DNR1 REGULATES APOPTOSIS: NEW INSIGHTS INTO MOSQUITO APOPTOSIS

by

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B.S., Kansas State University, 2007

A THESIS

submitted in partial fulfillment of the requirements for the degree

MASTER OF SCIENCE

Division of Biology
College of Arts And Sciences

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2009

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Abstract

Apoptosis, or programmed cell death, is a crucial conserved process among organisms for deleting damaged unwanted cells, as well as for development and viral defense, and plays an important role in multiple diseases. Too much apoptosis may lead to Alzheimer's disease, and too little may result in cancer. Therefore, the ability to understand this process is essential for improved medical knowledge today. Apoptosis has been explored in a number of species and pathways seem relatively conserved among most, with unique aspects contained in each, but little is known about apoptosis in mosquitoes. Improved knowledge and growing interest concerning apoptosis in mosquitoes is necessary considering the vast health effects seen across the globe as a result of diseases transferred by the mosquito vector. The Dengue virus mosquito vector *Aedes aegypti* was the focus here. A new player named *defense repressor 1* was discovered in *Drosophila melanogaster* (DmDnr1), shown to play a role in apoptosis, and the homolog discovered in *A. aegypti* (AeDnr1). Silencing *Dmdnr1* resulted in cells sensitized to apoptosis but was not enough to induce spontaneous apoptosis. In contrast, silencing *Aednr1* in the *A. aegypti* cell line, Aag2, led to spontaneously induced apoptosis. This showed the importance of AeDnr1 as a member of the apoptotic pathway in this species. Epistasis experiments showed that apoptosis induced by silencing *Aednr1* requires the initiator caspase Dronc and the effector caspase CASPS8, whereas apoptosis induced by silencing the inhibitor of apoptosis, *Aeiap1,* also requires Dronc but acts through the effector caspase CASPS7. Further epistasis experiments showed that apoptosis induced by silencing *Aednr1* requires the IAP antagonist Mx, but not IMP. This showed for the first time a gene regulating upstream of an IAP antagonist. Biochemical studies showed that AeDnr1 regulates active CASPS8 but not CASPS7, and interacts with Mx and CASPS8 but not AeDronc, CASPS7 nor AeIAP1. Studies also showed Mx competes effectively with CASPS8 but not CASPS7 for AeIAP1 binding, and IMP competes effectively with CASPS7 but not CASPS8 for AeIAP1 binding. An improved apoptosis pathway for the mosquito *A. aegypti* emerged involving a potential

feedback loop with explanations for the upstream IAP antagonist preference as well as the downstream effector caspase preference resulting from apoptosis induced by *Aednr1* silencing. Through the discussed research, multiple unique findings resulted. Studying the mosquito model will allow us to find certain gene relations that are more difficult to uncover in the *Drosophila* model. Because Dnr1 is found in most systems, this improved pathway may shed light not only on a potential role of Dnr1 in apoptosis in insects but higher organisms as well.

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Acknowledgments

Dr. Rollie Clem

I will always admire Dr. Clem for the way he runs his lab. I always felt motivated to give him more data. He is extraordinarily fair and wants his students to succeed. He is good at keeping open communication with his lab members, and handling any situation that might arise. He is an excellent teacher, communicating clearly to the student, while remaining approachable. One thing that I will always appreciate about Dr. Clem is his support in guiding students to their respective career goals. His attitude, advice, and help were refreshing. He also takes an interest in knowing his students outside the lab as I have had the pleasure of playing golf and softball with him. I am so thankful for the opportunity to join his lab and have loved the work I have been able to do as a result.

Dr. Lorena Passarelli

I find Dr. Passarelli a very motivational, naturally gifted teacher, and it has been ideal having her as a wonderful female professional role model. I feel she balances her work and home life very well, and that is something for everyone to look up to. I have appreciated all of her advice and comments throughout my graduate career, as well as the opportunity to know her outside the lab by playing volleyball weekly.

Dr. Chris Thorpe

I have been impressed by Dr. Thorpe's discussion inputs during MCDB, and have really appreciated his flexibility in joining my committee. I have enjoyed playing softball with him as well.

Dr. Gary Conrad

Dr. Conrad has an absolutely fantastic outlook on life. He is always encouraging students to work hard, be creative and unique in our ideas, but to also take the time to appreciate the outdoors, and therefore the big perspective of everything we are studying

in the lab. He will always remain dear to me as he has been a great motivator for life, as well as an excellent windsurfing teacher.

Dr. John Means

I cannot thank John enough. As the person who acclamated me to the lab and showed me the ropes he went above and beyond his job as a senior experienced guide to the lab. He was always available, willing, and eager to answer my increasingly many questions on theories and techniques. He taught me his protocols and preferences on techniques while taking a growing interest in the project. I will always remember our heated discussions regarding results and implications, his guidance in the lab, and all around consider him a good person and a close friend. He is a very unique person as his sense of humor fits perfectly with his hard work ethic, and his artistic side complements his science side. I am excited to see all life has in store for his talents.

Dr. Qingzhen Liu

Qingzhen was usually the first person I saw in the lab each day as well as the last. She always greeted me with a smile and was always sad to see me go. Also, I was happy she felt I was good luck for her data. I appreciate her friendship and was happy for her company in the lab.

Dr. Hua Wang

I am grateful for Hua's friendship and her beaming happiness each day. She was always refreshing to talk with and have in the lab. I am very appreciative for her helping save me hours by setting up and teaching me the tricks of Endnote, and for supplying me with reagents for experimental use. I will truly miss Hua.

Bart Bryant, Carolyn Delandre, Taryn Penabaz

It was fun biking and windsurfing with Bart, excellent teaching with Carolyn for a semester, and a pleasure to know Taryn and her family. I am happy for their friendships.

Lindsey Fallis and Katelyn O'Neill

I have really enjoyed knowing Lindsey and Katelyn. I know we will all stay connected throughout life and they are great roommates. It has been the perfect balance having their support and shared work ethic, as well as their eagerness to explore the outdoors, relax and enjoy our personal lives.

Dedication

Mom and Dad

I have enjoyed following the K-State tradition of our family and being close to home for these extra years. I truly love how close our family is and feel so blessed to have you both as my parents. I have had the perfect life and I owe so much of it to you. You both have raised three wonderful children and I love you both and am so blessed for all the fun we have had over the years. Thanks for being such wonderful examples.

Bryan, Kevin, and Lisa

I couldn't ask for better brothers. Bryan, I have extremely enjoyed our weekly hilarious phone conversations. Kevin, I am extremely happy that you have been the frontrunner towards the goal of keeping all of us kids together. You both have helped keep me motivated, and I am so glad we are so close, and for all the fun times growing up through today. Lisa, you are an awesome addition to our family, lots of fun, and you are one of the best people to go to for advice. I am excited for all of our future plans. Love you!

CHAPTER 1 - Introduction

"Equipped with his five senses, man explores the universe around him and calls the adventure Science."

-Edwin Powell Hubble

Apoptosis

Apoptosis, or programmed cell death, takes place in most, if not all, cells (Hengartner, 2000, Vaux & Strasser, 1996). It is a highly regulated series of events that leads to the destruction of unwanted cells and is necessary for survival. It was originally described as early as 1842, but it was not until 1972 that Kerr, Wyllie, and Currie characterized key differences between what they termed apoptosis and the previously known type of cell death necrosis (Kerr et al., 1972, Wyllie et al., 1980). The physical characteristics of apoptotic death not seen in necrosis include DNA fragmentation, nuclear condensation, plasma membrane blebbing, cell shrinkage and formation of apoptotic bodies that are recognized and quickly disposed of by phagocytes (Kerr et al., 1972, Thornberry & Lazebnik, 1998, Wyllie et al., 1980). Also, with apoptosis, the plasma membrane is not compromised, allowing cells to retain their cytoplasmic contents (Edinger & Thompson, 2004). However, with necrosis the integrity of the plasma membrane is lost causing the cytoplasmic contents to spill into the extracellular space, leading to an inflammatory response (Edinger & Thompson, 2004). Apoptosis is important in many processes including development, tissue homeostasis, DNA damage and viral defense. In many ways, it helps eliminate cells that have been compromised. Apoptosis is important in maintaining tissue homeostasis not only through development but also continually through life, regulating cells that are not dividing properly (Vaux & Korsmeyer, 1999). Excessive apoptosis can lead to neurological disorders such as Alzheimer's disease, while too little apoptosis can lead to cancer and autoimmune diseases (Hanahan & Weinberg, 2000, Thompson, 1995, Yuan & Yankner, 2000). There are several main players involved in apoptosis that will be discussed below including the family of cysteine proteases called caspases, inhibitor of apoptosis (IAP) proteins, and

IAP antagonists. It was in the nematode *Caenorhabditis elegans* that early strides were made in identifying the genes involved in regulating apoptosis (Hengartner & Horvitz, 1994b, Horvitz, 1999). These main players are conserved in metazoans but there are some differences in the regulation of apoptosis among different phyla. The organisms that will be discussed below are mammals, *C. elegans*, the fruit fly *Drosophila melanogaster*, and the yellow fever mosquito *Aedes aegypti*. A look at the apoptotic pathway reveals a general series of events where a death stimulus removes an inhibitor of apoptosis, allowing caspases to kill the cell by cleaving key cellular substrates that lead to the typical morphological changes associated with apoptosis (Budihardjo et al., 1999, Thornberry & Lazebnik, 1998). A general review will be presented as well as speculations made into important players noted in the current research, and their applications between organisms regarding apoptosis (Fig. 1.1).

Players in Apoptosis

Caspases

Caspases are the central executioners of apoptosis and are cysteine aspartate specific proteases (Hengartner, 2000, Shi, 2002b, Thornberry & Lazebnik, 1998). Caspases are present within the cell as inactive zymogens, and to become active proteolytic processing must occur (Cohen, 1997, Degterev et al., 2003, Fuentes-Prior & Salvesen, 2004). Caspases contain a key cysteine in their active site, recognize a specific set of four amino acids, P4-P3-P2-P1, and cleave substrates after an aspartic acid in the P1 position (Nicholson, 1999, Shi, 2002b). Although an aspartic acid is the generally accepted requirement for caspase cleavage, it has been determined that cleavage can also occur after a glutamate in the P1 position. For example, Dronc can cleave after aspartic acid residues, similar to other caspases, but it also cleaves *Drosophila* IAP1 and itself after a glutamate residue, the latter in order to autoactivate (Hawkins et al., 2000, Yan et al., 2004). In general, caspases contain an N-terminal prodomain as well as a large (P20) and small (P10) subunit (Hengartner, 2000, Riedl & Shi, 2004). The size of the prodomain specifies the category of caspases. A caspase with a long prodomain is considered an initiator caspase, and that with a small prodomain is considered an effector caspase (Shi, 2002b). Methods of cleavage and activation differ for initiator and effector

caspases and even within the initiator caspases, but a general sense of cleavage and activation can be described. Initially, cleavage occurs at specific internal aspartic acid residues separating the large (P20) and small (P10) subunits (Bratton & Cohen, 2001). For each caspase, two cleavages are required to form a fully active protease. The first cleavage separates the large and small subunit. After a conformational change subsequent cleavage will separate the prodomain, resulting in the active enzyme (Raff, 1998, Riedl & Shi, 2004).

Initiator Caspases

Initiator caspases are the first caspases to be activated, and are usually auto-activated with the help of oligomerizing factors (Bao & Shi, 2007, Ho & Hawkins, 2005). Initiator caspases contain, and are identified by, long prodomains. The long prodomain contains a domain important for protein-protein interactions. This domain can be either a CARD (Caspase Activation and Recruitment Domain) or a DED (Death Effector Domain) (Earnshaw et al., 1999, Fuentes-Prior & Salvesen, 2004, Ho & Hawkins, 2005, Park et al., 2007, Thornberry & Lazebnik, 1998, Weber & Vincenz, 2001). They are active as heterotetramers formed when initial cleavage after the aspartic acid residue separates each pair of small and large subunits, and these two subunits associate closely with one another to form a caspase heterodimer. Two heterodimers associate to form a heterotetramer, where the active caspase contains two active sites (Earnshaw et al., 1999, Thornberry & Lazebnik, 1998). It was originally reported that this cleavage formed active initiator caspases, but subsequent reports have suggested that cleavage is not necessary for initiator caspase activation (Srinivasula et al., 2001, Stennicke et al., 1999). This is based on the fact that autocatalytic cleavage of initiator caspases has minimal effect on their catalytic activity when compared to the effect seen with effector caspases (Rodriguez & Lazebnik, 1999, Srinivasula et al., 2001, Stennicke et al., 1999). The true mechanism of initiator caspase activation has not been identified. Two models identify the necessity for initiator caspases to come in close proximity of each other. The first is the induced-proximity model that states that auto-processing occurs among the initiator caspases once they are in close proximity to each other (Degterev et al., 2003, Salvesen & Dixit, 1999, Shi, 2004b). The second is the idea that dimerization is promoted by oligomeric complexes, namely apoptosome for Caspase-9 and DISC (Death Inducing

Signaling Complex) for Caspase-8, and these complexes auto-activate the initiator caspases (Boatright et al., 2003, Boatright & Salvesen, 2003, Ho & Hawkins, 2005, Shi, 2004a). Once active, these initiator caspases will cleave to activate downstream effector caspases (Boatright & Salvesen, 2003, Cohen, 1997, Raff, 1998).

The initiator caspases in mammals include Caspase-2, -8, -9, and -10. Caspase-9 appears to be the main initiator caspase involved in the intrinsic mammalian apoptotic pathway, and Caspase-8 appears to be the main initiator caspase involved in the extrinsic/receptor mediated pathway (Budihardjo et al., 1999, Li et al., 1997). Caspase-9 contains a CARD domain whereas Caspase-8 contains two DED domains in their respective long prodomains (Budihardjo et al., 1999). As oligomerizing factors are required for the activation of initiator caspases in mammals, apoptosis protease activating factor-1 (Apaf-1) has been found to be the adaptor protein for Caspase-9, whereas fas-associated protein with death domain (FADD) is the adapter protein for Caspase-8 (Boatright et al., 2003, Boatright & Salvesen, 2003, Ho & Hawkins, 2005, Li et al., 1997).

In *Drosophila melanogaster*, initiator caspases include Dronc, Dredd, and Strica where Dronc is the Caspase-9 homolog, Dredd is the Caspase-8 homolog, and Strica is the Caspase-10 homolog. Dronc is the main initiator caspase involved in the *Drosophila* apoptotic pathway, although it has also been found to play a role in non-apoptotic processes such as spermatogenesis (Huh et al., 2004a, Huh et al., 2004b). Dronc has the most sequence similarity to Caspase-2, but because it is the only initiator caspase in *Drosophila* that contains a CARD domain it has been characterized as the true Caspase-9 ortholog, confirmed by its functional similarity (Dorstyn et al., 1999, Kumar & Doumanis, 2000). Dredd, which contains two DED domains, has not been found to have significant relevance in the apoptotic pathway. The best characterized role of Dredd is its involvement in innate immunity (Leulier et al., 2000). Specifically, Dredd induces the expression of anti-microbial peptides in response to the presence of Gram-negative bacteria by cleavage of the NF-kB homolog Relish (Leulier et al., 2000).

CED-3 is the only caspase involved in the apoptotic pathway in *C. elegans*, although there are four total known caspases (Kumar, 2007, Shaham, 1998). CED-3 contains a CARD domain in its long prodomain, and is also able to autocatalyze its own

cleavage (Kumar, 2007). Because of these characteristics we can categorize this caspase as an initiator caspase, although it acts as an effector caspase.

In *A. aegypti*, there are six known initiator caspases including AeDronc and AeDredd (homologous to *Drosophila* Dronc and Dredd, respectively). AeDronc is the initiator caspase involved in apoptosis, and AeDredd is involved in the innate immune response (Cooper et al., 2007a, Cooper et al., 2007b). AeDronc has significant sequence homology to *Drosophila* Dronc and modest similarity to both mammalian Caspases-2 and -9, while AeDredd is homologous to both *Drosophila* Dredd and mammalian Caspase-8 (Cooper et al., 2007a, Cooper et al., 2007b). AeDronc contains a CARD domain, and AeDredd contains two DED domains (Cooper et al., 2007a, Cooper et al., 2007b).

Effector Caspases

Effector Caspases are the second type of caspases to be activated. They lack a long prodomain and therefore, the ability to autoactivate (Degterev et al., 2003). In order for effector caspases to become activated, they must be cleaved by initiator caspases (Degterev et al., 2003). Cleavage occurs at specific internal aspartic acid residues to separate the large and small subunit. Effector caspases exist as homodimers in both their active and zymogen form (Chai et al., 2001b, Riedl et al., 2001). Cleavage is required to achieve the active form, and creates a drastic difference in activation status when comparing an active effector caspase to its inactive form. Once active, these effector caspases cleave key cellular substrates that lead to apoptosis. These substrates include DNA repair enzymes, chromatin modifying enzymes including poly(ADP ribose) polymerase (PARP), structural proteins such as cytoplasmic actin and nuclear lamin, inhibitors of nucleases (such as DFF45 or ICAD), as well as additional proapoptotic proteins and caspases (Earnshaw et al., 1999, Enari et al., 1998, Fischer et al., 2003, Liu et al., 1997, Nagata, 2000, Sakahira et al., 1998, Yokoyama et al., 2000).

In mammals, the specific effector caspases are Caspases-3, -6, and -7. The caspase identified to be most important in the mammalian apoptotic pathway is Caspase-3, as it cleaves most of the cellular substrates in cells undergoing apoptosis (Cohen, 1997). In *Drosophila*, the effector caspases include Drice, Dcp-1, Decay, and Damm. The main effector caspase in *Drosophila* is Drice, although Drice and Dcp-1 appear to

have some redundancy in function (Fraser et al., 1997, Xu et al., 2006). In *C. elegans* CED-3 is the only caspase involved in apoptosis. Although it is considered to be an initiator caspase, it also functions as an effector caspase since it cleaves known effector caspase (DEVD) substrates (Yuan et al., 1993). In *A. aegypti*, two of the known effector caspases are CASPS7 and CASPS8 (Bryant et al., 2008).

