mice. This has lead to much confusion and reinterpretation of existing data and suggestions that caspases may not always be required for apoptosis. Nonetheless, caspase-2, -8, -9 and -10 are thought to be the initiator caspases, whereas caspase-3, and to a lesser extent caspase-6 and -7, serve as effector caspases (Figure 2; Table 2).[8,9] Caspase-9 is regarded as the canonical caspase in the intrinsic mitochondrial pathway and caspase-8 as the key initiator of death receptor-mediated apoptosis.[8,9] The role of caspase-2 remains somewhat controversial and enigmatic (see below).

Caspase-9. Caspase-9 is a key component of the mitochondrial death pathway (the intrinsic pathway) that is regulated primarily by the Bcl-2 family and the BH-3 (Bcl-2 homology domain-3)-only proteins.[8,9] In this pathway, cell death signals lead to cytochrome *c* release from the mitochondria, which binds and facilitates the formation of the septameric apoptosome that recruits and activates caspase-9.[58,68] The apoptosome-bound caspase-9 cleaves and activates caspase-3. Caspase gene KO in mouse emphasises the complexity of the caspase activation pathways. Depending upon the strain of mice, KO of caspase-9 results in embryonic or postnatal lethality.[69,70] In the mixed genetic background, the phenotypes of the *casp9*−/− and *casp3*−/− mice are quite similar suggesting that these caspases lie in the same pathway.[69–71] The most profound defect in *casp9* KO mice is hyperplasia in the brain resulting from decreased apoptosis.[69,70] Defective neural tube closure in the hindbrain region and enlarged proliferation zones in the fore and midbrain result in expansion and protrusion of cranial tissues. Cells from *casp9*-null mice show resistance to apoptosis induced by a range of cytotoxic drugs and irradiation.[69,70] Caspase-3 processing is deficient in *casp9* mutant brain tissue and lysates from caspase-9-deficient thymocytes or embryonic brains are unable to process caspase-3 even in the presence of cytochrome *c* and dATP. This activity can be restored by the addition of *in vitro* translated caspase-9 to the lysates.

As stated above, Apaf-1 (CED-4/ARK homologue) is required for caspase-9 activation. Overall, *apaf-1* KO animals have a phenotype similar to *casp9* KO animals, but they additionally show a delayed removal of interdigital webbing in embryos.[72,73] Caspase-3 activation is reduced in *apaf1*-deficient cells and mutant cells show resistance to a variety of apoptosis stimuli.[72,73] Additional defects seen in *apaf1* KO but not in *casp-9*−/− mutants indicate that Apaf-1 may function in developmental cell deaths that are independent of caspase-9 activity. Interestingly, Honarpour *et al*.[74] reported that around 5% of the *apaf-1*-deficient animals survive to adulthood. These animals show normal brain development, but are male sterile because of degeneration of spermatogonia. More recently, mixed background *apaf-1* KO animals, backcrossed into C57BL/6 background for more than five times, generated fertile males in the complete absence of Apaf-1.[75] When crossed with heterozygous *apaf-1* females, 45% *apaf-1* homozyous animals were born alive, many survived for more than eight weeks, some survived to adulthood and were fertile.[75] The Apaf-1/caspase-9 pathway also appears to be dispensable for the apoptosis of many haematopoietic lineages and loss of either caspase-9 or Apaf-1 only delays apoptosis but fails to provide clonogenic survival of cells.[76–79] These data suggest that caspase-9/Apaf-1 pathway is not essential for normal animal development or cell death and the genetic background of mice is a major factor in many of the observed phenotypes.

Table 2 A summary of studies employing loss-of-function mutations, gene knockout (KO) or RNAi to demonstrate caspase function *in vivo*

