Bcl-2 Function in *Drosophila*

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# DEDICATION

I would like to dedicate my dissertation thesis to my family. My parents provided me endless support (intellectual, emotional and financial) throughout my development and academic career. My education was always of utmost priority since childhood, and I learned early on that obtaining an education was invaluable and could never be taken away. They always told me that while personal belongings may come and go, my education is for life.

This dedication would be remiss if I didn't thank the most important person in my life: my big brother. Rene (Bubbo to me), provided, and continues to provide academic and personal guidance. His never-ending intellectual input, his constant concern for my well-being, both academically and personally, and lastly, his unconditional love and friendship make me the luckiest grad student around. Many times, he played the role of a third parent growing up, but more importantly and unforgettably, he continues to be my best friend. For that, I am ever grateful. I would not be the same person if he weren't such a pivotal role model in my life, and though it goes without saying, "I love you!"

I would also like to dedicate my thesis to my college advisor, Dr. Cimadevilla. Dr. C was responsible for my early exposure to basic scientific research since I was just in high school. He did a wonderful job of starting the MARC Program (Minority Access to Research Careers) at St. Mary's University, and I was proud to be the first freshman at St. Mary's to be a recipient of the MARC Fellowship. His goals were to recruit and expose young students, particularly minority students, to the possibility of entertaining a career in scientific research, and because of his ambitious efforts, many St. Mary's alumni have completed their Ph.D.'s in the basic sciences. To that, I will always be grateful. Less than two years ago, Dr. C lost his battle to esophageal cancer, and his memory and legacy will forever be remembered. Thank you Dr. C. I hope I've made you proud.

Lastly, I want to acknowledge my pets: Chandler, Robby, Squirt, Zoey and Twinky. Though they did not directly contribute to my academic career, they did keep me going when times got rough. Graduate school has been my most challenging academic endeavor to date, and countless times they made coming home to an empty house after a grueling days' work worth-while with their unconditional love and affection. My life would be empty without them.

# Bcl-2 Function in *Drosophila*

by

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# ABSTRACT

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Bcl-2 family members are pivotal regulators of programmed cell death (PCD). In mammals, pro-apoptotic Bcl-2 family members initiate early apoptotic signals by causing the release of cytochrome *c* from the mitochondria, a step necessary for the initiation of the caspase cascade. Worms and flies do not show a requirement for cytochrome *c* during apoptosis, but both model systems express pro- and anti-apoptotic Bcl-2 family members. *Drosophila* encodes two Bcl-2 family members, Debcl (pro-apoptotic) and Buffy (anti-apoptotic). To understand the role of Debcl in *Drosophila* apoptosis, we produced an authentic null allele at the Debcl locus. Although gross development and lifespans were unaffected, we

found that *debcl* was required for pruning cells in the developing central nervous system. *debcl* genetically interacted with the *ced-4/Apaf-1* counterpart, *dark*, but was not required for killing by RPR proteins. Surprisingly, in a model of caspase-independent cell death, we found that heterologous killing by *Murine* Bax required *debcl* to exert its pro-apoptotic activity. $debcl^{KO}$ mutants were also significantly affected for mitochondrial density. Taken together, these findings suggest that evolutionary functions impacting mitochondrial properties represent ancient activities which preceded the evolution of these proteins as central regulators of PCD.

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PRIOR PUBLICATIONS

Kathleen Galindo and Dean P. Smith. A Large Family of Divergent *Drosophila* Odorant-Binding Proteins Expressed in Gustatory and Olfactory Sensilla. *Genetics*. **159** 1059-1072 (2001)

PRESENT PUBLICATIONS

Kathleen A. Galindo, Jae Park and John M. Abrams. Debcl, the *Drosophila* Proapoptotic Bcl-2 family member, functions in developmental PCD and in Regulating Mitochondrial Density. (Manuscript in preparation)

Nicholas Joza, Kathleen Galindo, J. Andrew Pospisilik, Paule Benit, Manu Rangachari, Elisabeth E. Kanitz, Yuko Nakashima, Gregory G. Neely, Pierre Rustin, John M. Abrams, Guido Kroemer, and Josef M. Penninger. The molecular archaeology of a caspase-independent death effector: AIF in *Drosophila*. (Manuscript in Submission to Cell Death and Differentiation)

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LIST OF DEFINITIONS

AIF – Apoptosis inducing factor

BH3 – Bcl-2 homology domain 3

Ced – Cell death defective

CNS – Central nervous system

Dark - *Drosophila* Apaf-1 related killer

Debcl – *Drosophila* executioner bcl-2 family member

Diap-1 – *Drosophila* inhibitor of apoptosis

Dp53 – *Drosophila* p53

Drice – *Drosophila* Interleukin-1 converting enzyme

Dronc – *Drosophila* nedd2-like caspase

Drp-1 – Dynamin related protein

Eg – Eagle

Egl – Egg laying defective

Endo-G – endonuclease G

Hid – head involution defective

IAP – Inhibitors of Apoptosis

IR – Ionizing radiation

Kr – kruppel

Lbe – Lady bird early

Mfn-2 – Mitofusin

MOM – Mitochondrial outer membrane

PCD – Programmed Cell Death

Rpr - reaper

VNC – Ventral nerve cord

## CHAPTER ONE
## General Introduction

The elimination of unwanted and/or damaged cells is a necessary process of programmed cell death conserved throughout all metazoans. Apoptosis is critical during embryonic development for proper organogenesis and in the sculpting of multicellular tissues. Cell death is also important in maintaining adult cell and tissue homeostasis. Perturbations in this finely tuned mechanism can lead to cancer or autoimmunity when cells fail to die, or in the event of accelerated cell death, degenerative diseases or immunodeficiency. Over the past two decades, there has been an explosion of investigations exploring the molecular and biochemical mechanisms that participate in the fine-tuning of this delicately balanced process. There are several control points in cell death, where careful yet intricate explorations in worms, flies, and mammals have all proven valuable in illuminating specific signals. All 3 model systems contain the Bcl-2 family members, the apoptosome, and caspases to orchestrate the PCD pathway.

### *Programmed Cell Death in C. elegans*

Significant contributions were made in identifying the core cell death regulators in *C. elegans* (Ellis and Horvitz 1986). In this wonderfully utilized genetic model system, precisely 131 out of 1090 cells undergo programmed cell

death during development. The key regulators of cell death include the proapoptotic BH3-only Bcl-2 family homolog, *egl-1*, the anti-apoptotic Bcl-2 family member, *ced-9*, the Apaf-1 ortholog, *ced-4*, and the cysteine protease, *ced-3*. *ced-4* and *ced-3* are required for all cell deaths to occur, whereas, *ced-9* is required to prevent cell death (Hengartner and Horvitz 1994). In worms, CED-9 physically binds to and inhibits CED-4, preventing activation of CED-3. The cell death machinery is activated when EGL-1 binds to CED-9 at the mitochondria, displacing bound CED-4, which is now free to bind to and activate CED-3 (Conradt and Horvitz 1998).

*Programmed Cell Death in Drosophila: Critical Control Points*

In *Drosophila*, there exist several critical control points of apoptosis. Caspase regulation is the primary mode of apoptotic activation. Flies have 3 initiator/apical caspases, Dronc (Dorstyn, Read et al. 1999), Dredd (Chen, Rodriguez et al. 1998) and Strica (Vernooy, Copeland et al. 2000; Doumanis, Quinn et al. 2001) (characterized by the presence of a long N-terminal prodomains) and 4 effector caspases, Drice (Fraser, Mccarthy et al. 1997), Decay (Dorstyn, Read et al. 1999), Dcp-1 (Song, Mccall et al. 1997) and Damm (Vernooy, Copeland et al. 2000; Harvey, Daish et al. 2001) (characterized by short pro-domains). Of the three apical caspases, Dronc is the primary apical

caspase whose primary function is in the apoptotic process. Upon recruitment to the apoptosome, Dronc is processed and activated (Muro, Hay et al. 2002; Muro, Monser et al. 2004; Yan, Huh et al. 2006), forming oligomers to perpetuate a self-amplification signal (Yan, Huh et al. 2006). Studies on Dredd function have implicated its primary role in the activation of innate immunity (Chen, Rodriguez et al. 1998). Strica is unique in that it contains a serine- and threonine-rich prodomain, and although ectopic expression of this protein can induce cell death (Doumanis, Quinn et al. 2001), little is known about the biological process by which Strica participates. The effector caspases Dcp-1 and Drice are very similar to one another and share high homology to the mammalian effector caspase-3 and -7. *dcp-1* mutant animals are relatively healthy, exhibiting defects only in starvation induced cell death during oogenesis (Laundrie, Peterson et al. 2003). *drice* mutant animals exhibit marked reduced cell death in a variety of tissues with semi-lethal phenotypes; however, some cell death still occurs (Muro, Berry et al. 2006). Interestingly, animals doubly deficient for *dcp-1* and *drice* are lethal with exacerbated cell death phenotypes compared to *drice* alone, suggesting these two effector caspases share partially redundant roles in *Drosophila* (Muro, Berry et al. 2006).

A second critical control points in fly PCD involves the Inhibitors of apoptosis (IAPs). IAPs are central regulators of caspase activation, defined by the presence of a 70-amino-acid motif known as the baculovirus IAP repeat (BIR).

The *Drosophila* genome encodes 3 IAPs: Diap-1 (Hay, Wassarman et al. 1995), Diap-2 (Hay, Wassarman et al. 1995; Duckett, Nava et al. 1996; Uren, Pakusch et al. 1996; Lindsten, Ross et al. 2000) and dBruce (Vernooy, Chow et al. 2002). Most is known about the function of Diap-1, which has revealed a requirement to inhibit Dark-, Dronc-, and Drice-dependent cell death, suggesting it as an essential inhibitor of cell death (Hay, Wassarman et al. 1995; Wang, Hawkins et al. 1999; Goyal, McCall et al. 2000; Lisi, Mazzon et al. 2000; Igaki, Yamamoto-Goto et al. 2002; Muro, Hay et al. 2002; Rodriguez, Chen et al. 2002; Yoo, Huh et al. 2002; Zimmermann, Ricci et al. 2002; Huh, Guo et al. 2004; Yin and Thummel 2004; Yokokura, Dresnek et al. 2004; Xu, Li et al. 2005; Kiessling and Green 2006; Leulier, Lhocine et al. 2006; Muro, Berry et al. 2006). The proapoptotic activators, *rpr*, *hid* and *grim* (RHG proteins) reside in the genomic interval H99. When the RHG proteins are deleted, all developmental and γ-irradiated cell death is abolished (White, Grether et al. 1994; Chen, Lee et al. 1996). The RHG activators bind to IAPs to allow for caspase activation and execution of subsequent apoptotic events.

A third critical control point in *Drosophila* apoptosis involves formation of the apoptosome. *dark*, the *Drosophila* Apaf-1 homolog, contains WD40 repeats, a motif known for binding to cytochrome *c* in mammalian Apaf-1, and a caspase recruitment domain (CARD) which binds to and activates DRONC (Caspase-9 homolog). Dark and Dronc, combined with dATP form this multi-

protein complex referred to as the apoptosome. The deletion of *dark* (Rodriguez, Oliver et al. 1999) or *dronc* (Chew, Akdemir et al. 2004) results in lethality and abolishment of most cell deaths. The role of cytochrome *c* in *Drosophila* apoptosis has been a subject of intense controversy. Although dark has WD40 repeats, binding to cytochrome *c* is not required for formation of the fly apoptosome (Yu, Wang et al. 2006). In fact, cytochrome *c* is dispensable for activation of cell death with the exception of a minor role in caspase activation in spermatid individualization (Arama, Bader et al. 2006) and in the timely death of retinal cells (Mendes, Arama et al. 2006).

*The role of cytochrome c and the mitochondria in apoptosis*

Taken all this evidence to date, one begs to ask the question if there are upstream activators of the apoptotic machinery that work through the apoptosome that are Dronc-independent and/or independent or in parallel of *rpr*, *hid* and *grim* and the IAP pathway. In mammals, the Bcl-2 family members play important roles in the activation and regulation of cell death. They indirectly activate the apoptosome by causing changes in the mitochondrial outer-membrane (MOM) permeability, which is followed by the flood release of cytochrome *c* and other mitochondrial proteins into the cytoplasm. This cytoplasmic cytochrome *c* then binds to monomeric dATP/ATP-bound Apaf-1 through its WD40 repeats.

dATP/ATP is hydrolyzed inducing a conformational change in Apaf-1, allowing for assembly of a large heptameric ring structure referred to as the apoptosome, which is loaded with 7 dATP, cytochrome *c*, and Apaf-1 molecules (Jiang and Wang 2000). Though Dark contains WD40 repeats capable of binding to cytochrome *c*, there is little evidence to support release of cytochrome *c* from mitochondria in *Drosophila* cells (Rodriguez, Oliver et al. 1999; Varkey, Chen et al. 1999). Furthermore, cytochrome *c* is not necessary for Dark- or Dronc-dependent cell death or for apoptosome formation. Although early *in vitro* evidence suggested that cytochrome *c* stimulates caspase activation in cell culture (Kanuka, Sawamoto et al. 1999; Rodriguez, Oliver et al. 1999; Dorstyn, Read et al. 2002), recent structural evidence of the fly apoptosome (which contains eight dark molecules rather than seven in mammals) failed to bind to cytochrome *c* (Yu, Wang et al. 2006). Similarly, down-regulation of *Drosophila* cytochrome *c* by various methods also failed to reveal a role for this gene in caspase activation (Zimmermann, Ricci et al. 2002; Dorstyn, Mills et al. 2004; Means, Muro et al. 2006).

