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# Can't live without them, can live with them: roles of caspases during vital cellular processes

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**Abstract** Since the pioneering discovery that the genetic cell death program in *C. elegans* is executed by the cysteine-aspartate protease (caspase) CED3, caspase activation has become nearly synonymous with apoptosis. A critical mass of data accumulated in the past few years, have clearly established that apoptotic caspases can also participate in a variety of non-apoptotic processes. The roles of caspases during these processes and the regulatory mechanisms that prevent unrestrained caspase activity remain to be fully investigated, and may vary in different cellular contexts. Significantly, some of these processes, such as terminal differentiation of vertebrate lens fiber cells and red blood cells, as well as spermatid terminal differentiation and dendritic pruning of sensory neurons in *Drosophila*, all involve proteolytic degradation of major cellular compartments, and are conceptually, molecularly, biochemically, and morphologically reminiscent of apoptosis. Moreover, some of these model systems bear added values for the study of caspase activation/apoptosis. For example, the *Drosophila* sperm differentiation is the only system known in invertebrate which absolutely requires the mitochondrial pathway (i.e. Cyt c). The existence of testis-specific genes for many of the components in the electron transport chain, including Cyt c, facilitates the use of the *Drosophila* sperm system to investigate possible roles of these otherwise essential proteins in caspase activation. Caspases are also involved in a wide range of other vital processes of non-degenerative nature, indicating that these proteases play much more diverse roles than previously assumed. In this essay, we review genetic, cytological, and molecular studies conducted in *Drosophila*, vertebrate, and cultured cells, which underlie the foundations of this newly emerging field.

**Keywords** Caspase · Terminal differentiation · Apoptosis · Nonapoptotic process · Apoptosis-like process · Proteolytic degeneration · Compartmentalized activity

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## Introduction

Virtually all cells of higher metazoans contain a genetic death program called programmed cell death (PCD) that usually functions to eliminate unwanted and potentially dangerous cells [1–6]. The pathogenesis of many diseases, including cancer and neurodegenerative disorders is attributed to the malfunctioning of PCD [7–10]. Apoptosis, the most common form of PCD, is characterized by a conserved sequence of morphological, cytological, and biochemical events [11–15]. A key feature of apoptosis is the activation of a unique family of cysteine-dependent aspartate specific proteases called caspases. The mammalian genome encodes fourteen distinct caspases, seven of which were shown to function in apoptosis and will henceforth be termed apoptotic caspases or simply caspases [16, 17].

Caspases are synthesized as inactive proenzymes, which work in a precisely controlled proteolytic cascade to activate themselves and one another [18–24]. The apoptotic caspases are generally classified as initiators (also called apical) or effectors (also known as executioners), depending on their position in the proteolytic hierarchy. Initiator caspases are activated through dimerization facilitated at multi-protein complexes. Activation of caspase-9, the initiator caspase of the intrinsic pathway, involves its recruitment to the apoptosome by Apaf-1-Cyt c complex,

while the apical caspase of the extrinsic apoptotic pathway caspase-8 is activated within the death-inducing signaling complex (DISC) [25–29]. An adaptor-independent oligomerization mechanism for activation of caspase-8 was also suggested recently [30]. On the other hand, activation of effector caspases, such as caspase-3 and -7 occurs upon their cleavage at specific internal aspartic acid residues by initiator caspases [28, 29]. Downstream of this activational cascade, caspases cleave a variety of regulatory and structural proteins and important enzymes, ultimately leading to cell death. The morphological manifestations of apoptosis include cell detachment and shrinkage, nuclear condensation and segmentation, membrane blebbing, and disassembly into apoptotic bodies that are often engulfed by neighboring cells or phagocytes [13, 14, 31–34]. Some biochemical aspects occuring during apoptosis can also be detected using various methods. For example, DNA fragmentation can be detected by terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) assay [35, 36], caspase activity may be revealed by detecting cleavage of known endogenous caspase targets, such as the DNA repair enzyme poly(ADP-ribose) polymerase (PARP) and the nuclear intermediate filaments (lamins), or labeled synthetic substrates or inhibitors [37], whereas the cleaved and thus active form of caspases can be visualized using specific antibodies [38].

In addition to regulation by activating proteins, caspases are also negatively regulated by the inhibitor of apoptosis proteins (IAPs), which can bind to and inhibit caspases in both insects and mammals [39–42]. Studies in *Drosophila* provided important insights into the anti-apoptotic function of IAPs [43]. *Drosophila* IAP1 (Diap1) encodes an E3 ubiquitin ligase that ensures cell viability by preventing inappropriate caspase activation and apoptosis [44–46]. In living cells, Diap1 promotes ubiquitination and degradation of the apoptotic initiator caspase Dronc [47, 48] and blocks effector caspases by targeting them for polyubiquitynation and nonproteasomal inactivation [49]. Upon receipt of apoptotic stimuli, Diap1 is inactivated by Reaper-family proteins [44–46, 50]. Reaper stimulates the self-conjugation and degradation of Diap1, thereby irreversibly removing this critical caspase inhibitor [51]. In mammals, induction of apoptosis in thymocytes induces the auto-ubiquitination and degradation of IAPs [52], and targeted removal of the RING domain of XIAP leads to the stabilization of this caspase inhibitor [53]. Therefore, the physiological requirement of the IAPs' ubiquitin-ligase activity for the inhibition of caspases has been conserved in evolution (Fig. 1).

Here, we review evidence suggesting that in addition to their pivotal role in cell death, apoptotic caspases normally play an important role in a variety of non-apoptotic and apoptosis-like vital processes, including cell differentiation,