Caspase and the adaptor genes	Mutant phenotype	Physiological function from mutant analysis and RNAi data
C. elegans		
ced-3	Animals are viable but have extra 131 cells	Essential for the programmed death of almost all somatic cells
ced-4 (adaptor)	Same as *ced-3* mutants	Similar to *ced-3*
Drosophila		
Dronc	Pupal lethality, multiple cell death defects in embryos, larvae and prepupae	Essential for most programmed cell death (PCD) during development and for stress-induced apoptosis
ark (adaptor for *dronc*)	Essentially similar to *dronc* mutants	Essential for most developmental PCD and stress-induced apoptosis
Dredd	Normal development	No clear function in PCD. Required for innate immunity
Strica	Specific mutants not available	*strica* RNAi delays larval salivary gland removal
Drice	Pupal lethal. About 10–20% animals develop to adulthood. Reduced PCD in embryos, larvae and prepupae	Required for much of the developmental PCD and stress-induced apoptosis. Some redundancy with DCP-1
dcp-1	Mutants are viable and fertile	Relatively minor role in PCD that overlaps with DRICE function
drice/dcp-1	Prepupal lethal. Phenotype similar to *dronc* mutants	Some overlap in function with DCP-1. However, DRICE is the predominant effector caspase in fly
Decay	Mutants are viable and fertile	No clear function in PCD established as yet
Damm	Specific mutants not available	Physiological function not established
Mammals		
caspase-2	Mice are viable and fertile. MEFs show some resistance to killing by HS and specific drugs	Functionally implicated in many cell death and other pathways. However, the KO data do not support many of the *in vitro* studies
caspase-3	Perinatal lethal in mixed genetic background, but some animals can survive to adulthood. Hyperplasia in brain	Required for neuronal cell death in mixed genetic background. May play redundant function in other tissues
caspase-6	Normal development	No reported cell death defects in mutants
caspase-7	Mutants are viable and fertile	No apparent apoptotic defects in mutants
caspase-3/-7 DKO	Perinatal lethal. Defects in cardiac development. MEFs show resistance to apoptosis	Caspase-3 and -7 are required for mediating mitochondrial events of apoptosis
caspase-8	Embryonic lethal. Defects in cardiac and T-cell development. All death receptor-mediated apoptosis impaired in $-/-$ cells. Familial mutations in humans associated with immunodeficiency	Essential for apoptosis mediated by TNF family members. Has nonapoptotic functions in vasculature development and activation-induced lymphocyte proliferation
FADD (adaptor for caspase-8)	Essentially similar to caspase-8 KO mice	Essential for caspase-8 activation
caspase-9	Perinatal lethal, but some animals survive to adulthood. Defects in apoptosis in the CNS. Some cells resistant to stress-induced apoptosis	Required for efficient apoptosis in some cell types. Apparently not required for apoptosis in haematopoietic cells
Apaf-1 (adaptor for caspase-9)	Essentially similar to caspase-9 KO mice. Some animals survive to adulthood	Essential for caspase-9 activation. May have additional functions
caspase-10	There is no *caspase-10* homologue in mouse. Familial mutations in patients with autoimmune lymphoproliferative syndrome type II	Possibly required for apoptosis mediated by the death receptors

Please see text for citations and references

The cytochrome *c* knock-in mice in which the normal allele is replaced by a mutant cytochrome *c* (known as the KA allele), which retains normal respiratory function but does not activate Apaf-1, show embryonic or perinatal lethality and a phenotype somewhat similar to *apaf-1* KO, with brain abnormalities.[80] Interestingly, while the fibroblasts from the KA mice are resistant to apoptosis, their thymocytes are more sensitive to apoptosis than the *apaf-1*-null thymocytes. Following irradiation, KA thymocytes undergo apoptosis and caspase activation in the absence of apoptosome formation, suggesting the existence of an apoptosome-independent mechanism of caspase activation in thymocytes.[80] The KA mice confirm that cytochrome *c* is essential for Apaf-1 activation, and also provide evidence for alternative pathways of caspase activation.

Caspase-8. Caspase-8 is an essential component of the extrinsic cell death pathways initiated by the TNF family members.[81,82] In response to the activation of the death receptors of the TNF family, caspase-8 is recruited to the death-inducing signalling complex (DISC) via binding to the adaptor protein FADD.[81,82] This recruitment results in caspase-8 activation and cell death.[83,84] The loss of function of caspase-8 provides one of the clearest phenotypes to support its role in extrinsic pathway of cell death. *casp8* KO mice are embryonic lethal around E12 with