Inhibitor of Apoptosis (IAP) Proteins

IAP proteins act in opposition to caspases and were initially discovered in the baculoviruses, *Cydia pomonella* granulovirus and *Orgyia pseudotsugata* nucleopolyhedrovirus, and have since been discovered in a wide variety of organisms including mammals, insects, and other viruses (Birnbaum et al., 1994, Crook et al., 1993). They were identified when researchers discovered that genes from these viruses could protect cells from apoptosis induced by viral infection. IAPs have a specific domain that categorizes them in this family of regulatory proteins, which is the N-terminal BIR (Baculovirus IAP Repeat) domain (Hinds et al., 1999, Miller, 1999). This domain is important for protein-protein interactions that are necessary for binding to caspases or IAP antagonists. The binding of caspases can inhibit apoptosis by either sequestering caspases away from their substrates, or targeting caspases for degradation (Deveraux et al., 1999, Huang et al., 2001, Tenev et al., 2005). Therefore, mutations that affect caspase binding lead to a total loss of anti-apoptotic activity. All IAPs contain at least one BIR domain and can contain between one and three BIR domains, however, not all BIR domain-containing proteins are considered to be IAPs (Silke & Vaux, 2001, Uren et al., 1998). That is to say that not all BIR-containing proteins can inhibit apoptosis. Some have been shown to be involved in cell signaling and cell cycle regulation (Dubrez-Daloz et al., 2008, Richter & Duckett, 2000). A second domain is the C-terminal RING (Really Interesting New Gene) finger domain (Joazeiro & Weissman, 2000). This domain has been shown to possess E3-ubiquitin ligase activity that is important for regulating the activity of caspases and itself via the ubiquitin-conjugating pathway (Joazeiro & Weissman, 2000, Vaux & Silke, 2005b, Wilson et al., 2002, Yang et al., 2000). The RING domain therefore acts as an acceptor of ubiquitin, by binding E2 ubiquitin-conjugating enzymes, and transferring ubiquitin to internal lysine residues of

either itself or its target substrate, leading to subsequent degradation by the proteasome of itself, or itself and the target substrate respectively (Suzuki et al., 2001b, Vaux & Silke, 2005b, Weissman, 2001, Yang et al., 2000). However, not all IAPs contain a RING domain and not all ubiquitination results in targeted degradation but instead in the modification of enzyme activity or localization of a protein (Hicke, 2001, Srinivasula & Ashwell, 2008). IAPs are found in a wide range of organisms, stressing their importance, but have differing dynamics in their functions depending on the organisms in which they are found.

In mammals, several IAPs have been identified but XIAP, cIAP1 and cIAP2 seem to be important in regulating caspase activity. XIAP, cIAP1 and cIAP2 can bind to and inhibit Caspases-3, -7, and -9 (Deveraux & Reed, 1999, Deveraux et al., 1998, Deveraux et al., 1997, Roy et al., 1997). XIAP, which contains three BIR domains and a RING domain, seems to be the most important. XIAP is able to bind to and negatively inhibit initiator and effector caspases and has been shown to inhibit apoptosis when overexpressed in cells (Deveraux et al., 1997, Duckett et al., 1996). The BIR3 domain is used in binding to active Caspase-9, whereas BIR2 and a small segment N-terminal to BIR2 are used together in binding to Caspases-3 and -7 (Chai et al., 2001a, Fesik & Shi, 2001, Huang et al., 2001, Riedl et al., 2001, Shiozaki et al., 2003, Srinivasula et al., 2001, Sun et al., 2000, Suzuki et al., 2001a). The anti-apoptotic function of XIAP with regards to caspases, whether through direct inhibition or degradation, depends on the ability to bind to caspases. Therefore, a mutation in the BIR domain that allows XIAP to bind a particular caspase disrupts the ability of XIAP to inhibit that particular caspase (Suzuki et al., 2001a). Although cIAP1 and cIAP2, which each contain identical domains to XIAP with the addition of a CARD domain, have a lower affinity to caspases, they appear to be somewhat functionally redundant to XIAP. One piece of evidence to support this is that knockout mice of XIAP are normal but show higher levels of cIAP1 and cIAP2 that might compensate for the absence of XIAP (Harlin et al., 2001). XIAP is not the central regulator in mammalian apoptosis, as knocking down XIAP does not lead to spontaneous apoptosis (Harlin et al., 2001). However, it serves to help reduce the apoptotic effects seen if Caspase-9 is activated, and therefore is an important inhibitor once apoptosis is induced (Morizane et al., 2005). Also, its presence in the absence of apoptotic stimuli

appears to be important for the inhibition of any caspase that may accidently become activated by unintentional cytochrome c release from mitochondria.

In *Drosophila*, the main IAP is DIAP1. DIAP1 is an E3-ubiquitin ligase that inhibits programmed cell death by targeting the substrate Dronc and itself for degradation by ubiquitination (Muro et al., 2002, Wilson et al., 2002). DIAP1 also has the ability to reversibly bind, and therefore inhibit, the initiator caspase Dronc (through BIR2) as well as the effector caspase Drice (through BIR1) (Chai et al., 2003, Meier et al., 2000, Yan et al., 2004). Two specifications with these last interactions are that DIAP1 requires a functional RING domain to effectively inhibit caspases (RING-mutated DIAP1 will bind to but not inhibit caspases), and that prior cleavage of DIAP1 by Drice is required for DIAP1 to bind and inhibit Drice (Wilson et al., 2002). Specifically, in order for BIR1 to bind to Drice, it is a requirement that Drice first cleaves DIAP1 at the D20 amino acid position (Ditzel et al., 2003). Therefore, mutations at the D20 site prevent cleavage, and Drice and DIAP1 binding is inhibited. This cleavage is also important for DIAP1 to undergo N-end rule degradation (Ditzel et al., 2003, Varshavsky, 2003). As the proteasome degrades DIAP1, it does not distinguish between anything bound to DIAP1 and thus also degrades any bound target substrate making this process essential for DIAP1 to inhibit apoptosis. DIAP1 serves as the key regulator in programmed cell death as other mitochondrial factors, namely cytochrome c that are necessary for inducing apoptosis in mammals, is not required for Dronc activation (Dorstyn et al., 2004, Li et al., 1997, Means et al., 2006, Wang, 2001, Zimmermann et al., 2002). Removal of DIAP1 is enough to induce spontaneous apoptosis in *Drosophila* S2 cells, as it is no longer able to negatively regulate constitutively active Dronc (Goyal et al., 2000, Muro et al., 2002, Wang et al., 1999). Also, DIAP1 mutations in the fly are embryonic lethal. For example, DIAP1 RING mutants are embryonic lethal, again pointing to the essential role of DIAP1 as an E3 ubiquitin ligase (Goyal et al., 2000, Lisi et al., 2000, Wang et al., 1999, Yoo et al., 2002).

In *C. elegans*, there are homologous proteins to the IAPs, but none involved in regulating apoptosis. These IAP proteins appear to function only in mitosis (Fraser et al., 1999, Speliotes et al., 2000). In *A. aegypti*, the main IAP is AeIAP1. Silencing of AeIAP1 is sufficient for inducing spontaneous cell death (Devore et al., 2009,

unpublished data; Liu and Clem, 2009, unpublished data). AeIAP1 is the homolog of DIAP1, and AeIAP2 is the homolog of DIAP2 (Bryant et al., 2008). Slight structural differences include that two BIR domains as well as a RING domain make up both AeIAP1 and DIAP1, and three BIR domains as well as a RING domain make up both AeIAP2 and DIAP2 (Bryant et al., 2008).

IAP Antagonists

IAP antagonists are pro-apoptotic regulatory proteins that play a crucial role in regulating programmed cell death. They were initially discovered in *Drosophila*. In *Drosophila*, the IAP antagonists include Reaper, Head involution defective (Hid), Grim, Sickle, and Jafrac2, whereas the first ones discovered and most characterized are Reaper, Hid, and Grim (collectively referred to as the RHG proteins) (Chen et al., 1996, Christich et al., 2002, Grether et al., 1995, Tenev et al., 2002, White et al., 1994). The genes encoding these proteins are closely linked, and were discovered when a chromosomal deletion of the region (H99 locus) that contained Reaper, Hid, and Grim prevented almost all embryonic cell death (White et al., 1994). This showed the importance of these three genes in *Drosophila* apoptosis. IAP antagonists have very little sequence similarity, but all share a common N-terminal motif called the IAP-binding motif (IBM) (Chai et al., 2000, Liu et al., 2000, Silke et al., 2000, Wing et al., 2001, Wu et al., 2000). This motif is vital for the function of IAP antagonists to induce cell death and through which RHG proteins bind to a groove in the BIR domains of IAP proteins (Chai et al., 2000, Liu et al., 2000, Silke et al., 2000, Srinivasula et al., 2000, Vucic et al., 1998, Wang et al., 1999, Wu et al., 2000, Wu et al., 2001). However, an additional domain found in Reaper and Grim, the GH3 motif, has been shown to have apoptotic activity in the absence of the IBM motif (Claveria et al., 2002, Olson et al., 2003a). Reaper, Hid, and Grim all have unique ways of antagonizing DIAP1. Reaper, Hid and Grim bind to the BIR1 and/or BIR2 domain of DIAP1 and this is done through their IBM (Shi, 2002a, Wu et al., 2001, Zachariou et al., 2003). These BIR domains are the caspase-binding sites as well. In this way the RHG proteins bind to DIAP1 and compete for the particular caspase binding site, freeing the caspase to cleave key cellular substrates resulting in cell death. RHG-induced apoptosis is caspase-dependent since expression of P35 (a caspase inhibitor) can block

RHG-dependent apoptosis (Chen et al., 1996, Grether et al., 1995, White et al., 1996). Specifically, the BIR1 domain of DIAP1 inhibits Drice but is also the site where Reaper and Grim, but not Hid can bind (Tenev et al., 2005, Zachariou et al., 2003). The BIR2 domain of DIAP1 inhibits Dronc but is also the site where Reaper, Hid and Grim can bind (Chai et al., 2003, Zachariou et al., 2003). The ability of an IAP antagonist to bind to DIAP1 is dependent on the prior exposure of an alanine at the N-terminus of the IAP antagonist. In *Drosophila*, the initial methionine blocks exposure of the IBM motif and is efficiently removed by methionine aminopeptidase (Hay & Guo, 2006). Besides binding, Reaper, Hid, and Grim have also been shown to induce degradation of DIAP1 (Holley et al., 2002, Ryoo et al., 2002, Yoo et al., 2002). Hid-induced degradation of DIAP1 requires the RING domain of DIAP1 while Reaper and Grim do not require the RING domain and can degrade DIAP1 via ubiquitination *in trans* with other E3 ligases (Ditzel et al., 2003, Goyal et al., 2000, Hay & Guo, 2006, Lisi et al., 2000, Yokokura et al., 2004, Zachariou et al., 2003). In addition to binding DIAP1 and/or stimulating its ubiquitination, Reaper and Grim have the ability to decrease endogenous levels of DIAP1 via general protein translational shutdown (Holley et al., 2002, Yoo et al., 2002). This function of Reaper and Grim does not depend on the RING domain of IAP proteins. Because DIAP1 has a shorter half-life than caspases, Dronc becomes free, leading to Dronc-dependent apoptosis (Yoo et al., 2002).

However, a major point to emphasize is that along with RHG proteins regulating the degradation of IAP proteins, IAP proteins can also stimulate the ubiquitination and degradation of RHG proteins (Olson et al., 2003b). This degradation of the RHG proteins by the IAP proteins results in the co-degradation of IAP proteins and is dependent on the proteosome, the IBM motif, the ubiquitination sites of the IAP antagonists, the BIR motifs of IAPs and the RING domain of the IAP proteins (Vucic et al., 1998). If any of these are mutated or inhibited, degradation of the RHG protein by IAP proteins does not exist. Since the IBM motif regulates interaction between Reaper and DIAP1 this shows that DIAP1 ubiquitination of IAP antagonists depends on their interaction. Also, because ubiquitin is accepted at lysine residues, a lysine-deficient Reaper is more stable than wildtype and induces cell death more efficiently (Olson et al., 2003b). Thus, IAP proteins can target IAP antagonists for degradation but co-degrade

themselves in the process (Vaux & Silke, 2005a, Yang & Du, 2004). It is reasoned that this is effective only when tiny amounts of IAP antagonists are released so that levels of IAP proteins are not significantly reduced. This also supports the hypothesis that although removal of XIAP does not cause apoptosis in mammals, the presence of XIAP is important for regulating accidental release of IAP antagonists from the mitochondria (Vaux & Silke, 2005a). This interaction among IAP proteins and IAP antagonists leads to the significant decrease in IAP proteins, leading to apoptosis, when regulatory signals tip the scale in favor of a greater quantity of IAP antagonists. The exact mechanisms to this opposing degradation and regulation by IAPs and IAP antagonists remain unclear (Duckett, 2005).

It was originally hypothesized that IAP antagonists existed in mammals because Reaper, Hid and Grim expression in mammalian cells induced apoptosis (Haining et al., 1999, McCarthy & Dixit, 1998). IAP antagonists were later discovered in mammals and include Smac/Diablo (Du et al., 2000, Verhagen et al., 2000). Smac/Diablo contains an N-terminal IBM motif where, like all IAP antagonists, only the first four residues are semi-conserved (Shi, 2002a). However, unlike the RHG proteins that are cytosolic, Smac/Diablo is localized in and released from the mitochondria. The IBM motif of Smac/Diablo is revealed after mitochondrial import when the signal sequence is cleaved off (Du et al., 2000, Verhagen et al., 2000). In the presence of an apoptotic stimuli active Smac/Diablo will be released from the mitochondria into the cytosol and be able to bind XIAP, cIAP1, or cIAP2. Smac/Diablo has the same binding site (BIR3) on XIAP as the initiator caspase, Caspase-9, and in that way Smac/Diablo competitively binds to XIAP and frees Caspase-9 (Chai et al., 2000, Ekert et al., 2001, Liu et al., 2000, Srinivasula et al., 2000, Srinivasula et al., 2001). In contrast to *Drosophila*, Smac/Diablo has only been found to competively bind and in that way negatively regulates the IAP proteins (Du et al., 2000, Verhagen et al., 2000). For instance, it cannot signal the IAP proteins for degradation via ubiquitination, nor general protein translational shutdown.

IAP antagonists were also later discovered in mosquitoes. Michelob_X (Mx) was found in *Anopheles gambiae*, *Aedes albopictus*, and *A. aegypti* making it the first insect IAP antagonist discovered outside of *Drosophila* (Zhou et al., 2005). Mx was found to interact with DIAP1 and its ability to induce apoptosis was dependent on its IBM.

Research showed removing its IBM prevented Mx from killing (Zhou et al., 2005). In addition, another IAP antagonist was discovered in *A. aegypti* called IMP (IAP-antagonist Michelob_X-like Protein) (Bryant et al., 2008). Expression of IMP in *A. albopictus* C6/36 cells was able to induce apoptosis (Bryant et al., 2008).

Mammalian Apoptosis

In mammals, there are two known apoptotic pathways, the extrinsic or receptor-mediated, and the intrinsic pathway (Adams, 2003, Ashkenazi & Dixit, 1998). The intrinsic pathway will be the focus here (Fig.1.1). The intrinsic pathway, in contrast to the extrinsic (which relies on external signals for activation), is activated by a signal from within the cell, such as DNA damage or virus infection. Many of the same players, including initiator and effector caspases, IAP proteins, and IAP antagonists are at play in mammals but unique points of regulation are involved. The initiator caspase, Caspase-9, becomes active in the Apaf-1/cytochrome c complex called the apoptosome (Acehan et al., 2002, Cain et al., 2000, Cain et al., 1999, Li et al., 1997, Rodriguez & Lazebnik, 1999). Therefore, cytochrome c must be released from the mitochondria where it is localized. The release of cytochrome c is one of two key regulation points in mammals, so the intrinsic pathway is often referred to as the mitochondria- or cytochrome c-mediated pathway (Li et al., 1997, Wang, 2001). Cytochrome c release is controlled by the Bcl-2 family of proteins (Adams & Cory, 1998, Antonsson & Martinou, 2000, Green & Reed, 1998, Kluck et al., 1997). Bcl-2 proteins are characterized by having at least one Bcl-2 homology (BH) domain that is involved in protein-protein interactions. There are pro- and anti-apoptotic members of the Bcl-2 family, and all Bcl-2 proteins are located in the cytosol and outer mitochondrial membrane (Adams & Cory, 2001, Borner, 2003, Gross et al., 1999). Pro-apoptotic Bcl-2 proteins include Bad, Bid, Bax, and Bak. During apoptosis Bax and Bak localize on the surface of the mitochondria, oligomerize to form hetero and homodimers, and as a result disrupt the mitochondrial membrane integrity by forming pores (Kroemer, 1999). These pores allow the release of cytochrome c as well as other pro-apoptotic proteins (IAP antagonists) from the mitochondria (Adams & Cory, 1998, Antonsson & Martinou, 2000, Kuwana et al., 2002). Anti-apoptotic Bcl-2 family proteins will prevent the oligomerization of Bax and Bak by binding to and

antagonizing them (Cheng et al., 2001). Apaf-1, after successful release of cytochrome c, will be bound via its WD-40 region by cytochrome c, causing a conformational change that exposes its CARD domain and creates its active form (Acehan et al., 2002, Hu et al., 1998, Li et al., 1997, Wang, 2001). The Apaf-1/cytochrome c complex then binds dATP with high affinity (Jiang & Wang, 2000). Caspase-9 will then be recruited to the Apaf-1/cytochrome c apoptosome and each Caspase-9, via the CARD domain of either protein, will bind to an Apaf-1 facilitating Caspase-9 activation (Qin et al., 1999, Rodriguez & Lazebnik, 1999, Zou et al., 1999). This active Caspase-9 can now cleave downstream effector caspases, Caspase-3 and -7, which will then cleave key cellular substrates leading to apoptosis. In addition, there is a positive feedback loop that allows Caspase-3 to go back and cleave Caspase-9, enhancing the apoptotic effects (Zou et al., 2003). Another key player is the inhibitor of apoptosis, XIAP. XIAP will negatively regulate the initiator Caspase-9 (Srinivasula et al., 2001). Therefore, for cell death to occur, XIAP must be removed from the system. This is the second key regulation point in mammals and the IAP antagonist, Smac/Diablo fulfills this requirement. When pro-apoptotic Bcl-2 family proteins initiate cytochrome c release, Smac/Diablo is also released from the mitochondria (Du et al., 2000, Verhagen et al., 2000). These antagonists will serve to negatively regulate XIAP which enables the active and now free Caspase-9 to cleave downstream effector Caspase-3 (Liu et al., 2000, Srinivasula et al., 2001). Thus, there are two checks in play for the mammalian system. One in which cytochrome c must be released from the mitochondria to activate Apaf-1 (the oligomerizing factor) allowing it to activate the initiator Caspase-9 (Li et al., 1997, Zou et al., 1997). The second in which Smac/Diablo must be released from the mitochondria in order to negatively regulate the inhibitor of apoptosis, XIAP, that is normally inhibiting active Caspase-9 (Ekert et al., 2001, Srinivasula et al., 2001). These two elements are the upstream regulators of apoptosis that ensure programmed cell death in mammals is regulated at the pro-apoptotic signal level upstream of initiator caspases (Wang, 2001).