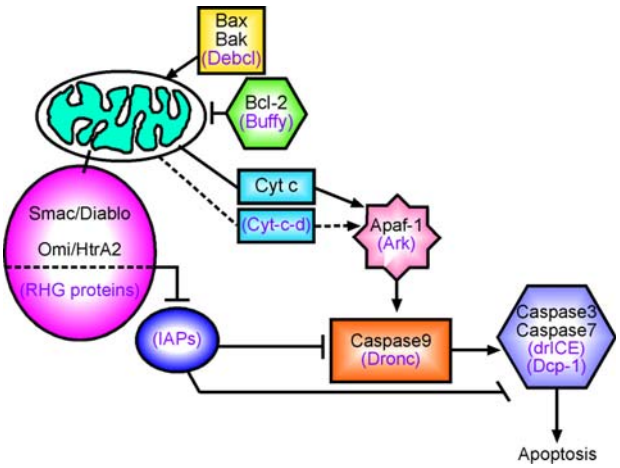


**Fig. 1** The conserved core apoptotic machinery in *Drosophila* and mammals. A schematic model of the major components in the conserved apoptotic machinery. Mammalian proteins are indicated in black, while their *Drosophila* counterparts are shown in purple and are flanked by parentheses. IAPs are found in both organisms. Arrows represent activation and T-shapes indicate inhibition. Dashed lines represent lack of biochemical evidence

cell signaling, and cellular remodeling. We also generally distinguish between the processes involving partial cellular degeneration, which are conceptually reminiscent of apoptosis and other processes that, at least superficially, do not seem to involve massive cellular proteolysis. Several recent reviews that cover some aspects of these processes are also available [23, 54–59]. In this essay, however, we focus and elaborate on the processes during which the apoptotic caspases are involved and, when relevant, indicate the molecular and morphological similarities among these processes and between them and conventional apoptosis. Although the mechanisms that prevent excessive caspase activity and undesirable cell death during these processes as well as those that restrict caspase activity to only a few substrates remained largely unexplained, in the concluding remarks section we discuss potential mechanisms based on rigorous survey of the relevant literature.

## Programmed elimination of cellular content

During apoptotic cell death, caspases orchestrate restricted proteolysis of several hundred proteins, resulting in demolition of cellular structures and organelles [60]. Given the ability of caspases to mediate cleavage of substrate proteins in a precisely controlled manner that is harmless to neighboring cells, it should not come as a complete surprise that some cells have developed mechanisms allowing the harnessing of these molecular knives to mediate only partial degeneration of their cellular content during normal

specialization. The early observations of such phenomenon can be dated back to the beginning of the twentieth century when researchers looked under a light microscope at differentiating lens fiber cells, sperm cells, and mammalian red blood cells, and noticed that these cells shed off much of their cellular content [61–63]. The more recent emergence of biochemical, histological, and genetic assays for detection and assessment of apoptotic mechanisms have facilitated the first comparative studies of these processes and apoptosis in both vertebrates and *Drosophila*.

## Loss of organelles and enucleation during terminal lens fiber cell differentiation

The role of vertebrate lens is to focus light on the retina. Lens fiber cells must therefore turn transparent with minimized potential light scatter from their cytoplasmic organelles. During embryonic development, organelle degradation is triggered in cells in the center of the lens, which is characterized by a rapid and coordinated disappearance of all membrane-bound organelles (Fig. 2a–c) [64]. Despite the absence of organelles, cells in the lens core continue to live in the sense that they continue to metabolize and persist throughout the life of an individual [65]. This unique terminal differentiation process is accompanied by a series of morphological and biochemical events that bear high resemblance to those that occur during apoptosis. For example, nuclear chromatin condenses, nuclear pores cluster, nucleolar structure changes, and eventually DNA fragments are produced that stain positively in a TUNEL assay (Fig. 2b) [66–69]. Other organelles, such as the endoplasmic reticulum and Golgi complex disintegrate, while the mitochondria fragment and fail to maintain membrane potential [70–72]. This process also involves proteolytic cleavage of known substrates of caspases, including PARP, lamin A/C and lamin B, and the actin-cross-linking protein Spectrin [67–69, 73]. Furthermore, denucleation and PARP cleavage in differentiating lens cells in culture were inhibited in the presence of peptide inhibitors of caspase-3, as well as upon addition of synthetic peptide inhibitors of caspase-1, -2, -4, -6, and -9 [69, 74]. Overexpression of the anti-apoptotic protein Bcl-2 in mice or chicken lenses also resulted in the abnormal retention of nuclei in the inner fiber cells [75, 76]. Although these studies support the notion that the apoptotic machinery is utilized during lens cell organelle degradation, lens fiber differentiation also exhibits many aspects that fundamentally differ from apoptosis. For example, whereas apoptotic cells usually exhibit membrane blebbing, there is no evidence of membrane blebbing or the formation of apoptotic bodies in lens fiber cells, but instead, aging lens fiber cells are compressed into the center of the lens, where they undergo cell-cell fusion and the formation of specialized membrane interdigitations [67, 73]. Moreover the caspase cleavage of the cytoskeletal protein $\alpha$-spectrin, which is believed to be important for membrane blebbing, results in a short-lived protein in apoptotic cells, but cleaved fragments of $\alpha$- and $\beta$- spectrins during terminal differentiation and aging of lens fiber cells appear to remain stable for the lifetime of the organism and may help maintain permanent remodeling of the membrane skeleton [73]. In addition, despite the superficial similarities, lens denucleation also appears to be distinctly different from classical apoptosis. In mature lens fibers, other cytoplasmic organelles have already disappeared 2–3 days before DNA fragmentation occurs [67]. In contrast, apoptosis in many cell types is characterized by the presence of organelles in the cytoplasm of cells in which the DNA has already been extensively degraded [77–80]. Furthermore, lens denucleation occurs over the course of 3–4 days from the onset of nuclear changes to the complete disappearance of the DNA, which is far slower than classical apoptosis that can be completed in about 3 h [81, 82]. Finally, a more recent study using knockout mice failed to demonstrate any significant alteration of this process in mice deficient for effector caspase-3, -6, -7, and the double mutant -3 and -6 [83]. One explanation for the discrepancy is that these caspase-like activities correspond to other caspases or other proteases with activities that partially overlap with caspases. Indeed, Bassnett and colleagues showed that caspase-6-like VEIDase activity is highly distributed throughout the lens from both wild-type and mice deficient for caspase-6, suggesting that another protease different from caspase-6 may exhibit this activity [83]. Additionally, increasing number of cellular proteins, including alpha- and beta-fodrin, calmodulin-dependent protein kinases, PARP and tau were found to be dually susceptible to both caspases and calpains [84], and the peptide Ac-DEVD-CHO was shown to inhibit not only caspase-3 activity, but also the activities of caspase-1, -6, -7, -8, -9, and -10 [85]. Thus, future identification of proteases that are involved in lens fiber cells differentiation may help to better define the extent of similarly and difference between this proteolytic process and apoptosis.

