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Annotation and expression profiling of apoptosis-related genes in the yellow fever mosquito, *Aedes aegypti*

Bart Bryant[1], **Carol D. Blair**[2], **Ken E. Olson**[2], and **Rollie J. Clem**[1,*]

1 *Molecular, Cellular, and Developmental Biology Program, Arthropod Genomics Center, Division of Biology, Kansas State University, Manhattan, KS*

2 *Arthropod-Borne and Infectious Diseases Laboratory, Department of Microbiology, Immunology, and Pathology, Colorado State University, Fort Collins, CO*

Abstract

Apoptosis has been extensively studied in *Drosophila* by both biochemical and genetic approaches, but there is a lack of knowledge about the mechanisms of apoptosis regulation in other insects. In mosquitoes, apoptosis occurs during *Plasmodium* and arbovirus infection in the midgut, suggesting that apoptosis plays a role in mosquito innate immunity. We searched the *Aedes aegypti* genome for apoptosis-related genes using *Drosophila* and *Anopheles gambiae* protein sequences as queries. In this study we have identified eleven caspases, three inhibitor of apoptosis (IAP) proteins, a previously unreported IAP antagonist, and orthologs of *Drosophila* Ark, Dnr1, and BG4 (also called dFadd). While most of these genes have been previously annotated, we have improved the annotation of several of them, and we also report the discovery of four previously unannotated apoptosis-related genes. We examined the developmental expression profile of these genes in *Ae. aegypti* larvae, pupae and adults, and we also studied the function of a novel IAP antagonist, IMP. Expression of IMP in mosquito cells caused apoptosis, indicating that it is a functional pro-death protein. Further characterization of these genes will help elucidate the molecular mechanisms of apoptosis regulation in *Ae. aegypti*.

Introduction

Apoptosis is a key pathway involved in normal processes such as development, tissue homeostasis, and DNA damage responses, as well as pathological processes including cancer, ischemia, neurological diseases, and defense against pathogens like viruses (Vaux and Korsmeyer, 1999; Opferman and Korsmeyer, 2003; James and Green, 2004; Clem, 2005). The process of apoptosis is largely carried out by a family of cysteine proteases called caspases. These enzymes are expressed as zymogens and are activated by multiple stimuli. There are two types of caspases, initiator and effector, which carry out different functions in apoptosis. A death insult first results in activation of one or more members of the initiator class, which then cleave and activate members of the effector class. The effector caspases cleave many cellular targets and dismantle the cell. All caspases consist of a prodomain and large and small catalytic domains, which are freed from each other by cleavage at aspartate residues. The large and small catalytic domains form a dimer, and two of these heterodimers associate to form the

*Corresponding author: Rollie J. Clem Kansas State University Division of Biology Ackert Hall Manhattan, KS 66506 Tel: 785–532–3172 Tel: 785–532–6653 Email: rclem@ksu.edu.

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active caspase molecule. Initiator caspases contain long prodomains, which are involved in interaction with adaptor proteins, while effector caspases contain short prodomains.

Most of what is known about the molecular mechanisms of apoptosis in insects comes from study of the fruitfly *Drosophila melanogaster* (reviewed in Hay and Guo, 2006). There are seven caspases encoded in the *Drosophila* genome, including the three initiator caspases Nc (also known as Dronc), Dredd, and dream (also known as Strica) and the four effector caspases Ice (also known as Drice), Dcp-1, decay, and Damm (also known as Daydream). Among the initiator caspases found in *Drosophila*, Nc appears to be the most important in carrying out apoptosis, while Dredd is important in the immune response, and dream is relatively uncharacterized. Among the effector caspases, Ice appears to be the most important for apoptosis, with Dcp-1 playing a supportive role.

Activation of initiator caspases by intrinsic signals involves the formation of a large protein complex called the apoptosome. Homologs of an adaptor protein that is an integral part of the apoptosome have been found in the nematode *Caenorhabditis elegans* (CED-4), in mammals (APAF-1), and in *Drosophila* (Ark). In mammals, cytochrome c binding to APAF-1 is required for apoptosome formation, but cytochrome c does not appear to be required for apoptosome formation in *Drosophila* (Zimmermann et al., 2002; Means et al., 2006; Yu et al., 2006; Bao and Shi, 2007).

Multiple gene products regulate caspases, either positively or negatively. Among the most important negative caspase inhibitors are the IAP (Inhibitor of Apoptosis) proteins. IAP proteins were first discovered in baculoviruses (Crook et al., 1993), but are now known to exist in cellular genomes ranging from yeast to mammals, where they play important roles in regulating apoptosis and cell division (Vaux and Silke, 2005). In *Drosophila,* the IAP protein that is most important in regulating apoptosis is thread (also known as DIAP1). Thread was first identified in an enhancer screen for apoptosis-regulating genes (Hay et al., 1995). Overexpression of thread inhibits apoptosis, while loss of thread leads to spontaneous apoptosis, both in the developing fly embryo and in cultured *Drosophila* cells (Hay and Guo, 2006). Thread has the ability to directly bind and inhibit effector caspases. It also can bind to Nc and causes its degradation via the ubiquitin-proteasome pathway (Wilson et al., 2002).

In addition to IAPs, a second type of negative caspase inhibitor was recently reported in *Drosophila*. The Dnr1 (defense repressor 1) protein was first identified in a cell-based screen for innate immunity, and was found to inhibit Dredd activity (Foley and O'Farrell, 2004). More recently, Dnr1 has been shown to inhibit apoptosis by causing a reduction in the level of Nc protein (Primrose et al., 2007).

In *Drosophila*, apoptosis depends on the expression of a set of proteins collectively referred to as IAP antagonists or RHG proteins (Hay et al., 2004). Four of these proteins, rpr (reaper), grim, W (also known as wrinkled or hid), and skl (sickle), are encoded by genes that are in a chromosomal region called the H99 interval (White et al., 1994; Grether et al., 1995; Chen et al., 1996). The RHG genes *rpr* and *grim* encode small proteins that are transcriptionally upregulated in cells that are destined to die (Kumar and Cakouros, 2004), while the *W* gene encodes a larger protein that is regulated post-translationally by phosphorylation (Bergmann et al., 1998). The protein encoded by *skl* is less well characterized. The RHG proteins each physically interact with thread through a short motif at their amino termini (Vucic et al., 1997; Vucic et al., 1998), and this interaction plays an important role in determining whether a cell lives or dies.

Apoptosis has been established as a component of the innate immune response in baculovirus infections of lepidopteran insects (Clem, 2005). In addition, cross-talk exists between innate immunity pathways and apoptosis pathways in insects. In *Drosophila,* Dredd (Elrod-Erickson

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et al., 2000; Leulier et al., 2000; Stoven et al., 2000), Iap2 (Gesellchen et al., 2005; Kleino et al., 2005), BG4 (also known as dFADD) (Zhou et al., 2005b) and Dnr1 (Foley and O'Farrell, 2004) have already been shown to play roles in innate immunity. At one day post infection with Sindbis virus, the midgut of *Aedes aegypti* exhibited an increase in expression of the *Ae. aegypti* ortholog of Dif, which is part of the Toll pathway in *Drosophila* (Sanders et al., 2005). In *Drosophila,* it has been shown that the protein MyD88 is a component of the Toll pathway, and MyD88 was shown to bind to BG4 (dFADD) and Dredd (Horng and Medzhitov, 2001). In mammals, FADD plays a role in activation of caspases through the extrinsic pathway (Chinnaiyan et al., 1996).

In mosquitoes, there are reports that arbovirus infection causes pathology resembling apoptosis in the midgut and salivary glands. This pathology has been found in *Ae. aegypti* infected with Semliki Forest virus (Mims et al., 1966), in *Culiseta melanura* infected with eastern equine encephalitis virus (Weaver et al., 1988), and in *Aedes albopictus* infected with Sindbis virus (Bowers et al., 2003). Long-term West Nile virus infection has also been shown to induce cell death in the salivary glands of *Culex pipiens quinquefasciatus* (Girard et al., 2005) and the same group later suggested that this late pathology affected transmission rates (Girard et al., 2007). Recently it has been shown that a lab-derived strain of *Culex pipiens pipiens* was refractory to infection with West Nile virus, and that infection with this virus caused extensive cell death in the midgut epithelial cells of these mosquitoes (Vaidyanathan and Scott, 2006). Besides viral infection, a number of apoptosis-related genes, as well as other immune response genes, are expressed in hemocytes of *Ae. aegypti* and *Armigeres subalbatus* infected with bacterial pathogens (Bartholomay et al., 2004). In addition, *Plasmodium* can elicit pathology resembling apoptosis in mosquito vectors. *Plasmodium berghei* infection causes apoptosis in midgut cells of *Anopheles stephensi* (Han et al., 2000) and *An. gambiae* (Vlachou et al., 2004), and activation of Ancaspase 7 in midgut cells of *An. stephensi* (Abraham et al., 2004). With *P. gallinaceum* and *Ae. aegypti,* ookinete infection of midgut cells has also been reported to activate caspases (Zieler and Dvorak, 2000).

Even though numerous reports suggest that cell death might play a role in certain infections of mosquitoes, knowledge of the basic mechanics of apoptosis in mosquito vectors is lacking. There have been reports of *IAP1* genes in *Aedes triseriatus* (Blitvich et al., 2002) and *Ae. albopictus* (Li et al., 2007). A recent study suggests that these genes are regulated by an alternative splicing mechanism (Beck et al., 2007). The initiator caspases Dredd and Dronc have been reported in *Ae. aegypti* (Cooper et al., 2007a; Cooper et al., 2007b). There has also been a report of an IAP-antagonist related to the rpr protein from *Drosophila* found in mosquitoes called Michelob_x (Zhou et al., 2005a). In addition, a number of caspases and IAP proteins were recently annotated in the *Ae. aegypti* genome (Waterhouse et al., 2007). We independently identified a number of apoptosis-related genes, using the available *Ae. aegypti* genome sequence. In most cases, our results agreed with these previous annotations, but we have made improvements to the annotation of several of these genes, and we have identified four additional apoptosis-related genes that were not previously annotated in *Ae. aegypti*.

