Characterization of Three *Drosophila melanogaster* Regulators of Programmed Cell Death that Lead to Caspase Activation

by

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ABSTRACT

Programmed cell death plays an important part in metazoan development as well as in disease. The morphology of cells undergoing programmed cell death has been carefully defined and found to be conserved between a variety of species. A number of key regulators of this process were first identified in the nematode *Caenorhabditis elegans* and then found to be conserved in *Drosophila* and mammals. In addition to the conserved components of the programmed cell death pathway, *Drosophila* contains three currently novel regulators: *reaper, head involution defective (hid)*, and *grim*.

The *C. elegans* gene *ced-4* is necessary for the proper execution of programmed cell deaths during development. A mammalian homolog, *Apaf-1*, has similar functions. Until recently, a *Drosophila* homolog for this component of the core cell death pathway had not been identified. We describe the cloning and characterization of a *Drosophila ced-4/Apaf-1* homolog, which we named *hac-1*. HAC-1, like CED-4 and Apaf-1 is required for some cell deaths during development, can help activate caspases *in vitro,* and appears to aid in the activation of procaspases overexpressed in the *Drosophila* eye. Interestingly, like *reaper*, *hac-1* transcription is rapidly upregulated in response to UV and X-irradiation.

An essential regulator of programmed cell death in *Drosophila* is *diap1*. Diap1 is part of a conserved family of Inhibitor of Apoptosis proteins (IAPs) that were first described in baculovirus. Loss of *diap1* function results in widespread apoptosis during early embryonic development. In addition to the morphological features of apoptosis, caspases are activated and physiological caspase substrates are cleaved. We show that some of the morphological changes observed can be explained by the cleavage of two caspase substrates, nuclear Lamin Dm_0 and Armadillo.

In order to identify additional components of the programmed cell death pathway and to further our understanding of how Reaper functions, we performed a yeast two-hybrid interactor screen using Reaper as the bait. We isolated ten potential interactors for which we determined the sequence, expression pattern, and cytological map position. None of these interactors has yet been shown to function in programmed cell death, but they provide leads to follow for future investigation.

Thesis Supervisor: Hermann Steller
Title: Professor of Neurobiology

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Chapter 1

Introduction to Programmed Cell Death

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OVERVIEW

Programmed cell death is an important mechanism for maintaining homeostasis in the multicellular organism. During development of most metazoans, more cells are generated than ultimately make up the adult organism. Programmed cell death plays an important role in eliminating cells that have a transient function once they have served their purpose in development, in eliminating cells with no apparent function, and in eliminating extraneous or damaged cells (reviewed by Jacobson et al., 1997; Vaux and Korsmeyer, 1999). An example of the elimination of cells with only a transient function is in the frog: as a tadpole it has a tail necessary during this stage of development, but the tail is lost via programmed cell death when the tadpole matures into the adult. An example of a tissue with no apparent function that is eliminated by programmed cell death is the interdigital webbing that is present early in development, but must be eliminated in animals with toes and fingers. In the developing nervous system, more cells than necessary are generated, but only the cells that find the proper target tissues continue to survive. The remaining neurons, though healthy, normal cells, are eliminated by programmed cell death. Similarly, in the immune system programmed cell death is used to protect the adult organism from potentially harmful self-recognizing immune cells by eliminating them. Furthermore, programmed cell death functions to protect the adult organism from damaged cells that no longer are subject to normal growth regulation and could potentially become cancerous, or those cells that become infected by a virus. In both cases, the cell would attempt to undergo apoptosis so that the damage is contained (Jacobson et al., 1997).

FEATURES OF APOPTOSIS

Most, if not all cells in a multicellular organism have the potential to undergo programmed cell death and do so using a conserved program that results in very characteristic morphological changes. These morphological changes have been carefully observed in several mammalian cell types and the term apoptosis was coined to distinguish these deaths from necrosis, a death that results from massive cellular damage. Necrotic cell death causes an uncontrolled release of the cellular contents into the surrounding environment resulting in inflammation (Kerr et al., 1972; Wyllie, 1980a). Apoptosis, in contrast, usually involves individual cells, and the death does not result in a release of the cellular contents. During apoptosis, the chromatin condenses and the nucleus appears to shrink and fragment. The cells round up and detach from their neighbors and the cytoplasm condenses. Organelles maintain their normal morphology, unlike in necrotic

cells, where the mitochondria swell and burst. The apoptotic cell finally begins to bleb, releasing membrane-bound vesicles, termed apoptotic bodies, that are phagocytosed by professional phagocytes or neighboring cells and degraded (Kerr et al., 1972; Wyllie, 1980a).

Besides undergoing specific morphological changes, apoptotic cells also go through cellular changes that are not visible under the microscope. The chromosomal DNA is first cleaved into 300 and/or 50 kbp fragments and then into a nucleosomal ladder, where the DNA appears to be cleaved in the linker region between nucleosomes, resulting in a ladder with 180-200 bp steps (Wyllie, 1980b; Oberhammer et al., 1993). In addition, the plasma membrane loses its asymmetric distribution of phospholipids causing phosphotidylserine to be exposed on the outer surface. The cells also are no longer able to exclude certain vital dyes such as acridine orange and nile blue (Abrams et al., 1993; Pazdera et al., 1998). In addition, while the mitochondria appear to remain morphologically normal, they change dramatically during apoptosis, resulting in the release of cytochrome *c* and possibly the loss of their membrane potential (Green and Reed, 1998; Martinou et al., 2000).

THE CORE CELL DEATH PATHWAY

The first genes in the programmed cell death pathway were identified using the nematode *Caenorhabditis elegans* (Ellis and Horvitz, 1986; Hengartner et al., 1992). The core pathway in *C. elegans* consists of four regulators of cell death, *egl-1, ced-3, ced-4* and *ced-9* , where egl stands for egg laying abnormal and ced for cell death abnormal (reviewed in Metzstein et al., 1998; Horvitz, 1999). The function of *egl-1, ced-3*, and *ced-4* is required for normal cell death to occur (Ellis and Horvitz, 1986; Conradt and Horvitz, 1998). These three genes are pro-apoptotic while the fourth gene, *ced-9*, acts as a protector from cell death (Hengartner et al., 1992). Loss-of-function mutations in either *ced-3, ced-4* or *egl-1* cause all 131 cells that normally undergo programmed cell death during *C. elegans* development to perdure. In contrast, loss-of-function mutations in *ced-9* result in the death of most cells in the embryo and are therefore lethal. Genetic and overexpression studies have ordered these genes into a pathway where *egl-1* acts upstream of *ced-9* to inhibit its activity, allowing *ced-4* to activate cell killing by *ced-3* (Hengartner et al., 1992; Shaham and Horvitz, 1996; Conradt and Horvitz, 1998). This pathway presents the most completely understood framework for how programmed cell death can be regulated and can also be applied to other experimental organisms.

CONSERVATION OF THE PROGRAMMED CELL DEATH PATHWAY BETWEEN *C. elegans* AND MAMMALS

In mammals, the core programmed cell death pathway is more complex than the linear model outlined above (Figure 1). However, functional homologs for each of the core pathway components in *C. elegans* have been identified in mammals. A brief overview of the mammalian homologs follows and will be expanded upon in later sections.

Based on the pathway in *C. elegans*, mammalian *egl-1* homologs are the most upstream components of the conserved programmed cell death pathway. *egl-1* encodes a protein that contains a BH3-like region (Bcl-2 homology region 3) (Conradt and Horvitz, 1998). Proteins containing only a BH3 domain are a subgroup in the large class of proteins that comprises the Bcl-2 family members (reviewed in Gross et al., 1999; Vander Heiden and Thompson, 1999). The BH3 region is likely to mediate protein-protein interactions between Bcl-2 family members and mammalian proteins with only the BH3 are usually pro-apoptotic, like EGL-1.

ced-9 is also a Bcl-2 family member, but it is part of the anti-apoptotic subgroup. The founding mammalian member of this group, Bcl-2, was identified as a gene frequently associated with B cell lymphoma, where a translocation results in the overexpression of Bcl-2. This overexpression causes inappropriate B-cell survival and overproliferation (reviewed in Gross et al., 1999; Vander Heiden and Thompson, 1999). At least nineteen Bcl-2 family members have been identified in mammals, but the mechanism by which they function is still open to much speculation. In brief, anti-apoptotic Bcl-2 family members may protect cells from apoptosis by either binding to the mammalian CED-4 homolog, probably Apaf-1 (Apoptotic Protease Activating Factor 1), and preventing it from activating downstream effectors or by regulating the release of apoptosis inducing factors from the mitochondria. This protein family is further discussed later in this chapter.

ced-4 was a novel gene until the identification of *Apaf-1*, which shares a region of similarity with *ced-4* (Zou et al., 1997). Both CED-4 and Apaf-1 appear to require dATP for their pro-apoptotic activity (Chinnaiyan et al., 1997a; Li et al., 1997). In addition, CED-4 and Apaf-1 both seem to be capable of binding to and activating caspases. Both proteins require dATP to help activate caspases and Apaf-1 requires binding to cytochrome *c* as well (Chinnaiyan et al., 1997a; Seshagiri and Miller, 1997b; Wu et al., 1997). While both Apaf-1 and CED-4 help activate caspases, it is not clear whether Apaf-1 is the true functional equivalent of CED-4 in mammalian cells. Apaf-1 is a considerably larger protein that contains an additional region with WD repeats, whose function is not entirely understood, but which are probably involved in protein-protein interactions with a

regulatory role, including those with cytochrome *c*. Furthermore, two other mammalian proteins with some similarity to Apaf-1 have been identified, Flash and Nod1/Card4, but their *in vivo* function needs to be further characterized to clearly define their role in programmed cell death (reviewed in Cecconi, 1999).

The pro-apoptotic gene *ced-3* is now part of a large family of cysteine proteases named caspases for cysteine aspartic acid-specific proteases (Yuan et al., 1993; Alnemri et al., 1996). A total of four caspases have been identified in *C. elegans*, but only CED-3 has been shown to be essential for all developmentally occurring programmed cell death (Ellis and Horvitz, 1986; Shaham, 1998). The mammalian founding member of this group is interleukin 1β converting enzyme (ICE), now known as caspase 1, a cysteine protease that cleaves the precursor of interleukin 1β to yield the active cytokine (Cerretti et al., 1992; Thornberry et al., 1992). Since then, at least 14 mammalian caspases have been identified, some of which appear to play some role in apoptosis, while others seem to mainly function in the activation of pro-inflammatory cytokines, like caspase 1 (Thornberry and Lazebnik, 1998; Kumar, 1999). These specific proteases are thought to act as the executioners in the cell, cleaving vital substrates within the cell once activated. The cleavage of these substrates ultimately leads to the death of the cell. Some of the potential substrates for caspases are lamins, kinases, DNA repair enzymes, and proteins involved in DNA replication and mRNA splicing (Thornberry and Lazebnik, 1998; Nicholson, 1999).

CELL DEATH IN *Drosophila*

Several central *Drosophila* regulators of programmed cell death have been identified, including homologs of CED-3, CED-4, and of CED-9/EGL-1. Three of these, Reaper, Hid, and Grim, have no clear homologs in other species at this point. They have, however, provided great insight into how the programmed cell death pathway can be regulated, including the involvement of p53, the Ras pathway, and steroid hormones (Figure 1) (Bergmann et al., 1998; Kurada and White, 1998; Brodsky et al., 2000; Jiang et al., 2000). In addition, genetic screens using *Drosophila* have yielded a large number of mutations in an essential Inhibitor of Apoptosis (IAP), Diap1, that have greatly enhanced our knowledge of IAP function (Goyal et al., 2000; Lisi et al., 2000). The completion of the *Drosophila* genome has resulted in the identification of several other potential homologs of known cell death regulators and effectors and *Drosophila* should continue to make a significant contribution to the apoptosis field (Rubin et al., 2000).

Reaper, Hid, and Grim

A screen for regions of the genome that, when deleted, affect the amount of programmed cell death in the developing *Drosophila* embryo identified one region on the third chromosome (75C1,2) that is essential for virtually all normally occurring cell death (White et al., 1994). The smallest deletion that retains the cell death defect is *Df(3l)H99* (or *H99*) and was found to contain three genes that function in programmed cell death: *reaper*, *head involution defective (hid)*, and *grim* (White et al., 1994; Grether et al., 1995; Chen et al., 1996b). Reaper, Hid and Grim are novel proteins that share limited homology to each other in the amino-terminal 14 amino acids (Figure 2) (Grether et al., 1995; Chen et al., 1996b). Reaper also shares limited similarity with Tumor Necrosis Factor Receptor 1 (TNFR1) and Fas, but the significance of this similarity has been questioned (Golstein et al., 1995; Chen et al., 1996a; Vucic et al., 1997b). Any one of the three genes, *reaper, hid,* or *grim,* is able to restore apoptosis in *H99* deletion embryos when overexpressed (White et al., 1994; Grether et al., 1995; Chen et al., 1996b). In addition, when either *reaper, hid* or *grim* are expressed under the eye specific GMR promoter, they cause an eye ablation phenotype which can be suppressed by the co-expression of GMR*p35* (Hay et al., 1994; Grether et al., 1995; Chen et al., 1996b; White et al., 1996). P35 is a baculovirus protein that has been shown to specifically bind to and inhibit caspases, suggesting that the eye ablation caused by the overexpression of *reaper, hid*, or *grim* results in apoptosis through caspase activation (Bump et al., 1995; Xue and Horvitz, 1995). Although it appears that these three genes can act independently of each other, all three genes appear to be required for the normal pattern of cell death in the embryonic central nervous system (Zhou et al., 1997; Wing et al., 1998). In the midline glia, expression of Reaper or Hid alone is insufficient to induce apoptosis, but expressing both proteins together results in a significant loss of midline glia cells, showing that these genes act synergistically in this cell type. The expression of Grim alone is sufficient to reduce the number of midline glia, but Grim also acts synergistically with Reaper and Hid (Wing et al., 1998).

No clear homologs of Reaper, Hid, and Grim have been found in other species, but they are predicted to exist based on the ability of Reaper, Hid, and Grim to activate apoptosis in mammalian tissue culture cells and of Reaper to induce morphological characteristics consistent with apoptosis in *Xenopus* extracts (Evans et al., 1997; McCarthy and Dixit, 1998; Haining et al., 1999).

Analysis of the three *Drosophila* genes, *reaper, hid*, and *grim* has provided some valuable insight into how a death-inducing stimulus feeds into the common cell death program. *reaper* and *grim* are specifically expressed in doomed cells approximately two hours before the cell shows an apoptotic morphology (White et al., 1994; Chen et al.,

1996b; Robinow et al., 1997). This observation suggests that these genes are not death effectors like caspases, but rather help integrate a pro-apoptotic signal into the central cell death machinery. The expression of *reaper* is regulated in response to a number of cell death-inducing stimuli, including X-irradiation, a block of cell differentiation, and steroid hormone signals (Nordstrom et al., 1996; Robinow et al., 1997; Jiang et al., 2000, A.-F. Lamblin and H. Steller, unpublished results). Recently, the analysis of the *reaper* promoter has identified two regions that integrate the death inducing stimuli and regulate *reaper* transcription. First, the promoter region of *reaper* contains a radiation-inducible enhancer that includes a p53 binding site from which the *Drosophila* p53 homolog can activate transcription (Brodsky et al., 2000). In addition, the *reaper* promoter contains a response element for the steroid hormone 20-hydroxyecdysone which appears to be essential for the proper transcription of *reaper* in larval salivary glands, a tissue that is removed by cell death during pupation (Jiang et al., 2000).

In contrast to *reaper* and *grim*, *hid* appears to be regulated on both the transcriptional and post-translational levels. This additional level of control was predicted based on the observation that *hid* is expressed not just in cells that are destined to undergo apoptosis, but also in cells that do not die (Grether et al., 1995). Mutations in genes that regulate the Ras pathway were identified in genetic screens for modifiers of the GMR-*hid* induced eye ablation phenotype (Bergmann et al., 1998; Kurada and White, 1998, J. Agapite, K. McCall, and H. Steller, unpublished results). These modifiers only affect *hid*, not *reaper* or *grim,* which would be consistent with a pathway regulating the activity of *hid* in those cells expressing it, but not doomed to die. The Ras pathway affects both *hid* expression as well as inhibiting Hid post-translationally through MAPK-mediated phosphorylation. This dual regulation provides a mechanism for inactivating existing Hid protein, in addition to preventing further Hid protein from being made (Bergmann et al., 1998; Kurada and White, 1998).

Most data suggest that Reaper, Hid, and Grim induce apoptosis by inhibiting the protective function of IAPs (see below), but they may utilize other mechanisms as well: Reaper and Grim have been shown to bind and inactivate Shaker-type voltage-gated K^+ potassium channels and may therefore initiate apoptosis in special circumstances, through membrane depolarization (Avdonin et al., 1998). Furthermore, Reaper, Hid and Grim have been shown to interact with Scythe, a pro-apoptotic *Xenopus* protein involved in the regulation of cytochrome *c* release (Thress et al., 1998; Thress et al., 1999). The amino-terminal region that is homologous between Reaper, Hid, and Grim is not required for the interaction with Scythe, unlike for the interaction with IAPs (Thress et al., 1999). This

observation suggests a way for the three *Drosophila* proteins to induce apoptosis that is distinct from their interaction with IAPs.

Inhibitor of Apoptosis Proteins

Drosophila contains a second class of apoptosis regulators, the homologs of IAPs or Inhibitor of Apoptosis Proteins (Hay et al., 1995; Duckett et al., 1996; Uren et al., 1996; Jones et al., 2000; Rubin et al., 2000; Wenzel et al., 2000). IAPs were first identified in baculovirus by their ability to functionally substitute for P35 in blocking apoptosis, although the IAP family now includes members in eukaryotic systems, or cellular IAPs (Crook et al., 1993; Birnbaum et al., 1994). IAPs consist of one, two, or three Baculovirus IAP Repeat (BIR) domains and usually a carboxy-terminal RING finger domain (Figure 3) (Deveraux and Reed, 1999). The BIR domain consists of a conserved arrangement of cysteines and histidines that bind a Zinc ion to form a zinc finger-like motif (Hinds et al., 1999). While IAPs contain BIR domains, not all proteins with BIR domains function in inhibiting apoptosis. BIR domain proteins, or BIRPs can also function in cell cycle regulation and cytokinesis (Li et al., 1998; Fraser et al., 1999; Uren et al., 1999; Li et al., 2000; Speliotes et al., 2000). In fact, *Saccharomyces cerevisiae* contains neither caspases nor an apoptotic program, but does contain a BIRP with a single BIR domain that functions in the regulation of cell division, suggesting that the BIRPs are a diverse family with only some members being anti-apoptotic (Li et al., 2000). Further support for diverse roles for BIRPs comes from the *C. elegans* BIR-1 protein, which appears to function during cell division in the regulation of chromosome behavior (Fraser et al., 1999; Speliotes et al., 2000).

Based on the genomic sequence, there are four BIRPs in *Drosophila* and all four (Diap1, Diap2, Deterin, and the *Drosophila* BRUCE homolog) are at least partially characterized (Hay et al., 1995; Duckett et al., 1996; Uren et al., 1996; Jones et al., 2000; Wenzel et al., 2000, J. Agapite and H. Steller, unpublished results).

Most BIRPs, including *Drosophila* IAP1 and 2 as well as Deterin have been shown to be anti-apoptotic (Hay et al., 1995; Duckett et al., 1996; Uren et al., 1996; Jones et al., 2000; Wenzel et al., 2000). Diap1 can block apoptosis induced by ectopic expression of *reaper, hid* or *grim* (Hay et al., 1995; Vucic et al., 1997a; Vucic et al., 1998; Lisi et al., 2000). In addition, Diap1 is able to block apoptosis induced by the expression of the *Drosophila* caspase drICE in insect cell culture as well as death induced by expression of Dcp-1 or drICE in *Saccharomyces* (Kaiser et al., 1998; Hawkins et al., 1999; Wang et al., 1999). Mammalian IAP homologs have also been shown to inhibit apoptosis in a variety of assays (Duckett et al., 1996; Liston et al., 1996; Uren et al., 1996; Ambrosini et al.,

1997; Deveraux et al., 1997). Furthermore, mutations in NAIP (Neuronal Apoptosis Inhibitory Protein) are thought to contribute to spinal muscular atrophy (SMA) which may be the result of a defect in inhibition of neuronal apoptosis, providing a link of IAPs to human disease (Roy et al., 1995).

IAPs are thought to function by binding to caspases and directly inhibiting their activation (Deveraux and Reed, 1999). This interaction with caspases is mediated through the BIR domains (Deveraux et al., 1997; Roy et al., 1997; Deveraux et al., 1998; Takahashi et al., 1998). Diap1 has been shown to bind the pro-domain of the *Drosophila* caspase Dronc (Meier et al., 2000). While Diap1 is unable to bind active drICE lacking the pro-domain in *Saccharomyces*, it is able to prevent death resulting from the expression of an artificially activated form of drICE that retains the pro-domain (Hawkins et al., 1999; Wang et al., 1999). In general, IAPs are unable to block apoptosis induced by active caspases, instead appearing to block at the step of caspase processing from the inactive, pro-domain containing zymogen, to the active enzyme (Roy et al., 1997; Seshagiri and Miller, 1997a; Deveraux et al., 1998; Kaiser et al., 1998; Tamm et al., 1998; Deveraux and Reed, 1999).

In addition to caspases, IAPs can also physically interact with Reaper, Hid, and Grim (Vucic et al., 1997a; Vucic et al., 1998). Genetic screens for modifiers of GMR-*reaper* and *hid* induced apoptosis yielded several alleles of *thread*, the locus that encodes Diap1 (Hay et al., 1995; Goyal et al., 2000; Lisi et al., 2000, J. Agapite, K. McCall, and H. Steller, unpublished results). These alleles fall into several classes, including loss-of-function and gain-of-function. The loss-of-function alleles are embryonic lethal, with the embryos developing normally up to the onset of gastrulation. At this point virtually all cells take on a rounded morphology, start to bleb and become TUNEL positive, indicating that they are undergoing apoptosis (Wang et al., 1999; Goyal et al., 2000; Lisi et al., 2000, J. Tittel and H. Steller, manuscript in preparation). Gain-of-function mutations map to the BIR domains of Diap1 and impair the binding of Diap1 to Reaper and Hid (Goyal et al., 2000; Lisi et al., 2000). These observations are consistent with a model where Reaper and Hid promote cell death by binding to Diap1 and interfering with its ability to prevent caspase activation. Therefore, gain-of-function mutations which reduce the ability of Reaper or Hid to bind Diap1 result in Diap1 continuing to be bound to the pro-caspase and blocking apoptosis from occurring even in the presence of Reaper and Hid activation.

Further evidence for IAPs being regulated through protein-protein interactions comes from a potential functional analog of Reaper, Hid and Grim, named Diablo/Smac, that was recently identified in mammalian cells (Du et al., 2000; Verhagen et al., 2000). This protein, while sharing no sequence homology to any of the three *Drosophila* genes, is also

able to bind IAPs and antagonize their ability to prevent caspase activation. Like Reaper, Hid, and Grim, Diablo/Smac binds the BIR domains of IAPs in a manner requiring the amino-terminus of the protein (Chai et al., 2000; Srinivasula et al., 2000). However, unlike the three *Drosophila* proteins, Diablo/Smac is not pro-apoptotic by itself when overexpressed, but instead requires a death-inducing stimulus to promote apoptosis.

Recent experiments showing that some IAPs have a potential ubiquitin ligase activity, or E3 activity, give further insight into how IAPs may function (Huang et al., 2000; Yang et al., 2000). The mammalian cellular IAPs cIAP-1 and XIAP appear to be degraded in a proteasome dependent manner prior to thymocyte apoptosis. These IAPs were shown to be capable of autoubiquitination, an activity shown to require the RING domain. XIAP lacking the RING domain is degraded more slowly and is a more potent inhibitor of apoptosis than wild-type XIAP in cell culture (Yang et al., 2000). Earlier Diap1 studies have also suggested that this protein is better able to protect from apoptosis in the absence of its RING domain, when overexpressed in the *Drosophila* eye (Hay et al., 1995). On the other hand, an *in vitro* analysis has shown that cIAP1 can promote the ubiquitination of several caspases, possibly marking them for degradation (Huang et al., 2000). The RING domain is required for this function of cIAP1, therefore the data would suggest that an IAP lacking the RING domain is not able to promote caspase degradation and thereby inhibit apoptosis. Further data using the baculovirus Op-IAP shows that this IAP forms homo-oligomers and that a version lacking the RING domain dominantly interferes with the ability of the full-length Op-IAP to protect from apoptosis, clouding the issue of RING domain function (Hozak et al., 2000). In addition, mutants in the RING domain of endogenous *diap1* can act as either enhancers or suppressors of apoptosis induced by the expression of *reaper, hid,* or *grim.* These mutants enhance killing induced by *reaper* or *grim* and suppress killing induced by *hid* (Lisi et al., 2000, J. Agapite, L. Goyal, K. McCall, and H. Steller, unpublished results). This would suggest that the RING domain serves an anti-apoptotic role in *reaper* and *grim* induced cell death, but a pro-apoptotic role in *hid* induced cell death. Despite the confusion about the function of the RING domain in IAPs, the involvement of the ubiquitination pathway is nevertheless intriguing. The *Drosophila* BRUCE homolog, which contains a single BIR domain as well as a functional ubiquitin conjugating domain, and is thus a potential E2 like protein, has been shown to be involved in protecting from Reaper induced programmed cell death (J. Agapite and H. Steller, unpublished results). Together, these observations suggest that ubiquitination and proteasome degradation are likely to play important roles in regulating programmed cell death.

Caspases: The effectors of cell death

The effectors of the programmed cell death pathway are thought to be caspases and *Drosophila* contains at least eight caspase homologs: Dcp-1, Dcp-2/Dredd, drICE, Dronc, and Decay, as well as three database sequences with homology to caspases (Fraser and Evan, 1997; Inohara et al., 1997; Song et al., 1997; Chen et al., 1998; Dorstyn et al., 1999a; Dorstyn et al., 1999b; Rubin et al., 2000; Vernooy et al., 2000). Caspases have been grouped into two classes based on their proteolytic specificities, with class 1 being caspases mainly involved in cytokine maturation and class 2 acting in apoptosis (reviewed in Nicholson, 1999). Class 2 caspases have been further subdivided into two groups: upstream or initiator caspases (group 1) and effector caspases (group 2). Initiator caspases are thought to be at the beginning of a caspase cascade where they remove the prodomains from downstream, effector caspases, activating them and also amplifying the pro-apoptotic signal. Initiator caspases tend to have longer pro-domains than effector caspases (Figure 4).

drICE, Dcp-1, and Decay have short pro-domains and are therefore likely to be effector caspases. Furthermore, Dcp-1 has a substrate specificity that is very similar to that of two effector caspases, mammalian caspase 3 and CED-3 (Song et al., 2000). Mutations in *dcp-1* show that, while loss of zygotic *dcp-1* results in no obvious cell death defects in the embryo, it does result in larval lethality and the appearance of melanotic tumors, indicating that *dcp-1* function is essential for development (Song et al., 1997). In addition, *dcp-1* is maternally required for the transfer of the nurse cell cytoplasm into the developing oocyte (McCall and Steller, 1998). The transfer of nurse cell cytoplasm is associated with changes in the actin cytoskeleton (Knowles and Cooley, 1994), and thus Dcp-1 activity may be required to cleave some actin-binding proteins. One possible candidate for cleavage is Armadillo, the *Drosophila* β-catenin homolog. Armadillo has multiple functions, including that of helping anchor the actin cytoskeleton to the plasma membrane (Gumbiner, 1996). In *diap1* loss-of-function mutants, most cells are undergoing apoptosis approximately four to five hours into development (Wang et al., 1999; Goyal et al., 2000; Lisi et al., 2000, J. Tittel and H. Steller, manuscript in preparation). It is also at this timepoint that Armadillo is cleaved in these embryos, providing a candidate substrate that could contribute to the changes in the actin cytoskeleton observed during apoptosis (J. Tittel and H. Steller, manuscript in preparation).

Both Dcp-2/Dredd and Dronc have been proposed to be initiator caspases, since both contain long pro-domains. Dronc also contains a potential caspase recruitment domain (CARD), supporting the idea that it is an initiator caspase (Dorstyn et al., 1999a). Dcp-2/Dredd contains a potential death effector domain (DED) which is present in some initiator

caspases and is thought to mediate critical protein interactions required for their activation (Inohara et al., 1997; Chen et al., 1998); however, the significance of the homology between known DEDs and the potential one in Dcp-2/Dredd has recently come into question, since it is quite weak (Meier et al., 2000; Vernooy et al., 2000). Furthermore, the identification of a *Drosophila* FADD (Fas Associated Death Domain containing protein) homolog that interacts through a novel domain distinct from the DED, that is conserved with Dcp-2/Dredd, suggests that Dcp-2/Dredd does not require a DED to potentially function in manner similar to mammalian initiator caspases (Hu and Yang, 2000). Mammalian FADD serves as an adaptor between membrane receptors and initiator caspases (Chinnaiyan et al., 1995). Also, Dcp-2/Dredd has a particularly interesting expression pattern. Unlike the widespread expression of most caspases, Dcp-2/Dredd appears to be specifically upregulated or stabilized in dying cells and the pattern of expression seems to be linked to *reaper, hid,* and *grim* expression (Chen et al., 1998). Dronc has been shown to interact both with Diap1 and with drICE and is able to induce an eye ablation phenotype upon expression in that tissue. This phenotype, however, cannot be rescued by concomitant expression of the caspase inhibitor P35 (Meier et al., 2000). The failure of P35 to block Dronc induced killing may be due to Dronc containing a novel active site for a caspase, with the amino acid sequence PFCRG instead of the usual QAC(R/Q/G)(G/E) (Dorstyn et al., 1999a). In addition, Dronc has recently been shown to have an unusual substrate specificity. The optimal cleavage site for Dronc is TATD and not DEVD as for caspase 3, DCP-1 and CED-3 (Hawkins et al., 2000; Song et al., 2000). *In vitro*, Dronc can also autoprocess after a glutamate residue, suggesting that Dronc is able to cleave after either an aspartate or a glutamate. So far it is the only caspase that has been demonstrated to have such a diverse substrate specificity (Hawkins et al., 2000).

The various caspases in *Drosophila* may play stage or tissue specific roles during development. More analysis is required to accurately order these caspases into a cascade, but it appears that *Drosophila* contains both initiator and effector caspases.

CED-4/Apaf-1: Activators of caspases

In both mammals and in *C. elegans*, caspases have been shown to be activated by Apaf-1/CED-4 (reviewed in Metzstein et al., 1998; Cecconi, 1999). Removing *ced-4* activity in *C. elegans* results in the survival of the 131 cells that normally undergo developmental programmed cell death, showing that *ced-4* is required for normal cell death in the worm. Similarly, removing *ced-3* results in a complete absence of programmed cell death during development. Overexpressing *ced-3* in a *ced-4* loss-of-function background results in cell death, although killing is less efficient than in a wild-type *ced-4* background. If *ced-3*

activity is reduced, overexpression of *ced-4* results in very inefficient killing. These experiments are consistent with *ced-4* acting upstream of *ced-3* and helping to activate *ced-3* (reviewed in Metzstein et al., 1998). Biochemical and cell culture interactions also support a model where CED-4 is required for the activation of CED-3 (Chinnaiyan et al., 1997a; Chinnaiyan et al., 1997b; Seshagiri and Miller, 1997b; Wu et al., 1997). Furthermore, a change in subcellular localization of endogenous CED-4 appears to be related to its function. CED-4 and CED-9 normally localize to the mitochondria, but when EGL-1 is expressed, CED-4 has been observed to translocate to the nuclear membrane in both *C. elegans* and cell culture. A gain-of-function mutation in CED-9 that constitutively protects from cell death prevents this translocalization of CED-4 in the presence of EGL-1 expression (Chen et al., 2000; del Peso et al., 2000). The translocation of CED-4 was shown to be independent of CED-3 function, suggesting that it occurs prior to caspase activation (Chen et al., 2000). These observations suggest a model where CED-9 localizes CED-4 to mitochondria and once EGL-1 displaces CED-4 from this complex, CED-4 is free to translocate to the nuclear membrane and activate CED-3 (See also the next section on the Bcl-2 family).

In mammals, the potential CED-4 homolog Apaf-1 has been shown to participate in the cytochrome *c* and dATP-dependent activation of an effector caspase (Li et al., 1997; Zou et al., 1997). Further support for a role for Apaf-1 in apoptosis comes from knock-out studies, where mice deficient for Apaf-1 have reduced cell death in certain tissues (Cecconi et al., 1998; Yoshida et al., 1998). In overexpression studies, Apaf-1, like CED-4, can also be found in a ternary complex with a Bcl-2 family member and a caspase, suggesting that CED-4 and Apaf-1 have similar functions in programmed cell death (Pan et al., 1998). However, under physiological conditions, all three proteins may not be able to interact simultaneously because of differences in subcellular localization.

Drosophila has recently been shown to also contain a CED-4/Apaf-1 homolog, Dapaf-1/HAC-1/Dark (Kanuka et al., 1999; Rodriguez et al., 1999; Zhou et al., 1999). This homolog contains a region that is homologous to CED-4 including the nucleotide binding motifs which bind dATP (Figure 5). However, Dapaf-1/HAC-1/Dark also contains WD repeats like Apaf-1 and is thus more structurally similar to Apaf-1. Like Apaf-1, Dapaf-1/HAC-1/Dark is able to bind cytochrome *c* (Kanuka et al., 1999; Rodriguez et al., 1999). In addition, dATP and cytochrome *c* enhance the ability of Dapaf-1/HAC-1/Dark to activate caspases (Kanuka et al., 1999; Rodriguez et al., 1999; Zhou et al., 1999). While a release of cytochrome *c* during programmed cell death has not been observed in *Drosophila*, the display of an apoptogenic form of cytochrome *c* is seen on *Drosophila* mitochondria prior to apoptosis (Varkey et al., 1999). It is possible that this exposed form of cytochrome *c* is

sufficient to activate Dapaf-1/HAC-1/Dark. Similar to Apaf-1 and CED-4, loss-of-function mutations in the *Drosophila ced-4/Apaf-1* homolog result in a reduction of developmental programmed cell death. In addition, removing both maternal and zygotic Dapaf-1/HAC-1/Dark by RNAi resulted in a severe reduction of programmed cell death in the injected part of the embryo (Zhou et al., 1999). Intriguingly, the transcription of the *Drosophila* CED-4/Apaf-1 homolog is upregulated in response to both X-ray and UV irradiation (Zhou et al., 1999), much like transcription of *reaper* (Nordstrom et al., 1996, A-F. Lamblin and H. Steller, unpublished observations). This suggests that irradiation may activate several pathways that feed into the core programmed cell death pathway.

The Bcl-2 family of proteins

Work in *C. elegans* established that *ced-9* is an essential protector from programmed cell death. CED-9 was found to share homology with members of the mammalian Bcl-2 family (Hengartner and Horvitz, 1994). Bcl-2 family members share most of their homology in four conserved regions (BH1, BH2, BH3, and BH4), though not every family member contains all four of these regions (Adams and Cory, 1998). CED-9 is thought to form a complex with CED-4 and prevent it from activating CED-3. A BH3 domain only protein, EGL-1, can interact with CED-9 and prevent it from complexing with CED-4, thereby leaving CED-4 free to help activate CED-3 (reviewed in Metzstein et al., 1998; Horvitz, 1999).

