GENETIC AND BIOCHEMICAL ANALYSES OF THE NECESSITY FOR CASPASE ACTIVATION BY THE CED4-DOMAIN PROTEINS, APAF-1 AND DARK

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DEDICATION

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GENETIC AND BIOCHEMICAL ANALYSES OF THE NECESSITY FOR CASPASE ACTIVATION BY THE CED4-DOMAIN PROTEINS, APAF-1 AND DARK

by

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Abstract

Activation of caspase proteases by Ced4 domain proteins is a critical step in the induction of programmed cell death, or apoptosis. Understanding of the genetic and biochemical regulation of the mammalian Ced-4 gene, Apaf-1, may be crucial to the understanding of autoimmune diseases, neurodegenerative disorders and cancer progression. Located in chromosomal band 12q22, Apaf-1 is in a locus frequently deleted in Male Germ Cell Tumors (GCT's). Though not homozygously inactivated in these tumors, Apaf-1 mediated caspase activation is impaired in GCT cell lines and may be a frequent event in other cancer types. Analysis of human genomic DNA facilitated the discovery of the homologous gene DARK, the Drosophila Apaf-1 Related Killer. Hypomorphic alleles of DARK cause developmental disruption, including wing defects, body wall defects, supernumerary bristles, male sterility and an enlarged nervous system. Mutation of DARK potently suppresses the apoptotic function of the genes reaper, grim, and hid. Recombinant Grim protein was shown to antagonize IAP-mediated caspase inhibition in vitro. Peptides corresponding to the conserved Amino-termini of the reaper, grim and hid genes could compete for a binding site also used by the mammalian anti-IAP protein SMAC to block IAP-caspase interaction. Despite these peptides' failure to allow for reconstitution of caspase activation in vitro, genetic inactivation of IAP's leads to significant activation of caspases in vivo. DARK plays a critical role in caspase activation in vivo, and mutations of DARK suppress several genetic measures of cell death due to IAP inactivation. These studies show DARK to be an important apoptosis gene in the fly and necessary for caspase amplification and apoptotic initiation in certain cell death pathways. Further understanding of the regulation of cell death in the genetically tractable Drosophila model may help shed light on the regulation of apoptosis in human cells and disease states as well.

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Apaf-1	Apoptotic Protease Activating Factor 1
AFC	7-amino-4-trifluoromethyl coumarin
ATP	Adenosine Triphosphate
BIR	Baculovirus Inhibitory Repeat
Bp	Base pairs
BSA	Bovine Serum Albumin
CARD	Caspase Recruitment Domain
Ced	Cell death (gene class)
CEPH	Centre d'Etudes Polymorphiques Human
CHO	Chinese Hamster Ovary
CytC	Cytochrome C
DARK	Drosophila Apaf-1 Related Killer
DMSO	Dimethyl Sulfoxide
DTT	dithiothreitol
dATP	Deoxy Adenosine Triphosphate
EST	Expressed Sequence Tag
FISH	Fluorescence In Situ Hybridization
GMR	Glass
GCT	Germ Cell Tumor
GST	Glutathione-S-Transferase
Grm	grim genetic symbol
Hid	Head Involution Defective gene
IAP	Inhibitor of Apoptosis
PCR	Polymerase Chain Reaction
PFU	Plaque Forming Units
RH	Radiation Hybrid
rpr	Reaper (genetic symbol)
SL2	Schneider's Cell Line number 2
SMAC	Second Mitochondrial Activator of Caspases
SSC	Succinate Socium Chloride Buffer
TUNEL	Terminal Uridine Nicked End Labeling
UTR	Untranslated Region

CHAPTER ONE
General Introduction

Many organisms in the metazoan lineage can selectively remove populations of their cells in a distinct, intentional manner dubbed apoptosis.[1] Apoptosis plays an essential role in the maintenance of a normal balance between cell types and of a normal number of cells. The process know as homeostasis envolves the removal of unnecessary cells during development, selecting and winnowing the immune system, and the sacking of damaged or diseased cells(Barres *et al.* 1992),. In perhaps its most common form, the culling of cells in a mature tissue to balance cellular proliferation, the process is so subtle that it was overlooked for many years. Recognized originally in 1972 as a histologically distinct sequence of events by Kerr, Wyllie, and Currie, apoptotic cells progressively detach from the surrounding parenchyma, undergo nuclear condensation, and finally fragment into small vesicles that are engulfed and cannibalized by their neighbors (Kerr et al, 1972). From its somewhat quiet discovery, apoptosis has emerged as an exquisitely regulated biological process that plays important roles in development, neural wiring, and regulation of the immune system. Because of these important roles, apoptosis and breakdowns in the apoptotic pathway are fundamental areas of research with relevance to such human diseases as autoimmune disorders, neurodegeneration, cancer and ischemic damage such as heart attacks or strokes.

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[1] Regarding the etymological debate over the pronunciation of the word "apoptosis", I quote the original paper (and footnote) on the subject.

> We are most grateful to Professor James Cormack of the Department of Greek, University of Aberdeen for suggesting this term. The word "apoptosis" (αποπτωσισ) is used in Greek to describe the "dropping off" or "falling off" of petals from flowers, or leaves from trees. To show the derivation clearly, we propose that the stress should be on the penultimate syllable, the second half of the word being pronounced like "ptosis" (with the "p" silent), which comes from the same route " to fall", and is already used to describe the drooping of the eyelid
>
> Kerr *et al.* 1972

In the simplest of models for apoptosis, the worm C. elegans, the system is completely nonessential and results in the seemingly innocuous phenotype of a total cell count in the adult that is increased by 100 or so cells (Ellis and Horvitz, 1986). Comparitively, several mutations in the apoptosis genes of larger and more complex flies (White *et al.* , 1994) and vertebrates cause developmental lethality or compromise the immune system. (Cecconi *et al.* , 1998; Yoshida *et al.* , 1998, Honarpour *et al.* 2000). While these specific differences may imply varying levels of importance to the role of apoptosis within a given species, they stress the ancient lineage of this pathway and its striking conservation through over 600 million years evolution. Throughout all of these systems, three components have proven central to the control and execution of apoptosis- the ced-4 family, the caspase family and the Bcl-2 family.

Paring the system down to its simplest scheme, the genetic system of *C. elegans* provided the initial appreciation of these preserved elements. Given the absolute conservation in body plan in the nematode line, extensive study generated a "fate map" that makes clear the results of all cell divisions within the worm (Sulston et al, 1983). A genetically controlled program ablates 131of 1090 somatic cells in every normally developing *C. elegans* nematode. Three mutations defective in this cell death or *ced* genetic pathway were identified as necessary regulators of these occurrences. Two genes, ced-3 and ced-4 were required for cell death to occur, while an additional loci, ced-9, negatively regulated these genes (Ellis and Horvitz, 1986, Hengartner et al, 1992). The cloning of these genes elucidated their correspondence with the orthologous vertebrate genes (Yuan *et al.* 1993, Hengartner and Horvitz, 1994, Yuan *et al.* 1992).

Mammalian homologues of these genes provided limited mechanistic insight into the proteins at first. It was recognized that the protein ced-3 was related to a family of proteases that is now called the caspases (Yuan *et al.* 1993). These enzymes have cysteine active site with substrate specificity for aspartic acid at the P1 site and were recognized at the time to be involved in processing of the cytokine IL-1Beta (Nicholson *et al.* 1995). Once the connection to apoptosis was made by ced-3, inhibitor studies of related proteases validated these proteases as having a clear role in mammalian cell death models (Los *et al.* 1995, Enari *et al.* 1995, Tewari and Dixit, 1995) and several groups cloned caspases that contributed to cell death in mammalian systems (Muzio *et al.* 1996, Fernandes-alnemri *et al.* 1994, 1995a, 1995b; Kumar *et al.* 1994 , Miura *et al.* 1993, Thornberry and Molineaux 1995, Wang *et al.* 1994, Vincenz and Dixit 1997). This deluge of cloning of mammalian homologues of ced-3 pointed out a crucial difference between the *C. elegans* and vertebrate pathways. Ced-3 is the only caspase necessary and involved in cell death in the worm (Yuan *et al.* 1993, Shasham 1998) whereas an expansion of the caspase family through evolution provides a large array of caspases with specialized functions.

This pattern of conservation and expansion was also seen with the pro-survival ced-9 gene. Independent work on B-cell follicular lymphomas identified a novel oncogene called Bcl-2 (Tsujimoto et al, 1984). Bcl-2 was a revolutionary oncogene at the time because it was the first gene shown to promote tumorigenesis by blocking cell death in lieu of promoting cell proliferation (Garcia *et al.* 1992). When ced-9 was cloned, it was shown to be homologous in structure and function to Bcl-2 (Hengartner and Horvitz, 1994) and Bcl-2 was even able to function to rescue the phenotype of ced-9 mutant worms (Vaux *et al.* 1992). Further work

again demonstrated an expansion in this family in the vertebrate lineage as additional members of the Bcl-2 family were cloned from the human genome (Boise et al, 1993, Lin *et al.* 1996). However, the function of this family soon would prove to a great riddle.

Bcl-2 localizes to the outer leaflet of the mitochondria and the nuclear envelope (Hockenbery et al, 1990). With no clear function, investigators searched for regulators of the anti-apoptotic gene and found, to the surprise of many, a novel Bcl-2 related gene called Bax (Oltvai et al, 1993) Bax could dimerize with itself and Bcl-2, and paradoxically accelerated the apoptosis that Bcl-2 prevented. The balance of these two proteins provides a rheostat for the cell, with the ratio being able to sensitize the cell to apoptotic stimuli. While this discovery provided a framework for research, critical evaluation of the model proved difficult until more light was shed on the third gene family identified in the *C. elegans* screen.

With this critical mass of information, a veritable chain reaction took place in the vertebrate apoptosis field over the next few years. Aided by the EST databases, researchers quickly expanded the number of players regulating apoptosis (Kiefer *et al.* 1995, Lin *et al.* 1996, Inohara *et al.* 1998, Guo *et al.* 2001, Ke et al, 2001). Now with the genome of representatives of the three studied lineages complete, the expanded list is believed to be complete (Aravind *et al.* 2001). The catalog shown in Table one is remarkable on several points. Firstly, one notices the economy of the *C. elegans* system which reports only one member of each class as being necessary for normal cell death. Yet, even this is a deceptive simplicity as there is an alternate splicing form of Ced-4, Ced-4s, which has been reported as an additional anti-apoptotic regulatory protein in this

Table one- Orthologous and homologues genes to the three central apoptosis-related protein families

Organism	Ced-4 domain proteins	Caspases	Bcl-2 family proteins
C. elegans	Ced-4l Ced-4s isoform	Long Prodomain Ced-3 Short-prodomain CRP1,2,3-(uncertain function in apoptosis)	Anti-apoptotic Ced-9 BH3 only Egl-1
D. melanogaster	DARK(dAPAF1,HAC) DapafS isoform	Long Prodomain Dronc Dredd STRICA Short-prodomain Dcp1 Drice DAMM Decay	Pro-apoptotic BORG(Buffy) DeBcl Anti-apoptotic Unknown if any are dual function BH3 only Unknown
Humans	Apaf-1 Apaf1-L isoform Apaf-1XL isoform	Long Prodomain Caspase-9 Caspase-8 Caspase-10 Caspase-11 Caspase-2L Short-prodomain Caspase-3 Caspase-6 Caspase-7 Caspase-12 Caspase-13 IL-1/Immune Subclass Caspase-1 Caspase-4 Caspase-5	Pro-apoptotic Bax Bak Bok Anti-apoptotic Bcl-2 Bcl-xl Mcl Bcl-w Bcl-G Bcl-B BH3 only Bid, Hrk, Bim, Puma, Noxa

pathway. Despite the presence of three caspase related proteins, known as csp-1, csp-2, csp-3 in the nematode which can cleave each other and Ced-3, only Ced-3 is necessary for cell death to occur (Shasham 1998). Also, a gene called egl-1 was identified as being essential for apoptosis, despite being missed in the primary screen. This was shown to be a member of a subset of the BCL-2 family known as the BH3 proteins that acts to regulate other BCL-2 members (Conradt *et al.* 1998). Because the regions of homology measure only 10-15 amino acid helix forming region with identified proteins (Zha *et al.* 1996), BH3 proteins have been the most difficult apoptotic regulators to identify. Several putatutive vertebrate BH3 only proteins have been identified, though no such Drosophila proteins have been found yet (Inohara *et al.* 1997, O'Connor *et al.* 1998, Yu *et al.* 2001, Oda *et al.* 2000, Nakano and Vousden 2001). The final number of true BH3 proteins and their role in apoptotic signaling remains unknown.

The Drosophila genome shows the initial trend of expansion of the core orthologs to Ced3, Ced-4 and Ced-9. This expansion occurs mainly in the small-prodomain caspases that function chiefly in the execution phase of apoptosis, but not apparently in the initiating phase (Aravind *et al.* 2001). Along with the vertebrate caspases, this pointed out a distinction that could be made based on caspases that contain only the catalytic domain with a small N-terminus, and caspases that contain a the catalytic domain with a longer N-terminus typically with a homophilic interaction domain. Drosophila also has a single pair of proteins that correspond to the initiating duo of Ced-4 and Ced-3 in the worms. As I will show in later chapters, this role is played by the Apaf-1/Ced-4 homolog called DARK and a caspase, Dronc. These proteins interact through a homophilic interaction domain termed the Caspase

Recruitment Domain (CARD). This single CARD:CARD pair of protease and activator is also present in Ced-4 and Ced-3 and is a conserved pattern even into the vertebrate line. The extensively studied Apaf-1/Caspase-9 interaction provides the paradigm for the other two mainly genetic systems. Folding into a six-helix bundle, the CARD domain has two surfaces that provide a conserved interaction motif. The Apaf-1 CARD uses a highly acidic patch that interacts with a basic patch on the caspase-9 counter-part (Qin *et al.* 1999). The CARD domain in the vertebrate lineage is present in an additional caspase and adapter molecule pair Raidd and Caspase-2 that play an unclear role in membrane receptor signaling (Chou et al, 1998). The CARD domain has also been co-opted by other kinase pathways that play a role in the innate immune system by regulating NF-KB (Hugot *et al.* 2001, Inohara *et al.* 1998, Koseki *et al.* 1998).

Two other long-prodomain caspases have been identified in Drosophila. The genome project identified an uncharacterized protein called STRICA (Doumanis *et al.* 2001) while more extensive analysis has been performed on a protein called Dredd (Rodriquez *et al.* 1998). Dredd's structure and function provide an interesting analogy for how these pathways have increased in complexity. Dredd appears to function in cell-death pathways due to its loss of function genetic phenotype which partially phenocopy defects seen in other apoptosis genes (Antony Rodriquez pc.) Additionally, overexpression in cell culture results in cell killing. Dredd, though a long prodomain protease, does not contain a CARD domain, but a novel domain. The addition of different prodomains has allowed the caspases to be recruited to novel signaling complexes beyond the prototypic Ced-4 domain.

The process has also occurred in the vertebrate system resulting in the generation of the Death Receptor class of proteins that can recruit caspases to ligand-bound receptor proteins. Through a domain called the Death Effector Domain (DED) which is strikingly similar in tertiary structure to the CARD domain, caspase-8 can be recruited to ligand bound receptors and activated. Adapter proteins, such as Fadd or Tradd, provide surfaces for homophilic interactions between these proteases and their cognate receptors and help form a large signaling complex around the receptor (Kischkel *et al.* 1995, Hsu *et al.* 1996). The model protein, Fadd contains a Death Effector Domain (DED) as well as a Death Domain (DD) (Chinnaiyan *et al.* 1995). A homophilic interaction between Death Domains on the adapter and the receptor allows for the clustering of the zymogen form of the proteases, and their autoactivation through what is currently believed to be an "induced proximity" model (Vincenz and Dixit 1997, Muzio *et al.* 1998). This model proposes that the local increase in concentration of proteases at the receptor complex is sufficient to allow the low activity present in the zymogen form to activate neighboring proteases and start a chain reaction. These pathways have been extensively used in the vertebrate immune system to regulate lymphocyte development, zones of immune protection, and in the removal of unnecessary lymphocytes after proliferation, and they have been studied extensively in the mouse models for autoimmune disease as the lpr (Fas receptor mutant) and gld (fas ligand mutant) strains (Griffith *et al.* 1995, Roths *et al.* 1984, Suda *et al.* 1993 Watanabe-Fukunaga *et al.* 1992). Mutations have been identified in patients suffering from type Ia autoimmune lymphoproliferative syndrome (Martin et al, 1999). These pathways also appear to regulate

development, as homozygous mutants of Caspase-8 and FADD are embryonic lethal due to dysfunction in cardiovascular formation and abdominal hemorrhage (Yeh *et al.* 1998). While a homolog of Fadd is clearly present in Drosophila, it is not clear if the entire Death Receptor pathway is intact (Hu and Yang, 2000). The prodomain of Dredd does have low levels of homology to the Death Domain, but no dipteran Death Receptors have been identified to date (Chen *et al.* 1998). But the known function of Dredd highlights how this class of proteases has expanded in function from being strictly apoptotic, to playing new cellular roles. Dredd has been identified genetically as being necessary for activating the innate immune response. . Without Dredd, a NF-Kb homologue, Relish, is not activated by cleavage (Stoven *et al.* 2000, Leulier *et al.* 2000). Such additional roles are also present in the vertebrate immune system where caspases also play an important role (Thornberry and Molineaux 1995). A subset of the mammalian caspases have been shown to play an important role in the processing of cytokines, specifically, IL-1beta. Thus, the expansion and diversification of the caspase family has allowed for more context specific roles for the proteins, and their participation in biological pathways beyond the pale of apoptosis. Dredd's many isoforms also point to the increasing complexities present in the system of more complicated organisms. Dredd is present in four isoforms with varying lengths of the prodomain portion of the molecule (Chen *et al.* 1998). Only one isoform has cell killing activity in transfection assays (See chapter 3). As the case of Ced-4 points out, the presence of different isoforms allows for dominant negative or competitive inhibitory forms. This may allow for a more subtle regulation of the sensitivity of a given cell type to an apoptotic stimulus or an immune response. Alternate splice forms with different activity have also been

reported for all three families in the mammalian apoptosis pathway including Apaf-1, ced-4, Bcl-x, and caspase-9 among others indicating alternate slpicing may play a role in the control of apoptosis (Seoh and Billiar, 1999, Hengartner and Horvitz 1994, Boise *et al.* 1993, Zou *et al.* 1998)).

In the subsequent chapters I will discuss my investigations into the importance of the Ced-4/Apaf-1 pathway in mammalian cells, and the identification of the Drosophila homologue of Ced-4 and Apaf-1, DARK. The Ced4 domain differs from the trend towards expansion seen in the other two domains. In all three genomes, only one protein with a Ced-4 domain has been identified. This points to the unique role of these Ced-4 proteins as a link between the many different signals that regulate the decision to commit to cell death and the effector molecules that execute the cell death program. Ced-4, identified genetically, provided the founding model of these proteins as cell death activators. The mammalian homologue, Apaf-1, was identified using biochemical means, and provided the first insight into the function of these proteins. Comparison of Ced-4 and Apaf-1 showed the overall conservation of the domain structure with an N-terminal CARD domain, followed by a Ced-4 domain characterized by signature nucleotide binding Walker A and Walker B motifs. The C-terminal regions of the proteins differ but are both proposed to be inhibitory regulatory domains. The model for the action of the Ced-4 proteins is now generally agreed upon as follows. In the basal state, an inactive form of the Ced-4 protein which masks its CARD domain is resident in most cells. Following signals regulated by the Bcl-2 family, the protein is activated to allow oligomerization and to expose the CARD domain and recruit a partner caspase zymogen. The zymogen is then activated by interaction with the Ced-4 protein and

promotes cleavage of subsequent proteins that induce the characteristic morphological features of cell death such as nuclear condensation and cellular blebbing. Recent structural studies have provided a fascinating picture of what this "apoptosome" looks like on a low resolution. Within this general picture each species appears to have unique variations on the mechanism of activation, as the biochemical characterization of these molecules in this thesis will document. With the majority of the components of the apoptotic regulatory pathway now identified, the stage is set to complete the characterization of the regulation of the CED-4 molecules in each system and appreciate the evolutionary similarities and deviations. The increasing understanding of the molecular sequence of events also offers the hope of best understanding which point in the apoptotic pathway is the best entry for intervention in efforts to alleviate the symptoms or pathology in such devastating diseases as Lou Gehrig's disease (ALS), heart attacks, drug-resistant cancer, strokes, and lupus (Nakagawa *et al.* 2000, Namura *et al.* 1998, Sanchez *et al.* 1999). Because of the importance of the Ced-4 pathway, I have focused my research into understanding this pathway in mammalian cells as a candidate tumor suppressor and understanding how the regulation of the evolutionary homologous Drosophila system can help shed more light on the on the vertebrate apoptotic cascade.

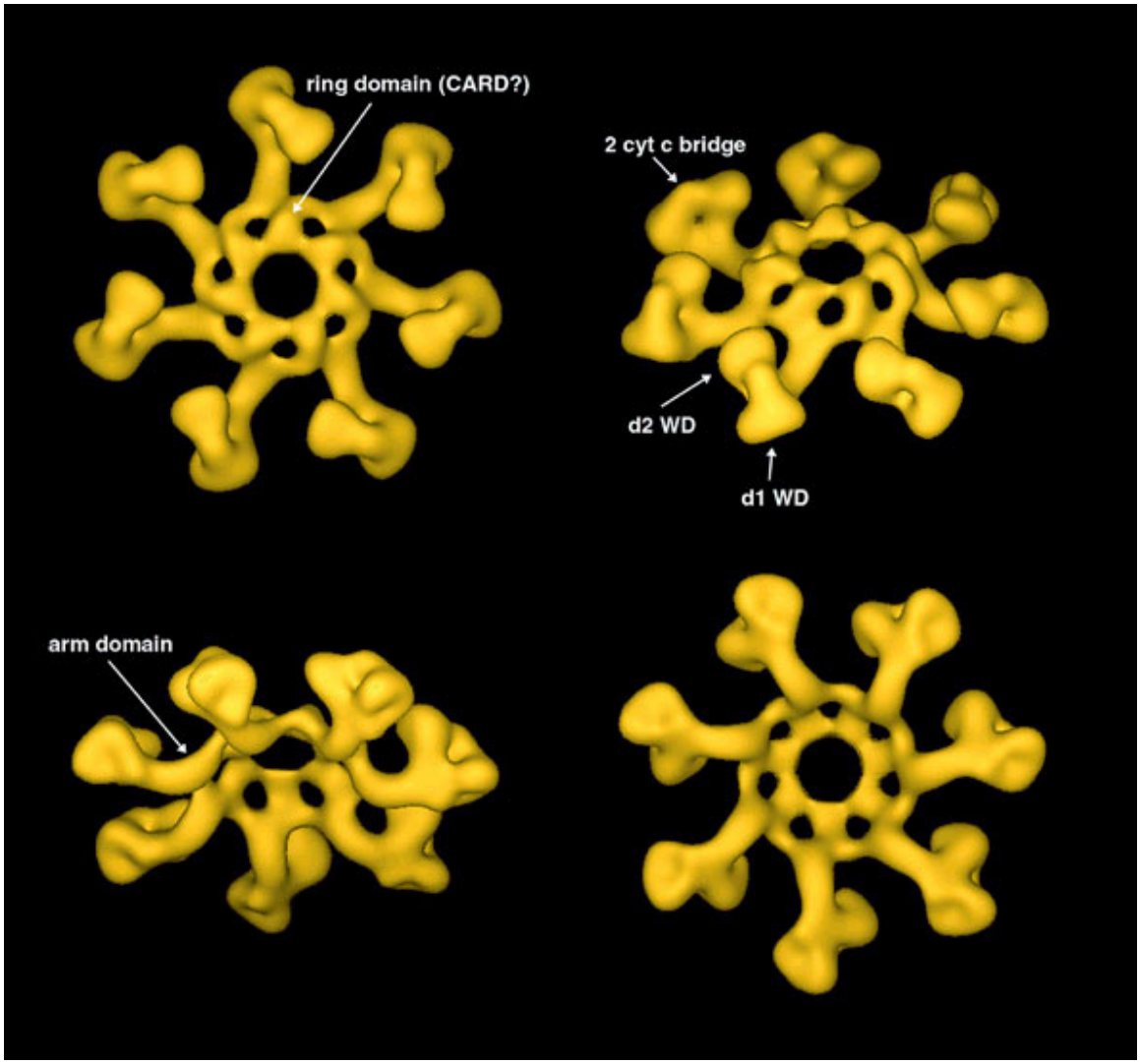


Figure One. Reconstruction of the Apaf-1-Cytochrome c complex from electron microscopy from Devrim et al 2002 (Courtesy Xuejun Jiang and X.W.)

CHAPTER TWO
Review of the Literature

While genetic studies unveiled the initial organization of the genetic program of apoptosis, biochemistry provided an unexpected insight that helped organize and direct the research of the field. In the mid-1990's the genetic relationships between the principal players seemed clear, the caspase proteases represented the effector arm of the pathway, and their activation from the zymogen form was positively regulated by ced-4 and negatively regulated by ced-9 gene (Yuan et al 1993, Vaux et al 1992). But this jump from inactive protease to active protease was the key step in apoptosis as it was understood at this time. While the mechanism leading to the activation of caspases by the death receptors, such as fas, were better understood (Enari et al 1995), the understanding of the mammalian pathway for caspase activation homologous to the genetic models was more unclear. Searching for activities that stimulated this step in apoptotic extracts initially yielded only the fact that the caspases could activate each other (Wang et al 1996, Pan et al. 1998a). This made activated caspases in apoptotic extracts overwhelming signals in the search for the initial activating steps. Thus, the initial report of a protein factor that stimulated caspase processing from a non-apoptotic cell lysate was quite a discovery. Shockingly, the protein factor isolated was one of the most studied proteins of all time, cytochrome c (Liu et al 1996). As controversial as this may have been, the observation would hold true and provide the key to unlocking the mechanism of caspase activity in mammalian cells.

Wang and colleagues identified dATP as a stimulatory compound that promoted the processing of procaspase-3 to its activated form in normal cell extracts (Liu et al. 1996).

Using this insight and a straightforward assay, the activity could be separated into three components. Cytochrome c was the first component identified, and the dATP-dependent activation of caspase could be clearly shown to be dependent on this protein. Extracts prepared with osmotically protected mitochondria were devoid of this activity unless purified cytochrome c was reintroduced (Liu et al. 1996). Cytochrome c was soon shown to be an excellent marker for apoptosis in vivo, as the mitochondria in apoptotic cell could be seen releasing cytochrome c into the cytosol (Yang et al 1997, Kluck et al 1997). This event also correlated with the expression of Bcl-2, with Bcl-2 overexpression resulting in both a block of apoptosis and a block of cytochrome c release. This would prove to be the first clear link between these biochemical findings and the genetic foundations laid previously.