*The role of the Bcl-2 family members in Programmed Cell Death*

In worms, the Bcl-2 family of proteins function to regulate PCD through direct physical interactions. Ced-9 physically binds to and represses Ced-4 from activating Ced-3. In mammals, Bcl-2's indirectly impact the apoptosome by

regulating MOM permeability, which precedes the flux of mitochondrial proteins into the cytoplasm. Mammals have several members of the both the anti- and proapoptotic family of Bcl-2 proteins. There also exists a family of proapoptotic Bcl-2 family members known as the BH3-only proteins. These proteins function to promote cell death by either inhibiting the anti-apoptotic Bcl-2 proteins directly, or by binding to and promoting the proapoptotic Bcl-2 members. Tissue specific transgenes and targeted gene deletions have aided in dissecting the fundamental roles of this gene family in programmed cell death and tissue homeostasis. The anti-apoptotic family of Bcl-2 proteins have been shown to play roles in preserving mitochondrial membrane integrity to prevent release of cytochrome *c* into the cytosol (Kluck, Bossywetzel et al. 1997; Yang, Liu et al. 1997). The proapoptotic Bcl-2 proteins have been shown to promote cytochrome c release from the mitochondria, which initiates formation of the apoptosome and subsequent activation of the caspase cascade (Jurgensmeier, Krajewski et al. 1997; Li, Zhu et al. 1998; Luo, Budihardjo et al. 1998; Kluck, Esposti et al. 1999; Wei, Zong et al. 2001). Single mutations in the proapoptotic genes *bak* or *bax* produce viable mice with no overt phenotypes, or with mild phenotypes, respectively (Knudson, Tung et al. 1995; Lindsten, Ross et al. 2000). Interestingly, double mutant animals for both *bax* and *bak* result in a plethora of defects in the mouse model (Lindsten, Ross et al. 2000). In particular, $bax^{-/-}bak^{-/-}$ mice are semi-lethal where less than 10% of these animals survive to adulthood,

showing various phenotypes that include interdigital webs and females with imperforate vaginas. These $bax^{-/-}bak^{-/-}$ mice also show the persistence of extra cells in the central nervous system (CNS), resulting in various neurologic abnormalities including deafness, circling behavior after stress exposure, and a failure in suckling behavior. Lastly, these mice also have a grossly abnormal immune system with elevated lymphocytes, resulting in massive enlargement of the spleen and lymph nodes. Additionally, cells from $bax^{-/-}bak^{-/-}$ mice showed resistance to various apoptotic stimuli such as nutrient deprivation, gamma-irradiation and etoposide treatment. These phenotypes were absent in $bax^{-/-}$ or $bak^{-/-}$ single mutants. These results implicate a major role for these proapoptotic Bcl-2 genes in developmental cell death and in the tissue homeostasis of adult organs (Lindsten, Ross et al. 2000). However, since some $bax^{-/-}bak^{-/-}$ animals still live and probably have some residual PCD, this raises the question about other pathways that engage cell death. The precise mechanism of how Bcl-2's cause changes in MOM permeability is still a topic of hot debate.

*The role of Bcl-2 proteins in non-apoptotic processes*

In addition to promoting apoptosis, the pro-apoptotic Bcl-2 members, Bax and Bak, also play essential non-apoptotic roles in regulating mitochondrial morphology. Bax and Bak oligomerize to forming pores in the MOM, resulting in mitochondrial shape change followed by the efflux of mitochondrial proteins

(Youle and Karbowski 2005). Mitochondria are very dynamic organelles with a normal flux between fusion and fission states. It's been reported that during apoptosis, Bax and Bak are involved in dictating mitochondrial fragmentation by co-localizing with and interacting with dynamin-related protein 1 (Drp1) (Karbowski, Lee et al. 2002). Recent studies have shown that in healthy cells, Bax and/or Bak are also required for proper mitochondrial fusion into elongated tubules by interacting with mitofusion 2 (Mfn2) (Karbowski, Norris et al. 2006). How they control the activities of Drp-1 and Mfn2 is unknown. Although mitochondrial morphology is perturbed in Bax/Bak double knockout (DKO) cells, mitochondrial membrane potential and ATP concentrations are not affected, suggesting that the mitochondrial dynamics, and not mitochondrial physiology, require Bax and Bak. It was recently shown that CED-9 and EGL-1 impact mitochondrial morphology in both worms and in mammalian cells, where mutants in these two genes are defective in mitochondrial remodeling (Delivani, Adrain et al. 2006). Other non-apoptotic roles for mammalian Bcl-2 family members include cell cycle regulation (Quinn and Richardson 2004) and autophagy (Maiuri, Zalckvar et al. 2007).

*Drosophila Bcl-2's*

On that note, what roles do the Bcl-2 family of proteins play in *Drosophila* apoptosis? *Drosophila* has two multi-domain Bcl-2 family members: the pro-

apoptotic member, *debcl*, and the anti-apoptotic member, *buffy*. Both Debcl and Buffy share the bcl-2 homology domains BH1, BH2, and BH3, as well as the C-terminal transmembrane domains. Buffy expression in embryos suppresses death due to the loss of Diap-1 (Quinn, Coombe et al. 2003); however, it is not known whether *debcl* or *buffy* impact cell death by activating or suppressing *dark* or *dronc*. Evidence for Debcl's proapoptotic activity includes ectopic overexpression analysis of Debcl in fly cells, as well as in various animal tissues (Brachmann, Jassim et al. 2000; Colussi, Quinn et al. 2000; Igaki, Kanuka et al. 2000). Specifically, overexpression of *debcl* in fly embryos resulted in increased cell death, suggesting its proapoptotic activity (Colussi, Quinn et al. 2000), and RNAi of *debcl* in embryos exhibited decreased cell death by TUNEL (Brachmann, Jassim et al. 2000), suggesting that Debcl is required for developmental PCD. Debcl was also shown to interact genetically with Dark and Diap1 (Colussi, Quinn et al. 2000), and Debcl also sensitizes cells to PCD in response to UV irradiation (Brachmann, Jassim et al. 2000). Additionally, DEBCL can bind to mammalian anti-apoptotic Bcl-2's by co-immunodepeletion experiments, but not to any pro-apoptotic Bcl-2's, further suggesting its proapoptotic activity (Colussi, Quinn et al. 2000). It is still amazing how so little is known about the fly Bcl-2's compared to the mammalian system. In order to elucidate the function of these genes in *Drosophila*, loss-of-function mutations are necessary in these genes.

# STATEMENT OF OBJECTIVES

*Project I*

One of the goals of my thesis research was to generate a null allele in the debcl gene and characterize the role of this gene in apoptotic and non-apoptotic contexts.

*Project II*

A second goal of my thesis research was to generate and characterize null animals for effector caspase Drice.

*Project III*

I characterized *dmAIF* $^{KO}$ flies during developmental PCD in collaboration with Gruido Kroemer's and Josef Penninger's groups.

# CHAPTER TWO

## Debcl, the Drosophila Pro-Apoptotic Bcl-2 Family Member, Plays a role in Developmental PCD and in the Regulation of Mitochondrial Density.

### Introduction

Apoptosis is a form of programmed cell death (PCD) that is required for proper development, maintenance of tissue homeostasis during adulthood, as well as in the elimination of damaged or unwanted cells. Though the core apoptotic machinery is conserved, distinct mechanistic differences in the activation and regulation of this process have evolved. In worms and mammals, both anti- and proapoptotic Bcl-2 family members play pivotal roles in regulating cell death early in the apoptotic pathway. In *C. elegans*, the anti-apoptotic Bcl-2 protein, CED-9, physically interacts with CED-4 to inhibit cell death. Upon detection of an apoptotic stimulus, the proapoptotic BH3 only protein, EGL-1, binds to CED-9, relieving suppression of CED-4 and allowing it to bind to and activate the caspase CED-3. In mammals, the BH3 only containing Bcl-2 family members activate apoptosis by either inhibiting the anti-apoptotic Bcl-2 members, or by directly activating the pro-apoptotic Bcl-2 members, such as Bax and Bak. Bax and Bak play major roles in apoptotic cell death (Lindsten, Ross et al. 2000). Unlike worms, where a direct physical link to the apoptosome is seen, regulation of apoptosis by the mammalian Bcl-2 gene family occurs indirectly through regulation of mitochondrial properties. For example, several pro- and anti-

apoptotic Bcl-2 proteins regulate cell death by impacting mitochondrial outer membrane (MOM) permeability (Kluck, Bossywetzel et al. 1997; Zou, Henzel et al. 1997), resulting in the release of cytochrome *c* and subsequent formation of the apoptosome (Green and Reed 1998; Gross, McDonnell et al. 1999).

Like worms and mammals, the *Drosophila* genome encodes at least two Bcl-2 family members (Chen and Abrams 2000). However, unlike mammals, but similar to worms, cytochrome *c* is not required during apoptosome formation. Hence, the role of *Drosophila* Bcl-2 family members as potential regulators of mitochondrial-dependent PCD remains unclear. Previous studies based on forced expression and RNAi reported proapoptotic functions for *debcl* (Brachmann, Jassim et al. 2000; Colussi, Quinn et al. 2000; Igaki, Kanuka et al. 2000; Zhang, Huang et al. 2000), and potentially anti-apoptotic functions for *buffy*, (Brachmann, Jassim et al. 2000; Quinn, Coombe et al. 2003). Very recently, genetic studies found very limited roles for either gene in apoptosis,(Sevrioukov, Burr et al. 2007). However, since partial *debcl* function may likely persist in these mutants, pivotal questions remain unresolved.

To illuminate the functional role of Bcl-2 proteins in PCD, we generated an unambiguous null allele at *debcl*. We exclude a global role for this gene in developmental PCD, but do find a limited role for *debcl* in regulating cell death and cell numbers in the CNS. *debcl* genetically interacted with the *ced-4/Apaf-1* counterpart, *dark*, but was not required for killing by RPR proteins. Surprisingly,

in a model of caspase-independent cell death, we found that heterologous killing by *Murine* Bax required *debcl* to exert its pro-apoptotic activity. *debcl*$^{KO}$ mutants were also significantly affected for mitochondrial density. We found no overt role for *debcl* in regulating stress-induced cell death, cell-cycle checkpoint kinetics, genomic instability, or autophagy. Taken together, these findings suggest that evolutionary functions impacting mitochondrial properties represent ancient activities which preceded the evolution of these proteins as central regulators of PCD.

**Materials and Methods**

**Ends-Out Donor Constructs.** Genomic DNA from *yw* flies was used as template for all PCR reactions. Primers 5'- ACA ATC ACA GCG GCC GCG CCT CAC TAA GAG AAA CTT ATG G -3' and 5'- CGG GGT ACC TAT TGT TGC TGC TGA GGC CTT TGT TGG -3', were used to PCR amplify the 3.84kb upstream flanking sequence (from - 3876 to - 37 bp upstream of the *debcl* start codon) to clone into the respective *Not*I and *Acc*651 sites in the pw25 donor plasmid. Primers 5'- TAT GGC GCG CCT GTT CTA GAT TCG CTT GGG ATC GCG TCG -3' and 5'- CGC CGT ACG ACA TCA ATG CGG ATG GAT TTC AAT GTG TGG G -3' were used to PCR amplify the 2.70kb downstream flanking sequence (including the 3'UTR of the *debcl*) to clone into the respective *Asc*I and *Bsi*WI pw25 vector sites. All constructs were transformed into the germ

line of *Drosophila melanogaster* by using standard methods (Rubin and Spradling 1982).

**Genetics and Heat Shocks.** Crosses for targeted recombination were performed in standard 25-mm-diameter vials, each carrying 3-5 females per vial and a corresponding number of the appropriate males. Males carrying the donor construct were crossed to virgin females carrying the heat-inducible (70Flp)(70 I-Sce I)/TM6. The adults were removed a couple days after egg laying, prior to heat shocks performed in a circulating water bath at 38$^{o}$C for 90 min and repeated for 3 consecutive days. A total of 100 vials were used for heat shock. Mosaic-eyed females were crossed *en mass* to males carrying the constitutively active P{70Flp} (stock center 6938).

**Verification of *debcl* $^{KO}$ alleles.**

**Southern Blotting.** For verification of targeting, genomic DNA was prepared from flies carrying the candidate targeted allele using Wizard Genomic Purification Kit (Promega). Genomic DNAs were digested as indicated (Fig. 1A), separated by 0.8% agarose gel electrophoresis, and transferred to positively charged nylon membranes. The membranes were probed with $P^{32}$ dCTP-labeled DNA from PCR product using primers 5'- GCT ACA GTC GAG TGT GCT GGG TTG TTT GCG- 3' and 5'-GAC GGC GGA TTC CAG ACG CTT TCA

GAA CGG -3' for verification of the left recombination arm and primers 5'- GCG TAC AAT TAG ACC AGC CGT TGT GTT GGC -3' and 5'- AAG AGG ACA ACA GCG AGG TGG AGG AGG ACG -3' for verification of the right recombination arm, and hybridized using Express Hyb Hybridization Solution (BD Biosciences).

