Identification of Novel Cell Death Regulators in *C. elegans* and *Drosophila*

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ABSTRACT

Apoptosis is a form of cell death executed by a class of cysteine proteases called caspases. Though caspases are well-conserved, the mechanisms by which caspases are regulated vary remarkably. This dissertation addresses three independent aspects of apoptosis and its regulation.

In the developing *Drosophila* eye, apoptosis is activated to remove extra cells that are initially present between ommatidia. Mutants for the gene *echinus* have a disorganized eye structure due to a failure of these cell deaths to occur. We demonstrate that *echinus* resembles a deubiquitinating enzyme, that it is expressed in the pupal eye during the time of cell death, and that *echinus* acts genetically upstream or independently of the death-inducing genes *head involution defective*, *reaper*, and *grim*. Based on *in vitro* assays and the fact that the Echinus enzyme lacks a catalytic cysteine residue, we propose that *echinus* and its orthologs constitute a novel class of inactive deubiquitinating enzymes, perhaps functioning in a dominant-negative manner to inhibit deubiquitination of specific substrates.

In *C. elegans*, the model for caspase inhibition is quite different from that in *Drosophila* and in mammals. To look for genes that directly inhibit the CED-3 caspase, we screened a

C. elegans cDNA library for CED-3 suppressors in the yeast *S. cerevisiae* and found several suppressors. Experiments in yeast suggest that one of these genes, Y39B6A.12, requires the prodomain of CED-3 for suppression, and ectopic expression in the *Drosophila* eye shows that it can suppress apoptosis induced by the Bcl2 family member *Debcl*.

In *Drosophila*, DIAP1 is the focal point in the regulation of apoptosis. To identify novel regulators of DIAP1, deficiency chromosomes spanning the *Drosophila* genome were screened for dominant modifiers of a *diap1* knockdown phenotype. Nine deficiencies were isolated that cover no known regulators, and two modifiers were mapped to small genomic regions. This screen has provided a starting point for identifying some of the many uncharacterized genes that are involved in regulating apoptosis.

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Death is essential to life. Programmed cell death, the removal of certain cells, allows multicellular organisms the ability to sculpt developing tissue or protect themselves from disease. The most studied form of programmed cell death is apoptosis, a highly conserved, intrinsic process characterized by a family of proteases called caspases. Initially formed as inactive zymogens, caspases themselves undergo several proteolytic cleavages to form active homomeric enzymes. Once activated, caspases induce gross morphological changes in the cell, such as DNA fragmentation and membrane blebbing.

Death in the nematode *Caenorhabditis elegans*

The initial studies in the nematode *Caenorhabditis elegans* showed that apoptosis is a carefully regulated genetic process. Genetic screens isolated a class of mutants, the so-called *ced* genes for CEll Death abnormality, that are important for the death of 131 somatic cell during *C. elegans* development (Ellis and Horvitz, 1986). Follow-up screens identified a total of 14 loci that fall into two broad functional catergories: cell killing or corpse removal. Epistatis analysis of the *ced* mutants revealed a basic genetic cascade wherein CED-3, CED-4, and EGL-1 are necessary for cell death, and CED-9 inhibits the function of CED-4. Characterization of the *C. elegans ced* genes showed the conserved

nature of the apoptotic machinery and laid the foundation for subsequent studies in other invertebrates and mammals (reviewed in Aravind et al., 2001; Danial and Korsmeyer, 2004). Part of a broad family, CED-3 encodes a caspase with its proteolytic cysteine residue and a long regulatory prodomain (Yuan et al, 1993). CED-4 promotes apoptosis by facilitating the oligomerization and activation of CED-3 and was later shown to be homologous to the mammalian Apaf-1 (Zou, et al, 1997; Yang et al., 1998). Loss of the anti-apoptotic *ced-9* causes ectopic cell death and sterility; *ced-9* is structurally similar to the proto-oncogene Bcl2 (Hengartner et al., 1992; Hengartner and Horvitz, 1994). EGL-1 is a BH3-domain protein, a subclass of the Bcl2 family, that promotes cell death by directly inhibiting the protective CED-9 (Conradt and Horvitz, 1998). The developmental regulation of cell death is critical and the invertebrates *C. elegans* and *Drosophila* provide important examples for understanding this regulation.

Cell death in *C. elegans* is transcriptionally activated

Two main points emerge from experiments in *C. elegans*. First, it is now clear that cells are primed to die. The apoptotic machinery, specifically CED-3 and CED-4, is present in most, if not all, cells, though these proteins are inhibited by CED-9. Loss of CED-9 relieves the inhibition of the death machinery and causes death in cells not normally slated to die (Hengartner et al., 1992). In fact, more cell death would be observed in *ced-9* mutants if it were not for the maternal deposit of CED-9 to the progeny. If all cells have the death machinery present and have the ability to kill themselves rapidly, these observations underscore the need for the careful regulation of CED-9 to maintain viability.

Secondly, studies show that death in *C. elegans* is transcriptionally activated by the upregulation of the CED-9 inhibitor EGL-1. The nervous system of *C. elegans* is a sexually dimorphic structure; for example, the HSN neurons die in embryonic males, but are essential for hermaphrodite egg laying. The sex determining factor TRA-1A is responsible for this dimorphism by altering the transcription of several genes. One target of TRA-1A is the pro-apoptotic *egl-1* gene. TRA-1A inhibits the transcription of *egl-1* in hermaphroditic HSNs, and therefore promotes their survival, while in embryonic male HSNs, TRA-1A is absent, causing the transcription of *egl-1* and the eventual activation of the CED-3 caspase (Conradt and Horvitz, 1999). Such transcriptional regulation of egl-1 and cell death has been also shown for the NSM sister cells (Thellmann et al., 2003).

The *Drosophila* model of apoptosis

Studies on the fruit fly *Drosophila melanogaster* have shown that only certain aspects of cell death are conserved. First of all, many of the genes involved in apoptosis are similar in *C. elegans* and *Drosophila* (reviewed in Vernooy et al., 2000). Though not all are involved in mediating apoptosis, the fly has seven caspases that can be grouped into two categories based on the presence of a regulator prodomain. The three upstream or apical caspases, *Dronc*, *Dream*, and *Dredd*, have long prodomains, and the prodomain of *Dronc* has a CARD domain also found in CED-3 and CED-4. The effector or downstream caspases, *DrICE, dcp-1, decay*, and *damm*, have short prodomains, and are the real mediators of apoptosis. Effector caspases are proteolytically activated by the apical group, which adds a

layer of regulation for the cell to maintain viability. Dark is functionally and structurally similar to CED-4; it is bound to the mitochondria and facilitates the activation and processing of the apical caspase Dronc (Rodriguez et al, 1999). The conservation of structure and function with CED-4 and Dark represents the most significant similarity between the *C. elegans* and *Drosophila* apoptotic machinery.

Here the similarity ends, and flies and worms have different mechanisms for the inhibition and activation of caspases. Because *Drosophila* has both the apical and effector caspase groups, caspases can assume different functions and show tissue specificity. The caspase Dredd for example is involved in the innate immune response (Elrod-Erickson et al., 2000). Secondly, in worms, the model of caspase inhibition is in part a consequence of CED-9 sequestering CED-4 to the mitochondria; in contrast, the *Drosophila* DIAP1 protein directly inhibits the processing of the Dronc zymogen and the active form of DrICE (Chai, et al., 2003; Yan et al., 2004). Finally, the activation of programmed cell death in *C. elegans* is mediated by the transcriptional upregulation of *egl-1*. Flies encode their own Bcl2 family members, *Debcl* and *Buffy*, which have been implicated in cell death, though the mechanism of their action is still unclear (Colussi et al., 2000; Quinn et al., 2003). To activate caspase-mediated cell death, *Drosophila* uses the small genes *head involution defective* (*hid*), *grim*, and *reaper*. HID, Grim, and Reaper have a small N-terminal motif that binds to DIAP1 and relieves the caspase inhibition (Wu et al., 2001). The mammalian proteins Smac/Diablo play a functionally similar role to HID, Grim, Reaper, though there is little sequence similarity between the proteins (Du, et al., 2000; Verhagen et al., 2000). Regulation of *hid*, *grim*, and *reaper* occurs by various mechanisms. The microRNA

bantam can control the translation of HID, though the developmental context of this regulation has yet to be found (Brennecke et al., 2003). The homeobox gene *Deformed* upregulates the transcription of *reaper* to cause death in the head segments in the developing embryo (Lohmann et al., 2002). Though discussed in more detail later, the EGF receptor pathway has been shown to attenuate HID activity both transcriptionally and post-transcriptionally (Kurada and White, 1998; Bergmann et al., 1998).

Programmed cell death in *Drosophila* development

The regulation of the death machinery in fly development has not been fully characterized. Cell death is important for the formation of several adult tissues as well as the removal of larval tissue during the pupal stage. Investigations of cell death during *Drosophila* development follow two approaches. Using a forward genetics approach, observation of developing tissues and characterization of mutants has yielded a class of genetic mutants. For example, *hid* was originally isolated as a mutant that displayed defects in male terminalia rotation and embryonic head involution (Abbott and Lengyel, 1991). Characterization of the long-standing mutants *roughest* (*rst*) and *echinus* (*ec*) showed that apoptosis is a natural part of the development of the adult retina (Wolff and Ready, 1991). Observations of wings from recently eclosed animals revealed that cell death was necessary for proper wing formation (Kimura et al, 2004).

The second approach involves the characterization of genes known to activate apoptosis by looking at gene expression patterns or, if available, the mutant phenotypes. The death activator *Dark* was cloned by its homology to CED-4 and then shown to be important for wing and scutellar bristle formation (Rodriguez et al., 1999). Gene expression patterns for *grim* revealed that the gene is transcribed just prior to the onset of cell death in embryonic tissues (Chen et al., 1996).

Steroid-induced cell death

Developmental cell death is particularly striking during *Drosophila* metamorphosis. During the pupal stage the tissues from the larval stage must make room for the proliferating cells that will make up the adult fly. At this time, the steroid hormone ecdysone is released in several bursts to facilitate the formation of the puparium and the destruction of the larval tissue. EcR and USP comprise the hormone receptor, which then relays a signal through FTZ-F1, BR-C, E74, and E93 to activate transcription (Jiang et al., 2000). Simultaneous to the destruction of the larval salivary gland and midgut, *E93* induces the transcription of the pro-apoptotic genes *Dronc*, *hid*, *reaper*, and *dArk* (Lee et al., 2000). Other caspases besides *Dronc* must be involved as the caspase inhibitor P35 can delay pupal cell death and *Dronc* is P35 insensitive (Jiang et al., 1997).

One curious observation of steroid-triggered cell death is that dying tissues display some non-apoptotic morphologies. Autophagic cell death is a less characterized mode of cell

death distinguished by large cytoplasmic vacuoles. Dying salivary glands display the autophagic vacuoles as well as markers for active caspases, like altered cytoskeleton proteins and DNA fragmentation (Martin and Baehrecke, 2004). Though the role of autophagic cell death in dying pupal tissue is not understood, caspases and their activators HID, Grim, and Reaper are essential.

Signaling and pattern formation in the eye

The developing adult eye presents a juxtaposition of various cellular signals that integrate cell differentiation, apoptosis, and cell adhesion. The adult eye is composed of approximately 750 repeating units called ommatidia. Three cell types comprise each ommatidium: the photoreceptors, the cone cells, and primary pigment cells. Interspacing the ommatidia are a collection of cells known as interommatidial cells (IOCs) and bristles. Adult eye formation begins in the third instar larval stage when the morphogenetic furrow sweeps anteriorly across the eye imaginal disc. The morphogenetic furrow synchronizes the cell cycle of the epithelial cells just prior to it, and then initiates the differentiation of those epithelial cells. The photoreceptors are the first cluster of cells to differentiate and can be visualized during the larval stage. The remaining cells differentiate during the pupal stage of development, with the cone cells first, followed by the primary pigment cells, then finally the IOCs.

In part, pattern formation of the fly eye is dependent on caspases and cell death. An understanding of the importance of cell death in late eye development began with the characterization of the *echinus* and *roughest* mutants. These two mutants display rough, non-ordered eyes due to the lack of apoptosis late in cell differentiation (Wolff and Ready, 1991). It was shown that after IOCs differentiate, a few unselected cells die in the normal course of development to complete the ordered ommatidia array. Later studies showed that pupal eye death can be blocked by overexpression of P35 or a mutation in *hid* (Hay et al., 1994; Yu et al., 2002). Mutants of *morgue*, a gene involved in ubiquitin-mediated protein degradation, show altered levels of DIAP1 and excess IOCs (Hays et al., 2002; Wing et al., 2002).

Experiments have also implicated several signaling pathways in late eye development. Most notably, a dominant-negative version of the EGF receptor (*DER*) disrupts normal differentiation of all eye cells including the IOCs, which underscores its necessity throughout eye development (Freeman, 1996). Likewise, overexpression of *Argos*, a natural inhibitor of the EGF receptor, mimics the phenotypes of dominant-negative *DER* (Sawamoto et al., 1998). Recent work also revealed the role of the transcription factor *lozenge* during late eye development. Loss of *lozenge* in late eye development decreases the amount of cell death, but not the differentiation of IOCs (Wildonger et al., 2005). Interestingly, *lozenge* was also shown to upregulate the transcription of *Argos*, providing a mechanism for control of retinal cell death by lozenge (Wildonger et al., 2005).

The other cellular factor involved in pupal eye apoptosis is cell adhesion. *roughest*, a mutant identified by a lack of cell death specifically in the eye, was later cloned as a novel immunoglobulin gene (Ramos et al., 1993). Antibodies revealed that proper localization of Roughest to the cell borders between primary pigment cells and IOCs was necessary for apoptosis and was dependent on the cell adhesion molecule DE-cadherin (Reiter et al., 1996; Grzeschik and Knust, 2005).

Clues are emerging as to how the retinal cells sense and integrate signals emitted during cell adhesion, cell differentiation, and cell death. The EGF receptor DER and the downstream component Ras have been shown to suppress the activity of HID by modulating its transcription and phosphorylation state (Kurada and White, 1998; Bergmann et al., 1998). These results suggest that DER/Ras provide a cell survival signal by inactivating HID and, therefore, the caspases. The Notch signaling pathway has been proposed either to promote IOC cell death or to inhibit cell survival (Miller and Cagan, 1998). One mechanism for Notch activity is through Roughest. Mutants for *Notch* affect the localization of Roughest in the pupal eye, which, as mentioned before, is necessary for retinal apoptosis (Gorski et al., 2000).

Despite all these gains, important questions remain about cell death in the pupal eye. It still remains unclear how the cell senses proper cell adhesion. Why is proper localization of Roughest to the border between IOCs and primary pigment cells critical for cell survival? Another remaining question revolves around the mutant *echinus*. *echinus* was originally isolated by Bridges in 1916 because of its rough eye phenotype, and subsequently it was

used as an X-linked marker (Lindsley and Zimm, 1992). Later studies show that mutants for *echinus* disrupt pupal eye cell death, much like *roughest* (Wolff and Ready, 1991). But since then *echinus* has remained uncharacterized. Finally, it is unknown what signals induce apparently equal interommatidial cells to either survive or commit cell suicide. It could be that improper or insufficient Roughest localization causes the activation of Lozenge, which in turn inhibits the EGFR cell survival signal, resulting in cell death. Another outstanding question that remains in *Drosophila* is how active caspases can be present in border cell migration in oogenesis and in sperm individualization, yet not induce cell death (Geisbrecht and Montell, 2004; Huh et al., 2004). Finally, it has been noticed that HID is present in cells of the primordial optic lobe that do not die (Grether et al., 1995). It is yet to be determined what prevents HID from activating cell death in this context.