An analysis of how Bcl-2 family members function in mammals has been complicated by the large number of proteins, at least fifteen to date, that share homology with Bcl-2 (reviewed in Gross et al., 1999; Vander Heiden and Thompson, 1999). As described in the previous section, one possible model for the apoptotic pathway is that the CED-4 homolog Apaf-1, in the presence of dATP and cytochrome *c,* promotes oligomerization and activation of the apical caspase 9. Once activated, caspase 9 processes downstream effector caspases which then cleave substrates that lead to apoptosis. The Bcl-2 family of proteins may function to amplify the caspase cascade by regulating the release of caspase activating factors. Given that Bcl-2 family members can homo- and hetero-dimerize through their conserved BH domains, the relative abundance of pro- and anti-apoptotic family members may determine the propensity for a cell to undergo programmed cell death.

The exact mechanism of how anti-apoptotic family members function to prevent apoptosis has been elusive to this point. Since human *bcl-2* can partially rescue cell death defects in *ced-9* loss-of-function worms, the two proteins are likely to share some functional similarities (Vaux et al., 1992; Hengartner and Horvitz, 1994). Based on the *C. elegans* model, it would seem logical that Bcl-2 should interact with Apaf-1 and thereby

prevent the activation of caspases, and this interaction has indeed been observed (Hu et al., 1998; Inohara et al., 1998; Pan et al., 1998; Song et al., 1999). In addition, endogenous CED-9 and CED-4 have both been shown to localize to the mitochondria in non-apoptotic cells and CED-9 was found to be necessary for this localization of CED-4 (Chen et al., 2000). However, at times this interaction has not been observed, such as when overexpressed Bcl-2 was assayed for interaction with Apaf-1, or when Bcl-X_L was added to cell-free apoptotic systems made from Jurkat cells or *Xenopus* eggs (Moriishi et al., 1999; Newmeyer et al., 2000). Furthermore, Bcl-2 and Bcl-X_L were shown to not localize to the same sub-cellular compartments as endogenous Apaf-1, raising doubts as to whether this interaction model with Apaf-1 is how Bcl-2 family members function (Hausmann et al., 2000). Other models for how the Bcl-2 family functions, which stress functions besides that of binding to Apaf-1, have been developed, including a model where Bcl-2 proteins can associate with, or form pores in, the mitochondrial membranes and thereby regulate the release of caspase activating substances such as cytochrome *c* (Green and Reed, 1998; Gross et al., 1999). Evidence for these models is provided by experiments that show that Bcl-2 can suppress the release of cytochrome *c* from isolated mitochondria (Kluck et al., 1997; Vander Heiden et al., 1997; Yang et al., 1997; Bossy-Wetzel et al., 1998). Bcl-2 family proteins also can potentially regulate the release of cytochrome *c* from the mitochondrial voltage-dependent anion channel (VDAC) (Shimizu et al., 1999). Finally, it has been proposed that Bax, a pro-apoptotic Bcl-2 family member, can destabilize mitochondrial membranes directly, leading to a release of apoptosis activating factors (Basañez et al., 1999). This diverse set of models for the function of Bcl-2 family members and often directly conflicting observations, makes it clear that more insight needs to be gained before the proper place for Bcl-2 family members can be established in the programmed cell death pathway.

The rate of mitochondrial release of cytochrome *c* in cells undergoing apoptosis was recently measured and found to be rapid (Goldstein et al., 2000): Most mitochondria released all their cytochrome *c* within a one minute period in a temperature-independent fashion over a range of 24°C to 37°C. The rapid and complete nature of the release and the lack of a temperature dependence suggests that an enzymatic transport mechanism is not involved. Further, various proteins besides cytochrome *c* are released from mitochondria, including apoptosis-inducing factor (AIF). AIF is normally confined to the mitochondria, but is released when apoptosis is induced and has been shown to be pro-apoptotic when microinjected into the cytoplasm of tissue culture cells (Susin et al., 1999). These data have been used to invoke a model involving non-specific pores forming in the mitochondrial membrane during apoptosis (Goldstein et al., 2000).

Neither mammalian model for the function of Bcl-2 family members is as clear as what has been determined in *C. elegans*. The inconsistencies seen in mammalian models are probably due to the greater number of family members which may have slightly different functions or interactions depending on the cell type.

Recently, two Bcl-2 family members have been identified in *Drosophila*:: the first, named Debcl/Drob-1/dBorg-1/DBok, and the second a largely uncharacterized member found in the genomic sequence (Figure 6) (Brachmann et al., 2000; Colussi et al., 2000; Igaki et al., 2000; Rubin et al., 2000; Zhang et al., 2000). Debcl/Drob-1/dBorg-1/DBok is most similar to Bok, a mammalian pro-apoptotic Bcl-2 related protein. It is also pro-apoptotic and can interact with most mammalian anti-apoptotic family members, but not with pro-apoptotic ones. The protein, like many mammalian Bcl-2 family members, localizes to mitochondria. While Colussi *et al.* (2000) report that the caspase inhibitor P35 is able to prevent Debcl induced apoptosis, Igaki *et al.* (2000) report that P35 is unable to block Drob-1 induced apoptosis and Zhang *et al.* (2000) were also unable to prevent cell death caused by DBok with peptide caspase inhibitors. These discrepancies may be due to the different constructs and experimental systems used and more analysis is needed to make definitive conclusions about whether expression of this gene results in caspase mediated cell death. While *debcl/drob-1/dborg-1/dbok* genetically interacts with *diap1* and *dapaf-1/hac-1/dark*, *Drosophila* Bok homolog-mediated apoptosis is not affected by the gene dosage of *reaper, hid*, and *grim* (Colussi et al., 2000). These observations suggest that this *Drosophila* Bcl-2 family member functions upstream or in parallel to Diap1 and Dapaf-1/HAC-1/Dark, but downstream of Reaper, Hid, and Grim. In addition, the overexpression of *debcl/drob-1/dborg-1/dbok* seems to sensitize the *Drosophila* pupal eye to cell killing induced by UV irradiation, suggesting that *debcl/drob-1/dborg-1/dbok* may synergize with the DNA damage response pathway (Brachmann et al., 2000). This observation is particularly intriguing since the transcription of both *dapaf-1/hac-1/dark* and *reaper* is upregulated in response to UV irradiation and the *Drosophila* p53 ortholog can regulate *reaper* transcription in response to DNA damage caused by X irradiation (Zhou et al., 1999; Brodsky et al., 2000, A.-F. Lamblin and H. Steller, unpublished results).

At this point, the data for the *Drosophila* Bok homolog would suggest that it could function in a similar manner to EGL-1 in *C. elegans*, but further analysis needs to be performed to support this prediction. Unlike EGL-1, this *Drosophila* Bcl-2 family member contains not just a BH3 domain, but also BH1, BH2, and possibly a BH4 domain. Furthermore, an anti-apoptotic Bcl-2 family member has not yet been characterized in *Drosophila*, but it is possible that the second Bcl-2-like gene identified in the genome performs this function.

Future directions

Most of the core programmed cell death pathway as defined in *C. elegans* has *Drosophila* counterparts. In addition, *Drosophila* appears to contain other key regulators including the IAPs, Reaper, Hid, and Grim (see Figure 1). The completion of the *Drosophila* genome has shown that many other players in programmed cell death also exist in *Drosophila*, including a death domain containing protein, dFADD(Hu and Yang, 2000), suggesting that cell death in *Drosophila* is regulated in a way that is very similar to that of mammals. The genetic manipulation of *Drosophila* is a powerful tool to study the function of these various genes in the context of the whole organism and allows for the analysis of how cell death shapes development. The pace of discovery is likely to increase now that cloning of specific genes is being facilitated by the continuing efforts of the genome project and *Drosophila* will undoubtedly continue to play a significant role in increasing our understanding of programmed cell death.

CASPASE SUBSTRATES

It is generally accepted that effector caspases are the executioners of programmed cell death, but it is less clear which of the many potential substrates in the cell are important for the changes observed during apoptosis. The current estimates for the number of caspase substrates are based on two-dimensional gel electrophoresis and suggest that approximately eighty proteins are cleaved during apoptosis (Brockstedt et al., 1998; Nicholson, 1999). Seventy of these substrates have been identified and in many cases, the rationale for cleaving the substrate can be predicted based on the contemporary knowledge of the cell death pathway and the morphological changes that a cell undergoes during apoptosis. Caspases disable a cell in various ways, including disabling the repair processes, blocking cell cycle progression, inactivating inhibitors of apoptosis, weakening the structural components of the cell, and marking the dying cell for engulfment (Nicholson, 1999).

A number of structural genes are cleaved during apoptosis, including the nuclear lamins, β-catenin, gelsolin and Gas2 (Brancolini et al., 1995; Lazebnik et al., 1995; Rao et al., 1996; Brancolini et al., 1997; Kothakota et al., 1997; Herren et al., 1998; Van de Craen et al., 1999). Given that the morphology of a cell changes dramatically during apoptosis, it is not surprising that some proteins vital for maintaining cell structure are degraded. It has been shown that the nuclear envelope shrinks and the chromatin condenses during apoptosis (Kerr et al., 1972; Wyllie, 1980a). Mutations in the potential caspase cleavage site in nuclear lamin result in a delay in the onset of apoptosis and an apparent lack of

nuclear envelope shrinkage, suggesting that lamin cleavage is essential for one aspect of the apoptotic morphology that has been observed (Rao et al., 1996). Cleavage of β-catenin, on the other hand, helps explain the loss of cell-cell contacts, as well as the reorganization of the actin cytoskeleton during apoptosis (Brancolini et al., 1997). The *Drosophila* β-catenin homolog, Armadillo, which helps anchor the actin cytoskeleton to the plasma membrane, is also likely to be cleaved by caspases. The Armadillo protein sequence contains two potential effector caspase cleavage sites (J. Tittel and H. Steller, manuscript in preparation) and a significant decrease in the level of Armadillo is seen in *Drosophila* follicle and nurse cells during apoptosis (Chao and Nagoshi, 1999). Furthermore, in *Drosophila* nurse cells deficient in *dcp-1*, the actin cytoskeleton does not reorganize properly during apoptosis (McCall and Steller, 1998). This observation would be consistent with caspase mediated cleavage of Armadillo being required for proper actin reorganization in this tissue. Finally, in *diap1* loss-of-function mutants, virtually all cells undergo apoptosis between four and five hours into development and at this time a significant decrease in the amount of Armadillo protein is seen (J. Tittel and H. Steller, manuscript in preparation).

The mammalian proteins CAD and ICAD provide a clear example of how caspase activation can result in characteristic degradation of the genomic DNA observed during apoptosis. CAD, caspase-activated deoxyribonuclease, can degrade DNA into an oligonucleosomal ladder in a caspase-dependent manner. Its inhibitor, ICAD, is cleaved by caspases, freeing up CAD to degrade the genomic DNA (Enari et al., 1998; Sakahira et al., 1998). While this is one mechanism by which DNA degradation can be achieved, *C. elegans* utilizes other DNAses for this purpose since both ICAD and CAD appear to be absent in the *C. elegans* genome and the DNAse II homolog NUC-1 is necessary for a step in DNA degradation seen during programmed cell death in *C. elegans* (Wu et al., 2000). Homologs of ICAD and CAD are predicted to exist in *Drosophila*, however (Rubin et al., 2000).

The above examples are but a small sampling of the potentially important caspase substrates. Up to this point, however, most of the caspase substrate data has come from *in vitro* cleavage experiments with limited verification *in vivo*. The *Drosophila* embryo has provided us with evidence that at least two of these substrates, lamin and Armadillo, are likely to be physiological substrates as well (J. Tittel and H. Steller, manuscript in preparation). Further analysis of the substrates cleaved during apoptosis should provide significant insight into how cells are disassembled during programmed cell death in an environment where the entire organism is undergoing apoptosis.

CONCLUSIONS

Our understanding of programmed cell death is still limited despite the identification of a number of essential genes involved in this process. It has become clear that a linear pathway is too simplistic. However, the identification of the core pathway will enable us to extend our understanding of the signals that lead into this pathway. We also need to further define the relevant caspase targets responsible for the changes we observe. Given the rapid increase in sequence information available, new assays utilizing that knowledge, such as DNA microarrays to look for differential expression, should further increase the pace of discovery in this field.

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Figure 1.

A Schematic Representation of the Core Programmed Cell Death Pathway

Multiple pathways lead to the activation of the executioners of death, the caspases, resulting in the cleavage of key substrates and the death of the cell. IAPs have been shown to block the conversion of pro-caspases into the active enzyme and Reaper, Hid, Grim, and Diablo/Smac prevent IAPs from carrying out this protective function. Caspases can also be activated with the aid of Apaf-1, which in turn appears to be regulated by cytochrome *c* and dATP. The Bcl-2 family appears to function in regulating the release of pro-apoptotic components from mitochondria as well as by possibly inhibiting Apaf-1 directly. This pathway integrates knowledge gained in multiple species, showing that apoptosis appears to be regulated in a similar manner regardless of the organism. One notable exception is that the *C. elegans* IAPs do not appear to function in programmed cell death.

Figure 1:

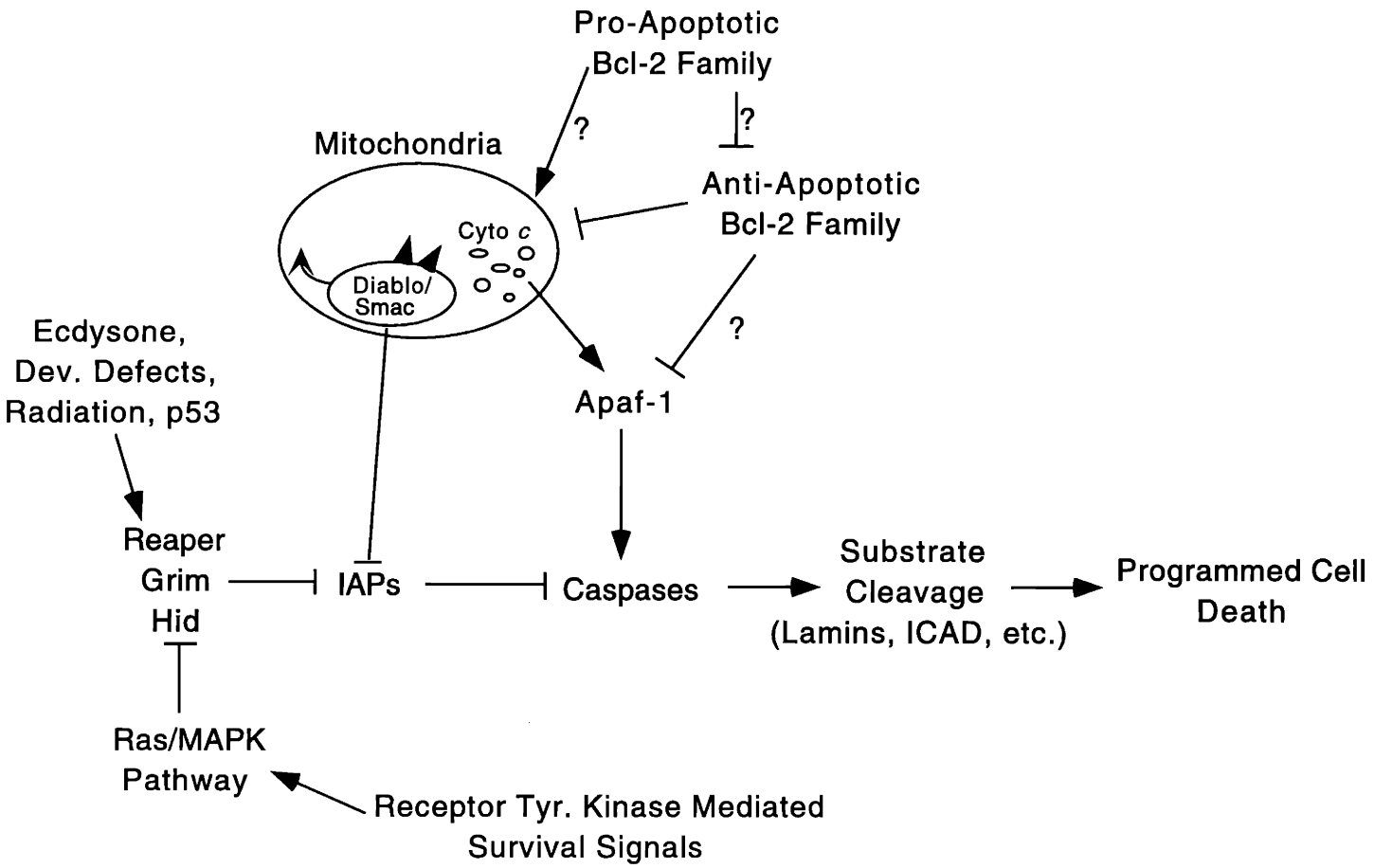
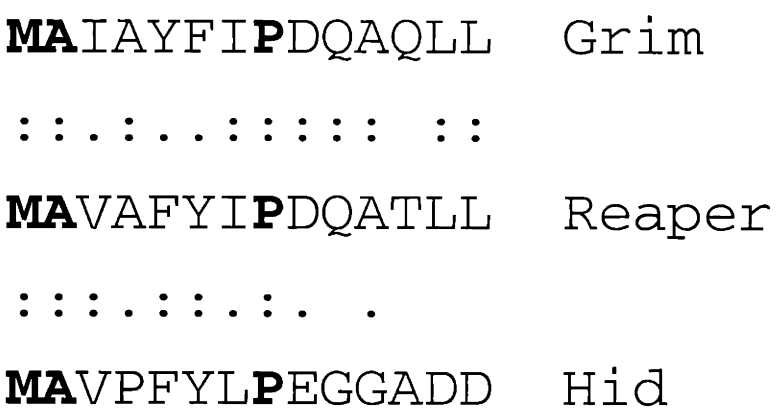

Pro-Apoptotic
Bcl-2 Family
?
?
Mitochondria
Anti-Apoptotic
Bcl-2 Family
Cyto c
Diablo/
Smac
?
Ecdysone,
Dev. Defects,
Radiation, p53
Apaf-1
Reaper
Grim
Hid
IAPs
Caspases
Substrate
Cleavage
(Lamins, ICAD, etc.)
Programmed Cell
Death
Ras/MAPK
Pathway
Receptor Tyr. Kinase Mediated
Survival Signals

Figure 2.

The Amino-Termini of Reaper, Hid, and Grim Share a Region of Homology

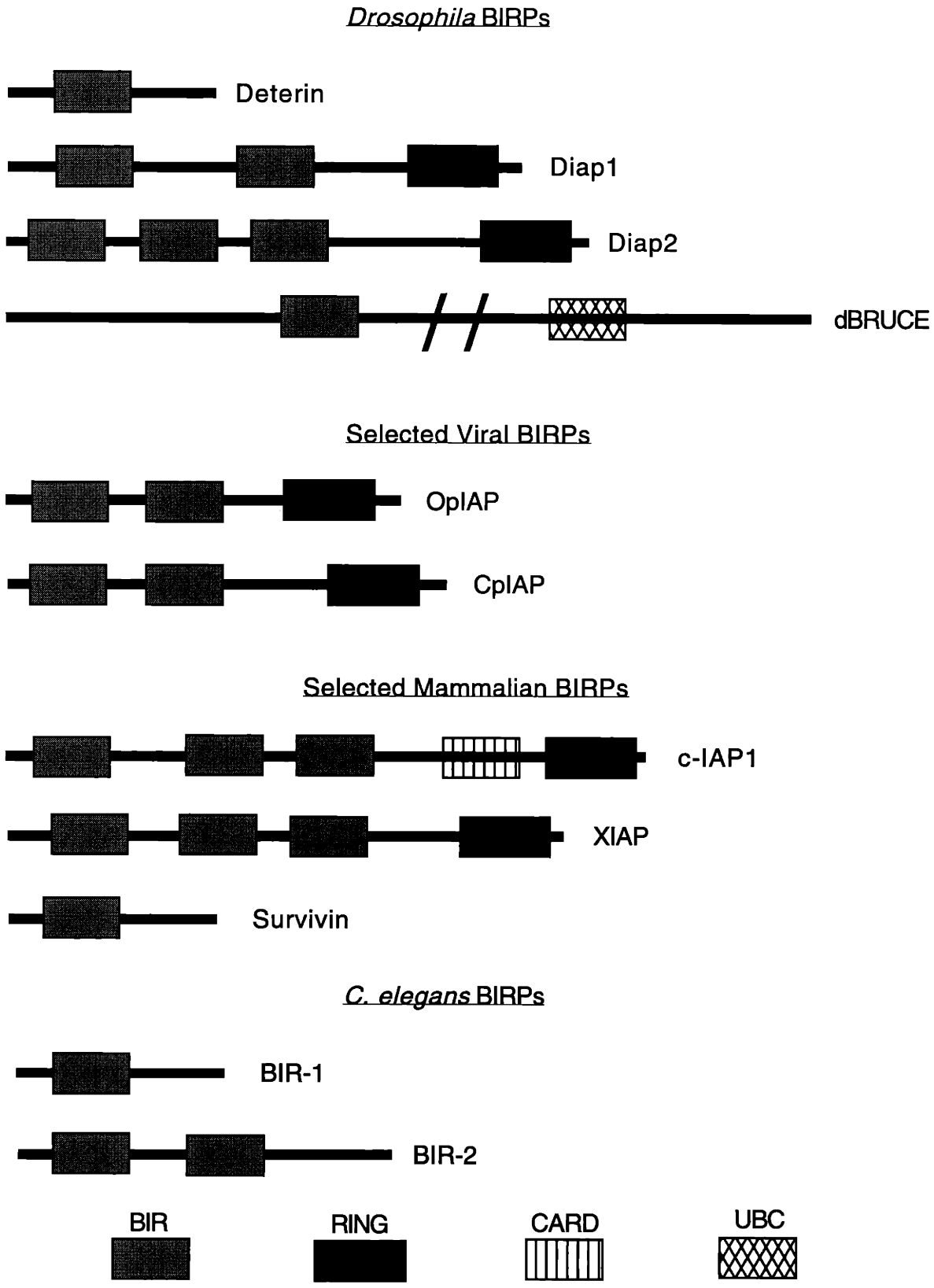


An alignment of the first 14 amino acids of Reaper, Hid, and Grim shows that these three proteins contain some similarity over this region. Amino acids that are identical in all three proteins are shown in bold. A colon (:) in the space between two proteins indicates that these amino acids are identical whereas a period (.) indicates that they are conserved.

Figure 3.

Schematic Representations of BIRPs in Several Species

BIR domain containing proteins (BIRPs) have at least one BIR domain that is involved in protein-protein interactions. Most BIRPs that function in inhibiting apoptosis also contain a carboxy-terminal RING finger which may function in the regulation of the protein. *Drosophila* contains four BIRPs, all of which appear to play some role in apoptosis. The *Drosophila* BRUCE homolog lacks a RING domain; however, it contains a ubiquitin conjugating (UBC) domain at its carboxy-terminus. *C. elegans* has two BIRPs, neither of which seems to function in apoptosis. One of these, BIR-1, functions in cell division and in regulating chromosome behavior. Some mammalian BIRPs contain a caspase recruitment domain (CARD).

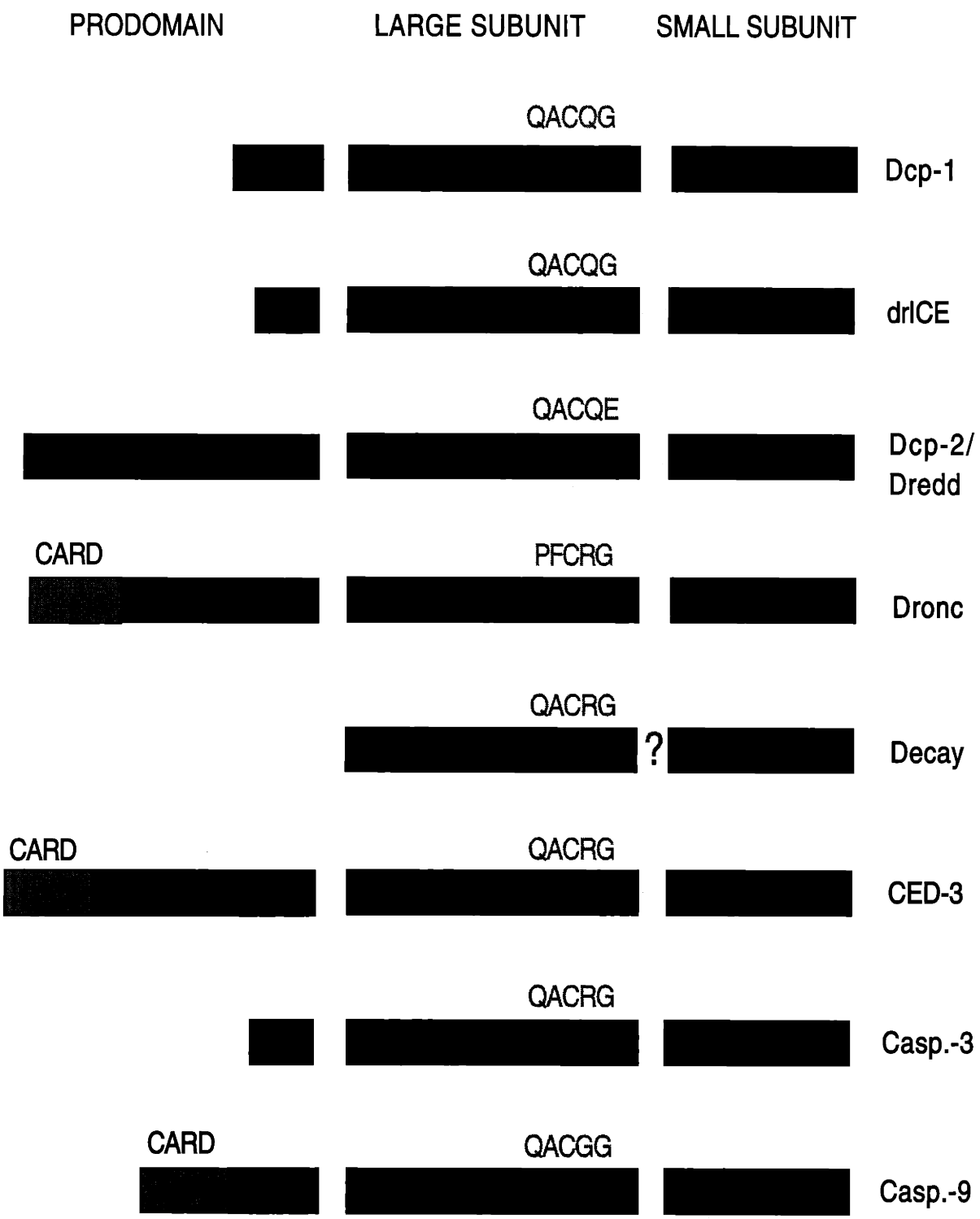


adapted from Vernooy et al., (2000) J. Cell Biol. *150*, F69-F75.

Figure 4.

Schematic Representations of the *Drosophila* Caspases and Several Other Caspases

Eight caspase-like sequences have been found in the *Drosophila* genome. A schematic representation of five characterized *Drosophila* caspases is shown in this figure. Three of these caspases (Dcp-1, drICE, and Decay) have short prodomains and are therefore likely to be effector caspases. The remaining two have long prodomains and are likely to function as initiator caspases. The amino acid sequence of the active site is shown above the large subunit in each case. The exact cleavage site between the large and small subunits of Decay is unclear as is indicated by the question mark. Dronc, like several other initiator caspases, contains a CARD domain that functions in caspase recruitment. For comparison, the *C. elegans* caspase CED-3 and two mammalian caspases (Caspase-3 and Caspase-9) are shown. CED-3 and Caspase-3 have a short prodomains and Caspase-9 has a long prodomain.

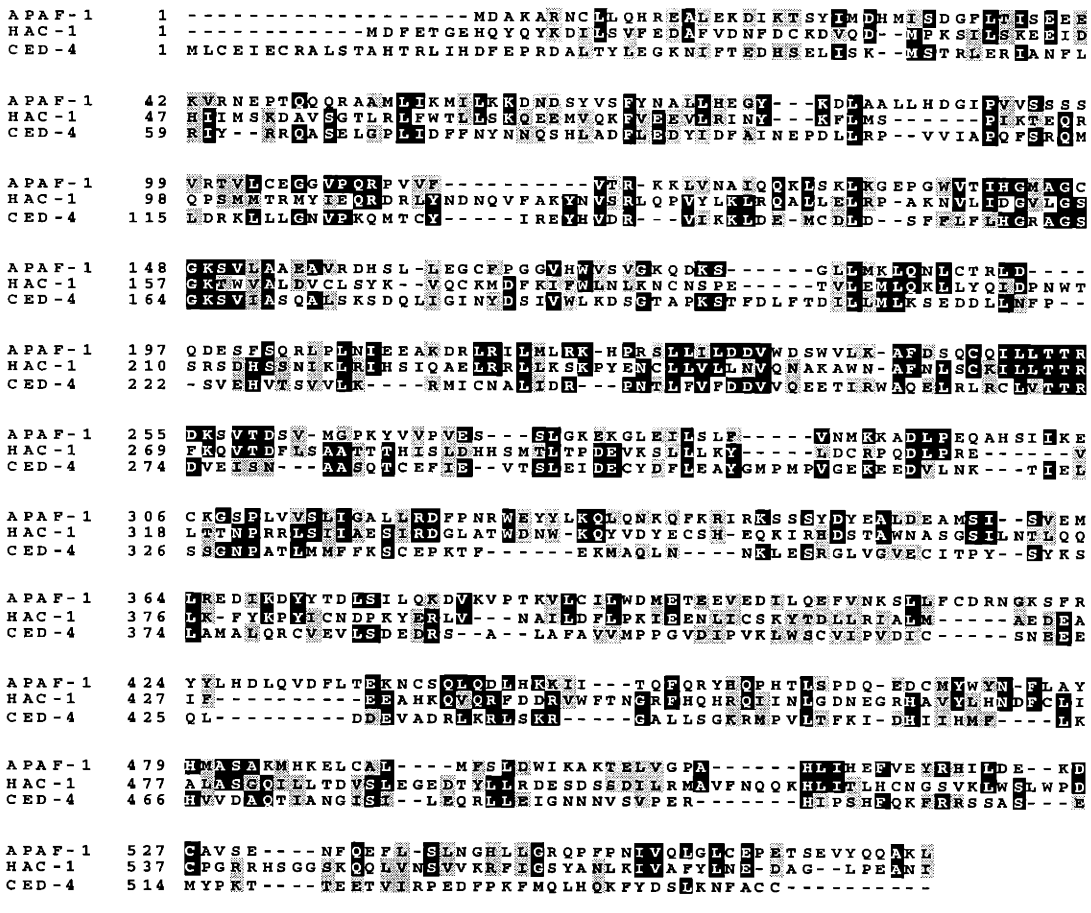


adapted from Vernooy et al., J. Cell Biol. (2000) *150*, F69-F75.

Figure 5.

Alignment of Apaf-1, HAC-1, and CED-4

Identical residues are shown in dark blocked letters, and conserved residues are shown in shadow blocked letters. HAC-1 is more similar to Apaf-1 than to CED-4, where the homology mainly lies in the kinase motifs of the nucleotide binding domains.



provided by Lei Zhou

Figure 6.

Schematic Representations of Several Bcl-2 Family Members

The structure of the pro-apoptotic *Drosophila* Bok homolog is shown. This protein contains three of the four Bcl-2 homology regions (BH). A second *Drosophila* Bcl-2 family member is present in the genome, but has not been extensively characterized to date. For comparison, two representative mammalian Bcl-2 family members are also shown. The mammalian Bcl-2 family contains at least fifteen members which are divided into pro- and anti-apoptotic sub-families. For example, Bcl-2 is anti-apoptotic and Bax appears to be pro-apoptotic and in *C.elegans*, CED-9 is anti-apoptotic and EGL-1 is pro-apoptotic.

Drosophila

Drob-1/Debcl/
dBorg-1/Dbok

Selected Mammalian

Bcl-2

Bax

C. elegans

CED-9

EGL-1

adapted from Vernooy et al., (2000) J. Cell Biol. *150*, F69-F75.

Chapter 2

HAC-1, a *Drosophila* Homolog of APAF-1 and CED-4, Functions in Developmental and Radiation-Induced Apoptosis.

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Running title: HAC-1 is required for apoptosis in *Drosophila*

My contributions to this paper were the RNAi experiments with ds *hac-1* mRNA and with ds *lacZ* mRNA shown in Figure 4 and the Appended Figure.

This paper, except for the appended figure which shows a ds *lacZ* RNA control, was published in Molecular Cell, Vol. 4, 745-755, November 1999.

SUMMARY

We have identified a *Drosophila* homolog of *Apaf-1* and *ced-4*, termed *hac-1*. Like mammalian APAF-1, HAC-1 can activate caspases in a dATP-dependent manner *in vitro*. During embryonic development, *hac-1* is prominently expressed in regions where cells undergo natural cell death. Significantly, *hac-1* mRNA expression is also rapidly induced upon ionizing irradiation, similar to the pro-apoptotic gene *reaper*. Loss of *hac-1* function causes reduced cell death, and reducing the dosage of *hac-1* suppresses the ectopic cell killing upon expression of the *dcp-1* procaspase in the retina, but has little effects on *reaper, hid* and *grim*-mediated killing. Our data indicate that caspase activation and apoptosis in *Drosophila* are independently controlled by at least two distinct regulatory pathways that converge at the level of caspase activation.

INTRODUCTION

Apoptosis, a morphologically distinct form of programmed cell death, is essential for normal development and tissue homeostasis in both invertebrates and vertebrates (Thompson, 1995; Jacobson et al., 1997; Raff, 1998; Vaux and Korsmeyer, 1999). During the last few years, rapid progress has been made in identifying some of the molecules that are responsible for the regulation and execution of apoptosis. Initially, genetic studies in the nematode *Caenorhabditis elegans* defined a core program for programmed cell death (Ellis and Horvitz, 1986; Ellis et al., 1991; Yuan et al., 1993; Hengartner and Horvitz, 1994b; Hengartner, 1996; Horvitz, 1999). In particular, this work revealed the importance of an unusual class of cysteine proteases, termed caspases (for cysteine aspartic acid-specific protease) for cell death (Yuan et al., 1993). Subsequently, a large number of mammalian caspases have been isolated and shown to play an important role in apoptosis (Takahashi and Earnshaw, 1996; Nicholson and Thornberry, 1997; Salvesen and Dixit, 1997; Thornberry et al., 1997). Caspases are synthesized as inactive zymogens which are widely expressed in both dying and live cells. The activation of caspases during apoptosis involves several internal cleavages in the pro-enzyme which lead to the removal of an inhibitory N-terminal prodomain and the generation of a large (p20) and small (p10) subunit. Caspases can be activated through cleavage by active "initiator" caspases in a caspase cascade (Li et al., 1997), and by auto-proteolysis following aggregation of two or more zymogen molecules (MacCorkle et al., 1998; Muzio et al., 1998; Yang et al., 1998). Upon activation of "executioner" caspases, a wide variety of specific intracellular proteins are cleaved in different cellular compartments, and it is thought that their breakdown ultimately leads to the characteristic morphological changes of apoptosis (Nicholson and Thornberry, 1997). The activation of caspases appears to be tightly controlled by both positive and negative regulators. On the one hand, members of the IAP (inhibitor of apoptosis protein) family can inhibit caspases and apoptosis in a variety of insect and vertebrate systems (Uren et al., 1998; Deveraux and Reed, 1999). On the other hand, caspase activation is stimulated by a family of proteins that include *C. elegans* CED-4 and mammalian apoptosis protease-activating factor-1, APAF-1 (Yuan and Horvitz, 1992; Zou et al., 1997). The *ced-4* gene is required for programmed cell death in *C. elegans*, and mice deficient for APAF-1 have reduced programmed cell death (Ellis and Horvitz, 1986; Cecconi et al., 1998; Yoshida et al., 1998). Furthermore, *ced-4* acts genetically upstream of *ced-3,* and CED-4 can physically interact with pro-CED-3 and certain mammalian procaspases (Shaham and Horvitz, 1996; Chinnaiyan et al., 1997; Seshagiri and Miller, 1997; Wu et al., 1997). APAF-1, which shares significant amino acid homology with CED-4, can bind to the prodomain of

procaspase-9 and activate it in the presence of cytochrome c and dATP in a cell free system (Li et al., 1997; Zou et al., 1997). These observations suggest that CED-4/APAF-1-like proteins have an important and direct function in the activation of caspases that has been conserved from nematodes to mammals.