**PCR and RT-PCR.** A PCR strategy was used to recognize candidate targeted recombination alleles. The primers 5'- GGT TAT CAT ACC ATT CCT GCT CTT TGG -3' (Primer C) and 5'- GTC CTG AAG GAG ATC TGC GAA GAG GAC AAC AGC -3' (Primer D) were used to amplify a PCR fragment unique to targeted recombination. The same strategy using sequence specific primers was used to verify the left recombination arm (data not shown). Additionally, the primers flanking the *debcl* ORF, 5'- AAC GAG AAC GGG AAC TCG AAA GAA CCT AGA TCG -3' (Primer A) and 5'- AAG ACG AAT TGT CGT ACT CAA AAT ATT GGC ACC -3', (Primer B) were used to distinguish native *debcl* or replacement by the *white*$^{+}$ gene. The genomic sequences from the PCR reactions were fully sequenced, and the sites of recombination at the right and left recombination arms match endogenous sequence. To access transcript levels, total RNA was prepared from *debcl*$^{KO}$ and *yw* L3 larvae using High Pure RNA Isolation Kit (Roche). Superscript One-step RT-PCR System w/ Platinum Taq (Invitrogen) was used for RT-PCR reactions. The following primers were used to

amplify transcript in *debcl* and neighboring genes: *rp49* (RT-PCR control) 5'-ATG ACC ATC CGC CCA GCA TAC A -3' and 5'- ACA AAT GTG TAT TCC GAC CAG G -3' , *fmo-2* 5'- ATC AAA ACT TCA GTG GAC AAG CGT CGT GTT TGC -3' and 5'- ATC GTA TAC TTG TTG CTC CTG TAC GTG TCC -3', *debcl* 5'- CCA AGT TCA AGT CCT CGT CGC TGG ACC -3' and 5'- GCG AAT CTA GAA CAG CAG CGA ATA CAG TTG ACC -3', *CG30443* 5'- GAG CTG GAC CAG TTC TAC TGC GAA ATA TGC -3' and 5'- AGC CAA TCT GTA ATA ACT TCC TCG CTG TGG -3', *geminin* 5'- CCA GGG TCT ACA TCC AAG TCG AGA CAG AGG -3' and 5'- TTG ACC TTG TCC TCG TCA CCC GTA GTG TCG -3'.

**Cell Death Assays.** Embryo TUNEL labeling was performed as in (White, Grether et al. 1994) using ApopTag Fluorescein *In Situ* Apoptosis Detection Kit (Chemicon International). Acridine Orange staining was perfomed as described in (Sogame, Kim et al. 2003).

**Fly Strains.** *dark* $^{CD4}$ was meiotically recombined with *debcl* $^{KO}$ to generate double knock-out flies for genetic interaction studies. The following stocks were also used for various analysis: *UAS-mito-GFP* (Cox and Spradling 2003), *pGMR-Gal4*, *da-GAL4,* pGMR-*rpr*, -*hid*, and -*grim4* (stock center).

**3rd Instar Larvae Dissections.** Wandering L3 larvae were dissected as described in (Chew, Akdemir et al. 2004).

**Immunohistochemistry.** Embryos or L3 larval wing discs were collected and treated as in Chew et al., (2004). Primary antibody was incubated overnight at 4°C (1:600 guinea pig α-*Kr*, (Kosman, Small et al. 1998), 1:800 α-*βGal* Ab (Promega), 1:500 α-*dHb9*, 1:1 α-*LBe*, 1:500 α-*Eg* (Rogulja-Ortmann, Luer et al. 2007), 1:200 rabbit α-PH3 (Upstate), 1:50 α-cleaved Caspase 3 (Cell Signaling)). Secondary antibodies from Molecular Probes were used at a 1:500 dilution.

**Microscopy and Imaging.** All embryos and imaginal disc imaging was done on a Zeiss LSM 510 META laser scanning confocal microscope, Leica TCS SP5 spectral confocal, or Zeiss Axioplan 2E digital light microscope. Images were processed using Image J and Adobe Photoshop software. Photographs of fly eyes were taken on a Zeiss SteREO Discovery.V12.

**Notched Wing Assays.** Wandering L3 larvae were treated with 0, 1500 or 2500 Rads of ionizing radiation. Adults with notched wings were scored as a percentage over the total number of adults counted.

**Lysotracker Assays.** Autophagy assays were performed as described in (Rusten, Lindmo et al. 2004). The following modifications were made: fat bodies were stained with LysoTracker Red DND-99 (Molecular Probes) diluted 1:4000 in PBS with 1μM Hoechst for 5-10 minutes. Fat bodies were washed several times in PBS and mounted in 70% glycerol/PBS and visualized immediately. Images were captured using a 40x dry lens. For quantification analysis, 3 fat body lobes from 5 independent animals of each genotype were obtained and imaged. The number of lysotracker-positive structures were quantified from Z-stacked images taken on a Zeiss Axioplan 2E digital light microscope and processed using ImageJ and Adobe Photoshop. A total of 800 sq. pixel area was quantified for each image. These results are expressed as mean values ± std. dev.

**Mitochondrial Assays.** Salivary glands from L3 larvae or pupae carrying the transgenes for *UAS-mito-GFP* and *Da-Gal4* were dissected in PBS and fixed for 15 minutes in 4% Formaldehyde (EM Grade). The salivary glands were mounted in 70% glycerol/PBS, visualized directly by confocal microscopy using a 63x oil objective lens, and imaged with a zoom factor of 5. Two cells per salivary gland were captured for thorough sampling. Quantification of mitochondrial properties was accomplished using Imaris software.

**Results**

*Generation of debcl mutant flies*.

To elucidate the biological function of *debcl*, we produced a null mutation at this locus. We applied ends-out homologous recombination (Gong and Golic 2003) to replace the endogenous *debcl* gene with the *white*$^{+}$ marker gene. The *debcl* gene is located on the right arm of the second chromosome 42C2, 4.0 Kb downstream of *fmo-2*, and 2.7 Kb upstream of *CG30443* (Fig. 1A). We screened 56,000 flies for recombination events and obtained 10 candidate alleles (Fig. 1B, top panel). Seven of these were confirmed for the predicted event by PCR and Southern Blot analysis (Fig. 1B, middle panel). RT-PCR was used to verify the absence of *debcl* transcript from these mutant alleles (Fig. 1B) (data shown for *debcl*$^{22}$). For most studies, a combination of trans-heterozygous individuals were examined. *debcl* $^{KO}$ animals are viable, fertile and exhibit a normal life-span (data not shown). Hence, *debcl* is not essential for survival or fertility. At variable penetrance, (25%-57%), extra scutellar bristles were found on the notum of *debcl* $^{KO}$ adults (Fig. 1C, white arrow). Notably, this extra bristle phenotype also occurs in *dark*, *dredd*, *dronc, dcp-1*, *cyto-c-d, and mir-9a* mutant adults (Chen, Rodriguez et al. 1998; Rodriguez, Oliver et al. 1999; Laundrie, Peterson et al. 2003; Xu, Li et al. 2005; Li, Wang et al. 2006). Other phenotypes, observed at lower penetrance, include rotated male genitalia, imperforate vaginas in females, melanotic-like cells in the maxillary palps, and patterning abnormalities of tergites and sternites.

*debcl impacts PCD and regulates proper cell number in the developing central nervous system.*

*debcl*$^{KO}$ embryos were examined for PCD, and compared to WT, a moderate decrease in the number of TUNEL positive cells was consistently observed (Fig. 2A, B). We also examined *debcl*$^{KO}$ embryos using well-established antibodies which mark cells that normally die in the nervous system, but that fail to die in PCD mutants (Rogulja-Ortmann, Luer et al. 2007). For instance, the number of *Kr*$^{+}$ cells in the ventral nerve cord (VNC) (Fig. 2C-D) was determined and quantification of these cells (Fig. 2E) in abdominal segments A2 and A3 showed no significant difference in cell number. However, extra *Kr*$^{+}$ cells were consistently present in segments A4 and A5 and in Bolwig's organ of *debcl* $^{KO}$ embryos (Fig. 2E-H). We also examined midline glia using two distinct markers for *slit* expressing cells ($\alpha$-*$\beta$gal* and a *slit*-LacZ reporter) and both detected supernumerary *slit*$^{+}$ cells in *debcl* $^{KO}$ embryos (Fig. 2I-L). Quantification of these cells is shown (Fig. 2M). Not all markers, however, exhibited extra cells in *debcl*$^{KO}$ animals. For example, antibodies directed against neuronal transcription factors Lbe, dHb9 and Eg showed wild type cell numbers in all segments (supplemental Figure 1). Likewise, examination of pupal eyes by staining for *discs large* displayed normal loss of interommatidial cells (data not shown).

*debcl is not required for apoptotic cell death in response to genotoxic stress.*

To test a possible role for *debcl* in stress-induced cell death, wing imaginal discs from larvae exposed to ionizing radiation (IR) were stained for apoptotic cells using Acridine Orange (AO). During larval development, low basal levels of PCD exist in the wing imaginal disc (Fig. 3, A-B). Upon treatment with IR, WT and *debcl* $^{KO}$ showed comparable increases in damage-induced cell death (Fig. 3, C-D). Likewise, no discernable difference in effector caspase levels between WT and *debcl* $^{KO}$ tissue was observed (Fig. 3 G, J). To evaluate the effects of IR on viability, larvae were treated with varying doses of IR and scored for survival (Fig. 3M). *debcl*$^{KO}$ animals were slightly compromised for viability after IR challenge, exhibiting a reduced $LD_{50}$ as shown in figure 3M, dashed line. In these experiments, especially at stronger IR doses, *debcl* mutants exhibited far greater tendencies toward some visible defects, scored here in the form of notched wing phenotypes (Fig. 3N).

We also examined irradiated wing imaginal discs for damage-induced checkpoint phenotypes using an anti-phosphohistone H3 antibody to label mitotic cells (Fig. 3H, I). After treatment with IR, proliferative arrest (see Fig. 3K, L), and resumption of proliferation (data not shown) occurred with similar kinetics in WT and *debcl*$^{KO}$ discs. Likewise, no genotype specific differences in wing sizes before and after IR stress were seen and using *mwh* as a loss of heterozygosity

readout, we found no evidence that *debcl* status affected levels of genomic instability (not shown).

*Interaction with other death genes: DEBCL is not required for killing by RPR proteins, but is required for heterologous killing by Murine BAX.*

We tested for genetic interactions between *debcl* and other members of the apoptotic pathway. Using wing blemishing as an indicator of defective PCD (Link, Chen et al. 2007), we found that the incidence of dark wing blemishing was clearly exacerbated in the $debcl^{KO}$, $dark^{CD4}$ double mutant background (Fig. 4E, F) compared to $dark^{CD4}$ (Fig. 4D). For example, $dark^{CD4}$ flies develop progressive blemishes that can be quantified as shown in Figure 4G. Similar positive genetic interactions have also been reported with $dark^{CD4}$, *dp53* (Sogame, Kim et al. 2003) and *dark* $^{CD4}$, $dronc^{\Delta 51}$ (Chew, Akdemir et al. 2004). Adult eyes of $debcl^{KO}$ flies show normal gross patterning (Fig. 5B). Furthermore, eye ablation phenotypes caused by forced expression of the pro-apoptotic genes *grim, rpr* and *hid* were unaffected by $debcl^{KO}$ genotypes (Fig. 5C-H). Hence, in the fly eye, killing activity by IAP antogonists is not dependent on *debcl* function. However, in related studies, we found that heterologous killing by *Bax* was strongly affected by *debcl* status. As previously shown (Gaumer, Guenal et al. 2000) *Bax* provoked an eye ablation phenotype (Fig. 5I). However, these phenotypes were almost completely suppressed in the *debcl* $^{KO}$ background (Fig.

5J-L), indicating that a *debcl*-dependent step is required for heterologous killing by Bax.

*debcl and autophagy*

We examined the possibility that *debcl*, like other Bcl-2 family members (Shimizu, Kanaseki et al. 2004), may play a role in autophagy. Using lysotracker as a marker for starvation-induced autophagy (Munafo and Colombo 2001), we examined larval fat bodies during fed and starved conditions. Autophagy was quantified in larval fat bodies, and, in all trials, no significant differences between WT and $debcl^{KO}$ animals were observed (Fig. 6B). Examples of fat body staining imaged for quantification analysis using lysotracker and Hoechst are shown (Fig. 6C-F). Likewise, WT and $debcl^{KO}$ larvae exhibit similar survival curves when transferred from fed to starved conditions (Fig. 6A), with both genotypes displaying an $LD_{50}$ of approximately 6.5 days after challenge.

*Debcl is a determinant for mitochondrial density.*

We examined the possibility that *debcl*, like other Bcl-2 family members (Karbowski, Norris et al. 2006), may function to specify mitochondrial properties. Using the *Gal4-UAS* system together with a GFP reporter (*mito-GFP*), we visualized mitochondria in salivary gland cells before and after metamorphosis. Mitochondria in $debcl^{KO}$ tissue from L3 larvae (Fig. 7B) appeared more elongated

compared to WT (Fig. 7A), and mitochondrial GFP fluorescence was brighter in *debcl*$^{KO}$ cells (Fig. 7B, D). Using unbiased image analysis software, we found that on a per cell basis, mitochondrial volume was similar (Fig. 7E), but the density of mitochondria per area was substantially increased in *debcl*$^{KO}$ animals (Fig. 7F).

## Discussion

Bcl-2 genes exert pivotal functions that specify PCD (Lindsten, 2000). In worms, the Bcl-2 protein, CED-9, directly regulates PCD by physically interacting with the apoptosome (Conradt and Horvitz 1998). In mammals, PCD signals are thought to funnel through the proapoptotic Bcl-2 members, Bax and Bak, which mobilize cytochrome *c* from mitochondria to promote formation of the apoptosome (Jurgensmeier, Krajewski et al. 1997; Li, Zhu et al. 1998; Luo, Budihardjo et al. 1998; Kluck, Esposti et al. 1999; Wei, Zong et al. 2001). The fly genome encodes two well-conserved Bcl-2 family members, yet in this animal, cytochrome *c* is not required for apoptosome activation (Yu, Wang et al. 2006). Combined, these observations suggest that insect Bcl-2 genes mediate cytochome *c* independent activities that may or may not be related to cell death. To clarify functional roles of this gene family in the *Drosophila* model, we

conducted loss-of-function analyses at Debcl, the fly ortholog of mammalian pro-apoptotic members Bax, Bak and Bok.