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Chapter 2: Echinus Shows Homology to Deubiquitinating Enzymes and is Required for Retinal Cell Death in *Drosophila*

2.1 Abstract

The *Drosophila* eye is a patterned epithelium of repeating units called ommatidia. The ommatidia consist of four cell types, with both cell differentiation and cell death important for the final arrangement of cells. Death occurs early in pupal development and removes the excess cells that surround each ommatidium. Mutants for the *echinus* (*ec*) gene are devoid of this pupal-stage apoptosis and extra cells are left between the ommatidia to create a rough eye in the adult fly. In this study, we characterized several *echinus* mutants and the Echinus protein resembles a deubiquitinating enzyme. The gene possesses a UCH domain, characteristic of deubiquitinating enzymes of the UBP family, though it lacks the necessary catalytic cysteine. Structure-function analysis revealed that the C-terminal poly-glutamine repeat is not important for function. Enhancer trap and *in situ* data corroborated the role of *echinus* in pupal eye development; expression of *echinus* was observed at the time of pupal eye apoptosis. Epistatis analysis suggested that *echinus* acts upstream or independently of the pro-apoptotic genes *hid*, *grim*, and *reaper*.

2.2 Introduction

The *Drosophila* eye is a beautiful hexagonal array of repeating units called ommatidia. The eye consists of approximately 750 ommatidia, which themselves are an ordered set of four cell types. Each ommatidium contains eight photoreceptor (R cells) neurons, four cone cells, and two primary pigment cells. The interommatidial cells (IOCs) surround each ommatidium and help pattern the cellular array. Differentiation of the retinal cells begins when the morphogenetic furrow (MF) progresses through the imaginal eye disc. From a layer of undifferentiated epithelium following the MF, the R8 cell develops first and then recruits the seven other R cells. Simultaneous to the differentiation of the R cells, the remaining cells undergo another round of mitosis to create the final pool of undifferentiated cells (Wolff and Ready, 1991b). The cone cells differentiate next, followed by the primary pigment cells, then finally the secondary and tertiary pigment cells, also known as the interommatidial cells (IOCs). During the time of IOC differentiation, at about 28 hours after pupal formation (APF), a wave of apoptosis occurs to remove excess IOCs, thereby completing the ordered array. How the IOCs commit to cell suicide or to differentiation is not well understood.

The core components of the apoptosis machinery are known to modulate the wave of death during pupal eye development. Active caspases are observed during IOC death (Yu et al., 2002); in addition, overexpression of the *Drosophila* caspase inhibitor *Diap1* causes the formation of excess IOCs (Hay et al., 1995). Likewise, ectopic expression of the pan-caspase inhibitor P35 interferes with cell death and results in the

differentiation of extra pigment cells (Hay et al., 1994). Mutants of the F-box/Ubc gene *morgue* have elevated levels of DIAP1 during pupal eye development and have superfluous IOCs (Hays et al., 2002; Wing et al., 2002).

Preliminary studies have also shown that both the *Egfr/Ras* and *Notch* signaling pathways are active during IOC differentiation or death. Activated alleles of both *Ras* and *Egfr* prevented cell loss, while expression of a dominant-negative version of Egfr had too few IOCs (Miller and Cagan, 1998; Freeman, 1996). Additionally, ectopic expression of *argos*, an inhibitor of the EGFR, increased the number of cells undergoing apoptosis (Sawamoto et al., 1998). Antibody studies also showed the presence of the Egfr in the IOCs (Miller and Cagan, 1998). Likewise the *Notch* signaling pathway is active during this stage of eye formation. Notch localizes to the IOCs during pupal development, and the mutant $N^{fa\text{-}g}$ allele shows disrupted primary cell morphology and an abundance of IOCs (Kooh et al, 1993; Cagan and Ready, 1989). It is not known whether either of these signaling pathway work by modulating apoptosis or the differentiation of the IOCs.

Deubiquitinating enzymes (DUBs) are ubiquitin-specific proteases that cleave ubiquitin at its C-terminus and have been shown to play many cellular roles (reviewed in Wilkinson, 1997). DUBs are categorized into two classes, the small- protein UCH family and the large-protein UBP family. A *Drosophila* member of the UCH family, UCH-L3, localizes to the PA700 regulatory region of the proteosome and is thought to remove ubiquitin moieties from proteosome-bound proteins (Holzl et al., 2000; Wojcik

and DeMartino, 2002). The proteins of the UBP family of deubiquitinating enzymes have little sequence similarity outside their UCH domain and are involved in a wide range of cellular processes. Two of the best characterized deubiquitinating enzymes are *fat facets* and HAUSP. The *Drosophila* gene *fat facets* (*faf*) mediates photoreceptor internalization by removing ubiquitin from *liquid facets*, the *Drosophila* epsin homolog (Cadavid et al., 2000). The UBP HAUSP opposes the ubiquitin ligase Mdm2 by hydrolyzing Ub from p53 (Li et al., 2002).

In this report, we characterize and clone the *Drosophila* gene *echinus*. *echinus* was originally isolated as a spontaneous mutant with rough eyes and subsequently used as a marker for the X-chromosome (Bridges, 1916, reiterated by Lindsley and Zimm, 1992). Loss-of-function alleles are deficient for apoptosis in the developing pupal eye and contain extra IOCs. *echinus* was cloned and found to show homology to deubiquitinating enzymes of the UBP family; however, Ec lacks residues thought to be critical for deubiquitinating activity. Homologous genes in humans, mice, and *Arabidopsis* show similarity only in the UCH domain. Enhancer-trap staining and *in situ* hybridizations show that *echinus* is expressed in the cone cells, the primary pigment cells, and the IOCs at developmental stages prior to and after the wave of cell death in the pupal eye. Genetic interactions with components of the death machinery show that *echinus* works upstream or independently of the core *Drosophila* apoptotic machinery.

2.3 Materials and Methods

Drosophila lines and genetics

Drosophila strains and crosses were performed at 25 ^{o}C. Pupal timing is expressed in hours, with the white prepupal stage defined as 0 hours after pupal formation (APF). Pupal dissections were performed at 42 hrs APF unless noted otherwise. The following strains were used: ec^{1} (Bloomington Stock Center, Indiana University), ec^{PlacZ}, GMR-hid, GMR-reaper, GMR-grim, GMR-dronc, GMR-strica. Generation of the series of *ec* deletion mutants were performed by imprecise P-element excision on the line EP(X)1343. RH68894 was obtained from Research Genetics/Invitrogen (Carlsbad, CA) and cloned into pGMR-1N. Interommatidial cell counts were made by counting the IOCs, minus the bristles, that surround two primary pigment cells. Three separate areas were counted per pupal eye discs, and at least five eye discs were counted for each genotype.

Constructs and transgenes

For RNAi, gene fragments were placed between two UAS control element on opposing strands, as previously described (Giordano, 2002). ec^{RNAi} (coding region amino acid 1237–1505) and CG2901^{RNAi} (coding region amino acid 1–171) were made using this technique. A third *echinus* fragment (coding region 232–398) was also introduced into this vector. Flies carrying this construct also phenocopy *echinus* loss-of-function (data not shown).

Full-length cDNA of *ec* was isolated from a larval-pupal cDNA library. A probe was generated against the UCH domain of CG2904. Several clones were isolated and sequenced. The longest cDNA was used to generate GMR and UAS constructs. Transformants were made by using microinjection into <1 hour w^{1118} embryos. Several transgenic lines were prepared for each construct.

GMR-*ec* (-polyQ) encodes the first 1389 amino acids of coding sequence and was generated by PCR amplification from *ec* cDNA. GMR-*ec* (UCH) was PCR amplified from *ec* cDNA and encodes the first 400 amino acids. The QuikChange protocol (Stratagene) was used to make a C134S mutation. GMR-USP53 was derived from a pGEX vector and placed into EcoRI-NotI site of pGMR-1N.

Microscopy, immunocytochemistry, and antibodies

Scanning electron microscope images were produced on a Hitachi machine. Flies were dehydrated in an ethanol series, incubated in hexamethyldisilazane (Sigma) overnight, and dried prior to use. Confocal microscopy was performed on a Leica (Deerfield, IL) DM IRBE microscope.

Pupal eye discs were dissected and immunostained according to standard procedures. Eye discs were fixed (20 minutes, room temperature) in 4% formaldehyde in 1x PBS (pH 7.2). Antibodies were used at the following concentration: mouse anti-Dlg (1:150) (DSHB, University of Iowa, Iowa City, IA), mouse anti-β-galactosidase (1:100)

(Promega). Alexa Fluor 488 mouse secondary antibodies were used (Molecular Probes). Eye discs were mounted in VectaShield medium (Vector, Burlingame, CA).

2.4 Results

Characterization of *echinus* mutants

A previous study on *echinus* has shown that the rough eye in the adult was due to the presence of excess interommatidial cells and a loss of apoptosis (Wolff and Ready, 1991a). Cell counts of the pupal eye disc and BrdU analysis showed no increase in cell number or proliferation (Wolff and Ready, 1991a). Based on these considerations, it was concluded that *echinus* is involved in the death of the interommatidial cells in the eye.

A new allele of *echinus*, caused by a P-element insertion, was isolated and mapped to the 3F3 region of the X-chromosome (Fig. 1). ec^{PlacZ} did not complement the original echinus allele (ec^{1}). Excisions of the ec^{PlacZ} P-element reverted the rough eye phenotype, confirming the role of this P-element in the echinus phenotype. This P-element is between the predicted genes CG2901 and CG2904. dsRNA targeted to CG2904 caused a rough eye phenotype in the adult, mimicking *echinus* (Fig. 3C, 3H). Pupal eye dissections of the RNAi line revealed about five excess IOCs per ommatidium.

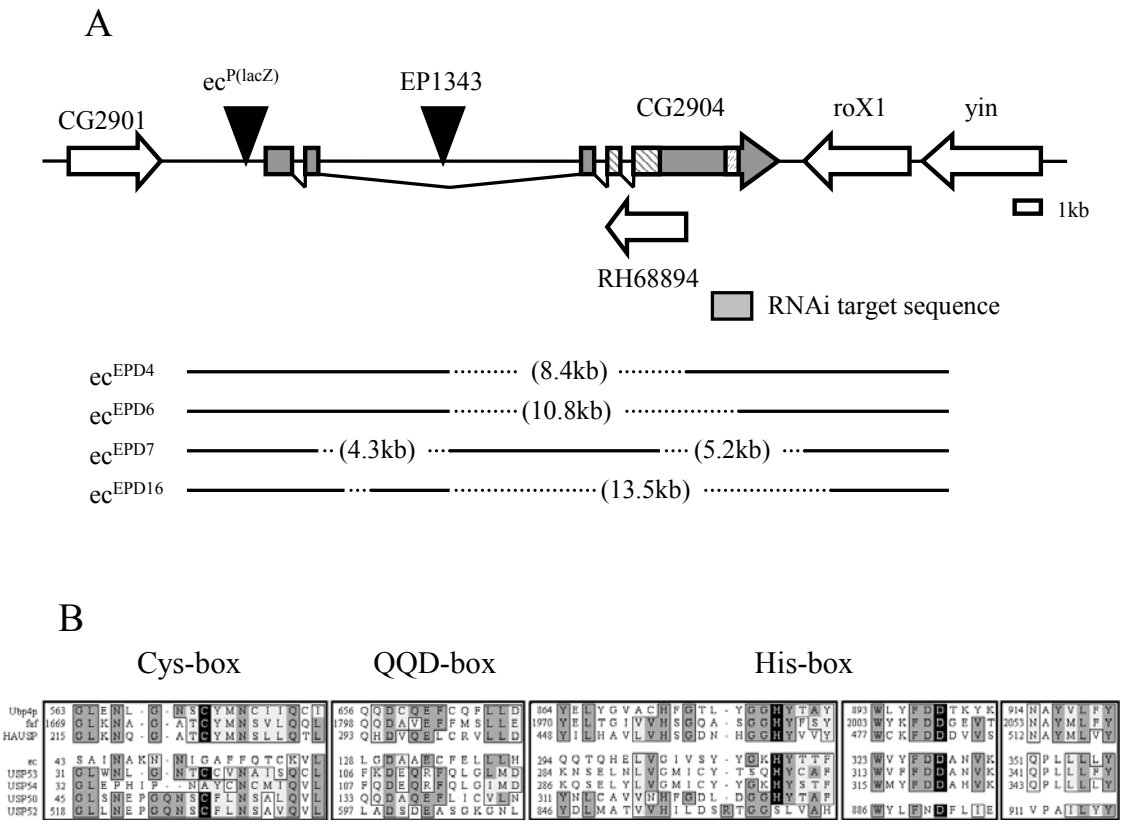


Figure 1. *echinus* encodes a protein with homology to deubiquitinating enzymes. A) Shown is the genomic region surrounding *echinus*, CG2904. The UCH domain is shown in diagonal stripes while the poly-glutamine stretch is shown in hatch marks. P-element insertions are represented by black triangles. Note that *ec*^{PlacZ} is an allele of *echinus*, while EP1343 is not. Shown below the genomic structure is a series of deletion alleles. Missing DNA sequences are shown as dotted lines with the parentheses indicating the size of the deletion. B) Sequence alignment of echinus to other deubiquitinating enzymes. The first three proteins, above the space, encode functional enzymes, while the five below, including Echinus are non-functional. The human Echinus orthologs, USP53 and USP54, are just below the Echinus protein sequence. Catalytic residues are shown in black, with grey representing conserved residues. Note the lack of catalytic cysteine in Ec.

Adult eyes were wild type in RNAi-mediated CG2901 knockdown lines (data not shown).

To generate additional alleles of *echinus*, imprecise P-element excision using the □2-3 transposase was performed. The EP line, EP1343, which lies in between the *ec* P-element and the predicted CG2904 gene, has a normal, wild-type eye. A series of *ec* alleles were generated that removed significant regions of the gene CG2904 by excising EP1343 (Figure 1). The $ec^{EP\square 4}$ allele was the smallest of these deletions, removing approximately 8kb of the predicted gene CG2904 (Fig. 2). Several EMS-induced point mutations were also isolated in the course of this study. ec^{3c3} is a loss-

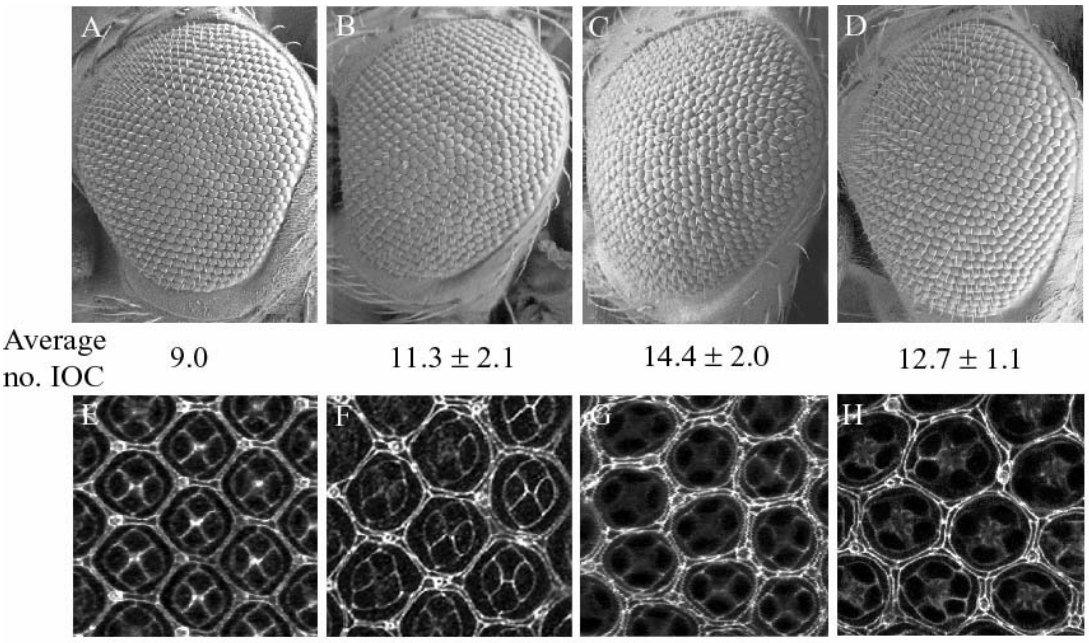


Figure 2. *echinus* mutants have rough eyes due to extra intraommatidial cells. Scanning electron micrographs of adult *Drosophila* eye (A-D) and confocal imaging of 42 hr APF pupal retinas (E-H). Cell borders are marked with anti-Discs large (Dlg) in confocal images. (A,E) Wild type. (B,F) *echinus*(1). (C,G) *echinus*(PlacZ). (D,H) *echinus*(EPΔ4).

of-function mutant carrying a lysine to missense mutation at amino acid 758. Later experiments showed that the original *echinus* mutant (ec^1) is a transposon insertion in the 5' UTR of CG2904 (I. Bosdet, personal communication). In all cases, each of the loss-of-function alleles gave adult eyes that were rough and disorganized. Upon dissection, the pupal eyes contained excessive interommatidial cells, and occasionally contained extra primary pigment cells.

echinus shows homology to deubiquitinating enzymes

These results demonstrate that CG2904 encodes *echinus*, a putative deubiquitinating enzyme. To isolate a full-length cDNA of this gene, a pupal-larval library was screened. Using a probe for the ubiquitin C-terminal hydrolase (UCH) domain, several cDNAs were isolated, all having a close resemblance to the predicted gene CG2904. The longest fragment was sequenced, and was shown to be a 6747 bp fragment, covering roughly 15 kb of the genomic region (GenBank accession number AY576488). Importantly, the 5' exon of the isolated cDNA lies 500 bp downstream of the mutant $ec^{P\ lacZ}$ allele, thus providing an explanation for how this allele disrupts Echinus function.