Drosophila Apoptosis

In *Drosophila*, resemblance to the mammalian extrinsic receptor-mediated cell death pathway exists through its innate immune pathway (Brandt et al., 2004, Igaki et al., 2002a, Kanda et al., 2002, Kauppila et al., 2003, Leulier et al., 2006a, Leulier et al., 2000, Schneider et al., 2007). The apoptotic pathway resembles the intrinsic pathway in mammals by having a cascade of caspase activation resulting from internal cellular signals, but many unique points will be touched on regarding apoptosis in this system (Fig. 1.1). In *Drosophila,* the initiator caspase is Dronc, the ortholog of Caspase-9 in mammals (Dorstyn et al., 1999). *dronc* RNAi blocks most apoptosis caused by a loss of DIAP1 (Leulier et al., 2006b, Muro et al., 2002). In contrast to mammals, Dronc is constitutively activated, but still requires the oligomerizing factor Dark (the homolog of Apaf-1) for activation (Dorstyn & Kumar, 2008, Muro et al., 2002, Quinn et al., 2000). *dark* silencing by RNAi in *Drosophila* S2 cells leads to an accumulation of full length (non-processed) Dronc, and decreases caspase-dependent cell death (Muro et al., 2002). In addition, *Drosophila* embryos that have a loss of function mutation in Dark can rescue defects caused by removal of DIAP1 (Quinn et al., 2000, Rodriguez et al., 2002). It is through their CARD domains that Dark and Dronc interact in *Drosophila*, and in mammals (Quinn et al., 2000, Yu et al., 2006). Although Dronc and Dark complex to form an apoptosome in much the same way as homologous proteins in mammals, and although Dark contains a C-terminal WD-40 domain like Apaf-1, cytochrome c is not required, and therefore the requirements for a functional active apoptosome are not conserved (Dorstyn et al., 2002, Means et al., 2006, Rodriguez & Lazebnik, 1999, Zimmermann et al., 2002). Supporting data shows that addition of cytochrome c to *Drosophila* cell extracts results in only modest caspase activation, whereas *Drosophila* cytochrome c added to mammalian cell extracts stimulates high levels of caspase activation (Dorstyn et al., 2004). In addition, RNAi to cytochrome c in *Drosophila* S2 cells does not affect apoptosis or caspase activation (Means et al., 2006, Zimmermann et al., 2002). However, in mammals, cytochrome c knockout cells are resistant to apoptosis induced by various stimuli. The only known Dronc substrates are Drice, Dcp-1, and DIAP1 (Hawkins et al., 2000, Muro et al., 2005, Yan et al., 2004). The effector caspases that Dronc will cleave in *Drosophila* are Drice and Dcp-1, and it is interesting to note that

these are not only highly homologous to each other but are also homologous to the mammalian Caspases-3 and -7 (Dorstyn et al., 2002, Fraser et al., 1997, Song et al., 1997). Current evidence suggests that Drice is the more important of these two caspases in the *Drosophila* apoptotic pathway (Fraser & Evan, 1997, Kumar & Doumanis, 2000). RNAi studies have shown that when *dcp-1* is silenced, there is still significant apoptosis (Leulier et al., 2006b). However, silencing *drice* substantially decreases the amount of Dronc-dependent apoptosis (Kilpatrick et al., 2005, Leulier et al., 2006b, Muro et al., 2006, Muro et al., 2002, Muro et al., 2004). In addition, overexpression of Drice in S2 cells induces apoptosis and Dronc activity is needed for cleavage and activation of Drice in S2 cells (Fraser & Evan, 1997, Hawkins et al., 2000, Leulier et al., 2006b, Muro et al., 2004). This suggests that apoptosis signaled through the initiator caspase Dronc acts through the effector caspase Drice, although Dcp-1 might be partially redundant (Leulier et al., 2006b, Xu et al., 2006). Although Dronc is constitutively activated, it is negatively regulated by DIAP1 (the homolog of XIAP), allowing cells to remain alive (Muro et al., 2002). This contributes to *Drosophila* having distinct points of control of apoptosis as compared to mammals. As opposed to mammals where cell death is determined by regulators upstream of the activation of initiator caspases (mitochondria-mediated), in *Drosophila*, cell death is determined by the "simple" interference of DIAP1-caspase interactions. Thus, DIAP1 is a crucial regulator of apoptosis in *Drosophila*. When DIAP1 is present, it will inhibit active Dronc, making it unable to cleave downstream effector caspases, and unable to start the caspase cascade. When DIAP1 binds to Dronc, it stimulates Dronc ubiquitination through its C-terminal RING domain (Wilson et al., 2002). When DIAP1 is removed by mutation or RNAi, massive cell death in both fly embryos and *Drosophila* S2 cells occurs (Igaki et al., 2002b, Leulier et al., 2006b, Muro et al., 2002, Wang et al., 1999). Although DIAP1 binds Drice as well, cleavage of DIAP1 at the D20 residue is required before DIAP1 can bind to Drice (Ditzel et al., 2003). This cleavage exposes the BIR1 domain allowing DIAP1 to recognize Drice. However, this cleavage also stimulates the degradation of DIAP1 through the N-end rule pathway (Ditzel et al., 2003, Varshavsky, 2003). So many processes partake in regulating the expression of each protein. In part, regulation of the key proteins Dark, Dronc, Drice, and DIAP1 can determine the outcome of DIAP1-caspase interactions, and

thus the outcome of the cell. Important contributors to the regulation of DIAP1-caspase interactions are the IAP antagonists Reaper, Hid, and Grim (homologous to Smac/Diablo) that with increased expression effectively bind DIAP1 and induce cell death (Chen et al., 1996, Grether et al., 1995, White et al., 1996). Reaper, Hid, and Grim are transcriptionally upregulated during apoptosis, and in development, a removal of the RHG proteins results in a total loss of apoptosis (Chai et al., 2003, Holley et al., 2002, Kornbluth & White, 2005, Wang et al., 1999, White et al., 1994, Yoo et al., 2002). Reaper, Hid, and Grim will competitively bind through their IBM motif to the caspase-binding BIR domain on DIAP1 and through this competitive binding block caspase binding to DIAP1 and negatively regulate the anti-apoptotic function of DIAP1 (Goyal et al., 2000, Wang et al., 1999). In this way, the IBM motif is required for RHG proteins to disrupt the IAP-caspase interactions in favor of apoptosis. IAP antagonists can also induce DIAP1 degradation through ubiquitination and in some cases via shutdown of general protein translation.

Caenorhabditis elegans Apoptosis

Many important discoveries about apoptosis were found using *C. elegans*. In *C. elegans* it was discovered that 131 cells are destined to die during development, and using this knowledge the lab of Robert Horvitz at M.I.T. was able to show that apoptosis is a carefully regulated genetic process (Conradt & Horvitz, 1998, Liu & Hengartner, 1999). Although apoptosis in *C. elegans* is somewhat unique compared to other model organisms (i.e., IAPs are not found to be involved in apoptosis, and there is only one known caspase to be involved), it still follows the basic blueprint of pro- and anti-apoptotic signals that regulate adaptor molecules (or oligomerizing factors) and caspases that cleave key cellular substrates leading to apoptosis (Fig. 1.1). In the case of *C. elegans* there are no initiator and effector caspase subgroups. Although there are four known caspases, there is only one protein homologous to caspases involved in apoptosis, called CED-3 (Cell death abnormal) (Ellis & Horvitz, 1986, Shaham, 1998, Yuan et al., 1993). Because CED-3 has a long prodomain (like initiator caspases) containing a CARD domain, and has an affinity for DEVD substrates (like effector caspases) it acts as both an initiator and effector caspase. CED-3 is auto-activated by the help of the adaptor

molecular, CED-4, which is homologous to Apaf-1 and Dark (Yang et al., 1998, Zou et al., 1997). When either CED-3 or CED-4 are mutated (loss of function), it results in the survival of all 131 cells that are otherwise destined to die (Ellis & Horvitz, 1986, Lettre & Hengartner, 2006). CED-4 binds to CED-3 via the CARD domains of each, and this promotes oligomerization, and therefore recruitment and activation of CED-3 (Lettre & Hengartner, 2006). Other regulatory players in *C. elegans* include CED-9 and EGL-1. CED-9 is an anti-apoptotic protein from the Bcl-2 family that directly binds to CED-4, interfering with the ability of CED-4 to form an apoptosome, and thereby inhibiting it from interacting with and activating CED-3 (Ellis & Horvitz, 1986, Hengartner, 1999, Hengartner et al., 1992, Hengartner & Horvitz, 1994a, Horvitz, 1999, Liu & Hengartner, 1999, Yan et al., 2005). Within the Bcl-2 family that contains pro- and anti-apoptotic proteins is a group of proteins that contains a BH3-domain alone. Among this group is the protein EGL-1. EGL-1 is a pro-apoptotic protein that binds to CED-9 (Conradt & Horvitz, 1998). This binding causes a conformational change that inhibits CED-9 from binding to CED-4. This in turn allows the freed CED-4 to activate CED-3, leading to apoptosis. Interestingly, no Bcl-2 proteins (like CED-9) have been found to inhibit the adaptor molecules in other systems (Hausmann et al., 2000, Pan et al., 1998).

Mosquito Apoptosis

The apoptotic pathway of mosquitoes has not been fully characterized, but conserved players have been found in many mosquito species including *A. aegypti*. AeDronc and AeDredd are the two initiator caspases that have been characterized and they are homologous to *Drosophila* Dronc and Dredd, respectively (Cooper et al., 2007a, Cooper et al., 2007b). The initiator caspase involved in mosquito apoptosis is AeDronc (Cooper et al., 2007b). Homologs of IAPs have also been identified in mosquitoes, and in *A. aegypti* there are five known IAP homologs (Bryant et al., 2008). AeIAP1 is a homolog of DIAP1 in *Drosophila* and (Bryant et al., 2008), the simple removal of AeIAP1 is enough to induce spontaneous apoptosis as in *Drosophila* (Devore et al., 2009, unpublished data, Liu and Clem, 2009, unpublished data). Therefore, genes that regulate AeIAP1-caspase interactions at any level are crucial to learn more about. The IAP antagonists include Mx and IMP, and Mx is known to negatively inhibit DIAP1-caspase

interactions (Zhou et al., 2005). Mx is the most characterized IAP antagonist found to play a role in mosquito apoptosis. IMP is another pro-apoptotic protein found to have an IBM domain recently discovered using Mx as a query and searching the genome of *A. aegypti* (Bryant et al., 2008). Overexpression of either Mx or IMP induces apoptosis, and Mx is known to do this via its IBM domain (Bryant et al., 2008, Zhou et al., 2005). Additional work in the mosquito model is required to decipher the mechanisms of key homologous players and previously unidentified players in the apoptotic pathway.

Drosophila Defense Repressor 1(Dnr1)

Defense Represssor 1 (Dnr1) is a 677 amino acid long protein originally identified in *Drosophila*. Dnr1 contains an N-terminal FERM domain, and a C-terminal RING finger domain (Foley & O'Farrell, 2004, Guntermann et al., 2009, Primrose et al., 2007). FERM domains are involved in the localization of proteins to the plasma membrane, and in protein-protein interactions (Chishti et al., 1998, Primrose et al., 2007). The C-terminal RING domain was found to be the most similar to the RING domain in the conserved family of proteins, IAPs (Foley & O'Farrell, 2004, Vaux & Silke, 2005b). In IAPs, the RING domain is important for E3 ubiquitin ligase activity and in some cases acts solely to regulate its own protein levels (Vaux & Silke, 2005b). The fact that Dnr1 appears to be a relatively unstable protein, shown by low expression levels, is consistent with the thought that its RING domain could be used for self regulation. A mutation leading to the loss of the RING domain E3 ubiquitin ligase activity leads to stabilization of *Drosophila* Dnr1, and N-terminal HA tagged Dnr1 shows a slightly higher molecular weight than C-terminal HA tagged Dnr1 suggesting N-terminal auto-processing is occurring (Foley & O'Farrell, 2004, Primrose et al., 2007).

Dnr1 was originally discovered to have a role inhibiting the initiator caspase Dredd (Foley & O'Farrell, 2004). Dredd, in the presence of a Gram negative microbial infection, cleaves the NF-kB homolog Relish. This transcription factor will translocate to the nucleus and activate anti-microbial peptides (Leulier et al., 2000, Stoven et al., 2000, Stoven et al., 2003). Addition of lipopolysaccharide (LPS) to *Drosophila* S2 cells increases the production of the antimicrobial peptide gene, *diptericin (dipt)* (Foley & O'Farrell, 2004). Research showed that similarly, the simple removal of *dnr1* by RNAi in

the absence of a microbial insult was enough to induce Dredd-dependent activation of Dipt (Foley & O'Farrell, 2004). Because Dnr1 has a RING finger domain similar to the IAPs that appears to be important for autoprocessing, it was hypothesized that Dnr1 would function like an IAP and act to inhibit the initiator caspase Dredd. This was verified by RNAi of *dredd*, which compared to other capsases, reduced Dnr1 protein levels (Foley & O'Farrell, 2004). The authors concluded that activation of Dredd stabilizes Dnr1 (allows accumulation) and that inactivation of Dredd decreases Dnr1 protein levels, and hypothesized that Dnr1 is regulated in a manner correlating to a negative feedback loop by Dredd (Foley & O'Farrell, 2004). Therefore, Dredd would be regulating its own inhibitor.

Further information sheds additional light on the role of Dnr1 in innate immunity. Research shows that Dnr1 directly interacts with Dredd (Guntermann et al., 2009). Also, as a loss of Dnr1 leads to Imd pathway activation, Dnr1 overexpression inhibits the activity of the Imd pathway (Guntermann et al., 2009). To support the idea that Dnr1 is regulating Imd pathway via interaction with the caspase Dredd, it was determined that Dnr1 regulates Dredd activity in a RING domain-dependent manner (Guntermann et al., 2009). In the adult fly, Dnr1 was able to suppress activation of the Imd pathway in response to Gram-negative bacteria *in vivo*, and a reduced viability after septic injury with *E. coli* is indistinguishable from infected flies containing a null allele of *relish* (Guntermann et al., 2009). In addition, loss of Dnr1 *in vivo* led to a temporary increase in the transcript levels of two antimicrobial peptides *dipt* and *attacin* (Guntermann et al., 2009).

Dnr1 was also shown to have a role in Dronc-dependent apoptosis. To determine the importance of Dnr1 in apoptosis, RNAi was used to knockdown *dnr1*. *dnr1* RNAi did not induce spontaneous apoptosis, but instead sensitized S2 cells to various apoptotic stimuli. Increased levels of full-length and PR2 isoform of Dronc (the fully active form) were reproducibly found in *dnr1*-silenced samples compared to the control (Primrose et al., 2007). Expression levels of a mutant form of Dnr1, in which the RING domain has been inactivated, were substantially increased as compared to wildtype Dnr1 (Primrose et al., 2007). This confirms earlier suggestions that Dnr1 regulates its own stability through its RING domain. Knocking down *dnr1* in the presence of actinomycin D also led to

earlier and greater detection of effector caspase activity (Primrose et al., 2007). This supports the idea that Dnr1 not only regulates Dronc protein levels, but also regulates Dronc-mediated apoptosis. In addition, overexpression of wildtype Dnr1 protects S2 cells from apoptosis induced by cytotoxic agents (Primrose et al., 2007). Overexpression of RING-domain mutated Dnr1 (which is expressed at a higher level due to its inability to autoregulate) did not protect against actinomycin D-induced apoptosis (Primrose et al., 2007). It was concluded that Dnr1 suppresses active caspases and induction of apoptosis, and that it does so in a RING-dependent manner. In addition, the authors tested whether Dnr1 overexpression would suppress *diap1* dsRNA-induced apoptosis (Primrose et al., 2007). Surprisingly, they found that both wildtype and RING-domain mutated Dnr1 suppressed *diap1* dsRNA-induced apoptosis (Primrose et al., 2007). This suggests that although the RING-domain of Dnr1 is important for autoregulation and suppression of apoptosis induced by cytotoxic agents, it is not important for suppression of *diap1* dsRNA-induced apoptosis. The authors also suggested that there is a mechanistic difference between the regulation of apoptosis induced by different signals. Specifically, which domains are essential for regulating Dronc protein levels came into question. They confirmed that the RING domain in Dnr1 is essential for controlling the levels of different Dronc isoforms, verifying that Dnr1 reduces Dronc protein levels in a RING-dependent manner (Primrose et al., 2007). In addition, the FERM domain was found to be required for Dronc destruction (Primrose et al., 2007). Therefore, Dnr1 relies on the RING domain as well as cytoplasmic localization in its ability to regulate Dronc protein expression. A physical interaction between Dnr1 and Dronc could not be detected, but the ability of Dnr1 to affect Dronc protein levels and Dronc-dependent caspase activity, and *dnr1* RNAi sensitizing S2 cells to apoptotic signals have all been shown (Primrose et al., 2007).