The dATP-dependent assay yielded two more essential proteins to the process of caspase activation that would close the loop. Cytochrome c dependent caspase-3 processing would require a known caspase zymogen, procaspase-9, and a novel protein, Apaf-1 that was the human homolog of ced-4 (Li et al 1997, Zou et al 1997). These findings immediately allowed for a clear model to emerge that has stood up to rigorous experimental validation. Apaf-1 resides in the cytoplasm in an inert state, as does procaspase-9 (Liu et al 1996, Li et al 1998). After apoptotic stimuli are integrated by the bcl-2 family members, the safety catch of Apaf-1/ced4 action is removed and allows for the activation of a caspase recruited by N-terminal CARD domains (Yang et al. 1996, Kluck et al 1996, Li et al 1998). This general outline, while providing a unifying explanation for these genetic modules in any system, would soon differentiate the different genetic systems when explained in detail. The

mammalian system could clearly be shown to be dependent on an activation step controlled by cytochrome c.

This finding that provided clarity in the vertebrate system proved confounding and unverified in the other two experimental models. No evidence of cytochrome c release to the cytosol could be found in Drosophila cells (Varkey et al 1999). Expression of the *C. elegans* proteins heterologously in mammalian cells indicated that Ced-9 bound directly to Ced-4 to prevent it from activating the paired caspase Ced-3 (del Paso et al 2000, Wu et al 1997a, Wu et al 1997b, Hisahara et al 1998). Several groups tried to show similar ternary complexes of the mammalian proteins (Song et al 1999, Pan et al 1998b, Hu et al 1998a), only later to have this data rigorously attacked and refuted by other researchers (Conus et al 2000, Newmeyer et al 2000, Haussman et al 2000). Because of the experimental limitations of each system, sorting out this discrepancy has proven difficult. No recapitulation of the dATP-dependent caspase assay has been achieved to date in *C. elegans* or Drosophila protein extracts. However, reverse genetics using murine homozygous deletion of the three proteins factors involved in the mammalian activity- procaspase-9, Apaf-1 and cytochrome c have all shown deficiencies in damage inducible cell death, and dATP- dependent caspase activation (Cecconi et al 1998, Honarpour et al 1999, Yoshida et al 1998, Kuida et al 1998, Hakem et al 1998, Li et al 2000). This tour-de-force has essentially quieted debate on the details of the ced-4 domain mediated caspase activation of the mammalian system, and it sets the general paradigm for future experimentation in the other model systems with a recognition that significant variation in some of the details may exist despite the extensive conservation of the core machinery of apoptosis.

Once the importance of cytochrome became apparent from in vitro experiments, examination of its movement in vivo was clearly an important question. The Bcl-2 family of proteins proved to be the deciding factors in the release of cytochrome c from the mitochondria (Yang et al 1997, Kluck et al 1997). While the list of pro-apoptotic and anti-apoptotic members grew and grew, the core question of how they regulated cytochrome c remained difficult to address. The mechanism could as easily be explained by the anti-apoptotic proteins preventing the deadly members from causing the release, as positing that the pro-apoptotic proteins disabled a critical function of the protective members that preserved mitochondrial integrity. The first clues came from following the movement of proteins within the cell.

Cell biology using antibodies and fluorescently tagged proteins pointed to a movement of the pro-apoptotic Bcl-2 members such as Bax onto the mitochondrial surface (Wolter et al 1997). Although Bax was often resident to some degree on the surface of mitochondria in normal cells, a certain fraction of the protein remained in the cytoplasm. This was unexpected for a protein with a transmembrane anchor, but structural studies would prove that the anchor could be tucked into a cleft on the protein's surface for storage (Suzuki et al 2000). After a treatment to induce cell death such as staurosporine, the Bax would mobilize from the cytosol and stably integrate into the outer membrane. Similar movements were also documented for the pro-apoptotic BH3 proteins such as Bid, Bim, or Bad which would move from the cytosol to the mitochondria after proper stimulation (Inohara et al 1997, Hegde et al 1998, Luo et al 1998, Li et al 1998, Yang et al 1995, O'Connor et al 1998, Puthalakath et al 1999). These observations pointed to two critical features, proapoptotic

protein addition to the mitochondria as the trigger, and the common BH3 helix as being an important trigger. Cytochrome c release could be reproduced in isolated mitochondria with the addition of Bax, Bid or even just BH3 peptides (Jurgensmeier et al 1998, Luo et al 1999).

An additional line of evidence would lead to a great controversy over the next steps in cytochrome c release. It had been observed that loss in the electrochemical proton gradient ($\Delta\Psi m$) across the mitochondrial inner membrane was associated with cell death (Zamzaniet al 1996). Loss of $\Delta\Psi m$ is also seen when a channel called the Permeability Transition Pore (PTP) is put in a high conductance state. Also, activators of the PTP, like atracyloside, could induce apoptosis and cytochrome c release while inhibitors such as cyclosporin A or bongkreic acid could inhibit the release (Zamzami et al 1996). While cytochrome c release could often be observed without any change in $\Delta\Psi m$, loss of $\Delta\Psi m$ could reproducibly cause cytochrome c release. The PTP is a complex structure composed of several proteins and spanning the inner and outer mitochondrial membranes. The main proteins include the Adenine Nucleotide Transporter (ANT), the Voltage Dependant Anion Channel (VDAC), as well as cyclophilin D. Evidence that Bax could interact with ANT provided an exciting opening to the field. The role of ANT was also supported by changes in the exchange of ATP and ADP when cytokine–dependent cells were deprived of IL3 and Bcl-xl could regulate this process (vanderHeiden et al 1999). ANT was also isolated in a screen for genes that caused apoptosis when overexpressed. Furthermore, homozygous mutation of Bax made cells resistant to treatment with atracyloside (Marzo et al 1998).

Various systems that tried to test this pathway more directly have yielded conflicting results and cast doubt on the relevance of this interaction. Attempts to analyze this system

using an artificial yeast phenotype led to contradictory findings. Bax overexpression in yeast could cause a toxic effect by targeting mitochondria, but whether this system truly recapitulates the effect of the protein in vertebrates has never been clarified. In the hands of different groups the system was alternatively used to prove or disprove the involvement of ANT, VDAC, and even F_O/F_1 ATPase in Bax induced cell death (Shimuzu et al 2000, Priault et al 1999, Matsuyama et al 1998, Gross et al 2000). The contradictions and lack of consistent results in the whole body of work raise concerns about the yeast system's relevancy. Attempts to reconstitute a pore to release cytochrome c also produced conflicting results. Tsujimoto and colleagues reconstituted the VDAC channel in liposomes, and demonstrated an effect by both Bcl-xl and Bax on the channel in method consistent with their roles in vivo. Other groups formed pores in liposomes which release cytochrome c or similarly sized markers using either Bax and ANT or Bax alone (Brenner et al. 2000, Shimuzu et al 1999). Another proposed model is that the proteins disrupt the normal function of the mitochondria through the ability of the proteins to form ion-conducting pores. This mechanism was suggested by the similarity in structure of the Bcl-2 members to bacterial pore-forming toxins. Aberrant ion conduction might cause a non-specific swelling and rupture, rather than relying on the proteins to form a direct protein conduction pore. Again, the varying camps have yet to agree on the necessary model to encompass all of these conflicting results.

Accumulating data with mouse genetics is providing a more consistent story. While individual mutation of a given proapoptotic member of the Bcl-2 family had little effect on normal development, closer examination could demonstrate clear defects. This was not

unexpected given the significant redundancy built into this expanded gene family. Crossing strains of mice with Bax and Bak mutations results in progeny with a profound defect in cell death throughout the animals (Lindsten et al 2001). Cell lines derived from these mice are resistant to many toxic stimuli including ionizing radiation, DNA damaging agents, and the BH3 protein tBID (Lindsten et al 2001, Wei et al, 2001). Sensitivity to similar agents and BH3 peptides could be restored only through the restoration of Bax or Bak, but not by the addition of BH3 only peptides (Zong et al. 2001). These new findings seem to organize the cell death pathway in the mitochondria around the activity of the pro-apoptotic members of the family such as Bax and Bak. Anti-apoptotic members can protect the cells by direct interaction with these proteins, and by indirect methods, possibly related to the proteins' ability to form ion conducting pores (Antonsson et al 1997, Schendel et al 1997). BH3 only proteins can trigger the activation of the pro-apoptotic Bax or Bak by dissociating them from the protective Bcl-2 or Bcl-xl, causing conformational changes in the pro-apoptotic members, and possibly inactivating the alternative mechanisms by which these proteins protect cells (Yang et al 1995, Kelekar et al. 1998, Eskes et al 2000). As discussed above, the final events that lead to the exodus of cytochrome c into the cytosol are still unclear although recent electron microscopy has identified large clusters of Bax or Bak on the edge of mitochondria in apoptotic cells that may be an intermediate step in cytochrome c release (Nechushtan et al 2001).

Because of its ready biochemical manipulation, the mammalian system has provided the most detailed studies of the mechanism of caspase activation by the ced-4 class of proteins after the decision-making by the Bcl-2 family. The ced-4 proteins maintain an

overall conserved layout with an N-terminal CARD domain followed by the ced-4 module and a more variable c-terminal region. Apaf-1's regulation is better characterized as this region contains 12-13 repeats of the WD module. This motif folds to form a propeller blade structure that can be assembled into fan-like structures with variable numbers of repeats (Garcia-Higuera et al. 1996). Apaf-1's WD repeat region has been shown to inhibit the ced-4 domains cell-killing activity. Two-hybrid analysis shows that the WD repeats can interact directly with the ced-4 domain, and co-expression of WD-repeats can suppress cell killing by a truncated protein that is constituitively active (Hu et al. 1998.) Apaf-1 can be cleaved between its Ced-4 domain and WD repeats by caspases during apoptosis (Lauber et al 2001), but the role of the truncated protein in vivo in unclear. The mechanism of this inhibition appears to be through reducing the affinity for adenosine trinucleotide binding to the Ced-4 or sterically hindering their binding (Jiang and Wang 2000). It is unclear if the WD repeats serve other functions in the fully assembled apoptosome, but truncation of the WD repeats cause the mutant protein problems in the turnover of caspase-9 on the complex (Saleh et al. 1998). Future structural studies are required to clarify this question.

The WD repeats mediate the binding of cytochrome c to Apaf-1. This binding utilizes a broad surface area of the cytochrome c protein. Mutational analysis of cytochrome c shows that basic residues dispersed around the three dimensional structure contribute to binding, specifically lysines 7, 8, 25, 39, and 72. These mutations as well as a loop structure and the lack of trimethylation of lysine 72 distinguish vertebrate cytochrome c from the highly conserved fungal protein in its ability to stimulate Apaf-1 activity (Kluck et al 2000, Yu et al 2000). The interaction of cytochrome c and Apaf-1 is stable (Purring-Kock and

McLendon 2000), and cytochrome c remains associated with Apaf-1 when it forms oligomeric structures (Zou et al 1999).

Once cytochrome c binds to the WD repeats, dATP binding is stimulated approximately five-fold (Jiang and Wang 2000). Co-addition of dATP and cytochrome c allow for the binding of procaspase-9 to form a holoenzyme that can activate bound procaspase-9, and then to activate soluble procaspase-3 (Rodriguez and Lazebnik 1999). Nucleotide binding is essential for the activation of pro-caspase-9, but its contribution appears to be through allosteric binding rather than through hydrolysis-driven conformational change. While most forms of non-hydrolysable adenosine triphosphates do not activate Apaf-1 (Hu et al 1999, Liu et al 1997), one form, ADPCP, is capable of allowing caspase activation. Pro-caspase-9 binding to Apaf-1 stimulates its dATP binding (Genini et al 2000). Some nucleotide compounds with potent Apaf-1 activating activity were already being used as chemotherapeutic treatments although their mechanism of action in vivo in unclear (Leoni et al 1998). Coupled with the evidence that there is no increase in ATP-hydrolysis rates by APAF-1 when added with cytochrome c and procaspase-9, individually or together, these findings point to a role for nucleotide binding to favor a conformation of Apaf-1 that permits its oligomerization (Jaroszewski et al 2000), and procaspase-9 binding (Srinivasula et al 1998, Zou et al 1999). Given the conservation of the nucleotide binding sequence between the identified Ced-4 homologues, this mechanism seems likely conserved though direct evidence in other systems is circumstantial. ATP nucleotides can be crosslinked with Ced-4, but not with Ced-4 which has the ATP-binding Walker A motif mutated.

The key step that nucleotide and cytochrome c binding regulates is oligomerization of the ced-4 domain of Apaf-1 (Zou et al 1999). Mutations that abrogate the oligomerization of Ced-4 first demonstrated the importance of this function (Yang et al 1998). The complex of cytochrome c, caspase-9 and Apaf-1 migrates as a large complex of approximately 1.4 megadaltons and is called the apoptosome. The exact size and stoichiometry are somewhat unclear as differently sized active complexes have been resolved using different conditions (Zou et al 1999, Cain et al 1999, 2000, Saleh et al 2000), but all reports confirm the co-migration of the three components. Caspase-3 can transiently bind to the apoptosome as a substrate of caspase-9 (Hu et al 1998), but it quickly dissociates into its normal dimeric form or a larger migrating complex that does not contain Apaf-1 (Cain et al. 2000). The complex appears to be necessary for Caspase-9 to adopt a functional conformation, as cleaved caspase-9 that is released from the complex has significantly lower activity. The intrinsic proteolytic activity of Caspase-9 is required for caspase-9 to be cleaved into a mature form on the complex because catalytically inactive mutated caspase-9 is not processed by Apaf-1 and cytochrome. However, cleavage of caspase-9 is not required for its activity when on the complex as mutant caspase-9 that cannot be matured still has that activity to process procaspase-3 when on the apoptosome (Srinivasula et al., 2001, Steinnicke et al. 1999)

Procaspase-9 is recruited to the apoptosome by the interaction of its CARD domain with the corresponding domain on Apaf-1. Apaf-1's CARD domain is not accessible in its monomeric form (Li et al, 1997). An acidic surface on the Caspase-9 CARD interacts tightly with a corresponding basic surface on Apaf-1 (Qin et al. 1999). Because competition for the CARD of either Apaf-1 or Caspase-9 can block recruitment of the caspase to the

apoptosome, several additional proteins which have CARD domains that can interact with either component have speculated to play a modulating role on apoptosis. Alternative splice versions of Caspase-9 that lack the catalytic site have also been proposed to play this role of competitive antagonists (Seol et al. 2000).

Paradoxically, some of these proteins have also been proposed to be stimulators of apoptosome formation. One class contains a CARD domain, a nucleotide binding domain and Leucine Rich Repeats (LRR) at their C-termini. Because of the domain organization, some investigators have suggested they may be homologs of Ced-4 as well, though the nucleotide binding domain does not bear the same regions of homology that are shared by Apaf-1, Dark, and Ced-4. The protein called NAC or DEFCAP has been proposed to associate with Apaf-1 and/or caspase to facilitate their oligomerization and activation leading to apoptosis (Hlaing et al 2001, Chu et al 2001, Poyet et al. 2001). Other proteins with a similar gene structure, Nod1 and Nod2, have been cloned and similarly proposed to regulate cell death through Caspase-9. However, the proteins share the ability to activate NF-Kb and appear to regulate the activation of IKK in response to bacterial products. An intriguing possibility is that these LRR proteins represent a separate lineage involved in the innate immune system related to the Ced-4 domain. Many plants contain multiple copies of so-called Resistance genes that share C-terminal Leucine rich repeats with an N-terminal domain with homology to the Ced-4 domain and respond to bacterial products (vander Bizen et al 1998). Although plants do not contain clear homologs of caspases or Bcl-2 genes, they can respond to infection by inducing cell death. Distantly related groups of genes termed paracaspases and metacaspases that play a role in an apparent immune response have been

identified in plants and lower animals and may be the precursors of the caspase family (Uren et al 2000, Wiens et al 2000). Because heterologous expression of the anti-apoptotic Bcl-2 genes can have protective effects against infection and even dehydration (Dickman et al 2001), some authors speculate that the apoptotic machinery may have ancient roots that extend even beyond the currently recognized boundary at the metazoan branch points (Uren et al 2000). The function of the Nod proteins in the immune system has also been confirmed genetically as mutations in Nod2 result in an increased risk of developing the autoimmune condition, Crohn's Disease (Hugot et al. 2001.) While the CARD domain is critical to the cell death associated with overexpression of these genes, the exact role of these proteins in apoptosis in vivo is not yet clear.

An additional class of inhibitors that play an important role in controlling caspase activation is the IAP family. Cloned as anti-apoptotic genes originally from baculovirus (Crook et al 1993), these genes were found to have homologs in Drosophila and humans that blocked cell death as well (Duckett et al 1996, Hay et al 1995, Rothe et al 1995, Liston et al. 1995, Uren et al 1996). The proteins contain 2-3 repeats of a domain called the Baculovirus Inhibitory Repeat or BIR, and a C-terminal Ring finger motif. The BIR domains are essential for blocking cell death in transfection assays (Vukic et al 1998), while the role of the Ring finger was initially obscure. The IAP proteins were shown to be direct inhibitors of caspases (Devereux et al 1997, Roy et al 1997) mediated by BIR domain inhibition directly against active caspases (Devereux et al. 1997). Single BIR domains could inhibit select caspases in some cases (Takahashi et al 1998) while some caspases require the BIR domain plus addition regions of the linker region between BIR proteins (Devereux et al 1999, Chai et

al 2001, Riedl et al 2001). Indeed, a new class of single BIR containing proteins has been identified including the genes, Survivin and Livin, which are overexpressed in some cancers (Ambrosini et al 1997, Kasof and Gomes, 2000). While these proteins can inhibit caspase activity, they have also been implicated in the regulation of cell division (Li et al 1999, Fraser et al 1999).

Overexpression of XIAP can alter the sensitivity of cell to caspases activated by the death receptor pathway showing that the IAP proteins can play a decisive role in changing a cell's apoptotic decision. The importance of IAP proteins to apoptosis seems to be a feature shared between vertebrates and Drosophila, but not with the *C. elegans* system. The only BIR containing proteins in the nematode are involved in cytokinesis and have no effect on apoptosis (Fraser et al. 1999). In insect cell lines, the BIR domain appeared to inhibit apoptosis by binding to the pro-apoptotic proteins Reaper, Grim and Hid (Vucic et al 1997, Vucic 1998). The purpose of the interaction between IAP's and the upstream inducers of cell death, Reaper, Grim, and Hid was not at all clear from previous studies. Investigating the role of IAP's in the regulation of apoptosis in the two systems should clarify the functions of these proteins and how they participate in apoptotic decision.

With the progress made in understanding the regulation of apoptosis in all three model systems, scientists have a solid foundation for exploring the links between the known proteins and other unrecognized pathways. The straightforward genetic understanding of *C. elegans* has now given way to a complex system of redundant proteins and pathways that are now being untangled in the insect and mammalian systems. Therefore, comparison of the

regulation between these organisms continues to give insight and help identify potential points of intervention for the many diseases related to apoptotic signaling.

CHAPTER THREE
Methodology

Fluorescence In Situ Hybridization

The probes (GSI16278 and GSI16279, GenomeSystems, Inc. St. Louis) was washed from the slides with a 5 minute wash in 2xSSC, pH 7.0 followed by a wash with 4xSSC-1%Tween20 at room temperature. Signal amplification was achieved by treating the slide with 2ug of Fluoresceinated Avidin (vector) in 200ul of 0.1%BSA4xSSC, 0.1%Tween20 for 45minutes at 47degrees after blocking the slide with 3% BSA 4xSSC, 0.1%Tween20 for 30 minutes at 37degrees. The slides are then washed three times in 4xSSC,) 0.1%Tween20at 42 degrees for three minutes with agitation followed by an amplification step using 10ug/ml biotinylated anti-avidin (Vector) in 4xSCC, 0.1% Tween20 for 45 minutes and then washed. The Avidin labeling and washing is repeated one time for 2 hours and the slides visualized under a cover slip with a 30ul volume of antifade solution (20mM Tris-HCl pH8, 1mg/ml p-phenylenediamine dihydrochloride, in 80% glycerol) with propidium iodide added to a concentration of 0.5ug/ml. Signal to noise was found to be acceptable and the slides were then G-banded.

Confirmation of Chromosomal localization and Radiation Hybrid mapping

Human chromosome hybrids with CHO cells, hamster genomic DNA, and mouse genomic DNA were obtained through the generosity of Dr. Anne Bowcock. The Genebridge Panel 4 (Research Genetics; Huntsville, AL) was screened using a PCR reaction using Apintron1 and Apintron2 primers. After denaturing from 2 minutes at 95 degrees, the

reaction was amplified using 30 cycles of 45 seconds at 94 degrees, 45 seconds at 57 degrees, 1 minute at 72 degrees and a 7 minute final extension at 72 degrees. Map data was analyzed using the WICGR server at the Whitehead Institute.

YAC clones

Yeast Artificial Chromosomes corresponding to the mapped region of Apaf-1 were collected from the CEPH library (courtesy of Dr. Glenn Evans) and grown using standard techniques on AHC media at 30 °C. Colonies were screened using colony PCR by resuspending a single yeast colony in 100 ul TE buffer and boiling for 15 minutes with 1 ul of the supernatant used as template for the Apintron1/Apintron2 PCR reaction.

Cloning of dark *cDNAs*

10^6 plaque-forming units (PFU) of *ZAPII* embryonic cDNA library (a gift from C. Thummel) were PCR-amplified with primers corresponding to the *dark* genomic region. The PCR product was further amplified using an internal primer pair. The resulting 2-kb PCR product was used as in (Chen et al. 1998) to screen the same library and obtain multiple cDNA isolates. At least 20 independent cDNA isolates were obtained. The longest clone was 3 kb long and contained the 5' end of *dark*, including 75 base pairs (bp) of 5' untranslated sequence. cDNAs representing the predicted 3' end of *dark* were cloned out by PCR-amplifying an embryonic cDNA library in λgt10 (Poole et al. 1985). A 1.8-kb PCR product was obtained containing 78 bp of 3' untranslated sequence. The predicted stop codon from this sequence was independently confirmed using 3' rapid amplification of cloned ends (RACE; Life Technology), according to the manufacturer's instructions, on messenger RNA

from 0–16-h embryos. A 1.9-kb PCR fragment was sequenced to confirm the stop codon and obtain the 3' untranslated region (UTR) sequence. 5' and 3' fragments of *dark* were ligated together using an internal *Eco*RI site.

Plasmids

PCR fragments corresponding to amino acids 1–411 of Dark with a C-terminal T7 tag or a 3´Myc tag and a 4.4-kb PCR-derived insert of the entire *dark* open reading frame (ORF) encoding an in-frame 3´Myc epitope tag at the C-terminal end were independently cloned into the *Drosophila* pRmHa.3 expression vector45. We fused the first 243 bp from expressed sequence tag (EST) clone GH10971 with the *dredd* ORF in plasmid pMT-Dredd-δ-Myc 21. The resulting plasmid, pMT-Dredd-δ*-Myc, contains extra 5' UTR sequence from the *dredd* locus. Note that pMT-Dredd-δ*-Myc makes use of an upstream start and thus encodes an extra 22 amino acids at the N terminus of the protein that were not included in a previous study21; this plasmid was able to induce cell death when expressed in cultured cells (Fig. 2). The cDNA originally considered full-length exhibited no killing activity but produced protein that was activated by apoptotic signaling (Chen et al. 1998). pMT-Dredd-δ*(C/A)-Myc includes a point mutation that substitutes the active-site cysteine for alanine. The site-directed mutant was obtained by substituting a *Hin*dIII fragment of pMT-Dredd-δ*-Myc with a *Hin*dIII-digested PCR product carrying the mutation (TGC to GCC).

Isolation of RNA and northern blotting

Total RNA was prepared with the Boehringer Mannheim kit according to the manufacturer's instructions. Blotting and hybridization with a *dark*-specific probe was done

as in (Maniatis et al 1989). The probe used was a PCR fragment spanning the region encoding the CED-4-like domain of Dark.

Transfections and cell-killing assays

Schneider L2 (SL2) cells were cultured and transfected using Cellfectin (Life Technology) as in Chen et al. 1998. Cell-killing assays were done as described47. Briefly, 2 μg of various forms of *dark* were co-transfected with 3 μg pRmHa.3 or mutant caspase constructs into 1´106 cells. As a control, 3 μg mutant Dredd-δ* was cotransfected with 2 μg pRmHa.3, and 2 μg of wild-type Dredd-δ* was co-transfected with 3 μg pRmHa.3. 0.2 μg pAct-*lacZ* was included in all transfections. 36 h after transfection, each well of cells was split into two identical halves, and copper (0.7 mM) was added to one of the splits. When caspase inhibitors were used, 50 mM of the indicated inhibitor was added to the cells simultaneously with copper. Cells were fixed and stained for LacZ-positive cells 16 h later. Blue cells in induced and control halves of the transfected cells were counted. Cell survival was scored 16 h later by the percentage of blue cells in copper-treated cells relative to LacZ-positive cells in the control cells that were not induced by copper. The data shown are average percentages ± s.d. from three experiments.

Co-immunoprecipitation and western analysis

10 μg of the indicated plasmids were transfected into 5 million SL2 cells. 36 h post-transfection, protein expression was induced for 4 h with 0.7 mM copper. The cells were subsequently lysed in 0.5% Triton- X100 in buffer A48 without dithiothreitol (DTT) on ice for 30 min, then centrifuged at 12,000*g* for 15 min. For detection of Dark interaction with caspases, the resulting supernatants (total lysates) were incubated overnight at 4 °C on a

rotator with anti-Myc agarose beads (Santa Cruz) that had been preincubated with SL2 lysate. The beads were washed five times with the same buffer, then mixed with SDS sample buffer and boiled for 5 min. The proteins were resolved on a 10% or 8% polyacrylamide gel. For detection of Dark interaction with cytochrome *c*, the cell cytosol was prepared from the total lysates as described in Liu et al 1996. 400 ml cytosol (including 1 mg protein) was mixed with 60 ml Myc beads and incubated at 4 °C overnight in a rotator. The beads were washed as above and eluted with 60 ml 0.5 M Myc peptide in buffer A. Aliquots of the eluates were subjected to 15% (to visualize cytochrome *c*) or 8% (to visualize Dark) SDS–PAGE. Cytochrome *c* and different epitope-tagged proteins were probed with appropriate antibodies (anti-T7-tag (Novagen), anti-Myc (a gift from R.G.W. Anderson), anti-FLAG (Sigma), anti-cytochrome *c* (Pharmingen)) and visualized by enhanced chemiluminescence (Amersham).