## Loss of organelles and enucleation during terminal erythrocyte differentiation

Erythropoiesis is the process by which mature red blood cells (erythrocytes) are produced [86]. As opposed to almost all vertebrates, maturation of mammalian erythrocytes involves extrusion of their nuclei and organelles, including mitochondria, endoplasmic reticulum, and cytoskeletal structures, providing more space for hemoglobin [63]. In an early work by Takano-Ohmur and colleagues, some of the apoptotic and non-apoptotic aspects of this

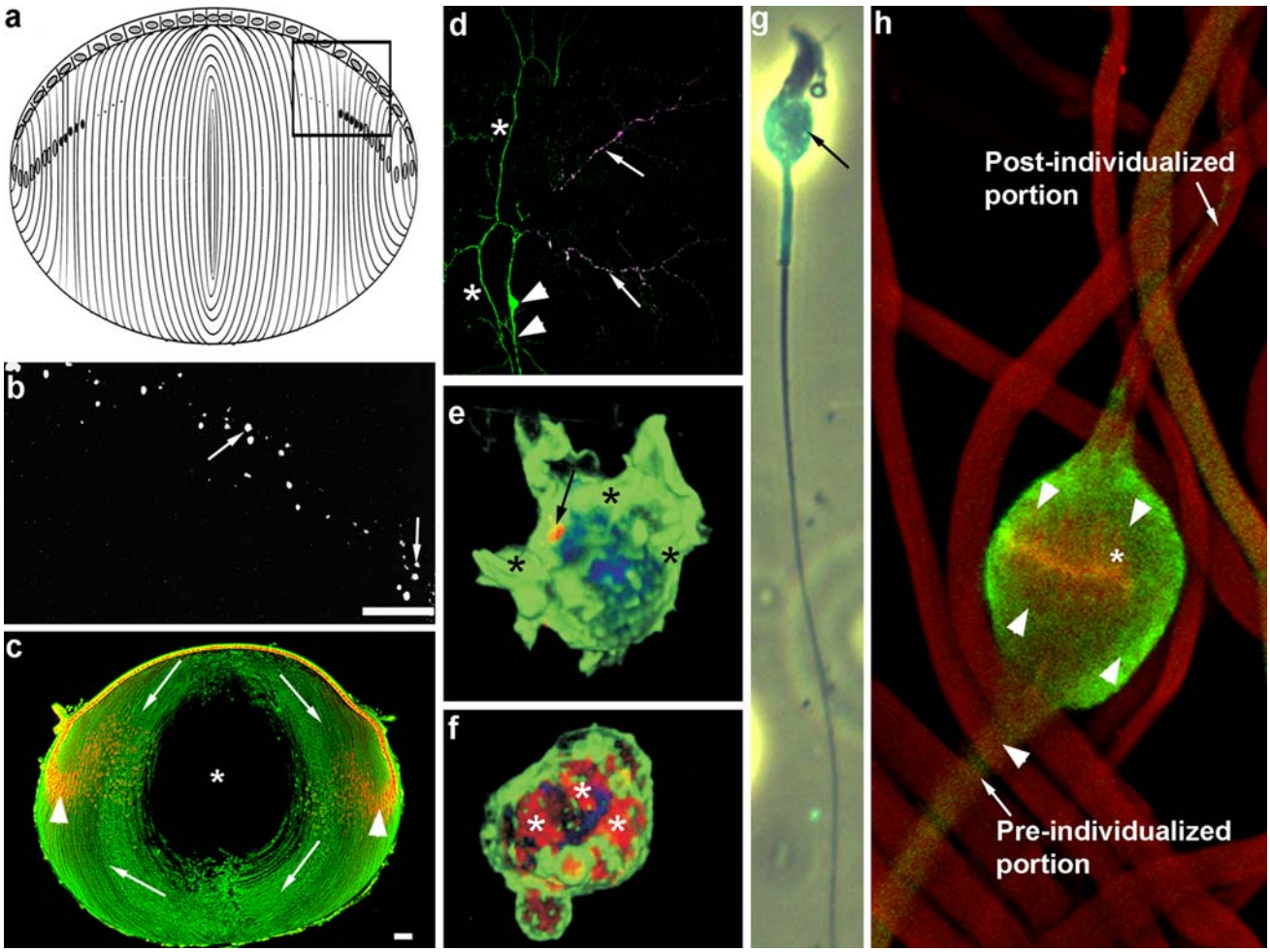


**Fig. 2** Roles of caspases during vital cellular processes. **a** Drawing of the rat lens. Lens epithelial cells cover the anterior half of the lens; those just above the equator proliferate, migrate inward, and differentiate into elongated postmitotic lens fibers, which eventually lose their nucleus. **b** Fluorescence micrograph of a frozen section of a post-natal day 11 rat lens in the region indicated with a square in **a**, labeled by TUNEL to detect fragmented DNA (white arrows pointing at two of the degrading nuclei). **c** Organelle degradation in the mouse lens from post-natal day 2. A midsagittal lens section stained with propidium iodide (arrowheads; orange), to label nuclei, and an antibody against protein disulfide isomerase (arrows; green), to visualize the distribution of the endoplasmic reticulum. Note the center of the lens from which the organelles have been eliminated (white asterisk). **d** Two dendritic branches of ddaC sensory neuron in *Drosophila* undergoing pruning and are positively labeled for caspase-3-like activity (arrows; magenta). mCD8 reveals the entire neuron (asterisks; green). Note that the cell body and the axon are intact and exhibit no caspase activity (arrowheads). **e** Active caspase-8 (arrow; red) colocalizes with discrete small foci within membrane lipid rafts (black asterisks; green) after T-cell antigen receptor (TCR) stimulation in Jurkat cells. **f** Fas stimulation of Jurkat T lymphocytes induces apoptosis. Note the much more profound activation of caspase-8 (white asterisks; red) that was exclusively cytosolic. **g** An elongated mouse spermatid displays active caspase-3 expression in the cytoplasm (black arrow; green). **h** *Drosophila* spermatids in the process of removing their bulk cytoplasm. The expelled cytoplasm is collected into a 'cystic bulge' (marked by the red filament staining; white asterisk) by an apoptosis-like process that requires caspase activity (activated effector caspase, arrowheads; green). Each thread-like structure is a bundle of 64 spermatids. Scale bars **b**, **c** = 100 μm. The figures were adopted with permission from the following **a**, **b** from Ishizaki et al. 1998. Originally published in [69]; **c** from Bassnett 2002. Originally published in [64]; **d** from Williams et al. 2006. Similar figure was originally published in [120]; **e**, **f** from Koenig et al. 2008. Originally published in [177]; **g** See in the main text; **h** from Arama et al. 2003; originally published in [98]. (Color figure online)

