THE REGULATION OF DROSOPHILA INHIBITOR OF APOPTOSIS PROTEIN I IN APOPTOSIS AND CELL SURVIVAL

by

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A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
(Molecular Cellular and Developmental Biology)
in The University of Michigan
2009

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The path to glory is always rugged

天将降大任于斯人也，必先苦其心志，劳其筋骨，饿其体肤，

空乏其身，行拂乱其所为，所以动心忍性，增益其所不能.

—孟子

To my mother and husband

ACKNOWLEDGEMENTS

It has been six years since I came to Ann Arbor to pursue my PhD degree. I would like to express my gratitude to all the people I met during this period. It has been a great journey.

First and foremost, I would like to thank my advisor, Dr. Ken Cadigan, for his continuous guidance, wholehearted support and enthusiastic encouragement. Ken has tremendous influence on me both inside and outside science. He has taught me an incredible amount about how to do research, how to envision 'the big picture' and then work out the details, and how to be a logical, critical and creative thinker, all of which are a truly invaluable treasure for me.

I am extremely grateful to the members of my thesis committee, Dr. Steve Clark, Dr. Eva Feldman, Dr. Ormond Macdougald and Dr. Jianming Li, for their insightful, thought provoking and constructive advice, which has greatly improved the quality of my dissertation work.

I would like to thank all current and previous Cadigan lab members for providing such an intellectual stimulating, supportive and enjoyable environment. I owe my gratitude to my friend and classmate, Yunyun Ni, who took the six-year journey together with me to achieve every milestone along the way, supporting me professionally and personally, and sharing openly with me happeniess and sadness. I am grateful to have

such a true friend in the lab. I would like to thank Dr. Ju Guan, who I worked with during my first year on the Dco project, and Dr. Jennifer Kennell and undergraduate student Liyu Tan, who also helped me with the Dco and Gish project. Thanks to Chandan Bhambhani and Dave Parker, my current and previous baymates, and Yan Liu, who shared a big bench with me. They always had interesting things to share with me, making my life in the lab very delightful. I also want to thank Mikyung Chang, JinHee Chang and Jennifer Kennell for their help and support. They were always so considerate and caring.

Thanks to our neighbors, Collins Lab, Csankovszki Lab and Raymond Lab, for their generosity for reagents, equipments and advice, as well as all the talks in the hallway and happy hours. During my first two years here, our lab didn't have neighbors, therefore, I really cherish our lovely third floor corner right now. I want to thank Dr. Clark for giving me the opportunity to rotate in his lab, and all people in Clark lab.

I would like to thank Dr. Santhadevi Jeyabalan, who I worked for as Graduate Student Instructor for three terms. She really helped me grow up as a confident instructor and start to love teaching. Special thanks to Dr. Diane Spillane, who I really should have known personally much earlier than when we taught Introductory Biology together. She generously helped me in the most difficult and depressing moment I had in the past several years.

I appreciate all the useful discussions on my projects with people in several joint group meetings our lab participated in.

Thanks to Ms. Mary Carr, who is always ready to help. She made my life here much easier. Thanks to Sobocinski Gregg for helping me with confocal microscopy. Also, thanks to all people in MCDB office.

I would like to sincerely thank my husband Weifeng Ye and my mom for their unconditional love and unwavering support. I am extremely grateful to all my friends for making my journey here so much enjoyable and rewarding.

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CHAPTER I

GENERAL INTRODUCTION

APOPTOSIS IN MULTI-CELLULAR ORGANISM

Living with Death

As in Taolism, the interconnected and interdependent balance between Yin and Yang achieves the ultimate path leading to a greater whole in nature. In an analogous fashion in biology, with Yin presenting death, and Yang presenting life, the ancient philosophy nicely describes how multi-cellular organisms balance the death and survival of cells, i.e., by achieving dynamic equilibrium between signals that drive cellular destruction and promote proliferation and differentiation (Lam and Greenberg 2000; Baehrecke 2002). In short, death shapes life during development, and death ensures life in adulthood.

Programmed cell death is an actively regulated cell death process

The destruction of cells was first observed during the seventeenth century, but physiological cell death was not clearly recognized as a normal component of life until

the middle of the twentieth century by A. Glücksmann (Glucksmann 1951). Studies beginning from 1950s conceptualized the term 'Programmed Cell Death' (PCD) which was originally used to describe a series of events that culminate in the death of a cell. Later on, PCD has been endued with a broad meaning for naturally occurring cell death that is carried out in an actively regulated process with defined morphological features (Baehrecke 2002; Vaux 2002; Zakeri and Lockshin 2008). PCD is distinct from necrosis which is induced by acute external cellular injury and provokes the leaking of the cell contents and inflammation (Baehrecke 2002; Gilbert 2003).

One major form of PCD in multi-cellular organisms is apoptosis. Coined in 1970s by Kerr and colleagues, the term 'apoptosis' was adopted from a Greek word meaning the process of leaves falling from trees or petals falling from flowers (Baehrecke 2002; Gilbert 2003). Apoptosis involves a series of biochemical events that lead to a step-wise morphological changes, involving chromosomal DNA fragmentation, nucleus and cytoplasm condensation, membrane blebbing, followed by cell fragmentation and phagocytosis by macrophages or neighboring cells (Figure 1.1) (Baehrecke 2002; Gilbert 2003; Lodish 2007). Apoptosis eliminates cells that are in excess or dangerous to achieve normal development and maintain homeostasis.

Autophagy is another abundant form of PCD with different molecular mechanisms and morphological changes (Levine and Kroemer 2008; Kourtis and Tavernarakis 2009). Although several studies indicate autophagy shares some key machinery with apoptosis,

e.g. during *Drosophila* midgut destruction in metamorphosis, the two processes are still considered to be distinct (Baehrecke 2002; Baehrecke 2003; Leulier et al. 2006; Kroemer et al. 2009).

Apoptosis has been implicated in development and adult homeostasis

Apoptosis is a normal part of development. Classical examples include the sculpting of digits in vertebrate limb bud (Zuzarte-Luis and Hurle 2002), the deletion of larval tissues during metamorphosis in amphibians and insects (in conjunction with autophagy) (Fox 1975; Shi and IshizuyaOka 1996; Baehrecke 2002), the elimination of male mouse mammary tissues (Kratochwil and Schwartz 1976), and the removal of excess neurons during the development of neuron system (Klambt et al. 1991; Robinow et al. 1993). Apoptosis also removes cells damaged, e.g. to DNA (Abrams et al. 1993; MacCallum et al. 1996), and cells that are potentially dangerous, e.g., lymphocytes that produce self-reactive receptors (Krammer 2000). In summary, apoptosis contributes significantly ensuring the appropriate shape, cell number and functions of developing organisms (Baehrecke, 2002; Gilbert, 2003).

In adulthood, apoptosis is essential for balancing proliferation, selection of the immune repertoire and defense of infected cells. An average adult human loses as many as 10^{11} cells via apoptosis each day, which means the accumulated mass that each person eliminates throughout a year roughly equals to the weight of the entire body (Gilbert 2003). Not surprisingly, deregulation of apoptosis has severe consequences.

Inappropriate apoptosis is reported to associate with degenerative neurological diseases, stoke, cardiac ischaemia and immune suppression that is implicated with AIDS; whereas suppression of naturally occurring apoptosis is linked with autoimmune diseases and cancers (Baehrecke 2002; Green and Evan 2002; Zakeri and Lockshin 2008).

Tremendous progress has been made in understanding the mechanism by which apoptosis is carried out and regulated; now apoptosis is widely believed to be the result of an evolutionarily conserved, dedicated cell destruction process regulated by a wide range of signals.

Apoptosis machinery is evolutionarily conserved

The core apoptosis machinery and mechanisms of regulation are evolutionarily conserved from the nematode *Caenorhabditis elegans*, the fruit fly *Drosophila melanogaster*, to mammals (Table 1.1).

The main executioner of apoptosis is a family of cysteine proteases called caspases. Caspases are highly selective proteases that preferentially cleave after Asp residues (Thornberry et al. 1997). As is true for most proteases, caspases are synthesized as enzymatically inactive zymogens, and they are activated by proteolytic processing. Based on their pro-domains as well as inherent substrate specificity, caspases can be divided into long pro-domain initiator caspases and short pro-domain effector caspases. Upon stimulation by upstream death signals, initiator caspases are activated first, leading to the cleavage and activation of effector caspases, which in turn process hundreds of cellular

proteins, resulting in a series well defined morphological changes that characterize apoptosis (Hengartner 2000; Salvesen and Abrams 2004).

In mammals, the caspase cascade can be initiated through two major branches, the intrinsic mitochondria pathway and external death receptor pathway. Intrinsic death stimuli trigger the release of cytochrome *c* from mitochondria, leading to the activation of Apaf-1 and its consequent oligomerization into an initiator caspase activation platform called the apoptosome. The apoptosome in turn recruits and facilities the activation of caspase-9 (Liu et al. 1996; Kluck et al. 1997; Li et al. 1997; Danial and Korsmeyer 2004). In the death receptor pathway, ligand-bound death receptors recruit the adaptor proteins Fadd, inducing the formation of a Death Inducing Signaling Complex (DISC). DISC recruits and activates initiator caspases, such as caspase-8 and caspase-10 (Danial and Korsmeyer 2004; Salvesen and Abrams 2004). The initiator caspases of both pathways cleave and thereby activate caspase 3 and caspase 7, therefore, the two pathways converge at the level of effector caspase activation (Danial and Korsmeyer 2004; Salvesen and Abrams 2004).

The comparison between the two pathways reveals a common theme that the activation of initiator caspases requires the formation of multimeric activation complexes with adaptor proteins as scaffolds. The protein-protein interaction modules that mediate the formation of those complexes are the Caspase Recruitment Domain (CARD) in Apaf-1 and caspase-9, and the Death Effector Domains (DED) in Fadd and caspase-8 / -10

(Hengartner 2000; Salvesen and Abrams 2004). CARD and DED share little sequence identity, but fold into very similar three-dimensional structures, suggesting they may derive from a common ancestral domain (Hofmann 1999; Yan and Shi 2005).

The fact that *C. elegans* has only one layer of caspase, CED-3, supports the idea that hierarchical activation of caspases was added to the ancient pathway during evolution to optimize the apoptosis system. The advantages of a caspase cascade may be threefold. First, it amplifies the apoptotic signal by generating substantial amounts of active effector caspases. Second, it provides more regulatory points before the final commitment to irreversible cell death. Third, it allows initiator caspases to be specialized to sense different types of upstream signals, yet integrate signals at the common execution phase at the level of effector caspases. Indeed, phylogenetic and statistic analyses suggest that effector caspases largely remain unchanged, while more initiator caspases and initial death signals are continuously recruited during the evolution of apoptosis pathway (Wang and Gu 2001).

Caspases is also subjected to negative regulation by endogenous inhibitors of caspases, the Inhibitor of Apoptosis Protein (IAP) family. The most extensively characterized IAPs include mammalian X-linked IAP (XIAP) (Duckett et al. 1996; Uren et al. 1996), c-IAP1 and c-IAP2 (Rothe et al. 1995), Survivin (Ambrosini et al. 1997) and *Drosophila* IAP1(DIAP1) (Hay et al. 1995; Vaux and Silke 2005; Srinivasula and Ashwell 2008). A significant body of evidence has shown that IAPs directly bind to and inhibit the

activation and activity of caspases through several distinct mechanisms (Hay et al. 1995; Vaux and Silke 2005; Srinivasula and Ashwell 2008). The anti-apoptotic function of IAPs is in turn antagonized by a group of pro-apoptotic proteins including SMAC/Diablo (Du et al. 2000; Verhagen et al. 2000) and Omi/HtrA2 (Suzuki et al. 2001) in mammals and Reaper (Rpr) (White et al. 1994; White et al. 1996), Head involution defective (Hid) (Grether et al. 1995) ,Grim (Chen et al. 1996), Sickle (Christich et al. 2002; Srinivasula et al. 2002; Wing et al. 2002), Jafrac-2 (Tenev et al. 2002) and dOmi (Khan et al. 2008) in flies, which are collectively referred to as RHG proteins. The regulation of IAPs is one of the important decisions for cells poised between life and death. Studies on DIAP1 and fly RHG proteins have contributed significantly to our current understanding of apoptosis regulation. The following discussion and the focus of the dissertation will be directed at the regulation of DIAP1 in both apoptotic and living cells in *Drosophila*.

APOPTOSIS REGULATION IN DROSOPHILA

The fruit fly *Drosophila* is a superb model system to explore the mechanisms by which apoptosis is carried out and regulated. First, apoptosis happens, and has been characterized, throughout fly development and adulthood, providing diverse and highly accessible research subjects. Secondly, the core machinery and regulation mechanism have been shown to be evolutionarily conserved, allowing similar findings to be extended to other model organisms. Third, the fly system is well known for its rich genetic and

molecular resources. Therefore, the fruit fly system provides outstanding opportunities to enrich our understanding on apoptosis regulation.

Apoptosis happens throughout fly development

Apoptosis happens throughout fly development. For instance, during embryogenesis, most of midline glial cells are eliminated by apoptosis to achieve a well circuited central neural system (Sonnenfeld and Jacobs 1995; Zhou et al. 1995). During metamorphosis, steroid hormone ecdysone triggers apoptosis and autophagy to remove the embryonic neural system, larval midgut, salivary glands and some other tissues that are no longer needed during adulthood (Jiang et al. 1997; Baehrecke 2003). In addition, apoptosis occurs during the development of fly compound eye in larva and pupal stages.

The fly retina provides a useful model to understand apoptosis events and to elucidate their physiological importance. The adult fly compound eye is composed of roughly 750 repeating hexagonal clusters called ommatidia. Each ommatidium consists of eight photoreceptors surrounded by pigment cells, bristle and cone cells (Figure 1.2). This highly organized structure is achieved by selective recruitment plus several waves of apoptosis which removes extra cells during larval and pupal stages (Kickson and Hafen 1993; Wolff and Ready 1993; Brachmann and Cagan 2003). At early larval life, eye disc is a sheet of undifferentiated cells which, later on, are specified into different cell fates via active recruitment of photoreceptors, cone cells, primary pigment cells and finally secondary and tertiary pigment cells. After the specification of photoreceptor cell clusters,

the undifferentiated cells furthest from these clusters undergo apoptosis (Cagan and Ready 1989; Bate and Arias 1993; Wolff and Ready 1993).

Another wave of apoptosis happens after the primary pigment cells adopt their cell fate around 20 hours after pupurium formation (APF, 25 ºC) (Cagan and Ready 1989; Bate and Arias 1993; Wolff and Ready 1993). During the following 16 hours, apoptosis eliminates approximately 2000 cells, which is about one third of the unspecified inter-ommatidia population at the time. The remaining cells are then sorted into secondary and tertiary pigment cells that resolve the precise inter-ommatidia lattice. There is a later occurrence of apoptosis that removes periphery ommatidia during mid-pupation, as discussed in detail below. These waves of apoptosis allow the precise rearrangement of ommatidia into a highly organized honeycomb-like structure, while imperfect alignment of ommatidia presumably affects the ability of the fly to see (Cagan and Ready 1989; Wolff and Ready 1991; Bate and Arias 1993; Kickson and Hafen 1993; Wolff and Ready 1993; Brachmann and Cagan 2003).

Apoptosis at the perimeter of pupal eye

Apoptosis eliminates about 80 to 100 ommatidia at the edge of the eye during mid-pupation (Wolff and Ready 1991; Bate and Arias 1993; Wolff and Ready 1993; Lin et al. 2004). These perimeter ommatidia often lack the full component of photoreceptors and support cells (Figure 1.3 A-H) (Lin et al. 2004).

What is physiological importance of this wave of apoptosis? The answer may lie in the precise organization of the fly retina and underlying lamina. The fly lamina is composed of an array of columns called cartridges, and each cartridge is positioned right underneath one retinal ommatidia. Interestingly, the six outer photoreceptors (R1-R6) of an ommatidium do not project axons into the underlying cartridge; rather, the bundle of six axons twists and diverges while it descends towards the lamina, and eventually each axon froms synapses with six distinct neighboring cartridges surrounding the underlying one (Figure 1.4). The process creates an interlocking network of interwoven receptor connections across the entire lamia, where each ommatidia form synapses with six cartridges in lamina. Such a precise network presumably fulfills the functional requirement of retinotopy of the fly compound eye (Meinertzhagen and Hanson 1993; Lin et al. 2004).

However, this strategy leads to a dilemma at the edge of the eye: on one hand, the precise arrangement described above requires one more circle of cartridges than ommatidia, so that ommatidia at outer rim have enough cartridges on the lamina to innervate; on the other hand, cartridges cannot be formed without the induction by the overlying ommatidia during larva and early pupae stage. It seems likely that flies solve the problem by first forming an extra circle of ommatidia, which induce the formation of underlying cartridges, and then eliminating these ommatidia through apoptosis after their mission is fulfilled. In short, perimeter ommatidia apoptosis is speculated to allow the precise synapse formation

between photoreceptors of the retina and cartridges of lamina (Meinertzhagen and Hanson 1993; Lin et al. 2004).

The molecular mechanism that triggers this apoptosis event has been partially uncovered by previous studies. It has been demonstrated that the secreted glycoprotein Wingless (Wg) elevates the expression of the RHG proteins Hid, Grim and Rpr, triggering apoptosis in perimeter ommatidia (Figure 1.4 I)(Lin et al. 2004). How Wg induces the RHG expression is unclear, although the snail family of transcription factors have been proposed to play a role in the process (Lim and Tomlinson 2006).

Perimeter ommatidia apoptosis serves as an outstanding system to study apoptotic mechanisms. First, the entire ommatidia are eliminated wholesale, rather than cell-by-cell elimination as in inter-ommatidia and embryonic midline cells apoptosis. It makes observation much easier, and does not require imaging techniques with single cell resolution. Secondly, ommatidia that are destined to die have a definite location; virtually, every ommatidia row ends in one of them (Wolff and Ready 1993). And Wg expression truthfully marks ommatidia that are destined to die (Lin et al. 2004). Therefore, cell fates can be predicted precisely, allowing early observations even before apoptosis takes places. In addition, apoptosis only eliminates part of the tissue, rather than the whole tissue as in larval midgut and salivary gland cell death. It is extremely useful for the observation of DIAP1 protein level changes during apoptosis, since interior ommatidia that continue to survive serve as natural internal controls. Furthermore, it has been demonstrated that the

core apoptosis machinery is required for the event (Lin et al. 2004; Mendes et al. 2006). All the reasons stated above make the periphery apoptosis an attractive model system to study naturally occurring apoptosis, and Chapter II in this dissertation presents one of the examples.

The apoptotic core machinery is composed of Dark, Dronc and Drice

The *Drosophila* genome encodes seven caspases, including three putative initiator caspases, Dronc (Dorstyn et al. 1999), Dredd (Chen et al. 1998) and Strica (Vernooy et al. 2000; Doumanis et al. 2001), and four putative effector caspase, Drice (Fraser and Evan 1997), Dcp-1(Song et al. 1997), Decay (Dorstyn et al. 1999) and Damm (Harvey et al. 2001). Among the seven caspases, the function of initiator caspase Dronc and effector caspase Drice are well established as critical factors for fly apoptosis (Salvesen and Abrams 2004; Hay and Guo 2006; Steller 2008). The activation of Dronc requires adaptor protein Dark (*Drosophila* Apaf-1 related killer) (Kanuka et al. 1999; Rodriguez et al. 1999; Zhou et al. 1999). Dark, Dronc and Drice initiate many, but not all, cell death in *Drosophila* (Hay and Guo 2006).

Dronc, the only CARD domain containing caspase in fly, is essential for bringing about apoptosis. Dronc is ubiquitously expressed during early development and dramatically upregulated at the transcription level by steroid hormone ecdyson in salivary glands and midgut before histolysis of these tissues during metamorphosis (Dorstyn et al. 1999). Animals that lacking *dronc* show reduced cell death during the developmental of

embryo, eye, wing, central nervous system and larval salivary gland, and in response to multiple stress conditions such as DNA damage (Chew et al. 2004; Daish et al. 2004; Waldhuber et al. 2005; Xu et al. 2005; Mendes et al. 2006). Similar results are also obtained by overexpression of the dominant-negative version of Dronc and *dronc* RNAi in flies or cultured embryonic cell line S2 cells (Hawkins et al. 2000; Meier et al. 2000; Quinn et al. 2000; Igaki et al. 2002; Muro et al. 2002; Muro et al. 2004; Kiessling and Green 2006; Leulier et al. 2006). The exact activation mechanism of Dronc is still under debate, but it is clear that the process is Dark-dependent.

Dark is the fly homologue of mammalian Apaf-1(Kanuka et al. 1999; Rodriguez et al. 1999; Zhou et al. 1999). Several loss of function analyses support its critical role in apoptosis. Reduced level of Dark by RNAi or in animals bearing *dark* hypomorphic alleles leads to decreased cell death in several contexts, including, embryo, larval brain, and central nervous system (Kanuka et al. 1999; Rodriguez et al. 1999; Srivastava et al. 2007). Both strong and null alleles of Dark blocks most, but not all, developmental apoptosis (Mills et al. 2006; Srivastava et al. 2007), including perimeter ommatidia apoptosis in developing eye (Mendes et al. 2006). Biochemical analysis and structural studies illustrated that Dark can interact with Dronc through its CARD domain to from a high-molecular-weight complex, a structure resembling the mammalian apoptosome, further supporting its function as a adaptor protein for Dronc activation (Quinn et al. 2000; Dorstyn et al. 2002; Yu et al. 2006; Dorstyn and Kumar 2008).

The effector caspase Drice is also important for many apoptotic events. As the case with *Dronc* mutants, loss of Drice results in reduced cell death in many contexts. Drice and another effector caspase Dcp-1 have partially overlapping functions to execute developmental apoptosis and apoptosis in response to stress such as X-irradiation (Leulier et al. 2006; Mendes et al. 2006; Muro et al. 2006; Xu et al. 2006). In contrast, *Dcp-1* null alleles are healthy and fertile (Xu et al. 2006). The only cell death phenotype reported for *Dcp-1* mutant is the lack of germline cell death during mid-oogenesis in response to nutrient deprivation. The comparison may imply Drice as a predominant effector caspase in the fly.

Drice is known to be cleaved and thereby activated by Dronc (Hawkins et al. 2000; Meier et al. 2000). Depletion of Drice inhibits Dronc-dependent cell death, suggesting Drice is genetically downstream of Dronc (Fraser and Evan 1997; Muro et al. 2004). The antibodies that recognize the cleaved, activated form of Drice, referred to as active-Drice antibody, are widely used to label apoptotic cells during development as well as cells exposed to other apoptotic stimuli (Yoo et al. 2002; Yu et al. 2002; Muro et al. 2006; Xu et al. 2006).

Clearly, Dark, Dronc, and Drice do not present the whole picture of the apoptosis execution in *Drosophila*. A number of apoptotic events have been shown to be independent of the three core factors in embryos and during metamorphosis (Chew et al. 2004; Daish et al. 2004; Xu et al. 2005; Xu et al. 2006; Srivastava et al. 2007), and some

animals lacking Dronc or Dark even survive to adulthood (Xu et al. 2005; Srivastava et al. 2007). It is likely that other relatively uncharacterized caspases, i.e. Strica, Damm, Decay and apoptosis regulators, i.e. adaptor proteins, may participate in apoptotic events. Another possibility is that other forms of cell death contribute to cell elimination in flies, as it is known that autophagy happens during metamorphosis (Jiang et al. 1997; Baehrecke 2003). Nonetheless, for the purpose of this dissertation, the simplified linear pathway that involves Dark dependent-activation of Dronc and consequent activation of Drice is used as a working model to represent the apoptosis machinery. This core machinery is subjected to negative regulation of DIAP1 which provides the last line of defense against inappropriate apoptosis.

INHIBITOR OF APOPTOSIS PROTEIN (IAP) IS AN ESSENTIAL INHIBITOR OF APOPTOSIS

IAPs were first identified by Miller and colleagues in baculoviruses where they inhibit apoptosis in virally infected insect cells (Crook et al. 1993). Since then, many IAP family members have been identified in yeast, nematodes, flies and higher vertebrates (Figure 1.5). The majority of IAPs can bind and inhibit caspases, preventing inappropriate apoptosis (Vaux and Silke 2005; Srinivasula and Ashwell 2008). In *Drosophila*, DIAP1 is absolutely essential for cell survival, as loss of DIAP1 results in rapid caspase-mediated cell death (Wang et al. 1999; Goyal et al. 2000; Lisi et al. 2000). Human IAPs, including

XIAP, c-IAP1, c-IAP2 and survivin, have been implicated in apoptosis related diseases such as cancer, and served as promising drug targets for anti-cancer therapy (LaCasse et al. 2008). Consistently, when over-expressed, mammalian IAPs XIAP, c-IAP1 and c-IAP2, inhibit apoptosis effectively by directly binding to and neutralizing caspases (Srinivasula and Ashwell 2008). Intriguingly, mice carrying mutants of XIAP, c-IAP1 and cIAP2 lack apoptosis-related phenotypes, which might be due to the functional redundancy of the eight IAPs identified by far in mouse (Ditzel and Meier 2002; Srinivasula and Ashwell 2008).

IAPs are characterized by the presence of the Baculavirus IAP Repeat (BIR) motif which is a sequence of about 70 amino acids holding a zinc ion via one histidine and three cysteine residues (Hinds et al. 1999; Sun et al. 1999). BIR motif mediates the interaction of IAPs with caspases and other pro-apoptotic factors such as RHG proteins, providing the basis for several models explaining how IAPs inhibit capases and how the inhibition is antagonized by RHGs in turn. These models will be further discussed in following sections.

Many of the IAPs also contain a C-terminal RING (Really Interesting New Gene) domain, which functions as an E3-uibquitin ligase in ubiquitination pathway. The first evidence supporting the importance of IAP's E3 ligase activity came from genetic studies on DIAP1, where point mutations that disrupt this activity resulted in an embryonic lethality in homozygotes, and affected cell death in different tissues in heterozygous flies (Wang et al. 1999; Lisi et al. 2000). Later, Yang *et. al.* conducted a breakthrough study in

thymocytes, illustrating that XIAP and c-IAP1 undergo auto-ubiquitination and consequent proteasomal degradation in a RING domain dependent manner, and the application of proteasome inhibitors prevents thymocyte death (Yang et al. 2000). Since then, significant efforts have been devoted to explore the roles of RING domain and ubiquitination in apoptosis regulation; however, it is extremely difficult to synthesize a simple model without controversy, probably due to the remarkable complexity of the regulation mechanisms.

The first level of complexity lies in the ultimate consequence of the ubiquitination by IAPs: cis-ubiquitination of IAP proteins themselves is pro-apoptotic, whereas trans-ubiquitination of substrates such as caspases or RHG proteins is anti-apoptotic. It is widely assumed that the relative levels of the two apparently opposite effects are clearly under regulatory control in living and apoptotic cells, which, however, remains to be elucidated.

Another level of complexity is that both degradative and nondegradative ubiquitination have been implicated in the regulation of apoptosis. Generally speaking, polyubiquitin chains assembled through lysine K48 of ubiquitin are a potent signal of ubiquitination-proteasome pathway, whereas polyubiquitin chains assembled through other lysine residues or monoubiquitin do not lead to degradation, rather they lead to a range of outcomes, including modification of protein activity (Vaux and Silke 2005). IAPs have been documented to mediate all the ubiquitination forms mentioned above

(Huang et al. 2000; Suzuki et al. 2001; Wilson et al. 2002; Tenev et al. 2005), raising the question what the intrinsic and/or extrinsic forces are that determine the forms of ubiquitination mediated by IAPs.

A further complexity is due to the potential crosstalk between IAPs and other E3 ligases. Accumulated evidence has brought into light some IAP interacting multi-subunit E3 ligase complexes (Hays et al. 2002; Wing et al. 2002; Ditzel et al. 2003), suggesting the existence of RING-independent ubiquitination. As it is ture for IAPs, the ubiquitination by these E3 complexes could be degratative or non-degratative, and the consequences could be pro- or anti-apoptotic, which makes the system more complicated (Hays et al. 2002; Wing et al. 2002).

To reveal the physiological functions and molecular mechanisms of IAPs in apoptosis regulaiton, intensive studies have been done on mammalian XIAP, c-IAP1, and cIAP2 and fly DIAP1. The role of IAPs in preventing inappropriate apoptosis is best illustrated by DIAP1.

DIAP1 is an essential inhibitor of apoptosis in *Drosophila*

DIAP1, the product of the *thread* (*th*) gene (Hay et al. 1995), is essential in preventing inappropriate apoptosis in *Drosophila*. *DIAP1* homozygous mutants die at very early stages of embryogenesis, with dramatically elevated levels of apoptosis (Wang et al. 1999; Goyal et al. 2000; Lisi et al. 2000). Loss of DIAP1 elicits cell death in variety of contexts, including homozygous clones in the germline cells and eye tissues, and RNAi

treated larval tissue, developing eye and cultured cells (Igaki et al. 2002; Muro et al. 2002; Zimmermann et al. 2002; Yokokura et al. 2004; Leulier et al. 2006). Consistent with the loss of function data, numerous lines of evidence have shown that overexpression of DIAP1 suppresses ectopic cell death induced by pro-apoptotic factors (Hay and Guo 2006).

The primary function of DIAP1 is to inhibit caspase activity. First of all, apoptosis caused by loss of DIAP1 can be suppressed by ectopic expression of the caspase inhibitor *p35* and by reducing the levels of Dark, Dronc, Drice and Dcp-1 (Igaki et al. 2002; Muro et al. 2002; Yoo et al. 2002; Zimmermann et al. 2002; Huh et al. 2004; Muro et al. 2004; Xu et al. 2005; Leulier et al. 2006; Muro et al. 2006; Xu et al. 2006). Overexpression of DIAP1 also reduces cell death induced by ectopic expression of activated versions of caspases (Wang et al. 1999; Hawkins et al. 2000; Meier et al. 2000). The same results were also obtained in yeast, a heterologous system thought to be unlikely to contain other counterparts of core death regulators of *Drosophila* (Wang et al. 1999; Hawkins et al. 2000). Finally, biochemical analyses illustrate that DIAP1 indirectly bind to and inhibit caspases through several distinct mechanisms.

DIAP1 regulates caspases through several mechanisms

The BIR1 domain of DIAP1 binds strongly with active Drice via the IAP-binding motif (IBM), and the interaction is critically important for apoptosis regulation. Cells with the interaction compromised, resulting from mutations on either IAP or Drice,

showed elevated apoptosis (Goyal et al. 2000; Lisi et al. 2000; Tenev et al. 2005). The exact mechanism by which DIAP1 inhibits Drice is unclear, although it has been proposed recently that the interaction between DIAP1 and Drice may lead to non-degradative ubiquitination of Drice, and the ubiquitin conjugation sterically interferes with substrate entry (Ditzel et al. 2008).

It should be noted that the binding by DIAP1 doesn't inhibit the catalytically activity of Drice. In fact, DIAP1 is cleaved at Asp20 by Drice upon the interaction (Tenev et al. 2005). The new N-terminal site created by the cleavage has been suggested to recruit the N-end rule ubiquitination machinery, leading to DIAP1 degradation, the process of which is referred to as the N-end rule degradation (Ditzel et al. 2003; Herman-Bachinsky et al. 2007). This mechanism would potentially remove any resistance from DIAP1, allowing apoptosis to proceed efficiently; however the physiological relevance of this positive feedback loop has yet to be established.

Interestingly, the IBM domain of Drice is only exposed after the removal of the pro-domain of the caspase, indicating effector caspases and DIAP1 are invisible to each other in absence of the Drice activation (Ditzel et al. 2003; Hay and Guo 2006; Ditzel et al. 2008). This, at some level, emphasizes the importance of the inhibition of initiator caspase Dronc by DIAP1 in preventing inappropriate apoptosis.

Accumulated evidence supports a working model that DIAP1 binds to Dronc, promoting Dronc ubiquitination through its RING domain. DIAP1 BIR2 domain

interacts with Dronc via a 12-residue sequence, which is structurally similar with a traditional IBM motif (Chai et al. 2003). *Dronc* mutants with disrupted IBM-like structure are unable to bind DIAP1 and show enhanced killing ability (Chai et al. 2003). DIAP1 has been shown to polyubiquitylate Dronc *in vitro* (Wilson et al. 2002; Chai et al. 2003), and the ubiquitination may lead to proteasome degradation, as elevated Dronc levels have been reported in cells harboring DIAP1 Ring domain mutants (Ryoo et al. 2004). Intriguingly, a recent study unveiled a new feedback inhibitory loop where Dronc and Dark lower their protein levels mutually, and the inter-inhibition is dependent on DIAP1 RING domain function (Shapiro et al. 2008), which indicates that DIAP1 may function at the apoptosome level to prevent uncontrolled Dronc activation.

Several interesting observations discussed below may help to supplement the current working model about Dronc regulation. One observation is that Dronc can cleave DIAP1 at Glu205 resulting one fragment containing BIR1 and the other containing BIR2 and RING domain (Yan et al. 2004; Muro et al. 2005). Mutation of Glu205 affects the ability of DIAP1 to interact with processed Dronc but not full the length version *in vitro*. However, little is known about the physiological relevance of the cleavage, and Chapter II in this dissertation may provide a clue to the issue. In addition, the IBM-like 12-residue sequence that mediates DIAP1-Dronc interaction is located upstream of the documented sites for Dronc processing, which raises the possibility that catalytically active Dronc is not inhibited through this binding, if it is regulated by DIAP1 at all (Chai et al. 2003; Hay and

Guo 2006). Moreover, the ubiquitination by DIAP1 has been reported not to result in Dronc degradation (Ditzel and Meier 2002; Vaux and Silke 2005; Lee et al. 2009). All the observations above suggest that the mechanism by which DIAP1 regulates Dronc has only been partially uncovered by far, and needs further exploration.

It seems that regulating caspases through distinct mechanisms is a common theme shared within the IAP family. For example, XIAP regulates caspase-7 (an effector caspase) by binding to the catalytic groove and functioning as a *bona fide* enzyme inhibitor, while it has been shown to poly-ubiquitinate and thereby degrade caspase-3, another effector caspase (Suzuki et al. 2001). XIAP inhibits initiator caspase 9 via binding which exerts steric hindrance to prevent apoptosome formation, maintaining caspase 9 in an inactive form. Besides, c-IAP2 is capable to mono-ubiquitylate caspase-3 and 7 *in vitro* (Huang et al. 2000), unveiling another mechanisms. Although IAPs may have different modes of action, the net outcome is the same: IAPs exert strong inhibition on caspases and apoptosis. This inhibition has to be counteracted to allow the occurrence of apoptosis, and RHG proteins are the key antagonists.

ACTIVATION OF APOPTOSIS BY RHG PROTEINS

RHG proteins are critical endogenous death activators. In *Drosophila*, the deletion of the genomic region that contains *rpr*, *hid* and *girm* (the *H99* defeciency) blocks almost all cell death during embryogenesis, providing compelling evidence that these proteins act

together to regulate developmental apoptosis (White et al. 1994). Overexpression of any one of the fly RHG proteins is sufficient to induce rapid caspase-dependent cell death in a number of fly tissues and cultured cells, as well as several heterologues system such as mammalian cells (White et al. 1994; Grether et al. 1995; Chen et al. 1996; Wing et al. 1998; Christich et al. 2002; Claveria et al. 2002; Srinivasula et al. 2002; Tenev et al. 2002; Wing et al. 2002; Olson et al. 2003; Claveria et al. 2004; Khan et al. 2008). Both loss of function and gain of function analyses strongly suggest that RHG proteins are partially redundant in the fly (White et al. 1994; Grether et al. 1995; Wing et al. 1998). Because of their critical role to elicit apoptosis, rigorous studies have been conducted to elucidate the mechanisms of action. The liberation model and degradation model for RHG action are two popular competing models.

Liberation Model—RHG proteins antagonize DIAP1 functions through their binding to BIR domains

An important mechanism that RHG proteins employ is to antagonize DIAP1 through direct binding. RHGs share very little similarity except for a short N-terminal IBM motif, which binds to the BIR domains of DIAP1. The binding competitively disrupts the BIR-IBM interaction between DIAP1and caspases, releasing the caspases to trigger apoptosis, which is commonly referred to as the liberation model.

The IBM motif is a hydrophobic short peptide, which has to be exposed at the N-terminal of the protein to mediate protein interactions (Figure 1.6). Cytoplasmic

proteins Hid, Rpr, Grim and Sickle expose their IBM domains after the removal of the initial methionine, which is thought to happen shortly after translation. Therefore, these proteins are subject to exquisite transcriptional regulation. Other RHGs are synthesized with N-terminal localization sequences, the removal of which exposes their IBM motif upon import to corresponding compartments. In response to death stimuli, they are released from the sequestering environment to antagonize IAPs. RHGs belonging to this group include Endoplasmic Reticulum (ER) localized Jafrac2 and mitochondrial localized dOmi in *Drosophila*, as well as Samc/Diablo and Omi/Htra2 in mammals.

Evidence supporting the libration model comes from a variety of assays. Structure analyses revealed that the IBM motif of RHGs occupies a conserved surface groove on BIR domains, which, remarkably, is also the binding site for caspases, and that the unlocked N-terminal Alanine (Ala) is critical to anchor IBM on the groove (Yan and Shi 2005). In addition, *in vitro* binding assays illustrated that RHG proteins disrupted the interaction between DIAP1 and caspases in a domain specific manner (Figure 1.6) (Zachariou et al. 2003). For instance, Hid binds to BIR2, not the BIR1 domain, with high affinity, thus it can only displace Dronc sequestered by DIAP1 BIR2, but not Drice bound by DIAP1 BIR1 domain (Zachariou et al. 2003). Moreover, deletions of IBM domain of RHGs have been shown to abolish the ability of Hid, Grim and Rpr to liberate caspases from DIAP1, and even the single mutation of Ala1 is found to greatly diminish the binding to DIAP1. Therefore it is not surprising that these mutations dramatically reduced RHGs'

capability to trigger cell death in fly tissues and in cultured cells. Conversely, mutations in DIAP1 BIR domains, for example, $th^{6\text{-}3s}$ with a BIR1 domain mutation (G88S) and $th^{23\text{-}4s}$ with a BIR2 mutation (G269S), exhibit reduced binding affinity with IBM, conferring cells that harbor the mutations the resistance to RHG induced cell death (Goyal et al. 2000). Consistent with the fly data, similar structural biology and biochemistry data on Smac/Diablo and Omi/HtrA2 also support the liberation model.

