



## Review

# Apoptosis without caspases: an inefficient molecular guillotine?

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## Abstract

Since the discovery that the cysteine protease CED-3 was essential for developmental death in the nematode *C. elegans*, the search has been on to identify homologous proteases governing mammalian apoptosis. Fourteen of these proteases, now called caspases, have been found to date, and studies with natural or chemical inhibitors, and more recently knock-out mice, confirmed the involvement of at least a subset of these proteases in various forms of mammalian apoptosis. However, there has been recent evidence that some apoptotic morphologies, such as cell shrinkage, membrane blebbing and nuclear condensation, are not blocked by caspase inhibitors and that the cells continue to die in a protracted and inefficient manner. This has led to the notion that caspases are not required for all aspects of apoptosis in mammals. Here we review the current knowledge about caspase-independent apoptosis, discuss the strengths and weaknesses of the reasoning that led to its proposition and provide insights into its possible regulation and physiological significance.

**Keywords:** apoptosis; programmed cell death; caspase-independent; Bcl-2; Bax; mitochondria; cytochrome c; *ced* genes; *C. elegans*; *Drosophila*

**Abbreviations:** CED, cell death abnormal; rpr, reaper; hid, head involution defective; IAP, inhibitor of apoptosis protein; DFF, DNA fragmentation factor; CAD, caspase activated Dnase; DED, death effector domain; CARD, caspase recruiting domain; FADD, Fas-associated death domain; RAIDD, RIP-associated Ich-1/CED-3 homologous protein with a death domain; TNF $\alpha$ , tumor necrosis factor  $\alpha$ ; Apaf-1, apoptotic protease activating factor 1; PT, permeability transition pore; ANT, adenine nucleotide translocator; ROS, reactive oxygen species; ER, endoplasmic reticulum; AIF, apoptosis inducing factor; NO, nitric oxide; PS, phosphatidylserine; SOD, superoxide dismutase; CAT, catalase; TEGT, testis

enhanced gene transcript; BI-1, Bax inhibitor-1; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling; Z-VAD.fmk, Z-Val-Ala-Asp.fluoromethylketone; BD.fmk, t-butoxy carbonyl-Asp.fluoromethylketone

## Apoptosis: a cell-intrinsic molecular guillotine

In the early 1970s, Kerr, Wyllie & Currie reported that cells undergo at least two distinct forms of death: a violent, rapid necrosis characterized by cytoplasmic swelling, membrane rupturing and organelle dissolution with relatively unremarkable nuclear changes and a more protracted, morphologically distinct apoptosis when cells shrink, dissociate from neighbors, leave their organelles mostly intact but show extensive nuclear/cellular fragmentation and expose novel surface molecules for rapid phagocytosis.<sup>1,2</sup> Ever since, cell deaths observed in culture and tissues have been classified into the simple dichotomy, apoptosis or necrosis. Today, we however know forms of cell death that cannot be readily fitted into either category, for example when cells die by cytoplasmic and membrane changes seen in apoptosis but do not exhibit DNA and/or nuclear fragmentation (see below). Even the initial dogma that apoptosis execution absolutely requires a specific class of proteases, called caspases, is about to tumble.

Apoptosis has been first recognized during vertebrate development as part of a natural process to remove superfluous or used-up cells.<sup>3,4</sup> It has since become evident that death morphologies closely resembling ontogenic deaths occur in post-developmental cells in response to various physiological, pathological or pharmacological agents.<sup>5,6</sup> Crucial roles of apoptotic death have thus been extended to morphogenesis, tissue homeostasis, immune regulation and the elimination of infected, mutated or damaged cells.<sup>5–7</sup> In all these cases, an afflicted cell senses that its environment or physical state has been compromised and by consequence undergoes a suicide process, using an intrinsic molecular death machine similar to the guillotine of the French revolution (Figure 1). Because every cell in a multicellular organism may once encounter an unfavorable situation, it is conceivable to propose that all living cells contain such a death machinery. As long as the cell survives, the guillotine hangs inactive on the rope. If a cell is to be removed, some death signal stimulates a 'trigger' molecule that, with the help of an efficient tool, progressively cuts into the rope until it breaks and unleashes the guillotine onto the cell (Figure 1). This process can be stopped by 'savers' up to the point of the final, guillotine releasing cut. As there can be many 'triggers' and 'savers', many molecules can activate or

block apoptosis via distinct intracellular signaling pathways (Figure 1). However, only one tool (for example an axe), and thus class of molecules, might be the most effective in the execution process. Does this account for all sorts of apoptosis in eukaryotic cells or can execution also be accomplished by less efficient tools, say a scissor?