Materials and Methods

Cells and insect rearing

ATC-10 (*Ae. aegypti*) and C6/36 (*Ae. albopictus*) cells were maintained in L-15 medium (Gibco) supplemented with 20% FBS (ATC-10) or 10% FBS (C6/36) at 25°C. *Ae. aegypti* mosquitoes (RexD strain) were reared at the Arthropod-Borne and Infectious Disease Laboratory at 26–28°C, 80–82% humidity, under a 10h dark/14h light regime. Adults were maintained on sucrose and naive adult females were collected at 4 days post-eclosion.

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Database mining

The *Ae. aegypti* genome was searched for apoptosis related genes using known proteins from *Drosophila* (obtained from FlyBase (http://flybase.bio.indiana.edu)) and predicted proteins from the *An. gambiae* genome as queries in BLAST searches. Supercontigs from the *Ae. aegypti* genome were obtained and small portions that were identified from the BLAST search were used as queries to search the EST_others database at NCBI using nucleotide-nucleotide BLAST. ESTs from *Ae. aegypti* identified after the initial BLAST search were then used as queries reiteratively until no new ESTs were obtained. The ESTs were then translated and aligned using Vector NTI to assemble mini-contigs for transcripts. Genscan (http://genes.mit.edu/GENSCAN.html) and fgenesh (http://www.SoftBerry.com) were used to predict genes and complete missing regions of some EST contigs.

Updated annotations for CASPS7, CASPS20, CASPS17, CASPS21, IAP2, DNR1, ARK, and IMP have been submitted to VectorBase (http://www.vectorbase.org) and are visible as part of the “Manual annotation track” in the genome browser. These sequences will be incorporated into the next VectorBase genebuild (Neil Lobo, personal communication).

Protein domain determination

Domains for the proteins were predicted using the programs ExPASY PROSITE (Hulo et al., 2006), SMART (Schultz et al., 1998), and Conserved Domain Database at NCBI (Marchler-Bauer et al., 2005).

Phylogenetic analysis

Amino acid sequences were aligned using the ClustalW algorithm with the default parameters found in MEGA 3.1 (Kumar et al., 2004). Phylogenetic trees were assembled using MEGA 3.1. Trees were built using neighbor end joining, complete deletion, and p-distance. Other parameters were set at default values.

Plasmid construction

Michelob_x cDNA was obtained as a generous gift from L. Zhou (University of Florida). EST clones DV326893, DV369010, DV323242, DV328064, DV330266 were obtained from D. Severson (University of Notre Dame). IMP and Michelob_x cDNAs were inserted into the expression vector pHSP70PLVI+Rpr-epi (Vucic et al., 1997), replacing the Rpr gene using BglII and SpeI, and the constructs were named pHSMichelob_x-c-EpiHisVI+ and pHSIMP-c-EpiHisVI+. This resulted in HA and His6 tags being fused to the C terminus of the inserted coding sequence. The plasmid pHSP70GFPBsu36I, expressing eGFP (enhanced green fluorescent protein), has been previously described (Clarke and Clem, 2002). All other genes were cloned into the pCRII vector (Invitrogen) for sequencing purposes. 5’ RACE was performed as previously published (Beck et al., 2007).

Transfections

C6/36 cells were plated at a density of $2x10^6$ cells per well in six-well plates and incubated overnight. The following day, cells were transfected using FuGENE 6 (Roche) following the manufacturer’s instructions. A total of 6 μg of DNA with 9μl of FuGENE 6 was used for each transfection. Under these conditions, transfection efficiency was approximately 20%.

Annexin V staining and caspase activity measurements

At 24 hours post transfection, cells were harvested and assayed for Annexin V staining and caspase activity. Cells were stained with Annexin V-PE (BD Pharmingen) following manufacturer’s instructions and analyzed by flow cytometry. Caspase activity was measured using N-acetyl-Asp-Glu-Val-Asp-7-amino-4-trifluoromethyl coumarin (Ac-DEVD-AFC)

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fluorescent substrate as previously described (Muro et al., 2004). The assays were done in triplicate. The caspase assay results include the results from all three assays, while the Annexin V staining results are from one representative assay.

Expression analysis

RNA was isolated from homogenized tissues of pooled individuals or lysed ATC-10 cells by Trizol (Invitrogen) and treated with Turbo DNA-free DNase (Ambion), following manufacturers instructions, to reduce the possibility of genomic DNA contamination. Approximately 2–3 μg of RNA was used to synthesize cDNA using M-MLV RT (Invitrogen) and an oligo dT-20 primer. The resulting cDNA was analyzed for expression of all eighteen genes (Table 1) as well as Michelob_x (GenBank accession number **ABD47742**) and actin 6 (*act-6*, GenBank accession number **DQ124691**). Expression was initially analyzed by semi-quantitative PCR and the amplicons were analyzed by agarose gel electrophoresis to verify their correct size. Real time PCR was performed using the BioRad iCycler Optical Module. Primers were designed by Beacon Designer 3 (Premier Biosoft) and are shown in Table III. Primers were tested by Tm gradient to determine the optimal Tm for each primer. iQ SYBR Green Supermix was used in all cycle threshold (Ct) determinations following manufacturers instructions (BioRad). To determine the concentration of cDNA needed for expression analysis, dilutions of the cDNA were assayed using *act-6* primers. For each of the five stages or tissues (L1/L2, L3/L4, pupae, female adult without midgut, and female adult midgut), three cDNA batches were made. All genes were assayed in duplicate using each cDNA batch. The PCR efficiency curves and melt curves were analyzed for each gene in each stage. For each cDNA batch that was analyzed, the RNA from each stage was used to determine the background for each pair of primers. Some primer sets gave a background Ct while others yielded no background Ct values. If the Ct from a certain gene was not below the background Ct, then the gene was considered not to be expressed.

Results

By searching the *Ae. aegypti* genome, we found numerous genes with homology to known apoptosis-related genes in *Drosophila*. The majority of these genes have been previously annotated (Waterhouse et al., 2007), but we were able to make improvements to some of these annotations (Table 1). We also identified four additional apoptosis-related genes, including an additional effector-type caspase, orthologs of *Drosophila* Ark and Dnr1, and an additional IAP antagonist, which we have named IMP (IAP-antagonist Micheob_x-like Protein). In keeping with the previously proposed genetic nomenclature (Waterhouse et al., 2007), we have named the new caspase CASPS21. Table 2 summarizes the bioinformatics analysis for each gene. If the EST overlap method yielded a full-length predicted transcript (as defined in Materials and Methods), and the existence of this transcript was verified by RT-PCR, then information about the gene is found in Table 2 and it is not discussed further here. Those that did not yield a full-length predicted transcript, however, are discussed further below.

Caspases

In NCBI, part of CASPS7 was represented by EAT35718, and this same fragment was also reported by Waterhouse et al. (2007). However, we determined that this was not the full-length transcript using the EST DV369010 (obtained from D. Severson). Portions of DV369010 are found on two supercontigs, 659 and 791. The portion represented by EAT35718, which includes the 3’ end of the transcript, is found on supercontig 659. Gene predictions, using GenScan, predicted that there were additional transcribed sequences further upstream of the gene in supercontig 791. When DV369010 was sequenced further, we found that the gene prediction program was correct, and this was verifed by RT-PCR from ATC-10 cells. This analysis added an additional 76 amino acids to the N-terminus of CASPS7.

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CASPS20 bioinformatics analysis yielded a full-length predicted transcript that is represented in NCBI as EAT33088, but the N-termini of the NCBI-predicted protein and the transcript constructed by overlapping ESTs are different. Waterhouse et al. (2007) reported the same sequence that is found in NCBI. 5' RACE was done on RNA from early stage (L1/L2) larvae and we found that the overlapping EST prediction for the transcript was correct. However, it is possible that there are alternatively spliced forms of CASPS20 present in other stages or tissues.

CASPS17 does not have a predicted protein in NCBI. Waterhouse et al. (2007) identified this gene, but their analysis did not include the full length transcript. CASPS17 is represented by EST DV323242 (obtained from D. Severson). DV323242 has a 3' stop codon, but further sequencing of DV323242 did not reveal an upstream stop codon in the same reading frame as the start codon. We were able to predict a potential 5' end of this transcript using the fgenesh gene prediction program, but we were unable to amplify this predicted transcript from ATC-10 cells, meaning that this transcript will require further verification. However, an interesting observation with this gene was that the 3'UTR (represented by DV323242) contained a repetitive DNA element approximately 35 bp in length that is found on multiple supercontigs and ESTs in *Ae. aegypti*. When this EST is used as a BLAST query against the genome, approximately 100 supercontigs result in hits. The exact nature of this repetitive sequence is unknown at this time.

Phylogenetic analysis of the caspases was done using MEGA 3.1 (Kumar et al., 2004), utilizing the full-length amino acid sequences to analyze caspases from *Ae. aegypti*, *An. gambiae* and *Drosophila*. It should be noted that the predicted caspases from *An. gambiae* have not been confirmed yet, and many are not full length. The results of the phylogenetic analysis are illustrated in Fig. 1A (effector caspases) and B (initiator caspases). *Drosophila* Damm and its mosquito orthologs, which are predicted to be effector caspases based on their short prodomains, actually aligned more closely with the initiator caspases, and so are included in the phylogenetic analysis with initiator caspases.

The effector caspases (Fig. 1A) are represented by two clades and an outlier caspase from *Ae. aegypti*, CASPS20. CASPS20 remained an outlier when all of the caspases were included in a single tree (not shown). Clade I includes only one caspase from *Drosophila*, decay. However, in both mosquito species, and especially in *An. gambiae*, there has been expansion of this gene. *An. gambiae* genes in Clade I include s3, s4, s5, s6, and s11, while *Ae. aegypti* is represented by CASPS19 and CASPS18. Also within Clade I there are additional caspases s1, s2 and s14 from *An. gambiae*. These are the only caspases in the analysis that have either serine or threonine instead of alanine in the active site sequence QAC(R/Q/G)(G/E) (Vernooy et al., 2000). Clade II includes caspases from all three species analyzed, but we were not able to distinguish clear orthologs for the effector caspases Ice and Dcp-1 from *Drosophila*, since Ice and Dcp-1 are more closely related to each other than to any of the mosquito caspases. However, CASPS7 and CASPS8 are the closest *Ae. aegypti* relatives to *Drosophila* Ice and Dcp-1.

