Genetic Analysis of Programmed Cell Death in *Drosophila melanogaster*

by

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Abstract

The correct regulation of programmed cell death, or apoptosis, is critical for proper development and prevention of disease. Components of the molecular mechanisms that govern apoptosis are conserved among organisms as diverse as *C. elegans*, *Drosophila*, and mammals. A central step in the execution of cell death is the activation of caspases, a conserved family of cysteine proteases. In *Drosophila*, the proteins, Reaper (Rpr), Head involution defective (Hid), and Grim, induce cell death via a mechanism that involves caspase activation. In order to further elucidate the mechanisms underlying the control of apoptosis, we conducted screens for genes involved in Rpr- or Hid-induced cell death. The analysis of the mutants isolated led to several new insights. The death inducing activity of Hid is post-transcriptionally down-regulated by the Ras/MAPK pathway. This is consistent with the pro-survival activity of this pathway and is probably mediated by direct phosphorylation of Hid. Furthermore, analysis of mutations in the gene encoding the Drosophila IAP, Diap1, led to a model for how Rpr, Hid and Grim activate caspases and induce cell death. In this model, Diap1 binds and inhibits caspases; Rpr, Hid, and Grim induce cell death by binding Diap1 and relieving caspases of Diap1-mediated inhibition. In addition, our mutants indicate that Diap1's RING finger domain, a domain found in proteins that function in ubiquitination, is required for inhibition of Rpr- and Grim-induced death but not Hid-induced death. Moreover, we identified a predicted ubiquitin conjugating enzyme, dBRUCE, which also functions to inhibit Rpr and Grim but not Hid. We propose that Diap1 and dBRUCE function together to inhibit Rpr- and Grim-induced death by ubiquitinating pro-apoptotic proteins, possibly caspases or Rpr and Grim themselves, and targeting them for degradation by the proteasome. These findings are likely applicable to mammalian systems, since both dBRUCE and Diap1 are conserved proteins with close homologs in murine and human genomes.

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This work is dedicated to my favorite scientist, Isabel Silverman, whose curiosity, careful experimentation and enthusiasm for discovery inspire me daily and also to my "three parents", Mimi and Giulio Agapite and Donata Pelini, whose love, support, encouragement and expert childcare made it all possible.

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Chapter 1.

Introduction

The appropriate removal of unwanted cells via programmed cell death, or apoptosis, is critical for proper development and protection against disease. Cell death plays an important role in sculpting parts of the body (like the formation of digits by the elimination of interdigital cells), in deleting unnecessary structures (like the tadpole tail during amphibian metamorphosis), and in adjusting cell number (as in matching the number of neurons with target cells) (Jacobson et al., 1997). Furthermore, apoptosis is crucial for eliminating cells that are harmful to the organism like cells infected by virus, cells undergoing unregulated division, or auto-reactive lymphocytes. Defects in apoptosis are therefore implicated in such diseases as AIDS, cancer, and autoimmune disorders (Thompson, 1995). Moreover, inappropriate cell death has been implicated in neurodegenerative disorders like Alzheimer's and Parkinson's diseases (Mattson et al., 2001). Thus, uncovering the molecular mechanisms underlying cell death is critical for understanding basic development as well as understanding and possibly treating human disease.

Genetic analysis of invertebrate model organisms has been instrumental in identifying key molecules involved in programmed cell death. In *C. elegans*, genetic studies have produced a core cell death pathway (Metzstein et al., 1998), the details of which have been further elaborated by molecular and biochemical analyses. The central step in the execution of cell death is the activation of the cysteine protease or caspase, Ced-3. This activation is facilitated through interaction with Ced-4. Normally Ced-4 is localized to the mitochondria via interaction with Ced-9, a member of the Bcl-2 family of cell death regulators, and is prevented from associating with Ced-3, which therefore remains inactive. Egl-1, which contains a BH3 (Bcl-2 homology 3) domain, activates cell death by binding and inhibiting Ced-9, thus allowing Ced-4 to translocate from the mitochondria to the nuclear membrane where it is presumably free to activate Ced-3 (Chen et al., 2000 and references therein).

The components of this core *C. elegans* pathway and elements of their function are conserved in mammals. The activation of the mammalian caspase-9 depends upon the binding of Apaf-1, a protein with some sequence similarity to Ced-4. Activation of caspase-9 by Apaf-1 relies on the release of cytochrome C from the mitochondria. BH3

domain-containing proteins can promote the release of cytochrome C, like Egl-1 promotes the release of Ced-4, while Ced-9-like Bcl-2 family members can block cytochrome C release (Adrain and Martin, 2001).

Although reverse genetics and genomics has recently identified similar molecules in another invertebrate model organism, *Drosophila melanogaster* (Vernooy et al., 2000), early genetic analysis in this organism led to the identification of novel cell death genes with no homologs in either worms or mammals (Chen et al., 1996b; Grether et al., 1995; White et al., 1994). A genetic screen for chromosomal regions involved in cell death identified the region, 75C1,2, as being required for virtually all cell deaths occurring in the developing embryo as well as the ectopic deaths induced by irradiation and developmental defects (White et al., 1994). Three genes that function in the activation of cell death, *reaper* (*rpr*) (White et al., 1994), *head involution defective* (*hid*) (Grether et al., 1995) and *grim* (Chen et al., 1996b), have been identified within this interval. The proteins encoded by these genes do not show significant homology to other known proteins. They do however possess a short 14 amino acid stretch at their N-termini that is conserved among all three (Chen et al., 1996b; Grether et al., 1995). All three genes can induce cell death when overexpressed in a variety of tissues as well as in cell culture (Chen et al., 1996a; Chen et al., 1996b; Grether et al., 1995; Hay et al., 1995; McNabb, 1997; Vucic et al., 1998a; Vucic et al., 1997a; White et al., 1996; Wing et al., 1998; Zhou et al., 1997). Furthermore, the conserved N-terminus is sufficient for this death inducing function (Vucic et al., 1998a).

The first link between these three *Drosophila* cell death genes and conserved elements of the *C. elegans* and mammalian apoptotic pathways came with the discovery that Rpr- Hid- and Grim-induced cell death involves the activity of caspases. Expression of *rpr*, *hid* or *grim* in the developing *Drosophila* eye results in widespread cell death and, consequently, an adult eye that is reduced in size and highly disorganized. Co-expression of p35, a baculoviral caspase inhibitor, blocks cell death and restores a nearly wildtype eye (Chen et al., 1996b; Grether et al., 1995; Hay et al., 1995; White et al., 1996). Furthermore, expression of *rpr*, *hid*, and *grim* in cell culture also induces cell death that is blocked by p35 (Vucic et al., 1998a; Vucic et al., 1997a).

These observations suggest that Rpr-, Hid- and Grim-induced cell death is mediated by caspases.

The involvement of caspases in cell death was initially revealed by the finding that *ced-3*, one of the *C. elegans* genes required for all cell deaths, encodes a caspase (Yuan et al., 1993). Since then much work has focused on the identification of caspases and on the analysis of their regulation and function. Caspases are a large family including 14 mammalian members and 4 *C. elegans* members (Aravind et al., 2001; Shaham, 1998). Not all of these however have been shown to have a role in cell death. Moreover, some caspases function in processes other than cell death. For example, caspase-1 functions in the processing of pro-inflammatory cytokines (Kuida et al., 1995; Thornberry et al., 1992).

Caspases cleave a wide variety of substrates. These include other proteins involved in apoptosis, the cleavage of which further promotes cell death. Caspases also cleave proteins involved in the cell cycle and DNA repair, perhaps shutting down normal cell division and repair mechanisms. Structural components of the cell, like nuclear lamins, are also targeted for cleavage by caspases. It is believed that these cleavage events bring about the ordered dismantling of the cell characteristic of apoptosis (Earnshaw et al., 1999).

In addition, caspases also cleave themselves. Caspases are initially synthesized as inactive or weakly active zymogens, consisting of an inhibitory N-terminal prodomain, followed by a large and then a small protease subunit. Caspase-mediated proteolytic cleavage leads to the formation of a tetrameric active enzyme composed of 2 large and 2 small subunits (Earnshaw et al., 1999).

Caspases fall into two classes, initiator caspases and effector caspases, based in part on the lengths of their prodomains and how they are activated. By cleaving various cellular substrates, effector caspases are believed to be the downstream mediators of programmed cell death. They are characterized by short prodomains and are activated by cleavage by upstream initiator caspases. Initiator caspases are characterized by their long prodomains. These prodomains mediate interaction between initiator caspases and their upstream regulators and this interaction promotes

activation of the initiator caspase (Earnshaw et al., 1999). For example, procaspase-9 is recruited into a multimeric complex with Apaf-1 via its prodomain. It is thought that once in this complex, procaspase-9 auto-activates by virtue of its weak proteolytic activity (Adrain and Martin, 2001). Thus, procaspase-9 is activated and is thought to set in motion a pro-apoptotic caspase cascade (Earnshaw et al., 1999).

Caspases play an important role in programmed cell death in *Drosophila*. This was first demonstrated by Hay and colleagues who showed that expression of the caspase inhibitor, p35, blocks the normal cell death that occurs during embryogenesis and during eye development, as well as the ectopic cell death induced by x-rays (Hay et al., 1994). As mentioned previously, p35 also blocks the death induced by expression of *rpr*, *hid*, and *grim* (Chen et al., 1996b; Grether et al., 1995; Hay et al., 1995; Vucic et al., 1998a; Vucic et al., 1997a; White et al., 1996) suggesting that *rpr*, *hid* and *grim* expression leads to the activation of caspases.

The *Drosophila* genome encodes seven caspases, Dcp-1, Drice, Dredd, Dronc, Decay, STRICA/Dream, and DAMM/Daydream. Dredd, Dronc, and STRICA/Dream have long prodomains and are thought to be initiator caspases, while Dcp-1, Drice, Decay and DAMM/Daydream have short prodomains and are thought to be effector caspases (Kumar and Doumanis, 2000; Vernooy et al., 2000). Most of the *Drosophila* caspases have been shown to possess caspase activity; they can cleave caspase substrates in vitro (Dorstyn et al., 1999a; Dorstyn et al., 1999b; Fraser and Evan, 1997; Harvey et al., 2001; Hawkins et al., 2000; Meier et al., 2000; Song et al., 2000; Song et al., 1997). Moreover, all have been shown to possess some ability to induce cell death either in vitro (Song et al., 1997), in cell culture (Dorstyn et al., 1999a; Dorstyn et al., 1999b; Doumanis et al., 2001; Fraser and Evan, 1997; Harvey et al., 2001; Meier et al., 2000; Quinn et al., 2000; Rodriguez et al., 1999), and/or in transgenic flies (Harvey et al., 2001; Hawkins et al., 2000; Meier et al., 2000; Quinn et al., 2000; Song et al., 2000). These results suggest that most if not all of the *Drosophila* caspases function in cell death.

However, there is only evidence for a critical role in cell death in vivo for one of the *Drosophila* caspases, Dronc. Diminishing *dronc* RNA levels through dsRNA-

mediated interference (RNAi) in the *Drosophila* embryo reduces the level of cell death normally observed during embryogenesis (Quinn et al., 2000) suggesting that *dronc* is required for this embryonic cell death. Verification of this RNAi –induced phenotype, however, awaits the analysis of *dronc* mutants.

Loss-of-function phenotypes have been described for two of the *Drosophila* caspases, *dcp-1* and *dredd*. Mutations in *dcp-1* reveal that *dcp-1* is required for "dumping", the transfer of nurse cell cytoplasmic contents to the oocyte during oocyte development. Specifically, *dcp-1* mutants are defective in the cytoskeletal reorganization and nuclear breakdown that occur during this process (McCall and Steller, 1998). Because cytoskeletal reorganization and nuclear breakdown also occur during apoptosis, this phenotype may reflect a defect in a specialized form of apoptosis. An apoptotic defect of *dredd* mutants has not been described. Rather, *dredd* mutants are reported to be defective in the anti-bacterial immune response (Elrod-Erickson et al., 2000; Leulier et al., 2000; Stoven et al., 2000). Failure to detect striking cell death defects in caspase mutants may reflect redundancy or roles for individual caspases in highly specific cell deaths, the failure of which may not produce overt defects.

Since Rpr-, Hid-, and Grim-induced cell death is mediated by caspases, at least one of the *Drosophila* caspases should function in the Rpr, Hid, and Grim pathways. Several of the *Drosophila* caspases are responsive to Rpr, Hid, and/or Grim in a variety of assays. For example, Drice is processed in *Drosophila* S2 cells undergoing Rpr-induced apoptosis (Fraser and Evan, 1997). The eye phenotype caused by the ectopic expression of *dcp-1* is dramatically enhanced by co-expression of *rpr* or *grim* (Song et al., 2000). Along the same lines, the eye phenotype caused by the ectopic expression of *dronc* is suppressed by decreasing the dosage of the *rpr*, *hid*, and *grim* genes (Quinn et al., 2000). Furthermore, *dredd* mRNA expression is responsive to *rpr*, *hid*, and *grim*. *dredd* mRNA normally accumulates in dying cells during embryogenesis. This accumulation is blocked in mutant embryos carrying a deletion of the 75C1,2 region where *rpr*, *hid*, and *grim* reside. Conversely, ectopic expression of *rpr*, *hid*, or *grim* results in the ectopic accumulation of *dredd* mRNA (Chen et al., 1998). Based on these results and others (Harvey et al., 2001; Hawkins et al., 2000), the caspases, Dcp-1,

Drice, Dredd, Dronc, and Damm are good candidates for involvement in the Rpr, Hid, and/or Grim cell death pathways.

Since caspases are potent cell death inducers and are widely expressed, it is imperative for cell survival that their activity be tightly regulated. Members of the Inhibitor of Apoptosis, or IAP, protein family have been shown to function as caspase inhibitors (Deveraux and Reed, 1999). IAPs were first identified in baculoviruses based on their ability to substitute for the baculoviral caspase inhibitor, p35 (Crook et al., 1993). Since then, a large number of cellular IAP-like proteins have been identified from a wide variety of organisms. IAPs are characterized by the presence of one to three N-terminal baculovirus IAP repeat, or BIR, domains. Many IAPs also contain a C-terminal RING domain (Deveraux and Reed, 1999).

While by definition all IAPs carry at least one BIR domain, not all BIR-containing proteins (BIRPs) are IAPs, i.e. not all BIRPs have been demonstrated to inhibit apoptosis (Uren et al., 1998). Moreover, some BIRPs may have roles in processes other than apoptosis. For example, BIRPs from *S. cerevisiae* (Bir2), *S. pombe* (Bir1) and *C. elegans* (Bir-1) appear to function in cell division (Fraser et al., 1999; Speliotes et al., 2000; Uren et al., 1999). The human BIRP, Survivin, has been implicated in both cell death and cell division (Li et al., 1999; Li et al., 1998; Reed and Reed, 1999; Tamm et al., 1998).

The *Drosophila* genome encodes 4 BIR containing proteins, Diap1, Diap2, Deterin, and dBRUCE (Vernooy et al., 2000). Diap1, Diap2, and Deterin have all been implicated in cell death. All can inhibit cell death in cell culture or in transgenic flies (Hay et al., 1995; Jones et al., 2000; Kaiser et al., 1998; Vucic et al., 1998a; Vucic et al., 1998b; Vucic et al., 1997a; Wing et al., 1998). However, only the phenotype of *diap1* mutants has been characterized. Loss-of-function *thread/diap1* mutants (*thread* is the locus that encodes Diap1) arrest early in embryogenesis and exhibit widespread cell death (Goyal et al., 2000; Lisi et al., 2000; Wang et al., 1999) indicating that Diap1 is critical for inhibition of apoptosis.

dBRUCE is the *Drosophila* ortholog of the mouse *BRUCE* and human *apollon* genes. The proteins these genes encode are notable because of their large size and

unique architecture; they are enormous (528 kD) and yet the only apparent homologies to known proteins or known motifs they carry are an N-terminal BIR domain and a C-terminal UBC domain (Chen et al., 1999; Hauser, 1998). *apollon* has been implicated in cell death due to its high expression levels in chemo-resistant human cancer cell lines. Antisense–mediated inhibition of *apollon* sensitizes these cells to chemotherapeutic drug induced apoptosis (Chen et al., 1999). Until the study described in this thesis, *dBRUCE* had not been characterized.

An important clue into how IAPs inhibit apoptosis came with the discovery that the mammalian IAPs, XIAP, c-IAP1 and c-IAP2 could directly bind and inhibit caspases-3, -7, and –9 (Deveraux et al., 1998; Deveraux et al., 1997; Roy et al., 1997). The interaction between XIAP and caspases has been best studied. XIAP contains three BIR domains and a RING finger domain. The region encompassing the second BIR domain (BIR2) is sufficient for both interaction with caspase-3 and –7 and inhibition of these caspases in vitro (Takahashi et al., 1998). Structural studies have shown that the BIR2 domain itself makes minimal contact with caspase-3 and-7 and may instead stabilize the linker region between BIR1 and BIR2, which makes extensive contacts with the caspase-3 and -7 substrate binding pockets. Binding by the XIAP linker region is expected to preclude substrate binding and perhaps represents the mode of XIAP inhibition of caspase-3 and –7 (Chai et al., 2001; Huang et al., 2001; Riedl et al., 2001; Sun et al., 1999). In contrast, the BIR3 domain of XIAP is sufficient for binding and inhibition of caspase-9 (Srinivasula et al., 2001; Sun et al., 2000). Thus, XIAP binds and inhibits different caspases differently.

Like XIAP, c-IAP-1 and c-IAP-2, Diap1 can also inhibit caspases directly. Diap1 has been shown to bind the caspases Drice, Dcp1, and Dronc (Kaiser et al., 1998; Meier et al., 2000; Quinn et al., 2000; Wang et al., 1999). Like XIAP binding and inhibition of caspase-3 and –7, the region encompassing the BIR2 domain of Diap1 is sufficient for Dronc binding (Meier et al., 2000). Furthermore, Diap1 can inhibit the activity of recombinant Dcp-1 in vitro (Hawkins et al., 1999; Wang et al., 1999). In addition to inhibiting caspase activity in vitro, there is evidence that Diap1 also inhibits caspase activity in vivo. Protein extracts made from *diap1* mutant embryos, which

exhibit widespread cell death, show increased caspase activity (Wang et al., 1999). However, these data do not necessarily indicate that Diap1 inhibits caspases directly in vivo.

Interestingly, in addition to binding caspases, several IAPs also bind Rpr, Hid, and Grim (McCarthy and Dixit, 1998; Vucic et al., 1998a; Vucic et al., 1997b). The ability of IAPs to inhibit Rpr-, Hid-, and Grim-induced death has been well documented in cell culture as well as in vivo (Goyal et al., 2000; Hay et al., 1995; Jones et al., 2000; Lisi et al., 2000; McCarthy and Dixit, 1998; Vucic et al., 1998a; Vucic et al., 1997b; Wing et al., 1998). The BIR2 domain of Diap1 is sufficient to bind Hid and Grim and the conserved N-termini of Hid and Grim are sufficient to bind the Diap1 BIR2 (Rpr has not been examined) (Wu et al., 2001). This binding suggests another possible mode of cell death inhibition by IAPs. Since the N-termini of Rpr, Hid and Grim possess death-inducing ability, IAPs may bind to this region and block Rpr-, Hid- and Grim-mediated cell killing.

The observation that IAPs bind to both caspases and the Rpr, Hid, and Grim death inducers led researchers to propose the "apopstat" model for IAP function (Kaiser et al., 1998). In this model, IAP levels dictate whether a cell lives or dies. When IAP levels are high, caspases and Rpr, Hid, and Grim are bound by IAPs and cells are resistant to apoptotic stimuli. When IAP levels drop, caspases and Rpr, Hid, and Grim are free to induce cell death. Also implicit in this model is the possibility that Rpr, Hid and Grim induce cell death by binding to IAPs and relieving caspases of IAP-mediated inhibition.

IAPs may possess yet additional activities that may contribute to their regulation of cell death. Many IAPs have a RING finger motif, a domain found in proteins involved in ubiquitination (Joazeiro and Weissman, 2000). Protein ubiquitination occurs in several steps. First ubiquitin is activated through covalent linkage with a ubiquitin activating enzyme, or E1. Ubiquitin is then transferred to a ubiquitin conjugating enzyme, a UBC or E2. Ubiquitin protein ligases, or E3s, bind both the E2 enzyme and protein substrate and facilitate transfer of ubiquitin from the E2 to substrate. In most cases ubiquitination of a protein targets it for proteasome-mediated degradation (Hershko and Ciechanover, 1998). Thus, the presence of a RING domain in IAPs

suggests that IAPs might inhibit cell death by targeting their pro-apoptotic binding partners for degradation by the proteasome. In fact, c-IAP-1 and XIAP have been shown to possess E3 ligase activity in vitro. c-IAP-1 and XIAP can promote ubiquitination of themselves and c-IAP-1 can also promote ubiquitination of caspase-3 and –7. All of these activities require an intact RING domain (Huang, 2000; Yang et al., 2000). It is unclear how each of the activities of IAPs contributes to cell death inhibition in vivo. IAPs can clearly inhibit caspase activity in vitro via direct binding without the addition of the ubiquitination and proteasome-mediated degradation machinery. But does this occur in vivo or does binding simply lead to ubiquitination? Binding and degradation may be two tiers of IAP-mediated caspase inhibition in vivo or these functions may be inseparable.

At the time the research that comprises this thesis was commenced, very little was known about cell death in *Drosophila*. The region 75C1,2 had been found to be essential for embryonic cell death and two of the cell death genes in this region, *rpr* and *hid*, had been identified (Grether et al., 1995; White et al., 1994). Early in the course of this work a third cell death gene in this region, *grim*, was identified and the involvement of caspases and IAPs in Rpr-, Grim- and Hid-induced cell death was uncovered (Chen et al., 1996b; Grether et al., 1995; Hay et al., 1995; White et al., 1996). The aim of the research presented here was to use genetic approaches to identify components of the Rpr, Hid and possibly also Grim cell death pathways. We expected that the elucidation of these pathways would help answer several important questions about cell death in *Drosophila*.

How similar are the Rpr, Hid and Grim pathways?

A number of observations suggest that Rpr, Hid and Grim induce cell death in a similar manner. All three can induce caspase-dependent cell death in cell culture and in the *Drosophila* eye (Chen et al., 1996b; Grether et al., 1995; Hay et al., 1995; Vucic et al., 1998a; Vucic et al., 1997a; White et al., 1996). The N-terminus of each protein contributes to the activation of cell death (Vucic et al., 1998a; Vucic et al., 1997a). Furthermore, all three proteins can interact with IAPs via their N-terminus (McCarthy

and Dixit, 1998; Vucic et al., 1998a; Vucic et al., 1998b; Vucic et al., 1997b). In addition to these similarities, a number of differences have been observed. While expression of *grim* alone can kill midline glia cells, expression of *rpr* or *hid* cannot (Wing et al., 1998; Zhou et al., 1997). While Rpr- and Hid-induced cell death in the *Drosophila* eye is blocked by Diap2, Grim-induced cell death is not (Hay et al., 1995; Wing et al., 1998). Furthermore, deletion of the N-terminus of Hid completely eliminates its cell killing function, while versions of Rpr and Grim lacking their N-termini can still induce cell death suggesting that Rpr and Grim possess multiple cell killing functions (Chen et al., 1996a; McCarthy and Dixit, 1998; Vucic et al., 1998a; Wing et al., 2001; Wing et al., 1998).

We performed genetic screens for components of the Rpr and Hid cell death pathways (described in chapter 2). Comparison of the effects of these mutants on Rpr-, Hid-, and Grim-induced death is expected to provide some indication of the degree of similarity in the Rpr, Hid, and Grim pathways. For example, if all mutants isolated affected only Rpr-induced death or Hid-induced death, then we would expect these pathways to be distinct. Alternatively, if all mutants affected Rpr-, Hid- and Grim-induced death, we would expect these pathways to be very similar.

How are Rpr, Grim and Hid regulated?

Since the pattern of *rpr* and *grim* expression closely mimics the pattern of cell death in the *Drosophila* embryo, *rpr* and *grim* are likely to be regulated, at least in part, at the level of transcription (Chen et al., 1996b; White et al., 1994). *hid*, on the other hand, is expressed rather widely in the embryo in cells that live, as well as in cells that die, suggesting that *hid* must be regulated post-transcriptionally (Grether et al., 1995). Our screens provided a clue into how *hid* may be regulated. We isolated components of a well-known survival signaling pathway that appears to be involved in negatively regulating *hid* post-transcriptionally (described in chapter 3). Interestingly, in similar screens others have found that this survival signaling pathway also regulates *hid* at the level of transcription (Kurada and White, 1998).

How does Diap1 function to inhibit Rpr-, Hid-, and Grim-induced death?

As discussed previously, Diap1 may inhibit cell death in *Drosophila* by binding Rpr, Hid, and Grim, by binding caspases, or both. Furthermore, like many IAPs, Diap1 contains a RING domain suggesting that it may function in protein ubiquitination and perhaps inhibit apoptosis by targeting pro-apoptotic proteins for degradation. We recovered many mutations in *thread*/d*iap1* in our screens. These mutants, as well as those recovered by others in similar screens (Lisi et al., 2000), provide insights into how Diap1 inhibits Rpr-, Hid -and Grim-induced cell death in vivo and are described in chapters 4 and 5.

Furthermore, we expected our screens to identify previously uncharacterized genes involved in cell death. The cloning and characterization of one such gene (described in chapter 5) further implicates protein ubiquitination in the regulation of Rpr-, Hid- and Grim-induced cell death in *Drosophila*. In summary, genetic analysis of programmed cell death in *Drosophila* has revealed important new insights into the regulation of cell death, which are likely applicable to the regulation of cell death in mammals as well.

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Chapter 2.

Genetic Screens to Identify Genes Involved in Reaper- and/or Hid- induced Programmed Cell Death in *Drosophila*.

The work presented in this chapter represents the efforts of a number of researchers, including Kim McCall, Chris Hynds and Andreas Bergmann. I played a leading role in many phases of the work especially in the deficiency and *GMRrpr* screens.

Abstract

Programmed cell death, or apoptosis, plays a critical role in normal development and protection from disease. Components of the molecular mechanisms that control apoptosis are conserved among *C. elegans*, *Drosophila*, and mammals. A central step in the induction of cell death involves the activation of members of the caspase family of cysteine proteases. In *Drosophila*, three genes, *reaper, head involution defective* (*hid*) and *grim*, function in the activation of cell death. Ectopic expression of *reaper*, *hid* or *grim* in the developing eye causes excess, caspase-dependent cell death and a rough, disorganized eye that is reduced in size. In order to determine how Reaper, Hid and Grim activate caspases and induce cell death, we performed a screen for dominant modifiers of these Reaper- and Hid-induced eye phenotypes. Approximately 500,000 progeny of mutagenized flies were screened, and 42 dominant enhancers and 175 dominant suppressors were isolated. Interestingly, components of the Ras/MAPK pathway and both gain- and loss-of-function alleles of the apoptosis inhibitor *thread/diap1* were isolated. At least one other, novel cell death regulator was also identified in these screens.

Introduction

The appropriate removal of cells via programmed cell death, or apoptosis, is essential for proper development, tissue homeostasis, and protection from disease. Apoptosis proceeds via a characteristic series of morphological changes including membrane blebbing, nuclear condensation, and nucleosomal laddering. It is a gene driven process that is highly conserved in animals as diverse as *C. elegans* and mammals (Jacobson et al., 1997).

Genetic analysis of programmed cell death in *C. elegans* and in baculoviruses has led to the identification of a number of large, conserved protein families involved in cell death. Two of these are caspases and inhibitor of apoptosis proteins, or IAPs. Caspases, a family of cysteine proteases, were originally implicated in cell death by the discovery that *ced-3*, a gene required for all cell deaths in *C. elegans*, encodes a caspase (Yuan et al., 1993). Caspases play a central role in executing the cell death process. They are initially synthesized as inactive zymogens and are processed to their active form by auto-cleavage or cleavage by other caspases. Once active, caspases cleave a variety of cellular substrates to bring about the typical morphological changes associated with apoptosis (Earnshaw et al., 1999). The activity of caspases is tightly controlled by a number of mechanisms including IAPs. Many IAPs have been shown to bind to active caspases directly and this binding correlates with their inhibition activity (Deveraux and Reed, 1999).

Genetic analysis of programmed cell death in *Drosophila* led to the identification of three novel genes, *reaper* (*rpr*), *head involution defective* (*hid*), and *grim*, that function in the activation of cell death (Chen et al., 1996b; Grether et al., 1995; White et al., 1994). In *Drosophila*, the genomic region 75C1,2 is required for both the cell death that normally occurs during embryogenesis and the ectopic cell death induced by X-rays or developmental abnormalities. Removal of this region in the chromosomal deletion *Df(3L)H99* eliminates virtually all of these deaths (White et al., 1994). *rpr*, *hid*, and *grim* reside within this interval (Chen et al., 1996b; Grether et al., 1995; White et al., 1994). The proteins encoded by these three genes do not share significant homology with

previously reported proteins. However, they are similar to each other; they share a short region of homology at their N-termini (Chen et al., 1996b; Grether et al., 1995).

Although Rpr, Hid, and Grim have no homologs in other species, the cell death pathways they activate include components that are conserved. All three genes, *rpr*, *hid*, and *grim* can induce cell death in a variety of cell types when expressed either in vivo or in culture. This death is abrogated by the caspase inhibitors p35 and IAPs indicating that it is mediated by caspases (Chen et al., 1996b; Grether et al., 1995; Hay et al., 1995; Vucic et al., 1998a; Vucic et al., 1997a; White et al., 1996). When this work was initiated, it was unclear how *rpr*, *hid*, and *grim* expression leads to the activation of caspases.

Although Rpr, Hid, and Grim appear to similarly activate cell death via caspases, there are also differences among Rpr, Hid, and Grim in the importance of their N-termini for killing, in how well they kill particular cell types, and in how they are regulated. Structure-function analysis based on cell culture models of Rpr, Hid, and Grim killing have shown that the conserved N-terminus contributes to the death inducing ability of all three (Vucic et al., 1998a; Vucic et al., 1997a). However, while the N-terminus of Hid is absolutely required, versions of Rpr and Grim with the N-terminal 14 amino acids deleted can still induce some degree of cell death (Chen et al., 1996a; McCarthy and Dixit, 1998; Vucic et al., 1998a; Vucic et al., 1997a; Wing et al., 2001; Wing et al., 1998). Furthermore, *grim* expression alone is sufficient to kill midline glia cells but expression of either *rpr* or *hid* alone is not (Wing et al., 1998; Zhou et al., 1997). Rpr, hid, and grim also appear to be regulated differently. *rpr* and *grim* expression in the embryo appears more or less restricted to cells that will die suggesting that these genes are transcriptionally regulated (Chen et al., 1996b; White et al., 1994). On the other hand, *hid* is expressed more widely in cells that die, as well as in cells that live, and must therefore be regulated, at least in part, post-transcriptionally (Grether et al., 1995).

In order to learn more about the mechanisms by which Rpr, Hid and Grim induce cell death and about how they are regulated, we conducted genetic screens designed to identify components of these pathways. Here we report the results of several screens for modifiers, suppressors and enhancers, of Rpr- and/or Hid-induced death. We report

the identification of 21 genomic regions found to be involved in Rpr- and/ or Hid-induced cell death, as well as the identification of 11 complementation groups that represent both known and novel genes.

Results

Isolation of dominant suppressors and enhancers of *GMRrpr* and *GMRhid*

For our genetic screens, we made use of reduced, rough eye phenotypes caused by the ectopic expression of *rpr* or *hid* under the control of *GMR* promoter elements (Grether et al., 1995; Hay et al., 1995; White et al., 1996). The roughness and reduction in size worsens with increasing dosage of *GMRrpr* or *GMRhid* transgenes suggesting that these eye phenotypes are ideal starting points for dominant modifier screens. The rationale behind dominant modifier screens is that small perturbations in the signaling pathways underlying such sensitized phenotypes, including a simple decrease in pathway component gene dosage, would result in detectable changes in the starting phenotype. Thus, both loss-of-function and gain-of-function mutations could be recovered in the F1 generation providing a relatively easy method for screening large numbers of progeny.

In our screens for dominant modifiers of *GMRrpr* and *GMRhid*, we expected loss-of-function mutations in cell death activators to decrease the degree of cell death and to appear as suppressors of the starting phenotypes. Conversely, we expected loss-of-function mutations in inhibitors of Rpr- and Hid-induced death to increase the degree of cell death and to appear as enhancers.

Dominant modifier screens have proven to be very useful for identifying signaling pathway components in the past. Among others, there are numerous examples of successful screens to identify components of Ras signaling pathways in the eye (e.g. Karim et al., 1996; Rebay et al., 2000; Simon et al., 1991). More importantly, this type of screen has been used to identify cell death genes. During the course of this work, Hay et al. reported the identification of the IAP, *diap1*, as a dominant enhancer of Rpr-induced death (Hay et al., 1995) proving that our strategy would indeed lead to the

identification of genes involved in the Rpr, Hid and possibly also Grim cell death pathways.

Prior to the publication of Hay et al. we sought to determine whether it was possible to obtain dominant modifiers of the *GMRrpr* and *GMRhid* phenotypes. For this reason, we first screened a set of chromosomal deficiencies collectively spanning 65% of the *Drosophila* genome. The benefit of this kind of screen is that one can quickly and relatively easily screen a large portion of the genome. We screened the entire collection by crossing *GMRrpr* or *GMRhid* to the deficiency strains and comparing deficiency and balancer progeny as outlined in Figure 1. Deficiencies on the X and 4th chromosomes are maintained over balancers with dominant eye phenotypes and these dominant eye markers interfered with the *GMRrpr* and *GMRhid* dominant eye phenotypes. Therefore, it was difficult to determine whether differences observed between deficiency and balancer progeny were due to modification by the deficiency or the balancer (data not shown). Thus, the results of these crosses were not considered and here we report only the results with 2nd and 3rd chromosome deficiencies (Table 1).

We identified 8 regions that behaved as suppressors of Rpr-induced death, 1 region that enhanced Rpr, 2 that enhanced Hid, 6 that suppressed both Rpr and Hid and 4 that enhanced both Rpr and Hid. Examples of the phenotypes produced by these modifiers are shown in Figure 2.

We next sought to identify the single genes responsible for the modification of the *GMRrpr* or *GMRhid* eye phenotypes observed. We found it especially intriguing that deletions in 75C1,2, where *rpr*, *hid*, and *grim* reside, were identified as modifiers. Three of the deficiencies tested were reported to remove 75C1,2 (Flybase). One of these suppressed both *GMRrpr* and *GMRhid*, one had no effect on either, and the third enhanced *GMRrpr*. Initially we speculated that these modification phenotypes were due to spurious mutations on the deletion chromosomes, since we expected that deletions of the same region should have the same phenotypes. But since this region was already implicated in cell death, we delved further and found that mutations in the gene *hid*, like *Df(3L)W10* (Table 1) and *Df(3L)H99*, suppressed *GMRrpr* and *GMRhid* (data

not shown). It is unknown whether *rpr* and *grim* may have similar effects since single gene mutations in *rpr* and *grim* did not exist.

In another case, the deficiencies *Df(2L)ast2* and *Df(2L)S3*, which were identified as enhancers of *GMRrpr* and *GMRhid*, were observed to have dominant rough eye phenotypes. Both of these deficiencies remove *Star*, which also shows a dominant rough eye phenotype when mutated (Lindsley and Grell, 1968). Thus, we reasoned that the modification observed resulted from the addition of *Star* and *GMRrpr* or *hid* rough eye phenotypes. *Star* mutations do enhance the *GMRrpr* and *GMRhid* phenotypes (data not shown) but it is unclear whether this enhancement is non-specific as initially expected or whether it indicates that *Star* has a role in cell death (see below).

Although two genes, *hid* and *Star*, were identified starting from deficiencies, moving from large deficiency to single gene proved difficult for various reasons. Some of the modifier phenotypes we observed did not appear to be due to the deficiencies. For example, a deficiency that removes 84D to 85B enhanced *GMRhid*, while two overlapping deficiencies that remove the same region do not (Table 1), suggesting that the modification was caused by either a mutation outside of 84D-85B or by the elimination of multiple genes in 84D-85B. Furthermore, identifying single genes tagged with P-elements that also had modifier phenotypes was difficult. Many P-element lines that we tested showed weak modifier effects (data not shown) and it was impossible to determine whether any of them were responsible for the modification observed with deficiencies in the same region. This difficulty was also reported by Karim and colleagues (Karim et al., 1996).

Thus, in order to isolate single gene mutations, gain-of-function as well as loss-of-function and to survey the entire genome, we performed two mutagenesis screens for dominant modifiers of *GMRrpr* and *GMRhid*. In one of our screens, we started with a moderate rough eye phenotype caused by two copies of *GMRrpr*. We screened about 170,000 F1 progeny of ENU and EMS mutagenized flies for dominant modifiers of the *GMRrpr* phenotype (Figure 3A) and recovered 42 enhancers and 5 suppressors. We also screened about 300,000 F1 progeny of ENU, EMS, and X-ray mutagenized flies for

suppressors of a severe *GMRhid* phenotype (Figure 3B) and recovered approximately 170 suppressors.

These modifiers were mapped to a chromosome, balanced, and their recessive phenotypes determined. We placed the modifiers into complementation groups by crossing mutants with similar recessive phenotypes on the same chromosome to each other and to deficiencies already isolated as modifiers. One hundred twenty eight mutants fell into eleven complementation groups and five of these groups corresponded to deficiencies (Tables 2 and 3). It should be noted that grouping the dominant modifiers in this way is based on the assumption that the dominant modifier phenotypes and recessive phenotypes are linked. The remaining mutants represent single hits, have no recessive phenotype or are otherwise uncharacterized. The dominant modifier phenotypes of some of these have been mapped by meiotic recombination and represent at least four groups. The map position of some suggests that they may be viable alleles of the lethal complementation groups already identified.

Secondary screens to identify mutants most likely to define cell death genes

It is possible that some of the mutants isolated in our screens modify the Rpr- and Hid-dependent eye phenotypes by merely affecting expression from the *GMR* promoter or eye development, rather than specifically affecting cell death. In order to determine which of the modifiers were more likely to define cell death genes and which were more likely to affect eye development or gene expression, we put these modifiers through two types of secondary screens (Tables 2 and 3). In the first type, we tested whether our mutants could also modify other eye phenotypes caused by the ectopic expression of genes with no known role in cell death, *phyllopod* (*phyl*) and *rho1*, under the control of the same *GMR* promoter elements (Chang et al., 1995; Hariharan et al., 1995). We expected genes involved in cell death to have no effect on *GMRphyl* and *GMRrho1* phenotypes, but genes affecting expression from *GMR* or eye development to also modify these phenotypes.

In another type of secondary screen, we tested whether suppressors of *GMRhid* could also suppress the lethality caused by expressing *hid* globally under the control of

the heat shock promoter (Grether et al., 1995) or the ablated wing phenotype caused by expressing *hid* in the wing under the control of the *vestigial* (*vg*) promoter (Figure 5, Tables 2 and 3). We expected genes involved in cell death to also affect these other phenotypes and genes that affect expression or eye development to have no effect.

We identified 4 groups and several single mutants that are not likely to be specifically involved in cell death. The first group corresponds to *glass*, which encodes the transcription factor that drives expression from *GMR* (Moses and Rubin, 1991). Two of the lethal complementation groups we isolated as suppressors of *GMRhid* correspond to *Su(GMR)2A* and *Su(GMR)3A*, two groups identified by K. Barrett and J. Settleman in a screen for dominant modifiers of *GMRrho1* (Barrett et al., 1997). K. Barrett and J. Settleman tested these mutants against a large panel of *GMR* phenotypes and found them to affect all of the *GMR* phenotypes non-specifically (personal communication). Like *glass*, these mutants are, therefore, likely to affect expression from *GMR*. We also recovered mutations in *Star* and *Delta* as enhancers of *GMRrpr*. Mutations in either *Star* or *Delta* result in dominant rough eye phenotypes (Lindsley and Grell, 1968) which likely lead to more severe phenotypes in combination with *GMRrpr* or other dominant rough eye phenotypes.

Interactions with other cell death genes

Because the mutants described here were isolated as modifiers of *GMRrpr* or suppressors of *GMRhid*, we wanted to determine whether they are specific to Rpr or Hid or whether they generally affect the death induced by Rpr, Hid and Grim. Therefore, we tested *GMRrpr* modifiers with *GMRhid* and vice versa. We also tested *GMRrpr* and *GMRhid* modifiers with *GMRgrim* (Chen et al., 1996b) (Tables 2 and 3).

Comparing the effects of the mutants on Rpr-, Hid-, and Grim-induced death allowed us to get some idea of how similar the Rpr, Grim, and Hid pathways are. Many mutants behaved similarly with Rpr, Grim and Hid indicating that the Rpr, Grim, and Hid pathways share some common components. However, while all of the mutants we tested behave similarly with Rpr and Grim, a number of mutants showed clear differences with Hid. For example, several enhancers of *GMRrpr* also enhanced

GMRgrim but had no effect on *GMRhid* (Table 2). Thus it appears that Rpr- and Grim-induced death are more similar to each other than to Hid-induced death.

Complementation groups

Regulators of Ras/MAPK signaling

We isolated mutations in *sprouty* (*spry*) and *Gap1*, two negative regulators of Ras/MAPK signaling (Gaul et al., 1992; Hacohen et al., 1998), as suppressors of *GMRhid*. Ras/MAPK signaling has been implicated in cell survival in mammalian systems (Downward, 1998). The isolation of mutations in negative regulators of Ras as suppressors of cell death suggests that Ras/MAPK signaling promotes survival in *Drosophila* as well. Furthermore, Hid contains five consensus MAPK phosphorylation sites (Grether et al., 1995) suggesting that Ras/MAPK signaling promotes survival by inhibiting Hid. These mutants and the implications outlined here are further explored and discussed in chapter 3.

thread/diap1

We expected to recover mutations in *thread/diap1* as enhancers of *GMRrpr* as reported by Hay and colleagues (Hay et al., 1995). We did in fact isolate loss-of-function mutations in *thread/diap1* that enhance Rpr-, Hid-, and Grim-induced death. However, we also recovered two classes of gain-of-function mutations. One class suppresses Rpr-, Hid-, and Grim-induced death. In addition to binding and inhibiting caspases, IAPs including Diap1 bind Rpr, Hid, and Grim (McCarthy and Dixit, 1998; Vucic et al., 1998a; Vucic et al., 1998b; Vucic et al., 1997b). The isolation of these *thread/diap1* mutants that are resistant to Rpr-, Hid- and Grim-induced death led to a model in which Rpr, Hid, and Grim activate caspases by binding to Diap1, thus, relieving them of Diap1-mediated inhibition. In this model, we expect these Diap1 mutants to be impaired in their ability to bind Rpr, Hid and Grim. The analysis of these mutants and a discussion of this model are presented in chapter 4. Another class of *thread/diap1* mutants enhances Rpr and Grim but suppresses Hid. We found this class

particularly interesting as it suggests Diap1 behaves differently in the Rpr and Grim pathways than in the Hid pathway. These mutants are discussed further in chapter 5.

mod86

Of the remaining complementation groups that defined previously uncharacterized genes, we found one to be particularly interesting. Most alleles of this group, which we named *mod86*, enhance Rpr and Grim and have little or no effect on Hid. One allele, however, enhances Rpr and Grim but suppresses Hid like one class of *thread/diap1* gain-of-function alleles. In order to gain insight into the molecular basis for these interesting properties, we cloned the *mod86* gene, which is discussed further in chapter 5.

Identification of modifiers linked to *GMRrpr*

Because males carrying the *GMRrpr* transgene were mutagenized (see Figure 1) in one of our screens, we expected to recover mutations in the transgene that inactivated it. Four modifiers were identified that were linked to *GMRrpr*. Among these we expected to find missense mutations in *rpr* that impaired its function thus, providing insights into what regions of *rpr* are critical for its function. First, we amplified the *rpr* ORF from *GMRrpr* specifically by PCR and then sequenced the *rpr* ORF. Modifier *5-1s* failed to give a PCR product and, thus, is likely to carry a rearrangement with a breakpoint within the region being amplified. Modifier *11-1e* may also carry a rearrangement. While this modifier did yield a PCR product, no mutation within the *rpr* ORF was detected. Since this modifier is an enhancer of *GMRrpr* it is likely that the mutation increases the expression of *rpr*. In addition this modifier also has a weaker eye color imparted by the P element vector than the parental *GMRrpr* suggesting that expression of the *white* gene is diminished. The simplest explanation is that this modifier carries a rearrangement that increases the expression of *rpr* and decreases the expression of *white*. The remaining two modifiers carried missense mutations in the *rpr* ORF. One, *7-2s*, results in conversion of the initiator methionine to isoleucine. Since Rpr has no other methionines, this mutant is not likely to produce any protein. Another,

3-4s, causes an isoleucine to methionine change in the N-terminus conserved among Rpr, Hid, and Grim (see figure 6A). This mutation severely impairs the ability of Rpr to kill as assayed in the eye (Figure 6B).

Discussion

We performed several screens for dominant modifiers of Rpr- and/or Hid-induced cell death. These screens were successful in that they identified genes known or suspected to be involved in cell death. For example, we identified mutations in *thread/diap1* as expected. We also identified negative regulators of Ras/MAPK signaling as suppressors of Hid-induced death suggesting that the Ras/MAPK pathway promotes cell survival in *Drosophila* as has been observed in mammalian systems (Downward, 1998). In addition to providing evidence for the effectiveness of our screens, the isolation of these mutants led to novel insights into the mechanisms of cell death regulation. The gain-of-function mutations in *thread/diap1* that we isolated led to models for how Diap1 inhibits cell death and how Rpr, Hid, and Grim overcome this inhibition (see chapters 4 and 5). The observation that Ras/MAPK signaling inhibits primarily Hid-induced death along with the observation that Hid contains MAPK consensus phosphorylation sites led to the idea that Ras/MAPK signaling promotes survival by inhibiting Hid (see chapter 3). We also isolated mutations in at least one previously uncharacterized gene, *mod86*. The cloning and characterization of mod86 is described and discussed in chapter 5.

Additionally, we discovered that mutations in *hid* suppress both *GMRhid* and *GMRrpr* phenotypes. *hid* is highly expressed in the developing eye (Grether, 1994), yet this expression is apparently not sufficient for cell death. In the wildtype eye, the death inducing activity of Hid protein may be downregulated by pro-survival factors, which are limiting in the cell. Thus, overexpression of *hid* or *rpr* may deplete these factors and cause increased cell death. Decreasing the dosage of endogenous *hid* decreases the total amount of pro-apoptotic factors, and, in turn, the degree of death observed.

An additional benefit of our *GMRrpr* screen is that besides isolating second site modifiers of *GMRrpr*, we identified mutations in *GMRrpr* itself. One mutation causes an

isoleucine to methionine change at residue 7 in the N-terminus conserved among Rpr, Grim, and Hid. This mutation strongly suppresses the *GMRrpr* eye phenotype (Figure 6B) suggesting that it drastically weakens the ability of Rpr to induce cell death. Single amino acid substitutions in Rpr's N-terminus generated in vitro have previously been shown to impair Rpr's death inducing function (Vucic et al., 1997a). Our mutation, generated in vivo, further highlights the importance of the N-terminus for Rpr function.

Yet despite their success, our screens had a number of difficulties. One impediment that we encountered was the isolation of a large number of *glass* alleles in the *GMRhid* screen. Sixty-nine mutants are confirmed alleles of *glass* based on non-complementation but there are almost certainly more. Many mutants show rough eye phenotypes, appear non-specific in our secondary screens, and map roughly to where *glass* maps (between *sr* and *e*). Since *glass* merely affects expression from *GMR*, the large number of *glass* alleles hindered the recovery and analysis of more interesting mutants. Given the large number of *glass* alleles, it is likely that these existed or arose in the background of one of the parental strains we used in the screen. The presence of *GMRhid* impairs the viability of the strain; while many individuals survive to adulthood, many die as pupae. *GMR* drives expression in some areas of the larval brain in addition to the developing eye (Flybase) and presumably it is this expression that affects the viability. Mutations in *glass* may improve the viability and confer a growth advantage. Thus, it is likely that many of the *glass* alleles we isolated arose in the parental *GMRhid* strain.

We also encountered difficulties in the secondary screens aimed at eliminating mutants that modified the *GMRrpr* and *GMRhid* phenotypes by virtue of their effect on gene expression or eye development. We eliminated *glass*, *Su(GMR)2A*, *Su(GMR)3A*, *Star*, and *Delta* based in part on these secondary screens but we also had other information that allowed us to evaluate the results of these screens. For example, *glass* was known to affect expression from *GMR*, *Su(GMR)2A* and *Su(GMR)3A* were isolated in unrelated *GMR* screens, and *Star* and *Delta* have dominant rough eye phenotypes. We had more difficulty in eliminating others based solely on the results of the secondary screens. Some modifiers that have a role in cell death, like *thread/diap1*, *spry* and

gap1, also have weak effects on *GMRphyl*. Although it was expected that mutants affecting cell death would have no effect on *GMRphyl*, the *GMRphyl* phenotype may have a cell death component and may also be affected by some cell death mutants. Conversely, one allele of *Su(GMR)2A*, a suppressor that is thought to affect *GMR* phenotypes generally, was judged to have no effect on *GMRphyl*. We have found *GMR* phenotypes to vary in their sensitivity. For example, mutations that modify weak *GMRhid* phenotypes sometimes do not modify strong *GMRhid* phenotypes. It is likely that we failed to detect an effect of *Su(GMR)2A* $^{29\text{-}4s}$ on *GMRphyl* for this reason.

Moreover, *Star* and *Delta* have dominant rough eye phenotypes and thus fail the secondary screens but these genes may nonetheless be involved in cell death. Star is involved in processing Spitz, a ligand for the EGF receptor, which activates the Ras/MAPK pathway (Klambt, 2002). Since the Ras pathway promotes survival, mutations that reduce signaling are expected to be enhancers as is observed with *Star*. Delta is a ligand for Notch, which has also been implicated in cell death. Miller and Cagan have found Notch to be involved in activating cell death in the developing eye (Miller, 1998). Although these mutants were eliminated from further study, we cannot rule out a role in Rpr- and/or Hid-induced cell death.

Only a subset of *GMRhid* suppressors that passed the *hshid* test passed the *vgGal4, UAShid* test. The reasons for this are unclear. Perhaps the *vgGal4, UAShid* phenotype is less sensitive to modification than *hshid*. The *hshid* test may also be more prone to false positives. It is interesting to note that modifiers that pass the *vgGal4, UAShid* test include *thread/diap1*, which encodes a protein that directly binds Rpr, Hid, and Grim. Therefore, other modifiers that pass the *vgGal4, UAShid* assay may also function closely with Rpr, Hid, and Grim.

Although we encountered some difficulties, these screens overall were very successful. They allowed us to identify new cell death related genes and characterize the role of known genes in programmed cell death. Each screen we performed was productive. The deficiency screen identified genomic regions involved in cell death and further analysis of these regions enabled us to identify *hid* and *Star* as modifiers. Furthermore, these deficiencies were used to quickly map lethal modifiers from our

mutagenesis screens leading to the rapid identification of the *Star*, *spry*, *Gap1* and *thread/diap1* complementation groups. Performing mutagenesis screens, in addition to the deficiency screen, with both *GMRhid* and *GMRrpr* allowed us to identify both gain-of-function and loss-of-function alleles of several genes including *thread/diap1* and *mod86*. Moreover, we identified from the mutagenesis, complementation groups that were not represented in the deficiency collection. Our analysis of the mutants generated in these screens has already led to a more thorough understanding of Rpr-, Hid- and Grim-induced cell death. Further analysis of the remaining uncharacterized mutants may lead to additional insights.

Experimental Procedures

Drosophila Stocks

GMRrpr 81, *GMRrpr 114* (White et al., 1996), *GMRrpr 34 CyO/Sco*, *GMRhid 1M* (Bergmann et al., 1998), *GMRhid 10*, *hshid 3* (Grether et al., 1995), *GMRgrim* (Chen et al., 1996b), *GMRphyl* (Chang et al., 1995), *GMRrho1* (Hariharan et al., 1995), *vgGal4* (F.M. Hoffmann, unpublished), *UAShid*.(McNabb, 1997; Zhou et al., 1997)
Deficiency stocks were obtained from the Bloomington Stock Center.
Flies were raised on standard cornmeal-molasses medium at 25°C unless otherwise indicated.

Dominant Modifier Screens

For the *GMRrpr* screen, *yw*; *GMRrpr 81* homozygous males were fed a solution of sucrose and 0.25mg/ml ENU or 25 mM EMS and then crossed to *yw*; *GMRrpr 81* homozygous females. The F1 progeny were screened for suppression or enhancement of the parental phenotype. Approximately 95% of the 170,000 F1 progeny screened were derived from ENU treated males, while 5% were from EMS treated males. For the *GMRhid* screen, *yw* males were fed a solution of sucrose and 0.25 mg/ml ENU or 25 mM EMS or treated with 4500 rad X-rays and then crossed to *GMRhid 10* homozygous females. The F1 progeny were screened for suppression of the *GMRhid 10* phenotype.

Approximately 49% of the 300,000 F1 progeny screened were derived from EMS treated males, 49% from X-ray treated males, and 2% from ENU treated males.

Complementation analysis was performed on mutants on the same chromosome exhibiting similar recessive phenotypes. Male sterility was assessed by mating twenty homozygous mutant males individually to Canton-S females. The mutants were considered to display some degree of sterility if fewer than 20% of the crosses gave rise to more than 20 progeny. Control crosses regularly gave rise to at least 40 progeny. Dominant modifiers on the third chromosome were mapped by meiotic recombination relative to the markers *th st cu sr e ca*.

Secondary screens

hshid

In the initial stages, *hshid/+* females were crossed to *suppressor/TM3* males and the resulting progeny were subjected to a 15 minute heat shock in the 1st instar larval stage. The ratio of *hshid/sup* and *hshid/TM3* relative to *sup/+* or *TM3/+* progeny was determined. Later, *hshid/TM3* females were crossed to *suppressor/TM3* males and the resulting progeny were subjected to a 15 minute heat shock in the 1st instar larval stage. The ratio of *hshid/sup* and *hshid/TM3* relative to *sup/TM3* progeny was determined.

vgGal4, UAShid

vgGal4, UAShid/CyO females were crossed to *suppressor/TM3* males. Progeny were raised at 25°C and 18°C and the wing phenotype of *vgGal4, UAShid/suppressor* progeny was compared to *vgGal4, UAShid/TM3*. Few suppressors were able to overcome the strong ablated wing phenotype observed when *vgGal4, UAShid* flies were grown at 25°C.

Analysis of 2nd chromosome *GMRrpr* modifiers

Because the 2nd chromosome carried an insertion of *GMRrpr*, all 2nd chromosome modifiers had to be recombined away from *GMRrpr* for further analysis. Modifiers that failed to separate from *GMRrpr* were candidates for carrying mutations in *GMRrpr* itself. To eliminate the possibility that these carried mutations in a gene that was close to

GMRrpr, we mobilized the *GMRrpr* transgene. Mutations in *GMRrpr* were inseparable from the transgene; excised chromosomes lacked the modifier phenotype while mobilized transgenes still carried the modified *GMRrpr* phenotype. The *rpr* ORF was specifically amplified by PCR using a 5' primer to the *rpr* 5' UTR and a 3' primer to the *GMR* vector. Products from duplicate PCR reactions were sequenced directly at the Biopolymers Facility, MIT.

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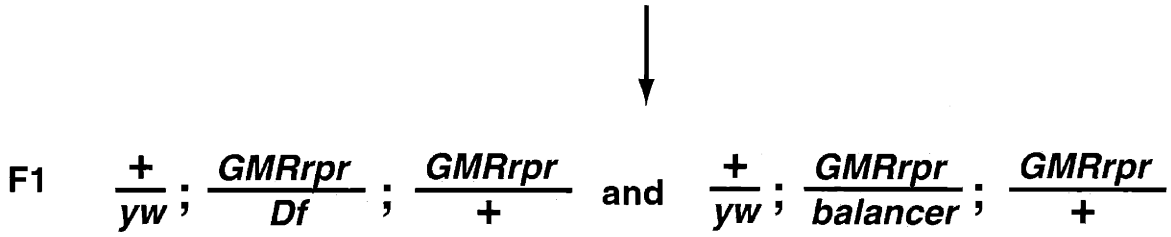
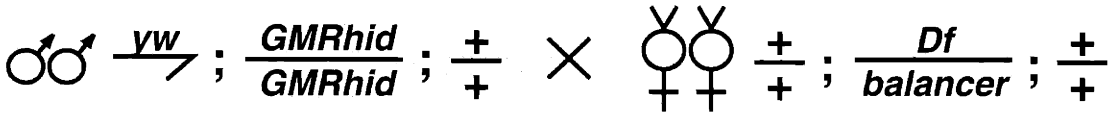
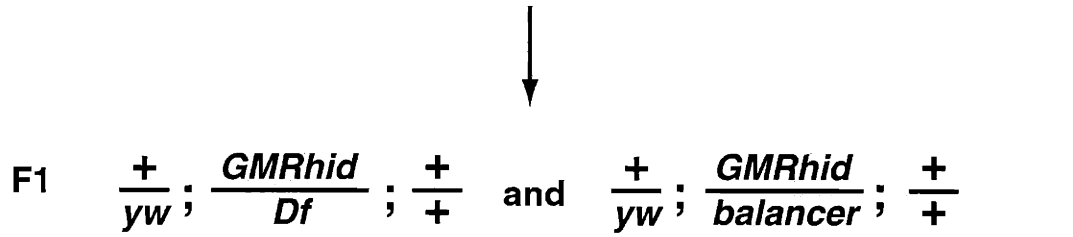
Figure 1. A general scheme for screening a collection of chromosomal deficiencies for modifiers of *GMRrpr* and *GMRhid*. Deficiency females were crossed to males carrying either 4 copies of the *GMRrpr* (A) or 2 copies of the *GMRhid 1M* (B) transgenes. In the F1 generation the eye phenotypes of deficiency bearing progeny were scored for suppression or enhancement as compared to progeny carrying the balancer chromosome.

