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Review

Caspase knockouts: matters of life and death

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Abstract

Apoptosis, the seemingly counter-intuitive act of physiological cell suicide, is accomplished by an evolutionarily conserved death program that is centered on the activation of a group of intracellular cysteine proteases known as caspases. It is now clear that both extra- and intra-cellular stimuli induce apoptosis by triggering the activation of these otherwise latent proteases in a process that culminates in caspase-mediated disintegration of cellular contents and their subsequent absorption by neighboring cells. While many elegant *in vitro* studies have demonstrated the requirement of caspase activities for the execution of most, if not all, apoptosis, the precise contribution of individual caspases *in vivo* and how they functionally relate to each other remain poorly elucidated. Fortunately, the generation of various caspase deficient mice through gene targeting has provided a unique window of opportunity to definitely examine the physiological function of these caspases *in vivo*. As the list of caspase knockouts grows, we considered it was time to review what we have been learned, from these studies about the exact role of individual caspases in mediating apoptotic events. We will also provide our prediction on the direction of future studies in this ever-growing field of caspases.

Keywords: caspase; apoptosis; gene targeting; development; neuronal cell death; negative selection

Abbreviations: AIF, apoptosis inducing factor; Apaf-1, apoptosis-activating factor 1; CAD, caspase-activated deoxyribonuclease; ICAD, inhibitor of CAD; ES cell, embryonic stem cell; ICE, interleukin 1β-converting enzyme; LPS, lipopolysaccharide; MEF, mouse embryonic fibroblast; TUNEL, TdT-mediated dUTP nick-end labeling

Introduction

In contrast to the world of single-celled organisms, cell death is an inseparable part of life for all multicellular organisms. The necessary removal of superfluous and dangerous cells by a cell suicidal program known as apoptosis is pivotal to tissue sculpting during development as well as maintenance of homeostasis in adulthood.[1–3] Dysregulations of apoptosis, including both excessive or insufficient cell death, can lead to devastating consequences ranging from tumorigenesis to the pathogenesis of various neurodegenerative diseases.[4]

How do cells commit such a seemingly counter-intuitive act of killing themselves? Thanks largely to genetic studies in *Caenorhabditis elegans* that allowed identification of crucial components (*ced-3*, *ced-4*, and *ced-9*) of the cell death pathway, as well as subsequent characterization of their mammalian counterparts, it is now evident that diverse apoptotic signals converge into a common death pathway that is evolutionarily conserved.[5] Central to its elaborate execution machinery, is a family of cysteinyl aspartate-specific proteinases known as caspases which normally exist as proenzymes with little, if any, catalytic activity, yet become activated following apoptotic stimulation.[6] The conservation of the apoptotic pathway during evolution is further reflected by the presence of both positive and negative regulators of caspase activation between *C. elegans* and mammals.[7]

The presence of multiple mammalian homologs of the *C. elegans* death genes *ced-3* and *ced-9*, however, clearly indicates that the cell death machinery has become more complex in 'higher' organisms. While the proto-caspase CED-3 is required for all the programmed cell death events in *C. elegans*, more than a dozen mammalian caspases have been identified.[6] Although the effective inhibition of apoptosis in response to a wide range of stimuli by both synthetic and viral inhibitors of caspases convincingly demonstrated the requirement of caspase activity for most, if not all, apoptotic events in mammals, the precise identities of the individual caspases involved remain uncertain. Furthermore, despite the fact that ectopic overexpression of all caspases individually can induce apoptosis *in vitro*, many questions pertaining to their exact contributions to the destruction of apoptotic cells *in vivo* have yet to be answered. For example, are caspases functionally redundant as suggested by the constitutively overlapping expression of multiple caspases in any given tissue or cell type, or does each caspase have its own distinct role(s)? And if so, what is their mode of action? Is it tissue specific, cascade-like or a combination of both? Given the limitation of most *in vitro* systems, several groups, including ours, have used gene-targeting technique to generate mice that are deficient in specific caspases so that their exact physiological functions can be examined definitively.

To date, caspase deficient mice have been generated for more than half of the 14 mammalian caspases identified so far with greatly varied phenotypes (Table 1). Instead of simply providing a list of the phenotypes of each caspase knockout mice, we thought it would be more fruitful to review what these studies revealed about the various aspects of how individual caspases function. More specifically, is there any tissue specific requirement for specific caspases during development and in fully differentiated cell types? Do caspases indeed function in a cascade fashion as suggested by *in vitro* studies and what caspases are directly responsible for the degradation of caspase targets during the final destruction of apoptotic cells? We hope that such an examination will provide some insight on the future direction in this, one of the most exciting fields of biological research.

Role of caspases during development

Cell death is a vital part of development in all metazoans. Just as cells that form the xylem in plants must die in order to conduct fluid; so must the cells that make up the limb webs and the extra neurons that fail to make proper target-dependent connections. The readily removal of these superfluous cells during development is critical for proper tissue modeling, cell number controlling and elimination of excessive or harmful cells.[1,2]

The requirement for caspase activity during development was first shown in *C. elegans* where mutations in the pro-caspase gene *ced-3* resulted in survival of almost all cells that normally die during *C. elegans* development.[5] Interestingly, such mutations do not seem to have any detrimental effects on the worms physiology and life span, at least under laboratory conditions. In contrast, caspase-mediated apoptosis plays a crucial role during *Drosophila* development as a loss of function mutation in one of the *Drosophila* caspase gene, Dcp-1, leads to female sterility and transgenic expression of the baculoviral inhibitor of caspases, p35, causes the survival of larval cells.[17,18]

As summarized in Table 1, targeted disruption of caspase genes in mice revealed differential requirements for individual caspases during mammalian development. While deficiency in either caspase-1 or -11, two caspases whose primary function is most likely mediating inflammatory responses, did not have any discernible effect on mouse development,[8,9,16] deletions of two of the upstream initiator caspases, caspase-8 and -9, resulted in embryonic and perinatal lethality, respectively.[13–15] Specifically, caspase-$8^{-/-}$ embryos died around E11 days with impaired heart development and abdominal hemorrhage; whereas caspase-$9^{-/-}$ mice died before or shortly after birth and exhibited severe brain abnormalities. On the contrary, mice carry a null mutation of another initiator caspase, caspase-2, did develop normally without an overt phenotype.[10] Interestingly, drastically different phenotypes were also observed when downstream effector phase caspase genes, caspase-3, -6 and -7, were deleted. While caspase-$6^{-/-}$ mice seemed to develop normally (Zheng & Flavell, unpublished results), caspase-$3^{-/-}$ mice were perinatally lethal as observed with caspase-$9^{-/-}$ mice[11,12] and caspase-$7^{-/-}$ embryos died early during embryogenesis (Kuida and Flavell, unpublished results).