Fig. 1B illustrates the phylogeny of the initiator caspases for the three dipteran species analyzed. The initiator caspases fall into three clades, Clades III through V. Clade III includes dream and Damm from *Drosophila* and their mosquito orthologs. It appears that *Drosophila* dream and Damm arose from a gene duplication event, which is particularly interesting since they differ in the lengths of their prodomains (dream has a long prodomain, while Damm has a short prodomain). The analysis suggests that these genes have been duplicated within each mosquito lineage. We note that the *An. gambiae* caspases s9 and s12 are extremely similar (98% nucleotide identity, including intron sequences). Thus we conclude these are alleles of the same gene, rather than two individual genes and so we have excluded s12 from our analysis. In

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contrast to Clade III, Clades IV and V include clear mosquito orthologs for *Drosophila* Nc and Dredd.

The caspase genes decay, Damm and dream all appear to have undergone duplication events in mosquitoes. Fig. 2 illustrates the genomic architecture for these duplications and how they compare in the three Dipteran species. CASPS15 and CASPS21 were found using gene prediction programs on supercontig 182, close to CASPS16 and CASPS17 (Fig. 2A). CASPS21 is a new caspase that was not previously identified. In *Ae. aegypti,* the predictions for CASPS16 and CASPS15 yielded introns with sizes of 16 and 11.5 Kbp. This is not surprising given that the *Ae. aegypti* genome contains a large number of transposable elements in the introns of genes, which has led to intron expansion (Nene et al., 2007). Accordingly, when we performed gene prediction analysis for the Damm and dream orthologs in *Ae. aegypti*, we found remnants of transposable elements in the introns of some of these genes. Fig. 2B shows the genome arrangements of expanded decay paralogs in mosquitoes.

Caspase Regulators

In addition to caspases, we also identified a number of genes that regulate caspases. This group includes proteins that are known in *Drosophila* to inhibit caspase activity or are involved in caspase activation. Fig. 3 illustrates the protein domains for the predicted *Ae. aegypti* proteins from the caspase regulator group and their *Drosophila* orthologs.

Among the genes in this category that we identified are members of the IAP family. We found five IAP homologs in the *Ae. aegypti* genome, but we only characterized three. Of the two that we did not characterize, one represents a dBruce ortholog and the other contains a single BIR domain, but determination of its 3' end was problematic due to the presence of transposable elements. The three IAP proteins that we characterized and their domains are illustrated in Fig. 3A-C. These *Ae. aegypti* IAPs have a protein architecture similar to that of the *Drosophila* IAPs. The first, which is designated as IAP1, is the ortholog of thread (th) and is represented in NCBI as ABK1289. The *Ae. aegypti* ortholog of Iap2 from *Drosophila* is represented in NCBI as EAT41756, but previous gene predictions do not appear to represent the full transcript because they do not include an initiating methionine. We employed GenScan and found an apparent initiator methionine in supercontig 214. We were able to amplify the predicted transcript from the ATC-10 cell line using RT-PCR, verifying the gene prediction results. This analysis added 90 amino acids to the N-terminus of *Ae. aegypti* IAP2. IAP5, the ortholog of *Drosophila* det (also called Deterin), is represented in NCBI as EAT33476. GenScan predictions did not result in additional sequences being included in this transcript.

In addition to members of the IAP family, we also identified the *Ae. aegypti* ortholog of the caspase inhibitor Dnr1. *Ae. aegypti* DNR1 has a predicted protein domain architecture similar to that of Dnr1 from *Drosophila*, as illustrated in Fig. 3D. The ESTs encoding the *Ae. aegypti* DNR1 did not contain an initiation codon, but did yield 3' stop codons. However the predicted protein in NCBI (EAT48387) has an initiation codon. We were able to verify the NCBI-predicted transcript by RT-PCR from ATC-10 cells.

We also identified genes that encode activators of caspases. The *Ae. aegypti* ortholog for Ark was found by using three domains found in Ark, the CARD (caspase recruitment domain), NB-ARC (nucleotide-binding adaptor shared by Apaf-1, certain R gene products and CED-4), and WD-40 domains, in individual BLAST searches against NCBI predicted proteins for *Ae. aegypti*. Two predicted proteins in NCBI, EAT48065 and EAT48066, contained these domains but were in separate predicted proteins found relatively close to each other. EAT48065 encodes CARD and NB-ARC domains, while EAT 48066 encodes WD-40 repeat domains. Primers were designed from the 5' end of EAT48065 and 3' end of EAT48066, and we were able to amplify a single continuous transcript from ATC-10 cells, thus confirming that these domains

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are all part of a single protein. Fig. 3E illustrates that *Ae. aegypti* ARK and *Drosophila* Ark are highly similar in their architecture, except for one fewer WD-40 domain in *Ae. aegypti* ARK. BG4 is also a known activator of caspases in *Drosophila*. The *Ae. aegypti* BG4 ortholog has been previously annotated (Waterhouse et al., 2007). Our bioinformatics analysis yielded a full-length transcript that is represented in NCBI as EAT46931. We were able to verify the full-length *Ae. aegypti* BG4 transcript by RT-PCR from ATC-10 cells.

Another group of proteins involved in caspase activation are the IAP antagonists, including rpr, W (wrinkled), grim and skl in *Drosophila*. These proteins do not share significant similarity, except for a small motif called the IAP Binding Motif (IBM) found at the N-terminus. By using Michelob_x as a query, we identified another protein containing an IBM in *Ae. aegypti*. The EST DV326893 (obtained from D. Severson) contained the transcript with 5’ and 3’ flanking initiation and stop codons. This gene, which we named IMP, is represented in NCBI as EAT44230.

Because of the low level of similarity between IAP antagonists, we sought to verify that IMP encodes a functional pro-apoptotic gene. The IMP cDNA was cloned into an expression vector and expressed in C6/36 cells by transient transfection. Cells overexpressing IMP were analyzed at 24 h post-transfection for Annexin V staining (a marker for early apoptosis) by flow cytometry (Fig. 4A) and for caspase activity by incubating lysate from transfected cells with the (human) caspase-3 substrate Ac-DEVD-AFC and analyzing AFC fluorescence (Fig. 4B). Expression of either Michelob_x or IMP, but not GFP, resulted in higher Annexin V staining (shown by a shift to the right) in a portion of the cells consistent with the level of transfection efficiency routinely observed using these cells (around 20%). There was also a decrease in cell size (shown by a downward shift) upon expression of Michelob_x or IMP, which is also characteristic of apoptotic cells.

In support of the flow cytometry results, we also observed increased effector caspase activity in C6/36 cells expressing Michelob_x or IMP. Lysates from cells expressing either Michelob_x or IMP cleaved significantly higher amounts of the effector caspase substrate Ac-DEVD-AFC during a 60 min incubation period than control cells expressing GFP (Fig. 4B). Cells expressing Michelob_x or IMP also exhibited blebbing morphology typical of apoptotic cells (data not shown). Together, these results verify that IMP is a pro-apoptotic protein similar to Michelob_x.

Expression Analysis

To examine the expression of these potential regulators of apoptosis throughout the life cycle of *Ae. aegypti*, we employed quantitative reverse transcriptase PCR (RT-PCR). We tested early larvae (pooled L1 and L2 larval stages), late larvae (pooled L3 and L4 larval stages), pupae, and adults. For the adults, we analyzed females only, and analyzed midguts separately from the rest of the adult insect.

Fig. 5 illustrates the quantitative RT-PCR results for the genes across the five stages and tissues, with the exception of CASPS15. Although we did not detect expression of CASPS15 in any of the samples we assayed, there are ESTs corresponding to CASPS15 in NCBI, indicating that it is an expressed gene. The RT-PCR results were normalized by comparison to *act-6* expression and are shown as $2^{-\Delta Ct}$. We observed that expression was highest in the adult female midgut for a majority of the genes, with exceptions being ARK, Dronc, CASPS8 and CASPS20, which showed the highest expression in pupae. The larval stages tended to have the lowest expression for most genes. Caspases were expressed at the highest levels in the midgut. ARK, CASPS17, CASPS21, CASPS20, CASPS19 and CASPS18 did not show Ct values above background in the adult body minus the midgut, while BG4, CASPS17, CASPS21, Dredd, CASPS19 and CASPS18 did not show Ct values above background in

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pupae. Michelob_x was the only gene that did not show expression above background levels in early larvae (L1/L2). In these cases where Ct values were not above background, it is still possible that these genes are expressed in these stages or tissues, but at low levels. These results suggest that different components of the apoptotic machinery are expressed at varying levels in different developmental stages and tissues in *Ae. aegypti*.

Discussion

This study reports the annotation of a number of potential apoptosis related genes in *Ae. aegypti.* To date, our understanding of apoptosis in insects is based almost entirely on studies from *Drosophila*. This study represents the most thorough attempt to date to identify apoptosis related genes in any other insect. With the exception of IAP1 (Beck et al., 2007), Dredd (Cooper et al., 2007a), Dronc (Cooper et al., 2007b), and Michelob_x (Zhou et al., 2005a), the genes reported here have not been previously verified by cDNA analysis. *In silico* analysis helps tremendously in annotating genes, but the importance of verifying cDNA sequences is illustrated by the differences we observed between predicted proteins and cDNA comparisons with ARK, CASPS7, CASPS20 and IAP2. We also identified one additional caspase (CASPS21) that has not been previously annotated. The genes identified in this study hold promise for improving our understanding of apoptotic regulation in the important disease vector *Ae. aegypti*. In addition to elucidating the mechanics of apoptotic regulation, identification of these genes promises to aide in improving our understanding of innate immunity in the yellow fever mosquito. Several of the genes examined in this study are thought to play important roles in *Drosophila* immunity, including Dredd, Iap2, BG4, and Dnr1. It is likely that these genes are also important in *Ae. aegypti* immunity, but further study is needed to determine their exact roles in this process.