A



B





Compare the rough eye phenotypes of the *Df* vs. *balancer* classes of progeny

Table 1. 2nd and 3rd chromosome deficiencies tested for interaction with *GMRrpr* and *GMRhid*				
Genotype	**Breakpoints**	***GMRrpr***	***GMRhid***	**Gene responsible**
Chromosome II				
Df(2L)net-PMF/SM6a	021A01;021B07-08	-	-	
Df(2L)al/In(2L)Cy, Cy[1]	021B08-C01;021C08-D01	ND	ND	
Df(2L)ast2/SM1	021D01-02;022B02-03	Enh	Enh	*Star*
Df(2L)S3/SM1	021D02-03;021F02-022A01	ND	Enh	*Star*
Df(2L)dp-79b, dp[DA] cn[1]/In(2LR)bw[V1], b[1]	022A02-03;022D05-E01	-	-	
Df(2L)ed1, al[1] b[1]/SM5	024A03-04;024D03-04	-	-	
Df(2L)sc19-8/SM6b?, Cy[1] Roi[1]; Dp(2;1)B19, y[1], ed[1] dp[o2] cl[1]	024C02-08;025C08-09	-	-	
Df(2L)cl-h3/SM6b?, Cy[1] Roi[1]	025D02-04;026B02-05	-	-	
Df(2L)GpdhA/CyO	025D07-E01;026A08-09	-	-	
Df(2L)J136-H52/SM5	027C02-09;028B03-04	-	-	
Df(2L)spd, al[1] dp[ov1]/CyO	027D-E;028C	-	-	
Df(2L)30C/CyO; w[1118]	029F07-030A01;030C02-05	-	-	
Df(2L)Mdh, cn[1]/Dp(2;2)Mdh3, cn[1]	030D-30F;031F	-	-	
Df(2L)J39/In(2L)Cy; Dp(2;Y)cb50, Dp(1;Y)B[S]Yy[+]/C(1)RM	031C-D;032D-E	-	ND	
Df(2L)Prl/CyO	032F01-03;033F01-02	-	-	
Df(2L)esc10, b pr/CyO	033A08-B01;033B02-03	-	-	
Df(2L)prd1.7, b[1] Adh[n2] pr[1] cn[1] sca[1]/CyO-vKa, P{ry[+t]=elav-lacZ.H}YH2*	033B02-03;034A01-02	-	-	
Df(2L)b87e25/In(2L)NS	034B12-C01;035B10-C01	Sup	-	
Df(2L)osp29, Adh[uf3] pr cn/CyO	035B01-03;035E06	Sup	-	
Df(2L)r10, cn[1]/CyO	035E01-02;036A06-07	-	-	
Df(2L)H20, b[1] pr[1] cn[1] sca[1]/CyO	036A08-09;036E01-02	-	-	
Df(2L)TW50, cn[1]/CyO, Dp(2;2)M(2)m[+]	036E04-F01;038A06-07	Enh	-	
Df(2L)A14, pr cn bw/SM5	037D02-07;039A04-07	-	ND	
Df(2L)E55, rdo hk pr/CyO	037D02-E01;037F05-38A01	-	-	
Df(2L)pr76, Sco/CyO	037D;038E	-	-	
Df(2L)TW84/CyO	037F05-38A01;039D03-E01	-	-	

Genotype	Breakpoints	*GMRrpr*	*GMRhid*	Gene responsible
Df(2L)TW161, cn[1] bw[1]/CyO	038A06-B01;040A04-B01	-	-	
Df(2R)M41A4/SM1	041A	-	-	
In(2R)pk78s/CyO	042C01-07;043F05-08 or 042B;042C max or 42F;43F+	-	-	
Df(2R)cn88b, cn/SM5	042C;042E	Wk Sup	-	
Df(2R)cn87e/In(2LR)bw[V1], ds[33k] b[1] bw[V1]	042B04-C01;043F-44A01	-	ND	
Df(2R)42, cn[1]/SM5	042C03-08;042D02-03	-	ND	
Df(2R)pk78k, Tp(2;2)CA30, sp[1]/CyO	042E03;043C03	Wk Sup	ND	
Df(2R)cn9/ Cy[1] Roi[1]	042E;044C	-	-	
Df(2R)44CE, al dp b pr/CyO	044C04-05;044E02-04	Sup	Sup	
Df(2R)eve1.27, cn/CyO	046C03-04;046C09-11	-	-	
Df(2R)X1/CyO, Adh[nB]	046C;047A01	Wk Sup	-	
Df(2R)en-A/CyO	047D03;048B02-05	-	-	
Df(2R)en30/SM5; Dp(1;Ybb[-])B[S]	048A03-04;048C06-08	Wk Sup	-	
Df(2R)en-SFX31/CyO	048A01;048B05-07	-	ND	
Df(2R)en28/SM5	048A01-02;048B- C01	-	ND	
Df(2R)vg135/CyO, S[*] bw[1]	049A-B;049D-E	-	-	
Df(2R)vg-C/SM5	049A04-13;049E07-F01	-	-	
Df(2R)CX1, b[1] pr[1]/SM1	049C01-04;050C23-D02	Enh	Enh	
Df(2R)vg-B/SM5	049D03-04;049F15-50A03	-	-	
Df(2R)trix/CyO	051A01-02;051B06	-	-	
Df(2R)Jp1/CyO; w[a] fa[g]	051C03;052F05-09	-	-	
Df(2R)Jp8, w[+]/CyO; w[a] fa[g]	052F05-09;052F10-53A01	-	-	
Df(2R)Pcl11B, al[1] dp[ov1] b[1] pr[1]/CyO-vKa, P{ry[+t]=elav-lacZ.H}YH2*	054F06-55A01;055C01-03	-	-	
Df(2R)PC4/CyO	055A;055F	-	-	
Df(2R)AA21, c[1] px[1] sp[1]/SM1	056F09-17;057D11-12	-	-	
Df(2R)Pu-D17, cn[1] bw[1] sp[1]/SM1	057B04;058B	-	-	
Df(2R)or-BR6, cn[1] bw[1] sp[1]/In(2LR)lt[G16L]bw[V 32gR]	059D05-10;060B03-08	-	-	
Df(2R)bw[VDe2L] Px[KR]/SM1	059D06-E01;060C-D	-	ND	
Df(2R)Px4, Dp(2L)Px4, In(2LR)Px4, dp b/CyO	060B;060D01-02, 021D01;022A03, 022A;060B;021C08;060 D;042A;058A	-	-	

Genotype	Breakpoints	*GMRrpr*	*GMRhid*	Gene responsible
Df(2R)Px2/SM5	060C05-06;060D09-10	-	-	
Df(2R)M-c33a/In(2LR)bw[V32g]	060E02-03;060E11-12	-	-	
Chromosome III				
Df(3L)Ar14-8, red[1]/TM2, emc[2] p[p] Ubx[130] e[s] ! = Df(3L)emc5	061C05-08;062A08	-	-	
Df(3L)R-G5, ve/TM6	062A10-B01;062C04-D01	-	-	
Df(3L)R-G7, ve[1]/TM6B, Tb[+]	062B08-09;062F02-05	-	-	
Df(3L)M21, ri[1] p[p]/In(3LR)T33[L]f19[R]	062F;063D	-	-	
Df(3L)HR370/Dp(3;3)T33[L] f19[R]	063A01;063D10, 062A;064C	-	-	
Df(3L)HR232/TM6B	063C01;063D03	Sup	Sup	*sprouty*
Df(3L)HR119/TM6B	063C06;063E	Sup	Sup	*sprouty*
w[1118]; Df(3L)GN50/TM8, l(3)DTS4[1] th[1] st[1] Sb[1] e[1]	063E01-02;064B17	Sup	-	
Df(3L)GN24/TM8, l(3)DTS4[1] th[1] st[1] Sb[1] e[1]	063F04-07;064C13-15	-	-	
Df(3L)ZN47, ry[506]/TM3	064C;065C	-	-	
Df(3L)pbl-X1/TM6B ! w[*] floating	065F03;066B10	-	-	
y[1] w[1] N[spl-1]; Df(3L)66C-G28/TM3	066B08-09;066C09-10	Sup	Sup	
Df(3L)h-i22, Ki[1] roe[1] p[p]/TM3	066D10-11;066E01-02	-	-	
Df(3L)29A6, ri[1] p[p]/TM3	066F05;067B01	-	-	
Df(3L)AC1, roe[1] p[p]/TM3, Sb[1]	067A02;067D07-13 or 067A05;067D09-13	Sup	Sup	*Gap1*
y[1?]; Df(3L)lxd6/TM3, Sb[1] Ser[1]	067E01-02;068C01-02	Sup	-	
Df(3L)vin2, ru[1] h[1] gl[2] e[4] ca[1]/TM3	067F02-03;068D06	Sup	Sup	*glass*
Df(3L)vin5, ru[1] h[1] gl[2] e[4] ca[1]/TM3, Sb[1] Ser[1]	068A02-03;069A01-03	Sup	Sup	*glass*
Df(3L)vin7, ru[1] h[1] gl[2] e[4] ca[1]/TM3, Sb[1] Ser[1]	068C08-11;069B04-05	Sup	Sup	*glass*
Df(3L)Ly, mwh/TM1, jv	070A02-03;070A05-06	-	-	
Df(3L)fz-GF3b, P{w[+tAR] ry[+t7.2AR]=wA[R]}66E/TM6B	070C01-02;070D04-05	-	-	

Genotype	Breakpoints	*GMRrpr*	*GMRhid*	Gene responsible
Df(3L)fz-M21, th[1] st[1]/TM6	070D02-03;071E04-05	Sup	-	
Df(3L)BK10, ru[1] Ly[1] red[1] cv-c[1] Sb[sbd-1] sr[1] e[1]/TM3	071C;071F	Sup	-	
Df(3L)brm11/TM6C, cu[1] Sb[1] ca[1]	071F01-04;072D01-10	Enh	Enh	*thread/diap1*
Df(3L)th102, h[1] kni[ri-1] e[s]/TM6C, cu[1] Sb[1] ca[1]	071F03-05;072D12	Enh	Enh	*thread/diap1*
Df(3L)st-f13, Ki[1] roe[1] p[p]/TM6B	072C01-D01;073A03-04	-	-	
Df(3L)81k19/TM6B	073A03;074F	-	-	
Df(3L)W10, ru[1] h[1] Sb[sbd-2]/TM6B	075A06-07;075C01-02	Sup	Sup	*hid*
Df(3L)Cat, ri[1] Sb[sbd-1] e[*]/TM3, Ser[1]	075B08;075F01	-	-	
Df(3L)W4, ru h e ca/TM6B =W[+R4]	075B10;075C01-02	Enh	-	
Df(3L)VW3/TM3	076A03;076B02	-	-	
Df(3L)rdgC-co2, th[1] st[1] in[1] ri[1] p[p]/TM6C, Sb[1] cu[1] Tb[1]	077A01;077D01	-	-	
Df(3L)ri-79c/TM3	077B-C;077F-78A	-	-	
Df(3L)Pc-MK/TM3	078A03;079E01-02	-	-	
Dp(3;1)2-2, w[1118]/?; Df(3R)2-2/TM3	081F;082F10-11	-	-	
Df(3R)Tpl10, Tp(3;3)Dfd[rv1], ri[1] p[p]/TM3	083C01-02;084B01-02	-	-	
Df(3R)Scr, p[p] e[s]/TM3	084A01-02;084B01-02	-	-	
Df(3R)Antp17/TM3	084B01-02;084D11-12 or A06,D14	-	-	
Df(3R)p712, red[1] e[1]/TM3	084D04-06;085B06	-	Enh	
Df(3R)dsx29/CxD	084C08-D01;084F06- 07	ND	-	
Df(3R)p40, red[1] e[1]/TM3, Sb[1]	084E08-09;085B06	ND	-	
Df(3R)p-XT103, ru[1] st[1] e[1] ca[1]/TM3	085A02;085C01-02	-	-	
Df(3R)by10, red[1] e[1]/TM3	085D08-12;085E07-F01	-	-	
Df(3R)by62, red[1] e[1]/TM1	085D11-14;085F16	-	-	
Df(3R)M-Kx1/TM3, Sb[1] Ser[1]	086C01;087B01-05	-	-	
Df(3R)T-32, (ri[1]) cu[1] sr[1] e[s]/MRS	086E02-04;087C06-07	Wk Enh	Enh	

Df(3R)ry615/TM3, Sb[1] Ser[1]	087B11-13;087E08-11	Sup	-	
Tp(3;Y)ry506-85C/MKRS	087D01-02;088E05-06;Y	-	-	
Df(3R)red1/TM1	088B01;088D03-04	-	-	
Genotype	**Breakpoints**	***GMRrpr***	***GMRhid***	**Gene responsible**
Df(3R)P115, e[11]/TM1; Dp(3;1)P115/+	089B07-08;089E07-08;020	-	-	
Df(3R)C4, p[*]/Dp(3;3)P5, Sb[1]	089E;090A	-	-	
Df(3R)P14, sr/T(2;3)ap[Xa]	090C02-D01;091A01-02	Sup	Sup	*glass*
Df(3R)Cha7/TM6B?	090F01-F04;091F05	Sup	Sup	*glass*
Df(3R)Dl-BX12, ss[1] e[4] ro[1]/TM6B	091F01-02;092D03-06	-	-	
Df(3R)e-R1, Ki[1]/TM3	093B03-05;093D02-04	-	-	
Df(3R)e-N19/TM2	093B;094	-	Enh	
Df(3R)e-F1, In(3R)C, sprd[1] cd[1]/cu[1] H[1] e[4]	093B06-07;093E01-02	-	Enh	
Df(3R)e-BS2, rsd[1]/TM3, Sb[1]	093C03-06;093F14-094A01	-	-	
Df(3R)crb87-4, st[1] e[1]/TM3, Ser[1]	095E08-F01;095F15	-	-	
Df(3R)crb87-5, st[1] e[1]/TM3, Ser[1]	095F07;096A17-18	-	-	
In(3R)Ubx[7LL]ats[R], asp[1] ats[1] p[p]/TM6B; y[1]/Dp(1;Y)y[+] ! = Df(3R)XS, Dp(3R)XS	096A01-07;096A21-25	-	-	
Df(3R)XTA1, th[1] st[1] ri[1] roe[1] p[1]/Dp(3;3)M95A[+]13, st[1] e[1]	096B;096D	-	-	
Df(3R)Tl-P, e[1] ca[1]/TM3, Ser[1]	097A;098A01-02	-	-	
w[1118]; Df(3R)3450/TM6B	098E03;099A06-08	-	-	
Df(3R)L127/TM6; Dp(3;1)B152	099B05-06;099E04-F01	-	-	
Ts(YLt;3Lt)B81, P{ry[+t7.2]=RP49}F2-80A e[1]/TM3; Dp(3;1)67A ! = Df(3R)B81	099C08;100F05	-	-	
Df(3R)awd-KRB, ca[1]/TM3, y[+] Sb[1] e[1] Ser[1]	100C;100D	-	-	
Sup, Suppressor; Enh, Enhancer; Wk, weak; -, no effect; ND, not done				

Figure 2. Suppression and enhancement of *GMRrpr* and *GMRhid* eye phenotypes by representative deficiencies. The *GMRrpr* and *GMRhid* conditions used produced moderate reduced, rough eye phenotypes (A and D). Deficiencies that suppressed *GMRhid* and *GMRrpr* improved the appearance of the eye, making it larger, smoother, and more organized (compare B to A and E to D). Deficiencies that enhanced *GMRhid* and *GMRrpr* worsened the appearance of the eye, making it smaller and devoid of structure (compare C to A and F to D). Genotypes are as follows- (A) *GMRhid* 1M/+; +/+; (B) *GMRhid 1M/+; Df(3L)HR119/+*; (C) *GMRhid 1M/+; Df(3L)brm11/+*; (D) *GMRrpr 81/+; GMRrpr 114/+*; (E) *GMRrpr 81/+; GMRrpr 114/Df(3L)HR119*; (F) *GMRrpr 81/+; GMRrpr 114/Df(3L)brm11*.

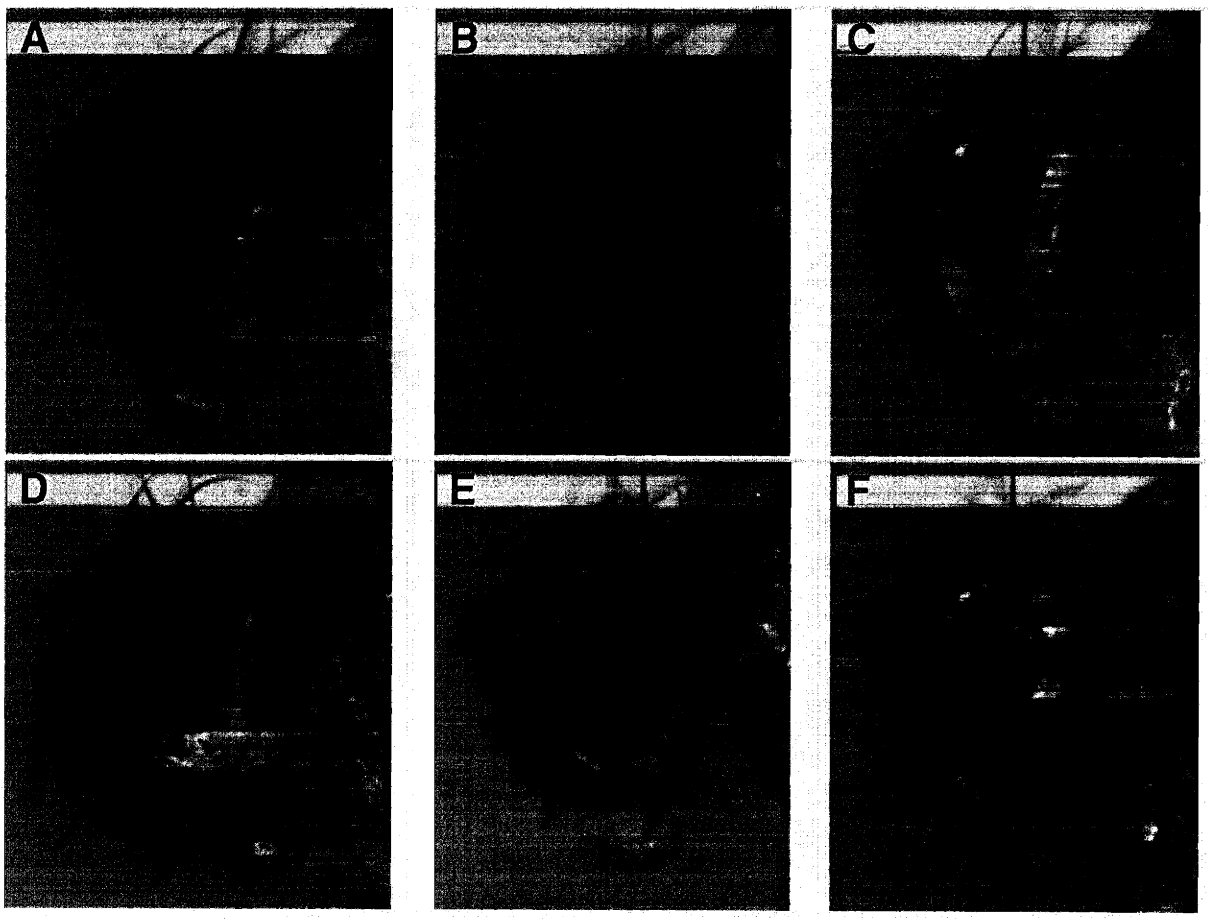
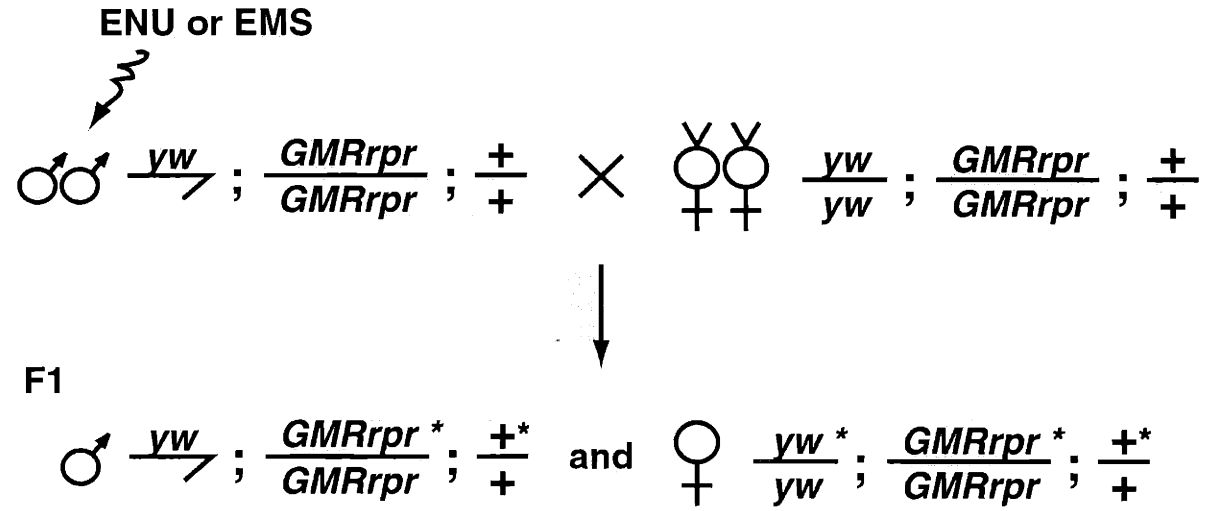

A
B
C
D
E
F

Figure 3. Scheme for isolating dominant modifiers of *GMRrpr* and *GMRhid*.

(A) Males carrying 2 copies of *GMRrpr* were mutagenized with ENU or EMS and mated to females of the same strain. The F1 progeny were screened for enhancers or suppressors of the parental moderate reduced, rough eye phenotype.

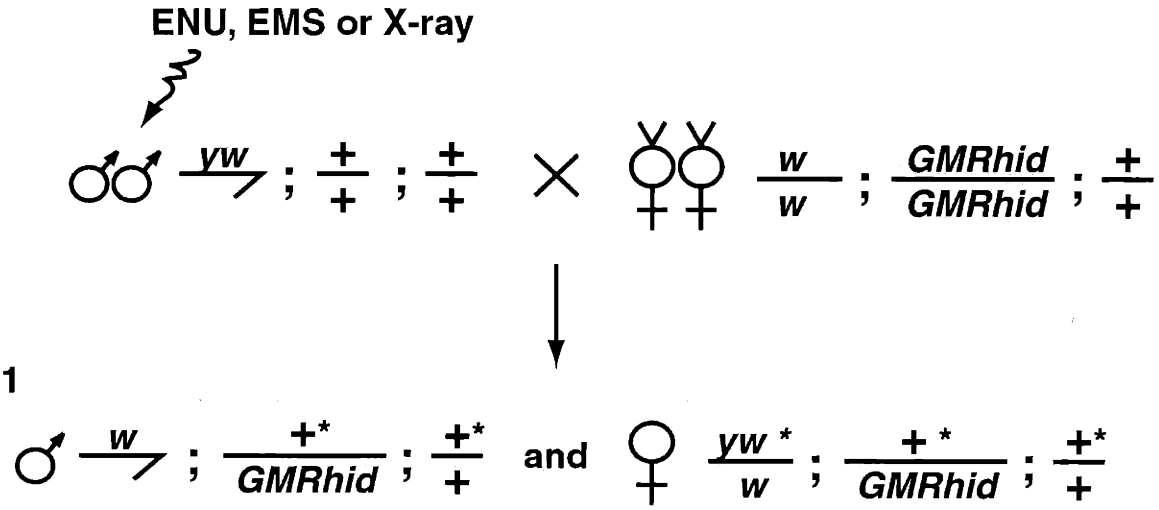
(B) *yw* males were mutagenized with ENU, EMS, or X-rays and mated to females carrying 2 copies of *GMRhid 10*. The F1 progeny were screened for suppressors of the parental ablated eye phenotype. Asterisks indicate mutagenized chromosomes.

A



Screen F1 progeny for enhancers and suppressors of *GMRrpr* rough eye phenotype

B



Screen F1 progeny for suppressors of *GMRhid* ablated eye phenotype

Table 2. *GMRrpr* modifiers: Summary of genetic interactions

Groups	No. of alleles	Map position	Alleles	Recessive phenotype	*GMRrpr*	*GMRhid*	*GMRgrim*	*GMRrho1*	*GMRphyl*
Star	5	21E2	*2-1e*	Lethal	Enh	ND	ND	ND	ND
			6-1e	Lethal	Enh	ND	ND	ND	ND
			10-2e	Lethal	Enh	ND	ND	ND	ND
			13-2e	Semi-lethal	Enh	ND	ND	ND	ND
			16-2e	Lethal	Enh	ND	ND	ND	ND
GMRrpr	4		*11-1e*	Rep	Enh	ND	ND	ND	ND
			3-4s	Viable	Sup	ND	ND	ND	ND
			5-1s	Viable	Sup	ND	ND	ND	ND
			7-2s	Lethal	Sup	ND	ND	ND	ND
thread/ diap1	2	72D1	*11-3e*	Lethal	Enh	Enh	Enh	-	-
			6-3s	Viable	Sup	Sup	ND	ND	-
mod86	11	86A	*2-2e*	Male sterile	Enh	-	Enh	-	-
			2-3e	Lethal	Enh	-	Enh	-	-
			3-1e	Male sterile	Enh	-	Enh	-	-
			3-5e	Male sterile	Enh	-	Enh	-	-
			4-1e	Male sterile	Enh	-	Enh	-	-
			8-1e	Male sterile	Enh	-	Enh	-	-
			10-1e	Male sterile	Enh	-	Enh	-	-
			10-4e	Male sterile	Enh	-	Enh	-	-
			10-5e	Male sterile	Enh	-	Enh	-	-
			13-6e	Male sterile	Enh	-	Enh	-	-
			15-3e	Male sterile	Enh	-	Enh	-	-
Delta	1	92A1-2	*10-12e*	Lethal	Enh	Enh	Enh	Enh	Enh
Other		*-th-st-*	*9-4e*	Viable	Enh	-	Enh	-	-
		-th-st-	*9-5e*	Viable	Enh	-	Enh	-	-
		-th-st-	*14-1e*	Viable	Enh	-	Enh	-	-
		-th-st-	*14-2e*	Viable	Enh	-	Enh	-	-
		-th-st-	*16-3e*	Viable	Enh	-	Enh	-	-
		sr-e	*5-2s*	Viable	Sup	Sup	ND	ND	Sup
		sr-e	*5-4e*	Viable	Enh	Enh	Enh	Enh	Lethal

Groups are named for the known gene to which they correspond. The group named "other" consists of mutants that were mapped roughly by meiotic recombination relative to the markers *th st cu sr e ca*; *-th-st-* indicates that the dominant modifier mutation maps roughly around the markers *th* and *st* and may be located on either side, *sr-e* indicates that the modifier maps between *sr* and *e*. Rep, reduced eye pigmentation; Sup, suppressor; Enh, enhancer; -, no effect; ND, not done. Interaction between *Star* alleles and *GMRhid*, *grim*, *rho1*, and *phyl* were not determined because the dominant rough eye phenotype of these mutants was expected to enhance all *GMR* phenotypes based on information from the Df screen. *Df(2L)ast2* was found to enhance *GMRhid* and *GMRphyl*.

Table 3. *GMRhid* suppressors: Summary of genetic interactions

Groups	No. of alleles	Map position	Alleles	Recessive phenotype	*GMRrpr*	*GMRgrim*	*GMRphyl*	*hshid*	*vgGal4, UAShid*
sprouty	5	63D1	*23-14s*	Lethal	Sup	Sup	-	Sup	-
			25-1s	Lethal	Sup	ND	-	Sup	-
			27-1s	Lethal	Sup	Sup	Wk sup	Sup	ND
			28-4s	Lethal	Sup	Sup	Wk sup	Sup	-
			30-5s	Lethal	Sup	Sup	Wk sup	ND	-
Gap1	5	67C2-3	*21-1s*	Ro, wv	Wk sup	Sup	-	Sup	-
			22-2s	Ro, wv	Wk sup	Sup	-	Sup	-
			23-9s	Ro, wv	ND	ND	ND	Sup	-
			24-6s	Ro, wv	Wk sup	Sup	Wk sup	Sup	-
			26-2s	Ro, wv	Sup	Sup	-	Sup	-
thread/ diap1	8	72D1	*21-2s*	Semi-lethal	Sup	Sup	-	Sup	Sup
			21-4s	Lethal	Enh	ND	-	Sup	Sup
			22-8s	Lethal	Enh	Enh	ND	Sup	-
			23-4s	Lethal	Sup	Sup	Wk sup	Sup	Sup
			23-8s	Lethal	Sup	Sup	-	Sup	Sup
			33-1s	Lethal	Enh	Enh	Wk enh	Sup	Sup
			41-8s	Lethal	Enh	ND	-	Sup	ND
			45-2s	Semi-lethal	Sup	ND	ND	ND	Sup
mod86	1	86A	*23-6s*	Lethal	Enh	Enh	Wk enh	Sup	Sup
glass	69	91A1-2	*23-3s*	Ro	Sup	Sup	Sup	-	-
Su(GMRhid)2A	3	2nd	*26-3s*	Lethal	Sup	ND	ND	ND	ND
			32-1s	Lethal	Sup	ND	ND	ND	ND
			43-4s	Lethal	ND	ND	ND	ND	ND
Su(GMR)2A	4	2nd	*22-1s*	Lethal	Sup	ND	Wk sup	ND	ND
			27-2s	Lethal	Sup	ND	Wk sup	ND	ND
			29-4s	Lethal	Sup	ND	-	ND	ND
			30-2s	Lethal	Sup	ND	Sup	ND	ND
Su(GMR)3A	5	3rd	*24-9s*	Lethal	Sup	ND	Wk sup	-	ND
			28-1s	Lethal	Sup	ND	Wk sup	-	-
			30-6s	Lethal	Sup	Sup	Lethal	ND	ND
			32-3s	Lethal	Sup	Sup	Sup	ND	ND
			32-8s	Lethal	Sup	Sup	Sup	Wk sup	ND

Groups	No. of alleles	Map position	Alleles	Recessive phenotype	*GMRrpr*	*GMRgrim*	*GMRphyl*	*hshid*	*vgGal4, UAShid*
Su(GMRhid)3A	2	*sr-e*	*23-15s*	Lethal	Sup	Sup	Wk sup	Sup	Sup
		3rd	*29-2s*	Lethal	Sup	ND	Sup	ND	-
Su(GMRhid)3B	8	*sr-e*	*24-3s*	Rep, ro	Sup	ND	Wk sup	Sup	-
		3rd	*38-5s*	Rep, ro	Sup	ND	ND	ND	-
		-sr-	*38-7s*	Rep, ro	Sup	ND	Sup	ND	-
		sr-e	*38-8s*	Rep, ro	ND	ND	Sup	ND	-
		-sr-e-	*38-11s*	Rep, ro	Sup	ND	Sup	-	-
		-sr-	*38-13s*	Rep, ro	Sup	ND	Sup	ND	-
		sr-e	*40-4s*	Rep, ro	Sup	ND	ND	ND	-
		3rd	*40-6s*	Rep, ro	Sup	ND	Sup	ND	-
Other		*-th-st-*	*24-4s*	Viable	ND	ND	ND	Sup	-
		th-st-	*41-1s*	Viable	ND	ND	ND	ND	-
		st-cu	*27-17s*	Lethal	ND	Sup	ND	Wk sup	ND
		-cu-	*21-3s*	Viable	Sup	Sup	-	Sup	Sup
		-cu-	*39-1s*	Ro	Sup	ND	-	Sup	-
		cu-sr	*23-5s*	Lethal	Sup	Sup	Wk sup	Wk sup	Wk sup
		-sr-	*22-6s*	Wv	Sup	ND	Sup	Sup	-
		-sr-	*24-8s*	Viable	Sup	ND	Wk sup	Sup	-
		-sr-	*30-4s*	Viable	ND	ND	ND	ND	-
		-sr-	*41-4s*	Ro	ND	ND	ND	ND	-
		sr-e	*24-2s*	Rep, ro	Sup	ND	Sup	Wk sup	-
		sr-e	*28-7s*	Rep, ro	Sup	ND	Sup	Sup	-
		sr-e	*40-5s*	Lethal	Sup	Sup	-	Wk sup	Sup
		sr-e	*41-2s*	Lethal	Sup	Sup	Sup	ND	Sup
		sr-e	*41-6s*	Ro	Sup	ND	Sup	ND	-
		sr-e	*41-7s*	Lethal	Sup	Sup	Sup	ND	Sup
		sr-e	*43-1s*	Wv	ND	ND	ND	ND	-
		sr-e	*43-5s*	Rep, ro	ND	ND	ND	ND	-

Groups are named for the known gene or previously identified complementation group to which they correspond. *Su(GMRhid)2A*, *Su(GMRhid)3A*, and *Su(GMRhid)3B* are complementation groups that have not been assigned to a previously characterized gene. The group named "other" consists of mutants that did not fall into any complementation group and that were mapped roughly by meiotic recombination relative to the markers *th st cu sr e ca*; *-th-st-*, *-cu-*, and *–sr-* indicate that the dominant modifier mutation maps roughly around the designated markers and may be located on either side, *st-cu*, *cu-sr*, and *sr-e* indicate that the modifier maps between the designated markers. Rep, reduced eye pigmentation; Ro, rough eye; Wv, extra wing veins; Wk, weak; Sup, suppressor; Enh, enhancer; -, no effect; ND, not done.

Figure 4. Suppression and enhancement of *GMRrpr* and *GMRhid* eye phenotypes by modifier mutations. For the *GMRrpr* mutagenesis screen, we used a moderate reduced, rough eye phenotype like that in (A). Suppressor mutations improved the appearance of the eye, making it larger, smoother, and more organized (compare B to A) while enhancers worsened the appearance of the eye, making it smaller and devoid of structure (compare C to A). For the *GMRhid* screen, we used the severe phenotype shown in (D). Suppressor mutations restore some eye structure (compare E and F to D). Genotypes are as follows- (A) *GMRrpr 34 CyO/Sco*; +/+; (B) *GMRrpr 34 CyO* /+; *6-3s/TM3*; (C) *GMRrpr 34 CyO* /+; *11-3e/CxD*; (D) *GMRhid 10*/+; *TM3*/+; (E) *GMRhid 10*/+; *23-15s/TM3*; (F) *GMRhid 10*/+; *23-8s/TM3*.

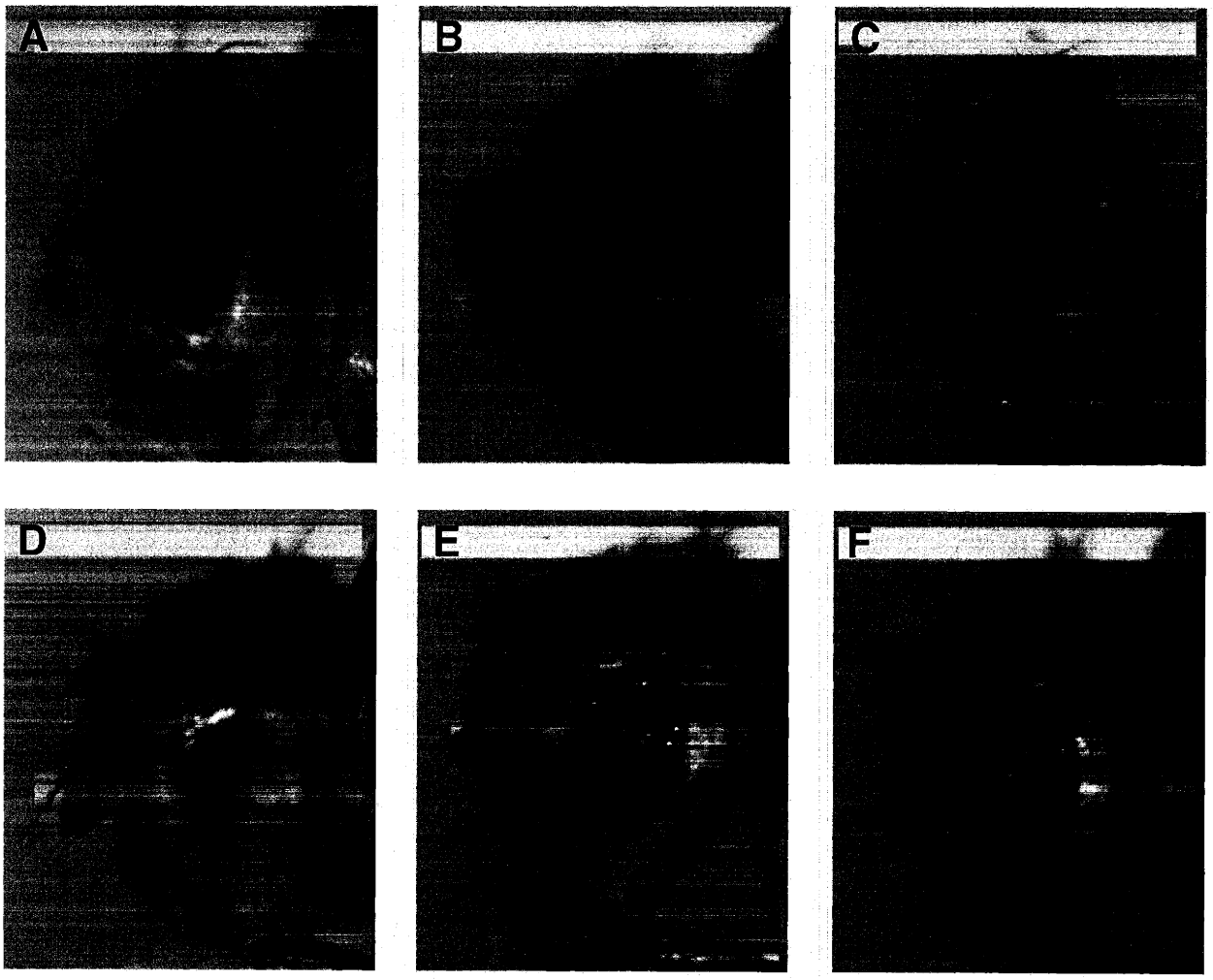
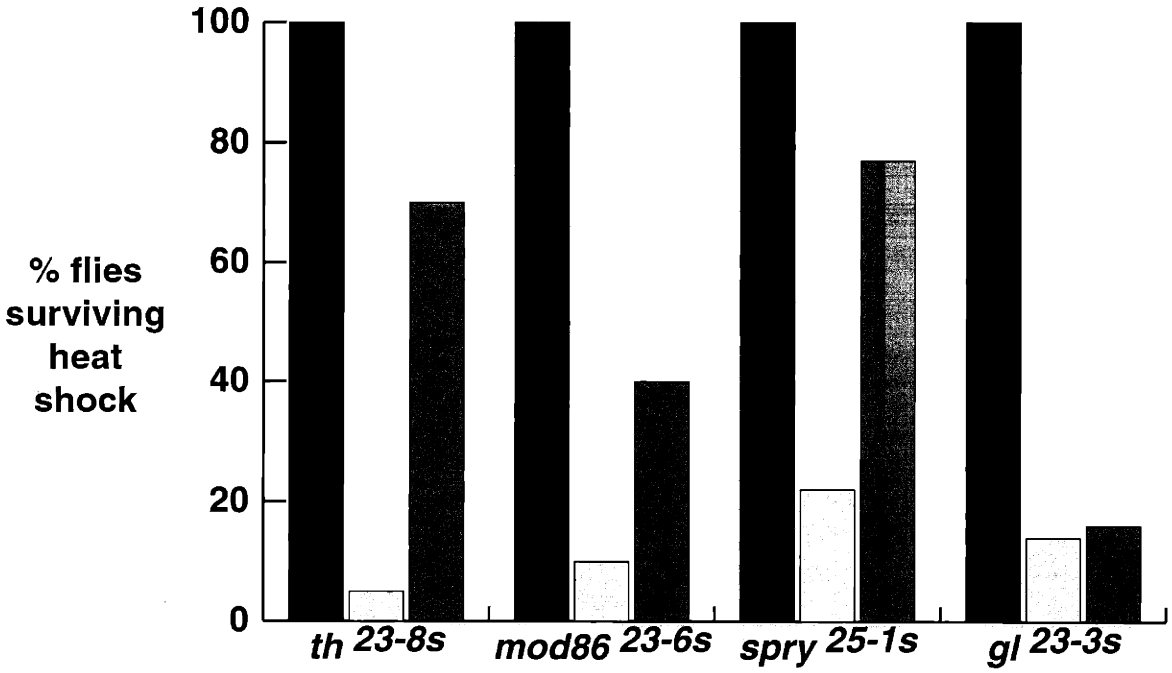

A
B
C
D
E
F

Figure 5. Dominant suppression of *hshid* induced lethality and *vgGal4, UAShid* induced wing phenotypes.

(A) Global expression of *hid* via a *hshid* transgene during the 1st instar larval stage results in significant organismal lethality. In the examples here, *hshid/+* females were crossed to *suppressor/TM3* males and the resulting progeny were subjected to a 15 minute heat shock. Twenty percent or less of *hshid/TM3* animals (light gray bars) survive heat shock compared to *+/TM3* siblings (black bars). With three of the mutants shown, *th* $^{23\text{-}8s}$, *mod86* $^{23\text{-}6s}$, and *spry* $^{25\text{-}1s}$, we observed increased survival of hs*hid*/suppressor progeny (dark gray bars) following heat shock. On the other hand, the mutation in *glass*, *gl* $^{23\text{-}3s}$, which suppresses the *GMRhid* phenotype by decreasing expression from the *GMR* transgene, has no effect on *hshid* induced lethality.

(B) Expression of *hid* in the developing wing in *vgGal4, UAShid* transgenic flies results in complete ablation of the wing (A) when raised at 25°C and in partial deletion of the wing (B) when raised at 18°C. Suppressors restore significant amounts of wing tissue (compare C to A and D to B). Genotypes are as follows- (A) *vgGal4, UAShid/+*; *+/+* raised at 25°C; (B) *vgGal4, UAShid/+*; *+/+* raised at 18°C; (C) *vgGal4, UAShid/+*; *45-2s/+* raised at 25°C; *vgGal4, UAShid/+*; *23-6s/+* raised at 18°C.

A


100
80
60
40
20
0
% flies surviving heat shock
th 23-8s
mod86 23-6s
spry 25-1s
gl 23-3s

B

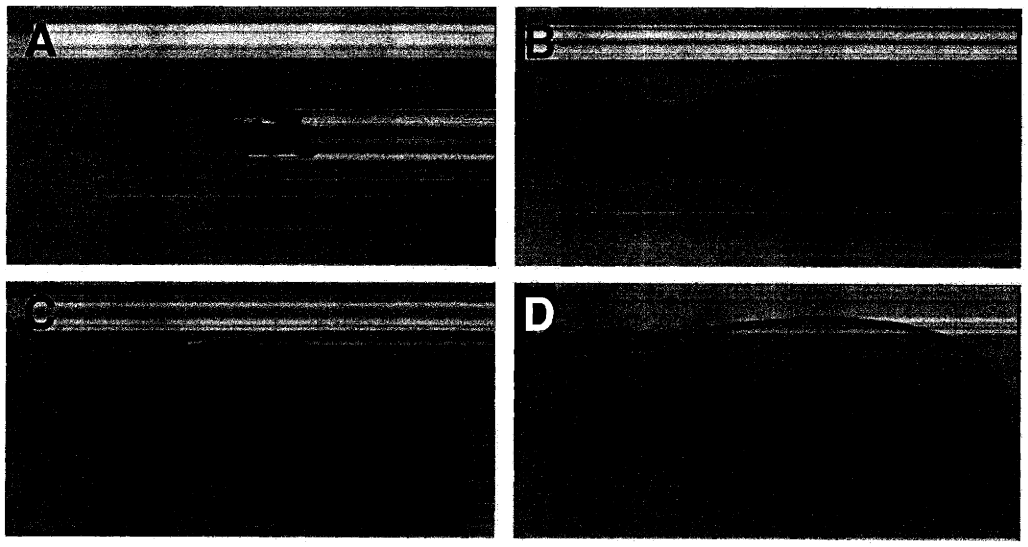
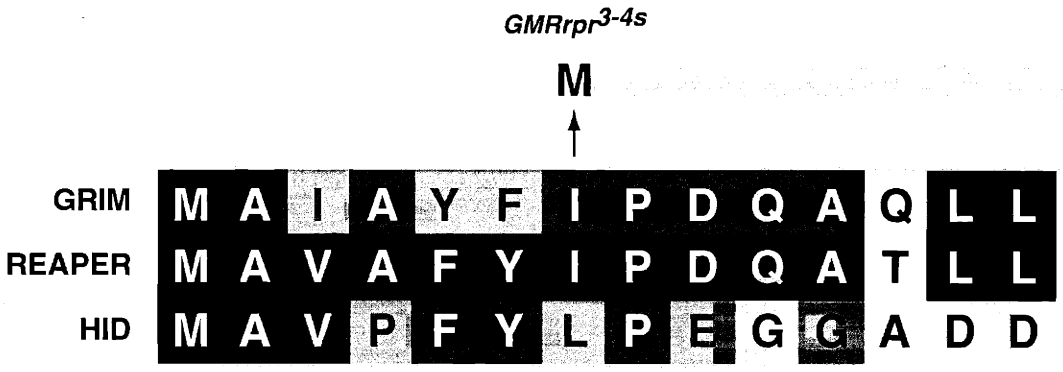

A
B
C
D

Figure 6. The mutation *GMRrpr* $^{3\text{-}4s}$ lies in the conserved N-terminus of Rpr and restores eye morphology to nearly wildtype levels.

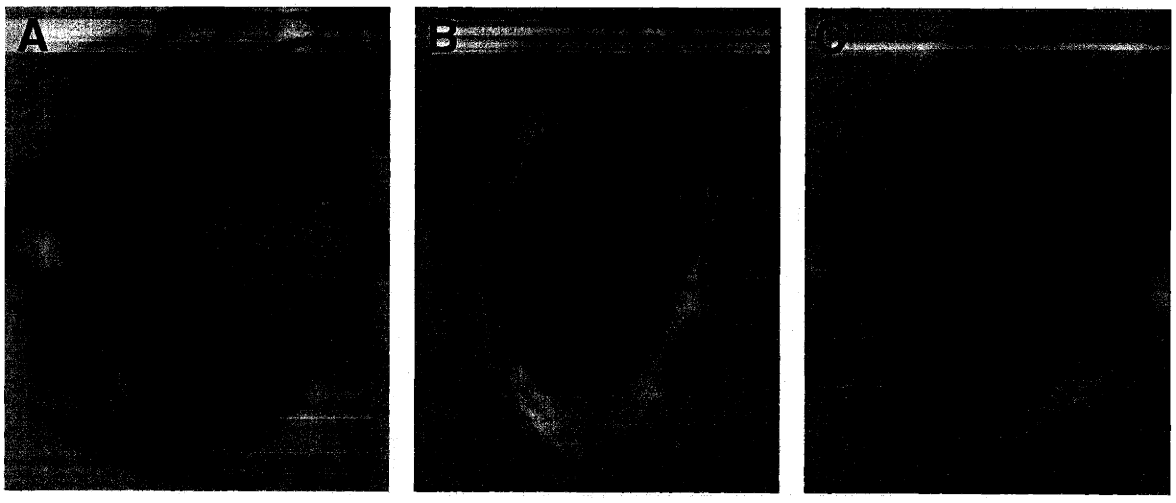
(A) Alignment of the N-terminal 15 amino acids of Rpr, Hid, and Grim taken from Chen et al. The mutation *GMRrpr* $^{3\text{-}4s}$ is predicted to result in the conversion of the isoleucine at residue 7 to methionine.

(B) *GMRrpr* $^{3\text{-}4s}$ causes a very mild phenotype as compared to *GMRrpr* ^{WT} (compare C to B). Genotypes are as follows- (A) Canton-S; (B) *GMRrpr* ^{WT} *81/GMRrpr* ^{WT} *81*; (C) *GMRrpr* $^{3\text{-}4s}$ *81/GMRrpr* $^{3\text{-}4s}$ *81*.

A


GMRrpr3-4s
M
GRIM M A I A Y F I P D Q A Q L L
REAPER M A V A F Y I P D Q A T L L
HID M A V P F Y L P E G G A D D

B


A
B
C

Chapter 3.

The *Drosophila* Gene *hid* Is a Direct Molecular Target of Ras-Dependent Survival Signaling

This chapter was previously published as Andreas Bergmann, Julie Agapite, Kimberly McCall, and Hermann Steller (1998) Cell 95, 331-341. My contribution to this chapter was the discovery that two suppressor of GMR*hid* complementation groups were allelic to *sprouty* and *Gap1*, negative regulators of Ras signaling.

Abstract

Extracellular growth factors are required for the survival of most animal cells. They often signal through the activation of the Ras pathway. However, the molecular mechanisms by which Ras signaling inhibits the intrinsic cell death machinery are not well understood. Here, we present evidence that in *Drosophila*, activation of the Ras pathway specifically inhibits the proapoptotic activity of the gene *head involution defective* (*hid*). By using transgenic animals and cultured cells, we show that MAPK phosphorylation sites in Hid are critical for this response. These findings define a novel mechanism by which growth factor signaling directly inactivates a critical component of the intrinsic cell death machinery. These studies provide further insights into the function of *ras* as an oncogene.

Introduction

The development and survival of multicellular organisms depend on the recruitment of a large number of different cell types into tissues and organs. A common principle for the development of tissues and organs appears to be the initial generation of an excess of cells prior to cell fate determination, after which surplus cells are removed by cell death. Physiological cell death occurs primarily through an evolutionarily conserved form of cell suicide called apoptosis. After development is complete, the survival of the organism depends largely on the maintenance and renewal of these cell types.

Apoptosis is regulated by a variety of extracellular and intracellular signals. In most tissues, cell survival is dependent on the constant supply of survival signals provided by neighboring cells and the extracellular matrix (Raff, 1992; Barres et al., 1993; Raff et al., 1993). Cells isolated in culture will undergo apoptosis in the absence of exogenous survival factors. In many cases, this form of cell death does not require protein synthesis, indicating that in these cells an intrinsic cell suicide program is present that operates by default unless active survival signaling suppresses it.

A number of peptide factors including the neurotrophins, insulin-like growth factor 1 (IGF-1), fibroblast growth factor (FGF), and epidermal growth factor (EGF) promote cell survival by suppressing the intrinsic cell death program (Raff et al., 1993; Gardner and Johnson, 1996; Parrizas et al., 1997; Yamada et al., 1997). The mechanisms by which survival factors inactivate the intrinsic cell death program are currently the subject of intensive investigation. The growth factors listed above bind to and activate receptor tyrosine kinases (RTKs) at the cell surface, which in turn stimulate the antiapoptotic activity of the proto-oncogene *ras* (reviewed in Downward, 1998). Ras controls the activity of a number of effector pathways, two of which result in activation of protein kinases known to mediate its antiapoptotic effect: the mitogen-activated protein kinase p42/44 (MAPK) of the ERK-type (extracellular signal-related kinase) via Raf (Xia et al., 1995; Gardner and Johnson, 1996; Parrizas et al., 1997) and the Akt kinase via Phosphoinositide 3-kinase (PI3-K; Yao and Cooper, 1995). Recently, it has been shown that activation of the antiapoptotic PI3-K/Akt-kinase pathway leads to phosphorylation of

Bad, a proapoptotic member of the Bcl-2 family, resulting in its binding to 14–3–3 as an inactive complex (Datta et al., 1997; del Peso et al., 1997). Activation of the Erk-type MAPK has been shown to be required to protect PC-12 cells from apoptosis induced by NGF withdrawal (Xia et al., 1995; Parrizas et al., 1997). However, a direct mechanistic link between the Raf/MAPK survival pathway and the cell death machinery has not been demonstrated thus far.

Molecular genetic analysis in the fruit fly *Drosophila melanogaster* might provide new insights into understanding the regulation of apoptosis by survival signaling pathways. Just like in vertebrates, large numbers of cells die during development of *Drosophila* (Abrams et al., 1993; Steller, 1995), and since many developmental mechanisms are conserved during evolution, it is likely that critical lessons learned by examination of survival signaling pathways in *Drosophila* will contribute significantly to our understanding of vertebrate survival signaling.

There is a *Drosophila* homolog of mammalian *N-ras*, *K-ras*, and *H-ras* termed Ras1 (Simon et al., 1991), and the *Drosophila* homolog of MAPK is encoded by the *rolled* (*rl*) locus (Biggs et al., 1994). The role Ras1/MAPK signaling plays in regulating cell proliferation and cell differentiation has been well established genetically (reviewed in Wassarman and Therrien, 1997). Recently, an important antiapoptotic function of Ras1 in *Drosophila* was revealed in cell ablation studies (Miller and Cagan, 1998) as well as by expressing genes that negatively regulate the Ras1 pathway in postmitotic cells (Sawamoto et al., 1998).

In *Drosophila*, molecular analysis has led to the isolation of three novel cell death genes, *reaper*, *head involution defective* (*hid*), and *grim*, which all appear to integrate different signals regulating apoptosis (White et al., 1994; Grether et al., 1995; Chen et al., 1996). Embryos homozygous mutant for these genes completely lack apoptosis (White et al., 1994; reviewed in McCall and Steller, 1997). When ectopically expressed, they induce apoptosis by activating a caspase pathway (Grether et al., 1995; Hay et al., 1995; Chen et al., 1996; White et al., 1996). The *reaper* and *grim* genes appear to be specifically expressed only in cells that are doomed to die (White et al., 1994; Chen et al., 1996; Robinow et al., 1997). In contrast, *hid* is expressed in many

cells that live as well as in cells that undergo apoptosis (Grether et al., 1995). This observation suggests that efficient posttranslational survival mechanisms operate in these cells to protect them from *hid*-induced apoptosis.

Here, the strong eye ablation phenotype caused by expressing *hid* under the control of an eye-specific promoter was used to perform a genetic screen aimed to identify components that regulate and mediate Hid activity. Mutations in genes that regulate the EGF receptor (EGFR)/Ras1 pathway were recovered as strong suppressors of Hid-induced apoptosis. The survival effect of the EGFR/Ras1 pathway is specific for Hid-induced apoptosis since neither Reaper- nor Grim-induced apoptosis is affected by the EGFR/Ras1 pathway. We show further in vivo and in cultured cells that the Ras1 pathway inhibits Hid activity apparently by direct phosphorylation of Hid by MAPK. We conclude that the *hid* gene in *Drosophila* provides a mechanistic link between the survival activity of Ras1 and the apoptotic machinery.

Results

Ectopic expression of *hid* under the control of the eye-specific glass multimer reporter construct (pGMR, Hay et al., 1994; construct designated *GMR-hid*) that drives expression in virtually all cells of the developing eye, beginning at the onset of differentiation in the morphogenetic furrow (Ellis et al., 1993), results in eyes that are severely reduced in size and devoid of most normal ommatidial morphology (Figure 1B; Grether et al., 1995). The severity of this eye ablation phenotype is dosage-sensitive; that is, flies carrying two copies of the *GMR-hid* transgene have significantly smaller eyes than flies carrying only one copy (data not shown). The correlation between the degree of *hid* activity and the strength of the induced phenotype suggests that a 50% reduction in the dose of a gene involved in *hid*-mediated apoptosis should result in the visible modification of the eye phenotype caused by *GMR-hid*. Therefore, using the sensitized *GMR-hid* background a genetic F1 screen was performed to isolate mutations in genes that dominantly suppress the *GMR-hid*-induced eye ablation phenotype. A similar approach has been highly successful for defining a genetic pathway for R7 cell fate determination in the *Drosophila* eye (Simon et al., 1991;

Dickson et al., 1996; Karim et al., 1996). Dominant suppressors were scored by looking for enlarged eye size compared to the unmodified *GMR-hid* phenotype (see Figure 1 for examples) and are expected to carry mutations in genes that are positively required for Hid activity. In this way, about 300,000 mutagenized F1 progeny were screened and a total of 120 dominant suppressors was isolated (J. A., K. M., and H. S., unpublished data).

Mutations in Components of the EGFR/Ras/MAPK Pathway Were Recovered as Dominant Modifiers in the *GMR-hid* Screen

Among known genes, five loss-of-function (lof) alleles of each *gap1* and *sprouty* (*spry*) were recovered as strong suppressors of *GMR-hid* in the screen (Figure 1C and Figure 1D). *gap1*, encoding a GTPase activating protein, was originally identified as a negative regulator of R7 photoreceptor development (Gaul et al., 1992) and appears to function by stimulating the GTPase activity of Ras1 causing Ras1 to hydrolyze GTP to GDP and thereby returning it to its inactive conformation (see Figure 1F). *Sprouty* was originally identified as an inhibitor of tracheal branching by antagonizing the *Drosophila* FGF RTK pathway and encodes a novel, presumably secreted polypeptide (Hacohen et al., 1998). Both genes are believed to negatively regulate RTK/Ras1 signaling (Figure 1F). Another gene, *argos* (*arg*), known to negatively regulate EGF receptor (EGFR) signaling (Freeman et al., 1992 ; Okano et al., 1992), was also found to suppress the *GMR-hid*-induced eye ablation phenotype (Figure 1E).

The wild-type function of the genes *gap1*, *spry*, and *arg* is required to inhibit EGFR/Ras1 signaling. Mutations in any one of these genes increase the signaling strength of the EGFR/Ras1 pathway. Thus, recovery of mutants in these genes as potent suppressors of the *GMR-hid*-induced eye phenotype indicates that the EGFR/Ras1-signaling pathway has an antiapoptotic effect by inhibiting *hid* activity. To test this notion, we studied the consequence of both lof and gain-of-function (gof) mutants of components of the Ras1 pathway on *GMR-hid*. Two different *GMR-hid* transgenic lines were used in this analysis. *GMR-hid* line 1M (*GMR-hid1M*) causes a mild eye ablation phenotype (Figure 2A) and was used to score for enhancement by

Ras1 pathway mutants. *GMR-hid* line 10 (*GMR-hid10*) causes a strong eye ablation phenotype (Figure 2G) and was used to score for suppression by Ras1 pathway mutants.