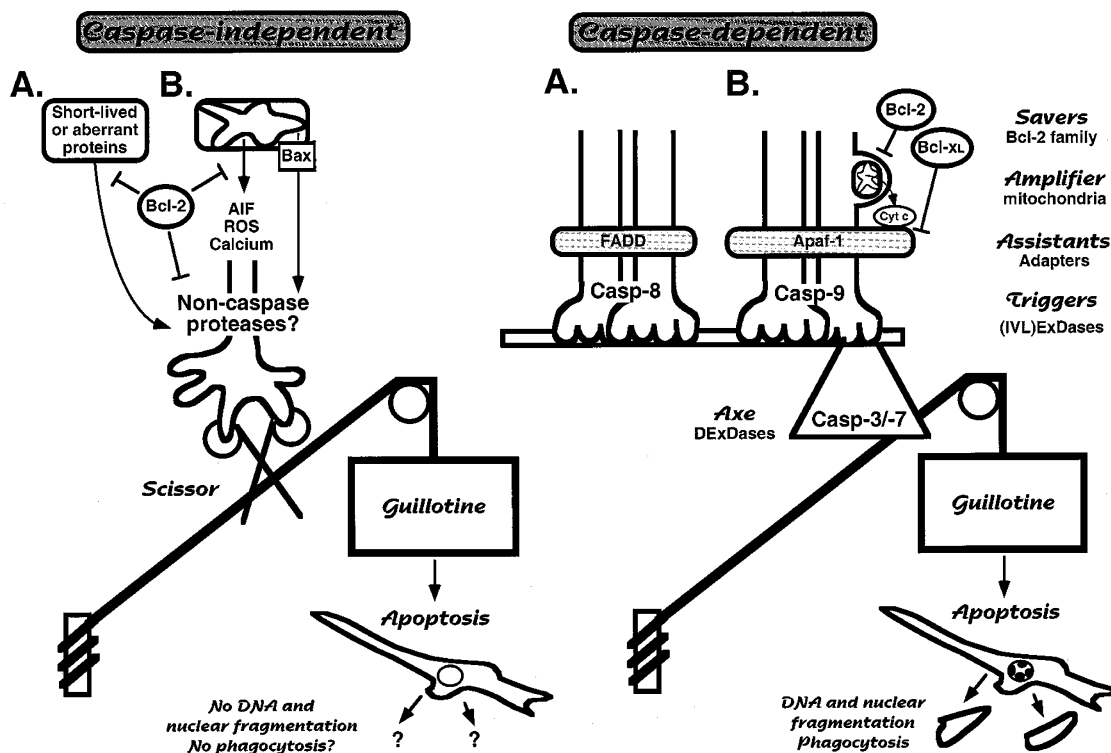
## The efficient CED-3 execution 'axe' of nematodes

Genetic analysis in the nematode *C. elegans* unveiled two gene products, CED-3 and CED-4, which are essential for developmental apoptosis, i.e. worms lacking either one of them or both contain at birth 131 superfluous cells.<sup>8,9</sup> CED-3 encodes a cysteine protease, called caspase, that cleaves cellular substrates after aspartate residues.<sup>9</sup> Although physiological substrates for CED-3 are not yet known, studies in higher eukaryotes revealed that CED-3 is probably the 'cutting axe' that progressively cleaves numerous cellular proteins until the rope of the guillotine breaks. This has been

elegantly proven by showing that a general caspase inhibitor from baculovirus, called p35, blocks developmental apoptosis to the same extent as in CED-3 null worms.<sup>10</sup> CED-3 is activated by the upstream 'trigger' CED-4.<sup>9</sup> This protein physically interacts with CED-3 as an oligomer causing the protease to aggregate and get autoactivated.<sup>11-14</sup>

## Apoptosis in the fly: The same 'cutting tool', but other 'triggers'

Widespread cell death occurs during *Drosophila* development and can additionally be induced by DNA damaging agents.<sup>15,16</sup> These deaths are morphologically identical to apoptosis suggesting a similar underlying mechanism as in worms. A first genetic screen had however not immediately identified CED-3 like execution caspases but three gene products, called reaper (*rpr*), head involution defective (*hid*) and grim, that function as apoptosis triggers rather than executioners.<sup>16-18</sup> Rewardingly, *rpr*, *hid* or *grim*-induced apoptosis was impeded by the co-expression of the caspase



**Figure 1** Caspase-dependent and -independent activation of the apoptotic 'guillotine'. Multicellular organisms primarily use caspase-dependent apoptosis (right panel) to eliminate superfluous, used-up and damaged cells. This ensures a rapid and efficient cleavage of nuclear and cellular structures as well as an immediate phagocytosis of the cell fragments. An apoptotic stimulus clusters 'assistant' molecules such as FADD to oligomerize and autoactivate 'trigger' caspases ((IVL)ExDases) which in turn launch the 'cutting axe', an efficient execution tool consisting of the DExDase caspases-3 and -7 (A). The 'axe' progressively cuts into the rope of the guillotine until the latter unleashes and fragments the cell. Execution can be amplified by triggers with strong 'muscles', i.e. mitochondrial components such as cytochrome c which enhance caspase activation via the 'assistant' Apaf-1 (B). The guillotine can be prevented from falling by 'savers' which block triggers or unsharpen the axe (caspase inhibitors, not shown) or disable the 'muscle' (cytochrome c release, Bcl-2) or 'assistants' (Apaf-1, Bcl-x<sub>L</sub>). If caspases are absent (yeast, enucleated cells, caspase knock-outs) or blocked (Z-VAD.fmk, baculovirus p35) the rope of the guillotine can still be cut, but by a less efficient 'scissor' (left panel). In this case, DNA and nuclear fragmentation does usually not occur but the cell continues to die with an otherwise apoptotic morphology without being necessarily phagocytosed. Trigger molecules may be accumulating short-lived and/or damaged proteins (A) or mitochondrial components such as AIF, ROS and calcium whose release into the cytoplasm could be under the control of the Bax channel (B). The 'scissors' may be non-caspase proteases such as serine proteases, calpains or cathepsins. Bcl-2 can still save the guillotine from falling. For details, see text