Apparent gene duplications have occurred with several caspase genes in both *Ae. aegypti* and *An. gambiae*. At this time it is not clear why mosquitoes possess more caspase genes than *Drosophila*. One possibility is that there is more of a need for caspases in regulating the innate immune responses in mosquitoes, since they may be exposed to more potential pathogens because of their hemophagic life style. For *Ae. aegypti*, these gene duplications are seen in Clade I (CASPS19 and CASPS18) and Clade III (CASPS16, CASPS15, CASPS17 and CASPS21). In Clade I, the *Drosophila* gene decay is expanded in both mosquito species analyzed. In *An. gambiae* there are eight homologs of this gene while *Ae. aegypti* has two homologs. The function of decay has not been studied in detail in *Drosophila* (Dorstyn et al., 1999), but there may be additional selective pressures for these caspases in mosquitoes. An interesting observation is that CASPS18 has a serine instead of a cysteine in its active site. This makes it unlikely that CASPS18 encodes a functional caspase. Similar levels of expression were observed for both CASPS19 and CASPS18 throughout the life cycle of *Ae. aegypti*. Thus it is possible that CASPS18 regulates CASPS19 in a dominant-negative manner, similar to what has been shown in humans with caspase-1 being regulated by Pseudo-ICE and ICEBERG (Druilhe et al., 2001).

Clade III includes duplications for both Damm and dream in the mosquitoes. The phylogeny of Damm and dream is interesting, since this appears to be a case where gene duplication occurred and one of the duplicated genes later either acquired or lost a long prodomain sequence. While dream appears to be an initiator caspase, whether Damm is an effector or initiator caspase is not entirely clear. The phylogeny results would suggest that Damm may be an unusual type of initiator caspase, although it is also possible that Damm may not be correctly annotated in any of these insect species. Damm and dream have also not been extensively studied in *Drosophila* (Doumanis et al., 2001; Harvey et al., 2001), and so studies of these duplicated genes in mosquitoes should not only lead to a better understanding of apoptosis and

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innate immunity in mosquitoes, but also may help in gaining a better understanding of the role of decay, Damm and dream in *Drosophila*.

When IMP is used as a BLAST query, a representative for this gene does not appear in other mosquitoes. In *Drosophila,* the RHG genes share very little similarity, so this is not surprising. However, when Michelob_x from *Ae. aegypti* is used as a query, one can easily find *An. gambiae* and *Ae. albopictus* orthologs of Michelob_x. Thus it appears that IMP is not as well conserved as Michelob_x in mosquitoes. Our results indicate that IMP is a pro-apoptotic gene that likely functions as an IAP antagonist, similar to Michelob_x. In *Drosophila*, there are at least four IAP antagonists (rpr, grim, W, and skl). Thus it is likely that there are additional IAP antagonists in the genomes of the mosquitoes, but their level of similarity is too low to detect by traditional BLAST searches.

Fig. 6 illustrates the differences between mosquitoes and *Drosophila* in the numbers of genes involved in the core apoptotic pathway, based on the pathway that has been established in *Drosophila*. In *Drosophila,* Nc is the main initiator caspase that activates Ice and Dcp-1, which ultimately leads to death. Thread has been shown to inhibit both Nc and Ice, and IAP antagonist interactions with IAP proteins have also been studied extensively (Chai et al., 2003;Yan et al., 2004). According to this simplified model, thread is required to prevent the accumulation of active Nc, which appears to be constitutively activated in an ARK-dependent manner. IAP antagonists interrupt the interaction between Nc and thread. This interaction frees Nc, which is then able to activate Ice, resulting in apoptosis (Hay and Guo, 2006). Fig. 6C illustrates the high amount of divergence among the IAP antagonists in different insects. However, even though these genes are extremely poorly conserved except for their IBM motifs, the genes still cluster in a species-specific manner. In Fig. 6D we illustrate the expansion that has occurred for the effector caspases in mosquitoes. Another interesting phenomenon is that *An. gambiae* has two paralogs of thread, while *Ae. aegypti* has only one version of this gene, as does *Drosophila*. While the effector caspases and IAP1 genes have duplications in mosquitoes, other genes that make up the core apoptotic pathway have not gone through a duplication event, such as the initiator caspase Nc and the adaptor protein ARK.

For a productive infection and transmission cycle to occur, pathogens such as arboviruses and *Plasmodium* must overcome many barriers in the mosquito vector. These pathogens must establish infection of the midgut epithelium and replicate in these cells, escape from this barrier by passing through the basal lamina, replicate efficiently in other organs, and finally infect the salivary glands and penetrate into the lumen of the salivary glands for transmission (Black et al., 2002). If any one of these barriers is not crossed, the pathogen cannot establish infection and be transmitted from mosquito to a vertebrate host. Interestingly, the midgut showed higher expression for many of the presumptive apoptotic regulating genes as compared to the other tissues or stages. Whether this apparent high level of expression is due to differences in *act-6* expression between tissues or stages is not known at this time (since transcript levels were normalized against *act-6*). In addition, it must be kept in mind that the level of protein may not correspond to the level of transcript. Especially intriguing is that expression of IAP1 was much higher in midgut than in any other tissue or stage analyzed. Based on the known crucial role of thread and SfIAP in preventing spontaneous apoptosis in *Drosophila* and *Spodoptera frugiperda* cells, respectively (Igaki et al., 2002; Muro et al., 2002; Zimmermann et al., 2002), it is tempting to speculate that *Ae. aegypti* IAP1 might have a similar role in regulating apoptosis. This needs to be determined, but if correct, then high levels of IAP1 expression may function to protect midgut cells from spontaneous apoptosis. It has been shown in *An. stephensi* (Han et al., 2000; Abraham et al., 2004), *An. gambiae* (Vlachou et al., 2004), and *Ae. aegypti* (Zieler and Dvorak, 2000) that apoptosis occurs during the establishment of infection in the midgut by different *Plasmodium spp*. It has also been demonstrated in *An. gambiae* by microarray analysis that transcript levels of the thread homologs AgIAP3 and

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AgIAP4 are up-regulated during midgut invasion by *P. berghei* and down-regulated after invasion (Vlachou et al., 2005). This suggests that, similar to thread and SfIAP, down regulation of AgIAP3 and AgIAP4 by *Plasmodium* infection may play a role in triggering apoptosis in midgut cells. Depending on the pathogen, regulation of IAP1 levels could possibly play a role in establishment of infection of *Ae. aegypti* midgut by *Plasmodium* and/or arboviruses.

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References

Abraham EG, Islam S, Srinivasan P, Ghosh AK, Valenzuela JG, Ribeiro JMC, Kafatos FC, Dimopoulos G, Jacobs-Lorena M. Analysis of the *Plasmodium* and *Anopholes* transcriptional repertoire during ookinete development and midgut invasion. J Biol Chem 2004;279:5573–5580. [PubMed: 14627712]

Bao Q, Shi Y. Apoptosome: a platform for the activation of initiator caspases. Cell Death Differ 2007;14:56–65. [PubMed: 16977332]

Bartholomay LC, Cho WL, Rocheleau TA, Boyle JP, Beck ET, Fuchs JF, Liss P, Rusch M, Butler KM, Wu RC, Lin SP, Kuo HY, Tsao IY, Huang CY, Liu TT, Hsiao KJ, Tsai SF, Yang UC, Nappi AJ, Perna NT, Chen CC, Christensen BM. Description of the transcriptomes of immune response-activated hemocytes from the mosquito vectors Aedes aegypti and Armigeres subalbatus. Infect Immun 2004;72:4114–4126. [PubMed: 15213157]

Beck ET, Blair CD, Black WCI, Beaty BJ, Blitvich BJ. Alternative splicing generates multiple transcripts of the inhibitor of apoptosis protein 1 in *Aedes* and *Culex spp*. mosquitoes. Insect Biochem Mol Biol. 2007in press

Bergmann A, Agapite J, McCall K, Steller H. The *Drosophila* gene *hid* is a direct molecular target of Ras-dependent survival signaling. Cell 1998;95:331–341. [PubMed: 9814704]

Black WCI, Bennett KE, Gorrochotegui-Escalante N, Barillas-Mury CV, Fernandez-Salas I, de Lourdes Munoz M, Farfan-Ale JA, Olson KE, Beaty BJ. Flavivirus susceptibility in *Aedes aegypti*. Arch Med Res 2002;33:379–388. [PubMed: 12234528]

Blitvich BJ, Blair CD, Kempf BJ, Hughes MT, Black WC, Mackie RS, Meredith CT, Beaty BJ, Rayms-Keller A. Developmental- and tissue-specific expression of an inhibitor of apoptosis protein 1 homologue from *Aedes triseriatus* mosquitoes. Insect Molec Biol 2002;11:431–442. [PubMed: 12230542]

Bowers DF, Coleman CG, Brown DT. Sindbis virus-associated pathology in Aedes albopictus (Diptera: Culicidae). J Med Entomol 2003;40:698–705. [PubMed: 14596286]

Chai J, Yan N, Huh JR, Wu J-W, Li W, Hay BA, Shi Y. Molecular mechanism of Reaper-Grim-Hid-mediated suppression of DIAP1-dependent Dronc ubiquitination. Nat Struct Biol 2003;10:892–898. [PubMed: 14517550]

Chen P, Nordstrom W, Gish B, Abrams J. *grim,* a novel cell death gene in *Drosophila*. Genes Devel 1996;10:1733–1782.