Drosophila *strains and histological methods*

Flies were raised at 25 °C under uncrowded conditions and embryos were staged as in Campos & Hartenstein 1997. TUNEL staining was done as described in Chen et al. 1996 except that, after proteinase-K treatment, samples were refixed in 0.5% glutaraldehyde and washed in PBS plus 0.3% Triton-X100. P[GMR-*grim*]-1 is described in Chen et al 1996, P[GMR-*reaper*]-97A in White et al. 1994 and P[GMR-*hid*]-1M in Bergmann et al 1998. Wandering third-instar larvae were stained for b-galactosidase as in Rodriguez et al. 1996. Scanning electron microscopy was done as described in Chen et al. 1996.

Isolation of dark *mutations*

The P1041 enhancer trap strain (PlacW l(2)k11502) was identified from the Berkeley *Drosophila* Genome Project. As precise excision of P1041 did not reverse lethality, we suspected that lethality was instead caused by a background lethal insertion. As expected, we were able to remove a lethal insertion mapping ~15 map units away from the P1041 insertion by recombination. Therefore, P1041 is not a homozygous lethal insertion and homozygotes are fully fertile and viable. Using P1041, we conducted a hybrid dysgenesis screen. Progeny derived from dysgenic P1041 flies were identified on the basis of dark-orange eye coloring. New P-insertions were screened by PCR for local re-insertions in *dark*, using primers specific to *dark* and the P-element. We recovered four candidate alleles and characterized three of these (*dark*CD4, *dark*CD8 and *dark*DD1) in greater detail. To safeguard against the possibility of newly introduced second-site P-element-induced mutations, we outcrossed animals expressing each of CD4, CD8 and DD1 with animals with a wild-type second chromosome and subsequently obtained several isogenic strains balanced over CyO. We used these strains for phenotypic analyses. There are no pre-existing chromosomal deletions for the 53F1-F2 genomic region.

Assay for Grim activation of procaspase-3 processing

Assay for Grim activation of procaspase-3 processing was performed as described in Chai et al 2000. Briefly, 1 microliter of S^{35} translated procaspase-3 (Liu et al 1996) was incubated with the relevant proteins in the presence of 10um dATP at 30°C for one hour in a final volume of 20ul in buffer A(20 mM HEPES-KOH [pH 7.5], 10 mM KCl, 1.5 mM MgCl2, 1 mM sodium EDTA, 1 mM sodium EGTA, 1 mM dithiothreitol, and 0.1 mM

PMSF). Each reaction 20ug of S-100 extract prepared from Hela cells (Liu et al 1996) and 1ug of GST-XIAP Bir2(residues 124-240) from the mouse cDNA , 500 nm recombinant SMAC or 1um recombinant grim where indicated.

Drosophila Embryo Caspase Activity assays

Embryos from a forty minute collection were aged three hours and twenty minutes at 25°C. After dechorionation in 50% bleach, pools of approximately 50 embryos were resuspended in 100ul of buffer A (20 mM HEPES-KOH [pH 7.5], 10 mM KCl, 1.5 mM MgCl2, 1 mM sodium EDTA, 1 mM sodium EGTA, 1 mM dithiothreitol, and 0.1 mM PMSF) and crushed with a disposable pestle in a 1.5ml eppendorf tube. Cellular debris and lipids were separated from the soluble fraction by 15 minute spin at 15,000g at 4degrees C, and the protein concentrations for all extracts were measured and normalized by addition of buffer A. Five micrograms of protein extract was incubated with 50uM AcDEVD-AFC substrate in a final volume of 20ul in a 384 plate. Fluorescence was monitored over time with excitation at 360nm and emission at 465nm in a SpectraFluor Plus plate reader (Tecan).

THREAD/DIAP-1 fails to enhance GRIM, and HID killing in Dark mutants.

All crosses and experiments were carried out at 25° C. For the GRIM eye killing experiments, females of the genotype y, w; dark-CD4, GMR-grim-1 / dark-CD4; th^5/+ were examined for suppression of GRIM killing in the eye using a Zeiss dissection microscope as compared to y, w; GMR-grim-1 /+ ; th-5/+ females. For HID experiments, females of the

genotype, y, w; dark-CD4, GMR-HID-1M / dark-CD4 ; th-5/+ were compared to y, w ; GMR-HID-1M /+ ; th-5/+ females. All crosses were carried in parallel at 25° C.

Dark functions as an initiator of caspase activity within the neuronal midline glia.

15- x hour old embryos were collected from flies of the genotype, y,w, P[slit-1.0 lacZ]; dark CD4/ dark CD4 and analyzed for Bgal expression. Control embryos from y,w, P[slit-1.0 lac-Z] flies were also examined in parallel. Bgal protein was detected using a mouse monoclonal antibody (Promega) following standard techniques for fixing and washing (Patel). To maximize the signal, the streptavidin-biotin kit from Vector labs was used. For detection, Nickel-enhanced DAB was used (Patel 1994).

DIAP-1-/- global cell death is blocked by Dark.

Previously, Hay et al, showed that maximal TUNEL cell death occurs in DIAP-1 null embryos occurs during late stage 9. Therefore, stage 9-10 (Wang et al 1999) embryos were collected and apoptosis assayed using TUNEL-labeling. Briefly, embryos were collected for 40 minutes from adults of the genotype, y,w; dark CD4 /dark CD4; TM6 / th^5which had previously been pre-cleared twice and the embryos were aged approximately 4 hours. DIAP1 - embryos were collected from adults of the genotype, y,w; TM6 / th^5in parallel as described above. TUNEL labeling was essentially as in, White et al. 94, except that an additional fixation step for 15 minutes in 1% glutaraldehyde was conducted and a proteinase K treatment in 50 ug /ml for 1 minute was conducted. In addition, for optimal staining and

microscopy, the DAB-peroxide signal was enhanced with Nickel and the embryos were cleared with methyl salicylate and viewed with a Zeiss microscope (Patel 1994.)

Chapter 4 Analysis of Apaf-1 as a Candidate Germ Cell Tumor suppresser

At the time of its identification, Apaf-1 represented the first mammalian ced-4 homologue and the only intracellular protein that could be shown to activate caspases zymogens in the absence of other activated proteases. Even with these preliminary studies the central role that this protein could play in initiating programmed cell death was quite clear. Several lines of evidence pointed to the fact that such a central gene involved in cell death would be a tempting target for transformed cells to inactivate and obtain a survival advantage. First, the initial apoptosis gene identified in mammalian cells, Bcl-2, established that protection against cell death was a viable cancer strategy as the gene was identified as an upregulated oncogene in follicular lymphoma. The observation that the oncogene protected against cell death rather than promoting cell death was revolutionary at the time, although this is now recognized as one of the many stepping stones in the pathway to tumorigenesis (reviewed in Hanahan and Weinberg 2000). Additionally, it had been shown that many different anti-tumor therapies, such as DNA damaging agents, ionizing radiation, steroid treatment and ligation of so-called Death receptors could induce cell death with the trademark phenotypes of apoptosis, such as DNA laddering, nuclear and cytoplasmic condensation and blebbing into apoptotic bodies of the soma. Genetic pathways related to pro-death apoptotic genes were also circumstantially linked to cancer at this time. One of the most commonly mutated tumor suppressor genes, p53, induced apoptosis and cell cycle arrest, and had been shown to be

a transactivator of the pro-apoptotic gene, Bax. Since this time several other stresses on the cancerous cell such as limited growth factor ability, hypoxia, and loss of cellular anchorage have all been associated with the induction of apoptosis. With apoptosis being such a powerful selective force on tumors, it was hypothesized that Apaf-1 would be a tumor suppressor gene (TSG). To pursue this line of research, I set out to map the genomic localization of Apaf-1, assess its role in cancers linked to mutations in this region, and search for inactivating mutations in these tumors.

I used two approaches to pin down the human genomic location of Apaf-1- using Fluorescence In Situ Hybridization (FISH) to obtain a location for the gene on the physical map and a Radiation Hybrid mapping approach to identify tightly linked genetic markers. These complementary approaches allowed for the search of cancer literature using both older cytogenetic markers for underrepresented areas of the genome and to utilize more detailed research using polymorphic markers that are used for the fine mapping of genetic lesions. To facilitate this FISH analysis, a PCR based assay was developed that could specifically amplify an Apaf-1 specific band from human genomic DNA. Using primers spanning a known splicing junction, a small intron was identified in the ced-4 portion of the Apaf-1 gene. This probe was used to isolate two PAC clones (GSI16278 and GSI16279) corresponding to Apaf-1. Restriction digest analysis of the independently isolated clones showed significant overlap between the two clones, and DNA sequencing of the PCR band from these clones confirmed the sequence as being from Apaf-1 and identified the nature of a natural splice variant identified during the cloning of Apaf-1 (Zou et al). Using biotinylated PAC DNA s probes of prometaphase spreads, the probes were visualized using fluorescently labeled avidin. Apaf-1 resides on

the long arm of a class C type chromosome. Based on the chromosomal G banding this location was determined to be Chromosome 12 band q22.

Using hamster hybrid cell lines with individual human chromosomes, the chromosomal localization of Apaf-1 was confirmed as Chromosome 12. A chromosome 12 containing hamster line was positive for the Apaf-1 specific PCR band, but this band was not seen in a Chromosome 1 containing cell hybrid, or in hamster or murine genomic DNA. The presence of a clear PCR assay in both hamster and human genomic DNA allowed for the screen of a radiation hybrid library. Radiation Hybrid libraries depend on stable hamster cell lines that contain pieces of human genomic DNA which have been fragmented using ionizing radiation. As the likelihood of a double stranded break occurring between any two loci increases with their separation, only closely linked markers share similar patterns of incorporation into the different cell lines. With a panel of 93 cell lines, the Genebridge 4 panel can localize markers into statistically linked bins and can show a rough order along the chromosome using the known location of specific markers. Apaf-1's Genebridge pattern is shown in Table , and the output of the mapping algorithm is shown in figure . The nearby markers showed Apaf-1 to be located on Chromosome 12, which confirmed the FISH determined localization. Apaf-1 was placed between the loci TMPO/PHC/D29485 proximally and D12S1098 distally. This region was notable for the fact that markers in this area frequently exhibited loss of heterozygosity (LOH) in male germ cell tumors (GCT). LOH is correlated with the deletion of tumor suppresser genes and surrounding markers due to the genetic instability in tumors and the selective pressure to inactivate tumor suppresser genes.

A high frequency zone of LOH was mapped using markers from a YAC contig of the 12q22 region. The presence of Apaf-1 in this region was verified using colony PCR on the yeast containing the YAC's from the map seen in figure . Apaf-1 was bounded between the end of YAC 781b3 and YAC 734b9, a region on the edge of the defined deletions.

To facilitate the screening of Apaf-1 as a candidate male germ cell tumor suppresser gene, BAC constructs from the 12q22 region were sequenced to locate the intron-exon boundaries of the gene. The Apaf-1 gene was found to consist of 27 exons and 26 introns (Table ,Genbank accession Nos. AF098868-AF09914). The coding region of Apaf-1 spanned 26 exons. The translation initiation site was located at nucleotide position 578 in exon 2 preceded by 5' untranslated region (UTR) within exon 1and exon 2, and the 3' end of the coding region ends at nucleotide position 4159 in exon 27. Exon 27 (3007 bp) encodes the last 49 amino acids, followed by a large 3'UTR of 2863 bp (Fig). Exon 18 represents an alternatively spliced exon that results in an additional WD repeat in the protean (Zou et al, 1999). The size of six small introns was determined by direct sequencing, with the remaining introns now determined by comparison with the complete genome sequence. The Apaf-1 gene spans about 55 kb of the genomic region.

Using a BAC clone corresponding to the Apaf-1 region (p373G19), FISH analysis showed that the APAF-1 region had a lower signal compared to Chromosome 12 centromeric probes in 2 of 8 GCT lines examined (268A,218A). RT-PCR utilizing overlapping sets of primers spanning the entire coding region was performed , followed by SSCP analysis on 17 GCT cell lines and 10 primary tumors; however, no mutations were found.

Northern analysis of poly-A RNA was performed on 8 GCT cell lines hybridized with a cDNA probe to assess the levels of *APAF-1* mRNA. No *APAF-1* mRNA was detected in any cell line, whereas the control □-actin showed abundant expression, suggesting undetectable levels or lack of expression of *APAF-1* mRNA in GCT cell lines (data not shown). To assess the *APAF-1* expression further, 35 cycles of multiplex RT-PCR were performed on cDNA using primers spanning 2 different exons of the gene. All 12 cell lines and 6 primary tumors analyzed showed detectable levels of *APAF-1* message (Fig. 2B).

To assess the levels of APAF-1 protein, Western blot analysis was performed on 17 GCT cell lines using polyclonal antibody directed against the CED-4 domain of APAF-1. All cell lines analyzed showed appropriate-size immunoreactive bands against APAF-1 antibody, with a considerable variation in protein levels. The Tera-2 and Tera-1 cell lines showed an abnormal protein of smaller molecular weight ('120 kD) in addition to a normal-size protein (Fig. 2C). That this variant represents an alternatively spliced form of *APAF-1* has not been ruled out. Variant forms of alternatively spliced *APAF-1* due to additional WD40 repeats have been reported in human (Hu et al., 1999; Zou et al., 1999) as well as in mouse (Cecconi et al., 1998) cells. WD-40 repeats in Apaf-1 play a role in recruiting procaspase-3 to the APAF-1-caspase-9 complex. Mutants carrying deletion in the WD-40 region fail to activate procaspase-3 and are deficient in apoptosis (Hu et al., 1999). Apaf-1 functions to mediate the activation of caspase-3 in response to exposure to cytochrome c and dATP (Li et al., 1997; Zou et al., 1997). To examine whether 12q22 deletions in GCT affect APAF-1 function, S-100 cytosolic extracts were assayed for dATP-induced cleavage of in vitro translated radio-labeled caspase-3 as a

marker for Apaf-1 activity. Of seven GCT cell lines tested, three (Tera-2, 218A, and 268A) showed lack or decrease in dATP-mediated caspase-3 cleavage (Fig. 3A,B). In Tera-2, the cell line that showed the aberrant protein, the cell extract exhibited undetectable or no activity at low concentrations of S-100 (10 and 20 μg) and a detectable level of activity was seen only when large amounts of protein (30 μg) were used. In the other cell lines (169A, 2102E-R, and 175A), a high level of activity was detected with as little as 10 μg protein (Fig. 3A). The cell lines 268A and 218A showed no dATP-mediated APAF-1 activity even after addition of large amounts of protein to the reaction mixture (80 μg for 268A and 120 μg for 218A) (Fig. 3B). This lack of APAF-1 activity correlates with genetic deletions seen by FISH in the cell lines 268A and 218A.

To determine whether this lack or decreased caspase-3 activity was due to disruption of Apaf-1 protein expression, Western blot analysis was performed confirming the presence of appropriately sized immunoreactive bands in all of the cell extracts examined (Fig. 2B and data not shown). Because the activation of caspase-3 is dependent on the presence of Apaf-1, cytochrome c, and caspase-9, these proteins were added back to S-100 to determine which components were deficient in the GCT cell lines that showed a lack of or decrease in activity. Figure 3 shows the results after supplementation of these protein components in cell extract from the 218A cell line. Similar results were obtained for 268A extracts (data not shown). Cytochrome c is abundantly present in the S-100 cytosolic fraction (Kim et al., 1997) and addition of cytochrome c had no effect on activation of dATP-dependent activity (Fig. 3C, lanes 1 and 2). Addition of recombinantly expressed APAF-1 showed only a marginal increase in dATP activation of caspase-3 cleavage (Fig. 3C, lanes 3 and 4). Introduction of

recombinant caspase 9 markedly increased the activity in the cell line (Fig. 3C, lanes 5 and 6). The activity of the recombinant protein is demonstrated by co-addition to the cell extract (Fig. 3, lanes 7 and 8). These data indicate that the deficiency in caspase-3 activation in these extracts is not due to the absence of active APAF-1 protein.

In view of the critical role played by the *APAF-1* gene in the apoptotic pathway, the gene was considered as a strong candidate TSG. I mapped the *APAF-1* gene immediately distal to the common region of deletion in GCTs. for 12q22 deletions and examined its role in GCT. The 12q22 band has been suggested to harbor a potential TSG in male GCTs (Murty et al., 1990; Samaniego et al., 1990; Murty et al., 1992, 1996, 1999; Rodgriguez et al., 1992). The same region has also been shown to be frequently deleted in pancreatic carcinomas (Hahn et al., 1995; Kimura et al., 1996, 1998). These studies strongly implicated the presence of a TSG at 12q22. The initial FISH analysis identified *APAF-1* deletions in 25% of GCT cell lines. These data provided evidence that the *APAF-1* gene is within a deleted segment of 12q22 in a certain proportion of GCTs, thus leading to further analyses of the gene in these tumors. It has previously been shown that one of the cell lines (218A) exhibited LOH at 12q22 (Murty et al., 1996). Similarly, 57% (four of seven) pancreatic carcinoma cell lines analyzed also showed deletions of the *APAF-1* gene (data not shown).

To find whether the *APAF-1* gene functions as a TSG, genetic and functional alterations of the gene were studied in GCT's. Lack of mutations in *APAF-1* suggested that the gene is not inactivated by mechanisms involving loss of function. Mutations were not found in any of 8 pancreatic carcinoma cell lines analyzed (data not shown). Consistent with this, no gross genomic alterations were found in the *APAF-1* gene in 45

GCT DNA's studied by Southern blot analysis (data not shown). Northern blot analysis of APAF-1 mRNA failed to find any detectable levels of expression in GCT cell lines. *APAF-1* mRNA expression was determined by RT-PCR, and the protein levels were examined by Western blot analysis. Both mRNA and proteins were detected in all GCTs analyzed, including the Tera-2 cell line that exhibited an aberrant-sized protein.

To determine whether the genetic deletions and the variations in mRNA and protein affect the functional activity of APAF-1, caspase-3 activation was assessed in GCT cell lines. The activity of dATP-dependent, APAF-1- mediated caspase-3 cleavage was measured in the Tera-2 cell line, which showed an aberrant-sized protein, at various concentrations of S-100 with different incubation periods. Caspase-3 was not activated at low concentrations of protein and at shorter incubation periods with these cells, in contrast to marked cleavage of caspase-3 in other cell lines (e.g., 169A). Detectable caspase-3 cleavage was seen in Tera-2 only after addition of a high concentration of protein and prolonged incubation times. In two other GCT cell lines, 218A and 268A, which exhibited *APAF-1* gene deletions, the dATP-dependent activation of caspase cleavage was found to be defective, without any detectable forms of active caspase-3 even at higher concentrations of protein with prolonged incubation periods (Fig. 4B). Utilizing similar conditions, the cell lines 2102E-R and 175A, that did not exhibit *APAF-1* genomic deletions, showed markedly higher levels of caspase-3 cleavage. Hence, these data suggest that the cell lines 218A and 268A are defective in the dATP-dependent apoptotic pathway.

To determine whether this decrease or lack of active caspase-3 is due to relative levels of APAF-1 or other components in the pathway, I added recombinant APAF-1 and

caspase-9 in these experiments. Addition of recombinant APAF-1 did not restore caspase activation in cell line 218A (Fig. 4C). Identical results were obtained with the cell line 268A (results not shown). Recombinant APAF-1 has been shown to promote the activation of caspase-3 in the presence of recombinant caspase-9 and pure cytochrome c or in by addition to Apaf-1 2/2 fibroblast extracts (Zou et al., 1999). These data, thus, suggest that the defect is not due to a disruption of the *APAF-1* gene itself. This latent activity was best demonstrated by addition of purified recombinant caspase-9, which allowed detectable active caspase-3 in the cell line 218A (Fig. 3C, lanes 5 and 6). Supplementation of caspase-9 with or without addition of APAF-1 re-stored the caspase-3 cleavage, although a marked increase occurred when both components were added. Because the caspase-9 protein was detectable in all cell lines by Western analysis (data not shown), these data suggest that the defect in dATP-mediated caspase activation may be in an endogenous regulatory pathway or due to a dominant- negative form of APAF-1 that might compete for caspase-9 recruitment. No evidence for dominant- negative mutations was found, however, by examination of the *APAF-1* mRNA transcript in these cells by SSCP analysis. Whereas the defect is not directly due to a disruption of *APAF-1* expression, the link between genomic deletions containing *APAF-1* and the dATP activation of caspase cleavage is intriguing. These deletions may also point to the regulated inactivation of caspase-9 or perhaps other components of the *APAF-1*-mediated pathway for caspase activation in response to disruption of another gene in the 12q22 region or else where in the genome. It is now known that at least one other gene, *RAIDD* (Rip-associated ICH1/CED3-homologous protein with death domain), which is involved with apoptosis, also maps to the 12q22 region. The growth factor-dependent regulation of

BAD phosphorylation and binding to Bcl-X demonstrate that such connections exist between cell cycle controls and apoptosis for other arms of the apoptotic control machinery (Datta et al., 1997; del Peso et al., 1997). Recently, a novel tumor associated protein, called TUCAN, has been proposed to have an inhibitory activity against caspase-9. Taken together, these data do not support a role for *APAF-1* as a candidate TSG targeting 12q22 deletion in GCT. It remains to be seen, however, whether other mechanisms involving downstream genes in the pathway play a role in tumorigenesis in these tumors. The genomic structure of the *APAF-1* gene generated in the present study would be of relevance in facilitating further studies in this direction in normal and disease states.

In light of subsequent investigations, the lack of Apaf-1 of mutations in candidate tumors point to an earlier point of regulation as a more essential checkpoint for the control of apoptosis. Instead the regulation of release of cytochrome c and other apoptogenic molecules from the mitochondria may prove the more common site of mutation in the intrinsic apoptotic pathway. While Apaf-1 is essential for dATP meditated activation of Caspase-3, homozygous mutation of this gene does not affect most normal development in the mouse (Cecconi et al, 1999.). Despite a delay in cell death, Apaf-1 knockout animals display normal cell death in all but a subset of neuronal progenitor cells. Several findings now point to the role of the mitochondria in providing redundancy in the induction of apoptosis.

Though the caspase activation downstream of apoptotic signals can be blocked genetically or pharmacologically, cell survival as measured by clonal culture often show the death signals to remain effective. One reason for this is the quick and nearly

complete release of cytochrome c from the mitochondria. When observed directly in Hela cells, cytochrome c release appears nearly complete and occurs with a period of approximately fifteen minutes. Additional studies have shown that the function of mitochondria from apoptotic cells is significantly impaired in ADP/ATP exchange and oxidative phosphorylation. Thus, the release of cytochrome c by itself may be enough to sentence a cell to death by disrupting the mitochondria, the main energy source of most cells.

Along with cytochrome c, several other apoptogenic proteins have also been shown to exit the mitochondria intermembrane space upon appropriate signaling. The proteins include the nucleases AIF and Endonuclease G which cleave DNA into 50-100kb fragments or oligonucleosomal fragments, respectively (Li et al,2001). A modifier of caspase function has also been identified. This protein, called SMAC or DIABLO, inactivates the IAP class of caspase inhibitors(Du et al). Similarly, a protein called OPI has a similar anti-IAP activity as well as a protease domain that may have other apoptotic signaling functions (Alnemri et al, Gordon Conference). The concomitant release of these factors along with cytochrome c may cause enough damage to a cell to kill it despite a block in the Apaf-1/Caspase-9 pathway.

Beyond the evidence here that a proportion of GCT's have alterations in the Apaf-1 pathway, new reports support the idea that inhibition of the Apaf-1 pathway occur in tumors and confer a genetic advantage. Defects in the dATP-induced signaling pathway have been identified in ovarian tumors lines and can be induced by selecting for UV-irradiation resistance in CHO cells (Min Fang, Deepak Nijhawan and Xiaodong Wang, unpublished data). Inactivation of Apaf-1 in MEF cells increases the rate of

transformation in soft agar assays in response to myc overexpression. Mutation of one copy of Apaf-1 with inactivation of the other by DNA methylation has been reported in malignant melanomas; thus, the relative effectiveness of inactivation of this gene may be limited to certain cell types. Despite the lack of Apaf-1 mutations in GCT, inactivation of Apaf-1/Caspase 9 pathway by diverse mechanisms appears to confer a selective advantage to some tumors.

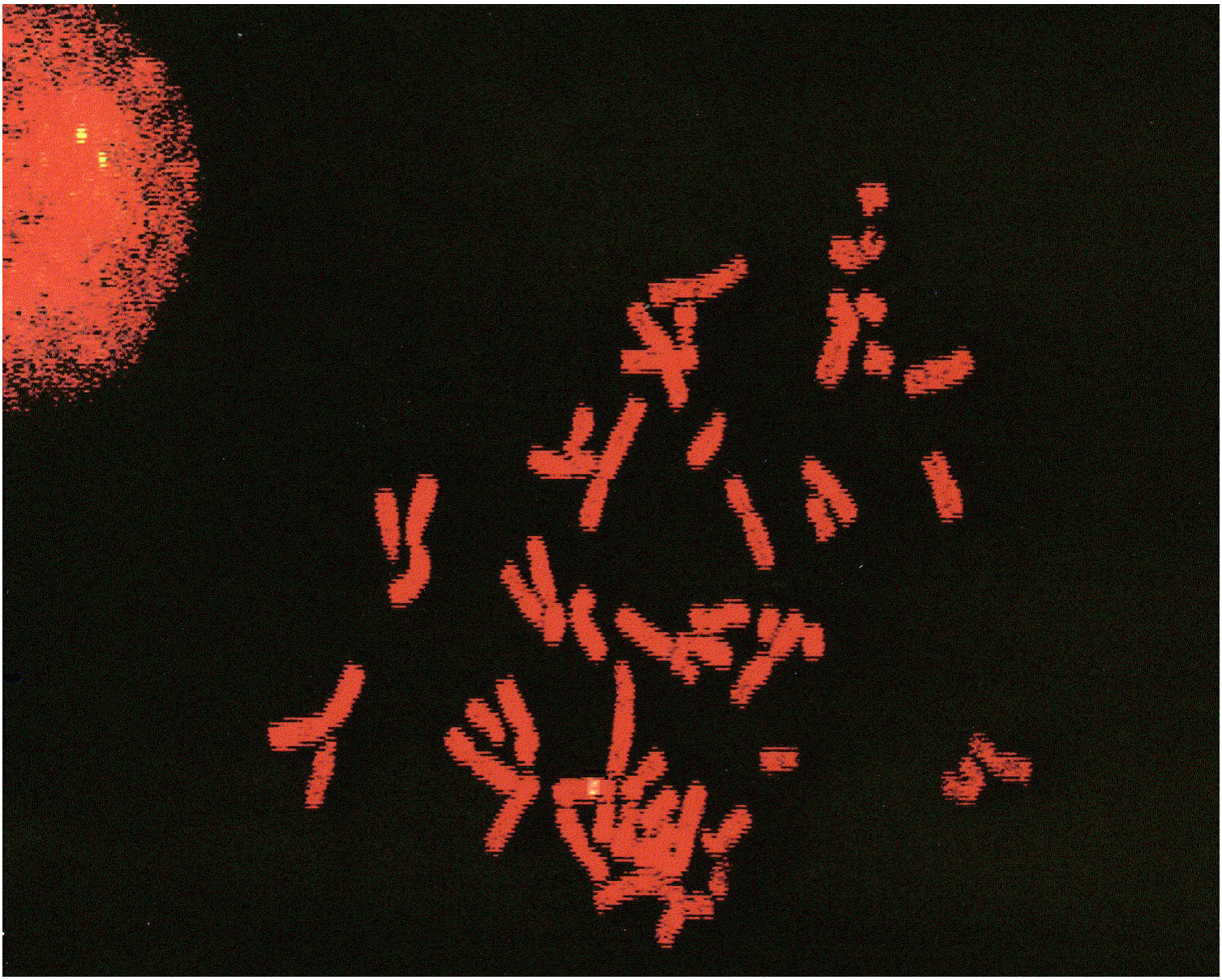


Figure 1 Fluorescence In Situ Fluorescence Chromosomal Localization of Apaf-1 to 12q.

