IDENTIFICATION OF APOPTOTIC REGULATORS IN *DROSOPHILA* AND THEIR NONAPOPTOTIC ROLES IN SPERMATOGENESIS: IMPLICATIONS FOR THE EXISTENCE OF A "CASPASE CASSETTE" WHICH REGULATES DIVERSE BIOLOGICAL PROCESSES

Thesis by

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In Partial Fulfillment of the Requirements

for the Degree of

Doctor of Philosophy

California Institute of Technology

Pasadena, California

2002

(Defended May 29, 2002)

Acknowledgments

I would like to thank Bruce Hay for being a phenomenal advisor and mentor. I have a tremendous amount of respect for him as both a scientist and a person, and have learned a great deal from him on both fronts. His insights helped drive the experiments in directions they would not otherwise have gone, and his energy and enthusiasm for science are infectious. I cannot imagine a better graduate experience than working on the forefront of a very fast-moving and exciting field under his guidance.

It has been a pleasure to interact with all members of the Hay lab, past and present; in particular I would like to recognize Chris Hawkins, Susan Wang and Soon Ji Yoo, without whose help and friendship my time at Caltech would not have been nearly as enjoyable as it was.

My family's support has been instrumental in helping me finish this degree, as it has been in everything I've done. I especially want to thank to my husband David for his love, patience and encouragement, and for being such a dedicated father. Finally, I'd like to dedicate this thesis to my son Ian, who gives me perspective and keeps my spirits up even on difficult days in the lab.

Abstract

Drosophila has long been an attractive, genetically tractable model system in which to study fundamental processes such as apoptosis which are common to higher eukaryotes. Following completion of the *Drosophila* genome sequence, we carried out comprehensive BLAST searches to annotate it with respect to apoptosis, and found sequence homologues of virtually all mammalian cell death genes with the exception of death receptors. The only *Drosophila* cell death genes for which mammalian homologues have not been identified are the cell death activators Rpr, Hid, and Grim. However, since proteins with similar activities are present in mammals and since their mechanisms are likely to be conserved even if true sequence homologues are not identified, understanding how Rpr, Hid, and Grim act to bring about death is an important area of research. To better understand their mechanisms of action, we carried out an overexpression screen to identify suppressors of Rpr-, Hid-, and Grim-induced death. We identified the strongest of these suppressors as dBruce, a large protein with an N-terminal baculovirus IAP repeat (BIR), characteristic of inhibitors of apoptosis (IAPs), and a C-terminal ubiquitin conjugation domain (E2). We show that it potently suppresses death induced by Rpr and Grim but not by Hid, and that this activity likely requires its E2 domain. It does not directly promote degradation of Rpr or Grim, but its antiapoptotic action requires that their N-termini, through which they interact with BIR2 of DIAP1, be intact. These data, combined with the inability of dBruce to block death induced by the apical

caspase Dronc or the proapoptotic Bcl-2 family member Debcl/Drob-1/dBorg-1/Dbok, suggest that dBruce regulates cell death at a novel point. Interestingly, dBruce mutant males are sterile, but a lack of increased caspase activity in these mutants suggests that dBruce may also play nonapoptotic roles. A closer look at *Drosophila* male testes revealed the surprising observation that high levels of caspases are present in wild type testes, along with the caspase activator Ark. This provokes speculation that core components of the cell death machinery can function to regulate processes other than apoptosis, such as spermatogenesis.

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CHAPTER 1

Introduction

Stephanie Y. Vernooy

For almost a century, *Drosophila* has been used as a model system for answering cellular and developmental questions (reviewed in Rubin and Lewis, 2000). The ability to combine powerful genetics with a molecular approach makes it an ideal organism in which to dissect fundamental processes common to higher eukaryotes. One such process is programmed cell death, or apoptosis. The core of the death machine consists of caspases, apoptotic activators and inhibitors. Caspases are cysteine proteases that act as the downstream effectors of apoptosis, cleaving a multitude of cellular substrates to bring about cell death (reviewed in Thornberry and Lazebnik, 1998). There are two different major pathways that lead to caspase activation. One, that of death receptor signaling, conveys proapoptotic signals from outside of the cell (reviewed in Ashkenazi and Dixit, 1999). The other inititates at the mitochondria as a result of cellular stress, such as DNA damage (reviewed in Wang, 2001). Numerous mechanisms of death inhibition exist upstream of caspase activation, including the action of antiapoptotic Bcl-2 family members (reviewed in Adams and Cory, 1998). Additionally, molecules known as inhibitor of apoptosis proteins (IAPs) function to block caspase activation or activity directly (reviewed in Stennicke et al., 2002).

Experimental evidence has made it clear for some time that *Drosophila* homologues exist for many mammalian cell death regulators and effectors (reviewed in Abrams, 1999). Upon completion of the *Drosophila* genome

sequence and before its public release, our lab undertook an annotation project to examine the full extent to which known mammalian apoptotic regulators were conserved in *Drosophila*. Our comparative analysis was based on exhaustive database searches for all known mammalian cell death genes and BLAST searches to identify homologues or conserved motifs in the *Drosophila* genome. My published review of the field of *Drosophila* apoptosis with a focus on information gained from the sequenced *Drosophila* genome is presented in Chapter 2 (Vernooy et al., 2000). Based on these searches, it is clear that *Drosophila* has homologues of essentially all mammalian death genes, with the possible exception of death receptors and antiapoptotic Bcl-2 family members. A *Drosophila* homologue of the mammalian adaptor FADD, which transduces death receptor mediated signals, has been identified (Hu and Yang, 2000). However, there is as of yet no evidence that death receptors themselves exist in the *Drosophila* genome.

With virtually all mammalian cell death genes aside from death receptors having counterparts in flies, the biggest remaining difference between the two with respect to apoptosis is the absence of mammalian homologues of the fly cell death activators Reaper (Rpr), Head involution defective (Hid), and Grim. Rpr, Hid, and Grim were the first cell death molecules identified in the fly, by virtue of a deficiency that removed them all and thereby abolished all normally occurring cell death (White et al., 1994; Grether et al., 1995; Chen et al., 1996). They share

homology only in their N-terminal 14 amino acids. Thus far, they are unique to *Drosophila*. Rpr, Hid, and Grim promote cell death through at least several mechanisms. All three, as well as the recently identified Sickle, can bind to the DIAP1 BIR2 domain through a short N-terminal motif, thereby inhibiting its ability to function as a caspase inhibitor (Wang et al., 1999; Wu et al., 2001; Christich et al., 2002; Wing et al., 2002; Srinivasula et al., 2002). Hid also stimulates DIAP1's function as a ubiquitin-protein ligase, promoting its polyubiquitination and degradation (Yoo et al., in press). Finally Rpr and Grim, but not Hid, promote a general decrease in protein translation, creating an imbalance between short lived DIAP1 and the much longer lived apical caspase DRONC, a critical target of DIAP1's prosurvival activity (Yoo et al., in press; Holley et al., in press). This latter activity may or may not be related to the ability of Rpr and Grim to promote cell death in the absence of their N-terminal DIAP1 interaction motif (Wing et al., 1998; Wing et al., 2001). Although true mammalian homologs of Rpr, Hid, and Grim have not yet been described, several proteins have been identified that disrupt IAP-caspase interactions through mechanistically similar interactions involving a Rpr-Hid-Grim-like N-terminal motif (reviewed in Shi, 2002). In addition, other observations point towards the existence of mammalian proteins that can stimulate IAP polyubiquitination and degradation (Yang et al., 2000). Thus, it is clear that the mechanisms by which Rpr, Hid, and Grim act, and are regulated, are conserved.

Therefore, it has been the goal of our lab as well as others to understand how Rpr, Hid, and Grim function to bring about cell death and how they are regulated. When I came to the lab early in my first year, I joined several others who were in the process of carrying out a genetic screen to identify suppressors of Rpr-, Hid- and Grim-induced death. Three SURF students, Julius Su, Koen Verbrugghe and Jennifer Yang, and a technician, Susannah Cole, began the screen looking for suppressors of Rpr. I, with the help of a technician, Asya Pogodina, carried out the Grim portion of the screen. Soon Ji Yoo screened for Hid suppressors. Technicians Becky Green-Marroquin and Paula Agguire helped with various aspects of screening. This screen, and a summary of its results, is discussed In Chapter 3. The suppressors identified in our screen will likely include components of the pathways described above, and may also shed light on other pathways through which Rpr, Hid, and Grim act.

I spent my second and third years trying to identify several of the suppressors that came out of the screen. This involved doing plasmid rescues and sequencing into the genomic DNA to find out where the P element had inserted into the genome, followed by the tedious process of using sequentially larger plasmid rescue fragments to screen cDNA libraries in the hopes of finding candidate genes. Eventually, I chose to narrow my focus to the strongest of the suppressors: SMF and VS3. For both I was able to identify candidate genes which were overexpressed in the developing eyes of suppressor flies. SMF in

particular was near a couple of genes that seemed quite interesting. One was a homolog of the yeast gene DPH5. DPH5 is a methyltransferase responsible for modifying a histidine residue in elongation factor 2, thereby inactivating it, in response to diptheria toxin (Chen and Bodley, 1988). The other, which was facing the opposite direction and which we thought might be knocked out in our suppressor line by means of RNAi, was a component of the COP9 signalsome (reviewed in Schwechheimer and Deng, 2001). However, when I made transgenic flies expressing either DPH5 or an RNAi construct corresponding to the COP9 subunit, I was not able to recreate the Rpr, Hid or Grim suppression. We finally discovered that there was an additional P element in this line, at the DIAP1 locus. That ended my work on SMF.

VS3 began as a similar story, in that the transgenic flies that I made to overexpress what I though was a reasonable candidate gene did not have a suppressor phenotype. It was at about this time that the *Drosophila* genome was completed, and proved invaluable to my work. Now I was able to scan the region of the P element insertion and immediately see genes as far away as I was interested in looking. When I did this, I found that there was a baculovirus IAP repeat (BIR)-containing protein roughly 20 kb downstream of the P element insertion. Since IAPs are inhibitors of apoptosis, this was an exciting candidate. It was farther away than we had imagined that our eye-specific promoter/enhancer could act, and without having the genome sequence we

possibly would never had made it that far away from the P element in our search. RNA in situs on eye imaginal discs showed that this gene was in fact overexpressed in the VS3 suppressor line. I spent the next couple of years working to characterize this suppressor, and present this analysis in Chapter 4. The gene responsible for the suppression phenotype is a homologue of mammalian Bruce, a large protein that contains an N-terminal baculovirus IAP repeat (BIR) and a C-terminal ubiquitin conjugation domain (E2) (Vernooy et al., in press). Ubiquitin-conjugating activity has been demonstrated for mouse Bruce (Hauser et al., 1998). Human Bruce upregulation occurs in some cancers, and may participate in chemoresistance of these cells (Chen et al., 1999). However, those two observations represent the entire literature on Bruce. The normal functions of Bruce and how it acts were unknown. My work demonstrates that *Drosophila* Bruce (dBruce) can potently inhibit cell death induced by Rpr and Grim, but not Hid, and that its E2 domain is likely required for this activity. I also explore its ability to inhibit death induced at various points in the apoptotic program in an attempt to identify its site of action.

Flies carrying mutations in dBruce are viable; however, homozygous males are sterile. This prompted us to look at *Drosophila* male testes, and led to the observation that there is a large amount of caspase activity in developing spermatids of wild-type male *Drosophila*. This was consistent with the earlier

observation that Jun Huh, a graduate student in the lab, had made, noticing that his antibodies against active caspases also recognize mature sperm.

I then examined testes for the presence of a number of death regulators during spermatogenesis (Vernooy et al., in preparation). These observations form the basis for Chapter 5. The lack of cell death in the presence of significant caspase activity as well as the presence of other apoptotic regulators suggests the possibility that a "caspase cassette," made up of core components of the apoptotic machinery, can regulate cellular processes other than apoptosis, and suggests interesting directions for future research.

References

Abrams, J. M. (1999). An emerging blueprint for apoptosis in *Drosophila*. *Trends Cell Biol. 9*:435-440. Review.

Adams, J. M. and Cory, S. (1998). The Bcl-2 protein family: arbiters of cell survival. *Science 281*, 1322-1326.

Ashkenazi, A. and Dixit, V. M. (1999). Apoptosis control by death and decoy receptors. *Curr. Opin. Cell Biol. 11*, 255-260.

Chen, J. Y. and Bodley, J. W. (1988). Biosynthesis of diphthamide in Saccharomyces cerevisiae. Partial purification and characterization of a specific S-adenosylmethionine:elongation factor 2 methyltransferase. *J. Biol. Chem. 263*:11692-11696.

Chen, P., Nordstrom, W., Gish, B. and Abrams, J. M. (1996). grim, a novel cell death gene in *Drosophila*. *Genes Dev. 10*:1773-1782.

Chen, Z., Naito, M., Hori, S., Mashima, T., Yamori, T. and Tsuruo, T. (1999). A human IAP-family gene, apollon, expressed in human brain cancer cells. *Biochem. Biophys. Res. Commun. 264*, 847-854.

Christich, A., Kauppila, S., Chen, P., Sogame, N., Ho, S. I. and Abrams, J. M. (2002). The damage-responsive *Drosophila* gene sickle encodes a novel IAP binding protein similar to but distinct from Reaper, Grim, and Hid. *Curr. Biol. 12*, 137-140.

Grether, M. E., Abrams, J. M., Agapite, J., White, K., and Steller, H. (1995). The head involution defective gene of *Drosophila melanogaster* functions in programmed cell death. *Genes Dev. 9*:1694-1708.

Hauser, H. P., Bardroff, M., Pyrowolakis, G. and Jentsch, S. (1998). A giant ubiquitin-conjugating enzyme related to IAP apoptosis inhibitors. *J. Cell Biol. 141*, 1415-1422.

Holley, C. L., Olson, M.R., Colon-Ramos, D. A. and Kornbluth, S. (2002). Reaper eliminates IAP proteins through stimulated IAP degradation and generalized translational inhibition. *Nature Cell Biol.* (in press).

Hu, S. and Yang, X. (2000). dFADD, a novel death domain-containing adapter protein for the *Drosophila* caspase DREDD. *J. Biol. Chem. 275*,30761-30764.

Rubin, G. M. and Lewis, E. B. (2000). A brief history of *Drosophila's* contributions to genome research. *Science 287,* 2216.

Schwechheimer, C. and Deng, X. W. (2001). COP9 signalosome revisited: a novel mediator of protein degradation. *Trends Cell Biol. 11*,420-426.

Shi, Y. (2002). Mechanisms of caspase activation and inhibition during apoptosis. *Mol. Cell 9*, 459-470.

Srinivasula, S. M., Datta, P., Kobayashi, M., Wu, J-W., Fujioka, M., Hegde, R., Zhang, Z., Mukattash, R., Fernandes-Alnemri, T., Shi, Y., Jaynes, J. B. and Alnemri, E. S. (2002). *sickle*, a novel *Drosophila* death gene in the *reaper/hid/grim* region, encodes an IAP-inhibitory protein. *Curr. Biol. 12*, 125-130.

Stennicke, H. R., Ryan, C. A. and Salvesen, G. S. (2002). Reprieval from execution: the molecular basis of caspase inhibition. *Trends Biochem. Sci. 27*, 94-101.

Thornberry, N. A. and Lazebnik, Y. (1998). Caspases: enemies within. *Science 281*, 1312-1316.

Vernooy, S. Y., Copeland, J., Ghaboosi, N., Griffin, E. E., Yoo, S. J. and Hay, B. A. Cell death regulation in *Drosophila*: conservation of mechanism and unique insights. (2000). *J. Cell Biol. 150*, F69-76.

Vernooy, S. Y., Chow, V. F., Su, J., Verbrugghe, K., Yang, J., Cole, S., Holley, C. L. and Hay, B. A. (2002). *Drosophila* Bruce (dBruce) is a potent suppressor of Rpr- and Grim-, but not Hid-dependent cell death. *Curr. Biol.* (Submitted).

Vernooy, S. Y., Huh, J. R. and Hay, B. A. (2002). *Drosophila* spermatogenesis is associated with high levels of caspase activity and requires multiple apoptosis regulators, including Ark and Bruce, in nonapoptotic roles. (In preparation).

Wang, S. L., Hawkins, C. J., Yoo, S. J., Muller, H. A. and Hay, B. A. The *Drosophila* caspase inhibitor DIAP1 is essential for cell survival and is negatively regulated by HID. (1999). *Cell 98*, 453-463.

Wang, X. (2001). The expanding role of mitochondria in apoptosis. *Genes Dev. 15*, 2922-2933.

White, K., Grether, M. E., Abrams, J. M., Young, L., Farrell, K., and Steller, H. (1994). Genetic control of programmed cell death in *Drosophila*. *Scienc. 264*, 677-683.

Wing, J. P., Zhou, L., Schwartz, L. M. and Nambu, J. R. (1998). Distinct cell killing properties of the *Drosophila reaper*, *head involution defective*, and *grim* genes. *Cell Death Differ. 5*, 930-939.

Wing, J. P., Schwartz, L. M. and Nambu, J. R. (2001). The RHG motifs of *Drosophila* Reaper and Grim are important for their distinct cell death-inducing abilities. *Mech. Dev. 102*, 193-203.

Wing, J. P., Karres, J. S., Ogdahl, J. L., Zhou, L., Schwartz, L. M. and Nambu, J. R. *Drosophila* sickle Is a novel Grim-Reaper cell death activator. *Curr. Biol. 12*, 131-135. (2002).

Wu, J. W., Cocina, A. E., Chai, J., Hay, B. A. and Shi, Y. (2001). Structural analysis of a functional DIAP1 fragment bound to Grim and Hid peptides. *Mol. Cell 8*, 95-104.

Yang, Y., Fang, S., Jensen, J. P., Weissman, A. M. and Ashwell, J. D. (2000). Ubiquitin protein ligase activity of IAPs and their degradation in proteasomes in response to apoptotic stimuli. *Science 288*, 874-877.

Yoo, S. J., Huh, J. R., Muro, I., Yu, H., Wang, L., Wang., S. L., Feldman, R. M. R., Clem, R. J., Muller, H. A. and Hay, B. A. (2002). Apoptosis inducers Hid, Rpr and Grim negatively regulate levels of the caspase inhibitor DIAP1 by distinct mechanisms. *Nature Cell Biol.* (in press).

CHAPTER 2

Cell death regulation in *Drosophila*: Conservation of mechanism and unique insights

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published in J. Cell Biol. 150(2), F69-76, July 2000

Abstract

Programmed cell death, or apoptosis, is a genetically encoded form of cell suicide that results in the orderly death and phagocytic removal of excess, damaged or dangerous cells during normal development and in the adult. The cellular machinery required to carry out apoptosis is present in most, if not all, cells, but is only activated in cells instructed to die (reviewed in Jacobson et al., 1997). Here we review cell death regulation in the fly in the context of a first pass look at the complete *Drosophila* genome and what is known about death regulation in other organisms, particularly worms and vertebrates.

Caspases: the core of the cell death machine

The caspase family of cysteine proteases is central to apoptotic signaling and cell execution in all animals that have been studied, including worms, flies, and vertebrates (Thornberry and Lazebnik, 1998). As with many proteases, caspases are synthesized as inactive zymogens known as procaspases, and are generally thought to be present in all cells at levels sufficient to induce apoptosis when activated. Death stimuli lead to one or more cleavages C-terminal to specific aspartate residues. These cleavage events separate the large and small subunits that make up the active caspase. Two sets of these subunits assemble to form the active caspase heterotetramer, which has two active sites. Frequently an N-terminal prodomain is also removed during caspase processing. An important point is that the sites cleaved to produce an active caspase often correspond to caspase target sites. Thus, once activated, caspases can participate in proteolytic cascades.

Caspases play two roles in bringing about the death of the cell. They transduce death signals that are generated in specific cellular compartments and they cleave a number of cellular proteins, resulting in the activation of some and the inactivation of others. These latter cleavage events are thought to lead, through a number of mechanisms, to many of the biochemical and morphological changes associated with apoptosis. Caspases that act as signal transducers (known as

apical or upstream caspases) have long prodomains. These regions contain specific sequence motifs (known as death effector domains (DEDs) or caspase recruitment domains (CARDs) that are thought to mediate procaspase recruitment into complexes in which caspase activation occurs in response to forced oligomerization (Budihardjo et al., 1999). Some caspases may also become activated as a consequence of prodomain-dependent homodimerization (Kumar and Colussi, 1999). Once activated, long prodomain caspases are thought to cleave and activate short prodomain caspases (known as downstream or executioner caspases) that rely on cleavage by other caspases for activation. This review focuses on caspases as cell death regulators. However, it is important to note that in mammals and flies mutant phenotypes suggest caspases can also play important nonapoptotic roles (Song et al., 1997; Zheng and Flavell, 2000), and the functions of a number of caspases are still unclear.

For much of our analysis of the *Drosophila* genome we used the BLAST search programs available through the BDGP (http://www.fruitfly.org/). Motif search programs were also sometimes used. Instances in which use of these latter programs resulted in the identification of proteins that were not identified using the standard BLAST server are indicated in the text. *Drosophila* encodes three long prodomain caspases, *dcp-2/dredd* (Inohara et al., 1997) (Chen et al., 1998), *dronc* (Dorstyn et al., 1999a), and *dream* (accession no. AF275814), as well as four caspases with short prodomains, *dcp-1* (Song et al., 1997), *drICE* (Fraser

and Evan, 1997), *decay* (Dorstyn et al., 1999b) and *daydream* (accession no. AF281077). An eighth *Drosophila* caspase, a head-to-head partial duplication of *daydream*, is likely to be nonfunctional because of numerous mutations (including premature stop codons and deletions). There is also good evidence that cell death in the fly is caspase-dependent (reviewed in Abrams, 1999). The *C. elegans* genome encodes three caspases, the known apoptosis inducer *ced-3* (Yuan et al., 1993), and *csp-1* and *csp-2* (Shaham, 1998), all of which have long prodomains. Fourteen caspases have been identified in mammals, 10 of which have long prodomains (Budihardjo et al., 1999).