The sequenced cDNA encodes a predicted 1712 amino acid protein with an N-terminal UCH motif and a C-terminal poly-glutamine stretch. UCH domains are the defining characteristic of deubiquitinating enzymes (DUBs), a large family of proteins involved in many biological processes. Like other members of the UBP family of DUBs,

Echinus is a large protein (188 kDa) and shares little resemblance to other deubuiquitinating enzymes outside its UCH domain.

DUBs are cysteine proteases that remove ubiquitin from ubiquitin-conjugated proteins (Wilkinson, 1997). Catalytic activity required three key resides: a cysteine, a histidine, and an aspartate. Based on homology to other DUBs, the catalytic histidine of Echinus is proposed to be at residue 310 and the aspartate at 328. The putative catalytic cysteine is difficult to pinpoint, as the sequence alignment to homologous proteins show that Echinus has a glycine in place of the active site cysteine. The alignment suggests either that Echinus encodes a non-functional deubiquitinating enzyme or that the putative catalytic cysteine lies elsewhere in the UCH domain. Four other putative catalytic cysteines exist within the UCH domain, at positions 79, 84, 87, and 134, though each shows only weak similarity to the consensus UCH motif.

The other noticeable feature of the Echinus protein is a stretch of glutamines near the C-terminus. This stretch of 156 amino acids contains 77 glutamines, or about 49%. One region contains a 34 amino acid poly-glutamine repeat. The significance of this region in Echinus and in other proteins is still unclear.

Echinus most closely resembles the two human genes USP53 and USP54 and two *Arabidopsis* genes At3g47900 and At3g47890. Specifically, the UCH domain of Echinus is 42% identical and 45% identical to its counterpart in USP53 and USP54,

respectively. Likewise, the two predicted plant genes (At3g47900 and At3g47890) are common to Echinus only within their UCH domain, both being 26% identical.

A bacterial assay has been used to show that the *Drosophila* deubiquitinating enzyme Fat Facet can cleave ubiquitin from a □-galactosidase protein conjugate (Huang et al., 1995). Bacterially expressed Echinus, however, failed to remove ubiquitin from the protein conjugate, unlike the control protein Ubp2p, a *S. cerevisiae* deubiquitinating enzyme (Table 1). Of interest, both human orthologs USP53 and USP54 also failed in this *in vitro* assay (Quesada et al, 2004).

Plasmid	Colony Color
pUb-Arg-β-Gal	Blue
pRB105	White
pRB-ec	White
pUb-Arg-β-Gal; pRB105	White
pUb-Arg-β-Gal; pRB-ec	Blue

Table1. Results of an in vitro assay to test for deubiquitination of a ubiquitin-β-galactosidase protein fusion. In pUb-Arg-β-gal ubiquitin is tied to β-galactosidase by the amino acid arginine. Cleavage of ubiquitin from β-gal exposes an N-terminal arginine residue on β-gal and promotes β-gal instability. Colony color is a measurement of β-gal activity of the X-gal substrate. Blue indicates an active β-gal enzyme. pRB105 encodes the yeast deubiquitinating enzyme Ubp, while pRB-ec encodes echinus.

CG2904 rescues *echinus*

Expression of the *echinus* cDNA under the control of the GMR enhancer looks wild type (Fig. 3D, 3I). All the pupal eye disc cells are present in their correct

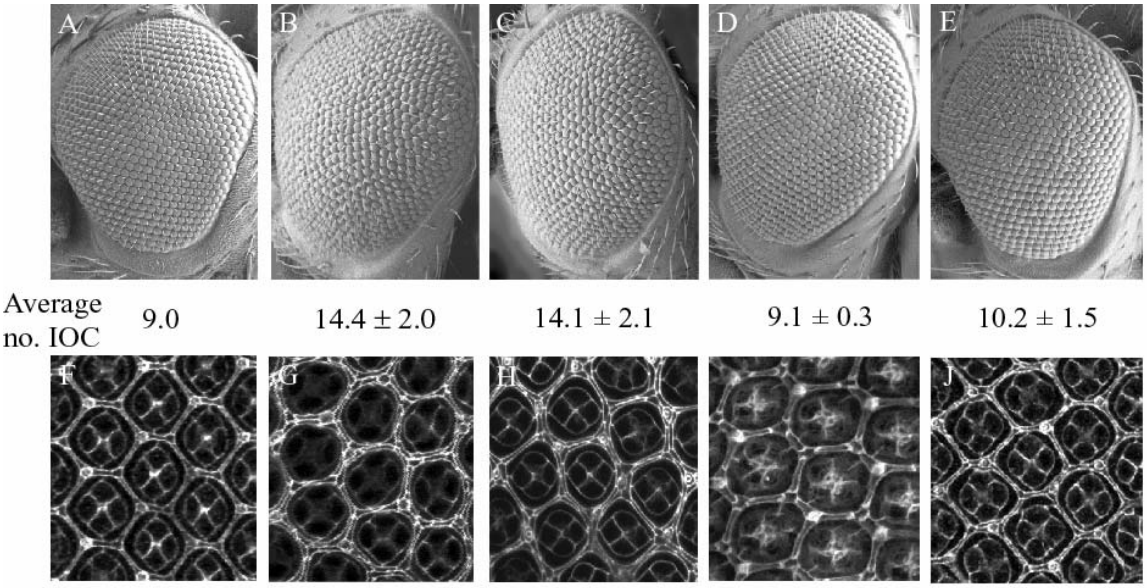


Figure 3. *echinus* resembles a deubiquitinating enzyme. Scanning electron micrographs of adult *Drosophila* eye (A-E) and confocal imaging of 42 hr APF pupal retinas (F-J). Cell borders are marked with anti-Discs large (Dlg) in confocal images. (A,F) Wild type. (B,G) *echinus*(PlacZ). (C,H) RNAi of the deubiquitinating enzyme CG2904. (D,I) Eye specific expression of CG2904 using the GMR enhancer. (E,J) GMR-CG2904 in an *echinus* loss-of-function background.

numbers and positions. When placed into an *echinus* loss-of-function background, GMR-CG2904 nearly rescues the rough eye phenotype (Fig. 3E, 3J). The adult eye is indistinguishable from wild type, and pupal eye dissections reveal about one extra IOC above wild type.

Several cDNAs have been isolated that overlap with the *echinus* coding region, though in the antisense direction (Fig. 1). To verify that these cDNAs do not mediate *echinus* function, we tested their ability to modulate the echinus phenotype. A representative of these cDNAs, RH68894, failed to rescue or enhance any *echinus* mutants (data not shown). Based on these observations, we conclude that *echinus* encodes a protein with

homology to a deubiquitinating enzyme. The roles, if any, of the antisense gene, RH68894 is unknown.

Echinus is expressed in pupal interommatidial cells

Both an enhancer trap line and *in situ* hybridizations were used to determine the

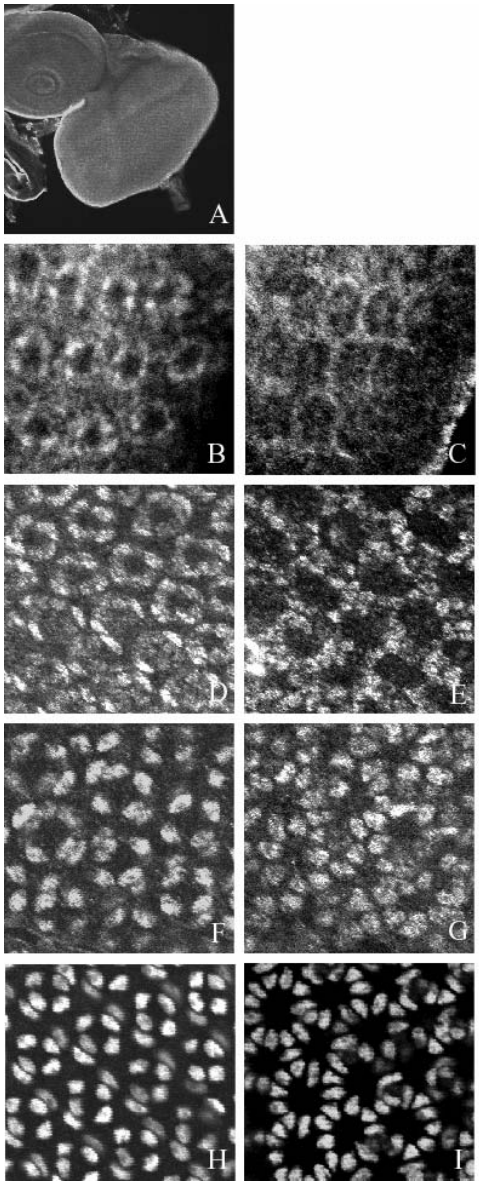
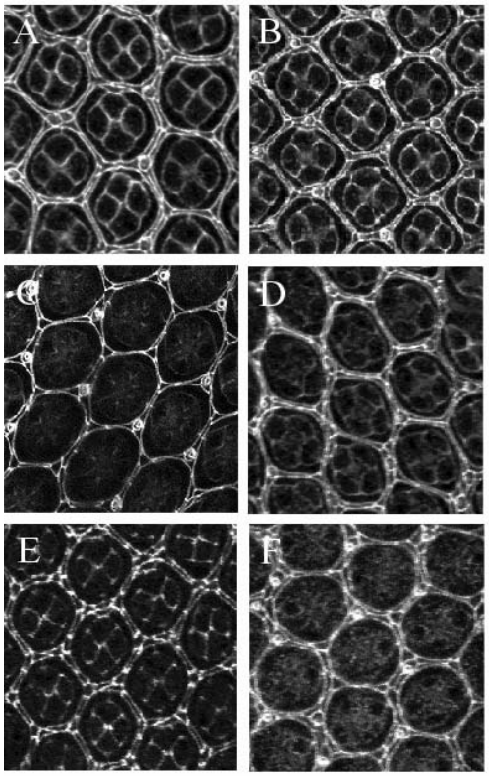


Figure 4. *echinus* is expressed in cone cells and pigment cells prior to and after during the onset of apoptosis in the *Drosophila* pupal eye. Echinus is visualized by anti-β-gal staining in $ec^{P\ lacZ}/+$ flies. A) No expression of *echinus* is seen in white prepupal stage (0 hr APF). (B, C) 20 hr APF. Faint expression is seen in cone cells and primary pigment cells (B) and IOCs (C). (D,E) 30 hr APF. Clearer echinus expression is seen in cone cells and primary pigment cells (D) and in IOCs (E) duringthis time of apoptosis. (F, G) 45 hr APF. echinus is still expressed in cones and primaries (F) and IOCs even after time of pigment cell removal (30 hr APF). (H,I) Enhancer trap staining using anti-β-gal antibody in the LX37 line. Cone cells, primary pigment cells (H) and IOCs (I) are visualized at 45 hr APF.

expression patterns of *echinus*. Flies heterozygous for the enhancer trap P-element, $ec^{P\ lacZ}$, were stained with a □-galactosidase antibody to detect *echinus* expression. At the white pre-pupal stage (0 hour APF), no □-galactosidase staining was seen (Fig 4A). Antibody staining was first detected at 20 hours APF in the cone cells, primary pigment cells, and the IOCs (Fig. 4B, C). Mutant analysis supports the notion that *echinus* acts within these cells, as *echinus* mutants have extra cells from each of these cell types. *ec* expression was detected in cone cells, primary pigment cells, and interommatidial cells at the time of cell death (30 hours APF) and afterwards (45 hours APF) (Fig 4). *In situ* hybridizations show the same expression pattern as the enhancer trap line (data not shown). Because *echinus* is expressed in the non-dying cone cells and primary pigment cells, it appears that *echinus* is not sufficient to induce death in the cells where it is expressed, but it is necessary for cell death as its absence prevents the loss of IOCs.

Structure-function analysis

Several mutant constructs of the *echinus* gene were made to determine which domains and amino acid residues were necessary for function. Of particular interest was the UCH domain, and in particular, the catalytic residues involved in ubiquitin C-hydrolysis. All *echinus* constructs were placed under the control of the GMR enhancer and tested for their ability to rescue the loss-of-function allele $ec^{P\ lacZ}$.



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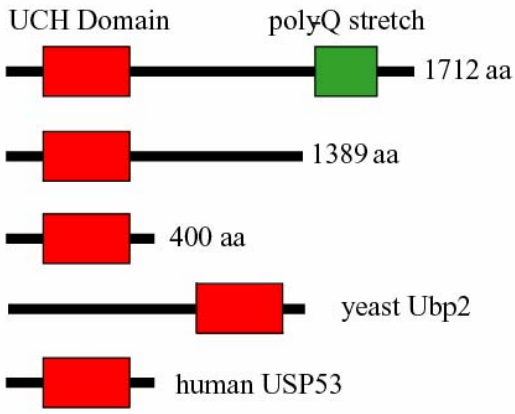

H

Genotype	Avg. Num. of IOC
WT	9
$ec^{P\ lacZ}$	14.1 ± 2.0
$ec^{P\ lacZ}$; GMR-ec	10.2 ± 1.5
$ec^{P\ lacZ}$; GMR-ec (-polyQ)	10.6 ± 0.9
$ec^{P\ lacZ}$; GMR-ec (UCH)	13.3 ± 1.4
$ec^{P\ lacZ}$; yUbp2	11.7 ± 1.2
$ec^{P\ lacZ}$; GMR-USP53	13.3 ± 1.5

Figure 5. Structure-function analysis of Echinus. A-F show pupal eye discs at 42 hr APF. Cell borders are visualized with anti-Dlg antibody. (A) *echinus* mutant (B) *echinus* mutant rescued by GMR-ec. (C) *echinus* mutant rescued by GMR-ec (-polyQ). (D) GMR-ec (UCH) fails to rescue *echinus*. (E) GMR-yUbp2 partially rescues *echinus*. (F) GMR-USP53, which overexpresses the UCH domain of USP53, fails to rescue *echinus*. (G) Structure of genes used in A-F. UCH domains are red, while the Echinus poly-glutamine stretch is green. Top three constructs are *echinus*, fourth is the *S. cerevisiae* gene Ubp2, and fifth is the human USP53 gene. (H) IOC number were counted and averaged over at least six different pupal eye discs. Standard deviations are shown.