inhibitor baculovirus p35 indicating that all three gene products acted upstream of some putative *Drosophila* execution caspase(s).<sup>17–20</sup> Three CED-3-like caspases, drICE, DCP-1 and DCP-2/DREDD have since been identified in the fly.<sup>21–23</sup> DCP-1 is required for early stages of *Drosophila* development as its loss of function leads to female sterility by inhibiting transfer of cytoplasm from apoptotic nurse cells to the primary oocyte.<sup>24</sup> Moreover zygotic loss of this caspase causes larval lethality and melanotic tumors.<sup>22</sup> Thus, as in *C. elegans*, caspases are an effective ‘cutting tool’ to execute physiological apoptosis in *Drosophila*. Interestingly, while the ‘cutting tool’ has been evolutionary conserved between worms and flies, the triggers appear to be distinct. Neither has a CED-4 homolog been found in *Drosophila* nor any of the activators *rpr*, *hid* and *grim* in *C. elegans*. Moreover different strategies are used to prevent the guillotine from falling. While *C. elegans* possesses a gene product, called CED-9 which interferes with developmental apoptosis by directly binding and neutralizing the ‘preferred trigger’ CED-4,<sup>11,12,25–28</sup> no such homolog has yet been found in *Drosophila*. The fly instead uses Inhibitors of Apoptosis Proteins (IAPs) that either associate and interfere with the triggers *rpr*, *hid* and *grim*<sup>29</sup> or with the activated caspase axe.<sup>30</sup>

### The guillotine of mammals: Further complexities to trigger, prevent and execute the ‘rope cutting’ process

There is no doubt that the regulation of life and death is more complex in mammals than worms and flies. More than 14 mammalian caspases have so far been identified, all of which are expressed as inactive proenzymes that are proteolytically activated to form a tetrameric catalytic complex.<sup>31</sup> They can be divided into three major groups based on their preference for a tetrapeptide recognition sequence in their substrates (DExDases, WEHDases, (IVL)ExDases).<sup>32,33</sup> While WEH-Dases function primarily as inflammatory mediators, the other two groups regulate apoptosis. At least two DExDases, caspase-3 and caspase-7, can function as axe of the mammalian guillotine (Figure 1) because all apoptotic stimuli activate one or the other enzyme, the substrate analog inhibitor DEVD-CHO blocks most forms of apoptosis and deletion of caspase-3 causes perinatal lethality in mice due to brain malformations.<sup>32–35</sup> Moreover, most of the caspase substrates identified so far are cleaved after DExD sequences.<sup>32,36</sup> Although we do not yet know which cleavages are essential for apoptosis execution, many facilitate cell disassembly (‘cutting the rope until the guillotine falls’). This includes the caspase-activated nuclease DFF40/CAD which cleaves the DNA into internucleosomal fragments,<sup>37,38</sup> and cytoskeletal proteins such as actin,  $\alpha$ -fodrin, gelsolin and the nuclear lamins whose cleavages may contribute to cell shrinkage, membrane blebbing and nuclear breakdown.<sup>39–42</sup>

If mammalian apoptosis is predominantly executed by DExDases, why is another subgroup of caspases needed? It turns out that the (IVL)ExDases are used to form a probably stimulus-specific proteolytic cascade (trigger chain) that ends up in the cleavage and activation of pro-

DExDases (Figure 1).<sup>31,32</sup> This is because mammalian DExDases contain an N-terminal inhibitory pro-domain that needs to be proteolytically removed for effective auto-activation. The connection between the death stimuli and the proteolytic cascades is ensured by adaptor proteins (trigger assistants). These proteins self-associate in response to an apoptotic stimulus and, by binding to a long N-terminal pro-domain present only in ‘trigger’ caspases (DED or CARD domain), oligomerize and autoactivate the first protease in the cascade (Figure 1).<sup>31,32</sup> For example, Fas/APO and other TNF $\alpha$ -like molecules use the adaptors FADD and RAIDD to oligomerize and autoactivate the ‘trigger’ pro-caspase-8 and pro-caspase-2, respectively.<sup>43,44</sup> Caspase-8 then directly cleaves and activates caspase-3 (Figure 1).<sup>45</sup> The proof that (IVL)ExDases are ‘trigger’ caspases on selected apoptotic pathways upstream of DExDases has been obtained by inhibitor and knock-out mice studies. For example caspase-8 null animals are resistant to Fas/APO and TNF $\alpha$ -induced apoptosis,<sup>46</sup> but are still susceptible to chemotherapeutic drugs, serum withdrawal and dexamethasone. By contrast, cells deficient in caspase-9, another ‘trigger’ caspase, remain sensitive to killing signaling by TNF or Fas/APO but show resistance towards  $\gamma$ -irradiation, chemotherapeutic drugs and dexamethasone.<sup>47,48</sup>

Thus, it appears that all what the mammalian execution ‘axe’ requires for its activation is an upstream cascade of ‘trigger’ caspases with their respective ‘assistants’ (adapters). What is then the function of CED-4 like molecules? The only so far identified mammalian CED-4 homolog, called Apaf-1, acts as a classical assistant as it binds to pro-caspase-9 and provokes self-association and activation of the protease (Figure 1).<sup>49–51</sup> This function of Apaf-1 is however not restricted to specific stimuli (such as FADD on the Fas/TNF-R pathway) but part of a commonly used apoptotic signaling pathway which involves mitochondrial components.<sup>52</sup> Interestingly, such a pathway has not yet been found in nematodes and is probably a recent evolutionary invention. Has CED-4 been deviated to function on a mitochondria-specific apoptotic pathway in mammals?