Using a targeted recombination strategy, we recovered 7 allellic strains amorphic for *debcl*. Our studies exclude a general requirement for *debcl* as a global apoptotic effector, which had been suggested from analyses of embryos injected with dsRNAs targeting this gene (Colussi, Quinn et al. 2000). Nevertheless, three compelling lines of evidence establish that *debcl* does function to regulate a limited number of developmental cell deaths. First, in every allelic combination tested, TUNEL labeling was consistently and markedly reduced (see Figure 2). Second, in every allele tested, *debcl* genetically interacted with a hypomorphic allele of the apoptosomal gene, Dark. Third, extra cells in *debcl* embryos were detected using the same markers that are commonly applied to visualize persisting or 'undead' cells in canonical PCD mutants (White, Grether et al. 1994; Rodriguez, Chen et al. 2002; Chew, Akdemir et al. 2004; Rogulja-Ortmann, Luer et al. 2007). While the impact caused by eliminating *debcl* was generally modest, we note that the quantified data represents consistent phenotypes observed in multiple alleles. Furthermore, these data represent a census that includes all positively marked cells, where only a small fraction of cells surveyed in the CNS (~25%) actually die (Rogulja-Ortmann, Luer et al. 2007). Hence, if we account for only cells that are eliminated, and compare these in isolation against the benchmark seen in animals defective for all PCD (e.g. H99

mutants), the effects caused by eliminating *debcl* are clearly significant *(If we say this, we need to show statistics, and they may weaken our argument)*. For example, in *H99* animals where no PCD is observed, a 37% excess of *Kr*+ cells is seen in Bolwig's organ (Link, Chen et al. 2007). By comparison, an excess of up to 23% *Kr*+ cells were seen in *debcl* mutants studied here (Figure 2). Differential effects along the anterior-posterior axis was another familiar phenotype detected here and was also previously reported for supernumerary motoneurons in *H99* mutants (Rogulja-Ortmann, Luer et al. 2007). Specifically, in cases where extra neuronal cells were observed, these cells tended to appear more commonly among the posterior segments *(*e.g. anti-*Kruppel,* see Figure 2), where this trend toward extra cells along the AP axis was not seen in populations of glia. In *debcl* $^{KO}$ animals, segments with extra cells averaged 17% excessive cells and, by comparison, mutations in apoptosomal genes, *dark* and *dronc* produced a range of 33-50% excessive cell numbers (Rodriguez, Chen et al. 2002; Chew, Akdemir et al. 2004). Other common examples of *Drosophila* PCD examined were notably unaffected by *debcl* status (e.g. interommatidial cells in the eye, salivary gland, $Cz^{+}$ neurons during the larval to pupal transition) and, to the extent represented by markers studied here, the impact of *debcl* status on PCD appears limited to certain neurons and glia. These combined studies establish a limited role for this gene in certain cell types and, at the same time, exclude a global requirement for *debcl* in *Drosophila* PCD.

Recently, Sevrioukov et al (2007) studied a *debcl*-associated mutation and, in agreement with this report, we found no evidence linking the action of *debcl* to PCD in the salivary gland or the pupal eye. However, in contrast to Sevrioukov et al (2007) we do find evidence linking *debcl* to PCD control in the embryonic CNS. Discrepancies between our respective analyses probably trace to at least two sources. First, the mutation studied by Sevrioukov et al (2007) likely corresponds to a hypomorphic allele. Their allele, $debcl^{E26}$, leaves at least half of the 5' UTR and the entire *debcl* open reading frame fully intact, and hence is unlikely to reflect the null condition (Sevrioukov, Burr et al. 2007). A second source of discrepancy can be traced to different sets of markers that were selected for analysis. For studies presented here, we chose well-established markers with precedents for detecting extra cells in *canonical PCD mutants, e.g. H99, dronc, dark* (Rodriguez, Chen et al. 2002; Chew, Akdemir et al. 2004; Rogulja-Ortmann, Luer et al. 2007). In contrast, Sevrioukov et al (2007) applied markers that highlight the pattern of axonal bundles ($Mab^+$ cells) and a glial cell marker ($repo^+$ cells), both of which were found to be unperturbed in *H99* mutants (Rogulja-Ortmann, Luer et al. 2007).

Studies here and in supplemental materials carefully examined other cellular processes in addition to PCD. *debcl* mutants showed normal levels of starvation-induced autophagy and were, likewise, unperturbed for radiation stress responses, including cell cycle arrest, and loss-of-heterozygosity post challenge.

Interestingly, however, we did find elevated levels of notch-wing phenotypes in *debcl* $^{KO}$ adults after IR treatment, and while the cellular basis for this observation is not clear, we suggest that *debcl* may be recruited to promote effective compensation when development is perturbed (Ryoo, Gorenc et al. 2004).

In a series of provocative studies, we also found that heterologous killing by Bax required normal Debcl function. In a wild type background, expression of *Murine* Bax in the eye disc kills retinal cells, producing a severely ablated eye condition. However, this phenotype is almost completely reversed in the absence of *debcl*. This observation that heterologous killing by Bax requires *debcl* to exert its proapoptotic activity suggests that Bax is engaging a pathway in flies that is normally inactive. Moreover, this specific requirement for *debcl* negates the notion that Bax killing in the fly is a result of general toxicity. Given that heterologous killing by Bax is not suppressible by p35 (Gaumer, Guenal et al. 2000) also suggests that Bax likely initiates a pathway through the mitochondria that is independent of caspases. These observations suggests that flies possess the machinery capable of exerting mitochondrial-dependent killing, but it requires the mammalian orthologue in a debcl-dependent fashion to become activated. Taken together, these observations strongly support the ancestral role of Bcl-2's in mitochondrial remodeling, and that this gene family evolved to engage mitochondria in the amplification of the death signal. Because mitochondrial remodeling and initiation of apoptosis are so closely intertwined in mammals, it is

difficult to isolate these two pathways in isolation. *Drosophila* now present a unique model to study modes of mitochondrial Bax regulation that are caspase-independent.

In numerous models of apoptosis, including flies, mitochondria are remodeled in association with PCD. In addition to apoptotic regulation, Bax and Bak also function to control mitochondrial remodeling (i.e. fusion and fission); however, whether this process is responsible for transducing the apoptotic signal remains elusive. Whereas Bax and Bak may indirectly regulate cell death by regulating mitochondrial remodeling, a topic that remains controversial, the worm Bcl-2 family members function to regulate PCD through direct physical interaction with the apoptosome (a process that does not require cytochrome *c* for its activation). The worm Bcl-2 relative, CED-9, promotes mitochondrial remodeling in mammalian cells and is suppressible by EGL-1 (Delivani, Adrain et al. 2006), however, CED-9 failed to block mitochondrial cytochrome *c* release or apoptosis in these cells when induced by various apoptotic stimuli. Likewise, expression of Egl-1 in mammalian cells failed to transduce the apoptotic signal (Delivani, Adrain et al. 2006). This suggests that sometime during evolution, the family of Bcl-2 proteins adapted the involvement of the mitochondria in perpetuating of the death signal. In *Drosophila* cells, mitochondria undergo fragmentation in response to apoptosis (Abdelwahid, Yokokura et al. 2007; Goyal, Fell et al. 2007) and Debcl overexpression in *Drosophila* and in

mammalian cells results in mitochondrial fragmentation (Igaki, Kanuka et al. 2000). Collectively, these data suggest a possible ancestral role for Drosophila Bcl-2's in the regulation of mitochondrial properties. Moreover, our studies suggest that heterologous killing by Bax requires Debcl to exert mitochondrial-dependent killing.

We investigated the impact of debcl on regulating mitochondrial properties in the larval salivary gland. We visualized mitochondrial populations (e.g. fragmented vs. tubular) in WT and *debcl* $^{KO}$ cells. In WT cells, both fragmented and elongated mitochondria are visible (see Figure 7). Initial observations suggested a higher population of elongated/tubular mitochondria in *debcl* $^{KO}$ cells compared WT. To quantify this observation, we used non-biased software analysis to measure various mitochondrial parameters, which consistently revealed a statistically significant increase in the mitochondrial density in *debcl* $^{KO}$ cells compared to WT (see Figure 7). This increase in mitochondrial density likely reflects the observed increase in elongated mitochondria. These results place *debcl* in the pathway that regulates mitochondrial properties, and suggests that *debcl* impacts mitochondrial fussion/fision. We also observed mitochondrial fragmentation during histolysis of the pupal salivary gland, supporting a change in mitochondrial dynamics linked to PCD (see Figure 7). These observations suggest that *debcl* impacts mitochondrial homeostasis, but is not required for PCD-induced mitochondrial fragmentation.

Together, these data also support an ancestral role for Bcl-2 genes in the regulation of mitochondrial dynamics across species. However, given that *debcl* does not exert a global role in PCD, nor do flies emphasize mitochondrial-dependent apoptosis, suggests that flies either evolved to deemphasize the Bcl-2's in PCD, or that mammals evolved to place a large emphasis on this gene family during apoptosis (Cory and Adams 2002; Danial and Korsmeyer 2004; Karbowski, Norris et al. 2006). This evidence also suggests that mammals evolved to engage mitochondria in the PCD pathway, a pathway that is present in flies, but otherwise inactive. Nevertheless, this is the first example of a *Drosophila* Bcl-2 family member that impacts developmental PCD, as well as mitochondrial properties, but whether this regulation of mitochondrial properties is upstream or downstream of apoptosis remains unclear. Together, these data support an ancestral role for Bcl-2 proteins involved in the regulation of mitochondrial properties and developmental PCD, but further studies will be needed to carefully dissect the mechanism of how *Drosophila* Bcl-2 proteins regulate these distinct pathways.

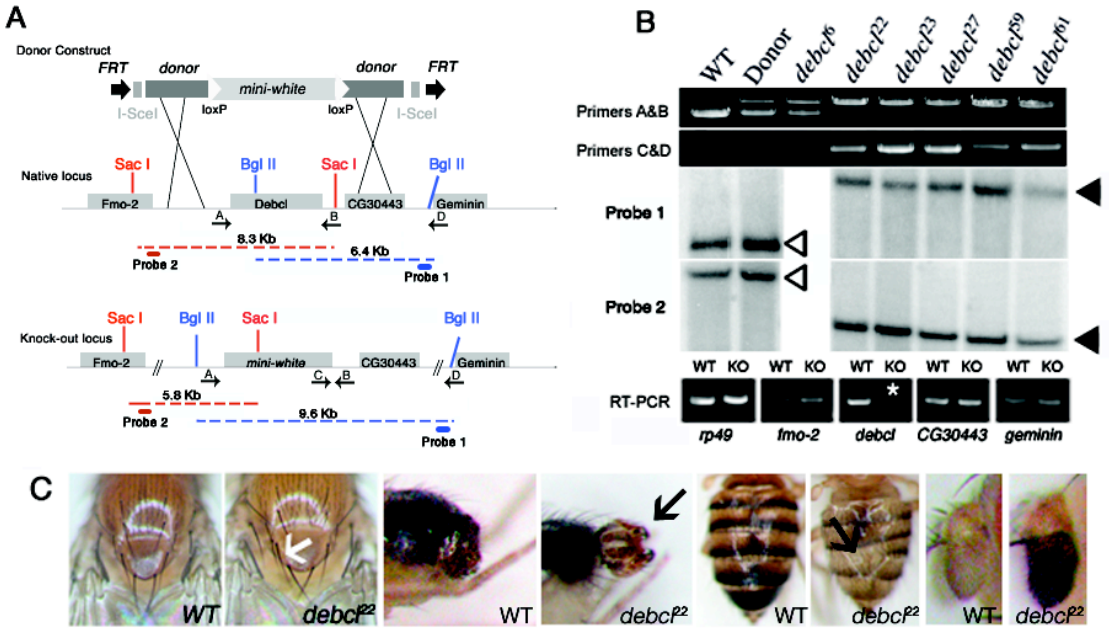



Figure 1. Generation and verification of a targeted *debcl* mutation. (A) Targeting scheme for the *debcl* gene. The donor construct was generated by insertional cloning of a 4Kb upstream genomic sequence of the *debcl* gene and a 2.7Kb downstream genomic sequence of the *debcl* gene into the targeting vector pW25. Upon chromosomal targeting, the *debcl* native gene is replaced with a *white*$^{+}$ marker gene. (B) Verification of the *debcl*-targeting event by PCR, Southern blot, and RT-PCR analysis. Primer pair A&B were used to amplify both the native and knock-out locus to confirm replacement of *debcl* (4.2Kb) with the white$^{+}$ marker gene (~5.2Kb). Primer pair C&D were used to verify the right arm of recombination during the screening process for potential recombinants. Primer pair E&F were used to confirm targeted recombination of the left arm of recombination (data not shown). WT refers to the *yw* parental strain, wild-type at the *debcl* locus on the second chromosome; Donor refers to the *debcl* donor construct on the third chromosome, wild-type at the *debcl* native locus on the second chromosome; debcl$^{6,22,23,27,59,61}$ represent different *debcl* knock-out alleles disrupted at the native locus. An additional *debcl* disruption allele, *debcl*$^{47/48}$, was also confirmed (data not shown). Southern blot analysis using genomic DNA from the indicated fly strains was used to confirm both the right (SBP1) and left (SBP2) arm of recombination. For SBP1 (Bgl II digest) and SBP2 (Sac I digest), the black arrow indicates the expected 6.4 Kb and 8.3 Kb genomic fragment in the WT and donor strains for SBP1 and SBP2, respectively. The arrowhead indicates

an aberrant genomic fragment of 9.6 Kb and 5.8 Kb indicative of gene-targeted replacement for SBP1 and SBP2, respectively. RNA from L3 larvae was used as a template to confirm abolishment of the *debcl* transcript in *debcl*$^{KO}$ flies, and confirm that transcript levels are unaffected in the neighboring genes *fmo-2*, *CG30443* and *geminin*. *rp49* was used as a control for RT-PCR. The white asterisk indicates the absence of *debcl* transcript from mutant flies. (C) Photograph of extra scutellar bristles in *debcl*$^{KO}$ flies (white arrow), rotated male genitalia, (black arrow), malformed sternites (black arrow), and dark masses in the maxillary palp.