The cell killing activity of *ced-4* is negatively regulated by *ced-9*, which encodes a protein homologous to the BCL-2 family of mammalian cell death regulators (Hengartner et al., 1992; Hengartner and Horvitz, 1994a; White, 1996; Reed, 1997b). Likewise, there is evidence that BCL-2-like proteins can control APAF-1 activity, but the precise mechanism has remained somewhat controversial. Because APAF-1 requires cytochrome c as a co-factor for caspase activation *in vitro,* it has been proposed that the release of cytochrome c from mitochondria into the cytosol is a critical regulatory step, and that this release is blocked by BCL-2-like proteins (Kim et al., 1997; Kluck et al., 1997; Li et al., 1997; Reed, 1997a; Reed, 1997b; Yang et al., 1997; Green and Reed, 1998). However, the conditions under which the release of cytochrome c is used to activate apoptosis *in situ* remain to be determined.

A major gap in our current understanding of apoptosis is how specific death-inducing signals turn on the death program. Not surprisingly, the control of programmed cell death is remarkably complex. Apoptosis can be induced by a wide variety of stimuli that may originate either from within cells or from their extracellular environment (Raff, 1992; Raff et al., 1993; Steller, 1995; Nagata, 1997; Pettmann and Henderson, 1998). Molecular genetic studies of programmed cell death in *Drosophila* offer unique opportunities for expanding our knowledge in this area. In *Drosophila*, like in vertebrates, the regulation of apoptosis is highly plastic and involves a wide variety of intracellular and extracellular signals (Steller and Grether, 1994; McCall and Steller, 1997; Bergmann et al., 1998b). The induction of apoptosis in *Drosophila* requires the activities of three closely linked genes, *reaper, grim* and *head involution defective (hid)*, which kill by activating a caspase pathway (White et al., 1994; Grether et al., 1995; Chen et al., 1996; White et al., 1996). Several *Drosophila* caspases have been identified (Fraser and Evan, 1997; Song et al., 1997; Chen et al., 1998; Dorstyn et al., 1999). Two of them, DCP-1 and DRICE, are very similar in structure and biochemical specificities. *reaper* and *grim* have been shown to be able to activate DCP-1 and DRICE *in vivo* (Song et al., submitted). Significantly, *reaper*, *hid* and *grim* are transcriptionally regulated by a variety of death-inducing stimuli. In particular, the expression of *reaper* is controlled by a range of different signals, including the steroid hormone ecdysone, segmentation and patterning genes, and DNA

damaging agents (White et al., 1994; Nordstrom et al., 1996; Jiang et al., 1997; Robinow et al., 1997; Draizen et al., 1999, Lamblin and Steller, submitted). Therefore, it appears that this gene acts as an integrator for relaying different death-inducing signals to the core death program.

The products of *reaper, grim* and *hid* share a short stretch of conserved amino acids at their N-terminus, but otherwise encode novel proteins without any significant homology to other known genes. However, expression of REAPER, GRIM or HID can induce apoptosis in mammalian cells, and recombinant REAPER can activate caspase activation and apoptosis-like events in a *Xenopus* cell-free system (Evans et al., 1997; Claveria et al., 1998; McCarthy and Dixit, 1998; Haining et al., 1999). Furthermore, REAPER, GRIM and HID interact physically and genetically with IAPs, a highly conserved family of anti-apoptotic proteins (Hay et al., 1995; Vucic et al., 1997a; Vucic et al., 1997b; Vucic et al., 1998; Goyal et al., 1999). Finally, the pro-apoptotic activity of HID is inactivated upon phosphorylation by MAP-kinase (Bergmann et al., 1998a). Collectively, these observations indicate that molecules homologous to REAPER, GRIM and HID may be used to regulate apoptosis in vertebrates. However, the apparent absence of *Drosophila* homologs of CED-4/APAF-1 and BCL-2-like molecules has previously raised concerns that the regulation of apoptosis in *Drosophila* may be substantially different from that in *C. elegans* and mammals (see, for example, Meier and Evan, 1998; Rich et al., 1999).

We now report the identification and characterization of a *Drosophila* homolog of CED-4 and APAF-1, *hac-1* (for homolog of *Apaf-1* and *ced-4*). We show that *hac-1* is structurally and functionally very similar to *Apaf-1*, and that it is required for normal cell death during embryonic development in *Drosophila*. Reducing *hac-1* activity in heterozygous animals suppresses cell killing by the *Drosophila* caspase *dcp-1*, but has little effects on *reaper, hid* and *grim*-induced ectopic apoptosis. Finally, we find that *hac-1* expression is regulated both in development and also by ionizing radiation. Our results indicate that death-inducing stimuli promote apoptosis in *Drosophila* by simultaneously activating two distinct regulatory pathways controlling caspase activation.

RESULTS

Identification of a *Drosophila* Homolog of APAF-1/CED-4

We identified *hac-1* (homolog of APAF-1 and CED-4) as a P1 sequence entry (GeneBank accession AC004335, located at cytological position 53F, (Kimmerly et al., 1996)) of the Berkeley *Drosophila* Genome Project (BDGP) using a novel database search scheme. This entry encodes a putative P-loop nucleotide binding motif (corresponding to CED-4 $_{155}$LFLHGRAGSGKSVIA) as well as a motif that is shared by CED-4, APAF-1, and a family of plant disease resistance proteins (corresponding to CED-4 $_{267}$RCLVTTRDVEISN, Figure 1 B) (van der Biezen and Jones, 1998). Computational analysis using the GENESCAN (Berge and Karlin, 1997) program suggested that this part of genomic sequence (about 600 bp) is likely to be an exon of a larger gene. Two EST entries (from cDNA clone GH17715 and GH23583, Berkeley *Drosophila* Genome Project/HHMI EST Project, unpublished) are derived from the 3' segment of this predicted gene. We also identified a lethal P-element insertion (Spradling et al., 1995), l(2)k11502, at the 5' end of this gene, immediately upstream of the presumptive TATA box (Figure 1C). This P-element insertion disrupts *hac-1* function and was subsequently used for phenotypic analyses (see below).

A combination of RT-PCR, genomic and cDNA sequencing was used to determine the nucleotide sequence of the corresponding 5.5kb mRNA. The results of this analysis predict an open reading frame, composed of nine exons, coding for a 1440 amino acid protein. The N-terminus of the deduced protein shares significant homology with APAF-1 (20% identity and 41% for amino acids 65-486) and the plant disease-resistant proteins (e.g. 19% identity, 34% similarity for amino acids 147-462 in comparing to RPR1, GeneBank accession AB019186). Like APAF-1, the *Drosophila* protein also has 9 WD repeat elements following the CED-4 homology region (Figure 1A). We termed this protein Homolog of APAF-1/CED-4 (HAC-1). The sequence homology between HAC-1 and CED-4 is more limited and mainly restricted to the two nucleotide binding motifs and the ARC domain (Figure 1B) (van der Biezen and Jones, 1998). Nevertheless, the biochemical and functional data presented below indicates that this homology is significant.

HAC-1 Can Induce Caspase Activation *In Vitro*

In order to investigate whether HAC-1 shares biochemical properties with APAF-1, we investigated the ability of the protein to stimulate *Drosophila* caspase activation *in vitro*. HAC-1 was expressed in 293T cells by transient transfection, and the cytoplasmic fraction

of these cells was tested for its ability to activate ^{35}S-labeled DCP-1 and DRICE zymogens (Figure 2) (Fraser and Evan, 1997; Song et al., 1997). While extracts from control cells had no detectable caspase-conversion activity (Fig. 2, lane 2), extracts from cells expressing *hac-1* were able to induce processing of both DRICE and DCP-1 pro-enzymes (Figure 2, lanes 3 and 4, and data not shown). Significantly, as previously observed for APAF-1 (Liu et al., 1996; Zou et al., 1997), this conversion was dependent on the presence of dATP (Figure 2, lanes 5 and 6). Therefore, HAC-1 appears to be similar to APAF-1 in its activity to stimulate caspase processing.

Distribution of *hac-1* during Embryogenesis

It is thought that the basic cell death program is constitutively expressed (Raff et al., 1993; Weil et al., 1996), and many cell death genes, including APAF-1, appear to be widely expressed (Zou et al., 1997). Figure 3 shows that *hac-1* mRNA is broadly distributed in pre-blastoderm embryos before the major onset of zygotic transcription, apparently due to maternal contribution (Figure 3A). However, by stage 7, the highest levels of *hac-1* mRNA were detected in the ventral neurogenic region, and around several invagination furrows (Figure 3B). Interestingly, these are regions in which prominent apoptosis occurs during subsequent development (Abrams et al., 1993; Nassif et al., 1998). Later, the highest levels of *hac-1* mRNA were found in the ectoderm and mesoderm of the procephalic region (Figure 3C). The expression of *hac-1* in the developing head overlaps significantly with that of the pro-apoptotic gene *hid* (Figures 3E and 3F; Grether et al., 1995). Again, abundant apoptosis is subsequently observed in this region. The mRNA distribution of *hac-1* is closely mirrored by the pattern of β-gal expression from the P-element insertion l(2)k11502, which carries a *lacZ* reporter gene. The l(2)k11502 P-element transposon is inserted near the transcriptional start site of *hac-1*, 81 bp upstream of the presumptive TATA box (Figure 1C). Although a basal level of *lacZ* expression could be detected in essentially all cells, high levels of expression were again seen in the procephalic region (Figure 3G). At later stages, both *lacZ* reporter expression and *hac-1* mRNA were detected in a segmentally repeated pattern. Finally, β-gal immunoreactivity was seen in macrophages starting from late stage 11 to the end of embryonic development. These observations indicate that *hac-1* is abundantly expressed in many but not all dying cells, and that its expression is transcriptionally regulated during development.

hac-1 Is Required for Normal Cell Death during Embryonic Development

The insertion of l(2)k11502 appears to disrupt the expression of *hac-1*, since embryos homozygous for the insertion displayed no head-specific *in situ* signals above the basal levels derived from maternally contributed mRNA (Figure 3D). Approximately 25% of l(2)k11502 homozygous embryos failed to hatch, and those that hatched had locomotion defects and died within 36 hours. Of those died as larvae, a significant portion developed melanotic tumor (data not shown), similar to what have been observed for the *dcp-1* zygotic mutant (Song et al., 1997). In order to examine whether zygotic *hac-1* function is required for normal cell death, embryos heterozygous or homozygous for the l(2)k11502 insertion were labeled with TUNEL to visualize apoptotic cells. In wild-type and heterozygous embryos (Figure 4A), a large number of TUNEL-positive cells were consistently observed in the head region of stage 12 embryos. In contrast, much fewer TUNEL positive cells were present in the head region of embryos homozygous for l(2)k11502 (Figures 4B and 4C). This phenotype was highly penetrant, but some variation among homozygous mutant embryos was seen (Figures 4B and 4C), possibly due to differences in the amount or perdurance of maternal *hac-1*. Therefore, although many cells can still die in homozygous mutant embryos, zygotic *hac-1* product is required for the normal pattern of apoptosis.

It is unlikely that elimination of zygotic *hac-1* function in l(2)k11502 homozygous embryos represents the true null phenotype, since *hac-1* mRNA is maternally contributed. To inactivate both zygotic and maternal *hac-1* mRNA, we used the RNA interference assay (RNAi). Previous work in *C. elegans* and, more recently, in *Drosophila* has demonstrated that RNAi can be an effective tool for studying gene function (Fire et al., 1998; Kennerdell and Carthew, 1998; Misquitta and Paterson, 1999). Double-stranded *hac-1* RNA corresponding to either the first 723 amino acids, or amino acids 134 to 332, encompassing the most closely conserved region between APAF-1 and CED-4, was made and injected into syncytial wild-type embryos. The patterns of cell death in these embryos were compared to uninjected and *lacZ* RNAi control embryos by TUNEL and acridine orange staining (Abrams et al., 1993; White et al., 1994). When injected into the posterior of the embryo, both *hac-1* dsRNAs significantly reduced the number of apoptotic cells in the injected part of the embryo (Figures 4D and 4E). Injecting the anterior of the embryo yielded similar results (data not shown), with the reduction of cell death restricted to the injected half of the embryo. Similar localized effects in RNAi experiments have been previously reported for other genes, such as *frizzled* and *frizzled-2* (Kennerdell and Carthew, 1998). In contrast, injecting a double-stranded *lacZ* RNA did not at all reduce the

amount of apoptosis (Figure 4F), but abolished the expression of β-galactosidase in the injected part of the embryo when compared to an uninjected control embryo (data not shown). Since RNAi produced a more severe reduction of apoptosis than elimination of zygotic *hac-1* function, it appears that maternal *hac-1* product contributes to the induction of cell death during normal embryonic development.

At least some of the "undead" cells in the l(2)k11502 mutant embryos appear to develop into extra neurons. When stained with the anti-Elav antibody, which recognizes all differentiated neurons, brains of l(2)k11502 homozygous mutant embryos contained more Elav-positive cells than wild-type (Figure 5). This phenotype is very similar to that seen in null mutants of the pro-apoptotic gene *head involution defective (hid)* (Grether et al., 1995). In addition, like loss of *hid* function, many *hac-1* mutant embryos showed defects in head involution (Figure 5). These findings suggest that *hac-1*, like *hid*, is required to eliminate cells for proper morphogenesis of the *Drosophila* embryo.

hac-1 and *reaper, hid* and *grim* Can Independently Promote Caspase Activation

The ectopic expression of *reaper, hid, grim* and several *Drosophila* caspases can lead to ectopic apoptosis. For example, when these genes are expressed in the developing retina under the control of an eye-specific promoter, GMR (Hay et al., 1994), cell death is induced in a dosage-dependent manner, and this produces various degrees of eye-ablation (reviewed in Bergmann et al., 1998b). This provides a highly sensitized background to investigate genetic interactions with other components of the cell death pathway. For example, the *Drosophila iap1* gene was identified, due to the ability of the heterozygous mutant to dominantly suppress the GMR-*reaper* eye ablation phenotype (Hay et al., 1995). We investigated potential interactions of *hac-1* with several other pro-apoptotic genes by crossing the l(2)k11502 P insertion strain with transgenic fly strains carrying GMR-*reaper*, GMR-*hid*, GMR-*grim*, and GMR-*dcp-1* (Figure 6) (Grether et al., 1995; Chen et al., 1996; White et al., 1996, Song et al., submitted). Heterozygosity for *hac-1* resulted in only a very mild suppression of the eye phenotypes of GMR-*reaper/hid/grim* (Figures 6A-6F). In contrast, l(2)k11502 robustly suppresses the eye phenotype of GMR-*dcp-1* (Figures 6G and 6H). This indicates that endogenous *hac-1* is rate-limiting for cell killing by DCP-1, but not for apoptosis induced by *reaper, hid* and *grim* under these circumstances. Similar effects were seen upon expression of a truncated form of *dcp-1* lacking the prodomain (GMR-*dcp-1-N,* data not shown). This suggests that *hac-1*

functions at a step either downstream of, or in parallel to the removal of the *dcp-1* prodomain.

Genetic evidence suggests that *reaper* and *grim* induce apoptosis by activating *dcp-1* (Song et al., submitted). For example, when both *reaper* and *dcp-1* are expressed in the eye, they synergize to induce cell death (compare Figures 6E, 6G, and 6I). Expression of full-length *dcp-1* results in a weak phenotype, presumably due to poor activation of the zymogen in these conditions. In contrast, co-expression of *reaper* and *grim* with *dcp-1* leads to an eye ablation phenotype far more severe than the expression of any of these genes alone (Song et al., submitted). We investigated whether this synergy depends on *hac-1*. Since the eye phenotype of GMR-*dcp-1* is suppressed by heterozygosity for *hac-1*, we expected to see a significant modification of the GMR-*reaper* GMR-*dcp-1* eye phenotype. However, this was not observed. Upon introduction of l(2)k11502 into a GMR-*reaper* GMR-*dcp-1* background, no significant changes in eye morphology could be detected (Figures 6I and 6J). We conclude that the activation of *dcp-1* by *reaper* is independent of *hac-1* function (see Figure 8).

hac-1 Is Induced by Irradiation

The mRNA distribution of *hac-1* during embryonic development suggests that the expression of this gene is developmentally regulated. This prompted us to examine whether *hac-1* expression can be induced by other death-inducing stimuli, such as DNA damaging agents. Ionizing radiation induces apoptosis and expression of *reaper* in *Drosophila* embryos (Abrams et al., 1993; White et al., 1994; Nordstrom et al., 1996). Significantly, X-ray and UV irradiation also induces *hac-1* expression (Figure 7). Embryos from wild-type or l(2)k11502/CyO flies were irradiated with X-ray (4000 Rads) or UV (50 mJ/cm^2 or 500mJ/cm^2). In control embryos, expression above basal levels was only detected in the head region (Figures 3A and 3C). In contrast, ectopic *hac-1* expression was detected within 45 minutes after radiation exposure in the ectoderm (Figure 7B), mesoderm in the trunk (Figure 7H), and endoderm (Figure 7J). In embryos carrying the l(2)k11502 P element insertion, the *lacZ* reporter gene of the P element was also induced by X-ray irradiation in an essentially identical pattern (Figure 7D). Interestingly, *hac-1* expression is normally not detected in mesodermal and endodermal cells (Figures 7G and 7I). This strongly suggests that the increased levels of *hac-1* mRNA are the result of *de novo* transcription, and not reduced mRNA turnover. Furthermore, the pattern of ectopic *hac-1* expression corresponded very well with the pattern of TUNEL labeling of embryos subjected to the same radiation treatment (compare Figures 7B, 7D, and 7F),

suggesting that the observed induction is functionally relevant. It appears that embryos between stage 8 and 11 are most sensitive to X-ray irradiation, since there was no detectable induction before stage 8, and induction in embryos after stage 12 was much weaker.

The expression of *hac-1* is also induced in response to UV irradiation (Figure 7 L). Because UV rays do not penetrate far into the embryo, *hac-1* expression was only induced on the exposed side (Figure 7 L). Again the ectopic *hac-1* expression correlated very well with TUNEL labeling of identically treated embryos (Figure 7N). However, there was one significant difference between UV and X-ray induction of *hac-1* expression. While X-ray irradiation failed to induce *hac-1* transcription prior to germ band elongation (stage 8), UV irradiation led to increased *hac-1* RNA as early as the blastoderm stage (data not shown). This suggests the possibility that distinct pathways are used for the induction of *hac-1* expression upon UV and X-ray irradiation.

DISCUSSION

Genetic analyses of programmed cell death in *C. elegans* and *Drosophila* have identified different pathways for controlling the activation of caspases (reviewed in Bergmann et al., 1998b). In the absence of known *Drosophila* homologs of CED-4/APAF-1 and CED-9/BCL-2-like proteins, it may have appeared that insects adopted a distinct mechanism for the activation of a caspase-based death program (Meier and Evan, 1998; Rich et al., 1999). However, the present study indicates that *Drosophila*, like *C. elegans* and mammals, also utilizes CED-4/APAF-1-like molecules to induce caspases and cell death. The same gene has been independently isolated by Kanuka et al. (1999)(this issue of *Molecular Cell*) and Rodriguez et al. (1999). Our initial characterization of *hac-1* also suggests that the activation of caspases can be simultaneously controlled by at least two separate pathways (Figure 8).

hac-1 Is a *Drosophila* Homolog of *Apaf-1* and *ced-4*

There are several reasons to conclude that *hac-1* is a *Drosophila* homolog of *Apaf-1* and *ced-4*. First, the predicted amino acid sequence of *hac-1* shares significant sequence homology with APAF-1, plant disease-resistant proteins, and CED-4. In particular, functionally important domains, including the nucleotide binding motifs, are conserved in HAC-1. Interestingly, HAC-1 is most similar to APAF-1, and it also contains 9 WD repeat elements in its C-terminal region that are not present in CED-4. Since it is thought that the WD repeat region of APAF-1 is the target for its activation by cytochrome c (Li et al., 1997; Hu et al., 1998; Hu et al., 1999), the conservation of this motif in HAC-1 raises the interesting possibility that a similar regulation may occur in *Drosophila*. However, at this point, there are no reports documenting the release of cytochrome c from mitochondria during apoptosis in *Drosophila*, and the functional significance of this domain remains to be investigated (Varkey et al., 1999).

HAC-1 is also functionally similar to APAF-1 and CED-4. Like previously described for APAF-1, extracts from cells expressing HAC-1 stimulate caspase conversion in a dATP-dependent manner *in vitro*. Most importantly, we provide several lines of evidence to show that *hac-1* is required for the normal pattern of apoptosis during *Drosophila* embryogenesis. The elimination of zygotic *hac-1* function leads to a prominent reduction of apoptosis, most noticeably in the head and brain region of the embryo. Furthermore, mutant embryos contain extra neurons, and a significant fraction of them display head involution defects that are strikingly similar to those previously described for

mutations in the pro-apoptotic gene *hid* (Grether et al., 1995). However, unlike *ced-4* mutants, many cells die in the absence of zygotic *hac-1* function. Likewise, mice deficient for APAF-1 retain significant levels of cell death (Cecconi et al., 1998; Yoshida et al., 1998). There are several reasons for why cells may die in the absence of zygotic *hac-1* activity. First, *Drosophila* may have additional homologs of CED-4/APAF-1 that have a partially redundant function. Since the zygotic expression of *hac-1* is restricted to specific regions of the embryo, in particular the head, it is possible that the induction of apoptosis in other regions involves other family members. Second, maternally contributed mRNA and protein may provide an adequate supply for the death of many cells. Consistent with this notion, we find that the phenotypes generated by RNAi injection experiments are stronger than those of the lethal P-element insertion. Because any maternally contributed protein would not have been removed in these experiments, even stronger phenotypes may result upon the complete elimination of all *hac-1* products from the *Drosophila* embryo. Finally, not all cells selected to undergo apoptosis during normal development may utilize APAF-1/CED-4-like proteins. As discussed below, it appears that *reaper, hid* and *grim* activate caspases by a pathway that is distinct from *hac-1*, and it is possible that certain cells rely primarily on one versus the other pathway for the induction of apoptosis (see Figure 8).

Two Distinct Pathways Converge for the Activation of Caspases in *Drosophila*

We observed strong genetic interactions between *hac-1* and the *Drosophila* caspase *dcp-1,* but only weak interactions with *reaper*, *grim* and *hid*. In particular, the dosage of endogenous *hac-1* was rate-limiting for ectopic cell killing in the fly retina induced by *dcp-1*, but had little effect on killing by *reaper, hid* and *grim*. On the other hand, *reaper* and *grim* interact genetically with *dcp-1* (Figure 6; Song et al., submitted). Furthermore, the synergy between *reaper* and *dcp-1* for the induction of cell death in the retina did not depend on the dosage of *hac-1*. These observations indicate that *hac-1* promotes caspase activation through a pathway that is distinct from the one used by *reaper, hid* and *grim*. We propose that the activation of caspases during apoptosis in *Drosophila*, and presumably also in mammals, is controlled by the simultaneous action of two separate pathways (Figure 8). On one hand, REAPER, HID and GRIM can activate caspases by inhibiting the anti-apoptotic activity of *diap1*, one of the *Drosophila* IAPs (Goyal et al., 1999). On the other hand, *hac-1* appears to promote caspase activation by a mechanism that is similar to APAF-1. If one activating pathway is strongly induced, for example by overexpression of REAPER, the activity of components in the other pathway may become less important.

This would explain why a reduction of *hac-1* dosage has little effect on *reaper, grim* and *hid*-induced eye-ablation. In addition, we consider it likely that the activation of *dcp-1* by *hac-1* involves one or more upstream caspases. A number of caspase-like sequences have been identified in *Drosophila* (Fraser and Evan, 1997; Song et al., 1997; Chen et al., 1998; Dorstyn et al., 1999), but the precise order in which they may act within a caspase cascade remains to be determined. Therefore, it is possible that *reaper, grim, hid* and *hac-1* initially activate distinct upstream caspases, but we have not incorporated this potential complexity in our model at this time (Figure 8).

We expect that the activity of HAC-1 may also be controlled in a manner similar to that of APAF-1 and CED-4. Specifically, HAC-1 may be regulated by CED-9/BCL-2-like proteins. The recent identification of BCL-2-like sequences in *Drosophila* (L. Z. and H. S., unpublished results) should allow a critical test of this hypothesis. Significantly, *hac-1* is also regulated at the transcriptional level. Our results demonstrate that zygotic *hac-1* expression is not at all uniform and becomes restricted to specific regions of the embryo. Expression of *hac-1* in the cephalic region is similar to the expression of *hid* and correlates overall well with regions in which major morphogenetic cell death occurs subsequently. Furthermore, *hac-1* transcription is induced by both X-ray and UV-irradiation in a manner similar to what has been previously reported for *reaper*. Therefore, it appears that at least some pro-apoptotic stimuli induce cell death by simultaneously turning on two different pathways for caspase activation (Figure 8).

EXPERIMENTAL PROCEDURES

Fly Stocks

Canton-S or yw67C23 strains were used as wild-type. The P-element insertion line l(2)k11502/CyO was obtained from the Bloomington stock center. To facilitate the identification of homozygous mutant, l(2)k11502 was also balanced with CyO{ftz-*lacZ*}. Transformant fly strains used in this study include: GMR-*reaper* (White et al., 1994), GMR-*grim* (Chen et al., 1996), GMR-*hid* (Grether et al., 1995), GMR-*dcp-1* and GMR-*dcp-1*,GMR-*rpr*/TM3 (Song et al., submitted).

Database Searching

Hidden Markov models of protein motifs were built using the alignment of identified families of cell death related proteins (Grundy et al., 1997). The specificity of these domains for searching databases was tested, and search parameters were trained empirically by searching these domains against the finished *C. elegans* genome sequence (The *C. elegans* sequencing consortium, 1998). For the CED-4/APAF-1 family, three separate models were built corresponding to the three kinase motifs of the nucleotide-binding domain (van der Biezen and Jones, 1998). FASTA files containing *Drosophila* genomic or EST sequence were downloaded from the Berkeley *Drosophila* Genome Project. A simple searching algorithm with O(n) complexity was chosen and implemented in standard C/C++. The program was compiled using Borland C++ Builder and executed on an IBM compatible microcomputer.

hac-1 cDNA Isolation

About 3 kb of *hac-1* sequence was obtained through RT-PCR. Primers for reverse transcription were designed based on the sequence of the cDNA clone GH17715. Superscript II (Gibco) was used at 50°C to minimize potential mRNA secondary structure. A mixture of Taq and Pwo polymerase was used for PCR reactions. RT-PCR products were cloned into Bluescript and sequenced. After comparison with the genomic sequence (AC004335), one clone of the RT-PCR products was chosen for further study because it only has two base pair differences with the genomic sequence, neither of which results in a coding change. The RT-PCR product was fused with cDNA clone GH17715 (Research Genetics) using a native Pvu I restriction site located in the overlapping region. The fused product was termed clone A2177 and used as the full-length cDNA for functional assays. The cDNA sequence corresponding to aa1-565, removing the WD repeats, was amplified using A2177 as template and used as *hac-1*ΔC for functional assays described below.

Double-Stranded RNA Construction:

For *hac-1*, either the sequence from Arg 143 to Arg 332 or the sequence from the start Met to Gly 723 plus 148 bases of 5' UTR was inserted into Bluescript SK+. The *lacZ* gene from pCaSpeR-AUG-β-gal (Thummel et al., 1988) was cloned into the BamH1 and Xba1 sites of pCS2+ (Rupp et al., 1994). dsRNA was synthesized essentially as described before (Kennerdell and Carthew, 1998). dsRNA was injected at concentrations between 0.6 and 1.0 mg/ml.

Injection Protocol

Canton S embryos were collected for 60 min at 25°C, dechorionated in 50% bleach, attached to a microscope slide with double-sided tape (3M Scotch), desiccated and covered with Voltalef 10S oil. Embryos were injected in the syncytial stage, either in the posterior or anterior. The injected volume was approximately 2-3% egg volume or 200-300 pL. Uninjected control embryos were also put on tape under oil, but not desiccated.

Cell Culture and Transfection:

293T cells were seeded in a 6 well plate, with 10^6 cells per well. After overnight culture in a 37°C incubator with 5% CO_2, the cells were transfected with 2 μg of pRK5-*hac-1* or pRK5-*hac-1*ΔC plasmid DNA per well with Lipofectamine(GIBCO/BRL). 48 hours after transfection the cells were collected and washed with ice-cold PBS twice by centrifugation in an Eppendorf tube. Packed cells (100 μl) were resuspended in 200 μl buffer A (Li et al., 1997) and homogenized in a Dounce glass homogenizer on ice. The cytoplasmic fraction was collected by centrifugation of the homogenate at 14,000 rpm for 15 min in an Eppendorf centrifuge. Ten microliters of this supernatant was mixed with 1 μl of ^{35}S-labeled DCP-1 or DRICE in the presence or absence of 1 mM dATP and incubated at 37°C for 1 hour. The DCP-1 and DRICE cleavage was analyzed by a 12% SDS-PAGE.

X-ray and UV Irradiation

Embryos were collected from wild-type or l(2)k11502/CyO flies and aged at 25°C for various times. X-ray irradiation was performed as described (White et al., 1994). A Stratalinker 2400 (Stratagene, La Jolla, CA) with 264nm UV source was used for UV-irradiation. Embryos on egg plates were put inside the Stratalinker and exposed to 50mJ/cm^2 or 500mJ/cm^2 irradiation. After UV-treatment, embryos were allowed to recover

for either 45 minutes (for *in situ* hybridization) or for 75 mins (for β-gal expression and TUNEL labeling) at 25°C, followed by 4% paraformaldehyde fixation.

Histology

In situ hybridization and immunocytochemistry were performed essentially as described before (Zhou et al., 1995; Zhou et al., 1997). The 9F8A9 anti-ELAV MAb (O'Neill et al., 1994)was obtained from the Developmental Studies Hybridoma Bank maintained by The University of Iowa, Dept. of Biological Science. In order to visualize the β-Gal expression pattern in l(2)k11502 embryos, monoclonal anti-β-Gal Ab was diluted 1:20,000. When the antibody was used at 1:1000 (Zhou et al., 1995), the embryo carrying the P insertion l(2)k11502 turned brown instantly and a specific pattern could not be observed, suggesting a high basal level of β-Gal activity in most, if not all, cells. Acridine orange and TUNEL labeling was performed as previously described (Abrams et al., 1993; White et al., 1994).

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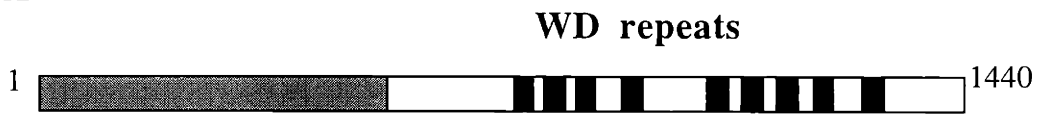
Figure 1.

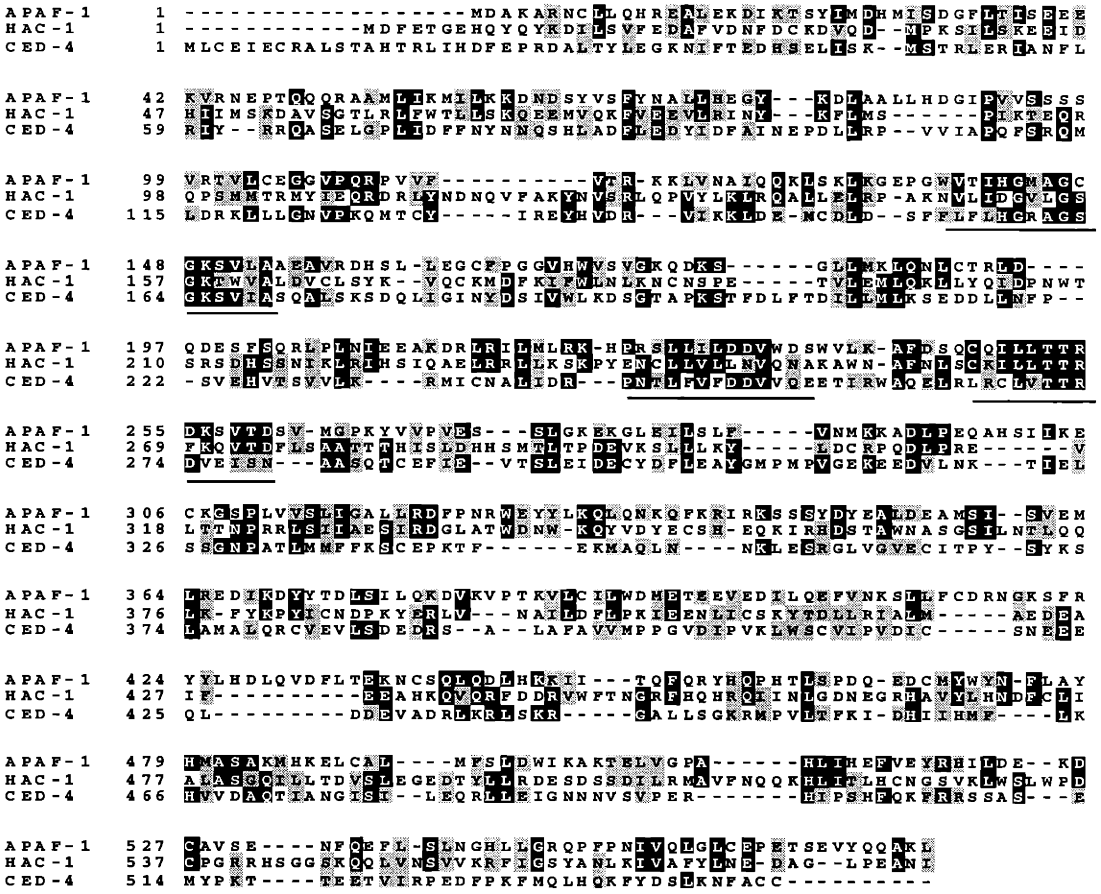
hac-1 Encodes a Homolog of *ced-4/Apaf-1*

(A) is a schematic representation showing the domain structure of the HAC-1 protein. (B) shows the sequence alignment of the N-termini of CED-4, APAF-1, and HAC-1. Identical and conserved residues are blocked as black or shadow, respectively. Of all the protein sequence in the database, HAC-1 is most similar to human APAF-1, and HAC-1 is less similar to CED-4, where the similarity is mainly limited to the three kinase motifs of the nucleotide binding domain (B, underlined). In (C), the location of the P insertion l(2)k11502 relative to the genomic sequence is indicated by the arrow. Only one sequence motif (underlined) consistent with the TATA box consensus (TATAA/TA) is present between the P insertion site and the first ATG of the ORF. The sequence corresponding to the 5' end of the longest RT-PCR product is shown in bold.

