APOPTOSIS DETERMINANTS IN *DROSOPHILA MELANOGASTER*

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DEDICATION

For love that bridges space and time

I dedicate this dissertation to my family

Mountains, oceans and years

Melt away when you are always with me

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APOPTOSIS DETERMINANTS IN *DROSOPHILA MELANOGASTER*

by

SU KIT CHEW

DISSERTATION

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APOPTOSIS DETERMINANTS IN *DROSOPHILA MELANOGASTER*

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Apoptosis is a form of programmed cell death (PCD) that is governed by a core set of genes conserved across diverse metazoan phyla. Cells dying by apoptosis exhibit a characteristic series of morphological and biochemical changes that is also conserved. This form of PCD plays pivotal roles in homeostatic regulation of cell numbers, developmental sculpting of organs, damage and infection responses; conversely, its disregulation has profound implications in diseases such as cancers, immune disorders, infertility and dystrophies. Common parallels in the regulation of the core apoptosis machinery have been elucidated in human and experimental model organisms, though

many fundamental questions in our understanding of its regulation remain. A conserved node in the apoptosis pathway is the apoptosome, comprising the apical caspase and its adaptor protein. To understand the functions of this node, I generated a null allele of the apical caspase *Dronc* in the experimental model organism *Drosophila melanogaster*. *Dronc* is required for developmentally regulated apoptosis in multiple tissues during embryogenesis and larval development. Failure of apoptosis correlated with tissue hyperplasia. Notably, the removal of *Dronc* eliminated the cellular apopototic response to stresses in cells. In some of the stress contexts tested, *Dronc* depletion partially rescued cell viability to the same levels as pan-caspase inhibition by small peptide inhibitors, suggesting that *Dronc* functions map specifically to caspase activation and apoptosis. These and similar observations in its adaptor protein *Dark* point to the apoptosome as a key node for apoptosis in *Drosophila*. From these observations, I sought to use the induced apoptosis cellular response as a means to identify novel components and regulators in the apoptosis pathway. I optimized a cell culture system for high-throughput cell-based screening using RNA interference (RNAi) mediated gene silencing and a synthetic antagonist of inhibitors of apoptosis proteins (IAPs). From a genome-wide *Drosophila* RNAi library, I identified 42 potential genes required for apoptosis, of which I characterized 13 highly validated targets for their requirements in multiple stress contexts. One of these hits, *Tango7*, regulates pro-Dronc protein and represents an unprecedented point of apoptosis regulation. Collectively, my studies bolster the model for the crucial requirement of the apoptosome in apoptosis and identify new regulation entry-points into the apoptosis pathway.

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PRIOR PUBLICATIONS

Chew, S. K.[*], Akdemir, F.[*], Chen, P.[*], Lu, W. J., Mills, K., Daish, T., Kumar, S., Rodriguez, A., and Abrams, J. M. (2004). The apical caspase Dronc governs programmed and unprogrammed cell death in Drosophila. Developmental Cell *7*, 897-907.

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[*] Denotes equal contribution from authors

This dissertation contains excerpts from the above manuscripts

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4NQO – 4-nitro-quinoline-N-oxide

APAF-1 – Apoptotic protease activating factor 1

BIR – Baculovirus IAP repeat

CARD – Caspase recruitment domain

Caspase – Cysteinyl aspartate-specific protease

CED-3 – Cell death defective 3

CED-4 – Cell death defective 4

Damm – Death associated molecule related to Mch2

Dark – Drosophila Apaf-1-related killer

Dcp-1 – Death caspase 1

Debcl – Death executioner Bcl-2 homologue

Decay – Death executioner caspase related to Apopain/Yama

DED – Death effector domain

Dredd – Death related ced-3/Nedd2-like protein

Drice – Drosophila ICE

Dronc – Drosophila Nedd2-like caspase

IAP – Inhibitor of apoptosis proteins

IBM – IAP binding domain

MMS – Methyl methanesulfonate

PCD – Programmed cell death

RING – Really interesting new gene

RNAi – RNA interference

SM – Smac mimetic

Smac – Second mitochondrial activator of caspases

Tango7 – Transport and golgi organization 7

zVAD.fmk – benzyloxycarbonyl-Val-Ala-Asp (OMe)-fluoromethylketone

CHAPTER ONE

INTRODUCTION

BACKGROUND

An overview of apoptosis

Programmed cell death (PCD) is a key physiological function in metazoans. Apoptosis is a form of PCD that is an active process originally defined by its distinctive characteristics in mammalian cells, these include nuclear condensation, cell shrinkage and blebbing leading to the formation of apoptotic bodies that are engulfed by surrounding cells (Kerr et al. 1972; Wyllie et al. 1980; Walker et al. 1988; Kerr and Harmon 1991). These phenotypic changes are effected by a set of genes that center on caspases, a family of cysteine proteinases that activate and execute key apoptotic processes during death. Caspases are synthesized as inactive zymogens that are activated in a proteolytic cascade mediated by both regulators and caspases themselves (Salvesen and Abrams 2004; Hay and Guo 2006; Kumar 2006; Bao and Shi 2007) (Figure 1.1). Many of the phenotypic cellular changes and genetic componenets in apoptosis are conserved across many taxa, including *Caenorhabditis elegans* and *Drosophila melanogaster* (Figure 1.1) (Meier et al. 2000a; Vernooy et al. 2000; Abrams 2002; Richardson and Kumar 2002; Hay and Guo 2006; Kumar 2007).

Apoptosis is involved in multiple aspects of physiology such as growth and development, morphogenesis, pathogen response and stress response. Apoptosis adjusts

cell populations through a dynamic process that balances proliferation and apoptosis (Jacobson et al. 1997; Abrams 2002), and can serve as an adaptive response to injury and immune challenges (Clem and Miller 1993; Canman et al. 1994; Spriggs 1996; Desmouliere et al. 1997; Teodoro and Branton 1997; Canbay et al. 2004). Conversely, the disregulation of apoptosis is implicated in diverse pathlogical states such as cancers (Green and Evan 2002; Johnstone et al. 2002; Reed 2003; Lowe et al. 2004) and dystrophies (Rudin and Thompson 1997; Martin 2001; Ferrer 2006).

An emergent model for apoptosis control that combines the collective observations of caspase regulation centers around the interplay between activating signals and repression (Salvesen and Abrams 2004; Hay and Guo 2006). Positive apoptogenic regulation impinges on APAF-1/Dark/CED-4 adaptor proteins to activate the caspases in tandem to the removal of negative repression by Inhibitor of Apoptosis Proteins (IAPs) (Figure 1.1). In this model, the contributions from positive signals and removal of negative signals determine the levels of caspase activity, and apoptosis is initiated by driving caspase activity beyond a threshold limit. At the beginning of my studies, existing data suggested that the relative contribution from each arm of the regulatory pathway differs across cell types and species. Eliminating the positive regulation components result in conspicuous PCD defects in the worm, fly and mouse (Yuan and Horvitz 1992a; Yuan et al. 1993a; Cecconi et al. 1998; Rodriguez et al. 1999b; Wei et al. 2001). Removing components in the negative regulation axis produces pronounced cell death phenotypes in the fly (White et al. 1994a; Grether et al. 1995; Chen et al. 1996b; Wang et al. 1999), but mice null for specific components in the same axis develop normally (Harlin et al. 2001; Okada et al. 2002; Martins et al. 2004). While it is possible some of

the mild phenotype in mice is due to rendundancy functions encoded by multiple genes in the mammalian genome, in at least some contexts of cancer cells though, de-repression of negative regulators (IAPs) take on more dominant roles, this is highlighted by Smac and Smac-mimetic compounds that have demonstrated tremendous promise in sensitizing cancer cells that would otherwise be refractory to induced apoptosis (Fulda et al. 2002; Arnt and Kaufmann 2003; Vogler et al. 2005; Zhou et al. 2005a). In contrast to mammals and the fly, this axis of control has not been identified in the worm.

While many of the conserved core apoptosis components and their roles have been elucidated across the different species (Abrams 1999; Vaux and Korsmeyer 1999), fundamental questions regarding the regulation of caspases and indications of hitherto unidentified regulators remain (Danial and Korsmeyer 2004; Hay and Guo 2006; Kumar 2006; Schafer and Kornbluth 2006). The use of genetic models such as *Drosophila melanogaster* and *Caenorhabditis elegans* with reduced complexity and redundancy provide valuable insights into the mechanisms of regulating this fundamental cellular process.

Understanding apoptosis in *Drosophila melanogaster*

There are seven caspases in the *Drosophila* genome. Structurally, *Dronc* and *Dredd* resemble classical apical/initiator caspases – they have long prodomains containing a caspase recruitment domain (CARD) and death effector domains (DEDs) respectively (Chen et al. 1998; Dorstyn et al. 1999b). *Strica* has a unique serine/threonine-rich prodomain and *Dcp-1*, *Drice*, *Decay* and *Damm* have short prodomains typical of effector/executioner caspases (Kumar and Doumanis 2000a;

Doumanis et al. 2001). Of the apical caspases, *Dredd* seems to primarily function in the innate immune response (Hu and Yang 2000; Leulier et al. 2000; Zhou et al. 2005b), although it has roles in both apoptotic and differentiation processes under specific contexts (Chen et al. 1998; Huh et al. 2004b). At the beginning of my studies, there were no mutant alleles of *Dronc*, but studies using chromosomal deficiencies, dominant-negative alleles and injected dsRNAs had implicated *Dronc* as the crucial apical caspase for apoptosis (Dorstyn et al. 1999a; Meier et al. 2000b; Quinn et al. 2000). More recently, it has also been shown to be involved in non-apoptotic functions such as compensatory proliferation, spermatogenesis and cell motility (Geisbrecht and Montell 2004; Huh et al. 2004a; Huh et al. 2004b; Kuranaga et al. 2006; Oshima et al. 2006). There were no described *Strica* mutants then too, though recent work published during the course of my studies describe partial redundant functions with *Dronc* during oogenesis with no other characterized developmental defects (Baum et al. 2007).

Analyses of the effector caspases *Dcp-1*, *Drice*, *Decay* and *Damm* have revealed functional redundancy, context-specific requirements and variable apoptogenic capacity on their own when overexpressed (Dorstyn et al. 1999c; Harvey et al. 2001; Laundrie et al. 2003; Kondo et al. 2006; Leulier et al. 2006b; Muro et al. 2006; Xu et al. 2006). *Drice* is required for both developmentally regulated and stress-induced apoptosis, and mutant animals exhibit multiple tissue defects (Kondo et al. 2006; Muro et al. 2006; Xu et al. 2006). *Dcp-1* mutant animals are compromised for starvation-induced death during oogenesis but are otherwise developmentally normal and capable of PCD (Laundrie et al. 2003; Kondo et al. 2006; Muro et al. 2006; Xu et al. 2006). However, *Drice/Dcp-1* double mutants exhibit tissue-specific enhancement of apoptosis defects observed in

Drice mutants (Xu et al. 2006), exemplifying the functional redundancy amongst capases. *Decay* mutant animals have no overt developmental PCD on its own, suggesting that it is functionally redundant in development (Kondo et al. 2006). No mutants for *Damm* have been described, although overexpression of either wildtype or inactive mutant Damm induces and suppresses cell death respectively (Harvey et al. 2001).

Studies have revealed several proximal regulators of the caspases. Analogous to the mammalian apical Caspase-9 and its adaptor protein Apaf-1 that form the apoptosome complex, Dark complexes with Dronc (Quinn et al. 2000; Dorstyn et al. 2002; Yu et al. 2006) and activates it (Kanuka et al. 1999; Rodriguez et al. 1999a; Zhou et al. 1999). In cultured cells, Dronc protein undergoes continual synthesis, processing and turnover. This cleavage of pro-Dronc zymogen depends on Dark and depletion of Dark levels results in accumulation of the pro-Dronc protein (Muro et al. 2002; Muro et al. 2004). Recently generated *Dark* null animals show profound requirements of *Dark* in apoptosis, with loss of almost all developmental and stress-induced apoptosis (Akdemir et al. 2006; Mills et al. 2006; Srivastava et al. 2006), suggesting that the fly apoptosome forms a key node in regulation of apoptosis.

When I began my studies, upstream regulators of the adaptor protein CED-4 (*C. elegans*) and Apaf-1 (mammals) had been established as key regulators of apoptosis in those species (Meier et al. 2000a; Danial and Korsmeyer 2004). In contrast, the definitive upstream regulators of *Dark* or *Dronc* had not been identified in *Drosophila*. The *Drosophila* genome encodes two annotated multidomain Bcl-2 family members, *debcl* and *buffy*. It was initially thought that *debcl* and *buffy* were the pro- and anti-apoptotic Bcl-2 proteins respectively, based on overexpression and *in vivo* RNAi studies

(Brachmann et al. 2000; Colussi et al. 2000; Igaki et al. 2000; Zhang et al. 2000; Quinn et al. 2003), but recently generated loss-of-function alleles of *debcl* and *buffy* show no indication of direct apoptosis regulation (Galindo, manuscript in preparation) (Sevrioukov et al. 2007). Additionally, while cytochrome *c* is firmly established as an important component of the mammalian apoptosome (Jiang and Wang 2004), there is little evidence to suggest that cytochrome *c* functions in a similar role in *Dark* mediated apoptosis beyond modest caspase activation in vitro (Kanuka et al. 1999). Rather, evidence from structural and loss of cytochrome *c* function studies argue for cytochrome *c* not being involved in *Drosophila* apoptosis (Zimmermann et al. 2002; Dorstyn et al. 2004; Means et al. 2006; Yu et al. 2006). These observations point to two possible models for positive death signals in *Drosophila,* either a constitutive flux of positive signaling through the apoptosome held only in check by the repression (IAP) regulation pathway, or hitherto unknown positive regulators that remain to be discovered.

The inhibitor of apoptosis proteins (IAPs) are important regulators of caspases which contain one or more BIR (baculovirus IAP repeat) domains that can physically bind active caspases, and can also contain a E3-ligase RING domain at the C-terminus (Hay 2000; Salvesen and Duckett 2002; Vaux and Silke 2005). Of the Drosophila IAPs, *DIAP1* is the main regulator of apoptosis. Forced expression of *DIAP1* blocks cell death while elimination results in concomitant activation of apoptosis (Hay et al. 1995; Kaiser et al. 1998; Wang et al. 1999; Igaki et al. 2002; Muro et al. 2002; Rodriguez et al. 2002; Yin and Thummel 2004). DIAP1 binds and targets pro-apoptotic proteins for degradation (Meier et al. 2000b; Wilson et al. 2002; Chai et al. 2003). Additionally, DIAP1 protein itself is also cleaved and targeted for degradation during apoptosis (Hays et al. 2002;

Ryoo et al. 2002; Ditzel et al. 2003). *DIAP2* governs immune functions and mutants have no apoptosis defects (Leulier et al. 2006a; Huh et al. 2007). The other two BIR containing IAPs *dBruce* and *Deterin* also have anti-apoptotic activities if ectopically expressed (Jones et al. 2000; Vernooy et al. 2002; Arama et al. 2003), though compared to DIAP1, relatively less is know about their roles and mechanisms.

The IAPs are in turn regulated by the IAP antagonists, encoded by *Reaper* (*Rpr*), *Head involution defective* (*Hid*), *grim*, *sickle* (*Skl*) and *Jafrac2* (White et al. 1994b; Grether et al. 1995; Chen et al. 1996b; Christich et al. 2002; Srinivasula et al. 2002; Tenev et al. 2002; Wing et al. 2002a). These apoptosis activators are collectively referred to as the RHG proteins and share a common RHG or IAP-binding motif (IBM) which mediates interaction with the BIR domains on DIAP1 and is proposed to displace DIAP1-bound proteins such as Drice, Dcp-1 and Dronc (Vucic et al. 1998b; Wu et al. 2001; Tenev et al. 2002; Chai et al. 2003; Zachariou et al. 2003; Yan et al. 2004). The RHG proteins also regulate the ubiquitination state (and thereby levels) of DIAP1 protein (Hays et al. 2002; Holley et al. 2002; Ryoo et al. 2002; Wing et al. 2002b; Yoo et al. 2002). Embryos containing the H99 chromosomal deficiency that deletes *Rpr*, *Hid* and *Grim* lack detectable developmental PCD (White et al. 1994a). Of note, part of the pro-apoptotic activity that Rpr and Grim exhibit maps outside of the IAP binding domain (Chen et al. 1996a; Vucic et al. 1998a; Wing et al. 1998; Wing et al. 2001; Chen et al. 2004), and Rpr or Grim lacking the IBM are able to repress translation (Holley et al. 2002; Yoo et al. 2002). An interesting parallel is observed in the mammalian orthologue Smac/DIABLO where a splice isoform of Smac lacking the N-terminal IAP binding

domain retains its proapoptotic potential (Roberts et al. 2001), although the mechanism of this pro-apoptotic activity remains unclear.

DISSERTATION OBJECTIVES

The aim of the studies presented in this dissertation is to understand the roles of apoptotic determinants and how they regulate apoptosis. To achieve this general aim, I proposed to study the functions of the apical caspase *Dronc* and complete a genome-wide screen to identify novel apoptotic determinants in the genetic model *Drosophila melanogaster*. The following chapters detail these specific aims:

Aim 1: Study the functions of the apical caspase *Dronc* – In order to understand the importance of apoptosis signal through the apoptosome, I isolated a null allele of *Dronc* and examined its roles in development and apoptosis. I also examined the role of *Dronc* under multiple contexts of stress-induced cell death.

Aim 2: Identify novel apoptotic determinants – I established a high-throughput cell-based screening platform using RNA interference (RNAi)-based silencing of gene functions. I screened the *Drosophila* genome and identified a collection of novel components required for apoptosis induced through the derepression of IAPs.

Aim 3: Characterization of *Tango7* - A novel gene identified from work done in aim 2, *Tango7* is required for apoptosis. I examined how *Tango7* affects the apoptosis pathway and its regulation.

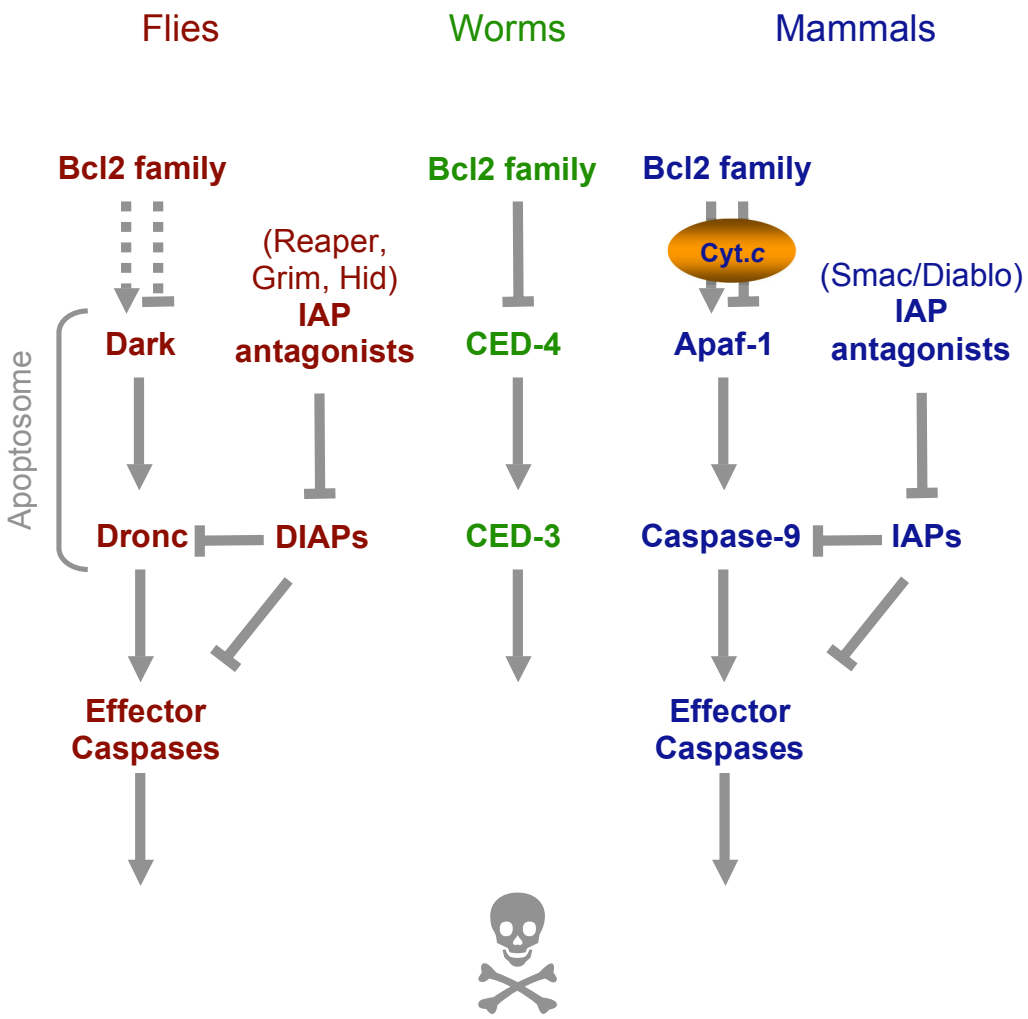


Figure 1.1 Conservation of the core apoptotic machinery across species
Schematic showing the conserved genes in the apoptotic pathway in parallel across mammals and experimental genetic models (the fruitfly *Drosophila melanogaster* and the roundworm *Caenorhabditis elegans*. The dotted line for *Drosophila* denotes the ambiguous role for regulation of the fly apoptosome).

CHAPTER TWO

FUNCTIONS OF THE APICAL CASPASE *Dronc*

ABSTRACT

Among the seven caspases encoded in the fly, only *Dronc* contains a caspase recruitment domain. To assess the function of this apical caspase in development and apoptosis, I isolated a null allele (*Dronc*^{51}) for *Dronc* using P-element mobilization to induce a deletion at the locus. Animals lacking *Dronc* exhibit defective developmental programmed cell death and arrest as early pupae. These mutants present a range of defects, including hyperplasia of imaginal and hematopoietic tissues, supernumerary neuronal cells, and head involution failure. Furthermore, studies of cells *ex vivo* from *Dronc*^{51} animals and cultured cells RNAi-depleted for *Dronc* are insensitive to induction of cell killing in multiple contexts of stressed-induced apoptosis, including macromolecular synthesis inhibition, DNA damage, alcohol toxicity, and Smac/Reaper induction. The observations presented here establish *Dronc* as a regulator of cell numbers in development and highlight a fundamental requirement for *Dronc* in cellular responses during stress-induced apoptosis.

INTRODUCTION

Caspases (cysteinyl aspartate-specific proteinases) are synthesized as inactive zymogens that undergo cleavage activation via networks of proteolytic cascades during apoptosis. The resultant activated caspases are the effectors of apoptosis that go on to execute the cellular death process (Salvesen 2002; Shi 2002b; Danial and Korsmeyer 2004; Hay and Guo 2006; Kumar 2007). Of the seven members of the caspase family encoded in the *Drosophila* genome (Kumar and Doumanis 2000b), *Dredd* (Chen et al. 1998) and *Dronc* (Dorstyn et al. 1999a) resemble apical or initiator caspases based on their prodomain structure. *Dredd* contains death effector-like domains (DEDs) and *Dronc* contains a caspase recruitment domain (CARD). *Strica* has a unique serine/threonine-rich prodomain and *Dcp-1*, *Drice*, *Decay* and *Damm* have short prodomains typical of effector/executioner caspases (Kumar and Doumanis 2000a; Doumanis et al. 2001). At the time of the studies presented here, the activation and cleavage relationships between the caspases were inferred from overexpression, *in vitro* or RNAi studies.

Dronc is the only CARD-domain containing caspase in the fly genome, and therefore postulated to be critical for apoptosis (Dorstyn et al. 1999a; Meier et al. 2000b). The other stereotypical apical caspase, *Dredd*, had been shown to be required for innate immunity (Leulier et al. 2000). Like the mammalian apoptosome that comprises the apical Caspase-9 and its adaptor protein Apaf-1, Dark complexes with Dronc (Quinn et al. 2000; Dorstyn et al. 2002; Yu et al. 2006) and activates it (Kanuka et al. 1999; Rodriguez et al. 1999a; Zhou et al. 1999).