All long prodomain caspases identified to date in mammals contain either CARD or DED sequences. In contrast, both *Drosophila* and *C. elegans* encode caspases that have long prodomains with unique sequences, as well as a single caspase with a CARD (Fig. 1). The unique prodomain sequences in these caspases may promote death-inducing caspase activation in response to unknown stimuli. Alternatively, they may regulate caspase activation in contexts other than cell death. Several *Drosophila* and *C.elegans* caspases, Dronc and Csp-1a and Csp-2a, respectively, are unique in a second way as well. Caspases are described as being specific for cleavage after aspartate, and typically have an active site that conforms to the consensus QAC(R/Q/G)(G/E) (catalytic cysteine is underlined). Dronc, Csp-1a and Csp-2a have active sites that differ in the first two positions.

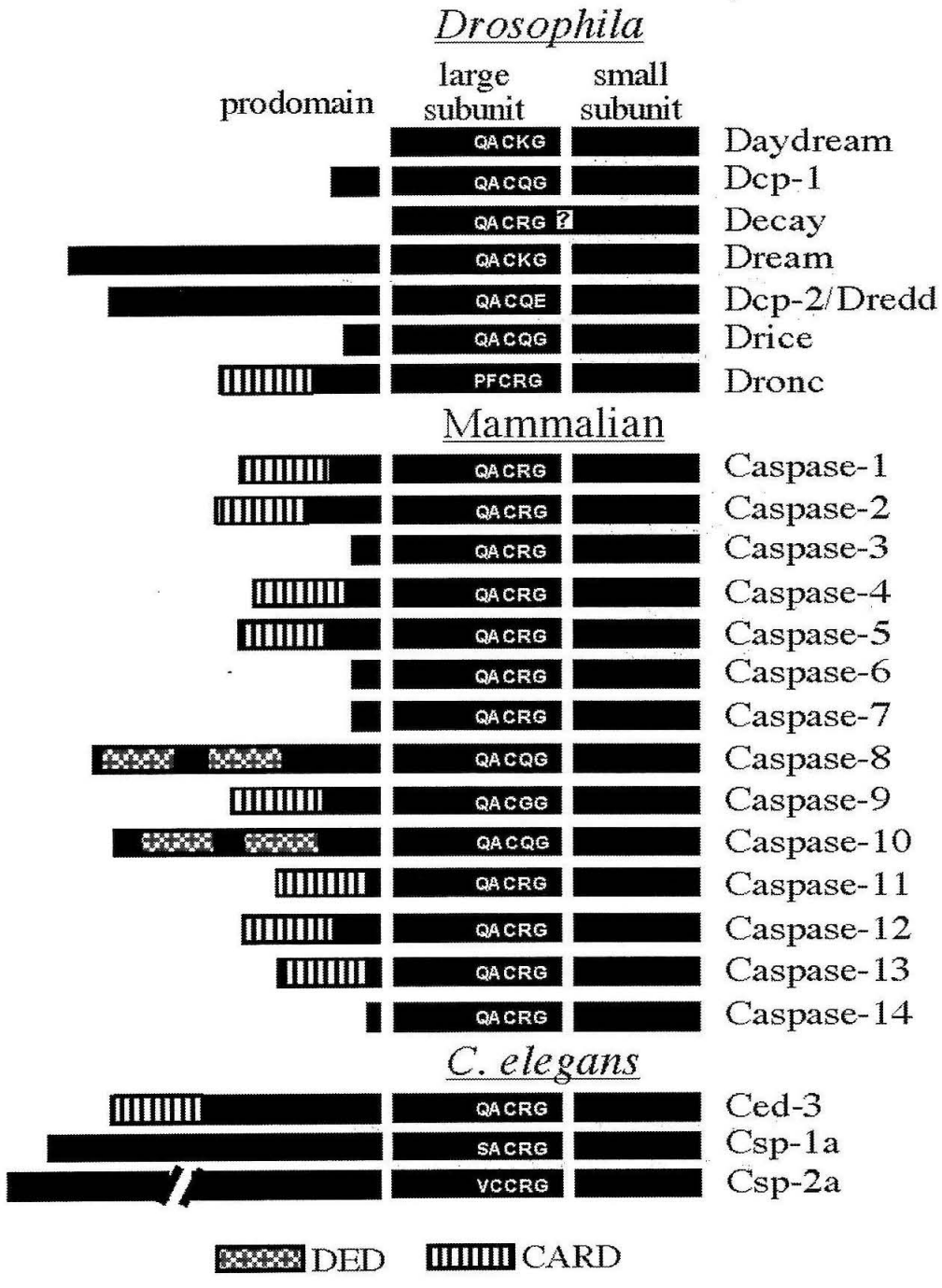

Drosophila
prodomain
large subunit
small subunit
QACKG
Daydream
QACQG
Dcp-1
QACRG ?
Decay
QACKG
Dream
QACQE
Dcp-2/Dredd
QACQG
Drice
PFCRG
Dronc
Mammalian
QACRG
Caspase-1
QACRG
Caspase-2
QACRG
Caspase-3
QACRG
Caspase-4
QACRG
Caspase-5
QACRG
Caspase-6
QACRG
Caspase-7
QACQG
Caspase-8
QACGG
Caspase-9
QACQG
Caspase-10
QACRG
Caspase-11
QACRG
Caspase-12
QACRG
Caspase-13
QACRG
Caspase-14
C. elegans
QACRG
Ced-3
SACRG
Csp-1a
VCCRG
Csp-2a
DED
CARD

Figure 1

Figure 1. Schematic representation of caspases with relative prodomain sizes is shown to highlight prodomain structure. The prodomain of Csp-2a, shown truncated, is about twice as long as that of Csp-1a. Csp-1 and Csp-2 also endcode other splice products which are not shown. Catalytic subunits are less variable in size, and are not shown exactly to scale. Active site sequences are indicated. Many caspases have a small linker seqence removed between the large and small subunits; linkers are not shown. Where prodomain cleavage sites were not known, they were chosen based on homology to caspases with known cleavage sites. A lack of aspartate residues for caspase cleavage in decay is indicated by (?). Caspase-1 through Caspase-10, Caspase-13 and Caspase-14 are human caspases. Caspase-11 and Caspase-12 are mouse caspases. Full sequence alignment of the caspases is available on the Web.

Because the glutamine at the first position of the active site pentapeptide QACRG is part of the substrate binding pocket, it is likely that caspases with different amino acids at this position will have unique cleavage preferences. In support of this hypothesis, Dronc, which has the active site sequence PFCRG, cleaves itself after glutamate rather than aspartate, and cleaves tetrapeptide substrates after glutamate as well as aspartate (Hawkins et al., 2000). Cleavage specificity data for Csp-1 and Csp-2 have not been reported. Why might these caspases have altered cleavage specificity? All are long prodomain caspases, suggesting that they act to transduce signals. One possibility is simply that these proteins have unique substrates (which may or may not be death related) that require an altered cleavage specificity. The altered cleavage specificity may also have evolved to be able to efficiently cleave the sequences present between their large and small caspase subunits, which contain sequences predicted to be very poor target sites for traditional caspases. An altered cleavage specificity, in conjunction with an absence of good target sites for other caspases in the linker region, may also serve as a way of making the activation of these caspases more strictly dependent on oligomerization rather than activation by other caspases.

Activating the caspase cascade

In mammals three pathways have been described that lead to caspase activation. In one pathway, which will not be discussed further, a serine protease,

granzyme B, is delivered directly into the cytoplasm of target cells from cytotoxic T cells, where it activates executioner caspases (Trapani et al., 2000). In the other two pathways, cytoplasmic adaptor proteins link a cell death signal transducer to a long prododmain caspase through homophilic receptor-adaptor and adaptor-caspase interactions, leading to caspase activation (Hofmann, 1999). In one pathway, initiating at the plasma membrane, caspase recruitment is initiated by the binding of ligands to receptors of the tumor necrosis factor/nerve growth factor receptor superfamily. The cytoplasmic region of these receptors contains a region known as the death domain (DD). Ligand-dependent receptor multimerization results in the recruitment of DD-containing-cytoplasmic adaptors such as Fas-associated death domain (FADD) through homophilic DD interactions. FADD and related adaptors also contain a second motif known as a death effector domain (DED), copies of which are also present in the prodomains of caspase-8 and caspase-10. Homophilic interactions between the DEDs present in receptor-bound adaptors and procaspases leads to caspase oligomerizaation and subsequent autoactivation. Other adaptors that include DD and CARD domains may also couple activated receptors to CARD domain-containing caspases.

We used the programs PFSCAN (http://www.isrec.isbsib.ch/software/ PFSCAN_form.html) and Pfam (http://www.sanger.ac.uk/Pfam/) to search for candidate death receptors (predicted type 1 transmembrane proteins containing

intracellular DDs) in the fly genome. We found a number of proteins or predicted proteins with DD homology, including the kinase pelle (accession no. AA540441), a *Drosophila* netrin receptor (accession no. AAF7419), a protein with a number of ankyrin repeats (accession no. CG7462), and three other proteins that lack significant similarity to other proteins (accession nos. CG2031, AF22205 and AF22206). (CG numbers refer to genes predicted by Celera Genomics.) However, none of these also shows DED or CARD homology. The prodomain of Dcp-2/Dredd does share weak homology with that of Caspase-8 (Chen et al., 1998), but the Dcp-2/Dredd prodomain is not itself identified in searches for *Drosophila* proteins with DEDs using PFSCAN or Pfam. In fact no *Drosophila* proteins with significant DED homology were identified in similar searches. These observations suggest several possibilities. One is that *Drosophila* lacks death receptor signaling pathways. A second possibility is that *Drosophila* has a death receptor pathway analogous to that found in mammals, but that the level of homology of these proteins with their mammalian counterparts is very low. Finally, *Drosophila* death receptors may incorporate a distinct set of oligomerization motifs. In the context of this possibility it will be interesting to identify proteins that interact with the Dream and Dcp-2/Dredd prodomains.

In a second major pathway of apical caspase activation in mammals, cellular stress of various sorts leads to the release of mitochondrial cytochrome c (cyto-c), which in conjunction with the cytosolic adapter protein Apaf-1, promotes

caspase-9 activation (reviewed in Budihardjo et al., 1999). Apaf-1 shows large regions of homology with the *C. elegans* apoptosis inducer Ced-4. In both organisms, caspase-activating adapter-caspase interactions are dependent on homophilic interactions between the two proteins, mediated at least in part by CARDs present at the N-terminus of Ced-4/Apaf-1 and in the caspase prodomain. In the case of worms, caspase activation by Ced-4 requires disruption of an association between Ced-4 and the apoptosis inhibitor and Bcl-2 family member Ced-9 by Egl-1, a second Bcl-2 family member that acts as an apoptosis inducer. Activation of Apaf-1 in mammals in vitro requires cyto-c, which stably interacts with WD-40 repeats present at the C-terminus of Apaf-1, but absent in Ced-4. The Apaf-1 WD-40 repeats inhibit its function, and this inhibition is relieved following cyto-c binding in the presence of ATP/dATP, allowing the formation of a multimeric Apaf-1/cyto-c complex. Procaspase-9 is recruited to this complex and activated through autocatalysis (reviewed in Budihardjo et al., 1999). Recently several Apaf-1-like genes have been identified in vertebrates (Cecconi, 1999). The proteins encoded by these genes contain distinct N- and C-terminal sequences, suggesting that they may activate other caspases through different upstream signaling pathways.

The *Drosophila* genome has one Ced-4/Apaf-1 homolog, variously known as *dapaf-1*(Kanuka et al., 1999), *dark* (Rodriguez et al., 1999), or *hac-1* (Zhou et al., 1999). Here we refer to this gene as *apaf-1-related killer* (*ark*), its designation in

the *Drosophila* on-line database (http:flybase.bio.indiana.edu/genes/). This gene encodes two splice forms. The long form most closely resembles Apaf-1, in that it contains a series of C-terminal WD-40 repeats that presumably mediate regulation by cyto-c. The short form most closely resembles CED-4, which lacks these repeats, and would thus be predicted to be constitutively active. Genetic evidence indicates that Ark is important for cell death induction in the fly (as well as other processes such as specification of photoreceptor number), and biochemical data point towards interactions between Ark, cyto-c and *Drosophila* caspases. Mitochondrial cyto-c is at least shifted in localization (Varkey et al., 1999), and perhaps released into the cytoplasm during apoptosis (Kanuka et al., 1999). Thus, the weight of evidence suggests that in *Drosophila*, as in vertebrates, cyto-c functions to transduce apoptotic signals through Apaf-1.

Keeping caspases in their place - the IAP family of cell death inhibitors

Since proteolysis is irreversible, and caspases have the potential to engage in amplifying cascades of proteolysis, caspase activation and activity must be carefully regulated in cells that normally live. The only known cellular caspase inhibitors are members of the inhibitor of apoptosis (IAP) family (reviewed in Deveraux and Reed, 1999; Miller, 1999). Genetic and biochemical evidence from *Drosophila* argues that IAP-dependent inhibition of caspase activity is essential

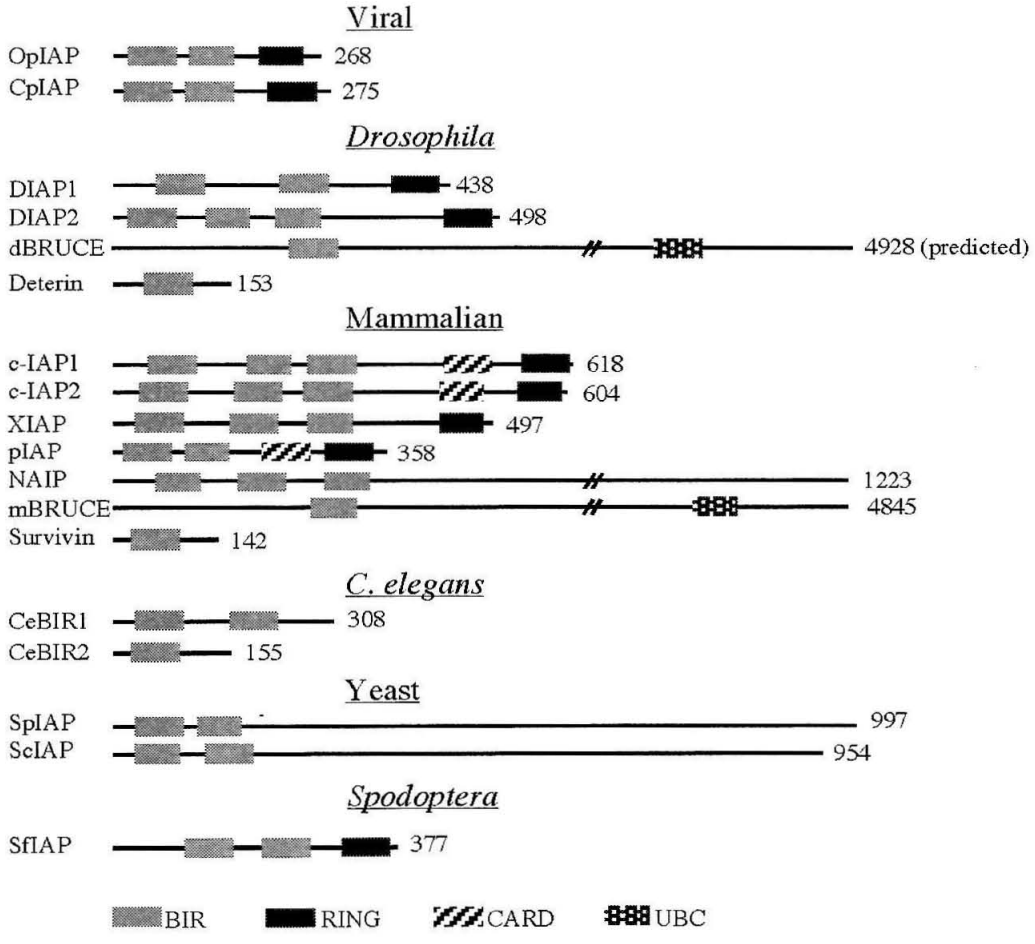

Viral
OpIAP
268
CpIAP
275
Drosophila
DIAP1
438
DIAP2
498
dBRUCE
4928 (predicted)
Deterin
153
Mammalian
c-IAP1
618
c-IAP2
604
XIAP
497
pIAP
358
NAIP
1223
mBRUCE
4845
Survivin
142
C. elegans
CeBIR1
308
CeBIR2
155
Yeast
SpIAP
997
ScIAP
954
Spodoptera
SfIAP
377
BIR
RING
CARD
UBC

Figure 2

Figure 2. Schematic representation of the structures of selected viral and cellular BIR-containing proteins. The approximate positions of BIR, RING, CARD, and UBC domains are shown with the total amino acid length shown to the right of each protein. In some proteins, RING domains confer E3 ubiquitin protein ligase activity on proteins which contain them; UBC refers to ubiquitin-conjugating domain predicted to have E2 activity. The presence of both of these domains in components of the apoptotic machinery suggests a link between apoptosis and protein degradation. The length of the *Drosophila* BRUCE homolog is not known as it derives solely from predicted sequence. Sequence alignment of the BIR repeat-containing proteins is available on the Web.

for cell survival and that one mechanism for cell death activation involves inhibition of IAP function (Wang et al., 1999; Goyal et al., 2000; Lisi et al., 2000).

IAPs were first identified as baculovirus encoded cell death inhibitors. These proteins contain several N-terminal repeats of a motif made up of approximately 70 amino acids and known as a baculovirus IAP repeat (BIR), as well as a C-terminal RING finger domain (reviewed in (Miller, 1999). RING fingers have since been found in proteins that function in a number of different contexts. For a number of proteins this domain confers E3 ubiquitin protein ligase activity (reviewed in Freemont, 2000). A number of cellular proteins that share homology with the viral IAPs based on the presence of one or more BIR repeats (referred to as BIR-repeat-containing proteins, or BIRPs) have now been identified in organisms ranging from yeast to humans (Uren et al., 1998) (Fig. 2). The *Drosophila* genome encodes four BIRPs, including *DIAP1*, the product of the *thread* locus (Hay et al., 1995), *DIAP2* (Hay et al., 1995; Duckett et al., 1996; Liston et al., 1996; Uren et al., 1996), *deterin*, a homolog of Survivin (Jones et al., 2000), and *dBRUCE*, a homolog of BRUCE (accession no. CG6303). A number of the cellular BIRPs, including XIAP, cIAP-1, cIAP-2, NAIP and Survivin in mammals, and DIAP1, DIAP2 and Deterin in *Drosophila*, have been tested and shown to act as cell death inhibitors. Notable exceptions are the BIRPs from *C. elegans* and yeast, which regulate cell division (Fraser et al., 1999; Uren et al., 1999). Thus, while all IAPs contain BIR repeats by definition, not all proteins with

BIRs are IAPs. Many of the death-inhibiting BIRPs, including XIAP, cIAP-1, cIAP-2, Survivin and DIAP1 have been shown to directly inhibit caspase activation or activity (reviewed in Deveraux and Reed, 1999). However, IAPs have been found to associate with a number of different proteins, and may have multiple mechanisms of action. This is particularly suggested in the case of those proteins that contain domains associated with ubiquitin conjugation.

Mitochondrial regulation of cell death

Mitochondria are necessary for cellular energy production, and are thus essential for cell survival. In vertebrates (and probably *Drosophila*), the mitochondria are an important site of integration for cell death and survival signals. As discussed above, the decision to release cyto-c constitutes one proapoptotic output of this calculation. A second proapoptotic protein released from mitochondria is apoptosis-inducing factor (AIF), which in mammals translocates from the mitochondria to the nucleus upon receipt of a death signal and causes large-scale fragmentation of the DNA (Susin et al., 1999). *Drosophila*, but not *C. elegans,* encodes a clear AIF homolog (accession no. CG7263).