Mitochondrial damage has been viewed as a typical sign of necrosis where the organelles swell, break open, prevent further ATP production and subsequently kill cells by disrupting ion homeostasis. Recently, significant but more subtle mitochondrial changes have also been noted during apoptosis.<sup>53–55</sup> They include enhanced production of oxygen radicals, calcium cycling, the fall in the transmembrane potential and an increased permeability of the outer mitochondrial membrane.<sup>53–55</sup> How these changes are triggered by apoptotic stimuli, whether they are causally linked and whether they are absolutely required for the activation of execution caspases is still a matter of large debate. What almost invariably occurs in response to various apoptotic stimuli is the release of cytochrome c from the intermembrane space of mitochondria to the cytoplasm<sup>56–59</sup> where it binds to the ‘assistant’ Apaf-1, leading to the sequential activation of pro-caspase-9 and -3 (Figure 1)<sup>49–52</sup> How important is this pathway for apoptosis

execution? Apaf-1 and caspase-9 knock out mice are severely compromised in brain development and die *in utero*.<sup>47,48,60,61</sup> However, morphogenesis in other parts of the body, such as for example the removal of interdigital webbing, was remarkably normal (although delayed by one day in Apaf-1 null mice). Also, apoptosis in response to Fas/APO and TNF $\alpha$  was largely unaffected and although caspase-3 activation and DNA fragmentation in response to other stimuli such as  $\gamma$ -irradiation, dexamethasone and chemotherapeutic drugs were blocked after a certain time, they may nevertheless occur later on.<sup>47,48,60,61</sup> Thus, the observed defects in these knock-out animals could be due to a delayed rather than abrogated apoptosis and the role of mitochondrial components would then be to amplify rather than execute the death process, as recently suggested.<sup>62</sup> In this respect, one could view mitochondrial contribution like having a trigger with strong muscles that launches the caspase-3 'axe' more effectively than a trigger with weak muscles (Figure 1). Such 'strong muscles' are especially important for the rapid and efficient removal of cells during embryonic development, thus explaining the brain defects in caspase-9 and Apaf-1 null mice.

How does a mammalian cell prevent its guillotine from falling? On one hand, it expresses several IAP proteins (NAIP, X-IAP, cIAPs) that either function as direct caspase inhibitors or interveners of Fas/TNF $\alpha$ -activated apoptotic pathways.<sup>29,30,63–65</sup> In addition, there are at least 16 human homologs of the nematodal CED-9 survival factor of which the proto-oncogene product Bcl-2 is the founding member.<sup>66,67</sup> Although Bcl-2 can mimic the death protective effect of CED-9 in worms and *vice versa*, the two proteins appear to function slightly differently.<sup>66–68</sup> While CED-9 directly binds to CED-4 and prevents the latter from activating CED-3,<sup>11,12,26,27</sup> Bcl-2 does not avidly interact with the CED-4 homolog Apaf-1 (own observation) but prevents the release of cytochrome c from mitochondria thereby blocking caspase-9/-3 activation upstream or independently of Apaf-1 (Figure 1).<sup>57,58</sup> How Bcl-2 performs this peculiar action on mitochondria has remained elusive. Perhaps it has acquired a new function to disable the 'muscle' rather than the 'assistant' of the 'trigger caspase' (Figure 1). That the original function of CED-9 has been nevertheless conserved is demonstrated by Bcl-x<sub>L</sub>, another potent anti-apoptotic member of the Bcl-2 family which, as expected, interacts with Apaf-1 and prevents Apaf-1 mediated caspase-9 activation (Figure 1).<sup>69,70</sup> Thus, during evolution several homologues 'CED-9 savers' have been created that each seems to act on different devices (muscles, assistants, triggers) to prevent the caspase 'axe' from cutting the rope.

## Apoptosis without caspases: 'Pros' and 'Contras'

If caspases are the only tool to exert the execution step of apoptosis, appropriate protease inhibitors should prevent apoptotic morphology and death and allow cells to survive in a functional state. This concept has been widely tested with the cell permeable tripeptide inhibitor Z-Val-Ala-Asp.fluoromethylketone (Z-VAD.fmk).<sup>74</sup> Due to an aspartate

residue mimicking the cleavage site and a fmk group forming a covalent inhibitor/enzyme complex, the inhibitor instantly and irreversibly binds to the catalytic site of caspases.<sup>33,75,76</sup> In addition, by replacing the caspase-specific P4 amino acid with a hydrophobic moiety (Z), the peptide is capable of occupying the catalytic pocket of all caspases thus making it a broad range caspase inhibitor. Indeed, Z-VAD.fmk and the related compound t-butoxy carbonyl-Asp.fluoromethylketone (BD.fmk) proved to be effective in blocking apoptosis in different animal disease models such as stroke, myocardial ischemia/reperfusion injury, liver disease and traumatic brain injury.<sup>77–80</sup> In both liver and ischemic brain models, the recovered cells were shown to be functional.<sup>77,78</sup>