smaller embryos displaying impaired heart muscle development and an accumulation of erythrocytes in the abdomen and in blood vessels in the trunk.[83] Excessive erythrocytosis is also seen in the liver of mutants although the mutants contain reduced haemopoetic stem cell numbers. *casp8*-null fibroblasts are resistant to apoptosis in response to the ligation of death-inducing TNFR family members, although cell death via the intrinsic pathway is not affected.[83] These results suggest that while caspase-8 plays nonredundant roles in normal embryonic development and apoptosis mediated by the TNFR family members, it is dispensable for other apoptotic pathways. There is some evidence to suggest that the loss of human caspase-8 leads to immunodeficiency. Chun *et al*.[85] described a kindred with inherited caspase-8 deficiency. Individuals homozygous for the allele show defective lymphocyte apoptosis and homeostasis. Additionally, defects in their T and B cells and natural killer cells were reported. Consistent with this finding, conditional deletion of *casp8* gene in mouse shows both apoptotic and nonapoptotic functions of caspase-8.[84] While *casp8* KO in hepatocytes resulted in resistance to Fas-induced apoptosis, KO in the bone marrow cells leads to a block in haematopoietic progenitor functional potential. Caspase-8 is also required for macrophage differentiation and in endothelial cells for the formation of yolk sac vasculature.[84]

Caspase-8 activation requires the adaptor FADD and the study of *fadd* KO animals recapitulates this nicely. The *fadd* KO in mice is lethal around E12.5.[86,87] The *fadd*-deficient animals display features of delayed development, similar to the *casp8* KO embryos. At E10.5, the ventricular myocardium is thinner than normal and the inner trabeculation is poorly developed, indicating that FADD plays a nonredundant role in heart development. The mutants also show abdominal haemorrhage, which is not caused by abnormal blood vessel development. The *fadd*-deficient mouse embryonic fibroblasts (MEFs) show resistance to apoptosis induced by TNF family members; however, apoptosis induced by the overexpression of oncogenes or by cytotoxic drugs is not affected, suggesting that the intrinsic pathway is intact. Conditional *fadd* KO data indicate that like caspase-8, FADD is required for T-cell development and peripheral T-cell homeostasis.[88,89] The similarity in the phenotypes of *casp8* and *fadd* mutant mice suggests that FADD and caspase-8 are essential for apoptosis mediated by many of the TNFRs, but also play context-dependent nonapoptotic functions (see article by Lamkanfi *et al*. in this issue).

Caspase-10. Caspase-10 is similar to caspase-8 and contains two DEDs like caspase-8,[8,9] however its function in apoptosis remains a matter of considerable debate. Interestingly, there is no mouse homologue of caspase-10. Some data suggest that human caspase-10 may have a function overlapping with caspase-8 in Fas ligand-mediated cell death pathway.[90,91] Similar to caspase-8, caspase-10 is recruited to CD95 DISC.[90] Furthermore, it can cleave Bid and activate the mitochondrial pathway *in vitro*.[91] Caspase-10 has also been shown to sensitise cells to TRAIL-induced apoptosis.[92] Recently, caspase-10, along with caspase-8, was shown to be essential for mediating NF-κB-dependent inflammatory responses in antiviral signalling.[93] Missense and inactivating mutations in *casp10* gene have been reported to be associated with some cases of the autoimmune lymphoproliferative syndrome II (ALPS II),[94] non-Hodgkin lymphoma[95] and gastric cancer,[96] however it is unclear whether these mutations directly contribute to the disease phenotype.

Caspase-2. Although caspase-2 was one of the first apoptotic caspases discovered, its physiological function remains a matter of considerable debate and somewhat of an enigma. Caspase-2 contains a CARD that facilitates dimerisation of procaspase-2 molecules,[97,98] interaction with CARD-containing protein RAIDD[99] or recruitment to large multiprotein complexes,[100] events that trigger procaspase-2 activation. Caspase-2 is recruited into a protein complex similar to the Apaf-1/caspase-9 apoptosome.[101] The adaptor proteins RAIDD and PIDD have been identified as members of this large complex designated the 'PIDDosome'.[101] An unusual feature of caspase-2 is its localisation to the nucleus and the Golgi in addition to cytosol, although its protein targets in these compartments remain obscure.[102–105] In some cells, caspase-2 was demonstrated to be required for mitochondrial outer membrane permeabilization (MOMP) and the release of apoptogenic factors in response to DNA-damaging agents.[106] Caspase-2 has also been shown to be necessary for TRAIL-mediated,[107,108] heat shock (HS)-induced apoptosis,[109,110] as well as oocyte cell death.[111] Recently, caspase-2 was shown to associate with Fas/CD95 DISC and get activated in that complex, but apparently not required for CD95-induced cell death.[112] Caspase-2 activation occurs rapidly and acute ablation of caspase-2 in a multitude of cell lines inhibits apoptosis in response to a range of stimuli including anticancer drugs.[106,113–120]