In some cells undergoing apoptosis caspase inhibitors are unable to prevent cell death. One cause of this caspase-independent death is thought to be due to

mitochondrial damage that occurs upstream of caspase activation (reviewed in Vander Heiden and Thompson, 1999). The Bcl-2 family of proteins constitutes a major family of cell death regulators, and many of their pro- and antiapoptotic functions in vertebrates can be traced to their effects on mitochondrial function. Currently 19 distinct vertebrate Bcl-2 family members have been identified that share up to four Bcl-2 homology domains (BH1-4). Some also have a hydrophobic C-terminus which targets them to membranes. An important aspect of Bcl-2 family member function is that pro- and antiapoptotic proteins can heterodimerize (though this is not always required for function), and a large body of evidence argues that they titrate each other's function. However, exactly how these proteins regulate cell death is still unclear. *Drosophila* encodes two clear Bcl-2 family members. The first is known variously as *drob-1* (Igaki et al., 2000), *dBorg-1* (Brachmann et al., 2000), *debcl* (Colussi et al., 2000), or *dbok* (Zhang et al., 2000). The second gene is known as *buffy* (Colussi et al., 2000; accession no. AF237864) or *dBorg-2* (Brachmann et al., 2000). Both proteins have BH1, BH2 and BH3 domains. Weak BH4 domain homology may also be present (Fig. 3). They show the greatest overall homology to the mammalian proapoptotic protein Bok/Mtd, and have proapoptotic function. Genes encoding candidate prosurvival Bcl-2 proteins are not apparent in the fly genome. One possibility is that prosurvival Bcl-2 proteins do not exist. Alternatively, prosurvival members may exist, but have such low homology that we were unable to identify them. Finally, prosurvival Bcl-2 function may be obtained from posttranslational

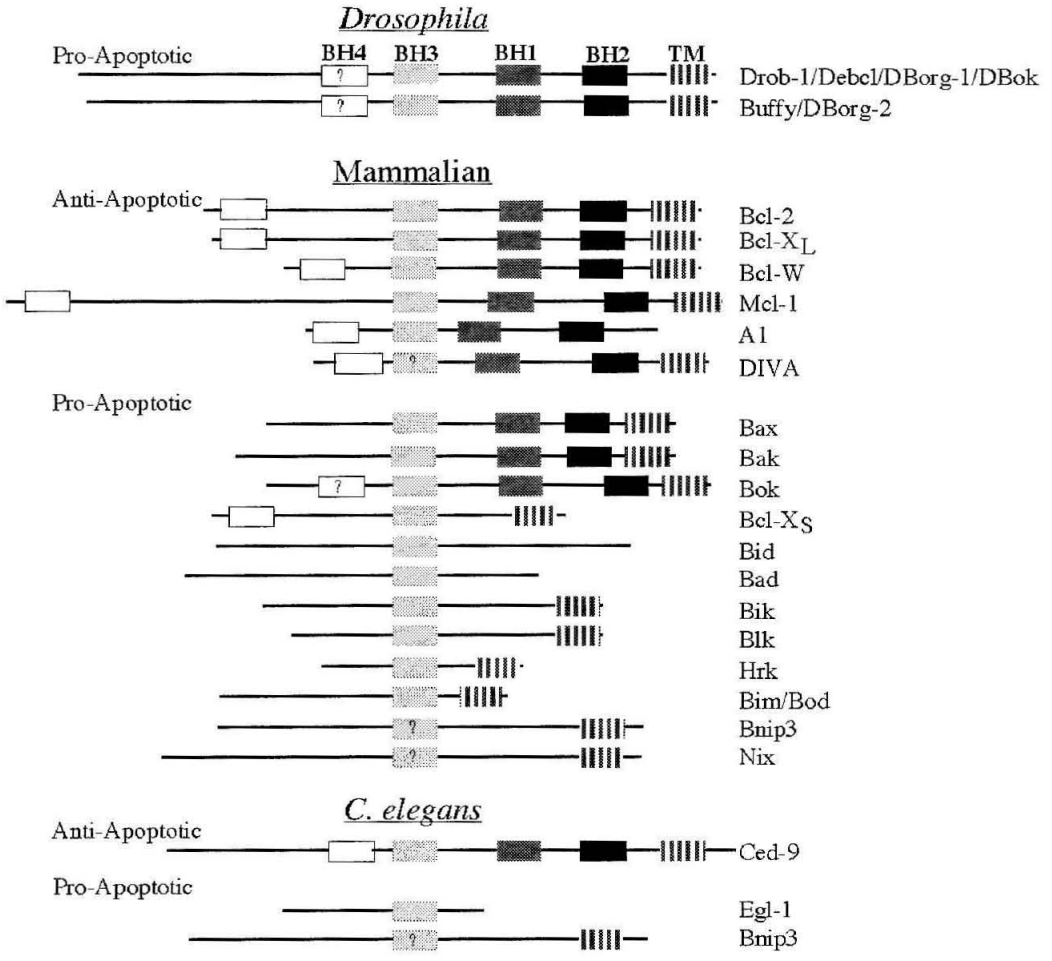

Drosophila
Pro-Apoptotic
BH4
BH3
BH1
BH2
TM
Drob-1/Debcl/DBorg-1/DBok
Buffy/DBorg-2
Mammalian
Anti-Apoptotic
Bcl-2
Bcl-XL
Bcl-W
Mcl-1
A1
DIVA
Pro-Apoptotic
Bax
Bak
Bok
Bcl-XS
Bid
Bad
Bik
Blk
Hrk
Bim/Bod
Bnip3
Nix
C. elegans
Anti-Apoptotic
Ced-9
Pro-Apoptotic
Egl-1
Bnip3

Figure 3

Figure 3. Schematic diagram of *Drosophila* Debcl/Drob-1/DBorg-1/DBok and Buffy/DBorg-2 and members of the mammalian and *C. elegans* Bcl-2 structural families. DBok initiates 86 residues C-terminal to the start codon predicted for Debcl/Drob/DBorg-1. BH1, BH2, BH3, BH4, and transmembrane domains are represented. The presence of (?) in some BH3 and BH4 domains indicates predicted BH domains based on weak sequence similarity. Debcl/Drob-1/DBorg-1/DBok and Buffy/DBorg-2 both contain BH1-4 domains as well as a transmembrane domain. Sequence alignment of the *Drosophila* Bcl-2 family members with selected mammalian and *C. elegans* members is available on the Web.

conversion of one or both of these proteins into an antiapoptotic form (Baker Brachmann et al., 2000).

Cell death in the nucleus

A common feature of apoptotic cell death is nuclear condensation and extensive DNA degradation. Apoptotic DNA degradation involves at least several steps. In vertebrates, the initial degradation of DNA is triggered by the caspase-dependent activation of a 40 kD nuclease known as CPAN/CAD/DFF. This protein is synthesized complexed to a specific chaperone/inhibitor known as DFF45/ICAD. Caspase cleavage of DFF45/ICAD by Caspase-3 releases CPAN/DFF40/CAD, which moves to the nucleus and cleaves DNA (reviewed in Nagata, 2000). Both DFF45/ICAD and CPAN/DFF40/CAD, as well as several other vertebrate proteins, contain a motif known as a CIDE domain. Experimental observations suggest that CIDE-CIDE interactions are important for regulation of CPAN/DFF40/CAD activity (Lugovskoy et al., 1999). Degradation of DNA following cell death also occurs in *Drosophila* and *C. elegans*. The fly genome encodes functional homologs of caspase-activated DNase (CAD) and CAD inhibitor (ICAD), as well as several other predicted proteins that have CIDE domains (Inohara et al., 1998; Inohara and Nunez, 1999; Yokoyama et al., 2000). CAD-like DNAses or other proteins with CIDE domains have not been identified in the *C. elegans* genome. However, DNA fragmentation occurs cell-

autonomously in a CED-3-dependent manner in dying cells, suggesting that a CAD-like activity is present (Wu et al., 2000). In a second step in apoptotic DNA degradation, which involves the participation of cells that engulf the dying cell, DNA is further processed by an acidic endonuclease. In mammals this activity is probably an acid lysozomal DNAse, either DNAse II or a DNAse II-like enzyme (McIlroy et al., 2000), and in *C. elegans* it is the product of the nuc-1 gene (Wu et al., 2000). *Drosophila* also encodes a DNAse-II-like protein (accession no. CG7780), and it seems likely that this form of DNA degradation occurs in flies as well.

Two other mammalian proteins that promote nuclear apoptotic events are AIF and acinus. AIF translocates from the mitochondria to cause chromatin condensation and large scale DNA fragmentation (Susin et al., 1999). Acinus, a DNA-condensing factor with no nuclease activity, localizes to the nucleus and is activated during apoptosis by combined caspase and serine protease cleavage (Sahara et al., 1999). *Drosophila*, but not *C. elegans*, encodes clear homologs of both these proteins (Acinus, accession no. CG10437; AIF, accession no. CG7263).

REAPER, HID, and GRIM. Insect-specific death regulators or conserved prophets of death?

One of the reasons for working with a model system such as the fly is the hope of being able to get a different perspective that will afford unique insight into a conserved, but complex process such as apoptosis. *Drosophila* has arguably been in this position for some time. An early genetic screen identified a genomic region at 75C that contained genes required for essentially all normally occuring cell deaths during *Drosophila* embryogenesis (White et al., 1994). Three genes within this region, *reaper* (*rpr*) (White et al., 1994), *head involution defective* (*hid*) (Grether et al., 1995), and *grim* (Chen et al., 1996), mediate this proapoptotic requirement, and a large body of evidence argues that they act to integrate and transduce many different cell death signals that ultimately lead to the activation of caspase-dependent cell death (reviewed in (Abrams, 1999). Rpr, Hid, and Grim have only very limited homology with each other (a short stretch of roughly 14 amino acids near their N-termini), and sequence homologs have not been identified in other organisms. However, recent observations argue that the mechanisms of action defined by these genes are likely to be conserved. First, each of these proteins induces apoptosis in mammalian cells, strongly suggesting that some aspect of their function is evolutionarily conserved (reviewed in (Abrams, 1999). Second, despite their very low level of homology with each other, they each interact with several different conserved death

regulators (Fig. 4). This suggests that putative mammalian homologs may also be quite divergent in sequence. For example, they each bind the *Drosophila* caspase inhibitor DIAP1 through interactions that require their N-termini (Vucic et al., 1997; Vucic et al., 1998), and genetic and biochemical data argue that one way they promote apoptosis is by inhibiting DIAP1's ability to prevent death-inducing caspase activity (Wang et al., 1999; Goyal et al., 2000). Since IAPs and caspases also function to regulate death in vertebrates, it seems reasonable that Rpr, Hid, and Grim orthologs exist that perform a similar death promoting function.* Rpr, Hid, and Grim also bind a *Xenopus* protein, Scythe, in an interaction that does not require their N-termini (Thress et al., 1999). In the case of at least Rpr this interaction leads to release of a Scythe-bound proapoptotic factor that promotes cyto-c release. *Drosophila* encodes a Scythe homolog (accession no. CG7546), suggesting that a similar pathway may exist in flies as well.

Cell death in the 21st century: why the fly?

This review has discussed a number of core cell death regulators found in worms, flies, and vertebrates. We have, however, only scratched the surface in terms of discussing all the genes and pathways that have been shown to regulate cell death in various system. In particular, we have not dealt with the extensive literature on survival factors, many of which lead to the activation of the

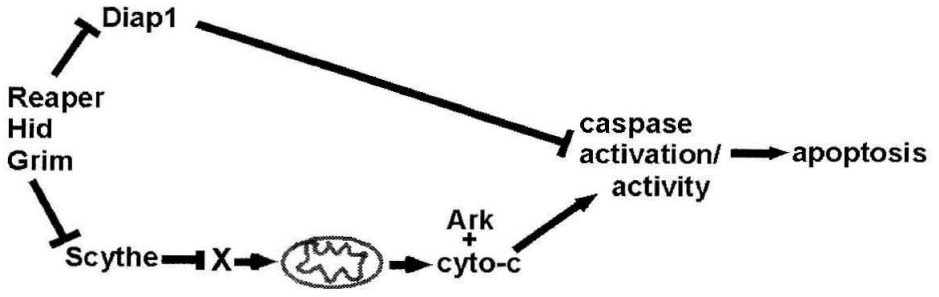


Figure 4

Figure 4. Mechanisms by which Rpr, Hid, and Grim are proposed to induce apoptosis. In one pathway, Rpr, Hid, and Grim bind to Diap1, suppressing its ability to inhibit caspase activation and/or activity. Diap2 also inhibits Rpr-, Hid-, and Grim-dependent cell death but has not been shown to act as a caspase inhibitor. Therefore it has not been included in this diagram. In a second pathway, Rpr, Hid, and Grim promote the release of a Scythe bound factor that promotes the release of apoptosis inducers such as cyto-c, which is necessary for the activation of one form of the *Drosophila* Apaf-1 homolog, Ark. See text under REAPER, HID, and GRIM for details.

Akt/PkB kinase (Datta et al., 1999). What is clear from our analysis is that *Drosophila* shares many of the molecules and pathways that are used by vertebrates to control cell death. A description of the full complement of *Drosophila* coding regions, in conjunction with mass spectroscopic analysis of protein complexes, will provide an important new approach to understanding how pieces of the death machine talk to each other, as will the use of DNA microarrays. However, *Drosophila* is likely to have its biggest impact on the cell death field in the 21st century, as in the 20th, through the continued use of genetics to carry out function-based screens. This is because the genetic approach to identifying cell death regulators makes few assumptions about the kinds of molecules and mechanisms that regulate this process and is thus well positioned to uncover new molecules and mechanisms. The completed genomic sequence provides an invaluable resource for this work because it tells us where the homologs of known death regulators are, and it greatly speeds the identification of novel genes identified in these screens.

Acknowledgments

This work was supported by grants to B. A. Hay from a Burroughs Wellcome Fund New Investigator Award in the Pharmacological Sciences, the Ellison Medical Foundation, and NIH grant GM057422-01 to B.A. Hay. S. J. Yoo is supported by a postdoctoral fellowship from the Jane Coffin Childs Foundation.

*Note added in proof:

A mammalian protein called Smac/DIABLO, which appears to play such a role, has recently been described (*Cell. 2000.* 102:33-42;*Cell. 2000.* 102:43-53).

References

Abrams, J. M. 1999. An emerging blueprint for apoptosis in *Drosophila. Trends Cell Biol.* 9:435-440.

Brachmann, C., O. W. Jassim, B. D. Wachsmuth, and R. L. Cagan. 2000. The *Drosophila* Bcl-2 family member dBorg-1 functions in the apoptotic response to UV-irradiation. *Current Biol.* 10:547-550.

Budihardjo, I., H. Oliver, M. Lutter, X. Luo, and X. Wang. 1999. Biochemical pathways of caspase activation during apoptosis. *Annu. Rev. Cell Biol.* 15:269-290.

Cecconi, F. 1999. Apaf1 and the apoptotic machinery. *Cell Death and Differentiation.* 6:1087-1098.

Chen, P., W. Nordstrom, B. Gish, and J. M. Abrams. 1996. grim, a novel cell death gene in *Drosophila. Genes Dev.* 10:1773-1782.

Chen, P., A. Rodriguez, R. Erskine, T. Thach, and J. M. Abrams. 1998. Dredd, a novel effector of the apoptosis activators reaper, grim, and hid in *Drosophila. Dev Biol.* 201:202-216.

Colussi, P. A., L. M. Quinn, D. C. Huang, M. Coombe, S. H. Read, H. Richardson, and S. Kumar. 2000. Debcl, a proapoptotic Bcl-2 homologue, is a component of the *Drosophila melanogaster* cell death machinery. *J. Cell Biol.* 148:703-714.

Datta, S. R., A. Brunet, and M. E. Greenberg. 1999. Cellular survival: a play in three Akts. *Genes Dev.* 13:2905-2927.

Deveraux, Q. L., and J. C. Reed. 1999. IAP family proteins--suppressors of apoptosis. *Genes Dev.* 13:239-252.

Dorstyn, L., P. A. Colussi, L. M. Quinn, H. Richardson, and S. Kumar. 1999a. DRONC, an ecdysone-inducible *Drosophila* caspase. *Proc Natl Acad Sci U S A.* 96:4307-4312.

Dorstyn, L., S. H. Read, L. M. Quinn, H. Richardson, and S. Kumar. 1999b. DECAY, a novel *Drosophila* caspase related to mammalian caspase-3 and caspase-7. *J Biol Chem.* 274:30778-30783.

Duckett, C. S., V. E. Nava, R. W. Gedrich, R. J. Clem, J. L. Van Dongen, M. C. Gilfillan, H. Shiels, J. M. Hardwick, and C. B. Thompson. 1996. A conserved

family of cellular genes related to the baculovirus iap gene and encoding apoptosis inhibitors. *Embo J.* 15:2685-2694.

Fraser, A. G., and G. I. Evan. 1997. Identification of a *Drosophila melanogaster* ICE/CED-3-related protease, drICE. *Embo J.* 16:2805-2813.

Fraser, A. G., C. James, G. I. Evan, and M. O. Hengartner. 1999. *Caenorhabditis elegans* inhibitor of apoptosis protein (IAP) homologue BIR-1 plays a conserved role in cytokinesis. *Curr Biol.* 9:292-301.

Freemont, P. S. 2000. Ubiquitination:RING for destruction? *Current Biology.* 10:84-87.

Goyal, L., K. McCall, J. Agapite, E. Hartwieg, and H. Steller. 2000. Induction of apoptosis by *Drosophila reaper*, *hid* and *grim* through inhibition of IAP function. *EMBO J.* 19:589-597.

Grether, M. E., J. M. Abrams, J. Agapite, K. White, and H. Steller. 1995. The head involution defective gene *of Drosophila melanogaster* functions in programmed cell death. *Genes Dev.* 9:1694-1708.

Hawkins, C. J., S. J. Yoo, E. P. Peterson, S. L. Wang, S. Y. Vernooy, and B. A. Hay. 2000. The *Drosopohila* caspase DRONC cleaves following glutamate and aspartate, and is regulated by DIAP1, HID and GRIM. *J. Biol. Chem.* 275:27084-27093.

Hay, B. A., D. A. Wassarman, and G. M. Rubin. 1995. *Drosophila* homologs of baculovirus inhibitor of apoptosis proteins function to block cell death. *Cell.* 83:1253-1262.

Hofmann, K. 1999. The modular nature of apoptotic signaling proteins. *Cell. Mol. Life Sci.* 55:1113-1128.

Igaki, T., H. Kanuka, N. Inohara, K. Sawamoto, G. Nunez, H. Okano, and M. Miura. 2000. Drob-1, a *Drosophila* member of the Bcl-2/CED-9 family that promotes cell death. *Proc. Natl. Acad. Sci. USA.* 97:662-667.

Inohara, N., T. Koseki, Y. Hu, S. Chen, and G. Nunez. 1997. CLARP, a death effector domain-containing protein interacts with caspase-8 and regulates apoptosis. *Proc Natl Acad Sci U S A.* 94:10717-10722.

Inohara, N., T. Koseki, S. Chen, X. Wu, and G. Nunez. 1998. CIDE, a novel family of cell death activators with homology to the 45 kDa subunit of the DNA fragmentation factor. *EMBO J.* 17:2526-2533.

Inohara, N., and G. Nunez. 1999. Genes with homology to DFF/CIDEs found in *Drosophila melanogaster. Cell Death Differ.* 6:823-824.

Jacobson, M. D., M. Weil, and M. C. Raff. 1997. Programmed cell death in animal development. *Cell.* 88:347-354.

Jones, G., D. Jones, L. Zhou, H. Steller, and Y. Chu. 2000. Deterin, a new inhibitor of apoptosis from *Drosophila melanogaster. J. Biol. Chem.* 275:22157-22165.

Kanuka, H., K. Sawamoto, N. Inohara, K. Matsuno, H. Okano, and M. Miura. 1999. Control of the cell death pathway by Dapaf-1, a *Drosophila* Apaf-1/CED-4-related caspase activator. *Molecular Cell.* 4:757-769.

Kumar, S., and P. A. Colussi. 1999. Prodomains-adaptors-oligomerization: the pursuit of caspase activation in apoptosis. *Trends in Biochem. Sci.* 24:1-4.

Lisi, S., L. Mazzon, and W. White. 2000. Diverse domains of THREAD/DIAP1 are required to inhibit apoptosis induced by REAPER and HID in *Drosophila*. *Genetics*. 154:669-678.

Liston, P., N. Roy, K. Tamai, C. Lefebvre, S. Baird, G. Cherton-Horvat, R. Farahani, M. McLean, J. E. Ikeda, A. MacKenzie, and R. G. Korneluk. 1996. Suppression of apoptosis in mammalian cells by NAIP and a related family of IAP genes. *Nature*. 379:349-353.

Lugovskoy, A. A., P. Zhou, J. J. Chou, J. S. McCarty, P. Li, and G. Wagner. 1999. Solution structure of the CIDE-N domain of CIDE-B and a model for CIDE-N/CIDE-N interactions in the DNA fragmentation pathway of apoptosis. *Cell*. 99:747-755.

McIlroy, D., M. Tanaka, H. Sakahira, H. Fukuyama, M. Suzuki, K. Yamamura, Y. Ohsawa, Y. Uchiyama, and S. Nagata. 2000. An auxiliary mode of apoptotic DNA fragmentation provided by phagocytes. *Genes Dev.* 14:549-558.

Miller, L.K. 1999. An exegesis of IAPs; salvation and surprises from BIR motifs. *Trends Cell Biol.* 9:323-328.

Nagata, S. 2000. Apoptotic DNA fragmentation. *Exp. Cell Res.* 256:12-18.

Rodriguez, A., H. Oliver, H. Zou, P. Chen, X. Wang, and J.M. Abrams. 1999. Dark is a *Drosophila* homologue of Apaf-1/CED-4 and functions in an evolutionarily conserved death pathway. *Nat Cell Biol.* 1:272-279.

Sahara, S., M. Aoto, Y. Eguchi, N. Imamoto, Y. Yoneda, and Y. Tsujimoto. 1999. Acinus is a caspase-3-activated protein required for apoptotic chromatin condensation. *Nature.* 401:168-173.

Shaham, S. 1998. Identification of multiple *Caenorhabditis elegans* caspases and their potential roles in proteolytic cascades. *J. Biol. Chem.* 273:35109-35117.

Song, Z., K. McCall, and H. Steller. 1997. DCP-1, a *Drosophila* cell death protease essential for development [published erratum appears in Science 1997 Jul 11;277(5323):167]. *Science.* 275:536-540.

Susin, S. A., H. K. Lorenzo, N. Zamzami, I. Marzo, B. E. Snow, G. M. Brothers, J. Mangion, E. Jacotot, P. Costantini, M. Loeffler, N. Larochette, D. R. Goodlett, R. Aebersold, D. P. Siderovski, J. M. Penninger, and G. Kroemer. 1999. Molecular characterization of mitochondrial apoptosis-inducing factor. *Nature.* 397:441-446.

Thornberry, N. A., and Y. Lazebnik. 1998. Caspases: enemies within. *Science.* 281:1312-1316.

Thress, K., E. K. Evans, and S. Kornbluth. 1999. Reaper-induced dissociation of a scythe-sequestered cytochrome c- releasing activity. *Embo J.* 18:5486-5493.