The results obtained are consistent with our previous findings. Reduction of Ras1 pathway activity leads to enhancement of *GMR-hid*-induced killing activity. Lof alleles of the *EGF receptor*, *ras1*, *raf*, and *rolled* (*rl*), the *Drosophila* MAPK homolog (referred to as *rl*/MAPK), enhance *GMR-hid1M*-induced apoptosis (Figure 2). The strongest enhancement of *GMR-hid1M*, however, was caused by a dominant negative allele of *ras1*, *ras1N17*, placed under eye-specific *sevenless* promoter control (*sev-Ras1N17*; Karim et al., 1996; Figure 2D).

The opposite effect was observed when gof alleles of *EGFR*, *ras1*, *raf*, and *rl*/MAPK were tested against *GMR-hid10*. The gof alleles of both Ras1 and Raf are transgenes that were placed under control of the eye-specific promoter of the *sevenless* gene (*sev-Ras1V12*, *sevRaftorso*). Their gof character was determined by the ability to induce supernumerary R7 cells in the absence of RTK signaling (Dickson et al., 1992 ; Fortini et al., 1992). The *sev-Ras1V12* transgenes contain a valine for glycine substitution at residue 12 that renders Ras1 constitutively active and bypasses the requirement for RTK activation (Fortini et al., 1992). Two independent *sev-Ras1V12* transformants, designated CR2 and T2B (Karim et al., 1996), showed very strong suppression of the *GMR-hid10*-induced eye phenotype (Figure 2I and Figure 2K). Activating Raf in *sev-Raftorso* is achieved by targeting Raf to the membrane by fusing the kinase domain of Raf to the transmembrane and extracellular domain of the RTK torso (Dickson et al., 1992). The *sev-Raftorso* transgene tested in this study shows a strong suppression of *GMR-hid10* (Figure 2L).

Whereas the gof alleles of Ras1 and Raf are transgenes, the gof alleles of the EGFR (*ElpE1*) and of *rl*/MAPK (*rlSem*, Sem-Sevenmaker) are mutations in the endogenous genes (Baker and Rubin, 1989; Brunner et al., 1994a). For instance, the RTK independent activation by the *Sevenmaker* allele of *rl*/MAPK is caused by a single amino acid substitution (Asp to Asn at position 334; Brunner et al., 1994a). Thus, it appears that the strong suppression of *GMR-hid10* by *rlSem*/MAPK (Figure 2M) is not

caused by a simple overexpression effect but rather by specific activation of the *rlSem*/MAPK gene product. We also saw strong suppression of *GMR-hid10*-induced apoptosis by expression of *secreted spitz*, the activated form of the EGF ligand encoded by the *spitz* gene (data not shown; Schweitzer et al., 1995).

In the *GMR-hid* suppression assay, we detected a slightly stronger suppression of *hid*-induced apoptosis by the *sev-Ras1V12* transgenes compared to the suppression obtained by *rlSem*/MAPK (compare Figure 2I and Figure 2K with Figure 2M). This suggests that in addition to MAPK signaling, other Ras1-dependent survival mechanisms may operate to inactivate *hid* activity. Active, GTP-bound Ras transduces signals through multiple intracellular targets including (among others) Raf (at the apex of the MAPK pathway), the p110 catalytic subunit of PI3-Kinase (activating the Akt-1 kinase), and Ral.GDS, the exchange factor for Ral.GTPases (Figure 3A; see Introduction). We investigated the relative contributions made by each of these effectors on suppression of *hid*-induced apoptosis. We used partial lof mutants located in the Ras effector loop that each activate only one of the downstream pathways mentioned above. Each mutant resides in a constitutively activated Ras1V12 background, such that the mutant Ras1V12S35 interacts with Raf but fails to interact with PI3-K or Ral.GDS; Ras1V12G37 interacts with Ral.GDS, but not with Raf or PI3-K; and the Ras1V12C40 mutant interacts with PI3-K, but not with Raf or Ral.GDS (Figure 3A; White et al., 1995; Rodriguez-Viciana et al., 1997; Karim and Rubin, 1998). We used the GAL4-UAS system (Brand and Perrimon, 1993) to express wild-type Ras1 and the Ras1 mutants under *sev* promoter control (*sev-GAL4*) specifically in eye imaginal discs. This analysis showed that the Raf/MAPK branch provides the major suppression of *hid*-induced apoptosis (Figure 3D). The Ral.GDS effector pathway failed to contribute to the survival activity of Ras1 (Figure 3E). A moderate suppression of *hid*-induced apoptosis was provided by the PI3-K/Akt-kinase branch (Figure 3F), consistent with its previously reported antiapoptotic function (Yao and Cooper, 1995; Kennedy et al., 1997; Staveley et al., 1998). Thus, it appears that Ras1 mediates its survival activity largely through the MAPK pathway, supplemented by a minor component of the PI3-K/Akt-kinase branch.

To further characterize the survival activity of the EGFR/Ras1/MAPK pathway on Hid-induced cell death, we sought an alternative assay in a different developmental context. Induction of *hid* under control of a heat shock promoter (*hs-hid*) very efficiently causes organismal lethality (Grether et al., 1995). Only about 2% of the animals containing a *hs-hid* transgene survive to adulthood compared to control (non-*hs-hid*) flies after they received a 30 min heat shock at 37°C during the first instar larval stage (Figure 4, see Experimental Procedures for details). A 40 min heat shock under the same conditions was sufficient to kill all *hs-hid*-containing animals.

However, in a heterozygous mutant background for the genes *gap1*, *spry*, and *arg*, the *hs-hid*-induced lethality is strongly reduced such that approximately 20%–40% of *hs-hid* transgenic animals survive even after a 60 min heat shock at 37°C during first instar larval stage (Figure 4). An even more striking rescue is observed if the *hs-hid* suppression assay is performed with the gof *rlSem*/MAPK allele (see above; Brunner et al., 1994a). After 60 min of heat shock 70%–80% of *hs-hid* transgenic flies survive (Figure 4)

In summary, the observed genetic interaction strongly suggests that activation of the EGFR/Ras1/MAPK pathway inactivates the death-inducing ability of the proapoptotic gene *hid*.

The EGFR/Ras1/MAPK Pathway Acts Specifically on Hid and Does Not Influence Reaper- and Grim-Induced Killing

We also studied the influence of the EGFR/Ras1/MAPK pathway on the other two known death effector genes in *Drosophila*, *reaper* and *grim*. In Figure 5, we compare the enhancing and suppressing effects of the dominant negative *sev-Ras1N17* allele and the gof *sev-Ras1V12* allele, respectively, on *GMR-reaper*- and *GMR-grim*-induced eye phenotypes. These two Ras1 alleles gave the strongest effects on *GMR-hid*-induced apoptosis (Figure 2D, Figure 2I, and Figure 2K). However, the eye ablation phenotypes of both *GMR-reaper* and *GMR-grim* are not significantly affected by the Ras1 mutants (Figure 5). This finding is further confirmed by analysis of other pathway mutants (data not shown). Thus, in summary, the antiapoptotic survival activity of the

Ras1 pathway is predominantly mediated by specific inactivation of the death effector gene *hid*. In the following, we address the molecular mechanisms of this effect.

Alteration of MAPK Sites of Hid Blocks the Survival Abilities of Ras1V12 and RlSem/MAPK in SL2 Cells

Since in both the *GMR-hid* and the *hs-hid* suppression assays, *hid* is placed under heterologous promoter control, we assumed that the EGFR/Ras1/MAPK pathway suppresses *hid* activity at a posttranslational level. The presence of five MAPK phosphorylation consensus sites in the *hid* protein (Figure 6) suggested the possibility that Hid is a direct target of MAPK phosphorylation. Three known phosphorylation targets of MAPK in *Drosophila* are Yan, which contains eight phosphorylation consensus sites (Rebay and Rubin, 1995), D-Jun (three consensus sites, Peverali et al., 1996), and Pointed P2 (one such sequence, Brunner et al., 1994b). Whereas D-Jun and Pointed P2 are activated via phosphorylation by MAPK, the Yan protein is inactivated in response to MAPK signaling (Brunner et al., 1994b; Rebay and Rubin, 1995; Peverali et al., 1996). By analogy to Yan, we considered that phosphorylation of Hid protein by MAPK leads to its inactivation and is the cause for the observed genetic effects.

To investigate this hypothesis, we used in vitro mutagenesis to replace the phospho-acceptor residues of the consensus sites with a nonphosphorylatable amino acid, alanine. If downregulation of *hid* activity occurs via phosphorylation by MAPK, then removal of the phosphorylation sites should result in a mutant form of Hid that fails to respond to Ras1/MAPK signaling. We constructed two different MAPK deficient mutants of *hid*. In *hidAla5* all five Ser/Thr were changed to Ala. In *hidAla3* only Thr-148, Thr-180, and Ser-251 were changed to Ala (indicated with asterisks in Figure 6).

First, we tested the biological activity of these mutant constructs in a rapid cell culture assay. Schneider line 2 cells (SL2), a *Drosophila* cell line (Schneider, 1972), were transiently transfected with *hidwt*, *hidAla3*, and *hidAla5*. The genes were expressed under control of the constitutively active *ie1* promoter from baculovirus (Jarvis et al., 1996). Transfection of the mutant constructs resulted in much stronger killing activity than transfection of the wild-type construct (Figure 7A). Therefore, the two

hid mutants behave as gof alleles of *hid*. To explain the gof characteristics of the *hid* mutants in SL2 cells we assume that they are not responsive to Ras1/MAPK signaling due to the change of the MAPK phosphorylation sites of Hid. The components of the Ras1 pathway are present in SL2 cells as shown by transcriptional assays of other target genes of the pathway like *pointed* and *yan* (O'Neill et al., 1994; Rebay and Rubin, 1995). Survival factors provided by the medium may trigger activation of the Ras1 pathway leading to inactivation of Hidwt. The Hid mutants, however, are not responsive to MAPK signaling anymore and thus behave as more efficient inducers of cell death in these cells.

To test the survival requirement of Ras1/MAPK on Hid-induced cell death, we transiently cotransfected the *hid* constructs with cDNAs encoding Ras1V12 and RlSem/MAPK under *ie1* promoter control into Schneider SL2 cells. Consistent with the genetic findings, both Ras1V12 and RlSem/MAPK suppress cell death induced by Hidwt in SL2 cells (Figure 7B and Figure 7C). The survival rescue provided by Ras1V12 is quantitatively much stronger than that provided by RlSem/MAPK. Increasing concentrations of Ras1V12 in this assay resulted in an up to 5-fold increase in the number of surviving SL2 cells, whereas RlSem/MAPK reduces the killing activity of Hidwt only about 2-fold (Figure 7B and Figure 7C). This difference in the survival abilities of Ras1 and Rl/MAPK has previously been observed in the *GMR-hid* suppression assay (Figure 2) and might reflect activation of the PI3-K/Akt effector branch of Ras1 as seen in Figure 3.

In the cotransfection experiment performed with the mutants HidAla3 and HidAla5, the survival ability of Ras1V12 on the mutants is significantly weaker as compared to Hidwt. The number of surviving SL2 cells is increased only 2.5-fold in the HidAla3/5 experiment compared to a 5-fold increase in the Hidwt experiment (Figure 7B). The weak rescue seen in this experiment might be the result of activation of the PI3-K/Akt-kinase effector branch (see Figure 3). However, alteration of the MAPK sites of Hid completely abrogates the survival ability of RlSem/MAPK on Hid-induced cell death in this assay (Figure 7C). The mutants HidAla3 and HidAla5 appear insensitive to MAPK signaling. These results indicate that changing the Ser/Thr residues in the MAPK

sites of Hid is sufficient to render the *hid* protein insensitive to RI/MAPK signaling under these assay conditions and is consistent with our assumption that the MAPK sites are critical for the observed survival activity of Ras1/MAPK signaling on *hid*-induced apoptosis.

Transgenic Analysis of *GMR-hidAla3* and *GMR-hidAla5*

In order to study the *hidAla3* and *hidAla5* mutants in vivo we generated GMR based constructs, designated *GMR-hidAla3* and *GMR-hidAla5*, and established transgenic lines using P element mediated transformation. The genetic analysis performed with *GMR-hidAla3* and *GMR-hidAla5* is consistent with the findings in SL2 cells and strongly supports a model according to which the activity of the cell death regulator *hid* is modulated by MAPK signaling in vivo.

In total, six *GMR-hidAla3* and four *GMR-hidAla5* transgenic lines were obtained. While the strength of the eye ablation phenotype caused by *GMR-hidwt* ranges from mild to severe defects (compare *GMR-hidwt-1M* with *GMR-hidwt-10* in Figure 2), all of the *GMR-hidAla3* and *GMR-hidAla5* lines produce a severe eye ablation phenotype (Figure 8), with some lines completely lacking eye structures (data not shown). These strong phenotypes are presumably caused by a failure of Ras1/MAPK signaling to suppress the MAPK deficient *GMR-hid* mutants, since Ras1 signaling plays an essential role during eye development (reviewed in Freeman, 1997).

To further confirm this notion, we analyzed the effect of the gof *rlSem*/MAPK allele on *GMR-hidAla3* and *GMR-hidAla5*. Based on the results obtained in SL2 cells (see Figure 7) we did not expect to detect a suppression of the *GMR-hidAla3*- and *GMR-hidAla5*-induced eye phenotype by activated forms of MAPK. Several lines were tested and gave identical results. Flies expressing *GMR-hidAla3* and *GMR-hidAla5* in a *rlSem*/MAPK mutant background show a mildly suppressed eye phenotype (Figure 8G and Figure 8M). The extent of this suppression is much weaker compared to the one obtained for *GMR-hidwt-10* (Figure 8B). Also, the *sev-Ras1V12* transgenes largely fail to suppress the eye ablation phenotype caused by the MAPK site deficient *GMR-hid* transformants (Figure 8H and Figure 8N). This result indicates that MAPK signaling

inactivates the cell death inducing activity of *hid*. However, the *GMR-hidAla3*- and *GMR-hidAla5*-induced eye phenotypes are still partially suppressed by activated forms of MAPK. This partial suppression might be caused by inactivation of the endogenous *hid* wild-type protein that is provided by the two genomic copies, which are widely expressed in the developing eye (Grether, 1994). Reduction of the endogenous *hid* gene dose by 50% (i.e., a heterozygous *hid* mutant background) resulted in weak suppression of the *GMR-hidwt*, *GMR-hidAla3*, and *GMR-hidAla5* eye phenotypes (Figure 8E, Figure 8K, and Figure 8P). Thus, the endogenous *hid* gene adds to the full *GMR-hid*-induced eye phenotype. Since its gene product is expected to be fully responsive to Ras1/MAPK signaling, it is likely that the weak suppression of *GMR-hidAla3* and *GMR-hidAla5* by activated forms of MAPK is caused by inactivation of the endogenous *hid* gene.

In control crosses, we tested transgenes of and mutations in specific cell death genes that are not involved in Ras1/MAPK signaling. The well-characterized gene *diap1* (*Drosophila* inhibitor of apoptosis protein) under GMR promoter control (*GMR-DIAP1*, Hay et al., 1995) suppressed the eye ablation phenotypes caused by *GMR-hidwt*, *GMR-hidAla3*, and *GMR-hidAla5* to a similar extent (Figure 8D, Figure 8I, and Figure 8O). Other control crosses included *GMR-p35*, a general inhibitor of apoptosis (Clem et al., 1991; Hay et al., 1994), dominant suppressors recovered in the *GMR-hid* suppressor screen, and mutations in *glass*, which encodes a transcription factor that activates transcription from the GMR promoter and is expected to influence GMR transgenes similarly. As with *GMR-DIAP1*, in these crosses, the eye ablation phenotypes caused by *GMR-hidwt*, *GMR-hidAla3*, and *GMR-hidAla5* are suppressed to a similar extent (data not shown). The findings in these control crosses further support the notion that the failure to suppress *GMR-hidAla3* and *GMR-hidAla5* by active MAPK is due to the lack of MAPK phospho-acceptor sites in Hid.

In summary, our mutational analysis provides strong evidence that the MAPK phosphorylation consensus sites of Hid are critical for the observed survival activity of the Ras1/MAPK pathway on Hid-induced apoptosis. Thus, it appears that active MAPK suppresses Hid by direct phosphorylation.

Discussion

Signaling via RTKs and the Ras/Raf/MAPK pathway has been implicated in the suppression of programmed cell death (reviewed in Downward, 1998). Recently, genetic evidence for the antiapoptotic function of the EGFR/Ras1/MAPK pathway in *Drosophila* has been provided (Miller and Cagan, 1998; Sawamoto et al., 1998). In this paper we reveal a molecular mechanism by which the EGFR/Ras1/MAPK pathway delivers an antiapoptotic signal via direct inhibition of a component of the intrinsic cell death machinery in *Drosophila*, *hid*.

hid is an important inducer of programmed cell death in *Drosophila* (Grether et al., 1995). *hid* mutant embryos have decreased levels of apoptosis and extra cells in the head. In ectopic expression studies, *hid* behaves as a very potent inducer of cell death (Grether et al., 1995; this study). However, the expression of the *hid* gene is not restricted to cells that are doomed to die; *hid* is also expressed in many cells that live (Grether et al., 1995). This observation, together with the efficient induction of apoptosis by *hid* in our assays, indicates that there must be very efficient posttranslational mechanisms present in these cells to protect them from *hid*-induced apoptosis.

Activation of the EGFR/Ras1/MAPK Pathway Delivers an Antiapoptotic Signal by Inhibition of *hid* Activity

In this paper we present genetic evidence that signaling via the EGFR/Ras1/MAPK pathway promotes survival by directly blocking *hid* from inducing apoptosis (Figure 9). It is striking that the survival activity of the EGFR/Ras1/MAPK pathway is specific for Hid, since we detected little or no effects on the other two known cell death regulators in *Drosophila*, *reaper* and *grim* (Figure 9). In our assays, *hid*-induced apoptosis, using either *GMR-hid* or *hs-hid*, is very efficiently blocked by mutations in genes that lead to overactivation of the EGFR/Ras1/MAPK pathway. This includes lof mutations in genes, which negatively regulate the pathway, such as *gap1*, *argos*, and *sprouty* (Figure 1), as well as gof mutations in components of the EGFR/Ras1/MAPK pathway such as *Elp*, *Ras1V12*, *Raftorso*, and *rlSem*/MAPK (Figure

2). In order to explain the observed genetic effects on *hid*-induced apoptosis by EGFR/Ras1/MAPK signaling, we propose a posttranslational inactivation mechanism of *hid* protein for three reasons. First, in our assays, *hid* is under control of heterologous promoters (GMR, hs, and ie1) such that a potential regulation at the level of the endogenous *hid* promoter cannot fully account for the observed genetic effects. However, such a mechanism might operate under different circumstances (see below). Second, since similar rescuing effects are not observed with *GMR-reaper* and *GMR-grim*, a transcriptional regulation at the level of the GMR promoter (and presumably the hs promoter) can be excluded as well. Third, there are five MAPK phosphorylation consensus sites present in the *hid* protein (Grether et al., 1995). In contrast, neither *reaper* nor *grim* proteins possess MAPK phosphorylation consensus sites (White et al., 1994; Chen et al., 1996). Our mutational analysis strongly suggests that the MAPK phosphorylation sites of Hid are critical for the response to EGFR/Ras1/MAPK signaling in both transgenic animals and cultured cells.

The EGFR/Ras1/MAPK signaling pathway has been implicated in controlling cell proliferation, cell differentiation, and cell death in both vertebrates and invertebrates (Wassarman and Therrien, 1997; Downward, 1998). Recently, Karim and Rubin, 1998 showed that ectopic expression of activated Ras1 (Ras1V12) during early imaginal disc development in *Drosophila* (during a stage when the imaginal discs are actively proliferating) induces ectopic cell proliferation and hyperplastic growth. Therefore, we were concerned that the observed survival activity of EGFR/Ras1/MAPK signaling on *hid*-induced apoptosis might actually be the result of a compensatory mitogenic effect of Ras1V12 rather than the result of a direct inhibition of the apoptosis inducing activity of *hid*. However, we excluded this possiblity for a number of reasons. First, a mitogenic response to the *sev-Ras1V12* transgenes used in this study has not been observed, presumably because the cells in which Ras1V12 is expressed are postmitotic (Karim and Rubin, 1998). Second, apoptosis induced by MAPK deficient mutants of *hid* is less well suppressed by Ras1 signaling. Third, if a mitogenic effect of the activated EGFR/Ras1/MAPK pathway accounts for the strong suppression of *GMR-hid*, then a similar effect should be detectable on *GMR-reaper*- and *GMR-grim*-induced apoptosis.

Finally, in control transfection experiments in SL2 cells, a potential mitogenic effect of Ras1V12 did not compensate for *hid*-induced cell death (data not shown). Thus, we conclude that the suppression of *hid*-induced apoptosis is the result of direct inhibition of the cell death–inducing activity of *hid* by the activated EGFR/Ras1/MAPK pathway.

We propose a model according to which the cell death–inducing activity of the Hid protein is specifically inactivated via phosphorylation of the *hid* protein by activated MAPK. The mutational analysis performed in transgenic animals and SL2 cells strongly supports such a mechanism. Unfortunately, in vitro kinase assays using recombinant Hid as a substrate have been hampered by its insolubility (unpublished data).

Our data show that phosphorylation of Hid by activated MAPK inhibits the cell killing activity of Hid, and we believe that this powerful control mechanism operates in most cells at all times (we tested different *Drosophila* tissues, developmental stages, and cultured cells). However, *hid* activity also appears to be regulated by the Ras1 pathway at the transcriptional level. Overexpression of *Ras1V12* during embryogenesis results in specific downregulation of *hid* mRNA expression (Kurada and White, 1998 [this issue of *Cell*]). Expression of *reaper*, *grim*, and *diap1* are not affected by this treatment. Thus, these findings suggest a regulation of *hid* activity by the Ras1 pathway at two different levels. We propose that in an acute response, *hid* protein is directly inactivated by phosphorylation via activated MAPK. This mechanism explains why the large number of cells that express *hid* are able to survive. In a second response, *hid* mRNA expression is downregulated by the Ras1 pathway (Kurada and White, 1998). In this way, the Ras1 pathway ensures that a cell that is selected to live will both inhibit existing *hid* protein pools and subsequently downregulate the transcription of *hid* and, thus, gain a safe distance from the "threshold of death." Taken together, both mechanisms can explain how cells that receive sufficient survival signals are stably selected to live.

Ras1-Dependent Inactivation of *hid*: Implications for Oncogenesis

Recent evidence suggests that the failure of cells to undergo apoptotic cell death might be involved in the pathogenesis of a variety of human diseases, including

cancer, autoimmune diseases, and viral infections (reviewed in Thompson, 1995). Mutational activation of *ras* oncogenes is associated with about 30% of all human tumors (Bos, 1989). An open question in cancer research is why transformation usually requires two or more cooperative oncogenic events to induce a neoplastic lesion. Harmful cells that have acquired genetic alterations that predispose them to uncontrolled cell proliferation are usually detected by the organism and subsequently removed by apoptosis. Thus, apoptosis appears to be a critical process that protects the organism from harmful cells. However, tumor cells as well as metastatic cells have a decreased ability to undergo apoptosis (Hoffman and Liebermann, 1994). The findings in this paper define a potential mechanism by which activated *ras* oncogenes decrease the susceptibility of cells to die by inactivation of a critical component of the intrinsic cell death machinery.

Although there are no mammalian homologs of *Drosophila hid* known to date, genetic and biochemical studies on *hid* might provide an important paradigm for the antiapoptotic function of *ras* oncogenes. In preliminary experiments, we found that *hid* kills mammalian cells very efficiently (N. Haining, A. B., and H. S., unpublished data), indicating that the mechanisms leading to *hid*-induced apoptosis might be conserved between vertebrates and invertebrates. Even if there is no *hid* homolog at the structural level, we propose that in mammals other cell death–inducing gene(s) exist that act as functional homologs to *hid* and that are similarly responsive to *ras*-induced inactivation. Thus, studies performed in *Drosophila* might provide new insights into the function of *ras* as an oncogene.

Experimental procedures

Fly Stocks

The following mutant and transgenic fly strains were used for phenotypical analysis and genetic interactions: *GMR-hid*, *hs-hid* 3, *hidWR+X1* (Grether et al., 1995), *GMR-hid10 sev-GAL4* (this study), *GMR-rpr* (White et al., 1996), *GMR-grim* (Chen et al., 1996), *GMR-DIAP1* 3–1 (Hay et al., 1995), *gap121-1s* and *spry28-4s* (this study) *argI7* (Freeman et al., 1992), *EGFR- = flbf2* (Nüsslein-Volhard et al., 1984), *rasDC40b*

and *raf11–29* (Hou et al., 1995), *rl10a* (Peverali et al., 1996), *ElpE1* (Baker and Rubin, 1989), *sev-Ras1V12* (Fortini et al., 1992), *sev-Ras1N17* (Karim et al., 1996), *sev-Raftorso* (Dickson et al., 1992), *rlSem*/MAPK (Brunner et al., 1994a), *UAS-Ras1+*, *UAS-Ras1V12S35*, *UAS-Ras1V12G37*, *UAS-Ras1V12C40* (Karim and Rubin, 1998). Flies carrying *GMR-hidAla3* and *GMR-hidAla5* were generated by P element–mediated transformation. The *GMR-hid10 sev-GAL4* line was obtained by meiotic recombination. All crosses were performed at 25°C.

hs-hid Suppression Assay

Offspring of crosses between *hs-hid*/TM3 and Sup/TM3 (Sup = *gap121-1s*, *spry28-4s*, *argI7*) were heat shocked at 37°C during first instar larval stage for 10, 20, 30, 40, 50, or 60 min. After recovery, the crosses were incubated at 25°C. After the flies eclosed, the ratio between *hs-hid*/Sup and Sup/TM3 animals was determined. For *rlSem*/MAPK, the procedure was similar except that the ratio between *rlSem*/+;*hshid*/+ and *rlSem*/+;+/TM3 was scored. In the control experiment, offspring of a cross between *hs-hid*/TM3 and +/+ animals were treated as described above, and the ratio between *hs-hid*/+ and +/TM3 animals was determined. The results presented in Figure 4 represent the average of three independently performed experiments.

Molecular Biology

In vitro mutagenesis of the MAPK phosphorylation consensus sites in *hid* was performed using PCR with specifically designed primers. Incorporation of the mutation was confirmed by sequencing. The constructs were subcloned into pGMR1 (Hay et al., 1994) for P element–mediated transformation and pIE1-3 (Novagen, Jarvis et al., 1996) for SL2 cell transfection experiments. The cDNAs encoding Ras1V12 and rlSem/MAPK were cloned into pIE1-3 using convenient restriction sites for cell transfections.

SL2 Cell Transient Transfection Experiments

SL2 cells (Schneider, 1972) were grown in Schneider's *Drosophila* Medium (GIBCO–BRL) supplemented with 10% NCS. In each experiment, 100 ng/ml of the

reporter plasmid pIE1-3-LacZ (kindly provided by Zhiwei Song) was transfected. Differences in the amount of tester plasmids were compensated for by the addition of empty vector pIE1-3. In three independent experiments, transfections were performed using the Cellfectin reagent according to the manufacturer's instructions (GIBCO–BRL) for 5 hr in serum-free medium in 24 well dishes in quadruplicates. Twenty four hours after transfection, cells were fixed and stained, and the number of surviving cells was determined.

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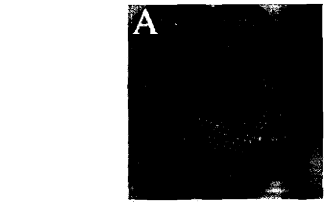
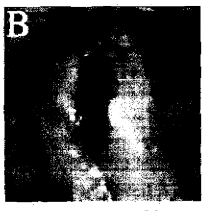
Figure 1. Mutations that Increase Ras1 Signaling Suppress Hid-Induced Apoptosis in the Compound Eye

gap1, *spry*, and *arg* encode genes that inhibit EGFR/Ras1 signaling. Mutations in these genes increase Ras1 signaling resulting in suppression of Hid-induced apoptosis. Flies in this and all other figures were incubated in parallel at 25°C throughout development. Compound eyes of females are shown. The genotypes of flies shown are indicated below each panel. All photographs were taken at the same magnification. (A) Wild-type. (B) Eye ablation phenotype caused by one copy of the *GMR-hid10* transgene. Note the strong reduction in eye size in comparison to A. (C–E) Dominant suppression of the *GMR-hid10*-induced eye phenotype by lof mutations in *gap121-1s*, *spry28-4s*, and *argI7*. (F) Schematic drawing of the EGFR/Ras1/MAPK signaling pathway and the relative position of the inhibitory genes *gap1*, *argos*, and *sprouty*. Arg and Spry are secreted polypeptides that inhibit EGFR activation. Gap1 promotes the GTPase activity of Ras1. Abbreviations used: EGF, epidermal growth factor; Drk, downstream of receptor kinase; Sos, Son of sevenless; Dsor, downstream suppressor of raf; MAPK, mitogen activated protein kinase; and MEK, MAPK-Erk kinase.

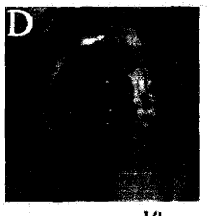


wild-type

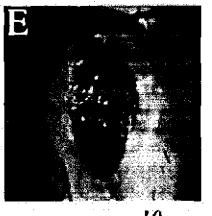


GMR-hid^{10}/+;
+/+

GMR-hid^{10}/+;
gap1/+



GMR-hid^{10}/+;
spry/+



GMR-hid^{10}/+;
arg/+

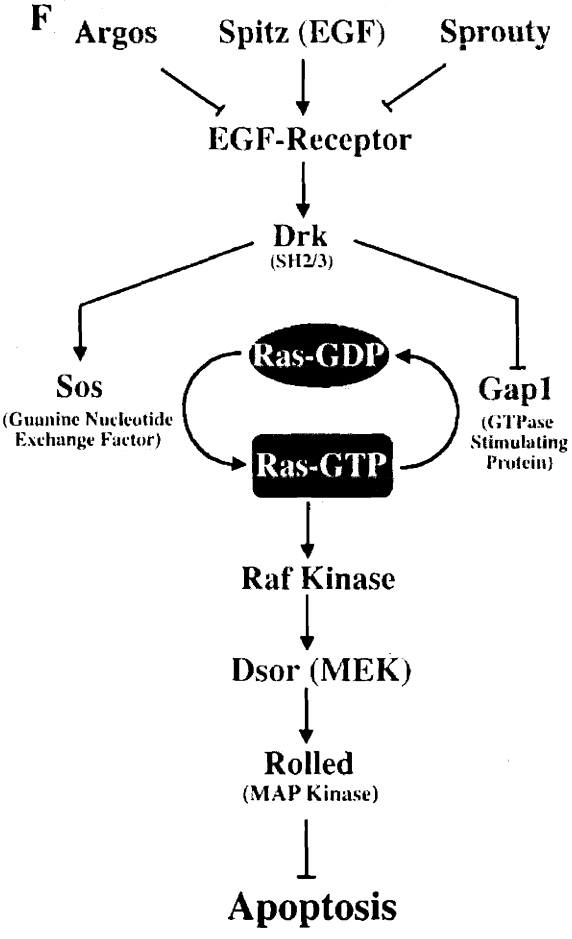


Figure 2. Genetic Interaction of EGFR/Ras1/MAPK Pathway Mutants with *GMR-hid*
The mild eye ablation phenotype of *GMR-hid1M* (A) was used to score for enhancement caused by lof mutants of *EGFR* (B), *ras1* (C), *raf* (E), and *rl*/MAPK (F) or caused by the dominant negative *sev-Ras1N17* transgene (D). Note the smaller eyes in (B)–(F) compared to the unmodified *GMR-hid1M* eye. The *sev-Ras1N17* transgene behaves as the strongest enhancer (D). The eye phenotypes of heterozygous *EGFR-*, *ras1-*, *raf-*, and *rl-*/MAPK flies alone are phenotypically wild-type (data not shown). The dominant negative *sev-Ras1N17* allele alone produces a mild rough eye phenotype as the result from the loss of R7 cells (Karim et al., 1996); the eye size, however, is not affected by *sev-Ras1N17*.

The strong eye ablation phenotype of *GMR-hid10* (G) was used to score for suppression caused by gof mutants and transgenes of *EGFR* (*ElpE1*, H), *sev-Ras1V12* (I and K), *sev-Raftorso* (L), and *rlSem*/MAPK. The strongest suppression is seen with the two *sev-Ras1V12* transgenes used (*CR2* and *T2B*). The genotypes of flies shown are indicated below each panel.

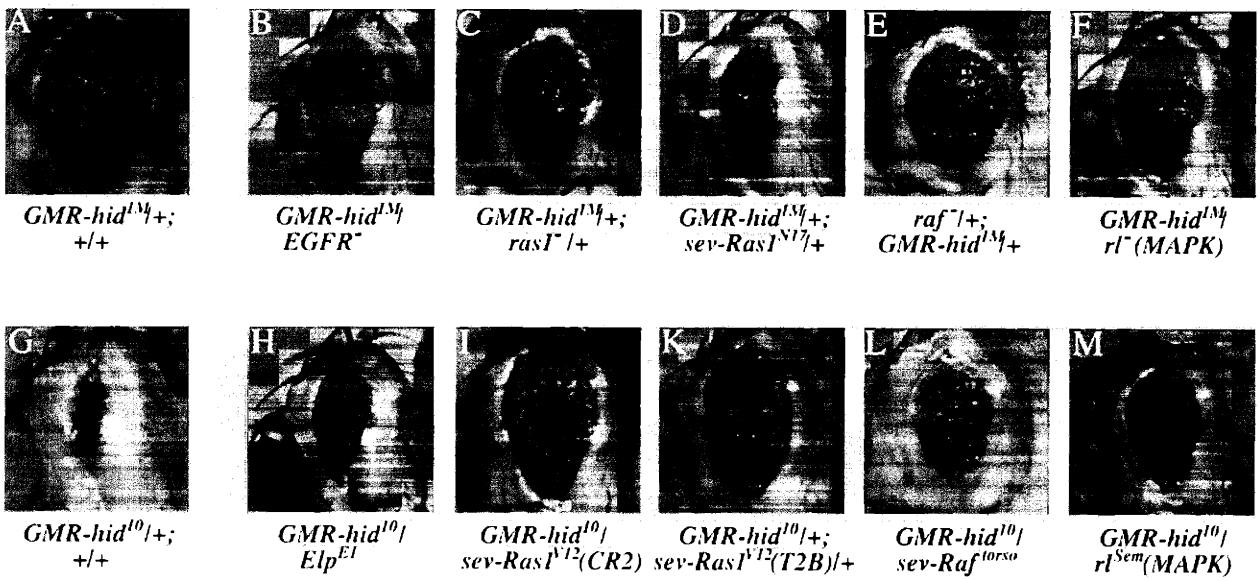

A
B
C
D
E
F
GMR-hid^{1M4}/+; +/+
GMR-hid^{1M4} EGFR^{-}
GMR-hid^{1M4}/+; ras1^{-}/+
GMR-hid^{1M4}/+; sev-Ras1^{N17}/+
raf^{-}/+; GMR-hid^{1M4}/+
GMR-hid^{1M4} rl^{-}(MAPK)
G
H
I
K
L
M
GMR-hid^{10}/+; +/+
GMR-hid^{10}/ Elp^{E1}
GMR-hid^{10}/ sev-Ras1^{V12}(CR2)
GMR-hid^{10}/+; sev-Ras1^{V12}(T2B)/+
GMR-hid^{10}/ sev-Raf^{torso}
GMR-hid^{10}/ rl^{Sem}(MAPK)

Figure 3. The Raf/MAPK Effector Branch of Ras1 Is the Major Pathway for Suppression of *GMR-hid*

(A) Schematic outline of the downstream effector pathways of Ras1 and of the effector loop mutations in Ras1 leading specifically to activation of only one effector branch. For instance, Ras1V12S35 interacts with Raf, but not with Ral-GDS and PI3-K; Ras1V12G37 only interacts with Ral-GDS; Ras1V12C40 only interacts with the p110 subunit of PI3-K. (B) Eye ablation phenotype caused by *GMR-hid10 sev-GAL4*. (C) Wild-type Ras1 does not modify the *GMR-hid10* eye phenotype. (D) Activation of the Raf/MAPK pathway by Ras1V12S35 results in strong suppression of *GMR-hid10*. The quantatively stronger suppression seen in this experiment compared to Figure 2M is due to stronger expression of Ras1V12S35 caused by the GAL4/UAS system. (E) The Ral.GDS effector pathway fails to suppress *GMR-hid10*. (F) Activation of the PI3-K/Akt pathway by Ras1V12C40 results in moderate suppression of *GMR-hid10*.

The genotypes are: (B) *GMR-hid10 sev-GAL4/+*, (C) *GMR-hid10 sev-GAL4/UAS-Ras1+*, (D) *GMR-hid10 sev-GAL4/UAS-Ras1V12S35*, (E) *GMR-hid10 sev-GAL4/UAS-Ras1V12G37*, and (F) *GMR-hid10 sev-GAL4/UAS-Ras1V12C40*.

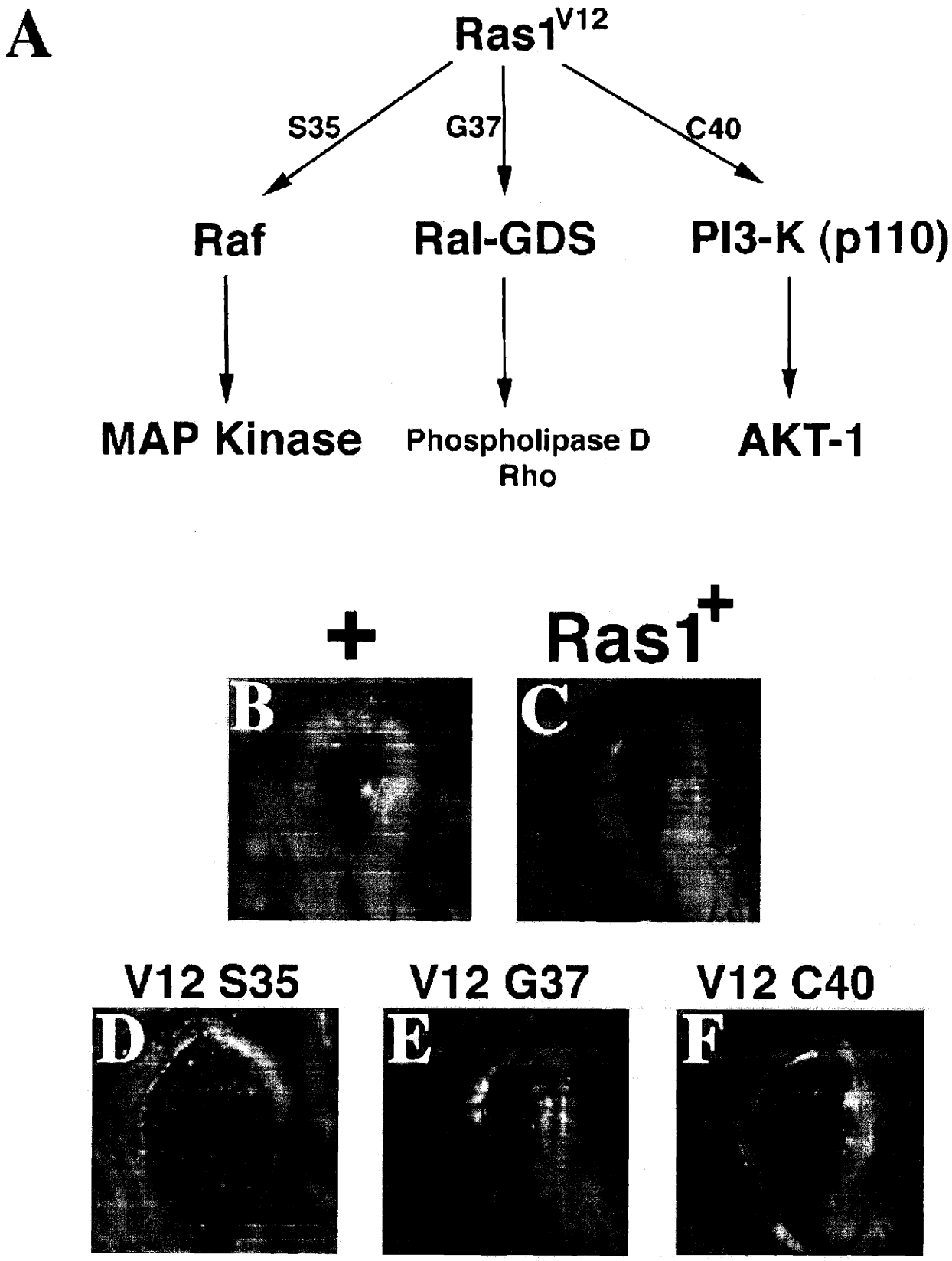

A
Ras1V12
S35
G37
C40
Raf
Ral-GDS
PI3-K (p110)
MAP Kinase
Phospholipase D
Rho
AKT-1
+
Ras1+
B
C
V12 S35
V12 G37
V12 C40
D
E
F

Figure 4. Dominant Suppression of *hs-hid*-Induced Lethality

Heat shock induction of *hid* via a *hs-hid* transgene during first instar larvae causes strong organismal lethality (Grether et al., 1995). After 30 min of heat shock, only 2% of the *hs-hid* animals survive compared to control (non-*hs-hid*) animals (see Experimental Procedures for details). Lof mutations in *gap1*, *spry*, and *arg* protect against *hs-hid*-induced lethality, such that about 20%–40% of Sup/*hs-hid* animals survive even a 60 min heat shock at 37°C. The gof mutation *rlSem*/MAPK has the strongest survival activity against *hs-hid*-induced lethality. Approximately 70% of *rlSem*; *hs-hid* animals survive a 60 min heat shock at 37°C.

The genotype of flies analyzed in this assay are +/*hshid*, *gap121-1s*/*hs-hid*, *spry28-4s*/*hs-hid*, *argI7*/*hs-hid*, and *rlSem*/+; *hs-hid*/+. The results shown represent the average of three independently performed experiments.

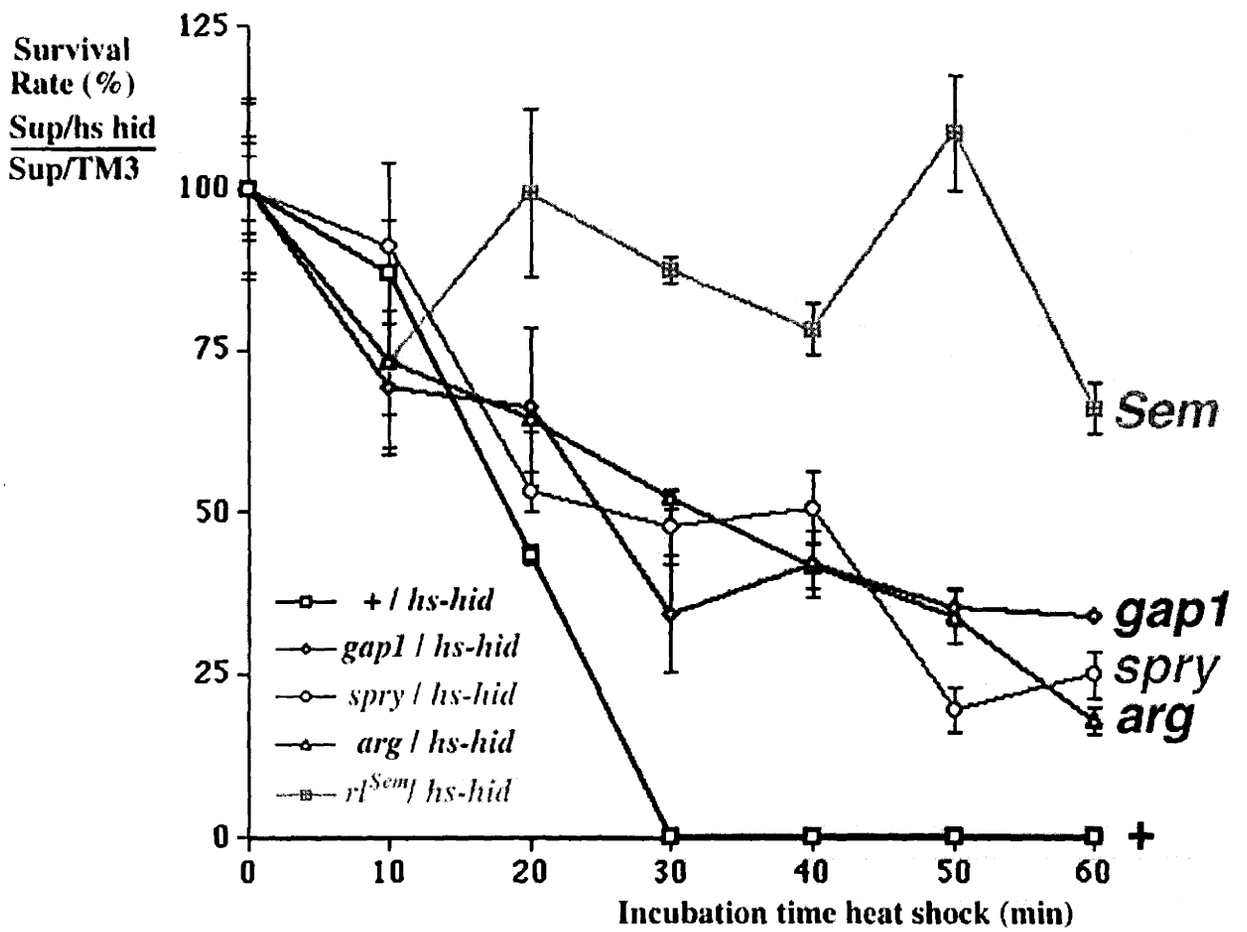

Survival Rate (%)
Sup/hs hid
Sup/TM3
125
100
75
50
25
0
0
10
20
30
40
50
60
Incubation time heat shock (min)
+ / hs-hid
gap1 / hs-hid
spry / hs-hid
arg / hs-hid
rl^Sem / hs-hid
Sem
gap1
spry
arg
+

Figure 5. *GMR-reaper*- and *GMR-grim*-Induced Eye Phenotypes Are Not Affected by EGFR/Ras1/MAPK Signaling

The mild eye ablation phenotypes caused by one copy of *GMR-reaper* (A) and *GMR-grim* (C) were used to score for an enhancement by the dominant negative Ras1 allele *sev-Ras1N17* (B and D). Stronger eye ablation phenotypes were produced by two copies of either *GMR-reaper* (E) or *GMR-grim* (G) and were used to test for suppression by the gof Ras1 allele *sev-Ras1V12* (F and H). Both Ras1 transgenes, *sev-Ras1N17* and *sev-Ras1V12*, do not or only weakly modify either the *GMR-rpr*- or *GMR-grim*-induced eye phenotypes. These Ras1 transgenes show striking effects on *GMR-hid*-induced apoptosis (see Figure 2D and Figure 2I). Other EGFR/Ras1/MAPK pathway mutants also fail to show a genetic interaction with *GMR-reaper* and *GMR-grim* (data not shown). We conclude that the antiapoptotic activity of the EGFR/Ras1/MAPK specifically counteracts *hid*-induced apoptosis. The genotypes of flies shown are indicated below each panel.

1x *GMR-reaper/ +*

1x *GMR-reaper/ sev-Ras1*^{N17}

1x *GMR-grim/ +*

1x *GMR-grim/ sev-Ras1*^{N17}

2x *GMR-reaper/ +*

2x *GMR-reaper/ sev-Ras1*^{V12}

2x *GMR-grim/ +*

2x *GMR-grim/ sev-Ras1*^{V12}

Figure 6. MAPK Phosphorylation Consensus Sites in Hid

The *hid* gene encodes a novel protein of 410 amino acid residues (Grether et al., 1995). The five MAPK phosphorylation consensus sites are indicated. The consensus site is defined as Pro-X-Ser/Thr-Pro, where X can be any residue exept Pro (Clark-Lewis et al., 1991). In *hidAla5*, all five phospho-acceptor residues are changed to a nonphosphorylatable residue, Ala. In *hidAla3*, only Thr-148, Thr-180, and Ser-251 are changed to Ala (indicated by asterisks), since the consensus sites of the remaining two phospho-acceptor residues, Ser-121 and Thr-228, contain a Pro in the X position.

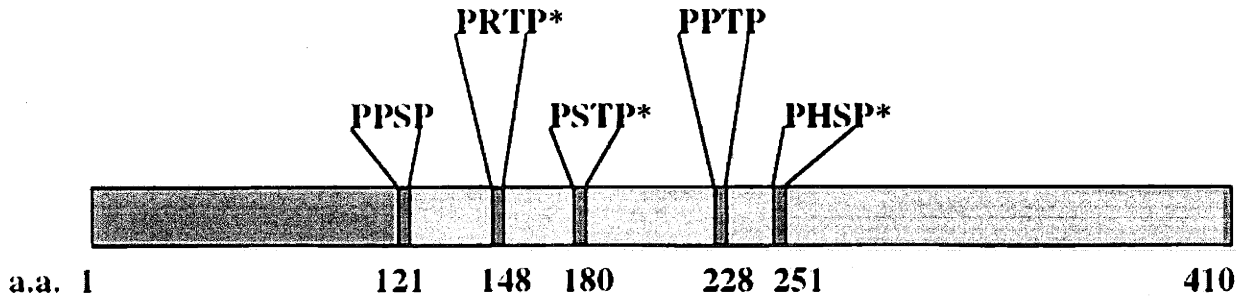
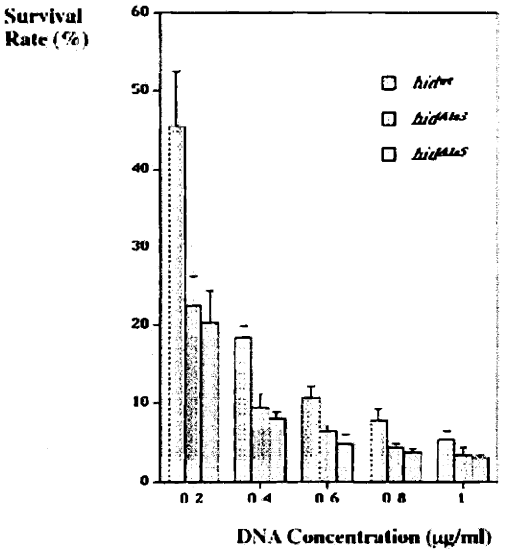

PRTP*
PPTP
PPSP
PSTP*
PHSP*
a.a. 1
121
148
180
228
251
410

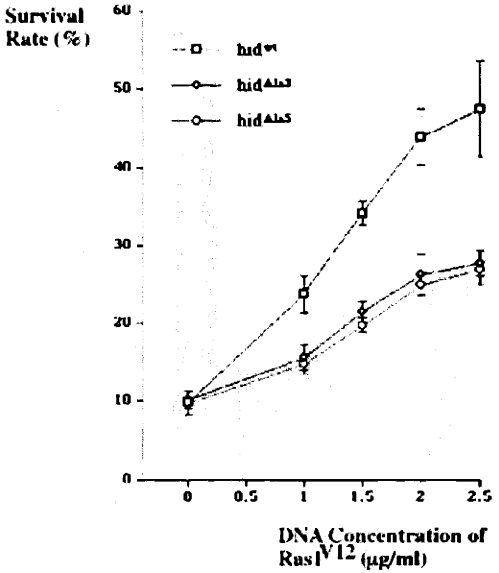
Figure 7. Effects of MAPK Phosphorylation in SL2 Cells

SL2 cells were transiently cotransfected with the indicated constructs. For details see Experimental Procedures. (A) The indicated amount of DNA of the Hid constructs was transfected into SL2 cells and tested for their killing activity. The two MAPK site deficient Hid mutants induce apoptotic death more efficiently than does wild-type Hid. (B) Comparison of the rescuing activity of Ras1V12 on Hidwt-, HidAla3-, and HidAla5-induced apoptosis in SL2 cells. Constant amounts of DNA of the Hid constructs were cotransfected with increasing amounts of Ras1V12 as indicated. Hidwt-induced apoptosis is efficiently blocked by Ras1V12, such that the survival rate goes up to about 50%. In contrast, the rescuing activity of Ras1V12 on HidAla3- and HidAla5-induced apoptosis is partially blocked. Only about 28%–30% of the cells survive. The amount of the Hid constructs (0.6 μg/ml Hidwt; 0.4 μg/ml HidAla3 and HidAla5) was determined by Figure 6A as the amount necessary to allow only 10% of SL2 cells to survive. (C) Similar experiment to Figure 7B except that increasing amounts of RlSem/MAPK instead of Ras1V12 were transfected with constant amounts of the Hid constructs (0.5 μg/ml each). The gof allele RlSem/MAPK behaves also in cultured cells as a suppressor of Hidwt-induced apoptosis. Alteration of the MAPK sites in Hid completely abolishes the rescuing activity of RlSem/MAPK on HidAla3- and HidAla5-induced apoptosis.

A


Survival Rate (%)
60
50
40
30
20
10
0
0.2
0.4
0.6
0.8
1
DNA Concentration (μg/ml)

B


Survival Rate (%)
60
50
40
30
20
10
0
0
0.5
1
1.5
2
2.5
DNA Concentration of Ras1V12 (μg/ml)

C

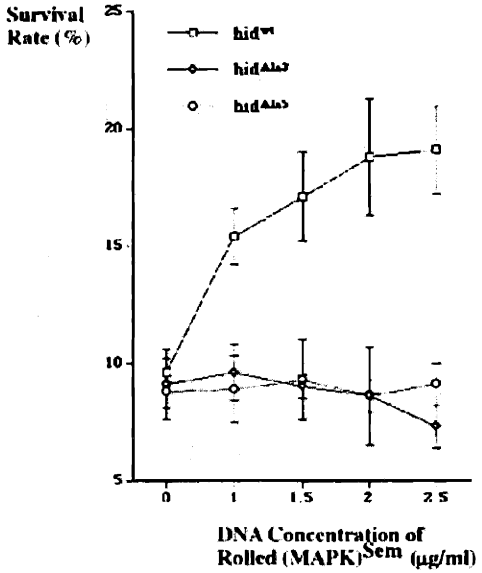

Survival Rate (%)
25
20
15
10
5
0
1
1.5
2
2.5
DNA Concentration of Rolled (MAPK)Sem (μg/ml)

Figure 8. Transgenic Analysis of *GMR-hidAla3* and *GMR-hidAla5*

The unmodified eye phenotypes of *GMR-hidwt-10* (A), *GMR-hidAla3* (F), and *GMR-hidAla5* (L) are of similar strength allowing direct comparison of the rescuing abilities of *rlSem*/MAPK (B, G, and M), *sev-Ras1V12* (line CR2; C, H, and N), *GMR-DIAP1* (D, I, and O), and *hid-* (E, K, and P). Note the block in the ability to suppress the eye ablation phenotype caused by the MAPK deficient *GMR-hid* transgenes by *rlSem*/MAPK (G and M) and *sev-Ras1V12* (H and N), whereas expression of the cell death inhibitor DIAP1 suppresses *hid*-induced apoptosis independently of the MAPK phosphorylation sites (D, I, and O). The weak suppression observed for *GMR-hidAla3* and *GMR-hidAla5* by *rlSem*/MAPK (G and M) might be the result of inhibiting endogenous wild-type *hid* protein, since removal of one genomic copy of *hid* results in a weak suppression, too (K and P; see text for explanations). The genotypes of flies shown are indicated below each panel. The *hid* allele used in (E), (K), and (P) is *hidWR+X1*.

GMR-hid$^{wt\text{-}10}$*/+;* *+/+*

GMR-hid$^{wt\text{-}10}$*/rl*^{Sem}

GMR-hid$^{wt\text{-}10}$*/sev-Ras1*^{V12}

GMR-hid$^{wt\text{-}10}$*/+;* *GMR-DIAP1/+*

GMR-hid$^{wt\text{-}10}$*/+; hid*$^{-}$*/+*

GMR-hid^{Ala3}*/+;* *+/+*

GMR-hid^{Ala3}*/rl*^{Sem}

GMR-hid^{Ala3}*/sev-Ras1*^{V12}

GMR-hid^{Ala3}*/+;* *GMR-DIAP1/+*

GMR-hid^{Ala3}*/+; hid*$^{-}$*/+*

GMR-hid^{Ala5}*/+;* *+/+*

GMR-hid^{Ala5}*/rl*^{Sem}

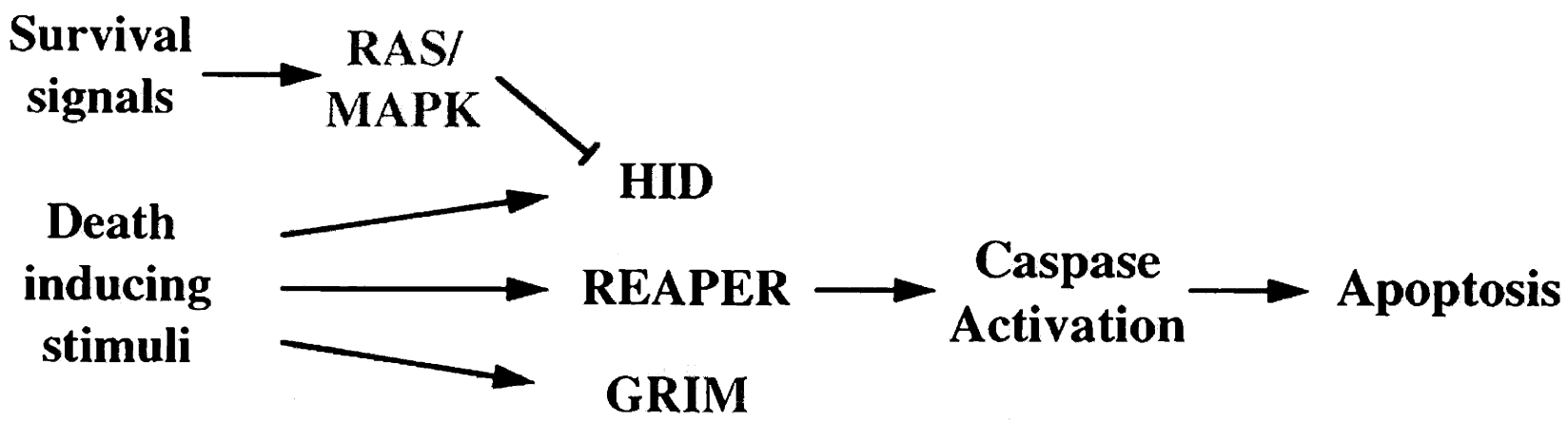
GMR-hid^{Ala5}*/sev-Ras1*^{V12}

GMR-hid^{Ala5}*/+;* *GMR-DIAP1/+*

GMR-hid^{Ala5}*/+; hid*$^{-}$*/+*

Figure 9. Specific Inhibition of Hid-Induced Apoptosis by Ras1/MAPK-Dependent Survival Pathways

The apoptotic inducers *hid*, *reaper*, and *grim* link the apoptotic program with many different death-inducing stimuli in *Drosophila*; these include cell lineage, cell–cell interactions, ecdysone, block of cell differentiation, deprivation of trophic factors, and radiation. Induction of apoptosis proceeds via a conserved caspase pathway. However, only the activity of *hid* is specifically inhibited by survival factors, which activate the Ras1/MAPK pathway.