Degradation Model—RHG proteins regulate DIAP1 level through ubiquitination and degradation

While the liberation model offers a simple scenario for the pro-apoptotic function of RHGs, it does not address the function of the RING domain in apoptosis. The observations that auto-ubiquitination of DIAP1 can be stimulated by RHG proteins have led to the view that the primary way RHG proteins promote apoptosis is by stimulating the RING-dependent auto-ubiquitination and degradation of DIAP1, releasing caspases to initiate the apoptotic program. This mechanism of RHG proteins' action is referred to as the degradation model.

Evidence supporting the degradation model can be summarized as follows. Overexpression of Hid, Rpr and Grim in cultured cells and several fly tissues, including embryos, developing wing and eye, stimulates apoptosis which is accompanied by degradation of DIAP1 (Hays et al. 2002; Ryoo et al. 2002; Yoo et al. 2002; Yokokura et al. 2004). RHG proteins have also been shown to trigger DIAP1 auto-ubiquitination *in vitro*

(Ryoo et al. 2002; Yoo et al. 2002) and in *Xenopus* egg extracts (Holley et al. 2002). In addition, RING domain mutants in DIAP1 that disrupt the E3 ligase activity can suppress the RHG-dependent degradation of DIAP1 in many contexts (Hays et al. 2002; Holley et al. 2002; Ryoo et al. 2002; Yoo et al. 2002). One major concern of the above mentioned studies is that they relied on over-expression assays in living tissues as well as reductionist biochemical approaches, causing uncertainty whether RHG-mediated degradation of DIAP1 occurs under physiological conditions and whether it is required for apoptosis initiation.

It should be noted that there is considerable controversy concerning the degradation model. Most studies have found that overexpression of RHGs can promote DIAP1 ubiquitination and down-regulation, but the effects with Hid and Rpr are variable in different fly tissues, leading to the suggestion that not all RHGs promote DIAP1 degradation (Hays et al. 2002; Ryoo et al. 2002). In addition, the importance of the RING domain in mediating DIAP1 down-regulation has not been universally observed *in vivo* (Wilson et al. 2002; Yoo et al. 2002). In some cases, RING domain mutants enhanced rather than suppressed apoptosis induced by RHG overexpression (Wilson et al. 2002). Moreover, there is no agreement in the reported *in vitro* studies that DIAP1 auto-ubiquitination leads to its own degradation (Ryoo et al. 2002; Yoo et al. 2002). Rather, it has been suggested that auto-ubiquitination attenuates DIAP's E3-ligase activity towards caspases (Herman-Bachinsky et al. 2007). Furthermore, suppression of DIAP1

degradation in cultured cells with a proteasome inhibitor failed to inhibit apoptosis (Yokokura et al. 2004), questioning the physiological importance of DIAP1 degradation. The controversy is likely due to the complexity of DIAP1 regulation in different tissues, as well as the inherent limitations of the overexpression and biochemical approaches used thus far.

Which function of RHG is essential for apoptosis initiation, the liberation of caspases or degradation of DIAP1? One hypothesis proposes that RHG-mediated DIAP1 degradation appears to happen after RHG's binding to DIAP1, and by then caspases have already been liberated from DIAP1. Under this assumption, DIAP1 degradation might not initiate apoptosis *per se*, rather, the total removal of DIAP1 would provide a fail-safe mechanism that prevents any DIAP1-caspase re-association, allowing apoptosis to proceed efficiently. Studies in Chapter II in this dissertation will provide new insights to address this debate and other questions around degradation model.

RHG proteins activate apoptosis through other mechanisms

Multiple lines of evidence suggest that RHG proteins may have other pro-apoptotic activities in addition to the two models discussed above.

First, Grim and Rpr may promote apoptosis by inhibiting general translation. The underlying mechanism is not clear, yet Rpr is identified as the first factor that binds directly to the 40S ribosomal subunit, modulating start-codon recognition during ribosome scanning along mRNA (Colon-Ramons 2006). General inhibition of protein synthesis

presumably affects short-lived proteins more than relatively stable ones. DIAP1 is reported to have a short half-life of approximately 30min (Holley et al. 2002; Yoo et al. 2002), which is much shorter than pro-apoptotic factors, for instance Dronc which has a approximately 3hr half-life (Yoo et al. 2002). Therefore, the general inhibition by Grim and Rpr may tip the balance between anti- and pro-apoptotic factors towards the latter, which ultimately leads to cell death.

In addition, Hid and Rpr might promote apoptosis by inducing mitochondria disruption. It is reported that Hid and Rpr rapidly localize to mitochondria after synthesis, resulting in changes in mitochondria ultrastructure and permeabilization. Inhibition of the mitochondria changes or disruption of Hid and Rpr mitochondria localization suppresses apoptosis in fly cultured cells (Abdelwahid et al. 2007). This is consistent with observations that overexpression of Rpr or Grim stimulate cytochrome *c* release in mammalian cells (Claveria et al. 2002; Olson et al. 2003; Claveria et al. 2004). Besides, the mitochondria localization of RHGs is dependent on a C-terminal GH3 domain for Rpr and Grim, and on the C-termini of Hid. Deletions of GH3 domains of Rpr or Grim, or Hid C-termini have been shown to attenuate their ability to induce apoptosis; conversely, overexpression of Rpr or Grim lacking IBM is able to kill fly and mammalian cells (Claveria et al. 2002; Olson et al. 2003; Claveria et al. 2004; Abdelwahid et al. 2007; Freel et al. 2008). Furthermore, it is known that loss of Dark partially suppresses RHG-induced apoptosis in flies (Rodriguez et al. 1999). One interpretation of these results is that RHGs

act through Dark, as the mammalian counterpart of Dark, Apaf-1, is well established to associate with mitochondria disruption during apoptosis initiation. Overall, these data support the idea that RHGs might function through mitochondria.

However, several concerns about the mitochondria hypothesis are definitely worth mentioning. Most important of all, all evidence above is based on massive over-expression of Hid, Rpr or Grim, which raises a considerable concern about the physiological significance of these functions. Moreover, even if part of RHG's function is associated with mitochondria, it might not be independent of IAP antagonism for three reasons: first, it is suggested mitochondria localization is important for promoting DIAP1 degradation in cultured cells (Freel et al. 2008); meanwhile, GH3 domain of Rpr is also required for its ability to inhibit IAP (Claveria et al. 2002); and the mitochondria disruption caused by Rpr and Hid is caspase-dependent (Abdelwahid et al. 2007). Finally, despite intense scrutiny over the past 10 years, the relationship between mitochondria changes and apoptosis onset in *Drosophila* remains a puzzle (Oberst et al. 2008). Nevertheless, RHG's function through mitochondria remains an interesting hypothesis, and deserves further exploration.

COORDINATION BETWEEN CELL PROLIFERATION AND APOPTOSIS

The development and survival of multi-cellular organisms are dependent on the dynamic equilibrium between cell proliferation, differentiation and death. A growing body of evidence illuminates these processes are intimately linked via several pathways.

The Hippo pathway mediates the coordination between cell death and proliferation

The Hippo pathway has recently emerged as a signaling pathway that regulates organ size by coordinating cell proliferation and apoptosis (reviewed in Harvey and Tapon 2007). The coordination is mediated by a complex composed of two serine/threonine kinases, Hippo (Harvey et al. 2003; Pantalacci et al. 2003; Udan et al. 2003; Wu et al. 2003) and Warts (Justice et al. 1995; Xu et al. 1995), and a scaffold protein Salvador (Kango-Singh et al. 2002; Tapon et al. 2002). Once activated, Hippo directly binds to and phosphorylates Warts (Pantalacci et al. 2003; Wu et al. 2003), which in turn phosphorylates and thereby inhibits a transcriptional co-activator Yorkie (Huang et al. 2005), leading to the suppression of putative target genes, notably the anti-apoptotic factors *diap1* and microRNA *bantam*, the cell-cycle regulator *cyclin E* (Huang et al. 2005; Nolo et al. 2006; Thompson and Cohen 2006). The microRNA Bantam inhibits apoptosis by down-regulating Hid translation (Brennecke et al. 2003). Not surprisingly, loss of Hippo, Warts or Salvador leads to dramatic tissue-overgrowth resulting from excessive proliferation and suppression of apoptosis (Xu et al. 1995; Kango-Singh et al. 2002; Tapon et al. 2002; Harvey et al. 2003; Pantalacci et al. 2003; Udan et al. 2003; Wu

et al. 2003). Studied initially in *Drosophila*, the Hippo pathway has been shown to be conserved evolutionarily (Harvey and Tapon 2007).

Many questions about the Hippo pathway remain unclear. For example, what are the upstream regulators of the Hippo complex? Recent genetic analyses revealed that Merlin, Expanded and the atypical cadherin Fat may be involved in the process, yet the link between these upstream factors and the Hippo complex is still missing (Bennett and Harvey 2006; Cho et al. 2006; Hamaratoglu et al. 2006; Silva et al. 2006; Willecke et al. 2006). Genetic studies also suggested that the fly casein kinase Iε, known as Discs overgrown (Dco), might function in the Hippo pathway (Cho et al. 2006; Mao et al. 2006). This is interesting as research in our lab (as in Chapter III) also reported the role of Dco in coordinating proliferation and apoptosis via activation of DIAP1, yet the DIAP1 regulation by Dco is post-transcriptional (Guan et al. 2007). Perhaps, as a pleiotropic protein, Dco may help to achieve the equilibrium of proliferation and cell death via different pathways.

Apoptosis induces compensatory proliferation

In addition to the Hippo pathway, it seems the coordination can be achieved through active communication between apoptotic cells and their surviving neighbors. Cell loss owing to stress or injury can induce proliferation of the surrounding cells, a process known as compensatory proliferation (Huh et al. 2004; Ryoo et al. 2004). Remarkably, it allows the organism to compensate a big loss of cells (up to 60% of cell

loss has been reported) and develop into tissues with normal pattern and size (reviewed in Domingos and Steller 2007; Fan and Bergmann 2008).

What is the underlying mechanism of compensatory proliferation? Recent studies revealed that caspases in apoptotic cells activate morphogenetic factors which stimulate the cell division of surrounding cells. For example, it has been reported that in the developing wing and eye discs, the initiator caspase Dronc activates the expression of Wingless (Wg), a *Drosophila* member of the Wnt family of secreted molecules, and decapentaplegic (Dpp), a *Drosophila* member of the TGFβ family, through mechanisms that are poorly understood (Huh et al. 2004; Ryoo et al. 2004; Kondo et al. 2006; Wells et al. 2006). Wg and Dpp move across the cell boarders to stimulate the proliferation of neighbor cells. Given that the function of Dronc in compensatory proliferation has been shown to be independent of effector caspases, this activity of Dronc is believed to be distinct from its apoptotic function (Ryoo et al. 2004; Kondo et al. 2006).

Very recently, the effector caspases Drice and Dcp-1 have been suggested to promote compensatory proliferation via activation of the Hedgehog (Hh) signaling pathway (Fan and Bergmann 2008). One intriguing explanation proposed to address the differences between the Dronc- and effector caspases-mediated compensatory proliferation is centered on the developmental potential of tissues studied, i.e., the first type seems engaged in proliferating tissues, whereas the second type in differentiating tissues (Fan and Bergmann 2008).

Although the precise mechanisms remain elusive, the studies of compensatory proliferation shed new light on how multi-cellular organisms orchestra cell death and proliferation to achieve normal development and homeostasis.

Caspases have other non-apoptotic functions

It has emerged in the last several years that caspases and apoptotic factors play essential roles in non-apoptotic contexts. In addition to mediating compensatory proliferation, caspases and their regulators have been implicated in the regulation of cell differentiation and cell shape.

Caspases can modify the shape of cells by destroying only part of the cells without resulting in apoptosis. This function of caspases is employed during dendrite pruning, a process needed to remodel the fly larval neuronal system to its adult form (Kuo et al. 2006; Williams et al. 2006). During dendrite pruning, caspases promote the local degeneration of dendrites, while the neural cell body is unaffected. The activation of Dronc and degradation of DIAP1 mediated by UbcD1, an E2 ubiquitn-conjugaing emzyme, are suggested to be required for dendrite pruning (Kuo et al. 2006; Williams et al. 2006). Another example is the local activation of caspases which is required to remove cytoplasm from developing spermatids during spermatid individualization (Arama et al. 2003). Multiple classical apoptotic factors Dronc, Drice, Dark, Dredd, dFadd, Dcp-1 and Hid have been suggested indispensible in this case (Arama et al. 2003; Huh et al. 2004; Arama et al. 2006; Muro et al. 2006; Arama et al. 2007).

Caspases also regulate cell differentiation, specifically, the specification of neural precursor cells in *Drosophila*. The macrochaete in the scutellum of the adult notum is derived from a single sensory organ precursor (SOP) cell (Kanuka et al. 2005). Inhibition of Dark and Dronc, and, conversely, activation of DIAP1 have been illustrated to convert extra cells into the SOP fate, leading to more macrochaetes (Kanuka et al. 2005; Mendes et al. 2006).

In addition, Dronc, Dark and DIAP1 are also reported to regulate the migration of border cells during fly oogenesis, as well as mediate the innate immunity in response to the infection of Gram-negative bacteria (Hultmark 2003; Tanji and Ip 2005; Domingos and Steller 2007).

Notably, each cell type discussed above can be induced to undergo caspase-dependent apoptosis; however, caspase activities do not trigger cell death during these processes, which strongly indicates the caspase activity is tightly restricted in non-apoptotic contexts. How is the restriction of caspase activity achieved? This outstanding question remains poorly understood at this point, while some studies may provide clues to it. During the SOP specification, the *Drosophila* IKK-related kinase (dmIKKε) has been shown to directly bind and phosphorylate DIAP1, promoting DIAP1 degradation. Loss of dmIKKε function results in up-regulation of DIAP1 and extra macrochaetes, and by contrast, no effects on cell death (Kuranaga et al. 2006). The finding of dmIKKε leads to a hypothesis that dmIKKε, and potentially other proteins,

may fine tune the levels of DIAP1, setting the threshold required for the caspase non-apoptotic functions. In addition, the dendrite-specific trafficking has been speculated to play a role in restricting the caspase activities locally during dendrite pruning (Geisbrecht and Montell 2004), indicating the sub-cellular localization of caspases may contribute to the regulation of their non-apoptotic functions.

It is interesting to speculate that the apoptotic and non-apoptotic functions of caspases can be beautifully reconciled at the level of cell fate coordination. Hypothetically, lower levels of caspases are employed to facilitate cell remodeling, proliferation and differentiation, while high levels lead to irreversible cell death. It emphasizes the importance of caspase regulation not only in eliminating unwanted cells, but in coordinating death and cell proliferation.

RATIONALE AND SPECIFIC AIMS

Given the importance of caspase regulation by IAP proteins for development and homeostasis, the goal of my dissertation is to advance the understanding of the regulation of DIAP1 in both apoptotic and surviving cells in *Drosophila*.

Specifically, the two major foci are:

1. Systematic analysis of DIAP1 down-regulation in developmental apoptosis in the pupal eye of *Drosophila* (Chapter II).

2. Characterization of novel factors that promote cell survival via activation of DIAP1 (Chapter III & IV).

In Chapter II, I investigated the down-regulation of DIAP1 during developmental apoptosis in perimeter ommatidia of the fly pupal eye. Using loss of function analyses, I provided evidence to support the physiological importance of the liberation and degradation models that have been proposed to explain how RHG proteins antagonize DIAP1. Strikingly, I found that the E3 ligase activity of DIAP1 RING domain may be dispensable for RHG-mediated degradation. In contrast, Dark and the initiator caspase Dronc are both required for DIAP1 down-regulation, suggesting that RHG proteins may act through Dark and Dronc.

In Chapter III, I directed my focus to DIAP1 regulation in living cells. The fly casein kinase Iε/δ, known as Discs Overgrown (Dco) was identified as an anti-apoptotic factor in a genetic screen. Further characterization illustrated that Dco is required for maintaining low levels of apoptosis via activation of DIAP1 post-transcriptionally. Dco also promotes cell division/growth. Therefore, we propose that Dco controls tissue size by stimulating proliferation and blocking apoptosis.

In Chapter IV, I carried out investigations in order to elucidate the molecular mechanism underlying the role of Dco. While doing this, I revealed a novel function of Gilgamesh (Gish), the fly CKIγ homologue, in DIAP1 regulation. Our results suggest Gish and Dco might be functionally redundant. At the end of the chapter, I initiated a chemical genetic screen to identify novel substrates of Dco.

FIGURES AND TABLES

Figure 1.1

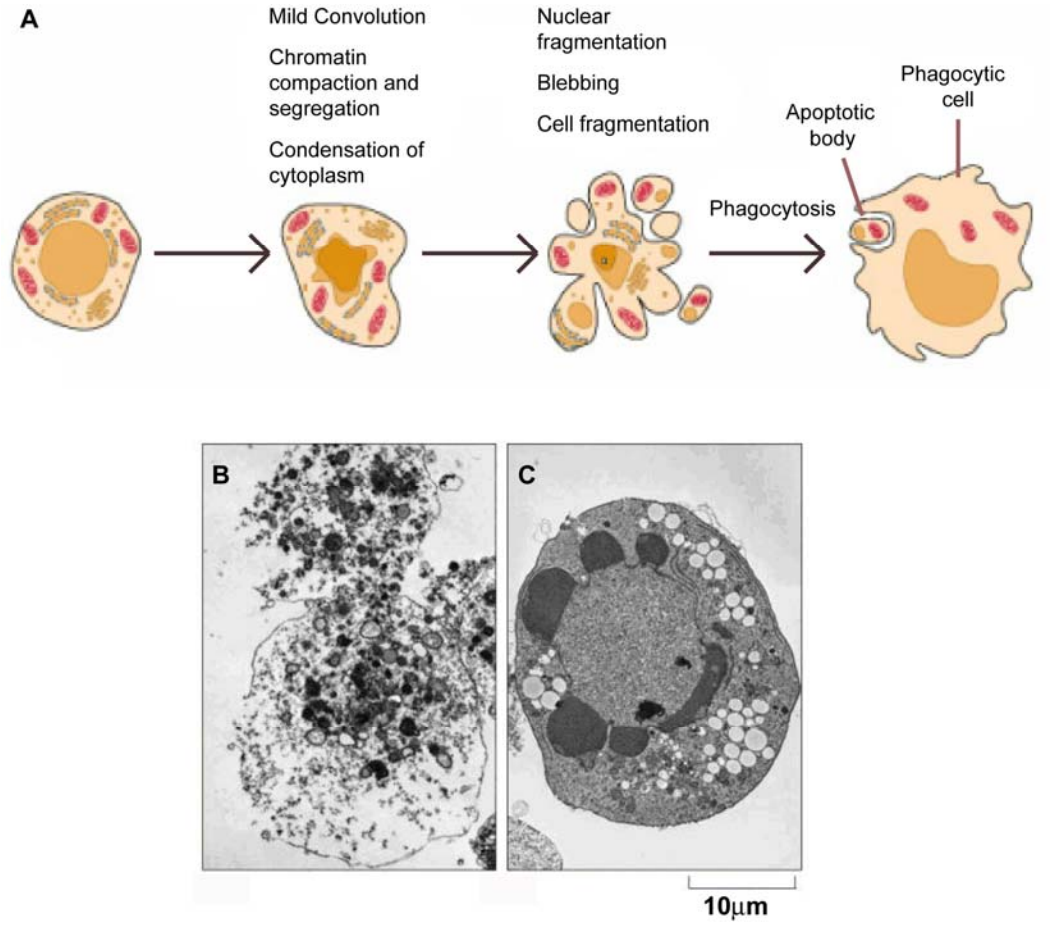


Figure 1.1 Ultrastructural features of cell death by apoptosis. (A) Schematic drawings illustrating the progression of morphologic changes observed in apoptotic cells. (B-C) Photomicrographs comparing a normal cell (B) and apoptotic cell (C). Clearly visible in the latter are dense spheres of compacted chromatin as the nucleus begins to fragment. (Adapted from Molecular Cell Biology, Lodish et al. 2007)

Figure 1.2

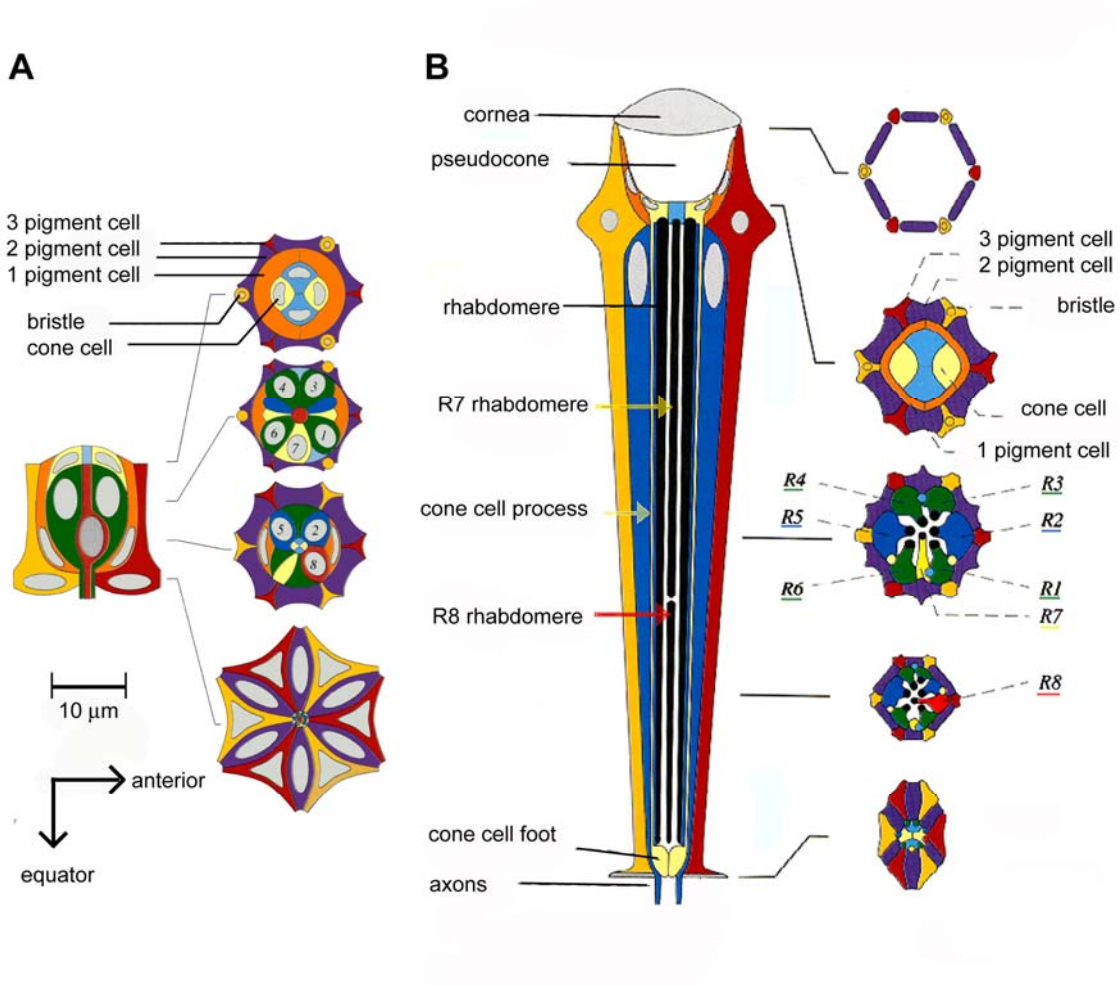


Figure 1.2 A schematic *Drosophila* Ommatidium. (A) Ommatidium at 33% (65hr at 20 ºC) of pupal life. Nuclear positions are stereotyped and distributed roughly into four planes as shown in the cross sections. The basal processes of the cone cells and their meeting at the floor are not shown in the side view. (B) Ommatidium in adult fly, drawn from a dorsal, right eye. (Adapted from Wolff and Ready 1993)

Figure 1.3

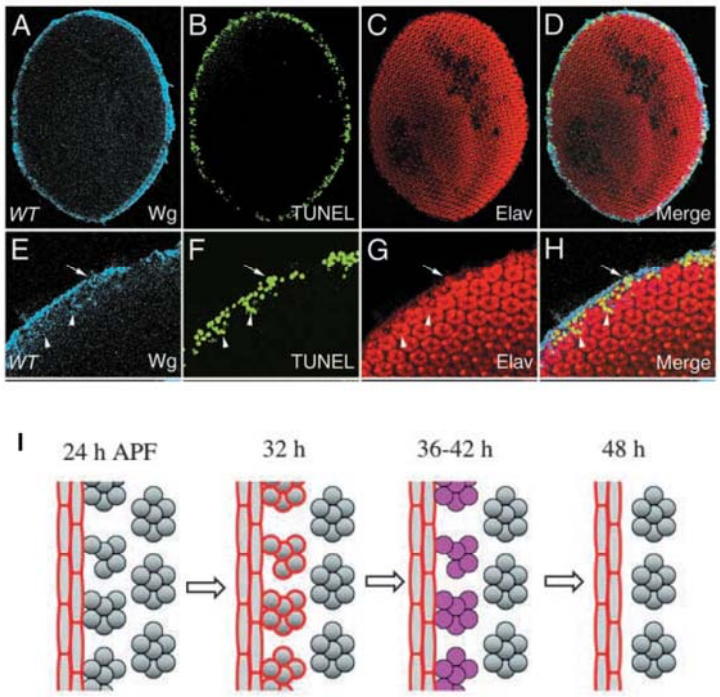


Figure 1.3 Periphery ommatidia apoptosis triggered by Wg. (A-D) Wild-type eye stained for Wg protein (blue), TUNEL (green) and Elav (red). Wg staining is coincident with the apoptotic cells at the edge of the eye. (E-H) close-up of the eye perimeter in (A-D). Most of the Wg protein is adjacent to the apoptotic cells, with lower levels in the dying cells, some of which are still Elav-positive (arrows). The edge of this eye is slightly curved, making some of the TUNEL-positive muclei appear to be present in interior Elav-positive photoreceptors in the optical stack (arrowheads). (I) model of periphery ommatidia apoptosis. Non-ommatidial cells at the edge of the eye are depicted as oblongs and ommatidial cells as circles. A red outline indicates Wg expression and purple represents *hid*, *grim* and *rpr* expression. At 24hr APF, Wg is found only in the edge cells. Between 28-32 hr APF, Wg expression is induced in the perimeter ommatidia, which are often lacking the full complement of cells. Wg signaling is necessary but not sufficient for this induction of ommatidial Wg expression. By 36hr APF, Wg expression in the ommatidia is fading and the expression of the pro-apoptotic genes *hid*, *grim* and *rpr* are triggering apoptosis, causing the elimination of these ommatidia. (Original data from Lin. et al 2004)

Figure 1.4

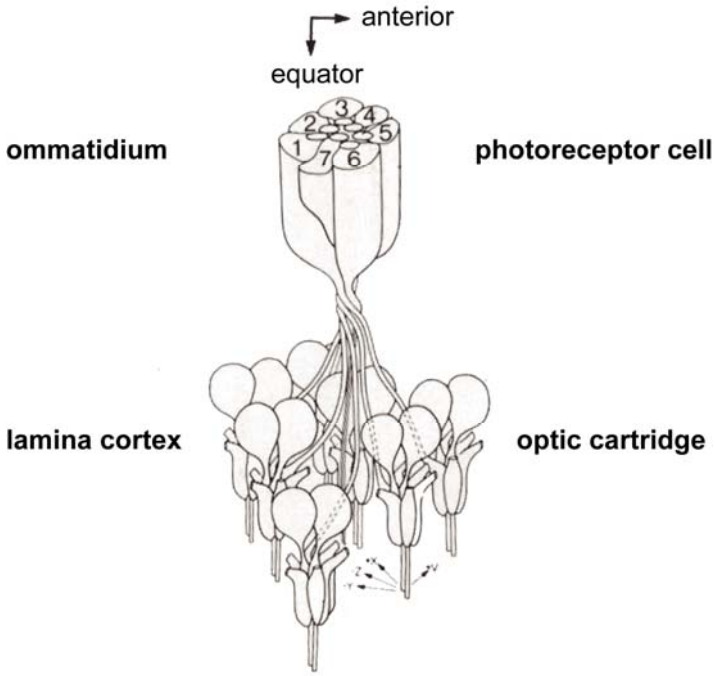


Figure 1.4 The projection of receptor axons from a single ommatidium. The axons of R1-R6 (labeled as 1-6) of a single ommatidium twist 180 degree as they descend to the lamina before diverging to their respective cartridges. R1-R6 in all the adjacent ommatidia also contribute axons to these cartridges through identical projections repeated in an overlapping mosaic, so that each cartridge receives not one but six receptor terminals. Therefore, an interlocking network of interwoven receptor connections is created across the entire lamina. The central cells R7 and R8 (not visible) share a single rhabdome, and their long visual fibers penetrate the lamina without terminating. (Adapted from Meinertzhagen and Hanson 1993)

Figure 1.5

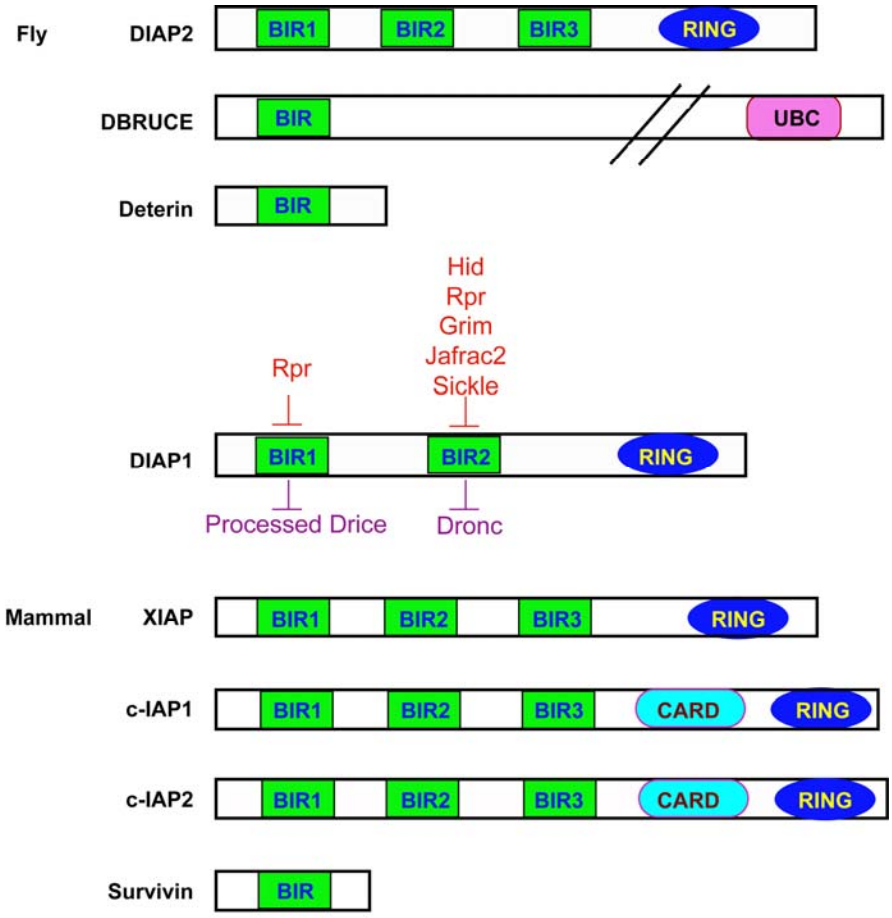


Figure 1.5 Schematic representation of IAP family members in mammal and fly. The functional motifs are shown in pictures. Abbreviations: BIR, bacularvirus IAP repeats; RING, really interesting new gene, CARD, caspase-associated recruitment domain; UBC, ubiquitin-conjugation; NOD, nucleotide-binding oligomerization domain; and LRR, leucine-rich repeats.

Figure 1.6

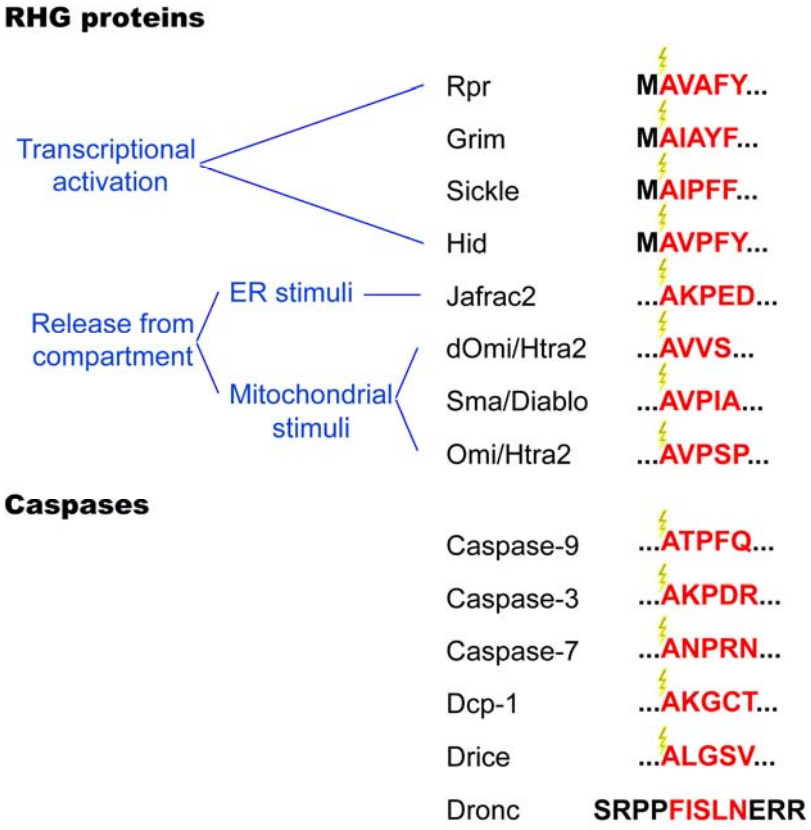


Figure 1.6 Fly and mammalian RHG proteins. RHG proteins are subjected to transcriptional and post-transcriptional regulation (left). RHG proteins and caspases contain IBM and IBM-like (in Dronc) motif, which provide molecular basis for the competitive binding to IAPs (right). (Adapted from Hay and Guo 2006)

Figure 1.7

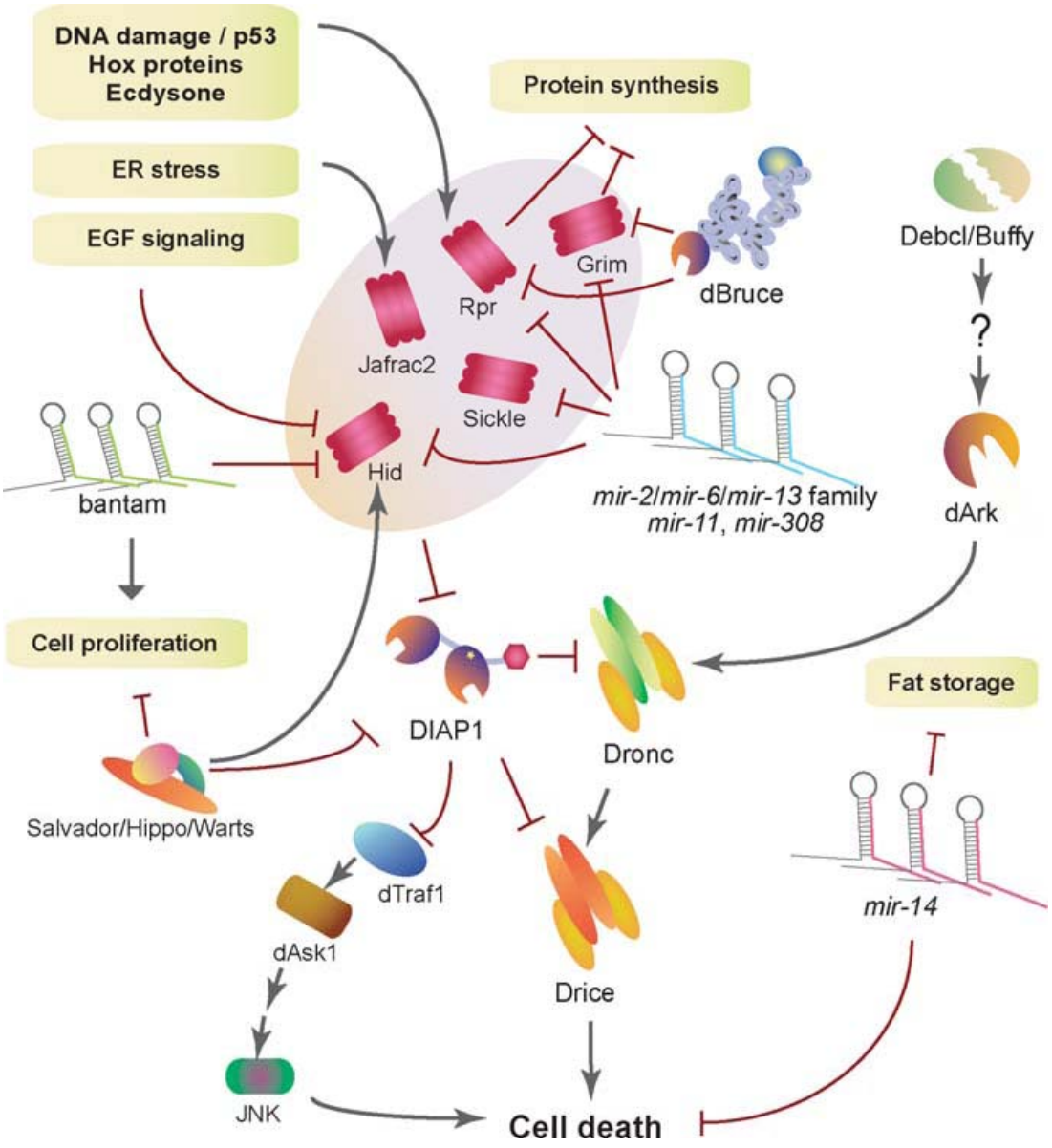
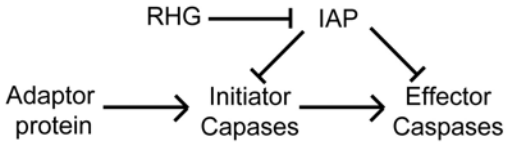


Figure 1.7 Regulators of caspase-dependent cell death in *Drosophila*. (Adapted from Hay and Guo 2006)

Table 1.1



	worm	fly	mammal
Caspases	CED-3 CSP-1 CSP-2	Dronc, Dredd, Strica*	caspase 2,8,9,10 caspase 1,4,5
		Dcp-1, Drice, Decay, Damm	caspase 3,6,7,14
Adaptor	CED-4	Dark	Apaf-1
IAP	Bir-1 Bir-2	DIAP1, DIAP2, dBruce, Deterin	XAIP, cIAP-1, cIAP2, ILP2, ML-IAP, NAIP, Survivin, Bruce
RHG		Hid, Grim, Reaper, Sickle, dOmi, Stritca	Smac/DIABLO Omi/HtrA2

Table 1.1 Core apoptosis machinery is concerved from the worm, fly to mammal. The conserved apoptosis regulation network is summarized on top of the table. The key factors from the worm, fly and mammal are listed in the table. (* Fly caspases Strica is listed as initiator caspase based on its long pro-domain. However, phylogenetic analysis based on the catalytic domain shows clustering with the effector caspases (Salvesen and Abrams 2004)).