Chinnaiyan AM, Tepper CG, Seldin MF, O'Rourke K, Kischkel FC, Hellbardt S, Krammer PH, Peter ME, Dixit VM. FADD/MORT1 is a common mediator of CD95 (Fas/APO-1) and tumor necrosis factor receptor-induced apoptosis. J Biol Chem 1996;271:4961–4965. [PubMed: 8617770]

Clarke TE, Clem RJ. Lack of involvement of haemocytes in the establishment and spread of infection in *Spodoptera frugiperda* larvae infected with the baculovirus *Autographa californica* M nucleopolyhedrovirus by intrahemocoelic injection. J Gen Virol 2002;83:1565–1572. [PubMed: 12075074]

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Clem RJ. The role of apoptosis in defense against baculovirus infection in insects. Curr Top Microbiol Immunol 2005;289:113–130. [PubMed: 15791953]

Cooper DM, Pio F, Thi EP, Theilmann D, Lowenberger C. Characterization of *Aedes* Dredd: a novel initiator caspase from the yellow fever mosquito, *Aedes aegypti*. Insect Biochem Mol Biol 2007a; 37:559–569. [PubMed: 17517333]

Cooper DM, Thi EP, Chamberlain CM, Pio F, Lowenberger C. *Aedes* Dronc: a novel ecdysone-inducible caspase in the yellow fever mosquito, *Aedes aegypti*. Insect Mol Biol. 2007bin press

Crook NE, Clem RJ, Miller LK. An apoptosis-inhibiting baculovirus gene with a zinc finger-like motif. J Virol 1993;67:2168–2174. [PubMed: 8445726]

Dorstyn L, Read SH, Quinn LM, Richardson H, Kumar S. DECAY, a novel *Drosophila* caspase related to mammalian caspase-3 and caspase-7. J Biol Chem 1999;274:30778–30783. [PubMed: 10521468]

Doumanis J, Quinn L, Richardson H, Kumar S. STRICA, a novel *Drosophila melanogaster* caspase with an unusual serine/threonine-rich prodomain, interacts with DIAP1 and DIAP2. Cell Death Differ 2001;8:387–394. [PubMed: 11550090]

Druilhe A, Srinivasula SM, Razmara M, Ahmad M, Alnemri ES. Regulation of IL-1beta generation by Pseudo-ICE and ICEBERG, two dominant negative caspase recruitment domain proteins. Cell Death Differ 2001;8:649–657. [PubMed: 11536016]

Elrod-Erickson M, Mishra S, Schneider D. Interactions between the cellular and humoral immune responses in *Drosophila*. Curr Biol 2000;10:781–784. [PubMed: 10898983]

Foley E, O'Farrell PH. Functional dissection of an innate immune response by a genome-wide RNAi screen. PLoS Biol 2004;2:E203. [PubMed: 15221030]

Gesellchen V, Kuttenkeuler D, Steckel M, Pelte N, Boutros M. An RNA interference screen identifies Inhibitor of Apoptosis Protein 2 as a regulator of innate immune signalling in *Drosophila*. EMBO Rep 2005;6:979–984. [PubMed: 16170305]

Girard YA, Popov V, Wen J, Han V, Higgs S. Ultrastructural study of West Nile virus pathogenesis in *Culex pipiens quinquefasciatus* (Diptera: Culicidae). J Med Entomol 2005;42:429–444. [PubMed: 15962797]

Girard YA, Schneider BS, McGee CE, Wen J, Han VC, Popov V, Mason PW, Higgs S. Salivary gland morphology and virus transmission during long-term cytopathologic West Nile virus infection in *Culex* mosquitoes. Am J Trop Med Hyg 2007;76:118–128. [PubMed: 17255239]

Grether ME, Abrams JM, Agapite J, White K, Steller H. The *head involution defective* gene of *Drosophila melanogaster* functions in programmed cell death. Genes Dev 1995;9:1694–1708. [PubMed: 7622034]

Han YS, Thompson J, Kafatos FC, Barillas-Mury C. Molecular interactions between *Anopheles stephensi* midgut cells and *Plasmodium berghei*: the time bomb theory of ookinete invasion of mosquitoes. EMBO J 2000;19:6030–6040. [PubMed: 11080150]

Harvey NL, Daish T, Mills K, Dorstyn L, Quinn LM, Read SH, Richardson H, Kumar S. Characterization of the *Drosophila* caspase, DAMM. J Biol Chem 2001;276:25342–25350. [PubMed: 11337486]

Hay BA, Guo M. Caspase-dependent cell death in *Drosophila*. Annu Rev Cell Dev Biol 2006;22:623–650. [PubMed: 16842034]

Hay BA, Huh JR, Guo M. The genetics of cell death: approaches, insights and opportunities in *Drosophila*. Nat Rev Genet 2004;5:911–922. [PubMed: 15573123]

Hay BA, Wassarman DA, Rubin GM. Drosophila homologs of baculovirus inhibitor of apoptosis proteins function to block cell death. Cell 1995;83:1253–1262. [PubMed: 8548811]

Horng T, Medzhitov R. *Drosophila* MyD88 is an adaptor in the Toll signaling pathway. Proc Natl Acad Sci USA 2001;98:12654–12658. [PubMed: 11606776]

Hulo N, Bairoch A, Bulliard V, Cerutti L, De Castro E, Langendijk-Genevaux PS, Pagni M, Sigrist CJA. The PROSITE database. Nucleic Acids Res 2006;34:D227–D230. [PubMed: 16381852]

Igaki T, Yamamoto-Goto Y, Tokushige N, Kanda H, Miura M. Downregulation of DIAP1 triggers a novel Drosophila cell death pathway mediated by Dark and Dronc. J Biol Chem 2002;277:23103–23106. [PubMed: 12011068]

James ER, Green DR. Manipulation of apoptosis in the host-parasite interaction. Trends Parasitol 2004;20:280–287. [PubMed: 15147679]

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Kleino A, Valanne S, Ulvila J, Kallio J, Myllymaki H, Enwald H, Stoven S, Poidevin M, Ueda R, Hultmark D, Lemaitre B, Ramet M. Inhibitor of apoptosis 2 and TAK1-binding protein are components of the *Drosophila*. Imd pathway EMBO J 2005;24:3423–3434.

Kumar S, Cakouros D. Transcriptional control of the core cell-death machinery. Trends Biochem Sci 2004;29:193–199. [PubMed: 15082313]

Kumar S, Tamura K, Nei M. *MEGA3*: Integrated software for molecular evolutionary genetics analysis and sequence alignment. Brief Bioinform 2004;5:150–163. [PubMed: 15260895]

Leulier F, Rodriguez A, Khush RS, Abrams JM, Lemaitre B. The *Drosophila* caspase Dredd is required to resist Gram-negative bacterial infection. EMBO Rep 2000;1:353–358. [PubMed: 11269502]

Li Q, Li H, Blitvich BJ, Zhang J. The *Aedes albopictus* inhibitor of apoptosis 1 gene protects vertebrate cells from bluetongue virus-induced apoptosis. Insect Mol Biol 2007;16:93–105. [PubMed: 17257212]

Marchler-Bauer A, Anderson JB, Cherukuri PF, DeWeese-Scott C, Geer LY, Gwadz M, He S, Hurwitz DI, Jackson JD, Ke Z, Lanczycki CJ, Liebert CA, Liu C, Lu F, Marchler GH, Mullokandov M, Shoemaker BA, Simonyan V, Song JS, Thiessen PA, Yamashita RA, Yin JJ, Bryant SH. CDD: a conserved domain database for protein classification. Nucleic Acids Res 2005;33:D192–D196. [PubMed: 15608175]

Means JC, Muro I, Clem RJ. Lack of involvement of mitochondrial factors in caspase activation in a *Drosophila* cell-free system. Cell Death Diff 2006;13:1222–1234.

Mims CA, Day MF, Marshall ID. Cytopathic effect of Semliki Forest virus in the mosquito *Aedes aegypti*. Am J Trop Med Hyg 1966;15:775–784. [PubMed: 5917634]

Muro I, Hay BA, Clem RJ. The *Drosophila* DIAP1 protein is required to prevent accumulation of a continuously generated, processed form of the apical caspase DRONC. J Biol Chem 2002;277:49644–49650. [PubMed: 12397080]

Muro I, Monser K, Clem RJ. Mechanism of Dronc activation in *Drosophila* cells. J Cell Sci 2004;117:5035–5041. [PubMed: 15383617]

Nene V, Wortman JR, Lawson D, Haas B, Kodira C, Tu ZJ, Loftus B, Xi Z, Megy K, Grabherr M, Ren Q, Zdobnov EM, Lobo NF, Campbell KS, Brown SE, Bonaldo MF, Zhu J, Sinkins SP, Hogenkamp DG, Amedeo P, Arensburger P, Atkinson PW, Bidwell S, Biedler J, Birney E, Bruggner RV, Costas J, Coy MR, Crabtree J, Crawford M, Debruyn B, Decaprio D, Eiglmeier K, Eisenstadt E, El-Dorry H, Gelbart WM, Gomes SL, Hammond M, Hannick LI, Hogan JR, Holmes MH, Jaffe D, Johnston JS, Kennedy RC, Koo H, Kravitz S, Kriventseva EV, Kulp D, Labutti K, Lee E, Li S, Lovin DD, Mao C, Mauceli E, Menck CF, Miller JR, Montgomery P, Mori A, Nascimento AL, Naveira HF, Nusbaum C, O'Leary S, Orvis J, Pertea M, Quesneville H, Reidenbach KR, Rogers YH, Roth CW, Schneider JR, Schatz M, Shumway M, Stanke M, Stinson EO, Tubio JM, Vanzee JP, Verjovski-Almeida S, Werner D, White O, Wyder S, Zeng Q, Zhao Q, Zhao Y, Hill CA, Raikhel AS, Soares MB, Knudson DL, Lee NH, Galagan J, Salzberg SL, Paulsen IT, Dimopoulos G, Collins FH, Birren B, Fraser-Liggett CM, Severson DW. Genome sequence of *Aedes aegypti*, a major arbovirus vector. Science 2007;316:1718–1723. [PubMed: 17510324]

Opferman JT, Korsmeyer SJ. Apoptosis in the development and maintenance of the immune system. Nat Immunol 2003;4:410–415. [PubMed: 12719730]

Primrose DA, Chaudhry S, Johnson AG, Hrdlicka A, Schindler A, Tran D, Foley E. Interactions of DNR1 with the apoptotic machinery of *Drosophila melanogaster*. J Cell Sci 2007;120:1189–1199. [PubMed: 17341581]

Sanders HR, Foy BD, Evans AM, Ross LS, Beaty BJ, Olson KE, Gill SS. Sindbis virus induces transport processes and alters expression of innate immunity pathway genes in the midgut of the disease vector, *Aedes aegypti*. Insect Biochem Mol Biol 2005;35:1293–1307. [PubMed: 16203210]

Schultz J, Milpetz F, Bork P, Ponting CP. SMART, a simple modular architecture research tool: Identification of signaling domains. Proc Natl Acad Sci U S A 1998;95:5857–5864. [PubMed: 9600884]

Stoven S, Ando I, Kadalayil L, Engstrom Y, Hultmark D. Activation of the *Drosophila* NF-kappaB factor Relish by rapid endoproteolytic cleavage. EMBO Rep 2000;1:347–352. [PubMed: 11269501]

Vaidyanathan R, Scott TW. Apoptosis in mosquito midgut epithelia associated with West Nile virus infection. Apoptosis 2006;11:1643–1651. [PubMed: 16820968]

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Vaux DL, Korsmeyer SJ. Cell death in development. Cell 1999;96:245–254. [PubMed: 9988219]

Vaux DL, Silke J. IAPs, RINGs and ubiquitylation. Nat Rev Molec Cell Biol 2005;6:287–297. [PubMed: 15803136]

Vernooy SY, Copeland J, Ghaboosi N, Griffin EE, Yoo SJ, Hay BA. Cell Death Regulation in *Drosophila*: Conservation of Mechanism and Unique Insights. The Jounal of Cell Biology 2000;150:F69–F75.