However, the studies on *Dronc* function had thus far relied on multigenic chromosomal deficiencies, overexpression studies, dominant negative alleles and injected dsRNAs. To elucidate the actual requirements for this apical caspase in development and PCD, P-element excision was used as a mutagenesis strategy to generate $Dronc^{51}$, a null allele. I isolated the allele in a mutagenesis effort started by Antony Rodriguez, and in collaboration primarily with Po Chen and Fatih Akdemir, we characterized the $Dronc^{51}$ allele (Chew et al. 2004). $Dronc^{51}$ embryos exhibit head involution defects reminiscent of the phenotype observed in mutants of the IAP antagonist *head involution defective* (*Hid*). $Dronc^{51}$ larvae show developmental delay and lethally arrest at the early pupal stage. They were defective in PCD in multiple tissues, and exhibited hyperplasia of blood, nerve and imaginal tissues. Somatic clonal tissue analyses revealed that removal of *Dronc* resulted in eye and wing development phenotypes. Using the adult wing as a phenotypic readout, *Dronc* also genetically interacts with its adaptor *Dark*. Furthermore, cells from $Dronc^{-}$ animals are insensitive to multiple contexts of stress-induced apoptosis. Using cultured cells, I show that *Dronc* depletion rescued cell viability to similar levels seen with pan-caspase pharmalogical inhibition, suggesting that *Dronc* functions is specific to caspases and apoptosis. These studies show *Dronc* regulation of cell numbers in development and requirements for this apical caspase in stress-induced apoptosis.

MATERIALS AND METHODS

Fly strains

Df(3L)AC1,rn,p/TM3,Sb, *P[y+w+=SUPor-P]KG02994* and *Hml-UAS:GAL4-GFP* were obtained from Bloomington stock center. *p[1.0Slit-LacZ]* was provided by John Nambu (Ma et al. 2000).

Mutagenesis and Screen for null allele of *Dronc*

KG02994 denotes *P[y+w+=SUPor-P]KG02994*. *KG02994* was crossed with *y,w;CyO,H{w[+mC]=PΔ2-3}HoP2.1/Bc,Egfr* to induce the mobilization of the KG02994 P-element. Mobilized P elements were identified among progeny by loss of yellow, white or both markers. Genomic DNA from 126 mobilization candidate lines were isolated and their *Dronc* locus were characterized by genomic PCR (Figure 2.1, Appendix A for primer sequences).

3rd-instar larva dissections

Wandering L3 are dissected with fine dissecting forceps (#5, Dumont) in a drop of phosphate buffered solution (PBS) or 0.1M phosphate buffer (72% Na_2HPO_4, 28% NaH_2PO_4). Dissected larval organs are fixed with 2% formaldehyde in PBS or 0.1M phosphate buffer for 10 min and washed in PBS.

Embryo collection

Embryos were collected and aged for the indicated lengths of time at 25°C. Embryos were dechorionated using 50% bleach, washed, and fixed for 15 minutes in 1:1 heptane-2% formaldehyde in PBS with vigorous shaking. The vitelline membranes were then cracked by replacing the aqueous fix with absolute methanol, followed by vigorous shaking for 15 seconds. The fixed embryos were transferred to fresh methanol, washed twice and stored in methanol at -20°C.

Immunohistochemistry

Fixed embryos in methanol or L3 larval eye discs in PBS are equilibrated to and washed in PBS with 0.1% Triton-X100 (PBT). PBT+NGS (PBT, 0.1% BSA, 5% normal goat serum) was used as a blocking solution for 1 hr before incubation with the primary antibody (1:600 guinea pig α-Kr, (Kosman et al. 1998); 1:500 α-Boss (Kramer et al. 1991); 1:800, α-β-Gal, Promega) overnight at 4°C. The labelling was visualized with either Vectastain ABC peroxidase kit or fluorochrome labelled secondary antibodies (Vector Laboratories). Labelled samples were washed in PBS and mounted using Vectorshield (Vector Laboratories) on glass slides.

Microscopy and imaging

Whole animal images were done on a dissecting binocular microscope (Zeiss), fluorescent light microscopy was done on an Axioscop microscope with an Axiocam MRC color digital camera (Zeiss). Confocal imaging was done on a TCSSP Spectral Confocal Microscope (Leica).

Hemocyte density counts

The assay for hemocyte density was modified from (Asha et al. 2003). L3 larvae are submerged in halocarbon oil, and the body wall was carefully pierced with a 250μm diameter tungsten needle to obtain a droplet of hemolymph. To calculate the number of cells per μL of hemolymph, the hemolymph droplets from 3-15 larvae were combined into a single drop, 1-4 μL of the hemolymph withdrawn with a fine pipette and diluted with PBS for counting using a hemocytometer on a fluorescent microscope.

Larval septic injury

1 ml culture of *Escherichia coli* (DH5alpha, Invitrogen) was pelleted at 9000 RCF for 1 min to obtain a bacteria cell pellet. Wandering 3rd instar larvae were stabbed with a 100μm diameter steel needle that had been dipped in the bacteria cell pellet, care was taken to puncture the body cavity without perforating internal organs.

RT-PCR

Total RNA was isolated from wandering 3rd instar larvae using High Pure RNA Isolation Kit (Roche). Superscript One-step RT-PCR System w/ Platium Taq (Invitrogen) was used for RT-PCR reactions (primer sequences in Appendix A).

Cell culture

S2R+ cells were cultured in Schneider's media with 10% Fetal Bovine Serum, 25U/mL penicillin, 25μg/mL streptomycin at 25°C (all cell culture reagents from Invitrogen). Unless otherwise indicated, media in all procedures refer to the above.

Haemocyte cultures

Wandering 3rd instar larvae were washed thoroughly for 3 minutes in each of the following solutions – water, 50% bleach, 70% ethanol and autoclaved water. For long term cultures, the media used was Schneider's with 10% FBS, 1xPSA (50 U/mL Penicillin, 50 μg/mL Streptomycin, 125 ng/mL Amphotericin B). Larvae were initially submerged and dissected in Schneider's cell culture media supplemented with 10% FBS, 10xPSA, the haemocytes were plated in 96-well microplates. The media was replaced with Schneider's with 10% FBS, 5xPSA on the second day, and then Schneider's with 10% FBS, 1xPSA on the third day.

RNAi

dsRNA synthesis and RNAi depletion of *Dronc* in S2R+ cells was carried out essentially as previously described (Clemens et al. 2000; Worby et al. 2001). For each intended gene target, cDNA sequences 200-800bp long were amplified with primers containing T7 promoter sequences at each end (see Appendix A for all primer sequences). The DNA template was gel purified and concentrated using the QiaQuick kit (Qiagen). RNA was synthesized from the template using the T7 Megascript kit (Ambion), and annealed by heating the RNAs to 95°C for 15 minutes and cooling gradually in a 1L beaker waterbath. The dsRNAs were precipitated in 10% sodium acetate, and washed 3x in 70% ethanol before air-drying and resuspension in nuclease-free water. Cultured cells were seeded in 15μg/ml dsRNA in serum-free media for 1 hour, recovered with twice volume of normal serum-containing media and incubated 72 hours to deplete target.

Stress-induced apoptosis

Cultured cells were treated with stressors as indicated to induced apoptosis. The total cell viability in each well was assessed 24 hours later using CellTiter-Glo (Promega) according to the manufacturer's instructions.

RESULTS

Mutagenesis at the *Dronc* locus to generate a null allele, *Dronc*^{51}

To generate novel alleles at the *Dronc* locus, I screened for null mutations in this gene. The strategy involved mobilization of a P transposon insertion, $P^{KG02994}$, mapping 113bp upstream of the *Dronc* transcription start site (Figure 2.1). The mobilized progeny were screened for genomic PCR fragment length polymorphism that would indicate a possible imprecise excision event resulting in deletion (Primers DRONCF1 and CG6674R1, primer sequences in Appendix A). The PCR reactions were done in pools of 10 candidates, and where length polymorphism was observed, individual lines were tested. Of the mobilized progeny, I identified line 51 (*Dronc*^{51}) as having a deletion event.

Together with Po Chen and Fatih Akdemir, we molecularly characterized the *Dronc*^{51} allele (Chew et al. 2004). We showed that *Dronc*^{51} is an imprecise excision of $P^{KG02994}$ that deletes the transposon through codon 303 (67%) of the *Dronc* open reading frame, leaving all sequences upstream of the insertion site unaffected (Figure 2.1). RT-PCR using primers spanning the remaining region of *Dronc* mRNA could not detect *any* transcribed *Dronc* mRNA, strongly indicative of *Dronc*^{51} being a null allele.

Dronc is required for PCD

To examine the roles of *Dronc* in developmentally regulated programmed cell death, we examined apoptosis in *Dronc*^{51} homozygous embryos. Maternal contribution occurs for *Dronc*, as *Dronc*^{51} homozygotes from heterozygous parents had normal

developmental cell death. In contrast, homozygous mutants from *Dronc*^{51}/*P*^{KG02994} parents showed reduced embryonic developmental apoptosis when assayed by TUNEL (White et al. 1994a) but not acridine orange staining (Abrams et al. 1993) (Figure 2.2), although mutants from either parents show head involution defects (Figures 2.2B, 2.3B) like other known apoptosis mutants (White et al. 1994a; Grether et al. 1995).

Dronc governs cell numbers and tissue patterning

In diverse models, cells from apoptosis mutants that fail to undergo developmental apoptosis may differentiate and produce tissue hyperplasia (Ellis et al. 1991; White et al. 1994a; Rodriguez et al. 1999b). Using the α-Kruppel antibody (Kosman et al. 1998), we found that *Dronc*^{51} embryos have additional cells associated with the optic organ (Figure 2.3A, B). Supernumerary optic organ cells and head involution failure (Figures 2.2B, 2.3B) parallels phenotypes described in mutants of another apoptotic determinant *hid* (Grether et al. 1995). Extra cells expressing a Slit-LacZ reporter were also observed in the midline of the embryonic central nervous system (CNS). Normally, 2-3 slit-expressing midline glial cells are detected within each segment (Zhou et al. 1995; Rodriguez et al. 2002) but, in *Dronc*^{51} mutants, we observed segments containing 4 or more *slit* positive cells (Figure 2.3C, D).

In wandering third instar larva, *Dronc*^{51} animals also often exhibit abnormalities of internal structures. For example, the brain lobes of these mutants are typically larger than wild type counterparts. Likewise, the imaginal tissues in these animals are enlarged and frequently deformed or mispatterned (Figure 2.4A, B, also 2.5B, C). Examination of circulating haemocytes (blood cells) also revealed conspicuous hyperplasia of blood cells

in *Dronc*^{51}. *Ex vivo* counts of all or *Hml-GAL4:UAS-GFP* marked hemocytes reveal a more than a 3-fold elevation in density (Figure 2.4C).

The severity of the hyperplasia phenotypes in 3rd instar larvae strongly correlated with larval size such that, in a given population of *Dronc*^{51} animals, these phenotypes were noticeably more severe among the larger sized larva. *Dronc*^{51} animals have a longer larval phase, and were typically larger by the time they progress to the wandering 3rd instar stage (Figure 2.5A). Despite these irregularities in organ size and tissue patterning, differentiation programs initiate and proceed normally, as the progression of the morphogenetic furrow through the eye disc appears unaffected in *Dronc*^{51} larvae even though they are larger than their wild type counter parts (Figure 2.5B, C).

Dronc^{51} larvae have normal immune response

In *Drosophila*, postembryonic haemopoiesis occurs in the larval lymph glands. The glands generate circulatory haemocytes consisting of presumptive primitive blast cells, plasmatocytes and crystal cells during an immune response (Lanot et al. 2001). To evaluate if the observed increase in circulating haemocytes in *Dronc*^{51} larvae could be due to activation of the lymph gland in an autoimmune response to the aberrant tissues, the immune status of *Dronc*^{51} larvae was evaluated. The transcription of immune response genes was not upregulated in *Dronc*^{51} larvae compared to wildtype larvae (Figure 2.5). Additionally, *Dronc*^{51} animals exhibit a normal upregulation of immune genes in response to septic injury, suggesting that the hyperplasia of haemocytes stem from a developmental rather than an immune process.

***Dronc*-null haemocytes resist apoptosis but exhibit no propensity for proliferation**

To evaluate if the lack of *Dronc* results in haemocytes of an aberrant differentiation state with different apoptotic and/or proliferative potential, both division and apopotosis of haemocytes *in vivo* and *ex vivo* were assayed. Notably, there was no significant difference in the percent of TUNEL-positive (Terminal transferase dUTP nicked end labeling, a marker for DNA fragmentation in apoptosis) circulatory haemocytes that are aspirated and assayed immediately. This is in contrast to the $Dronc^{51}$ haemocytes that are isolated and cultured *ex vivo* where no apoptosis is observed. To investigate if *Dronc*-null haemocytes could have extra proliferative potential that accounts for the hyperplasia, I assayed for phospho-HistoneH3 that marks mitotic cells. Both $Dronc^{51}$ and wildtype circulatory haemocytes have similarly low proliferative potential, with less than 1% of the cells being positive for phospho-HistoneH3. These observations argue for the source of extra cells being earlier in the haemopoietic lineage, in an earlier progenitor cell that has proliferative capacity.

To evaluate if the cell death block from removing *Dronc* could be exploited as a tool for facilitating the production of culture cell lines, I developed a long-term haemocyte culture protocol (see methods). Whereas wildtype haemocytes normally undergo apoptosis within ~3 days after isolation, I cultured *Dronc*-null haemocytes for up to 30 days with no detectable apoptosis. The *Dronc*-null haemocytes were morphologically normal, but there was no detectable cell division in the cultures. To investigate if a 'proliferative push' could be exerted using an additional mutation, I cultured haemocytes from animals that were $Dronc^{51}$ homozygous in trans to mutations in *Dap*, *Hop*, *l(3)Mbn*, *Oho31*, *prod*, and *Rbf* annotated as proliferation driving (Flybase).

Additionally, I also investigated the use of *Srp-Gal4* to drive pan-haemocyte overexpression of BcrAbl, HoxB4, *Dfd* (*DmHoxB4*) and *Scr* (*DmHoxA5*), as ectopic expression of HoxB4 has been shown to induce definitive haematopoietic stem cell proliferation in culture (Antonchuk et al. 2002; Kyba et al. 2002). While the haemocytes were also long-lived in these experiments (due to the lack of *Dronc*), no sustained proliferative capacity was observed in culture. These results show that *Dronc* is required in apoptosis, but do not affect proliferation in the conditions and contexts tested.

Dronc is required for stress-induced apoptosis

To expand on the observations that *Dronc*-null haemocytes are insensitive to multiple apoptotic stressors (Chew et al. 2004), I surveyed numerous additional stimuli assaying for efficient killing of cultured S2R+ cells (Table 2.1). These apoptotic stimuli represented imputs from different cellular processes, such as DNA damage, inhibition of macromolecular synthesis, de-repression of IAPs (Smac-mimetic) and apoptotic triggers that are not well understood molecularly (ethanol). To assess the role of *Dronc* in the apoptotic contexts that induced killing of S2R+ cells, I tested for the rescue of cell viability by RNA interference (RNAi) mediated depletion of *Dronc.* In all the contexts tested where *Dronc* depletion could rescue cell viability, the levels of rescue by *Dronc* depletion correlated with treatment by zVAD.fmk, a pan-caspase inhibitor. For example, both *Dronc* depletion and zVAD.fmk partially rescue cell viability to the same levels in cell death induced by 4-nitro-quinoline-N-oxide, and fully rescue cell viability for killing induced by a small molecule Smac-mimetic (Li et al. 2004) (Figure 2.7A). By comparison, neither *Dronc* depletion nor a pan-caspase block could rescue cell viability

in the context of methyl methanesulfonate (MMS), even though killing by MMS is dose-dependent (Figure 2.7B). These results support the model that *Dronc* functions as the apical caspase for activating the apoptotic caspase cascade, such that in PCD where cells die by apoptosis, the result of depleting *Dronc* is the same as blocking caspases generally.

DISCUSSION

Of the three apical caspases found in the fly genome, *Dronc* is the only CARD domain containing caspase orthologous to mammalian Caspase-9. Observations on *Dronc* function presented here, together with coincident and subsequent analyses of independently derived *Dronc* mutants (Daish et al. 2004; Waldhuber et al. 2005; Xu et al. 2005; Kondo et al. 2006) have established the crucial roles of *Dronc* as an apical caspase in *Drosophila* PCD. Our studies show that *Dronc* is an essential gene, as animals lacking *Dronc* do not progress beyond the early pupal stage. *Dronc* mutants exhibit many developmental phenotypes that have been described in mutants of other cell death regulators. For example, $Dronc^{51}$ embryos consistently exhibit head involution defects (Figures 2.2B, 2.3B) reminiscent to *hid*, a pro-apoptogenic IAP-antagonist (Grether et al. 1995). Extra scutellar bristles in viable *Dronc* hypomorphs have also been observed in mutants of the apical caspase adaptor *Dark* (Rodriguez et al. 1999b) and the apical caspase *Dredd* (Rodriguez et al. 2002).

Dronc mutants also exhibit extra cells that have been described in mutants of *dark* (Rodriguez et al. 1999b), *hid* and *grim* (Zhou et al. 1997). The source of extra cells likely maps to PCD failures, as cells that fail to undergo developmental apoptosis may differentiate and produce supernumerary cells (Ellis et al. 1991; White et al. 1994a; Rodriguez et al. 1999b). The normal progression of the morphogenetic furrow through the larval eye also bolsters the model that while loss of *Dronc* disregulates cell numbers, the underlying developmental progression is not affected (Figure 2.4B, C). The expression of genes in the H99 or *Reaper* region have been described to preceed death in

cells, including those labeled by the *Kruppel* and *Slit* neuronal markers (White et al. 1994a; Grether et al. 1995; Zhou et al. 1995; Wing et al. 1998; Christich et al. 2002), hence the presence of supernumerary cells positive for these markers in *Dronc* mutant embryos (Figure 2.2) corroborates the role of *Dronc* in PCD induced by the IAP-antagonists. This is also recapitulated the suppression of a Smac mimetic compound in cells lacking *Dronc* (Table 2.1, Figure 2.7) and in genetic interaction experiments where loss of one copy of *Dronc* can attenuate eye-ablation induced by overexpression of *Rpr*, *Grim* or *Hid* (Meier et al. 2000b; Quinn et al. 2000; Daish et al. 2004). These observations point to *Dronc* and the apoptosome being downstream of the IAP antagonists, though this is likely to be tissue- and context-specific as recent experiments using Ovo^{D} germ line clones to generate embryos that are maternal and zygotic nulls of *Dronc* or its adapter protein *Dark* resulted in embryos severely compromised in PCD that nevertheless still have rare apoptotic cells (Xu et al. 2005; Akdemir et al. 2006). This is in contrast to the almost complete loss of developmental PCD in H99 embryos where the IAP antagonists *Reaper*, *Grim*, and *Hid* are removed (White et al. 1994b), arguing for a model where the apoptosome is not the only means to activating caspase-dependent apoptosis. While mutations that block most developmental PCD have not been observed in mammals, it is interesting to note that the mutations that produce such phenotypes in the worm and fly are mutations of the CED-3/CED-4 apoptosome and the IAP antagonists *Rpr*/*Grim*/*Hid* respectively (Yuan and Horvitz 1992b; Yuan et al. 1993b; White et al. 1994b). This alludes to a paradigm where control nodes have diverged through evolution even when parallel components exist across different species. IAP antagonists are known to be crucial points of regulation by multiple signaling pathways

including microRNAs (Brennecke et al. 2003; Xu et al. 2003; Thompson and Cohen 2006) and tumor suppressors (Harvey et al. 2003; Pantalacci et al. 2003; Wu et al. 2003). Further studies could yield insights into how distinct components of the apoptotic machinery are engaged in regulation of cell numbers and tissue size.

The hyperplasia of haemopoietic tissue in *Dronc*^{51} animals is markedly more obvious than other tissues. The results presented here establish *Dronc* as an arbiter of haemocyte numbers, although the point in haemopoiesis where *Dronc* exerts this effect remains unknown. The *Dronc*-null circulatory haemocytes are inherently apoptosis resistant, as no apoptosis was observed when these cells are cultured *ex vivo*, yet the incidence of TUNEL-positive cells were the same as wildtype if haemocyte aspirates are assayed immediately after isolation from the animals. One possible explanation would be that the extra haemocytes in circulation are dying cells that do not exhibit overt apoptosis due to the lack of *Dronc*, even though aspects of cell death (DNA fragmentation resulting in TUNEL-positive cells) can occur. A second possibility is that *Dronc*^{51} haemocytes form 3rd instar larvae still contain maternally loaded *Dronc*, and *ex vivo* culturing allows turnover depletion of *Dronc* to uncover the requirement of *Dronc* in apoptosis.

Blocking cell death in cells that do not divide would have only mild effects on total cell numbers. Given the observation of gross hyperplasia in circulatory haemocyte numbers and that the *Dronc*^{51} haemocytes do not show higher proliferative potential than their wildtype counterparts, the point where *Dronc* acts in haemopoiesis likely impacts an earlier progenitor cell. Further work pursuing the role of *Dronc* in early haempoiesis in either the larval lymph gland or the embryonic haemocyte anlage could reveal insights into the biology of progenitor or stem-like cells.

As an extension of the observations in haemocytes, I investigated if the cell death block from *Dronc* removal could be coupled to a proliferative push to establish primary cell lines from haemocytes. Even when the proliferation mutations were introduced into the genotype of the haemocytes, at most a couple of cell divisions would be observed before the cells go quiescent in culture. There are several possible explanations for these observations. First, the *Dronc*-null haemocytes could represent a highly differentiated state that do not have the potential to proliferate, although this is unlikely as aberrant proliferation of circulatory haemocytes have been observed in multiple genetic backgrounds (Gateff et al. 1996; Asha et al. 2003). It is also a possibility that like mammalian cells, an additional block for senescence/quiescence exists for growth of primary cells. The haemocytes in culture did not exhibit gross morphological changes typically associated with senescence (such as appearance of stress fibers and extensive vacuolation), although arguably the changes might not be overt. I would propose that the limitations are likely metabolic or growth factor dependent, which leaves the possibility that further investigations on culture conditions could potentially yield a novel means for establishing cell lines in *Drosophila*.

The results presented here also indicate that *Dronc* has a fundamental role in stress-induced apoptosis. *Dronc* deficient cells from either $Dronc^{51}$ animals or RNAi depletion exhibit resistance to apoptosis in a wide range of stimuli contexts, from inhibition of macromolecular synthesis to DNA damage. This profound insensitivity stress-induced apoptosis is also recapitulated in *Dark* deficient cultured cells and null mutants (Akdemir et al. 2006). Parallels are observed in caspase-9 knockouts where multiple stress-induced signals converge on the mammalian apoptosome (Zheng et al.

1999). These results point to a generalisable principle for the apoptosome as a crucial node in stress-induced apoptotic responses. In the case of ethanol-induced PCD where the mechanism of cytotoxicity is not known, this represents a genetic entrée to future work for understanding the signaling and cellular response pathways involved.