process are examined using isolated embryonic erythroid cells from hamster [87]. Several apoptosis-like features are described, including DNA fragmentation (although non-specific), TUNEL positive nuclei, and a marked decrease in the levels of histone H1 and lamin B2. However, as also noted by the authors, erythrocyte maturation process is clearly deviated from apoptosis in several important ways. Similar to lens fiber cells, enucleation is relatively slow, taking nearly 3 days to complete. In addition, erythrocytes display expansion as opposed to the typical apoptosis-related shrinkage of the cytoplasm, nuclei never form lobules or become pyknotic, and no apoptotic bodies are formed [87]. Three subsequent studies have strongly supported the idea that apoptotic caspases are required for

human and murine erythroid cell differentiation in culture. These studies showed that Caspase-3, -9, -7, and -2, are transiently activated during erythroid differentiation, and that inhibition of caspases by peptide inhibitors, small interfering RNA (siRNA), and overexpression of the apoptosis inhibitor kinase Raf-1 all led to a dramatic reduction in erythroid differentiation with cells arresting at a relatively early stage as basophilic erythroblast [88–90]. Classical caspase substrates, including PARP, Lamin B, and Acinus, whose activation by cleavage is required for chromatin condensation, were also shown to be cleaved during erythroid differentiation [88–90]. Nonetheless, the significance of caspase activity for the enucleation process remains unclear, since the peak in the activity occurs relatively early during differentiation of these cells, suggesting that it may be required to prepare these cells for the enucleation process by, for example, modulating gene expression required for this process [91].

## Shedding of cytoplasm and organelles during spermatid terminal differentiation in *Drosophila* and mammals

Spermatogenesis is the process of sperm formation. In *Drosophila*, the male germ cells mature within individual units termed cysts that also contain two somatic cyst cells. After completion of four mitotic divisions and meiosis, each cyst contains 64 haploid round spermatids that are interconnected by cytoplasmic bridges due to incomplete cytokinesis. These spermatids mature synchronously as bundles of 64 members that undergo dramatic metamorphosis. The morphological changes include fusion of the mitochondria into a giant spherical structure called Nebenkern that extends the length of the tail, hypercondensation and structural remodeling of the nuclei that eventually assume a needle-like shape, and generation of flagellar axoneme that also dictates spermatid elongation [92, 93]. By the end of this cellular transformation, the 1.9 mm long bundle of spermatids undergoes the process of "individualization", during which the 64 interconnected spermatids are separated from each other by the caudal movement of an actin-based "individualization complex". This complex also drives the translocation of a membrane-bound sack, known as the "cystic bulge", into which the bulk of the spermatids' cytoplasm and organelles are collected and eventually discarded as a "waste bag" [94, 95]. Despite the early observations made in a variety of vertebrates and invertebrates that a mass of "residual protoplasm" is sloughed off late in spermatogenesis [96], the progress in understanding the mechanisms that mediate this process has been slow. In their early insightful work, Tokuyasu and colleagues suggested that the organelles removed by the individualization process might undergo degradation by mechanisms similar to lysosomal proteolysis [94], whereas others claimed that this type of degradation does not seem to be the primary process in spermatogenesis [97]. Our more recent study in *Drosophila* demonstrated that the cytoplasmic compartment in individualizing spermatids might be degraded by a process that is reminiscent of the proteolytic process of apoptosis, as "cystic bulges" and "waste bags" that contain the expelled cytoplasmic content of the spermatids were positively marked by acridine orange [98], which specifically stains apoptotic corpses in *Drosophila* [99]. Furthermore, activation of the effector caspase at the onset and during spermatid individualization was demonstrated, and inhibition of effector caspase activity in transgenic flies that ectopically expressed the baculoviral caspase inhibitor gene p35 or in primary testis cultures supplemented with a synthetic caspase-3 peptide inhibitor, both impaired proper movement of the "individualization complex" and the removal of bulk cytoplasm in differentiating spermatids (Fig. 2h) [98]. The *Drosophila* orthologues of the apoptosome components cytochrome C, Apaf-1 (Ark), and caspase-9 (Dronc) were also shown to be required in this process. Loss-of-function mutations in the testis-specific cytochrome C gene *cyt-c-d* caused male-sterility due to defects in effector caspase activation, resulting in a significant attenuation of the effector's (DEVDase) activity, and the subsequent failure to properly exclude the cellular content [98, 100]. Although the exact mechanism by which cytochrome C-d regulates caspase activation in spermatids is still obscure, it appears that it is not related to its function in ATP production (L. Ravid-Lustig. and E. A., unpublished results). On the other hand, hypomorphic allelic combinations of either *ark* or *dronc* mutants affected the proper removal of the spermatids cytoplasm [98, 100]. Similar phenotypes were also reported for spermatids expressing RNAi against *ark* and dominant-negative Dronc, and mutants for *drice*, *dredd* and *dfadd*, the *Drosophila* orthologues of caspase-3, -8 and Fadd, an adaptor that mediates recruitment of apical caspases to the DISC [101, 102]. Recently, we have reported a role of the ubiquitin pathway for caspase activation in this system. In a screen for mutants that abrogate effector caspase activation in spermatids, mutations were isolated in *cullin-3* and *klhl10*, two components of an E3 ubiquitin ligase complex [103]. It is noteworthy that several other ubiquitin pathway proteins were also found to play important roles for caspase regulation, indicating that the control of cell survival and death relies extensively on targeted degradation by the ubiquitin–proteasome pathway [43].