A


WD repeats
1
1440
CED-4/APAF-1 homo-
logous region

B



C

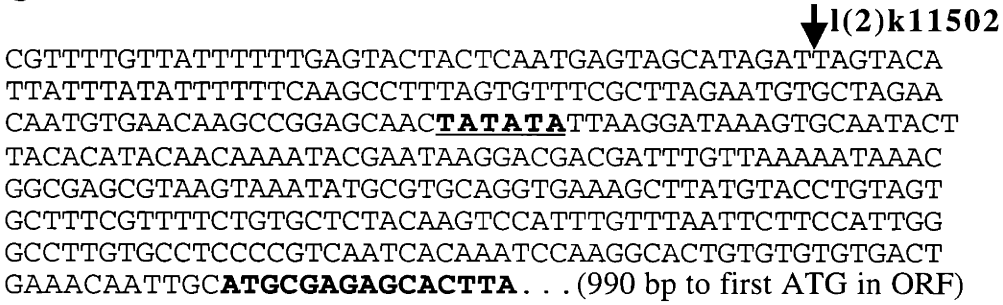

l(2)k11502
CGTTTTGTTATTTTTTGAGTACTACTCAATGAGTAGCATAGATTAGTACA
TTATTTATATTTTTTCAAGCCTTTAGTGTTTCGCTTAGAATGTGCTAGAA
CAATGTGAACAAGCCGGAGCAACTATATAATTAAGGATAAAGTGCAATACT
TACACATACAACAAAATACGAATAAGGACGACGATTTGTTAAAAATAAAC
GGCGAGCGTAAGTAAATATGCGTGCAGGTGAAAGCTTATGTACCTGTAGT
GCTTTCGTTTTCTGTGCTCTACAAGTCCATTTGTTTAATTCTTCCATTGG
GCCTTGTGCCTCCCCGTCAATCACAAATCCAAGGCACTGTGTGTGTGACT
GAAACAATTGCATGCGAGAGCACTTA. . . (990 bp to first ATG in ORF)

Figure 2.

HAC-1 Induces *Drosophila* Caspase Activation *in vitro*

The full length HAC-1 protein and a C-terminal truncated version of HAC-1, derived from 293T cells, activated DCP-1 and DRICE in a dATP-dependent manner. 293T cells were transiently transfected with expression vectors to overexpress the full length or a C-terminal truncated HAC-1. The cytosolic fractions from these transfected cells activated DCP-1 and DRICE in the presence of dATP. Lane 1, 1 µl ^{35}S-labeled DRICE in 10 µl of buffer A. Lane 2, 1 µl ^{35}S-labeled DRICE mixed with 10 µl of cytosol of untransfected 293T cells, in the presence of 1 mM dATP, as control. Lane 3, 1 µl ^{35}S-labeled DRICE mixed with 10 ml of cytosol of 293T cells transfected with pRK5-*hac-1* in the presence of 1 mM dATP. Lane 4, 1 µl ^{35}S-labeled DRICE mixed with 10 µl of cytosol of 293T cells transfected with pRK5-*hac-1ΔC* in the presence of 1 mM dATP. Lane 5, same as lane 3 except without dATP added. Lane 6, same as lane 4 except without dATP added. DCP-1 results are essentially identical (data not shown).

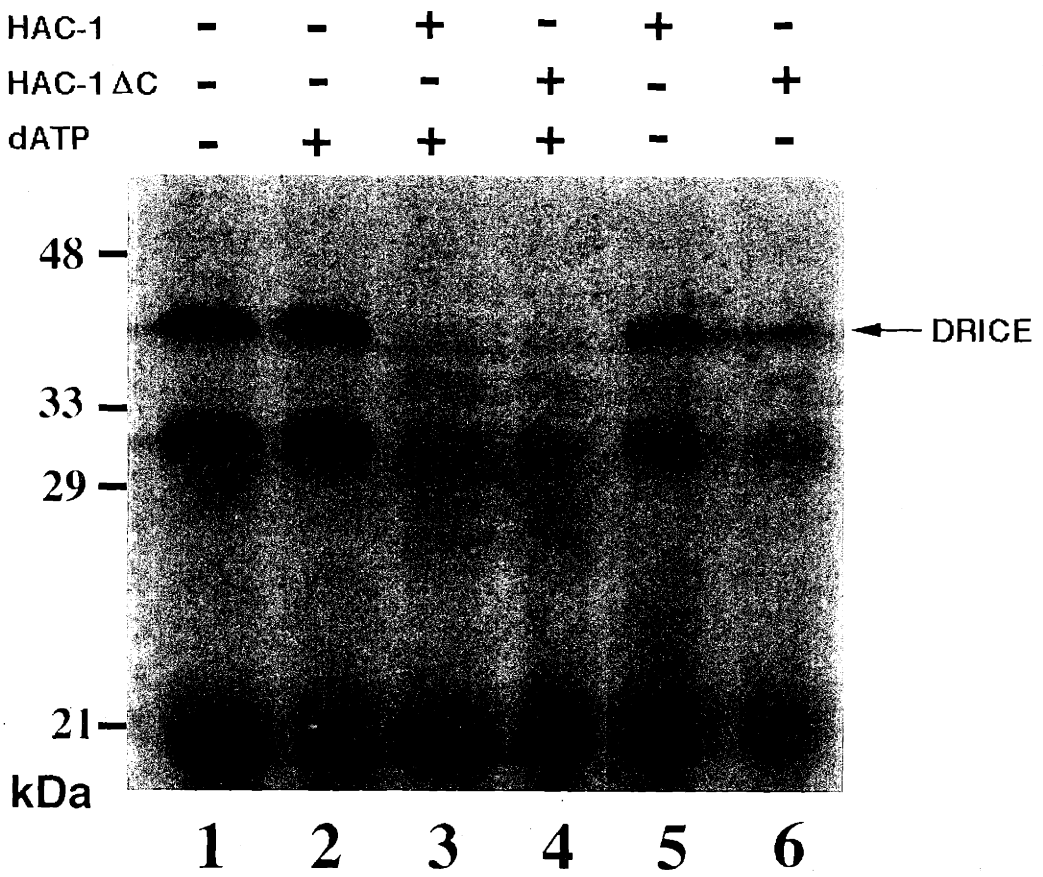

HAC-1 - - + - + -
HAC-1 ΔC - - - + - +
dATP - + + + - -
48
33
29
21
kDa
DRICE
1 2 3 4 5 6

Figure 3.

The Transcription of *hac-1* mRNA Is Regulated during Development

(A-C) show the distribution of *hac-1* mRNA in wild-type embryos (A, stage 4; B, stage 7; C, stage 10-11). The mRNA signal in the procephalic region (C) is absent in embryos homozygous for l(2)k11502 (D). (E and F) compare the distribution of *hac-1* (E) and *hid* (F) mRNA in the procephalic region of stage 11 embryos. The expression patterns partially overlap. However, *hid* expression is restricted to clusters of cells in the ectoderm, *hac-1* is expressed in the ectoderm as well as the mesoderm, which is a source of macrophages. (G-I) show anti-β-gal staining of l(2)k11502/CyO embryos, the β-gal expression pattern largely mimics the mRNA distribution (G, stage 10; H, stage 11; I, stage 12/1). Although a low level of β-gal immunoreactivity is present in most, if not all cells, cells in the procephalic region (G and H), and later in the head and macrophages (I) express much higher levels.

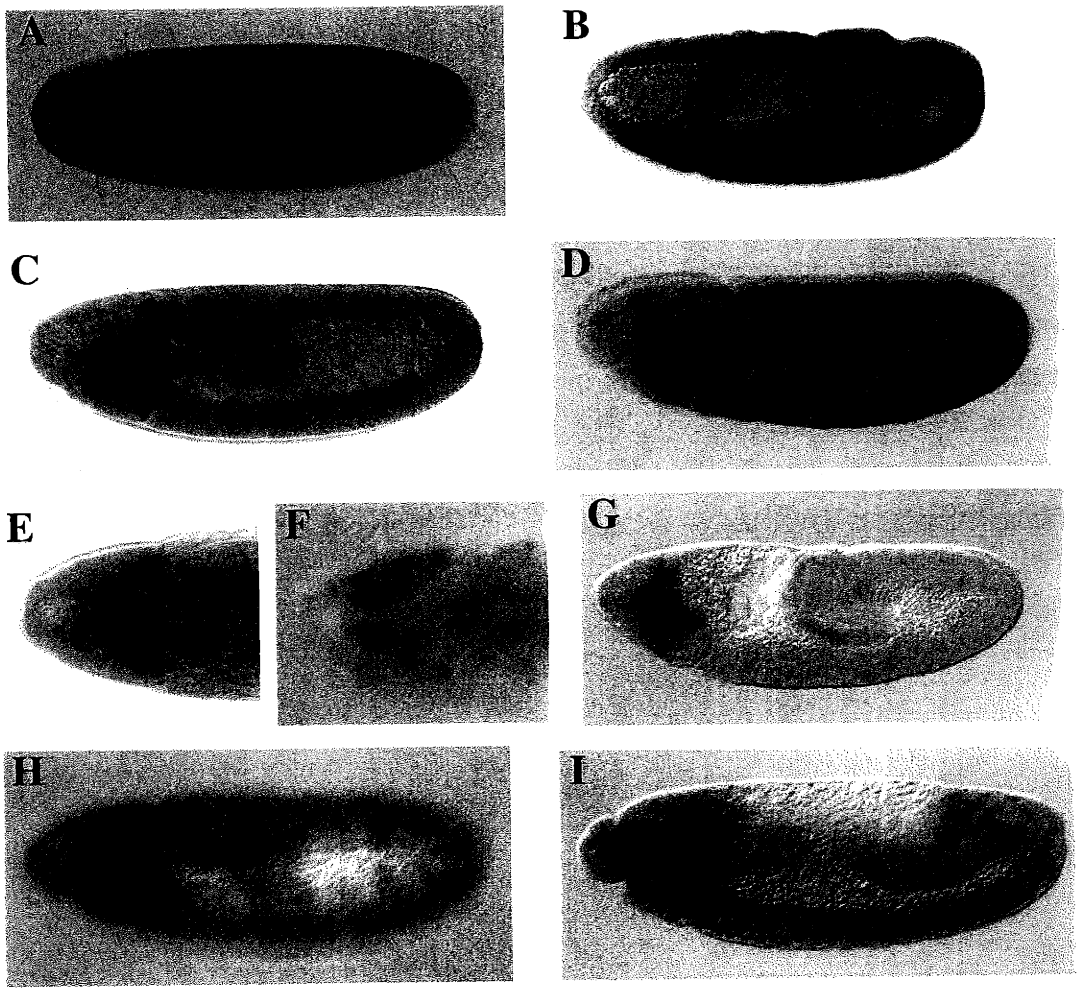

A
B
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Figure 4.

Apoptosis Is Reduced in *hac-1* Mutant Embryos

A-C depict TUNEL labeling of apoptotic cells in wild type (A, stage 12/1) and l(2)k11502 mutant (B and C, stage 12/1) embryos. Apoptotic cells first appear in the head as well as the germ band at later stage 10 to early stage 11 (White et al., 1994; Franc et al., 1999). The apoptotic cells in the procephalic region appears to be quickly phagocytosed by hemocytes/macrophages migrating away from the head mesoderm, as most of the TUNEL labeling is inside phagosomes within the macrophages (arrow heads) of both WT (A) and l(2)k11502 (B and C) embryos. However, fewer TUNEL positive cells are seen in the head region of l(2)k11502 mutant embryos (two representative mutant embryos are shown in (B and C). (D-F) depict acridine orange staining of stage 12 wild-type embryos. Injection of ds *hac-1* RNA, corresponding to Arg 143-Arg 332, posteriorly, significantly reduces the number of acridine orange-positive cells in the posterior third of the embryo (E) when compared to uninjected embryos (D). No reduction in acridine orange staining is seen in ds *lacZ* RNA posteriorly injected control embryos (F). Ten to twenty percent of ds *hac-1* RNA injected embryos showed a reduction of acridine orange staining to this extent seen in (E).

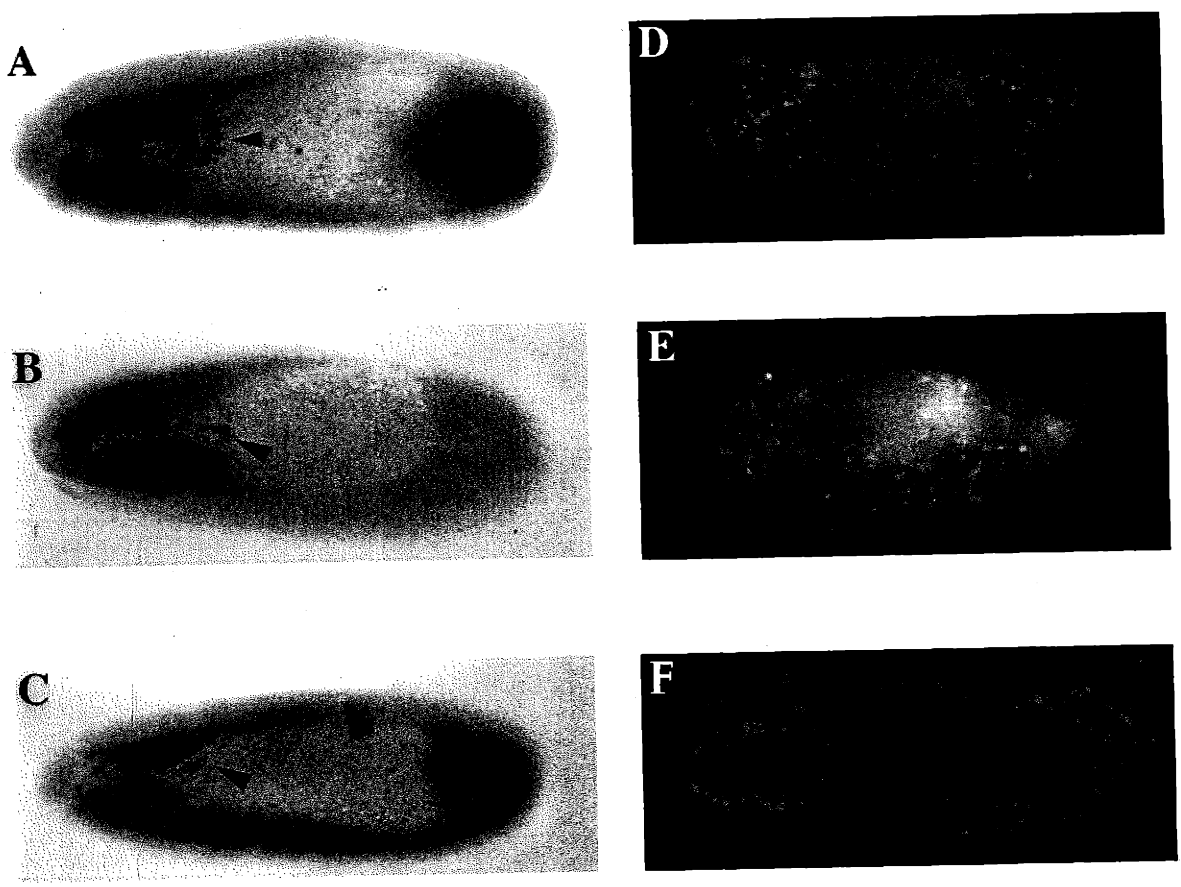
A
B
C
D
E
F

Figure 5.

Extra Neurons Are Present in *hac-1* Mutant Embryos

Elimination of zygotic *hac-1* function leads to increased size and cell number in the embryonic nervous system. An anti-ELAV antibody was used to stain neurons of wild-type (A and D), *hid* mutant (B and E) and l(2)k11502 (C and F) embryos. (A-C) are sagittal views to show the involution of the head; note the shape of the head segments (arrows) as well as the space between the head and the epidermis (A, asterisk), which is missing in *hid* and l(2)k11502 mutants. (D-F) are focused on the brain to demonstrate enlarged size and extra ELAV-positive cells in the *hid* and l(2)k11502 mutant embryos. The focal plane that has the greatest number of neurons was chosen in wild-type as well as mutant embryos.

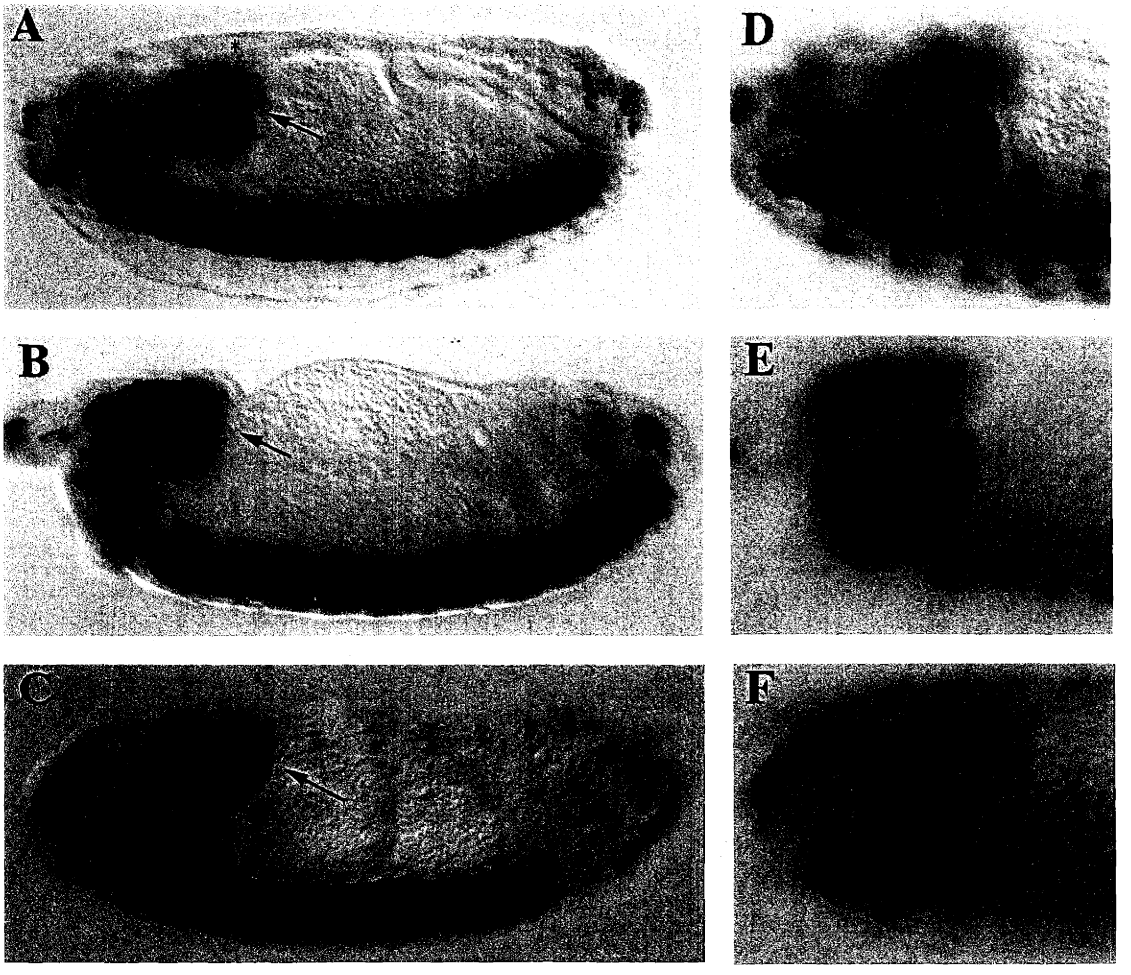
A
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Figure 6.

hac-1 and *reaper, hid*, and *grim* Can Independently Promote Caspase Activation

Heterozygous l(2)k11502 has little effect on the eye phenotype of transgenic flies carrying one copy of GMR-*hid* (A and B), GMR-*grim* (C and D) and GMR-*rpr* (E and F). In contrast, it dominantly modifies the eye phenotype of transgenic flies carrying two copies of GMR-*dcp-1* (G and H). A strong synergism in cell ablation is observed when both *rpr* and *dcp-1* are expressed in the eye (compare E, G and I) (Song et al., submitted). This activation of DCP-1 by RPR is independent of HAC-1 dosage since it is not modified by l(2)k11502 (J).

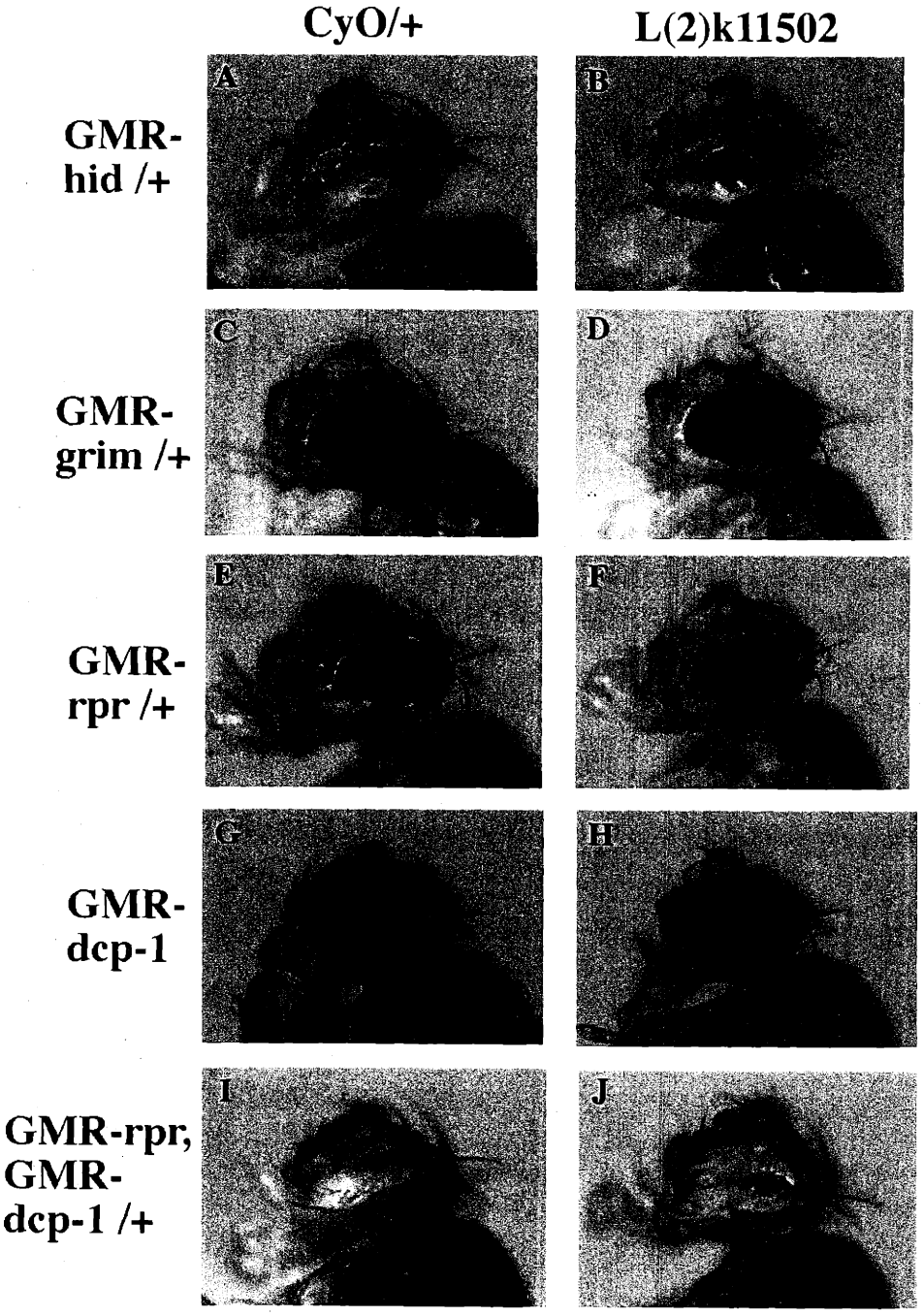
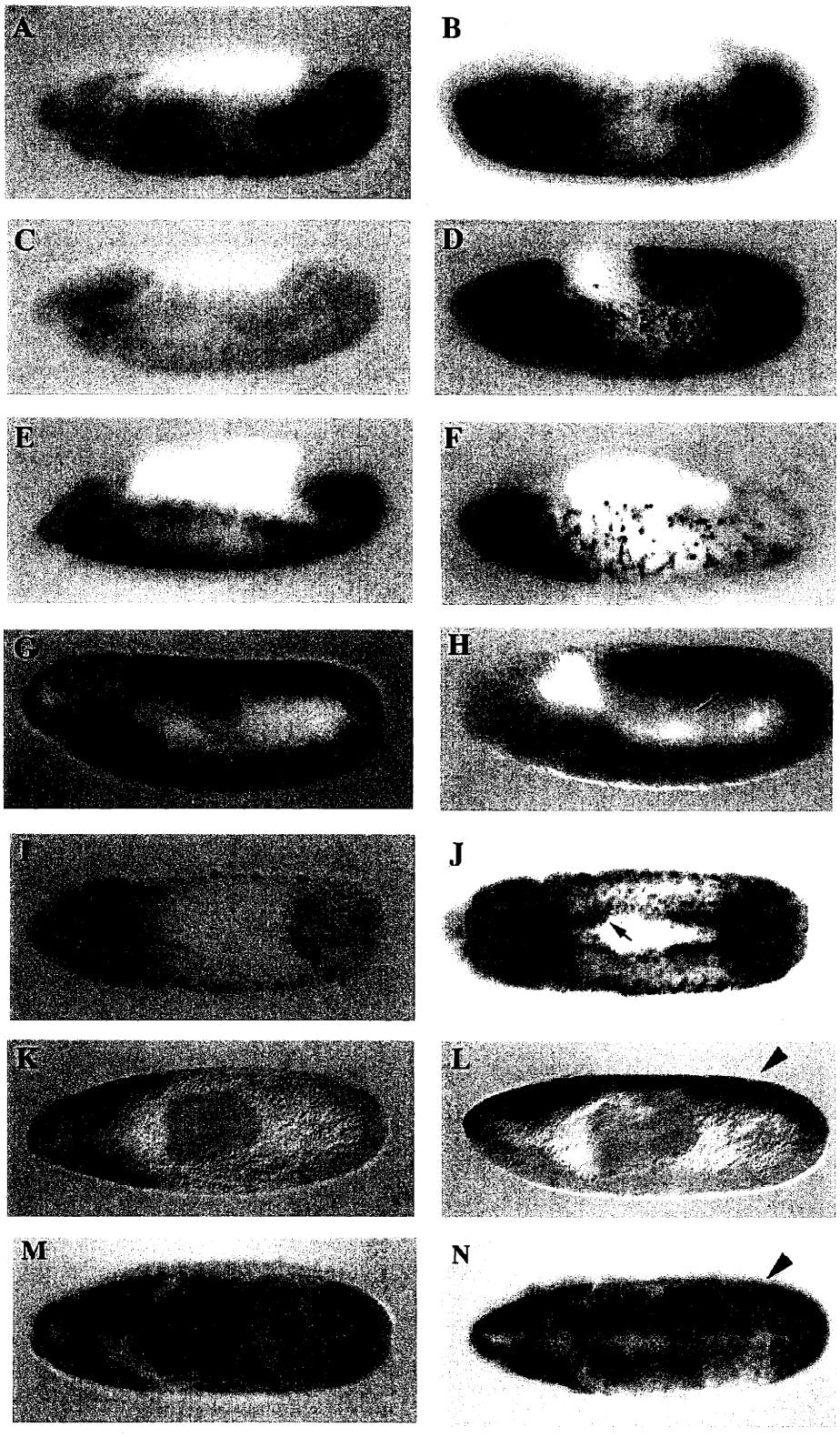

CyO/+
L(2)k11502
GMR-hid /+
GMR-grim /+
GMR-rpr /+
GMR-dcp-1
GMR-rpr, GMR-dcp-1 /+
A
B
C
D
E
F
G
H
I
J

Figure 7.

hac-1 Is Induced by X-rays and UV Irradiation

Embryos on the left side are nonirradiated control embryos that underwent the same histological analysis as their irradiated counterparts in the right panels. (A-F) depict stage 12 embryos. Many epidermal cells and neuroblasts express high levels of *hac-1* mRNA, 45 minutes after X-ray treatment (A and B; *in situ* hybridization). This is mimicked by the β-gal expression pattern in l(2)k11502/CyO embryos treated with X-ray (C and D; anti-β-gal staining). (F) shows TUNEL labeling of embryos treated with X-rays. X-ray treatment also induces ectopic *hac-1* expression in mesoderm of the trunk (arrow in (H), *in situ* hybridization of stage 10 embryo) and endoderm cells (arrow in J, β-gal staining of l(2)k11502/CyO stage 13 embryo), which normally have no detectable *hac-1* expression (G and I). UV irradiation induced *hac-1* expression on the exposed side (arrow-heads) of the embryo, which is paralleled by the *lacZ* expression from the P insertion (L, stage 9 embryo). The UV-induced *hac-1* expression pattern corresponds well with the pattern of TUNEL labeling in embryos treated identically (N, stage 9).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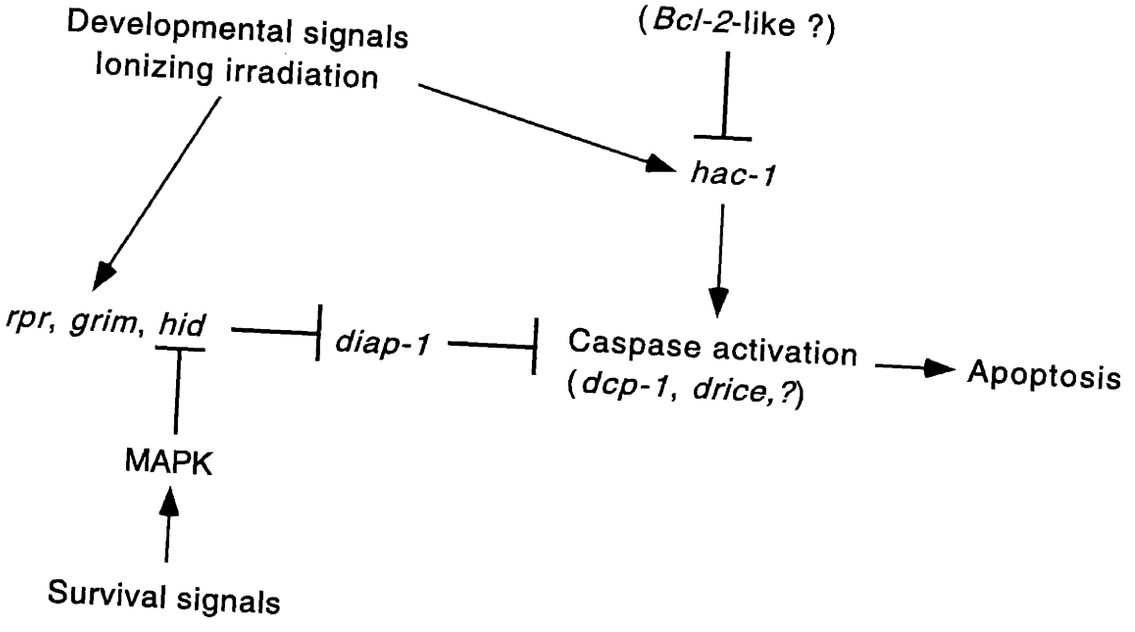
M
N

Figure 8.

Model for the Control of Caspase Activation in *Drosophila*.

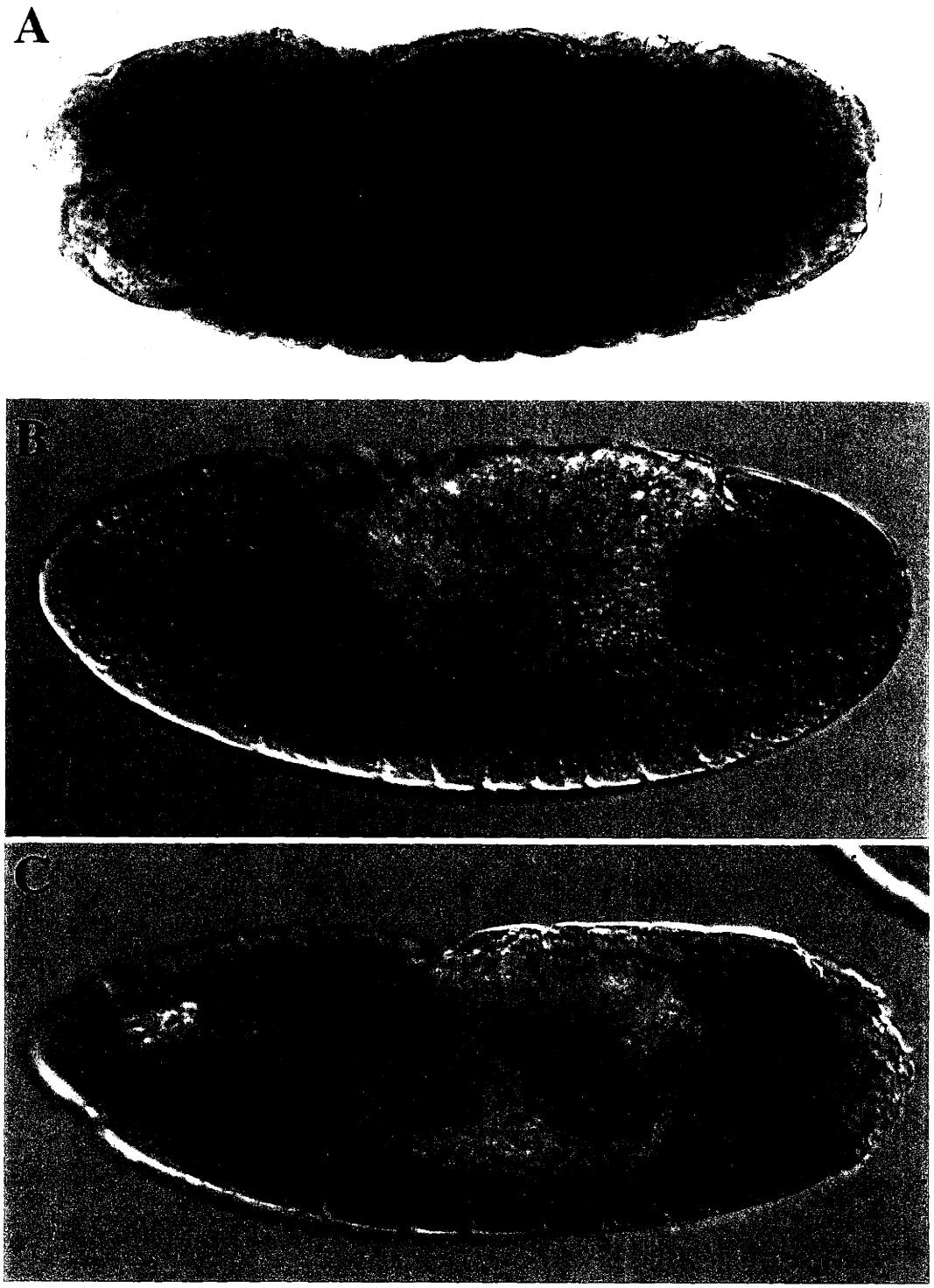
Genetic studies of cell death in *Drosophila* have initially defined *reaper, hid* and *grim* as key activators of a caspase-mediated death program. The products of these genes appear to kill by binding to and inhibiting that anti-apoptotic activity of the DIAP1 protein (Goyal et al., 1999). The *hac-1* gene, identified based on its homology with APAF-1 and CED-4, appears to act in a separate pathway to stimulate caspase activation and cell death. At least under some circumstances, both pathways are simultaneously used to induce cell death in *Drosophila*. The expression of *reaper, grim, hid* and *hac-1* is regulated at the transcriptional level during normal development, and transcription of both *reaper* and *hac-1* is rapidly induced by ionizing radiation. However, since *Drosophila* contains multiple caspases that may act in a proteolytic cascade, it is possible that *reaper, grim, hid* and *hac-1* activate initially distinct caspases upstream of presumptive effector caspases, such as *dcp-1*. This would explain why genetic interactions among *reaper, grim, hid*, and *hac-1* are very weak.