G-banded Partial Metaphase spreads of Chromosome are labeled with PAC GSI16279, (yellow) show paired signals on the long arm of an acrocentric chromosome, identified as Chromosome 12, by its size and banding pattern.

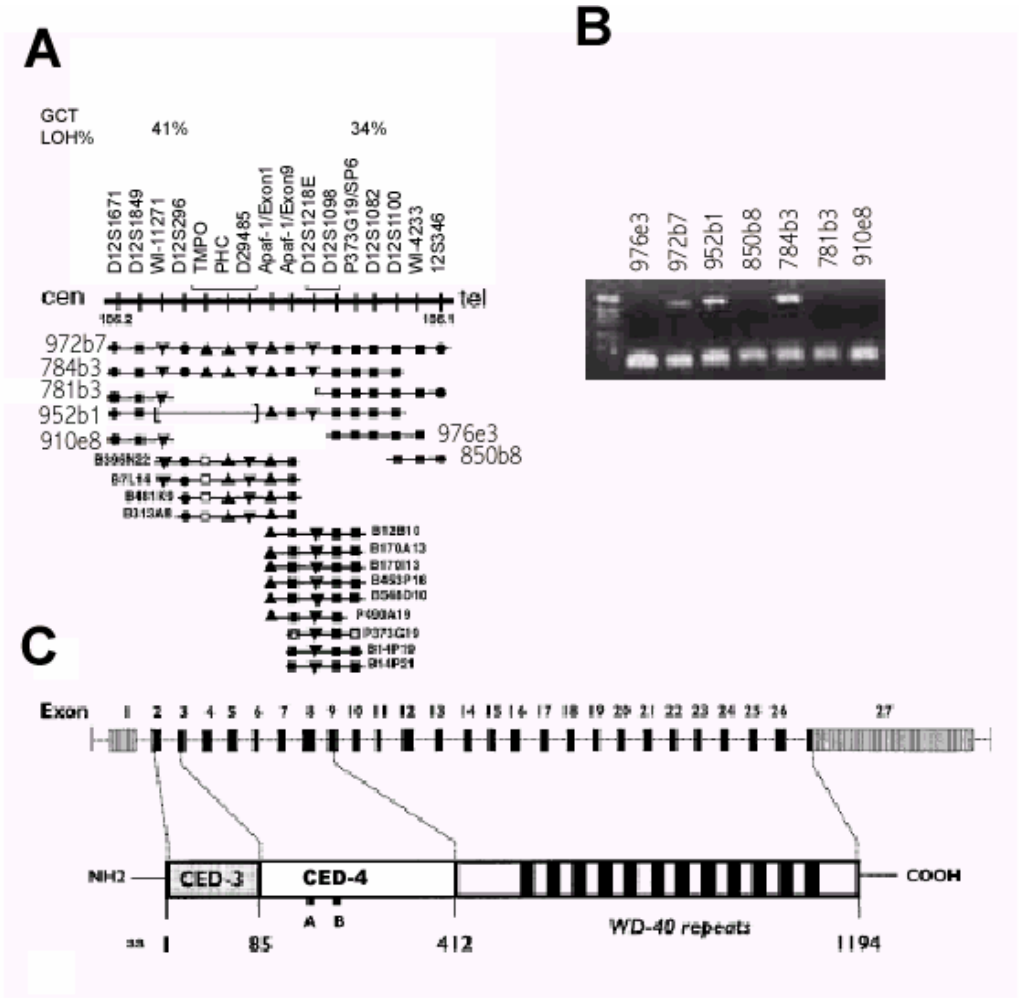
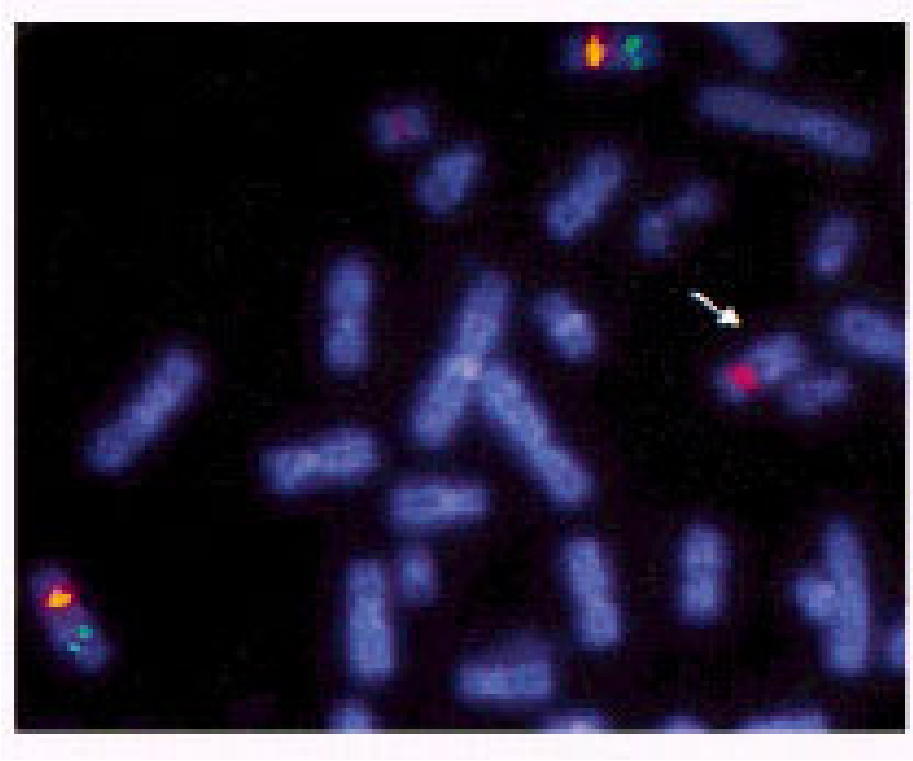

A
GCT
LOH%
41%
34%
D12S1671
D12S1849
WI-11271
D12S296
TMPO
PHC
D29485
Apaf-1/Exon1
Apaf-1/Exon9
D12S1218E
D12S1098
P373G19/SP6
D12S1082
D12S1100
WI-4233
12S346
cen
tel
972b7
784b3
781b3
952b1
910e8
976e3
850b8
B
976e3
972b7
952b1
850b8
784b3
781b3
910e8
C
Exon
NH2
CED-3
CED-4
COOH
A
B
WD-40 repeats
1
85
412
1194

Figure 2. Fine Mapping of *APAF-1* gene.

(A) Physical mapping of *APAF-1* gene between polymorphic markers D12S296 and D12S346. The map comprised of 5 YACs (denoted by 'y'), 11 BACs (denoted by 'B'), and 2 PACs (denoted by 'P'). Solid bar on the top represents chromosomal region in centromeric (cen) to telomeric (tel) orientation. The markers placed on the map are shown above the solid bar by vertical lines (distances on the map are not to scale). Thin brackets facing upward indicate that the relative order of the markers could not be determined. Numbers below the solid bar indicate the genetic map positions on chromosome 12. Solid circle, polymorphic marker; solid square, non-polymorphic marker; downward triangle, EST; upward triangle, gene; thin empty square, marker not tested; thick empty square, clone-end marker generated by sequencing of the end. A bracket within a YAC indicates an internal deletion. TMPO, thymopoietin; PHC, phosphate carrier mitochondrial; APAF-1, apoptotic protease activating factor-1. The frequency of deletion for two markers is shown as reported in Murty et al. 1996 **(**B) Yeast Colony PCR Assay for Apaf1 on YAC's covering the GCT common deleted region. (C) Schematic representation of genomic organization of *APAF-1* gene showing exons and the corresponding protein domains. Black boxes represent coding exons and hatched boxes noncoding exons (drawn to scale) (top), connected by dotted lines as intervening sequences (not drawn to scale). Protein structure at the bottom shows different domains, amino-acid (aa) positions, and their corresponding coding exons by broken vertical lines. A and B in the CED-4 domain indicate consensus sequences for nucleotide binding. Exon 18 represents an alternatively spiced exon resulting in an additional WD 40 repeat (not represented in the figure).



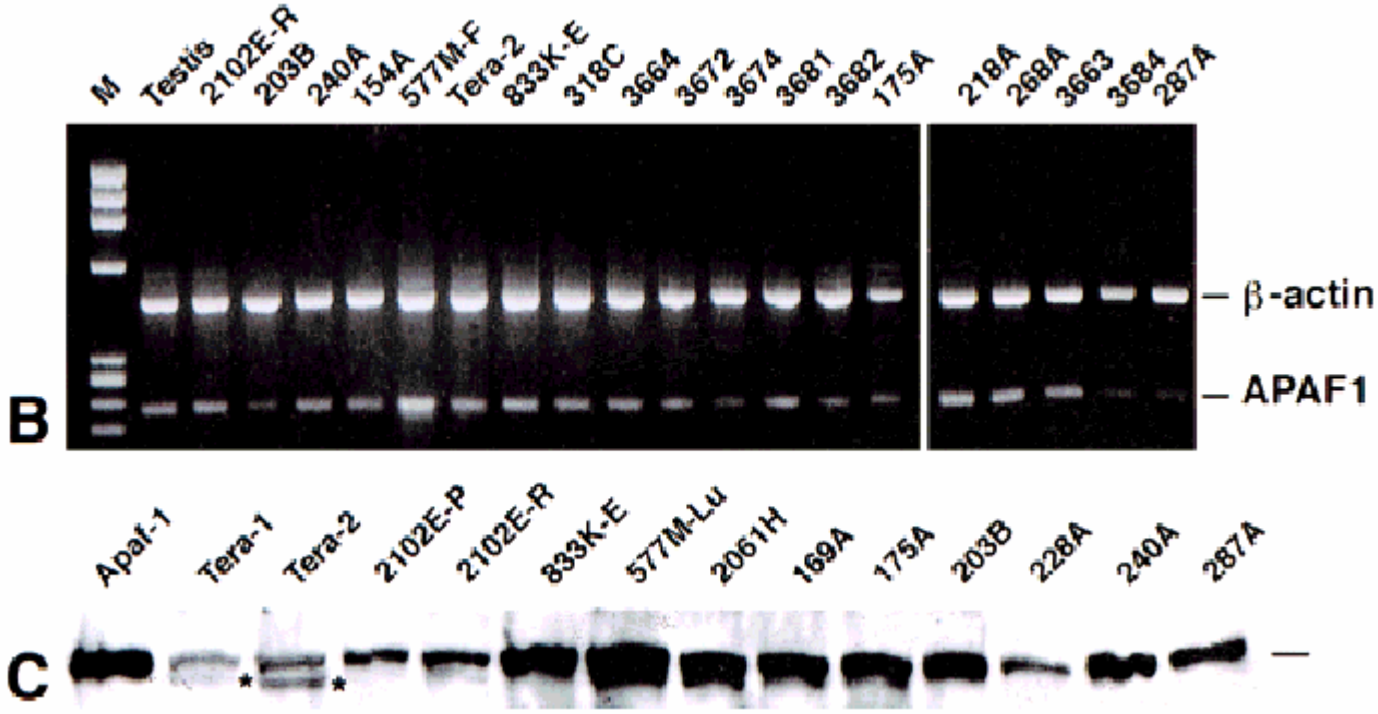

M
Testis
2102E-R
203B
240A
154A
577M-F
Tera-2
833K-E
318C
3664
3672
3674
3681
3682
175A
218A
268A
3663
3684
287A
β-actin
APAF1
B
Apaf-1
Tera-1
Tera-2
2102E-P
2102E-R
833K-E
577M-Lu
2061H
169A
175A
203B
228A
240A
287A
C

Figure 3. Genetic analysis of *APAF-1*.

(A) Identification of deletions at the 12q22 region containing *APAF-1* gene using a PAC clone 373G19 as probe by double color FISH. Partial metaphases with DAPI counter stain showing centromeric (orange) and P379G19 (green) signals. Cell lines 218A (left) and 268A (right) with deleted chromosome 12 are indicated by arrows. **(**B) RT-PCR analysis of *APAF-1* gene expression in GCTs. β-actin was used as an internal control. **(**C) Western blot analysis of APAF-1. Aliquots of 100 μg lysates were subjected to 6% SDS-PAGE and electroblotted, and then the filters were probed with a polyclonal anti-Apaf-1 antibody (see Methods). APAF-1 reactive bands were identified by comparing with the position of purified Apaf-1. The position of the APAF-1 is indicated on the gel. Asterisks indicate aberrant- size bands.(Bala et al 2000)

Table 2 Pattern of Apaf-1 positive PCR reactions from the Genebridge 4 panel

Negative amplification of an Apaf-1 specific PCR probe from a given well of the Genebridge panel is indicated by a zero, positive amplification and indeterminate results by a 2.

	1	2	3	4	5	6	7	8	9	10	11	12
A	0	0	1	0	0	0	0	0	0	1	0	0
B	0	0	0	0	0	0	0	1	0	1	0	1
C	1	0	0	1	1	0	0	0	1	1	0	0
D	1	0	1	0	0	0	0	0	0	1	0	0
E	0	0	1	0	0	0	0	2	1	0	0	0
F	0	0	0	0	0	0	1	0	1	0	0	1
G	0	1	1	0	0	0	0	0	0	0	2	2
H	1	0	1	0	0	0	0	0	2			

TABLE 3. Genomic Organization and Intron-Exon boundaries of Apaf-1

Exon	S Size (bp)	Intron- 5' **Exon** boundary — 3'**Exon**- intron boundary	CDNA location	Intron size
1	536	gat ttg act gtc cgc tgt cca gag gcg gag **AAG AAG AGG TAG– GCA AAG GCT TGG** gta agt tga cct cct cgc ttt tct ccc cga	1– 536	NA
2	179	ctg aca aat att ttg gtg ttt tgg ctg tag **CTC ATG GTT GAC– GTA AGA AAT GAG** gta aag ctc tct gaa gca gtc cac act tcc	537– 715	128
3	157	ttg tat act aaa cta ctt aat ttt ttt tag **CCC ACT CAA CAG– CTT CTT CCA GTG** gta aag att cag tta gtg gaa taa ctt cgt	716– 872	NA
4	198	cat tca tgc ttg ttt tgt ttt gga ttt tag **TAA GGA CAG TCC– CCC TTT TAG AAG** gta agt gtc tta tcc att tca tag tct agt	873– 1070	NA
5	184	cct taa gtt cat tat tct ttc cct cat tag **GTT GTT TCC CAG– CAA ACA CCC AAG** gta ccg atg gtc aaa ttt agt tgg tgt gtc	1071– 1254	NA
6	113	tta gtg att aat att ttt ttt tta aat tag **GTC TCT CTT GAT– ATT CAG TAA TGG** gta agg att aga cgt tta ctt ttt agt acc	1255– 1367	106
7	132	ctt act ttg tct tgt gat ttt tgt ttg cag **GTC CTA AAT ATG– AAG AAT GTA AAG** gta ttg tta ttt att tgt tta tga gga gat	1368– 1499	NA
8	239	tag cat agt gac ttc att ttt ttt tta aag **GCT CTC CCC TTG– GTG CCT ACA AAG** gta atg gga tca atg atc ctc atc att ggg	1500– 1738	399
9	168	ggc ata tta aat act tac aac aat tcc tag **GTG TTA TGT ATT– AGC CAG CTT CAG** gta ctt gca tct tgg ttt act ttt ttt ttt	1739– 1906	NA
10	132	tgg ctt ctg aaa cgt ttc att ggg ttg cag **GAT CTA CAT AAG– AAG ATG CAC AAG** gta aga tga ccc att tgg aaa tac ttt tat	1907– 2038	NA
11	114	gct gct gat act act ttt tgt ttt tta aag **GAA CTT TGT GCT– CTA GAT GAA AAG** gta tat ata tta aca tga aaa att agt gct	2039– 2152	446
12	185	tgt tta taa gag att tca gtt tat ttg tag **GAT TGT GCA GTC– CCT GGA ATG GAT** gta agt agg tta gga gag aaa cca aag gga	2153– 2337	NA
13	127	aat ttc tgt tca ttt ttt ccc tgt att tag **AAA CAA AAA AAA– AAA ACC TTA CAG** gta aaa cac atc tct tga gaa aaa tgc aaa	2338– 2464	NA
14	126	ata aaa aat att tta ttg tta ctt gtg cag **GTG TTC AAA GCT– AAA AAA GTG AAG** gta gga aaa tct ttt cct ctt gag ttg taa	2465– 2590	NA
15	132	att tgt aaa ttt ttt ctc ttt tct ctt tag **ATT TGG AAT TCT– TGC TTC CTC AAA** gta agt gtg gat att gag aat tag gta gat	2591– 2722	NA
16	126	tta att tat ctt ttt ttg ttt cac aaa tag **CTT TGG GAT TTG– GGA ACC TTA AAG** gta tgc ttt tgt aca cta tta aaa tag ttg	2723– 2848	NA
17	162	ctt ttt ctt ttt tat tac ttt aat tca aag **CTT TGG GAT GCT– AAT AAA ATC TTT** gta agt act tta aaa agc caa ctt cag tct	2849– 3010	NA
18	129	cct tca tag gat ctc tat tta tgt tga cag **CTT TTT GAC ATT– TAC TGT GTA GAG** gtg agt agt tga att tat tct gtg aag cct	*	NA
19	126	gat ttc cta tat gct gtg ttt att ctg tag **TTG TGG AAT ACA– CAG ACA ATC AGG** gtg aga aat att gag att ttc att ttg aac	3011– 3136	NA
20	120	gga atg ata att ttt tta tct ctt aat cag **CTC TGG GAG ACA– AGA CGT CTG CAA** gtg agt att ttt tag aaa aca att gga	3137– 3256	NA
21	117	aat aac tga ttt tgc ctc att ttt cat tag **CTC ATT AAT GGA– GGA GCC ATT GAG** gta ttc agt gct agt ctt cag aat ctt tct	3257– 3373	NA
22	126	ctt aat ttt gtg ggt ttt tct gct ttg aag **ATT TTA GAA CTT– GCT GAA ATT CAG** gtg aga ggg agg atg aac tct taa cat att	3374– 3499	NA
23	120	tta taa cag act tat ttc ttt gat att cag **GTA TGG AAT TGG– GGA ACA GTG AAG** gta att taa agt ata aat ttg ttt ttt gaa	3500– 3619	326
24	126	acc tcc aag tgt ttt ttt ttt ttt ttt aag **GTA TGG AAT ATT– AAG ACT GCA AAG** gta ggt caa tca att gaa acc atg cat aaa	3620– 3745	355
25	126	cat tgc taa aca atc cta att gcc ttc cag **ATC TGG AGT TTT– GGA GAA ATC AGG** gta ggc tgt ttg ctg aca tga aag cac tgc	3746– 3871	NA
26	144	gtt aat gaa ttg tgt atc atg ttt atg tag **ATA TGG AAT GTC– GGA TAT ATT AAG** gta aga gtt ccc caa gaa ctg tga aag aaa	3872– 4015	NA
27	3007	cta atg aga att tta ttt ttc tct gaa cag **TGG TGG AAC GTT– AAA ATT GTT TTG–**		

*Alternatively spliced exon derived from KIAA0413 sequence.

Figure 4

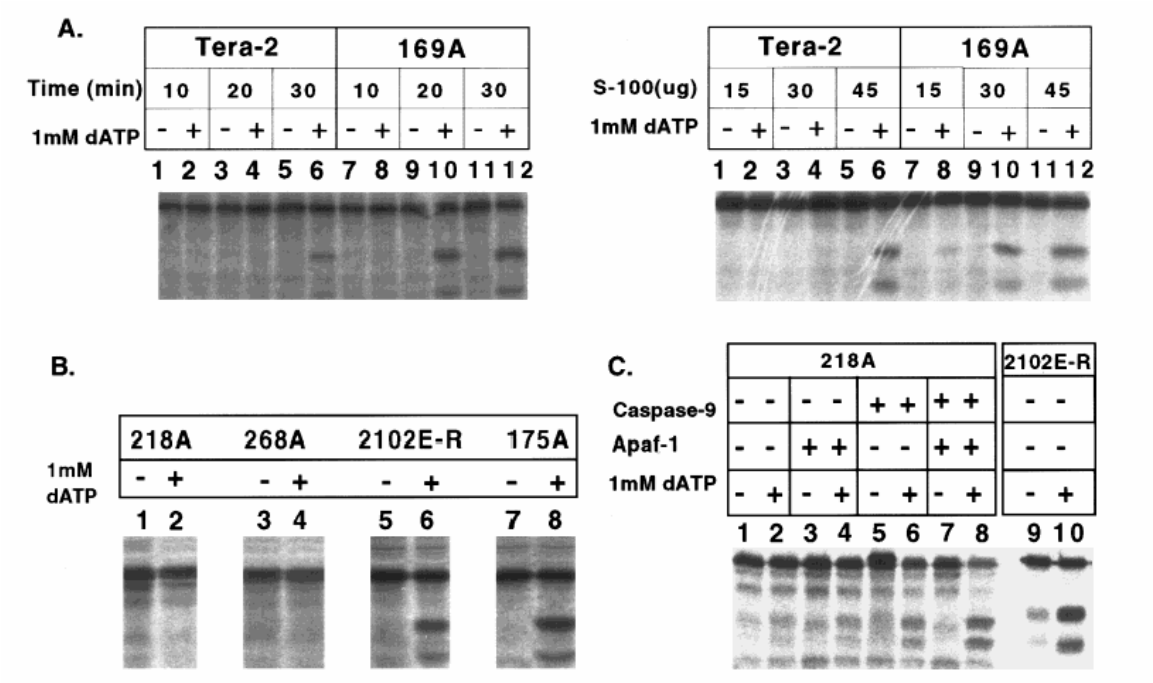

A.
Tera-2
169A
Time (min)
10 20 30 10 20 30
1mM dATP
- + - + - + - + - + - +
1 2 3 4 5 6 7 8 9 10 11 12
Tera-2
169A
S-100(ug)
15 30 45 15 30 45
1mM dATP
- + - + - + - + - + - +
1 2 3 4 5 6 7 8 9 10 11 12
B.
218A 268A 2102E-R 175A
1mM dATP
- + - + - + - +
1 2 3 4 5 6 7 8
C.
218A
2102E-R
Caspase-9
- - - - + + + + - -
Apaf-1
- - + + - - + + - -
1mM dATP
- + - + - + - + - +
1 2 3 4 5 6 7 8 9 10

Figure 4. Analysis of dATP-dependent caspase-3 activation in GCT cell lines.

Left panel: aliquots (2 μl) of in vitro-translated, 35 S-labeled, and affinity-purified caspase-3 were incubated at 30°C for 1.5 hr with 10 μg (lanes 1, 2, 7, 8), 20 μg (3, 4, 9, 10)or 30μg (5, 6, 11, 12) of S-100 from Tera-2 or 169A cell lines. Right panel: aliquots (2 μl) of 35 S caspase-3 were incubated at 30°C with 30 μg of S-100 from Tera-2 or 169A cell lines for 15 min (lanes 1, 2, 7, 8), 30 min (3, 4, 9, 10) or 45 min (5, 6, 11, 12) in the presence (lanes 2, 4, 6, 8, 10, 12) or absence (lanes 1, 3, 5, 7, 9, 11) of 1 mM dATP and 1 mM MgCl2 in a final volume of 32 μl. (B) Aliquots (2 μl) of in vitro-translated, 35 S-labeled, and affinity-purified caspase-3 were incubated at 30°C for 2.5 hr with 80 μg aliquots of GCT S-100 from cell lines 218A (lanes 1, 2), 268A (lanes 3, 4), 2102E-R (lanes 5, 6) or 175A (lanes 7, 8) in the presence (lanes 2, 4, 6, 8) or absence (lanes 1, 3, 5, 7) of 1 mM dATP and 1 mM MgCl2 in a final volume of 32 μl. (C) Aliquots (2 μl) of in vitro-translated, 35 S-labeled, and affinity-purified caspase-3 were incubated at 30°C for 4 hr with 30 μg aliquots of S-100 from cell lines 218A (lanes 1–8) or 2102E-R (lanes 9, 10) supplemented with 200 ng of cytochrome c (Sigma) in the presence (lanes 2, 4, 6, 8, 10) or absence (lanes 1, 3, 5, 7, 9) of 1 mM dATP and 1 mM MgCl2 in a final volume of 30 μl. Samples were additionally supplemented with either 300 ng of recombinant APAF-1 (lanes 3, 4, 7, 8) or 65 ng of recombinant caspase-9 (lanes 5–8). In all experiments, after incubation, samples were subjected to 15% SDS-PAGE and transferred to nitrocellulose filters. The filters were exposed overnight to a phosphorimaging plate at room temperature.