Survival signals
RAS/ MAPK
HID
Death inducing stimuli
REAPER
Caspase Activation
Apoptosis
GRIM

Chapter 4.

Induction of apoptosis by *Drosophila reaper, hid* and *grim* through inhibition of IAP function

This chapter was previously published as Lakshmi Goyal, Kimberly McCall, Julie Agapite, Erika Hartwieg and Hermann Steller (2000) EMBO J, 19, 589-597. My contribution to this chapter was the identification of different classes of *thread/diap1* alleles as modifiers of *GMRrpr* and *GMRhid*.

Abstract

Induction of apoptosis in *Drosophila* requires the activity of three closely linked genes, *reaper*, *hid* and *grim*. Here we show that the proteins encoded by *reaper*, *hid* and *grim* activate cell death by inhibiting the anti-apoptotic activity of the *Drosophila* IAP1 (*diap1*) protein. In a genetic modifier screen, both loss-of-function and gain-of-function alleles in the endogenous *diap1* gene were obtained, and the mutant proteins were functionally and biochemically characterized. Gain-of-function mutations in *diap1* strongly suppressed *reaper*-, *hid*- and *grim*-induced apoptosis. Sequence analysis of these alleles revealed that they were caused by single amino acid changes in the baculovirus IAP repeat domains of *diap1*, a domain implicated in binding REAPER, HID and GRIM. Significantly, the corresponding mutant DIAP1 proteins displayed greatly reduced binding of REAPER, HID and GRIM, indicating that REAPER, HID and GRIM kill by forming a complex with DIAP1. These data provide strong *in vivo* evidence for a previously published model of cell death regulation in *Drosophila*.

Introduction

Programmed cell death or apoptosis is crucial to normal embryonic development and metamorphosis of multicellular organisms (Thompson, 1995; Jacobson *et al.*, 1997; McCall and Steller, 1997). In *Drosophila*, the induction of apoptosis requires the products of *reaper*, *hid* and *grim*, genes encoded by the 75C1,2 region of the *Drosophila* third chromosome (White *et al.*, 1994, 1996; Grether *et al.*, 1995; Chen *et al.*, 1996). *reaper*, *hid* and *grim* are transcriptionally regulated in response to diverse death-inducing signals and appear to link distinct signaling pathways with the cell death program (Nordstrom *et al.*, 1996; Kurada and White, 1998). In addition, HID is post-transcriptionally regulated by the RAS-MAP kinase pathway in response to cell survival signals (Bergmann *et al.*, 1998a,b). Phosphorylation of HID by activated MAPK inhibits the pro-apoptotic activity of HID (Bergmann *et al.*, 1998a). Ectopic expression of *reaper*, *hid* and *grim* induces cell death in different tissues of transgenic animals and in cultured insect and mammalian cells (Grether *et al.*, 1995; Hay *et al.*, 1995; Chen *et al.*, 1996; Pronk *et al.*, 1996; White *et al.*, 1996; Vucic *et al.*, 1997, 1998a; McCarthy and Dixit, 1998; Haining *et al.*, 1999). This ectopic cell death is blocked by baculovirus p35, a caspase inhibitor protein (Bump *et al.*, 1995) and by chemical inhibitors of caspases (Hay *et al.*, 1994; Grether *et al.*, 1995; Chen *et al.*, 1996; White *et al.*, 1996; McCarthy and Dixit, 1998; Haining *et al.*, 1999), indicating that *reaper*, *hid* and *grim* induce apoptosis by activating a caspase pathway. Several caspase-like molecules have been identified in *Drosophila* (Fraser and Evan, 1997; Song *et al.*, 1997; Chen *et al.*, 1998; Dorstyn *et al.*, 1999), but the precise mechanism of their activation is not clear.

Apoptosis is negatively regulated by another conserved and important class of molecules, the inhibitor of apoptosis proteins (IAPs) (Deveraux and Reed, 1999). IAPs were first described in baculoviruses based on their ability to rescue the p35 mutant phenotype and were shown to exert anti-apoptotic activity (Crook *et al.*, 1993; Birnbaum *et al.*, 1994; Clem and Miller, 1994; Miller, 1997). Since then, several cellular homologs from both vertebrates and invertebrates have been described (Clem and Duckett, 1997; Deveraux and Reed, 1999). Typically, IAP family genes have at least one and often two or three tandem baculovirus IAP repeat (BIR) motifs and may have an additional C-

terminal RING finger domain (Clem and Duckett, 1997; Deveraux and Reed, 1999). Some mammalian IAPs have been implicated in human diseases, including spinal muscular atrophy (SMA) (Roy *et al.*, 1995) and cancer (Ambrosini *et al.*, 1997). In *Drosophila*, two IAP homologs have been reported: *diap1* and *diap2* (Hay *et al.*, 1995; Duckett *et al.*, 1996; Uren *et al.*, 1996). *diap1* is encoded by the *thread* (*th*) locus (Lindsley and Zimm, 1992), and loss-of-function mutations in *diap1* are lethal and enhance *reaper*-, *hid*- and *grim*-induced cell death (Hay *et al.*, 1995). Ectopic expression of either *diap1* or *diap2* suppresses apoptosis (Hay *et al.*, 1995; Vucic *et al.*, 1997, 1998a). Baculovirus, *Drosophila* and mammalian IAPs can physically interact with REAPER, HID and GRIM and antagonize their pro-apoptotic properties (Vucic *et al.*, 1997, 1998a; McCarthy and Dixit, 1998). Human XIAP, cIAP1 and cIAP2 can bind to and inhibit caspase 3, 7 and 9 *in vitro* (Deveraux *et al.*, 1997, 1998; Roy *et al.*, 1997). These observations have led to the proposal that IAPs are apoptosis antagonists exerting their inhibitory effect at several levels in the apoptotic cascade and, in *Drosophila*, inhibit REAPER, HID and GRIM from activating effectors downstream in the death program (McCarthy and Dixit, 1998; Vucic *et al.*, 1998b). More recently, however, results obtained primarily from a yeast expression system suggest that REAPER, HID and GRIM block DIAP1 from inhibiting caspase activity (Wang *et al.*, 1999). Consistent with this observation, loss-of-function *diap1* mutants are embryonic lethal, with the widespread induction of apoptosis (Wang *et al.*, 1999; this study). We now report the isolation and characterization of several gain-of-function and loss-of-function mutants in the endogenous *diap1* gene. The gain-of-function mutant DIAP1 proteins are impaired for binding to REAPER and HID. This suggests that the association of REAPER and HID with DIAP1 is critical for the induction of apoptosis by these pro-apoptotic genes *in vivo*. Collectively, these data provide strong support for the idea that REAPER, HID and GRIM kill by inhibiting DIAP1's ability to antagonize caspase function.

Results

Mutations in *diap1* modify *reaper*- and *hid*-induced cell death phenotypes

Ectopic expression of *reaper*, *hid* and *grim* under the control of an eye-specific promoter (*GMR*) results in ectopic cell death in the developing retina and causes rough and reduced eyes (Grether *et al.*, 1995; Hay *et al.*, 1995; Chen *et al.*, 1996; White *et al.*, 1996). The phenotypes caused by *GMR-reaper* and *GMR-hid* transgenes are dosage dependent and very sensitive to the dosage of other cell death genes acting downstream or in parallel pathways. This was the basis of a mutagenesis screen to isolate modifiers of *reaper*- and *hid*-induced cell death (J.Agapite, K.McCall and H.Steller, unpublished) (Bergmann *et al.*, 1998a). Among a large number of dominant modifiers of apoptosis obtained in this screen, at least 10 were mutations in *diap1*. As expected, loss-of-function *diap1* alleles were strong enhancers of both *reaper*- and *hid*-induced eye phenotypes (Hay *et al.*, 1995) (Figure 1; Table I). However, we also obtained several gain-of-function mutants of *diap1* that potently inhibited cell death and restored the eye morphology to near wild-type levels (Figure 1; Table I). Another class of mutants that enhance *reaper*-induced death but suppress the *hid* phenotype was also identified.

The gain-of-function mutants were also tested for suppression of *reaper*- and *hid*-induced cell death in the wing (J.Agapite and H.Steller, unpublished) and the suppression of larval lethality induced by the expression of *hid* under a heat shock promoter (Bergmann *et al.*, 1998a). In all assays tested, the *diap1* gain-of-function mutants suppressed *reaper*- and *hid*-induced cell death very efficiently (data not shown) and the *diap1* mutant phenotype was recapitulated outside the context of the developing eye.

Gain-of-function mutations in *diap1* map to the BIR domains

Molecular analysis revealed that all five gain-of-function alleles were associated with single amino acid changes in the BIR domain of DIAP1 (Table I; Figure 2). Significantly, this domain of DIAP1 has been implicated in the binding of REAPER, HID and GRIM (Vucic *et al.*, 1998a,b). The proline residue mutated to serine in $th^{21\text{-}2s}$ is

highly conserved among different BIR domains (Uren *et al.*, 1998). In $th^{6\text{-}3s}$ and $th^{45\text{-}2s}$ as well as in $th^{23\text{-}4s}$ and $th^{23\text{-}8s}$, amino acid substitution of a specific glycine residue in corresponding locations in BIR1 and BIR2, respectively, was found to cause the gain-of-function phenotype. The flanking amino acid of the mutated glycine is a conserved non-polar residue (valine, leucine or isoleucine) in 100% of all BIR domains (Uren *et al.*, 1998). Two previously reported loss-of-function *thread* alleles, th^4 and th^5 (Hay *et al.*, 1995), were also included in the molecular analysis. th^4 is a change in a highly conserved histidine residue (Uren *et al.*, 1998), and the th^5 mutation introduces a premature truncation resulting in the deletion of several amino acids at the C-terminus of DIAP1 (Figure 2).

To confirm that the missense mutation in the primary protein sequence of *diap1* was responsible for the observed phenotype, we employed a cell death assay in transfected culture cells. Expression of *reaper* and *hid* can induce apoptosis in several cultured cell types (Pronk *et al.*, 1996; Bergmann *et al.*, 1998a; McCarthy and Dixit, 1998; Vucic *et al.*, 1998a; Haining *et al.*, 1999), which is inhibited efficiently by co-transfecting IAPs (McCarthy and Dixit, 1998; Vucic *et al.*, 1998a; Haining *et al.*, 1999). Representative gain-of-function and loss-of-function *diap1* alleles were tested in *Drosophila* Schneider (S2) cells for their ability to overcome *reaper*- and *hid*-induced cell death. All *diap1* mutants tested expressed protein at levels comparable to wild-type, indicating that these mutations did not significantly affect the expression and stability of the protein (Figure 3). Gain-of-function mutants showed an ability to inhibit *reaper*- and *hid*-induced cell death by 40-50% above wild-type levels. All the mutants exhibited the same phenotypes as observed *in vivo* (Table I), except for the th^4 allele, which showed a near wild-type level of protection against *hid*-induced death (Figure 3, see below).

Gain-of-function mutants of DIAP1 show impaired binding to REAPER and HID

REAPER, HID and GRIM can bind to DIAP1 via the BIR domains (Vucic *et al.*, 1998b), and all the strong *diap1* gain-of-function suppressor mutations that we obtained in our modifier screen map to a specific region in the BIR domains (Figure 2). Therefore, we hypothesized that these mutations may have altered the binding of DIAP1 to

REAPER, HID and GRIM. Based on previous findings that REAPER, HID and GRIM can interact functionally and physically with IAPs (Vucic *et al.*, 1997, 1998a), two simple models can be proposed to explain the role of *diap1* in regulating apoptosis. In the first model, *diap1* acts upstream of *reaper*, *hid* and *grim* to prevent them from activating the cell death program (McCarthy and Dixit, 1998; Vucic *et al.*, 1998b). Alternatively, *reaper*, *hid* and *grim* may induce apoptosis by inhibiting *diap1* from blocking cell death (Wang *et al.*, 1999). According to the later model, mutations in *diap1* that inhibit binding of REAPER, HID and GRIM would be less susceptible to inactivation by the pro-apoptotic proteins and hence show increased anti-apoptotic activity. To test this hypothesis directly, we performed *in vitro* binding assays of wild-type and mutant DIAP1 protein to GST-REAPER and GST-HID. As predicted, gain-of-function mutants of *diap1* ($th^{6\text{-}3s}$ and $th^{23\text{-}4s}$) showed greatly diminished binding to both REAPER and HID as compared with wild-type DIAP1 (Figure 4). The effect of these single amino acid changes appears to be quite specific, since the mutant DIAP1 proteins retain potent anti-apoptotic activity (Figures 1 and 3; Table I), presumably by direct interaction with caspases (Deveraux *et al.*, 1997, 1998; Roy *et al.*, 1997; Hawkins *et al.*, 1999). Since the anti-apoptotic activity of IAPs requires a functional BIR domain, it is highly unlikely that these *diap1* gain-of-function mutations have a general effect on the overall structure of the BIR domains.

The gain-of-function mutations in *diap1* reduce binding to REAPER and HID up to 14-fold (Figure 4). The residual association with REAPER and HID may be due to the presence of one intact BIR domain in both the mutants (Figure 2). However, it is notable that mutations in either the first or the second BIR domain can reduce binding of HID and REAPER by >2-fold, suggesting that both BIR domains may cooperate for the efficient binding of REAPER and HID. On the other hand, we cannot exclude the possibility that other regions of DIAP1 are also involved in binding of REAPER, HID and GRIM, perhaps involving domains distinct from the N-terminal region of these proteins. In any event, our results show that gain-of-function alleles of *diap1* with increased anti-apoptotic activity are associated with reduced *in vitro* binding activity to REAPER and HID. These findings are not reconciled easily with the model that DIAP1 protects

against cell death by inhibiting the pro-apoptotic activity of REAPER and HID. Rather, they suggest that REAPER and HID activate apoptosis by binding to and inactivating DIAP1.

Not all BIR domain mutations have impaired binding to REAPER, HID and GRIM. The th^4 mutant, which harbors a single amino acid change in BIR2 (Figure 2), binds to REAPER and HID *in vitro* (Figure 4). Although this is a loss-of-function allele in *Drosophila*, presumably by lacking the ability to inhibit caspases, overexpression of th^4 in cultured cells can block *hid*-induced cell death (Figure 3). In order to explain this apparent paradox, we propose that overexpression of the mutant protein at unphysiologically high levels in cultured cells protects against *hid*-induced apoptosis by binding to and sequestering HID. While this proposed mechanism is not reflective of the normal function of DIAP1, it explains results from previous cell transfection studies suggesting that IAPs may function upstream of REAPER, HID and GRIM to inhibit apoptosis (McCarthy and Dixit, 1998; Vucic *et al.*, 1998b).

Loss of *diap1* results in the induction of apoptosis

Loss-of-function *diap1* mutants are embryonic lethal (Hay *et al.*, 1995) (Table I). Since *diap1* has anti-apoptotic activity, we investigated whether zygotic loss of *diap1* function may lead to excessive apoptosis during embryogenesis. Using the TUNEL technique to label apoptotic nuclei, essentially all nuclei of homozygous $th^{11\text{-}3e}$ embryos stained positive by 7-8 h after egg laying (AEL) (Figure 5). In contrast, only a few TUNEL-positive cells were observed in correspondingly aged wild-type embryos (stage 11), the first time point at which cell deaths are normally seen in *Drosophila* embryos (Figure 5A) (Abrams *et al.*, 1993). Due to the extraordinary amount of apoptosis, *thread* loss-of-function embryos appeared highly disorganized at this time (Figures 5B and E, and 6B-D). However, time course analyses revealed that these embryos develop normally until the cellular blastoderm stage and begin to gastrulate, but become abnormal shortly after the formation of the ventral furrow (J.Tittel and H.Steller, unpublished observations). Analysis of $th^{11\text{-}3e}$ embryos by electron microscopy revealed many nuclei with features characteristic of apoptosis, including condensed

chromatin and electron-dense nuclei (Figure 6B-D). These experiments reveal a striking dependence of cell survival on zygotic *diap1* function. We conclude that *diap1* is required to prevent apoptosis in most, if not all cells of the *Drosophila* embryo.

diap1 functions downstream of *reaper*, *hid* and *grim*

If *diap1* functions upstream of *reaper*, *hid* and *grim*, the excessive apoptosis that we observed in *diap1* loss-of-function embryos should be blocked in the absence of *reaper*, *hid* and *grim*. In contrast, if *reaper*, *hid* and *grim* function upstream of *diap1*, apoptosis in *diap1* loss-of-function embryos should be independent of these pro-apoptotic genes. To distinguish between these two models, we constructed double mutant embryos that are homozygous for both *H99* and $th^{11\text{-}3e}$, a deletion that removes *reaper*, *hid* and *grim*. Wild-type, $th^{11\text{-}3e}$ and *H99*,$th^{11\text{-}3e}$ embryos were analyzed for their phenotypic morphology and by TUNEL labeling as before. We found that the *H99*,$th^{11\text{-}3e}$ phenotype was indistinguishable from the $th^{11\text{-}3e}$ phenotype observed earlier (Figure 5). Similar results were reported recently by Wang *et al.* (1999). The failure of the *H99* deletion to inhibit cell death in *diap1* loss-of-function embryos is not consistent with the idea that DIAP1 blocks apoptosis by inhibiting REAPER, HID and GRIM.

Discussion

In this study, we report the isolation of novel gain-of-function and loss-of-function mutants in one of the *Drosophila* IAP genes, *diap1*. Gain-of-function mutants of *diap1* strongly suppressed *reaper*- and *hid*-induced cell death and correspond to single amino acid changes in the BIR domain of the DIAP1 protein. The gain-of-function mutant DIAP1 proteins displayed impaired binding to REAPER and HID, indicating that the ability of REAPER and HID to bind DIAP1 is critical for the regulation of DIAP1 *in vivo*. Loss-of-function mutations in *diap1* caused widespread induction of cell death resulting in embryonic lethality, and a deletion in *reaper*, *hid* and *grim* was unable to rescue the *diap1* loss-of-function phenotype (Wang *et al.*, 1999; this study). These results indicate that *diap1* is required for cell survival and is genetically downstream of *reaper*, *hid* and *grim*.

Based on overexpression studies in cultured cells, it was initially proposed that IAPs function upstream of *reaper*, *hid* and *grim* to prevent them from activating caspases (Vucic *et al.*, 1997, 1998a). Subsequently, IAPs were also reported to bind to and inhibit caspases (Deveraux *et al.*, 1997, 1998; Roy *et al.*, 1997; Kaiser *et al.*, 1998). In order to reconcile this apparent paradox, it was proposed that IAPs act on multiple targets and at different steps in the apoptotic pathway (Kaiser *et al.*, 1998; McCarthy and Dixit, 1998; Vucic *et al.*, 1998b). Based on data obtained primarily from a yeast expression system, Wang *et al.* (1999) have suggested a more simple linear model for the induction of apoptosis by *reaper*, *hid* and *grim*. According to this 'double inhibition model', REAPER, HID and GRIM induce apoptosis by binding directly to and preventing DIAP1 from inhibiting caspase activation (Figure 7). The *diap1* mutants analyzed in this study provide evidence for the function of *reaper* and *hid in vivo* and concur with the conclusions obtained by Wang *et al.* (1999) from their study in yeast. The decreased binding of REAPER and HID to DIAP1 correlates with a gain-of-function *diap1* phenotype, suggesting that the association with DIAP1 is important for the pro-apoptotic activity of REAPER, HID and GRIM. These data strongly support the idea that in *Drosophila*, REAPER and HID induce apoptosis by binding to and inhibiting DIAP1 function (Figure 7; Wang *et al.*, 1999). We believe that the previously proposed function of IAPs upstream of *reaper*, *hid* and *grim* is simply an artifact of unphysiologically high levels of protein expression in heterologous systems. When IAP expression constructs are introduced into cultured cells under the control of strong promoters and at high copy numbers, the levels of proteins expressed far exceed those of the endogenous cellular IAP proteins. Under these unphysiological conditions, cellular IAPs can display properties that do not reflect their normal mechanism of action. In particular, our results with th^4 and other *diap1* alleles (data not shown) demonstrate that mutant proteins that completely lack anti-apoptotic activity *in vivo* can still inhibit cell death *in vitro* as long as they can bind to REAPER, HID and GRIM (Figures 3 and 4). Conversely, gain-of-function *diap1* alleles that display reduced binding to REAPER, HID and GRIM have strongly increased anti-apoptotic function *in vivo* (Figures 1 and 4), but show reduced protection in heterologous cell transfection assays (data not shown). These results

clearly reveal the limitations of overexpression studies in cultured cells for determining the normal mechanism of action of these proteins in the cell death pathway.

A mutant of HID that fails to bind IAPs has no pro-apoptotic activity (Vucic *et al.*, 1998a; Haining *et al.*, 1999; Wang *et al.*, 1999), implying that HID functions primarily, if not exclusively, by inhibiting IAPs. Although the precise mechanism by which IAPs inhibit apoptosis is not known, there are strong reasons to believe that the primary mode of action is a direct inhibition of caspases and/or their activation (Hawkins *et al.*, 1999; Wang *et al.*, 1999; Meier *et al.*, 2000). Mammalian IAPs can inhibit caspases *in vitro* (Deveraux *et al.*, 1997, 1998; Roy *et al.*, 1997), and DIAP1 has been shown to interact with and inhibit *Drosophila* caspases DrICE and DCP1 in heterologous systems (Kaiser *et al.*, 1998; Hawkins *et al.*, 1999). Although these inhibitory activities have been detected with active caspases, we prefer the model that DIAP1 interacts with pro-caspases to prevent caspase activation (Meier *et al.*, 2000; Song *et al.*, 2000). In order to explain the *diap1* loss-of-function phenotype, we propose that in the absence of inhibition by DIAP1, caspases are activated either by a weak autocatalytic activity or by the presence of DARK/HAC-1/dAPAF-1, the *Drosophila* APAF1/CED4-like activator (Kanuka *et al.*, 1999; Rodriguez *et al.*, 1999; Zhou *et al.*, 1999).

How general is the proposed model of IAP regulation and function? In mammals, as in *Caenorhabditis elegans* and *Drosophila*, caspases are widely expressed as weakly active zymogens in virtually all cells. This includes cells that survive for the lifetime of the animal, such as neurons. Therefore, powerful control mechanisms must be in place to prevent the inappropriate activation of caspases in cells that should live, and it appears that some IAPs play a critical role in this regard. On the other hand, these `brakes on death' must somehow be removed in cells that are selected to die. In *Drosophila*, this appears to be mediated by REAPER, HID and GRIM. Although no mammalian homologs of *reaper*, *hid* and *grim* have been reported yet, we expect that a similar regulation must occur in mammalian cells. Consistent with this idea, *reaper*, *hid* and *grim* can activate apoptosis in mammalian cells, and REAPER has been shown to activate caspases and apoptosis-like events in a vertebrate cell-free system (Evans *et al.*, 1997; McCarthy and Dixit, 1998; Haining *et al.*, 1999). Many of the residues

involved in the *diap1* gain-of-function mutants have been highly conserved during evolution, indicating that the domain that is necessary for binding to REAPER/HID/GRIM is under considerable selective pressure. Therefore, we consider it likely that the inhibition of IAP function is a general mechanism of cell death induction. If so, gain-of-function mutations similar to those described here may contribute to aberrant regulation of apoptosis in human diseases, such as cancer.

Materials and methods

Genetic screen and fly stocks

The genetic screen performed to isolate modifiers of *GMR-hid* has been described previously (Bergmann *et al.*, 1998a). In a similar screen for dominant modifiers of *GMR-reaper*-induced eye ablation, 180 000 mutagenized F1 progeny were screened and 46 dominant modifiers were identified (J.Agapite, K.McCall and H.Steller, unpublished). The following mutant and transgenic fly strains were used for the genetic analysis: *GMR-hid* and *GMR-reaper* (Bergmann *et al.*, 1998a), $th^{11\text{-}3e}$ and $th^{6\text{-}3s}$ (this study). *H99*,$th^{11\text{-}3e}$ recombinants were generated by standard *Drosophila* genetic techniques. All crosses were performed at 25°C.

Molecular analysis and plasmid construction

Single embryos of the *diap1* mutants over a balancer with a LacZ marker were selected 4 h AEL and squashed in 10 μl of Gloor and Engel's buffer (10 mM Tris pH 8.2, 1 mM EDTA, 25 mM NaCl, 200 μg/ml proteinase K). The squashed single embryo was incubated at 37°C for 30 min and at 95°C for 2 min to inactivate proteinase K. A 4 μl aliquot of the single embryo DNA was used in each PCR with primers specific for the open reading frame (ORF) of *diap1*. Primers specific for LacZ were used in an independent PCR to identify homozygous *diap1* mutant embryos. The PCR product from homozygous mutant embryos was either sequenced directly or cloned into pIE1-3 vector (Novagen) with a FLAG epitope tag at the N-terminus using restriction sites engineered into the primers. Cleaned PCR product or plasmid DNA carrying the mutant *diap1* coding sequence was subjected to automated sequencing to determine the

molecular nature of the mutation. Mutations were confirmed by sequencing the *diap1* ORF from multiple independent homozygous mutant single embryos. Similar procedures were used to clone and sequence the ORF of wild-type *diap1* from the background strain as control.

Cell culture assays

SL2 cell transient transfection experiments were performed as described (Bergmann *et al.*, 1998a). Briefly, 0.15 μg of the FLAG epitope-tagged wild-type or mutant *diap1* cDNA in the pIE1-3 vector (Novagen) were co-transfected with 0.2 μg of *reaper* or *hid* cDNA in the pIE1-3 vector into each well of a 24-well plate of SL2 cells. pIE1-3 LacZ (18 ng) was included in all transfections as a reporter. Transfections were performed using Cellfectin (Gibco-BRL) according to the manufacturer's instructions. SL2 cells adhere poorly to culture dishes and apoptotic cells float up into the culture medium. At 24 h post-transfection, cells still adhering to the dish are viable. Therefore, 24 h post-transfection, the cells still adhering to the dish were fixed and stained for LacZ activity and the number of blue cells was counted as an indicator of cell survival. All plasmid combinations were transfected in triplicate and the mean and standard deviation calculated. For Western blots, extracts were made from SL2 cells transfected with FLAG-tagged wild-type or mutant pIE1-3 *diap1* plasmids and resolved by SDS-PAGE. After transfer of the proteins to nitrocellulose membranes, the blots were probed with a monoclonal antibody directed to the FLAG epitope tag (Kodak). An anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibody was used and the immune complexes were detected by enhanced chemiluminescence (Amersham).

TUNEL labeling of embryos

For TUNEL and Oligreen staining, Canton S, $th^{11\text{-}3e}$ or $H99,th^{11\text{-}3e}$ embryos were aged for 16 h at 18°C before processing. TUNEL was carried out as previously described (White *et al.*, 1994) with some modifications. Briefly, embryos were fixed in 4% paraformaldehyde, in phosphate-buffered saline (PBS) with heptane, and devitellinized with methanol. After several washes with PBS plus 0.1% Tween-20 (PBT), the embryos

were treated with proteinase K (10 μg/ml), washed in PBT and treated with reagents from the Apotag Plus kit (Oncorr). For detection, an alkaline phosphatase-conjugated antibody to digoxigenin and reagents from the genius kit (both from Boehringer Mannheim) were used. For Oligreen (Molecular Probes) staining to visualize the nuclei, fixed and washed embryos were incubated with a 1:5000 dilution of Oligreen in PBS. Embryos were examined and photographed on a Zeiss Axiophot microscope.

Electron microscopy

th$^{11\text{-}3e}$ homozygous mutant embryos were aged for 4-5 h AEL and selected under a dissection microscope. Embryos were dechorionated and fixed with 1.5% glutaraldehyde, 1% paraformaldehyde and 0.75% acrolein in 0.1 M cacodylate buffer (pH 7.4). Fixed embryos were devitellinized by hand peeling and post-fixed in 1% osmium tetroxide. Thin sections were cut after embedding in an eponate mixture and photographed on a Jeol 1200EX II microscope.

In vitro binding assays

Full-length coding sequences of *reaper* and *hid* in the pGEX vector (Amersham Pharmacia Biotech) (kindly provided by Vivian Hua and Andreas Bergmann) or the pGEX vector alone were used to generate recombinant GST, GST-REAPER and GST-HID proteins in *Escherichia coli.* [^{35}S]Methionine-labeled wild-type and mutant DIAP1 proteins were generated in an *in vitro* transcription-translation system (Promega) using T7 expression plasmids. Equivalent amounts of the labeled proteins, as determined by SDS-PAGE analysis of one-tenth of the *in vitro* translation mix, were incubated with 2 μg of the GST or GST fusion proteins immobilized on glutathione-Sepharose beads. The binding was performed for 2 h at 4°C in the buffer described previously (Vucic *et al.*, 1997) containing 1% NP-40 and washed four times with the same buffer. Protein-bound Sepharose beads were boiled in SDS-containing sample buffer and resolved on 10% SDS-PAGE gels. Gels were fixed, dried and autoradiographed.

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Table I. Phenotypes and molecular changes associated with the *diap1* alleles

Screen obtained from	*diap1* mutant	Modifier phenotype		Recessive phenotype	Mutation
		reaper	*hid*		
Gain-of-function					
hid	*45-2s*	strong suppressor	suppressor	semi-lethal[a]	G88D
reaper	*•6-3s*	strong suppressor	strong suppressor	semi-lethal[a]	G88S
hid	*21-2s*	weak suppressor	weak suppressor	semi-lethal[a]	P105S
hid	*•23-4s*	strong suppressor	strong suppressor	lethal	G269S
hid	*23-8s*	strong suppressor	strong suppressor	lethal	G269S
Loss-of-function					
-	*•th^4*	enhancer	enhancer	lethal	H283Y
-	*•th^5*	enhancer	enhancer	lethal	W273stop
reaper	*11-3e*	strong enhancer	strong enhancer	lethal	N117K

The amino acid changes deduced from the nucleotide sequence of the *diap1* gene of homozygous mutant embryos and their genetic properties are listed. All mutants except *th^4* and *th^5* were obtained from the modifier screens for GMR-*reaper*, and GMR-*hid*, *th4* and *th5* have been reported previously (Hay *et al.*, 1995) and were obtained from J.A.Kennison (NIH). Mutants representative of amino acid changes in both BIR domains were used in subsequent cell culture and biochemical studies and are indicated by a •.

[a] These gain-of-function mutants were semi-lethal with lethality ranging from 25 to 40% and observed mostly in the larval and pupal stages of development

Fig. 1. Mutations in *diap1* modify *reaper*- and *hid*-induced cell death. A gain-of-function mutation in *diap1*, $th^{6\text{-}3s}$, suppresses the *GMR-reaper* (B) (*GMR-rpr*/$th^{6\text{-}3s}$) and *GMR-hid* (E) (*GMR-hid*/$th^{6\text{-}3s}$) induced eye phenotypes. Reduction of *diap1* activity as in $th^{11\text{-}3e}$ enhances the eye phenotypes associated with *GMR-rpr* (C) (*GMR-rpr*/$th^{11\text{-}3e}$) and *GMR-hid* (F) (*GMR-hid*/$th^{11\text{-}3e}$). The unmodified phenotypes are shown in (A) (*GMR-rpr*/+) and (D) (*GMR-hid*/+). The wild-type eye (not shown) is similar to that in (B).

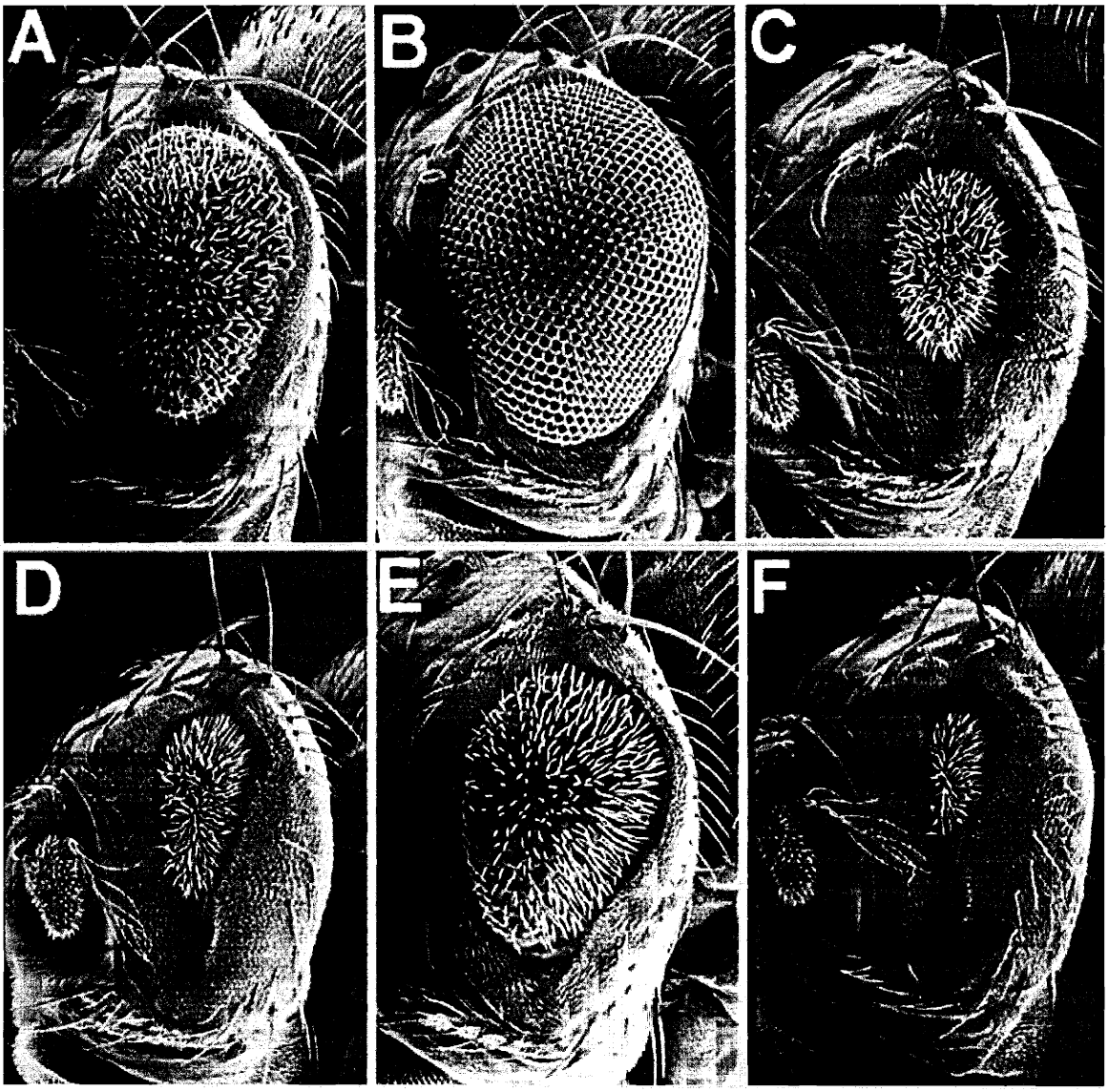

A
B
C
D
E
F

Fig. 2. Schematic representation of the *diap1* mutants. The DIAP1 protein is boxed, with the BIR domains indicated as cross-hatched regions and the RING domain in black. The gain-of-function mutants are indicated in bold and the loss-of-function mutants are in normal face. The figure is drawn to scale.

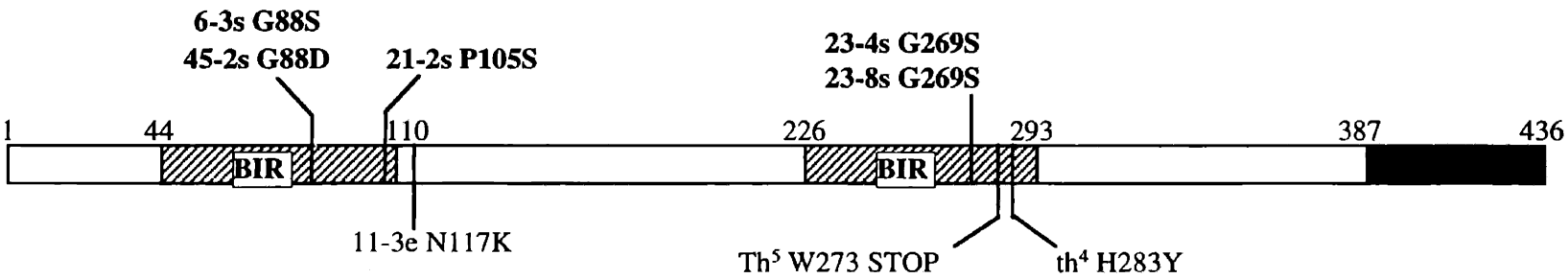
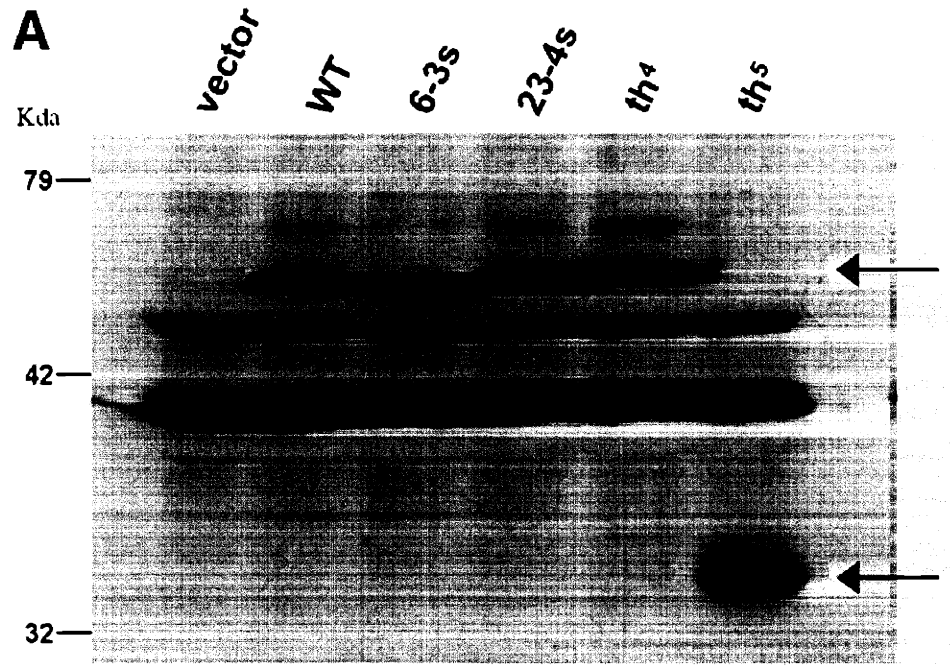

6-3s G88S
45-2s G88D
21-2s P105S
23-4s G269S
23-8s G269S
1
44
110
226
293
387
436
BIR
BIR
11-3e N117K
Th5 W273 STOP
th4 H283Y

Fig. 3. *diap1* mutant alleles maintain their phenotype when expressed in S2 cells. (A) The wild-type and mutants of *diap1* were expressed in S2 cells under the control of a viral promoter with a FLAG epitope tag at the N-terminus of the protein. Protein expression levels were analyzed by immunoblotting equal amounts of protein extracts using an epitope-specific antibody. Epitope-tagged versions of wild-type and mutant proteins are expressed at comparable levels and are indicated with arrows. The th^5 mutant is a truncated protein due to the introduction of a premature stop codon. (B) The wild-type and mutant *diap1* constructs were co-transfected with plasmids for the expression of REAPER and HID in a ratio of 3:4. A β-galactosidase plasmid was also co-transfected as a marker. The numbers of viable cells with β-galactosidase activity were counted and expressed as a percentage of the number of viable blue cells in the wild-type *diap1*-transfected wells, to assess the ability of the mutants of *diap1* to inhibit *reaper*- and *hid*-mediated apoptosis. Except for th^4, all the mutants maintained the phenotypes observed *in vivo* with respect to *reaper*- and *hid*-induced eye ablation (see Table I and text).


A
vector
WT
6-3s
23-4s
th4
th5
Kda
79
42
32

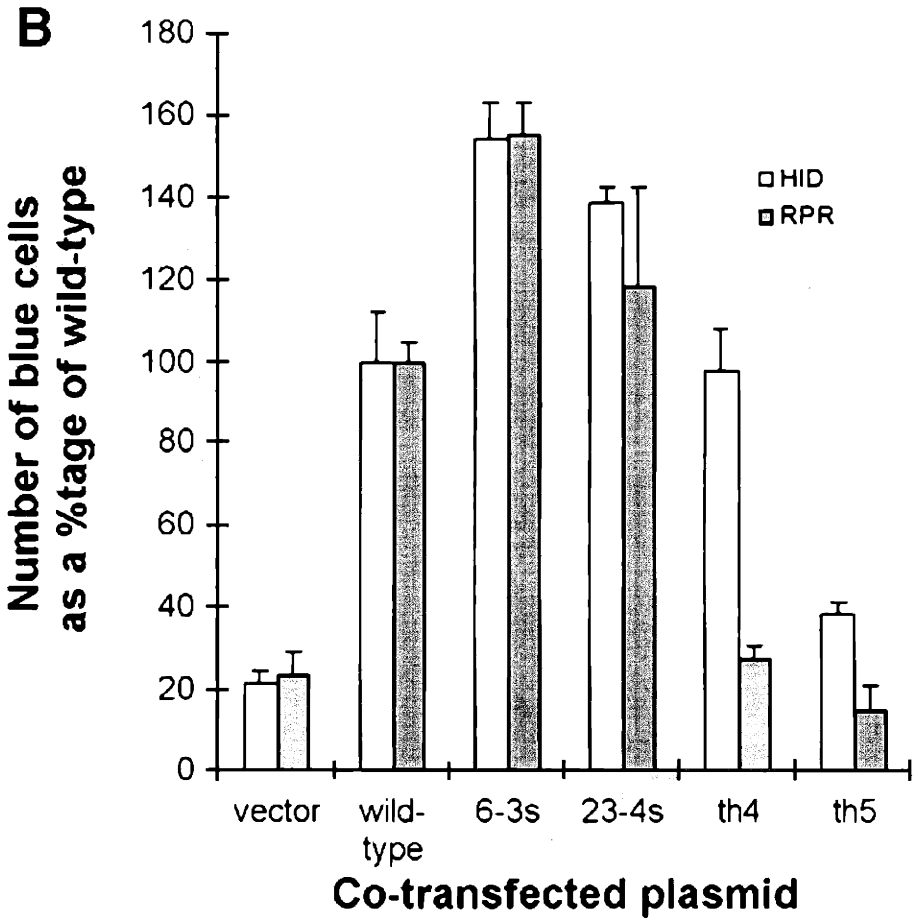

B
Number of blue cells as a %tage of wild-type
180
160
140
120
100
80
60
40
20
0
HID
RPR
vector
wild-type
6-3s
23-4s
th4
th5
Co-transfected plasmid

Fig. 4. Gain-of-function mutations in *diap1* diminish binding to REAPER and HID. Equal amounts of ^{35}S-labeled wild-type and mutant DIAP1 proteins (IVT input) were tested for association with GST, GST-REAPER and GST-HID in an *in vitro* binding assay. Wild-type DIAP1 protein shows specific binding to GST-REAPER and GST-HID. Mutant DIAP1 proteins encoded by $th^{6\text{-}3s}$ and $th^{23\text{-}4s}$ show significantly reduced binding of GST-REAPER and GST-HID as compared with wild-type DIAP1. In contrast, a protein corresponding to th^{4}, a loss-of-function allele of *diap1*, retains essentially normal affinity for REAPER and HID. The lower band visible in most lanes is due to initiation of translation at the second methionine in DIAP1 during *in vitro* synthesis. The th^{5} mutant translates into a truncated protein due to the introduction of a premature stop codon. The IVT input represents 10% of the *in vitro* translated protein used in each pull-down reaction.

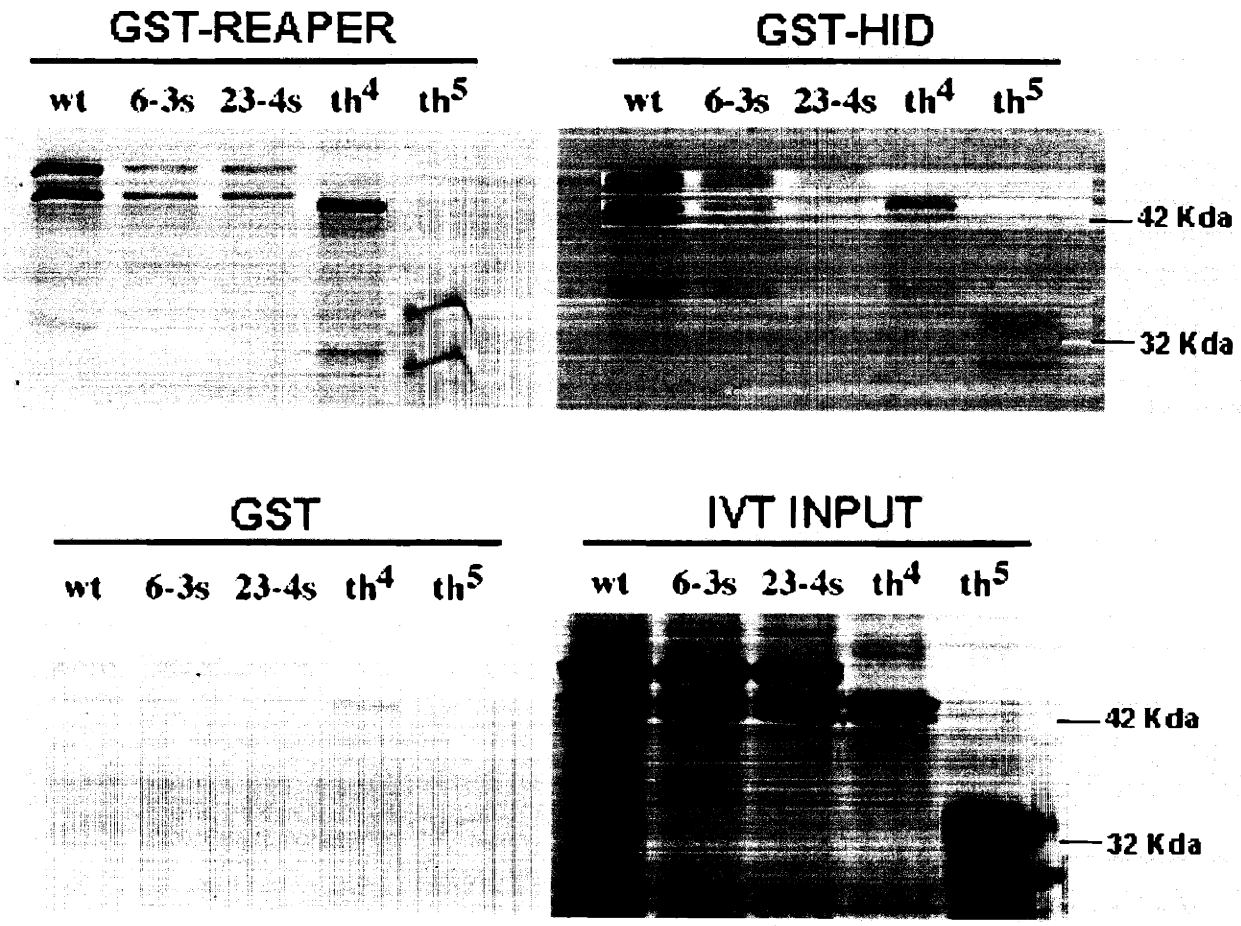

GST-REAPER
wt 6-3s 23-4s th4 th5
GST-HID
wt 6-3s 23-4s th4 th5
42 Kda
32 Kda
GST
wt 6-3s 23-4s th4 th5
IVT INPUT
wt 6-3s 23-4s th4 th5
42 Kda
32 Kda

Fig. 5. *diap1* function is essential to prevent apoptosis. Embryos were labeled using the TUNEL procedure (A, B and C) to detect apoptotic nuclei (White *et al.*, 1994) or with Oligreen (D, E and F) to stain DNA. (A) In wild-type embryos, the first evidence of apoptosis is seen at stage 11; the arrows point to clusters of apoptotic cells. (B) A $th^{11\text{-}3e}$ embryo aged to a physiological age of 7 h (7 h AEL). In *diap1* loss-of-function mutants, such as $th^{11\text{-}3e}$ and th^{5}, essentially all nuclei of embryos are TUNEL positive 7-8 h AEL. (C) $th^{11\text{-}3e}$ *Df(3L)H99* double mutant embryos. As with $th^{11\text{-}3e}$ embryos, essentially all nuclei of the early *H99* $th^{11\text{-}3e}$ double mutant embryos are TUNEL positive. Therefore, induction of apoptosis in $th^{11\text{-}3e}$ embryos does not require the activity of *reaper*, *hid* and *grim*, indicating that *diap1* functions downstream from these genes. (D) Stage 5 (cellular blastoderm) wild-type embryo. (E) $th^{11\text{-}3e}$ embryo aged to 7 h AEL. At this stage, mutant nuclei have condensed chromatin characteristic of apoptosis, and the embryo is organized abnormally. (F) $th^{11\text{-}3e}$ *Df(3L)H99* double mutant embryos. The absence of *reaper*, *hid* and *grim* does not attenuate the $th^{11\text{-}3e}$ phenotype, since the phenotype of $th^{11\text{-}3e}$ and the double mutant embryo is virtually indistinguishable (compare E and F).

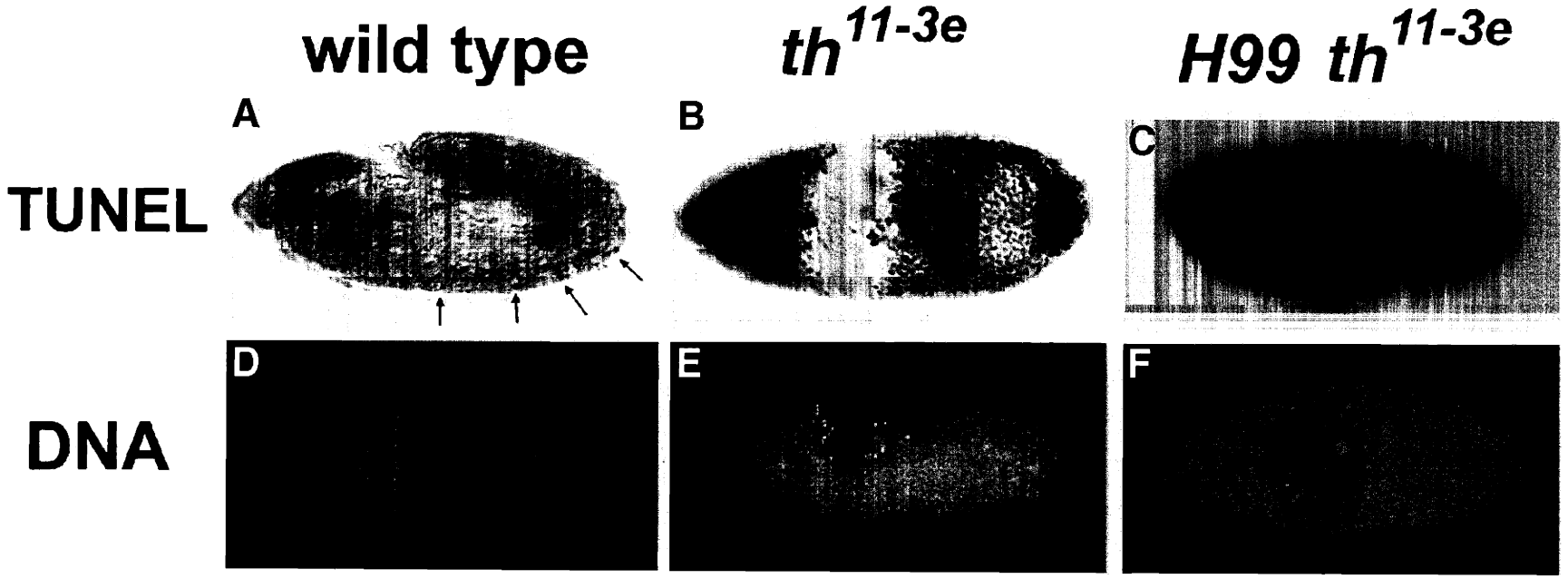

wild type
th11-3e
H99 th11-3e
A
B
C
TUNEL
D
E
F
DNA

Fig. 6. Cell deaths in the *diap1* mutant embryo display the ultrastructural features of apoptosis. (A) Electron micrograph of a stage 6 wild-type embryo. All nuclei have normal `healthy' morphology, and no signs of condensed chromatin can be detected until ~7 h AEL (stage 11) (Abrams *et al.*, 1993). (B-D) Homozygous $th^{11\text{-}3e}$ mutant embryos were selected by morphology 4 h after egg laying and processed for electron microscopy. Representative micrographs of electron-dense nuclei from $th^{11\text{-}3e}$ homozygous mutant embryos showing condensed chromatin characteristic of apoptosis. The scale bar as represented in (D) is 1 μm.

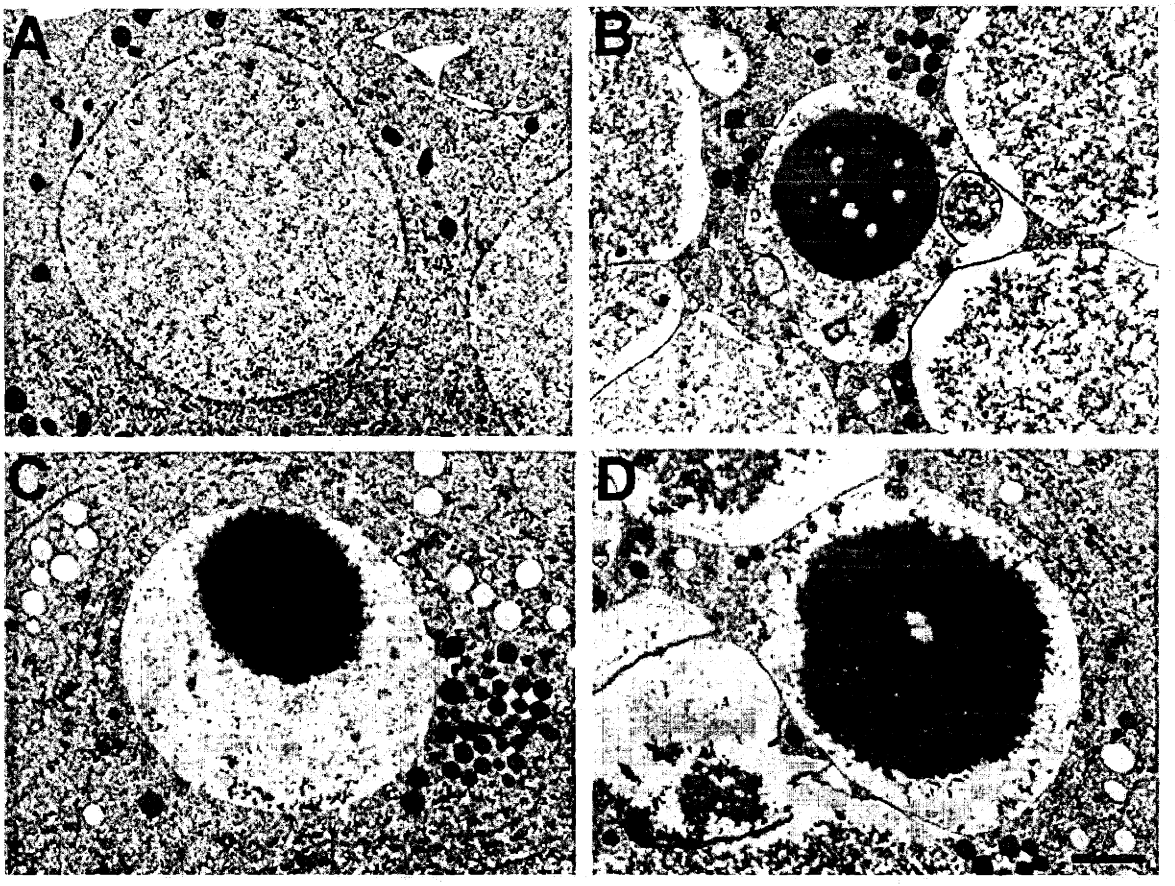
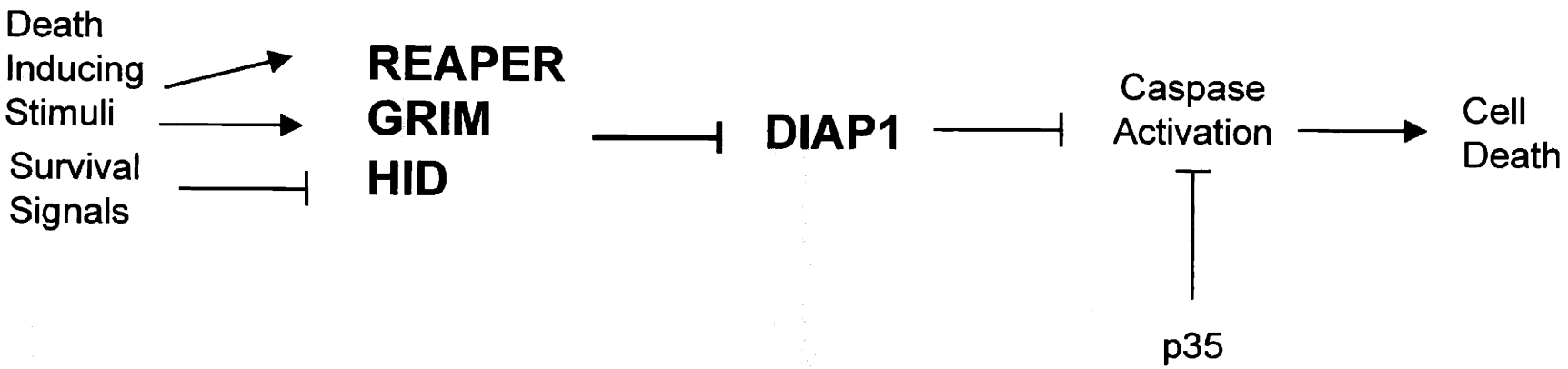

A
B
C
D

Fig. 7. *diap1* functions downstream of *reaper*, *hid* and *grim* to inhibit caspase activation. The `double inhibition' model presented here and by Wang *et al.* (1999) indicates that *diap1* is required to keep the caspases in check. The binding of *reaper*, *hid* and *grim* to *diap1* results in the release of inhibition and therefore the activation of caspases and apoptosis.