In conclusion, Dnr1 appears to play a role in the innate immune response and apoptosis in *Drosophila*. It appears to be working through the initiator caspases Dredd and Dronc, but more information will help unfold the true mechanism through which Dnr1 carries out its important role in each pathway. In addition, a homolog of *Drosophila* Dnr1 has been found in many higher eukaryotes, as well as the mosquito *A.*

aegypti (Bryant et al., 2008). In this study, the function of *A. aegypti* Dnr1 was examined.

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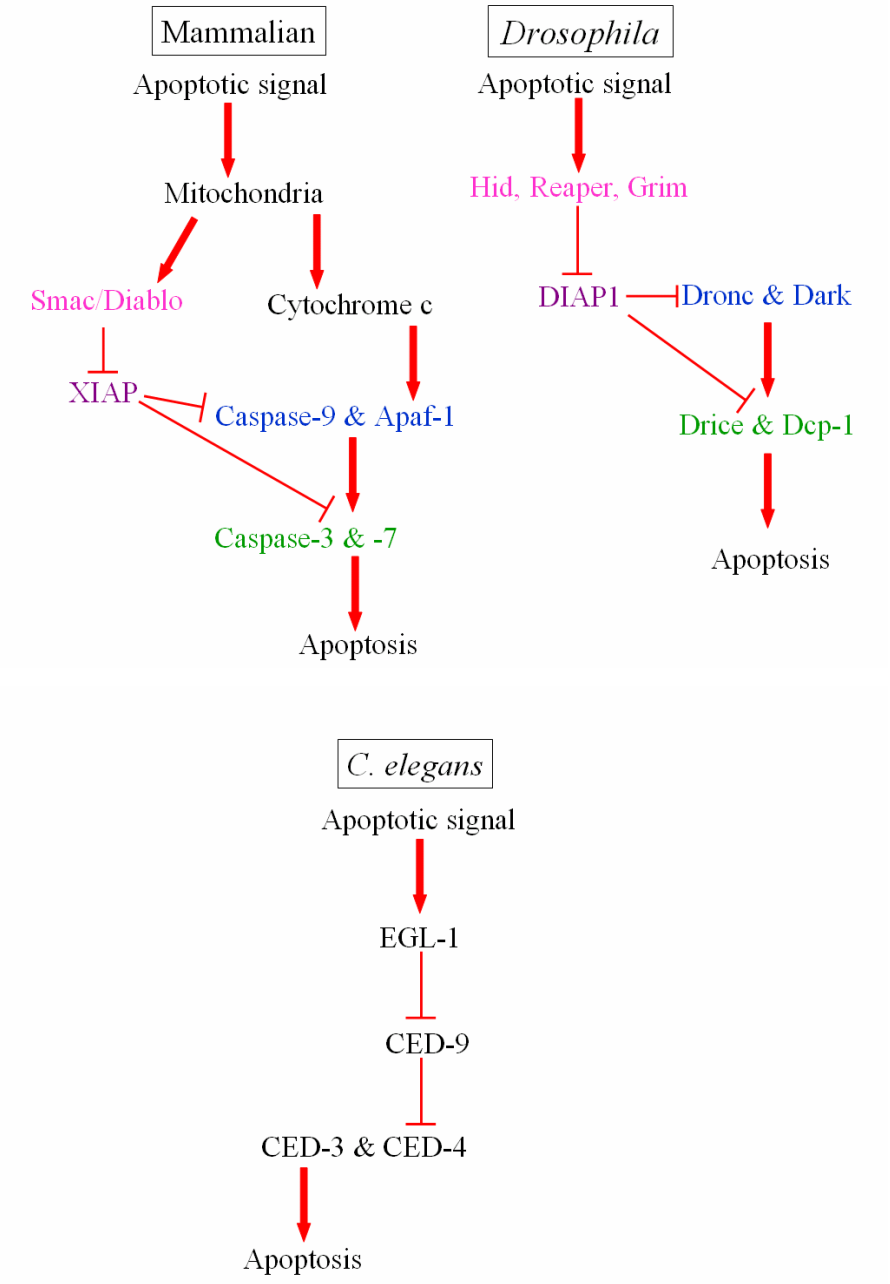
Figure 1.1 A Comparison of the Apoptosis Pathway models in Mammals, *Drosophila*, and *C. elegans*

Pointed arrows represent activation of respective proteins, while blunt arrows represent inhibition of respective proteins. Homologous proteins are color coded.



CHAPTER 2 - New insights into insect apoptosis: *Aedes aegypti* Dnr1 regulates apoptosis both upstream and downstream of IAP1 by targeting a specific IAP antagonist and a specific effector caspase

Abstract

The *dnr1* (*defense repressor-1*) gene was discovered in a genome-wide RNAi screen for genes that regulate immunity in *Drosophila melanogaster* and later shown to play a role in apoptosis regulation through its ability to regulate the initiator caspase Dronc. Silencing *dnr1* expression in *Drosophila* S2 cells results in increased Dronc levels and greater sensitivity to apoptotic stimuli, but does not cause outright apoptosis. In this study, we have explored the function of *dnr1* in the mosquito *Aedes aegypti*. In contrast to S2 cells, silencing *Aednr1* caused *A. aegypti* Aag2 cells to undergo spontaneous apoptosis. Epistasis experiments showed that apoptosis induced by *Aednr1* silencing required the initiator caspase AeDronc and the effector caspase CASPS8 but not the effector caspase CASPS7, whereas silencing *Aeiap1* induced apoptosis through AeDronc and CASPS7 but not CASPS8. Furthermore, epistatic and biochemical interaction experiments indicated that AeDnr1 acts through the IAP antagonist Michelob-X, but not IMP, and that AeDnr1 can bind to and directly inhibit CASPS8, but not CASPS7 or AeDronc. These results thus reveal novel aspects of the insect core apoptosis pathway, including the existence of inhibitors (AeIAP1 and AeDnr1) that specifically inhibit distinct effector caspases, and the identification of an inhibitor (AeDnr1) acting upstream of AeIAP1 by specifically targeting an IAP antagonist. Taking all this evidence into account, an improved model emerges for apoptosis regulation in insects.

Introduction

The mosquito *Aedes aegypti* is the principal vector for dengue and yellow fever viruses, which combined sicken tens of millions and kill tens of thousands of people each year. Dengue viruses, in particular, have expanded in range considerably in the past two decades, resulting in nearly two-fifths of the world's population now being at risk for dengue fever and dengue hemorrhagic fever. These and other alarming statistics explain why interrupting the transmission of mosquito-vectored viral diseases has become a public health priority.

The type of programmed cell death known as apoptosis serves a role in anti-viral defense in mammals and insects (Clarke & Clem, 2003, Clem & Miller, 1993, Scallan et al., 1997). Thus, there is interest in determining whether apoptosis can play a role in modulating virus transmission by mosquito vectors. At the molecular level, apoptosis is regulated by a core pathway that is fairly well conserved among nematodes, insects and mammals, but significant differences in how the pathway is regulated exist between these different phyla. In insects, apoptosis has mainly been studied in the model organism *Drosophila melanogaster*. In the *Drosophila* core apoptosis pathway, the initiator caspase Dronc (Daish et al., 2004) is constitutively activated at a low level with the help of the oligomerizing factor Dark (Rodriguez et al., 1999), and DIAP1 serves to prevent spontaneous apoptosis by binding to and inhibiting the activity of Dronc (Muro et al., 2002, Muro et al., 2005). Following an apoptotic stimulus, there is an increase in the levels and/or activity of IAP antagonists such as Hid, Reaper, and Grim, which displace DIAP1 from Dronc, allowing accumulation of active Dronc (Abrams, 1999, Bump et al., 1995, Holley et al., 2002, Salvesen & Abrams, 2004, White et al., 1996). Dronc then activates the effector caspase Drice, whose downstream cleavage of various cellular substrates leads to apoptosis (Muro et al., 2002). In addition to regulating Dronc, DIAP1 also has the ability to directly inhibit Drice activity. The mechanism of caspase inhibition by DIAP1 appears to be a combination of direct inhibition and ubiquitination via the RING domain in DIAP1 (Tenev et al., 2005, Wilson et al., 2002). Unlike in mammals, where IAP antagonists such as Smac/Diablo are released from mitochondria

after an apoptotic stimulus, Reaper, Hid and Grim are cytoplasmic proteins, and are regulated transcriptionally and post-transcriptionally by death stimuli (Du et al., 2000, Kornbluth & White, 2005). Reaper and Grim also have the ability to inhibit cellular translation, which probably also plays a role in removing DIAP1, which has a relatively short half-life (Holley et al., 2002, Yoo et al., 2002).

Our group has recently defined the core apoptotic pathway in *A. aegypti*, demonstrating that the pathway is well conserved compared to *Drosophila* (Q. Liu and R. Clem, submitted; H. Wang and R. Clem, submitted). The major players in this pathway include the initiator caspase AeDronc, the caspase activating protein AeArk, the effector caspases CASPS8 and CASPS7, the inhibitor of apoptosis (IAP) protein AeIAP1, and the IAP antagonists Michelob_x and Imp. Similar to DIAP1 in *Drosophila*, silencing of AeIAP1 results in spontaneous apoptosis, which is dependent on AeDronc and AeArk. Downstream of AeDronc, both CASPS7 and CASPS8 play roles in apoptosis triggered by various stimuli, while upstream, Mx and Imp are also both involved in apoptosis.

In addition to these main players in *Drosophila* apoptosis, another *Drosophila* gene that appears to have a role in apoptosis regulation, but was originally identified as an inhibitor of the immunity-related caspase Dredd, is *dnr1 (defense repressor-1)* (Foley & O'Farrell, 2004). The *Drosophila* Dnr1 (DmDnr1) protein contains an N-terminal FERM domain and a C-terminal RING domain, the latter being highly similar to those found in IAP proteins. In addition to having a role in suppressing the immune response, depletion of *Dmdnr1* from S2 cells results in an increase of Dronc protein levels and sensitizes cells to apoptotic signals (Primrose et al., 2007). In addition, DmDnr1 overexpression blocks apoptosis and reduces Dronc levels in a RING-dependent manner. It has been proposed that DmDnr1 directly regulates Dronc, presumably promoting its ubiquitination, although a direct interaction between DmDnr1 and Dronc has not been demonstrated (Primrose et al., 2007).

Dnr1 orthologs are found in most metazoans (with the notable exception of nematodes), but the function of Dnr1 has not been studied outside of *Drosophila*. In this study, we examined the role of *A. aegypti* Dnr1 (AeDnr1) in apoptosis regulation in *A. aegypti* cells. Our results reveal that Dnr1 plays a critical role in regulating apoptosis in *A. aegypti*, as depletion of *Aednr1* results in spontaneous apoptosis. We further

investigated the pathway regulated by AeDnr1, and our results show that both AeDnr1 and AeIAP1 are involved in regulating apoptosis, but these two inhibitors work through inhibiting distinct effector caspases and IAP antagonists. These results significantly strengthen our knowledge of the current model for apoptosis regulation in insects.

Materials and Methods

Cells and plasmids

Aag2 cells (Lan & Fallon, 1990) were grown in Schneider's medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS) (Atlanta Biologicals).

Plasmids pCRII/AeDnr1, pCRII/AeDronc, and pCRII/AeIAP1 were constructed using the pCRII vector and Dual Promoter TA Cloning Kit (Invitrogen) according to the protocol of the manufacturer.

RNA interference (RNAi)

Double-stranded RNA (dsRNA) corresponding to the open reading frames of the genes of interest was synthesized using the T7 High Yield Transcription Kit (Epicentre) according to the protocol of the manufacturer. The resulting dsRNA was annealed by incubating at 65 °C for 15 minutes, 37 °C for 15 minutes, and room temperature for 15 minutes. Aag2 cells were plated at 5 x 10^5 cells per 35mm well and allowed to attach to the plate for 4 hrs. The cells were washed with Schneider's media lacking FBS, and 40 µg dsRNA was then added to each well containing 1 ml of Schneider's media without FBS, followed by vigorous shaking. Five hrs after dsRNA addition, 1 ml of media containing 20% FBS was added to each well for a final FBS concentration of 10%. Photographs were taken 19 hrs after adding dsRNA and wells were harvested for either caspase activity or RT-PCR analysis 25 hrs after dsRNA addition. In some experiments, the pan-caspase inhibitor Z-Val-Ala-Asp-fluoromethylketone (Z-VAD-FMK) (MP Biomedicals) was added to the cells at a concentration of 50 µM at the time of dsRNA addition and maintained until cell harvesting.

RNAi epistasis experiments

When knocking down more than one gene at a time, the first dose of dsRNA added (40 μg) was for the respective caspase in each well. A second dose of dsRNA (40 μg) for each respective caspase was added 24 hrs later simultaneously with 40 μg dsRNA of either *Aednr1* or *Aeiap1*. *cat* dsRNA was used as a filler to maintain equal concentrations of dsRNA present in each well. In triple RNAi experiments, 40 μg of each IAP antagonist, or 20 μg of each if both were present, were added, followed 24 hrs later by addition of a second dose of dsRNA for each IAP antagonist, together with 40 μg of either *cat* or *Aednr1* dsRNA. Thus, each well contained a total of 80 μg dsRNA. Cells from these experiments were harvested at 19.5 hrs for caspase activity.

Caspase Activity

Caspase activity was measured using the substrates N-acetyl-Asp-Glu-Val-Asp - 7-amino-4-trifluoromethyl-coumarin (Ac-DEVD-AFC) or N-acetyl-Ile-Glu-Thr-Asp-AFC (Ac-IETD-AFC) (MP Biomedicals, Inc.) at a concentration of 20 mM. To detect caspase activity, Aag2 cells were harvested after dsRNA treatment, and centrifuged at 17900 x g for 2 min. The cell pellets were resuspended in 100 μl caspase buffer A (20 mM HEPES*KOH, pH 7.5, 50 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM DTT) and stored at -80 °C until use. Cells were lysed by three freeze-thaw cycles, then centrifuged at 17900 x g for 2 min and incubated for one hour at 37 °C. Caspase substrate was then added and the reaction was analyzed fluorometrically (excitation 405 nm, emission 535 nm) using a Victor3 1420 Multilabel Counter (Perkin Elmer) and activity was expressed as arbitrary fluorescence units.

RT-PCR

Aag2 cells were treated with dsRNA as described above. At 19.5 hrs after treatment, samples were resuspended in 100 μl Trizol reagent (Invitrogen) and total RNA was isolated according to the instructions of the manufacturer and as described previously (Means et al., 2003). Total RNA (0.5 μg) was used in a reverse transcription reaction with an oligo-dT primer. From the resulting 20 μl reaction, 2 μl cDNA was used as a template in a PCR reaction using primers specific for the gene of interest.

Viability Assay

Photomicrographs from three different fields of view were taken from each plate of samples done in duplicate or triplicate. Viable cells were enumerated from the pictures based on cell morphology. The average number of cells in the negative control wells was set at 100 percent.

Recombinant Protein Preparation

GST, GST-AeIAP1, AeDRONC-His_6, CASPS7-His_6, CASPS8-His_6, IMP-His_6, and Michelob_X- His_6, were expressed in BL21pLysS(DE)3 *Eschericia coli* (Stratagene). Cultures (50 ml) were grown overnight at 37 °C and the following day used to seed 1 liter cultures. The 1 liter cultures were grown at room temperature to $OD_{600} = 0.4$. Cultures were then induced with 0.1 M IPTG for 1 hour. The bacteria were sonicated in Lysis Buffer A (200 mM Tris-Cl pH 8.0, 0.4 M ammonium sulfate, 10 mM $MgCl_2$, 10% glycerol, and protease inhibitor cocktail (Roche)) and purified using either glutathione-sepharose-conjugated beads for GST fusion proteins or with Talon Metal Affinity Resin (Clontech) for the His_6-tagged proteins according to the instructions of the manufacturer.

Interaction Assays

^{35}S-labeled AeDnr1, AeDronc and AeIAP1 were synthesized using the TNT T7 Coupled Reticulocyte Lysate System (Promega). To examine the interaction between AeIAP1 and AeDnr1, ^{35}S-AeDnr1 or ^{35}S-AeDronc (25 µl of a 50 µl reaction made from 1 µg DNA template) was incubated with recombinant GST-AeIAP1, GST, or by itself (250 ng of recombinant protein). After incubation, the reactions were incubated with protein G-sepharose (Sigma) that had been incubated with mouse monoclonal anti-GST antibody (Santa Cruz Biotechnology) (200 µg/ml) diluted 1:100 by rocking overnight at 4 °C. The beads were washed three times with Caspase buffer A and bound protein was removed from the beads by heating samples at 100 °C in SDS-PAGE sample buffer for five minutes. Proteins were resolved by SDS-PAGE and visualized by autoradiography. To examine interactions between AeDnr1 or AeIAP1 and other proteins, ^{35}S-AeDnr1 or ^{35}S-AeIAP1 (25 µl of a 50 µl reaction made from 1 µg DNA template) was incubated for 4 hrs at 30 °C with control GST, AeDronc-His_6, CASPS7-His_6, CASPS8-His_6, IMP-His_6, and Mx-His_6 (250 ng each). Reactions were then incubated with protein G-sepharose

preincubated with anti-His antibody (Santa Cruz Biotechnology) (200 µg/ml) diluted 1:100 and rocked overnight at 4 °C. Bound proteins were examined as described above.

In vitro Caspase Assays

Aag2 whole cell lysate or recombinant caspases AeDronc, CASPS7, or CASPS8 (250 ng) were incubated with control CAT, AeDnr1 or AeIAP1 *in vitro* translated proteins (25 µl of a 50 µl reaction made from 1 µg of DNA template) or recombinant Mx and IMP (500 ng each), incubated for 2 hrs at 30 °C, and caspase activity was determined using Ac-DEVD-AFC as a substrate. All combinations were added simultaneously during incubation.