The poly-glutamine stretch does not appear to be important for Echinus function. GMR-ec transgenic flies were generated that contained the first 1389 amino acids and lacked the C-terminal poly-glutamine stretch. This construct, GMR-ec(-polyQ), rescued *echinus* mutants to the same degree as full-length GMR-*ec* clone (Fig. 5C). Rescued adult eyes are wildtype in appearance, with the ommatidia again in an ordered

array. The pupal eye discs occasionally show an extra IOC, at levels similar to those seen in the GMR-Ec rescue. In a wildtype background, GMR-ec(-polyQ) did not alter the adult eye morphology (data not shown). The UCH domain is not sufficient for rescue of *echinus* mutants. Transgenics of either the *echinus* UCH or the USP53 UCH domain, placed under the control of GMR, failed to rescue (Fig. 5D, 5F).

echinus acts upstream or independently of the core apoptotic machinery

echinus seems to be a pro-apoptotic gene, as recessive mutants show impaired apoptosis during pupal eye development. To test whether *echinus* is involved with the core components of the apoptosis machinery, crosses were made of *echinus* to the GMR driven pro-apoptotic genes. Specifically, either the recessive allele $ec^{P\ lacZ}$ or the GMR-ec construct was crossed to overexpressed *hid, grim, reaper*, *Dronc*, *Drice*, *Strica*, and *dcp-1*. Loss of *echinus* did not suppress any of these genes, suggesting that *echinus* acts upstream of these genes or is not directly involved in their activation (Fig 6). GMR-ec fails to activate apoptosis in the eye; the cell number is the same as that of wildtype. GMR-ec also did not enhance the death in response to the expression of the apoptotic machinery (Fig. 6). Crosses to other genes implicated in apoptosis, such as *morgue*, *ubcD1*, *hpo*, *wts*, and *sav*, did not change any of the phenotypes associated with *echinus*.

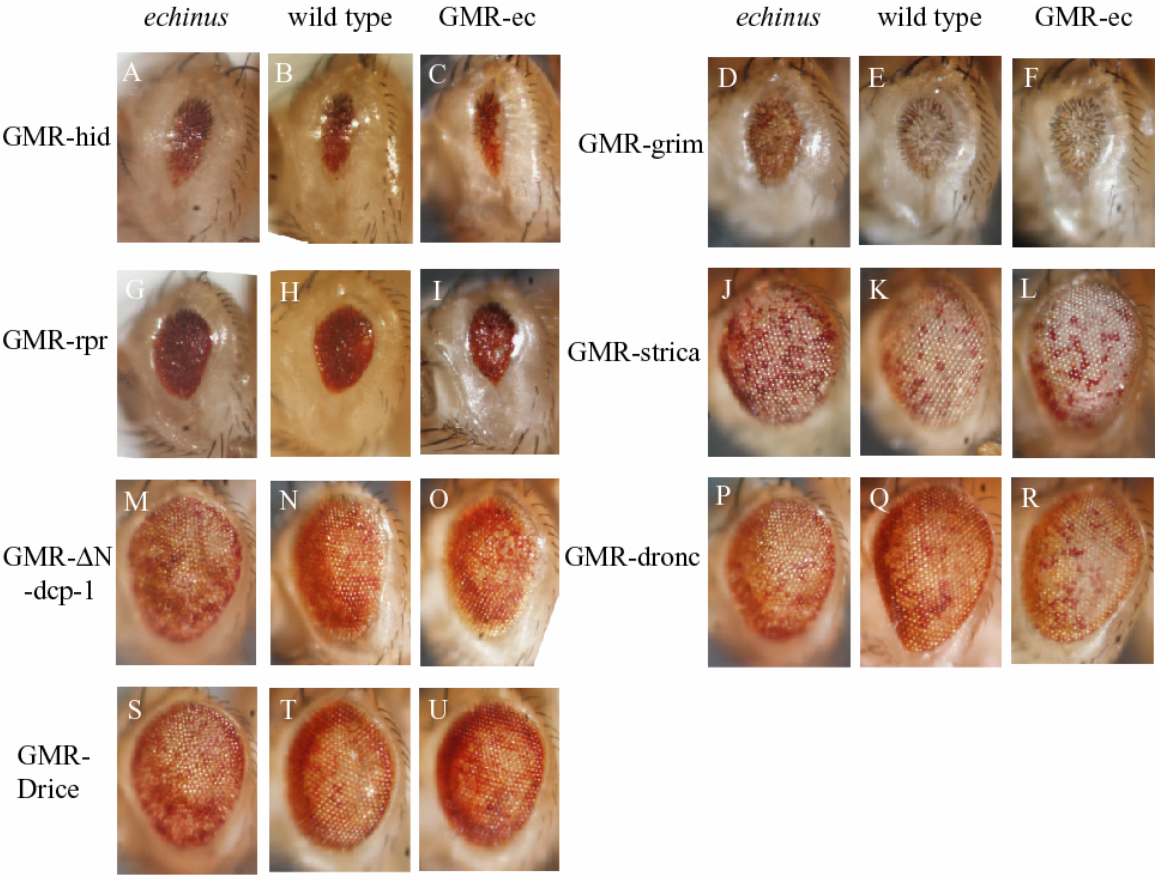

Figure 6. *echinus* acts upstream or independently of the core apoptotic machinery in *Drosophila*. *echinus* mutants (A, D, G, J, M, P, S), wildtype (B, E, H, K, N, Q, T) and GMR-*ec* (C, F, I, L, O, R, U) were crossed to GMR-*hid* (A-C), GMR-*grim* (D-F), or GMR-*rpr* (G-I). Overexpression of the caspases Strica (J-L), ΔN dcp-1 (M-O), Dronc (P-R), and Drice (S-U) were also tested.

2.5 Discussion

Echinus resembles a deubiquitinating enzyme, but lacks a catalytic cysteine

Mutants of *echinus*, produced by P-element mutagenesis or RNAi, have excess IOCs in the eye, while eye-specific expression of the CG2904 cDNA rescued the mutant echinus phenotype. Echinus looks like a deubiquitinating enzyme, except that it lacks a

critical catalytic cysteine residue. The UBP family of deubiquitinating enzymes is a loose organization of proteins united only by their UCH domain and ability to remove ubiquitin from protein conjugates. An *in vitro* assay in bacteria revealed that Echinus could not act as a deubiquitinating enzyme, much like its human orthologs USP53 and USP54. Structure-function analysis shows that a poly-glutamine stretch of the gene is not necessary for rescuing the mutant phenotypes, but the UCH domain is.

Predicting the correct active-site cysteine for Echinus is difficult. Sequence alignment of yeast deubiquitinating enzymes shows a rough consensus sequence surrounding the active site cysteine (GLV**N**MG**N**T**C**YM**N**SIL**Q**CL) (Wilkinson, 1997). The crystal structure of the deubiquitinating enzyme HAUSP shows that the surrounding asparagines and a following glutamine are important for activity (Hu, et al, 2002). Recently, additional human deubiquitinating enzymes were isolated and the alignment of the human DUBs revealed conservation of additional residues (Quesada, et al, 2004). Specifically, the active-site cysteine is always followed by a phenylalanine, tryptophan, or tyrosine. When Echinus was compared to its human orthologs, however, no cysteine within Echinus stood out as the catalytic residue. The best sequence alignments reveal a non-catalytic glycine (amino acid 52) in place of the catalytic cysteine.

Four other cysteines (C79, C84, C87 and C134) are potential alternative active-site residues. Cysteine 134 fits best within the criteria, as it is followed by a phenylalanine, and mutation of this residue prevents *echinus* from fully rescuing the mutant alleles (data not shown). Cysteine 134, however, might be a part of the ubiquitin-binding

region of *echinus*, based on similarity to the crystal structure of HAUSP. The general importance of this cysteine is also supported by the fact that the *echinus* orthologs share remarkable sequence similarity to cysteine 134 and the surrounding region. Thus, while the lack of rescue underscores the importance of this residue, it is not sufficient to conclude a catalytic role.

Whether Echinus is a deubiquitinating enzyme is still in question. Unlike the deubiquitinating enzyme *fat facets*, *echinus* loss-of-function mutants were not modified by heterozygous mutants of the proteosome or *ubcD1* (data not shown; Wu et al., 1999). Echinus, as well as the human orthologs USP53 and USP54 could not remove ubiquitin from a β-galactosidase reporter gene, though only the UCH domains of USP53 and USP54 were tested and this may have disrupted enzyme activity (Table 1; Quesada et al, 2004). The yeast gene Ulp1 has been shown to remove the ubiquitin-like gene Smt3 from protein conjugates, opening the possibility that Echinus encodes de-SUMOlytating or de-Neddylating enzymes (Li, 1999). Phenotypes of either the loss of *echinus* or its overexpression driven by GMR-gal4, however, were not modified by SUMO or Nedd8 mutants *Smt3*, *Cul1*, *Cul3*, and *Nedd8* (data not shown).

Several cDNAs have been isolated that overlap with *echinus* – however, in the opposite orientation. These cDNAs do not play role in Ec function. Eye-specific expression of RH68894, a representative of these cDNAs, has no phenotype and fails to rescue the mutant phenotype. Furthermore, overexpression of the cDNA fails to enhance any of the adult eye phenotypes, suggesting that the cDNA doesn't act as an antisense

inhibitor to *echinus*. No obvious gene or miRNA is predicted within this cDNA, making it unlikely that they play an important role in the echinus phenotype.

The role of *echinus* in apoptosis

Our results confirm that *echinus* plays a role in activating apoptosis in the developing *Drosophila* eye, though the context in which it acts is unresolved. This situation is quite similar to the genes *irregular chiasmC-roughest* (*irreC-rst*) and *nemo*. Like *echinus*, an *irreC-rst* mutant was isolated because it lacked pupal eye apoptosis (Wolff and Ready, 1991). *IrreC-rst* encodes a transmembrane protein of the immunoglobulin superfamily and the protein is localized to the cell membrane contacts (Ramos et al., 1993; Reiter et al., 1996). While *morgue*, an ubiquitin-conjugase-related protein modifies the *irreC-rst* mutant phenotype, and *Delta*/*Notch* perturb IrreC-rst protein localization, it is not clear how *IrreC-rst* acts to promote apoptosis or antagonize cell differentiation (Gorski et al., 2000; Hays et al., 2002). Viable *nemo* mutants have extra interommatidial cells and a decrease in apoptosis and in the developing eye (Mirkovic et al., 2002). *nemo* encodes a protein kinase, and homologues have been shown to be activated by dTak1, a component of the JNK signaling pathway (Choi and Benzer, 1994; Ishitani et al., 1999; Rocheleau et al., 1999). All three of these genes, *echinus*, *irreC-rst*, and *nemo*, remain somewhat mysterious.

Several signaling pathways and protein cascades have been shown to be mediated by ubiquitination. DIAP1 has been shown to protect cells by ubiquitinating the pro-apoptotic machinery (Wilson et al., 2002; Yan et al., 2004). However, our results show

that *echinus* does not mediate apoptosis by inhibiting DIAP1's function. *echinus* mutants alleles fail to suppress ectopic expression of *hid*, *grim*, or *reaper*, and *echinus* overexpression fails to enhance these genes as well (Fig. 6). Furthermore, loss of *diap1* specifically in the eye induces cell death resulting in a small eye. Loss of *echinus* or its overexpression did not attenuate this small-eye phenotype (data not shown).

While *echinus* does not appear within the core components of the apoptotic machinery, it might act as an upstream activator. The *Notch* signaling pathway has been implicated in promoting apoptosis in the developing retina (Miller and Cagan, 1997). *neur*, LNX, sel-10, and Su(dx) encode E3 ligases and have been shown to mediate protein degradation of components of the Notch signaling pathway, establishing the role of ubiquitination within the *Notch* pathway (reviewed in Lai, 2002).

A potential role for *echinus* in the *EGFR/ Ras* pathway is also possible, but is more remote. The EGF receptor is known to promote differentiation of R cells, cone cells, and primary pigment cells. Ectopic expression of a dominant-negative version of the receptor suggests a role in IOC formation as well (Freeman, 1996). *D-cbl* and orthologs have been shown to mediate ubiquitin-dependent degradation of receptor protein-tyrosine kinases (Miyake et al., 1998; Joazeiro et al., 1999). The possibility that the Egfr is a target of Echinus is slight as *echinus* mutants would lead to decreased Egfr levels and fewer cells in the developing pupal eye. Another possible target is the negative regulator Ttk88. Ttk88 protein levels have been shown to be mediated by the actions of either *ebi* or *phyl* and *sina* (Dong et al., 1999; Li et al., 1997; Tang et al.,

1997). However, *echinus* loss-of-function and ectopic expression fail to modulate ectopic expression of Ttk88 (data not shown).

The search for such potential targets of Echinus is limited by the fact that the gene is specific to the eye. Enhancer trap staining using the ec^{PlacZ} alleles failed to stain embryos and imaginal discs (data not shown). Yeast 2-hybrid screens would be ineffective since the *Drosophila* libraries are generated from embryos, ovaries, and imaginal discs.

Determining the enzymatic target of Echinus and its role is important because little is known of how the apoptotic machinery is activated in a developmental context. Echinus potentially lies at the junction of several signaling pathways and the activation of apoptosis. We have ruled out several possibilities, and for now the puzzle is unsolved.

2.6 Conclusions

echinus is necessary for cell death of the interommatidial cells in the developing *Drosophila* retina. All *echinus* mutants examined have excess IOCs due to the lack of apoptosis. CG2904 encodes *echinus* as mutants for the CG2904 phenocopy *echinus* and its overexpression rescues the mutant phenotype. Echinus belongs to an inactive subclass of deubiquitinating enzymes; Echinus and its orthologs share homology to other deubiquitinating enzymes, though they lack catalytic activity. *In situs* show that *echinus* is expressed in both dying and non-dying pupal eye cells suggesting that *echinus* is not sufficient to drive apoptosis.

2.6 Acknowledgments

We thank Victor Quesada and Carlos Lopez-Otin for the USP53 fragment, Ian Bosdet and Sharon Gorski for their collaboration in confirming *echinus* expression in pupal eye discs and echinus mutant sequencing, Janice Fischer for the yeast Ubp2 clone, Rohan Baker for donating plasmids for the bacterial assay.

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2.8 Supplementary Figure

Genetic crosses of *echinus* with signaling pathway component involved in *Drosophila* eye development. – refers to change in dominant phenotype.

***echinus* crosses with echinus and RH68894**

	$ec^{P\ lacZ}$	GMR-ec	GMR-gal4, UAS-ec
ec^{1}	fails to complement		
$ec^{P\ lacZ}$		rescue	same phenotype as GMR-gal4, UAS-ec
GMR-RH68894	-	-	-
UAS-RH68894(RNAi)			-

***echinus* crosses with the genes involved in protein ubiquitination**

	$ec^{P(lacZ)}$	GMR-LP2	GMR-gal4, UAS-LP2
$morgue^{19}$	-		
$ubcD1^{\Delta 173}$	-	-	-
l(3)73Ai	-	-	-

***echinus* crosses with the apoptotic machinery**

	$ec^{P(lacZ)}$	GMR-LP2	GMR-gal4, UAS-LP2
GMR-Hid	-	-	-
GMR-lg Hid	-	-	-
GMR-Reaper	-	-	-
GMR-lg Reaper			-
GMR-Grim	-	-	-
GMR-lg Grim			-
GMR-Debcl	-	-	-
GMR-Dronc	-	-	-
GMR-Dream	-	-	-
GMR-□N Dcp1	-	-	-
GMR-Drice	-	-	-
GMR-P35	no enhance.		-
GMR-diap1	no enhance.		-
H99 Deficiency	-		-
th^5		-	-
GMR-gal4, UAS(RNAi)-diap1	-	-	

echinus **crosses with the genes involved in the JNK pathway**

	$ec^{P(lacZ)}$	GMR-LP2	GMR-gal4, UAS-LP2
GMR-gal4, UAS-dTraf1		-	
GMR-Eiger	-		
GMR-dTak	-		
GMR-Hep	suppresses lethality no change in eye size		
hep^{r75} (hep fem)	-	-	-
GMR-Rac1	-		
$nemo^{j147-1}$	-	-	-
Jra^{IA109}	-	-	-
puc^{E69}			
bsk^{1}	-	-	-
GMR-gal4, UAS-bsk			
GMR-gal4, UAS-bsk^{dn}			-
msn^{102}	-		
GMR-gal4, UAS-msn			
peb^{EP55}	-	-	
peb^{1} (peb fem.)	-	-	

***echinus* crosses with the EGF pathway**

	$ec^{P(lacZ)}$	GMR-LP2	GMR-gal4, UAS-LP2
$Egfr^{E1}$	-	-	
$Egfr^{f24}$	-	-	-
GMR-gal4, UAS-$Egfr^{dn}$	-	-	-
$argos^{W11}$	-		-
$Dsor1^{LH110}$	-		
rl^{1}	-	-	-
rl^{SEM}	very slight enhancement in females	-	-
GMR-ras^{V12}	-	-	lethal
$elav^{C155}$-GMR-gal4, UAS-ras^{N17} (on X)	slight suppression in ec/+ female	slight suppression	slight suppression
ebi^{k16213}			-
GMR-ttk	-	-	-