Developmental signals
Ionizing irradiation
(Bcl-2-like ?)
hac-1
rpr, grim, hid
diap-1
Caspase activation
(dcp-1, drice, ?)
Apoptosis
MAPK
Survival signals

Appended Figure.

Expression of β-galactosidase Is Reduced in *lacZ* RNAi Embryos

Injecting ds *lacZ* mRNA into the posterior of embryos expressing a *lacZ* transgene results in a significant reduction of β-galactosidase activity in the injected part of the embryo. (A) shows an injected control embryo and (B and C) depict two representative *lacZ* RNAi embryos. These embryos are at developmental stage 11 to 12, approximately the same age as the *hac-1* RNAi embryos shown in Figure 4.


A
B
C

Chapter 3

Loss of Zygotic *diap1* Function Results in Severe Developmental Abnormalities at the Start of Zygotic Transcription and in the Cleavage of Physiological Caspase Substrates

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This chapter has been submitted for potential publication in Developmental Biology.

SUMMARY

diap1 is a key regulator of apoptosis in *Drosophila*. Loss of *diap1* function results in caspase activation and the induction of apoptosis during early embryonic development. We show that *diap1* loss-of-function embryos begin to develop abnormally shortly after entering mitotic cycle fourteen and that the onset of abnormal development occurs over a very narrow eight-minute time period. At this time, cells in the mutant embryos undergo a series of morphological changes that are consistent with cells undergoing apoptosis: the DNA condenses, nuclear lamin staining becomes diffuse, and filamentous actin becomes disorganized. In addition, the cells round up and fail to adhere well to each other. We show that two caspase substrates, nuclear Lamin Dm_0 and Armadillo, are cleaved and that cleavage of these two proteins can explain some of these observed morphological changes, linking caspase activation in a developing organism with the morphological changes that define apoptosis.

INTRODUCTION

Programmed cell death is an important process for normal metazoan development and functions in a number of ways in development, including to help sculpt or remove unnecessary structures, control cell number and eliminate abnormal or harmful cells (reviewed in Jacobson et al., 1997). The genes comprising the core programmed cell death pathway were identified using the nematode *Caenorhabditis elegans* (reviewed in Metzstein et al., 1998; Horvitz, 1999) and have since been found to be conserved in many species, including *Drosophila* and mammals (reviewed in Bergmann et al., 1998; Bangs and White, 2000). In *Drosophila*, normally-occurring developmental apoptosis requires at least three gene products, Reaper, Head Involution Defective (Hid), and Grim, all encoded by the 75C1,2 region of the third chromosome (White et al., 1994; Grether et al., 1995; Chen et al., 1996). Any one of these three genes is sufficient to induce caspase-dependent cell death when overexpressed in the developing *Drosophila* eye as well as in insect and mammalian cell culture (Grether et al., 1995; Hay et al., 1995; Chen et al., 1996; Pronk et al., 1996; White et al., 1996; Vucic et al., 1997; McCarthy and Dixit, 1998; Vucic et al., 1998; Haining et al., 1999).

Drosophila apoptosis is also negatively regulated by a conserved class of proteins, the Inhibitors of Apoptosis (IAPs), that were first identified in baculovirus (Crook et al., 1993; Birnbaum et al., 1994). IAPs contain either one, two, or three Baculovirus IAP Repeat (BIR) domains and often contain a carboxy-terminal RING domain (reviewed in Deveraux and Reed, 1999). Not all BIR containing proteins (BIRPs) have been shown to be inhibitors of apoptosis. In fact, yeast and *C. elegans* BIRPs seem to mainly function in cell cycle regulation and cytokinesis (Fraser et al., 1999; Uren et al., 1999; Li et al., 2000; Speliotes et al., 2000). *Drosophila* contains four BIRPs, three of which have so far been shown to suppress apoptosis (Hay et al., 1995; Duckett et al., 1996; Uren et al., 1996; Jones et al., 2000; Rubin et al., 2000; Wenzel et al., 2000). Of these proteins, *Drosophila* IAP 1 (Diap1), which is encoded by the *thread* locus (Lindsley and Zimm, 1992), has been most extensively characterized. Loss-of-function mutations in *diap1* are early embryonic lethal with virtually all cells becoming TUNEL positive at the time of gastrulation, showing that the DNA has become degraded in a manner consistent with apoptosis taking place (Hay et al., 1995; Wang et al., 1999; Goyal et al., 2000; Lisi et al., 2000). Interestingly, the onset of apoptosis in *diap1* loss-of-function embryos occurs approximately two hours before the first developmental cell deaths are normally observed in the wild-type *Drosophila* embryo (Abrams et al., 1993; Wang et al., 1999; Goyal et al., 2000; Lisi et al., 2000). Furthermore, the failure of attempts to make mitotic and germline clones of a loss-of-

function *thread/diap1* allele suggests that the loss of *diap1* function is essential for cell survival (Hay et al., 1995). Diap1 is also able to suppress the induction of apoptosis by overexpression of Reaper, Hid, or Grim in the developing *Drosophila* eye (Hay et al., 1995; Lisi et al., 2000). In addition, Diap1 has been shown to be anti-apoptotic in insect cell culture systems (Vucic et al., 1997; Vucic et al., 1998). In these studies, Diap1 was found to bind to Reaper, Hid, or Grim and the binding was shown to require the BIR domains of Diap1 and the N-terminal region of Reaper, Hid, or Grim that is conserved among these three otherwise divergent proteins (Vucic et al., 1997; Vucic et al., 1998).

Besides binding to Reaper, Hid, or Grim, IAPs have also been shown to bind and inhibit caspases (reviewed in Deveraux and Reed, 1999). Diap1 can bind the *Drosophila* caspases Dcp-1 and drICE and thereby protect from cell death (Kaiser et al., 1998; Hawkins et al., 1999; Wang et al., 1999). The identification of loss-of-function and gain-of-function mutants in *diap1* and an analysis of their binding affinities for Reaper and Hid has provided further support for a model where Reaper, Hid, and Grim bind the BIR domains of Diap1 and thereby prevent it from binding to caspases and blocking their activation (Goyal et al., 2000; Lisi et al., 2000). Consistent with this model is data showing that the *diap1* loss-of-function phenotype is not affected in embryos also lacking *reaper, hid,* and *grim*, placing *diap1* downstream of these three genes in the programmed cell death pathway (Wang et al., 1999; Goyal et al., 2000).

Caspases were shown to be active in extracts derived from *diap1* loss-of-function embryos (Wang et al., 1999). In this case, a fluorometric caspase substrate was readily cleaved; however, this indicator substrate is not present *in vivo* and is therefore of questionable physiological relevance. We now report that two caspase substrates normally present in cells, nuclear lamins and Armadillo, are cleaved in embryos that are deficient for *diap1*. Cleavage of these substrates explains some of the morphological changes observed in *diap1* loss-of-function embryos and therefore offers a direct correlation between caspase activation and morphological events seen during apoptosis. In addition, we report that *diap1* loss-of-function embryos develop relatively normally up to the point where zygotic transcription begins and that the onset of developmental abnormalities occurs over a far more narrow time window than previously thought. Together, these data further our understanding of the importance of *diap1* in *Drosophila* development and underline the key role that this gene plays in the regulation of programmed cell death, while also aiding in our understanding of caspase-mediated cleavage of specific substrates and their effect on the characteristic changes observed during programmed cell death.

MATERIALS AND METHODS

Fly Stocks and Manipulation

Canton-S was used as wild-type. The *diap1* loss-of-function alleles analyzed were th^5 (Hay et al., 1995) and $th^{11\text{-}3e}$ (Goyal et al., 2000). Fly stocks were maintained at 25°C on standard cornmeal and molasses medium. All staging of embryos is according to Campos-Ortega and Hartenstein (Campos-Ortega and Hartenstein, 1997). RNA injections were carried out as previously described (Zhou et al., 1999).

Immunohistochemistry and TUNEL Labeling

TUNEL labeling was carried out as previously described (White et al., 1994). Phalloidin and Bisbenzimide staining was performed as follows: Injected embryos and control uninjected embryos were removed from the double-sided tape in heptane (Sigma), fixed in 4% paraformaldehyde (Polysciences)/ heptane (1:1) for at least 30 minutes. They were rinsed with fresh heptane, dropped onto double-sided tape and covered with PBS+0.2% Tween-20 (PBT). The embryos were then hand-peeled with a drawn microcapillary tube to remove the vitelline membrane. Rhodamine-conjugated phalloidin (Molecular Probes R-415) was dissolved in methanol according to the manufacturer's directions, 20 µls were added to the embryos in 400 µls of PBT and these were incubated in the dark for 20 minutes. The embryos were rinsed twice with PBT and then incubated in 0.5 mls of 1µg/ml Bisbenzimide in PBT (Riedel-de Haën AG, H33258) for 4 minutes in the dark, rinsed twice in PBT for 10 minutes each and mounted in 1:1 glycerol/PBS for examination on the confocal microscope. Lamin antibody stainings were performed as previously described (Zhou et al., 1995). Anti-Lamin Dm_0 antibody (mAb 101, Paul Fisher) was diluted 1:1 in PBT supplemented with 5% Normal Horse Serum (Vector). For detection, the secondary antibody used was fluorescein isothiocyanate-conjugated horse anti-mouse (Vector) at a 1:100 dilution. Anti-phoshorylated Histone H3 (anti-PH3) staining was performed as follows: Injected embryos were removed from the tape, fixed and peeled as above. Embryos were blocked for 1 hour at room temperature in wash buffer (PBS containing 1% BSA, 0.3% Triton X-100 and 10% Normal Goat Serum (GibcoBRL)). The embryos were then incubated with the anti-PH3 antibody (Upstate Biotechnology #06-570) at room temperature overnight (1:500 in wash buffer), rinsed twice, washed four times for 15 minutes in wash buffer, then incubated for 3 hours at room temperature in Cy3-conjugated goat anti-rabbit secondary antibody (Jackson) diluted 1:100 in wash buffer.

The embryos were rinsed and washed as before, rinsed in PBS, incubated in Bisbenzimide as above and mounted in 1:1 glycerol/PBS. Stained embryos were observed on a Zeiss LSM confocal microscope.

Western Analysis

Injected embryos were removed from the double-sided tape as above, rinsed in heptane and squashed with a pestle inside a 0.5 ml Eppendorf tube in standard SDS sample buffer, boiled for one minute, put on ice and loaded onto standard SDS-PAGE (Sambrook et al., 1989). An amount representing ten embryos was loaded in each lane. The proteins were transferred onto Immobilon-P membranes (Millipore), blocked for at least one hour at room temperature in 5% nonfat dry milk in PBS and incubated overnight at 4°C with the following primary antibodies: anti-Armadillo (1:100; DSHB), anti-β-Tubulin (1:50; DSHB) and anti-Lamin Dm_0 (1:20; Paul Fisher), all diluted in Iscove's Modified Dulbecco's Medium (GibcoBRL) with 5% Normal Goat Serum (GibcoBRL). After washing five times in PBS+0.2% Tween-20, the membranes were incubated for one hour in block containing the goat anti-mouse HRP secondary antibody (1:10,000; Jackson). Detection was done using ECL reagents (Amersham). Kodak AR film was exposed for the following times: anti-β-Tubulin 5 minutes, anti-Lamin Dm_0 5 minutes, anti-Armadillo 4 hours.

ds RNA Construction

The entire 2 kb *diap1* cDNA containing the ORF and part of the 5' and 3' untranslated regions in pNB40 (obtained from Bruce Hay) was used to generate the ds *diap1* RNA. The sense and anti-sense strands were transcribed with Sp6 (Stratagene) and T7 (GibcoBRL) RNA polymerase rèspectively. The ds RNA was prepared as previously described (Kennerdell and Carthew, 1998). ds RNA was injected at a concentration of approximately 1.0 mg/ml.

RESULTS

The *diap1* Loss-of-Function Phenotype

The loss of zygotic *diap1* function in *Drosophila* results in early embryonic lethality (Hay et al., 1995). This earlier observation has been recently confirmed and the lethality has been shown to be due to widespread programmed cell death at the time of gastrulation (Wang et al., 1999; Goyal et al., 2000; Lisi et al., 2000). Consistent with most cells undergoing apoptosis at this stage is evidence of caspase activation from experiments where embryo extracts have been shown to cleave fluorometric caspase substrates (Wang et al., 1999). The exact timepoint at which developmental abnormalities begin had not been determined. The embryos appeared to develop normally up to the point of early germband extension, but they fail to normally progress to later morphological stages. The cells begin to round up and no longer adhere well to each other, suggesting that cytoskeletal and cell-cell adhesion changes are taking place. Eventually, a large bubble-like structure forms and moves to the surface of the embryo (Wang et al., 1999; Goyal et al., 2000) (Figure 1A). This bubble contains yolk granules, but lacks cells. Some variability was reported in the onset of developmental abnormalities based on the last recognizable normal developmental stage that the embryo reached (Wang et al., 1999). We set out to define more narrowly the time window in which development of *diap1* loss-of-function embryos begins to deviate from wild-type.

The *diap1* loss-of-function phenotype appears very early in embryonic development, shortly after the onset of zygotic transcription, making it difficult to mark mutant embryos. Further, *diap1* loss-of-function alleles are homozygous lethal, so the stocks have to be maintained as heterozygotes and only one quarter of the embryos from a cross would be homozygous mutant. To surmount this difficulty in knowing the genotype of the embryo as well as to determine if the maternal contribution of *diap1* mRNA affects the loss-of-function phenotype, we phenocopied the *diap1* loss-of-function phenotype by using RNA interference (RNAi). RNAi has been shown to be an effective technique for studying the mutant phenotype of genes in *Drosophila*, including genes involved in programmed cell death (Kennerdell and Carthew, 1998; Misquitta and Paterson, 1999; Zhou et al., 1999; Brachmann et al., 2000; Colussi et al., 2000). *diap1* RNAi embryos develop relatively normally up to early gastrulation and then proceed to display the same morphological abnormalities as *diap1* loss-of-function embryos, including the formation of the bubble-like structure, and virtually all cells becoming TUNEL positive (Figure 1). In addition, the timing of these changes is identical to that observed in *diap1* loss-of-function mutants,

showing that the *diap1* RNAi accurately reflects the *diap1* loss-of-function phenotype. The inability to see a difference between *diap1* RNAi embryos and *diap1* loss-of-function embryos suggests that the maternal contribution of *diap1* mRNA does not significantly affect the timing of the onset of abnormalities or the resultant phenotype. Utilizing the RNAi technique, the majority of injected embryos (64%, n=280) display the *diap1* loss-of-function phenotype.

In order to visualize the entry of cells into mitosis, we employed an antibody against phosphorylated histone H3 (anti-PH3) (Hendzel et al., 1997). Anti-PH3 antibody analysis shows that *diap1* RNAi embryos proceed through the first thirteen nuclear divisions with no major abnormalities and enter cell cycle fourteen, however, they usually begin to display abnormalities after mitotic domain four enters into fourteenth mitotic cycle and before mitotic domain eleven enters into mitosis fourteen (Figure 2). Some cells continue to undergo mitosis in four to five hour old *diap1* RNAi embryos, but by that point the embryo has become morphologically very abnormal and many cells already have rounded up and their nuclei look apoptotic based on morphology (Figure 3). Since mitotic domain four enters mitosis fourteen approximately 204 minutes after egg deposition at 25°C and domain eleven after approximately 212 minutes (early stage 8) (Foe et al., 1993), the timepoint at which the first abnormalities can be seen can be defined as an eight minute window.

One explanation for this precise transition to abnormal development is that the maternal contribution of Diap1 protein is sufficient to carry the embryo through early development to the stage where zygotic transcription begins. To address the effect of the maternal contribution, we injected ds *diap1* RNA into embryos laid by mothers heterozygous for *diap1*, which should contain significantly less maternally supplied *diap1* mRNA. A similar percentage of these embryos entered mitosis 14 (89%, n=18) as do wild-type embryos injected with ds *diap1* RNA (83%, n=24) or uninjected wild-type embryos (86%, n=35), suggesting that the maternal contribution of *diap1* mRNA does not affect the timepoint at which developmental abnormalities first become apparent. Alternatively, an inducer of apoptosis could be transcribed at this time and *diap1* function is required to block programmed cell death. Consistent with the latter explanation is the zygotic transcription of several caspases, including the initiator caspases *dcp-2/dredd* and *dronc* shortly after gastrulation (Chen et al., 1998; Dorstyn et al., 1999).

Filamentous Actin and Nuclear Lamins Become Disorganized in *diap1* Loss-of-Function Embryos

The morphology of the cells in *diap1* loss-of-function embryos changes dramatically at the time when normal development ceases. To define further the subcellular changes taking place in *diap1* loss-of-function embryos, we looked at filamentous actin and nuclear lamin organization. Filamentous actin is normally localized around the inner surface of the plasma membrane. In *diap1* loss-of-function mutants, actin becomes very disorganized approximately four to five hours into development (stage 9 to 10). At this point, it is no longer evenly distributed along the periphery of the cell, but rather clumps of actin are seen with an asymmetrical distribution (Figure 4). This observation suggests that, while actin appears to not be degraded, it is no longer being anchored to the rest of the cytoskeleton. In addition, blebbing of the plasma membrane can be seen, with many of these pinched off vesicles that contain significant amounts of actin. Likewise, the *Drosophila* nuclear lamin Dm_0 is normally evenly distributed along the periphery of the nuclear membrane, but the distribution becomes diffuse in *diap1* loss-of-function embryos after four to five hours of development, suggesting that the integrity of the nuclear envelope becomes compromised at this time (Figure 5).

Morphological Consequences of Caspase Activation in *diap1* Loss-of-Function Embryos

Numerous potential substrates for caspases have been identified in cell culture systems as well as in *in vitro* cleavage assays (Nicholson, 1999). The significance of most can be inferred, but the exact rationale for the cleavage of all of these substrates and not others and how this actually leads to apoptosis remains elusive in many cases. One clear example of cause and consequence includes the mammalian DNAse CAD and its inhibitor ICAD. ICAD is cleaved by caspases, leaving CAD free to degrade the genomic DNA (Enari et al., 1998; Sakahira et al., 1998). A second well established observation is the cleavage of nuclear lamins during apoptosis (Lazebnik et al., 1995; Neamati et al., 1995), where experiments in mammalian tissue culture cells altering the proposed caspase cleavage site in lamin show that lamins need to be cleaved for the normal morphological events of apoptosis to take place (Rao et al., 1996). While apoptosis eventually does take place, the nuclear envelope does not break down properly if lamins are not cleaved and there is a general delay in apoptosis. Further evidence that lamin disorganization is the result of caspase activation comes from observations of *Drosophila* nurse cell apoptosis. Nurse

cells deficient for the *Drosophila* caspase *dcp-1* fail to show the normal lamin disorganization during apoptosis (McCall and Steller, 1998).

We show that in *diap1* loss-of-function embryos lamin not only becomes disorganized during apoptosis (Figure 5), but at least some of the lamin is also cleaved (Figure 6A and B). This observation shows that lamin cleavage occurs in a physiological context during *Drosophila* apoptosis. Lamin cleavage is concurrent with the nuclei of the embryo taking on an apoptotic morphology and becoming TUNEL positive after four to five hours of development. No lamin cleavage products can be seen on a Western blot of protein extracts from *diap1* RNAi embryos that are younger than three and a half hours, but a distinct cleavage product of approximately the predicted 30 kDa size, based on the location of the likely caspase cleavage site, becomes visible in four to five hour old *diap1* RNAi embryos (Figure 6A). The same cleavage product is also apparent in embryos induced to undergo apoptosis by the overexpression of Hid protein (Figure 6B), showing that lamin cleavage is a general characteristic of apoptosis. We are therefore able to correlate a morphological event, the disorganization of lamin seen in apoptotic cells, with caspase activation in the developing organism.

A second example of a morphological feature of apoptosis being due to caspase activation comes from the observation that in *diap1* loss-of-function embryos, filamentous actin becomes disorganized and that cells no longer adhere well to each other, a phenotype that is consistent with the cleavage of the *Drosophila* β-catenin homolog, Armadillo. Filamentous actin is anchored to the plasma membrane through a complex containing several proteins, including α-catenin, β-catenin, and cadherins (Kemler, 1993; Gumbiner, 1996; Provost and Rimm, 1999; Steinberg and McNutt, 1999). β-catenin has been shown to be part of cell adherens junctions, and it also plays a role in Wnt/Wingless pathway signal transduction. Several reports have shown that β-catenin can be cleaved by caspases (Brancolini et al., 1997; Brancolini et al., 1998; Herren et al., 1998; Van de Craen et al., 1999; Steinhusen et al., 2000). In addition, in *Drosophila* Armadillo disappears during follicle and nurse cell apoptosis in the ovary (Chao and Nagoshi, 1999). Consistent with this observation are data from egg chambers mutant for the caspase *dcp-1*, in which actin fails to reorganize properly, possibly contributing to a defect in the transfer of the nurse cell cytoplasm to the developing oocyte (McCall and Steller, 1998). Embryos mutant for *armadillo* also display a loss of cell-cell adhesion and filamentous actin is no longer normally localized, with some actin aggregates becoming apparent (Peifer et al., 1993; Cox et al., 1996). Like the changes seen in *armadillo* mutants, cleavage of Armadillo is likely to disrupt its role in cell-cell adhesion and in actin organization. Armadillo contains potential

caspase cleavage sites, $_{86}$DQVD and $_{713}$DLQD, the locations of which, at the amino- and carboxy-termini, are consistent with the cleavage products observed with β-catenin.

We show that Armadillo protein levels also are significantly reduced at the time that the morphological changes associated with apoptosis occur in *diap1* RNAi embryos (Figure 6C). This disappearance is consistent with the protein being cleaved. Again, the disappearance of Armadillo is also observed in embryos undergoing apoptosis as a result of Hid overexpression (Figure 6C) and it thus seems to be a general feature of apoptosis. Cleavage of Armadillo can explain the loss of cell adherence and disorganization of filamentous actin: In order to mediate adherence between cells, cadherins must be complexed to the actin cytoskeleton via α- and β-catenin, where Armadillo/β-catenin serves as the link between cadherins at the plasma membrane and α-catenin, which binds actin (reviewed in Gumbiner, 1996). Thus, cleavage at the amino-terminus of Armadillo would sever the domain interacting with α-catenin from the domain interacting with cadherin and thereby result in cadherins no longer being complexed to the actin cytoskeleton and a subsequent loss of cell adhesion. No cleavage product is apparent on the Western blot, however, the caspase consensus site $_{86}$DQVD is in the 56 amino acid region of Armadillo against which the antibody was produced, such that the epitope recognized by the monoclonal antibody may be destroyed when Armadillo is cleaved.

The organization of filamentous actin in *diap1* RNAi embryos is not identical to that seen in *armadillo* mutants, but a key feature is maintained. In *diap1* RNAi embryos, like in *armadillo* mutants (Peifer et al., 1993; Cox et al., 1996), filamentous actin is now generally present in aggregates or clumps as can be seen as brightly staining orange spots in Figure 4E-G, and is no longer evenly distributed along the periphery of the cell. Other mutants that affect the cytoskeletal organization in early *Drosophila* embryos, such as *daughterless-abo-like*, *nuclear-fallout*, and *scrambled*, show defects in the actin cytoskeleton, but do not result in the uneven distribution and aggregation of filamentous actin at the time of cellularization (Sullivan et al., 1993).

As a control to show that loss of *diap1* function does not result in a general degradation of all structural proteins, we looked at β-tubulin. β-tubulin has been shown to remain intact in cells where various caspase substrates were being cleaved, indicating that it is not a caspase substrate (Brancolini et al., 1995). β-tubulin is also not cleaved in *diap1* RNAi embryos (Figure 6D), showing that Lamin Dm_0 and Armadillo cleavage observed in *diap1* RNAi embryos is not due to simply a non-specific degradation of structural proteins. This provides further evidence for a model where caspase-mediated cleavage of a limited number of key substrates results in the morphological features of apoptosis.

We show that two separate substrates are cleaved during apoptosis induced by the loss of *diap1* function and that this cleavage is likely due to caspases becoming activated. However, we were unable to rescue the *diap1* loss-of-function phenotype with the injection of *p35* sense mRNA or with the peptide caspase inhibitor BOC-fmk. The lack of rescue may be due to either the *p35* mRNA not being translated quickly enough to inhibit caspase activation or the levels of caspase inhibitor being insufficient to block all active caspases. Without a rescue of caspase activation, we were unable to address whether *diap1* has functions besides inhibiting apoptosis. In addition, one may consider the possibility that programmed cell death in *diap1* loss-of-function embryos is not directly related to the loss of *diap1*, but rather is a death by frustration such as those seen in developmental mutants such as *crumbs*. Evidence to counter this argument comes from the many experiments that clearly establish Diap1 as an anti-apoptotic protein: the overexpression of *diap1* can protect from apoptosis and that Diap1 can bind to and inhibit the activation of pro-caspases (reviewed in Deveraux and Reed, 1999).

DISCUSSION

diap1 Loss-of-Function Mutant Phenotype

In this study we show that *diap1* loss-of-function mutants progress normally through the first thirteen nuclear divisions, enter normally into mitosis fourteen, and that loss of *diap1* function results in the cleavage of two physiologically relevant caspase substrates, nuclear lamin Dm_0 and Armadillo. While many different proteins have been shown to be cleaved during apoptosis (Brockstedt et al., 1998; Nicholson, 1999), few have been looked at under physiological conditions and none has been shown to be cleaved in a developing organism as a result of the loss of a regulator of apoptosis. Furthermore, cleavage of nuclear lamins and of Armadillo correlates well with the morphological changes seen during programmed cell death.

In addition to playing a structural role in the adherens junction, Armadillo also functions in the Wingless signaling pathway (Cox et al., 1999). This second function of Armadillo would be interesting to pursue in the context of apoptosis. It is possible that caspase-mediated cleavage of Armadillo also affects transcription from the target genes of the Wingless pathway. Observations with the vertebrate homolog β-catenin show that cleavage of β-catenin by Caspase-3 results in proteolytic fragments that interfere with the transcription activation potential of β-catenin (Steinhusen et al., 2000).

diap1 Loss-of-Function Mutants Cellularize and Enter Mitosis 14

It has been reported that mutations in the *thread* locus affect cellularization, based on the inability to detect germ cells (Moore et al., 1998). While five to six hour old *diap1* loss-of-function are morphologically similar to blastoderm embryos prior to cellularization, this study and previous work provide evidence that cellularization is not affected in *diap1* loss-of-function embryos (Wang et al., 1999; Goyal et al., 2000). *diap1* loss-of-function embryos proceed through the complex morphological movements of early gastrulation and display lamin and filamentous actin organization that is indistinguishable from wild-type during early gastrulation, strongly suggesting that cellularization takes place normally.

A second possible function of Diap1 is in cell division and cytokinesis, since at least three BIR containing proteins have been shown to be involved in cell cycle regulation or in cell division (Li et al., 1998; Fraser et al., 1999; Li et al., 1999; Uren et al., 1999; Li et al., 2000; Speliotes et al., 2000). In addition, the *C. elegans* BIR-1 protein forms a complex with an Aurora-like kinase at the spindle midzone of chromosomes and both proteins are

required for Histone H3 phosphorylation (Speliotes et al., 2000). The stage at which the first developmental abnormalities occur in *diap1* loss-of-function embryos correlates well with entry into mitosis fourteen. Since in the *Drosophila* embryo the first thirteen nuclear divisions occur in a syncytium, without cell membranes present, it seemed reasonable to check whether the *diap1* loss-of-function embryos enter mitosis fourteen normally, as this is where a defect in cytokinesis would become apparent. A similar percentage of *diap1* RNAi embryos enter mitosis fourteen as wild-type control embryos, suggesting that Diap1 is not required for entry into mitosis fourteen; however, slightly fewer cells enter mitosis fourteen in the domains observed (Figure 2 C-F). Furthermore, staining with an antibody against phosphorylated Histone H3 was similar to wild-type during the first thirteen mitotic divisions. Even when development became abnormal during cell cycle fourteen, some cells still stained with the phosphorylated Histone H3 antibody (Figure 3), suggesting that the loss of *diap1* has effects that differ from those seen for the loss of *bir-1* in *C. elegans*. The reduction in the number of cells entering mitosis fourteen in each mitotic domain is likely due to some cells already being in the early stages of apoptosis, since numerous condensed nuclei are already present at this stage. In addition, no apparent difference was observed in the number of nuclei present in late blastoderm embryos, implying that the normal number of cells form in these embryos and that the developmental defects in *diap1* loss-of-function embryos manifest themselves after this timepoint.

The Role of *diap1* in Development

One explanation for why *diap1* loss-of-function embryos develop to this advanced stage before undergoing apoptosis is that the maternal contribution of Diap1 is sufficient until this point and that the induction of apoptosis is then due to the lack of zygotic *diap1* transcription. Work in *Xenopus* suggesting that zygotic transcription is required to block a maternal apoptotic program in the developing embryo supports this view: Apoptosis at the midblastula transition induced by the addition of the transcription inhibitor α-amanatin was blocked by injection of the anti-apoptotic Bcl-2 protein, which suggests that the zygotic transcription of anti-apoptotic genes is necessary for continued development at that stage (Sible et al., 1997). Combining this observation with the data in this paper suggests a model where zygotic *diap1* function is needed to protect the embryo from either a pre-existing maternally supplied protein or a newly transcribed pro-apoptotic gene. Likely candidates for this gene include the initiator caspases *dcp-2/dredd* and *dronc*. However, the formal possibility still exists that *diap1* has functions besides protecting from programmed cell death and this question cannot be definitively addressed until we are able

to specifically block cell death in *diap1* loss-of-function embryos and observe any consequent effects.

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Figure 1.

diap1 RNAi Phenocopies the *diap1* Loss-of-Function Morphology

(A-C) depict three *Drosophila* embryos after 5 to 6 hours of development. The anterior is oriented towards the left. These embryos were labeled with the TUNEL procedure to mark apoptotic nuclei (Gavrieli et al., 1992). (A) shows a homozygous *diap1* loss-of-function ($th^{11\text{-}3e}$) mutant embryo, where virtually all nuclei are TUNEL positive (Goyal et al., 2000). The empty space approximately one third into the embryo is a result of the bubble-like structure having moved towards the surface of the embryo. (B and C) show two representative *diap1* RNAi TUNEL-labeled embryos. Due to the orientation, the bubble-like structure is not easily visible in (B), but like in (A), virtually all nuclei are TUNEL positive. (C) shows a cross-section of an embryo to highlight the distinct bubble breaking the surface of the embryo.