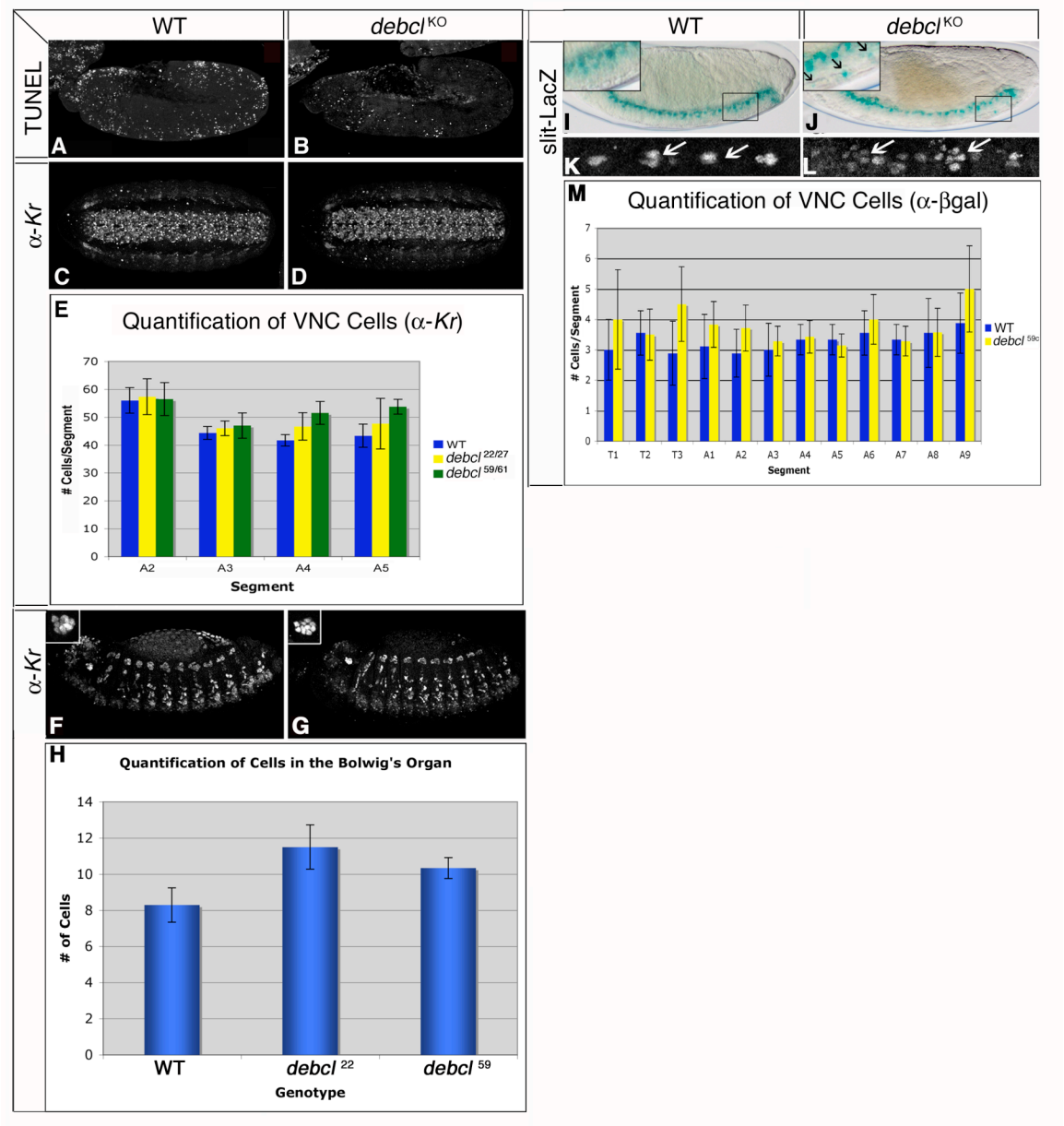



Figure 2. *debcl* plays a marginal role in developmental Programmed Cell Death. TUNEL staining is moderately reduced in *debcl* mutant embryos (B) (multiple alleles tested, $debcl^{22/27}$ shown) compared to WT (yw) embryos (A) (stage 11). The presence of extra cells during embryonic development was examined by anti-*Kr* (C-F) and *slit*-LacZ staining (I-L). Quantification of extra cells in the VNC by anti-*Kr* (E). Error bars show ± the standard deviation (n=6). Quantification of

cells in Bolwig's Organ (H). Error bars show ± the standard deviation (n=8). Quantification of VNC cells by anti-*βGal* staining (M). Error bars show ± standard deviation (n=8).

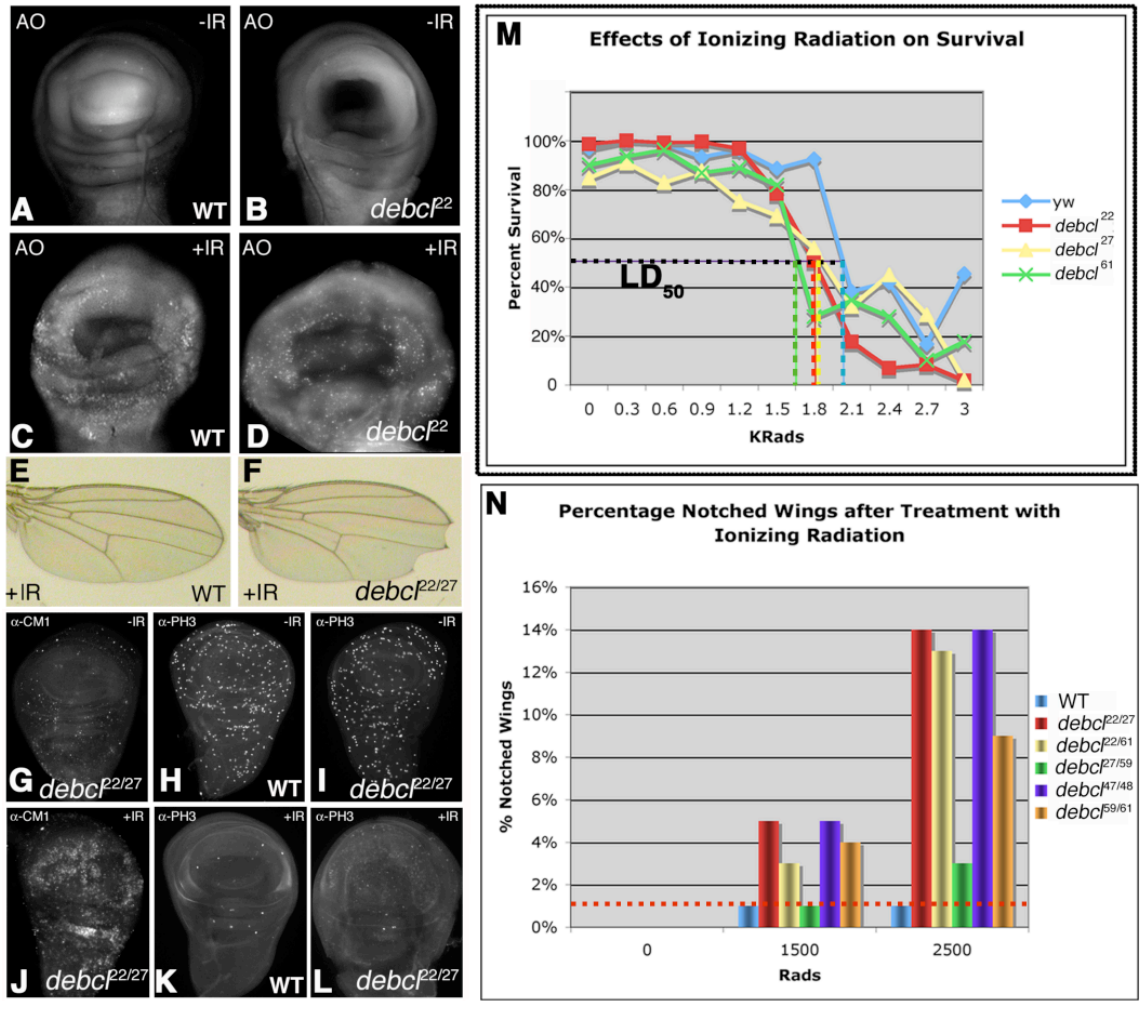



Figure 3. *debcl* plays a limited role in stress-induced cell death. Basal levels of cell death in the larval wing imaginal disc (A, B). After 4000 Rads of ionizing radiation (IR), robust cell death is observed after 4 hours (C, D). Adult survival curve after treatment with IR (M). The corresponding $LD_{50}$ is shown for each sample. Cell proliferation in the larval L3 wing imaginal disc was examined before (H, I) and after (K, L) treatment with 4000 Rads of IR labeled by phosphohistone H3 staining. Effector caspase levels labeled by anti-proCaspase3 staining (G) drastically increase after IR challenge in *debcl*$^{22/27}$ tissue (J) (WT not shown). Photograph of a wing from a WT (E) and *debcl*$^{22/27}$ (F) adult after challenge showing a notched wing phenotype in the *debcl* mutant. (N) Histogram

illustrating the penetrance of notched wings in WT (*yw*) and trans-heterozygous *debcl* mutant alleles at 0, 1500 and 2500 doses of IR.

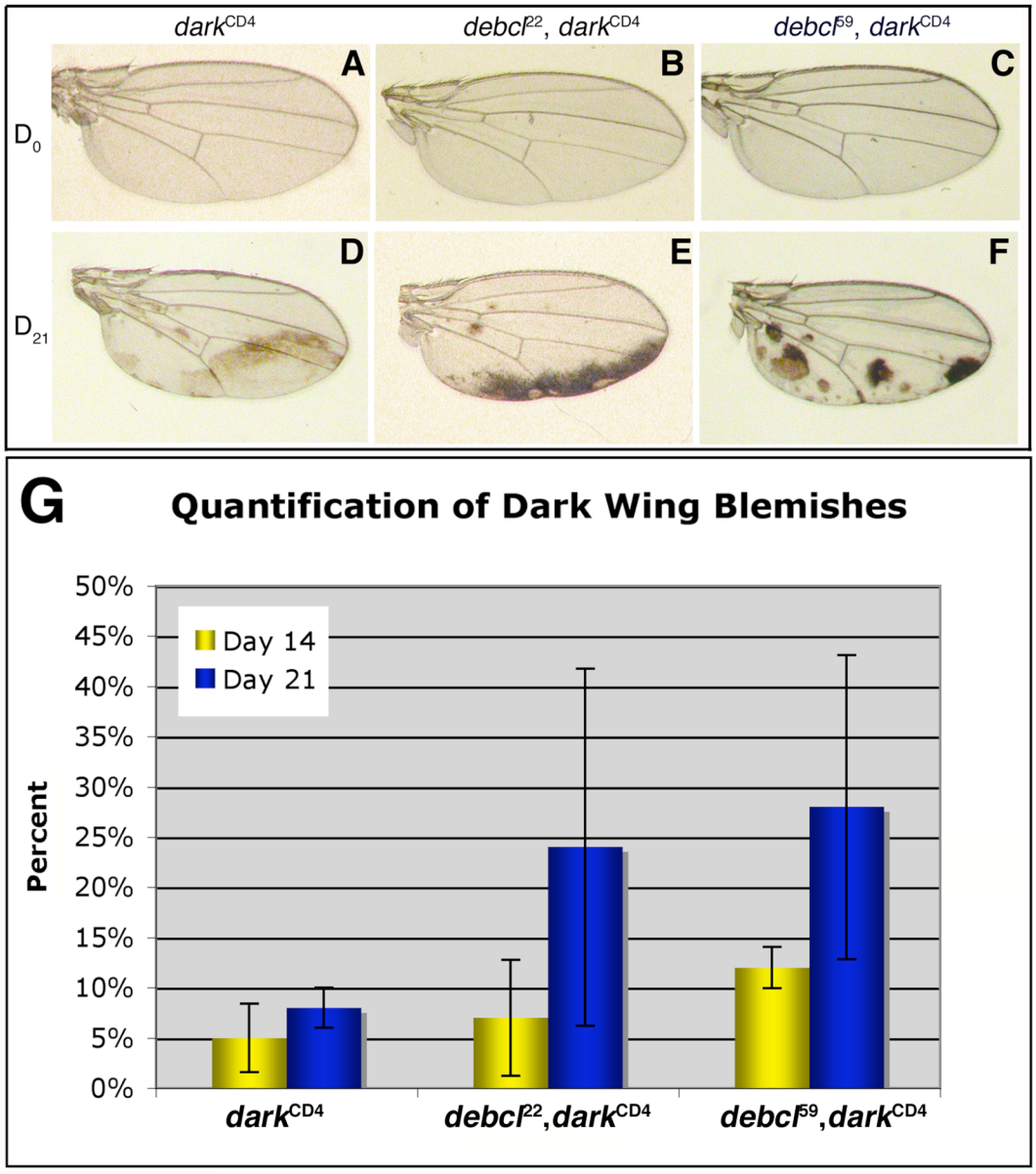



Figure 4. *debcl* genetically interacts with *dark*. (A-C) Adult wings are normal in morphology and appearance at the time of eclosion ($D_0$). (D-F) Wing blemishes are progressive in nature and more severe in *debcl*, $dark^{CD4}$ double mutants (E,F)

compared to *dark*$^{CD4}$ alone (D). Wings shown are 3 weeks old ($D_{21}$) (D-F). Histogram showing quantification of dark wing blemishing (G).