***echinus* crosses with the *Notch* pathway**

	$ec^{P(lacZ)}$	GMR-LP2	GMR-gal4, UAS-LP2
GMR-gal4, UAS-Dl	-	-	(combination of 2 phenotypes)
Dl^{RF}	-	-	-
DL^{6B}	-		
GMR-gal4, UAS-N^{ICN}	-	-	-
GMR-gal4, UAS-N^{dn}	stronger suppression in males	slight supp. more black in eye	pupal lethal
sev-N^{ICN}	-	-	
N^{fa-g}	- (in double het. females)	-	btw the 2 phenotypes; female = nothing
N^{spl-1}	-	-	-
$neur^{11}$	-	-	-

echinus* crosses with *hippo, salvador, warts

	$ec^{P(lacZ)}$	GMR-LP2	GMR-gal4, UAS-LP2
GMR-hpo	-	black spots in anterior region of eye	-
GMR-gal4, UAS-hpo	-		-
GMR-gal4, UAS-$hpo^{(KD)}$	rougher eyes		same phenotype as GMR-gal4, UAS-ec
hpo^{MGH3}	-	-	-
hpo^{42-47}	-	-	-
GMR-sav		-	-
sav^{3}	-	-	-
GMR-wts	-	-	variable roughness

***echinus* crosses with genes involved in cell cycle regulation**

	$ec^{P(lacZ)}$	GMR-LP2	GMR-gal4, UAS-LP2
dap^{4}	-	-	
$CycE^{AR95}$	-	-	
stg			-

Chapter 3: Novel Regulators of the *C. elegans* Caspase CED-3

3.1 Abstract

The hallmark of apoptosis is the presence of activated caspases, a conserved family of cysteine proteases. Proper regulation of caspases is crucial to maintain cell viability. IAPs are a class of proteins known to inhibit caspases in *Drosophila* and mammals, though they do not regulate the *C. elegans* caspase CED-3. To understand how CED-3 is regulated, we have performed a yeast-based screen for CED-3 suppressors and isolated two previously uncharacterized genes, Y39B6A.12 and T23G11.7b. Characterization of these genes in *Drosophila* shows that T23G11.7b and its ortholog CG7967 are regulators of gene expression and not caspases. Y39B6A.12, on the other hand, inhibits the Bcl-2 gene *Debcl*, but fails to regulate the pro-apoptotic genes *Grim*, *Hid*, or *Dronc*. It still remains unclear if Y39B6A.12 is a critical regulator of CED-3 in viable *C. elegans* cells.

3.2 Introduction

Programmed cell death is a regulated process used by an organism for the removal of deleterious cells or developmentally transient cells. Characterization of programmed cell death

began in the nematode *Caenorhabditis elegans* and led to the eventual identification of the genes involved (Ellis and Horvitz, 1986). *ced-3* and *ced-4* were shown to be necessary for cell death while the gene *ced-9* blocks their function (reviewed in Metzstein et al., 1998).

Later studies from other model systems showed that the *ced* genes were highly conserved in apoptosis. *ced-3* belongs to a family of proteases now known as caspases, which are the executioners of programmed cell death (Yuan et al., 1993). The mitochondrial localized CED-4 facilitates the irreversible activation of CED-3 (Seshagiri and Miller, 1997). Subsequent studies show that Apaf-1 is structurally similar to CED-4 and promotes the activation of the apical caspase Caspase-9 (Zou et al., 1997). *ced-9* is a member of the Bcl-2 family of mitochondrial proteins and inhibits ced-4 (Hentgartner and Horvitz, 1994).

While the genes involved in apoptosis are highly conserved, the inhibition of caspases appears to vary in different organisms. In flies and mammals, caspases are inhibited by the IAP group of proteins. Removal of IAPs causes rapid cellular destruction, thus proving that they are crucial for maintaining cell viability (Wang et al., 1999). Importantly, no functional caspase inhibitor has yet been identified in *C. elegans*. The two genes with structural motifs similar to other IAPs in worms, the genes *bir-1* and *bir-2*, are not involved in apoptosis, but instead are required for midzone spindle formation and cytokinesis (Fraser et al, 1999, Speliotes, et al, 2000). To explain how CED-3 is kept from chewing up the cell, two models have been proposed. One model states that an alternative anti-apoptotic splice form of CED-4 keeps CED-3 down, though this splice form is relatively rare when compared to the pro-apoptotic one (Shaham and Horvitz, 1996). Another model suggests that the Bcl-like protein CED-9 binds CED-3 and CED-4 in a ternary complex and prevents CED-3 activation (Xue and

Horvitz, 1997). A necessary tenet of this model is that CED-3 is localized to the mitochondria along with CED-4 and CED-9, yet no one has shown the localization pattern of CED-3. Furthermore, recent work in a heterologous assay has shown that CED-9 cannot inhibit CED-3 mediated cell death (Jabbour et al., 2004).

If these models are flawed, the question remains: What keeps CED-3 from killing the cell? To probe this question, we used a function-based assay in *S. cerevisiae* to screen for inhibitors of CED-3. Two *C. elegans* genes were isolated from this screen, though subsequent studies in *Drosophila* eliminated one candidate from consideration. The remaining candidate, Y39B6A.12, weakly suppressed death induced by the Bcl2 gene *Debcl* in the *Drosophila* eye. Though Y39B6A.12 still needs to be characterized further in *C. elegans*, this heterologous screen provides a novel way to screen for regulators of the caspase CED-3.

3.3 Materials and Methods

Yeast protocols

S. cerevisiae were transformed using a standard LiAc protocol. The W303α strain (*MATα, can1-100, leu2-3, his3-11, -15, ura3-1, ade2-1*) was grown on selective media containing 2% glucose or 2% galactose/1% raffinose for gene induction. *ced-3* and *ced-4* were induced using an 815 bp fragment of the Gal1 promoter region (Hawkins et al., 1997). The caspase-lacZ reporter assay used to test caspase suppression independent of galactose has been previously described (Hawkins et al., 1997).

C. elegans cDNA library construction

Mixed-stage *C. elegans* polyA+ RNA was converted into cDNA using the Superscript cDNA synthesis kit (GIBCO). cDNAs were size-fractioned to select clones greater than 500 bp, and were ligated into the NotI–SalI sites of the gpat-his vector. Transformation of the ligation mix into the bacteria strain DH10B (GIBCO) yielded approximately 5 x 10^5 colonies. The cDNA library was amplified by growing the colonies in LB with carbecillin and isolating their plasmid DNA (Qiagen).

Drosophila genetics

Drosophila strains were grown at 25 ^{0}C. CG7967, T23G11.7b, and Y39B6A.12 were subcloned into the UASt vector and injected using standard embryo microinjection techniques. Recombinants of the UAS transgenics with the GMR-gal4 chromosome were made. UAS-Q78 was kindly provided by N. Bonini. UAS-Wg and UAS-pygo were provided by K. Cadigan. Pictures of Drosophila retina were taken using an Olympus DP10 digital camera.

C. elegans RNAi

cDNAs for T23G11.7b and Y39B6A.12 were cloned into the BamHI–XbaI sites of pPD129.36. RNAi knockdown of these genes was conducted by feeding *C. elegans* on bacteria producing dsRNA from these constructs.

3.4 Results

Isolation of two *ced-3* suppressors in yeast

CED-3 is the only *C. elegans* caspase known to be involved in programmed cell death. To test whether *C. elegans* does encode a novel CED-3 inhibitor we screened an inducible C. elegans cDNA library for genes that suppress *ced-3* in *S. cerevisiae*. As previously reported, overexpression of *ced-3*, under the control of the galactose-inducible promoter, kills *S. cerevisiae* cells (Figure 1, Jabbour et al., 2004). This killing is complete when two Gal-*ced-3* plasmids are present. The *C. elegans* genes T23G11.7b and Y39B6A.12 were both repeatedly isolated as suppressors of CED-3 mediated death (Fig. 1B, C). Reintroduction of the cDNAs encoding Y39B6A.12 and T23G11.7b back into *S. cerevisiae* verified their suppression.

The CARD domain found in the prodomain of CED-3 is necessary for its regulation, though not essential for proteolytic activity. Overexpression of CED-3 lacking its CARD

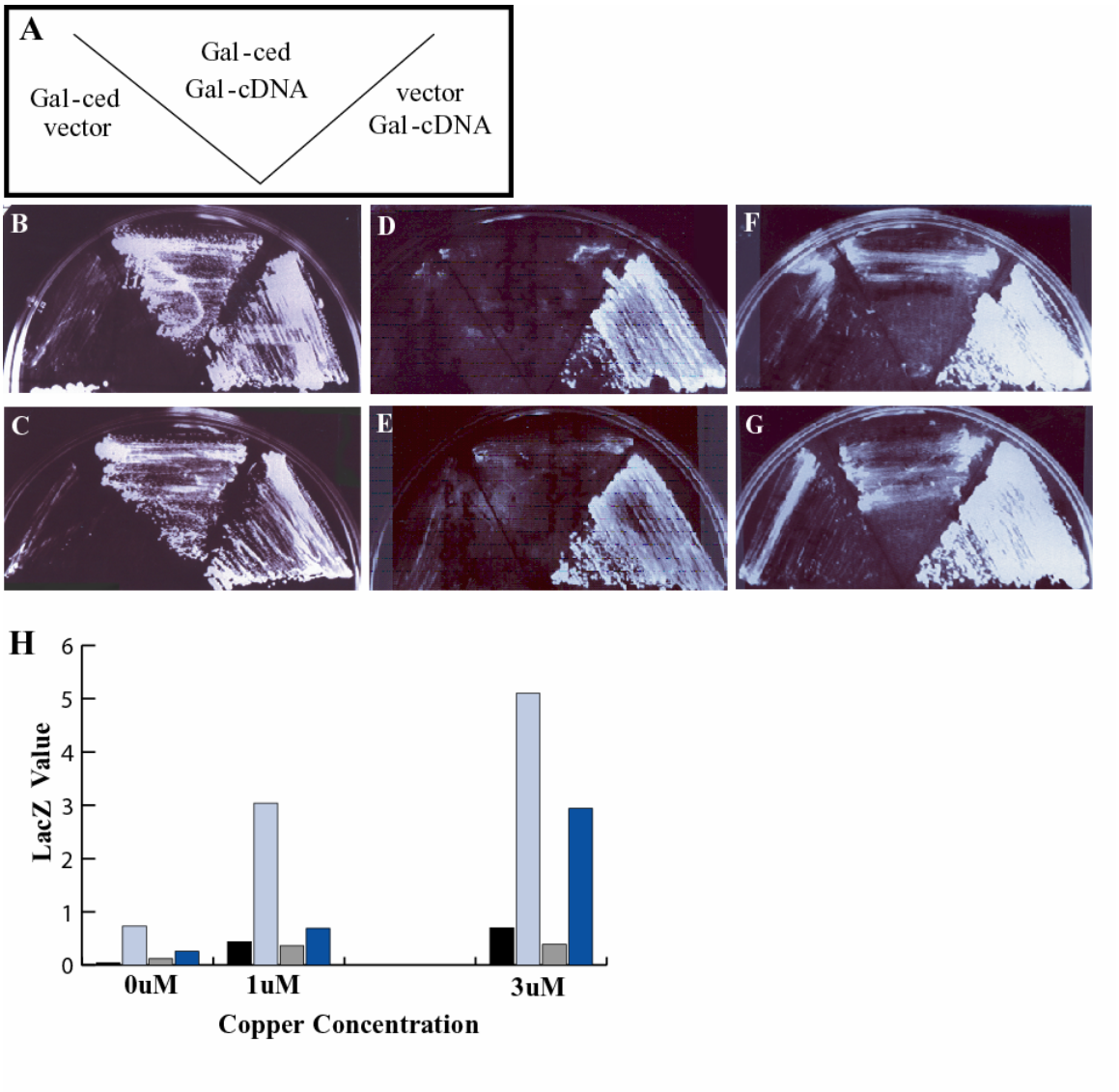

Figure 1. Y39B6A.12 and T23G11.7b were isolated as CED-3 suppressors in yeast. Panel A is the schematic for the plate growths in panels B-G where the 'ced' gene is either ced-3 (B,C), ced-3 lacking its prodomain (D,E), or ced-4 (F,G) and the cDNA are the C. elegans cDNAs Y39B6A.12 (B,D,F) or T23G11.7b (C,E,G). (B,C) Both cDNAs suppress CED-3-dependent death. cDNAs fail to suppress death induced by CED-3 lacking its prodomain (D,E) or death induced by CED-4 (F,G). (H) Caspase CED-3 was induced by the copper-inducible promoter and its activity measured by lacZ (see Materials and Methods for details). Black bars represent P35 suppression of CED-3 activity, light blue bars represent empty vector, light grey represent presence of Y39B6A.12, and dark blue represents T23G11.7b. Note that the isolated cDNAs suppress CED-3 activity independent of the Gal1 promoter, and that Y39B6A.12 suppresses as well as the viral P35.

domain (*ced-3* Δprodomain) can still kill yeast cells. Interestingly, both T23G11.7b and Y39B6A.12 can no longer function as CED-3 inhibitors when the CED-3 CARD domain is missing, suggesting that the CARD domain is important for regulation (Fig 1D, E). CED-4 also encodes a CARD domain, and CED-4 has been shown to activate apoptosis and kill *S. pombe* (James et al., 1997). In *S. cerevisiae*, galactose-induced *ced-4* can also kill, though this death cannot be suppressed by T23G11.7b or Y39B6A.12 (Fig 1F, G).

Because the two cDNAs failed to suppress CED-3 (Δprodomain) and CED-4 dependent death in yeast, it is reasonable to suggest that the cDNAs are CED-3 inhibitors and not suppressors of the galactose-inducible promoter. To test this hypothesis another way, we

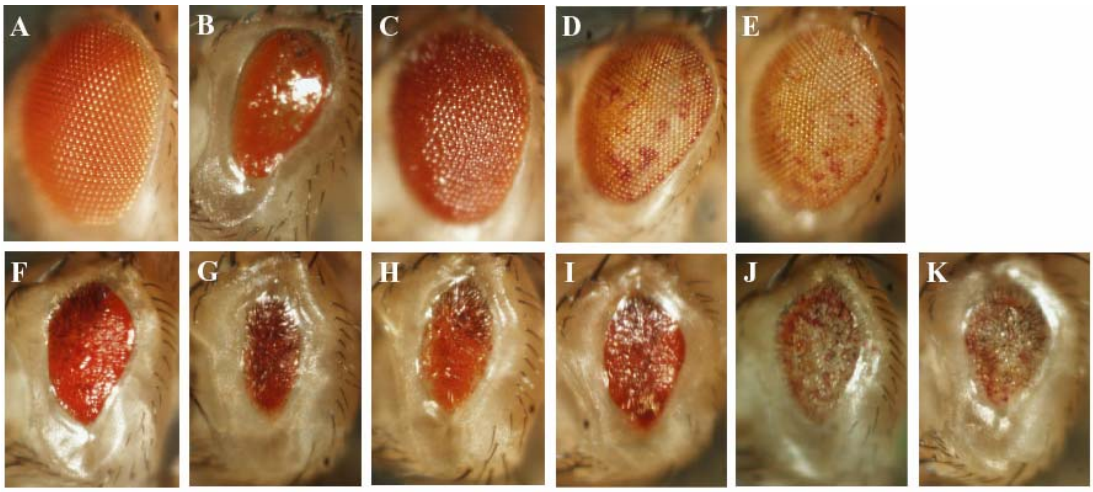

Figure 2. CG7967 is the *Drosophila* ortholog of *C. elegans* T23G11.7b and suppresses ectopic Debcl, but not GMR-dronc, GMR-hid, GMR-grim, GMR-reaper. (A) GMR-gal4, UAS-CG7967 shows no phenotype on its own. (B) GMR-gal4,UAS-Debcl (C) GMR-gal4, UAS-Debcl; UAS-CG7967 (D) GMR-gal4; GMR-dronc (E) GMR-gal4,UAS-CG7967; GMR-dronc (F) GMR-gal4; GMR-hid (G) GMR-gal4, UAS-CG7967; GMR-hid (H) GMR-gal4; GMR-reaper (I) GMR-gal4, UAS-CG7967; GMR-reaper (J) GMR-gal4; GMR-grim (K) GMR-gal4, UAS-CG7967; GMR-grim

tested whether T23G11.7b and Y39B6A.12 could suppress *ced-3* controlled by the copper-inducible promoter. Increasing the concentration of copper led to an increase in CED-3 activity. Y39B6A.12 and T23G11.7b, as well as the pan-caspase suppressor P35, suppressed CED-3 activity independent of the Gal1 promoter (Fig. 1H). Notably, Y39B6A.12 suppressed just as well as P35.