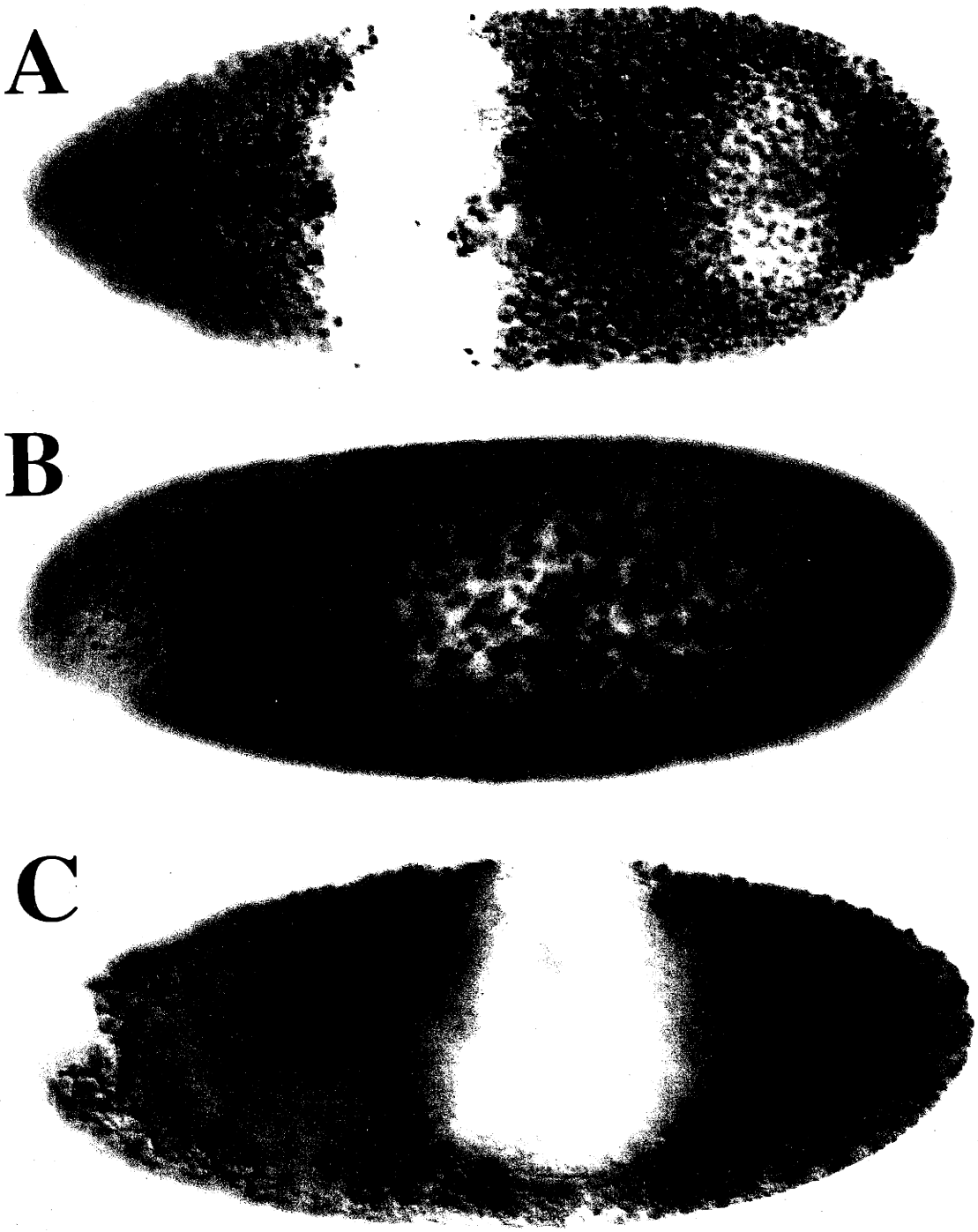
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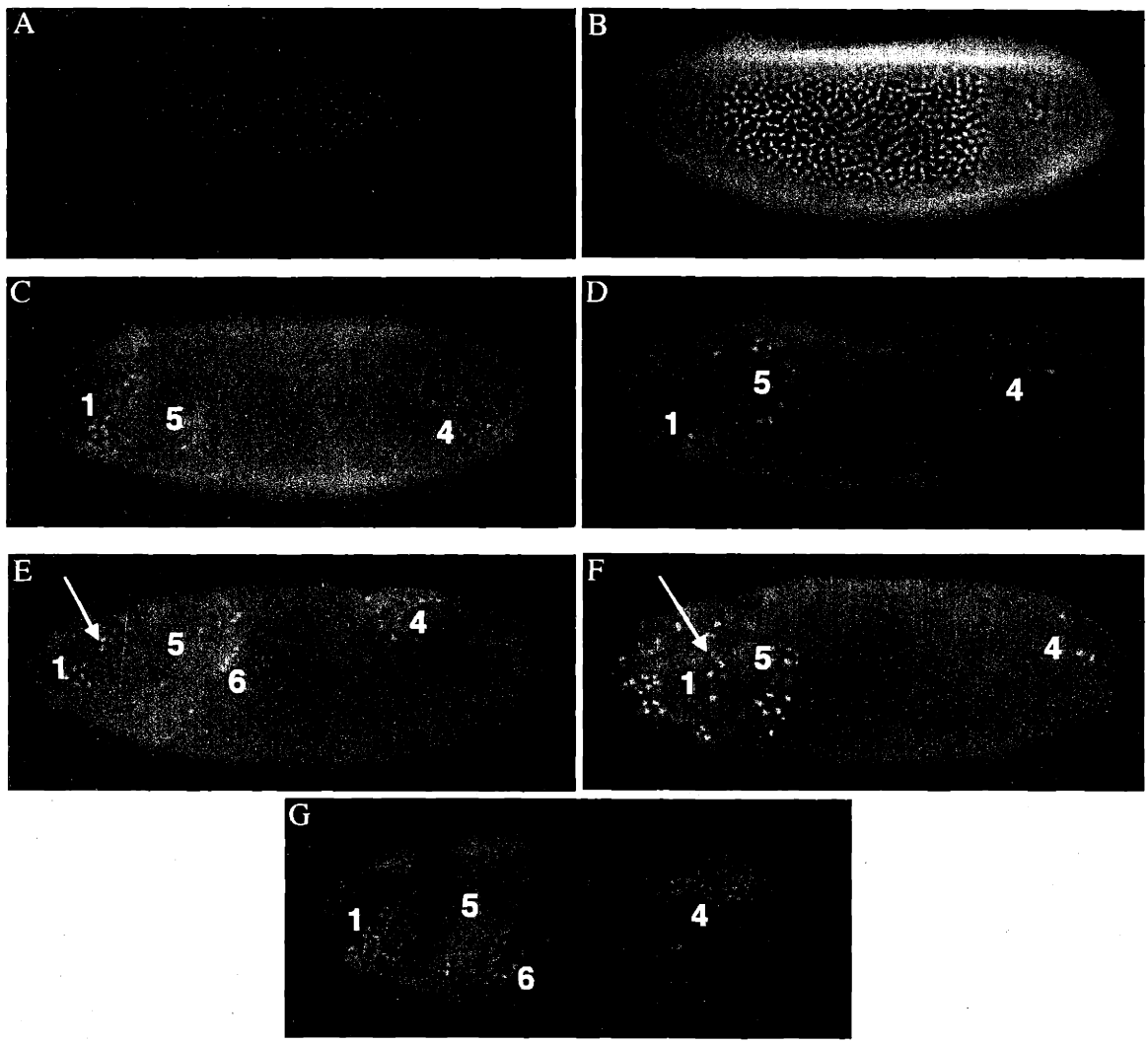
Figure 2.

diap1 Loss-of-Function Embryos Enter Mitotic Cycle 14

All embryos were stained with an antibody against phosphorylated histone H3. The stained mitotic domains are numbered in the individual panels. (A, C, and E) depict wild-type embryos at late blastoderm (A), early germband extension with mitotic domains 1, 4 and 5 visibly in mitosis (C), and slightly later in germband extension with mitotic domains 1, 4, 5 and 6 visibly in mitosis (E). (B, D, and F) show *diap1* RNAi embryos at late blastoderm (B), early germband extension (D), and slightly later in germband extension (F). The first thirteen nuclear divisions appear to proceed normally in *diap1* RNAi embryos (compare A and B). In addition, *diap1* RNAi embryos enter mitosis 14, as can be seen in panels (D and F). However, the mitotic domain structure is not as organized as in wild-type and this can be seen most clearly in domain 1, where the wild-type embryo shows a diagonal line of cells in mitosis (arrow in E), but the *diap1* RNAi embryo shows a more random organization of mitotic cells at this stage (arrow in F). Mitotic domain 4 is clearly evident at the tip of the extending germband in the wild-type embryo (E), but the *diap1* RNAi embryo (F), while having some cells in mitosis in domain 4, shows significant morphological abnormalities as is evidenced by the lack of visible germband its posterior. In addition, many cells in these embryos are beginning to show condensed nuclei, consistent with entry into apoptosis (data not shown). (G) shows a *lacZ* RNAi control embryo. Injection of double-stranded *lacZ* RNA does not affect entry into mitosis 14 or the morphology of the embryo (compare E and G).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4
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4
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5
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F
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5
4
6

Figure 3.

Limited Mitosis Continues in 4-5 Hour Old *diap1* Loss-of-Function Embryos

(A-C) depict *Drosophila* embryos stained with an antibody against phosphorylated histone H3 to label mitotic cells (orange) and bisbenzimide to label DNA (blue). In all cases, the arrows indicate cells in mitosis. (A) shows a stage 9/10 uninjected wild-type embryo, indicating normal nuclear morphology and some mitosis. (B and C) depict *diap1* RNAi embryos of similar age as (A), but the embryos have lost their normal morphology, note the absence of a clear cephalic furrow in (arrowhead in B and C) when compared with (arrowhead in A), and many nuclei are condensed and appear apoptotic, especially in the posterior of the embryo. However, some cells continue to enter into mitosis (arrows). The bubble-like structure is beginning to form at this stage, as can be seen in (C, asterisk), where a large gap in the nuclear staining is visible. This bubble will migrate to the surface within the next hour of development. By that point, all cells will be apoptotic and no further mitosis is observed.

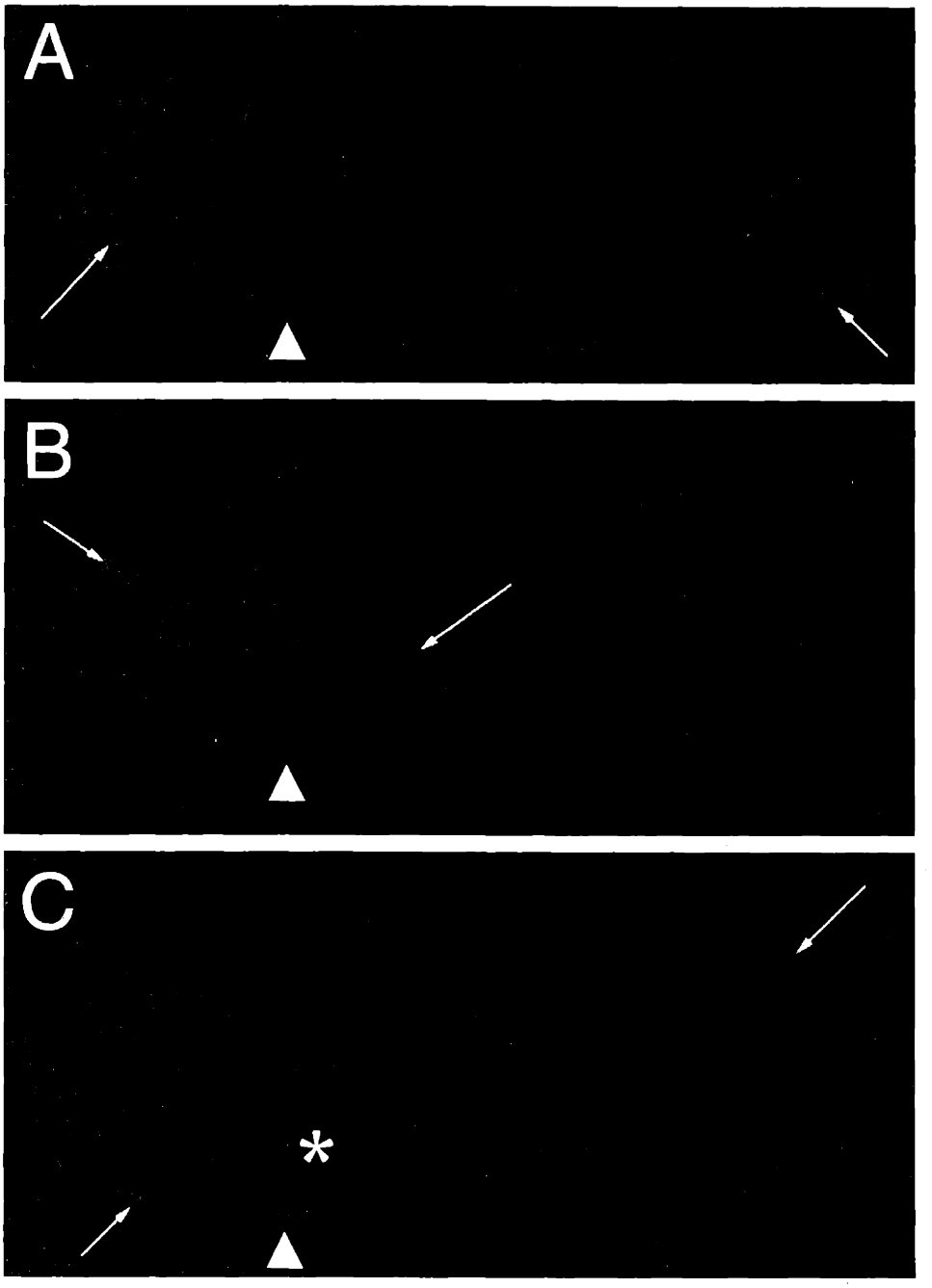
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Figure 4.

Filamentous Actin Becomes Disorganized in *diap1* Loss-of-Function Embryos

Rhodamine conjugated phalloidin was used to label filamentous actin (orange) and bisbenzimide was used to stain DNA (blue) in *Drosophila* embryos. (A and B) show late blastoderm embryos stained with phalloidin and bisbenzimide where (A), the uninjected wild-type and (B), the *diap1* RNAi embryo both show similar actin localization at this stage, with filamentous actin evenly distributed around the periphery of each cell. The nuclei are not condensed and occupy most of the space inside each cell. (D) shows a *diap1* RNAi embryo during early gastrulation, approximately 3 hours into development, with the ventral and cephalic furrows clearly visible. Development is essentially normal up to this point. (E-G) show *diap1* RNAi embryos that are five to six hours old. (E) depicts the entire embryo, showing that filamentous actin has become disorganized and now is present in clumps, as can be seen by the brightly orange staining spots, rather than being evenly distributed along the periphery of the cells (No DNA stain was used for this embryo). (F) focuses on the posterior of a *diap1* RNAi embryo. (G) depicts a close-up view of the area boxed in (F) to show that the nuclei are condensed and that smaller, smaller bright orange particles are present that appear to be fragmented cells, or blebs (arrow). The focal planes for the two lasers used to visualize actin and DNA are not exactly identical, preventing us from making any conclusions as to the position of the nucleus relative the actin staining in each cell. (C) shows an uninjected wild-type embryo of the same developmental age as the *diap1* RNAi embryos shown in (E-G). Here, filamentous actin remains evenly distributed along the periphery of each cell and no clumping of actin can be seen. The scale bar in (G) equals 10 μm.

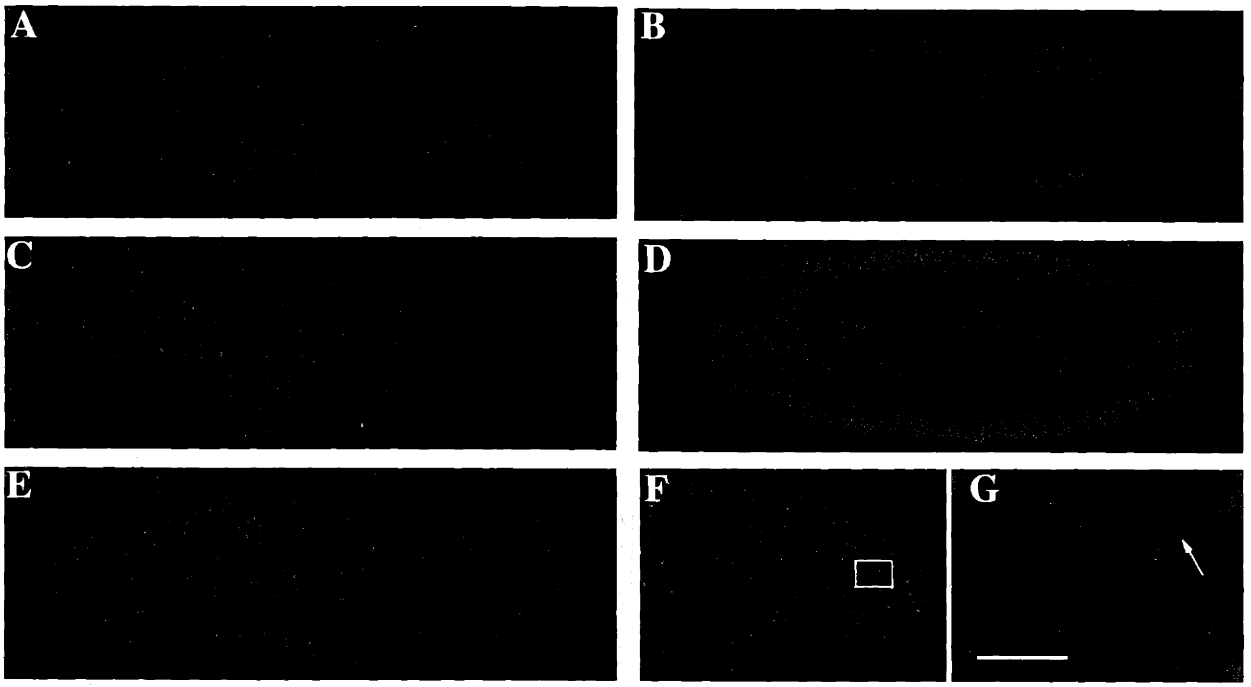
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Figure 5.

Nuclear Lamins Become Disorganized in *diap1* Loss-of-Function Embryos

An anti-lamin Dm_0 monoclonal antibody was used to label nuclear lamins in *diap1* loss-of-function embryos. (A) shows an embryo during late blastoderm where lamin is localized in a ring along the nuclear envelope. (B) depicts a *diap1* loss-of-function embryo during early gastrulation, with the ventral furrow visible along the midline of the embryo; again, lamin organization is similar to wild-type. (C and D) show 5 to 6 hour old *diap1* loss-of-function embryos. Here, lamin is evenly distributed inside the cells and no longer appears to be associated with the nuclear envelope.

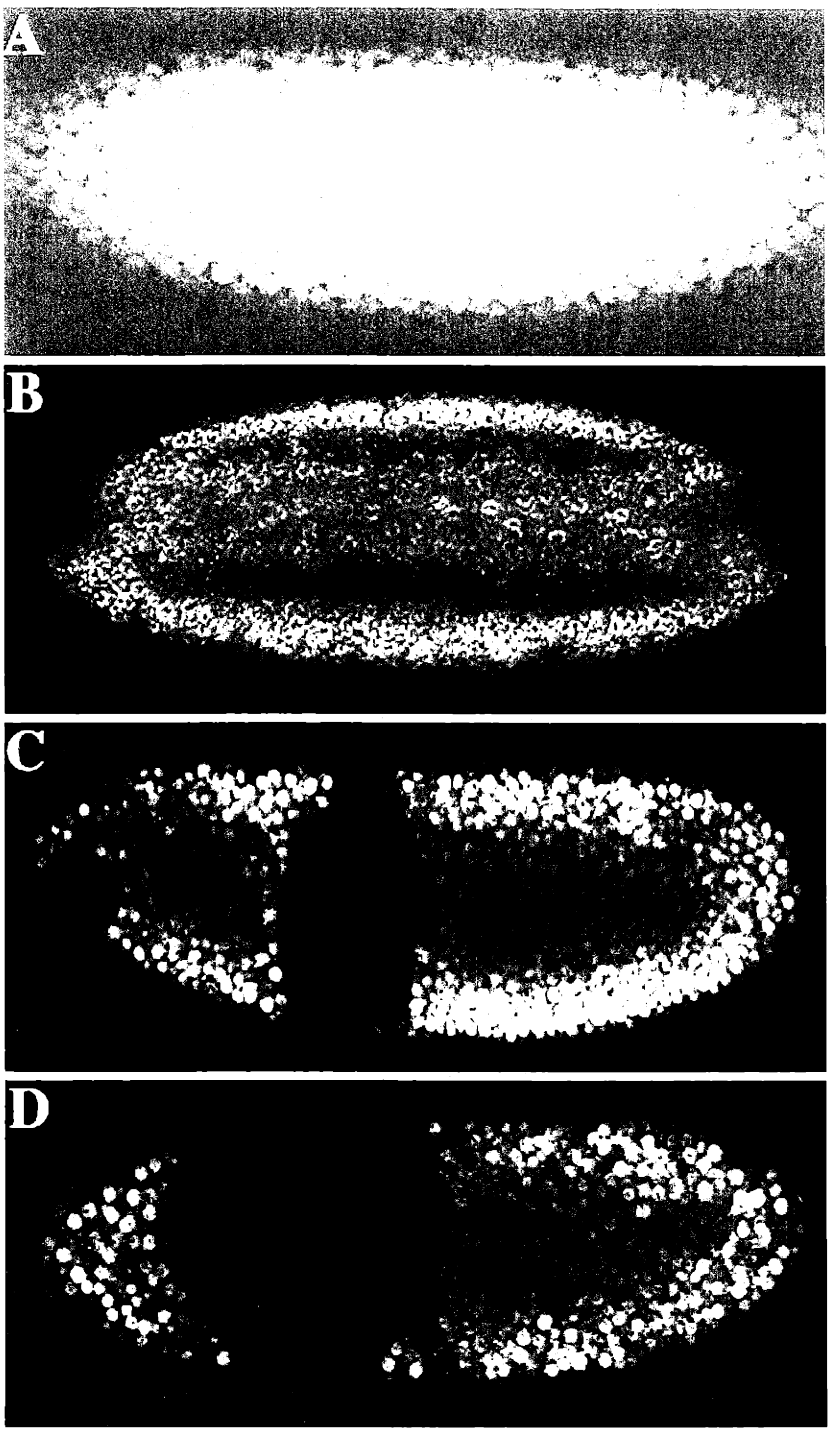
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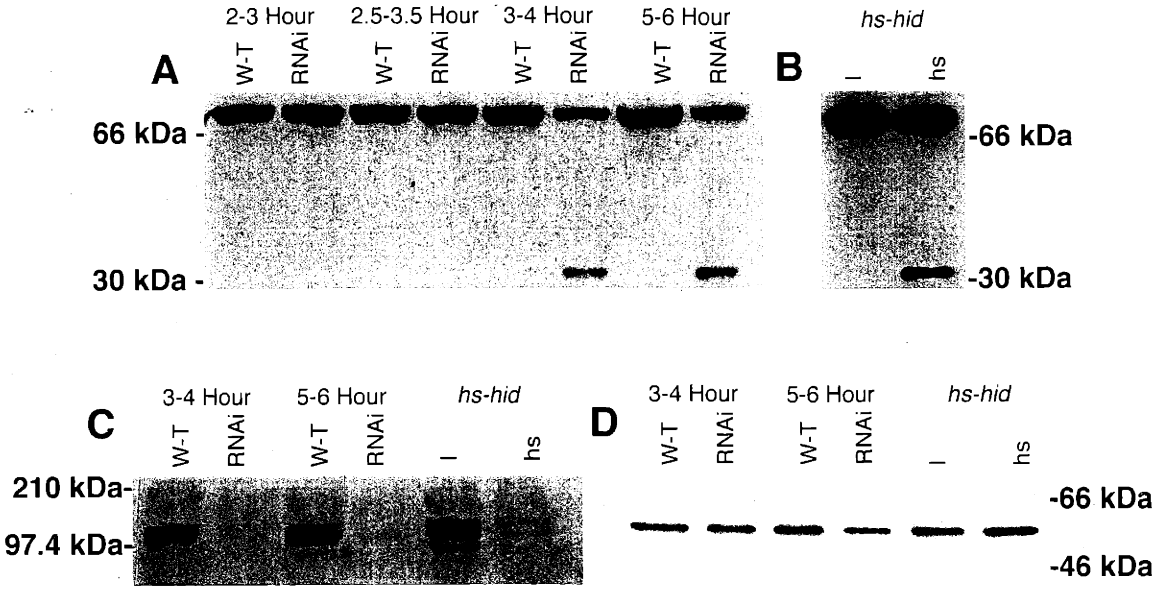
Figure 6.

Physiologically Relevant Caspase Substrates Are Cleaved in *diap1* Loss-of-Function Embryos

(A) is a Western blot showing a time course for nuclear lamin Dm_0 in uninjected wild-type and *diap1* RNAi wild-type embryos. The time in hours indicated above the lanes represents the developmental age of the embryos from which proteins extracts were made. A distinct lamin cleavage product of roughly 30 kDa becomes apparent after 4 hours of development in the *diap1* RNAi embryos, but is not seen in the uninjected control embryos. (B) is a Western blot stained with a lamin Dm_0 antibody showing untreated (-) and heat shocked (hs) embryos overexpressing *hid* to induce apoptosis. The same lamin cleavage product is visible in the embryos overexpressing *hid* as is seen in *diap1* RNAi embryos. *hid* was overexpressed using the heat-shock promoter as previously described (Grether et al., 1995). A Western blot using an antibody against Armadillo (C) shows that the full-length Armadillo protein disappears in *diap1* RNAi embryos. Again, Armadillo protein also disappears when apoptosis is induced by the overexpression of *hid*. (D) is a Western blot probed with a β-tubulin antibody showing that β-tubulin is not affected in *diap1* RNAi embryos and heat shocked *hs-hid* embryos.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RNAi
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30 kDa -
B
hs-hid
-
hs
-66 kDa
-30 kDa
C
3-4 Hour
5-6 Hour
hs-hid
210 kDa-
97.4 kDa-
D
-66 kDa
-46 kDa

Chapter 4

Searching for Additional Components of the Programmed Cell Death Pathway in *Drosophila melanogaster*

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SUMMARY

Reaper is a central regulator of apoptosis during *Drosophila melanogaster* development. A yeast two-hybrid screen for proteins interacting with Reaper yielded ten potential binding partners. The sequence, cytological location and embryonic expression pattern have been determined for these interactors. None has yet been clearly shown to play a role in apoptosis, however, several interactors warrant further attention since they are known to also interact with other proteins with potential roles in apoptosis. The best characterized of the favored interactors is Nap-1, a nucleosome assembly protein that also binds Granzyme A and the p127 lethal 2 giant larvae protein, both of which have been implicated in programmed cell death. Other interactors include a protein similar to one that binds Huntingtin, Translationally Controlled Tumor Protein (TCTP), and a transcription factor that functions in cell differentiation. In addition, three interactors remain novel and may deserve further study once more is known about their function or homology. This study provides a series of leads into how Reaper may function and is a resource for anyone wishing to undertake further analysis of these proteins.

RATIONALE

During the Summer of 1994, when this yeast two-hybrid screen was initiated, *reaper* and *hid* had recently been identified and little else was known about the programmed cell death pathway in *Drosophila*. The rationale for this project was to rapidly identify additional components of this pathway and correlate the results with a large scale mutagenesis project begun at the same time by Julie Agapite.

INTRODUCTION

Programmed cell death, or apoptosis, is a gene-directed mechanism that is crucial for the normal development of multicellular organisms (Thompson, 1995; Jacobson et al., 1997). In *Drosophila*, the induction of apoptosis requires the gene products of a small interval of the chromosomal region 75C1,2 that is removed by the H99 deletion (White et al., 1994). This genomic interval is essential for virtually all normal developmental cell deaths and its loss also provides significant protection from cell deaths induced by X-irradiation or those seen in developmental mutants. Three genes contained in this interval, *reaper*, *head involution defective (hid),* and *grim* have been shown to function in apoptosis (White et al., 1994; Grether et al., 1995; Chen et al., 1996b). Re-introducing the genomic clone containing *reaper* partially restores cell death to H99 mutant embryos, and overexpression of the *reaper* cDNA results in increased apoptosis (White et al., 1994; White et al., 1996). Exactly how *reaper* functions in programmed cell death is not known, but it is likely to be a cell death regulator and not an effector since H99 mutant embryos, which lack *reaper*, can be induced to undergo limited apoptosis when exposed to large doses of X-irradiation (White et al., 1994). The cells that die under these circumstances look morphologically like apoptotic cells and are stained by acridine orange, a dye that specifically labels apoptotic cells. These observations suggest that the basic cell death program can be activated in cells lacking *reaper* if the death promoting stimulus is of sufficient strength.

Since Reaper is a small protein with no obvious motifs providing clues to its function, finding binding partners for this protein became a high priority. The yeast two-hybrid system utilizes the modular nature of eukaryotic transcription factors, where the DNA binding domain can be functionally separated from the transcription activation domain, (Keegan et al., 1986; Ma and Ptashne, 1988; Fields and Song, 1989) and neither domain by itself is sufficient to activate transcription. Thus, only when two proteins fused to these domains interact can transcription be activated from reporter genes. This approach has been used with great success to identify various components of the programmed cell death

pathway, including proteins that bind the death domain of Tumor Necrosis Factor Receptor 1 (TNFR1) (Chinnaiyan et al., 1995; Sato et al., 1995; Stanger et al., 1995). The death domain is a conserved protein-protein interaction motif found in several regulators of apoptosis. Reaper seems to share limited similarity with the death domains of TNFR1 and Fas, and at the time the screen was initiated the similarity was deemed worth pursuing. The functional significance of this similarity has since come into question, however, since mutating conserved residues in the putative death domain does not affect the ability of Reaper to induce apoptosis when overexpressed (Golstein et al., 1995; Chen et al., 1996a; Vucic et al., 1997b). A yeast two-hybrid screen using Reaper as the bait yielded ten potential interactors that appeared to be specifically binding to Reaper. We obtained the sequence, embryonic expression pattern, and chromosome map position for these interactors and attempted to verify the specificity of the interactions with *in vitro* binding experiments for two of the interactors.

SCREEN RESULTS

In order to identify potential additional members of the programmed cell death pathway and to try and elucidate the mechanism by which Reaper is regulated, I used a LexA-Reaper fusion protein as a bait in a yeast two-hybrid system to screen two *Drosophila* cDNA libraries. The libraries chosen, a 0-12 hour embryonic library and a third instar larval imaginal disc library, were constructed from developmental stages where significant apoptosis normally takes place (Truman et al., 1992; Abrams et al., 1993). A pilot screen of approximately a quarter million transformants for each library yielded 84 potential interactors which were grouped into 19 classes based on restriction digest patterns from the PCR-amplified cDNA fragments in the library vector. Most interactors were isolated multiple times (Tables 1 and 2). Presented with this reasonable number of clones, I decided to further characterize these interactors rather than screen more transformants. After testing the potential interactors for specific binding to Reaper using non-related control bait plasmids, I was left with ten interactors that were specific to Reaper in this system.

Upon sequencing the clones, several looked potentially interesting based on homology to known genes (Table 2). Only one of these interactors, 5-15 or Nap-1, has been extensively studied. Nap-1 was initially described as functioning in nucleosome assembly, but has also been implicated in cell cycle regulation. Most importantly, it binds to two proteins that have potential roles in apoptosis, p127 and Granzyme A (Ishimi and Kikuchi, 1991; Kellogg et al., 1995; Kellogg and Murray, 1995; Ito et al., 1996; Beresford et al.,

1997, Bernard Mechler, personal communication). p127, is a protein recently reported to be required for salivary gland apoptosis in *Drosophila* (Farkas and Mechler, 2000). Granzyme A has been suggested to play a role in apoptosis in the immune system (Beresford et al., 1997). The p127 protein is encoded by *lethal(2)giant larvae (l(2)gl)*. Mutations in this gene cause a complex syndrome which includes the development of neuroblastomas in the larval brain as well as tumor-like overgrowth of the imaginal disc, suggesting that this gene functions to regulate cell proliferation and possibly differentiation (Mechler et al., 1985). The p127 protein has also been shown to be a component of the cytoskeleton and associates with a number of other proteins besides Nap-1, including nonmuscle myosin II heavy chain and a serine kinase that may regulate the attachment of p127 to the plasma membrane (Strand et al., 1994a; Strand et al., 1994b; Kalmes et al., 1996). Granzyme A is a trypsin-like enzyme that is expressed at high levels in cytotoxic T lymphocytes and appears to be able to synergize with perforin and Granzyme B to trigger apoptosis and DNA degradation (Nakajima et al., 1995; Kam et al., 2000).

The other interactors with a possible role in apoptosis are homologous to largely uncharacterized proteins. Interactor 6-15 is homologous (48-49% identical) to a class of translationally controlled tumor proteins (TCTPs) that have not been extensively studied (Chitpatima et al., 1988; Gross et al., 1989; Xu et al., 1999). Since tumor growth is facilitated by a reduction in, or elimination of, programmed cell death, a gene that is differentially expressed in tumor versus normal tissue could be involved in regulating apoptosis.

Another interactor, 6-19, shares limited homology (28-30% identical) with the Huntingtin interacting protein HIP-1 (Wanker et al., 1997). Huntington's disease is an inherited neurodegenerative disorder which is thought to be caused by an expansion of polyglutamine repeats in the Huntingtin protein (The Huntington's Disease Collaborative Research Group, 1993). Expression of polyglutamine-expanded human Huntingtin in the *Drosophila* eye causes degeneration of photoreceptor neurons (Jackson et al., 1998). Expression of another human polyglutamine-expanded protein, SCA3/MJD, also causes neuronal degeneration in *Drosophila*. While neuronal degeneration due to Huntingtin expression is not rescued by the co-expression of p35, a caspase inhibitor, degeneration caused by the expression of SCA3/MJD is mitigated by p35, indicating that the negative effects caused by polyglutamine-expanded proteins may be due to the activation of a caspase-dependent pathway (Warrick et al., 1998). Consistent with this idea are data showing that caspases in apoptotic cell extracts cleave *in vitro* translated Huntingtin protein, generating truncated proteins containing only the polyglutamine tract, which appear to be a key step leading to the pathogenesis of the disease (Goldberg et al., 1996; Wellington et

al., 1998). While *hip-1* is still largely uncharacterized, it is possible that it plays a role in the regulation of Huntingtin or its cleavage and by studying the *Drosophila* homolog, one may gain more rapid insight into its function.

Clone 9-24 is identical to a previously identified gene, *cryptocephal,* a spontaneous *Drosophila* mutant that was defined by its morphology. It also shares significant homology with the cyclic AMP-dependent transcription activating factor (ATF) family of basic leucine-zipper transcription factors which is involved in the transcriptional regulation of genes in response to changes in cAMP levels in the cell (Fristrom, 1965; Chevray and Nathans, 1992; Vallejo et al., 1993; Hewes et al., 2000). In addition, ATF family members have been shown to dimerize with the CAAT box/enhancer binding protein family (C/EBP), which can regulate the expression of genes during cell differentiation or in response to inflammatory cytokines (Vallejo et al., 1993). In *Drosophila, cryptocephal* seems to control molting and metamorphosis in response to ecdysone hormone signaling (Hewes et al., 2000). Interestingly, *reaper* transcription is regulated by an ecdysone response element (Jiang et al., 2000). Furthermore, the *C. elegans ces-2* (cell-death specification) gene, which is a transcriptional regulator of programmed cell death, also encodes a basic region leucine-zipper transcription factor (Metzstein et al., 1996). The significance of the interaction between 9-24 and Reaper is unclear, but it may provide a link between Reaper and the transcriptional regulation of genes involved in programmed cell death.

Interactor 6-8 contains a region of homology with prefoldin 4, one of a family of six proteins that act as chaperones, delivering unfolded proteins to other cytosolic chaperones (Vainberg et al., 1998). Given that heat-shock proteins have recently been implicated in the regulation of apoptosis (reviewed in Xanthoudakis and Nicholson, 2000), this protein may also function to control the levels of key programmed cell death regulators within the cell.

Of the remaining interactors, three are novel proteins (5-1, 5-21, 6-7) and two (5-2 and 9-6) are unlikely to be regulators of cell death based on their homology. Of the novel proteins, 5-21 is likely to be an artifact created during the construction of the library since the short open reading frame that is fused to the transcription activation domain in the library vector is not the normal reading frame of the novel gene encoded by 5-21.

Of the interactors of doubtful relevance, clone 5-2 is a precursor to fumarate hydratase that converts L-malate to fumarate as part of the TCA cycle (Suzuki et al., 1989; Walker et al., 1992), and clone 9-6 is identical to UB-3D, a fusion between ubiquitin and a ribosomal protein. This protein is likely to be localized to the ribosome and could act as a source of ubiquitin during ribosome assembly (Barrio et al., 1994). It is unclear how these two

interactors, given their homology to these known proteins, could act in programmed cell death.

I performed anti-sense mRNA *in situs* for interactors 5-1, 5-2, 5-21, 6-7, 6-8, and 6-15. The *in situs* revealed that these interactors are broadly expressed in the embryo and maternally deposited, which is consistent with the prediction for genes that are part of the cell death pathway, since every cell is thought to have the potential to die via apoptosis (Raff, 1992). The expression patterns for 9-6 and 5-15 were already known. 9-6, or *UB3-D* is known to be broadly expressed throughout development, while 5-15, or *nap-1*, is known to be broadly expressed in the embryo (Lee et al., 1988; Barrio et al., 1994; Ito et al., 1996).