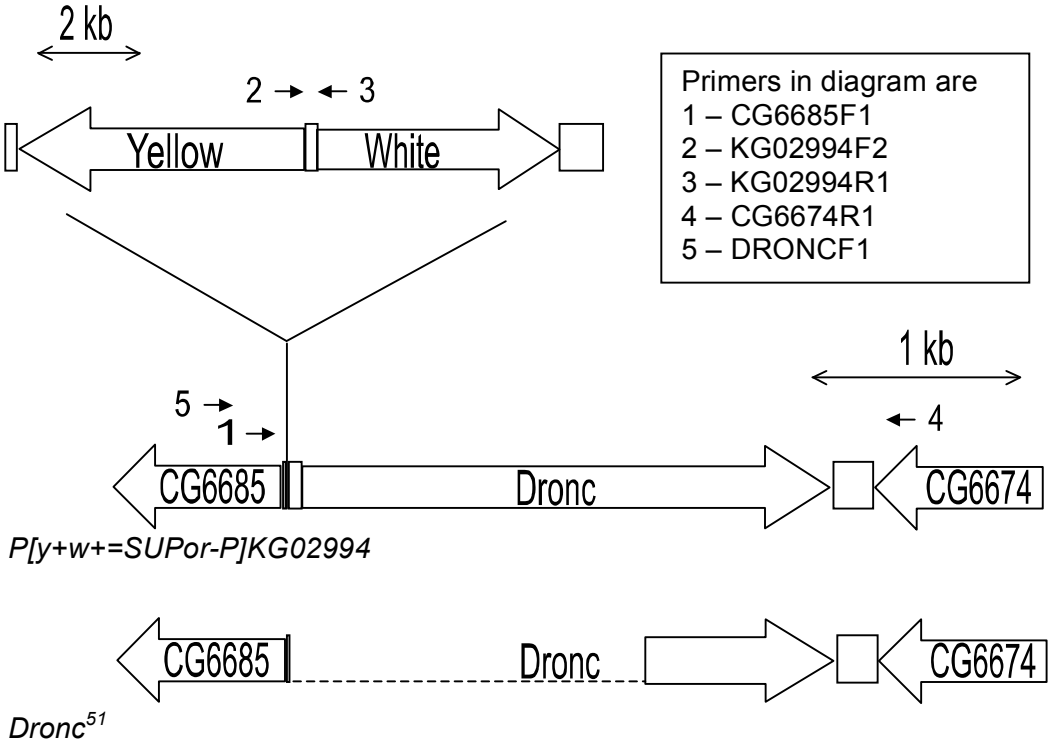


Figure 2.1 – Mutagenesis at the *Dronc* locus
Schematized genomic structure of the *dronc* locus. The starting P insertion *KG02994* and the resultant allele *dronc*^{51} is shown together with the primers used to screen for the deletion. *KG02994* is a transposon that maps between *dronc* and the neighboring gene CG6685 (upper schematic). *dronc*^{51} is the result of an imprecise excision of the mobilised P-element, producing a deletion that eliminates sequences from the P insertion site to codon 303 of the *dronc* ORF. The deletion eliminates the P element and most of the *dronc* locus (indicated by the dashed line), while leaving the CG6685 locus intact.

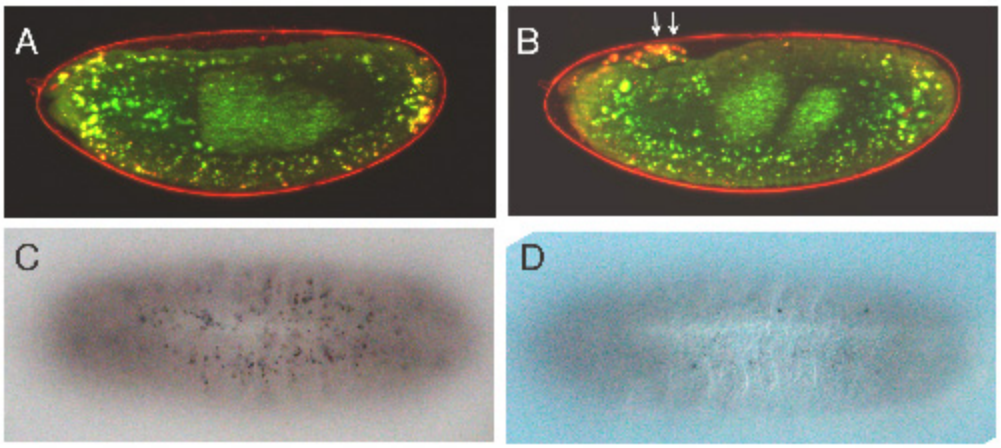


Figure 2.2 – *Dronc*$^{-}$ embryos are defective for programmed cell death (PCD) and head involution.
Embryos were stained with acridine orange (A and B) and TUNEL (C-F) to visualise PCD. Note that *Dronc51* embryos shown here were all progeny of *Dronc*^{51}*/KG02994* female flies.
(A and B) Comparably staged wild type *yw* (A) and *Dronc*^{51} (B) embryos. The arrows in (B) indicate ectopic tissue, persisting from the dorsal ridge, which is characteristic for the head involution defect.
(C and D) Ventral views of late stage 12 *yw* (C) and *Dronc*^{51} (D) embryos, showing the incidence of apoptosis detected by TUNEL in the epidermis.

(Figure by Po Chen, from Chew et al, 2004)

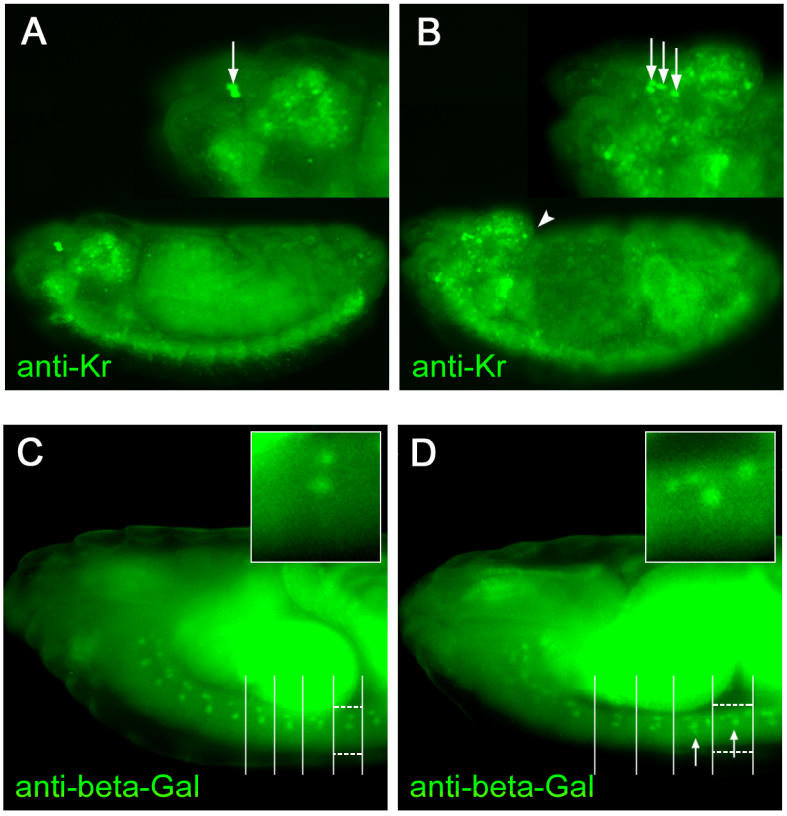


Figure 2.3 – *Dronc*^{51} embryos exhibit hyperplasia of neuronal tissues
(A and B) Stage 15 embryos labeled with α-kruppel antibody. Insets show enlarged dorso-anterior region of the same embryo. (A) wild type (*yw*) embryo with *Kruppel* immunoreactivity indicated in the optic organ (thin arrow). (B) *dronc*^{51} embryo with extra cells associated with the optic organ (arrows) and dorsal ridge abnormality associated with head involution defect (arrowhead).
(C and D) Late-stage embryos expressing the *p[1.0Slit-LacZ]* marker stained with α-β-Gal antibody to label *Slit* positive cells. Wild type embryo (C) and comparably staged *p[1.0Slit-LacZ]; dronc*^{51} embryo (D) are shown with segmental boundaries in the nerve cord (white lines). Insets show a single segment from the nerve cord of the same animal (boxed). Note that 2-3 *Slit-LacZ* positive cells are seen in each segment of wild type animals whereas some segments of *p[1.0Slit-LacZ]; dronc*^{51} embryos show 4-5 *Slit-LacZ* expressing cells (arrows in D). Inset in D shows a single segment with 4 *Slit-LacZ* positive cells. Fluorescence associated with gut is non-specific.

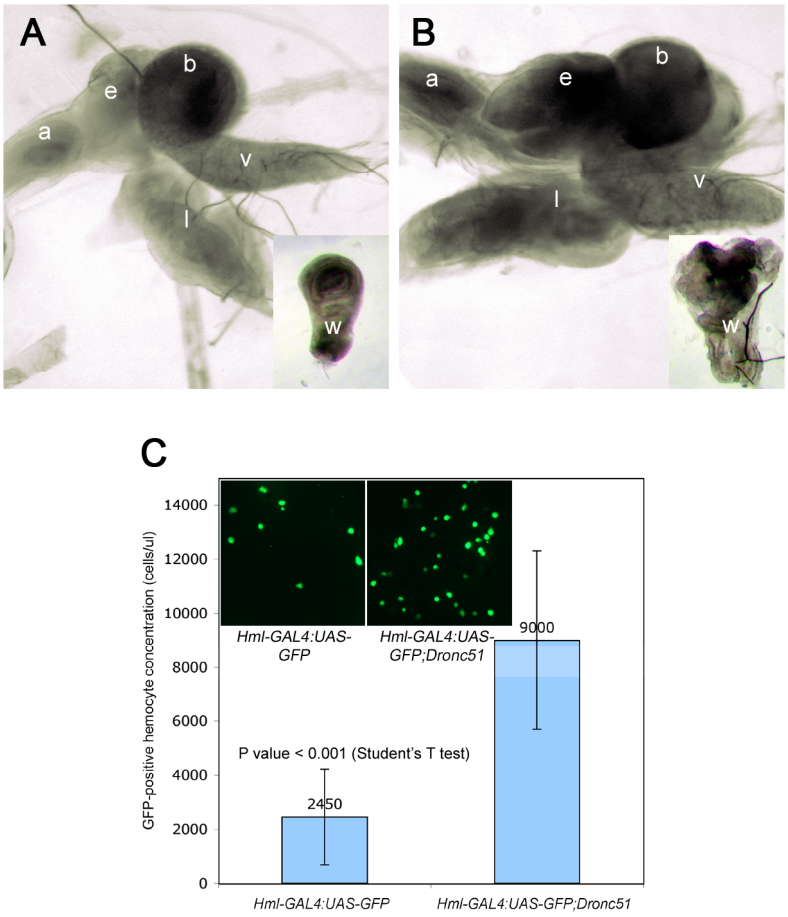


Figure 2.4 – *Dronc*^{51} larvae exhibit hyperplasia of imaginal organs and haemocytes
(A and B) *yw* (A) and *dronc*^{51} (B) wandering 3rd-instar larvae dissected to show the major central nervous system organs and imaginal discs. Labels are a, antenna disc; e, eye disc; b, brain; l, thoracic leg disc; v, ventral nerve ganglion; w, wing disc (inset).
(C) Hyperplasia of blood cells in *dronc* mutant animals was detected using *Hml-GAL4:UAS-GFP*, a marker chromosome for hemocytes. The figure quantifies GFP-expressing hemocyte concentration in wild type and *dronc*^{51} wandering 3rd-instar larvae. Insets are low magnification views of the GFP-expressing hemocytes from hemolymph slide spreads. Error bars denote standard deviation, *yw* n=8 independent dissections, *Dronc*^{51} n=6 independent dissections.

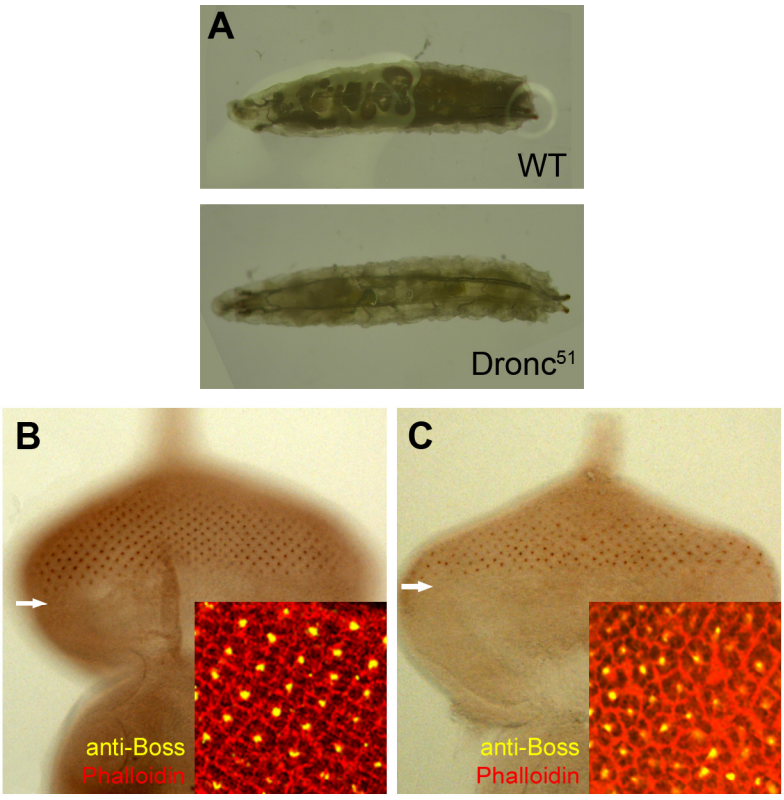


Figure 2.5 – *Dronc* impacts tissue patterning in the eye
(A) Whole mount of *yw* (wild type) and *Dronc*^{51} wandering 3rd-instar larva, *Dronc* larvae develop slower but eventually reach larger sizes than wild type larvae.
(B and C) Eye discs from yw (B) and dronc51 (C) wandering 3rd-instar larvae probed with antibody against Bride of Sevenless (Boss). Inset show close-up of cells double-labelled with α-Boss antibody (yellow) and phalloidin (red, which stains the actin cytoskeleton). Arrow shows approximate position of morphogenetic furrow progressing from posterior (top) to anterior (bottom), showing similar progression of eye development, even though the *Dronc*^{51} larva is older and larger than the wild type larva.

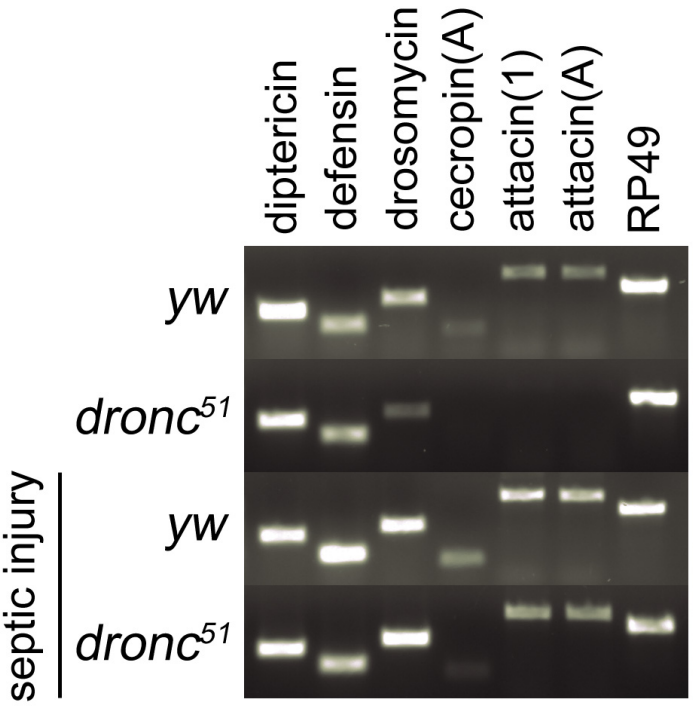


Figure 2.6 –*Dronc*^{51} larva have normal immune responses
Semi-quantitative RT-PCR of gene transcripts genes associated with immune responses (*RP49* is an unrelated loading control). RT-PCR was performed on RNA from *yw* (wildtype) and *Dronc*^{51} wandering 3rd-instar larvae as indicated. Total RNA was harvested from larvae that were untreated or 8-hours post-septic injury. Note that like wild type controls, *Dronc*^{51} animals qualitatively induced expression of the anti-microbial genes defensin, drosomycin, cecropin and attacin after septic injury.

Treatment	Dose	S2R+ killing	*Dronc* RNAi rescue
Actinomycin D	1-100μM	++	partial
Aphidicolin	1-100μg/mL	+++	no
Camptothecin	0.2-20μM	-	
Cycloheximide	10μM-1mM	+	yes
Dexamethasone	1-100μM	-	
Ecdysone	10-100μM	+	
Ethanol	0.1-5%	++	partial
Etoposide	10μM-1mM	+	
Hydrogen Peroxide	0.1-10mM	+	partial
Methyl methanesulfonate	0.001-2.5%	+++	no
Nicotine	10μM-10mM	-	
4-nitro-quinoline-N-oxide	1-1000ng/mL	+++	partial
Smac-mimetic	0.1-100μM	+++	yes
Staurosporine	0.5-50μM	++	
UVC 254nm	10-200mJ/cm^2	++	yes
X-ray ionising radiation	5-40kRads	-	

Table 2.1 – Summary of stress-induced death in S2R+ cells
In dose titration experiments, the relative killing potency measured by CellTiter-Glo viability assay after 24h treatment is denote by +s (+, detectable cell death; ++, significant cell death; +++, most cells killed). For moderate to strong death stimuli, the ability of *Dronc* RNAi to reverse cell viability relative to healthy untreated cells is tested.

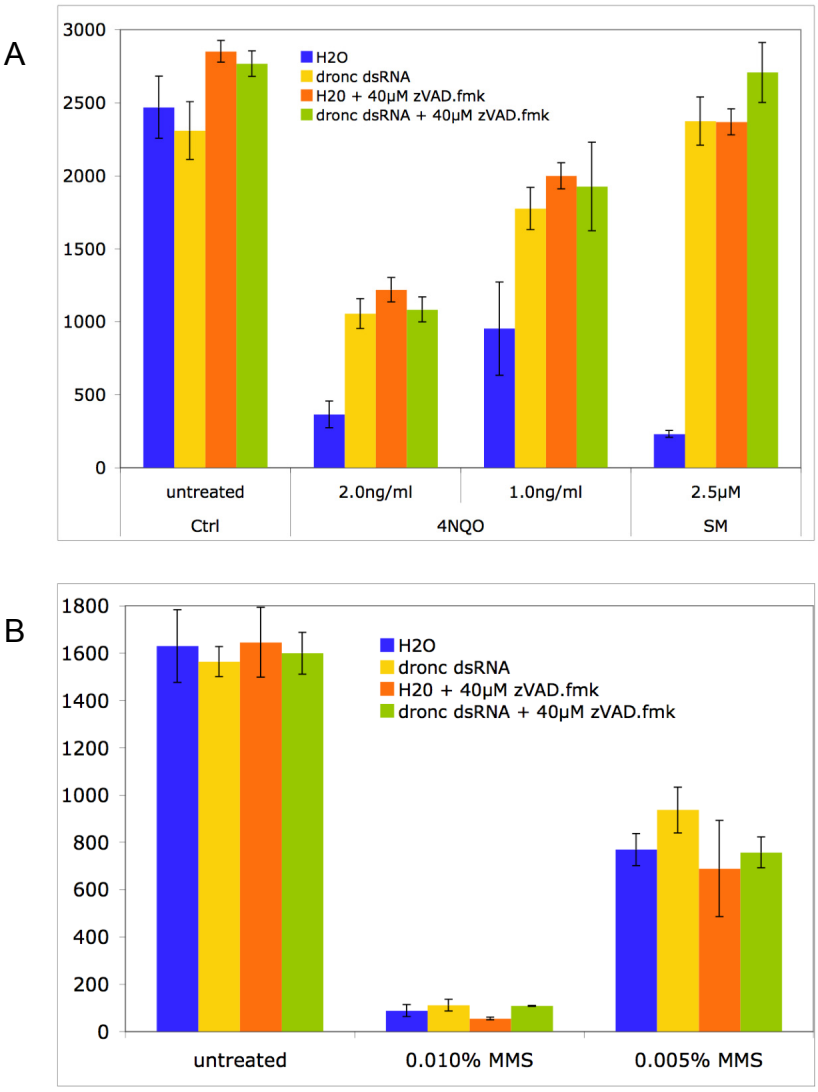


Figure 2.7 – *Dronc* functions in stress-induced apoptosis
Effects of *Dronc* depletion by RNAi in contexts of stress-induced cell death where blocking caspase-dependent apoptosis with zVAD.fmk results in full rescue (A) (Smac mimetic, SM), partial rescue (A) (4-nitro-quinoline-N-oxide, 4NQO) or no rescue (B) (methyl methanesulfonate, MMS). Y-axis in graphs measures cell viability, units are arbituary fluorescent units from assaying ATP levels in each well, error bars denote standard deviation, n=3 independent wells of cells in each experiment.

CHAPTER THREE

THE SEARCH FOR NOVEL APOPTOTIC DETERMINANTS

ABSTRACT

Apoptosis is a conserved form of programmed cell death (PCD) with pivotal importance in the regulation of cell numbers during development, aging and disease progression (Baehrecke 2002; Reed 2003; Danial and Korsmeyer 2004; Yuan 2006). Central to the core machinery of apoptosis are the caspases and their regulators that are conserved across flies, worms and mammals (Lee and Baehrecke 2000; Salvesen and Abrams 2004; Kumar 2006; Bao and Shi 2007). In order to obtain a comprehensive understanding of components that support caspase-dependent cell death, I used systematic RNA interference (RNAi) mediate gene silencing to query gene function in cultured cells treated with a chemical antagonist of Inhibitor of Apoptosis Proteins (IAPs) that simulates the action of native regulators in the Reaper/Smac family (Chai et al. 2000; Verhagen and Vaux 2002; Li et al. 2004). I conducted a pilot screen using a dsRNA library targeting phosphatases and kinases in the *Drosophila* genome, and subsequently a genome-wide screen. The genome-wide primary screen identified 42 potential hits. Using a different RNAi library in a secondary screen followed by secondary assays using distinct non-overlapping RNAi amplicons and distinct apoptotic stimuli, a highly validated set of targets necessary for death provoked by multiple stress contexts was identified.

INTRODUCTION

An emergent model for apoptosis control that coalesces the collective observations on caspase regulation centers around the interplay between activating signals and repression (Salvesen and Abrams 2004; Hay and Guo 2006). Positive apoptogenic regulation through APAF-1/Dark/CED-4 adaptor proteins to activate the caspase zymogens (Green 1998; Salvesen and Renatus 2002) occurs in combination with the removal of repression exerted by the Inhibitor of Apoptosis Proteins (IAP) pathway (Salvesen and Abrams 2004). While the roles of many conserved components have been elucidated, fundamental questions and indications of unidentified regulators remain (Thompson 1995; Jacobson et al. 1997; Danial and Korsmeyer 2004; Hay and Guo 2006; Schafer and Kornbluth 2006).

The recent emergence of RNA inteference in cultured cells as a screening platform has enabled high throughput screens interrogating for functional properties of interest (Boutros et al. 2004; Eggert et al. 2004; Cherry et al. 2005; Zhang et al. 2006; Goshima et al. 2007). Genome wide silencing platforms in *Drosophila* offer important advantages linked to reduced pathway complexities and reduced intervention since direct soaking methods can bypass transfection and its associated stresses (Clemens et al. 2000; Worby et al. 2001).

Additionally, several small molecule mimetics to components in the apoptosis pathway have been developed recently (Li et al. 2004; Sun et al. 2004; Perez-Galan et al. 2007). These small molecule mimetics present the potential to conceptually query

pathways by activating apoptosis using a mimetic, while potentially avoiding “noise” and secondary effects from upstream or parallel stimulus-specific signalling pathways.

With technical assistance from Kristi Pogue, I developed and conducted an RNAi screen to identify components needed for apoptosis in Drosophila S2R+ cells stimulated with a small molecule mimetic of Smac. The Smac mimetic is a functional mimetic of *Drosophila* IAP antagonist proteins in contexts related to cellular phenotype, molecular interactions with DIAP1 and genetic epistasis. The whole genome dsRNA library used in the primary screen covers annotated genes from *Drosophila* genome build 3. A secondary screen was done using a different dsRNA library, and I have gone on to prioritize a small set of genes for characterization using two further non-overlapping dsRNA amplicons in different assays and apoptosis triggers. This work captured a small set of highly validated hits that regulate apoptosis at different parts of the pathway and in different death contexts.

MATERIALS AND METHODS

Cell culture

S2R+ cells were cultured in Schneider's media with 10% Fetal Bovine Serum, 25U/mL penicillin, 25µg/mL streptomycin at 25°C (all cell culture reagents from Invitrogen except FBS, Atlas Biologicals). Unless otherwise indicated, media in all procedures refer to the above.

Primary whole genome RNAi screen

The whole genome RNAi library (Silencer, Ambion) was plated in 96-well microplates (Corning) using liquid handlers (Beckman FX). Each well contained 0.5µg dsRNA in 20µl of SF900 media. 18,000 cells in 30µl serum/antibiotic-free Schneider's media were seeded in each well and incubated for 1 hour before adding 100µl media. 3 days later, the media was replaced with 80µl media containing 2.5µM SM. Cell viability was assayed 2 days later using CellTiter-Glo (Promega) in a plate reader (Envision multimode).