Chapter 5 Identification of DARK, a Drosophila homologue of Apaf-1

Because the regulation of the Apaf-1 pathway and its upstream signals represent a prime target for the understanding of different diseases and cancers, a vigorous effort to dissect this problem is being advanced on all fronts. Studies of programmed cell death (PCD) in the nematode *C. elegans* led to the identification of at least three essential components of the apoptotic pathway, CED-3, CED-4 and CED-9 (Yaun et al. 1992, Yuan et al 1993, Hengartner and Horvitz 1994). Genetic evidence for regulation in *C. elegans* points to a straightforward pathway of transcriptional regulation of a single gene, egl-1, that is deterministically controlled. However, cell death in Drosophila has been shown to respond to ionizing radiation and developmental arrest in an adaptive fashion more similar to vertebrates. As such a greater knowledge of the regulation of the Drosophila homologue of Apaf-1 could help shed light on the vertebrate system. The recent systematic sequencing of the Drosophila genome allowed for reverse genetics as an entrée into the apoptotic signaling as classical genetics has identified three genes, reaper grim and hid. . Productive signaling by these death activators requires caspase action, but the mechanism(s) connecting these upstream activators to downstream protease effectors was not known Although a requirement for caspase function during apoptotic cell death is well documented in flies (McCall & Steller 1997, Rodriguez et al. 1998), no *Drosophila* homologues of the Bcl-2 or CED-4/Apaf-1 families have yet been identified. In a search for such proteins I identified a new *Drosophila* gene, *dark* (for *Drosophila* Apaf-1-related killer), which encodes a homologue of CED-4 and Apaf-1.

Dark is a Drosophila *CED-4/Apaf-1 homologue.*

A TBLASTN search of the high-throughput genomic sequence (HTGS) database using the first 600 amino acids of human Apaf-1 protein identified a segment of *Drosophila* genomic DNA that encodes a stretch of homology to the 'CED-4' domain. We used the predicted exon sequences of this DNA segment to isolate the full-length complementary DNA encoding *dark* (see Methods). Comparison of the cloned cDNA to the genomic sequence identified 11 exons spanning 6.5 kilobases (Fig. 1a). A portion of the deduced 1,440 amino-acid open reading frame of Dark (Fig. 1b) is aligned with Apaf-1 and CED-4 sequences (Fig. 1e). The three proteins share a homologous CED-4 domain of ~320 amino acids in length, but Apaf-1 and Dark also contain another region of homology of an extra 70 amino acids. The CED-4-domain homologies included the Walker A and B loops (Walker et al. 1982) thought to mediate essential ATP-driven functions and an extra conserved six amino-acid stretch that is conserved between Apaf-1 and CED-4 (Zou et al. 1997, Van der Biezen et al. 1998).

Despite their overall conservation of sequence and physical properties, Apaf-1 and CED-4 differ in two important ways. Apaf-1, unlike CED-4, requires cytochrome c, released from mitochondria, as a co-factor for its activity, and activates procaspases through a protease cascade, with caspase-9 as the apical caspase and caspase-3 and caspase- 7 as downstream caspases (Srinivasula et al. 1998, Li et al. 1997). Cytochrome *c* promotes the formation of a multimeric complex of Apaf-1 and cytochrome *c* in an energy-dependent manner that recruits and activates procaspase- 9 (Zou et al. 1999). This activity is regulated by functions that map to a WD-repeat region of Apaf-1 not found in CED-4. Deletion of this domain renders Apaf-1 constitutively active so that it functions

independently of ATP/dATP and cytochrome *c* (Srinivasula et al. 1998, Hu et al. 1998, Chinnaiyan et al. 1997).

Analysis using the PSA sequence-analysis server (Stultz et al. 1997) also predicts the presence of at least eight complete WD repeats in Dark (Fig. 1b, outlined). A region of Apaf-1 containing its last WD repeat could be aligned with 31% identity and 49% similarity to a 74-amino-acid area of Dark that contains its last WD repeat. Dark contains two groups of WD repeats separated by more than 200 amino acids in an arrangement similar to that in Apaf-1 (Fig. 1b, c). The presence of incomplete WD repeats, and tertiary-structure predictions (made using the PSA server) indicative of b-propeller structures over incomplete WD repeats from residues 700 to 950, indicates that extra WD folds not detected by the computer algorithm may exist.

Apaf-1 bears an amino-terminal CARD domain thought to be important for caspase recruitment and oligomerization (Srinivasula et al. 1998, Yang et al. 1998, Li et al. 1997, Hofmann & Bucher 1997)(Fig. 1c), and similar profiles of CED-4 uncover ‘‘subsignificant’’ similarities to this motif, though the domain in Ced-3 is significantly homologues(Hofmann and Bucher 1997). Four *Drosophila* members of the caspase family, Dcp-1 (Song et al. 1997), drICE (Fraser & Evan 1997), Dredd (Chen et al. 1998) and Dronc(Dorstyn et al. 1999), have been characterized and implicated in the regulation of PCD. Dcp-1 and drICE have short prodomains with features that resemble those of effector-type caspases. In contrast, Dredd and Dronc contain a long prodomain with features that resemble those of initiator or apical caspases (Chen et al. 1998, Dorstyn et al. 1999, Inohara et al 1997). The N terminus (amino acids 1–91) of Dark shows moderate similarities to both Apaf-1 and Ced-4, and identical and/or similar residues tend

to cluster at important core hydrophobic sites (Fig. 1d) predicted from the solution structure of a CARD (Chou et al. 1998). More important, like its counterparts in both worm and mammal, the minimal region of Dark necessary for caspase interaction (residues 1–411) includes this N-terminal 'CARD-like' domain (see below). Dark induces apoptosis and interacts with pro-Dredd. To determine whether Dark expression is sufficient to trigger cell death, we directed conditional expression of the protein in cultured fly cells. We transiently transfected epitope-tagged versions of Dark into *Drosophila* Schneider L2 (SL2) cells. We observed little or no cell death (<3%) resulting from transfection of 'empty vector' controls or plasmids expressing irrelevant proteins (for example, pMTAL-Hk (Kramer & Phistry 1996)), but robust killing (88%) was induced by expression of the apoptosis activator Grim (see also Chen et al. 1996). In parallel tests, moderate cell killing was associated with expression of full-length Dark, whereas a C-terminal truncation of the WD-repeat region, Dark(1– 411), showed markedly enhanced killing activity (Fig. 2). In both cases, cell killing was completely suppressed by the caspase inhibitor peptide Z-VAD and moderately attenuated by the Z-DEVD peptide. Thus Dark-mediated cell death requires caspase activity.

Coexpression of an active-site C408A mutant of the fly apical caspase, Dredd (producing Dredd(C/A)), substantially attenuated cell killing triggered by Dark. In contrast, a comparable C211A mutation in the putative effector caspase drICE (producing drICE(C/A)) did not have similar effects even though it was prominently expressed (Fig. 3b). Therefore, Dark-mediated cell killing was generally not suppressed by the coexpression of mutant caspases, and the effect of Dredd(C/A) was specific. These data indicated that the Dredd mutant might exert a dominant-negative effect through a

physical interaction with Dark. We therefore tested whether Dark associates with Dredd. We detected a strong interaction between these proteins when using either Dark(1–411) (Fig. 3a) or the full-length protein (data not shown). In the experiment shown in Fig. 3a, Dredd-δ*C/A was tagged with Myc and immunoprecipitated, and Dark(1–411) was tagged with T7 and immunoblotted from anti-Myc immunoprecipitates. The interaction was confirmed in reciprocal experiments with an alternate configuration of epitopes (T7-tagged Dredd and Myc-tagged Dark(1–411); Dark protein was precipitated and blotted for Dredd co-immunoprecipitation; data not shown). Similar tests with a comparable mutant form of drICE showed no evidence for an interaction between this caspase and Dark (Fig. 3b). These results do not address the question of whether a cofactor is necessary to regulate the Dark–Dredd interaction, as apoptotic SL2 cells may contain other proteins needed for their association. Nevertheless, Dark specifically interacts with the apical caspase Dredd but not with the effector caspase drICE. These data raise parallels to the binding observed between counterparts in the worm (CED-4 and CED-3) and in mammals (Apaf-1 and caspase-9).

Dark interacts with cytochrome c.

Release of cytochrome *c* from the mitochondrial compartment and its association with Apaf-1 is a common feature of apoptosis in mammalian cells (reviewed in Reed 1997, Green & Reed 1998). In *Drosophil*a, changes in cytochrome *c* also occur but the protein remains tethered to mitochondrial membranes, which are sufficient to trigger cytosolic caspase activation if isolated from apoptotic cells (Varkey et al. 1999). We therefore tested whether Dark, like Apaf-1, might similarly associate with fly cytochrome c. Considerable levels of cytochrome *c* co-precipitated from Dark-expressing cells but not

from parental cells (Fig. 3c). To determine whether Dark's C-terminal WD domain might be important for this association, we also tested an identically tagged (3´Myc) C-terminal truncation version of Dark(1–411). After transient transfection, substantial expression of Myc 3 –Dark(1–411) occurred but no co-immunoprecipitation with cytochrome *c* was observed (data not shown). Therefore, a specific association of Dark with cytochrome *c* requires residues mapping between residues 412 and 1,440 in the Dark protein. These results indicate that Dark and Apaf-1 share homologous functions engaged by cytochrome c, and that the apoptosis-inducing activity of cytochrome c, through Apaf-1/Dark-like molecules, may be broadly conserved. Consistent with this idea, purified *Drosophila* cytochrome *c* was able to substitute for human cytochrome *c* in activating caspases via Apaf-1 *in vitro* (data not shown). Future studies will determine the subcellular localization of Dark and the functional role of its interaction with cytochrome c.

dark loss-of-function mutations cause pleiotropic defects.

To determine the function of *dar*k, we screened for and isolated mutants defective at this locus. Using a nearby P-element (P1041), we initiated a genetic screen to obtain loss-of-function *dark* alleles (see Methods). From ~700 transposition events, we identified three bearing a P-element insertion within the *dark* locus. Genomic polymerase chain reaction (PCR) analysis revealed that these alleles *(dark* CD4 (CD4), *dark* CD8 (CD8) and *dark* DD1 (DD1)) contain an insertion in the first intron and retain the original P1041 insertion upstream of the *dark* promoter (Fig. 1a). Consistent with the differential severity of these alleles, CD4 and CD8 map several hundred base pairs downstream of DD1.

To characterize the nature of these mutations further, we studied RNA from CD4, CD8, DD1 and wild-type animals for *dark* expression by northern blot analysis. In the wild type, we detected a single *dark* transcript, migrating at ~5 kilobases (kb), at all stages examined (Fig. 4). Increased levels of *dark* occur at a time coincident with the histolysis of larval tissues (during the third-instar and early pupation stages), whereas lowest expression occurs in embryos and adults. In CD4 animals, *dark* messenger RNA was not detected even after long exposures of the blot. This mutation represents a strong loss-of-function hypomorphic allele given preliminary RT-PCR results (Antony Rodriguez and John Abrams p.c.). By this criterion, CD8 is a hypomorphic allele as a reduced amount of transcript was found. A slightly larger-sized transcript occurs in DD1 animals, but no significant changes in the levels of *dark* expression are seen, consistent with the idea that this insertion represents a weak *dark* allele.

Most animals homozygous for CD4 and CD8 survive to the adult stage, albeit at lower frequencies relative to heterozygous siblings. Many of these CD4/CD4 and CD8/CD8 animals derived from heterozygous parents exhibited melanotic tumors and abnormalities affecting the wings and/or bristles (Fig. 5a–f). Although these flies also had impaired viability and fecundity, progeny derived from homozygous parents suffered from notably increased penetrance and expression of similar defects. Substantial numbers of these F 1 homozygotes were either sterile and/or died prematurely within several days (Table 1). CD4 and CD8 animals showed the same classes of phenotypes (Fig. 5, Table 4), albeit at different frequencies.

Correlating with our molecular analysis, CD4 and CD8 show the strongest penetrance for all classes of abnormalities whereas DD1 animals (which express *dark*

RNA at wild-type levels) showed milder defects that are limited to extra bristles (no defective wings or melanotic tissue are seen in these mutants). Transheterozygous combinations of the stronger CD4 mutation with the CD8 allele failed to complement, showing that lesions at *dark* are the cause of the phenotypes observed. Wing abnormalities in CD4 and CD8 homozygotes fall into different classes. Most affected individuals exhibited severe wing defects similar to a 'gnarled' and or 'wrinkled' phenotype (Fig. 5a). Other afflicted flies had wing blisters (data not shown) or burnt 'notched' wings (Fig. 5d). At moderate frequencies, melanotic tumors were also observed protruding from the body of the animal next to the haltere (Fig. 5d, e). Melanotic tumors in *Drosophila* are thought to arise from abnormalities in hematopoietic blood cells during larval growth or autoimmune defects (Watson et al. 1991). Among *dark* mutants with extra bristles, most had one extra anterior scutellar macrochaetae while a minority had two extra such macrochaete and/or an extra posterior scutellar macrochaetae.

***dark* mutants are defective in programmed cell death**. Neuronal PCD is important in the patterning of a functionally mature nervous system. For instance, *Drosophila* Df(3L)H99 embryos, which lack cell death, suffer from a greatly enlarged central nervous system (CNS) (White et al. 1994), and mouse strains lacking Apaf-1 exhibit cell-death defects leading to hyperplasia of the nervous system (Yoshida et al. 1998, Cecconi et al. 1998).

To determine whether the CD4 insertion also causes defects in the CNS, we dissected the brain lobes and ventral ganglion from wandering third-instar CD4/CD4 larvae. Most *dark* mutants had a significantly overgrown CNS as compared with wild type (Fig. 5g, Table 4). Occasionally, hyperplasia of the CNS was observed in the brain

lobes only, while in other animals the ventral nerve cord (for example, the ganglion) was abnormally extended (Fig. 5g).

As the hyperplasia of the larval CNS might result from defective cell death earlier in development, we studied the patterns of apoptosis in CD4 embryos using the *in situ* TUNEL technique. Compared with wild-type embryos, CD4/CD4 homozygotes exhibited markedly reduced levels of apoptosis (Fig. 6). Although there was a general decrease in TUNEL labeling throughout these embryos, the reduction in number of apoptotic cells was most noticeable within the CNS and the epidermis. In contrast to wild-type stage 16 embryos, which exhibited 70 ±10 TUNEL-positive cells within the CNS, CD4 homozygotes had only 7±4 TUNEL-positive cells at this stage in this tissue. Reduced apoptosis was most easily detected within the ventral nerve cord of *dark* mutant embryos (Fig. 6, compare a, b), but patterns of cell death were diminished in other tissues as well. For instance, whereas a large number of ventral epidermal cells normally undergo PCD shortly after germ-band shortening, *dark* mutant embryos have a greatly reduced number of TUNEL-positive cells during this stage (Fig. 6, compare c, d).

Mutations in dark *suppress* reaper*-,* grim*- and* hid*-induced apoptosis.*

As *dark* embryos are defective in apoptosis, we sought to determine whether *reaper*, *grim* or *hid* signaling might require *dark* function. Directed expression of transgenes expressing *reaper* (P[GMR-*reaper*]), *grim* (P[GMR-*grim*]) and *hid* (P[GMR-*hid*]) in the eye disc triggers ectopic apoptosis and, depending on expression levels, the effect is seen in adults as phenotypes that can range in severity from complete ablation of eye tissue to milder 'rough' eye phenotypes. Alterations in the magnitude of cell-death signaling resulting from such transgenes are an effective means of identifying genetic

components that regulate apoptosis (Chen et al. 1998, Bergmann et al. 1998, Kurada & White 1998). We therefore compared cell-death phenotypes caused by P[GMR-*reaper*]-97A, P[GMR-*grim*]-1 and P[GMR-*hid*]-1M in backgrounds that were either wild-type, heterozygous or homozygous for *dark* CD4 .

Overt suppression of cell killing was not evident in flies heterozygous for *dark*. In contrast, substantial suppression was observed in homozygous individuals. The eyes of tester strains with two copies of P[GMR-*grim*]-1 were completely ablated (Fig. 7a and Chen et al. 1996), whereas homozygous *dark* flies with two copies of P[GMR-*grim*]-1 retained substantial retinal tissue with many surviving ommatidia that were properly pigmented (Fig. 7b). Similarly, the P[GMR-*hid*]-1M phenotype was dramatically suppressed in *dark* mutants and, compared with the wild-type tester strain, a large number of retinal cells that would have otherwise died persisted in the *dark* back-ground (Fig. 7, compare c, d). Parallel tests with P[GMR-*reaper*]-97A uncovered a similar suppressive effect on *reaper* signaling (Fig. 7, compare e, f). These results indicate that *dark* functions as a pro-apoptotic effector of *reaper*-, *grim*-, and *hid*-induced cell killing.

Discussion

These data favor a shared evolutionary lineage for *dark*, *Apaf-1* and *ced*-4. Among these, the fly and mammalian genes share considerable homologies not found in *ced*-4. Therefore, Dark and Apaf-1 probably share the most recent common ancestry. Accordingly, the WD-repeat domain, which may represent the site of engagement by mitochondrial signals, could be a recent acquisition or, alternatively, this domain may have been lost from the worm protein. Completed analysis of the genomes of the three organisms show that are no other 'truer' orthologues of *ced-4* in flies and mammals.

Functional studies also support a more recent lineage shared by Dark and Apaf-1. Dark, Apaf-1 and CED-4 each associate with apical caspases, but only the fly and mammalian counterparts are known to associate with cytochrome c, through a WD-repeat domain. Moreover, deletion of this region in Dark and Apaf-1 produces enhanced effects upon cell killing. Therefore, analogous to the scenario that has been proposed for Apaf-1 function, our results indicate that the association between cytochrome *c* and Dark may function to derepress an inhibitory effect imposed on Dark by the WD-repeat domain. One implication of these results is that an ancient mitochondrial circuit for propagating apoptotic signals (binding of cytochrome *c* to a WD-repeat domain) is preserved in insects and thus probably existed in a common ancestor of insects and mammals.

The identification of *dark* also raises important mechanistic questions. For instance, the *C. elegans* Bcl-2 homologue CED-9 can directly bind and repress the activity of CED-4 (Wu et al. 1997, Chinnaiyan et al. 1997, Spector et al. 1997, Wu et al. 1997). Although analogous interactions can occur between Bcl2-family members and Apaf-1, these findings are hotly contested (Hu et al. 1998, Sohg et al. 1999, Inohara et al. 1998). However, it is not yet known whether Bcl-2 proteins represent an 'obligate' component of the apoptosome; future studies on Dark and its regulators may shed light on this issue. It will also be interesting to determine whether Dark can engage and/or function to activate other fly caspases. An intriguing candidate in this regard is a newly discovered CARD-containing caspase, Dronc (Dorstyn et al. 1999), which, in initial tests, also associates with Dark in cultured SL2 cells (P.C. and J.M.A, unpublished observations).

Some aspects of the *dark* mutant phenotype suggest striking parallels to those reported for Apaf-1-deficient mice (Yoshida et al. 1998, Cecconi et al. 1998). In both cases, the CNS appears to be preferentially affected and a decrease in apoptosis leads to hyperplasia of this tissue. Although PCD is overtly compromised in *dark* CD4 embryos, clearly not all deaths are affected. Thus, certain apoptotic deaths may not use a *dar*k-dependent pathway; similar inferences have been drawn from study of the Apaf-1-deficient mouse embryos (where partial PCD suppression also occurs). As *dark* CD4 is not a null mutation, we cannot yet exclude an absolute requirement for *dark* activity in all PCDs. However, charcterization of a null allele is still fothcoming (A.R. and J.M.A., unpublished observations). This might argue for other CED-4/Apaf-1-like molecules with partially redundant functions to be present in flies; however, sequence analysis of the completed genome shows no additional regions homologous to DARK or the other ced-4 genes. Alternatively, this may indicate conservation with the upstream pathways causing mitochondria damage in mammalian systems or activation of parallel apoptogenic pathways coincident with the activation of DARK that may be able to compensate for the lack of DARK in some tissues.

Although the existence of an enlarged CNS can be easily reconciled with reduced apoptosis, the precise origins of other abnormalities are less obvious. Nevertheless, conspicuous parallels to phenotypes associated with mutations in caspases and other cell-death regulators in the fly are apparent. For example, larvae homozygous for loss-of-function mutations in the caspase gene *DCP-1* (Song et al. 1997) also suffer from melanotic tumors, and *dred*d-deficient flies have wing and bristle abnormalities (A.R. and J.M.A., unpublished observations). Bristle defects are also associated with mutations at

the *Drosophila inhibitor of apoptosis-1* gene and wing defects are associated with mutation of a *hid* allele *(Wrinkle*d) (Grether et al. 1995). Some of these defects (such as melanotic tumors) could arise from the incomplete histolysis of larval tissue during metamorphosis but, for the most part, many defects appear to be confined to tissues derived from the wing disc. It will be interesting to determine whether defects occur in tissues derived from other imaginal tissues.

Other questions to be addressed relate to the role of *dark* as an effector of signaling by the death activators *reape*r, *grim* and *hi*d. As these proteins can activate both pro-Dredd processing and the accumulation of *dredd* RNA in cells that are specified to die (Chen et al. 1998), it will be interesting to determine the precise role of *dark* in these pathways. For example, it should be possible to test directly whether the apoptosis activators function through Dark to trigger Dredd processing. The data in Fig. 7 show a function for *dark* as a pro-apoptotic effector of ectopic cell killing by *reape*r, *grim* and *hi*d. However, the effect of these cell-death activators was not entirely abolished in *dark* homozygotes and, therefore, *dar*k-independent apoptosis pathways, also triggered by *reape*r, *grim* and *hi*d, are implicated. Further investigation into the function of *dark* as it relates to signaling by *reape*r, *grim* and *hid* is now possible within the context of normal PCD and cell-type-specific fates.

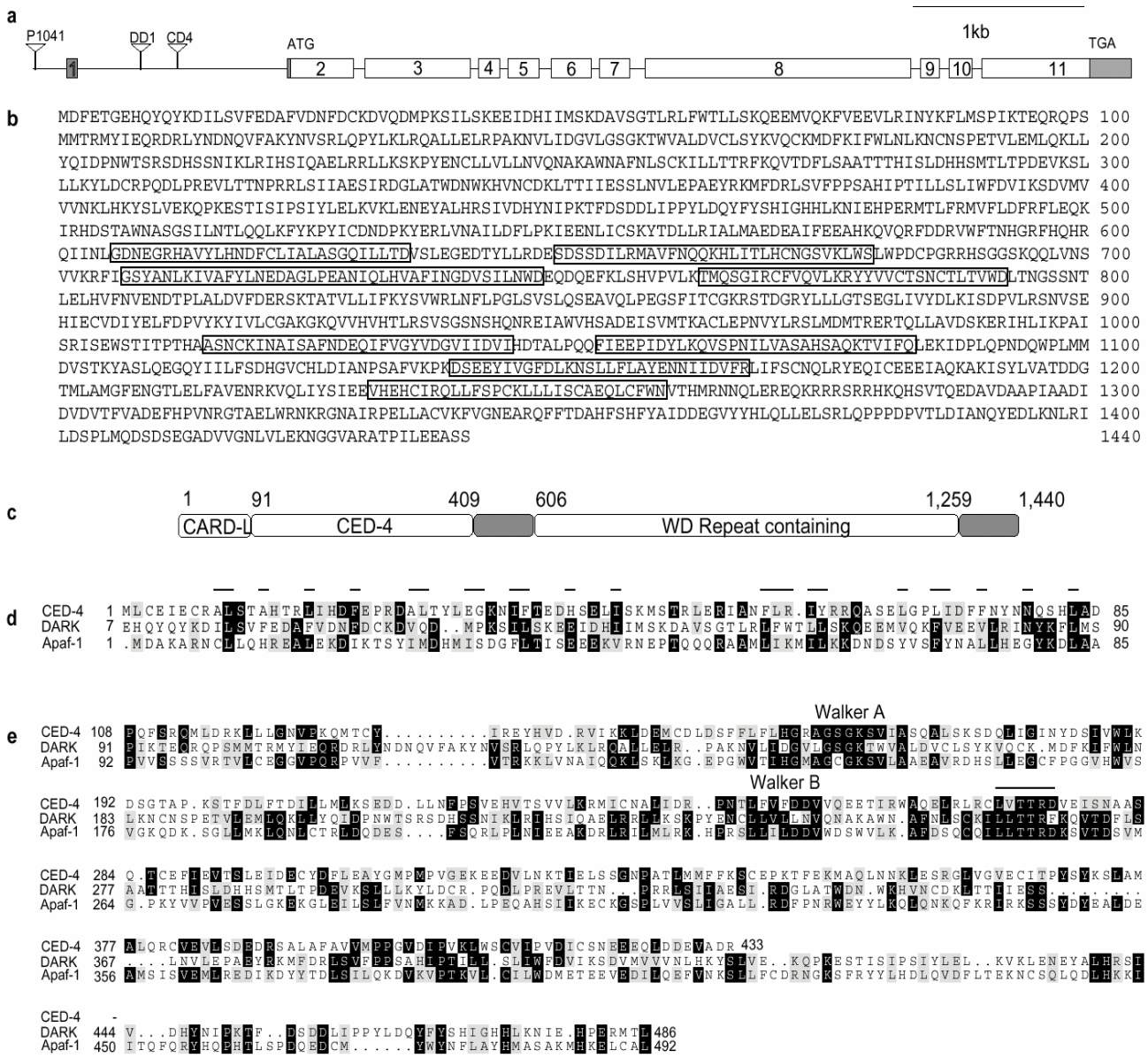

a
P1041
DD1
CD4
ATG
1kb
TGA
1
2
3
4
5
6
7
8
9
10
11
b
c
1
91
409
606
1,259
1,440
CARD-L
CED-4
WD Repeat containing
d
CED-4
DARK
Apaf-1
e
Walker A
Walker B

Figure 1 **The predicted amino-acid sequence of Dark and comparison to Apaf-1 and CED-4.** a, Genomic organization of the *dark* gene. The *dark* locus is transcribed towards the centromere of chromosome 2 and, on the basis of the flanking downstream gene, dRhoGEF, maps to 53F1-F2. The insertion sites of P-elements (P1041, DD1 and CD4) are indicated, and exons are numbered. b, Protein sequence of Dark. Two groups of WD repeats separated by a region of more than 200 amino acids were identified by the PSA search engine (WD repeats are outlined). c, Domains of the Dark protein. Three domains were delineated by sequence analysis and comparison: an amino-terminal CARD-like domain, a CED-4 domain, and a WD-repeat-containing domain. Amino-acid residues at domain boundaries are indicated at the top. d, Dark contains an N-terminal 'CARD-like' domain. An alignment of the N termini of Dark, Apaf-1 and CED-4 was made using Pileup (GCG, Wisconsin). Like CED-4, the N terminus of DARK shares subsignificant homology to the CARD domain. Identical and/or similar residues tend to cluster at important core hydrophobic sites of the CARD domain predicted from the partial solution structure of the death adaptor RAIDD; these sites are indicated by bold overlines. In this region, Dark shares 21 of 24 nonpolar or hydrophobic residues with this conserved hydrophobic core and, like other CARD domains, the region exhibits a high probability of helical secondary structure. Direct alignment between N termini allowed a 65-amino-acid overlap, with 20% identity and 38% similarity, between Apaf-1 and Dark. Direct alignment of Dark and CED-4 revealed 16% identity and 47% similarity over a region of 109 amino acids. e, A common CED-4 domain is shared by Apaf-1, CED-4 and Dark. CLUSTAL-W was used to generate an alignment of a minimal shared domain of 320 amino acids among these proteins, with a further 70-amino- acid homologous region identified between Apaf-1 and Dark from BLAST sequence comparison. Bars indicate highly conserved regions, including the ATP-binding Walker A (P-loop) and Walker B sites and a third region of unknown function. The presumed Mg 2+ -binding aspartate residues in the Walker B domain are not conserved. BLAST comparison of full-length Dark and Apaf-1 identified a region with 20% identical and 41% positive alignments spanning the conserved region. Although Apaf-1 shares ~20% identity with both CED-4 and Dark, the worm and fly proteins share only 14% amino-acid identity within this domain.