Trapani, J. A., J. Davis, V. R. Sutton, and M. J. Smyth. 2000. Proapoptotic functions of cytotoxic lymphocyyte granule constituents in vitro and in vivo. *Curr. Opin Immunology.* 12:323-329.

Uren, A. G., T. Beilharz, M. J. O'Connell, S. J. Bugg, R. van Driel, D. L. Vaux, and T. Lithgow. 1999. Role for yeast inhibitor of apoptosis (IAP)-like proteins in cell division. *Proc Natl Acad Sci U S A.* 96:10170-10175.

Uren, A. G., E. J. Coulson, and D. L. Vaux. 1998. Conservation of baculovirus inhibitor of apoptosis repeat proteins (BIRPs) in viruses, nematodes, vertebrates and yeasts. *Trends Biochem Sci.* 23:159-162.

Uren, A. G., M. Pakusch, C. J. Hawkins, K. L. Puls, and D. L. Vaux. 1996. Cloning and expression of apoptosis inhibitory protein homologs that function to inhibit apoptosis and/or bind tumor necrosis factor receptor-associated factors. *Proc Natl Acad Sci U S A.* 93:4974-4978.

Vander Heiden, M. G., and C. B. Thompson. 1999. Bcl-2 proteins: regulators of apoptosis or of mitochondrial homeostasis? *Nature Cell Biology*. 1:E209-E216.

Varkey, J., P. Chen, R. Jemmerson, and J. M. Abrams. 1999. Altered cytochrome c display precedes apoptotic cell death in *Drosophila*. *J. Cell Biol.* 144:701-710.

Vucic, D., W. J. Kaiser, A. J. Harvey, and L. K. Miller. 1997. Inhibition of reaper-induced apoptosis by interaction with inhibitor of apoptosis proteins (IAPs). *Proc. Natl. Acad. Sci. USA*. 94:10183-10188.

Vucic, D., W. J. Kaiser, and L. K. Miller. 1998. Inhibitor of apoptosis proteins physically interact with and block apoptosis induced by *Drosophila* proteins HID and GRIM. *Mol. Cell Biol.* 18:3300-3309.

Wang, S. L., C. J. Hawkins, S. J. Yoo, H. A. Muller, and B.A. Hay. 1999. The *Drosophila* caspase inhibitor DIAP1 is essential for cell survival and is negatively regulated by HID. *Cell*. 98:453-63.

White, K., M. E. Grether, J. M. Abrams, L. Young, K. Farrell, and H. Steller. 1994. Genetic control of programmed cell death in *Drosophila*. *Science*. 264:677-683.

Wu, Y.-C., G. M. Stanfield, and H. R. Horvitz. 2000. NUC-1, a *Caenorhabditis elegans* DNase II homolog, functions in an intermediate step of DNA degrdation during apoptosis. *Genes Dev.* 14:536-548.

Yokoyama, H., N. Mukae, H. Sakahira, K. Okawa, A. Iwamatsu, and S. Nagata. 2000. A novel activation mechanism of caspase-activated DNase from *Drosophila melanogaster*. *J. Biol. Chem.* 275:12978-12986.

Yuan, J., S. Shaham, S. Ledoux, H. M. Ellis, and H. R. Horvitz. 1993. The *C. elegans* cell death gene ced-3 encodes a protein similar to mammalian interleukin-1 beta-converting enzyme. *Cell.* 75:641-652.

Zhang, H., Q. Huang, N. Ke, S. Matsuyama, B. Hammock, A. Godzik, and J. C. Reed. 2000. *Drosophila* proapoptotic Bcl-2/Bax homologue reveals evolutionary conservation of cell death mechanisms. *J. Biol. Chem.* 275:27303-27306.

Zheng, T. S., and R. A. Flavell. 2000. Divinations and surprises: genetic analysis of caspase function in mice. *Exp. Cell. Res.* 256:67-73.

Zhou, L., Z. Song, J. Tittel, and H. Steller. 1999. HAC-1, a *Drosophila* homolog of APAF-1 and CED-4, functions in developmental and radiation-induced apoptosis. *Mol. Cell.* 4:745-755.

CHAPTER 3

An overexpression screen to identify modifiers of the *Drosophila* cell death activators Rpr, Hid, and Grim

Stephanie Y. Vernooy, Susannah Cole, Julius Su, Koen Verbrugghe, Jennifer Yang, Kimberly Walter, Soon Ji Yoo, Paula Agguire, Becky Green-Marroquin, Asya Pogodina, and Bruce A. Hay

The *Drosophila* cell death activators, Rpr, Hid, and Grim, are essential for normally occurring cell death in the fly (White et al., 1994; Grether et al., 1995; Chen et al., 1996). As discussed in Chapter 1, progress has been made over the last few years in understanding some of the mechanisms by which these molecules work to bring about the demise of a cell, and suggest that they act through multiple mechanisms. However, little is known about other molecules involved in these pathways or how they are regulated. The only known inhibitors of Rpr, Hid, and Grim activity are p35, a baculovirus-encoded protein, and the *Drosophila* IAP1 and IAP2 (Hay et al., 1994; Hay et al., 1995).

One method of identifying regulators of Rpr, Hid, and Grim activity involves the biochemical approach of looking for binding partners. However, this requires having full-length candidate cDNAs, as well as making assumptions about binding conditions. Another approach is to carry out a theory-independent genetic screen. This approach has the added benefit that it gives access to regulators that may not physically interact with Rpr, Hid, or Grim.

We chose to carry out a P element mediated, tissue-specific overexpression screen for several reasons. One is that it may cause phenotypes where mutational inactivation fails to. Though mutagenesis screens can be a powerful way to get at gene function, problems that can be avoided by doing a misexpression screen include the inability to recover mutations in genes that are required early in development, lack of mutant phenotypes for many genes under

laboratory conditions, and compensation by other molecules or pathways (reviewed in Miklos and Rubin, 1996). Another reason for doing a P element insertion screen is the ability to easily recover genomic sequence from the site of insertion by means of plasmid rescue sequences included in the P element construct. This is especially useful given that the *Drosophila* genome has now been completely sequenced, facilitating rapid identification of genomic location and nearby genes (Adams et al., 2000). Finally, an overexpression screen is a way to identify molecules that may be relevant to cancer biology. Oncogenic growth can result from hyperactivation of genes that stimulate cell growth. Overexpression is therefore physiological with respect to oncogenesis, and can be used to identify genes that are capable of modifying signaling pathways even if they do not do so under normal developmental conditions (Haupt et al., 1991; van Lohuizen et al., 1991).

Results and Discussion

Apoptosis is an important process in normal *Drosophila* eye development. This, in conjunction with the dispensability of the eye for both viability and fertility and the ease with which it is possible to score death phenotypes in this tissue, makes it an ideal tissue in which to conduct a misexpression screen for apoptotic regulators. We used as a mutagen the GMREP element, which carries a

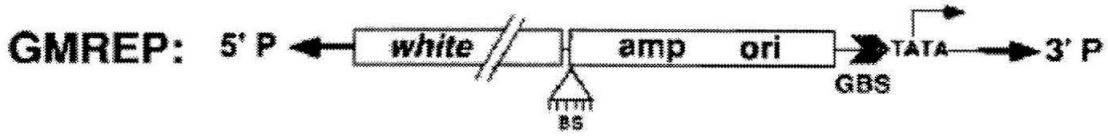


Figure 1

Figure 1. Structure of the GMREP element (Adapted from Hay et al., 1997). Ampicillin resistance (amp) and an origin of replication (ori) are included for plasmid rescue. BS refers to the bluescript polylinker. Glass binding sites (GBS) follow the plasmid rescue sequences. The white gene is used as a marker.

multimer of glass binding sites that drives expression posterior to the morphogenetic furrow in the developing eye, to screen for suppressors of Rpr-, Hid- and Grim-induced death (Figure 1) (Hay et al., 1997). Transgenic flies carrying the GMREP element were made using standard methods. We then crossed them to flies carrying the gene for P-transposase in order to mobilize the P element to other loci. The genetic scheme is shown in Figure 2. Using this method, we generated approximately 7000 independent insertion lines.

To identify genes capable of blocking death caused by overexpression of Rpr, Hid, or Grim, we crossed each of the 7000 lines to GMR-Rpr, GMR-Hid, and GMR-Grim flies. Each of these genes overexpressed in the eye causes a small eye phenotype due to massive cell death (Figure 3). We then looked for progeny that carried the Rpr, Hid, or Grim transgene, but had large eyes, presumably due to ectopic expression of a gene that could block their death-inducing effects. We eliminated false positives by crossing the Rpr, Hid, and Grim suppressors to flies that expressed unrelated molecules, including activated Ras (RasV12) and Tramtrack (Ttk). These give rough eye phenotypes, but not due to cell death. Those that suppressed all were discarded. Figure 4 shows phenotypes for an insertion at the DIAP1 locus, which is a suppressor of all three activators and does not suppress the small eye phenotypes caused by overexpression of RasV12 or Ttk.

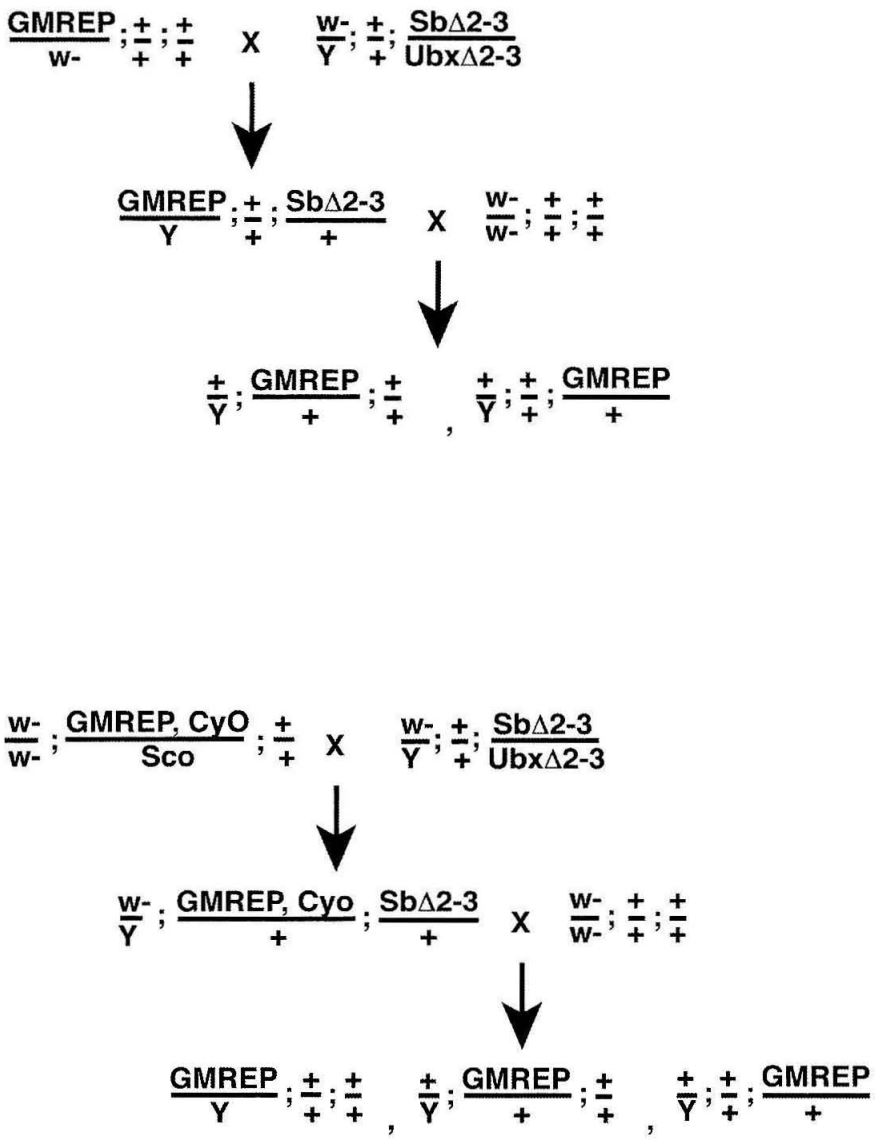

GMREP ; + ; + X w- ; + ; Sb∆2-3
w- + + Y + Ubx∆2-3
GMREP ; + ; Sb∆2-3 X w- ; + ; +
Y + + w- + +
+ ; GMREP ; + + ; + ; GMREP
Y + + Y + +
w- ; GMREP, CyO ; + X w- ; + ; Sb∆2-3
w- Sco + Y + Ubx∆2-3
w- ; GMREP, Cyo ; Sb∆2-3 X w- ; + ; +
Y + + w- + +
GMREP ; + ; + + ; GMREP ; + + ; + ; GMREP
Y + + Y + + Y + + +

Figure 2

Figure 2. Two genetic schemes are shown. In the first, GMREP is hopped off of the X chromosome. Females containing the P element are crossed to males expressing delta 2-3 transposase. The transposase-carrying chromosomes are marked with the dominant marker stubble (Sb) or Ultrabithorax (Ubx). In flies that contain both the P element and the transposase, the P element is mobilized. To isolate stable insertions, males carrying both are outcrossed to w- females, and red-eyed male progeny (indicating the presence of GMREP) lacking transposase are selected. Because males inherit a Y chromosome from their father and an X chromosome from their mother, the presence of GMREP (which was formerly on their father's X chromosome) is indicative of a hop to an autosome. In the second scheme, GMREP is similarly mobilized, but is hopped off of a chromosome marked with Curly (CyO) so that insertions on the X chromosome as well as on the autosomes can be recovered.

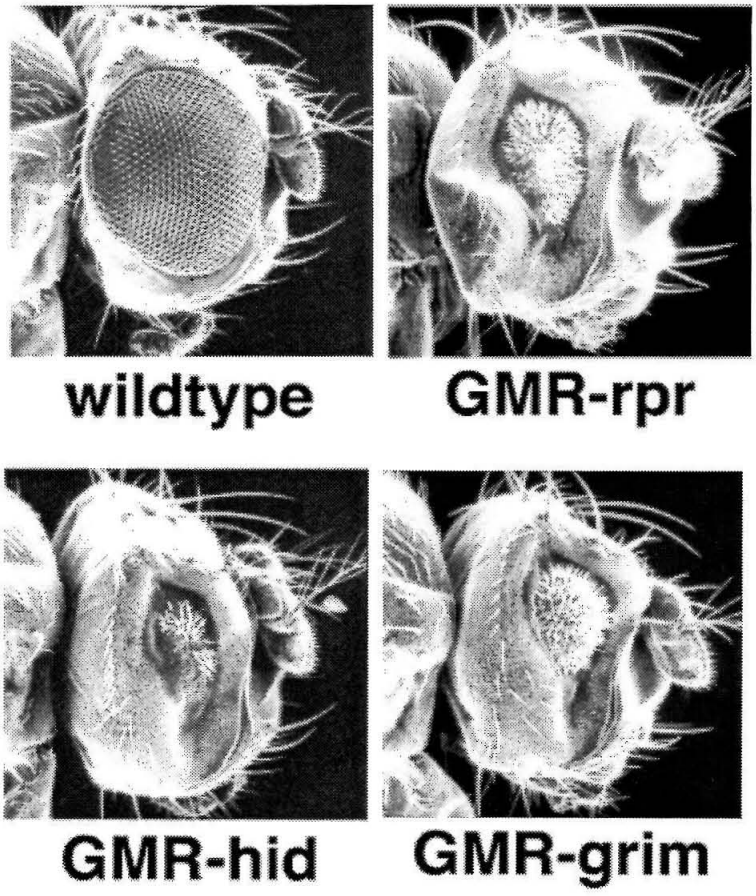


Figure 3

Figure 3. Scanning electron micrographs are shown, with wild-type for comparison. GMR-Rpr, GMR-Hid, and GMR-Grim cause a small eye phenotype due to ectopic cell death.

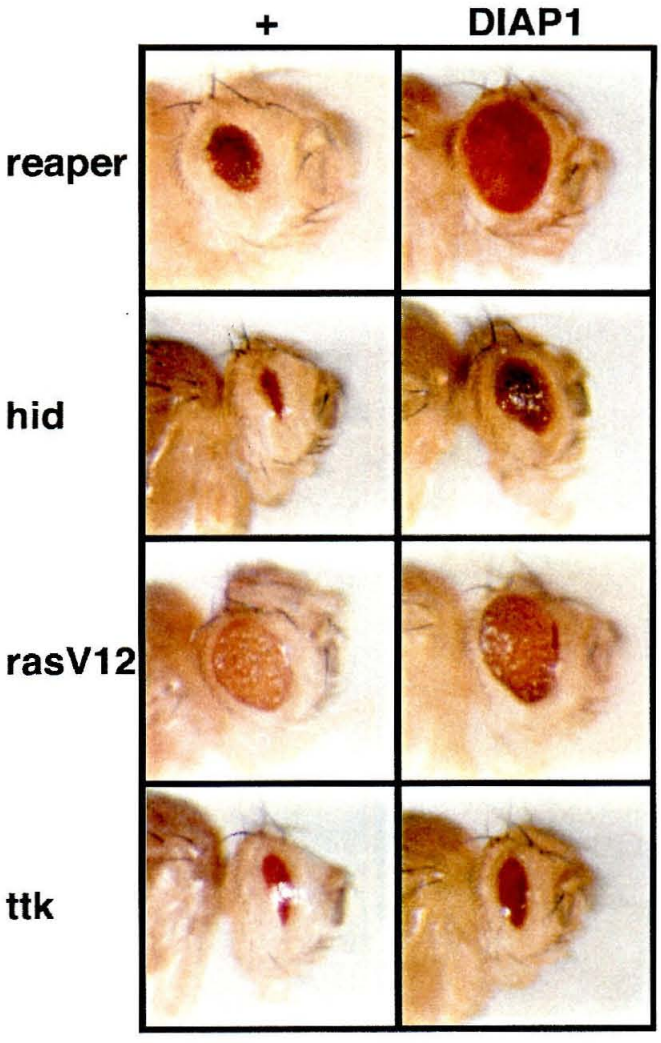


Figure 4

Figure 4. DIAP1 suppression phenotypes are shown as an example. DIAP1 suppresses GMR-Rpr and GMR-Hid, but has no effect on phenotypes caused by GMR-RasV12 or GMR-Ttk. GMR-RasV12 and GMR-Ttk flies were a gift from Gerry Rubin.

Table 1 summarizes the results of the screen. Interestingly, the suppressors fall mainly into two categories: those that suppress both Rpr and Grim, and those that suppress Hid. This is consistent with experimental results discussed in Chapter 1 that Rpr and Grim are mechanistically more similar to each other than to Hid, and suggests that the suppressors identified in this screen will in fact include components of these pathways. Crossing suppressor lines with flies that overexpress various downstream components of the apoptotic pathway, such as caspases, yields more information about where the suppressors are acting. Some of these crosses are summarized in Table 2. The varied results indicate that our screen has tapped into multiple apoptotic regulatory points.

The genomic loci represented by GS1 and GS5 are the only two that are represented by only one line; all of the other suppressor loci are represented multiple times (Table 3). By screening 7000 lines, we have reached the point of diminishing returns, if not the point of saturation. However, further screening with a transposable element that has different insertion preferences, such as the hobo transposable element, might be expected to uncover suppressor loci that may be refractory to P element insertion (Smith et al., 1993).

The completion of the *Drosophila* genome sequence has rendered unnecessary some of the analysis that we routinely undertook in the early stages of the screen, such as using sequentially larger plasmid rescue fragments to gain

suppressor name	genomic location	suppresses Rpr?	suppresses Grim?	suppresses Hid?
X.2	X	+	+	-
PEG	2R	+	+	-
KV1	2R	+	+	-
3.1	3L	+	+	-
VS3	3R	+	+	-
HS2	X	-	-	+
RS1	3L	-	-	+
H111	3L	-	-	+
GS5	3R	-	+	-
GS1	3R	+	+	+
DIAP1	3L	+	+	+

Table 1

Table 1. Suppression profiles and chromosomal locations are indicated for the strongest suppressors identified in the screen. Names (except for DIAP1) are not meaningful; even we do not recall all of their origins.

suppressor name	suppresses Dronc?	suppresses Strica?	suppresses Dcp-1(ΔN)?
X.2			
PEG	+	-	+
KV1	-	-	+
3.1	+	-	-
VS3	-	-	-
HS2	-	-	+
RS1	+	-	-
H111	-	-	
GS5	-	-	
GS1	+	+	
DIAP1	+	+	+

Table 2

Table 2. Suppressors were crossed to lines overexpressing caspases under the control of GMR. The version of Dcp-1 used here lacks its N-terminal prodomain, and was a gift from Hermann Steller. + indicates suppression; - indicates no effect. Those left blank were not tested.

suppressor name	number of independent lines
X.2	2
PEG	9
KV1	3
3.1	4
VS3	5
HS2	2
RS1	2
H111	3
GS5	1
GS1	1
DIAP1	30

Table 3

Table 3. The majority of suppressor loci were identified multiple times in the screen, indicating that we approached saturation.

access to the genomic region surrounding a P element insertion, and screening cDNA libraries with plasmid rescue fragments to identify candidate genes in the region. Plasmid rescue sequence now immediately translates to genomic location with a simple BLAST search. A map of known genes, ESTs, predicted genes, and *Drosophila* stocks carrying mutations in that genomic region is a mouse click away (www.fruitfly.org). Candidate cDNAs can be purchased and used for in situ hybridization to see if they represent genes that are in fact overexpressed in suppressor lines. This can be done in parallel with as many cDNAs as are available representing the nearby region. Carrying out this kind of analysis led to the important observation that GMREP can drive expression of genes at least as far away as 23 kb, upstream or downstream of GMREP, and facing in either direction. With this knowledge in hand and with the wealth of tools provided by the Berkeley *Drosophila* Genome Project, several of the suppressor lines are now being actively pursued in the lab. This will ultimately lead to a better understanding of how Rpr, Hid, and Grim, as well as potential mammalian homologues, act to bring about cell death. A description of the progress made in characterizing one of the suppressors follows in Chapter 4.