Given the ability of nematodes carrying mutations of their only caspase gene, *ced-3*, to develop and function normally, it is puzzling that deficiency in just one of the more than a dozen or so mammalian caspases could result in complete developmental blockage in mice. One contributing factor to this may be the fact that *C. elegans* is determined by strict adherence to the lineage of cells whereas mammals are plastic. It is also likely that, during evolution, as multicellular organisms become more complex and require more cell specialization as well as coordination, the necessity to use cell death as a means to regulate cell numbers by molding the plasticity of the developing mammalian embryo has also increased in order to ensure the proper development of various tissues and organs. As a result, multiple caspases, as well as regulators of caspase activation may have evolved to provide organisms with the ability to respond to more diverse stimuli, to control cell death in tissue-restricted settings, and to allow a more vigorous control over the apoptotic pathway. The absolute requirement of the caspase-9/caspase-3 pathway for neuronal cell death and of caspase-8 for the proper formation of heart muscle thus demonstrated a strict

Table 1 A survey of caspase knockouts

Caspases	Development	Apoptotic phenotype	References
Caspase-1	normal	Fas? (thymocytes)	Kuida *et al*,[8] Li *et al*[9]
Caspase-2	normal	germ cells	Bergeron *et al*[10]
Caspase-3	perinatally lethal*	neuroepithelial progenitors; lack of or delayed morphological changes and DNA fragmentation	Kuida *et al*,[11] Woo *et al*[12]
Caspase-6	normal unpublished	N/D	
Caspase-7	embryonic lethal unpublished	N/D	
Caspase-8	embryonic lethal	Death receptor (Fas, TNF, DR3) pathway (MEFs)	Varfolomeer *et al*[13]
Caspase-9	embryonic lethal**	neuroepithelial progenitors; mitochondrial (dexamethasone, staurosporin, etoposide, γ-radiation) pathways (thymocytes)	Kuida *et al*,[14] Hakem *et al*[15]
Caspase-11	normal	Fas? (thymocytes)	Wang *et al*[16]

*The perinatal lethality of caspase-3 knockout mice depends on genetic background (see text for discussion). **A very small percentage of caspase-9 knockout mice ($<2\%$) can survive through birth and develop normally. N/D, not determined

tissue-specific requirement for certain caspases during development.

Tissue-specific requirement of caspases

One of the early hypotheses proposed to explain how multiple caspases function *in vivo* was that individual caspases act in a tissue specific fashion. Although the simultaneous expression of multiple caspases in most, if not all, cell types rules out the possibility of any simplistic tissue-specific model for caspases, several caspase knockout mice did exhibit tissue-restricted phenotypes.

Caspase-9/caspase-3 pathway in neuronal development

Deletion of either caspase-3 or caspase-9 gene resulted in abnormal external appearance among mutant mice characterized by frequently observed prominent protrusions of the brain tissues which was associated with a skull defect, indicating that brain development in both caspase-$3^{-/-}$ and -$9^{-/-}$ mice were severely compromised.[11,14,15] Indeed, histological studies revealed many incidences of supernumerary cells in the brains of mutant animals and suggested that brain tissue expansion in these mice is likely due to ectopic cell masses that were mainly present in cortical areas, and to a lesser extent, in the cerebellum and the retinal neuroepithelium.[11] Importantly, immunohistological staining with neuronal and glial cell markers revealed similar cellular composition between these ectopic masses and the surrounding brain tissue, both of which failed to incorporate BrdU, thus clearly indicates that these supernumerary cells are postmitotic, terminally differentiated and not of any tumor origin.[11] Perhaps most significantly, further studies carried out in our studies and others have provided several lines of evidence indicating that developmental malformations such as hyperplasia observed in caspase-$3^{-/-}$ and -$9^{-/-}$ mice is due to reduced neuronal apoptosis during early embryogenesis. First, marked expansion of the periventricular zone, a place where active cell proliferation and death occur throughout brain development, were consistently observed in both mutant strains and led to ventricular disorganization. Second, both toluidine blue and TUNEL staining of brain sections from E12 caspase-$3^{-/-}$ and E10.5 to E16.5 caspase-$9^{-/-}$ embryos revealed a pronounced decrease in the number of apoptotic cells in the cortex and the neuroepithelium compared to wild-type mouse embryos.

The discovery that the absence of either capase-3 or caspase-9 function both led to a significant decrease in neuronal cell death during development and resulted in accumulation of superfluous neuronal cells has several important implications. Foremost, these studies definitely demonstrated that mutation of a mammalian caspase could alter cell death *in vivo*, casting out any remaining doubts on the importance of caspases in mediating mammalian apoptotic events. The similarities in the phenotypes observed in caspase-$3^{-/-}$ and caspase-$9^{-/-}$ also confirmed the presence of a caspase-9 dependent caspase-3 activation pathway that had been suggested from *in vitro* studies and revealed this pathway's essential role in mediating neuronal cell death that is required for proper brain development. Previously it has been argued that neuronal cell death in developing vertebrates was not genetically programmed, but mainly depended on cell-cell interactions.[19,20] One such example is the target-driven neuronal death which is believed to play a key role in matching postmitotic neurons with their targets to ensure the correct formation of synaptic connections. The altered cell death observed in caspase-$3^{-/-}$ and -$9^{-/-}$ embryos, however, was mainly of those actively dividing neuroepithelial progenitors located in the ventricular proliferative zone, indicating that the caspase-9/caspase-3 apoptotic pathway is involved in an earlier neuronal death event whose importance in the ontogeny of the brain was previously underappreciated.[21] Finally, the postmitotic and well-differentiated properties of supernumerary cells seen in caspase-$3^{-/-}$ and -$9^{-/-}$ mice are reminiscent of the survival neurons in *ced-3* mutant nematodes, suggesting that the basic machinery of programmed cell death is conserved evolutionarily.