Prior to their release to the lumen of the seminiferous tubules of the testis, inter-connected mammalian spermatids are also individualized and shed off excess cytoplasm. Ultra structural studies reported the presence of granules (basophilic bodies susceptible to staining by base dyes),

vacuolation, and a high electron density due to an increased condensation of organelles at both the distal cytoplasm of maturing spermatids and at "residual bodies" (anatomical equivalents of *Drosophila* "waste bags") [62, 104, 105]. These cellular features are reminiscent of apoptosis [11] and were later both confirmed and expanded in a study using a rat model. Membranes of isolated "residual bodies" were labeled with annexin V [106], a $Ca2^{+}$-dependent phospholipid-binding protein with high affinity for the phosphatidylserine exposed on the surface of apoptotic cells [107]. In addition, expression levels of c-jun and p53 which also exert apoptosis-related regulatory roles, gradually increased in the caudal cytoplasmic compartment of maturing spermatids, peaking in "residual bodies" [106]. As in *Drosophila* spermatogenesis, caspase-3 is also activated during the late stages of mammalian spermatogenesis (Fig. 2g; H. Kissel, E. A., and H. Steller, unpublished results) [108]. Furthermore, targeted deletion of the mouse *Sept4* genomic locus, which knocked out the pro-apoptotic protein ARTS, resulted in defects in the elimination of residual cytoplasm during sperm maturation [108]. Interestingly, as in *Drosophila*, mammalian KLHL10 and Cullin-3 can also interact in vitro and Cullin-3 is highly expressed during late murine spermatogenesis [109]. In addition, KLHL10 is exclusively expressed in developmentally advanced murine spermatids. Mice carrying a null *KLHL10* allele are infertile due to defects during late spermatid maturation [110], and a high frequency of mutations in KLHL10 was reported in infertile men displaying low sperm count [111]. These data indicate that a similar E3 complex may function in late mammalian spermatogenesis and that defects in the mammalian *KLHL10* mutant spermatids may be attributed to a lack of effector caspase activity, which might affect proper spermatid individualization and removal of the cytoplasmic content.

## Local degeneration during dendrite pruning in the *Drosophila* sensory neuron

Pruning is a process often used to selectively eliminate excessive neuronal projections (i.e. axons, dendrites, and synaptic connections), without the death of parent neurons. This process of limited cellular degeneration is important for normal development of the nervous system as well as in response to injury or disease in the adult [112]. During larval-adult transformation in *Drosophila*, several types of neurons undergo pruning, including axon pruning in the CNS mushroom body $\gamma$ neurons and pruning in class IV dendritic arborization (C4da) sensory neurons of the PNS [112–114]. Interestingly, although these two systems share some morphological and molecular characteristics, they also exhibit several distinct molecular features. For example, efficient axon pruning in the $\gamma$ neurons involves engulfment of the degenerating axons by glial cells in a mechanism that requires Draper, the *Drosophila* orthologue of the *ced-1* gene from *C. elegans*, which is essential for the clearance of apoptotic cells [115–118]. In accordance phagocytic blood cells can engulf neuronal debris and may assist efficient severing of branches that show signs of destabilization during dendrite pruning of the sensory neurons, and flies mutant for *draper* displayed strong suppression of branch removal after the initial severing from the cell body [119, 120].

Despite these anatomical similarities between axon and dendritic pruning, recent data suggest that dendritic pruning in C4da, but not axon pruning in $\gamma$ neurons, share mechanisms similar to apoptosis. These conclusions are mainly based on a variety of independent experiments aiming to block axon or dendritic pruning in the respective neurons. In $\gamma$ neurons, for example, axon pruning could not be blocked in flies carrying the H99 deletion which covers the three major apoptotic activators Reaper, Hid and Grim [121]. Likewise, mutations in either the initiator caspase Dronc [122], the apical caspase Dredd, or the effector caspase Drice (O. Schuldiner and L. Luo, personal communication), all failed to attenuate this type of pruning. In addition, overexpression in $\gamma$ neurons of the caspase inhibitor proteins P35 or Diap1 had no effect on axon pruning [117, 121], as well as no staining with the anti-cleaved caspase-3 antibody was detected [117]. On the other hand, dendritic pruning in C4da sensory neurons utilizes key components of the apoptotic machinery, including caspases [120, 122]. Localized activation of Dronc in the degeneration dendrites appear to be an important step in the execution of pruning in this system, as loss of function mosaic clones for *dronc*, flies homozygous for a *dronc* null allele, or expression of dominant-negative forms of Dronc, all suppressed this process. Similarly, inhibition of Dronc activity by overexpression of Diap1 or in flies that carry a gain-of-function *diap1* allele, or in mutants heterozygote for the adapter protein Ark (Apaf-1), all caused suppression of branch removal [120, 122]. Dronc activity, marked by staining with the anti-cleaved caspase-3 antibody [122], as well as caspase-3-like activity detected in vivo by using an artificial caspase-3 substrate (Fig. 2d) [120], were both restricted to the degenerating dendrites. Whereas Jan and colleagues could not detect any effect on dendrite pruning upon expression of the viral effector caspase inhibitor p35 [123], Truman and colleagues reported that p35 expression significantly halts the removal of dendritic branches [120]. Moreover, while the former work suggests that local activation of caspases destabilizes proximal dendritic branches and severs them from the soma, the latter supports a role for caspases only after the severing event [120]. This discrepancy is not only a matter of semantics, because if caspases are "dormant" before

detachment of dendrites from the cell body and only acquire a full-blown activity after the occurrence of initial severing, it may explain how dendrite-restricted caspase activity does not affect the soma.

In summary, whether the apoptotic machinery is completely irrelevant during axon pruning in $\gamma$ neurons is yet to be fully determined. The failure to observe an effect in mutants for several key apoptotic components and by analogy to the model of distal axon degeneration after transection where active caspases appear not to play a role [124] suggest that different mechanisms may govern pruning in these two systems. Finally, it is possible that pruning of $\gamma$ neurons dendrites differs from pruning of their axons and thus may require apoptotic factors similar to sensory neurons.

### The specialized form of megakaryocyte cell death that produces functional platelets

Blood platelets, or thrombocytes, are functional anucleated cell fragments derived from long and thin cytoplasmic extensions (proplatelets) of the progenitor megakaryocyte cells and play a key role in blood clotting [125, 126]. The idea that the apoptotic machinery might be involved in platelet formation first came from genetic studies of two members of the Bcl-2 family proteins during hematopoiesis in mice. In these studies, transgenic mice which ubiquitiously expressed the anti-apoptotic human *bcl-2* gene throughout their hematopoietic system or mice deficient for the pro-apoptotic gene *bim* both showed a reduction of about 50% in the number of platelets in their blood [127, 128]. Another work suggested that the terminal stage of megakaryocytopoeisis, during which platelets are produced represents a specialized form of apoptosis, as pro-apoptotic stimuli such as nitric oxide [129] or Fas ligation [130] triggered increased release of platelets from cultured megakaryocyte cells. In addition, after the release of platelets, the denuded megakaryocytes are subsequently engulfed by macrophages [131] and it was suggested that these so-called senescent megakaryocytes correspond to apoptotic cells [132].