Results

The *A. aegypti* ortholog of Dnr1 is 46% identical to *Drosophila* Dnr1 at the amino acid level, and includes well-conserved N-terminal FERM and C-terminal RING domains (Fig. 2.1). Silencing *dnr1* in *Drosophila* S2 cells sensitizes the cells to apoptotic stimuli, but does not result in spontaneous apoptosis (Primrose et al., 2007). To examine the role of Dnr1 in *A. aegypti* apoptosis, we silenced *Aednr1* expression using RNAi in the *A. aegypti* cell line Aag2. Silencing of *Aednr1* was verified by RT-PCR (Fig. 2.2A). In contrast to previous observations in S2 cells, Aag2 cells treated with *Aednr1* dsRNA underwent spontaneous apoptosis, similar to *Aeiap1* silencing (Fig. 2.2B). When either *Aednr1* dsRNA or *Aeiap1* dsRNA was added to cells, plasma membrane blebbing typical of apoptosis began within 3-4 hrs, and apoptotic body formation was observed. Treatment with the broad spectrum caspase inhibitor Z-VAD-FMK prevented cell blebbing and cell death (Fig. 2.2B), indicating that *Aednr1* RNAi induced caspase-dependent apoptosis in Aag2 cells, similar to that seen following RNAi of *Aeiap1*.

Caspase activity was assayed in these dsRNA-treated cells by measuring fluorescence resulting from cleavage of the effector caspase substrate Ac-DEVD-AFC. Effector caspase activity increased in both the *Aednr1* and *Aeiap1* RNAi samples compared to controls, and was reduced to basal levels when cells were simultaneously treated with the caspase inhibitor Z-VAD-FMK (Fig. 2.2C). These data indicate that apoptosis is spontaneously induced by depletion of *Aednr1* in Aag2 cells.

These results regarding AeDnr1 differ from those previously reported for DmDnr1 (Primrose et al., 2007). To verify this difference, we performed RNAi on *Dmdnr1* in S2 cells and examined the effects on caspase activity (Fig. 2.3A). Silencing of *Dmdnr1* was verified by RT-PCR (Fig. 2.3B). Consistent with previous reports, we observed no morphological evidence of apoptosis and no increase in caspase activity in S2 cells treated with *Dmdnr1* dsRNA. Apoptosis and caspase activity was observed in S2 cells treated with *diap1* dsRNA, consistent with previously published results.

Since silencing of *Dmdnr1* in S2 cells does not result in apoptosis, the observation that *Aednr1* depletion caused spontaneous apoptosis in Aag2 cells gave us a unique opportunity to determine where AeDnr1 functions in the apoptotic pathway. A series of RNAi epistasis experiments were performed using combinations of either *Aednr1* or *Aeiap1* dsRNA and the dsRNA corresponding to caspases that are important in the *A. aegypti* core apoptotic pathway. *Aedronc* is orthologous to *Drosophila* d*ronc*, while *casps7* and *casps8* are effector caspases that are most closely related to *drice* and *dcp-1* in *Drosophila*, although it is not possible to assign strict orthology for these effector caspases (Bryant et al., 2008). All three of these caspases play important roles in *A. aegypti* apoptosis, with *Aedronc* being essential, and *casps7* and *casps8* playing partially redundant roles (Q. Liu and R. J. Clem, submitted). We examined the effect of silencing *Aedronc, casps7, or casps8* on the apoptosis induced by silencing of *Aednr1* or *Aeiap1*. Silencing of each gene was effective, as assessed by RT-PCR (Fig. 2.4A and B). Silencing *Aedronc* inhibited apoptosis induced by silencing of either *Aednr1* or *Aeiap1* (Fig. 2.4C), indicating that both proteins function upstream of AeDronc. Interestingly, however, we observed a strict partition in the involvement of the effector caspases. Silencing *casps8,* but not *casps7,* suppressed *Aednr1* dsRNA-induced apoptosis (Fig. 2.4C). However, silencing *casps7,* but not *casps8*, suppressed *Aeiap1* dsRNA-induced apoptosis (Fig. 2.4C). These results were unexpected, as they suggest that AeDnr1 and AeIAP1 regulate apoptosis through two distinct pathways that utilize the same initiator caspase, but distinct downstream effector caspases.

These results were verified by measuring effector caspase activity (Fig. 2.4D) and by quantifying cell viability (Fig. 2.4E). Consistent with the cell morphology observations in Fig. 2.4C, high effector caspase activity and decreased cell viability were

observed in samples where *Aednr1* was silenced, unless either *Aedronc* or *casps8* were co-silenced. Meanwhile, silencing of *Aeiap1* caused effector caspase activation and diminished cell viability, except when either *Aedronc* or *casps7* were co-silenced.

To further examine the position of *Aednr1* in the *A. aegypti* apoptotic pathway, we performed another series of epistasis experiments to explore the possible interaction of *Aednr1* with the IAP antagonists *mx* and *imp*. Either *mx*, *imp*, or both genes were silenced, followed 24 hrs later by silencing of *Aednr1* (Fig. 2.5A). When *Aednr1* was co-silenced with *mx*, either alone or together with *imp*, cells were protected against apoptosis, but silencing of *imp* by itself had no effect on apoptosis induced by silencing of *Aednr1* (Fig. 2.5B). Caspase activity and cell viability assays were consistent with these results (Fig. 2.5C and D). These data imply that AeDnr1 functions upstream of or parallel to the IAP antagonist Mx, and further, that AeDnr1 function does not involve IMP.

To confirm the functions of AeDnr1 and AeIAP1 using a different approach, *in vitro* assays were performed where either *in vitro* translated (control chloramphenicol acetyl transferase (CAT) and AeDnr1) proteins or purified, bacterially expressed (Mx and IMP) proteins were incubated with lysate from normal Aag2 cells, followed by assaying for caspase activity (Fig. 2.6A). Baseline caspase activity was observed in Aag2 cell lysate alone or lysate incubated with the recombinant proteins CAT or AeDnr1. Addition of Mx or IMP protein to Aag2 lysate resulted in caspase activation, similar to what has been observed when recombinant IAP antagonists were added to *Drosophila* S2 lysate (Means et al., 2006). In *Drosophila*, it is thought that increased levels of IAP antagonist proteins results in decreased caspase binding by DIAP1, allowing caspase activation.

Addition of AeDnr1 protein prevented the caspase activation observed by addition of Mx, but had no effect on caspase activation caused by addition of IMP (Fig. 2.6A). Therefore, similar to the results seen in Fig. 2.5, AeDnr1 can suppress the caspase activation resulting from increased levels of Mx, but not IMP.

We also used *in vitro* assays to monitor the ability of *in vitro* translated AeDnr1 and AeIAP1 to directly inhibit various caspases. In Fig. 2.6B, we measured the activity of recombinant AeDronc using the substrate Ac-IETD-AFC. Bacterially expressed and purified AeDronc protein was inhibited by AeIAP1, but not by AeDnr1. In addition,

either Mx or IMP protein was able to prevent AeIAP1 from inhibiting AeDronc (Fig. 2.6B), indicating that both Mx and IMP can compete with AeDronc for binding to AeIAP1. Furthermore, addition of AeDnr1 to Mx and AeIAP1 again brought AeDronc activity down to baseline levels, but addition of AeDnr1 was not able to prevent IMP from antagonizing AeIAP1, again supporting the idea that AeDnr1 serves as a negative regulator of Mx function, but it does not act on IMP.

In Fig. 2.6C, we explored the effects of different recombinant proteins on the activity of the effector caspases CASPS7 and CASPS8. AeIAP1 was able to directly inhibit both CASPS7 and CASPS8. However, while AeDnr1 was able to partially inhibit the activity of CASPS8, it had no effect on CASPS7. Interestingly, Mx protein only partially antagonized the ability of AeIAP1 to inhibit CASPS7, but completely antagonized inhibition of CASPS8, while the opposite was true for IMP. These data indicate that Mx has a preference for competing with CASPS8 for binding to AeIAP1, while IMP has a preference for competing with CASPS7 for binding to AeIAP1.

Additional evidence as to the mechanisms of AeDnr1 and AeIAP1 function was obtained through biochemical interaction experiments. One possibility was that AeDnr1 functions by directly binding to AeIAP1, and somehow modulating AeIAP1 function. To examine this, we performed a co-immunoprecipitation experiment (Fig. 2.7A). *In vitro* translated, ^{35}S-labeled AeDronc or AeDnr1 were incubated with bacterially expressed and purified, GST-tagged AeIAP1 or GST alone, and the resulting protein complexes were immunoprecipitated using an antibody against GST. AeIAP1 interacted with AeDronc, but not with AeDnr1, suggesting that AeDnr1 does not achieve its anti-apoptotic function through direct interaction with AeIAP1. In Fig. 2.7B, *in vitro* translated, ^{35}S-labeled AeIAP1 was incubated with recombinant His-tagged AeDronc, Mx, IMP, CASPS7, or CASPS8, and protein complexes were immunoprecipitated with anti-His antibody. AeIAP1 interacted with each of these proteins, but the interaction with CASPS8 was significantly weaker than with the other proteins, which correlates with the above findings that AeIAP1 has a downstream effector caspase preference for CASPS7.

In vitro translated AeDnr1 was also tested for its interaction with His-tagged AeDronc, CASPS7, CASPS8, IMP, and Mx (Fig. 2.7C). No interaction was observed between AeDnr1 and AeDronc, again indicating that AeDnr1 does not directly act

through AeDronc. Strong interaction was observed between AeDnr1 and Mx, and also between AeDnr1 and CASPS8, but not between AeDnr1 and CASPS7 or IMP. These data are consistent with our RNAi epistasis experiments, in which AeDnr1 acts in a pathway involving Mx and CASPS8, but not IMP or CASPS7. We note that the Dnr1 protein in the CASPS8 reaction ran faster than full length Dnr1, suggesting that Dnr1 may be cleaved by CASPS8.

Discussion

Based on our results, we propose the following apoptosis pathway model in *A. aegypti*, which extends the known *Drosophila* apoptotic pathway (Fig. 2.8). In *A. aegypti*, AeDnr1 specifically binds to the IAP antagonist Mx, but not IMP, and functions upstream of AeIAP1 by preventing Mx from antagonizing AeIAP1. AeDnr1 is also able to bind and inhibit any activated effector caspase CASPS8, but not CASPS7 or the initiator caspase AeDronc. AeIAP1, on the other hand, binds and inhibits AeDronc and CASPS7, but only weakly binds and inhibits CASPS8. Furthermore, AeIAP1 can be antagonized by either Mx or IMP.

Silencing *Aeiap1* induces apoptosis by directly freeing AeDronc, which is then able to activate both CASPS7 and CASPS8. Since AeDnr1 can inhibit CASPS8 but not CASPS7, apoptosis is induced through CASPS7, explaining why silencing of *Aedronc* or *casps7* is more effective than silencing *casps8* in preventing apoptosis induced by silencing of *Aeiap1*. On the other hand, silencing of *Aednr1* results in the freeing of Mx, which is then able to antagonize AeIAP1 bound to AeDronc, resulting in activation of AeDronc, CASPS7, and CASPS8. Since Mx does not compete effectively with CASPS7 for binding to AeIAP1, AeIAP1 is presumably still able to inhibit CASPS7, but AeIAP1 is not a good inhibitor of CASPS8, and so apoptosis occurs through CASPS8. This explains why silencing of either *Aedronc* or *casps8*, but not *casps7*, inhibits apoptosis induced by silencing of *Aednr1*.

Our results indicate that AeDnr1 does not appear to interact with or directly inhibit AeDronc. In *Drosophila* S2 cells, silencing of *Dmdnr1* increases the level of Dronc protein, and overexpressing DmDnr1 decreases Dronc levels (Primrose et al., 2007). It should be pointed out, however, that no evidence exists which shows a direct

interaction between DmDnr1 and Dronc in *Drosophila*. Indeed, the effects of DmDnr1 on Dronc levels in S2 cells may be due to indirect effects similar to what we have reported here. In other words, silencing *Dmdnr1* may result in increased antagonism of DIAP1 by an IAP antagonist, indirectly resulting in higher Dronc levels, while overexpressing Dmdnr1 may result in less antagonism of DIAP1, causing a decrease in Dronc levels. It has also been shown that the ability to reduce Dronc levels requires the RING domain of DmDnr1 (Primrose et al., 2007). Thus it may be that DmDnr1 acts by targeting one or more IAP antagonists for ubiquitination.

As to why silencing of *Aednr1* causes spontaneous apoptosis in Aag2 cells but not in S2 cells, it is possible that the balance of IAP antagonists and IAP proteins may be different in these two cell lines. If there is higher expression of Mx in Aag2 cells relative to AeIAP1 than the relative expression of IAP antagonists to DIAP1 in S2 cells, then silencing *Aednr1* could be enough to overwhelm the available AeIAP1, tipping the balance towards apoptosis. Silencing *Dmdnr1* in S2 cells makes the cells more sensitive to apoptosis (Primrose et al., 2007), which is consistent with this possibility.

We observed possible cleavage of AeDnr1 by CASPS8. Although more work is needed to verify this result, there is precedence for caspases cleaving their inhibitors. A feedback loop exists between *Drosophila* Drice and DIAP1 where activated Drice cleaves its inhibitor DIAP1, and this cleavage is required for DIAP1 to recognize and inhibit Drice (Ditzel et al., 2003). It has also been suggested that a feedback loop exists between the initiator caspase Dredd and Dnr1 in *Drosophila*, as inhibiting Dredd activity or expression is accompanied by a decrease in Dnr1 protein levels (Foley & O'Farrell, 2004).

Our finding that there are preferred downstream effector caspases depending on the death stimulus (*Aednr1* RNAi versus *Aeiap1* RNAi) is of particular interest. Since relatively recent gene duplications have given rise to Drice and Dcp-1 in *D. melanogaster* and CASPS7 and CASPS8 in *A. aegypti*, and there has been an overall expansion in the number of effector caspases in mosquitoes compared to *D. melanogaster* (Bryant et al., 2008), it is impossible to assign strict orthology relationships to these caspases. In *Drosophila* it is known that silencing *Drice* can protect cells against the apoptosis induced by silencing of *diap1*, whereas silencing *dcp-1* cannot do so. In addition, it has

been shown that mutant flies lacking Drice have defects in apoptosis, while Dcp-1 mutants are largely normal, although Dcp-1 does seem to play a role in apoptosis regulation in the absence of Drice in certain cells (Muro et al., 2006, Xu et al., 2006). This has led many to believe that *Drice* is the main effector caspase involved in *Drosophila* apoptosis. Our data in this study and elsewhere (Q. Liu and R.J. Clem, submitted) suggest that the effector caspases CASPS7 and CASPS8 are equally important in *A. aegypti* apoptosis, as it appears that based on the death stimulus, apoptosis can function through either downstream caspase. This may be a difference in the apoptotic pathways of *Drosophila* and *A. aegypti*, or further investigation may result in new insights into the importance of both effector caspases in *Drosophila*, similar to what we have observed in *A. aegypti*.

The concept of AeDnr1 functioning through Mx is also unique, because it is the first example of a gene acting upstream of an IAP antagonist in insects. An upstream role for AeDnr1 is supported by our epistatic interaction evidence, *in vitro* assays, and co-immunoprecipitations, which showed no interaction of AeDnr1 with either AeDronc, AeIAP1, nor IMP, but did show interaction between AeDnr1 and Mx. To our knowledge, there is no previous evidence of genes acting upstream of IAP antagonists (other than genes involved in transcriptional regulation of IAP antagonists following a death stimulus). This finding may prove to be critical in deciphering the apoptotic pathway in *A. aegypti* as well as other models.

Interestingly, AeDnr1 appears to function entirely through Mx and not IMP. Mx and IMP share around 27 % amino acid sequence identity and 55 % similarity, which is high for IAP antagonists, either within or between species. Also, Mx orthologs are present in other mosquito species including *Aedes albopictus*, *Culex pipiens*, and *Anopheles gambiae*, but IMP is not found in these other mosquitoes, suggesting that IMP arose fairly recently by duplication in the *A. aegypti* lineage. The lack of interaction between AeDnr1 and IMP suggests that IMP has taken on a somewhat different role than Mx in *A. aegypti*. IMP does seem to play a role in *A. aegypti* apoptosis, as silencing IMP partially protects Aag2 cells against apoptosis (H. Wang and R.J. Clem, submitted). It will be interesting to determine whether Dnr1 and Mx have similar roles in other mosquito species.

In summary, AeDnr1 serves as a crucial negative regulator of apoptosis. Although AeDnr1 and AeIAP1 both function through AeDronc, AeDnr1 has a downstream effector caspase preference opposite of AeIAP1. Also, AeDnr1 acts upstream or in parallel to the AeIAP1 antagonist Mx in order to serve as a unique anti-apoptotic protein. Thus AeDnr1 appears to function by arbitrating the outcome in the battle between IAPs and IAP antagonists. Other regulatory factors could contribute as well. There are still many questions left unanswered, and future research on these topics will help uncover the exact regulatory roles of genes involved in insect apoptosis. Because Dnr1 is also found in higher animals, including humans, it will be interesting to explore its function in other systems and to determine whether it contributes to apoptosis regulation.

Acknowledgments

We thank Drs. John Means and A. Lorena Passarelli for intellectual input, Dr. Hua Wang for supplying constructs, and Dr. Alexander Beeser (Kansas State University) for helpful comments.

This research was supported by National Institutes of Health grants R21 AI067642 (to Rollie J. Clem), R21 AI063089 (to A. Lorena Passarelli), and P20 RR16475 from the BRIN Program of the National Center for Research Resources, by the Terry C. Johnson Center for Basic Cancer Research, and by the Kansas Agricultural Experiment Station.