T23G11.7b/ CG7967 suppresses the Gal4/UAS system and does regulate apoptosis

To determine whether the isolated cDNAs are genuine caspase suppressors *in vivo*, we used the *Drosophila* eye for our assays. Using the GMR (Glass multiple repeat)-gal4 line to drive gene expression in the eye, ectopic expression of CG7967, the *Drosophila* ortholog of T23G11.7b, showed no phenotype (Fig. 2A). *hid*, *grim*, and *reaper* are

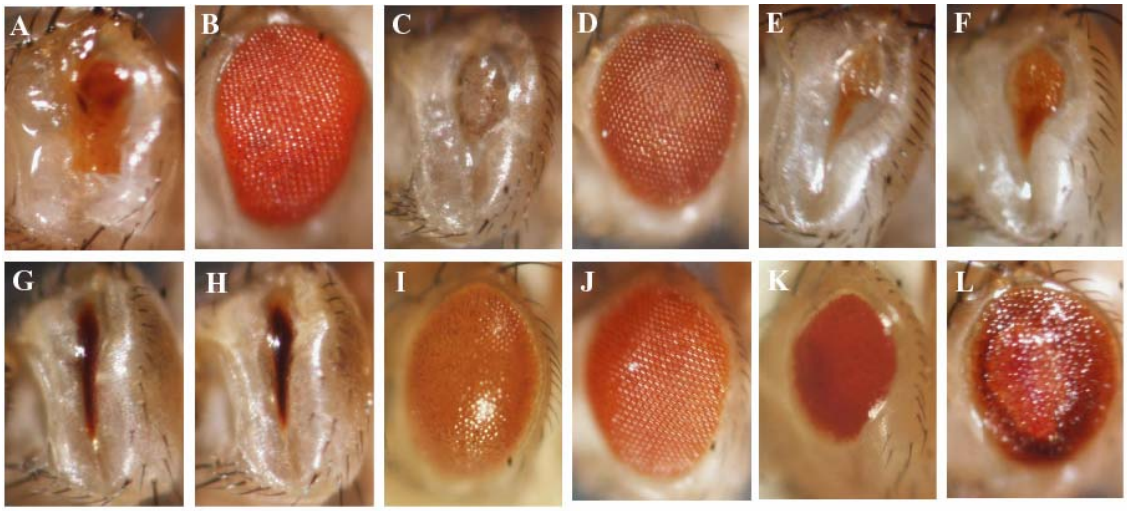

Figure 3. The Drosophila gene CG7967 suppresses members of the JNK pathway, *pygopus (pygo)*, and *fat facets (faf)*. (A) GMR-gal4; UAS-Eiger (B) GMR-gal4; UAS-Eiger/ UAS-CG7967 (C) GMR-gal4; UAS-dTak1 (D) GMR-gal4; UAS-dTak1/ UAS-CG7967 (E) GMR-gal4; UAS-hep (F) GMR-gal4; UAS-hep/ UAS-CG7967 (G) GMR-gal4; UAS-Wingless (Wg) (H) GMR-gal4; UAS-Wg/CG7967 (I) GMR-gal4; UAS-pygo (J) GMR-gal4; UAS-pygo/ UAS-CG7967 (K) GMR-gal4; *faf*(EP381) (L) GMR-gal4; *faf*(EP381)/ UAS-CG7967. CG7967 suppresses the ablated eye phenotype of all lines except Wingless (Wg).

activators of apoptosis and cause a small eye phenotype when overexpressed using the GMR enhancer. Crosses of GMR-gal4, UAS-CG7967 to GMR-hid, GMR-grim, and GMR-reaper showed no changes in eye size (Fig 2). In a similar fashion, expression of GMR-Dronc, which also induces death, could not be suppressed by GMR-gal4, UAS-CG7967 (Fig 2D, E). Though the Bcl proteins are much more extensively characterized in humans and mice, the *Drosophila* members, *Debcl* and *Buffy*, have been shown to regulate apoptosis. Ablation of the eye by overexpressed *Debcl* can be restored by

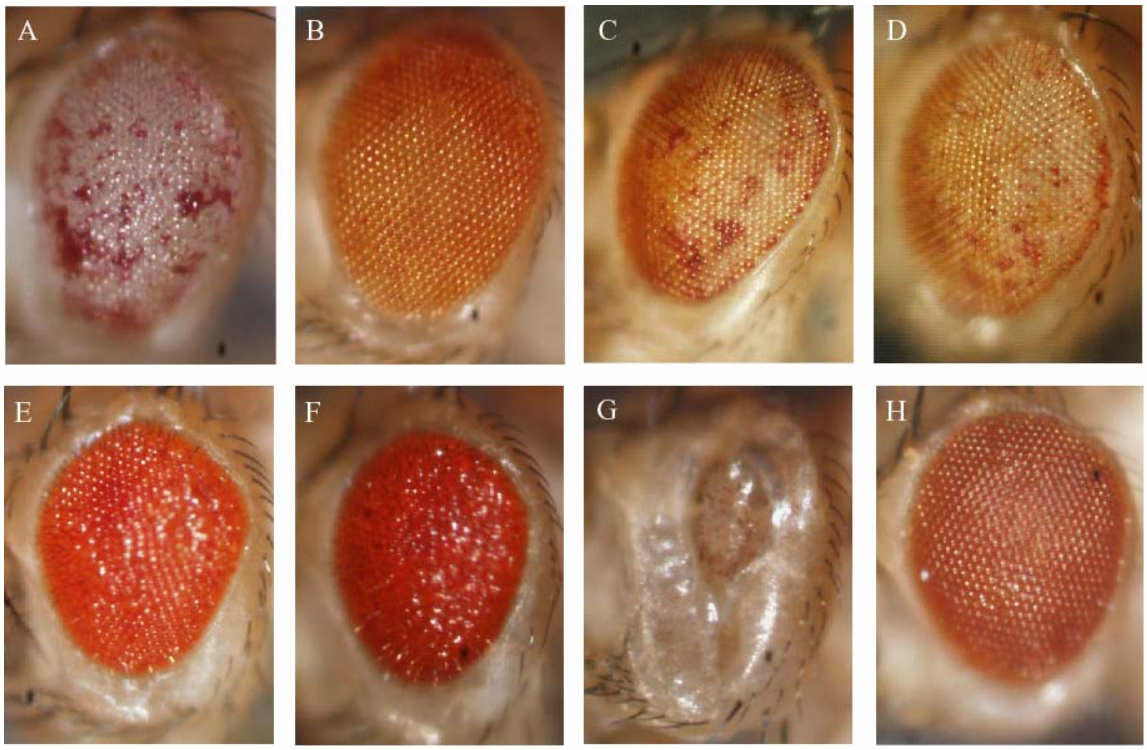

Figure 4. The *Drosophila* gene CG7967 regulates Gal4 dependent expression. CG7967 suppresses death induced by GMR-gal4, UAS-Dronc, but not GMR-Dronc (A-D). CG7967 also fails to suppress ectopically expressed mouse Tak1, but yet suppresses *Drosophila* dTak1 driven by GMR-gal4 (E-H). (A) GMR-gal4, UAS-Dronc (B) GMR-gal4, UAS-Dronc; UAS-CG7967 (C) GMR-gal4; GMR-Dronc (D) GMR-gal4; GMR-Dronc/ UAS-CG7967 (E) GMR-mTak1 (F) GMR-mTak1; GMR-gal4, UAS-CG7967 (G) GMR-gal4; UAS-dTak1 (H) GMR-gal4, UAS-CG7967; UAS-dTak1

overexpression of *Buffy* (Quinn et al., 2003). CG7967 can suppress excess death phenotypes associated with ectopically expressed *Debcl* both in the eye and the wing (Fig 2, data not shown).

During the course of experiments, it became apparent that CG7967 could suppress genes expressed using the Gal4/UAS system, but not genes controlled directly by the GMR enhancer (Fig 2, 3). Once again, to test whether CG7967 and T23G11.7b are genuine inhibitors of apoptosis, we tested their ability to suppress genes expressed using the GMR enhancer and the Gal4/UAS system. The caspase Dronc expressed using the Gal4/UAS system gives a stronger phenotype than GMR-Dronc. CG7967 could not suppress GMR-Dronc, as previously mentioned, though it could suppress GMR-gal4, UAS-Dronc (Fig. 4). Along with other members of the JNK pathway, overexpression of the kinase dTak1 and its mouse counterpart mTak1 activates apoptosis in the *Drosophila* eye. Overexpression of CG7967 failed to suppress GMR-mTak, while it suppressed GMR-gal4, UAS-dTak (Fig. 4). Taken together, these results show that CG7967, and, by implication, T23G11.7b are not genuine regulators of apoptosis.

Y39B6A.12, a possible caspase inhibitor

To determine whether Y39B6A.12 is a pan-caspase inhibitor, we resorted to the *Drosophila* eye once again. In yeast, Y39B6A.12 proved to be a CED-3 inhibitor. *Drosophila* encodes for seven caspases, with the downstream caspases *DrICE*, *decay*, and *dcp-1* showing homology to *ced-3* in their catalytic domains. Since at the time of this writing, recombinants of GMR-gal4; GMR-DrICE, -Decay, and –dcp-1 do not exist, we

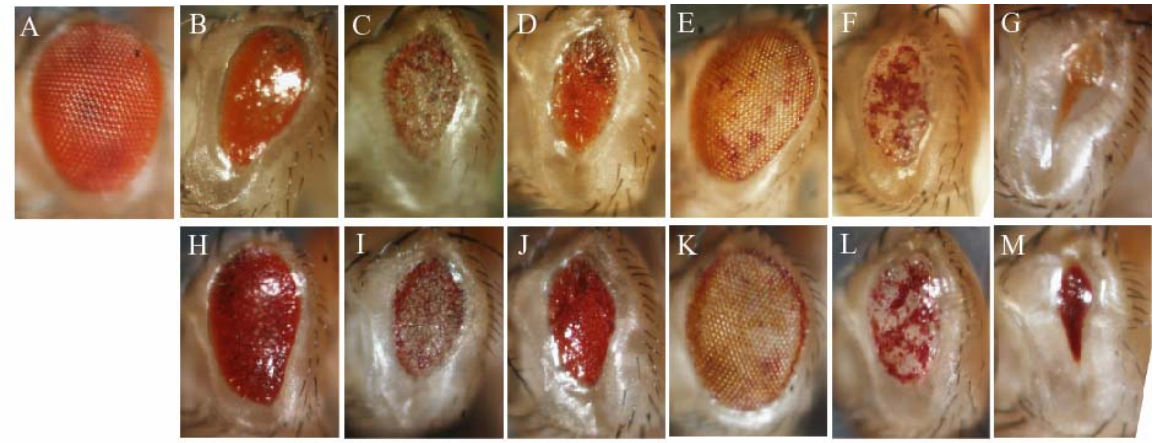

Figure 5. Ectopically expressed C. elegans Y39B6A.12 in the Drosophila eye suppresses Debcl. Y39B6A.12 shows no suppression of overexpressed grim, hid, Dronc, diap1(RNAi), or hep. UAS-Y39B6A.12 in panels H-M. (A) GMR-gal4, UAS-Y39B6A.12 (B) GMR-gal4; UAS-debcl (C) GMR-gal4; GMR-grim (D) GMR-gal4; GMR-hid (E) GMR-gal4; GMR-dronc (F) GMR-gal4, UAS-diap1(RNAi) (G) GMR-gal4; UAS-hep (H) GMR-gal4; UAS-debcl, UAS-Y39B6A.12 (I) GMR-gal4; GMR-grim, UAS-Y39B6A.12 (J) GMR-gal4; GMR-hid/ UAS-Y39B6A.12 (K) GMR-gal4; GMR-dronc, UAS-Y39B6A.12 (L) GMR-gal4, UAS-diap1(RNAi); UAS-Y39B6A.12 (M) GMR-gal4; UAS-hep/ UAS-Y39B6A.12

sought to test whether Y39B6A.12 could suppress activators of these caspases. GMR-gal4, UAS-Y39B6A.12 could not suppress ectopic expression of *grim* nor *hid* (Fig. 5). The upstream caspase Dronc contains an N-terminal CARD motif, though with little similarity to the CARD domain in CED-3, and has been shown to activate DrICE. Like *hid* and *grim*, overexpression of *Dronc* could not be suppressed by ectopically expressed Y39B6A.12. However, Y39B6A.12 did slightly suppress the ablated eye caused by overexpression of Debcl (Fig. 5). Finally, unlike T23G11.7b, Y39B6A.12 failed to counteract the JNK pathway components, *Eiger*, *hep*, and *dTak1* (Fig. 5, data not shown).

Crosses to establish the GMR-gal4, UAS-Y39B6A.12 recombinant are being made, so that we can test Y39B6A.12 suppression against the effector caspases *DrICE* and *dcp-1*.

To test for the function of Y39B6A.12 in *C. elegans*, worms were fed with bacteria expressing dsRNA to Y39B6A.12. RNAi knockdown of Y39B6A.12 in N2 worms caused a sterile phenotype that was not suppressed by mutants for *ced-3* or *ced-4* (B. Derry, personal communication). Loss of the upstream inhibitor *ced-9* shows partial sterility, among other effects (Hentgartner et al., 1992). The results of the Y39B6A.12 RNAi and epistasis analysis suggest that either Y39B6A.12 is a tissue-specific inhibitor of an unidentified caspase or that the sterility is not related to cell death genes at all.

Protein characteristics and homology

Y39B6A.12 encodes a previously uncharacterized gene. With an ortholog found only in *C. briggsae*, Y39B6A.12 is a nematode-specific gene characterized only by its BED Zn-finger domain. BED Zn-finger proteins are a diverse class of nuclear proteins thought to bind to DNA. *C. elegans* encodes several BED proteins, including *dpy-20* and the transposase *tam-3*. The best-characterized BED proteins in *Drosophila* are the transcriptional activator Dref and the chromatin insulator Beaf. How a DNA-binding protein like Y39B6A.12 functions in relation to CED-3 is yet to be determined.

3.5 Discussion

Y39B6A.12 shows anti-apoptotic activity in yeast and Drosophila

CED-3 remains the only characterized caspase in *C. elegans* apoptosis, and to date no proteins have been identified as CED-3 inhibitors. In this report, we describe the two genes identified in a yeast-based screen performed to isolate CED-3 suppressors. The *C. elegans* gene

T23G11.7b and its *Drosophila* ortholog proved to be general suppressors of gene induction, while preliminary work on the *C. elegans*-specific gene Y39B6A.12 shows conserved function, as it partially suppressed death induced by expression of the pro-apoptotic Bcl2 protein Debcl. Because Y39B6A.12 was isolated in *S. cerevisiae*, which is devoid of the core components of the apoptosis machinery, it stands to reason that Y39B6A.12 functions to inhibit caspases. Though Y39B6A.12 could not suppress the overexpressed *Drosophila* caspase Dronc, Y39B6A.12 may still inhibit caspases that have not been tested. *Drosophila* encodes seven caspases, and the downstream caspases DrICE, DCP-1, and Decay share the most sequence similarity to CED-3. Tests to determine if Y39B6A.12 is a pan-caspase inhibitor and can inhibit any *Drosophila* caspase are currently underway.