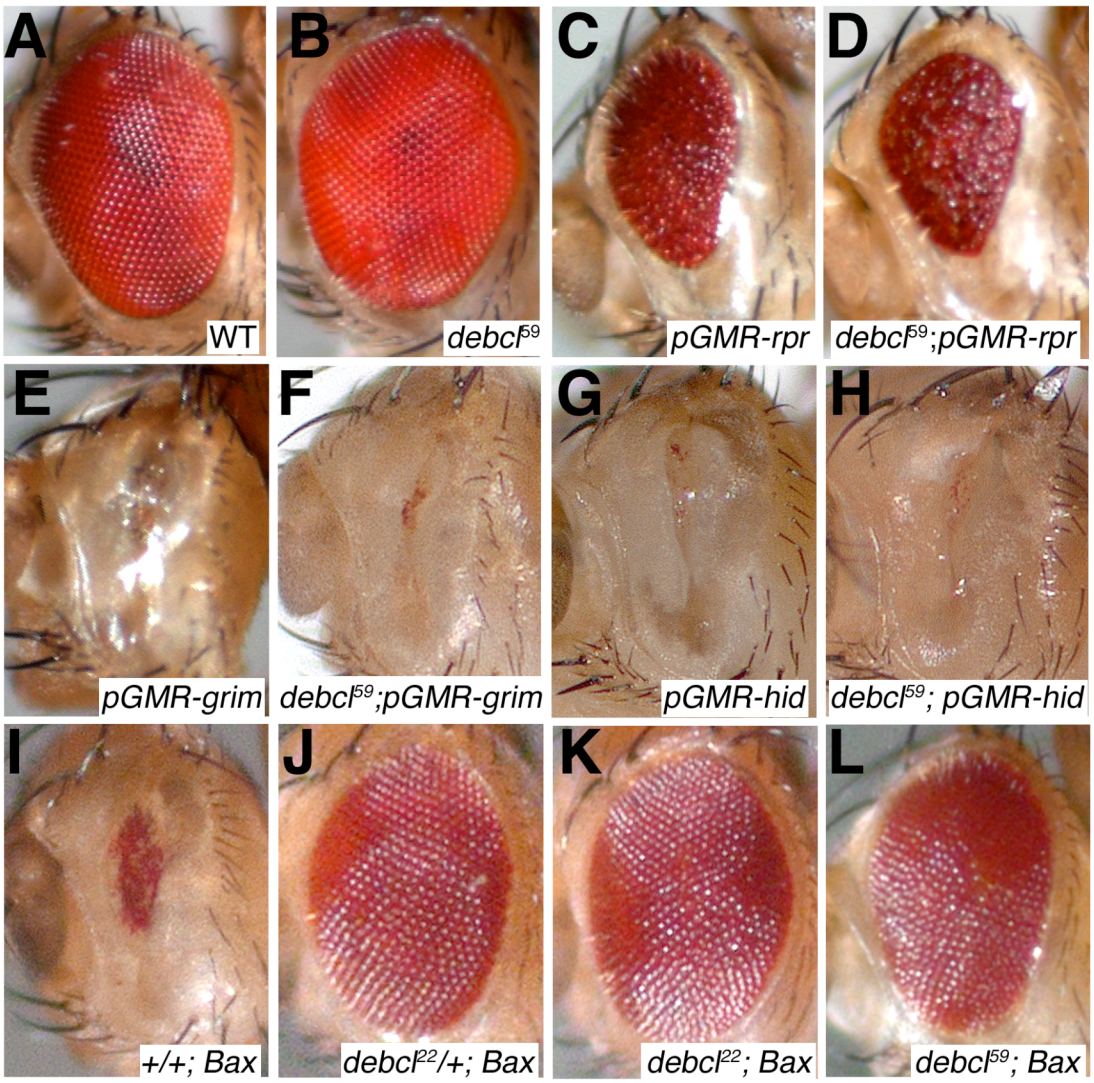
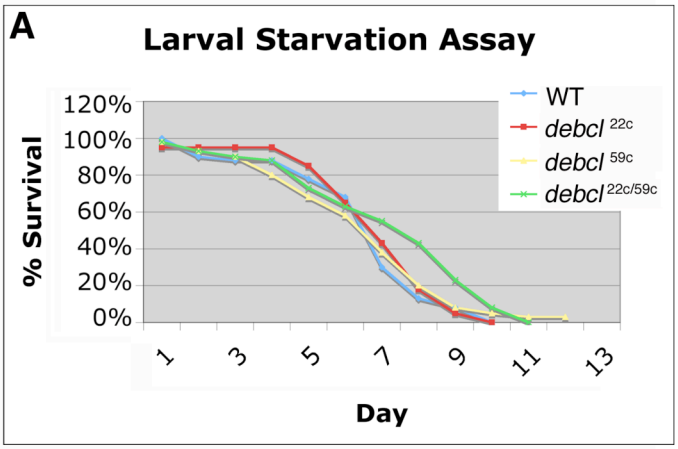



Figure 5. Ectopic expression of pro-apoptotic genes in *debcl* mutant animals. Photograph of a morphologically normal eye in WT (A) and *debcl*$^{59}$ (B). Killing by the RGH proteins is not dependent on *debcl* function. Photograph of pGMR-*rpr* (C, D), pGMR-*grim4* (E, F) and pGMR-*hid* (G, H) in the eye showing no effect on the ablated eye phenotype. (I) Photograph of *UAS-Bax1*, *GMR-Gal4*

showing severe ablation of the eye in the WT background. This severe eye phenotype is nearly completely reversed in the heterozygous *debcl*$^{22}$/+; *UAS-Bax1*, *GMR-Gal4* (J) mutant background, as well as in the homozygous *debcl*$^{22}$*; UAS-Bax1, GMR-Gal4* (K) and *debcl*$^{59}$; *UAS-Bax1, GMR-Gal4* mutant backgrounds (L).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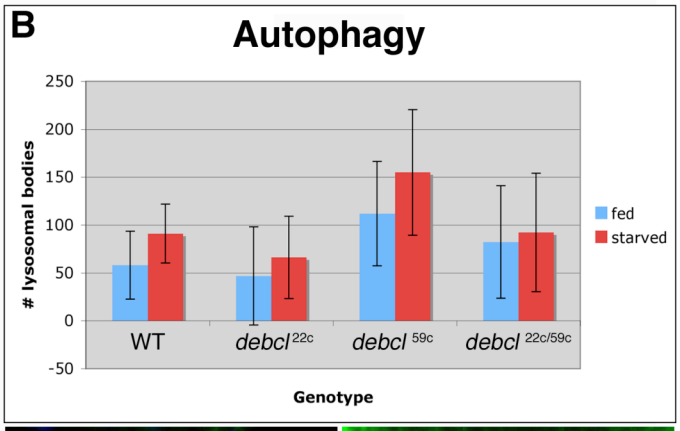
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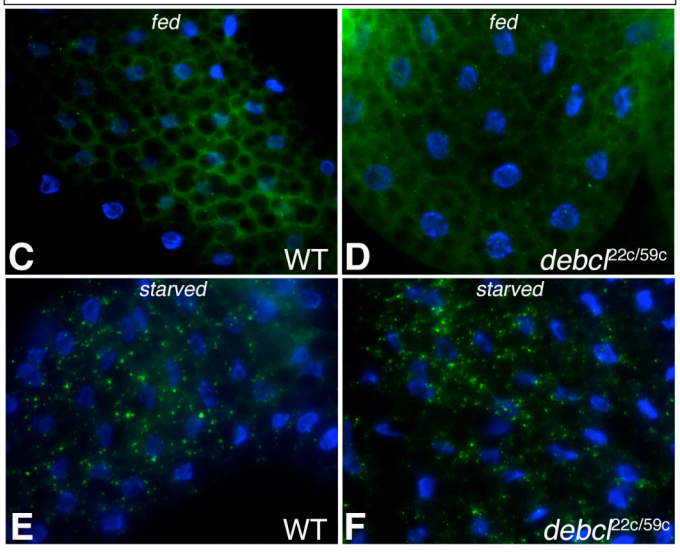
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Figure 6. *debcl* mutants display normal autophagy under starved conditions. (A) Histogram illustrating the survival curve of L2 larvae when placed under starved conditions (20% sucrose). Fat bodies of young L3 larvae were quantified for autophagic bodies under fed (wet yeast) and starved (20% sucrose) conditions using lysotracker as a marker for autophagy. Error bars show ± the standard deviation (n=5). (C-F) Photographs representing live fat bodies stained with Hoechst (blue) and lysotracker (green).

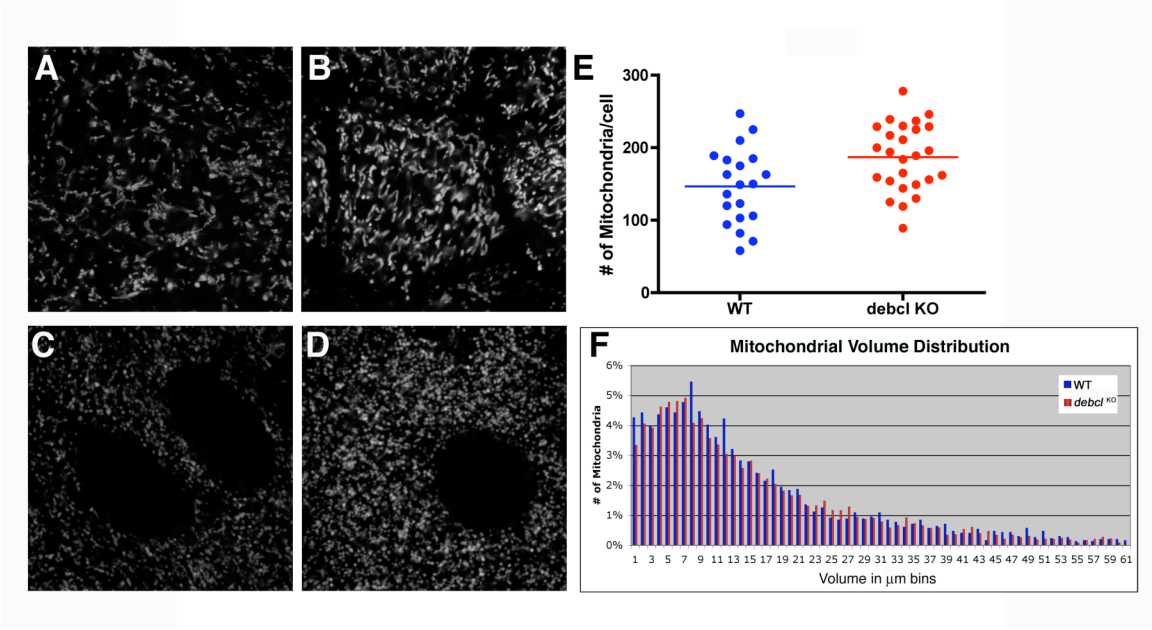
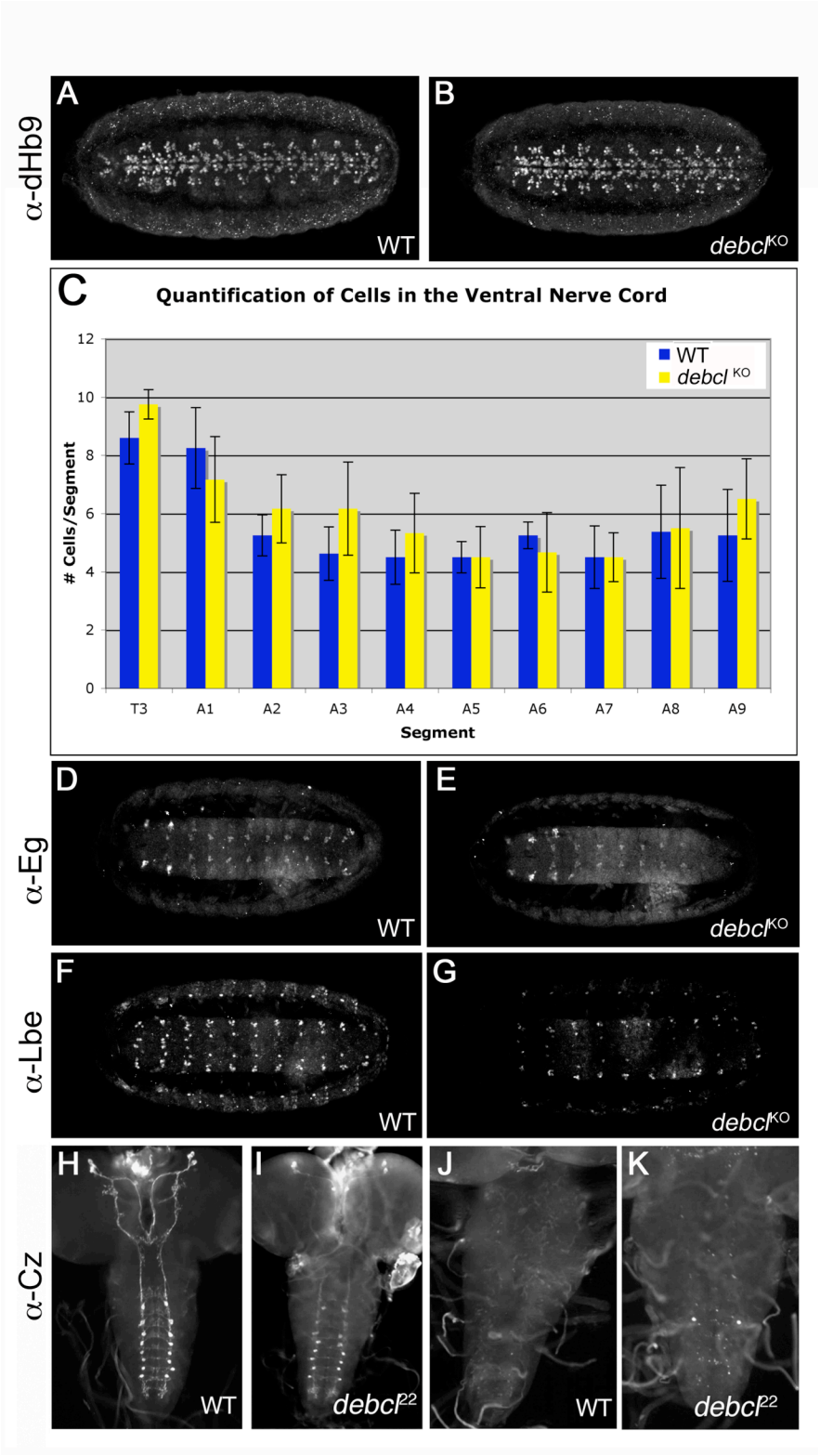