Statistical analyses

The plate mean centered *z*-score for each well is its luminescence value minus the plate average, divided by the plate standard deviation (n=92 sample wells). The Z-factor for each plate was calculated as described (Zhang et al. 1999), in this case $Z = 1 - [(3\sigma_s + 3\sigma_c) \div |(\mu_s - \mu_c)|]$ (where σ_s= variance of 92 samples; σ_c=variance of 4 dronc controls; μ_s=sample mean; μ_c=control mean). To correct for systematic bias/edge effects

(Malo et al. 2006), the position mean centered *z*-score for each well is calculated by its plate mean centered *z*-score minus the position average, divided by the position standard deviation (n=143 plates per triplicate). Genes with $z>3.1$ from either plate mean or position mean centered normalizations were considered together as primary candidates.

RNAi procedure

For secondary screens and subsequent RNAi experiments, dsRNAs synthesis and treatment was essentially as previously described (Clemens et al. 2000; Worby et al. 2001) (See Appendix A for T7 primers used in dsRNA synthesis). Cells were exposed to 15µg/ml dsRNA in serum-free media for 1 hour, recovered with twice volume of normal serum-containing media and incubated 72 hours to deplete target. Effector caspase (DEVDase) activity was measured using Caspase3/7-Glo (Promega) 6 hours after apoptosis stimulation according to manufacturer's instructions.

Pulldown experiments

Cells were lysed in 20 mM HEPES-KOH [pH 7.5], 10 mM KCl, 1.5 mM MgCl2, 1 mM sodium EDTA, 1 mM sodium EGTA, 1 mM dithiothreitol, 1% Triton-X100 buffer with protease inhibitors (#1836170 Roche). Biotinylated SM was incubated with avidin-conjugated beads and 10mg/ml BSA in PBS for 4 hours at 4 °C. The beads were then incubated with precleared lysate (~1-2 mg protein) overnight before analysis.

Western blot analysis

Proteins were separated by SDS PAGE, transferred to PDVF membranes, and probed with 1:1000 anti-DIAP1 (Lisi et al. 2000), 1:1000 anti-Dronc (Yoo et al. 2002), 1:1000 anti-Dark (Akdemir et al. 2006), 1:5000 anti-tubulin (E7, Developmental Studies Hybridoma Bank, University of Iowa).

RESULTS

A Smac-mimetic compound exhibits broad cross-species IAP antagonist activity

Mammalian Smac proteins are thought to be functional orthologues of the *Drosophila* IAP antagonists necessary for PCD, referred to as RHG proteins (*Reaper*, *Head Involution Defective*, *Grim, Sickle, Jafrac*) (Hay and Guo 2006). Therefore, we tested the possibility that a small molecule Smac-mimetic (SM) (Li et al. 2004) might also simulate the action of RHG proteins in *Drosophila* cells. RHG proteins have several defining traits. First, they tightly bind DIAP1, a central brake against caspases (Wu et al. 2001; Chai et al. 2003; Yan et al. 2004). Second, they induce rapid apoptosis when overexpressed (White et al. 1994a; Grether et al. 1995; Chen et al. 1996b). Third, RHG killing is prevented by caspase gene ablation or peptide inhibitors (White et al. 1994a; Grether et al. 1995; Chen et al. 1996b). Using avidin beads to pellet a biotinylated version of the SM compound in a pull-down assay, we found that, like RHG proteins (Shi 2002a; Shi 2004), the SM compound specifically bound DIAP1 (Fig. 3.1a). In *Drosophila* cultured cells, the SM compound also induced stereotypical apoptotic cell death (Fig. 3.1b, supplementary movies). I tested and observed killing in KC167, S2 and S2R+ cells (shown here). The SM-indcued killing was completely reversed either by peptide caspase inhibitors or RNAi depletion of the apical caspase *Dronc* (Fig. 3.1c). These results recapitulate observations on haemocytes mutated for apoptosomal components which also show resistance to SM induced killing (Chew et al. 2004; Akdemir et al. 2006). Together, these findings show that the SM compound is a molecular mimetic of RHG proteins and similarly constrained by canonical apoptosis determinants.

Assay development and pilot screen

In order to develop an assay in a format suitable for high throughput screening (HTS) using a whole genome dsRNA, I modified the standard RNAi procedure for target depletion in *Drosophila* S2 cells (Clemens et al. 2000; Worby et al. 2001). First, I used S2R+ cells as they are adherent, which facilitated manipulations during the screen. Second, I added an additional wash for the cells after typsinization to completely remove trypsin during seeding. Third, the protocol was scaled down to a 96-well microplate format and I used a lower seeding density (18,000 cells/well or ~56,000 cells/cm^2).

A pilot screen was conducted to evaluate the suitability of the assay for HTS (Figure 3.2a). A library targeting the phosphatases and kinases in the genome was used (Lum et al. 2003). Each experimental pass during the pilot screen had a control plate containing 10 wells of each control dsRNA targeting *Dronc*, *Dark* and GFP. The Z-factor was used as a metric for assay quality, based on its the signal separation, variation of the baseline measurement and variation of reference positive control (Zhang et al. 1999) (see methods). The quality of the assay was consistently high in the pilot screen plates treated with the SM compound. I also tested UV as a killing trigger in the pilot screen, but found less plate to plate consistency using this stimulus.

The contribution of each gene to an apoptotic response triggered by SM was measured by cell viability, and the *z*-score (a different measurement from Z-factor) is calculated by the number of standard deviations each well is from the global mean. The pilot screen did not produce many significant hits as there was only 1 gene with $z>3.1$ ($P<0.001$) for SM induced killing (Figure 3.2b) and 3 genes with $z>3.1$ for UV (Figure

3.2c). Gratifyingly, the top hit for SM induced killing *skittles* (a PIP-5-Kinase), was also a hit in UV, showing that the assay could consistently recover hits across different stimuli. *sktl* was subsequently recovered in the main screen and shown to affect caspase activation (Tables 3.1, 3.3).

A genome-wide RNAi screen identifies pro-apoptotic targets

I conducted an RNAi-based genome scale screen to identify essential components of the apoptosis pathway (Fig. 3.3a, methods). The assay rationale captured targets that prevent SM-induced killing when silenced (Fig. 3.3c). Each dsRNA was sampled in triplicate, and all assay plates included 4 wells containing *Dronc* dsRNA as benchmark controls. These internal reference standards permit self-contained appraisals of assay quality, monitoring both the dynamic range and the capacity for dsRNA-mediated protection in each plate. The assay quality, as quantified by the Z-factor was consistently high, of 429 sample plates assayed in the screen, 76% (325 plates) had excellent assay quality ($Z \geq 0.5$) and only 2% were unacceptable ($Z < 0$) (Zhang et al. 1999).

High reproducibility was observed for most dsRNAs tested in the library, with scatter plots for the triplicate samples showing impressive correlation coefficients between 0.674 and 0.696 (Fig. 3.3b). Using both plate and position mean centering analyses (see methods), I identified 42 candidate genes with protective activity meeting or exceeding a stringent threshold of *z*-score greater than 3.1 (Table 3.1). These included the expected benchmarks *Dronc* and *Dark*, as well as the effector caspase *Drice* (Fig. 3.3c, Table 3.1).

Secondary screen and characterization of candidate genes

To prioritize apoptogenic candidates, 33 of the 42 candidate genes with *z*-score greater than 3.1 were re-tested using a different dsRNA amplicon (Boutros et al. 2004). To evaluate the biological relevance of this threshold, a sample of 42 genes conferring less potent activity ($2.1<z<3.1$) were also included in this retest collection (Table 3.2). Notably, 19 candidates retested at least 3.1 standard deviations above the control mean, 16 of which had $z>3.1$ in the original screen, while only 3 that survived this retest standard originally had $2.1<z<3.1$. Hence, the *z* threshold was experimentally relevant and highly predictive for reproducibility across different dsRNA amplicons.

From these analyses, I selected a set of 13 high rank targets (Table 3.3) for further characterization based on conservation (Homologene, NCBI) and/or implied links to human disease or development (Online Mendelian Inheritance in Men, NCBI). For each member in this gene set, an additional pair of non-overlapping dsRNA amplicons was synthesized and used in subsequent experiments as additional controls to exclude off target effects (Kulkarni et al. 2006; Ma et al. 2006).

General apoptotic determinants should function outside of the original test system. Therefore, the strategy for biological validation applied both a different assay and different apoptotic triggers. Here, the effects of target silencing were assessed by measuring effector caspase activity provoked either by UV or cycloheximide (Table 3.3), two well-established apoptotic stimuli (Zimmermann et al. 2002; Kiessling and Green 2006). The data in this table permit assessment of the range of activity for a given target and discriminate context-specific versus general functions. Several gene classes emerged

from the results in Table 3.3 – genes that have substantial activity in multiple stimuli (*Drice*, *Hrb27C*, *enok*, *Tango7*), those with protective activities that varied with stimuli (*CG32626*, *CG7275*), and those that have mild but significantly consistent activity (*SNF4Agamma*, *eIF5*, *sktl*).

DISCUSSION

Even though parallels of the main conserved components of apoptosis are conserved across divergent species (Figure 1.1), the considerable divergence that occurs across the different phylogenies suggests that key regulatory points and nodes change (Kornbluth and White 2005; Hay and Guo 2006). Significantly, the main axes of regulation – positive activation through the apoptosome and derepression of the IAPs, are well conserved and highlight caspase activation as the focal point for apoptosis regulation. Model systems with reduced complexity like the fly and the worm are poised to answer fundamental questions about regulating caspase activation, such as whether the apoptosome is a fundamentally required complex for activating caspases. Removing components of the fly apoptosome results in loss of most but not all developmental PCD (Chew et al. 2004; Daish et al. 2004; Waldhuber et al. 2005; Xu et al. 2005; Akdemir et al. 2006; Kondo et al. 2006; Mills et al. 2006; Srivastava et al. 2006), in contrast to the total ablation seen in removal of the IAP antagonists *Reaper, Grim, Hid* (RHG) genes H99 animals (White et al. 1994a). This raises the possibility that there might be alternate pathways of bypassing the apoptosome to activate caspases. In mammals, a recent study using a 'knock-in' non-apoptogenic allele of cytochrome *c* in mouse showed that the positive apoptogenic signalling through the mammalian adapter Apaf-1 can bypass cytochrome *c* and apoptosome formation (Hao et al. 2005). Interestingly, while regulation of positive signals represents a crucial regulatory axis in worms and mammals (Meier et al. 2000a; Danial and Korsmeyer 2004), no definitive upstream regulators of *Dark* and *Dronc* have been identified. Both Bcl-2 proteins and cytochrome *c* show no direct

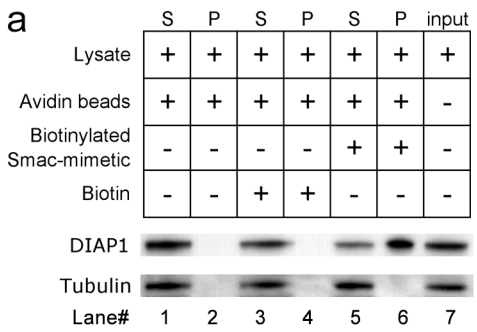
involvement in apoptosis regulation (Galindo, manuscript in preparation) (Zimmermann et al. 2002; Dorstyn et al. 2004; Means et al. 2006; Yu et al. 2006; Sevrioukov et al. 2007). Which brings to fore the question of what (if any) upstream regulators activate the apoptosome or caspases in *Drosophila*?

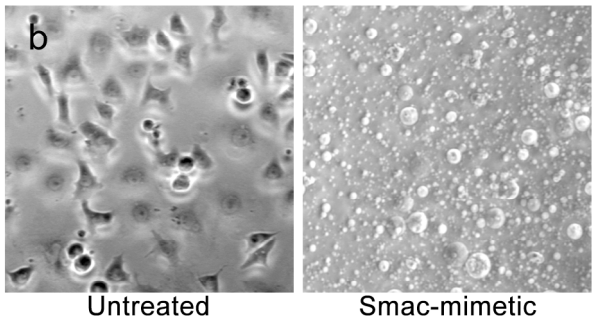
The screen presented here is a systematic effort to use RNAi silencing to identify genes required for apoptosis. The availability of the Smac-mimetic (SM) compound (Li et al. 2004) was crucial for this project, and is the result of extensive work from the Harran and Wang labs. Like any screen, genetic or chemical – the quality of the primary assay determines the stringency and ability of the screen in identifying hits (Zhang et al. 1999; Armknecht et al. 2005; Malo et al. 2006). Exhibiting molecular properties of a direct IAP antagonist, the SM represents a proximal activator of apoptosis. I originally proposed to use a separate stimuli (UV) in a second screen to generate multiple whole genome datasets for comparisons, but I found that UV as a stimulus for killing cells in the 96-well microplate format is variable from one irradiation exposure to the next, and therefore would obscure the signal to noise separation in a high throughput screen. This increased baseline could also arguably come from the fact that UV is a distant stimulus to caspase activation compared to an IAP antagonist, and signal transduction could add additional noise and variability to the system.

A secondary advantage of the SM compound as an apoptotic stimulus is that it theoretically activates apoptosis in an otherwise normal cell state. By comparison, activating apoptosis using genotoxic or cytotoxic stressors activate additional damage repair and signaling pathways, increasing the potential that the signal from a downstream apoptotic regulator can be masked or complicated by other responses. I have observed

that for many stress-induced apoptosis contexts, while eliminating *Dronc* or *Dark* can attenuate the apoptosis response, the cells still undergo arrest and/or other forms of cell death due to the secondary effects from the stress. In cells that have been treated with SM and blocked for cell death, removal of the SM compound results in growth and proliferation, lending sensitivity to the ability to detect genes required for apoptosis. These observations highlight not only the importance of developing a suitable assay in any screen, but also argues for the possibility of similar screens recovering different hits based on where the input stimulus impacts the pathway of interest. This underscores the the opportunities that the SM compound presents as a point activator of apoptosis.

The 13 high priority targets that I chose to characterize for their effects on caspase activation have not been implicated in *Drosophila* apoptosis and represent novel points of caspase regulation. Human lkb1 is known to associate with p53 and regulate p53-dependent apoptosis pathways (Karuman et al. 2001). Human MYBL2, which mostly closely resembles *Drosophila Myb*, has been shown to regulate Bcl-2 and survival (Lang et al. 2005). Interestingly, overexpression of PIP5KIalpha (homolog of *sktl*) has been shown to protect against caspase activation (Mejillano et al. 2001). For this subset of genes, the observations in mammalian systems illuminate a potential conservation of signaling pathways that impact apoptosis. The other genes in the high priority target list also have human homologs, some of which are implicated in disease pathologies with unknown mechanisms. Future work on these hits will likely yield immediate new insights on the proximal regulation of caspase activation.


a
S P S P S P input
Lysate + + + + + + +
Avidin beads + + + + + + -
Biotinylated Smac-mimetic - - - - + + -
Biotin - - + + - - -
DIAP1
Tubulin
Lane# 1 2 3 4 5 6 7


b
Untreated
Smac-mimetic

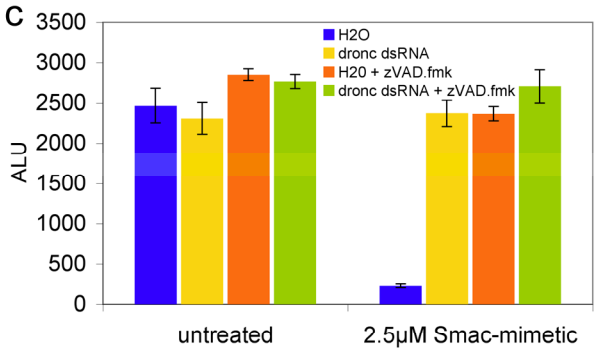

c
3500
3000
2500
2000
1500
1000
500
0
ALU
H2O
dronc dsRNA
H20 + zVAD.fmk
dronc dsRNA + zVAD.fmk
untreated
2.5μM Smac-mimetic

Figure 3.1 – A smac-mimetic compound exerts broad cross-species IAP antagonist activity

(a) A compound developed as a small molecule mimic for human Smac was biotinylated (Li et al. 2004) and incubated with cell free lysates. Binding to DIAP1 was detected in pellets of avidin-conjugated beads from whole cell lysates probed with anti- DIAP1 (lane 6). Neither the avidin-conjugated beads alone, with or without biotin bound to DIAP1 (lanes 2 and 4) (S – supernatant; P – avidin beads pellet). Blotting for tubulin is used as a control for specificity and for loading (lanes 1, 3, 5 and 7).

(b) The Smac-mimetic provokes acute apoptosis in Drosophila S2R+ cultured cells. Images here are after 24 hours, but overt apoptotic blebbing can be seen within 6 hours of treatment (Supplementary Movies). The compound triggers similar effects when applied to other fly cell lines (not shown) and primary cells cultured *ex vivo* (Chew et al. 2004; Akdemir et al. 2006).

(c) RNAi mediated depletion of *Dronc* (yellow) or the pan-caspase inhibitor, zVAD.fmk (orange) or a combination of both treatments (green) fully prevents killing triggered by the Smac-mimetic. The y-axis plots viability measured using the CellTiter-Glo assay (ALU denotes arbitrary luminescence units, error bars show ±SD, n=3 different wells of cells from the same experiment).

a

	Drosphila Phosphatase/Kinase dsRNAi library plate #				
	I	II	III	IV	V
Smac Mimetic	2 passes	2 passes	3 passes	3 passes	3 passes
UV	1 pass	1 pass	1 pass	1 pass	1 pass

b

Protection of viability – Smac mimetic challenge	
Gene	z-score average
sktl	3.93
HIPK	2.39
PI4K	2.28
CKIIb1	1.98
PIP5K 59B	1.94

c

Protection of viability – UV challenge	
Gene	z-Score
CKIa-like	4.05
CAKI	3.62
sktl	3.11
PELLE/IL-1	2.28
PIP5K 59B	2.27
p38a	2.21
CKI-like	2.18
HIPK	2.18

Figure 3.2 – Pilot screen using phosphatase-kinase dsRNA library
(a) Summary of the pilot screen done using a *Drosophila* phosphatase kinase library (Lum et al. 2003) that comprises 5 96-well microplates (numbered I –V), the number of trials each plate was assayed using either Smac-mimetic or UVC stimulation is indicated. (b and c) Genes from the pilot screen with the highest plate-mean centered *z*-scores for each stimuli are shown. (b) shows potential genes that rescue cell viability (assayed by CellTiter-Glo) after treatment with Smac-mimetic, the *z*-scores are averaged from the multiple passes as indicated in (a). (c) shows the z-scores of potential hits in the context of UV-induced death from the single pass of the library. Note that the *sktl* which is the gene with the highest *z*-score in the overlap of both stimuli is also capture in the whole genome screen and subsequent validations (See results and Tables 3.1, 3.3).

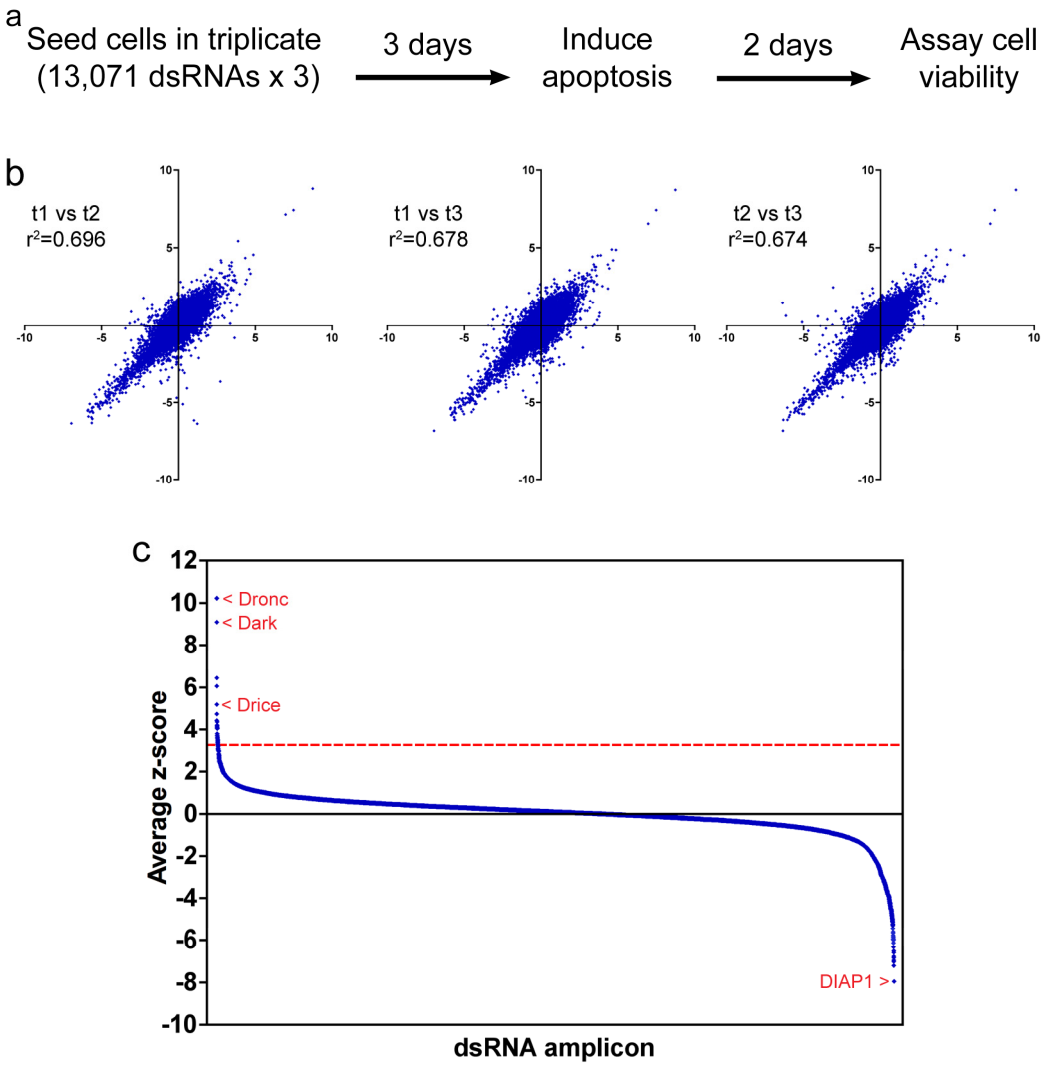


Figure 3.3 – A genome-wide screen captures apoptogenic effectors
(a) Screening protocol. 18,000 cells/well were seeded with media containing dsRNAs 3 days prior to challenge by the Smac-mimetic and were assayed for viability 2 days later.
(b) Scatter plots demonstrate high reproducibility among triplicated samples. The plate mean centered *z*-scores for triplicate samples of each dsRNA (t1, t2 and t3) are plotted against each other as shown (r^2 denotes correlation coefficient).
(c) The average position mean centered *z*-score for each amplicon was calculated from the individual z-scores of the triplicates and plotted in rank format as shown. Arrowheads highlight 'landmark genes' in the apoptosis pathway with highly significant pro- and anti-apoptotic activity. Dotted line show z=3.1.

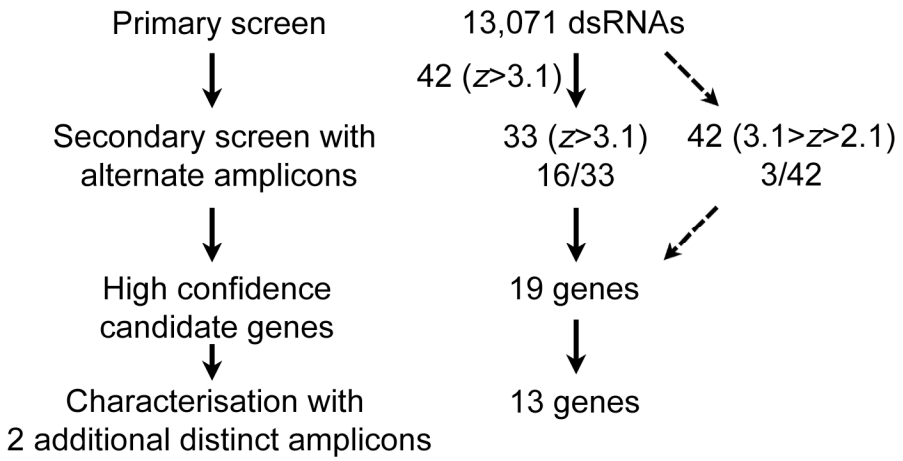


Figure 3.4 – Flow chart schematic for validation of candidates genes in the screen
From the primary genome-wide screen, 42 genes had plate mean or position mean centered z-scores greater than 3.1. 33 of these genes and a sample of 42 genes below the threshold were re-tested using distinct amplicons obtained from a different dsRNA collection (Boutros et al. 2004). 16/33 of the z>3.1 retested with at least 3.1 SDs above control mean (Table 3.3), while 3/42 of the z<3.1 genes retested 3.1 or more SDs above control mean (Table 3.4). Data from these secondary tests, combined with information on human homologs was used to select a collection of 13 high rank genes (see Table 3.2) for further characterization.