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CHAPTER II

RHG PROTEIN MEDIATED APOPTOSIS IN THE PUPAL EYE OF DROSOPHILA: DOWN-REGULATION OF DIAP1

ABSTRACT

Drosophila Inhibitor of apoptosis protein 1 (DIAP1) is an essential negative regulator of apoptosis in fly cells. DIAP1 binds to caspases and inhibits their activity. RHG proteins, e.g., Head Involution defective (Hid), Grim and Reaper (Rpr), can induce apoptosis by binding to DIAP1 and liberating the bound caspases. In addition, some RHG proteins can induce DIAP1 degradation. These studies are based on *in vivo* overexpression or *in vitro* biochemical assays, raising questions about which mechanism (liberation or degradation) is more physiologically relevant. In this report, we demonstrate significant down-regulation of DIAP1 protein in perimeter ommatidia undergoing apoptosis in the pupal eye. This regulation was dependent on Hid, Grim and Rpr. Interestingly, a partial reduction in RHG proteins suppressed DIAP1 degradation to undetectable levels. Under these conditions, apoptosis was initiated at the normal time, but was markedly reduced thereafter. These results argue that both liberation of caspases

and degradation of DIAP1 are required for timely initiation and progression of apoptosis in this tissue. In addition, we provide evidence that the E3 ligase activity of DIAP1's RING domain is dispensable for RHG-mediated degradation. In contrast, the *Drosophila* Apaf-1 related killer (Dark) and the initiator caspase Dronc are both required for DIAP1 down-regulation, suggesting that RHG proteins may act through Dark and Dronc.

INTRODUCTION

Apoptosis is an evolutionarily conserved cell death process that is essential for normal development and adult homeostasis. The key executioners of apoptosis are a family of cysteine proteases called caspases. Once activated, initiator caspases cleave and activate downstream effector caspases, which in turn proteolyze hundreds of cellular proteins, leading to cell death (Hengartner 2000; Hay and Guo 2006). In the fruit fly *Drosophila melanogaster*, the initiator caspase *Drosophila* interleukin-IB-converting enzyme (Dronc) and effector caspases *Drosophila* Nedd-2-like caspase (Drice) and death caspase-1 (Dcp-1) are core components of the apoptosis machinery(Hengartner 2000; Hay and Guo 2006).

Cells tightly regulate caspase activation and consequent apoptosis through several mechanisms. In vertebrates, the so-called "intrinsic pathway" initiates cell death through the release of cytochrome *c* from the intra-mitochondrial compartment. The released cytochrome *c* binds to a protein called Apaf-1, and this complex promotes activation of

caspase 9, an initiator caspase(Hengartner 2000; Salvesen and Abrams 2004). While the role of cytochrome *c* in fly apoptosis is still controversial(Mendes et al. 2006; Oberst et al. 2008), the Apaf-1 homolog Dark is required for activation of the initiator caspase Dronc(Hay and Guo 2006).

Cells also employ factors called Inhibitor of Apoptosis proteins (IAPs) to bind and inhibit caspases, thereby preventing the inappropriate onset of the apoptotic program(Salvesen and Abrams 2004; Hay and Guo 2006; Srinivasula and Ashwell 2008). IAPs are characterized by the presence of one or more Bacularvirus IAP Repeat (BIR) domains which mediate the interaction with caspases and other pro-apoptotic factors(Srinivasula and Ashwell 2008). The importance of IAPs in preventing inappropriate apoptosis is highlighted by the phenotype of *Drosophila* IAP1 (DIAP1). Loss of DIAP1 leads to global caspase-dependent apoptosis in multiple fly tissues and cultured cells, demonstrating that DIAP1 is an essential inhibitor of cell death in the fly (Hay and Guo 2006; Steller 2008).

The anti-apoptotic function of IAPs is antagonized by a group of pro-apoptotic proteins including SMAC/Diablo and Omi/HtrA2 in mammals and Reaper (Rpr), Head involution defective (Hid), Grim, Sickle and Jafrac-2 in flies, collectively referred to as RHG proteins(Salvesen and Abrams 2004; Hay and Guo 2006; Srinivasula and Ashwell 2008). In *Drosophila*, the deletion of the genomic region containing *hid*, *grim* and *rpr* (the *Df(3L)H99* deletion) prevents almost all developmental apoptosis in embryos(White

et al. 1994). Consistent with a positive role in promoting cell death, over-expression of RHG proteins induces massive apoptosis in many developmental contexts in flies(Hay and Guo 2006).

A major mechanism that RHG proteins employ to antagonize IAPs is through direct binding. RHGs share very little similarity except for a short N-terminal motif, called the IAP Binding Motif (IBM), which binds to the BIR domains of IAPs. RHG protein binding to IAPs competitively disrupts the IAP-caspase interaction, releasing caspases to trigger apoptosis. Evidence for displacement of IAPs from caspases by RHGs comes from studies of mammalian SMAC/DIABLO and Omi/HtrA2 as well as Hid and Rpr in flies (Du et al. 2000; Verhagen et al. 2000; Suzuki et al. 2001; Chai et al. 2003; Zachariou et al. 2003; Hay and Guo 2006). This mechanism to explain RHG protein action is commonly referred to as the liberation model.

While the liberation model offers a simple explanation of the pro-apoptotic function of RHGs, it does not take into account the fact that many IAPs contain a C-terminal RING domain that can function as an E3-ubiquitin ligase(Yang et al. 2000; Suzuki et al. 2001; Wilson et al. 2002; Yoo et al. 2002). The RING domain function of DIAP1 has been implicated both in ubiquitination and proteasomal degradation of the initiator caspase Dronc and in auto-ubiquitination of DIAP1(Hays et al. 2002; Holley et al. 2002; Ryoo et al. 2002; Wilson et al. 2002; Yoo et al. 2002). The auto-ubiquitination of DIAP1 can be stimulated by Rpr, Hid and Grim in a cell-context-dependent manner(Hays et al. 2002;

Holley et al. 2002; Ryoo et al. 2002; Wilson et al. 2002; Yoo et al. 2002). These data have led to the view that the primary way that RHG proteins promote apoptosis is by stimulating the RING-dependent auto-ubiquitination and degradation of DIAP1, releasing caspases to initiate the apoptotic program. This mechanism of RHG protein action is referred to as the degradation model.

Evidence supporting the degradation model of RHG antagonism of DIAP1 can be summarized as follows. Overexpression of RHG proteins in several fly tissues stimulates apoptosis and degradation of DIAP1(Hays et al. 2002; Ryoo et al. 2002; Yoo et al. 2002). *In vitro*, RHG proteins can trigger DIAP1 auto-ubiquitination(Ryoo et al. 2002; Yoo et al. 2002). In addition, RING domain mutants in DIAP1 that disrupt E3 ligase activity can suppress the RHG-dependent degradation of DIAP1 in some contexts(Hays et al. 2002; Holley et al. 2002; Ryoo et al. 2002; Yoo et al. 2002). One major drawback of the above mentioned studies is that they relied on over-expression assays in living tissues as well as reductionist biochemical approaches. While these are powerful for exploring molecular mechanism, they do not address whether RHG-mediated degradation of DIAP1 occurs under physiological conditions and whether this regulation contributes to the initiation of apoptosis.

There is considerable controversy concerning the degradation model. Most studies have found that overexpression of RHGs can promote DIAP1 ubiquitination and down-regulation, but the effects with Hid and Rpr are variable in different fly tissues,

leading to the suggestion that not all RHGs promote DIAP1 degradation(Hays et al. 2002; Ryoo et al. 2002). In addition, the importance of the RING domain in mediating DIAP1 downregulation has not been universally observed *in vivo*(Wilson et al. 2002; Yoo et al. 2002). In some cases, RING domain mutants enhanced rather than suppressed apoptosis induced by RHG overexpression(Wilson et al. 2002). Moreover, there is no agreement in the reported *in vitro* studies that DIAP1 auto-ubiquitination leads to its own degradation(Ryoo et al. 2002; Yoo et al. 2002). Rather, it has been suggested that auto-ubiquitination serves to attenuate DIAP's E3-ligase activity towards caspases(Herman-Bachinsky et al. 2007). Furthermore, suppression of DIAP1 degradation in cultured cells with a proteasome inhibitor failed to inhibit apoptosis (Yokokura et al. 2004). The controversy is likely due to the complexity of DIAP1 regulation in different tissues, as well as the inherent limitations of overexpression and biochemical approaches.

In this report, we address the physiological relevance of the DIAP1 degradation model by examining naturally occurring apoptosis of ommatidia at the perimeter of the pupal fly eye(Lin et al. 2004). This apoptotic event is triggered by the expression of RHG proteins and is dependent on Dronc and effector caspases (Lin et al. 2004; Mendes et al. 2006). We provide evidence, for the first time, that DIAP1 degradation occurs during developmental apoptosis. Hid, Grim and Rpr all contribute to this regulation. Interestingly, a partial reduction in Hid, Grim and Rpr activity reduced DIAP1 degradation

to undetectable levels, but apoptosis was still initiated at the normal time, though it then proceeded with much slower than normal kinetics. These results strongly suggest that the liberation model for RHG function contributes significantly in perimeter ommatidial apoptosis. We favor a model where the combination of degradation of DIAP1 and disruption of DIAP1-caspase complexes allows apoptosis to proceed in a timely fashion at the periphery of the pupal fly eye.

Our results also demonstrate that the E3 ligase activity of RING domain of DIAP1 is dispensable for RHG-mediated degradation. Instead we find that the Apaf-1 homolog Dark and the initiator caspase Dronc are required to achieve the full extent of DIAP1 degradation. These results suggest that RHG proteins may act through Dark and Dronc to promote DIAP1 down-regulation.

MATERIALS AND METHODS

Drosophila Genetics

Fly stocks were maintained on standard medium at 25°C. The following fly strains were used. Oregon R was our wild type stock. UAS-*p35* was from the Bloomington Stock Center (Indiana University). The stocks hid^{X14}, *Df(3f)H99*, *Df(3f)X25*, and *Df(3f)XR38* were from Kristin White (Massachusetts General Hospital). $dronc^{I29}$ P[*FRT, w*+]^{2A}, P[*FRT,hs-neo*]^{42D} $dark^{G8}$ and P[*GMR-Gal4*] on the third chromosome were from Andreas Bergmann (M. D. Anderson Cancer Center). *Eye-Flp*^{T11} and *Eye-Flp*^{T12} were from Barry

Dickson (I.M.P., Vienna). UAS-*GPI-fz2* was previously described(Cadigan et al. 1998; Lin et al. 2004). The DIAP (*th*) alleles th^{j5c8}, th^{6B}, $th^{33\text{-}1s}$, $th^{6\text{-}3s}$, $th^{23\text{-}4s}$ were from Kristin White and John Abrams (UT, Southwestern). Pupal developmental ages are expressed as hours after puparium formation (APF) at 25°C with white pre-pupae defined as 0 hour APF. Clonal analyses in the eye were performed as previously described(Lin et al. 2004) using $Eye\text{-}Flp^{T11}$ for $dark^{G8}$ and $Eye\text{-}Flp^{T12}$ for *th* alleles, $dronc^{I24}$ and *Df(3f) H99*.

Immunostainings and TUNEL

Immunostainings were performed as previously described(Lin et al. 2004). DIAP1 antibodies were mouse anti-DIAP1(1:200) (from B. Hay) and rabbit anti-DIAP1 (1:200) (from K. White), with the mouse antibody being used throughout this report. Other primary antibodies were rat anti-ELAV (1:100) (Developmental Studies Hybridoma Bank) and rabbit anti-*β*-galactosidase (1:500) (Cappel, ICN, Costa Mesa, CA). Cy3- and Cy5-conjugated (Jackson Immunochemicals) and Alexa Fluor 488-conjugated (Molecular Probes) secondary antibodies were applied. To perform co-staining of mouse anti-DIAP1 and mouse anti-Wg (1:100) (Developmental Studies Hybridoma Bank), samples were first incubated sequentially with anti-DIAP1 and anti-mouse secondary antibody. After washing off unbound secondary antibody, the samples were incubated with normal mouse serum and unconjugated anti-mouse Fab (1:200) (Jackson Immunochemicals) to ensure that all the mouse anti-DIAP1 antibody was blocked. The samples were then incubated with anti-Wg and Cy5-conjugated anti-mouse IgG_1(Jackson Immunochemicals) and

processed as normal. The TUNEL assay was performed using the Cell Death Detection kit (Fluorescein; Roche Diagnostics, Indianapolis, IN) as previously described(Lin et al. 2004).

Microscopy and image analysis

All fluorescent pictures were obtained with Leica confocal microscope DM6000 B (Leica) and processed in Adobe Photoshop CS. For DIAP1 fluorescence pixel intensity (PI) analysis, each ommatidium was traced manually around one ELAV positive cell cluster. Perimeter ommatidia were identified via Wg immunostaining, and the adjacent internal ommatidia lacking Wg signal were selected for comparison. Raw PI values were calculated by the Mean Intensity function of Analyze Particle tool in Scion Image (Scion Corporation, MD). All Raw PI values were normalized with the average PI values of internal ommatidia. Normalized PI values of all ommatidia from multiples discs were subjected to calculation of average, standard error of mean (S.E.M.) and a two-tailed Student's t-test. More than 20 perimeter/internal ommatidia pairs from at least 4 eyes were quantified for each experimental condition. Normalized PI values were shown with plus/minus S.E.M.. Differences were considered significant at $P < 0.05$.

RESULTS

DIAP1 is down-regulated post-transcriptionally in peripheral ommatidia that undergo apoptosis.

The fly compound eye is composed of approximately 800 repeated optical units called ommatidia(Wolff and Ready 1993). During mid-pupation, the ommatidia at the perimeter of the developing eye undergo apoptosis, starting around 36 hr after puparium formation (APF). This process is complete by 48 hr APF (Lin et al. 2004). This programmed cell death is controlled by Wingless (Wg) (Lin et al. 2004), a member of the Wnt family of signaling molecules(Cadigan 2008). Wg is expressed in the outer rim of the eye throughout pupal stages, and it is also activated in the cone cells of the peripheral ommatidia that are destined to die starting around 28 hr APF (Lin et al. 2004; Lim and Tomlinson 2006). Therefore, Wg immunostaining is used in this report to mark the perimeter ommatidia that are beginning to undergo apoptosis (e.g., Figure 2.1a).

We found that DIAP1 levels in the perimeter ommatidia are down-regulated preceding the onset of apoptosis. At 24 hr APF, all ommatidia throughout the pupal eye had similar levels of DIAP1 (data not shown). By 33 hr APF, there are lower DIAP1 levels in many peripheral ommatidia (Figure 2.2a-d). At 36hr APF, all Wg-positive ommatidia had less DIAP1 than ommatidia in the interior (Figure 2.1c). This down-regulation is also observed at later stages as apoptosis progresses (Figure 2.2e-g). The same results were also obtained by using an independent polyclonal antibody (Figure 2.2i-l), confirming that DIAP1 is down-regulated in developmental apoptosis.

To quantify the difference in DIAP1 levels between ommatidia that are undergoing apoptosis and interior ommatidia, the Mean Pixel Intensity (PI) values for the DIAP1 immunostaining in ommatidia were determined. The area of an ommatidia was defined by co-staining with ELAV, a marker for photoreceptors(Oneill et al. 1994). The PI of the Wg-positive perimeter ommatidia are expressed as a percentage of the PI of the adjacent Wg-negative ommatidia (the Normalized Mean Pixel Intensity (NPI) in Table 2.1). This analysis revealed that the level of DIAP1 signal in the ommatidia undergoing apoptosis at 36 hr APF was 47.6% of that found in interior ommatidia (Table 2.1).

The possibility that the observed down-regulation of DIAP1 protein levels was due to decreased *DIAP1* transcription was examined in flies carrying the DIAP1-lacZ allele th^{j5c8}. These flies contain an enhancer trap insert in the DIAP1 locus, placing the LacZ reporter under the control of the DIAP1 regulatory region (Hay et al. 1995; Ryoo et al. 2002). DIAP1-lacZ eyes displayed no difference in LacZ levels between the peripheral ommatidia and interior ones (Figure 2.1e). These results argue that the down-regulation of DIAP1 expression in perimeter ommatidia occurs at the post-transcriptional level.

DIAP1 down-regulation is independent of effector caspase activity but requires Wg signaling as well as Hid, Grim and Rpr.

The initiation of programmed cell death occurs through the activation of effector caspases (Hengartner 2000). These proteases cleave a wide variety of cellular substrates to promote the apoptotic cell program(Hengartner 2000). In addition, it has been

suggested that the cleavage of DIAP1 by effector caspases exposes a motif to recruit the N-end-rule-associated ubiquitination machinery and accelerate the degradation of DIAP1 (Ditzel et al. 2003). This raises the possibility that the down-regulation of DIAP1 observed in the pupal eye is effector caspase-dependent. This question was addressed by examining the expression of DIAP1 in eyes where effector caspase activity is inhibited by the baculavirus protein p35. Flies containing a GMR-Gal4 (expressing Gal4 in all eye cell types) and UAS-*p35* transgene did not show any perimeter ommatidia apoptosis (Lin et al. 2004) (data not shown). Despite the block in apoptosis, the down-regulation of DIAP1 still occurred at a similar level as in wild type (Figure 2.1g,h), with a NPI of 44.4% (Table 2.1). These results indicate that the lower DIAP1 levels in perimeter ommatidia occurred independently of effector caspase activity and is not an indirect result of the apoptotic cell program.

The expression of Wg in peripheral ommatidia triggers apoptosis by activating the expression of the RHG genes *hid*, *grim* and *rpr*(Lin et al. 2004). Expression of a *GPI-fz2* transgene, known to inhibit Wg signaling(Cadigan et al. 1998; Lin et al. 2004) , resulted in a complete block in DIAP1 down-regulation (Figure 2.3). To test if Hid, Grim and Rpr are also required for DIAP1 down-regulation, we examined eyes from flies that were heterozygous for *hid* (Figure 2.4a-d), *rpr* (Figure 2.4e-h), *hid* and *grim* (Figure 2.4i-l), and *hid* and *rpr* (Figure 2.4m-o). DIAP1 down-regulation was partially suppressed in all four backgrounds, and the NPI values obtained for the perimeter ommatidia in each background

were significantly higher than that of wild-type (Figure 2.4p and Table 2.1). Flies heterozygous for the *H99* deficiency (which removes *hid*, *grim* and *rpr*) blocked DIAP1 down-regulation (Figure 2.5a-d). The NPI in *H99*/+ perimeter ommatidia was not significantly different from internal ommatidia (97.3%, P>0.5). Taken together, these results indicate that Hid, Grim and Rpr are major factors contributing to DIAP1 down-regulation. The fact that removal of even one copy of *hid* or *rpr* caused a significant reduction in DIAP1 down-regulation indicates that the system is very sensitive to the amount of RHG protein present.

Apoptosis can occur in the absence of DIAP1 down-regulation in peripheral ommatidia.

RHG proteins are required for apoptosis (Lin et al. 2004) and down-regulation of DIAP1 in perimeter ommatidia (Figure 2.4; Figure 2.5a-f; Table 2.1). Loss of DIAP1 results in a dramatic increase in apoptosis in several fly tissues (Hay et al. 1995; Wang et al. 1999; Goyal et al. 2000; Lisi et al. 2000; Yokokura et al. 2004; Hay and Guo 2006). This suggests a model where RHG-dependent down-regulation of DIAP1 is the major cause of apoptosis in the peripheral ommatidia. However, these correlations do not rule out the competing model of RHG action, where RHGs liberate caspases from DIAP1 independently of DIAP1 down-regulation.

Reducing *hid*, *grim* and *rpr* activity by 50% (*H99*/+ flies) results in no detectable down-regulation of DIAP1 (Figure 2.5a-d; Table 2.1). Despite the absence of DIAP1

down-regulation, perimeter apoptosis was initiated at similar level as in wild type eyes, as judged by TUNEL staining at 36 hr APF (Figure 2.5g,h). Therefore, apoptosis in the peripheral ommatidia can still occur in the absence of DIAP1 down-regulation, perhaps by RHG proteins displacing DIAP1 bound to caspases, liberating them for apoptosis initiation.

Although apoptosis is initiated at the normal time in *H99* heterozygotes, programmed cell death proceeds much more slowly compared to with wild-type controls. TUNEL staining is much lower at 39, 42 and 45 hr APF in *H99* heterozygotes (Figure 2.5k,l, and Figure 2.6). In *H99*/+ eyes at 45 hr APF, TUNEL-positive ommatidia still contain unfragmented ELAV-positive photoreceptors (Figure 2.5 j), which is rarely observed in wild type eyes (Figure 2.5 i). This indicates that apoptosis has not progressed to the terminal stages in the *H99* heterozygotes and strongly argues that down-regulation of DIAP1 is required for the timely execution of programmed cell death.

Down-regulation of DIAP1 in the perimeter ommatidia depends on its BIR1 and BIR2 domains, but is independent of its RING domain

RHG proteins interact with DIAP1 through direct interactions between the IBM domain of RHGs and the BIR1 and BIR2 domains of DIAP1 (Chai et al. 2003; Zachariou et al. 2003; Hay and Guo 2006). The requirement for this interaction for DIAP1 down-regulation in the pupal eye was examined by utilizing DIAP1 mutants carrying point mutations in the BIR1 or BIR2 domains. The DIAP1 alleles $th^{6\text{-}3s}$ (G88S) and $th^{23\text{-}4s}$

(G269S) encode proteins with dramatically reduced binding to RHG proteins (Goyal et al. 2000; Zachariou et al. 2003). In peripheral ommatidia that are homozygous for either $th^{6\text{-}3s}$ or $th^{23\text{-}4s}$, the degree of down-regulation was significantly reduced compared to wild type eyes (Figure 2.7, Table 2.1). These results suggest that the IBM-BIR domain interaction of RHGs and DIAP1 contributes to DIAP1 down-regulation in perimeter ommatidia.

DIAP1 contains a RING domain at its C-terminus that can function as an E3-ubiquitin ligase and promote auto-ubiquitination and degradation (Yang et al. 2000; Suzuki et al. 2001; Wilson et al. 2002; Yoo et al. 2002). To explore whether the down-regulation of DIAP1 in peripheral ommatidia is mediated by its RING domain, we induced clones of the RING domain mutation th^{6B}. The C412Y mutation in th^{6B} removes one of the cysteines that coordinate zinc in the RING domain, and abolishes the E3 ligase activity in *in vitro* ubiquitination assays (Lisi et al. 2000; Yoo et al. 2002). Homozygous clones of th^{6B} in the eye failed to survive to mid-pupal stage even in a *GMR-p35* background, presumably due to lack of degradation of caspases. Therefore, these mosaic eyes contained a mixture of wild type cells and cells that are heterozygous for th^{6B} (Figure 2.8a). In internal ommatidia, cells harboring the th^{6B} allele had higher DIAP1 levels than wild type cells (Figure 2.8c, ** denotes +/+ cells and * denotes th^{6B}/+ cells). This suggests that loss of the RING domain results in stabilization the DIAP1 protein, due to loss of constitutively occurring RING domain-dependent auto-ubiquitination.

Surprisingly, this stabilized DIAP1 is still down-regulated in perimeter ommatidia (Figure 2.8c, arrowheads).

Consistent with the th^{6B} clonal analysis, flies that are heterozygous for th^{6B} or $th^{22\text{-}8}$, another allele that abolishes E3 ligase activity (Ryoo et al. 2002), also showed DIAP1 down-regulation in peripheral ommatidia (Figure 2.8 e-h, Table 2.1). These results support a model where RHG-dependent down-regulation of DIAP1 in peripheral apoptotic cells does not require auto-ubiquitination.

Removal of Dronc and Drak partially suppresses DIAP1 down-regulation.

If auto-ubiquitination is not responsible for DIAP1 down-regulation, what is? As part of our efforts to uncover this mechanism, we assessed the requirement of other pro-apoptotic components that are required for perimeter ommatidia cell death. These include Dark, the fly Apaf-1 homologue, and Dronc, an initiator caspase that is activated by Dark (Hay and Guo 2006). $dronc^{I29}$ and $dark^{G8}$ are strong loss-of-function or null alleles that are compromised for apoptosis (Xu et al. 2005; Srivastava et al. 2007). Interestingly, clones of $dronc^{I29}$ or $dark^{G8}$ exhibit incomplete down-regulation of DIAP1 (Figure 2.9; Table 2.1). These results indicate that Dark and Dronc are required for maximal down-regulation of DIAP1 and suggests that RHG may act through these factors.

DISCUSSION

Most developmental apoptosis in *Drosophila* is dependent on the Rpr, Hid and Grim(Hay and Guo 2006). These proteins are proposed to initiate apoptosis by inducing DIAP1 degradation, referred to as the degradation model. However, little is known about the physiological relevance of this model. In this report, we analyzed the regulation of DIAP1 protein levels during naturally occurring apoptosis at the perimeter of the fly pupal eye. Our data showed, for the first time, that DIAP1 is down-regulated post-transcriptionally by Hid, Grim and Rpr during developmental apoptosis (Figures 2.1-4). This allowed us to systematically examine how DIAP1 levels are regulated by pro-apoptotic proteins under physiological conditions, and how the RING and BIR domains of DIAP1 contribute to this down-regulation.

The degradation vs. the liberation model

RHG proteins have been proposed to activate apoptosis by promoting DIAP1 down-regulation (degradation model) or by disrupting DIAP1 binding to caspases (liberation model). When DIAP1 down-regulation was inhibited in *H99* heterozygous eyes, apoptosis was still initiated with similar intensity at 36hr APF (Figure 2.5 g, h), suggesting that RHG proteins are able to trigger apoptosis without down-regulating DIAP1. This is consistent with a previous report in fly cultured cells that blocking DIAP1 degradation did not alter the initiation of apoptosis by Hid or Rpr overexpression

(Yokokura et al. 2004). However, the absence of DIAP1 down-regulation clearly attenuated the progression apoptosis intensity (Figure 2.5k,l and Figure 2.6). This supports the physiological importance of DIAP1 degradation in apoptosis. This is consistent with data in mammalian thymocytes, where proteasome inhibitors prevented thymocyte death, presumably by suppressing XIAP and c-IAP1 degradation(Yang et al. 2000). Taken together, our data support a model where both the degradation and liberation models are required for RHG-induced apoptosis to occur with normal kinetics.

Our finding that a 50% reduction in Hid Grim and Rpr gene activity blocks DIAP1 down-regulation indicates that this regulation is a consequence of high level of RHGs. We speculate that when Wg signaling first activates RHG expression, the low RHG protein levels initiate apoptosis via the liberation model. As RHG levels increase, down-regulation of DIAP1 also occurs, facilitating the progression of apoptosis to completion.

In addition to the degradation model, Grim and Rpr have also been suggested to reduce DIAP1 levels by suppressing global translation (Holley et al. 2002; Yoo et al. 2002). While we provide evidence that DIAP1 down-regulation occurs post-transcriptionally in the pupal eye, we cannot rule out that decreased translation contributes to the down-regulation. Because Hid has not been shown to regulate translation, we believe that the DIAP1 down-regulation is at least partially due to degradation, if not entirely.

Role of the RING domain in DIAP1 down-regulation

The RING domain has been proposed to mediate the auto-ubiquitination and consequent degradation of DIAP1 during apoptosis (Hay and Guo 2006; Steller 2008). Our results showed that the RING domain mutant th^{6B} had elevated levels of DIAP1 in internal ommatidia (Figure 2.8c), suggesting that the E3 ligase activity of the RING domain contributes to the regulation of DIAP1 stability in these cells, presumably through auto-ubiquitination. Strikingly, the RING domain mutant was still down-regulated in peripheral ommatidia as wild type proteins (Figure 2.8c-h). These results suggest that auto-ubiquitination regulates DIAP1 levels in most ommatidial cells, but it is not required for down-regulation of DIAP1 in the apoptotic cells at the periphery.

One possibility is that DIAP1 degradation at the eye perimeter is mediated by other E3 ligases. Intriguingly, SkpA, an SCF (SKP-Cullin-F-box) multiunit E3 ligase complex component, has been implicated in Rpr- and Grim-induced apoptosis (Wing et al. 2002). SkpA has been shown to interact with the F-box protein Morgue, and Morgue can bind to DIAP1 and promote its degradation (Hays et al. 2002; Wing et al. 2002). These results suggest that an SCF E3 ligase complex could target DIAP1 for degradation in peripheral ommatidia, but this remains to be tested.

It has also been suggested that effector caspases cleave DIAP1 at its NH2 terminus, which exposes a binding motif for UBR-domain-bearing class of NH2-end-rule E3 ligases (Ditzel et al. 2003; Ditzel et al. 2008). However, we believe this class of E3 ligases is

irrelevant in our system, because our experiments were conducted in presence of *p35*, which blocks the activity of effector caspases. Our results are consistent with those showing that *p35* does not block the DIAP1 down-regulation caused by overexpression of RHGs (Ryoo et al. 2002; Yoo et al. 2002).

Role of the BIR domains in DIAP1 down-regulation

Both the BIR1 and BIR2 domains are required for DIAP1 down-regulation (Figure 2.7). Since these domains are required for interaction with RHGs *in vitro* (Zachariou et al. 2003; Hay and Guo 2006), this suggests that direct RHG-DIAP1 binding is required for DIAP1 down-regulation in perimeter ommatidia.

Our studies have also revealed an unexpected level of regulation of DIAP1 levels in the interior ommatidia of the pupal eye. A BIR2 mutation ($th^{23\text{-}4s}$) resulted in increased DIAP1 in internal ommatidia (Figure 2.7e). Likewise, mutations in the RING domain of DIAP1 also caused a large increase in DIAP1 protein levels in these ommatidia (Figure 2.8c). In contrast, even though *dronc* and *H99* (*hid*, *grim*, *rpr*) mutant clones suppressed DIAP1 down-regulation at the perimeter, they had no effect on DIAP1 levels in the interior ommatidia (Figure 2.9g and Figure 2.10). These results indicate a requirement for the BIR2 and RING domains of DIAP1 in regulating expression levels that is independent of Hid, Grim, Rpr and Dronc (which are crucial for regulation at the periphery). The mechanism of this regulation remains to be discovered.

Role of Dark and Dronc in DIAP1 down-regulation

Our results demonstrate that Dronc is required to achieve the full extent of DIAP1 down-regulation in peripheral ommatidia, (Figure 2.9e-h). Previous reports showed that active Dronc cleaves DIAP1 between the BIR1 and BIR2 domains in cultured fly cells as well as *in vitro* (Yan et al. 2004; Muro et al. 2005). We speculate that in the perimeter ommatidia of the pupal eye, Dronc cleavage of DIAP1 makes a significant contribution to DIAP1 down-regulation.

In addition to Dronc, we also found that Dark is partially required for DIAP down-regulation (Figure 2.9a-d). It is known that loss of Dark partially suppresses RHG-induced apoptosis in flies(Rodriguez et al. 1999). One interpretation of these results is that RHGs act through Dark to activate Dronc, leading to DIAP1 degradation. Indeed, Hid, Grim and Rpr have all been reported to localize to the mitochrondria (Haining et al. 1999; Claveria et al. 2002; Olson et al. 2003; Abdelwahid et al. 2007) and in the case of Rpr, this localization is important for promoting DIAP1 degradation in cultured cells (Freel et al. 2008). Interestingly, DIAP1 has also recently been shown to promote down-regulation of Dark and Dronc (Shapiro et al. 2008). This raises the possibility that RHGs could trigger a positive feedback loop where RHG binding to DIAP1 causes increased Dark and Dronc levels, which lead to DIAP degradation, thereby leading to even higher levels of Dark and Dronc. Further studies examining the levels of Dark and Dronc in the perimeter ommatidia will be required to explore this model.

ACKNOWLEDGEMENTS

I'd like thank Bruce Hay, Kristin White, Andreas Bergmann, Barry Dickson and John Abrams for providing fly stocks and antibodies. Thanks to Gregg Sobocinski for help with confocal microscopy. I am grateful to Eva Feldman and Steven Clark for discussion and advice, and also want to thank Yunyun Ni, Chandan Bhambhani, Hilary Archbold and other members of the Cadigan lab for critical reading and helpful discussions. This work was supported by NIH grant GM08894 to K. M. C.

This chapter has been submitted for publication as:

H. E. Li, and K. M. Cadigan "RHG protein mediated apoptosis in the pupal eye of *Drosophila*: down-regulation of DIAP1"

Figures and Tables

Figure 2.1

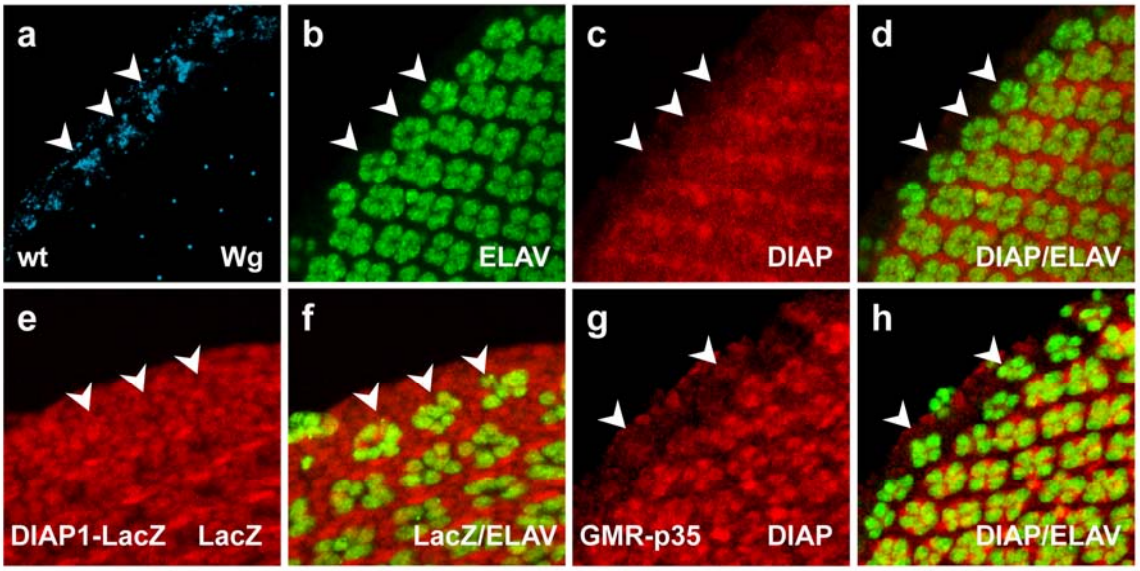

Figure 2.1 DIAP1 is down-regulated post-transcriptionally in peripheral ommatidia that undergo apoptosis. All images are of 36hr APF eyes. (**a-d**) Wild type eye stained for Wg (blue), ELAV (green) and DIAP1 (red). Wg marks the ommatidia that will be removed by apoptosis. DIAP1 levels in these peripheral ommatidia are lower than that of the Wg-negative internal ommatidia. (**e,f**) DIAP1-*lac*Z line (th^{j5c8}) stained for galactosidease (red) and ELAV (green). Peripheral ommatidia have the same galactosidease level as internal ones. (**h,i**) GMR-Gal4::UAS-*p35* eyes stained for DIAP1 (red) and ELAV (green). Down-regulation of DIAP1 is still observed in peripheral ommatidia.

Figure 2.2

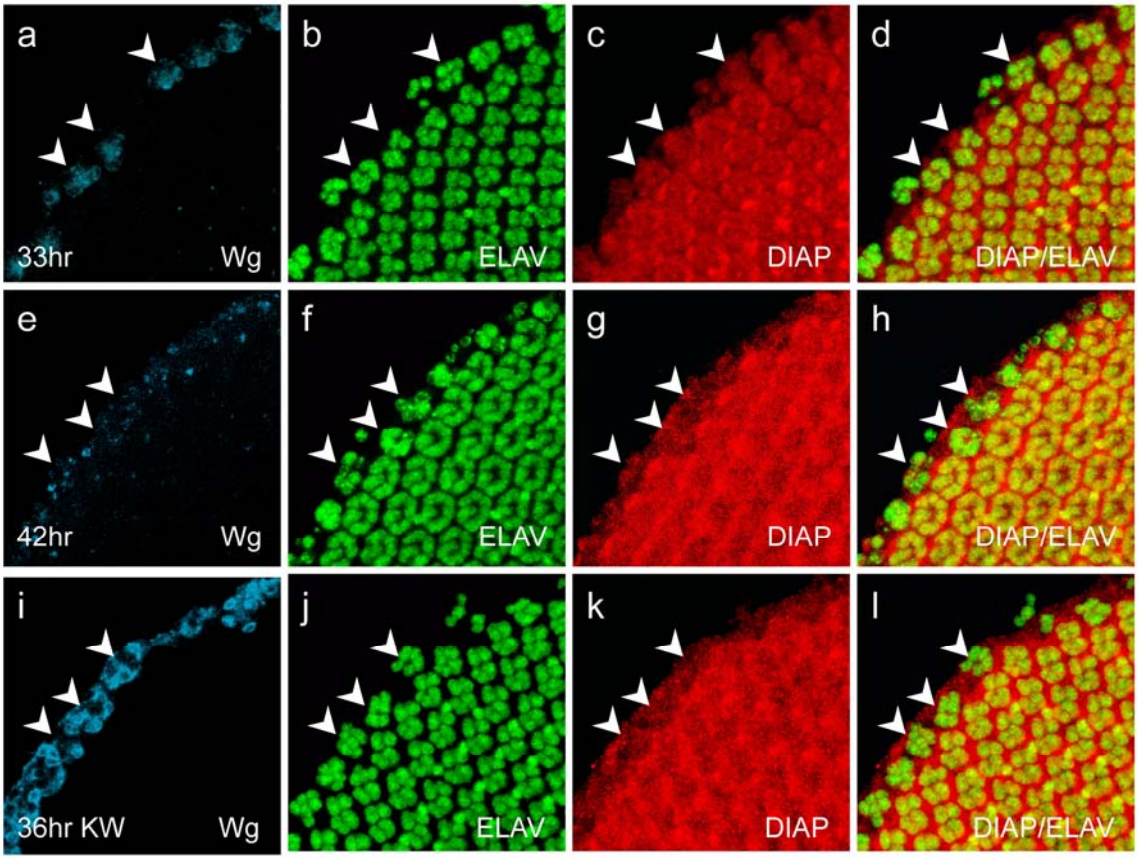

Figure 2.2 DIAP1 is down-regulated in peripheral ommatidia that undergo apoptosis. Wild type eye stained for Wg (blue), ELAV (green) and DIAP1 (red) at 33hr (**a-d**), 42hr (**e-h**) and 36hr (**i-l**). Wg marks the ommatidia that will be removed by apoptosis. DIAP1 levels start to decrease at some ommatidia at 33hr, and stay lower than that of the Wg-negative internal ommatidia at 42hr. (**i-l**) DIAP1 down-regulation is also observed at 36hr in wild type eye stained with an independent anti-DIAP1 antibody from K. White (KW) (**i**).