It must be noted that backcrossing of caspase-$3^{-/-}$ mice onto the C57BL/6J background led to drastically alleviated neuronal phenotype and greatly improved survival rate (Zheng & Flavell, unpublished data). This observation thus suggests the possible presence of strain-specific gene or genes that can actively suppress the phenotype caused by the absence of caspase-3. In fact, the existence of such a strain-specific element capable of suppressing apoptosis-related phenotypes has been suggested before. Previously it was found that the survival ability of Bcl-$2^{-/-}$ mice varied markedly, depending on the particular strain background. While Bcl-$2^{-/-}$ mice with C57BL/6J background could not live past 6 weeks of age, backcrossing onto the 129/Sv background dramatically prolonged the life span for these mutant mice (K Nakayama, personal communication). Such genetic component(s) could potentially be identified using a genome scanning strategy that has been successfully employed for the mapping of a genetic modifier affecting the embryonic lethality of TGFβ1-deficient mice,[22] and subsequently isolated through positional cloning. It would also be interesting to see whether this genetic modifier(s) affects the phenotype of caspase-$9^{-/-}$ mice so that we might learn where this modifier(s) exerts its function along the apoptotic pathway.

Caspase-8 in cardiac development and erythrocytosis

Perhaps the most unexpected discovery from studies of caspase knockouts was the requirement of caspase-8 for regulation of erythropoiesis and proper development of heart muscles during embryogenesis.[17] Caspase-$8^{-/-}$ embryos died around E11 *in utero* with impaired formation of cardiac muscles and conspicuously visible hemorrhage in the abdominal area, in particular the liver. Marked hyperemia was also evident in most major blood vessels and in other organs including the lung and the retina. Although the molecular basis underlying these developmental abnormalities are completely unknown, it is likely due to defective

signaling of death receptors in the absence of caspase-8 since the almost identical phenotypes were also observed in embryos that lacked FADD,[23] an adaptor protein that bridges death receptors and caspase-8. In fact, such interpretation is consistent with the observation that death receptors such as Fas and DR5 are constitutively expressed in cardiac muscle. Interestingly, it was recently demonstrated *in vitro* that Fas-FasL interaction might also be important in the regulation of red blood cell homeostasis, through both eliminating less mature erythroblasts, as well as halting erythroid differentiation.[24] Given the fact that lack of either Fas or TNF receptors does not lead to the calamitous phenotypes observed in caspase-8 or FADD knockout mice, the death receptor involved in cardiac development and erythrogenesis could be another death receptor such as DR3 or DR5, which also utilizes FADD and caspase-8. Alternatively, more than one death receptor can potentially deliver these signals either in a tissue-specific or compensatory fashion. Gene targeting of DR3 and DR5 should help to clarify their potential roles in mediating these FADD/caspase-8 dependent biological processes.

Caspase-2 as tissue specific positive and negative effectors of apoptosis

Caspase-2 is a unique caspase in that it has two splicing isoforms, caspase-2_L and caspase-2_S, that can induce and inhibit apoptosis, respectively.[25] It is therefore not surprising that deletion of the caspase-2 gene had both positive and negative effect on apoptosis in a tissue-specific fashion.[10] In female germ cells where the maternally derived caspase-2_L represents the only caspase-2 species detectable, caspase-2 deficiency led to excess numbers of oocytes in the ovaries and rendered them resistant to apoptosis induced by anti-cancer reagent doxorubicin. The defective apoptosis in response to doxorubicin is tissue specific since caspase-$2^{-/-}$ blastocysts remained sensitive to treatment with doxorubicin. Unexpectedly, lack of caspase-2 caused accelerated cell death of facial motor neurons during development, while having no apparent effect on the number of neurons in other places including the vestibular, geniculate, nodose, and superior cervical ganglia. Although the underlying molecular basis for this developmental abnormality is entirely unknown, it is tempting to suggest that such a cell type specific alteration in apoptosis could result from different ratios of the long-to-short form of caspase-2 mRNA in various regions during embryonic brain development.

Taken together, studies from caspase deficient mice confirmed previous suggestion that certain caspases might act in a tissue specific manner. This conclusion, however, does not contradict the current cascade model for stepwise activation of caspases. In fact, almost all the tissue-specific phenotypes were observed in mice deficient in initiator caspases including caspase-2, -8 and -9, supporting their role as apical caspases that respond to respective apoptotic stimuli and are responsible for the activation of multiple downstream effector caspases. It is likely that some of these death signals are required for the various critical cell death events during development; and deficiencies in these apical caspases cannot be compensated and therefore lead to the various developmental anomalies observed in these mutant animals. Such an interpretation then inadvertently begs the intriguing question of why lack of caspase-3, an effector caspase, caused abnormal development of the brain while its deficiency was apparently compensated in thymocytes by other effector caspases presumably such as caspase-7. Although it is possible that the apoptotic stimulus responsible for the removal of neuronal progenitors triggers a linear caspase-9/caspase-3 activation pathway, we think a more plausible cause for the failure of caspase-7 to compensate in the embryonic brain is the developmental regulation of caspase expression. It is conceivable that caspase-3 is the only effector caspase expressed in those neuronal progenitors at that particular developmental stage, a notion supported by our finding that, at least in adult animals, effector caspases caspase-3, -6 and -7 are not ubiquitously expressed, but rather in restricted subregions of the brain (Hunot and Flavell, unpublished data).