References

Adams, M. D. et al. (2000). The genome sequence of *Drosophila melanogaster. Science 287*, 2185-2195.

Chen, P., Nordstrom, W., Gish, B., and Abrams, J. M. (1996). grim, a novel cell death gene in *Drosophila. Genes Dev. 10*:1773-1782.

Grether, M. E., Abrams, J. M., Agapite, J., White, K, and Steller, H. (1995). The head involution defective gene of *Drosophila melanogaster* functions in programmed cell death. *Genes Dev. 9*:1694-1708.

Haupt, Y., Alexander, W. S., Barri, G., Klinken, P. S. and Adams, J. M. (1991). Novel zinc finger gene implicated as myc collaborator by retrovirally accelerated lymphomagenesis in E mu-myc transgenic mice. *Cell. 65*, 753–763.

Hay, B. A., Wolff, T. and Rubin, G. M. (1994). Expression of baculovirus P35 prevents cell death in *Drosophila. Development. 120*:2121-129.

Hay, B. A., Wassarman, D. A. and Rubin, G. M. (1995). *Drosophila* homologs of baculovirus inhibitor of apoptosis proteins function to block cell death. *Cell. 83*:1253-1262.

Hay, B. A., Maile, R. and Rubin, G. M. (1997). P element insertion-dependent gene activation in the *Drosophila* eye. Proc. Natl. Acad. Sci. USA *94*, 5195-5200.

Miklos, G. L. and Rubin, G. M. (1996). The role of the genome project in determining gene function: insights from model organisms. Cell *23*, 521-529.

Smith, D., Wohlgemuth, J., Calvi, B. R., Franklin, I. and Gelbart, W. M. (1993). hobo enhancer trapping mutagenesis in *Drosophila* reveals an insertion specificity different from P elements.

van Lohuizen, M., Verbeek, S., Scheijen, B., Wientjens, E., van der Gulden, H. and Berns, A. (1991). Identification of cooperating oncogenes in E mu-myc transgenic mice by means of provirus tagging. Cell *65*, 737–752.

White, K., Grether, M. E., Abrams, J. M., Young, L., Farrell, K. and Steller H. (1994). Genetic control of programmed cell death in *Drosophila*. Science. *264*:677-683.

CHAPTER 4

Drosophila Bruce (dBruce) can potently suppress Rpr- and Grim-, but not Hid-dependent cell death

Stephanie Y. Vernooy, Vivian Chow, Julius Su, Koen Verbrugghe, Jennifer Yang, Susannah Cole, Christopher L. Holley[1] and Bruce A. Hay

Current Biology, in press

Abstract

Bruce is a large protein (530 kDa) that contains an N-terminal baculovirus IAP repeat (BIR) and a C-terminal ubiquitin conjugation domain (E2) [1, 2]. Bruce upregulation occurs in some cancers, and contributes to the resistance of these cells to DNA-damaging chemotherapeutic drugs [2]. However, it has remained unknown whether Bruce inhibits apoptosis directly or instead plays some other more indirect role in mediating chemoresistance, such as by promoting drug export, decreasing the efficacy of DNA damage-dependent cell death signaling, or by promoting DNA repair. Here we demonstrate, using gain-of-function and deletion alleles, that *Drosophila* Bruce (dBruce) can potently inhibit cell death induced by the essential *Drosophila* cell death activators Reaper (Rpr) and Grim, but not Head involution defective (Hid). The dBruce BIR domain is not sufficient for this activity, and the E2 domain is likely required. dBruce does not promote Rpr or Grim degradation directly, but its antiapoptotic actions do require that their N-termini, required for interaction with DIAP1 BIR 2, be intact. dBruce does not block the activity of the apical cell death caspase Dronc or the proapoptotic bcl-2 family member Debcl/Drob-1/dBorg-1/Dbok. Together, these results argue that dBruce can regulate cell death at a novel point.

Results and Discussion

In *Drosophila*, the products of the *reaper* (*rpr*), *head involution defective* (*hid*) and *grim* genes are essential activators of caspase-dependent cell death (reviewed in [3]). We carried out a genetic screen for suppressors of Rpr-, Hid-, and Grim-dependent cell death to identify regulators of their activity. We generated approximately 7000 new insertion lines of the GMREP P element transposon [4]. GMREP contains an engineered eye-specific enhancer sequence (GMR). This sequence is sufficient to drive the expression of linked genes in and posterior to the morphogenetic furrow during eye development. Thus, insertion of GMREP within a region can lead to the eye-specific expression of nearby genes. Each insertion line was crossed to flies that had small eyes due to the eye-specific expression of Rpr (GMR-Rpr flies), Hid (GMR-Hid flies), or Grim (GMR-Grim flies), and the progeny scored for enhancement or suppression. A number of suppressors were identified (to be described elsewhere). Five lines (GMREP-86A-1-5) mapped to the 86A region (see Figure 1A), and each strongly suppressed cell death induced by eye-specific expression of Rpr (Figure 1A II, III) or Grim (Figure 1A IV, V), but not Hid (Figure 1A VI, VII). These lines mapped within a 6 Kb interval. We obtained a number of other lines with P element insertions located in the nearby region. Four of these, EP(3)0359, EP(3)0739, l(3)j8B6, and l(3)06142, mapped within 6 base pairs of the GMREP-86A-3-5 insertion sites (Figure 1B). None of these, or a fifth nearby line, l(3)06439, acted as suppressors of GMR-Rpr-, GMR-Grim-, or GMR-Hid-dependent cell death

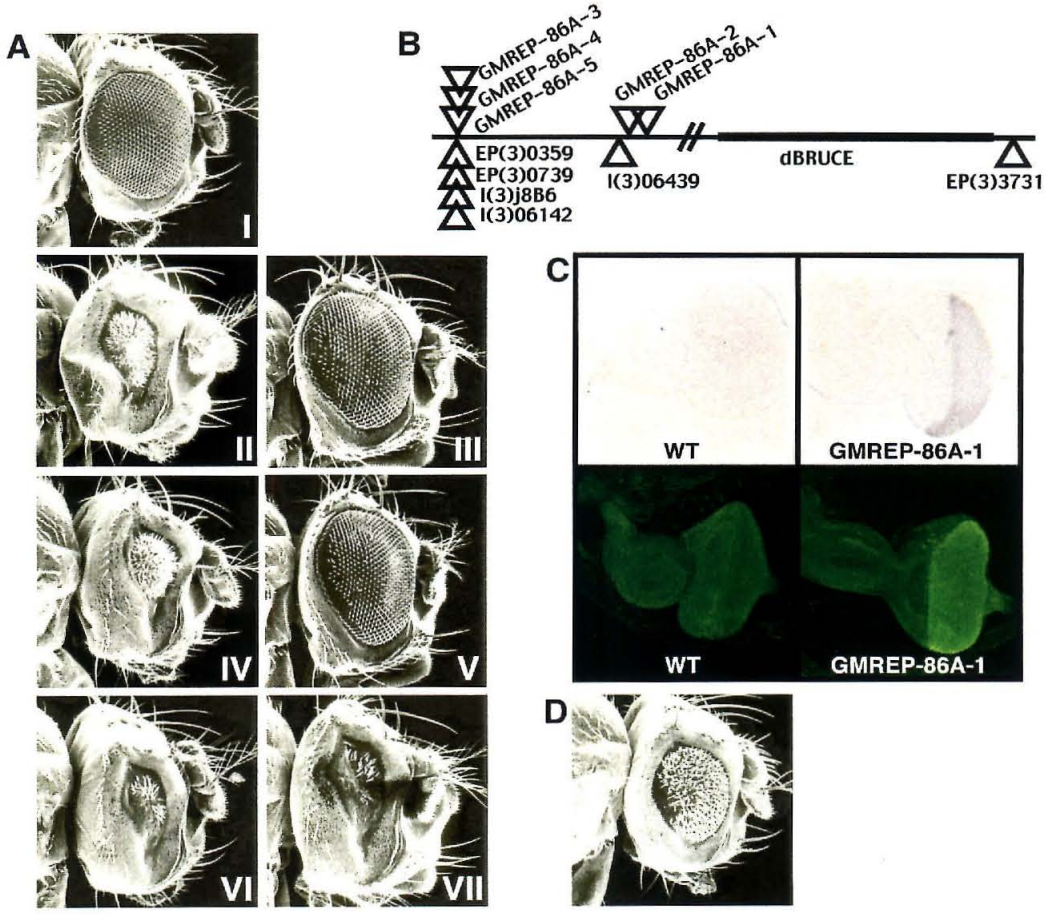

A
I
II
III
IV
V
VI
VII
B
GMREP-86A-3
GMREP-86A-4
GMREP-86A-5
GMREP-86A-2
GMREP-86A-1
EP(3)0359
EP(3)0739
l(3)j8B6
l(3)06142
l(3)06439
dBRUCE
EP(3)3731
C
WT
GMREP-86A-1
WT
GMREP-86A-1
D

Figure 1

Figure 1. dBruce expression suppresses cell death induced by Rpr and Grim, but not Hid.

(A) Scanning electron micrographs are shown. The genotypes are as follows: I, wild-type. II, GMR-Rpr/+. III, GMREP-86A-1/GMR-Rpr. IV, GMR-Grim/+. V, GMREP-86A-1/GMR-Grim. VI, GMR-Hid/+. VII, GMREP-86A-1/GMR-Hid. Each of the GMREP-86A insertion lines, which ectopically express dBruce in the eye (Figure 1C) act as strong suppressors of Rpr- and Grim-, but not Hid-dependent eye cell death. Representative examples are shown for one of these insertions, GMREP-86A-1. Scanning electron microscopy was performed as described in [22].

(B) Diagram of P element insertions in the 86A region. The P elements shown stacked on top of each other are all within 6 base pairs of each other, and are 23 kb upstream of the 5' end of the dBruce translation start codon. GMREP-86A-1 and –2 and l(3)06439 are within 1 kb of each other, and as a group are about 18 kb upstream of dBruce. Only the GMREP-86A-1-5 lines suppress GMR-Reaper- and GMR-Grim-induced death. EP(3)3731 is located 1 kb 3' to the dBruce translation stop codon.

(C) dBruce transcript and protein are ectopically expressed posterior to the morphogenetic furrow in eye discs from all five GMREP-86A lines. In situ hybridizations with a dBruce probe and immunolabelling with a dBruce-specific antibody on eye discs from wild-type larvae and GMREP-86A-1 larvae are shown. In situ hybridizations were performed as described in [22]. See Supplemental Material for immunolabelling details.

(D) GMREP-86A-1-dependent suppression of GMR-Rpr-induced death (compare with Figure 1A, III) is attenuated by co-expression of a dBruce RNAi construct. Scanning electron micrograph of a fly eye with the genotype GMREP-86A-1, GMR-Rpr/GMR-dBruce-RNAi is shown.

(data not shown). These results argued that the cell death suppression seen with the GMREP-86A lines was not due to a transposon-induced loss of function, but rather to the GMREP-dependent expression of a nearby gene. All of the GMREP-86A insertions were located 5' to a gene encoding the *Drosophila* homolog, dBruce, of murine Bruce [1] (also known as Apollon in humans [2]), suggesting this as an obvious candidate. The results of tissue in situ hybridizations with a dBruce probe and immunocytochemistry with a dBruce-specific antibody supported this possibility. dBruce transcript and protein were expressed at uniform low levels in wild-type eye discs. However, in the GMREP86A lines they were expressed at high levels in and posterior to the morphogenetic furrow of the eye disc, which is where the GMR-element drives expression [4] (Figure 1C).

To demonstrate that dBruce was responsible for the GMREP-86A-dependent suppression of Rpr-and Grim-dependent cell death, we specifically downregulated levels of the dBruce transcript in the eyes of flies carrying a GMR-Rpr transgene as well as a GMREP-86A element. We focused our analysis on one line, GMREP-86A-1, as all five lines behaved similarly with respect to cell death suppression and dBruce overexpression. We generated flies that carried a dBruce RNA interference (RNAi) construct driven under GMR control (GMR-dBruce-RNAi flies). The eyes of GMR-dBruce-RNAi flies were normal (data not shown). We crossed these animals to flies in which GMR-Rpr-dependent cell

death was suppressed by the presence of the GMREP-86A-1 transposon, and identified progeny from this cross that carried all three transgenes, GMR-dBruce-RNAi, GMR-Rpr, and GMREP-86A-1. We reasoned that if ectopic expression of dBruce in the eye, driven by the GMREP-86A-1 insertion, was responsible for the suppression of Rpr-dependent cell death, then expression of dBruce-RNAi should downregulate levels of dBruce sense transcript. This should lead to an attenuation of the GMR-EP-86A-1-dependent suppression of Rpr-dependent cell death, causing a decrease in eye size. Such an attenuation was in fact observed (Figure 1D, compare with Figure 1A,III). These observations, in conjunction with those obtained from studies with dBruce deletion mutants (Figure 2), argue that dBruce can suppress Rpr- and Grim-dependent cell death.

We sequenced cDNAs encompasing the dBruce coding region. This allowed us to assemble an accurate map of the dBruce exon-intron structure, which differs in some respects from that of the BDGP predicted gene (Figure 2A and genbank accession number#). Overall, dBruce is 30% identical to murine Bruce. However, the dBruce N-terminal BIR domain and the C-terminal E2 domain show much higher degrees of homology, 83 and 86 percent identity, respectively. *C. elegans* homologs of Bruce were not apparent. We generated mutations in the dBruce gene by carrying out imprecise excision of a P element, EP3731, located 3' to the dBruce transcript (Figure 2A). We generated two deletions that extended only in one direction, into the 3' end of the dBruce coding region. E12 deleted a relatively

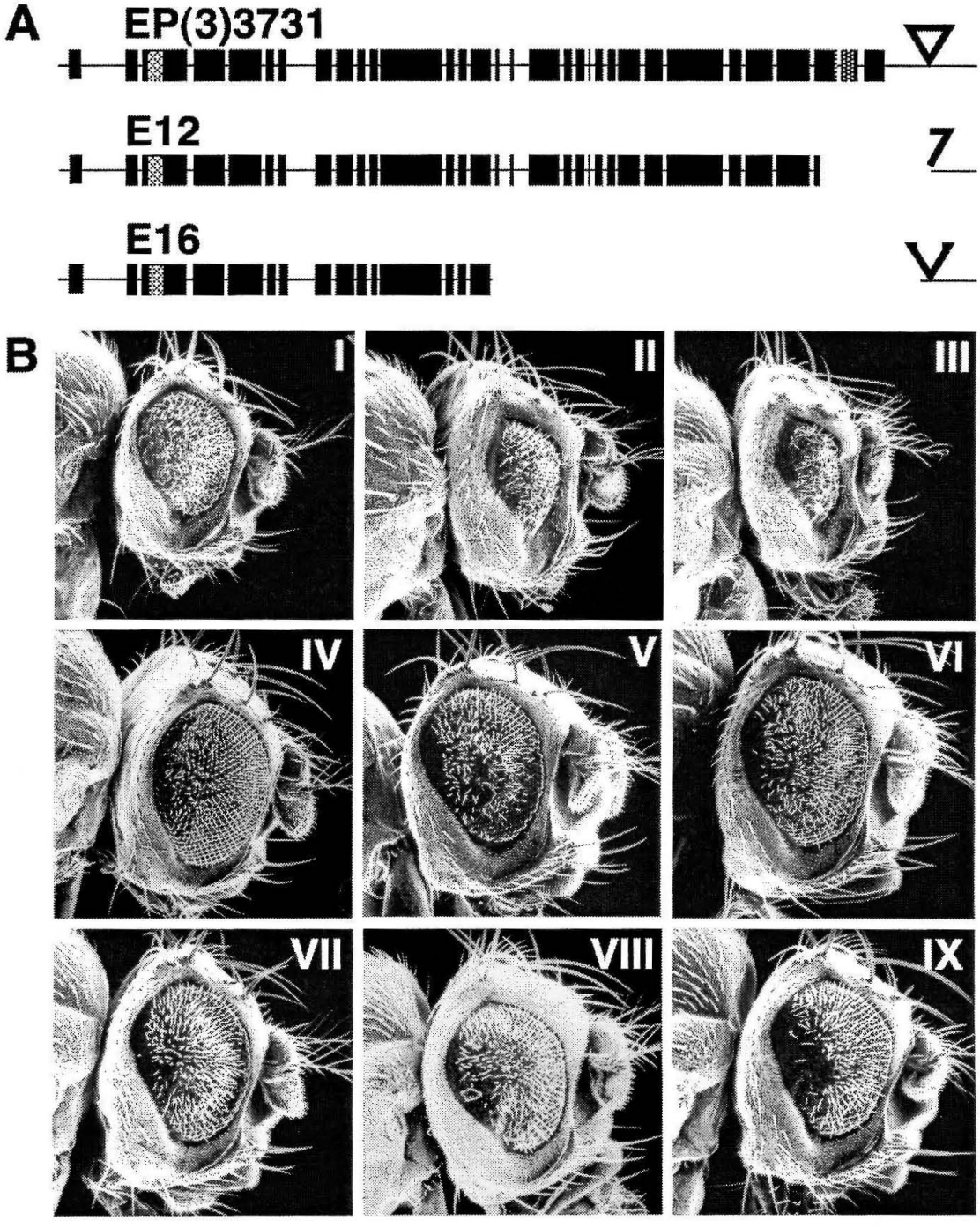


Figure 2

Figure 2. C-terminal deletion mutations of dBruce mutations enhance Rpr- and Grim-dependent cell death.

(A) Genomic structure of the dBruce coding region, and the regions removed in the deletion mutants E12 and E16, are shown. The patterned box in the third exon indicates the location of the BIR; the patterned boxes in the second and third exons from the 3' end of dBruce indicate the location of the ubiquitin conjugation domain. E12 removes 1.5 kb of dBRUCE genomic DNA, and E16 removes 10 kb. Both deletions remove the ubiquitin-conjugating domain. See Supplemental Material for details.

(B) dBruce deletion mutants enhance Reaper- and Grim-dependent death. Scanning electron micrographs are shown. The genotypes shown are as follows: (I) GMR-RprM/+. (II) E12/GMR-RprM. (III) E16/GMR-RprM. (IV) GMR-GrimM/+. (V) E12/GMR-GrimM. (VI) E16/GMR-GrimM. (VII) GMR-HidM/+. (VIII) E12/GMR-HidM. (IX) E16/GMR-HidM. GMR-RprM, GMR-GrimM, and GMR-HidM are lines that have slightly larger eyes than the GMR-Rpr, GMR-Grim, and GMR-Hid lines used in Figure 1, and are used here to score for enhancement.

small region of the C-terminus that includes the E2 domain, while E16 deleted approximately the C-terminal half of the dBruce coding region (Figure 2A). Both lines were homozygous viable but male sterile. We cannot exclude the possibility that E12 and E16 represent neomorphic mutations in dBruce. However, we favor the hypothesis that they represent hypomorphs or null mutations since they had the opposite phenotype to the GMREP-86A dBruce expression lines when in combination with GMR-Rpr, acting as enhancers rather than suppressors of Rpr-dependent cell death in the eye (Figure 2B, I-III). E12 and E16 also enhanced GMR-Grim, but this effect was much more modest (Figure 2B, IV-VI). E12 and E16 had no clear effect on cell death due to expression of Hid (Figure 2B, VII-IX).

These results argue that endogenous dBruce levels, at least in the eye, are sufficient to act as a brake on Rpr-, and to some extent Grim-dependent, cell death. How does dBruce suppress apoptosis? A number of observations argue that Rpr and Grim-dependent killing proceeds through distinct mechanisms, and/or is regulated differently than that due to Hid. These differences are manifest at multiple points. At the level of DIAP1, point mutations of DIAP1 have effects on Rpr and Grim-dependent cell death that are opposite to those due to Hid [5]. In addition, in a *Drosophila* extract, Hid, but not Rpr and Grim, promotes DIAP1 polyubiquitination [6]. In contrast, in a different set of assays Rpr and Grim, but not Hid, act as general inhibitors of protein translation [6, 7]. Finally, Rpr and Grim, but not Hid, show strong synergism with the effector caspase

DCP-1 in terms of their ability to induce cell death in the eye [8]. Each of these points defines a possible target for dBruce antiapoptotic action.

Because dBruce strongly suppressed cell death induced by Rpr and Grim, but not by Hid, one obvious possibility was that dBruce promoted Rpr and Grim ubiquitination and degradation. We tested this hypothesis by generating mutant versions of Grim and Rpr that lacked all lysines, the amino acid to which ubiquitin is added. We introduced these genes into flies under GMR control. GMR-Rpr-lys$^-$ and GMR-Grim-lys$^-$ flies have small eyes, indicating that these mutant proteins are effective cell death inducers. GMREP-86A-1-dependent dBruce expression suppressed this death very effectively, indicating that dBruce cannot be promoting ubiquitin-dependent degradation of Rpr or Grim (Figure 3). Interestingly, however, dBruce expression did not suppress cell death induced by expression of versions of Rpr (GMR-RprC) or Grim (GMR-GrimC) lacking their N-termini [9, 10], which are required for their IAP-caspase disrupting interactions with the DIAP1 BIR2 [11]. This result is important because it argues that dBruce does not act to regulate this relatively uncharacterized death pathway.