Figure 7. *debcl* plays a role in regulating mitochondrial density. Images of WT (A) and *debcl*$^{KO}$ (B) mitochondria (*UAS-mito-GFP; Da-Gal4*) in cells of the L3 salivary gland. Mitochondria from pupal salivary glands ~12 hrs APF are fragmented, with brighter GFP fluorescence in *debcl* $^{KO}$ cells (C) compared to WT cells (D). The increase in the mitochondria/area in *debcl* $^{KO}$ animals is statistically significant (E) (student t-test, p=0.0139; WT n=20, *debcl*$^{KO}$ n=26). The distribution of mitochondrial volume is comparable between WT and *debcl*$^{KO}$ animals (F).


α-dHb9
A
WT
B
debcl KO
C
Quantification of Cells in the Ventral Nerve Cord
WT
debcl KO
# Cells/Segment
0
2
4
6
8
10
12
T3
A1
A2
A3
A4
A5
A6
A7
A8
A9
Segment
α-Eg
D
WT
E
debcl KO
α-Lbe
F
WT
G
debcl KO
α-Cz
H
WT
I
debcl 22
J
WT
K
debcl 22

Supplemental Fig. 1. PCD of specific neuronal cells is unaffected by *debcl* status. Cell numbers in the VNC by α-*dHb9* antibody exhibit similar cell counts between *debcl* $^{KO}$ and WT (A-C). Cell numbers are similar by α-*Eg* and α-*Lbe* staining (D-G). PCD of cells in pupal CNS occur at similar rates (H-K) (L3 CNS H, I; 7hrs APF J,K)

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## CHAPTER THREE
## GENERATION OF LOSS-OF-FUNCTION MUTANTS IN DRICE

### Introduction

The family of caspase proteases play critical roles in the execution of PCD. A large body of evidence implicates the apical caspase Dronc as an important cell death activator that is recruited and cleaved by Dark in the formation of the apoptosome (Hay and Guo 2006). Dronc then cleaves and activates the two effector caspases Dcp-1 and Drice (Hawkins, Yoo et al. 2000; Meier, Silke et al. 2000; Muro, Hay et al. 2002). Dcp-1 and Drice share a high degree of sequence similarity and are most homologous to the mammalian effectors caspase-3 and caspase-7. Animals homozygous for a null allele in *dcp-1* were overall healthy, showing only mild defects in starvation-induced cell death during oogenesis (Laundrie, Peterson et al. 2003). Lines of evidence suggesting that Drice may be an important cell death effector included immunodepletion of drice from S2 cells resulting in inhibition of apoptosis in response to many stimuli (Fraser, Mccarthy et al. 1997; Muro, Hay et al. 2002; Muro, Monser et al. 2004). Additionally, antibodies that recognize cleaved Drice label dying cells during development (Yoo, Huh et al. 2002; Yu, Yoo et al. 2002) and when exposed to various death stimuli (Huh, Vernooy et al. 2004; Perez-Garijo, Martin et al. 2004). Based on this evidence, it was likely that Drice acted as a pivotal player in the execution of PCD; therefore, we sought to generate *drice* null animals. At the

time I started this project, there were no existing P-elements in the neighborhood of *drice* to employ P-element mobilization strategy for gene disruption. Therefore, we designed a knock-out strategy using ends-out homologous recombination.

**Materials and Methods**

**Ends-Out Donor Constructs.** Genomic DNA from *yw* flies was used as template for all PCR reactions. Primers 5'- ttg aga aaa tgg ctc taa tgt gaa aat gcg -3' and 5'-gcc tgc gaa aac tga tga gtc aca agc act -3', were used to PCR amplify the 5.7kb upstream flanking sequence to clone into the respective *Not*I and *Acc*651 sites in the pw25 donor plasmid. Primers 5'- ata aac caa gtc gca att aag tta gaa cc -3' and 5'-aac tcc aca gtt tca gaa att atg ttt cc -3' were used to PCR amplify the 2.3kb downstream flanking sequence to clone into the respective *Asc*I and *Bsi*WI pw25 vector sites. All constructs were transformed into the germ line of *Drosophila melanogaster* by using standard methods (Rubin and Spradling 1982).

**Genetics and Heat Shocks.** Crosses for targeted recombination were performed in standard 25-mm-diameter vials, each carrying 3-5 females per vial and a corresponding number of the appropriate males. Males carrying the donor construct were crossed to virgin females carrying the heat-inducible (70Flp)(70 I-

Sce I)/CyO. The adults were removed a couple days after egg laying and heat shocks were performed in a circulating water bath at 38$^{o}$C for 90 min, and repeated for 3 consecutive days. A total of 100 vials were used for heat shock. Mosaic-eyed females were crossed *en mass* to males carrying the constitutively active P{70Flp} (stock center 6938).

**Verification of *drice* $^{KO}$ alleles.**

**Southern Blotting.** For verification of targeting, genomic DNA was prepared from flies carrying the candidate targeted allele using Wizard Genomic Purification Kit (Promega). Genomic DNAs were digested as indicated (Fig. 8A), separated by 0.8% agarose gel electrophoresis, and transferred to positively charged nylon membranes. The membranes were probed with $P^{32}$ dCTP-labeled DNA fragment from PCR product using primers 5'- cgt aaa cac gga ctt tcc cca gaa cga agc - 3' and 5'- ata aaa cgg cat gtg gca cca atc tcg tcg -3' for verification of the left recombination arm and primers 5'- att cca atg ccc tgc atc agc aag -3' and 5'- aag cta tct cac ttg gct ccc act -3' for verification of the right recombination arm, and hybridized using Express Hyb Hybridization Solution (BD Biosciences).

**PCR and RT-PCR.** PCR strategy was used to obtain candidate targeted recombination alleles. The primers 5'- GGT TAT CAT ACC ATT CCT GCT

CTT TGG -3' and 5'- cgt tat ttt att tgt atg gga caa gga tgc-3' were used to amplify a PCR fragment unique to the right-arm of recombination (data not shown).

**Results and Discussion**

Obtaining *drice* $^{KO}$ flies proved challenging. During the first attempt to achieve targeted recombination, we screened ~26,000 flies and obtained 34 candidate recombination events based on chromosomal mapping. These flies were further screened by PCR to verify the right arm of recombination, and 10 alleles produced the proper PCR product. Unfortunately, all 10 alleles failed to pass the RT-PCR test, which resulted in a *drice* specific product in the homozygous mutant condition (verified by sequencing). Next, we repeated the screen using different donor flies and scaled up the number of flies that were screened. We screened ~50,000 flies and obtained 50 putative targeted recombinants based on chromosomal mapping. We used southern blot analysis to confirm targeted recombination on both the right arm (Hind III digest) and left arm (Sac II digest) of recombination. Three alleles were candidates for targeted recombination on both arms. I stripped and reprobed the Hind III digest blots with a *drice* ORF probe since these alleles were homozygous viable. Unfortunately, all 3 alleles were positive for the *drice* ORF. Possible explanations for this result include an unexplained recombination event such as a

tandem duplication or possible excision of *debcl* and then reinsertion into another locus.

While preparing to use a PCR strategy to examine the nature of the recombinant alleles, Bruce Hay's group published their own null allele in *drice* (Senoo-Matsuda, Igaki et al. 2005). They used a P-element excision strategy using a P-element strain from a Japanese company. At this point, we did not pursue characterization of our candidate alleles.

Results from Hay's work demonstrated that *drice* null animals showed 80% lethality during puparium formation. However, animals doubly deficient for *drice* and *dcp-1* were 100% lethal, showing some redundancy. Homozygous *drice* null adults that survived showed minor phenotypes, including rotated male genitalia, also seen in *hid* mutants. Adults had opaque wings and extra interommatidial cells. *drice* $^{KO}$ embryos also showed a modest reduction in TUNEL positive cells in the developing embryo and extra midline glia were detected. Additionally, *drice* is required for cell death upon irradiation challenge. Killing by RPR proteins requires *drice*, and lastly, *drice* $^{KO}$ testis showed a partial failure in spermatid individualization. Collectively, these data implicate a role for *drice* in developmental and stress-induced apoptosis.

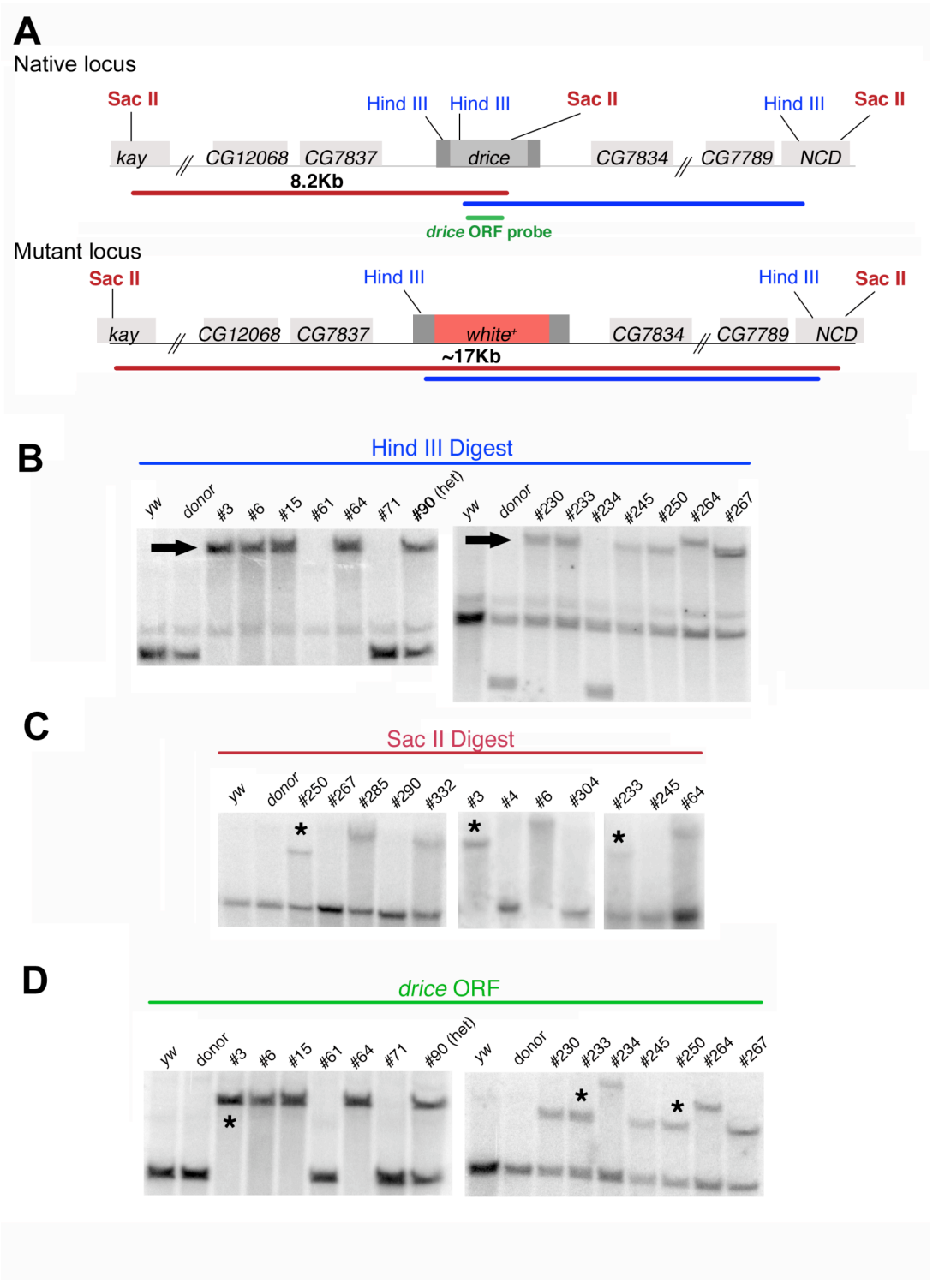
A
Native locus
Sac II
Hind III
Hind III
Sac II
Hind III
Sac II
kay
CG12068
CG7837
drice
CG7834
CG7789
NCD
8.2Kb
drice ORF probe
Mutant locus
Sac II
Hind III
Hind III
Sac II
kay
CG12068
CG7837
white+
CG7834
CG7789
NCD
~17Kb
B
Hind III Digest
yw
donor
#3
#6
#15
#61
#64
#71
#90 (het)
yw
donor
#230
#233
#234
#245
#250
#264
#267
C
Sac II Digest
yw
donor
#250
#267
#285
#290
#332
#3
#4
#6
#304
#233
#245
#64
D
drice ORF
yw
donor
#3
#6
#15
#61
#64
#71
#90 (het)
yw
donor
#230
#233
#234
#245
#250
#264
#267

Figure 8. Schematic of *drice* targeted recombination. (A) Native locus of *drice* with corresponding Sac II (red) and Hind III (blue) restriction digest sites for southern blot verification. Probe for *drice* ORF (green) to confirm abolishment of *drice* gene product. After targeted recombination, the mutant locus has a *white*$^{+}$ marker gene in place of *drice*. Corresponding Sac II (red) and Hind III (blue) restriction digest sites for southern blot analyses. (B) Southern blot verification by Hind III digest. Black arrows show bands of predicted recombination size. (C) Southern blot verification by Sac II digest. Black asterisks represent bands of targeted recombination size (~17Kb). (D) Southern blot using a probe specific to the *drice* ORF (green) to verify the abolishment of *drice* gene product. Black asterisks are alleles that were candidates for targeted recombination based on Sac II digest results (C).