Caspase cascade

Recent studies on both substrate specificity and prodomain function strongly suggest a stepwise mechanism for caspase activation during apoptosis that can be best exemplified in Fas-mediated and dexamethasone-induced apoptosis (Figure 1). Upon apoptotic stimulation, initiator caspases such as caspase-8 and -9 are recruited to their respective adaptors, such as FADD and Apaf-1, through homophilic interactions. Such recruitment probably results in oligomerization of the initiator caspases and presumably in an autocatalytic fashion, leads to their activation. Subsequently, activated initiator caspases proteolytically activate downstream effector caspases such as caspase-3 and -7 which in turn carry out the final destruction of the apoptotic cells.[26]

This overall framework of how caspases are activated in response to death signaling has been proven by studies carried out using cells derived from caspase knockout mice. For example, caspase-$9^{-/-}$ thymocytes exhibited striking resistance to diverse apoptotic stimuli including dexamethasone, etoposide, and γ-radiation, while they remained sensitive to apoptosis induced by UV, heat and osmotic shock, as well as by death receptors such as Fas and TNF receptor.[14,15] On the other hand, MEFs derived from caspase-$8^{-/-}$ mice were resistant to apoptosis mediated through p55 TNF receptor, Fas and DR3, but died rapidly when treated with dexamethasone, etoposide, UV-radiation, staurosporin, C6-ceramide and serum starvation. In contrast, cells deficient in caspase-3, -6, or -7 remain sensitive to all these above stimuli, indicating the presence of compensatory mechanisms among effector caspases[11,12] (Zheng and Flavell, unpublished data). These results, together with the finding that both brain and thymocyte extracts from caspase-$9^{-/-}$ mice failed to activate caspase-3 when incubated with dATP and cytochrome c *in vitro*, demonstrated the presence of a hierarchy of caspases consisting of initiators including caspase-8 and -9, as well as effectors including caspase-3, -6, and -7.[14,15] Importantly, the requirement of caspase-9 for the activation of caspase-3 is not simply an artifact of

the *in vitro* system, as we also demonstrated the lack of caspase-3 activation in caspase-$9^{-/-}$ neuroepithelial cells *in vivo*[14] and subsequently found that both caspase-3 and -7 activation was defective in dexamethasone, but not anti-Fas treated caspase-$9^{-/-}$ thymocytes (Zheng and Flavell, unpublished data).