CG number	Full Name	Symbol	Plate mean ctr z-average	Position mean ctr z-average	Secondary, SDs above ctrl
CG10002	fork head	fkh	**3.7**	2.2	-1.4
CG10377	Heterogeneous nuclear ribonucleoprotein at 27C	Hrb27C	**4.4**	**4.4**	**4.3**
CG11081	plexin A	plexA	1.7	**3.1**	
CG11290	enoki mushroom	enok	**3.3**	2.7	**3.4**
CG12837	Tetraspanin 42Er	Tsp42Er	3.1	**3.4**	0.6
CG13613	CG13613	CG13613	**3.5**	2.7	0.1
CG14543	CG14543	CG14543	**3.4**	**3.3**	**7.0**
CG1666	Helicase	Hlc	**3.2**	2.2	1.3
CG1703	CG1703	CG1703	**3.8**	**3.5**	1.1
CG17209	CG17209	CG17209	**3.4**	**3.7**	2.5
CG18817	Tetraspanin 42Ea	Tsp42Ea	**3.4**	2.2	-1.0
CG1882	CG1882	CG1882	2.4	**3.2**	-1.9
CG2221	lethal (1) G0289	l(1)G0289	**3.4**	2.8	-0.2
CG2238	Elongation factor 2b	Ef2b	**3.2**	**3.6**	1.0
CG2275	Jun-related antigen	Jra	**3.2**	3.1	1.7
CG30219	CG30219	CG30219	1.7	**4.1**	
CG31768	CG31768	CG31768	1.7	**3.5**	
CG31789	CG31789	CG31789	2.6	**3.2**	1.5
CG32504	CG32504	CG32504	**3.1**	2.4	
CG32626	CG32626	CG32626	**4.2**	**6.1**	**14.5, 12.7,** 0.7
CG3845	lethal (2) 01424	l(2)01424	2.4	**3.2**	**3.3**
CG40084	CG40084	CG40084	**3.4**	**4.1**	**7.1, 4.4**
CG40234	CG40234	CG40234	2.9	**3.7**	
CG4182	yellow-c	yellow-c	2.8	**4.2**	-2.1
CG5370	Death caspase-1	Dcp-1	3.0	**5.2**	1.6
CG5670	Na pump alpha subunit	Atpalpha	**6.9**	**6.4**	**11.8**
CG5778	CG5778	CG5778	1.2	**3.1**	
CG6755	Elongin A	EloA	2.3	**3.6**	-0.8
CG6829	Apaf-1-related-killer	Dark	**7.5**	**9.1**	**48.8**
CG7118	Jonah 66Ci	Jon66Ci	1.1	**3.3**	
CG7438	Myosin 31DF	Myo31DF	**3.7**	**4.2**	0.8
CG7756	Heat shock protein cognate 2	Hsc70-2	**3.1**	**3.6**	-1.5
CG7788	Ice	Drice	**4.6**	**4.7**	**9.4**
CG8091	Nedd2-like caspase	Dronc	**8.8**	**10.2**	**49.6**
CG8309	Tango7	Tango7	2.1	**3.4**	
CG8344	RNA polymerase III 128kD subunit	RpIII128	2.2	**3.5**	
CG8527	pickpocket 23	ppk23	**3.3**	**4.4**	**4.1**
CG8936	Arpc3B	Arpc3B	**3.6**	**3.4**	**3.4**
CG9045	Myb oncogene-like	Myb	**4.8**	**3.8**	**5.6**
CG9177	eIF5	eIF5	2.8	**4.0**	**9.7**
CG9374	Ikb1	Ikb1	**3.1**	1.3	**4.2**
CG9985	skittles	sktl	**4.1**	2.4	**5.3**

Table 3.1 – Primary candidates and calculated *z*-scores
Genes that had z-scores greater than 3.1 in plate mean centered or position mean centered normalizations are shown. Where tested in the secondary screen with alternate amplicons, the number of standard deviations above the control genes are also indicated. Data calculated from triplicates in primary screen and duplicates in secondary screen. Bold highlights numbers greater than 3.1.

CG number	Full Name	Symbol	Plate mean ctr z-average	Position mean ctr z-average	Secondary, SDs above ctrl
CG10234	Hs2st	Hs2st	2.6	1.6	1.6
CG10269	D19A	D19A	3.0	3.0	-2.1
CG1065	Succinyl coenzyme A synthetase alpha subunit	Scsalpha	2.5	2.2	-1.9
CG10685	CG10685	CG10685	2.7	1.5	-1.1
CG11008	CG11008	CG11008	2.4	1.8	-1.1
CG11276	Ribosomal protein S4	RpS4	2.4	1.4	0.0
CG11438	CG11438	CG11438	2.3	1.3	1.4
CG13826	CG13826	CG13826	2.8	2.8	-0.5
CG13828	CG13828	CG13828	2.8	3.0	-0.2
CG14401	CG14401	CG14401	2.3	1.1	0.4
CG14612	CG14612	CG14612	2.9	2.3	1.1
CG14735	CG14735	CG14735	2.8	2.2	3.0
CG15021	CG15021	CG15021	2.5	1.8	-2.5
CG15278	CG15278	CG15278	2.4	2.5	-2.3
CG15597	CG15597	CG15597	2.7	2.4	0.4
CG16849	CG16849	CG16849	2.3	1.4	0.8, 0.3
CG16874	Vitelline membrane 32E	Vm32E	2.5	1.7	-2.0
CG17090	CG17090	CG17090	2.3	1.7	0.5
CG17299	SNF4/AMP-activated protein kinase gamma subunit	SNF4Agamma	2.9	2.7	**9.1**, 3.0, -2.5
CG2246	CG2246	CG2246	2.6	1.9	-0.7
CG32282	drosomycin-4	dro4	2.4	3.0	-1.3
CG32304	CG32304	CG32304	2.4	1.8	-0.1
CG33147	CG33147	CG33147	2.4	2.4	2.1, -2.0, -7.6
CG3501	CG3501	CG3501	2.7	2.0	-1.0
CG40415	CG40415	CG40415	2.7	2.5	1.6
CG4074	CG4074	CG4074	2.4	1.2	0.5
CG4184	Mediator complex subunit 15	MED15	2.7	3.0	-0.7
CG4604	Glial Lazarillo	GLaz	2.4	2.5	0.3
CG5436	Heat shock protein 68	Hsp68	2.6	2.5	0.7
CG5476	CG5476	CG5476	2.4	3.0	-2.0
CG5699	CG5699	CG5699	2.7	1.6	-1.8
CG6143	Protein on ecdysone puffs	Pep	2.4	1.7	**6.1**
CG6364	CG6364	CG6364	2.6	2.3	-0.2
CG7275	CG7275	CG7275	2.4	1.6	**3.5**
CG7808	Ribosomal protein S8	RpS8	2.6	1.7	-0.4
CG8029	CG8029	CG8029	2.5	2.1	-0.9
CG8415	Ribosomal protein S23	RpS23	2.5	2.4	0.7
CG8765	CG8765	CG8765	2.5	2.0	-6.2
CG8950	CG8950	CG8950	2.9	3.0	0.7
CG9038	UBL3	UBL3	2.4	2.2	-4.9
CG9305	CG9305	CG9305	2.6	1.6	2.1
CG9753	CG9753	CG9753	2.5	1.6	-0.7

Table 3.2 - Sample of genes with lower z-scores that were retested in secondary screen

42 genes conferring lower rescue activity (plate mean centered $2.1<z<3.1$) that have been tested in the secondary screen with alternate amplicons are shown. Bold highlights numbers greater than 3.1.

% Caspase Inhibition[1] (*t*-Test[2])		UV		Cycloheximide	
Gene (Human)	**Description**	**amplicon 1**	**amplicon 2**	**amplicon 1**	**amplicon 2**
Control					
AmpR	Bacteria gene	0.0 ± 3.3		0.0 ± 3.5	
Known Benchmarks					
Dronc (CASP9)	Apical caspase	63.5 ± 4.9 (*P*=1.6E-10)		71.4 ± 1.8 (*P*=7.6E-13)	
Dark (APAF1)	Caspase activation adaptor	55.0 ± 7.8 (*P*=1.9E-08)		47.0 ± 19.8 (*P*=0.00019)	
Candidates					
Drice (CASP3)	Effector caspase	59.0 ± 7.5 (*P*=7.3E-09)	30.1 ± 8.5 (*P*=1.1E-05)	67.2 ± 12.4 (*P*=1.6E-07)	48.9 ± 24.2 (*P*=0.00063)
Hrb27C (DAZAP1)	Ribonucleoprotein	59.9 ± 6.5 (*P*=2.0E-09)	54.7 ± 4.5 (*P*=3.7E-10)	40.4 ± 16.8 (*P*=0.00019)	27.1 ± 29.5 (*P*=0.049)
enok (MYST3)	Histone acetyltransferase	57.7 ± 5.4 (*P*=7.6E-10)	38.1 ± 4.4 (*P*=1.2E-08)	47.6 ± 14.7 (*P*=1.6E-05)	24.1 ± 13.1 (*P*=0.0014)
SNF4Agamma (PRKAG1)	SNF4/AMP-activated kinase	12.0 ± 10.0 (*P*=0.019)	22.1 ± 3.5 (*P*=5.2E-07)	8.7 ± 6.3 (*P*=0.015)	17.0 ± 4.3 (*P*=2.1E-05)
CG32626 (AMPD2)	AMP deaminase	18.3 ± 4.4 (*P*=1.0E-05)	14.9 ± 2.4 (*P*=4.8E-06)	7.4 ± 3.9 (*P*=0.0062)	2.6 ± 7.6 (*P*=0.46)
CG40084 (CNNM2)	Cystathionine beta-synthase domain	9.6 ± 9.2 (*P*=0.037)	9.9 ± 2.9 (*P*=0.00026)	5.4 ± 3.3 (*P*=0.020)	6.1 ± 10.1 (*P*=0.19)
ATPalpha (ATP1A3)	Na+,K+ ATPase alpha subunit	22.1 ± 3.9 (*P*=1.0E-06)	-12.9 ± 28.8 (*P*=0.30)	4.8 ± 11.3 (*P*=0.34)	-2.7 ± 2.8 (*P*=0.17)
CG7275 (WDSOF1)	WD40, Sof1 domains	27.9 ± 1.2 (*P*=3.0E-09)	30.9 ± 4.0 (*P*=5.0E-08)	9.5 ± 7.6 (*P*=0.020)	14.4 ± 7.0 (*P*=0.0011)
Tango7 (PCID1)	PCI domain	66.0 ± 7.7 (*P*=3.2E-09)	68.0 ± 7.4 (*P*=1.7E-09)	58.4 ± 9.9 (*P*=8.6E-08)	60.9 ± 10.7 (*P*=1.1E-07)
Myb (MYBL2)	Myb oncogene like transcription factor	16.4 ± 4.3 (*P*=2.5E-05)	20.8 ± 3.1 (*P*=5.4E-07)	12.0 ± 9.8 (*P*=0.018)	10.8 ± 17.6 (*P*=0.17)
eIF5 (EIF5)	Translation initiation factor	28.4 ± 5.9 (*P*=1.2E-06)	13.1 ± 2.3 (*P*=1.3E-05)	19.0 ± 2.9 (*P*=1.2E-06)	9.7 ± 9.0 (*P*=0.033)
lkb1 (STK11)	Serine/threonine kinase	5.7 ± 5.4 (*P*=0.051)	36.7 ± 11.2 (*P*=1.7E-05)	3.4 ± 4.0 (*P*=0.15)	10.6 ± 22.1 (*P*=0.27)
sktl (PIP5K1A)	PIP5-kinase	16.1 ± 3.8 (*P*=1.4E-05)	19.4 ± 3.2 (*P*=1.3E-06)	17.7 ± 4.6 (*P*=2.1E-05)	16.2 ± 9.3 (*P*=0.0025)

Table 3.3 – Characterization of high rank candidate genes

13 high rank candidate genes were characterised for their role in apoptosis by measuring caspase activity levels in cells treated with UV or cycloheximide after RNAi depletion of the targets indicated. The annotated human homolog (Homologene, NCBI) of each gene is shown in paranthesis below the *Drosophila* gene name. [1] Caspase activity was measured for each well 6 hours after treatment with 90mJ/cm2 UVC or 250µM cycloheximide. Percent caspase activity was calculated relative to control mean (100%) for each experiment. Data shown are mean percent inhibition ± S.D. from 2 independent experiments (n=3 wells for each experiment). [2] P-values for 2-tail t-tests were calculated by comparing percent inhibition of each gene with the control for each treatment.

CHAPTER FOUR

CHARACTERIZATION OF *CG8309* (*Tango7*)

ABSTRACT

Among the novel apoptotic determinants identified from the whole genome RNAi screen described in the previous chapter, *Tango7* is presented here as a regulator of caspase activation. Cells depleted for *Tango7* resisted apoptosis at a step prior to induction of effector caspase activity. Unlike known PCD regulators in this animal (Kornbluth and White 2005; Hay and Guo 2006), *Tango7* does not influence stimulus-dependent loss of *Drosophila IAP1* (*DIAP1*). Instead, the action of *Tango7* impinged on the apical caspase *Dronc*. *In vivo* studies of *Tango7* functions show that it is involved in the same developmental processes as *Dronc*, *Dark* and other cell death mutants. Together, the work defines a new set of apoptogenic genes and reveals a novel axis of control exerted upon the fly apoptosome, bypassing a canonical brake on caspase function.

INTRODUCTION

The collection of high priority hits identified from the whole genome RNAi screen described in the previous chapter represents the beginning of many new avenues of investigations into the regulation of apoptosis. As discussed previously, their point of entry into the apoptosis pathway is of particular interest, because of collective observations that point to unknown direct regulators of caspases.

To survey the point of regulation by these candidates, I traced the pathway for apoptosis activation in *Drosophila* (Figure 1.1) by doing western blot analyses of *DIAP1*, *Dark* and *Dronc* protein levels in S2R+ cultured cells that have RNAi depleted for the respective genes, in both untreated and after apoptotic stimulation. With the exception of *Tango7*, the other genes had no effects on basal or stimulated changes in protein levels when depleted. As they impact csapase activation, these observations suggest that they could potentially represent direct regulators of effector caspases.

Tango7 (previously *CG8309*) suppressed stress-stimulated cell death and caspase activity induced by multiple triggers to levels comparable to our benchmarks *Dronc* and *Dark*. *Tango7* did not affect basal or stimulus-dependent loss of DIAP1 protein levels, nor steady state levels of the apoptosomal protein Dark. However, silencing of *Tango7* did affect steady-state leavels of pro-Dronc zymogen. These observations suggest that *Tango7* activity impinges proximally on apoptosis regulation at the level of the apoptosome, highlighting it as a regulator of the apoptosome.

MATERIALS AND METHODS

Fly strains

y^1w^{1118};P{vgM-GAL4.Exel}3 and *P{vgMQ-GAL4.Exel}1,y^1,w^{1118}* were obtained from Bloomington stock center. *pUAST-8309R1* was obtained from the National Institute of Genetics Fly Stock Center (Japan).

Cell culture

S2R+ cells were cultured in Schneider's media with 10% Fetal Bovine Serum, 25U/mL penicillin, 25µg/mL streptomycin at 25°C (all cell culture reagents from Invitrogen except FBS, Atlas Biologicals). Unless otherwise indicated, media in all procedures refer to the above.

RNAi procedure

For secondary screens and subsequent RNAi experiments, dsRNAs synthesis and treatment was essentially as previously described (Clemens et al. 2000; Worby et al. 2001) (See Appendix A for T7 primers used in dsRNA synthesis). Cells were exposed to 15µg/ml dsRNA in serum-free media for 1 hour, recovered with twice volume of normal serum-containing media and incubated 72 hours to deplete target. Effector caspase (DEVDase) activity was measured using Caspase3/7-Glo (Promega) 6 hours after apoptosis stimulation according to manufacturer's instructions.

Timelapse microscopy

Photomicrographs were captured at 6-minute intervals on a Zeiss Axiovert 200M inverted microscope equipped with Marzhauser programmable stage, Nikon DXM1200F camera and controlled by Metamorph software (Molecular Devices). Movie images were assembled and analysed using ImageJ (Abramoff et al. 2004).

Western blot analysis

Proteins were separated by SDS PAGE, transferred to PDVF membranes, and probed with 1:1000 anti-DIAP1 (Lisi et al. 2000), 1:1000 anti-Dronc (Yoo et al. 2002), 1:1000 anti-Dark (Akdemir et al. 2006), 1:5000 anti-tubulin (E7, Developmental Studies Hybridoma Bank, University of Iowa).

RT-PCR

Total RNA was isolated from cells using High Pure RNA Isolation Kit (Roche). Superscript One-step RT-PCR System w/ Platium Taq (Invitrogen) was used for RT-PCR reactions (See Appendix A for kdchk primers).

RESULTS

Tango7 is required for stress-induced cell death

Tango7 was identified in a whole genome RNAi screen as a gene that prevents loss of cell viability in Smac-mimetic (SM) induced cell death. To investigate the cellular effects of *Tango7* on the apoptotic process, I visualized *Tango7* depleted cells in timelapse tracking studies after treatment with SM for 12 hours (Figure 4.1A), and after UV irradiation for 22 hours (Figure 4.1B). Like cells silenced for *Dronc*, cells that were silenced for *Tango7* were strongly protected from apoptosis when directly visualized over these time periods (supplementary movies). This strongly contrasted with unrelated dsRNA targeting a control where over 95% of the cells died in the same time period.

Tango7 is required for stress-stimulated caspase activation

To evaluate the impact of *Tango7* on caspase activation, I assayed cells depleted for *Tango7* after treatment with SM and UV. Loss of *Tango7* strongly suppressed caspase activation in these contexts, comparable to the benchmark apoptotic genes *Dronc* and *Dark* (Figure 4.2A, B). A strong attenuation of capase activation was also observed in the context of cycloheximide induced apoptosis (Table 3.3). This pronounced effect on caspase activation was observed in non-overlapping dsRNA amplicons (Tango7#1 and Tango7#2) (Figure 4.2C). While Tango7#1 and Tango7#2 were effective at depleting *Tango7* transcript levels, they had no overt effects on *Dronc* transcript and *Dark* protein levels (Figures 4.2C, 3.2A).

The combined observations from multiple dsRNA amplicons (Tango7#1 and Tango7#2), multiple apoptosis stimuli (Smac mimetic, Cycloheximide and UV), and multiple ways of measuring impact on apoptosis (viability, caspase activation and apoptotic morphology) qualify *Tango7* as a general apoptogenic effector.

Tango7 depletion results in loss of pro-Dronc protein

To investigate the mode of apoptosis regulation by *Tango7*, molecular events leading to the activation of apoptosis were investigated. In contrast to canonical *Drosophila* apoptosis pathways where DIAP1 is a central point of control (Kornbluth and White 2005; Hay and Guo 2006), *Tango7* did not affect basal or UV stimulus-dependent loss of DIAP1 protein levels (Figure 4.3A). Likewise, steady state levels of the apoptosomal protein Dark also remained unchanged. Instead, the apoptosis associated cleavage of Dark protein (Akdemir et al. 2006) was attenuated, similar to that seen in *Dronc* silenced cells (Figure 4.3A). Interestingly, silencing of *Tango7* prompted loss of pro-Dronc zymogen, but had no overt effect on *Dronc* mRNA transcript levels (Figure 4.2C), implicating a post-transcriptional mechanism linking *Tango7* activity to Dronc protein levels. Dronc processing and turnover depends on Dark, RNAi silencing of *Dark* results in pro-Dronc accumulation (Muro et al. 2002; Muro et al. 2004). Co-silencing of both *Tango7* and *Dark* restored pro-Dronc, though not to levels comparable to effects of *Dark* dsRNAs alone. These combined findings suggest *Tango7* activity impinges proximally on apoptosis regulation at the level of the apoptosome.

Tango7 depletion affects wing development

PCD occurs in the wing blade after eclosion, apoptotic cells are cleared into the body cavity and the dorsal and ventral wing epithelial sheets fuse to form the wing blade (Kimura et al. 2004; Link et al. 2007). In mutants of cell death genes, this clearance fails to occur, causing incomplete or total failure of the bonding of the two epithelial wing sheets. For example, *Dark*, *Dronc* and other cell death mutants have been shown to affect the development of the adult wing (Rodriguez et al. 1999b; Chew et al. 2004; Xu et al. 2005; Link et al. 2007). The wings in these mutants exhibit blistered wing blades and/or progressive melanization of cells trapped in the epithelial sheets. To investigate the potential functions of *Tango7 in vivo*, I used two independently derived wing-specific *Vg-Gal4* transgenes to drive *8309R1*, an RNAi construct targeting *Tango7*. Depletion of *Tango7* using either of the *vestigial* driver transgenes results in a range of wing defects such as failure of wing blade extension, melanization and blistering (Figure 4.4), reminiscent of defects described in other cell death mutants. *Vg-Gal4/8309R1* animals also show a low penetrance of held out wings (Figure 4.4 arrowed), which has also been observed in rare homozygous escapers of the $Dronc^{I24}$ allele and other cell death mutants (Xu et al. 2005). This observation of *Tango7* requirement *in vivo* corroborates with *Tango7* being a component of the apoptosis pathway.

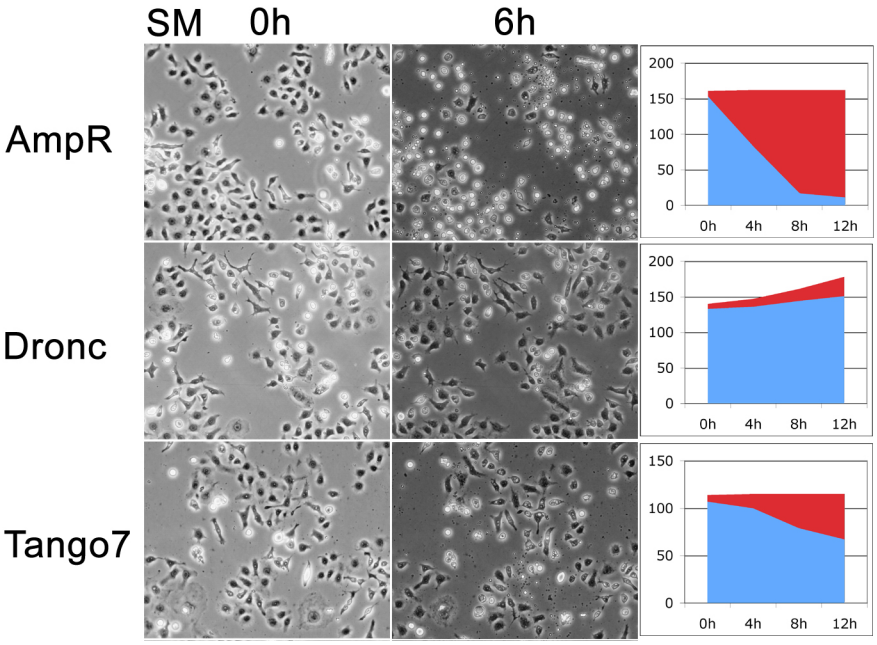
DISCUSSION

Direct upstream regulators of *Dronc* and *Dark* have not been decribed before in *Drosophila*. Hence, *Tango7* presents an interesting and novel point of entry into apoptosis regulation, as the data presented here implicate it in the regulation of pro-Dronc zymogen. The preliminary *in vivo* study showing *Tango7* involvement in adult wing maturation like other cell death mutants strongly corroborate observations in the cell culture model.