Given these straightforward results, it came as a surprise when two years ago, McCarthy *et al* reported that while cells treated with Z-VAD.fmk or BD.fmk did not display cleavages of caspase substrates, chromatin condensation or nucleosomal laddering in response to oncogenic and DNA damaging apoptotic stimuli, they continued to exert other apoptotic features such as cell shrinkage and membrane blebbing for hours and could not be rescued from the death fate.<sup>81</sup> This observation suggested that certain cytoplasmic hallmarks of apoptosis may be triggered by enzymes other than caspases, but that the nuclear events required caspase activity. Subsequent studies have lent support for such caspase-independent phases of apoptosis. For example, Z-VAD.fmk could not rescue cells from apoptosis induced by the overexpression of Bax although caspase-3 activation and nuclear fragmentation were clearly blocked.<sup>82,83</sup> Similarly, Z-VAD.fmk inhibited nuclear damage of target cells induced by exocytotic granules of cytotoxic T cells but did not prevent target cell lysis (as measured by Cr<sup>51</sup> release).<sup>84,85</sup> More recent examples include apoptosis induced by the GD3 ganglioside,<sup>86</sup> class I MHC antibodies,<sup>87</sup> puromycin,<sup>88</sup> polyamine analogues,<sup>89</sup> CD-2 and staurosporine,<sup>90,91</sup> intracellular acidification,<sup>92</sup> the retinoid AHPN,<sup>93</sup> E4orf4, a novel adenovirus death factor,<sup>94</sup> irradiation,<sup>95,96</sup> VP-16, dexamethasone and actinomycin D,<sup>97–99</sup> PML,<sup>100</sup> and nitric oxide (NO).<sup>101</sup> Even some aspects of Fas-induced apoptosis (when triggered with the adapter FADD) may occur caspase-independently although in this case necrotic morphologies were observed as well.<sup>102</sup> In our own hands, we have not seen any apoptotic system in culture (using various physiological and toxic stimuli) where Z-VAD.fmk could save cells from dying. The inhibitor reproducibly blocks the activation of execution caspases and nuclear fragmentation, but nuclei have irregular shapes and are partially condensed, the ER dilates, cells still shrink and display dramatic blebbing at the surface, and no rescue and/or clonogenic growth is possible at any time after removal of the inhibitor (own observations). Why then can cells be fully rescued with Z-VAD.fmk in models of ischemia and liver damage?<sup>77,78</sup> Either these are special cases of entirely caspase-dependent, apoptotic processes or the attenuation of damage by Z-VAD.fmk is a consequence of the inhibition of enzymes other than caspases. Alternatively, the rescue may not be as complete as thought. A recent report shows that neurons saved with Z-VAD.fmk

after an apoptotic challenge in low KCl stay in a hypoenergetic state, vulnerable to necrosis.<sup>103</sup>

Is there enough evidence to propose that mammalian cells can undergo apoptosis caspase-independently? Unfortunately most of the data proposing such a form of apoptosis have been collected from experiments using the artificial caspase inhibitor Z-VAD.fmk or related agents.<sup>81–103</sup> Although Z-VAD.fmk is known to block all so far known caspases,<sup>33</sup> we cannot exclude that yet unidentified caspases are resistant to this drug. We also do not know whether the inhibitor is sufficiently stable inside cells. It has recently been reported that inhibitory peptides based on their substrate recognition sequence (f. ex. DEVD) are not necessarily strong inhibitors because they are rapidly degraded.<sup>104</sup> Appropriate amino acid substitutions can convert the inhibitor into a stable compound (DQMD) that even allows clonogenic growth after removal.<sup>104</sup> Moreover, it is worrisome that Z-VAD.fmk can inhibit other cysteine proteases such as calpain<sup>105</sup> and cathepsin B<sup>106</sup> because such inhibitions may provoke additional cell death. Finally, the methylketone group of Z-VAD.fmk and related peptide inhibitors is fairly reactive and covalently labels intracellular lipids and proteins (including other proteases such as cathepsin H and B) at concentrations generally used to demonstrate the involvement of caspases ( $>100 \mu\text{M}$ ).<sup>106</sup> This may be another source of non-specific, cytotoxic side effects. Future work has therefore to focus on repeating the Z-VAD.fmk experiments with more natural caspase inhibitors.

One such inhibitor could be baculovirus p35 as it is quite effective in blocking caspase activation and nuclear fragmentation in many cell culture and animal models of apoptosis.<sup>10,17–20,71–73,107</sup> In some cases such as for example the degeneration of the *Drosophila* retina, p35 actually restored visual function to otherwise blind flies.<sup>107</sup> Its potency might be due to the fact that it forms a stable complex with the catalytic site of the caspase after being cleaved at a DQMD sequence.<sup>108</sup> However, only a subset of caspases (-1, -3, -6, -7, -8 and -10) have so far been demonstrated to be inhibited by recombinant p35 *in vitro*,<sup>109</sup> and there are at least three reported cases where p35 cannot rescue cells from apoptosis.<sup>84,101,110</sup> Of course, this could be due to p35 expression levels that are too low for caspase inhibition, as many investigators have problems to highly overexpress p35 due to its viral origin. Alternatively, p35 may not block all caspases. This would explain a recent study where Z-VAD.fmk, but not p35, blocked reaper-induced ceramide production although both inhibitors similarly interfered with downstream caspase activation and nuclear fragmentation.<sup>111</sup> Thus, to validate the existence of caspase-independent apoptosis, it will be necessary to develop and test other physiological caspase inhibitors or knock-out mice deficient in more than one execution caspase (caspases-3, -7, -6).