Death
Inducing
Stimuli
Survival
Signals
REAPER
GRIM
HID
DIAP1
Caspase
Activation
Cell
Death
p35

Chapter 5.

Genetic Identification of *Drosophila* BRUCE Implicates Ubiquitination in the Regulation of Rpr- and Grim-Induced Cell Death

Abstract

Protein ubiquitination and proteasome-mediated degradation has long been implicated in the regulation of programmed cell death, yet the precise role for this modification in established apoptotic pathways remains elusive. Here we describe the identification of the *Drosophila* homolog of BRUCE, a large baculovirus IAP repeat (BIR) and ubiquitin conjugation enzyme (UBC) domain containing protein. Mutations in *dBRUCE* enhance Reaper- and Grim-dependent cell death phenotypes indicating that this gene functions to inhibit Reaper- and Grim-induced apoptosis. Most *dBRUCE* mutants eliminate the UBC domain suggesting that dBRUCE-mediated protein ubiquitination plays a central role in the regulation of programmed cell death. Interestingly, most *dBRUCE* mutants do not affect Hid-induced death arguing that Hid function is not regulated by dBRUCE-mediated ubiquitination. These phenotypes are reminiscent of those observed in Diap1 RING mutants; thus we propose a model whereby Diap1 and dBRUCE function together as a ubiquitin E2/E3 complex that targets pro-apoptotic proteins, perhaps caspases or Reaper and Grim, for proteasome-mediated degradation.

Introduction

The removal of unnecessary or harmful cells via programmed cell death, or apoptosis, is critical for proper development and for protection against disease (Jacobson et al., 1997). In *Drosophila*, the genes *reaper* (*rpr*), *head involution defective* (*hid*), and *grim*, function in the activation of cell death (Chen et al., 1996b; Grether et al., 1995; White et al., 1994). The 75C1,2 region of the *Drosophila* genome, where these three genes are clustered, is required for cell death, as deletion of this region results in the elimination of virtually all cell deaths in the developing embryo (White et al., 1994). Furthermore, the expression of either *rpr*, *hid*, or *grim* is sufficient to induce apoptosis in a variety of *Drosophila* tissues as well as in cell culture (Chen et al., 1996a; Chen et al., 1996b; Grether et al., 1995; McNabb, 1997; Vucic et al., 1998; Vucic et al., 1997a; White et al., 1996; Wing et al., 1998; Zhou et al., 1997).

Rpr-, Hid- and Grim-induced apoptosis is mediated by caspases, members of a large, conserved family of cysteine proteases that play a central role in the execution of apoptosis (Hay, 2000). The cleavage of caspase substrates including nuclear lamins and actin contributes to the ordered dismantling of the cell characteristic of apoptotic cell death (Earnshaw et al., 1999). The involvement of caspases in Rpr-, Hid- and Grim-induced apoptosis was first demonstrated by the finding that p35, a caspase inhibitor from baculovirus, blocks Rpr-, Hid-, and Grim-dependent death (Chen et al., 1996b; Grether et al., 1995; Hay et al., 1995; Vucic et al., 1998; Vucic et al., 1997a; White et al., 1996). The *Drosophila* genome encodes seven caspases and Rpr, Hid, or Grim have been shown to activate some of these (Kumar and Doumanis, 2000).

The inhibitor of apoptosis proteins, or IAPs, play an important role in inhibiting caspase activity. These proteins were initially identified in baculoviruses based on their ability to substitute for the caspase inhibitor, p35 (Crook et al., 1993). Since that time, IAP homologs have been identified in a wide variety of organisms including *Drosophila* and mammals (Deveraux and Reed, 1999). Several of the mammalian IAPs have been shown to bind and inhibit caspases *in vitro* (Deveraux et al., 1998; Deveraux et al., 1997; Roy et al., 1997). IAP proteins are characterized by the presence of one or more baculovirus IAP repeats, or BIR, domains. The BIR domain of IAPs is required and is in

some cases sufficient for both binding and inhibition of caspases suggesting that inhibition is achieved through direct binding (Deveraux and Reed, 1999; Takahashi et al., 1998).

In *Drosophila*, caspase activation is negatively regulated by the IAP, Diap1. Loss of Diap1 function results in increased caspase activity and widespread cell death in the developing embryo (Goyal et al., 2000; Lisi et al., 2000; Wang et al., 1999). In addition, Diap1 blocks the death induced by the caspases, Dcp1, Drice, Dronc, STRICA/Dream, and DAMM/Daydream in transgenic flies, insect cells, yeast or in vitro (Doumanis et al., 2001; Harvey et al., 2001; Hawkins et al., 1999; Hawkins et al., 2000; Kaiser et al., 1998; Meier et al., 2000; Quinn et al., 2000; Song et al., 2000; Wang et al., 1999). Furthermore, a physical interaction has been shown between the Diap1 protein and the caspases, Drice and Dronc (Kaiser et al., 1998; Meier et al., 2000; Quinn et al., 2000). A fragment of Diap1 including one BIR domain is sufficient for the interaction between Diap1 and Dronc suggesting that the BIR domain of Diap1, like that of mammalian IAPs, is important for caspase binding (Meier et al., 2000). Interestingly, Diap1 also interacts with the cell death activators, Rpr, Hid and Grim via its BIR domains (Vucic et al., 1998; Vucic et al., 1997b; Wu et al., 2001).

Since Diap1 interacts with Rpr, Hid and Grim as well as with caspases, a model in which Rpr, Hid, and Grim induce apoptosis by binding to Diap1 and relieving caspases of Diap1-mediated inhibition has been proposed (Goyal et al., 2000; Kaiser et al., 1998; Lisi et al., 2000; Wang et al., 1999). In the absence of apoptotic signals, Diap1 binds to and inhibits the proteolytic activity of caspases. In order to induce programmed cell death, Rpr, Hid and Grim bind to Diap1 and relieve caspases of inhibition. Thus, caspases are activated and cell death is induced. This model is supported by the ability of Rpr, Hid and Grim to overcome Diap1-mediated caspase inhibition in a reconstituted system in yeast (Wang et al., 1999). The model is further supported by the isolation of Diap1mutants that show weakened interactions with Rpr and Hid and are less sensitive to Rpr and Hid induced cell death (Goyal et al., 2000). Although no mammalian Rpr, Hid, or Grim orthologs have been identified, the mammalian protein Smac/DIABLO is thought to function in an analogous manner, by

binding to IAP BIR domains and interfering with IAP-mediated caspase inhibition (Du et al., 2000; Verhagen et al., 2000).

Most IAPs including Diap1 contain a RING finger motif in addition to BIR domains (Hay, 2000). The relatively recent realization that many RING finger proteins function in ubiquitination has implicated RING containing IAPs in ubiquitination and proteasome-mediated protein degradation (Joazeiro and Weissman, 2000). In fact, such ubiquitination activity has been shown for the mammalian IAPs, XIAP and c-IAP-1 (Huang, 2000; Yang et al., 2000).

The ubiquitination of proteins involves a series of reactions catalyzed by different classes of enzymes. First, ubiquitin is activated, in an ATP dependent process, by covalent linkage to a ubiquitin-activating enzyme, or E1. Ubiquitin is then transferred to a ubiquitin-conjugating enzyme, or E2. A third class of enzymes, ubiquitin-protein ligases or E3s, interacts both with protein substrates and with E2 enzymes and is thought to bring the substrate into proximity with the E2 and facilitate the transfer of ubiquitin to the substrate. So far, the E3 proteins studied fall into two classes characterized by the type of motif they carry, HECT domain E3s or RING finger E3s. In most cases ubiquitination of a protein substrate leads to its proteasome mediated degradation (Hershko and Ciechanover, 1998).

The ubiquitin and proteasome-mediated protein degradation system has been implicated in cell death in a number of studies. An increase in the abundance of polyubiquitin mRNA, ubiquitin protein, and ubiquitin-protein conjugates during or just prior to cell death has been observed in a variety of systems. An increase in the abundance of proteasome subunits and proteasome activity has also been observed. Moreover, treating cells with proteasome inhibitors in some cell types induces apoptosis while in other cells such treatment blocks apoptosis (Orlowski, 1999).

Perhaps the most intriguing implication of the ubiquitin system in apoptosis has been the identification of the mouse gene encoding BRUCE and its human ortholog, Apollon. These proteins are unusual in that they are enormous (530kD) and they contain an N-terminal BIR domain and a C-terminal UBC domain while the rest of the protein shows no significant homology to other proteins in the database (Chen et al.,

1999; Hauser, 1998). The presence of a UBC domain, the catalytic domain of ubiquitin conjugating enzymes, or E2s, suggests that these proteins function as E2 enzymes. Consistent with this, the UBC domain of mBRUCE can be charged with ubiquitin in vitro (Hauser, 1998). The presence of the BIR domain, a domain found in IAPs, suggests that these proteins function in cell death. Treatment of chemotherapy resistant cells that express high levels of Apollon with antisense Apollon sensitizes them to apoptosis (Chen et al., 1999). Thus, mBRUCE and Apollon likely function as E2s in a process that inhibits cell death.

Here we present the identification and characterization of *dBRUCE*, the *Drosophila* ortholog of mBRUCE and Apollon. Loss-of-function *dBRUCE* mutants enhance Rpr- and Grim-mediated apoptosis but have little effect on Hid-induced death suggesting that dBRUCE functions to inhibit Rpr- and Grim-induced cell death. The presence of sequence motifs implicated in ubiquitination in both dBRUCE and Diap1 and their similar phenotypes suggests a model in which Diap1 and dBRUCE function together to inhibit Rpr- and Grim-induced cell death, presumably by ubiquitinating pro-apoptotic proteins and targeting them for degradation.

Results

mod86, a dominant modifier of Rpr- and Hid-induced cell death

In order to identify components of the Rpr and Hid apoptotic pathways, we performed genetic screens for dominant modifiers, suppressors and enhancers, of Rpr- and Hid-induced cell death. Dominant modifier screens have been used with great success in *Drosophila* to identify components of various signaling pathways (Karim, 1996; Rebay et al., 2000). These screens utilize a sensitized genetic background in which small perturbations in signaling can easily be detected as visible modification of the starting phenotype. A modest change in the dose of signaling components may sufficiently perturb signaling such that loss-of-function or gain-of-function mutations in the genes that encode signaling components can be recovered as dominant modifiers of the starting phenotype.

The ectopic expression of Rpr or Hid in the developing eye with the *GMR* promoter leads to excess cell death and consequently, an adult eye that is reduced in size, rough, and highly disorganized (Grether et al., 1995; Hay et al., 1995; White et al., 1996). These eye phenotypes are sensitive to the dosage of the *GMRrpr* and *GMRhid* transgenes; more copies of the transgenes lead to more dramatically reduced, rough eyes. The correlation between the severity of the eye phenotypes and the degree of Rpr and Hid activity suggests that the *GMRrpr* and *GMRhid* phenotypes provide appropriately sensitized genetic backgrounds for dominant modifier screens. Loss-of-function mutations in cell death activators are expected to decrease the amount of cell death and suppress the *GMRrpr* and *GMRhid* phenotypes. Loss-of-function mutations in cell death inhibitors, on the other hand, are expected to increase the amount of cell death and enhance the *GMRrpr* and *GMRhid* phenotypes.

We performed two dominant modifier screens aimed at identifying genes involved in Rpr- and Hid-induced cell death. In one of our screens, we started with a moderate rough eye phenotype caused by two copies of *GMRrpr*. We screened about 170,000 F1 progeny of ENU and EMS mutagenized flies for dominant modifiers of the *GMRrpr* phenotype and recovered 42 enhancers and 5 suppressors. We also screened about 300,000 F1 progeny of ENU, EMS, and X-ray mutagenized flies for suppressors of a severe *GMRhid* phenotype and recovered approximately 170 suppressors.

We examined whether these modifiers were specific to the Rpr or Hid pathways or whether they affected cell death more generally. We tested whether mutants isolated as modifiers of *GMRrpr* also modified *GMRhid* and vice versa. We further tested whether these modifiers affected *GMRgrim*, which produces a reduced, rough eye phenotype similar to *GMRrpr* and *GMRhid*.

Two particularly interesting complementation groups emerged from this analysis. The first group corresponds to the previously described gene *diap1/thread* (Hay et al., 1995). We isolated *diap1* loss-of-function mutations and two classes of gain-of-function alleles. Loss-of-function alleles enhance Rpr, Grim, and Hid (Goyal et al., 2000; Hay et al., 1995; Lisi et al., 2000) while one class of gain-of-function alleles suppresses Rpr, Grim, and Hid (Goyal et al., 2000). The second class of gain-of-function alleles

suppresses Hid but enhances Rpr and Grim (Goyal et al., 2000; Lisi et al., 2000). This duality is intriguing as it suggests that Diap1 acts differently in Hid-induced death than in Rpr- and Grim-induced death.

Interestingly, we also identified twelve alleles of another gene, this one previously uncharacterized, that can be mutated to enhance Rpr and Grim and suppress Hid. Eleven alleles of this gene, which we named *mod86*, were isolated as enhancers of *GMRrpr*. These alleles also enhance *GMRgrim* and have little or no effect on *GMRhid*. (Figure 1) One *mod86* allele was recovered as a suppressor of *GMRhid*. However, this allele enhances *GMRrpr* and *GMRgrim*. (Figure 1) We found it intriguing that *mod86*, like *diap1*, can be mutated to enhance Rpr and Grim but suppress Hid. This suggests that Mod86 functions in a manner similar to Diap1 or perhaps in a complex with Diap1. To investigate this idea further we cloned the *mod86* gene.

Cloning of *mod86*

We mapped two *mod86*-associated phenotypes, dominant *GMRrpr* enhancement and recessive male sterility, to the region between the P-element insertions 044505 and 04369 on the right arm of the third chromosome by standard meiotic recombination as well as P-element induced male recombination (Chen et al., 1998). We further mapped *mod86* with respect to deficiencies in this region that we generated by imprecise excision of the P-element j8B6. *Df(3R)JA-B* fails to complement *mod86* male sterility while *Df(3R)JA-C* does complement indicating that at least part of *mod86* lies within the interval delimited by the breakpoints of these deletions. (Figure 2A)

In order to facilitate molecular characterization, we isolated genomic lambda clones that span this interval. We used sequences adjacent to the breakpoints of *Df(3R)JA-B* as starting points for two converging chromosome walks. The analysis of these lambda clones led to the restriction map shown in Figure 2A and to the estimate that the *mod86* interval spans ~74Kb.

To further localize the *mod86* gene, we screened this ~74Kb interval by genomic Southern blotting for polymorphisms in our twelve *mod86* mutants relative to wildtype. We found one fragment, designated A in figure 2B, that gave rise to a polymorphism in

mod86$^{15\text{-}3e}$. Sequencing this fragment revealed sequence similarity to the C-terminus of the mouse gene *BRUCE*. Three BDGP (Berkeley *Drosophila* Genome Project) ESTs with sequence similarity to the N-terminus of BRUCE were identified. Three additional cDNAs were isolated from embryonic and testis cDNA libraries using genomic fragment A as a probe. A gap in sequence coverage by these two sets of cDNAs was filled by RT-PCR. Additional 5' sequence was obtained by 5' RACE.

We thus assembled a cDNA sequence of 15.5Kb. Comparison of this assembled sequence with the genomic sequence that was made available during the course of this work led to the intron-exon structure shown in Figure 2B. This gene is comprised of 31 exons and corresponds to predicted gene product CG6303/dBRUCE although the cDNA we assembled differs slightly with that predicted by the *Drosophila* genome project.

mod86 encodes an ortholog of mouse BRUCE and human Apollon

mod86 is predicted to encode dBRUCE, an enormous protein of 4852 amino acids with an N-terminal BIR domain and a C-terminal UBC domain (Figure 2C). Mod86 is 30% identical overall to mouse BRUCE (Figure 3) and human Apollon (data not shown). The degree of conservation between the *Drosophila* and mammalian genes is greatest in the BIR and UBC domains, showing 83% and 87% identity, respectively. Plotting percent identity of consecutive non-overlapping 50 a.a. fragments highlights several additional regions which are highly conserved between the *Drosophila* and mammalian genes (Figure 3B). In particular, the regions around amino acids 1750-1800, 4050-4150, and 4300-4350 show 60% identity or greater, suggesting other domains potentially important for BRUCE function. Comparison of these conserved regions to the database failed to reveal significant similarity to any other known protein motifs.

Molecular characterization of *mod86/dBRUCE* mutant alleles

In order to demonstrate that *dBRUCE* corresponds to *mod86*, we sequenced the *dBRUCE* open reading frame and intron-exon boundaries in ten of our *mod86* alleles.

We identified molecular lesions in nine of the ten alleles (Table 1 and Figure 4) indicating that *mod86* is *dBRUCE*.

Since *Df(3R)JA-B*, the deficiency that removes *dBRUCE*, behaves as a strong dominant enhancer of *GMRrpr* and *GMRgrim* and has little effect on *GMRhid*, the eleven alleles of *dBRUCE* that behave similarly are considered loss-of-function. Most of the loss-of-function *dBRUCE* mutations we identified are predicted to result in the loss of either the BIR domain or UBC domain indicating that these domains are critical for the proper function of dBRUCE. Allele $dBRUCE^{8\text{-}1e}$ carries an in-frame deletion that removes the BIR domain. Alleles $dBRUCE^{2\text{-}3e}$, $dBRUCE^{10\text{-}1e}$, $dBRUCE^{3\text{-}5e}$, $dBRUCE^{4\text{-}1e}$, $dBRUCE^{13\text{-}6e}$, and $dBRUCE^{3\text{-}1e}$ are all predicted to produce truncated proteins that do not have the UBC domain. Mutation *15-3e* results in an in-frame deletion that removes a region of 58 amino acids situated about 800 residues upstream of the UBC domain that is not particularly conserved between dBRUCE and the mammalian BRUCEs. This mutation might destabilize the dBRUCE protein or perhaps impair the formation of the proper structure of the UBC domain given the relative proximity. No mutations were found in the open reading frame or intron-exon boundaries of $dBRUCE^{2\text{-}2e}$ suggesting that the mutation associated with this allele may lie in a regulatory region.

Since deletion of *dBRUCE* has little effect and may weakly enhance Hid-induced death, the *dBRUCE* allele, *23-6s*, which suppresses *GMRhid* is considered gain-of-function. Interestingly, this allele is predicted to produce the shortest truncated protein of 1233 amino acids. This suggests that expression of the N-terminus of dBRUCE, including the BIR domain, can inhibit the function of Hid. (See discussion.)

Discussion

We isolated *dBRUCE* mutants in dominant modifier screens designed to identify components of the Rpr and Hid cell death pathways. Most of the *dBRUCE* alleles that we isolated enhance Rpr- and also Grim-induced cell death but have little or no effect on Hid-induced death. One particularly interesting *dBRUCE* allele enhances Rpr and Grim but suppresses Hid. This differential effect indicates that mutations in *dBRUCE* do not modify the Rpr and Grim dependent eye phenotypes by simply affecting expression

of the transgene or eye development. If this were the case, *dBRUCE* mutations would have similar effects on Rpr, Grim and Hid when in fact they have no effect or even the opposite effect on Hid. Additionally, *dBRUCE* mutants have no effect on eye phenotypes caused by the ectopic expression of *phyllopod* or *rho1* (Chang et al., 1995; Hariharan et al., 1995)(data not shown), two genes that are not known to be involved in cell death, further showing specificity to Rpr- and Grim-induced cell death. Moreover, since a deficiency that removes *dBRUCE*, like most of the *dBRUCE* mutants we isolated, strongly enhances Rpr and Grim but not Hid, dBRUCE is expected to function primarily to inhibit Rpr- and Grim-induced cell death.

dBRUCE is an ortholog of mouse BRUCE and human Apollon. The *Drosophila* protein shows 30% identity to the mammalian proteins overall but over 80% identity in the BIR and UBC domains. The high degree of conservation in these domains suggests that they are particularly important for BRUCE function. We identified the mutations in nine of our *dBRUCE* alleles. Most of these mutations eliminate the BIR or UBC domains, further indicating the importance of these domains for proper dBRUCE function.

The UBC domain is a highly conserved catalytic domain of ubiquitin conjugating enzymes, or E2 enzymes, that are involved in protein ubiquitination (Hershko and Ciechanover, 1998). The UBC domain of mBRUCE exhibits E2 activity in vitro (Hauser, 1998). The genetics of *dBRUCE* indicate that dBRUCE inhibits Rpr- and Grim-induced death. Thus, dBRUCE may inhibit cell death by ubiquitinating pro-apoptotic components of the Rpr and Grim pathways and thereby targeting them for degradation.

The BIR domain of IAPs functions as a protein-protein interaction domain and has been shown to be involved in IAP binding to the pro-apoptotic proteins, Rpr, Grim, Hid, Smac/DIABLO and caspases (Hay, 2000). The presence of a BIR domain in dBRUCE suggests that it may physically interact with these BIR-binding proteins. The presence of both BIR and UBC domains suggests that dBRUCE may recruit BIR binding proteins and ubiquitinate them. Since dBRUCE primarily inhibits Rpr- and Grim-induced death, potential substrates for dBRUCE include Rpr, Grim, or the caspases they activate.

Several observations suggest that dBRUCE may enlist the function of Diap1 as an E3 partner protein. Diap1 has a RING finger domain, a domain that is found in many E3 proteins and is indispensable for E3 function (Joazeiro and Weissman, 2000). In fact, other RING-containing IAPs have been found to display E3 activity *in vitro* (Huang, 2000; Yang et al., 2000). Both dBRUCE and Diap1 function in the same process, inhibition of Rpr- and Grim-induced death. Finally, Diap1 like *dBRUCE* can be mutated to enhance Rpr and Grim but suppress Hid. Together these observations suggest that dBRUCE and Diap1 function as an E2/E3 complex to inhibit Rpr- and Grim-induced cell death.

We therefore propose a model in which dBRUCE and Diap1 act together to recruit caspases, or Rpr and Grim themselves, via the BIR domain(s) and ubiquitinate them via the UBC and RING domains. Ubiquitination would then lead to the degradation of Rpr, Grim, and/or caspases by the proteasome, thereby limiting the activation of cell death.

A model for Diap1 function has previously been proposed in which Diap1 binds and inhibits caspases. In this model, Rpr, Hid and Grim activate cell death by binding Diap1 and relieving caspases of Diap1-mediated inhibition (Goyal et al., 2000; Kaiser et al., 1998; Lisi et al., 2000; Wang et al., 1999). This model is supported by the direct interactions observed between IAPs including Diap1 and caspases and Rpr, Grim and Hid (Hay, 2000). Additionally, Hay and colleagues have shown Diap1 can block caspase activation in yeast and that Rpr, Grim, and Hid can interfere with this inhibition (Hawkins et al., 1999; Hawkins et al., 2000; Wang et al., 1999). Furthermore, this model is supported by mutations in *diap1* that were isolated as modifiers of Rpr- and Hid-induced cell death. Gain-of-function *diap1* alleles that suppress Rpr-, Grim-, and Hid-induced death carry mutations in the BIR domains of Diap1 and these mutant proteins are impaired in their ability to bind Rpr, Hid and presumably Grim (Goyal et al., 2000).

This model suggests that Rpr, Grim and Hid function similarly to inhibit Diap1 and activate caspases. However, a third group of *diap1* alleles that enhances Rpr- and Grim-induced death but suppresses Hid-induced death suggests that Diap1 can also

function in a Rpr and Grim specific pathway. These mutations are in the RING finger (Lisi et al., 2000) suggesting ubiquitination activity is required for inhibition of Rpr- and Grim-induced death but not for inhibition of Hid-induced death. Interestingly, *dBRUCE* mutants also appear restricted to Rpr and Grim. Thus, it appears that Diap1 may inhibit death, in conjunction with dBRUCE, by ubiquitinating Rpr, Grim, and/or caspases in addition to inhibiting caspases by direct binding.

It is therefore attractive to extend the previous model in which Rpr, Grim and Hid bind Diap1 and relieve caspases of Diap1 mediated inhibition to include the ubiquitination activity of a possible dBRUCE/Diap1 complex. In this new revised model, Diap1 and dBRUCE are part of a complex that binds and ubiquitinates caspases thereby targeting them for degradation. Rpr and Grim bind the dBRUCE/Diap1 or dBRUCE/ Diap1/caspase complex such that it can no longer ubiquitinate caspases. Thus, caspases are stabilized and can activate cell death. Furthermore, Rpr and Grim may be decoy substrates for the dBRUCE/Diap1 E2/E3 complex. Perhaps when Rpr and Grim bind dBRUCE/Diap1, they themselves are ubiquitinated and degraded. This would explain the observation that the Rpr protein becomes unstable under conditions in which cell death is induced (Vucic et al., 1997b).

The mutations in *dBRUCE* or *diap1* that enhance Rpr- and Grim- but not Hid-induced death can be explained in the context of this model. Mutations in the Diap1 RING or in dBRUCE would impair the ubiquitination activity of the complex so that caspases aren't degraded as efficiently. These mutations would lead to increased caspase activity and would thus appear as enhancers. Since *dBRUCE* loss-of-function mutants have no affect on Hid-induced death, Hid is not expected to interact with a dBRUCE/Diap1 complex normally. Instead, Hid is likely to interfere primarily with the inhibition of caspases by direct binding of Diap1. Thus, loss-of-function mutations in *dBRUCE* or mutations in the Diap1 RING domain do not enhance Hid-induced death.

However, in addition to enhancing Rpr- and Grim-induced death, mutations in the Diap1 RING and one *dBRUCE* allele, which eliminates the C-terminal three quarters of dBRUCE including the UBC domain, suppress Hid-induced death. Since loss of neither *diap1* nor *dBRUCE* suppresses Hid-induced death, the ability of the Diap1 RING finger

mutants and the one dBRUCE allele to do so is considered to be a novel property of these mutant proteins. There are several possibilities for how this suppression comes about. Mutant Diap1 with the RING finger impaired may be a more potent inhibitor of caspase activity by for example, binding caspases more strongly. Hid then would be less able to overcome this inhibition to activate caspases. Alternatively, the mutant version may bind Hid less well, thus being less sensitive to Hid-mediated stimulation. The Hid suppressing *dBRUCE*$^{23\text{-}6s}$ allele, which is the shortest truncation, might now be able to bind Hid via its BIR and prevent it from binding a Diap1/caspase complex. Under normal conditions Hid could be prevented from binding the dBRUCE BIR due to steric factors. For example the geometry of dBRUCE may allow only small molecules like Rpr and Grim to access the BIR domain. Hid is significantly larger, 45kD, than Rpr, 7kD or Grim, 15kD. Interestingly, the next shortest dBRUCE protein, 1438 amino acids, produced by allele *3-1e*, has no effect on Hid. Perhaps this mutant protein is large enough to prevent Hid access to the BIR domain. This *dBRUCE* allele also has a second lesion, a T to I substitution at residue 404, just outside the BIR domain. This second lesion could destabilize the protein or alternatively, interfere with BIR function such that it does not bind Hid.

In summary, dBRUCE inhibits Rpr and Grim induced cell death probably by ubiquitinating caspases and/or Rpr and Grim themselves, thereby targeting them for proteasome mediated degradation. dBRUCE's mammalian counterparts, mBRUCE and Apollon, may function similarly. Although no structural homologs of Rpr and Grim have been described, the mammalian proteins Smac/DIABLO are thought to be functional analogs of Rpr, Hid and Grim. Therefore, mBRUCE/Apollon may inhibit Smac/DIABLO induced death by ubiquitinating caspases and/or Smac/DIABLO themselves and targeting them for proteasome mediated degradation.

Currently, proteasome inhibitors are being studied for their efficacy as cancer therapy. One key attribute of tumor growth is the uncontrolled division of transformed cells. Cells have numerous mechanisms to check uncontrolled cell division including the activation of apoptosis. For a tumor to develop, this apoptotic control of excess cell division must be lost. Many chemotherapeutic agents are designed to induce cell death

(Thompson, 1995). Yet, tumors often become resistant to these agents. Therefore, new therapies that can stimulate the death of cancer cells are of great interest. To this end, proteasome inhibitors are in clinical trials based on their known ability to block NF-κB activation, an inhibitor of programmed cell death (Rivett and Gardner, 2000). Perhaps, these drugs will be doubly beneficial because of their potential to inhibit BRUCE mediated ubiquitination and proteasome mediated degradation of apoptotic inducers.

Experimental Procedures

Drosophila Stocks

GMRrpr 81 (White et al., 1996), *GMRrpr 46*, *GMRhid 1M* (Bergmann et al., 1998), *GMRhid 10* (Grether et al., 1995), *GMRgrim* (Chen et al., 1996b), *GMRphyl* (Chang et al., 1995), *GMRrho1* (Hariharan et al., 1995).

l(3)j9A5, *l(3)04369*, and *l(3)j8B6* were obtained from the Bloomington Stock Center. *l(3)044505* was obtained from the Szeged Stock Center.

Flies were raised on standard cornmeal-molasses medium at 25°C.

Genetics

For the *GMRrpr* screen, *yw*; *GMRrpr 81* homozygous males were fed a solution of sucrose and 0.25mg/ml ENU or 25 mM EMS and then crossed to *yw*; *GMRrpr 81* homozygous females. The F1 progeny were screened for suppression or enhancement of the parental phenotype. Approximately 95% of the 170,000 F1 progeny screened were derived from ENU treated males, while 5% were from EMS treated males. For the *GMRhid* screen, *yw* males were fed a solution of sucrose and 0.25 mg/ml ENU or 25 mM EMS or treated with 4500 rad X-rays and then crossed to *GMRhid 10* homozygous females. The F1 progeny were screened for suppression of the *GMRhid 10* phenotype. Approximately 49% of the 300,000 F1 progeny screened were derived from EMS treated males, 49% from X-ray treated males, and 2% from ENU treated males.

Complementation analysis was performed on mutants on the same chromosome exhibiting similar recessive phenotypes. Male sterility was assessed by mating twenty

homozygous mutant males individually to Canton-S females. The mutants were considered to display some degree of sterility if fewer than 20% of the crosses gave rise to more than 20 progeny. Control crosses regularly gave rise to at least 40 progeny.

The dominant eye modification and recessive male sterility phenotypes associated with *mod86/dBRUCE* were mapped between P-element insertion *l(3)j9A5* and the visible marker *cu* by standard meiotic recombination and then between *l(3)044505* and *l(3)04369* by P-element induced male recombination.

Deletions in the *mod86/dBRUCE* region were isolated by imprecise excision of *l(3)j8B6*. Since P-element disruption or deletion of *mod86/dBRUCE* is expected to enhance *GMRrpr*, *l(3)j8B6/Δ2-3 Sb* males were crossed to *GMRrpr 46* homozygous females and the resulting progeny were screened for enhancers of the *GMRrpr 46* eye phenotype.

Scanning Electron Microscopy

Flies were raised in parallel on standard cornmeal-molasses medium at 25°C. Just prior to eclosion, pupae were transferred to empty vials. After eclosion, adult flies were anaesthetized and dehydrated through an ethanol series: 25, 50, 75, 2x100% ethanol. Critical point drying, sputter coating with gold palladium, and microscopy were performed at the Northeastern University SEM facility.

Molecular analysis of the *mod86/dBRUCE* region

The chromosome walks were initiated with probes flanking the breakpoints of *Df(3R)JA-B*. *Df(3R)JA-B* was isolated by mobilizing P-element *l(3)j8B6* and screening for possible P-element disruption or deletion of *mod86/dBRUCE* (see above). In *Df(3R)JA-B*, the P-element *l(3)j8B6* remained in its original position with proximal sequences deleted. Genomic sequences on either side of *l(3)j8B6* were isolated by plasmid rescue and used to probe a genomic lambda library by standard methods (library from Ron Davis). The genomic clones isolated were analyzed by restriction analysis and by Southern blot. After three rounds of screening with probes derived from the lambda clone ends, the two chromosome walks converged and a physical map spanning 110 Kb was

assembled. The proximal breakpoint of *Df(3R)JA-C* was localized on the physical map by genomic Southern analysis and the *mod86/dBRUCE* interval was estimated to be 74 Kb.

The 74 Kb *mod86/dBRUCE* interval was screened for polymorphisms in *mod86/dBRUCE* mutants relative to wildtype by genomic Southern blot. Genomic DNA was prepared from homozygous *mod86/dBRUCE* or *mod86/dBRUCE /Df(3R)JA-B* flies as well as parental *yw* and *yw; GMRrpr 81* flies for comparison. Genomic DNA from all twelve *mod86/dBRUCE* mutants and parental controls was subjected to three different restriction digests- BamHI and HindIII, BglII and DraI, and XhoI and SspI. Twenty-two individual EcoRI fragments spanning the 74Kb were used as probes.

cDNA clones were isolated from an embryonic library (LD, BDGP) and from an adult testis library (T. Hazelrigg). A 1.6 Kb BstXI, BglII subclone from fragment A in Figure 2 was used as a probe. Two cDNA clones were isolated from the LD library and one from the testis library. Sequence analysis indicated that the clones span 5Kb and include the 3' end of the gene.

The ESTs, LD31268, GH10317, and LP05246, were identified by the BLAST algorithm as encoding proteins homologous to the N-terminus of mouse BRUCE. These clones span 6Kb and do not contain any stop codons.

An additional 4Kb intervening the ESTs and cDNAs described above was isolated by RT-PCR. Total RNA from adult males was isolated (Trizol, GibcoBRL), treated with DNase I, and used as template for first strand cDNA synthesis using the Superscript Preamplification System (GibcoBRL). The reaction was primed with random hexamers. PCR was performed using the Elongase enzyme mix (GibcoBRL) according to the protocol provided.

Additional 5' sequence was obtained by 5' RACE using the SMART RACE cDNA Amplification kit (Clontech) and total RNA from adult males as template. The RACE product was cloned into pCR2.1 (Invitrogen) and ten individual clones were analyzed. The longest product analyzed extended the LD31268 sequence by about 160nt. This sequence contained stop codons and was therefore considered to represent the 5' end of the ORF.

Sequencing was mainly performed using an automated sequencer (Research Genetics). Genomic and cDNA clones were subcloned prior to sequencing. PCR products were purified with Qiaquick columns (Qiagen) and sequenced directly. Sequences were analyzed and assembled using Sequencher software (GeneCodes).

Mutation Detection

Genomic DNA was isolated from homozygous *mod86IdBRUCE* mutant adult flies as well as the *GMRrpr 81* parental strain. For *mod86IdBRUCE* alleles which were lethal or semi-lethal, *mod86IdBRUCE /Df(3R)JA-B* adults were used. The *mod86IdBRUCE* ORF was amplified by PCR by 35 primer pairs designed to produce overlapping products of approximately 700bp each. The PCR products were purified and sequenced as above.

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Figure 1. *mod86* mutants were recovered as enhancers of *GMRrpr* and as suppressors of *GMRhid*.

Eleven alleles, including the representative allele shown, *3-5e*, enhance *GMRrpr* (compare (D) to (A)) and *GMRgrim* (compare (E) to (B)) but have little or no effect on *GMRhid* (compare (F) to (C)). One allele, *23-6s*, suppresses *GMRhid* (compare (I) to (C)) but enhances *GMRrpr* (compare (G) to (A)) and *GMRgrim* (compare (H) to (B)). Genotypes are as follows- (A) *GMRrpr46/+*; (B) *GMRgrim/+*; (C) *GMRhid 1M/+*; *+/+*; (D) *GMRrpr 46/mod86*$^{3\text{-}5e}$; (E) *GMRgrim/ mod86*$^{3\text{-}5e}$; (F) *GMRhid 1M/+*; *mod86*$^{3\text{-}5e}$*/+*; (G) *GMRrpr 46/mod86*$^{23\text{-}6s}$; (H) *GMRgrim/ mod86*$^{23\text{-}6s}$; (I) *GMRhid1M/+*; *mod86*$^{23\text{-}6s}$*/+*.

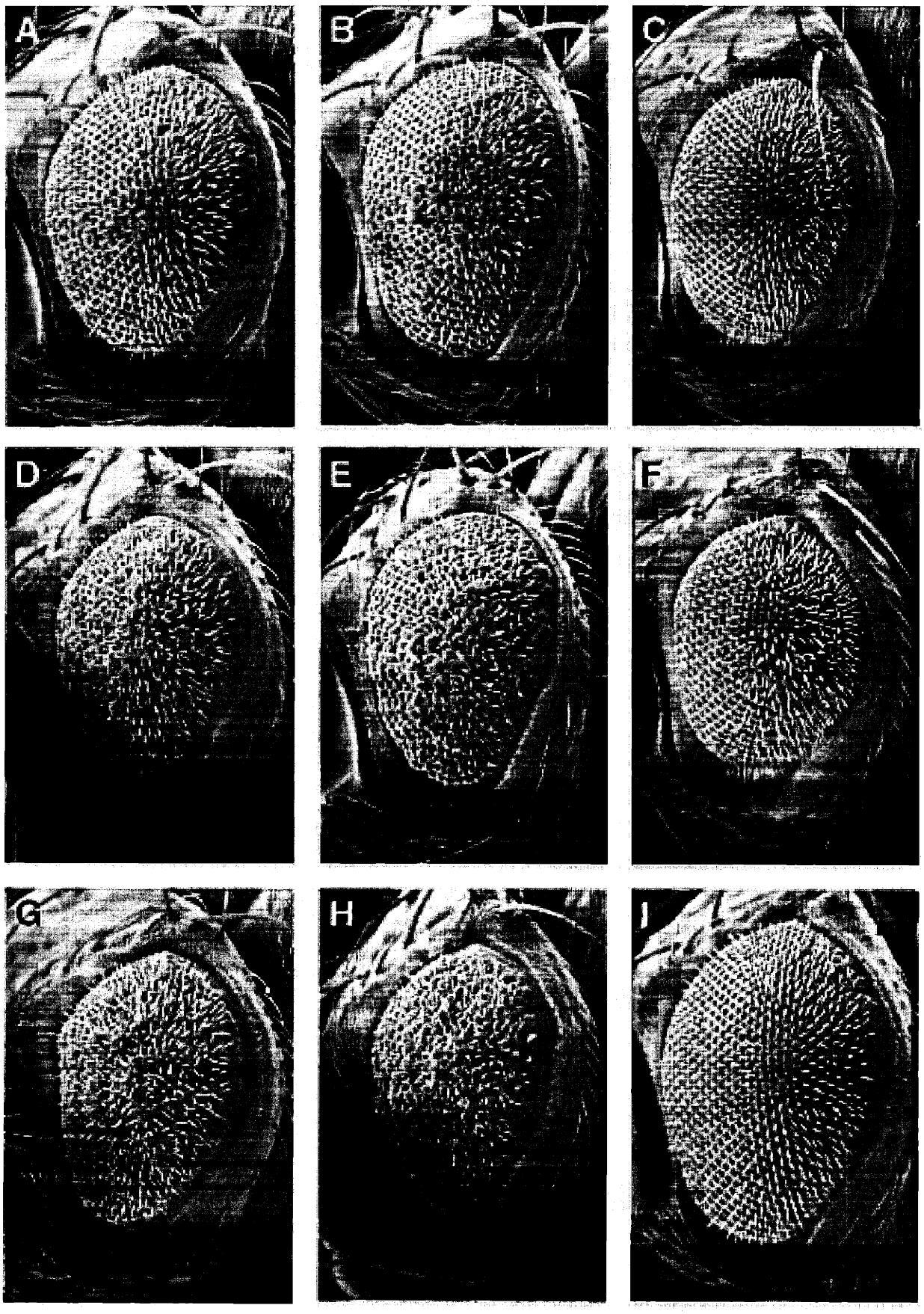
A
B
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Figure 2. Molecular characterization of the *mod86* locus.

(A) *mod86* was mapped to a 74Kb region delimited by the breakpoints of the deficiencies *Df(3R)JA-B* and *Df(3R)JA-C*. *Df(3R)JA-C* complements the male sterility associated with *mod86* while *Df(3R)JA-B* does not indicating that *mod86* lies in the region deleted by *Df(3R)JA-B* but not *Df(3R)JA-C*. 100Kb of the genomic region surrounding *mod86* is shown as a horizontal black line at the top of the panel. EcoRI sites are depicted as vertical bars above the line and the scale of the genomic map is shown below the line. The P-element *l(3)j8B6* that was used to generate the two deficiencies shown is depicted as an inverted triangle. The region deleted by the deficiencies is shown as horizontal black lines bounded by vertical bars below the genomic map. Uncertainty about the extent of the distal breakpoint of *Df(3R)JA-C* is indicated by a question mark. The genomic map reads left to right as proximal to distal.

(B) Identification of a *mod86* candidate gene. The EcoRI fragment labeled "A" gave rise to a polymorphism in a genomic Southern blot in the *mod86* allele *15-3e* relative to wildtype. Analysis of this fragment revealed a *mod86* candidate gene and led to the assembly of the cDNA depicted at the bottom of the panel. Exons are shown as thick black horizontal lines and introns are illustrated as the intervening triangles.

(C) *Mod86/dBRUCE* protein structure. The *mod86/dBRUCE* gene encodes an enormous protein of 4852 amino acids with an N-terminal BIR domain shown as a black box and a C-terminal UBC domain shown as a gray box.

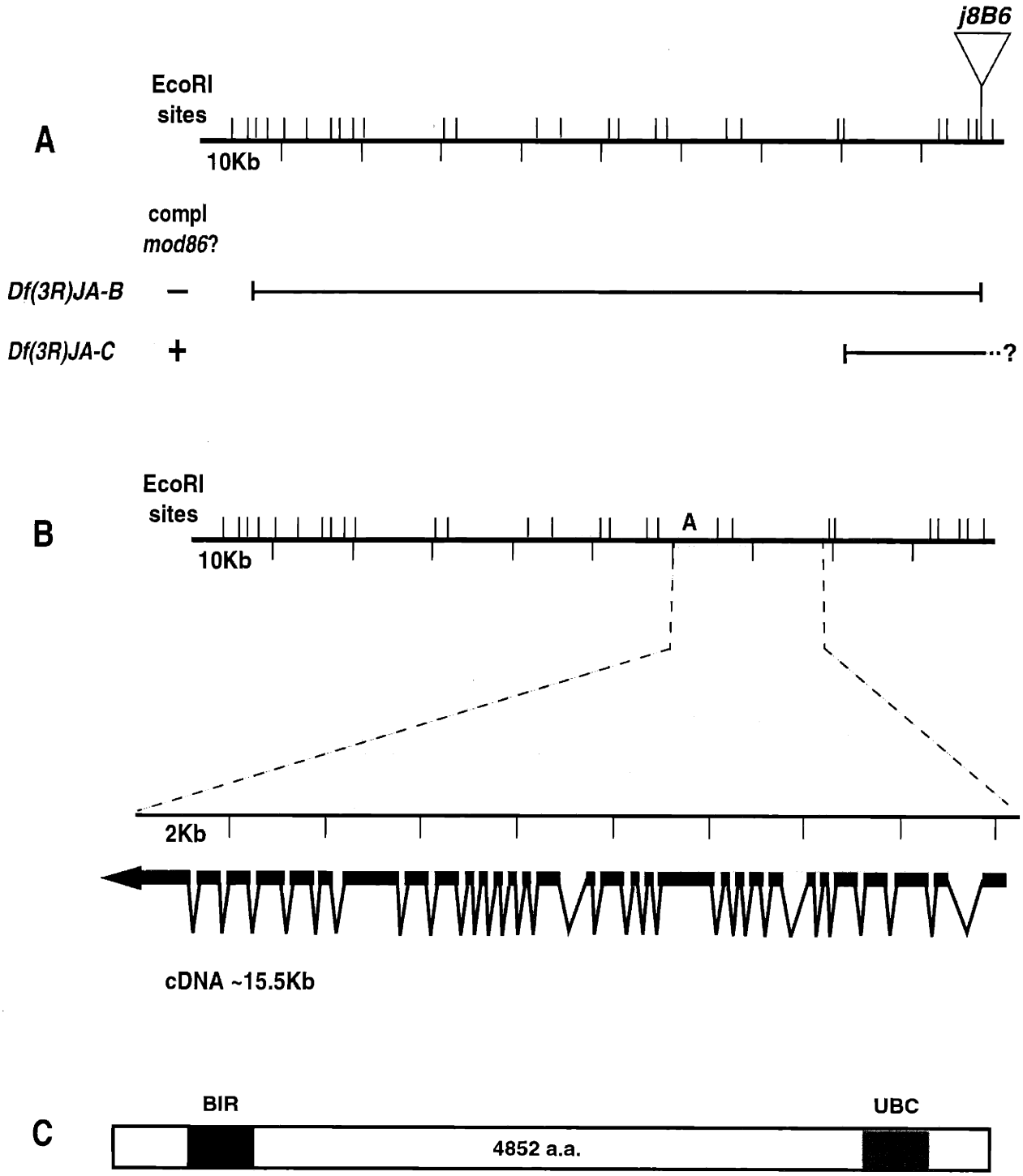
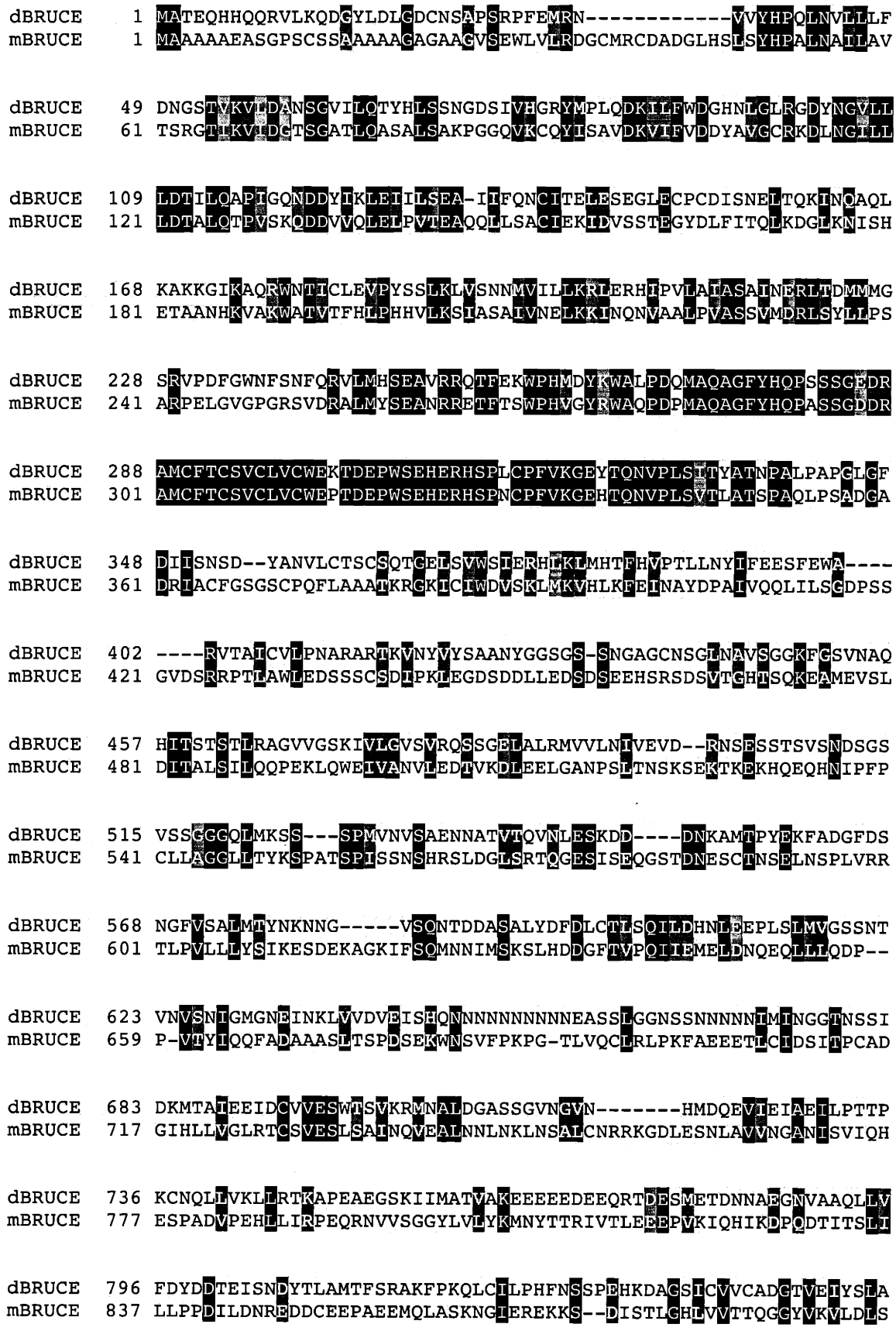

j8B6
A
EcoRI
sites
10Kb
compl
mod86?
Df(3R)JA-B
–
Df(3R)JA-C
+
···?
B
EcoRI
sites
A
10Kb
2Kb
cDNA ~15.5Kb
C
BIR
UBC
4852 a.a.

Figure 3. Comparison of *Drosophila* and mouse BRUCE proteins.

(A) Sequence alignment of dBRUCE and mBRUCE. Numbers indicate amino acid position. Identical residues are shaded in black. Similar residues are shaded in gray. Dashes indicate gaps in the alignment. ClustalW was used to make the alignment.