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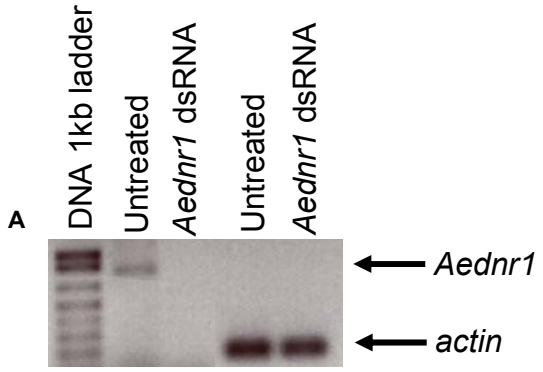
Figure 2.1. Alignment of the predicted amino acid sequences of *Drosophila* and *A. aegypti* Dnr1.

Yellow bars represent identical amino acids found in both sequences, while green bars represent similar amino acids. Bolded text represents N-terminal FERM and C-terminal RING domains. Sequence alignment was performed with Vector NTI (Invitrogen).

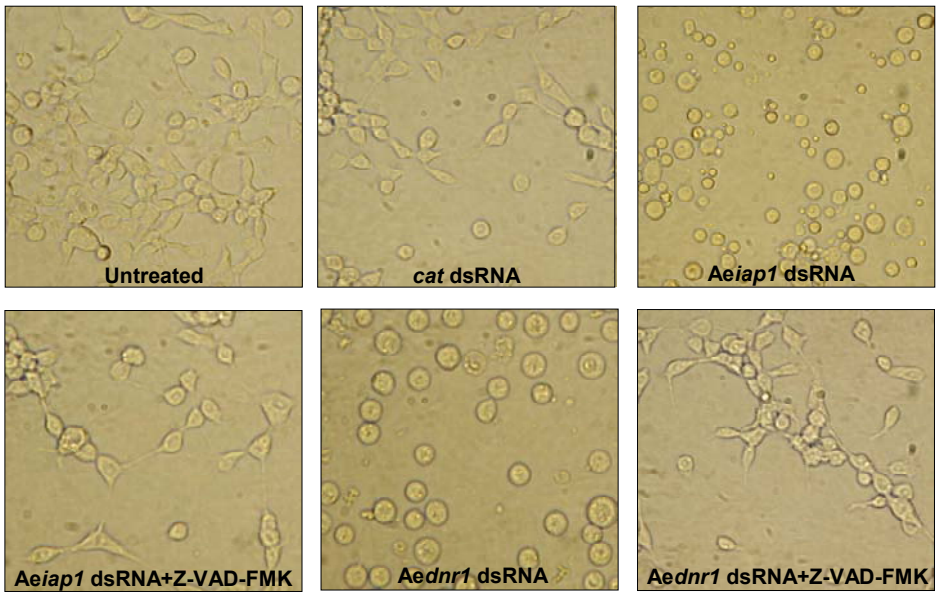
```
                1                                                  50
AeDnr1     (1)  MWCLINLPNGTTSGVQCDPKKNSQECLEKVCNDLGIICETDYFGLIPVRD
DmDnr1     (1)  MWCIVNLPNGTQQAVKWDPKANGQECLEKVCRAMNIICEMEYFGLEHWTP
               51                                                 100
AeDnr1    (51)  GDGAEVDECSAKQWINLRNPLSLHANDRHP---ILLSLRVKFWVPAHLIL
DmDnr1    (51)  NQ----KESQTRQWINLRNRLSGDSGSSGSGIQLMLALRVKFWVPVHFIL
              101                                                 150
AeDnr1    (98)  QDSVKKLFYMQARQELLDGHISAASWENAAHISALLLQADGYIYDPNKVF
DmDnr1    (97)  QESVRNLFYMQARRDLLEGRLTAPDWSGAAKLAALLCQADGLRFNESSLK
              151                                                 200
AeDnr1   (148)  ANLTGLERGSPLNTVDRR------QLR----------------RPSKRK-
DmDnr1   (147)  ADCPMRMRRELAQQQQQQQQQAQQHRLEQQRKEKEHVLSFKKRRLSKQKS
              201                                                 250
AeDnr1   (175)  ---------------------------------------------CSETDVK
DmDnr1   (197)  MEHIEVCTLPAASTTSCSLQPSPSASASASASASASASASTSACSQTHSN
              251                                                 300
AeDnr1   (182)  RGS----IASTVTVDDAVSETEPKNIYQTYIVRPKFEDVEPEPMPENFIQ
DmDnr1   (247)  SSSSSPSNSSSQTGLDERLASNPLRVYEEYFMQPSCEGE-P---PADYLR
              301                                                 350
AeDnr1   (228)  MIAKEHEVLSKIKMTASSAQYWVLEEISSLNGYGEEVFEGITINEPSVRC
DmDnr1   (293)  QIAVEHGKLAKLQMSLKTAKYWLLKSIQDLEGYGEELFSGVTTNESATRC
              351                                                 400
AeDnr1   (278)  KIGVSPHGLTIAKDDEKYNVPFTAVKAAKSIKRSFRLTYMNEEHEETHIE
DmDnr1   (343)  DIAVGAHGITVCRGGEKQSIPFGAIAAAKSLRRTFKLEYVDDHNDRKELE
              401                                                 450
AeDnr1   (328)  LKLPNHRTAACLYRAITEKHVFYSCETVRPVVTTQFIRDLKGTIVSMFNE
DmDnr1   (393)  IKLPKQPIAAGLYRSITERHAFYVCDKVRGVVTNQFTRDLKGTIASMFME
              451                                                 500
AeDnr1   (378)  DTELGKRYVFDIQRTCREVYDNARRVLHARG-----------------ME
DmDnr1   (443)  NTELGKRYVFDIQHTCREVHDQARRTLHERGGDLVAEGAEGCSAVAGGLG
              501                                                 550
AeDnr1   (411)  ISKVQKECPQAQAS-----------KLDQQLADEAEESNKLERLVEERL
DmDnr1   (493)  ASAVGEPGVSPWAMALTTGAGGSMAGKIDLAIREKEAREAAIERCVDTRI
              551                                                 600
AeDnr1   (449)  TEALTCIICADNMIDTMFLPCGHITACRQCAEQCDRCPLCRANIECVNKA
DmDnr1   (543)  SEAMQCKICMDRAINTVFNPCCHVIACAQCAARCSNCPNCRVKITSVVKI
              601                                                 650
AeDnr1   (499)  FLPPVMRTRSQTAAANASAQSIEAH------------------------
DmDnr1   (593)  YLPPELRTSQTGSGATTTSSSSIMGDGQVEEQLLQQQLDEISAAPASLEA
              651                                684
AeDnr1   (524)  ----------------------------------
DmDnr1   (643)  GADAGVGVGGMAVGAGAGAGATGPGGQPKVTTAA
```

Figure 2.2. Aag2 cells treated with *Aednr1* dsRNA undergo caspase-dependent apoptosis.

(A) Addition of dsRNA corresponding to *Aednr1* silences *Aednr1* expression. The presence of *Aednr1* and *actin* mRNA was determined by RT-PCR in untreated or *Aednr1* dsRNA-treated Aag2 cells at 25 hrs after dsRNA treatment. (B) Treatment of Aag2 cells with *Aednr1* or *Aeiap1* dsRNA induces apoptosis. Aag2 cells were left untreated or were treated with the indicated dsRNAs, with or without the pan caspase inhibitor Z-VAD-FMK. Images were taken at 400X magnification starting at 19 hrs after dsRNA addition. (C) Treatment of Aag2 cells with *Aednr1* or *Aeiap1* dsRNA induces caspase activity. Caspase activity was determined at 25 hrs after dsRNA treatment by measuring cleavage of caspase substrate Ac-DEVD-AFC, and the results were plotted as the mean +/- SE based on 3 replicates.



B


Untreated
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Aeiap1 dsRNA
Aeiap1 dsRNA+Z-VAD-FMK
Aednr1 dsRNA
Aednr1 dsRNA+Z-VAD-FMK

C

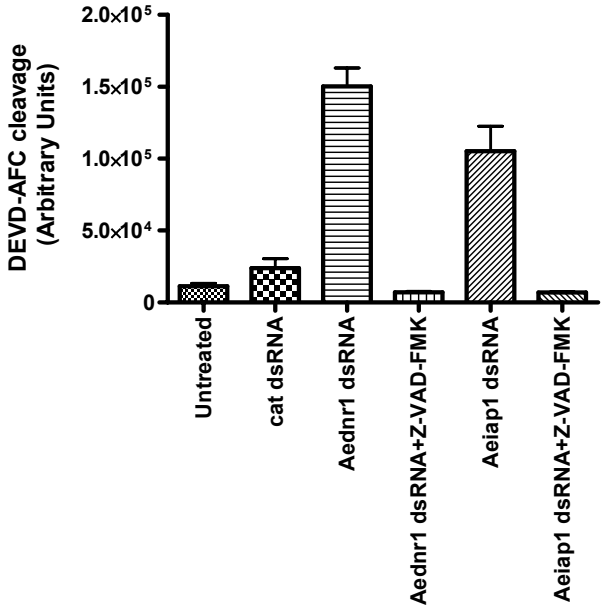
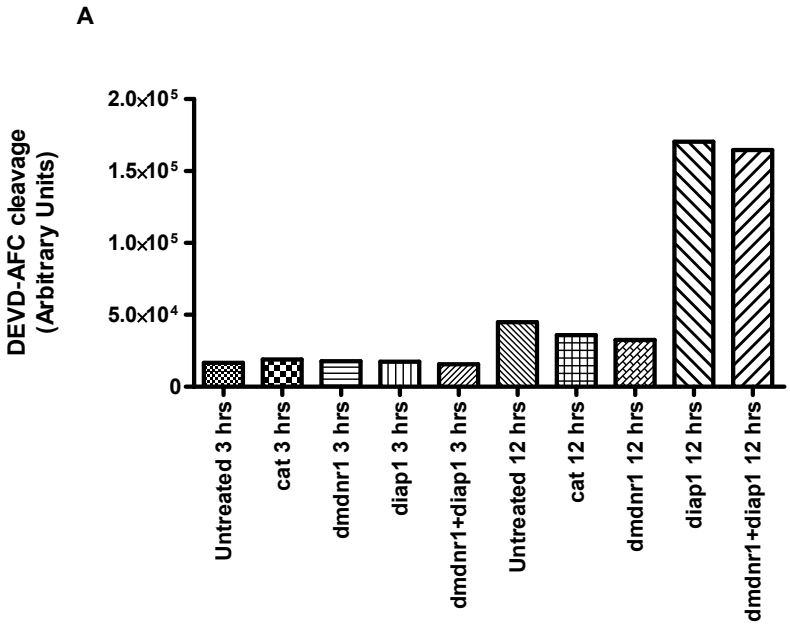

DEVD-AFC cleavage (Arbitrary Units)
2.0×10⁵
1.5×10⁵
1.0×10⁵
5.0×10⁴
0
Untreated
cat dsRNA
Aednr1 dsRNA
Aednr1 dsRNA+Z-VAD-FMK
Aeiap1 dsRNA
Aeiap1 dsRNA+Z-VAD-FMK

Figure 2.3. *Dmdnr1* RNAi does not induce spontaneous apoptosis in *Drosophila* S2 cells.

(A) S2 cells were treated with the indicated dsRNAs and harvested at 3 and 12 hrs after dsRNA addition. Caspase activity was determined using Ac-DEVD-AFC. (B) Addition of *Dmdnr1* dsRNA silences *Dmdnr1* expression in S2 cells. The presence of *Dmdnr1* and *actin* mRNA was determined by RT-PCR in *Dmdnr1* dsRNA-treated S2 cells.



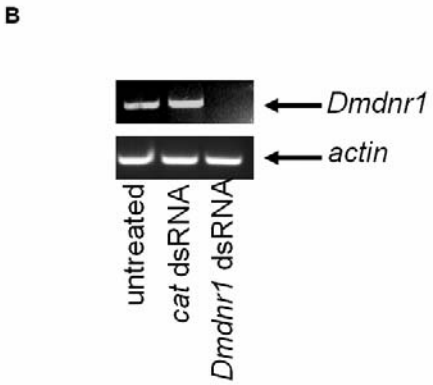
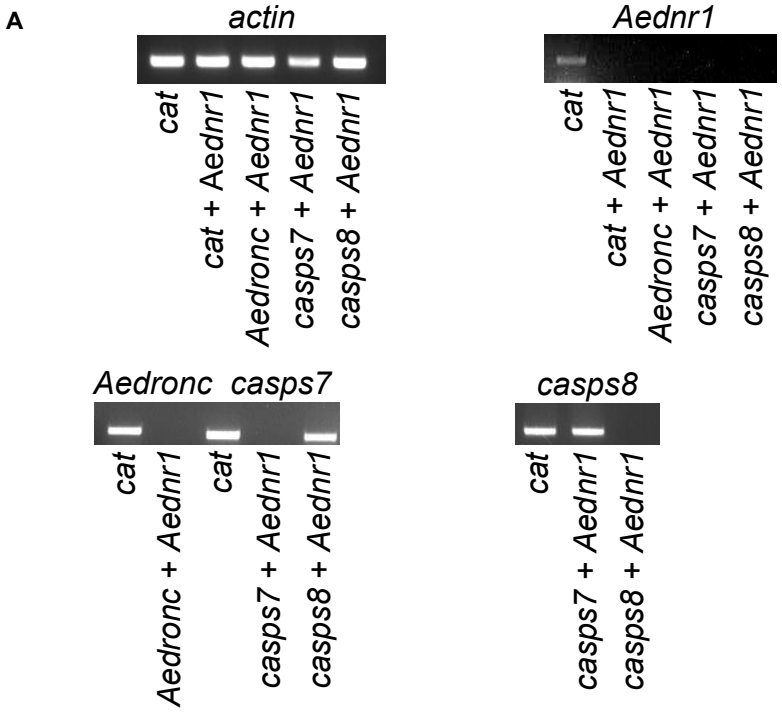
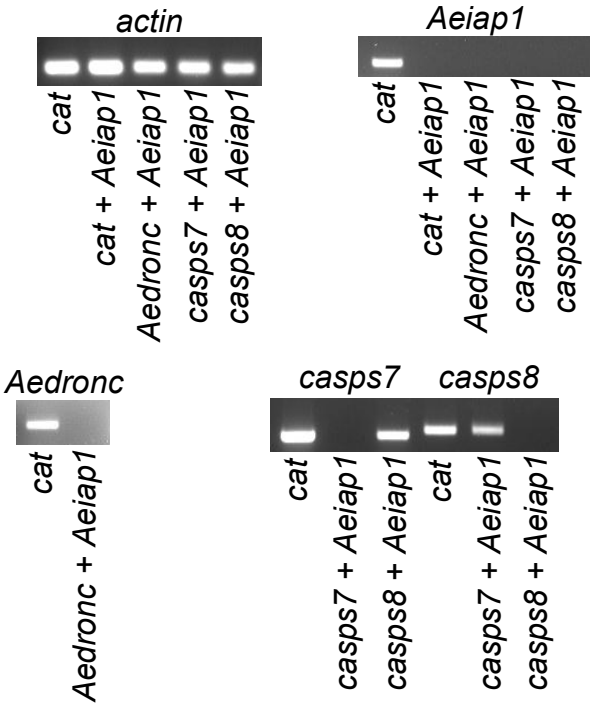


Figure 2.4. *Aedronc* and *casps8* RNAi suppresses *Aednr1* dsRNA-induced apoptosis, while *Aedronc* and *casps7* RNAi suppresses *Aeiap1* dsRNA-induced apoptosis.

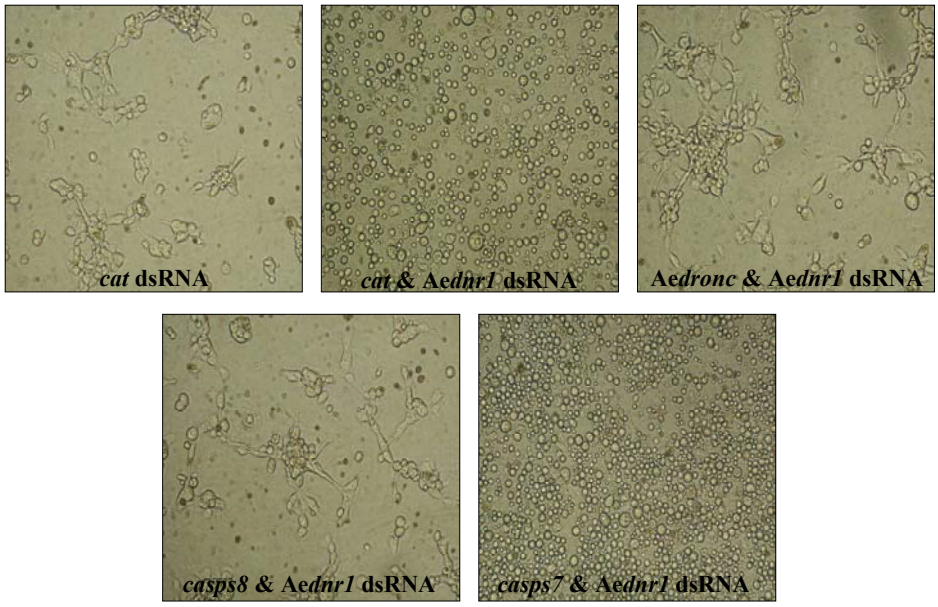
(A) RT-PCR results showing successful silencing of *Aednr1* and other target genes. The indicated dsRNAs were added to Aag2 cells, and RNA was harvested at 19.5 hrs and subjected to RT-PCR. (B) RT-PCR results showing successful silencing of *Aeiap1* and other target genes. (C) Apoptosis due to *Aednr1* silencing is inhibited by RNAi of *Aedronc* or *casps8*, but not *casps7*, while apoptosis due to *Aeiap1* silencing is inhibited by RNAi of *Aedronc* or *casps7*, but not *casps8*. Aag2 cells were treated with the indicated dsRNAs, and images were taken starting at 17 hrs at 400X magnification. (D) Caspase activity in RNAi-treated cells. Aag2 cells were treated with the dsRNAs shown and lysates were harvested and assayed for ability to cleave Ac-DEVD-AFC at 19.5 hrs. Shown are the mean values +/- SE based on 3 replicates. (E) Viability of RNAi-treated cells. Aag2 cells were treated with the indicated dsRNAs and cell viability was determined at 17 hrs. Shown are the mean values +/- SE based on 2 replicates.