# CHAPTER FOUR
# ANALYSIS OF *DROSOPHILA AIF* MUTANTS DURING DEVELOPMENT

## Introduction

Apoptosis-inducing factor (AIF) is a mitochondrial protein that plays roles in both the execution of cell death as well as in the maintenance of mitochondrial energy homeostasis (Cande, Cecconi et al. 2002; Lipton and Bossy-Wetzel 2002). It was originally identified from a biochemical assay where *AIF* was observed to induce apopotic killing (Susin, Lorenzo et al. 1999). In *C. elegans*, knock-down of the AIF ortholog, WAH-1, results in decreased cell death (Wang, Yang et al. 2002), and in yeast, *AIF* $^{KO}$ results in decreased cell death in response to oxidative stress and in response to aging (Wissing, Ludovico et al. 2004). Currently, the role of mitochondria in cell death remains a topic of hot debate. In mammals, cell death is executed when there is a loss in MOM potential, resulting in the release of mitochondrial factors into the cytoplasm (i.e. AIF, Endo G, Smac/Diablo and cytochrome *c*) (Green and Reed 1998; Youle and Karbowski 2005). Recent reports support a role for mitochondrial remodeling during *Drosophila* apoptosis (Abdelwahid, Yokokura et al. 2007; Goyal, Fell et al. 2007), where the only cytochrome *c* caspase-related role seems to be related to spermatid individualization (Arama, Bader et al. 2006). Characterization of the *Drosophila AIF* ortholog had not yet been accomplished. A collaboration between Guido

Kroemer's and Josef Penninger's groups generated a *DmAIF*$^{KO}$ allele by ends-in homologous recombination and showed that *DmAIF*$^{KO}$ larvae displayed severe growth retardation and are larval lethal, although organogenesis appeared normal. Enzymatic activity of respiratory chain complexes I and IV and ATP levels were severely reduced in *DmAIF* mutants and missexpression of *DmAIF* results in severe eye ablation, which is caspase-independent.

One critical piece of data missing in these studies was a direct link between *DmAIF* and cell death. As a collaborative effort between our group and Kroemer and Penninger's group, I obtained these mutant flies and analyzed them for defects in developmental PCD.

## Materials and Methods

### Immunohistochemistry and Cell Counts

Embryos were collected and treated by Acridine Orange (AO) staining as described in Chew et al, 2005. Primary antibody staining was carried out at 4$^{o}$C overnight (1:600 guinea pig α-*Kr* (Kosman, Small et al. 1998), 1:500 α-*dHb9* antibody (Rogulja-Ortmann, Luer et al. 2007). The labeling was visualized using fluorocrome labeled secondary antibodies 1:500 (Vector Laboratories). Embryos were imaged by confocal microscopy (Leica TCS SP5) and cell counts were quantified using ImageJ software. All the cells per segment of the Ventral Nerve Cord (VNC) labeled against α-*Kr* were quantified, and only the lateral VNC cells

peripheral to the horizontal lines labeled against α-*dHb9* antibody were quantified (yellow brackets). Error bars represent ± std. dev.

**Results**

*DmAIF Impacts Developmental PCD and Regulates Proper Cell Number in the CNS.*

We analyzed developmental programmed cell death (PCD) in mid-stage embryos by acridine orange staining. *dmAIF* $^{KO}$ embryos have lower incidence of developmental PCD (Fig. 9 B, D) compared to WT (Fig. 9 A, C) embryos (stage 12). Prompted by this finding, we investigated the pattern and persistence of extra cells in the central nervous system (CNS) using different neuronal markers. Although the embryonic CNS appears grossly normal, we found consistent evidence for supernumerary $Kr^{+}$ cells in the VNC and Bolwig's organ in *dmAIF* $^{KO}$ embryos (Fig. 9F, I) compared to WT (Fig. 9E, H). The number of $Kr^{+}$ cells was comparable in abdominal region A1, but the presence of extra cells increases in a posterior fashion in *dmAIF* $^{KO}$ embryos from abdominal segments A2-A6 compared to WT (Fig. 9G). *dmAIF* $^{KO}$ embryos have on average over 3 extra cells in the Bolwig's organ (Fig. 9J) ($p=0.05$, student t-test). Since *kruppel* is a pan-neuronal CNS marker, we looked at a specific subset of neurons in the VNC.

Antibody staining against dHb9 also revealed the presence of extra cells along the lateral edges of the VNC in *dmAIF* $^{KO}$ embryos (Fig. 9 L, M) compared to WT (Fig. 9K).

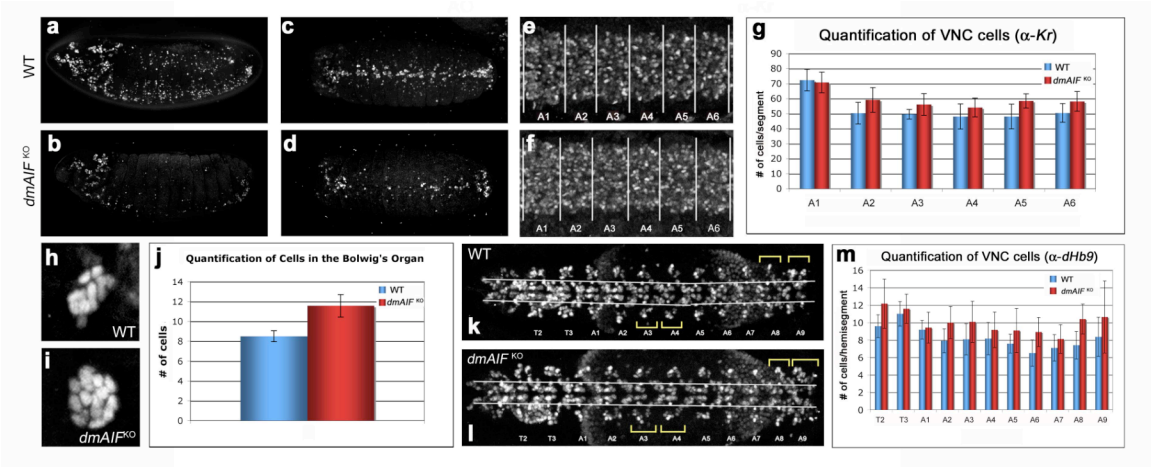


Figure 9. *dmAIF* $^{KO}$ embryos have reduced PCD and extra cells in the Central Nervous System. Acridine Orange staining on WT (A, C) and *dmAIF* $^{KO}$ embryos (B, D). *dmAIF* $^{KO}$ embryos (F) show extra cells by α-*Kr* antibody staining compared to WT (E). Histogram of extra cells in the ventral nerve cord (VNC) by α-*Kr* antibody staining (G) (n=8/group). *dmAIF* $^{KO}$ embryos have extra cells in the Bolwig's organ (I) compared to WT Bolwig's organ (H)(α-*Kr*). Histogram of cell counts in the Bolwig's organ (J) (n=6/group). *dmAIF* $^{KO}$ embryos (L) have extra cells in the VNC by α-*dHb9* antibody staining compared to WT (K) (yellow brackets). Histogram of extra VNC cells by α-*dHb9* antibody staining (n=6/group).

**Discussion**

These studies contributed to comprehensive analysis of AIF in *Drosophila*. Evolutionarily conserved functions for this gene in organismal growth and mitochondrial respiration are presented, with phenotypes similar to *Aif* mutant worms and mice. Overall, AIF plays a role in maintaining energy homeostasis, critical for proper growth and development; however, the precise role of AIF in modulating mitochondrial respiration remains unknown. Importantly, our studies provide evidence for *DmAIF's* proapoptotic function based on marked reduction in developmental PCD and extra neuronal cells in the CNS where PCD is known to occur. Given that these mutant animals arrest during the larval stage, it will be interesting to examine the effects of $dmAIF^{KO}$ if maternal contribution is removed.

## CHAPTER FIVE

## Conclusions and Future Directions

The Apoptosis field has exploded over the last two decades. A large emphasis is placed on Bcl-2 proteins and the mitochondria, but these studies have been predominantly in mammals. Our results provide the first evidence for a role of *Drosophila* Bcl-2 family members that links PCD with mitochondrial properties. To address the possibility of redundancy, we briefly examined animals doubly mutated for *debcl* and *buffy* using a deficiency that uncovers *buffy*, and did not detect a global role for these Bcl-2 family members in this context. Together with genetic evidence from Brachmann's group, it is clear that *Drosophila* has deemphasized the Bcl-2 family members as essential regulators of developmental PCD. Given that heterologous Bax killing requires native Debcl to exert its killing activity suggests that Bax initiates a program in flies that otherwise would not be activated. *Drosophila* now provide a new model to study modes of Bax killing that are caspase-independent, and could prove to be a useful genetic model to screen for novel cell death regulators that impact PCD through the mitochondria. This would be an entirely new field of exploration in the fly community.

Additionally, *DmAIF* also fits nicely into this paradigm of mitochondrial regulators impacting PCD. The mechanisms of how these two genes impact and regulate PCD remain unknown. It will be interesting to see if *DmAIF* killing is

affected by *debcl* status. These experiments are currently underway. This would provide the first genetic evidence that links *Drosophila* Bcl-2 genes with AIF, further emphasizing the role these genes play in mitochondrial-dependent cell death.

This is a very interesting time to be in the PCD field, given that more and more evidence suggests that Bcl-2 family members are not the exclusive determinants of developmental PCD as indicated by 'conventional wisdom'. In time, I predict that there will be even more emphasis put on novel regulators that impact PCD at the level of the apoptosome. Our lab is in the process of revealing potential novel regulators that are critical for PCD that interact with members of the apoptosome, such as Dark and Dronc. One of these new regulators includes Homeodomain Interacting Protein Kinase (Link, Chen et al. 2007), and many more candidates are currently being investigated by classical cell death and genetic-based assays. It will take time to unravel the precise mechanisms by which these novel regulators impact and regulate cell death, but nonetheless, this opens up a whole new pathway of cell death that has previously been unexplored, and will keep scientists busy for years to come. Ultimately, these future studies should shed light on regulators of cell death that impact cancer research, and could potentially be targets for drug therapy.

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**VITAE**

Kathleen Ann Galindo was born on February 11th, 1977 to the proud parents Mary Helen Galindo and Rodolfo Galindo. She was born and raised in San Antonio, TX, and has one older sibling, Rene Galindo, who is 8 years her senior. She attended Incarnate Word High School and graduated in the top 13% of her class in May, '95. She participated in basic scientific research in her junior and senior years in high school as a participant in the Howard Hughes Medical Institute High School Student Summer Research Program in the Laboratory of Dave Sharp, Ph.D.

She went on to attend college at St. Mary's University in San Antonio, where she was the first freshman to enter the Minority Access to Research Careers Program (MARC Fellow). She was a MARC fellow for 4 years, where she participated in several Summer Undergraduate Research Fellowship Programs (SURF) including The University of Wisconsin, Madison ('96), The Rockefeller University ('97), and The University of Texas Southwestern Medical Center at Dallas ('98). She attended several undergraduate research conferences where she co-won 1st place for outstanding oral presentation for her research at UW Madison at the Biennial Research Symposium for Women, Minorities, and the Medically Underserved. She also won 1st place for outstanding undergraduate poster presentation for her summer research at The Rockefeller University at the '97 SACNAS Conference (Society for the Advancement of Chicanos and Native Americans in Science).

She graduated from St. Mary's University in May, 1999, and continued her research interests by working as a Research Associate for Dean Smith, M.D., Ph.D., at UT Southwestern, and her work resulted in a first author publication in Genetics. She then entered the Graduate Program at UT Southwestern Medical Center at Dallas, where she was the recipient of the Ruth Kirschstein NIH Minority Fellowship from '03-'07, and joined the laboratory of John Abrams, Ph.D. in the Genetics and Development Program.

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