Another possibility is that Y39B6A.12 could function only with CED-3 and not show any activity in *Drosophila*. To test for *in vivo* function in *C. elegans*, we knocked-down Y39B6A.12 levels by the feeding RNAi method. If Y39B6A.12 inhibits apoptosis, then loss of its function would cause spurious cell death. Reduced Y39B6A.12 function caused sterility, though this phenotype could not be suppressed by mutants for *ced-3* or *ced-4* (B. Derry, personal communication). Whether this sterility is caused by ectopic cell death is still to be determined. The ability of Y39B6A.12 to prevent cell death in C. elegans is currently being examined.

Complete story of developmental apoptosis in *C. elegans* remains to be resolved

C. elegans was used first to understand the molecular components of programmed cell death. Screens pulled out 13 genes involved in killing the cell and in corpse removal. Even though worms have the simplest cell killing machinery it is not completely understood. For example it is not understood how the fragmentation of the mitochondria, important in mammalian

apoptosis, is involved in worm cell death (Jagasia et al., 2005). The homologous apoptotic components AIF (apoptosis inducing factor) and endonuclease G are important in *C. elegans* programmed cell death, though the mechanism of their mitochondrial release has not been determined (Wang et al., 2002; Parrish et al., 2001). Anecdotal evidence also suggests that even the list of players mediating *C. elegans* apoptosis is incomplete. A deficiency screen looking at abnormal corpse numbers isolated many genomic regions containing no previously characterized CED genes (Sugimoto et al., 2001). In addition, mutants for *ced-4* but not *ced-3* suppress the embryonic cell death caused by loss of *icd-1*, a mitochondrial localized βNAC ortholog. Finally, *ced-3* is not essential for the death of the male linker-cells, the so-called "murders"; linker-cell death requires the presence of another cell (Sulston and White, 1980, Ellis and Horvitz, 1986).

3.6 Conclusion

We report the isolation of *C. elegans* genes that can suppress the killing ability of the *C. elegans* caspase CED-3. One of these genes, T23G11.7b and its Drosophila ortholog CG7967 prove to be regulating Gal4 dependent gene expression. Y39B6A.12 is a nematode-specific gene containing a BED Zn-finger domain. In the *Drosophila* retina, Y39B6A.12 shows weak ability to suppress ectopically expressed *Debcl*. Further tests are being conducted to determine if Y39B6A.12 encodes a novel inhibitor of CED-3.

3.7 Acknowledgments

I would like to thank Chris Hawkins for providing most of the yeast constructs and establishing the caspase-lacZ reporter assay, Jackie Barton and Kim Copeland for use of their radioactive hoods, Brent Derry for performing RNAi analysis for Y39B6A.12 and T23G11.7b, and Jun Huh for providing the JNK pathway strains.

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Chapter 4: A Deficiency Screen to Isolate Novel Regulators of DIAP1

4.1 Abstract

DIAP1, a ubiquitin E3 ligase, is the key *Drosophila* regulator of the family of cysteine proteases known as caspases. Because DIAP1 is crucial for cell viability, determining how DIAP1 functions in healthy and dying cells is of importance. We have created an eye-specific *diap1 Drosophila* mutant using RNAi, and this mutant, which has a small eye due to increased cell death, provides a sensitized background in which to screen for modifiers of DIAP1. A deficiency screen of the *Drosophila* genome isolated two suppressors and seven enhancers. One enhancer was mapped to a narrow region within the genomic area 30F and tests are being performed to determine the possible role of the ribosomal protein RpL13. A suppressor in the region of 90F has been narrowed down to approximately ten candidate genes. Due to the fact that each deficiency isolated contains no known apoptotic regulators, this screen provides a means to identify novel regulators of cell death.

4.2 Introduction

Elimination of cells through programmed cell death is crucial for the development and viability of an organism. Though several mechanisms for cell death exist, apoptosis still remains the best characterized mode of cell death. Caspases, a family of cysteine proteases, comprise a key switch in activation of apoptosis. Activated caspases cleave several protein substrates, leading to gross morphological changes in the cell and eventual death. The first caspase identified was the *ced-3* gene in *C. elegans*, and caspases have since been found throughout metozoa.

In *Drosophila* and mammals, IAPs are the key caspase inhibitors. In part, IAPs are characterized by their BIR domains and some members also have the characteristic E3 ubiquitin ligase RING domain. DIAP1 in flies and XIAP in mammals contain RING domains and act to inhibit caspases through ubiquitin-mediated protein degradation (Suzuki et al., 2001; Wilson et al., 2002).

Several proteins promote cell death by counteracting the function of the anti-apoptotic IAPs. In *Drosophila*, the most famous of these are the small proteins HID, GRIM, REAPER, and SICKLE. Each contains a small hydrophobic motif that binds to a BIR domain (Wu et al., 2001). Once bound to these small proteins, DIAP1 promotes its own degradation and releases the caspase from its grasp (Wang et al., 1999; Yoo et al., 2002). UBCD1 and MORGUE, two components of the ubiquitin protein-degradation

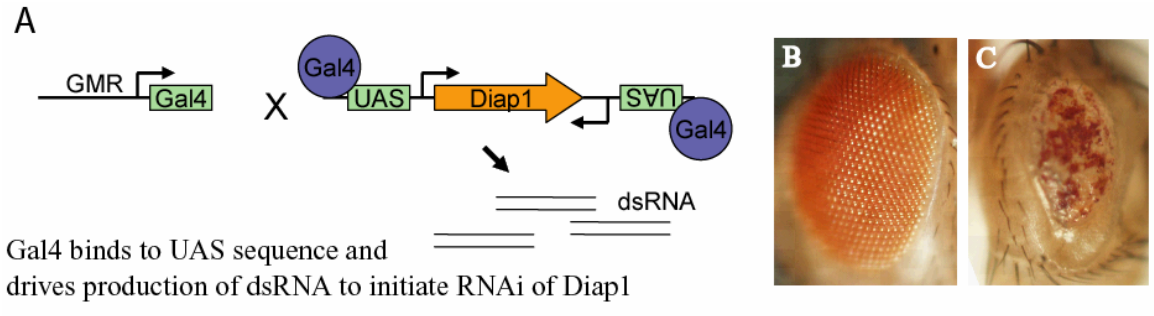

Figure 1. Diap1 RNAi knockdown in the *Drosophila* retina. A) Schematic of the production of dsRNA specifically in the eye by the GMR-gal4 driver. dsRNA serves as a substrate for the RNAi machinery to target and reduce DIAP1 levels. B) Adult wild type eye. C) Small eye phenotype in GMR-diap1(RNAi) flies.

machinery, have also been shown to promote the down regulation of DIAP1 protein levels (Ryoo et al, 2002; Hays et al., 2002; Wing et al., 2002).

While some of the regulators of DIAP1 in dying cells are known, two questions arise: Are there others and what factors aid DIAP1 function in healthy cells? To address these questions, we have screened for modulators of DIAP1 function in a sensitized background. Knockdown of *Diap1* by RNAi in the *Drosophila* eye gives a moderately small eye phenotype (Fig. 1C). By screening though a collection of genetic deficiencies, we were able to isolate several genomic regions that dominantly alter the eye size of the *diap1*(RNAi) mutant. Secondary screens eliminated many of the loci and left nine loci that contained genuine modifiers. Two genomic regions were selected in hopes of isolating one genetic enhancer and one suppressor.

4.3 Material and Methods

Fly Genetics

Drosophila stocks were maintained at 25 ^{o}C. Deficiencies and mutants were obtained from the Bloomington Stock Center (Bloomington, IN). UAS-Q78, UAS-EGFR(dn), and UAS-Debcl, encoding a pro-apoptotic Bcl family member, were obtained from N. Bonini, K. Moses, and S. Kumar, respectively, and recombined to either the GMR-gal4 or ptc-gal4 chromosome. GMR-ec(RNAi) was reported in a previous chapter. GMR-P35, GMR-Diap1, *thread*^{5} have been previously reported. Transgenic flies were created by microinjection into embryos. Constructs were injected by Rainbow Transgenic Flies (Newbury Park, CA). For each injected construct, several lines were balanced and tested for modification of the GMR-diap1(RNAi) small eye phenotype.

Molecular Biology

Mutant *diap1*(RNAi) flies were made by driving dsRNA production from UAS elements flanking the coding region (Giordano et al., 2002). Two *diap1*(RNAi) lines were generated. The strong line has a small eye phenotype, while the weaker has mild disruption in the eye ommatidia. To generate an eye-specific RNAi mutant of the ribosomal gene *RpL13*, a 22nt sequence from the gene replaced the stem sequence of the microRNA mir-6 (Table 1). The resulting miR-6::RpL13 hybrid was cloned into pGMR for gene expression in the eye. In a similar manner, GMR-miPP2A-B', GMR-miCG14314, GMR-miCG14316, GMR-miCG7940, GMR-miCG7985, GMR-miCG7156, GMR-miCG7183, GMR-miCG7215, GMR-miCG7993 were made (Table 1).

Gene	Sequence
RpL13	TGATCGTTCTGTCTACAACATC
PP2A-B'	GGAGCTGTTCATACAGAAGATC
CG14314	ACACTAGCTGTCAGCCCAAAAC
CG14316	GAACCCTTTTACAAAGTCTAAT
CG7940	TGAAAGCACCGAAAAATACATG
CG7985	GGATAATACGAATGTTAACAGC
CG7156	AGGCACTATTTGCTTTGCTATT
CG7183	AAAGGCATACCCTTCATTTATA
CG7215	AAATTTTACGGCAATTGCTATG
CG7993	TATGTCGCTTTTACGCATCAGG

Table 1. Genes and sequences used for RNAi as described in Materials and Methods section.

4.4 Results

Screen for diap1(RNAi) modifiers

DIAP1 is the key inhibitor of apoptosis in *Drosophila*. To screen for regulators of DIAP1 function, we created a sensitized background by RNAi knockdown of *Diap1* specifically in the eye. The coding region of *Diap1* was flanked by UAS enhancer elements to drive expression of dsRNA in the presence of a gal4 driver line (Fig. 1). Using the GMR-gal4 line, a mutant *diap1*(RNAi) line was created that resulted in a small eye phenotype and an increase of apoptosis. This line was used to screen a set of autosomal deficiencies covering approximately 80% of the *Drosophila* genome.

Initial results from this screen resulted in 19 genetic deletions that when present as heterozygotes modified the small eye phenotype (Table 2). The effectiveness of our screen was confirmed by the isolation of two enhancers that covered *thread* (*Diap1*). Interestingly, no deficiencies covering the caspase *Dronc* were isolated as modifiers.

To reduce the number of false positives from the list of modifying genomic loci, each deletion was crossed to GMR-gal4>UAS-Egfr(dn), GMR-gal4>UAS-Q78, and GMR-gal4>UAS-ec(RNAi). Though each of these lines has been implicated in regulating cell

Deficiency	Genomic Location	Enhancer/ Suppressor	GMR>ec(RNAi)	GMR>Q78	GMR>Egfr(dn)	Gene
Df(2L)net-PMF	21A1-21B8	E	-	-	-	
Df(2L)BSC37	22D2-22F2	E	+	+	+	
Df(2L)sc19-4	25A-25E5	E	+	nd	nd	
Df(2L)XE-3801	27E2-28D1	E	-	-	-	
Df(2L)BSC17	30C3-30F1	E	-	+	-	
Df(2L)TW161	38A-40AB1	E	-	-	-	
Df(2R)M41A4	41A	E	+	nd	+	
Df(2R)cn9	42E-44C	E	+	nd	nd	
Df(2R)stan1	46D7-47F16	E	+	nd	nd	
Df(2R)Jp8	52F-53A1	E	nd	-	+	
Df(2R)017/ Df(2R)AA21	56F 56F9-57D12	E	-	-	-	
Df(2R)X58-12	58D1-2;59A	E	+	+	+	
Df(2R)59AD	59A1-59D4	E	-	-	+	
Df(3L)BSC35	66F1-67B3	S	-	nd	nd	
Df(3L)vin7	68C8-69B5	E	-	-	-	
Df(3L)brm11/ Df(3L)st-f13	71F1-72D10 72C1-73A4	E	-	-	-	th
Df(3L)ri-XT1	77E4-78A4	E	-	-	-	
Df(3R)DG2/ Df(3R)Cha7	89E-91B2 90F1-91F5	S	-	+	+	
Df(3R)H-B79	92B3-92F13	E	-	-	-	

Table 2. A list of deficiencies isolated as modifiers of the GMR-diap1(RNAi) small eye phenotype. Deficiencies are listed as enhancers (E) or suppressors (S). GMR-ec(RNAi) is RNAi knockdown of the deubiquitinating enzyme Echinus, using the same RNAi technique as GMR-diap(RNAi). Both GMR-Q78 and GMR-EGFR(dn) use GMR-gal4 to express the UAS construct of each gene. Genetic interactions are listed as none (-), positive (+), or not determined (nd).

death in various ways, they provide a secondary screen to eliminate deletions that alter either GMR function or the gal4/UAS system. This round of crosses eliminated all but nine of the genomic loci – two suppressors and seven enhancers (Table 2).

Enhancer of *diap1*(RNAi), the Df(2L)BSC17 Deletion

One of the deletions isolated, Df(2)BSC17, enhanced the *diap1*(RNAi) small eye phenotype. When crossed to the *diap1*(RNAi) mutant, heterozygotes for Df(2)BSC17 resulted in pupal lethality. A weaker *diap1*(RNAi) mutant, which by itself causes a mild phenotype, was crossed to Df(2L)BSC17 and resulted in a smaller, rougher eye (Fig. 3).

Df(2)BSC17 spans the chromosomal 30 region and deletes approximately 100 genes. A series of small overlapping deficiencies narrowed the potential modifier to the right distal

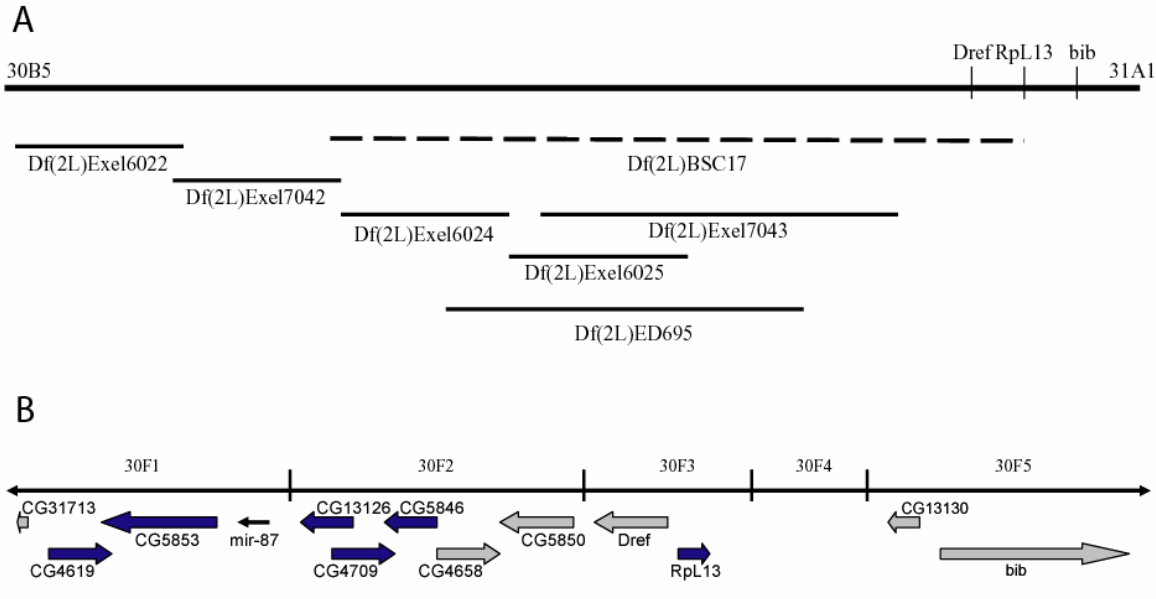

Figure 2. (A) Genomic region of Df(2L)BSC17. Df(2L)BSC17 is shown as a modifier (dashed line) while other non-interacting deficiencies (solid) are shown. Right breakpoint of Df(2L)BSC17 lies between the genes Dref and bib. (B) A view of the region containing the modifier. Genes in grey have been excluded. Genes in blue are candidates.

tip of Df(2)BSC17 (Fig. 2A). The right breakpoint of Df(2)BSC17 was mapped between the genes *Dref* and *bib* by the fact that *Dref* fails to complement and *bib* does complement. The modifier, thus, lies in the 30F region between CG31713, the last gene removed by Df(2L)Exel7043 and *bib* (Fig. 2B). Mutants for the genes CG13130, *Dref*, *bib*, CG5850, and CG4658 failed to phenocopy Df(2)BSC17.