The *Tango7* locus encodes a highly conserved PCI (Proteasome, COP9 signalosome and Initiation factor 3 associated) domain and was recently found in a collection of *Drosophila* genes implicated in protein trafficking and golgi organization (hence the name *Transport and Golgi Organization 7*) (Bard et al. 2006). Also, the annotated human homolog PCID1 has been implicated in Herpes Simplex Virus susceptibility in porcine and human cells (Perez et al. 2005; Perez-Romero et al. 2005). These previously attributed activities, combined with observations described here, raise intriguing possibilities for a novel pathway to engaging apical caspases and controlling apoptotic propensity that could be answered with further analyses of the *Tango7* function in cells and in mutant animal.

As caspases can have functions in non-apoptotic processes without causing cell death (e.g. immunity, spermatogenesis, motility) (Geisbrecht and Montell 2004; Huh et al. 2004b; Kuranaga et al. 2006; Oshima et al. 2006), *Tango7* could present new opportunities for understanding how cells remain viable despite upstream activation signals through IAPs or *Dark*.

A


SM
0h
6h
AmpR
Dronc
Tango7
200
150
100
50
0
0h
4h
8h
12h

B

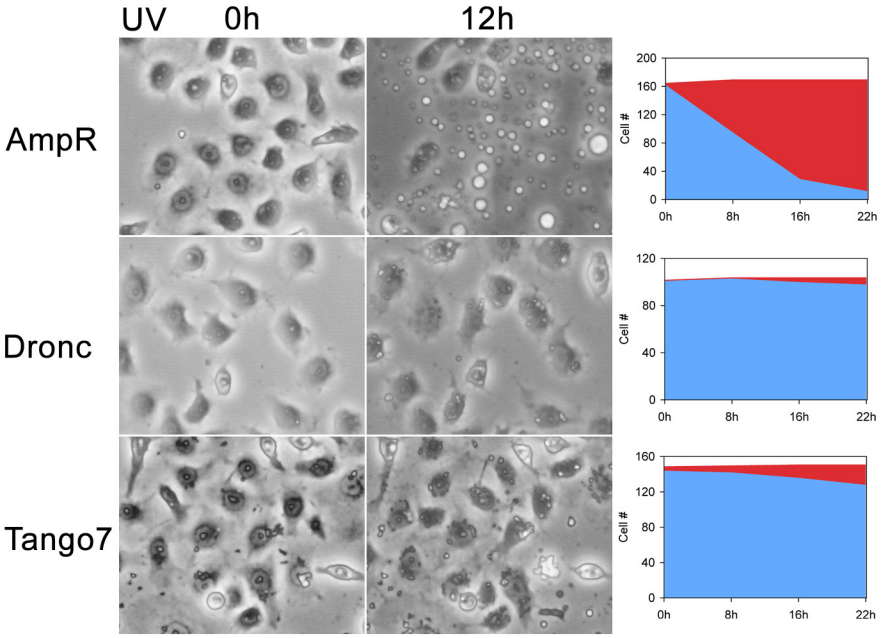
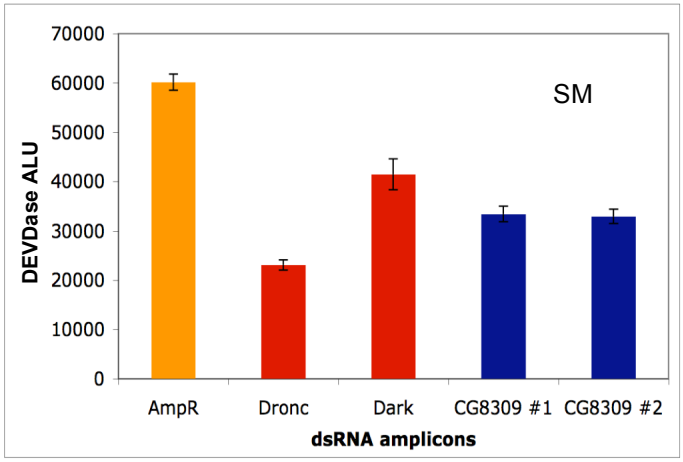
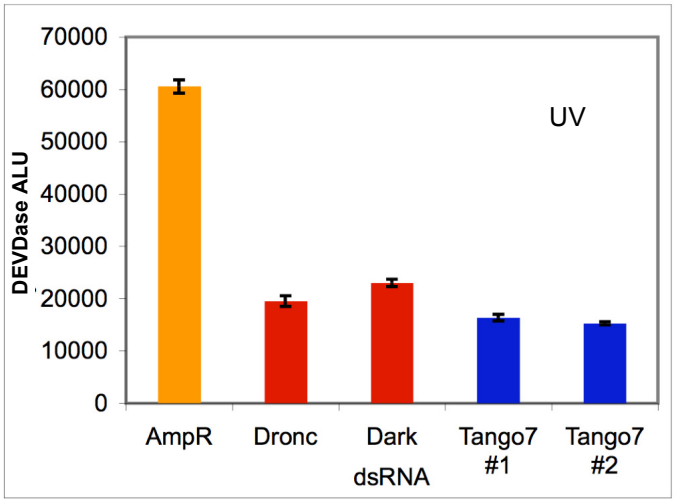

UV
0h
12h
AmpR
Dronc
Tango7
Cell #
200
160
120
80
40
0
0h
8h
16h
22h

Figure 4.1 – *Tango7* is required for stress-induced cell death
Cells pretreated with indicated dsRNAs were followed by timelapse microscopy for (A) 12 (2.5μM SM) or (B) 22 hrs (90mJ/cm^2 UVC) after exposure. Images are excerpts taken at times indicated. Tracking at least 100 cells in each treatment, graphs show cumulative counts of live (blue) or dead (red) cells over the course of these timelapse studies (y-axis indicates number of cells). Dead cells are scored by morphology of rounding up, blebbing or fragmentation into corpses. Shown here are cells treated with Tango7#1 dsRNAs. Similar results were obtained with Tango7#2 dsRNAs (not shown). Timelapse movies used in analysis are available in supplementary materials.

A


70000
60000
50000
40000
30000
20000
10000
0
DEVDase ALU
SM
AmpR
Dronc
Dark
CG8309 #1
CG8309 #2
dsRNA amplicons

B


70000
60000
50000
40000
30000
20000
10000
0
DEVDase ALU
UV
AmpR
Dronc
Dark
Tango7 #1
Tango7 #2
dsRNA

C

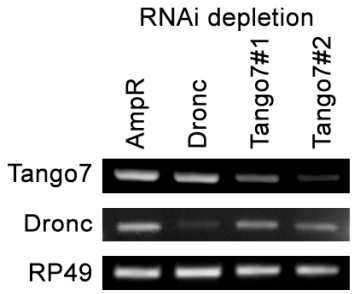

RNAi depletion
AmpR
Dronc
Tango7#1
Tango7#2
Tango7
Dronc
RP49

Figure 4.2 – Depletion of *Tango7* results in loss of stress-stimulated caspase activation

(A and B) Shows DEVDase caspase activity 6 hours after SM (A) or UV (B) treatment in cells RNAi depleted for their respective gene targets as indicated. Tango7#1 and Tango7#2 are 2 non-overlapping dsRNA amplicons that target different parts of the *Tango7* transcript (see methods, ALU denotes arbitrary luminescence units, error bars show ±SD, n=3 different wells of cells from the same experiment). dsRNAs targeting *Tango7* exhibit protection similar to 'benchmark activity' seen for dsRNAs silencing *Dronc*.

(C) Semi-quantitative rtPCR detects relative mRNA transcript levels of *Tango7*, *Dronc* and an unrelated gene *RP49* in cells treated with dsRNAs to deplete the transcripts of genes as indicated.

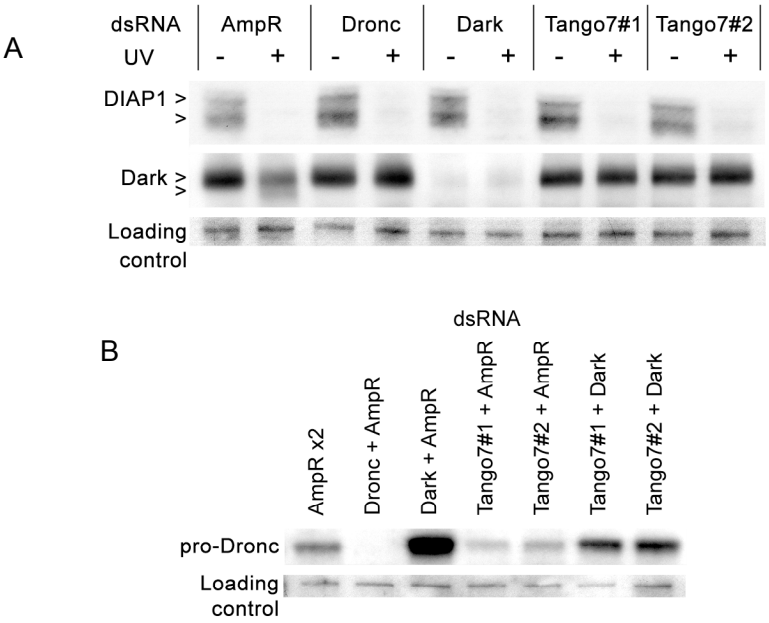


Figure 4.3 – *Tango7* depletion results in loss of pro-Dronc protein
(A) Western blot analyses of cells treated with dsRNAs as indicated. Cells were analysed either untreated or 6 hours after UV challenge. The same western blot was probed with both anti-DIAP1 and anti-Dark antibodies. The anti-DIAP1 antibody detects both full length and N-end processed forms (open arrowheads) (Ditzel et al. 2003). The anti-Dark antibody detects both full length and cleaved Dark proteins (open arrowheads) (Akdemir et al. 2006). A non-specific band detected by the anti-DIAP1 antibody is used as loading control.
(B) Western blot analyses of cells treated with dsRNAs as indicated and probed with anti-Dronc antibody that detects the full-length pro-Dronc zymogen. A non-specific band detected by the anti-Dronc antibody is used as loading control. Effects of Tango7 depletion on pro-Dronc in either single or co-silencing of 2 dsRNAs was observed in 6 independent experiments.

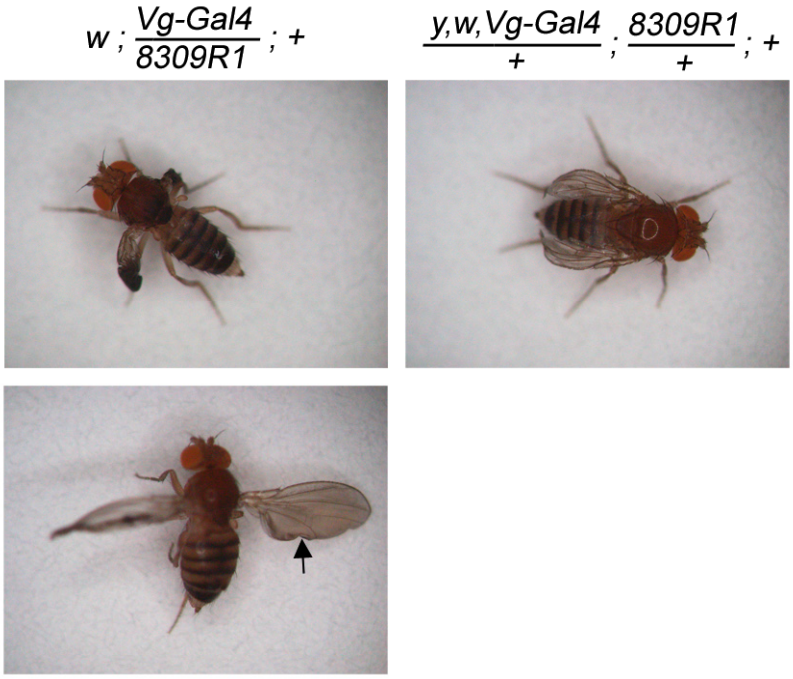


Figure 4.4 – *Tango7* depletion affects wing development
Figure shows whole adults of the genotypes indicated, *8309R1* denotes a GAL4-responsive transgene encoding a snap-back dsRNA transcript targeting *Tango7* (NIG, Japan). Note that animals with both independent transgenes of *Vg-Gal4* (on chromosomes I and II) can drive expression of the *8309R1* transgene. Animals of both genotypes show 100% penetrance of a wing development defect, with the range of severity in the wing phenotype shown here. The arrow shows an animal with a blistered wing and melanization along the edges of the blister, the held out wing phenotype is rare.

CHAPTER FIVE

CONCLUSIONS AND FUTURE DIRECTIONS

The last 100 years have seen the humble fruitfly play major and exciting roles in the field of biology, spanning structural biology at the atomic level to pan-continental ecology surveys. For the field of apoptosis, the *Drosophila* "apoptotic machinery" presents a bridge from the invariant cell death patterns and simpler genetic pathway of *C. elegans* to the dynamic compensatory system and complex networks in mammals. While studies in the cell death field over the past two decades have revealed a remarkable conservation of core components that employ recurring themes in their modes of regulation, divergences in the way these same core components regulate apoptosis have also been the source of much confusion and mystery (Danial and Korsmeyer 2004; Hay and Guo 2006; Schafer and Kornbluth 2006). The venerable worm and fly still play important roles in understanding how the individual components fit together in a regulatory pathway, not just because of their reduced complexity and redundancy, but also by providing a rich system of powerful and comprehensive toolkits for answering biological and genetic questions (Kornberg and Krasnow 2000; Rubin and Lewis 2000)

I started my dissertation studies with investigating the role of the apical caspase in development and apoptosis responses. The studies on *Dronc* highlight the pivotal hub that the apoptosome plays in both developmental and stimuli-response regulated apoptosis, which are results that could neither have been intuitive or obvious, given the

divergence between the absolute requirement in the worm and the complex redundancy in mammals. In addition to my work, other labs have described new pathways and signals that converge on *Dronc* in diverse cellular functions from coupling death and proliferation, differentiation and cell migration to cytoskeletal rearrangements (Geisbrecht and Montell 2004; Huh et al. 2004b; Kuranaga et al. 2006; Oshima et al. 2006).

A challenge that lies ahead would be understanding how the cell interprets these different signals and contexts. This context-dependence would surely necessitate a move away from responses of cultured cells *in vitro* towards fine dissection of cellular responses in tissue contexts within the animal. I expect the use of mutant somatic clones expressed in controlled tissue and developmental contexts to be extremely useful in these future investigations.

Saturating genome-wide screens using RNA interference in cultured cells is an emerging tool that has been used in multiple recent publications. These papers have added new candidates and genes of interest to many different fields (Boutros et al. 2004; Eggert et al. 2004; Cherry et al. 2005; Zhang et al. 2006; Goshima et al. 2007). While this technology is also directly applicable to human cells and has been used successfully in drug discovery and therapy-directed investigations, the genome wide RNAi platform in *Drosophila* still offers important opportuntities to answer fundamental questions in a simpler system with a rich array of followup tools.

I had the fortune of being able to develop a project at the conjunction of several key developments. First, developing a whole-genome RNAi high throughput screen (HTS) when the technology is still novel and represents an exciting endeavour, while at

the same time, the technology has matured enough where one is aware of the caveats and how to address them with thoughtful controls. Second, the development and generous gift of the small molecule Smac-mimetic by the Wang and Harran labs provided an unprecendented opportunity to query IAP derepression. Third, the development of the HTS lab and the purchase of the whole genome *Drosophila* RNAi library funded by the UTSW High Impact/High Risk Grant program. Each of the novel apoptosis determinants identified in the screen represents exciting opportunities to further illuminate a new aspect of apoptosis regulation. The next level to take the hits identified in the screen would be to elucidate biochemical mechanisms and to place them in the existing apoptosis pathway framework with mutant animal analyses. Studies in cell culture models and preliminary *in vivo* observations point to *Tango7* potentially highlighting a novel axis of regulation in *Drosophila* apoptosis, and I expect exciting new insights on regulators of the *Drosophila* apoptosome in the near future.

APPENDIX A

PRIMER SEQUENCES

Primer Name	Sequence
CG6685F1	GCGTAAATTGTTGCTCCTCCTCCATTTCTTT
KG02994F1	TAATGAGTCAGACATCACCTTTCGCTGGGT
KG02994F2	TGTGGTAATCGGGCGATAATCATTTAATAGTCGAC
KG02994R1	ACACCACAAATATACTGTTGCCGAGCACAATT
KG02994R2	AGACAATTTGATGTTGCAATCGCAGTTCCTATAGA
CG6674R1	AAGATCGAGTTCAAGAGGACCAGCAAACTCAG
PEINVRP	CGACGGGACCACCTTATGTTATTTCATCATG
T7 Dronc F	TTAATACGACTCACTATAGGGAGAGCCATATTGGGCACATATAAGATGCAATCACG
T7 Dronc R	TTAATACGACTCACTATAGGGAGAGTCTGTGTCGTGGGCATGATCGGCCATCACTTG
T7 Hid F	TTAATACGACTCACTATAGGGAGACCACAGAGCTTCACTTGGCC
T7 Hid R	TTAATACGACTCACTATAGGGAGATTGAGTCTCTTGCCAGTC
T7 Dark F	TTAATACGACTCACTATAGGGAGAGAAGCTAAGGCAGGCTCTTCTAGAACTTCG
T7 Dark R	TTAATACGACTCACTATAGGGAGACGGGCTCCAGAACGTTTAGAGAGCTCTCAA
T7 DIAP1 F	TTAATACGACTCACTATAGGGAGATTAAACCGCGAGGAGACGCGATTA
T7 DIAP1 R	TTAATACGACTCACTATAGGGAGAAATAATTGCCACTGGCGGCTGAAG
T7 Drice #1 F	TTAATACGACTCACTATAGGGAGAAGAGAACTTAGCCATGGACGCCACTAAC
T7 Drice #1 R	TTAATACGACTCACTATAGGGAGACTGGAATCTTGTAGCTCATCGAGGAGTC
T7 Drice #2 F	TTAATACGACTCACTATAGGGAGACCTACTCGACGGTTCCTGGATTCTATTC
T7 Drice #2 R	TTAATACGACTCACTATAGGGAGAATATAGATAAGGGTGGGCCGACATTCCT
Drice kdchk F	GACTTCGAAGTGACCGTGTACAAGGACT
Drice kdchk R	GTACCCACCGTTTGATCCATACCATTAG
T7 Hrb27C #1 F	TTAATACGACTCACTATAGGGAGAAGTTAATTTCTTTGCACCCCGTGTTAAT
T7 Hrb27C #1 R	TTAATACGACTCACTATAGGGAGACTTCTTCTTCTCCTGGTCGTACATGATAA
T7 Hrb27C #2 F	TTAATACGACTCACTATAGGGAGAGCTACGGCGGATACGACATGTATAACTC
T7 Hrb27C #2 R	TTAATACGACTCACTATAGGGAGACTTCTCTCTTTAGACAGCCTGCGAGGTT
Hrb27C kdchk F	CAAGGTCACCGAGGTGGTTATCATGTAC
Hrb27C kdchk R	GAGTTATACATGTCGTATCCGCCGTAGC
T7 enok #1 F	TTAATACGACTCACTATAGGGAGAAGTCCTTCGACCTCGAAACTTTGTGTCT
T7 enok #1 R	TTAATACGACTCACTATAGGGAGACTCTGTTAGATACGGAATGTCGTCCTCG
T7 enok #2 F	TTAATACGACTCACTATAGGGAGACGTACATGCTCCTGTGAATACGGAAAAG
T7 enok #2 R	TTAATACGACTCACTATAGGGAGAGTGTGATAGTTCGGCATCTGATTCAGTG
Enok kdchk F	GCCAGAAGGCTCGTCAAATATTAACCTC
Enok kdchk R	CTACTATTCGCCGTGGTTATCGAGTCCT
T7 SNF4Agamma #1 F	TTAATACGACTCACTATAGGGAGATCACAAGTGCTATGATCTGATACCCACC
T7 SNF4Agamma #1 R	TTAATACGACTCACTATAGGGAGAGGTCTCGATGTTGTTATAGGTGCCAATC
T7 SNF4Agamma #2 F	TTAATACGACTCACTATAGGGAGATATCTCGCTGTCCGATATACTGCTCTACC
T7 SNF4Agamma #2 R	TTAATACGACTCACTATAGGGAGACTGTTGTTGCTATCACCATTAGCTGTCG

SNF4Agamma kdchk F	GATTGGCACCTATAACAACATCGAGACC
SNF4Agamma kdchk R	GGACTTATTGTTATCACTGTCGGCATCG
T7CG32626 #1 F	TTAATACGACTCACTATAGGGAGAAGCACCGTCATGTTGGCGTACATATAGT
T7CG32626 #1 R	TTAATACGACTCACTATAGGGAGACATCGACAATGATGTGTACAGCTCGAAC
T7 CG32626 #2 F	TTAATACGACTCACTATAGGGAGACCATTCAGATAGTTGTCCGTCTTCAGGA
T7CG32626 #2 R	TTAATACGACTCACTATAGGGAGACGTCTCTGTTATCTGTCGTCCAAGTATCAG
CG32626 kdchk F	CATGATACCGTCCTCGTAATAGATGGGT
CG32626 kdchk R	CGAACAACTCCCTGTTCCTCAACTATCA
T7 CG40084 #1 F	TTAATACGACTCACTATAGGGAGAGCTGATGACACTAACTTCGAACTCATCTAGC
T7 CG40084 #1 R	TTAATACGACTCACTATAGGGAGAAGTCAGTAGCAACTGCACCAATGATGAC
T7 CG40084 #2 F	TTAATACGACTCACTATAGGGAGAAAGCAGCTGTCGTATCTTGTTCTCCACT
T7 CG40084 #2 R	TTAATACGACTCACTATAGGGAGACCTGATTACTCAGTTCGTGCTATATCGG
CG40084 kdchk F	GGTACTGGAAAGTAGCCAGTGTTAATTGAGG
CG40084 kdchk R	GTAAGAAAACTGTTGCGGACGTCATGAC
T7 ATPalpha #1 F	TTAATACGACTCACTATAGGGAGAAAGGAGATCCACCATTTCATCCACCTTA
T7 ATPalpha #1 R	TTAATACGACTCACTATAGGGAGAGTATTGAACACCCGACTGATCCTCAGTT
T7 ATPalpha #2 F	TTAATACGACTCACTATAGGGAGAGCTGGTTGGAGCAGGAGACCTACTATTA
T7 ATPalpha #2 R	TTAATACGACTCACTATAGGGAGAGTTGTAGACGTAGCTGTTGCTGTTGTTG
ATPalpha kdchk F	CGTCCCAATCCTCAAGAAAGAAGTCAGT
ATPalpha kdchk R	GATATAATACCGACAGACTTGGCGATGG
T7 CG7275 #1 F	TTAATACGACTCACTATAGGGAGAGAATCGTATCAGCTCTCGGAACTTTGTG
T7 CG7275 #1 R	TTAATACGACTCACTATAGGGAGAATAGGATATGGTGTGAAGGGTATCGACG
T7 CG7275 #2 F	TTAATACGACTCACTATAGGGAGACAGCTACGACAAGACCATTCGGATCTAC
T7 CG7275 #2 R	TTAATACGACTCACTATAGGGAGAGTTAGCAATCATCCTCACACCTCCTCCT
CG7275 kdchk F	ATGAGGTGCCGGTGAACACTATACTGAG
CG7275 kdchk R	GTAGATCCGAATGGTCTTGTCGTAGCTG
T7 CG8309 #1 F	TTAATACGACTCACTATAGGGAGAAATAATTTAGGTCGTCACTCCAGTGCCC
T7 CG8309 #1 R	TTAATACGACTCACTATAGGGAGAAGAACATGAAGAAGATGCGTCTGCTGAC
T7 CG8309 #2 F	TTAATACGACTCACTATAGGGAGAAGTTGACAAACTCCCTGTGATCCTCGTA
T7 CG8309 #2 R	TTAATACGACTCACTATAGGGAGATACCATGTGTACTACCACCTGGTCCAGG
CG8309 kdchk F	GTAGGTGCCCAGCAGCTCAATCATAAC
CG8309 kdchk R	CATCGGAGAAGTCCAATAAAGGTGTGG
T7 Myb #1 F	TTAATACGACTCACTATAGGGAGAGGGATCAATGACATGATCGTAGGTCAAG
T7 Myb #1 R	TTAATACGACTCACTATAGGGAGATCAGAACTCAACGCTAAGCACCGAATAC
T7 Myb #2 F	TTAATACGACTCACTATAGGGAGAGATCTCCATTCCAACGCGATTATAGACC
T7 Myb #2 R	TTAATACGACTCACTATAGGGAGACGAGATTATCTACCAGGCTCACTTGGAG
Myb kdchk F	CCGTTCAGATATCGTGCAATTAGTGTCC
Myb kdchk R	AGAGCCGGAGATCTATCACTTTTGTTCG
T7 eIF5 #1 F	TTAATACGACTCACTATAGGGAGATACTGGTTGGATTAGGGTAGCTGTTCTC
T7 eIF5 #1 R	TTAATACGACTCACTATAGGGAGAGAGCAAACTGTAGAGCTGCATAAGGGTA
T7 eIF5 #2 F	TTAATACGACTCACTATAGGGAGAGGATATCTATCCGTGCACATACCTTGTG
T7 eIf5 #2 R	TTAATACGACTCACTATAGGGAGAGTTAAGTAAGCAGCAGCGGCAACTACTC
eIF5 kdchk F	CATCCGAAATAGTCATACCCTTAGCACC
eIF5 kdchk R	ACAATCCGGAGACCAATTTAACTGTGTC

T7 lkb1 #1 F	TTAATACGACTCACTATAGGGAGAGCGGAGGTTTATGCAATGTTCTAGCTCT
T7 lkb1 #1 R	TTAATACGACTCACTATAGGGAGAGCGGAGGTTTATGCAATGTTCTAGCTCT
T7 lkb1 #2 F	TTAATACGACTCACTATAGGGAGACAACTCCACGGTGATACCTTACTTGGAA
T7 lkb1 #2 R	TTAATACGACTCACTATAGGGAGATCTATGTTTAGTGGAGGCATCTAAGGCG
lkb1 kdchk F	GAAGAGCATTAAGATGGTGGGCAAGTAC
lkb1 kdchk R	TAGATATTGTCGCCCTCGAAGGGATACT
T7 Rluc F	TTAATACGACTCACTATAGGGAGAAAGGAAACGGATGATAACTGGTCCGC
T7 Rluc R	TTAATACGACTCACTATAGGGAGACTAACGGGATTTCACGAGGCCATGAT
T7 Ampr F	TTAATACGACTCACTATAGGGAGATACATCGAACTGGATCTCAACAGCGG
T7 Ampr R	TTAATACGACTCACTATAGGGAGATCGTTCATCCATAGTTGCCTGACTCC

"T7" denotes that the primer contains the T7 promoter sequence, "kdchk" denotes primer pairs used in RT-PCR to assay RNAi depletion. "F" and "R" denote the forward and reverse primer of each pair, relative to the direction of the open reading frame.