Obtaining the cytological map position for the interactors using polytene chromosome *in situs* revealed that a number of them mapped to parts of the genome that had not been covered in previous screens for regions that modify Reaper or Hid induced apoptosis in the *Drosophila* eye (J. Abrams, K. White, J. Agapite, and H. Steller, unpublished observations). These screens exploit the reduced eye phenotype that results from the overexpression of *reaper* or *hid* under the eye-specific GMR promoter. Using this system, I tested available deficiencies removing appropriate parts of the genome to see whether the pGMR-*reaper/hid/grim* eye phenotype was either suppressed, indicating that a gene in the deficiency is pro-apoptotic, or enhanced, indicating that the interval contains an anti-apoptotic gene (Hay et al., 1994; Grether et al., 1995; Chen et al., 1996b; White et al., 1996). I identified three regions where a deficiency modifies the pGMR*reaper* eye-ablation phenotype, though these may not correlate with the putative interactors (Table 3). The modification seen in cytological position 69A is likely due to a mutation in *glass* that is present in the background on the deficiency chromosome. *glass* affects transcription from the GMR promoter, thereby potentially altering the level of *reaper* expression in the developing eye, resulting in a change in eye size that is unrelated to regulation of *reaper* function. Deficiency *(2R) b23lf* acts as a strong enhancer of Reaper-induced cell death. Single embryo PCR showed that this deficiency does not remove the gene encoding the interactor Nap-1, and deficiencies in the region that do remove *nap-1*, (see Table 3), do not modify the pGMR*reaper* eye phenotype suggesting that the enhancer is not *nap-1*. The third region that acts as a modifier is 92B-92F. Deficiency *(3R)B79* acts as a specific and strong suppressor of pGMR*reaper*, while not affecting pGMR*hid* or *grim* (Figure 1). However, the two-hybrid interactor that was originally mapped to that region has since been mapped to 86D7 by the Berkeley *Drosophila* Genome Project and deletions that uncover this region have no effect. Nevertheless, this deficiency warrants further study.

In order to test the specificity of the interaction with Reaper outside of the yeast two-hybrid system, I used GST-Reaper and GST-Hid fusion proteins and *in vitro* translated protein for two interactors, Nap-1 (5-15) (Figure 2) and TCTP (6-15) to perform pull-down assays. Neither protein bound specifically to Reaper, leading us to the conclusion that a number of the interactors are likely to be non-specific. The GST-Reaper and GST-Hid fusion proteins were successfully used to pull down Diap1 as a positive control. Further analysis of the specificity of the interactions would be necessary before any conclusions can be drawn about potential roles for the interactors in cell death.

REAPER SELF-AGGREGATION

In addition to the screen, I also used the yeast two-hybrid system to address whether Reaper interacts with itself. Reaper is an unusually small protein of only 65 amino acids, opening up the possibility that it needs to form multimers in order to function. Further, since expression of Reaper would be detrimental to a cell, its function should be tightly regulated. One way to accomplish such regulation is to have the monomer of the protein inactive, and to activate it once the intracellular concentration reaches a threshold where multimerization can occur. An example of such a control mechanism is the death domain-containing receptor family which appears to be activated upon forming trimers (reviewed in Ashkenazi and Dixit, 1998). Reaper shares rather limited homology with the TNFR1 death domain, (Golstein et al., 1995) so the possibility that Reaper may be similarly regulated needed to be investigated. Previous experiments using Reaper protein expressed in and purified from *E. coli* lent further support to the theory that Reaper can form multimers. Purified Reaper protein, either with or without a 6-Histidine tag, forms large and relatively uniform aggregates of approximately 100 subunits and multiple higher molecular weight forms of Reaper can also be seen on Western blots suggesting that the protein can self-aggregate (B. Raumann, C. Carboy-Newcomb, R. Sauer and H. Steller, unpublished results). I used several combinations of yeast two-hybrid vectors to address the issue of Reaper aggregation. In neither the LexA nor the GAL4 based system was I able to see Reaper interacting with itself. The fusion proteins are expressed as I was able to visualize the Gal4-Reaper and LexA-Reaper fusion proteins on a Western blot (Figure 3). The possibility that the proper binding sites for self-aggregation are inaccessible in the Reaper yeast two-hybrid baits remains.

DISCUSSION AND FUTURE DIRECTIONS

The yeast two-hybrid screen using Reaper as bait yielded ten potential interactors. Several of these are previously characterized proteins that could function in programmed cell death based on their interactions with other proteins, and would need to be further analyzed. Of these candidates, Nap-1 has been most closely studied, but its role in programmed cell death remains elusive and the limited *in vitro* binding analysis failed to confirm that this protein specifically interacts with Reaper.

Programmed cell death is negatively regulated by an important class of proteins first described in baculovirus, the Inhibitors of Apoptosis (IAPs) (Crook et al., 1993; Birnbaum et al., 1994). IAPs consist of one, two, or three Baculovirus IAP Repeat (BIR) domains and often a C-terminal RING finger domain (Deveraux and Reed, 1999). In *Drosophila*, four BIR containing proteins have been found and the anti-apoptotic function of three of these, Diap1, Diap2, and Deterin has begun to be verified (Hay et al., 1995; Duckett et al., 1996; Uren et al., 1996; Jones et al., 2000; Rubin et al., 2000; Wenzel et al., 2000). Reaper, Hid, and Grim can physically interact with and block the anti-apoptotic activities of IAPs from baculovirus, mammalian cells, and *Drosophila* (Vucic et al., 1997a; McCarthy and Dixit, 1998; Vucic et al., 1998).

Currently, if one were to undertake this screen, Diap1 would likely be identified as an interactor with a Reaper bait, as these proteins have been shown to interact *in vitro* (Vucic et al., 1997a). There are several explanations for not having isolated Diap1. It is possible that *diap1* was not present in the libraries, but a more likely reason is that the screen was not done to saturation, since it was limited to approximately half a million transformants. At that point, the screen had yielded ten interactors and it seemed prudent at the time to characterize these further rather than obtain an unmanageable number of potential interactors from further screening. Another possibility is that the Reaper protein does not have the proper binding sites exposed when complexed with LexA. The LexA DNA-binding domain is a 202 amino acid protein and is significantly larger than the 65 amino acid Reaper protein. In addition, LexA is fused to the N-terminus of Reaper as is the standard practice when making fusion proteins for two-hybrid screens. The N-terminus of Reaper is the only part of the protein that shares significant homology to Hid and Grim (Grether et al., 1995; Chen et al., 1996b), and it is this part of the protein that is also closely conserved between the *Drosophila melanogaster* and *Drosophila virilis* Reaper homologues (M. Grether, A.-F. Lamblin, R. Jespersen and H. Steller unpublished results). It was therefore thought to be important for protein function including, perhaps, for protein-protein interactions. Indeed, the N-terminus appears to be necessary for the

interaction between Reaper and Diap1 (Vucic et al., 1997a). Having the N-terminus of Reaper attached to the LexA DNA binding domain may interfere with this interaction. However, evidence to the contrary comes from a separate yeast two-hybrid screen using a Hid bait to screen the same *Drosophila* libraries (C. L. Wei and H. Steller, unpublished results) (Table 2). Two interactors from the Hid screen were also isolated with the Reaper bait. Since the only region of homology between Reaper and Hid is the extreme N-terminus, these interactors either bind to the N-terminus of both proteins or are non-specific. The interactors did not bind any of the control bait plasmids used, however, arguing that some level of binding specificity exists.

A second predicted interaction is that of Reaper with itself. Previous observations with bacterially expressed and purified Reaper protein showed that Reaper can form homomultimers (B. Raumann , C. Carboy-Newcomb, R. Sauer and H. Steller, unpublished results). However, I was unable to see any Reaper-Reaper interactions in the yeast two-hybrid system. It may be that Reaper multimers have no functional significance, but it is also possible that Reaper may not be able to form multimers in the yeast two-hybrid system.

When the yeast two-hybrid screen was initiated, the interaction between Reaper and Diap1 had not yet been identified; if so, I could have tested my Reaper baits with Diap1 as a positive control for my system. In addition, a mutagenesis screen for modifiers of the Reaper-induced eye phenotype isolated a point mutation in the N-terminus of Reaper in the pGMR-*reaper* transgene that reduces its ability to induce apoptosis (J. Agapite, K. McCall and H. Steller, unpublished results). Testing this mutation in the yeast two-hybrid system also could serve as a negative control, but these controls were not available until after I had finished the screens.

Characterizing the remaining positives from the screens would be facilitated now that more is known about Reaper and interactors since found by the use of other methods. Nevertheless, the interactors isolated in the two-hybrid screen are leads to follow in the continued study of programmed cell death in *Drosophila*. Finally, further attention should be paid to the region removed by *Df(3R)B79*, since it acts as a specific suppressor of Reaper-induced cell death in the eye. A careful analysis of this region could yield another member of the programmed cell death pathway.

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MATERIALS AND METHODS

The Yeast Two-Hybrid Screen

The Screen: The screening was conducted using Roger Brent's LexA-based system (Gyuris et al., 1993; Zervos et al., 1993). The vectors provided with the system and the yeast strain EGY 48 were used, except as noted below. Two *Drosophila* cDNA libraries, constructed by Russell Finley, were screened: RFLY1 (0-12 hour wild-type embryonic RNA) and RFLY5 (poly(A)+ selected disc RNA). Yeast transformations were done as described by Gietz, *et al.* (Gietz et al., 1992).

Bait plasmid construction: The PCR amplified *reaper* ORF cloned into the EcoR1 and Xho1 sites of pEG202 failed to enter the nucleus based on the test with the pJK101 plasmid provided with the system. We instead used the pNLex bait vector which includes a nuclear localization signal and inserted the PCR amplified *reaper* ORF into the EcoR1 site. The finished bait construct was sequenced and determined to be in frame and containing the correct sequence.

Control Baits: Those used were pRFHM12 (LexA-*Dros* cdc2 fusion), pRFHM7-3 (LexA-*ftz* homeodomain fusion), pRFHM∅ (No LexA control), and pLexIIBC (LexA-TFIIB C-terminal domain fusion), all provided by Roger Brent's laboratory.

Assay for Reaper-Reaper interactions: We used the Brent LexA-based system as well as both the Matchmaker 1 and 2 systems (Clontech). The Reaper ORF was cloned into the *Eco R1* site of pGBT9, pGAD 424, pAS2-1, and pACT2 and the finished constructs were sequenced and determined to be correct. Interactions were assayed using the β-galactosidase colony lift filter assay according to Clontech's instructions.

Sequencing

Sequencing of the double stranded cDNAs for the interactors in the pJG4-5 vector was accomplished using the BCO1 and BCO2 primers (sequence is provided in the Brent protocol) and Sequenase (U.S. Biochemical Corp.) according to the manufacturer's instructions. Homology searches were performed using BLASTn and BLASTp with the reading frame defined by the library vector. The non-redundant public databases, including the Berkeley *Drosophila* Genome Project database, were searched.

in situ Hybridization

Expression patterns: These were determined using whole mount *in situ* hybridization (Tautz and Pfeifle, 1989). Single stranded, digoxigenin labeled RNA probes were used according to a protocol communicated by R. Bodmer (U. of Michigan) and previously described

(White et al., 1994). Probes were generated using the entire insert that interacted with the pNLex*reaper* bait subcloned from pJG4-5 into pBluescript (Stratagene).

Chromosome *in situs*: Climbing third instar larvae were harvested from uncrowded bottles incubated at 18°C. The salivary glands were removed in a depression plate containing 45% acetic acid and most of the fat body was removed. The dissected salivary glands were then moved to a siliconized 22 mm, square cover slip containing a small drop of 3 parts acetic acid/ 2 parts ddH_2O/ 1 part lactic acid. A regular slide was lowered onto the cover slip and the salivary glands were squashed by tapping the cover slip with the tip of a dissecting needle and then going over the cover slip in a zigzag fashion with the dissecting needle. The slides were left at room temperature overnight and dehydrated the following day. To dehydrate the slides, three coplin jars were filled with 95% ethanol. The first jar was placed in dry ice to cool the ethanol. The slides were dipped into liquid nitrogen, the cover slip was popped off with a razor blade and the slides were immediately placed into the cold ethanol for 5 min. followed by 5 min. in each of the other two ethanol baths. The slides were then allowed to air dry. The chromosomes were prepared for denaturation by washing the slides in 2xSSC at 68°C for 30 min., a 10 min. wash in 70% ethanol at room temp and a 5 min. wash in 95% ethanol at room temp., followed by air drying. The slides were then incubated in 0.07 N NaOH for exactly 3 min. at room temp., followed by two 5 min. washes in 2xSSC, a 10 min. wash in 70% ethanol, a 5 min. wash in 95% ethanol all at room temp. and were then allowed to air dry. For three slides, 15µls of probe were added to 45µls of hybridization buffer warmed to 70°C (225µls deionized formamide, 50µls 20xSSC, 100µls 50% dextran sulfate, 125µls ddH2O). 18µls of the probe mix were dotted onto the area of the slide containing the chromosomes and a 22 mm square cover slip was added and sealed with rubber cement. The slides were incubated at 37°C in a moistened chamber, overnight. The sealed slides were then dipped into a beaker containing 2xSSC at 37°C and the cover slips were floated off. They were then washed twice at 37°C for 15 min. in 2xSSC, twice at room temp. for 15 min. in 2xSSC, once at room temp. for 10 min. in PBS (pH8.0), once at room temp. for 2 min. in PBT (PBS+0.1% Triton X-100) and twice at room temp. for 15 min. in PBS. The slides were then washed once with 75µls vectastain "Elite" solution (Vector) (1 ml PBS, 10µls vectastain solution A, 10µls vectastain solution B) and then incubated with another 75µls of solution, and covered with a 22x50 mm cover slip in a moistened chamber for 2 hours at 37°C. The cover slips were floated off in a beaker of PBS and the slides washed once for 2 min. at room temp. with PBT and twice for 20 min. at room temp. in PBS. The slides were developed by washing them twice with 100µls of DAB solution (100µls 5mg/ml DAB, 900µls PBS, 1µl 30%H_2O_2) and then incubating for 2-5 mins. in an additional 100µls of DAB solution.

They were then washed twice with PBS for 15 min. and counterstained with Giemsa (Fisher) (2.5 mls Giemsa, 50mls 10mM PO_4 buffer, pH 7.0) for 30 seconds followed by a 10 second rinse with water and allowed to air dry. The chromosomes were observed under a phase contrast microscope and mapped using cytological maps present in Lindsley and Zimm (Lindsley and Zimm, 1992).

Chromosome *in situ* probes: The plasmid DNA was linearized with the appropriate restriction enzyme and 30-50ng of the linearized DNA were boiled for 2 min., and put on ice. To this, 2µls of 10x Hexanucleotide mix (Boehringer-Mannheim), 3µls of each dATP, dCTP, and dGTP, 5µls bio-16-dUTP (Enzo diagnostics), 1µl Klenow, and ddH_2O were added to a final volume of 20µls. The reaction was incubated at room temp. overnight and precipitated with 1µl salmon sperm DNA (10mg/ml), 2.5µls 3M Sodium Acetate and 60µls 100% ethanol at -20°C for 20 min. The probe was pelleted at 14,000 rpm at 4°C in an Eppendorf microcentrifuge for 15 min, allowed to air dry, resuspended in 50µls hybridization solution and stored at -20°C.

Drosophila Stocks

Canton-S was used as wild-type. pGMRrpr46(White et al., 1996), pGMRhid (Bergmann et al., 1998), pGMRgrim (Chen et al., 1996b). *Df(2R)b23lf* and *Df(2R)S246.5* were obtained from Kristi Wharton; *Df(3L)w5.4* was obtained from Wayne Johnson; *Df(3L)ΔAK1* was obtained from Hugo Bellen; *Df(3R)B79* was obtained from James Posakony; *Df(3R)Espl5* was obtained from Spyros Artavanis-Tsakonas. All other stocks mentioned were obtained from the Bloomington *Drosophila* Stock Center.

in vitro Binding Assays

GST-Reaper, GST-Hid, and GST alone were used to assay binding to *in vitro* translated Nap-1 and TCTP protein as described in Goyal *et al.* (2000). Nap-1 was *in vitro* translated from pET25b+ containing the ORF of Nap-1 inserted into the *Xho1* and *Nde1* sites. 6-15 (TCTP) was *in vitro* translated from pOT2 GM13045 obtained from Research Genetics.

Single Embryo PCR

The adaptation of Gloor and Engel's single fly PCR method by Garozzo and Christensen was used (Gloor and Engels, 1992; Garozzo and Christensen, 1994). Briefly, single embryos were squashed in 10 µl of extraction buffer (10mM Tris pH 8.2, 1mM EDTA, 25mM NaCl, 200 µg/ml proteinase K) using a small pestle in a 0.5 ml Eppendorf tube. The mix was incubated at 37°C for 30 minutes and then at 95°C for 2 minutes after which it

was stored at 4°C. The PCR mix was assembled as follows: 1µl embryo mix, 10µls 10xPCR buffer, 2µls 10mM dNTPs, 0.2µl of 100 pmol/µl for each primer, 84.1µls ddH_2O. The PCR mix was incubated for 5 min. at 95°C and then 2µls of Taq polymerase were added. Thirty-five cycles were run as follows: 45 sec. 95°C, 45 sec. 56°C, 1 min. 72°C. A 7 min. extension at 72°C was then run before cooling to 4°C. PCR products were run out on a 3% agarose gel (for 100 mls: 2g regular agarose (Sigma A-1069) and 1 g NuSieve GTG agarose (FMC) in TBE).

Western Blotting

Proteins extracts were prepared by using 3 OD units of yeast (i.e. 3 mls of OD_{600}=1), spinning them down at setting 4 on an IEC tabletop centrifuge for 5 min., aspirating the supernatant and lysing the cells in 100µls lysis buffer (1.85 N NaOH, 7% β-mercaptoethanol). Then 100µls of TCA and the mix was spun in a refrigerated microfuge at 14,000 rpm for 10 min. The supernatant was aspirated and the pellet resuspended in 0.5 mls of 1M Tris Base (dissolved in H_2O, pH not adjusted). After spinning for 5 min. at 14,000 rpm in a refrigerated microfuge, the pellet was resuspended in 150µls protein buffer (Sambrook) and boiled for 4 min. before being put on ice and loaded onto standard SDS-PAGE (20µls/lane) (Sambrook et al., 1989). Rainbow colored protein molecular weight markers were used (Amersham). The proteins were transferred onto Immobilon-P membranes (Millipore) at 300 mA for 1 hour at 4°C, blocked for at least one hour at room temperature in 5% nonfat dry milk in PBS and incubated overnight at 4°C with the following primary antibodies: anti-LexA DNA binding domain (1:1000; Guarente Lab), anti-GAL4 transcription activation domain (GAL4AD) (1:2000; Santa Cruz Biotechnology) and anti-GAL4 DNA binding domain (GAL4 BD) (1:1000; Santa Cruz Biotechnology). All antibodies were diluted in Iscove's Modified Dulbecco's Medium (GibcoBRL) with 5% Fetal Bovine Serum (GibcoBRL). After washing five times in PBS+0.2% Tween-20, the membranes were incubated for one hour in block containing either goat anti-mouse HRP secondary antibody (1:10,000; Jackson) for the GAL4 AD and GAL4 BD antibodies or goat anti-rabbit HRP secondary antibody (1:10,000; Jackson) for the LexA BD antibody. Detection was done using ECL reagents (Amersham). Kodak AR film was exposed for the following times: anti-LexA BD 2 seconds, anti-GAL4 AD and GAL4BD 30 seconds.

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Table 1.

Primary Characterization of Clones Obtained from the LexA-Reaper Yeast Two-Hybrid Screen

RFLY1: 0-12 Hour *Drosophila* Embryonic cDNA Library
Number of clones screened= $2x10^5$
Total number of positives= 45, grouped into 6 classes.

RFLY5: *Drosophila* Third Instar Larval Imaginal Disc cDNA Library
Number of clones screened= $2.5x10^5$
Total number of positives= 39, grouped into 13 classes.

Of these classes, ten remained that did not interact with the control baits used (see Materials and Methods) and are listed below. These were characterized further.

Interactor	Number of times isolated
RFLY1 5-1	5
RFLY1 5-2	7
RFLY5 5-15	1
RFLY5 5-21	3
RFLY5 6-7	2
RFLY5 6-8	5
RFLY5 6-15	1
RFLY5 6-19	6
RFLY5 9-6	5
RFLY5 9-24	4

Table 2.

Yeast Two-Hybrid Clones Interacting with the Reaper Bait

These ten clones interact with the Reaper bait protein but not with any of the control baits. Four represent novel proteins and six are either identical or homologous to known proteins. Three clones also interact with Hid, two of which, 5-1 and 9-6, were also isolated in an independent yeast two-hybrid screen using Hid as the bait (C. L. Wei and H. Steller, unpublished results). The library from which the individual clones were isolated is indicated in parenthesis after the clone number. RFLY1 is a 0-12 Hour *Drosophila* embryonic cDNA library and RFLY5 is a *Drosophila* third instar larval imaginal disc library.

Table 2

Interactor	Interacts w/Hid	Chromosome Location	Homology/Identity
5-1(RFLY1)	Yes	75A4	Novel
5-2(RFLY1)	No	6C7	Fumarate Hydratase
5-15(RFLY5)	No	60A9	Nucleosome Assembly Protein-1
5-21(RFLY5)	No	83B6	Novel
6-7(RFLY5)	No	98E4	Novel
6-8(RFLY5)	No	64D3	Prefoldin 4
6-15(RFLY5)	No	86D7	Translationally Controlled Tumor Protein
6-19(RFLY5)	Yes	69E1	Huntingtin Interacting Protein-1
9-6(RFLY5)	Yes	31E	UB-3D
9-24(RFLY5)	No	39D2	Cryptocephal and cAMP-dependent Transcription Factor 4

Table 3.

Effects of Deficiencies Covering the Yeast Two-Hybrid Positives on Reaper-Induced Cell Death in the *Drosophila* Eye

These deficiencies were tested based on the cytological position of the two-hybrid positives determined by chromosome *in situs*. The location has since also been determined by the Berkeley *Drosophila* Genome Project and Celera. Whenever a difference in localization was observed after the genome project results were made public, the genome project position is given in parenthesis as "actual". Several deficiencies were tested for each region whenever possible. Where no deficiencies were available "Not Tested" is entered into the table. Deficiencies in the region of clone 5-1 were not tested since they often remove *reaper*, *hid* and *grim* as well, complicating the analysis. *Df(2R)b23lf* acts as a strong enhancer of Reaper-induced eye ablation. No other deficiencies in this region show this phenotype and *Df(2R)b23lf* does not remove *nap-1* based on single embryo PCR. Clone 6-15 was remapped to 86D1-E6, therefore *Df(3R)B79* is no longer relevant for this interactor: however, this deficiency is a strong suppressor of pGMR-*reaper* without affecting pGMR-*hid* or *grim* and would therefore be worthy of further study (Figure 1). Deficiencies in the 69A region carry a mutation in *glass* on the deficiency chromosome which can affect the expression of the pGMR-*reaper* transgene and thereby lead to an eye phenotype due to the level of *reaper* expression and not to the reduction of another component in the cell death pathway.

Table 3 Deficiencies covering regions containing the yeast two-hybrid positives
(Deficiencies marked with a "*" were tested by Julie Agapite)

Interactor	Location	Deficiencies	Region removed	Effect on pGMRrpr eye phenotype
5-1	75A4			Not Tested
5-2	6C7			Not Tested
5-15	60A9	*Df(2R)b23lf*	59F8;60A3	Strong Enhancer
		Df(2R)S246.5	60A1;60A3?	No Effect
		Df(2R)G10-BR27	59F3;60A8-16	No Effect
		Df(2R)orBR-11	59F6-8;60A8-16	No Effect
5-21	89B	*Df(3R)sbd105*	88F9-89A1;89B9-10	No Effect
		Df(3R)sbd26	89B9-10;89C7-89D1	No Effect
		Df(3R)sbd45	89B4;89B10	No Effect
	(actual 83B6)			Not Tested
6-7	98E4	*Df(3R)3450*	98E3;99A6-8	No Effect*
6-8	65C	*Df(3L)w5.4*	65A;65E1	No Effect
	(actual 64D3)	*Df(3L)ZN47*	64C;65C	No Effect *
6-15	92E	*Df(3R)B79*	92B3-11;92F8-13	Suppressor
		Df(3R)Espl5	92E9;92E15	No Effect
	(actual 86D7)	*Df(3R)M-Kx1*	86C1;87B5	No Effect *
		Df(3R)cu	86C1-2;86D8	No Effect *
6-19	69A	*Df(3L)vin7*	68C8-11;69B4-5	Weak Suppressor
		Df(3L)BK9	68E;69A1	Weak Suppressor
		Df(3L)vin6	68C8-11;69A4-5	Weak Suppressor
		Df(3L)vin5	68A3;69A1	Weak Suppressor *
	(actual 69E1)			Not Tested
9-6	31E	*Df(2L)J2*	31B;32A	No Effect
9-24	79F	*Df(3L)ΔAK1*	79F;80A	No Effect
	(actual 39D2)	*Df(2L)TW161*	38A6-B1;40A4-B1	No Effect*

Table 4 (a-j).

These ten tables show the primary sequence obtained from the yeast two-hybrid library vector for each of the interactors isolated as well as the open reading frame (ORF) in-frame with the transcription activation domain in the library vector. The *EcoR1* restriction site used to insert the cDNAs into the library vector as well as the linker attached to the cDNAs are marked on the nucleotide sequence. Since the *Drosophila* genome project has completed the genomic sequence, all genes have been assigned a number as indicated. Whenever possible the full-length open reading frame was used to search the databases for homologs in other species.

Table 4a.

Interacting Fragment **5-1** (279bp)

BDGP/Celera Gene CG5546

This protein is still novel to this date.

EcoR1 Linker

*GAATTC*GGCACGAGGCGCAGAACTTACCGGCGACAGGAATCCACGCTGCTCG
ACTCGGCCGGCCTGGAGACTTACGAGAAAAAGCACAAGAAGCAGAAACGCCA
TGAGGACGACAAGGAGCGCAAGAAGCCCAAAAAGGAGAAAAAGCGTAAAAAG
AAAAACCAGAGCCCCGAACCGGCGTGGGCTTGCTGCCCGGAGAGCGAGCTTG
GGCGCCGGCGGGTGTAATGGGAGCCACTAGTCTGGGATCGCTGGGAGGAGG
ACCTGGAGGACCCGGGCTGT

ORF in frame with transcription activation domain (91aa)

GTRRRTYRRQESTLLDSAGLETYEKKHKKQKRHEDDKERKKPKKEKKRKKKNQ
SPEPAWACCPESELGRRRVXWEPLVWDRWEEDLEDPGC

Table 4b.

Interacting Fragment **5-2** (1633bp)

BDGP/Celera gene CG4094

This protein is 60% identical and 66% similar to a vertebrate fumarate hydratase mitochondrial precursor (fumarase).

Eco R1 Linker

*GAATTC*GGCACGAGGCGGCTTCGCGGCGTTCAACAAATGACGAAACCCTGGG
CCGCCATTGGAAGTTTGCGTCTGGCCTCCCAGGAGTTTCGCGTGGAGAGCGAC
ACCTTTGGCGAACTGAAGGTGCCCGCGGATAAGTACTATGGCGCCCAAACGAT
GCGATCCCAGATCATTTCCCATCGGCGGACACCGCCGAACGGATGCCCAAACC
AGTGGTGCAGGCCATGGGCATCCTGAAGAAGGCCGCCGCCGAGGTGAACAAG
GAGTTCGGACTGGACAGCAAGGTTAGCGAGGCGATCTCGAAGGCGGCCGACG
ATGTGATGATCTCTGGCAAGCTATACGACGACCACTTCCGCTGGTCATCTGGCA
ACGGGCTCGGGCACGCAGAGCAACATGAATGTGAATGAGGTGATCAGCAATC
GTGCCATTGAGCTGCTGGGCGGCAAACTGGGCTCCAAGACGCCCGTGCATCCC
AACGATCATGTGAATAAATCGCAGAGCTCCAACGATACCTTCCCCACCGCCATT
CACATCTCGGTGGCGCTGGAGCTGAACAACAACCTTAAGCCGGCGATTAAGAC
GCTGCACGATGCGCTGCGTGCCAAGTCGGAGGAGTTCAAGGACATTATCAAGA
TCGGACGCACGCATACGAGGATGCGGTGCCATTGACGCTGGGCCAAGAGTTC
AGCGGCTATGCCCAGCAATTGCCTACGCCCAGGAGCGCATCGATGCCTGTCTG
CCGCGCGTCTATGAGCTGGCTCTGGGCGCACTGCCGTGGGTACGGGTCTGAA
CACACGCAAGGATTCGCCGAGAAGTGCGCTGCAAAGATCGCCGAGCTGACCA
GCCTGCCCTTCGTTACCGCGCCCAACAAGTTCGAGGCACTGGCTGCCCGCGAT
GCCATGGTGGAGGTGCATGGTGTGCTCAACACGATCGCCGTTAGCCTGATGAA
GATTGCCAACGATATTCGTTTCCTTGGCTCCGGACCGTTGCGGTTTGGGTGAGT
CTCGCTGCCGGAGAACGAGCCCGGTAGCCTCATCATGCCGGCAAGGTGAATCC
CACGCAATGCGAGTCGCTGACAATGCTCTCCGCCCAGGTGATGGGCAATCAGG
TGGCGGTGACCATCGGTGGCTCCAATGGGCACTTCGAGCTGAAGTATTCAAGC
CCCTGATCGTGTCCAATGTCTGCGCTCCATTCGGCTATTGTCTGATGGCAGCAG
GACCTTCACTGCCAACTGTGTTGAATGGCATCCAGGCGAACCGCGAGAATATT
GCCAAGATCATGAACGAGTCGCTTATGCTGGTGACCGCTCTGAATCCGCACATT
GGTTACGACAAGGCTGCGAAGATCGCAAAGACGGCGCACAGAATGGCACCAC
ACTCAAGGAGGAGGCCATCAATTTGGGCTACCTGACGGAGCAGCAATTCAACG
ACTGGGTGCGACCCGAACAGATGCTGGGACCCAAGTGAAGTGGAGCCAAGTA

GGCCGACGACGAAATCCATCATTCAATCATCGCTGTCATCGGAATCGCAGTGTA
ATTTTCATGACTCAGATCGTTTTGTTGTATATGAATCGCTCGGAAACACAGAACC
AATAATAATTGAAAATGCTGAAGCAAAAAAAAAAAAAAAAAAAA

ORF in frame with the transcription activation domain (77aa)

EFGTRRLRGVQQMTKPWAAIGSLRLASQEFRVESDTFGELKVPADKYYGAQTMRS
QIISHRRTPNGCPNQWCRPWAS

Table 4c.

Interacting Fragment **5-15** (497bp)

BDGP/Celera gene CG5330

Identical to NAP-1, the *Drosophila* homolog of the yeast and vertebrate nucleosome assembly protein 1.

EcoR1 Linker

*GAATTC*GGCACGAGGCGCAGCCCACCAGAGGTCCCGAGCGACCAGGAGGAAG
TCGACGACGACTCCCAGAGATCCTGGCCAGGAGGAAGTCGACGACGACTCCC
AGCAGTTCTGGCCACCGACTTCGAAATTGGTCACTTCTTGCGCGCCAGAATTAT
TCCAAAAGCAGTGCTTTACTACACTGGCGACATCGTCGACGATGAGGACGATG
AGGACGAAGAGGAGTATGACGAGAACGAGGAGGATGAGTACGACGACGACG
ATGCACCGCCACCAAAGGGCCCCCAAATCAGCCGGTATCAAGAAGCAGTCGCC
CAACGACTGCCCGAATCAGTAGGTTGATGTGGCTGGATTACACATGTGTAATGT
ATATACATAATTCAAATTTGATTTGAAATATATTTTTGGTGAATAAAAATAAAAAC
AACAATTACAAACGATATGAGCTGAATCCGAATAAACCTACATGATATTAGAAA
AAAAAAAAAAAAAAAAAA

ORF in frame with the transcription activation domain (113aa)

EFGTRRSPPEVPSDQEEVDDDSQRSWPGGSRRRLPAVLATDFEIGHFLRARIIPKAV
LYYTGDIVDDEDDEDEEEYDENEEDEYDDDDAPPPKGPQISRYQEAVAQRLPESVG

Table 4d.

Interacting Fragment **5-21** (933bp)

BDGP/Celera gene CG2907

The gene product encoded by this gene is novel to this date and is not identical to the open reading frame fused to the LexA DNA binding domain in the two-hybrid vector. The short open reading frame is therefore likely to not be physiologically expressed.

EcoR1 Linker

*GAATTC*CGGCCCGAGGCGCATAATTCTGGATCGGATCATGAATTTCGCGGGCA
AAGTGGTCTTTTTACGGGAGCAGCTCCGGAATCGGAGCTGCAACGCCATTAAT
TTGCCAAGTACGGCGCCTGTCTGGCTCCAATGGACGCATGTGGAGAACCTGAA
GAAGGTAGCCGCTGGAGTGCAGCAAGGTGGTGGGCGACATTGCCAAGGAGGC
GGACACCCAGAGGATTTGGTCGGAAACCCTGCAGCAGTACGGCAAATTGGATG
TGCTGGTCAACAATGCCGGAATCATCGAGACGGGCACCATCGAGACGACTAGC
CTGGAGCAGTACGACCGCGTCATGAACACCAACCTGAGGGCAATCTACCACCT
TACTATGCTGGCCACCCCCGAGCTGGTCAAGACCAAGGGCAACATCGTGAACG
TGTCCAGTGTCAATGGATTCGCTCCTTCCCTGGCGTTCTGGCCTACAACATATC
CAAAATGGGAGTGGATCAGTTCACCCGCTGTGTGGCGTTGGAGCTGGCTGCCA
AGGGTGTGCGCGTGAACTGCGTGAATCCCGGCGTGACGGTCACCAATCTGCAT
GCCCGCGGCGGCATGGATGCGGAGACGTACAAAAAGTTCCTGGAACACTCCA
AGACCACCCATGCCTTGGGTCGTCCTGGAGATGTCAAGGAGGTGGCTGCGGC
CATTGCCTTCCTGGCCAGCGATGAGGCCAGCTTCAGACTGGAGTCAGCCTGCC
GGTGGACGGCGGTCGCCATGCCATGTGCCCACGCTAATCCCACTGGACATGGA
ACTTTTAGTTTCCACTGCATGGATTCTTCCATCATGAGTAAATGCATTTAATCCG
ATTTTTTTAATTGTTATTTTGATTTACCTATTGTTTTGATTGTGTACATAAAACTGA
TTGCCATTTAAAAAAAAAAAAAAAAAA

ORF in frame with the transcription activation domain (52aa)

EFRPEAHNSGSDHEFRGQSGLXYGSSSGIGAATPLICQVRRLSGSNGRMWRT

Table 4e.

Interacting Fragment **6-7** (740 bp)

BDGP/Celera gene CG1646

This protein is novel to this date.