Figure 2.3

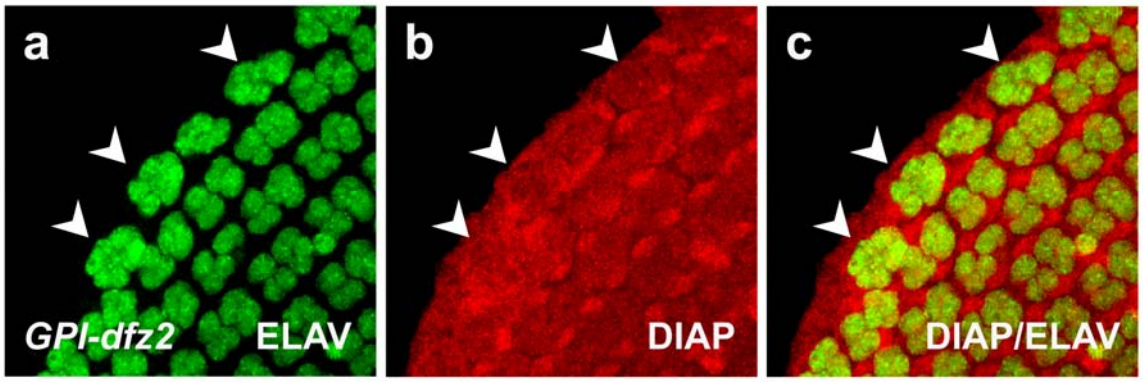

Figure 2.3 Down-regulation of DIAP1 is inhibited in peripheral ommatidia where Wg signaling is blocked by overexpression of *GPI-fz2*. GMR-Gal4::UAS-*GPI-fz2* eyes stained for DIAP1 (red) and ELAV (green).

Figure 2.4

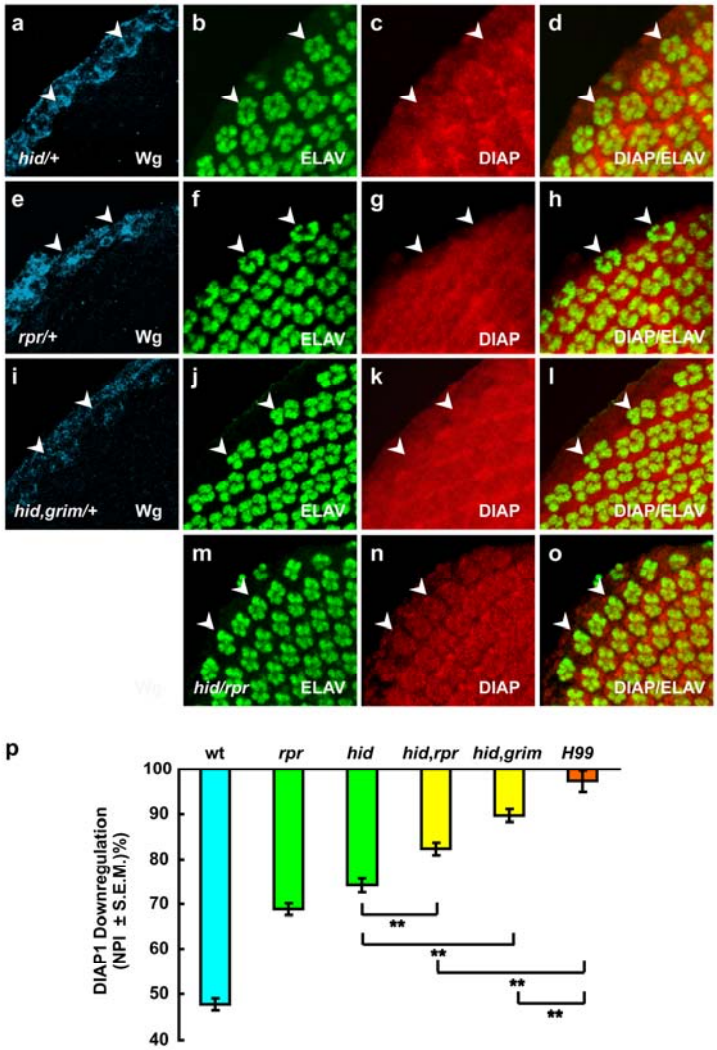

Figure 2.4 Hid, Grim and Rpr contribute to DIAP1 down-regulation in peripheral ommatidia. All images are of 36hr APF eyes. Eyes stained for Wg (blue), ELAV (green) and DIAP1 (red) from hid^{X14}/+ (**a-d**), *Df(3f)XR38*/+ (which removes one copy of *rpr*) (**e-h**), *Df(3f)X25*/+ (which removes one copy of *hid* and *grim*) (**i-l**), and a hid^{X14}/ *Df(3f)XR38* trans-heterozygote (**m-o**). DIAP1 down-regulation in peripheral ommatidia (arrowheads) is partially suppressed in all four genetic backgrounds. Quantification of DIAP1 protein levels in peripheral ommatidia are summarized in bar graph (**p**) and Table 2.1. Protein levels are presented as Normalized Mean Pixel Intensity (NPI), and shown as percentage of NPI of internal ommatidia in each genetic background. Two-tailed Student's t-test was performed between perimeter ommatidia of different background, and ** denotes $p < 0.001$.

Figure 2.5

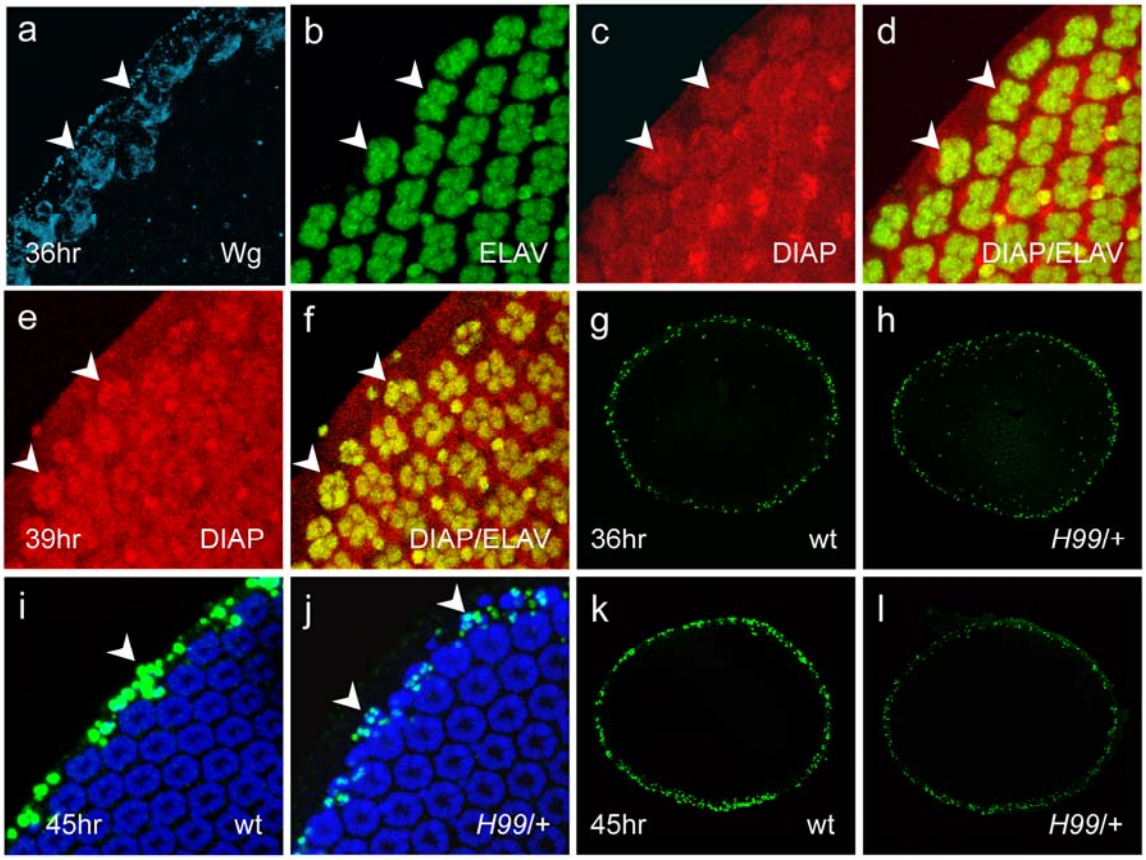

Figure 2.5 Apoptosis can occur in the absence of DIAP1 down-regulation in peripheral ommatidia. (**a-f**) Eyes from *Df(3f)H99*/+ (which removes one copy of *hid*, *rpr* and *grim*) stained for Wg (blue), ELAV (green) and DIAP1 (red) at 36hr APF (**a-d**) and 39hr APF (**e-f**). No significant difference in DIAP1 levels were observed between peripheral (arrowheads) and internal ommatidia, as shown in the representative samples (**c, e**) and summarized in Table1. (**g-l**) TUNEL staining of wild type (**g, i, k**) and *Df(3f)H99*/+ (**h, j, l**) at 36hr APF (**g-h**) and at 45hr APF (**i-l**). TUNEL signal is comparable between wild type and *Df(3f)H99*/+ at 36 hr, while the TUNEL signal is much greater in wild type at 45hr. At this time, *H99* heterozygous eyes still contain unfragmented ELAV-positive photoreceptors compared to the TUNEL-positive cells in wild type (arrowheads in **i, j**).

Figure 2.6

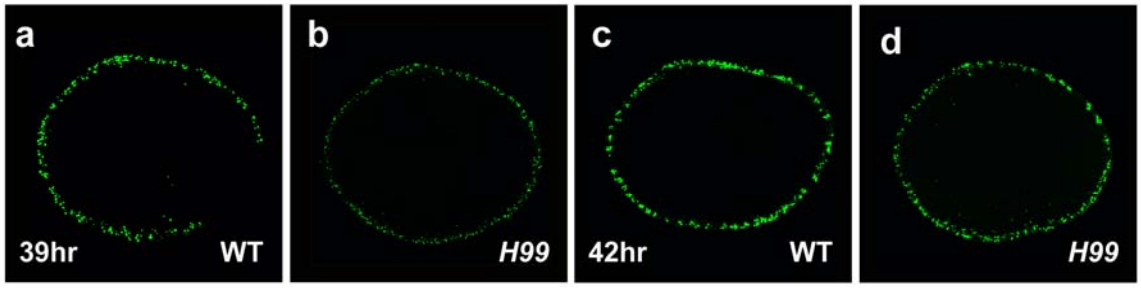

Figure 2.6 The absence of DIAP1 down-regulation attenuates the progression of apoptosis. TUNEL staining of wild type (**a, c**) and *Df(3f)H99*/+ (**b,d**) at 39hr APF (**a,b**) and at 42hr APF (**c,d**). TUNEL signals in wild type are more intensive than in *Df(3f)H99*/+.

Figure 2.7

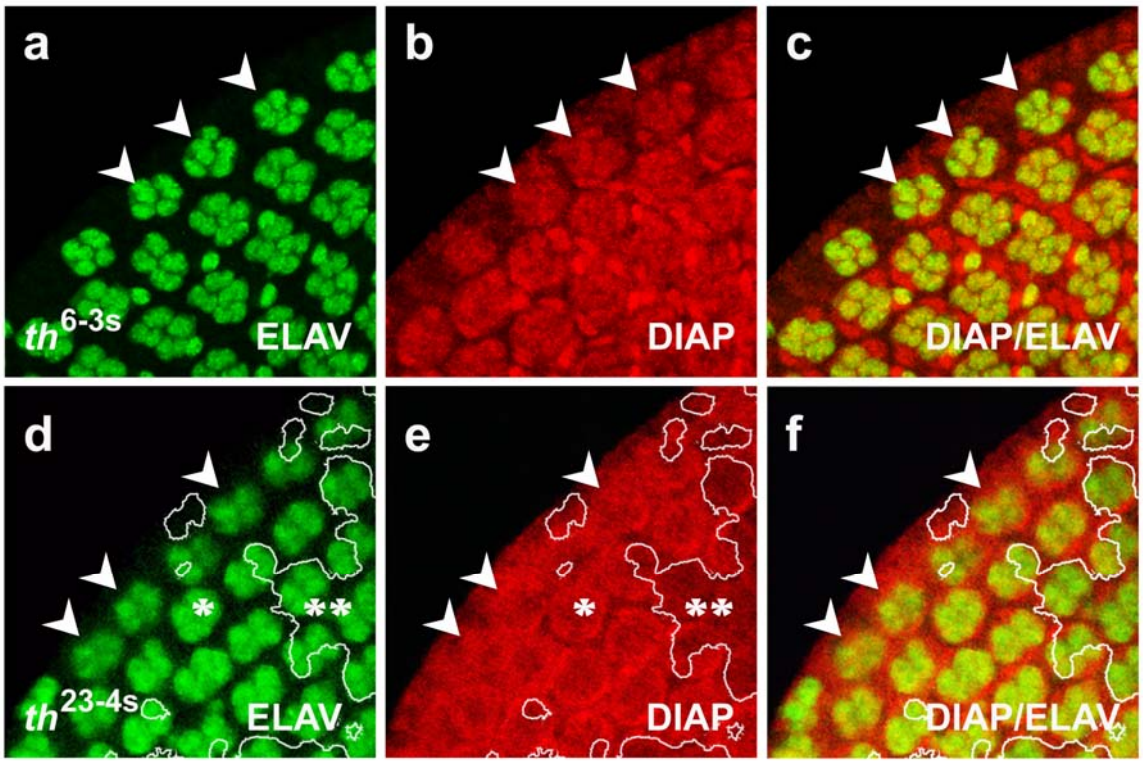

Figure 2.7 Down-regulation of DIAP1 in the peripheral ommatidia depends on its Bir1 and Bir2 domains. (**a-c**) Eye from a DIAP1 Bir1 domain homozygous mutant ($th^{6\text{-}3s}$) stained for ELAV (green) and DIAP1 (red). (**d-f**) Clone of DIAP1 Bir2 domain mutant ($th^{23\text{-}4s}$) stained as above. White lines represent the clonal boundaries of $th^{23\text{-}4s}$ mutant (*) and wild type cells (**). The cells with the dramatic increase in DIAP1 levels are inside the $th^{23\text{-}4s}$ mutant clone (*). DIAP1 down-regulation in peripheral ommatidia (arrowheads) is suppressed in both genetic backgrounds. Quantification of DIAP1 protein levels are summarized in Table 2.1.

Figure 2.8

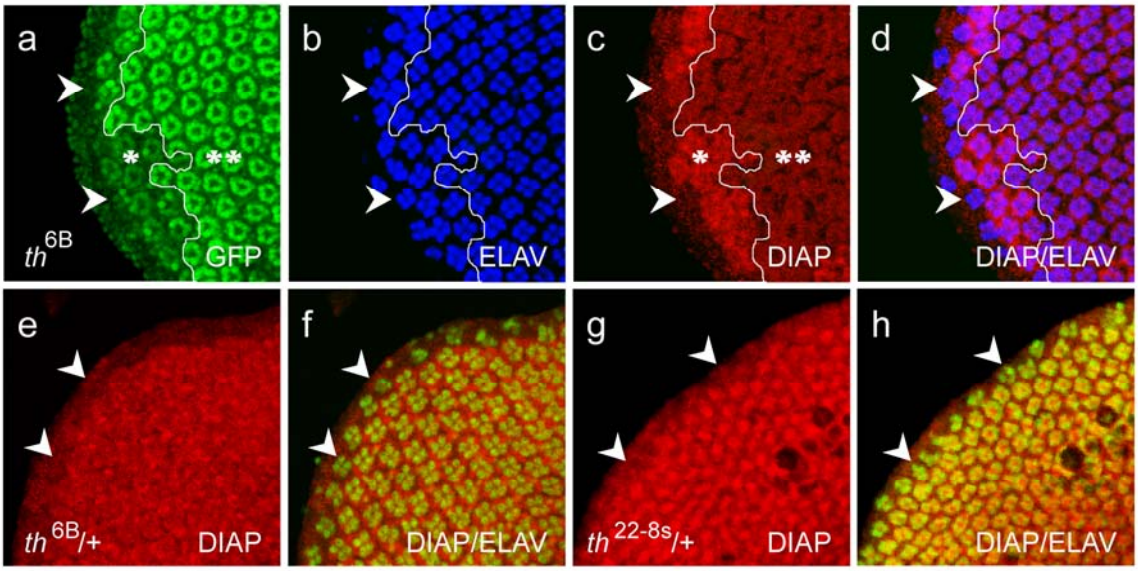

Figure 2.8 Downregulation of DIAP1 in peripheral ommatidia is independent of its RING domain. All images are of 36hr APF eyes. (**a-d**) Mosaic eye for DIAP1 RING domain mutant (th^{6B}) stained for ELAV (blue) and DIAP1 (red). High levels of GFP (**a**) mark the wild type cells (+/+), while low levels of GFP mark the th^{6B}/+ cells. White lines mark the boundary of the +/+ (**) and th^{6B}/+ cells (*). In internal ommatidia, cells containing the mutant allele (*) have much higher DIAP1 levels than wild type cells (**). However, down-regulation of DIAP1 still occurs in th^{6B}/+ peripheral ommatidia (arrowheads). (**e-h**) Eyes stained for DIAP1 (red) and ELAV (green) from th^{6B}/+ (**e**,**f**) and $th^{22\text{-}8s}$ /+ (**g**,**h**) animals. $th^{22\text{-}8s}$ is another DIAP1 Ring domain mutant distinct from th^{6B}. Down-regulation occurs in peripheral ommatidia in both th^{6B} /+ and $th^{22\text{-}8s}$ /+. Quantification of DIAP1 protein levels are summarized in Table 2.1.

Figure 2.9

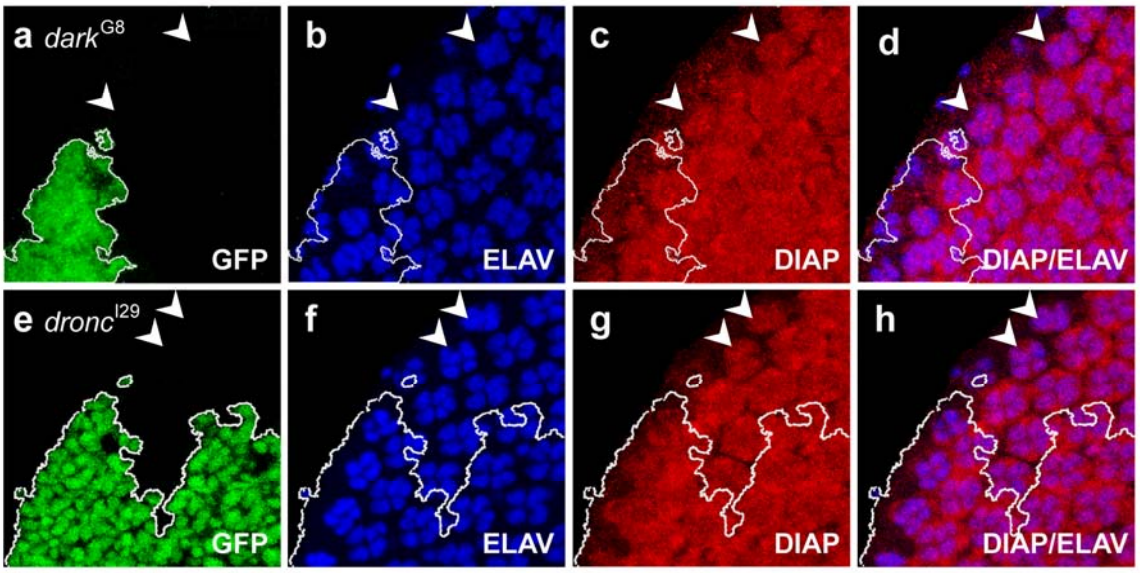

Figure 2.9 Dronc and Dark are required for DIAP1 down-regulation. All images are of 36hr APF eyes. Clones of *dark*^{G8} (**a-d**) and *dronc*^{I29} (**e-h**) stained for ELAV (blue) and DIAP1 (red). Clones are marked by lack of clonal maker GFP (**a, e**), and white lines represent the boundaries. DIAP1 down-regulation in peripheral ommatidia (arrowheads) is partially suppressed in *dronc* and *dark* clones. Quantification of DIAP1 protein levels are summarized in Table 2.1.

Figure 2.10

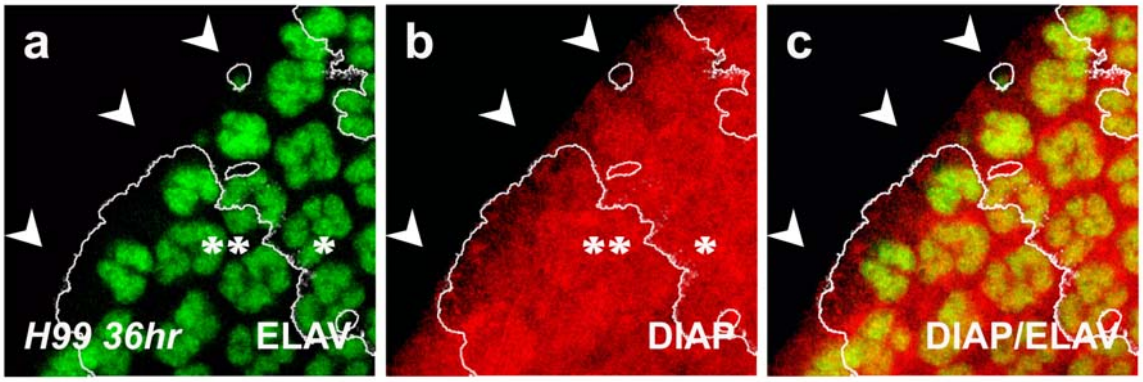

Figure 2.10 Removal of Hid, Rpr and Grim blocks DIAP1 down-regulation in peripheral ommatidia. Clones of *Df(3f)H99* stained for ELAV (green) and DIAP1 (red). White lines represent the boundaries of *H99* mutant cells (*) and wild type cells (**). DIAP1 down-regulation does not occur in H99 peripheral ommatidia. Note also that loss of *H99* has no detectable effect on DIAP1 levels in internal ommatidia.

Table 2.1

Genotype	Internal ommatidia (NPI ± S.E.M.)%	Periphery ommatidia (NPI ± S.E.M.)%
wild type	100.0 ± 0.6	47.7 ± 1.3 **
GMRp35	100.0 ± 0.9	44.4 ± 1.9 **
th $^{6\text{-}3\ -/-}$	100.0 ± 0.8	88.9 ± 1.1 *
th $^{23\text{-}4\ -/-}$	100.0 ± 1.0	91.3 ± 2.0 **
hid $^{+/-}$	100.0 ± 1.4	74.2 ± 1.5 **
rpr $^{+/-}$	100.0 ± 1.0	68.8 ± 1.3 **
hid $^{+/-}$, *rpr* $^{+/-}$	100.0 ± 1.0	82.4 ± 1.4 **
hid,grim $^{+/-}$	100.0 ± 1.3	89.7 ± 1.4 **
H99 $^{+/-}$	100.0 ± 0.9	97.3 ± 2.4 †
GMRp35; th $^{6B\ +/-}$ (clone)	100.0 ± 3.1	31.8 ± 3.8 **
GMRp35; th $^{6B\ +/-}$	100.0 ± 4.8	40.4 ± 3.5 **
GMRp35; th $^{22\text{-}8s}$	100.0 ± 2.2	43.9 ± 4.9 **
GMRp35; Dark $^{G8\ -/-}$	100.0 ± 2.0	83.4 ± 2.6 **
GMRp35; Dronc $^{I29\ -/-}$	100.0 ± 2.2	79.1 ± 1.0 **

GMRGal4::UASp35 is abbreviated as *GMRp35* in the table
Two-tailed Student's t-text between internal and periphery ommatidia
** P < 0.001 * P < 0.05 † P > 0.5

Table 2.1 Comparison of DIAP1 protein levels between periphery and internal ommatidia in different genetic backgrounds. Protein levels are presented as Normalized Mean Pixel Intensity (NPI), and shown as percentage of NPI of internal ommatidia in each genetic background. Each value is derived from at least 20 ommatidia from at least four different eyes.

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CHAPTER III

THE DROSOPHILA CASEIN KINASE I EPSILON DISCS OVERGROWN PROMOTES CELL SURVIVAL VIA ACTIVATION OF DIAP1 EXPRESSION

ABSTRACT

The proper number of cells in developing tissues is achieved by coordinating cell division with apoptosis. In *Drosophila*, the adult wing is derived from wing imaginal discs, which undergo a period of growth and proliferation during larval stages without much programmed cell death. In this report, we demonstrate that the *Drosophila* casein kinase Iε/δ, known as Discs overgrown (Dco), is required for maintaining this low level of apoptosis. Expression of *dco* can suppress the apoptotic activity of Head involution defective (Hid) in the developing eye. Loss of *dco* in the wing disc results in a dramatic reduction in expression of the caspase inhibitor DIAP1 and a concomitant activation of caspases. The regulation of DIAP1 by Dco occurs by a post-transcriptional mechanism

that is independent of *hid*. Mutant clones of *dco* are considerably smaller than controls even when apoptosis is inhibited, suggesting that Dco promotes cell division/growth in addition to its role in cell survival. The *dco* phenotype cannot be explained by defects Wingless (Wg) signaling. We propose that Dco coordinates tissue size by stimulating cell division/growth and blocking apoptosis via activation of DIAP1 expression.

INTRODUCTION

Apoptosis, or programmed cell death (PCD), is a tightly regulated mechanism of cell death that is essential to animal development and homeostasis of adult organs (Jacobson et al., 1997; Meier et al., 2000). In many developmental contexts, PCD is required for the removal of excess cells during morphogenesis. Apoptosis also serves as a quality control mechanism to eliminate abnormal or damaged cells from the organism. In addition, there is a link between cell growth/proliferation and PCD. Cells stimulated to grow and divide often also have an increased propensity for undergoing apoptosis (Abrams, 2002; Green and Evan, 2002). This relationship has been suggested to act as a "fail-safe" system, to prevent unwanted expansion of cell populations. Inhibition of this system is thought to occur during the multi-step process of oncogenesis (Evan and Vousden, 2001; Green and Evan, 2002; Hanahan and Weinberg, 2000).

PCD is executed by a family of cysteine proteases called caspases. Caspases are synthesized as inactive zymogens and are processed to their active form to cleave cellular

substrates, thus promoting the apoptotic pathway. Caspases and many of their regulatory components, such as the Bcl2 family proteins, are evolutionarily conserved from nematodes to mammals (Kaufmann and Hengartner, 2001). However, additional strategies have evolved in insects and higher organisms to provide greater flexibility of regulation. One example is the Inhibitor of Apoptosis (IAP) family of proteins. IAPs can bind to caspases, inhibiting their processing, catalytic activity or the ability of active caspase to bind to substrates (Deveraux and Reed, 1999; Tenev et al. 2005). IAPs can also facilitate caspase degradation by targeting them for ubiquitination via their E3 ubiquitin ligase activity (Wilson et al., 2002; Yang et al., 2000).

Studies of a *Drosophila* member of the IAP family, DIAP1, encoded by the *thread* (*th*) locus, have provided critical insights into the function and regulation of IAPs. DIAP1 can be bound and inhibited by Head involution defective (Hid), Grim and Reaper (Rpr) (Hay, 2000). Overexpression of these pro-apoptotic factors can down regulate DIAP1 levels by promoting its degradation via the ubiquitin ligase activity of DIAP1 as well as inhibiting DIAP1 translation (Hays et al., 2002; Holley et al., 2002; Ryoo et al., 2002; Yoo et al., 2002). Once caspases are activated, they can cleave DIAP1, stimulating its ubiquitination and degradation (Ditzel et al., 2003). This multilevel regulation of DIAP1 reflects the critical role it plays in cell survival, as evidenced by the massive activation of caspases and apoptosis observed in *DIAP1* mutants (Ryoo et al., 2004; Yoo et al., 2002).

The connection between cell growth/proliferation and cell death and pattern formation has been extensively studied in the *Drosophila* wing imaginal discs (Edgar, 1999; Serrano and O'Farrell, 1997; Tapon et al., 2001a). During the three larval stages, the wing discs undergo a rapid phase of cell growth and proliferation with very low levels of PCD (Milan et al., 1997). The *Drosophila* homologue of Casein Kinase Iε/δ (CKIε/δ), known as *discs overgrown*/*double time* (*dco*)**,** was shown to be essential for imaginal tissue development (Jursnich et al., 1990; Zilian et al., 1999). Weak *dco* mutants exhibit a lag in disc growth, while stronger alleles have small or severely degenerated discs. Mitotic clones of *dco* null mutant cells in the wing disc did not grow or survive when induced during first or second larval stages, though small clones could be observed if induced at later stages (Zilian et al., 1999). Electron micrographs of *dco* mutant discs demonstrated the presence of basally extruded cells with nuclear condensation, suggesting that PCD was occurring. Based on these observations it was concluded that *dco* is required for cell proliferation and/or survival through an undetermined mechanism.

In this report we demonstrate that Dco is an essential cell survival factor in the wing imaginal disc. We initially identified Dco as a potent suppressor of Hid induced cell death in the eye. This Hid antagonism requires Dco kinase activity. Loss of *dco* in the wing results in massive PCD, which is a major cause of the small disc phenotype seen in *dco* mutants. When apoptosis is inhibited, *dco* mutant wings are normally patterned, suggesting that *dco*'s effect on cell survival is not an indirect consequence of abnormal cell

specification. We demonstrate that *dco* mutant cells have a dramatic post-transcriptional reduction of DIAP1 protein levels, which is sufficient to explain the increase in PCD. This regulation of DIAP1 is independent of *hid* and not does appear to require the E3 ligase activity of DIAP1. We also provide results suggesting that Dco promotes cell growth/proliferation in the wing disc.

CKI family members, including CKIδ and CKIε, have been implicated in several developmentally important signaling pathways. In *Drosophila*, *dco* has been shown to act as a positive regulator of planar cell polarity in the larval eye and pupal wing (Strutt et al., 2006; Klein et al., 2006). In fly cell culture and the wing imaginal disc, *dco* has been shown to be required for Wnt/Wingless (Wg) signaling (Cong et al., 2004; Klein et al., 2006; Zhang et al., 2006). Wg signaling is known to promote cell survival in the wing imaginal discs (Giraldez and Cohen, 2003; Johnston and Sanders, 2003). However, we find that cells homozygous for a null *dco* allele have normal Wg signaling in the developing wing. Our data support a model where Dco promotes cell survival by activating DIAP1 protein levels independently of Wg signaling.

MATERIALS AND METHODS

Fly strains

The following fly strains were used. Oregon R was our wild type stock. The *GMR-hid*^{l0}, *GMR-hid*^{1M}, *GMR-rpr*^{2}, *GMR-rpr*^{5}, *GMR-grim*^{2} and *GMR-grim*^{3} (Chen et al.,

1996; Rodriguez et al., 1999) stocks were from J. Abrams. *GMR-hid*^{ala5} (Bergmann et al., 1998) was from A. Bergmann. UAS-*lacZ* (Brand and Perrimon, 1993), UAS-*p35* (Mergliano and Minden, 2003), *en*Gal4 (Neufeld et al., 1998) and *ptc*Gal4 (Johnson et al., 1995) and *th*^{j5c8} (Hay et al., 1995) were from the Bloomington Stock Center. UAS-*dco* (on the X chromosome; Price and Kalderon, 2002) was from M. Young. A wild-type UAS-*dco* stock and UAS-*dco*^{D132N} mutant (Klein et al., 2006) were obtained from M. Mlodzik and a UAS-*dco* and UAS-*dco*^{K38R} (Strutt et al., 2006) was provided by D. Strutt. *GMR*Gal4 (Freeman, 1996) was from M. Freeman. The *hid*^{05014}*and hid*^{X14} stocks (Grether et al., 1995) were from K. White, while the *Df(3L)H99* and UAS-*DIAP1* stocks (Hay et al., 1995) were from B. Hay. The *dco* mutant alleles *dco*^{P915}, *dco*^{P1396} and *dco*^{le88} (Zilian et al., 1999) were from M. Noll. The *th*^{6B} allele (Lisi et al., 2000) was from K. White. *FRT*^{82B} P[*w*+, *Ubi-GFP*^{nls}] and *FRT*^{82B} P[*w*+, *armlacZ*] (Tapon et al., 2001b) were from I. Hariharan while all other chromosomes for clonal analysis, *yw flp*^{122} , *FRT*^{82B} (Xu et al., 1993) were from the Bloomington Stock Center. The UAS-*dco*^{RNAi} transgene was constructed by cloning an inverted repeat of the C-terminal non-kinase domain coding sequence using the PCR primers 5'CCAGTCTAGACAGCGGCCGCAGGACG GAGCGGAC3' and 5'GCTATTTAGATCTAAACCGCAAGGGGTGCGGTGG3' into the pWIZ vector (Lee and Carthew, 2003). Transgenic flies were generated following the standard P element methodology.

Generation of dco clones

To generate *dco* clones with *p35* expressed in the posterior domain of the wing disc, the fly strain *yw*, flp^{122}; *en*Gal4; FRT^{82B} P[*armlacZ*]/TM6 was crossed to *w*, UAS-*p35*; FRT^{82B}*dco*^{le88}. For the *DIAP*-lacZ experiment, the following strain *yw*,flp^{122}; *en*Gal4; FRT^{82B} P[w+, Ubi-GFP^{nls}]/TM6 was crossed to UAS*p35*; *th*^{j5c8}, FRT^{82B}*dco*^{le88}/TM6. The progenies were heat shocked during early 2nd instar larval stage for 1 hour at 37°C. The larvae were dissected and fixed with 4% formaldehyde 60 to 72 hours later.

Immunostainings, TUNEL and in situ hybridization

Immunostainings were performed as previously described (Parker *et al*., 2002). Mouse anti-DIAP1 (1:200) and rabbit anti-active Drice (1:2,000) (Yoo et al., 2002) were both provided by B. Hay. Guinea pig anti-Sen (1:5000) was made by immunization with GST-Sen as previously described (Nolo et al., 2000). Rabbit anti-Dll (1:200) was from J. Kohtz and rabbit anti-Spalt (1:100) (Kuhnlein et al., 1994) was from R. Schuh and B. Mollereau. Rabbit anti-β-galactosidase (Cappel, ICN, Costa Mesa, CA) and mouse anti-β-galactosidase (Sigma, St. Louis, MO) were both used at 1:500. Rabbit anti-phospho-Histone 3 (1:150) was from Upstate (Lake Placid, NY). Monoclonal anti-En (1:20) and anti-Wg (1:100) were from the Developmental Studies Hybridoma Bank (Iowa City, IA). Cy3- and Alexa Fluor 488-conjugated secondary antibodies were from Jackson Immunochemicals (West Grove, PA) and Molecular Probes (Eugene, OR), respectively. The TUNEL assay was performed using the Cell Death Detection kit

(Roche Diagnostics, Indianapolis, IN) as previously described (Lin et al., 2004). In situ hybridization detection of hid, grim and rpr transcripts was performed as previously described (Lin et al., 2004). Pupal wings were stained as described (Axelrod, 2001) with Alexa568 phalloidin (Molecular Probes) at a concentration of 6.25U/ml.

Microscopy and image analysis

The adult eyes were photographed on a Leica M10 microscope as previously described (Parker et al., 2002). All fluorescent pictures were obtained with a Ziess Axiophot coupled to a Zeiss LSM510 confocal apparatus. The pixel values of the clone and twin areas in Figure 3.8 were calculated using the histogram tool in Photoshop 6.

RESULTS

Dco preferentially suppresses Hid induced cell death in the eye.

dco was identified in an EP misexpression screen (Rorth et al., 1998) for genes that interact with the Wg signaling pathway. When placed under the control of the eye-specific *GMR* promoter, Wg causes a severe reduction in eye size (Parker et al., 2002). This is due in part to an increase in apoptosis in the late larval/early pupal eye (Lin et al. 2006). We expected that some EP lines that could suppress the *GMR*/*wg* phenotype would be inhibitors of PCD. One such example was *EP(3)3280*, where the P-element is inserted into the first intron of *dco* (data not shown). Similar suppression of *GMR*/*wg* was

obtained with a UAS-*dco* line, confirming that *dco* was responsible for the effect. Misexpression of *dco* in several contexts outside the eye revealed no effect on Wg signaling (data not shown). However, expression of *dco* in the eye strongly suppressed the activity of the pro-apoptotic factor Hid (Figure 3.1B). *dco* had more subtle effects on the small eye phenotype caused by *GMR-rpr* (Figure 3.1D, F) and moderately suppressed *GMR-grim* (Figure 3.1H). Because *hid*, *grim* and *rpr* are expressed by the heterologous *GMR* promoter, the suppression is not due to transcriptional inhibition of these pro-apoptotic genes.

It has been previously shown that ectopic expression of activated Ras preferentially suppresses Hid induced cell death in the eye (Bergmann et al., 1998; Kurada and White, 1998). However, the ability of activated Ras to suppress the GMR-*hid* small eye phenotype was blocked when five potential MAP kinase phosphorylation sites were mutated to alanine (GMR-hid^{ala5}) (Bergmann et al., 1998). Both *EP(3)3280* and UAS-*dco* were able to suppress GMR-hid^{ala5} to the same extent as wild type GMR-*hid* (Figure 3.2B and data not shown), suggesting that *dco* overexpression is not working through a Ras/MAP kinase pathway.

To determine whether endogenous *dco* regulates apoptosis in the eye, we reduced *dco* expression using RNAi via a *dco* hairpin construct (UAS-dco^{RNAi}). Expression of this hairpin with the *GMR-Gal4* driver resulted in a roughened eye phenotype but did not reduce overall eye size (Figure 3.3B). However, dco^{RNAi} enhanced the small eye

phenotype of an intermediate *GMR-hid*^{1M} construct (Figure 3.3 C, D). *dco*^{RNAi} had much smaller effects on *GMR-rpr* and *GMR-grim* constructs (data not shown). These data indicate that endogenous *dco* influences the level of apoptosis caused by misexpression of *hid*.