B


actin
cat
cat + Aeiap1
Aedronc + Aeiap1
casps7 + Aeiap1
casps8 + Aeiap1
Aeiap1
cat
cat + Aeiap1
Aedronc + Aeiap1
casps7 + Aeiap1
casps8 + Aeiap1
Aedronc
cat
Aedronc + Aeiap1
casps7
casps8
cat
casps7 + Aeiap1
casps8 + Aeiap1
cat
casps7 + Aeiap1
casps8 + Aeiap1

c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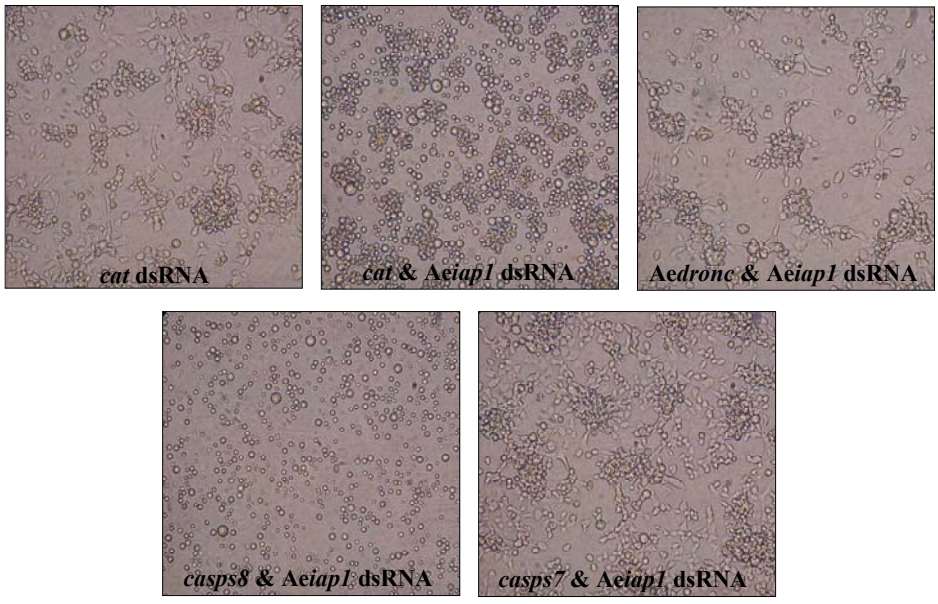
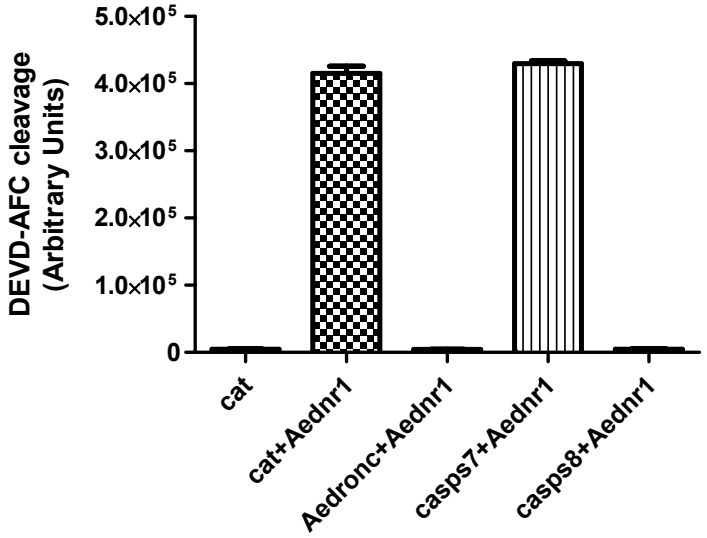
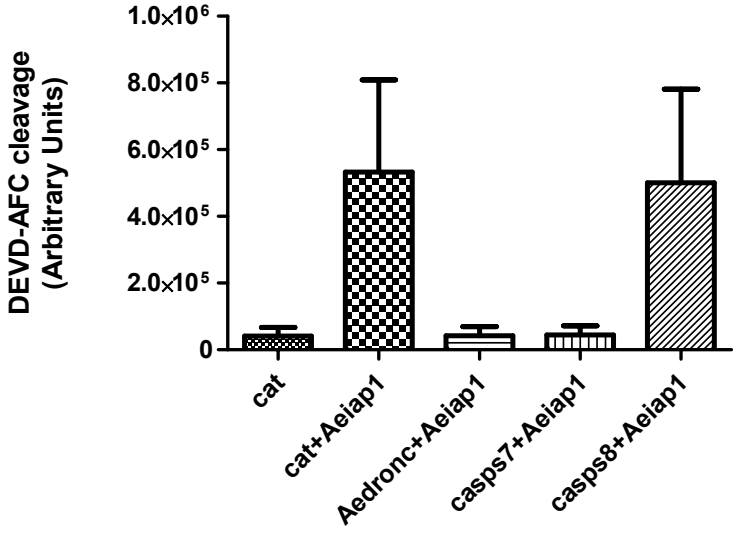
cat & Ae*dnr1* dsRNA
Ae*dronc* & Ae*dnr1* dsRNA
casps8 & Ae*dnr1* dsRNA
casps7 & Ae*dnr1* dsRNA

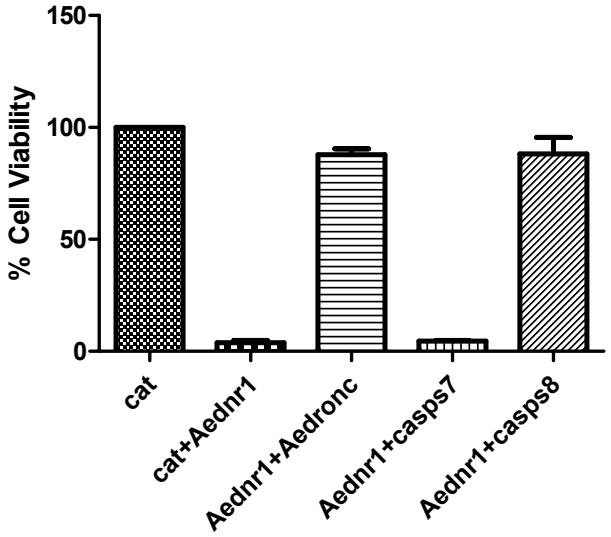

cat dsRNA
cat & Ae*iap1* dsRNA
Ae*dronc* & Ae*iap1* dsRNA
casps8 & Ae*iap1* dsRNA
casps7 & Ae*iap1* dsRNA

D


DEVD-AFC cleavage
(Arbitrary Units)
5.0x10^5
4.0x10^5
3.0x10^5
2.0x10^5
1.0x10^5
0
cat
cat+Aednr1
Aedronc+Aednr1
casps7+Aednr1
casps8+Aednr1


DEVD-AFC cleavage
(Arbitrary Units)
1.0x10^6
8.0x10^5
6.0x10^5
4.0x10^5
2.0x10^5
0
cat
cat+Aeiap1
Aedronc+Aeiap1
casps7+Aeiap1
casps8+Aeiap1

E


150
100
50
0
% Cell Viability
cat
cat+Aednr1
Aednr1+Aedronc
Aednr1+casps7
Aednr1+casps8

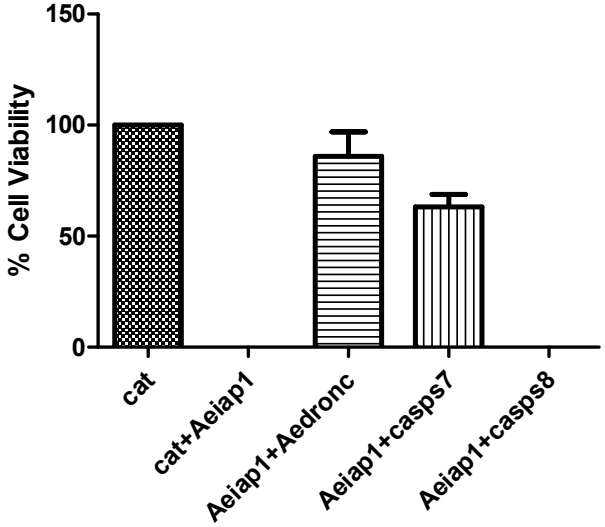
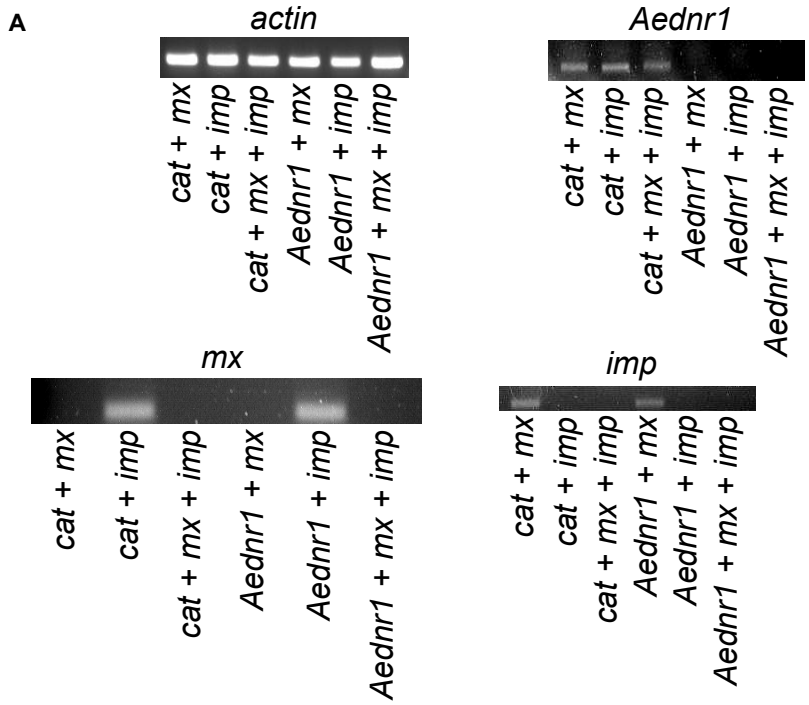
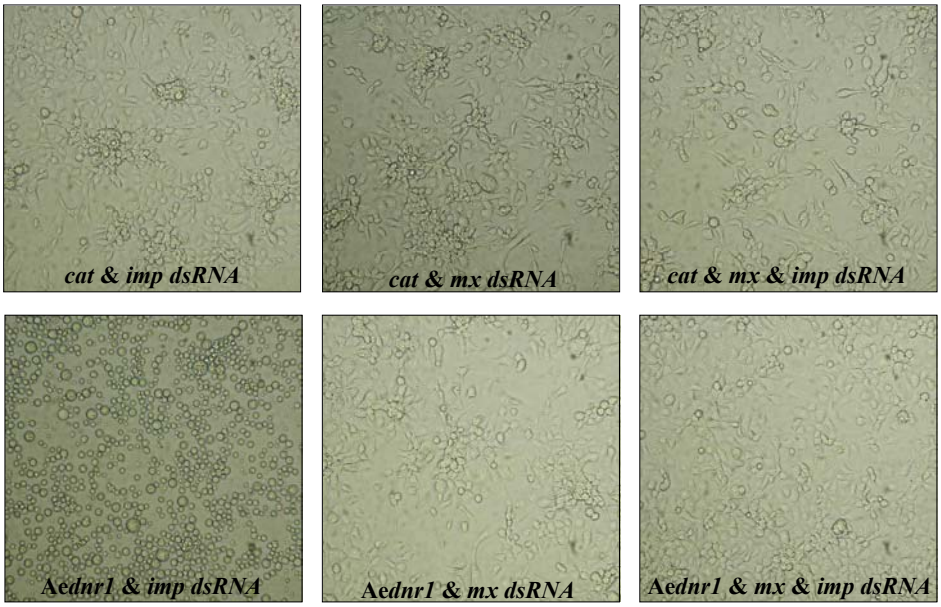

150
100
50
0
% Cell Viability
cat
cat+Aeiap1
Aeiap1+Aedronc
Aeiap1+casps7
Aeiap1+casps8

Figure 2.5. *mx* RNAi but not *imp* RNAi suppresses *Aednr1* dsRNA-induced apoptosis.

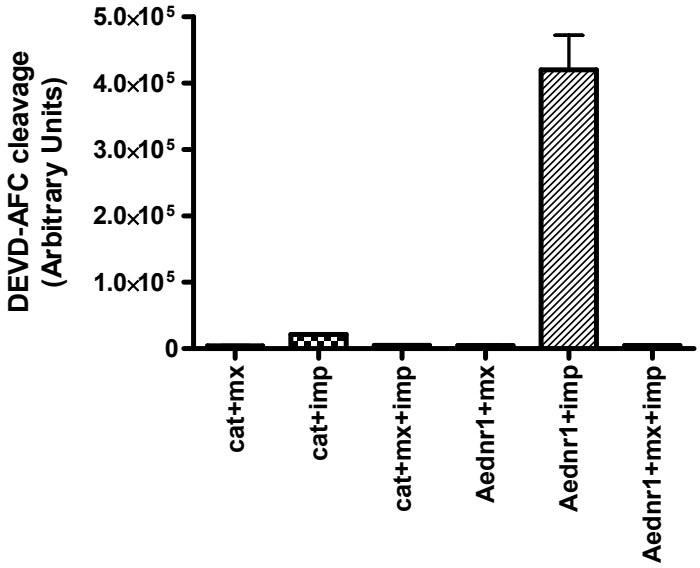
(A) Aag2 cells were treated with the indicated dsRNAs and silencing was confirmed by RT-PCR. (B) Aag2 cells were treated with the indicated dsRNAs and images were taken starting at 17 hrs at 400X magnification. (C) Caspase activity was measured in Aag2 cells treated with the indicated dsRNAs at 19.5 hrs. The data shown represent the mean +/- SE of 3 replicates. (d) Cell viability was determined at 17 hrs after treatment with the indicated dsRNAs. Shown are the mean +/- SE of 3 replicates.



B


cat & imp dsRNA
cat & mx dsRNA
cat & mx & imp dsRNA
Aednr1 & imp dsRNA
Aednr1 & mx dsRNA
Aednr1 & mx & imp dsRNA

C


DEVD-AFC cleavage
(Arbitrary Units)
5.0×10⁵
4.0×10⁵
3.0×10⁵
2.0×10⁵
1.0×10⁵
0
cat+mx
cat+imp
cat+mx+imp
Aednr1+mx
Aednr1+imp
Aednr1+mx+imp

D

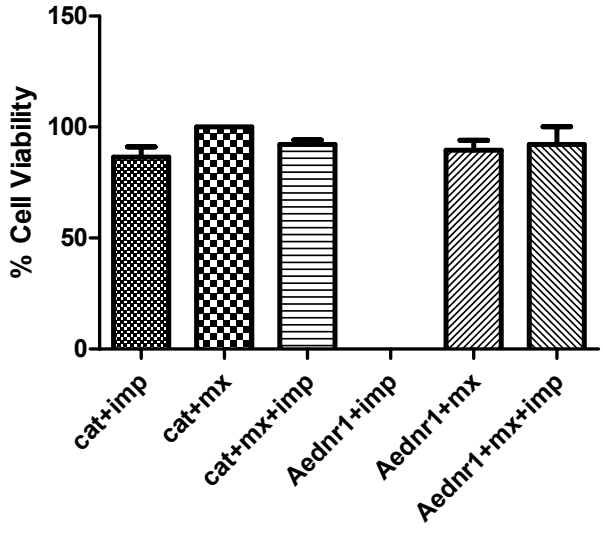
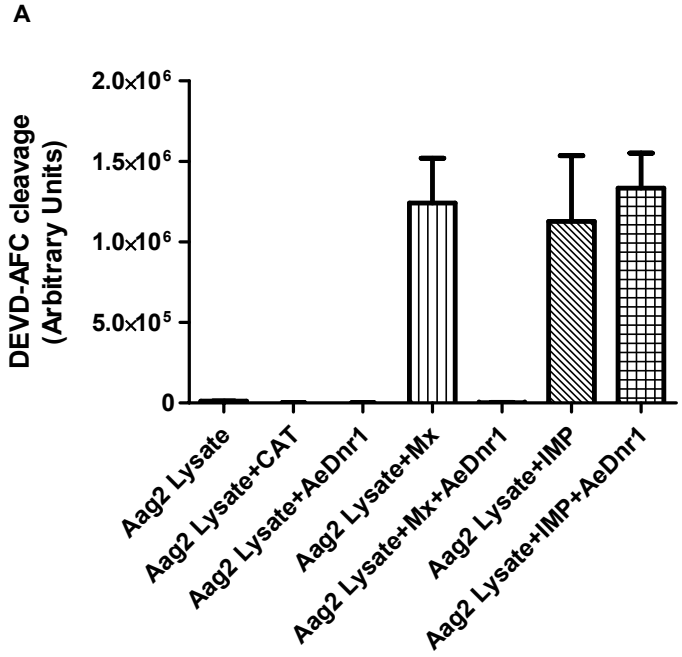
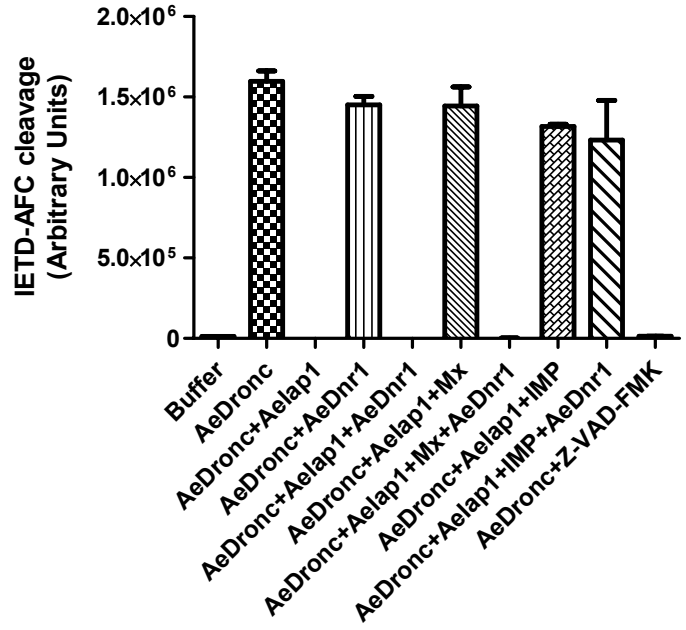

150
100
50
0
% Cell Viability
cat+imp
cat+mx
cat+mx+imp
Aednr1+imp
Aednr1+mx
Aednr1+mx+imp

Figure 2.6. Mx is regulated by AeDnr1 and acts through CASPS8, while IMP is not regulated by AeDnr1 and acts through CASPS7.