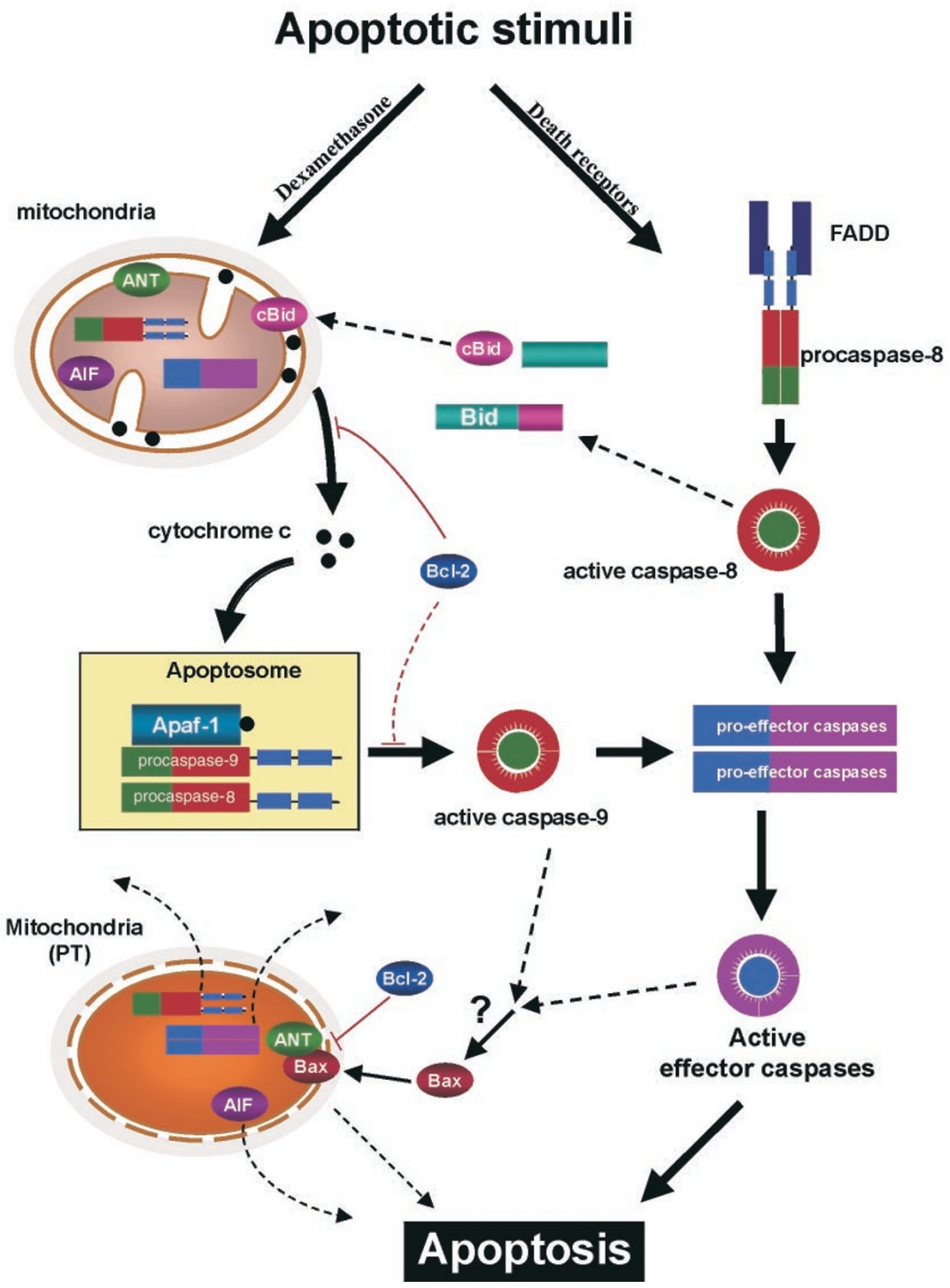

Figure 1 Apoptotic pathways and caspase activation. Studies using caspase knockout mice have confirmed the presence of at least two apoptotic pathways capable of initiating sequential activation of caspases. While death receptors most likely can directly activate the initiator caspase-8, activation of caspase-9, the upstream caspase in response to many other apoptotic stimuli requires release of cytochrome c into the cytosol from the mitochondria. In addition, mitochondrial participation provides an amplifying mechanism for further caspase activation through caspase-dependent release of multiple pro-apoptotic factors including additional cycochrome c, mitochondria-resident caspases and AIF

While these studies clearly demonstrated that caspases are not created equal, but play distinct roles in a proteolytic cascade, they also left us with a few unexpected findings and unanswered questions regarding caspase activation. (1) A number of *in vitro* biochemical experiments have suggested a linear caspase-9/Apaf-1/caspase-3 pathway,[27,28] deficiencies in these proteins, however, resulted in developmental abnormalities with different severities.[11,14,15] More importantly, Apaf-1$^{-/-}$ embryos displayed delayed removal of interdigital webs,[29,30] a phenomenon that was not observed in either caspase-3$^{-/-}$ or -9$^{-/-}$ mice, suggesting that Apaf-1 can either act as an adaptor for another initiator caspase, or participate in caspase-independent apoptosis. (2) It has been proposed that caspase-2 can associate with TNFR1 receptor complex via the adaptor protein RAIDD/CRADD and contribute to TNFR1 induced apoptosis. In light of the fact that caspase-8$^{-/-}$, but not caspase-2$^{-/-}$ MEFs, are resistant to TNF receptor-mediated death, it seems that, at least in MEFs, TNFα-induced apoptosis exclusively utilizes caspase-8 and the potential physiological relevance of caspase-2 in death receptor signaling in other cell types remain to be investigated. (3) Recent studies have revealed that the apparently unrelated dexamethasone and Fas pathways are in fact interconnected through caspase-8 mediated Bid cleavage, which results in the translocation of the C-terminal fragment of Bid into the mitochondria and subsequent release of cytochrome c into the cytosol.[31,32] Although this Bid-mediated pathway has been suggested to provide an amplification mechanism for the Fas signaling, no apparent alteration in the kinetics of cell death has been observed in anti-Fas treated caspase-9$^{-/-}$ or Apaf-1$^{-/-}$ thymocytes, thus indicating such an amplification pathway might function in cell-type specific settings. Given the scarce supply of mitochondria in thymocytes, it would be extremely interesting to examine whether the absence of caspase-9 would affect the Fas-pathway in mitochondria-rich tissues such as the liver. (4) The molecular ordering of the activation of effector caspases remains to be clarified. While *in vitro* approaches using relatively specific caspase inhibitors or antibody depletion have started to delineate such activating pathways,[33,34] definitive conclusions can only be drawn using cells derived from various caspase-deficient mice, especially when such activating sequences are cell type specific. Finally, it is worth noting that dexamethasone treated caspase-9$^{-/-}$ thymocytes eventually do die of apoptosis, exhibiting classic features such as PS flipping and DNA fragmentation. Since we have been unable to detect any caspase-3 or -7 activation in these thymocytes, the precise nature of this compensatory apoptotic mechanism remains a mystery. Interestingly, it has been historically shown that thymocytes treated with dexamethasone at a low concentration undergo a slow apoptotic death that is transcriptionally dependent;[35] the caspase-9 dependent rapid cell death induced by high concentrations of dexamethasone, however, is not affected by inhibitors of transcription (Zheng and Flavell, unpublished observation). It is therefore tempting to speculate that a transcription-dependent mechanism, whether caspase-dependent or not, might be responsible for the much delayed, residual cell death induced by dexamethasone in caspase-9$^{-/-}$ thymocytes. Since almost nothing is known about the possible connection between transcription-dependent apoptotic mechanisms and caspase activation, further investigation into this matter would be of great interest.

Caspases and mitochondria: the interconnection

In addition to activation of caspases, various mitochondrial alterations including release of cytochrome c into the cytosol and disruption of the mitochondrial potential ($\Delta\psi$m) are also frequently observed during the induction phase of mammalian apoptotic events.[36] While the precise mechanism underlying these processes is just beginning to unfold, emerging evidence indicates that these biochemical changes are not simply bystanders of the death program, but rather active participants in the cell death pathway. Through a series of elegant experiments using *in vitro* biochemical approaches, it was demonstrated that cytochrome c can be released from the intermembrane space of mitochondria upon apoptotic stimulation by a Bcl-2 inhibitable mechanism and triggers the formation of the apoptosome, the caspase-activating complex that initiates caspase proteolytic cascade.[27,37,38] The presence of this mitochondria-dependent caspase-activating pathway is further supported by the observation that dexamethasone-treated caspase-9$^{-/-}$, as well as Apaf-1$^{-/-}$ thymocytes, are defective in activating caspase-3 despite unaffected cytochrome c translocation, thus confirming the linear pathway of caspase-3 activation through cytochrome c mediated formation of the caspase-9/Apaf-1 complex.[14,15]