Vlachou D, Schlegelmilch T, Christophides GK, Kafatos FC. Functional genomic analysis of midgut epithelial responses in *Anopheles* during *Plasmodium* invasion. Curr Biol 2005;15:1185–1195. [PubMed: 16005290]

Vlachou D, Zimmermann T, Cantera R, Janse CJ, Waters AP, Kafatos FC. Real-time, in vivo analysis of malaria ookinete locomotion and mosquito midgut invasion. Cell Microbiol 2004;6:671–685. [PubMed: 15186403]

Vucic D, Kaiser WJ, Harvey AJ, Miller LK. Inhibition of Reaper-induced apoptosis by interaction with inhibitor of apoptosis proteins (IAPs). Proc Natl Acad Sci USA 1997;94:10183–10188. [PubMed: 9294184]

Vucic D, Kaiser WJ, Miller LK. Inhibitor of apoptosis proteins physically interact with and block apoptosis induced by *Drosophila* proteins HID and GRIM. Mol Cell Biol 1998;18:3300–3309. [PubMed: 9584170]

Waterhouse RM, Kriventseva EV, Meister S, Xi Z, Alvarez KS, Bartholomay LC, Barillas-Mury C, Bian G, Blandin S, Christensen BM, Dong Y, Jiang H, Kanost MR, Koutsos AC, Levashina EA, Li J, Ligoxygakis P, Maccallum RM, Mayhew GF, Mendes A, Michel K, Osta MA, Paskewitz S, Shin SW, Vlachou D, Wang L, Wei W, Zheng L, Zou Z, Severson DW, Raikhel AS, Kafatos FC, Dimopoulos G, Zdobnov EM, Christophides GK. Evolutionary dynamics of immune-related genes and pathways in disease-vector mosquitoes. Science 2007;316:1738–1743. [PubMed: 17588928]

Weaver SC, Scott TW, Lorenz LH, Lerdthusnee K, Romoser WS. Togavirus-associated pathologic changes in the midgut of a natural mosquito vector. J Virol 1988;62:2083–2090. [PubMed: 2896802]

White K, Grether ME, Abrams JM, Young L, Farrell K, Steller H. Genetic control of programmed cell death in *Drosophila*. Science 1994;264:677–683. [PubMed: 8171319]

Wilson R, Goyal L, Ditzel M, Zachariou A, Baker DA, Agapite J, Steller H, Meier P. The DIAP1 RING finger mediates ubiquitination of Dronc and is indispensable for regulating apoptosis. Nat Cell Biol 2002;4:445–450. [PubMed: 12021771]

Yan SJ, Wu JW, Chai J, Li W, Shi Y. Molecular mechanisms of DrICE inhibition by DIAP1 and removal of inhibition by Reaper, Hid and Grim. Nat Struct Mol Biol 2004;11:420–428. [PubMed: 15107838]

Yu X, Wang L, Acehan D, Wang X, Akey CW. Three-dimensional structure of a double apoptosome formed by the *Drosophila* Apaf-1 related killer. J Mol Biol 2006;355:577–589. [PubMed: 16310803]

Zhou L, Jiang G, Chan G, Santos CP, Severson DW, Xiao L. Michelob_x is the missing inhibitor of apoptosis protein antagonist in mosquito genomes. EMBO Rep 2005a;6:769–774. [PubMed: 16041319]

Zhou R, Silverman N, Hong M, Liao DS, Chung Y, Chen ZJ, Maniatis T. The role of ubiquitination in *Drosophila* innate immunity. J Biol Chem 2005b;280:34048–34055. [PubMed: 16081424]

Zieler H, Dvorak JA. Invasion *in vitro* of mosquito midgut cells by the malaria parasite proceeds by a conserved mechanism and results in death of the invaded midgut cells. Proc Natl Acad Sci USA 2000;97:11516–11521. [PubMed: 11027351]

Zimmermann KC, Ricci J-E, Droin NM, Green DR. The role of ARK in stress-induced apoptosis in *Drosophila* cells. J Cell Biol 2002;156:1077–1087. [PubMed: 11901172]

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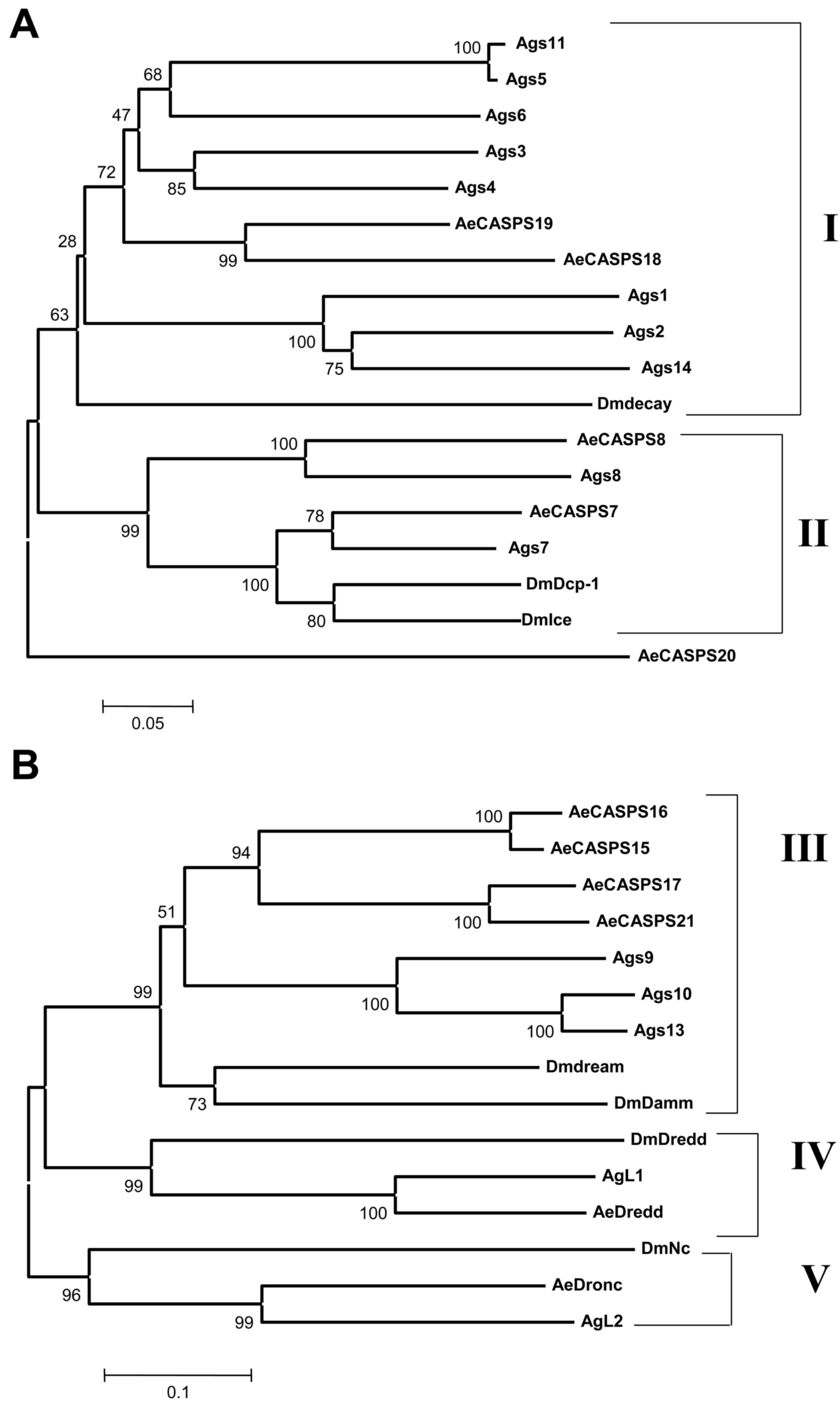

Figure 1.

Phylogenetic analysis of caspases in *Drosophila*, *An. gambiae*, and *Ae. aegypti.* Full length amino acid sequences were used to build phylogenetic trees using MEGA 3.1. Panel **A** includes effector caspases, while panel **B** includes initiator caspases and caspases related to Damm, which is predicted to be an effector caspase based on its short prodomain, but which groups with initiator caspases. Clades were determined by branching patterns and are represented as vertical lines on the right. Bootstrap values are shown.

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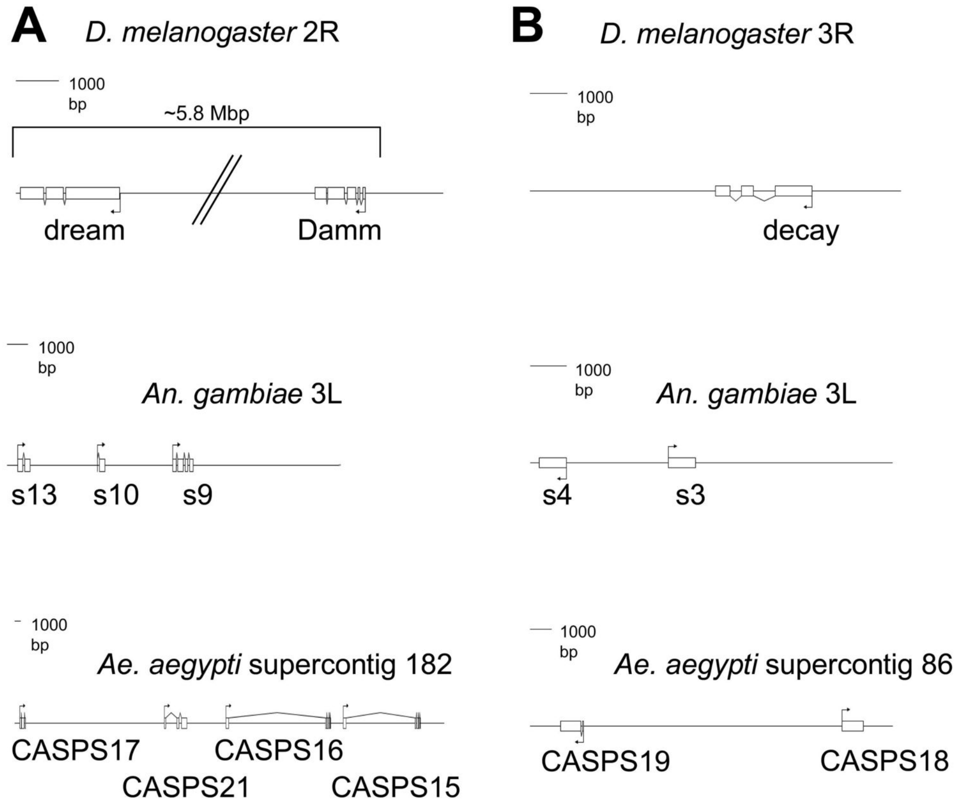

Figure 2.