EcoR1 Linker

*GAATTC*GGCACGAGGCGCCCAAAAGTCGATGAGCAGGAGGTAGTCGAAATTAT
GGATAAGTTCATGGCTCGTGCGGACATCGAACCCGATCAGAAGGTTTTGTTTG
CCCAGCGCAAAGTGGAGTTCCTTGAGGATTTCGGCAGCACGGCCAGGGGTCTA
CAAGACGCACAGCGCGCTCTGCAGCAGGCACTCACTAAAGCCAAGGAAGCAC
AAAAGAAGAGTGATGGCAGTCCTAGTCGCAAAAACTCATCAAGTAGCAAAGAA
GGTCTGTCCCCACAGGATCCGCAGCGGCAGCTTACAACAATGGTGGCTCGGCC
GCAGCAGTCGCAGGCTACAACTATGGAGCAGCAAATCCATACTATGGTCAGCA
GAATACCGCGGCAGCCTTATCCCTCACAGACGCCACAACAGGCGTCCTACGAC
TCTTACTACAATCAGTGGGGCTACGGTTCCGGTGGGGCCAGCGCCAACAGTGG
TGGTAGCGGCTACAACTACGGCACGTGGAGCGGTTATGGCAACTACTATTAAG
TTCGCTGCCTAGATGTAACCCACTCCACCACCTGTAGCTCTAAGACGCTACGGC
TTGTTTAGTTTTGCCTTGCTGCTCTGTTAAGTTGTACTCATCTAATGTAATTTTTT
ATCAACCCCTATTTGAGCCCACAAAATAACCCCACCATTGCGAAAAATAAAATA
TACCAGCTTTTTATCGGTCCACCATAAAAAAAAAAAAAAAAAAAAA

ORF in frame with the transcription activation domain (175aa)

EFGPRRPKVDEQEVVEIMDKFMARADIEPDQKVLFAQRKVEFLEDFGSTARGLQDA
QRALQQALTKAKEAQKKSDGSPSRKNSSSSKEGLSPQDPQRQLTTMVARPQQSQA
TTMEQQIHTMVSRIPRQPYPSQTPQQASYDSYYNQWGYGSGGASANSGGSGYNY
GQWSGYGNYY

Table 4f.

Interacting Fragment **6-8** (567bp)

BDGP/Celera gene CG10635

The gene product has a 120 aa region that is 37% identical and 64% similar to vertebrate Prefoldin 4.

EcoR1 Linker

*GAATTC*GGCACGAGGCGGTTTGTAAAATATCATAAATTATTTATTAAATTAATTA
AAACCAACGGGAAAATGGCAGCAAAAGTAGCGAGCGGCACGAACAAAGTGTT
CCAGAACCACGATGACGTGCACATCTCCTTCGAGGATCAGCAGCGGATCAACC
GCTTTGCGAAGCACAACGCCCGCATGGATGACTTCAAAGCGGAGCTGGAAACG
AAGCGCAATGAGCTGAAGAGCTTGGAGGAGGCGCTGGAGGAGATTGAGCTGT
TCGACGAGACGAGGACATACCGTTCCTGGTTGGCGAGGTGTTCCTCTCGCACA
AGCTGAAGAAAACACAGGACATGCTGAAGGAGACCAAGGAGCAGGTGCTCAA
GGAGTACGCCGTCGTGGAAGCCAAGGCGAAGGTTATCAAGGCGGAAATGGAC
GAGCTGAAGGCTCATCTGTATCAGCGATTCGGCAGCAATATCTCACTAGAGGC
CGAGGATTAATCCACCATTCATTGATCAATTGTTAATTTAACATTTTACAAAATA
GAGTACATTTAATTTAAACTCAAAAAAAAAAAAAAAA

ORF in frame with the transcription activation domain (161aa)

EFGTRRFVKYHKLFIKLIKTNGKMAAKVASGTNKVFQNHDDVHISFEDQQRINRF
AKHNARMDDFKADVETKRNELKSLEEALEEIELFDEDEDIPFLVGEVFLSHKLEKT
QDMLKETKEQVLKEIAVVEAKAKVIKAEMDELKAHLYQRFGSNISLEAED

Table 4g.

Interacting Fragment **6-15** (786bp)

BDGP/Celera gene CG4800

This gene product is 48-49% identical and 65-66% similar to vertebrate translationally controlled tumor protein (TCTP).

EcoR1 Linker

*GAATTC*GGCACGAGGCGCGCATCCGCCGAGGAGGCCGACGAGGGCACCGATA
TCACCTCGGAGTCTGGCGTGGATGTTGTGCTTAACCACCGACTGACGGAGTGC
TTCGCCTTCGGCACAAGAAGTCGTACACCCTCTACCTGAAGGACTACATGAAGA
AGGTGCTGGCCAAGCTGGAGGAGAAGTCGCCCGACCAGGTCGACATCTTCAA
GACCAACATGAACAAGGCCATGAAGGACATCCTTGGCCGCTTCAAGGAGCTGC
AGTTCTTCACCGGCGAATCTATGGACTGCGACGGCATGGTGGCCCTGGTGGAA
TACCGCGAAATCAACGGCGACAGCGTGCCCGTGCTCATGTTCTTCAAGCACGG
TCTGGAGGAGGAGAAATGCTAGATGGATCCCAATCCCCCAAACAAAACGGAAC
CTATACTCATGCATACCTTCTACACACATATACATATTTATCAGATGCTTAGTTAC
AATTTTGAAAGTCTTAAATTTATGTTTAAAAACGCTGACAAAAATGCCTGTCTTC
GATCAGTGCGATCTATATCTAATTTCTTTTTACCGGAGCGCTTATAAATTCGACA
TGTATGTTTATTGTTTAATCACAGCGAGCTAAACAATTCAATTGCGCCACCTATG
TAATTAAAATAATAATTTGATAGCAACACCTACAAATTAGTATGCATTTTCGCTA
AGATATCAAAAAGCAACGAAGACCGAAAATGGGAACACCAGTACTGAAATTAA
ACGGAACACCTATACAAAAAAAAAAAAAAAAAA

ORF in frame with the transcription activation domain (157aa)

EFGTRRASAEEADEGTDITSESGVDVVLNHRLTECFAFGTRSRTPLPEGLHEEGAG
QAGGEVARPGRHLQDQHEQGHEGHPWPLQGAAVLHRRIYGLRRHGGPGGIPRN
QRRQRARAHVLQARSGGGEMLDGSQSPKQNGTYTHAYLLHTYTYLSDA

Table 4h.

Interacting Fragment **6-19** (423bp)

BDGP/Celera gene CG10971

This protein is 28-30% identical and 45-49% similar to vertebrate Huntingtin Interacting Protein 1 (HIP-1).

EcoR1 Linker

*GAATTC*GGCACGAGGCGATTGGACGCAATGCAAAAAAATTTCCCCCCACTGCA
GCAAAGCTACAGACACGACGTTCAAGAGTTGCAACAGAACAATACTGTGCTGT
CAAATGATCTGGTCTTGGCGAGAGAAATGTGTGCCACCTTTCGGATGCAAAAT
GACGATCTTGAGATGCAACTGAATCAGAACCCAATTCTTTTACAAAAAGCTATG
GAGGAAGAAGAAAAGCACAAGTTATCGTCGGAAAAGTTTAATAAACTCAAAAC
TCTATACACAAAGATTCGGGATGAGCACATTCAGCTGCTGAGAGAGCAAAGTG
ATTGCAACAAGTCCTTAAATAAAGAGAAACAAGTAAACTCACAACTATTGTTGG
AAACTAAGGAGCTTACCAATGAAATTTCCATAACAAGAAACGTCGTAAAA

ORF in frame with the transcription activation domain (138aa)

EFGTRRLDAMQKNFXXLQQSYRHDVQELQQNNTVLSNDLVLAREMCATFRMQND
DLEMQLNQNPILLQKAMEEEEKHKLSSEKFNKLKTLYTKIRDEHIQLLREQSDCNK
SLNKEKQVNSQLLLETKELTNEISITRN

Table 4i.

Interacting Fragment **9-6** (235bp)

BDGP/Celera gene RpS27A

Identical to UB-3D, a ubiquitin-ribosomal protein fusion.

EcoR1 Linker

*GAATTC*GGCCCGAGGCGCGGCACGAGGCGTGAGGTCGAACCGTCGGATACAT
CGAAAATGTCAAAGCCAAGATTCAGGACAAAGAAGGAATTCCTCCAGATCAGC
AGCGTCTGATCTTCGCTGGCAAGCAGCTGGAAGATGGCCGCACCCTCTCTGAC
TATAACATTCAGAAGGAGTCCACTTTGCACTTGGTGCTCCGCCTGCGTGGTGGT
GCCAAGAAGCGCAAGAAGAAGAA

ORF in frame with the transcription activation domain (61aa)

VKAKIQDKEGIPPDQQRLIFAGKQLEDGRTLSDYNIQKESTLHLVLRLRGGAKKRK
KKNYS

Table 4j.

Interacting Fragment **9-24** (1247bp)

BDGP/Celera gene CG8669, *cryptocephal.* This *Drosophila* gene has a 60 aa stretch that is 50% identical and 66% similar to vertebrate cAMP dependent transcription factor 4.

EcoR1 Linker

*GAATTC*GGCCCGAGGCGAGACGCTGCTTCGCTTCCTTCTGCCGCTGATTACCA
GCTCAATCATGGCCCATCCCTTATTCTACAGCAATTAACGCCGCCGCAGTCTCC
GCCTCAATTCGATCCTTACAAACTGGCGGGCGATGCACAGCCAAAACCCGTGC
TCGTAAAGGCCGAGCAAAAGGTGCAATGCTATCCCCCTGATGTTACCCACGCA
GCATCTGCCACGCCCTTTAACTTTACCAACTGGGTAGGCGGCACGAAATTGCCC
GAGAGAACCAACTGGTCGATGATATGTCAATATCGTGCTAAGGAGCTCGAATT
GTCCACGAACTGGCAGCAGCTCAATGAGGACTGCGAGTCTCAGGCGTCTTCAT
CTTTAGACAGCAGAAGCACAGGAAGCGGAGTGTGCAGCAGCATCGCCGACGC
CGACGAGGACTGGGTTCCAGAGCTTATCAGCAGCTCCTCCTCGCCAGCTCCTA
CAACCATCGAGCAGTCTGCGTCCAGCCAAAAAAGCGTACATCGCACTACGGGC
GCGGGTGTAGAGGATCGAAAGATCAGGAAAAAGGAGCAGAACAAGAACGCTG
CCACACGATATCGCCAGAAAAAGAAACTTGAAATGGAGAATGTGCTGGGCGAG
GAGCATGTGCTGTCTAAAGAAAACGAGCAGCTTCGTCGCACTCTGCAGGAGCG
GCGCAACGAAATGCGCTATCTGCGCCAGCTGATCCGAGAGTTCTACCATGAAC
GCAAGCGCTAGGCTTTATGTTCTTTGAAATCAATTTTATCGTCGTTTGCAAAGTT
GTTATAGTTAAACCCAAGCCTCTTCCATAAACTAATTGTCATCATAATGTTTCAA
TGTAATACTATCAATAACAATCTCCGGGTTGCCCAAAATCTACAAATCACCAAA
GTACGCAGCCTGTCCTTTGTCCTACATTTTTGGCACTAATGACGAGTCTCCTGA
ATTCGGAAATATTTGTTGCCAGTGGGGCCTAAAGGCGACCCCATATCCCAAGC
AGCACGGTCCAATGTAAAAACACATTAATTGAAAAGCTCATTGATTTGTTTTTCT
GCAAATCGGCCAGTGTTGCGGCTGCTGTGCTATTTCAAAAACACTTATATTTTTA
ATCTGGTAGCATCTCGAAAGATGTAGATTTATTTTAATGTTTCATTATTTTTTTCA
TATTCAACATTGATCTGTAATAAATCAAAGGGTTTTTAAAAAAAAAAAAAAAAAA
AAAAAAAAAAAAA

ORF in frame with the transcription activation domain (204aa)

EFGPRRDAASLPSAADYQLNXGPSLILQQLTPPQSPPQFDXYKLAGDAQPKPVLVK
AEQKVQCYXPDVTHAASATPFNFTNWVGGTKLPERTNWSMICQYRAKELELSTN

WQQLNEDCESQASSSLDSRSTGSGVCSSIADADEDWVPELISSSSSPAPTTIEQSASS
QKSVHRTYGRGCRGSKDQEKGAEQERCHTISPEKET

Figure 1.

Df(3R)B79 Acts as a Strong, Specific Suppressor of the pGMR-*reaper* Eye Phenotype

pGMR-*hid*, -*reaper*, or -*grim* males were crossed to either wild-type (CS) or *Df(3R)B79* virgin females and the progeny was analyzed. Heterozygosity for the genomic region removed by the deficiency suppressed the ability of *reaper* to kill when overexpressed in the eye (compare **C** and **D**), but had no effect on *hid*- mediated (compare **A** and **B**) or *grim*-mediated (compare **E** and **F**) killing, showing that the region removed by *Df(3R)B79* (92B3-11;92F8-13) is likely to contain a gene that is a specific modifier of *reaper*.

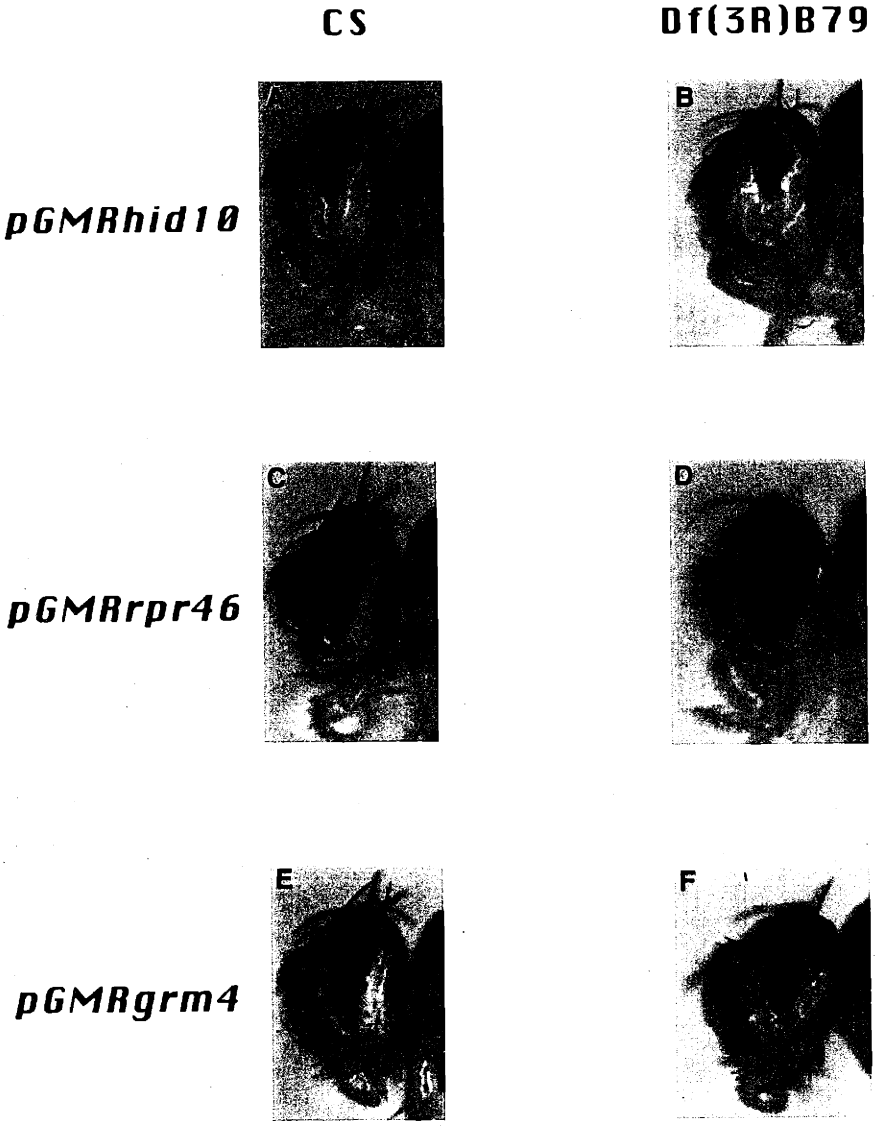

CS
Df(3R)B79
pGMRhid10
pGMRrpr46
pGMRgrm4
A
B
C
D
E
F

Figure 2.

in vitro Verification of the Reaper-Nap-1 Interaction

A shows 1/10th of the in vitro translation reaction run on 10% SDS-PAGE. Nap-1 has a predicted molecular weight of 56 kDa and the translation product of that molecular weight is marked by an arrow. **B** shows the results of a pull-down experiment run on 10% SDS-PAGE. Either GST alone, GST-Reaper, or GST-Hid was tested for binding to *in vitro* translated Nap-1. A low level of binding is seen in the GST-Reaper and GST-hid lanes (arrow), but Nap-1 does not bind to Hid in the yeast two-hybrid. Both GST-Reaper and GST-Hid pulled down *in vitro* translated Diap1 as a positive control (data not shown).

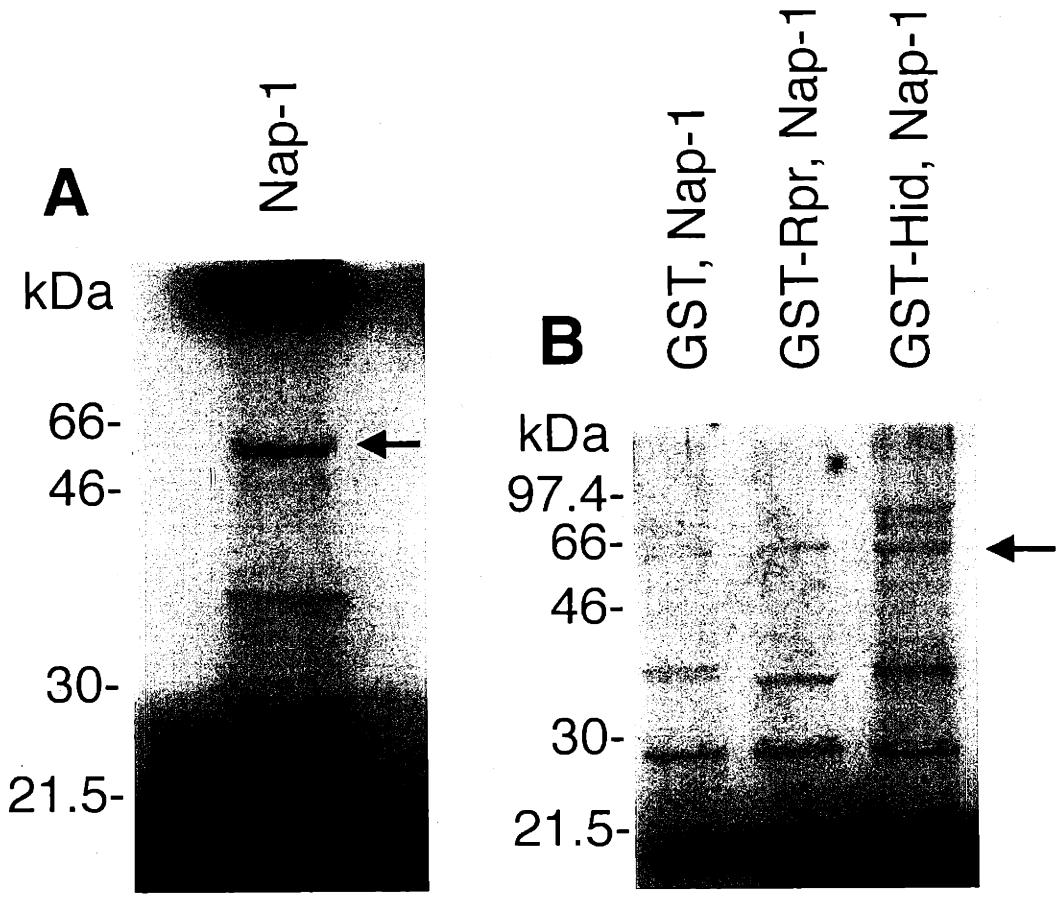
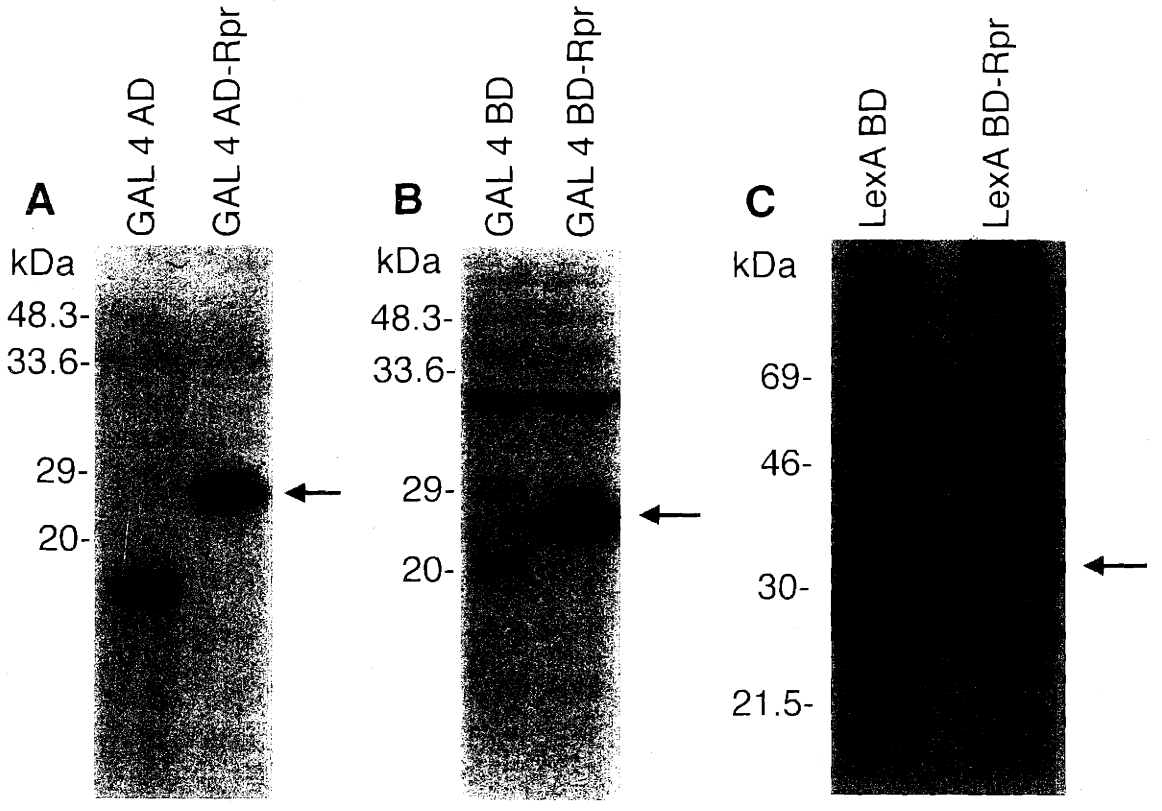

A
Nap-1
kDa
66-
46-
30-
21.5-
B
GST, Nap-1
GST-Rpr, Nap-1
GST-Hid, Nap-1
kDa
97.4-
66-
46-
30-
21.5-

Figure 3.

Expression of the Reaper Yeast Two-Hybrid Constructs.

A is a Western blot probed with an antibody against the GAL4 transcription activation domain (GAL4 AD) expressed from pACT 2 (Clontech). When the open reading frame of Reaper is fused to this protein, a larger protein is expressed (arrow). The upshift is slightly greater than the 7 kDa expected for the addition of Reaper. **B** shows a Western blot probed with an antibody against the DNA binding domain of GAL4 (GAL4 BD) expressed from pAS2-1 (Clontech). The addition of Reaper causes the expected 7 kDa upshift in the size of the GAL4 BD (arrow). **C** shows a Western blot probed with an antibody against the LexA DNA binding domain (LexA BD) expressed from pNLex (Roger Brent). Again, the addition of Reaper adds approximately 7 kDa to the apparent molecular weight of the LexA BD (arrow).


A
GAL 4 AD
GAL 4 AD-Rpr
kDa
48.3-
33.6-
29-
20-
B
GAL 4 BD
GAL 4 BD-Rpr
kDa
48.3-
33.6-
29-
20-
C
LexA BD
LexA BD-Rpr
kDa
69-
46-
30-
21.5-

Future Opportunities

Thoughts About Current Priorities Regarding the Three Projects

Jan N. Tittel

The three experimental chapters of the thesis bring up a number of key questions that should be addressed in the future. Some of these were already raised in the discussion of the individual chapters, but are expanded upon below.

CHARACTERIZATION OF HAC-1

The *Drosophila* CED-4/Apaf-1 homolog was independently identified in three different laboratories (Kanuka et al., 1999; Rodriguez et al., 1999; Zhou et al., 1999). The initial analysis revealed that this gene is essential for some developmental cell deaths and that it acts upstream of caspases and downstream or in parallel to *reaper*, *hid*, and *grim*. Based on the analysis already performed, several key areas warrant further attention.

First there are two different splice forms of the *Drosophila* CED-4/Apaf-1 homolog reported by Kanuka *et al.* They identified both a short form, lacking the WD repeats like CED-4, and a long form containing the WD repeats like Apaf-1; both forms are pro-apoptotic. Both Zhou *et al.* and Rodriguez *et al.* only characterized the CED-4/Apaf-1 long form. The short form is expressed at very low levels and was only detected in *Drosophila* embryos, but a similar short form of Apaf-1 has also been detected in mammalian embryos. In addition, CED-4 has also been shown to exist as both a short and a long form, but the two forms have different functions, unlike the short and long forms of the *Drosophila* CED-4/Apaf-1 homolog. While CED-4 short is the more abundant form and is pro-apoptotic, CED-4 long is anti-apoptotic (Shaham and Horvitz, 1996). Given that CED-4/Apaf-1 mediated regulation of programmed cell death is likely to be quite complex, additional analysis of the significance of the short form of the *Drosophila* CED-4/Apaf-1 homolog offers the potential of furthering our understanding of this key regulator of programmed cell death.

A second area of interest concerns the issue of cytochrome *c* release during *Drosophila* apoptosis. Apaf-1 can activate caspases when it is bound to both dATP and cytochrome *c*. *Drosophila* CED-4/Apaf-1 also requires dATP, and possibly cytochrome *c*, for activation of caspases and the WD repeats are thought to be involved in binding cytochrome *c*. Cytochrome *c* release has not been carefully looked at in *Drosophila*, but it is likely to occur since it is seen in *Drosophila* tissue culture cells when apoptosis is induced and an epitope is unmasked on cytochrome *c* in the mitochondria during apoptosis (Kanuka et al., 1999; Varkey et al., 1999).

A third question to further investigate is whether the *Drosophila* CED-4/Apaf-1 homolog also physically associates with a *Drosophila* Bcl-2 family member. Both Apaf-1 and CED-4 bind to anti-apoptotic Bcl-2 family members, so it stands to reason that the *Drosophila*

CED-4/Apaf-1 homolog may be able to do so as well. Only two Bcl-2 family members have so far been identified in *Drosophila*. The *Drosophila* Bok homolog is a pro-apoptotic Bcl-2 family member, and is thus unlikely to associate with the *Drosophila* CED-4/Apaf-1 homolog, but the second Bcl-2 family member in the database still needs to be characterized and may turn out to be anti-apoptotic. If so, an interaction between it and *Drosophila* CED-4/Apaf-1 would provide further evidence that the core programmed cell death pathway functions in a similar manner in *C. elegans*, *Drosophila*, and mammals.

Finally, the transcription of the *Drosophila* CED-4/Apaf-1 homolog is regulated by UV and X irradiation (Zhou et al., 1999). *reaper* transcription is also upregulated in response to irradiation and the *reaper* promoter contains a p53 response element. This shows that irradiation can directly affect two different regulators of programmed cell death. Finding the genes that regulate *Drosophila ced-4/Apaf-1* expression in response to irradiation will help us understand how upstream signals feed into the core cell death pathway.

diap1 LOSS-OF-FUNCTION ANALYSIS

Analysis of the *diap-1* loss-of-function phenotype has yielded further insight into how this gene is involved in apoptosis. Embryos lacking zygotic *diap1* begin to display noticeable developmental abnormalities between 204 and 212 minutes after egg deposition. Within the next hour, caspases are activated in these embryos and virtually all cells are TUNEL positive, no longer adhere well to each other, and display an apoptotic morphology.

IAPs are part of a family of proteins that is defined by the presence of a Baculovirus IAP Repeat (BIR) domain. While the family was first discovered and defined in the context of programmed cell death, some of these proteins have not been shown to function in apoptosis, but rather appear to regulate cell division, cytokinesis and chromosome behavior (Li et al., 1998; Fraser et al., 1999; Uren et al., 1999; Li et al., 2000; Speliotes et al., 2000). An earlier report stated that *diap1* mutants have defects in cellularization (Moore et al., 1998), so a key question was whether *diap1* has multiple functions. By looking carefully at the progression through development and at nuclear lamins and filamentous actin, it was apparent that normal cellularization takes place in *diap1* loss-of-function embryos. However, the question of whether *diap1* has functions besides inhibiting apoptosis remains open. The most direct experiment would be to specifically block apoptosis in *diap1* loss-of-function embryos and observe if these embryos now develop further and whether they show any other developmental abnormalities. To this end, I tried to inject *p35* sense mRNA and the peptide caspase inhibitor BOC-fmk (Enzyme Systems;

100 μM final concentration in the embryo). Neither inhibitor was able to rescue the *diap1* loss-of-function phenotype, however, this may be due to an insufficient level of caspase inhibitor in the embryo. Attempts to ectopically express *p35* early in the embryo through genetics have also been unsuccessful to this date (L. Goyal and H. Steller, unpublished results). In addition, I attempted to interfere with caspase expression using the RNAi technique. I injected ds RNA for *dcp-1*, *drice*, *dcp-2/dredd* and *dronc*. None of the four ds RNAs was able to rescue the *diap1* loss-of-function phenotype on its own, and a combination of *dcp-1* and *drice* also had no effect. More caspases have now been identified and further characterized in *Drosophila* and one could try hampering the expression in different combinations. In particular, a combination of the three potential initiator caspases, *dronc*, *dcp-2/dredd* and a yet uncharacterized one in the database, should be tried. Similarly, a combination of the five potential effector caspases (*dcp-1, drice, decay,* and two uncharacterized ones in the database) should be attempted. I did not look at expression of P35 protein from the injected *p35* mRNA, an additional control that should be performed. One of these approaches may at least partially rescue the *diap-1* loss-of-function phenotype. Since the phenotype is now well characterized, a subtle or even local rescue should be readily apparent.

The second priority in carrying this project further is to understand why the *diap-1* loss-of-function embryo begins to show abnormal development over such a narrow timeperiod. The abnormalities become visible at the time when zygotic transcription starts. One explanation for this precise transition to abnormal development is that the maternal contribution of Diap1 protein is sufficient to carry the embryo through early development to the stage where zygotic transcription begins. Alternatively, an inducer of apoptosis such as an initiator caspase could be transcribed at this time and *diap1* function is required to block programmed cell death. I have started to address this question by looking at embryos laid by a mother heterozygous for *diap1*. These embryos should contain half the amount of any maternally supplied *diap1* mRNA or protein. If the level of maternally supplied *diap1* determines when the embryo begins to develop abnormally, these embryos should show abnormalities earlier than wild-type embryos after being injected with ds *diap1* RNA. The current results suggest that the maternal contribution of *diap1* mRNA does not affect the timing of the onset of abnormalities (see Chapter 3), but I would like to verify that the level of *diap1* mRNA or Diap1 protein is actually reduced in embryos laid by heterozygous mothers. Another approach would be to block zygotic transcription using an inhibitor such as α-amanitin. This approach has potential problems since blocking transcription is likely to cause general developmental abnormalities and may even result in widespread apoptosis. Controls on wild-type embryos are therefore essential.

I currently favor a model where a zygotically transcribed gene(s) activates the programmed cell death pathway unless *diap1* is present to prevent this step over a model where the timing of the onset of developmental abnormalities is dependent on the amount of maternally supplied Diap1. This would explain why the timeperiod of the first detectable abnormalities is so narrow and why virtually every cell becomes apoptotic over a short period of time. The slow degradation of maternally supplied Diap1 is unlikely to be consistent between embryos and between cells within an embryo and thus would result in a wider spread in the timing of the observed onset of developmental abnormalities. If this model is correct, identifying the zygotically transcribed gene(s) responsible for the induction of apoptosis in *diap1* loss-of-function embryos would greatly enhance our understanding of *diap1* function and of the regulation of the programmed cell death pathway in general.

YEAST TWO-HYBRID INTERACTORS WITH REAPER

The yeast two-hybrid screen with Reaper as the bait yielded ten potential interactors that were further characterized. As discussed in the yeast two-hybrid chapter (Chapter 4), none of these interactors plays a clear role in programmed cell death at this point. Several, including 5-15(Nap-1), 6-8(Prefoldin 4), 6-15(TCTP), 6-19(Hip-1), and 9-24(ATF4) should continue to be followed in the literature because the homology of these positives points towards potential roles in programmed cell death. Also, some of the still novel interactors could turn out to be interesting once homologs are identified in other organisms, now that more genomic information is continuously being made available. I have successfully used RNAi to further our understanding of the *Drosophila ced-4/Apaf-1* homolog and of *diap1*, therefore I would also try to analyze the most interesting interactors using this technique. Since the mutant phenotype of the five interactors (5-15, 6-8, 6-15, 6-19, 9-24) whose sequence homology is particularly interesting is not known, RNAi would be a rapid assay system to determine if any affect the amount of developmental cell death in the embryo.

Given the current tools, I would first test the Reaper bait that I used against Diap1 as a positive control. Reaper has been shown to interact with IAPs with its amino-terminus (Vucic et al., 1997). One potential problem with the Reaper bait used is that the LexA DNA binding domain is fused to the amino-terminus of Reaper, possibly interfering with interactions between Reaper and potential binding partners. I tested a Reaper bait where the LexA DNA binding domain is fused to the carboxy-terminus of Reaper (pNLexA-Reaper; pNLexA was obtained from Roger Brent), but this bait was not expressed well in yeast. If

the Reaper bait used in the two-hybrid screen is able to interact with Diap1, I would have greater confidence that the interactors from the screen are significant. Likewise, I would want to test the interactors against a mutant form of Reaper that was isolated in the genetic screen for modifiers of Reaper induced death in the *Drosophila* eye. This mutation is actually in the *reaper* transgene used for the screen and results in a point mutation in the amino-terminus of Reaper (J. Agapite, K. McCall, and H. Steller, unpublished results). Since this mutant form of Reaper shows reduced death-promoting activity, it could serve as a useful negative control. Any clones from the screen that also interact with this form of Reaper would be deemed less interesting. Finally, I would test the full-length interactor proteins against GST-Reaper to verify the interactions. I tested two interactors, 5-15(Nap-1) and 6-15(TCTP), but I was unable to see specific binding to Reaper. Instead, the proteins appeared to bind the GST-Hid and possibly the GST alone controls as well. Further refining the technique could yield more insight as to whether the interactions observed in the two-hybrid are physiologically relevant.

Another interesting observation that was made during the analysis of the two-hybrid positives was that a deficiency on the third chromosome, *Df(3L)B79*, acts as a specific and strong suppressor of the pGMR-*reaper* induced eye-ablation phenotype. Both pGMR-*grim* and -*hid* are not modified by this deficiency. However, it would be worthwhile to test a weaker pGMR-*hid* line to see if the deficiency can modify that phenotype since the degree by which the eye is reduced in pGMR-*hid10* is quite severe. While none of the two-hybrid interactors map to this region, one should carefully look at all the genes removed by this deficiency to see if any could be part of the programmed cell death pathway.

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