Caspase-2 has also been shown to promote MAPK and NF-κB activation and may have some functions independent of its protease activity (see review by Lamkanfi *et al*. in this issue). Curiously, *casp2*-null mice develop normally with only minor apoptotic defects in some cell types, suggesting that the function of caspase-2 in developmental cell death and in the adult animal is either redundant, or compensated by other caspases in the KO animals.[102,121] Cells derived from the *casp2*-deficient mice show normal sensitivity to many apoptotic stimuli.[102,121] Recently Tu *et al*. reported that *casp2* $-/-$ MEFs are resistant to apoptosis induced by HS.[109] However, another study by Milleron and Bratton[122] failed to see any role for caspase-2 in HS-induced MEF cell death. The phenotype of *casp2/casp9* double KO is similar to *casp9* KO, suggesting that these two caspases are unlikely to be functionally redundant.[77] Although some support for a compensatory pathway in *casp2*-deficient cells comes from a report where the authors found that in *casp2*-deficient neurons, the NGF-deprivation induced caspase-2-dependent cell death becomes dependent on the caspase-9 pathway and *casp2*-null neurons show a three-fold compensatory elevation of caspase-9 expression.[123] Similar to the *casp9*-null thymocytes, the thymocytes derived from the *casp2/casp9* double knockout (DKO) are normally sensitive to cell death, suggesting that in these cells these caspases do not

compensate the loss of each other.[77] Furthermore, B and T cells from the DKO mice are normally sensitivity to a variety of apoptotic stimuli including cytokine withdrawal and dexamethasone.[77] These results suggest that caspase-2 is not essential for most physiological cell death, or plays a redundant role in apoptosis. Another possibility is that caspase-2 is primarily required for executing apoptosis of aberrant cells (such as transformed cells).

The exact mechanism of caspase-2 activation is still a matter of considerable debate. Some data suggest that dimerisation of caspase-2 molecules is sufficient to mediate its activation, and recruitment of caspase-2 to large apoptosome-like complexes, such as the PIDDosome, may facilitate caspase-2 dimerisation.[98,101] It is possible that caspase-2 activation occurs by more than one mechanism. Recent data suggest that phosphorylation of caspase-2 at Ser157 by protein kinase CK2 (PKCK2) keeps it inactive.[108] During TRAIL-mediated apoptosis, a lowering of PKCK2 levels results in dephosphorylation of caspase-2 that leads to caspase-2 dimerisation and activation in a PIDDosome-independent manner.[108] In *Xenopus* oocytes, CamKII-mediated phosphorylation of caspase-2 appears to prevent its activation.[111] These data are consistent with a model that predicts that the main event in caspase-2 activation is precursor dimerisation that is prevented in healthy cells by phosphorylation of caspase-2. Whether this is a general mechanism of caspase-2 activation or limited to specific cell death signalling remains to be determined. Owing to many contradictory and confusing publications, it is unclear how active caspase-2 induces apoptosis. However, one plausible model is that caspase-2 cleaves and activates Bid, a BH3-only protein, which in turn engages the mitochondrial pathway to induce apoptosis.[110]