Genome architecture of caspase genes within three dipteran species. **A**, genome organization of Damm and dream in *Drosophila* and their paralogs in *An. gambiae* and *Ae. aegypti*. **B**, genome organization of Decay in *Drosophila* and its paralogs in *An. gambiae* and *Ae. aegypti*. Genome information was obtained from websites for each organism, as explained in Materials and Methods. The illustrations were produced using GenePalette.

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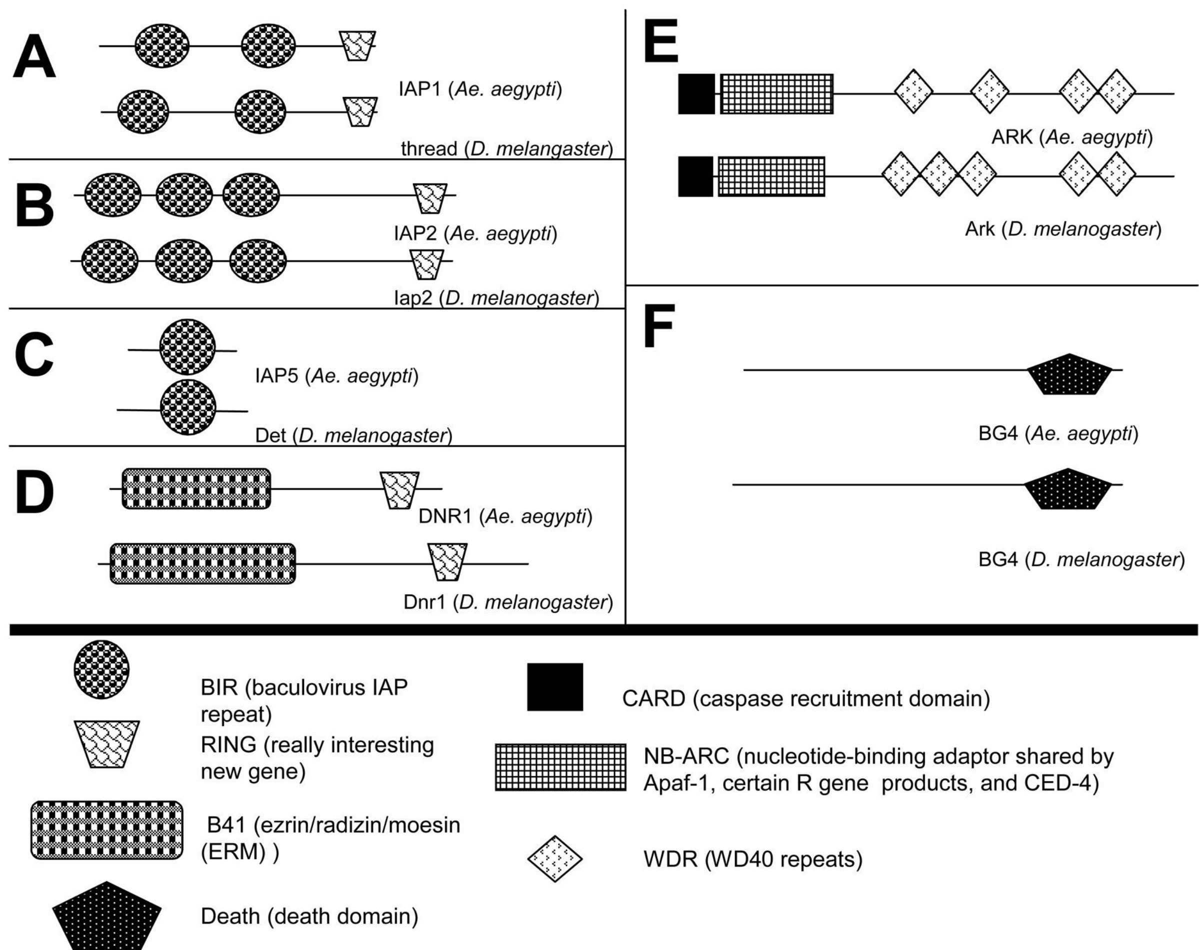

Figure 3.

Predicted domain architecture for some of the proteins identified in this study. Protein domains were predicted using SMART. Proteins for *Drosophila* were obtained from NCBI and FlyBase.

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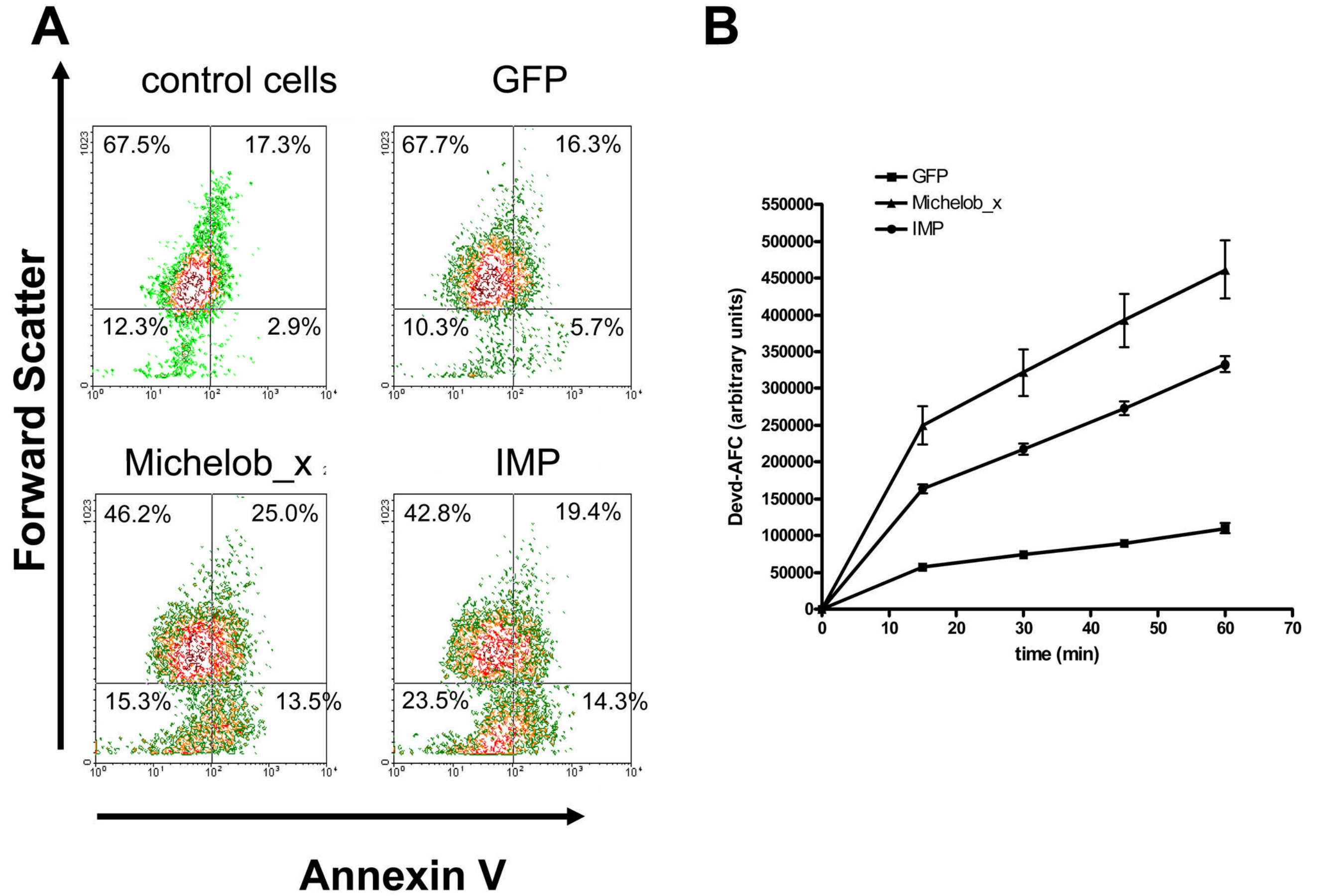

Figure 4.
Expression of IMP or Michelob_x causes apoptosis in C6/36 cells. **A**, AnnexinV staining (X-axis) versus forward scatter (Y-axis) of untransfected (control) cells or cells transfected with constructs expressing GFP, Michelob_x, or IMP, as analyzed by flow cytometry. Percentages are given for each quadrant. Each graph represents analysis of 10,000 cells. **B,** caspase activity in lysates from cells expressing GFP, Michelob_x, or IMP as determined by liberated AFC fluorescence over 60 min incubation with the caspase substrate Ac-DEVD-AFC. The data shown represent the combined results from three independent transfections.