Using a primary cell culture model of differentiating megakaryocytes as their experimental system, Debili and colleagues tested a possible involvement of apoptotic caspases in the process of proplatelet formation [133]. This work first showed that caspase peptide inhibitors, but not calpain inhibitor, could lead to an almost complete inhibition of proplatelet formation when added at a stage prior to the production of these cytoplasmic extensions. At the stage of platelet-shedding megakaryocytes, activation of caspase-9 and -3, as well as cleaved forms of the effector caspase substrates gelsolin and PARP, were detected. Interestingly, whereas a granular pattern of cleaved caspase-3 staining was detected during proplatelet formation and the cells preserved their DNA integrity, megakaryocytes that were induced to undergo apoptosis displayed a diffuse cleaved caspase-3 expression and had TUNEL-positive nuclei, suggesting that, at least in this system, compartmentalized activation of caspase-3 may be a major factor in the ability of these cells to deal with the danger of excessive caspase activity and cell death. Finally, cytochrome C release to the cytosol accompanied proplatelet formation, while overexpression of the *bcl-2* gene strongly attenuated this process, implying that the mitochondrial pathway may be required for caspase activation in this system. Taken together, these studies suggest that apoptosis-related mechanisms may also function during platelets formation in vivo.

## Roles of apoptotic caspases during non-degenerative processes

In the remaining sections, we discuss non-apoptotic roles of caspases in different cellular frameworks that do not exhibit compartmentalized degeneration. These less intuitive paradigms imply that the broad affinity of caspases towards hundreds of substrates must be modified, such that they could recognize and cleave only a few specific targets. In the last paragraph, we discuss mainly hypothetical mechanisms for how the activity of caspases can be controlled during these processes.

### Neural precursor development in *Drosophila*

The appearance of an extra few external sensory organs (macrochaetae) on the notum of flies mutant for Apaf-1, caspase-9, or cytochrome C orthologues (Ark, Dronc, or cytochrome C-d, respectively), implies that the apoptotic machinery might play a role in the formation of the sensory organ precursor (SOP) cells in *Drosophila* [134–137]. However, it was suggested that caspase activity in this system is required for a non-apoptotic process, as transient inhibition of caspase activity by overexpression of the viral effector caspase inhibitor p35 at a stage prior to specification of SOP cells led to the appearance of extra macrochaetae, and caspase-3-like activity was detected only at this early stage [138]. Therefore, as opposed to the above mentioned caspase-dependent cell terminal differentiation processes, in this system caspase activity prevents specification and differentiation of SOP cells. As previously reported [139], a deficiency screen for large chromosomal deletions that dominantly modify the number of macrochaetae in *dronc* mutants demonstrated the involvement of the Wingless (Wnt) signaling pathway in this process, including a fly-specific inactive orthologue (Sgg46) of the kinase GSK3B, an antagonist of the Wnt pathway [138]. Sgg46 was cleaved and activated following expression of

the effector and initiator caspases Drice or Dronc in *Drosophila* S2 cells, and *sgg* mutants induced extra macrochaetae and SOP cells [138]. Although it is still remained to be determined whether Sgg46 is indeed cleaved and activated within SOP cells, this study suggests that caspases may normally function in a non-apoptotic mode to inhibit the Wnt pathway during SOP cell specification. This idea was further fortified in a subsequent report by the same group [140]. In this work, knockdown of the *Drosophila* IKK-related kinase gene (*DmIKKε*) in the notum resulted in suppression of caspase-3-like activity in the proneural clusters (SOP primordial cells), extra SOP cells, and appearance of additional macrochaetae [140]. Significantly, although mutations in *DmIKKε* were originally identified as dominant suppressors of apoptosis induced by Reaper expression in the adult eye, subsequent genetic and cell culture studies showed that DmIKKε can phosphorylate the potent caspase inhibitor Diap1, leading to its proteasome-mediated degradation independently of the Reaper family proteins. Furthermore, no significant changes in embryonic cell death were detected in *DmIKKε* mutants and knockdown of *DmIKKε* in wing discs did not suppress naturally occurring cell death despite the marked increase in Diap1 levels [140]. Therefore, unlike Diap1 inhibition by the Reaper family proteins which leads to cell death, DmIKKε-dependent Diap1 inhibition does not cause apoptosis. Whether DmIKKε is a mediator of caspase activation during other non-apoptotic processes remains to be determined. The findings that DmIKKε can also act as a negative regulator of F-actin assembly during other caspase-dependent non-apoptotic processes ([141]; see also in the next section) suggest that this possibility shall be a good avenue to take. Moreover, the fact that the mammalian DmIKKε orthologue NAK can similarly mediate phosphorylation-dependent degradation of the apoptosis inhibitor XIAP, raises the attractive hypothesis that NAK may be involved in regulation of caspase-dependent non-apoptotic processes in mammals [140].

## Caspase-mediated F-actin dynamics during cellular morphogenesis and cell migration in *Drosophila*

The continuous turn over of F-actin filaments by concurrent growth and shortening at the opposite ends is essential for many of the actin cytoskeleton functions [142–144]. Extensive reorganization of the cytoskeleton is a major feature of apoptosis [145]. Importantly, caspase-3 can indirectly depolymerize actin filaments by cleaving Gelsolin and activating its actin-severing activity [146]. Three studies using different *Drosophila* cellular systems strongly support a critical role of the initiator caspase Dronc, but not of the effector caspase in the regulation of actin dynamics during cellular morphogenesis and cell motility, albeit the molecular mechanisms by which this caspase regulates F-actin dynamics are completely obscure. Branching of the antennal aristae was originally regarded as a cell death associated process, since flies carrying the classical weak allele ($th^1$) of the *thread* locus which encodes the caspase inhibitor Diap1 produced a branchless arista [147, 148]. Consistent with this idea, mutations in the pro-apoptotic gene *hid* led to numerous extra branches and a weak allele of the apoptosome adaptor Ark partially suppressed the $th^1$ branchless phenotype [148]. However, a failure to detect any effect following ectopic expression of the caspase inhibitor gene p35 and the finding that in $th^1$ antennal disc cleaved caspase-3 staining is much more widespread than TUNEL labeling in the aristal progenitor cluster [148] both implied that caspase activity in this system may not lead to cell death. Indeed, a more recent study showed that mutations in DmIKKε, a Diap1 inhibitor during the non-apoptotic process of SOP cell specification (see also previous paragraph), caused aberrantly branched morphology of tracheal terminal cells, bristles, and arista laterals, which require accurate F-actin assembly for their polarized elongation [141]. Consistent with the previous study on the arista [148], these phenotypes were sensitive to a change in the dosage of Diap1 and Dronc or its activator Ark, while exhibiting no effect upon inhibition of effector caspases by p35 [141]. Since there was no apparent change in cell viability, these studies strongly support a role of DmIKKε in the activation of the initiator caspase via negative regulation of Diap1, which in turn, through an as yet unknown effector caspase-independent mechanism, leads to turnover of F-actin and subsequent polarized elongation during cell morphogenesis. Therefore, it appears that DmIKKε exerts at least two non-apoptotic functions during macrochaetae development; an early role during specification of SOP cells which requires the effector caspase [140] and a later role in dictating the fidelity of polarized elongation, allowing the formation of smooth macrochaetae [141].