## Execution of caspase-independent apoptosis: Scissor instead of axe?

If caspase-independent apoptosis indeed exists what cellular components might be involved? In most apoptotic systems Z-

VAD.fmk does not block mitochondrial changes such as the fall of the membrane potential, the production of oxygen radicals or the release of apoptogenic factors like cytochrome c and apoptosis-inducing factor (AIF).<sup>53,54,82,90,94,98,99,101</sup> This means that despite effective caspase inhibition, the cell is continuously 'flooded' with factors that can still provoke apoptotic morphologies and death. Two factors may be important in this process (Figure 1): (i) AIF which has recently been cloned and identified as a mitochondrial interspace membrane protein with homology to bacterial NADH oxidoreductases.<sup>112</sup> In response to apoptotic stimuli, it is liberated, migrates to the nucleus and participates in the induction of chromatin condensation and the dissipation of the mitochondrial membrane potential (positive feedback loop) in a Z-VAD.fmk-insensitive manner. (ii) Bax and Bax-like proteins, pro-apoptotic homologs of the Bcl-2 family, which are known to form ion channels on artificial membranes and isolated mitochondria.<sup>67,113,114</sup> Bax is both a membrane and cytoplasmic protein and there is increasing evidence that the cytoplasmic form undergoes a conformational change and translocates to mitochondrial membranes in response to apoptotic stimuli.<sup>115–118</sup> There it may puncture the outer mitochondrial membrane releasing cytochrome c, AIF and other factors<sup>119,120</sup> or promote the opening of the mitochondrial permeability transition (PT) pore through its association with the adenine nucleotide translocator (ANT), a crucial component of the pore.<sup>121</sup>

How AIF and other released proteins trigger the apoptotic guillotine in the absence of caspases is unknown. An attractive possibility is that they activate other proteases such as serine proteases, calpains and cathepsins which can partially take over the job of caspases but in a less efficient way (acting as 'scissors' instead of 'axes') (Figure 1). Indeed, a recent report has shown that any type of protease, when loaded into cells, can provoke apoptosis-like morphologies.<sup>122</sup> In addition, there is a fair amount of literature implicating non-caspase proteases in apoptosis.<sup>123–125</sup> For example, serine proteases play a crucial role in the early cleavage of chromatin into 50–300 kb fragments before they are digested to nucleosome-sized pieces by DFF40/CAD,<sup>126</sup> and the former but not the latter activity may persist in the presence of Z-VAD.fmk. Another crucial serine protease is AP24 because its specific inhibition confers better protection against TNF $\alpha$  and UV-induced apoptosis than a caspase-3 inhibitor.<sup>127</sup> Furthermore, it has been postulated that the serine protease granzyme A may mediate caspase-independent CTL/target cell lysis as serine protease but not caspase inhibitors blocked this event.<sup>84,128</sup> Another strong candidate for a 'scissor' function in caspase-independent apoptosis are the calpains. Based on inhibitor studies, these proteases participate in apoptosis in response to hypoxia in hepatocytes,<sup>129</sup> in neuronal degeneration<sup>130</sup> and in irradiation and dexamethasone treatment of thymocytes.<sup>131</sup> Since they are calcium requiring enzymes, they may be activated in response to increased cytoplasmic calcium levels, for example due to mitochondrial damages (enhanced calcium cycling).<sup>55,132</sup> In addition, calpains are known to cleave the cytoskeletal protein fodrin upstream of caspases, which

eventually disrupts the cytoskeletal network and allows the persistent membrane blebbing seen in caspase-independent apoptosis.<sup>105</sup> Finally, there is a clear implication of the lysosomal protease cathepsin D in apoptosis induced by interferon- $\gamma$ , Fas/APO-1 and TNF $\alpha$ .<sup>125</sup> During cell death, the enzyme is processed into a proteolytically active, single chain form and itself provokes apoptosis when over-expressed. It is however yet unknown whether its pro-apoptotic action is within the lysosomal/endosomal system or whether it is released into the cytoplasm. This also accounts for other lysosomal components that have recently been implicated in TNF- $\alpha$ -induced apoptosis.<sup>133</sup> Why have these proteases attracted less attention for apoptosis regulation than caspases? Perhaps several types of proteases are simultaneously activated during apoptosis, and biochemical and morphological changes in response to non-caspase inhibitors may be more difficult to detect because of the predominant contribution of caspases. It might therefore be of advantage to have an apoptotic system in which caspases are not activated at all. Our group has recently identified such a system.<sup>134</sup>

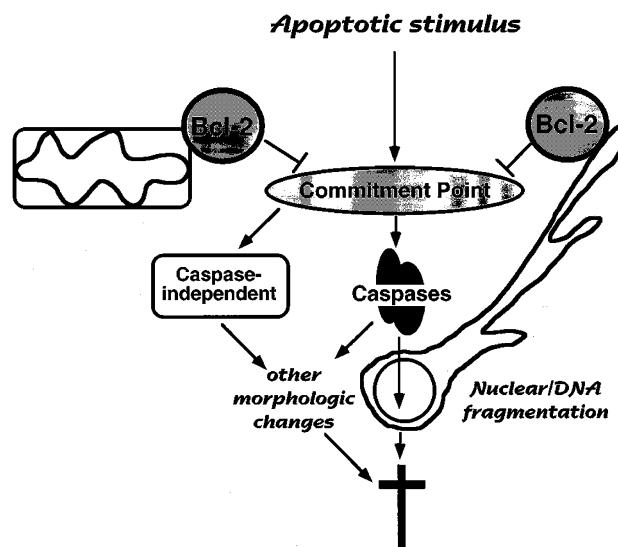
Ts20 fibroblasts containing a temperature-sensitive mutation in the ubiquitin-activating enzyme E1 die by apoptosis at the restrictive temperature because short-lived and damaged proteins accumulate due to decreased ubiquitination and degradation by the proteasome. Surprisingly, this type of cell death proceeds slowly, displays only marginal DNA condensation and cleavage, no nuclear fragmentation and is not blocked by Z-VAD.fmk. Western blot analysis and recent fluorogenic activity assays revealed that neither of the known execution caspases-3, -7 and -6 are activated at any time at the restrictive temperature. Thus, the ts20 system holds great promise to uncover molecules regulating caspase-independent apoptosis. Moreover, it may teach us more about the function of Bcl-2 because despite the absence of caspase activation, this cell survival factor blocked cell death (Figure 1). A caspase-independent death protection of Bcl-2 has also recently been reported in cells exposed to NO.<sup>101</sup> Perhaps Bcl-2 and homologous proteins act near a point (commitment point?) where caspase-dependent and -independent pathways bifurcate (Figure 2). Such a scenario agrees with previous reports (including our own observations) that Bcl-2 allows clonogenic growth of rescued cells and therefore saves them better from the death fate than caspase inhibitors.<sup>97-99</sup> Thus, while Bcl-2 successfully interferes with the execution 'axe' and the 'scissor', caspase inhibitors cannot prevent the 'scissors' from cutting the rope.