(B) *Drosophila* and mouse BRUCE proteins are most similar in the BIR and UBC domains but also show other regions of higher similarity. Plotting percent identity of non-overlapping 50 amino acid segments shows that regions around 1750-1800, 4050-4150, and 4300-4350 are also highly conserved.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mBRUCE 61 TSRGTIKVIDGTSGATLQASALSAKPGGQVKCQYISAVDKVIFVDDYAVGCRKDLNGILL
dBRUCE 109 LDTILQAPIGQNDDYIKLEIILSEA-IIFQNCITELESEGLECPCDISNELTQKINQAQL
mBRUCE 121 LDTALQTPVSKQDDVVQLELPVTEAQQLLSACIEKIDVSSTEGYDLFITQLKDGLKNISH
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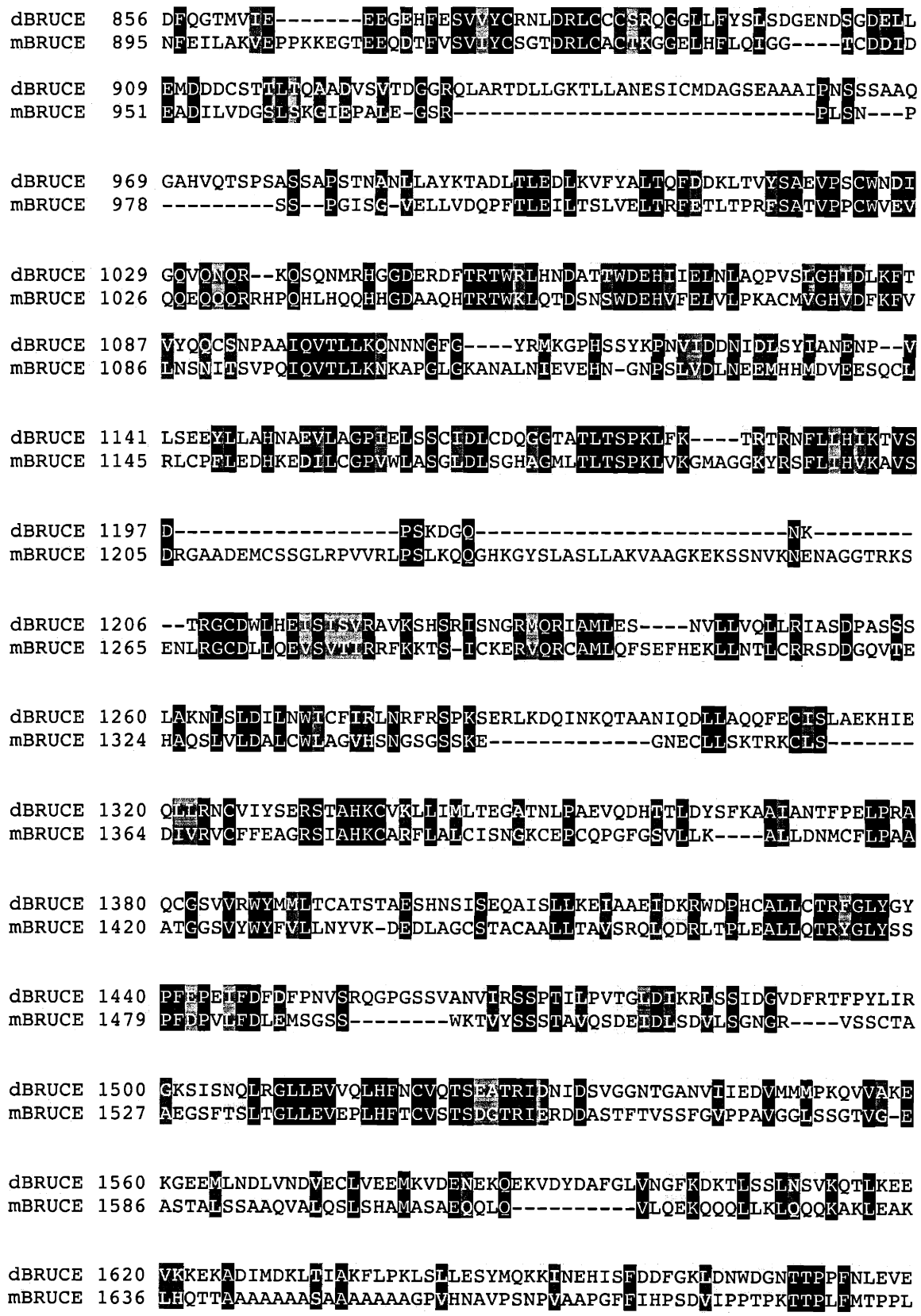
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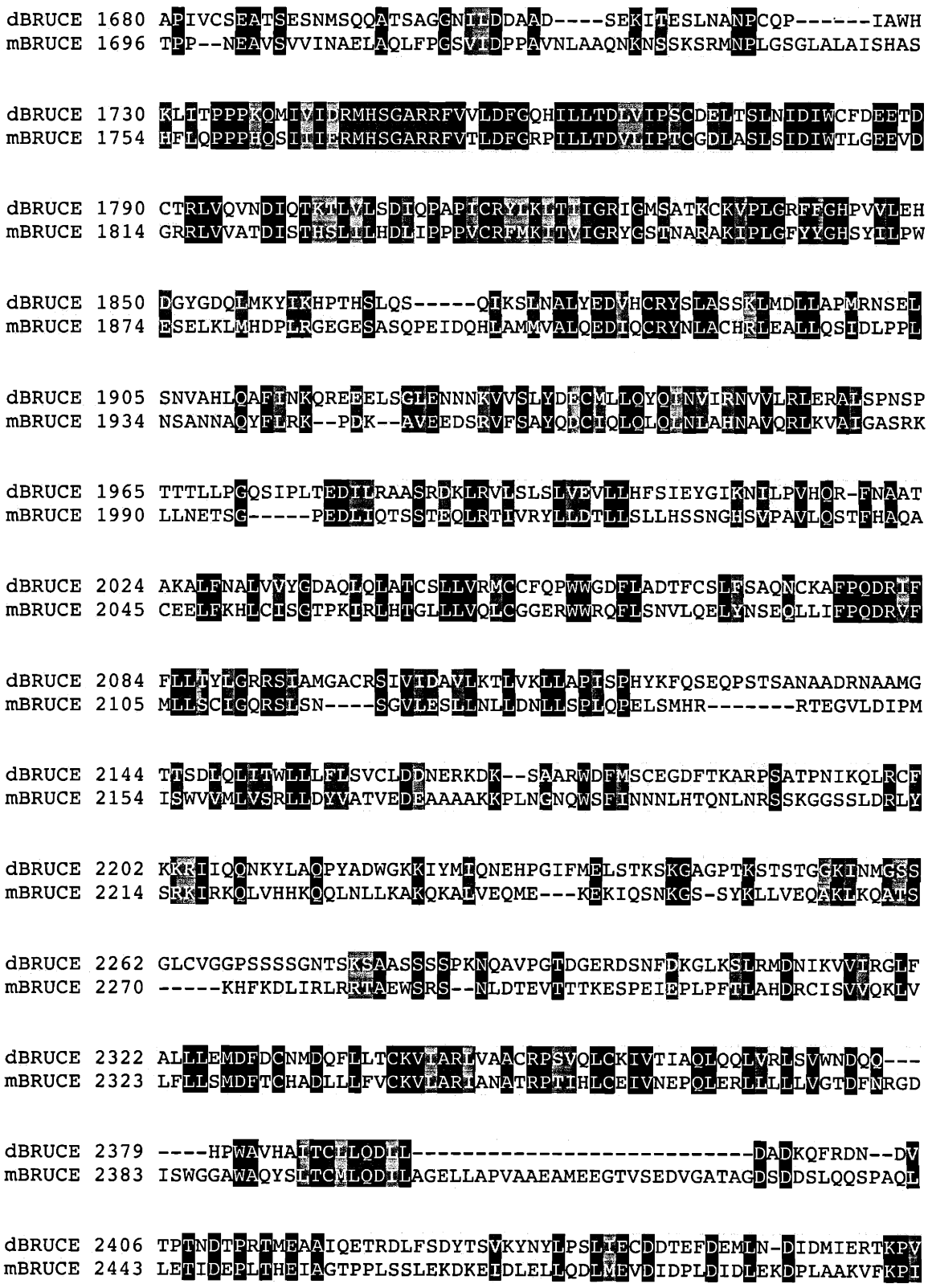
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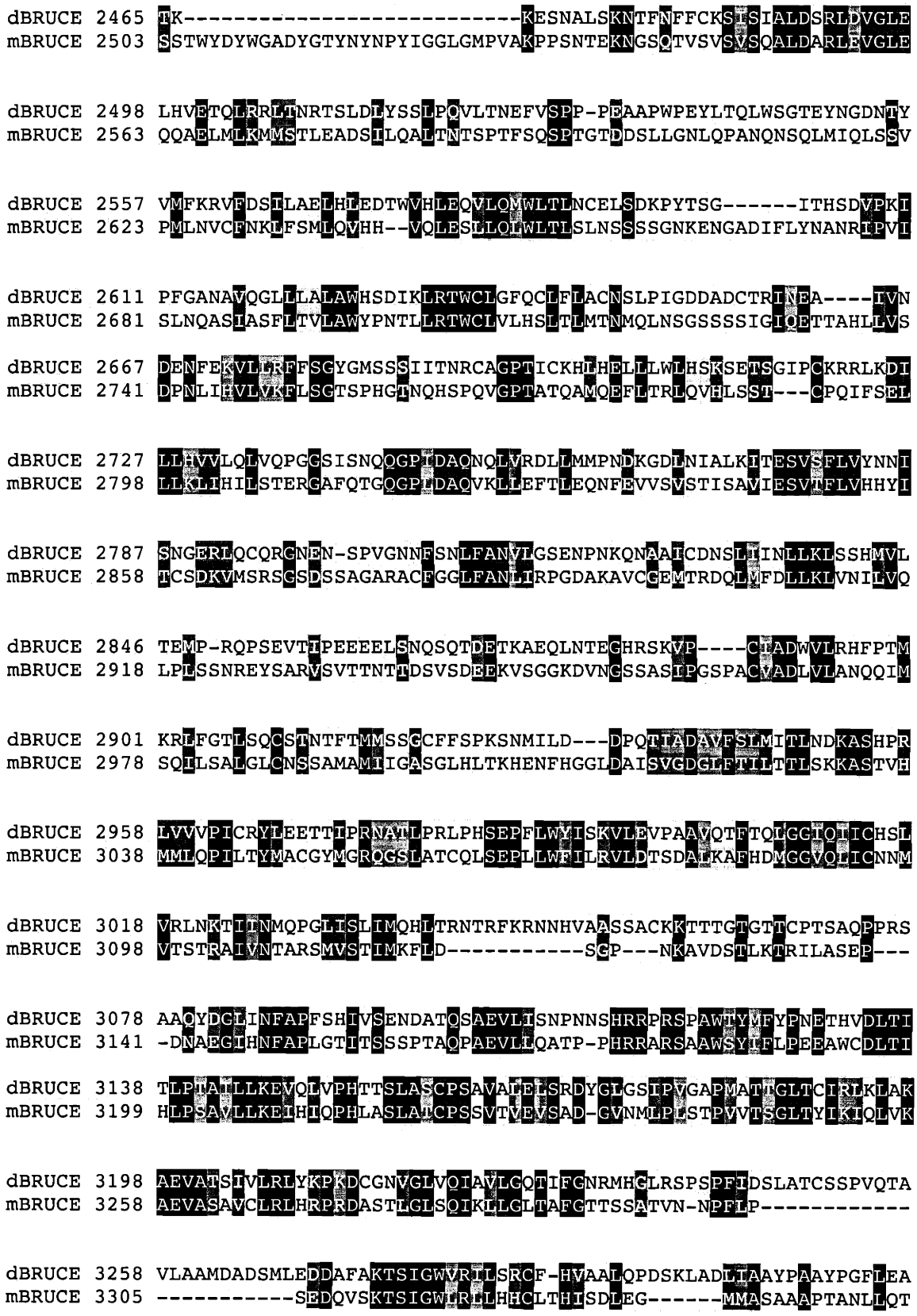
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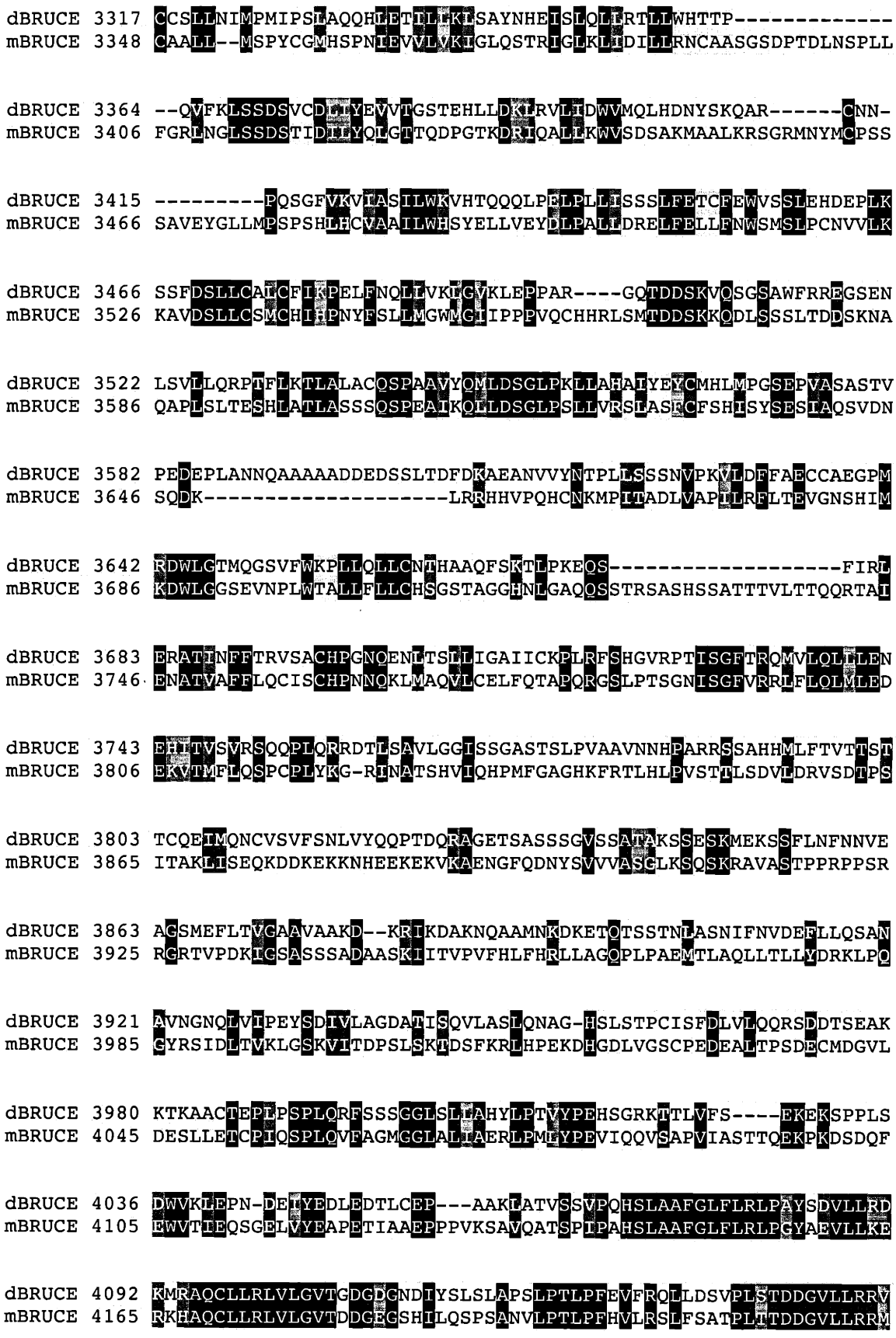
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dBRUCE 3522 LSVLLQRPTFLKTLALACQSPAAVYQMLDSGLPKLLAHAIYEYCMHLMPGSEPVASASTV
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dBRUCE 3582 PEDEPLANNQAAAAADDEDSSLTDFDKAEANVVYNTPLLSSSNVPKVLDFFAECCAEGPM
mBRUCE 3646 SQDK--------------------LRRHHVPQHCNKMPITADLVAPILRFLTEVGNSHIM
dBRUCE 3642 RDWLGTMQGSVFWKPLLQLLCNTHAAQFSKTLPKEQS------------------FIRL
mBRUCE 3686 KDWLGGSEVNPLWTALLFLLCHSGSTAGGHNLGAQQSSTRSASHSSATTTVLTTQQRTAI
dBRUCE 3683 ERATINFFTRVSACHPGNQENLTSLLIGAIICKPLRFSHGVRPTISGFTRQMVLQLLLEN
mBRUCE 3746 ENATVAFFLQCISCHPNNQKLMAQVLCELFQTAPQRGSLPTSGNISGFVRRLFLQLMLED
dBRUCE 3743 EHITVSVRSQQPLQRRDTLSAVLGGISSGASTSLPVAAVNNHPARRSSAHHMLFTVTTST
mBRUCE 3806 EKVTMFLQSPCPLYKG-RINATSHVIQHPMFGAGHKFRTLHLPVSTTLSDVLDRVSDTPS
dBRUCE 3803 TCQEIMQNCVSVFSNLVYQQPTDQRAGETSASSSGVSSATAKSSESKMEKSSFLNFNNVE
mBRUCE 3865 ITAKLISEQKDDKEKKNHEEKEKVKAENGFQDNYSVVVASCLKSQSKRAVASTPPRPPSR
dBRUCE 3863 AGSMEFLTVGAAVAAKD--KRIKDAKNQAAMNKDKETQTSSTNLASNIFNVDEFLLQSAN
mBRUCE 3925 RGRTVPDKIGSASSSADAASKIITVPVFHLFHRLLAGQPLPAEMTLAQLLTLLYDRKLPQ
dBRUCE 3921 AVNGNQLVIPEYSDIVLAGDATISQVLASLQNAG-HSLSTPCISFDLVLQQRSDDTSEAK
mBRUCE 3985 GYRSIDLTVKLGSKVITDPSLSKTDSFKRLHPEKDHGDLVGSCPEDEALTPSDECMDGVL
dBRUCE 3980 KTKAACTEPLPSPLQRFSSSGGLSLLAHYLPTVYPEHSGRKTTLVFS----EKEKSPPLS
mBRUCE 4045 DESLLETCPIQSPLQVFAGMGGLALIAERLPMLYPEVIQQVSAPVIASTTQEKPKDSDQF
dBRUCE 4036 DWVKLEPN-DEIYEDLEDTLCEP---AAKLATVSSVPQHSLAAFGLFLRLPAYSDVLLRD
mBRUCE 4105 EWVTIEQSGELVYEAPETIAAEPPPVKSAVQATSPIPAHSLAAFGLFLRLPGYAEVLLKE
dBRUCE 4092 KMRAQCLLRLVLGVTGDGDGNDIYSLSLAPSLPTLPFEVFRQLLDSVPLSTDDGVLLRRV
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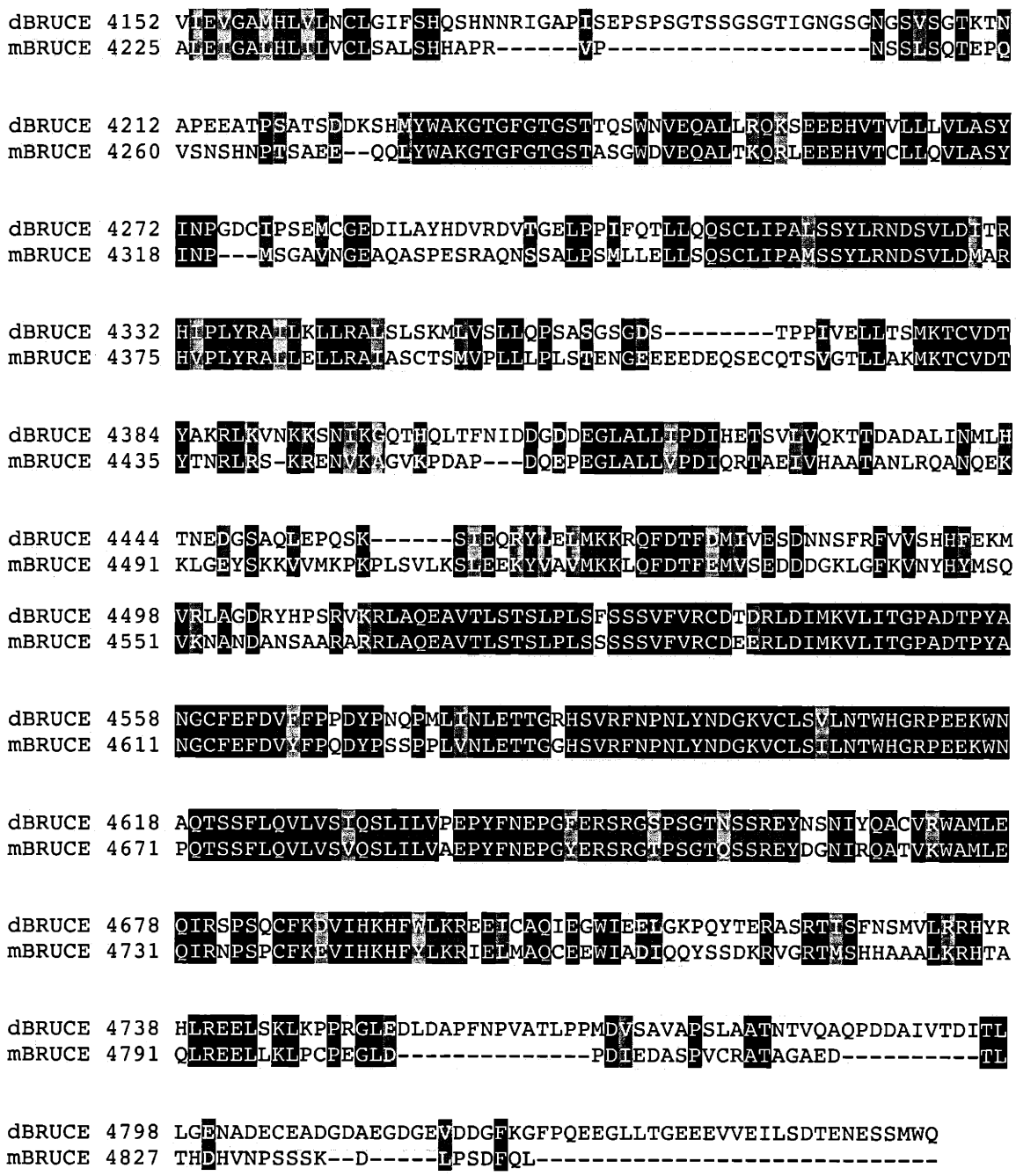
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dBRUCE 4212 APEEATPSATSDDKSHMYWAKGTGFGTGSTTQSWNVEQALLRQKSEEEHVTVLLLVLASY
mBRUCE 4260 VSNSHNPTSAEE--QQLYWAKGTGFGTGSTASGWDVEQALTKQRLEEEHVTCLLQVLASY
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mBRUCE 4318 INP---MSGAVNGEAQASPESRAQNSSALPSMLLELLSQSCLIPAMSSYLRNDSVLDMAR
dBRUCE 4332 HTPLYRALLKLLRALSLSKMLVSLLQPSASGSGDS--------TPPIVELLTSMKTCVDT
mBRUCE 4375 HVPLYRALLELLRAIASCTSMVPLLLPLSTENGEEEEDEQSECQTSVGTLLAKMKTCVDT
dBRUCE 4384 YAKRLKVNKKSNIKGQTHQLTFNIDDGDDEGLALLIPDIHETSVLVQKTTDADALINMLH
mBRUCE 4435 YTNRLRS-KRENVKAGVKPDAP---DQEPEGLALLVPDIQRTAELVHAATANLRQANQEK
dBRUCE 4444 TNEDGSAQLEPQSK------STEQRYLELMKKRQFDTFDMIVESDNNSFRFVVSHHFEKM
mBRUCE 4491 KLGEYSKKVVMKPKPLSVLKSLEEKYVAVMKKLQFDTFEMVSEDDDGKLGFKVNYHYMSQ
dBRUCE 4498 VRLAGDRYHPSRVKRLAQEAVTLSTSLPLSFSSSVFVRCDTDRLDIMKVLITGPADTPYA
mBRUCE 4551 VKNANDANSAARARRLAQEAVTLSTSLPLSSSSSVFVRCDEERLDIMKVLITGPADTPYA
dBRUCE 4558 NGCFEFDVFFPPDYPNQPMLINLETTGRHSVRFNPNLYNDGKVCLSVLNTWHGRPEEKWN
mBRUCE 4611 NGCFEFDVYFPQDYPSSPPLVNLETTGGHSVRFNPNLYNDGKVCLSILNTWHGRPEEKWN
dBRUCE 4618 AQTSSFLQVLVSIQSLILVPEPYFNEPGFERSRGSPSGTNSSREYNSNIYQACVRWAMLE
mBRUCE 4671 PQTSSFLQVLVSVQSLILVAEPYFNEPGYERSRGTPSGTQSSREYDGNIRQATVKWAMLE
dBRUCE 4678 QIRSPSQCFKDVIHKHFWLKREEICAQIEGWIEELGKPQYTERASRTISFNSMVLRRHYR
mBRUCE 4731 QIRNPSPCFKEVIHKHFYLKRIELMAQCEEWIADIQQYSSDKRVGRTMSHHAAALKRHTA
dBRUCE 4738 HLREELSKLKPPRGLEDLDAPFNPVATLPPMDVSAVAPSLAATNTVQAQPDDAIVTDITL
mBRUCE 4791 QLREELLKLPCPEGLD----------PDIEDASPVCRATAGAED----------TL
dBRUCE 4798 LGENADECEADGDAEGDGEVDDGFKGFPQEEGLLTGEEEVVEILSDTENESSMWQ
mBRUCE 4827 THDHVNPSSSK--D-----LPSDFQL----------

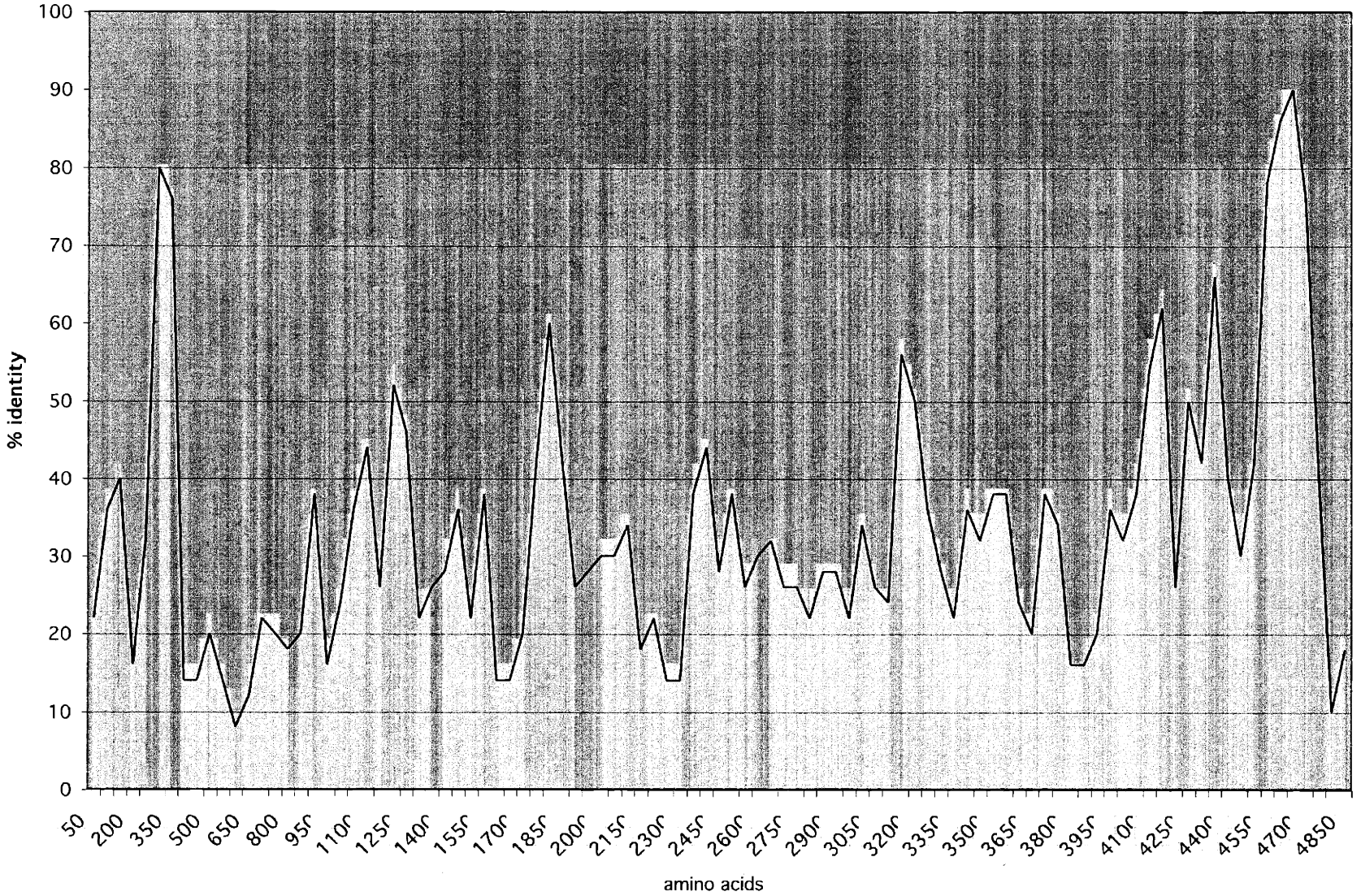
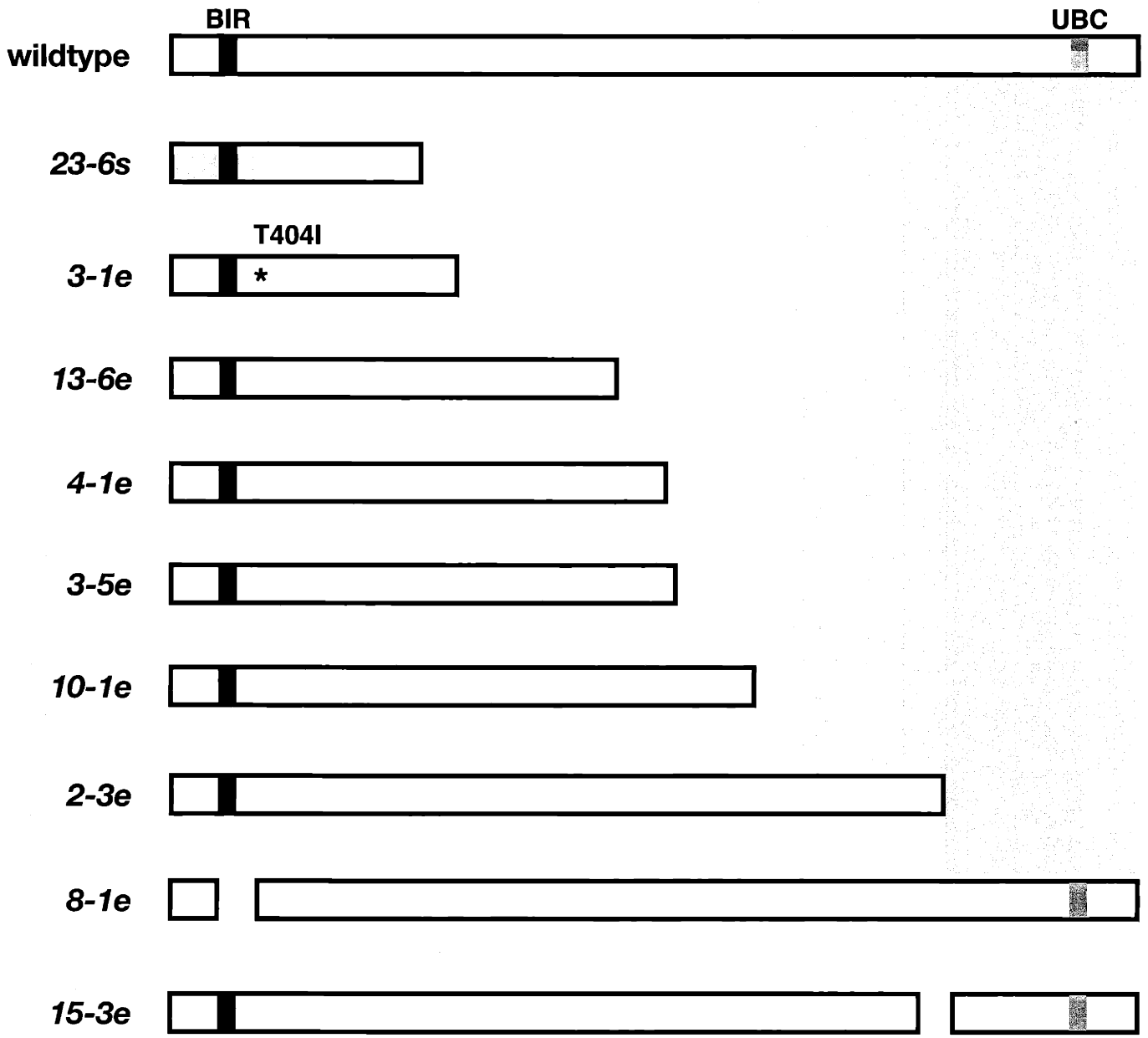

100
90
80
70
60
50
40
30
20
10
0
% identity
50
200
350
500
650
800
95
110
125
140
155
170
185
200
215
230
245
260
275
290
305
320
335
350
365
380
395
410
425
440
455
470
4850
amino acids

Table 1. Mutations associated with *mod86/dBRUCE* alleles		
Allele	**Nucleotide Changes**	**Resulting Amino Acid Changes**
8-1e	Deletion of 534 nt In frame	Deletion of residues 234-411
3-1e	ACT->ATT, TAT->TAA	T404I, Y1439stop
*23-6s**	CAG->TAG	Q1234stop
13-6e	AGgtg->AGatg splice donor	After residue E2229, 9 residues read from intron then a premature stop
4-1e	Deletion of 10 nt Frameshift	After D2514, 5 residues then a premature stop
3-5e	CAA->TAA	Q2543stop
10-1e	Deletion of 1 nt Frameshift	After P2956, 25 residues then a premature stop
15-3e	Deletion of 174 nt In frame	Deletion of residues 3729-3786
2-3e	Deletion of 11 nt Frameshift	After A3877, 4 residues then a premature stop
Introns are shown as lower case, amino acids as single-letter abbreviations. * This allele was isolated as a dominant suppressor of *GMRhid*		

Figure 4. Predicted products of *dBRUCE* mutants. The BIR domain is shown as a black box, the UBC domain as a gray box. Deletions are shown as gaps in the protein.


BIR
UBC
wildtype
23-6s
T404I
3-1e
*
13-6e
4-1e
3-5e
10-1e
2-3e
8-1e
15-3e

Chapter 6.

Concluding Remarks

We performed several screens for dominant modifiers of Rpr- or Hid-induced cell death. These screens were designed to identify components of the Rpr, Hid and possibly also Grim cell death pathways. Over one-hundred-fifty mutants were isolated that modify the cell death inducing activity of Rpr and/or Hid. A comparison of the effects caused by these mutants on Rpr-, Hid-, and Grim-induced death suggests that the Rpr, Hid and Grim death inducing pathways share some common components. Moreover, the Rpr and Grim cell death pathways are most similar to each other, while the Hid pathway is distinct in several respects. The screens described here have led to insights into the regulation of the Hid protein, as well as into the functions of Diap1 in cell death inhibition. Furthermore, the mutants we isolated suggest that Diap1 functions differently in Hid-induced death than it does in Rpr- and Grim-induced death. Finally, these screens have led to the identification of a previously uncharacterized gene that appears to function specifically to inhibit Rpr- and Grim-induced death.

Analysis of the mutants isolated in these screens and in similar screens (Kurada and White, 1998) led to the discovery that Ras/MAPK signaling is involved in negatively regulating Hid. The expectation that Hid is regulated, at least in part, post-transcriptionally, as well as the observation that the Hid protein has 5 MAPK consensus phosphorylation sites (Grether et al., 1995), led us to propose that Ras/MAPK signaling leads to phosphorylation of Hid and inhibition of Hid function. In fact, mutation of the MAPK phosphorylation sites turns Hid into a more potent apoptotic inducer and confers resistance to Ras/MAPK signaling, suggesting that phosphorylation of Hid is critical for inhibition of Hid activity. Since mutations in the gene encoding the *Drosophila* MAPK, Rolled, affect Hid-induced death, it is likely that Rolled phosphorylates Hid directly, although this remains to be shown. In addition, there are still many mechanistic questions yet to be addressed. For example, how does phosphorylation of Hid inhibit its activity? Perhaps phosphorylated Hid is unable to bind Diap1 and activate cell death, or alternatively, phosphorylated Hid could be targeted for degradation.

Diap1, and IAPs in general, inhibit cell death, perhaps by virtue of their ability to bind caspases and/or their ability to bind Rpr, Hid, or Grim (Hay, 2000). The ability of one class of *thread/diap1* mutation to suppress Rpr, Hid and Grim-induced death is

consistent with a model in which Rpr, Hid, and Grim activate caspases by binding to Diap1 and relieving caspases of Diap1-mediated inhibition. These mutants are impaired in their ability to bind Rpr and Hid and are presumably therefore more resistant to Rpr- and Hid-induced death. It is interesting to note that these mutations lie in both the BIR1 and BIR2 domains of Diap1. The BIR2 domain of Diap1 has been shown to be sufficient for interaction with Hid and Grim (Wu et al., 2001). The observation that BIR1 mutants show impaired binding suggests a role for BIR1 in this interaction. Although the BIR1 domain cannot bind Rpr, Hid, and Grim on its own and is not required for Diap1 binding in vitro (Wu et al., 2001), it is possible that this domain nonetheless contributes to binding perhaps by stabilizing the interaction between BIR2 and Rpr, Hid or Grim. Alternatively, Rpr, Hid, and Grim may bind BIR1 or BIR2 in vivo. Even though the phenotype of these Diap1 mutants is consistent with a simple model for Diap1 function, in which Diap1 binds and inhibits caspases and Rpr, Hid, and Grim, in turn, bind to and inhibit Diap1, our data do not exclude the possibility that Diap1 binds and inhibits other potential pro-apoptotic functions of Rpr, Hid, and Grim.

Since the N-termini of Hid and Grim are sufficient for binding the Diap1 BIR2 domain (Vucic et al., 1998; Wu et al., 2001), according to the model outlined above the pro-apoptotic activity of Rpr, Hid, and Grim should reside in their N-termini. While the N-termini of these proteins are sufficient to induce cell death, versions of Rpr and Grim lacking their N-termini can still induce cell death (Chen et al., 1996; McCarthy and Dixit, 1998; Vucic et al., 1998; Wing et al., 2001; Wing et al., 1998). Therefore, Rpr and Grim possibly induce apoptosis by a mechanism distinct from that proposed here. Interestingly, Rpr can induce apoptosis in Xenopus extracts (Evans et al., 1997). This pro-apoptotic activity requires the presence of a Xenopus protein, Scythe, which physically interacts with Rpr. The interaction between Rpr and Scythe does not require Rpr's N-terminus (Thress et al., 1999). Since the *Drosophila* genome encodes a Scythe homolog (Abrams, 1999), Rpr binding to Scythe may represent a alternate mechanism for Rpr induced apoptosis. Alternatively, the observation that N-terminal deletions of Rpr and Grim can still induce apoptosis, while N-terminal deletions of Hid cannot (Chen et al., 1996; McCarthy and Dixit, 1998; Vucic et al., 1998; Wing et al., 2001; Wing et al.,

1998), may reflect a more complex interaction between Diap1 and Rpr and Grim such that these mutant versions of Rpr and Grim can still bind Diap1 while an N-terminal Hid mutant cannot. In fact, a version of Grim lacking the N-terminus has been shown to retain its ability to bind Diap1 (Vucic et al., 1998).

Rpr, Hid, and Grim have all been shown to function as death inducers in mammalian cells (Claveria et al., 1998; Haining et al., 1999; McCarthy and Dixit, 1998), and thus it is likely that mammalian counterparts of Rpr, Hid, and Grim exist. Although not overtly similar to Rpr, Hid and Grim at the sequence level, the recently identified mammalian protein Smac/DIABLO is thought to be a functional analog of Rpr, Hid and Grim (Du et al., 2000; Verhagen et al., 2000). Like Rpr, Hid and Grim, Smac/DIABLO can bind IAPs and relieve caspases of IAP-mediated inhibition (Du et al., 2000; Verhagen et al., 2000). Structural and biochemical studies have revealed that Smac/DIABLO binds either the BIR2 or BIR3 domain of XIAP and that the N-terminal four amino acids are critical for binding (Chai et al., 2000; Liu et al., 2000; Wu et al., 2000). Interestingly, the N-terminal four amino acids of mature Smac/DIABLO are strikingly similar to the N-terminal four amino acids of Rpr, Hid, and Grim further supporting the idea that Smac/DIABLO is a mammalian counterpart to Rpr, Hid, and Grim (Liu et al., 2000; Wu et al., 2000).

Based on the details of the interaction between Smac/DIABLO and XIAP, Smac/DIABLO has been proposed to function in two ways to relieve caspases of XIAP-mediated inhibition (Goyal, 2001). XIAP binds caspase –3 and -7 via the linker region between the BIR1 and BIR2 domains (Chai et al., 2000; Huang et al., 2001; Riedl et al., 2001; Sun et al., 1999), thus Smac/DIABLO binding to the BIR2 adjacent to this region might destabilize the interaction between XIAP and the caspases. In contrast, both Smac/DIABLO and caspase-9 bind XIAP via the BIR3 domain and their binding is mutually exclusive (Chai et al., 2000; Liu et al., 2000; Srinivasula et al., 2001; Sun et al., 2000; Wu et al., 2000). Smac/DIABLO is thus thought to activate caspase-9 by competitive binding with XIAP.

The molecular details of how Rpr, Hid, and Grim relieve caspases of Diap1 mediated inhibition are not clear. One reason for this is that little is known about the

interaction between Diap1 and caspases. A region of Diap1 including the BIR2 domain and adjacent sequences has been shown to bind the caspase, Dronc (Meier et al., 2000). It is, however, unknown whether the BIR2 domain itself or the adjacent sequences are responsible for this interaction. Thus, Rpr, Hid, and Grim may compete with Dronc for binding the BIR2 domain or may destabilize an interaction between Dronc and DIAP1. Further refinement of the interaction between Diap1 and caspases, perhaps at the structural level, is necessary to distinguish between these models.

Since the N-terminal 4 amino acids of Smac/DIABLO, which are similar to the N-terminal 4 amino acids of Rpr, Hid, and Grim, contact the XIAP BIR3 domain (Liu et al., 2000; Wu et al., 2000), it is likely that these 4 amino acids in Rpr, Hid, and Grim are important for binding Diap1 BIR domains. Indeed, structural analysis of the Diap1 BIR2 domain bound to Hid or Grim N-terminal peptides has revealed that these 4 amino acids mimic Smac/DIABLO binding to XIAP. However, unlike Smac/DIABLO, the next 3 amino acids of Hid and Grim also contribute to the interaction (Wu et al., 2001). Assuming Rpr binds in the same way, we would predict that the *GMRrpr* $^{3\text{-}4s}$ mutant isolated in our screens, which results in an isoleucine to methionine change at residue 7, produces a protein that is impaired in its ability to bind Diap1. Thus, Rpr ^{I7M} is likely to be a weak death inducer because of this presumed impaired ability to relieve caspases of Diap1-mediated inhibition.

Much research and discussion has focused on the apoptotic regulatory role of IAP binding to caspases, as well as to Rpr, Hid, Grim and Smac/DIABLO. Yet, IAPs with RING motifs may also function as E3 ubiquitin ligases and the role of this potential activity in the regulation of cell death is more poorly studied. Analysis of the mutants isolated in our screens suggests that ubiquitination plays a key role in inhibiting Rpr- and Grim-induced death. Mutations in the Diap1 RING (this work and Lisi et al., 2000) as well as in the predicted ubiquitin-conjugating enzyme, dBRUCE, enhance only Rpr- and Grim-induced death, suggesting that these proteins specifically interfere with Rpr- and Grim-mediated apoptosis. Moreover, since dBRUCE is likely to possess E2 activity and Diap1 is likely to possess E3 activity, and E2 and E3 proteins typically function together in a complex, it is likely that Diap1 and dBRUCE function together to inhibit cell

death. Possible targets for this potential Diap1/dBRUCE ubiquitination activity include Rpr and Grim themselves, or caspases.

It is particularly attractive to incorporate ubiquitination into the model for Diap1 inhibition of caspases described above. Perhaps, Diap1 binding to caspases, along with dBRUCE, results in the ubiquitination of the caspases and their subsequent degradation by the proteasome. Thus, does Diap1 function simply as an E3 or does it function in other capacities to inhibit cell death, like inhibiting caspases through binding, without degradation?

Diap1 must have at least two functions. Eliminating the presumed E3 function through deletion of the RING impairs the ability of Diap1 to inhibit Rpr- and Grim- but doesn't impair the ability to inhibit Hid-induced death. Thus, while Diap1 may function solely as an E3 to inhibit Rpr and Grim, it must function differently to inhibit Hid. Perhaps in Hid-induced death Diap1 inhibits caspases via direct binding. Hid may then preferentially bind a Diap1/caspase complex to activate the caspase, while Rpr and Grim preferentially bind a Diap1/dBRUCE/caspase complex. This model is consistent with the modifier effects that have been observed. Complete elimination of Diap1 in both cases would lead to enhancement of Rpr, Grim, and Hid, while elimination of the Diap1 RING or dBRUCE would lead to enhancement of Rpr and Grim but not Hid.

However, not only do Diap1 RING mutants still support inhibition of Hid-induced death, they inhibit Hid-induced death better than wildtype. There are several potential explanations for this observation. Diap1 RING mutants may not bind Hid as well, thus becoming resistant to Hid-induced death. Alternatively, these mutants may better caspase inhibitors. Moreover, Diap1 may auto-ubiquitinate, like XIAP and c-IAP-1 (Yang et al., 2000), and be targeted for proteasome-mediated degradation; thus, deletion of the RING would block this activity and lead to an increase in the level of Diap1.

Although it is interesting to speculate about the possible functions of Diap1and dBRUCE, these models must be substantiated by further molecular and biochemical analysis. An important direction for future experiments will be to identify the targets of dBRUCE-mediated ubiquitination. Obvious candidates include the *Drosophila*

caspases. However, cell death phenotypes caused by expression of *dcp-1*, *drice*, and *dronc* (Meier et al., 2000; Quinn et al., 2000; Song et al., 2000) in the developing eye are not sensitive to levels of dBRUCE (data not shown). Thus, it seems unlikely that these particular caspases are dBRUCE targets. Furthermore, to support the idea that dBRUCE and Diap1 function together in a complex, protein-protein interaction studies will be required.

Do the models discussed here apply to mammalian IAPs as well? It will be interesting to learn if mBRUCE or Apollon functions in concert with any of the mammalian IAPs and if mBRUCE/Apollon can inhibit Smac/DIABLO-induced death. If so, it will make the parallels between Smac/DIABLO and Rpr and Grim even more striking. Also, it is curious that the *Drosophila* genome encodes at least three cell-death inducers that may function in part by inhibiting the inhibitors of cell death, Diap1 and dBRUCE, yet only one mammalian counterpart has been identified. Perhaps, mammals have more Rpr-, Hid-, and Grim-like death inducers but since the region of sequence homology between the *Drosophila* and mammalian counterparts consists of merely 4 amino acids, it will be difficult to identify these based on sequence alone.

In summary, the screens described in chapter two led to several avenues of research described in later chapters of this thesis. These studies (and those spawned by similar screens (Hay et al., 1995; Kurada and White, 1998; Lisi et al., 2000) have made significant contributions to understanding the regulation of cell death in *Drosophila*. Furthermore, much of what we have learned in flies is directly relevant to mammalian systems, especially with the similarities between Rpr-, Hid-, and Grim- and Smac/DIABLO-induced death. In addition to continuing the research described in chapters 3-5, further analysis of as yet uncharacterized mutants will undoubtedly advance our understanding of cell death. For example, a number of uncharacterized mutants are good candidates for being alleles of *thread/diap1* based on a rough estimate of map position. These mutants enhance Rpr and Grim and have no effect on Hid and thus may be yet another class of *thread/diap1* mutant. Furthermore, analysis of other uncharacterized mutants may lead to the identification of new cell death

regulators. Thus, the results of our screen will continue to lead to new insights into the regulation of cell death, in *Drosophila* and beyond.

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Appendix I.

The *head involution defective* gene of *Drosophila melanogaster* functions in programmed cell death.

This appendix was previously published as Megan E. Grether, John M. Abrams, Julie Agapite, Kristin White, and Hermann Steller (1995) Genes and Development, 9:1694-1708. My contribution was the construction of *pGMR-hid* transformants, SEM analysis of their eye phenotype, and suppression of the phenotype by coexpression of p35 shown in Figure 6.

Abstract

Deletions of the chromosomal region, 75C1.2 block virtually all programmed cell death (PCD) in the *Drosophila* embryo. We have identified a gene previously in this interval, *reaper (rpr),* which encodes an important regulator of PCD. Here we report the isolation of a second gene in this region, *head involution defective (hid),* which plays a similar role in PCD. *hid* mutant embryos have decreased levels of cell death and contain extra cells in the head. We have cloned the *hid* gene and find that its expression is sufficient to induce PCD in cell death defective mutants. The *hid* gene appears to encode a novel 410-amino-acid protein, and its mRNA is expressed in regions of the embryo where cell death occurs. Ectopic expression of *hid* in the *Drosophila* retina results in eye ablation. This phenotype can be suppressed completely by expression of the anti-apoptotic p35 protein from baculovirus, indicating that p35 may act genetically downstream from *hid.*

Cell deaths that occur during the development of essentially all metazoan animals display a characteristic ultrastructural morphology known as apoptosis (Kerr et al. 1972; Wyllie et al. 1980). Because it is thought that these natural cell deaths result from the execution of an active, gene-directed cell suicide program, the process of apoptosis is also referred to as programmed cell death (PCD). Strong support for this concept has come from genetic studies in the nematode *Caenorhabditis elegans,* where a large number of mutations affecting specific aspects of PCD have been isolated and ordered into a genetic pathway (for review, see Ellis et al. 1991; Hengartner and Horvitz 1994a,b). In particular, three genes, *ced-3, ced-4,* and *ced-9,* have been shown to control the onset of all somatic PCDs in the nematode. Interestingly, two of these genes, *ced-3* and *ced-9,* have mammalian homologs that are believed to play a similar function during apoptosis. The *ced-3* gene is homologous to a family of cysteine proteases that includes interleukin-1β converting enzyme, and *ced-9* is a member of the *bcl-2* family (Yuan et al. 1993; Hengartner and Horvitz 1994c; Wang et al. 1994). Moreover, expression of human Bcl-2 can suppress some PCD in C. *elegans* and can partially substitute for the loss of ced-9 function (Vaux et al. 1992; Hengartner and Horvitz 1994c). Therefore, at least some components of the cell death pathway have been conserved throughout evolution. This idea is also supported by reports that the baculovirus p35 gene can inhibit PCD in insects, nematodes, and mammalian neurons (Rabizadeh et al. 1993; Clem and Miller 1994; Hay et al. 1994; Sugimoto et al. 1994). However, despite considerable progress in identifying cell death genes, the molecular basis of apoptosis remains unknown. In addition, it is not clear how the death program is only activated in those cells that should be eliminated. Recent work on PCD in *Drosophila* indicates that this system may offer powerful new approaches for advancing our understanding in these areas.

In *Drosophila,* as in vertebrates, the induction of cell death depends on a variety of distinct signals, such as hormones, diffusible survival factors, intercellular interactions, ionizing radiation, and other cellular damage (see e.g., Fischbach and Technau 1984; Meyertholen et al. 1987; Steller et al. 1987; Bryant 1988; Magrassi and

Lawrence 1988; Kimura and Truman 1990; Wolff and Ready 1991; Campos et al. 1992; Truman et al. 1992; for review, see Steller and Grether 1994). Cell deaths in *Drosophila* display many of the morphological and biochemical hallmarks of mammalian apoptosis (Bryant 1988; Abrams et al. 1993; Robinow et al. 1994; White et al. 1994). A number of mutations have been isolated that affect PCD in specific cells or tissues (see, e.g., Fristrom 1969; Wolff and Ready 1991; Abrams 1993; Bonini et al 1993; Cheyette et al. 1994). More recently, a large fraction of the *Drosophila* genome has been systematically surveyed for genes that are required for PCD by analyzing the pattern of cell death in embryos homozygous for previously isolated chromosomal deletions (White et al. 1994). In this screen a single region on the third chromosome has been identified which is required for all PCDs that normally occur in the *Drosophila* embryo. Deletions in chromosomal region 75C1,2, such as *Df(3L)H99* (abbreviated *H99*), block virtually all PCD in response to several distinct death-inducing signals. Molecular analysis of this region has led to the identification of a gene, *reaper (rpr),* which appears to play a central control function for the induction of apoptosis in *Drosophila* (White et al. 1994). Expression of *rpr* is sufficient to restore PCD to *H99* mutant embryos, and *reaper* mRNA appears to be specifically expressed in cells that are destined to die. However, these results did not exclude the possible existence of another, functionally redundant cell death gene in this interval. In this paper, we present evidence that a second gene in the *H99* interval, *head involution defective (hid),* has a similar role in PCD as *rpr*. We find that *hid* mutants have reduced levels of apoptosis and show that expression of a *hid* cDNA under the control of the *hsp70* heat shock promoter is sufficient for the rapid and widespread induction of apoptosis in both wild-type and *H99* mutant embryos. Furthermore, ectopic expression of *hid* in the developing retina results in eye ablation. This phenotype can be completely suppresed by coexpression of the baculovirus p35 protein, which has been shown previously to suppress apoptosis in multiple contexts (Rabizadeh et al. 1993; Sugimoto et al. 1994; Clem and Miller 1994; Hay et al. 1994). The ability of *hid* to kill cells is independent of *rpr* function, and both genes appear to act at the same step in the cell death pathway. Interestingly, we find some limited amino acid similarity between the deduced polypeptide sequences of *hid* and *rpr*, suggesting

that the overall functional similarities between both genes may extend to the molecular level. Our results indicate that *hid* and *rpr* represent parallel functions for the induction of PCD in *Drosophila*.

Results

Genetic analysis of chromosomal region 75C1,2

We have reported previously that the genomic region included in *H99*, corresponding to polytene chromosome bands 75C1,2, is required for PCD (White et al. 1994). Embryos homozygous for *H99* lack all PCD that is normally induced in response to various death-inducing signals. To identify single gene mutations in this interval we performed a standard F_2 chemical mutagenesis for mutations that produced either lethal or visible phenotypes in combination with the *H99* deficiency (see Materials and methods). We isolated five alleles corresponding to only a single complementation group, and none of these alleles was fully cell death defective. One possible explanation for our failure to isolate single-gene mutants lacking PCD within the *H99* interval is the presence of multiple cell death genes with overlapping function. In this case, inactivation of any one gene would be insufficient to completely abolish apoptosis. All five alleles isolated in our screen failed to complement mutations in the *hid* locus identified previously (Abbott and Lengyel 1991) and displayed significantly reduced viability, *hid* mutants have a pronounced defect in the morphogenetic movements of head involution but otherwise develop an overall normally segmented cuticle and reach advanced embryonic stages (Abbott and Lengyel 1991). Because head involution is associated with extensive cell death, we considered the possibility that the *hid* phenotype results from decreased levels of PCD.

hid mutant embryos have decreased levels of PCD and extra cells in the head region

We analyzed the pattern of cell death in *hid* embryos using the TUNEL technique (Gavrieli et al. 1992). This method labels the nuclei of dying cells by allowing in situ detection of the DNA fragmentation that occurs during apoptosis. Because of the need

to use parents heterozygous for the *hid* mutation, only 25% of all embryos will be homozygous mutant (see Materials and methods). Therefore, it was important to distinguish *hid*$^{-}$ embryos from their siblings to unequivocally determine whether *hid* mutants have reduced levels of PCD. For this purpose, we combined the TUNEL labeling with an in situ hybridization protocol. Using an allele of *hid*, *hid*$^{WR+X1}$ which does not express *hid* mRNA (see below), we were able to identify *hid*$^{-}$ embryos based on their lack of an in situ hybridization signal (see Materials and methods). Wild-type control embryos used for comparison were taken through the same procedure, except that no *hid* hybridization probe was included, as the in situ hybridization signal can obscure the TUNEL labeling.

We found that there was less apoptosis in *hid* mutants when compared to wild type. There was a general decrease in TUNEL labeling throughout the *hid* embryos, and this phenotype. was most noticeable in the head region prior to completion of head involution (Fig. 1). This result is consistent with the striking defects in head morphogenesis, which result, in part, from a failure of the dorsal fold to migrate anteriorly in *hid* mutants (Abbott and Lengyel 1991; M.E. Grether and H. Steller, in prep.). It is possible that these morphogenetic defects result from the decrease of PCD in this region.

We have reported previously that *H99* embryos contain many additional cells in the nervous system (White et al. 1994). Using an antibody against Krüppel (Gaul et al. 1987), we found that *hid* mutant embryos have extra cells in the head region, in particular, extra larval photoreceptor cells (Fig. 2). Wild-type embryos have an average of 9.3 Krüppel^{+} cells per photoreceptor cluster (n = 60 clusters) and never > 13 cells in a single cluster. In contrast, in *hid* mutants we found an average of 15 cells per cluster (n = 156 clusters), and occasionally as many as 20 or more cells in a single cluster (n = 17). This increase in cell number is consistent with the observation that *hid* mutants had decreased amounts of cell death in the head region. In addition, we have also observed extra cells in the abdominal segments of *hid* mutants using an anti-*engrailed* antibody (Patel et al. 1989; data not shown). Taken together, these results indicate that *hid* function is required for the normal pattern of PCD in the *Drosophila* embryo.

Molecular characterization of the *hid* locus

We have speculated previously that the *H99* interval may contain more than one functionally redundant cell death genes, one of which is *rpr* (Steller et al. 1994; White et al. 1994). The finding that *hid* has reduced levels of PCD is consistent with the idea that *hid* may function in the control of apoptosis. One prediction of this model is that *hid* expression should be sufficient to restore PCD to cell death defective *H99* embryos. To test this prediction, we decided to clone the *hid* gene. For this purpose, we extended the chromosomal walk of Segraves and Hogness (1991) to include all the DNA encompassed by the *H99* deficiency and looked for the presence of transcripts derived from this region (see Materials and methods). In addition to *rpr,* we identified only three other transcription units that gave reproducible signals by Northern analysis. One of these corresponds *to hid* (see below). The embryonic expression pattern of the two other transcripts, as determined by in situ hybridization, showed no obvious correlation with the pattern of cell death in the *Drosophila* embryo. The unusually low density of transcripts in this region is consistent with the paucity of mutations producing a lethal or visible phenotype.

The molecular isolation of the *hid* locus was facilitated by the availability of a lethal P-element insertion, *l(3)05014,* in the 75C1,2 interval (Karpen and Spradling 1992; Berkeley *Drosophila* Genome Project, pers. comm.). We found that *l(3)05014* fails to complement *hid.* To determine whether the P-element insertion causes the *hid* mutation, we mobilized the 05014 P-element (see Materials and methods). We obtained many independent reversion events, indicating that the P-element insertion is responsible for the *hid* phenotype. DNA flanking the 05014 P-element insertion was cloned by plasmid rescue and mapped with respect to the chromosomal walk (see Materials and methods). These sequences hybridized to a single transcript of ~4.2 kb on Northern blots containing embryonic RNA. Several cDNA clones corresponding to this transcript were isolated from an eye disc cDNA library. The longest clone, 5A1, had an insert of 3.9 kb and contained an open reading frame (ORF) of 410 amino acids (see below). This clone was used for all further analyses.

The 5A1 cDNA was mapped to genomic clones in the interval to determine the genomic structure of this locus in wild-type and several *hid* alleles (Fig. 3A). A number of mutant alleles had chromosomal rearrangements that either broke within or deleted this transcription unit (Fig. 3A). One of these mutations, hid^{wR+xl}, deletes the 5' end of this gene and completely abolishes its transcript, as determined by in situ hybridization. This allowed us to identify *hid* mutant embryos in the aforementioned TUNEL experiments. We also sequenced the coding region and intron/exon boundaries of several independently isolated *hid* alleles (Table 1; Fig. 3B). This analysis revealed that the 05014 P element has inserted into the ORF between amino acids 105 and 106, thereby disrupting the putative coding sequence. No detectable P-element sequences remained in two independent reversion chromosomes that had been generated by mobilization of the 05014 P-element, restoring the ORF in the 5A1 transcript. In contrast, two chromosomes where the 05014 P element had excised without restoring *hid* function had residual insertions of 38 and 40 bp of P-element DNA that failed to restore the 5A1 ORF (Table 1). Finally, five of eight chemically induced alleles were associated with nucleotide changes that either introduced premature stop codons, missense mutations, or mutated a splice donor sequence (see Table 1). Taken together, these results demonstrate that the 5A1 transcript is essential for hid^{+} function. Therefore, we refer to it from now on as the 5A1 *hid* cDNA.

The sequence of the 5A1 cDNA is presented in Figure 3B. The longest ORF predicts a translation product of 410 amino acids with a deduced relative molecular mass of 43 kD. There are multiple translational stops in all three reading frames upstream of the relevant AUG start codon. The carboxy-terminal half of the predicted protein is fairly rich in charged residues, and the protein as a whole is relatively basic (predicted pI=8.27). Hydropathy analysis shows that the predicted protein is fairly hydrophilic, with no hydrophobic regions of sufficient length to serve as either signal sequences or transmembrane domains. Analysis using BLAST and FASTA sequence comparison algorithms revealed no significant similarities with nucleotide or amino acid sequences reported previously, *hid* does, however, have limited sequence similarity to the *rpr* gene (Fig. 3C). This is most striking in the first 11 amino acids, 6 of which are

identical and 4 of which are conserved substitutions between *hid* and *rpr*. Also, in this block of amino acids there is the highest degree of identity between the *rpr* genes of *Drosophila melanogaster* and a distantly related species, *Drosophila virilis* (M.E. Grether, R. Jespersen, and H. Steller, unpubl.).

Expression of *hid* is sufficient to induce cell death in *H99* embryos

To determine whether *hid* functions in PCD, we wanted to examine whether *hid* expression is capable of restoring apoptosis in *H99* embryos, which are deficient for both *hid* and *rpr*. Initially, we used a genomic cosmid clone containing the transcribed region of *hid to* generate germ-line transformants but failed to rescue *hid* mutants (data not shown). Presumably, this cosmid lacked 5' sequences required for *hid* expression. Unfortunately, the large size of the *hid* transcription unit (Fig. 3A) prohibits the use of cosmids that contain additional regulatory sequences and makes it technically impractical to use genomic DNA for rescue experiments. Therefore, we decided to examine the consequences of *hid* cDNA expression on PCD. For this purpose, we generated germ-line transformants expressing the 5A1 *hid* cDNA under the control of the *hsp70* heat shock promoter (P[*w; hs-hid*]). When *H99* embryos carrying the *hs-hid* construct were heat shocked, we observed very high levels of PCD within 2 hr upon heat shock induction (Fig. 4). These deaths appeared to occur by apoptosis, as judged by both acridine orange staining and TUNEL. No significant induction of PCD was observed in transformants without a heat shock, or in control embryos that were heat shocked but lacked the *hs-hid* transgene (data not shown). Furthermore, ectopic expression of several developmental control genes under the *hsp70* promoter, such as *hs-disco* (A. Campos and H. Steller, unpubl.), *hs-engrailed* (Poole and Komberg 1988), or *hs-hedgehog* (Tabata and Komberg 1994), all failed to induce PCD in *H99* embryos (data not shown). The only other construct that had a similar ability to induce apoptosis under these experimental conditions was a *hs-rpr* transgene (White et al. 1994; K. White and H. Steller, unpubl.). These results provide strong support for the idea that *hid* functions in PCD.

The *hs-hid* transgene also induces ectopic cell death in wild-type embryos

following heat shock. The cell death induced by the *hs-hid* transgene is lethal to wild-type embryos, and a single heat pulse during embryogenesis kills all flies bearing the *hs-hid* construct (data not shown). We considered the possibility that the ability of *hid* to activate PCD is enhanced by the presence of *rpr*. To test this idea, the amount of *hs-hid* induced cell death in a wild-type and an *H99/H99* genetic background was compared. No reproducible differences between *H99* and wild-type embryos were detected, indicating that the ability of *hid to* kill is not significantly augmented by the presence of an endogenous *rpr* gene. We also used in situ hybridization to examine whether *hs-hid*-induced apoptosis in a wild-type background would lead to *rpr* mRNA expression. This point deserved some attention, because *rpr is* normally expressed in all cells that will undergo apoptosis. However, the embryonic pattern of *rpr* mRNA was not significantly affected by ectopic expression of *hid,* even under conditions where large numbers of cells were dying (data not shown). We conclude that the induction of PCD by *hid* occurs independently of *rpr*.

hid mRNA is expressed in many regions where cell death occurs

The distribution of *hid* mRNA in embryos was determined using whole-mount in situ hybridization with digoxygenin-labeled RNA probes. We found that the pattern of *hid* expression is highly dynamic and complex throughout embryogenesis. Significantly, *hid* is expressed in many regions where cell death occurs (Fig. 5A-F). For example, in stage 11 embryos, we found both acridine orange staining and *hid* mRNA hybridization in the head and gnathal segments, as well as segmentally repeated throughout the extended germ band (Fig. 5A,B). In slightly older embryos undergoing early stages of head involution, we observed a correspondence between the patterns of cell death and *hid* mRNA expression, particularly in the head (Fig. 5C-F). We also found evidence of *hid* expression within macrophages, apparently confined to cell corpses that have been engulfed (Fig. 5G,H).

Although there is significant overlap between the patterns of *hid* expression and acridine orange staining, these patterns are not entirely coincident. For example, *hid* mRNA is found throughout the entire optic lobe primordium, but only some of these cells

undergo apoptosis (Fig. 5E,F). It is possible that post-translational control mechanisms restrict the production of active Hid protein to a subset of cells expressing *hid* RNA. Alternatively, not all cells may be equally sensitive to the amount of *hid* expression (see Discussion). In addition, although there is considerable cell death in the ventral nerve cord during late embryogenesis (Abrams et al.1993), little or no *hid* expression can be detected at this time (not shown). Perhaps *hid* is not required for these deaths; alternatively, *hid* may be expressed in the ventral nerve cord below our level of detection.