The exact contribution of the other prominent mitochondrial change associated with apoptosis, collapse of the mitochondrial inner membrane potential or permeability transition (PT), remains much less certain. Although it is well documented that inhibitors and stimulants of PT pore opening can deter or induce apoptosis, respectively, several *in vitro* experimental approaches have reached contradictory conclusions as to whether PT is an essential component of the apoptotic machinery or simply an accompanying phenomenon resulting from caspase activation.[39] When caspase-9$^{-/-}$ and Apaf-1$^{-/-}$ thymocytes were treated with apoptotic stimuli such as dexamethasone or staurosporin, no mitochondrial potential collapse was observed as measured by $DiOC_6$, a fluorescent dye whose incorporation is mitochondrial potential dependent.[15,30] These results thus indicate that, at least in these situations, permeability transition requires caspase activity and most likely functions as a downstream feedback mechanism. This interpretation is also consistent with the previous observations that mitochondrial potential collapse can be mediated by insertion of Bax into the mitochondrial membrane and that such targeting of Bax can be blocked by the caspase inhibitor zVAD.fmk.[40–42]

Given the conspicuous changes associated with mitochondria during apoptosis and the fact that many of the Bcl-2 superfamily members are localized in this organelle, it has been proposed that the mitochondrion,

rather than caspases, is the central executioner of mammalian apoptotic events.[43] While the cytochrome c-mediated formation of the apoptosome undoubtedly plays a crucial role in activating caspase-9, studies from caspase-$8^{-/-}$ and caspase-$9^{-/-}$ mice seem to suggest a minimal involvement of mitochondrial element(s) in apoptosis induced by death receptors such as Fas at least in lymphocytes. Thus, it is unlikely that effective induction and completion of apoptosis can be attributed to either caspase or mitochondria elements alone. Instead, it seems that caspase activation and mitochondrial alterations are interconnected to provide multiple feedback loops so as to ensure the speedy progression and possibly control of the apoptotic pathway. The interconnectedness of caspases and mitochondria is further supported by the unexpected finding that caspases can both positively and negatively regulate mitochondrial potential, as revealed in caspase knockout mice. For example, untreated caspase-$9^{-/-}$ thymocytes exhibited lower $\Delta\psi$m compared to untreated caspase-$3^{-/-}$ and wild type thymocytes.[15] In addition, we have also observed that in response to certain stimuli, caspase-$3^{-/-}$ thymocytes display accelerated collapse of mitochondria potential (Zheng and Flavell, unpublished data). While the molecular basis for these observations are completely unknown, it is tempting to speculate that it is the procaspases that are somehow involved in the regulation of PT, especially considering recent findings that several caspases including caspase-2, -3, and -9 are also localized in the mitochondria.[44,45]

Taken together, caspase knockout mice, particularly those of caspase-8 and -9, have revealed two major apoptotic pathways in mammals that ultimately lead to the activation of caspases. Although caspases are indeed indispensable for the phenotypic features of apoptotic cell death (see below), mitochondria acts as a crucial facilitator of caspase activation by both initiating caspase-9 activation and subsequently, in a caspase dependent manner, further amplifying the apoptotic process through release of various pro-apoptotic components including additional cytochrome c, mitochondrially resident caspases and AIF (Figure 1).

Role of caspases in mediating morphological and biochemical changes associated with apoptosis

The suicidal nature of apoptotic death is characterized by the various morphological and biochemical changes that allow the disassembly of cellular structures, decomposition of cellular contents and tagging of apoptotic cells for their engulfment by neighboring phagocytes.[46] Despite our lack of understanding of the underlying mechanism governing these processes, a large body of evidence indicates that caspases as a whole play an indispensable role in mediating these cellular events through proteolytically cleaving various cellular targets. It has been convincingly demonstrated that all of the diagnostic features of apoptosis, including cytoplasmic blebbing, chromatin condensation and internucleosomal DNA fragmentation, can be inhibited by pan-caspase inhibitors like zVAD.fmk.[47] To date, more than 60 proteins have been found to undergo caspase-dependent cleavage during apoptosis, most of which serendipitously.[48] While the relevance of most cleavages to apoptosis remains correlative, the cleavages of several cellular targets of caspases, including DFF45/ICAD, gelsolin and Pak2, have been shown to be critical for some of these morphological and biochemical changes. Based on studies using caspase inhibitors and *in vitro* cleavage assays, it is generally assumed that effector phase caspases (caspase-3, -6 and -7) are responsible for the proteolytic cleavage of these substrates.

Studies from caspase deficient mice, while supporting the general role of effector caspases in this process, unexpectedly revealed a strict requirement for caspase-3. In fact, diverse cell types including hepatocytes, thymocytes and MEFs from caspase-$3^{-/-}$ mice exhibited a set of altered morphological and biochemical features when undergoing apoptosis including absence or drastically delayed onset of cytoplasmic blebbing, nuclear condensation and breakdown, as well as DNA fragmentation.[12,49] Such aberrant apoptotic death, exhibiting some, but not all, classical features of apoptosis, was also observed in the human cell line MCF7 which has defective caspase-3 expression,[50] suggesting that the requirement for caspase-3 in mediating these events is evolutionarily conserved. Furthermore, studies carried out in our laboratory have also correlated the delayed onset or lack of these morphological and biochemical changes with delayed or lack of cleavage of several key substrates that have been implicated in these processes, including DFF45/ICAD, gelsolin, lamin and fodrinα.[49] A definitive conclusion on the cause-effect relationship between their cleavage and apoptotic-related changes, however, can only be drawn using mutant cell lines where individual substrates such as laminB or gelsolins has been replaced with their noncleavable mutants through gene 'knock-in' technique.