The N-terminal dBruce BIR lacks a number of residues thought to be important for binding of Rpr, Hid, and Grim to DIAP1 BIR2 [12]. Thus it seems unlikely that GMR-driven expression of dBruce inhibits cell death by simply titrating Rpr and Grim away from interactions with DIAP1 BIR2 as a result of similar interactions

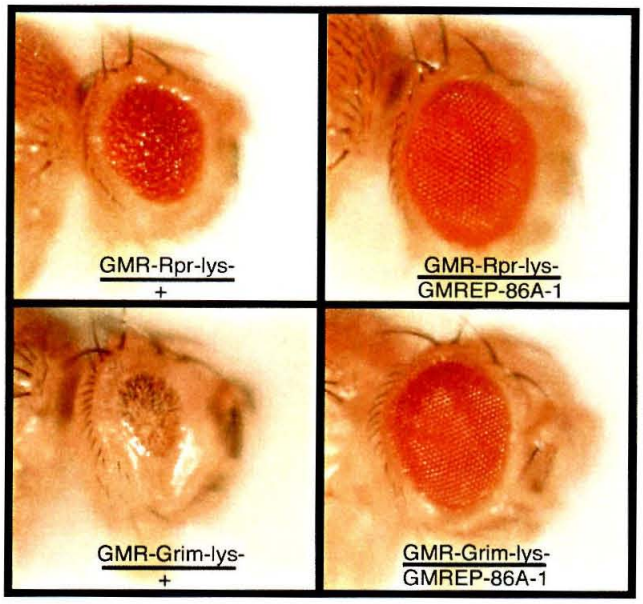


Figure 3

Figure 3. dBRUCE does not suppress Rpr-and Grim-dependent cell death by promoting Rpr and Grim ubiquitination.

GMREP-86A-1 suppresses death induced by overexpression of versions of Rpr and Grim that lack lysine residues, and thus cannot be ubiquitinated.

with the dBruce BIR. Nonetheless, the high degree of conservation between dBruce and mammalian Bruce in the BIR suggests that it is functionally important. To explore this role further we expressed under GMR control a fragment of dBruce that contained residues 1-531, including the BIR domain (aa 251-321). Flies carrying this construct, GMR-dBruce-BIR flies, had normal appearing eyes, and in crosses to flies expressing GMR-Rpr, -Hid, or -Grim, GMR-dBruce-BIR did not enhance or suppress these eye phenotypes (data not shown). These results do not rule out a role for the dBruce BIR in suppressing Rpr- and Grim-dependent cell death. However, they do suggest that the BIR alone is unlikely to mediate this inhibition.

dBruce overexpression in the eye also did not suppress cell death resulting from GMR-driven expression of the caspase Dronc, which is required for many apoptotic cell deaths in the fly, including those induced by expression of Rpr, Grim, and Hid [13-17] (Supplement Figure 1). Dronc most resembles mammalian caspase-9, and its activation is likely to involve interactions with the *Drosophila* Apaf-1 homolog Ark [16, 17]. Thus, this result strongly suggests that dBruce does not block Ark-dependent Dronc activation or Dronc activity. This result is also suggested by the observation that decreasing Ark or Dronc in the eye strongly suppressed Hid-dependent cell death [14-16, 18], which dBruce did not. A similar lack of cell death suppression was seen in the progeny of crosses between GMR-dBruce flies and flies expressing a second long prodomain caspase Strica

[19], whose mechanism of activation and normal functions are unknown. Finally, GMREP-86A-1 also failed to suppress the cell death due to GMR-dependent expression of the *Drosophila* proapoptotic Bcl-2 family member known variously as Debcl, Drob-1, dBorg-1 or Dbok (reviewed in [3]) (Supplement Figure 1).

Concluding remarks

The Bruce gene is found in mammals and flies, but not in the worm *C. elegans*. In humans it is upregulated in some cell lines derived from gliomas and an ovarian carcinoma, and the results of antisense inhibition of Bruce suggested that it contributes to the resistance of these cells to DNA-damaging chemotherapeutic drugs [2]. Here we showed that the *Drosophila* homolog of Bruce, dBruce, can potently inhibit cell death induced by Rpr and Grim, but not Hid. In addition, flies with C-terminal deletions that removed the Bruce ubiquitin conjugation domain, or much larger regions of the coding region, acted as dominant enhancers of Rpr- and Grim-, but not Hid-dependent cell death. Together, these observations clearly demonstrate that dBruce can function as a cell death suppressor. Our results with the deletion mutants suggest, but do not prove, that dBruce's death inhibiting activity requires its function as a ubiquitin-conjugating enzyme. Based on the general conservation of cell death regulatory mechanisms our results, in conjunction with those of Chen et al. [2], argue that mammalian Bruce is likely to facilitate oncogenesis by directly promoting cell survival in the face of specific death signals. One mechanism by which Rpr,

Grim, and Hid promote apoptosis is by binding to DIAP1, thereby blocking its ability to inhibit caspase activity [11]. It will be interesting to determine if mammalian Bruce also inhibits cell death induced by the expression of specific IAP binding proteins.

How does dBruce inhibit cell death? It does not promote the ubiquitination and degradation of Rpr and Grim directly. However, we cannot rule out the possibility that it somehow sequesters them from their proapoptotic targets. The fact that it does not inhibit cell death due to Hid or Dronc expression argues that it is unlikely to be acting on core apoptotic regulators such as Ark, Dronc or DIAP1, which are important for Hid-, Rpr-, and Grim-dependent cell death. An attractive hypothesis is that dBruce, perhaps in conjunction with apoptosis-inhibiting ubiquitin-protein ligases such as DIAP1 or DIAP2, promotes the ubiquitination and degradation of a component specific to Rpr- and Grim-dependent death signaling pathways. What might such a target be? Little is known about how Rpr- and Grim-dependent death signals differ from those due to Hid. However, one possibility is suggested by the recent observation that Rpr and Grim, but not Hid, can inhibit global protein translation [6, 7]. This creates an imbalance between levels of short-lived IAPs and the caspases they inhibit, thereby sensitizing cells to other death signals. Perhaps dBruce targets a protein(s) required for this activity.

Finally, Bruce is a very large protein and thus its coding region might be expected to be subject to a relatively high frequency of mutation. Truncation of dBruce through introduction of a stop codon or a frameshift is thus likely to be a relatively common form of Bruce mutation. The results of our deletion analysis show that C-terminal dBruce truncations act to enhance cell death in response to several different signals. Given this, it will be interesting to determine if human Bruce mutations are associated with predisposition to pathologies that involve an inappropriate increase in cell death.

Acknowledgments

We thank John Nambu and Sharad Kumar for providing fly stocks, Pat Koen for assistance with SEM, Lijuan Wang for assistance with protein purification, Hong Yu for assistance with Rpr construct generation, and Sally Kornbluth for the Rpr-lys- transgene. This work was supported by grants to B.A.H. from Amgen, and NIH grant GM057422.

References

1. Hauser, H. P., Bardroff, M., Pyrowolakis, G. and Jentsch, S. (1998). A giant ubiquitin-conjugating enzyme related to IAP apoptosis inhibitors. *J. Cell Biol. 141*, 1415-1422.

2. Chen, Z., Naito, M., Hori, S., Mashima, T., Yamori, T. and Tsuruo, T. (1999). A human IAP-family gene, apollon, expressed in human brain cancer cells. *Biochem. Biophys. Res. Commun. 264*, 847-854.

3.Vernooy, S. Y., Copeland, J., Ghaboosi, N., Griffin, E. E., Yoo, S. J. and Hay, B. A. Cell death regulation in *Drosophila*: conservation of mechanism and unique insights. (2000). *J. Cell Biol.* 150, F69-76.

4. Hay, B. A., Maile, R. and Rubin, G. M. (1997). P element insertion-dependent gene activation in the *Drosophila* eye. *Proc. Natl. Acad. Sci. USA 94*, 5195-5200.

5. Lisi, S., Mazzon, I. and White, K. (2000). Diverse domains of THREAD/DIAP1 are required to inhibit apoptosis induced by Reaper and Hid in *Drosophila*. *Genetics 154*, 669-678.

6. Yoo, S. J., Huh, J. R., Muro, I., Yu, H., Wang, L., Wang., S. L., Feldman, R. M. R., Clem, R. J., Muller, H. A. and Hay, B. A. (2002). Apoptosis inducers Hid, Rpr

and Grim negatively regulate levels of the caspase inhibitor DIAP1 by distinct mechanisms. *Nature Cell Biol.* (in press).

7. Holley, C. L., Olson, M. R., Colon-Ramos, D. A. and Kornbluth, S. (2002). Reaper eliminates IAP proteins through stimulated IAP degradation and generalized translational inhibition. *Nature Cell Biol.* (in press).

8. Song, Z., Guan, B., Bergman, A., Nicholson, D. W., Thornberry, N. A., Peterson, E. P. and Steller, H. (2000). Biochemical and genetic interactions between *Drosophila* caspases and the proapoptotic genes *Rpr, Hid, and Grim*. *Mol. Cell Biol. 20*, 2907-2914.

9. Wing, J. P., Zhou, L., Schwartz, L. M. and Nambu, J. R. (1998). Distinct cell killing properties of the *Drosophila reaper*, *head involution defective*, and *grim* genes. *Cell Death Differ. 5*, 930-939.

10. Wing, J. P., Schwartz, L. M. and Nambu, J. R. (2001). The RHG motifs of *Drosophila* Reaper and Grim are important for their distinct cell death-inducing abilities. *Mech. Dev. 102*, 193-203.

11. Shi, Y. (2002). Mechanisms of caspase activation and inhibition during apoptosis. *Mol. Cell 9*, 459-470.

12. Wu, J. W., Cocina, A. E., Chai, J., Hay, B. A. and Shi, Y. (2001). Structural analysis of a functional DIAP1 fragment bound to Grim and Hid peptides. Mol. *Cell 8*, 95-104.

13. Dorstyn, L., Colussi, P. A., Quinn, L. M., Richardson, H. and Kumar, S. (1999). DRONC, an ecdysone-inducible *Drosophila* caspase. *Proc. Natl. Acad. Sci. USA 96*, 4307-4312.

14. Meier, P., Silke, J., Leevers, S. J. and Evan, G. I. (2000). The *Drosophila* caspase DRONC is regulated by DIAP1. *Embo J. 19*, 598-611.

15. Hawkins, C. J., Yoo, S. J., Peterson, E. P., Wang, S. L., Vernooy, S. Y. and Hay, B. A. (2000). The *Drosophila* caspase DRONC cleaves following glutamate or aspartate and is regulated by DIAP1, Hid, and Grim. *J. Biol. Chem. 275*, 27084-27093.

16. Quinn, L. M., Dorstyn, L., Mills, K., Colussi, P. A., Chen, P., Coombe, M., Abrams, J., Kumar, S. and Richardson, H. An essential role for the caspase Dronc in developmentally programmed cell death in *Drosophila*. *J. Biol. Chem. 275*, 40416-40424.

17. Dorstyn, L., Read, S., Cakouros, D., Huh, J. R., Hay, B. A. and Kumar, S. (2002). The role of cytochrome c in caspase activation in *Drosophila melanogaster* cells. *J. Cell Biol. 156*, 1089-1098.

18. Rodriguez, A., Oliver, H., Zou, H., Chen, P., Wang, X. and Abrams, J. M. (1999). Dark is a *Drosophila* homologue of Apaf-1/CED-4 and functions in an evolutionarily conserved death pathway. *Nat. Cell Biol. 1*, 272-279.

19. Doumanis, J., Quinn, L., Richardson, H. and Kumar, S. (2001). STRICA, a novel *Drosophila melanogaster* caspase with an unusual serine/threonine-rich prodomain, interacts with DIAP1 and DIAP2. *Cell Death Differ. 8*, 387-394.

20. Colussi, P.A., Quinn, L. M., Huang, D. C., Coombe, M., Read, S. H., Richardson, H. and Kumar, S. (2000). Debcl, a proapoptotic Bcl-2 homologue, is a component of the *Drosophila melanogaster* cell death machinery. *J. Cell Biol. 148*, 703-714.

21. Giordano, E., Rendina, R., Peluso, I. and Furia, M. (2002). RNAi triggered by symmetrically transcribed transgenes in *Drosophila melanogaster*. *Genetics 160*, 637-648.

22. Hay, B. A., Wassarman, D. A. and Rubin, G. M. (1995). *Drosophila* homologs of baculovirus inhibitor of apoptosis proteins function to block cell death. *Cell 83*, 1253-1262.

CHAPTER 5

Drosophila spermatogenesis is associated with high levels of caspase activity and requires multiple apoptosis regulators, including Ark and Bruce, in nonapoptotic roles.

Stephanie Y. Vernooy, Jun R. Huh and Bruce A. Hay

(In preparation)

Abstract

Drosophila spermatogenesis is complex process that culminates in the generation of 64 haploid spermatids from a single spermatagonial cell. Spermatozoa mature within a germline syncytium in which cells are connected to each other by cytoplasmic bridges. Ultimately, however, each spermatid must become encapsulated by an independent plasma membrane through a process known as individualization [1, 2]. Here we demonstrate that individualization, which does not involve apoptosis, requires the activity of multiple cell death regulators. Spermatids undergoing individualization contain high levels of activated apoptptic effector caspases. They also express high levels of the apical caspase Dronc and the Dronc-activating cytoplasmic adaptor Ark. Importantly, males mutant for Ark or the cell death inhibitor dBruce are semisterile or sterile, respectively, with defects in sperm individualization. Together, these observations suggest that core components of the apoptotic caspase cascade can function as a cassette, regulating spermatid differentiation as well as apoptosis.

Results and Discussion

Most if not all cells have the potential to carry out the apoptotic cell death program [3, 4]. Key players in this process are a family of cysteine proteases known as caspases. Apical caspases transduce death signals by cleaving and activating effector caspases. These then cleave and alter the function of a number of cellular proteins, leading to the morphological and biochemical events associated with apoptosis [5]. Caspases have also been described as playing nonapoptotic roles in an increasing number of situations (reviewed in [6-8]). An interesting question is how cascades of caspase activation can be channeled, allowing them to perform both apoptotic and nonapoptotic functions. In *Drosophila*, the products of the *reaper* (*rpr*), *head involution defective* (*hid*), and *grim* genes are essential activators of caspase-dependent cell death (reviewed in [9]). dBruce, a very large protein with an N-terminal BIR repeat and a C-terminal ubiquitin conjugation domain, acts as a strong suppressor of Rpr- and Grim- but not Hid-dependent cell death [10]. dBruce mutant males are viable, but sterile. Postmeiotic spermatids in dBruce mutant testes elongate, but they remain clumped, fail to enter the seminal vesicle and never become motile [10]. However, this phenotype does not result from an increase in testes cell death. These observations suggested that dBruce has a nonapoptotic function during spermatogenesis [10]. To explore this role, and the possibility that normal spermatogenesis might have a more general requirement for components of the core cell death machine, we examined testes of various genotypes with several different probes.

Drice and Dcp-1 are *Drosophila* effector caspases [11, 12]. They, like other effector caspases [5], become activated in response to apoptosis-inducing signals that promote cleavage of the caspase zymogen into large and small subunits that heterodimerize and make up the active caspase. Drice and Dcp-1 are expressed relatively ubiquitously. However, their state of activation can be visualized using antibodies that recognize only the cleaved and therefore active versions of the protease. In the embryo, and in larval disc tissues, immunoreactivity with anti-active Drice antibodies correlates well with the presence of cells undergoing apoptosis [13]. We also generated antibodies designed to recognize only active versions of Dcp-1 (see legend to Figure 1). These antibodies also specifically recognize apoptotic cells in the *Drosophila* embryo and imaginal discs (data not shown). However, they recognize active versions of Drice as well as Dcp-1. These antibodies (anti-active DCP-1/Drice) are thus very useful for visualizing more generally the levels of active effector caspases, and we have used them in the analysis described below.

Spermatozoa are generated and mature within a germline syncytium, which is itself encapsulated by two somatic cyst cells (reviewed in [14]). Spermatogenesis initiates apically with the formation of a primary spermatogonial cell and proceeds basally. Typically cysts at multiple stages of development can be visualized in a single testis. At the end of meiosis each *Drosophila* cyst contains 64 haploid spermatids, each approximately 2 mm long. The 64 nuclei are located at the basal end of the testis, near the seminal vesicle, and the flagellar tails extend

apically, throughout the length of the testis. Somewhat to our suprise, elongated spermatids from wild-type testes showed very high levels of active DCP-1/Drice immunoreactivity along their length (Figure 1A). To confirm that this staining really reflected the presence of activated caspases, and was not simply due to the presence of a crossreactive epitope, we carried out experiments with a mechanistically distinct, active caspase-specific reagent, sulforhodamine-VAD-fluoromethyl ketone (SR-VAD-FMK) (see legend toFigure 1 for details). SR-VAD-FMK is a sulforhodamine-labeled, membrane permeable caspase substrate that becomes covalently attached to active caspases following substrate cleavage (see Figure 1 legend for details). As shown in Figure 1B, when living testes were incubated with SR-VAD-FMK, they accumulated a pattern of label similar to that seen with anti-active DCP-1/Drice. Anti-active Drice antibodies showed a pattern of immunoreactivity in elongated spermatids similar to that seen with anti-activeDCP-1/Drice (Supplemental Figure 1). Active Drice immunoreactivity was also present in premeiotic spermatocytes at low levels. However, the significance of the spermatocyte signal is not clear since it was not apparent in testes incubated with SR-VAD-FMK. Regardless, these observations demonstrate that activated caspases are present at high levels in elongated spermatids. In addition, since SR-VAD-FMK requires cleavage of the caspase substrate for visualization these experiments argue that not only are effector caspases present in an activated form in elongated spermatids, but also that they are active.

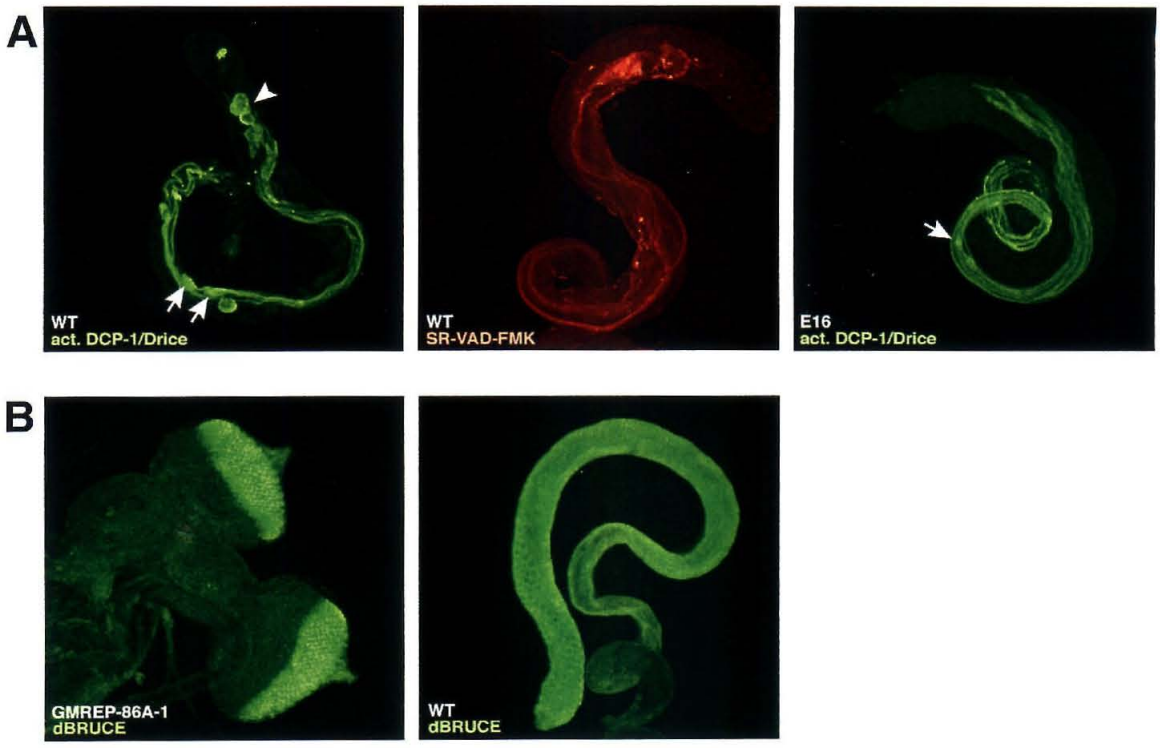

A
WT
act. DCP-1/Drice
WT
SR-VAD-FMK
E16
act. DCP-1/Drice
B
GMREP-86A-1
dBRUCE
WT
dBRUCE

Figure 1

Figure 1. *Drosophila* testes have high levels of activated caspases in elongated spermatids, and express high levels of dBruce throughout spermatogenesis.