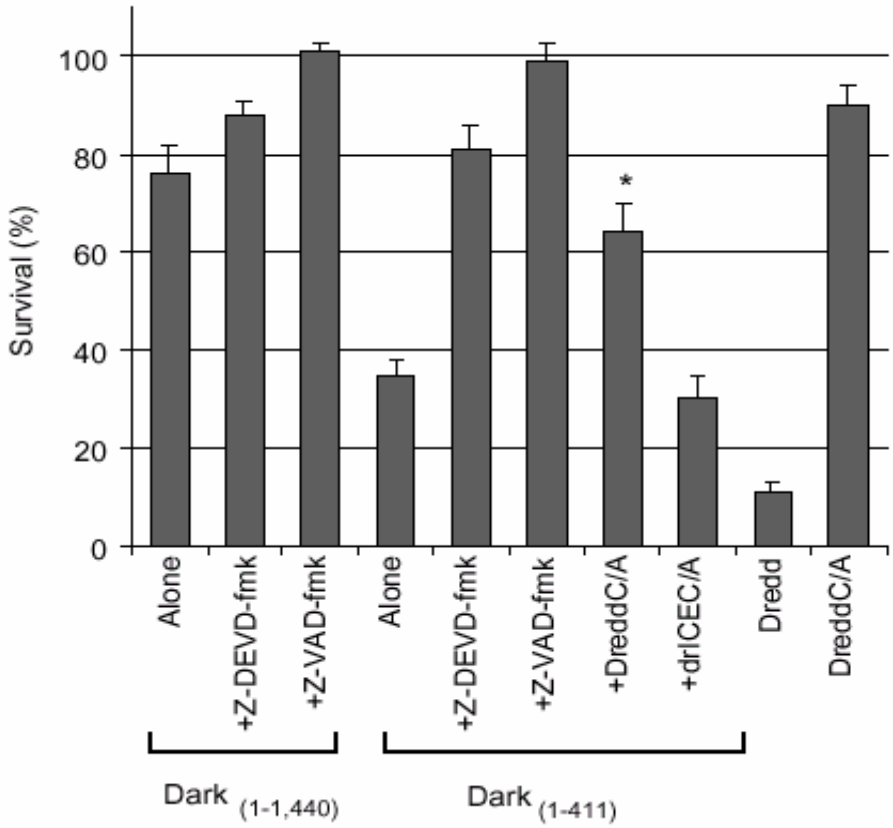
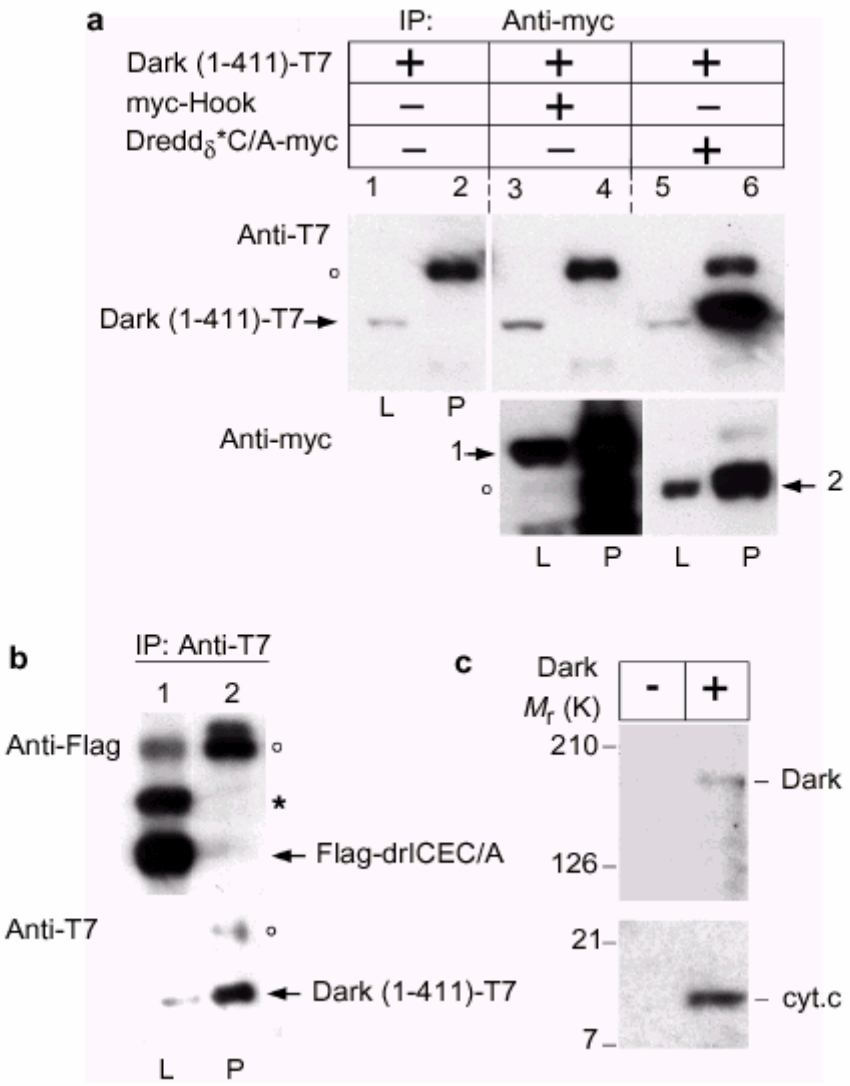

Survival (%)
100
80
60
40
20
0
*
Alone
+Z-DEVD-fmk
+Z-VAD-fmk
Alone
+Z-DEVD-fmk
+Z-VAD-fmk
+DreddC/A
+drICEC/A
Dredd
DreddC/A
Dark (1-1,440)
Dark (1-411)

Figure 2 **Dark-induced cell killing is suppressed by an active-site Dredd mutant.** Conditional expression of Dark in cultured *Drosophila* SL2 cells triggered modest levels of cell death that were substantially enhanced by removal of the C-terminal WD domain. Cell killing by full-length (Dark(1–1,440)) and C-terminally-truncated (Dark(1–411)) Dark was suppressed by the DEVD caspase-inhibitor peptide and blocked by the Z-VAD peptide (50 mM each). In coexpression studies, T7- tagged Dark(1–411) was transfected alone or together with Dredd-δ*(C/A)–Myc (DreddC/A) or FLAG–drICE(C/A) (drICEC/A) as indicated. Dark-mediated cell death was specifically attenuated by the mutant apical caspase, Dredd-δ*(C/A), but not by a comparable mutation in the effector caspase drICE despite robust expression of both constructs (Fig. 3). *P<0.005, two-tailed homoscedastic t-test. Note that Dredd-δ* used here contains an extra 22 amino acids at the N terminus of the previously described Dredd-δ(see Methods). Dredd-□is not competent to induce apoptosis when overexpressed 21 , but Dredd-δ* (Dredd) is sufficient to provoke cell killing (second bar from the right). (Po Chen and Hua Zou)


a
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Dark (1-411)-T7
myc-Hook
Dredd$_{\delta}$*C/A-myc
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Anti-T7
Dark (1-411)-T7
L P
Anti-myc
1
2
L P L P
b
IP: Anti-T7
1 2
Anti-Flag
Flag-drICEC/A
Anti-T7
Dark (1-411)-T7
L P
c
Dark
M_r (K)
210
126
21
7
Dark
cyt.c

Figure 3 **Dark interacts with the fly apical caspase, Dredd, and cytochrome c.** a, b, Lysates prepared from SL2 cells transfected with a, T7-tagged Dark(1–411) alone or with Myc-tagged Dredd-δ*C/A or control protein Myc–Hook, or b, T7-tagged Dark(1–411) plus FLAG-tagged drICE(C/A), were immunoprecipitated (IP) with anti-Myc or anti-T7 antibodies as indicated. Immunoprecipitated protein complexes (lanes labeled with P) and the original lysates (lanes labeled with L) were immunoblotted using the indicated antibodies **(**a, anti-T7 and anti-Myc; b, anti-FLAG and anti-T7). The positions of Dark(1–411)–T7 **(**a, b), Myc–Hook (band 1 in a), Myc–Dredd (band 2 in a), and FLAG–drICE(C/A) **(**b) are indicated. IgG heavy chain is indicated with a circle. Note that Dredd–Myc (lower panels in a) co-migrated with the IgG heavy chain, and in lane 5, three times more lysate was loaded in the lower panel to visualize Dredd– Myc better in the total lysate. The interaction between Dark and Dredd was confirmed in reciprocal assays using Dark(1–411)–Myc (pMT-Dark(1–411)–Myc3) together with Dredd(C/A)–T7 (pMT-Dredd-δ*(C/A)–T7) (data not shown). The band indicated with an asterisk in **b** represents a protein that crossreacts with anti-FLAG antibody. We estimate that >30% of Dark protein expressed in the total lysate was precipitated by Dredd. In contrast, no interaction was detected between Dark and a similarly Myc-tagged version of Hook 34 or Grim (data not shown). Use of full-length Dark–Myc3 (pMT-Dark–Myc3) together with pMT-Dredd-δ*(C/A)–T7, and of Dark(1– 411)–Myc together with FLAG–drICE(C/A), gave results (not shown) consistent with the conclusion above. c, Lysates from a stable line induced for pMT-Dark–Myc3 , or similarly treated parental SL2 cells, were incubated with anti-Myc antibody. Pelleted protein from immunoprecipitation was analyzed with anti-Myc and anti-cytochrome c monoclonal antibody. The positions of cytochrome *c* (cyt.c) and Dark–Myc3 (Dark) are indicated. Similar assays using lysates from cells transiently transfected with pMT-Dark(1–411)–Myc3 showed no co-immunoprecipitation of cytochrome *c* (data not shown). (Po Chen and Hua Zou)

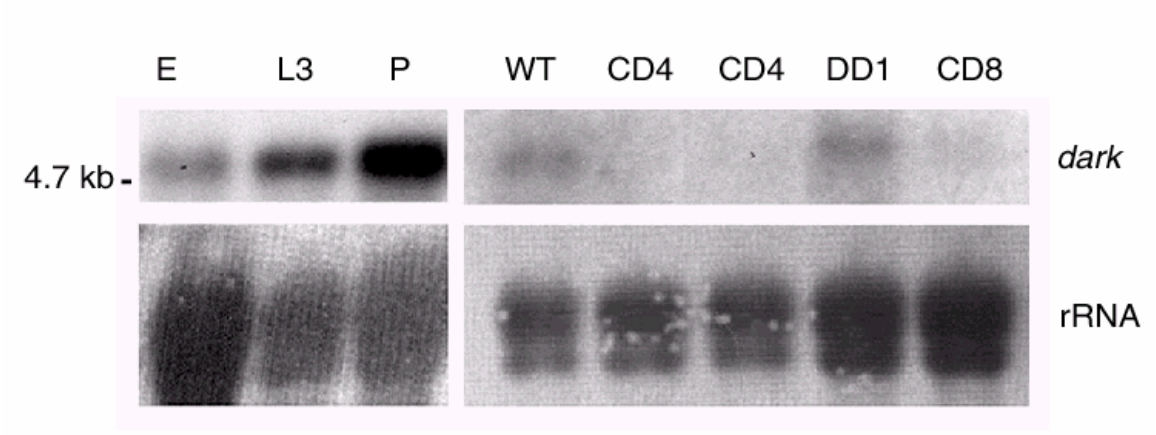
E
L3
P
WT
CD4
CD4
DD1
CD8
4.7 kb
dark
rRNA

Figure 4 **Expression of *dark* during development.** Upper panels, northern blot of total RNA from wild-type embryos (E, 0–24 h; L3, third-instar larvae; P, early pupae; WT, adults) and homozygous CD4, DD1 and CD8 flies. A single ~5-kb transcript is detected at all stages in wild-type animals. Increased *dark* expression is found in early pupae. *dark* is also detectable in wild-type adults but not in adults homozygous for CD4. Lower panels, the same blots stained with methylene blue to reveal the relative amounts of total ribosomal RNA loaded in each lane. Semiquantitative analyses from the phosphorimager indicate that *dark* transcript in CD8 animals occurs at ~25% of wild-type levels and is undetectable in CD4 animals (if any expression occurs at all, levels are <10% of wild type).

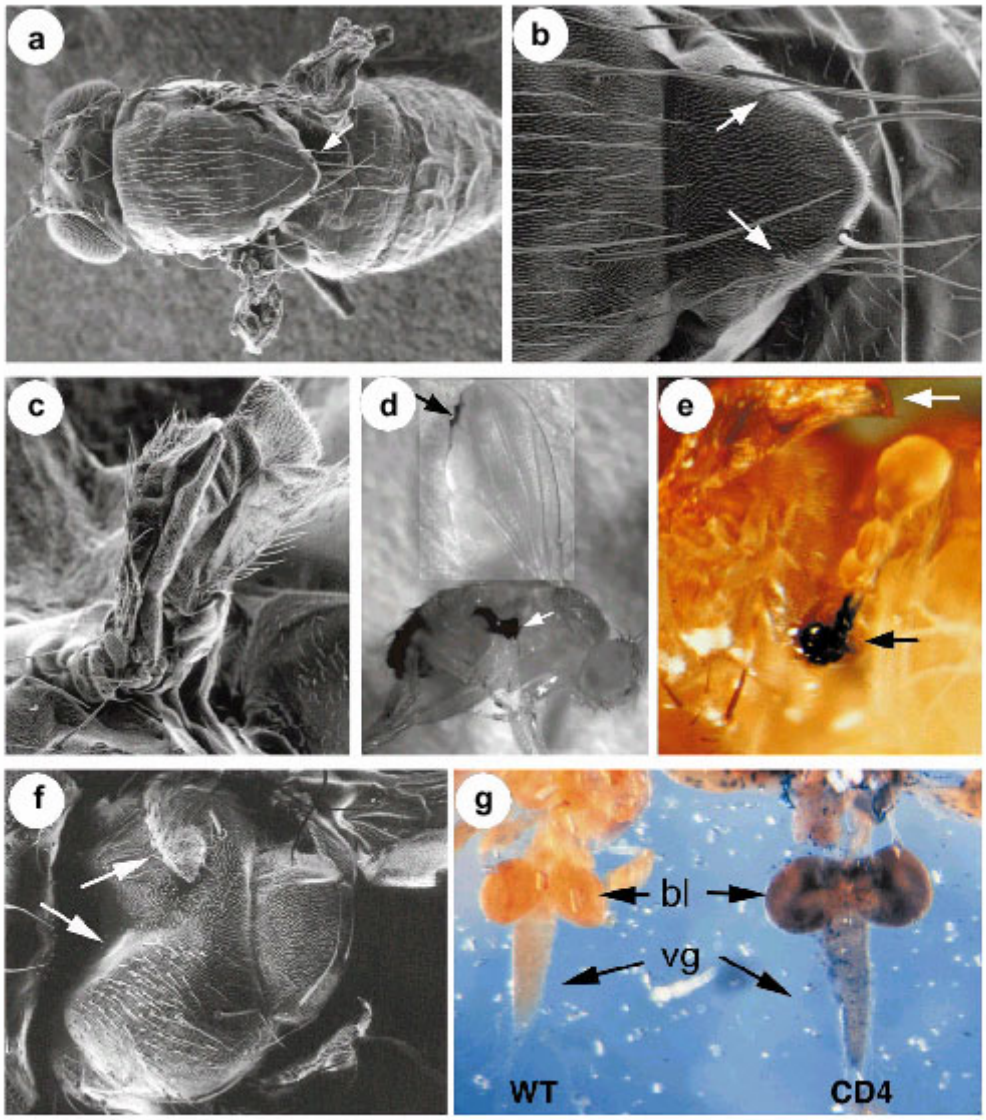
a
b
c
d
e
f
g
bl
vg
WT
CD4

Figure 5 ***dark* loss-of-function phenotypes.** Genotypes of all animals shown in **a–f** are *y*w; *dark* CD4 */dark* CD4 . a, Scanning electron micrograph of an adult fly with abnormally gnarled wings and one extra anterior scutellar macrochaete (arrow). b, c, High-magnification scanning electron micrographs of a mutant showing two extra anterior scutellar bristles (arrows in b) and an abnormal wing **(**c). d, e, Examples of *dark* CD4 flies with ectopic melanotic tumors outside the body. The tumor is indicated in **d** by a white arrow and in **e** by a black arrow. A nicked wing (black arrow in d) and a gnarled wing (white arrow in e) are also shown. f, Example of a rare homozygote with irregular bumps (white arrows) and severe bristle abnormalities in the notum. g, CNS dissections from wild-type (left) and *dark* CD4 / *dark* CD4 (right) third-instar larvae. Note the substantially larger size of the mutant brain lobes (bl) and ventral ganglion (vg) compared with the wild type, and also b-galactosidase staining from the CD4 enhancer trap in this tissue. (Antony Rodriguez)

Allele and genotype*	Total abnormal†	Abnormal wings only‡		Abnormal wings and extra bristles		Extra bristles only§	Melanotic tumours		*n*	Enlarged CNS¶	Male sterility#
		Both wings	One wing	Both wings	One wing		Outside body	Inside body			
CD4/CD4	35%	10%	15%	2%	2%	6%	7%	3%	285	70% *n*=23	47% *n*=21
CD8/CD8	21%	2%	5%	1%	4%	9%	8%	0%	127	n.d.**	n.d.
CD4/CD8	22%	2%	9%	1%	2%	8%	6%	0%	141	n.d.	n.d.

* The data for the CD4 and CD8 alleles came from study of F_1 flies derived from homozygous parents, whereas the CD4/CD8 data came from study of flies with heterozygous parents.
† Total number of afflicted flies is less than the summation of individual phenotypes because melanotic tumours occur only in the subsets of flies showing wing defects.
‡ 'Abnormal wings' refers to several defects, including wrinkled/gnarled wings, notches or wing blisters.
§ Additional bristles in the scutellum were scored and most flies had extra anterior scutellar macrochaetae.
¶ Third-instar larvae were scored for hyperplasia of the CNS relative to *yw* (*n*=20) controls.
\# Single homozygous males were mated to *yw* females in single vials and monitored for F_1 progeny for two weeks.
** n.d., not determined.

Table 4 Developmental abnormalities observed in* dark *loss-of-function mutants

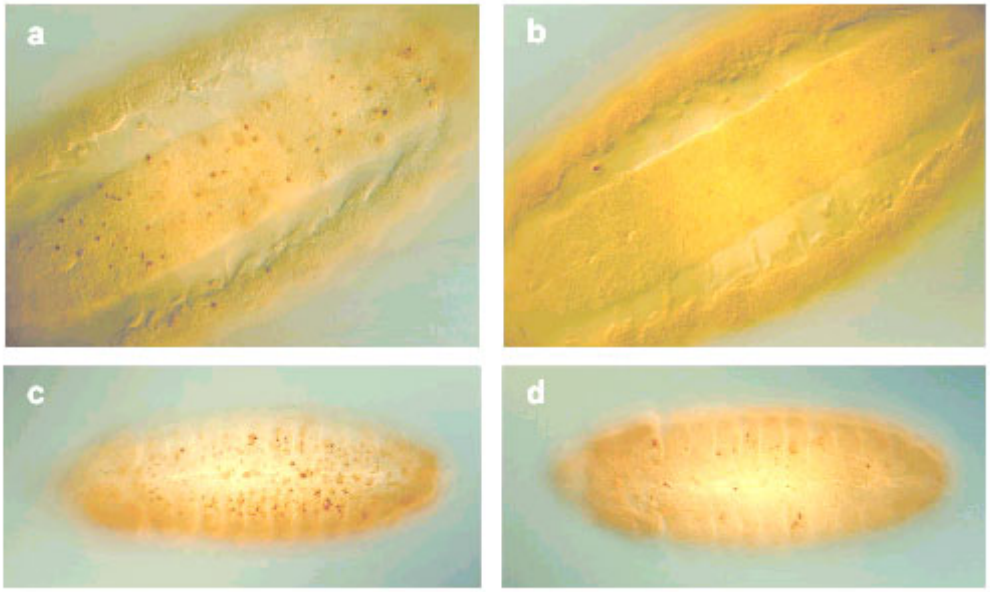
a
b
c
d

Figure 6 *Reduced apoptosis in* dark *mutants.* Embryos were stained with TUNEL to detect relative levels of PCD. a, b, Ventral views of comparably staged wild-type (a) and dark CD4 (b) embryos (stage 16). a, As the nerve cord condenses, prominent cell death is evident in wild-type embryos throughout the CNS. b, In contrast, dark CD4 mutant embryos show substantially less cell death in this tissue. c, d, Ventral views of comparably staged younger wild-type (c) and dark CD4 (d) embryos, showing the incidence of apoptosis in the epidermis. Note the widespread reduction in PCD in dark CD4 mutants. Hid (Antony Rodriguez)

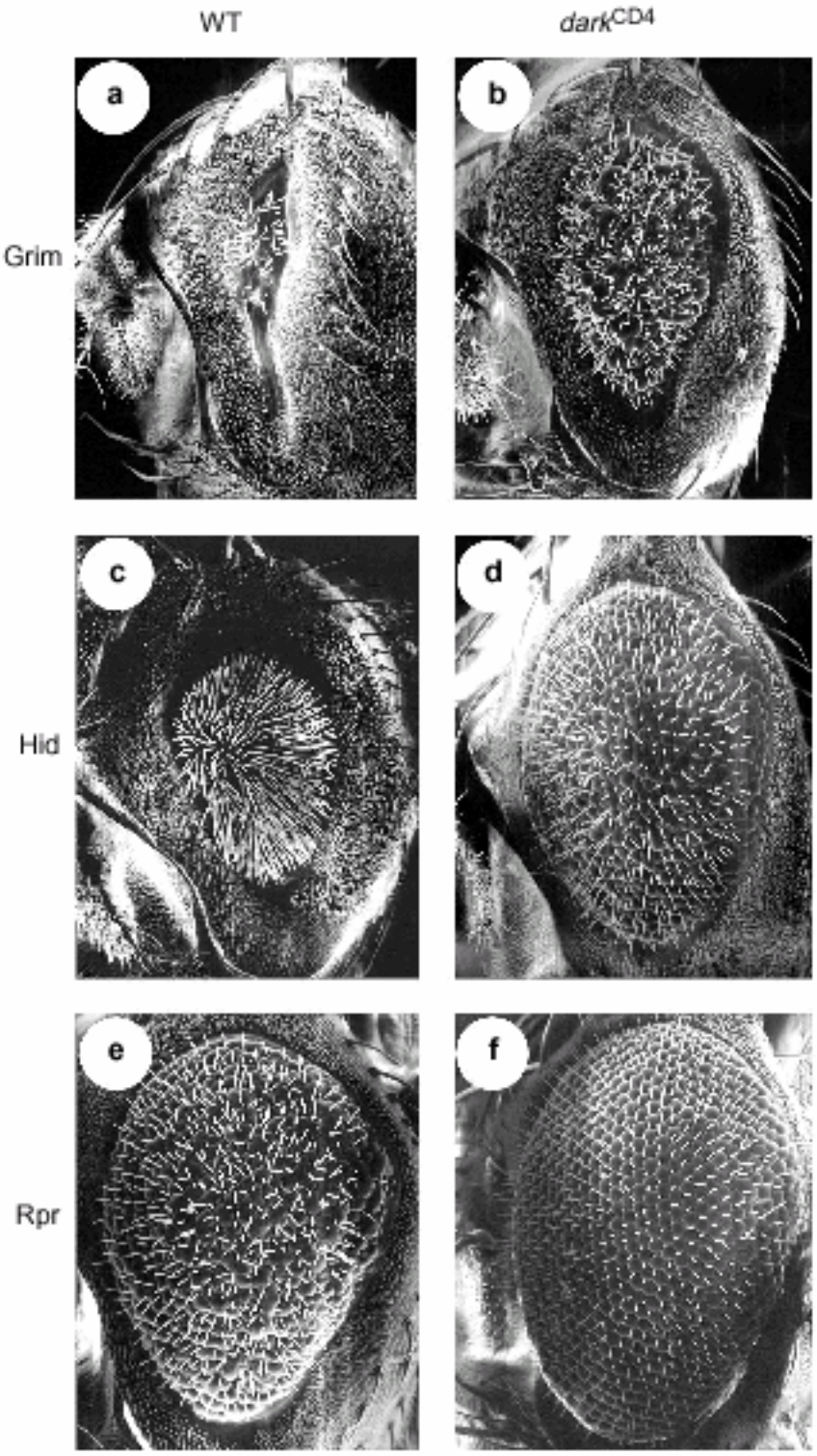
WT
darkCD4
a
b
Grim
c
d
Hid
e
f
Rpr

Figure 7 ***Dark* function is required for *reaper*-, *grim*-, and *hid*-induced cell death.** Scanning electron micrographs of adult fly eyes of the following genotypes: a, P[GMR-*gri*m]-1/P[GMR-*gri*m]-1; b, *dark* CD4 , P[GMR-*gri*m]-1/*dark* CD4 , P[GMR-*gri*m]-1; c, P[GMR-*hi*d]-1M/P[GMR-*hi*d]-1M; d, *dark* CD4 , P[GMR-*hi*d]-1M/*dark* CD4 , P[GMR-*hi*d]-1M; e, CyO/*dark* CD4 ; P[GMR-*reape*r]-97A/P[GMR-*reape*r]-97A; f, *dark* CD4 */dark* CD4 ; P[GMR-*reape*r]- 97A/P[GMR-*reape*r]-97A. a, Two copies of the P[GMR-*gri*m]-1 transgene completely eliminate ommatidia in flies wild-type for *dar*k. b, In a homozygous *dark* CD4 background, the *gri*m-induced phenotype (induced by two copies of this same P[GMR-*gri*m]-1 transgene) is suppressed. Note considerable rescue of organized ommatidia. c, Apoptosis induced by two copies of the P[GMR-*hi*d]-1M transgene is manifested as a severely roughened, small eye in a wild-type background. d, The phenotype caused by two copies of P[GMR-*hi*d]-1M is markedly suppressed in the *dark* CD4 mutant. e, Two copies of P[GMR-*reape*r]-97A in a background heterozygous for *dark* CD4 exhibit a markedly roughened phenotype. f, In a background homozygous for *dark* CD4 , the phenotype induced by two copies of P[GMR-*reape*r]-97A is strongly suppressed. (Antony Rodriguez)

Chapter 6 Examination of the mechanisms of Grim function and the role of DARK in Drosophila Apoptosis

The initiating events in apoptosis are tightly regulated and are regarded as one of the toughest remaining problems in the field. As the most irrevocable of all cell decisions, understanding of the process is crucial to the hopes of therapeutically intervening in the process. Despite the clear conservation of the main components in this decision (Ren et al 2001), the mechanism remains obscure and possibly is different in each model system. Comparison of the completed genomes of several animals highlights the manner in which conserved protein modules are specifically adapted within an organism. By looking at the proteins Dark and Grim which regulate caspase activation in Drosophila in comparison with their human orthologues, one can show how even functionally homologous molecules can have their role customized to fit the regulatory signals within a species. Armed with the knowledge of how the mammalian homologues of these proteins work, I examined these proteins as an intriguing entry point to this question.