Border cell migration in the *Drosophila* ovary is another example of a role for the initiator caspase in regulation of F-actin dynamics and cell migration. In the course of exploring the role of the small GTPase Rac during border cell migration in vivo, Geisbrecht and Montell have found that overexpression of Actin5C or Diap1 can suppress the border cell migration defect caused by dominant-negative Rac [149]. Interestingly, this migration defect of Rac mutant border cells appeared to be primarily due to effects on actin, as overexpression of Profilin, the protein required for maintenance of a pool of active monomeric actin, rescued this migration defect nearly as well as wild-type Rac. Furthermore, *diap1* mutant follicle epithelial cells displayed a reduction in F-actin and border cells failed to migrate. In addition, mutant alleles of both *profilin* and *diap1* showed strong dosage-sensitive genetic interaction and Rac, Profilin,

and Diap1 could form a complex in vitro. Finally, whereas weak *ark* mutants or overexpression of dominant-negative forms of Dronc rescued the migration defects in Rac mutant border-cells, no TUNEL-positive staining was detected in these cells and overexpression of p35 did not rescue their migration defect [149]. Therefore, although the underlying molecular mechanisms of F-actin regulation by Dronc are yet unknown, the authors hypothesized that Dronc may cleave one or more proteins required for Rac-mediated cell motility. Rac itself was shown to be cleaved and inactivated by caspase-3 in lymphocytes [150]. Collectively, these studies suggest antagonistic regulation of the Diap1-Dronc-dependent/effector caspase-independent pathway by Rac and DmIKKε, which in turn seem to result in opposite dynamics of F-actin. It is attractive to speculate that Rac might protect Diap1 against DmIKKε-mediated phosphorylation and degradation. It will be interesting to examine whether Rac is involved in regulation of F-actin-mediated cellular morphogenesis and if DmIKKε is required for proper dynamics of F-actin during border cell migration.

The role of caspases in the remodeling of the cytoskeleton is consistent with the major morphological changes constituting the hallmark of apoptosis [11]. Therefore, in addition to their roles in F-actin dynamics, caspases are likely to affect other components of the cytoskeleton during non-apoptotic processes. The effect of caspases on the morphology of PC12 cells, an in vitro model for neuronal differentiation, provides such an example [151]. Following plating on poly-D-Lysine/laminin-coated wells, PC12 cells normally stop growing as clumped colonies and adhere and disperse as a monolayer over time. Rohn et al. observed a transient activation of caspase-3 already at 1 h following plating with no effect on cell death, while upon treatment with a caspase peptide inhibitor or antisense caspase-3 oligonucleotides, these cells remained rounded in appearance and failed to disperse properly. Interestingly, the microtubule-associated protein that stabilizes the neuronal cytoskeleton tau was shown to be cleaved by caspase-3 following plating of PC12 cells [151]. These results suggest that transient caspase activity in these cells may allow for the proper disassembly of the cytoskeleton that is required for cells to disperse to their final destinations. However, the relevance of these findings to cytoskeletal remodeling-associated processes in vivo remains unclear.

## Caspase-mediated differentiation of monocytes to macrophages

The idea that vertebrate differentiation of monocytes into macrophages involves a non-apoptotic function of caspases came first from a study of a human leukemia cell line model for terminal monocytic differentiation. Treatment of these cells with phorbol ester led to the appearance of a mature differentiated phenotype which was associated with some characteristics of apoptosis, including cytochrome C release and caspase-3 activation [152]. Along these same lines, treatment of similar cells with tumor necrosis factor (TNF)–related apoptosis-inducing ligand (TRAIL) which can induce DISC formation and apoptosis [153], resulted in rapid caspase-dependent cytotoxicity associated with progressive maturation of the surviving cells along the monocytic lineage; and cytokine and TRAIL-treated primary normal hemopoietic progenitor cells displayed increased number of mature monocytes and macrophages [154]. In another study where the involvement of caspases was tested directly, active forms of caspase-3 and -9 were found to be associated with differentiation of normal human peripheral blood monocytes into macrophages following cytokine induction [155]. Furthermore, differentiation of a leukemia cell line into macrophages was inhibited upon treatment with either a pan-caspase peptide inhibitor, expression of the viral effector caspase inhibitor p35, or overexpression of the anti-apoptotic human mitochondrial protein Bcl-2. Caspase activation in these cells was accompanied by cytochrome C release and cleavage of the caspase-3 substrate Acinus, but not oligonucleosomal DNA fragmentation, PARP cleavage, nor annexin V labeling, suggesting that the mitochondrial pathway is involved in caspase activation during this non-apoptotic differentiation process [155]. These model systems for caspase-mediated monocyte-to-macrophage differentiation can produce functional macrophages, as was demonstrated by their ability to phagocytose live bacteria [156]. Moreover, in vivo evidence for the requirement of caspases in this system is also available, as conditional targeted deletion of caspase-8 in the myelomonocytic lineage led to arrest of differentiation into macrophages and to cell death [157]. Finally, proteomic analysis using the leukemia cell line system revealed several proteins that are potentially cleaved by caspases in monocytes undergoing differentiation into macrophages, including proteins involved in cytoskeletal regulation, such as α-tubulin, vinculin, β actin, and PAK2 [158]. Significantly the p21-activated kinase 2 (PAK2) was implicated in apoptosis and can be activated through binding to the monomeric G protein Rac [159] or following caspase-3-mediated removal of its autoinhibitory domain [160]. These findings raise the possibility that similar to Rac-mediated actin rearrangement during border cell migration in *Drosophila*, caspase activity during monocyte-to-macrophage differentiation may also function in the organization of the cytoskeleton.