BIBLIOGRAPHY

Abramoff, M.D., Magelhaes, P.J., and Ram, S.J. 2004. Image Processing with ImageJ. *Biophotonics International* **11**(7): 36-42.

Abrams, J.M. 1999. An emerging blueprint for apoptosis in Drosophila. *Trends in Cell Biology* **9**(11): 435-440.

-. 2002. Competition and compensation: coupled to death in development and cancer. *Cell* **110**(4): 403-406.

Abrams, J.M., White, K., Fessler, L., and Steller, H. 1993. Programmed cell death during *Drosophila* embryogenesis. *Development* **117**: 29-44.

Akdemir, F., Farkas, R., Chen, P., Juhasz, G., Medved'ova, L., Sass, M., Wang, L., Wang, X., Chittaranjan, S., Gorski, S.M., Rodriguez, A., and Abrams, J.M. 2006. Autophagy occurs upstream or parallel to the apoptosome during histolytic cell death. *Development* **133**(8): 1457-1465.

Antonchuk, J., Sauvageau, G., and Humphries, R.K. 2002. HOXB4-induced expansion of adult hematopoietic stem cells ex vivo. *Cell* **109**(1): 39-45.

Arama, E., Agapite, J., and Steller, H. 2003. Caspase activity and a specific cytochrome C are required for sperm differentiation in Drosophila. *Dev Cell* **4**(5): 687-697.

Armknecht, S., Boutros, M., Kiger, A., Nybakken, K., Mathey-Prevot, B., and Perrimon, N. 2005. High-throughput RNA interference screens in Drosophila tissue culture cells. *Methods Enzymol* **392**: 55-73.

Arnt, C.R. and Kaufmann, S.H. 2003. The saintly side of Smac/DIABLO: giving anticancer drug-induced apoptosis a boost. *Cell Death Differ* **10**(10): 1118-1120.

Asha, H., Nagy, I., Kovacs, G., Stetson, D., Ando, I., and Dearolf, C.R. 2003. Analysis of ras-induced overproliferation in Drosophila hemocytes. *Genetics* **163**(1): 203-215.

Baehrecke, E.H. 2002. How death shapes life during development. *Nat Rev Mol Cell Biol* **3**(10): 779-787.

Bao, Q. and Shi, Y. 2007. Apoptosome: a platform for the activation of initiator caspases. *Cell Death Differ* **14**(1): 56-65.

Bard, F., Casano, L., Mallabiabarrena, A., Wallace, E., Saito, K., Kitayama, H., Guizzunti, G., Hu, Y., Wendler, F., Dasgupta, R., Perrimon, N., and Malhotra, V. 2006. Functional genomics reveals genes involved in protein secretion and Golgi organization. *Nature* **439**(7076): 604-607.

Baum, J.S., Arama, E., Steller, H., and McCall, K. 2007. The Drosophila caspases Strica and Dronc function redundantly in programmed cell death during oogenesis. *Cell Death Differ*.

Boutros, M., Kiger, A.A., Armknecht, S., Kerr, K., Hild, M., Koch, B., Haas, S.A., Consortium, H.F., Paro, R., and Perrimon, N. 2004. Genome-wide RNAi analysis of growth and viability in Drosophila cells. *Science* **303**(5659): 832-835.

Brachmann, C.B., Jassim, O.W., Wachsmuth, B.D., and Cagan, R.L. 2000. The Drosophila Bcl-2 family member dBorg-1 functions in the apoptotic response to UV-irradiation. *Current Biology* **10**(9): 547-550.

Brennecke, J., Hipfner, D.R., Stark, A., Russell, R.B., and Cohen, S.M. 2003. bantam Encodes a Developmentally Regulated microRNA that Controls Cell Proliferation and Regulates the Proapoptotic Gene hid in Drosophila. *Cell* **113**(1): 25-36.

Canbay, A., Friedman, S., and Gores, G.J. 2004. Apoptosis: the nexus of liver injury and fibrosis. *Hepatology* **39**(2): 273-278.

Canman, C.E., Chen, C.Y., Lee, M.H., and Kastan, M.B. 1994. DNA damage responses: p53 induction, cell cycle perturbations, and apoptosis. [Review] [53 refs]. *Cold Spring Harb Symp Quant Biol* **59**(277): 277-286.

Cecconi, F., Alvarezbolado, G., Meyer, B.I., Roth, K.A., and Gruss, P. 1998. Apaf1 (Ced-4 Homolog) Regulates Programmed Cell Death in Mammalian Development. *Cell* **94**(6): 727-737.

Chai, J., Du, C., Wu, J.W., Kyin, S., Wang, X., and Shi, Y. 2000. Structural and biochemical basis of apoptotic activation by Smac/DIABLO. *Nature* **406**(6798): 855-862.

Chai, J., Yan, N., Huh, J.R., Wu, J.W., Li, W., Hay, B.A., and Shi, Y. 2003. Molecular mechanism of Reaper-Grim-Hid-mediated suppression of DIAP1-dependent Dronc ubiquitination. *Nat Struct Biol* **10**(11): 892-898.

Chen, P., Ho, S.I., Shi, Z., and Abrams, J.M. 2004. Bifunctional killing activity encoded by conserved reaper proteins. *Cell Death Differ* **11**(7): 704-713.

Chen, P., Lee, P., Otto, L., and Abrams, J. 1996a. Apoptotic activity of REAPER is distinct from signaling by the tumor necrosis factor receptor 1 death domain. *J Biol Chem* **271**(42): 25735-25737.

Chen, P., Nordstrom, W., Gish, B., and Abrams, J.M. 1996b. Grim, a novel cell death gene in drosophila. *Genes & Development* **10**(14): 1773-1782.

Chen, P., Rodriguez, A., Erskine, R., Thach, T., and Abrams, J.M. 1998. Dredd, a Novel Effector of the Apoptosis Activators Reaper, Grim, and Hid in Drosophila. *Developmental Biology* **201**(2): 202-216.

Cherry, S., Doukas, T., Armknecht, S., Whelan, S., Wang, H., Sarnow, P., and Perrimon, N. 2005. Genome-wide RNAi screen reveals a specific sensitivity of IRES-containing RNA viruses to host translation inhibition. *Genes Dev* **19**(4): 445-452.

Chew, S.K., Akdemir, F., Chen, P., Lu, W.J., Mills, K., Daish, T., Kumar, S., Rodriguez, A., and Abrams, J.M. 2004. The apical caspase dronc governs programmed and unprogrammed cell death in Drosophila. *Dev Cell* **7**(6): 897-907.

Christich, A., Kauppila, S., Chen, P., Sogame, N., Ho, S.I., and Abrams, J.M. 2002. The Damage-Responsive Drosophila Gene sickle Encodes a Novel IAP Binding Protein Similar to but Distinct from reaper, grim, and hid. *Curr Biol* **12**(2): 137-140.

Clem, R. and Miller, L.K. 1993. Apoptosis Reduces both the In Vitro Replication and the In Vivo Infectivity of a Baculovirus. *Journal of Virology* **67**: 3730-3738.

Clemens, J.C., Worby, C.A., Simonson-Leff, N., Muda, M., Maehama, T., Hemmings, B.A., and Dixon, J.E. 2000. Use of double-stranded RNA interference in Drosophila cell lines to dissect signal transduction pathways. *Proc Natl Acad Sci U S A* **97**(12): 6499-6503.

Colussi, P.A., Quinn, L.M., Huang, D.C.S., Coombe, M., Read, S.H., Richardson, H., and Kumar, S. 2000. Debcl, a proapoptotic Bcl-2 homologue, is a component of the Drosophila melanogaster cell death machinery. *Journal of Cell Biology* **148**(4): 703-714.

Daish, T.J., Mills, K., and Kumar, S. 2004. Drosophila caspase DRONC is required for specific developmental cell death pathways and stress-induced apoptosis. *Dev Cell* **7**(6): 909-915.

Danial, N.N. and Korsmeyer, S.J. 2004. Cell death: critical control points. *Cell* **116**(2): 205-219.

Desmouliere, A., Badid, C., Bochatonpiallat, M.L., and Gabbiani, G. 1997. APOPTOSIS DURING WOUND HEALING, FIBROCONTRACTIVE DISEASES AND VASCULAR WALL INJURY [Review]. *International Journal of Biochemistry & Cell Biology* **29**(1): 19-30.

Ditzel, M., Wilson, R., Tenev, T., Zachariou, A., Paul, A., Deas, E., and Meier, P. 2003. Degradation of DIAP1 by the N-end rule pathway is essential for regulating apoptosis. *Nat Cell Biol* **5**(5): 467-473.

Dorstyn, L., Colussi, P.A., Quinn, L.M., Richardson, H., and Kumar, S. 1999a. DRONC, an ecdysone-inducible Drosophila caspase. *Proceedings of the National Academy of Sciences of the United States of America* **96**(8): 4307-4312.

-. 1999b. DRONC, an ecdysone-inducible Drosophila caspase. *Proc Natl Acad Sci U S A* **96**(8): 4307-4312.

Dorstyn, L., Mills, K., Lazebnik, Y., and Kumar, S. 2004. The two cytochrome c species, DC3 and DC4, are not required for caspase activation and apoptosis in Drosophila cells. *J Cell Biol* **167**(3): 405-410.

Dorstyn, L., Read, S., Cakouros, D., Huh, J.R., Hay, B.A., and Kumar, S. 2002. The role of cytochrome c in caspase activation in Drosophila melanogaster cells. *J Cell Biol* **156**(6): 1089-1098.

Dorstyn, L., Read, S.H., Quinn, L.M., Richardson, H., and Kumar, S. 1999c. DECAY, a novel Drosophila caspase related to mammalian caspase-3 and caspase-7. *J Biol Chem* **274**(43): 30778-30783.

Doumanis, J., Quinn, L., Richardson, H., and Kumar, S. 2001. STRICA, a novel Drosophila melanogaster caspase with an unusual serine/threonine-rich

prodomain, interacts with DIAP1 and DIAP2. *Cell Death Differ* **8**(4): 387-394.

Eggert, U.S., Kiger, A.A., Richter, C., Perlman, Z.E., Perrimon, N., Mitchison, T.J., and Field, C.M. 2004. Parallel chemical genetic and genome-wide RNAi screens identify cytokinesis inhibitors and targets. *PLoS Biol* **2**(12): e379.

Ellis, R.E., Yuan, J., and Horvitz, H.R. 1991. Mechanisms and functions of cell death. *Annu Rev Cell Biol* **7**: 663-698.

Ferrer, I. 2006. Apoptosis: future targets for neuroprotective strategies. *Cerebrovasc Dis* **21 Suppl 2**: 9-20.

Fulda, S., Wick, W., Weller, M., and Debatin, K.M. 2002. Smac agonists sensitize for Apo2L/TRAIL- or anticancer drug-induced apoptosis and induce regression of malignant glioma in vivo. *Nat Med* **8**(8): 808-815.

Gateff, E., Wismar, J., Habtemichael, N., Loffler, T., Dreschers, S., Kaiser, S., and Protin, U. 1996. Functional analysis of Drosophila developmental genes instrumental in tumor suppression. *In Vivo* **10**(2): 211-215.

Geisbrecht, E.R. and Montell, D.J. 2004. A role for Drosophila IAP1-mediated caspase inhibition in Rac-dependent cell migration. *Cell* **118**(1): 111-125.

Goshima, G., Wollman, R., Goodwin, S.S., Zhang, N., Scholey, J.M., Vale, R.D., and Stuurman, N. 2007. Genes required for mitotic spindle assembly in Drosophila S2 cells. *Science* **316**(5823): 417-421.

Green, D.R. 1998. Apoptotic Pathways - the Roads to Ruin. *Cell* **94**(6): 695-698.

Green, D.R. and Evan, G.I. 2002. A matter of life and death. *Cancer Cell* **1**(1): 19-30.

Grether, M.E., Abrams, J.M., Agapite, J., White, K., and Steller, H. 1995. The *head involution defective* gene of *Drosophila melanogaster* functions in programmed cell death. *Genes & Development* **9**(14): 1694-1708.

Hao, Z., Duncan, G.S., Chang, C.C., Elia, A., Fang, M., Wakeham, A., Okada, H., Calzascia, T., Jang, Y., You-Ten, A., Yeh, W.C., Ohashi, P., Wang, X., and Mak, T.W. 2005. Specific ablation of the apoptotic functions of cytochrome C reveals a differential requirement for cytochrome C and Apaf-1 in apoptosis. *Cell* **121**(4): 579-591.

Harlin, H., Reffey, S.B., Duckett, C.S., Lindsten, T., and Thompson, C.B. 2001. Characterization of XIAP-deficient mice. *Mol Cell Biol* **21**(10): 3604-3608.

Harvey, K.F., Pfleger, C.M., and Hariharan, I.K. 2003. The Drosophila Mst ortholog, hippo, restricts growth and cell proliferation and promotes apoptosis. *Cell* **114**(4): 457-467.

Harvey, N.L., Daish, T., Mills, K., Dorstyn, L., Quinn, L.M., Read, S.H., Richardson, H., and Kumar, S. 2001. Characterization of the Drosophila caspase, DAMM. *J Biol Chem* **276**(27): 25342-25350.

Hay, B.A. 2000. Understanding IAP function and regulation: a view from Drosophila. *Cell Death & Differentiation* **7**(11): 1045-1056.

Hay, B.A. and Guo, M. 2006. Caspase-dependent cell death in Drosophila. *Annu Rev Cell Dev Biol* **22**: 623-650.

Hay, B.A., Wassarman, D.A., and Rubin, G.M. 1995. Drosophila homologs of baculovirus inhibitor of apoptosis proteins function to block cell death. *Cell* **83**(7): 1253-1262.

Hays, R., Wickline, L., and Cagan, R. 2002. Morgue mediates apoptosis in the Drosophila melanogaster retina by promoting degradation of DIAP1. *Nat Cell Biol* **4**(6): 425-431.

Holley, C.L., Olson, M.R., Colon-Ramos, D.A., and Kornbluth, S. 2002. Reaper eliminates IAP proteins through stimulated IAP degradation and generalized translational inhibition. *Nat Cell Biol* **4**(6): 439-444.

Hu, S.M. and Yang, X.L. 2000. dFADD, a novel death domain-containing adapter protein for the Drosophila caspase DREDD. *Journal of Biological Chemistry* **275**(40): 30761-30764.

Huh, J.R., Foe, I., Muro, I., Chen, C.H., Seol, J.H., Yoo, S.J., Guo, M., Park, J.M., and Hay, B.A. 2007. The Drosophila inhibitor of apoptosis (IAP) DIAP2 is dispensable for cell survival, required for the innate immune response to gram-negative bacterial infection, and can be negatively regulated by the reaper/hid/grim family of IAP-binding apoptosis inducers. *J Biol Chem* **282**(3): 2056-2068.

Huh, J.R., Guo, M., and Hay, B.A. 2004a. Compensatory proliferation induced by cell death in the Drosophila wing disc requires activity of the apical cell death caspase Dronc in a nonapoptotic role. *Curr Biol* **14**(14): 1262-1266.

Huh, J.R., Vernooy, S.Y., Yu, H., Yan, N., Shi, Y., Guo, M., and Hay, B.A. 2004b. Multiple apoptotic caspase cascades are required in nonapoptotic roles for Drosophila spermatid individualization. *PLoS Biol* **2**(1): E15.

Igaki, T., Kanuka, H., Inohara, N., Sawamoto, K., Nunez, G., Okano, H., and Miura, M. 2000. Drob-1, a Drosophila member of the Bcl-2/CED-9 family that promotes cell death. *Proceedings of the National Academy of Sciences of the United States of America* **97**(2): 662-667.

Igaki, T., Yamamoto-Goto, Y., Tokushige, N., Kanda, H., and Miura, M. 2002. Down-regulation of DIAP1 triggers a novel Drosophila cell death pathway mediated by Dark and DRONC. *J Biol Chem* **277**(26): 23103-23106.

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

Jiang, X. and Wang, X. 2004. Cytochrome C-mediated apoptosis. *Annu Rev Biochem* **73**: 87-106.

Johnstone, R.W., Ruefli, A.A., and Lowe, S.W. 2002. Apoptosis: a link between cancer genetics and chemotherapy. *Cell* **108**(2): 153-164.

Jones, G., Jones, D., Zhou, L., Steller, H., and Chu, Y. 2000. Deterin, a new inhibitor of apoptosis from Drosophila melanogaster. *J Biol Chem* **275**(29): 22157-22165.

Kaiser, W.J., Vucic, D., and Miller, L.K. 1998. The Drosophila inhibitor of apoptosis D-IAP1 suppresses cell death induced by the caspase drICE. *FEBS Letters* **440**(1-2): 243-248.

Kanuka, H., Sawamoto, K., Inohara, N., Matsuno, K., Okano, H., and Miura, M. 1999. Control of the cell death pathway by Dapaf-1, a Drosophila Apaf-1/CED-4-related caspase activator. *Molecular Cell* **4**(5): 757-769.

Karuman, P., Gozani, O., Odze, R.D., Zhou, X.C., Zhu, H., Shaw, R., Brien, T.P., Bozzuto, C.D., Ooi, D., Cantley, L.C., and Yuan, J. 2001. The Peutz-Jegher gene product LKB1 is a mediator of p53-dependent cell death. *Mol Cell* **7**(6): 1307-1319.

Kerr, J.F., Wyllie, A.H., and Currie, A.R. 1972. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br J Cancer* **26**(4): 239-257.

Kerr, J.F.R. and Harmon, B.V. 1991. Definition and incidence of apoptosis: an historical perspective. in *Apoptosis: the molecular basis of cell death*, pp. 5-29. Cold Spring Harbor Laboratory Press, New York.

Kiessling, S. and Green, D.R. 2006. Cell survival and proliferation in Drosophila S2 cells following apoptotic stress in the absence of the APAF-1 homolog, ARK, or downstream caspases. *Apoptosis*.

Kimura, K., Kodama, A., Hayasaka, Y., and Ohta, T. 2004. Activation of the cAMP/PKA signaling pathway is required for post-ecdysial cell death in wing epidermal cells of Drosophila melanogaster. *Development* **131**(7): 1597-1606.

Kondo, S., Senoo-Matsuda, N., Hiromi, Y., and Miura, M. 2006. DRONC coordinates cell death and compensatory proliferation. *Mol Cell Biol* **26**(19): 7258-7268.

Kornberg, T.B. and Krasnow, M.A. 2000. The Drosophila Genome Sequence: Implications for Biology and Medicine. *Science* **287**: 2218-2220.

Kornbluth, S. and White, K. 2005. Apoptosis in Drosophila: neither fish nor fowl (nor man, nor worm). *J Cell Sci* **118**(Pt 9): 1779-1787.

Kosman, D., Small, S., and Reinitz, J. 1998. Rapid preparation of a panel of polyclonal antibodies to Drosophila segmentation proteins. *Dev Genes Evol* **208**(5): 290-294.

Kramer, H., Cagan, R.L., and Zipursky, S.L. 1991. Interaction of bride of sevenless membrane-bound ligand and the sevenless tyrosine-kinase receptor. *Nature* **352**(6332): 207-212.

Kulkarni, M.M., Booker, M., Silver, S.J., Friedman, A., Hong, P., Perrimon, N., and Mathey-Prevot, B. 2006. Evidence of off-target effects associated with long dsRNAs in Drosophila melanogaster cell-based assays. *Nat Methods* **3**(10): 833-838.

Kumar, S. 2006. Caspases and their many biological functions. *Cell Death Differ*.

-. 2007. Caspase function in programmed cell death. *Cell Death Differ* **14**(1): 32-43.

Kumar, S. and Doumanis, J. 2000a. The fly caspases. *Cell Death Differ* **7**(11): 1039-1044.

-. 2000b. The fly caspases. *Cell Death & Differentiation* **7**(11): 1039-1044.

Kuranaga, E., Kanuka, H., Tonoki, A., Takemoto, K., Tomioka, T., Kobayashi, M., Hayashi, S., and Miura, M. 2006. Drosophila IKK-related kinase regulates nonapoptotic function of caspases via degradation of IAPs. *Cell* **126**(3): 583-596.

Kyba, M., Perlingeiro, R.C., and Daley, G.Q. 2002. HoxB4 confers definitive lymphoid-myeloid engraftment potential on embryonic stem cell and yolk sac hematopoietic progenitors. *Cell* **109**(1): 29-37.

Lang, G., Gombert, W.M., and Gould, H.J. 2005. A transcriptional regulatory element in the coding sequence of the human Bcl-2 gene. *Immunology* **114**(1): 25-36.

Lanot, R., Zachary, D., Holder, F., and Meister, M. 2001. Postembryonic hematopoiesis in Drosophila. *Dev Biol* **230**(2): 243-257.

Laundrie, B., Peterson, J.S., Baum, J.S., Chang, J.C., Fileppo, D., Thompson, S.R., and McCall, K. 2003. Germline cell death is inhibited by P-element insertions disrupting the dcp-1/pita nested gene pair in Drosophila. *Genetics* **165**(4): 1881-1888.

Lee, C.Y. and Baehrecke, E.H. 2000. Genetic regulation of programmed cell death in Drosophila. *Cell Res* **10**(3): 193-204.

Leulier, F., Lhocine, N., Lemaitre, B., and Meier, P. 2006a. The Drosophila inhibitor of apoptosis protein DIAP2 functions in innate immunity and is essential to resist gram-negative bacterial infection. *Mol Cell Biol* **26**(21): 7821-7831.

Leulier, F., Ribeiro, P.S., Palmer, E., Tenev, T., Takahashi, K., Robertson, D., Zachariou, A., Pichaud, F., Ueda, R., and Meier, P. 2006b. Systematic in vivo RNAi analysis of putative components of the Drosophila cell death machinery. *Cell Death Differ*.

Leulier, F., Rodriguez, A., Khush, R.S., Abrams, J.M., and Lemaitre, B. 2000. The Drosophila caspase Dredd is required to resist Gram-negative bacterial infection. *EMBO Reports* **1**(4): 353-358.