The significantly delayed internucleosomal cleavage of genomic DNA in both caspase-$3^{-/-}$ hepatocytes and thymocytes is intriguing. Based on the current model on how caspase activation leads to DFF40/CAD activation,[51–53] it is tempting to postulate that the attenuated cleavage of DFF45/ICAD in the absence of caspase-3 is likely responsible for delayed DNA ladder formation in caspase-$3^{-/-}$ cells. Careful examination of the data, however, revealed that the cleavage of DFF45/ICAD does not fully correlate with the internucleosomal cleavage of DNA[49] and therefore suggests that there might be more to the DNA degradation during apoptosis than the simplistic ICAD/CAD model. In fact, DFF40/CAD synthesized in the absence of DFF45/ICAD is not enzymatically active,[51] together with the finding that DFF45/ICAD deficient cells failed to undergo DNA fragmentation,[54] suggesting that DFF45/ICAD is required for both the activity and inhibition of CAD. Furthermore, there might well be more than one CAD protein, a possibility strengthened by the recent cloning of other DFF45/ICAD homologs with apoptosis-inducing activities.[55] It is therefore conceivable that other DFF45/ICAD and CAD complexes also exist and can be activated in the absence of caspase-3. It is also important to point out that two other groups, using either caspase-$3^{-/-}$ MEFs or MCF-7 have reported that no DNA fragmentation was observed in these mutant apoptotic

cells.[12,50] In our studies however, we were always able to detect some residual level of DNA fragmentation in both caspase-3$^{-/-}$ thymocytes and hepatocytes.[49] It is likely that this apparent discrepancy reflects differences in experimental systems such as cell types, apoptotic stimuli and the sensitivity of the assays used.

Cytoplasmic membrane changes associated with apoptosis include the formation of blebs and loss of PS membrane asymmetry. Although their inhibition by zVAD.fmk indicates that these alterations are caspase-dependent, the precise caspases involved are not known. Interestingly, PS flipping is defective in caspase-9$^{-/-}$ but not caspase-3$^{-/-}$ or -6$^{-/-}$ thymocytes treated with dexamethasone, indicating that either caspase-9 or another caspase-9 activated caspase (but not caspase-3 or -6) is responsible for the exposure of PS during apoptosis[14,15] (Zheng and Flavell, unpublished data). While the caspase target involved in PS flipping is entirely unknown, cleavage of many proteins, ranging from cytoskeletal structural proteins such as fodrin, actin, and Gas2 to signaling molecules like PAK2 and cdc42, has been suggested to play a role in the formation of cytoplasmic blebs. As apoptotic neutrophils lacking gelsolin are defective in forming blebs,[56] the greatly reduced cleavage of gelsolin could account for the lack of bleb formation in caspase-3$^{-/-}$ cells, where PAK2 and cdc42 cleavage remain unaffected during apoptosis (Zheng & Flavell, unpublished data). Interestingly, the cleavage of fodrinα, a spectrin-like cytoskeleton protein that has also been implicated in mediating bleb formation, is altered in caspase-3$^{-/-}$ cells as well.[49] It has been previously assumed that fodrinα undergoes sequential cleavage during apoptosis mediated by calpains followed by a caspase, although the precise identity of the caspase involved in the second step was controversial.[57,58] We and others, using caspase-3$^{-/-}$ thymocytes and MCF7 cells respectively, clearly demonstrated that caspase-3 is indeed required for the second-step cleavage of Fodrin-α.[49,59] Moreover, we have preliminary data indicating that at least in thymocytes, a caspase, not calpains, is responsible for the initial cleavage of fodrinα as zVAD.fmk could complete block both cleavage steps and most significantly, neither cleavage was observed in caspase-9$^{-/-}$ thymocytes treated with dexamethasone (Zheng and Flavell, unpublished data). While it is tempting to suggest that caspase-9 might directly cleave the protein, we can not rule out the possibility that another effector caspase, likely caspase-7, could be responsible for this first-step cleavage of fodrinα that is followed by a secondary cleavage by caspase-3. Therefore, it would be of great interest to directly test *in vitro* whether caspase-7 and -9 can mediate the initial cleavage of fodrinα.

It is important to recognize that one caveat of these studies is that they can not distinguish between substrate cleavage by caspase-3 directly or other caspases downstream of caspase-3. As a result, we can not conclude with absolute certainty that caspase-3 is directly responsible for the cleavage of fordrinα, DFF45/ICAD, laminB and gelsolin. However, given corroborating results from *in vitro* studies and the fact that the cleavages of these substrates are not affected at all in cells lacking caspase-6 and caspase-7 (Zheng and Flavell, unpublished data), we are convinced that caspase-3 is indeed the key caspase that directly mediates the proteolysis of these cellular targets. Since *in vitro* studies have always supported the notion that caspase-3 and -7 are biochemically indistinguishable, the apparent rigid requirement for caspase-3 in processing these substrates therefore begs the question of what is the exact *in vivo* function of caspase-7. Although it is possible that caspase-7 is activated later than caspase-3 during apoptosis and is responsible for the residual and delayed cleavage of DFF45/ICAD, gelsolin and laminB, our preliminary data suggests that caspase-7 activation is concurrent with that of caspase-3 (Zheng and Flavell, unpublished data). Instead, we favor the possibility that caspase-3 and -7 are differentially compartmentalized during apoptosis and thus perform distinct functions.

Role of caspases in mediating inflammation

While the only known function of the prototype caspase CED-3 is to execute the death program in *C. elegans*, certain mammalian caspases have apparently evolved to carry out other physiological functions. In fact, the very first mammalian caspase, caspase-1, was originally identified as interleukin-1β converting enzyme (ICE), an enzyme involved in mediating inflammatory responses.[60] Studies from caspase-1$^{-/-}$ mice confirmed the essential role of caspase-1 in mediating the processing and export of mature interleukin-1β as caspase-1 deficient mice were resistant to endotoxic shock induced by LPS challenge and caspase-1$^{-/-}$ monocytes failed to produce mature IL-1β following LPS/nigericin treatment.[8,9] Unexpectedly, the secretion, but not processing, of IL-1α was also defective in these caspase-1$^{-/-}$ cells, revealing a previously unknown function of caspase-1 in mediating the release of both IL-1α and -β, two cytokines who lack conventional signal peptide and are exported independent of the ER/Golgi secretory pathway. Although how caspase-1 mediates their secretion remains entirely unknown, it seems that caspases play a key role in the production of cytokines that lack the conventional signal peptide sequences. In addition to IL-1α and β, it was subsequently demonstrated that the processing of another such cytokine, IL-18, also required caspase-1.[61] More recently, *in vitro* studies have suggested that the processing and secretion of IL-16 might be mediated by caspase-3,[62] an intriguing possibility that is yet to be confirmed using caspase-3$^{-/-}$ T cells and is consistent with the emerging view that even effector caspases can be activated without inducing cell death.[63]