(A) *Drosophila* testes have high levels of active caspases in elongated spermatids. Scanning confocal micrographs are shown. An antibody that recognizes the active forms of both DCP-1 and Drice (act. DCP-1/Drice) highlights elongated spermatids in testes from wild-type (WT) and dBruce mutant (E16) males (green). Cystic bulges are indicated by arrows. Waste bags are indicated by the arrowhead. E16 mutant testes lack the large cystic bulges and waste bags present in wild-type. However, a few tiny bulges containing a small number of investment cones are seen. One example is indicated by the arrow. The fluorescent caspase inhibitor and substrate SR-VAD-FMK (a sulforhodamine analog of benzyloxycarbonylvalylalanylaspartic acid fluoromethyl ketone) also localized to elongated spermatids (red), providing an independent line of argument that these cells contain active caspases. Active DCP-1/Drice specific antibodies were raised in rabbits using a synthetic nonapeptide corresponding to the COOH terminus of the DCP-1 large subunit (LEKGVTETD) conjugated with keyhole limpet hemocyanin as the immunogen (Covance). Antibodies were then purified by sequential protein affinity purification. Antisera was first applied to an affinity column containing the DRICE large subunit ending at the caspase cleavage site MQRSQTETD (1-230). The flow-through from this column was applied to an affinity column containing the full length DCP-1 protein and the flow-through collected. Immunoblotting showed that the purified antibodies were specific for the cleaved large subunits of active DCP-1 and Drice. For immunolabeling, dissected testes were fixed in PBS + 4% formaldehyde for 20 minutes and then permeabilized in PBS+ 0.3% Trition X-100, 0.3% deoxycholate, 5% BSA for 1 hour. Following an overnight incubation in primary antibody (1:40), testes were washed for 1 hour in PBTB (PBS + 0.1% Trition X-100, 5% BSA) and incubated for 1 hour in secondary antibody. Testes were then washed for an

hour, mounted in Vectashield mounting medium (Vector Labs) and viewed on a Leica TCS-NT confocal microscope. All secondary antibodies were from Molecular Probes and were used at a concentration of 1:500. For staining with SR-VAD-FMK, testes were dissected in Schneider's complete medium. SR-VAD-FMK (Intergen) was added at 1x concentration and testes were incubated 1 hr at room temperature. Testes were then fixed for 15 minutes in PBS + 4% formaldehyde, permeabilized with PBS + 0.3% Triton X-100 for 1 hour, and mounted and viewed as above.

(B) dBRUCE is abundant in *Drosophila* testes. Third instar eye-antennal discs from the dBRUCE overexpression line, GMREP-86A-1 show high levels of dBruce protein specifically in the eye disc, in and posterior to the morphogenetic furrow, where dBRUCE expression is driven by GMR element (green) [10]. dBRUCE staining is present throughout wild-type testes, but is less concentrated in spermatid tails. Antibodies were raised in rabbits using a GST-fusion protein corresponding to amino acids 361-693 of dBRUCE. Antibodies were applied to an affinity column containing protein used as the immunogen, and bound antibodies were eluted using 100mM glycine, pH2.5. Purified rabbit anti-dBRUCE was used at a concentration of 1:100.

Elongated spermatids from testes of males homozygous for the dBruce mutant E16, which results in deletion of approximately the C-terminal half of the *dBruce* coding region [10], were also anti-active DCP-1/Drice reactive (Figure 1C). Importantly, however, ectopic caspase activation was not observed, consistent with the fact that dBruce mutant testes do not display increased levels of cell death. However, we did note several important differences with wild-type testes. During individualization, the 64 spermatids, which are linked to each other in a syncytium by cytoplasmic bridges, each acquire an independent plasma membrane and lose most of their cytoplasm (reviewed in [1, 2, 14]). This process initiates when an actin-cytoskeletal-membrane complex, known as an investment cone, assembles around each spermatid nucleus. These move synchronously along the length of the cyst towards the sperm tails. This results in elimination of the connections between spermatids, encasing each in an individual plasma membrane, and extrusion of most of the sperm cytoplasm. As the investment cones move down the length of a cyst, they cause a visible bulge, known as the cystic bulge, as the cytoplasm being extruded from between the sperm tails is pushed ahead. When the cystic bulge reaches the sperm tails, it is detached and becomes known as the waste bag [1]. In wild-type testes cystic bulges could be observed along the length of the anti-active DCP-1/Drice-stained testes. Waste bags were present as large, round bulges at the apical end of the testes (Figure 1, 2). In contrast, in testes from E16 mutants waste bags were never observed. Cystic bulges were present rarely, and only near the basal end of the testes,

suggesting that they were unable to proceed the entire spermatid length. To determine where dBruce was expressed during spermatogenesis, we generated anti-dBruce antibodies. The specificity of these antibodies is illustrated in Figure 1B, which shows dBruce staining in eye-antennal discs of GMREP-86A-1 larvae. These larvae ectopically express dBruce in and posterior to the morphogenetic furrow in the eye disc [10]. Eye discs from comparable widltype larvae showed only low uniform levels of dBruce staining (data not shown). In contrast, testes of wild-type adults showed high levels of dBruce expression. This first became apparent just basal to the most apical region of the testes, which contains the somatic and germline stem cells, and remained present throughout spermatogenesis (Figure 1B).

The defects present in E16 mutants - spermatid clumping, failure to enter the seminal vesicle, a paucity of cystic bulges, and no waste bags - are characteristic of mutants in which the process of sperm individualization has failed (cf. [2, 15-17]. Progress of the cystic bulges along the spermatids can be visualized using Alexa Fluor 488-labeled phalloidin, which highlights the investment cones present on each spermatid [2]. In testes from wild-type males, investment cones within a cyst moved synchronously as a tight cluster from basal to apical within the testes. They could be found throughout the testes, including at the site of the waste bag (Figure 2). In contrast, in testes from E16 males most investment cones were located in the basal region of the testes.

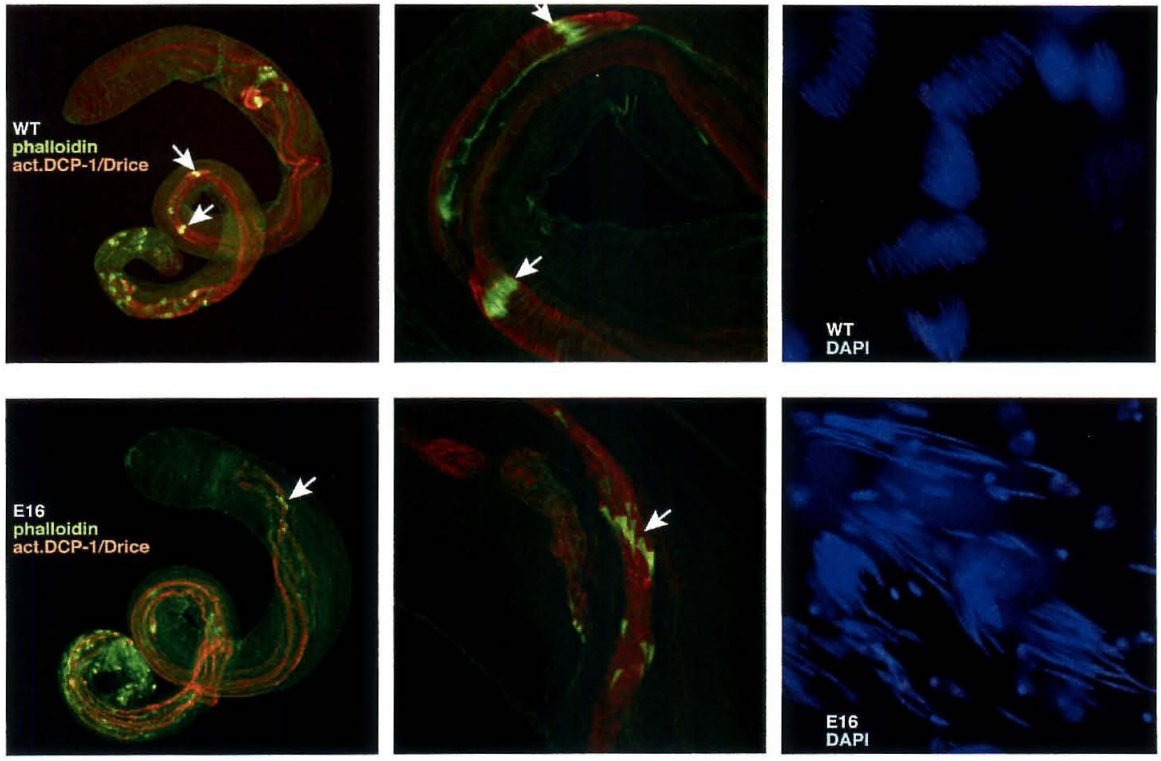

WT
phalloidin
act.DCP-1/Drice
WT
DAPI
E16
phalloidin
act.DCP-1/Drice
E16
DAPI

Figure 2

Figure 2. dBRUCE mutants are defective in individualization.

Wild-type (WT) and E16 mutant testes were double labeled with anti-active DCP-1/Drice antibody (red) and phalloidin (green). Phalloidin highlights the individual investment cones within the cystic bulges. In wild-type testes, the cones move in tightly coordinated bundles, indicated by the arrows and shown magnified in the adjacent panel. In the E16 mutant, the cones rarely leave the base of the testis, and when they do, they are scattered (indicated by the arrow and shown magnified in the adjacent panel.) Wild-type spermatid nuclei are tightly bundled and in register, whereas E16 spermatid nuclei are disordered and appear slightly longer than those of wild-type. For double labeling Alexa Fluor 488 phalloidin (Molecular Probes) was added at a final concentration of 4 units/ml along with the secondary antibody. Nuclei were visualized by mounting testes in Vectashield mounting medium with DAPI.

Investment cones moved apically in a few cysts, but in these they were invariably scattered over a wide area and they were never found at the spermatid tails (Figure 2). Nuclei from wild-type and E16 spermatids were visualized with DAPI. In the wild-type, spermatid nuclei within a cyst were arranged in register, and had a characteristic length and overall shape. In contrast, in the E16 mutant, spermatid nuclei within a cyst were often more loosely organized, and the nuclei themselves were often much longer and thinner.

The above observations, that effector caspase activity is prominent during individualization, and that loss of a caspase-dependent cell death inhibitor, dBruce, caused a defect in this process, suggested to us that individualization might involve the action of other core components of the *Drosophila* apoptosis program. One such core component is Dronc, an apical caspase whose activity is required for many different normally occurring and induced cell deaths, and which is able to cleave and activate Dcp-1 and Drice [18-22]. To test this hypothesis, we stained testes from wild-type and E16 mutants with anti-Dronc antibodies. Both showed elevated levels of Dronc protein in elongated spermatids (Figure 3A). Much, if not all, Dronc activation during apoptosis is likely to require the activity of Ark, the *Drosophila* homolog of the mammalian cytoplasmic adaptor protein, Apaf-1 [22-25]. Furthermore, Ark expression is transcriptionally regulated, and becomes prominent in multiple contexts in which high levels of apoptosis occur [23]. Thus, if Ark-dependent Dronc activity were important for individualization, one might expect that Ark expression would be

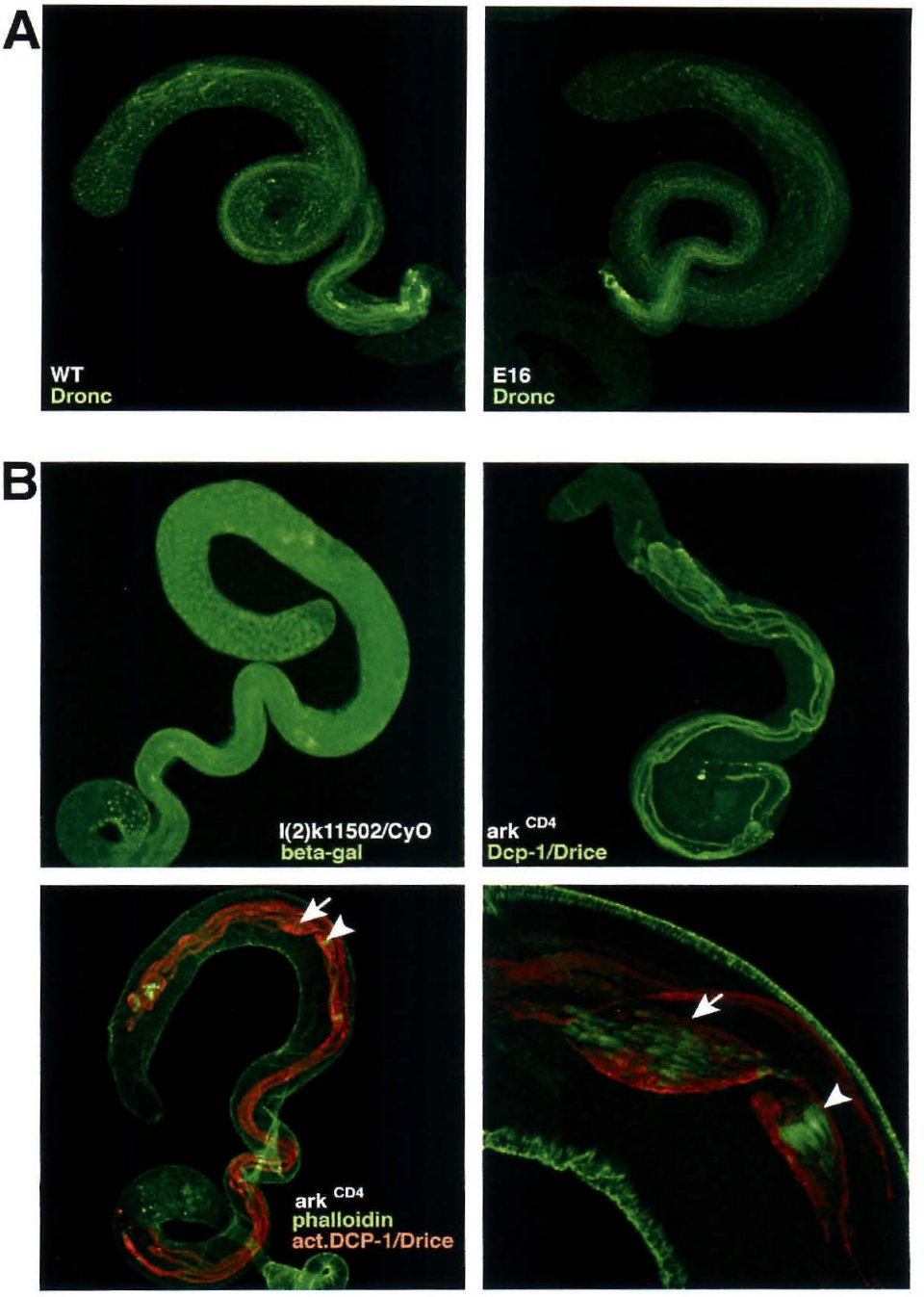

A
WT
Dronc
E16
Dronc
B
l(2)k11502/CyO
beta-gal
ark CD4
Dcp-1/Drice
ark CD4
phalloidin
act.DCP-1/Drice

Figure 3

Figure 3. Dronc and Ark are present in testes, and Ark is required for spermatid individualization.

(A) Anti-Dronc staining of wild-type (WT) and E16 testes shows that Dronc is concentrated in elongated spermatids (green). Rabbit anti-Dronc was used at a concentration of 1:400 and is described in [22].

(B) Ark, an activator of Dronc, is expressed at high levels during spermatogenesis, and is required for sperm individualization. Staining of the Ark enhancer trap line l(2)k11502/Cyo with an antibody against beta-galactosidase is shown (green). Ark mutants show levels of active DCP-1/Drice staining comparable to those of wild-type (green). The ark^{CD4} mutant has individualization defects (bottom panels). Active DCP-1/Drice is shown in red, and phalloidin in green. Some cystic bulges have tightly bundled investment cones as in wild-type, indicated by the arrowhead. However, others have scattered cones, indicated by the arrow. ark^{CD4} flies were a generous gift from John Abrams. l(2)k11502/Cyo was provided by G. M. Rubin.

elevated during spermatogenesis. Figure 2B shows that this is in fact the case. We monitored Ark expression using the l(2)k11502/Cyo line of flies, which carries a lacZ reporter gene under the control of the Ark promoter [23]. Ark was expressed in a pattern very similar to that of dBruce. It was expressed minimally in the most apical region of the testis, which contains the germ line and somatic stem cells. High level expression commenced shortly thereafter, and remained throughout the rest of spermatogenesis.

Thus, Ark is upregulated during spermatogenesis. Ark's known target, the apical caspase Dronc, is highly expressed in elongated spermatids. In addition, the Dronc targets Dcp-1 and Drice are specifically activated in elongated spermatids. Finally, mutations in dBruce, an inhibitor of caspase-dependent cell death, which is also expressed at high levels throughout spermatogenesis, are associated with defects in spermatid individualization. Together, these observations suggest, though they do not prove, that individualization requires the activity of a caspase cascade, initiated by Ark and Dronc, and regulated in some fashion by dBruce. Mutations in Dronc are not available, but mutations in Ark are. Ark^{CD4} males are homozygous viable, but approximately 40% are sterile [24]. Ark^{CD4} mutant testes contained elongated spermatids, and these did not show dramatically decreased levels of activated caspases, as might have been expected if this activity was only dependent on Ark/Dronc-dependent activation (figure 3B). However, because Ark^{CD4} mutants are due to a transposon insertion in the first intron, upstream of the initial methionine, it may

also be that Ark levels in Ark^{CD4} mutants are still sufficient to promote significant levels of Dronc-dependent effector caspase activation. Alternatively, there may be multiple pathways sufficient to promote effector caspase activation in elongated spermatids. Importantly, however, Ark^{CD4} males did show defects in sperm individualization. Some cysts appeared relatively normal, and investment cones could be seen moving synchronously towards the apical end of the testis. However, in other cysts investment cones, though present apically, were scattered over a large area, much like those present in the dBruce mutant (Figure 3B).

Concluding Remarks

Here we showed that normal *Drosophila* spermatogenesis is associated with elevated levels of multiple apoptosis regulators: Ark, Dronc, activated versions of the effector caspases Dcp-1 and Drice, and dBruce. For two of these components, Ark and dBruce, an apoptosis inducer and inhibitor, respectively, we demonstrated requirements during sperm individualization. We cannot exclude the hypothesis that Ark, Dronc, effector caspases and dBruce participate in independent processes required for spermatid individualization. However, we favor the hypothesis that they function together as a "caspase cassette," that is required for spermatid differentiation as well as apoptosis. An important test of this hypothesis will involve the characterization of animals in which caspase inhibitors such as baculovirus p35 or dominant negative versions of Dronc are expressed in different genetic backgrounds

under the control of spermatid-specific transcriptional and translational control elements (cf. [26]). Finally, while *Drosophila* and mammalian spermatogenesis are different in many ways, it is worth noting that mammalian spermatogenesis also requires that spermatids individualize from the syncytium in which they develop. Little is known about the molecular basis for this process, and whether there is any relationship between it and the high levels of spermatagonial cell apoptosis seen in normal and infertile individuals [27]. As insight is gained into individualization in *Drosophila,* it will be interesting to ask if mechanisms and molecules are conserved.

Regardless of the role caspase activity plays during individualization, our observations raise several interesting questions: how is it that elongated spermatids avoid apoptosis in the presence of activated caspases for periods of days? What are the caspase substrates? Are they different from those that promote apoptosis, or the same? If the former, then how are these components targeted specifically? If the latter, then how is the caspase cascade kept from promoting an apoptotic cell fate? Perhaps postmeiotic spermatids simply lack apoptosis-promoting caspase substrates. Alternatively, perhaps caspase-dependent cell death inhibitors sequester, modify or target particular caspase substrates for protection or degradation. In any case, it is clear that dBruce cannot be the only important such inhibitor of the apoptotic fate because dBruce mutants do not show increased levels of testes cell death. A number of mutants have been identified that show defects in spermatid individualization [2, 14-17]. It will be interesting to explore the relationship between these genes and the

apoptotic regulators described here. Perhaps novel cell death regulators are lurking in the male germline.

Acknowledgments

We thank John Abrams for providing the Ark^{CD4} flies and Lijuan Wang for protein purification. This work was supported by a National Institutes of Health grant GM057422-01 to B. A. H.

References

1. Tokuyasu, K., Peacock, W. J. and Hardy, R. W. (1972) Dynamics of spermiogenesis in *Drosophila melanogaster*. I. Individualization process. *Z. Zellforsch. 124*, 479-506.

2. Fabrizio, J. J., Hime, G., Lemmon, S. K. and Bazinet, C. (1998). Genetic dissection of sperm individualization in *Drosophila melanogaster*. *Development 125*, 1833-1843.

3. Weil, M. et al. (1996). Constitutive expression of the machinery for programmed cell death. *J. Cell Biol. 133*, 1053-1059.

4. Jacobson, M. D., Weil, M. and Raff, M. C. (1997). Programmed cell death in animal development. *Cell 88*, 347-354.

5. Thornberry, N. A. and Lazebnik, Y. (1998). Caspases: enemies within. *Science 281*, 1312-1316.

6. Zheng, T. S. and Flavell, R. A. (2000). Divinations and surprises: genetic analysis of caspase function in mice. *Exp. Cell Res. 256*, 67-73.

7. Los, M., Stroh, C., Janicke, R. U., Engels, I. H. and Schulze-Osthoff, K. (2001). Caspases: more than just killers? *Trends Immunol. 22*, 31-34.

8. Khush, R. S., Leulier, F. and Lemaitre, B. (2002). Pathogen Surveillance--the Flies Have It. *Science 296*, 273-275.

9. Vernooy, S. Y. et al. (2000). Cell death regulation in *Drosophila*: conservation of mechanism and unique insights. *J. Cell Biol. 150*, F69-76.

10. Vernooy, S. Y., Chow, V., Su, J., Verbrugghe, K., Yang, J., Cole, S., Holley, C. L. and Hay, B. A. (2002). *Drosophila* Bruce (dBruce) can potently suppress Reaper- and Grim-, but not Hid-dependent cell death. *Curr. Biol.* (In press).

11. Fraser, A. G., McCarthy, N. J. and Evan, G. I. (1997). drICE is an essential caspase required for apoptotic activity in *Drosophila* cells. *Embo J. 16*, 6192-6199.

12. Song, Z., McCall, K. and Steller, H. (1997). DCP-1, a *Drosophila* cell death protease essential for development. *Science 275*, 536-540.

13. Yoo, S. J. et al. (2002). Hid, Rpr and Grim negatively regulate levels DIAP1 by distinct mechanisms. *Nature Cell Biol.* (in press)

14. Fuller, M. (1993). Spermatogenesis. In The Development of *Drosophila melanogaster*, M. Bate and A. M. Arias, ed. (new York: Cold Spring Harbor Press), pp. 71-148

15. Castrillon, D. H. et al. (1993). Toward a molecular genetic analysis of spermatogenesis in *Drosophila melanogaster*: characterization of male-sterile mutants generated by single P element mutagenesis. *Genetics 135*, 489-505.