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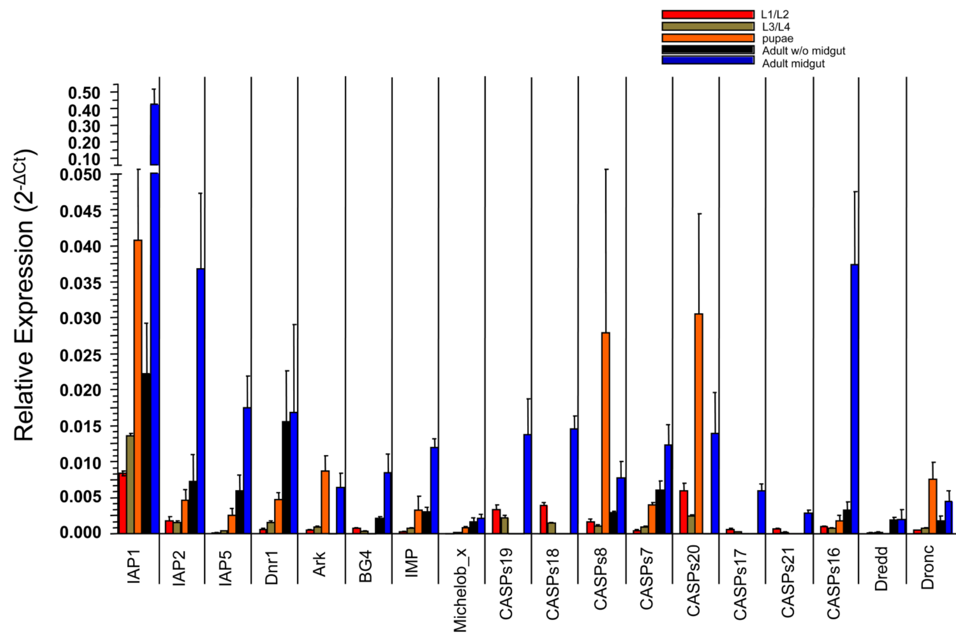

Figure 5.

Quantitative analysis of transcript levels for the annotated apoptosis regulatory genes in *Ae. aegypti* throughout the life cycle of the organism. The results of real time RT-PCR analysis in early larvae (L1/L2), late larvae (L3/L4), pupae, adult females without midgut, or adult female midgut are shown. Each data point represents the average $2^{-\Delta Ct}$ (+/−SE) obtained from three batches of cDNA made from each stage or tissue.

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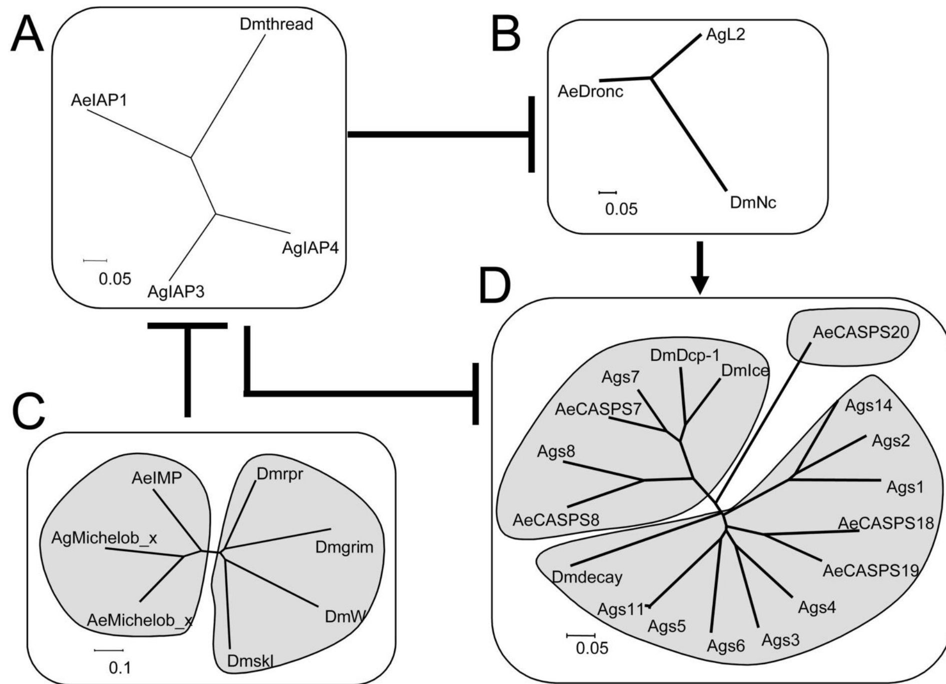

Figure 6.
Illustration of the conservation of core apoptosis regulatory genes within *Drosophila*, *An. gambiae* and *Ae. aegypti*. The unrooted trees were built using neighbor end joining, complete deletion, p-distance with full amino acid sequences. The pathway illustrated is based on information obtained from studies using *Drosophila*. In *Drosophila*, thread inhibits Nc and Ice, while thread itself is inhibited by the IBM-containing proteins rpr, W, and grim. The initiator caspase Nc is responsible for cleaving and activating effector caspases including Ice and Dcp-1. Nc is activated by Ark, which for simplicity is not shown but which is also conserved in *Ae. aegypti*.

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Table 1

Ae. aegypti apoptotic regulatory genes characterized in this study. Shown are the NCBI predicted proteins (if any), supercontigs the genes reside on, percent amino acid similarity and identity to the closest relative in *Drosophila*, and whether the confirmed sequence differs from that predicted in NCBI.

Gene	NCBI protein(s)	Supercontig(s)	%Similarity/Identity	Different from predicted?
IAP1	ABK01289	368	51.7/39.4 (th)	No
IAP2	EAT41756	214	46.8/34.3 (IAP2)	Yes
IAP5	EAT33476	1049	50.6/41 (det)	No
DNR1	EAT48387	11	45.9/35.1 (Dnr1)	No
ARK	EAT48065, EAT48066	18	30.4/18.8 (Ark)	Yes
BG4	EAT46931	46	35.1/21 (BG4)	No
IMP	EAT44230	117	10.9/7 (rpr)	No
CASPS19	EAT45302	86	53.8/37.8 (decay)	No
CASPS18	EAT45303	86	47.9/37.4 (decay)	No
CASPS8	EAT33369	1085	47.7/38.3 (Ice)	No
CASPS7	EAT35718	791, 659	61.1/52.5 (Ice)	Yes
CASPS20	EAT33088	1207	36.1/28.1 (Ice)	Yes
Dredd	EAT33580	1019	46.6/31.3 (Dredd)	No
Dronc	EAT36368	589	42.4/30.3 (Nc)	No
CASPS17	none	182	35.4/25.3 (Damm)	Yes
CASPS21	none	182	36.9/25 (Damm)	Yes
CASPS16	EAT42502	182	32.9/23.3 (dream)	No
CASPS15	EAT42503	182	34.2/24 (dream)	No

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Table 2

Bioinformatic analysis overview for the genes characterized in this study. Shown are the overlapping ESTs used to determine the transcript (if any), whether the ESTs yielded a full-length transcript, whether the transcript could be verified by RT-PCR from the ATC-10 cell line, and whether gene prediction programs were employed for each gene.

Gene	ESTs used	ESTs full- length?	Amplify from ATC- 10 cell line?	Gene prediction used?
IAP1	NA[a]	NA	Yes	No
IAP2	dv383781 eb090402	No	Yes	Yes/GenScan
IAP5	dv364268	No	Yes	Yes/GenScan
DNR1	dv237984 eg007533 eb089278 dw221134 dw205154	No	Yes	No
ARK	NA	NA	Yes	No
BG4	dv416615 dv416614 dv383790	Yes	Yes	No
IMP	dv326893	Yes	ND[b]	No
CASPS19	dv395012 dv330266	Yes	No	No
CASPS18	NA	NA	ND	No
CASPS8	dv250139 dv250137	Yes	Yes	No
CASPS7	dv369010	No	Yes	Yes/GenScan
CASPS20	dw202685 ee999223	Yes	ND	No
Dredd	dv382387 dv382385 dw190212	Yes	Yes	No
Dronc	dw220447 dw207394 dv343751 dv356662	Yes	Yes	No
CASPS17	dv323242	No	No	Yes/fgenesh
CASPS21	NA	NA	ND	Yes/fgenesh
CASPS16	dv241054 dv332892 dv328064	Yes	No	No
CASPS15	NA	NA	ND	Yes/fgenesh

[a]NA, not applicable

[b]ND, not determined

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Table 3

Primers utilized for expression analysis of presumptive apoptotic regulators of *Ae. aegypti.*

Gene	Forward	Reverse
IAP1	CTGAAACTAATGAAGGGCGAAGC	TTGAGATGACTGAAGCGAGGATG
IAP2	CCATTATCGTCGCCGTCTACC	CTTTCAGTCGTTTGTTCTCCTCTTC
IAP5	ACGACAAGGAGGACGAAGAC	TCCAGCAGGTTAAGCATTTCC
DNR1	GAAGATACTGAACTCGGCAAACG	CGGCAGGCGGTAATATGTCC
ARK	TGTCTAGCGTTTCGGTCTTGAG	GCGTTGGTTAGCCTGGATAATAATC
BG4	ACTTTGCCTGCTCAATTTCTTTCTC	GATACGCTGTTCTCCCTGTTGG
Michelob_x	CAACAGCAAAATCAGAACCAAATCAG	GCACAGCAGACATCGGGAAC
IMP	GCTGGACTGAGAACGCCTTC	ACGACTGATGAGAACAACAACAAC
CASPS19	CTCGCCGTGTGACATCATAAC	AAGCAAGGAAGTTCTCGTTTCTC
CASPS18	CTGTCTTGTGGTAGTTGTGATGTC	CGGATGCTTGTGATTCTTCTTCTC
CASPS8	TGGCAAAAGCAAACAGGAAGTC	GGGATGAAGGCGAAGTAATATACG
CASPS7	TTGGCAGAACGCACCGAAAC	CGAAAGTCAGCAGGGTCAGTAG
CASPS20	GCGGATTGCCTGATGGTATTC	ATGCTTGGACTATGAACAACTTCG
Dredd	AGAAGTATGTAATATCGTGGAAGAATGC	AGAACAGTGATGCGGCTCAAC
Dronc	CAACTTTCCAACTGCCTATAAATTGC	CTCCACCGTATCGTTATTGTTCTTAG
CASPS17	TGCCATTGATGAGAAGAGAATTTGAG	GCCTACTTGTCCCGTGTTACC
CASPS21	CGATTGTAATAAAACGGTTCCTAGTCC	CTATTGACATTTCTGGCATCTCTCTTAG
CASPS16	TCCGCTATCTTCATATTGTATCCTTTG	GACCCGCCACTGTATCTCTG
CASPS15	CCTAACTTGGGTTTGACGATTGC	AATGTCCGCTATCTTCATATTGTATCC
Act-6	AAGGCTAACCGTGAGAAGATGAC	GATTGGGACAGTGTGGGAGAC