To examine whether the kinase activity of Dco is required for Hid antagonism, two mutant forms of *dco* were co-expressed with *hid* in the eye. *dco*^{D132N} is predicted to compromise ATP binding (Peters et al., 1999; Zhu et al., 1998) while *dco*^{K38R} contains a mutation within the catalytic site (Peters et al., 1999; DeMaggio et al., 1992; Fish et al., 1995). Both mutants can act as dominant negatives for CKI function (Zhu et al., 1998; Peters et al., 1999; McKay et al., 2001; Klein et al., 2006; Strutt et al., 2006). However, Dco^{D132N} was clearly able to suppress the *GMR-hid* small eye phenotype (Figure 3.4B), while Dco^{K38R} had the opposite effect. The K38R form of Dco enhanced both a moderate/strong *GMR-hid* (Figure 3.4D) and a weaker *GMR-hid* transgene (Figure 3.4F). When expressed by *GMR*Gal4 in an otherwise wild-type eye, Dco^{K38R} caused a roughening of the eye, but did not effect eye size (data not shown). Because the D132N mutation has been reported to reduce, but not eliminate CKI kinase activity (Zhu et al., 1998), the data suggest that a partially active Dco can still suppress Hid-mediated apoptosis but some Dco kinase activity is required for antagonizing Hid activity.

dco mutant imaginal discs have elevated apoptosis

Although *dco* overexpression and RNAi knockdown effect the ability of Hid to promote apoptosis, mitotic clones homozygous for a *dco* null allele (dco^{le88}) are readily detectable in late larval eye discs and mid-pupal eyes (data not shown). *dco* mutant clones are not recovered in the adult eye, although some scarring near the twin spots was seen (data not shown). We have not been able to detect increased apoptosis in the larval or pupal clones. Apparantly, cells lacking *dco* are eliminated sometime in late pupal development.

To further explore whether *dco* is required for suppressing PCD, we examined several other imaginal discs. Using the TUNEL DNA fragmentation assay, we compared the level of apoptosis in imaginal discs from wild type and hypomorphic *dco* mutants ($dco^{P915/P1395}$). Throughout third instar larval development, TUNEL positive cells in the wing were detected at low levels in wild type, as shown in Figure 3.5A & D. In contrast, we found a dramatic increase in TUNEL signal in the *dco* mutant wing discs (Figure 3.5B, E). Note that the *dco* hypomorphs are developmentally delayed, and the discs were normalized for developmental stage by examining the pattern of Wg expression. Elevated TUNEL signal was also observed in the leg and haltere discs from *dco* mutants, but no increase was observed in eye imaginal discs (data not shown).

To determine whether the elevated PCD we observe is related to *hid*, *grim* and/or *rpr* function, we lowered the dose of these genes in a *dco* mutant background. Removal of

one copy of *hid, grim* and *rpr* (using the *Df(3L)H99* deficiency) caused a significant reduction of TUNEL signal in *dco* mutants (Figure 3.5E, F). Removal of one copy of *hid* alone did not significantly reduce the level of apoptosis, but a dramatic reduction was achieved by removing both copies of *hid* (data not shown). These data suggest that in *dco* mutant discs endogenous *hid*, *grim* and/or *rpr* are required for the elevated cell death.

As reported previously, $dco^{P915/P1395}$ mutant animals have a prolonged larval phase with slow imaginal disc growth (Zilian et al., 1999). These animals die in late larval or early pupal stages. We found that the decrease in cell death in *H99,* dco^{P915}*/+,* dco^{P1395} discs was correlated with marked acceleration in disc growth and larval development (from 8-10 days to 5-6 days). These *H99/+,* $dco^{P915/P1395}$ animals have increased survival into late pupal stages, and the veination pattern of the majority of these animals are similar to wildtype (Figure 3.5G, H). Occasional escapers survive to adulthood displaying normal legs and wrinkled or blistered wings (data not shown). These results suggest that the developmental delay in disc growth and larval development seen in *dco* mutants is largely caused by elevated apoptosis. In addition, the data in Figure 3.5H indicate that patterning of the wing is normal in *dco* mutants when PCD is reduced.

Dco activates DIAP1 expression in the wing imaginal disc

Both overexpression and loss of function studies with *dco* suggest that it encodes an anti-apoptotic factor in *Drosophila* imaginal tissues. Our initial observation that overexpression of *dco* differentially suppresses GMR-*hid* and GMR-*rpr* is reminiscent of

DIAP1 alleles containing mutations in its RING domain (Lisi et al., 2000; Wilson et al., 2002). It has been shown that these DIAP1 mutants lack ubiquitin ligase activity and may be present at higher levels due to stabilization of DIAP1. These findings prompted us to look at DIAP1 protein levels when *dco* expression is elevated (with UAS-*dco*) or reduced (UAS-dco^{RNAi}). Both UAS transgenes were driven by *patched* (*ptc*)-Gal4, which is active in a stripe on the anterior side of the anterior/posterior boundary of the wing imaginal disc (Johnson et al., 1995).

Overexpression of *dco* led to a moderate increase of DIAP1 protein levels in the *ptc* expression domain (Figure 3.6B). Conversely, DIAP1 expression was decreased upon RNAi-mediated knockdown of *dco* (Figure 3.6C). Using an antibody specific for the activated form of the effector caspase Drice (Yoo et al., 2002), we found that *dco* knockdown also caused a marked increased in Drice activation (Figure 3.6E). Z-axis projection of *ptc*-Gal4/UAS-dco^{RNAi} wing discs reveals that the Drice-positive cells are found basally in the epithelial sheet (Figure 3.6G) and that DIAP1 levels are low both apically and basally (Figure 3.6H). Loss of DIAP1 is known to cause apoptosis (Ryoo et al. 2004; Wang et al. 1999; Yoo et al. 2002), and the data suggest that Dco promotes cell survival by maintaining DIAP1 expression.

To determine whether *hid* was required for Dco regulation of DIAP1, the *ptc*-Gal4/UAS-dco^{RNAi} experiments were repeated in a *hid* mutant background. An obvious decrease in DIAP1 levels was still observed (Figure 3.6D) indicating that Dco can

activate DIAP1 independently of *hid*. The reduction in DIAP1 was also seen in a *H99*/+ background (data not shown). In contrast, the activation of Drice by dco^{RNAi} was greatly suppressed in *hid* mutants (Figure 3.6F) or *H99* heterozygotes (data not shown). This is consistent with the results from *dco* hypomorphic mutants (Figure 3.5C, F).

To rule out the possibility that Dco regulates the transcript levels of *hid*, *grim* or *rpr*, in situ hybridization was performed on *ptc*-Gal4/UAS-dco^{RNAi} wing imaginal discs. *hid* expression was not detectably effected (Figure 3.7A), but a small reduction in *grim* expression was sometimes observed within the Ptc domain (Figure 3.7B) while a small increase in *rpr* transcripts was also consistently seen (Figure 3.7C). These minor differences were not observed in wild-type discs (data not shown). We consider it highly unlikely that these minor and contradictory differences can explain the dramatic effect observed on DIAP1 protein levels.

We also examined the phenotype of *dco* null (dco^{le88}) mutant cells induced by mitotic recombination. As reported previously (Zilian et al., 1999), clones of this allele were not obtained when recombination was induced before third larval instar (data not shown). This experiment was repeated in discs where the baculovirus caspase inhibitor *p35* was constantly expressed in the posterior compartment via *engrailed* (*en*)-Gal4. As shown in Figure 3.8A, *dco* mutant clones were not detected in the anterior compartment (lacking *p35*), but were found in the posterior compartment expressing *p35*. Twin spots were visible in both compartments, indicating that mitotic recombination had occurred.

These data indicate that caspase-dependent PCD contributes significantly to the loss of cells in *dco* mutant clones.

Consistent with a role of Dco in promoting cell survival by maintaining DIAP1 levels, we found elevated active Drice and decreased DIAP1 in *dco* clones (Figure 3.8C-H). The activation of Drice is largely cell autonomous, though we occasionally see some additional signal adjacent to the clones (arrows in Figure3.8C-E). We speculate that this might represent dying cells engulfed by surrounding cells or macrophages. It has been reported in fly embryos that phagocytosis of cells containing activated caspases is not blocked by *p35* expression (Mergliano and Minden, 2003). The *dco* null clones have decreased DIAP1 levels regardless of the area in the wing discs (hinge, pouch, notum) where the clones are found, indicating that Dco regulates DIAP1 throughout the wing discs.

When *dco* clones were generated in a Minute/+ background, a strong decrease in DIAP1 levels were observed, but only a small increase in activated Drice was seen, most prominently in the basal area of the clone (Figure 3.9). This suggests that cell competition is involved in promoting PCD in *dco* mutant cells, though not in the primary effect on DIAP1 protein expression.

To examine whether this activation of DIAP1 by *dco* is at the transcriptional level, we looked at the expression of a *lacZ* reporter under the control of the *DIAP1* control

region (*DIAP1-lacZ*) in *dco* clones and found that is was not altered (Figure 3.8I-K). This suggests that Dco regulates DIAP1 by a post-transcriptional mechanism.

dco mutant clones are significantly smaller than their twins

A role for Dco in cell proliferation was suggested by the size of *dco* clones in the presence of the caspase inhibitor *p35*, which were consistently smaller than the twin spots (e.g., Figure 3.8A). When this was quantitated, we found that the *dco* clones were on average 4 times smaller than the twin spots in the posterior compartment (Figure 3.8B). Because the twins and clones originate from the two daughter cells of a single cell that underwent mitotic recombination, they grow for the same period of time in a similar location. Therefore the approximate 1:4 ratio of clone/twin size in *dco* mutants suggests a defect in the rate of growth/proliferation. To examine this further, we performed bromo deoxyuridine incorporation and immunostaining for phospho-Histone 3, which label cells that are undergoing DNA replication or mitosis, respectively. Neither analysis revealed any obvious differences between the clones and surrounding tissues (data not shown). These techniques are probably not sensitive enough to reveal subtle differences in cell proliferation that could contribute to the reduced clone size after several generations of cell division. The cells and nuclei in the clones also appear to be of similar size and shape as those in the surrounding tissue, suggesting that reduced cell size cannot account for the small size of *dco* clones.

The E3 ubiquitin ligase activity of DIAP1 is not required for its regulation by Dco

The C-terminus of DIAP1 contains a RING domain that has intrinsic E3 ubiquitin ligase activity which can promote its own degradation via auto-ubiquitination. Mutations that abolish this activity suppress Hid induced cell death but do not affect or enhance Rpr killing (Lisi et al., 2000; Wilson et al., 2002; Yokokura et al., 2004). This is consistent with the differential suppression of Hid and Rpr by ectopic expression of Dco (Figure 3.1A-F). It is conceivable that Dco regulates DIAP1 level through modulating the auto-ubiquitination process.

To explore the relationship between Dco and DIAP1 E3 ligase activity, we examined whether a point mutation in one of the essential cysteine residues in the RING domain (th^{6B}) disrupted the decrease in DIAP1 protein levels upon RNAi depletion of *dco*. One complication of monitoring DIAP1 levels in the RING domain mutant is the possibility that the mutant DIAP1 proteins are unusually stable compared to wild-type, due to lack of degradation by Hid or perhaps Rpr/Grim, since there are reports that these factors can also effect DIAP1 stability (Hays et al. 2002; Ryoo et al. 2002). However, mitotic clones of *Df(3L)H99* did not contain higher levels of DIAP1 immunostaining in the wing imaginal disc (Figure 3.10A-C). This was true of clones located throughout the wing pouch and notum (data not shown). While much of the tissue surrounding these clones are heterozygous for the *hid, grim and rpr* deficiency, the +/+ twins clones also had similar levels of DIAP1 staining compared to the *H99* clones (data not shown). These data

indicate that under normal conditions, endogenous Hid, Grim and Rpr do not significantly regulate the expression of DIAP1 in the wing.

As previously described, expression of dco^{RNAi} in the *ptc* domain of the wing disc results in a significant decrease in DIAP1 expression (Figure 3.6C) and an increase in Drice activation (Figure 3.6E). In a th^{6B}/+ background, similar results were obtained (Figure 10D-E), suggesting that the effect of Dco on DIAP1 protein levels does not require DIAP1's E3 ligase activity.

dco mutant clones have normal Wg and Dpp signaling

CKIε has been proposed to act both as a positive and negative regulator of Wnt signaling (Price, 2006). A positive role for Dco in Wg signaling has recently been demonstrated in cultured *Drosophila* S2 cells (Cong et al., 2004) and wing imaginal discs (Klein et al., 2006; Zhang et al., 2006). Wg signaling regulates cell proliferation and promotes cell survival in the wing imaginal discs (Giraldez and Cohen, 2003; Johnston and Sanders, 2003). Therefore, the phenotypes we have observed in *dco* mutant clones could be explained by a reduction in Wg signaling. However, we find that two targets of Wg signaling in wing imaginal discs, Senseless (Sen) and Distal-less (Dll) (Parker et al., 2002) are unaffected in cells (expressing *p35*) that are homozygous for the *dco* null mutation (Figure 3.12A-F). We conclude that the cell survival function of Dco in wing imaginal discs is not linked to the Wg pathway and that Dco is dispensable for Wg signaling under the conditions used in our study.

Like Wg, Dpp signaling has also been shown to promote cell proliferation and cell survival in the wing imaginal disc (Martin-Castellanos and Edgar, 2002; Moreno et al., 2002). CKIε has been shown to influence TGF-β signaling in mammalian cell culture, both negatively and positively, depending on the assay (Waddell et al. 2004). However, Dpp signaling appears to be normal in cells lacking *dco*, as judged by expression of the Dpp target Spalt (Figure 3.12G-I).

DISCUSSION

Dco promotes cell survival by activating DIAP1 expression

Dco is required for suppression of PCD in several developing tissues. Hypomorphic *dco* mutants have elevated apoptosis in the wing, leg and haltere discs (Figure 3.5 and data not shown). Reduction of *dco* with either RNAi or with mitotic clones of a null allele also caused activation of the effector caspase Drice in wing discs (Figure3.6E, 3.8D). In the eye disc and early pupal eye, clones of the null *dco* allele did not result in obvious activation of Drice or PCD (data not shown). However, reduction of endogenous *dco* with RNAi did enhance the ability of Hid to promote cell death in the eye (Figure 3.3C, D), indicating that Dco promotes cell survival in this tissue as well. It is possible that more subtle phenotype in the eye is caused by other CKI family members acting redundantly with Dco in this tissue.

In the wing disc, the loss of *dco* resulted in a dramatic reduction in DIAP1 expression (Figure 3.6, 3.8, 3.10). The loss of *DIAP1* in either fly embryos or the wing

imaginal discs leads to massive activation of caspases and cell death (Ryoo et al., 2004; Wang et al., 1999; Yoo et al., 2002). Therefore, the reduction in DIAP1 levels observed in *dco* mutants is sufficient to explain the elevated caspase activation and apoptosis in these cells. The reduction in DIAP1 expression is not a secondary consequence of activating the apoptotic machinery, since a strong reduction in DIAP1 levels was observed in the presence of a caspase inhibitor (Figure 3.8G), or in mutant backgrounds where active caspases are barely detectable (Figure 3.6D, F). These results argue that reduction in *dco* leads to a loss of DIAP1 expression, which results in caspase activation and PCD.

Though our data indicate that Dco activation of DIAP1 is important to suppress apoptosis, the possibility existed that this regulation was due to non-specific effects of mispatterning in *dco* mutants. However, the phenotype of *H99*, dco^{P915}/+, dco^{P1395} mutants argues strongly against this scenario. Heterozygosity of *H99* suppressed the elevated PCD in the wing (Figure 3.5C, F), shortened the developmental lag observed in *dco* hypomorphic mutants and increased the lethal phase of these mutants from early pupation to late pupal stages, with some escapers eclosing. The flies that emerged had normally patterned legs, but the pattern of the wings was difficult to analyze due to lack of unfolding and blistering. However, the veination pattern of the pupal wings (prior to folding) appeared normal (Figure 3.5H). This data, combined with the fact that Dpp and Wg signaling appear normal in *dco* null clones (Figure 3.12) strongly suggests that Dco does not have a significant role in patterning, but rather is a direct repressor of apoptosis.

The role of the IAP antagonists Hid, Grim and Rpr

dco interacts genetically with *hid*, *grim* and *rpr* in the eye (Figure 3.1-4) and the wing (Figure 3.5-6), suggesting a link between Dco and these pro-apoptotic factors. Dco appears to regulate DIAP1 at a post-transcriptional level (Figure 3.8J) and it has been reported that DIAP1 is inhibited by Hid, Grim and Rpr at both the translational level and by increased protein turnover (Hays et al., 2002; Holley et al., 2002; Ryoo et al., 2002; Yoo et al., 2002). This raises the possibility that Dco could activate DIAP1 expression through repression of one or more of these IAP inhibitors. Alternatively, Dco could activate DIAP1 though a pathway that acts in parallel to Hid, Grim and Rpr.

If Dco regulates DIAP expression by repressing IAP inhibitor activity, it cannot work solely through Hid. The elevated caspase activation and PCD observed upon reduction of *dco* gene activity is greatly suppressed in a *hid* mutant background (Figure 3.6F and data not shown). However, DIAP1 levels are still reduced with *dco* RNAi in *hid* mutants (Figure 3.6D). The apoptotic phenotype in *dco* mutant wing discs is suppressed by removal of one copy of *hid*, *grim* and *rpr* (Figure 3.5C,F), but not by removal of one copy of *hid* (data not shown). Therefore Dco would have to act by inhibiting Hid and Grim and/or Rpr. While Dco overexpression most effectively suppresses Hid activity (Figure 3.1B), it also moderately suppresses Grim (Figure 3.1H) and has a small effect on Rpr-induced PCD (Figure 3.1F).

If Dco regulates DIAP1 in parallel to the IAP inhibitors, then how to explain the suppression of caspase activation and PCD in *dco* mutants when *hid* is mutated (Figure 3.6F) or in *Df(3L)H99* heterozygotes (Figure 3.5C, F)? These data can be explained by a balance between negative regulators of apoptosis (Dco) and positive inputs (Hid/Grim/Rpr). Reduction of *dco* lowers DIAP1 levels, promoting caspase activation, however, the IAP inhibitors are known to bind to DIAP1 and sequester it from its normal function of caspase inhibition (Hay, 2000). So reduction of these DIAP1 inhibitors allows the lower levels of DIAP1 in the *dco* mutants to bind to caspases and prevent their activation.

In addition to binding to DIAP1 and inhibiting its ability to repress caspases, Hid, Grim and Rpr have been shown to down regulate DIAP1 expression (Hays et al., 2002; Holley et al., 2002; Ryoo et al., 2002; Yoo et al., 2002). Interestingly, we found that clones lacking *hid*, *grim* and *rpr* had no effect on DIAP1 protein levels in the wing disc (Figure 3.10A-C). These results suggest that under normal conditions, these IAP inhibitors are not major regulators of DIAP1 expression in the wing imaginal disc. A more informative experiment would be to examine DIAP1 expression in clones lacking hid, grim, rpr and dco, to determine whether the decrease in DIAP1 requires the three pro-apoptotic factors. Attempts to perform this experiment have thus far not been possible, due to technical difficulties.

Hid, Grim and Rpr have been suggested to downregulate DIAP1 though its intrinsic E3 ubiquitin ligase activity (Hays et al., 2002; Ryoo et al., 2002; Yoo et al., 2002). However, a mutation that disrupts enzymatic activity (th^{6B}) is still downregulated in dco^{RNAi} depleted cells (Figure 3.10D-F). This argues against DIAP1 auto-ubiquitination playing an important role in Dco regulation of DIAP1. This experiment could only be performed in a th^{6B} heterozygous background, because th^{6B} clones do not survive/grow.

Dco may promote cell growth/proliferation

In addition to its role in promoting cell survival, our clonal analysis indicates that *dco* may promote cell growth/proliferation. In the presence of *p35* caspase inhibitor, *dco* mutant clones are about four times smaller than their twins (Figure 3.8B). One explanation for this is that *p35* does not completely suppress the elevated apoptosis in the *dco* clone. However, the UAS-*p35* transgene used is able to fully suppress a strong GMR-*hid* phenotype in the eye, and no TUNEL positive cells are observed in our *dco* clones in the presence of *p35* (data not shown).

The conclusion that *dco* mutant cells grow/divide slower than controls is tempered by several caveats. Recent findings show that apoptotic cells in the wing disc can stimulate their neighbors to proliferate (de la Cova et al., 2004; Huh et al., 2004; Moreno and Basler, 2004; Ryoo et al., 2004). This phenomenon has been observed in cells expressing *p35*, suggesting that caspase activation but not PCD itself generates the proliferative signal (Huh et al., 2004; Ryoo et al., 2004; Perez-Garijo et al. 2005).

Induced expression of Wg in the dying cells has been proposed to be part of the mitogenic signal for this compensatory proliferation (Ryoo et al., 2004). While we observed substantial ectopic Wg staining in *dco* mutant wing discs (Figure 3.11), ectopic Wg was not observed in the *p35* expressing *dco* mutant clones (n=30, data not shown). Despite this, our current data cannot rule out that the low clone/twin ratio we observe for *dco* clones in Figure 3.8B could at least be partially due to increased proliferation of the twins.

Relation of Dco to Wg and other pathways affecting PCD/proliferation

In our study, no defect in Wg signaling was observed in mitotic clones for a *dco* null allele in wing imaginal discs (Figure 3.12A-F). A similar result has been recently reported, though a hypomorphic *dco* allele was used (Strutt et al., 2006). In contrast, another recent study generated large clones with a strong *dco* allele ($dco^{dbt\text{-}P}$) in a *Minute*/+ background, and reported no activation of caspases and a loss of Sens and adult wing margin, two readouts of Wg signaling (Klein et al., 2006). We have made dco^{le88} clones with the Minute technique, and find that they are infrequent and similar in size to the ones reported in Figure 3.8. The few at the dorsal/ventral boundary expressed Sens normally (data not shown), while clones throughout the wing pouch had a dramatic reduction in DIAP1 expression and only a small increase in activated Drice (Figure 3.9). Another report generating very large dco^{le88} clones using *MS1096*-Gal4/UAS-*FLP*, Minute and *p35* expression (Zhang et al. 2006) observed a strong decrease in Sens expression. This report also found evidence that other CKI family members could promote Wg signaling (Zhang et

al., 2006). Clone size and redundancy/compensation may explain the differences between our results and those of Klein et al. (2006) and Zhang et al. (2006). However, the primary phenotype observed when *dco* is removed under the conditions used in this study is a decrease in DIAP1 levels.

In our misexpression studies in the eye, Dco was able to suppress the ability of a Ras/MAPK resistant version of Hid (Bergmann et al., 1998) to induce apoptosis (Figure 3.2). At least in the eye, Dco appears to control cell survival independently from Ras/MAPK signaling.

The phenotype of *dco* is similar to that of *yorkie*, a nuclear protein that is required for DIAP1 expression and proliferation (Huang et al., 2005). Yorkie is inhibited by a complex containing the Lats and Hippo kinases, and the adaptor protein Salvador (Sav). Clones mutant for *lats*, *sav* or *hippo* display an opposite phenotype to *dco*, i.e., dramatic increases in proliferation and a block in PCD (see Edgar, 2006 for a review). Double mutant clones for *sav* and *dco* do not survive/grow (data not shown), suggesting that Dco may act downstream of the Wts/Sav/Hippo complex. However, the expression of *DIAP1*-lacZ is upregulated in *hippo* clones (Wu et al., 2003) and decreased in *yorkie* clones (Huang et al., 2005) but we observe no effect on this transgene in *dco* clones (Figure 3.8I-K). In addition, Wts/Sav/Hippo represses Cyclin E expression, but Dco does not (data not shown). Therefore, we consider it unlikely that Dco acts with Wts/Sav/Hippo in a simple linear pathway. Hippo has also been reported to phosphorylate DIAP1, leading

to instability (Harvey et al., 2003; Pantalacci et al., 2003), so the possibility remains that Dco may interact with Hippo in this regard.

CKIδ/ε in other systems

Our data supports a model where Dco promotes cell survival in fly imaginal discs by maintaining expression of DIAP1 through a novel pathway. Do casein kinase Iδ/ε family members have similar functions in other organisms? The yeast CKI gene Hrr25 is involved in DNA repair, cell growth and viability (Hoekstra et al., 1991). In mammalian cell lines, CKIε plays a protective role against apoptosis induced by extrinsic death signals (Desagher et al., 2001; Izeradjene et al. 2004). This cell survival activity was correlated with CKIε phosphorylation of the pro-apoptotic factor Bid (Desagher et al., 2001). Inhibition of CKIδ induces apoptosis in trophoblast cells (Stoter et al. 2005). Further studies will be required to determine whether there are commonalities between these different systems. The ability of Dco to inhibit apoptosis and possibly promote cell proliferation makes it an attractive candidate for functioning as an oncogene in tumorigenesis.

ACKNOWLEDGEMENTS

We would like to thank all the researchers who provided the reagents described in the Methods section, especially M. Noll for the *dco* stocks, J. Abrams for the GMR-*hid*,

GMR-*rpr* and GMR-*grim* stocks, K. White for the *hid* alleles, H. Steller, K. White and B. Hay for *th* alleles. Special thanks to D. Strutt and M. Mlodzik for providing UAS-dco mutant stocks shortly after publication. Thanks also to J. Kennell for generating one of the UAS-dcoRNAi stocks used in this study. We also would like to thank B. Hay for DIAP1 and active Drice antibodies, P. Meier for DIAP1 antibody, L. Saez and M. Young for the Dco antibody and the UAS-*dco* stock, and H. Richardson for the CycE antibodies. This work was supported by NIH grants RO1 GM59846 and RO1 CA95869 to K.M.C.

This chapter was published as:

Guan, J., **H. Li**, A. Rogulja, J. D. Axelrod and K. M. Cadigan (2007). "The *Drosophila* casein kinase I epsilon/delta Discs overgrown promotes cell survival via activation of DIAP1 expression." *Developmental Biology* **303**(1): 16-28.

In this project, I generated the data as below:

Figure 3.3;
Figure 3.4;
Figure3.6 G-I;
Figure 3.7;
Figure 3.9,
Figure 3.10

FIGURES

Figure 3.1

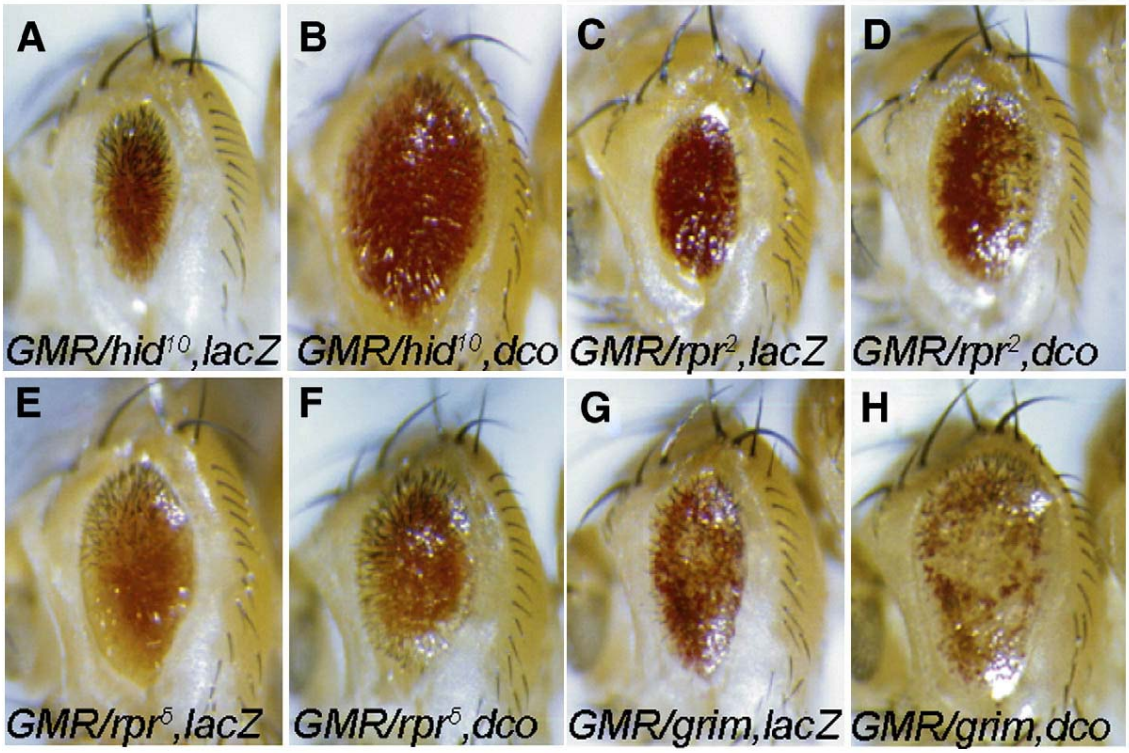

Figure 3.1. Ectopic expression of *dco* suppresses the small eye phenotype induced by overexpression of the proapoptotic genes *hid*, *rpr* and *grim*. Micrographs are of adult female eyes. All flies shown contain *GMR*-Gal4 and either UAS-*lacZ* (A, C, E, J) or UAS-*dco* (B, D, F, H). They contain two copies of *GMR-hid*^{1M} (A, B), one copy of *GMR-rpr*^{2} (C, D), *GMR-rpr*^{5} (E, F) or a copy each of *GMR-grim*^{2} and *GMR-grim*^{3} (G, H). Overexpression of *dco* strongly suppresses *GMR-hid*^{1M} and other *GMR-hid* strains (data not shown), while suppression of *GMR-rpr* and *GMR-grim* is weaker and variable.

Figure 3.2

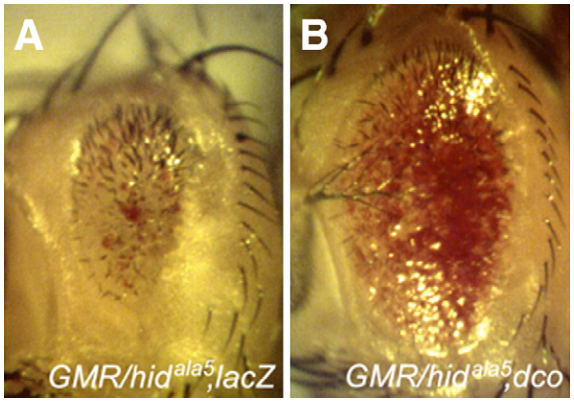

Figure 3.2. Ectopic expression of *dco* suppresses the small eye phenotype induced by a Ras/MAPK resistant form of Hid. Micrographs are of adult female eyes. Flies contain *GMR*-Gal4, *GMR-hid*^{ala5} and either UAS-*lacZ* (A) or UAS-*dco* (B). Overexpression of *dco* strongly suppresses the *GMR-hid*^{ala5} phenotype.

Figure 3.3

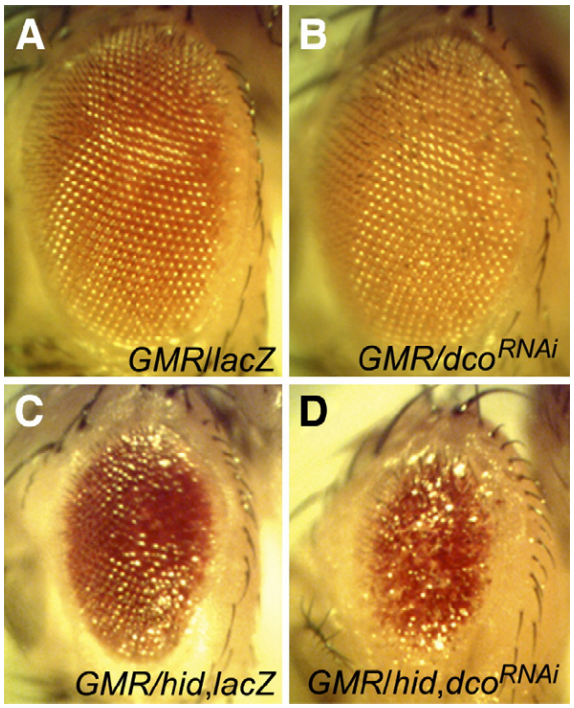

Figure 3.3. RNAi inhibition of *dco* enhances Hid-mediated apoptosis in the eye. Adult eyes from *GMR*-Gal4/UAS-*lacZ* (A, C), *GMR*-Gal4/UAS-dco^{RNAi} (B, D) without (A, B) or with one copy of *GMR-*hid^{1M} (C, D). Depletion of *dco* roughens the eye but does not cause a reduction in size in an otherwise wild-type background (B), but does significantly enhance the *GMR-*hid^{1M} phenotype (compare C and D).

Figure 3.4

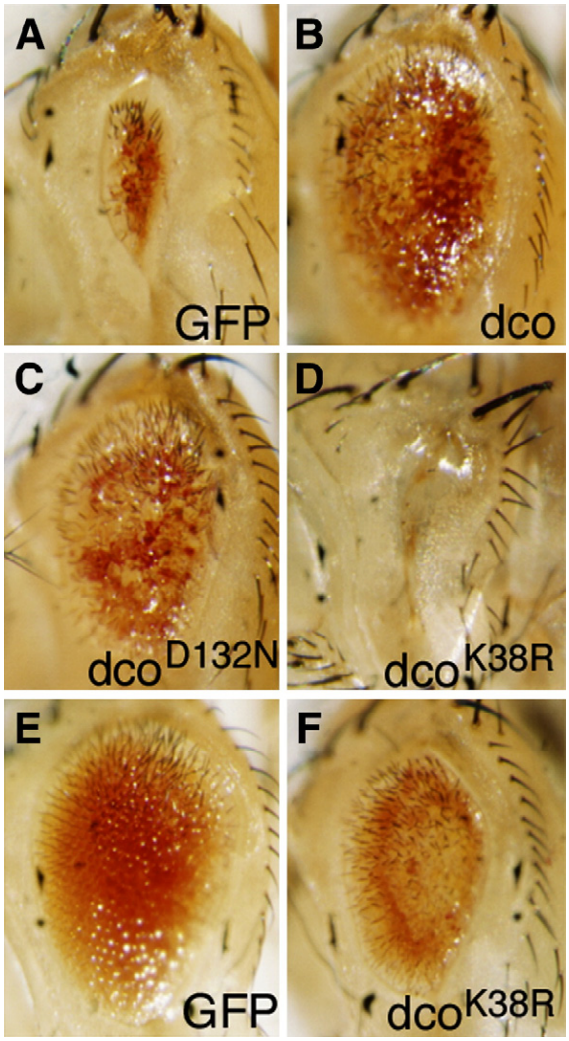

Figure 3.4. Kinase activity is required for *dco* to suppress Hid-mediated apoptosis in the eye. Adult eyes from *GMR*-Gal4, *GMR-hid*^{ala5} and either UAS-*GFP* (A), UAS-*dco* (B), UAS-*dco*^{D132N} (C) or UAS-*dco*^{K38R} (D). While the ATP-binding mutant *dco*^{D132N} suppresses Hid activity as well as wild-type *dco*, the kinase dead mutant *K38R* enhances the small eye phenotype. (E, F) Enhancement was also observed with a weaker *GMR-hid* transgene. *GMR*-Gal4, *GMR-hid*^{1M} and either UAS-*GFP* (E) or UAS-*dco*^{K38R} (F). Wild type *dco* and *dco*^{D132N} strongly suppressed *GMR-hid*^{1M} (data not shown).

Figure 3.5

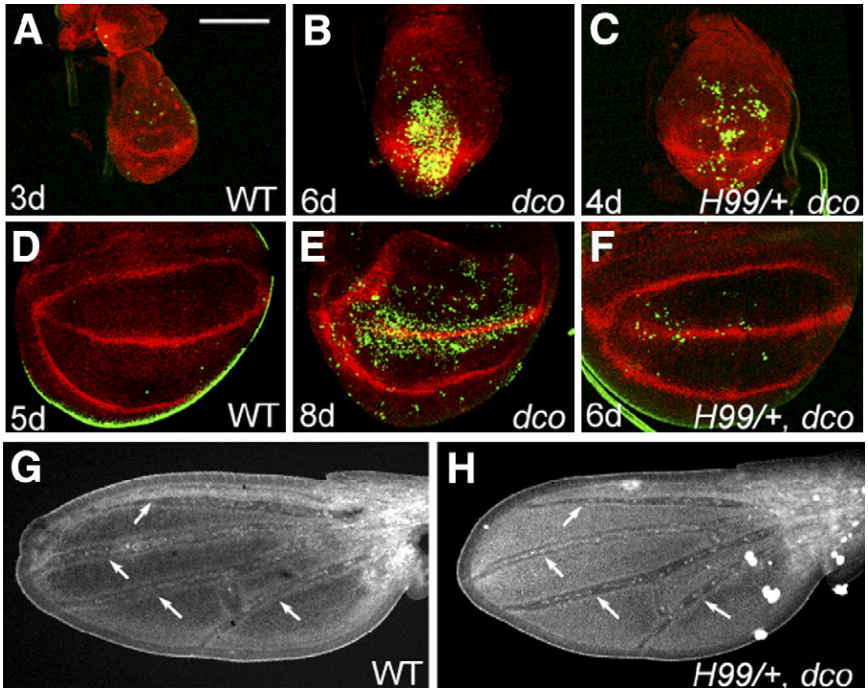

Figure 3.5. *dco* mutant wing discs have elevated cell death and growth delay that is largely suppressed by removing one copy of *hid*, *rpr* and *grim*. Confocal images of wing discs stained for TUNEL (green) to label apoptotic cells and Wg (red, apical optical section) to identify the developmental age of each disc. The discs in panels A-C are early third instar and those in panels D-F are late third instar. (A, D) Wild type discs 3 and 5 days after egg laying. (B, E) *dco* mutant discs (dco^{P915}/dco^{P1396}), 6 and 8 days after egg laying. (C, F) *Df(3L)H99*/+, *dco* (dco^{P915}/dco^{P1396}) discs 4 and 6 days after egg laying. *H99* is a deficiency that removes a chromosomal region including *hid*, *rpr* and *grim*. The green signals at the edge of the wing discs in D and F are non-specific staining of nuclei from the peripodal membrane. (G, H) Wild type (G) and *Df(3L)H99*/+, *dco* (H) pupal wings stained with phalloidin. The wings are 33 hr and 38 hr after the white prepupal stage, respectively. The white arrows denote the position of the longitudinal veins. The overall pattern of the *H99*/+; *dco* wings is identical to wild-type. Scale bar: 100μm.