16. Hicks, J. L., Deng, W. M., Rogat, A. D., Miller, K. G. and Bownes, M. (1999). Class VI unconventional myosin is required for spermatogenesis in *Drosophila*. *Mol. Biol. Cell 10*, 4341-4353.

17. Timakov, B. and Zhang, P. (2001). The hsp60B gene of *Drosophila melanogaster* is essential for the spermatid individualization process. *Cell Stress Chaperones 6*, 71-77.

18. Dorstyn, L., Colussi, P. A., Quinn, L. M., Richardson, H. and Kumar, S. (1999). DRONC, an ecdysone-inducible *Drosophila* caspase. *Proc. Natl. Acad. Sci. USA 96*, 4307-4312.

19. Meier, P., Silke, J., Leevers, S. J. and Evan, G. I. (2000). The *Drosophila* caspase DRONC is regulated by DIAP1. *Embo J. 19*, 598-611.

20. Hawkins, C. J. et al. (2000). The *Drosophila* caspase DRONC cleaves following glutamate or aspartate and is regulated by DIAP1, HID, and GRIM. *J. Biol. Chem. 275*, 27084-27093.

21. Quinn, L. M. et al. (2000). An essential role for the caspase Dronc in developmentally programmed cell death in *Drosophila*. *J. Biol. Chem. 275*, 40416-40424.

22. Dorstyn, L. et al. The role of cytochrome c in caspase activation in *Drosophila melanogaster* cells. *J. Cell Biol. 156*, 1089-1098. (2002).

23. Zhou, L., Song, Z., Tittel, J. and Steller, H. (1999). HAC-1, a *Drosophila* homolog of APAF-1 and CED-4 functions in developmental and radiation-induced apoptosis. *Mol. Cell 4*, 745-755.

24. Rodriguez, A. et al. (1999). Dark is a *Drosophila* homologue of Apaf-1/CED-4 and functions in an evolutionarily conserved death pathway. *Nat. Cell Biol. 1*, 272-279.

25. Kanuka, H. et al. (1999). Control of the cell death pathway by Dapaf-1, a *Drosophila* Apaf-1/CED-4- related caspase activator. *Mol. Cell 4*, 757-769.

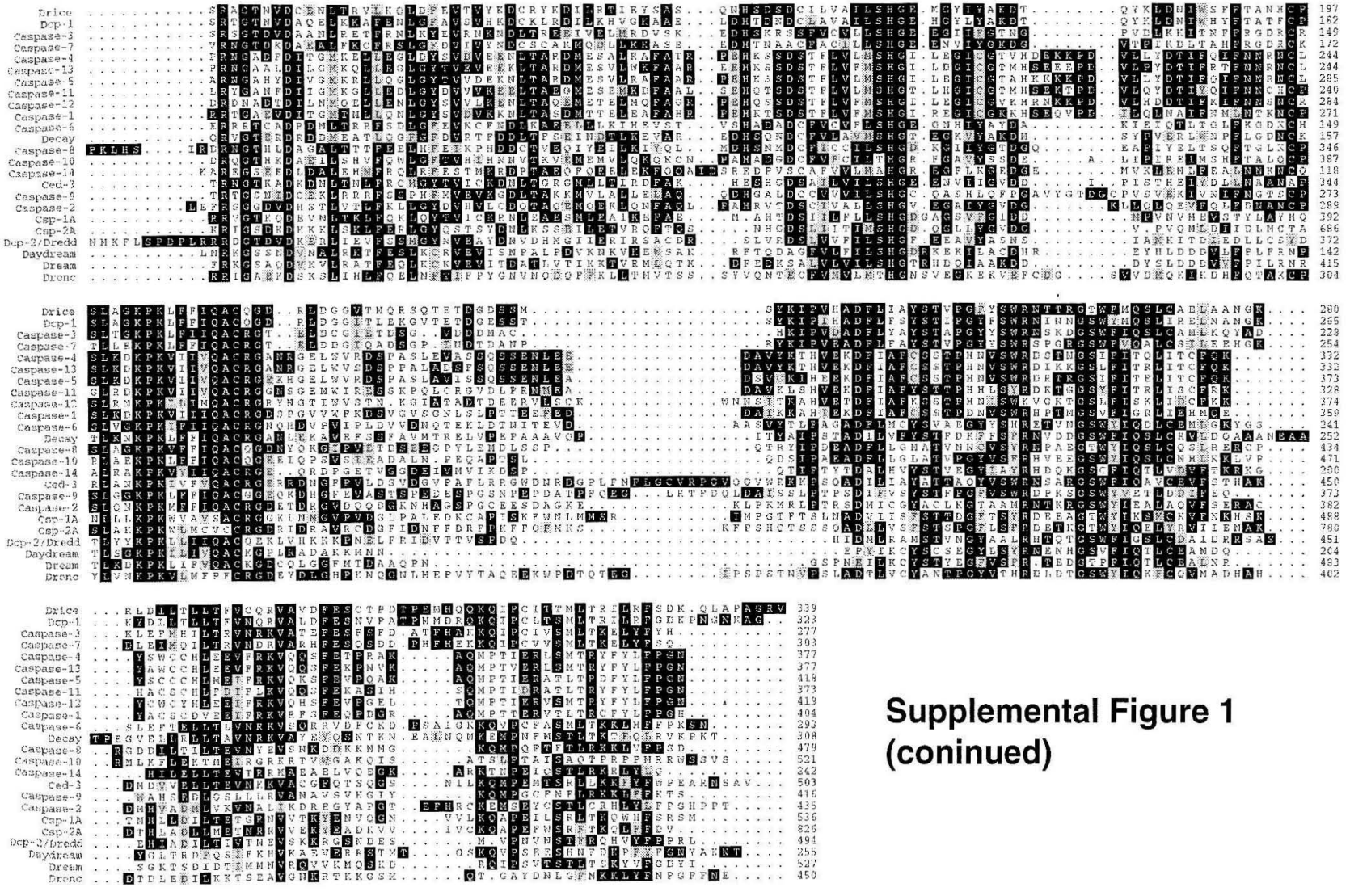
26. Santel, A., Kaufmann, J., Hyland, R. and Renkawitz-Pohl, R. (2000). The initiator element of the *Drosophila* beta2 tubulin gene core promoter contributes to gene expression in vivo but is not required for male germ-cell specific expression. *Nucleic Acids Res. 28*, 1439-1446.

27. Print, C. G. and Loveland, K. L. (2000). Germ cell suicide: new insights into apoptosis during spermatogenesis. *Bioessays 22*, 423-430.

Appendix I

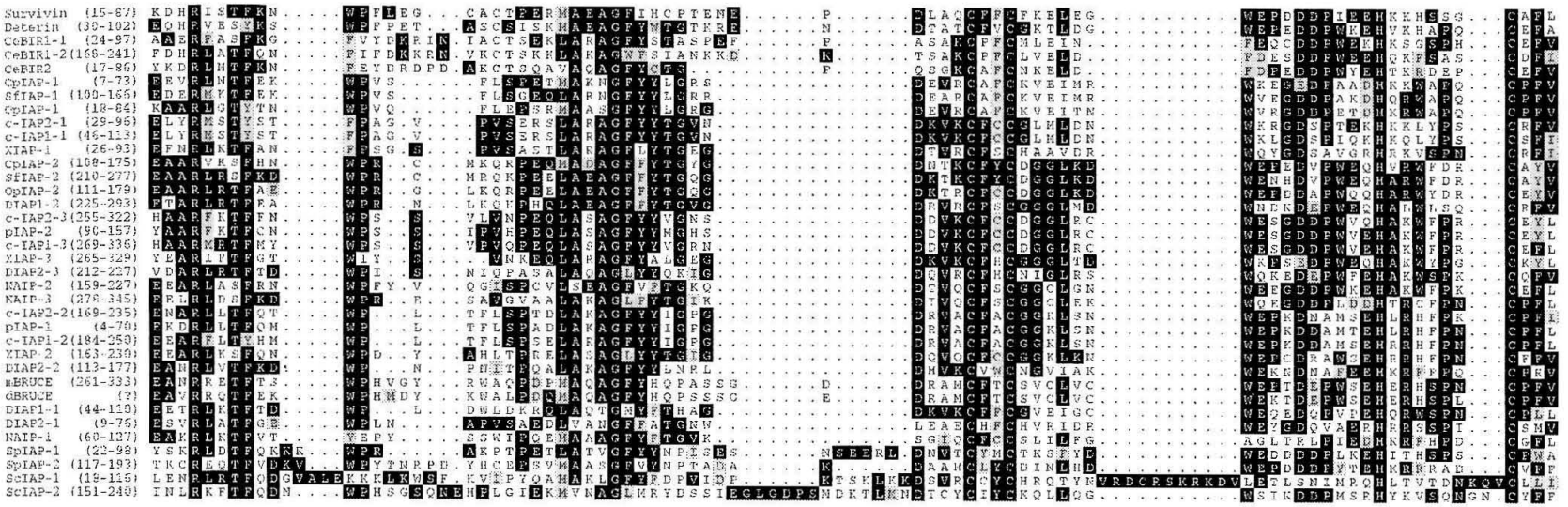
Supplemental Figures, Vernooy et al. 2000


Drice
Dcp-1
Caspase-3
Caspase-7
Caspase-4
Caspase-13
Caspase-5
Caspase-11
Caspase-12
Caspase-1
Caspase-6
Decay
Caspase-8
Caspase 10
Caspase-14
Ced-3
Caspase-9
Caspase-2
Csp-1A
Csp-2A
Dcp-2/Dredd
Daydream
Dream
Dronc



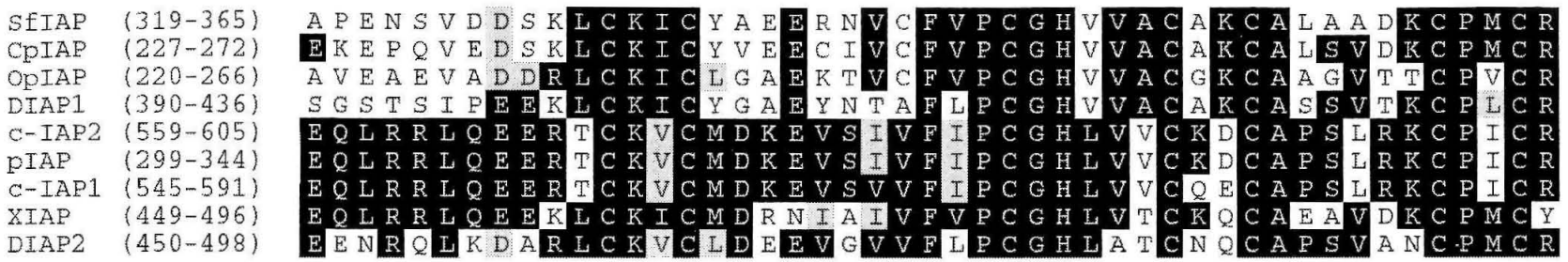
Supplemental Figure 1 (coninued)

Supplemental Figure 1. Caspase sequence alignments were done using ClustalW. The first 300 amino acids of the long prodomain caspase Csp-2 were removed for the alignment. Alignment includes *Drosophila* caspases Dronc (CAB53565), Drice (O01382), Dcp-2/Dredd (AAC33117), Decay (AAD54071), Dcp-1 (O02002), Dream (AF275814), and Daydream (AF281077), *C. elegans* caspases Ced-3 (P42573), Csp-1a (AAC98292), and Csp-2a (AAC98295), and mammalian caspases Caspase-1 (P29466), Caspase-2 (P42575), Caspase-3 (P42574), Caspase-4 (P49662), Caspase-5 (P51878), Caspase-6 (P55212), Caspase-7 (P55210), Caspase-8 (Q14790), Caspase-9 (P55211), Caspase-10 (Q92851), Caspase-11 (P70343), Caspase-12 (NP_033938), Caspase-13 (AAC28380), Caspase-14 (AAD16173). Caspase-1 through Caspase-10, Caspase-13 and Caspase-14 are human caspases; Caspase-11 and Caspase-12 are mouse caspases.



Supplemental Figure 2

Supplemental Figure 2. Alignment of the amino acid sequences of BIR motifs from selected BIR-containing proteins. Alignment was performed using ClustalW program with default parameters. For proteins containing multiple repeats, the BIRs are listed in order from the N-terminus to the C-terminus and are indicated by the hyphenated number to the right of the protein name. The amino acid position range for each BIR motif is shown to the left of the sequence. Alignment includes BIRs from OpIAP (P41437), CpIAP (P41436), DIAP1 (Q24306), DIAP2 (Q24307), predicted dBRUCE (CG6303), Deterin (cDNA sequence - A1260030), c-IAP1 (Q13490), c-IAP2 (Q13489), XIAP (P98170), pIAP (AAC39171), NAIP (AAC52047), mBRUCE (CAA76720), Survivin (O15392), CeBIR1 (AAD00182), CeBIR2 (AAB94330), SpIAP (CAA20434), ScIAP (AAB39312.1), and SfIAP (AAF35285).



Supplemental Figure 3

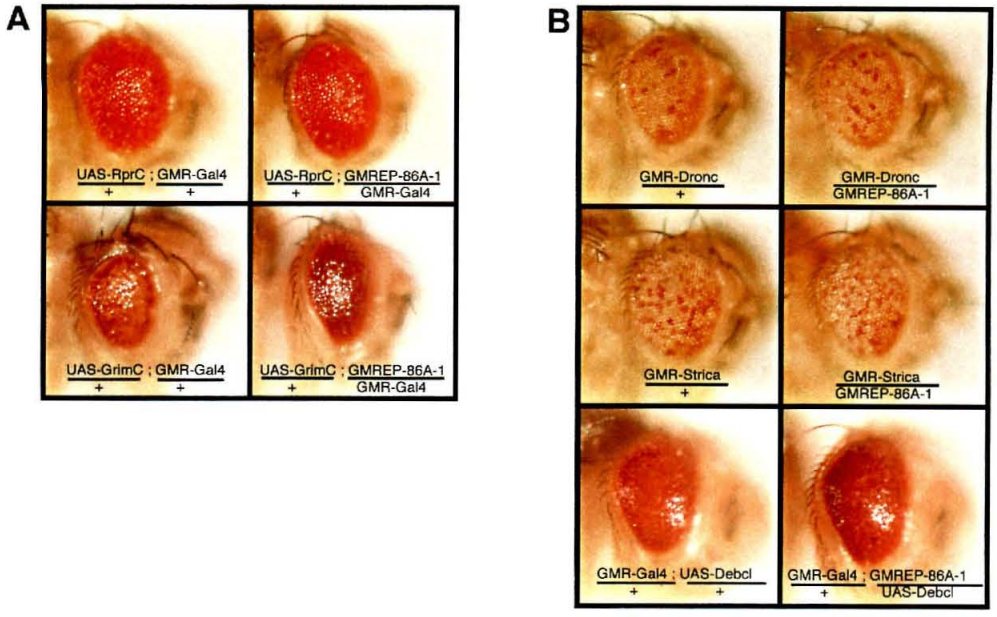
Supplemental Figure 3. ClustalW alignment of the RING finger motifs from the BIR proteins which contain them. The amino acid position range for each RING motif is shown to the left of the sequence. Genbank accession numbers are all included in legend for Supplemental Figure 2.

Supplemental Figure 4

Supplemental Figure 4. Pileup was used to align human Bcl-2 (P10415), Bcl-X_L (B47537), Bcl-W (Q92843), Bax (A47538), Bak (AAA74466), and Bok (AAD51719) and *C. elegans* Ced-9 (P41957) with Drob-1/Debcl/dBorg-1/DBok (BAA89603, AAF26289, AAF44714, not given) and Buffy/DBorg-2 (AAF44120, not given). Drob-1/Debcl/DBorg-1 and Buffy/DBorg-2 contain BH1-3 domains, a transmembrane domain and a potential BH4 domain with limited homology to other Bcl-2 family members. Both of these *Drosophila* genes are most closely related to Bok.

Appendix II

Supplemental Figures and Methods, Vernooy et al. 2002



Supplemental Figure 1

Supplemental Figure 1.

(A) GMREP-86A-1-driven dBruce does not suppress death induced by eye-specific expression of versions of Rpr (RprC) and Grim (GrimC) that lack their N-terminal DIAP1 interaction domains.

(B) GMREP-86A-1-driven dBruce does not suppress cell death induced by eye-specific expression of the long prodomain caspase Dronc, the long prodomain caspase Strica, or the proapoptotic Bcl-2 family member Debcl/Drob-1/dBorg-1/Dbok (referred to in the legend as Debcl). GMR-Dronc flies were previously described [15]. GMR-Gal4/CyO; UAS-Debcl/TM6B flies were a generous gift from Sharad Kumar [20]. Flies carrying UAS-RprC on the X chromosome or UAS-GrimC on the second chromosome were a generous gift from John Nambu [9, 10].

Supplemental methods

Construction of transgenic flies. GMR-Grim-lys$^-$ was made by PCR amplifying grim using a reverse primer that incorporated nucleotides specifying arginine (CGG) instead of the single endogenous lysine (AAG.) A version of Reaper with all five lysines mutated to arginine was made using site-directed mutagenesis and was a generous gift from Sally Kornbluth. Both Grim-lys$^-$ and Rpr-lys$^-$ were introduced into GMR. A GMR-RNAi construct was generated as described in [21], by PCR-amplifying the GMR promoter/enhancer sequence and reintroducing it into GMR, downstream of the existing promoter, in the opposite orientation. A 732 bp fragment of dBruce encompassing nucleotides (as determined from the dBruce start codon) 13,354-14,086 was introduced into the GMR-RNAi construct between the GMR promoters, so that both sense and antisense would be transcribed. GMR-Strica flies were generated by introducing the Strica coding region into GMR. Transformants were generated using standard techniques.

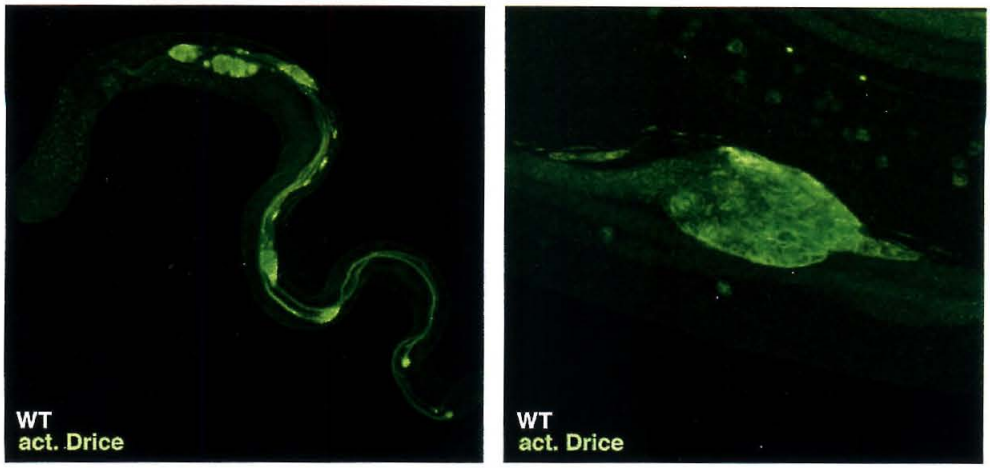
Characterization of the dBruce gene and generation of dBruce mutants. The exon-intron structure of dBruce was determined as follows: first-strand cDNA was made from *Drosophila* embryo mRNA (Clontech Cat no. 6947-1) using three different primers spaced 5 kb apart along the length of the predicted dBruce sequence. 1 kb fragments were PCR amplified from this cDNA using primers that spanned dBruce, based on the BDGP predicted sequence. These

fragments were sequenced and assembled into a contiguous dBruce coding sequence. To generate dBruce deletions E12 and E16, we first used recombination to remove a background lethal from the EP(3)3731 chromosome. The transposon was mobilized as described in [22], and balanced excision lines generated. Deletions were characterized by carrying out PCR on genomic DNA from the excision lines using sequential sets of primers spanning dBruce, and sequencing products that bridged the deletions.

Antibody Generation and Immunostaining. Antibodies were raised in rabbits using a GST-fusion protein corresponding to amino acids 361-693 of dBruce. Antisera were applied to an affinity column containing protein used as the immunogen, and bound antibodies were eluted using 100mM glycine, pH2.5. For immunolabeling, dissected larval eye discs were fixed in PBS + 4% formaldehyde for 20 minutes and then permeabilized in PBS+ 0.3% Trition X-100, 0.3% deoxycholate, 5% BSA for 1 hour. Following an overnight incubation with purified dBruce antibody (1:100), eye discs were washed for 1 hour in PBTB (PBS + 0.1% Trition X-100, 5% BSA) and incubated for 1 hour in Alexa Fluor 488 goat anti-rabbit IgG (1:500) (Molecular Probes). Discs were then washed for an hour, mounted in Vectashield mounting medium (Vector Labs) and viewed on a Leica TCS-NT confocal microscope.

Appendix III

Supplemental Figures, Vernooy et al. (In preparation)



Supplemental Figure 1

Supplemental Figure 1. *Drosophila* testes have high levels of activated Drice in elongated spermatids.

An antibody that recognizes the active form of Drice (act. Drice) highlights elongated spermatids in wild-type testes, in a similar pattern to that seen with an antibody that recognized active forms of both Dcp-1 and Drice. Unlike anti-active Dcp-1/Drice, however, anti-active Drice also shows low-level immunoreactivity with pre-meiotic spermatocytes. In the adjacent panel, a magnified view of a cystic bulge is shown. Purified anti-active Drice was used at a concentration of 1:40 and is described in [13].