## Is caspase-independent apoptosis physiological?

Since caspase-independent apoptosis has been mainly detected in cell cultures, it is important to know whether it is physiologically relevant. Recent analysis in *C. elegans* unveiled that CED-3 is probably not the only tool that 'cuts' the rope of the guillotine. Once born with 131 surplus cells, CED-3 null worms overall look and behave normally<sup>9</sup> and cells from these worms still die if a metabolic process required

for their survival is blocked (for example in response to irradiation, toxins or lack of nutrients).<sup>135</sup> These findings indicate that nematodal apoptosis can proceed CED-3-independently, either due to the activation of redundant CED-3-like caspases such as the recently identified caspase-related genes *csp-1/-2A/-2B*<sup>136</sup> or other proteolytic and/or degradative systems as described above. This also accounts for *Drosophila* flies as most embryonic cell deaths still occurred and larvae had an overall normal nervous system in the absence of the essential caspase DCP-1.<sup>22</sup> In mammals, evidence for physiological, caspase-independent apoptosis is still murky. Weil *et al* have recently reported that spontaneous apoptosis of sperms does not seem to require caspases, but unfortunately this was again based on a lack of death protection by Z-VAD.fmk.<sup>91</sup>

Where do we expect to see caspase-independent apoptosis in a multicellular organism and what should it look like? Since caspase-dependent apoptosis does not only involve rapid and irreversible killing but also immediate clearing of the apoptotic cell by phagocytosis, one could search for apoptotic cells that are not efficiently engulfed. In fact, a crucial signal for engulfment is the appearance of phosphatidylserine (PS) on the cell surface, a strictly caspase-dependent process.<sup>137</sup> In addition, we should look for apoptosis that proceeds slowly and in the absence of major nuclear fragmentation. These criteria may be fulfilled by enucleated cells such as erythrocytes or the outer layer of the epidermis. Artificially enucleated cells still show Bcl-2 blockable, apoptotic morphologies in response to various stimuli,<sup>138</sup> and as erythrocytes mature and lose



**Figure 2** Bcl-2 blocks caspase-dependent and -independent apoptosis. It is proposed that Bcl-2 acts from its site on mitochondrial and ER/nuclear membranes to interfere with a putative point of 'commitment' where caspase-dependent and -independent signaling pathways bifurcate. By this action, Bcl-2 can functionally rescue cells from both 'axe' and 'scissor' cutting actions while caspase inhibitors can only do the former. Thus, while cells rescued by Bcl-2 are capable of clonogenic growth, those rescued by caspase inhibitors continue to die

their nuclei, they also seem to get rid of their caspases.<sup>91</sup> In the epidermis, some dividing basal cells move up to the granular layer where they lose their nuclei and cytoplasmic organelles and are transformed into the keratinized squames of the upper epidermal layer. These cells finally flake off from the surface of the skin. The time from the birth of a cell in the basal layer to the shedding on the surface can take 2–4 weeks. Where on this outward journey apoptosis occurs and whether it involves caspases is not known. Targeted deletion of execution caspases in the epidermis will provide us the answer.

Other attractive physiological systems of caspase-independent apoptosis may be Huntington's and Alzheimer diseases. Jackson *et al* have reported a *Drosophila* model for Huntington's disease where transgenic baculovirus p35 was incapable of reducing the extent of neuronal degeneration.<sup>110</sup> Similarly, although neuronal cell death during Alzheimer's disease has been proposed to involve caspase-driven apoptosis because the implicated molecules  $\beta$ -amyloid and presenilin 1 and 2 can be cleaved by caspases,<sup>139,140</sup> there is no reported evidence that caspase inhibitors can interfere with disease generation or progression. Moreover, the number of degenerating neurons does not correlate with the number of cells showing TUNEL positive DNA fragmentation at any given time during the disease.<sup>141</sup> Also, neuronal cell death in Alzheimer occurs over a lengthy period (*ca.* 10 years) suggesting distinct mechanisms from the classical, caspase-dependent apoptotic process (which takes 16–24 h to complete).