Ectopic expression of *hid* driven by an eye-specific promoter results in eye ablation

If *hid* expression is sufficient to induce PCD, it should be possible to use *hid to* kill tissues that normally live. To test this possibility we used a tissue-specific promoter to direct expression of *hid to* the developing *Drosophila* eye. The compound eye is particularly well suited for this type of study because it is a nonessential tissue, and its different cell types and their mode of development have been characterized extensively. The *hid* cDNA was placed under the control of an eye-specific regulatory element in the pGMR transformation vector (Hay et al.1994), and the resulting construct, *pGMR-hid,* was used to generate germ-line transformants.

Transformants carrying a single copy of *pGMR-hid* displayed a dramatic eye ablation phenotype (Fig. 6). Normally, compound eyes consist of ~800 regular units, called ommatidia, each of which consists of several distinct cell types (Fig. 6A). In *pGMR-hid* transformants, only undifferentiated cuticle and a dense band of bristles remained in the places normally occupied by the compound eyes (Fig. 6B). It appears that these bristles represent the mechanosensory bristles normally found at the comer of each ommatidium. Apparently, these cells are less susceptible to *hid*-induced death. However, the number of these cells is severely reduced in transformants that are homozygous for the *pGMR-hid* transgene, indicating that their survival is sensitive to the dosage of *hid* expression. It is possible that the *hid* transgene is expressed only weakly in bristle precursors. Alternatively, bristle cells may be better protected against *hid*-

induced PCD. Interestingly, a very similar phenotype is obtained upon expression of *rpr* in the developing retina (K. White and H. Steller, unpubl.). Significantly, the *hid*-induced eye phenotype is completely suppressed by coexpression of the baculovirus p35 gene (Fig. 6D), which has been shown previously to inhibit PCD (Hay et al. 1994; Clem and Miller 1994; Rabizadeh et al. 1993; Sugimoto et al. 1994). Flies expressing both *hid* and p35 in the retina have an eye morphology that is very similar to transformants for p35 alone (Fig. 6C). This indicates that *hid* expression in the retina leads specifically to the induction of PCD but otherwise does not interfere with eye development. This result also suggests that protective functions, like p35, operate genetically downstream of *hid*.

Ectopic expression of *hid* in the developing retina appears to have no detrimental effects outside the visual system. Transformants that are homozygous for the *pGMR-hid* transgene have no other detectable phenotypes and are viable and fertile. In addition, the ability of *hid to* kill appears to be cell autonomous, at least on a global level, because mosaic animals expressing *hid* in only a portion of the retina have morphologically normal sectors of the eye abutting the ablated portions (J. Agapite and H. Steller, unpubl.). This indicates that tissue-specific expression of *hid* may represent an efficient tool for the ablation of specific cell types in *Drosophila*. Finally, as discussed below, the dosage sensitivity of the eye phenotype in *pGMR-hid* transformants suggests a powerful new genetic screen for isolating cell death genes in *Drosophila*.

Discussion

hid is sufficient for the induction of PCD

Deletions of chromosomal region 75C1,2, including *H99*, protect against the induction of PCD in response to many different death-inducing signals. Previous work has implicated one gene in this interval, *rpr* as a central control function for the induction of apoptosis in *Drosophila* (White et al. 1994). However, this earlier work did not exclude the possible existence of another cell death gene in this interval. We examined the DNA encompassed by the *H99* deficiency for other transcription units that might also serve cell death functions. The results from this study strongly support a model in

which the *hid* gene encodes another cell death function in 75C1,2 that acts at the same step in apoptosis as *rpr*. To our knowledge, *hid* and *rpr* are the only cell death genes in the *H99* interval.

Mutations in the *hid* gene lead to reduced levels of cell death and the presence of extra cells, most notably in the head region of the *Drosophila* embryo, *hid* mutant embryos also have a striking inability to retract the head into the thoracic region, a process called head involution (Abbott and Lengyel 1991). Because there is a considerable amount of cell death associated with the morphogenetic movements of head involution, it is possible that this head phenotype results from the decreased amount of cell death that we have observed in *hid* mutants. The *hid* phenotype has variable penetrance and some adult flies emerge. These rare survivors have other phenotypes, such as rotated genitalia and a wing defect (Abbott and Lengyel 1991) that may be attributable to a decrease of PCD during imaginal development. The wings of *hid* adult flies of all ages are opaque and resemble the wings of newly eclosed wild-type adults (data not shown). In wild-type flies, immediately prior to and after eclosion, all of the cells in the wing intervein regions die and the wing flattens into what is essentially two cuticular sheets (Johnson and Milner 1987). It is possible that the wings of old *hid* flies remain opaque because of a failure of the intervein cells to undergo PCD.

Strong evidence for a role of *hid* in PCD comes from the observation that *hid* expression is sufficient to induce high levels of cell death in *H99* embryos. Expression of a *hid* cDNA under the control of the heat-inducibie *hsp70* heat shock promoter *(hs-hid)* leads to rapid and widespread induction of PCD in both wild-type and *H99* embryos. There are two possible explanations for this result. First, *hid* may have a physiological role in the induction of apoptosis. Alternatively, the ectopic expression of *hid* could generate a developmental conflict that would subsequently activate the cell death program. However, there are strong reasons to believe that the latter explanation is highly unlikely. Embryos that are homozygous for the *H99* deletion are virtually resistant to the induction of apoptosis by different death-inducing signals (White et al. 1994). Not only do normal developmental cell deaths fail to occur in *H99* embryos, but ectopic cell deaths that are usually seen in many developmental mutants, such as *crumbs,* are

blocked as well. In addition, *H99* protects significantly against PCD induced by ionizing radiation, and the amount of apoptosis induced by *hid* far exceeds the levels that can be caused by X-rays. Therefore, we do not expect the misexpression of developmentally important genes to induce PCD in *H99* embryos. This notion has received direct experimental support, as we did not detect any significant levels of PCD in *H99* embryos upon ectopic expression of three such genes, *engrailed, disco,* and *hedgehog.* Furthermore, within the time course of our experiments, ectopic expression of these genes failed to induce ectopic PCD even in wild-type embryos. We conclude that the ability of *hs-hid to* induce PCD in these experiments reflects a direct and normal function of *hid* in apoptosis and is not attributable to indirect effects on development.

Because the *H99* deletion includes the *rpr* gene, the ability of *hid* to kill in an *H99* mutant background must be independent from rpr function. However, it remained possible that *hid* would activate *rpr* expression in wild-type embryos, and/or that rpr and *hid* act synergistically. Our results indicate that neither of these possibilities is correct. In situ hybridization experiments demonstrate that the pattern of *rpr* mRNA is not significantly altered by ectopic *hid* expression. Therefore, *rpr* does not need to be expressed during the activation of PCD induced by *hid.* In contrast, *rpr* expression is turned on by every other death-inducing stimulus tested to date. We also see no synergism between *rpr* and *hid,* because the ability of *hs-hid* and *pGMR-hid* to kill is not significantly affected by the presence of an endogenous *rpr* gene or *rpr* transgenes (data not shown). This suggests that *hid* and *rpr* encode functions that act in parallel to induce PCD.

We have also tested the ability of *hid* to kill cells that would normally survive by directing its expression to the developing retina. A single copy of a transgene that expresses *hid* during eye development results in essentially complete eye ablation. This phenotype is apparently attributable to the activation of PCD, because it can be completely suppressed by the coexpression of the anti-apoptotic baculovirus p35 protein. Therefore, *hid* expression appears to have no significant detrimental effects on eye development as long as the induction of PCD is blocked. This result indicates that the *hid*-induced eye ablation is not a secondary consequence of a developmental

conflict created by the misexpression of *hid* and provides strong evidence for a direct role of *hid* in the activation of PCD. The one cell type that largely escapes *hid*-mediated cell killing is the mechanosensory bristle of the compound eye. It is possible that these cells express relatively low levels of the transgene. Alternatively, these cells may either contain high levels of a negative regulator of PCD or low amounts of a cell death promoting factor acting downstream of *hid.* Interestingly, the survival of bristles appears to be sensitive to the dosage of *hid,* as their number is significantly reduced in animals containing two copies of the transgene. This dosage sensitivity may provide a powerful assay for identifying relatively subtle changes in the efficiency of PCD in *Drosophila* (see below).

The *hid* gene

Several lines of evidence confirm that we have molecularly identified the *hid* gene. The 05014 P-element insertion behaves as an allele of *hid,* and reversions of this allele were always associated with precise excision events. In contrast, imprecise excision of the P-element did not restore *hid* function. The 05014 P element is inserted within the longest ORF contained in the 4.2-kb *hid* RNA, between amino acids 105 and 106 of the putative Hid protein. In addition, one chemically and two X-ray-induced *hid* alleles were found to abolish or alter expression of the 4.2-kb *hid* transcript. Finally, four *hid* alleles were associated with single-nucleotide changes in the Hid ORF (Table 1). Two of these mutations introduce stop codons and are predicted to result in premature translational termination of the Hid protein. Taken together, these results demonstrate that the ORF encoded at this locus is required for *hid* function.

Conceptual translation of the *hid* amino acid sequence reveals that *hid* encodes a novel 410-amino-acid protein that is fairly basic and contains a high number of charged residues. There is no indication that *hid is* secreted, as it does not contain any obvious signal sequences. The expected intracellular localization of the Hid protein is also consistent with its apparent cell-autonomous function in PCD. Hid contains several potential phosphorylation sites, indicating that it may be subject to post-translational modification. Interestingly, we find some limited sequence similarity between the amino-

termini of the Hid and Rpr proteins (Fig. 3C). Given the overall functional correspondence of both genes, it is possible that this small block of similarity between Hid and Rpr has mechanistic significance. Consistent with this idea is the observation that the amino terminus of Rpr has been highly conserved between different *Drosophila* species. Furthermore, the two *hid* alleles that contain stop codons at amino acids 274 and 300, respectively, are weak mutations, suggesting that a significant amount of *hid* function is encoded in the amino-terminal part of the protein.

Models for the role of *hid* in apoptosis

The decision of whether a cell lives or undergoes apoptosis is determined by the interplay of both death-promoting and death-protective functions. Current evidence suggests that most, if not all, animal cells constitutively express all of the proteins necessary to execute the cell death program (Raff 1993; Jacobson et al. 1994). However, it appears that the inadvertant activation of cell death machinery is prevented by negative regulators of cell death, such as ced-9/Bcl-2 family members (Vaux et al. 1988; Hengartner et al. 1992; Boise et al. 1993; Oltvai et al. 1993; Hengartner and Horvitz 1994c). What are the genes involved in triggering the activation of the apoptotic program? We have proposed previously that *rpr* is a global regulator of PCD (White et al. 1994). The results from this study suggest that *hid* serves a similar function for the induction of PCD in *Drosophila*. We have strong reasons to believe that *hid* and *rpr* do not encode death effector proteins. When *H99* embryos are irradiated with very high doses of X-rays, some cell death can be induced (White et al. 1994). Significantly, the morphology of the few cell deaths that are observed under these circumstances is indistinguishable from apoptosis seen in wild-type embryos. This suggests that the basic cell death machinery is present in *H99* embryos but fails to be efficiently activated in the absence of *hid* and *rpr*.

The functions of *hid* and *rpr* appear to be at least partially redundant, as mutations in *hid* do not completely block PCD, and expression of either gene alone is sufficient to induce apoptosis in *H99* embryos. The ability of *hid* to cause cell death in the absence of endogenous *rpr* and *rpr to* cause cell death in the absence of

endogenous *hid* argues that neither gene acts through the other but, rather, that they act in parallel pathways or in parallel branches of a common pathway. Consistent with this idea, we did not find changes in the pattern of *rpr* mRNA expression in *hid* embryos (data not shown). Similarly, we could not detect changes in the pattern or levels of *rpr* expression in *hs-hid* embryos following heat shock, nor did we find alterations in *hid* expression in *hs-rpr* embryos (data not shown). Finally, we have also been unable to obtain any evidence for synergism between *rpr* and *hid* in animals containing both the *hs-rpr* and *hs-hid* or the *pGMR-rpr* and *pGMR-hid* transgenes.

In the *Drosophila* embryo, *hid* RNA is generally found in regions where cell death occurs. However, despite these striking correlations, there are also clear discrepancies between the pattern of PCD and *hid* mRNA distribution. Overall, *hid* RNA expression tends to be more widespread than the amount of cell death normally observed. This is in contrast to *rpr,* which appears to be selectively expressed in all cells that are destined to undergo apoptosis. There are several possible explanations for this discrepancy. Most notably, the production of active Hid may be subject to post-transcriptional modifications, such as translational regulation or post-translational modifications. Alternatively, different cells may express different amounts of protective functions and may therefore vary in their sensitivity to the amount of *hid* expression required to induce PCD. Finally, it is possible that certain cells have insufficient amounts of death-promoting functions that act downstream from *hid.* In any case, it is clear that overexpression of *hid* can largely override any normally operating regulatory mechanisms, as shown by the increased levels of PCD in *hs-hid* embryos, and the ability of *pGMR-hid* to ablate the compound eye. In these instances, high levels of *hid* expression may conceivably titrate out protective functions or bypass the need for auxiliary factors. The biochemical mechanism by which *hid* activates apoptosis remains to be determined. It is possible that Hid activates downstream targets that promote apoptosis, which may include ced-3/ICE or ced-4-like proteins (Yuan and Horvitz 1992; Yuan et al. 1993; Wang et al. 1994). Alternatively, Hid may block the action of negative regulators of cell death, such as Bcl-2/ced-9 family members. The fact that p35 expression can suppress *hid*-induced cell death indicates that *hid* acts genetically

upstream of death-inhibiting functions like p35.

The ability of *hid to* rapidly induce apoptosis indicates that this gene may be used for targeted cell ablation in *Drosophila.* Methods developed previously that rely on the expression of toxin genes have found only limited use in *Drosophila* because of the considerable lag between toxin expression and cell killing, and because of the difficulties in restricting toxic effects to the targeted cells (Kunes and Steller 1991; Bellen et al. 1992; Moffat et al. 1992; discussed in Steller 1993). The use of *hid* instead of toxin genes may largely overcome these limitations. First, *hid* expression leads to the rapid onset of PCD, apparently as quickly as 1 hr. Second, it appears that *hid* needs to be expressed above a critical threshold to induce PCD, because we have not seen detrimental effects in *hs-hid* transformants raised at 29°C. This threshold effect should greatly help restrict the specificity of ablation to the targeted cell type or tissue. By using the GAL4 system of Brand and Perrimon (1993), one may be able to ablate given tissues of interest with considerable facility. Because widespread expression of *hid* causes organismal lethality, the *hs-hid* transgene can also be used to select against offspring bearing specific chromosomes. Apparently, this method produces fewer escapers than when dominant temperature sensitive mutations are used for this purpose (L. Moore and R. Lehmann, pers. comm.).

Finally, *pGMR-hid* transformants may provide a powerful assay for identifying mutations in other cell death genes. Because the severity of the eye ablation phenotype is dosage-sensitive, relatively subtle changes in the level of PCD are expected to alter the eye phenotype. By screening for dominant genetic modifiers of *pGMR-hid,* it should be possible to isolate mutations in cell death genes acting downstream of *hid. A* similar strategy has been highly successful for defining a genetic pathway for cell fate determination in the *Drosophila* eye (Simon et al. 1991; Gaul et al. 1992; Brunner et al. 1994). Therefore, we expect that the *pGMR-hid* transformants will greatly facilitate the elucidation of a genetic pathway for apoptosis in *Drosophila.* Because *hid*-induced cell death can be blocked by p35, and because p35 expression can protect against PCD in several other systems (Rabizadeh et al. 1993; Sugimoto et al. 1994; Clem and Miller 1994; Hay et al. 1994), we expect that at least some of the genes in this pathway have

been conserved in evolution.

Materials and methods

Drosophila stocks

Df(3L)H99, hid^{A22}, hid^{A206}, hid^{A329}, hid^{H89}, $In(3L)hid^{WR+X1}$, hid^{WR+E1}, and hid^{WR+E4} were obtained from M. Abbott and J. Lengyel. *l(3)05014* (Karpen and Spradling 1992; Berkeley *Drosophila* Genome Project, pers. comm.) was provided by A. Spradling. hid^{40C} and hid^{8D} were generated by excision of the 05014 P element (see below). *X14, X20,* and *X25* were generated by White et al. (1994). The wild-type strain used was Canton-S. All genetic symbols are described in Lindsley and Zimm (1992).

Isolation of single gene mutations in 75C1,2

Males of the genotype *st e* were allowed to feed on a solution of 0.4 mg/ml of ENU with 1% sucrose for 24 hr. Mutagenized males were crossed en masse to virgin *Sb H/TM2* females. Individual **st e/Sb H* males were then crossed to *WR4/TM3Sb* females, and their progeny were scored for visible mutations uncovered by *WR4* and for lack of **st e/WR4* individuals, indicating the presence of a recessive lethal mutation when in combination with the *WR4* chromosome. Candidates for mutations in 75C1,2 were then tested for lethality or segregation of the visible phenotype by crossing them to *WR10* flies. Approximately 23,000 mutagenized third chromosomes were screened, and we identified five chromosomes that were lethal in combination with both *WR4* and *WR10.* These mutations were determined to be *hid* alleles by standard complementation analysis. When placed in *trans to* existing *hid* alleles, significant lethality was observed. Some escapers were also recovered, which then displayed the characteristic adult *hid* phenotypes (Abbott and Lengyel 1991). *st e* and *Sb H/TM2* stocks were provided by R. Lehmann, Massachusetts Institute of Technology, Cambridge. *WR4* and *WR10* are genomic deficiencies that uncover 75C1,2 (Segraves and Hogness 1990) and were provided by the Indiana Stock Center. The *H99* deficiency is entirely internal to the overlap defined by the deficiencies *WR4* and *WR10* (White et al. 1994).

Excision of the 05014 insertion

The 05014 P element was mobilized using Δ2-3(99B), a genomic source of P transposase (Robertson et al. 1988). Excision chromosomes were identified by loss of the *ry*$^{+}$ marker. Reversion of the *hid* phenotype was scored by placing each excision chromosome in *trans to H99*. Forty-five percent of all excision events resulted in reversion of the *hid* phenotype.

Histological methods

Embryos were staged according to Campos-Ortega and Hartenstein (1985). Whole-mount in situ hybridizations and TUNEL/in situ hybridization were performed as described in White et al. (1994). The probe used was generated by in vitro transcription of the antisense strand of the 5A1 cDNA clone according to the protocol of the Boehringer RNA labeling kit. No hybridization with this probe was detected in embryos homozygous for 75C1,2 deficiencies. For TUNEL/*hid* mRNA double labelings, *WR+X1/H99* and Canton-S embryos were collected and aged until they were 8-13.5 hr old at 25°C (stages 12-15). It is during this time that head involution occurs. The *hid* and Canton-S embryos were treated identically except that no *hid* mRNA probe was added to the Canton-S embryos. In a parallel experiment, Canton-S embryos were labeled with the *hid* probe to control for uniformity of labeling. Approximately 25% of the embryos from the *hid* egg collection did not label with the *hid* mRNA, confirming our expectation that these embryos were *hid* mutants. In contrast, 100% of Canton-S (wild-type) embryos taken through the in situ hybridization procedure in parallel did label with the *hid* mRNA. Because the amount of cell death in wild-type embryos changes considerably during development (Abrams et al. 1993), care was taken to ensure that patterns of cell death were compared between closely aged matched *hid* and wild-type embryos. Embryos were determined to be the same age by examining gut morphology, avoiding the problems associated with staging *hid* embryos by head morphology. To examine *rpr* expression in *WR +X1* embryos, pools of *WR +X1/H99* embryos were hybridized with both *hid* and *rpr* mRNA probes. The patterns of *hid* and *rpr* mRNA expression are sufficiently distinct to allow identification of embryos which hybridized

with the *rpr* probe but not the *hid* probe. Those embryos were assumed to be genotypically *hid* and were examined for alterations in *rpr* expression as compared to wild-type embryos that had been probed with *rpr* alone.

Krüppel staining was done as described in Gaul et al. (1987). Embryos were aged to 12-17 hr old (stages 15-17), by which time head involution is complete in wild-type embryos, *hid* embryos were identified as those in which head involution had failed. The alleles examined were *WR+X1* (average number of cells per cluster=13.6, *n*=42), *A206* (average number of cells per cluster=13.9, *n*=32), *X14/H99* (average number of cells per cluster =17.5, *n=36),* and *WR+X1/H99* (average number of cells per cluster =15.2, *n*=46). Extra Krüppel-positive cells were also found in *WR+E6, H89,* and *05014/H99* embryos, but systematic large-scale counts were not done on these samples.

Acridine orange staining was performed as in Abrams et al. (1993).

Identification of transcripts in *H99*

Genomic DNA in the *H99* interval was cloned by chromosomal walking using standard techniques (Sambrook et al. 1989). This DNA was probed with cDNA prepared from embryonic RNA to identify putative transcription units. Northern analysis was used to confirm the presence of transcribed sequences. Four transcripts were identified that gave reproducible Northern signals. One corresponded to *rpr,* and another to *hid.* The embryonic expression pattern of all four transcripts was determined by in situ hybridization.

Plasmid rescue of genomic DNA flanking the 05014 insertion

Molecular biological techniques were performed by standard procedures (Sambrook et al. 1989). Rescue of genomic sequences was performed basically as described in Steller and Pirrotta (1986). For plasmid rescue, genomic DNA from flies carrying the 05014 insertion was digested to completion with KpnI. Approximately 13 kb of genomic DNA flanking the site of insertion was recovered and mapped to a chromosomal walk in 75C (Segraves and Hogness 1990; Grether 1994) by Southern hybridization.

Isolation and sequencing of the 5A1 cDNA

The 8-kb EcoRI restriction fragment from phage 3506 (Segraves and Hogness 1990) was used to screen an eye disc cDNA library in λgt10 (constructed by A. Cowman). One clone, clone 5A1 was isolated, which was longer than 2 kb. The cDNA insert was subcloned into the EcoRI site of the pBluescript II SK$^+$ (Stratagene) cloning vector. Series of nested deletions for both strands of the cDNA were generated according to manufacturer's instructions using the Exo/Mung deletion kit (Stratagene). Sequencing of double-stranded DNA was carried out using Sequenase (U.S Biochemical Corp.) according to the manufacturer's instructions. Both strands were sequenced. Gaps in the series of nested deletions were filled by sequencing using oligonucleotide primers derived from the cDNA sequence determined previously. Determination of intron/exon boundaries and confirmation of the 5A1 sequence were done by sequencing portions of phage clones corresponding to the genomic *hid* locus. Sequencing of the genomic *hid* locus was done using [γ-^{33}P]ATP-labeled oligonucleotide primers and the Circumvent sequencing kit (New England Biolabs) according to the manufacturer's instructions.

Sequencing of the *hid* alleles

Flies of each allele to be sequenced were crossed to *H99*. When possible, DNA was isolated from adult flies of the genotype *hid/H99*. If no adults emerged from the cross, embryos with head involution defects were culled for DNA preparation. DNA was prepared as described in Ashbumer (1989). Fragments corresponding to each coding exon of the *hid* locus were amplified by the polymerase chain reaction (PCR). The primers used for amplification are as follows: exon 1, 5'-GTGCCAACAAAGTGCA-3' and 5'-AATGGATATCCTGATTAACCCACAC-3'; exon 2, 5'-GATTTTCTTATTATGTGCCAACTGT-3' and 5'-TGATTTGATTTGCACCACCCAGGC-3'; exon 3, 5'-TCCAAGTCAACCGTCTATATGT-3' and 5'-TTGTGCATTCCAAGATTGGTGGCC-3'; and exon 4, 5'-TTAAGATTTGC-CTTCACGTGCACC-3' and 5'-AACTAAGTTTAGATCGG-3'. For each PCR reaction, ~50 ng of genomic DNA was used as template. A standard 50-μl PCR reaction was set up according to protocols of Perkin-Elmer Cetus. Annealing temperatures for each set

of primers are as follows: exon 1, 52°C; exon 2, 56°C; exon 3, 60°C; exon 4, 50°C. Cycling profiles for all amplifications were as follows: 1 cycle for 1 min at 94°C, followed by 30 cycles of 1 min at 94°C, 2 min at the annealing temperature, 2 min 15 sec at 72°C with 2 sec added per cycle. This was followed by 10 min at 72°C. Fifty percent of each reaction was loaded onto a 1% SeaPlaque GTG grade (FMC) low melting agarose gel in 1x TAE. Bands of the appropriate size were excised from the gel and diluted two- to threefold with water. Five microliters of this was used as sequencing template. Sequencing was done using [γ-^{33}P]ATP-labeled oligonucleotide primers and the Circumvent sequencing kit (New England Biolabs). For each polymorphism identified, a second, independent PCR reaction was performed and the allele resequenced.

Construction of the *hs-hid* transgene, genomic transformation, and phenotypic analysis

A 450-bp BamHI-EcoRI fragment containing the *Drosophila hsp70* promoter from pUC hsB —> R (H. Steller) was cloned into the polylinker of pCaSpeR (Pirrotta 1988). An EcoRI fragment containing the *hid* cDNA was then cloned into the EcoRI fragment in the pCaSpeR/*hsp70* construct in the sense orientation. Orientation was determined using an asymmetric restriction site in the *hid* cDNA. DNA used for transformation was purified by the Qiagen plasmid purification kit and resuspended at a concentration of 1 mg/ml. Cesium chloride-purified pπ25.7wc (Karess and Rubin 1984) was used as the source of transposase and mixed with the plasmid DNA at a final concentration of 150 μg/ml. Embryos (3090) were injected, and four independent transformed lines were isolated, *hs-hid 1* and *hs-hid 3* map to the third chromosome and *hs-hid 2* and *hs-hid 4* map to the X. The X chromosome insertions were independently crossed in to the *H99* background. Embryos were collected at 22°C for *4-6* hr from flies of the genotype *hs-hid; H99/TM3 Sb*. They were aged at 18°C until they were 8-12 hr old and heat shocked in a water bath for 1 hr at 39°C. They were allowed to recover for 1 hr at 25°C and then stained with acridine orange as in Abrams et al. (1993). Untransformed flies were also subjected to the same heat shock regimen; a few acridine orange-positive cells can be found in untransformed *H99* embryos. Excess acridine orange staining was not

observed in embryos that were not heat shocked.

To check that the effects observed were specific to *hid* expression and not to the transformation vector, or the heat shock promoter itself, three other heat shock constructs were tested: *hs-disco* (A. Campos and H. Steller, unpubl.), *hs-engrailed* (Poole and Komberg 1988), and *hs-hedgehog* (Tabata and Kom-berg 1994). These insertions were crossed independently into the *H99* background. For these experiments, embryos were collected from flies of the genotype *hs-disco* or *hs-engiailed* or *hs-bedgebog/+*; *H99/+* and subjected to the heat shock regimen described above to the *hs-hid* flies. No acridine orange staining was observed above background levels in flies bearing any of these constructs.

To check for evidence of synergism between *hid* and *rpr,* lines were constructed that contained either two copies of *hs-hid,* two copies of *hs-rpr,* or one copy of each transgene (two transgenes total). Embryos were collected from these flies and subjected to the heat shock regimen described for the *hs-hid* experiment except that heat shocks were done at 32°C, 33°C, and 37°C (in independent experiments). Embryos were stained with acridine orange and scored blind by two independent observers. No significant difference among the samples was observed.

Construction of the *pGMR-hid* construct, genomic transformation, and phenotypic analysis

A 3.9-kb EcoRI fragment containing the *hid* cDNA subclone 5A1B was inserted into the EcoRI cloning site of the pGMR vector (Hay et al. 1994) creating *pGMR-hid. pGMR-hid* and the helper plasmid pπ25.7wc (Karess and Rubin 1984) were purified using the Qiagen plasmid purification kit. For transformation, *pGMR-hid* and pπ25.7wc were mixed at a ratio of 10:1 such that the final total DNA concentration was 1 mg/ml. yw^{67c23} embryos (1800) were injected, and one transformed line was isolated in which *pGMR-hid* maps to the second chromosome. Scanning electron microscopy was performed on adult flies that were dehydrated through an ethanol series, critical point dried, and coated with gold-palladium.

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Note added in proof

The sequence data for *hid* have been deposited in the GenBank data library under accession no. U31226.

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Figure 1. PCD in wild-type and *hid* mutant embryos. Lateral view of the heads of embryos labeled with TUNEL (see Materials and methods) to assay the relative levels of cell death in wild-type (A,C) and *WR+X1/H99* (B,D) embryos. In this and all subsequent figures, anterior is to the left and dorsal to the top. (A,B and C,D). Equivalent focal planes in aged matched stage 12 embryos. In A, cell death predominates in the ventral head in the migrating gnathal segments. Little or no cell death is seen in this location in the *hid* embryo (B). Although cell death is found close to the site of dorsal fold migration in wild type (C), cell death is not found in the corresponding head region in *hid* (D). Overall, note how there is much less cell death in the *hid* mutant embryos (B,D) than in the corresponding wild-type embryos (A,C). Bar, 25 μm.

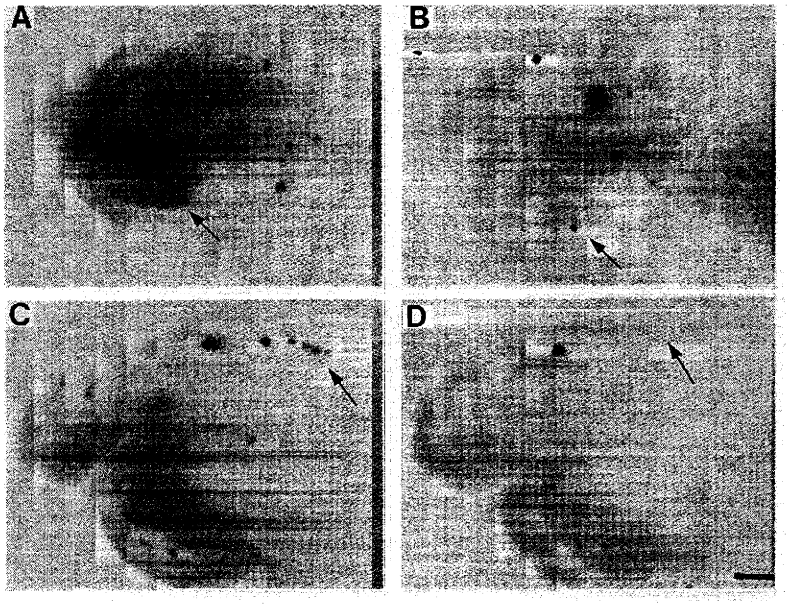
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Figure 2. *hid* mutant embryos have extra cells in the larval photoreceptor organ. Dorsal view of embryos stained with anti-Krüppel antibody to visualize the cells of the larval photoreceptor organ (Gaul et al. 1987). All embryos are of an equivalent stage. (A) Wild-type embryo; (B-D) the *hid* mutants *WR+X1, A206,* and *l(3)05014/H99,* respectively. Arrows indicate those cells that will become the larval photoreceptors. Wild-type embryos have an average of 9.3 cells per photoreceptor cell cluster. In the embryo shown in A, five cells can be seen in this focal plane, *hid* mutants have an average of 15 cells per cluster. In the *hid* mutants (B-D), 15, 17, and 15 cells are visible in each photoreceptor cell cluster, respectively. Bar, 25 μm.

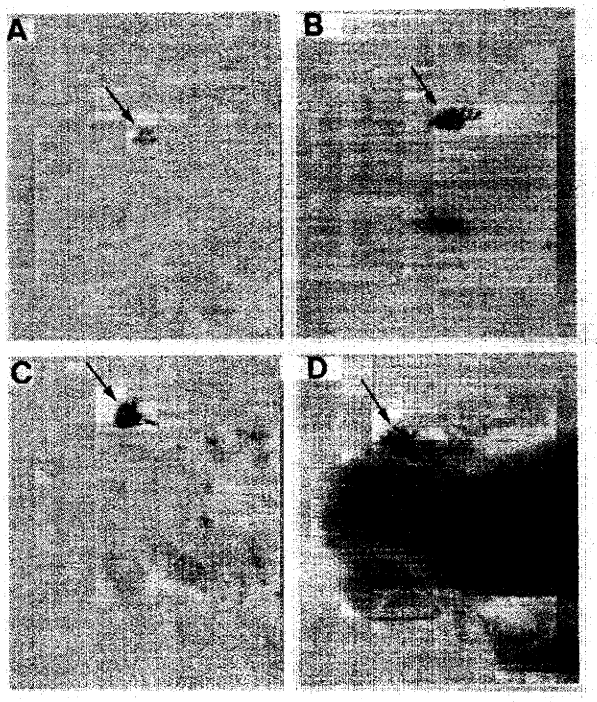
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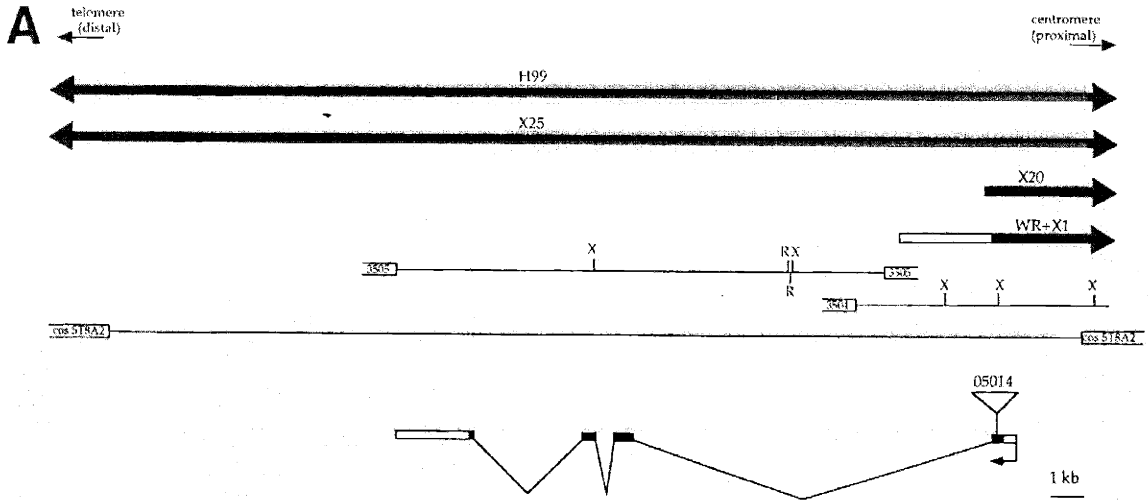
Figure 3. Molecular characterization of the *hid* locus. (A) Genomic map of the *hid* transcript. Clones 3505 and 3504 are genomic phages isolated by Segraves and Hogness (1990). The intron/exon structure of the *hid* transcript was determined by Southern blot analysis and DNA sequencing (see Materials and methods). In the transcript, solid bars represent coding exons, open bars, untranslated sequences. The site of the 05014 insertion and direction of transcription are also shown. Large arrows above the genomic map represent genomic deficiencies in 75C1,2 which extend in the direction shown by the arrows. All deficiencies either break within or delete this transcript entirely. In *WR+X1,* the open portion of the arrow marks the genomic region in which the distal breakpoint must lie. (R) *Eco*RI; (X) *Xho*I. (B) Sequence of *hid* cDNA 5A1. The deduced amino acid sequence of the longest potential ORF is shown beneath the nucleotide sequence. Two upstream ATGs are underlined: Five potential MAP kinase consensus phosphorylation sites are underlined beneath the amino acid sequence. Intron positions are marked by open arrows. The site of the 05014 insertion is marked with an arrow. The polymorphisms identified in the sequenced *hid* alleles are shown in uppercase letters next to the name of each allele, above the cDNA sequence. (C) Comparison between the first 11 amino acids of *hid* and *rpr*. The predicted protein sequences of *hid* and *rpr* have some limited sequence similarity at their amino termini that is not shared with any other proteins in the data base. Bars indicate identities; double dots indicate conserved amino acid substitutions.



B

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1    TGACAAAAAATAAAAAACGAAATCCATCGTGAACAGTTTTGTGTTTTTTAAATCAGTTCTAAACACGAAAAGGGTTGATGAAAAAACGCAGAAGAATCCGAAAAACTAACTAACCGAGCAAA  120
121  AACTTGACTTCAGTGTTGTTTGACAAATCAGGAAAGATAAAAAACAAATCATAAGAAAAAACTGCACGAAAAATGAAAAAGTTTCTAATATTCAAAATCTTGCACAAGAAATACAAAATC  240
241  AATTAAAGTGAACTCTAACCAAAAGTTGTACACAAAATAAAAAGCAAAACAAAGCAGCGAACAACAATCACAACAAGAGCAAAGTGCCAACAAAGTGCAGGAAGGAAGTAAGCGGATAAG  360
361  GACAAAAAGCAAGCCAGCACACACACACACCCACACAATGGCCGTGCCGTTTTATTTGCCCGAGGGCGGCGCCGATGACGTAGCGTCGAGTTCATCGGGAGCCTCGGGCAACTCCTCC  480
                                                                  M  A  V  P  F  Y  L  P  E  G  G  A  D  D  V  A  S  S  S  S  G  A  S  G  N  S  S   27

481  CCCCACAACCACCCACTTCCCTCGAGCGCATCCTCGTCCGTCTCCTCCTCGGGCGTGTCCTCGGCCTCCGCCTCCTCGGCCTCATCTTCGTCATCCGCATCGTCGGACGGCGCCAGCAGC  600
 28  P  H  N  H  P  L  P  S  S  A  S  S  S  V  S  S  S  G  V  S  S  A  S  A  S  S  A  S  S  S  S  S  A  S  S  D  G  A  S  S   67

                                                                                                                  1(3)05014: ↓
601  GCCGCCTCGCAATCGCCGAACACCACCACCTCGTCGGCCACGCAGACGCCGATGCAGTCTCCACTGCCCACCGACCAAGTGCTATACGCCCTCTACGAGTGGGTCAGGATGTACCAGAGC  720
 66  A  A  S  Q  S  P  N  T  T  T  S  S  A  T  Q  T  P  M  Q  S  P  L  P  T  D  Q  V  L  Y  A  L  Y  E  W  V  R  M  Y  Q  S  107

     ⇓
721  CAGCAGAGTGCCCCGCAAATCTTCCAGTATCCGCCGCCAAGCCCCTCTTGCAATTTCACTGGCGGCGATGTGTTCTTTCCGCACGGCCATCCGAATCCGAACTCGAATCCCCATCCGCGC  840
106  Q  Q  S  A  P  Q  I  F  Q  Y  P  P  P  S  P  S  C  N  F  T  G  G  D  V  F  F  P  H  G  H  P  N  P  N  S  N  P  H  P  R  147

                                                                  A22: T
841  ACCCCCCGAACCAGCGTGAGCTTCTCCTCCGGCGAGGAGTACAACTTCTTCCGGCAGCAGCAGCCCCAACCACATCCGTCATATCCGGCGCCATCAACAGCCGCAGCCAATGCCACCGCAG  960
146  T  P  R  T  S  V  S  F  S  S  G  E  E  Y  N  F  F  R  Q  Q  Q  P  Q  P  H  F  S  Y  P  A  P  S  T  P  Q  P  M  P  P  Q  187

961  TCAGCGCCGCCGATGCACTGCAGCCACAGCTACCCGCAGCAGTCGGCGCACATGATGCCACACCATTCCGCTCCCTTCGGAATGGGCGGTACCTACTACGCCGGCTACACGCCACCACCC  1080
186  S  A  P  P  M  H  C  S  H  S  Y  P  Q  Q  S  A  H  M  M  P  H  H  S  A  P  F  G  H  G  G  T  Y  Y  A  G  Y  T  P  P  227

                                                                                                  A206: T
1081 ACTCCGAACACGGCCAGTGCGGGCACCTCCAGCTCATCGGCGGCCTTCGGCTGGCACGGCCACCCCCACAGCCCCTTCACGTCGACCTCCACGCCGTTATCGGCGCCAGTGGCGCCCAAG  1200
226  T  P  N  T  A  S  A  G  T  S  S  S  S  A  A  F  G  W  H  G  H  P  H  S  P  F  T  S  T  S  T  P  L  S  A  P  V  A  P  K  267

          A206: T    ⇓                                                                                  A329: A
1201 ATGCGCCTGCAGCGCAGCCAGTCGGATGCGGCCAGACGCAAGCGATTGACCTCGACGGGCGAGGATGAGCGCGAGTACCAGAGCGATCATGAGGCCACTTGGGACGAGTTTGGCGATCGC  1320
266  M  R  L  Q  R  S  Q  S  D  A  A  R  R  K  R  L  T  S  T  G  E  D  E  R  E  Y  Q  S  D  H  E  A  T  W  D  E  F  G  D  R  307

1321 TACGACAACTTTACGGGCCGGCCGGGAGCGTCTGCAGGAGTTCAATGGACGCATCCCGCCCCGGAAGAAGAAGAGCTCCAATAGCCACTCGAGCAGCAGCAATAATCCAGTCTGCCATACC  1440
306  Y  D  N  F  T  A  G  R  E  R  L  Q  E  F  N  G  R  I  P  P  R  K  K  K  S  S  N  S  H  S  S  S  S  N  N  P  V  C  H  T  347

                                                                                                                  ⇓
1441 GACAGCCAGTCCGGTGGTACATCCCAAGCGGAGAGCGGTGCCATCCATGGCCACATCAGTCAGCAGCGACAGGTGGAGCGAGAACGACAAAAGGCGAAGGCCGAGAAGAAGAACCACAG  1560
346  D  S  Q  S  G  G  T  S  Q  A  E  S  G  A  I  H  G  H  I  S  Q  Q  R  Q  V  E  R  E  R  Q  K  A  K  A  E  K  K  K  P  Q  387