One candidate modifier in the 30F region is the gene encoding the ribosomal protein *RpL13*. An eye-specific RNAi mutant was created by replacing the stem structure of the microRNA mir-6 with a 22 nt sequence specific for *RpL13*, and placing the hybrid sequence under the control of the GMR enhancer elements. Transgenic flies for the *RpL13* knockdown, miRpL13, showed small eye phenotype (Fig. 3). The miRpL13 mutant causes lethality in both the strong and weak *diap1* RNAi lines. These results underscore that loss of *RpL13* enhances loss of *diap1* and remains a potential regulator of DIAP1 function. Transgenic flies with overexpressed *RpL13* are now being generated to eventually test for rescue in a *diap1* mutant fly.

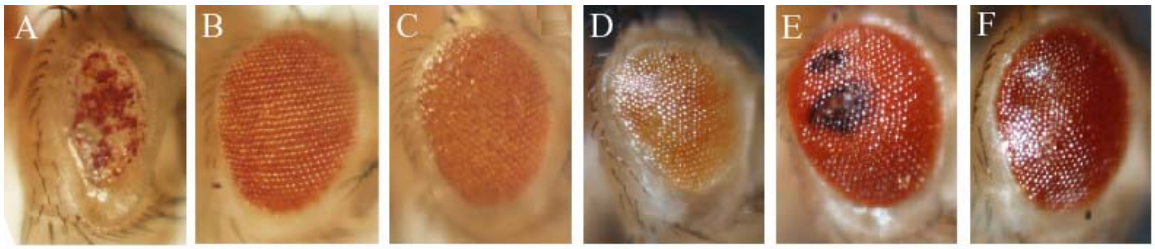

Figure 3. RNAi of the ribosomal gene RpL13 causes a small eye phenotype that can partially suppressed by Diap1 and p35. miRpL13 causes lethality when in trans to GMR-diap1(RNAi). Fly eye pictures of the interaction between Df(2L)BSC17 and RpL13 with GMR-diap1(RNAi). (A) GMR-diap1(RNAi) [strong] (B) GMR-diap1(RNAi) [weak] (C) GMR-diap1(RNAi) [weak]; Df(2L)BSC17/+ (D) GMR-miRpL13 (E) GMR-p35; GMR-miRpL13 (F) GMR-diap1; GMR-miRpL13

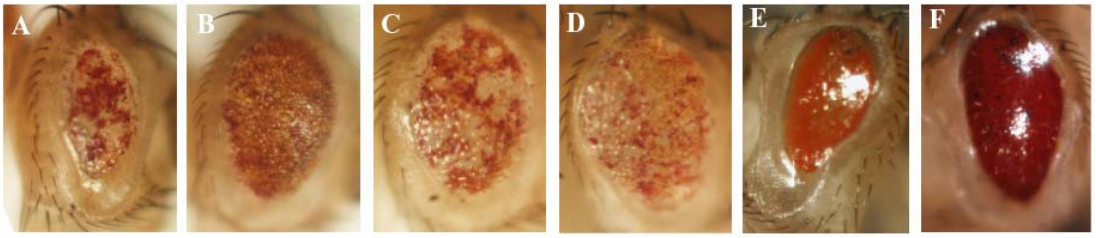

Figure 4. Deficiency Df(3R)DG2 contains two suppressors of the GMR-diap1(RNAi) small eye phenotype. Both *glass* and Df(3R)DG4 overlap Df(3R)DG2, but complement each other. Fly eyes showing the interactions between GMR-diap1(RNAi) and deficiencies of the 90F region. (A) GMR-diap1(RNAi) (B) GMR-diap1(RNAi); Df(3R)DG2/+ (C) GMR-diap1(RNAi); Df(3R)DG4/+ (D) GMR-diap1(RNAi); *gl*[60j]/+ (E) GMR-gal4; UAS-debcl (F) GMR-gal4; UAS-debcl/ Df(3R)DG2

A potential suppressor in 90F that is not *glass*

In the screen both the Df(3R)DG2 and Df(3R)Cha7 deficiencies were isolated as moderate suppressors of the eye phenotype (Fig. 4B, data not shown for Df(3R)Cha7). Both Df(3R)DG2 and Df(3R)Cha7 include *glass* (*gl*), a transcription factor used to drive gene expression in GMR, as both deficiencies failed to complement the *glass* mutant gl^{60j}. As loss of *gl* function would prevent the GMR construct from driving gene expression, we tested whether *glass* mutants could suppress the *diap1*(RNAi) construct. Heterozygotes for gl^{60j} moderately increased the eye size in the *diap1* knockdown flies, proving that disruption of the GMR enhancer element can alter the eye size in GMR-diap1(RNAi) mutants (Fig. 4C).

A second modifier might exist within these modifying chromosomes, despite the fact that both Df(3R)DG2 and Df(3R)Cha7 span the *glass* locus. A series of smaller deficiencies covering the entire 90 chromosomal region, but importantly not covering the *glass* locus, were obtained and tested for suppression of *diap1*(RNAi). Interestingly, Df(3R)DG4 acted similar to Df(3R)Cha7, but complemented *gl*. Based upon crosses with other

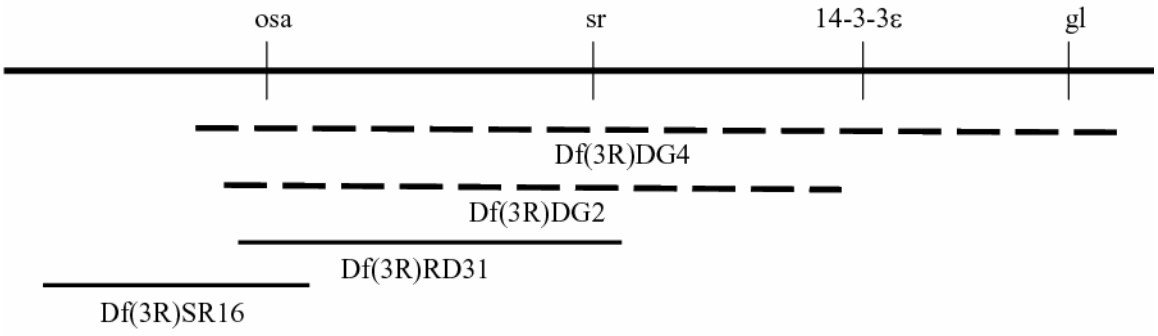

Figure 5 Genomic region of Df(3R)DG2. Df(3R)DG2 and Df(3R)DG4, suppressors of the GMR-diap1(RNAi) eye phenotype, are shown as dashed lines while non-suppressing deficiencies are shown as solid lines.

deficiencies and mutants, the potential suppressor was narrowed down to the genomic area between the genes *stripe* and 14-3-3ε (Fig. 5). Hypomorphic mutants for *stripe*,

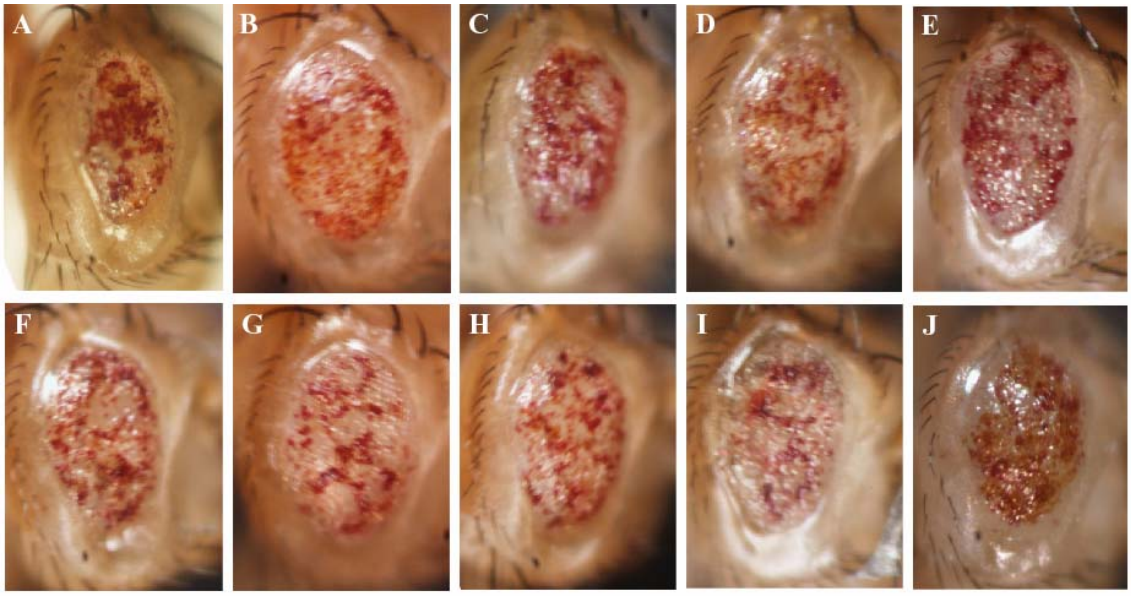

Figure 6 Fly eye pictures of candidate genes in 90F. Each candidate has been knockdown in the eye using the microRNA method described in text. No candidate RNAi lines had dominant eye phenotypes. (A) GMR-diap1(RNAi) (B) GMR-diap1(RNAi); GMR-CG14314 (C) GMR-diap1 (RNAi); GMR-miPP2A-B' (D) GMR-diap1(RNAi); GMR-CG7940 (E) GMR-diap1(RNAi); GMR-CG7215 (F) GMR-diap1(RNAi); GMR-mi7156 (G) GMR-diap1(RNAi); GMR-CG14316 (H) GMR-diap1(RNAi); GMR-CG7183 (I) GMR-diap1(RNAi); GMR-CG7993 (J) GMR-diap1(RNAi); GMR-CG7985

cadmus, *Trap80*, *Ssdp*, *repo*, and *14-3-3ε* failed to phenocopy the suppression by Df(3R)DG4. Ectopic expression of CG14315, CG7217, CG7208, CG7998 with P-elements carrying UAS elements also failed to modify the small eye phenotype. RNAi gene knockdown was employed to test several other genes in the region using the microRNA hybrid method mentioned above. Testing nine of them, however, showed no modification of the *diap1*(RNAi) eye phenotype (Fig. 6).

4.5 Discussion

In this study a deficiency screen was performed to isolate dominant modifiers of a *diap1*(RNAi) eye phenotype. After a secondary screen to remove regulators of the Gal4/UAS system and RNAi machinery, two suppressors and seven enhancers were isolated. Two overlapping deletions covering *thread*, which encodes the *Diap1* gene, were pulled out of the screen, thus confirming the ability of the screen to identify modifiers.

Sensitized screens are commonly employed as a means of uncovering novel regulators of gene function. In this situation lowered levels of *Diap1* by RNAi caused an increase in apoptosis and reduced the size of the eye, allowing for the recovery of dominant modifiers that regulate DIAP1 function. Deficiency screens provide a quick and easy means of screening the genome, but it is tedious to pinpoint the actual modifier. A P-element screen would be a better means for screening due to the relative ease in identifying the transposon genomic location and thus a smaller number of candidate genes. P-element screens,

though, do not necessarily span the entire genome and are more labor intensive. A small P-element screen has recently been performed in the lab with the results pending.

RpL13, the potential enhancer in Df(2L)BSC17

In our screen, we isolated seven different enhancers, all of which are lethal when combined with GMR-diap1(RNAi). The deficiency Df(2L)BSC17 was a strong enhancer, and the potential modifying gene was narrowed down to a group of seven candidates in the 30F genomic region. RNAi of the ribosomal gene *RpL13*, one of the seven candidates, showed a small eye phenotype, making *RpL13* a good candidate modifier. Loss of *RpL13* specifically in the eye enhanced both the strong and weak loss of *diap1* lines, which further proves that *RpL13* is the responsible modifier in Df(2L)BSC17. Finally, overexpression of *Diap1* or the viral caspase inhibitor P35 slightly rescued the mutant *RpL13* eye phenotype. Tests are currently underway to determine whether overexpression of *RpL13* can rescue loss of *diap1*. Nevertheless, based on the observation that loss of *RpL13* phenocopies heterozygosity of Df(2L)BSC17, we strongly suspect that *RpL13* is the responsible gene for modulating DIAP1 levels.

It is interesting to hypothesize about the connection between apoptosis and ribosomal proteins. A recent study in zebra fish showed that eleven ribosomal proteins were haploinsufficient tumor suppressors, including the ortholog for RpL13 (Amsterdam et al., 2004). In *Drosophila* cell culture, depletion of ribosomal proteins by RNAi did not kill the cells, and even prevented the entry of internal ribosome entry site (IRES) viruses (Cherry et al., 2005). In both cases novel roles for the ribosome were discovered. In the zebra fish

study, loss of ribosome led to tumor formation, though the role of the ribosome in apoptosis or even growth control remains unclear. An interesting hypothesis would be that loss of ribosome serves as a protection against apoptosis. Thus, loss of the ribosome allows the cell to bypass growth controls and to initiate tumorigenesis. In support of this model, our lab has seen that the loss of some ribosomal proteins protects S2 cells against *diap1* RNAi induced cell death (I. Muro, unpublished data). This model, however, contradicts the observation that *RpL13* knockdown in the *Drosophila* retina either prohibits cell proliferation or promotes cell death. Does loss of the ribosome promote uncontrolled growth or promote apoptosis, or is there a context in which both these models hold true? For one thing, nothing is known mechanistically of how loss of the ribosome promotes tumor formation in zebra fish or inhibits proper *Drosophila* eye development. Secondly, we do not know if these observations depend on the tissue assayed or the specific ribosomal proteins lost. In both S2 cell lines and in the zebra fish experiments, proliferating cells were depleted of ribosomes, while in the *Drosophila* retina, the cells are essentially post-mitotic. These observations do however point to the fact that there is an uncharacterized connection between the ribosome and control of apoptosis, and the isolation of RpL13 as a modifier in the deficiency screen adds another tie.

A suppressor in the 90F region

From the screen, we also isolated a potential pro-apoptotic molecule in the 90F region. The deficiency Df(3R)DG4 suppressed the *diap*(RNAi) eye phenotype, while several overlapping deficiencies showed no suppression. Df(3R)DG4 complemented both 14-3-3ε and *glass*, and only the genes located between *stripe* and 14-3-3ε were left as candidates.

This genomic deficiency also suppressed the phenotype of ectopically expressed *Debcl*, a pro-apoptotic Bcl family member whose function is still unknown.

The phosphatase PP2A-B' is a particularly interesting candidate in the region between *stripe* and 14-3-3ε. This enzyme belongs to the class of regulatory subunits for the PP2A phosphatase, and has been shown to inhibit Bcl2 function in mammalian cell culture (Chiang et al., 2001; Deng et al., 1998). The phosphatase, however, is not the suppressor; both RNAi knockdown and a P-element mutant, though located in an intron, show no genetic interaction with loss of *diap1* in the eye (Fig. 6, data not shown). Other candidates were excluded because their knockdown by RNAi failed to alter the small eye phenotype. All ten remaining genes encode novel genes so their likely function and potential connection to regulation of apoptosis remains elusive. Constructs to generate knockdown lines of these remaining candidates are currently being made.

4.6 Conclusions

In this study, loss of *diap1* in the eye by RNAi provided a sensitized background for a deficiency screen. With the exception of the deletions spanning the Diap1 locus *thread*, the isolated deficiencies cover novel regulators of apoptosis. One enhancer shows a potential role for ribosomal proteins in mediating cell death, while another genomic region containing a pro-apoptotic molecule has been narrowed down to ten candidates.

4.7 Acknowledgments

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