Apaf-1's function and regulation during apoptosis are the best understood of all of the Ced-4 proteins, yet its function as an initiator or an amplifier is still debatable (Finkel 2000). Inert in the absence of cytochrome c and an adenosine-triphosphate nucleoside at its normal levels of expression, Apaf-1 complexes together with Pro-caspase-9 after the release of cytochrome c from the mitochondria to start the flow of caspases that shrinks and dices the cell into bite-size packets for phagocytic cells (Li et al 1997). While clearly a critical point of caspase activation, this event is not the irrevocable step in apoptosis in most cases (Cecconi et al 1998, Yoshida et al. 1998, Honarpour et al 2000). The release of cytochrome c from the mitochondria appears to be the most important decision for the

cell given that the lack of sufficient gene dosage of pro-apoptotic Bcl-2 members gives a much more severe phenotype than that of the Apaf-1 knockout (Lindsten et al 2001, Wei et al 2001, Zong et al 2001). Apaf-1 is essential for caspase activation in response to many cell death stimuli (Cecconi et al 1998, Yoshida et al 1998) and can be said to be an initiator of cell death in the neuronal precursor region of the brain that surrounds the ependymal region (Honarpour et al 2000).

By comparison the upstream events that play a role in the early stages of caspase activation in Drosophila remain mostly unknown. The genetic analyses of the fruitfly yielded a tightly linked cluster of genes that are that species' apical regulators of programmed cell death- Reaper, Grim and Hid (White et al 1994, Grether et al 1995,Chen et al 1996). With no apparent homology to any known protein, the function of these proteins has remained elusive. One tantalizing hint has come from the fact that the conserved N-terminus of these proteins interacts with the BIR domains of the IAP proteins, DIAP1 and DIAP2 from Drosophila (Vucic et al 1997). With the discovery that the IAP proteins can directly inhibit caspases (Devereux et al 1998), one speculation has been that the proteins interact with IAP's to interfere with their inhibition of caspases. However, not all of the killing activity of Reaper and Grim resides in the N-terminal IAP binding motifs (Chen et al. 1997, Wing et al 2001). Alternatively, the binding of Reaper, Grim and Hid to IAP's is more important for the sequestration and degradation of the pro-apoptotic proteins. The IAP proteins contain a c-terminal RING finger motif that has been implicated in the targeted degradation of IAP's and their associated proteins in vivo (Wang et al.2000). While the RING finger motif is not essential for IAP's to block Reaper, Grim or Hid induced death in transfection experiments, mutation of the RING

fingers in the Thread-6 allele of DIAP1 results in a homozygous lethal mutation (Lisi et al. 2000). Despite efforts to address this question with peptides or protein fragments (Hawkins et al 2000, Deveraux et al 1998), the function of the pro-apoptotic proteins has not been assayed directly due to the lack of suitable biochemical system in Drosophila.

Using a variant Hela cell line that has reduced dATP-dependent activation of caspase-3 due to the overexpression of IAP's, a mammalian anti-IAP factor was isolated called SMAC (Du et al 2000, Verhagen et al 2000). SMAC is a mitochondrial protein that can overcome the inhibition of caspase-3 and caspase-9 by IAP such as c-IAP-1, c-IAP-2, and XIAP. To test the function of recombinant Grim, I used a similar mammalian protein based assay that is IAP dependent. As seen in figure 1A, addition of dATP and cytochrome c to the Hela cell extract activates caspase-3 processing (Figure 1,Lane 1). Addition of the Bir2 domain of XIAP to the extract results in a decreased level of caspase-3 processing (Lane2). Addition of SMAC to the extract overcomes the inhibition by IAP and restores a normal level of caspase-3 processing (Figure 1, Lane 3). Although bacterial expression of the Reaper, Grim or Hid proteins is difficult due to aggregation, renaturing of Grim protein with a C-terminal histidine tag allows for the purification of the protein with a stable protein fold as assayed by Circular Dichroic Spectroscopy (Matt Junker, Personal communication). Using this renatured Grim protein, the IAP-dependent inhibition of dATP mediated could also be reversed (Figure 1, Lane 4).

To show this activity was due to IAP's more directly, a completely purified and reconstituted system of proteins was used. Baculovirus-expressed Apaf-1 and caspase-9, and purified horse cytochrome c form an apoptosome that processes S^{35}-labelled in vitro translated Procaspase-3 in the presence of dATP(Zou et al 1997). This activity was

inhibited using a bacterially expressed construct that contains the three BIR domains of XIAP. Both the addition of bacterially expressed SMAC or bacterially expressed Grim show a dose-dependent increase in caspase-3 processing (Figure 1B).

Structural analysis of the SMAC-BIR interface shows that the N-terminus of SMAC is essential to its binding to the BIR domain. This portion of the protein is generated by proteolytic maturation of the protein as it is translocated to the mitochondria to generate a peptide sequence beginning with the amino acids AVPIA(Du et al 2001). Mutations that affect the sequence significant block the ability of SMAC to bind to the BIR domain(Liu et al 2000, Wu et al 2000). Comparison of this region of the protein with the conserved portion of Reaper, Grim and Hid shows similarity between the first amino acids of the RGH domain after the starting methionine (cf. Hid M-AVPFY). To test whether this similarity had functional significance peptides corresponding to the first six amino acids of the N-terminal regions of Reaper, Grim, and Hid were synthesized to test for competition for the SMAC binding site. Because of the tightly packed alanine residue of SMAC's binding site with the BIR domain (Wu et al 2000), the peptides were also generated without methionine to see what role this first residue may have on the peptides' binding. Using a bacterial expressed version of the BIR3 domain of XIAP fused to GST, SMAC binding to BIR3 was assayed. In Figure 2, SMAC can be precipitated from solution with the GST-BIR3 protein. SMAC's binding blocked with the peptide corresponding to the N-terminal seven amino acids of mature SMAC. While the hexapeptides corresponding to the translated sequences of Reaper, Grim and Hid could not compete for SMAC binding, peptides without the N-terminal methionine were

effective competitors for the SMAC binding site allowing for SMAC protein to remain in solution.

The amino terminal methionine of many proteins are cleaved from proteins depending on their second amino acids. Small second amino-acids such as alanine, serine, or glycine predispose proteins to being processed by methionyl aminopeptidases. There are four proteins in Drosophila that are homologous to the methionyl amino peptidase enzymes identified in other organisms. While mutations of one of these enzymes DMAP2 have been identified genetically as the *uninitiated* gene (Cutforth and Gaul 1999), genetic analysis of the strongest DMAP2 allele (DMAPΔ34) had no effect on cell death as examined by acridine orange staining of embryos or by crossing the mutation into flies expressing Reaper, Grim, or Hid in the eye under control of the GMR promoter (data not shown).

Genetic analysis of the role of IAPs in Drosophila has positioned the gene as functioning downstream of *reaper*, *grim* and *hid*. Loss-of function alleles of DIAP1 can sensitize fly cells to apoptosis caused by these genes while gain-of-function mutants can suppress *reaper*-, *grim*- or *hid*-induced cell death (Goyal et al 2000). If the IAP and Grim interact solely to interfere with caspase inhibition, and thereby launch the apoptotic events in Drosophila, one would expect that counteracting the IAP's using pharmacological means would also be able to activate caspases. However, treatment of extracts from Drosophila embryos and two Drosophila cell lines with peptides corresponding to first five amino acids or reaper, grim and hid could not activate DEVD cleaving caspases nor could the addition of recombinant SMAC or Grim protein(Data not shown).

To better understand the downstream events after the deactivation of IAP proteins, I examined the biochemical activity of Drosophila embryo extracts in embryos with a severe loss-of-function allele of DIAP1, *thread-5 (th^5)*. DIAP1 is an essential gene, and loss-of-function alleles are embryonic lethal (Hawkins et al. 2000). th^5/th^5 embryos have a severe cell death phenotype with massive TUNEL staining and increased caspase activation. Because both DIAP-1 mutations and homozygous $dark_{CD4}$(CD4) mutations are epistatic to cell death caused by ectopically expressed *reaper*, *grim* and *hid*, the necessity for Dark function downstream of DIAP1 inactivation was examined. As seen in figure 3, embryo lysates from populations containing homozygous th^5 mutations have a significant increase in caspase activity versus wild-type strains as measured using a fluorescent DEVD substrate in four to four and half hour embryos. As seen in figure 4a, endogenous levels of caspase activity are very low in the background yellow/white (y/w) strain with a strong increase in the caspase seen if embryo populations containing $+/th^5$ and th^5/th^5. Homozygosity with the CD4 allele of DARK significantly suppressed the levels of caspase activity in the th^5population of flies and the y/w strain. The degree of suppression was 75-90% with variability possibly due to differing ratios of the embryonic genotypes in each group combined into a single lysis event. Although this suppression did not rescue the embryos, the effects on downstream apoptotic pathways could be assessed by examining TUNEL labeling of DNA. Homozygous CD4 mutations caused a significant decrease in the TUNEL staining in th^5/th^5 embryos (Figure 4c)

The CD4 mutation did not block the ability of caspases to autoactivate their zymogen forms. Dilution of 1ul of extract containing caspases activated by the th^5 mutation into 10ul of both wildtype y/w embryonic lysate, or homozygous cd4 extract

allowed for a non additive increase in the level of effector caspase(Figure 3b). The mixture of quiescent extract with the active caspase activity effectively tripled the level of caspase activity seen in with extract alone regardless of the presence of a mutation in DARK. The final level of caspase activity of the mixed extracts remained below the level th^5extract even after a 1 hr preincubation at 30 degrees Celsius.

The requirement for Dark function in cell death pathways regulated by DIAP-1 can also be verified genetically As Dark is a powerful supressor of reaper, grim, and hid induced apoptosis in vivo (Rodriguez et al 1999) we sought to determine whether a 2 fold reduction in DIAP1 (heterozygozity at DIAP1 locus) might neutralize this effect. Expression of reaper, grim, and hid under the eye specific promoter, GMR, is manifested as a rough eye ablation phenotype which is highly reproducible and modifiable depending on the genotypes of other cell death genes in the pathway. The cell killing phenotypes caused by P[GMR-hid]-1M (GMR-hid) and P[GMR-grim]-1 (GMR-grim) are very sensitive to DIAP1 gene dosage (Meier et al 2000). Data from a number of labs have shown that loss-of-function DIAP1 alleles act as strong dominant enhancers of reaper-, hid, and grim-induced cell killing in the eye whereas DIAP1 gain-of-function alleles can suppress cell killing by these cell death initiators (Goyal et al 2000, Lisi et al 2000). To determine if Dark could modify the genetic interaction between a loss-of-function DIAP1 allele and hid- or grim- induced cell killing, we generated a stable dark ^{CD4} ; th^5 double mutant strain and crossed it to either a dark ^{CD4}, GMR-hid or dark ^{CD4}, GMR-grim tester strain (see Materials and Methods). While DIAP1 is still an effective dominant enhancer of grim- or hid-induced cell killing in flies heterozygous for dark (data not shown), it fails to modify grim or hid killing in flies homozygous for Dark (Fig

4). These results suggest that Dark functions genetically downstream or parrallel to from DIAP1 in grim and hid signaling pathways.

From the previous results it seemed likely that Dark would function downstream or at least parallel to DIAP1 in apoptosis. However, the epistatic experiments in the eye are not ideal since DIAP1 is at best heterozygous under those conditions and *rpr, grim* and *hid* are overexpressed. Ideally, we would want to determine if dark is epistatic to DIAP1 in a DIAP1 null situation. This is complicated by the fact that DIAP1 is essential for survival and homozygous eye clones are impossible to obtain. One might predict that if Dark were epistatic to DIAP1, then it might also be possible to alleviate or suppress a DIAP1 null phenotype. To get around this problem, we used the strain, described above, which is homozygous for dark and balanced for th^5 to obtain double mutant embryos and determine if dark can rescue some or all of the DIAP1 null embryonic phenotypes.

Previously, it was proposed that the disruption of caspase-IAP interactions is central to the initiation of cell death during Drosophila embryogenesis. In this model, IAPs would be expected to continually hold caspases in check from an ever-present death signal. Consistent with this hypothesis DIAP1 null animals die of severe morphological abnormalities and most, if not all, cells show signs of DNA fragmentation early during embryogenesis. We tested whether dark might modify these DIAP1 -/- phenotypes by labeling embryos with TUNEL and observing them by Nomarski optics.

We find that dark CD4 ; th^5 embryos show a striking suppression of the TUNEL and also the blastoderm-arrested phenotypes which are seen in DIAP1 embryos (see below). As reported by Hay et al, we find plenty of examples of blastoderm-arrested TUNEL positive th^5 embryos (Figure 5B). In contrast, $dark^{CD4}$; th^5 double mutants never

show this phenotype. Moreover, the vast majority of dark^{CD4} ; th^{5} embryos have a phenotype indicative of some normal cell differentiation and cell migration such that many of the morphological characteristics utilized for staging purposes are discernable (Figure 5: compare panel A with panel C; Hawkins et al 2000). Interestingly, the presence of embryos such as depicted in Figure5 panel B indicate that dark can rescue the early morphogenetic arrest at the beginning of germband extension which is reported to always occur in th^{5} embryos (Hawkins et al 2000). Nevertheless, while all of these double mutant embryos appear to be spared from an early morphogenetic arrest they show a significant amount of TUNEL labeling and are not spared from less severe morphological abnormalities later in development (compare Figures 5A with 5B with regard to TUNEL staining and morphology). Also, on occasion we could find blastoderm-arrested double mutants which failed to stain significantly for TUNEL (Figure 5: panel D). While these embryos were less frequent, their occurrence could possibly mean that the morphological 'rescue' we saw in most dark CD4; th^{5} embryos may not be necessarily related to a suppression of TUNEL cell death per se.

The robust suppression of the DIAP1 embryonic phenotype by Dark prompted us to check if other DIAP1 phenotypes in other tissues might also be rescued. Since DIAP1 null mutations are embryonic lethal we took advantage of the fact that DIAP1 mutants transhomozygous for th^{6} and th^{8} were reported to live to the adult stage but suffer from infertility (Lisi et al 2000). We verified that th^{6} / th^{8} transhomozygous flies were indeed viable but sterile when mated to each other. To make sure that our th^{6} and th^{8} strains were indeed DIAP1 alleles, were also tested them for non-complementation with th^{5} (see Materials and Methods). Since the molecular nature of the th^{8} mutation is still undefined

we wanted to safeguard against the possibility that it might represent a mutation at a loci other than DIAP1. Therefore we tested the th^8 strain for non-complementation against DfX (a large deletion which removes the DIAP1 gene) and also for dominant suppression or enhancement of GMR-hid, and P[GMR-rpr]-M (GMR-reaper) cell killing phenotypes in the eye. While we find that th^8 is lethal in trans to DFX we failed to see any modification of cell killing with GMR-hid and GMR-rpr (data not shown), suggesting that it might represent a very weak DIAP1 mutation. The th^6 (also known as th^{6B}) allele has a C412Y missense mutation within the RING domain of DIAP1 and is believed to represent a type 1 gain-of-function mutation because it enhances induced cell killing by grim and reaper (Lisi et al 2000).

Closer examination of th^6/ th^8 adults was conducted and the percentage of females which failed to lay eggs determined. As depicted in Table 5 the vast majority (>90%) of th^6/ th^8 mutant females fail to lay any eggs in the food when mated to wild type males (see Materials and Methods). To determine if dark might alleviate this defect, we generated $dark^{CD4}$; th^6/ th^8 double mutant flies and similarly scored them for egg laying. As evidenced from the data in Table 5, heterozygozity or homozygozity for Dark greatly rescues this egg laying defect. This suggested some oogenesis defect which would prevent most th^6/ th^8 females from laying embryos might be alleviated in $dark^{CD4}$; th^6 / th^8 females. Therefore we dissected ovaries from th^6 / th^8 flies and compared them to ovaries from $dark^{CD4}$; th^6 / th^8 mutants. In the majority of cases th^6 / th^8 flies have extremely small or severely stunted and irregular shaped ovaries (data not shown). In comparison, $dark^{CD4}$; th^6 / th^8 females had ovaries which were generally larger and more normal in size (data not shown). After only 4-5 days post-eclosion individually dissected

ovarioles from th^6 / th^8 females display a severe drop in egg chamber production and late stage egg chambers frequently undergo degeneration (Fig 6A). In stark contrast, $dark^{CD4}$; th^6 / th^8 egg chambers are normal in appearance and number (Fig6B).

The strong suppression of DIAP phenotypes by Dark begged the question as to whether or not Dark would function simply as an amplifier to alter the time-course or intensity of caspase activity downstream or parallel to DIAP1 signaling or if could also function as an initiator of cell death downstream from reaper, grim, and hid. Previously, we showed that Dark mutants show greatly reduced levels of cell death during embryogenesis(Rodrigues et al 1998). To determine if a disruption in mitochondrial/dark-signaling would result in the survival of cells that would otherwise have been killed off by reaper, grim, and hid signaling during normal development we utilized the reporter strain, P[slit-lacZ] to follow PCD of the midline glia (Figure 7).

Discussion

In order to better understand the mechanisms and sequence of events involved in the initiation of apoptosis in Drosophila, the mechanism of IAP inactivation and the events that occur downstream of IAP inactivation were investigated using biochemical and genetic readouts. Using recombinant Grim protein, it was shown definitively that Grim can function to counteract the inhibition of caspases caused by IAP proteins. The N-terminal conserved motif of Reaper, Grim and Hid could compete for the same binding site on IAP's that is used by SMAC, and this N-terminus of these proteins must be modified by removal of the starting methionine to bind to this site. These experiments show that SMAC and Grim are functionally homologous proteins despite their overall lack of similarity. Structural investigation

of this interaction show the interactions to be a network of van der Wahls interactions and a tight network of hydrogen bonds formed by the amino terminal alanine. Because the necessary binding site to the BIR domain is only a few amino-acids, this shared functionality is most likely a result of convergent evolution. A similar sequence (ATFS) is used by XIAP's binding to activated caspase-9 (Srinivasula et al. 2000). The necessity for the amino-terminal alanine explains the increased affinity of IAP's for activated caspases as this primary contact site requires the protease to be cleaved at its activation site to reveal the alanine.

Despite the mechanistic similarity in binding to the BIR domain, the role of SMAC in mammalian apoptosis seems to be less essential that that of *reaper*, *grim* and *hid.* While induction of *reaper*, *grim* and *hid* can cause cell death in Drosophila tissues or tissue culture, the mitochondrial localization of SMAC ensures that SMAC expression is non-toxic and acts merely to increase cell death in response to another stimulus such as UV irradiation (Du et al,2001 Verhagen et al 2000). Although heterologous expression of *reaper* can induce cell death in mammalian cell lines, there are no reports as to whether SMAC artificially expressed as a mature cytosolic protein can induce cell death by inhibiting IAP's in non-apoptotic cells.

While Grim, and by extension, Hid and Reaper, can interact with the BIR domain in such a way as to block its caspase inhibition, it is not clear that this is the only role for these proteins. Reaper and Grim can both induce cell death even without the N-terminal BIR interaction region albeit at a lower efficiency(Wing et al 1998, 2001). A second IAP interaction domain other than the N-terminus has been proposed for reaper, but it remains unknown if these proteins can engage signaling pathways other the IAP's to induce cell

death. The interaction may play more of role in the reverse regulation of *reaper*, *grim* and *hid* by IAP proteins. The RING finger motif of the IAP's has been shown to function as an E3 ligase in other proteins. Examination of mammalian IAP's have show that they can be ubiquinated leading to the degradation of both themselves and activated caspases (Yang et al 2000. Clem et al 2000) The sequestration and degradation of the apical killing proteins may be an important anti-apoptotic function of DIAP1 and DIAP2. Indeed, the th-6 allele of DIAP has a mutation in the RING finger motif while maintaining the functional BIR domains (Vucic et al. 1998). Similar constructs were effective in blocking cell death in transfection assays, yet the mutation is homozygous lethal in the animal.(Lisi et al. 2000). Additionally, it is not yet clear to what extent the IAP proteins play roles in signaling pathways through interactions with other proteins. The human homologues of the IAP's were first identified as proteins binding to the TRAF1 proteins (Chu et al 1996), and XIAP has been shown to activate NFKb activation through IKK (Yamaguchi et al 1999). These TRAF complexes help stimulate NF-Kb downstream of membrane receptors such as the TNF or IL-1 receptors, thus the mechanism of caspase activation downstream of IAP mutation may be due to spontaneous autoactivation of caspases, or a disruption of other protective features of the protein which result in stimulated caspase activation.

The ability of the CD4 mutation in Dark to significantly suppress the caspase activity seen in th^5embryoes and otherwise wildtype embryoes indicates that DARK is an essential component in the normal levels of caspase activation. This is due to at least three possible scenarios. First, DARK, even in an unactivated cellular state, could allow for the processing of a low level of procaspase. This smoldering basal activity would

normally be suppressed by IAP's but in the th^5 mutant would be allowed to amplify itself into a robust caspase response. Alternatively, after low-levels of effector caspases could be ectopically activated by the absence of DIAP-1, engaging a caspase dependent mechanism to activate Dark and allowing for the further amplification of effector caspases to a higher level. This pathway would be analogous to the cleavage of the BH3 protein Bid by caspase-8 in the mammalian system that allows for the activation of Apaf-1 via Cytochrome c release. Finally, the disruption of *diap-1* may cause a developmental arrest or may deinhibit unknown regulatory pathways thereby triggering an induction of apoptotic stimuli that directly activate caspases via Dark. In this case, residual effector caspase activity seen in the CD4/th^5 double mutant is due to an incomplete elimination of Dark activity by the CD4 allele.

Dark clearly can participate in the amplification of caspase activity in Drosophila, and this role is apparently essential to initiate cell death in both normal development in the nervous system and in ectopic circumstances in the eye or ovary. Whether DARK is absolutely required for caspase activation remains unsolved, as the CD4 allele does express very low levels of mRNA (Rodriguez et al 1999). Targeted mutation of the mouse Apaf-1 gene can ablate the caspase activation induced by many stimuli; however, this does not necessarily block cell death in many mammalian cells (Cecconi et al 1998, Yoshida et al 1998, Honarpour et al 2000). While Apaf-1, Caspase-9 and Caspase-3 function are required for normal cell death in the neural progenitor region of the brain, many other cell deaths such as the interdigital cells in the extremities of the animals are completed, though at a delayed rate (Chautan et al 1999). This cell death appears to be due to the mitochondrial damage caused during cytochrome c release and the release of

additional apoptogenic molecules from the mitochondria such as AIF and endonuclease G (Li et al 2001, Suzin et al 2000). The role of mitochondria in Drosophila apoptosis remains unclear, but the fact that homozygous mutant strains of *dark* can still produce some adult flies points to redundant pathways of cell death in the fly as well (Rodriguez et al 1999). Mutation of the fly homologues of the Bcl-2 genes have not been reported, but should shed light on whether they play the essential role in orchestrating the parallel apoptotic pathways as they do by controlling mammalian mitochondria permeablization.

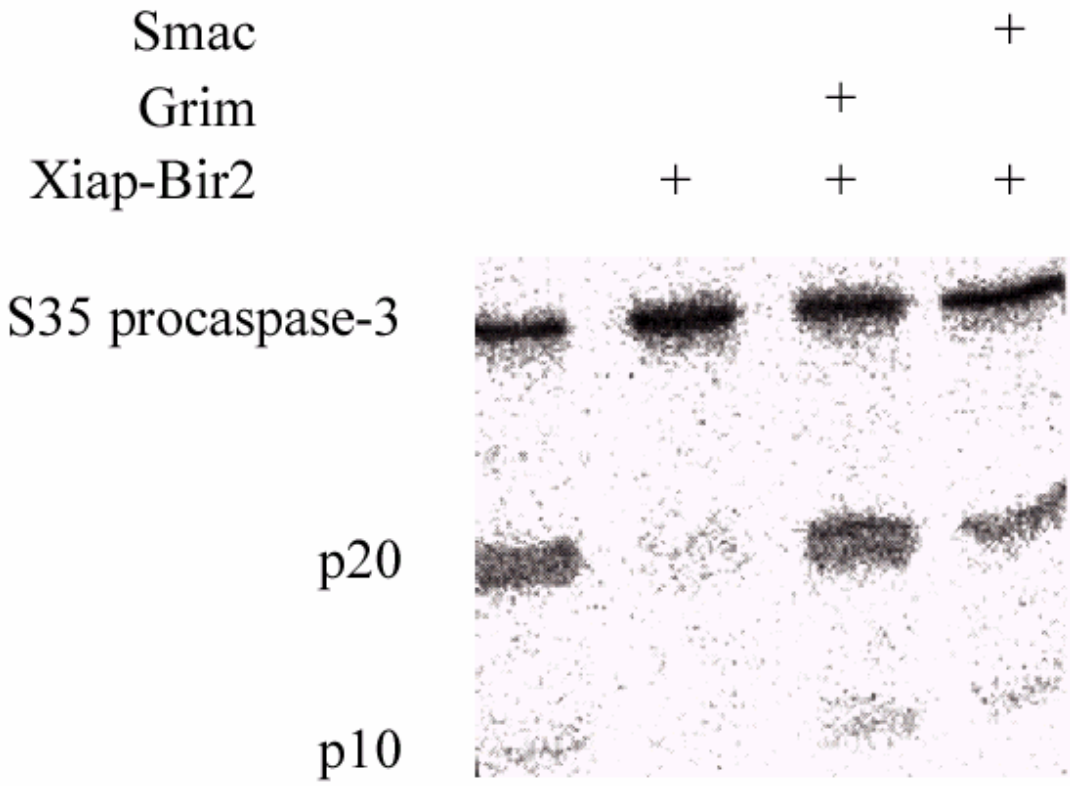
Smac
Grim
Xiap-Bir2
+
+
+
+
S35 procaspase-3
p20
p10

Figure 1. Recombinant Grim can overcome BIR mediated suppression of dATP dependent caspase activation. 20 ug of Hela S-100 was incubated with 1mM dATP and 1ug of cytochrome c to induce cleavage of S35 labeled Procaspase-3 (lanes 1-4). Inclusion of 10ug GST-XIAPBir2 (lanes 2-4) could inhibit cleavage of Procaspase-3 unless co-incubated recombinant SMAC (500nM, lane 3) or recombinant Grim (1uM, lane4).