1561 AGCTTCACTTGGCCAACTGTTGTGACCGTTTTCGTTTTGGCCATGGGCTGTGGCTTCTTTGCGGCGCGATGAAAGCGCAGGAGACGTGTAATCGAATGATCTATAGTGAAATCAGCTAGC  1680
386  S  F  T  W  P  T  V  V  T  V  F  V  L  A  M  G  C  G  F  F  A  A  R  *                                                    411
1681 CCTTAAGATATATGCCGATCTAAACATAGTTGTAGTTAAACCGTACATAAGTGCAACGAATTTATTGAACTGCAGGAGCGAAAGCAGAAAGTCATTAATTCGTAAACGGATTGTTAGATA  1800
1801 CACAAACAGCCAACATACACGAAGAGTGTGCCTAAGATTAAGAAGTTGACGGGACACAAGAACAATATATTCTATCTGTCATGTAACTGCATTGTATTTCTAAAACGAAACCGAAG  1920
1921 ATAACAATCTTAACTGCTCAAAGTAATGAAAACTCTTAGACTGGCAAGAGACTCAAATCACACTTATTTTTTTGCTGATCCATATTTTTGTACAACCTTTGGAGCGATATTTACAAATTA  2040
2041 TACTAGTACAAAAAAAAGAGAGAGAGATAAGCAAAAGAAAACTGCCACTTTTGAGATACTTTTGATTTGCAGTTTATCATTTCCACACTTGCATTTTTATAAACAAC  2160
2161 AAACAAAATTACTTCCATTGTAGAACAAAGTAACTGCAATTTCAATGTCTTCGCATTTGTAATTCCGAATTGCAACAAAACAAAAATATTTTAAATATNTTTAACTAGTACAATTTT  2280
2281 TAAACGTAAGTCCACAAAACAAGCACATCTAGCTTTAATTGTTGAAACAAAAGCAGAAAAAAGCCACAACAAAAAAATAAATGAAAATCATAAAATTAATTTTTATATAGTTTTTATGCC  2400
2401 ATTTTTGTGATGTTTTGTGTCTACGGTTTATGTCATGTTATTTTAGTTAAATTTCTTATGATTTATGTTTATTTTTAATATTTTTGTCATTGTTTGTTCATCATCATATTCAAATTGGT  2520
2521 CTCACAATATAATAGTTTTAAGCTCCACGCCCGGAGATTGATGGCAAAACGATTGAAATTTGGCCAGAAGAGAGATAGTTTTCCCCATTCGTACACAGTCTTTTTGGAATGCACATTA  2640
2641 ATGATCTCTCACAATGCAAATTAATGAAATTGATCTCCCGCAGCTAGCCAAAGTTAAAAAAGAAATGGAAGAGGAAAACATATTCTATAGCAATTTCACTATATGCTAGAATTTCCCNG  2760
2761 GCGTTTCAATGCTAATCGAATACAGTGACATGAAAGCAAACATAGCGAAAATATTAAGAAAATCAATCAAAAAGAAAGAAAACCAATTCCCAAAATCCCATTGATCTCATGGATTTAT  2880
2881 ACAATACAATTACATCAACCGTTTTTTTACAATGAGAAATGTTATAAAAAGCAGAAAGTGAAACACAGAAACATAAACAAAATTAACGAAAAGCTTAGATATAAGTTCGCCAAGCGTTT  3000
3001 TAGTTCTATTTTCTAGAATGCTAAGTCGGTTAGTGAGTTATAATAGCTGTCTTCGCACAAGTTTATTTGTATATAAGCAATATTATTTGTGTAGCCTAAGTGACAGTCCCAATCAA  3120
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3721 CAATTTAAGAAACATTTCTGTTGTAGTTTAGTGATTGCTAGCAGAAGCACTTTGTTTAATGTACATTTTATATTATGCTGTAATATTTTAATATACATAAATATCATTATTGATCTCA  3840
3841 TGAATATGTTCATAAGACAACAAAAATTACATATATGAATACATCTATGTGTATGTGTAA                                                              3902
```

C

Table 1. Molecularly characterized *hid* alleles

Allele			
1) *l(3) 05014*	P-element insertion at nucleotide 714, between amino acids 105 and 106		
2) *40C*	imprecise excision of l(3)05014		
3) *8D*	imprecise excision of l(3)05014		
Canton-S	698	GAGTGGGTCA**GGATGTAC**	CAGAGCCAGC
40C	698	GAGTGGGTCA**GGATGTACC**ATGATGAAATAACAT	T TTATTTCATCATG**GGATGTAC**CAGAGCCAGC
8D	698	GAGTGGGTCA**GGATGTACC**ATGATGAAATAACATA	TGTTATTTCATCATG**GGATGTAC**CAGAGCCAGC
		target duplication 5'P	3'P target duplication
4) *A22*	amino acid 170	CCA to TCA Pro Ser	
5) *A206*	amino acid 261	TCG to TTG Ser Leu	
6) *A206*	amino acid 274	CAG to TAG Gln End	
7) *A329*	amino acid 301	TGG to TGA	
8) *ML66*	splice donor mutation - junction between exons 3 and 4		
Canton-S		AG/gt... tcag/AA	
ML66		Ag/ t... tcag/AA	

All *hid* alleles for which polymorphisms were identified by sequencing are shown *l(3)05014* is a P-element insertion, and *40C* and *8D* were generated by imprecise excision of this insertion. *A22*, *A206*, and *A329* were generated by Abbott and Lengyel (1991). *A206* and *A329* are derived from the same parental chromosome *(ru h th ri e). A22* was induced on a distinct parental chromosome *(red* e). *ML66* was generated in this study on the *st e* parental chromosome. The wild-type chromosomes sequences were derived from Canton-S and *st e* flies. None of the polymorphisms identified in the *hid* mutants was shared with either wild-type chromosome.

Figure 4. Patterns of cell death in heat-shocked wild-type, *H99,* and *hs-hid* embryos. Lateral views of 10- to 14-hr-old embryos stained with acridine orange to visualize apoptosis. All embryos were subjected to an identical heat shock regimen (see Materials and methods). Each brightly staining small dot represents a dead or dying cell or cell fragment. (A) Cell death in a heat shocked wild-type embryo. Following heat shock, the pattern of cell death in wild-type embryos is still characteristically patterned and virtually undistinguishable from the pattern of cell death seen in non-heat-shocked wild-type embryos. The large brightly stained area in the center of the embryo is the yolk. (B) Cell death in a heat-shocked *H99* embryo. Heat shock alone cannot induce cell death in the fully cell death-defective *H99* mutant embryos. Other than the yolk, which can be seen to autofluoresce, there is a conspicuous lack of any acridine orange staining in this heat-shocked *H99* embryo. (C-F) Embryos from a collection of either *hs-hid4; H99/TM3* (C,D) or *hs-hid2; H99/TM3 (E,F)* flies. Following heat shock, all embryos carring the *hs-hid* transgene (here, examples from two independent transgenic lines are shown) have significantly more cell death than wild type (cf. A). In addition, the heat-shocked *hs-hid* embryos display abnormal morphology and fail to develop properly. Equivalent levels of cell death are induced in both heat-shocked wild-type and heat-shocked *H99* mutant embryos when both carry the *hs-hid* transgene. Bar, 50 μm.

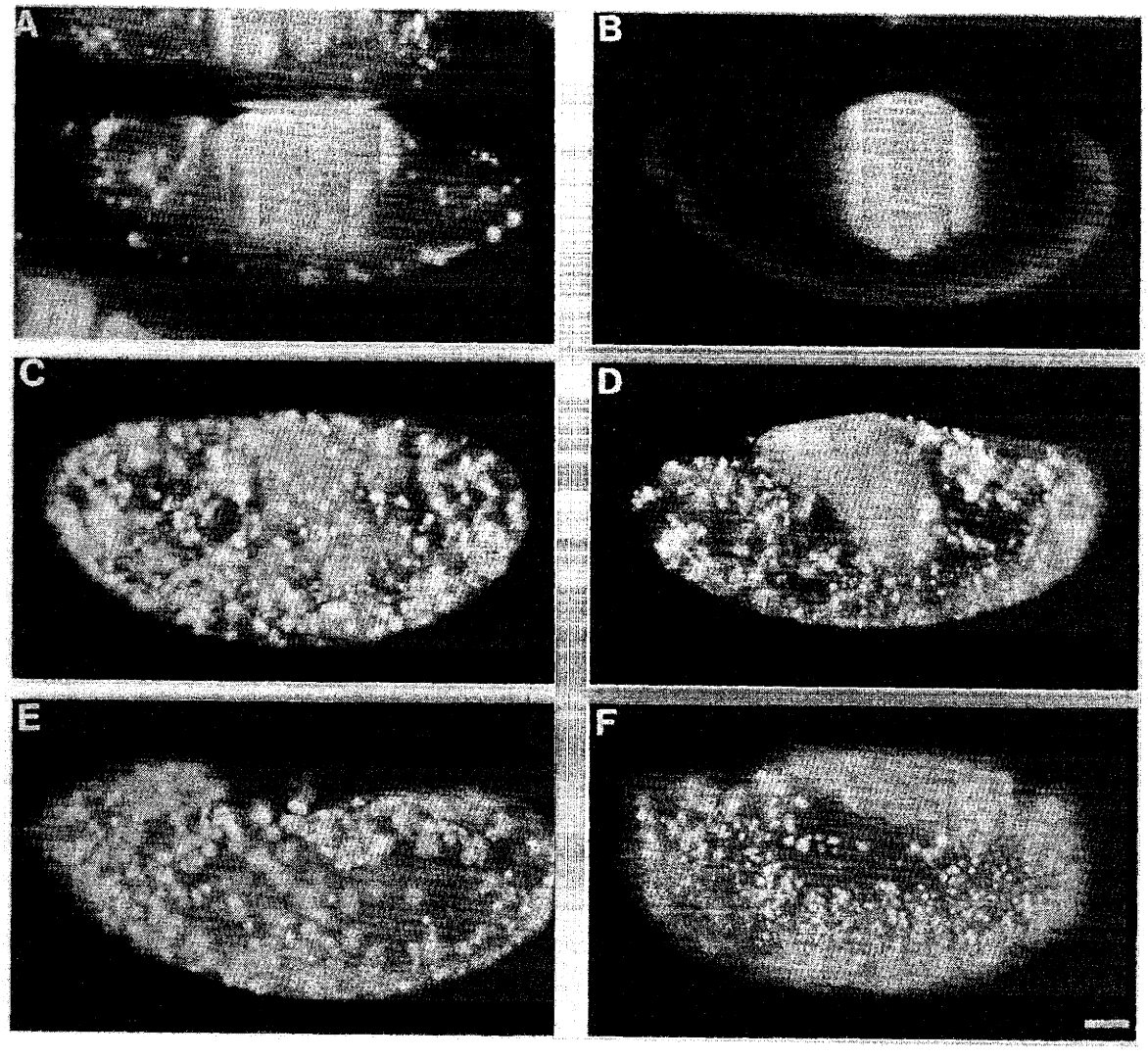
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Figure 5. Comparison of the tissue distribution of *hid* mRNA and the pattern of PCD in the embryo. The *hid* transcript was detected in whole-mount embryos by in situ hybridization with digoxigenin-labeled RNA probes (A,C,E,G,H). Cell death was visualized by acridine orange staining (B,D,F). Throughout all stages of embryonic development, there are many similarities among the patterns of *hid* mRNA expression and PCD. (A,B) Lateral view of stage 11 embryos, *hid* mRNA and acridine orange staining are found in the head and gnathal segments, and are segmentally repeated throughout the extended germband. (C,D) Stage 12 embryos. The correspondence between the pattern of *hid* mRNA expression and cell death is particularly striking in the head region, e.g., in the optic lobe region (arrow). (E,F) Dorsal view of late stage 12 embryos. Both *hid* mRNA and cell death are found in the clypeolabrum, the invaginating optic lobe primordia (arrowheads), and a portion of the hindgut (arrow). (G,H) *hid* expression is found in cell corpses engulfed by macrophages (arrows), indicating that at least some *hid*-expressing cells undergo PCD and are recognized by phagocytic cells. Bar, (A-F) 50 μm; (G,H) 17 μm.

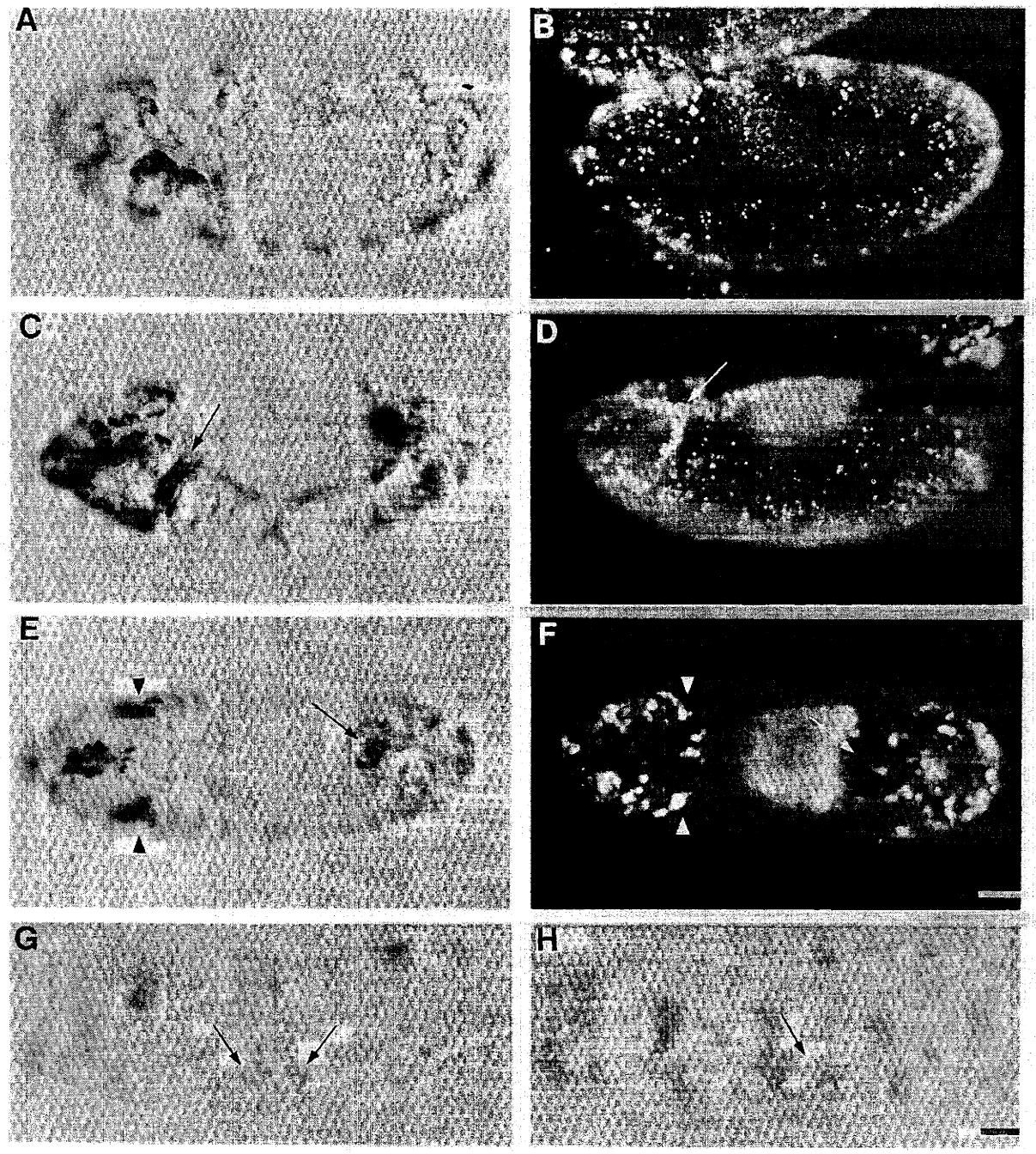
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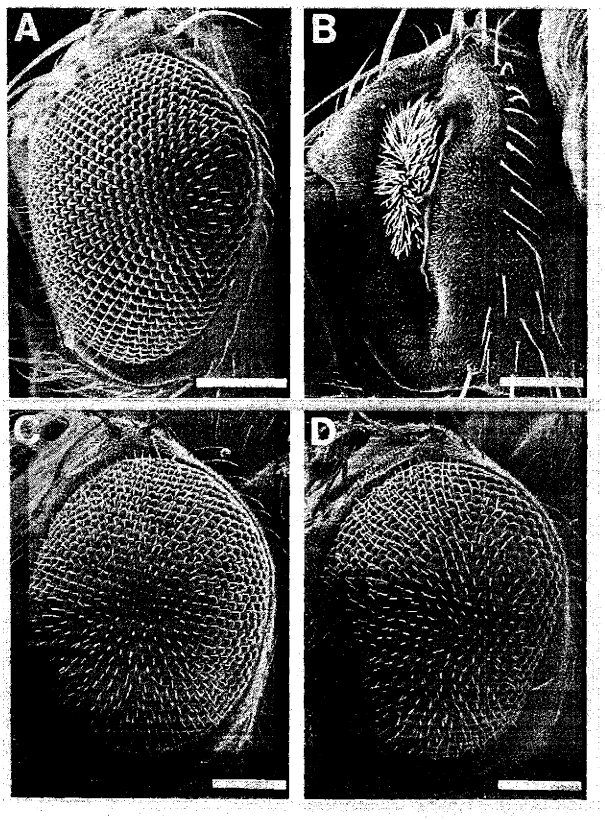
Figure 6. Scanning electron micrographs (SEMs) of the compound eye of wild-type and *pGMR-hid* flies. Expression of *hid* in the developing retina causes eye ablation, which is suppressed by coexpression of the anti-apoptotic p35 protein. SEMs of the compound eye of wild type (A), *pGMR-hidl* + (B), *pGMR-p35/pGMR-p35* (C), and *pGMR-hid/pGMR-p35* (D) flies are shown. In wild-type flies (A), the compound eye is composed of regularly spaced ommatidia with mechanosensory bristles protruding from each ommatidium. In *pGMR-hid* transformants (B), no ommatidial units are visible and undifferentiated cuticle and bristles fill the space normally occupied by ommatidia. These bristles are likely to be the mechanosensory bristles normally found at the corner of each ommatidium. The presence of a single copy of the *pGMR-p35* transgene restores the ommatidia that are slightly disorganized (D). A similar disorganization is seen in flies expressing p35 alone (C, see also Hay et al. 1991), apparently because of the suppression of cell death that normally occurs during retinal development (Wolff and Ready 1991; Hay et al. 1994). Bar, 100 μm.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Appendix II.

Cooperative functions of the *reaper* and *head involution defective* genes in the programmed cell death of *Drosophila* central nervous system midline cells.

This appendix was previously published as L. Zhou*, A. Schnitzler*, J. Agapite, L. M. Schwartz*, H. Steller, and J. R. Nambu*, *Proc. Natl. Acad. Sci. USA* Vol. 94, pp. 5131-5136, 1997. My contribution was the construction of the *UASrpr* and *UAShid* transgenic flies.

ABSTRACT

In *Drosophila*, the chromosomal region 75C1-2 contains at least three genes, *reaper* (*rpr*), *head involution defective* (*hid*), and *grim*, that have important functions in the activation of programmed cell death. To better understand how cells are killed by these genes, we have utilized a well defined set of embryonic central nervous system midline cells that normally exhibit a specific pattern of glial cell death. In this study we show that both *rpr* and *hid* are expressed in dying midline cells and that the normal pattern of midline cell death requires the function of multiple genes in the 75C1-2 interval. We also utilized the P[UAS]/P[Gal4] system to target expression of *rpr* and *hid* to midline cells. Targeted expression of *rpr* or *hid* alone was not sufficient to induce ectopic midline cell death. However, expression of both *rpr* and *hid* together rapidly induced ectopic midline cell death that resulted in axon scaffold defects characteristic of mutants with abnormal midline cell development. Midline-targeted expression of the baculovirus p35 protein, a caspase inhibitor, blocked both normal and ectopic *rpr*- and *hid*-induced cell death. Taken together, our results suggest that *rpr* and *hid* are expressed together and cooperate to induce programmed cell death during development of the central nervous system midline.

INTRODUCTION

Programmed cell death is a tightly regulated process that serves to eliminate unnecessary and/or deleterious cell types in a variety of developmental, physiological, and pathological contexts. The most well studied form of programmed cell death is apoptosis, which is characterized by distinctive morphological and molecular changes that include cell shrinkage, membrane blebbing, chromatin condensation, and the generation of nucleosomal ladders (1). Whereas a wide range of distinct signaling mechanisms can be used to elicit apoptosis, the cell death machinery itself appears to be highly conserved (reviewed in refs. 2-4). In *Drosophila*, genetic studies have shown that the 75C1,2 region of the third chromosome is essential for apoptosis, as deficiency strains that lack this region result in the blockade of essentially all embryonic cell deaths (5). Three different genes that map in this region, *reaper* (*rpr*), *head involution defective* (*hid*), and *grim*, are involved in regulation of apoptosis (5-7). These genes are all expressed in dying cells and can induce death in 75C1,2 deletion mutant embryos. In addition, ectopic expression of these genes results in stage-specific lethality and induces excess cell death in embryos and the adult eye (5-9). *rpr*, *hid*, and *grim* all function upstream of one or more caspases, as their ability to induce cell death is blocked by caspase inhibitors, including the baculovirus anti-apoptosis protein, p35, and *N*-benzyloxycarbonyl-Val-Ala-Asp fluoromethyl ketone (6-10). RPR is a small, 65-amino acid, protein which has homology to the cytoplasmic death domains of the vertebrate FAS and TNFR (tumor necrosis factor receptor) proteins, both of which play key roles in induction of apoptosis (5, 11, 12). Death domains are also found in several other proteins which dimerize with FAS and/or TNFR (13-15). Alternately, HID and GRIM are both novel proteins that do not exhibit extensive homology to other known proteins. RPR, HID, and GRIM do all share a similar 14-amino acid stretch at their amino termini (6, 7). The presence of these multiple closely linked cell death genes raises the question of whether they may functionally interact. For example, do these genes act combinatorially to regulate the elimination of individual cells, or do they each regulate the killing of distinct cells or cell types?

One cellular system that provides a useful model for addressing this issue is the central nervous system (CNS) midline of the *Drosophila* embryo. CNS midline cells derive from two stripes of mesectoderm in the blastoderm, which come together at the ventral midline during gastrulation and go on to generate a distinctive set of 6-8 CNS midline nerve cell precursor (CMP) cells per segment (16, 17). These CMP cells later differentiate into approximately 25 neurons and glia (17, 18). The midline cells play crucial roles in several distinct developmental events, including organization of the axon scaffold (16, 17), differentiation of the ventral epidermis (19), and migration of phagocytic macrophages (20). Previous studies have shown that most of the developing midline glia die and are quickly phagocytosed by migrating macrophages, whereas none of the ventral unpaired median (VUM) neurons die during embryogenesis (20, 21). 75C1,2 deficiencies, such as *Df(3L)H99*, that eliminate *rpr*, *hid*, and *grim* (5, 7), result in a blockade of all these deaths and an accumulation of ectopic glia (20, 21). The relative contributions of *rpr*, *hid*, and *grim* in midline cell death have not yet been defined.

In this study we examine the specific functions of the *rpr*, *hid*, and *grim* genes in regulating midline cell death. We show that *rpr* and *hid* are expressed in midline cells that normally die and that *hid*, as well as *rpr* and *grim*, appears to be required for normal patterns of midline cell death. Ectopic midline expression of *rpr* or *hid* alone did not efficiently induce ectopic midline cell death during embryogenesis. In contrast, coexpression of both *rpr* and *hid* induced very rapid midline cell death, indicating synergistic actions between these two death regulators.

MATERIALS AND METHODS

Fly Strains and Genetic Crosses.

The P[52A-Gal4] strain was isolated in the Nambu laboratory by means of an enhancer trap screen of approximately 400 independent P[GawB] (22) insertions. P[52A-Gal4] has a 2nd chromosome lethal insertion that drives strong embryonic Gal4 expression in the midline glia as well as most of the VUM neurons from stage 11 onward (stages defined in ref. 23). The P[UAS-*rpr*] and P[UAS-*hid*] strains were generated by cloning *rpr* cDNA clone 40KA or 13B2, and *hid* cDNA clone 5A1B, into the

*Eco*RI site of the pUAST vector (22), and microinjecting these DNA constructs along with 25.7wc helper DNA (24) into *w1118* or *yw67c23* embryos. Two independent P[UAS-*rpr*] insertions were obtained that mapped to the X chromosome (using 40KA) and the 3rd chromosome (using 13B2), while one P[UAS-*hid*] insertion was obtained that mapped to the 3rd chromosome. Additional P[UAS-*rpr*] and P[UAS-*hid*] strains were generated by *P* element mobilization using the 2-3 source of transposase (25). Four strains*y, w*,P[UAS-*hid*], *y, w*,P[UAS-*rpr*], *w*;;P[UAS-*rpr*]/TM3, and *w*;;P[UAS-*hid*]were used in these experiments. Two P[UAS-*lacZ*] strains, one on the 2nd and one on the 3rd chromosome, were kindly provided by Andrea Brand (Univ. of Cambridge, Cambridge, U.K.). The P[1.0*slit-lacZ*] strain contains an X-linked insertion which drives strong expression of *lacZ* in developing midline glia from stage 11 onward (26). Two P[UAS-p35] strains with insertions on the 2nd and 3rd chromosomes were kindly provided by Bruce Hay (California Institute of Technology, Pasadena).

To test for cooperative functions of *rpr* and *hid*, six strains were generated:

(*i*) *w*, P[UAS-*hid*];;P[UAS-*hid*], P[UAS-*lacZ*];

(*ii*) *w*, P[UAS-*rpr*];;P[UAS-*rpr*], P[UAS-*lacZ*]/TM3;

(*iii*) *w*, P[UAS-*hid*];;P[UAS-*rpr*], P[UAS-*lacZ*]/TM3;

(*iv*) *w*, P[UAS-*rpr*];;P[UAS-*hid*], P[UAS-*lacZ*];

(*v*) *w*, P[UAS-*hid*], P[UAS-*rpr*];;P[UAS-*lacZ*]; and

(*vi*) *w*, P[UAS-*lacZ*];;P[UAS-*hid*], P[UAS-*rpr*]/TM3.

Embryos were collected from crosses between the P[52A-Gal4] strain and the above six strains. All crosses were performed at room temperature or 25°C.

Immunocytochemistry and *in Situ* Hybridization.

Embryos were collected and processed for immunocytochemistry and *in situ* hybridization as described previously (20). The monoclonal antibody BP102 (obtained from the Developmental Studies Hybridoma Bank) was used to visualize axon pathways and a mouse monoclonal anti--galactosidase antibody (Promega) was used to detect *lacZ* expression. cDNA clones 13B2 (*rpr*) and 5A1B (*hid*) were used to generate single-

stranded digoxigenin-labeled cRNA probes using the DIG RNA Labeling Kit (Boehringer Mannheim).

Cell Counting and Statistical Analysis.

Analysis of P[1.0*slit-lacZ*]-expressing midline cells in stage 16 wild-type embryos indicated no significant difference among segments T1 to A8. *lacZ*-expressing cells in segments T2, A1, and A5 were counted for individual wild-type and mutant embryos, and the mean of *lacZ*-expressing cells per segment was calculated for each embryo. Twelve embryos were counted for each genotype. Cell counting data were then analyzed using the Statistical Analysis System software (version 6.11; SAS Institute, Cary, NC).

For analysis of ectopic *rpr* and *hid* killing of midline cells, the number of *lacZ*-expressing cells was monitored in embryos bearing P[52A-Gal4], P[UAS-*lacZ*], P[UAS-*rpr*], and/or P[UAS-*hid*]. For analysis of midline glia, the number of *lacZ*-expressing glia was determined in segments T2 and A5, while for analysis of the VUM neurons, cells in segments T1 to A8 were monitored. Because of the high number and close proximity of the labeled VUM neurons, calculations on the effects of ectopic *rpr* and *hid* expression were assessed by determining the number of segments in each embryo that had fewer than three *lacZ*-expressing VUM neurons. No differences were observed among segments T1 to A8 in the elimination of midline glia or neurons (n = 10 embryos for each cross).

RESULTS

Both *rpr* and *hid* Are Expressed in Dying Midline Cells.

It was previously shown (20, 21) that extensive cell death occurs among developing midline glia and that these deaths are blocked by 75C1,2 deficiencies that eliminate *rpr*, *hid*, and *grim*. These three genes each exhibit dynamic and complex patterns of expression in both dying and nondying cells throughout the embryo (5-7). In this study we specifically analyzed the pattern of *rpr* and *hid* expression in CNS midline

cells. This was accomplished by *in situ* hybridization of *rpr* and *hid* cRNA probes, derived from *rpr* and *hid* cDNA clones, to wild-type embryos.

The *hid* gene exhibited expression in many midline cells from stages 12-14 (Fig. 1*A*). Interestingly, *hid* expression was largely restricted to midline cells that reside adjacent to a series of basement membrane midline pores through which phagocytic macrophages migrate and engulf dying midline cells. *hid*-expressing midline cells decrease in number after stage 14, and in stage 16 embryos few *hid*-expressing midline cells are detected (Fig. 1*B*). This reduction in *hid*-expressing midline cells correlates well with the timing of normal midline cell death. In addition, use of P[52A-Gal4] and P[UAS-p35] strains (see *Materials and Methods*) to drive ectopic midline expression of p35, an anti-apoptotic protein (27, 28), prevented the death of midline cells, resulting in ectopic *hid*-expressing cells along the dorsal surface of the nerve cord and alongside the midline pores (Fig. 1*C*). This further suggests that *hid* is normally expressed in dying midline cells.

The midline expression of *rpr* was more restricted and difficult to detect. One or two *rpr*-expressing midline cells per segment were detected in stage 12 embryos, and we were unable to detect *rpr* expression in midline cells at later stages (Fig. 1 *D* and *E*). This might suggest a more transient function for *rpr* than *hid* in dying midline cells; however, no data are currently available regarding the translation pattern of the corresponding proteins. Strong midline *rpr* expression was detected in P[52A-Gal4]/P[UAS-p35] embryos (Fig. 1*F*), providing additional evidence that *rpr*, like *hid*, is normally expressed in dying midline cells. These ectopic *rpr*-expressing cells were located at the same positions as the ectopic *hid*-expressing cells, suggesting that at least some of these rescued cells express both genes. We also examined *rpr* expression in *Df(3L)X25* embryos, where *hid* and *grim* but not *rpr* are removed (7). These embryos exhibited blockade of many, though not all, midline cell deaths (see below). In *Df(3L)X25* mutant embryos there was an accumulation of ectopic *rpr*-expressing midline cells (Fig. 1 *D* and *G*). However, there were still fewer *rpr*-expressing midline cells than in P[52A-Gal4]/P[UAS-p35] embryos. This result indicates that *rpr*

alone was not sufficient for the normal pattern of midline cell death and suggests that *hid* and/or *grim* functions are also required.

Normal Patterns of Midline Glial Death Require *hid*.

To further address whether *rpr*, *hid*, and *grim* all function in midline cell death, we utilized a P[1.0*slit-lacZ*] marker chromosome (26) to examine development of the midline glia in a series of cell death mutations, including the following: *Df(3L)H99*, which eliminates *rpr*, *hid*, and *grim* (5-7); hid^{WR+X1} and hid^{WR+E6} null mutants (6); and *Df(3L)X25* (see above). In stage 15 *Df(3L)H99* mutant embryos there was a 3-fold increase in the normal number of *lacZ*-expressing midline glia measured in segments T2 and A5 (Fig. 2 and refs. 20 and 21). In both hid^{WR+X1} and hid^{WR+E6} mutant embryos there was a 2-fold increase in the number of *lacZ*-expressing cells, indicating that *hid* is essential for many, but not all, midline cell deaths. *Df(3L)X25* mutant embryos exhibited a slight increase in the number of *lacZ*-expressing midline cells compared with *hid* mutants, though there were still fewer *lacZ*-expressing cells than in *Df(3L)H99* mutants (Fig. 2). This suggests that *rpr* and *grim* are also essential for some midline cell deaths. Thus, the normal pattern of midline cell deaths may require the functions of all three of these death genes.

***rpr* and *hid* Cooperate to Kill Midline Cells.**

We then analyzed the ability of ectopic *rpr* and *hid* expression to kill midline cells that normally survive. Targeted expression of *rpr* and *hid* was achieved by crossing the P[52A-Gal4] strain to P[UAS-*rpr*] and P[UAS-*hid*] strains. To visualize the developing midline neurons and glia, a P[UAS-*lacZ*] reporter was introduced into the strains containing P[UAS-*rpr*] and/or P[UAS-*hid*] (see *Materials and Methods*). Embryos were collected from several different crosses and stained by anti-β-galactosidase immunocytochemistry. In embryos where two copies of either *rpr* or *hid* alone were targeted to the midline, most or all of the midline cells appeared normal (Fig. 3*A-C*). [In some embryos bearing two copies of P[UAS-*rpr*] there was a slight reduction of midline glia in a few segments (Fig. 3*B*, Table 1).] This analysis indicated that prolonged

expression of *rpr* or *hid* alone was not sufficient to induce substantial apoptosis in embryonic CNS midline cells. In contrast, when one copy each of both P[UAS-*rpr*] and P[UAS-*hid*] were driven together by P[52A-Gal4], a striking loss of midline glia was detected (Fig. 3*D*). Loss of midline glia was apparent by stage 12, 1-2 hr after ectopic expression of *rpr* and *hid* was first detected, and by stage 14 most of the midline glia were eliminated. Interestingly, the VUM neurons exhibited a greatly reduced sensitivity to the effects of *rpr* and *hid* coexpression, as these cells appeared normal in position, number, and morphology in most or all segments (Fig. 3*D*, Table 1).

These results suggest that *rpr* and *hid* can cooperate to induce ectopic midline cell death, since the simultaneous targeted expression of both genes produced a much stronger effect than expression of two copies of either gene alone. The onset of ectopic midline glial cell death is similar to the timing of normal midline glial cell death, and the weak effect on the VUM neurons is also consistent with the lack of death that normally occurs in these cells. To examine whether the effects of targeted *rpr* and *hid* expression in the midline cells is dosage sensitive, crosses were performed to generate embryos where two copies of P[UAS-*rpr*] and P[UAS-*hid*] were driven by P[52A-Gal4]. These embryos displayed a complete elimination of the midline glia and dramatic loss of VUM neurons (Fig. 3*E*; Table 1). This result indicates that all midline cells are capable of undergoing cell death, and it suggests that the midline neurons and glia may have different sensitivities to *rpr* and *hid* expression.

To determine whether the ectopic midline cell deaths occur by a caspase-dependent mechanism, we tested whether they could be blocked by p35. Crosses were carried out to generate embryos where P[52A-Gal4] drove the simultaneous midline expression of *rpr*, *hid*, *lacZ*, and p35. These embryos exhibited an absence of ectopic cell deaths (Fig. 3*F*), demonstrating that midline cell deaths induced by ectopic *rpr* and *hid* require the functions of one or more caspases. Interestingly, the *Drosophila* caspase gene, *DCP-1*, exhibits strong CNS midline expression (29). Expression of p35 was able to block both *rpr*- and *hid*-induced as well as normal midline cell death, as stage 16 embryos exhibited greater than normal numbers of midline cells and no engulfed midline cells were detected in phagocytic macrophages.

To analyze the developmental consequences of the *rpr*- and *hid*-induced ectopic midline cell death, we monitored ventral nerve cord organization by means of immunostaining with the monoclonal antibody BP102, which labels all CNS axons (30). Midline expression of one or two copies of *rpr* or *hid* alone did not result in detectable axon scaffold defects (Fig. 4*A-C*), suggesting that the midline cells were not only surviving but were capable of carrying out normal axon guidance functions. In contrast, in embryos where both *rpr* and *hid* were expressed, significant defects in axon scaffold organization were detected. In several segments, the anterior and posterior commissures exhibited partial fusion and the two longitudinal connectives were narrowed (Fig. 4*D*). Embryos bearing two copies of both *rpr* and *hid* exhibited more severe disruption of axon scaffold organization, including fusion of the commissures in every segment and severe narrowing of the longitudinals (Fig. 4*E*). This CNS phenotype is similar to that of *single-minded*, *slit*, and *spitz* class mutants, where some or all midline cells fail to develop or maintain their normal positions (see ref. 17). These axonal defects imply that the death of midline cells by means of targeted expression of *rpr* and *hid* was quite rapid and occurred over a time course comparable to that seen with targeted neuronal expression of metabolic toxins, such as ricin (31).

DISCUSSION

In this study we have analyzed the cell death functions of the *rpr*, *hid*, and *grim* genes in the embryonic CNS midline cells. Both *rpr* and *hid* are expressed in dying midline cells, and it will be of interest to identify the mechanisms used to regulate midline transcription of these genes. Analyses of *hid* null and *Df(3L)XL25* mutant embryos suggested both that multiple 75C1,2 genes are required for the normal pattern of midline cell death, and that *rpr*, *hid*, and *grim* may all be essential. Definitive resolution of this issue will likely require isolation of specific *rpr* and *grim* mutants. The mechanism through which these genes may interact to regulate midline cell death is not yet clear. While *rpr* and *hid* were able to synergistically induce midline cell death, it is not known whether RPR and HID proteins interact directly or simply function in converging pathways. In vertebrates, the FAS and TNFR transmembrane proteins form

oligomers and their cytoplasmic death domains interact with death domains present on adapter proteins that, in turn, are linked to a caspase cascade (32, 33). The RPR protein, which possesses some similarity to death domains, is capable of forming multimers *in vitro* (Cerinda Carboy-Newcomb, Chia-Lin Wei, and H.S., unpublished results), suggesting that it may interact with related adapter proteins in *Drosophila*. However it should be noted that two recent studies suggest that the homology between RPR and vertebrate death domain proteins may not be indicative of conserved functions (34, 35). Neither HID nor GRIM possesses death domain homology; however, they, along with RPR, do possess a related 14-amino acid stretch at their amino termini (6, 7). Perhaps this shared region of the three proteins allows for homotypic or heterotypic interactions among themselves, or with common intermediaries. While *in vitro* biochemical studies will clearly facilitate analysis of *rpr* and *hid* interactions, the requirement of both *rpr* and *hid* to kill CNS midline cells may provide an *in vivo* system for mapping which regions of the RPR and HID proteins are essential for their cooperative functions.

Our results confirm and extend previous findings (5-7) that multiple genes in the 75C1,2 region are required for the proper patterns of cell death during embryogenesis. By focusing on a discrete subset of CNS midline cells, we have uncovered synergistic actions of the *rpr* and *hid* genes and also found that these genes may exhibit different efficiencies in killing midline neurons and glia. Our findings raise interesting possibilities, given that (*i*) it was previously shown that expression of *rpr*, *hid*, or *grim* alone was sufficient to induce cell death in wild-type or *Df(3L)H99* embryos (5-7, 9), and (*ii*) synergistic functions of *rpr* and *hid* were not detected in targeted expression in the adult eye (6). Collectively, these data suggest that different cell types may have distinct requirements for 75C1,2 cell death genes during normal development and distinct sensitivities to ectopic *rpr*, *hid*, and *grim* expression. Thus, in previous cell killing experiments that analyzed the effects of ubiquitous embryonic expression of these genes (5-7), it was not determined whether the same cells were killed by each gene, or whether each gene participated in the death of a distinct subset of cells. Additionally, while *rpr* and *hid* can act synergistically to induce CNS midline cell deaths, their ability

to do so may depend upon specific cellular contexts. One attractive hypothesis is that these three genes may act in a combinatorial fashion to regulate cell death in different tissues.

It will also be of interest to determine the effectiveness of ectopic *grim* expression in killing midline cells and to analyze its potential functional interactions with *rpr* and *hid*. This is a particularly interesting question, given that ectopic *grim* is capable of inducing cell death at earlier embryonic stages than either *rpr* or *hid* (7). Ultimately, additional studies of the functional interactions between these key death proteins will likely have relevance not only for the regulation of cell death during normal development and homeostasis but also for how ectopic cell death occurs during disease and injury.

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Fig. 1. Both *hid* and *rpr* are expressed in dying midline cells. *hid* is expressed in several dorsally placed midline cells in stage 13 wild-type embryos (*A*). Most of this expression was eliminated by stage 16 (*B*), and residual *hid* expression above midline pores was inside macrophages (arrow). The broken lines indicate the interface between midline cells and the midline pore, a basement membrane structure separating segmentally reiterated clusters of midline cells. In stage 16 P[52A-Gal4]/P[UAS-p35] embryos there were numerous ectopic *hid*-expressing midline cells localized along the dorsal surface of the nerve cord and alongside of the midline pore (*C*). *rpr* expression was detected in only 1-2 midline cells per segment at stage 12 wild-type embryos (*D*) and was not detected in midline at later stages (arrowheads, *E*). In P[52A-Gal4]/P[UAS-p35] embryos (*F*), there were many ectopic *rpr*-expressing cells in the midline (arrowheads). In *Df(3L)X25* mutant embryos (*G*) there were also greater than normal numbers of *rpr*-expressing midline cells (arrow). *A-D* and *G* are sagittal views with anterior to left. *E* and *F* are ventral views with anterior up. (*A-D* and *G*, x200; *E* and *F*, x100.)

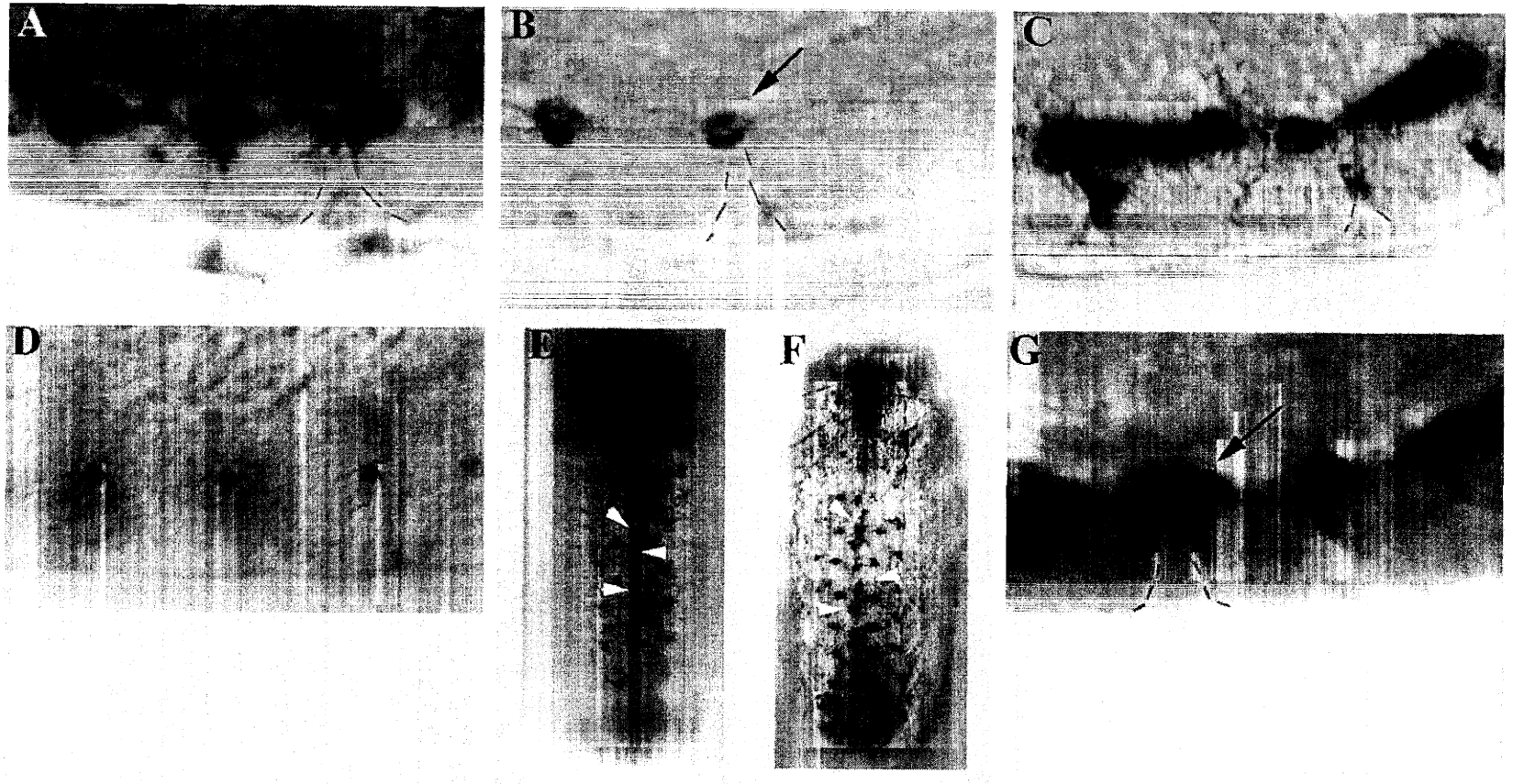

A
B
C
D
E
F
G

Fig. 2. Numbers of P[1.0*slit-lacZ*]-expressing cells in stage 16 wild-type, hid^{WR+X1}, *Df(3L)X25*, and *Df(3L)H99* embryos. ANOVA testing placed the phenotypes of three mutants at different statistical rankings (α, β, and γ) using the wild-type value as the control group.

* See *Materials and Methods* for cell counting and statistical analysis.

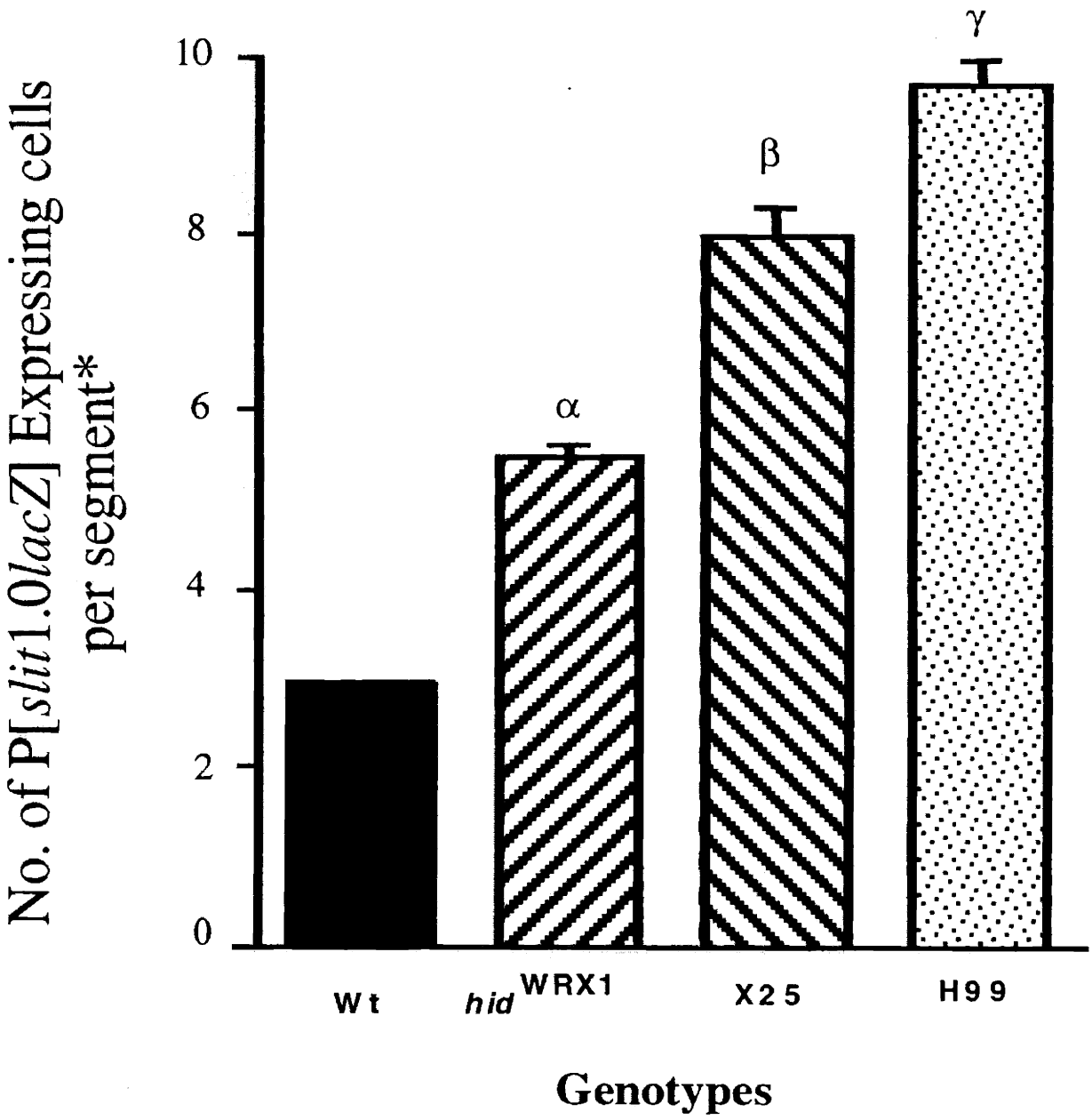

No. of P[slit1.0lacZ] Expressing cells per segment*
10
8
6
4
2
0
α
β
γ
Wt
hid WRX1
X25
H99
Genotypes

Table 1. Quantitation of *rpr*- and *hid*-induced midline cell deaths

Ectopically expressed cell death genes	No. of midline glia in segments T2 and A5	No. of segments with fewer than 3 VUM neuron*
0 copies of *rpr* or *hid*	2.30 ± 0.15	0.00 ± 0.00
2 copies of *hid*	2.00 ± 0.21	0.10 ± 0.10
2 copies of *rpr*	1.80 ± 0.25	0.00 ± 0.00
1 copy of *rpr* and *hid*	0.30 ± 0.21	1.10 ± 0.35
2 copies of *rpr* and *hid*	0.00 ± 0.00	7.80 ± 0.47

Numbers of *lacZ*-expressing midline cells in embryos where P[52A-Gal4] is used to drive P[UAS-*lacZ*] along with 0, 1, or 2 copies of *rpr* and/or *hid*. n = 10 for each group. All numbers are mean ± SE.

*Segments T1 to A8 scored

Fig. 3. *rpr* and *hid* act cooperatively to kill CNS midline cells. Shown are anti--galactosidase staining of stage 16 embryos bearing only P[52A-Gal4] and P[UAS-*lacZ*] (*A*), or additionally bearing two copies of P[UAS-*rpr*] (*B*), two copies of P[UAS-*hid*] (*C*), one copy of P[UAS-*rpr*] and one copy of P[UAS-*hid*] (*D*), two copies of P[UAS-*rpr*] and two copies of P[UAS-*hid*] (*E*), or one copy each of P[UAS-*rpr*], P[UAS-*hid*], and P[UAS-p35] (*F*). (*A*) P[52A-Gal4]/P[UAS-*lacZ*] embryos exhibited -galactosidase expression in 2-3 midline glia (arrow) and the VUM neurons (arrowhead) in each segment. In embryos containing two copies of P[UAS-*rpr*] (*B*) or two copies of P[UAS-*hid*] (*C*), essentially normal numbers of midline glia and VUM neurons were present, indicating that these genes were not individually sufficient to kill midline cells. In embryos containing one copy each of P[UAS-*rpr*] and P[UAS-*hid*] (*D*), there was a dramatic loss of midline glia, although most of the VUM neurons remained present. In embryos containing two copies of P[UAS-*rpr*] and P[UAS-*hid*] (*E*), there was more severe loss of midline glia, as well as elimination of most of the VUM neurons. In embryos containing one copy of P[UAS-*rpr*] and P[UAS-*hid*] as well as one copy of P[UAS-p35], both ectopic and normal midline cell deaths were blocked. Note that many of the ectopic midline cells aggregated at the dorsal surface of the nerve cord (star). All views sagittal with anterior to left. (x100.)

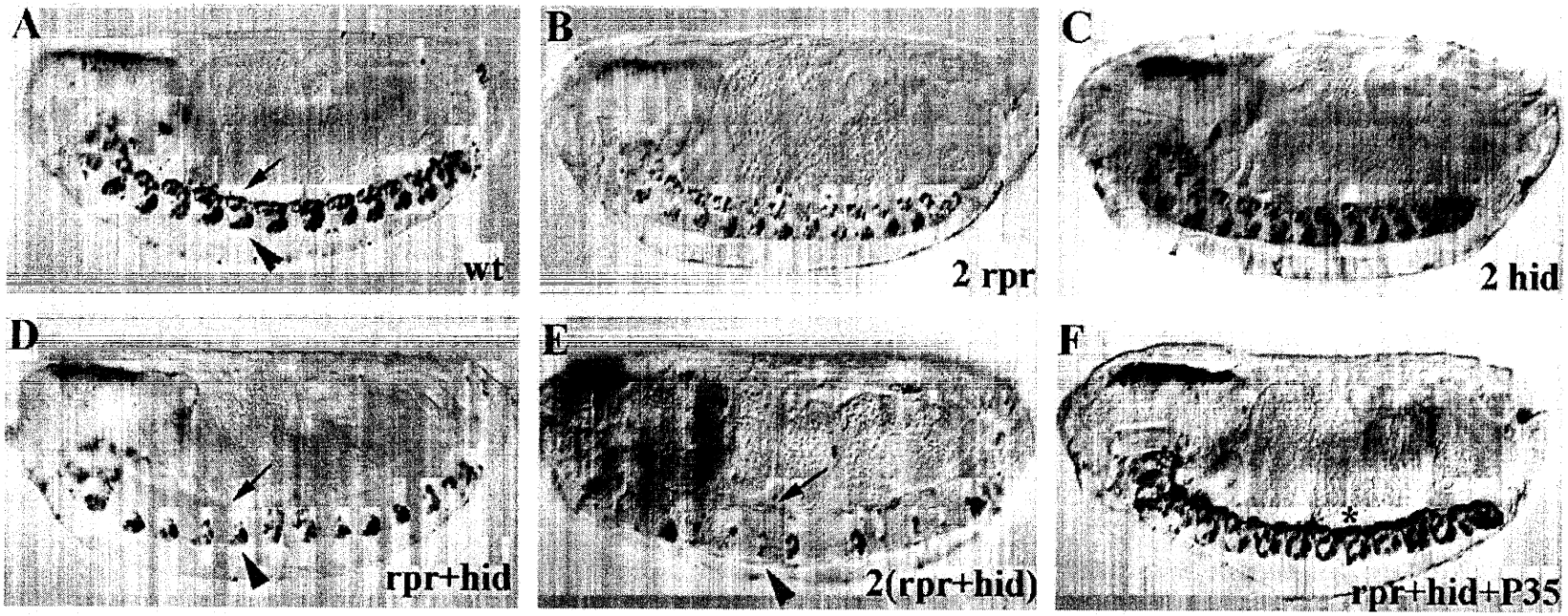
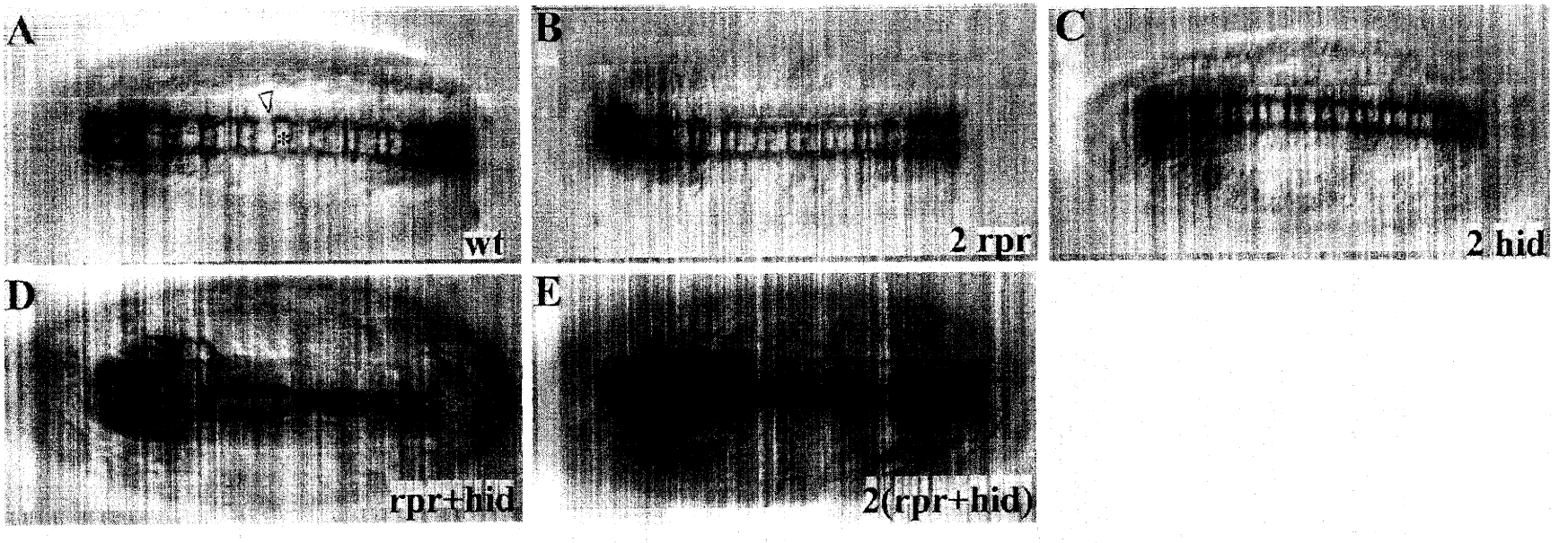

A
wt
B
2 rpr
C
2 hid
D
rpr+hid
E
2(rpr+hid)
F
*
rpr+hid+P35

Fig. 4. Ectopic midline cell deaths result in axon scaffold defects. (*A*) Stage 16 wild-type embryo stained with monoclonal antibody BP102 to visualize the CNS axon scaffold (star indicates commissural axon tracts, arrowhead indicates longitudinal connectives). (*B* and *C*) Similarly stained stage 16 embryos in which two copies of P[UAS-*rpr*] (*B*) or P[UAS-*hid*] (*C*) were driven in the CNS midline cells by P[52A-Gal4]. Note normal CNS organization. (*D*) Stage 16 embryo in which one copy each of both *rpr* and *hid* were driven by P[52A-Gal4]. Note that several segments exhibited fusion of commissures and narrowing of longitudinal connectives. (*E*) Stage 16 embryo in which two copies each of both *rpr* and *hid* were driven by P[52A-Gal4]. Note severe commissure fusions and narrowing of longitudinals in all segments. All views ventral with anterior to left. (x100.)


A
wt
B
2 rpr
C
2 hid
D
rpr+hid
E
2(rpr+hid)

Appendix III.

Analysis of *dBRUCE* expression and preliminary phenotypic analysis of *dBRUCE* mutants.

Figure1. The pattern of *dBRUCE* mRNA expression during embryogenesis. *dBRUCE* transcripts are maternally deposited (A). The maternal transcripts dissipate (B and C) and completely disappear by the cellular blastoderm stage (D). Interestingly, *dBRUCE* mRNA persists slightly longer in the posterior pole, where the pole cells are located, than in the rest of the embryo (arrow in C). *dBRUCE* transcripts are again detected weakly and ubiquitously during gastrulation (E) and then strongly by the fully extended germ band stage (F). During late stages of embryogenesis, i.e. post dorsal closure, expression of *dBRUCE* becomes restricted to the CNS (H) and gonad (arrows in H) . It is interesting to note that cell death and *rpr* and *grim* expression in the embryo have also been described to commence at or just prior to the fully extended germ band stage and at late stages are also restricted to the CNS (Abrams et al., 1993; Chen et al., 1996; White et al., 1994). This correlation between *dBRUCE* expression and cell death is consistent with a role for *dBRUCE* in Rpr- and Grim-induced cell death during embryogenesis.

Whole mount in situ hybridization was performed as described in White et al. (1994) on 0-16hr Canton-S embryos. Sense and antisense digoxygenin labeled probes were generated by in vitro transcription using a linearized *dBRUCE* cDNA subclone, LV1, as template. LV1 is a 1.7 kb EcoRV-XhoI subclone of the BDGP EST, LD31268, in pOT2. No staining was observed with the control sense probe (data not shown). In all panels, anterior is to the left, dorsal is up. The stages are (A,B) blastoderm, (C,D) cellular blastoderm, (E) gastrulation, stage 8, (F) stage 10, (G) stage 14, (H) post dorsal closure.

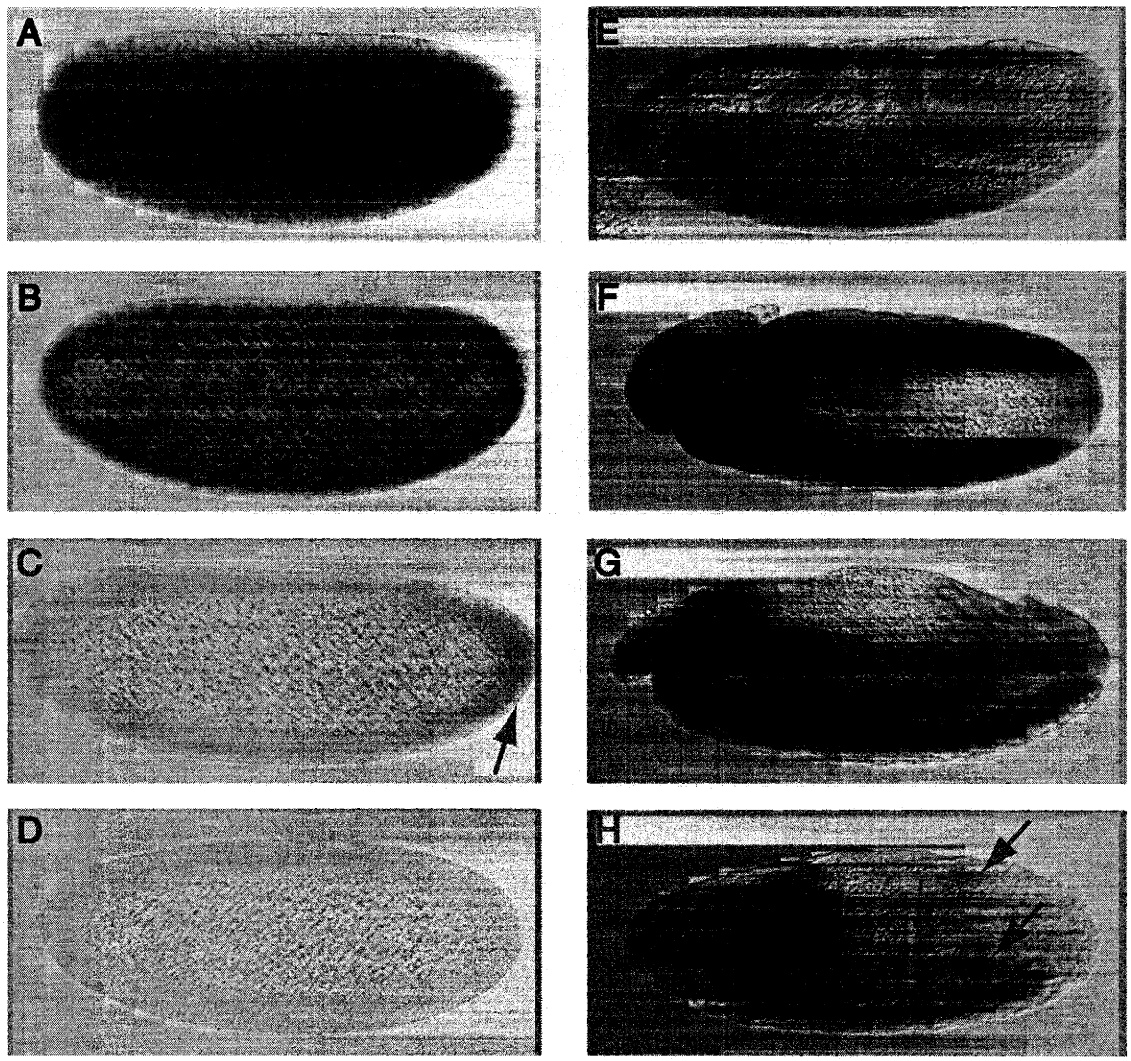

A
B
C
D
E
F
G
H

Figure 2. *dBRUCE* expression in larval tissues and in the adult testis.
dBRUCE is expressed ubiquitously in 3rd instar larval eye-antennal (A), wing (D), and leg (E) imaginal discs and brain (B). *dBRUCE* is also expressed in the adult testis (C). Significant amounts of *dBRUCE* transcripts are not found at the apical tip of the testis, where spermatogonia are formed from stem cells and, subsequently, undergo 4 rounds of mitosis, followed by meiosis. Instead, *dBRUCE* expression commences during or just prior to spermatid differentiation and appears to persist until the release of mature sperm into the seminal vesicle. This expression, along with the observation that *dBRUCE* mutants are male sterile, suggests a role for *dBRUCE* in spermatogenesis, or perhaps more specifically in spermatid differentiation.

Larval tissues were dissected in PBS from Canton-S wandering 3rd instar larvae raised at 25°C. Testes were dissected in PBS from 0-1 day old adult Canton-S males. In situ hybridization was performed as in Figure 1.
(A) eye-antennal imaginal disc, (B) 1 lobe of the larval brain, (C) adult testis, (D) wing imaginal disc, (E) leg imaginal disc.

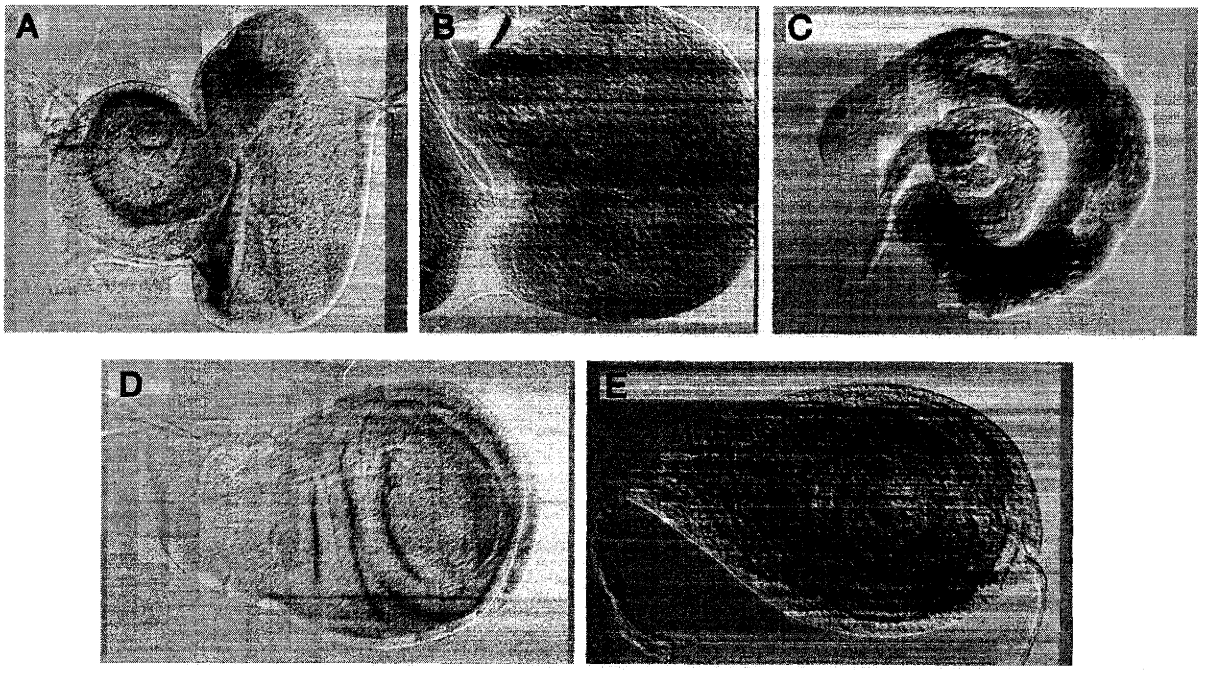
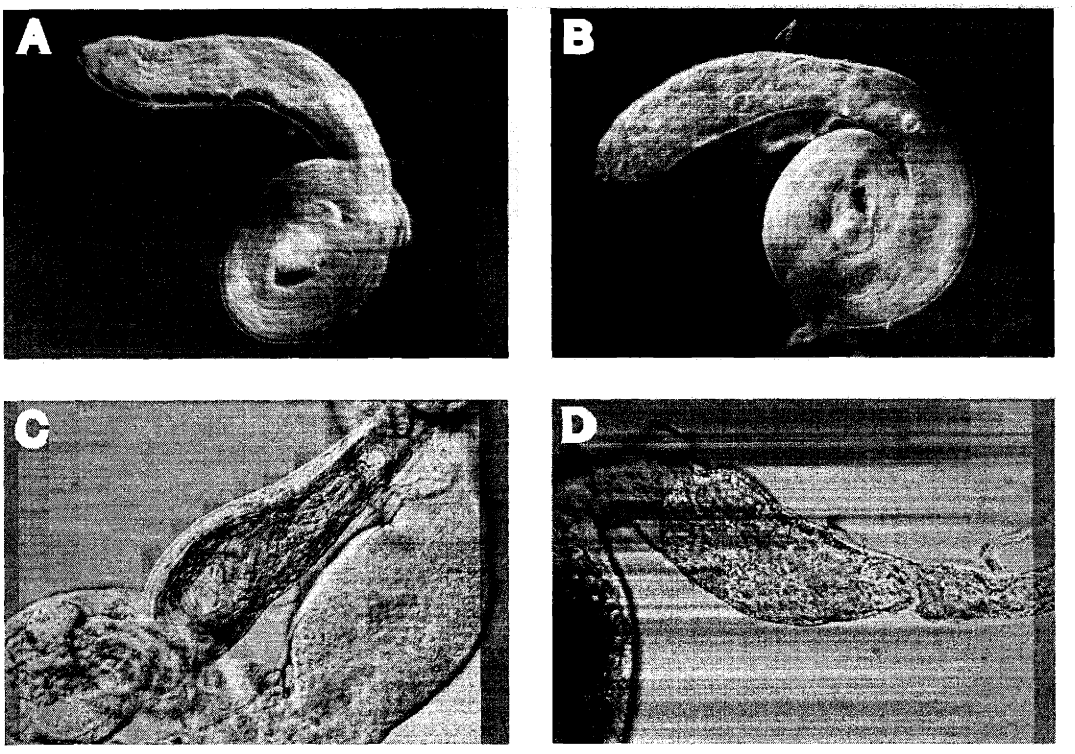

A
B
C
D
E

Figure 3. *dBRUCE* mutants fail to accumulate sperm in the seminal vesicle.
Testes from adult male *dBRUCE* mutants are similar to those from wildtype males in gross overall appearance (compare B to A). However, while bundles of sperm can be seen in the seminal vesicles of wildtype males (C), the seminal vesicles of the *dBRUCE* mutants observed were empty (D). This phenotype suggests a defect in spermatid differentiation or possibly transport to the seminal vesicle, and further suggests a role for *dBRUCE* in these processes.
Genotypes are as follows- (A) and (C) *yw*, (B) $dBRUCE^{3\text{-}5e}$, (D) $dBRUCE^{2\text{-}3e}$.
Testes were dissected from 0-1 day old adult males raised at 25°C in Ringer's buffer. Testes were placed on a glass slide in a drop of Ringer's and viewed with phase contrast microscopy.


A
B
C
D

References

Abrams, J. M., White, K., Fessler, L. I., and Steller, H. (1993). Programmed cell death during *Drosophila* embryogenesis. Development *117*, 29-43.

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

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.

Appendix IV.

Preliminary molecular characterization of the *GMRhid* suppressor, *21-3s*.

Figure 1. *21-3s* mutants carry an insertion which may disrupt a predicted Ser/Thr kinase.

The mutation, *21-3s*, was isolated in the genetic screens described in chapter 2. This mutation dominantly suppresses Rpr-, Hid-, and Grim-induced eye phenotypes. It also suppresses Hid-induced wing phenotypes and organismal lethality. The dominant suppressor phenotype associated with *21-3s* was mapped to the right arm of the 3rd chromosome near the visible marker, *curled*. *dBRUCE* also maps in this general area. However, unlike *dBRUCE* homozygotes, which are male sterile, *21-3s* homozygotes are fertile with no obvious phenotype.

Despite the differences in *21-3s* and *dBRUCE* phenotypes, we thought that *21-3s* might be a mutation in *dBRUCE* because of its proximity. For this reason, we included *21-3s* in the analysis described in chapter 5 that led to the cloning of *dBRUCE*. Briefly, we mapped *dBRUCE* to a 74Kb interval (shown in (A)) and screened this interval for polymorphisms in *dBRUCE* mutants relative to wildtype by genomic Southern, using individual EcoRI fragments as probes. The 10Kb fragment labeled "B" in (A) revealed a polymorphism in *21-3s* relative to wildtype. This 10Kb genomic fragment was partially sequenced and the *21-3s* polymorphism further localized by PCR. PCR primer pairs were designed to give 1 Kb products on average that spanned the entire 10Kb fragment. PCR was performed using both wildtype and *21-3s* genomic DNA as template. One primer pair yielded a 1Kb product from the wildtype and a 3Kb product from the *21-3s* template suggesting that *21-3s* mutants carry a 2Kb insertion. These PCR products were partially sequenced and part of this sequence in shown in (B). The sequence of the *21-3s* product diverged from that of wildtype (underlined) further suggesting that *21-3s* mutants carry an insertion in this region.

BLAST analysis with sequences adjacent to this insertion reveals that this insertion falls within a predicted intron of the predicted gene, CG11870 (BDGP). CG11870 is predicted to encode a protein Ser/Thr kinase. Interestingly, this kinase is also reported to contain a CARD (BDGP), or caspase recruitment domain, which is found in a number of cell death regulators. It is unclear whether the *21-3s* insertion disrupts proper splicing of CG11870 and it is also unclear whether this insertion is the

cause of the dominant suppressor phenotypes we observe with the *21-3s* mutation. However, the possible presence of a CARD domain in CG11870 is sufficiently intriguing to warrant further study.

Note: Further information about CG11870 can be obtained using the Gadfly tool on the Berkeley Drosophila Genome Project web page- http://www.fruitfly.org/.

A

B

WT	CATCATCCCAACATCATCCACATCTACGAAGGTAATTGAGTTTAAGC
21-3s	CATCATCCCAACATCATCCACATCTACGAAGGTAATTGAGTTTAAGC

WT	ACAGCTTAAATATACATAGATACAAATTGAATGCCTAAACACTTGCAG
21-3s	ACAGCTTAAATATACATAGATACAAATTCATATATAATAATGCA