Figure 3.6

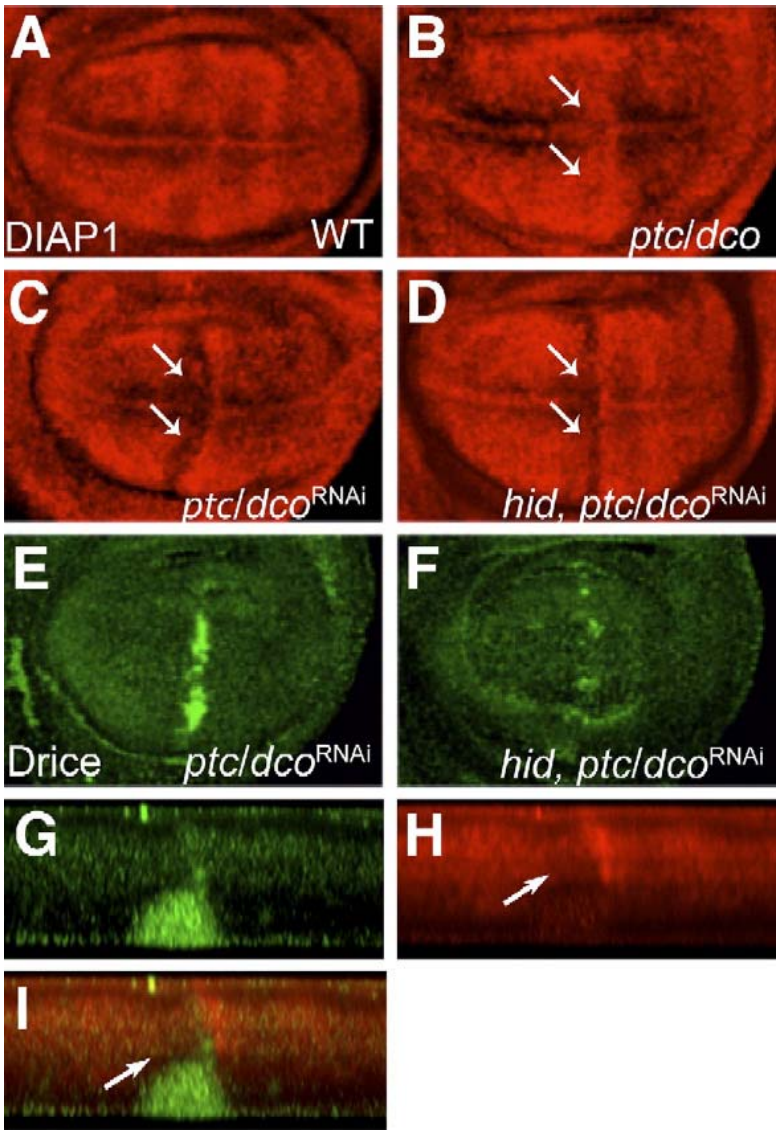

Figure 3.6. Dco regulates DIAP1 protein levels in the wing imaginal disc. Confocal images of late third instar larval wing discs stained for DIAP1 in red (A-D,H,I) and activated Drice in green (E-G,I). *ptc*Gal4 is expressed at an anterior strip next to the anterior-posterior boundary (indicated by arrows in B-D). All discs are oriented anterior to the left. (A) Wild type. (B) *ptc*-Gal4/UAS-*dco*. (C, E) *ptc*-Gal4/UAS-dco^{RNAi}. (D, F) *ptc*-Gal4/UAS-dco^{RNAi}; hid^{05014}/hid^{X14}. (G-I) Z axis projection of *ptc*-Gal4/UAS-dco^{RNAi} disc showing basal localization of Drice positive cells. Arrows indicate the reduction in DIAP1 staining in the *ptc* expression domain.

Figure 3.7

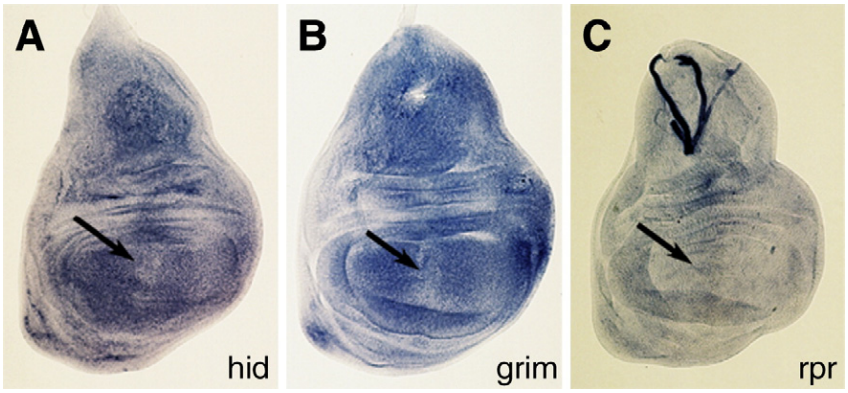

Figure 3.7. Inhibition of *dco* has little effect on *hid*, *grim* or *rpr* transcript levels. *ptc*-Gal4/UAS-dco^{RNAi} discs stained for hid (A), grim (B) and rpr (C) transcripts. The location of the ptc expression domain is indicated by the arrows. There is no consistent change in *hid* expression by dco^{RNAi}, but a small decrease in *grim* was sometimes observed, as was a small increase in *rpr* expression.

Figure 3.8

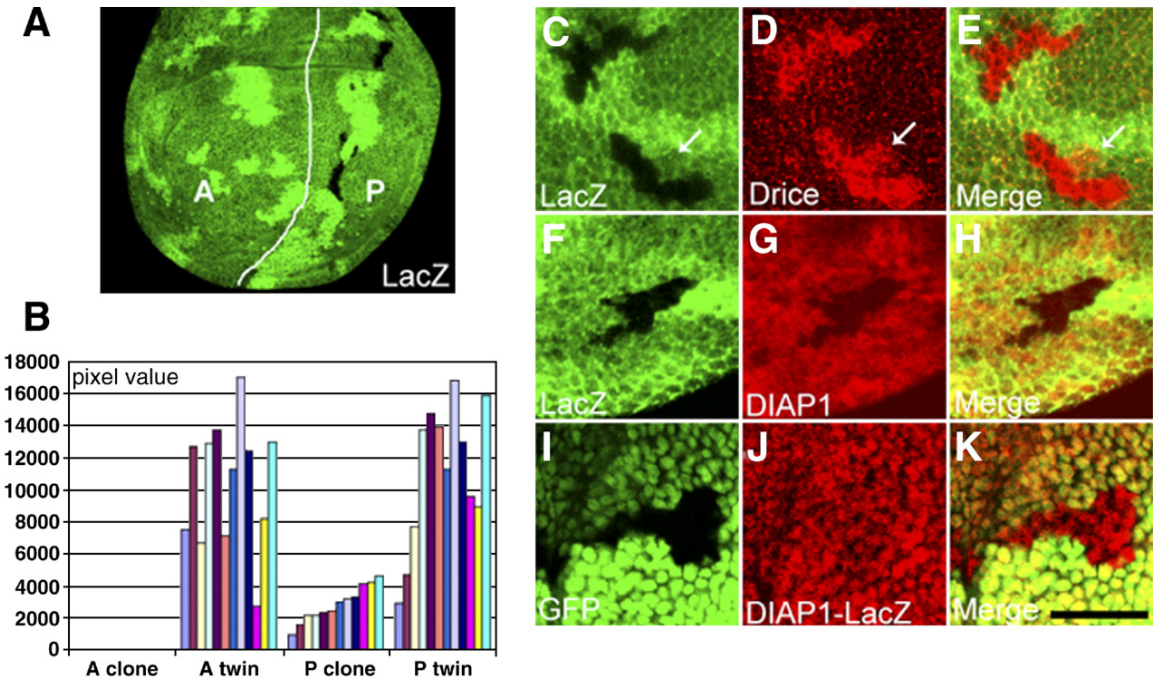

Figure 3.8. *dco* mutant clones are rescued by inhibition of caspases and exhibit increased Drice activation and decreased DIAP1 protein, but not DIAP-lacZ. *dco* null (*dco*^{le88}) mutant clones were generated in an *en*-Gal4/UAS-*p35* background and are marked by the absence of lacZ staining (green; A,C-H) or GFP (green; I-K) as indicated. (A) *dco* clones are found in the posterior (P) compartment where *p35* is expressed by *en*-Gal4, but the anterior (A) clones are not observed. The white line marks the AP boundary. The presence of the twins at the anterior indicates that mitotic recombination had been induced. (B) Quantification of the *dco* clone/twin area. The pixel value of each bar represents the total area of clones or twins in A or P in one disc, and 12 discs containing a total of more than 30 individual clones were analyzed. The difference between the areas of A twins (average: 10451 pixels/disc) and P twins (average: 11138 pixels/disc) is not statistically significant ($p>0.20$ by the Mann-Whitney test). The average size of the P clones (2855 pixels/disc) is 25.6% of the size of the P twins. (C-E) Active Drice (red) is elevated in *dco* clones. Occasionally active Drice is observed adjacent to the clones (arrows). (F-H) DIAP1 protein (red) is decreased in *dco* clones. (I-K) *dco* clones marked by the absence of GFP were generated in a DIAP-lacZ background (*th*^{i5c8}). DIAP-LacZ (red) is not altered in the clones. Scale bar: 20μm.

Figure 3.9

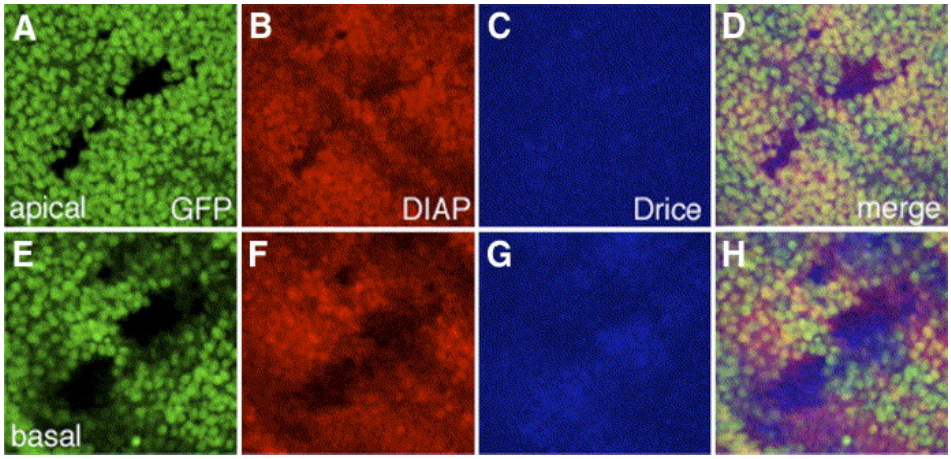

Figure 3.9 Mitotic clones of *dco* in a *Minute*/+ background result in loss of DIAP1, but only modest Drice activation. A apical (A-D) and basal (E-H) optical slice through a clone of dco^{le88} stained for DIAP1 (B,F) and active Drice (C,G). Clonal boundaries were marked with GFP (A,D). A strong reduction in DIAP1 is observed in the clones, while only a modest elevation of active Drice was observed in the basal portion of the clones. Note that there is appreciable Drice activation outside of the clones in the basal portion of the disc.

Figure 3.10

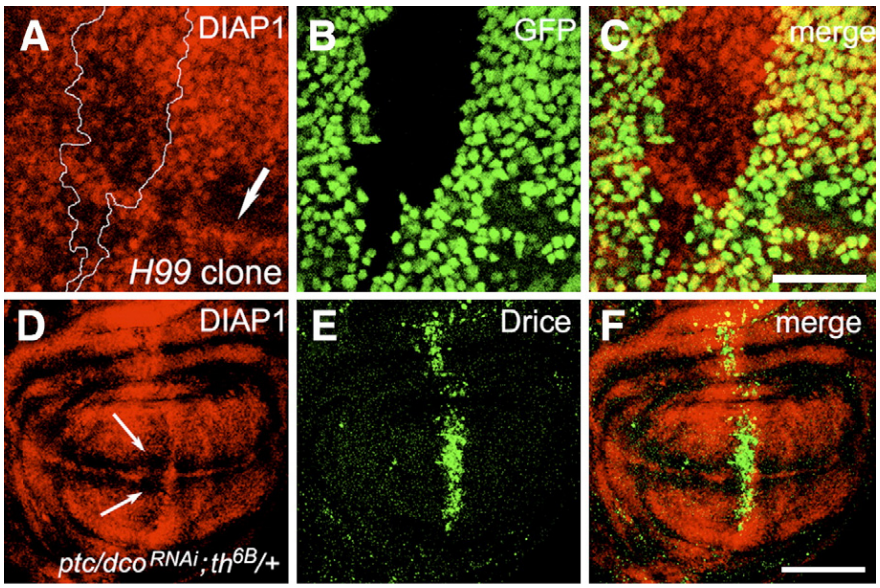

Figure 3.10. Hid, Grim and Rpr do not regulate DIAP1 expression in the wing disc and DIAP1 E3 ligase activity is not required for Dco regulation of DIAP1. (A-C) Confocal images of late third wing imaginal discs containing clones of *Df(3L)H99* (A-C), stained for DIAP1 (red). Clones were marked by GFP (green) and the location of the D/V boundary is indicated by the white arrow. The expression pattern of DIAP1 is unaffected in the clones. (D-F) Confocal images of wing imaginal discs stained for DIAP1 (red) and activated Drice (green) from *ptc*-Gal4/UAS-dco^{RNAi} ; th^{6B}/+ animals. As in *ptc*-Gal4/UAS-dco^{RNAi} controls (see Figure 3.4), a strong decrease in DIAP1 levels and increase in activated Drice is observed in the *ptc* stripe. The scale bars are 20 μM in C and 100μM in F.

Figure 3.11

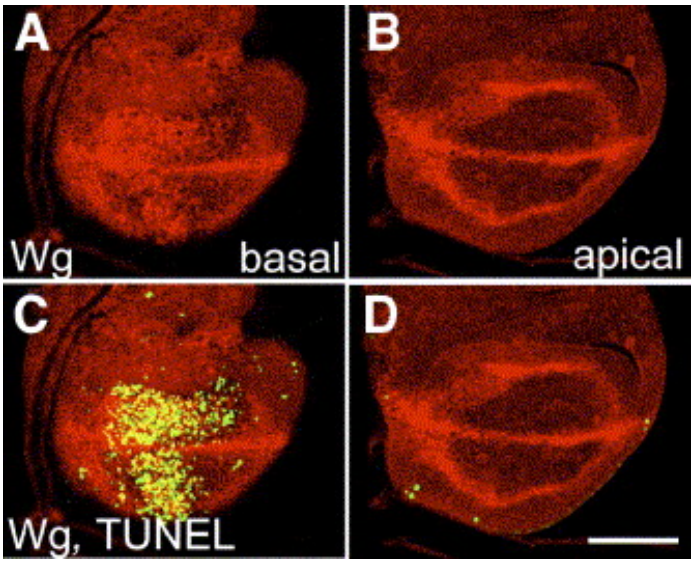

Figure 3.11 The dying cells in *dco* mutant wing discs are basally extruded and express Wg. TUNEL (green) labels dying cells and Wg is stained in red. All four panels are confocal images of the same wing disc from a dco^{P915}/dco^{P1396} larva. (A, C) Basal focal plane. (B, D) Apical focal plane. Wg retains a relatively normal pattern in the apical view but is ectopically expressed in the dying cells at the basal epithelium. Scale bar: 100μm.

Figure 3.12

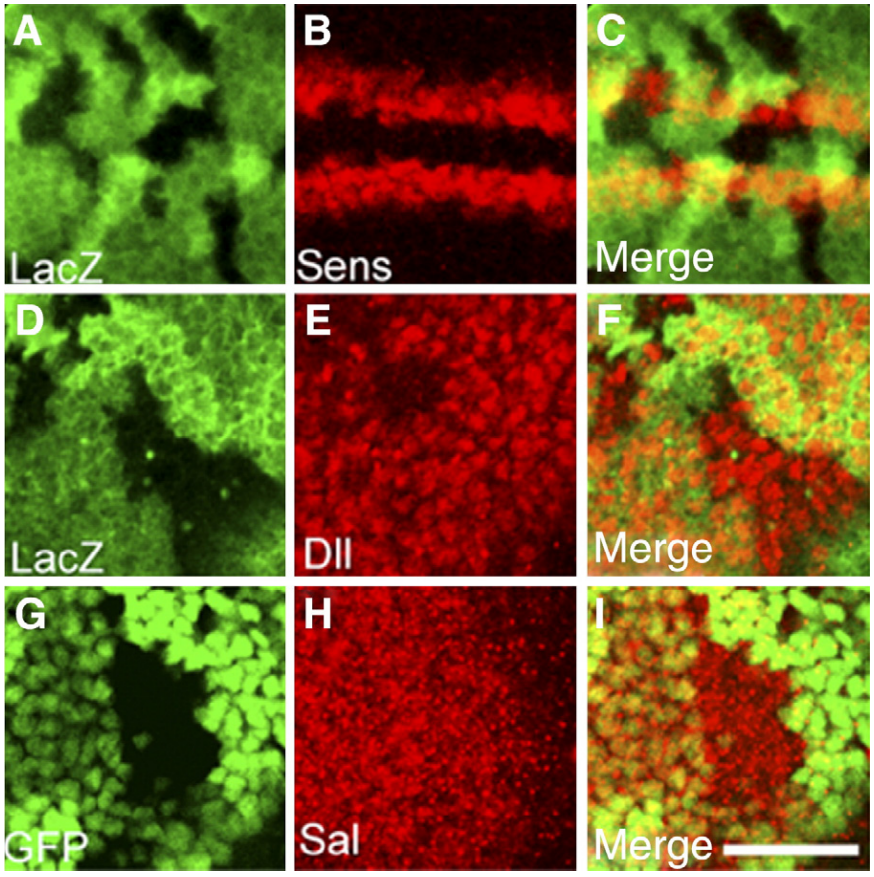

Figure 3.12. The *dco* mutant cells have unaffected Wg and Dpp signaling. Confocal images of late third instar wing imaginal discs containing dco^{le88} null clones, generated as described in Figure 3.5. Clones are marked by the lack of lacZ staining (green) and the Wg targets Sens and Dll and Dpp target Spalt are stained in red. Sens (A-C) and Dll (D-F) and Spalt (G-I) expression are normal in *dco* clones.

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CHAPTER IV

EXPLORATION OF THE REGULATION MECHANISM OF APOPTOSIS AND THE WINGLESS PATHWAY BY DROSOPHILA CASEIN KINASE I FAMILY PROTEINS

ABSTRACT

The Casein Kinase I (CKI) family of proteins has been implicated in a variety of cellular processes, including apoptosis and the Wingless (Wg) signaling pathway. The *Drosophila* CKIε/δ homologue, known as Discs Overgrown (Dco), has been demonstrated to regulate apoptosis via activation of the endogenous caspase inhibitor DIAP1 *in vivo*, yet the molecular mechanisms remain to be elucidated. In this report, we explored the molecular mechanism of Dco in cultured cells. We conducted two small scale screens of *Drosophila* CKI members in the adult fly eye and cultured cells. The screens and further characterization revealed a novel function of Gilgamesh (Gish), the fly CKIγ homologue, in DIAP1 regulation. Our results suggest Gish and Dco might be functionally redundant. In order to address the controversy around the role of Dco in Wg signaling, we extended our study into the Wg pathway. Our results strongly support Dco acting as a positive regulator of the pathway. We also initiated a chemical genetic screen to identify novel substrates of Dco.

INTRODUCTION

Introduction to Casein Kinase I family

The Casein Kinase I protein family (CKI) is a large family of monomeric serine/threonine protein kinases which contain a highly conserved central kinase domain (53–98% identical), flanked by diverse amino- and carboxyl-termini (Vielhaber and Virshup 2001). CKI members have been implicated in a diversity of cellular progresses, including the Wnt/Wingless (Wg) signaling pathway, apoptosis, circadian rhythm, cell division and membrane transport (reviewed in Vielhaber and Virshup 2001; Knippschild et al. 2005).

Family members of CKI are found in eukaryotic organisms, from yeast to mammals. So far, seven mammalian CKI isoforms (α, β, γ1, γ2, γ3, δ and ε) have been characterized. The examinations of substrate specificity revealed a substrate recognition motif S/T(P)-X_{1-2}-S/T or D_n-X_{1-2}-S/T, where S/T(P) indicates phosphor-serine or threonine, X is any amino acid, and D is aspartic acid (Vielhaber and Virshup 2001). As this motif is relatively common, the substrate selection is thought to be regulated via subcellular localization and docking sites in substrates (Price 2006).

In the fruitfly *Drosophila melanogaster*, eight family members have been predicted based on sequence homology (Figure 4.1A). Among them, CKIα (Santos et al. 1996), the *Drosophila* CKIγ Gilgamesh (Gish) (Hummel et al. 2002), and the *Drosophila* CKIε/δ Discs overgrown (Dco) (Kloss et al. 1998), have been characterized in multiple cellular

processes (Figure 4.1B) (Vielhaber and Virshup 2001; Knippschild et al. 2005). Dco is also known as double-time (DBT), and as the name implies, it plays an important role in circadian rhythm (Knippschild et al. 2005). Interestingly, all of the three isoforms have been implicated in the Wg pathway (discussed in details below) (Price 2006). Recently, we reported that Dco regulates apoptosis (Guan et al. 2007)(Chapter III of this dissertation).

Apoptosis and CKI

Apoptosis is a highly conserved cell death process that is essential for development and adult homeostasis. The main executioners of apoptosis are called caspases, whose proteolytic activity results in cleavage of numerous cellular substrates, leading to cell death. Synthesized as zymogens, caspases are subject to tight regulation at the activation level by several pathways, including the intrinsic mitochondria pathway and extrinsic death receptor pathway (Hengartner 2000; Salvesen and Abrams 2004). There is another level of caspase regulation, carried out by the Inhibitor of Apoptosis Proteins (IAP), which bind to caspases to prevent their activation and/or enzymatic activity (Srinivasula and Ashwell 2008). IAPs are in turn inhibited by a class of proteins termed RHG proteins, e.g. Hid in *Drosophila*, and Smac in mammals. Expression of RHG proteins can rapidly induce apoptosis by antagonizing *Drosophila* IAP1(DIAP1) in the fly (reviewed in Salvesen and Abrams 2004; Hay and Guo 2006; Steller 2008).

Data from our laboratory demonstrated that Dco promotes cell survival via the activation of DIAP1 during larval stages (Guan et al. 2007). Expression of Dco blocks Hid-dependent apoptosis in the developing eye, whereas loss of Dco leads to a dramatic reduction in expression of DIAP1 and activation of caspases (Guan et al. 2007). Since the observation cannot be explained by Dco's activity in other pathways, we believe that we have uncovered a novel mechanism of DIAP1 regulation.

Several questions remained unanswered about the underlying mechanism by which Dco regulates DIAP1 activation. First, what is the substrate of Dco in apoptosis regulation? As discussed in Chapter III, Dco probably regulates DIAP1 post-transcriptionally in a kinase activity-dependent manner (Guan et al. 2007). The simplest scenario would be that Dco phosphorylates and thereby stabilizes DIAP1. In fact, several putative CKI substrate recognition motifs are found in DIAP1, and DIAP1 protein levels have been reported to be regulated via phosphorylation (Kuranaga et al. 2006). However, our attempts to show DIAP1 being the substrate of Dco was negative. In order to identify the substrate of Dco in apoptosis regulation, we initiated a chemical genetic screen.

Dco has been shown to regulate DIAP1 protein levels in wing imaginal discs, however the same effect has not been observed in eye discs (Guan et al. 2007). One hypothesis is that the loss of Dco in eye discs is compensated by a redundant partner, presumably another CKI. Therefore, efforts have been made to identify CKI family

members that contribute to apoptosis regulation, and Gish appeared as a promising candidate, as discussed in this chapter.

Wingless signaling and CKI

The *Drosophila* Wingless (Wg) protein belongs to the Wnt family of secreted glycoproteins that are important for a wide range of developmental events throughout the animal kingdom (Cadigan and Nusse 1997; Cadigan 2008). A major Wg downstream player is cytoplasmic Armadillo (Arm), the homologue of mammalian β-catenin. In the absence of Wg signaling (Figure 4.2A), Arm is constitutively expressed, but the cytoplasmic pool of Arm is continuously phosphorylated and subsequently degraded by a degradation complex, which contains two kinases GSK3β and CKIα, scaffold proteins Axin and APC, and protein phosphatase 2A (PP2A). Upon Wg stimuli (Figure 4.2B), the signal crosses the plasma membrane through the Frizzled receptors Frizzleds (Fz&Fz2) and the members of the low-density-lipoprotein receptor-related family protein Arrow (in mammals LRP5 and LRP6) to inactivate the degradation complex. Disheveled (Dsh) mediates the signal transduction from receptors to the degradation complex via mechanisms that are not well understood (Cadigan and Liu 2006). The stabilized Arm then translocates into the nucleus, where it interacts with the transcription factors TCF, and converts TCF from a transcriptional repressor into an activator, leading to activation of Wg target genes (Cadigan 2008).

The *Drosophila* CKI members, CKIα, Gish and Dco have been implicated in the Wg pathway regulation in the past decade. Three key phosphorylation events have been described. Phosphorylation of Arm by CKIα, which primes the consequent phosphorylation by GSK3β, leads to Arm degradation (Amit et al. 2002; Liu et al. 2002). In the presence of Wg signaling, Gish has been recently demonstrated to phosphorylate Arrow, which contributes to the recruitment of Axin to membrane, leading to the inactivation of the degradation complex (Davidson et al. 2005; Zeng et al. 2005). In addition, there are several reports suggest that the phosphorylation of Dsh by Dco is an important event to activate the pathway; however, the mechanisms still remain a mystery (reviewed in Wharton 2003; Price 2006). The three events have been shown conserved from flies to mammals.

Several other functions of CKIε have also been suggested, which leads to the controversy about the molecular mechanism of CKIε in regulating the Wnt/Wg pathway (reviewed in Vielhaber and Virshup 2001; Knippschild et al. 2005; Price 2006). Briefly, both positive and negative roles have been proposed, and some genetic data suggests that Dco/CKIε is not required for Wg/Wnt signaling in some tissues. Making the situation more complex, several components throughout the Wnt/Wg pathway, i.e. Disheveled, APC, Axin, Arm/β-catenin, PP2A and TCF have been suggested to be modified by Dco/CKI ε (Figure 4.2). To clarify this puzzle in a physiological context, we are exploring the role of Dco in the *Drosophila* embryonic cell line Kc167 (Kc) in this chapter.

MATERIALS AND METHODS

Cell Culture, RNAi treatment, DNA transfection, and conditional media treatment

Drosophila embryonic cell lines were cultured at room temperature in Schneider's *Drosophila* media (Invitrogen) containing Penicillin/ Streptomycin and 10% FBS for S2 cells and 5% FBS for Kc cells.

RNAi-mediated gene knockdowns were performed as described (Fang et al. 2006; Liu et al. 2008). Double-strand RNAs with a typical length of 400–700 bp were synthesized using the MEGAscript T7 *in vitro* transcription kit (Ambion). The sequences of the PCR primers for the dsRNA synthesis are as follows:

dco 3' UTR 5'TAATACGACTCACTATAGGGCGTTGAATGTATCCAAGCGGCAGG,

3'TAATACGACTCACTATAGGGTAGGTTACAATGTGGGTGCCTTGC;

gish –5'TAATACGACTCACTATAGGGCTAGACGGTGTACAATGTAGTCCA,

3' TAATACGACTCACTATAGGGCGGTGTGTTTGTAGTTGGT;

CKIa –5' TAATACGACTCACTATAGGGCGTGGATGCGTGGATGTAAA,

3' TAATACGACTCACTATAGGGGATGAGAGTGGGTGAGTGTGT;

CG7094-1–5' TAATACGACTCACTATAGGGCCACTCCAACATGAAGTAGT,

3' TAATACGACTCACTATAGGGGCAGGGAACAGATTCTGGAA;

CG7094-2–5' TAATACGACTCACTATAGGGCGTGATGCTTAGTCCGAGATA,

3' TAATACGACTCACTATAGGGGGCAGTAAAGTCACAA;

CG2577–5' TAATACGACTCACTATAGGGCGATAAGCCCAACTACGACT ,

3’ TAATACGACTCACTATAGGGGTTGTGAGGGGAGTAATTCT;

CG12147-1–5’ TAATACGACTCACTATAGGGGTGGCAACAATAGATCGCCA ,

3’ TAATACGACTCACTATAGGGGGTCCATCACCATGACATT;

CG12147-2–5’ TAATACGACTCACTATAGGGCAGATGCCCTGTCTTTGA ,

3’ TAATACGACTCACTATAGGGCCAAGTTTCCACGGTATCGA;

CG9962-1–5’ TAATACGACTCACTATAGGGGAACGACTACGAACTGGA,

3’ TAATACGACTCACTATAGGGCAGTAACTCCATGACCAGCA;

CG9962-2–5’ TAATACGACTCACTATAGGGGAACAGGCATGTGCCACAA ,

3’ TAATACGACTCACTATAGGGCAGCAAACTCAAGAAGGTGCA;

CG11533-2–5’ TAATACGACTCACTATAGGGGTGCATCTAAGCCACTTGAGT,

3’ TAATACGACTCACTATAGGGGCACTGTTCTGGAGCCTATA.

Transient transfections were performed with Fugene 6 (Roche Applied Science) according to the manufacturer’s instruction. *pMT-Hid-flag, pAc-GFP and pAc-DIAP1-flag* were from P. Meier lab. *pAc-Gish-flag* was from C. Niehrs lab. *pAc-Dco-flag* was cloned from Dco cDNA. *pAc-Arm** was previously described (Fang et al. 2006). Mutant forms of Dco, including DcoK38R, M82G, M82A, M82V and M82C were generated using QuikChange® Site-Directed Mutagenesis Kit (Strategene, Catalog #200518) using *pAc-Dco-flag* as the template. Hid is under the control of a copper inducible metallothionein promoter. The expression of Hid was induced 24hr after transfection by the application of 0.7mM $CuSO_4$ at the final concentration. When both

transfection and RNAi were performed, cells were typically treated with RNAis for 4 days, diluted to 10^6 cells/ml and transfected with plasmids.

Wg-CM and control medium were prepared as described (Fang et al. 2006; Liu et al. 2008). 10^6 Kc cells were treated with 400 μl of Wg-CM for 5 hr prior to harvesting.

Cell survival assay and flow cytometry

A cell survival assay was employed to quantitatively describe cell death intensity (Goyal et al. 2000). GFP proteins were co-expressed with other proteins to label transfected cells. The transfection efficiency is around 10-15% for S2 cells and 15-20% for Kc cells. The percentage of GFP positive cells over total cell numbers was utilized to reflect cell survival rate. Cell death is induced by ectopic expression of Hid as described above. The cell numbers were counted either manually or by flow cytometry.

Flow cytometry was performed 60hr after the induction of Hid expression. Cells were spinned down at 1500rpm for 2min, and resuspended in 0.5ml 0.4% formaldehyde for 30min at room temperature. Then cells were spinned down and wash with sterile PBS twice, and stayed at 4 degree for 12hr before be filtered into the Falcon tubes for analysis.

Real-time quantitative RT-PCR (qRT-PCR)

qRT-PCR was performed as previously described (Fang et al. 2006). Briefly, Trizol (Invitrogen) was used to extract total RNA from 1-5×10^6 cells. Reverse transcription was performed using Stratascript reverse transcriptase (Stratagene) followed

by qPCR analysis using an iCycler iQ real-time PCR detection system. β-tubulin was used to normalize transcript levels. qPCR primers were designed by using the online program Primer3. Primers for *nkd* and *CG6234* were described previously (Fang et al. 2006). Primers for *dco* to detect *dco* RNAi knockdown efficiency was designed across the 3' boundary of *dco*UTR RNAi targeted region, i.e. 5' primer (GCATATGCCACACAGCAAGG) is upstream of the boundary, while 3' (TGCACGTGGTAATCGCAATC) is downstream.

Fly Genetics

The following fly strains were used. Oregon R was our wild type stock. The *GMR-hid*^{lM} and *GMR-hid*^{10} stocks were from J. Abrams. *GMR-hid*^{ala5} was from A. Bergmann. UAS-*lacZ*, UAS-*p35*, *dpp*Gal4 and *ptc*Gal4 and *th*^{j5c8} were from the Bloomington Stock Center. UAS*gish*, UAS*CG2577*, UAS*CG7094*, UAS*CG9962* and UAS*CG12147* were from J. Jiang. *GMR*Gal4 was from M. Freeman. The *Df(3L)H99* and UAS-*DIAP1* stocks were from B. Hay. The *dco* mutant allele *dco*^{le88} were from M. Noll. The FRT82B P[w+, Ubi-GFP^{nls}] was from I. Hariharan. The *gish* null allele mutant gish^{null} was generated by Dr. Jennifer Kennell in Cadigan lab. All other reagents for clonal analysis, *yw*flp^{122} , FRT^{82B}, FRT^{82B} P[w+, *armlacZ*] were from the Bloomington Stock Center. The UAS*dco* $^{RNAi-J}$ transgene on the third chromosome was generated by mobilizing the transgene from UAS*dco* ^{RNAi} (Guan et al. 2007).

Immunostainings and Western blotting

Immunostainings were performed as previously described (Lin et al. 2004). Antibodies used were mouse anti-DIAP1(1:200) (from B. Hay) and rabbit anti-β-galactosidase (1:500) (Cappel, ICN, Costa Mesa, CA). Cy3 conjugated (Jackson Immunochemicals) and Alexa Fluor 488-conjugated (Molecular Probes) secondary antibodies were applied after primary antibody.

Western blotting was performed as standard protocol. Antibodies used include anti-Flag (M2) (1:5000)(Sigma), anti-anti-DIAP1 (1:1000) (from B. Hay), rat anti-Dco (1:1000)(from M. Young), and mouse anti-tubulin (1:5000) (Sigma). HRP-anti-rabbit IgG and HRP-anti-mouse IgG are used as secondary antibody (Amersham Bioscience). Signal was detected using Amersham ECL Plus Western Blotting Detection Reagents kit (GE Healthcare).

Whole-mount staining and microscopy

The adult eye, notum and wing were photographed on a Leica M10 microscope as previously described (Parker et al., 2002). All fluorescent pictures were obtained with a Leica confocal microscope DM6000 B (Leica) and processed in Adobe Photoshop CS.

RESULTS

Exploration of the anti-apoptosis function of Dco in S2 cells

The *Drosophila* embryonic cell line S2 has been widely utilized to explore apoptosis regulation mechanism. Ectopic expression of pro-apoptotic protein Hid has been shown to rapidly trigger apoptosis, which can be suppressed by over-expression of DIAP1 in S2 cells (Goyal et al. 2000). This potentially provides a promising system to elucidate the mechanism by which Dco suppresses apoptosis.

Our results of cell survival assays illustrated that the anti-apoptotic function of Dco can be recapitulated in S2 cells. Over-expression of Hid reduces the survival rate to 25% of that in control cells, and the reduction was effectively suppressed by DIAP1 (Figure 4.3 A, B), consistent with previous reports (Goyal et al. 2000). In this system, Dco partially suppresses Hid-induced cell death (Figure 4.3 A, B); conversely, dominant negative version of Dco, i.e. DcoK38R, and *dco* RNAi enhances Hid-induced cell death (Figure 4.3 C, D), supporting the anti-apoptotic function of Dco in S2 cells.

Previous report showed that Dco depletion led to DIAP1 down-regulation in fly wing imaginal discs (Guan et al. 2007). However, no detectable reduction of DIAP1 was observed in S2 cells that were either treated with *dco* RNAi (Figure 4.4 A) or transfected with the dominant negative mutant DcoK38R (Figure 4.4B). DIAP1 is a short-lived protein that is degraded via the ubiquitination-proteasome pathway. As expected, DIAP1 was very sensitive to the treatment of the proteasome inhibitor lactacystin (LAC) and the

ribosomal inhibitor cycloheximide (CHX), supported by the results that LAC stabilized DIAP1 while CHX reduced it. Even in these sensitized backgrounds, *dco* RNAi didn't influence DIAP1 level. In addition, no *dco* RNAi induced reduction of endogenous DIAP1 was detected in another fly embryonic cell line Kc167, or the wing imaginal disc-derived cell line Clone8 (Data not shown). Similar results were obtained when exogenous DIAP1 was introduced into cells by transfection (Data not shown).

The possibility that Dco may phosphorylate and thereby enhance DIAP1 stability was examined by *in vitro* phosphorylation, where bacterially produced MBP-DIAP1 was used as substrate, and kinases were expressed and immunoprecipitated (IP) from S2 cells. In our system, wild type Dco was able to phosphorylate casein, a known substrate of Dco, while the kinase dead mutant DcoK38R showed dramatically reduced activity. However, no detectable phosphorylation of DIAP1 was observed in the same assay (Figure 4.5). In addition, the attempts to detect the interaction between Dco and DIAP1 in S2 cell lysates via co-IP were unsuccessful (date not shown).

Overall, although Dco shows anti-apoptotic activity in S2 cells, its role in regulating DIAP1 levels was not established in cultured cells, which might be due to the existence of potential redundant partners.

Exploration of potential redundant partner of Dco

Dco belongs to the casein kinase I family, composed of eight members in *Drosophila* (Figure 4.1 A). Given that redundancies among CKI family members have

been reported repeatedly in different signaling pathways (Vielhaber and Virshup 2001; Price 2006), small scale screens were conducted in the fly eye and S2 cells to determine whether other CKI members are potential redundant partners of Dco.

It has been previously shown that expression of Dco strongly suppresses the small eye phenotype induced by wild type *hid* alleles hid^{10}, hid^{IM}, and a Ras/MAPK resistant form hid^{ala5} (Guan et al. 2007). Thus Dco redundant partner(s) are expected to show similar suppression. In the screen, *hid* alleles and UAS lines of CKI members were under the control of an eye-specific promoter GMR. The screen results were summarized in Figure 4.6B, and the results with hid^{ala5} were shown as examples in Figure 4.6A. Our data showed that Gish, the fly homologue of casein kinase Iγ, was able to suppress all three alleles, and thereby selected as a putative Dco redundant partner for further characterization.

Another screen conducted in cultured cells also indicated Gish as a redundant partner based on its ability to down-regulate DIAP1 (Figure 4.7). In the screen, Kc cells that stably expressing exogenous DIAP1-flag (DIAP1-Kc) were treated with double-stranded RNA targeting one of the eight CKI family members, and the levels of DIAP1-flag were examined. It is worth mentioning here that all eight CKIs are expressed in S2 cells, judged by the existence of the corresponding cDNA generated from the cell lysate. Cells treated with *gish* RNAi showed lower DIAP1 levels compared with control cells (Figure 4.7A). The down-regulation was further confirmed with the same cell line

(Figure 4.7C) and with endogenous DIAP1 in S2 cells (Figure 4.7B). Consistently, over-expression of Gish stabilized ectopically expressed DIAP1 (Figure 4.7E). Overall, results in the fly eye and cultured cells indicate Gish as a putative anti-apoptotic factor working through DIAP1

It should be noted that the effects of DIAP1 down-regulation by *gish* RNAi were variable, and sometimes not reproducible (data not shown). Thus, the ability of Gish to regulate DIAP1 stability definitely requires further validation.