(A) AeDnr1 suppresses caspase activity induced by Mx but not IMP in Aag2 cell lysate. Aag2 cell lysate was incubated with either no protein, reticulocyte lysates containing *in vitro* translated CAT or AeDnr1, or bacterially expressed and purified Mx, or IMP protein. After incubation, caspase activity was determined. Shown are the mean +/- SE of 2 replicates. (B) AeIAP1 but not AeDnr1 inhibits AeDronc activity, and AeIAP1 inhibition of AeDronc can be antagonized by either Mx or IMP. *In vitro* translated AeDnr1 or AeIAP1 protein was mixed with bacterially expressed and purified AeDronc, Mx or IMP, and AeDronc activity was measured using Ac-IETD-AFC. The values shown are the mean +/- SE based on 2 replicates. (C) AeDnr1 inhibits CASPS8 more efficiently than CASPS7. In addition, Mx has a preference for antagonizing AeIAP1 that is bound to CASPS8, while IMP has a preference for antagonizing AeIAP1 that is bound to CASPS7. *In vitro* translated AeDnr1 or AeIAP1 was mixed with bacterially expressed and purified CASPS7, CASPS8, Mx and/or IMP. Caspase activity was measured by Ac-DEVD-AFC cleavage. Shown are the mean +/- SE of 2 replicates.



B


IETD-AFC cleavage
(Arbitrary Units)
2.0×10^6
1.5×10^6
1.0×10^6
5.0×10^5
0
Buffer
AeDronc
AeDronc+Aelap1
AeDronc+AeDnr1
AeDronc+Aelap1+AeDnr1
AeDronc+Aelap1+Mx
AeDronc+Aelap1+Mx+AeDnr1
AeDronc+Aelap1+IMP
AeDronc+Aelap1+IMP+AeDnr1
AeDronc+Z-VAD-FMK

C

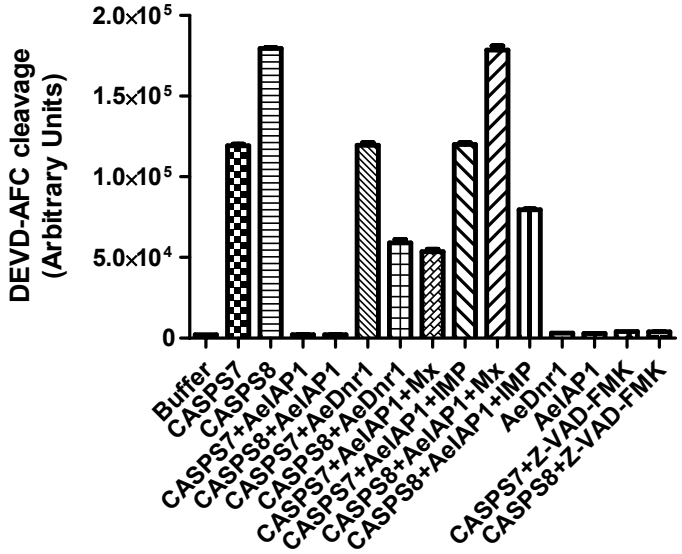
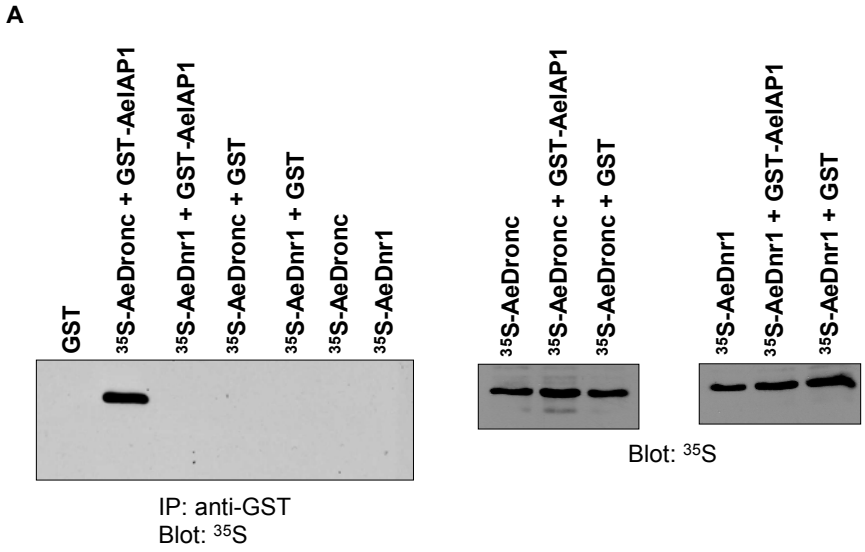
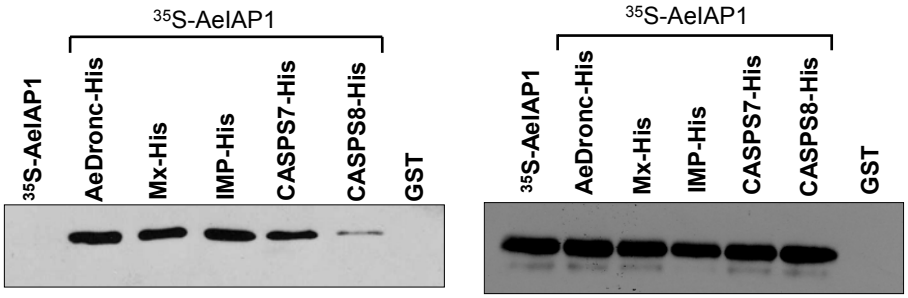

DEVD-AFC cleavage
(Arbitrary Units)
2.0×10^5
1.5×10^5
1.0×10^5
5.0×10^4
0
Buffer
CASPS7
CASPS8
CASPS7+AelAP1
CASPS8+AelAP1
CASPS7+AeDnr1
CASPS8+AeDnr1
CASPS7+AelAP1+Mx
CASPS7+AelAP1+IMP
CASPS8+AelAP1+Mx
CASPS8+AelAP1+IMP
AeDnr1
AelAP1
CASPS7+Z-VAD-FMK
CASPS8+Z-VAD-FMK

Figure 2.7. AeDnr1 interacts with Mx and CASPS8 but not AeIAP1, AeDronc, IMP, or CASPS7.

(a) AeDronc, but not AeDnr1, directly interacts with AeIAP1. *In vitro* translated, ^{35}S-labeled AeDronc or AeDnr1 was incubated with bacterially expressed and purified GST or GST-AeIAP1, and protein complexes were immunoprecipated using anti-GST antibody and visualized by autoradiography. The right blots show the ^{35}S-labeled AeDnr1 input (10% of total). (B) ^{35}S-labeled AeIAP1 was incubated with recombinant His-tagged AeDronc, Mx, IMP, CASPS7 or CASPS8. Protein complexes were immunoprecipitated with anti-His antibody and visualized by autoradiography. The right blot shows the ^{35}S-labeled AeIAP1 input (10% of total). (C) ^{35}S-labeled Dnr1 was incubated with recombinant GST, or His-tagged AeDronc, CASPS7, CASPS8, IMP or Mx, and immunoprecipitated with anti-His antibody and visualized by autoradiography.



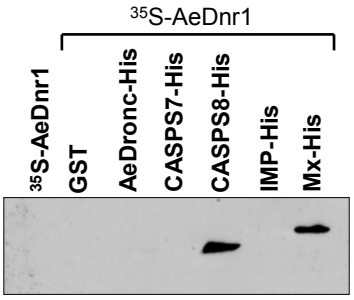
B



IP: anti-His
Blot: ^{35}S

Blot: ^{35}S

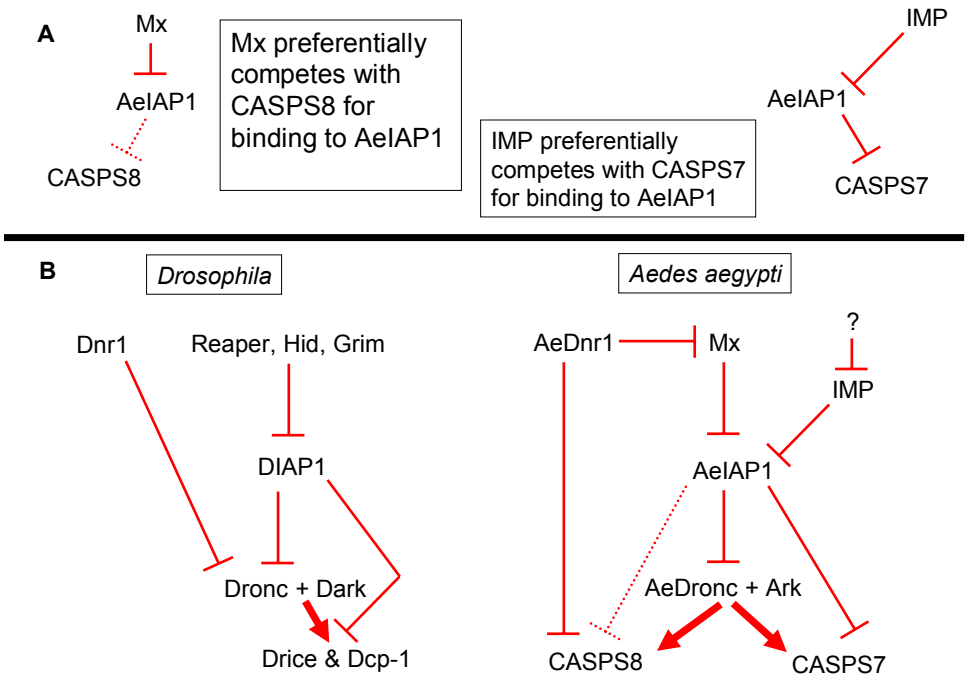
C



IP: anti-His
Blot: ^{35}S

Figure 2.8. The apoptotic pathway of *Drosophila melanogaster* and *Aedes aegypti*.

Models showing (A) biochemical interactions and (B) genetic interactions for the current pathway in *Drosophila* and the proposed model for *A. aegypti*. Pointed arrows indicate positive genetic regulation. Blunt arrows indicate negative genetic regulation.



CHAPTER 3 - Conclusion

In conclusion, there is still much to learn about the mosquito apoptotic pathway. In particular, AeDnr1 is a newly characterized anti-apoptotic protein with a major role in regulating mosquito apoptosis. We have shown that AeDnr1 plays an essential role in regulating apoptosis, and that its function is upstream of and dependent on Mx and CASPS8 but not IMP and CASPS7. More studies need to be done in order to fully characterize this protein in mosquitoes, and to see if similar dynamic effects exist for this protein in other systems.

Future work includes first that it would be interesting to discover what regions are necessary for the function of AeDnr1 in *A. aegypti*. For instance, we could mutate the RING domain and see if the stabilization of AeDnr1 or its ability to regulate Mx or CASPS8 is RING-dependent. As seen in *Drosophila*, IAP antagonists Reaper and Grim can regulate DIAP1 with or without direct binding (Claveria et al., 2002, Holley et al., 2002, Olson et al., 2003a, Yoo et al., 2002). Since function and direct binding do not always correlate we could also detect whether the direct binding of AeDnr1 to Mx or CASPS8 is required for its function, and what residues are required for binding. If the RING domain mutant inhibits AeDnr1 from regulating Mx or CASPS8 we could see if ubiquitination is the method of regulation. We could test this in a number of ways, including ubiquitination effects on Mx by mutation of the lysine residues (inhibiting the acceptance of ubiquitin) and determining whether the Mx mutant is more stable compared to wildtype, and if the mutated protein leads to increased apoptosis compared to wildtype. To detect if this is AeDnr1-specific we could also perform *in vitro* ubiquitination studies comparing the ability of the RING-mutant and wildtype AeDnr1 to ubiquitinate Mx or CASPS8. With regards to the FERM domain, it appears to be important in *Drosophila* apoptosis and was shown to lead to a mislocalization of the Dnr1 protein (Primrose et al., 2007). It would be interesting to note if the importance of the FERM domain is in promoting correct localization of AeDnr1, or whether its importance lies in its role in protein-protein interactions. Also to add to our knowledge in *A.* aegyp*ti*,

we could determine whether AeDnr1 affects the expression level of key proteins, including the two proteins it is directly involved with, Mx and CASPS8. It would also be extremely interesting to confirm the role of AeDnr1 in regulating AeDronc. In *Drosophila*, although direct binding was not detected, an increased level of Dronc protein resulted from knocking down *Dmdnr1* (Primrose et al., 2007). We could silence *Aednr1* and see if Dronc protein levels are affected in *A. aegypti*. In addition, if AeDnr1 does affect protein levels of any of the genes, it would be interesting to detect if this is RING- or FERM domain-dependent. Also, just as knocking down AeDnr1 induces apoptosis, it is necessary to overexpress AeDnr1 and see if we inhibit apoptosis via different stimuli including chemicals and UV light, and especially RNAi to *Aeiap1*. If so, again determine whether this ability is RING- or FERM domain-dependent. It would also be interesting to confirm the possible existence of a feedback loop between CASPS8 and AeDnr1. We could see if there is more AeDnr1 accumulation with active CASPS8 than with inactive, suggesting there exists a negative feedback loop similar to Dredd and Dnr1 in *Drosophila*. We could also see if AeDnr1 mutated at potential caspase cleavage sites can inhibit CASPS8 activity, suggesting there exists a negative feedback loop similar to Drice and DIAP1 in *Drosophila*. Likewise, we could also detect if blocking the cleavage of AeDnr1 by CASPS8 would stop the function of AeDnr1 from inhibiting IAP antagonists, in this way creating a positive feedback loop. By using mutant constructs we could also detect the important sequences required for a feedback loop to occur.

In terms of the whole organism, we could silence *Aednr1* expression in the mosquito and see what effects may occur. Flies with a disruption in *Dmdnr1* are viable, showing that *Drosophila* Dnr1 is not a major regulator of apoptosis like DIAP1 (FlyBase). Because of the role of AeDnr1 in cells, we expect AeDnr1 to have an effect in the whole mosquito. We could also determine whether *Aednr1* RNAi helps virus replication within the mosquito.

As a homolog of Dnr1 has been found in nearly every system it would be interesting to expand our knowledge of Dnr1 in these systems. Specifically, it would be extremely interesting to determine if Dnr1 has a role in mammalian apoptosis, and to add further knowledge to the role of Dnr1 in *Drosophila*. Starting with mammals, we could first knockdown *dnr1* in a mammalian cell line and determine if there are any effects,

such as inducing apoptosis or making cells more sensitized to undergo apoptosis. Correlating, we could see whether these possible effects were a result of Dnr1 alone or also dependent on key checkpoint breakdowns such as causing cytochrome c release and/or upregulation of IAP antagonists. If so, which domain is this effect dependent on and what are the different stimuli that would induce apoptosis and/or affect caspase activity? We could also overexpress Dnr1 and determine if this inhibited apoptosis in a mammalian cell line. To explore a mechanism for this possible effect on apoptosis, we could look to see if Dnr1 has a role in regulating IAP antagonists. We could determine if Dnr1 binds directly to Smac/Diablo, or if it decreases Smac/Diablo protein levels and if so in both cases determine whether this was dependent on a RING and/or FERM domain. If Dnr1 does either of these effects we could determine whether ubiquitination, competitive binding, or some other method was the mechanism used. As *Aednr1* RNAi-mediated apoptosis acts through CASPS8 and not CASPS7 in *A. aegypti*, it would be interesting to see (if Dnr1 has an effect in mammals) whether Dnr1 acted through Caspase-3 or Caspase-7. Also, we could determine if Dnr1 has a role in the extrinsic pathway in mammals. With this thought, it would be interesting to study the role of the FERM domain in localization of proteins to the plasma membrane. Dnr1 could perhaps play a role in detecting a proapoptotic signal, and therefore be involved in the extrinsic pathway in mammals. This would make sense because DmDnr1 has been shown to interact with Dredd (the receptor mediated Caspase-8 homolog) (Guntermann et al., 2009). We could also see if Dnr1 affects the protein levels of any key players involved in apoptosis.

In *Drosophila*, we could determine whether Dnr1 binds directly to Hid, Reaper, and/or Grim, and if so, what domains are required on each of the proteins for the respective binding. If so, using mutant proteins that do not interact we could determine whether sensitivity to apoptosis is dependent on these proteins interacting. One possibility is that perhaps Dnr1 is regulating some IAP antagonists and not others. As our data show, AeDnr1 inhibits Mx but not IMP. In addition, we could overexpress AeDnr1 in *Drosophila* S2 cells and see if this inhibits apoptosis to determine a possible conservation of function. It would also be interesting to try another *Drosophila* cell line,

besides S2, to see if there is a difference in the role of Dnr1 in other cell types. This same line of thinking could be used in all organisms.

Overall, I think the most interesting direction to take next is determining whether Dnr1 interacts with either Hid, Reaper, and/or Grim in *Drosophila* or Smac/Diablo in mammals and if it does to discover the functional mechanism. Also, to address the question of whether, in mammals, Dnr1 might induce or sensitize cells to undergo apoptosis when knocked down, or protect cells from apoptosis when overexpressed. Finally, it would be important to ask whether inhibition of IAP antagonists or protein localization serves a functional role for Dnr1 in these higher systems.