Interestingly, studies from caspase-11 deficient mice revealed that deficiency in caspase-11 resulted in a similar phenotype as in caspase-1$^{-/-}$ mice that is characterized by resistance to septic shock and defective production of IL-1α and -β.[16] Given the fact that caspase-11 does not process either caspase-1 or IL-1β *in vitro*, together with the demonstration that caspase-11 physically interacts with caspase-1 and is required for caspase-1 activation, it seems that in parallel to the caspase activation cascade following apoptotic stimulation, there also exists a hierarchy

of caspases during inflammatory responses consisting of at least the upstream caspase-11 and the effector caspase-1. Since caspase-1 is also required for IL-18 processing, it would be of great interest to examine whether IL-18 production is affected in caspase-11$^{-/-}$ mice.

Concluding remarks and future perspectives

The presence of multiple mammalian homologs of the *C. elegans* death genes *ced-3* and *ced-9* clearly indicates that the cell death machinery has been not only conserved, but has also acquired greater complexity during the evolution of the mammals. While the proto-caspase CED-3 in *C. elegans* likely functions as both an initiator and an effector, these two functions have apparently diverged during evolution to provide organisms with the ability to respond to more diverse stimuli, as well as to allow a more vigorous control over the apoptotic pathway. The emerging evidence for an elaborately programmed intracellular cascade that centers on caspase activation thus presents a major challenge to the cell death field which is how each caspase participates in and contributes to the dismantling of apoptotic cells. As discussed in this review, through studies using various caspase knockout mice, we have begun to dissect the precise involvement of individual caspases in mammalian apoptosis, as well as how different caspases functionally relate to each other. Like most scientific endeavors, however, these studies have probably raised more questions than they have answered. Just as our discovery that caspase-3 is crucial in neuronal development leads to the possible existence of an unidentified genetic modifier, revelation of unexpected requirement for caspase-8 in heart muscle development unavoidably invites questions about its underlying molecular basis. The answers to many of these newly raised questions, we believe, lie in a fundamental understanding of the very basic aspects of caspase expression and activation: what, where and when?

It is widely assumed that most caspases are concurrently expressed in all cell types with little, if any, transcriptional regulation. While that might be the case for adult tissues, the expression of various caspases during mammalian development is undoubtedly regulated, at both the developmental stage and location in the embryo. In fact, we speculate that the apparent lack of compensation by caspase-7 during the early development of caspase-3$^{-/-}$ brain is due to the absence of caspase-7 in those neuronal progenitors rather than lack of its activation, as discussed before. Furthermore, we have also found that, even in adult animals, many murine caspases (including caspase-3, -6 and -7) exhibit differential expression patterns of their mRNA isoforms in different tissues (Zheng and Flavell, unpublished data). Considering the previous finding that two caspase-2 isoforms have opposing effects on apoptosis[25] and that multiple species of a single caspase can be identified during apoptotic events,[64] the possibility that one caspase might in fact exert different functions in different tissues must be taken seriously. Thus, a systematic examination needs to be undertaken to ascertain whether these different isoforms translate to functional differences and to assess where and when these caspase isoforms are expressed in adult tissues and during development. Such information is not only of great intellectual importance to our basic understanding of caspases, but also has a significant bearing on development of strategies for therapeutic interventions that would allow selective regulation of caspase activities.

Although a large body of evidence supports the idea that any given apoptotic stimulus will lead to the activation of multiple caspases, the exact identities of those caspases remain poorly studied. Much less is known about the difference in the pool of caspases that are activated in response to different stimuli. Meanwhile, despite recent attempts from our laboratory and others to unveil the caspase activating sequence in dexamethasone- and Fas-mediated apoptosis, the caspase cascades induced by a wide variety of other well-characterized apoptotic stimuli, such as p53 and ceramide, are yet to be delineated. Since such knowledge is paramount to our basic understanding of the apoptotic machinery as well as our ability to potentially regulate its activation, a comprehensive approach must be taken to find out what caspases are activated and more importantly, when they are activated under various physiological and pathological conditions.

Perhaps a more challenging issue, is determining the intracellular location of various caspases and their translocations upon apoptotic stimulation. It has been reported that in addition to their localization in the cytosol, many if not all caspases, are also present in other subcellular organelles such as the mitochondria, the nucleus and the endoplasmic reticulum (ER). Furthermore, several caspases have been shown to undergo intracellular translocation during apoptosis. For example, both caspase-2 and -9 translocate from mitochondria to the cytosol whereas caspase-7 does the exact opposite.[45,65] These observations therefore present the intriguing possibility that compartmentalization of caspases is yet another mechanism by which caspase activation can be regulated. The defective cleavage of several caspase-7 cleavable substrates in caspase-3 deficient cells, despite the activation of caspase-7, further supports the notion that ability of a given caspase to cleave a cellular target not only depends on its substrate specificity, but also its accessibility to that target *in vivo*. Determining where caspases and their cofactors are located intracellularly will contribute to a better understanding of caspase regulation and likely offer yet another new window of opportunity to achieving proper control of apoptosis.

In a short span of 5 years since the first caspase, ICE, was implicated in mammalian apoptosis, we have witnessed an explosion in interest and knowledge in the field of cell death and its central executioners, caspases. Despite the tremendous progress, the ultimate goal of translating our basic understanding of the apoptotic pathway into therapeutic interventions remains distant. We are convinced, however, that a more vigorous investigation into the what, when and where questions of caspase activation will provide important guidance to the development of more effective, small-molecule-based cell-perme-

able inhibitors and bring us closer to the final goal of controlling apoptosis.

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