Exploration of the redundancy between Gish and Dco

To test the hypothesis that redundancy may cause the lack of DIAP1 reduction in the developing eye and culture cells where Dco is reduced, we simultaneously reduced the levels of Dco and its putative redundant partner Gish in both S2 cells (Figure 4.7 D) and eye imaginal discs (Figure 4.8). *gish* RNAi alone decreased the level of DIAP1 in S2 cells, yet no synergetic effect was observed in cells with both Dco and Gish depleted (Figure 4.7 D). Negative result was also obtained in eye discs where double mutant cells of *dco* and *gish* didn't show lower levels of DIAP1. Interestingly, double mutant clones were considerably smaller than *dco* single mutant clones, indicating cell survival was affected in absence of *gish*, which is consistent with its putative anti-apoptotic role (Figure 4.8).

To explore whether Gish is a redundant partner of Dco, we examined the ability of Gish to rescue *dco* RNAi induced defects in the adult notum (Figure 4.9 A), adult wing (Figure 4.9 B) and the larval wing imagnial disc (Figure 4.9 C). UAS-*dco* RNAi driven

by *dpp*-Gal4 resulted in less macrochaetes in the scutellum of the adult notum, which was partially reversed by over-expression of *gish*. And over-expression of *gish* alone led to more macrochaetes (Figure 4.9 A). The macrochaete phenotype caused by *dco* RNAi was not suppressed in a *H99* heterozygous background, where the proapoptotic factors Hid Rpr and Grim were reduced (Figure 4.9 A). This suggests the macrochaete phenotype is not a consequence of increased apoptosis due to elevated RHG proteins. In the fly adult wing, *dco* RNAi distorted the vein pattern, which was unexpectedly enhanced by *gish* expression, and this phenotype was not attenuated in the *H99* heterozygous background (Figure 4.9B). The wing imaginal discs are the tissue where the function of Dco to regulate DIAP1 was originally reported, thus the inspection of this phenotype would directly address the question whether Gish is a redundant partner of Dco in apoptosis regulation. Preliminary results showed no consistent suppression of DIAP1 reduction by *gish* (Figure 4.9 C), arguing against the redundancy.

Exploration of Dco's function in the Wingless signaling pathway

Dco and its vertebrate homologue CKIε have been implicated in Wnt/ Wg signaling in the past decade, however, the molecular mechanism still remains controversial. To clarify the function of Dco/CKIε in a physiological context, we are exploring the role of Dco in the *Drosophila* embryonic cell line Kc167 (Kc), mainly using a loss of function approach.

Kc cells have been well established as a system to study the Wg signaling pathway in Cadigan lab, as they are responsive to Wg signaling, judged by luciferase reporter genes and several endogenous target genes (Fang et al. 2006). Readouts used in this study include a luciferase reporter gene, SuperTopFlash (STF), which is composed of 12 copies of TCF binding sites, and the transcript levels of two endogenous genes, *nkd* and *CG6234* , which has been demonstrated as direct target genes of Wg signaling (Fang et al. 2006). Wg conditional medium (Wg-CM) can activate all three readouts effectively, compared with control medium (Figure 4.8 A, B, D).

In order to address the requirement of Dco in Wg signaling, we inhibited endogenous Dco by IC261, a CKIε specific inhibitor (Mashhoon et al. 2000), or RNAi. The treatment of IC261, suppressed Wg CM induced activation of STF reporters (Figure 4.10 A). Cells depleted for *dco* with RNAi showed 3-4 fold reduction of Wg signaling, as judged by the activation of STF reporters (Figure 4.10B), and the transcript levels of *nkd* (Figure 4.10C) and *CG6234* (Figure 4.10D), which strongly support Dco as a positive regulator of Wg signaling. Consistently, expression of Dco, not DcoK38R, activated STF in a dosage dependent manner (Figure 4.10E), indicating that the kinase activity is required for Dco to stimulate Wg signaling. To illustrate the specificity of the RNAi treatment, a rescue experiment was performed as follows. Dco was knocked down by RNAi that specifically targets the 3'UTR, which presumably would not inhibit Dco that were expressed from an exogenous plasmid only containing the coding region. Exogenous Dco

was transfected at such a low amount (10ng) that it, on its own, could not activate Wg signaling (Figure 4.10 E, F). Under these conditions, exogenous Dco partially rescued the suppression caused by *dco* RNAi (Figure 4.10 F), supporting the specificity of *dco* RNAi. The lack of full rescue may be due to the relatively low levels of exogenous Dco. Overall, our results support the positive role of Dco in the Wg pathway, at least in Kc cells.

Where does Dco function in the Wg pathway? We conducted epistasis analyses to address this question. The Wg pathway can be stimulated remarkably by over-expresion of a stabilized form of Arm (Arm*) and by knocking down two negative regulators Axin and CKI*a*, which are components of the Arm degradation complex (Figure 4.11). Dco knockdown did not reproducibly suppress the activation of STF reporters induced by any of the three approaches, (Figure 4.11 and data not shown), placing Dco upstream of Arm and the Arm degradation complex.

To explore the possibility that Dco positively regulates Wg signaling by stabilizing Arm, the protein levels of Arm were compared between *dco* RNAi and control RNAi treated cells which are stimulated by Wg CM. The treatment of *dco* RNAi effectively reduced *dco* transcripts to approximately 10% of its normal level, leading to 4-5 fold suppression of the *nkd* activation in presence of Wg signaling (Figure 4.12 A). Unexpectedly, in the same cells, no reduction of total Arm (Figure 4.12 B) or nuclear Arm levels (Figure 4.12 C) were reproducibly observed, indicating Dco might not regulate Arm protein levels or Arm nuclear translocation. These results apparently contradict to the

epistasis analyses, and reconciliation of all the data requires a better understanding of the pathway (see discussion).

To characterize the functions of all CKI isoforms in Wg signaling, cells were depleted for a single CKI member, or together with *dco*. *gish* RNAi attenuated Wg activation, and interestingly it showed an additive effect with *dco* RNAi (Figure 4.13 A). Gish/CKIγ has been reported to phosphorylate the Wnt co-receptor Arrow/LRP6, promoting the disassociation of the Arm degradation complex (Davidson et al. 2005; Zeng et al. 2005), which is distinct from all documented functions of Dco/CKIε. As Dco/CKIε has been proposed to regulate Dsh, we speculate the additive effect may be due to the paralleled functions of Dsh and Arrow in traducing the Wg signal from membrane to downstream. Consistently, Dco expression didn't rescue Wg suppression by *gish* RNAi, nor vice versa, arguing against Gish as the redundant partner of Dco in the Wg pathway.

Attempted identification of Dco substrates by the Shocat substrate screen

We have shown that the kinase activity is required for Dco to regulate apoptosis and the Wg pathway (Figure 3.4, Figure 4. 10E). Therefore, the identification of its novel substrates could undoubtedly expand our knowledge of the kinase and the regulation mechanism.

To identify the novel substrates of Dco, we have employed a chemical genetic screen developed by Shokat and colleagues, using an engineered kinase with larger ATP binding pocket and a bulky ATP analogue, which is referred to as the Shokat screen thereafter

(Shah et al. 1997; Liu et al. 1998). This method has been shown to specifically label direct substrates of a particular serine/threonine protein kinase in a mixture of cellular proteins, and the application of the Shokat screen has successfully identified many novel substrates of several kinases belonging to different families (Elphick et al. 2007).

In the Shokat screen, the ATP binding pocket in the kinase of interest is modified to accept an ATP analogue with a bulky substitution at the N-6 position of the purine ring (Figure 4.14A) (Shah et al. 1997; Liu et al. 1998). Specifically, one or more conserved residue that comes into close contact with the N-6 position of ATP is mutated to a smaller amino acid residue. Then, ATP analogues with different N-6 substitutions are screened to identify the one that the mutant kinase can transfer most efficiently. Thereby, the mutant kinase, but not other cellular kinases, can utilize a particular N-6 modified ATP to phosphorylate substrates. Theoretically, when radio-labeled ATP analog is used in a kinase reaction with the mutant kinase and cell lysates, only direct substrates of the kinase become radio-labeled and consequently identified.

To apply the Shokat screen to Dco, we first examined the structure of CKI ATP binding pocket. The crystal structure of Dco has not been solved, but several structures of CKI family members in other organisms have been documented in Protein Data Bank (PDB). Among them, the structure of CKI from *Schizosaccharomyces pombe* (PDB ID: 1CSN) (Xu et al. 1995) was utilized as a model to represent Dco, mainly because the

protein was co-crystalized with ATP, which is extremely useful to identify amino acids that in close contact with N-6 position.

In order to engineer the ATP pocket so that it can accomendate N-6 modified ATP, the following criteria were summarized based on published examples (Shah et al. 1997; Liu et al. 1998; Eblen et al. 2003; Hindley et al. 2004): 1) the candidate mutation residue(s) should locate in the ATP pocket, but should NOT be an essential residue to transfer the phosphate; 2) the site should not be a charged residue; 3) the candidate residue should be in close proximity with N-6 position; specifically the distance between the nearest atom of the candidate residue and ATP N6 should be around 5.5 Å; 4) the distance between the backbone of the candidate residue and ATP N6 atom should be 7.5 Å or above, so that when the candidate residue is mutated to glysine, the space should be big enough to accommodate N6-modified ATP analogues; 5) the residue should be located in the area where the N-6 substitution is predicted to occupy. A careful inspection revealed that I85 was the only residue in CKI that meets all criteria (Figure 4.14 B). Sequence alignment showed that it corresponds to M82 of Dco.

Dco M82 was mutated to G, A, V or C, the four amino acids with shorter side chains. As in published examples, the mutant kinases should be able to utilize wild type ATP, indicating they may be functional *in vivo*. However, all of the four mutants showed very low catalytic activity, if any, as judged by the STF activation assay (Figure 4.14C), raising doubt about the feasibility of this approach.

DISCUSSION

How does Dco regulate DIAP1 protein levels?

Previous genetic studies have demonstrated the anti-apoptotic role of Dco via DIAP1 activation (Guan et al. 2007), yet the underlying molecular mechanism remains to be elucidated. The *Drosophila* S2 cell line has been nicely established as a useful system to study apoptosis mechanism, and the core apoptosis machinery in *Drosophila,* including Dark, Dronc, Drice and DIAP1, has been demonstrated to mediate apoptosis regulation in S2 cells (Hay and Guo 2006). Therefore, we took advantage of S2 cells to explore the molecular mechanism of Dco.

We have provided evidence that the anti-apoptotic function of Dco *in vivo* can be recapitulated in S2 cells (Figure 4.3). However, DIAP1 protein levels seem not under the control of Dco in S2 cells (Figure 4.4). These data reminded us of the situation in the fly developing eye, where Dco regulates Hid-induced apoptosis in both overexpression (Figure 3. 1, 3.2) and loss of function analyses (Figure 3. 3), yet loss of Dco does not lead to any detectable modification of DIAP1 levels (Figure 4.8). The similarity might imply some cell-specific mechanism that we still don't understand.

Our results also showed that Dco did not phospharylate DIAP1 *in vitro* (Figure 4.5). It is possible that bacterially produced DIAP1 may lack certain modifications that are indispensable for Dco phosphorylation, given that CKI members often require a so called 'priming' phosphorylation, which means substrates have to be phosphorylated by another

kinase beforehand so as to be recoganized by CKIs (Vielhaber and Virshup 2001). Alternatively, DIAP1 could simply not be the direct substrate of Dco. Instead, the function of Dco might be mediated by other factor(s), for instance, RHG proteins which directly regulate DIAP1, or by multiple components composing an unidentified pathway. The identification of Dco's substrate would help uncover the molecular mechanism.

Putative function of Gish in DIAP1 regulation

Our results may have uncovered a novel function of Gish in DIAP1 regulation, as we illustrated that depletion of *gish* caused DIAP1 down-regulation in S2 cells (Figure 4.7). What is the physiological importance of the regulation? The macrochaete phenotype might provide a clue (Figure 4.9). It has been proposed that the number of macrochaetes is determined by a nonapoptotic function of caspases, and proper DIAP1 levels set a threshold for the caspase activity in this context (Kanuka et al. 2005; Kuranaga et al. 2006). By integrating the above information, we speculate that Gish might play a role in maintaining proper DIAP1 levels and consequently regulating the non-apoptotic function of caspase.

It is quite possible that Gish regulates DIAP1 in apoptotic contexts, which is supported by the ability of Gish to suppress Hid-induced small eye phenotype (Figure 4.6). Nevertheless, the putative anti-apoptotic function of Gish has yet to be established. Whether *gish* mutant cells show elevated apoptosis levels *in vivo* and whether Gish suppresses Hid induced cell death in S2 cells are among the first questions to be addressed.

It should be noted that the lack of reproducibility is a concern with the DIAP1 level changes upon *gish* depletion in S2 cells. Although the effects have been observed several times in independent experiments, they did display variations ranging from dramatic effects to almost none, sometimes even within the same experimental set. In Figure 4.7, it is also clear that the levels of the loading control Tubulin fluctuated for uncertain reasons. While this could be due to technical reasons, it seems possible that *gish* RNAi treatment might affect cell healthiness, which may be associated with the putative anti-apoptotic role of Gish, although no reproducible effects on cell survival were observed (data not shown). In conclusion, Gish's ability to modulate DIAP1 levels needs further validation with careful monitoring of cell conditions.

Dco as a positive regulator of Wg signaling pathway

Through both loss and gain of function analyses, our results in S2 cells clearly suggest that Dco regulates the Wg signaling pathway positively (Figure 4.10). However, how Dco regulates Wg signaling remains inconclusive. Epistasis analysis indicates that Dco functions upstream of Arm (Figure 4.11), while the fact that Dco reduction did not result in a reproducible decrease of Arm levels argues against the upstream position of Gish (Figure 4.12). Curiously, the latter observation provokes an interesting question whether the protein level of Arm correlates with the activation fold of target genes in a linear manner. Take the result in Figure 4.12 as an example, the question can be elaborated as whether cells with the 5-fold reduction of *nkd* transcripts harbor a 5-fold

reduction of Arm protein level. If not, is it possible that the change of Arm level might be much below the detection threshold of Western blotting, a semi-quantative approach? Another explanation to the paradox is that the negative regulation by *dco* RNAi becomes undetectable when Arm is accumulated to an unnaturally high level, as in cells treated with *axin* RNAi, *CKIa* RNAi and Arm*. In this scenario, the epistasis analysis may be somewhat misleading. Also, Dco may regulate Arm activity rather than its stability. Thus far, it still remains inconclusive how Dco regulates the Wg pathway. We are still open to the possibility that Dco regulates several components throughout the Wg pathway.

Does Dco have a redundant partner?

In signal transduction pathways, many crucial factors are frequently associated with functional redundancy, with many examples of redundant isoforms of kinases (Kafri et al. 2009). Numerous examples reveal that typical redundant partners have partially overlapped functions, exhibit either spatially or temporally different expression patterns, show synergistic effects when expresses together, and moreover, one can compensate the other's loss (Kafri et al. 2009). These shared attributes have been widely referred to as functional redundancy.

According to the definition stated in the paragraph above, we carefully inspected if Dco and Gish are redundant partners in apoptosis regulation. So far, no synergistic effects on DIAP1 levels have been observed when *dco* and *gish* are simultaneously depleted in S2 cells or eye discs. Gish does not rescue DIAP1 downregulation caused by the loss of Dco

in wing discs. Although Gish partially rescues the loss of macrochaetes resulting from Dco depletion, the defects may not be apoptosis-related (Kuranaga et al. 2006). These data strongly argue against the redundancy for Dco and Gish in apoptosis regulation. However, we are still open to this possibility, mainly because Gish has been shown as a suppressor of the Hid induced small eye phenotype, and as a putative regulator of DIAP1, which are functions shared by Dco. It would be interesting to examine if *gish* mutant cells display low DIAP1 levels in wing discs as *dco* mutants, and if expression of Dco can suppress the decrease of DIAP1 resulted from Gish knockdown in S2 cells. It is also possible that Dco and Gish act redundantly in non-apoptotic contexts. Ultimately, experiments elucidating the molecular mechanisms, for instance, the kinase substrates and consequence of phosphorylation, would help address this issue conclusively.

Attempted identification of Dco kinase substrates

In the efforts to identify direct substrates of Dco, we initiated a genetic chemical screen, referred to as the Shokat screen. We engineered the ATP binding pocket which potentially enlarges the space to accommodate N-6 substitutions of ATP. The M82 site we chose by examining the protein structure is also listed as the only recommended site by Shokat lab, the information of which we obtained afterwards. Unfortunately, all the DcoM82 mutants showed low enzymatic activities in functional assays carried out in S2 cells. Three speculations, if validated, may potentially rescue the application of the Shokat screen for Dco. First of all, although DcoM82 mutants are not efficient users of

wild type ATP, they might be able to transfer the bulky ATP analogue better to phosphorylate substrates. Secondly, as M82V and M82C mutants showed limited activity *in vivo*, it is possible that when applied with a considerable amount of ATP *in vitro*, they may be able to phosphorylate enough substrates to the level for detection. Thirdly, it is possible that mutations of other residues located away from the ATP binding sites would modify the overall three-dimensional protein structure and partially rescue Dco's catalytic activity, as kindly suggested by David L. Akey, a structural biologist at the University of Michigan. Detailed structural analysis might provide clues to this possibility.

ACKNOWLEDGEMENTS

I would like to thank Dr. Ju Guan for initiating this project, generating Figure 4.4A and collaborating with me to generate Figure 4.3 and Figure 4.5. I'd like to thank Dr. Ming Fang for the initial analysis on the fly CKI family members, and Figure 4.1 is modified from the phylogenetic tree generated by him. I also want to thank Dr. Jennifer Kennel for discussion and advice on the Gish project. Dr. Kennel first reported the inhibition of Hid induced small eye phenotype by EP676, and generated $dco^{RNAi\text{-}J}$ for Figure 4.9 and $gish^{null}$ for Figure 4.10.

I'd like to thank all members from Cadigan lab who made a tremendous effort to establish the Kc cell system to study the Wg signaling pathway. I'd like to thank B. Hay, K. White, A. Bergmann, P. Meier, C. Niehrs, J. Jiang, I. Hariharan, M. Young and J.

Abrams for providing fly stocks and antibodies. Thanks to David L. Akey for the insightful advice about the Shokat screen. Thanks to Gregg Sobocinski for help with confocal microscopy. Thanks to the flow cytometry facility at the University of Michigan.

FIGURES

Figure 4.1

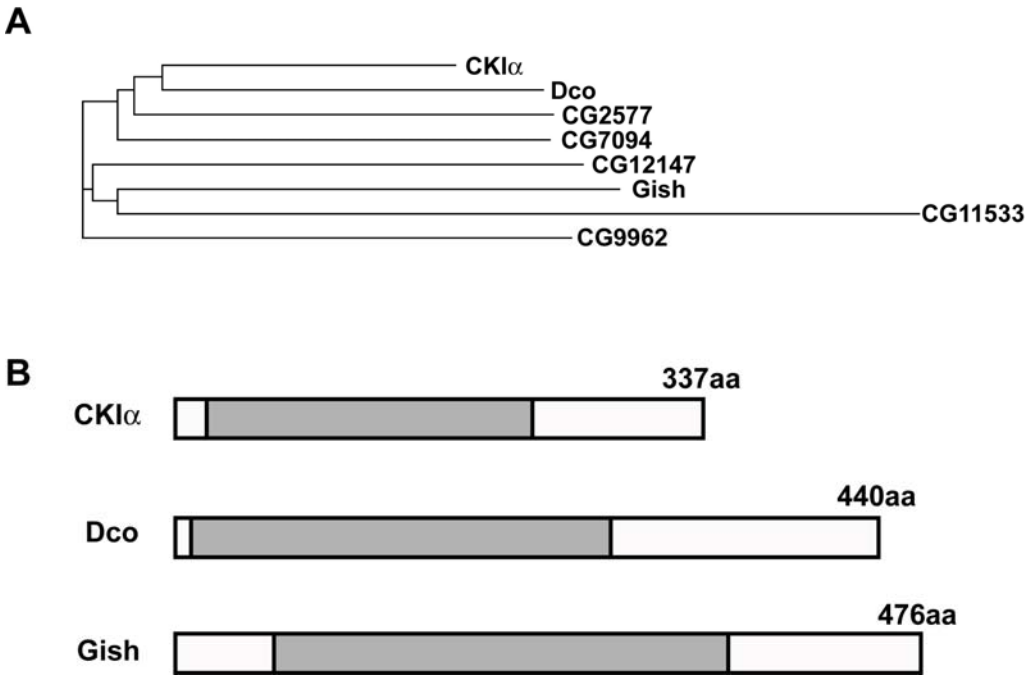


Figure 1.1 Casein Kinase I family members in *Drosophila*. (A) Phylogenetic tree of eight CKI family members in *Drosophila*. (B) Schematic presentation of CKIα, Dco and Gish. The lengths of proteins are labeled on the left. Grey boxes represent the conserved kinase domains.

Figure 4.2

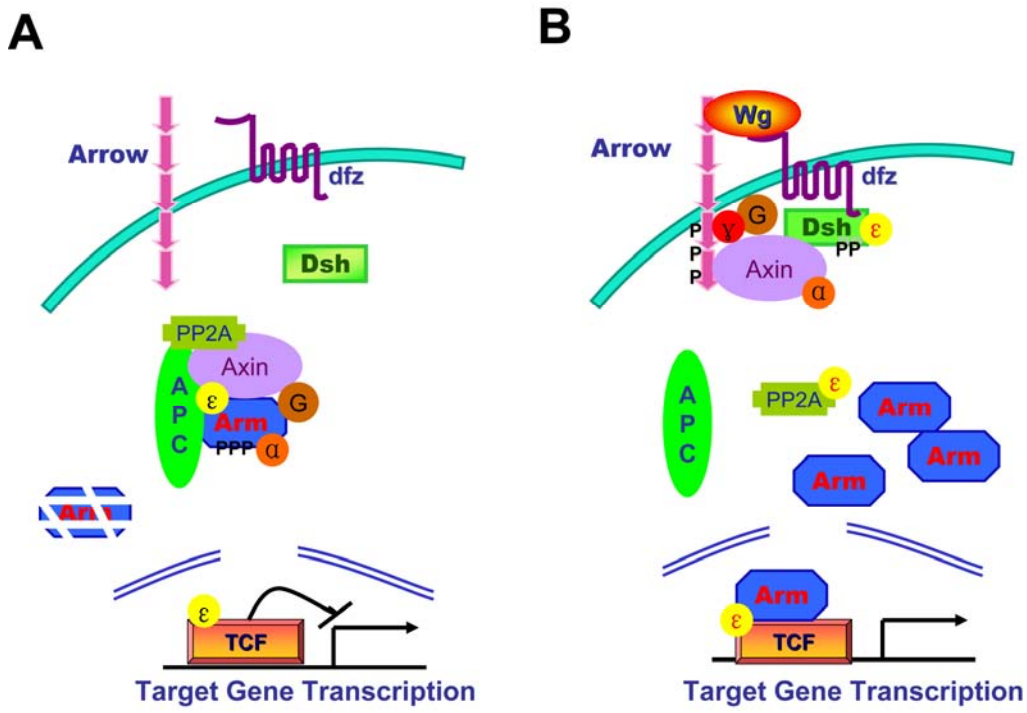


Figure 4.2 Model of the Wg signaling pathway and proposed phosphorylation events by CKIs. (A) In the absence of Wg ligand, Arm is polyubiquitinated and degraded. This degradation requires Axin and APC, and requires phosphorylation of Arm by CKIα ⓐand GSK3β Ⓖ. (B) Upon binding of Wg to its coreceptors dFz and Arrow, Arrow becomes phosphorylated by CKIγ⍟(Gish in the fly), and Dsh becomes phosphorylated by CKIε Ⓔ (Dco in the fly). This recruits Axin to the receptor complex and stabilizes it, leading to the disassociation of the degradation complex which results in Arm stabilization. Arm is accumulated and migrates to the nucleus where it complexes with TCF to activate expression of Wg-target genes. The above mentioned key phosphorylation events are labeled in the figure as **P**. In addition, other phosphorylation events by CKIε are proposed according to studies in the fly and mammalian cells. The positive roles as presented in red Ⓔ include (1) CKIε binds and phosphorylates mammalian Tcf3, leading to the enhanced interaction between TCF and β-catenin (mammalian homologue of Arm); (2) CKIε phosphorylates PP2A, leading to reduced affinity of PP2A with axin and β-catenin which facilitates the disassociation of degradation complex. The negative roles presented in black Ⓔ include (1) CKIε binds and phosphorylates Axin and APC to reinforce the degradation complex; (2) CKIε works redundantly to phosphorylate β-catenin; (3) CKIε phosphorylates LEF-1, a mammalian TCF family member, leading to the disruption of LEF-1and β-catenin complex. (Price 2006; Cadigan 2008)

Figure 4.3

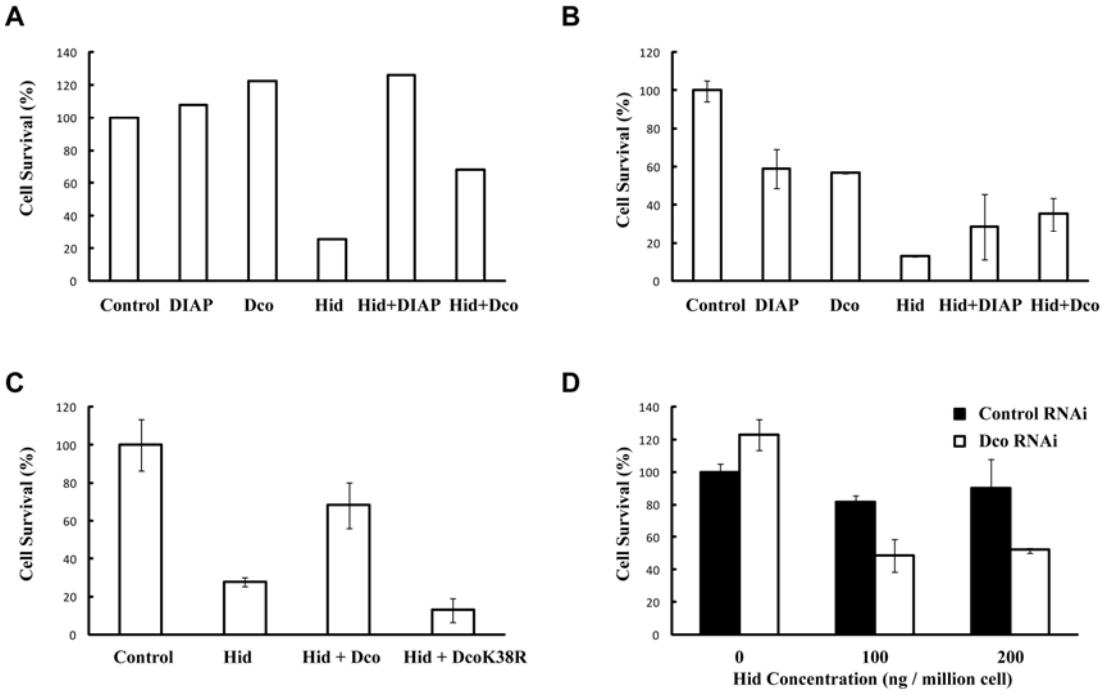


Figure 4.3 Dco suppresses Hid induced cell death in S2 cells. (A-B) Dco suppresses Hid induced cell death. Vectors indicated in the figure were cotranfected with GFP. 24hr after transfection, cells were treated with copper to induce Hid expression. 48 hr later, cells were inspected for the percentage of GFP positive cells by manual cell counting (A) or flow cytometry (B). The percentage of GFP positive cells is normalized to that in control cells, and presented as cell survival rate (control cells is 100%). (C) The kinase activity of Dco is required for its anti-apoptotic function. DcoK38R is a kinase dead mutant, and it has comparable expression levels as wild type. (D) Dco depletion enhances Hid induced cell death. The amount of Hid vector transfected in (D) is much less than that in (A-C) to provide a sensitized backgroud.

Figure 4.4

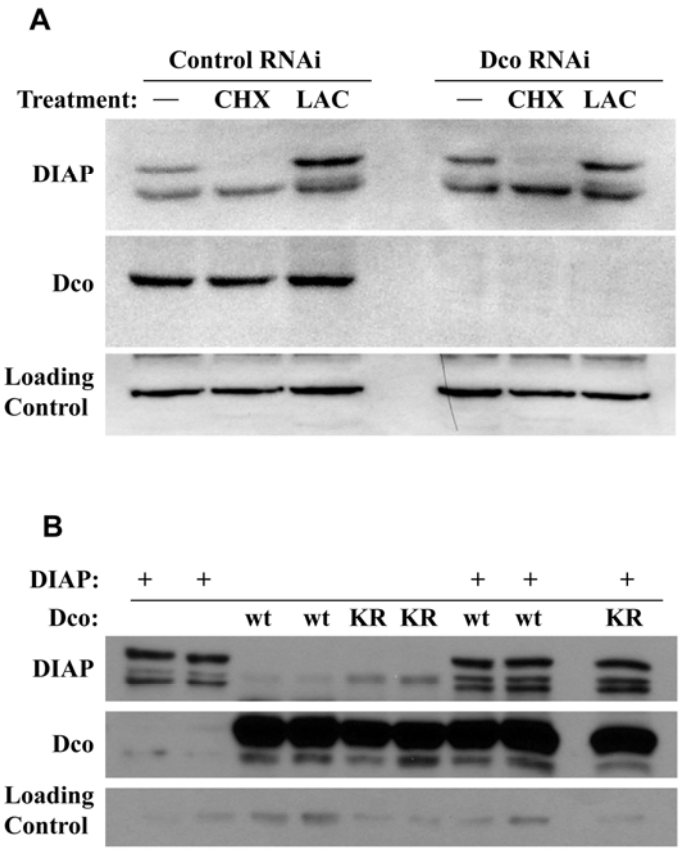


Figure 4.4 Dco does not regulate DIAP1 protein levels in S2 cells. (A) Dco knockdown does not regulate DIAP1 protein level. Cells were incubated with control or *dco* RNAi for 4 days and subsequently treated with carrier DMSO (-), the ribosomal inhibitor cycloheximide (CHX) and the proteosome inhibitor Lactacystin (LAC) for 2 hours. Cells were harvested and analyzed by SDS-PAGE followed by western blotting for DIAP1 and Dco. A background band in the DIAP1 blot was used as a loading control. (B) Overexpression of either wild type Dco or the dominant negative version (KR) does not affect exogenous DIAP1 level. DIAP1-flag, Dco-flag or DcoK38R-flag were transfected as indicated. Three days after transfection, cells were harvested and analyzed by western blotting for DIAP1 and Dco using anti-flag antibody (M2 antibody). Endogenous tubulin was used as loading control.

Figure 4.5

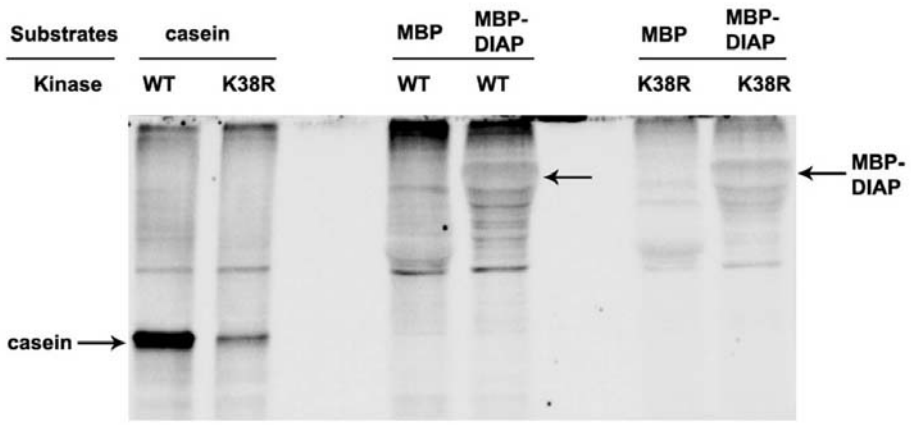
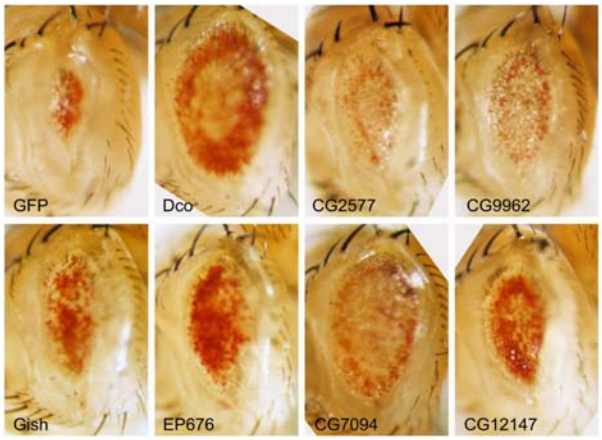


Figure 4.5 Dco does not phosphorylate DIAP1 *in vitro*. Wild type Dco, but not kinase dead mutant DcoK38R, effectively phosphorylates casein, a known substrate of casein kinase I. No phosphorylation of MBP-DIAP1 (indicated by arrows) by wild type Dco was detected. Both wild type and kinase dead version of Dco were immuno- precipitated with anti-flag (M2) antibody from S2 cells transfected with corresponding vectors. Dco and DcoK38R were eluted with flag peptide for immediate use in *in vitro* phosphorylation assay. MBP-DIAP1 was bacterially produced and used as substrates. Casein pre-treated with phosphotase was used as positive controls for kinase activities.

Figure 4.6

A



B

	Hid ^{ala5}	Hid ^{10}	Hid ^{IM}
Dco	Yes	Yes	Yes
Gish	Yes	Slightly	Yes
EP676	Yes	Yes	Yes
CG2577	Yes	Slightly	No
CG7094	Yes	Slightly	No
CG9962	Yes	Slightly	No
CG12147	Yes	Slightly	No

Figure 4.6 Suppression of Hid induced small eye phenotype by CKI family members. (A) Ectopic expression of CKI family members suppresses the small eye phenotype induced by *hid*^{ala5}, a Ras/MAPK resistant form of Hid. *hid*^{ala5} and UAS-*CKI*s were expressed driven by *GMR*-Gal4. (B) Summary of the suppression of Hid induced phenotypes. *hid*^{10} and *hid*^{IM} express wild type protein with different strength, resulting in a severe and intermediate small eye phenotypes respectively.

Figure 4.7

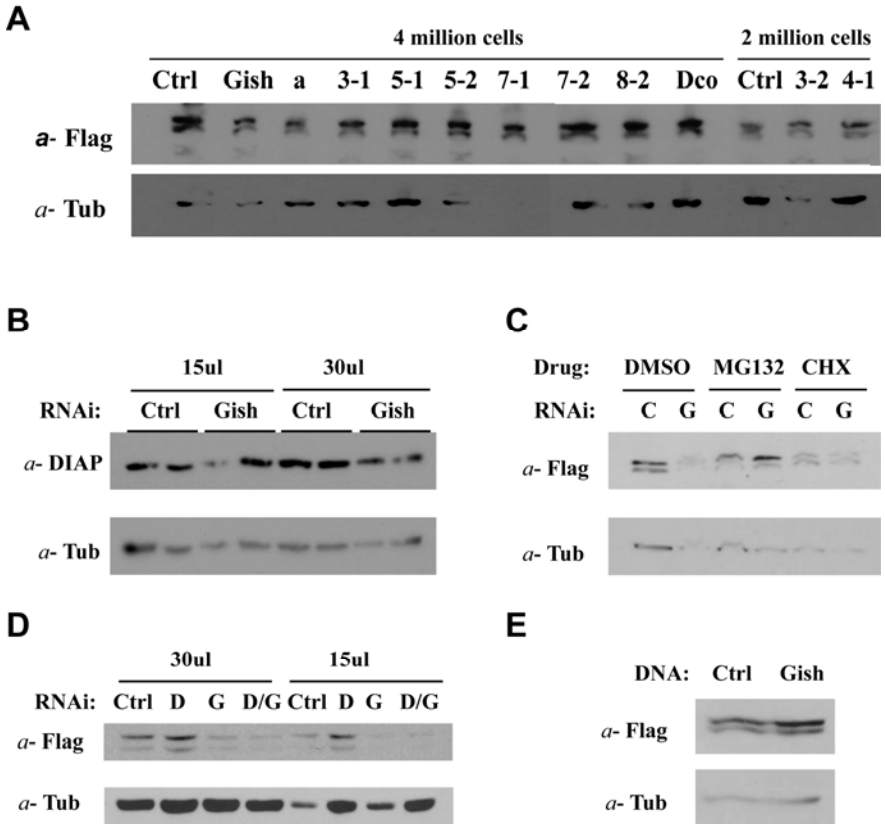


Figure 4.7 Identification of Gish as putative redundant partner of Dco in DIAP1 regulation. (A) RNAi based screen of CKIs reveals the ability of Gish to decrease DIAP1 level. Cells stably expressing DIAP1-flag (DIAP1-Kc) were treated with dsRNA targeting corresponding CKIs for 4 days. DIAP1 was detected by anti-flag (M2) antibody. RNAis target *gish*, *CKIa* (a), *CG7094* (3-1,3-2), *CG2577* (4-1), *CG12147* (5-1, 5-2), *CG9962* (7-1, 7-2) and *CG11533* (8-2) as indicated. (B, C) Depletion of *gish* reduces DIAP1 protein level in S2 cells (B) or DIAP1-Kc cells (C). Cells were treated with RNAis as indicated for 4 days. Cells in (C) were subsequently treated with carrier DMSO (-), the ribosomal inhibitor cycloheximide (CHX) and the proteosome inhibitor MG132 for 5 hours before harvesting. DIAP1 levels were detected by either *a*-DIAP1 antibody (B) or *a*-flag (C). (D) Simultaneous knockdown of Dco and Gish do not show synergistic effects. Cells were treated with either single or double RNAis targeting *dco* (D) and *gish* (G) for 4 days before harvesting. (E) Overexpression of Gish increases DIAP1 levels. Cells were transfected with vectors expressing DIAP1-flag and Gish and harvested after three days. DIAP1 levels were detected by anti-flag antibody.