## Caspase-mediated differentiation of stem cells

Stem cells are defined as cells that, at the single-cell level, are capable of self-renewal and differentiation to specialized cell types [161]. Several studies using different tissue-specific

stem cells provide evidence for a role of caspase-3 in mediating stem cell differentiation. Mice deficient for caspase-3 [162, 163] displayed attenuated osteogenic differentiation of bone marrow stromal stem cells [164], attenuated neuronal differentiation of primary derived neuronal stem cells [165], accelerated proliferation and retarded differentiation of adult hematopoietic stem cells [166], and marked differentiation defects in caspase-3 knockout embryonic stem cells [167]. Caspase-3 activity in all of these cells was examined either directly or indirectly using caspase peptide inhibitors. At least in embryonic stem cells, caspase activation may depend on the mitochondrial pathway, as enforced Bcl-2 expression in mouse embryonic stem cells was sufficient for their self-renewal in serum- and feeder-free conditions when supplemented with the leukemia inhibitory factor (LIF) [168]. Whereas the molecular mechanisms by which caspase-3 regulates differentiation of these cells are still obscure, altered expression of several components in relevant signaling pathways was reported [164–166]. Interestingly, induced expression of caspase-3 stimulated embryonic stem cell differentiation which coincided with a reduction in the levels of Nanog, one of the small core set of transcription factors which work together to maintain the pluripotent state of these cells. Moreover, Nanog was reported to be a direct target of caspase-3 (and perhaps also caspase-9) both in vitro and in vivo, and forced expression of a caspase cleavage-resistant Nanog form in embryonic stem cells readily promoted self-renewal [167]. Further studies are required in order to determine whether caspases-3 exerts common molecular mechanisms to direct differentiation of different types of stem cells or that this protease may cleave distinct protein substrates in each stem cell type.

### Caspase-3 in learning and memory

Memory relates to a diverse set of cognitive capacities by which an organism retains information and reconstructs past experiences. A recent study on song-specific habituation in adult zebra finches provides a direct evidence for caspase-3 activation by animal training [169]. Following exposure to tape-recorded birdsong, the concentration of the active form of caspase-3 sites was shown to increase within minutes in dendritic spines within the auditory forebrain of these birds. This rapid activation of caspase-3 was probably not due to de novo synthesis of this protease, but rather to a relief of inhibition of the active caspase form by the inhibitor of apoptosis protein XIAP. Perhaps the most informative piece of data came from auditory forebrain infusion studies using a cell-permeable caspase-3 inhibitor; taking advantage of a molecular marker assay for habituation, birds from the caspase-3 inhibition group but not wild-type controls "forgot" a song, which they repeatedly heard a day earlier and responded as though the song was novel to them. Although nothing is known about the mechanism by which caspase-3 affects learning and memory in these birds, it is possible that it may involve cleavage of AMPA receptors and thereby reduce glutamatergic responses and prolong spike habituation [170]. It remains to be seen, however, whether this possibility holds true in vivo and whether there are other affected pathways.

## Concluding remarks

The weight of evidence presented in this review counters the dogma that cells expressing active apoptotic caspases are doomed to die but leaves unexplained the mechanisms that prevent excessive caspase activity and unwanted killing of these cells. The ability of cells to evade apoptotic cell death is an essential "hallmark of cancer". Furthermore because of their aberrant behaviors, cancer cells are believed to constantly express active caspases, implying that cancer cells must have developed a way to counteract this "killing" activity [171]. Indeed, aberrant expression and/or function of the inhibitor of apoptosis proteins (IAPs) are found in many human cancers and have been implied in resistance to current treatment approaches [172, 173]. Likewise, IAPs may also play a role in attenuation of caspase activity during non-apoptotic processes. In *Drosophila*, mutations in the giant IAP *dbruce* lead to male sterility due to spermatid individualization defects [98]. Although it still remains to be demonstrated, the phenotype of degenerate and hypercondensed nuclei in these mutant spermatids suggests that dBruce might protect spermatid nuclei against excessive caspase activity. The high expression level of XIAP and its ability to bind to caspase-3 in zebra finch auditory forebrain extracts also suggest a similar role of this inhibitor in restraining caspase activity in this cellular system [169]. Another mechanism which can limit the exposure time of these cells to caspase activity appears to involve a transient activation of caspases as opposed to their full blown activity during conventional apoptosis. Indeed, transient caspase activation has been observed during differentiation of erythrocytes [91], lens fiber cells [65], and PC12 cells [151]. Along the same lines, caspases may possess different affinities towards different substrates [174], hence, transient or low level of caspase activity may result in cleavage of only a few substrates that exhibit the highest affinity. Notably, some caspase substrates change their functions from anti- to pro-apoptotic as caspase activity increases, while others turn anti-apoptotic by cleavage at a low level of caspase activity and further cleavage leads to a pro-apoptotic conversion [175, 176]. Localized expression of active caspases may be a mechanism by which caspase activity is confined to specific subcellular compartments. Consistent with this idea, granular labeling of

cleaved caspase-3 was detected in megakaryocytes during platelet formation, whereas megakaryocytes that were induced to die displayed a more uniform diffused staining [133]. Furthermore, a recent work demonstrated marked differences in spatial expression of active caspase-8 following T-cell activation versus cell death [177]. Following T-cell antigen receptor (TCR) signaling, caspase-8 is activated in aggregates within membrane lipid rafts (Fig. 2e) [177], probably mediating proliferation of these cells [178, 179]. In contrast, apoptosis induction by Fas stimulation of T cells resulted in a much more profound activation of caspase-8 that was exclusively cytosolic (Fig. 2f) [177]. This elegant experiment suggests that active caspase-8 is sequestered in different cellular compartments following activation for proliferation versus apoptosis, and that this spatial difference may reflect altered access to caspase-8 substrates during the non-apoptotic process. Other roles of caspases in the induction of compensatory proliferation in *Drosophila* were reported [48, 180–184]. It will be interesting to determine whether active caspases display similar compartmentalized distribution during these processes. Finally, post-translational modifications of caspases and/or binding to modulating proteins may alter their affinity towards different substrates, which may result in modified forms of caspases that recognize specific subset of substrates [185–188]. The emergence of accurate tools for detection of caspase activity in vivo will help to shed light on the exact mechanisms by which caspases exert non-apoptotic functions.

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