Finally, we may get mechanistic insights into caspase-independent apoptosis from studying the process of aging. Many changes occur as an animal ages. Proteins become modified and cross-linked, somatic mutations accumulate, stress resistance decreases and the probability of death increases.<sup>142</sup> A major reason for these changes is thought to be oxidative damage to macromolecules and lipid membranes, caused by superoxides and other free radicals that originate mainly from aerobic metabolism.<sup>143</sup> Consistent with this notion, flies overexpressing both superoxide dismutase (SOD) and catalase (CAT), two important antioxidant enzymes, exhibited increases in life span and stress resistance.<sup>144</sup> Similarly, a mutation in an important component of the mitochondrial electron transport, called *clk-1*, doubles the life span of the worm *C. elegans*,<sup>145</sup> presumably because the metabolic rate is slowed down with an accompanying decrease in the production of oxygen radicals. Given the implication of mitochondria, oxygen radicals and DNA damage in apoptosis,<sup>53,54</sup> it is tempting to propose that an aged cell finally succumbs to a classical way of caspase-dependent apoptosis. Several observations however speak against such a possibility. (i) the morphology of aged cells is not necessarily apoptotic, (ii) the process is slow, accumulating mistakes over the years before a cell finally dies, and (iii) worms and flies knocked out in caspases do not have an altered life span.<sup>8,9,22,24</sup> Thus, it is more likely that aging involves components which are also used for caspase-independent apoptosis. Again Bcl-2 may help us to identify these components as it can act as an antioxidant,<sup>146,147</sup>

and its deletion in mice leads to some phenotypes that superficially resemble aging.<sup>148</sup>

## Apoptosis in lower eukaryotes: genetically amenable systems to uncover caspase-independent regulators

Lower eukaryotes such as yeast and *Dictyostelium* may be ideal systems to isolate components of caspase-independent apoptosis. They are both genetically amenable, their cells die with a morphology partially resembling apoptosis and at least in the case of yeast, caspases are missing from the genome.<sup>28,149–151</sup> *Dictyostelium* multiplies as a unicellular organism but upon starvation aggregates and differentiates into a fungus-like sorocarp of which spores are viable and stalk cells display apoptotic morphologies except nuclear fragmentation.<sup>152,153</sup> Based on Z-VAD.fmk inhibition experiments, stalk cell apoptosis proceeds caspase-independently although we do not know whether caspases exist in *Dictyostelium*.<sup>152</sup>

Yeast can be killed by various means such as oxidative stress, metabolic block, irradiation and other toxic substances, but cell death does not resemble apoptosis and there is no endogenous expression of known apoptosis regulators of the CED-3/caspase, CED-4 or Bcl-2/Bax families. Nevertheless, forced overexpression of Bax, Bak or CED-4 provokes vacuolarization and focal chromatin condensation as seen in mammalian apoptosis,<sup>28,150,151,154,155</sup> indicating these death factors can indeed provoke a caspase-independent form of apoptosis via components that are already present in a unicellular organism. To identify these components, a mammalian expression cDNA library has recently been screened for clones that rescue yeast from Bax-mediated cell death. The search unveiled a novel Bax inhibitor (BI-1) homologous to the Testis Enhanced Gene Transcript (TEGT) that interacts with Bcl-2 (but not Bax), localizes to the ER/nuclear membrane and inhibits apoptosis in response to some but not all stimuli.<sup>156</sup> Moreover, by using genetic complementation in a Bax-resistant yeast mutant, Matsuyama *et al* isolated a gene encoding a subunit of the yeast  $F_0F_1$ -ATPase and showed that its product was required for Bax-induced apoptosis in yeast and mammalian cells.<sup>157</sup> As the  $F_0F_1$ -ATPase is a mitochondrial proton pump required for aerobic respiration, it might cooperate with the channel function of Bax to transport ions and/or to release apoptogenic factors from mitochondria. Two other ER proteins appear to be attractive regulators of yeast cell death: the calnexin homologue Cnx1 which is necessary for yeast lethality mediated by Bak<sup>154</sup> and CDC48, a highly conserved ATPase with essential functions in cell division and homotypic fusion of ER vesicles whose loss of function provokes not only chromatin condensation, but strikingly also PS exposure, membrane blebbing and DNA fragmentation.<sup>158</sup> Even Bcl-2 can function in yeast as it improves the viability of a mutant defective in SOD1 under some growth conditions.<sup>147</sup> This indicates that Bcl-2 can block caspase-independent apoptosis via its antioxidant activity. Although yeast holds great promise to isolate further death regulators it remains to be seen whether they indeed make

up an ancestral form of caspase-independent apoptosis regulation. If caspases have been only created to accelerate and/or improve the basic 'scissor' like guillotine, their reconstitution in yeast should add the 'axe' to effectively kill unicellular organisms via a full blown apoptotic morphology. Expression of caspase cascades in yeast has just been started.<sup>159</sup>

## Conclusions and future perspectives

There is no doubt that multicellular organisms use a caspase 'axe' for effective cell removal by apoptosis. However, certain basic executionary processes may not necessarily require caspases and it is crucial to uncover the molecules involved. This will tell us more about ancient forms of apoptosis and probably help to understand how sperms and enucleated cells die, how epidermal surfaces shed their outer cell layer and how multicellular organisms age. It will certainly also aid in defining the function of Bcl-2-like proteins as they seem to control both caspase-dependent and -independent pathways in a yet mysterious way. Finally, studying this cell death is important for the development of further drugs for therapeutic intervention of various apoptosis-based diseases. Although broad specificity caspase inhibitors seem to be effective against ischemia, stroke and fulminate liver disease, this may not be the case for other diseases as we cannot yet firmly say whether the dying cells are functionally rescued. If such cells would still die but slowly and without the possibility of being phagocytosed, disease progression may even be worse in the presence of caspase inhibitors.

Future research on physiological model systems of caspase-independent apoptosis, the exploitation of genetically amenable lower eukaryotes, the cloning of more caspases and the development of more general, non-toxic caspase inhibitors will all help us to face the next challenge of apoptosis research: the identification of alternative rope cutters of the cell's guillotine.

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