Figure 4.8

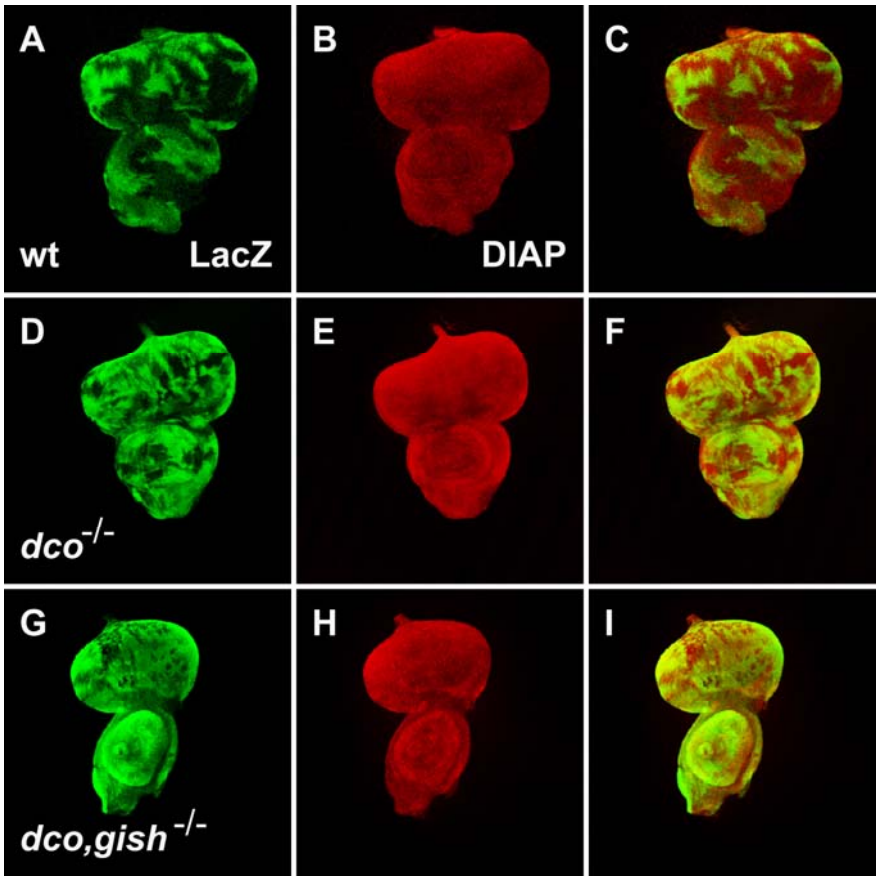


Figure 4.8 *dco gish* double null mutant clones exhibit no change on DIAP1 level. Confocal images of late third instar larval eye and antenna discs stained for DIAP1 in red (B,E,H) and the clonal marker LacZ in green (A,D,G). Clones analyzed are wild type (A-C), *dco*^{le88} (D-F) and *dco*^{le88}, *gish*^{null} double mutants(G-I). Clones are indicated by the lack of LacZ staining. The clones in *dco*^{le88}, *gish*^{null} double mutants are smaller than those in *dco*^{le88}, which are smaller than wild type.

Figure 4.9

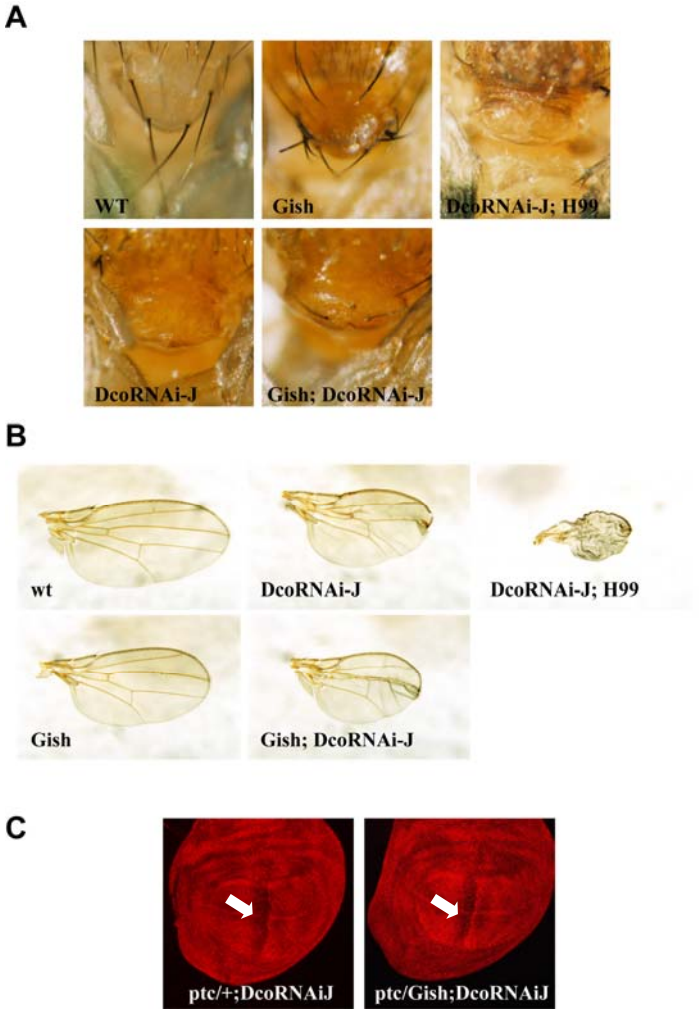


Figure 4.9 Exploration of the ability of Gish to rescue DcoRNAi phenotypes. (A) Overexpression of Gish partially suppresses the reduction of macrochaetea in the scutellum of the adult notum induced by *dco* RNAi. Photos are of notums from wild type, *dpp-Gal4;UAS-gish*, *dpp-Gal4;dco*$^{RNAi\text{-}J}$, *dpp-Gal4/UASgish;UASdco*$^{RNAi\text{-}J}$, *and dpp-Gal4;dco*$^{RNAi\text{-}J}$*,Df(3L)H99/-* as indicated. Over-expression of Gish results in more macrochaetes, while *dco* RNAi causes less. Reduction of pro-apoptotic factors Hid, Rpr and Grim by *Df(3L)H99* deficiency does not reverse the less macrochaete phenotype by *dco* RNAi, suggesting the phenotype may not be a consequence of increased apoptosis. (B) Over-expression of Gish enhances the vain pattern defects of adult wings. Genotypes are the same as in (A). (C) Preliminary data shows no producible rescue of DIAP1 reduction resulting from *dco* depletion by over-expression of Gish. Photos are of late third instar wing imaginal discs from *patchedGal4;UASdco*$^{RNAi\text{-}J}$ and *patchedGal4/UASgish*; *UASdco*$^{RNAi\text{-}J}$. DIAP1 level reduction in *pathched* domain is indicated by arrows.

Figure 4.10

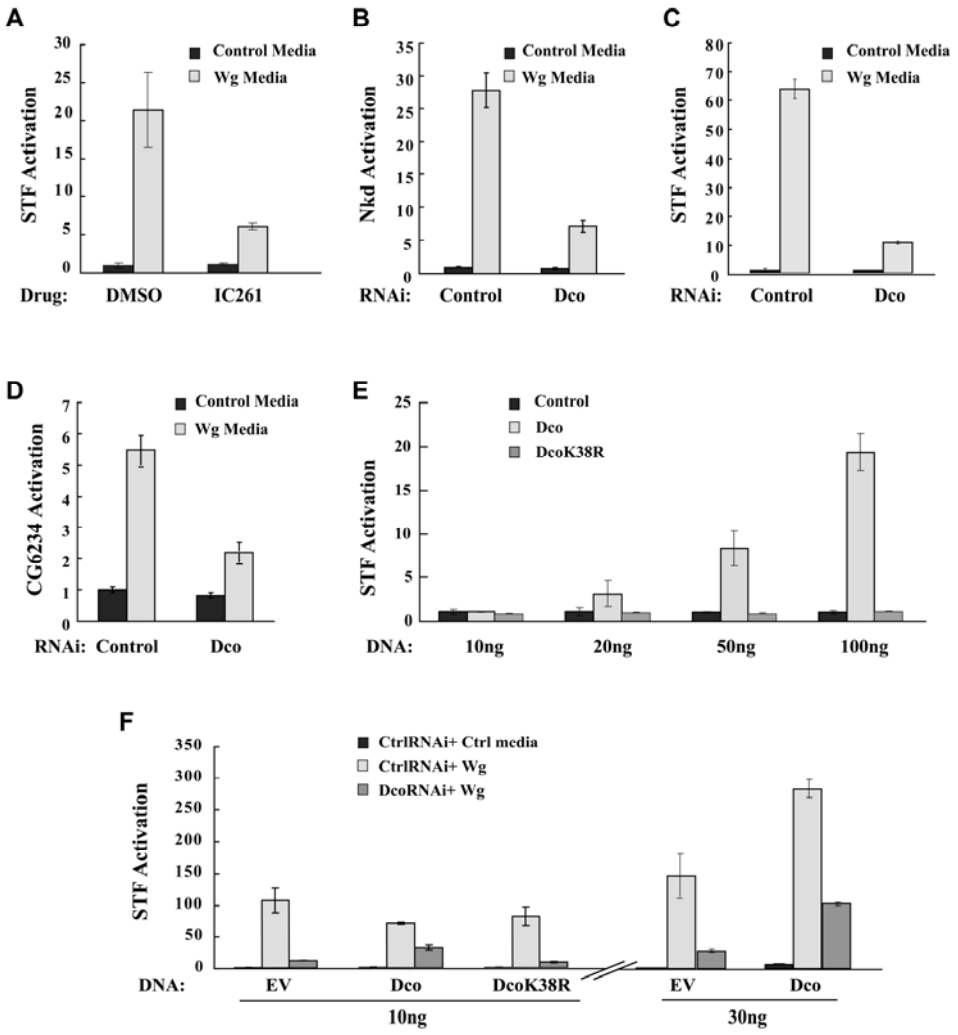


Figure 4.10 Dco is a positive regulator of the Wg signaling pathway in Kc cells. (A-D) Inhibition of Dco suppresses the activation of Wg signaling reporter SuperTopFlush (STF) (A,C), Wg endogenous target genes *nkd* and *CG6234*. Cells were treated with DMSO or the CKIε specific inhibitor IC261 for 2 hours (A), or with control or *dco* RNAi for 4 days (B-D) before the treatment of control or Wg media for 4 h before harvesting. (E) Dosage dependent activation of STF reporters by overexpression of Dco. (F) Expression of Dco rescues the suppression of STF reporter activation resulting from *dco* RNAi. 10ng and 30ng were chosen because they showed minimal activation when expressed alone. STF activation (A,C,E,F) was measured by standard luciferase assays, while *nkd* and *CG6234* activation was reflected by their transcripts level, judged by quantitative RT-PCR.

Figure 4.11

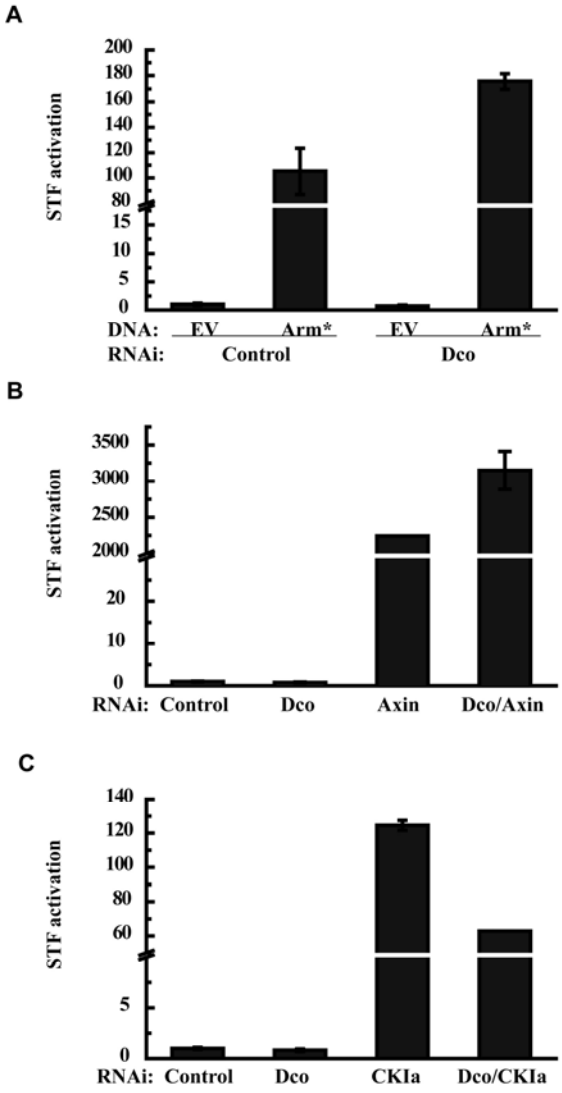


Figure 4.11 Dco regulates the Wg pathway upstream of Axin CKIα and Arm in Kc cells. *dco* RNAi does not suppress the STF activation induced by stabilized Arm (Arm*) (A), *axin* RNAi (B) and *CKIα* RNAi (C). (A) Cells were treated with RNAi for 4 days before the transfection of empty vectors (EV) or vectors expressing Arm*, and harvested 48hrs later. (B,C) Cells were treated with RNAi as indicated for 5 days before harvesting.

Figure 4.12

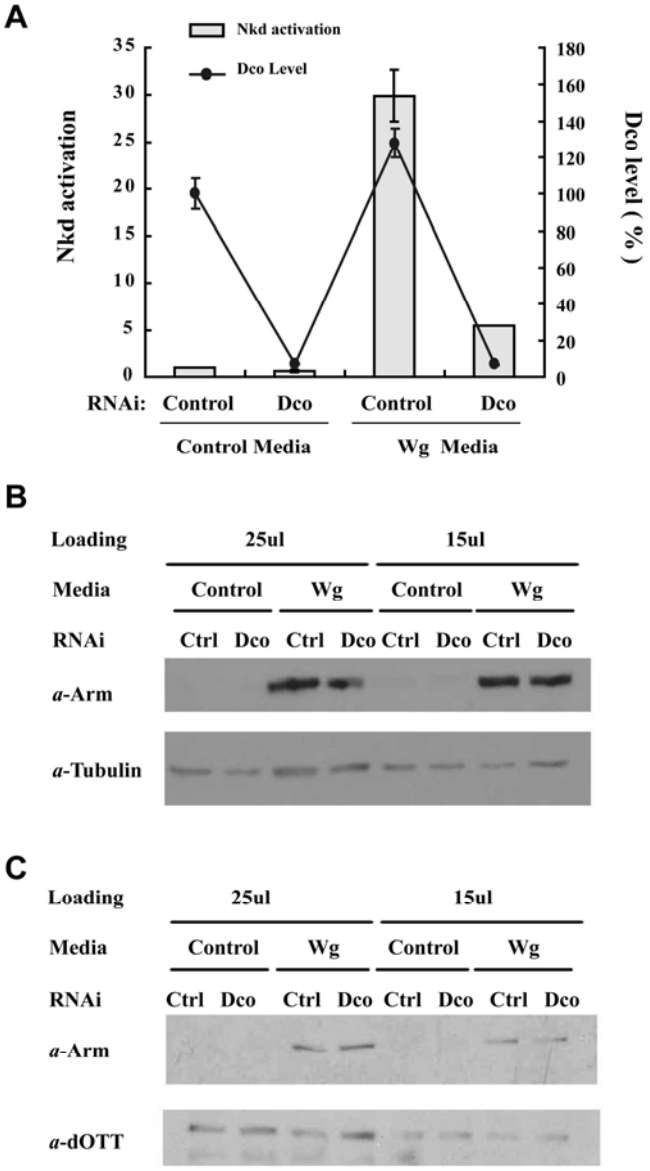


Figure 4.12 Exploration of the ability of Dco to regulate Arm stability. Cells were treated with RNAi for 4 days and with control or Wg media for 5 hours. After harvesting, cells were split into three portions for analyses of *dco* and *nkd* transcript levels (A), endogenous total Arm protein levels (B) and nuclear Arm levels (C). Tubulin and a predominately nuclear protein dOTT were used as loading controls respectively. *dco* RNAi effectively deceases *dco* transcript levels, leading to the reduction of *nkd* activation. However, no reproducible reduction of Arm or nuclear Arm levels were detected in this and several other similar experiment sets.

Figure 4.13

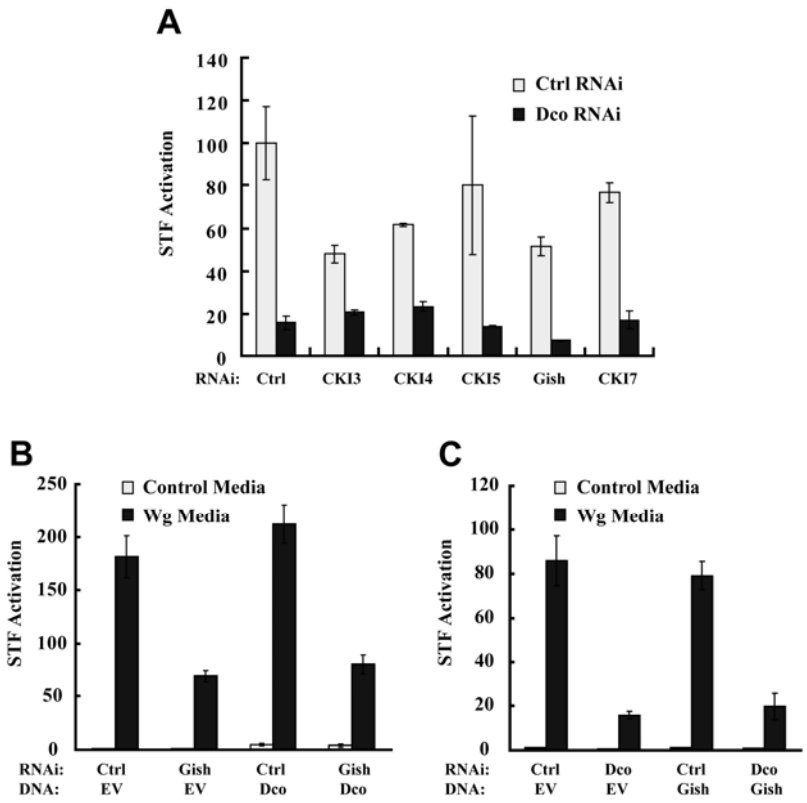


Figure 4.13 Gish is not the redundant partner of Dco in the Wg pathway.
(A) RNAi-based screen for redundant partners of Dco in the Wg pathway. Simultaneous knockdown of *dco* and *gish* shows additive effects to suppress STF activation. (B-C) Gish and Dco fail to rescue the other's loss. Cells were treated with corresponding RNAi for 4 days, and transfected with indicated vectors. 48 hours later, cells were incubated with either control or Wg mediate for 5 hour, followed by standard luciferase assays.

Figure 4.14

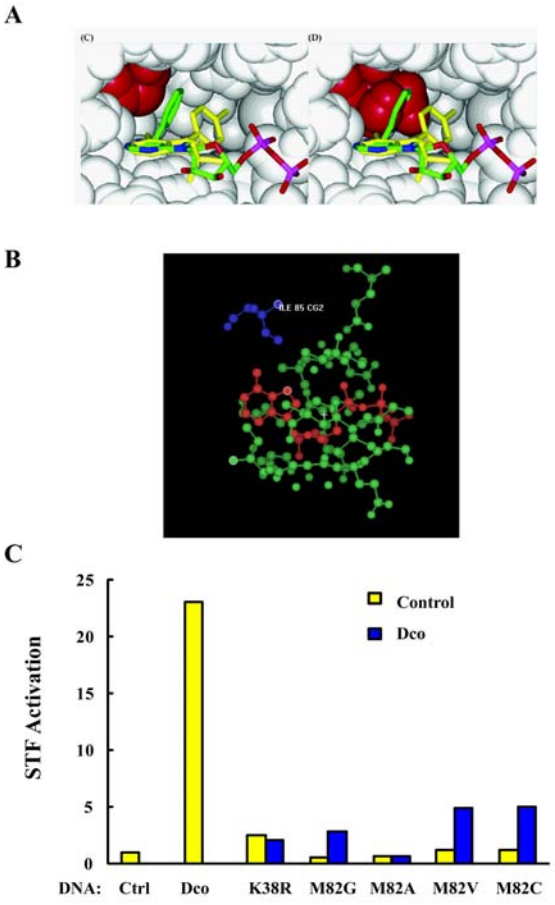


Figure 4.14 Attempted identification of Dco substrate by the Shocat Substrate Screen. (A) Schematic drawings illustrating how engineered ATP binding pocket accommodates bulky ATP analog. Amino acids in the ATP pocket are shown in white filled-space mode, and ATP analog is shown in bar structure. The amimo acid in red shows steric clash with the ATP analog in right panel. When it is mutated with shorter side chain, the clash is removed as in left panel. (B) Partial view of the ATP binding pocket of the yeast CKI (PDB id:1SCN). Through systematic analysis of the CKI structure, I85 (M82 in Dco) was identified as the putative mutation site for the Shokat screen. ATP is in red, the targeted mutation site ILE85 in blue, and amino acids of ATP binding site in green, (C) Dco mutants carrying shorter side chains at the amino acid 82 show dramatically reduced kinase activity, judged by the activation of STF reporters. Dco mutants were expressed alone (yellow bars) or co-expressed with wide type Dco (blue bars) to exam if they act as dominant negative manner (Panel A is adapted from Kraybill et al. 2002)

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CHAPTER V

CONCLUDING REMARKS

SUMMARY OF CONTRIBUTIONS

Apoptosis is an evolutionarily conserved, actively regulated cell death process that plays an essential role during development and in adulthood. Apoptosis is subject to the negative regulation of Inhibitor of Apoptosis Proteins (IAPs). The function of IAPs has to be antagonized to allow the occurrence of apoptosis, while the activation of IAPs promotes cell survival. The goal of my dissertation research was to advance the understanding of IAP regulation during development. Specifically, the two major foci were: a. systematic analysis of *Drosophila* IAP1 (DIAP1) down-regulation in developmental apoptosis in the fly pupal eye; and b. characterization of novel factors that promote cell survival via activation of DIAP1.

RHG protein mediated apoptosis in the pupal eye of *Drosophila*: down-regulation of DIAP1

Drosophila Inhibitor of apoptosis protein 1 (DIAP1) is an essential negative regulator of apoptosis in fly cells. DIAP1 binds to caspases and inhibits their activity. The antagonism of DIAP1 by RHG proteins, e.g., Head Involution defective (Hid), Grim

and Reaper (Rpr), is a central theme in cells committed to apoptosis. Previous studies revealed two major hypotheses, generally referred to as the liberation model and degradation model, which explain how RHG proteins counteract DIAP1's anti-apoptotic function. The liberation model proposes that RHGs induce apoptosis through binding to DIAP1 and replacing the caspases, whereas the degradation model emphasizes the ability of RHGs to induce DIAP1 degradation, releasing the inhibition on caspases. However, these two models were mainly based on *in vivo* overexpression or *in vitro* biochemical assays, provoking an important question which mechanism (liberation or degradation) is more physiologically relevant.

In this dissertation, I demonstrated significant down-regulation of DIAP1 levels in naturally occurring apoptosis in the perimeter ommatidia of the fly pupal eye. Further characterization revealed that the down-regulation is post-transcriptional and independent of effector caspase activity. Using a loss of function approach, I illustrated that the regulation was dependent on Hid, Grim and Rpr. This is the first time that RHG protein dependent DIAP1 down-regulation has been illustrated in naturally occurring apoptosis.

My results also help to resolve the debate around the two competing models. I showed that a partial reduction in RHG proteins suppressed DIAP1 degradation to undetectable levels. Under these conditions, apoptosis was surprisingly initiated at the normal time with comparable strength, but was markedly reduced thereafter. These results argue that the liberation of caspases plays a significant role in apoptosis initiation,

whereas the degradation of DIAP1 is required for timely progression of apoptosis in this tissue.

In addition, I provided evidence that the E3 ligase activity of DIAP1's RING domain is dispensable for RHG-mediated degradation. The data was unexpected as auto-ubiquitination and consequent degradation has been proposed as an important approach to eliminate DIAP1. In contrast, I showed the *Drosophila* Apaf-1 related killer (Dark) and the initiator caspase Dronc are both required for DIAP1 down-regulation, suggesting that RHG proteins may act through Dark and Dronc.

In summary, by studying DIAP1 down-regulation under physiological conditions, I provided new insights regarding the mechanism underlying DIAP1 antagonism in apoptotic cells.

The *Drosophila* casein kinase Iε/δ Discs overgrown promotes cell survival via activation of DIAP1 expression

The development and survival of multi-cellular organisms are dependent on the dynamic equilibrium between cell proliferation and death. For tissue to achieve fast growth, apoptosis has to be kept at low levels while cell division needs to be promoted. Through a genetic screen, the *Drosophila* casein kinase Iε/δ, known as Discs overgrown (Dco), was identified as a negative regulator of apoptosis. Further characterized by Dr. Ju Guan in the Cadigan lab, Dco has been demonstrated to promote cell survival via activation of DIAP1.

In order to reveal the regulation mechanism, I demonstrated Dco mutants don't affect the levels of Hid, Grim and Rpr mRNA, suggesting the regulation is probably at the protein level. I also demonstrated that the kinase activity is required for Dco to suppress apoptosis.

To uncover the underlying molecular mechanism, I established a cell culture system using fly embryonic cell line S2 to study apoptosis and DIAP1 regulation. I showed that the anti-apoptotic role of Dco could be recapitulated in S2 cells, however no influence on DIAP1 levels was observed under a variety of conditions. One attractive hypothesis to explain the absence of DIAP1 regulation was the existence of redundant partners, which possibly is another casein kinase I (CKI).

To identify redundant partners of Dco in apoptosis regulation, I conducted two small scale screens in the fly eye and cultured cells by knocking down expression of the other fly CKI members. Both screens indicated Gilgamesh (Gish), a homologue of mammalian CKIγ, can influence apoptosis. Characterization of Gish showed that it may down-regulate DIAP1 in S2 cells, and partially rescue some phenotypes caused by *dco* depletion in the fly. Further analyses are required to establish the anti-apoptotic role of Gish as well as the redundancy of Dco and Gish.

In summary, via studies on Dco and Gish, I have made contribution to understand how DIAP1 function is reinforced to secure the survival fate of cells. Further studies on

Dco and Gish can potentially uncover a novel regulation pathway that coordinates cell survival and apoptosis.

FUTURE DIRECTIONS

Exploration of the apoptosis regulation via the ubiquitination pathway

The RING domain has been proposed to mediate the auto-ubiquitination and consequent degradation of DIAP1 during apoptosis (Hay and Guo 2006; Steller 2008). Surprisingly, our results suggest the E3 ligase activity of DIAP1's RING domain may be dispensable for RHG-mediated degradation.

One concern with my experimental data is that I examined DIAP1 downregulation in flies that were heterozygous for the RING domain mutant th^{6B}. Mammalian IAPs have been reported to dimerize through the RING domains (Vaux and Silke 2005), raising the possibility that wild type DIAP1 present in the heterozygous cells may ubiquitinate the RING domain mutant *in trans*, leading to the consequent degradation. To address this concern, we need to overcome the technical difficulty to generate homozygous clones of th^{6B}. I am currently using MarcM system to achieve the goal, and the fly stocks needed for the experiments are under construction. If the down-regulation of DIAP1 is still observed in cells homozygous for th^{6B}, it will strongly reinforce our argument that DIAP1's RING domain is dispensable for RHG-mediated degradation. Conversely, if downregulation is not observed when both copies of DIAP1 have RING domain mutations,

this would suggest that DIAP1 may employ a similar dimerization mechanism as its mammalian counterparts. In this case, it would be interesting to conduct biochemical analyses to illustrate the interaction and map the corresponding interaction domain. If the interaction domain is the RING domain as for mammalian IAPs, the physiological importance of the putative dimerization can be easily tested in flies heterozygous for the RING domain deletion $th^{33\text{-}1s}$ (Wilson et al. 2002).

If the RING domain is confirmed to be dispensable, it is likely that DIAP1 degradation in apoptotic cells is mediated by other E3 ligases. Intriguingly, SkpA, an SCF (SKP-Cullin-F-box) multiunit E3 ligase complex component, has been implicated in Rpr- and Grim-induced apoptosis (Wing et al. 2002). SkpA has been shown to interact with an F-box protein Morgue, and Morgue can bind to DIAP1 and promote its degradation (Hays et al. 2002; Wing et al. 2002). It suggests that an SCF E3 ligase complex could target DIAP1 for degradation in peripheral ommatidia. One approach to test this possibility is to examine DIAP1 down-regulation in ommatidia depleted of *skpA* or *morgue* by RNAi. *UAS-skpA* RNAi and *UAS-morgue* RNAi have been obtained from Vienna Drosophila RNAi Center, and they can suppress the small eye phenotype induced by *GMRGal4; UAS-Rpr* (data not shown). Preliminary data showed that DIAP1 down-regulation is partially suppressed by *skpA* and *morgue* RNAi. Further validation is needed to support the hypothesis that DIAP1 down-regulation is facilitated via the *trans*-ubiquitination by the SkpA containing multi-unit E3 complex.

One interesting question is how DIAP1 directs its E3 ligase activity toward different substrates, including Dronc, RHG proteins and DIAP1 itself. A simple scenario which has been suggested is that DIAP1 ubiquitinates Dronc in living cells, while it ubiquitinates itself in apoptotic cells (Chai et al. 2003). Our results clearly suggest the physiological situation is far more complicated, as we found E3 ligase activity of the RING domain contributes to the DIAP1 stability in cells that have a survival fate, whereas it seems dispensable in apoptotic cells. It would be interesting to examine the stability of Dronc in both apoptotic and living cells carrying th^{6B}. This information will be helpful to understand how the E3 ligase activity of DIAP1 is regulated differently in cells with different fates.

In addition, several ubiquitination pathway factors have been implicated in apoptosis regulation, including the E1 ubiquitin-activating enzyme Uba1 (Wing et al. 2002), the E2 ubiquitin-conjugating enzyme UbcD1(Ryoo et al. 2002), the de-ubiquitinating enzyme Fat facets (Wing et al. 2002), as well as SkpA and Mogure. To address the physiological relevance of these factors in apoptosis, it would be informative to observe whether the DIAP1 stability and the occurrence of apoptosis in perimeter ommatidia are affected in flies with these factors either mutated or depleted by RNAi.

DIAP1 regulation in other developmental apoptosis

In Chapter II, I utilized the naturally occuring apoptosis in the developing pupal eye as a model system to address the physiological importance of several popular models about

DIAP1 regulation mechanisms. I obtained several unexpected results which are inconsistent with some of the tested models. This is not totally surprising, since the discrepancy is likely due to the inherent limits of *in vitro* and over-expression analyses that these models are based on. From this perspective, my data illustrates the significance of studies using naturally occurring apoptosis as model systems.

I am currently developing another system that is based on developmental apoptosis during embryogenesis, where most of the developing midline glial cells are eliminated by apoptosis to achieve a well circuited central neural system (Sonnenfeld and Jacobs 1995; Zhou et al. 1995). Hid and Rpr have been proposed to cooperate with each other in a synergistic fashion to achieve this apoptosis (Zhou et al. 1997). It would be interesting to see if DIAP1 is regulated differently during the midline glial apoptosis compared to the pupal eye.

To outline the midline cells, I will express CD8-GFP under the control of a CNS midline precursor cell specific promoter (Bloomington Stock No.9150). Preliminary data illustrated the midline glial apoptosis happens between stage 11 to stage 14 of the fly embryo. However, because cells with survival fates cannot be distinguished from those committed to die, it is hard to judge whether DIAP1 is down-regulated in apoptotic cells (data not shown). To address this issue, I will ectopically express the caspase inhibitor *p35* to produce "undead cells". If DIAP1 is down-regulated during the midline cell apoptosis, lower DIAP1 levels would be observed in some of the GFP positive cells, which

presumably would have died without *p35*. Positive data would extand the physiological importance of DIAP1 degradation during apoptosis, while negative results would be equally interesting as it might indicate that the fly may employ tissue-specific mechanisms to regulate DIAP1 and apoptosis.

Another event that can be potentially developed as a model system to study DIAP1 regulation is the inter-ommatidia cell death that removes extra secondary and tertiary pigment cells in the early pupal eye (Cagan and Ready 1989; Brachmann and Cagan 2003). DIAP1 staining of pupal eyes at 24hr APF shows a highly ordered pattern with different intensities of DIAP1 in inter-ommatidia areas, indicating DIAP1 levels might be regulated at this stage (data not shown). To specifically label pigment cells, homothorax will be used as a marker (Mendes et al. 2006).

Studies in these two apoptosis events may further confirm our results in Chapter II, and may potentially provide new mechanistic insights on how RHG proteins trigger apoptosis via DIAP1 regulation.

Characterization of Gilgamesh (Gish) in DIAP1 regulation

In Chapter III, our results suggest that Gish may regulate DIAP1 stability in S2 cells. Before further characterization, DIAP1 down-regulation resulting from the depletion of *gish* needs to be confirmed. After validation, it would be interesting to determine the physiological importance of this regulation. Our results illustrated that over-expression of Gish causes extra macrochaetes in the scutellum of the adult notum, a phenotype

considered as a consequence of decreased non-apoptotic activity of capases. Therefore, I speculate Gish may regulate DIAP1 in both apoptotic and non-apoptotic contexts.

To explore the anti-apoptotic function of Gish, the levels of DIAP1, active-Drice and TUNEL will be tested in clones of *gish*^{null} in wing imaginal discs. Elevated TUNEL and active-Drice would suggest the role of Gish in promoting cell survival via activation of DIAP1. If *gish*^{null} cells can not survive to the third instar larval stage, *p35* would be expressed to block apoptosis, and in this case only active-Drice level, but not TUNEL, would be informative. One caveat about active-Drice is that it is also activated in non-apoptotic contexts, although the level of Drice activation has been reported to be considerably low (Kuranaga et al. 2006). Meanwhile, the cell survival assay can be performed in S2 cells to show the ability of Gish to suppress Hid-induced cell death.

To test the possibility that Gish regulates DIAP1 in a non-apoptotic context, I would start with the macrochaete phenotype. Previous studies illustrated that DIAP1 over-expression resulted in extra SOP cells labeled by anti-senseless antibody, ultimately causing the extra macrochaete phenotype (Kuranaga et al. 2006). It would be interesting to examine the number of SOP cells in wing discs of *dppGal4;UASgish*, which showed extra macrochaetes in adult previously (Figure 4.9A). *Scabrous-Gal4; UASgish* would be tested too, as *Scabrous-Gal4* drives expression in proneural clusters of the wing discs, and *Scabrous-Gal4; UASDIAP1* shows extra macrochaetes in adult (Kuranaga et al. 2006). If Gish over-expression leads to more SOP cells, loss of function analyses through either

RNAi knockdown or mutant clones would be performed to show the physiological relevance. If positive results are obtained for the non-apoptotic role, it would be interesting to examine the genetic interaction between Gish and dmIKKε, the known DIAP1 regulator during SOP specification (Kuranaga et al. 2006).

At the same time, biochemical assays would be conducted to elucidate the molecular mechanism, for instance, co-immnoprecipitation between DIAP1 and Gish, and *in vitro* kinase assay of Gish with DIAP1 as the putative substrate.

Identification of Dco substrates

In Chapter IV, I initiated a chemical genetic screen, referred to as the Shokat screen, to identify the substrates of Dco. I engineered the ATP binding pocket which potentially enlarges the space to accommodate N-6 substitutions of ATP. Unfortunately, all the DcoM82 mutants show low enzymatic activities in a functional assay carried out in S2 cells (Figure 4.14), causing me to abandon this approach at that time.

I think it is worthwhile considering resuming the Shokat screen, as there are some possibilities to potentially produce a functional Dco mutant for the screen. First of all, DcoM82 mutants cannot use normal ATP, but they might be able to utilize N-6 modified ATP analogues. Thus, we may apply multiple N-6 modified ATP analogues to DcoM82 mutants in *in vitro* kinase assays to identify the best match of ATP and mutants that leads to acceptable kinase activity. In addition, DcoM82V and M82C show some residual kinase activities in the functional assay (Figure 4.14C). Perhaps a high concentration of the right

ATP analogue in *in vitro* kinase assay with DcoM82V or M82C would enable an acceptable level of kinase activity to generate enough phosphorylated proteins for detection. Finally, I am also interested in developing a strategy to rescue the kinase activity by mutating one or two amino acids that are located away from the ATP binding pocket. With helps from an experienced structural biologist, we can evaluate the feasibility of this idea.

In the most optimistic scenario that one of the approaches rescues the Shokat screen, I would like to propose following experiments. First of all, it would be very informative if any known apoptotic factors or DIAP1 regulators are identified from the screen. In this case, the genetic and molecular interactions between Dco and these factors will be investigated after the phosphorylation is confirmed. Meanwhile, other factors will be subject to an RNAi-based functional screen, in order to narrow down to those who affect cell survival and/or DIAP1 stability. Positive picks will be bacterially produced, and verified as Dco substrates in *in vitro* kinase assay. The confirmed factors will be examined whether they can either phenocopy Dco or show the opposite effects in a series of experiments in S2 cells and fly tissues. Finally, the phosphorylation sites will be mapped and consequently mutated to illustrate the importance of the phosphorylation in apoptosis regulation.

Dco/CKIε substrates are missing in several other pathways, for example the Hippo pathway. Thus, if any known factors in these pathways are identified from the screen,

they will be of interests too. Presumably, the success of the screen can be easily extended to other CKI members, for example, Gish.

PROSPECTS

At the very beginning of this dissertation, I emphasized that life and death are two opposite yet interconnected and interdependent forces in multi-cellular organisms. From the perspective of my dissertation, it is interesting to speculate that DIAP1 serves as a coordinator of the two forces, as the activation of DIAP1 promotes cell survival, while the inhibition leads to cell death.

Our knowledge of DIAP1 regulation in apoptosis has accumulated for over a decade. With all great information in hand, I think it is time to synthesize all the information in the context of physiological importance. Luckily, my dissertation study takes a small step towards that direction. In addition, I think the studies on DIAP1 regulation under physiologal conditions hold the promise to uncover how cells control caspases to pursue either apoptotic or non-apoptotic function.

As major regulators of DIAP1, RHG proteins have been established as essential players to initiate apoptosis in *Drosophila*. I think the putative connection of RHG proteins with mitochondria, as well as their potential role to trigger Dark-dependent Dronc activation will continue to attract considerable attention in the near future. As Hid, Grim and Rpr are elegantly regulated at the transcription level, I would like to predict that

studying the *cis*- and *trans*-regulatory elements of RHG genes and how they integrate a variety of death and survival signals may become a new focus of the field.

To take the speculation one step further, I would like to envision that the regulation of IAPs in living cells is far more complex than what we have known: the IAP family should not be a lonely fighter against caspases, and there may be a network of factors supporting and regulating their roles. Our limited understanding of Dco might serve as an example to show how much is missing with our current knowledge on this issue. Future investigations on the regulation of IAP proteins in both apoptosis and cell survival would advance our understanding on how multi-cellular organisms balance the death and survival of cells.

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