Caspase-3, -6 and -7. Caspase-3 is the main downstream effector caspase that cleaves the majority of the cellular substrates in apoptotic cells.[124] It is activated following cleavage by caspase-8 or -9, but not by caspase-2.[8,9,124] Caspase-7 is highly similar to caspase-3 and has similar substrate specificity.[8,9] *casp3* mutant mice in genetically mixed 129/SvJ $\times$ C57BL/6 background die prenatally and show a phenotype somewhat similar to *casp9* KO with hyperplasia of brain tissue.[71] In these animals, reduced cell death and increased cell number in the central nervous system (CNS) of casp3-null animals becomes apparent by E12. Protrusions of the neuroepithelium in the retina are also visible due to reduced apoptosis. Cells derived from these animals show DNA fragmentation defects following treatment with cytotoxic agents, although most cells eventually die by apoptosis.[71] However, in pure C57BL/6 background, *casp3*-null animals are viable but show reduced fertility, suggesting that caspase-3 is redundant for most developmental cell death.[125] *Casp7*-null animals also develop normally and cells derived from these animals are sensitive to apoptosis induced by a range of agents.[125] In C57BL/6 background, *Casp3/Casp7* DKO mice die rapidly after birth but most animals show normal appearance.[125] The DKO mice display defects in heart development. Additionally, the MEFs derived from DKO animals show resistance to apoptosis induced by UV, staurosporine, FasL and TNF, suggesting that combined function of caspase-3 and -7 is required for cell death execution.[125] The DKO thymocytes show resistance to apoptosis induced via the intrinsic pathway (treatment with staurosporine and etoposide), but, interestingly, not to apoptosis induced by the death receptor pathway. The DKO cells also display delayed Bax translocation and cytochrome *c* release from mitochondria, prompting the suggestion that caspase-3 and -7 function in amplifying mitochondrial caspase activation signalling.[125]

Although structurally similar to caspase-3 and -7, caspase-6 has a somewhat different substrate specificity with optimal substrate being VEHD, rather than DEVD that is preferred by caspase-3 and -7.[126] The function of caspase-6 remains a mystery as it does not seem to be essential for apoptosis induced by various agents. In earlier studies, caspase-6 was shown to be required for the apoptotic cleavage of lamin A.[127] However, caspase-6 KO mice develop normally[128] and caspase-6-deficient DT40 chicken lymphoma cells are sensitive to killing by cytotoxic drugs.[129]

The caspase mutant and KO data suggest that while some caspases play essential roles in specific cell death pathways, others are either redundant, or compensated in the KO situations (Table 2). The studies also show that individual caspases may participate in apoptosis execution in a context-specific manner.

Regulators of Mammalian Caspases

Given that active caspases execute cell dismantling, caspase activation is tightly regulated in various ways. Some of these regulatory mechanisms and caspase inhibitors are covered in a review by Callus and Vaux in this issue; however, caspase regulation by FLIP and the inhibitor of apoptosis proteins (IAPs) is worth a mention here given the important functions of these molecules in regulation of apoptosis. The gamma-herpesvirus encodes v-FLIPs (viral FLICE-inhibitory proteins), which interfere with apoptosis signalling through the death receptors.[130] The mammalian homologue of v-FLIP is cellular FLIP (cFLIP or FLIP).[131,132] Both viral and cellular FLIPs contain two DEDs, which interact with the DED in FADD, thus inhibiting the recruitment and activation of caspase-8 by TNFR family members.[130–132] cFLIP is a key regulator of extrinsic pathway of caspase-8 activation. FLIP is upregulated by the NF-κB pathway and this leads to cell survival in response to TNF.[133] Recent data suggest that TNFα-mediated JNK activation accelerates turnover of the NF-κB-induced FLIP.[134] JNK acts via phosphorylation and activation of the ubiquitin ligase Itch, which then ubiquitinates FLIP, leading to its proteosomal degradation, thus sensitising cells to apoptosis. The *flip* KO mice die around E10.5 and, similar to *fadd* and *casp8* KO, show impaired heart development.[135] However, *flip* KO MEFs are highly sensitive to apoptosis induced by Fas ligand or TNF and show rapid caspase activation, suggesting that FLIP is necessary for protecting cells against caspase-8-mediated apoptosis mediated by TNFR family members.[135]