Li, L., Thomas, R.M., Suzuki, H., De Brabander, J.K., Wang, X., and Harran, P.G. 2004. A small molecule Smac mimic potentiates TRAIL- and TNFalpha-mediated cell death. *Science* **305**(5689): 1471-1474.

Link, N., Chen, P., Lu, W.J., Pogue, K., Chuong, A., Mata, M., Checketts, J., and Abrams, J.M. 2007. A collective form of cell death requires homeodomain interacting protein kinase. *J Cell Biol* **178**(4): 567-574.

Lisi, S., Mazzon, I., and White, K. 2000. Diverse domains of THREAD/DIAP1 are required to inhibit apoptosis induced by REAPER and HID in drosophila. *Genetics* **154**(2): 669-678.

Lowe, S.W., Cepero, E., and Evan, G. 2004. Intrinsic tumour suppression. *Nature* **432**(7015): 307-315.

Lum, L., Yao, S., Mozer, B., Rovescalli, A., Von Kessler, D., Nirenberg, M., and Beachy, P.A. 2003. Identification of Hedgehog pathway components by RNAi in Drosophila cultured cells. *Science* **299**(5615): 2039-2045.

Ma, Y., Certel, K., Gao, Y., Niemitz, E., Mosher, J., Mukherjee, A., Mutsuddi, M., Huseinovic, N., Crews, S.T., Johnson, W.A., and Nambu, J.R. 2000. Functional interactions between Drosophila bHLH/PAS, Sox, and POU transcription factors regulate CNS midline expression of the slit gene. *J Neurosci* **20**(12): 4596-4605.

Ma, Y., Creanga, A., Lum, L., and Beachy, P.A. 2006. Prevalence of off-target effects in Drosophila RNA interference screens. *Nature* **443**(7109): 359-363.

Malo, N., Hanley, J.A., Cerquozzi, S., Pelletier, J., and Nadon, R. 2006. Statistical practice in high-throughput screening data analysis. *Nat Biotechnol* **24**(2): 167-175.

Martin, L.J. 2001. Neuronal cell death in nervous system development, disease, and injury (Review). *Int J Mol Med* **7**(5): 455-478.

Martins, L.M., Morrison, A., Klupsch, K., Fedele, V., Moisoi, N., Teismann, P., Abuin, A., Grau, E., Geppert, M., Livi, G.P., Creasy, C.L., Martin, A., Hargreaves, I., Heales, S.J., Okada, H., Brandner, S., Schulz, J.B., Mak,

T., and Downward, J. 2004. Neuroprotective role of the Reaper-related serine protease HtrA2/Omi revealed by targeted deletion in mice. *Mol Cell Biol* **24**(22): 9848-9862.

Means, J.C., Muro, I., and Clem, R.J. 2006. Lack of involvement of mitochondrial factors in caspase activation in a Drosophila cell-free system. *Cell Death Differ* **13**(7): 1222-1234.

Meier, P., Finch, A., and Evan, G. 2000a. Apoptosis in development. *Nature* **407**(6805): 796-801.

Meier, P., Silke, J., Leevers, S.J., and Evan, G.I. 2000b. The Drosophila caspase DRONC is regulated by DIAP1. *EMBO Journal* **19**(4): 598-611.

Mejillano, M., Yamamoto, M., Rozelle, A.L., Sun, H.Q., Wang, X., and Yin, H.L. 2001. Regulation of apoptosis by phosphatidylinositol 4,5-bisphosphate inhibition of caspases, and caspase inactivation of phosphatidylinositol phosphate 5-kinases. *J Biol Chem* **276**(3): 1865-1872.

Mills, K., Daish, T., Harvey, K.F., Pfleger, C.M., Hariharan, I.K., and Kumar, S. 2006. The Drosophila melanogaster Apaf-1 homologue ARK is required for most, but not all, programmed cell death. *J Cell Biol* **172**(6): 809-815.

Muro, I., Berry, D.L., Huh, J.R., Chen, C.H., Huang, H., Yoo, S.J., Guo, M., Baehrecke, E.H., and Hay, B.A. 2006. The Drosophila caspase Ice is important for many apoptotic cell deaths and for spermatid individualization, a nonapoptotic process. *Development* **133**(17): 3305-3315.

Muro, I., Hay, B.A., and Clem, R.J. 2002. The Drosophila DIAP1 protein is required to prevent accumulation of a continuously generated, processed form of the apical caspase DRONC. *J Biol Chem* **277**(51): 49644-49650.

Muro, I., Monser, K., and Clem, R.J. 2004. Mechanism of Dronc activation in Drosophila cells. *J Cell Sci* **117**(Pt 21): 5035-5041.

Okada, H., Suh, W.K., Jin, J., Woo, M., Du, C., Elia, A., Duncan, G.S., Wakeham, A., Itie, A., Lowe, S.W., Wang, X., and Mak, T.W. 2002. Generation and characterization of Smac/DIABLO-deficient mice. *Mol Cell Biol* **22**(10): 3509-3517.

Oshima, K., Takeda, M., Kuranaga, E., Ueda, R., Aigaki, T., Miura, M., and Hayashi, S. 2006. IKK epsilon regulates F actin assembly and interacts

with Drosophila IAP1 in cellular morphogenesis. *Curr Biol* **16**(15): 1531-1537.

Pantalacci, S., Tapon, N., and Leopold, P. 2003. The Salvador partner Hippo promotes apoptosis and cell-cycle exit in Drosophila. *Nat Cell Biol* **5**(10): 921-927.

Perez, A., Li, Q.X., Perez-Romero, P., Delassus, G., Lopez, S.R., Sutter, S., McLaren, N., and Fuller, A.O. 2005. A new class of receptor for herpes simplex virus has heptad repeat motifs that are common to membrane fusion proteins. *J Virol* **79**(12): 7419-7430.

Perez-Galan, P., Roue, G., Villamor, N., Campo, E., and Colomer, D. 2007. The BH3-mimetic GX15-070 synergizes with bortezomib in mantle cell lymphoma by enhancing Noxa-mediated activation of Bak. *Blood* **109**(10): 4441-4449.

Perez-Romero, P., Perez, A., Capul, A., Montgomery, R., and Fuller, A.O. 2005. Herpes simplex virus entry mediator associates in infected cells in a complex with viral proteins gD and at least gH. *J Virol* **79**(7): 4540-4544.

Quinn, L., Coombe, M., Mills, K., Daish, T., Colussi, P., Kumar, S., and Richardson, H. 2003. Buffy, a Drosophila Bcl-2 protein, has anti-apoptotic and cell cycle inhibitory functions. *Embo J* **22**(14): 3568-3579.

Quinn, L.M., Dorstyn, L., Mills, K., Colussi, P.A., Chen, P., Coombe, M., Abrams, J., Kumar, S., and Richardson, H. 2000. An essential role for the caspase dronc in developmentally programmed cell death in Drosophila. *J Biol Chem* **275**(51): 40416-40424.

Reed, J.C. 2003. Apoptosis-targeted therapies for cancer. *Cancer Cell* **3**(1): 17-22.

Richardson, H. and Kumar, S. 2002. Death to flies: Drosophila as a model system to study programmed cell death. *J Immunol Methods* **265**(1-2): 21-38.

Roberts, D.L., Merrison, W., MacFarlane, M., and Cohen, G.M. 2001. The inhibitor of apoptosis protein-binding domain of Smac is not essential for its proapoptotic activity. *Journal of Cell Biology* **153**(1): 221-227.

Rodriguez, A., Chen, P., Oliver, H., and Abrams, J.M. 2002. Unrestrained caspase-dependent cell death caused by loss of Diap1 function requires the Drosophila Apaf-1 homolog, Dark. *Embo J* **21**(9): 2189-2197.

Rodriguez, A., Oliver, H., Zou, H., Chen, P., Wang, X., and Abrams, J.M. 1999a. Dark is a Drosophila homologue of Apaf-1/CED-4 and functions in an evolutionarily conserved death pathway. *Nat Cell Biol* **1**(5): 272-279.

Rodriguez, A., Oliver, H., Zou, H., Chen, P., Wang, X.D., and Abrams, J.M. 1999b. Dark is a Drosophila homologue of Apaf-1/CED-4 and functions in an evolutionarily conserved death pathway. *Nature Cell Biology* **1**(5): 272-279.

Rubin, G.M. and Lewis, E.B. 2000. A brief history of Drosophila's contributions to genome research. *Science* **287**(5461): 2216-2218.

Rudin, C.M. and Thompson, C.B. 1997. APOPTOSIS AND DISEASE - REGULATION AND CLINICAL RELEVANCE OF PROGRAMMED CELL DEATH [Review]. *Annual Review of Medicine* **48**(267): 267-281.

Ryoo, H.D., Bergmann, A., Gonen, H., Ciechanover, A., and Steller, H. 2002. Regulation of Drosophila IAP1 degradation and apoptosis by reaper and ubcD1. *Nat Cell Biol* **4**(6): 432-438.

Salvesen, G.S. 2002. Caspases and apoptosis. *Essays Biochem* **38**: 9-19.

Salvesen, G.S. and Abrams, J.M. 2004. Caspase activation - stepping on the gas or releasing the brakes? Lessons from humans and flies. *Oncogene* **23**(16): 2774-2784.

Salvesen, G.S. and Duckett, C.S. 2002. IAP proteins: blocking the road to death's door. *Nat Rev Mol Cell Biol* **3**(6): 401-410.

Salvesen, G.S. and Renatus, M. 2002. Apoptosome: the seven-spoked death machine. *Dev Cell* **2**(3): 256-257.

Schafer, Z.T. and Kornbluth, S. 2006. The apoptosome: physiological, developmental, and pathological modes of regulation. *Dev Cell* **10**(5): 549-561.

Sevrioukov, E.A., Burr, J., Huang, E.W., Assi, H.H., Monserrate, J.P., Purves, D.C., Wu, J.N., Song, E.J., and Brachmann, C.B. 2007. Drosophila Bcl-2 proteins participate in stress-induced apoptosis, but are not required for normal development. *Genesis* **45**(4): 184-193.

Shi, Y. 2002a. Mechanisms of caspase activation and inhibition during apoptosis. *Mol Cell* **9**(3): 459-470.

-. 2002b. Mechanisms of Caspase Activation and Inhibition during Apoptosis. *Mol Cell* **9**(3): 459-470.

-. 2004. Caspase activation, inhibition, and reactivation: a mechanistic view. *Protein Sci* **13**(8): 1979-1987.

Spriggs, M.K. 1996. ONE STEP AHEAD OF THE GAME - VIRAL IMMUNOMODULATORY MOLECULES [Review]. *Annual Review of Immunology* **14**(101): 101-130.

Srinivasula, S.M., Datta, P., Kobayashi, M., Wu, J.W., Fujioka, M., Hegde, R., Zhang, Z., Mukattash, R., Fernandes-Alnemri, T., Shi, Y., Jaynes, J.B., and Alnemri, E.S. 2002. sickle, a Novel Drosophila Death Gene in the reaper/hid/grim Region, Encodes an IAP-Inhibitory Protein. *Curr Biol* **12**(2): 125-130.

Srivastava, M., Scherr, H., Lackey, M., Xu, D., Chen, Z., Lu, J., and Bergmann, A. 2006. ARK, the Apaf-1 related killer in Drosophila, requires diverse domains for its apoptotic activity. *Cell Death Differ*.

Sun, H., Nikolovska-Coleska, Z., Yang, C.Y., Xu, L., Tomita, Y., Krajewski, K., Roller, P.P., and Wang, S. 2004. Structure-based design, synthesis, and evaluation of conformationally constrained mimetics of the second mitochondria-derived activator of caspase that target the X-linked inhibitor of apoptosis protein/caspase-9 interaction site. *J Med Chem* **47**(17): 4147-4150.

Tenev, T., Zachariou, A., Wilson, R., Paul, A., and Meier, P. 2002. Jafrac2 is an IAP antagonist that promotes cell death by liberating Dronc from DIAP1. *Embo J* **21**(19): 5118-5129.

Teodoro, J.G. and Branton, P.E. 1997. REGULATION OF APOPTOSIS BY VIRAL GENE PRODUCTS [Review]. *Journal of Virology* **71**(3): 1739-1746.

Thompson, B.J. and Cohen, S.M. 2006. The Hippo pathway regulates the bantam microRNA to control cell proliferation and apoptosis in Drosophila. *Cell* **126**(4): 767-774.

Thompson, C.B. 1995. Apoptosis in the pathogenesis and treatment of disease. [Review] [84 refs]. *Science* **267**(5203): 1456-1462.

Vaux, D.L. and Korsmeyer, S.J. 1999. Cell death in development. *Cell* **96**(2): 245-254.

Vaux, D.L. and Silke, J. 2005. IAPs, RINGs and ubiquitylation. *Nat Rev Mol Cell Biol* **6**(4): 287-297.

Verhagen, A.M. and Vaux, D.L. 2002. Cell death regulation by the mammalian IAP antagonist Diablo/Smac. *Apoptosis* **7**(2): 163-166.

Vernooy, S.Y., Chow, V., Su, J., Verbrugghe, K., Yang, J., Cole, S., Olson, M.R., and Hay, B.A. 2002. Drosophila Bruce can potently suppress Rpr- and Grim-dependent but not Hid-dependent cell death. *Curr Biol* **12**(13): 1164-1168.

Vernooy, S.Y., Copeland, J., Ghaboosi, N., Griffin, E.E., Yoo, S.J., and Hay, B.A. 2000. Cell death regulation in Drosophila: conservation of mechanism and unique insights. *J Cell Biol* **150**(2): F69-76.

Vogler, M., Giagkousiklidis, S., Genze, F., Gschwend, J.E., Debatin, K.M., and Fulda, S. 2005. Inhibition of clonogenic tumor growth: a novel function of Smac contributing to its antitumor activity. *Oncogene* **24**(48): 7190-7202.

Vucic, D., Kaiser, W.J., and Miller, L.K. 1998a. A mutational analysis of the baculovirus inhibitor of apoptosis Op-IAP. *Journal of Biological Chemistry* **273**(51): 33915-33921.

-. 1998b. Inhibitor of Apoptosis Proteins Physically Interact With and Block Apoptosis Induced By Drosophila Proteins Hid and Grim. *Molecular & Cellular Biology* **18**(6): 3300-3309.

Waldhuber, M., Emoto, K., and Petritsch, C. 2005. The Drosophila caspase DRONC is required for metamorphosis and cell death in response to irradiation and developmental signals. *Mech Dev* **122**(7-8): 914-927.

Walker, N.I., Harmon, B.V., Gobe, G.C., and Kerr, J.F. 1988. Patterns of cell death. *Methods Achiev Exp Pathol* **13**: 18-54.

Wang, S.L., Hawkins, C.J., Yoo, S.J., Muller, H.A., and Hay, B.A. 1999. The Drosophila caspase inhibitor DIAP1 is essential for cell survival and is negatively regulated by HID. *Cell* **98**(4): 453-463.

Wei, M.C., Zong, W.X., Cheng, E.H., Lindsten, T., Panoutsakopoulou, V., Ross, A.J., Roth, K.A., MacGregor, G.R., Thompson, C.B., and Korsmeyer, S.J.

2001. Proapoptotic BAX and BAK: a requisite gateway to mitochondrial dysfunction and death. *Science* **292**(5517): 727-730.

White, K., Grether, M., Abrams, J.M., Young, L., Farrell, K., and Steller, H. 1994a. Genetic Control of Programmed Cell Death in *Drosophila*. *Science* **264**: 677-683.

White, K., Grether, M.E., Abrams, J.M., Young, L., Farrell, K., and Steller, H. 1994b. Genetic control of programmed cell death in Drosophila. *Science* **264**(5159): 677-683.

Wilson, R., Goyal, L., Ditzel, M., Zachariou, A., Baker, D.A., Agapite, J., Steller, H., and Meier, P. 2002. The DIAP1 RING finger mediates ubiquitination of Dronc and is indispensable for regulating apoptosis. *Nat Cell Biol* **4**(6): 445-450.

Wing, J.P., Karres, J.S., Ogdahl, J.L., Zhou, L., Schwartz, L.M., and Nambu, J.R. 2002a. Drosophila sickle Is a Novel grim-reaper Cell Death Activator. *Curr Biol* **12**(2): 131-135.

Wing, J.P., Schreader, B.A., Yokokura, T., Wang, Y., Andrews, P.S., Huseinovic, N., Dong, C.K., Ogdahl, J.L., Schwartz, L.M., White, K., and Nambu, J.R. 2002b. Drosophila Morgue is an F box/ubiquitin conjugase domain protein important for grim-reaper mediated apoptosis. *Nat Cell Biol* **4**(6): 451-456.

Wing, J.P., Schwartz, L.M., and Nambu, J.R. 2001. The RHG motifs of Drosophila Reaper and Grim are important for their distinct cell death-inducing abilities. *Mech Dev* **102**(1-2): 193-203.

Wing, J.P., Zhou, L., Schwartz, L.M., and Nambu, J.R. 1998. Distinct Cell Killing Properties of the Drosophila Reaper, Head Involution Defective, and Grim Genes. *Cell Death & Differentiation* **5**(11): 930-939.

Worby, C.A., Simonson-Leff, N., and Dixon, J.E. 2001. RNA interference of gene expression (RNAi) in cultured Drosophila cells. *Sci STKE* **2001**(95): PL1.

Wu, J.W., Cocina, A.E., Chai, J., Hay, B.A., and Shi, Y. 2001. Structural analysis of a functional DIAP1 fragment bound to grim and hid peptides. *Mol Cell* **8**(1): 95-104.

Wu, S., Huang, J., Dong, J., and Pan, D. 2003. hippo encodes a Ste-20 family protein kinase that restricts cell proliferation and promotes apoptosis in conjunction with salvador and warts. *Cell* **114**(4): 445-456.

Wyllie, A.H., Kerr, J.F., and Currie, A.R. 1980. Cell death: the significance of apoptosis. *Int Rev Cytol* **68**: 251-306.

Xu, D., Li, Y., Arcaro, M., Lackey, M., and Bergmann, A. 2005. The CARD-carrying caspase Dronc is essential for most, but not all, developmental cell death in Drosophila. *Development* **132**(9): 2125-2134.

Xu, D., Wang, Y., Willecke, R., Chen, Z., Ding, T., and Bergmann, A. 2006. The effector caspases drICE and dcp-1 have partially overlapping functions in the apoptotic pathway in Drosophila. *Cell Death Differ* **13**(10): 1697-1706.

Xu, P., Vernooy, S.Y., Guo, M., and Hay, B.A. 2003. The Drosophila MicroRNA Mir-14 Suppresses Cell Death and Is Required for Normal Fat Metabolism. *Curr Biol* **13**(9): 790-795.

Yan, N., Wu, J.W., Chai, J., Li, W., and Shi, Y. 2004. Molecular mechanisms of DrICE inhibition by DIAP1 and removal of inhibition by Reaper, Hid and Grim. *Nat Struct Mol Biol* **11**(5): 420-428.

Yin, V.P. and Thummel, C.S. 2004. A balance between the diap1 death inhibitor and reaper and hid death inducers controls steroid-triggered cell death in Drosophila. *Proc Natl Acad Sci U S A* **101**(21): 8022-8027.

Yoo, S.J., Huh, J.R., Muro, I., Yu, H., Wang, L., Wang, S.L., Feldman, R.M., Clem, R.J., Muller, H.A., and Hay, B.A. 2002. Hid, Rpr and Grim negatively regulate DIAP1 levels through distinct mechanisms. *Nat Cell Biol* **4**(6): 416-424.

Yu, X., Wang, L., Acehan, D., Wang, X., and Akey, C.W. 2006. Three-dimensional structure of a double apoptosome formed by the Drosophila Apaf-1 related killer. *J Mol Biol* **355**(3): 577-589.

Yuan, J. 2006. Divergence from a dedicated cellular suicide mechanism: exploring the evolution of cell death. *Mol Cell* **23**(1): 1-12.

Yuan, J. and Horvitz, H.R. 1992a. The Caenorhabditis elegans cell death gene ced-4 encodes a novel protein and is expressed during the period of extensive programmed cell death. *Development* **116**(2): 309-320.

-. 1992b. The *Caenorhabditis elegans* cell death gene *ced-4* encodes a novel protein and is expressed during the period of extensive programmed cell death. *Development* **116**: 309-320.

Yuan, J., Shaham, S., Ledoux, S., Ellis, H.M., and Horvitz, H.R. 1993a. The C. elegans cell death gene ced-3 encodes a protein similar to mammalian interleukin-1 beta-converting enzyme. *Cell* **75**(4): 641-652.

-. 1993b. The C. elegans cell death gene *ced-3* encodes a protein similar to mammalian Interleukin-1B-converting enzyme. *Cell* **75**: 641-652.

Zachariou, A., Tenev, T., Goyal, L., Agapite, J., Steller, H., and Meier, P. 2003. IAP-antagonists exhibit non-redundant modes of action through differential DIAP1 binding. *Embo J* **22**(24): 6642-6652.

Zhang, H., Huang, Q., Ke, N., Matsuyama, S., Hammock, B., Godzik, A., and Reed, J.C. 2000. Drosophila pro-apoptotic Bcl-2/Bax homologue reveals evolutionary conservation of cell death mechanisms. *J Biol Chem* **275**(35): 27303-27306.

Zhang, J.H., Chung, T.D., and Oldenburg, K.R. 1999. A Simple Statistical Parameter for Use in Evaluation and Validation of High Throughput Screening Assays. *J Biomol Screen* **4**(2): 67-73.

Zhang, S.L., Yeromin, A.V., Zhang, X.H., Yu, Y., Safrina, O., Penna, A., Roos, J., Stauderman, K.A., and Cahalan, M.D. 2006. Genome-wide RNAi screen of Ca(2+) influx identifies genes that regulate Ca(2+) release-activated Ca(2+) channel activity. *Proc Natl Acad Sci U S A* **103**(24): 9357-9362.

Zheng, T.S., Hunot, S., Kuida, K., and Flavell, R.A. 1999. Caspase knockouts: matters of life and death. *Cell Death Differ* **6**(11): 1043-1053.

Zhou, L., Hashimi, H., Schwartz, L.M., and Nambu, J.R. 1995. Programmed cell death in the Drosophila central nervous system midline. *Current Biology* **5**(7): 784-790.

Zhou, L., Schnitzler, A., Agapite, J., Schwartz, L.M., Steller, H., and Nambu, J.R. 1997. Cooperative functions of the reaper and head involution defective genes in the programmed cell death of Drosophila central nervous system midline cells. *Proc Natl Acad Sci U S A* **94**(10): 5131-5136.

Zhou, L., Song, Z., Tittel, J., and Steller, H. 1999. HAC-1, a Drosophila homolog of APAF-1 and CED-4 functions in developmental and radiation-induced apoptosis. *Mol Cell* **4**(5): 745-755.

Zhou, L.L., Zhou, L.Y., Luo, K.Q., and Chang, D.C. 2005a. Smac/DIABLO and cytochrome c are released from mitochondria through a similar mechanism during UV-induced apoptosis. *Apoptosis* **10**(2): 289-299.

Zhou, R., Silverman, N., Hong, M., Liao, D.S., Chung, Y., Chen, Z.J., and Maniatis, T. 2005b. The role of ubiquitination in Drosophila innate immunity. *J Biol Chem* **280**(40): 34048-34055.

Zimmermann, K.C., Ricci, J.E., Droin, N.M., and Green, D.R. 2002. The role of ARK in stress-induced apoptosis in Drosophila cells. *J Cell Biol* **156**(6): 1077-1087.

VITAE

Su Kit Chew was born in Singapore on the 18th of June, 1978, the son of Weng Chew and Ah Mui Pang. He has an older sister Kia Yin Chew. After graduating from Victoria Junior College in 1996, he served in the Singapore Armed Forces from December of 1996 to June of 1999, during which he was commissioned as an infantry officer. Su Kit left for England in the fall of 1999 to read biological sciences at Brasenose College, Oxford University. While at Oxford, he was tutored by Dr. Malcolm M. Campbell at Brasenose College and received his Finals Honours thesis training under Dr. Janneke Balk in the laboratory of Professor Chris J. Leaver. He received a Bachelor of Arts degree with Upper Second Class Honours in June 2001. He moved to Dallas, Texas in August of 2001 to attend the Graduate School of Biomedical Sciences at the University of Texas Southwestern Medical Center. In May of 2002, he joined the laboratory of Professor John M. Abrams under the Genetics and Development program to pursue his dissertation work.

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