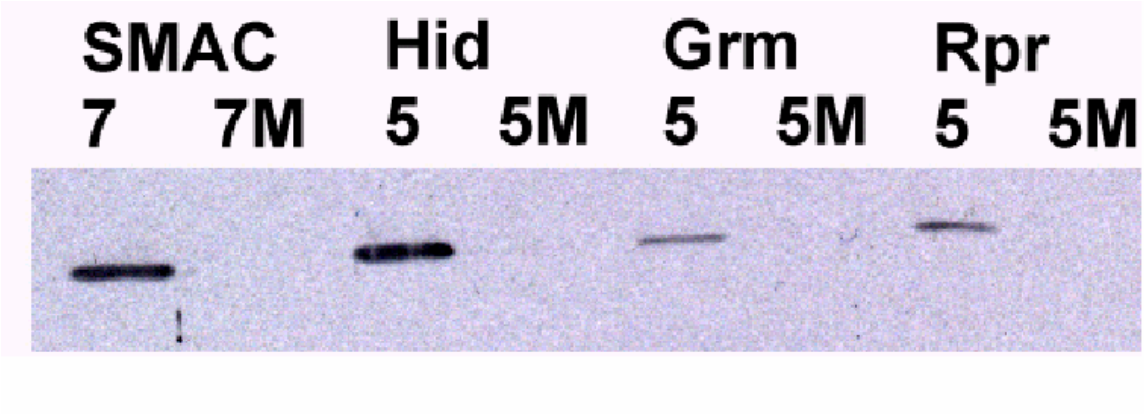
SMAC
Hid
Grm
Rpr
7
7M
5
5M
5
5M
5
5M

Figure 2. Peptides corresponding to the RGH motif compete for SMAC binding to the BIR domain of XIAP.

25 micrograms of bacterially expressed SMAC protein was incubated in 100 ul with 10 ug of GST-BIR3 protein and precipitated with Glutathione-Sepharose in the presence of peptides corresponding to the N-terminal regions with and without the start methionine of mature SMAC(7aa), Reaper, Grim or Hid (5aa each, final concentration 1mM). Soluble protein was resolved by SDS-PAGE and recognized with anti-SMAC Western Blot.

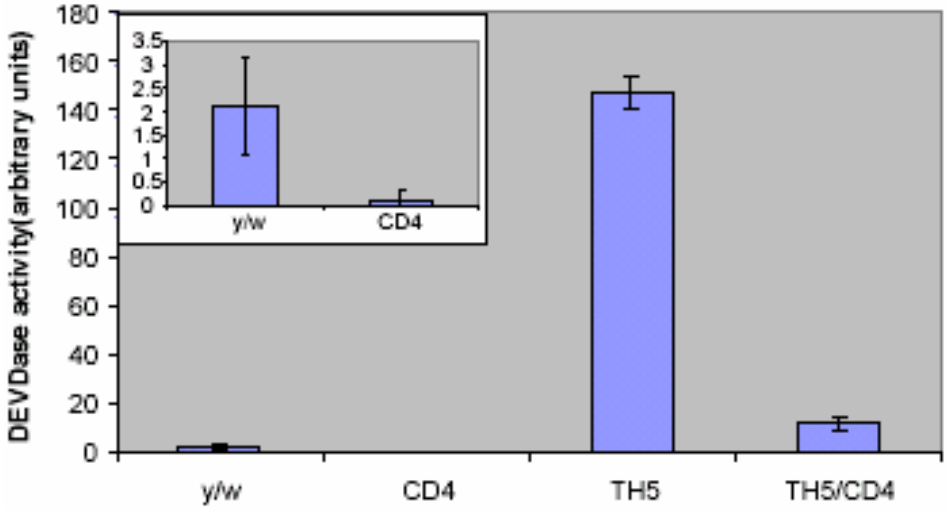
DEVDase activity(arbitrary units)
180
160
140
120
100
80
60
40
20
0
y/w
CD4
TH5
TH5/CD4
3.5
3
2.5
2
1.5
1
0.5
0
y/w
CD4

Figure 3. *dark* inhibits DIAP1-dependent caspase hyperactivation. Dark suppresses ectopic caspase activation in thread mutant extracts. Caspase 'hyperactivation' fails to occur in dark; thread double mutant embryo extracts.

A pool of 50-200 stage 9-10 embryos were collected from each of yw, yw; dark CD4, yw; TM6 / th 5, and the yw; dark CD4 ; TM6 / th^5 stocks respectively. 5 ug of embryo protein extract was analyzed for caspase activity by measuring Ac-DEVD-AFC fluorescence after cleavage over time. Each result was verified at least three times, and data represent the average slope of three separate pools of embryo lysates. $P<0.005$, two homoscedastic t-test.

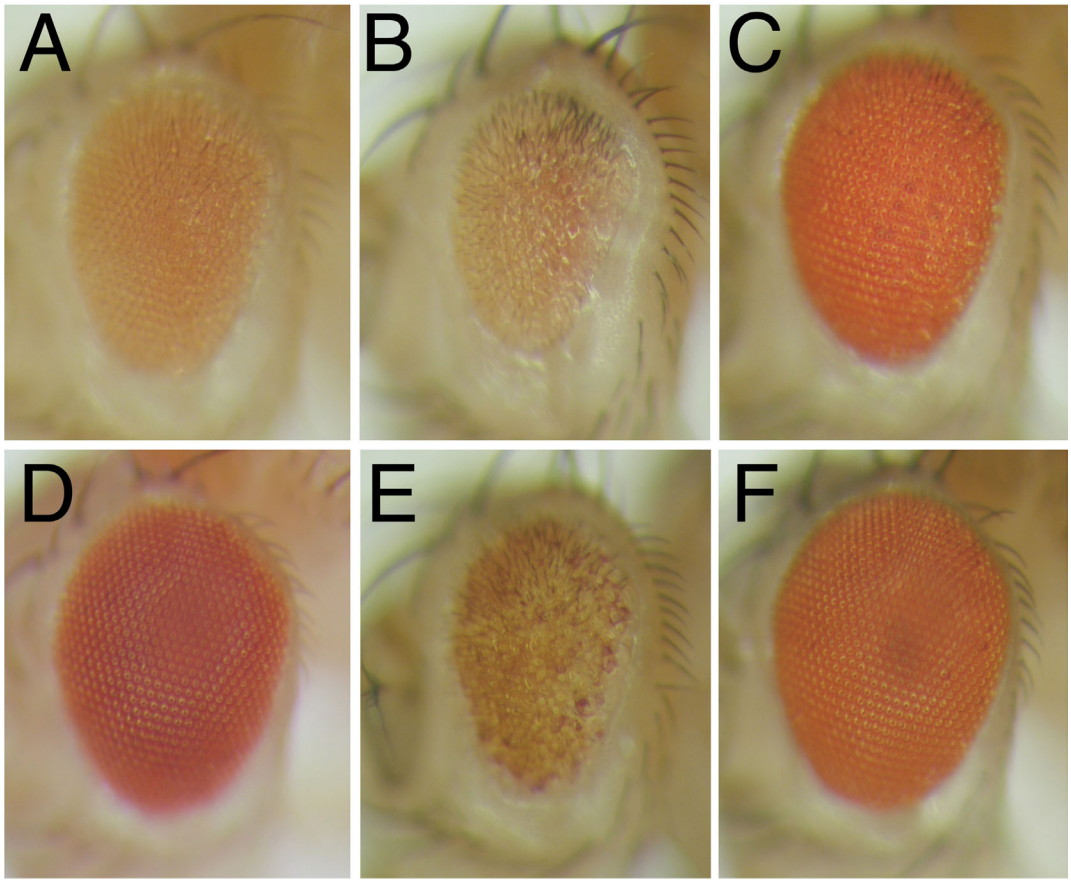
A
B
C
D
E
F

Figure 4. A loss of function Dark mutation mitigates the ability for DIAP1 to enhance Hid- and Grim- induced cell killing. Loss of function DIAP1 mutations fail to enhance HID or GRIM cell killing in the absence of Dark signaling. DIAP1 fails to enhance HID- and GRIM-induced cell killing in a Dark mutant.

Light microscopy micrographs of GMR-hid / + (A), GMR-hid / + ; th^5 / + (B), dark ^{CD4}, GMR-hid-1M / dark ^{CD4} ; th^5 / + (C), GMR-grim / + (D), GMR-grim / + ; th^5 / + (E), and dark ^{CD4}, GMR-grim / dark ^{CD4} ; th^5 / + (F) fly eyes. A single copy of GMR-hid or GMR-grim in a background heterozygous for DIAP1 gives rise to a less severe eye phenotype when placed in a background homozygous for Dark (compare B with C and E with F respectively).

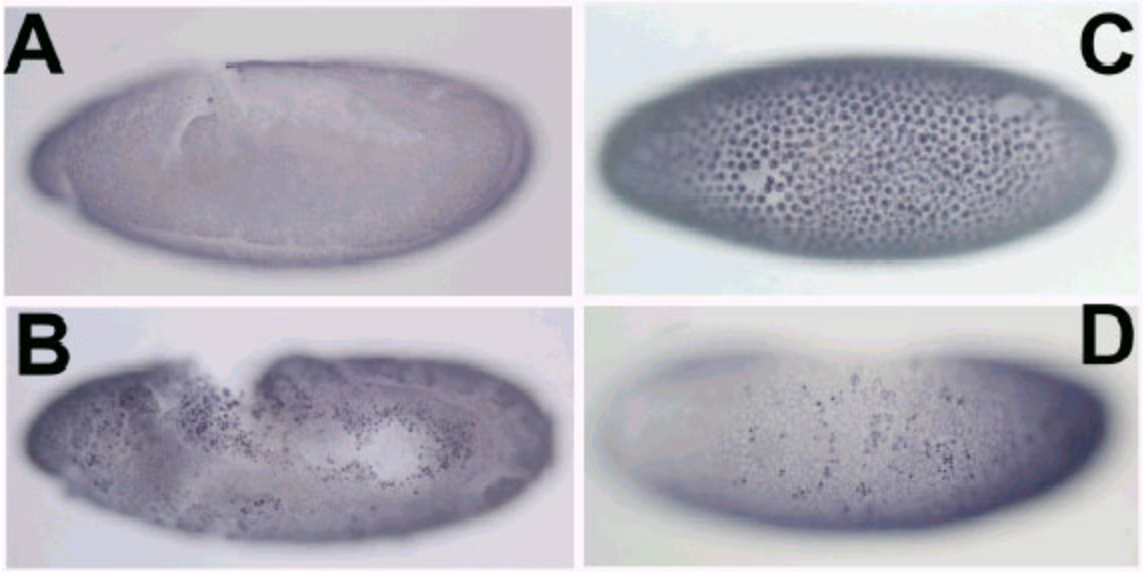
A
C
B
D

Figure 5. Suppression of the zygotic DIAP1 loss of function phenotypes by dark. Dark suppresses the zygotic loss of function DIAP1 phenotype in the embryo. Dark suppresses the global early morphogenesis defect and cell death seen in DIAP1 mutant embryos (Antony Rodriguez)

Nomarski micrographs of wild-type (A), th^5 (B), and $dark^{CD4}$; th^5, (C-D) embryos labeled by TUNEL. (A) Wild type embryos show few programmed cell deaths at the extended germband stage (early stage 9). (B) Stage 9-10 th^5 embryos show nuclear TUNEL labeling in virtually all cells and also a rounded blastoderm-like morphology. (C) In comparison, most $dark^{CD4}$; th^5 mutant embryos are considerably improved in development and TUNEL labeling (large arrows indicate the periodic bulges seen during stage 10). Note that there are still some signs of irregularities in embryonic development (arrowhead indicates a large gaping hole in the posterior midgut primordium). (D) Shows a $dark^{CD4}$; th^5 mutant embryo which is not rescued for the morphological abnormalities but shows TUNEL labeling in only a few cells. (Antony Rodriguez)

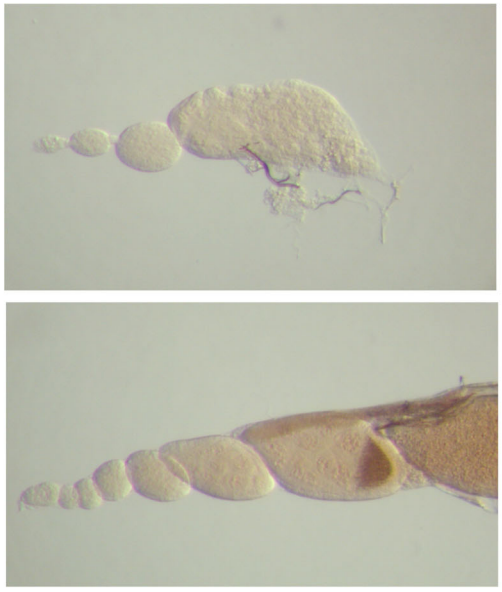

Figure 6. DIAP1 mutant phenotype in the ovary is suppressed by mutations at the Dark locus. th^6/th^8 mutant phenotype in the ovary is suppressed by $dark^{CD4}$. Dark alleviates the mutant phenotype which is seen in th^6/ th^8 transhomozygous ovaries.

Nomarski color micrographs of th^6/ th^8 (A), and dark CD4 / dark CD4 ; th^6/ th^8 (B) mutant egg chambers. (A) th^6/ th^8 mutant females have poorly developed and abnormal ovarioles. Note that the late egg chamber (arrow) shows abnormal morphology and signs of degeneration. (B) By comparison, double mutant ovarioles show improved distribution of egg chambers and even maturation of the fertilized oocyte (arrow). (Antony Rodriguez)

Female Genotype[1]	Fail to lay Eggs[2]	*n*
th^6/th^8	91%	34
$dark^{CD4}/+$; th^6/th^8	69 %	54
$dark^{CD4}/dark^{CD4}$; th^6/th^8	47%	63

1 Single pair matings with yw males were set up using females of the genotype shown.

2 Individual vials with females were monitored for up to 14 days for eggs in food.

Table 5 DarkCD4 suppresses th^6/th^8 egg laying deficiency

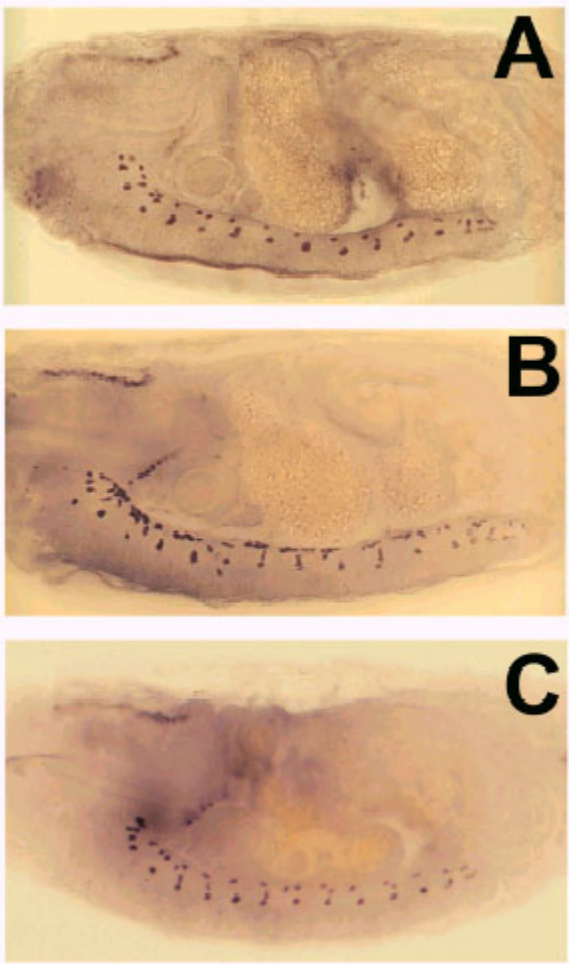
A
B
C

Figure 7. Dark functions as an initiator of programmed cell death in the neuronal midline. Embryos were collected from flies of the genotype, (A) y,w, P[slit-1.0 lacZ];(B) $dark^{CD4}$/ $dark^{CD4}$ P[slit-1.0 lacZ] and (C)dredd-/- P[slit-1.0 lacZ] were analyzed for Bgal expression. Control embryos contained 3 LacZ positive neurons per segment whereas dark mutant embryoes contained an average of seven neurons(n=15). (Antony Rodriguez)

Chapter 7 General Conclusions

The altruistic decision of a cell to undergo apoptosis is weighed heavily against the efforts a cell will make to survive. Rigorously proofreading its DNA to correct mutations or eking the most mileage out of the available growth signals, a cell will do whatever is possible to continue. When the final decision is made, the termination is swift. The genetic program of apoptosis ensures that these cells have a well regulated system to integrate the many inputs in the decision and a well sheathed sword ready at a moments notice. Increasing understanding of the field has brought new paradigms to the field of biology and a new landscape to those hoping to chart pathways to intervene in the breakdowns in the pathways that occur in autoimmune disease, neurodegeneration and cancer. By looking at the function of one off the key players in apoptosis, I strove to better understand the role of the Ced-4 proteins and their potential as an interventional site in therapy. Using genetic analysis, phylogenetic comparison and biochemical assays, the regulation, activation and inhibition of the ced4 module studied here can now be evaluated along side other data in the field to help form this picture.

Given the central role that caspases play in orchestrating the morphological events involved in apoptosis, blocking the activation of caspases via the intrinsic Apaf-1pathway would seem a logical means for tumors to escape cell death. Despite this simple logic no tumor type has been found to have homozygous mutation of Apaf-1 gene. Even in the panel of male germ cell tumors that have a high frequency of deletion of a single copy of the gene due to a neighboring tumor suppressor gene, Apaf-1 does not seem to be under great selection for inactivation. Integrating the genetic comparisons of loss of function alleles in mice and Drosophila, this result can now be better understood. While causing

defects in select tissues, mutations in these ced-4 genes still allow most cell deaths during normal development to occur. The greatest exception to this observation seems to be the nervous system. In both flies and vertebrates, excessive neural tissue is present in the stocks with mutated ced-4 related genes. This tissue specificity might explain the lack of Apaf-1 mutations in male germ cell tumors, and why the only tumor which has been clearly shown to down regulate Apaf-1 by epigenetic mechanisms is the neural crest derived melanoma. Interestingly, melanotic tumors are also present in a subset of fruit flies with the homozygous CD4 mutation.

The fact that targeted Apaf-1mutation does permit increased colony formation in ES cells makes the lack of Apaf-1 mutations in tumors still somewhat puzzling. Genetic evidence from Drosophila shows here also points to situations where blocking ced-4 mediated caspase activation can protect a cell from dying. Given that truncated versions of the protein that lack the WD repeats can be constitutively active, over half of the possible nonsense or frameshift mutations might actually be toxic to tumor cells. This could provide a counter-selection to any possible benefit that might be obtained by mutating Apaf-1. This hypothesis might also explain the difference in regulatory mechanisms that are seen between *C. elegans* where ced-4 is regulated by interaction directly with ced-9 and DARK/Apaf-1 which seems to be regulated by an inhibitory C-terminus. Given the shorter life span and greater specificity in cell fate, *C. elegans* cells may not need as complicated a regulatory mechanism to prevent oncogenic transformation.

While direct mutation of Apaf-1 seems to be an infrequent event in oncogenesis, a growing body of literature supports the idea that inhibition of caspase activation is

frequently seen in different cancers. Ovarian cancers, certain leukemia types, and melanomas have all been demonstrated to have defects in dATP-mediated caspase activation in addition to the defects reported here in male germ cell tumors. Because of the great number of IAPs, CARD containing proteins, and other proteins such as heat shock proteins that could potentially act as negative regulators, many different possible mutations could result in the same biochemical phenotype. With an increasing number of biochemical assays related to apoptosis such as cytochrome c release, IAP inhibition, and caspase activation, future studies may be able these assays to help classify the pathways that are affected in particular tumors. This might help steer future drug development to overcome the defect as adjunctive therapy.

The recent genetic studies show the primacy of pro-apoptotic Bcl-2 proteins such as Bax and Bak in determining mitochondria permeability also can help explain the lack of Apaf-1 mutations. While the lack of Apaf-1 might prevent caspase activation and markers of cell death that are frequently used to score cell death assays, the true test of cell death is clonal survival of cells. Most cells that are stimulated to release cytochrome c are doomed because of the damage to mitochondria even though caspases might not be activated. A growing body of evidence also show that additional apoptogenic proteins are released from the mitochondria along with cytochrome c. Even with SMAC, AIF, and the recent discovery of Endonuclease G, the list may yet be incomplete. Recent evidence from *C. elegans* also indicates that the homologues of some of these genes can assist a cell with an uncertain fate in dying. These accessory factors can even function in a non-autonomous fashion as the phagocytic machinery responsible for clearing corpses in normal circumstances can hasten the death of a cell with only a weak apoptotic signal.

These accessory cell death pathways can result in caspase independent cell death, and compensate for the lack of Apaf-1 or Dark. Because of these pathways any therapeutic intervention aiming to maintain cells will either have to prevent cell death upstream of mitochondrial release, or be targeted to tissue that lack the alternative pathways. Because of the requirement for ced-4 proteins in neuronal cell death in both murine and insect genetic models, the targeted inhibition of Apaf-1 mediated caspase activation might still be effective against some neurodegenerative diseases.

In examining other potential points of intervention in the apoptotic machinery, IAP proteins make for a promising target. The effort to sensitize cells to apoptosis is tricky because of the necessity for selective toxicity. Treatments that promote apoptosis must preferentially have their effects on diseased cells, or else they would produce toxic side effects. This is most classical demonstrated by the failed attempts to use Tumor Necrosis Factor to induce cell death in treating cancers because of unavoidable damage to the liver. Because IAPs preferentially bind to activated caspases, IAP function against caspases is needed only where there are ongoing generation of active caspases. This is more likely to occur in cancers where aberrant cell cycle progression, DNA damage, and growth factor withdrawal can all induce caspase activation absent in most normal cells. The frequent upregulation of IAP's in various tumors indicates the selective advantage of this genetic change and make its targeting even more tempting. The nature of the interface between the BIR domain and either their targets, caspases, or their inhibitor, SMAC, also make them an appealing drug target. The cleft interface surface is a better drug target than more diffuse surface interactions.

Comparative studies of the biochemical regulation in Drosophila and vertebrate systems point to the importance of this interaction; however, the genetic comparison offers some concerns. Wide spread inactivation of the DIAP1 gene causes a massive cell death effect. Because of the many homologous IAP genes, single gene inactivation has not been informative as to which other pathways might be affected in the vertebrate system. Differing possibilities in the upstream initiation of caspase activity might make any subtle differences between IAP's irrelevant, if Drosophila has more spontaneous auto-activation of caspases or constitutive activation of Dark. The alternate hypothesis that IAP's regulate other cellular signaling pathways might complicate their use as therapeutic targets. The IAP's are known to interact with the TRAF receptor signaling complexes, and XIAP has been shown to mediate interactions between TRAF proteins and the TAB/TAK kinase complex which stimulates both the NFKb and MKK6 pathways.

While clear genetic and biochemical evidence exists to demonstrate the importance of the IAP's and Ced-4 domain protein Dark in regulating Drosophila apoptosis, the evidence to support a conserved role for cytochrome C in the process is still at best circumstantial. The argument is bolstered by the binding of cytochrome c to Dark, the Apaf-1-Dark conservation of the WD protein domains which facilitate Apaf-1 binding to cytochrome c and conservation of the regulators of mammalian cytochrome, pro-apoptotic and anti-apoptotic members of the Bcl-2 family. However, the key regulatory role of IAP proteins and the IAP inhibitory proteins Reaper, Grim, Hid, and Sickle may indicate that suppression of constitutive caspase activity is the more important control switch in Drosophila, with the Bcl-2 proteins regulating release of anti-IAP

molecules such as SMAC and OMI rather than cytochrome c. Given the lack of cytochrome c release in Drosophila and the failure of cytochrome c to augment in vitro activation of caspases through Dark, I currently favor the latter view.

In my doctoral studies, I have used genetic and biochemical comparison of the Drosophila and vertebrate apoptotic pathways to gain better understanding of the mechanisms controlling the cellular decision to undergo programmed cell death. This fruitful approach shows the value of using multiple experimental paradigms in tackling a problem, and helps one appreciate their relative strengths and weaknesses. Continued exploration of these systems should help shed light on an area of tremendous scientific and medical importance.

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CURRICULUM VITAE

George (Holt) Reinhold Oliver was born in Birmingham, Alabama on June 7, 1973, the son of Dr. Robert I. Oliver and Marsha Oliver. Graduating from Mountain Brook High School in 1991, the intrepid youth traveled to the cold hinterlands of New Jersey to matriculate at Princeton University. He performed summer research at the University of Alabama-Birmingham, and the Pasteur Institute in Paris. His undergraduate research thesis on the topic on the bacterial stress response was under the guidance of Thomas J. Silhavy, Ph.D., and he graduated magnum cum laude in the Department of Molecular Biology with an A.B. degree from Princeton University in 1991.The following summer he returned to the South to thaw and begin his work on an M.D./Ph.D. dual degree program at the University of Texas-Southwestern Medical Center at Dallas. Through this program he met his future wife, Qian Zhou from Shanghai, P.R. of China. They married in 2001 and lived happily ever after.

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