The mammalian IAP-like proteins include XIAP, cIAP-1, cIAP-2, NAIP, ML-IAP, ILP-2 and survivin (reviewed by Callus and Vaux in this issue). Among these, only XIAP, c-IAP-1 and -2 can physically interact with caspases and inhibit mature

caspase-3, -7 and -9.[57] However, only XIAP inhibits caspases at physiological concentrations. Structural studies suggest that the N-terminal BIR2 linker region of XIAP occupies the catalytic site in the active caspase-3 and -7, whereas the adjacent BIR2 may help to stabilise the interaction.[57] In contrast, the XIAP BIR3 domain is required for inhibiting caspase-9.[57] During apoptotic signaling, SMAC/DIABLO and HtrA2 are released from the mitochondria into the cytoplasm where they bind to XIAP and prevent its ability to inhibit caspase activity.[136] Mature SMAC/HtrA2 contain the characteristic IAP-binding REAPER/HID/GRIM (RHG) motif present in *Drosophila* RPR, HID and GRIM that binds to the BIR domain of IAPs. Structual data show that the binding of SMAC to the BIR2 and BIR3 domains of XIAP disrupts its ability to inhibit the caspases by steric hindrance.[136] Although the presence of IAPs and RHG (functional homologues of RPR, HID and GRIM) proteins in mammals suggest conserved mechanisms of caspase regulation, unlike in *Drosophila*, the function of these proteins in mammals does not appear to be essential for apoptosis regulation. The *smac* KO mice develop normally and *smac*-deficient cells are sensitive to apoptosis induced via both intrinsic and extrinsic pathways.[137] *HtrA2* KO animals die around 30 days after birth owing to a neurodegenerative disorder, but cells derived from these animals fail to show any resistance to apoptosis.[138] Combined deficiency of HtrA2 and SMAC leads to a phenotype similar to HtrA2 deficiency alone, suggesting that these two proteins are not compensating for each other.[138] *Xiap* KO mice are also phenotypically normal and fail to show any defects in apoptosis.[139] Although it is possible that in the absence of XIAP, SMAC and HtrA2, other yet unknown mechanisms can compensate for these proteins, the KO phenotypes suggest that these proteins are themselves not essential for developmental PCD. However, elevated levels of IAPs are often associated with resistance to apoptosis in human cancer cells and lowering IAPs using SMAC mimetics is known to sensitise cells to cytotoxic agents.[140,141]

Perspectives

It has been around 15 years since the first caspase was discovered. During this time, the pendulum has shifted from earlier predictions that all caspases play some role in the execution of apoptosis, to caspases may not be required for many forms cell deaths. The evolutionary conservation of caspases, and the fact that the loss of CED-3 (or its activator CED-4) results in a complete block in the developmental death of all somatic cells that are programmed to die, clearly suggest that caspases have evolved to be essential components of the cell death machinery. This concept is further supported by biochemical, cellular and genetic studies in *Drosophila*, where a single CED-3/caspase-9 homologue DRONC is essential for most developmental cell death and all stress-induced apoptosis. However, genetic and *in vivo* studies have also provided us the knowledge that some cell death and caspase activation can occur in the absence of DRONC, and apoptotic caspases can also function in some nonapoptotic pathways. Clearly, some caspases have no roles in apoptosis, some have both apoptotic and nonapoptotic functions, whereas others are primarily involved in executing apoptosis (see also other reviews in this issue). Based on *C. elegans* and fly studies one could argue that all apoptosis and most (if not all) developmental PCD require caspase function. It is probably the same in mammals, but mammals may have evolved alternative strategies to delete unwanted cells when caspases or caspase function is abrogated (such as in KO mice). These strategies may involve the alternate use of caspases or other pathways that are usually not involved in apoptosis.

A number of important issues and controversies remain unresolved in caspase biology. These include (but not limited to) (i) how to interpret and explain caspase redundancy and compensation in mammals and the KO phenotypes in mixed genetic backgrounds, (ii) how some caspases can function in both apoptotic and nonapoptotic pathways and factors that regulate the alternative caspase function, (iii) what pathways of caspase activation exist in the absence of initiator caspases, (iv) whether physiological cell death in the complete absence of caspases is indeed programmed or entirely different from apoptosis, (v) what caspase targets are essential for dismantling apoptotic cells and for the characteristic apoptotic phenotype and (vi) how precisely are caspases activated and regulated in response to different apoptotic and nonapoptotic signals. Hopefully, the ongoing genetic studies using various model systems and careful *in vitro* biochemical and molecular analyses coupled with more reliable reagents will help to explore the unresolved issues and clarify some of the controversies in the caspase field.

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